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Article *in* Acta Neurobiologiae Experimentalis · October 2020

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Acta Neurobiol Exp 2020, 80: 322–330
DOI: 10.21307/ane-2020-028

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Diazepam and electrical stimulation of paleocerebellar cortex inhibits seizures in pentylenetetrazol-kindled rats

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The cerebellum is a potent anti-epileptic target for deep brain stimulation in patients with drug-resistant epilepsy. The effects of such stimulation, however, may also favor seizure activity. Our goal was to investigate the effect of cerebellar electrical stimulation (ES) alone and in combination with the anti-epileptic drug diazepam (DIA) on seizure outcome. We used a rat model of pentylenetetrazol kindling, which is characterized by seizures followed by deteriorations in central benzodiazepine-GABAA (BDZ-GABAA) receptors. We tested the effects of ES alone and in combination with DIA (0.1 and 1.0 mg/kg) on seizures. Our data demonstrated: 20 ES trials can prevent the recurrence of clonic-tonic kindled seizures, administration of either DIA-0.1 or ES (5 trials) alone is ineffective on seizures, and combining DIA-0.1 and 5 ES or DIA-1.0 and 5 ES caused an additive effect, prolonged the latency to seizure onset, and prevented recurrence of clonic-tonic seizures. We also observed that ES alone produced either facilitation or inhibition of seizures on EEG. In contrast, the same ES inhibited EEG seizures when delivered after a combination of DIA-1.0 and 5 ES and ultimately prevented the facilitation of the discharges. Lastly, we demonstrated that seizure suppression is intensified when cortical ES is performed after DIA administration. Our data supported the hypothesis that both BDZ-GABAA receptor activity along with cerebellar output comprise the potential mechanisms underlying the peculiar effects of deep brain stimulation in the cerebellum on seizures.

Key words: paleocerebellum, deep brain stimulation, benzodiazepines, kindling, pentylenetetrazol

INTRODUCTION

Epilepsy is a neurological disease characterized by the emergence of spontaneous recurrent seizures leading to long-term disability or death (Fisher et al., 2014). Traditional pharmacological treatment leads to complete control of seizures in approximately half of epilepsy patients and to a substantial reduction of manifestations in 20-30% of the patients (Fisher et al., 2014; Fisher and Velasco, 2014). The remaining population of patients with epilepsy are resistant to anti-epileptic drugs and require alternative therapeutic approaches (Kim et al., 2017).

Cerebellar nuclei and paleocortex are known as anti-convulsive targets for electrical stimulation (ES) (Reimer et al., 1967; Ojemann 1980; Velasco et al., 2005; Davis, 2009; Fountas et al., 2010; Fisher and Velasco, 2014; Klinger and Mittal, 2016; Kim et al., 2017; Sprengers et al., 2017). Yet, pro-convulsive responses of these targets to ES were also described (Reimer et al., 1967; Hutton et al., 1972; Hablitz et al., 1975; Ojemann 1980; Wyckhuys et al., 2009).

Previously, optogenetic stimulations of Purkinje cells (PC), in conjunction with intrahippocampal kainic acid administration, clearly inhibited seizure activity in mice (Krook-Magnuson et al., 2014). Taking into con-

Received 13 November 2019, accepted 12 June 2020

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sideration the GABAergic character of cerebellar cortex output (Wisden et al., 2009) and its diminishment caused by abolishing benzodiazepine (BDZ) receptors in cerebellar cortical neurons (Komulainen et al., 1995), it is expected that the combined use of BDZ agonists (diazepam, DIA) and cerebellar cortical ES could effectively counter seizures, especially of kindled origin, which are characterized by deteriorations in BDZ-receptors – both in the dentate gyrus (Clark et al., 1994) and the cerebellum (Bazyan et al., 2001) in pentylenetetrazole (PTZ)-kindled animals.

BDZs are approved by the FDA as a drug of choice for status epilepticus and seizures associated with post-anoxic insult treatment (Riss et al., 2008). However, tolerance, withdrawal symptoms, cognitive disturbances, and sedation limit their usage. Additionally, drug interactions restrict the possibility of combined pharmacological treatments. The limitations above leave a narrow window for BDZ prescription in practical epileptology (Verotti et al., 2015). Nevertheless, the combined use of anti-epileptic drugs (AEDs), including BDZs and ES of brain structures, has revealed potentiated seizure-preventing effects on lithium-pilocarpine-induced status epilepticus (Cuellar-Herrera et al., 2004, 2010; Besio et al., 2013; Gersner et al., 2016). These data point to the potential for the development of new and better therapies for drug-resistant seizures (Asgari et al., 2014, 2016).

