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Research Article

Cerebellum Neurons Involvement in Apoptosis Observed in Rats, after Single and Intermittent Epilepsy Seizures with Comet Assay Method

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Abstract

Neuronal apoptosis was observed in the rat cerebellum in two experimental models of human Temporal Lobe Epilepsy (TLE). Three hours after one hippocampal kindling stimulation, a marked increase of in situ DNA damage (alkaline comet assay) of fragmented DNA was observed in nuclei located within border of the granule cell layer. Fifty kindling stimulations with 3-min interval produced higher numbers of nuclei as compared with one stimulation. The increase of DNA single-strand breaks, modifications to the method allow detection of DNA double-strand breaks was prevented by the protein inhibitor cycloheximide but not affected by the Dizocilpine, known as MK-801. Kainic acid-induced seizures causes the formation of a pattern similar to the one produced by kindling. A large proportion of cells displaying DNA damage was observed, as slides are neutralised to pH 7, stained with a DNA specific fluorescent stain and analysed using a microscope with an attached CCD (device such as digital camera) that is connected to the computer with image analysis software, showing the neuronal characteristics identity during apoptosis of cells. 50 kindling stimulations also caused a clear rise of the number of cells double-labeled with bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU, BUdR, BrdUrd, broxuridine) on the hilar border of the cerebellum cell layer. The present data show that seizures, short in time length, induce both apoptotic death and prolifecation of dentate cerebellum. We gather the evidence that these processes, which normally do happen in early stages of epileptogenesis, are pretty forerunner in the development of hippocampal pathology in animals, and this fact can be extended to the human patients in temporal lobe epilepsy.

Keywords: Kindling; Rat; Comet Assay Method; Temporal Lobe Epilepsy; Apoptosis

Introduction

Human temporal lobe epilepsy is frequently associated with a marked loss of hippocampal and cerebellum neurons. This degenecation, called Hippocampal sclerosis (HS) which is a neuropathological condition with severe neuronal cell loss and gliosis in the hippocampus, specifically in the CA-1 (Cornu Ammonis area 1) and subiculum of the hippocampus, was recognized early on [1–3], it is still difficult to say what is the real cause of neuronal death. The reason is that it is fairly possible that the epilepsy brings about the neuronal death, as well as the epilepsy following phenomenon in prolonged seizures. Recent experimental evidence makes the point that cell degenecation can be the very result of activities during seizure, causing further progress of the epileptic disease [4]. It has been shown many times that severe sustained seizures, known as epilepticus, bring about the neuronal degenecation in human hippocampus after hours of its start and it should be factored in as the consequences are so important in the overall impact of epileptic disease [5]. Furthermore, epilepticus, produced in the animal experiments, resembles and imitates the same neuronal loss process observed in the human [6]. Also, Seizure-induced cell death was discussed in details at the backdrop of excitotoxic mechanisms and degeneration necrosis [7,8]. However, there can be observed the apoptosis actually contributes to the whole phenomenon of degenecation after the epilepticus in rats [9–12]. It is yet unclear whether the same pattern of degenecation and loss of hippocampal neuron death are observable in much shorter length of times, as this is the case with chaotic or single seizure, in the ranges of minute instead of hours. It seems pretty important to see this connection, explored above, as chaotic, short lived seizure attacks, or just simply single seizure happens frequently in patients with Temporal Lobe Epilepsy (TLE). Connected with this, kindling, which is a permanent epileptic state, can be formed by the induction of a series of short seizures by electrical simulation, which is done in a very routine way for this kind of situation [13]. This simulation of seizure and the consequences of the seizure, which go through some well defined stages, shall make the work of detailing the whole assessment of cellular degenecation during epileptic attach, which is known as epileptogenesis. It is interesting to pay attention to the fact that previous studies fell short of detecting any cell loss in that important short period of time, just after the epileptic attach, as they were using the conventional method for estimating the cell number [14,15]. However, at later stages, much after the start of epileptic seizure, there have been observed the hyperecitability, accompanied by tonic-clonic convulsions, as well as, a modecate cell loss, starting in

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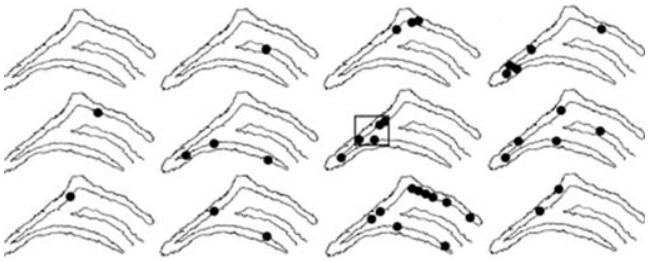

Figure 1:

the dental gyrus [14,15], though it's still hard to get a confident result out of these experiments, especially as the cerebellum region never effectively targeted in them [16–20]. However, the fact that there is such a change in the hippocampal cell number is truly complex because of the dentate granule cell neurogenesis as well as cerebellum region neuronal complexity, which are present in nonhuman primates [21], and can be sharply increased by the very status of epilepticus [22].

