

Activation of extrasynaptic GABA_A receptors inhibits cyclothiazide-induced epileptiform activity in hippocampal CA1 neurons

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ABSTRACT

Extrasynaptic GABA_A receptors (GABA_ARs)-mediated tonic inhibition is reported to involve in the pathogenesis of epilepsy. In this study, we used cyclothiazide (CTZ)-induced *in vitro* brain slice seizure model to explore the effect of selective activation of extrasynaptic GABA_ARs by 4,5,6,7-tetrahydroisoxazolo[5,4-c] pyridine-3-ol (THIP) on the CTZ-induced epileptiform activity in hippocampal neurons. Perfusion with CTZ dose-dependently induced multiple epileptiform peaks of evoked population spikes (PSs) in CA1 pyramidal neurons, and treatment with THIP (5 μmol/L) significantly reduced the multiple PS peaks induced by CTZ stimulation. Western blot showed that the δ-subunit of the GABA_AR, an extrasynaptic specific GABA_AR subunit, was also significantly down-regulated in the cell membrane 2 h after CTZ treatment. Our results suggest that the CTZ-induced epileptiform activity in hippocampal CA1 neurons is suppressed by the activation of extrasynaptic GABA_ARs, and further support the hypothesis that tonic inhibition mediated by extrasynaptic GABA_ARs plays a prominent role in seizure generation.

Keywords: GABA_ARs; tonic inhibition; epilepsy; population spike; cyclothiazide; hippocampal CA1 neurons

INTRODUCTION

Epilepsy is a common neurological disorder, and various

factors such as brain trauma, infection, and genetic factors contribute to its pathogenesis^[1]. This disorder entails abnormal behavior caused by sudden, overriding, and synchronized electrical activity of certain neuronal groups in the central nervous system. Although the exact mechanisms that lead to this abnormal firing are not yet fully understood, a functional imbalance of GABAergic inhibition and glutamatergic excitation is considered to be one of the fundamental etiologies.

Hitherto, many antiepileptic drugs have targeted GABA_A receptors (GABA_ARs)^[2–4]. Current studies show that GABA_ARs are present in, but not confined to the synapse; they are also abundant at extrasynaptic sites, although these receptors contain different subunits^[5, 6]. Synaptic GABA_ARs have relatively a low affinity for GABA, and are principally activated by neurotransmitters released into the perisynaptic space, mainly mediating fast synaptic inhibition. Conversely, extrasynaptic GABA_ARs have a high affinity for GABA and are persistently activated by low concentrations, resulting from the extrasynaptic leakage of the neurotransmitter, and mediate ‘tonic’ inhibition. The δ-subunit-containing GABA_AR is the major extrasynaptic form, particularly localized in the hippocampal area and the cerebellum^[7, 8]. Extrasynaptic GABA_ARs are not sensitive to most of the benzodiazepines; however, they are highly sensitive to 4,5,6,7-tetra-hydroisoxazolo[5,4-c] pyridine-3-ol (THIP)^[5, 9]. The role of synaptic GABA_AR-mediated phasic inhibition in epileptogenesis has been well investigated^[10, 11], yet recent research efforts have also revealed that extrasynaptic GABA_AR-mediated tonic inhibition plays

an equivalent or even more critical role in the regulation of epilepsy^[12, 13]. Moreover, clinical studies have shown that there is a significant down-regulation of δ -subunit-containing GABA_ARs in brain samples from patients with temporal lobe epilepsy^[14], and that mutation of the δ -subunit is one of the pathogenic mechanisms of epilepsy^[15]. Our recent study demonstrated that enhancing tonic inhibition by overexpressing either the α 5- or the δ -subunit-containing extrasynaptic GABA_ARs substantially inhibits the formation of epileptiform activity in hippocampal cultures. Furthermore, the injection of the selective extrasynaptic GABA_AR agonist THIP inhibits both epileptiform burst activity in anesthetized rats and seizure behavior in freely-moving rats^[12, 13].

In this work, we further investigated whether δ -subunit-containing GABA_ARs were deficient during cyclothiazide (CTZ)-induced epileptogenesis in hippocampal brain slices, and whether activation of the extrasynaptic GABA_ARs could reverse the CTZ-induced epileptiform activity.

MATERIALS AND METHODS

Experimental Animals and Hippocampal Slice Preparation

Brain slices were prepared from P21–28 male Sprague-Dawley rats provided by the Shanghai Institutes for Biological Sciences Experimental Animal Center. The rats were housed in a regulated environment ($22 \pm 1^\circ\text{C}$) with a 12 h light–dark cycle, and food and water were available *ad libitum*. All experiments were carried out in accordance with the local animal protection law, and approved by the Experimental Animal Ethics Committee of Fudan University.

Rats were anesthetized by intraperitoneal injection of 1.25% pentobarbital sodium at 0.1 mL per 100 g body weight. After full anesthesia, the rats were decapitated and the brain was removed and cooled in iced artificial cerebrospinal fluid (ACSF; in mmol/L: NaCl 124, KCl 3.3, KH₂PO₄ 1.2, NaHCO₃ 26, CaCl₂ 2.5, MgSO₄ 2.4, glucose 10) for 1 min, then the hippocampus was exposed on ice. After that, the brain was fixed on a vibrating cryotome and bathed in iced ACSF throughout the slicing process. The thickness of the slices used in both field potential recording and western blot was 350 μm . The slices were transferred to ACSF at room temperature, and later to a 33°C water

bath for 30 min, in order to restore neuronal function before they were allowed to recover in room temperature ACSF for 1 h. At the end of this process, the slices were ready for pharmacological treatment and electrophysiological recording.

