■ Original article

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Epileptic Disorders

Cu-Zn SOD suppresses epilepsy in pilocarpine-treated rats and alters *SCN2A/Nrf2/HO-1* expression

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ABSTRACT

Objective. Copper-zinc superoxide dismutase (Cu-Zn SOD) is downregulated in epilepsy, however, the role of Cu-Zn SOD in epilepsy remains unclear.

Methods. Based on the pilocarpine hydrochloride-induced rat model of epilepsy, cortical-striatum brain slices of rats were examined based on field excitatory post-synaptic potentials. Pathological changes were observed by transmission electron microscope. Also using SH-SY5Y cells, flow cytometry and TUNEL staining were applied to investigate cell apoptosis, and ELISA was applied to detect SOD activity. In addition, qRT-PCR and western blot were performed to detect *SCN2A*/*Nrf2*/*HO-1* gene and protein expression levels, respectively.

Results. Cu-Zn SOD over-expression suppressed epilepsy *in vivo*. In addition, Cu-Zn SOD knockdown notably decreased SOD activity and induced apoptosis in SH-SY5Y cells. Moreover, Cu-Zn SOD silencing decreased the levels of *SCN2A*, *Nrf2* and *HO-1*. Lastly, Cu-Zn SOD was shown to modulate the NaV1.2/Nrf2/HO-1 axis in rats.

Significance. In this model, Cu-Zn SOD attenuated epilepsy and was shown to alter the expression level of proteins of the NaV1.2 /Nrf2/HO-1 signalling pathway, indicating that Cu-Zn SOD might be a target for the treatment of epilepsy.

Key words: Cu-Zn SOD, EP, Nrf2/HO-1 signalling pathway, *SCN2A*

Epilepsy is the second most common disease in the nervous system resulting from recurrent and complicated central nervous system (CNS) dysfunction, due to abnormal brain neurons [1, 2]. Due to its repeated, irregular, and sudden onset, patients are prone to physical injury and mental stress, which poses a serious threat to global health [3, 4]. The incidence of epilepsy in China appears to be increasing each year [5, 6]. Moreover, paediatric epilepsy can lead to serious comorbidities (language disability, intellectual disability, *etc.*) [7]. Since the pathogenesis of epilepsy has not been fully elucidated, new strategies are urgently needed for the treatment of epilepsy.

Voltage-dependent sodium channels are transmembrane proteins that play a crucial role in propagation of action potentials which can regulate neuron function [8, 9]. For instance, sodium channels are often open during the depolarization of neurons [10, 11]. There are 11 genes encoding human sodium channel α subunits, of which only *SCN1A*, *SCN2A*, *SCN3A* and

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SCN8A are expressed in the CNS, and *SCN1A* and *SCN2A* are known to be one of the important targets for epilepsy [12-14].

Copper-zinc superoxide dismutase (Cu-Zn SOD) catalyses the disproportionation of superoxide and is converted into hydrogen peroxide. Cu-Zn SOD is upregulated in epilepsy and promotes the progression of epilepsy [15, 16]. The Nrf2/HO-1 pathway is an important mediator in cellular process which has been reported to inhibit inflammatory responses [17]. Moreover, Dai *et al*. reported that Nrf2/HO-1 is involved in progression of epilepsy [18]. However, the function of Cu-Zn SOD in epilepsy and the correlation with NaV1.2 (encoded by *SCN2A*) and Nrf2/HO-1 signalling are largely unknown.

In the current study, we reveal that Cu-Zn SOD inhibited epilepsy based on the pilocarpine hydrochloride-induced rat model. In addition, Cu-Zn SOD was shown to alter the level of proteins of the NaV1.2/Nrf2/HO-1 axis. We hope this finding may be of value for future approaches aimed at treating epilepsy.

Materials and methods

Animals

Fifteen Wistar rats (eight weeks old, 160-200 g, both male and female) were obtained from Chinese Academy of Sciences (Shanghai, China). Rats were maintained in specific pathogen-free (SPF) conditions. This animal research was approved by the Medical Ethics Committee of The Second Xiangya Hospital of Central South University.

In vivo study

Rats were randomly divided into three groups: control (treated with saline), model group (pilocarpine-treated rats treated with saline) and Cu Zn-SOD group (pilocarpine-treated rats treated with viral vector expressing Cu-Zn-SOD [*SOD1*] via the brain cavity). As noted in a previous publication [19], rats were given 200 mg/kg nicotine (Beyotime, Shanghai, China) subcutaneously to combat the surrounding cholinergic effect. After 30 minutes, rats were administrated with pilocarpine hydrochloride (Beyotime)-to induce epilepsy- except for control group, accordingly [20]. Rats in the model and Cu Zn-SOD group exhibited convulsions and fainting. If there was no epilepsy, pilocarpine was reinjected after 60 minutes until induction of epilepsy. After 60 minutes, rats in the Cu Zn-SOD group were injected with viral vector expressing the Cu-Zn-SOD (*SOD1*) gene (200 µL, 2×10^{11} MOI adenoviruses, pAV-TBG-Cu-Zn-SOD) via the cerebellar ventricle for four weeks. At the end of the study, rats were sacrificed and brain tissues and blood were collected for the following investigations.

