Neurotherapeutics (2023) 20:399–418
https://doi.org/10.1007/s13311-023-01342-y

ORIGINAL ARTICLE

Cerebellar α6GABA$_A$ Receptors as a Therapeutic Target for Essential Tremor: Proof-of-Concept Study with Ethanol and Pyrazoloquinolinones

Ya-Hsien Huang[1] · Ming Tatt Lee[1,6] · Han-Yun Hsueh[1] · Daniel E. Knutson[3] · James Cook[3] · Marko D. Mihovilovic[4] · Werner Sieghart[5] · Lih-Chu Chiou[1,2,7]

Accepted: 29 December 2022 / Published online: 25 January 2023

Abstract

Ethanol has been shown to suppress essential tremor (ET) in patients at low-to-moderate doses, but its mechanism(s) of action remain unknown. One of the ET hypotheses attributes the ET tremorgenesis to the over-activated firing of inferior olivary neurons, causing synchronic rhythmic firings of cerebellar Purkinje cells. Purkinje cells, however, also receive excitatory inputs from granule cells where the α6 subunit-containing GABA$_A$ receptors (α6GABA$_A$Rs) are abundantly expressed. Since ethanol is a positive allosteric modulator (PAM) of α6GABA$_A$Rs, such action may mediate its anti-tremor effect. Employing the harmaline-induced ET model in male ICR mice, we evaluated the possible anti-tremor effects of ethanol and α6GABA$_A$R-selective pyrazoloquinolinone PAMs. The burrowing activity, an indicator of well-being in rodents, was measured concurrently. Ethanol significantly and dose-dependently attenuated action tremor at non-sedative doses (0.4-2.4 g/kg, *i.p.*). Propranolol and α6GABA$_A$R-selective pyrazoloquinolinones also significantly suppressed tremor activity. Neither ethanol nor propranolol, but only pyrazoloquinolinones, restored burrowing activity in harmaline-treated mice. Importantly, intra-cerebellar micro-injection of furosemide (an α6GABA$_A$R antagonist) had a trend of blocking the effect of pyrazoloquinolinone Compound 6 or ethanol on harmaline-induced tremor. In addition, the anti-tremor effects of Compound 6 and ethanol were synergistic. These results suggest that low doses of ethanol and α6GABA$_A$R-selective PAMs can attenuate action tremor, at least partially by modulating cerebellar α6GABA$_A$Rs. Thus, α6GABA$_A$Rs are potential therapeutic targets for ET, and α6GABA$_A$R-selective PAMs may be a potential mono- or add-on therapy.

Keywords GABRA6 · GABA$_A$ receptor α6 subunit · Essential tremor · Ethanol · Cerebellum · Harmaline

Introduction

Essential tremor (ET) is a neurological disorder with symptoms characterized by uncontrollable rhythmic shaking of one or more body parts [1, 2]. It is one of the most common movement disorders, especially in the elderly [3–6], and a potential risk factor for other neuropsychiatric conditions, such as depression and anxiety [3, 7]. The socio-economic burden inflicted by ET is insurmountable, as it negatively affects the well-being and productivity of patients and their

✉ Lih-Chu Chiou
lcchiou@ntu.edu.tw

1 Department and Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., Section 1, Taipei 10051, Taiwan

2 Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei 10051, Taiwan

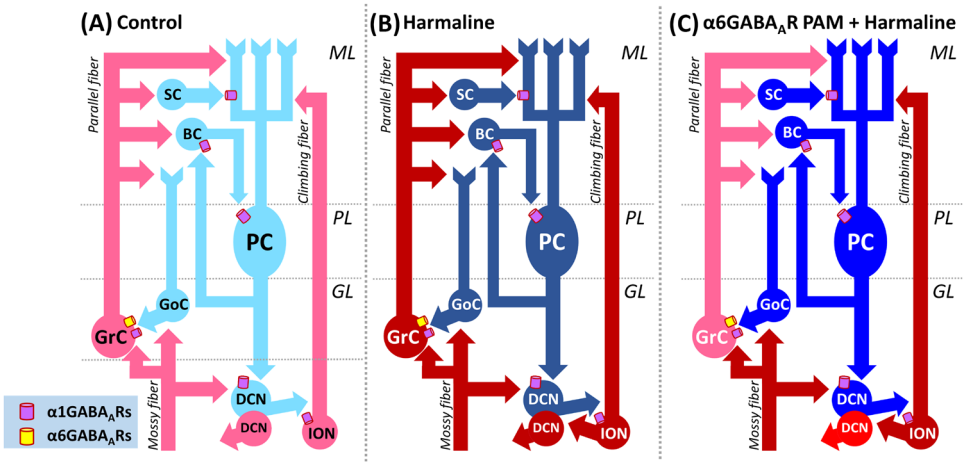
3 Department of Chemistry and Biochemistry, Milwaukee Institute for Drug Discovery, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA

4 Institute of Applied Synthetic Chemistry, TU Wien, Vienna 1060, Austria

5 Center for Brain Research, Department of Molecular Neurosciences, Medical University Vienna, Vienna 1090, Austria

6 Faculty of Pharmaceutical Sciences, UCSI University, Kuala Lumpur 56000, Malaysia

7 Graduate Institute of Acupuncture Science, China Medical University, Taichung 40402, Taiwan



Scheme 1 The cerebellar microcircuit proposed to be involved in **A** normal condition, **B** in the tremorgenic mechanism of harmaline and ET, and **C** in the possible tremorlytic effect of positive modulation of α6 subunit-containing $GABA_A$ receptors (α6$GABA_A$Rs). **A** In the cerebellar cortex, there are five main neuronal cell types, granule cells (GrCs), Golgi cells (GoCs), Purkinje cells (PCs), stellate cells (SCs), and basket cells (BCs). Among these, GrCs are glutamatergic (red) whereas other cerebellar neurons are GABAergic (blue). These cells are segregated in three distinct layers: GrCs and GoCs in the granular layer (GL), PCs in the Purkinje layer (PL), SCs and BCs in the molecular layer (ML). GrCs receive GABAergic synaptic transmission from GoCs via α1$GABA_A$Rs (purple cylinder) and α6$GABA_A$Rs (yellow cylinder). Note that α6$GABA_A$Rs are only found in GrCs [46]. The color intensity represents the degree of activation of the relevant neurons. **A** Under normal condition, PCs receive GABAergic transmission from SCs and BCs via α1$GABA_A$Rs and in turn send GABAergic output feedback to BCs via α1 subunit-containing $GABA_A$ receptors (α1$GABA_A$Rs) [103, 131]. PCs also send GABAergic projections to neurons in the output nuclei, the deep cerebellar nuclei (DCN) that consists of mixed glutamatergic and GABAergic neurons expressing α1$GABA_A$Rs [103, 131]. On the other hand, all GABAergic neurons in the cerebellar cortex receive glutamatergic inputs from GrCs via parallel fibers. PCs and DCN neurons also receive glutamatergic innervation from the input nuclei of cerebellum, the inferior olivary nuclei (ION), via climbing fibers. Thus, the input ION can modulate the cerebellum activity by both directly exciting and indirectly inhibiting (through PCs) the output DCN, and DCN subsequently transmit the cerebellar output signals to the other brain regions, e.g., thalamus for further processing. **B** During the condition of action tremor induced by harmaline, the hyperactivity of the ION leads to synchronized rhythmic firings of cerebellar PCs, through glutamatergic climbing fibers inputs, and subsequently causes marked rhythmic, alternating hyperpolarization and rebound bursting in DCN neurons, resulting in action tremor. Note that darker blue and red colored neurons represent higher neuronal activity. **C** Upon administration of α6$GABA_A$R PAMs, like low-to-moderate doses of ethanol or α6$GABA_A$R-selective pyrazoloquinolinones PAMs, the tonic and phasic GABAergic inhibitions on GrCs are pharmacologically potentiated, via extrasynaptic α6ββδ$GABA_A$Rs and synaptic α6βγ2$GABA_A$Rs at GoC-GrC synapses, respectively. These actions would ultimately reduce the excitatory input from GrCs onto PCs and decrease the neuronal activity of PCs, and thereby contribute to tremor suppression.

caregivers [8]. Current therapeutic agents for ET are limited and often hampered by either inadequate efficacies or intolerable side effects [9].

The pathogenesis of ET is not fully understood. Several human studies suggest a general hypo-function of GABAergic transmission in the brains of ET patients. ET patients had lower GABA, but higher glutamate concentrations in the cerebrospinal fluid (CSF) than normal controls [10]. Consistent with a reduced GABAergic inhibition, ET patients have been reported to have excessive cerebellar activity as revealed by neuroimaging studies [11]. Postmortem studies in ET patients revealed a significant loss of cerebellar Purkinje cells (PCs) [12, 13] and their dendritic arborization [12, 14, 15], possibly caused by an overexcitation of PCs and excessive cerebellar activity [16].

As shown in Scheme 1, the cerebellar cortex is a three-layered structure with GrCs and GoCs in the granular layer, PCs in the Purkinje layer, and stellate cells (SCs) and basket cells (BCs) in the molecular layer, and receives two excitatory inputs via mossy and climbing fibers, respectively [17]. Mossy fibers relay the sensory and contextual information from the brain stem and spinal cord to GrCs that elicit the coordinated motor programs via PCs. Climbing fibers, originated from the inferior olive nucleus (ION) transmit signals from the spinal cord, and provide error-correction signals to PCs for precise timing control of motor function [18]. PCs are the only output neurons of the cerebellar cortex, providing the cerebellar coordinated inhibitory signals [19] to precisely control the neuronal excitability of the downstream deep cerebellar nuclei (DCN) [20]. GoCs are GABAergic interneurons, providing direct feedforward and indirect feedback inhibition, respectively, on excitatory GrCs to sharpen their signals [21]. The axons of GrCs go ascending to the molecular layer, extending as parallel fibers that form excitatory synapses on the dendritic field of PCs, and inhibitory interneurons, BCs and SCs (Scheme 1). The glutamatergic

unipolar brush cells that also play a role in mediating signals from mossy fibers to GrCs [22] are not included in the scheme.

In agreement with their central role in the function of the cerebellum, PCs were recently reported to be significantly involved in the excessive cerebellar activity and play an important role in the pathogenesis of ET. In an ET animal model induced by harmaline, a β-carboline neurotoxin [23], it was demonstrated that the onset of tremor coincides with the abnormal rhythmic firing of cerebellar PCs and bursting firing of the downstream DCN neurons (Scheme 1B), and that a genetic reduction of PC-DCN transmission reduces tremor activity [24]. This conclusion was also supported by another study indicating that misfiring of cerebellar PCs elicited by synaptic pruning deficits of climbing fiber-to-PC synapses caused by PC-specific glutamate receptor subunit insufficiency, cause excessive cerebellar oscillations and might be responsible for tremor generation [25].

Ethanol, in low-to-moderate doses, has long been shown in ET case reports to relieve tremor in patients [26–28]. Clinical studies also indicated that ethanol significantly suppressed the magnitude and frequency of tremor [29, 30] and the gait ataxia [31] in ET patients. The anti-tremor effect of ethanol was comparable [32] or even superior [33] to propranolol, a first-line medication for ET [34]. However, the mechanism(s) how ethanol suppresses tremor remain unclear. Although the clinical use of ethanol for tremor relief in ET patients is generally discouraged due to the abuse potential of ethanol [35], ethanol is self-medicated in 20% of ET patients [36]. Thus, elucidation of the anti-tremor mechanism of ethanol might lead to the development of new drugs that are devoid of the adverse effects of ethanol for ET treatment.

Positron emission tomography (PET) studies have demonstrated that a single low dose (0.5 g/kg) of alcohol drastically suppressed the blood flow in the cerebellum in healthy volunteers [37–39], but not in other motor-related brain regions, like the striatum and cortex. Another PET study indicated that ethanol, at a blood alcohol concentration (BAC) of 35 mg/dl, which is below the legal driving limit in USA (80 mg/dl, 0.08% or 17 mM) [40], significantly reduced the tremor-associated excessive activity in bilateral cerebellar hemispheres of ET patients [41]. A high-density electroencephalography study in ET patients also supports the cerebellum as the site of action of alcohol in relieving action tremor [42]. Since ethanol is a positive allosteric modulator (PAM) of GABA$_A$Rs [43], it is likely that increasing cerebellar GABAergic transmission plays a role in its anti-tremor effect.

GABA$_A$Rs are pentameric ligand-gated chloride channels. A total of 6α, 3β, 3γ, δ, ε, π, θ, and 3ρ subunits have been identified in the mammalian nervous system [44]. The majority of GABA$_A$Rs consists of two α, two β, and one γ or δ subunits, i.e., αβγ- or αβδ GABA$_A$Rs [45]. GABA$_A$Rs containing α6 subunits (α6GABA$_A$Rs) are especially enriched in the cerebellum, where they are exclusively located in GrCs (Scheme 1). Whereas α6βγ2GABA$_A$Rs are located at the inhibitory GoC-GrC synapses and at extrasynaptic sites, α6ββδGABA$_A$Rs are exclusively located at extrasynaptic dendritic and somatic membranes [46] as well as at GrC axons and parallel fibers where they are modulated by GABA tonically released from glia cells [47]. The involvement of extrasynaptic α6ββδGABA$_A$Rs in the action of ethanol has been suggested by the finding that ethanol at blood concentrations above the legal driving limit impairs motor coordination by enhancing tonic inhibition of cerebellar α6β3δGABA$_A$Rs. In addition, a high-affinity binding site for ethanol has been demonstrated at the α6+ β3- interface of α6β3δGABA$_A$Rs [48, 49], which could be the site of action of low doses of ethanol. However, only some [50–53] but no other groups [54–57] could confirm the high potency of ethanol for modulation of recombinant α6β3δGABA$_A$Rs.

Recently, we have identified several pyrazoloquinolinones (PQs) [58] and their deuterated derivatives [59] to be highly α6GABA$_A$R-selective PAMs. Using PQ Compound 6 (originally coded as PZ-II-029), the compound with the highest efficacy for modulating α6GABA$_A$Rs [58], and its derivatives as positive controls, here we examined whether low-to-moderate doses of ethanol can attenuate action tremor, at least in part, by positively modulating cerebellar α6GABA$_A$R in a mouse model of ET induced by harmaline.

