BRAIN RESEARCH 1634 (2016) 179–186

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Brain Research

Research Report

Relationship between seizure frequency and number of neuronal and non-neuronal cells in the hippocampus throughout the life of rats with epilepsy

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ARTICLE INFO

Article history:
Accepted 26 December 2015
Available online 4 January 2016

Keywords:
Epilepsy
Seizure frequency
Hippocampus
Neuronal cell
Non-neuronal cell

ABSTRACT

The relationship between seizure frequency and cell death has been a subject of controversy. To tackle this issue, we determined the frequency of seizures and the total number of hippocampal cells throughout the life of rats with epilepsy using the pilocarpine model. Seizure frequency varied in animals with epilepsy according to which period of life they were in, with a progressive increase in the number of seizures until 180 days (sixth months) of epileptic life followed by a decrease (330 days-eleventh month) and subsequently stabilization of seizures. Cell counts by means of isotropic fractionation showed a reduction in the number of hippocampal neuronal cells following 30, 90, 180 and 360 days of spontaneous recurrent seizures (SRS) in rats compared to their controls (about 25%–30% of neuronal cell reduction). In addition, animals with 360 days of SRS showed a reduction in the number of neuronal cells when compared with animals with 90 and 180 days of seizures. The total number of hippocampal non-neuronal cells was reduced in rats with epilepsy after 30 days of SRS, but no significant alteration was observed on the 90th, 180th and 360th days. The total number of neuronal cells was negatively correlated with seizure frequency, indicating an association between occurrence of epileptic seizures throughout

Abbreviations: SE, *status epilepticus*; SRS, spontaneous recurrent seizures; PBS, phosphate-buffered saline; PB, phosphate-buffered; DAPI, 4′-6-diamidino-2-phenylindole dihydrochloride; NeuN, neuron-specific nuclear protein; MRI, magnetic resonance imaging; DG, dentate gyrus; GFAP, glial fibrillary acidic protein

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http://dx.doi.org/10.1016/j.brainres.2015.12.055

life and neuronal loss. In sum, our results add novel data to the literature concerning the time-course of SRS and hippocampal cell number throughout epileptic life.

1. Introduction

Temporal lobe epilepsy (TLE) is a neurological disorder that involves disruption of the brain's normal activity due to neuronal hyperexcitability in temporal lobe regions (Schwartzkroin, 1986). Histopathological analyses of surgically removed tissue from refractory TLE patients have pointed to hippocampal sclerosis as the most common finding. Hippocampal sclerosis is the combination of atrophy and astrogliosis of the hippocampus, amygdala, parahippocampal gyrus and entorhinal cortex; often characterized by decreased pyramidal and hilar neurons, and losses in other extratemporal regions (Cendes et al., 1993; Thom et al., 2009). Animal models for seizures and epilepsy induction have been widely used to understand the physiological and behavioral changes associated with human epilepsy (Kharatishvili et al., 2006; Pitkanen and McIntosh, 2006; Arida et al., 2007). The pilocarpine model of epilepsy is probably the most commonly studied model for TLE, since it reproduces the main features of human TLE in rats and mice (Cavalheiro, 1995). Pilocarpine-induced *status epilepticus* (SE) leads to cell loss in several hippocampal and extrahippocampal structures and spontaneous recurrent seizures (SRS) (Turski et al., 1983; Leite et al., 1990).

