α-linolenic acid ameliorates pentylenetetrazol-induced neuron apoptosis and neurological impairment in mice with seizures via down-regulating JAK2/STAT3 pathway

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Abstract

Epilepsy ranks fourth among neurological diseases, featuring spontaneous seizures and behavioral and cognitive impairments. Although anti-epileptic drugs are currently available clinically, 30% of epilepsy patients are still ineffective in treatment, and 52% of patients experience serious adverse reactions. In this work, the neuroprotective effect of α-linolenic acid (ALA, a nutrient) in mice and its potential molecular mechanisms exposed to pentylenetetrazol was assessed. The mice were injected with pentetrazol 37 mg/kg, and ALA was intra-gastrically administered for 40 days. The treatment with ALA significantly reduced the overall frequency of epileptic seizures and improved the behavior impairment and cognitive disorder caused by pentetrazol toxicity. In addition, ALA can not only reduce the apoptosis rate of brain neurons in epileptic mice, but also significantly reduce the content of brain inflammatory factors (IL-6, IL-1, and TNF-α). Furthermore, we predicted that the possible targets of ALA in the treatment of epilepsy were JAK2 and STAT3 through molecular docking. Finally, through molecular docking and Western Blot studies, we revealed the potential mechanism of ALA ameliorates pentylenetetrazol-induced neuron apoptosis and neurological impairment in mice with seizures by downregulating the JAK2/STAT3 pathway. This study aimed to investigate the antiepileptic and neuroprotective effects of ALA, as well as explore its potential mechanisms, through the construction of a chronic ignition mouse model via intraperitoneal PTZ injection. The findings of this research provide crucial scientific support for subsequent clinical application studies in this field.

Keywords: α-linolenic acid; epilepsy; JAK2/STAT3 signalling pathway; neuron apoptosis; neuroinflammation; pentylenetetrazol toxicity

1. Introduction

Epilepsy ranks fourth among neurological diseases, featuring spontaneous seizures and behavioral and cognitive impairments; it affects approximately 65 million people worldwide. Research indicates axonal damage, neuroinflammation, and oligodendrocyte loss may increase morbidity in epilepsy. Despite the availability of antiepileptic drugs, 30% of epileptics are resistant to treatment and >52% have serious adverse events [(1)]. Therefore, new practical approaches to epilepsy management are urgently required.

ALA (Figure 1A), a polyunsaturated fatty acid abundant in walnut and canola oil, is the only omega-3 fatty acid produced by vegetables. Accumulating evidence suggests ALA is essential in the proper operation of the central nervous system (CNS) [(2)]. ALA consumption alleviates various neuropathological conditions. Studies have found that ALA exhibits anticonvulsant effects, and possible mechanisms may include altering membrane composition of nerve cells, activating peroxisome proliferator-activated receptors, and reducing inflammation[(3, 4)]. However, the results of clinical studies are inconsistent, and there is limited research on the effects and mechanisms of ALA in relation to epilepsy.

Status epilepticus (SE)-associated brain inflammation further aggravates SE, with induced neuronal dysfunction [(5)]. During development and after brain damage, JAK2-STAT3 signaling regulates genes controlling cell survival and proliferation, the cell cycle, and angiogenesis [(6)]. Recently, the potential role of the JAK-STAT pathway in CNS disorders has been investigated [(7, 8)]. Researchers firstly demonstrated the effect of STAT3 polymorphism on epilepsy [(9)]. Additional research identified a direct bond between IL-6 and CD5, resulting in STAT3 activation through gp130 and JAK2, its downstream kinase [(10)]. JAK/STAT signaling, a key player in inflammation, can exert major effects on neuronal degeneration, memory formation, and synaptic plasticity in the CNS [(11)]. JAK2-STAT3 pathway induction was detected after traumatic brain damage, pilocarpine, and kainic acid-induced SE and ischemia, indicating this pathway could be targeted to prevent and treat SE [(12)]. However, it is currently unknown whether ALA can affect SE through the JAK2/STAT3 pathway.

Pentylenetetrazol (PTZ) is a GABA receptor antagonist that induces epilepsy by inhibiting

chloride ion channels in downstream signaling pathways. The PTZ model is capable of replicating myoclonic seizures observed in humans, offering a rapid disease model generation process and a low mortality rate. This model has been extensively utilized in antiepileptic drug research[13]. KM mice, a natural strain without artificial selection or genetic modification, possess a stable genetic background and display neural structure and functionality similarities to humans. As a result, they serve as an ideal model for investigating the development and characteristics of human epilepsy[14]. In this study, we employed PTZ to induce epilepsy in mice and utilized this model to explore the ameliorative effect of ALA and its underlying mechanisms.

2. Materials and methods

2.1 Chemicals and reagents

α-linolenic acid (>98% purity) and pentylenetetrazol (PTZ) were provided by Sinopharm Chemical Reagent (China) and Sigma (USA), respectively. All Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Nanjing Jiancheng Bioengineering Institue (Nanjing, China). JAK2, STAT3, p-JAK2, p-STAT3 and β-actin antibodies was provided from Bioss Biotechnology Co. Ltd. (Beijing, China). Secondary antibody, horse radish peroxidase-conjugated goat anti-rabbit IgG was obtained from Jackson (Lancaster, China).

2.2 Animals

Thirty male KM mice (18–22g) from Wuhan University Laboratory Animal Centre were housed at 23.2°C under a 12-h photoperiod, with adequate food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Wuhan University and carried in accordance with the requirements of the ARRIVE guidelines. All efforts were made to minimize the number of animals used and animal suffering in this study. All animals were intact and unmedicated prior to the experiment.

2.3 Experimental group

Thirty KM mice were randomly selected and assigned to 3 weight-matched groups (n=10, the sample size was calculated according to the resource equation method) [15, 16], including the normal control, model and intervention groups (Figure 1B), with the following treatments.

(1) The normal control group was injected with 0.9% saline daily. (2) The model group received intraperitoneal administration of PTZ (37 mg/kg) in 0.9% saline daily until level 4-5 epileptic seizures according to the Racine scale. (3) The intervention group was treated with PTZ as model mice, and administered ALA at 4 ml/kg/d (37 mg/10g/d), based on preliminary studies.

2.4 Body weight and survival of the mice

Body weights and survival rate of mice during the modeling process are important indicators reflecting the state of mice. Body weights were obtained every other day to assess animal fitness and survival rates were determined at the end of the study.

2.5 Behavioral grading of seizures with the Racine scale

Epilepsy grade is an important indicator for evaluating the ameliorative effect of ALA. Following PTZ injections, mice were placed in empty cages for 30 minutes for behavioral assessment of seizure development by a blinded, independent volunteer as 0 (no abnormalities), 1 (mouth and facial movements), 2 (head nodding), 3 (forelimb clonus), 4 (rearing) and 5 (rearing followed by falling or death) points. The latency and score of each seizure were obtained.

2.6 Functional tests

Behavioural diseases, e.g., depression and learning and memory impairments, occur in mice administered PTZ [17]. Multiple assays were performed with Shenzhen RWD Instruments to assess behavioural changes. Light intensity was determined by the experimental setting.

2.6.1 Open Field Test (OFT)

The OFT was performed for assessing psychomotor results and exploratory behaviour in a 45 cm x 45 cm x 40 cm black acrylic box arranged into 25 squares, as reported previously [18].

2.6.2 Forced Swimming Test (FST)

The FST was performed to detect behavior associated with depression [19], in a 140 mm × 200 mm Plexiglas cylinder containing 150 mm of water at 23-25°C, for 8 min. A blinded investigator scored the mouse's last 6 min of immobility.

2.6.3 Tail Suspension Test (TST)

The TST also measures immobility, an indicator of depression, and was performed as described in a previous report [20]. The entire immobility duration was computed using the software's event count mode.

2.6.4 Morris Water Maze

This assay assesses spatial learning and reference memory, and was carried out in accordance with the reported methodology [21]. The number of platform crossings and distance travelled in 60s were analysed.

2.7 H&E and Nissl staining

At study end, the animals underwent euthanasia (n = 6). Three mice per group were utilized for histological analyses, while the remaining three were used for Western blot and ELISA. For H&E and Nissl staining, the animals were perfused with 0.9% NaCl and 4% paraformaldehyde. Brain tissue specimens underwent overnight fixation with 4% paraformaldehyde and paraffin embedding, deparaffinization with xylene, and rehydration with ethanol gradient. This was followed by hematoxylin and eosin (H&E) staining or Nissl staining (0.1% cresyl violet for 3 minutes), and dehydration. Histopathological hippocampal alterations were observed under an Olympus microscope.

