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Propylparaben applied after pilocarpine-induced status epilepticus modifies hippocampal excitability and glutamate release in rats

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ABSTRACT

Propylparaben (PPB) induces cardioprotection after ischemia–reperfusion injury by inhibiting voltage-dependent Na^+ channels. The present study focuses on investigating whether the i.p. application of 178 mg/kg PPB after pilocarpine-induced status epilepticus (SE) reduces the acute and long-term consequences of seizure activity. Initially, we investigated the effects of a single administration of PPB after SE. Our results revealed that compared to rats receiving diazepam (DZP) plus vehicle after 2 h of SE, animals receiving a single dose of PPB 1 h after DZP injection presented 126% ($p < 0.001$) lower extracellular levels of glutamate in the hippocampus. This effect was associated with an increased potency of low-frequency oscillations (0.1–13 Hz bands, $p < 0.001$), a reduced potency of 30–250 Hz bands ($p < 0.001$) and less neuronal damage in the hippocampus. The second experiment examined whether the subchronic administration of PPB during the post-SE period is able to prevent the long-term consequences of seizure activity. In comparison to animals that were treated subchronically with vehicle after SE, rats administered with PPB for 5 days presented lower hippocampal excitability and interictal glutamate release, astrogliosis, and neuroprotection in the dentate gyrus. Our data indicate that PPB, when applied after SE, can be used as a therapeutic strategy to reduce the consequences of seizure activity.

1. Introduction

The antimicrobial agent propylparaben (PPB) has been widely used as a preservative in drugs, cosmetics and food for more than 60 years (Soni et al., 2001). Using the Central Nervous System Multiparameter Optimization and Desirability Score (CNS MPO) developed by Wager et al. (2010), it was predicted that PPB can act in the brain due to its physicochemical properties, i.e., favorable permeability, metabolic stability and low P-glycoprotein efflux. This prediction and previous evidence obtained in dogs indicate that PPB reaches the brain tissue when applied systemically (Jones et al., 1956).

Studies have revealed that PPB blocks voltage-gated Na^+ channels in cardiomyocytes and induces cardioprotection in rats after ischemia–reperfusion injury (Ji et al., 2004). The data obtained in our laboratory show that PPB blocks Na^+ channels and decreases neuronal excitability in vitro (Lara-Valderrábano et al., 2016). Because Na^+ channels participate in the generation of nerve impulses that trigger the release of neurotransmitters such as glutamate, their blockage by PPB may induce inhibitory effects in the brain (Talevi et al., 2007). In addition, PPB may induce neuroprotection under conditions in which high glutamatergic neurotransmission leads to neuronal hyperexcitability and damage due to the overactivation of Na^+ channels.

Status epilepticus (SE) and epilepsy are associated with augmented extracellular levels of glutamate (Santana-Gómez et al., 2015; Soukupova et al., 2015). High glutamate levels and glutamatergic receptor activation have been associated with excitotoxicity and neuronal death in several structures of subjects experiencing SE (Fujikawa, 1996; Isokawa and Mello, 1991). However, at present, no effective pharmacological treatment is available to prevent neuronal damage after seizure activity (Kobow

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et al., 2012; Pitkänen and Lukasiuk, 2011; Rogawski and Löscher, 2004).

The present study investigated whether a single administration of PPB lessens the augmented release of glutamate and neuronal damage in hippocampus that are produced by pilocarpine-induced SE. We also investigated whether subchronic treatment with PPB after pilocarpine-induced SE can prevent the elevated glutamate release, neuronal loss, and hyperexcitability that occur in the hippocampus during chronic epileptic periods.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were maintained individually in acrylic cages under controlled environmental conditions (12-h light/dark cycles; temperature, 22 °C) with access to food and water ad libitum. All experiments were performed in accordance with the Mexican Official Norm (NOM-062-ZOO-1999) and with the approval of the Internal Committee for the Care and Use of Laboratory Animals of the Center for Research and Advanced Studies (project 512-12). All efforts were made to reduce the number of animals used and to minimize their suffering.

2.2. Surgery

The animals were anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (20 mg/kg, i.m.). Next, the rats were stereotactically implanted with a guide cannula attached to a bipolar electrode that comprised two twisted strands of stainless steel wire, which were insulated except at the cross-section of their tips, into the right ventral hippocampus. The following coordinates relative to bregma were used: anteroposterior, −5.3 mm; lateral, −5.2 mm; and skull surface depth, −7.5 mm for the electrode and −4.5 mm for the cannula (Paxinos and Watson, 1998). The electrode was attached to male connector pins. Stainless steel screws were threaded into the cranium over the frontal cortex to fix the electrode assembly to the skull with dental acrylic. The animals were allowed to recover for 7 days before further manipulation. After the recovery period, the rats received a daily administration of saline solution (1 ml/kg, i.p.) for 5 days to habituate them to manipulation.

2.3. Experiment 1: effects of PPB on the short-term consequences of SE

This experiment was designed to determine whether a single dose of PPB after diazepam (DZP) administration can modify the pilocarpine-induced electrographic activity, high release of glutamate and neuronal damage in the hippocampus of rats receiving this treatment (Fig. 1).

2.3.1. SE-PPB group (n = 5)

Following habituation, a microdialysis probe, which was constructed according to Maidment et al. (1989) and designed to protrude 3 mm beyond the cannula tip in the ventral hippocampus, was inserted into the guide cannula and then fixed to the socket with dental acrylic. A polyacrylonitrile membrane (molecular weight cutoff of 40,000 Da) was the active part of the dialysis probe. The dialysis system was continuously perfused with fresh artificial cerebrospinal fluid (previously filtered and sterilized; concentrations in mM: sodium chloride 125, potassium chloride 2.5, sodium dihydrogen phosphate 0.5, sodium hydrogen phosphate 5, magnesium chloride 1, ascorbic acid 0.2, calcium chloride 1.2; pH 7.4) during the microdialysis experiment at a flow

