FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2014/2015

José Luís da Silva Quesado

Loss of hippocampal neurons after kainate treatment correlates with behavioral deficits

março, 2015

FMUP

FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

José Luís da Silva Quesado

Loss of Hippocampal Neurons after Kainate Treatment Correlates with Behavioral Deficits

Mestrado Integrado em Medicina

Área: Neurociências

Tipologia: Dissertação

Trabalho efetuado sob a Orientação de:

Doutor Nikolay Lukoyanov

Trabalho organizado de acordo com as normas da revista:

PLoS One

março, 2015

FMUP

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO | MONOGRAFIA

Projeto de Opção do 6º ano - DECLARAÇÃO DE INTEGRIDADE

Eu, José Luís da Silva Quesado, abaixo assinado, nº mecanográfico 200908689, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

Neste sentido, confirmo que **NÃO** incorri em plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria de um determinado trabalho intelectual, ou partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores, foram referenciadas, ou redigidas com novas palavras, tendo colocado, neste caso, a citação da fonte bibliográfica.

Faculdade de Medicina da Universidade do Porto, 9 / 3 / 2015

Assinatura conforme cartão de identificação:

José Luís da Silva Quesado

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
PROJETO DE OPÇÃO

DECLARAÇÃO DE DIVULGAÇÃO

NOME

José Luís da Silva Quesado

CARTÃO DE CIDADÃO OU PASSAPORTE (SE ESTRANGEIRO)	E-MAIL	TELEFONE OU TELEMÓVEL
13370797 0 ZY5	joselsquesado@gmail.com	967520147

NÚMERO DE ESTUDANTE	DATA DE CONCLUSÃO
200908689	2015

DESIGNAÇÃO DA ÁREA DO PROJECTO

Neurociências

TÍTULO DISSERTAÇÃO/MONOGRAFIA (riscar o que não interessa)

Loss of Hippocampal Neurons after Kainate Treatment Correlates with Behavioral Deficits

ORIENTADOR

Prof. Doutor Nikolay Lukoyanov

COORIENTADOR (se aplicável)

Declaro, para os devidos efeitos, que concedo autorização para divulgar o trabalho-supra identificado, apresentado no âmbito do Ciclo de Estudos Integrado do Mestrado em Medicina, que disponibilizo no formato e nas conduções abaixo indicadas.
A subscrição da presente declaração não implica a renúncia à titularidade dos direitos de autor, o direito de usar a obra em trabalhos futuros, os quais são pertença do seu criador intelectual.

ASSINALAR AS OPÇÕES APLICÁVEIS EM 1, 2 E 3.

1. TIPO DE DIVULGAÇÃO	2. ÂMBITO DE DIVULGAÇÃO	3. FORMATO DO FICHEIRO
[X] TOTAL	[ ] REPOSITÓRIO FMUP	[ ] PAPEL
[ ] RESUMO/ABSTRACT	[X] REPOSITÓRIO UP	[X] DIGITAL

Data: 9 / 3 / 2016

José Luís da Silva Quesado

(Assinatura do Estudante conforme cartão de identificação)

Ao meu avô.

Ao meu primo Nuno.

OPEN ACCESS Freely available online

PLOS ONE

Loss of Hippocampal Neurons after Kainate Treatment Correlates with Behavioral Deficits

Gisela H. Maia[1,2,3], José L. Quesado[1], Joana I. Soares[2,3], Joana M. do Carmo[3], Pedro A. Andrade[3], José P. Andrade[1], Nikolai V. Lukoyanov[1,2,3]*

1 Departamento de Anatomia, Faculdade de Medicina da Universidade do Porto, Porto, Portugal, **2** Neural Networks Group, Instituto de Biologia Molecular e Celular da Universidade do Porto, Porto, Portugal, **3** Programa Doutoral em Neurociências, Universidade do Porto, Porto, Portugal

Abstract

Treating rats with kainic acid induces status epilepticus (SE) and leads to the development of behavioral deficits and spontaneous recurrent seizures later in life. However, in a subset of rats, kainic acid treatment does not induce overt behaviorally obvious acute SE. The goal of this study was to compare the neuroanatomical and behavioral changes induced by kainate in rats that developed convulsive SE to those who did not. Adult male Wistar rats were treated with kainic acid and tested behaviorally 5 months later. Rats that had experienced convulsive SE showed impaired performance on the spatial water maze and passive avoidance tasks, and on the context and tone retention tests following fear conditioning. In addition, they exhibited less anxiety-like behaviors than controls on the open-field and elevated plus-maze tests. Histologically, convulsive SE was associated with marked neuron loss in the hippocampal CA3 and CA1 fields, and in the dentate hilus. Rats that had not experienced convulsive SE after kainate treatment showed less severe, but significant impairments on the spatial water maze and passive avoidance tasks. These rats had fewer neurons than control rats in the dentate hilus, but not in the hippocampal CA3 and CA1 fields. Correlational analyses revealed significant relationships between spatial memory indices of rats and neuronal numbers in the dentate hilus and CA3 pyramidal field. These results show that a part of the animals that do not display intense behavioral seizures (convulsive SE) immediately after an epileptogenic treatment, later in life, they may still have noticeable structural and functional changes in the brain.

Citation: Maia GH, Quesado JL, Soares JI, do Carmo JM, Andrade PA, et al. (2014) Loss of Hippocampal Neurons after Kainate Treatment Correlates with Behavioral Deficits. PLoS ONE 9(1): e84722. doi:10.1371/journal.pone.0084722

Editor: Liset Menendez de la Prida, Consejo Superior de Investigaciones Cientificas - Instituto Cajal, Spain

Received June 26, 2013; **Accepted** November 18, 2013; **Published** January 7, 2014

Copyright: © 2014 Maia et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by FEDER Funds through the Programa Operacional Factores de Competitividade – COMPETE and National Funds through FCT - Fundação para a Ciência e a Tecnologia within the scope of the Project FCOMP-01-0124-FEDER-015919– PTDC/SAU-NSC/115506/2009, Strategic Project Centro de Morfologia Experimental (CME/FM/UP) – 2011–2012, Project PEst-OE/SAU/UI0121/2011. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: luk@med.up.pt

Introduction

Temporal lobe epilepsy (TLE) can be associated with hippocampal cell loss (hippocampal sclerosis) and other structural changes in the medial temporal lobe region, such as fiber sprouting and synaptic reorganization [1,2,3]. It has also been repeatedly reported that TLE patients with hippocampal sclerosis may show learning and memory deficits, increased anxiety, depression and mood disorders [4,5,6]. Thus, it seems reasonable to assume that these behavioral changes are secondary to the TLE-related hippocampal neurodegeneration. However, it is also possible that they are not so straightforwardly related to classical hippocampal sclerosis, but may rather reflect some subtle functional or structural alterations in neuronal circuits, either associated with or even unrelated to epileptic seizures.

Most of the neuropathological aspects of TLE can be reproduced using appropriate animal models. For example, many studies have shown that the treatment of rats with kainic acid (KA), a specific agonist for glutamate receptors, induces acute status epilepticus (SE), accompanied by severe convulsive (motor) seizures, and that later in life these animals show spontaneous recurrent seizures, hippocampal cell loss and memory deficits [7,8,9]. However, in this epilepsy model some rats are considerably more resistant to KA-induced convulsive SE than the others and, for that reason, are usually excluded from the studies [9,10,11,12,13]. Thus, given that the research on the neuronal effects of KA in this subset of animals has been scarce [14], which represents an important gap in the understanding of epilepsy-related neuropathology, the present study was designed to directly compare the behavioral and neuroanatomical outcomes in rats that developed convulsive SE after kainate treatment to those that did not. We thought that the presence of behavioral deficits in KA-treated rats that had not experienced convulsive SE would support the hypothesis that at least some of the behavioral impairments seen in epilepsy can be explained by relatively mild structural or functional changes in the hippocampal region. To address this issue, we assessed the behavioral profile of KA-treated rats of both phenotypes using the Morris water maze [15], passive avoidance and fear conditioning tests, as well as in the open field and elevated plus maze tests. Additionally, absolute numbers of neurons in the dentate gyrus hilus and pyramidal layers of the hippocampus proper were estimated using stereological approaches in order to compare the hippocampal cell loss between the two groups.

Materials and Methods

The handling and care of the animals were conducted according to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experimental protocol has been approved by the Ethics Committee of the Faculty of Medicine of Porto and the General Veterinary Direction (03.04.2012) for the FCT application grant PTDC/SAU-NSC/115506/2009. All efforts were made to minimize the number of animals used and their suffering.

Animals and Treatments

Male Wistar rats, maintained under standard laboratory conditions, were used in this study. At 10 weeks of age (mean body weight 323±69 g), they were randomly divided into two groups: KA group (n = 28) and control group (n = 8). In the first group, rats were treated with KA according to the protocol developed by Dudek and coworkers and described in detail elsewhere [16,17]. Briefly, the rats were injected every hour with 5 mg/kg of KA (i.p., Sigma) until the onset of convulsive SE, which was defined as the appearance of behavioral symptoms corresponding to stage 3, 4 or 5 seizures on the Racine scale [18], i.e. forelimb clonus, rearing, and rearing with falling. When necessary, treatment was continued in order to assure that SE lasted for at least 3 hours. All rats in this group demonstrated numerous wet-dog-shake seizures after either the second or third injection of KA. The SE, lasting 3–6 hours, was detected in 17 rats after 3–5 injections, with the mean cumulative dose of KA equal to 21±3 mg/kg. These rats were ascribed to the "behavioral status epilepticus" group (BSE; n = 17). Remaining 11 rats after 4 to 6 KA injections either became motionless (unchanged position in the observation cage for tens of minutes, weak reactivity to touching by experimenter's hand), or showed excessive activity (long runs, jumping). Because we and others [17] have previously observed that continuation of the treatment of animals in these states often leads to their death, the treatment was terminated. The mean cumulative dose of KA received by these rats, which were ascribed to the "no behavioral status epilepticus" group (no-BSE; n = 11) was 24±4 mg/kg. It was previously reported that the treatment protocol employed in our study is associated with a relatively low mortality rate of the animals when compared to the single high-dose kainate treatments [16,17]. However, because animal mortality is a prominent cause of bias in quantitative evaluation of neuronal loss [19], special efforts were made in order to further improve the survival rate in the kainate-treated rats. In particular, the animals were periodically injected with saline (s.c.) during the first 48 hours of the recovery period. On the following days, the rat diet was supplemented with apples that were sliced and left at the bottom of the cage. The rats that refused to eat or drink were hand-fed using a plastic syringe. Rats in the control group were injected with saline.

Following the treatments, all animals were given a 5-month recovery period. In the last week of each month, the behavior of rats in their home cages was videorecorded during the light phase of the 12-h light/dark cycle (between 08:00 h and 20:00 h) over a period of 5 consecutive days. The recording was performed using the multichannel video-tracking system EthoVision 8.5 (Noldus, The Netherlands). The video files generated by the system and stored on the hard disk of the computer were then analyzed in a fast-motion mode by a person blind to treatment groups using the VLC media player (VideoLAN, France). In addition, the rats were daily (except weekends) observed for spontaneous behavioral seizures during 1-h intervals between 09:00 h and 10:00 h, as well as during all the procedures associated with handling and behavioral testing.

Open-field and Elevated Plus-maze Tests

Behavioral testing began when animals were 7 months old. Animals were handled 3 minutes per day during 5 days and subjected to the open-field test and plus-maze as previously described in detail [20]. The rats were counterbalanced so that, in each group, half received the open-field test first and the other half received the plus-maze test first. There was a 3-day interval between the two tests.

The behavior of rats in the open field (100×100×40 cm) was tested during 5-minute sessions. Distances traveled in the outer zone of the open field, defined as 20 cm from any wall, and in its inner zone were measured using the computerized EthoVision video-tracking system.

The elevated plus maze was arranged as a cross with two opposite open and two opposite closed arms (50×12 cm), connected by a common central square (12×12 cm). The rats were allowed to explore the apparatus for 5 minutes. Behavior of rats was recorded and analyzed using the video-tracking system. The percentages of time spent by rats in the open arms, closed arms, and in the central square were measured. The number of entries into the arms of the apparatus was also registered.

Step-through Passive Avoidance and Fear Conditioning

Following a 3-day interval, all rats were submitted to the passive avoidance and fear conditioning tests. The rats were counterbalanced so that, in each group, half received the passive avoidance test first and the other half received the fear conditioning first. There was a 3-day interval between the two tests.

