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# Regulation of epileptiform activity by two distinct subtypes of extrasynaptic GABA<sub>A</sub> receptors

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## Abstract

**Background:** GABAergic deficit is one of the major mechanisms underlying epileptic seizures. Previous studies have mainly focused on alterations of synaptic GABAergic inhibition during epileptogenesis. Recent work suggested that tonic inhibition may also play a role in regulating epileptogenesis, but the underlying mechanism is not well understood.

**Results:** We employed molecular and pharmacological tools to investigate the role of tonic inhibition during epileptogenesis both *in vitro* and *in vivo*. We overexpressed two distinct subtypes of extrasynaptic GABA<sub>A</sub> receptors,  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  receptors, in cultured hippocampal neurons. We demonstrated that overexpression of both  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  receptors enhanced tonic inhibition and reduced epileptiform activity *in vitro*. We then showed that injection of THIP (5  $\mu$ M), a selective agonist for extrasynaptic GABA<sub>A</sub> receptors at low concentration, into rat brain also suppressed epileptiform burst activity and behavioral seizures *in vivo*. Mechanistically, we discovered that low concentration of THIP had no effect on GABAergic synaptic transmission and did not affect the basal level of action potentials, but significantly inhibited high frequency neuronal activity induced by epileptogenic agents.

**Conclusions:** Our studies suggest that extrasynaptic GABA<sub>A</sub> receptors play an important role in controlling hyperexcitatory activity, such as that during epileptogenesis, but a less prominent role in modulating a low level of basal activity. We propose that tonic inhibition may play a greater role under pathological conditions than in physiological conditions in terms of modulating neural network activity.

**Keywords:** Extrasynaptic GABA<sub>A</sub> receptor,  $\alpha 5$  subunit,  $\delta$  subunit, Tonic inhibition, Epileptogenesis, Epileptiform activity, THIP, Seizure behavior

## Background

Many antiepileptic drugs are targeting GABAergic synaptic transmission, but may cause certain side effects [1,2]. GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs) are abundant not only at synaptic sites, but also at extrasynaptic sites. Synaptic GABA<sub>A</sub>-Rs have low affinity for GABA, are activated in a transient manner by GABA released from presynaptic vesicles, and primarily mediate phasic inhibitory transmission. In contrast, extrasynaptic GABA<sub>A</sub>-Rs exhibit high affinity for GABA, are persistently activated by low concentration of ambient GABA, and mediate tonic inhibition [3-5]. There are two distinct subtypes of extrasynaptic

GABA<sub>A</sub>-Rs in the brain, one contains the  $\alpha 5$  subunit [6-10] and the other contains the  $\delta$  subunit [11-13]. The  $\alpha 5$ -GABA<sub>A</sub>-Rs are sensitive to a specific inverse agonist L-655,708 [14,15], while the  $\delta$ -GABA<sub>A</sub>-Rs are typically insensitive to benzodiazepine [16,17] but highly sensitive to THIP (gaboxadol) [18,19].

Functional deficit of synaptic GABAergic inhibition plays an important role in the etiology of epilepsy [2,20-22]. Recent studies revealed a possible role of tonic inhibition in modulating epileptic seizures [23,24]. A significant reduction of GABA<sub>A</sub>-R  $\alpha 5$  and  $\delta$  subunit level has been reported in the hippocampus of animals with temporal lobe epilepsy (TLE) [8,25,26]. Interestingly, the decrease of  $\delta$  subunit may be compensated by an increase of  $\alpha 4$  and  $\gamma 2$  subunits [26-28]. Mutations in the  $\delta$  subunit of GABA<sub>A</sub>-Rs have been mapped in human epilepsy patients [29,30]. Increased  $\delta$  subunit level during diestrus stage of ovarian cycle has been

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associated with less seizure activities in kainic acid-induced epilepsy models [31]. However, in the pyramidal neurons of hippocampal CA1 region, the  $\alpha 5$  GABA<sub>A</sub>-R mediated tonic current was reduced but overall tonic inhibition was not changed or even increased in pilocarpine epilepsy model [32]. Furthermore, enhanced tonic inhibition in thalamocortical neurons was reported to induce absence seizure [33], suggesting that different tonic inhibition may play different roles in different brain regions.

Here we investigated the functional role of two distinctly different subtypes of extrasynaptic GABA<sub>A</sub>-Rs in hippocampal epileptogenesis. We demonstrated that enhancing tonic inhibition by overexpressing either the  $\alpha 5\beta 3\gamma 2$  or  $\alpha 6\beta 3\delta$  extrasynaptic GABA<sub>A</sub> receptors significantly inhibited the formation of epileptiform activity in hippocampal cultures. Furthermore, *in vivo* injection of selective extrasynaptic GABA<sub>A</sub>-R agonist THIP also inhibited epileptiform bursting activity in anesthetized rats and seizure behaviors in freely moving rats. Interestingly, low concentration of THIP did not affect basal level of neuronal activity, but significantly suppressed higher frequency neuronal firing. Therefore, our data suggest that tonic inhibition mediated by extrasynaptic GABA<sub>A</sub>-Rs may play a more prominent function in pathological conditions such as during epileptogenesis.

## Results

### Molecular enhancement of tonic GABA currents after overexpressing $\alpha 5\beta 3\gamma 2$ GABA<sub>A</sub> receptors

We have previously demonstrated that epileptiform activity downregulates tonic inhibition mediated by extrasynaptic GABA<sub>A</sub> receptors [34]. In this study, we investigated the effect of enhanced tonic inhibition on epileptiform activity. The majority of extrasynaptic GABA<sub>A</sub> receptors (GABA<sub>A</sub>-Rs) can be categorized into two distinct subtypes, containing either the  $\alpha 5$  or the  $\delta$  subunit. The  $\alpha 5$ -GABA<sub>A</sub>-Rs are mainly expressed in the hippocampus of adult brain, while  $\delta$ -GABA<sub>A</sub>-Rs are mostly expressed in dentate and cerebellar granule cells as well as in the thalamus. To understand which subtype of extrasynaptic GABA<sub>A</sub>-Rs may affect epileptiform activity, we overexpressed both the  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  GABA<sub>A</sub>-Rs in hippocampal neurons to enhance tonic inhibition and tested their effects on epileptiform activity. Since  $\alpha 5$  subunit is normally expressed in hippocampal pyramidal neurons [6], we first tested the effect of  $\alpha 5\beta 3\gamma 2$  receptors on epileptiform activity. We have tested several  $\alpha 5$  subunits and found a right one that can give large GABA-evoked current when coexpressed with the  $\beta 3$  and  $\gamma 2$  subunits in HEK 293T cells (Figure 1A). The inverse agonist L-655,708 (100 nM) specific for the  $\alpha 5$  subunit significantly inhibited the GABA-evoked currents (Figure 1A-B; Control,  $437 \pm 63$  pA,  $n = 10$ ; L-655,708,  $143 \pm 26$  pA,  $n = 10$ ; \*\*\*,  $p < 0.001$ ; Student's *t* test),

