

BETA-TUBULIN EXPRESSION IN THE HIPPOCAMPUS OF PATIENTS WITH MESIAL TEMPORAL LOBE EPILEPSY

EXPRESSÃO DE BETA-TUBULINA NO HIPOCAMPO DE PACIENTES COM EPILEPSIA DO LOBO TEMPORAL MESIAL

EXPRESIÓN DE BETA-TUBULINA EN EL HIPOCAMPO DE LOS PACIENTES CON EPILEPSIA DEL LÓBULO TEMPORAL MESIAL

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ABSTRACT

Introduction: The neuronal loss and abnormal mossy fibers sprouting are frequently observed in patients with mesial temporal lobe epilepsy (MTLE). Beta-tubulin, a cytoskeleton protein, is critical for the maintenance of the neuritic structure. **Objective:** Considering the axonal reorganization in patients with MTLE, our objective was to analyze the beta-tubulin expression in the hippocampus of these patients. **Methods:** We evaluated the hippocampus of 38 MTLE patients and seven control cases. Histological sections were submitted to neo-Timm histochemistry to evaluate the sprouting of mossy fiber, and to immunohistochemistry for neuronal density evaluation (NeuN) and beta-tubulin expression. **Results:** The MTLE group showed lower neuronal density than the control group in the granular layer (GL), hilus, CA4, CA3, CA1, and presubiculum. The MTLE group showed higher gray value on the neo-Timm staining when compared to the control group in GL, IML, and outer molecular layer (OML), and sprouting of thicker mossy fibers in the IML. When compared to the control group, group MTLE showed higher beta-tubulin expression in GL and lower expression in CA3 region. The aberrant sprouting of mossy fibers correlated inversely with the beta-tubulin expression in several subfields of the hippocampal formation. **Conclusions:** The differential expression of beta-tubulin in the regions CA3 and GL of the MTLE group, as well as its correlation with neuronal loss and the mossy fiber sprouting, suggests a possible role of this protein in the neuropathological changes that occur in the hippocampus in chronic cases of MTLE.

Keywords: Temporal lobe epilepsy; Cytoskeleton; Tubulin; Hippocampus.

RESUMO

Introdução: A perda neuronal e o brotamento anormal de fibras musgosas são observados com frequência em pacientes com epilepsia do lobo temporal mesial (ELTM). A beta-tubulina, uma proteína do citoesqueleto, é essencial para a manutenção da estrutura neurítica. **Objetivo:** Considerando a reorganização axonal nos pacientes com ELTM, nosso objetivo foi analisar a expressão de beta-tubulina no hipocampo desses pacientes. **Métodos:** Foram avaliados 38 hipocampos de pacientes com ELTM e sete casos controle. Cortes histológicos foram submetidos à histoquímica de neo-Timm para avaliação do neobrotamento de fibras musgosas e à imuno-histoquímica para avaliações da densidade neuronal (NeuN) e da expressão de beta-tubulina. **Resultados:** O grupo ELTM apresentou menor densidade neuronal do que o grupo controle na camada granular (CG), hilo, CA4, CA3, CA1 e no pró-subículo. O grupo ELTM apresentou maior valor de cinza na coloração neo-Timm com relação ao grupo controle na CG, CMI e camada molecular externa (CME) e neobrotamento mais espesso de fibras musgosas na CMI. O grupo ELTM apresentou maior expressão de beta-tubulina na CG e menor expressão na região de CA3, quando comparado ao grupo controle. O neobrotamento aberrante de fibras musgosas correlacionou-se inversamente com a expressão de beta-tubulina em diversos subcampos da formação hipocámpal. **Conclusões:** A expressão diferencial da beta-tubulina nas regiões da CA3 e CG do grupo ELTM, assim como suas correlações com a perda neuronal e o neobrotamento de fibras musgosas sugerem uma possível participação dessa proteína nas alterações neuropatológicas que ocorrem no hipocampo nos casos crônicos de ELTM.

Descritores: Epilepsia do lobo temporal; Citoesqueleto; Tubulina; Hipocampo.

