Exp Brain Res (2004) 154: 192–200
DOI 10.1007/s00221-003-1658-3

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Selective loss of hilar neurons and impairment of initial learning in rats after repeated administration of electroconvulsive shock seizures

Received: 11 April 2003 / Accepted: 30 July 2003 / Published online: 14 October 2003

Abstract Prolonged seizures induced by neurotoxins or intracranial electrical stimulation provoke death of hippocampal neurons, which results in conspicuous learning and memory deficits. We examined whether repeated brief seizures elicited by electroconvulsive shock (ECS) can also deteriorate hippocampal structure and function. Adult Wistar rats were administered six ECS seizures, the first five of which were 24 h apart, whilst the last two were spaced by a 2-h interval. Following a 2-month recovery period, the cognitive status of the animals was assessed using the water maze task. ECS-treated animals were incapable of learning the constant platform position version of this task during the first 4 days of training, but performed similarly to control rats throughout the rest of the acquisition period, on the probe trial, and on the variable platform position and visible platform tasks. The results of the morphological analysis showed that the total number of hippocampal pyramidal neurons and dentate gyrus granule cells were similar in control and ECS-treated rats. However, ECS treatment caused loss of approximately 17% of cells in the hilus of the dentate gyrus, which was accompanied by significant mossy fiber sprouting into the dentate inner molecular layer. In addition, we found that the ECS-induced decrease in the total number of hilar cells was not due to loss of inhibitory interneurons immunoreactive to somatostatin. These findings support the view that ECS-induced seizures can produce a number of morphological and functional changes in the rat hippocampal formation, which qualitatively resemble those previously described in other seizure models.

Keywords Electroshock seizures · Epilepsy · Neuronal loss · Synaptic reorganization · Dentate hilus · Spatial learning and memory · Stereology · Morris water maze

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Introduction

Temporal lobe epilepsy is often associated with loss of neurons in the hippocampal formation, thus leading to progressive impairments of learning and memory (Hauser 1983; Armstrong 1993). Although the etiology of epilepsy-related changes in the hippocampal formation remains elusive, it is commonly believed that seizure activity by itself can trigger damage to hippocampal neurons (Salmenperä et al. 1998; McNamara 1999). This issue has been thoroughly explored in animal studies using various seizure models. For example, it has been reported that epileptic activity induced by either neurotoxic agents, such as kainic acid and pilocarpine, or intracranial electrical stimulation provokes death of hippocampal neurons, which results in conspicuous memory deficits (Ben-Ari et al. 1980; Turski et al. 1985; Lopes da Silva et al. 1986; Sloviter 1987; Ylinen et al. 1991; Sperk 1994; Sutula et al. 1994; Kelsey et al. 2000; Hannesson et al. 2001). However, it has also been demonstrated that the extent of hippocampal damage induced by seizures is critically dependent on their duration and frequency, and that significant cell loss occurs only after prolonged (tens or hundreds of minutes), but not brief, temporally spaced seizures (Sloviter 1987; Bertram and Lothman 1993; Lemos and Cavalheiro 1995; Hsieh 1999).

Electroconvulsive shock (ECS) is widely used in animal studies as a model of brief generalized seizures (Fisher 1989). Recent evidence indicates that repeated ECS seizures cause a number of subtle morphological alterations in the hippocampal formation of adult rats, such as enhanced neurogenesis of dentate gyrus granule cells

(Scott et al. 2000) and aberrant sprouting of their axons, the mossy fibers (Gombos et al. 1999; Vaidya et al. 1999), but no evident cell loss (Devanand et al. 1994; Vaidya et al. 1999). The finding that, unlike other seizure models, ECS does not cause noticeable death of hippocampal neurons is not surprising taking into consideration that ECS seizures last for only a few seconds and, once induced repetitively, are typically spaced by either 24-h or 48-h intervals (Rowley et al. 1997; Gombos et al. 1999; Vaidya et al. 1999). It is, however, possible that the loss of a small number of hippocampal neurons might have been overlooked in prior studies in which the neuroanatomical sequelae of ECS seizures were assessed solely from the qualitative point of view. In the present study we have re-examined the neuropathological consequences of repeatedly elicited ECS seizures using quantitative stereological methods. Moreover, we stimulated rats in accordance with a modified protocol such that the animals were administered six ECS, the first five of which were spaced by 24-h intervals, whilst the last two were only 2 h apart. This protocol is based on the findings reported by Rowley et al. (1997), who showed that, following induction of five ECS seizures over a 10-day period, there is a transient, lasting a minimum of 2 h, post-ictal reduction in the capacity of the hippocampal amino acid reuptake system to maintain extracellular glutamate concentrations at normal level, a condition known to render hippocampal neurons highly vulnerable to excitotoxic damage (Meldrum 1993). In addition, we have also assessed spatial learning abilities of the animals, which are known to be dependent on the structural integrity of hippocampal circuits.

