Epilepsy Research 126 (2016) 16–25

Contents lists available at www.sciencedirect.com

Epilepsy Research

journal homepage: www.elsevier.com/locate/epilepsyres

Seizures triggered by pentylenetetrazol in marmosets made chronically epileptic with pilocarpine show greater refractoriness to treatment

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ARTICLE INFO

Article history:
Received 12 March 2016
Received in revised form 29 May 2016
Accepted 25 June 2016
Available online 27 June 2016

Keywords:
Epilepsy
Pharmacoresistance
Pentylenetetrazole
Pilocarpine
Anti-epileptic drugs
Animal model

ABSTRACT

The efficiency of most of the new antiepileptic drugs (AEDs) on clinical trials still falls short the success reported in pre-clinical studies, possibly because the validity of the animal models is insufficient to fully represent the human pathology. To improve the translational value for testing AEDs, we propose the use of non-human primates. Here, we suggest that triggering limbic seizures with low doses of PTZ in pilocarpine-treated marmosets might provide a more effective basis for the development of AED. Marmosets with epileptic background were more susceptible to seizures induced by PTZ, which were at least 3 times longer and more severe (about 6 times greater frequency of generalized seizures) in comparison to naïve peers. Accordingly, PTZ-induced seizures were remarkably less attenuated by AEDs in epileptic than naïve marmosets. While phenobarbital (40 mg/kg) virtually abolished seizures regardless of the animal's background, carbamazepine (120 mg/kg) and valproic acid (400 mg/kg) could not prevent PTZ-induced seizures in epileptic animals with the same efficiency as observed in naïve peers. VPA was less effective regarding the duration of individual seizures in epileptic animals, as assessed in ECoG ($p = 0.05$). Similarly following CBZ treatment, the behavioral manifestation of generalized seizures lasted longer in epileptic ($p < 0.05$), which were also more frequent than in the naïve group ($p < 0.05$). As expected, epileptic marmosets experiencing stronger seizures showed more NPY- and ΔFosB-immunostained neurons in a number of brain areas associated with the generation and spread of limbic seizures. Our results suggest that PTZ induced seizures over an already existing epileptic background constitutes a reliable and controllable mean for the screening of new AEDs.

1. Introduction

Epilepsy is a common neurological disorder that affects at least 50 million people worldwide (Loscher and Schmidt, 2002). Despite recent developments of anti-epileptic drugs and innovative approaches in the epilepsy treatment (Loscher et al., 2013) 30% of patients with temporal lobe epilepsy (TLE) still are unable to have their seizures controlled as a consequence of pharmacoresistance (McKeown and McNamara, 2001) and also do not meet the criteria for surgical intervention (Centeno et al., 2006). In the more recent years, as a first choice approach for seizure control, pharmacology has not brought into clinical practice the same successes reported in pre-clinical trials (Loscher and Schmidt, 2002). One possible reason for such failure relies on the fact that most AEDs are tested in animal models that not completely mimic human TLE and are mostly based in acute, chemically- or electrically-evoked seizures (Bialer and White 2010; Loscher and Schmidt, 2011; White and Loscher, 2014).

In contrast, one of the key hallmarks of epilepsy, including TLE, is the occurrence of unpredicted, spontaneous (not evoked) and recurrent seizures (SRSs). In animal models of TLE, spontaneous seizures are often observed in the chronic phase that ensues

Abbreviations: AEDs, antiepileptic drugs; CBZ, carbamazepine; ECoG, electrocorticogram; LFP, local field potential; PB, phenobarbital; PFA, paraformaldehyde; PSD, power spectrum density; PTZ, pentylenetetrazole; SE, status epilepticus; SNr, substantia nigra pars reticulate; SRF, serum response factor; SRSs, spontaneous recurrent seizures; TLE, temporal lobe epilepsy; VPA, valproic acid.

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http://dx.doi.org/10.1016/j.eplepsyres.2016.06.012
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after *status epilepticus* (*SE*), which can be induced by chemoconvulsants, such as the cholinergic agonist pilocarpine (Lemos and Cavalheiro, 1995). The pilocarpine model of TLE is characterized by pathological features of the human condition, such as mossy fiber sprouting, hippocampal gliosis and cognitive impairments (Mello et al., 1992). However, the unpredictable nature and variable profile of SRSs in post-*SE* models make it inadequate, expensive and time-consuming for the testing of new potential AEDs. One strategy to overcome this limitation is to acutely evoke seizures (using PTZ) in rats made epileptic with pilocarpine (Blanco et al., 2009). However, a caveat of this rodent model is the extensive injury, encompassing hippocampus, entorhinal and piriform cortices, amygdala, thalamic and hypothalamic nuclei, claustrum and bed nucleus of stria terminalis (Covolan et al., 2000; Mello et al., 1993). This contrasts significantly with the more restricted damage seen in human TLE patients (Engel, 1996). The injection of pilocarpine in marmosets (Perez-Mendes et al., 2011) has recently been shown to result in more restricted neurodegeneration, ressembling the human pathology, with neuronal damage and abnormal plasticity mainly restricted to the limbic system, particularly the hippocampus (Perez-Mendes et al., 2011). Here we examined the effect of carbamazepine (CBZ), phenobarbital (PB) and valproic acid (VPA), three clinically relevant and widely used AED, in a non-human primate model where predictable acutely elicited seizures (by means of PTZ) were generated in an epileptic brain (yielded epileptic by means of SE induction with pilocarpine). We aimed at providing an animal model that would more closely mimic the human condition and provide the ease of acutely induced seizures.

2. Methods

2.1. Animals

Here we used 35 adult marmosets (*Callithrix jacchus*) of both genders (2–8 years-old; 250–400 g) from the primate facility of the Federal University of São Paulo (authorization number 36120-1). Each experimental group was composed by equal proportion of males and females (50%–50%). At the beginning of the experiments, animals were individually housed in wired cages (50 cm × 50 cm × 50 cm) under controlled conditions. Room temperature was kept stable (25 ± 2 °C) and the light-dark cycle followed a 12-h periodicity with lights on at 7:00 am. Cage environment was enriched with branches across the cage, colored wooden swings and a plastic box nest. The diet was based on chow supplemented with fresh fruits and vegetables. All protocols were consistent with ARRIVE guidelines and were carried out according to the National Institutes of Health guide for the care and use of Laboratory animals. Protocols also complied with Brazilian legislation and were previously approved by the Animal Care and Use Committee of the Federal University of São Paulo (authorization number 0147/10). Substantial efforts were made to reduce the number of experimental animals used.

2.2. Electrode implantation and electrocorticography analysis

After sedation with diazepam (1 mg/kg; i.m.) and anesthesia with ketamine 10% (10 mg/kg; i.m.) and xylazine 2% (0.5 mg/kg; i.m.), we placed a bipolar electrode (TA11CTAF40; *Data Sciences International, St. Paul, MN*) over the motor cortex and the associated WiFi transmitter was attached to the abdominal wall. All animals were treated with a broad spectrum veterinarian antibiotic (pentabiotic® 0.1 mL/kg; i.m. – a combination of various forms of penicillin and streptomycin) and flunixin meglumine as analgesic (Banamine®, 1 mg/kg; i.m.).