2.8 TUNEL

Frozen sections were subjected to TUNEL analysis upon fixation with 4% paraformaldehyde. The avidin-labeled fluorescein or ABC kit (Vector) was utilized for this assay.

2.9 IL-1β, IL-6 and TNF-α levels

Euthanasia was performed after the behavioural trials, and hippocampal specimens were obtained (n=3). The specimens underwent homogenization (10% w/v) in cold potassium phosphate buffer (pH 7.4). Upon centrifugation (3000 rpm for 10 minutes) at 4°C, the resulting supernatants were obtained for ELISA assessment of IL-1β, IL-6, and TNF-α with specific kits [22, 23].

2.10 Molecular Docking Analysis

ALA's 3D structure was drawn using Chem3D and imported into Discovery Studio (2019) for pre-processing of small molecules. At the same time, we downloaded the JAK2 (PDB ID: 5AEP), STAT3 (PDB ID: 5AX3) proteins from the Uniport database and imported them into Discovery Studio (2019). Each imported protein was treated separately to remove unwanted water molecules and structures, followed by hydro-processing protein modification, and selection of docking sites. After the small molecule and protein were processed in ALA, docking of the half flexible molecule between the protein and ALA was carried out. Selected the association with the highest score for analysis.

2.11 Immunoblot

Equal amounts of total protein (20 μ g) were resolved by 10% SDS-PAGE, followed by transfer unto polyvinylidene difluoride (PVDF) membranes. The samples underwent overnight incubation at 4°C with rabbit anti-JAK2 (1:500; Bioss Biotechnology, China), anti-p-JAK2 (1:500), anti-STAT3 (1:500; Boster Biotechnology, China), and anti—actin (1:10000; TDY Biotechnology, China) primary antibodies, respectively. Then, the membranes were treated with goat anti-rabbit IgG linked to HRP at ambient for 120 minutes (1:10000; Aspen, China). Electrochemiluminescence (ECL Plus) was utilized to identify and quantify signals [24].

2.12 Immunofluorescence

After three PBS rinses, brain slices were incubated with PBS containing 10% BSA and 0.3% Triton X-100 for 1 hour. The samples underwent successive incubations with rabbit polyclonal antibodies targeting p-JAK2 and p-STAT3 (1:500; Boster Biotechnology), respectively (overnight) and FITC-conjugated goat anti-rabbit secondary antibodies (1:1000; Beyotime) for 1 hour. Mounting was performed with Prolong Gold antifade reagent (Invitrogen). An Olympus microscope and the Image-Pro software were utilized for analysis [(25)].

2.13 Statistical analysis

SPSS 19 was utilized for data analysis by unpaired Student's t-test, one-way ANOVA, or two-way ANOVA with post hoc Bonferroni test. Prism 6.0 (GraphPad Software, USA) was utilized for further statistical analyses. The Log-rank (Mantel-Cox) test was utilized for comparing mouse survival. Data are showed as mean ± SD.

3. Results

3.1 α-linolenic acid enhances mouse survival and body weights in mice

Deaths occurred during the course of the experiment, and this was a serious adverse reaction in both the model and intervention groups, and every effort was made to alleviate the animals' suffering. Mouse survival is shown in Supplementary Fig.1A. Survival was 60% in pentylenetetrazol treated animals at 40 days, versus 100% in control mice and 80% in the ALA group. In addition, bodyweight changes of mice were recorded during the modeling (Supplementary Fig. 1B). In control animals, mean body weight steadily increased from 19.92g to 32.21g, indicating a 61.8% weight gain. However, the PTZ group had markedly reduced mean weight from 19.81g to 15.71g (20.7% reduction; $p<0.05$). In the PTZ+ALA group, the average body weight exhibited an upward trend from 20.12g to 26.41g ($p<0.01$ versus control group). These findings indicated that pretreatment with ALA by gavage reduced mortality reversed PTZ-induced body weight loss.

3.2 α-linolenic acid decreases the frequency of epileptic attacks

Mice displayed classic signs after pentylenetetrazol injection, including early facial and mouth movements, rearing, and significant convulsions, which were assessed using the Racine scale [(26)]. On Day 40, the average seizure level in the model group peaked at 4-5, indicating a propensity toward upgrading. Following therapy with ALA, seizures decreased significantly from Days 20 to 40 ($p<0.05$). This was not the case for the pentylenetetrazol-induced group. In control mice, no seizures were seen (Fig. 2A). The PTZ group had starkly reduced latency of seizures compared with control mice ($p<0.001$; Fig. 2B). Interestingly ALA administration significantly increased the latency of seizures after PTZ treatment ($p<0.05$).

3.3 Effect of α-linolenic acid on depression-like behavior

The effects of ALA on depression-like behavior in animals with seizures were investigated by the forced swimming and tail suspension tests. Pentylenetetrazol treatment resulted in a substantial elevation of immobility time ($p<0.05$) in the TST, which was reversed by ALA ($p<0.01$). (Fig. 3A). In the FST, the model group demonstrated considerably increased immobility time compared with controls ($p<0.01$), and this effect was also reversed by ALA ($p< 0.05$). (Fig. 3B). Jointly, these findings suggested ALA has significant antidepressant effects.

3.4 α-linolenic acid enhances animal exploration behavior

In Kunming mice, a unique open-field activity box was employed to assess spontaneous motor activity and adaptability to a new environment. The model group had considerably fewer total crossings compared with controls ($p<0.05$). A marked increase was found after ALA treatment ($p<0.05$; Fig. 3C), indicating that ALA increased motor skills in epileptic mice. Pentylenetetrazol-treated animals exhibited increased uneasiness and moved about the box more than control mice, with reduced number of center crossings and time spent in the center ($p<0.05$ and $p<0.01$, respectively). Pretreatment with ALA starkly enhanced these parameters ($p<0.01$; Fig. 3D and E). The above findings indicated ALA enhanced exploratory behavior in epileptic mice.

3.5 Effect of α-linolenic acid on spatial cognition and memory

The Morris water maze was used to investigate ALA's effects on spatial learning and memory (Fig. 4A-G). All groups were comparable before treatments. Escape latency in the PTZ group increased from Day 2 compared with control animals. This effect was particularly noticeable on the last training day ($p<0.05$ and $p<0.001$ on days 2-3 and 4, respectively). ALA administration reduced escape latency in PTZ treated animals ($p<0.01$ and $p<0.05$ on days 2 and 3-4, respectively). In addition, PTZ decreased crossing times considerably versus control mice ($p<0.05$). Meanwhile, ALA increased platform crossing significantly in PTZ treated mice ($p<0.05$; Fig. 4B). In comparison with control animals, the model group spent less time in the target quadrant ($p<0.01$). Meanwhile, pre-treatment with ALA significantly reversed this effect ($p<0.01$, Fig. 4C). In terms of the distance traveled inside the quadrant, the model group showed starkly lower values than controls ($p<0.05$). ALA increased the traveled distance substantially in epileptic animals ($p<0.05$; Fig. 4D). In the MWM, representative photographs of mouse movements in the probe trial task were collected (Fig. 4E-G). These data indicated impaired spatial learning and memory in mice induced by pentylenetetrazol might be improved by ALA.

3.6 Effects of ALA on neuronal damage and neuron apoptosis

Next, the effects of ALA on neuronal damage and apoptosis were investigated. Histological investigation of hippocampal slices indicated normal cellular structure in control mice. In contrast, overt damage was found in the PTZ group, whose brain sections had overtly decreased cell volume, nuclear condensation, cell reduction, and disarray, notably in the CA1 area. This nerve cell damage was reversed by ALA (Fig. 5B-D). Then, neuronal loss in brain specimens from mice with seizures were investigated by Nissl staining (Fig. 6A-C and M). The amounts of neurons in CA1 were obtained, demonstrating PTZ-induced injury was linked with severe CA1 neuronal degeneration. Treatment with ALA could counteract this degeneration, as the PTZ+ALA group had more neurons in comparison with the model group ($p<0.001$). TUNEL was used to examine the effect of ALA on apoptosis (Fig. 6D-L and N). While apoptosis was enhanced in the PTZ group versus control animals, ALA reduced the number of

TUNEL-positive cells in the hippocampus of model mice ($p< 0.001$). These findings suggested ALA could prevent neuronal necrosis and apoptosis in the hippocampus for a long time.