The apparatus used in the passive avoidance task was comprised of two adjacent compartments separated by a guillotine door [20]. The larger compartment (45×45×45 cm) was brightly lit and the smaller compartment (30×16×16 cm) was dark. The floor of both compartments was composed of stainless steel bars, 0.5 cm in diameter and spaced 1.2 cm apart, but only the floor of the dark compartment was wired to the stimulus generator (Hugo-Sachs Elektronik, Germany). On the first day, the rats were allowed to explore the apparatus with the guillotine door open for 5 minutes. The following day, each rat was placed into the brightly lit compartment and the latency to enter into the dark compartment was recorded. Upon entry into the dark compartment, the door was lowered and a 1-mA, 1-s footshock was delivered 3 times at 5-s intervals. Twenty four hours later, this procedure was repeated, with the exception that no footshock was delivered, and the latency to enter the dark compartment was again recorded.

All rats were given a single session of fear conditioning as previously described in detail [20]. The conditioning chamber (San Diego Instruments, USA) was equipped with a metal grid floor, wired to the stimulus generator and acoustic stimulus unit. The grid floor was composed of stainless steel bars, 0.6 cm in diameter, spaced 1.4 cm apart. The chamber was scented with 1% acetic acid solution (placed into base pan underneath the grid floor) in order to provide a distinct context odor. Rats were placed inside the apparatus and left undisturbed for 3 minutes. During next 3-minute period they received 5 tone-footshock conditioning trials, with 30-s intervals. Each conditioning trial consisted of a 10-s tone conditioned stimulus (80 dB, 2.8 kHz), which coterminated with a 1-s footshock unconditioned stimulus (0.8 mA) delivered via the grid floor.

One day later, all rats were submitted to retention tests. The order of testing was counterbalanced so that half of the rats in each

group were tested for retention of the conditioned context by repeating the procedure employed during training (in the absence of the tone or footshock), whereas remaining rats were tested for retention of the conditioned tone in a different context. In the later test, the rats were placed into a novel chamber which was located in a novel behavioral room. The floor of the new chamber was composed of a piece of a black carpet. The chamber was scented with lemon instead of acetic acid. The animals remained in the chamber for a period of 6 minutes and the conditioned stimulus (10-s tone) was presented 5 times during the last 3 minutes of this period. Four hours later, these rats were tested for retention of the context as described above, whereas the animals from the context test were now subjected to the tone retention test.

All training and testing trials were recorded with a video camera connected to a SVHS recorder for subsequent analysis. The rat's behavior was analyzed by an observer blinded with respect to experimental group. Freezing (defined as the absence of all movement other than that required for breathing) was scored if the rat remained inactive for at least 3 seconds. The percentage of accumulated time spent freezing was calculated.

Morris Water Maze Test

The procedure of the water maze training, previously described in detail [21], initiated one week following the termination of the passive avoidance and fear conditioning tests. The maze consisted of a black circular tank, 180 cm in diameter and 50 cm deep, and was located in a corner of a room containing extramaze cues. The apparatus was filled with water at room temperature ($21\pm1^{\circ}C$) to a depth of approximately 35 cm. The water was made opaque by adding a non-toxic paint. The maze was divided, by imaginary lines, into four equal-size quadrants. An escape platform, 10 cm in diameter, was placed in the center of one of the quadrants. It was located 2 cm below the surface of the water. The animals were trained to find the escape platform during 14 consecutive days with two 60-s daily trials separated by a 30-s inter-trial interval. Each rat was placed in the water facing the pool wall at one of the four starting points that were used in a pseudo-random order so that each position was used once in each block of four trials. However, in each trial the starting positions were the same for all animals. If the rats did not find the escape platform within 60 s, the experimenter guided them to the platform where they were allowed to remain for 15 s. The platform location was not changed during the 14-day training period. The swim path length and swim speed were registered using the EthoVision video-tracking system. One day after completion of the acquisition, animals were submitted to a single 60-s probe trial in which the platform was removed from the pool. The number of times the rats swam through the zone where the platform had been located (platform crossings) and the percentage of time spent by rats swimming in the training quadrant were recorded.

Performance of animals on the visible platform task was assessed during a 2-day period. The rats were given 1 block of 4 trials per day separated by 30-s inter-trial intervals. The platform, painted in white, was exposed 3 cm above the water surface. The position of the platform was different in each trial. The distances swum to locate the platform were recorded and averaged across 8 trials.

Tissue Preparation

Following the completion of the behavioral experiments, six animals in each group, selected at random, were deeply anesthetized with pentobarbital (90 mg/kg) and injected intracardially with 0.1 mL of a heparin solution, followed by 1 mL of 1% sodium nitrite in saline. Then, they were perfused transcardially with 150 mL of 0.1 M phosphate buffer (pH 7.4) for vascular rinse, followed by 250 mL of a fixative solution containing 4% paraformaldehyde in phosphate buffer. The brains were removed from the skulls, immersed for 2 hours in the fixative, and infiltrated during 36 h in 10% sucrose solution at $4^{\circ}C$. After the frontal poles were trimmed away, the blocks were mounted on a vibratome and sectioned in the coronal plane at 40 μm. Every sixth section containing the hippocampal formation was collected using a systematic random sampling procedure [22], which provided an average of 14 sections per animal, mounted serially on gelatin-coated slides and air-dried. The sections were stained with a Giemsa, dehydrated in a series of ethanol solutions (50%, 70%, 90% and 100%) and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA).

Morphological Analysis

The Giemsa-stained tissue sections were visualized and imaged using an Axio Scope.A1 microscope equipped with a AxioCam MRc5 digital camera (Zeiss, Germany). The total numbers of neurons were estimated by applying the optical fractionator method [23]. The boundaries of the hilus of the dentate gyrus and of the CA3 and CA1 hippocampal fields were consistently defined at all levels along the rostrocaudal axis of the brain on the basis of cell morphology and cytoarchitectonic criteria [24]. Neurons belonging to the CA2 hippocampal field were included in the CA3 region. Estimations were carried out using the C.A.S.T.-Grid System (Olympus, Denmark). Beginning at a random starting position, visual fields were systematically sampled along the x and y axes, using a raster pattern procedure. Neurons were counted in every frame using the optical disector at a final magnification of ×2,000. Tissue thickness was estimated at each counting frame and guard zones of 2 μm were implemented. The nucleus of the neurons was used as the counting unit. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. [25] and it ranged between 0.06 and 0.09.

Statistical Analysis

Before conducting statistical comparisons, data were tested for normality using the Shapiro-Wilk's W test, which is known to be the most powerful normality test, especially in the case of small sample sizes [26]. Because all data samples passed the normality test ($p>0.05$), they were analyzed for statistical significance using parametric tests. Data derived from the passive avoidance test, acquisition trials of the water maze task, and training trials and tone retention trials of the fear conditioning test were analyzed using repeated measures ANOVA. The remaining data were analyzed using one-way ANOVA. Newman-Keuls post hoc test was used where appropriate. Because some of the behavioral tests were presented in a counterbalanced manner, the data derived from these tests were collapsed across order of presentation. Pearson's product moment regression model was used to analyze correlations between the neuron numbers and the two behavioral indices derived from the water maze test: the percentage of savings assessed by the difference in the mean distance swum between the first four trials and the last four trials (index of learning), and the percentage of time spent by rats swimming in the quadrant where the platform had been located during training (index of retention). All behavioral data are presented as the mean±SEM, while morphological results are expressed as the mean±SD. Differences were considered as significant at the $p<0.05$ level.

Results

Monitoring during the Recovery Period

Despite all the efforts made to reduce the mortality rate in KA-treated rats (see above), a significant proportion of them have died during the recovery period. In particular, from the 17 rats that had experienced convulsive SE 3 rats have died within 48 h following the treatment and 3 rats have died during the first month of the recovery period. From the 11 rats that did not show motor manifestations of SE 2 rats have died within 48 h following the treatment. Repeated behavioral seizures of stage 3 to 5 on the Racine scale were observed in a total of 9 rats: by the end of the first month of recovery – in just 1 rat; by the end of the second and third months – additionally in 3 and 5 rats, respectively. Interestingly, during the treatment with KA, all of these rats showed a typical pattern of behavioral SE, that is, continuous stage 3 to 5 seizures on the Racine scale. Three of the rats in this group had motor seizure attacks during the behavioral tests: 1 rat – during the water maze training (twice) and 2 rats during the tone test of the fear conditioning procedure. The respective trials were repeated for these animals on the next day. None of the rats had motor seizures during the 1-h intervals preceding or following the behavioral tests. The remaining 2 rats, which experienced convulsive SE during the treatment with KA, showed no spontaneous stage 3, 4 or 5 seizures during the entire 6-month observation period (including the period of behavioral tests). However, one of the experimenters detected in these 2 rats repeated mouth and facial movements (stage 1 seizures on the Racine scale), but this observation was not confirmed by other experimenters. Thus, the final group size for BSE rats was n = 11. No signs of behavioral seizures (stages 1–5 on the Racine scale) were detected in the remaining 9 KA-treated rats. These rats, in contrast, did not show clearly expressed motor signs of SE during the treatment with KA (no-BSE group; final n = 9). No behavioral abnormalities were observed in the control group.

Behavioral Effects of KA Treatment in the Open-field and Elevated Plus-maze

The results obtained in the open-field tests are shown in Fig. 1A. ANOVA revealed a significant effect of the treatment on the locomotion activity of rats both in the outer zone of the apparatus ($F_{2,25} = 4.70$, $p<0.05$) and in its inner zone ($F_{2,25} = 4.07$, $p<0.05$). Post-hoc comparisons showed that animals from BSE group traveled significantly longer distances in the peripheral zone of the open field than did control rats and no-BSE rats ($p<0.05$). Similarly, rats from BSE group showed increased locomotion in the inner part of the open field. However, in this zone, the difference was significant only when BSE group was compared to no-BSE group ($p<0.05$), but it did not reach the level of significance when compared to control group ($p = 0.08$).

Rats in all groups showed clear preference to protected areas of the plus maze, as they spent more time in the closed arms and central zone of the apparatus in comparison to its open arms (Fig. 1B). However, although the percentages of time spent by rats in the closed arms and central zone did not differ between the groups ($Fs<1$, $p>0.05$), ANOVA revealed a significant effect of the treatment on the time spent by rats in the open arms ($F_{2,25} = 3.31$, $p<0.05$). Particularly, rats in the BSE group spent significantly more time in the open arms than those from control group and no-BSE group ($p<0.05$). No significant differences were detected between the groups regarding the total number of entries into the arms of the apparatus.

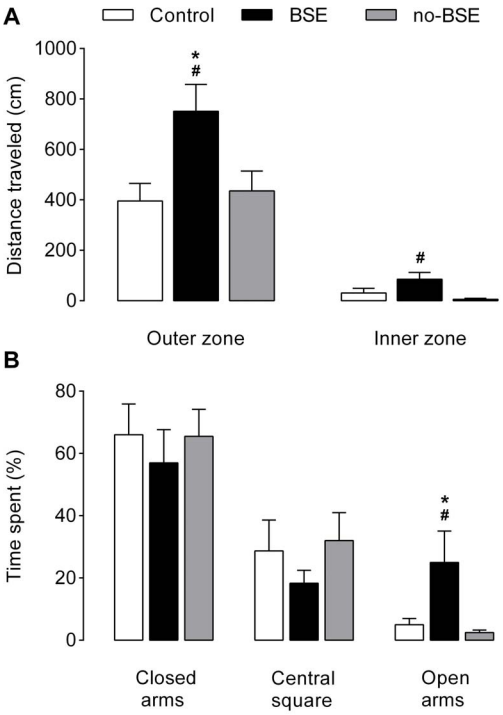


Figure 1. Behavior of kainate-treated rats in the open-field (A) and elevated plus-maze (B) tests. The y-axis represents the distances travelled by rats during the 5-min testing sessions in the outer and inner zones of the open-field apparatus, and in the central zone and open and closed arms of the plus-maze apparatus. Note that rats who had experienced behavioral status epilepticus (BSE group) traveled longer distances in the outer and inner zones of the open field when compared to control rats and rats that had not experienced behavioral status epilepticus (no-BSE group), respectively. In the plus-maze test, rats in the BSE group spent more time in the open arms of the apparatus than rats in the other two groups. *$p<0.05$ vs. control group and $^{\#}p<0.05$ vs. no-BSE group. Data are presented as the mean±SEM.
doi:10.1371/journal.pone.0084722.g001

Effects of KA Treatment on Passive Avoidance and Conditioned Freezing Behavior

The results of the passive avoidance testing are shown in Fig. 2. Repeated measures ANOVA did not reveal a significant main effect of treatment on the performance of rats on this task ($F_{2,25} = 1.47$, $P = 0.25$). However, there was a significant effect of training ($F_{1,25} = 14.30$, $p<0.001$) and a significant interaction between these two effects ($F_{2,25} = 5.09$, $p<0.01$). Post-hoc comparisons showed that, although rats in no-BSE group did not differ from control rats in the baseline latency to enter the dark compartment, on the retention trial, the entry latencies were significantly reduced in this group relative to controls ($p<0.05$). The latencies to enter the dark compartment on the retention test were not significantly different between control group and BSE group. However, the baseline latency was significantly increased in BSE rats relative to control rats ($p<0.05$), suggesting that the high entry latencies registered in this group on the retention test might be related to a decreased motivation to enter the dark compartment of the apparatus, for example, due to reduced levels of anxiety.