Cell culture and transfection

SH-SY5Y cells were obtained from ATCC (USA) and cultured in an incubator with DMEM (Thermo Fisher Scientific, Waltham, MA, USA) +10% FBS +100 units/mL penicillin and streptomycin (Thermo Fisher Scientific) at 37°C, 5% CO_2 and 95% air. For cell transfection, SH-SY5Y cells were cultured in DMEM supplemented with 10% FBS and streptomycin and penicillin (100 U/mL) at 37°C and 5% CO_2. SH-SY5Y cells were transfected with si-negative control (NC), pcDNA3.1, pcDNA3.1-*SCN2A* (*SCN2A* over-expression) or si-SOD (*SOD* knockdown) using the Lipofectamine 2000 for four hours. All vectors were obtained from Genepharma.

Field excitatory post-synaptic potential detection

Cortical-striatum brain slices were prepared and recorded via clamping whole cells. Under urethane (1.2 g/kg) anaesthesia, a double-barrel glass micropipette (Borosilicate, outer diameter: 1.5 mm, length: 10 cm; World Precision Instruments) was inserted into the granule cell layer of the Dentate Gyrus (DG) in the right hemisphere (from the bregma: anteroposterior: −3.0 mm; mediolateral: 2.15 mm; dorsoventral: 2.5–3 mm below the dura). The recording barrel was filled with 3 M NaCl (tip resistance: 2–10 Mς) for recording of field potentials. The other was filled with saline or fasudil and was connected to a Hamilton syringe (25 µL) driven by a syringe pump (Stoelting Co., Wood Dale, IL, USA). A bipolar tungsten electrode (stainless steel, Tefon-coated, 127 µm in diameter, insulated except at its tips) was used to stimulate the medial perforant path (PP) (from the bregma: anteroposterior: −6.5 mm; mediolateral: 3.8 mm; dorsoventral: 2–2.5 mm below the dura) of the right hemisphere. The depth of recording and stimulating electrodes (dorsoventral coordinate) was adjusted to obtain a large positive excitatory post-synaptic potential (EPSP) followed by a negative population spike (PS) in response to the PP stimulation. After obtaining an input–output curve and a 15-minutes baseline recording, plasticity was induced by application of a tetanic stimulation consisting of four trains of high-frequency stimuli (HFS) (100 Hz, 1-second duration) or by application of low-frequency stimulation (LFS) (1 Hz, 900 pulses). Infusions of 0.9% saline solution or fasudil (10 µM) were given for one hour starting from the induction, using a Hamilton pump (a 20-µL volume, at a rate of 0.33 µL/min). The ratio of 5-minutes averages of the EPSP slopes and PS amplitudes at the end of recording was used as a measure of the magnitude of LTP.

qRT-PCR

Total RNA was extracted from SH-SY5Y cell lines or brain tissues using TRIzol reagent (TaKaRa, Tokyo, Japan). PrimeScript RT reagent Kit (Takara) was applied to synthesize first-strand cDNA. RT-qPCR was performed using an ABI7500 system and SYBR Green methods. RT-qPCR was performed three times for 2 minutes at 95°C, followed by 40 cycles (30 seconds at 90°C and 30 seconds at 60°C). The primers were obtained from GenePharma (Shanghai, China). The $2^{-\Delta\Delta Ct}$ method was applied for quantifying the data. GAPDH was used for normalization.

Pathological observation

Brain tissues were sliced and digested for 30 minutes at 37°C. Then, cell suspensions were collected after filtration. Subsequently, neurons were isolated by using magnetic beads (BD). The pathological changes in striatum nerve cells were observed under a transmission electron microscope (TEM; Huaxian Co. Ltd, Shenzhen, China).

Western blot

RIPA buffer was used to isolate total protein. Protein was quantified using a BCA protein kit (Thermo Fisher Scientific). Proteins were then separated with a SDS-PAGE gel (10%), followed by transferring onto PVDF membranes. Membranes were then incubated with primary antibodies after they were blocked with 3% non-fat milk for one hour. Subsequently, membranes were incubated with secondary anti-rabbit antibody (ab288151, 1:5000) for one hour. Finally, membranes were analysed with Image J software. The primary antibodies were as follows: anti-NaV1.2 (ab93616, 1:1000), anti-Nrf2 (ab62352, 1:1000), anti-HO-1 (ab68477, 1:1000) and anti-GAPDH (ab8245, 1:1000). GAPDH was used for quantification. All antibodies were purchased from Abcam (Cambridge, UK).