Materials and methods

Animals and treatment

Thirty-two male Wistar rats obtained from the Gulbenkian Institute of Science (Oeiras, Portugal) were used in the present study. Animals were housed two to three per cage, maintained under standard laboratory conditions (20–22°C and a 12:12 h light-dark cycle), and had free access to food and water. At 2 months of age, 16 rats were selected at random and received a course of five ECS seizures, administered on a 24-h schedule. However, 2 h after the fifth stimulation, each of the animals received one additional ECS seizure. ECS was administered as previously described by Rowley et al. (1997) via ear-clip electrodes wired to a stimulus generator (model 215/IZ, Hugo-Sachs Elektronik, Germany). Each stimulation (constant voltage of 200 V for 2 s) produced full tonic-clonic seizure with hind-limb extension lasting for 5–10 s. The remaining 16 rats (control group) received handling identical to that of experimental rats, including attachment of ear-clip electrodes for 1 min, but were not stimulated. After the end of treatment, animals were allowed to recover for 2 months. During the entire period of recovery, ECS-treated rats were observed in their home cages for 4 h/day and no signs of spontaneous behavioral seizures were detected. During the last 5 days of the recovery period, all rats were handled for 5 min/day. At 4 months of age, 12 animals from each group were selected at random and assigned to behavioral testing. All the rats were killed at approximately 5 months of age.

The handling and care of the animals were conducted according to the European Communities Council guidelines in animal research (86/609/UE) and Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985).

Behavioral tests

The behavioral consequences of repeated treatment with ECS were evaluated using the water maze paradigm. 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, i.e., three posters of different size and shape, and a computer desk. The apparatus was filled with water at room temperature (21±1°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. The swim path was recorded by a computerized video-tracking system (EthoVision V1.90, Noldus, The Netherlands).

In order to familiarize the animals with the general task requirements and to minimize the potentially confounding influence of non-associative factors such as reactivity to stress, each rat received two sessions of shaping trials in which no spatial learning was required. In this procedure, a black acrylic alley, measuring 130×65×15 cm, was placed on the bottom of the maze so that its walls extended 30 cm above the surface of the water. A black escape platform, 10 cm in diameter, was placed inside the alley, approximately 25 cm from one of its ends. It was located 2 cm below the surface of the water. Each rat was placed in the water and allowed to swim to the platform and to climb on it. If the rats did not find the escape platform within 20 s, the experimenter guided them to the platform. Each session consisted of three trials, spaced by 15-s intertrial intervals, in which the rats were released in the water from three different locations, i.e., 30, 60 and 90 cm from the position of the platform. The interval between the two shaping sessions was 24 h. No extramaze cues were present during this phase of training.

In the classical Morris reference task (Morris 1984), the animals were trained to find the submerged escape platform, which was now located in the center of one of the quadrants. For acquisition, rats were given two trials on each day for 10 consecutive days. 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. 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. After the first daily trial, the animals were placed in a clean cage, and a 30-s interval was imposed before the beginning of the next trial. The platform location was not changed during the acquisition period. The swim path length was calculated. 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 in the training quadrant were recorded.

Three days after the probe trial, the rats were tested on the variable platform position task. This procedure was similar to the reference task except that the position of the platform in the maze was different for each session, which consisted of two consecutive trials. The duration of each trial and intertrial interval remained the same as in the reference task. During the first 2 days of the experiment, the rats were given two sessions per day separated by a 3-h intersession interval (pre-training stage). This procedure trained the rats to search for a novel position of the platform on each session. Following pre-training, the rats were tested on the variable platform position task for 5 successive days using the same procedure, except that only one session was given per day. The distances swum to locate the platform were recorded and the percentage of savings from the first to the second trial was calculated.

Performance of animals on the visible platform task was assessed 1 day after the end of the testing on the variable platform position task. In this task, the rats were given one block of four trials with 30-s intertrial 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.

All the experiments were performed after at least 30-min habituation of animals to the testing room. Testing was done at the same time of day, beginning at 14.00 h.