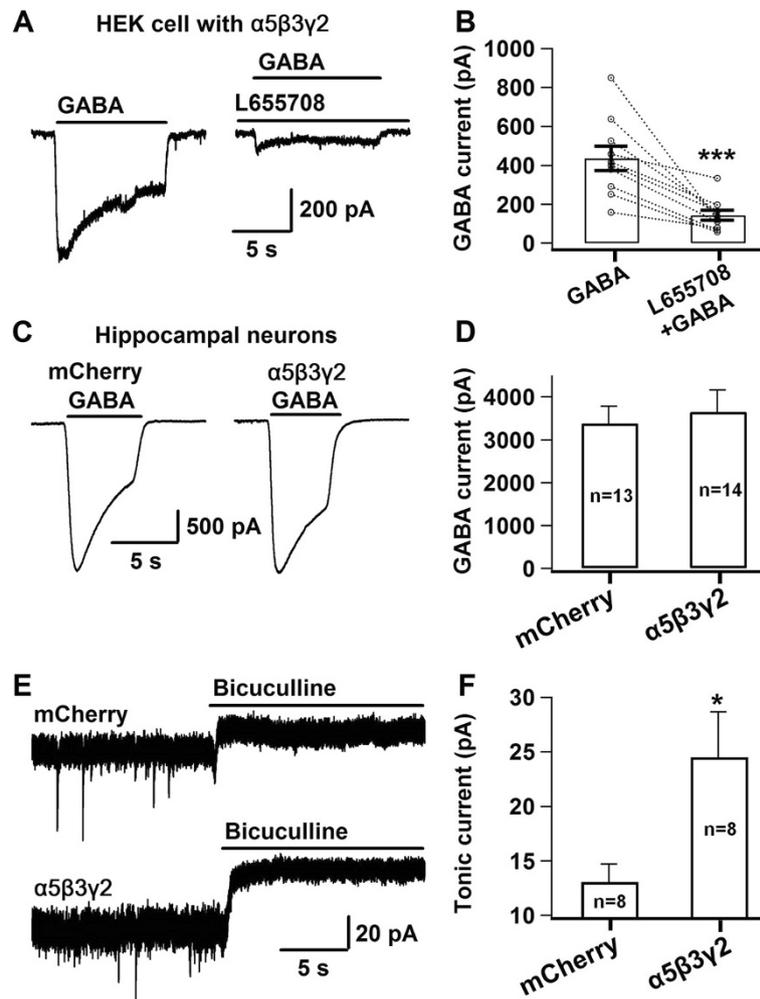
confirming that the GABA current was mediated by  $\alpha 5$  subunit-containing GABA<sub>A</sub>-Rs. We next overexpressed the  $\alpha 5\beta 3\gamma 2$  receptors in cultured hippocampal pyramidal neurons. The GABA-evoked whole-cell currents appeared to be similar between neurons transfected with the  $\alpha 5\beta 3\gamma 2$  receptors and the mCherry for control (Figure 1C-D; mCherry,  $3380 \pm 408$  pA,  $n = 13$ ;  $\alpha 5\beta 3\gamma 2$ ,  $3646 \pm 518$  pA,  $n = 14$ ;  $p > 0.5$ ). However, tonic GABA currents revealed by acute application of GABA<sub>A</sub>-R blocker bicuculline (Bic, 40  $\mu$ M; together with 1  $\mu$ M TTX and 10  $\mu$ M DNQX) [9,34,35], were significantly increased in  $\alpha 5\beta 3\gamma 2$ -transfected neurons compared to mCherry-transfected control neurons (Figure 1E-F; mCherry,  $13.1 \pm 1.7$  pA,  $n = 8$ ;  $\alpha 5\beta 3\gamma 2$ ,  $24.5 \pm 4.2$  pA,  $n = 8$ ;  $p < 0.05$ ). These data demonstrated that overexpression of the  $\alpha 5\beta 3\gamma 2$  receptors enhanced tonic GABA currents in hippocampal neurons.

### Tonic inhibition mediated by the $\alpha 5\beta 3\gamma 2$ receptors suppresses epileptiform activity

We further tested whether epileptiform activity was affected by the enhanced tonic GABA current in neurons overexpressing the  $\alpha 5\beta 3\gamma 2$  receptors. We previously established a unique cyclothiazide (CTZ) induced epilepsy model both *in vitro* and *in vivo* [36-39]. The advantage of CTZ model is its reliability in inducing epileptiform activity without significant cell death [36]. We treated hippocampal culture with CTZ (5  $\mu$ M, 24 hr) to induce epileptiform activity, similar to reported before [36]. Control neurons transfected with mCherry showed robust epileptiform activity after CTZ-treatment, with a train of high-frequency action potentials overlaying on the plateau of large depolarization shifts (Figure 2A). In contrast, the majority of neurons transfected with the  $\alpha 5\beta 3\gamma 2$  receptors did not show clear epileptiform activity, except some sparse action potentials on top of small depolarization bursts (Figure 2B). Quantitatively, about 90% of control neurons showed epileptiform activity after CTZ-treatment (26 out of 29 neurons), whereas only about 33% of neurons transfected with the  $\alpha 5\beta 3\gamma 2$  receptors showed epileptiform activity (11 out of 33 neurons) (Figure 2C). The average frequency of epileptiform bursts was also significantly reduced in  $\alpha 5\beta 3\gamma 2$ -transfected neurons (Figure 2D; mCherry,  $1.83 \pm 0.35$  per min,  $n = 29$ ;  $\alpha 5\beta 3\gamma 2$ ,  $0.49 \pm 0.16$  per min,  $n = 33$ ;  $p < 0.001$ ). Together, our experiments demonstrated that enhanced tonic inhibition mediated by the  $\alpha 5\beta 3\gamma 2$  receptors significantly suppressed the formation of epileptiform activity in hippocampal neurons.

### Inhibition of epileptiform activity by the $\alpha 6\beta 3\delta$ receptors

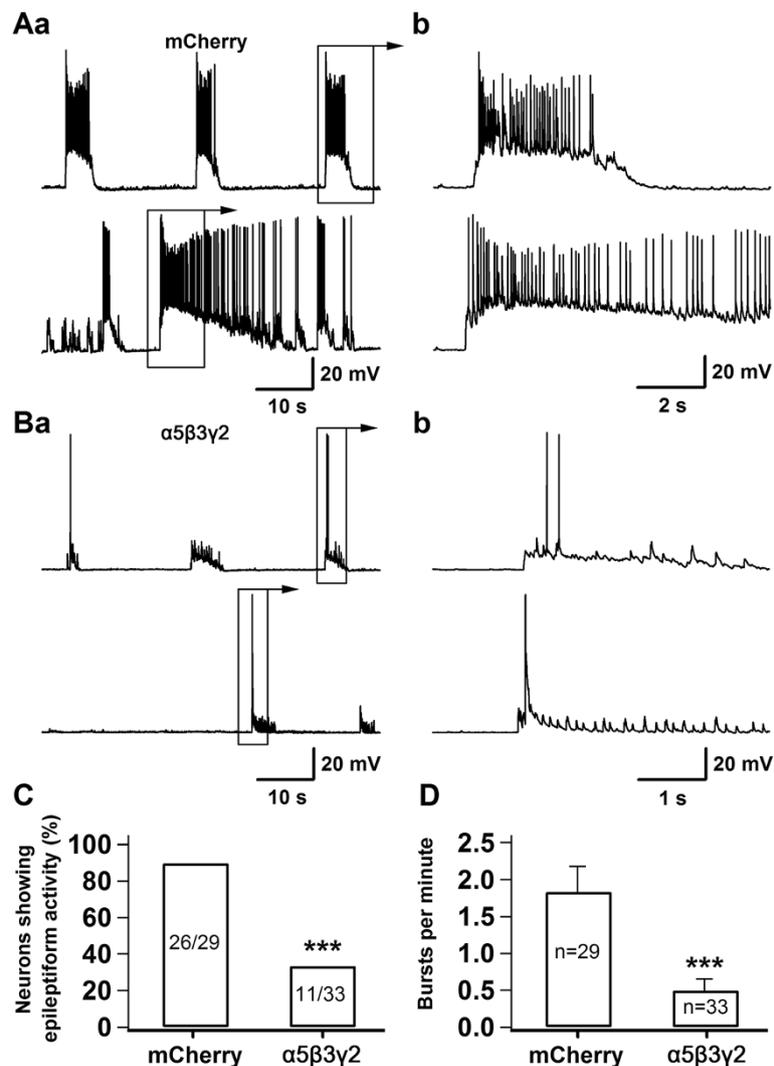
We next examined a distinctly different subtype of extrasynaptic GABA<sub>A</sub>-Rs, the  $\alpha 6\beta 3\delta$  receptors [11,12], in the regulation of epileptiform activity. We first examined