Studies have investigated the progression of neuronal damage after pilocarpine induction over time (Fujikawa, 1996; Sankar et al., 1998) and findings regarding hippocampal cell loss after SE and during the chronic phase of the model have been inconsistent. For instance, a study conducted by Liu et al. (1994) did not observe progressive neuronal loss in CA1 and CA3 hippocampal regions three, six and twelve weeks after pilocarpine injection during which animals presented spontaneous seizures. Chen et al. (2013) observed that one hour of pilocarpine-induced SE in mice evolved to SRS but did not cause neuronal death. In another study, cell loss after pilocarpine-SE induction did not increase over time, even with SRS stability (Bertram et al., 1990). Conversely, Cavarsan et al. (2013) observed a lower density of neurons in aged animals with epilepsy (20 months). The divergence of the experimental findings could be related to variables utilized by the different authors, such as the pilocarpine dosage, time elapsed between SE-induction and anatomical evaluation, different histological methodologies and time window during the phase of spontaneous recurrent seizures in which the pathological observation was performed. Therefore, it is not clear whether the cell loss is progressive throughout life and whether there is a correlation between hippocampal cell loss and seizure frequency. Accordingly, the present study was designed to examine the time-course of epileptic seizures at different ages (30, 90, 180 and 360 days after the first spontaneous seizure) of adult animals and its relationship with cell quantification (neuronal and non-neuronal) in the hippocampal formation, using an isotropic fractionation method (Herculano-Houzel and Lent, 2005).

2. Results

2.1. Behavioral analysis

The behavioral manifestation of pilocarpine-induced seizures in rats was similar to that reported previously (Turski et al., 1983; Cavalheiro et al., 1987). Briefly, pilocarpine injection sequentially induced behavioral changes such as akinesia, facial automatisms, and limbic seizures consisting of forelimb clonus with rearing, salivation, masticatory jaw movements and falling. This type of behavior built up progressively into motor limbic seizures that recurred repeatedly and rapidly evolved to SE. The mean latency to reach SE (time in minutes between the pilocarpine injection and the beginning of SE) was 29.4 $\pm$10.2 min. The duration of the latent period (time in days between the SE-induction and the occurrence of the first spontaneous seizure) was 22.1$\pm$8.9 days (mean$\pm$SD, n=37).

All animals that developed SE evolved to the chronic phase of the model with SRS. After the first spontaneous seizure, the number of subsequent seizures increased progressively peaking at 180 days. However, it is important to point out that increasing in seizure frequency was not statistically different among every month during this period, i.e., until 180 days. Significant differences were found between first month (5.6 seizures/month) and fourth month (mean value: 10.7 seizures/month), corresponding to about 91% of increase; and also between second month (6.9 seizures/month) and fourth month, corresponding to about 55% of increase. Besides, a significant increase in seizure frequency between first month and sixth month (11.6 seizures/month), corresponding to about 107% of increase; and also between second month and sixth month, corresponding to about 68% of increase were observed. After peaking, the number of SRSs decreased again to reach a plateau around 300–360 days (Fig. 1).

Repeated measures one-way ANOVA revealed difference between periods of 30 days ($F_{(11,\ 99)}$=7.551; p<0.001). As observed in Fig. 1, a significant increase of seizure number was noted at 120 and 180 days when compared to the 30 (p=0.001 and p=0.006, respectively), 60 (p=0.006 and p=0.005, respectively) and 330 (p=0.012 and p=0.002, respectively) days after the first spontaneous seizure, possibly attributable to the progressive nature of epileptogenesis in this epilepsy model. Although our analysis has been predominantly made via behavioral video confirmation of the seizures, we observed that the overall aspect of the seizures was not significantly modified during the whole study, i.e.

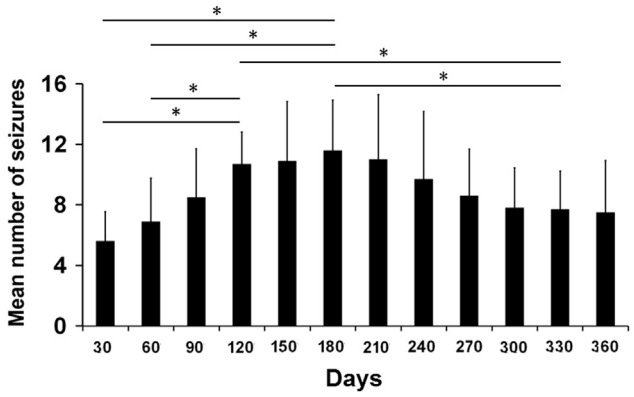

Fig. 1 – Number of seizures in animals submitted to 360 days of behavioral observation after the first spontaneous seizure. *Significative difference from respective control groups: 30 ($p=0.001$, $p=0.006$ respectively), 60 ($p=0.006$, $p=0.005$ respectively) and 330 ($p=0.012$, $p=0.002$ respectively) days. Results are presented as mean ± standard deviation.

