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ARTICLE

Inflachromene attenuates seizure severity in mouse epilepsy models via inhibiting HMGB1 translocation

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Epilepsy is not well controlled by current anti-seizure drugs (ASDs). High mobility group box 1 (HMGB1) is a DNA-binding protein in the nucleus regulating transcriptional activity and maintaining chromatin structure and DNA repair. In epileptic brains, HMGB1 is released by activated glia and neurons, interacting with various receptors like Toll-like receptor 4 (TLR4) and downstream glutamatergic NMDA receptor, thus enhancing neural excitability. But there is a lack of small-molecule drugs targeting the HMGB1-related pathways. In this study we evaluated the therapeutic potential of inflachromene (ICM), an HMGB-targeting small-molecule inhibitor, in mouse epilepsy models. Pentylenetetrazol-, kainic acid- and kindling-induced epilepsy models were established in mice. The mice were pre-treated with ICM (3, 10 mg/kg, i.p.). We showed that ICM pretreatment significantly reduced the severity of epileptic seizures in all the three epilepsy models. ICM (10 mg/kg) exerted the most apparent anti-seizure effect in kainic acid-induced epileptic status (SE) model. By immunohistochemical analysis of brain sections from kainic acid-induced SE mice, we found that kainic acid greatly enhanced HMGB1 translocation in the hippocampus, which was attenuated by ICM pretreatment in subregion- and cell type-dependent manners. Notably, in CA1 region, the seizure focus, ICM pretreatment mainly inhibited HMGB1 translocation in microglia. Furthermore, the anti-seizure effect of ICM was related to HMGB1 targeting, as pre-injection of anti-HMGB1 monoclonal antibody (5 mg/kg, i.p.) blocked the seizure-suppressing effect of ICM in kainic acid-induced SE model. In addition, ICM pretreatment significantly alleviated pyramidal neuronal loss and granule cell dispersion in kainic acid-induced SE model. These results demonstrate that ICM is an HMGB-targeting small molecule with anti-seizure potential, which may help develop a potential drug for treating epilepsy.

Keywords: epilepsy; neuroinflammation; anti-seizure drugs; HMGB1 inhibitor; inflachromene; anti-HMGB1 monoclonal antibody

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INTRODUCTION

Epilepsy is a chronic brain disorder characterized by recurrent, excessive abnormal neuronal discharges in the brain [1]. Anti-seizure drugs (ASDs) are mainly used to control seizure symptoms in clinic, but they are still ineffective in about one-third of patients [2]. Apart from epileptic seizures, epilepsy patients are also accompanied by pathological changes and suffer from other complications, which are also resistant to ASDs. Temporal lobe epilepsy (TLE), one of the most common types of epilepsy, usually has an etiology of hippocampal sclerosis distinguished by hippocampal mossy fiber sprouting, loss of neurons in the CA1, CA3, and dentate gyrus (DG) regions of the hippocampus, diffuse granule cell layers as well as astrocyte cell proliferation [3]. In addition, up to 75% of patients with TLE may develop refractory epilepsy [4]. Furthermore, current ASDs have several drawbacks, including side effects and drug-drug interactions. Therefore, further investigation of the pathological mechanisms of epilepsy and the development of new drug targets are crucial to improve the quality of epilepsy patients.

Epilepsy is mediated by neuroinflammation, and high mobility group box 1 protein (HMGB1) signaling is one of the critical factors in neuroinflammation. HMGB1 is highly conserved evolutionarily and plays a vital role in regulating transcriptional activity and maintaining chromatin structure and DNA repair. Under normal circumstances, HMGB1 acts as a DNA-binding protein in the nucleus to perform normal physiological functions, and the level of extracellular HMGB1 is very low [5]. Under pathological conditions including epilepsy, HMGB1 is translocated from the nucleus to the cytoplasm, and then released to the outside of the cell. Extracellular HMGB1 acts as a damage-associated molecular patterns (DAMPs) mediating various physiological and pathological responses, such as inflammation, immunity, cell migration, proliferation and differentiation, and tissue regeneration. In epileptic brains, HMGB1 can be released by activated glia cells and neurons, interacting with various receptors like Toll-like receptor 4 (TLR4) and downstream glutamatergic receptor NMDA, which mediate enhanced neural excitability [6–10]. We previously demonstrated that anti-HMGB1

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monoclonal antibody (mAb) is beneficial for epileptic seizure and shows a disease-modifying anti-epileptogenetic effect on epileptogenesis [11]. However, there remains a lack of small-molecule regulators targeting the HMGB1-related pathways. To exert desired pharmacological effects, small-molecule inhibitors have various advantages, including good druggability and pharmacokinetic properties, potential permeability toward biological membranes, and interaction with intracellular and extracellular molecular targets. As the inflachromene (ICM) was discovered as an HMGB-targeting small-molecule inhibitor with anti-inflammatory and neuroprotective effects [12], the present study investigates the anti-seizure potential of ICM in different epilepsy models. The highlight of this study is the discovery of a small molecule inhibitor ICM with potential anti-seizure and neuroprotective effects, which is dependent on HMGB1 signaling. This study confirmed that ICM holds promise as a potential therapeutic agent against epileptic seizures.

MATERIALS AND METHODS

Animals

The animals used in this study were all C57BL/6 wild-type male mice, weighing 25–30 g, SPF grade, provided by the Zhejiang University Laboratory Animal Center (Hangzhou, China). For the housing environment, the animals were fed with water freely, with a daily 12 h light-dark cycle (8:00–20:00 light), and daily behavioral experiments were controlled between 9:00 and 18:00. Experiments were approved by the ethical committee of Zhejiang University (Hangzhou, China) and were conducted with regulations on the Care and Use of Laboratory Animals (National Institutes of Health, MD, USA).

Reagents

ICM was gifted by Prof Seung Bum Park group of Chemistry Department at Seoul National University. The structure of ICM is shown in Fig. 1a. ICM (3 mg/kg and 10 mg/kg) was dissolved in 58% ddH_2O, 40% PEG-400, and 2% DMSO. The dosage design of ICM was referred to in the study before [12]. The aqueous solubility of ICM was ~23 μg/mL, and the hepatic microsomal stability was ~0.41% and ~0.82% in mice and humans, respectively. After intraperitoneal injection in mice, the T_{max} was ~0.14 h, the C_{max} was ~380 μg/mL, the AUC was ~327 $\mu g \cdot mL^{-1} \cdot h$, and the $T_{1/2}$ was ~4 h [13]. KA (0.2 μg in 0.4 μL, ab120100, Abcam, Cambridge, UK) and PTZ (80 mg/kg, MKCJ57, Sigma-Aldrich, MO, USA) were dissolved in normal saline to a working concentration before being injected into mice. ICM and PTZ were administered by intraperitoneal (i.p.) injection. Anti-HMGB1 mAb (gifted by Prof Masahiro Nishibori in Okayama University, Okayama, Japan) was dissolved in phosphate-buffered saline at a concentration of 2 mg/mL and stored at −80 °C until use. Anti-HMGB1 mAb was administered 3 h before KA injection by i.p. injection. The dosage design is referred to the dosing paradigms used in seizure models [11]. Except for the hippocampal-kindled seizure model, the mice in other experiments were divided into groups by simple randomization.

