

Clptm1 Downregulation Exerts Antiepileptic Activity by Regulating GABAAR-mediated Inhibitory Synaptic Transmission in PTZ-induced Epileptic Model

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Research Article

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Abstract

Background: Disruption of GABA_AR synaptic clustering and a decrease number in their cell surface are thought to contribute to the alteration in the balance between excitatory and inhibitory neurotransmission, which contributes to seizure induction and propagation. Cleft lip and palate transmembrane protein 1 (Clptm1), a multi-pass transmembrane protein, has been showed that it is an intracellular molecule that controls forward trafficking of GABA_AR. Clptm1 downregulating increased miniature inhibitory postsynaptic current (mIPSC) in vivo. Thus, Clptm1 controls phasic and tonic inhibitory transmission in brain. In this study, we hypothesized that Clptm1 may be involved in epileptic seizure by regulating GABA_AR-mediated inhibitory synaptic transmission in epileptic model.

Methods and Results: In PTZ-induced epileptic model, we found that Clptm1 was increased in temporal lobe epilepsy (TLE) patients as well as in epileptic model. Then, we showed that Clptm1

downregulation exerted antiepileptic activities in epileptic model, which was associated to the increased surface GABA_ARγ2 expression and mIPSCs amplitudes.

Conclusions: Clptm1 downregulation exerted antiepileptic activities in epileptic model, thus, it may be a promising target for antiepileptic treatments.

Introduction

TLE is the most prevalent form of focal epilepsy. It is characterized as a form of adult focal onset epilepsy and is highly associated with refractory epilepsy (1). Abnormal neuronal discharges in patients with TLE is due to failure of inhibitory and/or excitatory neurotransmission. Gamma-amino butyric acid (GABA) receptors (GABA_AR), the main inhibitory neurotransmitter, may be involved in seizure induction and propagation (2). Of all of the GABAR, the GABA_A receptors (GABA_ARs) are considered as the most important in TLE induction and propagation (3), which persistently activated GABA_ARs can suppress neuronal voltage responses towards incoming excitation (4). Thus, it is important to investigate the molecular mechanisms underlying the effects of GABA_AR-mediated inhibitory synaptic transmission on epileptic seizure.

Clptm1 is identified by association of a chromosomal translocation with cleft lip and palate (5). It modulates postsynaptic assembly of GABA_ARs. Knocking down Clptm1 increased the number of GABA_ARγ2 in cell surface and mIPSC amplitudes in neurons (6). However, whether Clptm1 regulates epileptic seizure by regulating GABA_AR-mediated inhibitory synaptic transmission has not been established.

In this study, we examined the role of Clptm1 in epilepsy. We demonstrated that Clptm1 is increased in TLE patients as well as in epileptic models. Then, we showed that Clptm1 downregulation attenuated seizure susceptibility and severity in PTZ mouse and that these changes are associated with the increase

of the number of GABA_AR γ 2 in cell surface and mIPSCs amplitudes. These results show the significance role of Clptm1 in GABA_AR-mediated inhibitory synaptic transmission and involved in epileptic seizure. Therefore, Clptm1 is a promising target for antiepileptic treatments.

Materials And Methods

Ethical statement

This study was conducted following the guidelines for the National Institutes of Health of China and the Committee on Human Research at Chongqing Medical University. Written informed consent for human brain tissues were collected. All protocols for the performing animal experiments were admitted by the Commission of Chongqing Medical University for Ethics in Animal Experiments (Approval number:0002648).

Human samples

Ten samples of TLE patients and ten normal samples who experienced head trauma were obtained as our previous publication (7). Clinical characteristics of patients are shown in Table1.

Mouse model of seizure or epilepsy

All animal were obtained from the Laboratory Animal Center of Chongqing Medical University. We established PTZ-induced epileptic model, which is considered an appropriate animal model for preclinical research on the roles of GABA_ARs in epilepsy (8-10). Acute and chronic epileptic models were established as our previous publication (7).

Behavioural tests

The behaviours were scored as previous study (11). For the shRNA-clptm1+ PTX intervention, mice were administered with shRNA-clptm1 or LV-GFP two weeks before inducing PTZ and thereafter with PTX (1 mg/kg. Ip. 5 min before PTZ inducing).