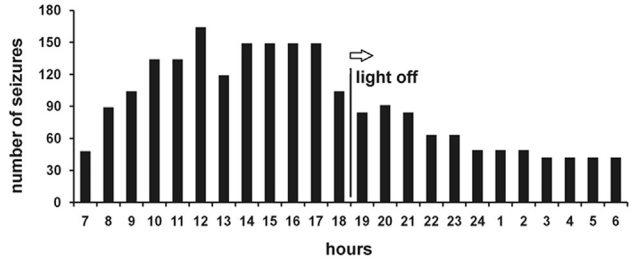

Fig. 2 – Pattern of distribution of spontaneous recurrent seizures (2195 seizures) recorded for 37 animals during 365 days of observation during the light:dark cycle. The frequency of seizures over the diurnal period (07:00–18:59 h) were significantly higher ($p<0.001$) when compared to the nocturnal period (19:00–06:59 h).

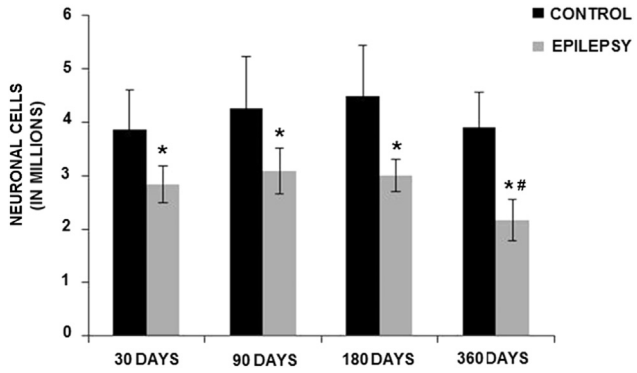

Fig. 3 – Number of neuronal cells in the hippocampal formation at different ages. *different from control groups ($p<0.05$); #different from 90 and 180 days epilepsy groups ($p<0.05$). Results presented as mean ± standard deviation.

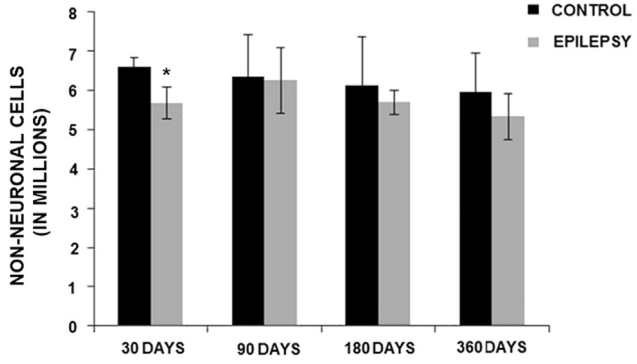

Fig. 4 – Number of non-neuronal cells in the hippocampal formation at different ages. Results represent both sides of hippocampus. *different from control group ($p<0.05$). Results presented as mean ± standard deviation.

most of the seizures could be classified as stages 4–5 of Racine with approximate duration of 60–90 sec. Concerning the light/dark cycle, our results showed that the number of seizures occurring in the light period of the cycle (7:00 am–6:59 pm) was significantly higher in comparison with the number of seizures observed in the dark period of the cycle (7:00 pm–6:59 am) ($F_{(1,\ 23)}=36.206$; $p<0.001$) (Fig. 2).