Cu-Zn SOD activity detection

The serum of rats was collected by centrifugation (200 g, 20 min). Cu-Zn SOD activity in the serum of rats was detected using an ELISA kit (Multisciences [Lianke] Biotech Co., Ltd, Hangzhou, China).

Analysis of cell apoptosis

SH-SY5Y cells were seeded in six-well plates (1×10^6/well). Cells were then trypsinized, washed and stained with 5 µL FITC and propidium (PI; BD, Franklin Lake, NJ, USA) in the dark for 15 minutes. The rate of cell apoptosis was calculated using flow cytometer (BD, Franklin Lake, NJ, USA).

TUNEL staining

SH-SY5Y cells were washed, fixed, permeabilized and then incubated with TUNEL reaction mixtures for 60 minutes at 37°C with no light. Cells were then incubated with POD, rinsed with PBS, and then incubated with 50 µL DAB for 10 minutes. Finally, cells were observed under a microscope. Image J (NIH) was used to quantify the data.

Statistical analysis

Differences between multiple groups were compared by one-way analysis of variance (ANOVA, Bonferroni), and the Student's t-test was used for two samples. The Pearson chi-square test was used to analyse sex differences. All data were statistically evaluated using GraphPad Prism 8.0 software. The experiments were conducted three times, and the data were presented as mean ± SD; $p<0.05$ was considered to be statistically significant.

