Ferrostatin-1 obviates seizures and associated cognitive deficits in ferric chloride-induced posttraumatic epilepsy via suppressing ferroptosis

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Abstract

Background and Purpose: Posttraumatic epilepsy (PTE) is a prevalent complication of brain trauma. Current anti-epileptic drugs available do not have satisfactory response to PTE. It is of desperate need to explore novel therapeutic approaches for curing PTE. Our prior work revealed that ferroptosis, a recently discovered mode of cell death, occurs in rodent model of PTE. In the present study, we aimed to further investigate the effect of ferrostatin-1 (Fer-1), a specific ferroptosis inhibitor, on seizure behavior and cognitive deficit in a mouse model of PTE. Experimental approach: The preparation of PTE was performed by stereotaxical injection in the somatosensory cortex region of 50 mM FeCl3. Seizure activity was assessed via Racine scoring and electroencephalogram analysis. PTE-related cognitive function was evaluated by novel object recognition and Morris water maze tests. Ferroptosis-related indices including glutathione peroxidase (GPx) activity and protein expressions of 4-hydroxynonenal (4-HNE) were detected using a commercial kit and immunofluorescence, respectively. Key Results: It was found that treatment with Fer-1 significantly exerted protective effects against acute seizure and memory decline, although no evident effect on epileptic progression. Fer-1 also exhibited good tolerability and safety as we observed that it hardly influenced the body weight. Furthermore, it was noted that administration of Fer-1 suppressed ferroptosis-related indices including GPx activity and protein expressions of 4-HNE in hippocampus. Conclusion and Implications: These data altogether indicate that Fer-1 has potent therapeutic effects against seizures and cognitive impairment following PTE-induced brain insult. Fer-1 may act as a promising drug for curing PTE patients.

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Running Title: Therapeutic effect of ferrostatin-1 against PTE

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Abstract

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Experimental approach: The preparation of PTE was performed by stereotaxical injection in the somatosensory cortex region of $FeCl_3$. Seizure activity was assessed via Racine scoring and electroencephalogram analysis. PTE-related cognitive function was evaluated by novel object recognition and Morris water maze tests. Ferroptosis-related indices including glutathione peroxidase (GPx) activity and protein expressions of 4-hydroxynonenal (4-HNE) were detected using a commercial kit and immunofluorescence, respectively.

Key Results: It was found that treatment with Fer-1 significantly exerted protective effects against acute seizure and memory decline, although no evident effect on epileptic progression. Fer-1 also exhibited good tolerability and safety as we observed that it hardly influenced the body weight. Furthermore, it was noted that administration of Fer-1 suppressed ferroptosis-related indices including GPx activity and protein expressions of 4-HNE in hippocampus.

Conclusion and Implications: These data altogether indicate that Fer-1 has potent therapeutic effects against seizures and cognitive impairment following PTE-induced brain insult. Fer-1 may act as a promising drug for curing PTE patients.

Keywords: posttraumatic epilepsy, seizure, cognitive impairment, ferroptosis, ferrostatin-1, therapy

Abbreviations

AP, anteroposterior; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; EEG, electroencephalogram; $FeCl_3$, ferric chloride; Fer-1, ferrostatin-1; F, the time exploring familiar object; GSH, glutathione; GPx, glutathione peroxidase; 4-HNE, 4-hydroxynonenal; KA, kainic acid; MDA, malonaldehyde; ML, mediolateral; MWM, Morris water maze; NOR, novel object recognition; N, the time exploring novel object; PTE, posttraumatic epilepsy; PBS, phosphate buffer saline; PTZ, pentylenetetrazole; ROS, reactive oxygen species; SRS, spontaneous recurrent seizure; TBI, traumatic brain injury; V, ventral.

1. Introduction

Posttraumatic epilepsy (PTE) is considered as a recurrent seizure disorder and has been described as one of the most serious complications associated with traumatic brain injury (TBI) (Pitkänen et al., 2006), with an estimated incidence ranging from 2% to more than 50% (Keith et al., 2019). It is well recognized that the incidence of PTE positively correlates with the severity of TBI (Annegers et al., 1998). Generally, PTE not only has detrimental effects on physical activity, cognitive performance and emotionality, but also increases the risk of mortality in patients with TBI (Xu et al., 2017). The direct evidence arises from the results showing that patients with PTE exhibit nearly 2.5 fold higher risk of mortality than those without PTE in

a population-based cohort study (Lin et al., 2019). Despite progress in the research of this pathologic state, mechanistic bases are not well characterized and treatments are lacking.

Iron-induced epilepsy is a well-known model that resembles human PTE, which is firstly proposed by Willmore and his colleagues (Willmore et al., 1978a). In principle, injection of iron salts such as ferrous or ferric chloride ($FeCl_3$) into the somatosensory cortex simulates the release of iron from breakdown of hemoglobin in the red blood cells after brain injury (Willmore et al., 1978b). Nowadays, this model is widely applicable for the investigation of post-traumatic epileptogenesis and therapeutic intervention. There is no doubt that repetitive seizures in PTE can activate cell death signaling (Dingledine et al., 2014). Ferroptosis is a recently discovered type of cell death by Stockwell lab, which is characterized with iron dependence and cumulative lethal lipid peroxides (Dixon et al., 2012). This cell death mode is distinct from other types of cell death paradigms such as apoptosis, necroptosis, autophagy and so on with the aspect of morphology, genetics and metabolic traits. Remarkably, ferroptotic cells often exhibit lipid reactive oxygen species (ROS) overproduction (Dixon et al., 2012). In our previous investigations, we revealed for the first time that ferroptosis occurred in $FeCl_3$-induced mouse model of PTE (Li et al., 2019). Therefore, we currently comprehensively evaluated the therapeutic potential of ferrostatin-1 (Fer-1), a potent and selective ferroptosis inhibitor (Dixon et al., 2012), against seizure and associated cognitive deficits during PTE.

Our present work illustrated that Fer-1 alleviated seizures and cognitive impairment in a mouse model of $FeCl_3$-induced PTE. We also disclosed good tolerability for Fer-1 because no toxic effects on body weight were observed in our present study. Besides, ferroptosis process reflected by the inhibition of the indices associated with lipid peroxides including 4-hydroxynonenal (4-HNE) expression and glutathione peroxidase (GPx) activity were also found to be suppressed in this model after Fer-1 treatment. We believe that Fer-1 may serve as a promising drug in counteracting PTE in the future.

