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Synergistic action of CB_1 and 5-HT_{2B} receptors in preventing pilocarpine-induced status epilepticus in rats

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ABSTRACT

Endocannabinoids (eCBs) and serotonin (5-HT) play a neuromodulatory role in the central nervous system. Both eCBs and 5-HT regulate neuronal excitability and their pharmacological potentiation has been shown to control seizures in pre-clinical and human studies. Compelling evidence indicates that eCB and 5-HT systems interact to modulate several physiological and pathological brain functions, such as food intake, pain, drug addiction, depression, and anxiety. Nevertheless, there is no evidence of an eCB/5-HT interaction in experimental and human epilepsies, including status epilepticus (SE). Here, we performed video-EEG recording in behaving rats treated with the pro-convulsant agent pilocarpine (PILO), in order to study the effect of the activation of CB_1/5-HT_2 receptors and their interaction on SE. Synthetic cannabinoid agonist WIN55,212–2 (WIN) decreased behavioral seizure severity of PILO-induced SE at 2 mg/kg (but not at 1 and 5 mg/kg, i.p.), while 5-$HT_{2B/2C}$ receptor agonist RO60–0175 (RO; 1, 3, 10 mg/kg, i.p.) was devoid of any effect. RO 3 mg/kg was instead capable of potentiating the effect of WIN 2 mg/kg on the Racine scale score. Surprisingly, neither WIN 2 mg/kg nor RO 3 mg/kg had any effect on the incidence and the intensity of EEG seizures when administered alone. However, WIN + RO co-administration reduced the incidence and the severity of EEG SE and increased the latency to SE onset after PILO injection. WIN + RO effects were blocked by the selective CB_1R antagonist AM251 and the 5-HT_{2B}R antagonist RS127445, but not by the 5-HT_{2C}R antagonist SB242084 or the 5-HT_{2A}R antagonist MDL11,939.

These data revealed a synergistic interaction between CB_1R/5-HT_{2B}R in the expression of PILO-induced SE.

1. Introduction

Status epilepticus (SE) is considered the most extreme form of a seizure and has serious long-term consequences, including neuronal death, neuronal injury, alteration of neuronal networks and development of temporal lobe epilepsy (TLE) and mesial temporal sclerosis (Trinka et al., 2015). Currently, benzodiazepines or barbiturates represent the first choice of management of SE, both which enhance inhibitory GABAergic activity (Meierkord et al., 2010). Unfortunately, up to 30–40% of SE patients are refractory to these treatments (Marawar et al., 2018). For this reason, much effort has been made to further our understanding of the pathological mechanisms underlying the development of the SE, with the aim of discovering alternative drugs for its treatments. Animal models of chemically-induced TLE have been very useful in this field, especially the pilocarpine (PILO)-induced SE (Curia et al., 2008; Levesque et al., 2016). Endocannabinoid (eCB) and serotonin (5-HT) systems play an important role in the control of neuronal excitability and epilepsy. The cannabinoid type 1 receptor (CB_1R) has been shown to mediate the anticonvulsant effects of both exogenous and endogenous CBs in different animal models of seizure and epilepsy (Katona, 2015). Synthetic cannabinoid agonist WIN55,212 (WIN) (Howlett et al., 2002) showed a CB_1R-mediated anti-seizure effect in the maximal dentate activation (MDA) model of limbic seizure in rats (Colangeli et al., 2017), an increased seizure threshold in the acute

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model of pentylenetetrazole (PTZ)-induced seizure in mice (Naderi et al., 2012), and a reduced incidence of spontaneous recurrent seizures following PILO-induced SE in rats (Wallace et al., 2001). Consistently, blockade of CB_1R activity increases seizure severity in different seizure models (Kow et al., 2014; Marsicano et al., 2003). However, other studies noted a pro-convulsant effect of WIN, likely due to the different doses, models of epilepsy and animal strain utilized (Katona, 2015). Limited evidence exists regarding the role of the cannabinoid system in generalized convulsive SE. Two studies showed that WIN was effective in preventing SE activity induced by low Mg^{2+} levels in primary hippocampal neuronal cultures (Blair et al., 2006) as well as in reducing the severity of behavioral seizures of PILO-induced SE in rats (Di Maio et al., 2015). However, CB_1R agonist CP55940 did not show any anti-epileptic effect in PILO-induced SE in mice (Kow et al., 2014).

Compelling evidence also indicates that 5-HT neurotransmission modulates a wide variety of experimentally-induced seizures (Bagdy et al., 2007). Generally, reduced 5-HT neurotransmission has been found to facilitate convulsive responses in several models of seizure induction (Bagdy et al., 2007). Moreover, nonselective 5-$HT_{1/2}R$ agonists reduced seizure activity in both the MDA model of mesial TLE in rats (Orban et al., 2014; Orban et al., 2013) and in the Genetic Absence Epilepsy Rats from Strasbourg (GAERS) (Venzi et al., 2016). The existence of crosstalk between the eCB and the 5-HT system has been previously demonstrated (Haj-Dahmane and Shen, 2011; Howlett et al., 2002; Mendiguren et al., 2018). For instance, 5-$HT_{2A}R$ activation induces eCB release which in turn activates CB_1Rs resulting in synapse inhibition in the inferior olive nucleus (Best and Regehr, 2008). Additionally, CB_1R knockout mice (KO) exhibit impaired serotonergic negative feedback and altered expression and functionality of 5-HT_2Rs in several brain regions (Aso et al., 2009). Moreover, the existence of functional CB_1R/5-$HT_{2A}R$ heteromers has been reported that seem only to be responsible for the cognitive deficits induced by CB_1R activation (Vinals et al., 2015).

Here, we investigated the involvement of the CB_1R/5-HT_2R interaction in the prevention of SE induced by PILO administration in rats. CB_1R/5-HT_2R interactions were explored by pharmacological manipulation of these two systems using the $CB_{1/2}R$ agonist WIN and CB_1R antagonist AM251 (AM), in addition to the 5-$HT_{2B/2C}R$ agonist RO60–0175 (RO) and 5-$HT_{2A}R$, 5-$HT_{2B}R$, 5-$HT_{2C}R$ selective antagonists, MDL11,939 (MDL), RS127445 (RS) and SB242084 (SB), respectively. We revealed a synergistic interaction between CB_1R/5-$HT_{2B}R$ and excluded an involvement of 5-$HT_{2A}Rs$ and 5-$HT_{2C}Rs$ in halting the SE. Single CB_1R activation was effective in controlling behavioral but not electrographic seizures, and 5-$HT_{2B/C}R$ had no effect in blocking either behavioral or electrographic SE.

2. Methods

2.1. Animals

Experiments were performed using adult male Sprague-Dawley rats (Charles Rivers, IT) weighing between 250 and 300 g. Rats were housed with controlled lighting (lights on 06:30–18:30 h), with free access to food and water. Procedures involving animals and their care were in accordance with the European Council Directive 2010/63/EU, the UK Animals Scientific Procedures Act 1986, scientific international guideline (Lidster et al., 2016) and local regulations regarding animals in research.

2.2. Drugs

(*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (*R*)-(+)-WIN 55,212-2), (αS)-6-Chloro-5-fluoro-α methyl-1H-indole-1-ethanamine fumarate (RO60-0175), 6-Chloro-2,3-dihydro-5-methyl-N [6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxyamide dihydrochloride (SB242084), 4-(4-Fluoro-1-naphthalenyl)-6-(1-methylethyl)-2-pyrimidinamine hydrochloride (RS127445), diazepam and ketamine were purchased from Tocris Cookson Ltd. (Bristol, United Kingdom). N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1-Hpyrazole-3 carboxamide (AM251) was purchased from abcam (Cambridge, United Kingdom). (3*S*,4*R*)-4,5-Dihydro-3-ethyl-4-(1-methyl-1H-imidazol-5-ylmethyl)-2(3H)-furanone hydrochloride, (Pilocarpine), (−)Scopolamine methyl nitrate and pentobarbital sodium salt were purchased from Sigma-Aldrich.