The local field potential (LFP) was recorded in freely moving marmosets with a telemetry system (*Data Sciences International*) at a 500 Hz sampling rate and were further analyzed on Matlab and Art 4.0 software. Seizures recorded in the electrocorticogram (ECoG) were classified as *SE*-like whenever ictal events occurred as chains of seizures lacking the post-ictal depression and animals' behavior suggested continuous unconsciousness.

2.3. SE *induced by pilocarpine*

SE was induced as described elsewhere (Perez-Mendes et al., 2011). In brief, scopolamine methyl bromide (1 mg/kg, i.p.) was followed 20 min later by pilocarpine (250 mg/kg i.p.) administration and thereafter seizures were halted 10 min after *SE* onset with diazepam (1.25 mg/kg, i.p.) to reduce mortality. Fourteen of the 20 pilocarpine-injected marmosets developed *SE* and were pooled as the epileptic group. The remaining six animals, that did not develop *SE* (hence non-epileptic), were omitted in data presentation and discussion for the sake of clarity. For simplicity pilocarpine-induced epileptic animals are further referred to as merely epileptic marmosets. Despite intensive care, three animals died because of *SE*. Fifteen naïve animals (naïve group) were subject to the same procedures, except that pilocarpine was replaced by an equal volume of saline solution.

2.4. Ictal and non-ictal behaviors

We quantified the frequency and duration of natural behaviors (Stevenson and Poole 1976) at 30 min long epochs (starting approximately at 8:00 am) previous to the first drug testing session. This assessment comprised locomotion and scent marking events; remaining in the nest box and remaining still in specific quadrants of the cage; moving around the cage; food examination and mastication; scratching; and grooming. A similar assessment was also performed for ictal or pre-ictal behaviors, such as frequency of tongue and mouth automatisms, mouth cleaning and head shakes, as well as repetitive orofacial movements, and intense salivation (the latter being also a likely consequence of peripheral cholinergic activation by pilocarpine). At last, the behavioral manifestation of seizures was evaluated for six hours after PTZ injection and seizures were characterized according to seizure severity as adapted from the Racine scale (Bachiega et al., 2008). In this context, type III and IV/V seizures are presented as partial and generalized events, respectively. The behaviors of the marmosets were acquired and categorized by means of The Observer XT 8.0 software.

2.5. Drug testing

Drugs were tested three months after TLE induction to allow full recovery of the marmosets. As illustrated in Fig. 1, animals were subjected to five sessions of drug testing, in which a compound was given orally (gavage) preceding the acute seizure induction by PTZ (40 mg/kg, i.p.). Sessions started approximately at 8:00 am and the tested compound was given 30 min later. For the first and fifth drug testing sessions the compound was invariably saline, whereas phenobarbital (PB, 40 mg/kg), carbamazepine (CBZ, 120 mg/kg) and valproic acid (VPA, 400 mg/kg) were randomly sorted in the remaining sessions. The dosage for PTZ, PB and CBZ administration were previously defined for the current experiments (Bachiega et al., 2008), whereas the dose of VPA was based on previous experience with rat (Blanco et al., 2009). The time interval between each AED injection and PTZ administration reflected differences in pharmacokinetics of each different AED: 20 min for CBZ and 30 min for VPA (Lemos and Cavalheiro, 1995; Patsalos, 2005), PB and saline. Drug testing sessions were segregated by a week washout and dur-

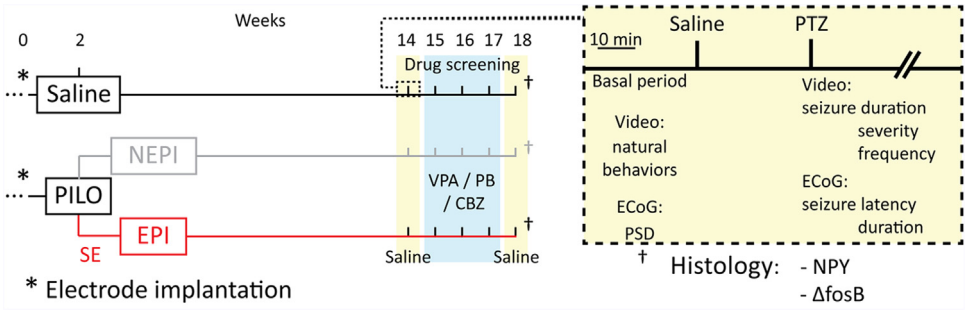

Fig. 1. Experimental design. Animals were implanted with telemetry-associated electrodes over the motor cortex and were then injected with either saline or pilocarpine. Pilocarpine could lead to the onset of SE (*status epilepticus*), in which case animals comprised the group EPI (epileptic), or not (NEPI group; which were omitted in data presentation and discussion). Three months later testing of 3 antiepileptic drugs against pentylenetetrazol (PTZ) seizures was carried out for 5 weeks. For first and fifth sessions the compound was invariably saline, whereas valproic acid (VPA), phenobarbital (PB) and carbamazepine (CBZ) were sorted in remaining sessions. After last testing session, the brain of these animals were fixed and processed for histology and immunohistochemistry.

ing each session animals were continuously recorded by video and ECoG.

2.6. Histological processing and immunohistochemistry

Euthanasia was performed one week after the fifth session of drug testing (in which all animals received saline followed by PTZ). The one week washout period was likely enough to proteolytically eliminate any fluctuation in ΔFosB's levels that might have been caused by PTZ one week before, hence preventing the bias from previous sessions of drug testing over the quantification of ΔFosB-expressing cells. For this, marmosets were deeply anesthetized (thiopental, 30 mg/Kg, intraperitoneal) and perfused through the heart with 200 mL phosphate buffer, pH 7.4 followed by 400 mL 4% paraformaldehyde (PFA) in 0.01 M PBS, pH 7.2. Next, brains were sequentially immersed in PFA and 30% (w/v) sucrose solution for 48 h each. At last, 30 μm thick brain coronal sections were cut on a cryostat.

Immunohistochemistry was carried out as previously described (Hoffman et al., 2008). In brief, primary antibodies were titrated in PBS (1:15,000 for neuropeptide y, NPY – Sigma-Aldrich, # N9528; and 1:2000 for ΔFosB – Sigma-Aldrich, # AV32519) and triton X-100, whereas biotinylated secondary antibodies were used in the presence of 0.5% (v/v) goat serum. Next, horseradish peroxidase was coupled to antibodies' biotin by means of ABC elite kit and the colorimetric reaction was based on the oxidation of diaminobenzidine for 20 min.

