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REST/NRSF transcription factor is overexpressed in hippocampus of patients with drug-resistant mesial temporal lobe epilepsy

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ABSTRACT

The Repressor Element-1 Silencing Transcription factor or Neuron-Restrictive Silencer Factor (REST/NRSF) is a zinc finger repressor transcription factor of the Kruppel family. Several studies in experimental models have shown that overexpression of REST/NRSF occurs after the induction of seizures. In the present study, the expression of REST/NRSF (messenger ribonucleic acid (mRNA) and protein) was evaluated in the hippocampus of 28 patients with drug-resistant mesial temporal lobe epilepsy (MTLE) and their correlation with clinical variables and comorbid anxiety and depression. The REST/NRSF protein expression was augmented in an age-dependent manner in the hippocampus of autopsied subjects. However, this condition was not observed in patients with MTLE, in whom overexpression of this transcription factor occurred at both the mRNA and protein levels. The correlations with clinical variables showed that the frequency of epileptic seizures was proportional to the protein expression of REST/NRSF. The results revealed that the overexpression of REST/NRSF was more evident in patients with MTLE without anxiety and depression. Our data indicate that the expression of REST/NRSF is modified in patients with MTLE. This condition has implications in the pathophysiology of this disorder, making it a potential candidate for the optimization of clinical treatments.

1. Introduction

The Repressor Element-1 Silencing Transcription factor or Neuron-Restrictive Silencer Factor (REST/NRSF) is a protein of approximately 121 kDa that belongs to the family of transcription-factors type GLi-Krüppel Repressor Class of Zinc Fingers [1]. The REST/NRSF has a deoxyribonucleic acid (DNA)-binding domain consisting of eight zinc fingers and carries out its repressor function by binding its consensus sequence (Repressor Element 1/Neuron-Restrictive Silencing Element (RE1/NRSE)), a 17–33 bp site found in the regulatory regions of several genes [2]. Once REST/NRSF binds the RE1/NRSE site, it recruits repressor cofactors, histone deacetylases, and histone methyltransferases, which induce chromatin remodeling around the target genes [3,4].

The expression of REST/NRSF is high in nonneural tissues and in undifferentiated neuronal precursors during brain development, and decreases as the development process progresses [5]. The REST/NRSF expression is low in the adult brain and is associated with neuroprotective processes [6]. The REST/NRSF is found in brain areas such as olfactory system, cerebral cortex, hippocampal formation, diencephalon, midbrain, brain stem, and cerebellum [5]. It is a master regulator in the expression of more than 2000 neuronal genes [7]. Some of these genes are involved in axonal growth, vesicular transport and release, and ionic conductance [8,9].

Studies support that REST/NRSF represses genes related to the depression- and anxiety-like behaviors [10]. Indeed, REST/NRSF expression is low in subjects with cognitive deterioration whereas its high expression is associated with a low incidence of psychiatric disorders such as major depression [11,12]. Other studies indicate that the repression of genes induced by REST/NRSF is associated with neurological disorders such as the Parkinson's disease [13,14], Huntington's disease [15,16] and the reduced dendritic growth and complexity in Down syndrome [17]. This information indicates that REST/NRSF is involved in different process, and its effects depend on the cell type-specific necessities and the genetic context.

Studies indicate that REST/NRSF is overexpressed as a consequence of seizure activity [5,18] and exacerbates the epileptogenesis process, a situation associated with the low expression of miR-124 [19]. Indeed, the overexpression of REST/NRSF may facilitate the neuronal

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excitability due to the transcriptional silencing of genes such as hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (*HCN1*), glutamate ionotropic receptor AMPA type subunit 2 (*GluA2*), calbindin 1 (*Calb1*), glycine receptor alpha 2 (*Glra2*) and potassium voltage-gated channel subfamily C member 2 (*Kcnc2*) [20–22]. Other studies support that the overexpression of REST/NRSF contributes to antiepileptic effects. Concerning this issue, it is described that REST/NRSF prevents hyperexcitability in mature hippocampal neurons by the reduced expression of voltage-gated Na^+ channels [23]. The REST/NRSF participates in the antiepileptogenic effects of 2-deoxyglucose through the repression of genes, such as brain derived neurotrophic factor (*BDNF*) and neurotrophic receptor tyrosine kinase 2 (*TrkB*) [4]. In addition, the seizure-induced overexpression of REST/NRSF represses some genes related to epileptogenesis in the mature rat hippocampus [22]. The information described above supports the notion that REST/NRSF is associated in both pro- and antiepileptic processes.