Double immunofluorescence labelling

Immunofluorescence (IF) staining was operated as previously described (7). The antibodies were mouse anti-Clptm1 (1:50, sc-374619; Santa Cruz), mouse anti-GABA_AR γ 2 (1:50, cat no.: MABN875) and chicken anti-MAP2 (1:100, catalogue number: MAB377) both from Millipore, Billerica, MA, USA as well as rabbit anti-gliial fibrillary acidic protein (GFAP) (1:50, cat no: [16825-1-AP](#) ; Wuhan, China).

Biochemical Measurement of Surface-expressed Receptors and Immunoblotting

According to ProteoExtract™ Native Membrane Protein Extraction Kit (M-PEK Kit, Merck Millipore, USA) instructions, we extracted membrane-associated proteins. Then, bicinchoninic acid protein (BCA) assay was determined the concentration of pure protein. SDS-polyacrylamide gel electrophoresis separated proteins and transferred to a polyvinylidene fluoride (PVDF) membrane. Next, the PVDF membrane was incubated with a polyclonal mouse anti- Clptm1 (1:50, sc-374619; SANTA CRUZ) or a mouse anti-GABA_ARγ2 (1:50, catalogue number: MABN875, Millipore, Billerica, MA, USA). The next day, the membrane incubated with a goat anti-rabbit IgG antibody (1:1500, Proteintech, Wuhan, China) or a goat anti-mouse IgG antibody (1:2000, Proteintech, Wuhan, China) for 1.5 h at 37 °C.

Coimmunoprecipitation

Co-immunoprecipitation was conducted as described in our previous study (7). The antibodies were mouse anti- Clptm1 (1:50, sc-374619; SANTA CRUZ), GABA_ARγ2 (1:50, catalogue number: MABN875, Millipore, Billerica, MA, USA). Immunoblotting assays were conducted with anti-GABA_ARγ2 or anti- Clptm1 antibodies.

Establishment of the Lentiviral Vector and Stereotactic Injection

LV vectors with Clptm1 constructs shRNA-Clptm1 were constructed by GeneChem company (GeneChem Co., Ltd. Shanghai, China). We microinjected Clptm1-shRNA or scramble-shRNA into the bilateral CA1 region of every mouse.

Electrophysiological Assessments

Mouse cortical brain slices (300 μm thick) were cut and immersed in an ice-cold buffer. Then brain sections were immediately placed in artificial cerebrospinal fluid (ACSF) for 1.5 h at 34°C. Cells in the CA1 region of the hippocampus were recordings under an inverted phase contrast microscope (Nikon, Japan). When stable mIPSCs were obtained, recordings were sustained for at least 3 min. Then, GABA (100 or 500 μM, Sigma, USA) was added to the brain slices to assess whether the mIPSCs required GABA activity.

Statistics

All data are shown as mean ± SEM. Student's t test was conducted for between group comparisons and LSD tests if three or four groups were compared. ANOVA repeated measures were used for comparisons

of sequential measurements to a single control measurement followed by Dennett's test. $P < 0.05$ was the cut-off for significance.

Results

Clptm1 is localized in neuron and interacted with GABA_AR γ 2 in epileptic brain

We first detected whether Clptm1 protein is localized in neuron, which epilepsy is known to be characterized by neuronal hyperexcitability. Confocal microscopy analyses revealed that Clptm1 immunoreactivity is located at cortical neurons of TLE patients (Fig. 1A-1C), and we showed that Clptm1 is located at the cortex as well as hippocampal of the epileptic mouse. Clptm1 co-localize with the specific neural marker MAP2. However, it did not co-localize with the specific astrocyte marker GFAP. Additionally, we found Clptm1 co-localizes with GABA_AR $\beta^{2/3}$ in epileptic brain, and co-immunoprecipitation showed that Clptm1 is interacted with GABA_AR γ 2 in TLE patients, and the hippocampus and cortex of the epileptic mouse are also (Fig. 1D-1F). These findings suggest that Clptm1 localized in neuron and interacted with GABA_AR γ 2 in epileptic brain.