2.7. Cell counting

Cell counting from different labeling procedures followed similar sampling of plates, whose quantification was adapted to the abundance of each event. In all cases, the cellular content of the frontal cortex was computed as the average of 8 plates (from 1 to 13.8 mm anterior to bregma), whereas temporal and entorhinal cortex comprised 6 plates (from 4.5 to 10 mm anterior to bregma). Similarly, the cell counting in CA1, CA3 and hilus of the hippocampus encompassed 4 plates (from bregma +3 to −3 mm). Estimation of the neuronal damage in the hippocampus of marmosets was based on Nissl-stained sections. To this end, we acquired images from sampled plates at 40 x magnification and only stained neurons superposed by 10 × 10 grid were computed. Neurons were identified based on morphological criteria. To quantify NPY and ΔFosB-positive neurons we summed every immunostained cells present in images from sampled plates at 10× and 40× magnification, respectively. For all quantification, the neuronal content was computed as an average of both hemispheres and expressed as cells/mm^2.

2.8. Statistical analysis

Data, which fit the normal distribution (according to Kolmogorov-Smirnov test) was analyzed by T-test or ANOVA, otherwise we used the Wilcoxon test or Kruskal Wallis. In parametric analysis, the hypothesis test was often followed by post-hoc Tukey-Kramer or Fisher's LSD to confirm effects and deepen data interpretation. Multiple regression analysis was also executed to correlate the amount of immunostained cells with ictal activity as recorded by ECoG. Finally, Chi-squared was computed to test differences regarding seizure incidence between different conditions treated with the same AED. For all analysis, it was considered $\alpha = 0.05$. In addition to $p > 0.05$, a statistical power higher than 0.7 was required to discard effects, otherwise analysis was considered inconclusive. For clarity, the statistical power was omitted in statistic presentation, but inconclusive analysis were locally indicated.

Natural behaviors were quantified in the first basal period, whereas ictal behaviors (as well as the influence of the epileptic condition on ictal profile) were assessed after the first drug testing session. Each AED was assumed to suppress seizures, when the ensuing ictal activity was significantly less than that after saline treatment (this analysis is presented in Supporting information). The influence of the epileptic neural background on the efficacy of AEDs was tested as the effect of experimental condition when each AED was analyzed individually. The behavioral analysis considered the summation of seizure incidence and duration over a six hours period following PTZ administration. In the same way, the analysis of seizures recorded by ECoG provided the duration of seizures as the summation of ictal events along 6 uninterrupted hours following PTZ administration.

The analysis of NPY- and ΔFosB-expressing neurons also took into account two additional groups: naïve and epileptic animals that had not been subject to sessions of drug testing. This approach was aimed to discard a potential influence of the AEDs (despite a one week washout) over NPY and ΔFosB expression. Notwithstanding, some groups were omitted in data presentation for the sake of clarity.

3. Results

3.1. Pilocarpine-treated animals are characterized by an altered "epileptic" background

In resting awakened animals, the LFP of epileptic marmosets was often dominated by oscillations around 3 Hz, whereas periods with faster oscillations (8–12 Hz) were more frequent in naïve counterparts (Fig. 2A). As a result, when longer periods were

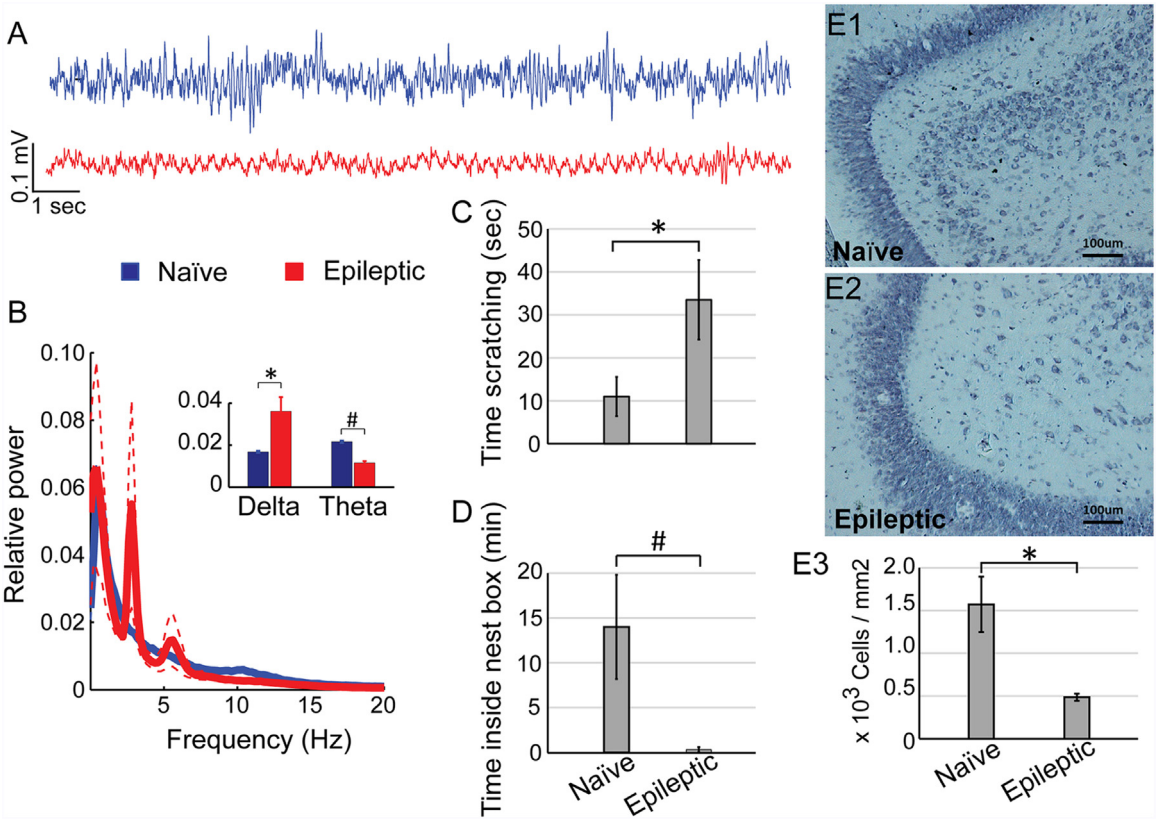

Fig. 2. Pilocarpine leads to anatomical and functional changes. A) Fifteen seconds of LFP acquired in a resting awake animal. Theta oscillation was abundant in naïve animals' LFP (blue), whereas a delta oscillation prevailed in the epochs from epileptic marmosets (red). B) The mean normalized power spectrum density (PSD) from one hour of resting awake in naïve (blue) and epileptic (red) groups. Dashed lines illustrate the standard errors of the mean (SEM). The inset in the upper right corner highlights the mean power integrated in delta and theta range. Error bars represent SEM, *indicates $p < 0.05$ and # indicates $p < 0.000001$, both from t-test. C-D) Time spent scratching and the time animals were sheltered inside the nest box, respectively, both on basal condition, in which animals were devoid of ictal activity and were not manipulated. Error bars represent SEM and * indicates $p < 0.05$, according to Tukey-Kramer. E) Representative images from Nissl staining of the hilus of the dentate gyrus from a naïve animal (E1) and an epileptic marmoset (E2). In E3, the mean neuronal density in the hilus from naïve and epileptic groups. Error bars illustrate SEM and * indicates $p < 0.05$ from t-test.