Conditioning produced an increase in the amount of freezing time in either group ($F_{1,25} = 156.11$, $p<0.0001$; Fig. 3A) and examination of the data revealed no significant differences between the groups. Once placed into the training chamber 1

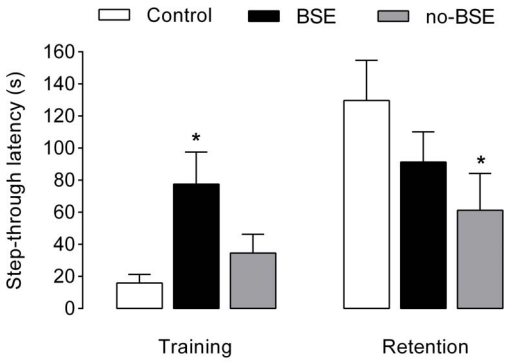


Figure 2. Performance of kainate-treated rats on the passive avoidance test. The y-axis represents the mean (±SEM) step-through latencies during training trial as well as on the retention trial given 24 h later. Note that rats which did not show behavioral signs of status epilepticus following the treatment with kainate (no-BSE group) showed lower retention scores than did control rats. In contrast, rats that had experienced behavioral status epilepticus (BSE group) showed increased baseline entry latency, but appeared normal on the retention test. *$p<0.05$ vs. control group.
doi:10.1371/journal.pone.0084722.g002

day later, the animals from all groups displayed increased levels of freezing behavior (Fig. 3B). However, the amount of freezing differed between the groups ($F_{2,25} = 4.68$, $p<0.05$), so that rats in the BSE group froze to the familiar context significantly less than control rats and no-BSE rats ($p<0.05$). However, no-BSE and control groups did not differ significantly in this test. In the novel context, the levels of freezing displayed by all groups in the absence of the tone were inferior to those observed during the tone presentation ($F_{1,25} = 99.42$, $p<0.0001$; Fig. 3C). Repeated-measures ANOVA of these data did not reveal a significant main effect of treatment, but yielded a significant treatment by tone presentation interaction ($F_{2,25} = 3.29$, $p<0.05$). Post-hoc tests showed that, although in the absence of the tone the 3 groups did not differ with respect to the amount of freezing, during the tone presentation, the rats in BSE group showed relatively low levels of fear, freezing significantly less than control rats ($p<0.05$).

Water Maze Navigation

The mean distances swum by rats in the control, BSE and no-BSE groups to locate the hidden platform in the place learning task are presented in Fig. 4A. The statistical analysis by repeated measures ANOVA showed that the rats included in this experiment progressively improved their ability to find the platform over the 14 days of acquisition ($F_{6,150} = 25.980$ $p<0.00001$). However, the overall performance of the rats on this task differed among the groups studied, as indicated by a significant main effect of treatment ($F_{2,25} = 12.89$, $p<0.0001$) and significant treatment × trial block interaction ($F_{12,150} = 1.86$, $p<0.05$). Post-hoc tests for the main effect of treatment revealed that animals from the BSE group and no-BSE group performed the task less well than did control animals ($p<0.001$ and $p<0.01$, respectively), but animals in the BSE group were more severely impaired than those in the no-BSE group ($p<0.05$). Post-hoc tests for the treatment × trial block interaction showed that BSE group performed significantly worse than control group on the 2nd to 7th trial blocks ($p<0.0001$). In turn, rats in the no-BSE group also traveled greater distances than did control rats on the 2nd to 7th trial blocks ($p<0.001$). Moreover, rats from the BSE group were less efficient in finding the escape platform than rats from the no-BSE group on the 4th, 5th, and 7th trial blocks ($p<0.05$).

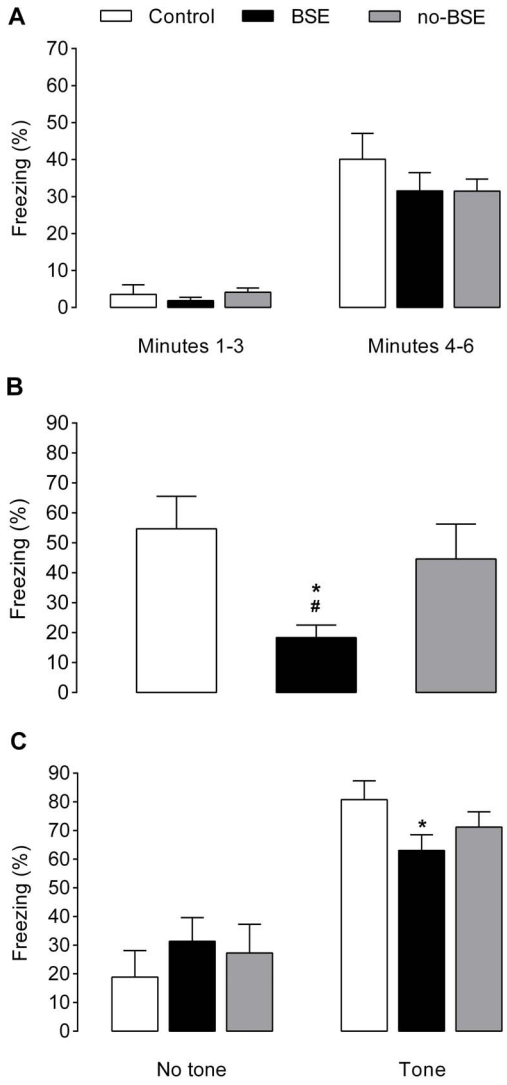


Figure 3. Effects of kainate treatment on the acquisition and retention of conditioned fear. The y-axes represent percentage of freezing time for each of the 3-minute periods of the acquisition session (**A**), during the context retention test (**B**), and during each of the 3-minute periods of the tone retention test which was performed in a novel context (**C**). No tone or footshock was delivered during the first 3-minute periods of the acquisition session (**A**) and of the tone retention test (**C**). Note that rats that had experienced behavioral status epilepticus (BSE group) showed reduced levels of fear, freezing significantly less than control rats on both retention tests. However, no-BSE group (composed of rats that had not experienced behavioral status epilepticus after kainate treatment) and control group did not differ significantly on these tests. *$p<0.05$ vs. control group and #$p<0.05$ vs. no-SE group. Data are presented as the mean±SEM.
doi:10.1371/journal.pone.0084722.g003

Behavioral measures obtained on the probe trial are shown in Fig. 4B. ANOVA revealed a significant effect of treatment on the total percentages of time spent by rats swimming in the training quadrant ($F_{2,25} = 7.01$, $p<0.01$) and a significant effect of treatment on the number of times they crossed the former location of the escape platform (platform crossings; $F_{2,25} = 6.40$, $p<0.01$). Post-hoc tests showed that both control and no-BSE rats spent more time in the training quadrant than did rats in the BSE group ($p<0.01$ and $p<0.05$, respectively). However, the no-BSE group did not differ from the control group on this measure, suggesting

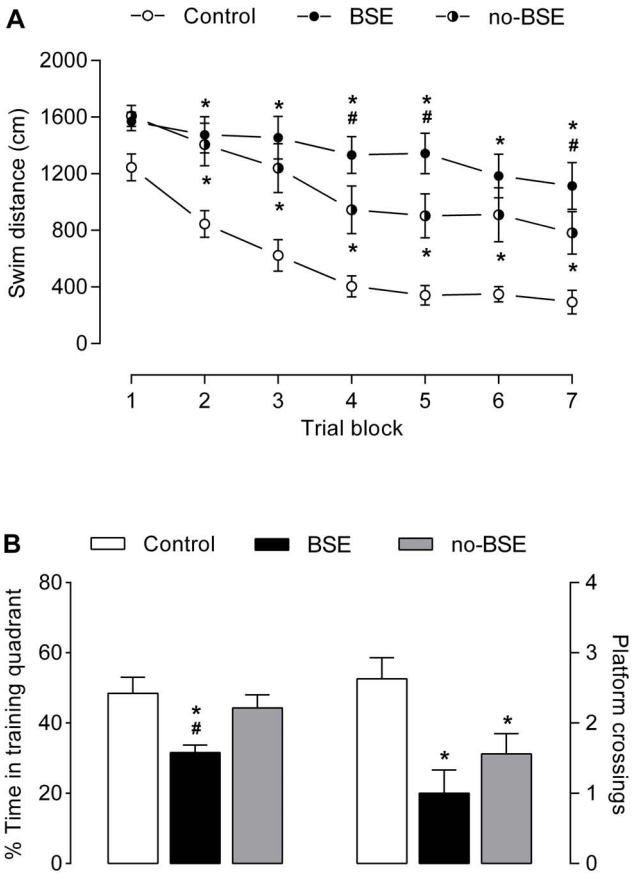


Figure 4. Effects of kainate treatment on the performance of rats on the water maze. (**A**) The y-axis represents the mean swim distance (±SEM) in centimeters to reach a hidden platform throughout the acquisition of the water maze task. The data were averaged across seven blocks of four consecutive trials each (the x-axis). Note that the performance of rats that had experienced behavioral status epilepticus (BSE group) on this task was extremely poor. However, spatial learning of the rats that showed no convulsive status epilepticus (no-BSE group) was also considerably impaired. *$p<0.0001$ for BSE group vs. control group and $p<0.001$ for no-BSE group vs. control group; $^{\#}p<0.05$ vs. no-BSE group. (**B**) The y-axes represent the percentage of time spent by rats in the training quadrant of the water maze during the probe trial and the number of times they swam through the zone where the platform had been located (platform crossings). Unlike control rats, rats in the BSE group did not show a preference for the training quadrant of the water maze and were impaired in their ability to localize the former platform position. However, the acuity of spatial search was also impaired in the no-BSE rats, as indicated by the fact that they crossed the former platform position less frequently than did control rats. *$p<0.05$ for no-BSE group vs. control group and $p<0.01$ for BSE group vs. control group; $^{\#}p<0.05$ vs. no-BSE group. Data are presented as the mean±SEM.
doi:10.1371/journal.pone.0084722.g004

that rats in both these groups, unlike those in the BSE group, were able to learn a spatial strategy when searching for the escape platform. Nevertheless, the acuity of spatial search was impaired in both KA-treated groups, as indicated by the fact that control rats crossed the former platform position more frequently when compared to both BSE group ($p<0.01$) and no-BSE group ($p<0.05$).

ANOVA revealed that there was a significant main effect of treatment on the speed of swimming averaged across all trials of the water maze acquisition ($F_{2,25}=3.29$, $p<0.05$). The animals from the BSE and no-BSE groups swam somewhat faster than control rats (24.7±1.2 and 22.3±1.5 cm/s vs. 20.1±1.1 cm/s, respectively). However, only BSE rats differed significantly from control rats on this measure ($p<0.05$). Animals in all groups were able to learn the visible platform task. The distances swum to locate the platform, averaged over eight trials, were 398±102 cm in the control group, 513±115 cm in the BSE group and 466±95 cm in the no-BSE group. ANOVA failed to reveal a significant effect of treatment on this parameter. These data indicate that, with respect to sensorimotor functions, KA-treated rats were not impaired when compared to control rats.