2.3. Surgery

Animals were anesthetized with a 5% isoflurane, maintained between 1% and 2% and positioned in a David Kopf stereotaxic frame. Body temperature was maintained by a heating pad and a temperature controller unit (Temperature Control Unit HB 101/2, Letica Scientific Instruments). Lidocaine was additionally subcutaneously injected at the incision site to minimize any local pain. Epidural screw electrodes were implanted bilaterally above the frontal and parietal cortex to record fronto-parietal EEG. Two additional screws were implanted above the cerebellum and used as ground electrodes. Electrodes were connected to a miniature connector (Straight PCB Socket PCB PRECI-DIP SA) and then secured to the skull with dental acrylic cement. All animals were housed individually after surgery and were allowed to recover for at least 10 days before the experiments.

2.4. Experimental protocol, drug preparation, and treatments

All drugs were dissolved in a vehicle composed of 20% DMSO in saline in a dose volume of 2 ml/kg. An equal volume of vehicle and compound was administered in each experimental condition. Pilocarpine (PILO; 360 mg/kg, i.p.) and scopolamine methyl nitrate (SCOP; 1 mg/kg, i.p.) were dissolved in saline. SCOP was administered 30 min before PILO treatment. Vehicle, WIN (1, 2, 5 mg/kg, i.p.) and RO (1, 3, 10 mg/kg, i.p.) either alone, or in combination (WIN + RO), were administered 15 min prior to SCOP injection. The 5-$HT_{2C}R$ antagonist SB (2 mg/kg, i.p.) the CB_1R antagonist AM (2 mg/kg, i.p.), the 5-$HT_{2A}R$ antagonist MDL (1 mg/kg, i.p.) or the 5-$HT_{2B}R$ antagonist RS (2 mg/kg, i.p.) were pre-administered 15 min before WIN + RO treatment or vehicle treatment. All the doses of the drugs used here were taken from previous studies (Colangeli et al., 2017; Di Maio et al., 2015; Orban et al., 2014; Venzi et al., 2016) or preliminary experiments (i.e., RS).

2.5. Pilocarpine treatment

Behavioral studies were conducted to choose effective doses of WIN and RO in blocking the PILO-induced SE. After PILO injection, ~85% of animals were motionless, displaying oro-facial movements, salivation, eye-blinking, twitching of vibrissae, and yawning. Discontinuous seizures, lasting up to 30–40 min from PILO injection, were then observed before animals displayed SE, which was characterized by intense salivation, rearing, forelimb clonus, and falling. The severity (in grades of the Racine Scale (Racine, 1972)), of SE was evaluated in the different groups of treated animals as shown previously (Di Maio et al., 2015). Behavioral studies were conducted for 2 h observation after PILO injection. SE was terminated straight after using diazepam (5 mg/kg, s.c.) and ketamine (50 mg/kg, s.c.) (Martin and Kapur, 2008). For EEG studies, the SE state was defined by a sustained and continuous seizure activity associated with large increase in the root mean square (RMS) power (Phelan et al., 2015; Turski et al., 1987). Mortality during SE was also recorded. Rats from behavioral and electrographic studies were euthanized with an overdose of pentobarbital sodium (100 mg/kg, i.p.) at the end of the experiments.

2.6. Data acquisition and analysis

For EEG recording, a Supertech Universal Biological Amplifier SBA4-v6 (Supertech Ltd., Pecs, Hungary, high pass: 0.16 Hz, low pass: 150 Hz, gain: 1000) was used and the video was captured by webcam. Both the EEG and the video were digitized by a CED 1401 plus analog-digital converter (Cambridge Electronic Design Ltd., Cambridge, UK), stored on a computer and analyzed offline using Spike2 7.11 software. The sampling rate was set to 300 Hz.

EEG activities were analyzed using a fast Fourier transform (FFT) algorithm (SUDSA22 script, Spike2 software; CED). Power spectra were generated for every 60 s epoch of the EEG, through the analysis of overlapping 1024-sample segments that were windowed with a raised cosine (Hanning) and subjected to an FFT. Each 60 s epoch was analyzed for absolute power in six bandwidths: full bandwidth (0.5–150 Hz), delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), beta (12–30 Hz), and gamma (30–70 Hz). The RMS of the absolute power was then calculated in each bandwidth, normalized respective to the baseline period (pre-drug condition) and expressed as a percentage.

2.7. Statistical analysis

Behavioral seizure severity scores data were analyzed with Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post hoc test. To compare the incidence of the PILO-induced SE between groups the $\chi 2$ test followed by Fisher's exact test was used. Normally distributed data were analyzed by either one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or repeated measures ANOVA followed by Tukey's post-hoc test when appropriate. The significance was set at $p < .05$.

3. Results

3.1. Dose-dependent effect of WIN55,212–2 and RO60–0175 as well as their coadministration on PILO-induced behavioral SE

As shown in Fig. 1A–C, Kruskal-Wallis one way ANOVA revealed significant treatment effects ($H_{10} = 119.0$; $p < .001$) on the behavioral severity of PILO-induced seizures. Rats pre-treated with the CB_1R agonist WIN, administered alone at different doses, exhibited significantly reduced severity of PILO-induced seizure (measured by the Racine scale) at the dose of 2 mg/kg ($n = 25$), but not with 1 mg/kg (n = 25) or 5 mg/kg doses (n = 25) ($p < .001$, Dunn's post-hoc test, Fig. 1A). Such results indicate an inverted U-shaped dose dependent anti-seizure action, in agreement with published evidence (Di Maio et al., 2015). When injected alone, the non-selective 5-$HT_{2B/2C}R$ agonist RO had no effect on the behavioral severity of PILO-induced seizures at any of the doses tested here (RO 0 mg/kg, $n = 18$; RO 1 mg/kg; n = 18; RO 3 mg/kg; n = 18; RO 10 mg/kg; n = 18; $p > .1$, Fig. 1B). Intriguingly, when administered in combination with the 2 mg/kg WIN dose, only the 3 mg/kg dose of RO potentiated the effect of WIN in reducing the severity of seizures (WIN + RO; n = 25; $p < .05$, Fig. 1C). In agreement to previous evidence (Di Maio et al., 2015), these results confirm that WIN is capable of reducing behavioral seizure severity and, for the first time, show a positive addictive effect of co-targeting CB_1 and 5-HT_2 receptors.

3.2. WIN + RO co-administration, but not WIN or RO injected alone, significantly reduced the electrographic incidence of the PILO-induced SE

Successively, in another group of animals implanted for EEG recording, we tested whether treatment with the most effective dose of WIN (2 mg/kg), administered alone or in combination with RO (3 mg/kg), was able to prevent the occurrence of the electrographic PILO-induced SE (Fig. 2A-D). In vehicle pre-treated rats, PILO caused electrographic SE in 87.5% of animals tested (14 rats out of 16, Fig. 2E). Contingency analysis ($p < .05$, $\chi 2$ test) revealed a significant treatment effect among the group analyzed (Fig. 2E–G). Interestingly, Fisher's exact test indicated that neither the cannabinoid agonist WIN (2 mg/kg; 9 rats out of 12; $p = .623$ vs vehicle) nor the serotonergic agonist RO (3 mg/kg; 11 rats out of 13; $p = 1.000$ vs vehicle) had any effect on the electrographic incidence of PILO-induced SE (Fig.2E). However, when these two compounds were co-administered, the incidence of the SE was significantly reduced as PILO-induced SE