**Figure 1** Tonic GABA current increased after the overexpression of  $\alpha 5\beta 3\gamma 2$  GABA<sub>A</sub> receptors. **A**, Typical GABA (100  $\mu$ M) induced currents in HEK293T cells transfected with  $\alpha 5\beta 3\gamma 2$  subunits (left panel), which could be largely blocked by  $\alpha 5$  subunit-specific inverse agonist L655,708 (100 nM, right panel). **B**, Summarized data showing GABA-induced  $\alpha 5\beta 3\gamma 2$  receptor currents in HEK293T cells significantly inhibited by L655,708 (Control,  $437.1 \pm 63.1$  pA,  $n = 10$ ; L655,708,  $142.8 \pm 25.7$  pA,  $n = 10$ ; \*\*\*,  $p < 0.001$ ). **C**, Typical GABA current traces in cultured hippocampal neurons transfected with mCherry or plus the  $\alpha 5\beta 3\gamma 2$  subunits. **D**, Bar graphs showing no significant difference between the total whole-cell GABA currents in neurons transfected with mCherry or plus the  $\alpha 5\beta 3\gamma 2$  subunits. **E**, Representative tonic GABA currents revealed by rapid application of GABA<sub>A</sub>-R blocker bicuculline (100  $\mu$ M) in hippocampal neurons transfected with mCherry or plus the  $\alpha 5\beta 3\gamma 2$  subunits. **F**, Summarized data showing that tonic GABA current in  $\alpha 5\beta 3\gamma 2$ -transfected neurons ( $24.5 \pm 4.2$  pA,  $n = 8$ ) was significantly increased in comparison with the control neurons ( $13.1 \pm 1.7$  pA,  $n = 8$ ; \*,  $p < 0.05$ ).

whole-cell GABA currents after overexpressing  $\alpha 6\beta 3\delta$  receptors in hippocampal cultures. GABA-evoked whole-cell currents showed no difference between GFP- and  $\alpha 6\beta 3\delta$ -transfected neurons (Figure 3A). Quantitatively, the average GABA-evoked current in  $\alpha 6\beta 3\delta$ -transfected neurons was  $3327 \pm 206$  pA ( $n = 10$ ), which was not significantly different from the GFP control ( $3967 \pm 328$  pA,  $n = 10$ ,  $p > 0.1$ ; Figure 3B). To confirm functional expression of the  $\alpha 6\beta 3\delta$  receptors in hippocampal neurons, we examined tonic GABA current after Bic treatment and found that the tonic GABA current was greatly increased in  $\alpha 6\beta 3\delta$ -transfected neurons (Figure 3C). Quantitatively, the average amplitude of tonic GABA currents in  $\alpha 6\beta 3\delta$ -

transfected neurons was  $28.1 \pm 3.6$  pA ( $n = 10$ ), significantly larger than that of control neurons ( $7.4 \pm 1.1$  pA,  $n = 11$ ,  $p < 0.0001$ ) (Figure 3D). Furthermore, we employed THIP (5  $\mu$ M), a relatively specific agonist for  $\delta$  subunit containing GABA<sub>A</sub>-Rs at low concentration, to investigate tonic currents in control and  $\alpha 6\beta 3\delta$ -transfected neurons. THIP activated a small non-desensitizing tonic current in GFP-transfected neurons, accompanied with an increase of the baseline noise (Figure 3E, top trace). In  $\alpha 6\beta 3\delta$ -transfected neurons, however, the THIP-induced tonic current was markedly increased (Figure 3E, bottom trace). The average amplitude of THIP current in control neurons was  $35.3 \pm 7.4$  pA ( $n = 11$ ), and greatly increased to

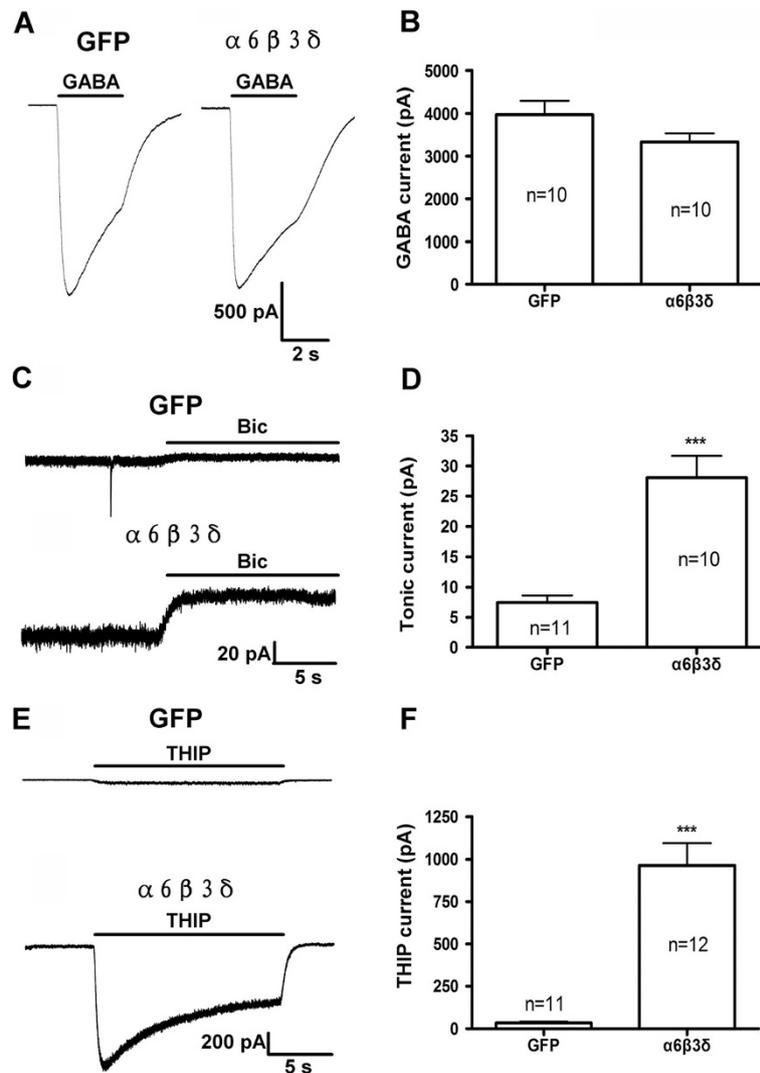


**Figure 2 Inhibition of epileptiform activity in cultured hippocampal neurons overexpressing  $\alpha 5\beta 3\gamma 2$  receptors.** **A**, Typical traces from two hippocampal neurons showing the epileptiform burst activity after chronic CTZ treatment (5  $\mu$ M, 24 h). Panel b shows the expanded view of a single epileptiform burst from panel a. Epileptiform burst is characterized by a train of action potentials on a large depolarization shift. **B**, Representative traces showing the lack of epileptiform bursts in two hippocampal neurons transfected with the  $\alpha 5\beta 3\gamma 2$  receptors. **C**, Bar graphs illustrating that overexpression of  $\alpha 5\beta 3\gamma 2$  receptors significantly reduced the percentage of neurons showing epileptiform activity after chronic CTZ treatment (mCherry, ~90%, n = 29;  $\alpha 5\beta 3\gamma 2$ , ~33%, n = 33; \*\*\*, p < 0.001). **D**, Neurons transfected with  $\alpha 5\beta 3\gamma 2$  receptors showing lower burst frequency (0.49  $\pm$  0.16 per min, n = 33), compared to mCherry controls after CTZ treatment (1.83  $\pm$  0.35 per min, n = 29; \*\*\*, p < 0.001).

