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ORIGINAL ARTICLE

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Tremorolytic effect of 5′-chloro-5′-deoxy-(±)-ENBA, a potent and selective adenosine A1 receptor agonist, evaluated in the harmaline-induced model in rats

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Summary

Aim: The aim of this study was to examine the role of adenosine A_1 receptors in the harmaline-induced tremor in rats using 5′-chloro-5′-deoxy-(±)-ENBA (5′Cl5′d-(±)-ENBA), a brain-penetrant, potent, and selective adenosine A_1 receptor agonist.

Methods: Harmaline was injected at a dose of 15 mg/kg ip and tremor was measured automatically in force-plate actimeters by an increased averaged power in the frequency band of 9-15 Hz (AP2) and by tremor index (a difference in power between AP2 and averaged power in the frequency band of 0-8 Hz). The *zif-268* mRNA expression was additionally analyzed by in situ hybridization in several brain structures.

Results: 5′Cl5′d-(±)-ENBA (0.05-0.5 mg/kg ip) dose dependently reduced the harmaline-induced tremor and this effect was reversed by 8-cyclopentyl-1,3-dipropyl xanthine (DPCPX), a selective antagonist of adenosine A_1 receptors (1 mg/kg ip). Harmaline increased the *zif-268* mRNA expression in the inferior olive, cerebellar cortex, ventroanterior/ventrolateral thalamic nuclei, and motor cortex. 5′Cl5′d-(±)-ENBA reversed these increases in all the above structures. DPCPX reduced the effect of 5′Cl5′d-(±)-ENBA on *zif-268* mRNA in the motor cortex.

Conclusion: This study suggests that adenosine A_1 receptors may be a potential target for the treatment of essential tremor.

KEYWORDS

adenosine A1 receptor agonist, essential tremor, harmaline, tremorolytic effect, zif-268

1 | INTRODUCTION

Harmaline (a β-carboline derivative) is a natural agent of plant origin, which induces tremor in humans[1] and is commonly used to model essential tremor (ET) in animals.[2] Similar to ET,[3] the harmaline-induced tremor is postural and kinetic, and its peak frequency in rats is 10-12 Hz.[2,4-9] It has been suggested that the harmaline-induced tremor is related to rhythmic firing of inferior olive neurons,[2,10] activation of the olivo-cerebellar glutamatergic climbing fibers,[2] elevation of glutamate release in the cerebellum,[11,12] and an increase in complex spike discharges of Purkinje cells (PCs) of the cerebellar cortex.[10] Moreover, harmaline induces oscillations in deep cerebellar nuclei, medullary reticular formation, and spinal cord[10,13,14] and activates c-Fos expression in the olivo-cerebellar system and in the basal ganglia.[11,15,16]

Besides phenomenological similarities between ET and the harmaline-induced tremor, their pharmacological treatments are also analogical.[3,6,8,9] Moreover, a recent study has shown that, similar to its beneficial effect in ET,[17] high-frequency stimulation of the ventrolateral thalamic nucleus in mice (a region receiving glutamatergic projections from deep cerebellar nuclei[18,19]) reduced the harmaline-induced tremor.[20] The latter result emphasizes importance of the cerebellothalamic connectivity for both kinds of tremor.

Adenosine, a neuromodulatory nucleoside, is involved in a wide variety of physiological and pathological processes, and its G protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3) are targets for therapeutic intervention

in several central and systemic disorders.[21] With regard to tremor therapy, A_{2A} but not A_1 receptor antagonists, such as DPCPX at doses which showed no in vivo occupancy of A_{2A} receptors, reduced tremulous jaw movements (a model of parkinsonian tremor) induced by cholinolytics or dopamine antagonists in rodents[22,23] and alleviated the resting tremor in parkinsonian patients.[24] On the other hand, an intrathalamic infusion of an A_1 receptor agonist decreased the harmaline-induced tremor in mice, and A_1 receptors have been suggested to contribute to the therapeutic effect of the high-frequency stimulation in this model.[20]

The aim of this study was to examine the role of adenosine A_1 receptors in the harmaline-induced model of ET. To this aim, we used 5′-chloro-5′-deoxy-(±)-ENBA (5′Cl5′d-(±)-ENBA), a brain-penetrant, potent, and selective adenosine A_1 receptor agonist; the affinity of which for human A_1 receptors (Ki=0.51 nmol/L) has been reported to be 2500-5300 times higher than for A_{2A}, A_{2B}, or A_3 receptors.[25-27] Systemic injection of 5′Cl5′d-(±)-ENBA induced antinociception in the formalin model,[25] reduced neuropathic pain,[26] reduced locomotor activity and L-DOPA-induced dyskinesia[27] in mice, as well as influenced microglia physiology.[28] In contrast to other A_1 receptor agonists, peripheral side effects of 5′Cl5′d-(±)-ENBA were reported to be limited because at a dose of 0.5 mg/kg ip, it did not affect heart rate or systolic blood pressure in mice.[26]

In order to search for brain targets for potential tremorolytic effect of 5′Cl5′d-(±)-ENBA, we analyzed the *zif-268* mRNA expression in different brain structures. *Zif-268*, similar to *c-fos*, belongs to the class of inducible immediate early genes (IEGs) that encode regulatory transcription factors and that have been implicated in various processes, including cell growth, differentiation, and apoptosis.[29] The *zif-268* mRNA and protein are constitutively expressed in several rat brain structures[29,30] and can be rapidly induced by a variety of physiological and pharmacological stimuli, including neurotransmitters (eg, glutamate), growth factors, seizures, ischemia, or cellular stress.[29] Therefore, it represents a sensitive neurochemical marker useful in the evaluation of neuronal responses.

2 | METHODS

2.1 | Animals

The experiments were carried out according to the EU Directive 2010/63/EU for animal experiments and were approved by the local ethics committee at the Institute of Pharmacology. All efforts were made to minimize the number and suffering of animals used. Male Wistar rats (240-350 g) were kept under a 12-h/12-h light/dark cycle (the light on from 7 AM to 7 PM) with free access to food and water. All experiments were carried out during the light period.

2.2 | Drugs

Harmaline hydrochloride dihydrate (Sigma-Aldrich, St Louis, MO, USA) was dissolved in redistilled water and administered as previously described[6-8] at a dose of 15 mg/kg ip, immediately before tremor measurements, which lasted 60 minutes. 5′-Chloro-5′-deoxy-N^6-(±)-(*endo*-norborn-2-yl)adenosine (5′Cl5′d-(±)-ENBA, Tocris Cookson Ltd., Bristol, UK) was dissolved in 0.5% DMSO in physiological saline and administered at doses of 0.01-0.5 mg/kg ip 30 minutes before harmaline. The doses of 5′Cl5′d-(±)-ENBA were chosen according to the lack of peripheral side effects, reported earlier in mice.[26] 8-Cycl opentyl-1,3-dipropylxanthine, a selective antagonist of adenosine A_1 receptors (DPCPX, Tocris Cookson Ltd., Bristol, UK),[31] was dissolved in 10% DMSO in physiological saline and administered at a dose of 1 mg/kg ip 10 minutes before 5′Cl5′d-(±)-ENBA (40 minutes before harmaline). Physiological saline was used as the control for harmaline, 0.5% DMSO for 5′Cl5′d-(±)-ENBA, and 10% DMSO for DPCPX.

