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Different levels of MT-I/II between patients with MTLE with or without seizure generalization: does hippocampal MT-I/II affects seizure spread, or does seizure spread promotes differential expression of MT-I/II?

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Different Levels of MT-I/II Between Patients With MTLE With or Without Seizure Generalization: Does Hippocampal MT-I/II Affects Seizure Spread, or Does Seizure Spread Promotes Differential Expression of MT-I/II?

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ABSTRACT

In the central nervous system, zinc is released along with glutamate during neurotransmission and, in excess, can promote neuronal death. Experimental studies have shown that metallothioneins I/II (MT-I/II), which chelate free zinc, can affect seizures and reduce neuronal death after *status epilepticus*. Our aim was to evaluate the expression of MT-I/II in the hippocampus of patients with temporal lobe epilepsy (TLE). Hippocampi from patients with pharmacoresistant mesial temporal lobe epilepsy (MTLE) were evaluated for expression of MT-I/II and for neuronal, astroglial, and microglial populations. Compared to control cases, MTLE group displayed widespread increase in MT-I/II expression, astrogliosis and reduced neuronal population. MT-I/II levels did not correlate with any clinical variables, but patients with secondary generalized seizures (SGS) had less MT-I/II than patients without SGS. In conclusion, MT-I/II expression was increased in hippocampi from MTLE patients and our data suggest that it may be associated with different seizure spread patterns.

Keywords: Metallothioneins; zinc homeostasis; gliosis; epilepsy; neuronal density.

RESUMO

Níveis diferentes de MT-I/II entre pacientes com MTLE com ou sem crise generalizada: os níveis hipocampais de MT-I/II afetam o alastramento das crises, ou o alastramento das crises promove expressão diferencial de MT-I/II?

No sistema nervoso central, o zinco é liberado juntamente com o glutamato durante a neurotransmissão e, quando liberado em excesso, pode promover morte neuronal. Estudos indicam que as metalotioneínas I/II (MT-I/II), proteínas quelantes de zinco livre, podem afetar parâmetros relacionados às crises e reduzir a morte neuronal subsequente a um *status epilepticus*. Nosso objetivo foi avaliar a expressão de MT-I/II no hipocampo de pacientes com epilepsia do lobo temporal (ELT). Hipocampos de pacientes com ELT mesial (ELTM) resistente ao tratamento farmacológico foram avaliados para a expressão de MT-I/II e para as populações neuronal e astroglial. Quando comparadas com o grupo controle, pacientes com ELTM apresentaram aumento na expressão de MT-I/II, astrogliose e redução na densidade neuronal. Não foram observadas correlações entre os níveis de MT-I/II e as características clínicas dos pacientes, mas pacientes com crises secundariamente generalizadas apresentaram um aumento menor nos níveis de MT-I/II que os pacientes sem estas crises. Em resumo, um aumento na expressão de MT-I/II é observado em pacientes com ELTM e nossos dados sugerem que o aumento pode estar associado a diferentes padrões de crises epilépticas.

Unitermos: Metalotioneínas; homeostase do zinco; gliose; epilepsia; densidade neuronal.

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INTRODUCTION

Zinc (Zn^{2+}) is an important modulator of glutamatergic transmission in the central nervous system (CNS).[1-3] Zn^{2+} is concentrated in presynaptic vesicles, along with glutamate, and released during normal neurotransmission.[4-8] Hippocampal neurons are specially rich in vesicular Zn^{2+}, particularly in the axonal boutons of granule cells, CA3 and CA1 pyramidal cells and prosubicular neurons.[5-7,9,10] In temporal lobe epilepsy (TLE), one of the most frequent drug-resistant epilepsies in adults, the hippocampus is associated with seizure generation.[11,12] The intense neuronal activity during seizures can release high amounts of Zn^{2+} in the synaptic cleft,[13,14] promoting reactive oxygen species (ROS) production,[15] which can ultimately lead to hippocampal neuronal death.[13-17] In fact, studies in hippocampi from TLE patients who underwent epilepsy surgery have shown neuronal loss,[18-20] increased glial reaction[21-24] and reorganization of mossy fibers axon collaterals into the inner molecular layer of the granule cell dendrites.[19,25] This synaptic reorganization of Zn^{2+}-enriched terminals has been hypothesized to contribute to synchronous firing and epileptiform activity.[19]