First, we examined the possible anti-tremor effects of various doses of ethanol and α6GABA$_A$R-selective PQs. The effect of propranolol, a first-line anti-tremor agent, was examined as a positive control. To substantiate the involvement of cerebellar α6GABA$_A$Rs, the anti-tremor effects of tested compounds were challenged with furosemide, a selective blocker of α6GABA$_A$Rs [60], given by intracerebellar (*i.cb.*) microinjection. In addition to the tremor activity in harmaline-treated mice, we also measured their burrowing activity, which is one of the "activities of daily living (ADL)" in laboratory rodents and has been employed as an indicator of well-being in rodents [61].

Materials and Methods

Animals

The animal care and experimental procedures reported in this study were approved by the Institutional Animal Care and Use Committee of National Taiwan University, College of Medicine, Taipei, Taiwan. Male adult mice (ICR strain) purchased from BioLASCO (Taiwan Co., Ltd) were housed in a holding room with a 12 h light-dark reversed cycle and access to food and water *ad libitum*. On the experimental

day, mice (8-10 weeks) were moved with their home cages to a behavior room and acclimated there for at least 1 h before testing.

Intracerebellar (*i.cb.*), Intraperitoneal (*i.p.*), and Subcutaneous (*s.c.*) Injections

The *i.cb.* cannulation procedure was performed as reported in our previous studies [62, 63] with modifications. Briefly, mice were anaesthetized with sodium pentobarbital (60 mg/kg, *i.p.*) and placed in a stereotaxic frame keeping the bregma-lambda axis horizontal. A 24-gauge stainless-steel guide cannula was implanted directly toward the vermis (−7.0 mm caudal, −0.4 mm ventral from bregma) according to the stereotaxic coordinate of the mouse [64]. After cannulation, the mouse was allowed to recover for at least 1 week. On the day for behavioral tests, a 30-gauge injection cannula connected to a 1-µl Hamilton syringe via a 50-cm polyethylene tube was inserted into the guide cannula for drug injection. The drug solution of 0.5 µl was slowly infused with a microinfusion pump (KDS311, KD Scientific Inc.) for 3 min with a further "hold" time for 2 min, while the mouse was allowed to freely move in an open field arena. The microinjection site was confirmed by the positive staining of trypan blue, which was injected through the cannula after the behavioral tests. For *i.p.* and *s.c.* injection, the drug solution was injected at a volume of 10 ml/kg.

Spontaneous Locomotor Activity

In ethanol-treated mice, their spontaneous locomotor activity was assessed by their total distance travelled and movement speed in an open field ($40 \times 40 \times 40$ cm^3 cm), which were videotaped for 60 min and analyzed by the Smart 3.0 software (Panlab-Harvard Bioscience Inc., Massachusetts, USA). In *i.cb.* furosemide-treated mice, their spontaneous locomotor activity was measured by the open field test in a grid arena ($48 \times 48 \times 40$ cm) divided into 36 squares as reported previously [65]. After *i.cb.* microinjection of furosemide, each mouse was placed in the arena. The number of squares the mouse transpassed with all paws (number of crossing) and the time that the mouse stood up with two paws on the floor (number of rearing) were counted for 5 min.

Tremor Induction and Measurement

Action tremor was induced in mice by harmaline in accordance with the protocol described previously with minor modifications [66, 67]. The frequency and intensity of tremor were measured and analyzed using a Tremor Monitor (San Diego Instruments, CA, USA) as reported previously [68], which can accurately differentiate tremor activity from ambulatory/stereotyped movements and global motion activity [69]. Each mouse was placed in the chamber for acclimatization, and then the baseline motion power was recorded for 10 min. Tested drugs were administered by *i.p.* or *i.cb.* injection 5 min before harmaline injection. Each raw trace of the movement activity recorded by Tremor Monitor was converted by a fast Fourier transform to generate a frequency domain-based motion power spectrum with the bin size at 1 Hz. The tremor activity in each mouse was measured as the ratio of the motion power at 10-16 Hz over the tremor frequency (0-34 Hz) of the global motion power over a 10-min duration (Fig. 1A). The overall tremor activity was expressed by the area under the curve (AUC) derived from time-dependent tremor responses. To ensure that the harmaline-induced tremor detected in our setups was indeed action tremor, we videotaped the motion of harmaline-treated mice in the tremor chamber and subsequently analyzed their action/immobile phases using the SMART Video Tracking System (Harvard Apparatus, MA, USA) (Fig. 1B).

Burrowing Activity Assessment

The burrowing activity of a mouse was measured as described previously [70, 71] with modifications. One day before the test, an empty burrowing tube was placed into the home cage to have all five mice acclimatized to the tube. On the testing day, the mouse was placed in a testing cage equipped with a burrowing tube filled with 200 g of food pellets. The burrowing activity was calculated as the weight of the total expelled food pellet by subtracting the weight of the food pellet left at the end of the experiment (Fig. 2A) from the initial weight (200 g).

Experimental Protocol

As depicted in Fig. 2A, 10 min after harmaline (*s.c.*) injection, the mouse was placed in the tremor chamber and its tremor activity was measured for 10 min (pink shade). Right after the 10-min tremor measurement, the mouse was placed back to its home cage (Fig. 3A) or in a designated cage with a pellet-prefilled burrowing tube, and its burrowing activity was measured for another 10 min (grey shade, Figs. 4A, 5A, 6A, 7A, and 8A–C). Then, the mouse was returned to the tremor chamber for tremor measurement. This tremor-burrowing measurement alternation was repeated for several cycles as indicated in each experiment. As harmaline-induced tremor is an action tremor, alternations were designed to refresh the alertness of the mouse once back in the tremor chamber, enhancing tremor consistency by replacing the rest period described in previous studies [66, 67] with the burrowing activity measurement.

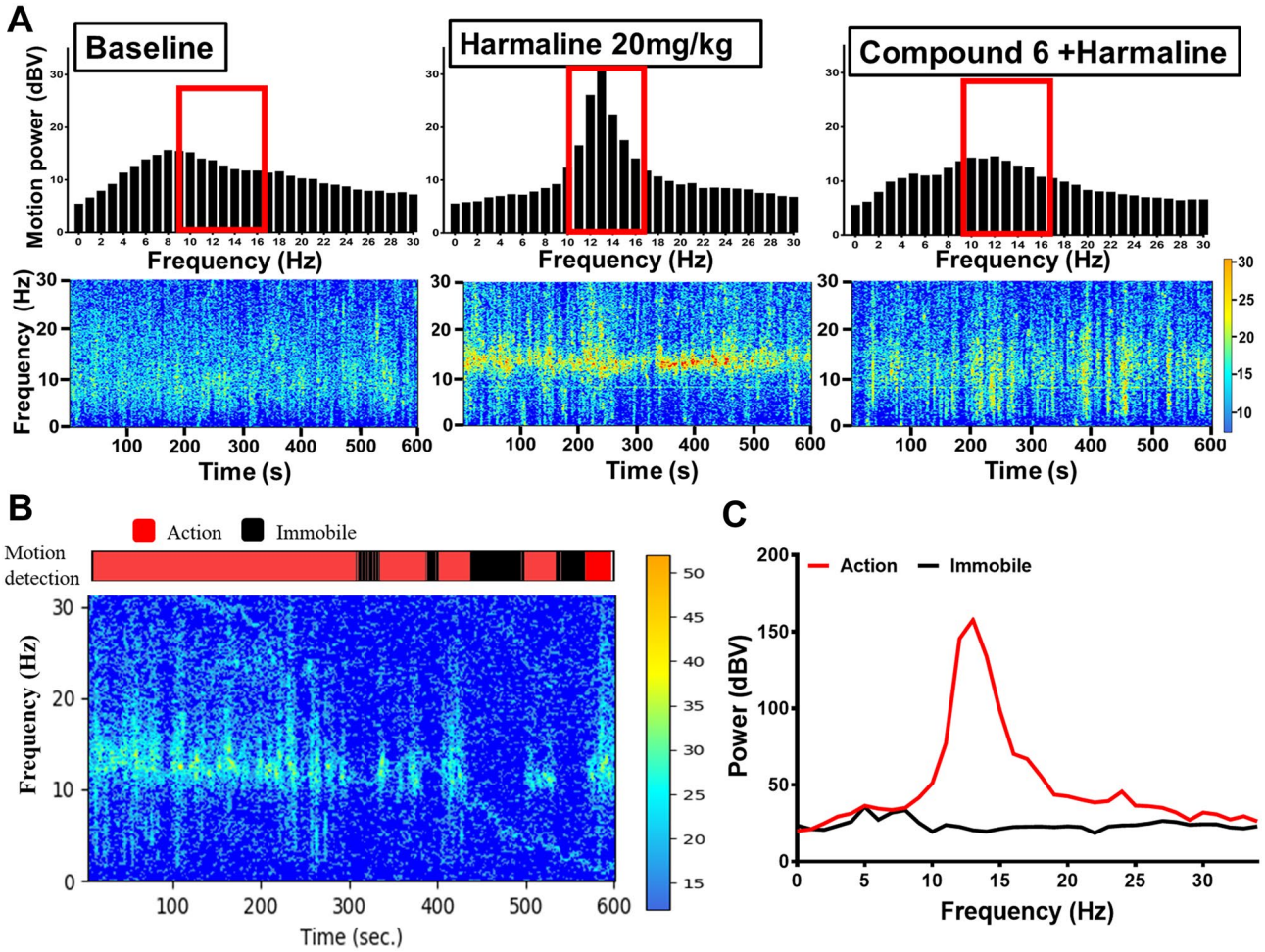


Fig. 1 Representative figures of frequency domain-based motion power spectra and motion power spectrograms in mice treated with saline, harmaline and Compound 6 plus harmaline. **A** The motion waves in mice measured by a Tremor Monitor (San Diego Instruments, CA, USA) were converted into frequency domain motion power spectra (upper panels) and motion power heatmap spectrograms (lower panels) by a fast Fourier transformer. Note that harmaline induced tremor response at the frequency of 10-16 Hz in ICR mice, and Compound 6 attenuated harmaline-induced tremor activity. **B** A sample of 10-min motion power spectrograms (lower panel) and concurrent motor activity detected by SMART motion detector showing that the tremor activity at the frequency of 10-16 Hz. **C** Occurs during movement (red region), but not immobile (black region) periods, suggesting that harmaline induces action tremor, but not rest tremor, in mice

Drugs and Chemicals

Compound 6, LAU 463, DK-I-56-1, and DK-I-58-1 were synthesized as previously described [59]. Ethanol (99%), harmaline, and propranolol were purchased from Sigma-Aldrich (St. Louis, MO, USA) and furosemide from Tocris Bioscience (Bristol, UK). Compound 6, LAU 463, DK-I-56-1, DK-I-58-1 (Supplementary Fig. S1A–D, respectively) and propranolol were dissolved in a vehicle containing 20% DMSO, 20% Cremophor® EL (polyoxyethylene castor; Sigma-Aldrich) and 60% normal saline. Ethanol and harmaline were dissolved in normal saline. Furosemide administered by *i.cb.* microinjection was dissolved in DMSO as reported previously [62, 63].

Statistical Analysis

Data were expressed as the mean ± *S.E.M.*, and the *n* number indicates the number of animals used. The two-way ANOVA followed by Holm-Sidak's *post hoc* test was employed to examine the difference in the tremor intensity among different treatment groups across time. The one-way ANOVA followed by Holm-Sidak's *post hoc* test was employed to examine the differences among treatment groups. However, if inhomogeneity was found in one-way ANOVA, as demonstrated in a significant Brown-Forsythe test, non-parametric Kruskal–Wallis followed by Dunn's *post hoc* test was employed. Statistical differences were considered significant if $P < 0.05$. The individual data for

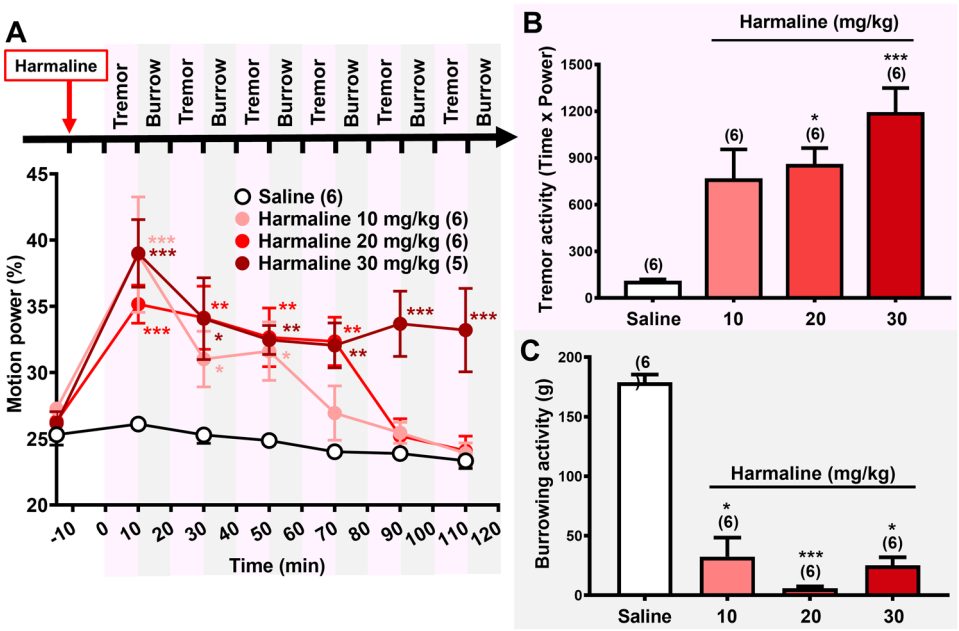


Fig. 2 Dose-dependent effects of harmaline on tremor activity and burrowing activity in mice. **A** Time courses of the tremor activity induced by saline and 10, 20, and 30 mg/kg (*s.c.*) of harmaline. The red arrow indicates the point of harmaline injection, i.e., 10 min before first tremor measurement. The tremor activity was measured for 10 min (pink shade), 15 min before, 5 min after, and then every 20 min after harmaline injection. Immediately after the tremor activity measurement, the mouse was placed in a chamber and its burrowing activity (grey shade) was measured for 10 min. Tremor activity and burrowing activity were measured alternatively for 120 min. The means of tremor activity for each 10-min period were plotted against time and compared among treatment groups. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. **B** The total tremor activity, as measured by the area under curve (AUC) of the tremor activity against time, and **C** the burrowing activity, as measured by total displaced pellets, during 120 min-recording period in saline- or harmaline-treated mice. $^{*}P<0.05$, $^{***}P<0.001$ *vs.* Saline group, Kruskal–Wallis test followed by Dunn's *post hoc* test. The numbers in the parentheses denoted the *n* number of mice tested in each group. Data are expressed as mean ± *S.E.M.*

all bar graphs in the manuscript are tabulated in Supplementary Table S1.