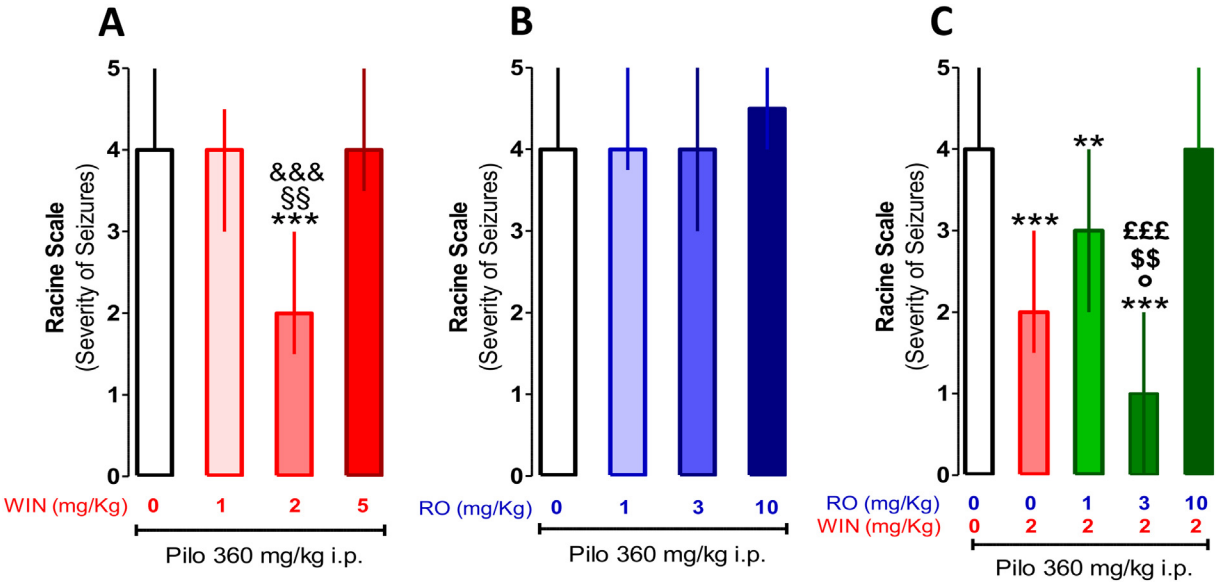

Fig. 1. Dose-response effect of the CB1 receptor agonist WIN (0, 1, 2, 5 mg/kg) (A) the 5-$HT_{2B/C}$ receptor agonist RO (0, 1, 3, 10 mg/kg) (B) or their combination (WIN + RO) (C) on behavioral seizure activity induced by intraperitoneal injection of PILO (360 mg/kg). The behavioral seizure activity was assessed using the Racine's scale (Racine, 1972); stage 0 = no seizures, 1 = freezing, 2 = orofacial clonus, 3 = unilateral forelimb clonus, 4 = bilateral forelimb clonus and rearing, 5 = tonic-clonic seizures with falling). Data are expressed as median and 25th and 75th percentiles. Kruskal Wallis test followed by Dunn's multiple comparison post-hoc test, $^{***}p < .001$ vs vehicle; $^{§§}p < .01$ vs WIN 1 mg/kg; $^{\&\&\&}p < .001$ vs WIN 5 mg/kg; $^{\circ}p < .05$ vs WIN 2 mg/kg; $^{\$\$}p < .01$ vs WIN 2 mg/kg + RO 1 mg/kg $^{£££}p < .001$ vs WIN 2 mg/kg + RO 10 mg/kg.

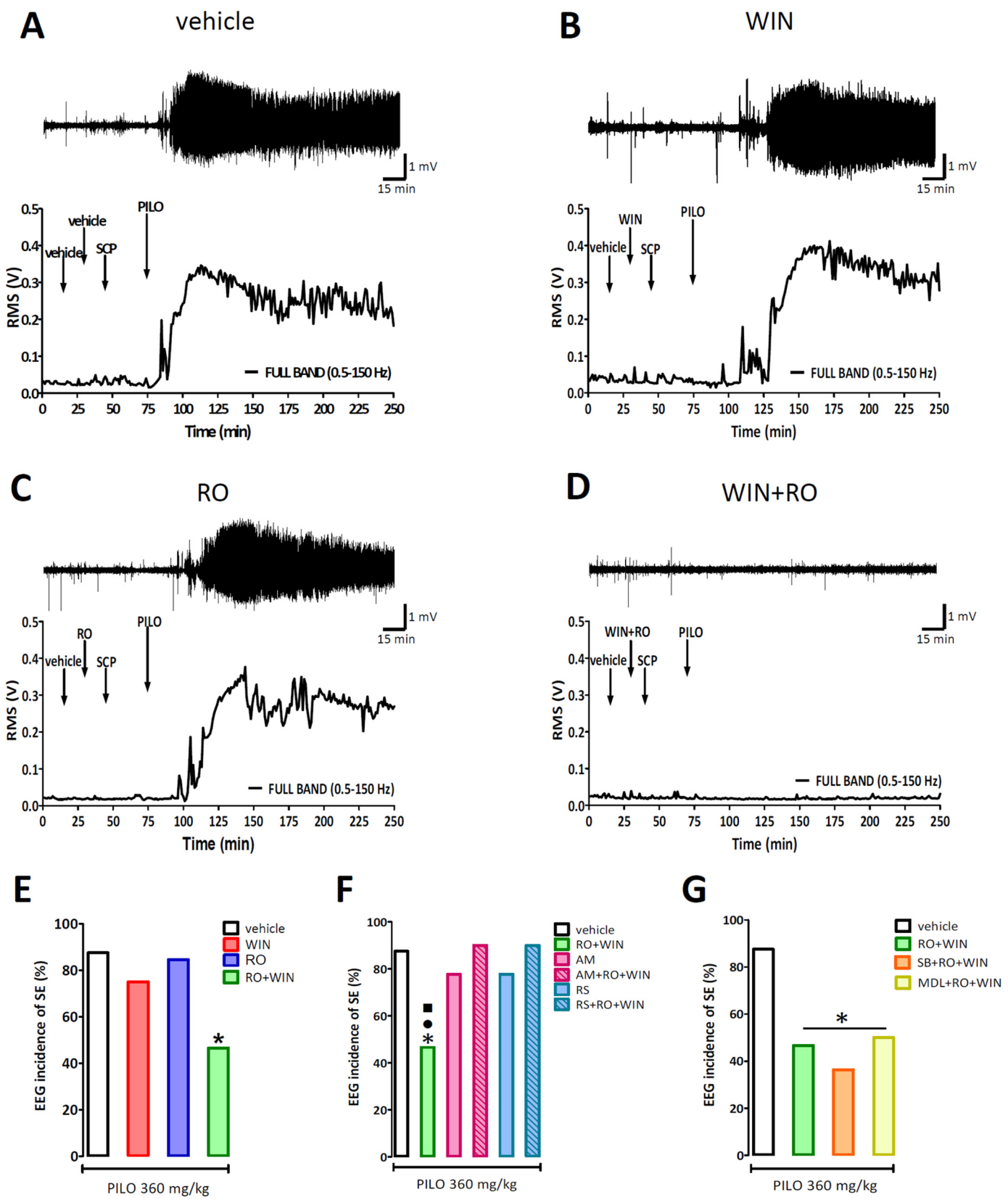

Fig. 2. Representative EEG recordings with the corresponding RMS power value of the EEG signals before and after PILO treatment (360 mg/kg) in vehicle (A), WIN (2 mg/kg) (B); RO (3 mg/kg) (C) and WIN + RO (2 mg/kg + 3 mg/kg, respectively) pre-treated rats (D). The incidence, expressed as a percentage of PILO-induced SE (87.5%) in WIN (75%), RO (84.6%) and WIN + RO pre-treated rats (46.6%) is shown in E. The CB1 receptor antagonist AM (77.7%) and the 5-HT_{2B} receptor antagonist RS (77.7%) had no effect when injected alone, whilst they were able to prevent the WIN + RO antiseizure effect (AM + WIN + RO, 90%; RS + RO + WIN 90%) as shown as a bar graph in F. The bar graph in G shows the failure of the 5-HT_{2C} receptor antagonist SB (36.3%) and the 5-HT_{2A} receptor antagonist MDL (50%) in preventing the WIN + RO antiseizure effect. Fisher exact test: *$p < .05$ vs vehicle; •$p < .05$ vs AM + WIN + RO; ■$p < .05$ vs RS + WIN + RO.

occurred in only 46.6% of the rats tested (7 rats out of 15; $p < .05$, WIN + RO vs vehicle, Fig. 2E). The CB_1R antagonist AM (2 mg/kg), administered 15 min prior to WIN + RO co-treatment completely prevented the WIN + RO anti-seizure effect (9 rats out of 10; $p < .05$, AM + WIN + RO vs WIN + RO; $p = 1.000$, AM + WIN + RO vs vehicle), whilst having no effect on the incidence of PILO-induced SE when administered alone (7 rats out of 9; $p = .601$, AM vs vehicle, Fig. 2F). Interestingly, the 5-HT_{2B}R antagonist RS (2 mg/kg) also prevented the RO + WIN anti-seizure effect (9 rats out of 10; $p < .05$, RS + RO + WIN vs WIN + RO, $p = 1.000$ RS + RO + WIN vs vehicle, Fig.2F), indicating the crucial role of CB_1R/5-HT_{2B}R co-activation in preventing SE. RS had no effect when injected alone (7 rats out of 9, $p = .601$ vs vehicle). On the other hand, pre-treatment with the 5-HT_{2C}R antagonist SB (2 mg/kg; 4 rats out of 11) and the 5-HT_{2A}R antagonist MDL (1 mg/kg; 5 out of 10) were ineffective in blocking the WIN + RO effect ($p = .701$, SB + WIN + RO vs WIN + RO; $p < .05$, SB + WIN + RO vs vehicle; $p = 1.0000$; MDL + WIN + RO vs WIN + RO and $p < .05$, MDL + WIN + RO vs vehicle), demonstrating the crucial role of CB_1R/5-HT_{2B}R co-activation in preventing PILO-induced SE (Fig.2G). The mortality rate associated with SE was not significantly affected by RO + WIN treatment (Fisher's exact test, $p = .521$), although all WIN + RO treated animals survived to SE (0 out of 7) compared to the vehicle-treated animals (3 out of 14) (data not shown).