Effects of KA Treatment on Hippocampal Morphology

Representative photographs of brain sections illustrating treatment-related morphological changes in the hippocampal formation of rats in the BSE and no-BSE groups are shown in Fig. 5 and Fig 6. The qualitative observation of the sections inferred that the neuronal density of the CA3 and CA1 pyramidal cells was considerably smaller in BSE-rats than in control rats and no-BSE rats. In some hippocampal regions, CA1 pyramids were practically absent in BSE rats (Fig. 5B). In addition, the dentate gyrus was deformed and its granule cell layer was dispersed in animals that experienced behavioral SE. Consistent with prior anatomical studies [27,28], the density of hilar neurons in control rats was higher in the sections obtained from the temporal (ventral) pole of the dentate gyrus when compared to those obtained from its septal (dorsal) pole (Fig. 5A and Fig. 6A). The density of hilar neurons was reduced in KA-treated rats that experienced convulsive SE throughout the entire dentate gyrus, but neuron loss was particularly extensive in the ventral hilus (Fig. 6B). However, in rats from the no-BSE group hilar neuron loss was less extensive and most of the neurons of the ventral hilus were preserved (Fig. 6C).

The estimates of the total numbers of neurons in the hilus of the dentate gyrus, and in the CA3 and CA1 fields of the hippocampus proper are shown in Fig. 7. Analysis of these data revealed that there was a significant main effect of seizures on neuron numbers in all three hippocampal subdivisions: dentate hilus ($F_{2,15}=6.77$, $p<0.01$), pyramidal CA3 field ($F_{2,15}=31.13$, $p<0.0001$) and pyramidal CA1 field ($F_{2,15}=30.72$, $p<0.0001$). Post-hoc comparisons showed that in rats in which KA treatment resulted in the development of behavioral SE there was a significant neuron loss in the dentate hilus (40%; $p<0.01$), and hippocampal CA3 (50%; $p<0.001$) and CA1 (80%; $p<0.001$) fields. In sharp contrast, in rats from the no-BSE group there was a significant neuron loss only in the dentate hilus (30%; $p<0.05$), but not in the hippocampal pyramidal fields.

Relationship between KA-induced Neuron Loss and Spatial Learning and Memory

Correlational analyses were conducted to determine whether individual variations in learning and memory abilities of rats are related to KA-induced loss of hippocampal neurons. The analyses were conducted separately for hilar neurons, and CA3 and CA1 hippocampal pyramidal neurons. Data obtained from 6 animals from each group, whose brains were randomly selected for histological assessment, were included in the analysis (final n = 18). The results of the correlational analyses, shown in Fig. 8, revealed significant correlations between the total number of hilar neurons and two behavioral indexes of spatial learning and memory, i.e. the percentage of savings between the first four trials and the last four trials of the water maze acquisition ($r=0.57$, $p<0.01$; Fig. 8A) and the percentage of time spent in the training quadrant during the probe trial ($r=0.49$, $p<0.05$; Fig. 8B). Interestingly, both behavioral measures tend to exhibit even stronger correlations

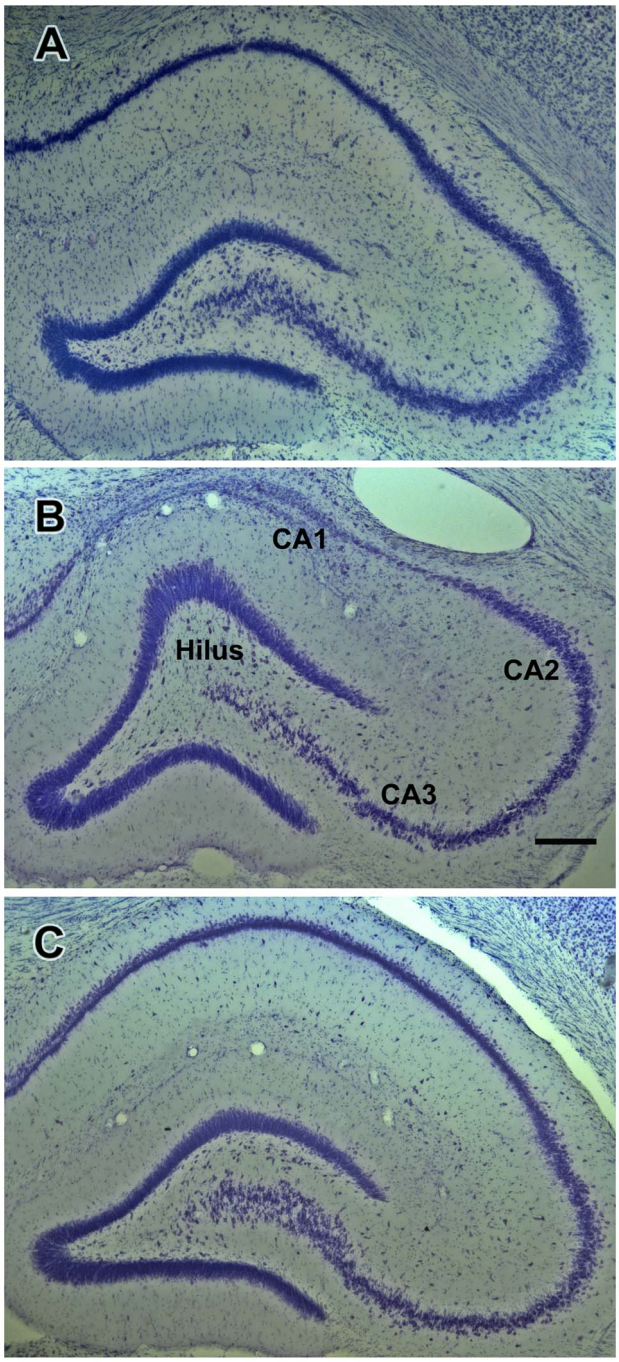


Figure 5. Effects of kainate treatment on hippocampal morphology. Photomicrographs of representative Nissl-stained coronal sections containing the dentate gyrus, CA3 and CA1 hippocampal fields taken from a control rat (**A**), from a rat in the behavioral status epilepticus (BSE) group (**B**) and from a kainate-treated rat that had not experienced behavioral status epilepticus (no-BSE; **C**). Note that the density of neurons in the dentate hilus is considerably reduced in the rats with (**B**) and without (**C**) behavioral SE when compared to the control rat (**A**). Note also dramatic loss of the CA3 and especially CA1 neurons in the rat from the BSE group; neurons in the CA2 field are relatively preserved (**B**). Scale bar shown in (**B**) = 200 μm.
doi:10.1371/journal.pone.0084722.g005

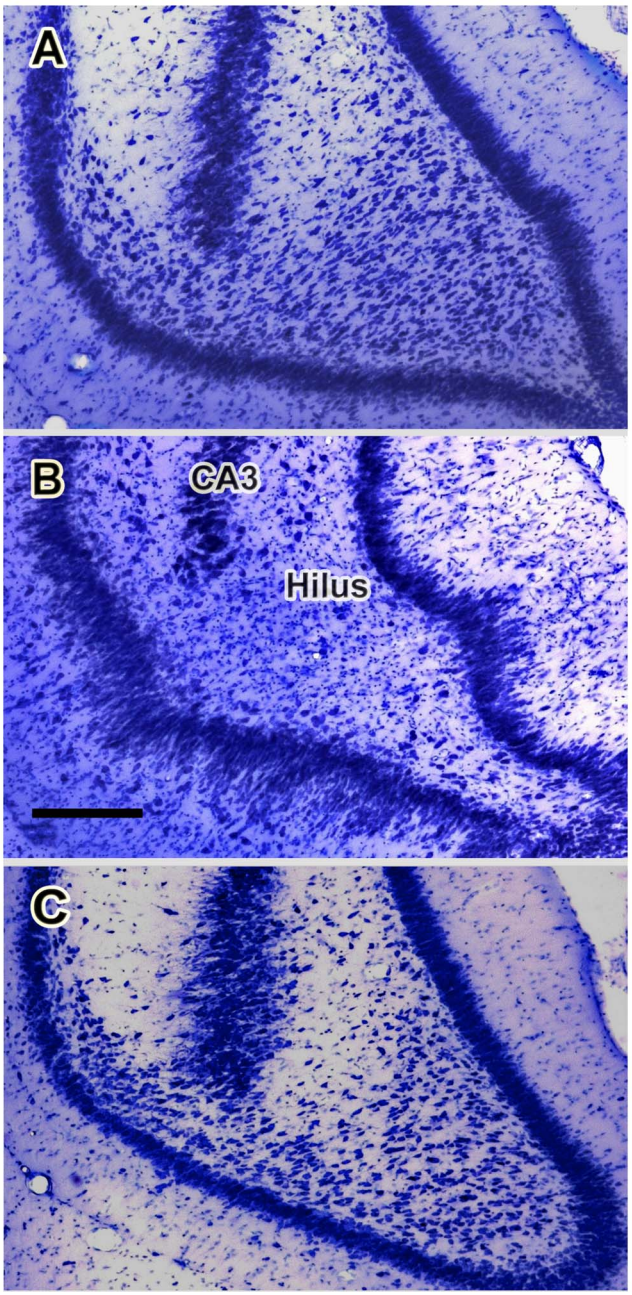


Figure 6. Kainate-induced loss of neurons in the ventral dentate hilus. Photomicrographs of representative Nissl-stained coronal sections containing the ventral portion of the dentate gyrus taken from a control rat (**A**), from a rat in the behavioral status epilepticus (BSE) group (**B**) and from a kainate-treated rat that had not experienced behavioral status epilepticus (no-BSE; **C**). Note that the density of neurons in the dentate hilus is dramatically reduced in the rat that experienced behavioral SE (**B**). In striking contrast, most of the neurons of the ventral hilus are preserved in the rat from the no-BSE group (**C**). Scale bar shown in (**B**) = 250 μm.
doi:10.1371/journal.pone.0084722.g006

with the total number of neurons in the CA3 hippocampal field ($r = 0.74$, $p < 0.0005$ and $r = 0.6$, $p < 0.01$ for the percentage of savings and percentage of time in the training quadrant, respectively; Fig. 8C, D). In contrast, we found no significant correlations between the two behavioral indexes and the total number of the CA1 pyramidal neurons (Fig. 8E, F).

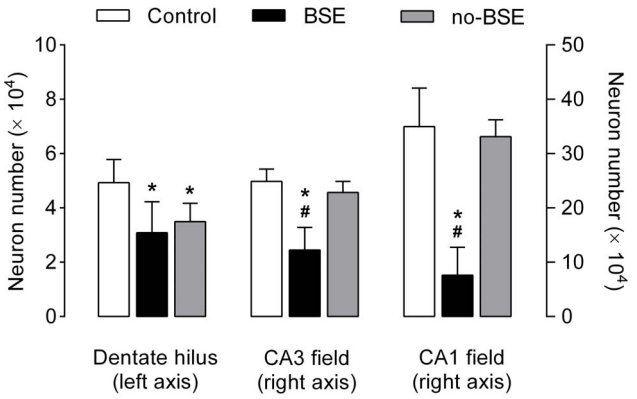


Figure 7. Loss of hippocampal neurons in kainate-treated rats. The y-axes represent the total numbers of neurons (mean±SD) in the dentate hilus and hippocampal CA3 and CA1 pyramidal fields in control rats and in kainate-treated rats with and without behavioral status epilepticus (BSE group and no-BSE group, respectively). The number of hilar neurons is reduced in both groups of kainate-treated rats when compared to control group. Note that the number of cells in the CA3 and CA1 pyramidal regions was strikingly reduced in rats from the BSE group, but not in those from the no-BSE group. Dentate hilus: $^{*}p<0.05$ for no-BSE group vs. control group and $p<0.01$ for BSE group vs. control group; CA3 and CA1 fields: $^{*}p<0.001$ vs. control group and $^{\#}p<0.001$ vs. no-BSE group.
doi:10.1371/journal.pone.0084722.g007

Discussion

The results of this experiment show that rats treated with similar doses of KA may have two quite distinct sets of brain changes. In particular, the neuropathological sequelae of the treatment were different in the following ways: (1) in one group of rats KA induced a clear pattern of convulsive SE including repeated stage 4 and 5 seizures on the Racine scale, but this was not observed in remaining rats; (2) majority of the rats that had experienced behavioral SE developed spontaneous motor seizures 1 to 3 months later, whereas the rats in the other group did not; (3) histological analysis revealed massive loss of hippocampal pyramidal neurons in rats that had experienced behavioral SE, whereas no pyramidal cell loss was detected in rats who did not show motor signs of SE; in either group of KA-treated rats, the total number of neurons in the hilus of the dentate gyrus was reduced; (4) although rats in both KA-treated groups were impaired in behavioral tests, the impairments were significantly more profound and wide-ranging in the BSE group. These data are consistent with previous studies showing that animals of the same strain may have different seizure susceptibility [14,29,30,31], which is believed to have both genetic [29,30] and environmental [32] underpinnings.