PTZ-induced seizure model

PTZ induced seizure model was conducted as our previous study [11]. Stainless steel screws were screwed into the dorsal aspect of the anterior fontanelle, the bilateral sensory cortex and the ventral aspect of the posterior fontanelle, and fixed with dental cement. The anterior and posterior screws were used as cortical EEG recording and grounding, respectively. Mice were treated with 3 and 10 mg/kg ICM or vehicle 15 min before the injection of PTZ (80 mg/kg, i.p.). EEG activity was recorded with a LabChart system (AD Instruments, Australia) when the mice were placed into polyvinyl chloride boxes immediately after being injected with PTZ. Their behaviors were observed over a period of 30 min. Seizure behavior was classified into 6 stages according to the Racine score to assess the severity of seizure: 1, mouth and facial movement; 2, head nodding; 3, forelimb clonus; 4, rearing with forelimb clonus; 5, rearing and falling with forelimb clonus; 6, fully tonic-clonic seizure. Stages 1–3 were defined as focal seizure (FS), and stages 4–6 were defined as generalized seizure (GS) [14]. A trained observer, who was unaware of the experimental groupings, scored seizure severity. For each mouse, the seizure stage, latency to focal seizure (FS), latency to generalized seizure (GS), duration of GS (GSD) and number of GS were recorded. If the mice did not develop GS in 30 min, the latency to GS was recorded as 30 min.

Hippocampal-kindled seizure model

Hippocampal-kindled seizure model was conducted as our previous studies [15–17]. Briefly, mice were anesthetized by injection of 1% pentobarbital sodium (60 mg/kg) and fixed in a stereotaxic instrument (68043, RWD Life Science Co., Ltd, Shenzhen, China). Stainless steel screws were screwed into the dorsal aspect of the anterior fontanelle, the bilateral sensory cortex and the ventral aspect of the posterior fontanelle, respectively, and the anterior and posterior screws were used as reference electrodes and grounding, respectively. Referring to the Franklin and Paxinos mouse brain map (2001) [18], bipolar-electrodes (795500, each 0.125 mm in diameter; A-M Systems, WA, USA) were inserted in the dorsal CA3 region of the hippocampus (AP: −2.9 mm; ML: −3.2 mm; DV: −3.2 mm) for EEG recording and kindling and fixed with dental cement.

After one week of recovery, the after-discharge threshold (ADT) of each mouse was determined (monophasic square-wave pulses, 20 Hz, 1 ms/pulse, 40 pulses) with an electrical stimulator (SEN-7203, SS-202J; Nihon Kohden, Japan) and EEGs were recorded with a digital amplifier (NuAmps, Neuroscan system, VA, USA). The stimulation intensity was started at 40 μA and was then increased in 20 μA steps every 1 min. The minimal intensity that produced at least a 5 s after discharge duration (ADD) was defined as the ADT for that animal, and this intensity was used thereafter for the grouping. All mice received 10 kindling stimulations daily (400 μA, 20 Hz, 2 s trains, 1 ms monophasic square-wave pulses). The interval of each stimulation was 30 min. Mice were considered to be fully kindled when they had experienced three consecutive seizures with stage five according to the Racine classification (1, mouth and facial movement; 2, head nodding; 3, forelimb clonus; 4, rearing with forelimb clonus; 5, rearing and falling with forelimb clonus). Mice were randomly allocated into two groups and given 3 and 10 mg/kg ICM 15 min before the next kindling. We recorded the seizure stage, latency to GS, GSD, and ADD. After the behavioral studies, the electrode locations were histologically verified in all mice.

KA-induced SE model

KA-induced SE model was conducted as our previous studies [19–21]. Mice were anesthetized by injection (i.p.) of 1% pentobarbital sodium (60 mg/kg) and fixed in a stereotaxic instrument (68043, RWD Life Science Co., Ltd, Shenzhen, China). Stainless steel screws were screwed into the dorsal aspect of the anterior fontanelle, the bilateral sensory cortex and the ventral aspect of the posterior fontanelle, respectively, and the anterior and posterior screws were used as reference electrodes and grounding, respectively. Referring to the Franklin and Paxinos mouse brain map (2001) [18], bipolar-electrodes (795500, each 0.125 mm in diameter; A-M Systems, WA, USA) were inserted in the dorsal CA3 region of the hippocampus (AP: −2.9 mm; ML: −3.2 mm; DV: −3.2 mm), using anterior fontanelle as the coordinate origin, for EEG recording. A cannula (62004, RWD Life Science Co., Ltd, Shenzhen, China) was inserted in the CA1 of the ipsilateral dorsal hippocampus (AP: −2.0 mm; ML: −1.3 mm; DV: −1.6 mm) for KA injection and fixed with dental cement.

After one week of recovery, the mice were randomly divided into two groups: vehicle group and ICM group. Baseline EEG was firstly recorded for 3 min, then mice were i.p. injected with 10 mg/kg ICM. Fifteen min later, KA (0.5 μg/μL, 0.5 μL) was administered in CA1 of the dorsal hippocampus through a infusion needle

