Research report

Seizure-induced structural and functional changes in the rat hippocampal formation: Comparison between brief seizures and status epilepticus

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ABSTRACT

Prolonged seizures produce death of hippocampal neurons, which is thought to initiate epileptogenesis and cause a disruption of hippocampally mediated behaviors. This study aimed to evaluate behavioral and neuroanatomical changes induced by brief seizures and to compare them with changes induced by prolonged seizures. Adult rats were administered 6 brief seizures, elicited by electroshock (ECS). Prolonged seizures (status epilepticus, SE) were induced by pilocarpine. Two months later, the rats' behavior was tested using the Morris water maze, passive avoidance and active avoidance tests. The number of neurons in the hippocampal formation was estimated using stereological methods. ECS seizures produced loss of neurons, ranging between 14% and 26%, in the dentate hilus, subiculum, presubiculum, parasubiculum, and entorhinal layers III and V/VI. However, the neuron loss caused by SE in the same structures, as well as in the hippocampal CA3 and CA1 fields, ranged between 34% and 50%. SE additionally killed many neurons in the dentate granular layer, postsubiculum and entorhinal layer II. ECS treatment caused mild impairments in spatial learning and passive avoidance, but it was not associated with spontaneous motor seizures. In contrast, SE produced a severe disruption of spatial learning, passive and active avoidance, and led to the development of spontaneous seizures. These data show that both prolonged seizure activity and brief seizures result in structural and functional alterations in the temporal lobe circuits, but those caused by prolonged seizures are considerably more severe. Hippocampal damage elicited by brief seizures does not necessarily lead to spontaneous motor seizures.

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1. Introduction

Growing evidence indicates that incident seizures can cause potentially epileptogenic brain damage and that uncontrolled seizure activity may be associated with extension of the initial lesion, thus rendering the course of the disease progressive and ultimately resulting in various behavioral impairments [1–5]. In particular, it is known that both febrile seizures [6–8] and status epilepticus (SE) caused, for example, by acute domoic acid intoxication [9,10] may lead to the development of temporal lobe epilepsy (TLE), one of the most widespread seizure disorders in humans. Furthermore, it is also well documented that recurrent seizures in TLE patients are associated with progressive atrophic changes in the cerebral cortex, which are particularly robust in the hippocampal and parahippocampal regions [11–13]. Consistent with these findings, studies in animals have shown that seizure activity elicited by either chemoconvulsants [14–18] or electrical stimulation [5,19,20] induces neuronal death and that the pattern of seizure-induced neurodegeneration in animals resembles, partly at least, hippocampal sclerosis observed in human TLE.

Despite the large number of studies demonstrating neuropathological changes in TLE patients and respective animal models, the effects of seizures on brain structure and function are not yet completely understood. In particular, it is not clear whether repeated seizures of short duration are capable of affecting hippocampal circuits to such an extent that it would lead to epileptogenesis and/or to significant cognitive deficits. Indeed, the vast majority of studies related to this issue have used models in which animals are subjected to prolonged seizures, associated with large lesions [15–17,21], whereas a few studies have focused on neuronal effects of brief seizures [5,22–31]. The present study, therefore, was specifically aimed at evaluating the effects of repeated brief seizures on the structural integrity of hippocampal circuits and hippocampal-dependent behaviors and at comparing them to the respective effects of prolonged seizures. To this end, we estimated, using stereological methods, the total neuronal numbers in the dentate gyrus, hippocampus proper, subicular complex and entorhinal cortex of rats which were given a course of 6 seizures elicited by electroshock (ECS, model of brief seizures), treated with pilocarpine to induce SE (model of prolonged seizures), or given sham-treatments (controls). The behavioral profile of all rats was assessed with the Morris

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water maze, passive avoidance and active avoidance tests. The occurrence of spontaneous behavioral seizures was also monitored in all groups.

2. Material and methods

2.1. Animals and treatments

Male Wistar rats, maintained under standard laboratory conditions, were used in this study. At 10 weeks of age, they were randomly divided into four groups. In the ECS group ($n = 10$), rats received a course of 5 ECS seizures, administered on a 24-h schedule. Each stimulus (50 Hz, 60 mA for 1 s) was delivered via ear-clip electrodes wired to a stimulus generator (Model 215/IZ, Hugo-Sachs Elektronik, Germany). Two hours after the fifth stimulation, each of the animals received one additional ECS seizure. ECS produced full tonic–clonic seizures with hindlimb extension lasting 5–10 s. In the SE group ($n = 10$), rats were pretreated with scopolamine methyl bromide (1 mg/kg, s.c.; Sigma) to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high dose of pilocarpine (350 mg/kg, i.p.; Sigma). The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4 or 5 seizures on the Racine scale [32], i.e. rearing, falling and generalized convulsions. SE onset was detected usually 30–60 min following the pilocarpine injection. It has been previously reported that pilocarpine-induced SE, if lasting several hours, can be associated with high mortality rates ranging between 15 and 50% depending on the dose of pilocarpine and other experimental conditions [33]. Therefore, because animal mortality is a prominent cause of bias in quantitative evaluations of neuronal loss [34], special efforts were made to improve the survival rate of the animals in SE group. In particular, 2 h after the beginning of SE, the rats were injected with diazepam (2.5 mg/kg, i.p.) in order to cease the convulsive manifestations of SE. However, seizure activity, albeit considerably reduced in severity, was not completely stopped by the single dose of diazepam. Thus, an additional dose of diazepam (2.5 mg/kg) was given to the rats 30 min after the first injection. The animals were periodically injected with saline (s.c.) during the first 24 h of the recovery period in order to improve their survival rate. Five rats in the sham-ECS group received handling identical to that of ECS-treated rats, but were not stimulated. The other five rats (sham-SE group) received handling and treatment identical to that received by rats in the SE group, including injections of scopolamine and diazepam, but were not treated with pilocarpine. However, the animals from the latter two groups were pooled into a single control group ($n = 10$), because, in our previous studies [22,23,35], we have never found any behavioral or neuroanatomical differences between the sham-ECS groups and sham-SE groups.