considered, the power of delta oscillations was enhanced in epileptic marmosets followed by a reduction in the power of theta oscillations, as compared to controls (Fig. 2B; t-test, $p = 0.0149$ and $p < 0.000001$, respectively). The contrast between the epileptic and naïve neural backgrounds was also manifested in natural behaviors. The time animals spent scratching was influenced by the epileptic condition (Fig. 2C; ANOVA, $F = 4.39$, $p = 0.03$), which was further confirmed by longer scratching by epileptic marmosets as compared to the naïve group (Tukey-Kramer, $p < 0.05$). Similarly, the time sheltered inside the nest box also depended on the epileptic condition (Fig. 2D; ANOVA, $F = 5.48$, $p = 0.02$). Indeed naïve animals remained longer periods inside the nest box than the epileptic group (Tukey-Kramer, $p < 0.05$). In addition structural changes induced by pilocarpine contributed to differentiate epileptic marmosets from naïve animals. Epileptic marmosets presented extensive mossy fiber sprouting and dispersion of the granular layer of the dentate gyrus (Supporting information, Figs. S1 and S2, respectively). At last, the abundance of neurons in the hilus of the dentate gyrus was reduced in epileptic marmosets as compared to naïve peers (Fig. 2E; t-test, $p < 0.02$), suggesting hippocampal atrophy. Altogether these effects confirm that changes induced by pilocarpine constitutively differentiate the epileptic neural background from the naïve condition regarding various histological aspects, brain rhythms and behavior.

3.2. Expression of neuropeptide Y is enhanced in epileptic marmosets

NPY is an inhibitory neuromodulator, whose expression is augmented under aberrant electrical activity, hence this is considered as an endogenous anti-epileptic response. Accordingly, the frequency of seizures reflected the amount of NPY-positive cells in the frontal cortex ($R = 0.69$, $p = 0.01$). We could not identify any influence of the epileptic condition on the number of NPY-positive cells in entorhinal cortex and CA3 (ANOVA, $p = 0.43$, for entorhinal cortex; $p = 0.12$ for CA3; inconclusive due to insufficient statistical power). In the hilus of the hippocampus, the influence of the epileptic background was not conclusive (because insufficient statistical power; ANOVA, $p = 0.06$). On the other hand, in frontal (Fig. 3A) and temporal cortices as well as in CA1 of hippocampus, the quantity of NPY-positive cells depended on the epileptic condition (ANOVA, $p < 0.001$ for frontal cortex; $p = 0.015$ for temporal cortex; and $p = 0.02$ for CA1), given that these cells were more abundant in epileptic marmosets as compared to naïve peers (Fig. 3B and C; Tukey-Kramer, $p = 0.0002$, $p = 0.015$, $p = 0.019$, respectively).

3.3. PTZ-elicited seizures were more frequent and last longer in already epileptic animals

Individual PTZ-induced seizures from both naïve (Fig. 4A) and epileptic marmosets (Fig. 4B) showed a similar ECoG architecture, with three stages. After a moderate beginning, as evaluated by

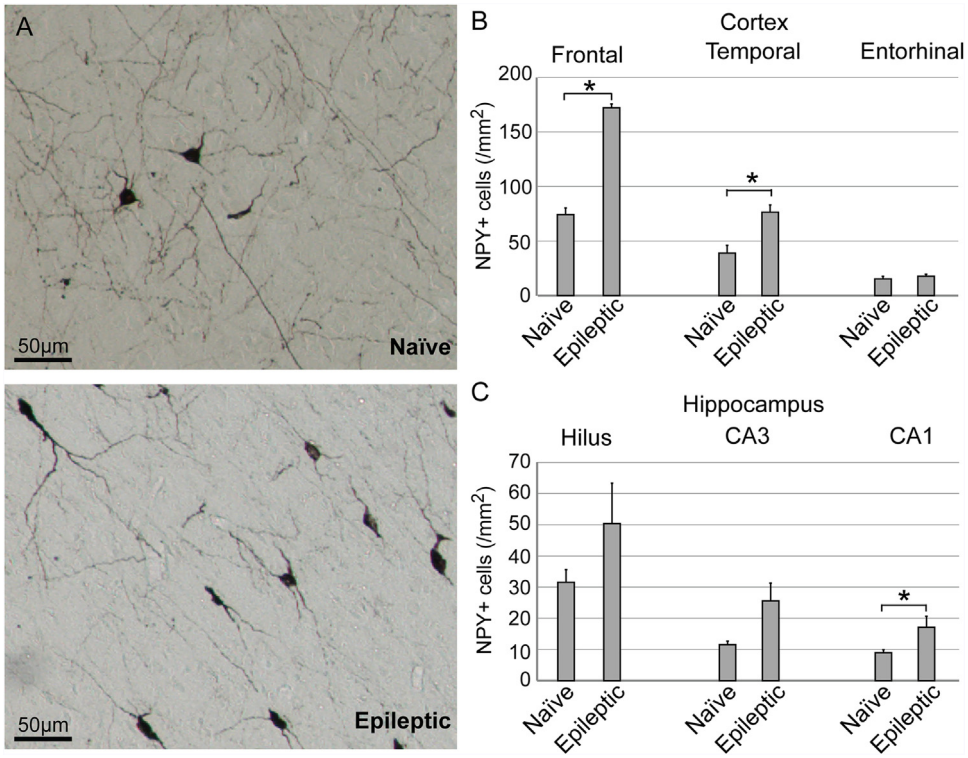

Fig. 3. Quantification of NPY-positive neurons. Animals, whose data are depicted in A, B and C were not subjected to AED drug testing, hence spared from the influence of PTZ. A-1) The image exemplifies the immunohistochemistry for NPY in frontal cortex of a naïve marmoset, whereas that of an epileptic peer is represented in A-2. Quantified immunostained cells are represented as group mean in B and C for neocortex and hippocampus, respectively. Error bars illustrate SEM and *indicates $p < 0.05$ from Tukey-Kramer.

means of power analysis, seizures evolved to a sustained period with high power centered around 10 Hz. Thereafter this diminished in frequency towards the termination phase. Seizure ending was marked by high power slow oscillations (around 2 Hz), whose valleys were concomitant with faster oscillations. At last a post-ictal depression marked the end of each ictal event. The seizure exemplified in Fig. 4B may suggest that seizures emerged with faster oscillations in epileptic marmosets, although this feature was less clear when seizures are averaged (data not shown).