Tissue preparation

Five animals from each group were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.12 M PB (pH 7.4) followed by a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in PB. The brains were removed from the skulls, codified to allow blind estimations, and placed in fresh fixative for 30 days. The brains were then separated by a midsagittal cut into right and left hemispheres. The blocks of tissue containing the hippocampal formations, alternately sampled from the left and right hemispheres, were dehydrated through a graded series of ethanol solutions and embedded in glycolmethacrylate, as described in detail elsewhere (West et al. 1991). These blocks were serially sectioned in the horizontal plane at a nominal thickness of 40 μm using a microtome. Every tenth section was collected using a systematic random sampling procedure (Gundersen and Jensen 1987), mounted serially on gelatin-coated slides, stained with a modified Giemsa solution (West et al. 1991), and coverslipped with Histomount. The glycolmethacrylate-embedded material was used to estimate the total number of hippocampal neurons and the volumes of main hippocampal layers.

Other two groups of five control and five ECS-treated animals were anesthetized 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 with PB, followed by a fixative solution containing 4% paraformaldehyde in PB. Brains were removed immediately after the perfusion, codified to allow blind estimations, post-fixed for 2 h at 4°C, and infiltrated overnight at 4°C in 10% sucrose solution. After removal of the frontal and occipital poles, the brains were cut at a vibratome into 50-μm coronal sections. Every fifth section was collected as free-floating, washed twice with PBS (pH 7.4), treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase, and incubated overnight at 4°C in primary antibody against somatostatin (SS) diluted 1:10,000 in 0.5% Triton X-100 solution in PBS (PBS/T). The primary antibody was obtained as a gift from Dr. Ruud Buijs (The Netherlands Institute for Brain Research, Amsterdam). After being rinsed in PBS/T for 30 min, the sections were incubated for 1 h in biotinylated goat anti-rabbit antibody (1:400, Vector Laboratories, Burlingame, CA, USA) diluted in PBS/T. After more rinses in PBS/T, the sections were treated with avidin-biotin peroxidase complex (1:800, Vector Laboratories) for 1 h, and rinsed again in PBS/T for 30 min. The tissue sections were then incubated in chromogen solution consisting of 0.02% diaminobenzidine and 0.01% H_2O_2 in PBS. Finally, the sections were rinsed in PBS, mounted, air dried, dehydrated, and coverslipped. These sections were used to estimate the total number of hilar neurons immunoreactive to SS.

Remaining animals, six from each group, were used for histochemical staining with the Timm sulfide silver method (Danscher and Zimmer 1978). They were anesthetized as described above and perfused with a buffered sodium sulfide solution, followed by 3% glutaraldehyde in 0.15 M Sörensen phosphate buffer at pH 7.4. The brains were removed from the skulls and codified to allow blind estimations. Then, the hippocampal formations were isolated and infiltrated overnight in 30% sucrose solution. After being frozen with gaseous carbon dioxide, the hippocampal formations, alternately sampled from the left and right hemispheres, were sectioned in the horizontal plane at a nominal thickness of 50 μm. Every fifth section was collected, mounted, subjected to physical development for 80 min, and coverslipped.

Estimation of neuron numbers

The total number of neurons in the different regions of the hippocampal formation was estimated using the optical fractionator method (West et al. 1991). The granular layer and hilus of the dentate gyrus, and the pyramidal cell layers of the CA1 and CA3 hippocampal fields were consistently defined at all levels along the septotemporal axis of the hippocampal formation on the basis of cell morphology. Neurons belonging to the CA2 hippocampal field were included in the CA3 region. Neuron counting was carried out using the Olympus CAST Grid System (Denmark). Beginning at a random starting position, visual fields were systematically sampled along the x and y axes, using a raster pattern procedure (for details, see Table 1). Neurons were counted in every frame using the optical disector at a final magnification, at the level of the monitor, of 2,000×. Glial cells, identified according to the criteria described by Ling et al. (1973), were not included in the estimations. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. (1999). The same procedure was used to estimate the total number of hilar neurons immunoreactive to SS.

Table 1 Summary of the stereological parameters used for the estimation of neuronal numbers and volumes of corresponding hippocampal layers [*a (frame)* area of the counting frame, *a (point)* area per point in grid used for volume estimates, *asf* area sampling fraction, *CE (N)* and *CE (P)* mean coefficients of error (for neuronal numbers and volumes of layers, respectively), *h* height of the optical disector, *ssf* section sampling fraction, *tsf* thickness sampling fraction, *x-step* and *y-step* predetermined distances used along the *x* and *y*-axes of the section to sample, $\sum P$ number of points on each neuronal layer; $\sum Q^-$ total number of neurons counted in each neuronal layer]