3.7 Effect of ALA on hippocampal inflammatory response in PTZ-treated mice

IL-1β, IL-6, and TNF-α amounts were evaluated to determine ALA's effect on inflammatory response. The model group had starkly elevated IL-1β amounts compared with control animals ($p<0.001$; Fig. 7A). However, the PTZ+ALA group had considerably lower levels of IL-1β ($p<0.05$). Furthermore, in comparison with control mice, the model group had markedly increased IL-6 amounts ($p<0.05$, Fig. 7B), and this effect was significantly alleviated by ALA ($p<0.01$). Furthermore, PTZ induced a considerable rise in TNF-α level ($p<0.001$; Fig. 7C), which was significantly reduced by ALA ($p<0.01$). The findings implied ALA regulated inflammation by considerably lowering IL-6, IL-1β, and TNF-α amounts.

3.8 Molecular Docking of Relation Proteins of Epilepsy

Using molecular docking, we performed an extensive search for the effects ALA has on the expression of potential downstream mediators. Table 1 shows the binding energy values of the ALA proteins and the relationship proteins obtained from the DS 3.0 binding energy program. The interactions between ALA and JAK2 and STAT3 were shown to stable and powerful, with binding energies of -71.35 Kcal/mol and -59.98 Kcal/mol, respectively (Fig.8).

As can be seen in figure, we have shown how amino acid residues in the JAK2 binding site interact with ALA in the following manner: ARG897 (slat bridge); PTR1008 and VAL1000 (conventional hydrogen bond); LEU997 (alkyl). The amino acid residue interactions at the STAT3 binding site with ALA were as follows: LYS45 (attractive charge) TYR27 (conventional hydrogen bond and carbon hydrogen bond), ILF22 (alkyl). Results showed that ALA binds to tyrosine, phosphoserine or valine of JAK2 and STAT3 proteins. Therefore, we speculated that ALA could affect the expression of JAK2 and STAT3, thereby exerting ameliorative effects in epilepsy.

3.9 ALA potently regulates JAK2-STAT3 signaling

In order to further elucidate the neuroprotective effects of ALA on epileptic seizures, we conducted an investigation involving the murine hippocampal tissue. We examined the protein expressions of p-JAK2, JAK2, p-STAT3, and STAT3. Representative bands of western blot were shown in Fig. 9A, and relative protein expression levels were displayed in Fig. 9B. PTZ-activated JAK2-STAT3 phosphorylation was blocked by ALA treatment. Immunofluorescence assays demonstrate that the expression of proteins in signaling pathways dramatically decreased with ALA treatment (Fig. S2A-S). There was a marked decline in the phosphorylation of JAK2 and STAT3 in the hippocampus, being $p < 0.01$ and $p < 0.05$, respectively, once α-linolenic acid had been administered. These findings revealed that ALA inhibited PTZ-dependent JAK2 and STAT3 phosphorylation. Taken together, the ALA suppression of the onset of epileptic seizures may be via inhibiting the JAK2/STAT3 pathway.

4. Discussion

Epilepsy is a persistent medical condition characterised by recurrent seizures and the degeneration of brain cells, resulting in cognitive impairments[27]. The majority of patients necessitate ongoing therapy, resulting in substantial stress and suffering. Consequently, investigating efficacious drugs for the prevention and treatment of epilepsy carries substantial social and therapeutic consequences.

While ALA shows promise as a natural dietary ingredient with potential therapeutic effects, it is not a standalone medicine. Therefore, its usefulness in treating epilepsy still requires validation through research. Moreover, due to the limited number of studies and inconsistent results, more data is needed to confirm the therapeutic potential of ALA. Our research provides more evidence that ALA can successfully regulate the occurrence and intensity of seizures by inhibiting the JAK2/STAT3 signalling pathway, which reduces neuroinflammation. This discovery has consequences for using ALA as a dietary intervention for patients with epilepsy. The results of this study illustrate the subsequent impacts of ALA: (a) Significant reduction in the severity and duration of convulsions in mice. (b) Reversal of the loss or apoptosis of

hippocampal neurons induced by PTZ toxicity, along with decreased levels of inflammatory markers such as IL-6, TNF-α, and IL-1β. Additionally, ALA treatment notably improved cognitive and functional impairments in epileptic mice. (c) The neuroprotective effect of ALA on epileptic mice is attributed to the activation of the JAK2-STAT3 signaling pathway.

In order to determine whether therapeutic interventions improve neurological function following epileptic convulsions, the rate and extent of neurological function recovery must be assessed. A range of functional assessment techniques were employed to evaluate the behavioural and functional recovery subsequent to the administration of ALA. These techniques included the OFT, TST, FST, and MWM.[28]. Research has reported that ALA can significantly improve the antidepressant effect in PTZ-induced toxic mice, possibly due to the upregulation of mature brain-derived neurotrophic factor during the forced swim task in the hippocampus[29, 30]. BDNF has been proven to have antidepressant and neuroprotective effects, and is closely related to neuroinflammation[31]. A decrease in motor activity might correspond to the emergence of symptoms resembling depression. As indicated by the reduced number of crossings, PTZ-induced epileptic mice exhibited substantially diminished motor activity in comparison to control mice, preferring to remain in the corners of the open field test box. The number of crossings increased substantially following ALA administration, indicating that the behavioural and cognitive disorder induced by pentetrazol in rats was ameliorated.

The impact of apoptosis or necrosis on seizure activity has been extensively demonstrated [32]. Apoptosis and inflammatory response play crucial roles in the onset and progression of epilepsy, although their underlying mechanisms have yet to be fully elucidated. In this study, the administration of ALA significantly reduced the number of TUNEL-positive cells after seizures, indicating a reduction in neuronal damage. Additionally, the administration of ALA facilitated the formation of Nissl bodies, which serve as a marker for the preservation of neuronal structure. The main objective of this inquiry was to study the CA1 area of the hippocampus, which is continuously and severely impacted by seizures in experimental animals. Additionally, ALA treatment was associated with increased neurogenesis and the

presence of mature neurons in the sub-granular zone of the dentate gyrus within a span of 30 days[33-35]. Further experiments are warranted to evaluate the impact of ALA on the hippocampal DG region.

Neuroinflammation plays a crucial regulatory role in the occurrence and progression of epilepsy. Inhibiting inflammation can reduce neuronal cell toxicity, improve neuronal apoptosis and neurofunctional impairment, enhance learning and memory abilities, and alleviate symptoms of epilepsy[36]. ALA is an omega-3 polyunsaturated fatty acid that possesses significant antioxidant capacity and anti-inflammatory effects[37]. Although ALA, as an unsaturated fatty acid, has significant physiological functions, its impact on epilepsy remains unclear. The physiological function of ALA is based on its conversion into EPA and DHA. ALA competes with linoleic acid for metabolic space, leading to a decrease in the level of arachidonic acid, as well as the quantity of class II prostaglandins and leukotrienes in tissue phospholipids[38]. By competing with cyclooxygenase and lipoxygenase, ALA and its derivatives (EPA and DHA) inhibit the synthesis of class I prostaglandins and leukotrienes, weaken the physiological activity of thromboxane, and occupy thromboxane receptors, thereby inhibiting the production of inflammatory factors[39]. Research has reported its ability to significantly decrease the mRNA levels and protein content of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β, improve spatial learning and memory abilities, and exert neuroprotective functions[40]. In our current study, ALA exhibited significant reductions in the levels of TNF-α, IL-6, and IL-1β. Hence, we hypothesize that ALA may mitigate epileptic seizures induced by pentetrazol intoxication through its anti-neuroinflammatory effects.