2. Materials and methods

2.1. Chemicals and reagents

$FeCl_3$ was purchased from Sigma-Aldrich (157740, St. Louis, MO, USA). Fer-1 was obtained from Selleck Chemicals (S7243, Houston, TX, USA). The commercial kit for the determination of GPx (S0056) was purchased from Beyotime Biotechnology, China.

2.2. Preparation of PTE mouse model and drug treatment

Male C57BL/6J mice (6-8 weeks of age) weighing 18-22 g were obtained from the Experimental Animal Center of Central South University. The mice were housed in a specific pathogen-free environment and provided with free food and water. All of the animal procedures followed the National Institutes of Health guidelines and the experimental procedures were approved by the Animal Care and Use Committee of Xiangya Hospital of Central South University. PTE mouse model was prepared according to our previous descriptions (Li et al., 2019). In brief, the mice were anesthetized with intraperitoneal injection of 10% (v/w) chloral hydrate and carefully placed in a stereotaxic apparatus. 5 μl of a 50 mM solution of $FeCl_3$ dissolved in phosphate buffer saline (PBS) was stereotaxically injected into the somatosensory cortex (coordinates AP-1.3 mm; ML-2.0 mm; V-1.6 mm) within 5 min using an injector cannula (62004, RWD Life Science) while the control group received an equal volume of PBS. Thereafter, we allocated the mice into four different groups as follows: Control group (injection with equal volume of PBS) (n=6); $FeCl_3$ group (injection with 50 mM $FeCl_3$) (n=6); $FeCl_3$+Fer-1 group (injection with 50 mM $FeCl_3$ and 1 pmol Fer-1) (n=6) and Fer-1 group (injection with 1 pmol Fer-1) (n=6).

2.3. Racine score

The behavioral score was evaluated for 90 min according to Racing scale (Racine et al., 1972) after $FeCl_3$ injection. The standards of seizure scoring were shown as we previously described (Li et al., 2019): Stage 0, no response; stage 1, facial and whisker rhythmic twitching; stage 2, head bobbing and circling; stage 3, myoclonic and spasm in multiple limbs; stage 4, uncontrolled rearing and falling; stage 5, general tonic-clonic seizures with running and jumping and stage 6, death. Mice with stage 3 or higher were deemed to

be successfully induced. Throughout the seizure score assessment, two participants were enrolled who were blind to the experimental design.

2.4. Electroencephalogram (EEG) recording

In order to collect EEG data in the acute and chronic stages of PTE (Pitkänen et al., 2014), mice underwent electrode implantation prior to seizure induction by KA. A bipolar electrode was implanted into CA3 region (AP-2.9 mm; ML-3.0 mm; V-3.0 mm) for EEG recording. After seven days' recovery, the baseline EEG trace was recorded 30 min before $FeCl_3$ injection. Subsequently, an injection tube (62204, RWD Life Science) was inserted into the guide cannula which was placed in the somatosensory cortex (AP-1.3 mm; ML-2.0 mm; V-1.6 mm) for injection with 5 μl of a 50 mM solution of $FeCl_3$ or 1 pmol Fer-1. Five minutes later, mice were subjected to 2 h EEG monitoring session. EEG data in the acute and chronic stages of PTE were both collected for 3 d with 2 h per day. Electrical seizures were defined as regular spike clusters lasting more than 10 s, spike frequency greater than 3 Hz and amplitude at least three times higher than the baseline according to the previous investigation (Zhao et al., 2020).

2.5. Nissl staining

The mice from each group were deeply anesthetized by intraperitoneal injection of 10% chloral hydrate and then subjected to transcardial perfusion with 0.9% NaCl, followed by fixation with 4% paraformaldehyde in 0.1 M phosphate buffer. Brain slices (5 μm) were deparaffinized and immersed in cresyl violet (C0117, Beyotime Biotechnology) for 10 min at room temperature. Brain sections were then dehydrated in graded alcohol, mounted with neutral balsam and observed under a light microscope. The hippocampal subregions including CA1, CA3 and DG were selected and viable neurons were calculated, respectively, from different groups.

2.6. Measurements of GPx activity

Hippocampus tissue samples were collected and the activity of GPx, which is responsible for the detoxification of hydroperoxides, was measured at the wavelength of 340 nm using a commercial assay kit (S0056, Beyotime Biotechnology, China)

2.7. Immunofluorescence

After deparaffinating, brain sections were processed for antigen retrieval by heat mediation in a citrate buffer for 20 min. For immunostaining with 4-HNE, slices were blocked with 10% donkey serum for 30 min followed by incubation with the primary antibody against 4-HNE overnight (ab46545, 1:50 dilution, Abcam, UK). Following three washes with PBS, samples were probed with Alexafluor 488 secondary antibody (A21206, 1:200, Thermo Fisher Scientific, United States) for 1 h. Negative control was performed by omitting the primary antibody. Fluorescent signals were captured using AXIOPHOT Zeiss microscope. Data acquisition was carried out by Leica Application Suite 4.9.0 program.

2.8. Novel Object Recognition (NOR) test

On the twenty fourth days after $FeCl_3$ injection, NOR test was performed to assess the effects of Fer-1 on the short-term recognition memory of mice according to the previous investigation (Lueptow, 2017). Briefly, in the habituation phase, mice were placed in a polyvinyl chloride chamber (45 cm × 45 cm × 45 cm) for familiarization. On the next day, mice were allowed to freely explore the arena with two identical objects in the center for 10 min. Twenty-four hours following the training trial, animals were placed in the chamber in which one of the objects was replaced with a novel object with different color and shape. Mice were allowed for 10 min exploratory trial. The exploration time, which meant that the time when the mice explored the familiar or novel object, was recorded in the NOR test. The apparatus and objects were cleaned thoroughly with 75% ethanol solution prior to each trial. The behavioral traits of each mouse were recorded by a video tracking system. The time exploring novel object (N) and exploring familiar object (F) were recorded carefully. The discrimination index was analyzed using the following formula: (N-F)/(N+F) ×100%.

2.9. Morris Water Maze (MWM) test

On the twenty ninth day after $FeCl_3$ injection, MWM test was carried out to evaluate the effects of Fer-1 on the spatial memory performance as previously described (Mao et al., 2014). For short, the water maze pool (120 cm in diameter) was filled with water with the temperature at 24 ± 2°C. During training trials, mice were subjected to find the hidden platform (10 cm in diameter), which was located in the center of one quadrant and hidden 1 cm under the water surface for 5 consecutive days. The mice from each group experienced three consecutive daily training trials for 5 days, with an inter-trial interval more than 1 h. In each trial, the escape latency (s) and the mean path length (cm) to find the platform were analyzed. Swimming speed was measured by dividing the path length by the time to find the platform. For probe trials, the platform was removed 24 h after the last training trial. Passing times, which referred to the number of times the mice crossed the once hidden platform, were measured within 60 s.

