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Tremor and Other Hyperkinetic Movements

Brief Reports

Suppression of Harmaline Tremor by Activation of an Extrasynaptic $GABA_A$ Receptor: Implications for Essential Tremor

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Abstract

Background: Metabolic imaging has revealed excessive cerebellar activity in essential tremor patients. Golgi cells control cerebellar activity by releasing gamma-aminobutyric acid (GABA) onto synaptic and extrasynaptic receptors on cerebellar granule cells. We postulated that the extrasynaptic $GABA_A$ receptor-specific agonist THIP (gaboxadol; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) would suppress tremor in the harmaline model of essential tremor and, since cerebellar extrasynaptic receptors contain α6 and δ subunits, would fail to do so in mice lacking either subunit.

Methods: Digitally measured motion power, expressed as 10–16 Hz power (the tremor bandwidth) divided by background 8–32 Hz motion power, was accessed during pre-harmaline baseline, pre-THIP harmaline exposure, and after THIP administration (0, 2, or 3 mg/kg). These low doses were chosen as they did not impair performance on the straight wire test, a sensitive test for psychomotor impairment. Littermate δ wild-type and knockout (*Gabrd*$^{+/+}$, *Gabrd*$^{-/-}$) and littermate α6 wild-type and knockout (*Gabra6*$^{+/+}$, *Gabra6*$^{-/-}$) mice were tested.

Results: *Gabrd*$^{+/+}$ mice displayed tremor reduction at 3 mg/kg THIP but not 2 mg/kg, and *Gabra6*$^{+/+}$ mice showed tremor reduction at 2 and 3 mg/kg. Their respective subunit knockout littermates displayed no tremor reduction compared with vehicle controls at either dose.

Discussion: The loss of anti-tremor efficacy with deletion of either δ or α6 $GABA_A$ receptor subunits indicates that extrasynaptic receptors containing both subunits, most likely located on cerebellar granule cells where they are highly expressed, mediate tremor suppression by THIP. A medication designed to activate only these receptors may display a favorable profile for treating essential tremor.

Keywords: Harmaline, GABA, cerebellum, essential tremor

Citation: Handforth A, Kadam PA, Kosoyan HP, Eslami P. Suppression of harmaline tremor by activation of an extrasynaptic $GABA_A$ receptor: implications for essential tremor. Tremor Other Hyperkinet Mov. 2018; 8. doi: 10.7916/D8JW9X9K

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Editor: Elan D. Louis, Yale University, USA

Received: February 7, 2018 **Accepted:** June 5, 2018 **Published:** June 26, 2018

Funding: Supported by International Essential Tremor Foundation and by Veterans Affairs.

Financial Disclosures: None.

Conflicts of Interest: The authors report no conflict of interest.

Ethics Statement: All procedures conformed to the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal protocols were approved by the Veterans Affairs Greater Los Angeles Institutional Animal Care and Use Committee.

Introduction

Tremor expression in essential tremor (ET) involves an oscillatory network that includes the cerebellum, thalamus, motor cortex, and brainstem, as shown by magnetoencephalography.[1] Thalamic deep brain stimulation (DBS) therapy acts by affecting cerebellar outflow to the cerebrum. ET patients exhibit numerous anomalies of cerebellar function, including imbalance, irregular finger tapping, and eye movement anomalies,[2] some of which improve with successful tremor treatment. Transcranial magnetic stimulation of the cerebellum improves tremor.[3] These and other observations indicate the cerebellum's critical role in ET.

Purkinje cells provide the sole output from cerebellar cortex; these cells receive a massive excitatory input via parallel fibers from granule cells, which in turn receive massive excitatory inputs from ascending mossy fibers. Evidence that this entire cerebellar cortical system is excessively active in ET is provided by positron imaging scans that

demonstrate increased blood flow in the cerebellum.[4] The notion of cerebellar cortical over-activity is further reinforced by clinical observations that tremor in ET is abolished by strokes involving the pons,[5] where excitatory afferent mossy fibers originate.