Results

Harmaline Dose-Dependently Induced Action Tremor in Mice

Subcutaneous injection (*s.c.*) of harmaline at doses of 10, 20, and 30 mg/kg significantly induced tremor activity in mice dose-dependently (Fig. 2A and B). A two-way ANOVA with repeated measures over time showed main effects of time [$F(6,120)=14.7$, $P<0.001$] and treatment [$F(3,20)=11.78$, $P<0.001$], and a significant interaction between time and treatment [$F(18,120)=2.822$, $P<0.001$] (Fig. 2A). The tremor activity peaked at 10–16 Hz as demonstrated in the motion power–frequency distribution plot and heatmap spectrogram (Fig. 1A). As shown in Fig. 1B, tremor activity was detected during mobile (red periods), but not during the immobile phase (black periods) of the harmaline-treated mice, suggesting that harmaline-induced tremor is an action tremor peaked at 10-16 Hz (Fig. 1C). Harmaline-induced tremor activity reached its peak at the first assessing time interval, 10-20 min after harmaline injection, and was similar among different dosage groups, but the time-dependent analysis with two-way ANOVA showed a significant difference among treatment groups (Fig. 2A). The tremor activity declined gradually and dose-dependently. The peak tremor activity lasted for at least 50, 70, and 110 min, respectively, induced by 10, 20, and 30 mg/kg harmaline. The AUC of the tremor activity over the 120 min-measuring period showed that the tremor-inducing effect of harmaline was dose-dependent (Fig. 2B).

The Burrowing Activity Was Markedly Reduced in Harmaline-Treated Mice

Besides inducing tremor, harmaline significantly suppressed the burrowing activity in mice at three tested doses, 10, 20,

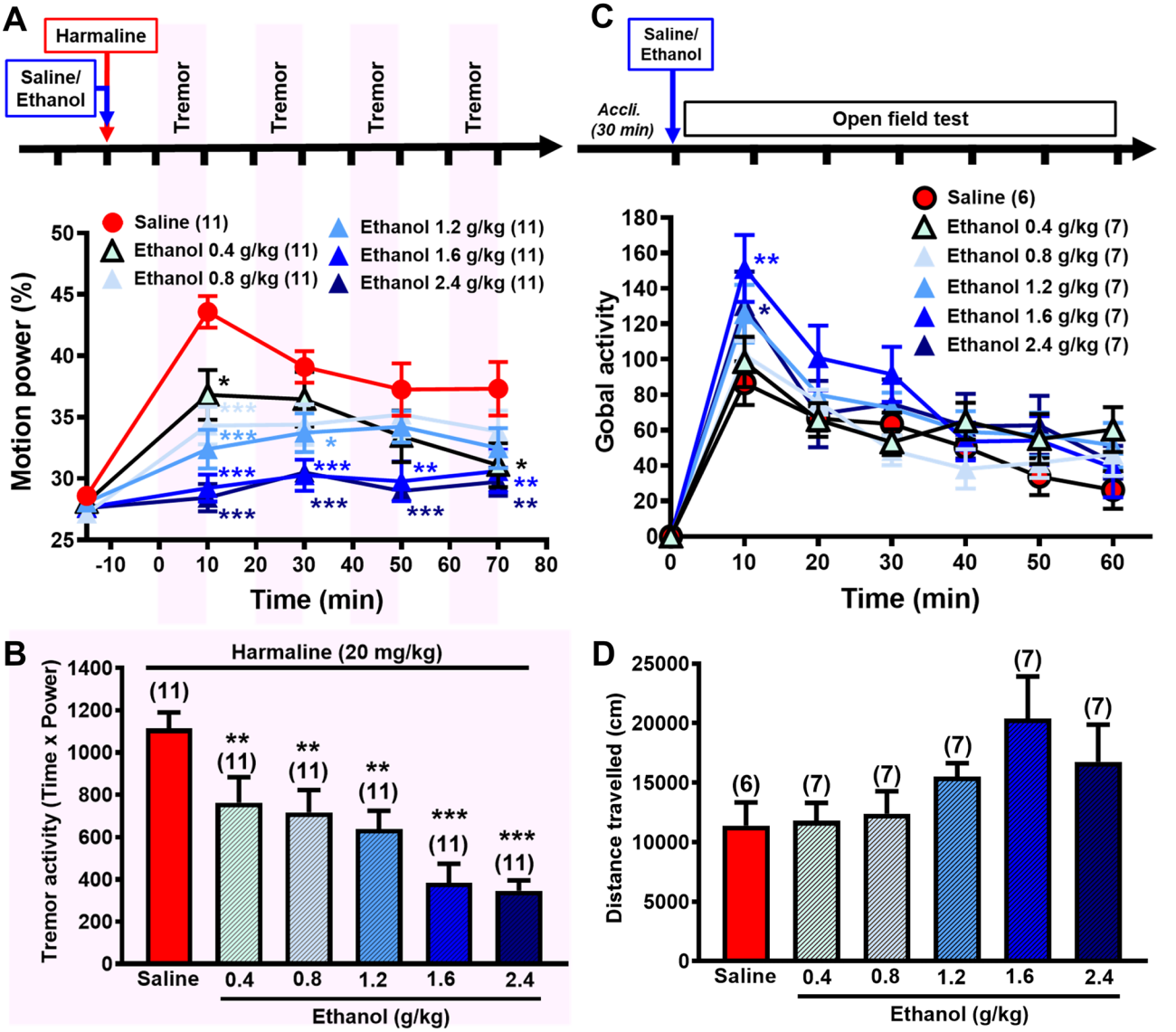


Fig. 3 Effects of low-to-moderate doses of ethanol on harmaline-induced tremor and locomotor activity in mice. **A** Time courses of effects of 0.4, 0.8, 1.2, 1.6, and 2.4 g/kg (*i.p.*) of ethanol and saline on harmaline (20 mg/kg, *s.c.*)-induced tremor. The tremor activity was measured for 10-min in the tremor monitor chamber with alternating 10 min in the home cage, for a total of 80 min. Ethanol and saline (blue arrow) were co-injected (*i.p.*) with harmaline (red arrow) 10 min before the first tremor measurement. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**B**) and the burrowing activity (**C**) were measured as described in Fig. 2 in harmaline-treated mice in various treatment groups. $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Saline group (**B**); $^{***}P<0.001$ *vs.* Control group (**C**), one-way ANOVA followed by Holm-Sidak's *post hoc* test. **D** Time courses of effects of 0.4, 0.8, 1.2, 1.6, and 2.4 g/kg (*i.p.*) of ethanol and saline on locomotor activity effect of ICR mice, measured via global activity in 60 min of open field test. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. **E** Total distance travelled by mice treated with 0.4, 0.8, 1.2, 1.6, and 2.4 g/kg (*i.p.*) of ethanol or saline in 60-min session of open field test. Note that at these low-to-moderate doses of ethanol, no significant effect on locomotor activity was observed. The numbers in the parentheses denoted the *n* number of mice tested in each group. Data are expressed as mean ± *S.E.M.*

and 30 mg/kg (Fig. 2C). At 20 mg/kg, harmaline produced the maximal suppression (96.7%) of the burrowing activity of mice. Thus, harmaline at the dose of 20 mg/kg (*s.c.*) was chosen to induce tremor and burrowing activity deficit for subsequent pharmacological studies, with the measuring period limited to the first 80 min after harmaline injection.

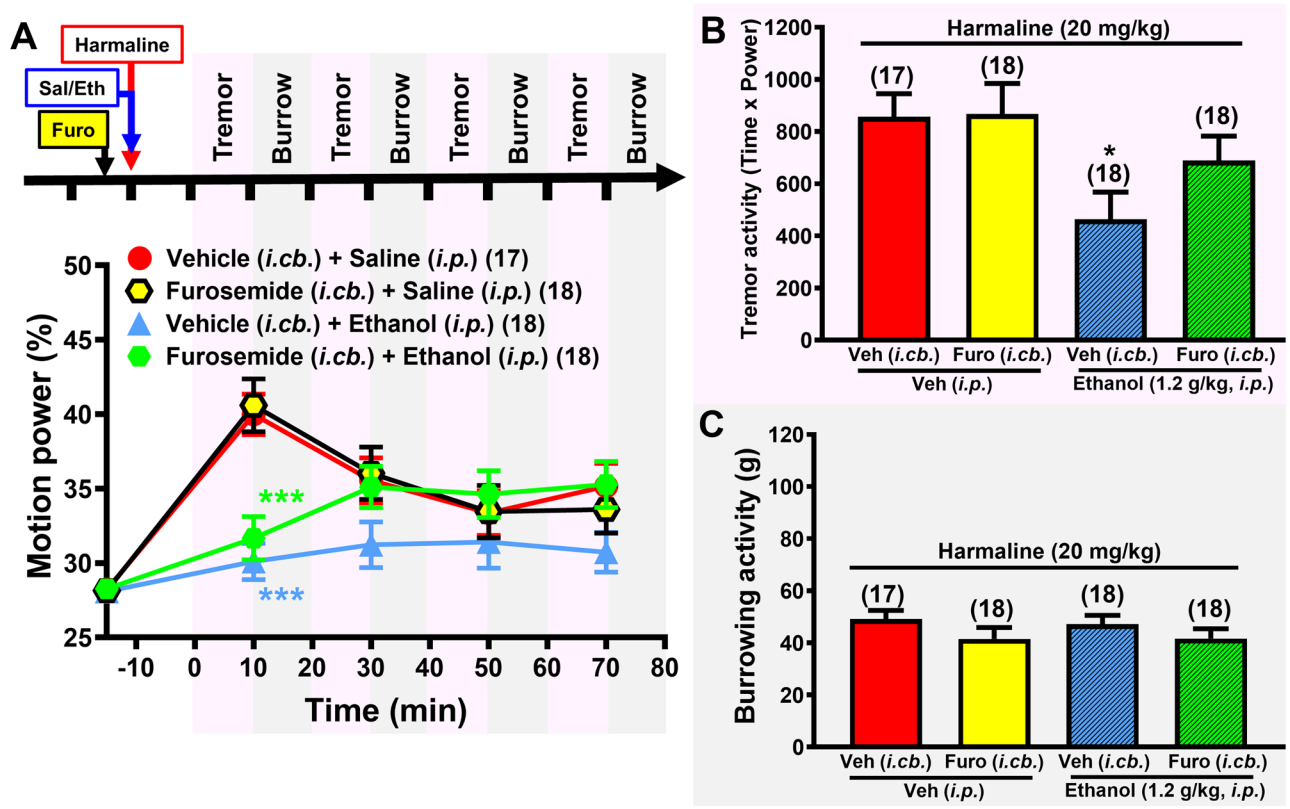


Fig. 4 Effects of intra-cerebellar microinjection (*i.cb.*) of furosemide, an α6GABA$_A$R antagonist, on anti-tremor effect of ethanol in harmaline-treated mice. **A** Time courses of effects of 1.2 g/kg (*i.p.*) of ethanol and saline without and with *i.cb.* furosemide (10 nmol) co-treatment on the tremor activity of harmaline-treated mice. Ethanol and saline (blue arrow) were co-injected (*i.p.*) with harmaline (red arrow) 10 min before the first tremor measurement, whereas *i.cb.* furosemide (black arrow) was administered 5 min before ethanol/saline and harmaline injections. Tremor activity and burrowing activity were measured alternatively for 80 min as described in Fig. 2A. ***$P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**B**) and burrowing activity (**C**) were measured as described in Fig. 3 in harmaline-treated mice pretreated with Compound 6 alone or in combination with *i.cb.* furosemide. *$P<0.05$, **$P<0.01$ *vs.* Vehicle group, one-way ANOVA followed by Holm-Sidak's *post hoc* test. Furo: furosemide, Veh: vehicle

Low to Moderate Doses of Ethanol Suppressed Harmaline-Induced Tremor Dose-Dependently

Similar to the protocol described in Fig. 2A, various doses (0.4, 0.8, 1.2, 1.6, and 2.4 g/kg) of ethanol or saline were intraperitoneally (*i.p.*) administered immediately after harmaline (20 mg/kg, *s.c.*) injection (Fig. 3A). A two-way ANOVA with repeated measures over time showed main effects of time [$F(20,240)=29.65$, $P<0.001$] and treatment [$F(5,60)=8.353$, $P<0.001$], and a significant interaction between time and treatment [$F(20,240)=3.223$, $P<0.001$]. In particular, at the first time point of 10 min after ethanol administration, ethanol dose-dependently suppressed tremor activity; the suppression was significant at the dose as low as 0.4 g/kg, gradually increased with increased doses, and reached the maximal at 1.6 g/kg (Fig. 3A). The AUC derived from the time-dependent analysis over 80 min confirmed that ethanol at a dose as low as 0.4 g/kg exerted significant anti-tremor effect, and the effect was dose-dependent and maximal at 1.6 g/kg (Fig. 3B). One-way ANOVA showed a significant difference among treatment groups [$F(5,60)=9.622$, $P<0.001$]. The ED_{50} of the anti-tremor effect of ethanol was estimated to be 1.386 g/kg (Supplementary Fig. S2). Interestingly, the burrowing activity that was significantly reduced by harmaline was not significantly restored by ethanol at 1.2 g/kg (Fig. 3C).

To further substantiate that the anti-tremor effect of ethanol in harmaline-treated mice is not a confounding effect elicited by its sedative or motor-impairing activity, we examined its effect on spontaneous locomotor activity. At the doses tested (0.4, 0.8, 1.2, 1.6, 2.4 g/kg, *i.p.*), ethanol did not reduce spontaneous locomotor activity but tended to increase motor activity at 1.6 or 2.4 g/kg (Fig. 3D, E). In subsequent experiments on the anti-tremor effects of ethanol, we thus avoided the use of these higher ethanol doses.