Evoked Population Spike Recording in Hippocampal Slices

Freshly-prepared hippocampal slices were superfused with normal ACSF using a Peri-star double-channel perfusion system (World Precision Instruments, Sarasota, FL), and the perfusate was continuously bubbled with 95% O₂ and 5% CO₂. The recording pipettes were pulled from borosilicate glass on a P97 microelectrode puller (Sutter Instruments, Novato, CA). The pipette was filled with normal ACSF and the impedance was 4–8 M Ω . Bipolar tungsten electrodes were used for stimulation. The recording electrodes were placed in the CA1 pyramidal layer, while the tungsten electrodes were placed across the Schaffer collaterals. The stimulation strength was set to evoke 60% of the maximal response, and the frequency was set to one per 30 s. The signal was amplified and filtered using the NeuroLog system (Digitimer Ltd, Hartford, UK), and was acquired using the CED1401 data acquisition system and Spike 2 software (CED Electronics, Cambridge, UK). After 30 min of baseline recording, either DMSO (0.1%) or one of the convulsants [(CTZ, kainic acid (KA), bicuculline (BIC), or Mg²⁺-free solution (0-Mg²⁺)] was added to the ACSF, and the recording was continued for another 2 h. In some experiments, after 2 h recording with CTZ (200 $\mu\text{mol/L}$) (Tocris, Northpoint, Bristol, UK), the perfusate was replaced with either DMSO (0.1%) or THIP (5 $\mu\text{mol/L}$) (Sigma Aldrich Chemical Co., Poole, Dorset, UK) for another hour.

Whole-cell Patch-clamp Recording

Whole-cell recordings were performed in voltage-clamp mode using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were pulled from borosilicate glass and fire-polished (2–6 M Ω). Before the pipettes were immersed in solution, positive pressure was applied to prevent tip blockage. When approaching target cells, the pressure was withdrawn to form a high-impedance seal (>1 G Ω) between the membrane and the pipette. Meanwhile, the membrane potential was held at around -70 mV to facilitate the seal. After the seal

stabilized, appropriate negative pressure was applied to break the cell for whole-cell recording. Data were acquired using pClamp 10 software, sampled at 2–10 kHz, and filtered at 1 kHz. Off-line analysis was done with Clampfit 10 software. Based on previous work, a large depolarization resembling a paroxysmal depolarization shift was defined as ≥ 10 mV depolarization, and ≥ 300 ms duration. And an epileptiform burst in a single neuron was defined by at least five consecutive action potentials superimposed on a large depolarization shift^[16].

Immunoblotting

Slices were dissected to preserve only the hippocampus under a dissecting microscope on ice, and then quickly homogenized in pre-cooled lysis buffer (#K268-50, Biovision, Milpitas, CA). The plasma membrane protein fraction was prepared from the homogenate following the standard procedure using a membrane protein extraction kit (#K268-50, Biovision). The membrane fraction was dissolved in 0.5 % Triton X-100 in PBS, and incubated at 45°C with SDS sample buffer for 45 min for inactivation. Membrane proteins were separated on SDS-PAGE, electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), then incubated with primary antibodies raised against the GABA_AR δ -subunit (#AB9752, 1:800; Millipore) or β -actin (#4967, 1:1 000; Cell Signaling Technology, Danvers, MA) in 5% skimmed milk-TBS-T (20 mmol/L Tris, pH 7.6, 137 mmol/L NaCl, 0.05% Tween 20) overnight at 4°C, followed by incubation with peroxidase-conjugated Affinipure goat anti-rabbit (#111-035-003, 1:20 000; Jackson, Noida, India) or rabbit anti-goat (#305-035-003, 1:20 000; Jackson) secondary antibody in TBS-T buffer. Bands were visualized using an ECL detection system (Pierce, Rockford, IL). The immunoreactivity of an individual band was measured by Imagepro plus and normalized to β -actin.

Data Analysis

Group data are expressed as mean $\pm$ SEM. Across different groups of data, statistically significant differences between means were determined using one-way ANOVA with Tukey's HSD *post hoc* analysis. Comparison within a group was carried out using a paired or unpaired *t* test. All analyses were performed using the statistics software Stata 7. The significance level was set at $P < 0.05$.

RESULTS

CTZ-induced Epileptiform Activity in CA1 Pyramidal Neurons of Hippocampal Slices

Previous studies have shown that CTZ induces progressive epileptiform activity including multiple evoked peaks followed by spontaneous epileptiform spike activity, and eventually highly-synchronized burst activity, in rat hippocampal CA1 neurons^[16, 17]. In this study, we first tried to establish a hippocampal slice model of seizures induced by CTZ. Under control condition, a single population spike (PS), without any spontaneous activity, was evoked in CA1 pyramidal neurons after stimulation of the Schaffer collaterals (Fig. 1Aa). However, slices treated with CTZ (50 or 200 μ mol/L, dissolved in 1% DMSO) dose-dependently induced multiple epileptiform PS peaks in CA1 pyramidal neurons, similar to those reported in anesthetized rats *in vivo*^[16] (Fig. 1Ab, Ac). The latency to the appearance of the second PS peak in the 50 μ mol/L CTZ group was 68.1 ± 4.0 min ($n = 9$), which was significantly longer than that of the 200 μ mol/L CTZ group (18.3 ± 1.5 min, $n = 6$; $P < 0.001$) (Fig. 1B, Table 1). In addition, CTZ at 200 μ mol/L induced multiple PS peaks (3 or more) (Fig. 1Ac), while 50 μ mol/L CTZ failed to induce three or more peaks during our 2-h recording paradigm (Fig. 1Ab). These results indicated that CTZ induces concentration-dependent epileptiform activity in hippocampal slices.