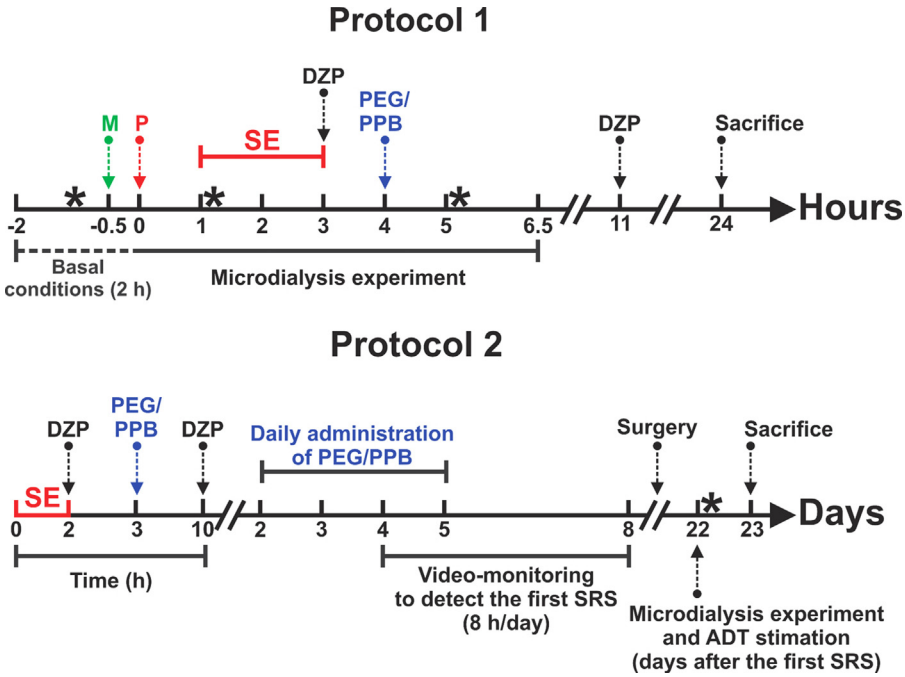

Fig. 1. Schematic representation of the experimental protocols used to determine the effects of PPB during the post-SE period (Protocol 1) and the chronic epileptic period (Protocol 2). Protocol 1: Rats previously implanted with a bipolar electrode attached to a microdialysis cannula in the hippocampus were submitted to a microdialysis experiment (see Section 2.3.1). The diagram illustrates the experimental protocol used to administer the various drugs to induce SE (methylscopolamine, M; pilocarpine, P) and stop the convulsive activity (DZP). Notice that PPB or vehicle was applied 1 h after the first administration of DZP. Protocol 2: Animals previously submitted to pilocarpine-induced SE received subchronic PPB or vehicle for 5 days. The animals were then recorded using a video-monitoring system to identify the first SRS, after which they were implanted with a cannula/electrode in the hippocampus. Microdialysis experiments and ADT estimation were carried out 22 days following the occurrence of the first SRS. All animals were killed 24 h after the microdialysis experiment. Asterisks indicate the schedule used to obtain 1 min electrographic recordings for subsequent analysis using Fast Fourier Transform methods (see Section 2.6) for Protocol 1 (during the basal condition, 5 min after the establishment of SE and 60 min following PPB administration) and Protocol 2 (during the interictal period).

rate of 2 μl/min. Following a stabilization period (2 h), dialysates were recovered every 30 min during a 2-h period to evaluate extracellular glutamate levels under baseline conditions. At the end of the basal period, the animals were administered a muscarinic receptor antagonist (methylscopolamine, 2 mg/kg, i.p.) to minimize the peripheral cholinergic effects induced by pilocarpine. Pilocarpine (300 mg/kg, i.p.) was injected 30 min after the methylscopolamine. The latency to the establishment of SE was then evaluated. The onset of SE was determined as the time at which the animals showed seizures continuously for more than 2 min without recovery between them. To stop the behavioral convulsive seizures, the rats were given DZP at 2 and 10 h after the onset of SE (2.5 and 1.25 mg/kg, i.m., respectively). This treatment reduces the mortality rate induced by SE via DZP-induced myorelaxant effects but does not revert the epileptiform activity or elevated glutamate release in the hippocampus (François et al., 2011; Roch et al., 2002; Santana-Gómez et al., 2015). One hour after the first DZP administration, a single 178-mg/kg dose of PPB diluted in polyethylene glycol (PEG) 30% was administered i.p. in a total volume of 10 ml/kg. This dose was chosen based on a preliminary study in our laboratory, in which we evaluated the dose-response anticonvulsant effects of PPB when applied 30 min before pilocarpine injection. The results obtained from those experiments revealed that PPB pretreatment does not modify the expression of clonic seizures but does avoid tonic-clonic convulsions in 50% of animals when administered at 178 mg/kg, i.p. (data not shown). Electrographic activity in the ventral hippocampus was recorded during the entire observation period (from basal conditions to 6.5 h after pilocarpine injection). The results were evaluated for one-minute periods under different experimental conditions using Fast Fourier Transforms (see Section 2.6): basal condition, 5 min after the establishment of SE, and 60 min following PPB administration (corresponding to 4 h after the establishment of SE). After recovery, the perfusates were diluted with perchloric acid ($HClO_4$, 2 N) (1:20) and assayed to determine extracellular glutamate levels (see Section 2.6). Twenty-four hours after pilocarpine administration, the animals were anesthetized and perfused, and their brains were used in subsequent histological procedures (see Section 2.9) (Fig. 1).

2.3.2. SE-PEG group (n = 7)

The animals were manipulated as described above for the SE-PPB group, except that the animals were given PEG (10 ml/kg, i.p.) instead of PPB (Fig. 1).

2.4. Experiment 2: effects of subchronic PPB administration on the long-term consequences of SE

It is known that over the long term after SE, animals present spontaneous recurrent seizures (SRS), a condition that is associated with high neuronal excitability and elevated glutamate release, as well as neuronal damage and gliosis in the hippocampus. This experiment was designed to determine whether the subchronic administration of PPB during the post-SE period can modify these consequences in the hippocampus during the chronic epileptic period (Fig. 1).

2.4.1. SRS-PPB group (n = 5)

Following habituation, rats were subjected to SE (see Section 2.3.1). Two hours after SE, the convulsive behavioral activity was stopped using two doses of DZP as previously described (see Section 2.3.1). PPB (178 mg/kg, i.p.) was injected daily for 5 days starting one hour after the first DZP injection. The latency to the onset of the first SRS was determined by video-monitoring the animal's behavior for 8 h each day. Seven days after the onset of the first SRS, the rats were implanted with a bipolar electrode and a guide cannula (see Section 2.2). The animals were subjected to microdialysis experiments (see Section 2.3.1) 22 days after the occurrence of the first SRS to determine the glutamate released during the interictal period (the period between seizures). Throughout the microdialysis procedure, electrographic recordings were obtained and used to calculate power spectra (see Section 2.6). The afterdischarge threshold (ADT) of the ventral hippocampus was determined to estimate neuronal excitability in this brain area (see Section 2.7) (Racine, 1972). Twenty-four hours after the microdialysis experiment, the animals were killed, and the brain was examined in subsequent histological procedures (see Section 2.9) (Fig. 1).

2.4.2. SRS-PEG group (n = 6)

The animals were handled in the same way as that described for the SE-PPB group (Section 2.4.1), except they were given subchronic PEG (10 ml/kg, i.p.) instead of PPB (Fig. 1).

2.5. Control group (n = 8)

The rats were implanted and submitted to manipulation as described above (see Section 2.3.1) except that they received only saline solution during the microdialysis experiment. The animals were evaluated in terms of electrographic activity, ADT, glutamate release and neuronal damage in the hippocampus and were also examined under basal conditions.

2.6. Evaluation of hippocampal electrographic activity

Hippocampal electrographic activity was recorded using a model P511 amplifier (Grass, MA, USA); the signals were amplified, band-pass filtered between 0.1 and 250 Hz, digitized at 1000 samples/s and stored on an optical disk. Off-line spectral analyses using Fast Fourier Transform methods (one-minute periods) at each stage of interest were performed using the ADQ8 software, which was developed at the National Institute of Psychiatry "Ramon de la Fuente Muñiz", Mexico (Fernández-Mas et al., 1998; Valdés-Cruz et al., 2012). The signals were undersampled to the final power spectra bandwidth. We applied a finite impulse response filter tuned to the stimulation frequency to remove associated artifacts. Artifact-free recordings were segmented in consecutive epochs of 60 s for each subject and experimental condition. Power spectra were then calculated for each epoch and averaged. To eliminate interindividual variance in the absolute electrographic power, the spectra were normalized by expressing each power estimation for each frequency step (0.1–4 Hz, 4–8 Hz, 8–13 Hz, 13–30 Hz, 30–90 Hz and 90–250 Hz) as a percentage of total power (the mean of all power estimates) in a frequency window. This power estimation is termed relative power. Power spectra were normalized separately for each subject and experimental condition. The values are presented as relative power and evaluated by one-way ANOVA test followed by a Fisher's LSD post-hoc (acute study) or by t-Student analysis (chronic experiment).