Following the respective treatments, the rats were daily observed for spontaneous behavioral seizures at random times between 08:00 h and 20:00 h, during at least 2 h. 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. All efforts were made to minimize the number of animals used and their suffering.

2.2. Behavioral procedures

Behavioral testing began when animals were 4.5 months old. All behavioral assessments were conducted by experimenters blinded to the treatment received by the rats. Before testing, animals were handled for 5 consecutive days. 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 13:00 h.

2.2.1. Step-through passive avoidance test

The apparatus used in this task comprised of two adjacent compartments separated by a guillotine door. The larger, open topped compartment (45 cm × 45 cm × 45 cm; opaque acrylic walls) was brightly lit by two 60-W fluorescent bulbs mounted on the ceiling of the testing room and the smaller compartment (30 cm × 16 cm × 16 cm; black acrylic walls and top) was dark. The floor of both compartments was composed of stainless steel bars, 0.5 cm in diameter and spaced 1.2 cm apart (center-to-center), but only the floor of the dark compartment was wired to the stimulus generator. On the first day, the rats were allowed to explore the apparatus with the guillotine door open for 5 min. The following day, each rat was placed into the brightly lit compartment facing away from the closed door. When it turned around to face the dark compartment, the door was manually raised and the latency for the rat to enter 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. Ten seconds after the last shock, the rat was removed from the apparatus and returned to its home cage. 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 (up to a maximum of 5 min).

2.2.2. Morris water maze test

After the retention trial of the passive avoidance test, the rats were given a 3-day resting period prior to testing in the Morris water maze [36]. 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 ($21 \pm 1\,^\circ$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. A black 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 swim path was recorded by a computerized video-tracking system (EthoVision V3.0, Noldus, The Netherlands). In the place learning task, the animals were trained to find the submerged escape platform and to climb on it. For acquisition, rats were given two trials on each day for 14 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 in each trial 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) provided a measure of accuracy in recalling the former position of the platform. The percentage of time spent by rats swimming in the training and opposite quadrants (quadrant preference scores) was also recorded.

Performance of animals on the visible platform task was assessed during a 2-day period following the day on which the probe trials were carried out. In this task, the rats were given 1 block of four 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 eight trials.

2.2.3. Two-way active avoidance test

Testing in the shuttle-box began 5 days following the visible platform task in the water maze. The box consisted of two equally sized compartments (25 cm × 23 cm × 24 cm), connected by an opening (10 cm × 14 cm). The lower 3.5 cm of the opening connecting the two compartments was closed with a 0.5-mm thin stainless hurdle, thus, preventing the rats from staying there for more than a few seconds [37]. Three walls and the ceiling of the compartments consisted of black acrylic and the front door consisted of clear acrylic. The floor was composed of stainless steel bars, 0.5 cm in diameter and spaced 1.2 cm apart (center-to-center), wired to the stimulus generator. Small halogen bulbs (10 W) were mounted on the ceiling of each compartment. The apparatus was located in a quiet experimental room which was dimly illuminated (indirect light from one 25-W fluorescent bulb mounted on the wall). On the first day of testing, the rats were familiarized with the apparatus for 15 min. One day later, each rat was placed into one of the compartments and left undisturbed for 5 min. After this period, 45 training trials were administered. Each trial consisted of a 6-s conditioned stimulus (light) followed by unconditioned footshock stimulus (0.5 mA). Both stimuli terminated simultaneously after 20 s. The intertrial interval varied randomly from 20 s to 60 s. Crossing into the opposite (dark) compartment during the first 6 s of the trial, i.e. before the onset of the shock, was registered as a correct avoidance response. In addition, a total time taken by the rat from the onset of the conditioned stimulus to enter the dark compartment (response latency) was measured.

2.3. Tissue preparation

Following the completion of the behavioral experiments, six animals in each group, selected at random, were deeply anesthetized with sodium pentobarbital and killed by transcardial perfusion with a fixative solution containing 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer. The brains were removed from the skulls, weighed, codified, and placed in fresh fixative for 60 days. The cerebral hemispheres were then separated by a midsagittal cut and alternately sampled for further processing. After removal of the frontal and occipital poles, the blocks containing the hippocampal formations and the adjacent neocortical shell were dehydrated through a graded series of ethanol solutions and embedded in glycol-methacrylate (Technovit 7100, Kulzer and Co., Wehrheim, Germany), as described in detail elsewhere [38]. These blocks were then sectioned in the coronal plane at a nominal thickness of 40 μm using a Jung Multicut microtome. Every fifth section was collected using a systematic random sampling procedure [39], mounted serially and stained with a Giemsa solution modified for use in glycolmethacrylate-embedded material [38].

2.4. Estimation of total neuron numbers

The total numbers of neurons were estimated on Giemsa-stained sections by applying the optical fractionator method [38]. The boundaries of the granular layer of the dentate gyrus and its hilus, the CA3 and CA1 hippocampal fields, the subiculum, presubiculum, parasubiculum and postsubiculum, and the entorhinal cortex were consistently defined at all levels along the rostrocaudal axis of the brain on the basis of cell morphology and cytoarchitectonic criteria [40–45]. Neurons belonging to the CA2 hippocampal field were included in the CA3 region. The transition between the CA1 field and neuronal layer of the subiculum was defined at the point