Comparison of the Latency to Evoke Epileptiform Multiple PS Peaks among Different *in vitro* Epilepsy Models

Previous studies have reported that KA, BIC, and 0-Mg²⁺ induce epileptiform activity in hippocampal slices, and all are commonly used in the study of epilepsy^[18–20]. We performed a study to compare these models with CTZ. Hippocampal slices continuously superfused with ACSF containing KA (0.5 μ mol/L), BIC (2 μ mol/L), or 0-Mg²⁺ all rapidly displayed double or even multiple PS peaks (Fig. 2 C–E), while this process was much slower with CTZ (50/200 μ mol/L). The CTZ model also induced relatively fewer peaks overall compared with the other three models (Fig. 2A, B). The latencies of the double peaks in these four models were: CTZ (50 μ mol/L), 68.1 ± 4.0 min, $n = 9$; CTZ (200 μ mol/L), 18.3 ± 1.5 min, $n = 6$; KA (0.5 μ mol/L), 3.6 ± 0.4 min, $n = 6$; BIC (2 μ mol/L), 2.5 ± 0.2 min, $n = 5$; 0-Mg²⁺, 7.4 ± 0.9 min, $n = 5$ (Fig. 2F). These results showed that the

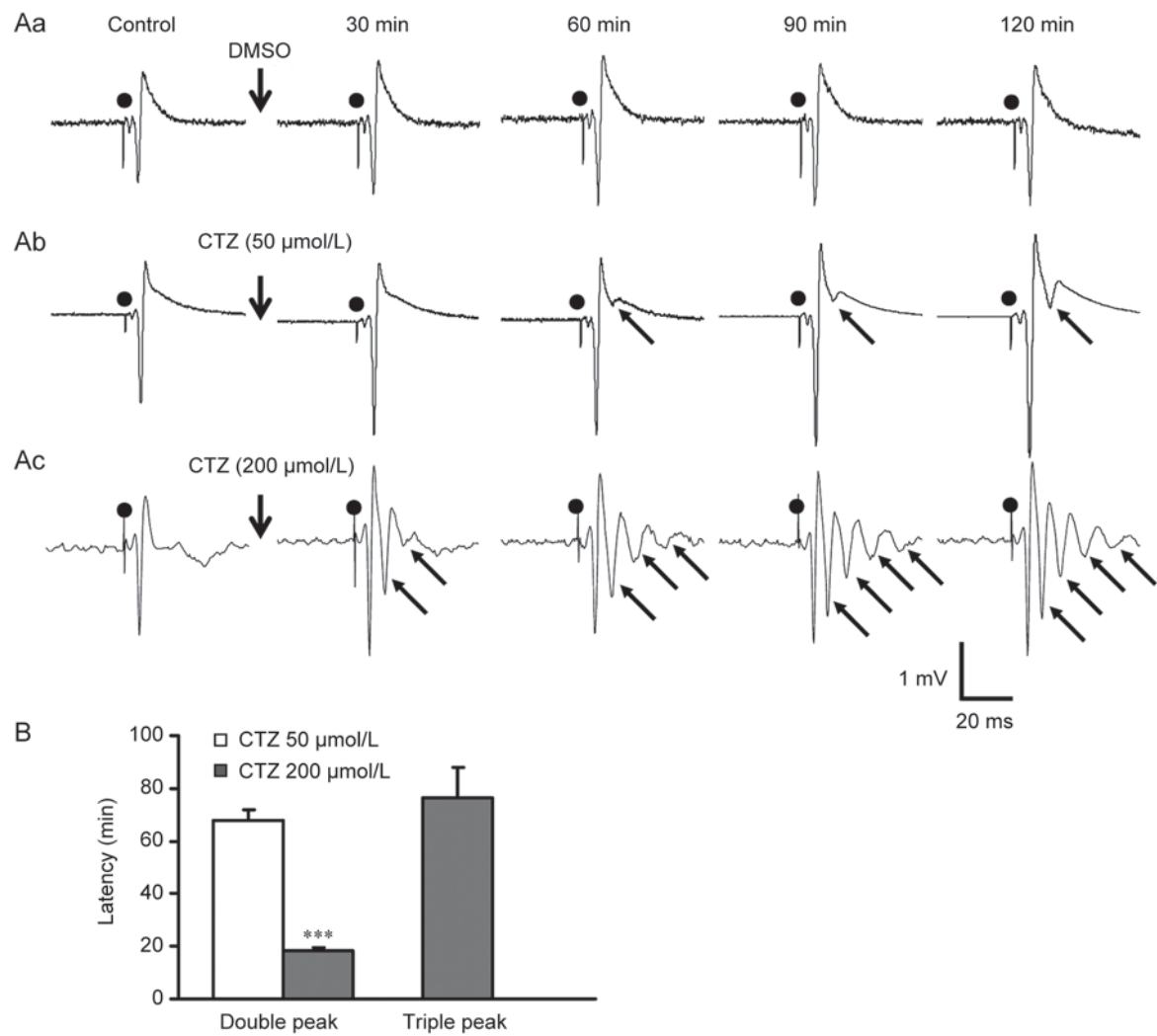


Fig. 1. Progressive change of hippocampal CA1 evoked potentials induced by CTZ in a brain slice. **A:** CTZ-induced time- and concentration-dependent progressive change of the evoked population spikes (PSs) in a hippocampal CA1 slice. **Aa:** PS was not significantly changed when superfused with DMSO as control ($n = 4$); **Ab–Ac:** PS peak number gradually increased after continuous superfusion with either 50 $\mu\text{mol/L}$ ($n = 9$) (**b**) or 200 $\mu\text{mol/L}$ ($n = 6$) (**c**) CTZ. **B:** Bar graph showing the latency to evoke double and triple peaks at different CTZ concentrations. Additional peaks are indicated by the arrows; “•” indicates the stimulus artifact.