The epileptic condition also did not affect the latency for the first seizure after PTZ administration (data not shown). However, the epileptic condition influenced the frequency and duration in which these PTZ-induced seizures emerged, favoring the occurrence of seizures, which emerged as longer sequences of ictal events (classified as *SE*-like seizures; Fig. 4C; Kruskal-Wallis, $p = 0.05$) in contrast to the scarcity of these sequences in naïve marmosets. Indeed, no naïve marmoset showed these *SE*-like seizures after PTZ administration as opposed to 55% of the epileptic cohort (table S1 in Supporting information; Chi-squared, $p < 0.05$). In addition, the frequency of the behavioral manifestation of seizures, depended on the epileptic condition (Fig. 4D; ANOVA, $F = 6{,}40$, $p = 0.01$), given the incidence of ictal events was greater in epileptic marmosets as compared to naïve counterparts (Tukey-Kramer, $p < 0.05$). This higher frequency was also associated with longer behavioral expression of seizures in epileptic marmosets (Fig. 4D; ANOVA, $p = 0.03$), which was further confirmed by post-hoc analysis (Tukey-Kramer, $p = 0.02$). The epileptic condition seemed to worsen the severity of the ictal profile elicited by PTZ, given that seizure intensity was concentrated in type IV/V seizures (ANOVA, $F = 4.42$, $p = 0.03$ and Tukey-Kramer, $p < 0.05$ for frequency; ANOVA, $F = 4.27$, $p = 0.04$ and Fisher's LSD, $p < 0.05$, for duration). In line with the longer behavioral expression of seizures, epileptic marmosets had longer durations of seizure-related orofacial movements as compared to the naïve group (Fig. 4D-3; Kruskal-Wallis, $p = 0.02$). Vocalization, spasms of limbs and visual disorientation may also account for differences in the behavioral expression of seizures between epileptic and control marmosets, although these effects were not statistically significant (Kruskal-Wallis $p = 0.07$ and Wilcoxon $p = 0.06$ for the latter). Given that the influence of the epileptic condition over seizures elicited by PTZ relied mostly on the frequency and duration of these events, the subsequent investigation of the effects of AEDs was centered in these two parameters.

3.4. Seizures in epileptic animals engaged a larger neuronal network than seizures in naïve animals

ΔFosB is a longer stable indirect marker of neuronal activation, hence suitable to identify neuronal populations engaged in seizure precipitation. Under the present conditions, we observed that seizure induction by PTZ altered the number of ΔFosB-positive cells, in a manner that was specific to the epileptic condition, in all analyzed brain areas (Fig. 5B and C; ANOVA, $p < 0.001$ for frontal, temporal and entorhinal cortices, CA3 and CA1; $p = 0.03$ for the hilus of the hippocampus). This effect is confirmed by the greater abundance of immunostained cells in epileptic marmosets as compared to naïve animals (Tukey-Kramer, all values of $p \leq 0.05$). Epileptic marmosets presented a higher number of ΔFosB-positive cells in the neocortex irrespective of ictal activity induced by PTZ (data not shown). Yet the differences, in this brain area, as compared to naive animals, is largely increased as a result of PTZ seizure induction, given that only epileptic marmosets had a further increase in the abundance of ΔFosB-immunostained cells after PTZ. On the other hand the number of ΔFosB-expressing neurons in the CA1 and CA3 of the hippocampus surpassed that of naïve peers only following acute seizure induction by PTZ (data not shown). Altogether these

