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ORIGINAL RESEARCH

ATP Synthase Subunit Beta Immunostaining is Reduced in the Sclerotic Hippocampus of Epilepsy Patients

Marcelo Vilas Boas Mota[1] · Bruna Cunha Zaidan[1] · Amanda Morato do Canto[2] · Enrico Ghizoni[3] · Helder Tedeschi[3] · Luciano de Souza Queiroz[1] · Marina K. M. Alvim[3] · Fernando Cendes[3] · Iscia Lopes-Cendes[2] · André Almeida Schenka[4] · André Schwambach Vieira[5] · Fabio Rogerio[1]

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Abstract
Epilepsy is a common disease presenting with recurrent seizures. Hippocampal sclerosis (HS) is the commonest histopathological alteration in patients with temporal lobe epilepsy (TLE) undergoing surgery. HS physiopathogenesis is debatable. We have recently studied, by using mass spectrometry-based proteomics, an experimental model of TLE induced by electrical stimulation. Specifically, protein expressions of both the beta subunit of mitochondrial ATP synthase (ATP5B) and of membrane ATPases were found to be reduced. Here, we investigated tissue distribution of ATP5B and sodium/potassium-transporting ATPase subunit alpha-3 (NKAα3), a protein associated with neuromuscular excitability disorders, in human hippocampi resected "en bloc" for HS treatment ($n = 15$). We used immunohistochemistry and the stained area was digitally evaluated (increase in binary contrast of microscopic fields) in the hippocampal sectors (CA1–CA4) and dentate gyrus. All HS samples were classified as Type 1, according to the International League Against Epilepsy (ILAE) 2013 Classification (predominant cell loss in CA1 and CA4). ATP5B was significantly decreased in all sectors and dentate gyrus of HS patients compared with individuals submitted to necropsy and without history of neurological alterations ($n = 10$). NKAα3 expression showed no difference. Moreover, we identified a negative correlation between frequency of pre-operative seizures and number of neurons in CA1. In conclusion, our data showed similarity between changes in protein expression in a model of TLE and individuals with HS. ATP5B reduction would be at least in part due to neuronal loss. Future investigations on ATP5B activity could provide insights into the process of such cell loss.

Keywords Epilepsy · Hippocampal sclerosis · Proteomics · Immunohistochemistry · Mitochondrial ATP synthase · Sodium/potassium ATPase

Introduction

Epilepsy is the most common neurological disease and, according to the International League Against Epilepsy (ILAE), is defined by one of the following conditions: at least two unprovoked (or reflex) seizures occurring more than 24 h apart; unprovoked (or reflex) seizure and a probability of more seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures occurring over the subsequent 10 years; or diagnosis of an epilepsy syndrome (benign childhood epilepsy with centrotemporal spikes, for instance) (Fisher et al. 2014; WHO 2006). Epilepsy may result from a wide range of pathological conditions. In most cases, patients show satisfactory seizure control (including remission) with use of medication. However, 30% of individuals with epilepsy do not respond to pharmacological treatment (refractory epilepsy) and become candidates for surgical intervention (Blümcke et al. 2017).

Hippocampal sclerosis (HS) is the most frequently diagnosed histopathological abnormality in surgical specimens of patients with epilepsy, this condition being found in about 36.4% of all cases (Blümcke et al. 2017). HS is characterized by neuronal loss in one or more hippocampal sectors, dispersion of granular cells in the dentate gyrus, and gliosis. Recently, the ILAE presented a proposal to classify HS, based on semi-quantitative microscopic evaluation, aimed at facilitating reproducibility among different epilepsy research and treatment institutions. Such classification presents

✉ Fabio Rogerio
fabio@fcm.unicamp.br

Extended author information available on the last page of the article

distinct histopathological types of HS considering the fact that hippocampal regions (CA1–4 sectors and the dentate gyrus) show different patterns of cell loss and astrocytic response. Particularly, HS ILAE Type 1, which corresponds to around 60–80% of the cases, is characterized by severe neuronal loss and gliosis in CA1 and CA4 sectors. Less frequent HS ILAE Types 2 and 3 correspond to neuronal loss and gliosis predominantly in CA1 or CA4, respectively. Hippocampus showing only gliosis (without significant neuronal loss) is an uncommon type and classified as No-HS/gliosis only (Blümcke et al. 2013).

Research on HS has used not only surgical specimens of patients but also animal models. Through different experimental approaches, it is possible to investigate etiopathogenic factors, cellular and/or molecular changes in the hippocampus, as well as possible biological markers predictive of clinical evolution (Engel 1996).

Our research group has recently studied, by using mass spectrometry-based proteomics, differential expression of proteins in a model of temporal lobe epilepsy. Specifically, we evaluated the dorsal and ventral dentate gyrus of the hippocampus of rats submitted to electrical stimulation of the perforant pathway (Norwood et al. 2010). After spectral counting analysis, protein expressions of both the beta subunit of mitochondrial ATP synthase (ATP5B) and of membrane ATPases were found to be reduced (submitted data).

In this study, we investigated tissue distribution and expression of ATP5B and sodium/potassium-transporting ATPase subunit alpha-3 (NKAα3) in human hippocampi resected ("en bloc") for treatment of HS. The latter protein is a membrane ATPase associated with epileptiform activity in murine hippocampus and neural excitability disorders (Vaillend et al. 2002; Heinzen et al. 2014; Rosewich et al. 2014). As far as we know, there are no previous reports on the expressions of ATP5B and NKAα3 in surgical specimens of patients with HS. Therefore, we aimed at performing a translational study to evaluate such expressions in human samples to contribute to a better knowledge of a relevant cause of epilepsy.

Materials and Methods

This study was based on a retrospective analysis of surgical specimens obtained from patients undergoing epilepsy surgery due to HS at our Institution. Only samples stored in paraffin blocks were analyzed and the individuals, whose identities were kept anonymous, were not submitted to any additional procedure associated with the present investigation. The study design was approved by the Research Ethics Committee (protocol#1313817). We evaluated 25 individuals ($n=25$) divided into two groups as follows: (1) controls consisting of hippocampi obtained from autopsied adults ($n=10$) with neither underlying neurological disease nor history of seizures/epilepsy, and (2) HS group consisting of 15 patients ($n=15$). Control samples were collected between 6 and 12 h after death; only samples that showed no signs of autolysis that could interfere with microscopic assessment (pyknotic nuclei and eosinophilic cytoplasm) were submitted to immunohistochemical evaluation. Moreover, the following clinical data were obtained: age at the moment of surgery; age at seizure onset; frequency of seizures in the pre-operative period; epilepsy family history; personal history of febrile seizures; and post-operative outcome based on the Engel Epilepsy Surgery Outcome Scale (Engel et al. 1993). Regarding this last clinical information, individuals with HS that showed no seizures after surgery were classified as Engel class IA. HS patients with post-operative seizures were grouped into a single class ("non IA"). This was done because a reduced frequency of individuals in Engel classes other than IA could weaken the statistical analyses.