Table 1. Summary of double or triple PS peak latency induced by different concentrations of CTZ

Group	Double Peaks (min)	Triple Peaks (min)
DMSO	>120 ($n = 4$)	>120 ($n = 4$)
CTZ (50 $\mu\text{mol/L}$)	68.1 $\pm$ 4.0 ($n = 9$)	>120 ($n = 9$)
CTZ (200 $\mu\text{mol/L}$)	18.3 $\pm$ 1.5*** ($n = 6$)	76.7 $\pm$ 11.3 ($n = 6$)

*** $P < 0.001$ compared with the 50 $\mu\text{mol/L}$ CTZ group.

onset of abnormal epileptiform PSs induced by CTZ was significantly slower ($P < 0.001$, Fig. 2F) than that seen with the classical experimental convulsants. The slow onset of action of CTZ is comparable to that reported in cultured hippocampal neurons^[16].

CTZ-induced Epileptiform Firing in Single Hippocampal CA1 Neurons

A previous study showed that the additional PS peaks

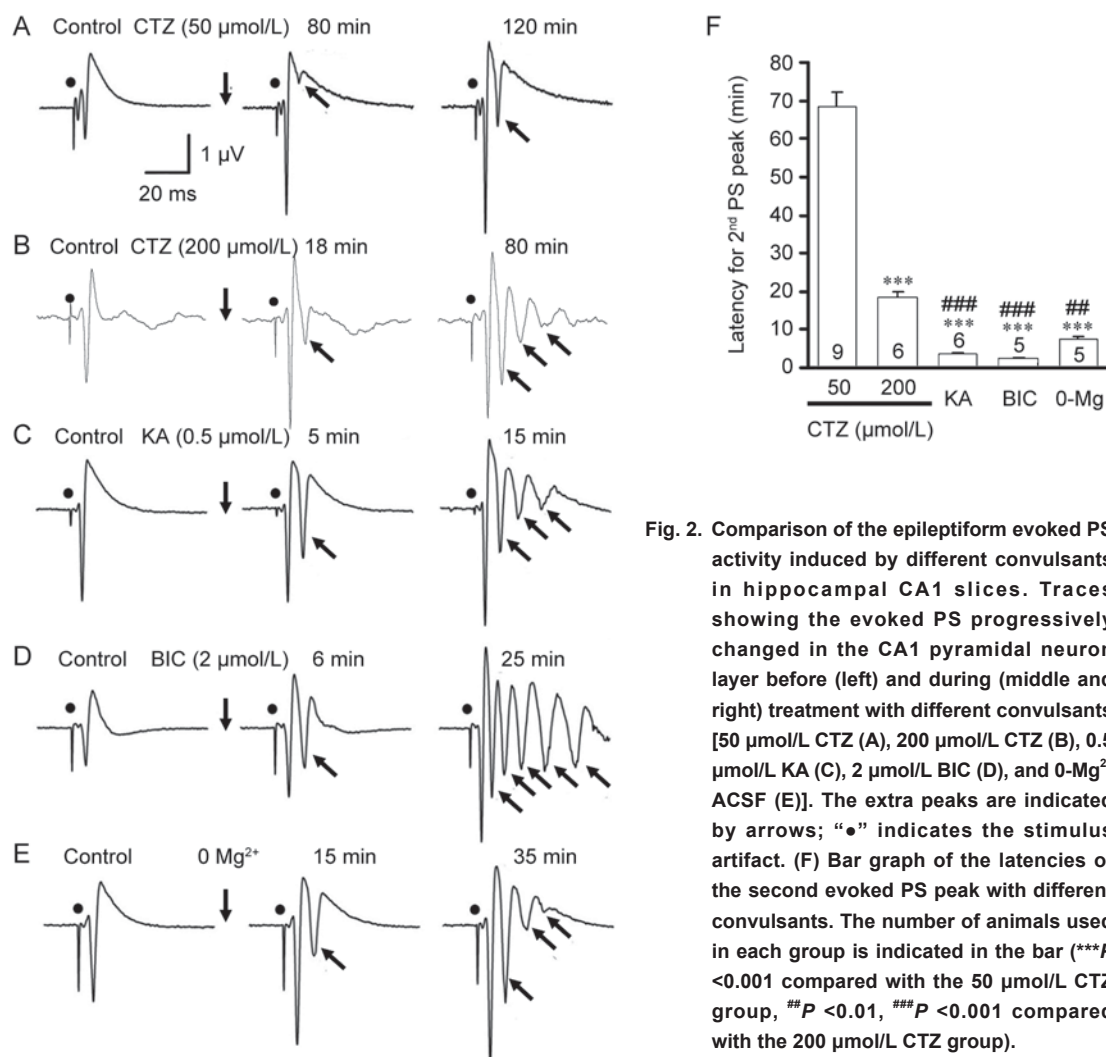


Fig. 2. Comparison of the epileptiform evoked PS activity induced by different convulsants in hippocampal CA1 slices. Traces showing the evoked PS progressively changed in the CA1 pyramidal neuron layer before (left) and during (middle and right) treatment with different convulsants [50 μ mol/L CTZ (A), 200 μ mol/L CTZ (B), 0.5 μ mol/L KA (C), 2 μ mol/L BIC (D), and 0- Mg^{2+} ACSF (E)]. The extra peaks are indicated by arrows; “•” indicates the stimulus artifact. (F) Bar graph of the latencies of the second evoked PS peak with different convulsants. The number of animals used in each group is indicated in the bar (*** P < 0.001 compared with the 50 μ mol/L CTZ group, ** P < 0.01, ### P < 0.001 compared with the 200 μ mol/L CTZ group).