2.2. *Number of hippocampal neuronal cells*

Numbers of cortical and hippocampal cells were assessed by the isotropic fractionator method, an unbiased technique designed to determine the absolute cellular composition of different brain regions (Herculano-Houzel and Lent, 2005). Two-way ANOVA demonstrated a significant effect of group ($F_{(1,\ 74)}=80.442$; $p<0.001$), age ($F_{(3,\ 74)}=4.911$; $p=0.004$), but not interaction between groups and age ($F_{(1,\ 74)}=1.177$; $p=0.325$) in the number of hippocampal neuronal cells. No difference in the number of hippocampal neuronal cells was observed throughout the life of control rats (maximum mean value in control group 180 days – 4.494.932 neurons; minimum mean value in control group 30 days – 3.849.773 neurons ($p=0.226$). The mean neuronal cell number for control group 90 days was 4.251.946 neurons, and for control group 360 days was 3.902.168 neurons) ($p=1.000$). Rats with epilepsy, however, presented a reduction in the total number of hippocampal neuronal cells, as evidenced in the counting undertaken at 30 (mean value: 2.835.262 neurons; about 26% of reduction compared to control group 30 days), 90 (3.092.023 neurons; about 27% of reduction compared to control group 90 days), 180 (3.009.610 neurons; about 33% of reduction compared to control group 180 days) and 360 (2.172.441 neurons; about 44% of reduction compared to control group 360 days) days after the occurrence of the first spontaneous seizure ($p<0.001$ when to compared to control rats) (Fig. 3). The group of animals with 360 days of SRS also showed a reduction in the number of neuronal cells when compared with animals with 90 ($p=0.017$; about 30% of reduction) and 180 ($p=0.037$; about 28% of reduction) days of seizures. All the mean values presented are referred to cell count in both hippocampal sides together.

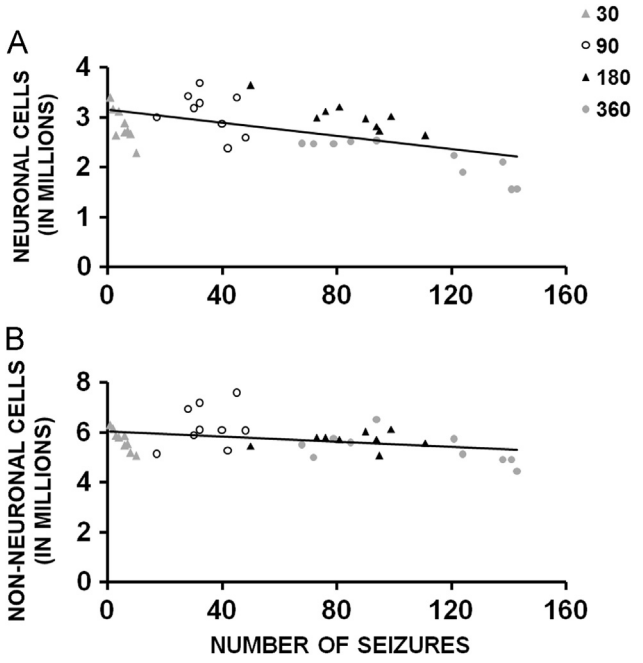

Fig. 5 – (A) Correlation between seizures and neuronal cells number. A medium negative correlation ($r=-0.49$; $p=0.002$). (B) Correlation between seizures and non-neuronal cells number. A weak negative correlation ($r=-0.36$; $p=0.029$). Results represent both sides of hippocampus.

2.3. *Number of hippocampal non-neuronal cells*

Two-way ANOVA demonstrated a significant effect of group ($F_{(1, 74)}=7.941$; $p=0.006$), but not of age ($F_{(3, 74)}=2.460$; $p=0.070$) and interaction between group and age ($F_{(1, 74)}=0.865$; $p=0.464$) in the number of hippocampal non-neuronal cells. Rats with 30 days of SRS presented a reduction in the total number of non-neuronal cells (mean value: 947.625 non-neuronal cells; about 14% of reduction) ($p=0.016$) when compared with the corresponding control animals (Fig. 4). No difference in the number of hippocampal non-neuronal cells was observed between control and epilepsy groups in all other periods of study.