A critical control point for cerebellar cortical activity is the Golgi cell, which receives excitatory inputs from granule cells, mossy fibers, and climbing fiber collaterals, and releases gamma-aminobutyric acid (GABA) onto granule cells. $GABA_A$ receptors may be divided into two main classes. Synaptic receptors are pentameric structures containing two α, two β, and one γ subunit, and mediate fast signaling. Extrasynaptic receptors are also pentameric but utilize a δ rather than a γ subunit, usually contain $\alpha4$ or $\alpha6$ subunits, exhibit sensitivity to low GABA levels, and exert tonic rather than phasic inhibition. These properties permit granule cell extrasynaptic $GABA_A$ receptors to exert powerful control over cerebellar cortical activity. In contrast to δ $GABA_A$ receptors elsewhere in the brain that usually contain $\alpha4$, those of cerebellar granule cells utilize $\alpha6$ subunits.[6]

Based on these considerations, we postulated that if a drug were to suppress tremor and require the presence of δ and $\alpha6$ $GABA_A$ receptor subunits for this action, such a finding would indicate the potential for extrasynaptic $\alpha6\beta\delta$ $GABA_A$ receptor activation to treat ET, most likely on cerebellar granule cells.

Considerable research has shown that THIP (gaboxadol; 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) is a highly selective agonist for extrasynaptic $GABA_A$ receptors at low doses; it activates recombinant $\alpha4\beta\delta$ and $\alpha6\beta\delta$ receptors at concentrations 1,000 times lower than those required to activate $\alpha4\beta\gamma2$ and $\alpha6\beta\gamma2$ receptors.[7] Slices taken from the thalamus or cerebellum of wild-type (WT) mice, but not those from δ-knockout (KO) mice, respond to low THIP concentrations with tonic inhibitory currents.[7,8] Doses of THIP that cause incoordination on the rotarod test in WT mice fail to do so in δ-KO mice.[8] Given this specificity of low-dose THIP for extrasynaptic receptors, we utilized this drug in the harmaline model of ET to evaluate the hypothesis that activation of $GABA_A$ receptors containing δ and $\alpha6$ subunits may suppress tremor.

Methods

Animals

Gabrd–/– mice (also referred to as $\delta^{-/-}$), generated with a disruption of exon 4,[9] and backcrossed with C57BL/6J mice for over 11 generations, were kindly donated by Dr. Martin Wallner at the University of California at Los Angeles. *Gabra6*$^{-/-}$ mice (also referred to as $\alpha6^{-/-}$) were obtained from Jackson Laboratories (#002710, Bar Harbor, ME). These mice were created with a disruption of exon 8 of the *gabra6* gene, with background strains of C57BL/6J and 129/SvJ,[10] and were backcrossed with C57BL/6J mice in our laboratory for five generations. Heterozygotes of each genotype were generated, which were then interbred to produce littermates ($\delta^{+/+}$, $\delta^{-/-}$; and $\alpha6^{+/+}$, $\alpha6^{-/-}$) for harmaline experiments. Genotypes were determined commercially from ear biopsies using real-time polymerase chain reaction with specific probes designed for each gene (Transnetyx, Inc., Cordova, TN). Male and female mice were used, and had *ad libitum* access to food and water.

All procedures conformed to the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used. Animal protocols were approved by the Veterans Affairs Greater Los Angeles Institutional Animal Care and Use Committee.

Test procedures

In preliminary experiments, the ability of WT mice not receiving harmaline to pass the straight wire test at various doses of THIP was assessed, as previously described.[11] This simple test is highly sensitive to psychomotor impairment.[12] In this test, a mouse is suspended by its front paws from a rigid, 2-mm diameter wire and the time it takes the mouse to bring a hind paw up to the wire is noted. To pass, a mouse had to stay on the wire for 10 seconds and touch the wire with a hind paw within that time, and pass on all tests conducted at 10-minute intervals for 1 hour following drug administration. Only doses at which six out of six mice passed all tests were utilized in harmaline experiments.