Histological Processing and Immunohistochemical Reactions

Histological sections (4 µm) were submitted to routine staining (hematoxylin and eosin, H&E), or immunohistochemical reactions. For immunostaining, the sections were incubated with anti-ATP5B (1:100, polyclonal, Aviva SysBio, cat # OAAB01752, San Diego, CA, USA) or anti-NKAα3 primary antibody (1:100, clone XVIF9-G10, Abcam, cat # ab2826, Cambridge, MA, USA) for 18 h at 4 °C. Then, the detection system containing the secondary antibody and peroxidase (AdvanceTMHRP®, Dako, cat # K4068, Glostrup, Denmark) was added for 30 min at 37 °C. 3,3′-diaminobenzidine (DAB) was used as chromogenic substrate. Counterstaining with hematoxylin was not used to avoid interference with digital analysis of the immunostaining (see "Analysis of Beta Subunit of Mitochondrial ATP Synthase and of Sodium/Potassium-Transporting ATPase Subunit Alpha-3 Immunostaining"). We successfully performed previous tests to evaluate the specificity of each primary antibody by using tissues considered positive controls by the manufacturers, that is, human neocortical neurons and human colonic tissue for anti-ATP5B and anti-NKAα3, respectively. Positive and negative controls (omission of the primary antibody) were used in all immunohistochemical reactions.

Histopathological Analysis and Hippocampal Sclerosis Classification

In control and HS groups, neuronal loss was evaluated by counting neurons in 5 high-power fields (HPF; x400) in each of the hippocampal sectors (CA1–CA4) and the dentate gyrus (DG) in H&E-stained sections. Specifically, the

specimens were classified following the ILAE criteria for HS (Blümcke et al. 2013).

Analysis of Beta Subunit of Mitochondrial ATP Synthase and of Sodium/Potassium-Transporting ATPase Subunit Alpha-3 Immunostaining

To evaluate beta subunit of mitochondrial ATP synthase (ATP5B) and sodium/potassium-transporting ATPase subunit alpha-3 (NKAα3) protein expression, pictures of five randomly selected microscopic fields (x200) from each of the hippocampal sectors (CA1, CA2, CA3, and CA4) and dentate gyrus were used for both groups. The pictures (TIFF format) were obtained with digital camera (Axiocam 105 Color, Zeiss) coupled with a microscope (Axio Lab.A1, Zeiss) and connected to a computer, allowing the concomitant visualization of each field by using a software (Zen Zeiss). Afterwards, each photographed field was analyzed through a digital technique to increase binary contrast (thresholding) by using the ImageJ® software (version 1.50i). Briefly, the colored picture to be analyzed was initially converted into a gray-scale image and then into binary information (that is, black and white). For such conversion, the software operator visually defined a cut-off point in the gray-scale (threshold) that maintained, as reliably as possible, the immunostaining features (intensity and contrast) observed in the colored images without counterstaining (Fig. 1). This gray-scale corresponds to numerical values ranging from 0 (black) to 255 (white).

Specifically, image processing was performed through the following steps: (i) grouping of the five immunostaining images of each hippocampal region in a single window; (ii) conversion of each immunostaining image to an 8-bit grayscale; (iii) creation of a binary image of each original immunostaining image by using the threshold tool (Fig. 1a', b'); (iv) complete conversion of each binary image; and (v) selection of the analysis parameter *Mean gray value*; by using this parameter, the software sums the gray values of all pixels of one single window, divides the total amount by the total number of pixels of the same window and provides a value defined as Mean (Reinking 2007). Thus, the immunostaining evaluation focused on the distribution of positivity over one sampled area.

Statistical Analysis

To describe the profile of the samples, absolute (*n*) and relative (%) frequency values for the categorical variables were presented, as well as the descriptive statistics of the numerical variables (mean, standard deviation and median (Md)). Mann–Whitney test was used to compare cell counting and immunostaining (Mean value) between control and HS groups, since variables showed no normal distribution. Mann–Whitney test (comparison between 2 groups) and the Spearman's correlation coefficient (relationship between numerical variables) were used to analyze the relationship between cell counting, immunostaining (Mean value) and clinical data. For Mann–Whitney test comparisons, a linear

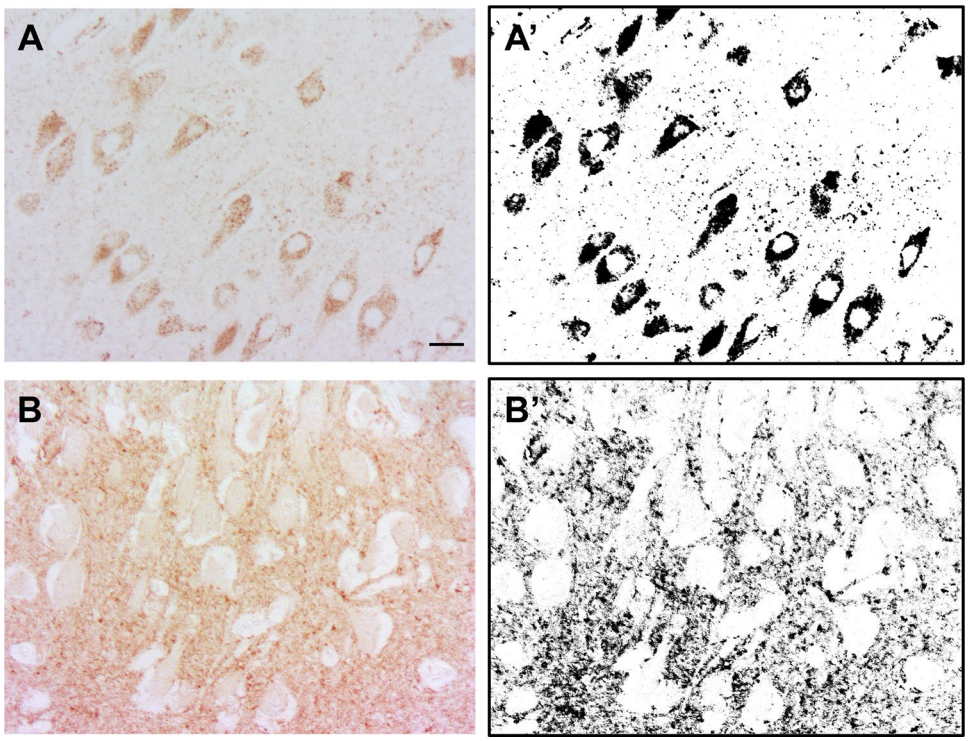

Fig. 1 Immunostaining analysis for beta subunit of mitochondrial ATP synthase (**a, a'**) and sodium/potassium-transporting ATPase subunit alpha-3 (**b, b'**). The fields observed on the right (gray-scale images) are the same as those shown on the left (colored images without counterstaining) after image processing. The immunostaining features (tissue distribution, intensity and contrast) were maintained as reliable as possible by the software operator. Immunoperoxidase. Scale bar (**a, a'**, **b, b'**): 20 µm

rank was performed by ordering the values obtained after cell counting and immunohistochemical analyses from lowest to highest. The significance level adopted for the statistical tests was 5% ($p < 0.05$). SAS 9.2 Software for Windows (SAS Institute Inc, 2002–2008, Cary, NC, USA) was used for statistical analysis.