3.3. WIN + RO co-administration significantly reduced the EEG intensity of SE

It has recently been shown that the behavioral severity of SE and the intensity of EEG signals during the SE phase do not always correlate (Phelan et al., 2015), thus we performed direct analysis of RMS power during SE to determine any significant effect of WIN (2 mg/kg), and RO (3 mg/kg), or their combined use, on the EEG intensity of SE. For this purpose, we performed a time course analysis of the EEG signals of rats which exhibited SE and then compared the RMS power during the baseline (pre-drug) period to that 60 min after the onset of SE. As shown in Fig. 3A–D, PILO-induced electrographic SE was characterized by a dramatic increase of RMS when compared to the pre-PILO baseline phase. As shown in Fig. 3E–G, repeated measure ANOVA revealed a significant PILO × Treatment interaction ($F_{9/64} = 3.38$; $p < .001$). Tukey's post-hoc analysis revealed that neither WIN (2 mg/kg, $n = 8$) nor RO (3 mg/kg, $n = 11$) significantly altered the PILO-induced effect on RMS cortical power respective to the vehicle-treated group (n = 11), while WIN + RO ($n = 7$) co-administration significantly reduced the EEG intensity of SE ($p < .001$, WIN + RO vs vehicle; p = n.s., WIN vs vehicle; p = n.s., RO vs vehicle; Fig. 3E). The combined effect of WIN + RO effect on RMS power was completely prevented by CB_1R antagonist AM ($n = 8$) and 5-HT_{2B}R antagonist RS pre-treatment ($n = 9$) ($p < .01$, WIN + RO vs AM + WIN + RO; $p < .05$, WIN + RO vs RS + WIN + RO, Fig. 3F), but not by 5-HT_{2C}R agonist SB ($n = 4$) or 5-HT_{2A}R antagonist MDL pre-treatment ($n = 5$) (p = n.s., WIN + RO vs SB + WIN + RO; p = n.s., WIN + RO vs MDL + WIN + RO, Fig. 3G).

3.4. WIN + RO co-administration significantly reduced PILO-induced increase of alpha band during SE

To better investigate the effect of WIN + RO co-administration on the PILO-induced increase of EEG intensity, we performed frequency band analysis, selecting individual bandwidths, 60 min after the SE onset. Fig. 4A,B shows a 5-min EEG recording with the corresponding spectral analysis performed 60 min after the SE for a representative (A) vehicle- and (B) WIN + RO-treated rat. As shown in Fig. 4C, PILO-induced increase of EEG activity involved the entire spectrum of the frequencies analyzed here (0.5–70 Hz), particularly those at the higher frequency, from theta (4–8 Hz) to beta (12–30 Hz), with the strongest increase occurring in the range of the alpha band (9–12 Hz; Fig.4C). One-way ANOVA revealed a significant treatment effect for alpha ($F_{9/73} = 5.067$; $p < .001$) and the beta ($F_{9/73} = 2.487$; $p < .0069$) bands (Fig. 4D–F). WIN + RO co-administration ($n = 7$) significantly reduced the PILO-induced increase of alpha activity ($p < .05$, WIN + RO vs vehicle; Fig. 4D), which was completely prevented by either AM ($n = 8$) or RS ($n = 9$) pre-treatment ($p < .01$ WIN + RO vs AM + WIN + RO; $p < .05$, WIN + RO vs RS + WIN + RO), whilst AM ($n = 5$) and RS ($n = 6$) per se were not effective (Fig. 4E). Moreover, SB ($n = 4$) or MDL (n = 5) pre-treatment did not block the changes induced by WIN + RO (p = n.s., WIN + RO vs SB + WIN + RO; p = n.s., WIN + RO vs MDL + WIN + RO, Fig. 4F). Interestingly, WIN + RO treatment caused a reduction of beta activity during SE when compared to WIN treatment (n = 8) ($p < .05$,WIN vs WIN + RO; Fig. 4D).

3.5. WIN + RO co-administration, but not WIN alone, significantly delay the time to first seizure appearance and the time to SE onset

The time between PILO administration and SE onset (latency to SE) is shown in Fig. 5 for a representative vehicle pre-treated rat (A) and WIN-RO pre-treated rat (B). The latency to SE is characterized by brief short-lasting seizures which occur until SE is full established. As shown in Fig. 5C–E, one-way ANOVA revealed a significant treatment effect for both the time to 1st seizure appearance ($F_{9/76} = 5.906$, $p < .001$) and the time to SE onset ($F_{9/76} = 6.146$, $p < .001$). Tukey's post-hoc analysis showed that WIN, when administered alone ($n = 8$), caused a shift of the time to 1st seizure and the time to SE onset with respect to the control group ($n = 12$) (time to 1st seizure, p = n.s, WIN vs vehicle; time to SE, p = n.s., WIN vs vehicle; Fig. 5C,F), although not statistically significant. Conversely, WIN + RO co-administration ($n = 7$) significantly increased both the latency to the 1st seizure ($p < .05$, WIN + RO vs vehicle) and the latency to SE with respect to the vehicle group ($p < .05$, WIN + RO vs vehicle; Fig. 5C,F), this effect was completely prevented by AM ($n = 9$) (1st seizure $p < .05$; time to SE $p < .05$; Fig. 5D,G) but not by RS (n = 9) pre-treatment (time to 1st seizure: p = n.s. RS + RO + WIN vs RO + WIN; p = n.s. RS + RO + WIN vs vehicle; time to SE: p = n.s. RS + RO + WIN vs RO + WIN; p = n.s. RS + RO + WIN vs vehicle, Fig. 5D,G). AM ($n = 6$) and RS (n = 6) did not change the latency to the 1st seizure and the latency to SE compared to the vehicle group (Fig. 5D,G). Neither SB ($n = 4$) nor MDL ($n = 5$) pre-treatments were able to prevent the WIN + RO effect during latency to the 1st seizure and the latency to SE (time to 1st seizure: p = n.s., WIN + RO vs SB + WIN + RO; time to SE: p = n.s., WIN + RO vs MDL + WIN + RO, Fig. 5E,H).

