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Urolithin A pretreatment protects against seizures through IGF-1R Tyr1166/1167 phosphorylation inhibition and enhances the antiseizure effect of valproic acid

Ming Li^{1#}, Jiabin Zong^{2#}, Yu Zhang^{1,4#}, Minghua Zou^{1,4}, Fu Lu^{1,4}, Songqing Pan¹, Lily Zhang^{1,4}, Liu Yang^{1,4}, Heyang Qin^{1,4}, Zhouhua Li^{1,4}, Haiyan Wen^{3*}, Wanbin Huang^{1,4*}, Zheman Xiao^{1,4*}

¹ Department of Neurology, Renmin Hospital of Wuhan University, Wuhan 430060, China

² Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

³ Department of Pharmacy, Renmin Hospital of Wuhan University, Wuhan 430060, China

⁴ Department of Encephalopathy in Traditional Chinese Medicine, Renmin Hospital of Wuhan University, Wuhan 430060, China

ABSTRACT: Urolithin A (UA), a metabolite derived from gut microbiota that originates from ellagitannins found in pomegranate, berries, and walnuts, has emerged as a promising postbiotic exhibiting mitophagy-inducing and neuroprotective properties. This study examined the antiseizure effects of UA pretreatment and its potential to enhance the efficacy of valproic acid (VPA), a first-line antiseizure medication, using a pilocarpine-induced status epilepticus (SE) rat model. UA significantly reduced seizure stage, prolonged latency, shortened duration, and decreased incidence of seizures, while alleviating SE-induced behavioral and cognitive impairments. Network pharmacology revealed insulin-like growth factor 1 receptor (IGF-1R) as a critical target of UA. Subsequent experiments demonstrated that UA exerts its therapeutic effects by inhibiting the phosphorylation of IGF-1R. Importantly, the combination of UA and VPA yielded enhanced antiseizure effects compared to either treatment alone in mitigating seizures and behavioral comorbidities. These findings suggest that UA may serve as a valuable adjuvant to conventional antiseizure therapy. Additionally, this study elucidates the antiseizure potential of UA as a pomegranate-derived postbiotic, providing experimental evidence from an animal model to support a UA-VPA combination strategy aimed at improving the efficacy of epilepsy treatment.

Keywords: urolithin A; epilepsy; combination therapy; IGF-1R

1. Introduction

Epilepsy, one of the most prevalent chronic neurological disorders, affects approximately 50 million individuals globally and accounts for 13 million disability-adjusted life years annually^[1]. The pathogenesis of epilepsy is multifactorial, encompassing an imbalance between excitatory and inhibitory neurotransmission, ion channel dysfunction, neuroinflammation, metabolic disturbances, and blood-brain barrier (BBB) disruption, which collectively lower the seizure threshold and promote recurrent spontaneous seizures^[2, 3]. Status epilepticus (SE), a life-threatening neurological emergency characterized by prolonged and excessive

These authors contributed equally to this work.

*Corresponding author

Zheman Xiao, zmxiao@whu.edu.cn;

Wanbin Huang, hwbwhu@163.com;

Haiyan Wen, why123@whu.edu.cn.

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neuronal excitation, may result in excitotoxic neuronal damage, cognitive dysfunction, and long-term behavioral abnormalities, representing a significant risk factor for the development of acquired epilepsy^[2, 4]. Despite the availability of various antiseizure medications (ASMs), approximately 30% of patients demonstrate inadequate responses, developing drug-resistant epilepsy and highlighting a major unmet therapeutic need^[5]. This urgent need is particularly evident in the context of SE, which has an estimated incidence of 10 to 40 cases per 100,000 persons annually and an overall mortality rate of 20% to 30%^[6]. Notably, about 20%-43% of SE cases progress to refractory SE, necessitating anesthetic coma induction—a condition associated with a short-term mortality rate of approximately 16%-39%, posing significant risks to patient life and health^[7, 8]. Consequently, the exploration of novel strategies to enhance conventional therapy remains an urgent clinical priority.

Among the signaling pathways implicated in epilepsy, the insulin-like growth factor 1 receptor (IGF-1R) and its downstream extracellular signal-regulated kinase (ERK) cascade have received increasing attention. IGF-1R, a transmembrane tyrosine kinase receptor widely expressed in the central nervous system, undergoes autophosphorylation upon ligand binding, activating key tyrosine residues in the kinase activation loop essential for full catalytic activation and the recruitment of insulin receptor substrate proteins, thus engaging downstream signaling cascades. In temporal lobe epilepsy, phosphorylation of IGF-1R is upregulated in human epileptogenic tissues and in rat models induced by pilocarpine and pentylenetetrazole^[9]. Following SE, robust activation of IGF-1R-mediated ERK signaling occurs in the hippocampus^[9]. Furthermore, constitutive neuronal ERK activation alone can induce spontaneous epileptic seizures by enhancing NMDA receptor function, and pharmacological inhibition of ERK signaling demonstrates anticonvulsant and neuroprotective effects across various seizure models^[10, 11]. These findings suggest that suppressing aberrant IGF-1R/ERK signaling could represent a promising disease-modifying strategy, particularly for seizures resistant to conventional ASMs. However, a critical knowledge gap persists: while elevated IGF-1R phosphorylation has been documented in epileptic tissues and animal models, no studies have yet employed site-directed mutagenesis to dissect the functional contributions of autophosphorylation sites to epileptogenesis.

Urolithin A (UA) is a metabolite produced by gut microbiota from ellagitannins (ETs) and ellagic acid (EA), which are naturally found in pomegranate, berries, walnuts, and certain medicinal plants^[12-15]. UA demonstrates favorable pharmacokinetic properties, including the ability to penetrate the BBB and accumulate in brain tissue, which provides a mechanistic basis for its central nervous system effects^[16]. Therapeutic benefits of UA have been reported in experimental models of Alzheimer's disease, Parkinson's disease, traumatic brain injury, cerebral ischemia, and age-related cognitive decline^[13, 17, 18]. Additionally, UA possesses an excellent safety profile, having received Generally Recognized as Safe (GRAS) status from the U.S. Food and Drug Administration in 2018 (GRAS Notice No. 791). Despite these compelling characteristics, the antiseizure potential of UA has not been systematically investigated.

Network pharmacology offers a systems biology approach to elucidate multi-target interactions between drugs and diseases. This study employed network pharmacology to predict potential targets of UA against

epilepsy, validated through *in vitro* and *in vivo* experiments. Evidence shows that UA exerts antiepileptic effects by targeting IGF-1R and suppressing IGF-1R phosphorylation at Tyr1165/1166 (corresponding to Tyr1135/1136 in the mature β -subunit). Using site-directed mutagenesis, activation-deficient (Y1136F) and phospho-mimetic (Y1136E) mutants were generated to directly validate the functional role of this site. Phosphorylation at the Tyr1166/1167 site is critical for the functional activation of IGF-1R, and UA-mediated inhibition of this key phosphorylation event disrupts downstream signaling. Importantly, the combination of UA with valproic acid (VPA) produced enhanced therapeutic effects that surpassed those of either agent alone, providing a novel strategy to improve epilepsy treatment outcomes.

2. Materials and Methods

2.1 Animals

Animal protocols received approval from the Institutional Animal Care and Use Committee of Wuhan University Renmin Hospital (Approval No. 2025000156, Wuhan, China) in accordance with the *Guide for the Care and Use of Laboratory Animals*^[19]. Eight-week-old male Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Center of Wuhan University Renmin Hospital. Rats were group-housed (three per cage) under a 12-hour light/dark cycle with ad libitum access to standard chow and water. Following a 7-day acclimatization period, rats were randomized into experimental groups using a computer-generated randomization sequence. All efforts were made to minimize animal numbers and suffering, adhering to the 3Rs principles.

2.2 Establishment of status epilepticus model and drug administration

The lithium-pilocarpine-induced SE model was established as per published protocols^[20]. Rats received an intraperitoneal (i.p.) injection of lithium chloride at a dose of 127 mg/kg (MedChemExpress, USA, HY-Y0649). After an interval of 18–24 hours, 1 mg/kg atropine sulfate was administered i.p. to mitigate peripheral cholinergic side effects. Different concentrations of UA (MedChemExpress, USA, HY-100599) or 300 mg/kg VPA (MedChemExpress, USA, HY-10585A) were also injected i.p. to assess their effects on epilepsy. UA was dissolved in DMSO (25 mg/mL) and subsequently diluted in phosphate-buffered saline (PBS, pH 7.4), resulting in a final DMSO concentration of 2% (v/v); the vehicle control contained an equivalent concentration of DMSO. Control rats received identical doses of lithium chloride and atropine sulfate, with pilocarpine replaced by an equivalent volume of vehicle. VPA was dissolved in saline. Thirty minutes later, 35 mg/kg pilocarpine hydrochloride (MedChemExpress, USA, HY-B0726) was injected i.p. to induce SE. SCH772984 ((MedChemExpress, USA, HY-50846, 10 μ g/g brain), dissolved in aCSF containing $\leq 5\%$ DMSO, or an equal volume of vehicle was injected into the cerebral ventricle 2 h before pilocarpine injection^[21]. All drug administrations preceded the pilocarpine injection; thus, the experimental design followed a pretreatment (prophylactic) paradigm, and the observed effects reflect preventive/protective rather than therapeutic actions.

Seizure severity was evaluated using the modified Racine scale: no abnormal behavior (Stage 0); orofacial clonus and chewing (Stage 1); rhythmic head nodding (Stage 2); sustained unilateral forelimb clonus (Stage 3); rearing with hindlimb support (Stage 4); rearing followed by loss of balance and falling (Stage 5); tonic-clonic seizures with postural loss (Stage 6)^[22]. Following 1 hour of continuous convulsions, 10 mg/kg diazepam was injected i.p. to terminate seizures. Control rats received the same doses of lithium chloride and atropine sulfate, with pilocarpine replaced by an equal volume of vehicle. Seizure severity assessments were conducted by research staff blinded to the experimental groups.