are likely due to the non-synchronized composition of the enhanced electrical activity from different neuronal layers^[21]. Enhanced neuronal activity for epileptiform burst firing^[16], which is at the core of epileptogenesis and network spread^[22–24], has been reported in cultured hippocampal neurons after treatment with CTZ. Therefore, we further explored changes in the firing pattern at the level of single hippocampal CA1 neurons in CTZ-treated hippocampal slices using the patch-clamp technique. Spontaneous action potentials were only occasionally recorded from CA1 pyramidal neurons when the membrane potential was current-clamped at -70 mV (Fig. 3, left). However, neuronal activity was enhanced after the application of CTZ (50 μ mol/L) (Fig. 3, middle), which eventually induced epileptiform

burst activity (Fig. 3, right), analogous to that reported in cultured hippocampal neurons^[16]. The percentage of neurons that displayed epileptiform burst firing in slices treated with 50 μ mol/L CTZ for 2 h was 55.6% ($n = 9$). The above results point towards the possibility of a correlation between epileptiform burst firing in single neurons and synchronization in the neuronal network after CTZ perfusion.

Incubation with CTZ Downregulates Neuronal Cell Membrane Expression of Extrasynaptic GABA_A Receptors in Hippocampal Slices

Extrasynaptic GABA_ARs have been suggested to exert a regulatory function during epileptogenesis and could be

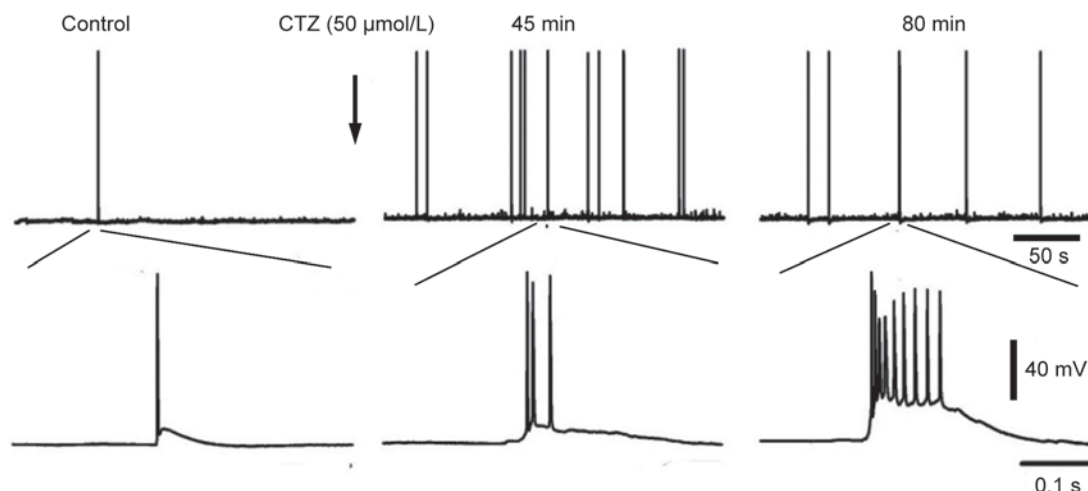


Fig. 3. CTZ-induced robust epileptiform burst activity in a CA1 pyramidal neuron in a rat hippocampal slice. Traces from current-clamp recordings (membrane potential held at -70 mV) in a CA1 pyramidal neuron showing that CTZ (50 $\mu\text{mol/L}$) transformed the firing pattern from single action potentials (left), to grouped (middle), and finally burst-like activity (right).

novel antiepileptic drug targets^[13]. Hence, we went on to examine the variation in expression of the δ subunit, one of the major extrasynaptic GABA_AR subunits, on the neuronal cell membrane after treatment of hippocampal slices with CTZ. Freshly-prepared slices were incubated in ACSF containing 50 $\mu\text{mol/L}$ CTZ or 1% DMSO (solvent control) for 2 h, after which membrane protein was extracted for western blot analysis. Two hours after treatment with CTZ (50 $\mu\text{mol/L}$), the relative level of the GABA_AR δ subunit was markedly decreased to $55.0 \pm 4.3\%$ (Fig. 4) of the DMSO control level. This result demonstrated that the extrasynaptic expression of GABA_ARs is reduced in CTZ-induced epileptogenesis in hippocampal slices.