The whole objective of this study is to make it clear whether just after the epileptic seizure, apoptosis process will begin which further brings about the apoptotic cell death and all of the experiment, as in opposition which some other ones, are done by comet assay method, which is very novel and would give much better assessment of the process, through hippocampal kindling stimulations. More than this, we compared the whole pattern of apoptosis after kindling with the ones done in other models of limbic epilepsy, which is systematic kainic acid treatment, causing status epilepticus. Finally, it is shown that there is such a possibility of neurogenesis in the cerebellum region, which is very promising for further experiments in this region.

Materials and Methods

Kindling procedure

Animal care procedures were done by following Iranian national health administration ethical and procedural codes, to ensure a high standard work and therefore accurate measurements. Animal care procedures followed local health care guidelines. A male rat, which was selected among the experimental fit ones in order to carry the experiment and the weight of the subject, was 4 kg and it was anesthetized according the procedure.

Hydrogen peroxide treatment and gel electrophoresis: After doing the decapitation, brains were immediately placed on a cool place, i.e. dry ice, in order to prevent any damage to the brains. Coronal cryostat sections (14μm) were processed according to the comet assay method [27]. DNA damage was induced *ex vivo* by exposing the leukocytes to a range of H_2O_2concentcations (0–250 μmol/L diluted in PBSa) to determine the optimal level of H_2O_2 required to induce a significant increase in DNA damage above background endogenous DNA damage levels. Leukocytes were thawed rapidly in a 35°C water bath, washed twice in PBSa, centrifuged at 600 × g for 10min and re suspended in PBSa at 2 × 105/mL. Cells were re suspended in 0, 15, 55, 105 and 255 μmol/L H_2O_2 in PBSa and incubated on ice for 6 min. Treatment on ice minimizes the possibility of cellular DNA repair.

Two layers of agarose were prepared. For the first layer, 84 μl 1% (w/v) high-melting point (HMP) agarose (Sigma) prepared at 95°C in PBSa was pipetted onto fully frosted microscope slides, covered with an 18 × 18-mm coverslip and allowed to set at 4°C for 10min. Untreated and hydrogen peroxide–treated leukocytes were washed twice in PBSa, centrifuged at 700 × g for 15min and re suspended at 2×10^5 in 85μl 1% (w/v) low melting point (LMP) agarose (Sigma). The cell suspension was then pipetted over the set HMP agarose layer, covered with an 18 × 18-mm coverslip and allowed to set at 4°C for 10min. After the coverslips were removed, the slides were immersed in prechilled lysis solution [2.5 M NaCl, 100mM sodium EDTA, 10mM Tris, pH adjusted to 10 using NaOH pellets, 1% Triton X-100 (v/v) (added immediately before use)] for 60 min at 4°C to remove cellular proteins.

Following lysis, slides were placed in a gel electrophoresis unit and incubated in fresh alkaline electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13) for 40min at room temperature, before being electrophoresed at 25V (300 mA) for 30 min at 4°C. All the above procedures were conducted in the dark to minimize extraneous sources of DNA damage. Following electrophoresis, the slides were immersed in neutralization buffer (0.4M Tris–HCl, pH 7.5) and gently washed three times for 5min at 4°C to remove alkalis and detergents. SYBR Green (50μL; Trevigen, Gaithersburg, MD) was added to each slide to stain the DNA, then covered with a coverslip and kept in the dark in an air-tight moist container before viewing. SYBR Green was chosen for staining damaged DNA following studies by Ward and Marples [19], demonstrating improved detection sensitivity and assay resolution of SYBR Green over alternative DNA stains.

Animal treatment schedule

Adult female Wistar rats weighing 190g maintained on a 12-h light/dark cycle and at a constant temperature of 24°C were kept in macrolone cages. Animals were fed a standard diet for laboratory rodents (Pliva, Zagreb, Croatia) and had free access to water. The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Tehran, according to current laws of the Islamic Republic of Iran.

The rats were randomly assigned to nine groups of 5 animals each receiving either OTA (0.5mg/kg body weight) dissolved in Tris buffer or solvent only (1.0 mL/kg, negative control animals) intraperitoneally (ip) every day for up to three weeks. The ip treatment was chosen because it is considered to be a valuable tool for clarifying the mechanism of action of mycotoxins [9]. Positive control animals were treated with methyl methane sulfonate (40 mg/kg body weight) according to the same schedule.