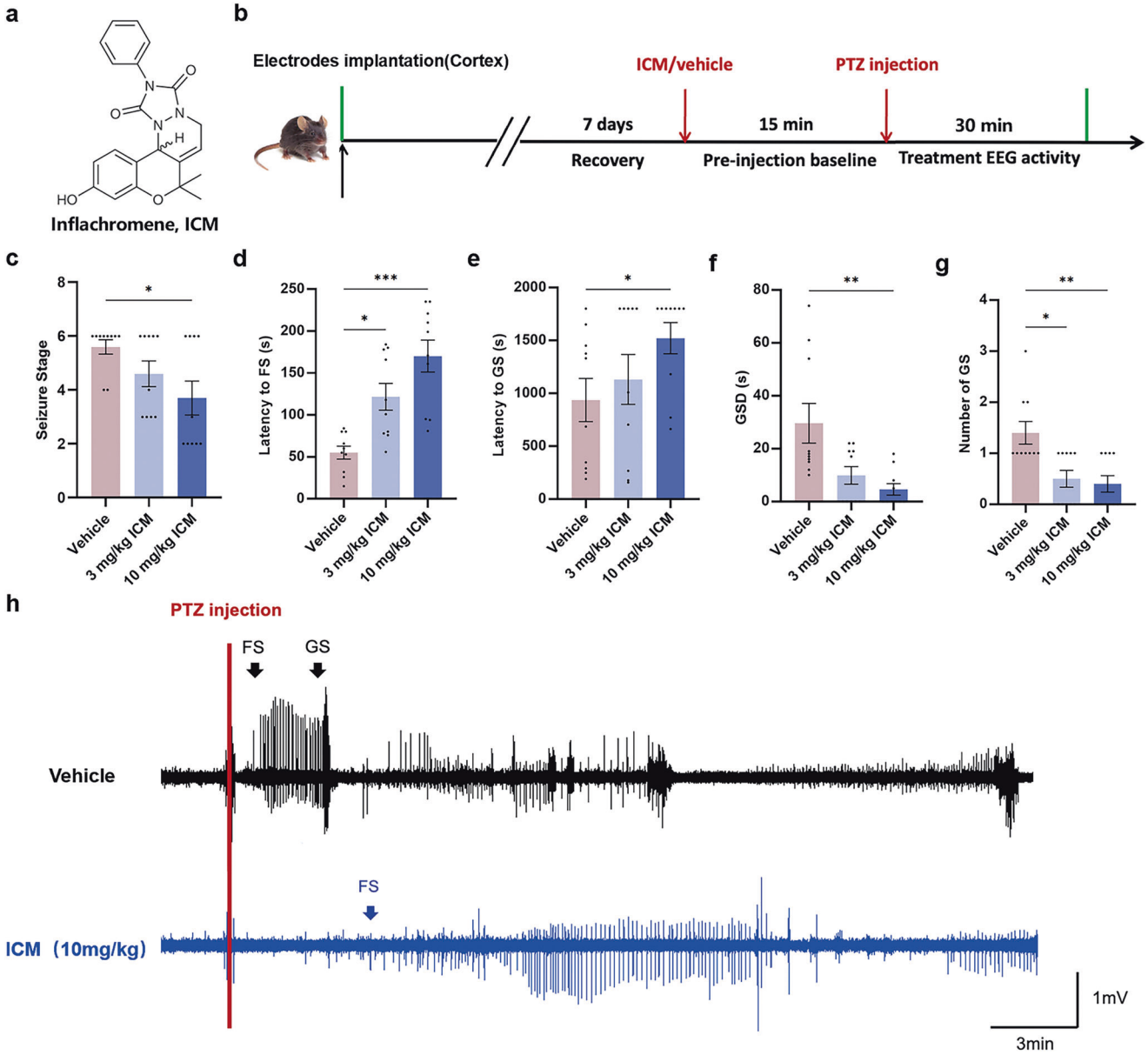

Fig. 1 ICM alleviates seizure severity in PTZ-induced seizure model. a Chemical structure of ICM. **b** Experimental paradigm of PTZ-induced seizure model. **c**–**g** Effects of ICM on seizure stage (**c**), latency to focal seizure (FS, **d**), latency to generalized seizure (GS, **e**), duration of GS (GSD, **f**), and number of GS (**g**) in PTZ-induced seizure model. ICM was i.p. injected 15 min before PTZ test. $n = 10$ for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, one-way ANOVA combined with *post hoc* Dunnett test. **h** Typical EEGs of Vehicle group and 10 mg/kg ICM group. All the data are presented as mean ± SEM.

(62204, RWD Life Science Co., Ltd, Shenzhen, China) and slowly pushed for 2 min, and the needle was left in place for an additional 3 min. EEG and behavioral scoring were continued to be recorded with a LabChart system (AD Instruments, Australia) for 90 min. Behavioral Racine score was recorded, and latency to FS, latency to GS as well as GSD were recorded. To test whether anti-seizure effect of ICM was dependent on HMGB1 signaling, anti-HMGB1 mAb were administered (i.p.) 3 h before KA injection and then ICM was injected 15 min before KA injection. After the behavioral studies, the electrode and cannula locations were histologically verified in all mice.

Immunohistochemistry

An immunohistochemistry experiment was conducted as in our previous studies [22, 23]. Mice were deeply anesthetized by sodium pentobarbital and perfused sequentially with saline, followed by buffered 4% paraformaldehyde. Brains were removed and fixed by immersion in 4% paraformaldehyde for 24 h. After dehydration with 30% sucrose for 48 h, we cut coronal 20-μm sections on a sliding freezing microtome (Cryostar NX70, Thermo Fisher Scientific, CA, USA). For immunostaining with HMGB1 and neuron/microglia/astrocyte markers (NeuN/Iba-1/GFAP), slices were incubated for 15 min in 0.1% Triton X-100 to facilitate perforation, followed by a 2 h incubation in 5% donkey serum. NeuN/Iba-1/GFAP or HMGB1 were subsequently visualized by detection with Alexafluor 488 or 594 secondary antibodies (711–545–152/712–005–153, Molecular Probes, OR, USA) after adding the primary antibodies to HMGB1, NeuN (MABN140, Millipore, MA, USA), Iba-1 (ab5076, Abcam, Cambridge, UK), or GFAP (BA0056, Boster, Wuhan, China). Image slices were mounted with Fluoroshield™ with DAPI (F6057, Sigma-Aldrich, MO, USA) before image capture with a fluorescence microscope (Olympus DP70, Tokyo, Japan).

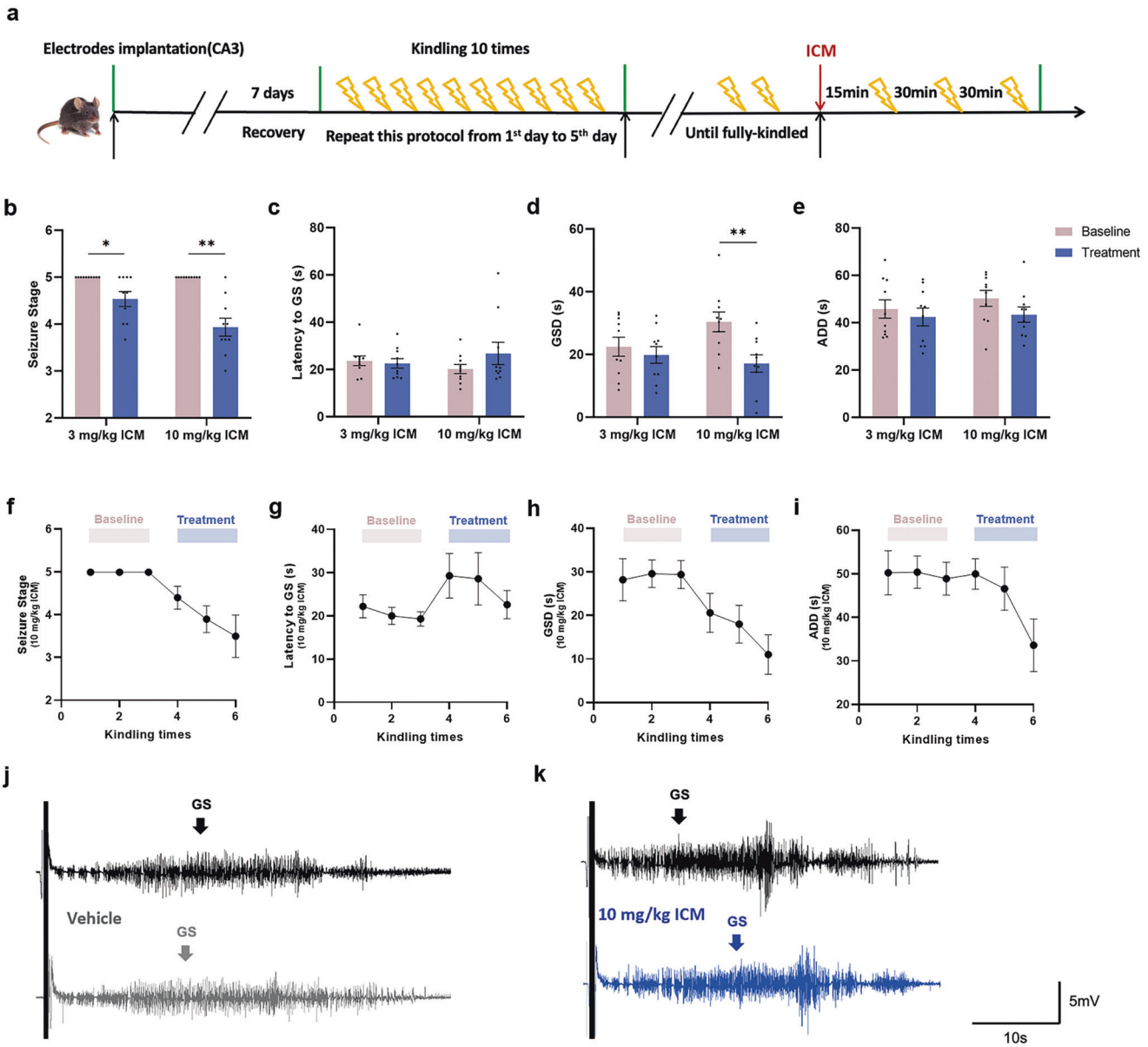

Fig. 2 ICM alleviates seizure severity in hippocampal-kindled seizure model. a Experimental paradigm of hippocampal-kindled seizure model. **b**–**e** Effects of ICM on seizure stage (**b**), latency to generalized seizure (GS, **c**), duration of GS (GSD, **d**), and after-discharge duration (ADD, **e**) in hippocampal-kindled seizure model. ICM was i.p. injected 15 min before the first kindling stimulation in fully kindled mice. $n = 10$ mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney test. **f**–**i** Timeline of anti-seizure effect of ICM (10 mg/kg) in hippocampal kindled seizure model. **j**, **k** Typical EEGs in Vehicle group and 10 mg/kg ICM group. All the data are presented as mean ± SEM.