2.7. Estimation of hippocampal excitability

Hippocampal ADT values were used to estimate the excitability of this brain area. ADT is defined as the minimum current necessary to produce a behavioral response and/or electroencephalographic afterdischarge with a duration of at least 3 s. ADT was determined by delivering a series of stimulations to the right ventral hippocampus (1 ms rectangular pulses, 60 Hz for 1 s) at 1 min intervals, beginning at 10 mA and increasing in increments of approximately 20% of the previous current (Racine, 1972). The ADT values were analyzed using one-way ANOVA tests followed by a

post hoc Fisher's LSD test, and the results are expressed as the means ± SEM of the current required (μA) for ADT generation.

2.8. High-performance liquid chromatography (HPLC)

For the separation and quantification of glutamate, 16 μl of the test mixture (perfusate-$HClO_4$) was mixed with 6 μl of o-phthalaldehyde (OPA), agitated for 30 s and injected into the solvent stream of an HPLC system 1.5 min later. The HPLC system comprised a fluorescence detector operating at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The HPLC fluorometric detection procedure for amino acid quantitation required the OPA-amino acids to be separated on a reversed-phase 3.9 mm × 150 mm column (Nova-Pack, 4 μm, C_{18}, Waters®) using solution A (sodium acetate dissolved in 90% milli-Q® water and 10% methanol; pH 5.75) as aqueous solvent and solution B (20% solution A and 80% methanol; pH 6.75) as the other mobile phase at a flow rate of 0.5 ml/min (Waters® model 474). Glutamate was quantified by comparing peak height measurements of samples against those of standard solutions (Kendrick et al., 1988). Glutamate levels were expressed in units of μM and as percent changes with respect to basal conditions. The glutamate release results were analyzed using one-way ANOVA followed by a post hoc Fisher's LSD test.

2.9. Histological analysis

The animals were overdosed on pentobarbital and were first transcardially perfused with saline solution (0.9%) containing heparin (1 mg/l) and then with 4% paraformaldehyde in phosphate buffer solution (monobasic sodium phosphate and sodium hydroxide; pH 7.4). The brain was removed, post-fixed for one week at 4 °C, and then embedded in paraffin. Coronal serial sections of 5 μm thickness were cut with a microtome at the level of the dorsal and ventral hippocampus. The sections were deparaffinized and hydrated in water for subsequent processing to evaluate the site of cannula and electrode implantation (Nissl staining) and to visualize neuron nuclei (NeuN immunofluorescence) and neurons undergoing damage (Fluoro-Jade B). Astrogliosis (GFAP immunofluorescence) was evaluated only in cerebral sections obtained from animals in the control, SRS-PEG and SRS-PPB groups.

2.9.1. NeuN-GFAP immunofluorescence

The slides were exposed to citrate buffer (sodium citrate and citric acid; pH 6.0) for 2 min and stirred for 20 min. The slides were then washed three times (5 min each time) in phosphate-buffered saline (PBS; containing sodium dihydrogen phosphate, sodium chloride, potassium chloride and potassium hydrogen phosphate; pH 7.4) and incubated in a blocking solution (goat serum 5% and bovine serum albumin (BSA) 3%) containing Triton X (0.3%) for 2 h. Sections were incubated overnight at 4 °C with primary antibodies (anti-NeuN (1:100, #MAB3077 Millipore) and anti-GFAP (1:500, # Z0334 DAKO)). Thereafter, the slides were rinsed three times with PBS and incubated with secondary antibodies (anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 648 Molecular Probes®, Invitrogen™) for 2 h at room temperature. The sections were counterstained with 4,6-diamino-2-phenylindole dihydrochloride (DAPI) and coverslipped with Vectashield (Vector Labs).

2.9.2. Fluoro-Jade B staining

The slides were immersed in a solution containing 1% sodium hydroxide and 80% alcohol for 5 min, followed by 70% ethanol for 2 min and distilled water for 2 min. The slides were then transferred to a 0.06% potassium permanganate solution for 20 min and then rinsed in distilled water for 2 min. Next, the slides were incubated in a Fluoro-Jade B solution (0.0004%, Histo-Chem, Inc.) for 24 h. After rinsing 3 times (1 min each time) in distilled water, the slides were dried. Finally, the slides were immersed in xylene for 1 min and then mounted in a synthetic resin (Entellan®, Merck Millipore) (Schmued et al., 1997).

2.9.3. Cell counting

Two investigators who were blinded to the classification of the tissue performed the cell count. Sections were examined under a Zeiss Axiovert 40 CFL microscope, and digitized images were obtained using Axiovision 4.8 software (Carl Zeiss MicroImaging, Germany). The average cell density per unit of volume was determined in the dentate gyrus (DG), hilus, CA1, and CA3 in the dorsal and ventral hippocampus using the optical fractional counting method (Besio et al., 2013; West et al., 1991). Briefly, three serial sections (5 μm in thickness) of the dorsal and ventral hippocampus were evaluated. In this case, the sampling fraction (*ssf*) was 1/5. The area sampling fraction (*asf*) = area (frame)/area (*x*, *y* step) was calculated, and this fraction was considered the counting frame (0.460 mm × 0.600 mm). Then, the thickness

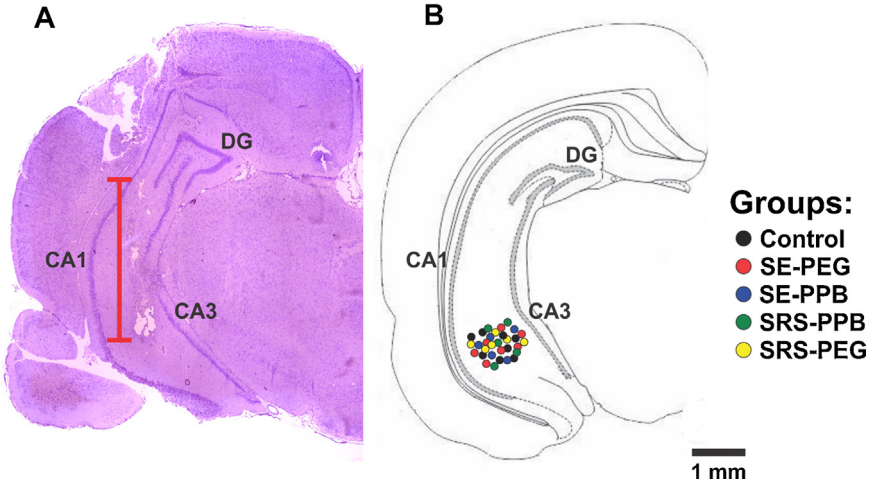

Fig. 2. (A) Representative microphotograph of a coronal section labeled with Nissl staining showing the area of electrode-cannula implantation in the ventral hippocampus. The red line indicates the placement of the microdialysis membrane. (B) A diagram indicating the area at −5.28 mm from Bregma (modified from Paxinos and Watson, 1998), indicating that the electrode-cannula tips were implanted in the ventral hippocampus of all animals in the evaluated experimental groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