Drug pretreatment

In a sepacate experiment, cycloheximide (Sigma–Aldrich; 2.0mg/kg s.c. in PBS, pH 7.4; volume 1.0ml/kg) was given to four rats 40min before a series of 40 rapid kindling stimulations. Another four rats received vehicle injections. Cycloheximide- and vehicle-pretreated rats together with 4 electrode-implanted, nonstimulated rats were killed 4h after termination of stimulations. At the dose used in this study, cycloheximide effectively blocks ribosomal protein synthesis for more than 12h [26]. In another experiment, 7 rats received MK-801 (Research Biochemicals, Natick, MA; 1mg/kg i.p.), and

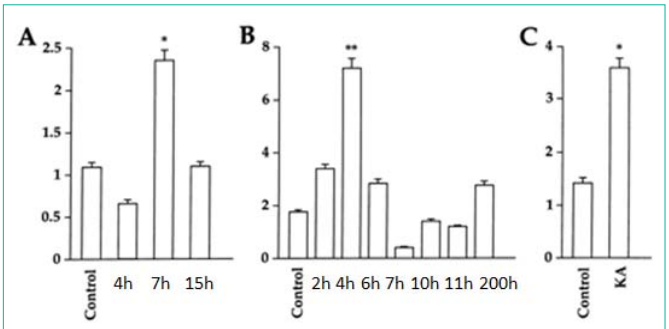

Figure 2:

7 animals were given vehicle injections 40min before 50 kindling stimulations and were decapitated 4h thereafter. In addition, MK-801 or vehicle was administered to electrode-implanted rats, which were nonstimulated (n = 5 in each group).

Kainic acid treatment

Rats (90–110g; n = 4) were given kainic acid (Sigma-Aldrich; 10mg/kg) s.c. and observed for 2h to confirm the presence of convulsions. Animals were decapitated 4h after onset of convulsions.

Silver staining

Six rats were given kainic acid (7mg/kg s.c.) and killed at 8h (n = 5) or 15h (n= 5) after the onset of convulsions. Two vehicle-injected animals served as controls (one at each time point). Another 8 animals were given 1 (n = 4) or 40 (n = 4) kindling stimulations and killed at 7 or 5h, respectively, after the last stimulation. Visualization of degenerating cells was performed according to Nadler and Evenson [29]. Briefly, rats were perfused transcardially with 50 ml of 0.9% NaCl followed by 250ml para formaldehyde in 0.1M phosphate buffer (pH 7.2). One hour after perfusion, the brains were removed from the skull and post fixed in the same solution for at least 24h. After cryoprotection of the tissue in 40% sucrose, 40-µm-thick sections were cut on a freezing microtome. Sections were washed in 0.1M Tris buffer (pH 7.6) followed by washing three times for 7min in dH_2O. After pretreatment in 4.5% NaOH and 10% NH_4NO_3, sections were incubated in impregnation solution (5.4% NaOH/6.4% NH_4NO_3/0.2% $AgNO_3$ in dH_2O) for 10 min and then washed in 31.6% ethanol/0.5% Na_2CO_3/0.12% NH_4NO_3. The staining was developed in 0.05% citric acid/0.55% formaldehyde/9.5% ethanol/0.12% NH_4NO_3 for 3–5min. Finally, sections were rinsed in 0.1M Tris buffer (pH 7.6), mounted, dehydrated, coverslipped, and analyzed by light microscopy. Later, the number of cells were done in the cerebellum region was quantified by four independent observers.

Analysis of cell prolifecation

Eighteen electrode-implanted rats were either left unstimulated or given 1 or 50 kindling stimulations (n = 6 in each group). Six days later, all rats received four injections of bromodeoxyuridine (BrdU; Sigma–Aldrich; 37.5mg/kg i.p.) with 8-h intervals. Three weeks later, animals were perfused and brains were processed for immunocytochemistry with antibodies against NeuN and BrdU. In brief, free-floating sections were denatured by incubation for 2h in 50% formamide/2× SSC (0.3 M NaCl/0.03M sodium citcate) at 65°C, rinsed for 5min in 2×SSC, followed by incubation in 2M HCl for 30min at 37°C. Sections were rinsed three times for 10min in PBS, preincubated in blocking solution (0.25% Triton X-100/2% normal donkey serum/2% normal horse serum in PBS) for 1h at room temperature and incubated overnight at 4°C with pooled primary antibodies (cat anti-BrdU, 1:100, Harlan Sera-lab, Crawley Down, U.K.; and mouse anti-NeuN, 1:100, Chemicon). The sections were then rinsed three times for 10min in blocking solution and two secondary antibodies conjugated to Cy3 (donkey anti-cat-IgG, 1:400, Jackson Immuno Research) and biotin (horse anti-mouse, 1:200, Chemicon), respectively, were applied for 4h in the dark at room temperature. After rinsing three times for 5min in PBS, fluoresce in avidin D (1:250, Vector Laboratories) diluted in PBS with 0.25% Triton X-100 was applied for 2h in the dark. The sections were rinsed three times for 20min in PBS, mounted, coverslipped with Vectashield, and analyzed by conventional epifluorescence light and confocal laser scanning microscopy.