Metallothioneins (MTs) are low molecular weight, cystein-enriched proteins that bound Zn^{2+} and cadmium. They can be found in various tissues, in four isoforms.[26] Isoforms I, II and III are found in the central nervous system (CNS), where the isoforms I and II are coexpressed in astrocytes and the isoform III is expressed in neurons.[27,28] MTs participate in Zn^{2+} homeostasis, scavenging ROS in the brain[29] and stimulate the expression of several neurotrophic and antiinflamatory factors.[30] Studies on rodent models of TLE have shown that MT expression is increased in the hippocampal formation shortly after seizures[31,32] and that high levels of MTs I and II are associated with reduced neuronal death after seizure-induced damage.[32-34]

Since MT-I/II levels may be associated with neuron survival after seizures, we hypothesize that MT-I/II expression is altered in TLE and can be associated with the preservation of neuronal density in the hippocampus of TLE patients. Therefore, in this study we evaluated the immunoexpression of MT-I/II and its correlation with hippocampal neuron density in hippocampi of patients with chronic TLE.

MATERIALS AND METHODS

Patients and clinical data

Patients with drug-resistant epilepsy were evaluated at the University of São Paulo Epilepsy Surgical Centre in Ribeirão Preto (Brazil), according to standard protocols published elsewhere.[35] MTLE patients (n=69) were patients with hippocampal atrophy or with normal hippocampal volume at MRI without other lesions associated with TLE.

For comparison purposes in the neuropathology studies, autopsy controls (Ctrl, n=20) were obtained from autopsy cases without history of neurological diseases, with no sign of CNS pathologies in *post mortem* pathological evaluation, and with less than 10 hours *post mortem*.

Medical records of all evaluated patients were assessed for clinical data analysis. The clinical variables investigated were age at death and cause of death for Ctrl patients and age at surgery, epilepsy duration, age at the first recurrent seizure, seizure frequency per month, presence of secondary generalized seizures, and neuropathological evaluation for MTLE patients. This study followed the principles of the Declaration of Helsinki, was registered in Brazilian's Health Ministry and was approved by our local ethics committee (processes HCRP 9370/2003 and HCRP 2634/2008).

Tissue collection and immunohistochemistry

Hippocampi from surgery or autopsy were cut in coronal sections and placed in 10% (vol/vol) buffered formaldehyde for one week, followed by paraffin embedding. Immunohistochemistry was performed in $8\,\mu m$ sections at the level of hippocampal body for evaluation of neuronal and astroglial populations and for MT-I/II expression with antibodies against, respectively, NeuN, GFAP and MT-I/II. The sections were submitted to endogenous peroxidase blocking with 4.5% H_2O_2 in 50 mM phosphate-saline buffer (PSB) pH 7.4, for 15 minutes, followed by microwave antigenic retrieval in 10mM sodium citrate buffer pH 6.0 (for GFAP) or 50 mM Tris-HCl pH 9.6 (for NeuN and MT-I/II). After achieving room temperature, the sections went through blocking free aldehyde groups with Tris-glycine 0.1 M pH 7.4 for 45 minutes, followed by blocking buffer with 5% defatted milk and 15% goat serum (# S-1000, Vector) in Triton buffer (PTB, 20mM phosphate + 0.45M NaCl, pH 7.4, with 0.3% Triton X-100) for four hours. The sections were then incubated with primary antibodies in blocking buffer for 16 hours. We used primary monoclonal antibodies raised in mouse anti-human GFAP (clone 6F2, #M0761, Dako), anti-murine NeuN (clone A60, #MAB377, Chemicon) and anti-equine MT-I/II (clone E9, #M0639, Dako), diluted in blocking buffer at concentrations of 1:500. The primary antibodies were detected using biotinylated rabbit anti-murine IgG (#E0354, Dako), at 1:200 dilution in blocking buffer, for one hour, followed by revelation with avidin-biotin-peroxidase system (Vectastain Elite ABC kit, #PK6100, Vector) and diaminobenzidine as chromogen (DAB, #34001, Pierce Biotechnology). The development times in DAB solution were 10.5 minutes for NeuN and 8 minutes for MT-I/II and GFAP.

Immunohistochemistry analysis

Images of all hippocampal regions were obtained with a video monochrome charge-coupled device camera (CCD;

Hamamatsu Photonics Model 2400, Japan) attached to an Olympus microscope (Model BX60, Melville, NY), and captured, averaged, and digitized using a frame grabber (Scion Corporation, Frederick, MD) on a Macintosh computer (Model G3, Cupertino, CA). Illuminance was uniformly maintained and regularly checked using optical density standards (Kodak, Rochester, NY). After captured, the image was analyzed using image system software (ImageJ, version 1.37c).