2.10. Statistical analysis

All experiments were carried out at least three independent replicates. The analyses and statistical significance were performed in a blinded manner and calculated using GraphPad Prism 8.0 software. The normal distribution of the data was evaluated with Shapiro-Wilk test. One-way ANOVA or RM-two-way ANOVA followed by Tukey's tests, Sidak's tests or unpaired t tests was used for multiple comparisons to normally distributed data. Non-normal data were analyzed by Kruskal-Wallis test or Mann Whitney U test. The details of statistical test for each experiment were summarized in Table 1. All results were presented as the mean ± SEM. P values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Fer-1 exhibits an anticonvulsant effect in the acute stage of PTE

In order to evaluate the therapeutic effect of Fer-1 on PTE, we firstly investigated whether Fer-1 exerted anticonvulsant effects in a mouse model of $FeCl_3$-induced epilepsy. The injected site for Fer-1 was the somatosensory cortex as marked in Figure 2A. It was found that stereotactic injection of Fer-1 at the dose of 1 pmol significantly decreased the seizure score in this mouse model compared with vehicle-treated group, notably at the interval time of 70-90 min (Figure 2B). Furthermore, EEG power analysis also revealed that the power per day and total power were both evidently diminished in mice subjected to PTE when treatment with Fer-1 (Figure 2C, Figure 2D and Figure 2F), despite no significant difference was found in the aspect of baseline power among all groups before $FeCl_3$ or Fer-1 injection (Figure 2E). Epileptiform activity usually includes spikes (Aarts et al., 1984). The results of Figure 2G showed that spikes were remarkably suppressed in PTE on the day 1, day 2 and day 3 after administration of Fer-1, indicating the suppression of epileptiform discharge in PTE by Fer-1. Besides, in an acute stage of a mouse model of $FeCl_3$ injection, treatment with Fer-1 obviously reduced seizures per day (Figure 2H) and the number of seizures (Figure 2J), increased seizure latency (Figure 2I) and reduced time in seizure (Figure 2K). Taken together, these date suggest that Fer-1 exerts potent anticonvulsant effects in an acute model of PTE.

3.2. Fer-1 hardly affects epileptic progression in PTE mice model

Next, we wondered whether Fer-1 blocked epileptogenic progression in PTE mice model. On the day 19 after $FeCl_3$ injection, we monitored EEG for three consecutive days. The recurrent epileptic seizures were observed in mice with PTE (Figure 3A). However, treatment with Fer-1 was found to hardly influence EEG power per day (Figure 3B), total power (Figure 3C) and spikes (Figure 3D). In addition, seizures per day for each mouse in the chronic stage of PTE and cumulative spontaneous recurrent seizure (SRS) duration were also not different between Vehicle- and Fer-1-treated PTE mice (Figure 3E and Figure 3F). Collectively, these findings suggest that Fer-1 has little effect on the epileptogenesis.

3.3. Fer-1 improves PTE-associated cognitive deficits in mice

Notably, previous reports have shown that over 20% of PTE patients experience cognitive impairments (Mazzini et al., 2003). Therefore, we attempted to explore the effects of Fer-1 on the learning and memory performance in $FeCl_3$-induced PTE in vivo. In this study, NOR and MWM tests are selected as they are widely applied for evaluation of cognitive function (Lueptow, 2017; Mao et al., 2014). The timeline of the

NOR test was indicated in Figure 4A. It was demonstrated that control or Fer-1 injection groups exhibited the normal ability to discriminate the new object as the exploration time, which meant the time when the mice explored the familiar or novel object, was greatly different in Con or Fer-1 group (Figure 4B). However, it was difficult for the mice with PTE to discriminate the new object since the exploration time was nearly the same when exploring the familiar and novel objects. Treatment with Fer-1 spent more time to explore the novel object (Figure 4B and Figure 4C), indicating the improvement of cognitive function in PTE mice after Fer-1 treatment. In MWM test, we confirmed there was no difference in the swimming speed of all mice (Figure 4D). Representative swimming traces concerning five days of training trials among different groups was shown in Figure 4E. Fer-1 treatment in PTE mice decreased the tendency in the aspects of escape latency (Figure 4F) and mean path length (Figure 4G). Additionally, the results of probe trial illustrated that mice with PTE exhibited a decrease of passing times (Figure 4H and Figure 4I). However, treatment with Fer-1 significantly increased the index. Collectively, these data suggest that Fer-1 ameliorates cognitive deficits in PTE in vivo.

3.4. Fer-1 does not display evident side effects on mice

The results mentioned above reveal that Fer-1 may serve as a candidate for the treatment of PTE. We further monitored the body weight of the mice every week to evaluate its safety. As shown in Figure 5A and Figure 5B, treatment with Fer-1 caused no significant difference in body weight compared with untreated mice. Thus, Fer-1 dose not display evident side effects on mice.

3.5. Fer-1 inhibits ferroptosis process in an acute stage of PTE

Fer-1 is a well-known ferroptosis inhibitor, therefore, in the present work, we also explored ferroptotic events are involved in $FeCl_3$-induced PTE. The indices reflecting lipid peroxidation which include 4-HNE level and GPx activity are analyzed. It was noteworthy that $FeCl_3$ injection in the mice resulted in a remarkable increase of 4-HNE distribution in the hippocampus, especially in the CA3 subregion and this phenomenon was significantly reversed in $FeCl_3$-induced PTE after Fer-1 treatment (Figure 6A). The mean intensity of CA3 region was measured and statistical analysis showed that $FeCl_3$ operated a remarkable increase in 4-HNE level in the hippocampus of mice in the CA3 region, and administration of Fer-1 significantly reduced it in the acute stage but not the chronic stage of $FeCl_3$-induced PTE (Figure 6B and Figure 6C). With the aspect of GPx, it was intriguing that enhanced GPx activity was found in the acute stage of PTE while administration of Fer-1 blocked its enzymatic activity (Figure 6D). Besides, although there was an obvious elevation of GPx activity in the chronic stage of PTE, Fer-1 treatment hardly affected $FeCl_3$-induced increase of GPx activity (Figure 6E). Altogether, these results indicate that Fer-1 suppresses ferroptosis process, especially in the acute stage of PTE.