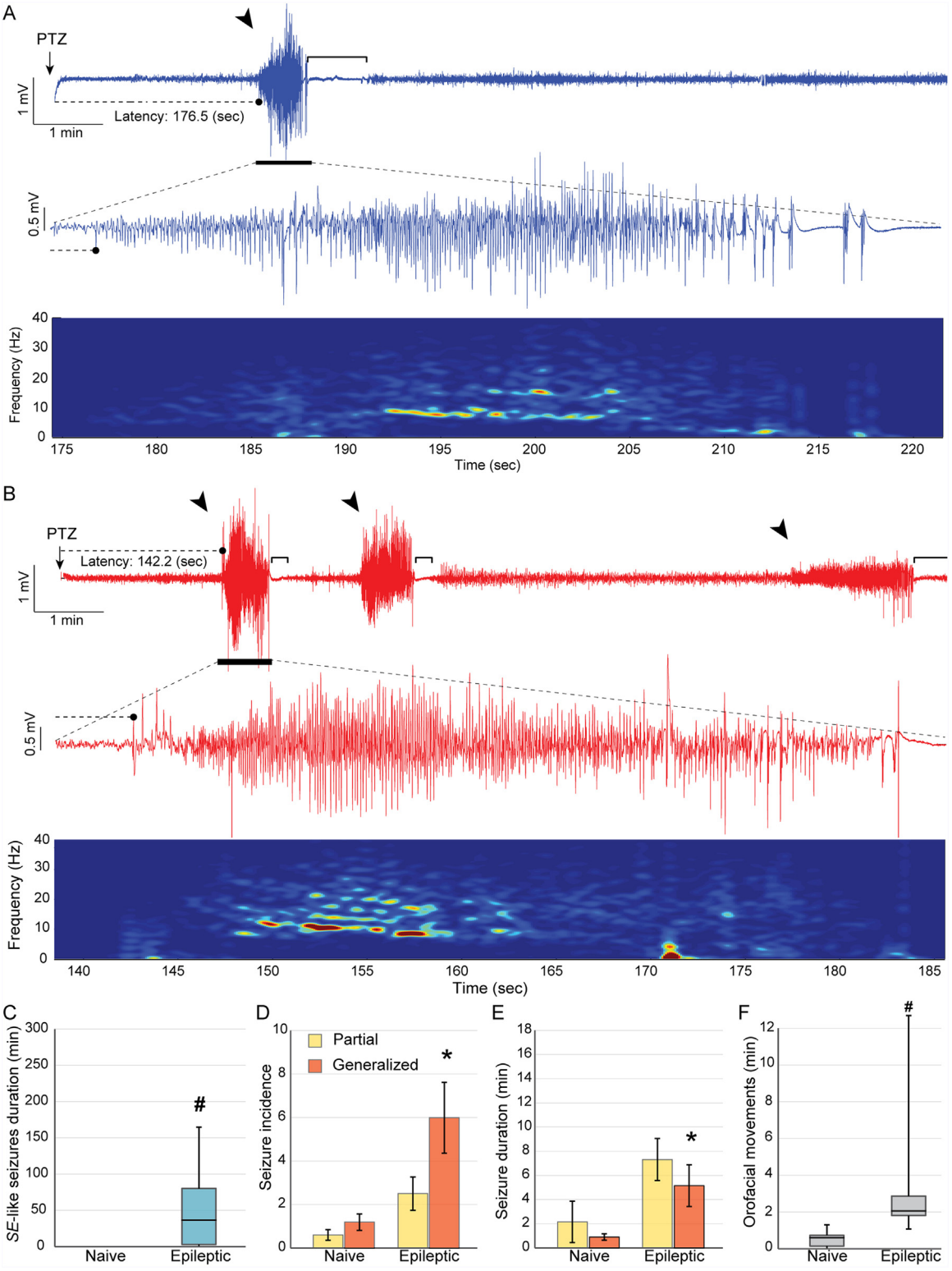

Fig. 4. The influence of chronic epileptic induction by pilocarpine over acute seizure evocation by PTZ. A) Upper panel: 13 min of LFP from a naïve marmoset starting at PTZ administration, showing one seizure, which is indicated with an arrowhead and is expanded in the middle panel, with corresponding PSD plotted bellow (lower panel). B) Similar analysis as shown in A but now for an epileptic marmoset. C) The boxplot illustrates median, quartiles, minimum and maximum values for the duration of chains of seizures (i.e., seizures not interrupted either by post-ictal depression or behavioral manifestation of consciousness. # indicates $p \leq 0.05$ according to Kruskal-Wallis, D-E) Behavioral evaluation of seizures elicited by PTZ during six hours following PTZ administration. Seizures were first categorized, according to severity following an adapted Racine scale: seizure types III and IV/V are presented as partial and generalized events, respectively. Seizure frequency is plotted in D and duration is presented in E. Colorful bars illustrate group means, whereas error bars represent the SEM. * indicate $p < 0.05$ according to Tukey-Kramer. F) Duration of orofacial movements accompanying seizures, represented as median, quartiles, minimum and maximum values. # indicates $p \leq 0.05$ according to Kruskal-Wallis.

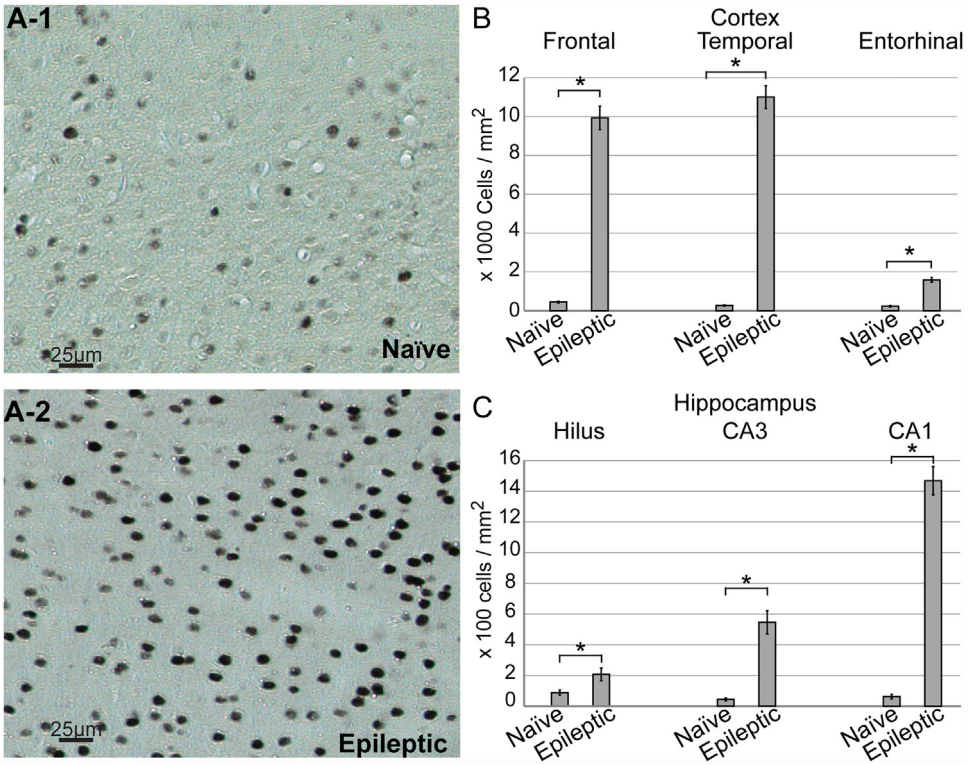

Fig. 5. Quantification of ΔFosB-positive neurons. The data depicted in A, B and C were collected following the last drug testing session, in which saline preceded PTZ, one week after last AED treatment. A-1) The image exemplifies the immunohistochemistry for ΔFosB in the frontal cortex of a naïve marmoset, whereas that of an epileptic peer is represented in A-2. The immunostained cells where quantified and are represented as group mean in B and C, for neocortex and hippocampus, respectively. Error bars illustrate SEM and * indicates $p < 0.05$ from Tukey-Kramer.

evidences indicate that seizure induction by PTZ engages a larger neuronal network in animals with an epileptic background.

3.5. *Efficacy of AEDs was less in epileptic animals*

In general, epileptic animals were less responsive than naïve animals to the anticonvulsant effects of the tested drugs. The exception was PB, which was equally effective on the control of every evaluated ictal parameter in both epileptic marmosets and naïve peers (Fig. 6B-1–B-4). In contrast, VPA was less effective in epileptic animals, regarding the duration of individual seizures, as assessed in ECoG (Fig. 4A-3; Kruskal-Wallis, $p = 0.05$).

The effect of CBZ on the duration of both isolated and *SE*-like seizures also depended on the epileptic background (Fig. 6C-3 and C-4; Kruskal-Wallis, $p = 0.02$ and $p = 0.03$, respectively). Similarly the epileptic condition influenced the effect of CBZ on the behavioral manifestation of the more severe generalized seizures (type IV/V; Duration: Fig. 6A-3; ANOVA, $F = 5.85$, $p = 0.01$. Frequency: Fig. 6B-3; ANOVA, $F = 4.07$, $p = 0.04$). Seizure suppression by CBZ was less evident in epileptic marmosets. As a result, following CBZ treatment, the behavioral manifestation of type IV/V seizures lasted longer in epileptic than in the naïve group (Fig. 6B-3; Tukey-Kramer, $p < 0.05$) and type IV/V seizures were more frequent in epileptic marmosets than in naïve animals (Fig. 6A-3; Fisher's LSD, $p < 0.05$). The effect of CBZ on seizure frequency also depended on the epileptic condition when all behavioral ictal observations were considered, regardless of seizure severity (Fig. 6A-3; ANOVA, $F = 6.07$, $p = 0.01$), given that the frequency of all types of behavioral seizures was greater in epileptic than in the naïve group (Fisher's LSD, $p < 0.05$).

4. Discussion

Here we observed that, in the absence of AEDs, the acute seizures evoked by PTZ were accentuated in epileptic marmosets. In general, all AEDs suppressed seizures, even though with different degrees of success. PB was the only evaluated AED to markedly abolish severe seizures even in epileptic animals. In turn the seizure blocking effect of CBZ and VPA were mostly restricted to naïve marmosets. Moreover, the epileptic condition was associated with different natural and ictal behaviors. These epileptic marmosets also presented greater numbers of NPY-positive cells in frontal and temporal cortices as well as in CA1 of hippocampus. In addition, the abundance of ΔFosB-positive cells was greater in epileptic marmosets in brain areas associated with genesis and propagation of seizures. Altogether these findings substantiate the hyperexcitability conferred by the epileptic background, over which there was a diminished efficacy of classical AEDs.

