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Full paper

3,4,5-Trimethoxycinnamic acid, one of the constituents of Polygalae Radix exerts anti-seizure effects by modulating GABAAergic systems in mice

Chang-Yuan Chen [a, 1], Xu-Dong Wei [b, 1], Chang-Rui Chen [c, *]

[a] Department of Cardiovascular Diseases, First Hospital, Lanzhou University, Lanzhou 730043, Gansu, PR China
[b] Department of Otolaryngology Head and Neck Surgery, Gansu Provincial Hospital, Lanzhou 730000, Gansu, PR China
[c] Department of Pharmacology, Shanghai Medical College, Fudan University, Shanghai 200032, PR China

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ABSTRACT

Polygalae Radix is an important medicinal plant that is widely used in most of Africa. 3,4,5-Trimethoxycinnamic acid (TMCA) is one of the constituents of Polygalae Radix. Until now, the mechanisms involved in the anti-seizure property of TMCA are still unclear. We examined the anti-seizure effect of TMCA. TMCA administered at doses of 5, 10 and 20 mg/kg and evaluated anti-seizure effects by maximal electroshock (MES) and pentylenetetrazol (PTZ) models in mice. TMCA administered at doses of 10 and 20 mg/kg significantly reduced the incidence of MES-induced tonic hindlimb extension (THE). TMCA significantly delayed the onset of myoclonic jerks (MJ), and decreased the seizure severity and mortality compared with the vehicle-treated animals in PTZ seizure model. TMCA 10 and 20 mg/kg treated groups also did not determined generalized clonic seizures (GCS).

Pretreatment with a GABAA/benzodiazepine (BZ) receptor antagonist flumazenil blocked the anti-seizure effects of TMCA. These data support the further investigation of TMCA as a GABAA/BZ receptor agonist for anti-seizure therapy.

1. Introduction

Epilepsy is one of the most common and heterogeneous neurological disorders (1). It has been reported that more than 50 million people worldwide suffer from epilepsy, current anti-epileptic drugs are only effective in 60–70% of individuals (2). Anti-epileptic drugs used to treat epilepsy can cause severe, life threatening side effects. BZs are highly prescribed anti-epileptic drugs and of great clinical significance; however, the development of tolerance restricts their usefulness (3). A number of newer anti-epileptic drugs have been developed in the last few years to improve the treatment outcomes in epilepsy (4). But resistance to anti-epileptic drugs as well as intolerability led to serious demands for developing new drugs for epilepsy treatment (5).

In traditional Chinese medicine, Radix Polygalae (the root of *Polygala tenuifolia*) have been known to be an important herb that exhibits sedative effects in insomnia, and is widely used as an tranquillizer (6). 3,4,5-Trimethoxycinnamic acid (TMCA), one of the constituents of Polygalae Radix, has been reported prolonged sleeping time induced by pentobarbital or decreased by stress (7,8). Furthermore, TMCA also could decrease the locomotor activity in mice (7). The current study was conducted to investigate the anti-seizure properties of TMCA in the PTZ- and maximal electroshock-induced seizure models and to investigate mechanisms underlying the anti-seizure property of TMCA. The GABAA receptors are Cl^- channel selective ligand-gated ion channels that mediate fast inhibition in the CNS and are important targets for benzodiazepines (BZs) (9). BZ binding with GABAA receptor complex, enhancing Cl^- channel conductance primarily by increasing the frequency of receptor channel opening (10,11). The GABAA receptor γ subunit, in particular $\gamma 2$ subunit is essential for the formation of the binding site, binding efficacy for BZ (9,12,13). TMCA increased Cl^- channel influx and the expressions of γ-subunit of GABAA receptors in the cerebellar granule cells (7). These findings suggest that TMCA might have important effect on GABAergic system.

The current study was conducted to investigate the anti-seizure properties of TMCA in the PTZ and the maximal electroshock

* Corresponding author. Tel.: +86 21 54237380.
E-mail address: changruichen@fudan.edu.cn (C.-R. Chen).
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[1] Both authors equally contributed to this work.

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induced seizure models and to investigate mechanisms underlying the anti-seizure property of TMCA. We showed that TMCA exerted an anti-seizure activity. These effects were attenuated by pre-treatment with flumazenil, a BZ receptor antagonist. The above studies suggest that TMCA may exert anti-seizure activity by acting at the GABAA/BZ receptor complex.