962  $\pm$  130 pA in  $\alpha 6\beta 3\delta$ -transfected neurons (n = 12, p < 0.0001; Figure 3F). Thus, overexpression of  $\alpha 6\beta 3\delta$  receptors in hippocampal neurons significantly increased tonic GABA currents, similar to the overexpression of  $\alpha 5\beta 3\gamma 2$  receptors.

We then examined whether overexpression of the  $\alpha 6\beta 3\delta$  receptors has any effect on epileptiform activity. In GFP-transfected control neurons, CTZ-treatment (5  $\mu$ M, 24 hr) induced robust epileptiform activity as expected (Figure 4A). In contrast, hippocampal neurons transfected with  $\alpha 6\beta 3\delta$  receptors showed a significant attenuation of epileptiform activity (Figure 4B). Quantitatively, the

percentage of neurons displaying epileptiform activity was 84% (21 out of 25) in GFP-transfected controls, but reduced to 31% (9 out of 29) in  $\alpha 6\beta 3\delta$ -transfected neurons (Figure 4C; p < 0.001). Furthermore, the frequency of epileptiform bursts in  $\alpha 6\beta 3\delta$ -transfected neurons also reduced significantly (0.56  $\pm$  0.2 bursts per min, n = 29, p < 0.003), compared to that in GFP control neurons (1.64  $\pm$  0.28 bursts per min, n = 25) (Figure 4D). We investigated whether the overexpression of extrasynaptic GABA<sub>A</sub>-Rs would alter neuronal intrinsic properties, but found no changes in resting membrane potential (mCherry, -52.3  $\pm$  1.3 mV, n = 15;  $\alpha 5\beta 3\gamma 2$ , -50.1  $\pm$  1.6 mV,

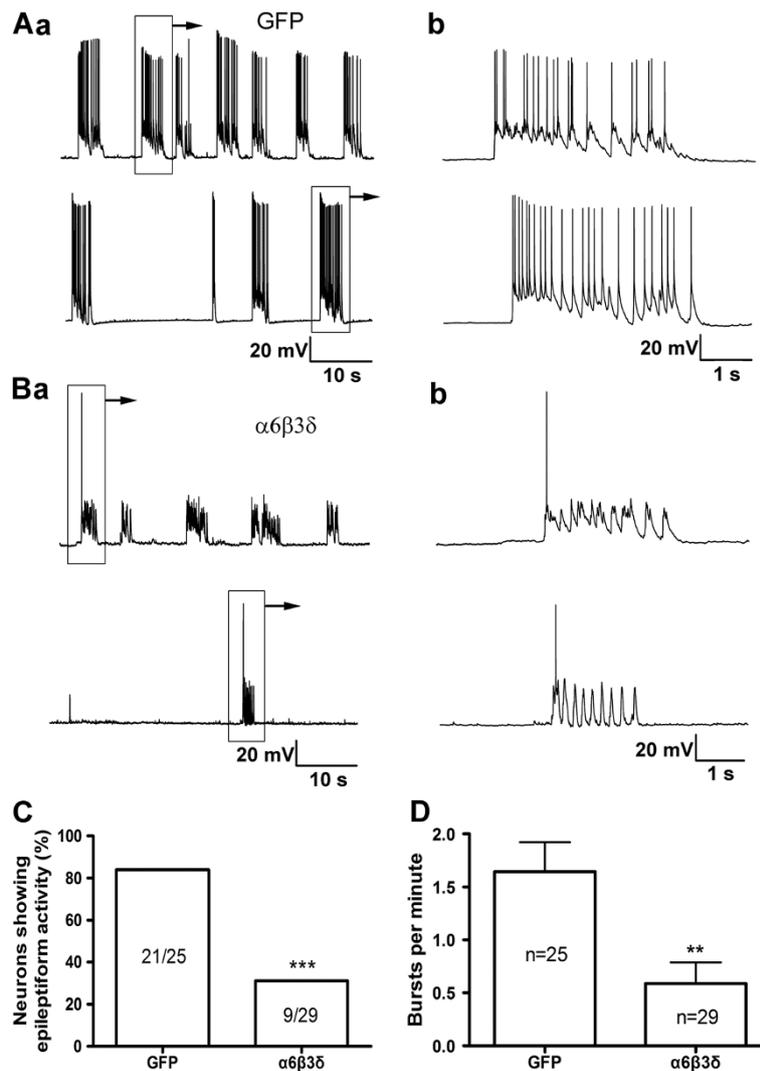


**Figure 3** Overexpression of  $\alpha 6 \beta 3 \delta$  subunits results in large tonic GABA current in cultured hippocampal neurons. **A**, Typical recordings showing whole-cell currents induced by rapid application of GABA (20  $\mu$ M) in GFP control and  $\alpha 6 \beta 3 \delta$ -transfected neurons in the presence of TTX (1  $\mu$ M) and DNQX (10  $\mu$ M). **B**, Summarized data showing no significant difference in whole-cell GABA currents between the two groups. **C**, Typical traces of tonic GABA currents, revealed by application of Bic (40  $\mu$ M) in the presence of TTX (1  $\mu$ M) and DNQX (10  $\mu$ M), recorded from a GFP control neuron and a  $\alpha 6 \beta 3 \delta$ -transfected neuron. Holding potential =  $-70$  mV. **D**, Summarized data showing that the average amplitude of tonic GABA currents was significantly increased in  $\alpha 6 \beta 3 \delta$ -transfected neurons ( $28.1 \pm 3.6$  pA,  $n = 10$ ), compared to the GFP controls ( $7.4 \pm 1.1$  pA,  $n = 11$ ;  $***, p < 0.0001$ ). **E**, Tonic currents activated by THIP (5  $\mu$ M) in GFP control and  $\alpha 6 \beta 3 \delta$ -transfected neurons. **F**, Summarized data showing a significant increase of THIP-induced tonic currents after transfection of  $\alpha 6 \beta 3 \delta$  subunits ( $962 \pm 130$  pA,  $n = 12$ ), compared to the GFP controls ( $35.3 \pm 7.4$  pA,  $n = 11$ ;  $***, p < 0.0001$ ).

$n = 12$ ;  $\alpha 6 \beta 3 \delta$ ,  $-51.5 \pm 1.4$  mV,  $n = 10$ ;  $p > 0.5$ , one way ANOVA), membrane resistance (mCherry,  $204 \pm 22$  M $\Omega$ ,  $n = 15$ ;  $\alpha 5 \beta 3 \gamma 2$ ,  $177 \pm 17$  M $\Omega$ ,  $n = 12$ ;  $\alpha 6 \beta 3 \delta$ ,  $237 \pm 24$  M $\Omega$ ,  $n = 10$ ;  $p > 0.4$ ), or membrane capacitance (mCherry,  $96.3 \pm 7.8$  pF,  $n = 15$ ;  $\alpha 5 \beta 3 \gamma 2$ ,  $114.6 \pm 10.8$  pF,  $n = 12$ ;  $\alpha 6 \beta 3 \delta$ ,  $95.7 \pm 16.1$  pF,  $n = 10$ ;  $p > 0.19$ ) in different transfected groups. Together, our data demonstrated that both  $\alpha 6 \beta 3 \delta$  and  $\alpha 5 \beta 3 \gamma 2$  extrasynaptic GABA<sub>A</sub>-Rs have an important role in regulating hippocampal epileptogenesis *in vitro*.