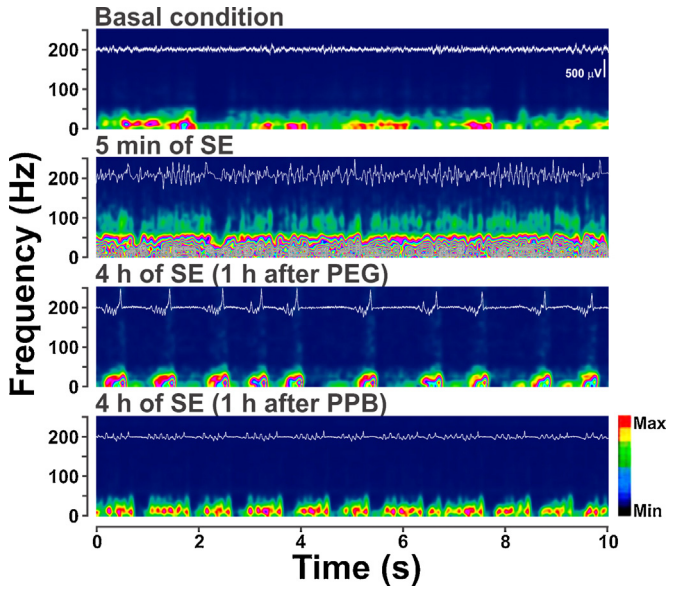

Fig. 3. Time–frequency representation of the power spectrum (0.1–250 Hz) of the electrographic activity measured under different experimental conditions. Red indicates the highest energy and blue indicates the lowest. In comparison with basal conditions (first panel), the electrical activity presents a significant enhancement at all frequencies when evaluated at 5 min after the establishment of the SE (second panel). The activity at high voltage and frequency is maintained at 4 h after the beginning of SE despite the administration of DZP plus vehicle (third panel). In contrast, animals that received DZP plus PPB showed reduced hypersynchronous high activity at low voltage at 4 h after the beginning of the SE (lower panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sampling fraction was estimated as the dissector height (h) relative to the section thickness t (t/h). The number of cells was calculated using the following formula: $N = (\sum Q^{-}) \times (t/h) \times (1/asf) \times (1/ssf)$. In this formula, Q^{-} represents the number of cells in a known volume fraction of each area of the hippocampus (West et al., 1991). The cell count values were analyzed using one-way ANOVA followed by a Bonferroni post hoc multiple comparison test (NeuN staining) and Student's t-test (Fluoro-Jade B staining). Values are expressed as the means ± SEM number of cells per mm^3.

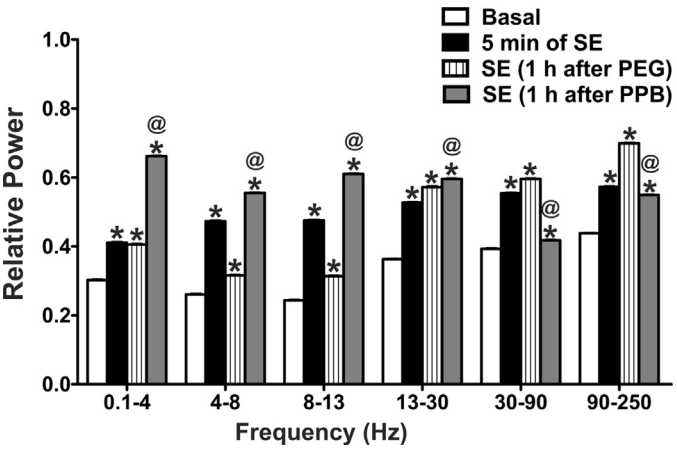

Fig. 4. Representation of relative power spectral analyses of the hippocampal electrical activity under various experimental conditions: basal conditions (open bars); 5 min after the establishment of SE (black bars); and 4 h after the beginning of SE in animals that received DZP plus vehicle (bars with vertical lines) or DZP plus PPB (gray bars). Note the increase in the relative power of low-frequency oscillations in the electrographic activity of animals that were treated with DZP plus PPB; this compares with the SE-PEG group, wherein high-frequency oscillations prevailed. Values represent means ± standard errors, $^{*}p < 0.001$ compared with basal conditions; $^{@}p < 0.001$ compared with the SE-PEG group.

3. Results

Histological analysis showed that the tips of the electrodes and the microdialysis cannula were implanted within the ventral hippocampus in all animals evaluated in the present study (Fig. 2).

3.1. Control group

Rats in the control group did not show behavioral or electrographic changes during the experimental procedure. Hippocampal electrographic activity demonstrated a homogeneous prevalence of all bands analyzed (Figs. 3 and 4). ADT values were 143 ± 35 μA, and extracellular levels of glutamate (1.20 ± 0.30 μM) were stable throughout the microdialysis experiments (Fig. 5). Histological analysis revealed NeuN immunopositive cells in the dorsal (DG, 5397 ± 331; hilus, 1078 ± 19; CA1, 1293 ± 59 and CA3, 1442 ± 192 neuronal nuclei per mm^3) and ventral hippocampus (DG, 3857 ± 375; hilus, 1036 ± 38; CA1, 1629 ± 73 and CA3, 1161 ± 41 neuronal nuclei per mm^3). Scarce neurons undergoing death

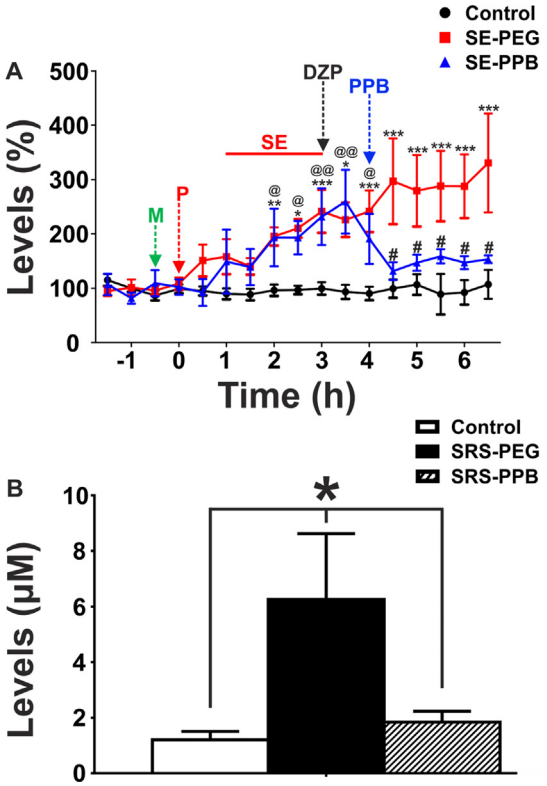

Fig. 5. (A) Glutamate release in the hippocampi of rats in the control, SE-PEG and SE-PPB groups. In the SE-PEG group, glutamate release is progressively augmented after pilocarpine administration, an effect that was maintained despite DZP injection. In contrast, PPB administration after DZP injection reduced glutamate release in the SE-PPB group. Values represent means ± standard errors of the percentage of change from release under basal conditions. SE-PEG group: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to basal conditions. SE-PPB group: $^{@}p < 0.01$, $^{@@}p < 0.001$ compared to basal conditions. $^{\#}p < 0.001$ compared to the SE-PEG group under similar experimental conditions. M, methylscopolamine administration; P, pilocarpine administration; SE period; DZP administration; PPB administration. (B) Extracellular levels (μM) of glutamate in the hippocampi of control rats and during the interictal period of animals with SRS that were treated subchronically with vehicle or PPB (the SRS-PEG and SRS-PPB groups, respectively). Note the higher levels of glutamate in the SRS-PEG group compared with that in the control and SRS-PPB groups. Values represent means ± standard errors. $^{*}p < 0.05$ compared to the control and SRS-PPB groups.