THIP Suppresses the Epileptiform PSs Induced by CTZ in Hippocampal Slices

Our previous study showed that the sustained tonic inhibition mediated by extrasynaptic GABA_ARs has a significant effect on epileptiform activity^[13]. Therefore, we further tested whether enhancing tonic GABA inhibition by THIP, an agonist specific for the GABA_AR δ -subunit, could suppress the epileptiform activity in hippocampal slices. Perfusion with 200 $\mu\text{mol/L}$ CTZ induced multiple PS peaks in all slices as shown above. After 2 h of continuous superfusion, the induced multiple PS peaks had reached a stable stage with an average of 2.3 ± 0.2 ($n = 9$) extra

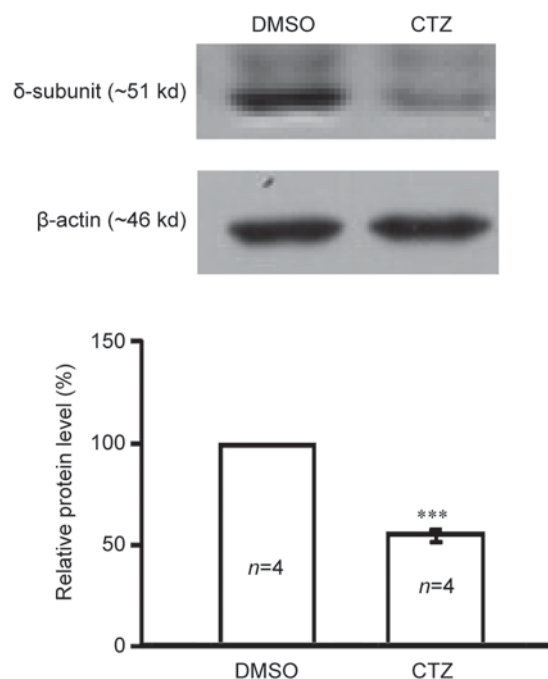


Fig. 4. CTZ treatment suppressed expression of the GABA_AR δ -subunit in the hippocampal cell membrane. A: Western blots showing the GABA_AR δ -subunit (upper) and β -actin (lower) in DMSO- and CTZ-treated (2 h) hippocampal slices. B: Bar diagrams showing the group data of the change of the GABA_AR δ -subunit after CTZ treatment. The number of animals in each group are indicated in the bar ($***P < 0.001$ compared with the DMSO group).

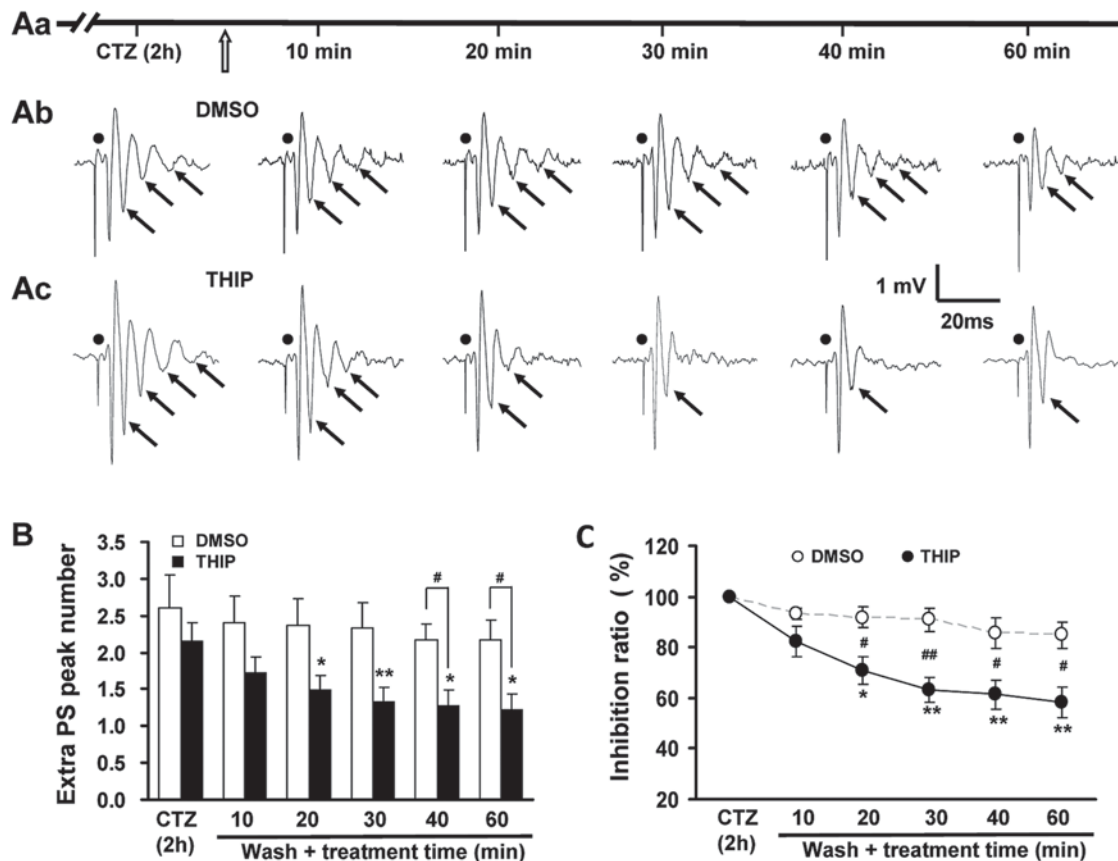


Fig. 5. Stimulation of extrasynaptic GABA_A receptors with THIP suppressed CTZ-induced epileptiform population spike (PS) peaks in hippocampal CA1 region. **Aa:** Line to show the protocol (arrow indicates the superfusate change from CTZ to either DMSO or THIP). **Ab–Ac:** Raw traces showing the change in the extra number of PS peaks (arrows) after the superfusate was changed to either DMSO (**Ab**) or THIP (**Ac**). **B:** Bar diagram showing that THIP (5 μ mol/L, $n = 6$) suppressed the CTZ (200 μ mol/L, 2 h)-induced multiple PS peaks compared with the DMSO control ($n = 3$) ($P < 0.001$, two-way ANOVA). **C:** Graph showing the reduction ratio of the extra PS peaks in the THIP (filled circles) and DMSO (unfilled circles) groups (* $P < 0.05$, ** $P < 0.01$ compared with 2-h CTZ control, paired t -test; # $P < 0.05$, ## $P < 0.01$ compared with time-matched DMSO control, unpaired t -test).