This study presents the first analysis of BDZ-GABAA-dependent mechanisms of seizure inhibition in response to paleocerebellar ES. To test this, we used the PTZ-induced kindling rat model since PTZ is a selective antagonist of the GABAA receptor-chloride ionophore complex (Erkec and Arihan, 2015).

Our first aim was to demonstrate the inhibitory effects of ES in the paleocerebellar cortex on fully developed PTZ-kindled seizures. Our second aim was to test the hypothesis that ES delivered after DIA administration potentiates the inhibitory effect on fully developed PTZ-kindled seizures.

METHODS

Animals

In this experiment, 96 male Wistar rats, aged 6 to 9 months, were used. Female rats were excluded from this study since the effectiveness of GABAA agonists in releasing GABA and glutamate of isolated neuronal terminals can be hindered by estradiol and progesterone (Fleischmann et al., 1995).

Animals were randomly allocated in experimental groups. Animals were kept at a constant room temperature of 23°C, a relative humidity of 60%, on 12 h light/dark cycles, on a standard diet, and were given tap water *ad libitum* in accordance to international laws and policies (EU Council Directive 2010/63/EU for animal experiments; National Institutes of Health Guide for Care and Use of Laboratory Animals, US National Research Council, 1996 P.21-55). Odessa National Medical University Bioethics Committee (UBC) approval (No 17) dated 22/03/2016 was obtained before the start of the study.

Kindling procedure

The rats were subjected to PTZ administration for 21 days. Kindling was induced *via* a subthreshold dose of PTZ (30.0 mg/kg, i.p., Sigma-Aldrich, USA) every 24 h over 21 days (Shandra and Godlevsky, 1990; Godlevsky et al., 2014). All seizure scoring was conducted from 12 to 6: 00 p.m., and all animals were monitored for 30 min after each kindled PTZ injection. Only rats with generalized clonic-tonic seizures as a response to each of the last three trials of PTZ administration continued in the study. Fifteen rats were excluded at this stage of the study, as they did not demonstrate the development of fully developed kindled seizures (7 rats) or did not respond with generalized clonic-tonic seizures during the last three PTZ administrations (8 rats).

The latent period of first seizures was measured, and severity of seizures was scored using the following scale: 0 – no response; 1 – mouth and muscle jerks; 2 – myoclonus of groups of body muscles; 3 – forelimb serial clonic seizures with rearing; 4 – rearing, loss of vertical posture, hindlimb clonus, and forelimb tonus; and 5 – repeated score 4 seizures or tonic extension of the hindlimb and/or death.

Experimental groups

24 h after the last PTZ administration, animals were randomly assigned to the following groups: 9 rats with implanted electrodes, sham-stimulated, and i.p. saline (control group); 9 rats with 5 trials of ES and i.p. saline; 8 rats with 20 trials of ES and i.p. saline; 7 rats with DIA treatment in a dose of 0.1 mg/kg i.p., sham stimulated; 8 rats with DIA treatment in a dose of 1.0 mg/kg, i.p., sham stimulated; 9 rats with 5 trials of ES and DIA administration in a dose of 0.1 mg/kg, i.p.; 8 rats with 5 trials of ES and DIA administration in a dose of 1.0 mg/kg i.p.

In total, 58 rats were included in the final study, while another 38 animals were excluded based on surgical outcomes described below.

Study design

In all groups, kindled rats were subjected to testing PTZ administrations (Fig. 1). We scored the seizures following the last (21st) PTZ administration twice in control and experimental (Fig. 1A) groups. The first session was performed 24 h after the last (21st) PTZ administration for scoring behavioral seizures only (Fig. 1A). The second session was conducted for scoring EEG-responses to ES in the frontal cortex and hippocampus 3 days after behavioral seizure scoring (Fig. 1B).

We used the following criteria to assess the responses to ES: facilitation of seizure discharge frequency (Fig. 2A), neutral effect: no modification of frequency and amplitude of discharges (Fig. 2B), and inhibition of seizure discharges, which was identified as the reduction of seizure discharge frequency during ES of no less than two times relative to the pre-stimulation period (Fig. 2C). The total number of responses (facilitation, neutral effect or inhibition) per group of animals was measured for statistical evaluation. The number of rats in each group with the same type of response to all three episodes of ES testing