3.6. WIN + RO co-administration significantly reduced PILO-induced increase of gamma band during the latency to 1st seizure period

To further investigate the effect of WIN + RO combination during the latency period, we performed a frequency band analysis of the power spectrum during the 5 min preceding the appearance of the first seizure. Fig. 6 shows a 5-min EEG recording with the corresponding spectral analysis for a representative vehicle (A) and WIN + RO (B) treated rat. As shown in Fig. 6C, PILO injection induced a suppression of the full bandwidth power except for gamma (30–70 Hz) activity, which was instead increased with respect to the pre-PILO phase. Fig. 6D shows the prevention of PILO-induced gamma increase by WIN + RO treatment in a representative rat. One-way ANOVA revealed a significant treatment effect in gamma activity ($F_{9/76} = 10.83$; $p < .001$, Fig. 6E–G), and Tukey's post-hoc showed that WIN + RO co-administration (n = 7), but not WIN (n = 8) or RO ($n = 11$) injected alone, was able to prevent the PILO-induced gamma activity increase seen in vehicle group (n = 12; $p < .001$ WIN + RO vs vehicle, $p < .001$ WIN + RO vs WIN, $p < .001$ WIN + RO vs RO Fig. 6E). Both the CB_1R antagonist AM (n = 9) and the 5-HT_{2B}R antagonist RS (n = 9) reverted the WIN + RO effect on gamma activity ($p < .001$ WIN + RO vs AM + WIN + RO, $p < .001$ WIN + RO vs RS + WIN + RO, Fig. 6F), while having no effect per se (AM n = 6; RS n = 6). Neither the 5-HT_{2C}R

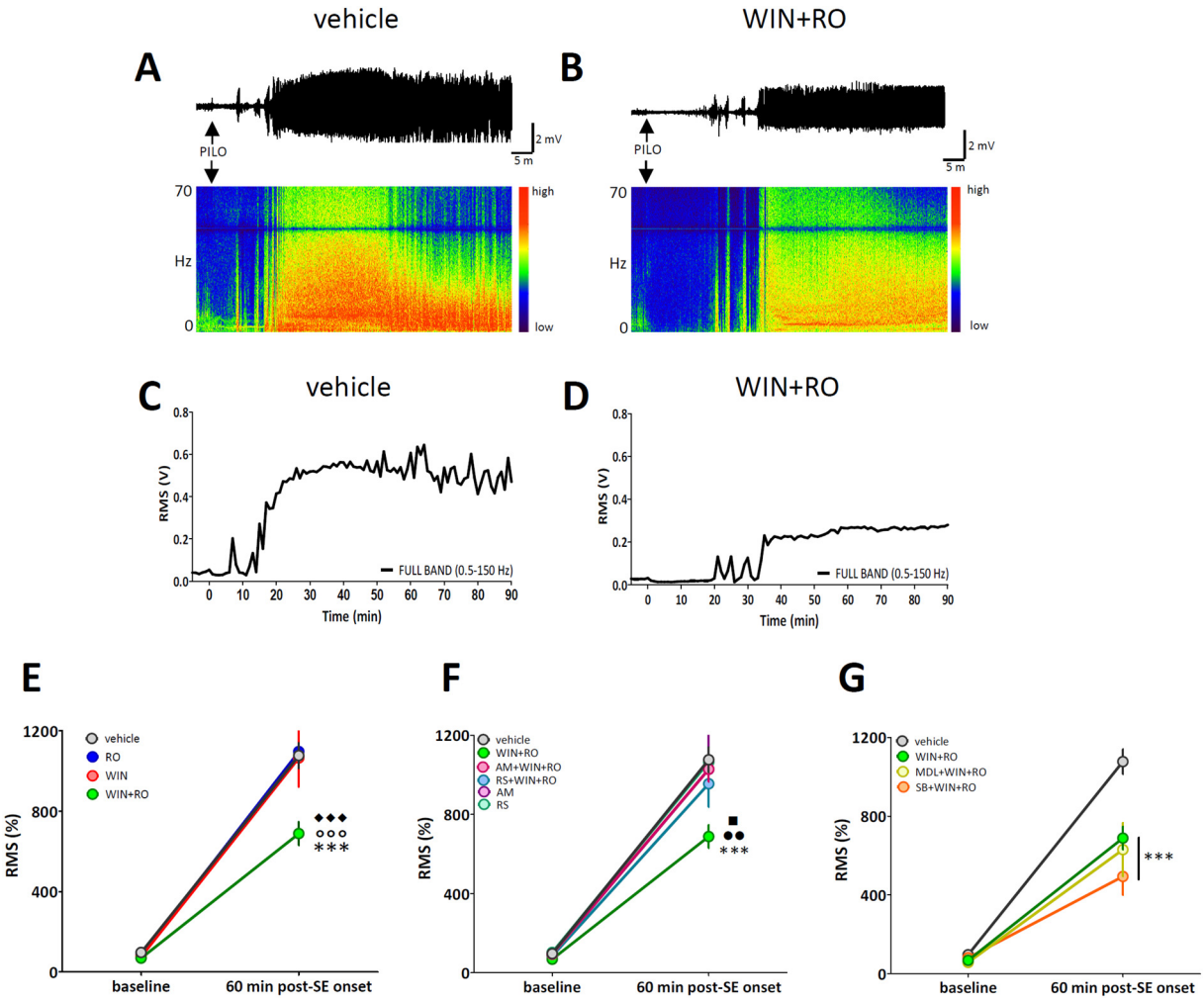

Fig. 3. EEG signals and the relative spectrograms on the same timescale from a representative vehicle treated rat (A) and a WIN + RO treated rat (B). The absolute RMS power of the full bandwidth corresponding to the vehicle-treated rat represented in A and the WIN + RO treated rat represented in B, are plotted in C and D, respectively. The graph in E shows the synergistic effect of WIN + RO on the RMS power, expressed as a percentage of the baseline phase. The WIN + RO effect was abolished by AM and RS (F), but not by the MDL or SB (G). Data are expressed as mean and SEM. Repeated measure ANOVA followed by Tukey's post hoc test: ***p < .001 vs vehicle; °°°p < .001 vs WIN; ◆◆◆p < .001 vs RO; ¨p < .01 vs AM + WIN + RO; ■p < .05 vs RS + WIN + RO.

antagonist SB (n = 4), nor the 5-HT_{2C}R antagonist MDL (n = 5) were able to prevent WIN + RO effect on the gamma wave (p < .001 MDL + WIN + RO vs vehicle, *p* < .01 SB + WIN + RO vs vehicle, p = n.s. WIN + RO vs SB + WIN + RO, p = n.s. WIN + RO vs MDL + WIN + RO, Fig. 6G).

4. Discussion

The main results of this study are threefold. Firstly, cannabinoid CB_1R activation was only capable of blocking behavioral but not electrographic PILO-induced SE. Secondly, we found that co-activation of CB_1Rs and 5-HT_{2B}Rs, via RO and WIN co-administration, significantly reduced the incidence of PILO-induced SE, strongly attenuated the behavioral and electrographic seizure intensity and significantly delayed the seizure onset. Thirdly, we showed that 5-$HT_{2A/2C}$Rs are not involved in the WIN + RO antiepileptic effect.

In the last two decades many studies have reported a protective role for both CB_1R (Katona, 2015) and 5-HT_2R (Bagdy et al., 2007), mainly the subtype 5-HT_{2C}R (Di Giovanni and De Deurwaerdere, 2016; Svob Strac et al., 2016; Venzi et al., 2016), in different seizures and epilepsy models. However, the effectiveness of CB_1R and 5-HT_{2C}R activation on the prevention of SE is controversial. As far as the CB_1Rs are concerned, behavioral sensitivity and severity of PILO-induced seizures were not altered by CP55940 in mice (Kow et al., 2014), while they were decreased by WIN in rats (Di Maio et al., 2015), further supporting the difference between the response of two rodent species to convulsants and/or the two CB_1R agonists' signal transduction (Lauckner et al., 2005). In addition, CB_1R indirect activation by increasing eCB brain concentration via blockade of the fatty acid amide hydrolase (FAAH) alleviated kainic acid (KA)-induced behavioral SE, but not electrographic SE in guinea pigs (Shubina et al., 2015; Shubina et al., 2017). Moreover, consistent proconvulsant effects have been obtained in CB_1 KO mice in PILO-induced SE (Kow et al., 2014) and in conditional KO mice lacking CB_1Rs on principal glutamatergic cells, but not GABA-$CB1^{-/-}$ mice in KA-induced SE (Monory et al., 2006).