2.3 | Force-plate actimeters (FPA) according to references[6-8,32]

Immediately after harmaline injections, rats were placed in the FPA. An animal was placed on a 44 cm×44 cm plate covered by a Plexiglas enclosure (33 cm high) and put into a ventilated sound-attenuating chamber. The FPA tracked the rat movements across a plate. Four force transducers below the corners of the plate recorded its position on a Cartesian plane and measured the force exerted on the plate at each time point. Data were collected during time units of 10.24 seconds ("frames") with the sampling frequency of 100 points/s, and accompanying software analyzed specific behaviors of interest.

Tremor was analyzed using fast Fourier transform (FFT) on each frame of the experiment. Then, the resulting power spectra were $\log_{10}$-transformed and averaged over two consecutive 180-frame series [two time periods of ca. 30-minute each (30.72 minutes)] to give the following parameters: AP1—averaged power in the frequency band I (0-8 Hz), AP2,—averaged power in the frequency band II (9-15 Hz; Figure 1A,B), and the tremor index defined as a difference in power between AP2 and AP1. The total distance traveled during two consecutive 180-frame series in millimeter was used as a measure of locomotor activity. Because vibration noise causes the measured position of the animal to fluctuate, this parameter could be artificially increased.

To analyze the relationship between tremor and motility of rats, the distance in millimeter was further divided by 10 000 and the ratio AP2/distance was calculated.

2.4 | In situ hybridization of *zif-268* mRNA

Quantitative in situ hybridization of *zif-268* mRNA in rat brain structures was performed according to standard procedures used in our laboratory.[7]

Rats were decapitated 1 hour after harmaline injections. Coronal sections (10 μm) were thaw-mounted on gelatin-coated microscopic slides, postfixed in 4% paraformaldehyde, dehydrated, delipidated, rehydrated, air-dried, and processed for in situ hybridization.

A 45-mer synthetic oligonucleotide probe complementary to bases 3-47 of the *zif-268* gene mRNA (GenBank accession number NM_012551.2, gi: 148747152) was labeled with [^{35}S]dATP (1000 Ci/mmol, Hartmann Analytic GmbH, Germany) by terminal deoxynucleotidyl transferase enzyme (Thermo Fisher Scientific Inc., Walthan, MA, USA) to obtain a specific activity of about 5-6×10^5 cpm/μL.[33]

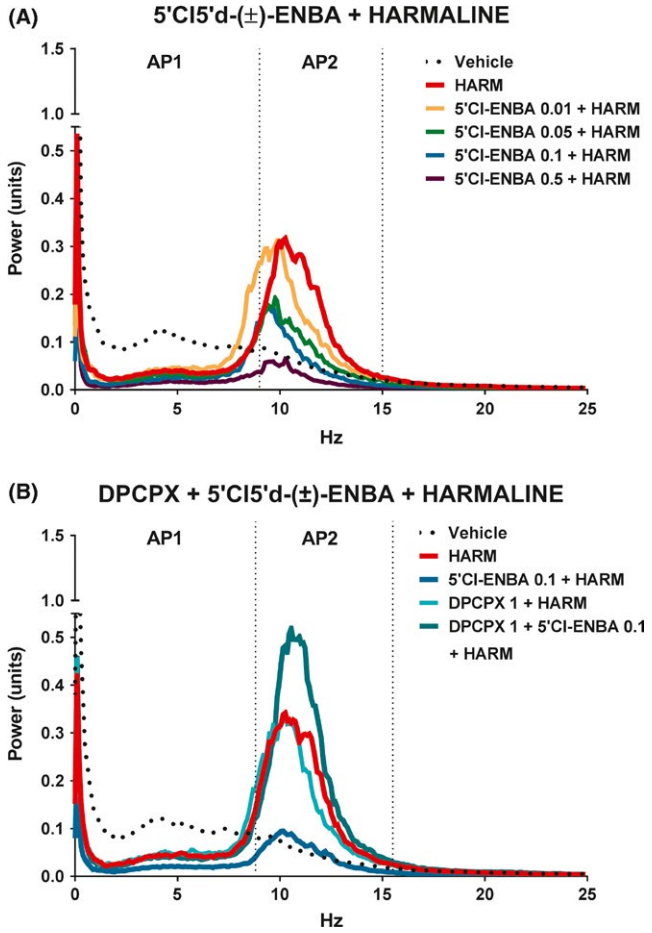

FIGURE 1 The effect of 5′Cl5′d-(±)-ENBA (A and B) and DPCPX (B) on the power spectrum related to the harmaline-induced tremor. The power spectrum within a range of 0-25 Hz averaged over the whole measurement period (0-60 min) for all animals is shown. AP1, power in the 0-8 Hz band; AP2, power in the 9-15 Hz band; HARM, harmaline 15 mg/kg; 5′Cl-ENBA 0.01, 0.05, 0.1, and 0.5, 5′Cl5′d-(±)-ENBA 0.01, 0.05, 0.1, and 0.5 mg/kg; DPCPX 1, DPCPX 1 mg/kg. The number of rats in A: vehicle, n=19; HARM, n=22; 5′Cl-ENBA 0.01+HARM, n=8; 5′Cl-ENBA 0.05+HARM, n=7; 5′Cl-ENBA 0.1+HARM, n=8; 5′Cl-ENBA 0.5+HARM, n=12. The number of rats in B: vehicle, n=10; HARM, n=10; 5′Cl-ENBA 0.1+HARM, n=12; DPCPX 1+HARM, n=6; DPCPX 1+5′Cl-ENBA 0.1+HARM, n=12

The tissue sections were incubated in a hybridization buffer with the radiolabeled oligonucleotide (5×10^5 cpm per tissue section) for 20 hours at 37°C in humidified chambers, washed (3×20 minutes in 2×SSC at 42°C, 1×15 minutes in 1×SSC at a room temperature), dehydrated, air-dried, and exposed to a Kodak BioMax MR film (Sigma-Aldrich) for 4 weeks at 4°C. The film was developed with a Dectol developer (Kodak; Sigma-Aldrich), fixed with a GBX fixer/replenisher (Kodak, Sigma-Aldrich), and dried.

Signal density [the mean optical density minus background (Q-BG) per unit area (pixel^2)] was measured in the scanned images using Multi Gauge 3.0 program (Fujifilm Europe, GmbH, Warsaw, Poland). The mRNA expression was estimated in the motor cortex at four consecutive levels (level I: from A=1.68 to 0.96 mm; level II: from A=−0.72 to −1.20 mm; level III: from A=−1.44 to −2.28 mm; level IV: from A=−2.64 to −3.60 mm), ventroanterior/ventrolateral thalamic nuclei (VA/VL) at two different levels (level III: from A=−1.44 to −2.28 mm; level IV: from A=−2.64 to −3.60 mm), cerebellum (lobules 1-10) at levels from A=−10.08 to −13.68 mm, and in the inferior olive at levels from A=−13.08 to −13.68 mm from the bregma, according to the Paxinos and Watson stereotaxic atlas[34]).

