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Decreased expression of proteins involved in energy metabolism in the hippocampal granular layer of rats submitted to the pilocarpine epilepsy model

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HIGHLIGHTS

- Decrease in expression of proteins involved in energy metabolism in the hippocampal granular layer.
- For the first time we identify new molecular insights into the altered protein profile of the epileptogenic dentate gyrus.
- The proteins described could be used as further biomarkers or therapeutic targets for epilepsy treatment.

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ABSTRACT

Long-term structural and functional changes in the hippocampus have been identified as the primary physiopathological basis for temporal lobe epilepsy. These changes include reactive gliosis and granule cell axonal sprouting within the dentate gyrus. The intimate mechanisms of these changes are beginning to be revealed. Here, we show the possibility of using laser capture microdissection (LCM) to isolate the dentate granular cell layer of Wistar rats submitted to the pilocarpine model of epilepsy. Using two-dimensional gel electrophoresis (2-D PAGE) and mass spectrometry for laser-captured cells, we identified molecular events that could be altered as part of the epileptic pathogenic process. According to our results, eight proteins related to energy metabolism were differentially expressed between both the control and pilocarpine-treated animals. These results provide, for the first time, new molecular insights into the altered protein profile of the epileptogenic dentate gyrus and can contribute to a better understanding of the phenomena involved in the genesis and maintenance of the epileptic state.

1. Introduction

The mechanism underlying the process of transforming a previously normal brain into a seizure-prone brain has been called epileptogenesis and identifying the molecular and structural changes that take place during this process may be the key to understanding epilepsy. Among the brain structures affected by epileptogenesis, the hippocampal region is certainly the best studied. The most significant histopathological changes found in the human sclerotic hippocampus (HSH) are the selective neuronal loss of CA1, CA3 and hilar region [1], and the sprouting of the mossy fibers of granule cells [2]. The region of the dentate gyrus, in particular the granular cell layer, seems to play a key role in the epileptogenic process [3]. Despite the large number of studies

Abbreviations: [K^+], potassium concentration; 2-D PAGE, two-dimensional gel electrophoresis; ATP, adenosine triphosphate; SUCLA2, succinyl-CoA ligase [ADP-forming] subunit beta; ACO2, aconitate hydratase; SLC25A12, calcium-binding mitochondrial carrier protein Aralar1; (GOT2), mitochondrial aspartate aminotransferase; FH1, mitochondrial fumarate hydratase; (CKB), creatine kinase B-type; Na^+/K^+ ATPase, sodium potassium pump ATPase; NAA, N-acetyl aspartate; SE, *Status Epilepticus*; SRS, spontaneous recurrent seizures; LCM, laser capture microdissection; SCOT, succinyl-CoA:3-ketoacid-coenzyme A transferase 1; HSH, human sclerotic hippocampus.

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to attempting to unravel the role of the granular layer in epilepsy, there is a controversy in the literature about which is the real role of this layer in the excitatory activity of the intra-hippocampal circuit underlying seizures [4–6].

It is known that energetic metabolism has to be adjusted in proportion to the activity of the neuronal cell, which needs a high level of energy to sustain normal and pathological neuronal activities, such as during epilepsy. Energetic metabolism is modulated mainly, though not exclusively, by mitochondria, small organelles involved in many cellular functions such as adenosine triphosphate (ATP) synthesis, calcium homeostasis and regulation of apoptosis [7,8]. Malfunctions in mitochondria are already associated with several reported cases of epilepsy [9–12].

Here we aim to consider a number of issues: first that epilepsy induces changes in the expression of proteins involved in energy metabolism in the hippocampal granular layer of rats submitted to the pilocarpine epilepsy model; and secondly that these affected peptides could be related, at least in part, to the genesis and maintenance of epilepsy.

2. Materials and methods

2.1. Pilocarpine model

Male Wistar rats (2 months) were submitted to the pilocarpine model of epilepsy according to Leite et al. [13]. In summary, they received an intraperitoneal injection (i.p.) of atropine methyl nitrate (1 mg/kg in saline, Sigma, St. Louis, MO), then 30 min later, SE was elicited with an intraperitoneal injection (i.p.) of pilocarpine (350 mg/kg in saline, Sigma, St. Louis, MO). SE was terminated after 3 h by diazepam (10 mg/kg, i.p.) to achieve uniform SE duration in both strains. Control animals were injected with atropine methyl nitrate (1 mg/kg) and then with saline, instead of pilocarpine. All experiments were performed with the approval of the Ethical Committee of UNIFESP and all protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. Behavioral analysis

Following SE, the animals were continuously video-monitored over 24 h for signs of the first spontaneous recurrent seizures (SRS). After the first SRS had been detected, animals were monitored over 30 days. Infrared emitting lights were used during the dark periods to allow video recording of animal activity during the night. To identify the seizures, two observers were recruited for the whole behavioral analysis. Seizures ranging from stages 3–5 according to Racine [14] were considered as spontaneous seizures. After 30 days from the first SRS, only experimental animals ($n = 6$) with an age-matched control group ($n = 6$) were used in the study.