2. Materials and methods

2.1. Animals

Ault male KunMing-strain mice (Experimental Animal Center, Fudan University, Shanghai, China) weighing 18–20 g were used. Animals were housed in cages at room temperature on a 12: 12 h day/night cycle and given ad libitum access to food and water. On days prior to seizure induction, animals were habituated to the test environment.

The experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Fudan, Shanghai Medical College (Permit Number: 20110307–049).

2.2. Drugs

TMCA, PTZ and flumazenil were purchased from Sigma–Aldrich Co. (St Louis, MO, USA), dissolved in saline containing 0.5% dimethylsulfoxide (DMSO), and administrated intraperitoneally (i.p.) in a constant volume of 10 ml/kg. The dosage selections, route of drug administration, and injection time of different compounds were based on preliminary experiments and pharmacokinetic considerations (7,14).

2.3. Seizure models

2.3.1. Maximal electroshock seizure (MES) method

The MES method produces reproducible tonic convulsion characterized by tonic hindlimb extension (THE). THE is the hindlimbs of animals outstretched 180°to the plane of the body (15). Electroconvulsions were produced by a current (fixed current intensity of 70 mA, 0.4 s stimulus duration), delivered by an electric stimulator (2-MU-2; Shanghai Medical College, China) (16,17). For the control (vehicle) group, animals received vehicle. For the positive control group, animals received diazepam (2 mg/kg, i.p.). For the test groups, mice were respectively injected with TMCA at 5, 10, 20 mg/kg. Thirty minutes later all the mice were treated with electrical stimulation. Animals were considered protected if they did not exhibit THE. The protective efficacy of TMCA was determined as ability to protect 50% of animals against the maximal electroshock-induced THE and expressed as respective values of the median effective dose (ED50) (18).

2.4. PTZ-induced convulsion

Animals were placed individually in Plexiglas boxes and seizure behaviors were observed for 60 min after PTZ injection (60 mg/kg, i.p.). The seizure behavior was evaluated as follows (14,17,19,20): Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, myoclonic jerks (MJ); Stage 3, clonic forelimb convulsions; Stage 4, generalized clonic seizures (GCS), with turning to a side position; and Stage 5, generalized clonic-tonic seizures (GTCS) or death within 60 min. Latency to the onset of MJ and GCS, the seizure stage were measured. The mice were divided into 5 groups and were injected i.p. with vehicle, TMCA at a dose of 5, 10, 20 mg/kg and diazepam 2 mg/kg; and 30 min later were injected with PTZ (60 mg/kg, i.p.).

2.5. Study on receptor mechanism involved in anti-seizure effects of TMCA

Flumazenil was chosen to probe the role of the GABAA/BZ receptors in the anti-seizure effects of TMCA. Flumazenil 1 and 5 mg/kg was given 60 min before TMCA administration. The mice were given TMCA 20 mg/kg 30 min before PTZ injection or exposition for MES procedure.

2.6. Statistical analysis

All data were expressed as the mean ± S.E.M. (n = 5–10). Seizure stages were evaluated by using the Kruskal–Wallis test followed by Nemenyi. For parametric data, single comparisons were tested using the t-test, whereas multiple comparisons among groups were analyzed using one-way or two-way ANOVA followed by LSD's test. Fisher's exact test followed by bonferroni was used to determine the overall differences in the incidence of THE due to convulsions. All statistical analyses were carried out by using SPSS 17.0 for Windows. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Anti-seizure property of TMCA

The protective effect of TMCA against maximal electroshock seizure (MES)-induced THE was observed 30 min after TMCA administration. In the control group, 100% of the mice exhibited THE. The tonic flexion of the limbs occurred immediately after the shock and progressed into tonic extension of the hind limbs followed by stupor and recovery. TMCA given at 10 and 20 mg/kg significantly decreased the incidence of MES-induced THE to 50% and 20% of the value of the vehicle controls (n = 10, Fig. 1). However, TMCA given at 5 mg/kg decreased the incidence of MES-induced THE to only 80%. The ED_{50} for the anti-convulsion effect of TMCA was 10 mg/kg. No mice died in both vehicle- and TMCA-treated groups. Diazepam as a positive control significantly decreased the incidence of MES-induced THE to 10%. However, we did not observed any prolongation on duration of THE induced by TMCA. These results indicate that TMCA given at 10 and 20 mg/kg doses has potent anti-seizure effects against MES-induced seizures.