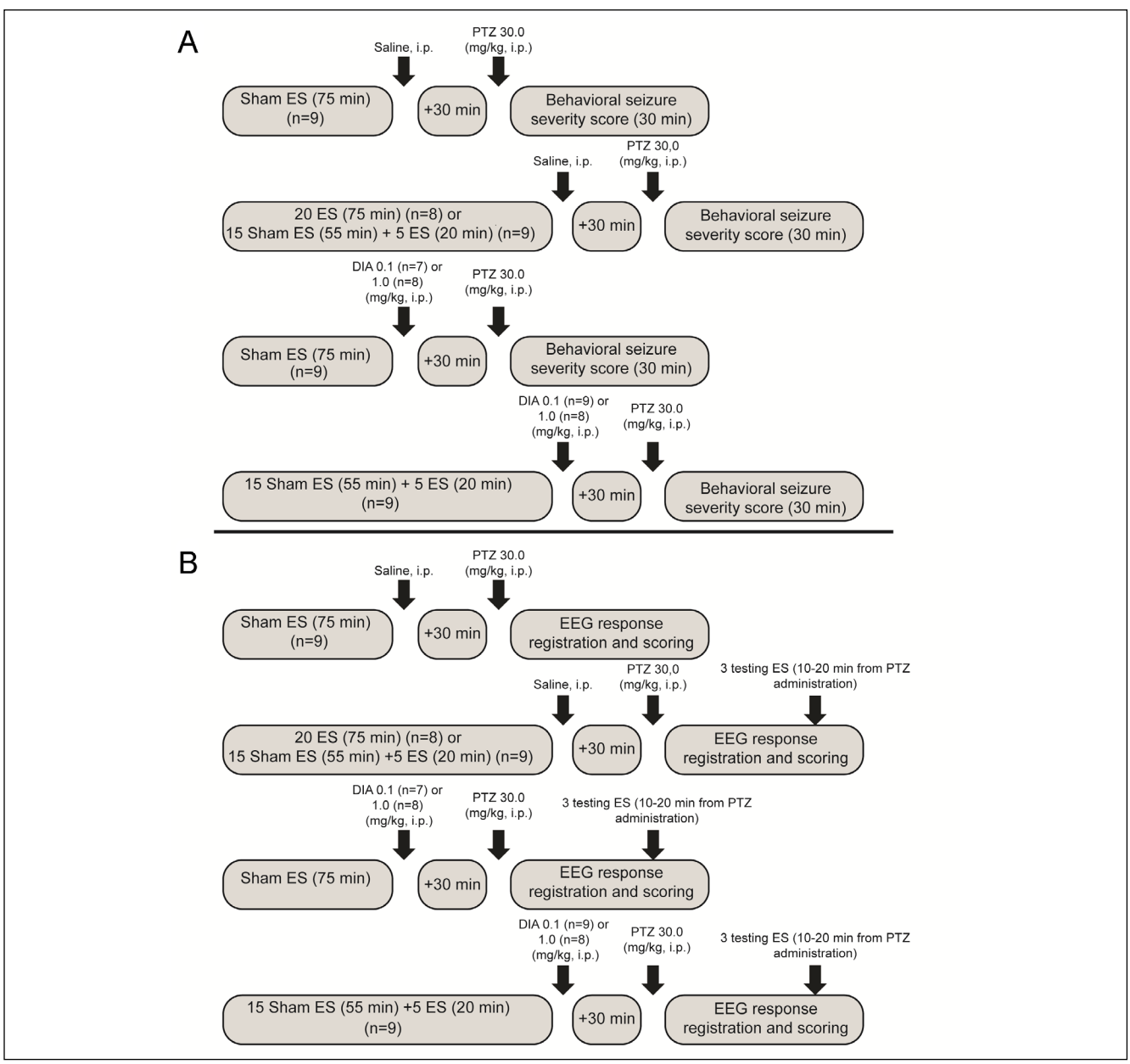

Fig. 1. Study design. (A) scoring of behavioral seizures 24 h from the 21st PTZ administration in the control group and (B) verification of types of electrographic epileptiform activity responses on three testing ES – three days after "a". DIA – diazepam; PTZ – pentylenetetrazol; i.p. – intraperitoneal; ES – electrical stimulation; min – minutes.

(inhibition, facilitation, or absence of reaction) was also registered as a marker of ES effects.

Surgery

Animals were single-housed to prevent aggression and elevated corticosterone levels (Kamakura et al., 2016). Anesthesia was performed with i.p. ketamine administration (100 mg/kg, Farmak, Ukraine). Rats were placed in a stereotactic apparatus "SEZh-5" (Kyiv, Ukraine). A 0.5% Novocain solution infiltration (Darnitsa, Ukraine) was used for local anesthesia before incision. The head was shaved and cleaned with iodine before incision. After skin dissection (2 cm along a middle sagittal axis) and removal of all soft tissues from the skull surface, EEG acquisition and stimulation electrodes were placed in relation to the bregma, where the parietal and frontal bones converge. A 1.5-2.0 mm burr hole for EEG acquisition and 4.0 mm hole for stimulation electrodes were drilled through the cranium with a standard dental portable drill (Colt 1, Charkov, Ukraine).