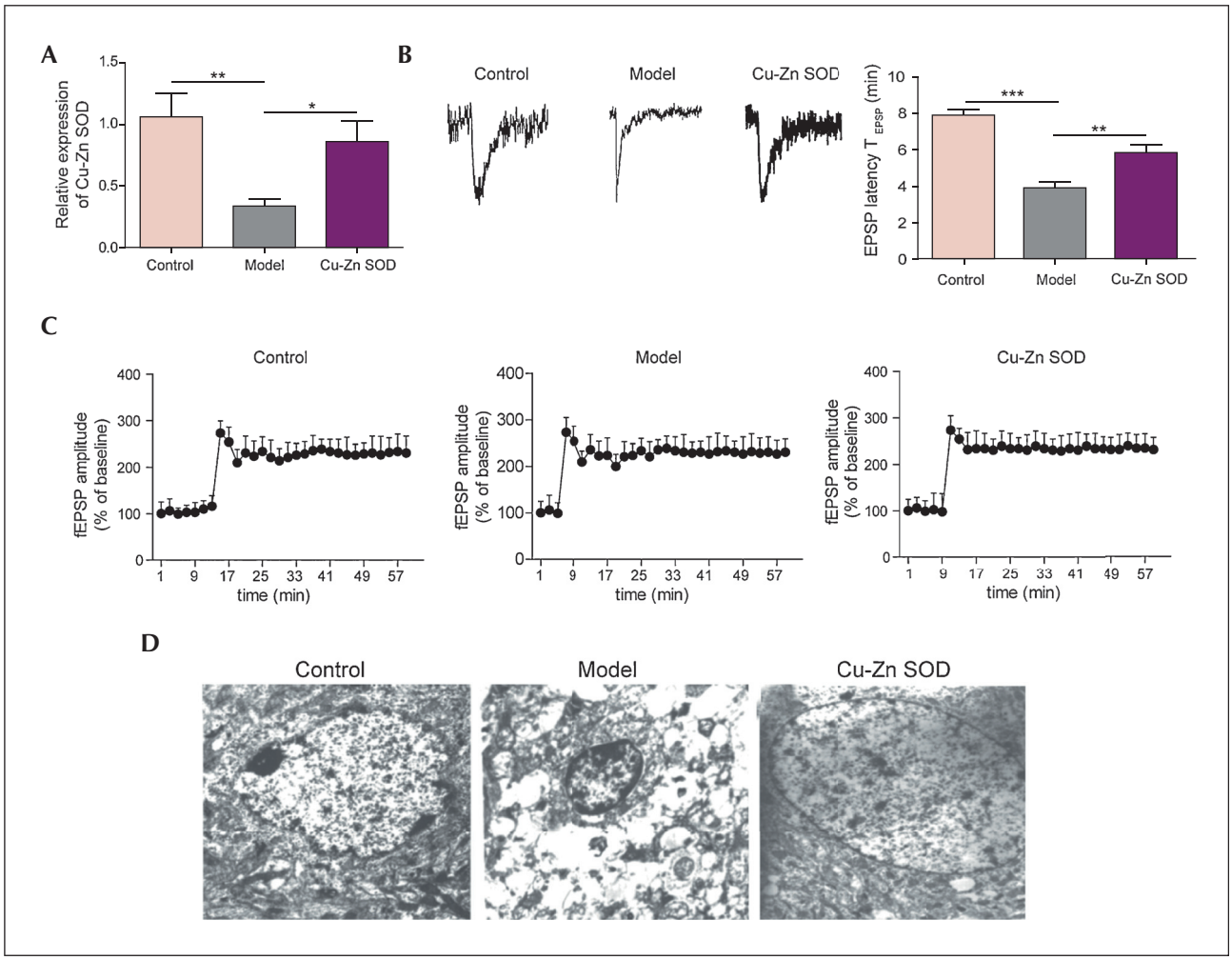
Results

Cu-Zn SOD suppressed induced epilepsy *in vivo*

The rat model of epilepsy was constructed to assess the function of Cu-Zn SOD in epilepsy. As indicated in *figure 1A*, the expression of Cu-Zn SOD was decreased in epileptic rats, while this phenomenon was partially reversed after Cu-Zn SOD over-expression for two days. In addition, the latency of post-synaptic potentials in epileptic rats was notably increased by Cu-Zn SOD (*figure 1B*). Moreover, the initial slope of fEPSP was increased in model rats, which was inhibited after Cu-Zn SOD over-expression (*figure 1C*). Furthermore, increased apoptotic cells, reduced cell volume, condensed nuclei, chromatin distribution along the nuclear membrane, and swollen and fluid-filled mitochondria were observed in epileptic rats, while Cu-Zn SOD notably rescued these changes (*figure 1D*). Altogether, over-expression of Cu-Zn SOD significantly delayed the onset of fEPSP.

Cu-Zn SOD silencing induced SH-SY5Y cell apoptosis

To investigate the function of Cu-Zn SOD in SH-SY5Y cells, cells were transfected with Cu-Zn SOD siRNA. As revealed in *figure 2A*, *B*, the mRNA and protein level of Cu-Zn SOD in SH-SY5Y cells were notably decreased after Cu-Zn SOD silencing. In addition,



■ **Figure 1.** Cu-Zn SOD suppressed epilepsy *in vivo*. (A) Cu-Zn SOD level in tissues of rats was assessed by qRT-PCR. (B) Analysis of the post-synaptic potential in rats. (C) The slope of fEPSP based on post-synaptic potential analysis. (D) Pathological changes in brain tissues of rats observed by TEM. n=8 (the number of rats), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

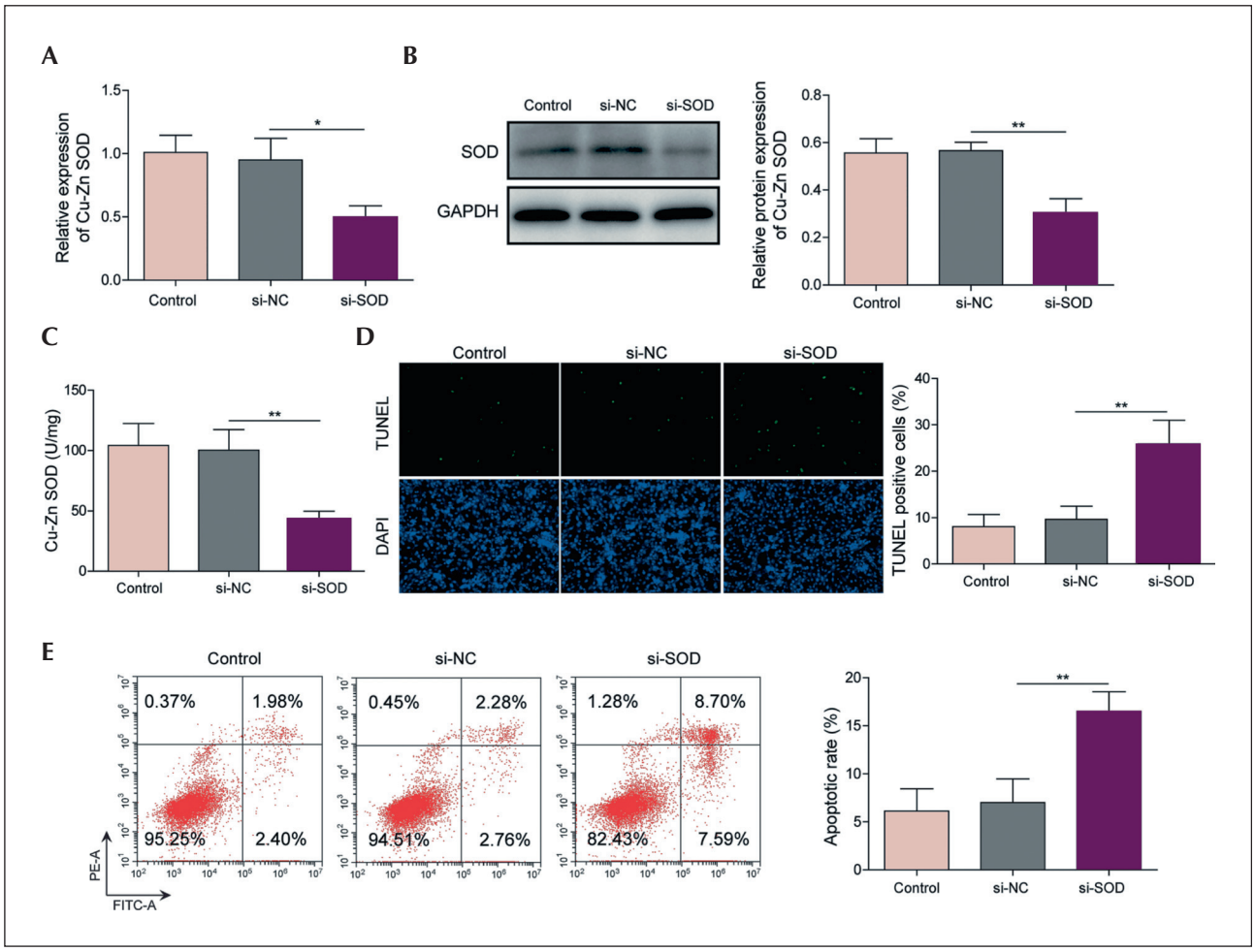
Cu-Zn SOD activity in SH-SY5Y cells was also decreased by Cu-Zn SOD silencing (*figure 2C*). Moreover, knockdown of Cu-Zn SOD notably induced SH-SY5Y cell apoptosis (*figure 2D, E*).