At present, it is unknown whether REST/NRSF is overexpressed in the hippocampus of patients with drug-resistant mesial temporal lobe epilepsy (MTLE), a frequently neurological disorder [24] with a high psychiatric comorbidity [25–27]. This situation is relevant because the hippocampus of subjects with MTLE exhibits changes that facilitate the epileptic activity and mood disorders [28–30]. Some of these changes include the modulation of genes [31], which can be associated with the severity of the seizure activity, clinical variables, and/or psychiatric comorbidities. The aim of the present study was to elucidate changes in the messenger ribonucleic acid (mRNA) and protein expression of REST/NRSF in the hippocampus of patients with drug-resistant MTLE and their correlation with clinical variables and comorbid anxiety and depression.

2. Materials and methods

2.1. Patient criteria and surgical samples

Hippocampal samples were obtained from 28 patients diagnosed with drug-resistant MTLE (15 males and 13 females) undergoing surgery according to the protocol established by the Epilepsy Surgery Program of the National Institute of Neurology and Neurosurgery in Mexico City. The patients underwent a presurgical evaluation consisting of neurological, neuropsychiatric, and neuropsychological inspections, as well as electroencephalogram (EEG)-video recording and magnetic resonance imaging (MRI). In all patients, the presence of hippocampal mesial sclerosis was confirmed through histopathological and MRI evaluation.

Hippocampal biopsies were collected immediately after resection during epilepsy surgery, frozen rapidly in powdered dry ice and stored at −70 °C until processing. The experimental protocol was previously approved by the scientific committees of the institutions involved in the present study, and informed consent was obtained from each patient. Table 1 presents a summary of clinical data of the patients included in this study.

As a control condition, hippocampus samples were obtained from the autopsies of 13 subjects (7 males and 6 females) who were deceased because of different causes, without evidence of neurological diseases (Table 1). After resection, the samples were handled and stored as previously described.

2.2. mRNA evaluation for REST/NRSF

2.2.1. mRNA extraction and cDNA synthesis

The hippocampal tissue (100 mg) was homogenized in 0.5 ml of phenol and guanidine isothiocyanate solution (TRIzol®, Invitrogen, Cat. No.15596026), and the procedure was performed according to the manufacturer's protocol. Each mRNA sample was quantified spectrophotometrically (Biotech Epoch™ spectrophotometer), and the purity was determined by the 260/280 nm ratio, with an acceptable index in the range of 1.8 to 2.0. All samples exhibited an index between 1.9 and 2.0. The integrity of samples was also evaluated by agarose/formaldehyde gel electrophoresis, which allowed observation of the presence of the eukaryotic ribosomal subunits 18S and 28S, indicating high integrity of the mRNA samples. To ensure integrity and purity, repurification was performed using the Agencourt® RNAClean™ XP kit (Beckman Coulter) according to the manufacturer's protocol.

Complementary DNA (cDNA) was synthesized from 1 μg of mRNA and random hexamers in a total volume of 20 μl, according to the manufacturer's protocol, using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega).

2.2.2. Real-time PCR

Real time quantitative PCR was performed on a LightCycler 2.0 Real-time Polymerase chain reaction (PCR) platform (Roche), where each reaction contained a volume equivalent to 500 ng of cDNA, 0.5 μg of each primer, 0.2 μM TaqMan probe, 2 μl of TaqMan Master Mix (Roche) and ddH_2O to a final volume of 10 μl. The primers used for the amplification of *REST/NRSF* were as follows: Fw: 5′-GCATCAGGTGCTCCAGATATT-3′, Rv: 3′-GCTTCATATTGGCATGGCTTAC-5′. Additionally, enolase-2 (*ENO2*, Fw: 5′-CAGGACTTTGTCAGGGACTATC-3′, Rv: 3′-CCCTACATTGGCTGTG AACT-5′) was amplified as an endogenous gene. The amplification program consisted of 1 cycle at 95 °C held for 10 min, followed by 45 cycles at 95 °C for 10 s, 62 °C for 30 s, and 72 °C for 1 s. Standard qPCR curves were prepared with dilutions at known concentrations of gBlocks® fragments (Integrated DNA Technologies) of the sequences to be amplified for *REST/NRSF* and *ENO2*, which had been previously transiently transfected in chemocompetent DH5-α *Escherichia coli*. The detection of transcripts was sensitive to concentrations of 30 ng to 3 fg. Normalization of the expression values was conducted by calculating the changes in expression relative to the *ENO2* gene. The experimental data were analyzed using LightCycler software version 4.05 (Roche).

Table 1

Summary of clinical data of patients with MTLE and autopsied subjects and their correlations with REST/NRSF expression.