The method for tissue collection has previously been described^[23]. Rats were anesthetized with isoflurane and subjected to perfusion with 4 °C PBS. The hippocampus was then collected for further analysis. Major visceral organs, including lung, liver, spleen, kidney, and heart, were harvested from experimental rats for frozen section staining to evaluate *in vivo* visceral toxicity induced by UA.

To verify that UA alleviates epilepsy by inhibiting IGF-1R phosphorylation at Tyr1166/Tyr1167, recombinant adenovirus vectors encoding rat *Igf1r* wild-type (ADV-CMV-*Igf1r*-wt), a phosphomimetic mutation (tyrosines substituted with glutamic acid; ADV-CMV-*Igf1r*-Y1166E/Y1167E), and a phosphorylation-deficient mutation (tyrosines substituted with phenylalanine; ADV-CMV-*Igf1r*-Y1166F/Y1167F) were generated, along with an empty control vector. Rats underwent stereotaxic intrahippocampal injection of each respective virus. One week after viral injection, SE models were established, and hippocampal tissues were harvested as previously described.

2.3 Assessment of anxiety-like behavior

Anxiety-associated behavior was evaluated using two established paradigms: the light-dark transition test and the open-field test^[24, 25]. The light-dark box apparatus (100 cm × 50 cm × 40 cm) comprises a dimly lit dark compartment (48 cm × 48 cm × 40 cm) and a brightly illuminated light compartment of identical dimensions. Each rat was initially placed in the dark compartment and allowed to explore both zones for a 5-minute testing period. Two primary metrics were quantified: total transitions and cumulative residence time in the light region. For the open-field test, a square arena measuring 100 × 100 × 40 cm was utilized, allowing rats to explore for 5 minutes, while the total time spent in the central zone was recorded for statistical comparison. Behavioral assessments were conducted by staff blinded to the experimental group allocation.

Prior to behavioral testing, all animals underwent motor function evaluation, including the rotarod test and locomotion velocity analysis, to exclude potential confounding effects of motor deficits or incomplete postictal recovery on behavioral outcomes. Only animals passing this motor screening were included in subsequent assessments.

2.4 Assessment of cognitive function

Spatial and recognition cognitive functions were assessed through the Y-maze test and the novel object recognition (NOR) test, as described in previous research^[26]. The Y-maze apparatus consisted of three identical arms (50 cm × 10 cm × 15 cm) arranged at 120° angles. Each rat was allowed unrestricted exploration for 5 minutes, and the spontaneous alternation percentage was calculated to reflect spatial working

memory capacity. In the NOR test, a cubic arena measuring $40 \times 40 \times 40$ cm was used. Animals underwent a 30-minute habituation period in the empty chamber to reduce environmental novelty stress. During the following 5-minute training trial, two identical objects were symmetrically placed within the arena. After the training interval, a testing phase of 5 minutes was conducted, during which one familiar object was retained and a novel object introduced. A discrimination index, derived from exploration durations of the objects, quantified recognition memory performance. For the Y-maze test, only rats completing ≥ 8 total arm entries during the 5-minute session were included in the spontaneous alternation analysis^[27, 28]. In the NOR test, animals were required to accumulate ≥ 20 seconds of total exploration time for both objects in the training and test sessions; those failing to meet this criterion were excluded^[29]. Behavioral assessments were conducted by staff blinded to the experimental group allocation.

2.5 Network pharmacology analyses

2.5.1 Acquisition of UA targets and epilepsy-associated targets

The structural information of UA, including its canonical SMILES notation and SDF file, was retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). To comprehensively identify the potential molecular targets of UA, four independent target-prediction platforms were utilized: PharmMapper, SwissTargetPrediction, SuperPred, and TargetNet. Epilepsy-associated targets were subsequently obtained from GeneCards, OpenTargets, OMIM, and the Therapeutic Target Database (TTD) using "epilepsy" as the search term. All compound-predicted and disease-associated targets were standardized to their official gene symbols via the UniProt database (<https://www.uniprot.org/>), with species restricted to *Homo sapiens*. Duplicate entries within each dataset were removed. The standardized drug and disease targets were intersected using the jvenn online platform (<https://jvenn.toulouse.inra.fr/app/index.html>), with the overlapping targets visualized in a Venn diagram. These intersecting targets were considered candidate targets that may mediate the therapeutic effects of UA against epilepsy and were selected for further analyses. Based on these candidate targets, a "drug–target–disease" network was constructed and visualized using Cytoscape software (version 3.10.4).

2.5.2 Protein–Protein Interaction (PPI) Network Analysis

To prioritize core hub genes, candidate targets were imported into the STRING database for PPI data, which were then analyzed topologically and visualized in Cytoscape^[30]. Hub genes were identified based on node degree and other topological parameters. The top 10 genes were extracted separately, and their intersection was determined using a Venn diagram to identify potential core targets of UA for epilepsy treatment.

2.5.3 GO and KEGG Enrichment Analyses

Gene Ontology (GO) functional annotation, encompassing biological process (BP), cellular component (CC), and molecular function (MF), along with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, were conducted using the DAVID Bioinformatics Resources

(<https://davidbioinformatics.nih.gov/>). Terms and pathways with a P -value < 0.05 were considered statistically significant. The enrichment results were visualized utilizing the Weishengxin platform (<https://www.bioinformatics.com.cn/>).

2.6 Blood collection and measurements

Blood samples were collected from anesthetized rats via the retro-orbital venous sinus^[31]. Serum biochemical markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr), were quantified using a FUJI DRI-CHEM NX500iVC biochemical analyzer. Hematological profiles, specifically red blood cells (RBC), white blood cells (WBC), and platelets (PLT), were assessed on a Mindray BC-2800 Vet veterinary automatic hematology analyzer.

2.7 Western blotting (WB)

WB was performed following previously established protocols^[32]. Proteins were extracted from the hippocampus and cells using RIPA buffer (P0013B, Beyotime) containing protease cocktail inhibitors (Selleck). Equal amounts of protein from different groups were loaded onto Tris-SDS gels and transferred onto PVDF membranes (Bio-Rad, USA). The membranes were blocked in 5% skim milk at room temperature (RT) for 1 hour. After blocking, membranes were incubated overnight at 4 °C with primary antibodies: IGF-1R (1:1000, Abclonal, A0243), p-IGF-1R (1:1000, Proteintech, 31133-1-AP), ERK (1:2000, Proteintech, 11257-1-AP), p-ERK (1:1000, Proteintech, 28733-1-AP), and GAPDH (1:5000, Proteintech, 10494-1-AP). Following incubation, PVDF membranes were exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, Proteintech) at RT for 1 hour. Protein bands were captured using ECL solution on the ChemiScope S6 (CLINX, China) and analyzed semi-quantitatively with ImageJ software.

2.8 Immunofluorescence staining

Immunofluorescence staining was conducted as previously described^[33]. The hippocampus was fixed in 4% paraformaldehyde (PFA). Frozen sections were prepared based on the rat brain atlas. Tissue sections underwent antigen retrieval in sodium citrate buffer (pH 6.0) and were then permeabilized in 0.2% Triton X-100 for 10 minutes at RT, followed by a 1-hour blocking step with 5% bovine serum albumin (BSA). Sections were incubated overnight at 4 °C with primary antibodies: NeuN (1:100, Servibio, GB11138), GFAP (1:300, GeneTex, GTX40988), and Iba-1 (1:100, Servibio, GB15105). The following day, slides were brought to RT for 30 minutes before thorough rinsing. Samples were then incubated for 1 hour with fluorophore-labeled secondary antibodies: CoraLite®488-conjugated donkey anti-rabbit IgG (1:100, Proteintech, SA00013-6) and CoraLite®488-conjugated donkey anti-mouse IgG (1:100, Proteintech, SA00013-5). TUNEL staining was performed according to the kit instructions (Servibio, G1504). After the final wash, all sections were counterstained with DAPI nuclear dye. Fluorescent signals were captured using an Olympus BX53 fluorescence microscope.

2.9 H&E staining

The H&E staining procedures adhered to previously published protocols^[34]. Frozen sections were equilibrated at RT for 10 minutes and fixed with PFA for 15 minutes. Sections were stained with hematoxylin reagent (Servicebio, G1005-1) for 3–5 minutes, followed by rinsing with running tap water. Brief differentiation (2–5 seconds) was performed using the corresponding differentiation solution (Servicebio, G1039), followed by additional water washing. Sections were neutralized with bluing reagent (Servicebio, G1040) and thoroughly rinsed, then dehydrated sequentially in 85% and 95% ethanol for 5 minutes each, prior to staining with eosin solution (Servicebio, G1005-2) for 5 minutes. Anhydrous ethanol dehydration and xylene clearing were then conducted, and slides were mounted with neutral balsam for coverslip sealing.

2.10 Drug Affinity-Responsive Target Stability (DARTS)

Recombinant rat IGF-1R protein (MedChemExpress, HY-P78724) was incubated with UA (0, 10, 20, or 40 μ M) in reaction buffer for 1 hour at 37 °C. Following incubation, protein-drug complexes underwent proteolysis by adding Pronase solution (0.1 mg/mL; MedChemExpress, HY-114158) and incubating for 15 minutes at 37 °C. Control samples without Pronase treatment were processed in parallel. The proteolysis reaction was terminated by adding 5 $\times$ SDS loading buffer and boiling at 100 °C for 10 minutes. Protein degradation levels were analyzed using WB analysis^[35], and the input level of IGF-1R in the absence of Pronase digestion was detected in parallel as a loading control.

2.11 Statistical analysis

All statistical computations were performed with GraphPad Prism software, and quantitative results were presented as mean $\pm$ standard error of the mean (SEM). The Shapiro–Wilk test was employed to assess the normal distribution of raw data. Unpaired two-tailed Student's t-test was used for intergroup comparisons between two experimental groups. For datasets containing three or more groups, one-way analysis of variance (ANOVA) with Tukey's post-hoc multiple comparison test was applied. Non-normally distributed data were analyzed using the Kruskal–Wallis H test, followed by Dunn's test for subsequent pairwise comparisons. All statistical analyses were conducted with a two-tailed design, and a significance threshold of $P < 0.05$ was set for this study.