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RESUMEN

Introducción: La pérdida neuronal y la brotación anormal de fibras musgosas se observan con frecuencia en los pacientes con epilepsia del lóbulo temporal mesial (ELTM). La beta-tubulina, una proteína del citoesqueleto, es crítica para el mantenimiento de la estructura neurítica. **Objetivo:** Teniendo en cuenta la reorganización axonal en pacientes con ELTM, nuestro objetivo fue analizar la expresión de beta-tubulina en el hipocampo de estos pacientes. **Métodos:** Se evaluó el hipocampo de 38 pacientes con ELTM y siete casos de control. Cortes histológicos fueron sometidos a la histoquímica neo-Timm para evaluar la brotación de fibras musgosas, y a inmunohistoquímica para la evaluación de la densidad neuronal (NeuN) y la expresión de beta-tubulina. **Resultados:** El grupo ELTM mostró una menor densidad neuronal que el grupo control en la capa granular (CG), hilo, CA4, CA3, CA1 y pró-subículo. El grupo ELTM mostró mayor valor de gris en la tinción neo-Timm en comparación con el grupo control en CG, CMI y en la capa externa molecular (CME), y la brotación de fibras musgosas más gruesas en la CMI. El grupo ELTM mostró una mayor expresión de beta-tubulina en CG y expresión más baja en la región CA3, cuando se compara con el grupo control. La brotación aberrante de fibras musgosa está inversamente correlacionada con la expresión de beta-tubulina en varios subcampos de la formación del hipocampo. **Conclusiones:** La expresión diferencial de beta-tubulina en las regiones CA3 y CG del grupo ELTM, así como su correlación con la pérdida neuronal y el surgimiento de fibras musgosas, sugiere un posible papel de esta proteína en los cambios neuropatológicos que se producen en el hipocampo en los casos crónicos de ELTM.

Descriptores: Epilepsia del lóbulo temporal; Citoesqueleto; Tubulina; Hipocampo.

INTRODUCTION

Mesial temporal lobe epilepsy (MTLE) is the most common form of drug-resistant epilepsy in adults. Hippocampal sclerosis is often found in MTLE and is characterized by neuronal loss, gliosis, and mossy fiber sprouting¹⁻³. The specific neuronal loss in hilus and CA3 subfields is intrinsically associated with sprouting of the mossy fiber to the molecular layers of *fascia dentata*⁴. This rearrangement in the neuronal circuitry is believed to contribute to hippocampal hyperexcitability^{5,6}. Studies in animal models and MTLE patients indicate the participation of the cytoskeleton in mossy fiber sprouting⁷⁻¹⁰.

The cytoskeleton is a highly dynamic structure that participates in plasticity process, cellular transport, cell morphology, and in the development and stabilization of axons and dendrites^{11,12}. Changes in the cytoskeleton can impair neuronal performance¹³. Microtubules are an important component of the cytoskeleton, participating in chromosome segregation, axonal transport, and neuronal polarity^{11,14}. Since beta-tubulin protein is an essential element of the microtubules, our objective was to analyze beta-tubulin expression in MTLE patients.

MATERIALS AND METHODS

Patients

We analyzed hippocampi from 38 patients with MTLE, submitted to epilepsy surgery at the Ribeirao Preto Epilepsy Surgery Program. Tissue collection and processing were conducted according to a protocol approved by our institution's Research Ethics Board (process HCRP 093/2008). For comparison, we used seven control hippocampi from necropsy.

Inclusion criteria for MTLE were: (I) seizure semiology consistent with MTLE; (II) pre-surgical investigation confirming the seizure onset zone in the temporal lobe; (III) anterior and mesial temporal interictal spikes on EEG; (IV) no lesions other than uni- or bilateral hippocampal atrophy on high-resolution magnetic resonance imaging scans; (V) clinical histopathological examination compatible with HS; and (VI) no evidence of dual pathology identifiable by any of the assessment methods described (clinical, electrophysiology, neuroimaging, and histopathology). Exclusion criteria were: (I) focal neurological ab-

normalities on physical examination; (II) generalized or extra-temporal EEG spikes; and (III) marked cognitive impairment indicating dysfunction¹⁵.

For the control group, inclusion criteria were: (I) age at death between 18 and 60 years; (II) *post mortem* time \leq 10 hours. The exclusion criteria were: (I) History of neurological disease; (II) Brain pathology present at necropsy evaluation; and (III) *post mortem* time $>$ 10 hours.

Tissue collection and hippocampal tissue processing

The hippocampal tissue was collected at the surgery center or autopsy room and sectioned into 1 cm thick coronal blocks. For the immunohistochemistries, blocks were fixed in formaldehyde, dehydrated, and paraffin-embedded. Blocks for neo-Timm histochemistry were fixed in glutaraldehyde with sodium sulfite, cryoprotected, and frozen.