GABA_AR γ 2 expression is decreased in epileptic brain

Disruption of GABA_AR-mediated inhibitory synaptic transmission is related to a post synaptic mechanism in epilepsy, thus, we first detected GABA_AR γ 2 levels in the epileptic brain. As shown in figures 2A, 2B and 2C, the immunoreactivity of GABA_AR γ 2 in the cortex of patients with TLE and in chronic epileptic model showed a significant decrease compared to the control. These changes may underlie the disrupted GABAergic transmission in epilepsy.

Clptm1 expression is increased in epileptic brain

To observe whether Clptm1 plays a role in epilepsy, we first detected the Clptm1 expression in the brains of the TLE patients and epileptic mouse model. Immunoblotting showed that Clptm1 expression was increased in TLE patients compared to the control (Fig. 2D), and in hippocampal and cortical tissue of the epileptic mouse model were also increased (Fig. 2E-2F). These findings suggested that Clptm1 upregulation may be involved in epileptic seizure.

Clptm1 downregulation attenuates PTZ-induced Seizure

Subsequently, to identify the relation between the increasing of Clptm1 expression and epilepsy, we used Clptm1-shRNA to downregulate Clptm1 proteins and assessed the effects of Clptm1 on PTZ-

induced seizures. Two weeks post-administration of the exogenous recombinant lentivirus vector (LV), we found green fluorescent protein (LV-GFP)-positive cells in the hippocampus (Fig. 3A). Moreover, immunoblotting revealed Clptm1 expression is decreased after initial administering (Fig. 3B). These findings suggesting that Clptm1-shRNA was transfected successfully in epileptic model. Subsequently, we observed behavioural alterations after PTZ-induced seizures (Fig. 3C), we found seizure susceptibility and severity were markedly decreased in Clptm1-shRNA group compared to the control (Fig. 3D). Taken together, we found Clptm1 regulates epileptic seizures and that Clptm1 downregulation exerts anti-epileptic activities in epileptic model.

Clptm1 modulates GABA_AR-mediated inhibitory synaptic transmission in epileptic mouse model

Since Clptm1 limits GABA_AR forward trafficking and synaptic inhibition by altering surface GABA_AR γ 2 expression (6), we studied whether this mechanism is involved in epilepsy. Biochemical measurements of surface-expressed receptors showed the surface GABA_AR γ 2 expression is increased in the Clptm1-shRNA group compared to the control (Fig. 4A). Moreover, to determine whether these effects of Clptm1 downregulation on surface GABA_AR γ 2 numbers were involved in the efficacy of synaptic suppression, mIPSCs were recorded from mouse treated with either PTZ alone or Clptm1-shRNA. As shown in Fig. 4B, the mIPSC amplitudes were increased in Clptm1-shRNA group compared to the control, but mIPSC frequencies were not changed. Taken together, these findings indicate that Clptm1 regulate GABA_AR-mediated inhibitory synaptic transmission in epileptic model.

Discussion

Epilepsy frequently due to an imbalance in inhibition and excitation, which is a failure of neurotransmission. The evidence between epilepsy and dysfunctional GABA_AR-mediated inhibitory synaptic transmission is substantial and has been extensively reported (12-16). A decrease in the number of GABA_ARs present on the cell surface increased excitability in animal models of TLE (17-20). The epileptic animals which experienced spontaneous seizures also show failed inhibitory neurotransmission in the chronic stage (21). Association between GABA_AR and regulatory proteins regulate synaptic transmission and plasticity via multiple pathways. Firstly, GABA_AR exits the endoplasmic reticulum (ER) and is traffic to the plasma membrane. Receptor trafficking and accumulation is regulated by associations between GABA_AR and several binding partners. These associations lead to the formation of mature receptor complexes, which enables their proper localization and functions at cell surfaces and in synapses (22,23). Recently, Ge et al confirmed that Clptm1, a negative regulator that traps GABA_ARs within intracellular compartments, could limit their expression on the cell surface (6). In this study, we examined the role of clptm1 function and dysregulation of GABAergic neurotransmission in epilepsy.