3.6. Fer-1 attenuates seizure-induced hippocampal damage in $FeCl_3$-induced PTE

Activation of ferroptosis process always triggers cell death (Ueda et al., 2003). Thus, in our current work, Nissl staining was performed to evaluate the effect of Fer-1 on neuron death following PTE. It was evident that PTE in the acute stage (day 3 after $FeCl_3$injection) induced enormous neuron death, especially in hippocampal DG region and treatment with Fer-1 obviously improved cell viability (Figure 7A, Figure 7B, Figure 7C and Figure 7D). Interestingly, in the chronic stage of PTE (day 34 after $FeCl_3$ injection), Nissl staining results demonstrated that Fer-1 prevented neuron death in hippocampal CA1 subregion of mice subjected to PTE. There was no significant difference in the number of surviving neurons between each group in CA3 and DG regions (Figure 7E, Figure 7F, Figure 7G and Figure 7H). Taken together, these findings implicate that Fer-1 ameliorates hippocampal neuron death in $FeCl_3$-induced PTE.

4. Discussion

The major findings of our present work demonstrate that Fer-1 could alleviate seizures in the acute stage and improve cognitive deficit in a mouse model of PTE with good safety and tolerability. Suppression of ferroptosis process may involve in the therapeutic effect of Fer-1 against PTE.

PTE is a very common acquired epilepsy, which is usually caused by TBI. Injection of $FeCl_3$ in different

brain regions such as somatosensory cortex (Willmore et al., 1978b) and amygdalar nuclear complex (Ueda et al., 2003) of rodents is useful to study the behavior and neuropathological characteristics of PTE. Notably, as early as 1978, Willmore et al. for the first time reported that unilateral injection of $FeCl_3$ into the somatosensory cortex triggered the onset of seizures and epileptogenesis (Willmore et al., 1978b). Generally, epileptic discharges from the sensorimotor cortex are induced 15 minutes after $FeCl_3$ injection and can last for more than 10 months (Moriwaki et al., 1992). Posttraumatic seizures are classified into three categories depending on the time delay from the TBI to the occurrence of the first seizure as follows: (1) immediate (<24 h); (2) early (1-7 d) and (3) late seizures (>7 d after TBI) (Zimmermann et al., 2017). Until now, the mechanism underlying the seizure generation is not well characterized. Our previous investigation has for the first time illustrated that ferroptosis, a novel type of cell death which is distinct from apoptosis, necroptosis, autophagy and so on, occurs in $FeCl_3$-induced PTE (Li et al., 2019). In the present work, we revealed that Fer-1, a potent and selective inhibitor of ferroptosis (Dixon et al., 2012), ameliorated early seizures as it was observed that epileptiform discharge was significantly suppressed from day 1 to day 3 after $FeCl_3$injection followed by Fer-1 treatment (Figure 2G). Similarly, our prior work also illustrated the protective effects of Fer-1 against seizure in various rodent models of epilepsy induced by pilocarpine or pentylenetetrazole (PTZ) (Mao et al., 2019). In vivo seizure model induced by kainic acid (KA), there are controversial results which show that Fer-1 counteracts KA-induced seizures in our previous study (Jia et al., 2020) while spontaneous seizures are not attenuated by Ye et al (Ye et al., 2019). It indicates that Fer-1 has different effects on distinct seizure models induced by different chemical reagents. In addition, it was also noted in our present work that treatment with Fer-1 did not evidently affect late seizures, indicating that Fer-1 hardly affects epileptogenesis. This is due to the notion that patients with chronic epilepsy usually exhibit neuropathological traits such as astrogliosis, mossy fiber and hippocampal sclerosis (Boison, 2008; Deshpande et al., 2020; Thom et al., 2011). On this occasion, cell death may not be dominant at this chronic stage.

Cognitive deficits are always very prevalent in patients with PTE (Jensen, 2009). The hippocampus is usually more vulnerable than other brain regions to memory processes (Cho et al., 2015; Fortin et al., 2002; Voss et al., 2017). In the present study, hippocampus-dependent learning and memory function was evaluated using two techniques, namely, NOR and MWM. Our findings demonstrated that Fer-1 ameliorated cognitive dysfunction in a mouse model of PTE. However, no significant difference was found in the aspect of spontaneous seizure on the chronic stage after Fer-1 treatment. It indicates the improvement of cognitive deficits by Fer-1 in PTE is not attributable to affecting spontaneous seizure. In line with our results, previous study in vivo KA-induced temporal lobe epilepsy, treatment with Fer-1 was also reported to attenuate cognitive dysfunction without suppressing SRS (Ye et al., 2019). Given that no evident toxic effect of Fer-1 on the normal mice, it indicates that Fer-1 may serve as a good candidate for improving cognitive deficits in PTE.

Ferroptosis is a novel type of regulatory cell death featured by lethal lipid peroxidation (Dixon et al., 2012). In our present work, toxic lipid hydroperoxide by-product 4-HNE was analyzed. It was demonstrated that elevation of 4-HNE level was detected in PTE. However, treatment with Fer-1 significantly decreased this index, indicating that inhibition of ferroptosis process in PTE when treatment with Fer-1. It was surprising that the activity of GPx, key enzymes converting toxic lipid hydroperoxides into non-toxic lipid alcohols, was increased in PTE while Fer-1 diminished GPx activity. It is possible that following PTE, enhanced GPx activity in order to counteract overproduction of lethal lipid hydroperoxides. In fact, prior work also illustrated the increase of GPx in TBI (Alim et al., 2019). Further investigation is indispensable to clarify this speculation.

In summary, we report that Fer-1 ameliorates seizure behavior in the acute stage of PTE and abrogates cognitive impairment in the chronic stage of PTE. Together with the results that Fer-1 exhibits no significant side effects in our present work, it indicates that Fer-1 may serve as a promising drug for curing patients with PTE. Besides, ferroptotic process may be associated with the protective effect of Fer-1 against seizure-induced brain damage. It warrants further investigation to explore the molecular mechanism underlying therapeutic potential of Fer-1 against PTE.

Author contributions

Xiao-Yuan Mao designed the manuscript; Kang-Ni Chen and Xi Xi-Yin wrote the manuscript; Qi-Wen Guan analyzed the data; Xiao-Yuan Mao, Zhao-Jun Wang and Hong-Hao Zhou revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no potential conflict of interest.