2.5 | Statistics

Statistical analyses were carried out using the software Statistica v.10 (StatSoft Inc., Tulsa, OK, USA). In the behavioral experiments, ANOVA for repeated measures was used followed by LSD post hoc test for individual comparisons. In situ hybridization data were analyzed by one-way ANOVA and LSD post hoc test.

3 | RESULTS

3.1 | The harmaline-induced behaviors in rats

As reported previously,[6-8] harmaline induced generalized tremor of the whole body which started as early as a few minutes after its administration and was manifested by an increase in power within the frequency band of 9-15 HZ (AP2), and in the tremor index (Figures 1, 2 and 4). Moreover, a decrease in the power within the frequency band of 0-8 Hz (AP1) was noted within the first 30 minutes of measurement (Figures 1, 2 and 4).

Harmaline altered additionally locomotor activity of rats, measured by the total distance traveled. In agreement with our previous study,[7,8] this drug reduced exploratory activity during the first 30 minutes after its injection but later (30-60 minutes) it increased motility of rats (Figures 2 and 4). This hyperactivity was characterized by episodic slow locomotor movements, general agitation, and sniffing and was accompanied by ataxia, balance disturbances, and tremor.

3.2 | The effect of 5′Cl5′d-(±)-ENBA on the harmaline-induced tremor

5′Cl5′d-(±)-ENBA (0.01-0.5 mg/kg *ip*) dose dependently alleviated the tremor induced by harmaline (Figures 1 and 2). A significant effect was demonstrated using ANOVA for repeated measures with regard to the tremor index or AP2 (treatment effect: $F[3,33]=4.293$-31.966, $F[2,29]=21.266$-27.445, $F[2,28]=5.770$-60.599, $P=.000$-$.012$). The LSD post hoc test revealed that 5′Cl5′d-(±)-ENBA reversed the harmaline-induced increases in these parameters at doses of 0.05-0.5 mg/kg. In contrast, the lowest dose of 5′Cl5′d-(±)-ENBA (0.01 mg/kg ip) was ineffective in this respect (Figures 1 and 2).

None of the doses of 5′Cl5′d-(±)-ENBA influenced AP1 lowered by harmaline (Figures 1 and 2).

3.3 | The effect of 5′Cl5′d-(±)-ENBA on locomotor activity

5′Cl5′d-(±)-ENBA influenced locomotor activity of rats (treatment effect: $F[3,26]=3.834$, $F[1,14]=68.18$, $P=.000$-$.021$). The LSD post

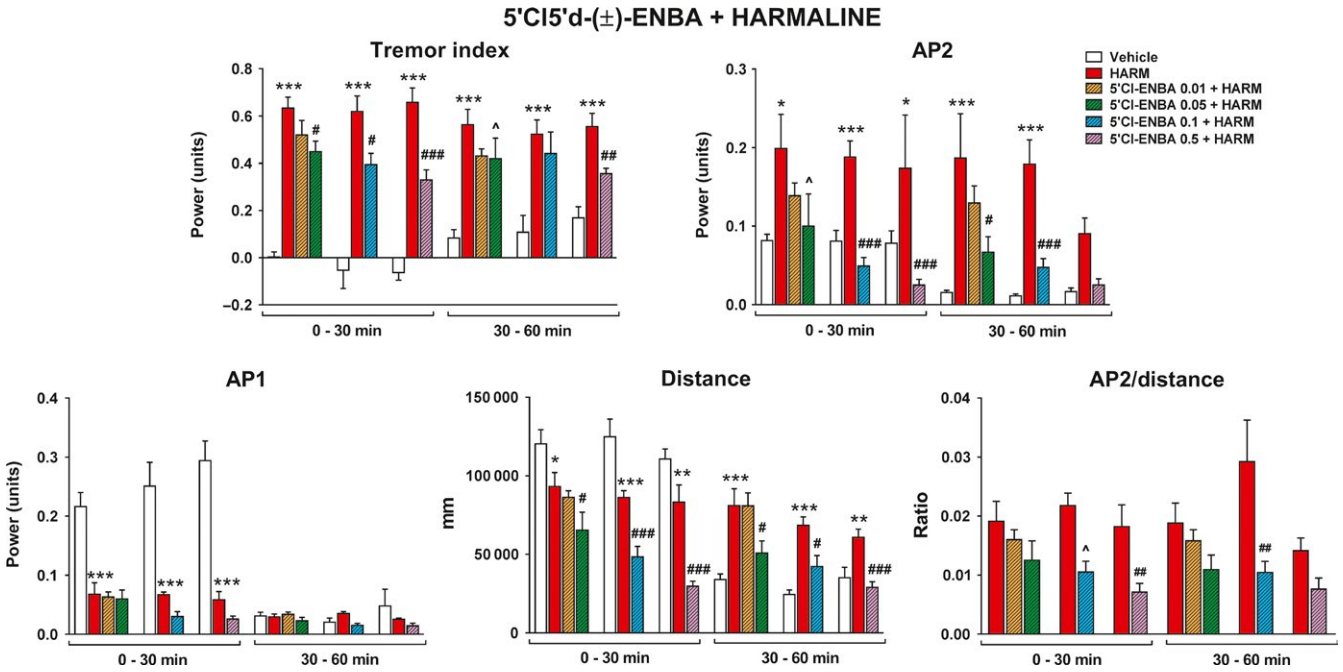

FIGURE 2 The effect of 5′Cl5′d-(±)-ENBA (0.01-0.5 mg/kg) on the harmaline-induced tremor and its influence on the distance traveled. Tremor index is defined as the difference between AP2 and AP1. The data are shown as the means±SEM. The number of animals: vehicle, n=9-10; HARM, n=10-12; 5′Cl-ENBA 0.01+HARM, n=8; 5′Cl-ENBA 0.05+HARM, n=7; 5′Cl-ENBA 0.1+HARM, n=12; 5′Cl-ENBA 0.5+HARM, n=12. Statistics: ANOVA for repeated measures+LSD post hoc test. **P*<.05, ***P*<.01, ****P*<.001 vs vehicle. $^{\#}P$<.05, $^{\#\#}P$<.01, $^{\#\#\#}P$<.001 vs HARM; $^{\wedge}P$=.066 vs HARM. For further details, see Figure 1

hoc test revealed that this compound at all tested doses decreased the distance traveled (exploratory activity) during the first period of measurements (0-30 minutes) in control animals (Figure 3). Rats treated with 5′Cl5′d-(±)-ENBA at the highest dose (0.5 mg/kg) were strongly disturbed. Animals were lying flat and seemed to be flaccid and cooler. However, these symptoms gradually disappeared along with lowering of doses, and general appearance of rats treated with the lowest one (0.01 mg/kg) was normal. Moreover, 5′Cl5′d-(±)-ENBA at higher doses (0.05, 0.1, and 0.5 mg/kg) deepened hypoactivity (0-30 minutes) and reversed hyperactivity (30-60 minutes) induced by harmaline, but the lowest dose was ineffective in this respect (ANOVA—treatment effect: 0.01 and 0.05 mg/kg—F[3,33]=2.29, P=.097; 0.1 mg/kg—F[2,29]=8.39, P=.002; 0.5 mg/kg—F[2,28]=27.045, P=.000; LSD significant effect for 0.05, 0.1, and 0.5 mg/kg; Figure 2).