In the KA-induced SE model, 6 mice were taken from each group, and three lays of brain slices were selected. The co-staining rates of NeuN, GFAP, Iba-1 and HMGB1 were counted in hippocampal CA1, CA3 and DG subregions ipsilateral to the cannula, and HMGB1 translocation ratio was averaged and then statistically analyzed. In addition, 4 mice were taken from each group, and the number of neurons in the CA1 and the widths of the granule cell layer in the DG of the ventral hippocampus on the ipsilateral side of the cannula were measured and statistically analyzed.

Data statistics and analysis

All data in this experiment are expressed as mean ± SEM. A number of experimental replicates (n) are indicated in Figures or Figure legends and refers to the number of experimental subjects used and independently treated in each experimental condition. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., CA, USA) and detail information was described in the figure legend. A two-tailed $P < 0.05$ is considered a significant difference.

RESULTS

ICM alleviates seizure severity in PTZ-induced seizure model

The PTZ-induced seizure model was first used to initially screen the anti-seizure effect of ICM (Fig. 1b). ICM dose-dependently lowered seizure stage ($P = 0.036$, vehicle: 5.60 ± 0.27; 10 mg/kg ICM: 3.70 ± 0.63; Fig. 1c), and significantly increased the latency to FS ($P = 0.038$, 0.0001, respectively; 55.10 ± 7.61 vs. 121.70 ± 15.95 vs. 170.20 ± 19.12 s; Fig. 1d) and GS ($P = 0.049$, vehicle: 936.60 ± 203.40 s; 10 mg/kg ICM: 1521.00 ± 147.70 s, Fig. 1e). ICM also decreased GSD ($P = 0.0072$, vehicle: 29.60 ± 7.49 s; 10 mg/kg ICM: 4.60 ± 2.18 s; Fig. 1f) and number of GS ($P = 0.019$, 0.0056, respectively; 1.40 ± 0.22 vs. 0.50 ± 0.17 vs. 0.40 ± 0.16; Fig. 1g)

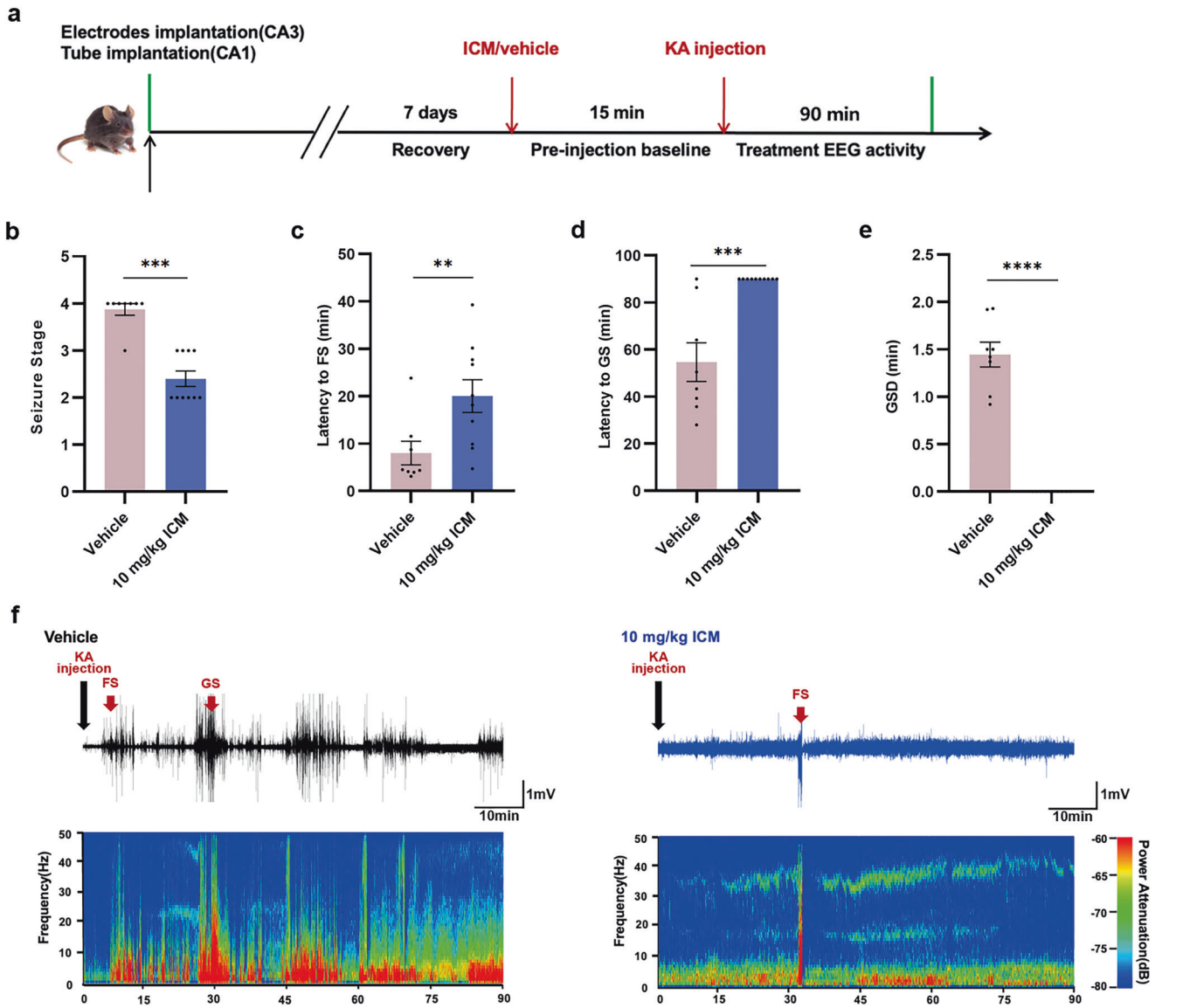

Fig. 3 ICM alleviates seizure severity in KA-induced SE model. a Experimental paradigm of KA-induced SE model. **b**–**e** Effects of ICM on seizure stage (**b**), latency to focal seizure (FS, **c**), latency to generalized seizure (GS, **d**) and duration of GS (GSD, **e**) in KA-induced SE model. ICM was *i.p.* injected 15 min before KA injection. $n = 8$ mice for Vehicle group, $n = 10$ mice for ICM group. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, Mann-Whitney test. **f** Typical EEGs and corresponding spectrum of seizures in vehicle group and 10 mg/kg ICM group in KA-induced SE model. All the data are presented as mean ± SEM.

during the observation period. Typical EEGs also reflected the anti-seizure effect of ICM in the PTZ-induced seizure model (Fig. 1h). All these results indicated that ICM alleviates the severity of PTZ-induced seizures in a dose-dependent manner.

ICM alleviates seizure severity in hippocampal kindled seizure model

The anti-seizure effect of ICM was further evaluated in a chronic hippocampal-kindled seizure model, in which a state of hyperexcitability (kindled seizure state) was induced in mice with an increased number of repeated electrical kindling stimuli [24]. To minimally reduce variation among different animals, we used self-control comparison here (Fig. 2a). ICM treated mice showed a dose-dependent significant reduction in the seizure stage ($P = 0.031$, 0.0039, respectively; 3 mg/kg ICM: 4.53 ± 0.16; 10 mg/kg ICM: 3.93 ± 0.19; Fig. 2b). Meanwhile, 10 mg/kg ICM significantly reduced GSD ($P = 0.0039$, 30.37 ± 3.16 vs. 17.10 ± 2.78 s; Fig. 2d). However, ICM failed to alter the latency to GS and ADD (Fig. 2c, e), suggesting that ICM might be sensitive to the maintenance of GS. The line graph during the kindling test reflects a gradual decrease in the seizure stage and GSD after the treatment of 10 mg/kg ICM compared to the baseline state (Fig. 2f–i). Typical hippocampal EEGs also reflected the anti-seizure effect of ICM in the hippocampal-kindled seizure model (Fig. 2j, k). These results suggested that ICM alleviates seizure severity in the hippocampal kindled seizure model, mainly in terms of reducing seizure stage and shortening GSD.