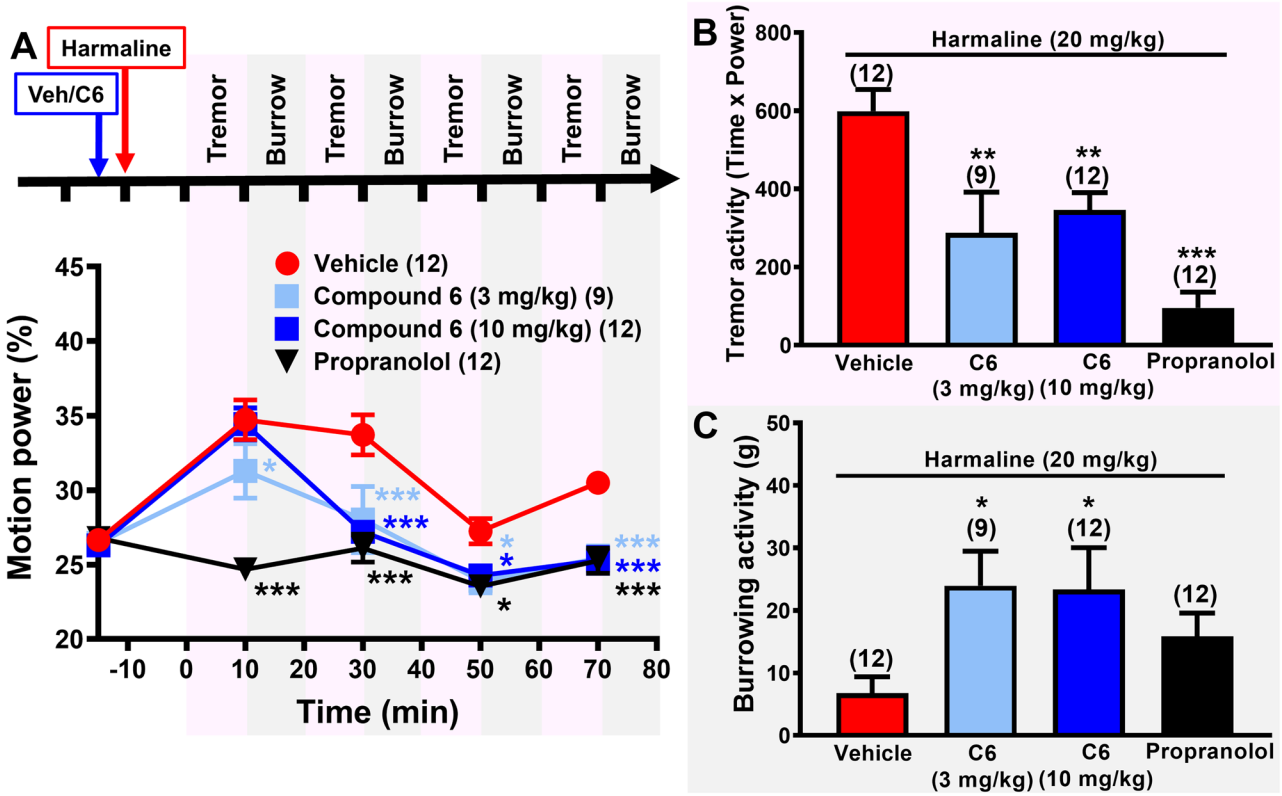


Fig. 5 Effects of Compound 6, an α6GABA$_A$R-selective PAM, and propranolol on harmaline-induced tremor and burrowing impairment in mice. **A** Time courses of effects of 3 and 10 mg/kg (*i.p.*) of Compound 6 and its vehicle, and propranolol (20 mg/kg) on harmaline (20 mg/kg, *s.c.*)-induced tremor. The tremor activity and burrowing activity were measured alternatively for 80 min as described in Fig. 2A. Compound 6 and vehicle (blue arrow) were injected (*i.p.*) 5 min before harmaline treatment (red arrow), followed by the first tremor measurement at 10 min later. $^*P<0.05$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**B**) and burrowing activity (**C**) were measured as described in Fig. 3 in harmaline-treated mice in various treatment groups. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Vehicle group, one-way ANOVA followed by Holm-Sidak's *post hoc* test. The numbers in the parentheses denoted the *n* number of mice tested in each group. Data are expressed as mean ± *S.E.M.* Veh: vehicle, C6: Compound 6

Furosemide (*i.cb.*) Antagonized the Anti-tremor Effect of Ethanol

To discern whether cerebellar α6GABA$_A$Rs are involved in the anti-tremor effect of ethanol, we co-treated mice with intra-cerebellar (*i.cb.*) microinjection of furosemide (10 nmol), an α6GABA$_A$R antagonist, and *i.p.* injection of ethanol (1.2 g/kg) immediately after harmaline injection (Fig. 4A). Two-way ANOVA with repeated measures over time showed main effects of time [$F(4,164)=41.59$, $P<0.001$] and treatment [$F(3,41)=12.57$, $P<0.001$], and a significant interaction between time and treatment [$F(12,164)=8.327$, $P<0.001$] (Fig. 4A). In both time-dependent analyses (yellow diamonds, Fig. 4A) and the derived AUC of motion power (yellow bar, Fig. 4B) in harmaline-treated mice, *i.cb.* furosemide alone had no significant effect on the tremor activity. This *i.cb.* dose of furosemide (10 nmol) also did not alter the spontaneous locomotor activity of mice (Supplementary Fig. S3). However, in the *i.cb.* furosemide-co-treated group, ethanol (1.2 g/kg, *i.p.*) failed to attenuate the tremor activity 30-70 min, but not 10 min, after harmaline injection (green diamonds, Fig. 4A). The derived AUC of motion power also showed that *i.cb.* cotreatment with furosemide, but not its vehicle, restored the tremor activity that had been suppressed by ethanol in harmaline-treated mice (green *vs.* blue bars Fig. 4B). Neither *i.cb.* microinjection of furosemide alone, nor in combination with *i.p.* injection of ethanol, improved the impaired burrowing-activity in harmaline-treated mice, nor in two respective vehicle-treated groups (Fig. 4C).

Compound 6 (*i.p.*) Suppressed Tremor and Restored Burrowing Activity in Harmaline-Treated Mice

We next treated mice with Compound 6, an α6GABA$_A$R-selective PAM, instead of ethanol in harmaline-treated mice. The clinically effective anti-tremor agent, propranolol, was also tested as a positive control in

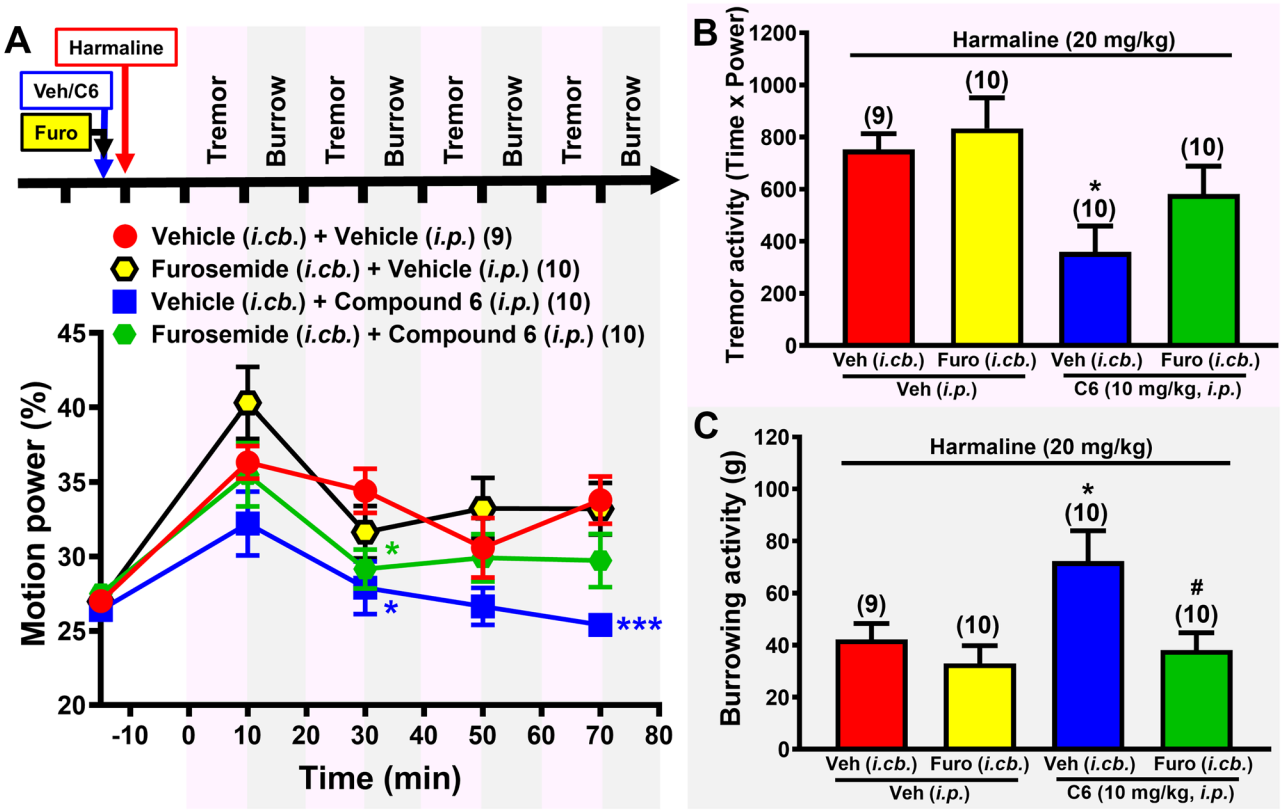


Fig. 6 Effects of intra-cerebellar microinjection (*i.cb.*) of furosemide, an α6GABA$_A$R antagonist, on anti-tremor and burrow-restorative effects of Compound 6 in harmaline-treated mice. **A** Time courses of effects of 10 mg/kg (*i.p.*) of Compound 6 and its vehicle (blue arrow) without and with *i.cb.* furosemide (10 nmol, black arrow) co-treatment on the tremor activity of harmaline-treated mice. Tremor activity and burrowing activity were measured alternatively for 80 min as described in Fig. 5A. $^{*}P<0.05$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**B**) and burrowing activity (**C**) were measured as described in Fig. 3 in harmaline-treated mice pretreated with Compound 6 alone or in combination with *i.cb.* furosemide. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Vehicle group, $^{\#}P<0.05$ *vs.* Veh (*i.cb.*) + C6 group, one-way ANOVA followed by Holm-Sidak's *post hoc* test. Furo: furosemide, Veh: vehicle, C6: Compound 6

harmaline-treated mice. Compound 6 (3 or 10 mg/kg) or propranolol were *i.p.* administered to mice 5 min prior to harmaline (20 mg/kg, *s.c.*) injection (Fig. 5A). Two-way ANOVA with repeated measures over time showed main effects of time [$F(4,164)=41.59$, $P<0.001$] and treatment [$F(3,41)=12.57$, $P<0.001$], and a significant interaction between time and treatment [$F(12,164)=8.327$, $P<0.001$]. As compared with the vehicle-treated group, Compound 6, at doses (3 and 10 mg/kg) that did not affect the spontaneous locomotor activity [72], reduced the tremor activity significantly 30 min after harmaline injection while propranolol (20 mg/kg) displayed anti-tremor activity more quickly (Fig. 5A). One-way ANOVA of the AUC of the tremor activity showed a significant difference among treatment groups [$F(3,41)=12.85$, $P<0.001$]. Compound 6 exerted significant and comparable anti-tremor effects at 3 and 10 mg/kg (Fig. 5B) (tremor inhibition: 51.91% and 42.14%, respectively). Propranolol showed a higher tremor-suppressive effect (84.15% inhibition) than Compound 6, in either the 3 mg/kg or 10 mg/kg (Fig. 5B). Interestingly, Compound 6 at both 3 and10 mg/kg significantly restored the burrowing activity in mice that had been impaired by harmaline. However, propranolol failed to restore the burrowing activity in harmaline-treated mice (Fig. 5C, one-way ANOVA, Holm-Sidak *post hoc*).

Furosemide (*i.cb.*) Antagonized Effects of Compound 6 (*i.p.*) on Tremor and Burrowing Activities in Harmaline-Treated Mice

Next, we examined whether *i.cb.* furosemide can antagonize the anti-tremor effect of *i.p.* Compound 6. Mice were co-treated with *i.cb.* furosemide (10 nmol) and *i.p.* Compound 6 (10 mg/kg) 5 min before harmaline injection (Fig. 6A). Two-way ANOVA with repeated measures over time showed main effects of time [$F(4,164)=41.59$, $P<0.001$] and treatment [$F(3,41)=12.57$, $P<0.001$], and a significant interaction between time and treatment