The AP2/distance ratio was also significantly lowered in rats treated jointly with 5′Cl5′d-(±)-ENBA (0.1 or 0.5 mg/kg) and harmaline in comparison with harmaline alone (treatment effect: F[1,20]=5.308-8.385, P=.01-.032; Figure 2).

3.4 | Reversal of inhibitory effects of 5′Cl5′d-(±)-ENBA in the harmaline-treated rats by DPCPX

In the next experiment, where the influence of DPCPX on 5′Cl5′d-(±)-ENBA effects in the harmaline model was tested (Figure 4), ANOVA for repeated measures revealed significant treatment effects with

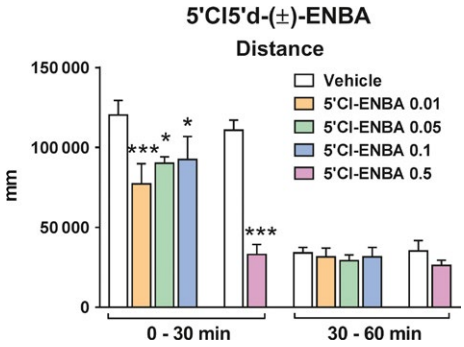

FIGURE 3 The effect of 5′Cl5′d-(±)-ENBA (0.01-0.5 mg/kg) on the distance traveled. The number of animals: vehicle, n=9-10; 5′Cl-ENBA 0.01, n=8; 5′Cl-ENBA 0.05, n=6; 5′Cl-ENBA 0.1, n=6; 5′Cl-ENBA 0.5, n=7. Statistics: ANOVA for repeated measures+LSD post hoc test. **P*<.05, ****P*<.001 vs vehicle. For further details, see Figures 1 and 2

regard to tremor index, AP2, AP1, and distance (treatment effect: F[3,38]=5.30-16.58, P=.0000-.0043). The LSD post hoc test confirmed the inhibitory influence of 5′Cl5′d-(±)-ENBA at the dose of 0.1 mg/kg ip on the harmaline-elevated AP2 and tremor index (Figure 4; for power spectrum, see Figure 1B). Moreover, it revealed that pretreatment of rats with DPCPX (1 mg/kg ip) reversed these 5′Cl5′d-(±)-ENBA effects (Figure 4; for power spectrum, see Figure 1B). Furthermore, 5′Cl5′d-(±)-ENBA at the above dose deepened the harmaline-induced hypomotility (in the first period of measurement—0-30 minutes) and reversed hyperactivity in the second period (30-60 minutes). Both

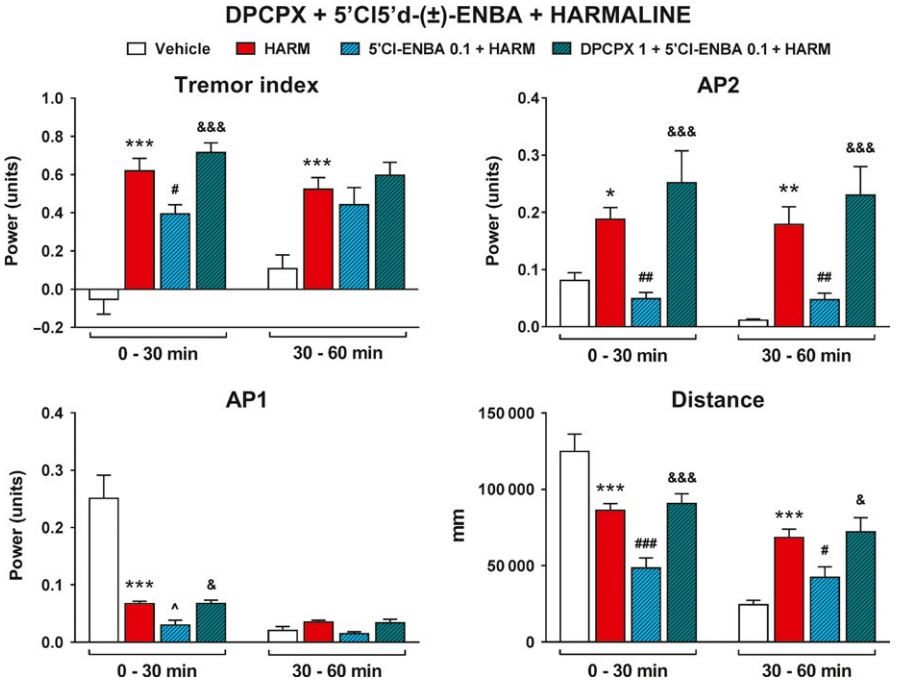

FIGURE 4 Reversal of the inhibitory effects of 5′Cl5′d-(±)-ENBA (0.1 mg/kg) on the harmaline-induced tremor and hyperactivity by DPCPX (1 mg/kg). The number of animals: vehicle, n=10; HARM, n=10; 5′Cl-ENBA 0.1+HARM, n=12; DPCPX 1+5′Cl-ENBA 0.1+HARM, n=12. Statistics: ANOVA for repeated measures+LSD post hoc test. $^{*}P<.05$, $^{**}P<.01$, $^{***}P<.001$ vs vehicle; $^{\#}P<.05$, $^{\#\#}P<.01$, $^{\#\#\#}P<.001$ vs HARM, $^{\wedge}P=.065$ vs HARM; $^{\&}P<.05$, $^{\&\&\&}P<.001$ vs 5′Cl-ENBA 0.1+HARM. For further details, see Figures 1 and 2

these 5′Cl5′d-(±)-ENBA effects were abolished by DPCPX (1 mg/kg ip; Figure 4).

DPCPX (1 mg/kg ip) given alone 40 minutes before harmaline influenced none of the behaviors induced by the latter agent (Figure 1B; data concerning motility are not shown).

3.5 | The inhibitory effect of 5′Cl5′d-(±)-ENBA on the harmaline-induced *zif-268* mRNA expression in brain structures; a differential influence of DPCPX

Harmaline increased the *zif-268* mRNA expression in the inferior olive, cerebellum, VA/VL, and motor cortex (one-way ANOVA, treatment effect: *F*[5,89]-*F*[5,92]=2.99-22.384, *P*=.000-.015; *F*[5,41]-*F*[5,43]=2.8195-9.4712, *P*=.0000-.0273; LSD significant effect; Figures 5 and 6 and S1).

5′Cl5′d-(±)-ENBA (0.1 mg/kg) which per se did not influence the *zif-268* mRNA expression significantly reversed the harmaline-evoked effect in all examined structures except for the level IV of VA/VL and motor cortex, and lobules 1, 7, 8, 9 of the cerebellum. In the lobules 2 and 5, a nonsignificant trend (*P*=.065-.069) was observed, respectively (Figures 6 and S1).

DPCPX reversed the antagonistic effect of 5′Cl5′d-(±)-ENBA on the harmaline-induced increase in *zif-268* mRNA expression in the motor cortex (level I and II) and enhanced per se the effect of harmaline in this structure (levels I-III). No influence of DPCPX on *zif-268* mRNA in any other brain regions was observed (Figures 6 and S1).