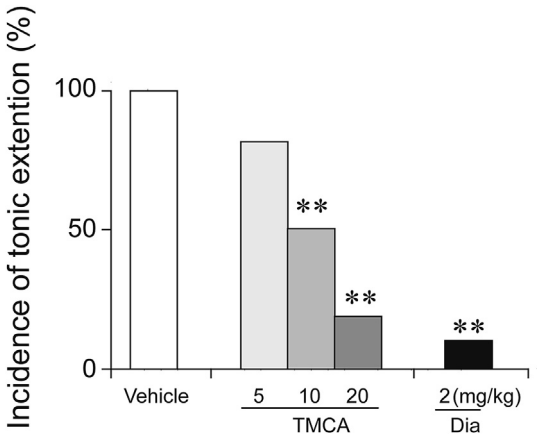

Fig. 1. Incidence of tonic extension (%) after treatment with TMCA and diazepam in the MES model. Each value represents the incidence of tonic hindlimb extension (THE) as a percentage (n = 10). $^{**}P < 0.01$, compared with the control group (Fisher's exact test followed by bonferroni for correction).

3.2. Involvement of GABAA/BZ in the anti-seizure property of TMCA in the MES model

To further investigate the role of GABAA/BZ receptor in the anti-seizure property of TMCA, we administrated GABAA/BZ receptor antagonist flumazenil. We found that 100% of the mice exhibited MES-induced THE in the vehicle treated group. When mice were injected with TMCA at 20 mg/kg, only 20% of the mice exhibited THE (n = 10, Fig. 2). As a competitive antagonist of GABAA/BZ, flumazenil significantly reversed the protective effect of TMCA on MES-induced convulsions. When pretreated with flumazenil 5 mg/kg, the incidence of THE increased from 20% to 90% in the mice given with TMCA 20 mg/kg (n = 10, $P > 0.05$), suggesting that flumazenil at 5 mg/kg significantly antagonized the effects of TMCA. Flumazenil injected with 1 mg/kg prior to TMCA 20 mg/kg treatment lead the incidence of THE enhanced to 50% ($P < 0.05$) (Fig. 2). These findings indicate that the GABAA/BZ receptor mediates the anti-convulsion effects of TMCA in this mouse MES model.

3.3. Anti-seizure property of TMCA in PTZ-induced seizure assessed by behavioral evaluation

As a non-competitive antagonist of GABAA receptor, pentylenetetrazol (PTZ) is often used to induce seizure in the animal model of epilepsy. To investigate the anti-seizure effects of TMCA on behavior, the behavioral seizure stage score and the latency to onset of myoclonic jerks (MJ) and generalized clonic seizures (GCS) were examined after PTZ injection. Within 30 min, PTZ caused a sequence of characteristic behavioral symptoms such as tremor of the vibrissae and muscles, MJ, GCS or GTCS followed by recovery or some animals may succumb to death. Seizure latency prolongation and stage decrease will be taken as index of protection. In the vehicle-treated mice, the latency to MJ and GCS was 62.7 ± 5.5 s and 133.3 ± 17.5 s, respectively. The latency of MJ was significantly delayed to 118.2 ± 6.4 s and 182.3 ± 18.8 s by TMCA (10 and 20 mg/kg) groups compared with the vehicle group (n = 6, $F_{(3, 23)} = 27.6$, $P < 0.001$) (Fig. 3A). Mice did not determined GCS behavioral in 10, 20 mg/kg of TMCA and 2 mg/kg of diazepam treated groups. TMCA administered at 5 mg/kg did not cause any significant change in the latency to MJ, and to GCS in animals that display GCS behavioral