Neo-Timm histochemistry and Immunohistochemistry

For neo-Timm staining¹, sections were processed in batches containing at least one HS and two controls cases (for standardization). Slices were immersed in a developer solution (10 mL 50% Arabic gum, 30 mL of citric acid 1.3 M and sodium citrate 0.9 M, 90 mL of hydroquinone 0.5 M, and 1.5 mL of silver nitrate 17%) for 40 min (light staining) or 50 min (dark staining). Sections were washed, dried, dehydrated, xylene-cleared, and mounted in Krystalon (EM Science, Gibbstown, USA).

Immunohistochemistry was performed according to previously published protocols², with antibodies against NeuN (Chemicon-Millipore, Billerica, MA, USA) and beta-tubulin protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted at 1:1000 and 1:25, respectively. After the revelation, sections were dehydrated, cleared with xylene, and mounted with Krystalon.

Neuron count and neo-Timm quantification

The hippocampal subfields were subdivided according to Lorente de No's classification¹⁶, and included: inner (IML) and outer molecular layer of *fascia dentata* (OML); granular layer of fascia dentate (GL), hilus, CA4, CA3, CA2, CA1, prosubiculum, subiculum, parasubiculum, and layer III of entorhinal cortex. Mossy fiber sprouting was evaluated in neo-Timm

stained sections in the hilus, granular layer, IML, and OML. Neuronal counting was performed in sections submitted to NeuN immunohistochemistry, in GL, hilus, CA4, CA3, CA2, CA1, prosubiculum, subiculum, parasubiculum, and layer III of entorhinal cortex. Neuron density was estimated according to Abercrombie's method¹⁷. Beta-tubulin expression was evaluated as an immunoreactive area for beta-tubulin antigen, following published protocols¹⁸⁻²⁰.

All measurements were done in Image J analysis system (NIH, USA, public domain). The statistical analyzes were realized by SigmaPlot (version 11) program. We used test t for comparison between MTLE and control groups. Statistical significance was set at $p < 0.05$.

RESULTS

Clinical Findings

The clinical variables of MTLE and control groups are presented in Table 1. The age of the control group was higher than MTLE group ($p = 0.048$, Fisher's exact test). The two groups of patients had no differences in gender or collected side.

Histological evaluation

MTLE group had reduced neuron density in GL, hilus, CA4, CA3, CA1, and prosubiculum when compared to control ($p \leq 0.009$; Figure 1). There were no differences between control and MTLE in CA2, subiculum, parasubiculum, and entorhinal cortex.

Neo-Timm staining was significantly higher in GL, IML, and OML of MTLE group, compared to controls ($p < 0.001$; Figure 2).

There was no difference between MTLE and control groups in the hilus. The length of mossy fiber sprouting in the IML of MTLE was of $222.02 \pm 76.58 \mu\text{m}$, whereas control cases had no mossy fiber sprouting in this region (Figure 2 D).

Beta-tubulin expression was seen in cell bodies, axons, and dendrites. MTLE patients had increased immunoreactive area for beta-tubulin in GL and decreased in CA3 when compared to the control group ($p < 0.005$; Figure 3).

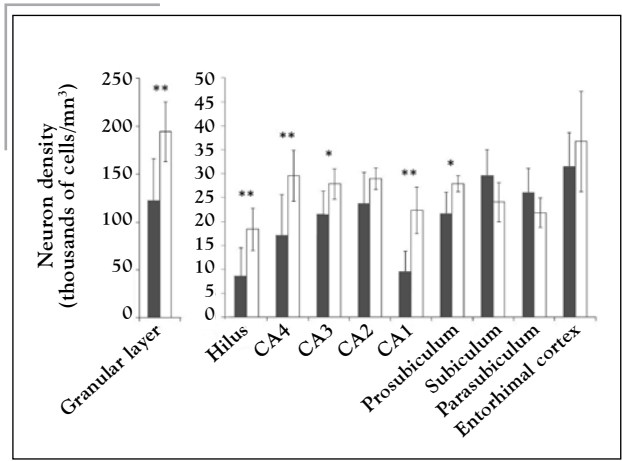


Figure 1. Neuron density in human hippocampal formation. The MTLE group (black bars) had lower neuron densities than the control group (white bars) in GL, hilus, CA4, CA3, CA1 e prosubiculum. * $p < 0.05$; ** $p < 0.001$.