Clptm1 was originally reported in a study of gene mutations in a cleft lip and palate patient family and has a role in intrathymic T-cell development (5, 24, 25). Very recently, Clptm1 has been shown to modulates forward trafficking of GABA_AR and inhibitory transmission in neuron (5). However, the function of clptm1 is not fully clear in central nervous system diseases. Few studies showed that Clptm1 is associated with Alzheimer disease (AD) and low-density lipoprotein cholesterol levels (26,27). In this study, we confirmed that Clptm1 regulates epileptic seizures by modulating the number of GABA_ARγ2 in the plasma membrane and efficacy of inhibitory synaptic. We demonstrated that Clptm1 was upregulated in chronic epileptic animal models and in TLE patients. Then we observed that Clptm1 downregulation increased the number of GABA_ARγ2 in the plasma membrane and mIPSCs amplitudes. Taken together, we showed that Clptm1 downregulation may be a molecular target for treatment of epilepsy.

The γ2 subunit is distributed throughout the central nervous system and is near-ubiquitous at synaptic GABA_A receptors (28,29). Thus, mutations in this receptor fundamentally change synaptic GABA_A receptor-mediated phasic inhibition. γ2 subunit mutations have been involved in severe myoclonic epilepsy in infancy (SMEI), Dravet syndrome (DS), genetic epilepsy syndromes, simple febrile seizures (FS) and childhood absence epilepsy (CAE) (30). γ2 mutations disrupt inhibitory synaptic transmission by aggravating IPSCs decay rates, whereas the other four mutations all reduce the number of GABA_ARs in the plasma membrane (30-36).

In summary, GABA_A receptors determine the efficacy of GABA-targeting drugs in TLE patients (37 -40). Understandably, elucidating the cellular mechanisms that modulates their assembly on the cell surface is of considerable interest. This study showed that Clptm1 is likely to modulate GABA_AR-mediated inhibitory synaptic transmission throughout the brain by regulating the number of GABA_ARs at the plasma membrane in epilepsy. Nevertheless, elucidation of molecular association networks involving Clptm1 and GABA_ARs is necessary.

Abbreviations

GABA _A Rs	γ-aminobutyric acid type A receptor
Clptm1	Cleft lip and palate transmembrane protein 1
PTZ	Pentylentetrazol
mIPSC	Miniature inhibitory post-synaptic current
shRNA	Short hairpin RNA

Declarations

Ethical considerations

All patients provided written informed consents. Chongqing Medical University admitted this study.

Consents for publication

Not applicable.

Availability of data and materials

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author Contribution Statement

Rong Li designed the study and wrote the manuscript, Ruifeng Wu, Qing Shen, Lianglin Wang and Menghao Zhang performed or assisted with all experiments, Peng Zhang collected human samples, Rong Mei and Fengli Zhang analysed the data, Sha Ma and Lan lin revised the manuscript. Yangmei Chen provided funding for the project. This manuscript was approved by all authors.

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Tables

Table 1-1 Clinical characteristics of patients with TLE

No.	Sex (M/F)	Age (years)	Duration	AEDs (years)	Resected tissue	Pathology result
1	M	7	3	OXC, CLB, VPA, TPM	LTN	G
2	F	12	5	OXC, VPA, GBP	LIN	G, NL
3	F	23	8	OXC, VPA, IPM	RIN	G, NL
4	F	17	5	VPA, CBZ, IPM	RIN	G, NL, ND
5	M	27	5	CBZ, VPA, CLB	LIN	G, NL, ND
6	M	13	7	LTG, IPM, CBZ	RIN	G
7	F	21	4	VPA, PB, CBZ, LEV	LIN	G, NL, ND
8	M	36	18	CBZ, VPA, CLB, IPM	LIN	G, NL, ND
9	M	16	7	OXC, VPA, PHI	LIN	G, NL
10	F	28	11	VPA, CBZ, PHI	RIN	G, NL

