Article

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The Role of the Orexin System in Craniocerebral Trauma-Induced Epilepsy in Mice

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Background: Following traumatic brain injury (TBI), an imbalance arises in the central nervous system within the hippocampus region, resulting in the proliferation of mossy cell fibers, causing abnormal membrane discharge. Moreover, disruptions in cellular neurotransmitter secretion induce post-traumatic epilepsy. Extensive experimental and clinical data indicate that the orexin system plays a regulatory role in the hippocampal central nervous system, but the specific regulatory effects are unclear. Therefore, further experimental evaluation of its relevance is needed.

Objective: This study aims to investigate the effects of orexin receptor agonists (OXA) on the seizure threshold and intensity in controlled cortical impact (CCI) mice, and to understand the role of the orexin system in post-traumatic epilepsy (PTE).

Methods: Male C57BL/6 mice weighing 18–22 g were randomly divided into three groups: Sham, CCI, and CCI+OXA. The three groups of mice were sequentially constructed with models, implanted with electrodes, and established drug-delivery cannulas. After a 30-day recovery, the Sham and CCI groups were injected with physiological saline through the administration cannulas, while the CCI+OXA group was injected with OXA. Subsequently, all mice underwent electrical stimulation every 30 minutes for a total of 15 times. Epileptic susceptibility, duration, intensity, and cognitive changes were observed. Concurrently, the expression levels and changes of GABAergic neurons in the hippocampus of each group were examined by immunofluorescence.

Results: Injecting OXA into hippocampal CA1 reduces the threshold of post-traumatic seizures, prolongs the post-discharge duration, prolongs seizure duration, reduces cognitive ability, and exacerbates the loss of GABAergic neurons in the hippocampal region.

Conclusions: Based on the results, we can find that injecting OXA antagonists into the CA1 region of the hippocampus can treat or prevent the occurrence and progression of post-traumatic epilepsy.

Keywords: hippocampal CA1; post-traumatic epilepsy; neuronal damage; orexin receptor

Introduction

Post-traumatic epilepsy (PTE) is the most prevalent form of secondary epilepsy, representing a severe complication following brain trauma. Approximately 30%–50% of pediatric epilepsy cases and 20% of adult epilepsy cases are associated with traumatic brain injury (TBI) [1]. The susceptibility to epilepsy significantly rises in patients with severe TBI [2], resulting in a 30-fold higher incidence compared to the general population. Undoubtedly, TBI-induced PTE seriously affects patients' quality of life, with 20% of PTE cases progressing to refractory epilepsy, resisting effective control [3].

The perifornical/lateral hypothalamus secretes two types of orexins, orexin-A and orexin-B. Orexin and its hippocampal receptor-expressing neurons have roles in sleep, learning, and memory regulation [4], and they also play a key role in TBI-induced PTE [5]. There are two types of orexin receptors, with the dental gyrus and CA1 being the primary sites of orexin type 1 receptor (OX1R) expression [6]. Activation of the orexin type 1 receptor induces synaptic responses in the hippocampus which plays a major role in learning, memory, and epilepsy [7]. Currently, the mechanism by which hippocampal orexin and its receptor, orexin type 1 receptor, regulate PTE remains unclear. Some studies suggest that the orexin system can promote the pathological process of epilepsy. For example, orexin receptor blocking can prevent an increase in respiratory rate while simultaneously reducing seizure incidence induced by methylcholine (Mch) in mice [8]. Contrarily, some studies suggest that the orexin system can inhibit epileptic-like electrical activity [9,10]. Orexin receptor agonists (OXA), when added to the CA1 region of the isolated hippocampus, can inhibit spontaneous epileptic-like discharges induced by berberine methionine. In addition, endogenous cannabinoids can bind to cannabinoid receptor 1 (CB1R) and inhibit nerve conduction. Research has shown that various cannabinoid receptor 1 agonists have anti-epileptic effects [11]. Meanwhile, study indicate that CB1R and OX1R have similar action sites and can form isomers [12]. Orexin receptor neurons can release endogenous cannabinoid 2-AG,