Harmaline tremor was assessed as previously described,[11,13,14] but with the modification that mice were placed on an elevated platform on top of a cylinder that rested on the chamber floor fitted with a motion detector. In brief, each mouse was placed on an 8.1-cm-diameter mesh on top of a 24.1-cm-high cylinder. The cylinder rested on a Convuls-1 replacement sensing platform model 1335-1A (Columbus Instruments, Columbus, OH), which was fitted with a load sensor and 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. The *motion power percentage* (MPP) is the tremor bandwidth divided by overall motion power (10–16 Hz power)/(8–32 Hz power) $\times$ 100.

Mice were acclimated to the platform; then, 15 minutes of pre-harmaline baseline motion data were collected, and then harmaline (Sigma-Aldrich, St. Louis, MO), 20 mg/kg in 4 mL saline/kg was injected subcutaneously. Once the tremor had stabilized, motion power was assessed for 30 minutes interrupted at 15 minutes by 5 minutes of rest in the home cage. THIP (Tocris, Minneapolis, MN) was then administered in doses of 0, 2, or 3 mg/kg in saline, 10 mL/kg intraperitoneally, and motion power accessed starting 10 minutes later for a total of 45 minutes, with 5-minute interruptions at 15-minute intervals for rest periods in the home cage. Tremor past this time was not analyzed, as it became highly variable due to the harmaline wearing off. The technique of placing each mouse on an elevated platform promotes alert behavior that is associated with action tremor. Rest periods were designed to refresh the alert behavior once back on the platform, enhancing tremor consistency. There were seven to 13 mice per group.

Statistics

Statistical comparisons of post-treatment MPP values between groups that received 0, 2, or 3 mg/kg were performed. Statistical significance between groups was determined using one-way analysis of

variance, followed by post hoc Student's *t* tests comparing THIP-treated groups with the vehicle-treated group using the Bonferroni correction, employing Excel (Microsoft). The two-tailed significance level was 0.05.

Results

Role of $GABA_A$ receptor δ subunit in tremor suppression by THIP

Gabrd$^{-/-}$ and *gabra6*$^{-/-}$ mice, as previously described in the literature,[9,10] display normal behavior without ataxia and are indistinguishable from littermate $^{+/+}$ mice. The THIP dose 3 mg/kg was chosen as the highest tested dose, as six out of six WT mice passed all straight wire tests at this dose; this dose is below the dose of 4 mg/kg reported to affect the electroencephalogram (EEG) in mice.[15]

During pre-harmaline baseline, the MPP was approximately 25–33% on average, reflecting the percentage of motion power that happens to fall within the 10–16 Hz tremor range during normal movement. With harmaline administration to *Gabrd*$^{+/+}$ mice, motion power became dominated by tremor at 10–16 Hz, so that MPP approximated 80% (Figure 1A). After THIP injection, motion power barely changed in *Gabrd*$^{+/+}$ mice administered 0 or 2 mg/kg (n = 7, 8, respectively). In contrast, the 3 mg/kg dose caused a significant reduction of motion power, reflecting suppression of tremor (n = 10, p = 0.0005, two-tailed Student's *t* test), and lasted during the period of tremor measurement.

Gabrd$^{-/-}$ mice displayed pre-harmaline baseline and pre-THIP harmaline MPP values similar to those of littermate $\delta^{+/+}$ mice, indicating no alteration in overall motion activity or tremor response to harmaline. THIP, 2 mg/kg, did not affect tremor in $\delta^{-/-}$ mice compared with mice given 0 mg/kg (n = 11 and 13 respectively, Figure 1B), nor did the 3 mg/kg dose reduce MPP significantly (n = 7, p = 0.14, two-tailed Student's *t* test). This result indicates that the presence of the δ subunit is required for tremor suppression by low-dose THIP.