Results

Clinical Data

Fifteen individuals ($n = 15$) with HS, ages ranging from 13 to 52 years (35.53 ± 9.91; mean ± standard deviation) were studied, of which seven were females (46.67%) and eight males (53.33%). Four patients had familial history of epilepsy (26.66%), seven denied (46.67%), and four did not know (26.66%). Three patients had febrile seizures (20%), seven did not (46.67%), and five were unaware (33.33%). The mean age at seizure onset was 15.21 years (± 9.88), ranging from 2 months to 36 years (Md = 16.00 years). The pre-operative period in which seizures occurred was 20.27 years (± 11.79) on average, with a minimum of 4 years and a maximum of 45 years (Md = 20.00 years). The mean frequency of pre-operative seizures per month ranged from 2 to 34, with a mean of 9.8 (± 9.56) (Md = 8.00). With regard to post-operative control of seizures (Engel Scale), 10 patients (66.67%) were classified as IA, while five (33.33%) were grouped into the "non IA" category (IB (non disabling simple partial seizures only since surgery; $n = 2$), IC (some disabling seizures after surgery, but free of disabling seizures for at least 2 years; $n = 1$), and IIB (rare disabling seizures since surgery; $n = 2$)).

Neuronal Counting and Hippocampal Sclerosis Classification According to ILAE Criteria

Hematoxylin and eosin-stained slides from HS individuals showed neuronal loss in all hippocampal regions in comparison with controls, more intense in CA1 and CA4 (CA1 (HS: 1.25 (mean) ± 1.97 (standard deviation), Md = 0.40 vs. control (C): 30.68 ± 9.87, Md = 27.90, $Z = 4.16$; $p < 0.001$); CA2 (HS: 9.56 ± 4.54, Md = 8.60 vs. C: 33.02 ± 5.28, Md = 32.40, $Z = 4.13$; $p < 0.001$); CA3 (HS: 6.68 ± 6.03, Md = 5.00 vs. C: 32.82 ± 5.29, Md = 31.70, $Z = 4.13$; $p < 0.001$); CA4 (HS: 4.00 ± 4.12, Md = 2.60 vs. C: 20.24 ± 4.02, Md = 20.10, $Z = 4.14$; $p < 0.001$) and DG (HS: 80.31 ± 19.59, Md = 85.80 vs. C: 111.38 ± 24.71, Md = 103.50, $Z = 2.97$; $p = 0.003$)). Thus, there were significant differences between both groups in CA1, CA2, CA3 and CA4 sectors and DG (Figs. 2, 3, 4). Considering the ILAE criteria, all samples with HS were classified as Type 1, that is, they presented more severe neuronal loss in CA1 and CA4.

Tissue Expression Analysis of Beta Subunit of Mitochondrial ATP Synthase and of Sodium/Potassium-Transporting ATPase Subunit Alpha-3

Beta Subunit of Mitochondrial ATP Synthase (ATP5B)

The cellular staining pattern observed for ATP5B was cytoplasmic and granular, no qualitative difference being identified between control and HS groups (Figs. 5, 6). Regarding tissue expression of ATP5B, the following data were observed in each one of the hippocampal regions evaluated in both groups: CA1 (HS: 9197.10 (mean) ± 4219.80 (standard deviation), Md = 9143.80 vs. C: 18695.00 ± 7960.00, Md = 19486.00, $Z = 3.02$; $p = 0.002$); CA2 (HS: 9181.60 ± 4969.10, Md = 8122.80 vs. C: 18571.00 ± 10903.00, Md = 15923.00, $Z = 2.52$; $p = 0.011$); CA3 (HS: 8069.40 ± 3996.00, Md = 6870.30 vs. C: 18524.00 ± 8075.10, Md = 18462.00, $Z = 3.13$; $p = 0.002$); CA4 (HS: 7475.00 ± 2988.00, Md = 7678.60 vs. C: 11357.00 ± 5087.90, Md = 12543.00, $Z = 2.02$; $p = 0.04$) and DG (HS: 14957.00 ± 5985.70, Md = 16196.00 vs. C: 21556.00 ± 7324.10, Md = 20697.00, $Z = 2.25$; $p = 0.023$). Therefore, significant differences were verified between both groups in relation to tissue expression of ATP5B in each sector (CA1, CA2, CA3, and CA4) and in the DG. The smaller values were found in the HS group.

Sodium/Potassium-Transporting ATPase Subunit Alpha-3 (NKAα3)

NKAα3 staining pattern was predominantly verified in the neuropil, with a granular aspect. No qualitative difference was detected between the two groups (Figs. 7, 8). Regarding tissue expression of NKAα3, the following data were observed in each of the hippocampal areas evaluated: CA1 (HS: 16342.00 (mean) ± 10604.00 (standard deviation), Md = 14488.00 vs. C: 21244.00 ± 11975.00, Md = 16315.00, $Z = 1.19$; $p = 0.222$); CA2 (HS: 19702.00 ± 11768.00, Md = 14906.00 vs. C: 20107.00 ± 7404.30, Md = 19081.00, $Z = 0.75$; $p = 0.437$); CA3 (HS: 19267.00 ± 11997.00, Md = 14732.00 vs. C: 22953.00 ± 10413.00, Md = 22297.00, $Z = 1.14$; $p = 0.244$); CA4 (HS: 16106.00 ± 5413.00, Md = 17275.00 vs. C: 19890.00 ± 10424.00, Md = 20173.00, $Z = 1.14$; $p = 0.244$) and DG (HS: 20630.00 ± 8980.10, Md = 19798.00 vs. C: 17166.00 ± 9031.50, Md = 16639.00, $Z = 0.69$; $p = 0.471$). Thus, there was no significant difference between both groups regarding tissue expression of NKAα3 in all hippocampal sectors and DG.