3. Results

3.1 UA pretreatment attenuates seizure severity and improves behavioral comorbidities after SE

To evaluate the therapeutic effect of UA pretreatment (Fig. 1A) for SE, an acute SE model in SD rats was established using lithium-pilocarpine, with pre-treatments of UA administered at doses of 0, 2.5, 5, and 10 mg/kg. Compared to SE rats in the vehicle group, treatment with 5 or 10 mg/kg UA significantly reduced the Racine seizure stage (Fig. 1B), prolonged latency to stage 3 seizures (Fig. 1C), shortened total seizure duration (Fig. 1D), and lowered the incidence of stage 3 seizures (Fig. 1E), with no significant differences observed between the 5 and 10 mg/kg UA groups. The 2.5 mg/kg dose produced measurable but weaker effects, showing no significant difference compared to SE rats in the vehicle group.

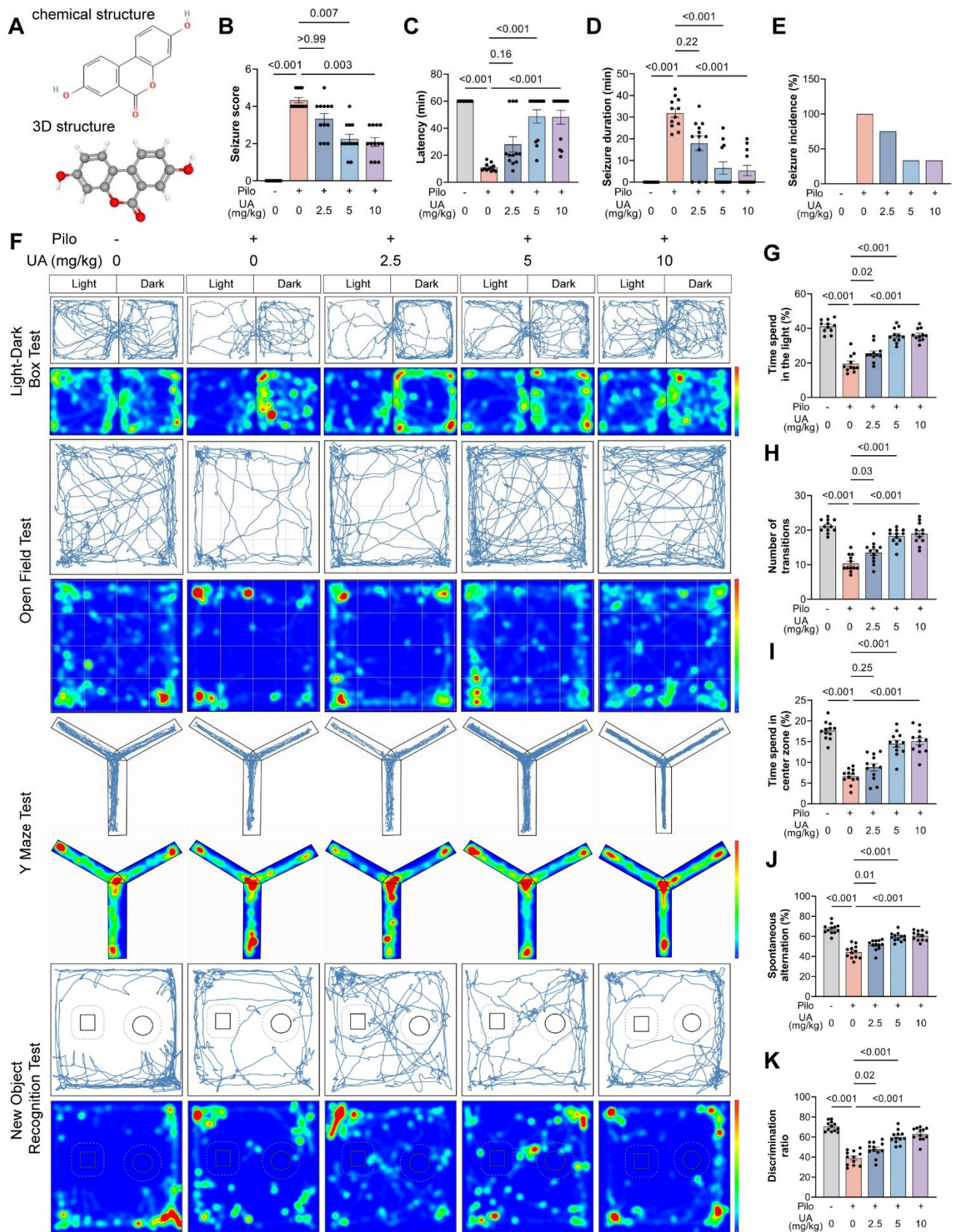


Fig. 1. UA improves seizure severity and cognitive-affective behaviors in an SE rat model.

(A) Chemical structure of UA. (B) Seizure stage scores in different groups. (C) Latency to the first stage 3 clonic seizure (min). (D) Cumulative duration of stage ≥ 3 seizures (min). (E) Incidence of stage ≥ 3 seizures. (F) Representative trajectories and heat maps of the light–dark box, open field, Y-maze, and NOR tests. (G) Quantitative analysis of the time spent in the light compartment (%). (H) Quantitative analysis of the number of transitions between the light and dark compartments. (I) Quantitative analysis of the percentage of residence time in the central region (%). (J) Quantitative analysis of spontaneous alternation (%). (K) Quantitative analysis of the percentage of exploration frequency for the novel object in the NOR test. All behavioral indicators (B–K) were assessed with $n = 12$ rats in each group.

Accumulating evidence confirms that SE induces behavioral and cognitive impairments^[36, 37]. Two behavioral paradigms, the light-dark box and open-field assays, were utilized to quantify anxiety-associated phenotypes. Relative to control rats, SE animals exhibited markedly reduced residence time in the light compartment, fewer light-dark crossings, and diminished occupancy in the central arena, indicating heightened anxiety traits (Fig. 1G–I). Administration of 5 or 10 mg/kg UA substantially ameliorated these anxiety-related behaviors (Fig. 1G–I). Cognitive functions were further assessed using the Y-maze and NOR tests. SE rats demonstrated decreased spontaneous alternation percentages and lower discrimination indices compared to control animals (Fig. 1J–K). Cognitive impairments were restored following intervention with 5 or 10 mg/kg UA. Consistent with observations regarding seizure severity, 2.5 mg/kg UA pretreatment yielded measurable benefits, albeit weaker than the higher doses (Fig. 1F–K). These findings suggest that UA has a positive effect on both seizure severity and behavioral comorbidities following SE, prompting the selection of 5 mg/kg as the dose for subsequent experiments.

Safety remains a key consideration for any candidate drug intended for neurological applications. Administration of 5 mg/kg UA did not result in significant differences in complete blood counts (RBC, WBC, and PLT) or body weight (Fig. S1A–B). H&E staining revealed no evident histopathological lesions in the lung, liver, spleen, kidney, or heart after UA treatment (Fig. S1C). Additionally, serum biochemical indicators for liver and kidney function, including ALT, AST, Cr, and BUN, remained stable across groups (Fig. S1D), indicating that UA exhibits no evident systemic toxicity at the tested dosage.

3.2 UA pretreatment protects hippocampal neuronal damage and restrains glial cell activation

SE triggers various pathological events within the hippocampus, primarily characterized by neuronal injury and excessive glial activation^[38]. NeuN immunofluorescence staining demonstrated severe neuronal loss across the CA1, CA3, and dentate gyrus (DG) subregions in SE rats (Fig. 2A–B). UA treatment preserved neuronal numbers in all three regions (Fig. 2A–B). TUNEL staining further confirmed alterations in neuronal apoptosis, indicating that the proportion of TUNEL-positive apoptotic cells increased post-SE, while UA pretreatment significantly alleviated this apoptotic phenotype (Fig. 2C–D).

Reactive gliosis serves as a biomarker for the epileptic hippocampus and contributes to network hyperexcitability^[39]. GFAP immunostaining revealed increased astrocytic activation after SE, which was attenuated by UA treatment across CA1, CA3, and DG (Fig. 2E–F). Iba-1 immunostaining reflected a similar pattern of microglial activation (Fig. 2G–H). Thus, UA pretreatment not only mitigates SE-induced neuronal loss but also suppresses the persistent activation of glial cells.