ICM alleviates seizure severity in KA-induced SE model

We further evaluated the anti-seizure effect of ICM in the KA-induced SE model (Fig. 3a), which is usually accompanied by sizeable neuronal loss in the following days [25]. ICM at the dose of 10 mg/kg significantly lowered seizure stage ($P = 0.0001$, 3.88 ± 0.13 vs. 2.40 ± 0.16; Fig. 3b), increased the latency to FS ($P = 0.0062$, 8.00 ± 2.48 vs. 20.04 ± 3.44 min; Fig. 3c) and GS ($P = 0.0003$, 54.66 ± 8.23 vs. 90.00 min; Fig. 3d) compared with the vehicle group. Notably, ICM nearly eliminated GSD ($P = 0.0001$,

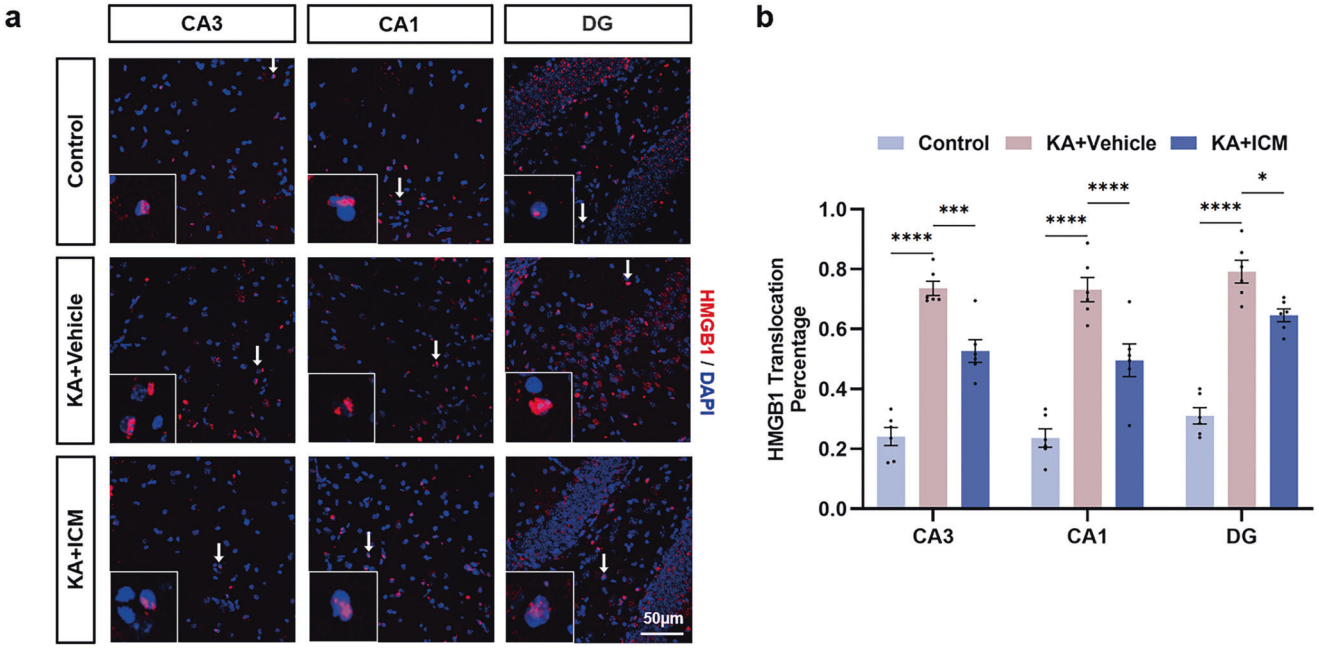

Fig. 4 ICM attenuates the increased HMGB1 translocation in KA-induced SE model. a Typical immunohistochemical images of HMGB1 (HMGB1 in red; DAPI in blue). Scal bar, 50 μm. **b** Statistical data of translocation percentage (extranuclear percentage) of HMGB1 in control group, KA + vehicle group and KA + 10 mg/kg ICM group in hippocampal CA3, CA1, and DG. $n = 6$ mice for each group. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, One-way ANOVA followed by post hoc Tukey test. All the data are presented as mean ± SEM.

1.45 ± 0.13 vs. 0.00 min; Fig. 3e), further verifying that ICM might be sensitive to the maintenance of GS. Typical hippocampal EEGs also reflected the anti-seizure effect of ICM in SE model (Fig. 3f). These results indicated that ICM alleviates seizure severity in the KA-induced SE model.

ICM attenuates the increased HMGB1 translocation

Next, we further aimed to investigate whether ICM affects the translocation of HMGB1, as ICM blocks the sequential processes of cytoplasmic localization and extracellular release of HMGBs by perturbing its post-translational modification [12]. Since ICM has the most apparent anti-seizure effect in the KA-induced SE model, we used brain sections after behavioral experiments in the KA-induced SE model and performed immunohistochemical staining. We found that SE significantly induced the translocation of HMGB1 in the hippocampus, including the CA3, CA1, and DG subregions. Furthermore, ICM treatment significantly attenuated the increased percentage of HMGB1 translocation in these subregions (Fig. 4). Next, we tested whether the translocation of HMGB1 shows cell-type specificity. In the CA3 subregion, ICM treatment significantly decreased the percentage of HMGB1 translocation in both neurons ($P = 0.048$, 0.89 ± 0.073 vs. 0.67 ± 0.028; Fig. 5b) and astrocytes ($P = 0.0087$, 0.66 ± 0.045 vs. 0.45 ± 0.032; Fig. 5f). In the CA1 subregion, ICM treatment significantly decreased the percentage of HMGB1 translocation in microglia ($P = 0.0022$, 0.63 ± 0.075 vs. 0.26 ± 0.061; Fig. 5d). While, in the DG subregion, ICM treatment significantly decreased the percentage of HMGB1 translocation mainly in astrocytes ($P = 0.043$, 0.79 ± 0.043 vs. 0.55 ± 0.088; Fig. 5f).

In addition, to test whether ICM would directly affect neuronal excitability, we preformed in vitro electrophysiological experiment and found that ICM could not reduce the action potential firing of CA1 pyramidal neurons ($P = 0.25$, 4.21 ± 0.62 vs. 3.43 ± 0.55; Supplementary Fig. S1a), and did not change the basic membrane properties such as resting membrane potential ($P = 0.20$, −63.85 ± 0.58 vs. −64.90 ± 0.59; Supplementary Fig. S1b) and rheobase ($P = 0.49$, 63.00 ± 8.11 vs. 72.50 ± 8.79; Supplementary Fig. S1c), indicating that ICM may not directly affect the excitability of CA1 pyramidal neurons.

These above results showed that ICM attenuated HMGB1 translocation in manners of the both region- and cell-type specificity.

Anti-HMGB1 mAb abolishes the anti-seizure effect of ICM on KA-induced SE

To further investigate whether the anti-seizure effect of ICM is correlated with HMGB1 signaling, we used anti-HMGB1 mAb in combination with ICM in the KA-induced SE model. The results showed that in the condition of anti-HMGB1 mAb pretreatment, ICM did not significantly decrease the seizure stage and GSD, nor did it significantly increase the latency to FS and GS (Fig. 6b–e). Typical EEGs are shown in Fig. 6f.

As ICM is an anti-inflammatory agent that directly binds both HMGB1 and HMGB2, we further investigated whether HMGB2 is involved in anti-seizure effect of ICM. We found that intra-hippocampal injection of anti-HMGB2 mAb did not reduce the severity of seizures in KA-induced acute SE model. Meanwhile, ICM still significantly reduced the severity of KA-induced seizures in the presence of anti-HMGB2 mAb (Supplementary Fig. S2), indicating that the anti-seizure effect of ICM is not dependent on HMGB2.

