



# Search for Novel Therapies for Essential Tremor Based on Positive Modulation of $\alpha 6$ -Containing GABA<sub>A</sub> Receptors

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ARTICLE

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## ABSTRACT

**Background:** Prior work using GABA<sub>A</sub> receptor subunit knockouts and the harmaline model has indicated that low-dose alcohol, gaboxadol, and ganaxolone suppress tremor via  $\alpha 6\beta\delta$  GABA<sub>A</sub> receptors. This suggests that drugs specifically enhancing the action of  $\alpha 6\beta\delta$  or  $\alpha 6\beta\gamma 2$  GABA<sub>A</sub> receptors, both predominantly expressed on cerebellar granule cells, would be effective against tremor. We thus examined three drugs described by *in vitro* studies as selective  $\alpha 6\beta\delta$  (ketamine) or  $\alpha 6\beta\gamma 2$  (Compound 6, flumazenil) receptor modulators.

**Methods:** In the first step of evaluation, the maximal dose was sought at which 6/6 mice pass straight wire testing, a sensitive test for psychomotor impairment. Only non-impairing doses were used to evaluate for anti-tremor efficacy in the harmaline model, which was assessed in wildtype and  $\alpha 6$  subunit knockout littermates.

**Results:** Ketamine, in maximally tolerated doses of 2.0 and 3.5 mg/kg had minimal effect on harmaline tremor in both genotypes. Compound 6, at well-tolerated doses of 1–10 mg/kg, effectively suppressed tremor in both genotypes. Flumazenil suppressed tremor in wildtype mice at doses (0.015–0.05 mg/kg) far lower than those causing straight wire impairment, and did not suppress tremor in  $\alpha 6$  knockout mice.

**Discussion:** Modulators of  $\alpha 6\beta\delta$  and  $\alpha 6\beta\gamma 2$  GABA<sub>A</sub> receptors warrant attention for novel therapies as they are anticipated to be effective and well-tolerated. Ketamine likely failed to attain  $\alpha 6\beta\delta$ -active levels. Compound 6 is an attractive candidate, but further study is needed to clarify its mechanism of action. The flumazenil results provide proof of principle that targeting  $\alpha 6\beta\gamma 2$  receptors represents a worthy strategy for developing essential tremor therapies.

## HIGHLIGHTS

We tested for harmaline tremor suppression drugs previously described as *in vitro*  $\alpha 6\beta\delta$  or  $\alpha 6\beta\gamma 2$  GABA<sub>A</sub> receptor-selective modulators. Well-tolerated flumazenil doses suppressed tremor in  $\alpha 6$ -wildtype but not  $\alpha 6$ -knockout mice. Compound 6 and ketamine failed to display this profile, likely from off-target effects. Selective  $\alpha 6$  modulators hold promise as tremor therapy.

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## INTRODUCTION

A large fraction of GABA<sub>A</sub> receptors is composed of two  $\alpha$  (of which there are 6 types), two  $\beta$  subunits (3 types), and a  $\delta$  (one type) or a  $\gamma$  subunit (3 types). Most brain GABA<sub>A</sub> receptors incorporate  $\gamma$ , and are synaptic, exerting phasic inhibition in response to GABA. Those that contain  $\delta$  are extra-synaptic, and exert tonic inhibition. In the latter receptors,  $\delta$  is usually associated with  $\alpha 4$  subunits throughout the brain, but in cerebellar granule cell (CGC) GABA<sub>A</sub> receptors,  $\alpha 6$  is the associated partner and is intensely expressed here, whereas  $\alpha 4$  levels in the cerebellum are much lower, being expressed in the Purkinje cell (PC) layer and the molecular layer in mice [1, 2]. CGCs from  $\alpha 6$  knockout (KO,  $\alpha 6^{-/-}$ ) mice lack GABA-mediated tonic inhibition [3]. The location of  $\alpha 6\beta\delta$  receptors on CGCs, where they respond to GABA released by Golgi neurons, provides a mechanism for controlling the excitatory CGC drive to PCs, which in turn are the sole output neurons for the cerebellar cortex.

The identification of new therapies for essential tremor (ET) has lagged, in part due to lack of known molecular targets. One strategy to address this problem is to “reverse engineer” a substance, such as alcohol, that reduces tremor in some ET cases, to identify molecular targets, then find more specific ligands for these targets with the goal of creating new drugs with better efficacy and tolerability. We previously found that low-dose alcohol suppresses tremor in the harmaline model of ET in wildtype (WT) mice, but fails to do so in KO littermates lacking either the  $\delta$  or the  $\alpha 6$  subunit [4]. Moreover, we found that ganaxolone and gaboxadol, which activate extra-synaptic GABA<sub>A</sub> receptors allosterically or directly respectively, also each suppresses harmaline tremor, but not if either the  $\delta$  or  $\alpha 6$  subunit is lacking [4, 5].

These observations with alcohol, well-known to suppress tremor in ET patients in low doses, are consistent with findings that low concentrations of alcohol enhance GABA-mediated currents in *Xenopus* oocytes expressing recombinant  $\alpha 6\beta\delta$  receptors, and tonic currents in CGC slices, but not if the slices are taken from  $\delta^{-/-}$  mice [6–9]. Given the predominant location of  $\alpha 6\beta\delta$  GABA<sub>A</sub> receptors on CGCs, these observations are also consistent with the finding from high-density electroencephalography (EEG) in ET subjects that tremor reduction by alcohol correlates with alterations of cerebellar activity [10], and with a blood flow study finding that a low dose of alcohol reduces the cerebellar hypermetabolism that occurs in ET towards normal levels [11, 12], an observation that suggests that drugs that do this selectively should be well-tolerated, as it is a normalizing physiological action that suppresses tremor.

Within the cerebellum,  $\alpha 6$  expression is virtually limited to the CGC layer [2]. Given the intense and relatively selective expression of  $\alpha 6\beta\delta$  receptors on CGCs, they represent a promising target for ET therapy. Unfortunately, alcohol, ganaxolone, and gaboxadol also act on  $\alpha 4\beta\delta$  GABA<sub>A</sub> receptors that are expressed throughout much of the brain, causing unwanted effects; such effects may limit the dosage of these agents that can be administered, in turn limiting the maximal efficacy that can be achieved. For example, gaboxadol at 10 mg/kg causes sedation, impaired rotarod performance, and increases tail flick latency, but not if the mice are  $\alpha 4$  KO, although in these mice the drug is free to act on  $\alpha 6\beta\delta$  GABA<sub>A</sub> receptors [13]. These observations suggest that a drug that selectively activates or positively modulates  $\alpha 6\beta\delta$  receptors without involving  $\alpha 4\beta\delta$  GABA<sub>A</sub> receptors should be well tolerated, permitting robust anti-tremor efficacy. A search for drugs that enhance  $\alpha 6\beta\delta$  receptor action selectively is justified. In addition, as CGCs express  $\alpha 6\beta\gamma 2$  GABA<sub>A</sub> receptors intensely [1], these represent an additional, potentially attractive target. Outside the cerebellum,  $\alpha 6$  is expressed in the trigeminal ganglion [14], cochlear nuclei [15, 16], and faintly in the spinal trigeminal nucleus [2]. This limited extra-cerebellar localization also suggests that selective modulation of  $\alpha 6$  GABA<sub>A</sub> receptors should be well-tolerated.

In a search for  $\alpha 6$ -selective drugs, we examined low-dose ketamine, Compound 6, and flumazenil. Ketamine has been reported to selectively enhance GABA-induced currents via *Xenopus* oocyte-expressed  $\alpha 6\beta 2\delta$  receptors, and to enhance tonic currents in CGCs in slices, but not if the slices are taken from  $\alpha 6$ - or  $\delta$ -subunit KO mice [17]. Low-dose ketamine has been reported to ameliorate Parkinson’s tremor [18]. Ketamine has significant limitations in that it is an anesthetic that has other receptor actions, nonetheless we wondered whether low doses could suppress tremor through a selective  $\alpha 6$  GABA<sub>A</sub> receptor action.

Compound 6 (PZ-II-029) is a pyrazoloquinolinone, a class of compounds that exert very little sedation [19]. In oocyte recombinant systems, Compound 6 binds to an extracellular  $\alpha$ - $\beta$  site of  $\alpha 3\beta 2\gamma 2$  receptors, displaying high selectivity for the  $\alpha 6$  subunit among  $\alpha$  subtypes. It also modulates  $\alpha 2\beta 2\gamma 2$  receptors to a lesser degree [20, 21]. In addition, it binds silently to the  $\alpha$ - $\gamma$  benzodiazepine binding site. Intraperitoneal (i.p.) injection of Compound 6 in mice blocks the disruption of prepulse inhibition caused by methamphetamine, whereas this effect of Compound 6 is prevented by intracerebellar injection of furosemide, which blocks  $\alpha 6$  GABA<sub>A</sub> receptors [22], suggesting that Compound 6 modulates  $\alpha 6\beta 2\gamma 2$  receptors *in vivo*. Limitations are that it has not been studied in receptor subunit KO mice or tissue, and the extent of non-GABA<sub>A</sub> receptor brain binding sites has not been fully delineated. Given this lack of knowledge,

there is a potential for binding to non-GABA<sub>A</sub> receptor sites to interfere with testing the hypothesis of  $\alpha 6$ -dependent tremor suppression.