Here, in agreement with previous evidence (Di Maio et al., 2015), we have found that WIN pre-treatment reduced the behavioral severity of SE in an inverted U-shaped dose-dependent fashion, with the lowest 1 mg/kg and highest 5 mg/kg doses found ineffective. U-shaped effects of WIN and other cannabinoid drugs such as URB597, AM404, JZL184 have been consistently described, for example, in memory and anxiety studies (Morena et al., 2014; Ratano et al., 2018; Rey et al., 2012). WIN anticonvulsant activity is likely CB_1R-mediated since it is blocked by 1 mg/kg AM pre-treatment (Di Maio et al., 2015). Strikingly, here we found that the dose of 2 mg/kg WIN, effective also in different seizures (Colangeli et al., 2017), reduced motor seizure severity but had no

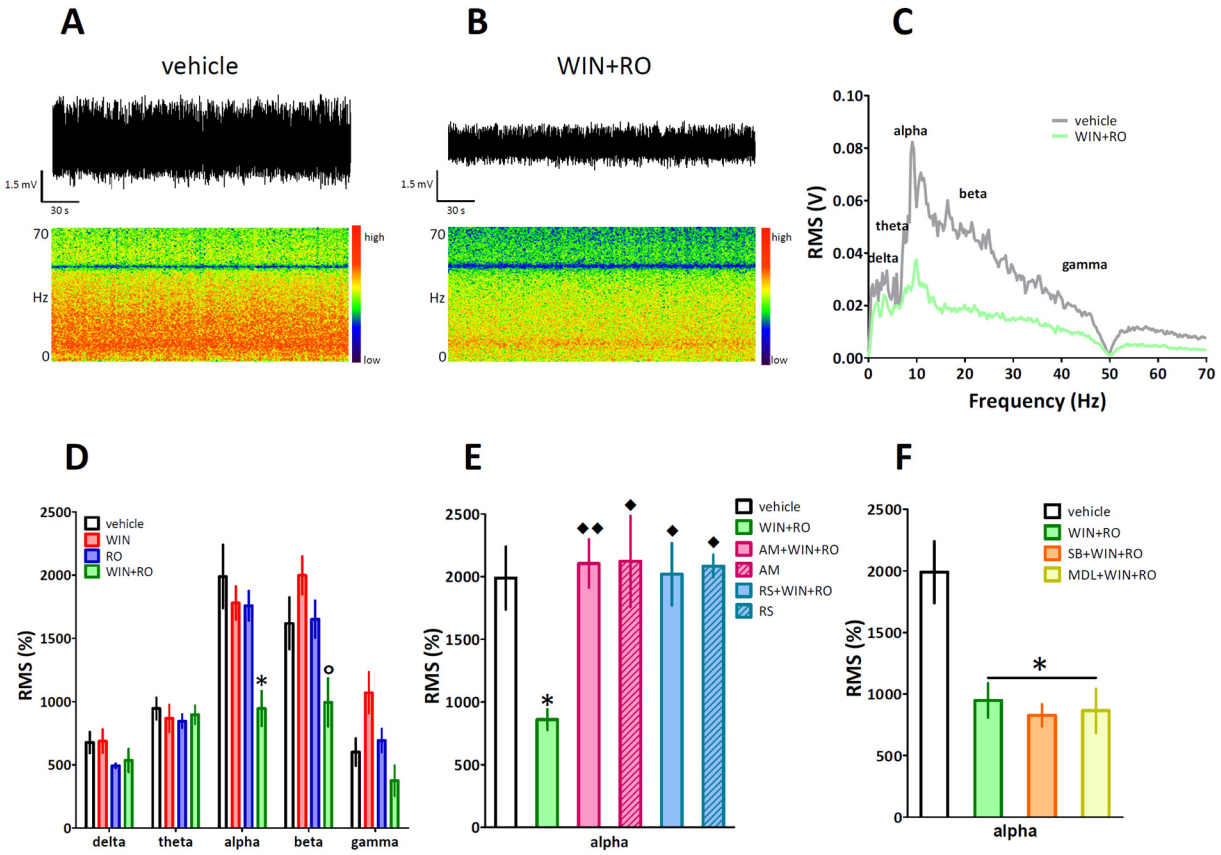

Fig. 4. EEG signals and the relative spectrograms on the same timescale from a representative vehicle treated rat (A) and a WIN + RO treated rat (B) are shown during the SE phase. Amplitude spectrum from fast Fourier transform (FFT) of time-domain EEG signal of the vehicle rat shown in A and the WIN + RO treated rat shown in B are plotted together in (C). Graph in D shows the effect of vehicle, WIN (2 mg/kg), RO (3 mg/kg) and WIN + RO on the relative RMS power analyzed in five bandwidths (delta 0.5–4 Hz; theta 4–8 Hz; alpha 8–12 Hz; beta 12–30 Hz; gamma 30–70 Hz) 60 min after SE onset. The effects of the antagonism of WIN + RO on the alpha band are shown in E and F. Data are expressed as mean and SEM. One-way ANOVA followed by Tukey's post hoc test: *$p < .05$ vs vehicle; °$p < .05$ vs WIN; ◆$p < .05$ vs RO + WIN. ◆◆$p < .01$ vs RO + WIN.

effect on either the electrographic intensity, measured as RMS power, or the incidence of rats displaying SE after PILO injection. Our data are in line with a previous observation that shows that the both FAAH inhibitor URB597 and the anandamide reuptake inhibitor AM404 reduce the behavioral severity of SE but do not affect EEG intensity of KA-induced SE in guinea pigs (Shubina et al., 2015). It is possible that the 2 mg/kg WIN used here which is not capable of affecting the paroxoistic activity in the cortex, might be effective on the seizures propagation from cortical to subcortical areas and the brainstem involved in the second generalization of the limbic seizures (Browning and Nelson, 1986; Hamani et al., 2004; Turski et al., 1987), thus preventing excessive behavioral manifestations.

To study the interaction of eCB with the 5-HT system we used the 5-HTR agonist RO, a common pharmacological tool for studying 5-HT_{2C}R function (Di Giovanni and De Deurwaerdere, 2016), although it has equal affinity for 5-HT_{2B}R and binds 5-HT_{2A}Rs with lower affinity (Di Giovanni and De Deurwaerdere, 2016; Martin et al., 1998).

Interestingly, when CB_1R was co-activated with 5-$HT_{2B/2C}$R by WIN and RO co-treatment, the anticonvulsant effect was visible both behaviourally and electrographically. The incidence of motor and EEG SE was significantly reduced by 50%, and in those rats in which SE occurred, both the behavioral severity and the EEG intensity of seizure were dramatically reduced compared to the action of the respective agonists tested alone. Several studies have demonstrated a functional interaction between eCB and 5-HT (Darmani et al., 2003; Egashira et al., 2002; Haj-Dahmane and Shen, 2011; Howlett et al., 2002; Mendiguren et al., 2018) as well as CB_1R and 5-HT_2R, which can be manifested in a synergistic fashion. For instance, a strong interplay between CB_1R and 5-HT_{2A}R has been shown, and this seems to occur through both functional and physical interaction (Vinals et al., 2015). Moreover, endogenous 5-HT is capable of activating postsynaptic 5-HT_{2A}Rs which evoke eCB production and release (Best and Regehr, 2008; Burattini et al., 2014), leading to an eCB-dependent LTD in the nucleus accumbens (Burattini et al., 2014) whilst decreasing glutamate release in the olive nucleus (Best and Regehr, 2008).

Surprisingly, neither the 5-HT_{2C}R nor the 5-HT_{2A}R antagonisms were able to prevent the CB_1R/5-HT_2R synergistic action on the induction of SE.

This lack of effect of RO-presumed activation of the 5-HT_{2C}Rs in PILO-induced behavioral and electrographic SE is in line with its ineffectivenes in the MDA model of TLE (Orban et al., 2014). Interestingly, when rats were pretreated with the selective 5-HT_{2C}R antagonist SB, RO induced a significant antiepileptic effect, suggesting that activation of the 5-HT_{2C}Rs is in reality pro-epileptic in this model of TLE (Orban et al., 2014). Moreover, the MDA epileptic rats showed a reduction of 5-HT_{2C}R-immunoreactivity in the hippocampus and enthorhinal cortex 4 h after the acute kindling protocol (Orban et al., 2014). On the other hand, a number of other findings displayed an upper-regulation of 5-HT_{2C}Rs in the hippocampus of chronic epileptic rats showing recurrent seizures after PILO-induced SE (Krishnakumar et al., 2009) and a general anticonvulsant effect of 5-HT_{2C}Rs in different type of epilepsies (Bagdy et al., 2007; Isaac, 2005; Venzi et al., 2016). In addition, increased audiogenic seizure susceptibility has been reported in mutant mouse models lacking functional 5-HT_{2C}Rs (Applegate and Tecott, 1998). This contrasting evidence suggests that 5-HT_{2C}Rs might play pro- or antiepileptic roles depending on the types of epilepsies.