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Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions

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Figure legends

Figure 1. Experimental design. Briefly, mice were subjected to surgery and recovered for 7 days. Thereafter, $FeCl_3$ was stereotaxically injected into the somatosensory cortex, followed by Fer-1 or vehicle. After the mice from different groups were waken up, seizure scoring was carried out for 90 min according to Racing scale. Then Electroencephalogram (EEG) recording from day 1 to day 3 after $FeCl_3$ injection was recorded, which was considered as the acute stage of PTE. In the chronic stage of PTE (from day 19 to day 21 after $FeCl_3$ injection), EEG recording was also captured and meanwhile, the cognitive performance was evaluated via novel object recognition (NOR) test (from day 24 to day 26 after $FeCl_3$ injection) and Morris Water Maze (MWM) test (from day 29 to day 34 after $FeCl_3$ injection). Additionally, on the day 3 and day 34 after $FeCl_3$ injection, brain tissue samples were both harvested for biochemical detection or pathological analysis. isc, injection in the somatosensory cortex.

Figure 2. Fer-1 has an anticonvulsant effect against $FeCl_3$-induced PTE. (A) Illustration of the injected site for Fer-1 and the location of electrode implantation for EEG recording; (B) Mean seizure scores after $FeCl_3$ injection at the interval time of 90 min; (C) Representative EEG recordings and power spectrum density in the acute stage of PTE; (D) Quantification of EEG power spectra from day 1 to day 3 after $FeCl_3$injection in different groups. The baseline EEG trace was monitored 30 min before $FeCl_3$ injection; (E-F) Cumulative analysis of baseline power and total power; (G) Effects of Fer-1 on the epileptiform spike frequency (marked with spikes per 10 min) from day 1 to day 3 after $FeCl_3$ injection in different groups; (H) Indication of heat map of seizure activity over time (per day) per mouse treated with either vehicle or Fer-1 in the acute stage of PTE; (I-K) Effects of Fer-1 on the seizure onset, number of seizures and time in seizure in $FeCl_3$-induced PTE. Data were shown as mean ± SEM (n=6), $^{*}p<0.05$ versus control group, $^{**}p<0.01$ versus control group, $^{***}p<0.001$ versus control,$^{\#}p<0.05$ versus $FeCl_3$+Vehicle group,$^{\#\#}p<0.01$ versus $FeCl_3$+Vehicle group and$^{\#\#\#}p<0.001$ versus $FeCl_3$+Vehicle group.

Figure 3. Fer-1 hardly affects epileptic progression in $FeCl_3$-induced PTE.

(A) Representative EEG recordings and power spectrum density in the chronic stage of PTE; (B) Quantification of EEG power spectra from day 19 to day 21 after $FeCl_3$ injection in different groups; (C) Cumulative analysis of the effects of Fer-1 on the total power; (D) Effects of Fer-1 on the epileptiform spike frequency (marked with spikes per 10 min) from day 19 to day 21 after $FeCl_3$ injection in different groups; (E) Indication of heat map of seizure activity over time (per day) per mouse treated with either vehicle or Fer-1 in the chronic stage of PTE; (F) Effects of Fer-1 on the cumulative spontaneous recurrent seizure (SRS) duration from day 19 to day 21 after $FeCl_3$ injection. Data were expressed as mean ± SEM. $^{*}p<0.05$ versus control group.

Figure 4. Fer-1 improves PTE-associated cognitive deficits in mice.

(A) Experiment procedure of NOR test; (B) Statistical analysis of the exploration time, which means that the time when the mice explored the familiar or novel object, is recorded in the NOR test. N represents the novel object, and F represents the familiar object; (C) Statistical analysis of discrimination index, which was calculated using the following formula: (N-F)/(N+F) ×100%. The higher percentage of discrimination index indicates improvement of the ability to discriminate the novel object; (D) Illustration of effects of Fer-1 on swimming speed from different groups; (E) Indication of swimming traces from day 29 to day 33 after $FeCl_3$ injection in different groups; (F-G) Indication of effects of Fer-1 on escape latency and mean path

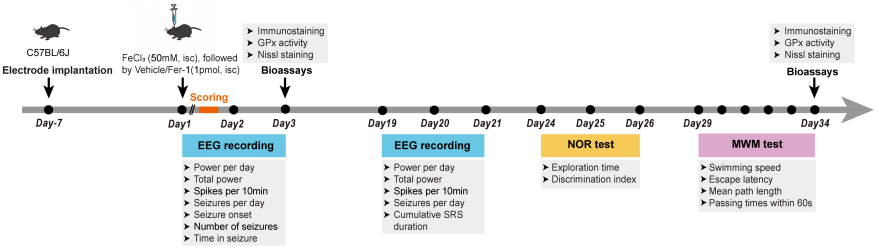
length, respectively in $FeCl_3$-induced PTE; (H-I) Indicate representative traces in the probe trial on the day 34 after $FeCl_3$ injection in different groups and analysis of the number of times of crossing platform. Data were expressed as mean ± SEM (n=6), *p<0.05 versus control group, **p<0.01 versus control group and #p<0.05 versus $FeCl_3$+Vehicle group.

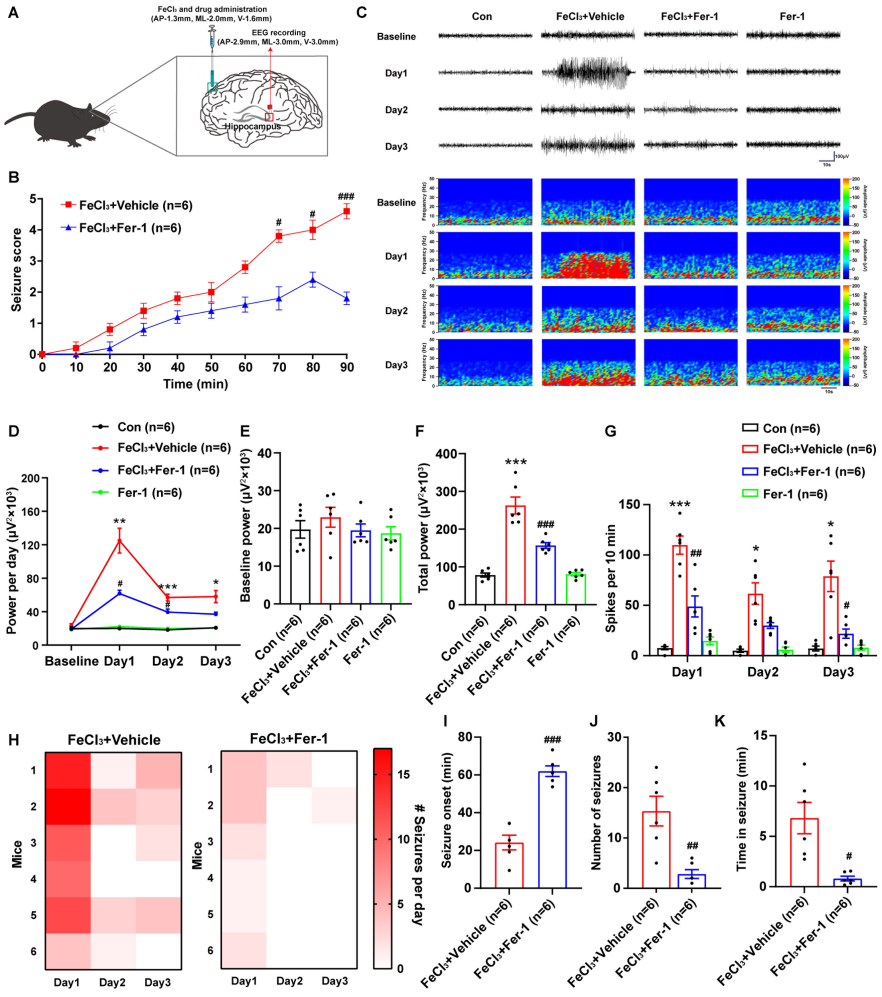
Figure 5. Fer-l does not display evident side effects on mice.(A) Time course of effects of Fer-1 on the body weigh from day 1 to day 34 after $FeCl_3$ injection; (B) Cumulative analysis of effects of Fer-1 on body weight. Data were expressed as mean ± SEM (n=6).