Table 1-2 Clinical characteristics of the control group

No.	Sex	Age (years)	Etiology diagnosis	Resected tissue	Seizure	Pathologic result
1	F	13	Brain trauma	LTN	None	Normal
2	M	24	Brain trauma	LTN	None	Normal
3	M	20	Brain trauma	RTN	None	Normal
4	M	25	Brain trauma	LTN	None	Normal
5	F	25	Brain trauma	RTN	None	Normal
6	M	31	Brain trauma	LTN	None	Normal
7	M	18	Brain trauma	LTN	None	Normal
8	M	44	Brain trauma	RTN	None	Normal
9	F	36	Brain trauma	LTN	None	Normal
10	F	33	Brain trauma	RTN	None	Normal

F, female; M, male; AEDs, anti-epileptic drugs; OXC, oxcarbazepine; CLB, clonazepam; VPA, valproic acid; TPM, topiramate; GBP, gabapentin; CBZ, carbamazepine; LTG, lamotrigine; PB, phenobarbital; LEV, levetiracetam; PHT, phenytoin; RTN, right temporal neocortex; LTN, left temporal neocortex; NL, neuronal loss; ND, neuronal degeneration; G, gliosis.

Figures

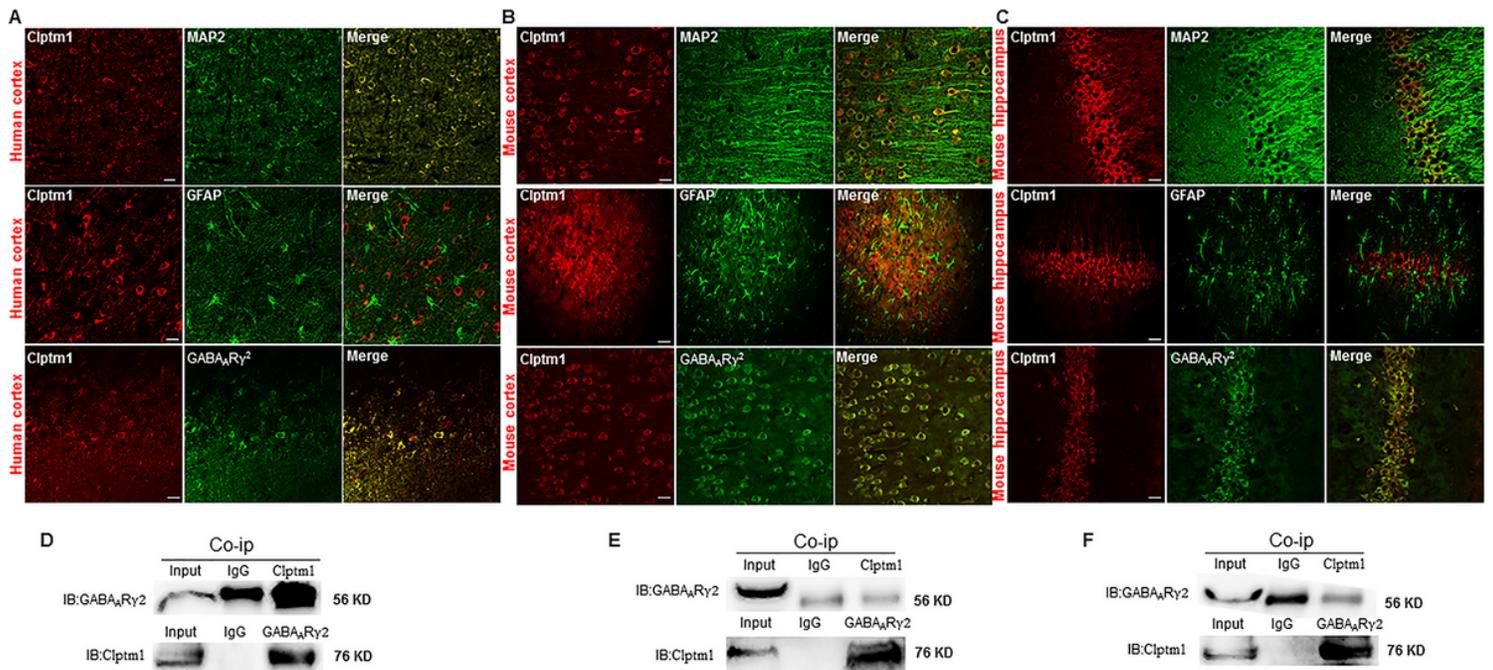


Figure 1

Clptm1 is localized in neuron and interacted with GABA_ARy2 in epileptic brain

A, B, C: The location of Clptm1 in epileptic brain. Immunofluorescent labelling showed that Clptm1 (red) co-localized with MAP2 (green) in TLE patients and both at cortex or hippocampus of epileptic mouse, but Clptm1 (red) are not co-localized with GFAP (green). Additionally, Clptm1 (red) co-localized with GABA_ARy2 (green) in epileptic brain. Scale bar = 50 μm (400×).

D, E, F: The interaction of Clptm1 and GABA_ARy2 in epileptic brain. Coimmunoprecipitation showed that Clptm1 is interacted with GABA_ARy2 in TLE patients, and the cortex as well as hippocampal neuron of the epileptic mouse are also.

Figure 2

GABA_ARy2 and Clptm1 expression are altered in epileptic brain

A. GABA_ARy2 expression in TLE patients and control. Western blot demonstrated that GABA_ARy2 expression in TLE patients was decreased compared to the control (n=12 pairs, **P < 0.01).

B, C. GABA_ARy2 expression in epileptic model and normal mouse. In chronic epileptic model, Clptm1 was decreased at hippocampus and cortex in epileptic model compared to the control (**P < 0.01, n=12 pairs).

D: Clptm1 expression in TLE patients and control. Western blot demonstrated that Clptm1 expression in TLE patients was increased compared to the control. (n=12 pairs, **P < 0.01).

E, F: Clptm1 expression in epileptic brain and normal mouse. Also, in chronic epileptic model, Clptm1 was increased at the hippocampus and cortex compared to the control. (n=12 pairs, *P < 0.05).