Rats in the BSE group were not only impaired in learning the precise platform position in the water maze, but were, in fact, unable to adopt a spatial strategy to solve the task. In addition, they showed severely impaired performance on the passive avoidance task and on the fear conditioning tests. Rats in this group were hyperactive in the open-field test and spent more time walking on the open arms of the elevated plus maze, suggesting a deficient emotional responsiveness. Our results are consistent with many prior studies in which behavioral changes were detected in animals following SE induced by either electrical stimulation [11,33] or injections of chemoconvulsants (pilocarpine [34,35], lithium-pilocarpine [9,13,36], and kainate [8,9,11,12,13,37,38] models). However, the behavioral deficits observed in the present study appear to be somewhat more pronounced than those reported in prior studies using the same model of SE [9,39,40]. This discrepancy is likely due to differences in experimental conditions between different studies. For example, it has been reported that the induction of SE in adult rats is followed by more robust behavioral deficits than in premature rats [8,37,38] and that old rats, in turn, are more susceptible to SE when compared to adult rats [12]. In addition, it has been shown that the seizure-related behavioral impairments are strain-specific [9,13,31,41]. Furthermore, it is also well documented that different treatment protocols, e.g. duration of SE, may be associated with very different neuropathological outcomes in experimental animals [9,16,42]. It may be also noted that we performed behavioral testing of rats approximately 5 months after the induction of SE, that is, at a time when seizure frequency is maximal [43]. For comparison, studies on animals just 2 or 3 months post-SE, when seizures are relatively less frequent and neuroanatomical changes are likely to be less dramatic, generally reveal less severe behavioral deficits [9,40]. However, it remains to be seen whether SE-related behavioral deficits actually worsen with increased duration of epileptic state. In fact, it has been recently reported that animals tested two months and twenty months after the induction of SE showed similar cognitive impairments [44].

There are a few prior studies in which animals that did not develop convulsive SE after the respective treatments were evaluated behaviorally in parallel to those who did. In particular, Szyndler et al. [36] reported that lithium-pilocarpine-treated rats resistant to develop SE did not differ behaviorally from control rats, whereas rats that developed SE were impaired in the fear conditioning and open-field tests. Müller et al. [34] have shown that pilocarpine-treated mice without motor signs of SE differed significantly from control mice in several measures derived from the open-field, hole-board and light-dark transition tests (mainly related to their increased activity), but were intact, unlike mice which had behavioral SE, on the elevated plus-maze, novel object exploration and spatial water maze tests. However, histological analysis carried out in the latter study revealed significant loss of hippocampal neurons only in mice that developed convulsive SE [34]. Interestingly, it has been also reported that treating rats with KA during early postnatal period induces electrographic and mild behavioral seizures, but does not evoke a typical pattern of SE, i.e. stage 4–5 seizures on the Racine scale [37,38]. Yet, later in life, these rats show significant cognitive deficits accompanied by long-term impairments in hippocampal plasticity [37,38]. In this respect, it is worth noting the results of a recent study by Suárez et al. [14] who reported that treating rats with KA, even if it induces neither motor SE, nor spontaneous seizures later in life, may lead to enduring changes in synaptic plasticity. In the present study, KA-treated rats that did not develop motor SE had cell loss in only one of the hippocampal subdivisions, the dentate hilus, but were impaired on the passive avoidance and spatial water maze tasks. Thus, taken together, these findings indicate that a part of the animals that do not manifest intense behavioral seizures immediately after an epileptogenic treatment, later in life, they may still have conspicuous structural and functional changes in the brain.

It is well documented that epilepsy models based on the induction of SE are typically associated with marked neuronal loss in various brain regions, such as the hippocampal formation, amygdala, thalamus, piriform, entorhinal, perirhinal, retrosplenial and olfactory cortices [7,9,10,11,33,35,45,46,47], many of which are implicated in cognitive and emotional processes [15,48,49,50,51,52]. Therefore, it is likely that brain damage induced by SE and subsequent spontaneous seizures is directly responsible for the behavioral deficits observed in these models. In

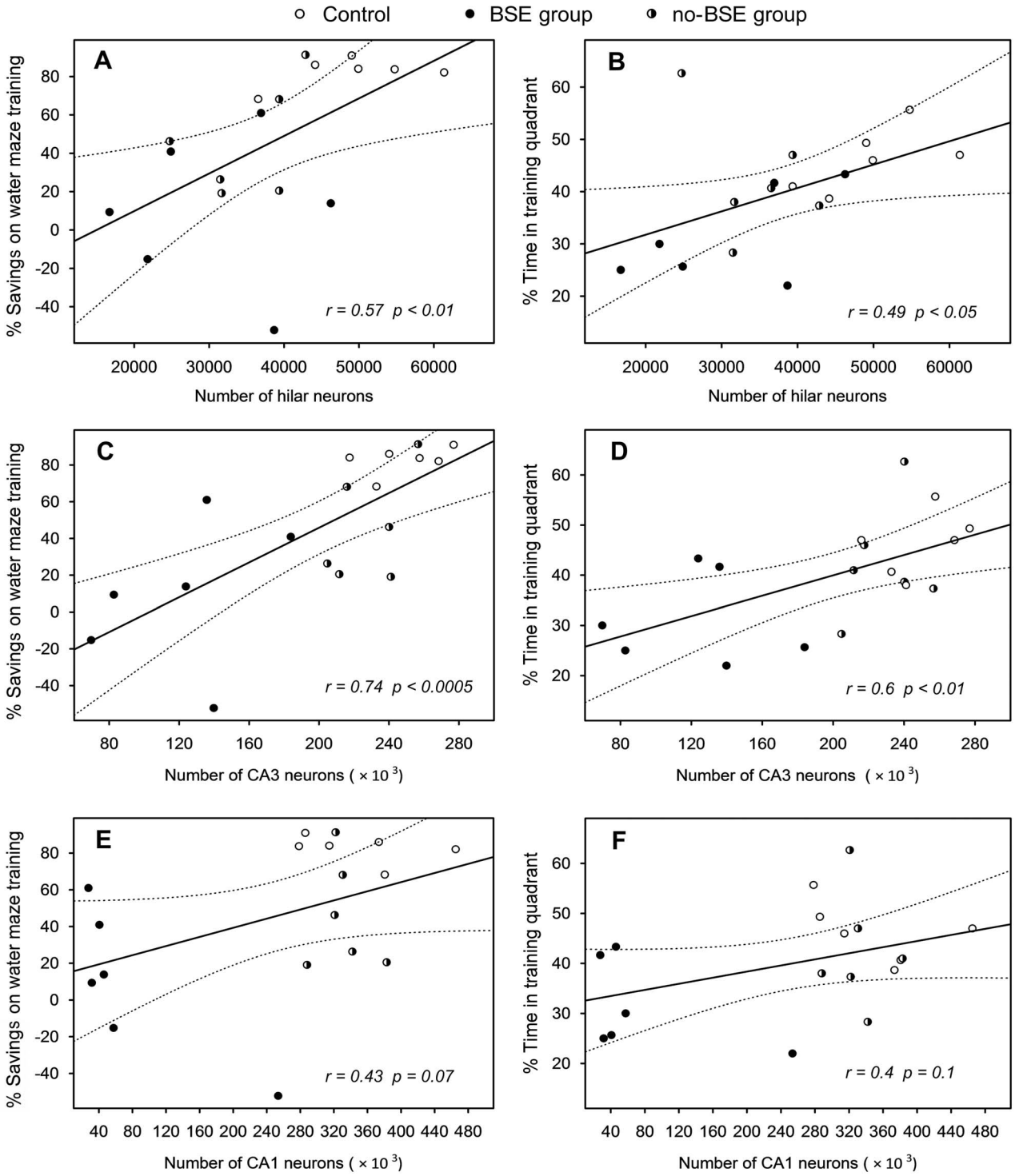


Figure 8. Correlations between hippocampal neuron numbers and spatial learning and memory indices. The x-axes represent the total numbers of neurons in the dentate hilus (**A**, **B**), and hippocampal CA3 (**C**, **D**) and CA1 (**E**, **F**) pyramidal fields in control and kainate-treated rats. The y-axes represent the percentages of savings during water maze training, i.e. the differences, in %, between the mean distances swum by rats on the first four trials and on the last four trials (**A**, **C**, **E**), and the percentages of time spent by rats in the training quadrant of the water maze during the probe trial (**B**, **D**, **F**). Data obtained from 6 animals randomly selected from each group (final n = 18) were used in the correlational analyses. Correlation coefficients are shown at the bottom of each plot and dashed lines represent 95% confidence intervals.
doi:10.1371/journal.pone.0084722.g008

effect, it has been recently shown that hippocampal volume of epileptic rats significantly correlates with their learning and memory abilities [9,53]. Similar relationships were found between the volumetric estimates of the amygdala and the indices of emotionality [9,53]. In this study, we detected marked loss of hippocampal CA3 and CA1 pyramidal neurons in rats that had

experienced behavioral SE, whereas in rats without behavioral manifestations of SE these neuronal populations were preserved. However, the loss of hilar neurons was detected in both KA-treated groups, even though it was predictably greater in BSE group than in no-BSE group. Thus, our data suggest that hilar cell loss alone may be sufficient to cause functional deficits in hippocampal circuits as indicated by the impaired performance of no-BSE rats on the water maze and passive avoidance tests. This suggestion is consistent with the evidence that hilar neurons may influence mnemonic processes by synchronizing the activity of the dentate gyrus granule cells and, more generally, by modulating the transmission of information from the entorhinal cortex to the hippocampal pyramidal fields [54,55]. This possibility is also supported by the results of the correlational analyses which revealed significant correlations between individual spatial memory indices of rats used in this experiment and the total number of hilar neurons, but not of CA1 pyramidal neurons. The strongest correlation, however, was found between the cognitive indices and the total number of CA3 neurons. This result may appear surprising given that significant neuronal loss in this region was observed in only one of the KA-treated group, namely in rats that had experienced convulsive SE. However, it is possible that even small loss of CA3 neurons, perhaps, undetectable in small-size samples (such as those used in the present study), may have a substantial impact on hippocampal function. This possibility is consistent with accepted theoretical models of hippocampal circuits [56,57,58] and experimental studies [59,60,61] implicating the CA3 region of the hippocampus in key cognitive processes underlying episodic memory, such as pattern separation (reducing the overlap between similar neuronal representations) and pattern completion (converging neuronal representations into a single pattern, thus, facilitating their retrieval using only partial cues). Therefore, it is plausible that the memory deficits observed in KA-treated rats in this study can be accounted for, partly at least, by the disruption of cognitive processes in CA3 neuronal networks, which may be associated with the loss of neurons in this region.

Although in this study we have not estimated neuronal numbers separately for the dorsal and ventral parts of the hippocampal formation, visual inspection of the brain sections clearly showed that neuronal loss associated with behavioral SE was more marked in the ventral division of the dentate hilus than in its dorsal part (Fig. 6). This finding is in line with a previous study demonstrating that kainate-induced SE produces more severe loss at the temporal (ventral) pole of the dentate hilus when compared to the septal (dorsal) pole [28]. However, in the no-BSE group, the neurons of the ventral hilus were less severely affected. This observation is important for the interpretation of the finding that rats in the no-BSE group, unlike those that had experienced motor SE, showed normal behavior in the open-field, elevated plus-maze and fear-conditioning tests. Indeed, considerable evidence suggests that the ventral hippocampus has a preferential role in the regulation of emotional behaviors in rodents [49]. Therefore, the different extents of cell loss in the ventral hilus of rats from the BSE and no-BSE groups can be related to the differences in their performance on the anxiety-related tests. This explanation, however, should be viewed with caution given that brain regions other than hippocampus, e.g. the amygdala, play important roles in emotional behaviors and were not analyzed histologically in the present study. Another implication of this morphological observation is that the greater loss of neurons in the ventral dentate hilus of BSE rats may be associated with the increased susceptibility to the development of spontaneous motor seizures. Relevant to this hypothesis is the finding by Inostrosa et al [9], who showed that kainate-induced convulsive SE produces significant mossy fiber sprouting only in the ventral part of the dentate gyrus, suggesting an enhanced susceptibility of this hippocampal subdivision to epileptogenesis [28]. Furthermore, it has been previously reported that KA injection into the ventral hippocampus induces local electrographic seizures, which rapidly propagate to the dorsal hippocampus and cingulate cortex and are accompanied by severe motor manifestations including stage 4 and 5 seizures on the Racine scale [62]. In contrast, injection of KA into the dorsal hippocampus induces only local electrographic seizures, which do not propagate to the cingulate cortex and do not evoke behavioral seizures [62]. Therefore, it is possible that the absence of spontaneous motor seizures in no-BSE rats may be related to the fact that the ventral hippocampus was better preserved in this group when compared to the BSE group, in which all rats showed spontaneous motor seizures. This, of course, does not mean that the absence of convulsive SE after the treatment with KA precludes the development of epilepsy later in life. Indeed, because in the present study we did not perform EEG monitoring, spontaneous subconvulsive seizures might have occurred in the no-BSE group, but were not registered. In addition, the latency between the kainate treatment and the onset of the first spontaneous seizures may exceed the 5-month period of observation implemented in the current study [16].