peaks (excluding the first PS) (Fig. 5Ab, Ac). Then slices were randomly washed with ACSF either containing 5 μ mol/L THIP ($n = 6$) or 1% DMSO ($n = 3$) as solvent control (Fig. 5Aa). The results showed that superfusion with THIP suppressed the epileptiform extra PS peaks in a time-dependent manner from an average of 2.2 ± 0.3 to 1.3 ± 0.2 at 30 min ($P < 0.01$) and 1.2 ± 0.2 at 60 min ($P < 0.05$), while those superfused with DMSO showed no significant effect on the PS peaks (Fig. 5B). Further analyses showed that, compared with the time-matched DMSO control group, the extra number of peaks was reduced at 40 min (1.3 ± 0.2 versus 2.2 ± 0.2 , $P < 0.05$), and at 60 min (1.2 ± 0.2 versus 2.2 ± 0.3 , $P < 0.05$) after CTZ was washed out with THIP

(Fig. 5B). The reduction ratio of the extra PS peak number in the THIP group was greater than that in the DMSO group ($P < 0.001$, Fig. 5C). These results showed that, although δ -GABA_ARs were down-regulated, the extrasynaptic GABA_AR agonist THIP was still capable of suppressing the CTZ-induced epileptiform activity in hippocampal slices.

DISCUSSION

In this study, we established a novel brain-slice epilepsy model that used the previously-characterized convulsant CTZ^[16]. Using this model, our results demonstrated that extrasynaptic GABA_ARs were downregulated by ~50%

during early CTZ-induced epileptogenesis, and selective stimulation of the remaining extrasynaptic GABA_ARs by THIP was still capable of suppressing the epileptiform activity established by CTZ.

Recently, several studies have shown that CTZ is a potent convulsant capable of inducing epileptiform activity both *in vivo* and in cultured hippocampal neurons^[16, 17, 25], as well as evoking seizure behavior in freely-moving animals^[26]. In the current study, we further extended this epilepsy model to hippocampal slices. CTZ has long been known as a blocker of AMPA receptor desensitization and was also recently found to be a GABA_AR inhibitor^[27]. Although the previous *in vitro* studies on cultured hippocampal neurons have shown that neurons treated with CTZ have a long-lasting alteration in neuronal firing pattern, including the induction of epileptiform burst activity^[16], the change in network activity after CTZ treatment was still not clear. The brain slice is a simple and convenient method that has been widely used in anticonvulsant drug development, and in experimental studies on the neuroprotective roles of these drugs during epilepsy^[28–30]. Thus, the brain-slice model is of special value in the study of epilepsy. Using the hippocampal slice preparation, we showed that not only were individual hippocampal pyramidal neurons induced to generate epileptiform burst firing but also, at the local network level, epileptiform activity was triggered in the form of multiple evoked PS peaks. We also demonstrated that CTZ is a potent convulsant in hippocampal slices, and provided another useful *in vitro* model for studying the mechanisms underlying seizures along with the development of potential anticonvulsant drugs. While the *in vivo* model requires injection of CTZ into the cerebral ventricles due to its inability to cross the blood-brain-barrier, the brain-slice model of epilepsy induced by CTZ is not only easy to establish, but also retains relatively intact neuronal networks. In addition, our previous *in vitro* work has demonstrated that, unlike other convulsants (e.g. kainate), CTZ does not appear to be neurotoxic^[16], and our recent study on a CTZ-induced chronic rat seizure model (data not shown) also indicated that intracerebroventricular application of CTZ, which gives rise to recurrent seizure behavior, has a mild effect on neuronal apoptosis. Thus, the CTZ seizure model has special advantages in the study of epilepsy.

Our previous studies both in anesthetized animals

and in cultured neurons indicated that the process of CTZ-induced epilepsy is relatively moderate with a rather long latency to the induction of the acute phases of seizure^[16]. We further characterized this property by comparing the latency to the onset of epileptiform activity in hippocampal slices with the commonly-used convulsants KA, BIC, and 0-Mg²⁺. We showed that the onset latency to evoke the extra PS peaks in CA1 was significantly longer than any of those convulsants, even after the CTZ concentration had been increased to 200 μmol/L. This discrepancy in latency provides a much longer window period, up to 1 h in the 50 μmol/L CTZ group, as compared to only few minutes with either KA, BIC, or 0-Mg²⁺, to explore the initial seizure induction mechanism. Thus, we have extended the use of the novel convulsant CTZ to this model for epilepsy research. Previous research on anesthetized rats showed that CTZ-induced epilepsy in hippocampal CA1 neurons has a characteristic 3-stage pattern consisting of multiple evoked PS peaks, followed by the appearance of spontaneous high-amplitude spikes that later synchronize to generate high-frequency bursting activity. In the current study on hippocampal slices, we noted that CTZ induced multiple evoked PS peaks, yet no spontaneous epileptiform activity was observed, in contrast with the high-K⁺ model in hippocampal slices reported by Liu and colleagues^[31]. The reason for this disparity may lie in the different experimental conditions; the limited recording time (recording for 2 h after CTZ treatment), and a lower recording temperature (room temperature (25°C) rather than body temperature (~37°C) *in vivo*). Researchers have pointed out that temperature greatly influences the occurrence of spontaneous activity *in vitro* in the brain slice. It has also been noted that the probability of detecting burst-like activity is reduced at a certain range of low temperatures^[32, 33]. Thus, the limited spontaneous epileptiform activity observed in our slice recording is comprehensible. Future studies with longer recording times and higher recording temperatures are required for comparison. However, under our conditions, the pattern of CTZ-induced multiple PS peaks in the slice model was similar to that reported in field potential recordings from anesthetized rats^[16, 17]. The evoked PSs were composed of evoked action potentials from multiple neurons, and are considered to be an important electrophysiological index in the study of excitation/inhibition relationships in the CNS. During the early phase