Quantification of the immunohistochemistry was performed with threshold tool, with the investigator blind to the group allocation. After the selection of the region of interest (ROI), the software calculated the immunopositive area by counting all pixels with gray intensity equal or superior to the threshold of staining. A complete protocol for threshold tool can be found at rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html. The threshold was defined for each protein evaluated, based on the mean immunopositivity of all control cases. The evaluated regions were outer molecular layer (OML), inner molecular layer (IML), granule cell layer (GCL), subgranular zone (SGZ), the hilus (HIL) and the stratus piramidale of CA4, CA3, CA2, CA1, prosubiculum (PRO) and subiculum (SUB). The characterization of hippocampal regions was based on the Lorente de Nó's classification.[36] Results were shown as percentage of immunopositive area/total area.

Additionally, neuronal density was evaluated in the NeuN stained sections. Neuronal count was processed in ImageJ 1.37c software with a 520x magnification for granule cell layer and 260x for pyramidal neurons of CA4, CA3, CA2, CA1, prosubiculum and subiculum. Neuronal densities were estimated with the correction of Abercrombie[37], which permits to estimate the neuronal density through mathematical method, and the results were shown as thousands of cells per cubic millimeter.

Statistical analysis

Statistics were carried out in SigmaStat 3.1 software. Tests for normality and homogeneity of variances were performed to define data distribution. For parametric variables, t-test was performed and, for the non-parametric variables, Mann-Whitney test was used. Correlation between MT expression and clinical variables was performed using Pearson's test. All results were considered significant at $p<0.05$.

RESULTS

Clinical data

The clinical characteristics of study participants are summarized in Table 1. Patients with MTLE and Ctrl patients have the same age ($p=0.175$). Epilepsy duration was 25 ± 10 years, and the age at onset was 13 ± 1. MTLE patients had seven seizures by month, being one of those a secondary generalized seizure (SGS).

Table 1. Clinical history of patients with MTLE and Ctrl cases

Group	Ctrl	MTLE
Age at evaluation* (years)	42±16	38±10
Epilepsy duration (years)	_	25±10
Age at epilepsy onset (years)	_	13±1
Minimal seizure frequency (per month)	_	7
Number of secondary generalizations (per month)	_	1
Frequency of secondary generalization (%)	_	59

* Age of death for Ctrl and age at surgery for TLE.

Immunohistochemistry evaluation

Neuronal density, estimated with the count of NeuN positive cells, was reduced in the granule cell layer, CA4, CA3, CA2, CA1, prosubiculum and subiculum of the MTLE group, when compared to Ctrl ($p < 0.03$). Increased astrogliosis was observed in all hippocampal regions of MTLE, compared to Ctrl ($p < 0.001$). MT-I/II expression, observed in astrocyte-like cells, was increased in all hippocampal subfields evaluated ($p < 0.032$). For further details, see Table 2.

Table 2. Neuronal density and percentage of immunopositive area for GFAP and MT-I/II in patients with MTLE and Ctrl

Region	NeuN*			GFAP**			MT-I/II**		
	MTLE	Ctrl	p	MTLE	Ctrl	p	MTLE	Ctrl	p
OML	_	_	_	59	4	<0.001	46	10	<0.001
IML				58	6	<0.001	58±31	34±30	0.003
GCL	187.5	375	<0.001	57±18	22±18	<0.001	46±26	28±26	0.008
SGZ	_	_	_	90	69	<0.001	48	11	<0.001
HIL	_	_	_	86	28	<0.001	30	7	<0.001
CA4	9.0	35.9	<0.001	72±22	18±21	<0.001	30	12	0.031
CA3	19±11	47±6	<0.001	60	6	<0.001	55±28	27±28	<0.001
CA2	29±9	41±4	<0.001	52	5	<0.001	62±25	30±29	<0.001
CA1	4.5	41.9	<0.001	87	2	<0.001	66±28	31±31	<0.001
PRO	19±8	32±3	<0.001	78	1	<0.001	64±29	34±32	<0.001
SUB	30±6	35±3	0.029	23	0.2	<0.001	66	31	<0.001

* Neuronal density, as thousands of cells per cubic millimeter.
** Percentage of immunopositive area in the amostral area.

The results are shown as median (for Mann Whitney test) or mean ± standard deviation (for Student's t-test). OML = outer molecular layer; IML = inner molecular layer; GCL = granule cell layer; SGZ = subgranule zone; HIL = hilus; PRO = prosubiculum; SUB = subiculum.