A bipolar stimulation electrode was placed into cerebellar vermis cortex (AP=-14 from bregma or -5.5 from lambda; ML=1.0; DV=-2.0) (Paxinos and Watson, 1982). The custom-made nichrome electrodes had a diameter of 0.15 mm, had an interelectrode distance of 0.25 to 0.30 mm, and, except for the tip, were insulated with 25 μm polyesterimide. The nichrome monopolar electrodes were implanted in the ventral hippocampus (AP=-4.3; ML=4.5; DV=-8.0), and frontal cortex (AP=1.7; ML=2.0; DV=-1.0) of both hemispheres, according to the rat brain atlas (Paxinos and Watson, 1982). The reference electrode was located in the nasal bones. The electrodes were fixed on the cranial surface with dental cement. To prevent dehydration,

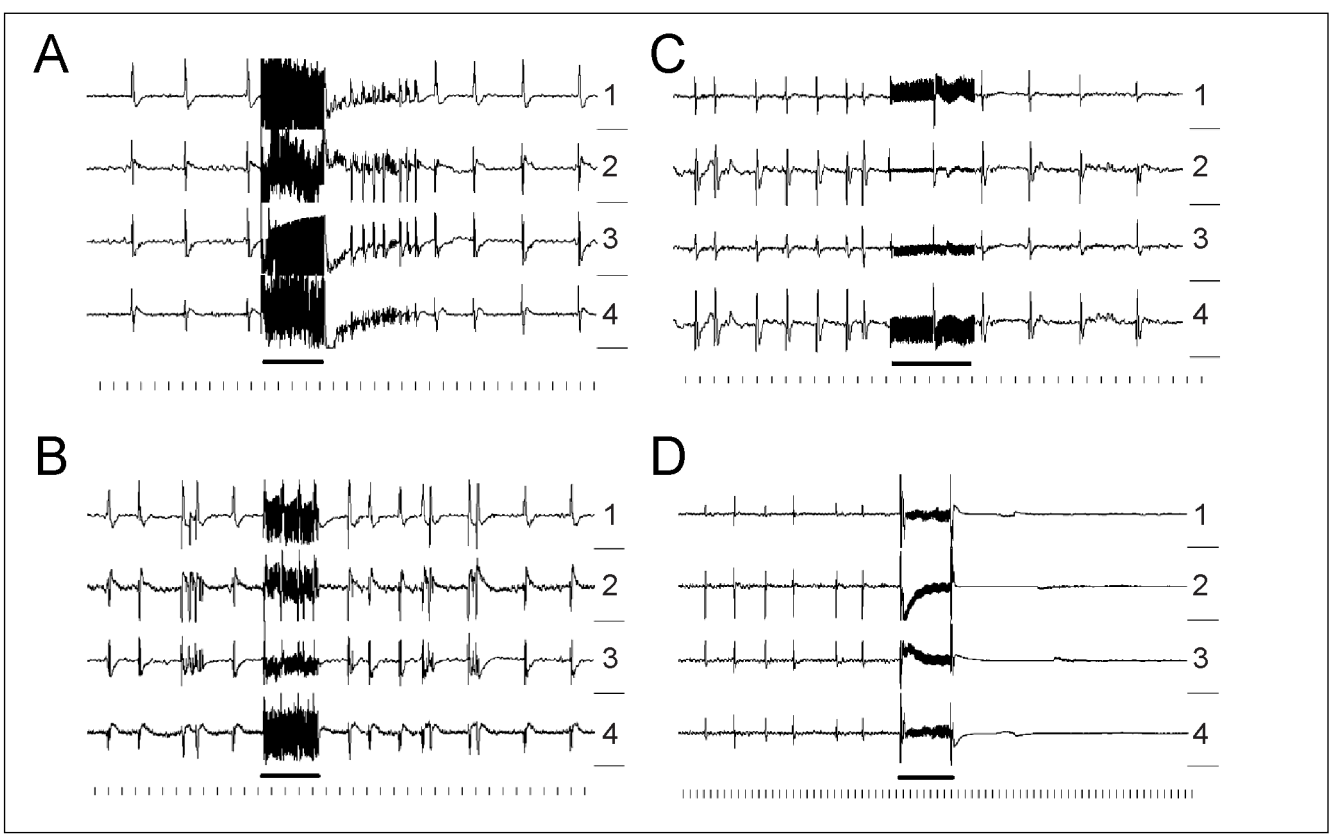

Fig. 2. Effect of ES on seizures. (A) Facilitation of kindling-induced seizures after ES of the paleocerebellar cortex. Stimulation was performed 16.5 min after the moment of kindled PTZ (30.0 mg/kg, i.p.) administration and 5.5 min after the moment of cessation of generalized ictal EEG. The intensity of stimulation was 75.0 mcA. (B) Absence of modulatory action by ES of paleocerebellum on kindling-induced seizures. Stimulation was performed 18.0 min after the moment of kindled PTZ (30.0 mg/kg, i.p.) administration and 4.0 min after the moment of cessation of generalized ictal EEG discharge. The rat was treated with DIA (0.1 mg/kg, i.p.). The intensity of stimulation was 100.0 mcA. (C) Inhibition of kindling-induced seizures after ES of the paleocerebellar cortex. Stimulation was performed 20.0 min after the moment of kindled PTZ (30.0 mg/kg, i.p.) administration and 4.0 min after the moment of cessation of generalized ictal EEG discharge. The intensity of stimulation was 100.0 mcA. (D) ES of paleocerebellum after DIA administration inhibited spike activity. Stimulation was performed 17.5 min after the moment of kindled PTZ (30.0 mg/kg, i.p.) administration and 3.5 min after the moment of cessation of generalized ictal EEG discharge. The rat was treated with DIA (1.0 mg/kg, i.p.). The intensity of stimulation was at the lower border (50.0 mcA). For A–C: 1 and 3 indicate frontal cortex of the left and right hemispheres; 2 and 4 indicate ventral hippocampus of corresponding hemispheres.

5.0 ml of 0.9% NaCl solution heated to 35°C was injected i.p. at the end of surgery. Penicillin potassium salt (100,000 IU/kg, intramuscular injection) was also administered every 12 h during 48 h post-surgery to prevent infection.

Animals were allowed to recover for 10 to 14 days after surgery before observations. At this stage, a total of 11 rats were excluded from further observation due to perioperative complications: infection (5 rats), poor electrodes fixation (3 rats), and death (3 rats).

Diazepam administration