Silencing of Cu-Zn SOD notably inhibited *SCN2A/Nrf2/HO-1* expression

We next sought to detect the impact of Cu-Zn SOD on *SCN2A* expression. The data revealed that knockdown of Cu-Zn SOD notably inhibited the level of *SCN2A* in SH-SY5Y cells (*figure 3A*). Consistently, *HO-1* and *Nrf2* levels in SH-SY5Y cells were also significantly decreased by silencing of Cu-Zn SOD (*figure 3B, C*). The protein levels of NaV1.2, HO-1 and Nrf2 in SH-SY5Y cells were notably decreased after treatment with Cu-Zn SOD siRNA (*figure 3D*). To sum up, silencing of Cu-Zn SOD notably inhibited the expression of *SCN2A/Nrf2/HO-1*.

Over-expression of *SCN2A* significantly protected cells against Cu-Zn SOD siRNA-induced apoptosis

To further confirm the relationship between Cu-Zn SOD and *SCN2A*, qRT-PCR was used to determine the respective levels of gene expression. The data showed that the Cu-Zn SOD siRNA-induced decrease of *SCN2A*, *Nrf2* and *HO-1* mRNA was reversed by *SCN2A* over-expression (*figure 4A-4C*). Consistently, the NaV1.2, HO-1 and Nrf2 protein levels in Cu-Zn SOD



■ **Figure 2.** Knockdown of Cu-Zn SOD significantly induced SH-SY5Y cell apoptosis. (A) SH-SY5Y cells treated with NC or Cu-Zn SOD siRNA for 48 hours; efficiency of cell transfection was investigated by qRT-PCR. (B) Cu-Zn SOD level was assessed by western blot; GAPDH was applied for normalization. (C) Activity of Cu-Zn SOD. (D) SH-SY5Y cell apoptosis tested by TUNEL staining; green fluorescence indicates TUNEL and blue fluorescence indicates DAPI. (E) Flow cytometry to assess cell apoptosis. Three independent experiments were performed in each group, $^{*}p<0.05$, $^{**}p<0.01$.