Tissue alterations and seizures

In MTLE group, patients without SGS had increased MT-I/II immunopositivity, when compared with patients with SGS, in the inner molecular layer ($p=0.037$), granule cell layer ($p=0.018$), subgranule zone ($p=0.004$), CA2 ($p=0.039$) and CA1 ($p<0.043$). A trend to increased MT-I/II immunopositivity was observed in the outer molecular layer ($p=0.072$), CA4 ($p=0.076$) and subiculum ($p=0.068$). No differences in neuronal or astroglial populations were observed between MTLE patients with or without SGS. Frequency of seizures did not correlate with NeuN, GFAP or MT-I/II in all hippocampal subfields.

DISCUSSION

In the present study, we found an increased MT-I/II expression in all hippocampal subfields of MTLE patients. In the CNS, MT-I/II are expressed mainly by astrocytes[38] and, when the tissue suffers an injury, increased MT-I/II expression is observed in astrocytes and microglias.[28,38] We also observed that higher degree of MT-I/II expression was observed in regions with higher astrogliosis. Increased glial population is a common finding in TLE[21-24] and is associated with the degree of neuronal death.[22-24,39] In our study, an increased expression of MT-I/II was observed in astrocytes and in a few neurons of some patients. Thus, our data support the notion that MT-I/II changes are essentially related to astroglial population.

Studies in rodents with kainic acid-induced SE showed an association between MT-I/II expression and neuronal protection. Transgenic mice over-expressing MT-I/II have reduced neuronal death, compared to wild type animals.[34] In addition, mice with reduced MT-I/II expression[32] or knockouts for MT-I/II[33] had increased neuronal death following SE, compared to wild type mice. In our study, however, the higher MT-I/II expression was observed in regions with lower neuronal density, indicating that MT-I/II was not associated with neuronal survival. In agreement with our data, an association between the severity of tissue damage and the increase in MT-I/II expression has been reported in mice subjected to soman-induced *status epilepticus* (SE).[31]

Data have shown that the increased MT-I/II immunoreactivity observed in animal models of TLE can also be a factor associated with the seizure generation process. Transgenic mice over-expressing MT-I, have increased seizure duration, a tendency to reduced latency, but similar number of seizures after KA administration.[34]

Since MT-I/II act chelating free Zn^{2+}[14,27] and Zn^{2+} chelation increases tissue excitability and facilitates seizure generation[40], excessive MT-I/II levels can reduce free Zn^{2+} in the synaptic cleft, increasing neuronal excitability and affecting seizure generation. We found no correlation between seizure frequency and MT-I/II expression in TLE.[34]

In MTLE, we found increased levels of MT-I/II in patients without SGS, when compared with those with SGS. This could indicate that MT-I/II is associated with different seizure spread patterns from the epileptogenic hippocampus to other brain regions. It is important to point out that no difference in neurons or glial cells was observed between MTLE with and without SGS. Studies from different groups also observed no association between changes in the hippocampus and SGS.[41-43] All those observations suggest that the increased MT-I/II expression in patients without SGS is not an effect of gliosis, but it is independently associated with SGS. Further studies with animal models of TLE must evaluate more closely the relationship between MT-I/II expression and seizure susceptibility.

Some limitations of our study must be pointed out. So far, studies about MT-I/II expression in animal models of TLE only evaluated the acute period following SE. Considering that our study was performed in patients with chronic epilepsy, it is difficult to establish comparisons between human and animal data. The lack of correlation between seizure frequency and MT-I/II expression does not exclude an association between seizures and MT-I/II expression. Other seizure characteristics, such as seizure duration and time between the last seizure and the surgery, could better correlate with MT-I/II expression than isolate seizure frequency.

Finally, our study may have translational implications in the future. The role of MTs in antiinflamatory response, neurotrophic factor expression, and protection against ROS and heavy metals make those proteins interesting for clinical applications. Studies have shown that EmtinB, a synthethic peptide that mimics the actions of MTs, attenuates KA-induced seizures and protects neurons from excitotoxic death.[30] Further studies with EmtinB and MTs should be done to evaluate the role of these proteins in neuronal survival and seizure susceptibility.

In summary, our data indicate that increased MT-I/II expression is a plastic alteration of chronic TLE, primarily related to the astrogliosis, a common finding in chronic TLE. Our findings suggest that increase MT-I/II expression may contribute to the control of the brain hyperexcitability.

ACKNOWLEDGMENTS

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