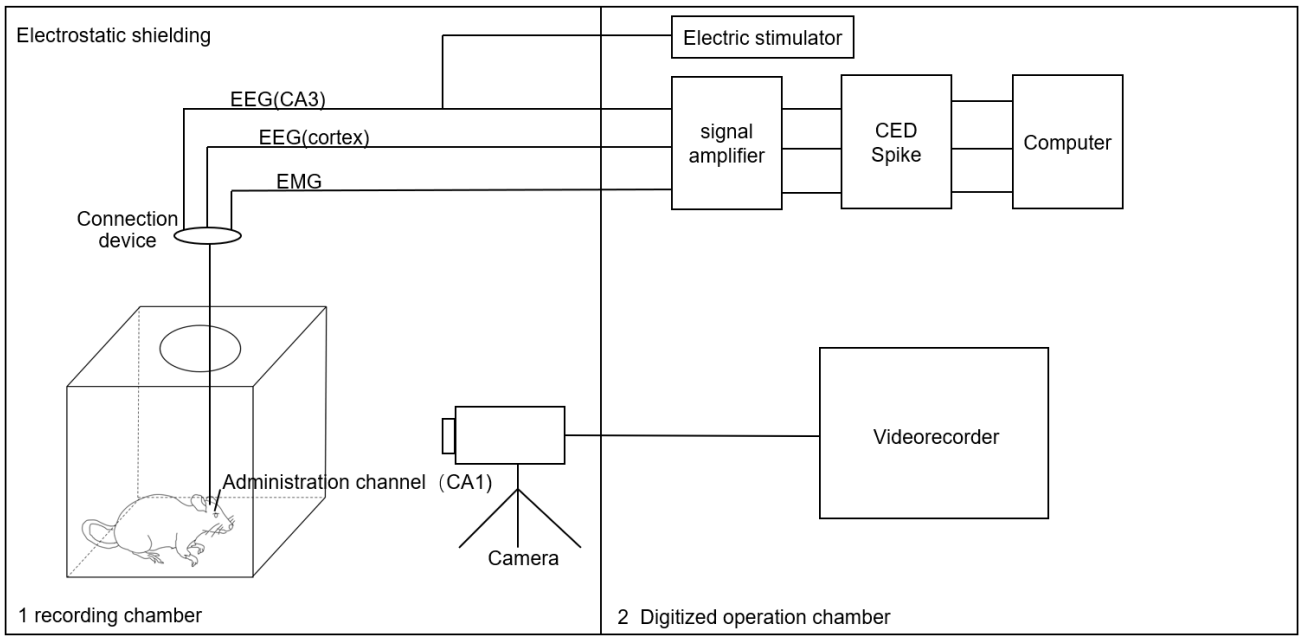

Fig. 1. Mouse electroencephalogram (EEG) recording and analysis system. Room 1 is the mouse EEG recording room. The room is well-ventilated, quiet, and electrostatically shielded, which allows the mice to move freely and eat and drink freely. Recorded electrical signals are sent to Room 2's digital operating room. As soon as the signal is amplified and filtered by the amplifier, it is collected by an analog-to-digital converter, which converts the electrical signal into a digital signal and collects it in a computer for recording and analysis. In addition, the smart camera records and saves the mouse's activities, while the electric stimulator ignites the CA3 region of the mouse hippocampus (The figure was drawn by Adobe Photoshop 2021, Adobe, San Jose, CA, USA).

which acts on presynaptic CB1R and activates phospholipase C to exert synergistic regulatory effects. OXA has been found to inhibit spontaneous epileptic discharge induced by bicuculline methionine and reduce the firing frequency of neurons in CA3, suggesting an anti-epileptic effect [13]. Therefore, to clarify the role of the orexin system in PTE, we electrically stimulated the CA1 region of the hippocampus in the mentioned mice groups, simulated post-traumatic seizures, and evaluated OXA's effects on seizures and emotions in mice.

Materials and Methods

Experimental Animals and Groups

Twenty-four male C57BL/6 mice (4–6 months old, weighing 18–22 g) were procured from Lanzhou University, adhering to specific criteria. All mice were kept in a controlled environment with a 12-hour light-dark cycle, 180 Lux of light, and a temperature of 23 °C, with free access to food and water. Animals were divided into three groups, with eight animals in each group: ① Sham operation group: only the skull was removed from the surgical area, ② controlled cortical impact (CCI) group, and ③ CCI+OXA group: the CA1 region of the hippocampal cortex was injected with OXA. Euthanasia was performed within 2 days post-experiment using pentobarbital sodium, ensuring a dose not less than 250 mg/kg for humane death, followed by proper waste disposal at the Gansu Provincial Hazardous Waste Center. During the experiment, measures were taken to the well-being of mice, including sufficient diet and rest to reduce pain and malignant stimuli. Ethical considerations were upheld by obtaining approval from the Experimental Animal Welfare Ethics Committee of Lanzhou University Second Hospital (Approval number: D2022-190).

Instruments and Reagents

The brain stereotaxic instrument (RWD, 68030, Shenzhen, China) and analog-to-digital converter CED1401 MK II (Cambridge Electronic Design, CED, London, UK) were purchased for this study. The mouse electroencephalogram recording and analysis system [14] was sourced from the Institute of Neuroscience, Lanzhou University, as shown in Fig. 1. Additionally, Orexin-A (205640-90-0, TOCRIS, Bristol, UK) and recombinant Anti ABAT/GABA-T antibody [EPR20842] (Abcam ab216465, Cambridge, UK) were acquired for the present study.

Mouse CCI Model

Experimental mice were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) and fixed in a prone position on the striking platform, ensuring protection against non-experimental injuries. Each mouse's position was stabilized using ear rods and elastic mouth

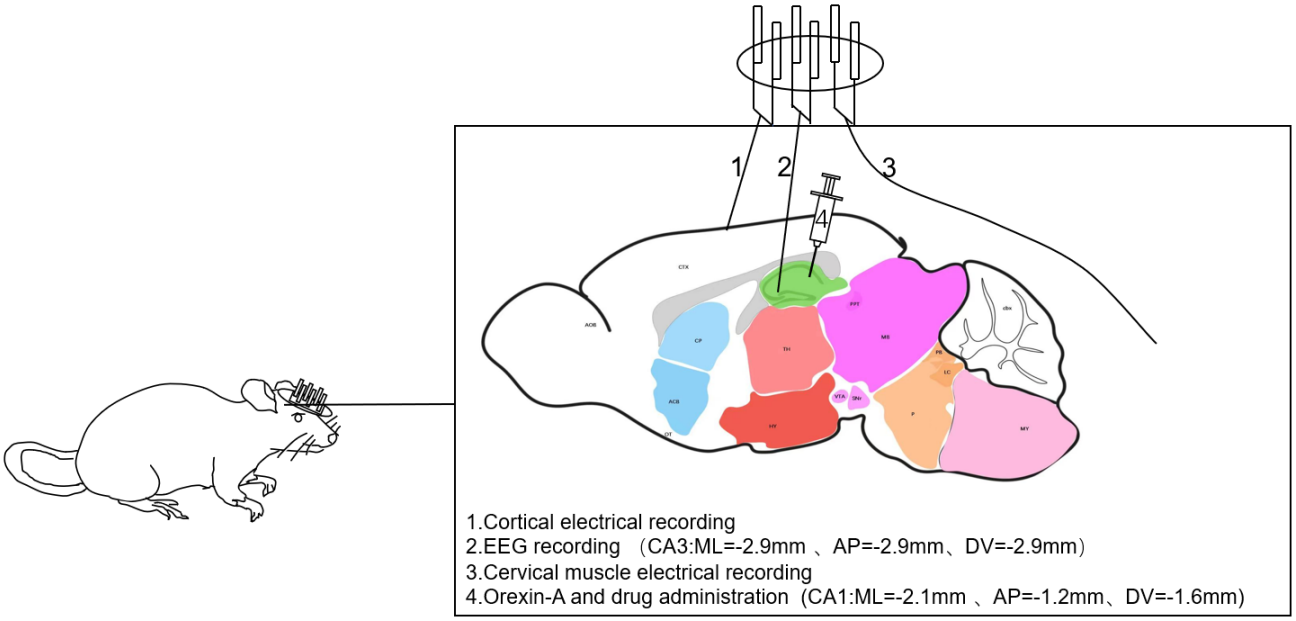

Fig. 2. Reference map of the location of relevant nuclei and regions in mice. (1) Implantation of EEG electrodes in the cerebral cortex of mice. (2) Implantation of EEG electrodes in the CA3 nucleus of the mouse hippocampal cortex. (3) Implantation of Electromyography (EMG) electrodes in mouse cervical muscles. (4) Implantation of a cannula in the mouse CA1 area (The figure was drawn by Adobe Photoshop 2021, Adobe, San Jose, CA, USA).