#### Tonic inhibition on *in vivo* epileptic seizures

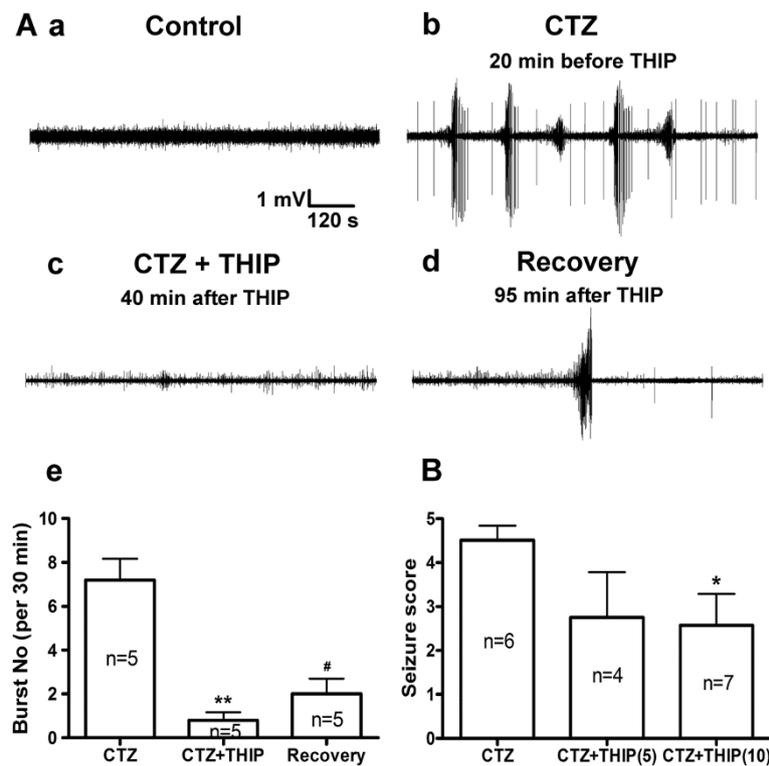
After performing *in vitro* studies, we decided to further investigate the effect of tonic inhibition on epileptiform activity in *in vivo* condition. We recorded field potentials in the hippocampal CA1 pyramidal layer from 5 urethane-anaesthetized rats. In all 5 rats studied, the evoked responses following low frequency stimulation of CA3 region consisted of a large EPSP and a single population spike (PS) during control recordings (data not shown, see [36]), and the baseline activity was virtually



**Figure 4** Overexpression of  $\alpha 6 \beta 3 \delta$  receptors inhibits epileptiform bursting activity in cultured hippocampal neurons. **Aa**, Representative traces showing the typical recurrent epileptiform bursts after chronic pretreatment with CTZ (5  $\mu$ M, 24 h) in two different GFP-transfected pyramidal neurons. **Ab**, A single epileptiform burst in (a) was expanded to show a train of action potentials overlaying on a large depolarization shift. **Ba**, Representative traces showing the lack of typical epileptiform bursts in two  $\alpha 6 \beta 3 \delta$ -transfected hippocampal neurons. **Bb**, Expanded view of the boxed activity in (a). **C**, Bar graph showing the percentage of neurons with epileptiform bursting activity after chronic treatment with CTZ (5  $\mu$ M for 24 h). \*\*\*  $p < 0.001$ , Pearson Chi-Square test. **D**, Bar graph showing a significant reduction of the average epileptiform burst frequency in neurons transfected with  $\alpha 6 \beta 3 \delta$  receptors, comparing to GFP controls after CTZ treatment. \*\*  $p < 0.01$ .

'silent' (Figure 5Aa). Following intra-cerebroventricular (i.c.v.) injection of CTZ (5  $\mu$ mol in 5  $\mu$ l), the single-peaked PS gradually transformed into a multiple-peaked event and the spontaneous recurrent epileptiform bursts were stable for at least 30 min in all 5 rats tested (Figure 5Ab). To enhance tonic inhibition *in vivo*, we chose to use agonist specific for extrasynaptic GABA<sub>A</sub>-Rs. For the  $\alpha 5$  subunit-containing GABA<sub>A</sub>-Rs, there is no specific agonist available except inverse agonists that reduce receptor responses. However, THIP at low concentration is a selective agonist for the  $\delta$  subunit-containing GABA<sub>A</sub>-Rs and widely used to elicit tonic

inhibition both *in vitro* and *in vivo* [40-43]. Therefore, we delivered THIP (4 mg/kg) through the cannula pre-implanted in the lateral tail vein after the induction of epileptiform activity. Compared with the epileptiform burst number ( $7.2 \pm 1.0$  per 30 min) before THIP injection, the mean burst number after THIP administration was significantly reduced to  $0.8 \pm 0.4$  ( $p < 0.003$ ) over a 30 min analysis period (Figure 5Ac). Interestingly, the inhibitory effect of THIP on epileptiform activity was reversible, as indicated by a gradual increase of the mean burst number ( $2.0 \pm 0.7$  per 30 min) during a prolonged recovery period after the THIP injection



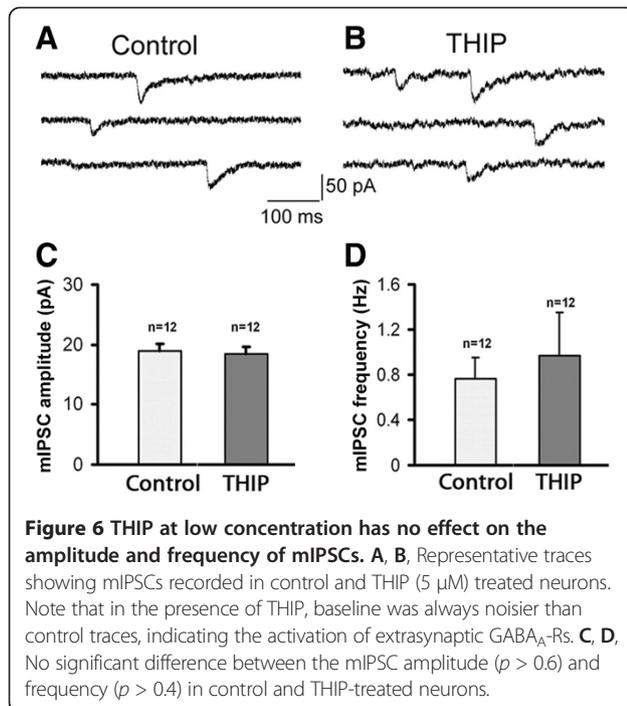
**Figure 5 THIP inhibits CTZ-induced epileptiform activity and seizure behavior.** Aa-d, Typical traces showing 'silent' baseline activity of hippocampal CA1 neurons in control condition (a), synchronized epileptiform bursting activities induced by CTZ (5  $\mu$ mol) (b), inhibition of THIP (4 mg/kg) on burst activity (c), and the recovery after the THIP injection (d). Ae, Bar histogram showing group data of THIP inhibition on CTZ-induced epileptiform burst activities. B, Bar histogram showing group data of CTZ-induced (5  $\mu$ mol) seizure behavioral score and its significant attenuation by pre-treatment with THIP (10 mg/kg). THIP(5): 5 mg/kg THIP; THIP(10): 10 mg/kg THIP. \*  $p < 0.05$  and \*\*  $p < 0.01$  in comparison with CTZ injection alone; #  $p < 0.05$  for recovery, in comparison with CTZ + THIP.