2.4. *Correlation between seizures and cell number*

Considering all animals with epilepsy, a medium negative correlation was observed (Fig. 5A) between the number of neuronal cells and number of seizures ($r=-0.49$; $p=0.002$). A weak negative correlation was observed between the number of non-neuronal cells and number of seizures ($r=-0.36$; $p=0.029$) (Fig. 5B).

3. Discussion

The present study reports on the long-term observation of seizure frequency in the pilocarpine model of epilepsy in adult Wistar rats, the absolute number of neuronal and non-neuronal cells in the hippocampal formation at different time points of the observation period and estimates the possible relationship between these two findings. Most of the literature related to experimental epilepsy is devoted to observing seizure frequency and epileptogenesis-related brain changes for short periods of time. To our knowledge, this is the first study to investigate the time course of SRS and its relationship with the number of neuronal and non-neuronal cells in the hippocampal formation of adult Wistar rats for periods as long as 360 days after the establishment of the epileptic state. In a previous study by our group, the course of untreated seizures in the pilocarpine model of epilepsy was analyzed for 135 days (4.5 months) after the observation of the first spontaneous seizure (Arida et al., 1999). In that study, we observed a significant increase in the number of seizures during the initial period of the chronic phase of the model (Arida et al., 1999), a finding that reproduces the classic study of the long-term effects of pilocarpine in rats conducted by Cavalheiro et al. (1991) which also described the initial increase in the number of SRS. This finding was also reported by Bertram and Cornett (1994) using a different model of experimental epilepsy via continuous electrical stimulation of the hippocampus. Our data are in line with these previous experimental studies suggesting a maturation process in the early phases of epilepsy.

We would like to point out that several investigations that analyzed the temporal evolution of seizures used short observational periods of seizure monitoring to detect seizure frequency, that is, the periodic video monitoring was not detailed (Pitkanen et al., 2002; Cavarsan et al., 2013), or varied between studies [(2 h/day, (Sharma, 2007 #49); 3 h/day, (Liu et al., 1994); 8 h/day, (Furtado et al., 2011); 12 h/day, (Polli et al., 2014); 8 h/week, (Hattiangady and Shetty, 2010)]. In addition, although several experimental epilepsy studies have monitored behavior (by video or mutual video–electroencephalography recording) to detect spontaneous seizures (Cavalheiro et al., 1991; Quigg et al., 1998; Hellier and Dudek, 1999), this analysis has not been performed either for long periods or continually. In our work, we carefully monitored the animals continuously over 24 h/day until 360 days after the first detected spontaneous seizure. Assessment of seizure rates is very important for behavioral analysis in epilepsy models because short-term monitoring may lead to an under- or overestimation in the observation of seizure frequency.

The assessment of seizure occurrence over the light/dark cycle has been extensively reported (Arida et al., 1999; Hellier and Dudek, 1999; Raedt et al., 2009; Bajorat et al., 2011). Both in clinical seizures and experimental models of epilepsy the daily periodicity is more frequent during rest periods (Shouse et al., 1996; Quigg et al., 1998; Hellier and Dudek, 1999). Similar to findings reported in the literature, we observed that seizure occurrence obeyed a well-defined circadian pattern, reinforcing the hypothesis that seizures are more prominent in periods of inactivity or sleep.

The association between seizures and neuronal loss has long been recognized. However, several researchers have debated whether seizures are the cause or effect of neuronal death. A number of investigations by several laboratories have postulated that repeated seizures can induce neuronal loss (Turski et al., 1983; Cavalheiro et al., 1991; Mello et al., 1993; Pollard et al., 1994). In humans, the relationship

between seizures and hippocampal atrophy or volume loss is better defined. Histopathological analysis of patients with refractory epilepsy suggests that initial precipitating injury as well as SRS may lead to a progressive process of neuronal damage (Mathern et al., 1995). This issue has also been addressed in magnetic resonance imaging (MRI) studies. MRI investigations have demonstrated hippocampal asymmetry in patients with increased seizure number (Kalviainen and Salmenpera, 2002) or seizures with longer duration (Fuerst et al., 2001). Although studies have reported that hippocampal damage is associated with lifetime and number of seizures (Van Paesschen et al., 1997), contrasting findings have also been reported. For instance, Thom et al. (2005) did not observe a correlation between seizures and hippocampal neuronal loss.