The continuous activation of the JAK2-STAT3 signaling pathway is closely associated with various inflammatory and immune diseases, including rheumatoid arthritis, inflammatory bowel disease, sepsis, and tumor-related diseases. During the inflammatory process, JAK2-STAT3 is the main signaling pathway regulated by cytokines, with a relatively simple signal transduction mechanism. JAK2 belongs to the tyrosine protein kinase family and is activated by certain cytokines and interleukins that act on transmembrane receptors during neural injury[41]. Activated JAK2 can recognize the SH2 domain in STAT3 and induce its

phosphorylation and activation. Research reports have shown that after phosphorylated STAT3 undergoes nuclear translocation, it can stimulate the expression of inflammatory genes and release inflammatory cytokines such as TNF-α, IL-1β, and IL-6, thereby exacerbating the inflammatory response[42]. Inhibiting JAK2/STAT3 pathway activation can significantly alleviate the inflammatory response and reduce inflammation damage. Furthermore, JAK2-STAT3 signaling is largely involved in the development and protection of neurons and glia, as well as brain inflammation[43]. Activated STAT3, for example, has been detected in numerous CNS cells and linked to neuronal growth and regeneration JAK2 and STAT3 have both been found to control hippocampal synaptic plasticity, which is associated with memory and learning[44]. JAK2-STAT3 signaling could be targeted for the treatment of epileptic seizures and other CNS illnesses, including depression, anxiety, and Alzheimer's disease [45]. Due to its strong link with the CNS immune system, investigators have responded to the JAK2-STAT3 pathway's essential significance in treating epileptic seizures by encouraging its application for the treatment of mental illness. Therefore, it is speculated that downregulating the JAK2/STAT3 pathway to inhibit inflammation may be the pharmacological mechanism by which ALA alleviates epileptic symptoms. In our study, we also utilized molecular docking and Western Blot studies to further confirm that ALA can downregulate the JAK2/STAT3 signaling pathway. This downregulation leads to a reduction in inflammatory factor levels and improvement in PTZ-induced neuron apoptosis and neurological impairment in mice. Moreover, ALA enhanced the cognitive function of mice with epilepsy and effectively alleviated seizure occurrences.

5. Conclusion

In conclusion, this study discovered ALA therapy reduces the severity of epileptic convulsions and reversed PTZ-induced necrosis or apoptosis of hippocampus neurons. Furthermore, pretreatment with ALA downregulated inflammatory markers, including IL-6, TNF-a, and IL-1, and suppressed JAK2/STAT3 signaling. Several functional tests were conducted, with the findings demonstrating ALA therapy enhanced neurological function considerably. These findings provide not only insights into the underlying mechanism of ALA, which appears to

block hippocampal JAK2-STAT3 signaling, but also additional evidence for ALA's therapeutic use in epileptic seizures. In addition, our study suggested that ALA might be a safe and effective candidate for neuroprotection in the therapy of epilepsy.

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Xin Zeng and Fei Luo were responsible for the whole experiment implementation and wrote the paper, Yahong Cheng and Jiefang Gao checked all the statistical analyses, Hong Ding did final proof reading and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Abbreviations: ALA, α-linolenic acid; IL6, interleukin 6; IL1β, interleukin-1β; TNF-α, tumor necrosis factor-alpha; JAK2, Janus kinase2; STAT3, Signal Transducer and Activator of Transcription3;PTZ, pentylenetetrazol; CNS, central nervous system; SE, status epilepticus; gp130, glycoprotein130; OFT, open Field Test; FST, Forced Swimming Test; TST, Tail Suspension Test; MWM, Morris Water Maze; H&E, hematoxylin-eosin; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling; PBS, Phosphate Buffered Saline; PVDF, polyvinylidene difluoride; CA1, cornu ammonis; DG, dentate gyrus; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; BDNF, brain derived neurotrophic factor.

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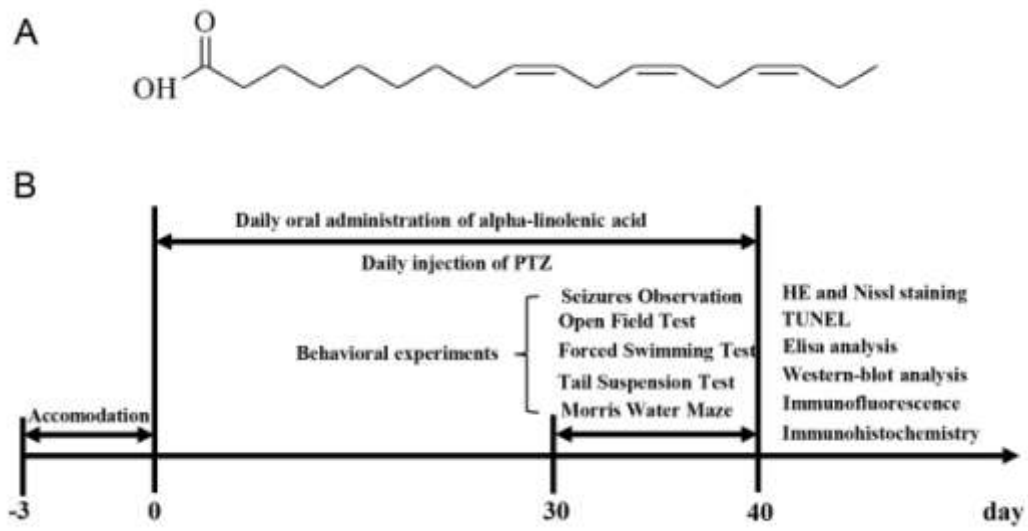


Fig. 1. Structure of α-linolenic acid and diagram flow. (A). The structure of α-linolenic. (B). The diagram flow of experiments.

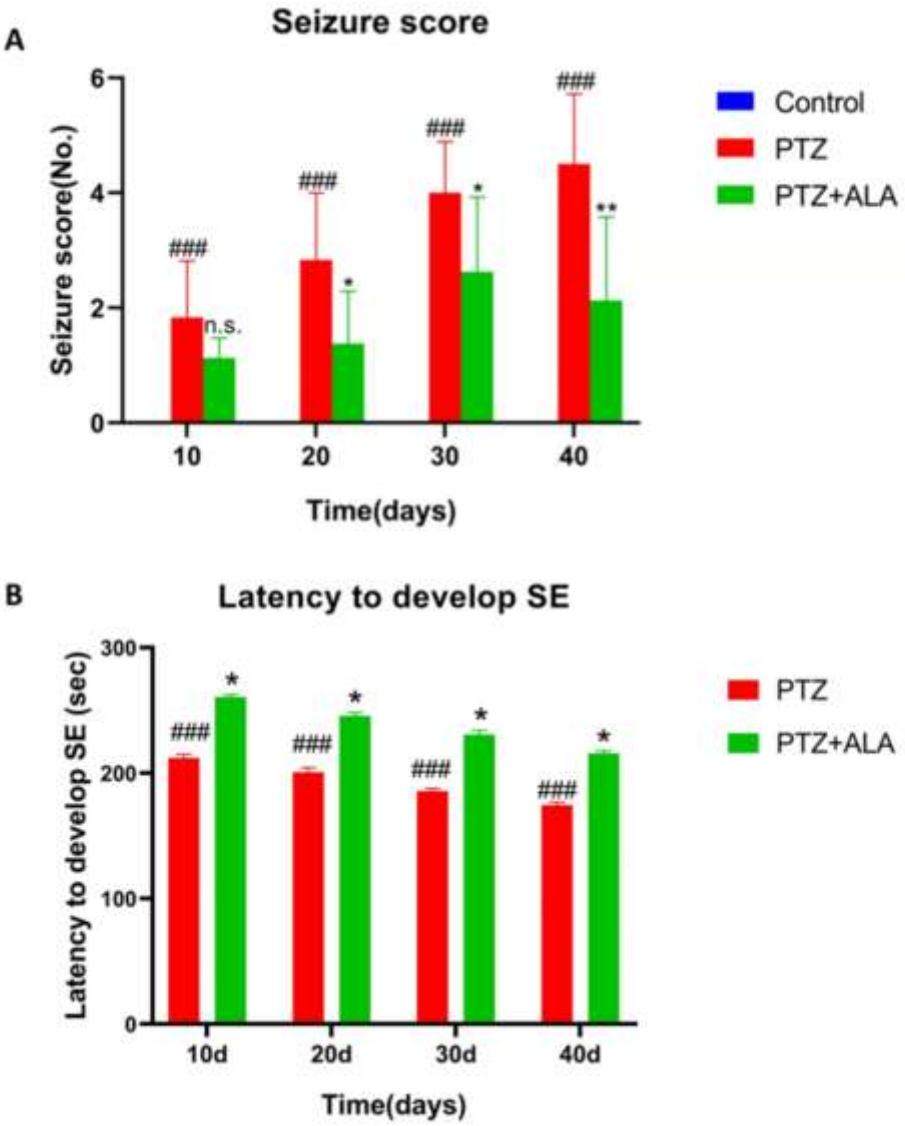


Fig. 2. Anticonvulsive properties of ALA in PTZ-induced epilepsy model. In a PTZ-induced epilepsy paradigm, ALA's effects on seizure score (A) and seizure latency (B) are shown. A significant group-by-day interaction was seen in two-way ANOVA for seizure score and latency to develop seizures. Data are mean ±standard deviation (N=6 in the PTZ group and N=10 in the other groups). n.s., no significance; ###p<0.001 vs Control group; *p<0.05 and **p<0.01 vs PTZ group.