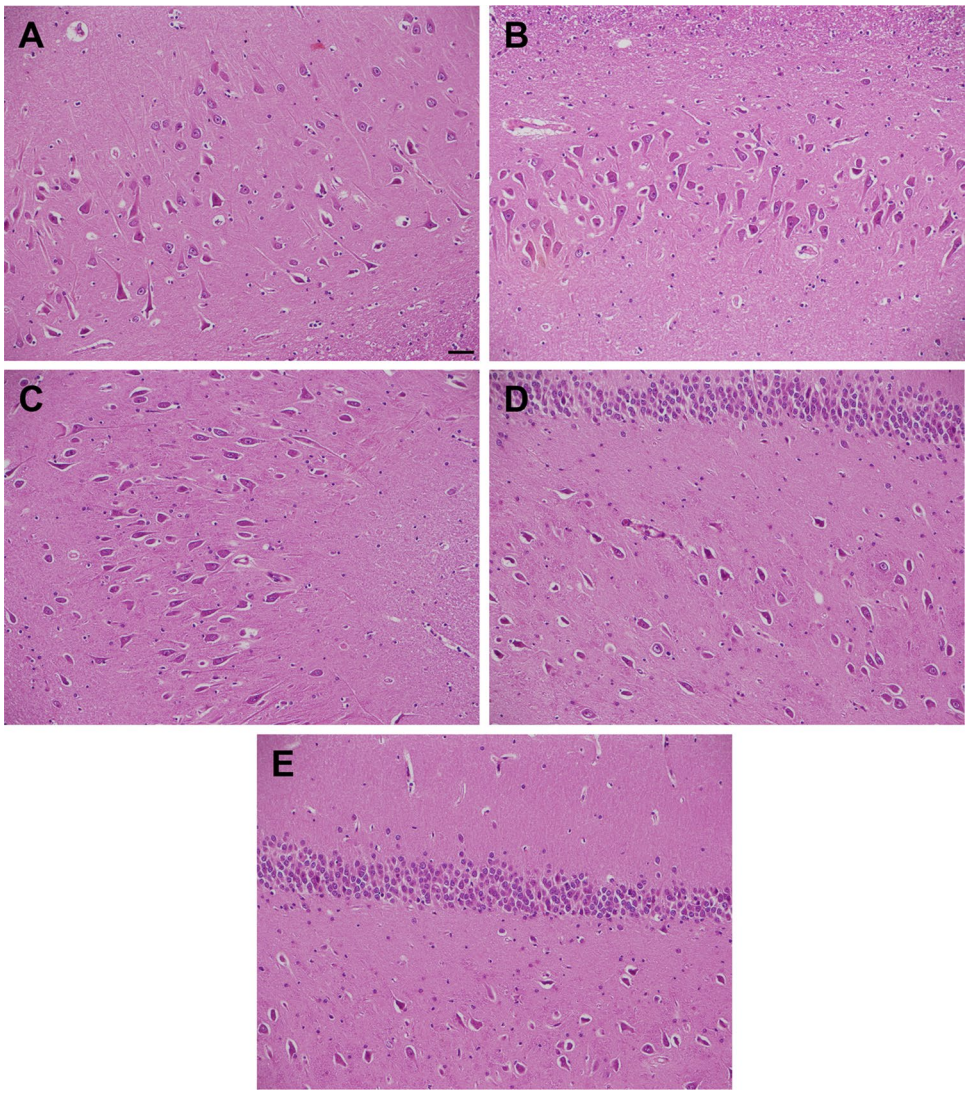

Fig. 2 Morphological findings in hippocampal sectors and dentate gyrus of controls. Cell density and features (shape and orientation) of the pyramidal neurons of each hippocampal sector (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and dentate gyrus (**e**) are representative of the normal spectrum observed in the different samples of this group. Hematoxylin and eosin. Scale bar (**a**–**e**): 50 µm

Comparative Analysis Between Clinical Data, Neuronal Counting and Tissue Expression of Beta Subunit of Mitochondrial ATP Synthase and of Sodium/Potassium-Transporting ATPase Subunit Alpha-3

Regarding HS group, no association was identified between the number of neurons (in hippocampal sectors or dentate gyrus) and the following clinical data: gender, familial cases of epilepsy, personal antecedent of febrile seizures, and Engel classification. Similarly, there was no association between tissue expression of beta subunit of mitochondrial ATP synthase (ATP5B) or of sodium/potassium-transporting ATPase subunit alpha-3 (NKAα3) and the same clinical data. On the other hand, there was a significant correlation between the frequency of pre-operative seizures and the number of neurons counted in the CA1 sector, that is, the higher the pre-operative seizure frequency, the smaller the number of neurons present in the CA1 sector ($r = -0.52444$, $p = 0.00448$).

Discussion

In the present study, we evaluated neuronal loss in the hippocampus of individuals with TLE due to HS and investigated tissue distribution of proteins whose expressions were altered in an experimental model of TLE. Regarding cell loss, individuals with HS showed a more intense neuronal depletion in CA1, followed by CA4, CA3, and CA2. Therefore, all surgical specimens were classified as Type 1, considering the criteria proposed in the last ILAE classification (Blümcke et al. 2013). These data are similar to those described by other authors. Indeed, in the international consensus that proposed such histopathological classification

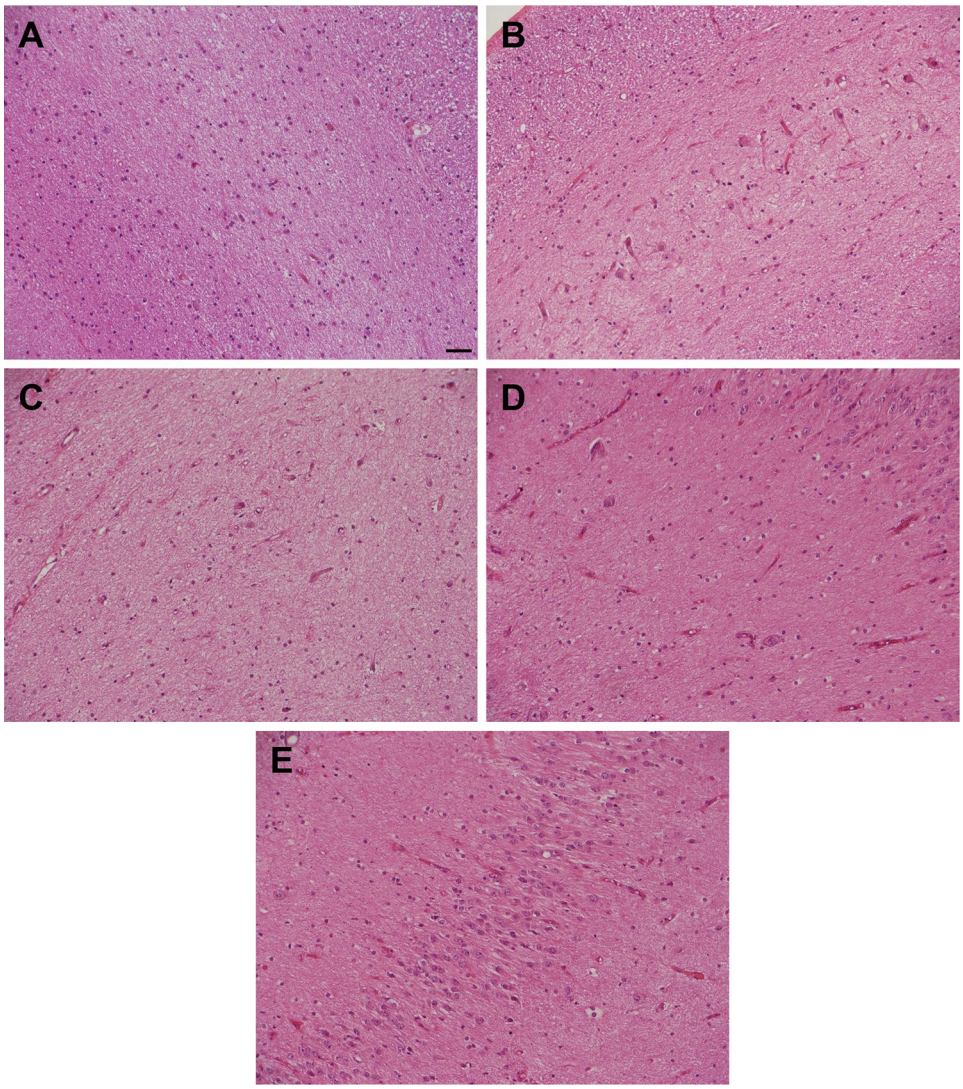

Fig. 3 Morphological findings in hippocampal sectors and dentate gyrus of patients with hippocampal sclerosis. Note a reduction in the number of neurons in the hippocampal sectors (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and dentate gyrus (**e**), with predominance of pyramidal cell loss in CA1 and CA4. Gliosis is observed in all regions. Hematoxylin and eosin. Scale bar (**a**–**e**): 50 µm

for HS, Blümcke et al. (2013) reported Type 1 as the most common, in which neuronal loss predominates in CA1 and CA4 (more than 80% and 40–90%, respectively) and is associated with intense gliosis. Those authors also stated that the other sectors showed gliosis and expressive neuronal loss, that is, about 30–50% of pyramidal neurons in CA2 and 30–90% in CA3. Moreover, the dentate gyrus showed loss of approximately 50–60% of granular cells (Blümcke et al. 2013). Thus, we conclude that ILAE diagnostic criteria for HS classification are both easy to apply and facilitate comparisons between results from different institutions.