2.3. Laser capture microdissection

For cell capture, animals were killed by decapitation (to avoid any interference of seizures *per se* on the expression of proteins, all epileptic animals were euthanized after an interval of 8 h from their last spontaneous seizures ranging from stages 3–5 according to Racine [14]) and the brains were quickly removed from the skull and immediately frozen with isopentane. Subsequently, they were cut coronally with a cryostat in 8-μm-thick sections collected in series. These sections were directly mounted onto previously autoclaved glass slides, dehydrated and then fixed with xylene. After fixation, samples were subjected to LCM with the microscope Palm Microbeam IP Z capture system (Carl Zeiss, Munich, Germany).

2.4. Two-dimensional gel electrophoresis (2D-PAGE)

The microdissected cells, isolated by LCM methodology (Fig. 1), were treated with a buffer solution consisting of 7 M urea, 2 M thiourea, 4% chaps, 1% protease inhibitor cocktail–EDTA free from Roche, 100 mM DTT, 0.01% bromophenol blue, 0.5% ampholyte pH 3–10. Then, the tubes containing the samples were centrifuged at 4000 rpm at 4 °C. The material was subsequently sonicated at 10 °C for 10 min, mixed and centrifuged at 12,000 rpm at 4 °C for 30 min. The supernatant was transferred to a sterile tube for protein quantitation according to the experimental method of Bradford [15]. One hundred and fifty micrograms of protein were used for the construction of each gel (3 control and 3 experimental). Eighteen cm linear pH 3–11 Immobilized pH gradient strips (GE Healthcare) were used for the first dimension. Passive rehydration was carried out over 12 h. Isoelectric focusing was performed using a Protean IEF cell (BioRad Laboratories, Hercules, CA, USA). Focusing was initiated at 300 V, and after 1 hour the voltage was gradually increased to 500 V in a linear mode for 60 min and, finally, 500 V was applied until 65 kV h was reached. The temperature was kept at 18 °C. After isoelectric focusing, the strips were equilibrated in equilibration buffer I (10 mg/ml DTT; 50 mM Tris–HCL 1.5 M pH 8.8; 6 M urea; 30% glycerol; 2% SDS, 0.01% bromophenol blue) and equilibration buffer II (40 mg/ml iodoacetamide; 50 mM Tris–HCL 1.5 M pH 8.8; 6 M urea; 30% glycerol; 2% SDS, 0.01% bromophenol blue) for 15 min each sequentially. The equilibrated strips were then placed onto second dimension 12.5% SDS–PAGE gels. The SDS–PAGE was conducted in a standard Tris–Glycine–SDS buffer in Protean II xi Multi-Gel (BioRad Laboratories, Hercules, CA, USA) at a constant current setting of 1.6 W/gel for 1 h, then at 16 W/gel until the bromophenol blue dye reached the end of the gel. Gels were stained with PageBlue Protein Stainin Solution according to the manufacturer's instructions (Thermo Fisher Scientific, USA).

2.5. Image analysis and determination of proteome differences

Stained gels were scanned by a GS-710 calibrated densitometer (BioRad, USA) and PDQuest 2D-gel analysis software (Version 8.0.1, BioRad, USA) was used to process and analyze the gel images. For each data analysis set, we used three technical replicates for each biological replicate of control and experimental protein extracts. Following the automatic detection mode, spots were manually edited so that any spots that were not present in all replicated gels were excluded from the analysis. Scatter diagrams were created using the Scatter Plot function of PDQuest software and the correlation coefficient was created. For quantitative comparisons, protein spots observed in the experimental and control groups were normalized for the total density of each gel after calibration by manual indication of the lowest and highest density spots. Normalized density values were used for comparisons, and spots exhibiting different expression (control *vs* pilocarpine) were identified by mass spectrometry.

2.6. In-gel digestion

Excised protein spots were subjected to in-gel trypsin digestion. The spots were briefly washed three times by adding and removing alternating 25 mM $(NH_4)_2CO_3$, 50% acetonitrile and 50 mM $(NH_4)_2CO_3$ solutions. After removing the supernatant, the protein spots were overlaid by 200 ng trypsin in 50 mM $(NH_4)_2CO_3$, 10% acetonitrile in H2O. Incubation was carried out overnight at 37 °C and was stopped by adding 0.5 volumes of 2% formic acid. After incubation for 1 h the supernatant was transferred to a new reaction for subsequent analysis by mass spectrometry.