fixers. Once fixed, hair was removed from the surgical area and disinfected with 75% alcohol. The right cranial parietal bone was fully exposed by cutting off the scalp, gently peeling away the subcutaneous tissue, and removing the periosteum from the skull surface. Assuming the Bregma (anterior fontanel) point as the coordinate system origin with a brain stereotaxic instrument, a window was gently drilled in the right parietal bone (ML = 2.5 mm, AP = –2.5 mm) with a 4.0 mm diameter, while leaving the dura mater intact. The brain stereotactic device (Jialichuang, BACA-C2G1-D16-L1050, Shenzhen, China) was vertically connected to a sterile solid round rod with a 3.0-mm diameter. Vertical axis parameters of the stereotactic instrument were set (DV = –2.0 mm), and the initial velocity upon contact with the dura mater was set to 1.5 m/s, to complete the impact [15]. Following impact, a sterile cotton ball was used to cover the surface of the damaged brain tissue to stop bleeding. The wound was sutured upon bleeding cessation. Mice were placed on an electric blanket at a constant temperature and then raised for recovery in the cage after regaining consciousness. Daily observations on postoperative conditions were conducted, allowing for a 30-day recovery period before future experiments. The modeling process proceeded smoothly, with 96% of mice completing the modeling and meeting the modeling requirements. Mice that failed modeling were euthanized according to the method described earlier in the article. ML is the X-axis, AP is the Y-axis, and DV is the Z-axis.

Mouse Head Cannula, Head and Neck Electroencephalogram (EEG)/Electromyography (EMG) Electrode Implantation

Following a 30-day recovery from the CCI surgery model, an intraperitoneal sodium pentobarbital (50 mg/kg) injection was administered for anesthesia. Hair was removed from each mouse's surgical area and secured to a stereotaxic instrument. The surgical area was cleaned with 75% alcohol, followed by the gentle removal of the scalp's subcutaneous tissue. The anterior and posterior fontanels, coronal suture, sagittal suture, and lambdoid suture were exposed after removing the periosteum on the skull surface. A stereotaxic device was used to ensure the anterior and posterior fontanelles were at the same height. Using the Fraklin and Paxinos mouse brain atlases, the relevant nuclei were localized in three dimensions (Fig. 2).

Mouse CA1 Area Implanted with a Cannula

Using the Bregma point as the reference (0 point), a cannula (0.25 mm, 304-316L, Baogang, shanghai, China) was embedded into the CA1 area (ML = –2.1 mm, AP = –1.2 mm, DV = –1.6 mm) of the hippocampus and fixed with dental cement for subsequent drug administration.

Implantation of EEG Electrodes in the Cerebral Cortex of Mice

Drilling was performed on all animals at the point of intersection 1 mm in front of the Bregma point and 1 mm to the right of the midline. Additionally, an incision was made at the intersection of 1 mm in front of Lambda (pos-

terior fontanel) and 1mm lateral to the skull's midline. The EEG electrode should be positioned deep enough to penetrate the skull and contact the dura mater. The screw for the electrode, designed to collect cerebral cortex electricity, was attached to the skull drill hole, and the electrode was secured with dental cement.

Implantation of EEG Electrodes in the CA3 Nucleus of the Mouse Hippocampal Cortex

Using the Bregma point as the origin of the coordinate system, holes were drilled in the CA3 nuclear area (ML = –2.9 mm, AP = –2.9 mm, DV = –2.9 mm). An EEG electrode was installed and fixed with dental cement to facilitate electrical stimulation of the mice and record hippocampal activity.

Implantation of EMG Electrodes in Mouse Cervical Muscles

The myoelectric activity was recorded by two electromyographic collection electrodes buried between the neck muscles.

Hippocampal CA3 Rapid Electric Ignition Model

A week after the implanted electrodes were restored, the experimental mice's EEG was recorded using the Spike2 system (Spike2, CED, London, UK). The first step involved determining each animal's after-discharge threshold (ADT). The stimulation parameters included a continuous wave with 60 Hz frequency and 2 s duration. Starting at 50 A, the stimulation current increased every minute until three after-discharge signals larger than 5 seconds were induced. The current at which this occurred represented the animal's ADT. Mice with a discharge threshold of 200 μA ± 10 μA were selected to reduce mouse individualization errors. The hippocampus CA3 post-discharge threshold was stimulated 15 times a day at 30-minute intervals, each lasting two seconds. Mouse's behavior was observed and brain electricity was recorded. Electrical ignition was noted when the mouse's epilepsy level reached Level V three times in a row [16]. To determine the level of epileptic seizures based on behavior, we used the Racine scale standard: Level 0: No response; Level I: Facial clonus; Level II: Rhythmic nodding; Level III: Single limb twitching, forelimb clonus; Level IV: Multiple limb twitching; Level V: Generalized tonic-clonic seizure, loss of postural control [17].

Nissl Staining to Screen the Nuclear Injection Site

Following an intraperitoneal injection of sodium pentobarbital (50 mg/kg), mice received cardiac perfusion with 4 °C 0.9% normal saline. After the liver turned white, the head was decapitated, and the brain was then extracted. Subsequently, the brain was fixed for two hours and preserved in a 30% sucrose solution until it was adequately dehydrated. Frozen sections were prepared, and Nissl stain was applied through two 15-minute immersions in the staining solution, followed by three 5-minute washes with pure water. The sections were dehydrated using ethanol concentrations of 75%, 95%, and 100%, and subsequently immersed in xylene to achieve transparency. Finally, the sections were sealed with neutral gum. The positioning of the cannula tip was validated using a microscope, and any data lacking the appropriate tip placement was excluded.