The above results suggest that anti-HMGB1 mAb abolishes the anti-seizure effect of ICM in the KA-induced SE model, and the anti-seizure effect of ICM is dependent on the intracellular translocation of HMGB1.

ICM alleviates neuronal loss and dispersion after KA-induced SE

As an anti-inflammatory inhibitor ICM exhibited neuroprotective effects in previous studies [12], we further aimed to investigate whether ICM can alleviate the pathological features of epilepsy. Pyramidal neuronal loss and granule cell dispersion were measured by the number of pyramidal neurons in the CA1 and the width of the granule cell layer in the DG regions of the hippocampus after KA-induced SE. The results showed that the number of pyramidal neurons in the CA1 region of the hippocampus was decreased after KA-induced SE compared with normal mice, and ICM could significantly alleviate this decrement ($P = 0.029$, 0.024, respectively; 4160.00 ± 231.40 vs. 2481.00 ± 374.20 vs. 4410.00 ± 189.70 per mm^2; Fig. 7a, b). The width of the granule cell layer in the DG region of the

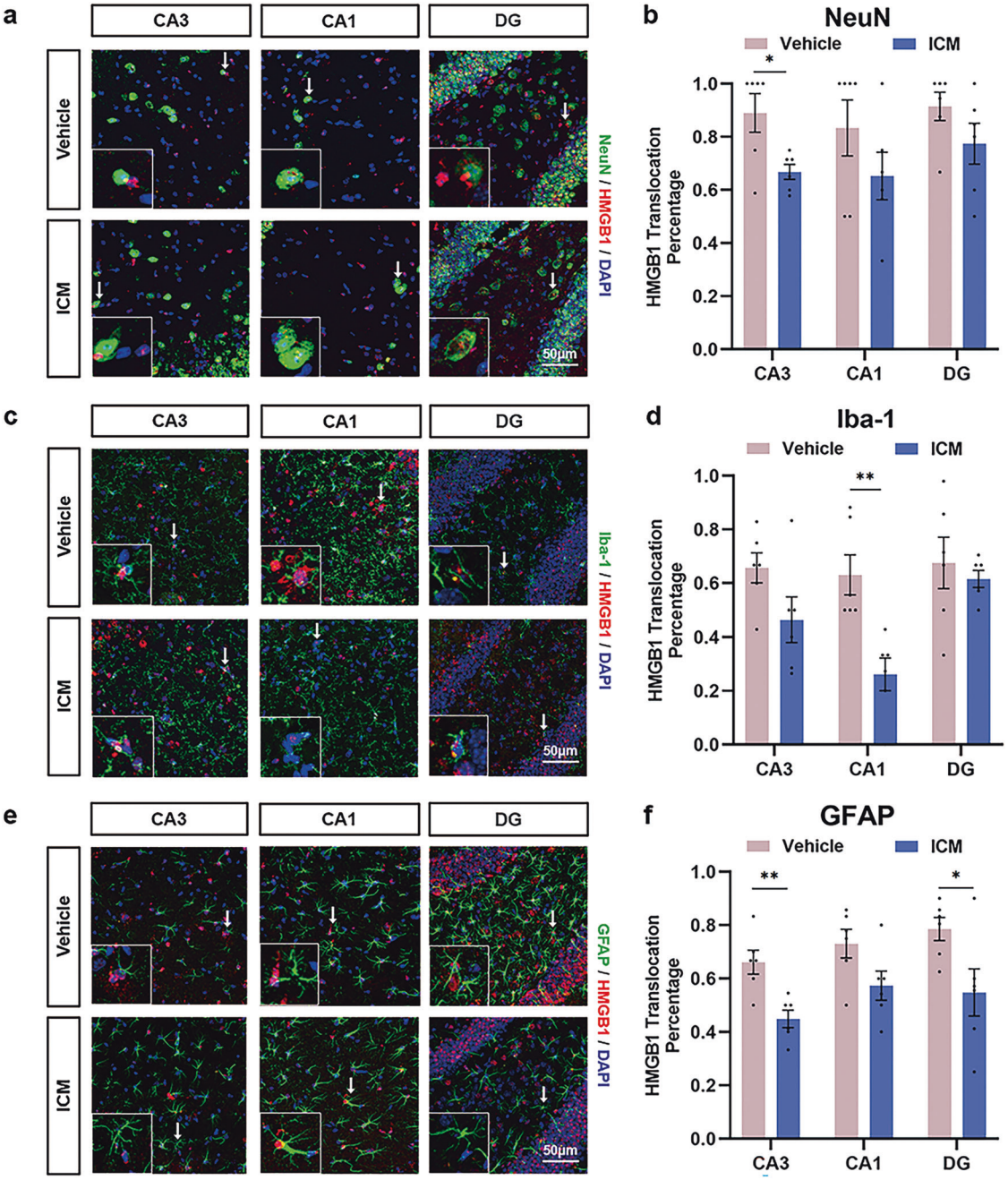

Fig. 5 ICM attenuates the increased translocation of HMGB1 with cell-type specificity in different subregions of hippocampus. **a**, **c**, **e** Typical immunohistochemical images of neurons (**a**), microglia (**c**), and astrocytes (**e**) co-stained with HMGB1 (NeuN / Iba-1 / GFAP in green; HMGB1 in red; DAPI in blue). Scal bar, 50 μm. **b**, **d**, **f** Statistical data of translocation percentage (extranuclear percentage) of HMGB1 in neurons (**b**), microglia (**d**) and astrocytes (**f**) in vehicle group and 10 mg/kg ICM group in hippocampal CA3, CA1, and DG. $n = 6$ mice for each group. $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney test. All the data are presented as mean ± SEM.

hippocampus was increased after KA-induced SE compared with normal mice, and this increment could be significantly alleviated by ICM ($P = 0.032$, 0.029, respectively; 88.57 ± 2.39 vs. 145.40 ± 2.69 vs. 91.09 ± 4.04 μm; Fig. 7c, d). These results suggest that ICM can alleviate pyramidal neuronal loss and granule cell dispersion after KA-induced SE, suggesting that ICM is protective against neurotoxicity after SE.

DISCUSSION

Epilepsy is not well controlled by current treatment. Many studies in recent years have confirmed the prevalence of HMGB1-mediated inflammatory pathways in neurological disorders, including epilepsy [7, 8]. However, there remains a lack of regulatory small molecules targeting HMGB1-related pathways. ICM, a novel small molecule inhibitor of HMGB, is discovered by target identification using fluorescence differences in two-dimensional gel electrophoresis. Here, we evaluated the anti-seizure effect of ICM using PTZ-, hippocampal-kindled, and KA-induced epilepsy models. We demonstrated that ICM has a good therapeutic effect on all three seizure models, among which ICM has the most obvious anti-seizure effect in the KA-induced SE model. Furthermore, ICM inhibits the enhanced intracellular translocation of HMGB1 in the hippocampus, and anti-HMGB1 mAb, but not anti-HMGB2 mAb, can