DIA (Calmpose, Ranbaxy Diagnostics, India) was administered in a single injection in doses of 0.1 and 1.0 mg/kg, i.p. (DIA-0.1 and DIA-1.0, respectively). These doses are classified as ineffective and semi-effective, respectively, against PTZ-induced kindled seizures (Ishizawa et al., 2000). The compound was dissolved in 0.9% saline and 5.0% dextrose solution and administered in a volume of 0.3-0.5 ml 30 min before the testing PTZ injection performed after generalized clonic-tonic kindled seizures establishment. Control animals were treated with the same volume of 0.9% saline solution only.

Electrical stimulation

ES was conducted using the electrical stimulator ESU-2 (universal electrical stimulator, FSU), with a 100 Hz impulse frequency, 0.25 ms monophasic pulse duration, 50-100 mcA intensity, and 4.5-5.0 s ES duration, manually controlled. The intensity of ES was 20% less than the threshold for behavioral reactions (arrests of on-going locomotor activity). That intensity was adjusted individually during the second to third day after the last (21st) kindled PTZ administration. Stimulations were repeated every 3.5 to 4.0 min; however, no behavioral reactions or interruption of locomotor activity resulting from ES were observed (Godlevsky et al., 2014). Sham stimulations were conducted by connecting the animal's electrodes to the stimulator without delivering electrical current.

Behavioral and EEG data acquisition and analysis

The analog data were acquired using computer electroencephalograph DX-5000 (Charkov, Ukraine), and the data were digitized at a 256 Hz sampling rate. The time constant was 0.1, and the low-pass filter was set at 70 Hz. Extremely low frequency and high amplitude (excessive) waves on EEG synchronously appearing in all leads and coinciding with behavioral movements were treated as artifacts and were excluded from the analysis. The polygraph recordings were analyzed off-line visually, and epochs containing artifacts were eliminated.

Visual control of stimulation electrodes location

All experimental animals were euthanized with Nembutal (100.0 mg/kg, i.p.). Upon completion of the experiment, the visual quality control of the electrode placement was *ex tempore* on the gently removed tissue (no transcardial perfusion). For this purpose, electrocoagulation was performed in the electrode placement area, applying the direct current with an amplitude of 5.0 mA over 30 s, and using the electrodes as an anode (Godlevsky et al., 2014). The visual control of the location of stimulation electrodes confirmed their placement in a medial part of the vermis cortex with their location in merges of VI lobule between AP – from -12.5 to -15.0, while lateral locations were ±1.0 from the midline. Animals with electrodes inserted deeper than 0.5 mm from the surface or located beyond coordinates mentioned above were excluded. Seven animals were excluded due to incorrect placement of stimulation electrode: 1 rat from the control group, 1 rat from the group with 5 trials of ES, 2 rats from the group with 20 trials of ES, 1 rat from the group with 5 trials of ES + DIA-0.1, and 2 rats from the group with 5 trials of ES and DIA-1.0.