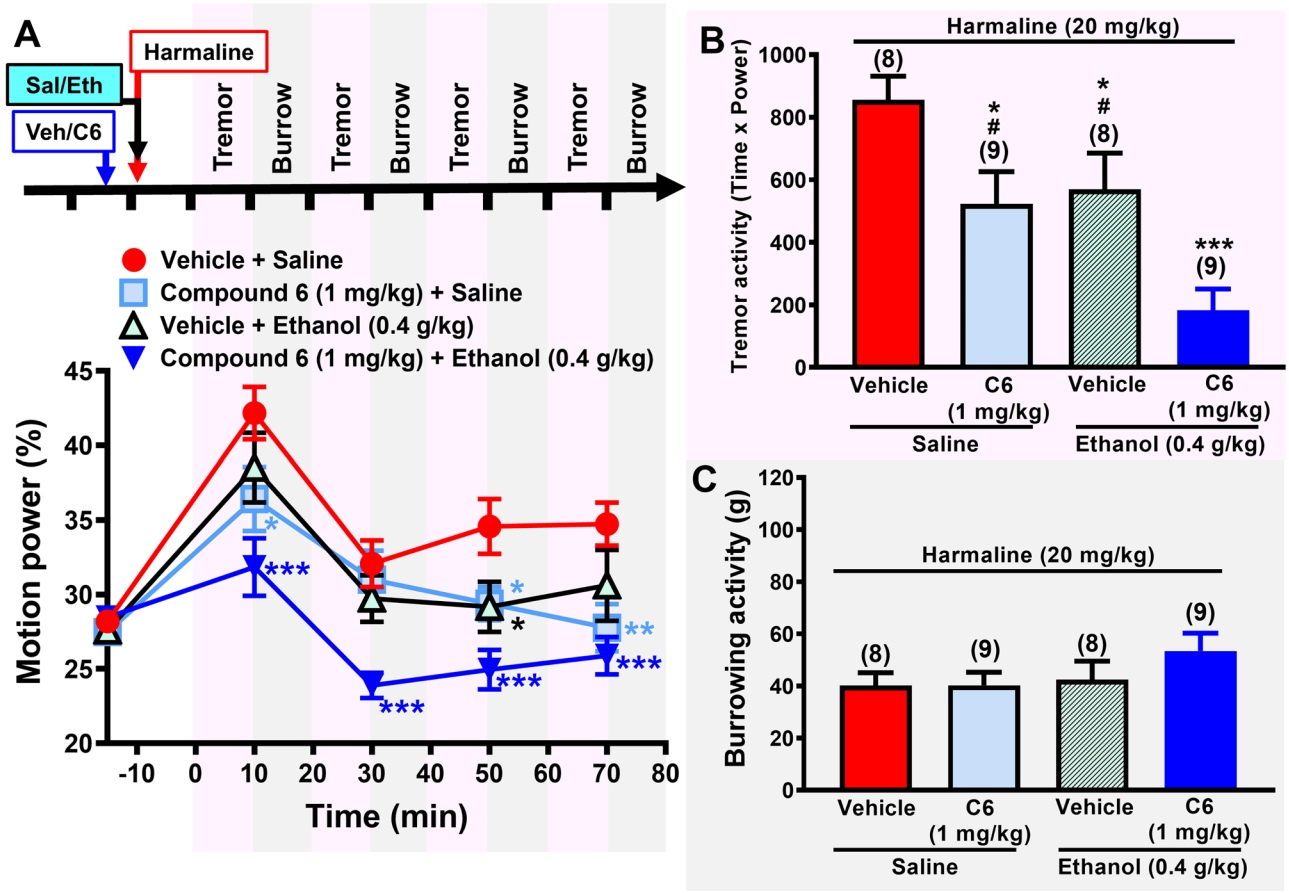


Fig. 7 Synergistic anti-tremor effect of low doses of ethanol and Compound 6, an α6GABA$_A$R-selective PAM in harmaline-treated mice. **A** Time courses of effects of 1 mg/kg (*i.p.*) of Compound 6 and its vehicle, and ethanol (0.4 g/kg) on harmaline (20 mg/kg, *s.c.*)-induced tremor. The tremor activity and burrowing activity were measured alternatively for 80 min as described in Fig. 2A. Compound 6 and vehicle (blue arrow) were injected (*i.p.*) 5 min prior harmaline treatment (red arrow), while ethanol and saline (black arrow) were co-administered with harmaline treatment. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**B**) and burrowing activity (**C**) were measured as described in Fig. 3 in harmaline-treated mice in various treatment groups. $^*P<0.05$, $^{***}P<0.001$ *vs.* Vehicle + Saline group, # $P<0.05$ *vs.* C6 + Ethanol group, one-way ANOVA followed by Holm-Sidak's *post hoc* test. The numbers in the parentheses denoted the *n* number of mice tested in each group. Data are expressed as mean ± *S.E.M.* C6: Compound 6

[$F(12,164)=8.327$, $P<0.001$] (Fig. 6A). Similarly, *i.cb.* furosemide alone had no effect on the tremor activity (yellow bar *vs.* red bar). Compound 6 (10 mg/kg, *i.p.*) significantly reduced harmaline-induced tremor activity, as compared to the vehicle-treated group (blue bar *vs.* red bar, Fig. 6B). However, in the *i.cb.* furosemide-co-treated group, Compound 6 no longer significantly attenuated the tremor activity (green bar *vs.* yellow bar, Fig. 6B). Interestingly, the restorative effect of Compound 6 on burrowing-activity in harmaline-treated mice was also completely reversed by *i.cb.* furosemide (green bar, Fig. 6C) while *i.cb.* furosemide *per se* did not affect the burrowing activity (yellow bar, Fig. 6C).

Synergistic Interaction Between Compound 6 and Ethanol

We further examined whether Compound 6 and ethanol could interact synergistically in their anti-tremor effects at marginal or minimal effective doses, i.e., 1 mg/kg and 0.4 g/kg, respectively. As shown in Fig. 7A, two-way ANOVA with repeated measures over time showed main effects of time [$F(4,120)=32.01$, $P<0.001$] and treatment [$F(3,30)=9.03$, $P=0.0002$], and a significant interaction between time and treatment [$F(12,120)=2.536$, $P=0.0051$]. The AUC of tremor activity over time showed that Compound 6 (1 mg/kg) or ethanol (0.4 g/kg) alone reduced tremor activity by

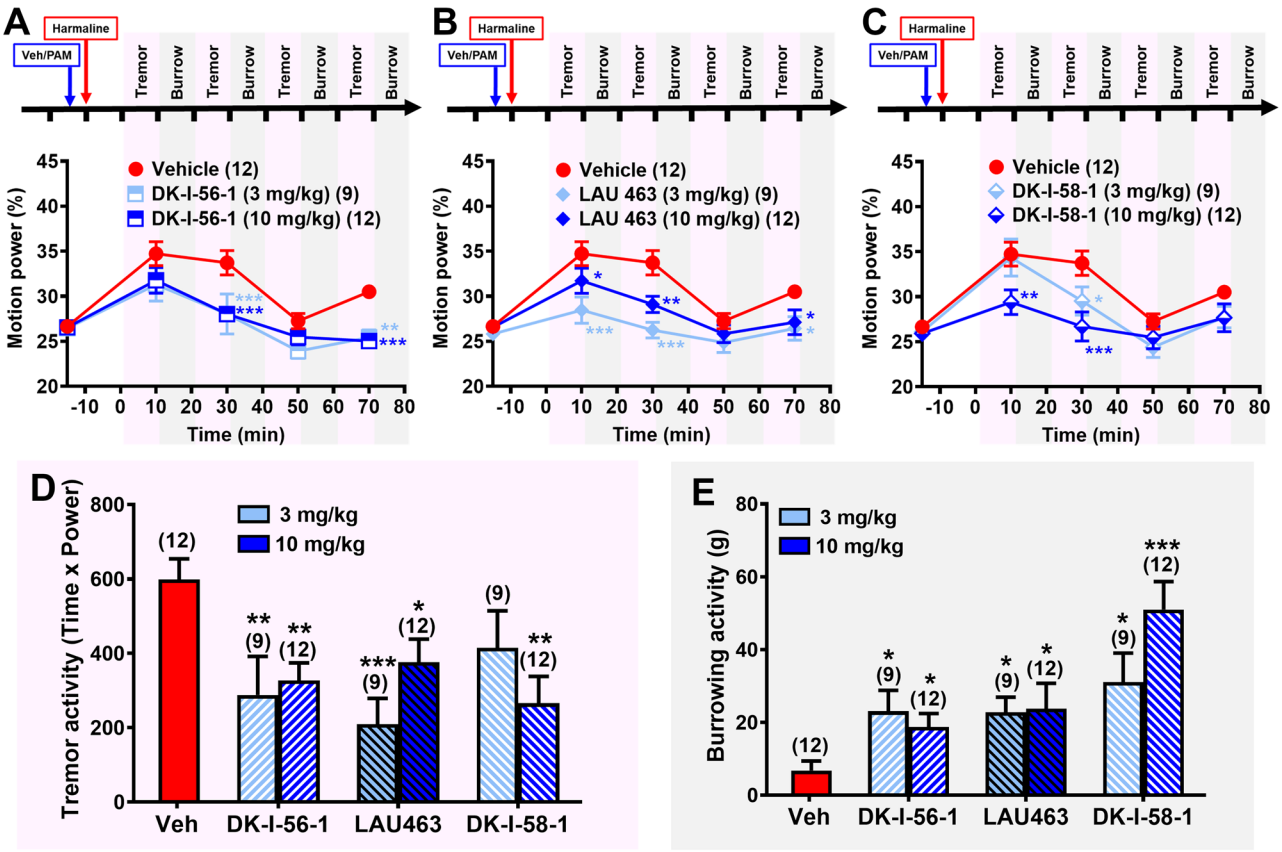


Fig. 8 Effects of DK-I-56-1, LAU 463, and DK-I-58-1, structural analogues of Compound 6, on harmaline-treated mice. LAU463 is a structural analogue of Compound 6. DK-I-58-1 and DK-I-56-1 are their respective deuterated derivatives (see Supplementary Fig. S1). These compounds are all α6GABA$_A$R PAMs with similar α6GABA$_A$R-selectivity and efficacy while deuterated derivatives have longer half-lives. Similar procedure as described in Fig. 5A were performed with Compound 6 replaced with (**A**) DK-I-56-1 (half-filled square symbols), (**B**) LAU 463 (diamond symbols), or (**C**) DK-I-58-1 (half-filled diamond symbols), at doses of 3 and 10 mg/kg (*i.p.*). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Saline group, two-way ANOVA with repeated measures over time followed by Holm-Sidak's *post hoc* test. The total tremor activity (**D**) and burrowing activity (**E**) were measured alternatively for 80 min as described in Fig. 3 in various treatment groups. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* Vehicle group, one-way ANOVA followed by Holm-Sidak's *post hoc* test. Veh: vehicle, PAM: positive allosteric modulator

31.3% and 27.4%, respectively, while together suppressing the tremor activity at a magnitude (79.8%) that is significantly greater than produced by Compound 6 ($p=0.035$) or ethanol ($p=0.023$) alone (Fig. 7B) (one-way ANOVA followed by Holm-Sidak's *post hoc* test). On the other hand, the burrowing activity impaired by harmaline in mice was not significantly restored ($p=0.345$) by a combination of Compound 6 (1 mg/kg) ($p=0.988$) and ethanol (0.4 kg/g) ($p=0.958$) (Fig. 7C) (One-way ANOVA followed by Holm-Sidak's *post hoc* test).

Compound 6 Analogues and Deuterated Derivatives Displayed Similar Anti-tremor and Burrowing-Restorative Effects in Harmaline-Treated Mice

Compound 6 and its structural analogue, LAU463, as well as their respective deuterated derivatives, DK-I-56-1 and DK-I-58-1, were previously demonstrated to possess similar selectivity towards α6GABA$_A$R as PAMs. In the present study, the administration of DK-I-56-1 (half-filled square symbols, Fig. 8A), LAU-463 (filled diamonds, Fig. 8B) and DK-I-58-1 (half-filled diamonds, Fig. 8C) at the of 3 and 10 mg/kg (*i.p.*) demonstrated time and treatment-dependent suppression of harmaline-induced tremor in mice, lasting for almost 1 h. By comparing the AUC derived from time-dependent analysis, we found that each drug group significantly suppressed tremor activity as compared with the vehicle group, except DK-I-58-1 at the dose of 3 mg/kg. Interestingly, as compared with the vehicle group within each drug group, all treatment groups significantly restored burrowing activity in harmaline-treated mice Fig. 8E), similar to the effect of Compound 6, but not of propranolol (Fig. 5C) or ethanol (Fig. 3C).

Discussion

In this study, we found that ethanol, at low-to-moderate doses (0.4-1.2 g/kg, *i.p.*) that did not affect locomotor activity, significantly and dose-dependently attenuated harmaline-induced action tremor in mice. The anti-tremor effect of ethanol was not significant when ethanol was co-administered with *i.cb.* furosemide, an α6GABA$_A$R-selective antagonist, suggesting the involvement of cerebellar α6GABA$_A$Rs. The finding that α6GABA$_A$R-selective PAMs attenuated harmaline-induced tremor further supports the conclusion that positive modulation of cerebellar α6GABA$_A$Rs can relieve action tremor.

Harmaline Induces Action Tremor in Male ICR Mice

Harmaline is a β-carboline toxin that can induce action tremor with a frequency range at 10-16 Hz in mice [67], 8-12 Hz in rats [67], 8-12 Hz in cats [73], and 6-8 Hz in monkeys [74], resembling the 4-12 Hz action tremor in ET patients [75]. Thus, the harmaline-induced action tremor is recognized as an animal model for screening potential anti-tremor agents for ET [67]. Here, we found that harmaline can induce significant tremor at 10, 20, and 30 mg/kg (*s.c.*) with a similar maximal tremor activity but a dose-dependent action duration (Fig. 2). The tremor was at the frequency of 10-16 Hz (Fig. 1A), absent at rest, maximal during movement (Fig. 1B), attenuated during posture maintenance, and often accentuated at the termination of movement, suggesting it is a type of action tremor. Thus, we have established the harmaline-induced action tremor in male ICR mice, mimicking the 4-112 Hz action tremor manifested in ET patients [76], as reported in female ICR mice [67] and in C57BL/6 mice with both sexes [66]. The predictive validity of harmaline-induced tremor in modeling ET in patients has been strongly supported by previous findings that it was reduced by clinically used ET-relieving agents, including propranolol, primidone, alcohol, benzodiazepines, gabapentin, gammahydroxybutyrate, 1-octanol, and zonisamide, and was exacerbated by drugs that worsen ET, such as tricyclics and caffeine [77]. In the current ET model, we confirmed that propranolol can be a positive control, which at 20 mg/kg (*i.p.*) inhibited harmaline-induced action tremor (Fig. 5).

Ethanol Suppressed Harmaline-Induced Tremor at Non-motor Impairing Doses

Here, we found that ethanol significantly reduced harmaline-induced tremor at a dose as low as 0.4 g/kg (*i.p.*) (Fig. 3), which could achieve a BAC of 0.05% (10.87 mM) 5 min after administration in the same strain/sex mice [78]. This concentration is below the driving legal limit in humans, i.e., 0.08% in the USA [40]. This is in line with clinical observations that "a glass of wine" [79] can suppress tremor in ET patients with the BAC of 0.03-0.06% [29, 32, 80–82]. In C57BL/6 mice, ethanol was also shown to reduce harmaline-induced tremor at 0.1 g/kg [83].

Ethanol, at the tremor-relieving doses, 0.4-2.4 g/kg (*i.p.*) that are expected to achieve 0.05-0.3% BAC in tested mice [78], did not reduce the spontaneous locomotor activity (Fig. 3D, E). This suggests that the anti-tremor effect of ethanol is not a confounding outcome due to its motor-impairing activity. Instead, ethanol at doses higher than 1.2 g/kg tended to, though insignificantly, increase the spontaneous locomotor activity. Effects of ethanol on the locomotor activity are dose- and strain-dependent. In Swiss mice, ethanol induced hyper-locomotion at 1.5-2.5 g/kg (*i.p.*) but hypo-locomotion at higher doses (≥ 3-4 g/kg) [84]. However, it induced only hypo-locomotion in C57BL/6 mice from 0.75 to 2.25 g/kg while produced only hyper-locomotion in BALB/cJ mice at the same dose range [85].