	Granular layer	CA3 field	CA1 field	Hilus (Giemsa)	Hilus (somatostatin)
No. sections	14	14	14	14	18
ssf	0.1	0.1	0.1	0.1	0.2
x-step (μm)	200	200	200	180	200
y-step (μm)	200	200	200	180	200
a (frame) (μm^2)	197	495	495	2,675	3,000
asf	0.00493	0.0123	0.0123	0.0825	0.075
h (μm)	10	15	15	15	10
tsf	0.3	0.46	0.46	0.46	0.64
$\sum Q^-$	168	190	204	148	170
CE (*N*)	0.08	0.08	0.10	0.09	0.09
a (point) (mm^2)	0.0155	0.0154	0.0241	0.0241	-
$\sum P$	320.5	271.1	194.0	172.3	-
CE (*P*)	0.09	0.08	0.07	0.08	-

The stereological parameters used for the estimations of cell numbers are summarized in Table 1.

Estimation of volumes

The volumes of the different neuronal layers of the hippocampal formation were estimated by using the principle of Cavalieri (Gundersen et al. 1988; Regeur and Pakkenberg 1989). All the sampled glycolmethacrylate-embedded sections containing the hippocampal formation were used. In each section the cross-sectional area of the neuronal layers was estimated by point counting (Gundersen and Jensen 1987), at a final magnification of 80×, using an adequate grid of test points (CAST Grid, Olympus, Denmark). The volumes of the neuronal layers were calculated from the total number of points that fell on each layer and the distances between the sections. The parameters used for volume estimations are shown in Table 1.

Estimation of mossy fiber sprouting

Mossy fiber synaptic reorganization was assessed by rating the distribution of Timm granules within the supragranular zone of the dentate gyrus. The density of the granules was rated semiquantitatively on a scale of 0 to 4 based upon a previously validated scoring method (Cavazos et al. 1991): 0, no or only occasional granules; 1, sparse granules in a patchy distribution; 2, more numerous granules in a continuous distribution; 3, prominent granules in a continuous distribution with occasional patches of confluent granules; 4, prominent granules that form a confluent dense laminar band. For each rat, nine systematically sampled sections were analyzed by the reviewer, who was blind to treatment group. The nine scores were averaged to yield a mean Timm score for each animal.

Statistical analysis

Distances swum to find the platform derived from the acquisition trials of the water maze experiment, averaged over two daily trials each, and from the variable platform position task trials, averaged across five testing days, were analyzed using repeated measures ANOVA followed by Newman-Keuls post hoc test where appropriate. Timm staining scores were compared using non-parametric Mann-Whitney *U*-test. The remaining data were analyzed using Student's *t*-test. All behavioral data are presented as the mean ± SE, while morphological results are expressed as the mean ± SD. Differences were considered as significant at the $P<0.05$ level.

Results

Water maze

The mean distances swum to locate the hidden platform in the classical Morris reference task are shown in Fig. 1A. The overall repeated measures ANOVA showed that the rats in both groups progressively improved their ability to find the platform over the 10 days of acquisition ($F_{(9,198)}=64.45$, $P<0.0001$). However, although there was no significant effect of treatment, the animals in the two groups learned the task at different rates, as indicated by significant group × day interaction ($F_{(9,198)}=2.49$, $P<0.01$). Post hoc comparisons for this interaction revealed that control animals learned the task significantly faster than the animals in the ECS group at the beginning of the acquisition ($P<0.01$ for the third and fourth days of training), but performed similarly to them throughout the rest of the acquisition period. The two measures of retention derived from the probe trial, i.e., the platform crossings and the percentage of time spent swimming in the training quadrant, did not significantly differ between control and ECS-treated groups (Fig. 1B).

On the variable platform position task, ECS-treated rats performed similarly to control animals, consistently swimming shorter distances on the second trial ($F_{(1,22)}=44.62$, $P<0.0001$; Table 2). Although there was a slight difference between groups in the distances swum to find the platform on both trials, the difference was not significant ($F_{(1,22)}=3.01$, $P=0.09$). Likewise, ANOVA did not reveal a significant treatment × trial interaction nor a significant effect of treatment on the percentage of savings between the first and the second trials, indicating unimpaired performance of ECS-treated rats on this task.