(Figure 5Ad). The quantification of the burst frequency of CTZ, CTZ + THIP, and the recovery group was summarized in Figure 5Ae. These results indicate that acute application of THIP in *in vivo* condition can effectively suppress epileptiform activity.

Besides epileptiform activity, we further studied whether THIP can directly modulate CTZ-induced seizure behavior in freely moving rats [38]. CTZ was injected repeatedly each day (0.25  $\mu$ mol i.c.v. for 3 consecutive days, total dose of 0.75  $\mu$ mol) to induce seizure behavior without or with a pre-injection of THIP. The administration of THIP (5 or 10 mg/kg, i.p.) at 10 min before CTZ injection dose-dependently attenuated the convulsant seizures induced by CTZ. The seizure score was  $4.5 \pm 0.3$  ( $n = 6$ ) after CTZ injection alone, and significantly reduced to  $2.6 \pm 0.7$  ( $n = 7$ ) in THIP-preinjected (10 mg/kg) animals ( $p < 0.05$ ; Figure 5B). The lower dose of THIP pretreatment (5 mg/kg) also reduced seizure score but not reaching statistical significance. Therefore, THIP may be used as a potential anti-convulsant drug to suppress seizure behaviors in living animals.

#### Tonic inhibition and basal GABAergic neurotransmission

While previous studies have linked tonic inhibition with seizure threshold [31,44], the underlying mechanism is not fully understood. We decided to investigate the mechanism of tonic inhibition in regulating epileptiform activity. One question we addressed is whether tonic inhibition has any direct effect on basal GABAergic synaptic transmission in rat hippocampal cultures. Spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of TTX (1  $\mu$ M) and CNQX (10  $\mu$ M) to block action potentials and glutamatergic responses, respectively. We have previously shown that THIP at micromolar concentrations elicited a tonic current (10–30 pA) in hippocampal neurons [34]. Here, we found that application of 5  $\mu$ M THIP had no significant effect on both the mIPSC amplitude (control,  $18.9 \pm 1.1$  pA,  $n = 12$ ; THIP,  $18.3 \pm 1.3$  pA,  $n = 12$ ;  $p > 0.6$ ) and the frequency (control,  $0.76 \pm 0.18$  Hz,  $n = 12$ ; THIP,  $0.96 \pm 0.30$  Hz,  $n = 12$ ;  $p > 0.4$ ) (Figure 6). We did notice that the baseline noise in the presence of THIP was always larger than the controls, indicating the tonic activation of extrasynaptic GABA<sub>A</sub>-Rs by low concentration of THIP.



The null effect of 5  $\mu$ M THIP on mIPSCs suggested that synaptic GABA<sub>A</sub>-Rs are not significantly affected at this low concentration of THIP.

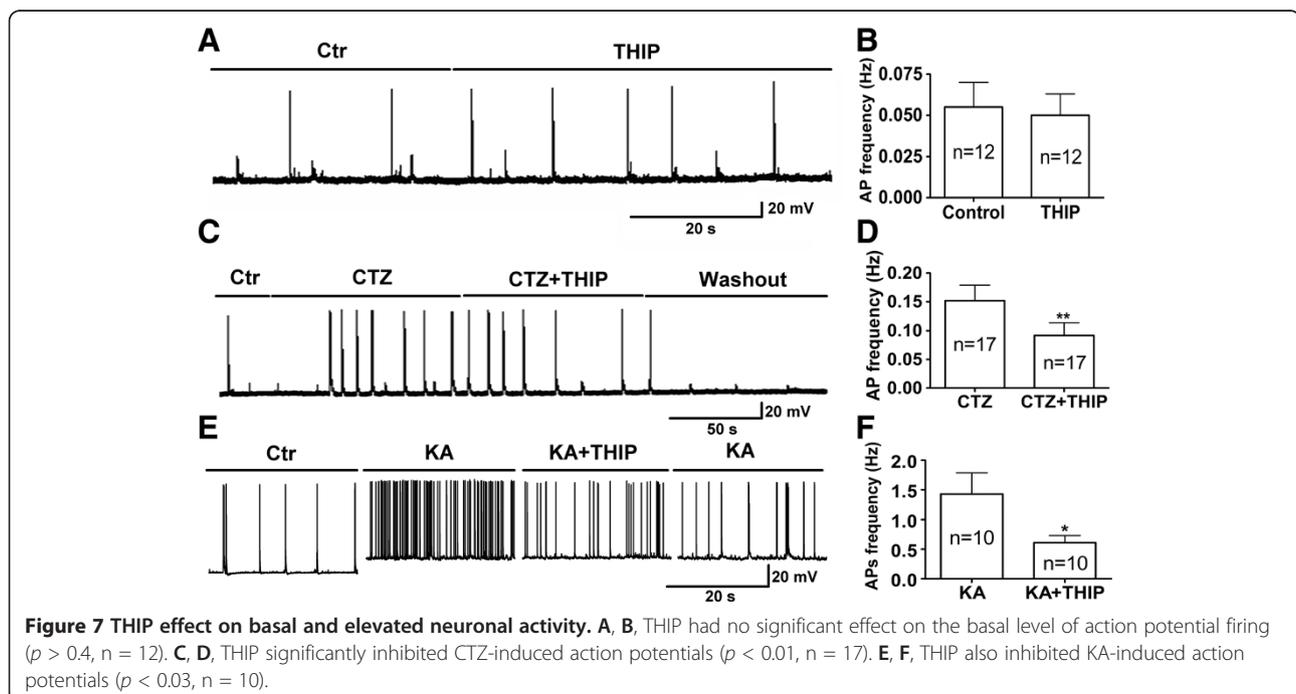
#### Mechanism of tonic inhibition in modulating neuronal activity

We next examined the effect of THIP on neuronal activity at basal level as well as under stimulated conditions. In

normal bathing solution, application of low concentration of THIP (5  $\mu$ M) showed no significant effect on action potential firing in the majority of neurons tested (Figure 7A-B; control,  $0.055 \pm 0.015$  Hz; THIP,  $0.050 \pm 0.013$  Hz;  $n = 12$ ,  $p > 0.4$ , paired  $t$  test). However, when neuronal activity was elevated by CTZ (5  $\mu$ M) as demonstrated previously [36], THIP exerted a significant inhibitory effect on neuronal activity (Figure 7C-D). Action potential firing frequency dropped significantly from  $0.152 \pm 0.027$  Hz under CTZ application to  $0.092 \pm 0.022$  Hz when THIP was applied together with CTZ ( $n = 17$ ,  $p < 0.01$ , paired  $t$  test). To confirm that THIP has greater effect when neuronal activity is elevated, we further stimulated neurons with low concentration of kainic acid (KA). Bath application of KA (2.5  $\mu$ M) induced a large membrane depolarization and a remarkable increase of action potential firing (Figure 7E). Importantly, co-application of 5  $\mu$ M THIP together with KA significantly decreased the firing frequency in all of the neurons recorded (Figure 7E-F; KA,  $1.4 \pm 0.4$  Hz; KA+THIP,  $0.6 \pm 0.1$  Hz;  $n = 10$ ,  $p < 0.03$ , paired  $t$  test). Together, our results suggest that tonic inhibition may have greater modulatory effect on neural networks with hyperexcitatory activity, such as that under epileptogenic stimulation, making extrasynaptic GABA<sub>A</sub>-Rs ideal for developing novel anti-convulsant drugs.