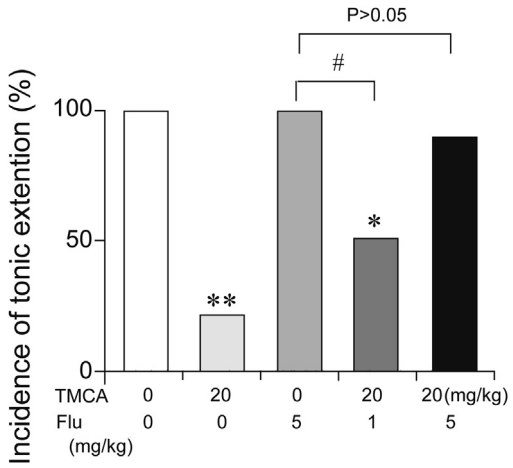

Fig. 2. Effect of pretreatment with flumazenil on anti-seizure effect of TMCA in the MES model. Flumazenil given at 0, 1 and 5 mg/kg attenuated the decrease in incidence of tonic hindlimb extension (THE) caused by TMCA (n = 10). $^{*}P < 0.05$,$^{**}P < 0.01$, compared with the control group; #$P < 0.05$, compared with flumazenil at 5 mg/kg group (Fisher's exact test followed by bonferroni for correction).

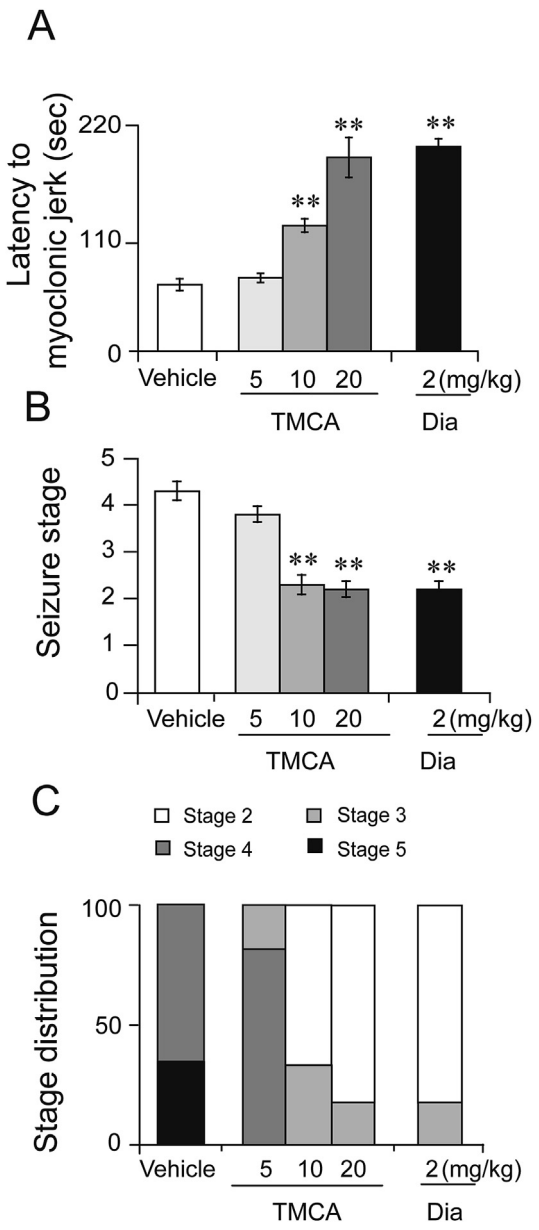

Fig. 3. TMCA prolonged behavior seizure latency and decreased seizure stage in the PTZ (60 mg/kg)-induced convulsions. (A) TMCA prolonged the latency to the onset of myoclonic jerks (n = 6) and (B) TMCA also decreased the mean seizure stage in the model (n = 6). Open, light gray, medium gray, dark gray, and dark bars stand for the groups treated with vehicle, TMCA at doses of 5, 10, 20 mg/kg, and diazepam at 2 mg/kg respectively. (C) Distributions of each stage after TMCA treatment. The divisions within each bar are defined above the bar graph (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the vehicle group, as assessed by one-way ANOVA followed by LSD's test. Kruskal–Wallis followed by Nemenyi test for seizure stage comparison.