(Fluoro-Jade B staining) and astroglial cells (GFAP immunofluorescence) were detected in the areas evaluated (Figs. 6–8).

3.2. A single dose of PPB decreases the acute consequences of SE

3.2.1. SE-PEG group

Under basal conditions, the electrographic activity of rats in the SE-PEG group was similar to that of rats in the control group. The rats reached the SE at 43.2 ± 2.5 min after pilocarpine administration, a situation that was associated with faster, high-voltage rhythmic spikes, an increase in spectral potency in 0.1–250 Hz bands ($p < 0.001$, compared with basal conditions), and a progressive rise in glutamate release. After the first DZP injection, the animals showed reduced convulsive activity, diminished amplitude and frequency of the epileptiform activity and reduced power in the 4–8 and 8–13 Hz bands ($p < 0.001$). However, increases in the high-frequency oscillations bands (13–250 Hz, $p < 0.001$) and glutamate release (230% compared with basal conditions, $p < 0.001$) were still evident at the end of the experiment, 5.5 h after SE establishment (2.5 h after PEG) (Figs. 3–5).

Compared with the rats in the control group, those in the SE-PEG group showed a decrease in the number of NeuN immunopositive cells in the hippocampus (Dorsal: DG, 29%, $p = 0.0301$; hilus, 47%, $p = 0.012$; CA1, 67%, $p = 0.003$ and CA3, 57%, $p = 0.015$; Ventral: DG, 63%, $p = 0.008$; hilus, 73%, $p < 0.001$; CA1, 65%, $p = 0.002$ and CA3, 64%, $p = 0.013$). A high number of Fluoro-Jade B-staining cells were also seen in the hippocampus (Dorsal: DG, 1040.29 ± 829.65; hilus, 536.31 ± 182.27; CA1, 608.30 ± 271.00 and CA3, 600.96 ± 203.16 neurons per mm^3; Ventral: DG, 671.00 ± 307.25; hilus, 429.78 ± 141.76; CA1, 634.75 ± 412.55 and CA3, 514.76 ± 148.11 neurons per mm^3) (Figs. 6 and 7).

3.2.2. SE-PPB group

Animals in the SE-PPB group achieved SE at 40.3 ± 2.0 min after pilocarpine injection. During SE, animals in the SE-PPB group showed a pattern of glutamate release and electrographic activity that was similar to that seen in the SE-PEG group. The epileptiform activity progressively decreased when PPB was applied. This effect was correlated with the following changes: (a) an increase in the power of low-frequency activity (0.1–13 Hz bands, $p < 0.001$); (b) a reduced potency of high-frequency activity (30–250 Hz bands, $p < 0.001$); and (c) a significant decrease in the release of glutamate (126% lower with respect to the SE-PEG group, $p < 0.001$). These changes were evident from 30 min to 2.5 h after PPB injection (i.e., 5.5 h after SE establishment) (Figs. 3–5).

When compared with rats in the SE-PEG group, animals in the SE-PPB group showed higher numbers of NeuN-immunopositive cells in the hippocampus (Dorsal: DG, 30%, $p = 0.13$; hilus, 115%, $p = 0.003$; CA1, 197%, $p = 0.004$ and CA3, 170%, $p = 0.004$; Ventral: DG, 117%, $p = 0.046$; hilus, 245%, $p < 0.001$; CA1, 174%, $p = 0.003$ and CA3, 171%, $p = 0.016$) and presented lower numbers of cells undergoing damage (Fluoro-Jade B staining) in this brain area (Dorsal: DG, 81%, $p < 0.001$; hilus, 55%, $p < 0.001$; CA1, 98%,

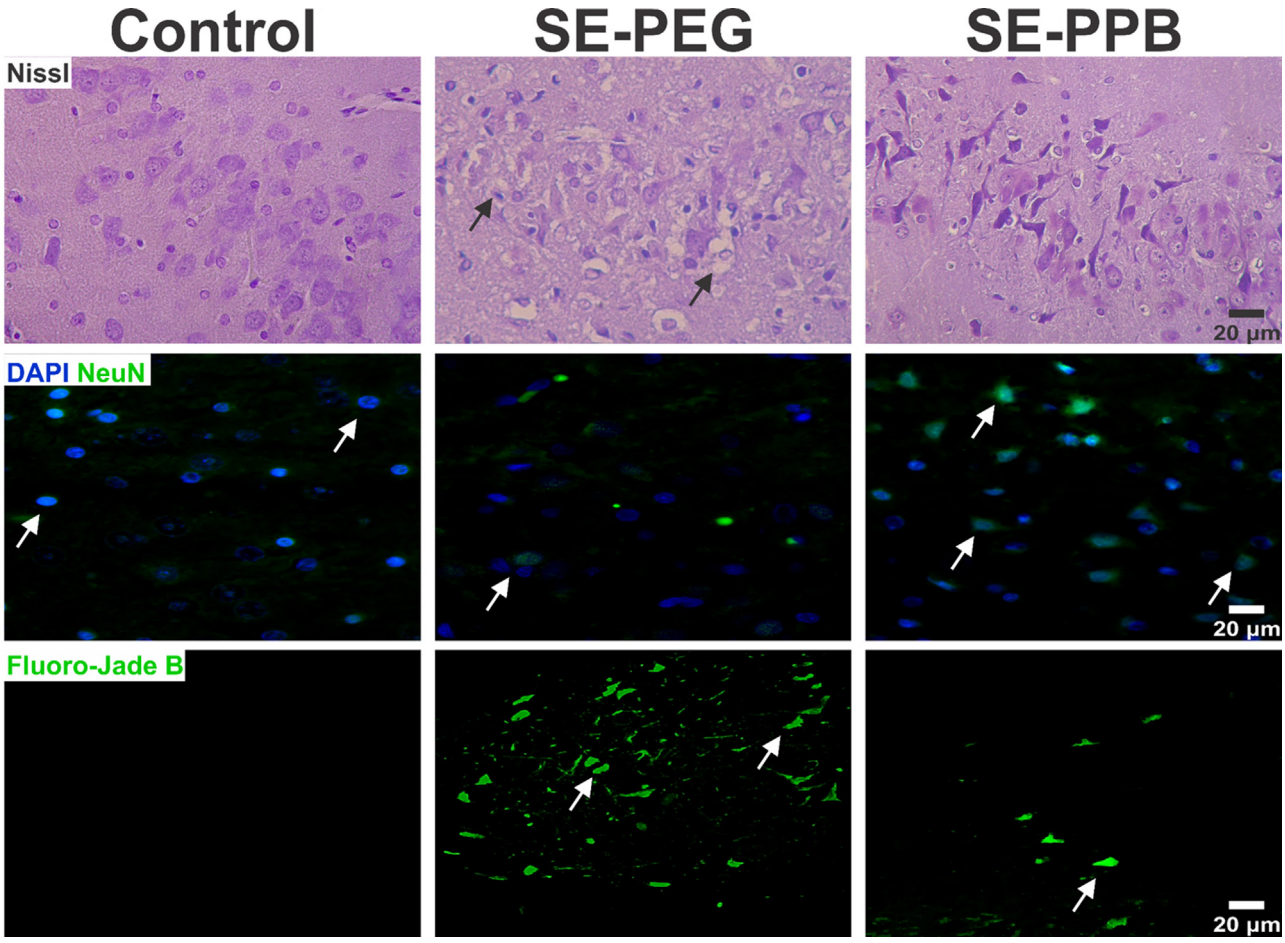

Fig. 6. Representative microphotographs of coronal sections of hilus at the dorsal hippocampus of rats in the control group and in animals receiving DZP plus vehicle (SE-PEG group) or DZP plus PPB (SE-PPB group) during the post-SE period. The sections were stained with Nissl (upper panels), NeuN-DAPI (middle panels) or Fluoro-Jade B (bottom panels). In contrast with the control tissue, SE-PEG animals showed decreased neuronal preservation (NeuN-DAPI), a condition that is associated with augmented cellular damage (Fluoro-Jade B). These changes were less evident in the sections obtained from animals in the SE-PPB group.