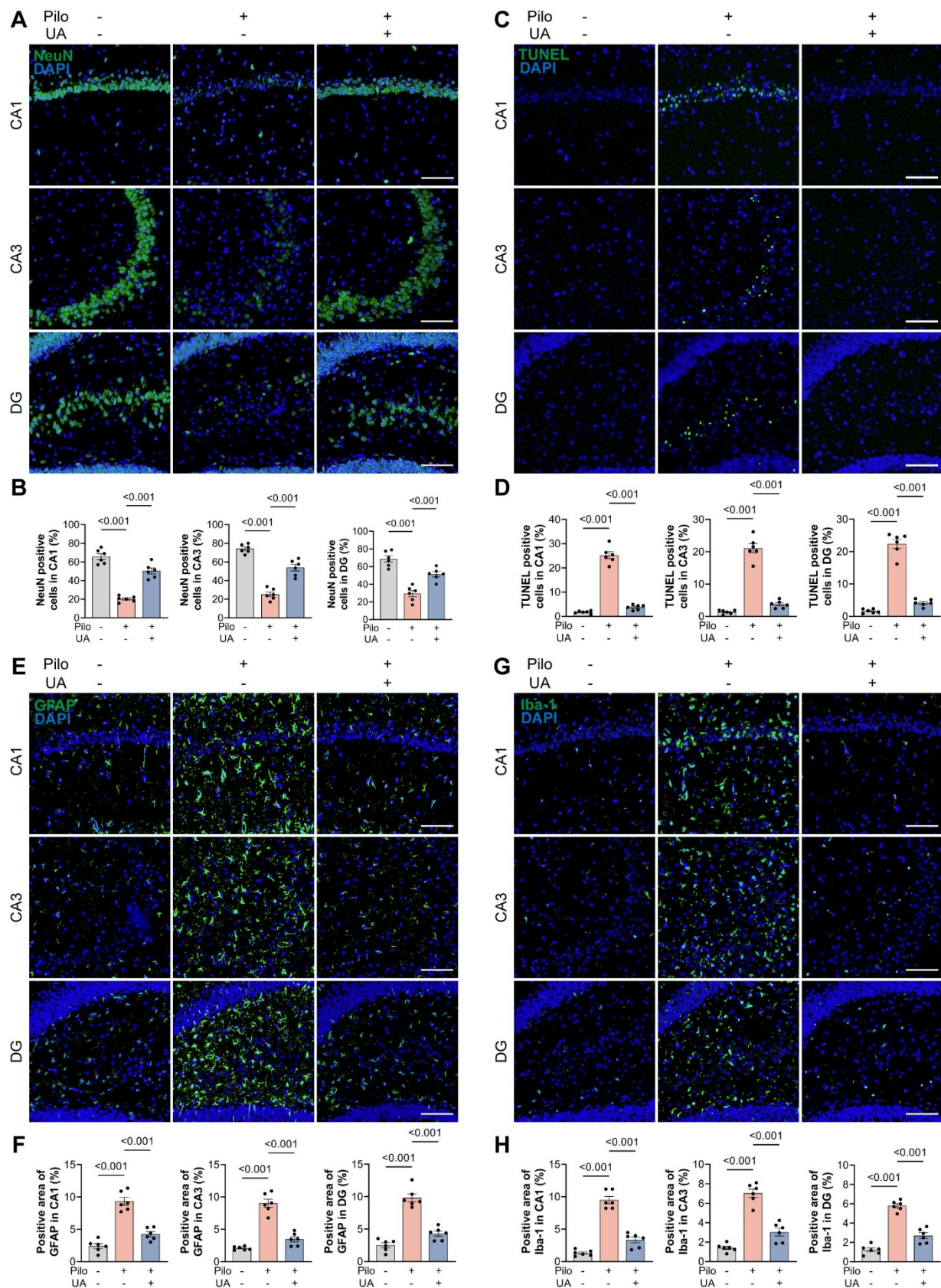


Fig. 2. UA reduces neuronal loss and activation of glial cells in an SE rat model.

(A) Representative NeuN immunofluorescence images in the hippocampus of rats treated with vehicle, pilocarpine, or UA.

(B) Statistical quantification of the proportion of NeuN-positive cells ($n = 6$ per group). (C) Representative TUNEL immunofluorescence images in the hippocampus of rats treated with vehicle, pilocarpine, or UA. (D) Statistical quantification of the proportion of TUNEL-positive cells ($n = 6$ per group). (E) Representative GFAP immunofluorescence images in the hippocampus of rats treated with vehicle, pilocarpine, or UA. (F) Statistical quantification of the proportion of GFAP-positive area ($n = 6$ per group). (G) Representative Iba-1 immunofluorescence images in the hippocampus of rats treated with vehicle, pilocarpine, or UA. (H) Statistical quantification of the proportion of Iba-1-positive area ($n = 6$ per group). Scale bar: 100 μm .

3.3 Network pharmacological analysis of potential targets of UA against epilepsy

A total of 197 UA-related targets were identified from the SwissTargetPrediction, PharmMapper, SuperPred, and TargetNet databases after duplicate entries were removed. Concurrently, epilepsy-associated targets were retrieved from GeneCards, OMIM, OpenTargets, and the TTD, resulting in 2,403 targets after data consolidation. The intersection of these two datasets, illustrated in a Venn diagram, revealed 37 overlapping targets shared between UA and epilepsy (Fig. 3A). These common targets were subsequently imported into Cytoscape to construct a UA–epilepsy target network comprising 39 nodes and 74 edges (Fig. 3B). The overlapping targets were also submitted to the STRING database to generate a PPI network, which was visualized in Cytoscape (Fig. 3C). In this network, nodes represented target proteins and edges denoted their interactions; a higher degree of connectivity indicated a more central role of the corresponding gene in UA-mediated epilepsy treatment. To identify core targets, the CytoHubba plugin in Cytoscape was utilized to rank the targets according to seven algorithms (MCC, Degree, EPC, MNC, Radiality, BottleNeck, and Closeness). The top 10 nodes from each algorithm were extracted, and their intersection was determined using another Venn diagram. This process revealed four common core genes (IGF-1R, AKT1, MAOA, and APP) that may serve as key targets for UA in the treatment of epilepsy (Fig. 3F). GO enrichment analysis indicated that these targets were primarily involved in the regulation of protein autophosphorylation and the insulin receptor complex (Fig. 3D). KEGG pathway analysis highlighted significant pathways, including the FoxO, MAPK, and Rap1 signaling pathways (Fig. 3E). Integrated analysis of the GO and KEGG enrichment results showed that many pathways are closely associated with IGF-1R. Collectively, these findings suggest that UA may alleviate epilepsy by targeting IGF-1R.

3.4 UA directly binds to IGF-1R

Following the network pharmacology prediction, IGF-1R was identified as the core therapeutic target of UA. To test this hypothesis, target identification technology using the DARTS method was employed to investigate the engagement of UA with IGF-1R. The results demonstrated that UA significantly promoted the stabilization of IGF-1R during pronase-induced degradation in a concentration-dependent manner (Fig. 3G-H), suggesting that UA directly binds to IGF-1R.