Flumazenil is marketed for reversal of benzodiazepine sedation, an action that is due to its silent binding at the  $\alpha$ - $\gamma$  benzodiazepine binding site within  $\alpha\beta\gamma 2$  receptors. Flumazenil is also a partial positive modulator with some  $\alpha$ -subunit selectivity. It enhances  $\alpha 6\beta\gamma 2$ ,  $\alpha 3\beta\gamma 2$ , and  $\alpha 2\beta\gamma 2$  GABA<sub>A</sub> receptor action *in vitro* at physiologically relevant levels, but not  $\alpha 1\beta\gamma 2$  receptors [23]. In humans, intravenous flumazenil, 1 mg, reduces anxiety [24] and improves finger tapping speed in Parkinson's subjects [25], yet does not affect psychomotor performance at 0.1 mg/kg [26] or affect the EEG after 10 mg given intravenously [27]. This profile suggests that if flumazenil exerts  $\alpha 6$ -mediated tremor suppression in ET, this action may be well-tolerated. Unlike Compound 6, it is not a pyrazoloquinolinone, so that any binding to non-GABA receptor sites will differ. A potential limitation is that its positive modulation is only partial, so that maximal efficacy may not be marked.

In the present study, we sought to test the hypothesis that drugs that selectively or preferentially modulate  $\alpha 6$ -containing GABA<sub>A</sub> receptors *in vitro* will exert anti-tremor effects in the harmaline model *in vivo* at well-tolerated doses, and in  $\alpha 6$ -dependent fashion, as assessed by comparing results from WT mice with those from  $\alpha 6$  KO littermates. (The term "WT mice" is meant to indicate they possess normal copies of the gene that is deleted in KO mice.) It should be noted that in contrast to our earlier studies with alcohol, ganaxolone, and gaboxadol [4, 5], none of the three candidate drugs in the present study have had prior supportive genetic GABA<sub>A</sub> receptor animal behavior studies. Insofar as many drugs bind to multiple, often unknown, sites, binding to a non-GABA<sub>A</sub> receptor site may lead to failure to translate from *in vitro*  $\alpha 6$  activity to  $\alpha 6$ -dependent anti-tremor efficacy. For such anti-tremor efficacy to occur, each candidate drug had to pass two tests. In *Test 1*, the drug had to suppress tremor meaningfully in doses not associated with psychomotor impairment. In *Test 2*, anti-tremor efficacy that is demonstrable in WT mice must be abolished in  $\alpha 6$  KO mice. Failures may occur because non-GABA<sub>A</sub> receptor binding induces psychomotor impairment that prevents administration of doses sufficient to affect tremor, such as may happen if levels are too low to modulate  $\alpha 6$  receptors (*Test 1* failure), or because activation of a non- $\alpha 6$  GABA<sub>A</sub> receptor site suppresses tremor in  $\alpha 6$  KO mice (*Test 2* failure). On the other hand, if a given drug passes both *Test 1* and *Test 2*, so that it suppresses tremor in doses that do not cause psychomotor impairment, an effect abolished in  $\alpha 6$  KO mice, then such a finding will support the hypothesis that an  $\alpha 6$ -GABA<sub>A</sub> receptor modulating drug can suppress tremor in tolerated doses.

## METHODS

### STUDY DESIGN

We evaluated each drug in a two-step process. In the first, we sought to determine the maximum dose at which 6/6 mice pass the straight wire test, a sensitive test of psychomotor impairment [28]. For example, we previously found that the maximum dose of alcohol at which 6/6 mice pass is 0.575 g/kg, a dose estimated to produce a blood level that is below the driving level limit of 0.080 g/dL [4]. Only a dose associated with 6/6 mice passing the straight wire test or lower doses were used in step two: effect of drug on harmaline tremor. Each drug was assessed for its ability to suppress tremor in WT mice and in littermates lacking the  $\alpha 6$  receptor subunit. If a drug were to suppress tremor in WT but not in  $\alpha 6$  KO mice, that would suggest the drug suppresses tremor by activating or modulating  $\alpha 6$ -containing GABA<sub>A</sub> receptors. Each mouse received any drug or harmaline only once.

The harmaline model of ET was utilized. Harmaline elicits tremor by driving rhythmic, coupled inferior olivary bursting [29]. It is a symptom model, in which the brain areas activated during harmaline tremor overlap with the tremor circuit revealed by magnetoencephalography in ET [29–31]. This extensive circuitry overlap is consistent with considerable pharmacologic overlap, in which many drugs exert similar actions on ET and harmaline tremor [32].

Mice were assigned randomly to dosing groups, and the quantitation was performed by automated software. Animal protocols conformed to the National Institute of Health's Guide for the Care and Use of Laboratory Animals (Eighth Edition, Washington DC, from the National Research Council, published in 2011), and were approved by the Veterans Affairs Greater Los Angeles Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### ANIMALS

$\alpha 6$  KO ( $\alpha 6^{-/-}$ , *Gabra6*<sup>-/-</sup>) mice were obtained from Jackson Laboratories (strain B6;129-*Gabra6*<sup>tm1Geh</sup>/J, #002710, Bar Harbor, ME). These had been generated with a 129 × 1/SvJ × 129S1/Sv cell line inserted into a C57BL6/J blastocyst [33] and were backcrossed with  $\delta^{+/+}$  mice in our laboratory for 10 generations; the  $\delta^{+/+}$  mice had been backcrossed with C57BL/6J for 11 generations as previously described [4]. Heterozygote mice were interbred to produce offspring that were genotyped with polymerase chain reaction (Transnetyx, Memphis, TN) and  $\alpha 6^{+/+}$ ,  $\alpha 6^{-/-}$  littermates used for experiments. Both sexes were used as adults, and mice had *ad libitum* access to food and water.

## TEST PROCEDURES

*Straight wire testing.* In this test, a mouse is suspended by the front paws from a rigid wire; to pass it must stay on the wire at least 10 seconds and touch the wire with a hind paw within those 10 seconds, and do so on each test conducted at 10-minute intervals for one hour following drug administration. A given drug dose passed if all 6 of 6 tested mice passed all tests; the highest dose at which 6/6 mice passed was sought.

*Harmaline-induced tremor.* Each mouse was placed on an 8.1-cm diameter mesh on top of a 24.1-cm high cylinder that rested on a Convuls-1 Replacement Sensing Platform model 1335-1A (Columbus Instruments, Columbus, OH), fitted with a load sensor, connected to a Grass model P511 AC amplifier (Grass Instruments, West Warwick, RI) with 1 and 70 Hz filter settings. Digitally recorded motion power was analyzed using Spike2 software (Cambridge Electronic Design; UK) to perform Fourier transformation of the data into frequency spectra. Data were sampled at 128 Hz. As previously described, harmaline-induced tremor occurs at 9–16 Hz, creating a corresponding motion power peak on digital frequency spectra [34, 35]. To control for tremor power changes due to activity level fluctuation, this tremor-associated motion power bandwidth was divided by background overall activity motion power to form the measure of analysis, *motion power percentage* (MPP): (9–16 Hz motion power)/(0.25–32 Hz motion power) × 100 [35]. The use of such a ratio reduces variation compared to measuring tremor-associated motion power alone [35]. Placing each mouse on an elevated exposed small platform with intermittent rest periods in the home cage promotes vigilance and sustained tremor during motion power accession.

Mice were acclimated to the platform, then 15 minutes of pre-harmaline baseline motion data collected, then harmaline, 20 mg/kg, administered subcutaneously as 4 ml/kg. Once tremor had developed, within 10 minutes, motion power was again assessed during a 15-minute epoch. Ketamine, Compound 6, flumazenil, or corresponding vehicle was then injected i.p., 10 ml/kg. Motion power accession was re-initiated 10 minutes after injection for five more 15-minute epochs on the elevated platform (E1 to E5), with intervening 5-minute rests in the home cage.

## DRUGS

Harmaline (Sigma-Aldrich, St. Louis, MO) and ketamine (Tocris Bio-Techne, Minneapolis, MN) were dissolved in saline. Compound 6 (synthesized in the laboratory of Marko D. Mihovilovic, Vienna Technical University, Vienna, Austria as previously described [36]) was dissolved by sonicating and warming in a mixture of 85% distilled water, 14% propylene glycol, and 1% Tween 80 (Sigma-Aldrich).

Flumazenil (Tocris) was dissolved with warming in 1.25% alcohol (Thermo Fisher, Canoga Park, CA), 5% cremophor (Sigma-Aldrich), 93.75% saline with a drop of Tween 80.

## STATISTICAL ANALYSES

Mean motion power percentage (MPP) values were compared using a repeated measure (mixed) analysis of variance (ANOVA) model. A repeated measure model is needed since the same animal is measured repeatedly across 7 time periods (baseline, H, E1, E2, E3, E4, E5). Residual errors were examined using normal quantile plots (not shown) to confirm that the errors have a normal distribution, as required by this parametric model. The Shapiro-Wilk test for normality also confirmed that the errors followed a normal distribution. The model-based means and pooled standard errors (SEs) were calculated as well as p values for dose comparisons at each genotype-receptor and time and p values for genotype-receptor comparisons at each dose and time. The p values were deemed significant using the Fisher least significant difference (Fisher LSD) criterion.

Computations were carried out using R 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>).