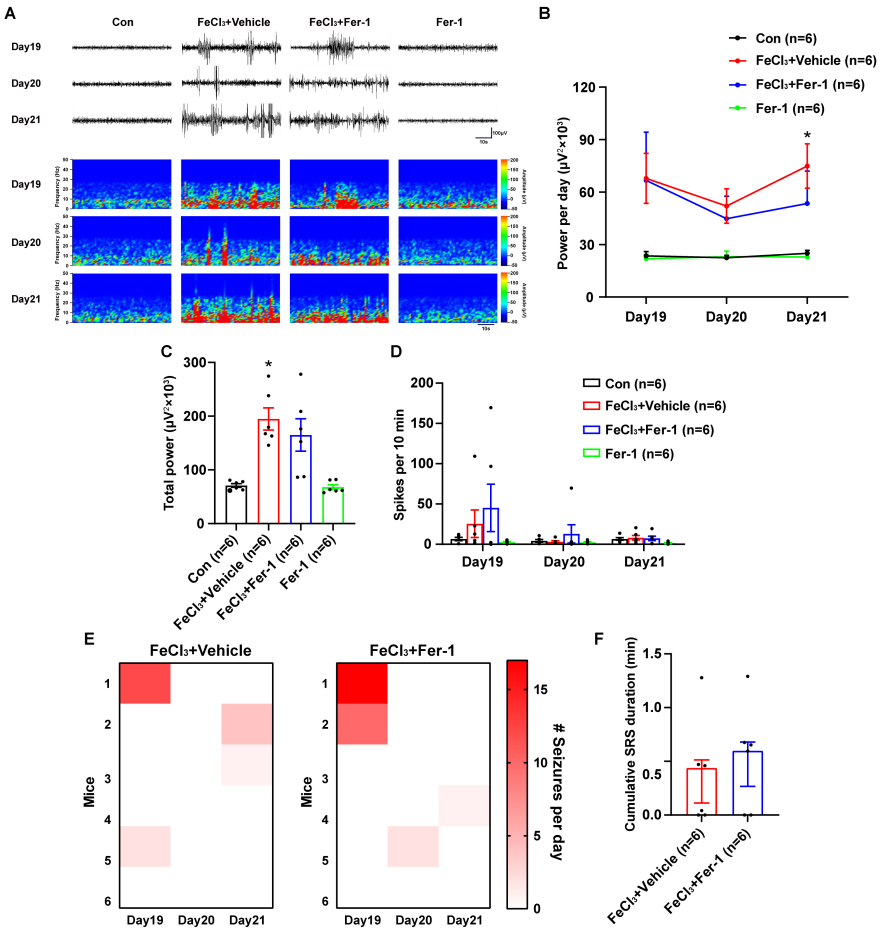
Figure 6. Fer-l inhibits ferroptosis process in a mouse model of PTE. (A) Illustration of 4-HNE immunostaining in hippocampal subregions including CA1, CA3 and DG in different groups on the day 3 or day 34 after $FeCl_3$ injection followed by Fer-1 treatment, Scale bar: 100 μm; (B-C) Quantitative analysis of optical intensity of 4-HNE in hippocampal CA3 regions on the day 3 and day 34 among different groups; (D-E) Measurements of effects of Fer-1 on GPx activity in $FeCl_3$-induced PTE. Data were expressed as mean ± SEM (n=3) **p<0.01 versus control group, ***p<0.001 versus control and $^{\#\#}$p<0.01 versus $FeCl_3$+Vehicle group.