4 | DISCUSSION

The present study showed that 5′Cl5′d-(±)-ENBA, a potent and selective agonist of adenosine A_1 receptors administered systemically in rats, strongly and dose dependently reduced the tremor induced by harmaline. This effect was evidenced by reversal of the harmaline-induced increase in two tremor parameters: AP2 and the tremor index. Dependence of this tremorolytic effect of 5′Cl5′d-(±)-ENBA on adenosine A_1 receptors was proven by demonstration that it was abolished by DPCPX, a selective antagonist of these receptors.[31] However, DPCPX administered alone did not influence the harmaline-induced tremor which indicated the lack of a modulating effect of endogenous adenosine on this disturbance. These results suggest that adenosine A_1 receptors may be a new target for ET therapy.

Our present study additionally revealed that, like other agonists of A_1 receptors[35] and its effects in mice,[25,27] 5′Cl5′d-(±)-ENBA in all tested doses decreased exploratory locomotor activity of rats measured as a distance traveled. Moreover, in higher doses it deepened the hypomotility and reversed hyperactivity induced by harmaline in the first (0-30 minutes) and second (30-60 minutes) periods after the latter agent injection, respectively. These effects of 5′Cl5′d-(±)-ENBA were abolished by DPCPX which supported the contribution of adenosine A_1 receptors.

It is well known that the tremor evoked by harmaline is action dependent.[2] Therefore, as 5′Cl5′d-(±)-ENBA induced parallel decreases in tremor and motility in the harmaline-treated rats, the former effect was likely to be secondary to its sedative properties. In the present study, however, we tested the hypothesis that these decreases were partly independent. To this aim, we calculated a ratio of AP2 (a parameter indicating the tremor intensity) to distance (a parameter reflecting motility). We have already applied the same method to propranolol (a drug well known to be efficient in treating essential tremor in humans[3]) and found that this drug decreased AP2/distance ratio in the harmaline-treated animals which indicated its markedly stronger tremorolytic than sedative effect,[8] which was in line with its clinical efficiency.[3] In the present study, although 5′Cl5′d-(±)-ENBA at a low dose

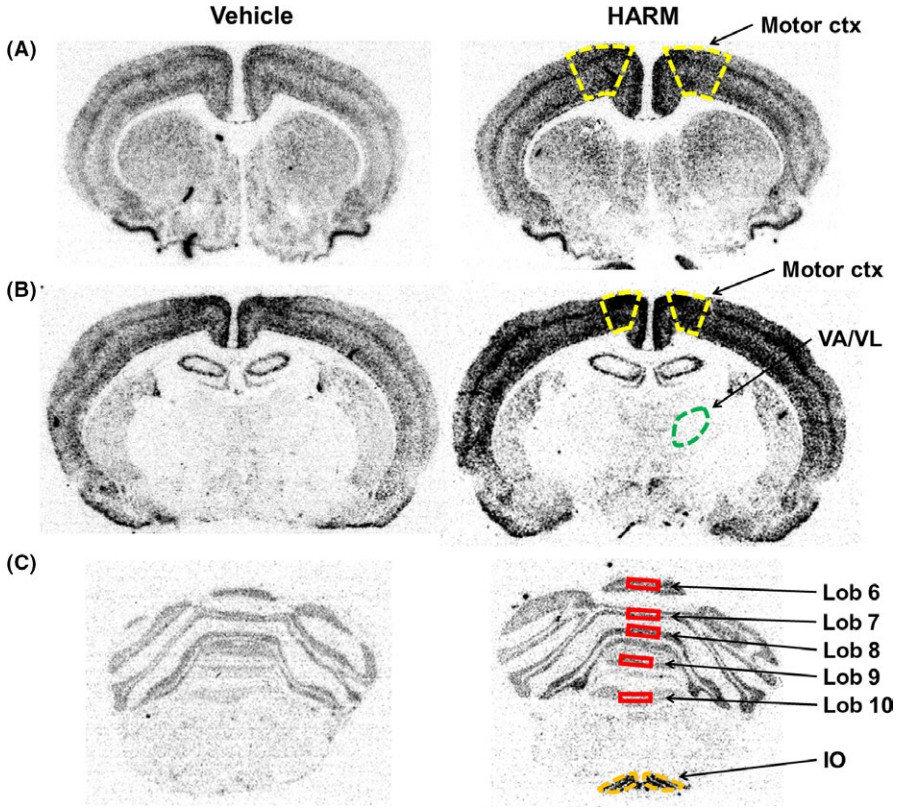

FIGURE 5 Representative autoradiograms showing *zif-268* mRNA expression in frontal sections of the level I of the motor cortex (A), level III of the motor cortex and VA/VL (B), and lobules 6-10 of the cerebellum and inferior olive (C). Outlines show the regions studied. ctx, cortex; HARM, harmaline 15 mg/kg; IO, inferior olive; lob 6-10, lobules 6-10 of the cerebellum; VA/VL, ventroanterior/ventrolateral nuclei of the thalamus