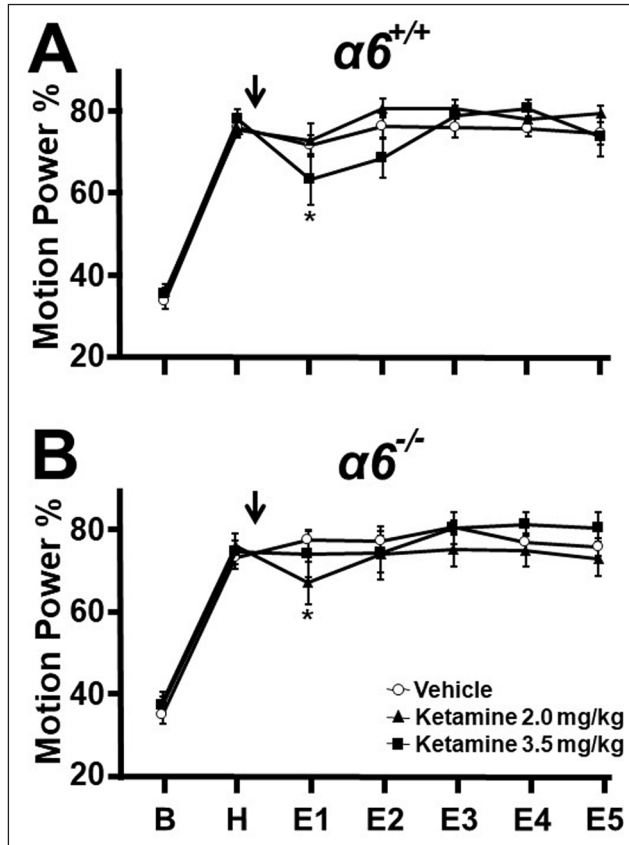
## RESULTS

### KETAMINE

The highest dose at which 6/6 passed all straight wire tests over an hour after ketamine administration was 3.5 mg/kg; at 3.75 mg/kg, not all 6 of 6 mice passed. Accordingly, the doses chosen for evaluation were 3.5 and 2.0 mg/kg.

In  $\alpha 6^{+/+}$  mice, the motion power percentage (MPP) that fell by chance within the 9–16 Hz bandwidth approximated 30–40% during the 15-minute pre-harmaline baseline (B) (Figure 1A). With harmaline administration, the MPP increased to 75–80%, so that tremor dominated motion power during the 15-minute harmaline pre-treatment epoch (H). Mice were then given vehicle or ketamine, 3.5 or 2.0 mg/kg i.p., n = 16 per group. After vehicle injection, harmaline tremor-associated elevated MPP was sustained for five more 15-minute epochs (Figure 1A). In comparison with the vehicle control group, ketamine at 3.5 mg/kg reduced tremor mildly in the first post-injection epoch (E1, vehicle vs 3.5 mg/kg means: 73.1 vs 64.6, p = 0.043), but not at 2.0 mg/kg (vehicle vs 2.0 mg/kg means: 73.1 vs 74.3, p = 0.782) or in post-injection epochs E2 to E5.

Littermate  $\alpha 6^{-/-}$  mice are indistinguishable from  $\alpha 6^{+/+}$  mice, and displayed pre-harmaline baseline and pre-treatment harmaline MPP values comparable to those of  $\alpha 6^{+/+}$  mice, indicating no alteration in harmaline tremor

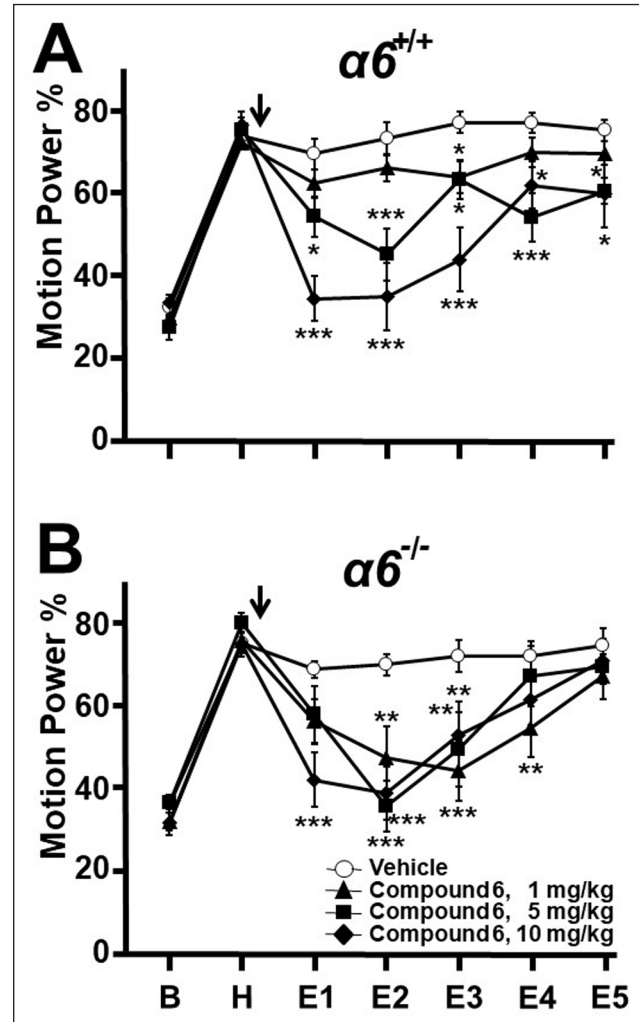


**Figure 1** Effect of ketamine on harmaline tremor in  $\alpha 6^{+/+}$  ( $\alpha 6$  WT) and littermate  $\alpha 6^{-/-}$  ( $\alpha 6$  KO) mice. Motion power in groups of mice was followed sequentially during 15-minute epochs at baseline (B), pre-treatment harmaline (H), and after vehicle or ketamine injection (arrow, E1-E5). **A.**  $\alpha 6^{+/+}$  **B.**  $\alpha 6^{-/-}$  Ketamine in doses that did not cause psychomotor impairment on the straight wire test, 3.5 and 2.0 mg/kg, had minimal effect in both genotypes compared to vehicle controls. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ANOVA with Fisher least significant difference criterion.

response. Figure 1B displays motion power in 12, 10, 10  $\alpha 6^{-/-}$  mice receiving vehicle or ketamine 2.0 or 3.5 mg/kg. The dose 2.0 mg/kg caused mild suppression during E1 (vehicle vs 2.0 mg/kg means: 77.6 vs 67.0,  $p = 0.039$ ) whereas 3.5 mg had no effect (vehicle vs 3.5 mg/kg means: 77.6 vs 74.1,  $p = 0.491$ ). No effect was seen in subsequent post-injection epochs. These findings indicate that low doses of ketamine that avoid psychomotor impairment exert only minimal effects on tremor that are transient and likely not related to the presence of the  $\alpha 6$  subunit. These results are interpreted as a Test 1 failure, in that off-target effects of drug that caused psychomotor impairment limited the testable dose to levels that did not induce meaningful tremor suppression.

## COMPOUND 6

At the highest dose tested, 20 mg/kg, all 6/6  $\alpha 6^{+/+}$  mice passed straight wire testing. Limited solubility required



**Figure 2** Effect of Compound 6 on harmaline tremor in  $\alpha 6^{+/+}$  and littermate  $\alpha 6^{-/-}$  mice. Motion power was followed sequentially during 15-minute epochs as described in Figure 1. **A.**  $\alpha 6^{+/+}$  Compound 6 in well tolerated doses suppressed tremor compared to vehicle controls. **B.**  $\alpha 6^{-/-}$  Despite lacking the  $\alpha 6$  GABA<sub>A</sub> receptor subunit, these mice also responded to Compound 6 with tremor reduction. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ANOVA with Fisher least significant difference criterion.

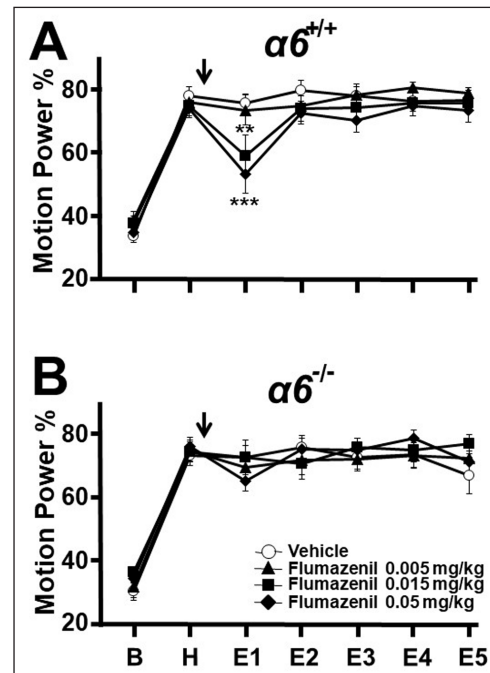
that this dose be given as 20 ml/kg. Higher doses could not be given as this would have exceeded permissible limits on injection volumes. Following the harmaline pre-treatment epoch (H),  $\alpha 6^{+/+}$  mice were administered vehicle or Compound 6 in doses of 1, 5 and 10 mg/kg, all as 10 ml/kg ( $n = 16, 17, 11, 11$  respectively). Compound 6 exerted tremor suppression that was dose-related (Figure 2A). At 10 mg/kg, Compound 6 suppressed tremor markedly, with effects seen in post-injection epochs E1 to E5 (vehicle vs 10 mg/kg means: 69.3 vs 34.0,  $p < 0.0001$ ; 73.3 vs 34.4,  $p < 0.0001$ ; 77.0 vs 43.5,  $p < 0.0001$ ; 77.0 vs 61.7,  $p = 0.019$ ; 75.1 vs 59.6,  $p = 0.039$  respectively). At 5 mg/kg, tremor suppression was less marked but

statistically significant from E1 to E5 (vehicle vs 5 mg/kg means: 69.3 vs 54.0,  $p = 0.019$ ; 73.3 vs 44.8,  $p < 0.0001$ ; 77.0 vs 62.9,  $p = 0.030$ ; 77.0 vs 53.7,  $p < 0.0005$ ; 75.1 vs 60.3,  $p = 0.036$  respectively). At 1 mg/kg, mild tremor suppression occurred during E3 only (vehicle vs 1 mg/kg means; 77.0 vs 63.7,  $p = 0.022$ ).