Statistical analysis

Values were compared using one-way ANOVA and Newman-Keuls test for latency of seizures; Kruskal-Wallis followed with a *post hoc* test was used for seizure severity; and "z" criteria for comparing two proportions. The severity of seizures was presented as median (Me) and quartiles – first and third: Me (Q1-Q3). The latency of seizures was presented as a mean value (M) ± sigma deviation (SD). P values <0.05 were considered significant. To avoid the influence of outliers, only observations falling between median ±3.0 SD of the sample were included in the dataset. This procedure was only used for the latent period of first seizures and after exclusion of outlined data. The Shapiro-Wilk test for normality was used for the latent period. Five cases out of the total number of latency determination subjects were excluded due to their extreme length. Automatically, the data on seizure severity for those subjects was also excluded.

RESULTS

PTZ administration caused kindled seizures

Kindling development was demonstrated by an initial phase of "minor" epileptiform manifestations characterized as absence-like seizures observed after 2 to 7 PTZ administrations (van Luijtelaar et al., 2016). The following 2 to 4 PTZ administrations increased the intensity of twitching to the level of myoclonic seizure and caused rearing with clonic seizures of forelimbs of the rats. The generalized clonic-tonic seizures emerged in most experimental animals after 8 to 17 PTZ injections. During seizures, rats lost their balance, fell on their side, and demonstrated post-seizure depression on the EEG. The following 4 to 7 PTZ injections induced generalized seizures in all experimental animals.

In the course of daily PTZ-kindled administrations, characteristic shortening of latency to first seizure as well as increased seizure severity were observed. Consequently, after the last (21st) PTZ injection, latency was 85.4±12.4 s. At the same time, all 9 rats demonstrated generalized clonic-tonic seizures, which reiterated in 8 animals.

Diazepam and ES increased the latent period of behavioral seizures and prevented generalized seizures

Testing PTZ injection performed after 5 trials of ES delivery was followed with generalized clonic–tonic seizures in 8 out of 9 rats (z=0.008, P=0.993), and latency of first seizures did not differ from PTZ sham-stimulated rats ($F_{(1,17)}$=0.07, P>0.05; Table I). 20 ES of the cerebellar cortex increased the latent period of the first seizures by 34.0% compared to PTZ-treated sham-stimulated rats ($F_{(1,16)}$=12.73, P<0.01; Table I). PTZ-induced generalized seizures were registered in 5 out of 8 animals in the group with 20 ES, with recurrent seizures in 3 rats. In the sham-stimulated PTZ-treated group, 8 out of 9 animals demonstrated recurrent generalized seizures (z=1.700, P=0.089).

The administration of DIA-1.0 increased the latency of seizures by 21.0% compared to the sham-stimulated PTZ-treated rats ($F_{(1,15)}$=0.68, P>0.05) and prevented repeated generalized seizures in 7 out of 8 animals (z=2.659, P=0.008; Table I). DIA-0.1 did not have a significant effect on the investigated variables (Table I).

Rats treated with DIA-0.1 after 5 trials of ES followed by PTZ administration demonstrated a 43.8% increase of latent period before the first seizure, compared to the control group ($F_{(1,17)}$=13.05, P<0.01; Table I). In this group, 2 out of 9 animals developed repeated generalized seizures (z=2.369, P=0.018).

DIA-1.0 administered after 5 ES increased the latent period of seizures by 1.7 times compared to a similar index in the control group ($F_{(1,16)}$=27.39, P<0.001), completely prevented generalized repeated seizures (z=3.175, P=0.001), and significantly decreased seizure severity (H=12.802, P<0.05; Table I). Neither indices differed from those noted in groups treated with DIA-1.0, 20 ES or treated with DIA-0.1 after delivering 5 ES, although they were significantly different for other groups (Table I).

Table I. Behavioral characteristics of PTZ-induced seizures in kindled rats under conditions of paleocerebellar ES and diazepam administration.