$p < 0.001$ and CA3, 86%, $p < 0.001$; Ventral: DG, 70%, $p = 0.006$; hilus, 91%, $p < 0.001$; CA1, 96%, $p < 0.001$ and CA3, 88%, $p = 0.004$) (Figs. 6 and 7).

3.3. Subchronic administration of PPB during the early post-SE period reduces long-term hippocampal hyperexcitability and glutamate release

3.3.1. SRS-PEG group

Animals in the SRS-PEG group presented the first spontaneous motor seizure at 4.7 ± 1.6 days after the SE with a seizure frequency of 8.7 ± 1.7 seizures per day. Analysis of the electrographic activity during the chronic epileptic period demonstrated interictal spontaneous high voltage spikes in the hippocampus with a high prevalence of all oscillations evaluated. This condition was associated with high extracellular levels of glutamate (a 420% increase relative to the control group, $p = 0.021$) and augmented hippocampal excitability revealed by low ADT values (36.67 ± 12.18 μA; 74% lower than the control group, $p = 0.029$) (Figs. 5 and 9).

The histological analysis indicated lower numbers of NeuN immunopositive cells in the hippocampus (Dorsal: DG, 38%, $p = 0.003$; hilus, 61%, $p = 0.005$; CA1, 77%, $p = 0.001$ and CA3, 54%, $p = 0.005$; Ventral: DG, 65%, $p = 0.027$; hilus, 77%, $p = 0.001$; CA1, 73%, $p = 0.001$ and CA3, 66%, $p < 0.001$) compared with the control group. A high number of Fluoro-Jade B-positive cells were also seen in the hippocampus (Dorsal: DG, 61 ± 3; hilus, 29 ± 23; CA1, 244 ± 63 and CA3, 101 ± 12, cells per mm^3; Ventral: DG, 56 ± 10; hilus, 21 ± 22; CA1 836 ± 69 and CA3, 330 ± 66 cells per mm^3). Strong GFAP-immunoreactivity, which is characteristic of astrogliosis, was detected in the evaluated areas, mainly in the hilar region (Figs. 7 and 8).

3.3.2. SRS-PPB group

Animals in the SRS-PPB group presented the first spontaneous behavioral convulsion at a similar latency (5.2 ± 1.8 days after SE, $p = 0.395$) but the daily seizure frequency was significantly lower (3.3 ± 0.9 seizures per day, 62% less, $p = 0.0161$, when compared with SRS-PEG group). The analysis of interictal hippocampal electrographic activity showed a lower relative power of all analyzed bands ($p < 0.001$) (Fig. 9). In this experimental group, interictal extracellular levels of glutamate and ADT values achieved control conditions (1.84 ± 0.39 μM, $p = 0.22$ and 161 ± 32 μA, $p = 0.131$, respectively) (Fig. 5).

Histological analysis revealed that in the dorsal and ventral DG of rats in the SRS-PPB group, the numbers of NeuN-immunopositive cells and neurons labeled with Fluoro-Jade B were similar to those of rats in the control group. On the other hand, hilus, CA1 and CA3 of the dorsal hippocampus showed fewer NeuN-immunopositive cells and greater Fluoro-Jade B staining. Indeed, values obtained for hilus and CA3 were significantly higher than those found in the SRS-PEG group (238%, $p = 0.038$ and 206%, $p = 0.004$, respectively). In the ventral hippocampus, the numbers of NeuN-immunopositive cells in hilus, CA1 and CA3 were similar to those in the control group. However, Fluoro-Jade B staining revealed that a high number of these cells were undergoing death. GFAP immunoreactivity in all hippocampal areas evaluated in the SRS-PPB group was similar to that detected under control conditions (Figs. 5 and 8).

4. Discussion

Extracellular levels of glutamate are increased during ictal and interictal periods in the brains of subjects with SE or epilepsy (During and Spencer, 1993; Luna-Munguia et al., 2011; Santana-Gómez et al., 2015). The increased glutamatergic neurotransmission of subjects with SE is involved in NMDA receptor-mediated excitotoxicity, especially in pyramidal and granular cells of the hippocampus (Fujikawa, 1995; Isokawa and Mello, 1991). This situation has been associated with neuronal damage, the development of SRS (epileptogenesis) and neurocognitive impairment (Cunha et al., 2009; Roch et al., 2002).

Our experiments revealed that the administration of DZP combined with PPB lessened the increased glutamate release and neuronal excitability that are induced by SE. It is known that the blockage of Na^+ channels reduces glutamate release and attenuates cellular damage (Calabresi et al., 2003; Xie et al., 1994). The inhibition of voltage-dependent Na^+ channels in hippocampal neurons by PPB (Lara-Valderrábano et al., 2016) can participate in