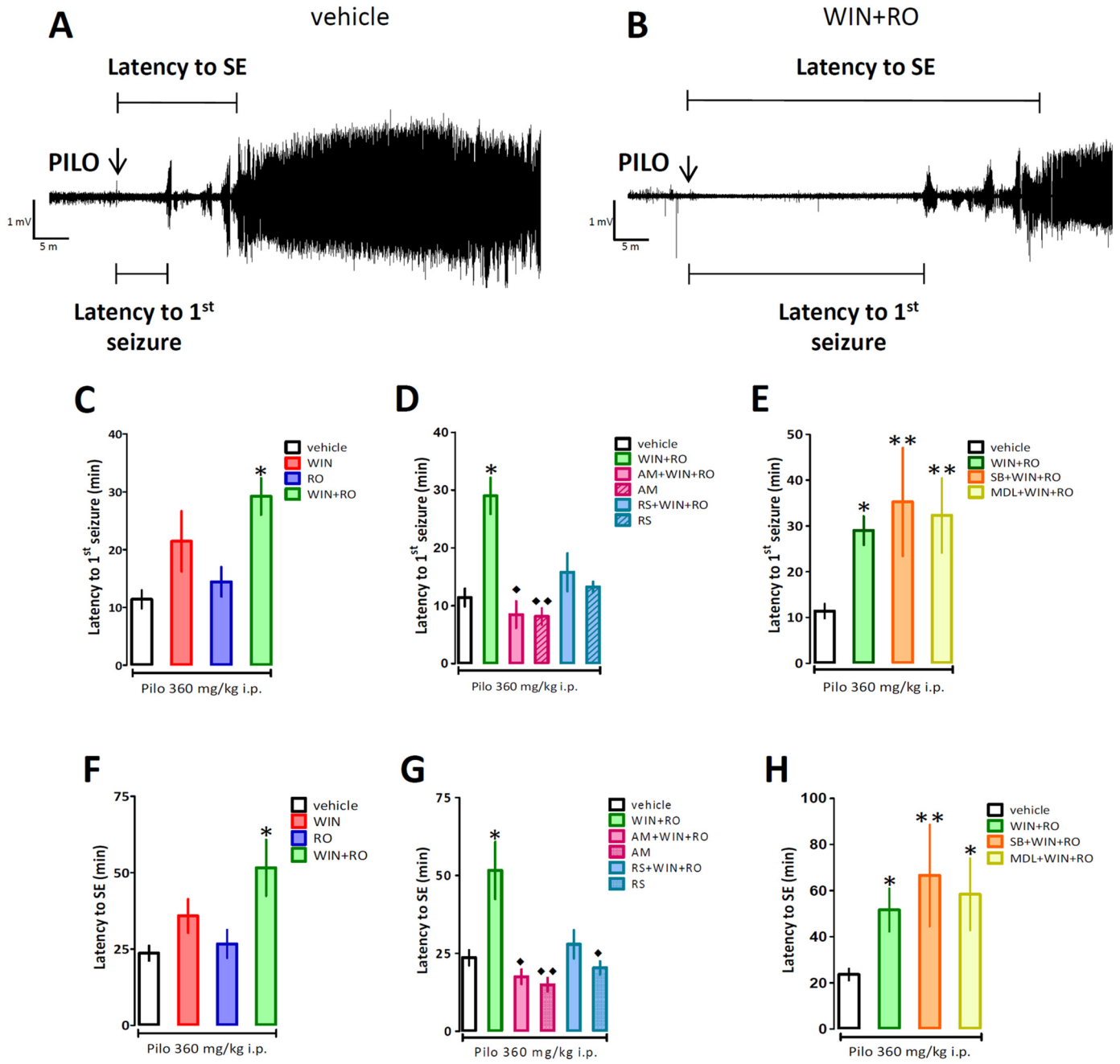

Fig. 5. Latency to the 1st seizure and the latency to SE onset are shown for a representative vehicle (A) and WIN + RO (B) treated-rat. The latency to the 1st seizure is the time measured from the PILO injection and the appearance of the 1st self-terminated epileptic-like discharge, while the latency to SE is the time measured from the PILO injection to the onset of continuous seizures occurring close together without recovery. The effect of the vehicle, WIN (2 mg/kg), RO (3 mg/kg) or their combination (WIN + RO) on the latency to 1st seizure and latency to SE are shown in C. The effects of the antagonism of WIN + RO on the latency to 1st seizure are shown in D and E. The effects of vehicle, WIN, RO and WIN + RO and the antagonism of WIN + RO on the latency to SE are shown in F, G and H, respectively. Data are expressed as mean and SEM. One-way ANOVA followed by Tukey's post hoc test: **p < .01 vs vehicle; *p < .05 vs vehicle; ◆p < .05 vs RO + WIN. ◆◆p < .01 vs RO + WIN.

Moreover, it is unknown if the acute and chronic 5-HT_{2C}R expression changes, observed in different epileptic brain areas, is an epiphenomenon, or a causative factor in TLE.

Here, we showed that instead 5-HT_{2B}R antagonism completely blocked the anticonvulsant effect of WIN + RO administration.

The role of the 5-HT_{2B}R in the brain is largely unknown, particularly in terms of seizures and epilepsy. To our knowledge, only one study has tested the effect of this receptor on seizure by using the PTZ-model of acute seizure with no appreciable results (Upton et al., 1998). 5-HT_{2B}R seems to be expressed presynaptically on 5-HT neurons and, contrary to other 5-HT autoreceptors which provide inhibitory feedback to 5-HT neurons, it acts as a stimulatory autoreceptor, thus promoting 5-HT release (Belmer et al., 2018; Diaz et al., 2012); consistently, 5-HT_{2B}R KO mice display a hyposerotonergic phenotype (Belmer et al., 2018). It is known that 5-HT neurons from the dorsal raphe heavily project to inhibitory interneurons in several brain regions crucially involved in seizure generation and seizure activity, such as the hippocampus, amygdala and cortex (Ciranna, 2006). It is likely that the stimulatory effect of 5-HT_{2B}R autoreceptors on 5-HT release potentiates GABA inhibitory activity in those brain regions during seizures. On the other