Open Field Test (OFT) Experiment

Prior to conducting the experiment, the experimental animals underwent a daily 20-minute adaptation process to acclimate to the testing environment for three consecutive days. Following the adaptation period, the animals were placed into the designated test box and subjected to testing based on the software parameters, which included information such as the animal's identification number, date of testing, and status. Afterward, we observed and recorded the total distance data of their movements within a 5-minute period. Additionally, the equipment was thoroughly cleaned between experiments to prevent any potential contamination from animal residues.

Object Recognition Test (ORT) Experiment

Prepare three cubes with a side length of 5 cm, labeled as A, B, and C. The colors A and B are red, and C is yellow. Then position the mouse with its back facing the cubes A and B. Release the mouse to move freely and start recording the contact between the mouse and the object. This recording was conducted within a five-minute timeframe. Subsequently, after the mouse had rested for one hour, object B was substituted with object C, and contact between the two objects within a five-minute interval was recorded. In this step, the recognition index (RI) and cognitive abilities of the mice were calculated. RI = new object/(new object + old object).

Hippocampal Region Fluorescence Immunity

Frozen sections of the hippocampus were utilized, which were dewaxed and sterilized before adding pepsin and incubating for 15 minutes. Following three washes, the cells were dehydrated using ethanol and subsequently dried. The fluorescently labeled gamma-aminobutyric acid receptor was pre-denatured at a temperature of 85 °C for five minutes, followed by overnight incubation at 37 °C before introducing the probe. The next day, samples were washed three times, dried in a light-restricted environment, and subjected to 4',6-diamidino-2-phenylindole (DAPI) counterstaining. The fluorescence intensity was then observed using a fluorescence microscope, and the results were subsequently analyzed.

Statistical Methods

Blind analysis and statistical significance calculations were performed using GraphPad Prism 9.51 (Dotmatics,

Boston, MA, USA). Both groups were compared using the independent samples *t*-test. Parametric tests, such as one-way Analysis of variance terminology (ANOVA) or Welch's ANOVA, were used for multiple group comparisons if the data adhered to a normal distribution. $p < 0.05$ was set as the level of statistical significance, and experimental data were described as mean ± standard error of the mean (SEM).

Results

Comparison of EEG between Normal Mice and Simulated PTE Mice

To accurately simulate post-traumatic epilepsy in mice, we subjected them to hippocampal CA3 rapid electrical ignition treatment. CCI mice underwent 10 daily stimulations with threshold current after hippocampal CA3 discharge, each lasting two seconds with a 30-minute interval for 3 days. During the electric ignition period, mouse behavior was observed, and their electroencephalograms were recorded and compared with those of normal mice to confirm the simulation of PTE seizures. Among them, we mainly recorded the electroencephalogram of the hippocampal CA3 region and the cerebral cortex in mice, as shown in Fig. 3. The horizontal axis of the ABCD four graphs represents time (S), and the numerical axis represents the amplitude of the potential (Volt) (Fig. 3A,B). A normal mouse cortical electroencephalogram and hippocampal CA3 region electroencephalogram, recording normal life activity waveforms [18] (Fig. 3C,D). The waveform recorded by the cerebral cortex electroencephalogram and hippocampal CA3 electroencephalogram of CCI mice during electrical ignition exhibited significant differences from normal mice. In the recorded electroencephalograms of the hippocampal CA3 region and the cerebral cortex, the electrical signal of the post-discharge threshold current stimulation (region a of Fig. 3C,D) for two seconds is first recorded. Subsequently, the post-discharge potential [19] was recorded, which exhibited abnormally high frequency and amplitude. The emergence of this potential coincided with observable epileptic behavior in the mice, graded according to the Racine scale standard (region b of Fig. 3C,D), classified as Level V on the Racine scale. Finally, the post-suppression potential was measured, indicating a low-amplitude suppression signal (region c of Fig. 3C,D) appearing after the cessation of the high-frequency and high-amplitude electrical signal. The amplitude of this electrical signal was even lower than the electrical signal recorded during normal life activities (Fig. 3E). An electrical signal was recorded during normal mouse neck muscle activity, with a low amplitude and regular frequency (Fig. 3F). The electrical signal was recorded during neck muscle activity in mice during electric ignition, with a higher amplitude and irregular frequency compared to normal mice.