In conclusion, this study was designed to compare the behavioral and neuroanatomical outcomes in rats that developed convulsive SE after kainate treatment to those that did not. It was found that those rats in which KA did not induce motor signs of SE were, however, impaired in hippocampal-dependent memory tasks later in life, which can be explained by long-term functional abnormalities in hippocampal circuits associated with the loss of hilar cells and, probably, CA3 pyramidal neurons. However, the extent of neuronal loss and the severity of cognitive deficits in this group were far less striking than those observed in the rats that had experienced motor SE. Thus, our findings add further weight to the evidence from former studies in animal models [9,53] and humans [4,6,63] showing that behavioral deficits in TLE correlate with the extent of hippocampal damage.

Author Contributions

Conceived and designed the experiments: GHM NVL. Performed the experiments: GHM JIS JMC. Analyzed the data: GHM JLQ PAA JAP NVL. Contributed reagents/materials/analysis tools: JPA NVL. Wrote the paper: GHM NVL.

References

1. Margerison JH, Corsellis JA (1966) Epilepsy and the temporal lobes. A clinical, electroencephalographic and neuropathological study of the brain in epilepsy, with particular reference to the temporal lobes. Brain 89: 499–530.
2. Sutula T, Cascino G, Cavazos J, Parada I, Ramirez L (1989) Mossy fiber synaptic reorganization in the epileptic human temporal lobe. Ann Neurol 26: 321–330.
3. Mathern GW, Babb TL, Pretorius JK, Leite JP (1995) Reactive synaptogenesis and neuron densities for neuropeptide Y, somatostatin, and glutamate decarboxylase immunoreactivity in the epileptogenic human fascia dentata. J Neurosci 15: 3990–4004.
4. Aldenkamp AP, Overweg J, Gutter T, Beun AM, Diepman L, et al. (1996) Effect of epilepsy, seizures and epileptiform EEG discharges on cognitive function. Acta Neurol Scand 93: 253–259.
5. Piazzini A, Canevini MP, Maggiori G, Canger R (2001) Depression and anxiety in patients with epilepsy. Epilepsy Behav 2: 481–489.
6. Alessio A, Damasceno BP, Camargo CH, Kobayashi E, Guerreiro CA, et al. (2004) Differences in memory performance and other clinical characteristics in patients with mesial temporal lobe epilepsy with and without hippocampal atrophy. Epilepsy Behav 5: 22–27.

7. Ben-Ari Y, Tremblay E, Ottersen OP, Meldrum BS (1980) The role of epileptic activity in hippocampal and "remote" cerebral lesions induced by kainic acid. Brain Res 191: 79–97.
8. Stafstrom CE, Chronopoulos A, Thurber S, Thompson JL, Holmes GL (1993) Age-dependent cognitive and behavioral deficits after kainic acid seizures. Epilepsia 34: 420–432.
9. Inostroza M, Cid E, Brotons-Mas J, Gal B, Aivar P, et al. (2011) Hippocampal-dependent spatial memory in the water maze is preserved in an experimental model of temporal lobe epilepsy in rats. PLoS ONE 6: e22372.
10. Covolan L, Mello LE (2000) Temporal profile of neuronal injury following pilocarpine or kainic acid-induced status epilepticus. Epilepsy Res 39: 133–152.
11. Kemppainen EJ, Nissinen J, Pitkänen A (2006) Fear conditioning is impaired in systemic kainic acid and amygdala-stimulation models of epilepsy. Epilepsia 47: 820–829.
12. Hattiangady B, Kuruba R, Shetty AK (2011) Acute seizures in old age leads to a greater loss of CA1 pyramidal neurons, an increased propensity for developing chronic TLE and a severe cognitive dysfunction. Aging Dis 2: 1–18.
13. Inostroza M, Cid E, Menendez de la Prida L, Sandi C (2012) Different emotional disturbances in two experimental models of temporal lobe epilepsy in rats. PLoS ONE 7: e38959.
14. Suárez LM, Cid E, Gal B, Inostroza M, Brotons-Mas JR, et al. (2012) Systemic injection of kainic acid differently affects LTP magnitude depending on its epileptogenic efficiency. PLoS ONE 7: e48128.
15. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47–60.
16. Hellier JL, Patrylo PR, Buckmaster PS, Dudek FE (1998) Recurrent spontaneous motor seizures after repeated low-dose systemic treatment with kainate: assessment of a rat model of temporal lobe epilepsy. Epilepsy Res 31: 73–84.
17. Hellier JL, Dudek FE (1999) Spontaneous motor seizures of rats with kainate-induced epilepsy: effect of time of day and activity state. Epilepsy Res 35: 47–57.
18. Racine RJ (1972) Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32: 281–294.
19. Herguido MJ, Carceller F, Roda JM, Avendaño C (1999) Hippocampal cell loss in transient global cerebral ischemia in rats: a critical assessment. Neuroscience 93: 71–80.
20. Lukoyanov NV, Lukoyanova EA (2006) Retrosplenial cortex lesions impair acquisition of active avoidance while sparing fear-based emotional memory. Behav Brain Res 173: 229–236.
21. Lukoyanov NV, Lukoyanova EA, Andrade JP, Paula-Barbosa MM (2005) Impaired water maze navigation of Wistar rats with retrosplenial cortex lesions: effect of nonspatial pretraining. Behav Brain Res 158: 175–182.
22. Gundersen HJ, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147: 229–263.
23. West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231: 482–497.
24. Amaral D, Witter M (1995) Hippocampal formation. In: Paxinos G, editor. The rat nervous system. San Diego: Academic Press. 443–493.
25. Gundersen HJ, Jensen EB, Kiêu K, Nielsen J (1999) The efficiency of systematic sampling in stereology-reconsidered. J Microsc 193: 199–211.
26. Shapiro SS, Wilk MB, Chen HJ (1968) A Comparative Study of Various Tests for Normality. J Am Stat Assoc 63: 1343–1372.
27. Gaarskjaer FB (1978) Organization of the mossy fiber system of the rat studied in extended hippocampi. I. Terminal area related to number of granule and pyramidal cells. J Comp Neurol 178: 49–72.
28. Buckmaster PS, Dudek FE (1997) Neuron loss, granule cell axon reorganization, and functional changes in the dentate gyrus of epileptic kainate-treated rats. J Comp Neurol 385: 385–404.
29. Gilby KL, Da Silva AG, McIntyre DC (2005) Differential GABA(A) subunit expression following status epilepticus in seizure-prone and seizure-resistant rats: a putative mechanism for refractory drug response. Epilepsia 46 Suppl 5: 3–9.
30. McIntyre DC, Gilby KL (2007) Genetically seizure-prone or seizure-resistant phenotypes and their associated behavioral comorbidities. Epilepsia 48 Suppl 9: 30–32.
31. Langer M, Brandt C, Löscher W (2011) Marked strain and substrain differences in induction of status epilepticus and subsequent development of neurodegeneration, epilepsy, and behavioral alterations in rats. Epilepsy Res 96: 207–224.
32. Gilby KL, Sydserff S, Patey AM, Thorne V, St-Onge V, et al. (2009) Postnatal epigenetic influences on seizure susceptibility in seizure-prone versus seizure-resistant rat strains. Behav Neurosci 123: 337–346.
33. Brandt C, Gastens AM, Sun M, Hausknecht M, Löscher W (2006) Treatment with valproate after status epilepticus: effect on neuronal damage, epileptogenesis, and behavioral alterations in rats. Neuropharmacology 51: 789–804.
34. Müller CJ, Gröticke I, Bankstahl M, Löscher W (2009) Behavioral and cognitive alterations, spontaneous seizures, and neuropathology developing after a pilocarpine-induced status epilepticus in C57BL/6 mice. Exp Neurol 219: 284–297.
35. Cardoso A, Lukoyanova EA, Madeira MD, Lukoyanov NV (2011) Seizure-induced structural and functional changes in the rat hippocampal formation: comparison between brief seizures and status epilepticus. Behav Brain Res 225: 538–546.
36. Szyndler J, Wierzba-Bobrowicz T, Skórzewska A, Maciejak P, Walkowiak J, et al. (2005) Behavioral, biochemical and histological studies in a model of pilocarpine-induced spontaneous recurrent seizures. Pharmacol Biochem Behav 81: 15–23.
37. Sayin U, Sutula TP, Stafstrom CE (2004) Seizures in the developing brain cause adverse long-term effects on spatial learning and anxiety. Epilepsia 45: 1539–1548.
38. Lynch M, Sayin U, Bownds J, Janumpalli S, Sutula T (2000) Long-term consequences of early postnatal seizures on hippocampal learning and plasticity. Eur J Neurosci 12: 2252–2264.
39. Gayoso MJ, Primo C, al-Majdalawi A, Fernandez JM, Garrosa M, et al. (1994) Brain lesions and water-maze learning deficits after systemic administration of kainic acid to adult rats. Brain Res 653: 92–100.
40. Barkas L, Redhead E, Taylor M, Shtaya A, Hamilton DA, et al. (2012) Fluoxetine restores spatial learning but not accelerated forgetting in mesial temporal lobe epilepsy. Brain 135: 2358–2374.
41. Hort J, Brozek G, Komárek V, Langmeier M, Mares P (2000) Interstrain differences in cognitive functions in rats in relation to status epilepticus. Behav Brain Res 112: 77–83.
42. Sloviter RS (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. Science 235: 73–76.
43. Williams PA, White AM, Clark S, Ferraro DJ, Swiercz W, et al. (2009) Development of spontaneous recurrent seizures after kainate-induced status epilepticus. J Neurosci 29: 2103–2112.
44. Cavarsan CF, Queiroz CM, Dos Santos JG Jr, Xavier GF, Mello LE, et al. (2013) Reduced hippocampal dentate cell proliferation and impaired spatial memory performance in aged-epileptic rats. Front Neurol doi: 10.3389/fneur.2013.00106.
45. Tuunanen J, Lukasiuk K, Halonen T, Pitkänen A (1999) Status epilepticus-induced neuronal damage in the rat amygdaloid complex: distribution, time-course and mechanisms. Neuroscience 94: 473–495.
46. Chen S, Buckmaster PS (2005) Stereological analysis of forebrain regions in kainate-treated epileptic rats. Brain Res 1057: 141–152.
47. Cardoso A, Madeira MD, Paula-Barbosa MM, Lukoyanov NV (2008) Retrosplenial granular b cortex in normal and epileptic rats: a stereological study. Brain Res 1218: 206–214.
48. Olton DS (1983) Memory functions and the hippocampus. In: Seifert W, editor. Neurobiology of the hippocampus. New York: Academic Press. 335–374.
49. LeDoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23: 155–184.
50. Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, et al. (2004) Regional dissociations within the hippocampus - memory and anxiety. Neurosci Biobehav Rev 28: 273–283.
51. Furtak SC, Wei SM, Agster KL, Burwell RD (2007) Functional neuroanatomy of the parahippocampal region in the rat: the perirhinal and postrhinal cortices. Hippocampus 17: 709–722.
52. Aggleton JP, O'Mara SM, Vann SD, Wright NF, Tsanov M, et al. (2010) Hippocampal-anterior thalamic pathways for memory: uncovering a network of direct and indirect actions. Eur J Neurosci 31: 2292–2307.
53. Kubová H, Mareš P (2013) Are morphologic and functional consequences of status epilepticus in infant rats progressive? Neuroscience 235: 232–249.
54. Paulsen O, Moser EI (1998) A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. Trends Neurosci 21: 273–278.
55. Vogt KE, Nicoll RA (1999) Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. Proc Natl Acad Sci U S A 96: 1118–1122.
56. Marr D (1971) Simple memory: a theory for archicortex. Philos Trans R Soc Lond B Biol Sci 262: 23–81.
57. Treves A, Rolls ET (1994) Computational analysis of the role of the hippocampus in memory. Hippocampus 4: 374–391.
58. O'Reilly RC, Bhattacharyya R, Howard MD, Ketz N (2011) Complementary Learning Systems. Cogn Sci doi: 10.1111/j.1551–6709.2011.01214.x.
59. Vazdarjanova A, Guzowski JF (2004) Differences in hippocampal neuronal population responses to modifications of an environmental context: evidence for distinct, yet complementary, functions of CA3 and CA1 ensembles. J Neurosci 24: 6489–6496.
60. Leutgeb S, Leutgeb JK, Treves A, Moser MB, Moser EI (2004) Distinct ensemble codes in hippocampal areas CA3 and CA1. Science 305: 1295–1298.
61. Bakker A, Kirwan CB, Miller M, Stark CE (2008) Pattern separation in the human hippocampal CA3 and dentate gyrus. Science 319: 1640–1642.
62. Akaike K, Tanaka S, Tojo H, Fukumoto S, Imamura S, et al. (2001) Kainic acid-induced dorsal and ventral hippocampal seizures in rats. Brain Res 900: 65–71.
63. Bonilha L, Alessio A, Rorden C, Baylis G, Damasceno BP, et al. (2007) Extrahippocampal gray matter atrophy and memory impairment in patients with medial temporal lobe epilepsy. Hum Brain Mapp 28: 1376–1390.