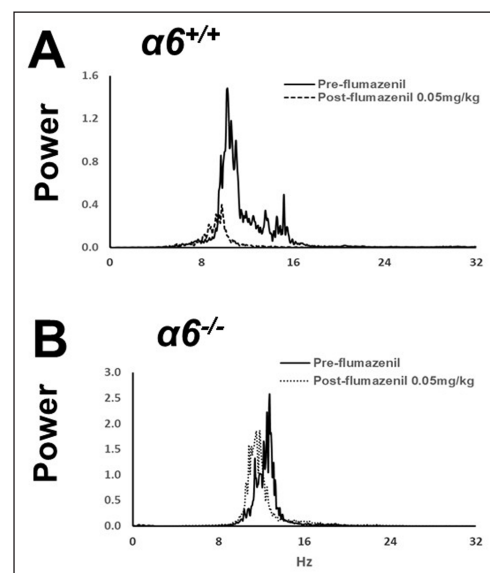
Littermate  $\alpha 6^{-/-}$  mice received vehicle or Compound 6 in doses of 1, 5, 10 mg/kg ( $n = 11, 14, 11, 11$ ). Although prior *in vitro* studies indicated a selective  $\alpha 6$  action,  $\alpha 6^{-/-}$  mice receiving Compound 6 displayed significant tremor suppression (Figure 2B). The dose 10 mg/kg suppressed tremor markedly in E1 to E3 (vehicle vs 10 mg/kg means: 68.6 vs 42.0,  $p = 0.0002$ ; 69.6 vs 38.8,  $p < 0.0001$ ; 71.7 vs 52.8,  $p = 0.0079$  respectively), while 5 mg/kg reduced tremor less markedly in E2 and E3 (vehicle vs 5 mg/kg means: 69.6 vs 35.6,  $p < 0.0001$ ; 71.7 vs 49.3,  $p = 0.0018$  respectively), while 1 mg/kg suppressed tremor in E2 to E4 (vehicle vs 1 mg/kg means: 69.6 vs 47.2,  $p = 0.0010$ ; 71.7 vs 44.3,  $p < 0.0001$ ; 71.7 vs 54.3,  $p = 0.0099$  respectively). In between-genotype comparisons, the MPP values for each epoch E1 to E5 did not differ significantly between KO and WT mice at 5 and 10 mg/kg, but were lower during E2 to E4 at 1 mg/kg in KO mice (KO vs WT means: 47.2 vs 65.9,  $p = 0.0021$ ; 44.3 vs 63.7,  $p = 0.0014$ ; 54.3 vs 69.7,  $p = 0.0104$  respectively). These results indicate that in  $\alpha 6^{-/-}$  mice, Compound 6 at 5 and 10 mg/kg suppresses tremor to a degree comparable to that seen in  $\alpha 6^{+/+}$  mice, but at 1 mg/kg exerts more tremor suppression in the KO mice. The failure to show abolition of tremor suppression in KO mice represents a Test 2 failure, in that specificity of the anti-tremor action to the  $\alpha 6$  receptor was not demonstrated.

## FLUMAZENIL

At 112.5 mg/kg, all 6/6  $\alpha 6^{+/+}$  mice passed straight wire testing, whereas not all passed at 125 mg/kg. Pilot testing indicated that far lower doses were associated with tremor reduction. In harmaline tremor experiments,  $\alpha 6^{+/+}$  mice were injected with vehicle or flumazenil, 0.005, 0.015, or 0.05 mg/kg ( $n = 12, 10, 11, 12$ ). Any effect on tremor was expected only in post-injection epoch E1, as flumazenil is cleared rapidly. It was found that flumazenil displayed dose-dependent tremor suppression, with 0.05 and 0.015 mg/kg moderately suppressing tremor during E1 (vehicle vs dose means: 75.6 vs 53.1,  $p < 0.0001$ ; 75.6 vs 59.0,  $p = 0.0016$  respectively), while 0.005 mg/kg had no significant effect (Figure 3A). Tremor during subsequent post-injection epochs was not affected by flumazenil. A display of motion power according to motion frequency is shown in Figure 4A for an example  $\alpha 6^{+/+}$  mouse during harmaline tremor before and after treatment with flumazenil, 0.05 mg/kg, in E1 and demonstrates suppression of the motion power peak associated with tremor.



**Figure 3** Effect of flumazenil on harmaline tremor in  $\alpha 6^{+/+}$  and littermate  $\alpha 6^{-/-}$  mice. Motion power was followed sequentially during 15-minute epochs as in Figure 1. **A.**  $\alpha 6^{+/+}$  Flumazenil in well tolerated doses suppressed tremor dose-dependently compared to vehicle controls. **B.**  $\alpha 6^{-/-}$  Littermates lacking the  $\alpha 6$  GABA<sub>A</sub> receptor subunit did not respond to flumazenil with tremor reduction. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , ANOVA with Fisher least significant difference criterion.



**Figure 4** Examples of motion power spectra of harmaline tremor in  $\alpha 6^{+/+}$  and  $\alpha 6^{-/-}$  mice before and after receiving flumazenil, 0.05 mg/kg i.p. **A.**  $\alpha 6^{+/+}$  In this example, the dominant motion power peak corresponds to harmaline-induced tremor. This peak is reduced upon administration of flumazenil. **B.**  $\alpha 6^{-/-}$  This example shows a comparable harmaline-associated motion power peak. In the absence of the  $\alpha 6$  subunit, flumazenil had little effect on this motion power peak.

When  $\alpha 6^{-/-}$  littermate mice were similarly treated with vehicle or flumazenil, 0.005, 0.015, or 0.05 mg/kg ( $n = 12, 12, 12, 14$ ), there was no significant reduction of tremor during E1 or other epochs at any dose (Figures 3B, 4B), indicating that flumazenil requires the presence of  $\alpha 6$ -containing GABA<sub>A</sub> receptors to exert tremor suppression. In summary, flumazenil passed both Test 1 and Test 2, displaying anti-tremor efficacy at well-tolerated doses and a requirement for  $\alpha 6$  GABA<sub>A</sub> receptors. In light of prior evidence that flumazenil exerts modulation of  $\alpha 6$  GABA<sub>A</sub> receptors *in vitro*, these findings support the hypothesis that drugs with such a profile may suppress tremor in well-tolerated doses.

## DISCUSSION

We previously found that gaboxadol, low-dose ethanol, and the neurosteroid ganaxolone each suppresses harmaline tremor in doses that do not cause impairment on the straight wire test, but had no effect on tremor in  $\delta$  KO or in  $\alpha 6$  KO mice [4, 5], suggesting that these drugs acted on  $\alpha 6\beta\delta$  GABA<sub>A</sub> receptors, most likely on CGCs, where they are intensely expressed. However, the concurrent activation or modulation of  $\alpha 4\beta\delta$  GABA<sub>A</sub> receptors that are expressed in numerous brain sites outside the cerebellum compromises the potential utility of these agents, as their enhanced action would induce unwanted effects. For example, doses of gaboxadol higher than 3 mg/kg could not be tested on tremor, as they caused impairment on straight wire testing [5]. Doses of 4–5 mg/kg affect the EEG in mice, but not in  $\delta$  KO mice [37], an effect likely mediated by  $\alpha 4\beta\delta$  GABA<sub>A</sub> receptors in the cerebral cortex.

We considered low-dose ketamine as a candidate drug of interest. Hevers et al [17]. reported that ketamine enhances GABA-induced currents by  $\alpha 6\beta 2/3\delta$  receptors, with specificity for the  $\alpha 6$ ,  $\beta 2$ ,  $\beta 3$ , and  $\delta$  subunits. Moreover, they found that ketamine induced tonic currents in CGCs, an action that depended on  $\alpha 6$  and  $\delta$  subunits [17]. Ketamine's most-studied action is blockade of N-methyl-D-aspartate (NMDA) receptors [38, 39]. However, Hevers et al. argued that the action of ketamine on CGCs may be clinically relevant, citing evidence that modulation of GABA<sub>A</sub> receptors occurs at levels below those that block NMDA receptors [17]. We therefore examined whether subanesthetic, non-impairing doses could suppress tremor, and do so in an  $\alpha 6$ -selective fashion. We found that the highest dose passed by all WT mice on the straight wire test was 3.5 mg/kg. At this dose or at 2.0 mg/kg there was only minimal and transient suppression of harmaline tremor in  $\alpha 6$  WT and KO littermates. Ketamine has multiple actions, as summarized by Hevers et al [17]

and it appears that these effects permitted only very low doses to be assessed that do not cause psychomotor impairment. Based on a pharmacokinetic study of ketamine administered to mice [40], it can be estimated that a dose of 3.5 mg/kg i.p. will result in a brain level of 1.6  $\mu$ M, well below the range of 10–100  $\mu$ M found by Hevers et al [17]. to enhance GABA currents via  $\alpha 6\beta 2\delta$  receptors in oocytes. The lack of efficacy by ketamine thus represents a Test 1 failure. Nonetheless, these receptors remain an inviting therapeutic target awaiting a selective positive allosteric modulator.