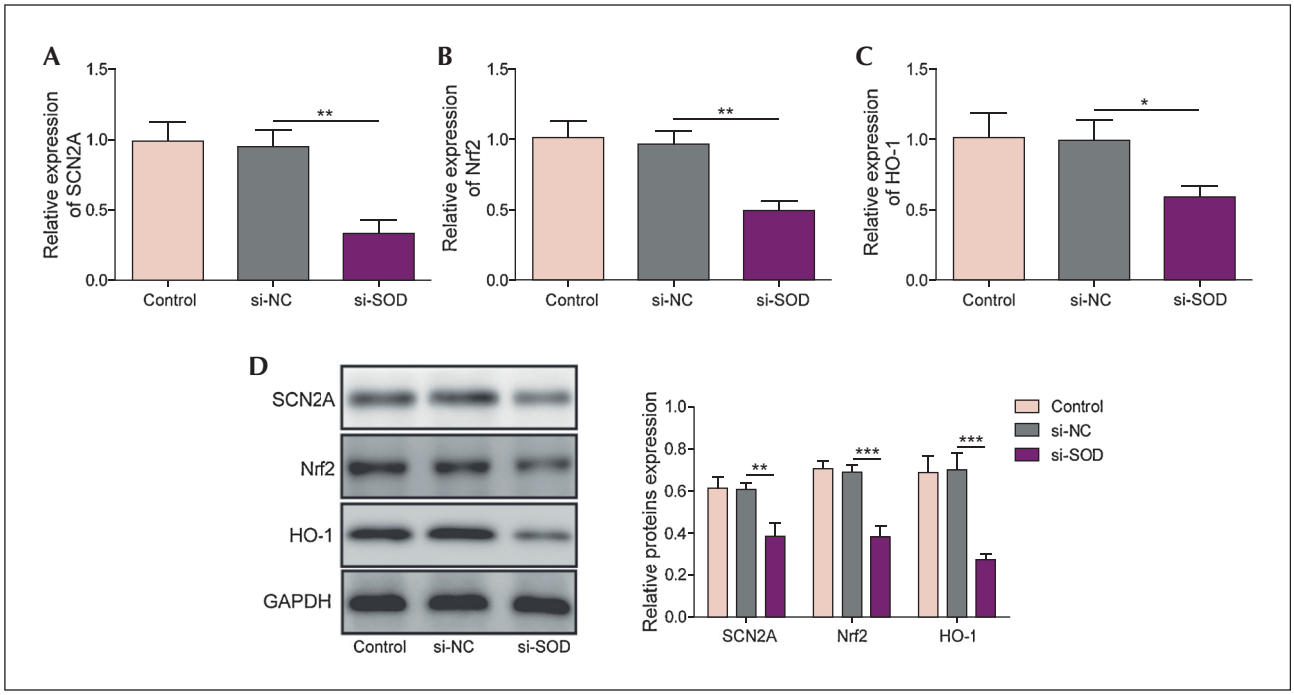
siRNA-treated SH-SY5Y cells were significantly increased by over-expression of *SCN2A* (*figure 4D*). Moreover, the data from flow cytometry and TUNEL staining demonstrated that *SCN2A* over-expression notably inhibited SH-SY5Y cell apoptosis induced by silencing of Cu-Zn SOD (*figure 4E, F*). Taken together, over-expression of *SCN2A* significantly protected cells against Cu-Zn SOD siRNA-induced apoptosis.

Modulation of the NaV1.2/Nrf2/HO-1 axis by Cu-Zn SOD

As shown in *figure 5A*, the activity of Cu-Zn SOD in epileptic rats was decreased but notably increased after Cu-Zn SOD over-expression. Additionally, Cu-Zn SOD level in epileptic rats was increased by over-expression of Cu-Zn SOD (*figure 5B*). However, *SCN2A*, *HO-1* and *Nrf2* levels were suppressed in epileptic rats, but notably rescued by Cu-Zn SOD over-expression (*figure 5C, D*). Taken together, Cu-Zn SOD may mediate the NaV1.2/Nrf2/HO-1 axis.

Discussion

Progression of epilepsy often leads to brain dysfunction, limb convulsions, and behavioural disorders or sensory disturbances, which seriously disturb patients