Even though cell loss and gliosis are known to be typical features of HS, its etiology and physiopathogenesis are still debatable (Thom 2014). In this sense, several experimental models have been used to better understand the mechanisms underlying HS (Engel 1996). Norwood et al. (2010) described a model of TLE in which restricted and consecutive electrical stimulation is applied to the perforant path, the main hippocampal input, in rats. The authors reported both recurrent seizures and neuropathological alterations similar to those identified in the hippocampus of patients with HS.

By using the same experimental model and mass spectrometry-based proteomics our group identified proteins differentially expressed in the dorsal and ventral dentate gyrus of the hippocampus (submitted data). In rodents, these regions correspond phylogenetically to the tail and head of the human hippocampus, respectively (Strange et al. 2014). Therefore, it may be hypothesized that protein expression changes detected in rodents could also be verified over the hippocampus (including the body) of humans with HS. The body is the hippocampal region that should be evaluated to identify neuronal loss and gliosis, as recommended by the ILAE (Blümcke et al. 2013). In our hands, analyses of the experimental samples by spectral counting showed that the mitochondrial ATP synthase subunit beta (ATP5B) and membrane ATPases were downregulated, an observation

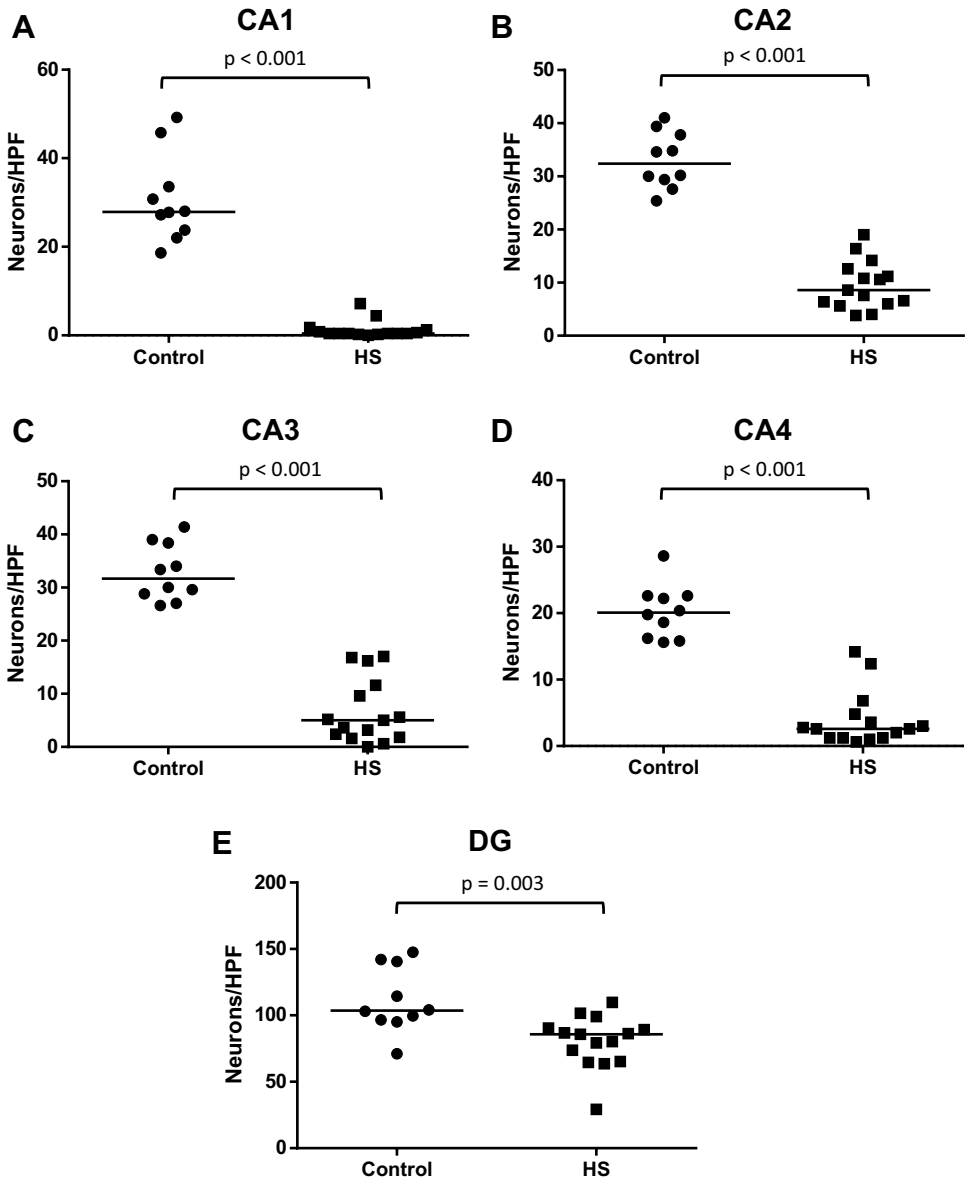

Fig. 4 Number of neurons counted in hippocampal sectors and dentate gyrus (DG) of controls and patients with hippocampal sclerosis. The counting was performed in five high-power fields representative of each sector (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) or the DG (**e**) in hematoxylin and eosin-stained sections. Each dot corresponds to the mean value of five high-power fields of each individual. The full line parallel to the *X*-axis corresponds to the median of each group. In A (CA1) the full line of the HS group coincides with the *X*-axis. Significant differences between hippocampal sclerosis (HS) and control groups were verified in each hippocampal sector (CA1–4) and in the DG. Please refer to the text for details on quantitative data; *p* values are shown in the Figure

that could be associated with the induction of seizures in animals. Then, we proceeded with the translational investigation in surgical samples.

ATP5B is a subunit of the mitochondrial ATP synthase that catalyzes ATP production during oxidative phosphorylation by using an electrochemical gradient of protons. This process is the main source of ATP in neurons, thus correlating with cellular activity (Filosto et al. 2011). As far as we know, there are no previous reports on ATP5B expression changes in surgical specimens of individuals undergoing HS treatment. However, altered expression of this protein has been described in other neurological conditions in humans. In fact, Xu and Li (2016) reported a significant increase in ATP5B expression in both neoplastic glial and endothelial cells of glioblastoma in comparison with normal brain tissue. Such increase was detected both through quantification of messenger RNA and immunohistochemistry and would be associated with the high demand for ATP by the neoplastic cells.