	Patients			Autopsies		
Clinical variables	Subjects (n = 28)	mRNA Correlation (r)	Protein Correlation (r)	Subjects (n = 13)	mRNA Correlation (r)	Protein Correlation (r)
Gender (M:F)	15:13	–	–	7:6	–	–
Age (years)	32 ± 1.4	−0.227	0.0779	34.5 ± 3.2	−0.417	0.646*
Age of onset of seizures (years)	13.3 ± 2.05	0.0505	0.227	DNA	–	–
Duration of epilepsy (years)	20.2 ± 2.3	0.0406	−0.151	DNA	–	–
Seizure frequency (per month)	13.8 ± 3.3	−0.176	0.658*	DNA	–	–
Focus laterality (R:L)	14:14	–	–	DNA	–	–
Number of drugs prior to surgery	4.4 ± 0.48	0.111	0.0977	DNA	–	–
Post-Mortem Interval (h)	DNA	–	–	15.58 ± 0.8	0.435	−0.0704

M, male; F, female; R, right; L, left; h, hours; DNA, Does not apply. Values are mean ± SEM, and values *r* are Pearson correlation Coefficients.

* $p < 0.05$ indicated by bold values.

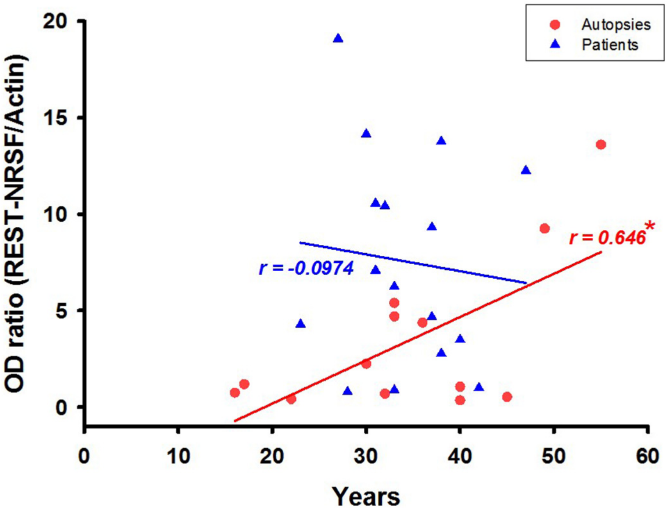

Fig. 1. Correlation of age with the REST/NRSF mRNA expression in human hippocampus. The graph shows the correlation of the relative expression of REST/NRSF mRNA with the age of autopsies and patients. Notice that the significant correlation found in autopsies is not evident in patients with drug-resistant MTLE. *r* indicates the value of the Pearson coefficient, *p < 0.05.

2.3. Evaluation of REST/NRSF protein expression

2.3.1. Protein extraction

The hippocampal tissue was mechanically homogenized in a 1:5 ratio (weight/volume) with NP-40 lysis buffer supplemented with the cOmplete (Roche) cocktail of protease inhibitors. The homogenate was centrifuged at 12,000×*g* for 15 min at 4 °C, and the supernatant was separated and stored at −80 °C until processing. The integrity was determinated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Coomassie blue staining, which allowed the evaluation of bands of all molecular weights. The concentration of proteins was determined by the Bradford method, using the Pierce™ Coomassie Protein Assay kit (Thermo Scientific) according to the manufacturer's protocol.

2.3.2. Western blot

A volume equivalent to 100 μg of protein was separated by electrophoresis on an 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, which was blocked with albumin and skim milk (1:5) diluted in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against REST/NRSF (150 kDa, 1:1000, ab75785, Abcam) and β-actin as loading control (42 kDa, 1:1000, sc-69879, Santa Cruz Biotechnology) at 4 °C overnight. After three washes with TBST, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase in TBST (1:5000) for 90 min at room temperature. After three washes with TBST, the membranes were washed with a solution of sodium chloride (NaCl) (0.5 M) and reacted for 2 min with the chemiluminescence reagent Luminata Crescendo Western HRP substrate (Millipore). Images were acquired with a FUSION-FX7 Advanced photodocumenter (Thermlab). The optical density of the bands was determined using ImageJ software (National Institutes of Health, NIH).

2.4. Statistical analysis

The values obtained from patients and autopsied subjects were compared using Mann–Whitney test. Pearson's correlation coefficients between the expression of REST/NRSF (mRNA and protein) and clinical factors were determined. The REST/NRSF mRNA and protein expression was also estimated according to psychiatric comorbidities and analyzed by one-way analysis of variance (ANOVA) with a post hoc Dunn–Sidak test (Bonferroni). SigmaPlot 12.5 software was used for all statistical analyses. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of the experimental subjects

The clinical data of the autopsies were as follows ((mean ± Standard Error of the Mean (SEM)): age, 34.5 ± 3.2 years and the postmortem interval (PMI), 15.58 ± 0.88 h. The age of the autopsied subjects was not significantly different when compared with that of patients with MTLE (32 ± 1.4 years, p > 0.05) (Table 1).