Table 1. Clinical variables from MTLE and control groups.

Clinical variable		MTLE	Control	Statistics
Gender (n)	Male	17	4	No difference
	Female	21	3	
IPI (n)	Present	15	n.a.	n.a.
	Absent	22	n.a.	n.a.
Age of first seizure (years)		6.9 ± 7.9	n.a.	n.a.
Age of recurrent seizures (years)		11.5 ± 7.5	n.a.	n.a.
Type of seizure (n)	CPS	13	n.a.	n.a.
	CPSG	25	n.a.	n.a.
Seizure Frequency (Seizures by month)		15 ± 19.8	n.a.	n.a.
Hand dominance (n)	Right	35	n.a.	n.a.
	Left	2	n.a.	
	Bilateral	1	n.a.	
Verbal memory tasks (n)	Average or above	9	n.a.	n.a.
	Below average	23	n.a.	
Non-verbal memory tasks (n)	Average or above	10	n.a.	n.a.
	Below average	22	n.a.	
Full-scale IQ		84.2 ± 11.3	n.a.	n.a.
Years at school		5.7 ± 4.2	n.a.	n.a.
Age at procedure* (years)		38.7 ± 7.1	48.1 ± 18.9	$p = 0.048$
Epilepsy duration (years)		27.5 ± 10.1	n.a.	n.a.
Collected side (n)	Right	19	3	No difference
	Left	19	4	

Values presented as mean ± standard deviation. IPI: initial precipitant insult; CPS: complex partial seizure; CPSG: complex partial seizure with secondary generalization; HS: hippocampal sclerosis; IQ: intelligence quotient; n = number of cases; n.a.: not applicable; *Age at surgery for MTLE and at death for control cases.

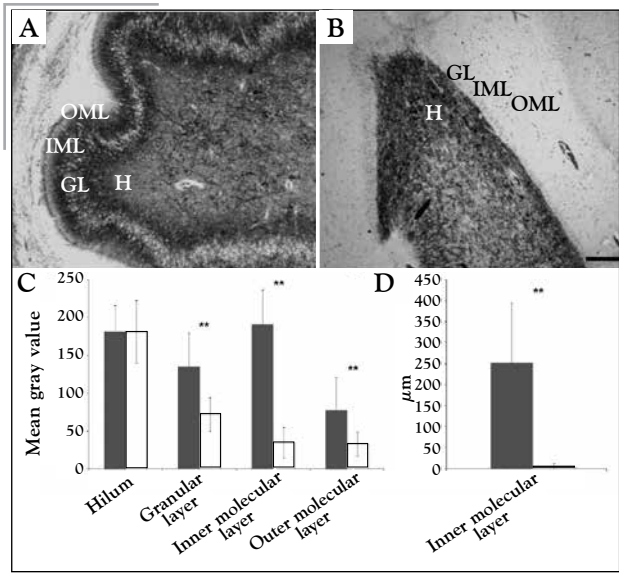


Figure 2. Mossy fiber sprouting. Illustrative image of neo-Timm histochemistry in MTLE (A) and control (B) patients GL: granular layer; H: hilus; IML: inner molecular layer and OML: outer molecular layer. C: Gray value graph of MTLE (black bars) and control (white bars) groups. D: Length of mossy fiber sprouting graph in MTLE and control groups. Asterisks: $p < 0.001$. Bar (A-B): $200 \mu\text{m}$.