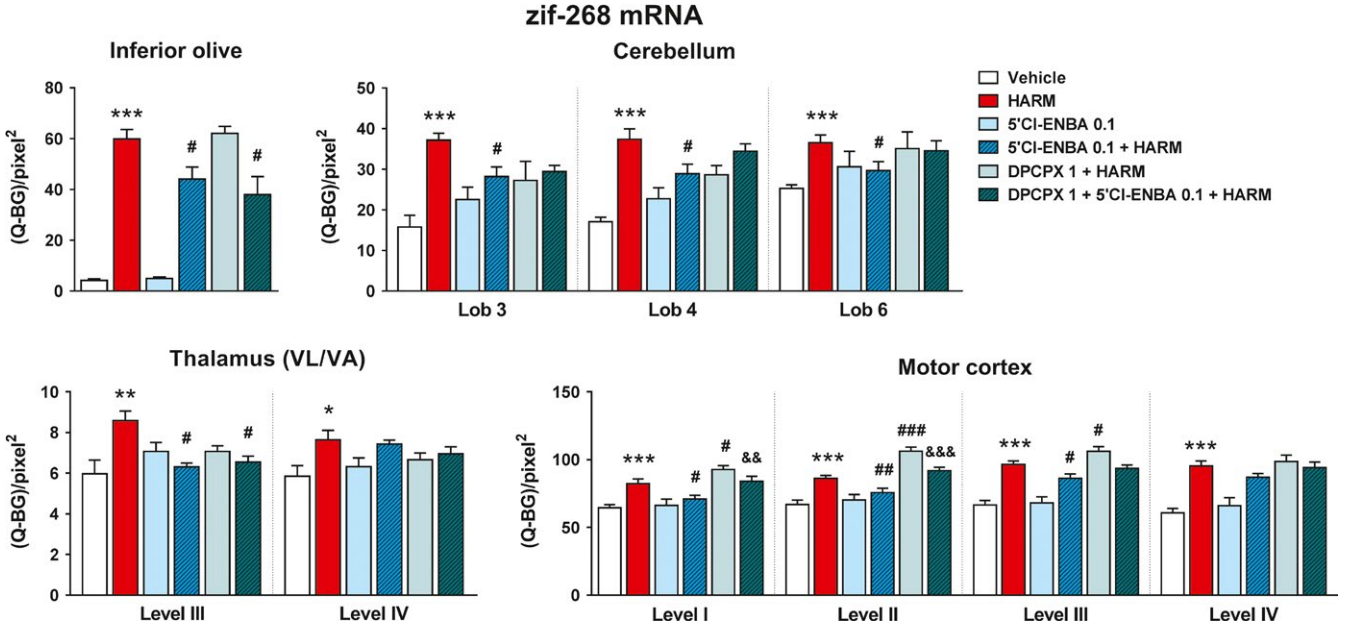

FIGURE 6 Reversal of the harmaline-induced *zif-268* mRNA expression by 5′Cl5′d-(±)-ENBA (0.1 mg/kg); influence of DPCPX (1 mg/kg) (Q-BG)/pixel^2—the mean optical density minus background per unit area. The number of animals: vehicle, n=6-8; HARM, n=9-10; 5′Cl-ENBA 0.1, n=5-6; 5′Cl-ENBA 0.1+HARM, n=9-10; DPCPX 1+HARM, n=5-6; DPCPX 1+5′Cl-ENBA 0.1+HARM, n=9-10. Statistics: one-way ANOVA+LSD post hoc test. $^{*}P$<.05, $^{**}P$<.01, $^{***}P$<.001 vs vehicle; $^{\#}P$<.05, $^{\#\#}P$<.01, $^{\#\#\#}P$<.001 vs HARM; $^{\&\&}P$<.01, $^{\&\&\&}P$<.001 vs 5′Cl-ENBA 0.1+HARM. For further details, see Figures 1, 2, and 5

(0.05 mg/kg) did not change the AP2/distance ratio in the harmaline-treated rats, which reflected its parallel influence on tremor and motility, at higher doses (0.1-0.5 mg) this drug lowered it significantly. Therefore, it seems that, at least at higher doses of 5′Cl5′d-(±)-ENBA, a certain independence of the mechanisms involved in its antitremor and antikinetic actions may be expected.

Adenosine *via* A_1 receptors reduces the excitatory synaptic transmission, glutamate release, and spontaneous neuronal activity in different brain structures.[20,36-39] Such mechanism may be responsible for A_1 receptor-induced inhibition of the harmaline-induced tremor as the role of an increased glutamatergic transmission in this phenomenon has been suggested. In fact, synchronous activation of glutamatergic olivo-cerebellar climbing fibers and an increase in glutamate release in the cerebellum,[2,11,12] which trigger complex spikes in the PCs and oscillations in deep cerebellar nuclei, are generally accepted to be the primary cause of tremor induced by this agent. Furthermore, this phenomenon was inhibited by NMDA and AMPA receptor antagonists.[4,5,9]

In agreement with the above glutamatergic mechanisms, we found that harmaline evoked *zif-268* mRNA expression, which is a marker of increased neuronal activity,[29,30] in the inferior olive, cerebellar cortex, VA/VL nuclei of the thalamus, and the motor cortex. The increase in *zif-268* expression in the inferior olive and cerebellar cortex was similar to that of c-Fos, reported earlier in these regions,[11,15,16] and was in line with the aforementioned activation of the olivocerebellar climbing fibers.[2,11,12] Similarly, it may be supposed that the harmaline-induced increase in *zif-268* mRNA expression in the motor nuclei of the thalamus and the motor cortex resulted from activation of the cerebellothalamic[18,19] and thalamocortical[40] glutamatergic projections conveying neuronal impulses from the cerebellum.

The present study showed additionally that 5′Cl5′d-(±)-ENBA reversed the harmaline-increased *zif-268* expression in all the above structures. As adenosine A_1 receptors are located in all these regions, with high levels in the cerebellar cortex, thalamus, and the cerebral cortex,[41,42] their inhibitory influence on neuronal activity may be supposed to contribute to the tremorolytic action of 5′Cl5′d-(±)-ENBA. The above hypothesis is supported by the study by Bekar and coworkers[20] showing that adenosine or another A_1 receptor agonist CCPA administered directly into the ventrolateral region of the thalamus decreased the harmaline-induced tremor in mice. Moreover, as far as the cerebellum is concerned, adenosine *via* A_1 receptors reduced excitatory postsynaptic currents (EPSCs) in the PCs induced by the stimulation of climbing fibers,[37,38] as well as their spontaneous firing.[36,43]

The motor cortex in humans is a part of oscillatory network active in ET.[44] The present study showing the harmaline-induced increase in *zif-268* expression in this structure and its reversal by 5′Cl5′d-(±)-ENBA may suggest that cortical adenosine A_1 receptors are involved in tremorolytic effect of the latter compound. This suggestion was further supported by a parallel antagonism, by DPCPX, of both inhibitory effects of 5′Cl5′d-(±)-ENBA on the harmaline-evoked tremor and cortical *zif-268* expression. However, in contrast to ET,[44] harmaline has not been reported to induce oscillatory neuronal activity in the motor cortex in mice.[45] Therefore, a putative involvement of cortical A_1 receptors in the harmaline model may be related rather to its modulatory influence on the activity of the olivo-cerebellar system executed by a disynaptic cerebrocerebellar projection.[46,47]

Surprisingly, in contrast to the motor cortex, DPCPX did not antagonize the 5′Cl5′d-(±)-ENBA effect on the harmaline-induced increase in *zif-268* mRNA expression in the inferior olive, cerebellum, and thalamus. As DPCPX has already been shown to inhibit, like an adenosine A_1 receptor agonist, the enhanced release of glutamate in rats,[39] a similar antiglutamatergic effect of this compound might contribute to the loss of its antagonistic properties vs 5′Cl5′d-(±)-ENBA in the above case. Although the mechanisms underlying such situation are unknown, they may involve complex interactions between adenosine receptors in conditions of enhanced level of endogenous adenosine. In fact, harmaline has been reported to activate 5′-nucleotidase (an intracellular enzyme which hydrolyzes 5′-AMP to adenosine),[48] suggesting an increase in adenosine level and release. Adenosine A_1 receptors form heteromers with A_{2A} or A_{2B} receptors and with other purinergic receptors, for example, P2Y1 and others, which alters responses mediated by constituent receptors, their sensitivity to endogenous adenosine, ligand binding, and may induce their cross-antagonism.[49-53] As some of adenosine heteromers, for example, A_1 with A_{2A}, and A_{2B} receptors are localized on glutamatergic terminals,[53,54] their activation by enhanced endogenous adenosine may mask the antagonistic effect of DPCPX on adenosine A_1 receptor in some brain regions where *zif-268* was analyzed.

The question arises, why in spite of reversing of tremorolytic effect of 5′Cl5′d-(±)-ENBA, DPCPX did not antagonize the inhibitory effect of this agonist on *zif-268* mRNA in brain structures known to be involved in the harmaline-induced tremor: inferior olive, cerebellum, and VA/VL nuclei of the thalamus.[2,10,20] It should be stressed, however, that *zif-268* expression measured in the present study was a resultant of activity of all neurons in rather wide brain areas. It is highly probable that these regions contain not only neurons involved in tremor-related networks, but also other nonrelated ones.