The epileptic condition was associated with longer time scratching, a natural behavior related to anxiety in marmosets (Barros et al., 2000). Accordingly, the anxiolytics lorazepam (Schino et al., 1991), midazolam (Maestripieri et al., 1991) and diazepam (Cilia and Piper, 1997) have been shown to reduce scratching in anxiety models in nonhuman primates. Also, the shorter time spent in the nest box by epileptic animals may reflect enhanced anxiety, a mood disorder, which affects twice as many persons with epilepsy as in the general population and deteriorates the life quality of these patients (Beyenburg et al., 2005). Indeed it has been suggested that increased locomotor activity in marmosets (Barros et al., 2000), might be seen as indicative of anxiety.

The induction of a chronic epileptic condition by pilocarpine turned marmosets more responsive to PTZ, accentuating duration and frequency especially of more severe seizures (generalized, type IV/V). This effect was rather subtle in rats (under a similar

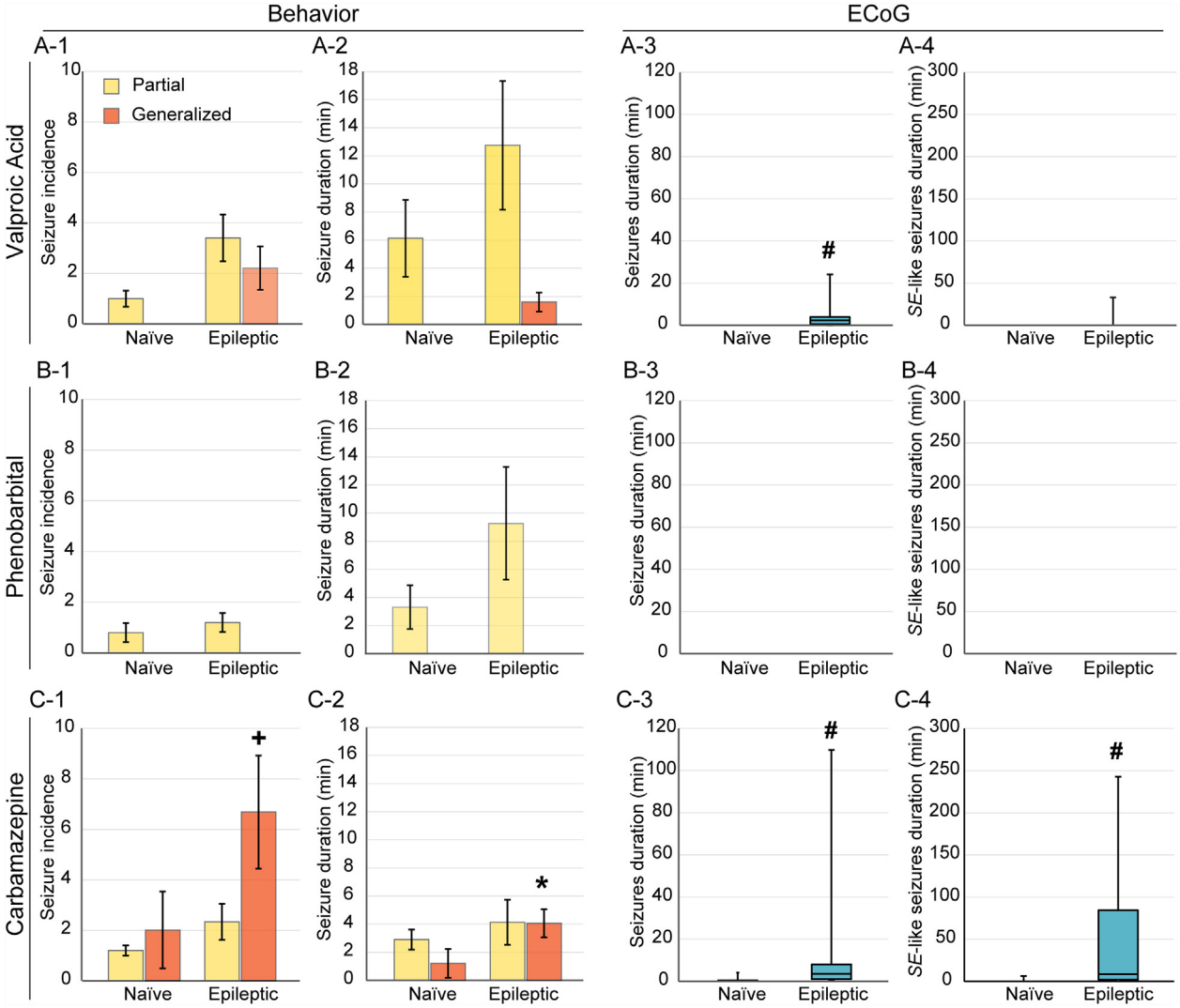

Fig. 6. The efficacy of AEDs, according to the epileptic condition. Rows A, B and C represent parameters from seizures evoked by PTZ in animals previously treated with valproic acid, phenobarbital and carbamazepine, respectively. Columns 1 and 2 represent, respectively, the summation of frequency and duration of the behavioral manifestation of seizures over six hours after PTZ administration. Seizures were classified as partial or generalized events, reflecting type III or IV/V of an adapted Racine scale. Color bars illustrate the mean of the group, whereas error bars indicate SEM. + and * indicates $p \leq 0.05$ according to Fisher's LSD and Tukey-Kramer, respectively. Column 3 represents the summation of the duration of isolated seizures as recorded in ECoG along 6 uninterrupted hours after PTZ administration. The absence of post-ictal depression and lacking behavioral evidence of consciousness characterized the chains of seizures as *SE*-like events, whose durations are presented in column 4 as a sum. The boxplots (blue) illustrate median, quartiles, minimum and maximum values. # indicates $p \leq 0.05$ according to Kruskal-Wallis.

pilocarpine/PTZ protocol), which presented similar profile of partial and generalized seizures in both epileptic and naïve rodents (Blanco et al., 2009). Importantly, this enhanced ictal prone condition might have undermined the efficacy of AEDs, given the suppression of seizures by AEDs was less in the chronic epileptic condition. Indeed, whereas the occurrence of seizures elicited by PTZ was completely abolished by VPA in naïve subjects, the same ictal event still persisted in 20% of epileptic marmosets after VPA. This was comparable to seizure inhibition in spontaneously seizing rats, whose epileptiform activity was triggered by electrical stimulation of the amygdala (Nissinen and Pitkanen, 2007). Yet, the present experimental design adds the convenience to concentrate data collection, given the seizure measurements are defined by the experimenter upon the administration of PTZ.