In the current study, we observed a significant decrease in the immunostaining of ATP5B in CA1, CA2, CA3, CA4 sectors and DG of the hippocampus of patients with HS compared with the control group. Such findings are similar to those that we found in the DG of rats submitted to consecutive electrical stimuli of the perforant pathway and that presented with extensive hippocampal cell death. These observations suggest that neuronal cell maintenance may be related to ATP synthase levels detected in the hippocampus. In accordance with this possibility, Danis et al. (2011) reported no change in the expression of another subunit of ATP synthase (delta) after using proteomics to investigate the hippocampus of rats genetically modified to display

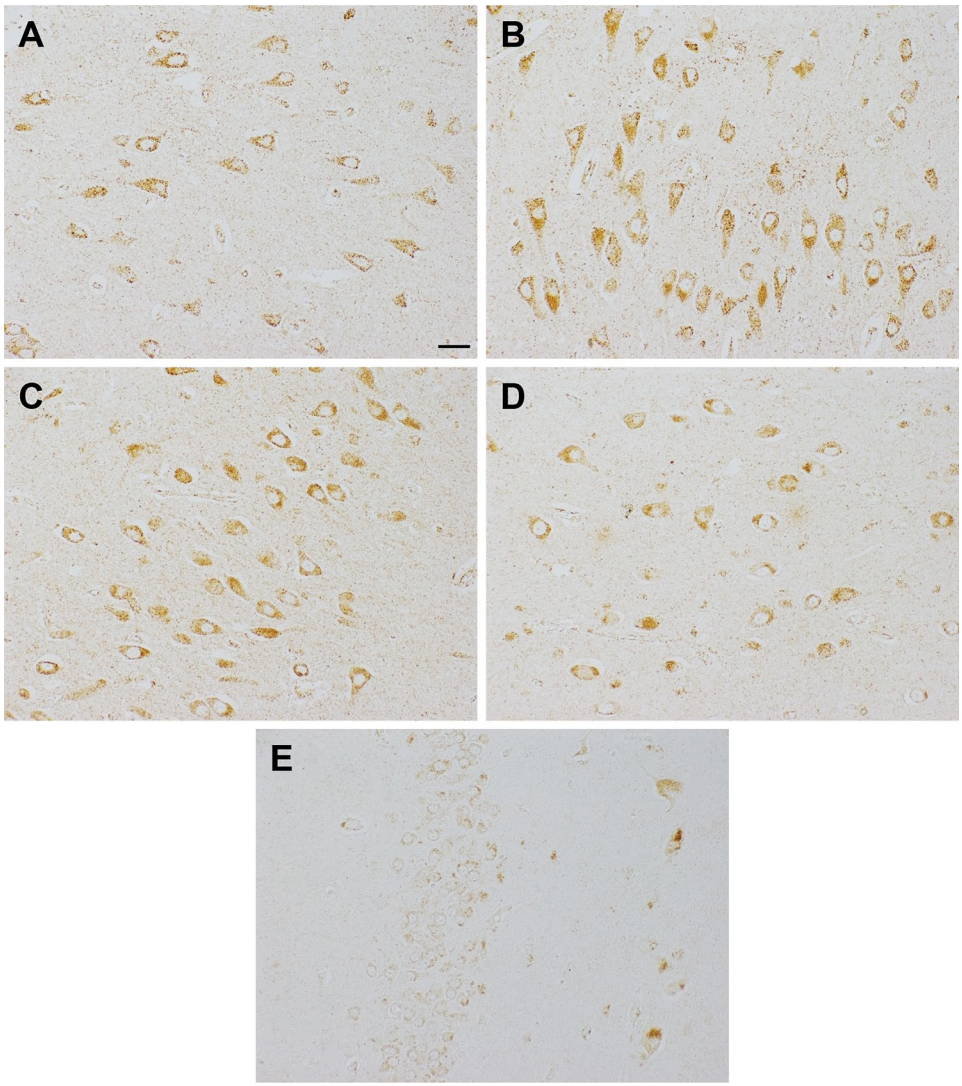

Fig. 5 Morphological and immunostaining findings for beta subunit of mitochondrial ATP synthase in the hippocampal sectors and dentate gyrus of controls. Note granular cytoplasmic staining in pyramidal (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and dentate gyrus (**e**) neurons. Immunoperoxidase. Scale bar (**a**–**e**): 40 µm

electrophysiological and behavioral features similar to those observed in human absence epilepsy. In this model (genetic absence epilepsy rats from Strasbourg), neither cellular structural alteration (including cell loss) is detectable nor the hippocampus is considered an epileptogenic region. Moreover, Noh et al. (2004) reported increase in gene expression of the beta subunit of mitochondrial ATP synthase in the hippocampus of rats submitted to ketogenic diet (KD) for 1 month compared with animals that were not fed on KD. The authors associated such increase with neuronal survival as KD may enhance ATP production and has been shown to be protective against hippocampal cell death induced by kainic acid. Thus, considering (1) the role of ATP5B in generating energy under physiological and pathological conditions, (2) the results that we had in the present study, and (3) the findings reported by others, it is possible to hypothesize that the reduction in ATP5B expression that we detected in all sectors and DG of human sclerotic hippocampi may be associated with neuronal loss. Finally, although it has been recognized that CA1-4 sectors and DG present distinctive patterns of cellular loss in HS, the fact that we evaluated end-stage sclerotic hippocampi with significant reduction in ATP5B expression in all those regions (CAs and DG) does not allow us to exclude previous interregional differences regarding the level of this protein.

Membrane ATPases have been associated with altered cellular excitability and/or epileptogenesis (Vaillend et al. 2002; Heinzen et al. 2014; Rosewich et al. 2014). We investigated the alpha-3 subunit of sodium / potassium pump ATPase (NKAα3) as this subunit, encoded by the ATP1A3 gene, is predominantly expressed in neurons. Under physiological conditions, NKAα3 takes part in the establishment and maintenance of the electrochemical gradients of sodium and potassium ions across the plasma membrane. These gradients are essential for the regulation of osmolarity and electrical excitability (Dobretsov and Stimers 2005). Furthermore, neurological diseases have been associated with ATP1A3 gene mutations. Even though the mechanisms are