Ethanol Suppressed Tremor at Least Partially via Acting as an α6GABA$_A$R PAM in the Cerebellum

The site of the tremor reducing action of ethanol has long been known to be located centrally [27] and mainly in the cerebellum [42]. The present finding that the ethanol-induced reduction of the harmaline-induced tremor no longer is significant after *i.cb.* microinjection of furosemide, an α6GABA$_A$R-selective antagonist [60] (Fig. 4A, B) suggests that cerebellar α6GABA$_A$Rs, at least partially, mediate the anti-tremor action of low-to-moderate doses of ethanol. Although furosemide can also inhibit the Na-K-Cl cotransporter (NKCC), this effect was observed at 20 times higher concentrations with a long duration until action develops (> 20 min) in brain slice electrophysiological studies [86, 87]. In addition, to the best of our knowledge, there is no report for a direct activity of low-to-moderate doses of ethanol on NKCCs. Due to its selective ability to inhibit α6GABA$_A$Rs [60], furosemide has thus been utilized as a pharmacological tool to differentiate between α6 subunit-containing and non-α6 subunit-containing GABA$_A$Rs [88].

An involvement of α6β3δGABA$_A$Rs in the anti-tremor action of ethanol can be also supported by previous findings that ethanol is a cerebellar α6GABA$_A$R PAM [43, 79] and that α6GABA$_A$Rs containing α6, β3 and δ subunits responded to ethanol at a concentration as low as 3 mM [89]. An anti-tremor action mediated by α6β3δGABA$_A$Rs was recently demonstrated by the finding that gaboxadol, a selective agonist of the δ-subunit containing GABA$_A$Rs, significantly suppressed harmaline-induced tremor in wild type, but not in *Gabra6*$^{-/-}$ or *Gabrd*$^{-/-}$ mice [66].

Higher Doses of Ethanol May Relieve Tremor via Additional Mechanisms

It is noteworthy that *i.cb.* furosemide could not completely block the anti-tremor effect produced by *i.p.* 1.2 g/kg ethanol 10 min after injection (Fig. 4A). At this time point, the plasma concentration would be about 0.15% (33 mM), based on the ethanol levels measured in ICR mice [78]. This is higher than the effective concentration (17 mM) that affects α6GABA$_A$Rs [90]. Thus, mechanism(s) other than the furosemide-sensitive α6GABA$_A$R PAM effect may be involved in the anti-tremor effect of ethanol here, such as positive modulation of α1GABA$_A$Rs [89] that are abundant on DCN and ION neurons (Scheme 1). The finding that the estimated ED_{50} of the anti-tremor effect of ethanol is about 1.4 g/kg (Supplementary Fig. S2) also suggests that ethanol may relieve tremor at higher doses via mechanism(s) not mediated by α6GABA$_A$Rs. Other proposed action mechanisms of ethanol at higher doses, like glutamatergic dysregulation [91], cerebellar cyclic GMP attenuation [83], nitric oxide synthase inhibition [92] and gap junction inactivation [93], may also be involved in the anti-tremor action of ethanol.

α6GABA$_A$R-Selective PAMs Suppressed Harmaline-Induced Action Tremor

The hypothesis that a positive modulation of cerebellar α6GABA$_A$Rs can suppress essential tremor is further supported by the anti-tremor effect of all tested PQ compounds, which are highly α6GABA$_A$R-selective PAMs (Figs. 6 and 8, Supplementary Fig. S1).

In previous electrophysiological studies on *Xenopus* oocytes expressing various recombinant GABA$_A$R subtypes, we demonstrated that Compound 6 and its structural derivative LAU 463, exerted their PAM activity at both α6βγ2- and α6ββδGABA$_A$Rs although having a lower efficacy at the α6ββδ subtype [62, 94]. Thus, Compound 6, LAU 463, and their deuterated derivatives may act at α6ββδGABA$_A$Rs on cerebellar GCs to exert their anti-tremor effect, although a contribution of α6βγ2GABA$_A$Rs cannot be excluded.

Given that Compound 6 (Fig. 5) and other α6GABA$_A$R-selective PAMs (Fig. 8) showed a significant suppressive effect in harmaline-induced tremor, and α6GABA$_A$Rs are most extensively expressed in cerebellar granule cells, it is reasonable to speculate that cerebellar α6GABA$_A$Rs may have a role, at least partially, in suppressing the action tremor induced by harmaline. Nevertheless, the anti-tremor effects of the α6GABA$_A$R-selective Compound 6 were reduced but not completely reversed after *i.cb.* microinjection of furosemide (Fig. 6B), possibly indicating that α6GABA$_A$Rs in brain regions outside the cerebellum [94] might have contributed to the anti-tremor effects of the systemically applied Compound 6. Such effects cannot be blocked by the *i.cb.* microinjection of furosemide.

Interestingly, the anti-tremor effects of ethanol and Compound 6 were synergistic, but not occlusive, although both compounds seem to exert their effects by acting via the same target, the cerebellar α6GABA$_A$Rs (Fig. 7). In these experiments, however, both compounds were applied at sub-maximal doses. Mutual inhibition can only be expected when applied at their maximally effective doses and when ethanol and Compound 6 cause their anti-tremor effects via the same binding site at α6GABA$_A$Rs. This not necessarily is the case. There are multiple allosteric binding sites at GABA$_A$ receptors that in detail might be different for ethanol [48] and Compound 6 [58]. In addition, as ethanol may have more than one mechanism of anti-tremor action, increasing its dose to reach the maximal effects would further increase the heterogeneity of its mechanism of action, leading to data that cannot be interpreted.

The Possible Tremolytic Mechanism of α6GABA$_A$R PAMs in Harmaline-Induced Action Tremor

The cerebellar microcircuit proposed to be involved in the tremorgenic mechanism of harmaline and in the anti-tremor effect of α6GABA$_A$R PAMs is depicted in Scheme 1. It is believed that harmaline-induced tremor originates from its enhancement of the ION activity [95]. In vivo electrophysiological recordings demonstrated that harmaline can change the complex spikes of PCs, which originate from ION-climbing fiber inputs [96, 97], from a low frequency one (~ 1 Hz) to a rhythmic one at 6-12 Hz [98, 99]. These synchronous complex spikes on PCs can provide effective phase-locking of DCN neuronal activity [100], leading to marked rhythmic, alternating hyperpolarization and rebound bursting in DCN neurons [24]. This exaggerated synchrony between PCs and DCN as well as the subsequent excessive synchrony between ION and PCs are hypothesized to contribute to both harmaline-induced tremor and tremor in ET patients (Scheme 1B) [24, 95].

Besides, a c-fos mapping study indicated that harmaline can also activate cerebellar GrCs [101]. Overly active GrCs would increase the activity of PCs and hence increase the inhibitory drive from PCs onto the small GABAergic neurons in the DCN, which project to the ION [102] and provide an inhibitory control on the synchrony of climbing fibers [103, 104] (Scheme 1A), ultimately leading to disinhibition of ION-climbing fiber inputs back to PCs. Thus. through this "double inhibitory pathway" in the PC-DCN-ION circuit [95], harmaline can also enhance the synchronized rhythmic firing of PCs, inducing tremor (Scheme 1B).

α6GABA$_A$R PAMs could enhance tonic and phasic GABAergic inhibition on GrCs, via extrasynaptic α6ββδGABA$_A$Rs and synaptic α6βγ2GABA$_A$Rs at GoC-GrC

synapses, respectively [46], ultimately reducing the excitatory input of GrCs onto PC and decreasing the neuronal activity of PCs. This may result in a reduction of the inhibitory synaptic transmission onto the ION-projecting GABAergic neurons in the DCN, i.e., suppression of the double inhibitory pathway in the PC-DCN-ION circuit, and thereby contribute to tremor suppression (Scheme 1C) [95].

A PET study in ethanol-responsive ET patients demonstrated that alcohol at the BAC below the driving limit can suppress tremor and reduce both ipsilateral and contralateral cerebellar cortical activities, but only reduced the ipsilateral cerebellar activation in normal subjects during a passive wrist oscillation at the tremor frequency [41]. Furthermore, ethanol increased ION activity in patients but not in controls [41]. These results support the notion that low doses of ethanol, probably via acting as an α6GABA$_A$R PAM, suppress the double inhibitory pathway of the PC-DCN-ION circuit in ET patients.

α6GABA$_A$R-Selective PAMs, but Not Ethanol and Propranolol, Restored Burrowing Activity in Harmaline-Treated Mice

The burrowing activity in laboratory rodents is one of their "ADL" [71], and has been used as an indicator of well-being [61], because it is negatively associated with stress [105] or chronic pain [106] in rodents. A deficit of the burrowing activity has also been reported in the mouse model of Alzheimer's disease [107] or Parkinson's disease [108]. The finding that harmaline markedly disrupted the burrowing activity in mice (Fig. 2C) suggests that this ET animal model mimics not only the action tremor but also the reduced ADL scores manifested in ET patients.

Unexpectedly, ethanol (1.2 g/kg) did not restore the burrowing activity in harmaline-treated mice (Fig. 4C), despite its significant anti-tremor efficacy (Fig. 4A, B). Conversely, α6GABA$_A$R-selective PQ compounds significantly restored the burrowing activity in harmaline-treated mice (Fig. 5C) in addition to attenuating their tremor activity (Fig. 5A, B). Furthermore, the synergism between Compound 6 and ethanol in the anti-tremor effect (Fig. 7A, B) was not shown in the burrowing restoring activity (Fig. 7C). It is thus possible that this beneficial effect of Compound 6 may be clouded by unspecific effects of ethanol. Interestingly, propranolol, although having markedly anti-tremor activity, did not restore the burrowing activity in harmaline-treated mice (Fig. 5C). This is probably due to its reduction of motor activity [109]; it also causes muscle weakness in humans [110].

Currently, neither the mechanism involved in the reduced ADL scores in ET patients, nor that of the reduction of the burrowing activity in rodents by harmaline is known. The results obtained in this study, however, suggest that tremor reduction does not automatically lead to an increase in the burrowing activity in harmaline-treated mice. The mechanism of harmaline-induced reduction of the burrowing activity in mice may be caused by its non-tremor related effects, *eg.* as an NMDA inverse agonist [111], monoamine oxidase A inhibitor [112], histamine-N-methyltransferase inhibitor [113], etc. Further studies need to be conducted to discern a possible involvement of α6GABA$_A$Rs in restoring the ADL in rodents. α6GABA$_A$Rs in the CNS, depending on the region of expression, may participate in various motor, sensory and cognitive functions [94].

Since α6GABA$_A$R PAMs can both reduce tremor activity and restore burrowing activity in harmaline-treated mice, they may be viable candidates to complement current anti-tremor drugs. The findings that combined sub-effective doses of Compound 6 with ethanol (Fig. 7A, B) exerted a synergistic anti-tremor effect further shed light on this notion.

In addition to Compound 6, its chemical derivatives also exhibited similar anti-tremor effects. Compound 6 and LAU 463 are structural analogues with different functional groups on the A-ring, whereas DK-I-56-1 and DK-I-58-1 are their respective derivatives with a deuterated methoxy group on the D-ring (Supplementary Fig. S1). These deuterated derivatives, compared with their parent compounds, had a similar potency and the same α6GABA$_A$R-selectivity in enhancing GABA currents of recombinant α6βγGABA$_A$Rs [59, 114] but displayed longer half-lives in rodents [59]. Nonetheless, the merit of longer half-lives of deuterated compounds cannot be observed in these behavioral assays that only lasted for 90 min.

Current Essential Tremor Therapy Is Insufficient

Currently, essential tremor is symptomatically treated with medicines that can suppress tremor activity, like ß-blockers, anti-epileptics, neuron stabilizers and GABA-related drugs [9, 115]. Among these, propranolol and primidone remain the first-line medications for relieving tremor in patients with essential tremor, even decades after their initial applications [9]. A combination of these two drugs yields a better response than using either of them alone [116]. However, one third of patients developed drug resistance [110], and some patients became even refractory to both medications [3]. The reported adverse effects differ between these two drugs with acute reactions more common with primidone and chronic reactions with propranolol [117]. The acute side effects associated with primidone are sedation, nausea, and vertigo, manifest after the first dose and tend to subside on chronic administrations [118, 119]. On the other hand, chronic propranolol may lead to bradycardia, hypotension, and breathlessness, especially when higher doses are used. Therefore, propranolol is contraindicated in conditions such

as chronic obstructive pulmonary disease, asthma, severe peripheral vascular disease, and diabetes [120].

Limited data from randomized controlled trials are available to support the use of other medications in essential tremor, including topiramate, alprazolam, gabapentin, and other beta-blockers besides propranolol (*e.g.*, atenolol, nadolol, and sotalol) [2, 110, 121, 122]. Randomized controlled trials have shown no significant benefit for several other drugs for essential tremor, including levetiracetam, amifampridine, flunarizine, trazodone, pindolol, acetazolamide, mirtazapine, nifedipine, and verapamil [122]. As a result, the need for an effective anti-tremor drug that is well-tolerated is urgent and obvious. Ethanol is not recognized as a main-stream pharmacotherapy for essential tremor, in fact, it remains controversial as high correlation was reported between alcohol abuse and ET [35, 123]. The abuse potential of ethanol is probably due to its positive modulatory effect on benzodiazepine-sensitive $GABA_A$Rs [124, 125]. Cerebellar a6$GABA_A$Rs, which are benzodiazepine-insensitive, may mediate the anti-tremor effects of low-to-moderate doses of ethanol. Thus, a6$GABA_A$R-selective PAMs may have a tremolytic effect without abuse liability. Indeed, we have demonstrated that the a6$GABA_A$R-selective PAM is free of addictive potential using the conditioned-place preference test [126].