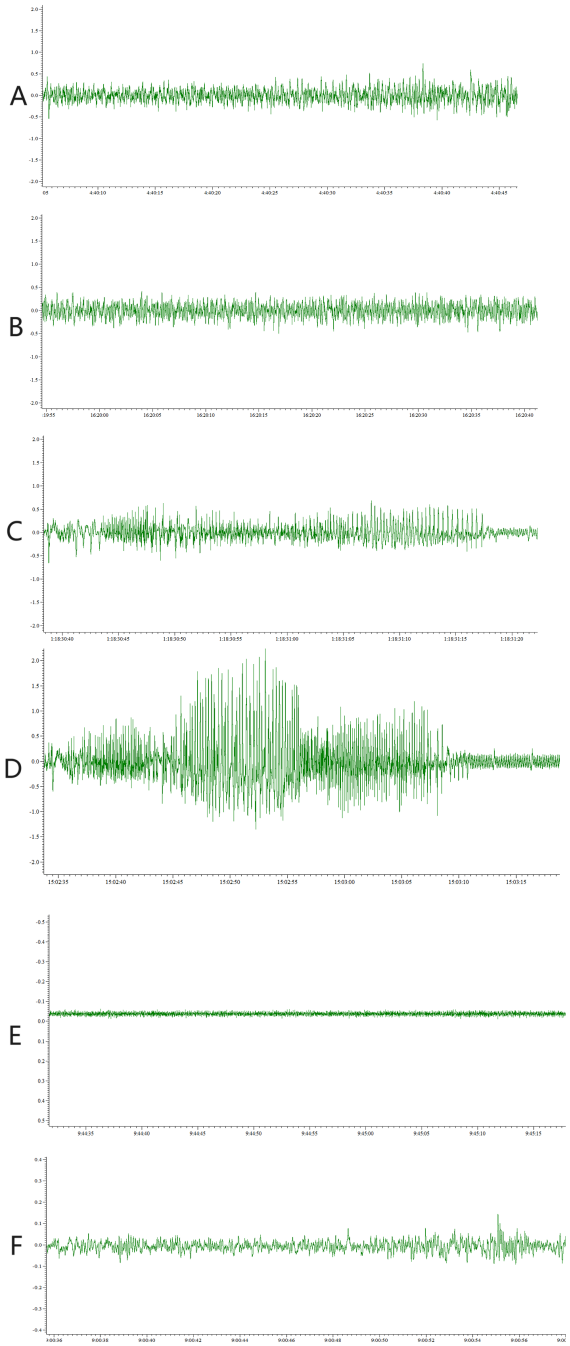

Fig. 3. Comparison of EEG between normal mice and controlled cortical impact (CCI) mice. (A) Normal mouse cortical electroencephalogram. (B) Normal mouse hippocampal CA3 region electroencephalogram. (C) Electroencephalogram of cerebral cortex in CCI mice during electric ignition. (D) Electroencephalogram of hippocampal CA3 in CCI mice during electric ignition. (E) Normal mouse neck muscle activity electrical signal. (F) Electrical signals of neck muscle activity in CCI mice during electric ignition. The above EEG was analyzed and recorded using the Spike2 system (Spike2, CED, London, UK).

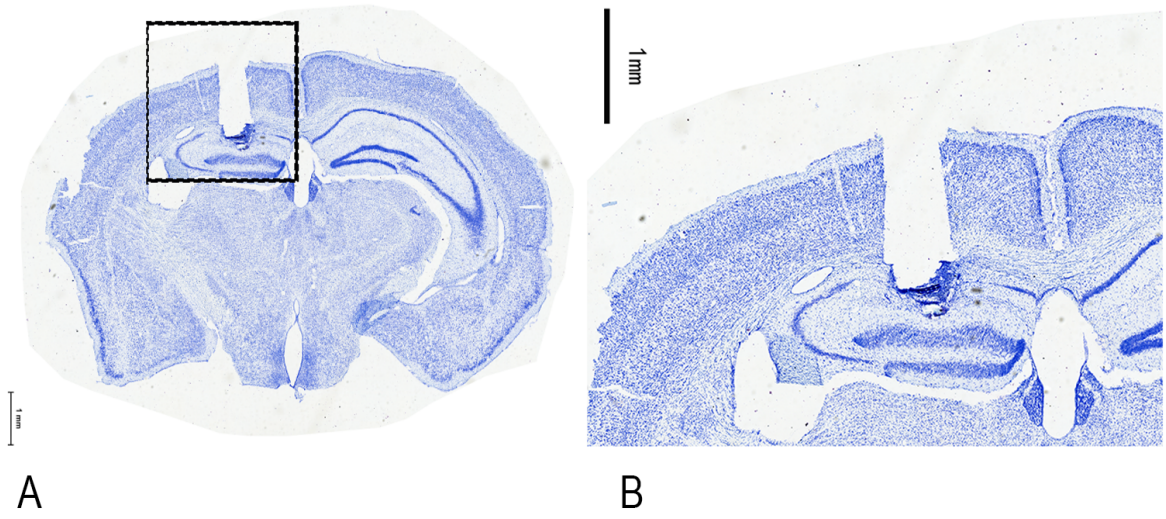

Fig. 4. Verification of the CA1 nuclear injection. (A) Nissl staining verifies the injection site of the drug delivery channel (Compared with Fraklin-Paxinos mouse brain atlas CA1 region [20]). (B) Magnified view of the dashed box.

The Nucleomass Injection Location Was Verified with Nissl Staining

The nuclear injection site was verified using Nissl staining, and CCI mice with accurate locations were included in the analysis. Results showed that the injection location of the CA1 nucleus was accurate and consistent with the published literature [20] (Fig. 4).

The Effect of OXA on Epilepsy Susceptibility in CCI Model Mice

The susceptibility of mice to epilepsy was assessed based on the number of electrical stimuli needed to induce an epileptic state, with a lower count indicating higher susceptibility. The results showed that the numbers of electrical stimuli required for the three groups of mice to reach the ignited state were (11.38 $\pm$ 2.26), (6.25 $\pm$ 1.16), and (3.63 $\pm$ 0.74) times for the Sham, CCI, and CCI+OXA groups, respectively (Fig. 5A). The CCI group ignited mice using the hippocampal CA3 rapid electrical kindling model, requiring less electrical stimulation than mice in the Sham group ($p < 0.0001$). The CCI+OXA group mice significantly reduced the number of required electrical stimuli when compared to the CCI group ($p < 0.01$). This suggests that the Sham, CCI, and CCI+OXA groups exhibit decreasing thresholds for external stimulation leading to epileptic seizures, with OXA notably enhancing susceptibility and promoting epileptic behavior.

The Effect of OXA on the Duration of Post-Discharge in CCI Model Mice

Post-discharge refers to the spontaneous synchronous discharge of relevant neurons, specifically in the hippocampal CA3 region, in experimental mice after the cessation of electrical stimulation. The post-discharge potential is consistent with epileptic discharge and can diffuse to surrounding brain tissue. Longer post-discharge durations indicate prolonged and more severe induced epilepsy. The post-discharge durations of the Sham, CCI, and CCI+OXA groups were (34.83 $\pm$ 6.32), (46.98 $\pm$ 5.98), and (61.54 $\pm$ 3.63) seconds, respectively. The CCI group exhibited a longer post-discharge duration than the Sham group ($p < 0.001$), and the CCI+OXA group had a longer duration than both other groups ($p < 0.0001$). The above results suggest that OXA can exacerbate the severity of seizures in mice (Fig. 5B).

The Effect of OXA on the Duration of Epileptic Seizures in CCI Model Mice

Following the conclusion of the post-discharge potential, mice transition into the epileptic seizure phase, characterized by observable epileptic EEG patterns and behaviors (Fig. 5C). Seizure durations for the Sham, CCI, and CCI+OXA groups were (22.88 $\pm$ 3.88), (34.24 $\pm$ 3.65), and (49.79 $\pm$ 4.20) seconds, respectively. The CCI group exhibited prolonged seizure duration ($p < 0.0001$). Compared with the CCI group, the duration of epileptic seizures in the CCI+OXA group was once again prolonged ($p < 0.0001$). This result indicates that OXA can prolong the duration of seizures in CCI mice.