(n = 5–6, $P > 0.05$) compared to vehicle. TMCA also significantly decreased the mean seizure stage (n = 6, $F_{(3, 23)} = 29.1$, $P < 0.001$) (Fig. 3B). The mean seizure stage was 4.3 ± 0.2 in vehicle-treated group. TMCA given at 20 or 10 mg/kg, decreased the mean seizure stage to 2.2 ± 0.17 ($P < 0.01$) or 2.3 ± 0.21 ($P < 0.01$), respectively, compared to the vehicle (n = 6, Fig. 3B). The stage-distribution analysis (n = 6, Fig. 3C) revealed that all vehicle-treated mice developed GCS (stage 4), with turning to a side position; and 33.3% of the mice also reached GTCS (stage 5) and died within 30 min, the mortality rate is 33.3%, whereas none of the TMCA-treated mice developed GTCS (stage 5). The incidence of

generalized clonic convulsions (stage 4) disappeared after TMCA was given at doses of both 10 and 20 mg/kg. However, TMCA administered at 5 mg/kg did not cause any significant change in the mean seizure stage (n = 6, $P > 0.05$) when compared to vehicle (n = 6, Fig. 3B). These results indicate that TMCA attenuated the seizure severity and prolonged the latency of seizure onset in the PTZ-induced seizure mouse model.

3.4. Involvement of the GABAA/BZ receptor in the anti-seizure effect of TMCA in the PTZ-induced seizure model

As shown in Fig. 4, the latency to onset of MJ and GCS in mice treated by vehicle plus 5 mg/kg flumazenil was 54.7 ± 4 s (n = 6, $P > 0.05$) and 87 ± 8.8 s (n = 6, $P > 0.05$), with 33.3% of the mice died, which did not cause any significant change in comparison to vehicle treatment alone. Pretreatment with flumazenil 5 mg/kg completely antagonized the prolonged onset MJ latency as well as the mean seizure stage that had been lowered by TMCA at the 20 mg/kg dose. TMCA administered at 20 mg/kg did not cause any significant change in the latency to GCS compared to vehicle when pretreated with flumazenil 5 mg/kg, and 16.7% of the mice even died. This suggests that the GABAA/BZ receptor is involved in the anti-seizure effect of TMCA in the PTZ-induced model.

4. Discussion

The results of the current study show that TMCA has anti-seizure properties. The GABAA/BZ receptor antagonist, flumazenil, reversed these effects, indicating that TMCA may exert its anti-seizure property through the GABAA/BZ receptor.

DMSO is an amphipathic molecule widely used to solubilize water-insoluble compounds. DMSO could exert different effects on seizure dependent on dosage. It was reported that low doses of DMSO decreased whereas high doses of DMSO increased the absence-like epileptic activity of WAG/Rij rats (21). However, DMSO at high doses (above 50%) also reduced the electrically induced paroxysmal activity (22). In our present and previous reports, 0.5% DMSO didn't shown significant influence on MES or PTZ-induced seizures.

Several *in vivo* and *in vitro* studies support our finding that TMCA might elicit anti-seizure effects by acting on the GABAA/BZ receptor complex. It was interesting that TMCA prolonged pentobarbital-induced sleep behaviors, and these results were similar to those of muscimol, a GABAA receptor agonist (7). TMCA increased the activation of glutamic acid decarboxylase (GAD) and the expressions of γ-subunit of GABAA receptors in the cerebellar granule cells and increases Cl-influx (7). In agreement with previous experiments, we have found here that the anti-seizure effects

