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

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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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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-