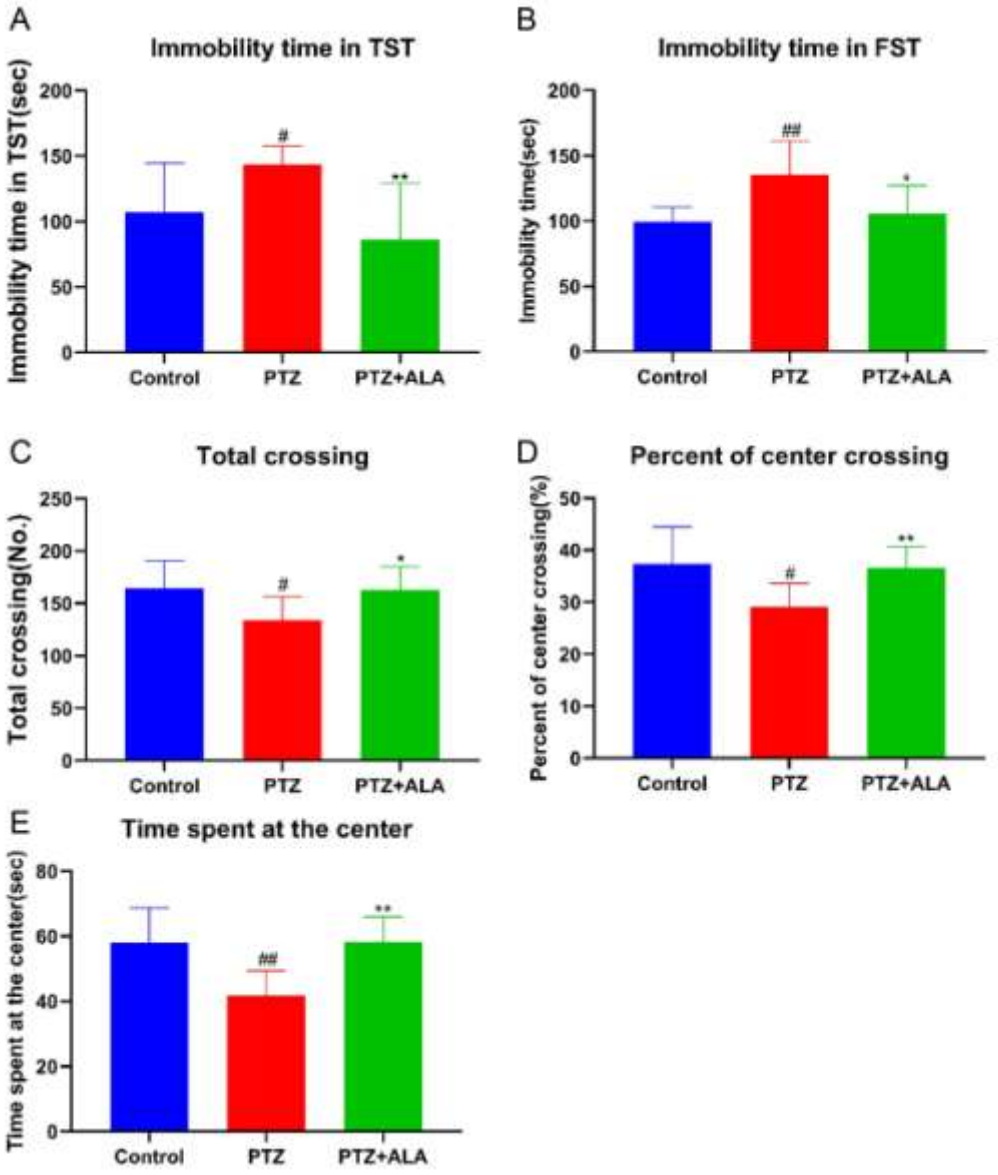


Fig. 3. Influence by α-linolenic acid on depression-like and exploration behavior. (A) tail suspension tests; (B) forced swimming tests;(C) Total numbers of crossings in an open field; (D) Percentage of open field crossings in the center; (E) Time spent in the open field's center. Data are mean ±standard deviation (N=6 in the PTZ group and N=10 in the other groups), with #p<0.05 and ##p<0.01 compared to Control group, and *p<0.05 and **p<0.01 compared to PTZ group. One-way ANOVA was carried out with Bonferroni post-test (A-E).