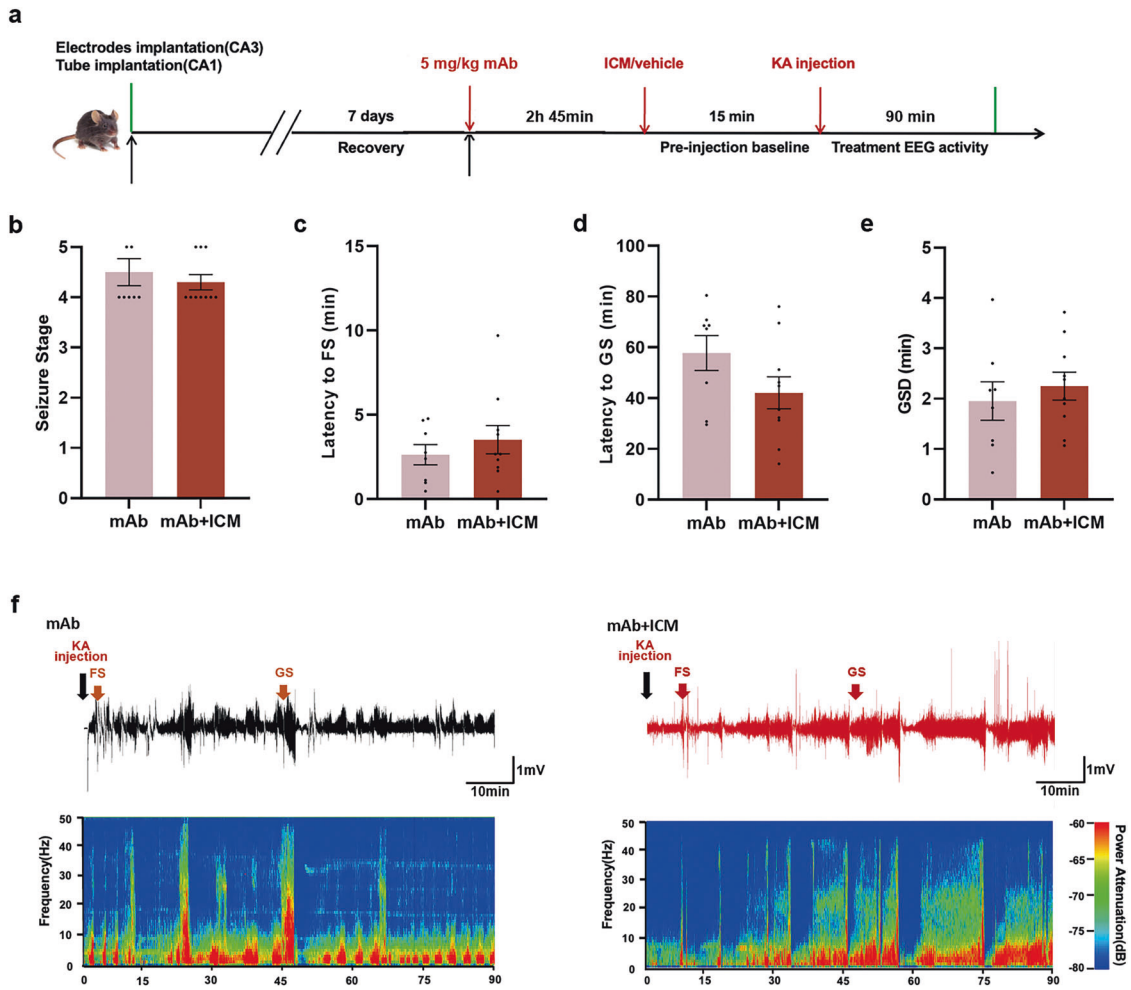

Fig. 6 Anti-HMGB1 mAb abolishes the anti-seizure effect of ICM in KA-induced SE model. a Experimental paradigm of KA-induced SE model. **b**–**e** Effects of ICM on seizure stage (**b**), latency to focal seizure (FS, **c**), latency to generalized seizure (GS, **d**) and duration of GS (GSD, **e**) in KA-induced SE model, in the pretreatment of anti-HMGB1 mAb. $n = 8$ for mAb group and $n = 10$ for mAb + ICM group, Mann-Whitney test. **f** Typical EEGs and corresponding spectrum of seizures in mAb group and mAb + ICM group in KA-induced SE model. All the data are presented as mean ± SEM.

abolish the anti-seizure effect of ICM in the KA-induced SE model, suggesting the anti-seizure effect of ICM is related to HMGB1 targeting. In addition, ICM is protective against neurotoxicity after SE. Our study confirms the critical role of HMGB1 signaling in epilepsy and broadens the scope of potential disease treatment with ICM.

Firstly, we applied several classical seizure models reported in previous studies [26–31] to evaluate the therapeutic efficacy of ICM. In the PTZ model, ICM dose-dependently lowered the seizure stage, increased the latency to FS and GS, and decreased GSD (Fig. 1). In kindled seizure model, ICM significantly reduced the severity of GS. In the KA-induced SE model, ICM at the dose of 10 mg/kg significantly lowered the seizure stage and increased the latency to FS and GS (Fig. 2). These suggested the anti-seizure effects of ICM in all three seizure models. Interestingly, ICM has the most apparent anti-seizure effect in the KA-induced SE model, as ICM nearly eliminated GSD (Fig. 3). This can be due to the possibility that different epilepsy models have different pro-seizure mechanism and KA-induced SE model usually showed more severe seizure activity and neuronal loss with neuroinflammation. KA is an agonist of glutamatergic KA receptor. The KA-induced acute SE model is mainly associated with large amount of neuroinflammation, and neuropathological changes, including the neuronal loss in the hippocampus [26]. While, PTZ is a $GABA_A$ receptor antagonist, it produces acute seizure mainly via inhibition on GABAergic transmission [29]. The kindling model obtains permanent hyperexcitability through electrical stimulation mainly via enhanced excitatory synaptic transmission [32]. These two models usually have short seizure duration and less neuroinflammation as that in the KA-induced acute SE model. ICM attenuated neuroinflammation by anti-HMGB1 and was able to reverse neurotoxicity in the KA-induced acute SE model, so the effect may be apprarent in the KA-induced acute SE model. Inhibiting the intracellular translocation of HMGB1 can also exert anti-inflammatory and anti-excitotoxic effects [33]. Here, ICM significantly reduced KA-induced pyramidal neuronal loss and granule cell dispersion (Fig. 7), suggesting that ICM is protective against neurotoxicity caused by seizures. In addtion, we found that the severity of GS, especially the GSD, is the most sensitive index in response to ICM treatment in all three seizure models, suggesting ICM might be critical to control the maintenance of GS. It is possible that severity of GS may be positively correlated with the increased neuroinflammation that could be easily intervened with anti-HMGB1 [34–39]. Together, our study reports the anti-seizure effects of ICM in three mice epilepsy models, particularly the suppression of GS, and the neuroprotective effects of ICM after SE. As HMGB1 and it signaling pathway are also important potential targets for the treatment of epileptogenesis, further