Pyrazoloquinolinones have been investigated as potential anxiolytics with an exceptionally low potential for sedation [19]. Certain members of this class are functionally selective for  $\alpha 6\beta 2/3\gamma 2$  receptors in recombinant systems, including Compound 6 [21]. It binds silently to the  $\alpha$ - $\gamma$  benzodiazepine site, consistent with the finding that, at well-tolerated doses, it blocks the ataxic effect of the benzodiazepine diazepam in rats [41]. Through its binding to an extracellular  $\alpha$ - $\beta$  site on  $\alpha 6\beta 3\gamma 2$  receptors, it is highly selective for the  $\alpha 6$  subunit over other  $\alpha$  subunit types. It also modulates  $\alpha 6\beta 2\gamma 2$  receptors to a lesser degree [21]. A cerebellar site of action has been inferred by Chiou et al., [22] who showed that intracerebellar injection of the  $\alpha 6$ -containing receptor antagonist furosemide prevents systemically administered Compound 6 (3 or 10 mg/kg) from blocking methamphetamine-induced prepulse inhibition disruption. A deuterated analog of Compound 6 has been found effective in a rat model of trigeminal neuralgia [42], possibly due to modulation of  $\alpha 6\beta 3\gamma 2$  GABA<sub>A</sub> receptors located in certain cells of the trigeminal ganglion [14]. Comparison of results with  $\alpha 6$  KO mice has not previously been described.

Due to limitations of solubility and permissible volumes of injection, 20 mg/kg was the highest dose that could be tested on straight wire testing; at this dose all 6/6 mice passed. Thus the dose that causes psychomotor impairment is not known, but is more than 20 mg/kg in mice. In harmaline tremor studies with WT mice, Compound 6 exerted dose-dependent tremor suppression at 1–10 mg/kg. These doses are comparable to those employed by Chiou et al [22]. We found that  $\alpha 6$  KO mice displayed comparable tremor suppression at 5 and 10 mg/kg and more tremor suppression than WT mice at 1 mg/kg. In interpreting these results, a consideration is whether Compound 6 might affect the compensating changes present in  $\alpha 6$  KO mice. In these mice, 50% of cerebellar GABA<sub>A</sub> receptors are lost, including both synaptic and extra-synaptic receptors [43, 44]. Yet  $\alpha 6$  KO mice exhibit no motor deficits [43, 44], and have normal harmaline tremor. Voltage-independent potassium conductance is upregulated in CGCs of these mice, appearing to underlie

at least part of the compensation [3]. Other, unknown, compensations may also be present in  $\alpha 6$  KO mice.

In summary, the results with Compound 6 represent an instance of Test 2 failure, in which  $\alpha 6$  KO mice have failed to lose the tremor suppression seen in WT littermates, so that the drug cannot be concluded to suppress tremor by an  $\alpha 6$ -dependent action. This may be secondary to an action by Compound 6 on a non-GABA<sub>A</sub> receptor target, either in  $\alpha 6$  KO mice alone, or in both WT and KO mice. Further work is needed to understand Compound 6's *in vivo* mechanism of action, but its high anti-tremor efficacy at well-tolerated doses suggests that this compound and others of its class hold promise for ET.

Flumazenil is a near silent binder with antagonistic effects at diazepam sensitive (DS)  $\alpha$ - $\gamma$  benzodiazepine binding sites, and acts as a positive GABA<sub>A</sub> receptor modulator in diazepam-insensitive  $\alpha 6\beta 3\gamma 2$  receptors [23]. In humans, it exerts various therapeutic effects in doses well below those associated with impairment [24–27]. Flumazenil is short-acting, with an elimination half-life in humans of 70 minutes [27].

We found that 112.5 mg/kg was the highest dose passed on the straight wire test by 6/6 mice. In harmaline experiments, doses of 0.05 and 0.015 mg/kg were found to suppress tremor in WT mice during the first post-injection epoch. In contrast,  $\alpha 6$  KO mice displayed no tremor suppression in response to flumazenil. These results suggest that flumazenil suppresses tremor by modulating  $\alpha 6\beta \gamma 2$  GABA<sub>A</sub> receptors on CGCs, and does so with striking tolerability, so that doses 2000 times higher than anti-tremor doses are required to cause psychomotor impairment. Although flumazenil is a positive modulator of  $\alpha 2\beta \gamma 2$  and  $\alpha 3\beta \gamma 2$  GABA<sub>A</sub> receptors [23], CGCs do not express these receptors [1], and flumazenil lacks activity for  $\alpha 1\beta \gamma 2$  receptors [23], so that the ability of this drug to suppress tremor in WT but not in  $\alpha 6$  KO mice is consistent with an action on CGC  $\alpha 6\beta \gamma 2$  receptors.

In summary, flumazenil passed Tests 1 and 2, displaying tremor suppression in highly tolerated doses in WT mice, but not in  $\alpha 6$  KO littermates. These findings, combined with the *in vitro* evidence, support the hypothesis that  $\alpha 6$  GABA<sub>A</sub> receptor-selective medications can suppress tremor effectively in well-tolerated doses, providing that off-target binding does not interfere. Flumazenil is the fourth drug that we have found to suppress tremor in  $\alpha 6$ -dependent fashion, the others being low-dose alcohol, ganaxolone, and gaboxadol [4, 5], but the latter three modulate  $\alpha 6\beta \delta$  and also  $\alpha 4\beta \delta$  GABA<sub>A</sub> receptors, so that achievable doses are limited by psychomotor effects, whereas flumazenil's relative selective action on  $\alpha 6\beta \gamma 2$  receptors enables a favorable tolerability/efficacy profile. Flumazenil is on the market, but its rapid clearance would require that

a prolonged delivery system be used or the compound undergo molecular modification to slow clearance if it were to be used as ET therapy. Another potential limitation is that in the harmaline model, tremor suppression was only moderate, likely due to a positive allosteric modulatory effect on  $\alpha 6\beta \gamma 2$  GABA<sub>A</sub> receptors that is partial [23]. Thus a compound with stronger positive modulation at  $\alpha 6\beta \gamma 2$  receptors, and longer duration of action, may possess greater anti-tremor efficacy. It may also be noted that patients treated chronically with flumazenil (or Compound 6) could not be administered benzodiazepines due to the blockade of the GABA<sub>A</sub> receptor benzodiazepine binding site by these drugs.

How would positive modulation of CGC  $\alpha 6$  GABA<sub>A</sub> receptors suppress tremor? As summarized in a review [29], it is postulated that the level of CGC parallel fiber-induced PC simple spike (SS) activity affects the synchrony of PC complex spike (CS) firing, deep cerebellar nucleus (DCN) burst-firing synchrony, downstream thalamic synchrony, and thus tremor. CSs are spike bursts triggered at inferior olivary (IO) climbing fiber synapses on PCs [45]. CSs among multiple PCs are synchronized by coupled clusters of projecting IO neurons [46], so that the degree of PC CS synchrony is controlled by the degree of IO coupling. The convergent action of synchronized PC CSs potentially inhibits DCN neurons [47, 48], provoking hyperpolarization-induced rebound bursting [49] that is transmitted to the thalamus; thus the degree of PC CS synchrony is important for movement amplitude and tremor. When IO coupling is increased by local injection of the GABA<sub>A</sub> receptor antagonist picrotoxin, increased PC CS synchrony and increased movement amplitude ensues [50]; and, in some animals, tremor occurs [51]. Likewise, systemic harmaline and intra-IO serotonin receptor 2a agonists increase IO coupling [52–54], increase PC CS synchrony [54, 55], and induce tremor [53, 56]. Larger ensembles of IO increase PC CS synchrony [54, 55], and induce tremor [53, 56]. In contrast, intra-IO GABA release inhibits coupling, thereby reducing PC CS synchrony [51, 57]. The main source of GABA in the IO is the massive GABAergic projection from DCN [58]. These IO-projecting DCN neurons in turn are inhibited by GABA released by PC terminals as PCs engage in SS activity [59, 60]. These structures thus form a tri-synaptic circuit, in which PCs that respond with SSs to CGC parallel fibers project GABAergic fibers to DCN neurons that in turn project GABAergic fibers to IO neurons that control PC CS synchrony within the same territory affected by parallel fiber input. Application of the GABA<sub>A</sub> receptor agonist muscimol to rat cerebellar cortex reduces PC SS firing, disinhibiting DCN neurons so that they release more GABA within IO, reducing coupling and therefore PC CS synchrony [61]. As we postulate that excess PC CS synchrony may be associated with tremor [28], such



DRUG	SUPPRESSED TREMOR WELL IN		PASSED		INTERPRETATION
	$\alpha 6^{+/+}$	$\alpha 6^{-/-}$	TEST 1	TEST 2	
Ketamine	No	No	No	NA	Binding to non-GABA <sub>A</sub> sites prevented testing of doses high enough to affect $\alpha 6$ receptors.
Compound 6	Yes	Yes	Yes	No	By suppressing tremor in $\alpha 6^{-/-}$ mice, it failed to display $\alpha 6$ selectivity, likely by binding to an unknown non-GABA <sub>A</sub> receptor site.
Flumazenil	Yes	No	Yes	Yes	This result supports the hypothesis that a drug with $\alpha 6$ GABA <sub>A</sub> receptor selectivity <i>in vitro</i> may suppress tremor with $\alpha 6$ selectivity.