Animal group	Latency of first seizures (seconds) (M±SD)	Number of rats with convulsions of stage						Seizure severity: Median (Me) (Q1-Q3)
		0	1	2	3	4	5	
Sham-stimulated (control) (n=9)	85.44±12.62	0	0	0	0	1	8	5.0 (5.0-5.0)
5 ES (n=9)	87.55±20.06	0	0	0	1	1	7	5.0 (5.0-5.0)
20 ES (n=8)	114.5±20.5	0	0	2	1	2	3	4.0 (2.75-5.0)
DIA-0.1 (n=7)	90.85±16.96	0	0	0	0	2	5	5.0 (5.0-5.0)
DIA-1.0 (n=8)	103.4±18.33	0	0	4	1	2	1***	2.5 (2.0-4.0)
5 ES+DIA-0.1 (n=9)	122.89±28.42*#	0	0	2	2	3	2**	4.0 (3.0-4.0)
5 ES+DIA-1.0 (n=8)	145.5±31.83*#	0	1	3	2	2	0***	2.5 (2.0-3.25)*

Notes: *P<0.05, **P<0.02, ***P<0.01 *vs.* control, and #P<0.05 *vs.* group with 5 electrical stimulations (ES). ANOVA-derived statistics for latency followed by Newman-Keuls test; for proportion of rats with generalized repeated seizures – z test; for seizure severity – by Kruskal-Wallis tests followed by Dunn test.

Effects of ES on electrographic PTZ-induced seizures

The number of inhibitory responses registered in sham-stimulated kindled rats was 26.7% compared to the total number of test trials (Fig. 1A, Table II).

After 20 ES, half of all trials were followed by inhibition of PTZ-induced epileptic discharges (z=1.478, P=0.139). At the same time, facilitative responses were observed in 20.8% of the total number of trials. The comparable ratio of inhibitory and facilitative responses was registered after DIA-1.0 administration: up to 45.8 (z=0.651, P=0.515) and 16.7%, respectively.

In rats treated with DIA (0.1 mg/kg) after 5 ES, inhibition of seizure discharges was observed in 55.6% trials (z=1,950, P=0.051). Facilitation was observed in 11.1% trials, which was three times less when compared with the sham-stimulated rats treated with DIA-0.1 (z=1.422, P=0.155).

ES performed after DIA-1.0 administration was followed by inhibition of seizure discharges generated in 66.7% of trials, which significantly exceeded analogous indices in groups with sham-stimulations (z=2.664, P=0.008) and the group with 5 ES (z=2.101, P=0.036; Table II). The facilitating response was completely abolished as well, and this effect was also significant when compared with sham-stimulated and 5 ES- groups (z=2.572, P=0.01 and z=2.276, P=0.023, correspondingly) (Table II). The pronounced inhibition was characterized by complete inhibition of spikes both in the neocortex and the hippocampus during ES and after its cessation (Fig. 2B).

Interestingly, in all ES trials that were followed by the facilitation of EEG signs, the animal did not display behavioral seizure.

DISCUSSION

For the first time, we have established that ES (100 Hz, 20 trials of ES) of the paleocerebellar cortex (lobule VI) prevented generalized clonic-tonic seizure seizures induced by a testing dose of PTZ (30.0 mg/kg) in PTZ-kindled rats. Fewer ES (5 trials) did not prevent the emergence of seizures. DIA also effectively prevented PTZ-induced generalized seizures when administered at a dose of 1.0 mg/kg, but not at a lower dose (0.1 mg/kg). When the ineffective doses of DIA and ES were combined, apparent anti-seizure effects were revealed. This result indicates an additive interaction between DIA- and paleocerebellar ES-induced mechanisms on kindled generalized seizures. The increased ability of GABA inhibition to intensify the seizure-preventing effect of low-frequency brain stimulation upon amygdala-kindled seizures shown by Asgari et al. (2014, 2016) is in line with our data. Also, previously shown anxiolytic effects of VI lobule ES in kindled rats (Godlevsky et al., 2014) supports its role as a site of intensification of anti-seizure effects with DIA.

Epileptic discharges induced by PTZ administration in both the cortex and ventral hippocampus responded to paleocerebellar ES (100 Hz) with three types of responses – inhibition, facilitation, and absence of changes. Quantitative estimation of each response revealed

Table II. Paleocerebellar ES-induced changes of epileptic activity in brain structures of kindled animals in conditions of diazepam administration.