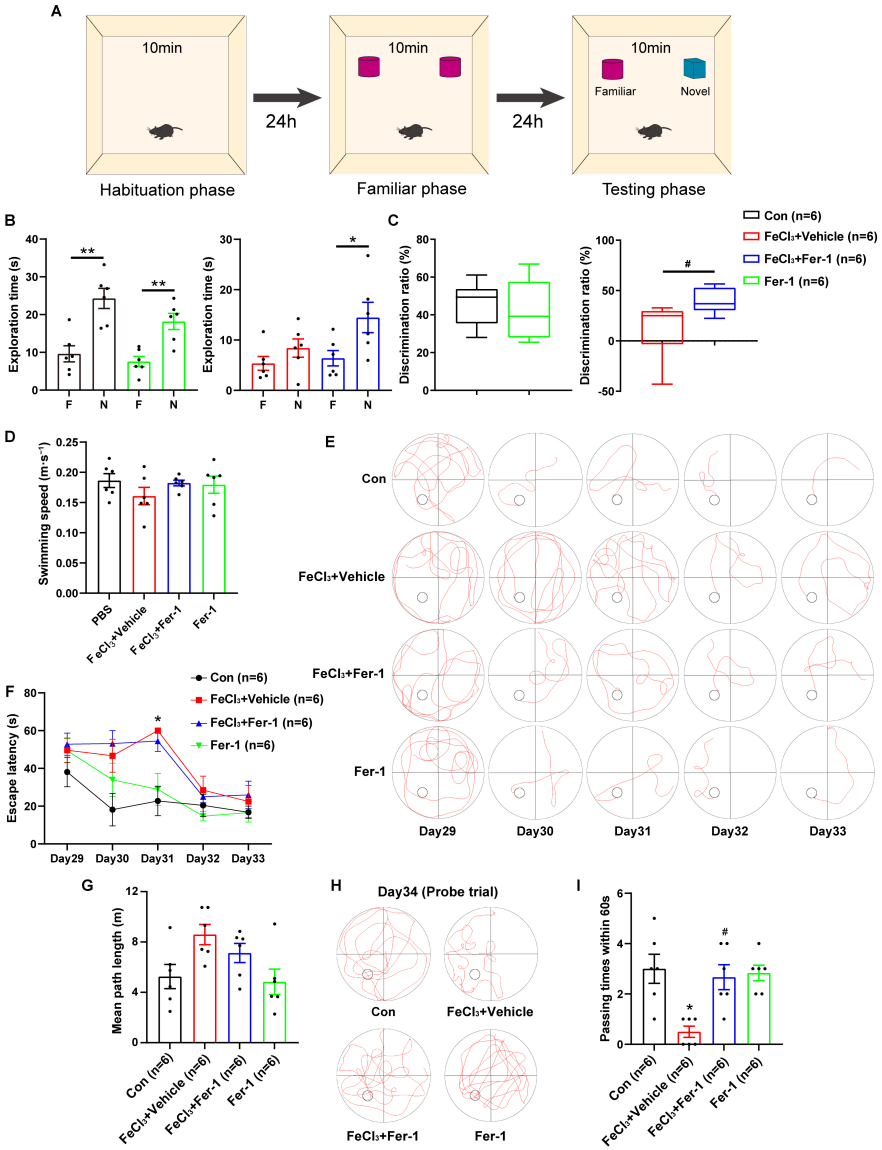
Figure 7. Fer-1 attenuates seizure-induced hippocampal damage in FeCl3-induced PTE. (A) Illustration of Nissl staining in hippocampal subregions including CA1, CA3 and DG in different groups on the day 3 after $FeCl_3$ injection followed by Fer-1 treatment; (B-D) Statistical analysis of viable neurons in CA1, CA3 and DG regions on the day 3 among different groups; (E) Representative Nissl staining images in CA1, CA3 and DG regions on the day 34 after $FeCl_3$injection followed by Fer-1 treatment; (F-H) Statistical analysis of viable neurons in CA1, CA3 and DG regions on the day 34 among different groups. Arrow head indicates dead neurons. Scale bar: 100 μm

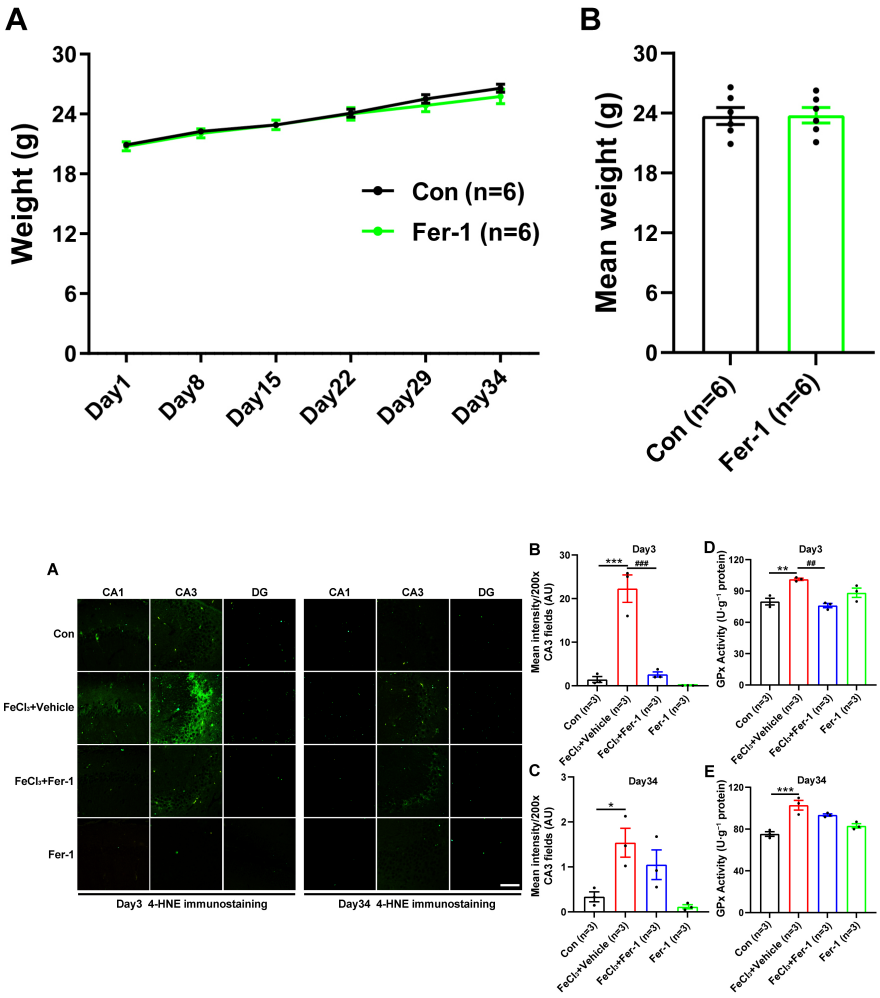
Figure 8. Working model. It demonstrated that Fer-1 ameliorates seizures and improves cognitive impairments in $FeCl_3$-induced PTE. Inhibition of ferroptosis process including decreases of 4-HNE level and GPx activity were possibly involved in Fer-1's neuroprotection.