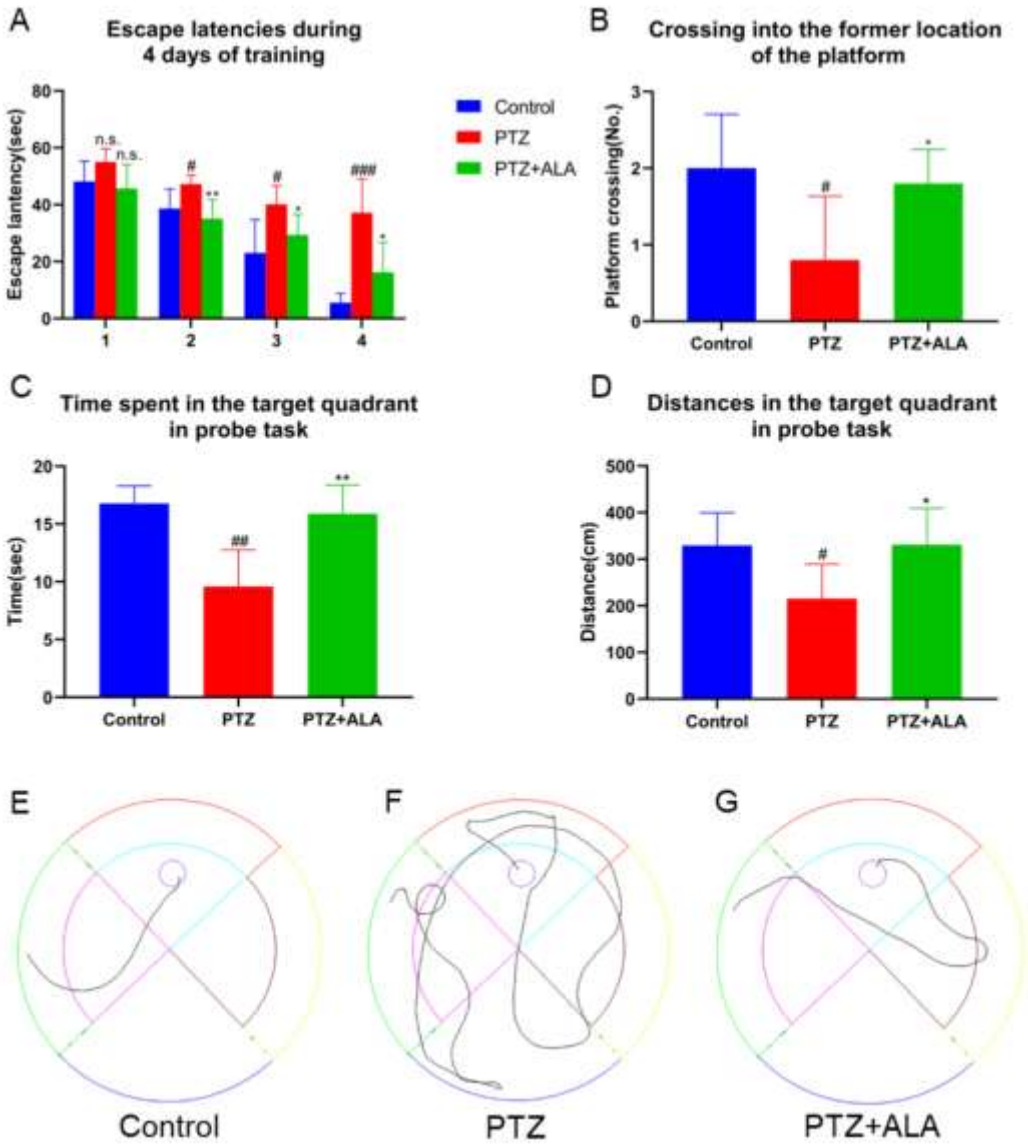


Fig. 4. Effect of α-linolenic acid (ALA) on spatial cognition and memory. The Morris Water Maze was used to determine ALA's effects on pentylenetetrazol-induced spatial cognition and memory deficits. (A) For escape latency, two-way ANOVA is displayed as the mean of trials over 4 days. Crossover into the old site of the submerged platform (B). time spent in the target quadrant (C) and distances traveled in the target quadrant (D) during the probing trial test. Swimming tracks obtained with a video tracking camera system are shown (E-G). Data were mean ± standard deviation (N=6 in the PTZ group and N=10 in the other groups). n.s., no significance; #p<0.05, ##p<0.01, and ###p<0.001 versus Control group; *p<0.05 and **p<0.01 versus PTZ group. One-way ANOVA was utilized with Bonferroni post-test (B-D).

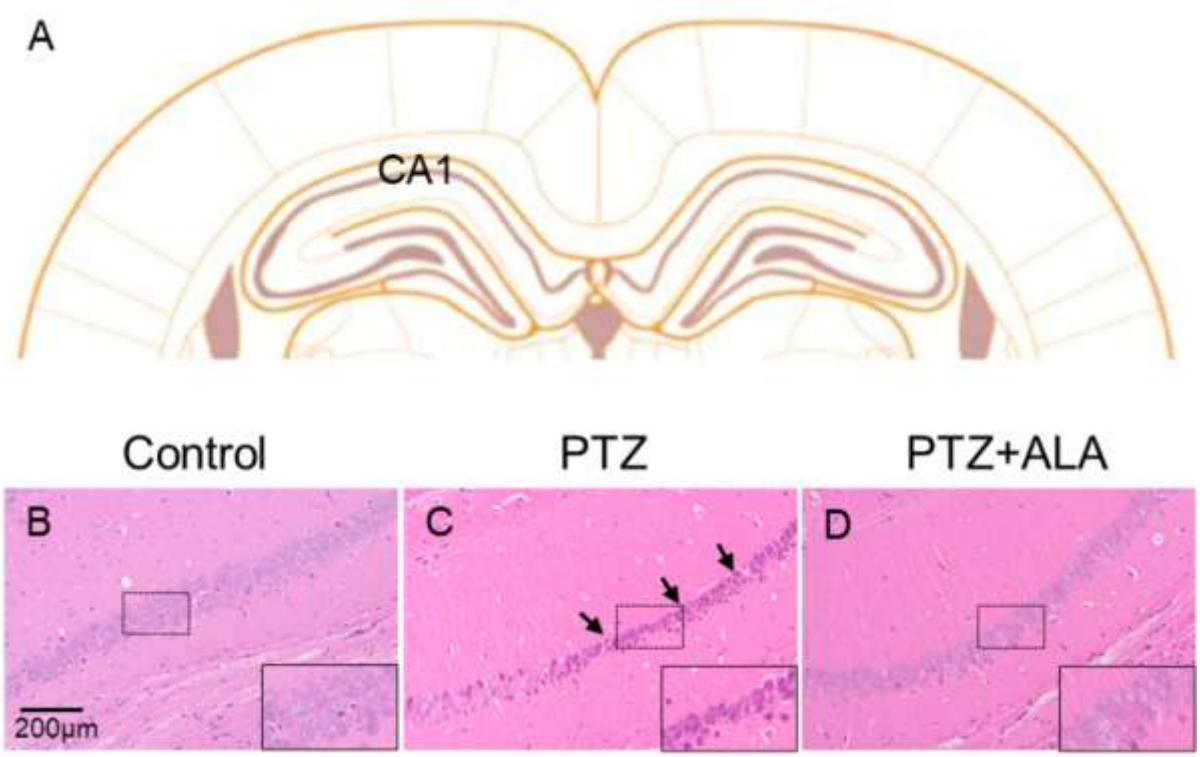


Fig. 5. Effects of α-linolenic acid on neuronal damage (H&E staining). Anatomical schematic representation of coronal brain sections (A). Histological analysis of hippocampal samples from control mice had normal cellular architecture (B). Meanwhile, PTZ-treated animals had the most severe damage among all groups, with brain sections exhibiting cell deflation, nuclear condensation, cell number decrease and disorganization, particularly in CA1 (C). However, α-linolenic acid markedly reversed nerve cell injury (D). Arrow heads indicate damaged cells. Magnification of originals: 200 ×. Magnification of insets: 400 ×. Scale bars represent 200 μm. N=3.

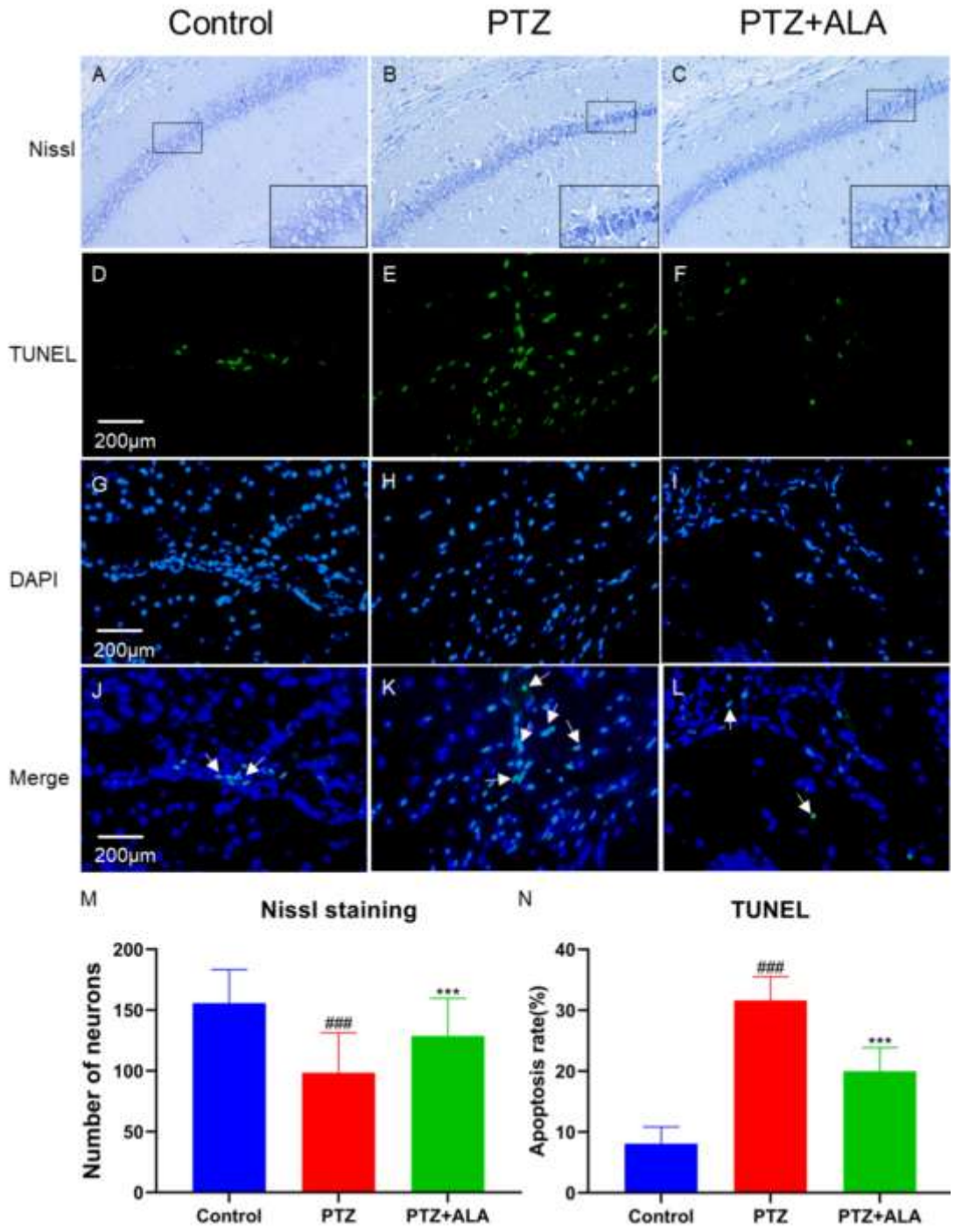


Fig. 6. α-linolenic acid's effects on neuronal loss and neuron apoptosis. Effects of α-linolenic acid on neuronal loss (Nissl staining) (A-C and M) and apoptosis (TUNEL) (D-L and N) in the hippocampal CA1 region. Magnification of originals: 200 ×. Magnification of insets: 400 ×. Scale bars represent 200 μm. White arrows, apoptotic cells. Data are mean ± standard deviation (SD) (N=3). ###p<0.001 versus Control group; ***p<0.001 versus PTZ group. One-way ANOVA was performed with Bonferroni post-test for M and N.