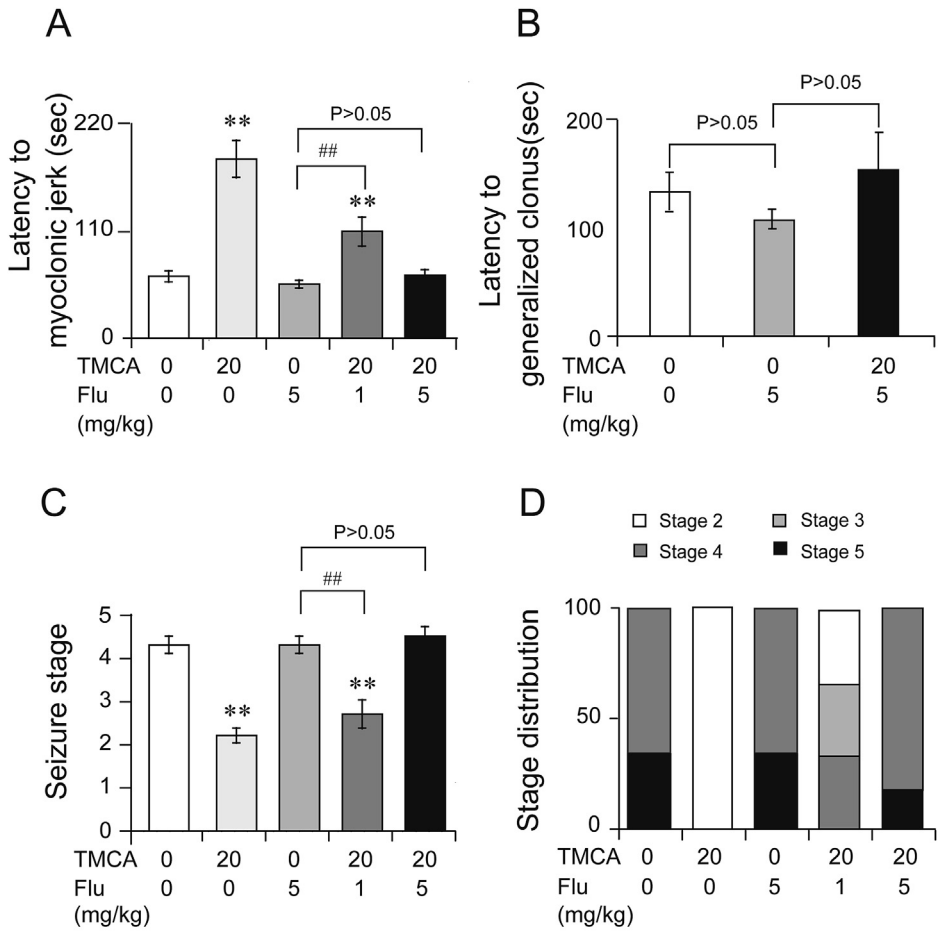

Fig. 4. Flumazenil antagonized the prolonged latency to myoclonic jerks (A, n = 6) and generalized clonus (B, n = 6) and increased the seizure stage reduced by TMCA in the PTZ seizure model (C, n = 6). (D) Distributions of each stage after TMCA at 20 mg/kg combined with flumazenil (0, 1, 5 mg/kg) treatment. The divisions within each bar are defined above the bar graph (n = 6). $^{**}P < 0.01$, compared with the vehicle group; $^{\#\#}P < 0.01$, compared with flumazenil at 5 mg/kg group, as assessed by two-way ANOVA followed by LSD's test. Kruskal–Wallis followed by Nemenyi test for seizure stage comparison.

of TMCA were reversed by flumazenil. Besides, Radix Polygalae extract exerts rapid-onset antidepressant effects. Radix Polygalae extract could decrease tail suspension tasks, and the immobility reduction in tail suspension task was blocked by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPA) receptor antagonist NBQX (23), suggesting TMCA may have modulatory roles at glutamatergic synapses.

γ-Aminobutyric acid (GABA) is the most prominent of the inhibiting neurotransmitters in the brain. It exerts its main action through GABAA receptors (24). GABAA receptor is an pentameric assembly formed by 19 subunits in humans: α1-6, β1-3, γ1-3, δ, ε, π, θ and ρ1-3, and form chloride ion selective channels (25). Molecular and genetic studies have revealed that BZs bind at the interface between an α and γ subunit of GABAA receptor, preferentially enhancing synaptic receptors largely composed of α (1–3,5), β3, and γ2 subunits (26). Flumazenil is typically classified as a non-selective BZ antagonist, acts at subunits α1, α2, α3, α5 and γ2 or partial agonist at subunits α4, α6 (27–29).

TMCA increased the expressions of γ-subunit of GABAA receptors in the cerebellar granule cells (7). However, α- and β-subunits proteins of GABAA receptors were not increased (7). Recent genetic studies revealed the importance of the mutations of GABAA receptor γ2 subunit in the development of febrile seizures (30–32). The heterozygous mutation of GABAA γ2 subunit was higher in patients with febrile seizure (31). TMCA may exert anti-seizure effects by regulating trafficking or expression of γ-subunit of GABAA/BZ receptors. .

In a conclusion, TMCA may exerts anti-seizure effects through interactions with the GABAA receptor complex.

Conflicts of interest

Except as noted in the acknowledgment, all other authors declare that no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. All authors declare that this manuscript/data, or parts of it, has not been submitted or published elsewhere for publication, and all the listed authors have read and approved the submitted manuscript.

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