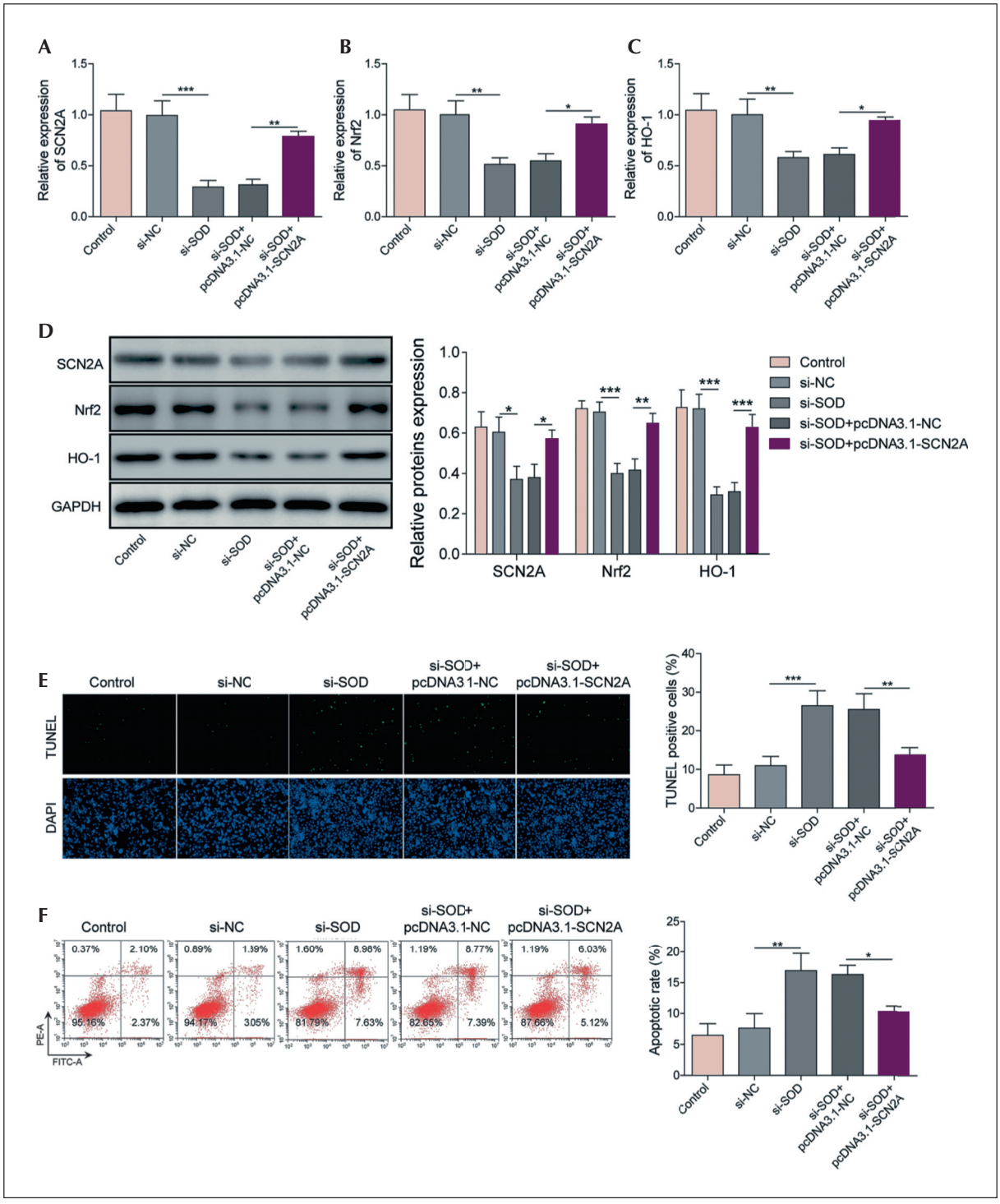


■ **Figure 3.** Silencing of Cu-Zn SOD notably inhibited *SCN2A*/*Nrf2*/*HO-1* expression in SH-SY5Y cells. (A-C) mRNA expression levels of *SCN2A*, *Nrf2* and *HO-1* detected by qRT-PCR. (D) Western blot to assess NaV1.2, HO-1 and Nrf2 protein levels, GAPDH was applied for normalization. Three independent experiments were performed in each group, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

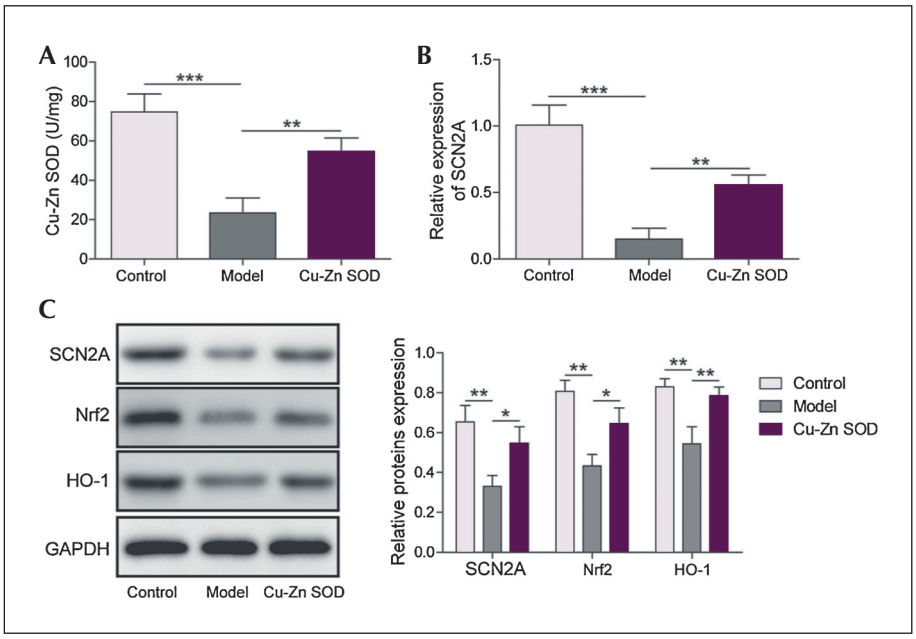
[21, 22]. According to the literature, development of epilepsy is known to result in the increase of oxidative stress, which leads to excessive neurotransmitter excitation, neuronal apoptosis or metabolic structural damage [23, 24]. The present study found that Cu-Zn SOD suppresses epilepsy and alters the NaV1.2/Nrf2/HO-1 axis. Xiang *et al*. reported that regulation of Cu-Zn SOD can modulate *SCN2A* expression in epilepsy [25], supporting our finding that Cu-Zn SOD positively regulated *SCN2A* in the epilepsy model. A recent study indicated increased Cu-Zn SOD activity as an oxidative stress marker during rehabilitation following total hip or knee replacement [26]. Thus, the functions of Cu-Zn SOD in biological processes need to be explored in the future.