Investigations with animal models of epilepsy have assessed the different time points after SE onset; however, few studies have evaluated the cell loss process in animals with epilepsy over long periods of spontaneous seizures (Cavalheiro et al., 1991; Obenaus et al., 1993; Liu et al., 1994; Buckmaster and Dudek, 1997). In our study, animals with epilepsy from all groups presented a reduction in neuronal cell number throughout life. In addition, a reduction in the number of neuronal cells was noted in animals euthanized 360 days after the first spontaneous seizure compared with those at 90 and 180 days. The medium negative correlation between the number of neuronal cells and seizures, evaluated by Spearman's correlation coefficient in our study, allows us to say that over time the occurrence of SRS causes loss of neuronal cells. Some experimental studies have shown that, following SE, further seizures may induce progressive neuronal loss, whereas others have not found this progressive decrease in neurons number. Extensive data in literature have shown that repeated seizures induced by kindling led to neuronal loss (Bengzon et al., 1997; Zhang et al., 1998; Kotloski et al., 2002).). For instance, a progressive neuronal loss in different regions of the hippocampal formation is reported after three (neuronal loss between 17% and 24%), 30 (neuronal loss between 17% and 39%), or 150 (neuronal loss between 16% and 49%) kindled generalized tonic-clonic seizures (Cavazos et al., 1994). Other groups have also reported that repeated seizures induced by kindling led to apoptotic neuronal death in the hippocampal area (Bengzon et al., 1997; Zhang et al., 1998), suggesting that seizures induce neuronal loss. Compared with other experimental models, such as electrically induced SE, Pitkanen et al. (2002) did not find fluoro-Jade B (which is a fluorochrome used for detecting neuronal degeneration) (Schmued et al., 1997) positive cells in the hilus of the dentate gyrus six months after SE. Using the same SE model, Gorter et al. (2003) reported that neuronal death was related to SE, rather than to the frequency of spontaneous seizures. Thus, neuron loss does not appear to occur in the hippocampus of spontaneously epileptic EL mice (Drage et al., 2002). Therefore, the divergence of experimental findings may be due to the different animal models employed to induce SE.

Detection of neuronal loss using the available cell counting methods also has a large variability in the pilocarpine model (Guillery and August, 2002; West, 2002). In the study by Liu et al. (1994), using stereological estimates with cresyl violet staining, no progressive neuronal loss in the hippocampus was noted at three, six and 12 weeks after spontaneous seizures. A recent study reported a reduction of NeuN positive cells in the hilus of the dentate gyrus (DG) only in animals with long epileptic life (Cavarsan et al., 2013). However, few studies have investigated cell loss in the later life of animals with epilepsy. The scarcity of studies of aged animals is due to intensifying infirmity and their high cost. From this limited information, Liang et al. (2007) demonstrated a decrease in CA1 and CA3 cells in 18–19 month old rats, seven days after the kainate injection. In a study that investigated the morphometric changes in young adult, middle-aged, and aged rats after unilateral intracerebroventricular kainic acid injection showed more prominent loss in hilar neurons in 24-month-old (aged) rats than four-month-old (young adult) rats (Shetty and Turner, 1999). Although in our work a reduced number of neuronal cells in the hippocampal formation occurred at all ages of animals with epilepsy compared with their respective controls, this loss was more evident at 12 months (360 days) after the first spontaneous seizure, suggesting that the vulnerability of neuronal loss is more prominent in later life epilepsy.