Several agonists of adenosine A_1 receptors have been developed as candidates for antiarrhythmic drugs because of their negative dromo- and chronotropic properties in humans.[55] These compounds slow down the heart rate and decrease systolic blood pressure in animals,[56,57] which may be regarded as their side effects when used for other than cardiovascular indications. 5′Cl5′d-(±)-ENBA has been proposed to be superior to other A_1 receptor agonists because when administered in mice at the dose of 0.5 mg/kg ip, which inhibited mechanical and thermal allodynia, it neither induced cardiovascular effects nor disturbed motor coordination.[26] In the present study, 5′Cl5′d-(±)-ENBA reduced the harmaline-induced tremor of rats at doses ranging from 0.05 to 0.5 mg/kg. However, we noticed that the highest dose of this compound strongly depressed motor behavior of rats, which did not seem to result solely from its strong sedative effect. Moreover, although we did not measure body temperature of rats, we noted by palpation a slight hypothermia induced by this dose which was in agreement with a recent study in mice.[58] Appearance of these disturbances of 5′Cl5′d-(±)-ENBA in rats which were not described (with the exception for the above hypothermia) in mice[26] may suggest species differences in metabolism of this drug. However, lower doses of 5′Cl5′d-(±)-ENBA were safer and induced only mild behavioral disturbances per se in rats which seems to be promising for future potential therapeutic use of adenosine A_1 receptor agonists as tremorolytic drugs.

Summing up, the present study suggests that adenosine A_1 receptors may be a potential therapeutic target for the treatment of ET. However, as activation of these receptors induces several unwanted effects, namely sedation, cardiovascular depression, and hypothermia, clinical use of their agonists may be limited.

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CONFLICT OF INTEREST

The authors state that any conflict of interest which might bias the present study does not exist.

REFERENCES

1. Frison G, Favretto D, Zancanaro F, Fazzin G, Ferrara SD. A case of β-carboline alkaloid intoxication following ingestion of Peganum harmala seed extract. *Forensic Sci Int*. 2008;179:e37-e43.
2. Handforth A. Harmaline tremor: underlying mechanisms in a potential animal model of essential tremor. *Tremor Other Hyperkinetic Mov (NY)*. 2012;2. pii. 02-92-769-1.
3. Zappia A, Albanese A, Bruno E. Treatment of essential tremor: a systematic review of evidence and recommendations from the Italian Movement Disorders Association. *J Neurol*. 2013;260:714-740.
4. Eblen F, Löschmann PA, Wüllner U, Turski L, Klockgether T. Effects of 7-nitroindazole, NG-nitro-L-arginine, and D-CPPene on harmaline-induced postural tremor, N-methyl-D-aspartate-induced seizures, and lisuride-induced rotations in rats with nigral 6-hydroxydopamine lesions. *Eur J Pharmacol*. 1996;299:9-16.
5. Iseri PK, Karson A, Gullu KM, et al. The effect of memantine in harmaline-induced tremor and neurodegeneration. *Neuropharmacology*. 2011;61:715-723.
6. Kosmowska B, Wardas J, Głowacka U, Ananthan S, Ossowska K. Pramipexole at a low dose induces beneficial effects in the harmaline-induced model of essential tremor in rats. *CNS Neurosci Ther*. 2016;22:53-62.
7. Ossowska K, Wardas J, Berghauzen-Maciejewska K, et al. Lu AF21934, a positive allosteric modulator of mGlu4 receptors, reduces the harmaline-induced hyperactivity but not tremor in rats. *Neuropharmacology*. 2014;83:28-35.
8. Ossowska K, Głowacka U, Kosmowska B, Wardas J. Apomorphine enhances harmaline-induced tremor in rats. *Pharmacol Rep*. 2015;67:435-441.
9. Paterson NE, Malekiani SA, Foreman MM, Olivier B, Hanania T. Pharmacological characterization of harmaline-induced tremor activity. *Eur J Pharmacol*. 2009;616:73-80.
10. Lamarre Y, De Montigny C, Dumont M, Weiss M. Harmaline-induced rhythmic activity of cerebellar and lower brain stem neurons. *Brain Res*. 1971;32:246-250.
11. 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 olivo-cerebellar pathway. *J Neurocytol*. 2004;33:49-74.
12. Gołembiowska K, Berghauzen-Maciejewska K, Górska A, Kamińska K, Ossowska K. A partial lesion of the substantia nigra pars compacta and retrorubral field decreases the harmaline-induced glutamate release in the rat cerebellum. *Brain Res*. 2013;1537:303-311.
13. Batini C, Bernard JF, Buisseret-Delmas C, Conrath-Verrier M, Horcholle-Bossavit G. Harmaline-induced tremor. II. Unit activity correlation in the interposito-rubral and oculomotor systems of cat. *Exp Brain Res*. 1981;42:383-391.
14. Lamarre Y, Weiss M. Harmaline-induced rhythmic activity of alpha and gamma motoneurons in the cat. *Brain Res*. 1973;63:430-434.
15. Miwa H, Nishi K, Fuwa T, Mizuno Y. Differential expression of c-Fos following administration of two tremorgenic agents: harmaline and oxotremorine. *NeuroReport*. 2000;11:2385-2390.
16. Tian JB, Bishop GA. Stimulus-dependent activation of c-Fos in neurons and glia in the rat cerebellum. *J Chem Neuroanat*. 2002;23:157-170.
17. Larson PS. Deep Brain Stimulation for movement disorders. *Neurotherapeutics*. 2014;11:465-474.
18. Haroian AJ, Massopust LC, Young PA. Cerebellothalamic projection in the rat: an autoradiographic and degeneration study. *J Comp Neurol*. 1981;197:217-236.
19. Nieoullon A, Kerkerian L, Dusticier N. High affinity glutamate uptake in the red nucleus and ventrolateral thalamus after lesion of the cerebellum in the adult cat: biochemical evidence for functional changes in the deafferented structures. *Exp Brain Res*. 1984;55:409-419.
20. Bekar L, Libionka W, Tian G-F, et al. Adenosine is crucial for deep brain stimulation-mediated attenuation of tremor. *Nat Med*. 2008;14:75-80.
21. Borea PA, Gessi S, Merighi S, Varani K. Adenosine as a multi-signalling guardian angel in human diseases: when and how does it exert its protective effects? *Trends Pharmacol Sci*. 2016;37:419-434.
22. Pinna A, Morelli M. A critical evaluation of behavioral rodent models of motor impairment used for screening of antiparkinsonian activity: the case of adenosine A2A receptor antagonists. *Neurotox Res*. 2014;25:392-401.
23. Collins LE, Galtieri DJ, Brennum LT, et al. Oral tremor induced by the muscarinic agonist pilocarpine is suppressed by the adenosine A2A antagonists MSX-3 and SCH58261, but not the adenosine A1 antagonist DPCPX. *Pharmacol Biochem Behav*. 2010;94:561-569.
24. Bara-Jimenez W, Sherzai A, Dimitrova T, et al. Adenosine A2A receptor antagonist treatment of Parkinson's disease. *Neurology*. 2003;61:293-296.
25. Franchetti P, Cappellacci L, Vita P, et al. N^6-Cycloalkyl- and N^6-bicycloalkyl-C5′(C2′)-modified adenosine derivatives as high-affinity and selective agonists at the human A1 adenosine receptor with antinociceptive effects in mice. *J Med Chem*. 2009;52:2393-2406.
26. Luongo L, Petrelli R, Gatta L, et al. 5′Chloro-5′-deoxy-(±)-ENBA, a potent and selective adenosine A_1 receptor agonist, alleviates neuropathic pain in mice through functional glial and microglial changes without affecting motor or cardiovascular functions. *Molecules*. 2012;17:13712-13726.
27. Mango D, Bonito-Oliva A, Ledonne A, et al. Adenosine A1 receptor stimulation reduces D1 receptor-mediated GABAergic transmission from striato-nigral terminals and attenuates L-DOPA-induced dyskinesia in dopamine-denervated mice. *Exp Neurol*. 2014;261:733-743.
28. Luongo L, Guida F, Imperatore R, et al. The A1 adenosine receptor as a new player in microglia physiology. *Glia*. 2014;62:122-132.
29. Beckmann AM, Wilce PA. Egr transcription factors in the nervous system. *Neurochem Int*. 1997;31:477-510.
30. Worley PF, Christy BA, Nakabeppu Y, Bhat RV, Cole AJ, Baraban JM. Constitutive expression of zif268 in neocortex is regulated by synaptic activity. *Proc Natl Acad Sci USA*. 1991;88:5106-5110.
31. Lohse MJ, Klotz K-N, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA. 8-cyclopentyl-1,2-dipropylxanthine (DPCPX) –