Thus, under the chronic epileptic condition induced by pilocarpine there is a failure of VPA in suppressing seizures. The mechanism underlying this pharmacoresistance might be related in part to GABAergic neurons from substantia nigra pars reticulata (Snr) (McNamara et al., 1984), whose firing rate is reduced by VPA. This decline, on pharmacoresistant amygdala-kindled rats (Tollner et al., 2011), is possibly due to reduced activity of GABA-synthesizing enzyme glutamate decarboxylase (Loscher and Schwark, 1985). Thus, chronic epileptic animals seem to lack GABAergic inhibition in SNr to mediate seizure suppression by VPA.

Similarly, under CBZ treatment, epileptic marmosets developed longer *SE*-like seizures, which reflected longer, more frequent and severe behavioral manifestations (generalized, type IV/V seizures) than naïve subjects. CBZ abolished *SE*-like seizures in naïve animals, whereas these events were largely not attenuated in epileptic marmosets. These evidences indicate that epileptic induction by pilocarpine also lead to pharmacoresistance to CBZ. The mechanism of action reported for CBZ relies on the reduction of neuronal excitability through the blockage of voltage-gated Na+ channels (Courtney and Etter, 1983). Indeed, these channels become insensitive to CBZ in CA1 neurons of the hippocampus of kindled rats (Vreugdenhil and Wadman, 1999), and in human patients with epilepsy (Vreugdenhil et al., 1998). Thus the pharmacoresistance to CBZ shown here might be associated to the fading of an anti-

epileptic mechanism caused by longer ictal activity, which only occurs in chronic models. Thus, similarly to VPA, the expected CBZ efficacy, based on data from acute models, does not show up when the treatment is assessed in an epileptic background.

PB was once the most widely used first-line antiepileptic drug, but its use has declined in wealthy countries given a number of side effects, chiefly cognitive and functional impairments (Elafros et al., 2014). The adverse effects of PB include sedation, hyperactivity, impaired cognition, depressed mood and affect, anemia, hepatotoxicity and teratogenicity (Kwan and Brodie, 2004). In our experimental conditions, PB was remarkably efficient in the overall control of seizures regardless of the epileptic background over which the drug was being tested. The higher efficacy of PB in the present conditions contrasts the meta-analysis, in which PB is considered as having comparable efficacy to other AEDs, such as VPA (Kwan and Brodie, 2004). This might be associated with both PTZ and PB acting on the same GABA-A channel. PB binds to the GABA-A receptor in the site for benzodiazepine, which extends the time the chloride channel remains opened (Twyman et al., 1989), whereas PTZ blocks the chloride influx by means of the binding to the picrotoxin site of the same GABA-A channel (Huang et al., 2001). Even though the antagonism between PTZ and PB does not rely on a competition for the same binding site, the previous PB administration may prompt conformational changes, which might reduce the responsiveness of GABA-A channel to PTZ. However, the efficacy of PB presented here corroborates the anticonvulsant effect on similar combined pilocarpine-PTZ in mice (Tollner et al., 2016) but contrasts with its negligible effect in an equivalent model in rats (Blanco et al., 2009). This divergence might be related to the P-glycoprotein, a drug efflux transporter located in the blood-brain barrier (BBB). P-glycoprotein was shown to reduce the concentration of PB in the brain, hence leading to loss of efficacy (Loscher and Potschka, 2005) and its expression is 3 folds higher in the rat brain as compared to marmosets and humans (Hoshi et al., 2013). The comparable BBB permeability between humans and marmosets (Hoshi et al., 2013) seems to consist another advantage to accurately forecast the efficacy of AEDs in humans when using these animals for testing.

The increased number of NPY-positive cells in frontal and temporal cortices and CA1 of the hippocampus of animals made chronically epileptic by pilocarpine corroborates similar findings in other models (Husum et al., 2000; Vezzani and Sperk, 2004). Indeed NPY is an inhibitory neuromodulator, whose synthesis is prompted by seizures (Vezzani and Sperk, 2004), and is thus considered as part of an endogenous antiepileptic mechanism (Richichi et al., 2004). Accordingly, here NPY expression increased in the temporal cortex of naïve marmosets as a consequence of repetitive PTZ administration, inherent to the drug testing protocol. In contrast, the expression of NPY in the epileptic animals was indifferent to PTZ, as though the epileptic condition had already saturated this endogenous anti-epileptic system. In any event the over-expression of NPY in epileptic as compared to naïve animals is a clear indication of the altered background neuronal network of epileptic animals.

The marmosets made chronically epileptic by pilocarpine presented more ΔFosB-positive cells in CA1, CA3 and the hilus of the dentate gyrus, corroborating the increased expression found in the hippocampus of rats with SRSs induced by kainic acid (Bing et al., 1997). Increased ΔFosB expression in the frontal cortex was already shown to follow repeated electroconvulsive seizures (Hiroi et al., 1998). Here we found that a similar increment also took place in temporal and entorhinal cortices of marmosets as a consequence of the chronic epileptic condition due to pilocarpine, hence generalizing the enhanced ΔFosB expression to other areas of seizure propagation. ΔFosB expression is conditioned to the activation of serum response factor (SRF) in response to repetitive stimulation (Morris et al., 2000). Accordingly, SRF was found to be increased after epileptic induction by pilocarpine (Nadler, 2003). ΔFosB differs from the remaining products of the immediate early gene *fosB* because the C-terminus truncation, which renders to ΔFosB increased stability: 10 h in culture (Ulery et al., 2006). Yet, the turnover of ΔFosB can be reduced by phosphorylation in the brain. As the result ΔFosB levels originated from five days of increased expression may persist for up to 10 days (Ulery-Reynolds et al., 2009). Thus it seems prudent to assume that ΔFosB induced by acute events, such as AEDs and PTZ administration in each session of drug testing, would not persist after a week washout. In this sense, the number of cells expressing ΔFosB might less likely reflect the influence of previous sessions of drug screening, and might reflect the outcome of acute seizure induction by PTZ. In addition, a possible bias from a cumulative effect of the drug testing sessions is rather unlikely, given all ictal parameters remained unchanged following the last administration of PTZ as compared to the first one (e.g., there was no kindling effect). Therefore, the greater expression of ΔFosB in epileptic as compared to naïve animals indicates that genesis and propagation of seizure induced by PTZ likely appropriates a much larger neuronal network in the former. This result might help to explain the enhanced expression of PTZ seizures in the epileptic animals, over which AEDs were less efficient.

Supported by histological evidence, here we show that the greater seizure propensity seen in epileptic animals was also associated to a diminished effectiveness of AEDs in these animals. We forward that induction of PTZ seizures in marmosets made chronically epileptic by pilocarpine might constitute a relevant means for investigating potential new AED.

Conflict of interest

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPQ), Brazil, for financial support. Every step of this work is devoid of influence by funding sources. Moreover, authors confirm the absence of conflicts of interest.

Acknowledgements

We acknowledge Evaldo Caral Junior and Fernanda de Paula for technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eplepsyres.2016.06.012.

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