Role of $GABA_A$ receptor α6 subunit in tremor suppression by THIP

Gabra6$^{+/+}$ mice displayed MPP values during baseline and pre-THIP harmaline periods, and after 0 mg/kg THIP (n =10, Figure 2A), that were similar to those of *Gabrd*$^{+/+}$ mice. THIP significantly reduced MPP after THIP at doses of 2 and 3 mg/kg compared with vehicle-treated controls (n = 11, p = 0.006; n = 10, p = 0.024 respectively, two-tailed Student's *t* test).

Gabra6$^{-/-}$ mice, like *Gabrd*$^{-/-}$ mice, displayed MPP values during pre-harmaline baseline and pre-THIP harmaline periods similar to those of their WT littermates, indicating no alteration in general motility or in their tremor response to harmaline (Figure 2B). However, compared with the 0 mg/kg THIP group (n = 10), animals receiving 2 or 3 mg/kg displayed no reduction in MPP values (n = 9, p = 0.59; n = 10, p = 0.67, respectively; two-tailed Student's *t* test), indicating a failure of low-dose THIP to affect tremor in the absence of the α6 subunit.

Discussion

Cerebellar $GABA_A$ receptors containing α6, both synaptic with γ2 and extrasynaptic with δ subunits, are highly expressed on granule cells.

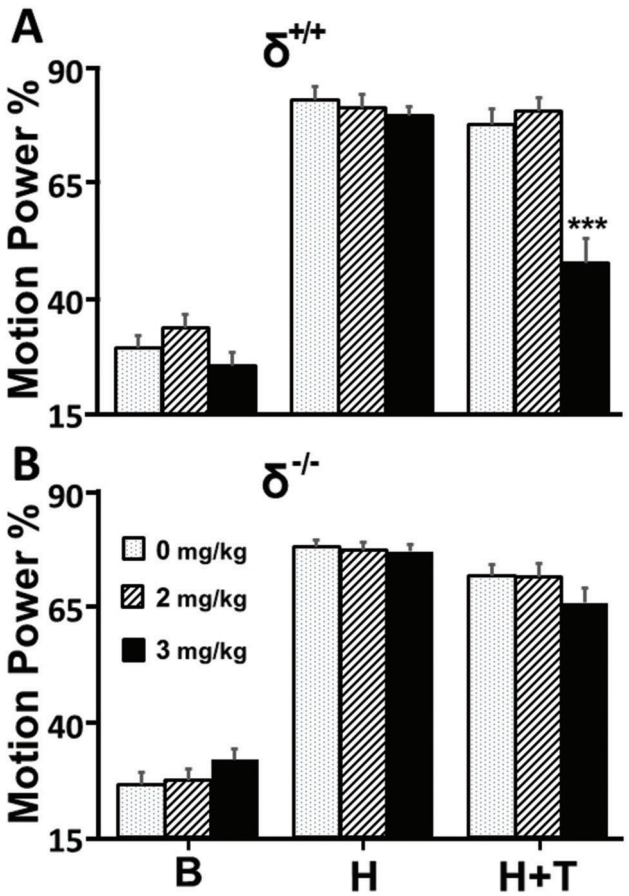

Figure 1. **Effect of THIP on Harmaline-induced Tremor in *Gabrd*$^{+/+}$ and $^{-/-}$ Littermates.** (A) Motion power percentage, representing the portion of 8–32 Hz motion power falling within the 10–16 Hz tremor bandwidth is shown during pre-harmaline baseline (B), followed by pre-THIP exposure to harmaline (H), and then following THIP injection (H + T) in *Gabrd*$^{+/+}$ mice that received 0, 2, or 3 mg/kg. These wild-type mice displayed robust 10–16 Hz harmaline tremor that was reduced by THIP, 3 mg/kg. (B) In contrast, littermate mice lacking the δ subunit failed to display any tremor suppression by THIP. Mean and standard error of the mean are shown; ***$p < 0.001$, two-tailed Student *t* test.