**Table 1** Outcomes of tremor testing in drugs displaying *in vitro*  $\alpha 6$  GABA<sub>A</sub> receptor selectivity.

an action, as with a modulatory drug action on  $\alpha 6\beta 3\delta$  or  $\alpha 6\beta 3\gamma 2$  GABA<sub>A</sub> receptors on CGCs, would be to exert anti-tremor effects. This conceptualization posits that increased PC CS synchrony underlies tremor. That this is the case is suggested by the finding that ET cases display cerebellar oscillations in field potential recordings [62]. These also occur in the *hotfoot17* mouse model, which displays pathologic features of ET, and are synchronous with olivary firing [62]. The *hotfoot17* and harmaline models of tremor share a number of features, including the required integrity of IO, climbing fiber-PC synapses, and of PC-DCN synapses [29, 62].

A potential limitation in this work is that we did not confirm the lack of  $\alpha 6$  gene product in  $\alpha 6$  KO mice with Western blot. However, this strain has long been established [33], and in contrast to WT littermates these mice were found to lack tremor suppression in response to alcohol, gaboxadol, ganaxolone, and in the present experiments, to flumazenil. It is thus unlikely that the results seen with Compound 6 and ketamine in KO mice were due to preserved  $\alpha 6$  expression.

Another potential limitation is that the harmaline model may potentially produce a false positive result. The circuit activated by harmaline matches the overall circuit activated in ET as revealed by magnetoencephalography [30], including DCN, thalamus, motor cortex, and cerebellar cortex (PCs, CGCs), and brainstem [11, 29, 31, 63]. Thalamic deep brain stimulation suppresses harmaline tremor as well as tremor in ET [64]. The shared anatomy in the model and in ET is in concordance with a similar pharmacologic response profile [32]. Indeed, the harmaline model has successfully predicted clinical efficacy by 1-octanol [65, 66], T-type calcium channel antagonists [34, 67, 68], and AMPA receptor antagonists [69, 70] for ET. However, there is some non-overlap between ET and harmaline model circuitry, as some drugs affect harmaline tremor that are not clinically effective, such as dopaminergic drugs [32].

In conclusion, we examined three compounds for anti-tremor efficacy based on their described *in vitro* activity as positive modulators of  $\alpha 6$  GABA<sub>A</sub> receptors. The prediction that this approach would be fruitful in identifying new anti-tremor compounds was based on our previous findings that low-dose alcohol, ganaxolone, and gaboxadol depend on  $\alpha 6$  GABA<sub>A</sub> receptors for anti-tremor efficacy. However, *in vitro* activity on  $\alpha 6$  receptors may fail to translate into an  $\alpha 6$ -dependent action on tremor if the tolerated dose is too low to affect  $\alpha 6$  receptors and tremor (Test 1 failure), or if the compound acts on another, unknown target to affect tremor in non- $\alpha 6$ -dependent fashion (Test 2 failure). Table 1 summarizes the present findings. Ketamine was tolerated only in low doses, so that brain levels likely did not achieve levels of 10–100  $\mu$ M needed to modulate  $\alpha 6$  receptors [16], leading to failure to display anti-tremor efficacy. An  $\alpha 6\beta 2/3\delta$ -selective positive modulator of GABA<sub>A</sub> receptors remains to be identified, but is an attractive target for tremor suppression. Compound 6, a pyrazoloquinolinone, suppressed tremor in both  $\alpha 6^{+/+}$  and  $\alpha 6^{-/-}$  mice, and was thus a Test 2 failure by not showing an  $\alpha 6$ -dependent action. Nonetheless, it exerted robust tremor suppression at well-tolerated doses, suggesting it may have potential clinical utility. Flumazenil's tremor suppression was  $\alpha 6$ -dependent, and displayed a very high behavioral toxicity to tremor efficacy ratio. The flumazenil findings support the hypothesis that an  $\alpha 6$  GABA<sub>A</sub> receptor-selective modulator may suppress tremor at well-tolerated doses, as long as its binding profile exhibits freedom from interfering effects of off-target binding. These results suggest that a continued search for GABA<sub>A</sub>  $\alpha 6\beta 3\delta$ - and  $\alpha 6\beta 3\gamma 2$ -receptor-selective positive modulators as potential novel therapies for ET is justified.

## DATA ACCESSIBILITY STATEMENTS

Data are available from the corresponding author upon reasonable request.

## ETHICS AND CONSENT

Statement of Human and Animal Rights: All performed experiments conformed to the National Institute of Health's Guide for the Care and Use of Laboratory Animals (Eighth Edition, Washington DC, from the National Research Council, published in 2011), in protocols approved by the Veterans Affairs Greater Los Angeles Healthcare System Institutional Animal Care and Use Committee.

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## COMPETING INTERESTS


The authors have no competing interests to declare.

## AUTHOR CONTRIBUTIONS

A Handforth, M Ernst, M Treven and R Singh participated in the conception of the research project, and A Handforth and R Singh participated in the organization and execution of the project, the statistical analysis, and manuscript preparation. M Ernst and M Treven contributed to manuscript preparation.

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## REFERENCES

1. **Pörtl A, Hauer B, Fuchs K, Tretter V, Sieghart W.** Subunit composition and quantitative importance of GABA(A) receptor subtypes in the cerebellum of mouse and rat. *J Neurochem.* 2003; 87: 1444–1455. DOI: <https://doi.org/10.1046/j.1471-4159.2003.02135.x>
2. **Hortnagl H, Tasan RO, Wieselthaler A, Kirchmair E, Sieghart W, Sperk G.** Patterns of mRNA and protein expression for 12 GABA<sub>A</sub> receptor subunits in the mouse brain. *Neuroscience.* 2013; 236: 345–372. DOI: <https://doi.org/10.1016/j.neuroscience.2013.01.008>
3. **Brickley SG, Revilla V, Cull-Candy SG, Wisden W, Farrant M.** Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature.* 2001; 409: 88–92. DOI: <https://doi.org/10.1038/35051086>
4. **Handforth A, Kosoyan HP, Kadam PA, Singh RP.** Alcohol and ganaxolone suppress tremor via extra-synaptic GABA<sub>A</sub> receptors in the harmaline Model of essential tremor. *Tremor Other Hyperkinet Mov (N Y).* 2023; 13: 18. DOI: <https://doi.org/10.5334/tohm.760>
5. **Handforth A, Kadam PA, Kosoyan HP, Eslami P.** Suppression of harmaline tremor by activation of an extrasynaptic GABA<sub>A</sub> receptor: implications for essential tremor. *Tremor Other Hyperkinet Mov (N Y).* 2018; 8: 546. DOI: <https://doi.org/10.5334/tohm.407>
6. **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: 15218–15223. DOI: <https://doi.org/10.1073/pnas.2435171100>
7. **Hanchar HJ, Dodson PD, Olsen RW, Otis TS, Wallner M.** Alcohol-induced motor impairment caused by increased extrasynaptic GABA<sub>A</sub> receptor activity. *Nat Neurosci.* 2005; 8: 339–345. DOI: <https://doi.org/10.1038/nn1398>