The rats in both groups quickly learned to find the visible platform. The distances swum to locate the visible platform position, averaged across four testing trials, were 233±89 cm for controls and 211±124 cm for ECS-treated rats. No significant effect of treatment on this measure was

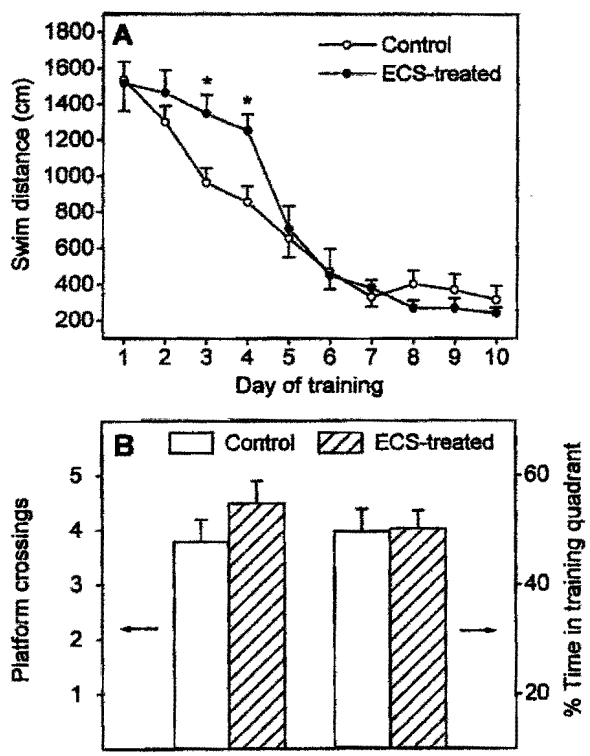

Fig. 1A, B The swim distance in centimeters to reach a hidden platform for each training day throughout the acquisition of the water maze task (**A**). Note that the animals that were treated with ECS were slower than controls to learn the task at the beginning of training but showed equivalent asymptote performance. *$P<0.01$ vs. control group. The number of times the rats crossed the former platform position (platform crossings) and the percentage of time spent in the training quadrant during the probe trial (**B**). No significant differences between ECS-treated and control groups were detected in this test

Table 2 The swim distance in centimeters to locate a submerged platform and the percentage of savings derived from the variable platform position trials. Values represent mean ± SE. No significant effect of treatment on the performance of the rats in this task was detected

	First trial	Second trial	% Savings
Control	1,136±62	655±106	47.1±7
ECS-treated	1,333±101	840±92	44±5

revealed, showing that rats in either group had comparable sensorimotor abilities.

Morphometric analysis

The qualitative observation of the Giemsa-stained material obtained from ECS-treated and control rats revealed no treatment-related cytological abnormalities in the granule cell layer of the dentate gyrus as well as in the pyramidal cell layers of the CA3 and CA1 hippocampal fields. However, the density of cells in the hilus of the dentate gyrus appeared to be smaller in ECS-treated rats than in controls (Fig. 2). The estimates of the total number of neurons in the subdivisions of the hippocampal formation and of the volumes of these subdivisions are shown in Table 3. Consistent with the qualitative observations, these data show that neither the volumes of the principal hippocampal layers nor the number of the dentate granule cells and hippocampal pyramidal neurons differed significantly between the groups. In contrast, the total number of hilar cells was significantly reduced in ECS-treated rats relative to control rats ($P<0.01$). This reduction was not due to a loss of somatostatinergic interneurons because the total number of hilar cells immunoreactive to this neuropeptide remained unchanged after repeated treatment with ECS (Table 3).

The distribution pattern of the mossy fibers and their terminals within the dentate gyrus of control and ECS-treated rats, as visualized by Timm staining, is shown in Fig. 3. As expected, the vast majority of these fibers,

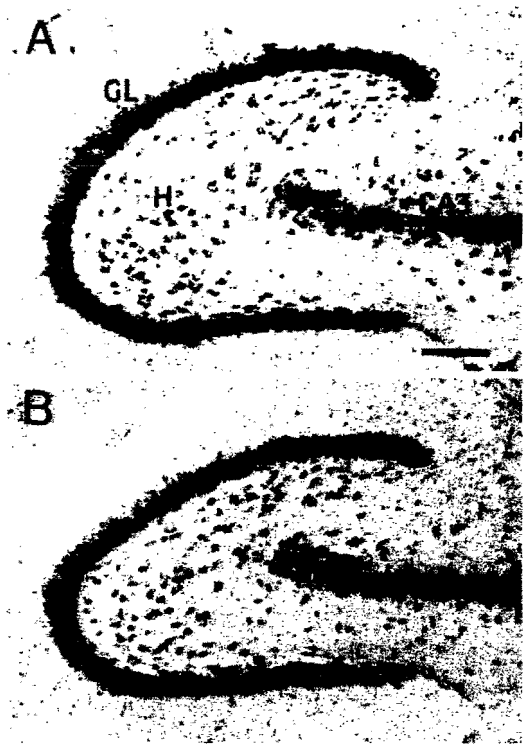

Fig. 2 Photomicrographs of Giemsa-stained horizontal sections of the hippocampal formations, cut at the midseptotemporal level, of a control rat (**A**) and of a rat that received a course of ECS treatment (**B**). The density of cells in the hilus of the dentate gyrus appears to be somewhat lower in the ECS rat than in the control rat (*GL* granule cell layer of the dentate gyrus, *H* hilus, *CA3* pyramidal cell layer of the CA3 hippocampal field). *Scale bar* 200 μm