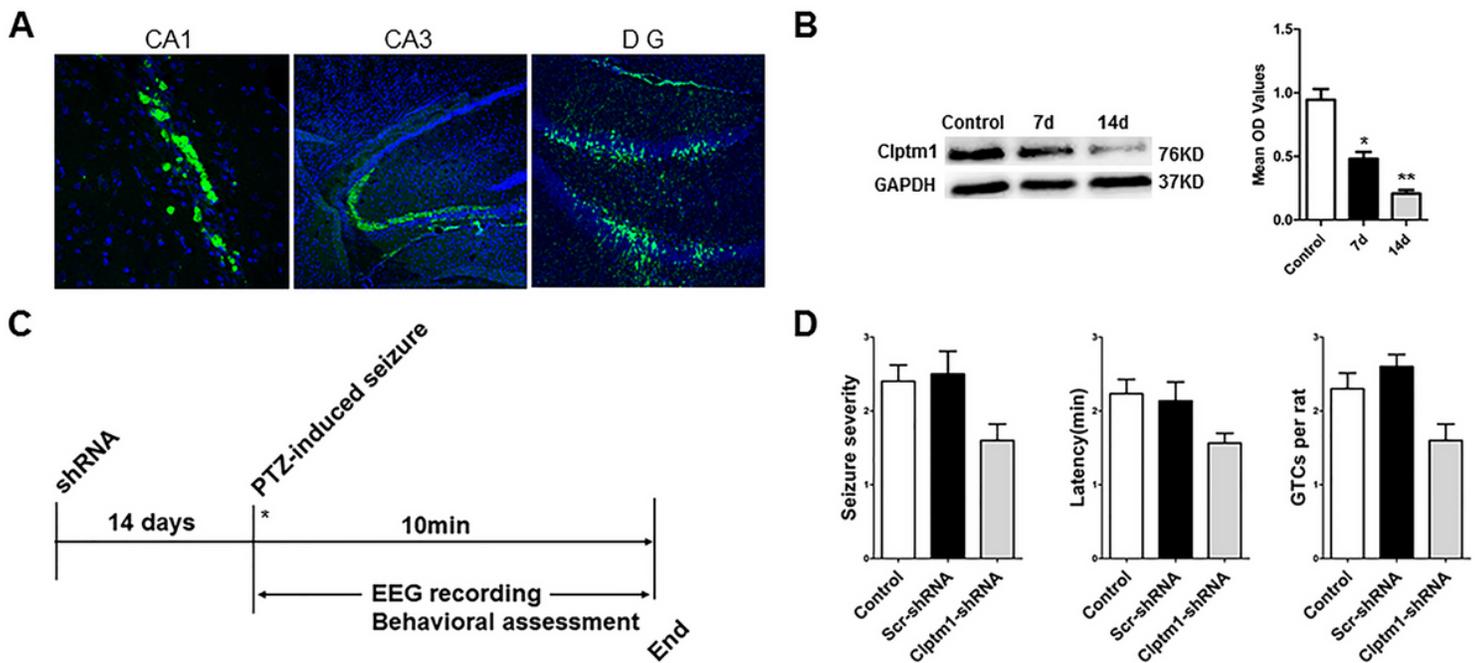


Figure 3

Clptm1 regulated PTZ-induced Seizure

A: Exogenous LV-GFP in the mouse hippocampus. Fluorescence (FITC)-labelled cells were transfected with exogenous LV-GFP. Scale bar = 50 μ m (400 \times).

B: Clptm1 expression in Clptm1-shRNA mouse. Western blot demonstrated that Clptm1 was decreased at 7 and 14 d post Clptm1-shRNA administration compared to the control (n=5, *P<0.05).

C: Schematic overview of Clptm1-shRNA +PTZ administration *in vivo*.

D: Behavior evaluation in Clptm1-shRNA. The severity and susceptibility of seizures were decreased in the Clptm1-shRNA compare to controls (Thirty mice were randomized into 3 groups of 10 each, *P < 0.05).