The total number of BrdU-labeled cells and the number of BrdU-positive, NeuN-positive cells within the dentate gyrus granule cell layer and sub granular zone was counted in three to seven sections per animal.

Statistics: The number of nuclei positive for DNA fragmentation in the different groups and the effect of cycloheximide and MK-801 on DNA fragmentation were analyzed statistically using one-way ANOVA followed by Bonferroni–Dunn post-hoc test. Comparisons of the number of nuclei positive for DNA fragmentation between the left and right side, of the number of proliferating cells between the groups, and of seizure characteristics were performed using Student's paired or unpaired t test. All values are given as means ± SEM.

Comet assays

Alkaline comet assays were performed via the protocol from Comet Assay TM ES unit (Trevigen, Inc.) Briefly, patient derived EBV transformed lymphocytes were grown as described. Cells were obtained prior to treatment or exposed to 100µM hydrogen peroxide (Sigma) or 10µM camptothecin (Sigma) for 30 and 60 minutes respectively at 37°C. After exposure, cells were immediately collected for 0min recovery time point or washed once, re suspended in growth media and incubated at 37°C. Cells were subsequently collected at times described. Cells were embedded in low melt agarose, plated upon microscope slides, lysed, treated with alkaline solution, and slides electrophoresed in alkaline solution at 1Volt/cm (21V) with ~300m Amps for 30 minutes. Slides were washed, dried, and DNA stained with SYBR green. Images of nuclei and tails were taken with a Nikon TE2000-E fluorescent microscope with CCD camera and % Tail DNA determined with Comet Score 1.5 software (TriTek, Corp.).

Results

In each section from electrode-implanted but nonstimulated control animals, a mean of 1.1 ± 0.2 nuclei in cerebellum with fragmented DNA, as visualized using the Comet assay technique, was observed within the granule cell layer or immediately infragranular in the polymorphic layer of the hilus (left and right side pooled; Figures. 1A and 2A). This observation is consistent with previous findings showing turnover of granule neurons at a low rate in the normal adult hippocampus and elimination of cells through apoptosis [30,31]. In regions outside the dentate gyrus and cerebellum region as well, no consistent in situ DNA fragmentation was seen.

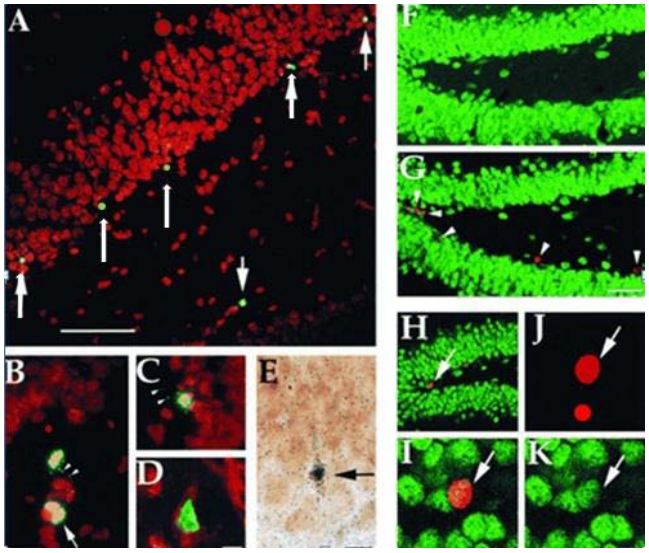

Figure 3:

Fifty rapid kindling stimulations brought about with 15-min stimuli in between, which interval resulted in 2.6 ± 0.8 generalized (grade 4–5) seizures. The mean duration for after discharge in the hippocampus was 37 ± 8 s. not extremely, but rather, moderate numbers of nuclei were observed within the cerebellum after fifty seizures, compared to dentate gyrus, however still it was higher as compared with one seizure (Figures 1C,2B,and 3A). Four hours after the last stimulation, the number of nuclei displaying fragmented DNA was 518 ± 87% of control (left and right side pooled; $P < 0.001$). A big increase in the number of cells was captured at this very time, although a trend toward higher numbers was registered before at 0.5 and 6h after the last seizure. Interestingly, because the 50 stimulations were given during 4h and 15min, the increase seen at 2h following the last seizure coincides with that observed at 8 h following a single stimulation. Also after 50 seizures, increased numbers of labeled nuclei were confined to the dentate gyrus and cerebellum granule cell layer and hilus. There were no significant differences between the left and right cerebellum in the number of labeled nuclei after either 1 or 50 kindling-evoked seizures. This finding indicates that the increased labeling was due to seizure activity and not caused by the electrical current from the unilateral stimulating electrode.

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