of treatment with the CTZ convulsant, the PS peaks changed remarkably over time; their amplitude and number gradually increased. The PS peaks, which represent the non-synchronized neuronal activity from different layers, changed from a single peak under control conditions to double or even multiple peaks, which parallel the onset of epilepsy and arrive at a seizure-prone state^[21, 34, 35]. Thus, in this study, we used the PS peak number as a marker for studying the epileptiform activity in hippocampal slices.

Epilepsy itself also has an impact on the inhibition mediated by GABA_ARs; long-term epilepsy may alter the expression of GABA_AR subunits^[36]. It is well known that a functional deficit of synaptic GABAergic inhibition plays an important role in the pathogenesis of epilepsy^[37–40]. Recent studies have also revealed the possible role of ‘tonic’ GABA inhibition in the modulation of epileptic seizures^[41–43]. Tonic inhibition has been reported in hippocampal dentate gyrus (DG) granule cells^[43], CA1 pyramidal neurons^[44], and hippocampal interneurons^[45]; however, in these three areas, the receptors mediating tonic inhibition are of different composition. Most extrasynaptic GABA_ARs in CA1 pyramidal neurons contain $\alpha 5$ and γ subunits, while in DG cells they mainly contain $\alpha 4$ and δ subunits. In interneurons, δ -GABA_ARs are the main mediator of tonic inhibition^[8, 44, 45]. Our western blot results showed that the δ -subunit-containing GABA_ARs on the cell membrane were significantly down-regulated by up to 50% during CTZ stimulation in hippocampal slices. This downregulation may be due to activation of the BDNF-TrkB signaling pathway, as our previous study showed that CTZ, as well as other classic convulsants, induces epileptiform activity via enhanced TrkB receptor-mediated BDNF function^[17, 46]. In addition, BDNF is a known modulator of the surface expression of δ subunits^[36]. This suggests that the function of the extrasynaptic GABA_ARs was largely impaired and is consistent with previous studies. In animal models of temporal lobe epilepsy, there have been reports of a significant reduction of GABA_AR $\alpha 5$ and δ subunit levels in the hippocampal area^[11, 14, 47]. Moreover, δ -subunit mutations have been mapped in epilepsy patients^[15, 48]. Furthermore, increased δ -subunit levels during the diestrus stage of the ovarian cycle have been associated with less seizure activity in KA-induced epilepsy models^[49]. All these pieces of evidence suggest that tonic inhibition, mediated by extrasynaptic GABA_ARs, play an essential

role in the process of epileptogenesis. Indeed, our recent study demonstrated that enhancing tonic inhibition by increasing the expression of either $\alpha 5$ - or δ -subunit-containing GABA_ARs effectively suppresses epileptiform activity in cultured hippocampal neurons^[13]. *In vivo* studies have further demonstrated that both seizure behavior and epileptiform activity are attenuated by enhancing tonic inhibition in hippocampal neurons^[13]. Based on previous studies and our own research, we therefore hypothesize that enhancing ‘tonic’ GABA inhibition inhibits epileptiform activity, while reducing tonic inhibition increases the susceptibility to epileptic seizures^[49, 50].

THIP is a GABA_AR agonist that can interact with the receptor at sites different from those usually occupied by benzodiazepines, non-benzodiazepines, and barbiturates. Our recent study showed that THIP at a relatively low concentration selectively activates extrasynaptic GABA_ARs^[13]. In light of this finding, we used THIP to test whether selective activation of the extrasynaptic GABA_ARs could reverse the CTZ-induced epileptiform activity. Indeed, we found that, despite the down-regulation of the membrane δ -subunit of GABA_ARs, THIP effectively suppressed the CTZ-induced multiple PS peaks. One simple explanation could be that THIP, by acting on the remaining extrasynaptic GABA_ARs, enhances tonic inhibition in the whole hippocampal neuronal network and inhibits epileptiform activity. In addition, our previous study disclosed a strong inhibitory effect of THIP on the pronounced neuronal activity induced by both CTZ and KA^[13]. This effect could be due to the increased neuronal activity induced by the convulsants, releasing a substantial amount of GABA^[51], which in turn acts concomitantly with THIP to enhance tonic inhibition and suppress neuronal activity. Furthermore, it is also possible that the tonic current may be an outward rectifying current at depolarized membrane potentials^[52], making the effect of THIP more potent when neurons are hyperexcited. Thus, our findings related to the inhibitory effect of THIP on epileptiform activity in hippocampal slices further support views on the significance of extrasynaptic GABA_ARs in epileptogenesis.

In conclusion, we developed a novel CTZ-induced brain slice seizure model, and using this model, we further demonstrated that the activation of extrasynaptic GABA_ARs with THIP effectively suppressed the progress of epileptogenesis. These results support the notion that

extrasynaptic GABA_ARs may be valuable drug targets for novel antiepileptic drug therapy.

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