Mice lacking δ subunits display no reduction of cerebellar α6; however, these mice display increased γ2, reflecting a compensatory increase in $\alpha6\beta\gamma2$ receptors.[16,17] A deletion of α6, by contrast, results in a 50% loss of cerebellar $GABA_A$ receptors, with severe depletion of both synaptic and extrasynaptic receptors as shown by depletion of the δ and γ2 subunits.[18,19] It may be noted that levels of α4, which is found in extrasynaptic $GABA_A$ receptors in most brain regions, are very low in cerebellum, and do not compensate for loss of α6.[20] Despite the loss of cerebellar granule cell GABA-mediated tonic currents,[21] α6 KO mice show no motor deficits, are agile, and perform well on the rotating rod.[10,18,19,21] The compensation appears to be due to powerful compensatory mechanisms in global KO animals, including upregulation of a voltage-independent potassium conductance in cerebellar granule cells. The latter compensatory change results in normalized

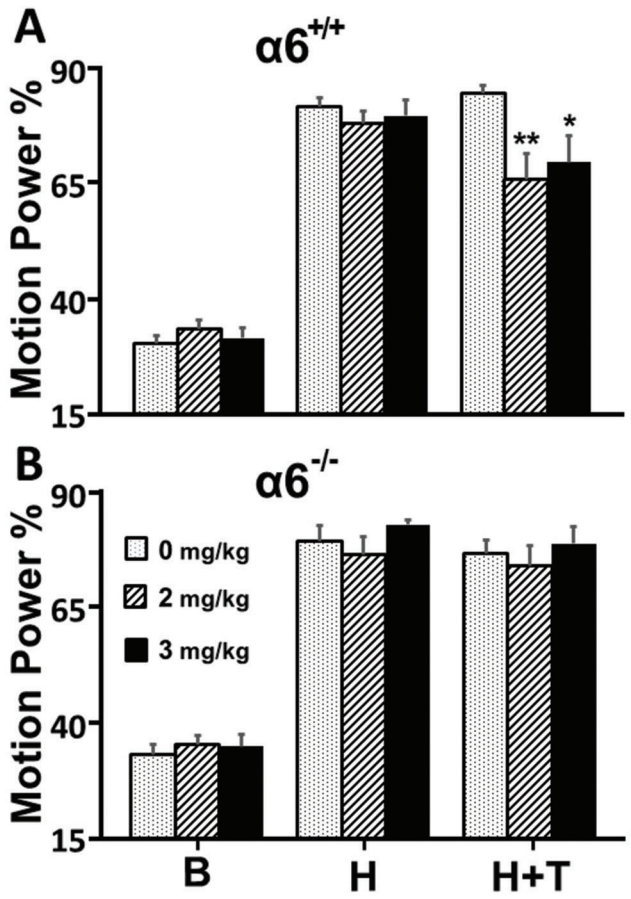

Figure 2. **Effect of THIP on Harmaline-induced Tremor in *Gabra6*$^{+/+}$ and $^{-/-}$ Littermates.** (A) Motion power percentages are displayed as in Figure 1 in *Gabra6*$^{+/+}$ mice that received 0, 2, or 3 mg/kg. These wild-type mice displayed robust 10–16 Hz harmaline tremor that was reduced by THIP, 2 and 3 mg/kg, compared with the 0 mg/kg group. (B) In contrast, littermate mice lacking the α6 subunit failed to display any tremor suppression by THIP. Mean and standard error of the mean are shown; *$p < 0.05$, **$p < 0.01$, two-tailed Student *t* test.

granule cell excitability.[21] The relatively normal response by cerebellar granule cells to multiple excitatory inputs thus may explain normal motor agility and tremor response to harmaline in α6- and δ-null mice; however, the absence of these subunits permits the testing of drugs whose behavioral action is hypothesized to depend on these subunits.