Agradecimentos

A elaboração deste trabalho ultrapassa largamente o manuscrito final que aqui apresento. Foi em 2011 que se deu início a esta aventura no Serviço de Anatomia e, como tal, justifica-se um agradecimento especial aos que me acompanharam.

Agradeço ao meu orientador, professor Nikolay Lukoyanov, por toda a ajuda, empenho, dedicação, espírito crítico, disponibilidade em me acompanhar e boa disposição durante estes 4 anos.

À Dr.ª Gisela Maia, por tudo aquilo que me ensinou, por toda a paciência que teve, por toda a ajuda que me deu e por todas as conversas interessantes que me proporcionou o meu muito obrigado.

Aos meus colegas e amigos (de Barcelos, dos Arcos de Valdevez, de Arcozelo de Baixo, Aveiro, Caminha, Chaves, Espinho, Esposende, Gaia, Gondomar, Guimarães, Ponte de Lima, Paços de Ferreira, Penafiel, do Porto, da Póvoa de Varzim, de Resende, Rio Tinto, Santa Maria da Feira, Vila Nova de Cerveira,…) pois, mesmo não tendo contribuído ativamente para este manuscrito, acompanharam-me ao longo destes importantes anos da minha vida e fazem de mim, bem ou mal, aquilo que eu sou.

A toda a minha família, em especial à minha mãe, ao meu pai e à minha irmã por me terem proporcionado as condições para que tudo isto fosse possível, por me apoiarem incondicionalmente nas minhas decisões, sem, no entanto, perderem o espírito crítico e cederem à minha teimosia e por serem a melhor família que eu podia desejar.

PLOS ONE Manuscript Guidelines

1. Format Requirements
2. Guidelines for Standard Sections
   - Title
   - Authors and Affiliations
   - Abstract
   - Introduction
   - Materials and Methods
   - Results, Discussion, and Conclusions
   - Acknowledgments
   - References
   - Figure Legends
   - Supporting Information Captions
   - Data Reporting Guidelines
   - Accession Numbers
   - Striking Images
   - Tables
3. Specific Reporting Guidelines
   - Human Subject Research
   - Clinical Trials
   - Animal Research
   - Observational and Field Studies
   - Cell Line Research
   - Blots and Gels
   - Antibodies
   - Systematic Review/Meta-Analysis
   - Paleontology and Archaeology Research
   - Software Papers
   - Database Papers
   - New Zoological Taxon
   - New Botanical Taxon
   - New Fungal Taxon
   - Qualitative Research

1. Format Requirements

PLOS ONE does **not** consider presubmission inquiries. All submissions should be prepared with the following files:

- Cover letter
- Manuscript, including tables and figure legends
- Figures (guidelines for preparing figures can be found at the Figure and Table Guidelines)
  Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service." Submissions are **not** copyedited before publication.

  In addition to the guidelines below, please refer to our downloadable sample files to make sure that your submission meets our formatting requirements:

- Download sample title, author list, and affiliations page (PDF)
- Download full manuscript sample (PDF)
  Submissions that do not meet the *PLOS ONE* Publication Criterion for language standards may be rejected.

Cover Letter

You should supply an approximately one page cover letter that:

- Concisely summarizes why your paper is a valuable addition to the scientific literature
- Briefly relates your study to previously published work
- Specifies the type of article you are submitting (for example, research article, systematic review, meta-analysis, clinical trial)
- Describes any prior interactions with PLOS regarding the submitted manuscript
- Suggests appropriate *PLOS ONE* Academic Editors to handle your manuscript (view a complete listing of our academic editors)
- Lists any opposed reviewers
  Your cover letter should **not** include requests to reduce or waive publication fees. Should your manuscript be accepted, you will have the opportunity to include your requests at that time. See *PLOS ONE* Editorial Policy for more information regarding publication fees.

Manuscript Organization

PLOS ONE considers manuscripts of any length. There are no explicit restrictions for the number of words, figures, or the length of the supporting information, although we encourage a concise and accessible writing style. We will **not** consider monographs.

All manuscripts should be double-spaced and include line numbers and page numbers.

Manuscripts should begin with the ordered sections:

- Title
- Authors
- Affiliations
- Abstract
- Introduction
  and end with the sections of:

- Acknowledgments
- References
- Supporting Information Captions
  Figures should be cited in ascending numeric order upon first appearance. Each figure caption should then be inserted immediately after the first paragraph in which it is cited in the article file.

  Figures should not be included in the main manuscript file. Each figure must be prepared and submitted as an individual file. Find more information about preparing figures here.

  Tables should be cited in ascending numeric order upon first appearance. Each table should then be inserted immmediately after the first paragraph in which it is cited in the article file.

  The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

  There are no explicit requirements for section organization between these beginning and ending sections. Articles may be organized in different ways and with different section titles, according to the authors' preference. In most cases, internal sections include:

- Materials and Methods
- Results
- Discussion
- Conclusions (optional)
  PLOS ONE has no specific requirements for the order of these sections, and in some cases it may be appropriate to combine sections. Guidelines for individual sections can be found below.

  Abbreviations should be kept to a minimum and defined upon first use in the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

  Standardized nomenclature should be used as appropriate, including appropriate usage of species names and SI units.

  PLOS articles do not support text footnotes. If your accepted submission contains footnotes, you will be asked to move that material into either the main text or the reference list, depending on the content.

Manuscript File Requirements

Authors may submit their manuscript files in Word (as .doc or .docx), LaTeX (as .pdf), or RTF format. Word files must not be protected.

LaTeX Submissions. If you would like to submit your manuscript using LaTeX, you must author your article using the *PLOS ONE* LaTeX template and BibTeX style sheet. Articles prepared in LaTeX may be submitted in PDF format for use during the review process. After acceptance, however, .tex files will be required. Please consult our LaTeX guidelines for a list of what will be required.

Microsoft Word Submissions with Equations. If your manuscript is or will be in Microsoft Word and contains equations, you must follow the instructions below to make sure that your equations are editable when the file enters production.

1. Format display equations only in MathType (http://www.dessci.com/en/products/mathtype/).
2. Inline equations should be completely input via MathType. Do not include an equation that is part text, part MathType.
3. Do not use graphic objects.
   If you have already composed your article in Microsoft Word and used its built-in equation editing tool, your equations will become unusable during the typesetting process. To resolve this problem, re-key your equations using MathType.

If you do not follow these instructions, PLOS will not be able to accept your file.

2. Guidelines for Standard Sections

Title

Manuscripts must be submitted with both a full title and a short title, which will appear at the top of the PDF upon publication if accepted. Only the full title should be included in the manuscript file; the short title will be entered during the online submission process.

The full title must be 250 characters or fewer. It should be specific, descriptive, concise, and comprehensible to readers outside the subject field. Avoid abbreviations if possible. Where appropriate, authors should include the species or model system used (for biological papers) or type of study design (for clinical papers).

Examples:

- Impact of Cigarette Smoke Exposure on Innate Immunity: A *Caenorhabditis elegans* Model
- Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

The short title must be 50 characters or fewer and should state the topic of the paper.

Authors and Affiliations

All author names should be listed in the following order:

- First names (or initials, if used),
- Middle names (or initials, if used), and
- Last names (surname, family name)
  Each author should list an associated department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

  This information cannot be changed after initial submission, so please ensure that it is correct.

  To qualify for authorship, one should contribute to **all** of the following:

1. Conception and design of the work, acquisition of data, or analysis and interpretation of data
2. Drafting the article or revising it critically for important intellectual content
3. Final approval of the version to be published
4. Agreement to be accountable for all aspects of the work
   All persons designated as authors should qualify for authorship, and all those who qualify should be listed. Each author must have participated sufficiently in the work to take public responsibility for appropriate portions of the content. Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments.

   When a large group or center has conducted the work, the author list should include the individuals whose contributions meet the criteria defined above, as well as the group name.

   All authors must approve the final manuscript before submission. PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission of the manuscript.

   One author should be designated as the corresponding author, and his or her email address or other contact information should be included on the manuscript cover page. This information will be published with the article if accepted.

   See the PLOS Editorial and Publishing Policies for more information.

Abstract

The abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should **not** include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

This section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

We encourage authors to submit detailed protocols for newer or less well-established methods as Supporting Information. Further information about formatting Supporting Information files, can be found here.

Methods sections of papers on research using **human or animal subjects and/or tissue or field sampling** must include required ethics statements. See theReporting Guidelines for human research, clinical trials, animal research, and observational and field studies for more information.

Methods sections of papers with **data that should be deposited in a publicly available database** should specify where the data have been deposited and provide the relevant accession numbers and version numbers, if appropriate. Accession numbers should be provided in parentheses after the entity on first use. If the accession numbers have not yet been obtained at the time of submission, please state that they will be provided during review. They must be provided prior to publication. A list of recommended repositories for different types of data can be found here.

Methods sections of papers using **cell lines** must state the origin of the cell lines used. See the Reporting Guidelines for cell line research for more information.

Methods sections of papers adding **new taxon names** to the literature must follow the Reporting Guidelines below for a new zoological taxon, botanical taxon, or fungal taxon.

Results, Discussion, and Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn. Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE*Publication Criteria for more information.

Acknowledgments

People who contributed to the work but do not fit the *PLOS ONE* authorship criteria should be listed in the acknowledgments, along with their contributions. You must ensure that anyone named in the acknowledgments agrees to being so named.

Funding sources should **not** be included in the acknowledgments, or anywhere in the manuscript file. You will provide this information during the manuscript submission process.

References

General guidelines

- PLOS uses the reference style as outlined in the ICMJE sample references, also referred to as the “Vancouver” style.
- References must be listed at the end of the manuscript and numbered in the order that they appear in the text.
- In the text, citations should be indicated by the reference number in brackets.
- Authors may cite any and all available works in the reference list.
- Authors may not cite unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”).
- If an article is submitted to a journal and also publicly available as a pre-print, the pre-print may be cited.
- If related work has been submitted to PLOS ONE or elsewhere, authors should include a copy with the submitted article as confidential supplementary information, for review purposes only.
- Authors should not state 'unpublished work' or 'data not shown,' but instead include those data as supplementary material or deposit the data in a publicly available database.

- Journal name abbreviations should be those found in the NCBI databases.

Reference formatting

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial. References should be formatted as follows:

Published papers
1. Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun, B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588.

Note: Use of a DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers:

Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005

Accepted, unpublished papers
Same as above, but “In press” appears instead of the page numbers or DOI.

Websites or online articles
1. Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available: http://www.globalizationandhealth.com/content/1/1/14.

Books
1. Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Book chapters
1. Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.