#### Discussion

In this study, we have demonstrated that two distinct subtypes of extrasynaptic GABA<sub>A</sub>-Rs both play an important role in regulating the formation of epileptiform



activity in hippocampal cultures. Enhancing tonic inhibition in living animals *in vivo* also modulates epileptiform activity and behavioral seizures in a dose-dependent manner. More importantly, we demonstrated that tonic inhibition potently inhibits high frequency action potentials under stimulated conditions but not at basal low frequency firing condition, suggesting a novel mechanism of tonic inhibition in regulating neural network activity.

#### **Extrasynaptic GABA<sub>A</sub>-Rs regulate epileptogenesis**

Due to its continuous activation by ambient GABA, the charge transfer of tonic currents mediated by extrasynaptic GABA<sub>A</sub>-Rs have been estimated to be several folds more than phasic currents mediated by synaptic GABA<sub>A</sub>-Rs [4,45,46]. Tonic GABA conductance controls the overall gain of neuronal input–output [5,45–48]. Therefore, when GABA<sub>A</sub> receptor  $\alpha 5$  and  $\delta$  subunits were found significantly reduced in the hippocampus of animal TLE models [8,25,49,50], it was realized that downregulation of tonic inhibition might have contributed to epileptogenesis. Consistently, our previous work has also demonstrated that tonic GABA inhibition was downregulated after chronic epileptogenic stimulation in cultured hippocampal neurons [34]. However, later studies found that despite a reduction of  $\alpha 5$  and  $\delta$  subunit expression in epileptic mice, tonic inhibition in the hippocampus was largely maintained or even increased, possibly mediated by increased expression of  $\alpha 4\gamma 2$ -containing GABA<sub>A</sub>-Rs [26,28,32]. Thus, tonic inhibition might have undergone homeostatic changes during and after epileptogenesis [51]. Our current study demonstrated that enhancing tonic inhibition by increasing the expression level of either  $\alpha 5\beta 3\gamma 2$  or  $\alpha 6\beta 3\delta$  receptors can effectively suppress epileptiform activity. We have further demonstrated that seizure behaviors are attenuated by enhancing tonic inhibition *in vivo*. Based on previous and our own studies, we attribute an important role to tonic inhibition in modulating hippocampal epileptogenesis: enhancing tonic inhibition will inhibit epileptiform activity, while reducing tonic inhibition will increase the susceptibility of epileptic seizures [31,44].

#### **Overexpression of extrasynaptic GABA<sub>A</sub>-Rs regulates epileptiform activity**

While THIP has been used previously to modulate epileptiform activity, the target receptors were usually not specifically identified because higher concentrations of THIP might activate different combinations of synaptic and extrasynaptic GABA<sub>A</sub>-Rs. Our current study provides more direct evidence on extrasynaptic regulation of epileptogenesis by demonstrating that overexpression of both  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  receptors can effectively attenuate epileptiform activity. We showed that neurons transfected with both  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  receptors had

enhanced tonic currents compared to control neurons. Mutations in the  $\delta$  subunit of GABA<sub>A</sub> receptors have been mapped in human epilepsy patients [29,30], indicating the clinical relevance of  $\delta$ -GABA<sub>A</sub> receptors. Our molecular expression studies suggest that extrasynaptic GABA<sub>A</sub>-Rs may be a potential therapeutic target for developing antiepileptic drugs to treat TLE.

#### **THIP regulation of neuronal activity**

Previous studies have reported that THIP may inhibit neuronal activity [52–56]. However, these studies used concentrations much higher than our current work. It is known that high concentration of THIP may directly activate  $\gamma 2$ -containing synaptic GABA<sub>A</sub>-Rs [42,57,58]. In this study, we used a low concentration of THIP (5  $\mu$ M) that did not affect mIPSCs, indicating that at this concentration THIP did not activate synaptic GABA<sub>A</sub>-Rs. Correspondingly, we found that 5  $\mu$ M THIP did not affect basal neuronal firing in the majority of neurons tested. More importantly, we discovered a strong inhibitory effect of THIP on elevated neuronal activity induced by both CTZ and KA. It is possible that elevated neuronal activity may induce substantial release of GABA [46], which will act together with THIP to enhance tonic inhibition and reduce neuronal activity. Another possibility is that tonic current may be outward rectifying at depolarized membrane potential [59], making the effect of THIP more potent when neurons are hyperexcitatory. Our discovery of the preferential inhibition of THIP on elevated activity makes it an ideal candidate for anticonvulsant drug, because it may have less side effects comparing to those affecting basal neural activity.

#### **Conclusions**

We employed both molecular and pharmacological tools to demonstrate that tonic inhibition modulates epileptiform activity both *in vitro* and *in vivo*. The overexpression of both  $\alpha 5\beta 3\gamma 2$  and  $\alpha 6\beta 3\delta$  receptors inhibited the formation of epileptiform activity in hippocampal neurons, establishing unambiguously a solid ground for extrasynaptic modulation of epileptogenesis. Furthermore, we discovered a more prominent role of tonic inhibition in inhibiting hyperexcitatory activity rather than low frequency basal activity, suggesting that extrasynaptic GABA<sub>A</sub>-Rs are ideal drug targets for developing anti-convulsant drugs that may specifically act against epileptiform activity without much side effect on normal brain functions.

#### **Methods**

##### **Primary neuronal culture**

Primary hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rat embryos of either sex, similar to our previous work with modifications

[34]. Briefly, after dissection of the hippocampi, the tissue was rinsed in cold HBS and then digested with 0.05% trypsin-EDTA for 20 min at 37°C, followed by trituration with pipettes in the plating medium (DMEM with 10% FBS and 10% F12). After rinsing for twice, cells were counted and plated onto coverslips precoated with 0.1 mg/ml poly-D-lysine (Sigma). After culturing for 1 day, media were changed into neuronal culture media (neurobasal media containing 2 mM GlutaMAX™-I Supplement and 2% B-27). AraC (1 μM, Sigma) was added 6–8 days after plating, and cells were fed twice weekly thereafter and maintained at 37°C and in 5% CO<sub>2</sub> incubators. Trypsin-EDTA, DMEM, FBS, F12, Neurobasal media, GlutaMAX™-I Supplement and B-27 were purchased from Invitrogen Corporation. Some experiments were also performed using mouse hippocampal cultures.

#### DNA transfection

Calcium-phosphate transfection was performed similar to the protocol previously described [60]. Neurons were transfected at 10 days *in vitro* (DIV). The plasmids of rat α5, α6, β3, γ2, δ subunits of GABA<sub>A</sub> receptors (gifts from Drs. Robert Macdonald, Matthias Kneussel, and Dr. Bernhard Luscher) were co-transfected with pEGFP or mCherry (Clontech). Transfection with EGFP or mCherry alone served as controls. Most of the experiments were performed around 2 weeks of culture unless otherwise indicated.