To our knowledge, there are no available drugs specific for activating $\alpha6\beta\delta$ $GABA_A$ receptors in vivo. Instead there are several agents that activate extrasynaptic receptors more generally, including $\alpha1\beta\delta$, $\alpha4\beta\delta$, and $\alpha6\beta\delta$ receptors. These include low-dose alcohol and neurosteroids, which act as positive allosteric modulators, and THIP, which acts as an agonist on δ receptors. In recombinant subunit-expressing cells, THIP activates $\alpha4\beta3\delta$ and $\alpha6\beta3\delta$ receptors at sub-micromolar levels, several orders of magnitude lower than those required to activate $\alpha4/6\beta3\gamma2$ receptors.[7] Slices of cerebellar granule cells, which express $\alpha6\beta\delta$ receptors, respond with tonic currents to low levels of THIP but fail to do so if taken from mice lacking δ or α6 subunits.[7,21]

In brain regions outside the cerebellum, such as the thalamus, where $\alpha4\beta\delta$ receptors are expressed, the tonic inhibitory response to low-level THIP requires the presence of δ and α4 subunits, as shown by using the corresponding KO mice.[8,22] The specificity of low-dose THIP for extrasynaptic receptors $GABA_A$ receptors compared with synaptic receptors has been confirmed at the behavioral level. WT mice display impairment on the rotarod test after administration of THIP, 10 mg/kg, but not in δ-subunit KO mice,[8] whereas δ subunit upregulation in the hippocampus is associated with increased seizure suppression by THIP.[23] THIP at low doses of 4 and 6 mg/kg affects the EEG in WT mice but not in δ-subunit KO mice.[15]

In the present study, we employed the harmaline model of tremor to test the hypotheses that $GABA_A$ receptors containing δ and α6 subunits mediate tremor suppression by THIP. The harmaline model is not a model of the disease ET, but of tremor that is the main symptom of ET. Harmaline induces action tremor in rodents that responds to alcohol and to standard ET therapies, and shares numerous pharmacological and physiological features with ET.[24] Harmaline acts by promoting inferior olive burst-firing, which then recruits circuits that overlap with those implicated in ET. Like ET, harmaline causes activation of components of the cerebellum, including Purkinje cells, the inferior olive, and the deep cerebellar nuclei. As in ET, harmaline tremor is suppressed by electrical stimulation of the thalamus.[25] Cerebellar granule cells are also activated during harmaline tremor, as indicated by c-fos labeling.[26,27] False positives have been reported, in which drugs that suppress harmaline tremor do not reduce tremor in ET patients;[24] at least some of these disparities were likely due to the use of doses that caused non-specific psychomotor impairment in the harmaline model.

We found that low-dose THIP caused partial but definite reduction in harmaline tremor in WT mice. The WT littermates for the α6 colony differed from those of the δ colony in displaying less marked tremor suppression, yet significant suppression at the lower dose of 2 mg/kg. These differences may have arisen from the less complete back-crossing of the α6 colony. Nonetheless, each genotype provided an important finding: tremor suppression by THIP that occurred in WT mice was absent in littermate mice that lacked the δ subunit or in those that lacked the α6 subunit.

The complete absence of any tremor suppression by low-dose THIP in δ KO mice indicates that low-dose THIP was unable to activate $\alpha4\beta\gamma$ or $\alpha6\beta\gamma$ $GABA_A$ receptors to suppress tremor, although these receptors are upregulated in δ KO mice.[16,17] Moreover, the complete absence of tremor suppression by low-dose THIP in α6 KO signifies that in the absence of this subunit, low-dose THIP was not able to suppress tremor by activating any δ or γ $GABA_A$ receptors that did not contain the α6 subunit. Together, these results are best interpreted as indicating that THIP's proximate anti-tremor mechanism involves the activation of $\alpha6\beta\delta$ receptors, mainly expressed in the brain on cerebellar granule cells.