Deposited articles (preprints, e-prints, or arXiv)
1. Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available: arXiv:1403.3301v1. Accessed 17 March 2014.

Published media (print or online newspapers and magazine articles)
1. Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html. Accessed 17 March 2014.

New media (blogs, websites, or other written works)
1. Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.

Masters' theses or doctoral dissertations
1. Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc.

Thesis, The University of Sheffield. 1999. Available: http://cumincad.scix.net/cgi-bin/works/Show?2e09.

Databases and repositories (Figshare, arXiv)
1. Roberts SB. QPX Genome Browser Feature Tracks; 2013. Database: figshare [Internet]. Accessed: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214.

Multimedia (videos, movies, or TV shows)
1. Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Figure Legends

Figures should **not** be included in the manuscript file, but figure legends should be. Guidelines for preparing figures can be found here.

Figure legends should describe the key messages of a figure. Legends should have a short title of 15 words or less. The full legend should have a description of the figure and allow readers to understand the figure without referring to the text. The legend itself should be succinct, avoid lengthy descriptions of methods, and define all non-standard symbols and abbreviations.

Figures should be cited in ascending numeric order upon first appearance. Each figure caption should be inserted immediately after the first paragraph in which they are cited in the article file. Further information about figure captions can be found in the Figure Guidelines.

Supporting Information Captions

Because Supporting Information is accessed via a hyperlink attached to its captions, captions must be listed in the article file. Do not submit a separate caption file. It is acceptable to have them in the file itself in addition, but they must be in the article file for access to be possible in the published version.

The file category name and number is required, and a one-line title is highly recommended. A legend can also be included but is not required. Supporting Information captions should be formatted as follows.

S1 Text. Title is strongly recommended. Legend is optional.

Please see our Supporting Information guidelines for more details.

Data Reporting Guidelines

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article. Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (eg, GenBank for sequences and PDB for structures), general, or

institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CCBY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include: Dryad and figshare. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

Deposit data in the integrated repository of choice. Once deposition is final and complete, the repository will provide the author with a dataset DOI (provisional) and private URL for reviewers to gain access to the data. Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS Submission form. Then provide the URL passcode in the Attach Files section. If you have any questions, please contact us at plosone@plos.org

Accession Numbers

All appropriate datasets, images, and information should be deposited in public resources. Please provide the relevant accession numbers (and version numbers, if appropriate). Accession numbers should be provided in parentheses after the entity on first use. Suggested databases include, but are not limited to:

- ArrayExpress
- BioModels Database
- Database of Interacting Proteins
- DNA Data Bank of Japan [DDBJ]
- DRYAD
- EMBL Nucleotide Sequence Database
- GenBank
- Gene Expression Omnibus [GEO]
- Protein Data Bank
- UniProtKB/Swiss-Prot
- ClinicalTrials.gov
  In addition, as much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene

- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem
  Providing accession numbers allows linking to and from established databases and integrates your article with a broader collection of scientific information.

Striking Images

Authors are encouraged to upload a "striking image" that may be used to represent their paper online in places like the journal homepage or in search results. The striking image must be derived from a figure or supporting information file from the paper, ie. a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows. If no striking image is uploaded, a figure from the paper will be designated as the striking image.

Please keep in mind that PLOS's Creative Commons Attribution License applies to striking images. As such, do not submit any figures or photos that have been previously copyrighted unless you have express written permission from the copyright holder to publish under the CCAL license. Note that all published materials in PLOS ONE are freely available online, and any third party is permitted to read, download, copy, distribute, and use these materials in any way, even commercially, with proper attribution.

Care should be taken with the following image types in particular:

1. PLOS ONE is unable to publish any images generated by Google software (Google Maps, Street View, and Earth)
2. Maps in general are usually copyrighted, especially satellite maps
3. Photographs
4. Commercial or government images, slogans, or logos
5. Images from Facebook or Twitter
   Authors must also take special care when submitting manuscripts that contain potentially identifying images of people. Identifying information should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF).

For license inquiries, e-mail license [at] plos.org.

Tables

Tables should be cited in ascending numeric order upon first appearance. Each table should be inserted immediately after the first paragraph in which it is cited in the article file. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as Supporting

Information. Please ensure that table formatting conforms to our Guidelines for table preparation.

3. Specific Reporting Guidelines

Human Subject Research

Methods sections of papers on research using human subject or samples must include ethics statements that specify:

- The name of the approving institutional review board or equivalent committee(s). If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- Whether informed consent was written or oral. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: "Caucasian" should be changed to "white" or "of [Western] European descent" (as appropriate); "cancer victims" should be changed to "patients with cancer."

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal (PDF), which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subject research, see the Publication Criteria and Editorial Policies.

Clinical Trials

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement website. Before the paper can enter peer review, authors must:

1. Provide the registry name and number in the methods section of the manuscript
2. Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as Supporting Information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
3. Include the CONSORT flow diagram as the manuscript's "Fig. 1"
Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

For more information about *PLOS ONE* policies regarding clinical trials, see the Editorial Policies.

Animal Research

Methods sections of manuscripts reporting results of animal research must include required ethics statements that specify:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s) (where ethical approval is not required, the manuscript should include a clear statement of this and the reason why)
- Relevant details for efforts taken to ameliorate animal suffering
For example:

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The organism(s) studied should always be stated in the abstract. Where research may be confused as pertaining to clinical research, the animal model should also be stated in the title.

We ask authors to follow the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information. Please note that inclusion of a completed ARRIVE Checklist will be a formal requirement for publication at a later date.

For more information about *PLOS ONE* policies regarding animal research, see the Publication Criteria and Editorial Policies.

Observational and Field Studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

For more information about *PLOS ONE* policies regarding observational and field studies, see the Publication Criteria and Editorial Policies.

Cell Line Research

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate. Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers a gift, authors must follow our policies for human subject research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended - e.g. by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis - and may be required during peer review or after publication.

Blots and Gels

Authors of manuscripts reporting results from blots (including Western blots) and electrophoretic gels should follow these guidelines:

- In accordance with *PLOS ONE*'s policy on image manipulation, the image should not be adjusted in any way that could affect the scientific information displayed, e.g. by modifying the background or contrast

- All blots and gels that support results reported in the manuscript should be provided
- Original uncropped and unadjusted blots and gels, including molecular size markers, should be provided in either the figures or the supplementary files
- Lanes should not be overcropped around the bands; the image should show most or all of the blot or gel. Any non-specific bands should be shown and an explanation of their nature should be given
- The image should include all relevant controls, and controls should be run on the same blot or gel as the samples
- A figure panel should not include composite images of bands originating from different blots or gels. If the figure shows non-adjacent bands from the same blot or gel, this should be clearly denoted by vertical black lines and the figure legend should provide details of how the figure was made

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species
- The commercial supplier or source laboratory
- The catalogue or clone number and, if known, the batch number
- The antigen(s) used to raise the antibody
- For established antibodies, authors are encouraged to supply a stable public identifier from the Antibody Registry (www.antibodyregistry.org).

Authors should also report the following experimental details:

- The final antibody concentration or dilution
- A reference to the validation study if the antibody was previously validated, and if not, details of how the authors validated the antibody for the applications and species used. Authors should consider adding information on new validations to a publicly available database such as Antibodypediaor CiteAb.

Systematic Review/Meta-Analysis

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document
  Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as Supporting Information and provide the registry number in the abstract.

If your article is a Systematic Review or a Meta-Analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flowchart as Fig. 1 (required where applicable)
- Include the PRISMA checklist as Supporting Information

Meta-Analysis of Genetic Association Studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in “Systematic Reviews of Genetic Association Studies” by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Paleontology and Archaeology Research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.
See the *PLOS ONE* Editorial Policies for more information regarding manuscripts describing paleontology and archaeology research.

Software Papers

Manuscripts describing software should provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

See the *PLOS ONE* Editorial Policies for more information about submitting manuscripts.

Database Papers

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

See the *PLOS ONE* Editorial Policies for more information about submitting manuscripts describing databases.

New Zoological Taxon

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*
You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "http://zoobank.org/". The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic

edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS [author to insert any additional repositories].
All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

New Botanical Taxon

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). In association with the International Plant Names Index (IPNI), the following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a *PLOS ONE* article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> **Solanum aspersum** S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

PLOS ONE staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording:

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].
All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

New Fungal Taxon

When publishing papers that describe a new fungal taxon name, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific fungal name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, "the description or diagnosis required for valid publication of the name of a new taxon" can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of a *PLOS ONE* article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Hymenogaster huthii. Stielow et al. 2010, sp. nov.
[urn:lsid:indexfungorum.org:names:518624]
You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called "Nomenclature" using the following wording (this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum):

The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the

electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (PubMed Central, LOCKSS etc)].
All *PLOS ONE* articles are deposited in PubMed Central and LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative Research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

Hippocampal neurodegeneration in kainate-treated rats with and without recurrent motor seizures

J. Quesado, G. Maia, N.Lukoyanov

Department of Anatomy, Faculty of Medicine, University of Porto, Portugal.

Temporal lobe epilepsy (TLE) is often associated with hippocampal sclerosis (cell loss) and other structural changes in the medial temporal lobe region. Generalized motor seizures are present in severe cases of TLE. As TLE is progressive this seizures can become intractable. It has also been repeatedly reported that TLE patients with hippocampal sclerosis may show learning and memory deficits, increased anxiety, depression and mood disorders. Thus, it seems reasonable that these behavioral changes are secondary to the TLE-related hippocampal neurodegeneration.

Animal models of TLE replicate the majority of the neuropathological aspects of TLE. Kainic acid (KA) is a specific agonist for the ionotropic glutamate receptor. When injected in rats, KA induces acute *status epilepticus* (SE). However, a large percentage of the injected animals don't develop RMS and are usually excluded from further analyses. The purpose of this study was to directly compare the neuroanatomical outcomes in KA-treated rats that developed RMS to those that did not. To address this issue, absolute numbers of neurons in the dentate gyrus hilus and pyramidal layers of the hippocampus proper were estimated using stereological approaches in order to compare the hippocampal cell loss among the two groups.

Male Wistar rats were injected with 5 mg/kg of KA until the onset of SE (n=28). Rats in the control group were injected with saline (n = 8). Across the 5-month recovery period the behavior of rats was videorecorded. If a rat showed two or more spontaneous motor seizures of stage 4 or 5 on the Racine scale, it was assigned to the RMS group. Remaining animals were assigned to the no-RMS group. From the 28 rats that were treated with KA, 8 rats have died and 9 rats developed spontaneous motor seizures (assigned to RMS group). No behavioral seizures were detected in the 11 remaining KA-treated rats (assigned to no-RMS group). After perfusion of the animals, their brains were sectioned in the coronal plane with 40 μm. The sections were stained with Giemsa and used for histological analysis.

The total number of neurons was estimated applying the optical fractionator method. The boundaries of the hilus of the dentate gyrus and the CA3 and CA1 hippocampal fields were consistently defined at all levels along the rostrocaudal axis of the brain based on the cell morphology and cytoarchitectonic criteria. Estimations were carried out using the C.A.S.T.-Grid System (Olympus, Denmark).

It was found that the total number of neurons in the hilus of the dentate gyrus and in the CA3 and CA1 fields of the hippocampus proper were significantly different in rats with or without seizures. Post-hoc comparisons showed that in animals from the RMS group there was a significant neuron loss in the dentate hilus and hippocampal CA3 and CA1 fields. In sharp contrast, in rats from the no-RMS group there was a significant neuron loss only in the dentate hilus, but not in the hippocampal pyramidal fields.

This work was supported by FEDER Funds through the Programa Operacional Factores de Competitividade – COMPETE and National Funds through FCT - Fundação para a Ciência e a Tecnologia within the scope of the Project FCOMP-01-0124-FEDER-015919 (PTDC/SAU-NSC/115506/2009), Strategic Project Centro de Morfologia Experimental (CME/FM/UP) - 2011-2012, Project PEst-OE/SAU/UI0121/2011.

U.PORTO

Certifica-se que **José Luís Quesado** esteve presente no IJUP'13 – 6º *Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 13, 14 e 15 de fevereiro de 2013, na Reitoria da Universidade do Porto, tendo apresentado a comunicação oral "**Hippocampal neurodegeneration in kainate-treated rats with and without recurrent motor seizures**".

Pela Comissão Organizadora

(O Vice-Reitor, Prof. Doutor Jorge Gonçalves)