The Effect of OXA on Hippocampal Cells of PTE in CCI Model Mice

After injecting OXA into the CA1 region of CCI model mice, we found that the cell layers of the hippocampal dentate gyrus and CA123 region of the model mice without OXA injection showed clear staining textures, tightly arranged rules, and orderly layered distribution. In contrast, model mice injected with OXA displayed abnormal cell layer structures in these hippocampal regions, notably characterized by the diffusion and widening of granulosa cells.

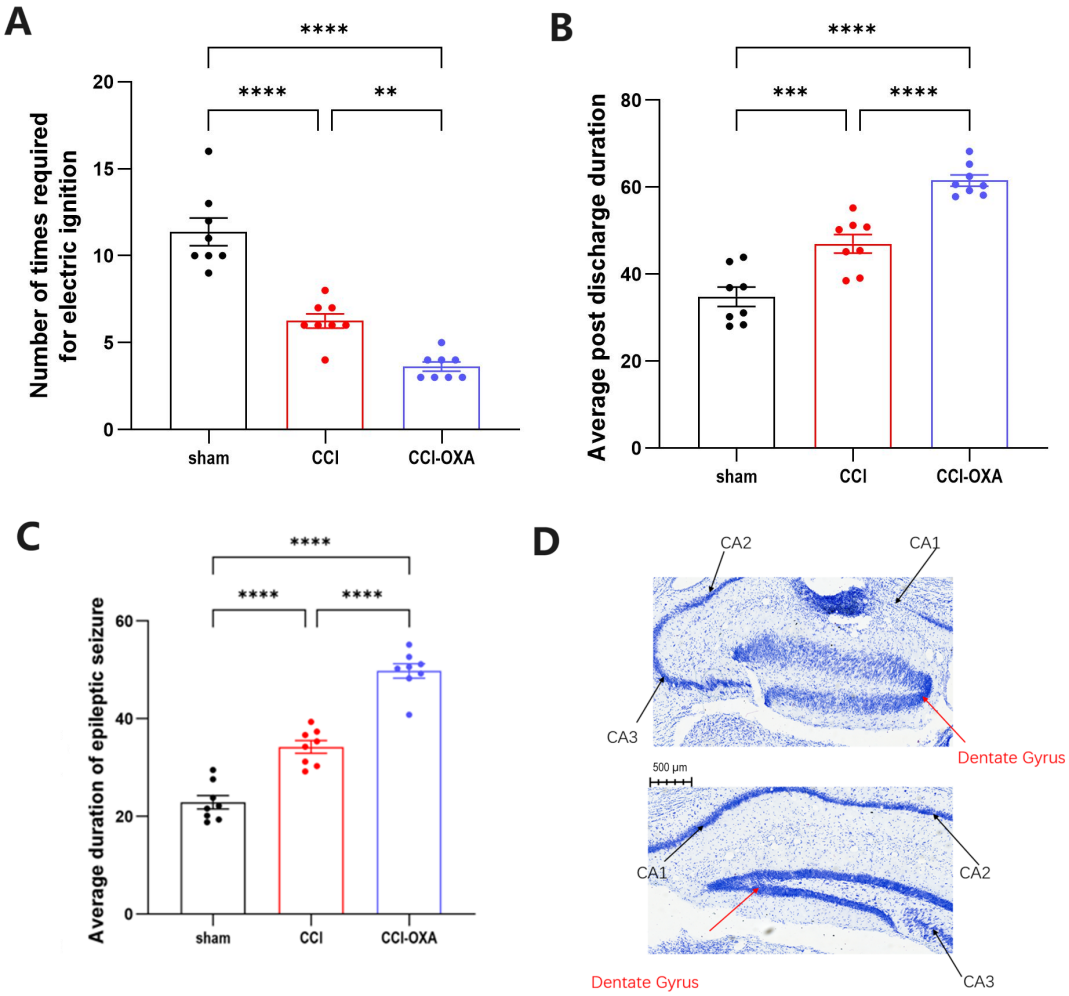

Fig. 5. The effect of orexin receptor agonists (OXA) on CCI model mice. (A) The effect of injection of Orexin-A into the CA1 region on the susceptibility to epilepsy in CCI model mice. (B) The effect of Orexin-A on the duration of post-discharge in CCI model mice. (C) The effect of Orexin-A on the duration of epileptic seizures in CCI model mice. (D) Orexin-A induced changes in hippocampal cells of post-traumatic epilepsy (PTE) in CCI model mice. Each tree graph represents mean ± standard error of the mean (SEM). Each group has n = 8, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

This observed effect suggests that OXA promotes epileptic seizures and disrupts the normal migration of granulosa cells, resulting in blurred boundaries between the granulosa layer and molecules, and a significantly wider width of granulosa cells in the dentate gyrus compared to the control group (Fig. 5D).

The Effect of OXA on the Movement and Cognitive Abilities of CCI Model Mice

The OFT and ORT experimental results established a total distance within 5 minutes was (1164.46 ± 7.04), (771.33 ± 37.20), and (366.31 ± 1.69) cm for the Sham, CCI, and CCI+OXA groups, respectively (Fig. 6A). Notably, CCI caused a decrease in mouse vitality ($p < 0.001$), which was further exacerbated by OXA ($p < 0.0001$). Moreover, the cognitive ability index also gradually decreased across the three groups: (0.76 ± 0.03), (0.51 ± 0.02), and (0.37 ± 0.04) (Fig. 6B). This indicates that CCI can reduce the cognitive ability of mice ($p < 0.001$), and injection of Orexin-A into the CA1 region can exacerbate the cognitive decline ($p < 0.01$).