a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmiedeberg's Arch Pharmacol*. 1987;336:204-210.
32. Fowler SC, Berkestrand BR, Chen R, et al. A force-plate actometer for quantitating rodent behaviors: illustrative data on locomotion, rotation, spatial patterning, stereotypies, and tremor. *J Neurosci Methods*. 2001;107:107-124.
33. Ziółkowska B, Gieryk A, Solecki W, Przewłocki R. Temporal and anatomic patterns of immediate-early gene expression in the forebrain of c57bl/6 and dba/2 mice after morphine administration. *Neuroscience*. 2015;284:107-124.
34. Paxinos G, Watson C. *The Rat Brain*, 6th edn. Amsterdam: Elsevier; 2007.
35. Karcz-Kubicha M, Antoniou K, Terasmaa A, et al. Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. *Neuropsychopharmacology*. 2003;28:1281-1291.
36. Dunwiddie TV, Basile AS, Palmer MR. Electrophysiological responses to adenosine analogs in rat hippocampus and cerebellum: evidence for mediation by adenosine receptors of the A_1 subtype. *Life Sci*. 1984;34:37-47.
37. Hashimoto K, Kano M. Presynaptic origin of paired-pulse depression at climbing fibre-Purkinje cell synapses in the rat cerebellum. *J Physiol*. 1998;506:391-405.
38. Takahashi M, Kovalchuk Y, Attwell D. Pre- and postsynaptic determinants of EPSC waveform at cerebellar climbing fiber and parallel fiber to Purkinje cell. *J Neurosci*. 1995;15:5693-5702.
39. Simpson RE, O'Regan MH, Perkins LM, Phillis JW. Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: effect of adenosine receptor agonists and antagonists. *J Neurochem*. 1992;58:1683-1690.
40. Kuramoto E, Furuta T, Nakamura KC, Unzai T, Hioki H, Kaneko T. Two types of thalamocortical projections from the motor thalamic nuclei of the rat; a single neuron-tracing study using viral vectors. *Cereb Cortex*. 2009;19:2065-2077.
41. Bisserbe JC, Pascal O, Deckert J, Maziere B. Potential use of DPCPX as probe for in vivo localization of brain A1 adenosine receptors. *Brain Res*. 1992;599:6-12.
42. Rivekees SA, Price SL, Zhou FC. Immunohistochemical detection of A1 adenosine receptors in rat brain with emphasis on localization in the hippocampal formation, cerebral cortex, and basal ganglia. *Brain Res*. 1995;677:193-203.
43. Kessler M, Kiliman B, Humes C, Arai AC. Spontaneous activity in Purkinje cells: multi-electrode recording from organotypic cerebellar slice cultures. *Brain Res*. 2008;1218:54-69.
44. Muthuraman M, Heute U, Deuschl G, Raethjen J. The central oscillatory network of essential tremor. *Conf Proc IEEE Eng Med Biol Soc*. 2010;201:154-157.
45. Park YG, Choi JH, Lee C, et al. Heterogeneity of tremor mechanisms assessed by tremor-related cortical potential in mice. *Mol Brain*. 2015;8:3.
46. Coffman KA, Dum RP, Strick PL. Cerebellar vermis is a target of projections from the motor areas in the cerebral cortex. *PNAS*. 2011;108:16068-16073.
47. Suzuki L, Coulon P, Sabel-Goedknegt EH, Ruigrok TJH. Organization of cerebral projections to identified cerebellar zones in the posterior cerebellum of the rat. *J Neurosci*. 2012;32:10854-10869.
48. Balaban CD, Wurpel JN, Severs WB. A specific harmaline-evoked increase in cerebellar 5′-nucleotidase activity. *Neurosci Lett*. 1984;50:111-116.
49. Franco R, Lluis C, Canela EI, et al. Receptor–receptor interactions involving adenosine A1 or dopamine D1 receptors and accessory proteins. *J Neural Transm*. 2007;114:93-104.
50. Yoshioka K, Saitoh O, Nakata H. Heteromeric association creates a P2Y-like adenosine receptor. *PNAS*. 2001;98:7617-7622.
51. Cristovao-Ferreira S, Navarro G, Brugarolas M, et al. A_1R-$A_{2A}R$ heteromers coupled to G_s and $G_{i/o}$ proteins modulate GABA transport into astrocytes. *Purinergic Signalling*. 2013;9:433-449.
52. Lopes LV, Cunha RA, Ribeiro JA. Cross talk between A(1) and A(2A) adenosine receptors in the hippocampus and cortex of young adult and old rats. *J Neurophysiol*. 1999;82:3196-3203.
53. Goncalves FQ, Pires J, Pliassova A, et al. Adenosine A_{2b} receptors control A_1 receptor-mediated inhibition of synaptic transmission in the mouse hippocampus. *Eur J Neurosci*. 2015;41:878-888.
54. Ciruela F, Casado V, Rodrigues RJ, et al. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A_1-A_{2A} receptor heteromers. *J Neurosci*. 2006;26:2080-2087.
55. Elzein E, Zablocki J. A1 adenosine receptor agonists and their potential therapeutic applications. *Expert Opin Investig Drugs*. 2008;17:1901-1910.
56. Curros-Criado M, Herrero JF. The antinociceptive effects of the systemic adenosine A1 receptor agonist CPA in the absence and in the presence of spinal cord sensitization. *Pharmacol Biochem Behav*. 2005;82:721-726.
57. Schindler CW, Karcz-Kubicha M, Thorndike EB, et al. Role of central and peripheral adenosine receptors in the cardiovascular responses to intraperitoneal injections of adenosine A1 and A2A subtype receptor agonists. *Br J Pharmacol*. 2005;144:642-650.
58. Carlin JL, Jain S, Gizewski E, et al. Hypothermia in mouse is caused by adenosine A1 and A3 receptor agonist and AMP via three distinct mechanisms. *Neuropharmacology*. 2017;114:101-113.

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