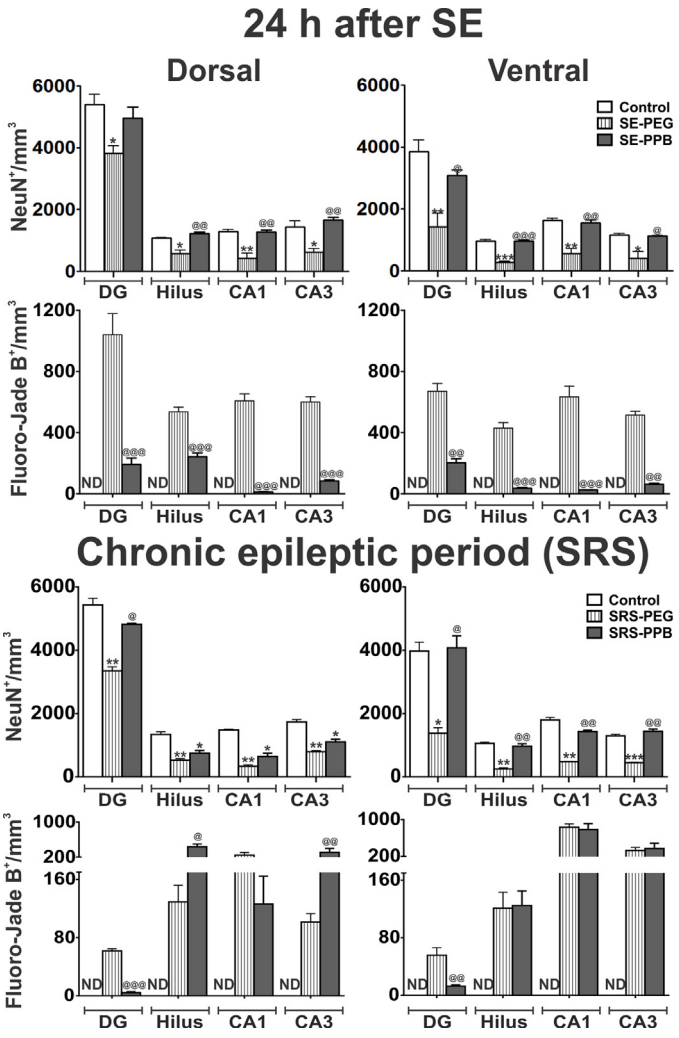

Fig. 7. Upper panel: a graphic representation of neuronal preservation and damage in the hippocampus as evaluated at 24 h after SE induction. Note the low neuronal count (NeuN staining) and the high number of cells undergoing death (Fluoro-Jade B staining) in all areas of the dorsal and ventral hippocampus of animals in the SE-PEG group that were killed at 24 h after SE. In contrast, rats from the SE-PEG group demonstrated neuronal preservation similar to that seen in the control group and a low number of cells undergoing damage. Lower panel: illustration of neuronal preservation and damage in the dorsal and ventral hippocampus of animals that were evaluated during the chronic epileptic period. Animals in the SRS-PEG group presented low neuronal counts and high numbers of cells undergoing death in all areas of the hippocampus. Dorsal and ventral DG of rats in the SRS-PPB group showed neuronal preservation that was similar to that seen in rats in the control group, which was associated with a low number of cells labeled with Fluoro-Jade B. In contrast, hilus, CA1 and CA3 of these animals presented high numbers of cells undergoing death. Values represent means ± standard errors per mm^3. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group. $^@p < 0.05$, $^{@@}p < 0.01$, $^{@@@}p < 0.001$ compared with the SE-PEG and SRS-PEG groups. ND, not determined.

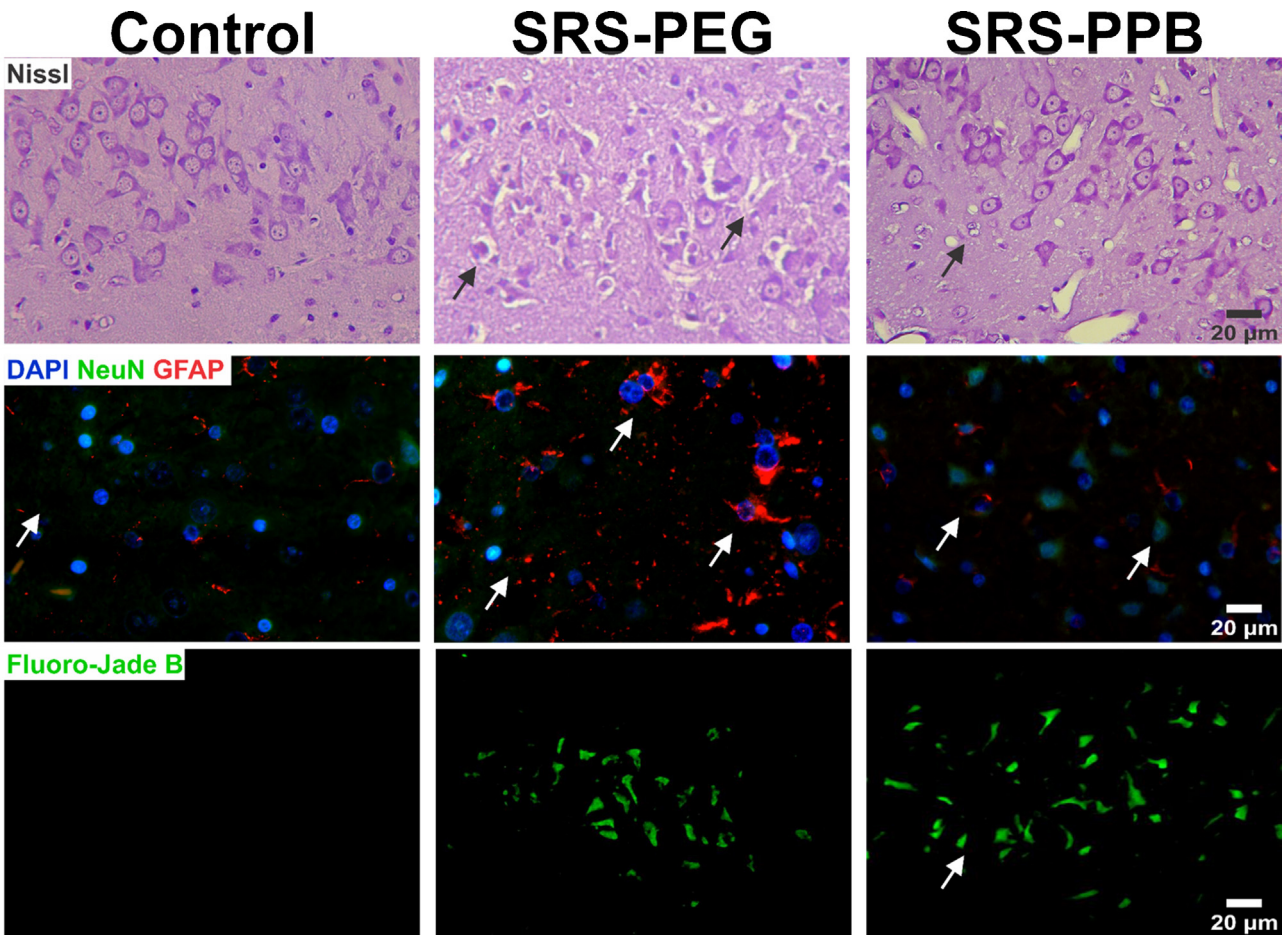

Fig. 8. Representative microphotographs of coronal sections of hilus at the dorsal hippocampus of rats in the control, SRS-PEG, and SRS-PPB groups during the chronic epileptic period PPB. The sections were stained with Nissl (upper panels), NeuN-DAPI-GFAP (middle panels) and Fluoro-Jade B (bottom panels). Note the decreased neuronal density and increased astrogliosis (GFAP expression) in the SRS-PEG group. These changes were less evident in the SRS-PPB group.

the decreased release of glutamate and some of the neuroprotective effects that were observed in this study.

At present, the interaction between PPB and Na^+ channels is unknown, but it may share some mechanisms with phenytoin, a drug that induces anticonvulsant and neuroprotective effects by blocking Na^+ channels (Raftopoulos et al., 2016). Studies indicate that hydrogen bonds are established between the carbonyl and amino groups of phenytoin and the protein structure of the Na^+ channel (Poupaert et al., 1984). Concerning the molecular structure of PPB, the presence of free electrons in the ester group (Cashman and Warshaw, 2005) may allow the formation of hydrogen bonds with Na^+ channels.

During pilocarpine-induced SE, the hippocampus presents epileptiform activity and high-frequency oscillations (90–250 Hz) that persist despite DZP administration (Karunakaran et al., 2012; Santana-Gómez et al., 2015). Our results indicate that the administration of DZP in combination with PPB decreases the epileptiform activity and high-frequency oscillations associated with SE more effectively. Similarly, drugs with anesthetic effects (ketamine, thiopental, and morphine) reduce the high-frequency brain activity that is evoked by chemical or electrical stimulation (Faulkner et al., 1998; Whittington et al., 2000). However, Fourier analysis revealed that the combination of DZP and PPB increased the potency of low-frequency oscillations (0.1–30 Hz), an effect that has been associated with reduced epileptiform activity. These results support the notion that the high prevalence of oscillations at low frequencies can be associated with antiepileptic effects (La Grutta and Sabatino, 1988; Miller et al., 1994).