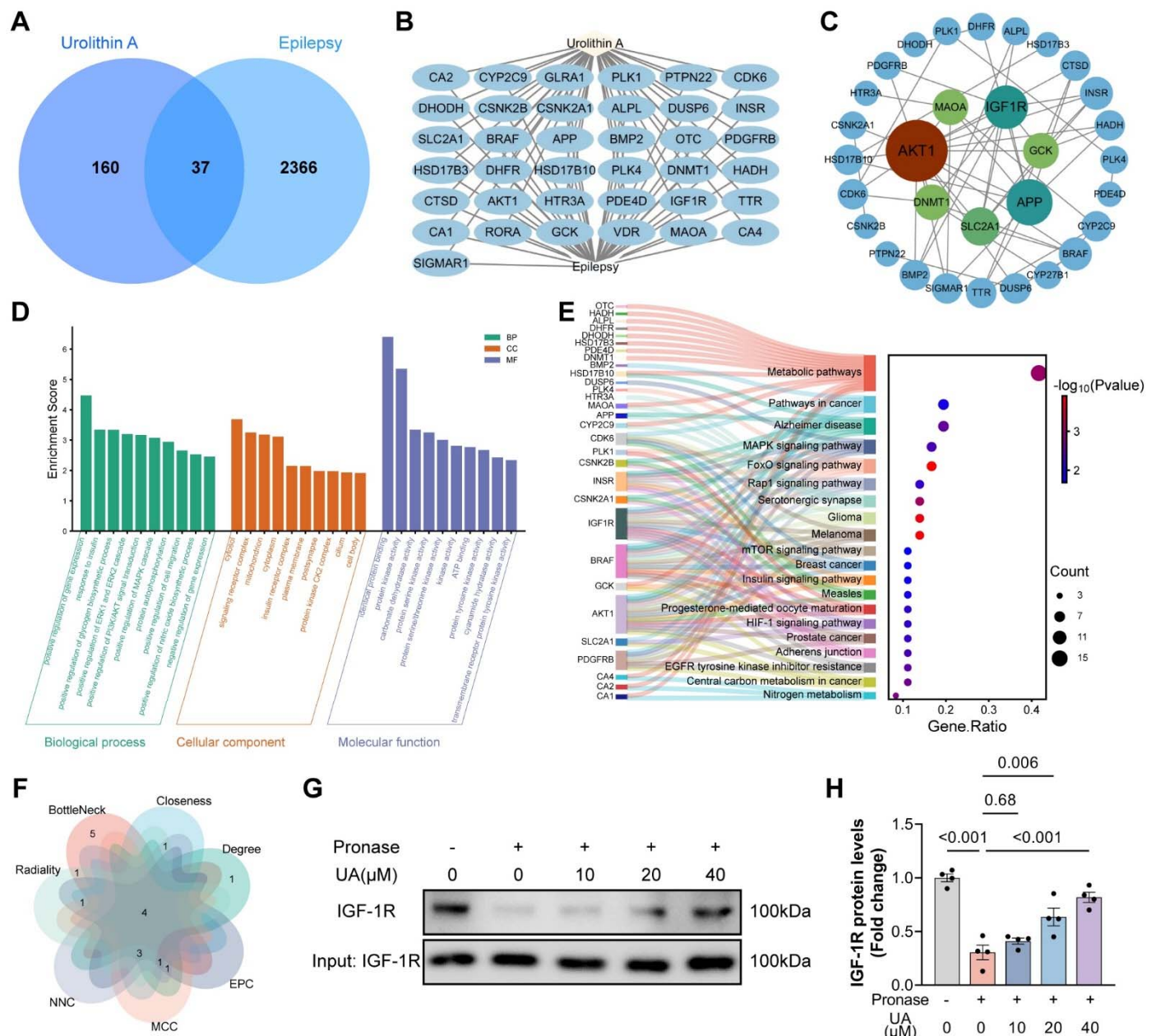


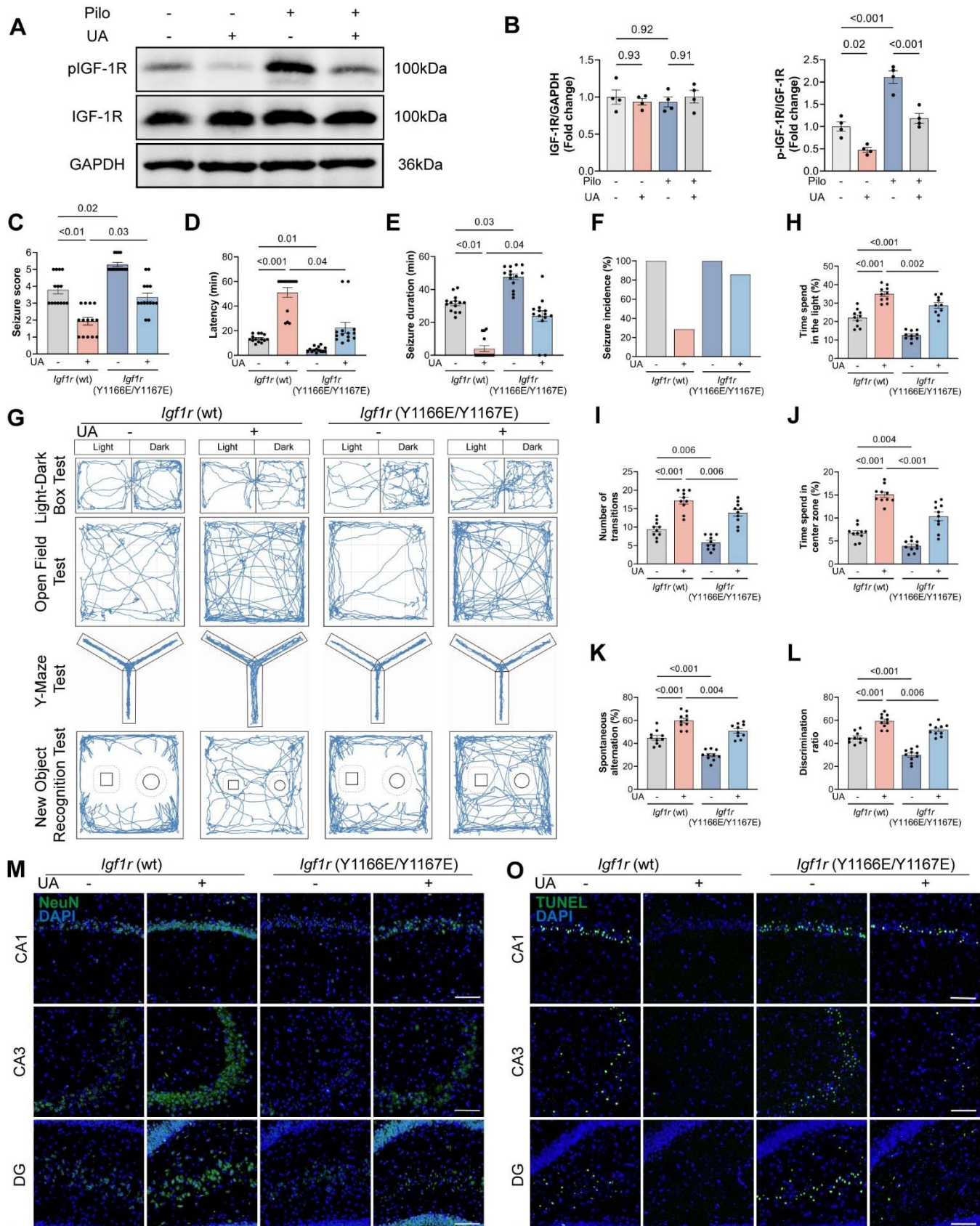
Fig. 3. Network pharmacology analysis of UA against epilepsy.

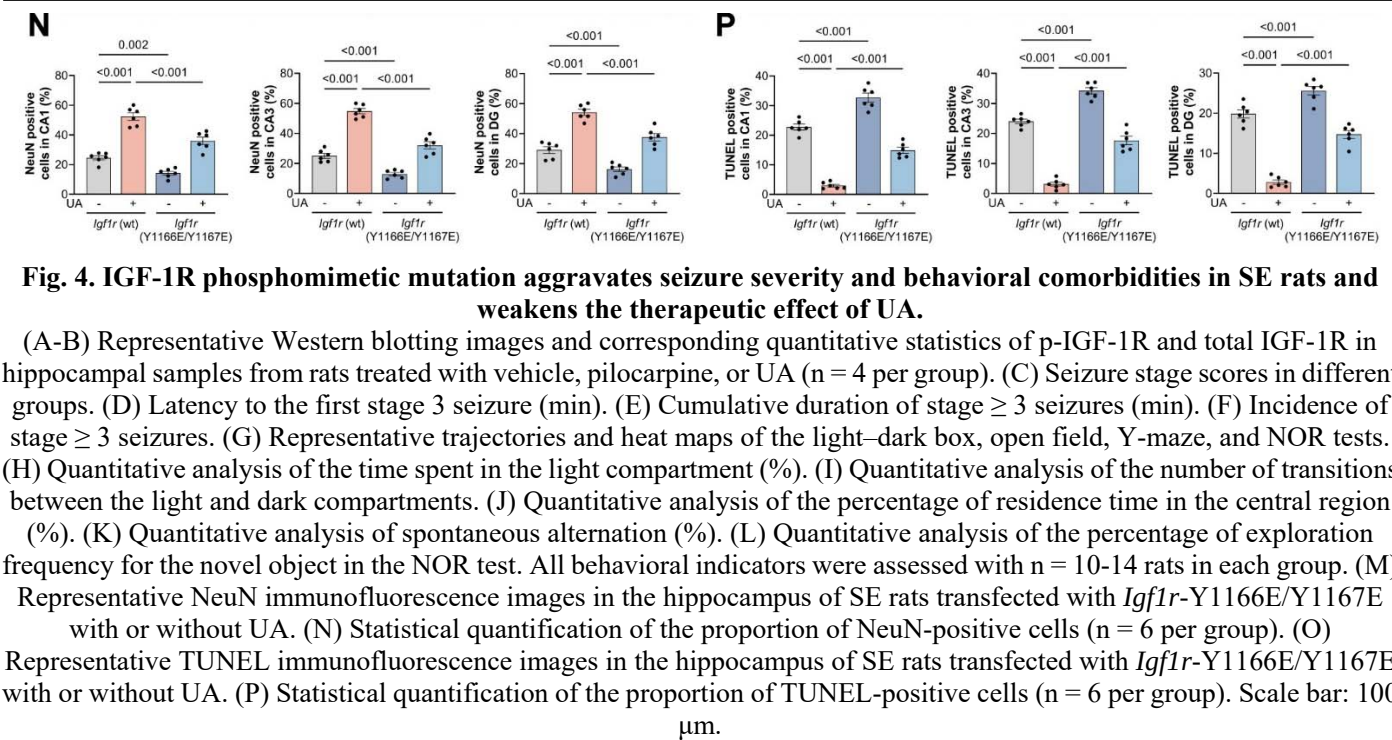
(A) Venn diagram of UA and epilepsy targets. (B) UA–epilepsy target network. (C) PPI network of common targets. (D) The top 10 GO terms ranked by P value. (E) The top 20 KEGG pathways ranked by P value. (F) Venn diagram of four core targets identified from the overlap. (G–H) Representative Western blotting images and corresponding quantitative statistics of DARTS analysis measuring the binding ability of UA to IGF-1R.

3.5 UA inhibits the phosphorylation of IGF-1R at Tyr1166/1167 (Y1166/1167)

Phosphorylation is a critical step for IGF-1R to exert its biological functions^[40]. Dysregulated phosphorylation of IGF-1R has been implicated in multiple neurological disorders^[41, 42]. By integrating predicted phosphorylation sites from PhosphositePlus (<https://www.phosphosite.org/>) with previous research^[9], it is hypothesized that UA may block the downstream effects of IGF-1R by modulating the phosphorylation of IGF-1R at Tyr1166/1167 (Y1166/1167). To verify whether UA affects the protein level of IGF-1R in hippocampal tissues, WB assays were performed. Notably, SE led to increased phosphorylation of IGF-1R at Y1166/1167 in the hippocampus of rats compared to the control group, while total IGF-1R protein levels remained unchanged (Fig. 4A–B). UA pretreatment did not significantly affect the total protein

abundance of IGF-1R; however, it markedly suppressed the phosphorylation level of IGF-1R protein in rats (Fig. 4A-B). Moreover, UA significantly inhibited the phosphorylation of IGF-1R at Y1166/1167 induced by pilocarpine (Fig. 4A-B).





3.6 IGF-1R phosphomimetic mutation at Tyr1166/1167 weakens the antiseizure effect of UA

To investigate the role of phosphorylation of IGF-1R at Y1166/1167 after UA treatment in epilepsy, adenovirus vectors (*Igf1r*-wt or *Igf1r*-Y1166E/Y1167E) were delivered via stereotaxic injection into the hippocampus of rats. Transfection with the *Igf1r*-Y1166E/Y1167E mutation vectors aggravated the seizure phenotype, evident by an increase in seizure stage, decreased latency, prolonged seizure duration, and elevated seizure frequency, thus weakening the antiseizure effect of UA (Fig. 4C–F, Fig. S2). Consistent results were observed in behavioral comorbidity assessments: the *Igf1r*-Y1166E/Y1167E mutation exacerbated anxiety-like behavior and cognitive impairments across various tests, including the light–dark box, open-field test, Y-maze, and NOR tests (Fig. 4G–L, Fig. S2).

Histologically, the *Igf1r*-Y1166E/Y1167E mutation was associated with increased apoptosis in hippocampal neurons (Fig. 4M–P) and amplified astrocyte and microglial activation (Fig. S3A–D) throughout the CA1, CA3, and DG subregions. Importantly, the protective effects of UA against these pathological changes were significantly inhibited following transfection with *Igf1r*-Y1166E/Y1167E (Fig. 4M–P, Fig. S3A–D).

Overall, these experiments demonstrate that the antiseizure effects of UA are dependent on its ability to suppress IGF-1R phosphorylation at Y1166/1167, with the antiseizure effect of UA diminished in the presence of the *Igf1r*-Y1166E/Y1167E mutation.