8. **Wallner M, Olsen RW.** Physiology and pharmacology of alcohol: the imidazobenzodiazepine alcohol antagonist site on subtypes of GABA<sub>A</sub> receptors as an opportunity for drug development? *Br J Pharmacol.* 2008; 154: 2882-98. DOI: <https://doi.org/10.1038/bjp.2008.32>
9. **Santhakumar V, Meera P, Karakossian MH, Otis TS.** A reinforcing circuit action of extrasynaptic GABA<sub>A</sub> receptor modulators on cerebellar granule cell inhibition. *PLoS One.* 2013; 8: e72976. DOI: <https://doi.org/10.1371/journal.pone.0072976>
10. **Pedrosa DJ, Nelles C, Brown P, Volz LJ, Pelzer EA, Tittgemeyer M,** et al. The differentiated networks related to essential tremor onset and its amplitude modulation after alcohol intake. *Exp Neurol.* 2017; 297: 50–61. DOI: <https://doi.org/10.1016/j.expneurol.2017.07.013>
11. **Boecker H, Wills AJ, Ceballos-Baumann A, Samuel M, Thompson PD, Findley LJ** et al. The effect of ethanol on alcohol-responsive essential tremor: a positron emission tomography study. *Ann Neurol.* 1996; 39: 650–658. DOI: <https://doi.org/10.1002/ana.410390515>
12. **Jenkins IH, Bain PG, Colebatch JG, Thompson PD, Findley LJ, Frackowiak RS,** et al. A positron emission tomography study of essential tremor: evidence for overactivity of cerebellar connections. *Ann Neurol.* 1993; 34: 82–90. DOI: <https://doi.org/10.1002/ana.410340115>
13. **Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF,** et al. GABA<sub>A</sub> receptor  $\alpha 4$  subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci U S A.* 2006; 103: 15230–15235. DOI: <https://doi.org/10.1073/pnas.0604304103>
14. **Hayasaki H, Sohma Y, Kanbara K, Maemura K, Kubota T, Watanabe M.** A local GABAergic system within rat trigeminal ganglion cells. *Eur J Neurosci.* 2006; 23: 745–757. DOI: <https://doi.org/10.1111/j.1460-9568.2006.04602.x>
15. **Campos ML, de Cabo C, Wisden W, Juiz JM, Merlo D.** Expression of GABA<sub>A</sub> receptor subunits in rat brainstem auditory pathways: cochlear nuclei, superior olivary complex and nucleus of the lateral lemniscus. *Neuroscience.* 2001; 102: 625–638. DOI: [https://doi.org/10.1016/S0306-4522\(00\)00525-X](https://doi.org/10.1016/S0306-4522(00)00525-X)
16. **Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G.** GABA<sub>A</sub> receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience.* 2000; 101: 815–850. DOI: [https://doi.org/10.1016/S0306-4522\(00\)00442-5](https://doi.org/10.1016/S0306-4522(00)00442-5)
17. **Hevers W, Hadley SH, Lüddens H, Amin J.** Ketamine, but not phencyclidine, selectively modulates cerebellar GABA<sub>A</sub> receptors containing  $\alpha 6$  and  $\delta$  subunits. *J Neurosci.* 2008; 28: 5383–5393. DOI: <https://doi.org/10.1523/JNEUROSCI.5443-07.2008>
18. **Wright JJ, Goodnight PD, McEvoy MD.** The utility of ketamine for the preoperative management of a patient with Parkinson's disease. *Anesth Analg.* 2009; 108: 980–982. DOI: <https://doi.org/10.1213/ane.0b013e3181924025>
19. **Williams M, Bennett DA, Loo PS, Braunwalder AF, Amrick CL, Wilson DE,** et al. CGS 20625, a novel pyrazolopyridine anxiolytic. *J Pharmacol Exp Ther.* 1989; 248: 89–96.
20. **Treven M, Siebert DCB, Holzinger R, Bampali K, Fabjan J, Varagic Z,** et al. Towards functional selectivity for  $\alpha 6\beta 3\gamma 2$  GABA<sub>A</sub> receptors: a series of novel pyrazoloquinolinones. *Br J Pharmacol.* 2018; 175: 419–428. DOI: <https://doi.org/10.1111/bph.14087>
21. **Varagic Z, Ramerstorfer J, Huang S, Rallapalli S, Sarto-Jackson I, Cook J,** et al. Subtype selectivity of  $\alpha + \beta$ - site ligands of GABA<sub>A</sub> receptors: identification of the first highly specific positive modulators at  $\alpha 6\beta 2/3\gamma 2$  receptors. *Br J Pharmacol.* 2013; 169: 384–399. DOI: <https://doi.org/10.1111/bph.12153>
22. **Chiou LC, Lee HJ, Ernst M, Huang WJ, Chou JF, Chen HL,** et al. Cerebellar  $\alpha 6$ -subunit-containing GABA<sub>A</sub> receptors: a novel therapeutic target for disrupted prepulse inhibition in neuropsychiatric disorders. *Br J Pharmacol.* 2018; 175: 2414–2427. DOI: <https://doi.org/10.1111/bph.14198>
23. **Ramerstorfer J, Furtmüller R, Vogel E, Huck S, Sieghart W.** The point mutation gamma 2F77I changes the potency and efficacy of benzodiazepine site ligands in different GABA<sub>A</sub> receptor subtypes. *Eur J Pharmacol.* 2010; 636: 18–27. DOI: <https://doi.org/10.1016/j.ejphar.2010.03.015>
24. **Kapczinski F, Curran HV, Gray J, Lader M.** Flumazenil has an anxiolytic effect in simulated stress. *Psychopharmacology (Berl).* 1994; 114: 187–189. DOI: <https://doi.org/10.1007/BF02245463>
25. **Ondo WG, Silay YS.** Intravenous flumazenil for Parkinson's disease: a single dose, double blind, placebo controlled, cross-over trial. *Mov Disord.* 2006; 21: 1614–1617. DOI: <https://doi.org/10.1002/mds.21022>
26. **Forster A, Crettenand G, Klopfenstein CE, Morel DR.** Absence of agonist effects of high-dose flumazenil on ventilation and psychometric performance in human volunteers. *Anesth Analg.* 1993; 77: 980–984. DOI: <https://doi.org/10.1213/00000539-199311000-00018>
27. **Breimer LT, Hennis PJ, Burm AG, Danhof M, Bovill JG, Spierdijk J,** et al. Pharmacokinetics and EEG effects of flumazenil in volunteers. *Clin Pharmacokinet.* 1991; 20: 491–496. DOI: <https://doi.org/10.2165/00003088-199120060-00005>
28. **Vanover KE, Suruki M, Robledo S, Huber M, Wieland S, Lan NC,** et al. Positive allosteric modulators of the GABA(A) receptor: differential interaction of benzodiazepines and neuroactive steroids with ethanol. *Psychopharmacology (Berl).* 1999; 141: 77–82. DOI: <https://doi.org/10.1007/s002130050809>
29. **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: 266–281. DOI: <https://doi.org/10.1007/s12311-020-01197-5>
30. **Schnitzler A, Münks C, Butz M, Timmermann L, Gross J.** Synchronized brain network associated with essential tremor as revealed by magnetoencephalography. *Mov Disord*. 2009; 24: 1629–1635. DOI: <https://doi.org/10.1002/mds.22633>
  31. **Batini C, Buisseret-Delmas C, Conrath-Verrier M.** Harmaline-induced tremor. I. Regional metabolic activity as revealed by [<sup>14</sup>C]2-deoxyglucose in cat. *Exp Brain Res*. 1981; 42: 371–382. DOI: <https://doi.org/10.1007/BF00237502>
  32. **Handforth A.** Harmaline tremor: underlying mechanisms in a potential animal model of essential tremor. *Tremor Other Hyperkinet Mov (N Y)*. 2012; 2: 02-92-769-1. DOI: <https://doi.org/10.5334/tohm.108>
  33. **Homanics GE, Ferguson C, Quinlan JJ, Daggett J, Snyder K, Lagenaur C,** et al. Gene knockout of the alpha6 subunit of the gamma-aminobutyric acid type A receptor: lack of effect on responses to ethanol, pentobarbital, and general anesthetics. *Mol Pharmacol*. 1997; 51: 588–596. DOI: <https://doi.org/10.1124/mol.51.4.588>
  34. **Handforth A, Homanics GE, Covey DF, Krishnan K, Lee JY, Sakimura K,** et al. T-type calcium channel antagonists suppress tremor in two mouse models of essential tremor. *Neuropharmacology*. 2010; 59: 380–387. DOI: <https://doi.org/10.1016/j.neuropharm.2010.05.012>
  35. **Martin FC, Le AT, Handforth A.** Harmaline-induced tremor as a pre-clinical screening method for potential essential tremor medications. *Mov Disord*. 2005; 20: 298–305. DOI: <https://doi.org/10.1002/mds.20331>
  36. **Simeone X, Iorio MT, Siebert DCB, Rehman S, Schnürch M, Mihovilovic MD,** et al. Defined concatenated  $\alpha 6\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptor constructs reveal dual action of pyrazoloquinolinone allosteric modulators. *Bioorg Med Chem*. 2019; 27: 3167–3178. Erratum in: *Bioorg Med Chem*. 2020; 28: 115417. DOI: <https://doi.org/10.1016/j.bmc.2020.115417>
  37. **Winsky-Sommerer R, Vyazovskiy VV, Homanics GE, Tobler I.** The EEG effects of THIP (Gaboxadol) on sleep and waking are mediated by the GABA<sub>A</sub>  $\delta$ -subunit-containing receptors. *Eur J Neurosci*. 2007; 25: 1893–1899. DOI: <https://doi.org/10.1111/j.1460-9568.2007.05455.x>
  38. **Anis NA, Berry SC, Burton NR, Lodge D.** The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methylaspartate. *Br J Pharmacol*. 1983; 79: 565–575. DOI: <https://doi.org/10.1111/j.1476-5381.1983.tb11031.x>
  39. **MacDonald JF, Bartlett MC, Mody I, Pahapill P, Reynolds JN, Salter MW,** et al. Actions of ketamine, phencyclidine and MK-801 on NMDA receptor currents in cultured mouse hippocampal neurones. *J Physiol (Lond)*. 1991; 432: 483–508. DOI: <https://doi.org/10.1113/jphysiol.1991.sp018396>
  40. **Ganguly S, Panetta JC, Roberts JK, Schuetz EG.** Ketamine Pharmacokinetics and Pharmacodynamics Are Altered by P-Glycoprotein and Breast Cancer Resistance Protein Efflux Transporters in Mice. *Drug Metab Dispos*. 2018; 46: 1014–1022. DOI: <https://doi.org/10.1124/dmd.117.078360>
  41. **Divović Matović B, Knutson D, Mitrović J, Stevanović V, Stanojević B, Savić S,** et al. Behavioural interaction of pyrazoloquinolinone positive allosteric modulators at  $\alpha 6$  GABA<sub>A</sub> receptors and diazepam in rats: Anti-diazepam-induced ataxia action as a structure-dependent feature. *Basic Clin Pharmacol Toxicol*. 2022; 131: 514–524. DOI: <https://doi.org/10.1111/bcpt.13801>
  42. **Vasović D, Divović B, Treven M, Knutson DE, Steudle F, Scholze P,** et al. Trigeminal neuropathic pain development and maintenance in rats are suppressed by a positive modulator of  $\alpha 6$  GABA<sub>A</sub> receptors. *Eur J Pain*. 2019; 23: 973–984. DOI: <https://doi.org/10.1002/ejp.1365>
  43. **Jones A, Korpi ER, McKernan RM, Pelz R, Nusser Z, Mäkelä R,** et al. Ligand-gated ion channel subunit partnerships: GABA<sub>A</sub> receptor  $\alpha 6$  subunit gene inactivation inhibits  $\delta$  subunit expression. *J Neurosci*. 1997; 17: 1350–1362. DOI: <https://doi.org/10.1523/JNEUROSCI.17-04-01350.1997>
  44. **Nusser Z, Ahmad Z, Tretter V, Fuchs K, Wisden W, Sieghart W,** et al. Alterations in the expression of GABA<sub>A</sub> receptor subunits in cerebellar granule cells after the disruption of the  $\alpha 6$  subunit gene. *Eur J Neurosci*. 1999; 11: 1685–1697. DOI: <https://doi.org/10.1046/j.1460-9568.1999.00581.x>
  45. **Eccles JC, Llinás R, Sasaki K.** The excitatory synaptic action of climbing fibres on the Purkinje cells of the cerebellum. *J Physiol*. 1966; 182: 268–296. DOI: <https://doi.org/10.1113/jphysiol.1966.sp007824>
  46. **Blenkinsop TA, Lang EJ.** Block of inferior olive gap junctional coupling decreases Purkinje cell complex spike synchrony and rhythmicity. *J Neurosci*. 2006; 26: 1739–1748. DOI: <https://doi.org/10.1523/JNEUROSCI.3677-05.2006>
  47. **Lang EJ, Blenkinsop TA.** Control of cerebellar nuclear cells: a direct role for complex spikes? *Cerebellum*. 2011; 10: 694–701. DOI: <https://doi.org/10.1007/s12311-011-0261-6>
  48. **Tang T, Blenkinsop TA, Lang EJ.** Complex spike synchrony dependent modulation of rat deep cerebellar nuclear activity. *Elife*. 2019; 8. pii: e40101. DOI: <https://doi.org/10.7554/eLife.40101>
  49. **Dykstra S, Engbers JD, Bartoletti TM, Turner RW.** Determinants of rebound burst responses in rat cerebellar nuclear neurons to physiological stimuli. *J Physiol*. 2016; 594: 985–1003. DOI: <https://doi.org/10.1113/JP271894>
  50. **Lang EJ, Sugihara I, Llinás R.** Olivocerebellar modulation of motor cortex ability to generate vibrissal movements in rats. *J Physiol (Lond)*. 2006; 571: 101–120. DOI: <https://doi.org/10.1113/jphysiol.2005.102764>
  51. **Lang EJ, Sugihara I, Llinás R.** GABAergic modulation of complex spike activity by the cerebellar nucleoolivary