The Effect of OXA on GABAergic Neurons

According to Nepovimova's [21] research, the Orexin system can regulate the function of the central nervous system by interacting with the GABAergic neuronal system . Therefore, to clarify how OXA affects GABAergic neurons during the seizure process of CCI mice, we counted GABAergic neurons in the hippocampus and observed the changes in the number of GABAergic neurons

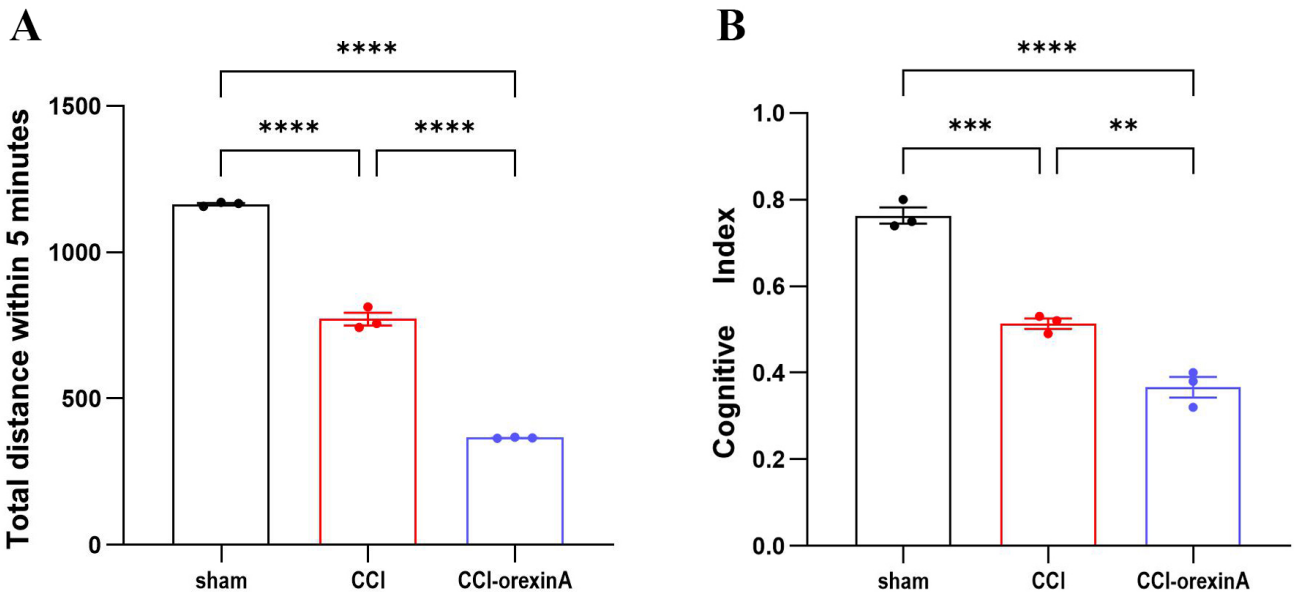

Fig. 6. Statistical chart of behavior comparison among different groups of mice. (A) The effect of injection of OXA into the CA1 region on mouse movement. (B) The effect of Orexin-A on cognitive ability in mice. Each tree graph represents mean ± SEM. Each group has n = 3, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

(Fig. 7A). The results revealed a notable decrease in the number of GABA neurons in the hippocampus of the CCI group compared to the Sham group ($p < 0.01$). Additionally, the CCI+OXA group exhibited a lower number of GABA neurons in the hippocampus compared to the CCI group (Fig. 7B) ($p < 0.05$). Therefore, we believe that OXA amplifies the loss of GABAergic neurons post-CCI, weakens the inhibitory effect of GABA on abnormal neuronal excitability, and thus exacerbates the seizure status of PTE.

Discussion

PTE involves a brain network alienation process, with the limbic system playing an important role in the development of PTE [22]. Notably, there is a growing focus on pathological alterations in the cell population of the hippocampal formation following trauma.

Substantial evidence suggests that orexins have excitatory effects on the central nervous system, and they may even induce seizures. For example, Kortunay [23] found that the injection of 100 pmol of OXA into the cortex can induce seizures and significantly enhance the spike number and amplitude induced by cortical penicillin and epileptic activity. Conversely, numerous studies have shown that blocking the Orexin receptor with Orexin receptor antagonists yields opposite outcomes. For example, Kordi injected the Orexin receptor antagonist SB-334867 into the lateral ventricle of Pentetrazol (PTZ) induced epileptic rats and found that the Orexin receptor antagonist can reduce the intensity of epileptic seizures in PTZ-induced epileptic rats, shorten the duration of each seizure phase [24], and improve their anxiety-like behavior [25]. In further research, Socala [26] induced epilepsy in mice through maximum electric shock and intravenous injection of PTZ, and the injection of SB-334867 into the lateral ventricle significantly increased the seizure threshold induced by PTZ. The mechanism of its occurrence may be related to SB-334867 blocking hippocampal OX1R, reducing total glutamate in the hippocampus and increasing total GABA content [27].

The two main inhibitory and excitatory neurotransmitters in the central nervous system are GABA and Glutamate. There is a common belief that the imbalance between excitation and inhibition, representing a disorder of neurotransmitter metabolism, is a key factor in inducing epilepsy. OXA is speculated to induce neuronal excitation by regulating the transmission of glutamate, GABAergy, aminergic, and cholinergic energy [28]. In this study, the susceptibility of CCI model mice to epilepsy significantly increased compared to the Sham group mice. After the CA1 injection of OXA, the severity of epileptic seizures increased. Moreover, immunofluorescence results showed a significant decrease in GABAergic neurons in the hippocampus. Therefore, we have reason to believe that OXA weakens the inhibitory effect of the central nervous system by reducing the number of GABA neurons, enhances the excitability of the central nervous system, and consequently exacerbates seizures.