We also aimed to examine changes in non-neuronal cells in animals with epilepsy throughout life. Several studies have reported the number of non-neuronal cells (for instance, glial cells) in chronic epileptic animals. However, our study is the first to investigate this subject in different time points and over long periods of spontaneous seizures. We observed a reduction in the number of non-neuronal cells in the group of rats with 30 days of spontaneous seizures compared with their controls. On the other hand, no difference was observed between animals with epilepsy and their controls at all other periods. Glial cells influence neuronal function by their role in extracellular ion homeostasis, brain metabolism, blood–brain-barrier and immune function (Middeldorp and Hol, 2011) dysregulation of glial functions can induce hyperexcitability. Reactive gliosis is a marked feature of temporal lobe epilepsy in humans and in several animal models. This alteration can be found by examining the expression of glial fibrillary acidic protein (GFAP), an indicator of reactive astrogliosis. Astrogliosis has been extensively analyzed after SE induction or in the early phase of SRS (Babb et al., 1996; Schmidt-Kastner and Ingvar, 1996; Foresti et al., 2011), which is in contrast to the current study which evaluated all non-neuronal cells over a long period of the epileptic state. Kang et al. (2006) reported neuronal degeneration in the hilus seven days after SE and reduced GFAP positive astrocytes in the dentate gyrus 14 days after SE. Similarly, Kim et al. (2008) found astroglial loss in the CA1 region and entorhinal cortex four weeks after SE. The reduced number of non-neuronal cells found in our study 30 days after the first spontaneous seizure is in agreement with the above findings which reported reduced astrocytes in the initial periods after pilocarpine-induced SE. We would like to point out that the cell quantification method used in our study (isotropic fractionator technique) is limited to identifying the total number of non-neuronal cells. Nuclei cellular staining with DAPI does not distinguish specific non-neuronal cells. One limitation of our work is concerned with EEG analysis. We could not perform continuous EEG, i.e., EEG for animals monitored for

360 days because the device to EEG records (implanted electrodes) cannot be fixed for long periods for the same animal. There is a high risk of losing or damaging the implanted electrodes and local infection in the implanted area (losing the animal or altering the cell counting) when the device remains for long periods in the animals' head.

It is also important to remark that seizure-induced damage is not restricted to the hippocampus. Morphological changes in extrahippocampal limbic regions as a result of repeated seizures may lead to cell damage and structural reorganization. We must also take into account that altered cell proliferation in the DG co-exists with cell loss. In animal models, increased neurogenesis is observed in the initial phase of SE (Parent et al., 1997; Scharfman and Gray, 2007) and reduced neurogenesis is noted in the chronic period of TLE (Hattiangady et al., 2004; Hattiangady and Shetty, 2008). This issue is outside the scope of this work (for a detailed review see Hattiangady and Shetty, 2010).

In sum, our results add novel data to the literature concerning the time-course of SRS and cell quantification in the hippocampal formation throughout epileptic life. Here, we demonstrate that seizure frequency varies in animals with epilepsy according to the period of life, with a progressive increase of seizures in initial periods followed by a decrease and later stabilization of seizures. We also provide evidence that repeated spontaneous seizures induce neuronal loss. Accurate long-term analyses may be required in animal models of chronic epilepsy to reinforce our findings.

4. Methods

4.1. *Induction of epilepsy*

One hundred and seventeen male Wistar rats (280±30 g; 60-days-old) were used in this study. All experimental protocols described below were approved by the ethics committee of the Universidade Federal de São Paulo (protocol #203573/13) and all efforts were made to minimize animal suffering in accordance with the guidelines for animal research of the Society for Neuroscience. The colony room was maintained at 21±2 °C with a 12 h light/dark schedule, and food and water *ad libitum* throughout the experiments. Rats were randomly divided into control and epilepsy groups. The latter group was submitted to temporal lobe epilepsy induced by a single administration of pilocarpine hydrochloride (350 mg/kg i.p.; Sigma, Lot#MKBS0848V) (Turski et al., 1984). Scopolamine methylnitrate (1 mg/kg s.c.; Sigma, Lot#BCBG3138V) was administered 30 minutes before pilocarpine injection to limit peripheral cholinergic effects (Turski et al., 1984). The systemic administration of the muscarinic agonist pilocarpine in rats promotes sequential behavioral and electrographic changes that build up progressively into a limbic SE which lasts 24 h. In order to end or limit behavioral seizures induced by pilocarpine, Diazepam (10 mg/kg, sc; Teuto, Lot2493046) was administered 4 h after the onset of SE. Following this acute period of the epilepsy model, the surviving animals (37 from 80) were continuously monitored over 24 h per day for detection of spontaneous recurrent seizures (SRS), using a video system (Intelbras VT 4 S 120). Seizure number was monitored for 30 days ($n=9$); 90 days ($n=9$); 180 days ($n=9$) and 360 days ($n=10$) after the first spontaneous seizure.