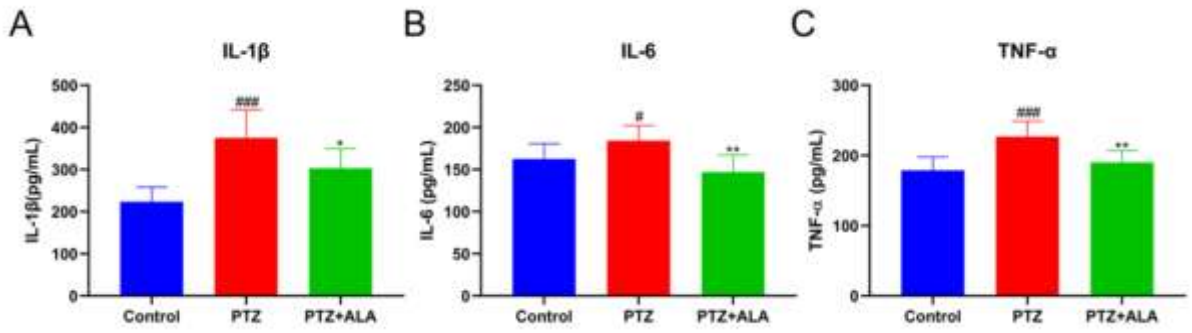


Fig. 7. Effect of α-linolenic acid on inflammatory response. The effect of ALA on inflammatory response was examined. The hippocampal levels of IL-1β (A), IL-6 (B) and TNF-α (C) in PTZ-exposed mice are shown. Data are mean ± standard deviation (N=3). ###p<0.001 and #p<0.05 versus Control group; *p<0.05 and **p<0.01 versus PTZ group. A Spark microplate reader was used to read absorbance at 450 nm (Tecan). One-way ANOVA was carried out with Bonferroni post-test (A-C).

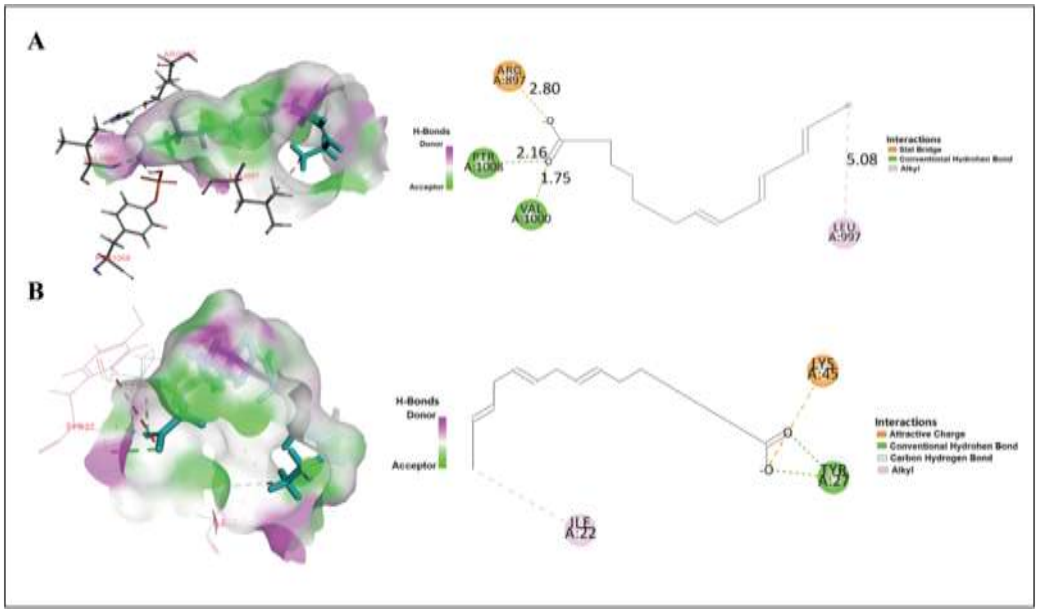


Fig. 8. Molecular Docking of Relation Proteins of Epilepsy. The specific interactions between ALA and JAK2 or ATST3 after automated docking of ALA to the JAK2 or ATST3 binding site. Forecasting 3D structure of the JAK2 (Protein Data Bank; PDB ID: 5AEP)- ALA complex and 2D diagram A. Forecasting 3D structure of the STAT3 (PDB ID: 5AX3)- ALA complex and 2D diagram B.

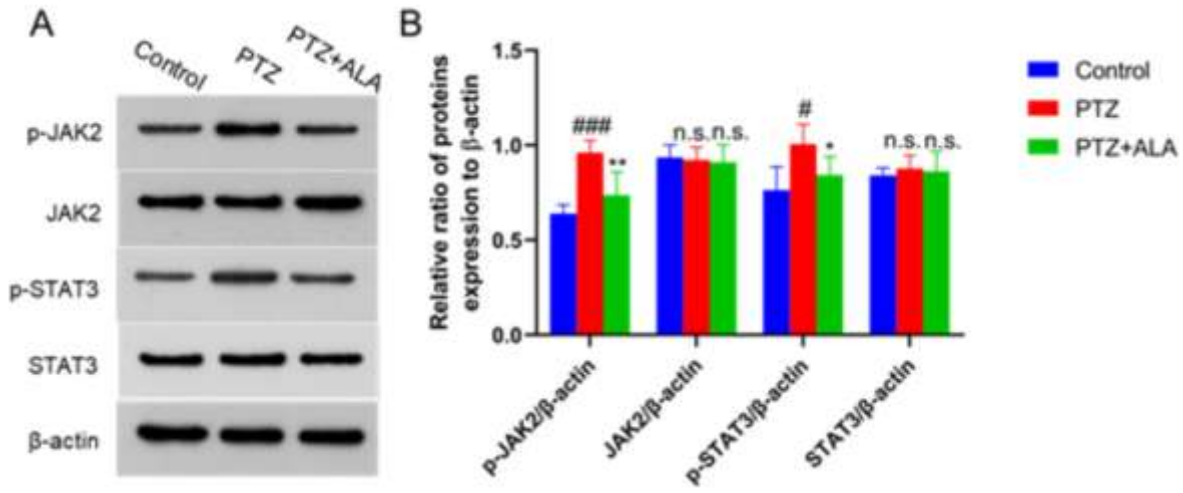


Fig. 9. JAK2/STAT3 signaling is involved in PTZ-associated seizures. The protein amounts of JAK2, p-JAK2, STAT3 and p-STAT3 in the hippocampus were measured by Western blot. Data are mean ± standard deviation (N=3). #p<0.05 and ###p<0.001 versus Control group; *p<0.05 versus PTZ group. One-way ANOVA was carried out with Bonferroni post-test (B).