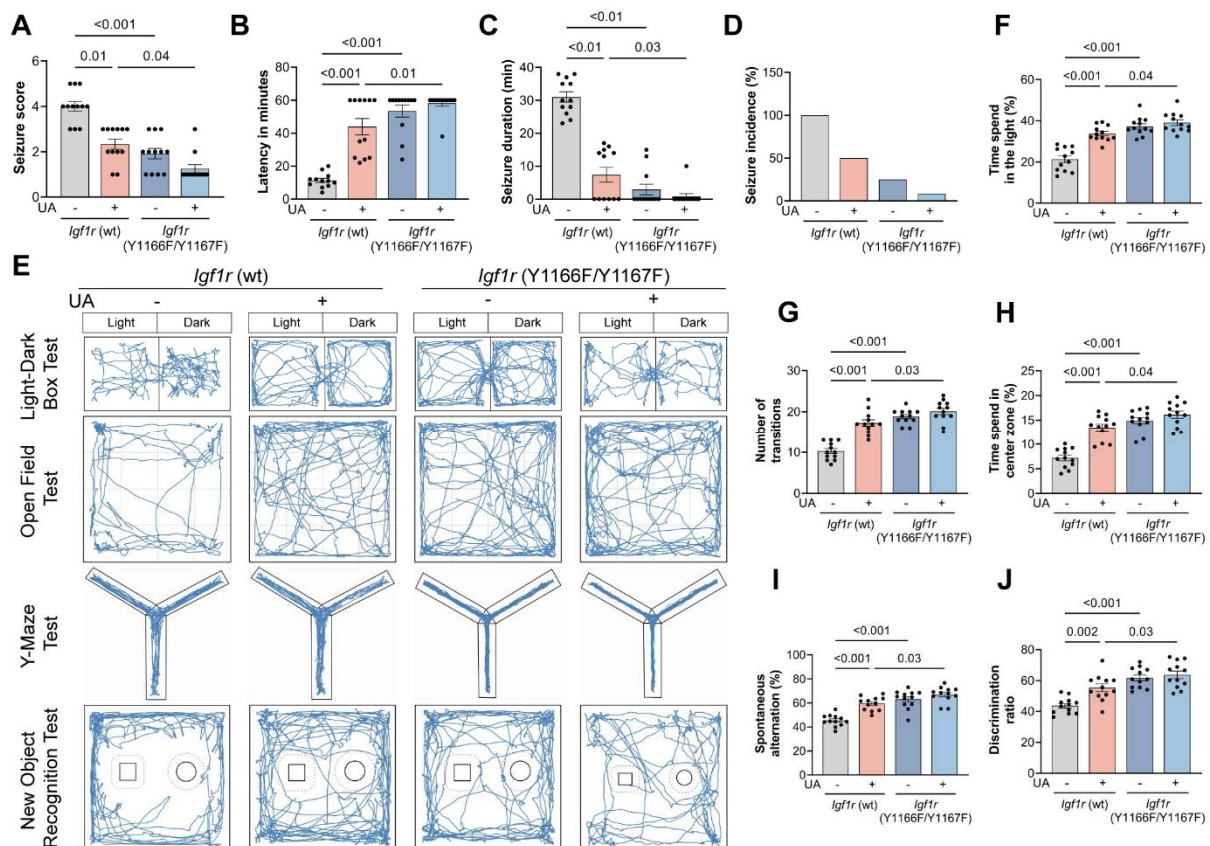
3.7 IGF-1R non-phosphorylatable mutation at Tyr1166/1167 simulates the antiseizure effect of UA

To further examine whether the non-phosphorylatable mutation of IGF-1R at Y1166/1167 could sufficiently account for the antiseizure effect of UA, adenovirus vectors (*Igf1r*-wt or *Igf1r*-Y1166F/Y1167F) were also delivered via stereotaxic injection into the hippocampus. The IGF-1R non-phosphorylatable mutation at Tyr1166/1167 mimicked the antiseizure effect of UA. Transfection with *Igf1r*-Y1166F/Y1167F

mutation vectors led to an improvement in seizure phenotypes, characterized by reduced seizure scores, prolonged latency to stage 3 seizures, shortened total seizure duration, and decreased incidence of stage 3 seizures (Fig. 5A-D). Behavioral assessments revealed similar improvements, with the *Igf1r*-Y1166F/Y1167F mutation alleviating anxiety-like behavior and cognitive impairments across the light-dark, open-field, Y-maze, and NOR tests (Fig. 5E-J, Fig. S4). Notably, the combination of the *Igf1r*-Y1166F/Y1167F mutation and UA resulted in even greater improvements in seizure severity and behavioral comorbidities after SE (Fig. 5A-J, Fig. S4).

Histologically, the *Igf1r*-Y1166F/Y1167F mutation led to reduced neuronal apoptosis in the hippocampus (Fig. 5K-N) and suppressed astrocytic and microglial activation (Fig. S5A-D) across the CA1, CA3, and DG subregions. Moreover, the combination treatment of the *Igf1r*-Y1166F/Y1167F mutation and UA produced enhanced protective effects on pathological changes compared to either treatment alone (Fig. 5K-N, Fig. S5A-D).

In conclusion, UA provides greater therapeutic benefits in the presence of the IGF-1R non-phosphorylatable mutation and loses efficacy when combined with the IGF-1R phosphomimetic mutation. These complementary experimental approaches suggest that UA exerts its antiepileptic effects through the inhibition of IGF-1R phosphorylation.



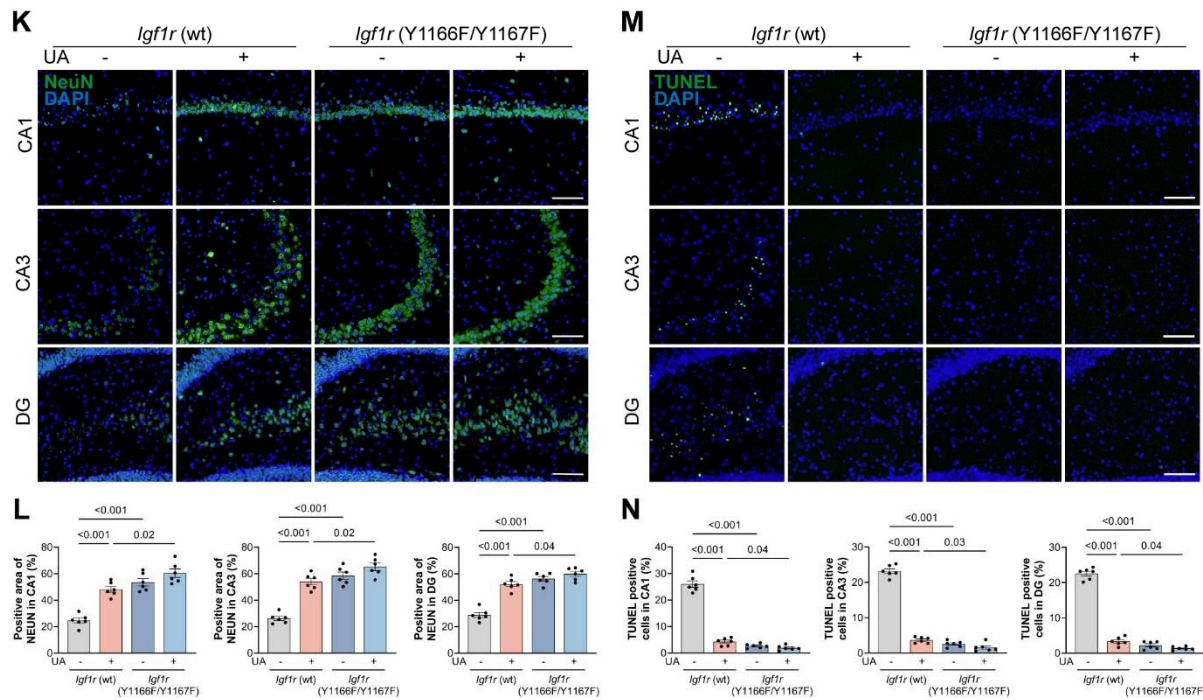


Fig. 5. IGF-1R non-phosphorylatable mutation improves pathophysiology in SE rats.

(A) Seizure stage scores in different groups. (B) Latency to the first stage 3 clonic seizure (min). (C) Cumulative duration of stage ≥ 3 seizures (min). (D) Incidence of stage ≥ 3 seizures. (E) Representative trajectories and heat maps of the light–dark box, open field, Y-maze, and NOR tests. (F) Quantitative analysis of the time spent in the light compartment (%). (G) Quantitative analysis of the number of transitions between the light and dark compartments. (H) Quantitative analysis of the percentage of residence time in the central region (%). (I) Quantitative analysis of spontaneous alternation (%). (J) Quantitative analysis of the percentage of exploration frequency for the novel object in the NOR test. All behavioral indicators were assessed with $n = 12$ rats in each group. (K) Representative NeuN immunofluorescence images in the hippocampus of SE rats transfected with *Igf1r*-Y1166F/Y1167F with or without UA. (L) Statistical quantification of the proportion of NeuN-positive cells ($n = 6$ per group). (M) Representative TUNEL immunofluorescence images in the hippocampus of SE rats transfected with *Igf1r*-Y1166F/Y1167F with or without UA. (N) Statistical quantification of the proportion of TUNEL-positive cells ($n = 6$ per group).

3.8 UA suppresses ERK hyperphosphorylation via inhibiting IGF-1R Tyr1166/1167 phosphorylation

Previous findings have established that UA alleviates epilepsy by inhibiting IGF-1R phosphorylation at the Y1166/1167 sites. However, the specific signaling pathway responsible for this reduction in IGF-1R phosphorylation and its subsequent effect on epilepsy remains to be elucidated. Prior research has identified ERK as a key downstream effector within the IGF-1R signaling cascade^[43]. Therefore, it is hypothesized that UA exerts its antiseizure effects by blocking IGF-1R-mediated phosphorylation of the downstream target ERK. Elevated ERK phosphorylation was observed in the hippocampus following pilocarpine-induced seizures, while UA treatment effectively reversed this seizure-triggered ERK hyperphosphorylation (Fig. 6A–B).