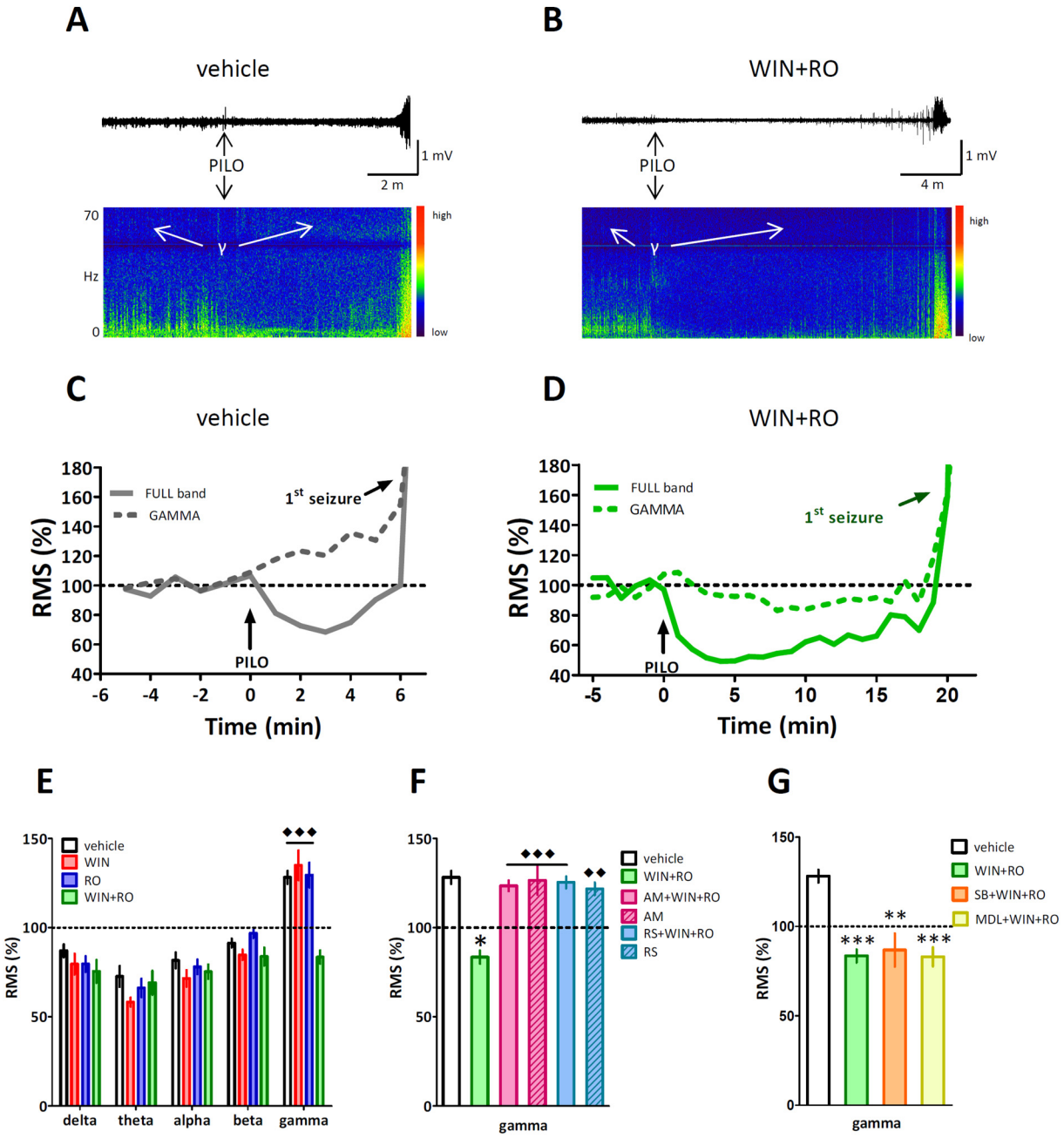

Fig. 6. EEG signals and the relative spectrograms on the same timescale for a representative vehicle treated rat (A) and a WIN + RO treated rat (B) are shown over time from PILO injection to appearance of the 1st self-terminated epileptic-like discharge. White arrows indicate the activity on the gamma range (30–70 Hz) on the spectrograms. Continuous RMS power analysis for full bandwidth and gamma band on the same time scale corresponding to the vehicle rat shown in A and WIN + RO treated rat shown in B are plotted together in C and D, respectively. Graph in E shows the effect of WIN (2 mg/kg), RO (3 mg/kg) and WIN + RO on the relative RMS power analyzed in five bandwidths (delta 0.5–4 Hz; theta 4–8 Hz; alpha 8–12 Hz; beta 12–30 Hz; gamma 30–70 Hz) 5 min before the appearance of the 1st self-terminated epileptic-like discharge. The effects of the antagonism of WIN + RO on the gamma band are shown in F and G. Data are expressed as mean and SEM. One-way ANOVA followed by Tukey's post hoc test: *** < 0.001 vs vehicle **p < .01 vs vehicle *p < .05 vs vehicle; ◆◆◆p < .001 vs RO + WIN; ◆◆p < .01 vs RO + WIN.

hand, although CB_1Rs can inhibit both glutamate and GABA release, broad CB_1R activation has been shown to cause a net reduction of neuronal excitability (Monory et al., 2006). In this scenario, it is tentative to speculate that the CB_1R/5-HT_{2B}R synergistic effect on PILO-induced SE might result from increased GABA inhibitory activity, triggered by enhanced 5-HT release through 5-HT_{2B}R activation, combined with a CB_1R-mediated reduction of glutamate release by WIN. Obviously, we cannot exclude an involvement of other neurotransmitter systems in the 5-HT_{2B}R effect, since it is very well known the 5-HT has a modulatory role in the CNS (De Deurwaerdere and Di Giovanni, 2017; Di Mascio et al., 1999).

To better investigate the CB_1R/5-HT_{2B}R synergistic antiseizure action, we performed RMS power analysis of EEG signals for individual bands during SE. Contrary to other studies (Phelan et al., 2014; Phelan et al., 2017), but in line with Terrone et al. (2018), we found that PILO increased the power of the entire spectrum of the frequencies analyzed here (0.5–70 Hz) with a predominant effect on alpha oscillatory activity (8–12 Hz). Interestingly, we found that alpha activity was the most affected band by the CB_1R/5-HT_{2B}R co-activation. Intriguingly, Terrone et al. (2018) also found that the eCB 2-AG hydrolysis inhibitor

administered after KA treatment caused a significant reduction of the alpha band (8–12 Hz), which was only partly mediated by CB_1R (Terrone et al., 2018). Further studies are needed to determine the physiological significance of alpha activity during the SE and the reason for the selective effect CB_1R/5-$HT_{2B}R$ co-activation on this oscillatory activity. Nevertheless, the relationship between the severity of SE and brain damage with consequent development of epilepsy has been reported (Lemos and Cavalheiro, 1995). Therefore, with regards to CB_1R/5-$HT_{2B}R$ co-activation, it would be interesting to investigate whether the reduction in EEG intensity, particularly the alpha oscillatory activity during the SE phase, might have a protective effect on the SE-induced functional changes that lead to chronic neuronal damage and the development of epilepsy.

Finally, we found that WIN + RO co-administration was anticonvulsive also by delaying the latency to the first seizure and the latency to SE after PILO injection, with respect to the agonists tested alone. One possible explanation for the delay in SE is that WIN + RO administration may have protected the blood-brain barrier, thereby slowing access of pilocarpine to the brain. Indeed, it has been shown that both WIN (Chi et al., 2012) and AP-267, a 5-HT_2R agonist (Sharma et al., 2006), were able to prevent blood-brain barrier dysfunction in rats.

To better investigate the CB_1R/5-$HT_{2B}R$ synergistic action on epileptogenesis, we analyzed the RMS power analysis of EEG signals for individual bands from PILO injection to the appearance of the first seizure. According to previous evidence (Phelan et al., 2014), we found a selective increase in the gamma band (30–70 Hz) induced by PILO. Interestingly, WIN + RO administration was able to prevent the PILO-induced increase in gamma oscillations which precede the appearance of the first seizure. The increased gamma activity after PILO administration is thought to trigger the consequent behavioral convulsion (Phelan et al., 2017), thereby it is possible that increased latency to first seizure found in WIN + RO treated rats might result from the action of CB_1R/5-$HT_{2B}R$ co-activation on gamma activity.

In contrast to earlier findings (Kow et al., 2014), but in agreement with others (Di Maio et al., 2015; Shubina et al., 2015), we did not find any pro-convulsant effect of the block of CB_1Rs by AM on the EEG severity of SE, although there was a trend toward a higher rate of the mortality induced by SE.

It is evident that co-stimulation of CB_1R and 5-$HT_{2B}R$ is essential to obtain the anticonvulsant effect, while single antagonism at CB_1R or 5-$HT_{2B}R$ can block this effect. Thus, it is possible that CB_1R and 5-$HT_{2B}R$ physically interact, leading to heteromer formation, and that activation of these heteromers produces the antiepileptic effect. This would not be surprising since CB_1Rs make heterodimers with other different G protein-coupled receptors (Hudson et al., 2010), including the 5-$HT_{2A}Rs$ (Vinals et al., 2015).

Co-targeting CB_1R or 5-$HT_{2B}R$ might also have some therapeutic relevance in treating SE given that propofol, effective in treating refractory SE (Brown and Levin, 1998), both activates the 5-$HT_{2B}Rs$ in the micromolar range (Matsunaga et al., 2015) and increases the production of eCBs (Hauer et al., 2011; Patel et al., 2003). Nonetheless, further studies are needed to clarify this important issue.

In conclusion, for the first time this study demonstrated an involvement of CB_1R/5-$HT_{2B}R$ interaction in the behavioral and electrographic expression of PILO-induced SE and established that these receptors are required for the genesis of this seizure activity. The contextual activation of CB_1R and 5-$HT_{2B}Rs$ results in a potent anticonvulsant effect by preventing SE and increasing the latency to seizure initiation induced by PILO treatment.

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Disclosure of conflict of interests

None of the other authors have any conflict of interest to disclose.

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