#### Electrophysiological recordings in cultured hippocampal neurons

Whole-cell recordings were performed in current- or voltage-clamp mode using a MultiClamp 700B amplifier (Axon Instruments). Patch pipettes were pulled from borosilicate glass (Sutter Instrument, BF150-86-10) and fire polished (4–6 MΩ). The recording chamber was continuously perfused with a bath solution consisting of (mM): 128 NaCl, 30 Glucose, 25 Hepes, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, pH 7.3 adjusted with NaOH. The pipette solution for recording action potentials and mEPSCs contained (mM): 125 K-gluconate, 10 KCl, 5 EGTA, 10 Hepes, 10 Tris-phosphocreatine, 4 MgATP, 0.5 NaGTP, pH 7.3 adjusted with KOH. For tonic GABA currents and GABA-induced whole-cell currents, patch pipettes were filled with (mM): 135 KCl, 10 Tris-phosphocreatine, 2 EGTA, 10 Hepes, 4 MgATP, 0.5 NaGTP, pH 7.3 adjusted with KOH. Liquid junction potentials were always corrected before forming giga-ohm seal. The series resistance was typically 10–20 MΩ and partially compensated by 30–50%. Data were acquired using pClamp 10.2 software (Axon Instruments), sampled at 2–10 kHz, and filtered at 1 kHz. Off-line analysis was done with Clampfit 10.2 software (Axon Instruments). Miniature events were analyzed using Mini Analysis software (Synaptosoft). Large depolarization

shift resembling paroxysmal depolarization shift is defined here as ≥ 10 mV depolarization and ≥ 300 ms in duration. An epileptiform burst is defined by at least five consecutive action potentials overlaying on top of the large depolarization shift. When quantifying the percentage of neurons showing epileptiform activity, the criterion is at least two epileptiform bursts occurring during 10 min of recording. All of the drugs used were freshly diluted in bath solution to their final concentrations before experiments.

#### Electrophysiological recordings in anesthetized rats

Adult male Sprague–Dawley rats weighing between 250–350 g were maintained on an *ad libitum* feeding schedule and kept on a 12 hr on/off light cycle. During electrophysiological study, rats were anesthetized with urethane (1.2 g/kg, i.p.) and the level of anesthesia was assessed by the absence of a withdrawal reflex, and additional anesthetic (urethane, 0.2–0.6 mg/kg, i.p.) was administered as necessary. Body temperature was maintained at 37 ± 0.5°C with a Harvard Homeothermic Blanket (Harvard Apparatus Limited, Kent, UK). At the end of experiments, animals were killed with an overdose of urethane. All animal experiments were approved by the local committee of Laboratory Animals, Fudan University and carried out in accordance with Chinese National Science Foundation animal research regulation. Animal preparation was similar to previously reported [36,37,39]. Briefly, all the animals had their lateral tail vein cannulated for drug administration and then mounted in a stereotaxic frame. An incision was made in the midline of the head to expose the top part of the skull for the implantation of i.c.v. guide cannula (22GA, Plastics One, USA) into the lateral ventricle (0.3 mm posterior to bregma, 1.3 mm lateral to the midline, and 4 mm below the skull surface), and then secured by the dental cement. For recording and stimulating, a large burr hole was made in the left side of the incised skull above the hippocampal area, and the dura was pierced and removed. A concentric bipolar stimulating electrode (Harvard Apparatus) was placed close to the CA3 region (3.8–4.5 mm posterior to bregma, 3.5–4.0 mm lateral to the midline, and 3.0–3.8 mm below the brain surface) in order to stimulate the Shaffer collateral pathway. For recording in the CA1 pyramidal cell layer, a tungsten electrode (0.5 MΩ, WPI, USA) was placed 3.5–4.2 mm posterior to bregma, 2.0–3.0 mm lateral to the midline. The depth of the recording electrode was approximately 2.0–2.5 mm below the brain surface as determined by the sudden change of electrical noise and the shape of the evoked field excitatory postsynaptic potentials (fEPSPs) and population spike (PS). For stimulation, a constant current generator passed a square-wave pulse (0.2 ms in duration) through the stimulating electrode (test pulse) and the stimulation frequency was set at

once per minute. The electrophysiological signals were amplified and filtered (0.3–3 kHz) using a NeuroLog System (Digitimer Ltd., Hearts, UK) and visualized and stored in a PC computer through an A-D converter, CED 1401 micro (Cambridge Electronic Design, Cambridge, UK). After both electrodes were in the right place, the fEPSPs and PS were monitored for at least 30 min until a stable recording was achieved. Following a 30 min baseline recording, CTZ (5  $\mu$ mol, 5  $\mu$ L) was administered i.c.v. via the pre-implanted guide cannula into the left lateral ventricle. Pharmacologically induced seizure-like activity was monitored after CTZ injection by observing the change of the evoked potentials transforming from single PS into a multi-peaked display, and spontaneous seizure burst activity in CA1 pyramidal neurons [37,39,61]. After the epileptiform burst activity was stable for at least 30 min, THIP (4 mg/kg in 1 mL/kg) or vehicles were delivered through the cannula pre-implanted in the lateral tail vein. To confirm correct placement of the electrode and cannula, the brain was taken for histological validation of the injection and recording/stimulating sites. Epileptiform activity within CA1 pyramidal cells was analyzed offline using Spike2 software (an analyzing program for CED 1401, Cambridge Electronics, UK) and specific scripts designed for this study with Spike2. The highly synchronized bursting activity was defined as having high frequency multiple high amplitude spikes (>0.5 mV) with an initial interspike interval of less than 0.1 s, a minimum of 5 spikes, and burst duration over 1 s [37].

#### Behavioral test in freely moving rats

CTZ induced seizure behavioral test was carried out similarly as previously reported [38]. Briefly, under general anesthetics with sodium pentobarbital (60 mg/kg, i.p.), a guide cannula was pre-implanted into left lateral ventricle (0.3 mm posterior to bregma, 1.3 mm lateral to the midline, and 4 mm below the skull surface) at least 5 days before the behavioral test. Cannula-implanted animals were randomly divided into following experimental groups: 1) CTZ group: 0.25  $\mu$ mol (i.c.v.) for one injection per day, three consecutive days; 2) THIP + CTZ group one: 5 mg/kg (i.p.) THIP + 0.25  $\mu$ mol (i.c.v.) CTZ for one injection per day, three consecutive days; 3) THIP + CTZ group two: 10 mg/kg (i.p.) THIP + 0.25  $\mu$ mol (i.c.v.) CTZ for one injection per day, three consecutive days. All behavioral tests were carried out between 2:00 pm and 7:00 pm. The animals were first placed in a plastic cage and acclimatized for at least half an hour before experiments. Before and after drug injection, animal behavior was continuously monitored for a period of 1 and 3 hours with video recording, respectively. Behavioral seizures were scored using 5-graded Racine Score system [62]. Briefly, Racine score I, facial clonus; score II, head nodding; score III, unilateral

forelimb clonus; score IV, rearing with bilateral forelimb clonus; score V, rearing and falling (loss of postural control).

#### Data analysis

Group data were expressed as mean  $\pm$  SEM. Across different groups of data, statistical significance between means was determined using one-way ANOVA with Tukey HSD post hoc analysis. Comparison within a group used a paired or unpaired *t* test (GraphPad Prism, GraphPad Software Inc.). Pearson Chi-Square test was used for statistical analysis of percentage (SPSS). Significance level was set at  $p < 0.05$ .

#### Drugs and solutions

Cyclothiazide (CTZ) and L655708 were purchased from Tocris (Northpoint, Bristol). THIP (4,5,6,7-tetrahydroisoxazolo[4,5-c] pyridine-3-ol and urethane (25% in distilled water) were purchased from Sigma Aldrich Chemical Co. (Poole, Dorset).

#### Competing interest

The authors declare that they have no competing interests.

#### Authors' contributions

YS carried out most electrophysiological analysis in cultured neurons and in anesthetized animals. ZW and DJ performed experiments of overexpressing  $\alpha 5/\beta 3/\gamma 2$  receptors in hippocampal cultures. SK analyzed THIP effect on animal seizure behaviors, and AP studied THIP effect on mIPSCs and epileptiform activity in cultured neurons. GC conceived and designed the project. GC and YS wrote the manuscript. YW supervised the in vivo and part of in vitro electrophysiology experiments and revised the manuscript. All authors read and approved the final manuscript.

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