It is noteworthy that, apart from GABAergic neurons, the regulation of the central nervous system is also related to glutamate, amine, and cholinergic systems. Kodama's [29] research demonstrated that intravenous injection of OXA can cause Glu release in the locus coeruleus region. Additionally, Kaslin *et al.* [30] showed that the ORX system can innervate the raphe, locus coeruleus, pontine area,

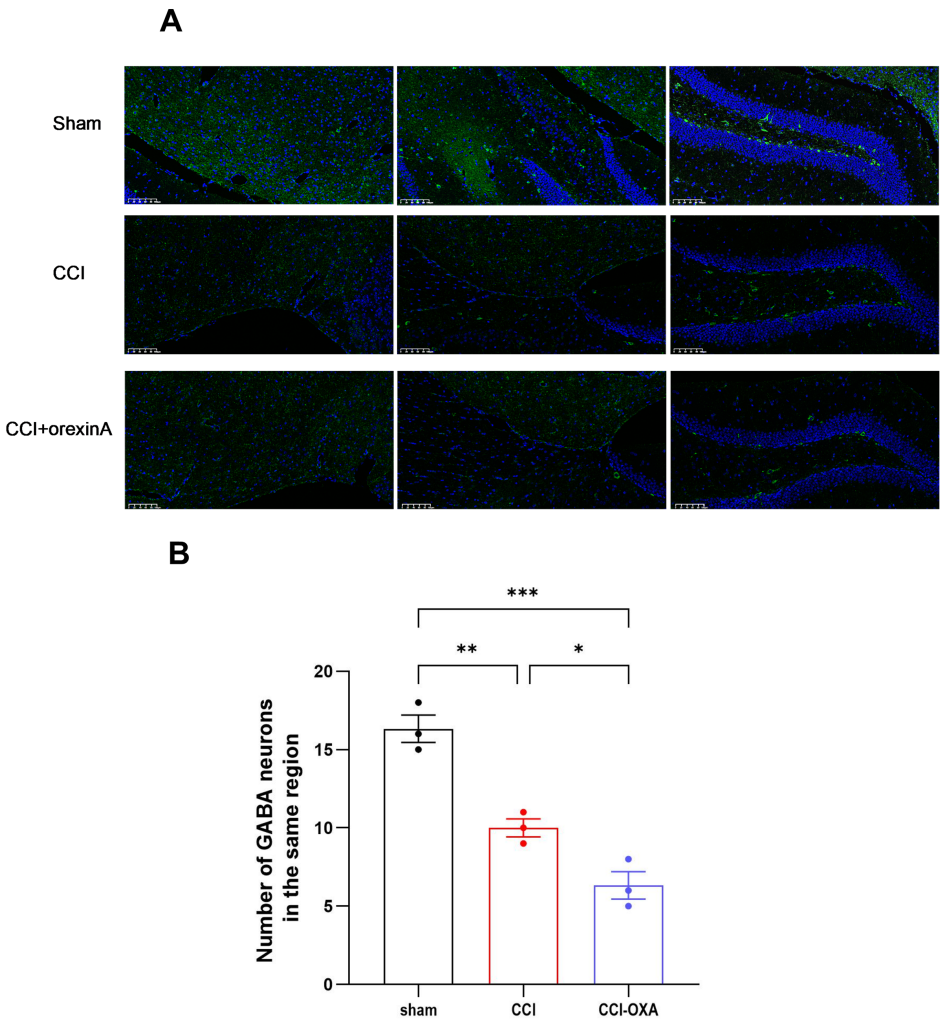

Fig. 7. Map of GABAergic neurons in the hippocampus of different groups of mice. (A) Immunofluorescence maps of GABAergic neurons in the same area of the hippocampus of mice in the Sham, CCI, and CCI+OXA groups (sharing the same scale, scale bar = 100 μm). (B) Statistical maps of GABAergic neurons in the same area of the hippocampus of mice in the Sham, CCI, and CCI+OXA groups. Each tree graph represents mean ± SEM. Each group has n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

dopaminergic, and histaminergic neurons to regulate wakefulness and central nervous system homeostasis. Regarding cholinergic, Ferrari [31] believes that there are prerequisites for Orexin-A to regulate cholinergic to regulate central nervous system homeostasis, which depends on the membrane potential of basal forebrain neurons. At –40 mV, the net effect of Orexin-A is to inhibit cholinergic activity through dynorphin-A. At a membrane potential of –70 mV, Orexin-A dominates the cholinergic excitability.

Of note, this experiment only studied the effect of OXA on GABAergic neurons in the hippocampus and did not involve GABAergic neurons in other parts of the central nervous system. Moreover, limited research exists on other central neurotransmitters and neurons. Current and previous studies have shown that OXA is a neuropeptide that promotes post-traumatic epilepsy, and OX1R antagonists can serve as potential candidate drugs for the prevention and treatment of PTE. At the same time, more research experiments are needed to clarify the precise role of the Orexin system in regulating the release of various neurons and their neurotransmitters, and its role in PTE.

Conclusions

In this experiment, we simulated human TBI to establish a controlled cortical injury model in mice. Subsequently, we induced seizures in mice through hippocampal CA3 electrical kindling, with the experimental group receiving OXA injection into the hippocampal CA1 region. During this process, we found that CCI increased the susceptibility of experimental animals to seizures, reducing the threshold and increasing seizure duration. Additionally, be-

havioral experiments assessing emotion and cognition revealed that CCI led to diminished cognitive abilities in experimental mice, manifesting in reduced mood and signs of depression. These findings are consistent with the symptoms observed in patients with PTE induced by TBI [32]. After further exploring the potential mechanism of injecting OXA into the CA1 region of the hippocampus to regulate seizures, the experimental results showed several key observations. OXA demonstrated the ability to further reduce the seizure threshold after CCI, prolong the seizure duration, prolong the post-discharge duration, and exacerbate both depression and post-traumatic PTE seizures. These findings align with the earlier mentioned results of OXA enhancing CA1 spontaneous epileptic discharge. Furthermore, the experimental results showed a decrease in the number of GABA neurons in the hippocampus of CCI model mice. Moreover, after the injection of OXA, the number of GABAergic neurons in the hippocampus further decreased. Therefore, we believe that OXA can reduce GABAergic neurons in the hippocampus after CCI, thereby weakening the inhibitory effect of GABA in the neural network and promoting seizures.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

KFZ is the first author responsible for experimental design, data analysis, paper writing, and all aspects of the experiment. GZY is mainly responsible for partial data analysis of this experiment and making critical revisions to the paper. BRH is mainly responsible for improving experimental design, data collection acquisition and revising the paper. HJR is mainly responsible for data analysis, interpretation, discussion of results and revising the paper. All authors have read and approved the final version, and agree to be responsible for all aspects of the work.

Ethics Approval and Consent to Participate

Ethical considerations were upheld by obtaining approval from the Experimental Animal Welfare Ethics Committee of Lanzhou University Second Hospital (Approval number: D2022-190).

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Conflict of Interest

The authors declare no conflict of interest.

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