known to be the axons of the granule cells, were localized within the hilus where they formed a densely packed plexus. In both groups, some Timm granules were also observed within the supragranular layer of the dentate gyrus. However, the density of these granules appeared to be higher in ECS rats than in control rats, suggesting that repeated treatment with ECS resulted in enhanced sprouting of recurrent mossy fibers into the inner molec-

Table 3 Number of hippocampal neurons and volumes of corresponding hippocampal layers found in control and ECS-treated rats. Values represent mean ± SD. $n=5$ in each group

	Control	ECS-treated
Granule cell layer		
Total number of cells	1,154,630±68,361	1,080,209±153,566
Volume (mm^3)	1.612±0.282	1.665±0.156
CA3 pyramidal field		
Total number of cells	225,769±13,354	213,645±17,305
Volume (mm^3)	2.151±0.177	2.065±0.155
CA1 pyramidal field		
Total number of cells	352,540±26,009	348,832±22,171
Volume (mm^3)	1.345±0.077	1.327±0.068
Hilus		
Total number of cells	43,872±5,229	36,488±1,386*
Number of SS-positive cells	18,567±2,858	18,859±1,193
Volume (mm^3)	1.446±0.112	1.263±0.194

*$P<0.01$

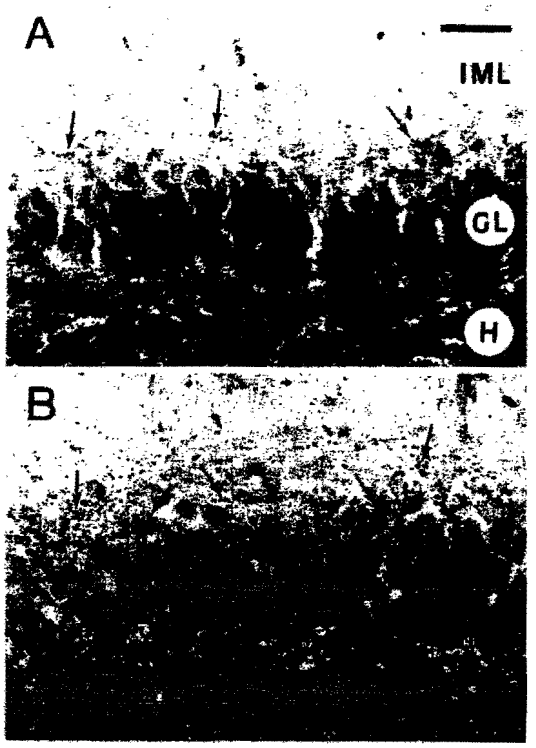

Fig. 3 Representative Timm-stained sections of the dentate gyrus from control (**A**) and ECS-treated (**B**) rats. The *arrows* point toward Timm granules in the supragranular layer (*H* hilus, *GL* granule cell layer, *IML* inner molecular layer). *Scale bar* 40 μm

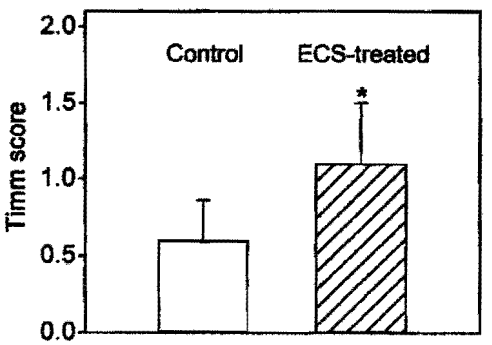

Fig. 4 The effect of repeated ECS seizures on the presence of Timm granules in the supragranular layer of the dentate gyrus. Note that ECS-treated animals exhibited a significant increase in the density of Timm granules relative to the animals from the control group. n=6 in each group. *P<0.05 vs. control

ular layer of the dentate gyrus. This observation was confirmed by the results of the semiquantitative analysis, which showed that the density of Timm granules in the supragranular layer was approximately two times higher in ECS-treated rats than in control rats (P<0.05; Fig. 4).