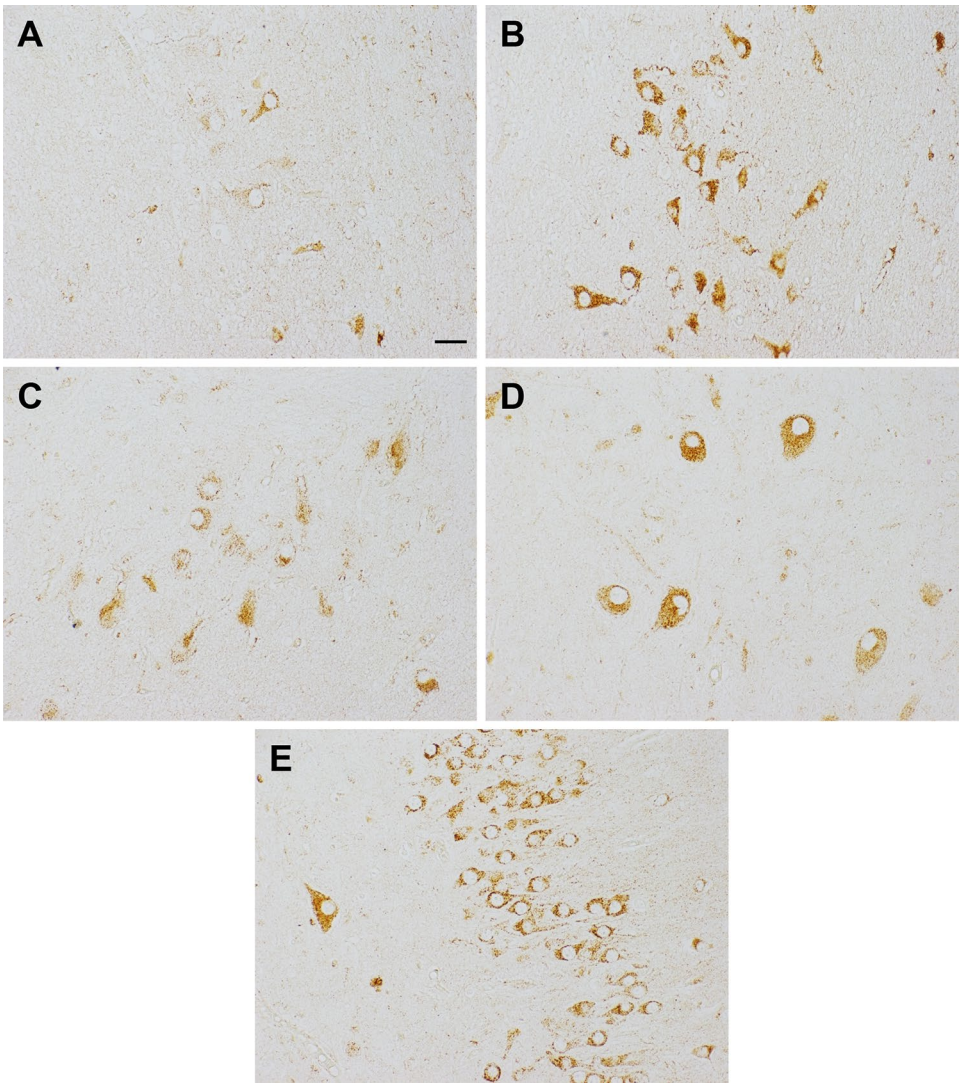

Fig. 6 Morphological and immunostaining findings for beta subunit of mitochondrial ATP synthase (ATP5B) in the hippocampal sectors and dentate gyrus of patients with hippocampal sclerosis. Granular cytoplasmic staining in pyramidal (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and dentate gyrus (**e**) neurons. No morphological differences were noted in the pattern of staining in comparison with control group. However, ATP5B tissue expression was significantly different between the two groups as detected by performing digital analyses in all hippocampal regions. Please refer to "Tissue Expression Analysis of Beta Subunit of Mitochondrial ATP Synthase (ATP5B)" for details. Immunoperoxidase. Scale bar (**a**–**e**): 40 µm

unknown, it has been considered that such mutations are involved with conditions characterized by alteration of neuronal excitability, such as epilepsy. In fact, Vaillend et al. (2002) observed a decrease in sodium/potassium ATPase activity and occurrence of epileptiform activity in the CA1 area of rat hippocampus slice.

In the present study, tissue expression of NKAα3 did not alter in HS specimens in comparison with controls. It is possible that, in the human hippocampus, changes in the expression of this enzyme may not be determinant of neuronal loss. However, since we studied patients with long history of seizures, whose specimens showed extensive cellular loss, it is not possible to rule out that NKAα3 expression alterations interfered previously with ionic homeostasis/neuronal excitability, thus taking part in the process of neuronal demise.

Finally, our data show that there is a negative correlation between the frequency of pre-operative seizures and the number of neurons in CA1. This observation supports the fact that the CA1 sector is the most susceptible to deleterious conditions, such as ischemia and frontotemporal dementia (Hatanpaa et al. 2014). The mechanisms underlying the selective vulnerability of hippocampal neurons are still unknown but some hypotheses have been put forward. In the hypoxic-ischemic injury there is strong evidence supporting the role of glutamate excitotoxicity. In fact, high concentrations of this neurotransmitter lead to excessive and uncontrolled intracellular levels of calcium and apoptosis (Hatanpaa et al. 2014). In experimental studies with rodents, it has been shown that there is a greater amount of glutamate receptors in CA1 cells compared with other hippocampal sectors (Butler et al. 2010). Additionally and mainly related to neurodegenerative diseases, CA1 neurons would be more vulnerable to proteasomes inefficient at degrading damaged

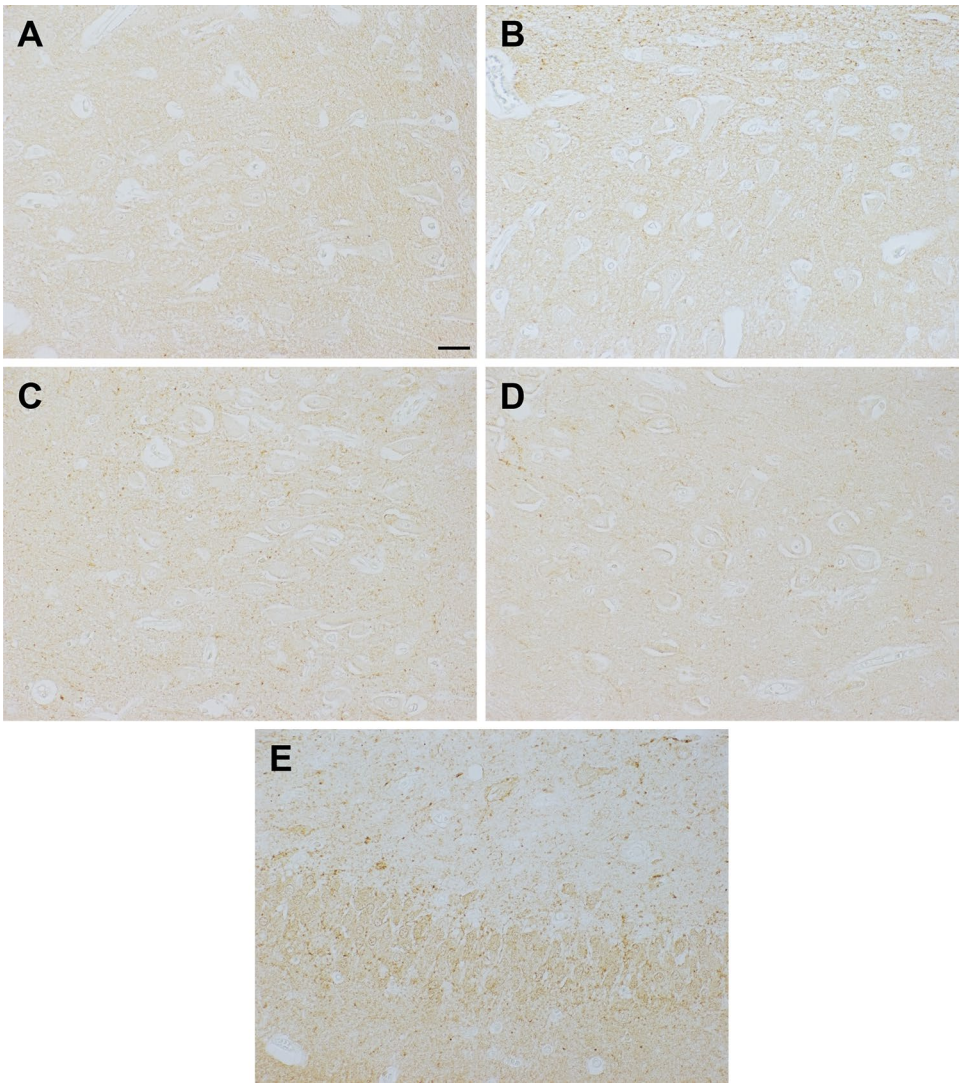

Fig. 7 Morphological and immunostaining findings for sodium/potassium-transporting ATPase subunit alpha-3 in the hippocampal sectors and dentate gyrus of controls. Note diffuse granular staining predominantly in the neuropil of all hippocampal sectors (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and the dentate gyrus (**e**). Immunoperoxidase. Scale bar (**a**–**e**): 40 µm

proteins, which eventually accumulate inside the cell and cause apoptosis (Hatanpaa et al. 2014).