α6$GABA_A$R as a Potential Therapeutic Target for Essential Tremor

We have previously demonstrated that Compound 6 and its deuterated derivative, DK-I-56-1 [58, 59], via acting at the α6$GABA_A$Rs in trigeminal ganglia, significantly inhibited nociceptive activation of the trigeminovascular system and migraine-like grimaces in animal models of migraine [126, 127]. DK-I-56-1 can also prevent and reduce nociceptive responses in a rat model of trigeminal neuropathic pain [128]. Besides, we found that Compound 6, via acting as a PAM of cerebellar α6$GABA_A$Rs, can ameliorate disrupted prepulse inhibition (PPI) of the startle response, social withdrawal and cognitive impairment in animal models mimicking schizophrenia [62, 94]. Here, we further substantiated the effectiveness of α6$GABA_A$R-selective PQ compounds in an animal model of essential tremor. The effective dose range (3-10 mg/kg, *i.p.*) of PQ Compounds in suppressing tremor was similar to that in mouse models of schizophrenia and migraine [62, 94, 126]. At these doses, Compound 6 did not impair motor functions in rats, nor display sedative or hypolocomotor activity in mice [59, 72, 94]. In contrast to the GABA binding site agonist, gaboxadol that directly activates all α4βδ and α6βδ $GABA_A$Rs in neuronal tissues [24, 129] and exhibits sedation and motor-incoordination [130], α6$GABA_A$R-selective PAMs can only allosterically enhance the activity of those α6βδ and α6βγ2 $GABA_A$Rs that are activated by GABA in certain tasks, explaining the absence of those side effects. In contrast to benzodiazepines and gaboxadol, α6$GABA_A$R-selective PQ compounds can be potential anti-tremor agents without sedative or motor-impairing activity. In addition, the synergistic effect of low doses of Compound 6 and ethanol in their anti-tremor effects suggests the potential of α6$GABA_A$R PAMs as an adjunct therapy to other anti-tremor drugs to lower the required doses and hence reduce the emergence of adverse effects. Therefore, the α6$GABA_A$R-selective PAMs have the potential to be an alternative mono- or add-on therapy for ET.

Abbreviations α1$GABA_A$R: α1 Subunit containing $GABA_A$ receptor; α6$GABA_A$R: α6 Subunit containing $GABA_A$ receptor; ADL: Activities of Daily Living; AUC: Area under curve; BAC: Blood alcohol concentration; BC: Basket cell; CSF: Cerebrospinal fluid; Compound 6: 7-Methoxy-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; DCN: Deep cerebellar nuclei; DK-I-56–1: 7-Methoxy-2-(4-methoxy-d3-phenyl)-2,5-dihydro-3Hpyrazolo-[4,3-c]quinolin-3-one; DK-I-58–1: 7-Bromo-2-(4-methoxy-d3-phenyl)-2,5-dihydro-3Hpyrazolo[4,3-c]quinolin-3-one; ET: Essential tremor; GrC: Granule cell; GoC: Golgi cell; ION: Inferior olive nucleus; *i.cb.*: Intracerebellar; *i.p.*: Intraperitoneal; LAU 463: 7-Bromo-2-(4-methoxyphenyl)-2,5-dihydro-3H-pyrazolo[4,3-c]-quinolin-3-one; PAM: Positive allosteric modulator; PC: Purkinje cell; PET: Positron emission tomography; PQ: Pyrazoloquinolinone; *s.c.*: Subcutaneous; PPI: Prepulse inhibition; SC: Stellate cell

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13311-023-01342-y.

Acknowledgements We thank the Milwaukee Institute of Drug Discovery for the mass spectroscopy. We also thank Mr. Ben Juan for participating in certain experiments.

Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

Author Contribution YHH: performed research, analyzed data, wrote the paper; MTL: analyzed data, wrote the paper; HYH: performed research and analyzed data; DEK: contributed new reagents or analytic tools; JC: contributed new reagents or analytic tools; MM: contributed new reagents or analytic tools; WS: wrote the paper; LCC: designed research, analyzed data, contributed new reagents or analytic tools, wrote the paper.

Funding This study was supported by grants from the Ministry of Science and Technology, Taiwan (MOST 104–2923-B-002–006-MY3, MOST 108–2320-B-002–029-MY3 and MOST 109–2320-B-002–042-MY3 to LCC), National Health Research Institutes, Taiwan (NHRI-EX111-11114NI to LCC), Ministry of Education, Taiwan (107M4022-3 to LCC), Fundamental Research Grant Scheme, Ministry of Higher Education, Malaysia (FRGS/1/2021/WAB13/UCSI/02/1 to MTL), UCSI University Research Excellence and Innovation Grant (REIG-FPS-2020/065 to MTL), National Institutes of Health, USA (R01AA029023 and R01MH096463 to JC), and the National Science Foundation, USA, Division of Chemistry (CHE-1625735 to JC).

Data Availability The data that reported in this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of Interest YHH, HYH, and MTL declare no conflict of interest. A patent application has been jointly filed by DEK, MM, JC, WS, and LCC for the chemical structures and therapeutic applications of α6GABA$_A$R-modulating PQ compounds, but the patent right is co-owned by the inventors' institutions. Some of the results presented in this study have been deposited in the *Biorxiv* as a preprint (https://doi.org/10.1101/2021.04.19.440397).

References

1. Deuschl G, Bain P, Brin M. Consensus statement of the movement disorder society on tremor. Ad hoc scientific committee. Mov Disord. 1998;13(Suppl 3):2–23.
2. Haubenberger D, Hallett M. Essential Tremor. N Engl J Med. 2018;379(6):596–7.
3. Louis ED. Essential tremor as a neuropsychiatric disorder. J Neurol Sci. 2010;289(1–2):144–8.
4. Sepúlveda Soto MC, Fasano A. Essential tremor: new advances. Clin Parkinsonism Relat Disord. 2020;3:100031.
5. Song P, Zhang Y, Zha M, et al. The global prevalence of essential tremor, with emphasis on age and sex: a meta-analysis. J Glob Health. 2021;11:04028.
6. Louis ED, McCreary M. How common is essential tremor? Update on the worldwide prevalence of essential tremor. Tremor Other Hyperkinet Mov (N Y). 2021;11:28.
7. Huang H, Yang X, Zhao Q, et al. Prevalence and risk factors of depression and anxiety in essential tremor patients: a cross-sectional study in southwest China. Front Neurol. 2019;10:1194.
8. Monin JK, Gutierrez J, Kellner S, et al. Psychological suffering in essential tremor: a study of patients and those who are close to them. Tremor Other Hyperkinet Mov (N Y). 2017;7:526.
9. Shanker V. Essential tremor: diagnosis and management. BMJ. 2019;366:l4485.
10. Mally J, Baranyi M, Vizi ES. Change in the concentrations of amino acids in CSF and serum of patients with essential tremor. J Neural Transm (Vienna). 1996;103(5):555–60.
11. Sharifi S, Nederveen AJ, Booij J, van Rootselaar AF. Neuroimaging essentials in essential tremor: a systematic review. Neuroimage Clin. 2014;5:217–31.
12. Louis ED, Faust PL, Vonsattel JP, et al. Neuropathological changes in essential tremor: 33 cases compared with 21 controls. Brain. 2007;130(Pt 12):3297–307.
13. Louis ED, Babij R, Lee M, Cortes E, Vonsattel JP. Quantification of cerebellar hemispheric purkinje cell linear density: 32 ET cases versus 16 controls. Mov Disord. 2013;28(13):1854–9.
14. Kuo SH, Wang J, Tate WJ, et al. Cerebellar pathology in early onset and late onset essential tremor. Cerebellum. 2017;16(2):473–82.
15. Louis ED, Lee M, Babij R, et al. Reduced Purkinje cell dendritic arborization and loss of dendritic spines in essential tremor. Brain. 2014;137(Pt 12):3142–8.
16. Louis ED, Faust PL. Essential tremor pathology: neurodegeneration and reorganization of neuronal connections. Nat Rev Neurol. 2020;16(2):69–83.
17. D'Angelo E. Physiology of the cerebellum. Handb Clin Neurol. 2018;154:85–108.
18. D'Angelo E, Solinas S, Mapelli J, et al. The cerebellar Golgi cell and spatiotemporal organization of granular layer activity. Front Neural Circuits. 2013;7:93.
19. Pugh JR, Raman IM. Nothing can be coincidence: synaptic inhibition and plasticity in the cerebellar nuclei. Trends Neurosci. 2009;32(3):170–7.
20. Moini J, Piran P. Chapter 16 - Cerebellum. In: Moini J, Piran P, editors. Functional and Clinical Neuroanatomy: Academic Press; 2020. p. 497–517.
21. Mapelli L, Solinas S, D'Angelo E. Integration and regulation of glomerular inhibition in the cerebellar granular layer circuit. Front Cell Neurosci. 2014;8:55.
22. Mugnaini E, Sekerkova G, Martina M. The unipolar brush cell: a remarkable neuron finally receiving deserved attention. Brain Res Rev. 2011;66(1–2):220–45.
23. Lavita SI, Aro R, Kiss B, Manto M, Duez P. The role of beta-carboline alkaloids in the pathogenesis of essential tremor. Cerebellum. 2016;15(3):276–84.
24. Brown AM, White JJ, van der Heijden ME, et al. Purkinje cell misfiring generates high-amplitude action tremors that are corrected by cerebellar deep brain stimulation. Elife. 2020;9.
25. Pan MK, Li YS, Wong SB, et al. Cerebellar oscillations driven by synaptic pruning deficits of cerebellar climbing fibers contribute to tremor pathophysiology. Sci Transl Med. 2020;12(526).
26. Critchley M. Observations on essential (heredofamial) tremor. Brain. 1949;72(Pt. 2):113–39.
27. Growdon JH, Shahani BT, Young RR. The effect of alcohol on essential tremor. Neurology. 1975;25(3):259–62.
28. Marshall J. Observations on essential tremor. J Neurol Neurosurg Psychiatry. 1962;25:122–5.
29. Hopfner F, Erhart T, Knudsen K, et al. Testing for alcohol sensitivity of tremor amplitude in a large cohort with essential tremor. Parkinsonism Relat Disord. 2015;21(8):848–51.
30. Hopfner F, Haubenberger D, Galpern WR, et al. Knowledge gaps and research recommendations for essential tremor. Parkinsonism Relat Disord. 2016;33:27–35.
31. Klebe S, Stolze H, Grensing K, et al. Influence of alcohol on gait in patients with essential tremor. Neurology. 2005;65(1):96–101.
32. Rajput AH, Jamieson H, Hirsh S, Quraishi A. Relative efficacy of alcohol and propranolol in action tremor. Can J Neurol Sci. 1975;2(1):31–5.
33. Koller WC. Pharmacologic trials in the treatment of cerebellar tremor. Arch Neurol. 1984;41(3):280–1.
34. Ferreira JJ, Mestre TA, Lyons KE, et al. MDS evidence-based review of treatments for essential tremor. Mov Disord. 2019;34(7):950–8.
35. Hess CW, Saunders-Pullman R. Movement disorders and alcohol misuse. Addict Biol. 2006;11(2):117–25.
36. Silva D, Matias C, Bourne S, et al. Effects of chronic alcohol consumption on long-term outcomes of thalamic deep brain stimulation for essential tremor. J Clin Neurosci. 2016;31:142–6.
37. Volkow ND, Ma Y, Zhu W, et al. Moderate doses of alcohol disrupt the functional organization of the human brain. Psychiatry Res. 2008;162(3):205–13.
38. Volkow ND, Mullani N, Gould L, et al. Effects of acute alcohol intoxication on cerebral blood flow measured with PET. Psychiatry Res. 1988;24(2):201–9.
39. Volkow ND, Wang GJ, Franceschi D, et al. Low doses of alcohol substantially decrease glucose metabolism in the human brain. Neuroimage. 2006;29(1):295–301.
40. Mostile G, Jankovic J. Alcohol in essential tremor and other movement disorders. Mov Disord. 2010;25(14):2274–84.
41. Boecker H, Wills AJ, Ceballos-Baumann A, et al. The effect of ethanol on alcohol-responsive essential tremor: a positron emission tomography study. Ann Neurol. 1996;39(5):650–8.
42. Pedrosa DJ, Nelles C, Brown P, et al. The differentiated networks related to essential tremor onset and its amplitude modulation after alcohol intake. Exp Neurol. 2017;297:50–61.
43. Olsen RW. GABAA receptor: Positive and negative allosteric modulators. Neuropharmacology. 2018;136(Pt A):10–22.
44. Olsen RW, Sieghart W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors:

classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev. 2008;60(3):243–60.
45. Sieghart W, Savic MM. International Union of Basic and Clinical Pharmacology. CVI: GABAA receptor subtype- and function-selective ligands: key issues in translation to humans. Pharmacol Rev. 2018;70(4):836–78.
46. Nusser Z, Sieghart W, Somogyi P. Segregation of different GABAA receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci. 1998;18(5):1693–703.
47. Lee S, Yoon BE, Berglund K, et al. Channel-mediated tonic GABA release from glia. Science. 2010;330(6005):790–6.
48. Hanchar HJ, Chutsrinopkun P, Meera P, et al. Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to alpha4/6beta3delta GABAA receptors. Proc Natl Acad Sci U S A. 2006;103(22):8546–51.
49. Wallner M, Hanchar HJ, Olsen RW. Alcohol selectivity of beta3-containing GABAA receptors: evidence for a unique extracellular alcohol/imidazobenzodiazepine Ro15-4513 binding site at the alpha+beta- subunit interface in alphabeta3delta GABAA receptors. Neurochem Res. 2014;39(6):1118–26.
50. Fleming RL, Wilson WA, Swartzwelder HS. Magnitude and ethanol sensitivity of tonic GABAA receptor-mediated inhibition in dentate gyrus changes from adolescence to adulthood. J Neurophysiol. 2007;97(5):3806–11.
51. Mody I, Glykys J, Wei W. A new meaning for "Gin & Tonic": tonic inhibition as the target for ethanol action in the brain. Alcohol. 2007;41(3):145–53.
52. Sundstrom-Poromaa I, Smith DH, Gong QH, et al. Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol. Nat Neurosci. 2002;5(8):721–2.
53. Wei W, Faria LC, Mody I. Low ethanol concentrations selectively augment the tonic inhibition mediated by delta subunit-containing GABAA receptors in hippocampal neurons. J Neurosci. 2004;24(38):8379–82.
54. Baur R, Kaur KH, Sigel E. Structure of alpha6 beta3 delta GABA(A) receptors and their lack of ethanol sensitivity. J Neurochem. 2009;111(5):1172–81.
55. Borghese CM, Storustovu S, Ebert B, et al. The delta subunit of gamma-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. J Pharmacol Exp Ther. 2006;316(3):1360–8.
56. Botta P, Radcliffe RA, Carta M, et al. Modulation of GABAA receptors in cerebellar granule neurons by ethanol: a review of genetic and electrophysiological studies. Alcohol. 2007;41(3):187–99.
57. Korpi ER, Debus F, Linden AM, et al. Does ethanol act preferentially via selected brain GABAA receptor subtypes? the current evidence is ambiguous. Alcohol. 2007;41(3):163–76.
58. Varagic Z, Ramerstorfer J, Huang S, et al. Subtype selectivity of alpha+beta- site ligands of GABAA receptors: identification of the first highly specific positive modulators at alpha6beta2/3gamma2 receptors. Br J Pharmacol. 2013;169 (2):384–99.
59. Knutson DE, Kodali R, Divovic B, et al. Design and synthesis of novel deuterated ligands functionally selective for the gamma-aminobutyric acid Type a receptor (GABAAR) alpha6 subtype with improved metabolic stability and enhanced bioavailability. J Med Chem. 2018;61(6):2422–46.
60. Korpi ER, Luddens H. Furosemide interactions with brain GABAA receptors. Br J Pharmacol. 1997;120(5):741–8.
61. Jirkof P. Burrowing and nest building behavior as indicators of well-being in mice. J Neurosci Methods. 2014;234:139–46.
62. Chiou LC, Lee HJ, Ernst M, et al. Cerebellar alpha6 -subunit-containing GABAA receptors: a novel therapeutic target for disrupted prepulse inhibition in neuropsychiatric disorders. Br J Pharmacol. 2018;175(12):2414–27.
63. Liao YH, Lee HJ, Huang WJ, Fan PC, Chiou LC. Hispidulin alleviated methamphetamine-induced hyperlocomotion by acting at alpha6 subunit-containing GABAA receptors in the cerebellum. Psychopharmacology. 2016;233(17):3187–99.
64. Franklin KBJ, Paxinos G. The mouse brain in stereotaxic coordinates: Academic Press; 1997.
65. Hwang LL, Wang CH, Li TL, et al. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. Obesity. 2010;18(3):463–9.
66. Handforth A, Kadam PA, Kosoyan HP, Eslami P. Suppression of harmaline tremor by activation of an extrasynaptic GABAA receptor: implications for essential tremor. Tremor Other Hyperkinet Mov (N Y). 2018;8:546.
67. Martin FC, Le Thu A, Handforth A. Harmaline-induced tremor as a potential preclinical screening method for essential tremor medications. Mov Disord. 2005;20(3):298–305.
68. White JJ, Arancillo M, King A, et al. Pathogenesis of severe ataxia and tremor without the typical signs of neurodegeneration. Neurobiol Dis. 2016;86:86–98.
69. Paterson NE, Malekiani SA, Foreman MM, Olivier B, Hanania T. Pharmacological characterization of harmaline-induced tremor activity in mice. Eur J Pharmacol. 2009;616(1–3):73–80.
70. Ballon Romero SS, Lee YC, Fuh LJ, et al. Analgesic and neuroprotective effects of electroacupuncture in a dental pulp injury model-a basic research. Int J Mol Sci. 2020;21(7).
71. Deacon R. Assessing burrowing, nest construction, and hoarding in mice. J Vis Exp. 2012;59:e2607.
72. Lee MT, Mouri A, Kubota H, et al. Targeting alpha6GABAA receptors as a novel therapy for schizophrenia: a proof-of-concept preclinical study using various animal models. Biomed Pharmacother. 2022;150:113022.
73. Lamarre Y, Mercier LA. Neurophysiological studies of harmaline-induced tremor in the cat. Can J Physiol Pharmacol. 1971;49(12):1049–58.
74. Ohye C, Bouchard R, Larochelle L, et al. Effect of dorsal rhizotomy on postural tremor in the monkey. Exp Brain Res. 1970;10(2):140–50.
75. Kuo SH, Louis ED, Faust PL, et al. Current opinions and consensus for studying tremor in animal models. Cerebellum. 2019;18(6):1036–63.
76. Metzer WS. Essential tremor: an overview. J Ark Med Soc. 1994;90(12):587–90.
77. Handforth A. Harmaline tremor: underlying mechanisms in a potential animal model of essential tremor. Tremor Other Hyperkinet Mov (N Y). 2012;2.
78. Gao Y, Li P, Ma LX, et al. Effects of acute administration of ethanol on experimental arrhythmia. Chin J Physiol. 2012;55(5):307–13.
79. Olsen RW, Hanchar HJ, Meera P, Wallner M. GABAA receptor subtypes: the "one glass of wine" receptors. Alcohol. 2007;41 (3):201–9.
80. de Haas SL, Zoethout RW, Van Dyck K, et al. The effects of TPA023, a GABAAalpha2,3 subtype-selective partial agonist, on essential tremor in comparison to alcohol. J Psychopharmacol. 2012;26(2):282–91.
81. Zeuner KE, Molloy FM, Shoge RO, et al. Effect of ethanol on the central oscillator in essential tremor. Mov Disord. 2003;18(11):1280–5.
82. Zoethout RW, Iannone R, Bloem BR, et al. The effects of a novel histamine-3 receptor inverse agonist on essential tremor in comparison to stable levels of alcohol. J Psychopharmacol. 2012;26(2):292–302.
83. Rappaport MS, Gentry RT, Schneider DR, Dole VP. Ethanol effects on harmaline-induced tremor and increase of cerebellar cyclic GMP. Life Sci. 1984;34(1):49–56.
84. Tambour S, Didone V, Tirelli E, Quertemont E. Locomotor effects of ethanol and acetaldehyde after peripheral and intraventricular injections in Swiss and C57BL/6J mice. Behav Brain Res. 2006;172(1):145–54.

85. Randall CL, Carpenter JA, Lester D, Friedman HJ. Ethanol-induced mouse strain differences in locomotor activity. Pharmacol Biochem Behav. 1975;3(3):533–5.
86. Misgeld U, Deisz RA, Dodt HU, Lux HD. The role of chloride transport in postsynaptic inhibition of hippocampal neurons. Science. 1986;232(4756):1413–5.
87. Wall MJ. Furosemide reveals heterogeneous GABA(A) receptor expression at adult rat Golgi cell to granule cell synapses. Neuropharmacology. 2002;43(4):737–49.
88. Accardi MV, Brown PM, Miraucourt LS, Orser BA, Bowie D. alpha6-containing GABAA receptors are the principal mediators of inhibitory synapse strengthening by insulin in cerebellar granule cells. J Neurosci. 2015;35(26):9676–88.
89. Wallner M, Hanchar HJ, Olsen RW. Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. Proc Natl Acad Sci U S A. 2003;100(25):15218–23.
90. Smith SS, Gong QH. Ethanol effects on GABA-gated current in a model of increased alpha4betadelta GABAA receptor expression depend on time course and preexposure to low concentrations of the drug. Alcohol (Fayetteville, NY). 2007;41(3):223–31.
91. Manto M, Laute MA. A possible mechanism for the beneficial effect of ethanol in essential tremor. Eur J Neurol. 2008;15 (7):697–705.
92. Kaplan JS, Mohr C, Rossi DJ. Opposite actions of alcohol on tonic GABA(A) receptor currents mediated by nNOS and PKC activity. Nat Neurosci. 2013;16(12):1783–93.
93. Wentlandt K, Kushnir M, Naus CC, Carlen PL. Ethanol inhibits gap-junctional coupling between P19 cells. Alcohol Clin Exp Res. 2004;28(9):1284–90.
94. Sieghart W, Chiou LC, Ernst M, et al. alpha6-containing GABAA receptors: functional roles and therapeutic potentials. Pharmacol Rev. 2022;74(1):238–70.
95. Handforth A, Lang EJ. Increased Purkinje cell complex spike and deep cerebellar nucleus synchrony as a potential basis for syndromic essential tremor. A review and synthesis of the literature. Cerebellum. 2021;20(2):266–81.
96. Eccles JC, Llinas R, Sasaki K. The excitatory synaptic action of climbing fibres on the Purkinje cells of the cerebellum. J Physiol. 1966;182(2):268–96.
97. Palmer LM, Clark BA, Grundemann J, et al. Initiation of simple and complex spikes in cerebellar Purkinje cells. J Physiol. 2010;588(Pt 10):1709–17.
98. Llinas R, Volkind RA. The olivo-cerebellar system: functional properties as revealed by harmaline-induced tremor. Exp Brain Res. 1973;18(1):69–87.
99. Sillitoe RV, Fu Y, Watson C. Chapter 11 - Cerebellum. In: Watson C, Paxinos G, Puelles L, editors. The mouse nervous system. San Diego: Academic Press; 2012. p. 360–97.
100. Person AL, Raman IM. Synchrony and neural coding in cerebellar circuits. Front Neural Circuits. 2012;6:97.
101. Miwa H, Kubo T, Suzuki A, Kihira T, Kondo T. A species-specific difference in the effects of harmaline on the rodent olivocerebellar system. Brain Res. 2006;1068(1):94–101.
102. Nelson BJ, Mugnaini E, editors. Origins of GABAergic inputs to the inferior olive. 1989.
103. Hirano T. GABA and synaptic transmission in the cerebellum. In: Manto M, Schmahmann JD, Rossi F, Gruol DL, Koibuchi N, editors. Handbook of the cerebellum and cerebellar disorders. Dordrecht: Springer, Netherlands; 2013. p. 881–93.
104. Najac M, Raman IM. Integration of Purkinje cell inhibition by cerebellar nucleo-olivary neurons. J Neurosci. 2015;35(2):544–9.
105. Van Loo PL, Kuin N, Sommer R, et al. Impact of "living apart together" on postoperative recovery of mice compared with social and individual housing. Lab Anim. 2007;41(4):441–55.
106. Huang W, Calvo M, Karu K, et al. A clinically relevant rodent model of the HIV antiretroviral drug stavudine induced painful peripheral neuropathy. Pain. 2013;154(4):560–75.
107. Geiszler PC, Barron MR, Pardon MC. Impaired burrowing is the most prominent behavioral deficit of aging htau mice. Neuroscience. 2016;329:98–111.
108. Baumann A, Moreira CG, Morawska MM, et al. Preliminary evidence of apathetic-like behavior in aged vesicular monoamine transporter 2 deficient mice. Front Hum Neurosci. 2016;10:587.
109. Poli A, Palermo-Neto J. Effects of d, l-propranolol on open field behavior of rats. Psychopharmacology. 1985;86(1–2):153–5.
110. Deuschl G, Raethjen J, Hellriegel H, Elble R. Treatment of patients with essential tremor. Lancet Neurol. 2011;10(2):148–61.
111. Du W, Harvey JA. Harmaline-induced tremor and impairment of learning are both blocked by dizocilpine in the rabbit. Brain Res. 1997;745(1–2):183–8.
112. Schwarz MJ, Houghton PJ, Rose S, Jenner P, Lees AD. Activities of extract and constituents of Banisteriopsis caapi relevant to parkinsonism. Pharmacol Biochem Behav. 2003;75(3):627–33.
113. Cumming P, Vincent SR. Inhibition of histamine-N-methyltransferase (HNMT) by fragments of 9-amino-1,2,3,4-tetrahydroacridine (tacrine) and by beta-carbolines. Biochem Pharmacol. 1992;44(5):989–92.
114. Treven M, Siebert DCB, Holzinger R, et al. Towards functional selectivity for alpha6beta3gamma2 GABAA receptors: a series of novel pyrazoloquinolinones. Br J Pharmacol. 2018;175(3):419–28.
115. Gironell A. The GABA hypothesis in essential tremor: lights and shadows. Tremor Other Hyperkinet Mov (N Y). 2014;4:254.
116. Zesiewicz TA, Elble R, Louis ED, et al. Practice parameter: therapies for essential tremor: report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology. 2005;64(12):2008–20.
117. Bain PG. The effectiveness of treatments for essential tremor. Neurologist. 1997;3(5):305–21.
118. Koller WC, Royse VL. Efficacy of primidone in essential tremor. Neurology. 1986;36(1):121–4.
119. Sasso E, Perucca E, Fava R, Calzetti S. Primidone in the long-term treatment of essential tremor: a prospective study with computerized quantitative analysis. Clin Neuropharmacol. 1990;13(1):67–76.
120. Winkler GF, Young RR. Efficacy of chronic propranolol therapy in action tremors of the familial, senile or essential varieties. N Engl J Med. 1974;290(18):984–8.
121. Schaefer SM, Vives Rodriguez A, Louis ED. Brain circuits and neurochemical systems in essential tremor: insights into current and future pharmacotherapeutic approaches. Expert Rev Neurother. 2018;18(2):101–10.
122. Zesiewicz TA, Elble RJ, Louis ED, et al. Evidence-based guideline update: treatment of essential tremor: report of the Quality Standards subcommittee of the American Academy of Neurology. Neurology. 2011;77(19):1752–5.
123. Levin J, Mehrkens J, Gerbes A, Botzel K. Essential tremor leading to toxic liver damage successfully treated with deep brain stimulation. Acta Neurochir (Wien). 2009;151(10):1305–7.
124. Ruedi-Bettschen D, Rowlett JK, Rallapalli S, et al. Modulation of alpha5 subunit-containing GABAA receptors alters alcohol drinking by rhesus monkeys. Alcohol Clin Exp Res. 2013;37(4):624–34.
125. Tan KR, Brown M, Labouebe G, et al. Neural bases for addictive properties of benzodiazepines. Nature. 2010;463(7282):769–74.
126. Tzeng HR, Lee MT, Fan PC, et al. alpha6GABAA receptor positive modulators alleviate migraine-like grimaces in mice via compensating GABAergic deficits in trigeminal ganglia. Neurotherapeutics. 2021;18(1):569–85.
127. Fan PC, Lai TH, Hor CC, et al. The alpha6 subunit-containing GABAA receptor: a novel drug target for inhibition of trigeminal activation. Neuropharmacology. 2018;140:1–13.

128. Vasovic D, Divovic B, Treven M, et al. Trigeminal neuropathic pain development and maintenance in rats are suppressed by a positive modulator of alpha6 GABAA receptors. Eur J Pain. 2019;23(5):973–84.
129. Meera P, Wallner M, Otis TS. Molecular basis for the high THIP/gaboxadol sensitivity of extrasynaptic GABA(A) receptors. J Neurophysiol. 2011;106(4):2057–64.
130. Wafford KA, Ebert B. Gaboxadol–a new awakening in sleep. Curr Opin Pharmacol. 2006;6(1):30–6.
131. Wisden W, Korpi ER, Bahn S. The cerebellum: a model system for studying GABAA receptor diversity. Neuropharmacology. 1996;35(9–10):1139–60.