To establish a causal link between IGF-1R/ERK signaling and the SE phenotype, the ERK-specific inhibitor SCH772984 was administered to rats with either wild-type IGF-1R (*Igf1r*-wt) or the phosphomimetic mutant IGF-1R (*Igf1r*-Y1166E/Y1167E) delivered via adenovirus vectors. The IGF-1R phosphomimetic mutation increased ERK phosphorylation, while SCH772984 treatment effectively reversed its induced ERK hyperphosphorylation (Fig. S6A–B). ERK inhibition significantly reduced seizure severity, as indicated by decreased seizure scores, prolonged latency to stage 3 seizures, shortened total seizure

duration, and lowered incidence of stage 3 seizures, thus partially mitigating the exacerbation caused by *Igf1r*-Y116E/Y1167E overexpression (Fig. S6C-F). In behavioral assessments, including the light-dark box, open field, Y-maze, and NOR tests, SCH772984 also ameliorated anxiety-like behavior and cognitive impairments, alleviating the adverse effects of the phosphomimetic mutant (Fig. S6G-K).

To ascertain whether UA regulates ERK activity through an IGF-1R phosphorylation-dependent mechanism, adenoviral vectors containing IGF-1R mutants were utilized. The phosphomimetic *Igf1r*-Y1166E/Y1167E mutant abolished the inhibitory effect of UA on ERK phosphorylation (Fig. 6C-D). Conversely, the phosphorylation-deficient *Igf1r*-Y1166F/Y1167F mutant reduced excessive ERK phosphorylation induced by seizures (Fig. 6E-F). Notably, expression of *Igf1r*-Y1166F/Y1167F further augmented UA-mediated suppression of ERK phosphorylation (Fig. 6E-F). These findings demonstrate that UA inhibits seizure-associated ERK hyperactivation by repressing IGF-1R phosphorylation at Tyr1166/1167.

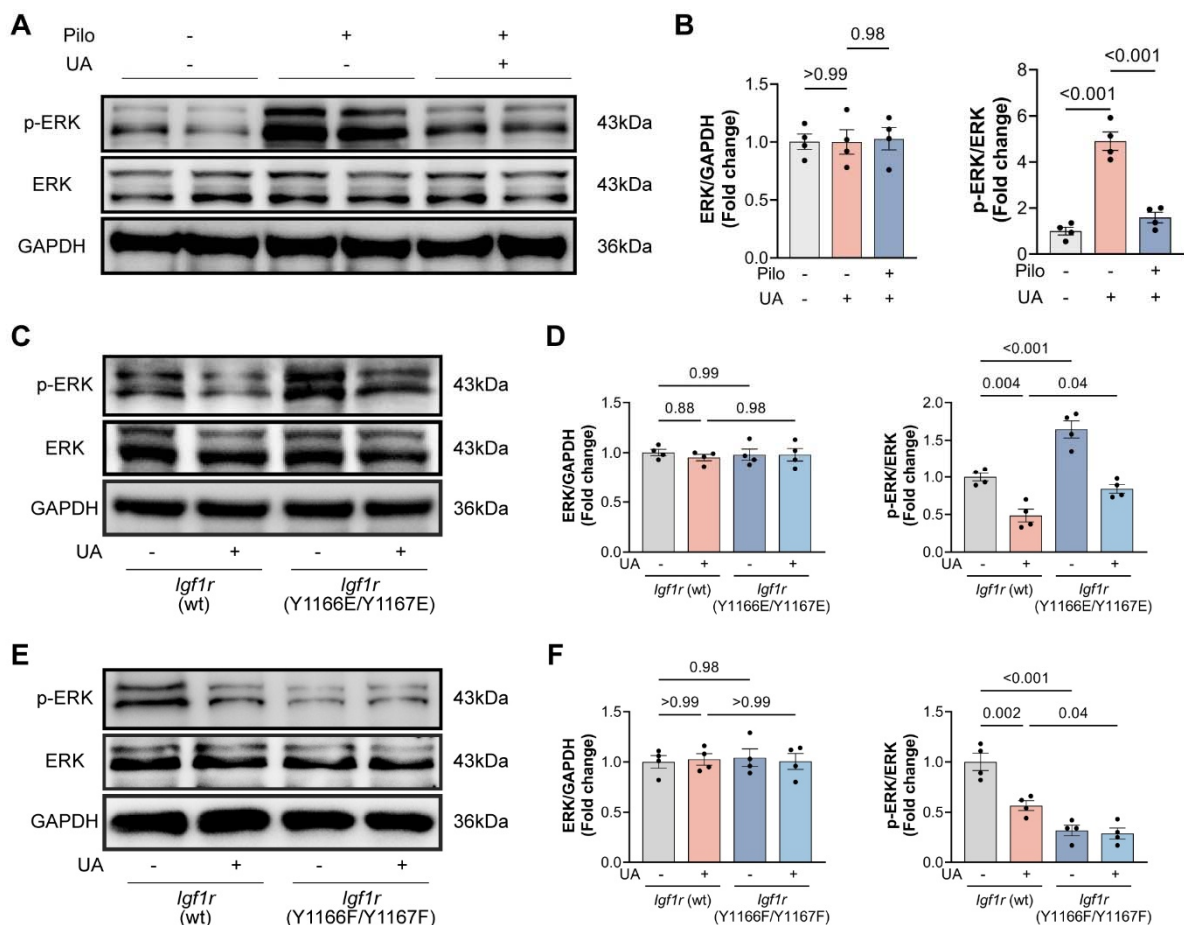


Fig. 6. UA suppresses ERK hyperphosphorylation by inhibiting IGF-1R Tyr1166/1167 phosphorylation.

(A-B) Representative immunoblot bands and statistical quantification of p-ERK and ERK proteins in the hippocampus of rats treated with vehicle, pilocarpine, or UA (n = 4 per group). (C-D) Western blotting profiles and quantitative data of p-ERK and ERK in the hippocampus of SE rats transfected with *Igf1r*-Y1166E/Y1167E with or without UA (n = 4 per group). (E-F) Representative Western blotting images and corresponding quantitative statistics of p-ERK and ERK in the hippocampus of SE rats transfected with *Igf1r*-Y1166F/Y1167F with or without UA (n = 4 per group).

3.9 Pretreatment with the combination of UA and VPA produces enhanced antiseizure effects in SE rats

The further development of UA as a potential candidate drug for epilepsy patients necessitates a deeper understanding of its use in conjunction with approved ASMs. The antiseizure effects of UA in combination

with VPA were investigated in an SE model of rats^[44]. UA pretreatment significantly enhanced the antiseizure efficacy of VPA, as evidenced by decreased seizure scores, prolonged latency to stage 3 seizures, shortened total seizure duration, and reduced incidence of stage 3 seizures (Fig. 7A-D). Similar improvements were observed in behavioral comorbidities: VPA reduced anxiety-like behavior and cognitive impairment in light-dark, open-field, Y-maze, and NOR tests (Fig. 7E-I), with enhancements noted in rats treated with UA (Fig. 7E-I).

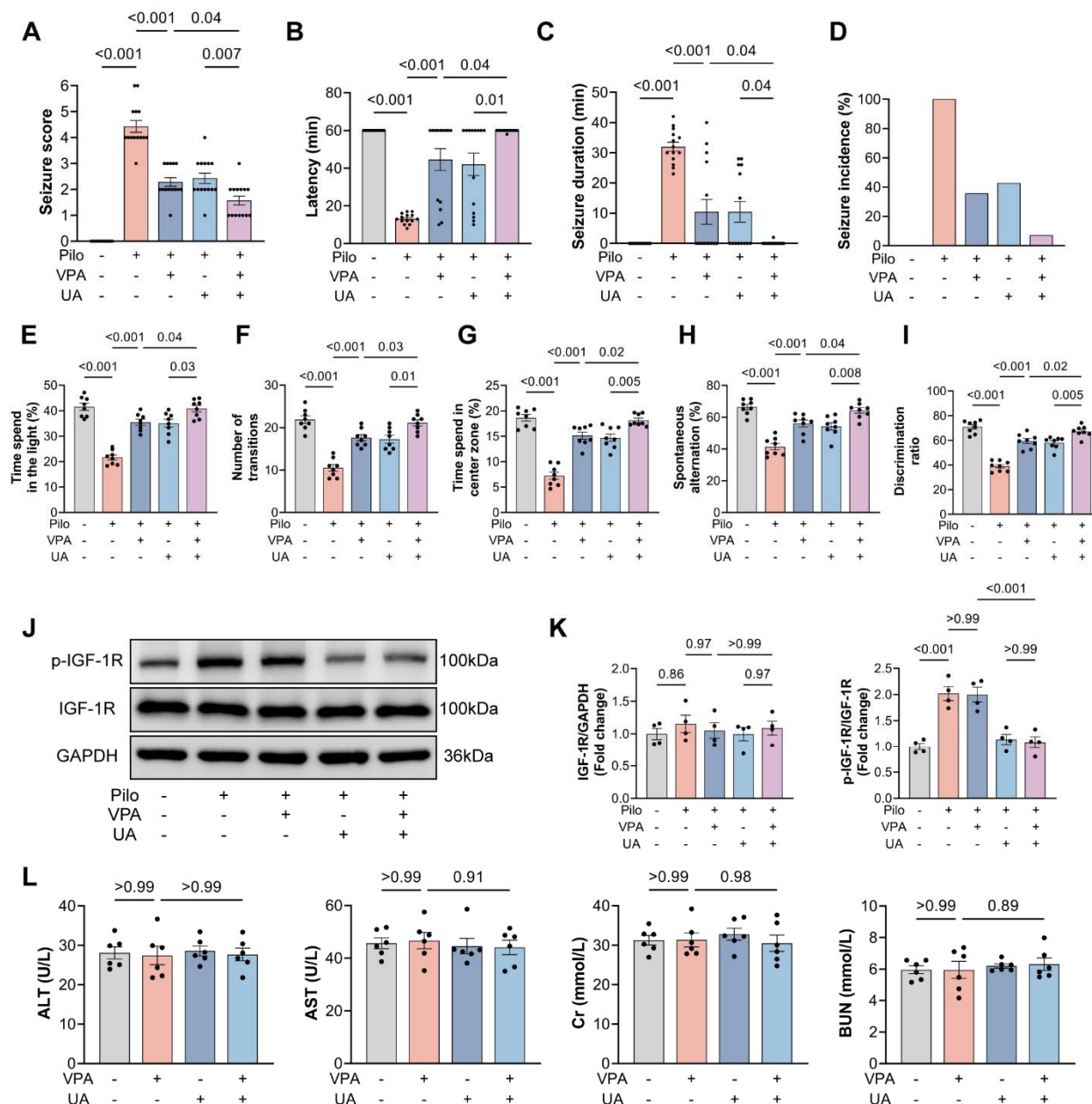


Fig. 7. UA enhances the therapeutic effect of VPA in an SE rat model.