Discussion

We found that the administration of six consecutive ECS seizures, the first five of which were separated from each other by 24-h intervals whereas the last 2 were 2 h apart, causes loss of approximately 17% of cells in the hilus of the dentate gyrus. Interestingly, we found no treatment-related changes in the total number of neurons in other regions of the hippocampal formation, namely in the CA3 and CA1 pyramidal layers, as well as in the dentate gyrus granular layer, suggesting that the hilar cells are selectively vulnerable to death in response to ECS-induced seizures. Such a selective and relatively moderate injury may, however, contribute to the deterioration of hippocampal function, as indicated by the impaired spatial learning scores shown by ECS-treated rats on the water maze test.

Previous studies in rodents showed that prolonged seizure activity causes death of hippocampal neurons, which, in turn, affects various hippocampal functions, including processing of spatial information (Lopes da Silva et al. 1986; Ylinen et al. 1991; Gilbert et al. 1996; Kelsey et al. 2000; Hannesson et al. 2001). With respect to the morphological changes induced by seizures, a marked cell loss in the CA3 and CA1 hippocampal pyramidal layers, in the hilus, and in the granular layer of the dentate gyrus was found in both kainic acid and pilocarpine models of status epilepticus (Ben-Ari et al. 1980; Turski et al. 1985; Bouilleret et al. 2000). Studies employing continuous stimulation models of status epilepticus yielded similar results and additionally demonstrated that, among hippocampal neuronal populations, mossy cells and inhibitory interneurons located in the hilus of the dentate gyrus are particularly vulnerable to seizure-induced death (Sloviter 1987; Freund et al. 1992; Schwarzer et al. 1996; Hsieh 1999; Sloviter et al. 2003). Recently, it has been reported that at least a small subset of dentate granule cells is also highly vulnerable to seizure-induced hyperexcitation (Sloviter et al. 1996; Bengzon et al. 1997; Zhang et al. 1998). In the present study, using a noninvasive type of stimulation, i.e., not requiring administration of neurotoxic agents or intracerebral electrode implantation, we found that repeated induction of brief generalized seizures also causes a significant cell loss in the hilus of the dentate gyrus. Furthermore, our results do not preclude the possibility that some granule cells were also injured by ECS seizures. Indeed, it is theoretically possible that the total number of neurons located in the dentate granular layer remained unchanged after ECS treatment because the granule cells that might have been lost were replaced by newly formed neurons as a result of neurogenesis that is known to be enhanced by ECS seizures (Scott et al. 2000). While this hypothesis remains to be examined in future experiments, the present findings provide considerable support to the notion that hippocampal degeneration can be triggered by seizures and that loss of hilar neurons appears to be one of the initial morphological events in this process.

Neuroanatomical sequelae following brief seizures were previously assessed in studies utilizing the kindling model

of epilepsy, in which seizure activity is triggered by a repeated subthreshold stimulation of different temporal lobe structures, such as the hippocampus proper, perforant path or amygdala (Binder and McNamara 1998). For example, it has been reported that the cell density in the rat dentate hilus is significantly reduced after three to five stage V kindling seizures (Cavazos and Sutula 1990; Cavazos et al. 1994). However, it has also been demonstrated that, depending on experimental conditions, the decreased hilar cell density induced by intermittent kindling seizures is not necessarily related to actual loss of neurons, but, instead, may be accounted for by a proportional increase in hilar area (Bertram and Lothman 1993; Adams et al. 1997; Tuunanen and Pitkänen 2000). Notwithstanding the distinct patterns of neuronal activation induced by kindling and ECS seizures, the likelihood that the loss of hilar neurons detected in ECS-treated rats in the present study is due to an enlargement of the hilus is very low, because we assessed the total number of hilar neurons rather than their density, and, furthermore, because we found that repeated induction of ECS seizures does not affect hilar volume.