In conclusion, our study shows altered expression of ATP5B in all hippocampal sectors and dentate gyrus of patients with HS ILAE Type 1. In this context, it is possible that the seizures may have reduced the neuronal population and ATP5B levels. Further studies may specifically address the role of ATP5B on the neuronal loss process characteristic of HS in experimental models. In addition, biochemical evaluation in HS surgical specimens may provide insights into the activity of this enzyme and oxidative metabolism in the remaining hippocampal cells, possibly contributing to a better understanding of the mechanisms leading to seizures in this frequent cause of epilepsy.

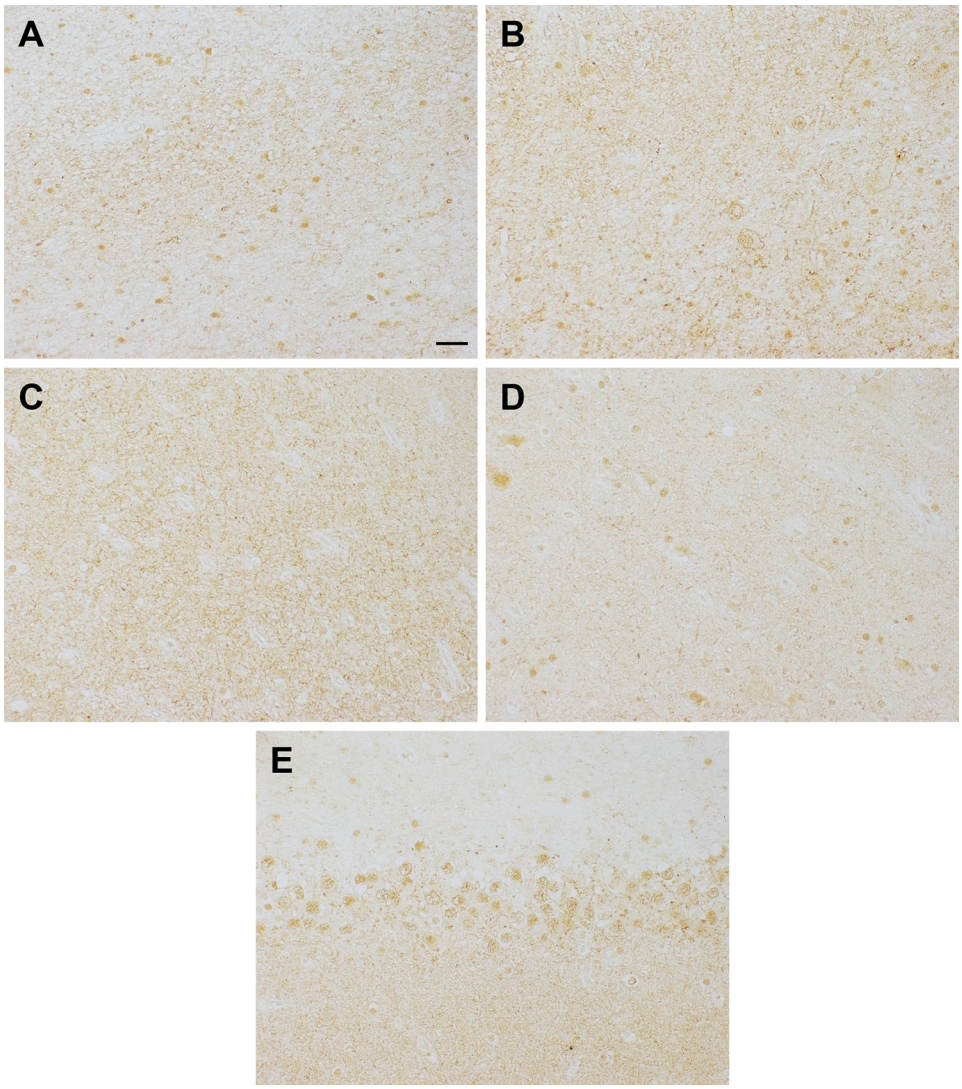

Fig. 8 Morphological and immunostaining findings for sodium/potassium-transporting ATPase subunit alpha-3 in the hippocampal sectors and dentate gyrus of patients with HS. Diffuse granular staining predominantly in the neuropil of all hippocampal sectors (CA1 (**a**), CA2 (**b**), CA3 (**c**), CA4 (**d**)) and the dentate gyrus (**e**), as observed in controls. No difference was verified between control and sclerotic hippocampus after performing digital analyses. Immunoperoxidase. Scale bar (**a**–**e**): 40 µm

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Conflict of interest Dr Iscia Lopes-Cendes was an ad-hoc specialist for Biomarin (Dec. 2017). The other authors declare that they have no conflict of interest.

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Affiliations

Marcelo Vilas Boas Mota[1] · Bruna Cunha Zaidan[1] · Amanda Morato do Canto[2] · Enrico Ghizoni[3] · Helder Tedeschi[3] · Luciano de Souza Queiroz[1] · Marina K. M. Alvim[3] · Fernando Cendes[3] · Iscia Lopes-Cendes[2] · André Almeida Schenka[4] · André Schwambach Vieira[5] · Fabio Rogerio[1]

Marcelo Vilas Boas Mota
motabuarque@yahoo.com.br

Bruna Cunha Zaidan
zaidanbruna@yahoo.com.br

Amanda Morato do Canto
amanda.morato.canto@gmail.com

Enrico Ghizoni
ghizonie@gmail.com

Helder Tedeschi
htedeschi@hotmail.com

Luciano de Souza Queiroz
gradanat@fcm.unicamp.br

Marina K. M. Alvim
marinakma@gmail.com

Fernando Cendes
fcendes@unicamp.br

Iscia Lopes-Cendes
icendes@unicamp.br

André Almeida Schenka
schenka@fcm.unicamp.br

André Schwambach Vieira
vieira.as@gmail.com

[1] Department of Anatomical Pathology, School of Medical Sciences, University of Campinas (UNICAMP), Av. Tessália Vieira de Camargo, 126, Campinas, SP CEP 13083-887, Brazil

[2] Department of Medical Genetics, School of Medical Sciences, University of Campinas (UNICAMP), Av. Tessália Vieira de Camargo, 126, Campinas, SP CEP 13083-887, Brazil

[3] Department of Neurology, School of Medical Sciences, University of Campinas (UNICAMP), Av. Tessália Vieira de Camargo, 126, Campinas, SP CEP 13083-887, Brazil

[4] Department of Pharmacology, School of Medical Sciences, University of Campinas (UNICAMP), Av. Tessália Vieira de Camargo, 126, Campinas, SP CEP 13083-887, Brazil

[5] Department of Structural and Functional Biology, Institute of Biology, University of Campinas (UNICAMP), R. Monteiro Lobato, 255, Campinas, SP CEP 13083-852, Brazil