$GABA_A$ $\alpha6\beta\delta$ receptors are also found on adult turtle spinal motoneurons, where they affect the monosynaptic reflex.[28] Activation by THIP of these receptors, if present in mice, would not be expected to

affect tremor, as harmaline tremor, like ET, is centrally generated. $GABA_A$ $\alpha6\beta\delta$ receptors are also located on locus coeruleus neurons in mice.[29] Activation of these receptors by THIP would be expected to enhance, not suppress, tremor, as lesions of this nucleus exacerbate harmaline tremor, while electrical stimulation suppresses tremor in rodents.[30] The most likely site of THIP's anti-tremor action via $GABA_A$ $\alpha6\beta\delta$ receptors is the cerebellar granule cell, where they are most abundantly expressed. A cerebellar site of action is consistent with imaging evidence of cerebellar cortical involvement in the harmaline model,[26,27] and the finding that mutant mice lacking Purkinje neurons display reduced harmaline tremor.[31] It is also consistent with the wealth of observations that the cerebellum is implicated in the pathophysiology of ET,[2] whereas several interventions that reduce tremor act on the cerebellum, including transcranial magnetic stimulation[3] and low-dose alcohol.[4,32]

Activated cerebellar granule cell $\alpha6\beta\delta$ $GABA_A$ receptors are well positioned to reduce cerebellar cortical hyperactivity inferred in metabolic imaging studies in ET subjects,[4] not only by inducing tonic inhibition, but by indirectly increasing Golgi cell firing and synaptic GABA release.[33] Our findings suggest that a drug that activates the $\alpha6\beta\delta$ $GABA_A$ receptor would be effective in treating tremor in patients with ET. Given the expression of $\alpha6\beta\delta$ receptors in other sites, there is a potential for off-target effects. Alternatively, a drug that activates $\alpha6\beta\gamma2$ receptors may also be anticipated to exert anti-tremor effects, as these receptors are also abundant on cerebellar granule cells. However, they are also present in the rat cochlear nucleus.[34] Further, $GABA_A$ receptors containing $\alpha6$ are present in the hippocampus and trigeminal ganglia in rats;[35,36] and thus may also have potential for off-target effects.

The present results suggest that other activators of $\alpha6\beta\delta$ receptors, such as neuroactive steroids and low-dose alcohol, should also suppress harmaline tremor, especially the latter in view of clinical observations that many ET patients report tremor reduction with low-dose alcohol. We are currently studying these agents. However, because such agents, as well as THIP itself, non-specifically activate $\alpha4\beta\delta$ and $\alpha6\beta\delta$ receptors, they are likely not ideally suitable for treating tremor, as the widespread cerebral distribution of $\alpha4\beta\delta$ receptors provides the basis of unwanted, off-target adverse effects. Indeed, THIP was evaluated for non-tremor indications and abandoned. In the present study, we were compelled to use low doses, 2–3 mg/kg, to avoid adverse effects. It is likely that such low doses underestimated the ability of selective $\alpha6\beta\delta$ receptor activation to suppress tremor. At 4–6 mg/kg, THIP alters the EEG but not in *Gabrd*$^{-/-}$ mice.[15] As the EEG is based mainly on cerebral hemisphere activity, where $\alpha4\beta\delta$ receptors dominate, it is likely that THIP's effect on the EEG is mainly $\alpha4\beta\delta$ mediated. More strikingly, it has been shown that, at 10 mg/kg, THIP causes sedation, increased tail flick latency, and incoordination on the rotarod test: all effects that are abolished in $\alpha4$ KO mice.[22] Thus, when THIP was in effect rendered $\alpha6\beta\delta$ selective by using $\alpha4$ KO mice, these mice passed the rotarod test after treatment with a10 mg/kg dose, which is much higher than the 3 mg/kg dose that was the maximal dose tolerated in our straight wire testing. These observations suggest that a drug that is specific for $\alpha6\beta\delta$, without affecting $\alpha4\beta\delta$ $GABA_A$ receptors, would not only provide high specificity in treating tremor but is likely to be well tolerated. Moreover, such a medication may not elicit tolerance or withdrawal effects, as these were not encountered with chronic dosing in rats or in a clinical trial of THIP for insomnia.[37,38]

Acknowledgement

We thank David I. Smith, M.S., for assistance with data analysis.

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