The present findings are in contrast with the results of a number of prior studies in rats, in which no gross anatomical changes within the hippocampal formation were found following repeated administration of ECS (Devanand et al. 1994; Gombos et al. 1999). Notably, Vaidya et al. (1999), using a sensitive silver staining technique, failed to detect degenerating neurons in the hilus of the dentate gyrus of rats treated with ECS once daily for 10 consecutive days. However, unlike our study, the previous studies were carried out in rats treated with ECS according to the sparse stimulation protocol and using qualitative methods of morphological assessment, which, as already mentioned, can contribute to this discrepancy. On the other hand, our observation that repeated induction of ECS seizures is associated with enhanced sprouting of recurrent mossy fibers into the inner molecular layer of the dentate gyrus is in agreement, both qualitatively and quantitatively, with the previous reports from other laboratories (Gombos et al. 1999; Vaidya et al. 1999). Interestingly, close scrutiny of data on the seizure-related brain damage suggests that the enhanced sprouting of the mossy fibers into the inner molecular layer can result from the loss of hilar mossy cells (Tauck and Nadler 1985; Cavazos and Sutula 1990; Sloviter 1991; Buckmaster and Dudek 1997), whose axons normally terminate in this area (Ribak et al. 1985; Amaral and Witter 1989; Frotscher et al 1991). This intriguing hypothesis is, however, difficult to test directly because, to the best of our knowledge, there are no specific markers for mossy cells (He et al. 1998; Sloviter et al. 2001). In this study, we therefore examined the effect of ECS seizures on the total number of hilar cells immunoreactive to SS, the most numerous subpopulation of hilar inhibitory interneurons (Houser and Esclapez 1996; Buckmaster and Jongen-Rêlo 1999), which, along with mossy cells, also possess high vulnerability to prolonged seizures (Buckmaster and Jongen-Rêlo 1999; Sloviter 1987; Sloviter et al. 2003). Consistent with the data previously reported by Dalby et al. (1996), we found that repeated administration of ECS seizures does not lead to a loss of SS-positive cells in the hilus. Thus, because mossy cells constitute approximately 64% of the total number of hilar neurons (Buckmaster and Jongen-Rêlo 1999) and because the majority of the remaining vulnerable cells, i.e., somatostatinergic inhibitory interneurons, apparently survived the administration of ECS seizures, it is likely that the decrease in the total number of hilar cells found in ECS-treated rats is, at least in part, due to the loss of mossy cells. Taken together, these data indicate that ECS-induced morphological changes in the rat hippocampal formation, namely sprouting of recurrent mossy fiber collaterals and death of neurons in the dentate hilus, presumably mossy cells, qualitatively resemble those previously described in other seizure models.

Impairments of spatial learning and memory are perhaps the best described behavioral deficits associated with hippocampal lesions (Olton 1983; Morris 1984). It has been shown consistently that the loss of hippocampal neurons caused by prolonged seizures results in robust and long-lasting impairments of this form of cognition (Lopes da Silva et al. 1986; Sutula et al. 1995; Gilbert et al. 1996; Kelsey et al. 2000; Hannesson et al. 2001). The results of the present study show for the first time that repeated elicitation of brief generalized ECS seizures can also lead to enduring, probably permanent, deficits in spatial learning, as indicated by the impaired performance of ECS-treated rats on the spatial version of the Morris water maze task despite the fact that behavioral testing began 2 months after the last stimulation. However, inspection of the behavioral data additionally shows that rats that received ECS treatment were incapable of learning the task as efficiently as controls only during the first 4 days of training. On day 5, there was an abrupt improvement in their learning scores and, since then, animals in experimental and control groups acquired the task at identical rates. Furthermore, performance of ECS-treated rats did not differ from that of control rats during the probe trial as well as on the variable platform position task, suggesting that, by the end of the training, animals in either group acquired an approximately equal amount of spatial information. The behavioral deficits detected in ECS-treated rats may plausibly be related to the degenerative changes caused by ECS seizures in the hippocampal formation, namely to the loss of hilar neurons. Indeed, although the precise function of this hippocampal region is still poorly understood, several lines of evidence indicate 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 CA3 pyramidal field (Bragin et al. 1995; Paulsen and Moser 1998; Deller et al. 1999; Vogt and Nicoll 1999; Vida and Frotscher 2000). Moreover, it has been suggested that the specific contribution of this neuronal circuitry to spatial learning is to compare the expected context with that currently experienced (Mizumori et al. 1999). In other

words, this model predicts that destabilization of the hilar/CA3 neuronal network can disrupt acquisition of spatial information during the initial stages of learning, i.e., when the difference between the expected and current contexts is greatest, whereas it can have little or no effect on the asymptote performance (Mizumori et al. 1999). The results of the present experiment are thus consistent with this view, because we found that even partial loss of hilar neurons impairs the ability of rats to form new spatial representations, but does not affect their performance in a familiar environment.

To our knowledge, this is the first study demonstrating that even a few generalized ECS seizures cause loss of approximately 17% of neurons in the hilus of the dentate gyrus. This relatively moderate injury was, however, sufficient to destabilize hippocampal neuronal circuits, as suggested by the impaired performance of ECS-treated rats during the initial phase of the water maze learning. The authors believe that these findings shed new light on the neurological consequences occurring at the earliest stages of seizure-induced brain damage.

Acknowledgements This work was supported by Fundação para a Ciência e a Tecnologia, Unit 121/94, Grant SFRH/BPD/1583/2000. The authors would like to thank Dr. J.P. Andrade and Dr. R.M. Mesquita for their assistance with the immunocytochemistry.

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