NaV1.2 plays a key role in epilepsy progression [27, 28]. For instance, Zhang *et al*. revealed that upregulation of NaV1.2 leads to inhibition of epilepsy development [29]. Our data are consistent and indicate that Cu-Zn SOD knockdown positively regulates NaV1.2 expression in the epilepsy model. During child development, 70% sodium current in the CNS is due to NaV1.2 sodium channels, encoded by *SCN2A* [30]. Moreover, previous studies have reported that *SCN2A* and Cu/Zn SOD have a potential link in cortical tissue of TLE patients [25]. In human neuroblastoma cells, NaV1.2 inactivation causes inhibition of Cu/Zn SOD, leading to changes in cell membrane structure permeability [29, 31]. Moreover, Cu/Zn SOD over-expression also enhances *SCN2A* gene expression following *SCN2A* knockdown in SH-SY5Y cells [25].

This study also shows that Cu/Zn SOD suppresses Nrf2/HO-1 expression based on our epilepsy model. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important regulator of oxidative stress [32]. Under oxidative stress, it regulates HO-1 and activates a series of enzymes that enhance the antioxidant capacity of cells, thereby improving oxidative stress and inhibiting inflammation [33, 34]. It has been demonstrated that inactivation of Nrf2/HO-1 signalling could lead to the inhibition of epilepsy [18]. In fact, decrease in Cu-Zn SOD has previously been documented to lead to apoptosis via the nitric oxide pathway [35]. Thus, Cu-Zn SOD may regulate the NaV1.2/Nrf2/HO-1 signalling pathway by inducing apoptosis. In addition to NaV1.2 and Nrf2/HO-1 signalling, PIK3/Akt signalling plays an important role in epilepsy [36], and since PI3K signalling is confirmed to closely associate with the Nrf2/HO-1 axis [18], the effect of Cu-Zn SOD on PI3K/Akt signalling should be explored in the future.



■ **Figure 4.** *SCN2A* significantly protected SH-SY5Y cells against Cu-Zn SOD siRNA-induced apoptosis. (A-C) SH-SY5Y cells were treated with si-SOD, siSOD+pcDNA3.1 or siSOD+pcDNA3.1-*SCN2A*, and mRNA expression levels of *SCN2A*, *Nrf2* and *HO-1* were then examined by qRT-PCR. (D) Protein expression of NaV1.2, Nrf2 and HO-1 by western blot; GAPDH was applied for normalization. (E) SH-SY5Y cell apoptosis examined by TUNEL staining. (F) Flow cytometry to examine cell apoptosis. Three independent experiments were performed in each group, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.



■ **Figure 5.** Modulation of the NaV1.2/Nrf2/HO-1 axis by Cu-Zn SOD in rats. (A) Cu-Zn SOD activity. (B) Cu-Zn SOD level in the tissues of rats assessed by qRT-PCR. (C) NaV1.2, HO-1 and Nrf2 protein level in the tissues of rats, assessed by western blot; GAPDH was applied for normalization. Eight rats were used for experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

In conclusion, Cu-Zn SOD was shown to suppress cell apoptosis, alter the level of proteins of the NaV1.2/Nrf2/HO-1 signalling pathway, and suppress epilepsy in pilocarpine hydrochloride-induced rats. Therefore, Cu-Zn SOD might be a target for epilepsy treatment. ■

Key points

- Cu-Zn SOD over-expression suppressed epilepsy in pilocarpine hydrochloride-induced rats.
- Knockdown of Cu-Zn SOD significantly induced apoptosis and altered *SCN2A*/*Nrf2*/*HO-1* expression in SH-SY5Y cells.
- Over-expression of Cu-Zn SOD modulated the NaV1.2/Nrf2/HO-1 axis in pilocarpine hydrochloride-induced rats.

Supplementary material.
Summary slides accompanying the manuscript are available at www.epilepticdisorders.com.

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Ethics statement.
This animal research was approved by the Medical Ethics Committee of The Second Xiangya Hospital of Central South University.

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TEST YOURSELF

(1) Which of the following genes is decreased in the epilepsy rat model?
A. *Cu-Zn SOD*
B. *SCN2A*
C. *Nrf2*
D. *HO-1*
E. *GAPDH*

(2) Which of the following genes is inhibited after Cu-Zn SOD knockdown in SH-SY5Y cells?
A. *SCN2A*
B. *Nrf2*
C. *HO-1*
D. *GAPDH*

(3) Which of the following genes is affected after *SCN2A* over-expression in SH-SY5Y cells?
A. *Cu-Zn SOD*
B. *SCN2A*
C. *Nrf2*
D. *HO-1*
E. *GAPDH*

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com.