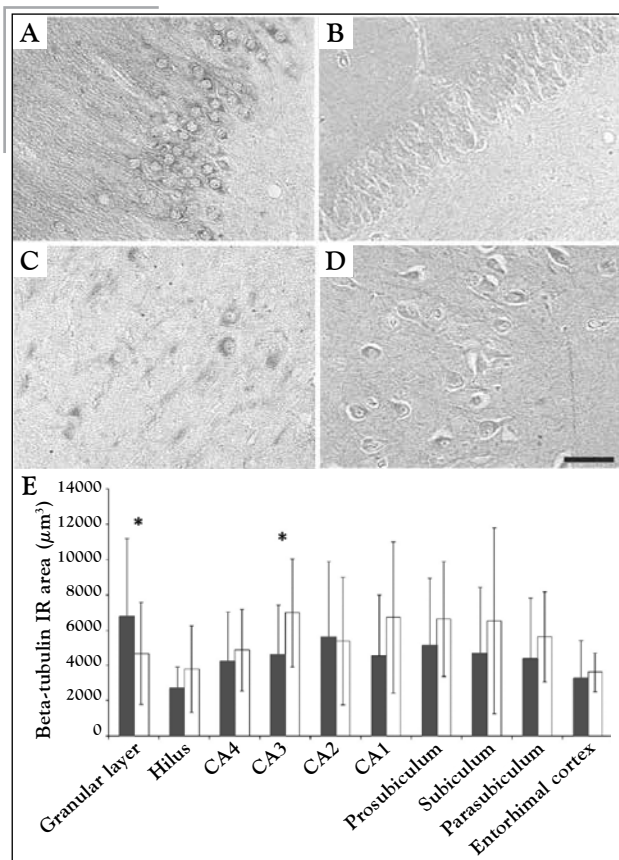


Figure 3. Beta-tubulin expression in hippocampal subfields of MTLE and control cases. In granular layer, the MTLE group (A and E) had higher beta-tubulin expression than the control group (B and E). In CA3 the MTLE group (C and E) had less beta-tubulin expression than the control group (D and E). The black bars in E represent MTLE group and white bars represent the control group. * $p < 0.05$. Bar (A-D) = $50 \mu\text{m}$.

Correlations

In the MTLE group, we found positive correlations between beta-tubulin expression in the entorhinal cortex and CA1 ($p = 0.002$; $r = 0.71$) and subiculum ($p = 0.005$; $r = 0.70$). Neuron density and beta-tubulin expression correlated positively in the subiculum ($p = 0.03$; $r = 0.45$). Neo-Timm gray values in IML showed a negative correlation with beta-tubulin expression in CA4 ($p = 0.04$; $r = -0.65$) and gray values in OML showed negative correlation with beta-tubulin expression in the entorhinal cortex ($p = 0.05$; $r = -0.76$). The expression of beta-tubulin in the hilus correlated negatively with the length of GL ($p = 0.03$; $r = -0.65$) and the length of IML ($p = 0.0005$; $r = -0.87$). In the control group, we found only a positive correlation between age and beta-tubulin expression in hilus ($p = 0.04$; $r = 0.75$).

DISCUSSION

The MTLE group had lower neuronal density than the control group in GL, hilus, CA4, CA3, CA2, CA1, and the prosubiculum. This pattern of cell loss corresponds to the classical HS (HS type 1 according to the new ILAE classification) described in the literature²¹⁻²⁵. The CA2 and the subiculum showed preserved neuronal density^{26,27}. We found a positive correlation between the neuronal density and beta-tubulin expression in subiculum, suggesting a preservation of both neuron density and cell morphology. The positive correlation between the beta-tubulin expression in CA1 and the entorhinal cortex might indicate the preservation of the alvear pathway, a fiber pathway of entorhinal axons that make synapses with CA1 neurons²⁸.

Mossy fiber sprouting and reorganization of axon collaterals are important histopathological changes observed in MTLE^{123,29}. After intense neuronal loss in hilus, CA4, and CA3 regions, the mossy fibers reorganize toward the molecular layers of fascia dentata, resulting in new synaptic terminals with dendrites of local interneurons and also granule cells²⁹. We found increased neo-Timm staining in IML and OML, indicating mossy fiber sprouting into the molecular layers of fascia dentata. Furthermore, we saw that the lower the beta-tubulin expression in the hilus and CA4, the higher the degree of mossy fiber sprouting in the molecular layer, corroborating the association between neuron loss and axonal reorganization in MTLE.

The decreased beta-tubulin expression in CA3 of MTLE patients might be related to neuronal loss and degenerative changes that occur in this region. Neuropathological studies in patients and animal models of MTLE showed significant neuronal degeneration and decreased dendritic arborization in CA3 subfield^{7,30-32}. In the MTLE group, we found increased beta-tubulin expression in GL, even with neuronal loss in this region. The increased beta-tubulin expression in the GL can be related to the mossy fiber sprouting seen in our MTLE patients.

In summary, our data indicate changes in beta-tubulin expression in the hippocampus of MTLE patients. We found correlations between beta-tubulin expression, neuronal loss, and mossy fiber sprouting. Our study suggests that changes in beta-tubulin expression could be an indicative of neuron loss and mossy fiber sprouting.

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