- pathway in rat. *J Neurophysiol.* 1996; 76: 255–275. DOI: <https://doi.org/10.1152/jn.1996.76.1.255>
52. **De Montigny C, Lamarre Y.** Rhythmic activity induced by harmaline in the olivocerebellar system of the cat. *Brain Res.* 1973; 53: 8195. DOI: [https://doi.org/10.1016/0006-8993\(73\)90768-3](https://doi.org/10.1016/0006-8993(73)90768-3)
  53. **Llinás R, Volkind RA.** The olivo-cerebellar system: functional properties as revealed by harmaline-induced tremor. *Exp Brain Res.* 1973; 18: 69–87. DOI: <https://doi.org/10.1007/BF00236557>
  54. **Sugihara I, Lang EJ, Llinás R.** Serotonin modulation of inferior olivary oscillations and synchronicity: a multiple-electrode study in the rat cerebellum. *Eur J Neurosci.* 1995; 7: 521–534. DOI: <https://doi.org/10.1111/j.1460-9568.1995.tb00657.x>
  55. **Beitz AJ, Saxon D.** Harmaline-induced climbing fiber activation causes amino acid and peptide release in the rodent cerebellar cortex and a unique temporal pattern of Fos expression in the olivocerebellar pathway. *J Neurocytol.* 2004; 33: 4974. DOI: <https://doi.org/10.1023/B:NEUR.0000029648.81071.20>
  56. **Barragan LA, Delhaye-Bouchaud N, Laget P.** Drug-induced activation of the inferior olivary nucleus in young rabbits. Differential effects of harmaline and quipazine. *Neuropharmacology.* 1985; 24: 645–654. DOI: [https://doi.org/10.1016/0028-3908\(85\)90107-8](https://doi.org/10.1016/0028-3908(85)90107-8)
  57. **De Zeeuw CI, Lang EJ, Sugihara I, Ruigrok TJ, Eisenman LM, Mugnaini E,** et al. Morphological correlates of bilateral synchrony in the rat cerebellar cortex. *J Neurosci.* 1996; 16: 3412–3426. DOI: <https://doi.org/10.1523/JNEUROSCI.16-10-03412.1996>
  58. **Nelson BJ, Mugnaini E.** Origins of GABAergic inputs to the inferior olive. In: Strata P (ed.), *The Olivocerebellar System in Motor Control.* 1989; 86–107. Berlin: Springer-Verlag. DOI: [https://doi.org/10.1007/978-3-642-73920-0\\_9](https://doi.org/10.1007/978-3-642-73920-0_9)
  59. **Najac M, Raman IM.** Integration of Purkinje cell inhibition by cerebellar nucleo-olivary neurons. *J Neurosci.* 2015; 35: 544–549. DOI: <https://doi.org/10.1523/JNEUROSCI.3583-14.2015>
  60. **Uusisaari M, Obata K, Knopfel T.** Morphological and electrophysiological properties of GABAergic and non-GABAergic cells in the deep cerebellar nuclei. *J Neurophysiol.* 2007; 97: 901–911. DOI: <https://doi.org/10.1152/jn.00974.2006>
  61. **Marshall SP, Lang EJ.** Local changes in the excitability of the cerebellar cortex produce spatially restricted changes in complex spike synchrony. *J Neurosci.* 2009; 29: 14352–14362. DOI: <https://doi.org/10.1523/JNEUROSCI.3498-09.2009>
  62. **Pan MK, Li YS, Wong SB, Ni CL, Wang YM, Liu WC.** Cerebellar oscillations driven by synaptic pruning deficits of cerebellar climbing fibers contribute to tremor pathophysiology. *Sci Transl Med.* 2020; 12(526): eaay1769. DOI: <https://doi.org/10.1126/scitranslmed.aay1769>
  63. **Miwa H, Nishi K, Fuwa T, Mizuno Y.** Differential expression of cfos following administration of two tremorgenic agents: harmaline and oxotremorine. *Neuroreport.* 2000; 11: 238590. DOI: <https://doi.org/10.1097/00001756-200008030-00010>
  64. **Bekar L, Libionka W, Tian GF, Xu Q, Torres A, Wang X,** et al. Adenosine is crucial for deep brain stimulation-mediated attenuation of tremor. *Nat Med.* 2008; 14: 75–80. DOI: <https://doi.org/10.1038/nm1693>
  65. **Sinton CM, Krosser BI, Walton KD, Llinás RR.** The effectiveness of different isomers of octanol as blockers of harmaline-induced tremor. *Pflugers Arch.* 1989; 414: 31–6. DOI: <https://doi.org/10.1007/BF00585623>
  66. **Nahab FB, Wittevrongel L, Ippolito D, Toro C, Grimes GJ, Starling J,** et al. An open-label, single-dose, crossover study of the pharmacokinetics and metabolism of two oral formulations of 1-octanol in patients with essential tremor. *Neurotherapeutics.* 2011; 8: 753–62. DOI: <https://doi.org/10.1007/s13311-011-0045-1>
  67. **Ondo WG.** Current and emerging treatments of essential tremor. *Neural Clin.* 2020; 38: 309–23. DOI: <https://doi.org/10.1016/j.ncl.2020.01.002>
  68. **Papapetropoulos S, Lee MS, Versavel S, Newbold E, Jinnah HA, Pahwa R,** et al. A Phase 2 proof-of-concept, randomized, Placebo-controlled trial of CX-8998 in essential tremor. *Mov Disord.* 2021; 36: 1944–1949. DOI: <https://doi.org/10.1002/mds.28584>
  69. **Handforth A, Tse W, Elble RJ.** A pilot double-blind randomized trial of perampanel for essential tremor. *Mov Disord Clin Pract.* 2020; 7: 399–404. DOI: <https://doi.org/10.1002/mdc3.12927>
  70. **Paterson NE, Malekiani SA, Foreman MM, Olivier B, Hanania T.** Pharmacological characterization of harmaline-induced tremor activity in mice. *Eur J Pharmacol.* 2009; 616: 73–80. DOI: <https://doi.org/10.1016/j.ejphar.2009.05.031>

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