4.2. *Quantification of hippocampal cells*

The total number of neuronal and non-neuronal cells in the hippocampal formation of experimental animals was investigated at 30 ($n=9$), 90 ($n=9$), 180 ($n=9$), and 360 ($n=10$) days after the observation of the first spontaneous seizure. For each animal with epilepsy, the same number of age-matched animal was used as control. Total number of cells was estimated as described previously using the isotropic fractionator method (Herculano-Houzel and Lent, 2005), a simple, fast and low-cost technique which estimates the total number of cells in determined tissue (whole brain or any dissectible structure). This technique does not require specific software for use but follows the same principles employed in stereology analysis (Herculano-Houzel et. al., 2015). Since the isotropic fractionator revealed about 100% of the numbers produced by unbiased stereology, this technique was validated to determine the absolute number of neuronal and non-neuronal cells in the brain (Bahney and von Bartheld, 2014).

Briefly, the rats were deeply anesthetized (Tionembutal, 50 mg-kg, i.p.) and perfused transcardially with a solution of 0.01 M phosphate-buffered saline (PBS), followed by a solution containing 4% formaldehyde in 0.1 M phosphate-buffered (PB), pH 7.4. After perfusion, the rat brains were removed immediately from the skull and postfixed in 4% paraformaldehyde in PB for 24 h. Then, the hippocampal formation was dissected and mechanically dissociated in a saline solution with 1% Triton X-100 and turned into an isotropic suspension of isolated nuclei, kept homogeneous by agitation. The total number of cells was estimated by determining the number of nuclei in small aliquots stained with the fluorescent DNA marker 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:1000; Novus Biological; Lot 033M4064V) utilizing a Zeiss Axiovert 100 microscope with a 40x objective, using a hemocytometer for quantification (Neubauer chamber, Loptik Labor). To determine neuronal and non-neuronal cell number, the samples were then incubated with the primary antibody against the neuron-specific nuclear protein (NeuN; 1:100; Novus Biological, Lot 121712-APC) at 4 °C overnight and, subsequently, with secondary antibody conjugated to AlexaFluor®594 (Molecular Probes; Lot 982384) diluted in PBS (1:200) and 10% normal goat serum for 2 h. The neuronal fraction in each sample was estimated by counting NeuN-labeled nuclei in at least 500 DAPI-stained nuclei and the number of non-neuronal nuclei was obtained by subtraction.

4.3. *Statistical analysis*

Longitudinal seizure number was compared by analysis of variance for repeated measures and the number of neuronal and non-neuronal cells for the different groups was compared using two-way ANOVA, both followed by Bonferroni *post hoc* test. The relationship between non-neuronal/neuronal cell numbers and seizures was verified by Spearman's correlation coefficient. All results were presented as means

and standard deviation (M±SD) and significance was established at $p<0.05$ level. All analyses were performed using an IBM SPSS version 20.0 (IBM, Chicago, Armonk, NY, USA).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

RMA, EAC, SGdS and GML designed the research. GML, DVC and AAdA performed the experiments. GML, DVC, SGdS, AAdA, RL, EAC and RMA analysed the data. All authors discussed the results on the manuscript at all stages. RMA is the coordinator of the project.

Acknowledgments

Research supported by CAPES, FAPESP, CNPq and Instituto Nacional de Neurociência Translacional (INNT)

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