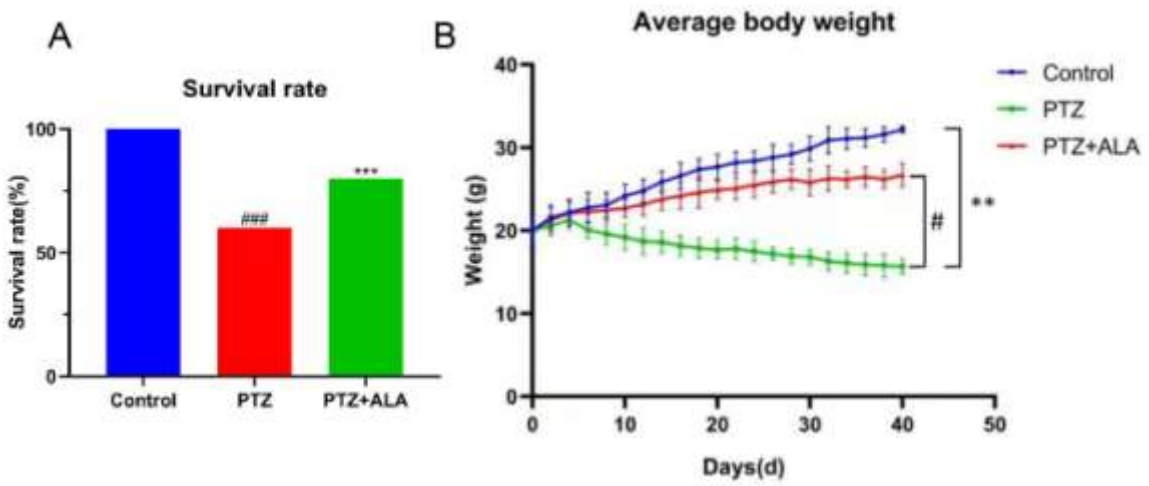


Fig. S1. Effects of α-linolenic acid on survival rate and body weight. (A). Survival rate of mice; (B). Body weight. All results are mean ± standard deviation (N=6 in the PTZ group and N=10 in the other groups). n.s., no significance; #p<0.05 vs Control group; **p<0.01 vs PTZ group.

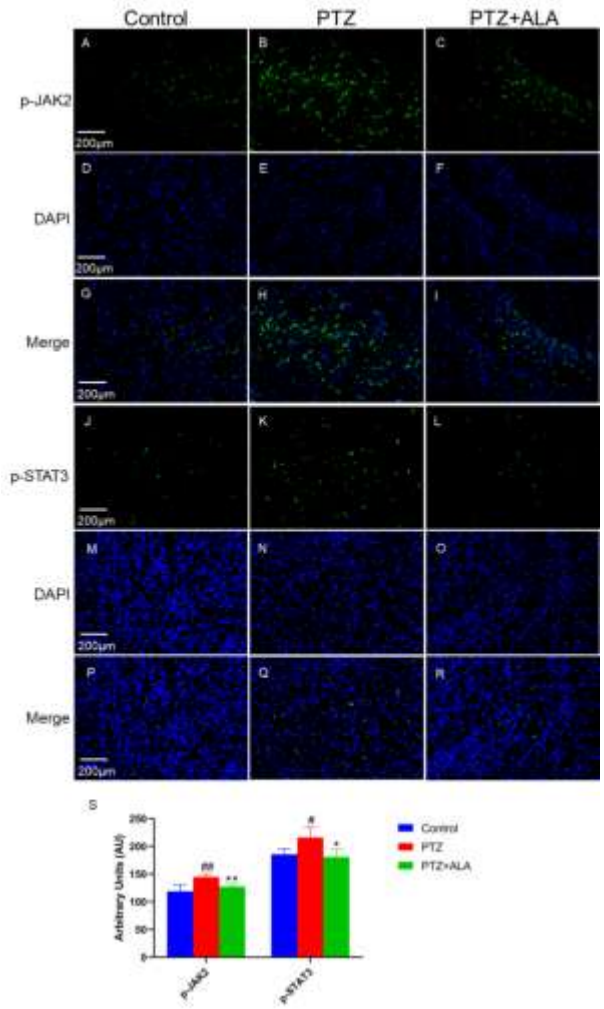
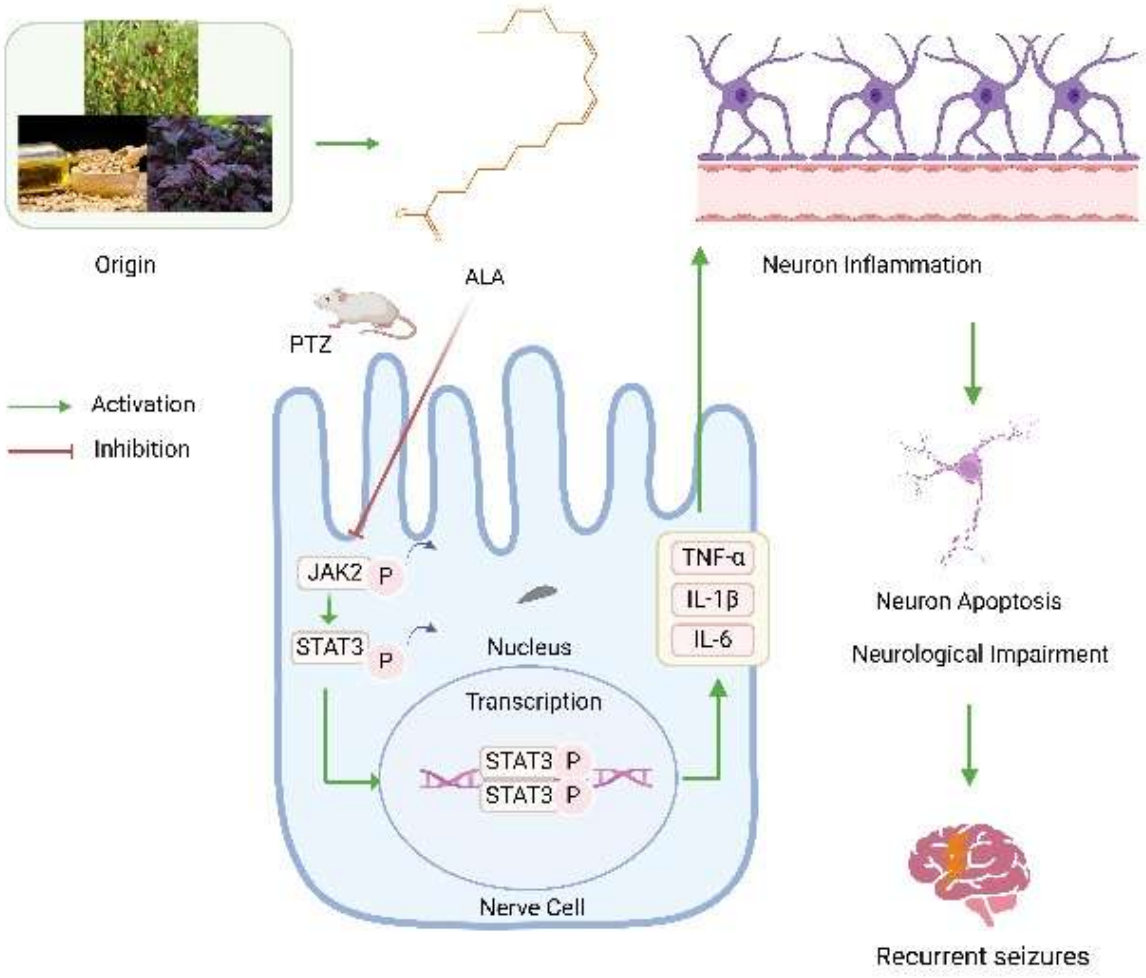


Fig. S2. Immunofluorescence shows that ALA treatment significantly reduces the expression of proteins in signaling pathways. (A-R) Immunofluorescent images depicting p-JAK2 and p-STAT3 expression in the hippocampal CA1 area. Scale bars indicate 200 meters. The graph depicted a quantitative examination of immunofluorescent intensities for p-JAK2 and p-STAT3 (S). Data are mean ± standard deviation (N=3). #p<0.05 and ##p<0.01 versus Control group; *p<0.05 and **p<0.01 versus PTZ group. One-way ANOVA was performed with Bonferroni post-test (S).



Graphical abstract ALA improves epilepsy symptoms by inhibiting the JAK2-STAT3 inflammatory pathway, reducing levels of inflammatory factors such as IL1β, IL6, TNFα, attenuating neuronal apoptosis and damage.

Tab. S1. Binding energy values between ALA and different proteins.

Protein	PDBID	Binding Energy (Kcal/mol)
JAK2	5AEP	-71.35
STAT3	5AX3	-59.98