PPB is used as a preservative in some oral pharmaceutical forms of antiepileptic drugs, such as levetiracetam (Keppra), carbamazepine (Tegretol), oxcarbazepine (Trileptal), DZP (Actavis, UK LTD) and gabapentin (Rosemont Pharmaceuticals Limited) (https://www.medicines.org.uk/emc/). Considering that PPB induces inhibitory effects in the CNS (Matthews et al., 1956; Lara-Valderrábano et al., 2016), it is possible to suggest that its combination with antiepileptic drugs may result in a more effective therapy to reduce seizure activity. However, additional experiments are necessary to support this hypothesis.

The results obtained revealed that the acute administration of PPB induces neuroprotective effects in DG, hilus, CA1 and CA3, 24 h after SE. In contrast, subchronic administration of PPB during the post-SE period did not prevent the occurrence of SRS or the cell loss in hilus, CA1, and CA3 through the chronic epileptic period. These results indicate that the subchronic treatment of PPB during the post-SE period was ineffective at preventing the epileptogenic process and neuronal damage in these brain areas. Similarly, cell loss is also found after the subchronic administration of antiepileptic drugs such as vigabatrin, levetiracetam, topiramate and DZP in animals that undergo SE (André et al., 2001; Brandt et al., 2007; François et al., 2006). Further experiments are necessary to determine whether antiepileptogenic and neuroprotective effects can be achieved using PPB at higher doses or with longer treatments.

DG plays a role as a *gate* by reducing seizure propagation from the hippocampus to other brain areas (Cohen et al., 2003; Krook-Magnuson et al., 2015). We found that animals receiving chronic PPB administration after SE presented a decrease in astrogliosis,

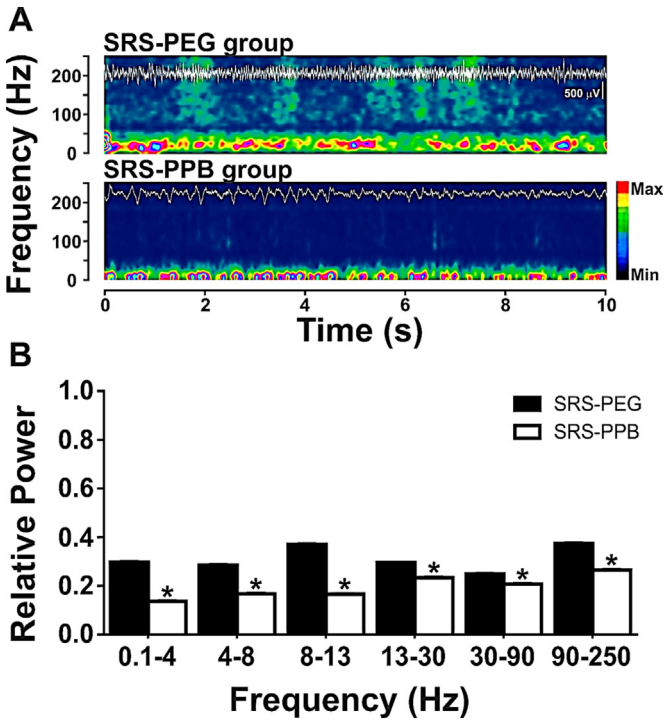

Fig. 9. (A) Time–frequency representation of the power spectrum (0.1–250 Hz) of the hippocampal electrographic activity during the interictal period of animals with SRS that were treated subchronically with vehicle or PPB (the SRS-PEG and SRS-PPB groups, respectively). The notations are as described for Fig. 3. Note an increase in the electrical activity at all evaluated frequencies for the SRS-PEG group. These changes were less evident in the recordings obtained for animals in the SRS-PPB group. (B) Representative relative power spectral analysis of the hippocampal electrical activity during the interictal period. Note that the relative power activity obtained for the SRS-PPB group (black bars) was significantly lower than that obtained for the SRS-PEG group (open bars). Values represent means ± standard errors, $^{*}p < 0.001$.

glutamate release and neuronal excitability during the chronic epileptic period, conditions that have been associated with neuroprotective effects that are restricted to DG. Parabens, including PPB, produce weak estrogenic effects (30,000-fold less potent than 17 β-estradiol) (Blair et al., 2000; Routledge et al., 1998). The administration of 17 β-estradiol induces neuroprotection in both SE and ischemic animal models (Dubal et al., 1998; Simpkins et al., 1997; Velísková et al., 2000). This effect is associated with antioxidant mechanisms (Behl et al., 1997), the blockage of Ca^{2+} voltage-dependent channels (Mermelstein et al., 1996), reductions in the activation of NMDA receptors (Weaver et al., 1997) and the enhanced binding of γ-aminobutyric acid (GABA) to $GABA_A$ receptors (Maggi and Perez, 1986). It is possible to suggest that the high benzodiazepine/$GABA_A$ receptor binding that is induced in DG during the epileptogenic process (Kamphuis et al., 1995; Rocha and Ondarza-Rovira, 1999) facilitates the neuroprotection mediated by PPB. Indeed, this would explain the lower seizure frequency and neuronal excitability in animals with SRS. Further studies are necessary to support this idea.

Studies have revealed that the estrogenic activity of parabens induces several important side effects (Byford et al., 2002; Darbre et al., 2003; Okubo et al., 2001) that may restrict their use as pharmacological neuroprotectants. These side effects include the development of breast cancer (Darbre and Harvey, 2014), the activation of genes and transductional mechanisms that are associated with cell proliferation (Wróbel and Gregoraszczuk, 2014a,b, 2015) and uterotrophic alterations (Lemini et al., 2004). Before proposing the administration of PPB for use as a new neuroprotective treatment, it is important to test for any neurotoxic effects; ongoing experiments in our laboratory using Fluoro-Jade B staining indicate that a daily PPB dose from 30 to 150 mg/kg, i.p., for 6 days does not induce neuronal damage. However, additional experiments are essential to establish safe conditions for chronic PPB administration.

5. Conclusion

PPB has a wide margin of security that allows its administration with a low probability of inducing toxic effects at low doses. In addition, the pharmacokinetic aspects of PPB are widely known (Soni et al., 2001).

The results obtained in this study suggest that the systemic administration of PPB could represent an appropriate therapeutic strategy to reduce the neuronal damage and neuronal excitability induced by SE and SRS. Future studies must be conducted to investigate if the PPB associated with sub-effective doses of antiepileptic drugs represent a novel pharmacological strategy to reduce the consequences of SE and epilepsy.

Conflict of interest

Dr. Santana-Gómez and Dr. Rocha report grants from National Council for Science and Technology of Mexico (CONACyT) and UC-MEXUS, during the conduct of the study. Dr. Rocha, Dr. Santana-Gómez, Dr. Orozco-Suárez, Dr. Talevi, and Dr. Bruno-Blanch have a patent WO 2015/049608 A1 pending. Dr. Magdaleno-Madrigal and Dr. Fernández-Mas have nothing to disclose.

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