To evaluate the prophylactic effects, rats were pretreated with VPA (300 mg/kg, i.p.) and/or UA (5 mg/kg, i.p.) 30 min before intraperitoneal injection of pilocarpine (Pilo, 35 mg/kg, i.p.) to induce SE. The control group received an equal volume of vehicle. (A) Seizure stage scores in different groups. (B) Latency to the first stage 3 clonic seizure (min). (C) Cumulative duration of stage ≥ 3 seizures (min). (D) Incidence of stage ≥ 3 seizures. (E) Quantitative analysis of the time spent in the light compartment (%). (F) Quantitative analysis of the number of transitions between the light and dark compartments. (G) Quantitative analysis of the percentage of residence time in the central region (%). (H) Quantitative analysis of spontaneous alternation (%). (I) Quantitative analysis of the percentage of exploration frequency for the novel object in the NOR test. All behavioral indicators were assessed with $n = 8-14$ rats in each group. (J-K) Representative immunoblot bands and statistical quantification of p-IGF-1R and IGF-1R in the hippocampus of SE rats treated with VPA or UA ($n = 4$ per group). (L) Serum levels of ALT, AST, Cr, and BUN in rats treated with VPA or UA ($n = 6$ per group).

To determine whether the enhanced antiseizure effect of the UA-VPA combination resulted from enhanced inhibition of IGF-1R phosphorylation, WB analysis was conducted to examine VPA's impact on IGF-1R phosphorylation levels. Notably, VPA did not alter IGF-1R phosphorylation compared to the vehicle group (Fig. 7J-K), which may explain the lack of efficacy in some patients. Consequently, the combination of VPA with UA, which actively reduces IGF-1R phosphorylation, presents a prospective treatment option for patients who experience limited efficacy with VPA alone, without incurring additional side effects (Fig. 7L).

4. Discussion

This study systematically evaluated the antiseizure effects of UA, a pomegranate-derived postbiotic, and investigated its potential to enhance the antiseizure efficacy of VPA, a first-line ASM. All drug administrations occurred prior to pilocarpine injection, establishing a pretreatment paradigm, with observed effects reflecting preventive or protective rather than therapeutic actions. Results indicate that UA pretreatment, as a gut microbiota metabolite of dietary ETs^[45, 46], effectively alleviates seizure symptoms and cognitive impairments by suppressing IGF-1R phosphorylation at Tyr1166/1167, while the UA-VPA combination further enhances antiseizure outcomes. These findings offer novel insights into improving epilepsy treatment through food-derived postbiotic adjuvant therapies.

Epilepsy is characterized by abnormal synchronized neuronal discharge, with SE representing its most severe manifestation^[47]. Using a pilocarpine-induced SE rat model, UA substantially reduced seizure stage, prolonged latency, shortened seizure duration, and mitigated anxiety-like behaviors and cognitive deficits. The employed UA dose (5 mg/kg) was within safety and efficacy ranges established in human studies, suggesting potential for translational use in seizure prevention.

The hippocampus plays a pivotal role in seizure regulation and cognitive function. Neurodegeneration and glial activation following SE are fundamental pathological features that drive epileptogenesis. This study demonstrated that UA effectively protects neurons in the CA1, CA3, and DG regions of the hippocampus, reduces TUNEL-positive cell ratios, and suppresses astrocytic (GFAP) and microglial (Iba-1) activation.

A critical prerequisite for the central antiepileptic effects of UA is its systemic bioavailability and ability to cross the BBB. UA exhibits good systemic bioavailability, with previous pharmacokinetic studies detecting UA in plasma following both oral and intraperitoneal administration^[16, 48, 49]. The dose administered in this study (5 mg/kg, i.p.) was selected based on these reports and has been proven safe and effective in other animal models^[50, 51]. Additionally, UA can cross the BBB; after intraperitoneal injection of UA (5 mg/kg) in rodents, free UA was identified in both plasma and brain tissue via mass spectrometry^[49]. *In vitro* BBB models further confirm that UA crosses the barrier and modulates cellular signaling^[16]. Although UA concentrations in plasma or brain tissue were not measured in this study, existing evidence supports that systemically administered UA reaches the hippocampus and regulates the IGF-1R/ERK signaling pathway.

The combination of UA and VPA resulted in significantly enhanced antiseizure effects compared to either treatment administered alone. The selection of VPA as the partner drug for combination therapy with UA was informed by several key considerations. First, VPA serves as a broad-spectrum, first-line ASM

effective against nearly all seizure types, including generalized tonic–clonic, absence, myoclonic, and focal seizures. This broad efficacy, stemming from multiple mechanisms of action, has solidified VPA's status as a standard first-line agent in current guidelines for both generalized and focal epilepsies^[52]. Its broad-spectrum properties provide a baseline antiseizure coverage regardless of seizure phenotype, rendering VPA a rational "backbone" drug for constructing combination regimens. Second, VPA possesses a favorable and well-characterized safety and interaction profile in polytherapy contexts. After over five decades of clinical use, its adverse effects are both predictable and manageable, especially when accompanied by therapeutic drug monitoring^[53, 54]. VPA has one of the most extensively documented interaction profiles among ASMs, such as inhibition of lamotrigine glucuronidation and displacement of phenytoin from plasma proteins^[54]. Furthermore, VPA is primarily metabolized by uridine diphosphate glucuronosyltransferases and mitochondrial β -oxidation, with minimal involvement of the cytochrome P450 system, making its disposition comparatively insensitive to enzyme-inducing ASMs^[55]. These characteristics substantiate its role as the most widely utilized partner drug in clinical polytherapy. Third, combination therapy is the standard of care for patients with drug-resistant epilepsy^[56], guided by the principle of combining ASMs with complementary, rather than overlapping, mechanisms of action^[57]. The anticonvulsant effects of VPA involve GABAergic enhancement, blockade of voltage-gated sodium channels, and histone deacetylase (HDAC) inhibition^[58]. This study demonstrates that UA exerts its antiseizure effects via the IGF-1R pathway, while VPA does not target this pathway. Such observations emphasize the engagement of independent, non-overlapping targets and provide mechanistic support for the UA-VPA combination. Additionally, numerous natural bioactive compounds have been reported to selectively enhance VPA's antiseizure efficacy in animal models^[59–61], offering experimental precedent for evaluating the UA-VPA combination. Therefore, the combination of VPA and UA presents a rational strategy, with the present study designed as an exploratory evaluation of this concept.

Contextualizing these findings within the realm of nutritional neuroscience and functional foods is essential. UA is not typically administered as a pure drug in diets; instead, it is produced by gut microbiota from ETs and EA found in pomegranates, berries, and walnuts^[16]. Consequently, its bioavailability is highly dependent on gut microbiome composition. Only individuals with a UA-producing metatype, characterized by specific bacterial consortia (e.g., *Gordonibacter urolithinifaciens* and *Enterocloster* spp.), can effectively convert dietary ETs into UA^[62]. Although ET-rich diets offer benefits, plasma UA concentrations vary greatly among individuals, leading to questions about whether dietary sources alone can achieve the exposure levels observed in this study's pretreatment regimen^[63]. Future research should address the reliance on endogenous microbial conversion, bridging the gap between a functional food strategy and precise pharmacological interventions. Currently, UA has received FDA approval and demonstrated good safety in human clinical trials, suggesting that direct UA supplementation may be a favorable option.

Several limitations in this study should be acknowledged. Firstly, experiments were conducted using an acute lithium-pilocarpine-induced SE model. While this model is useful for investigating acute seizure

mechanisms, it may not fully capture the pathophysiology of chronic epilepsy or other seizure types, such as spontaneous recurrent seizures. Secondly, UA administration in this study was prophylactic (pre-treatment), and therapeutic efficacy after seizure onset was not assessed, which is critical for clinical application. Furthermore, although VPA was selected for its first-line clinical status and broad activity spectrum, other ASMs (e.g., levetiracetam, lamotrigine) might exhibit different interaction profiles with UA. Systematic dose-ratio optimization and formal interaction analyses of UA in combination with multiple ASMs will be vital directions for future research. Additionally, the study lacks direct pharmacokinetic measurements, and UA concentrations in the brain have not been verified. Future work should establish exposure–response relationships and definitively correlate brain UA levels with the observed modulation of hippocampal IGF-1R/ERK and antiseizure effects. Finally, the oral bioavailability of UA and its effective dosage in humans are yet to be determined, with significant metabolic and gut microbiota differences between rodents and humans indicating a translational gap that must be bridged for clinical applicability.

5. Conclusion

This study demonstrates the antiseizure effects of pomegranate-derived UA and its mechanism involving IGF-1R phosphorylation inhibition. Notably, pretreatment with UA in combination with VPA produced enhanced antiseizure effects compared to either agent alone. These findings contribute to a deeper understanding of food-derived postbiotics in managing neurological diseases and provide essential evidence for combination therapy strategies to improve epilepsy treatment. Future studies should aim to validate the efficacy and safety of this UA-VPA combination in clinical settings.

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Conflicts of interest

The authors declare no conflict of interest.

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