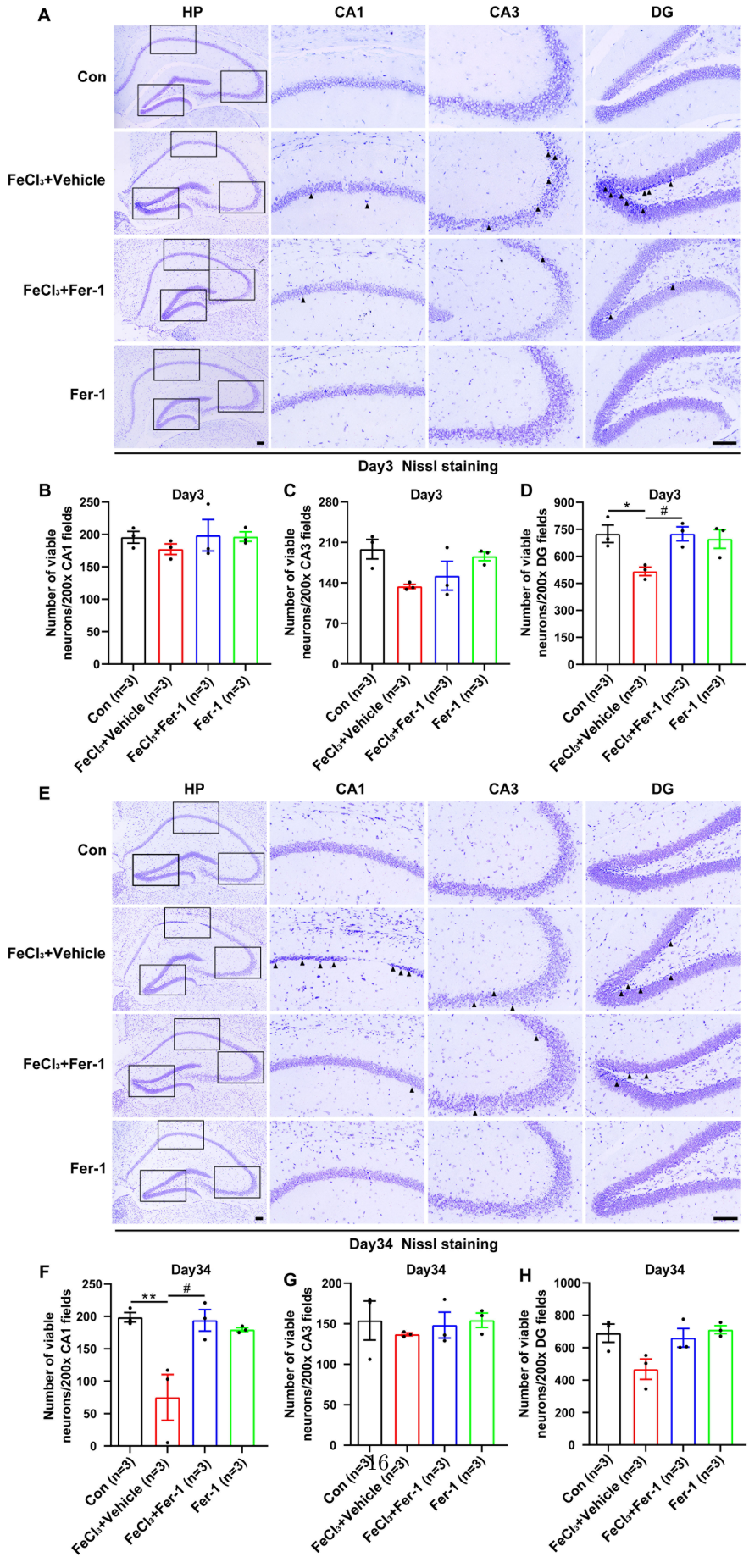












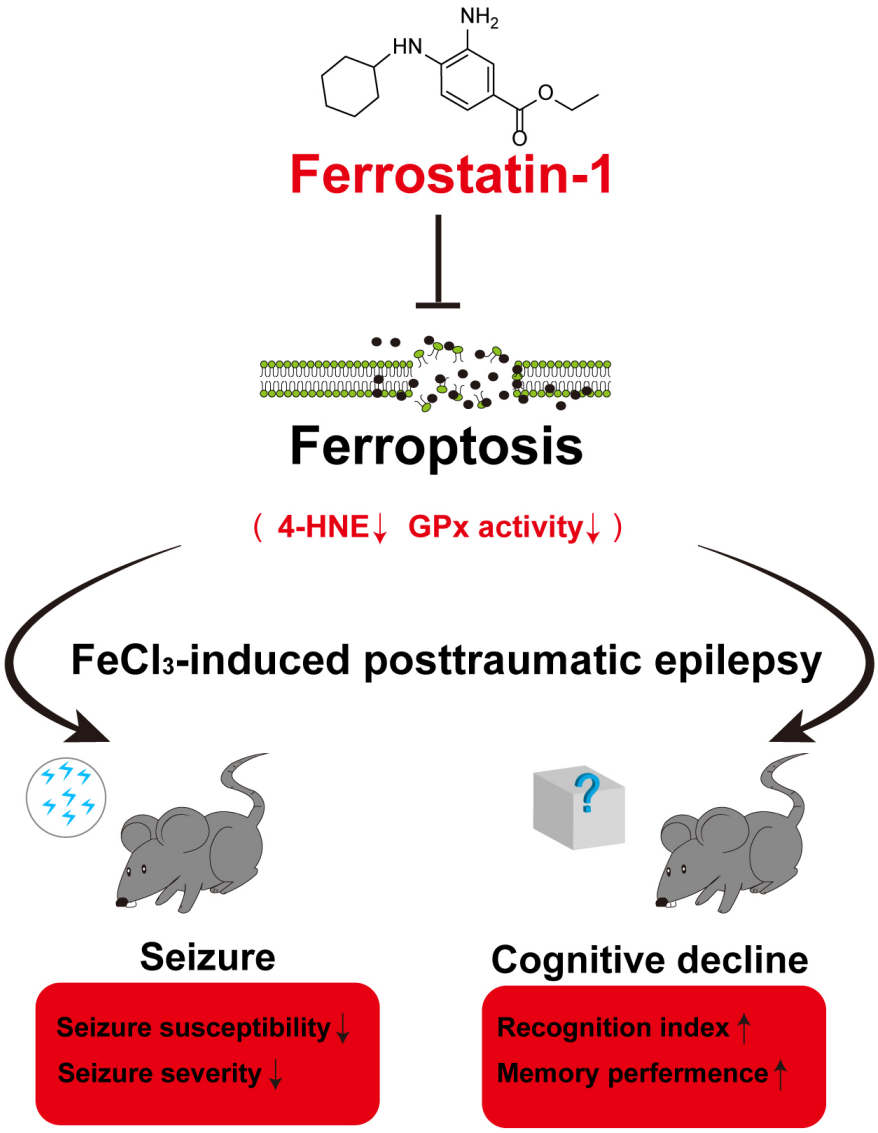


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Table 1. Summary of statistical tests.doc available at https://authorea.com/users/734145/articles/711359-ferrostatin-1-obviates-seizures-and-associated-cognitive-deficits-in-ferric-chloride-induced-posttraumatic-epilepsy-via-suppressing-ferroptosis