Animal group	Number of trials (ES)	Number of observed responses (%)		
		Inhibition	Changes are absent	Facilitation
Sham-stimulated (control) (n=9)	27	7 (25.9)	12 (44.4)	8 (29.6)
5 ES (n=9)	27	9 (33.3)	11 (40.7)	7 (25.9)
20 ES (n=8)	24	12 (50.0)	7 (29.2)	5 (20.8)
DIA-0.1 (n=7)	21	6 (28.6)	8 (38.1)	7 (33.3)
DIA-1.0 (n=8)	24	11 (45.8)	9 (37.5)	4 (16.7)
5 ES+DIA-0.1 (n=9)	27	15 (55.6)	9 (33.3)	3 (11.1)
5 ES+DIA-1.0 (n=8)	24	16 (66.7) (P<0.01)* (P<0.05)#	8 (33.3)	0 (P<0.01)* (P<0.05)#

Notes: *P<0.05 *vs.* control, and #P<0.05 *vs.* group with 5 ES. Statistics were derived by comparing two proportions (z test).

that inhibition was observed in 25.9%, while facilitation was observed in 29.6% out of 30 testing ES trials. Five trials consisted of preliminarily administered DIA (1.0 mg/kg) followed by ES, which presented significant inhibition (observed in 66.7% of ES trials), while facilitative effects were absent. Thus, the effectiveness of paleocerebellar anti-seizure ES with DIA was also demonstrated in electrographic kindled seizures.

The potentiation of paleocerebellar ES-induced inhibition of discharges in the cortex and the hippocampus with DIA points to the presence of diametrically opposite responses to ES in the underlying mechanisms: inhibition and facilitation. These opposing reactions could be explained by the different networks triggered by the stimulation, with a crucial role being played by functionally preserved inhibitory interneurons in the epileptogenic zone (Ojemann, 1980). This difference may also be due to opposite changes in neuronal activity originating from the zone of stimulation during ES and in the post-stimulatory period. During ES of the cortex, PC activity is drastically reduced due to the intrinsic prevalence of inhibitory control in the cerebellar cortex network, as a principle of its functionality (Dauth et al., 1978; Wisden et al., 2009). Inversely, after ES and disinhibition of PCs, a rebound in their activity is strongly expected. This assumption is in line with data on inhibition of spontaneous temporal lobe seizures caused by the optogenetic increase of PC activity (Krook-Magnuson et al., 2014).

It is unclear though which mechanism drives the inhibition of seizure discharges observed during ES when PC activity is reduced or even absent. Thus, antidromic activation of disinhibited nuclei might have resulted from cortical ES (Bantli et al., 1976; Daskalakis et al., 2004). Such an activation might be responsible for anti-seizure effects as far as direct ES of cerebellar nuclei can induce seizure inhibition (Maiti and Snider, 1975; Shandra and Godlevsky, 1990; Wang et al., 2008). The anti-seizure role of cerebellar nuclei is supported by research that found a correlation between progressive amygdala-kindled seizures and the reduced functional activity of cerebellar nuclei (Rijkers et al., 2015). Hence, an increased functional state of cerebellar nuclei might direct the inhibition of epileptic discharges observed during the vermis cortex ES.

The pathway from the dentate nucleus to motor thalamic nuclei, including ventral, anterior, lateral, and medial, is composed of excitatory afferent nerve fibers (Groenewegen and Witter, 2004). This allows high-frequency impulses (conveyed from the zone of vermis ES) to induce prolonged depolarization of thalamic neurons and disrupt thalamocortical oscillations (Salt and Copeland, 2017). Disruption of networks that support synchronization of epileptic discharges, namely the thalamocortical oscillatory system, might be considered the leading mechanism responsible for the inhibition of seizure discharges observed during the period of ES delivered to the vermis cortex. This assumption is congruent with the anti-seizure mechanism of DIA action, which likely occurs *via* inhibition of the oscillatory thalamocortical system (Christian et al., 2013).

CONCLUSION

We identified that ES (100 Hz) of the cerebellar vermis cortex (lobule VI) performed before PTZ administration prevented repeated PTZ-kindled clonic-tonic seizures in 5 out of 8 animals. Administration of either DIA-0.1 or 5 trials of ES alone was ineffective on seizures. Yet, when combined, they significantly prolonged the latent period to the first PTZ-kindled seizure and prevented repeated PTZ clonic-tonic seizures in 7 out of 9 animals. The combination of DIA-1.0 and 5 ES increased latency to seizure onset, prevented the recurrence of clonic-tonic seizures and reduced seizure severity in kindled rats. Electrographic signs of kindled seizures registered in the frontal cortex and ventral hippocampus demonstrated different dynamics during ES: facilitation by increasing the seizure discharge frequency to ictal-like discharge levels, neutral effect with the absent modification of frequency and amplitude of discharges, and inhibition of seizure discharges. DIA-1.0 with 5 trials of ES significantly increased the number of inhibitory responses, induced during ES with complete prevention of facilitative responses.

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