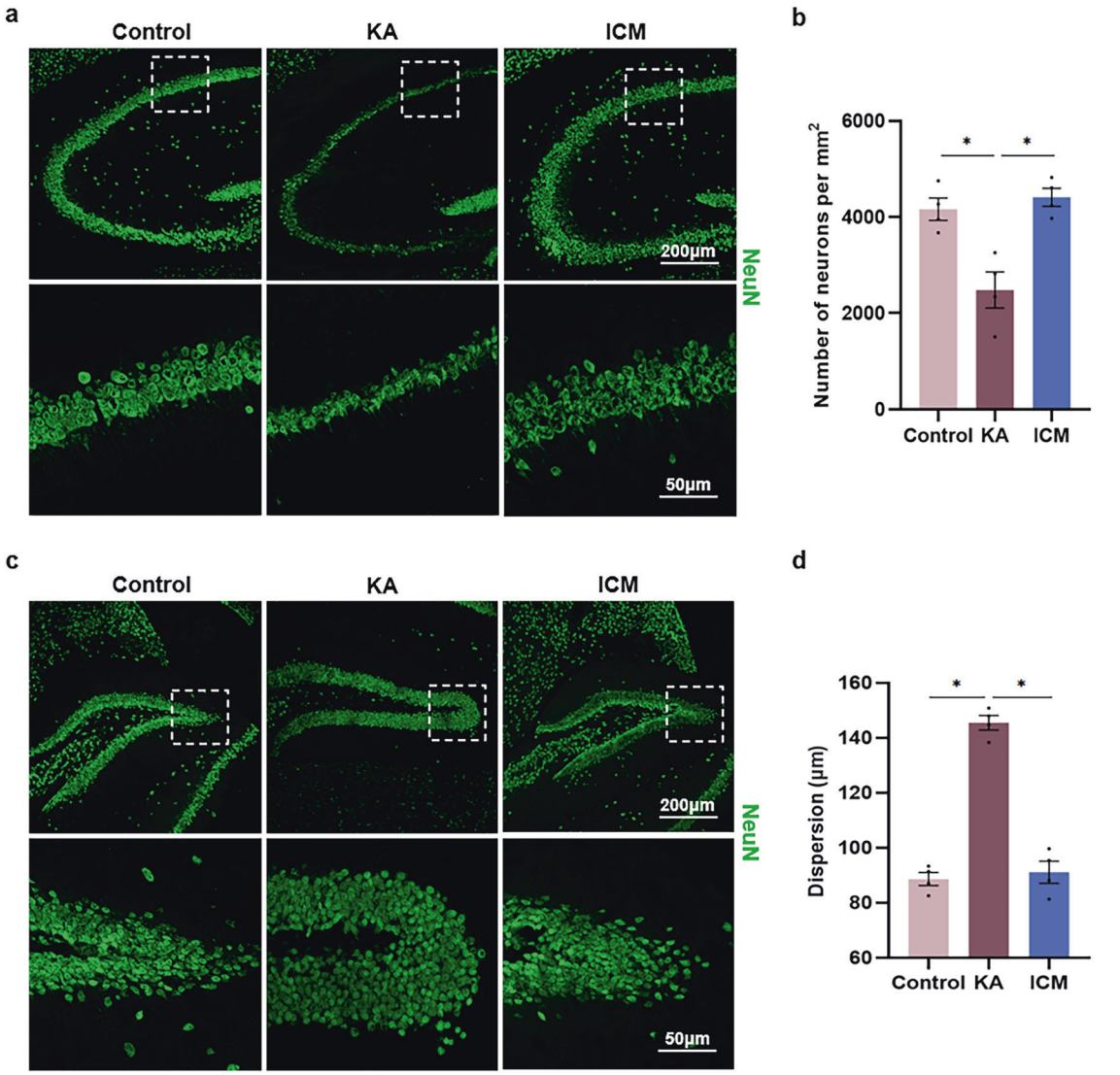

Fig. 7 ICM alleviates pyramidal neuronal loss and granule cell dispersion after KA-induced SE. a, **b** Typical immunohistochemical images and statistical data of pyramidal neuronal loss in the CA1 region in control group (naïve mice), KA group and 10 mg/kg ICM group. **c**, **d** Typical immunohistochemical images and statistical data of granule cell dispersion in the DG region in control group, KA group and 10 mg/kg ICM group. $n = 4$ for each group. *$P < 0.05$, one-way ANOVA followed with *post hoc* Dunnett test. All the data are presented as mean ± SEM.

investigation of the anti-epileptogenic action of ICM during the development of epileptogenesis would broaden the scope of therapeutic potential of ICM in epilepsy.

Next, we further explored whether ICM affects the intracellular translocation of HMGB1, as ICM was discovered as a small-molecule inhibitor targeting HMGBs [12]. First, we found that ICM attenuates the enhanced translocation of HMGB1 in different subregions of hippocampus in the KA-induced SE model (Fig. 4). Interestingly, the proportion of HMGB1 translocation in each brain region decreased by ICM treatment showed cell type-specific changes. In particular, the proportion of HMGB1 translocation in neurons of CA3, microglia of CA1, and astrocytes of CA3 and DG was significantly decreased, with the most significant decrease in microglia of CA1, region for KA injection that is usually considered as seizure focus (Fig. 5). This may be because the KA-induced acute SE model is closely related to neuroinflammation, and CA1 is the injection site of KA (the focus of seizure initiation), and the inflammatory response mediated by microglia is the most obvious, so ICM may have the most significant inhibitory effect on it. In other words, the anti-seizure effect of ICM may be divided into direct (early stage in acute seizure onset) and indirect effects (spread of seizure activity). During the onset of epileptic seizures, HMGB1 translocation occurs in microglia, and local excitability is further increased, leading to seizure spread to CA3 and DG, and promotes further HMGB1 translocation. As one of the main receptors of HMGB1, TLR4 -mediated signaling further promoted seizures and subsequent HMGB1 translocation [35]. This forms a positive feedback loop. As there may be different mechanisms for HMGB1 translocation and TLR4 receptor expression in different brain regions and different cell types, the translocation of HMGB1 in different types of cells has different causal status correlated with ICM. Since our study found that ICM can prolong the focal seizure latency in PTZ-induced seizure model and KA-induced acute SE model, it means that ICM is involved in the early stage of epilepsy. Further electrophysiological results showed that ICM does not directly affect neuronal excitability (Supplementary Fig. S1). Therefore, ICM may directly affect the translocation of HMGB1 in microglia in the early stage of seizures, and the subsequent increase in HMGB1 translocation is the result mediated by other cells such as microglia. Previous studies have reported the critical role of dysfunctional microglia in epilepsy, including the abnormal morphology of microglia, enhanced phagocytosis of microglia, enhanced microglia-mediated neuroinflammation or synaptic strip

etc. [40–47]. Notably, HMGB1-mediated microglia activation via the TLR4/NF-κB pathway promotes seizures [48]. ICM mainly inhibits microglia-mediated neuroinflammation by binding to HMGBs [12], indicating ICM may exert anti-seizure effect mainly by inhibiting HMGB1 translocation of microglia in hippocampal CA1 subregion.

In addition, activated microglia can promote neuronal death after seizures. It has also been found that minocycline, a tetracycline antibiotic commonly used to inhibit microglia activation, inhibited neurodegeneration in hippocampal CA1 and CA3 subregions in the KA-induced mTLE mouse model [49], which may be the reason for the neuroprotective effect of ICM. Furthermore, our results showed that anti-HMGB1 mAb abolishes the anti-seizure effect of ICM in the KA-induced SE model (Fig. 6), further suggesting that ICM may exert anti-seizure effects by targeting HMGB1. ICM has been shown to act as a post-translational modification modulator of the translocation process of HMGB1 and HMGB2 [12]. We also excluded the possibility that HMGB2 is involved in the anti-seizure mechanism of ICM (Supplementary Fig. S2). We found that intra-hippocampal injection of anti-HMGB2 mAb did not reduce the severity of seizures and ICM still significantly reduced the severity of KA-induced seizures in the presence of anti-HMGB2 mAb, indicating that the anti-seizure effect of ICM is dependent on HMGB1, but not HMGB2. Although we have previously reported that anti-HMGB1 is effective in anti-seizure treatment [11], these mAbs are currently not easily produced and seem to be more expensive. ICM, being a novel small molecule compound, has the advantage of good druggability with easy availability and better pharmacokinetic properties crossing biological membranes and BBB [13].

In summary, this study demonstrates for the first time that ICM, a small molecule inhibitor of HMGBs, has significant therapeutic effects on different epilepsy models, including attenuating seizure severity and neuroprotective potential. This study may help develop a potential therapeutic agent for treating epilepsy.

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AUTHOR CONTRIBUTIONS

ZC, YW, and SBP designed the research. SJD, YYS, MQY, and XYQ conducted the experiments. SJD, YZ, and YW conducted the data analysis. YZ, JYSun, ZSL, JYShi, CLX, WSC, SY, SBP, MN, YW, and ZC provided technical guidance and contributed to the data discussion. SJD, YYS, YZ, and YW wrote the manuscript. YW and ZC supervised all aspects of the work.

ADDITIONAL INFORMATION

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