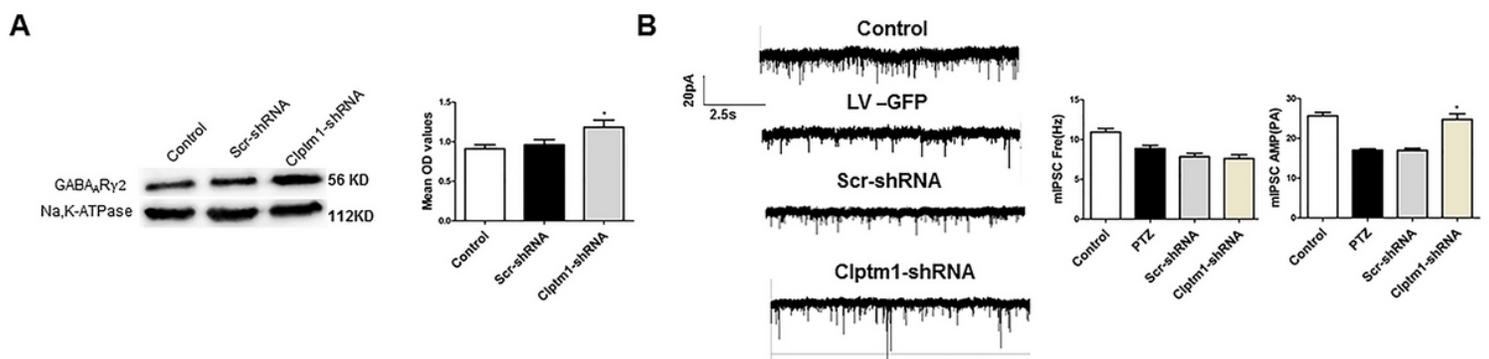


Figure 4

Clptm1 regulates GABA_AR-mediated inhibitory synaptic transmission in epileptic model

A: GABA_AR γ 2 expression in Clptm1-shRNA and control. Western blot demonstrated that the surface GABA_AR γ 2 expression in the Clptm1-shRNA group was increased compared to the control (n=5, *P <0.05).

B: mIPSCs in Clptm1-shRNA and control. mIPSCs amplitudes were increased in Clptm1-shRNA group compared to the control (n=15, **P < 0.01). No difference in mIPSCs frequencies.

Error bars denote mean \pm SEM.