Patients with MTLE used 2 or more antiepileptic drugs (AEDs) during the presurgical period. Their clinical data were as follows: age of disorder onset, 13.3 ± 2.05 years; duration of epilepsy, 20.2 ± 2.3 years; and frequency of epileptic seizures, 13.8 ± 3.3 per month (Table 1).

3.2. Expression of REST/NRSF in the hippocampus of autopsied subjects

No significant correlation was found between the PMI and the REST/NRSF mRNA and protein expression values ($r = 0.435$, $p > 0.05$; and 0.0704, $p > 0.05$, respectively) (Table 1). These results indicate that the PMI range of the autopsied subjects evaluated in the present study did not influence the expression of REST/NRSF.

The protein expression of REST/NRSF was positively correlated with the age of the autopsied subjects ($r = 0.646$, $p < 0.05$), i.e., the older the age, the higher the protein expression of REST/NRSF in hippocampus. This condition was not evident for mRNA expression ($r = -0.417$, $p > 0.05$) (Table 1, Fig. 1). No other significant correlations were found.

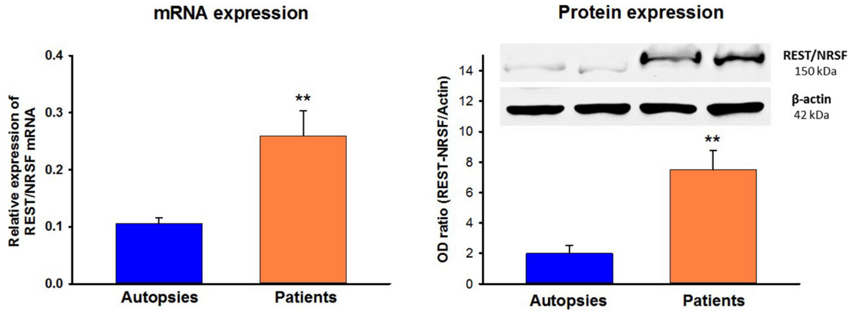

Fig. 2. REST/NRSF expression in the hippocampus of autopsies and patients with pharmacoresistant MTLE. The left graph shows the relative expression of REST/NRSF mRNA. The right graph presents the optical density ratio between protein expression of REST/NRSF and the β-actin, and representative images obtained from the western blots. The results are expressed as mean ± SEM. T-test *p < 0.05, **p < 0.01.

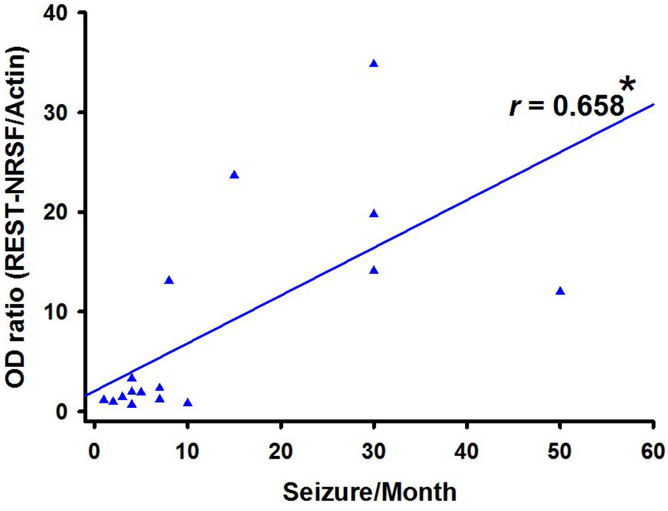

Fig. 3. Correlation between the frequency of epileptic seizures and the protein expression of REST/NRSF in the hippocampus of patients with pharmacoresistant MTLE. The graph shows the correlation between the protein expression of REST/NRSF expressed as optical density and the frequency of seizures per month. *r* indicates the value of the Pearson coefficient, $^{*}p < 0.05$.

3.3. Expression of REST/NRSF in the hippocampus of patients with MTLE

Compared with autopsied subjects, the hippocampus of patients with MTLE showed an increase in REST/NRSF expression for both mRNA (146%, $p < 0.01$) and protein (273%, $p < 0.01$) (Fig. 2).

The correlation analysis of the results obtained with the clinical variables revealed that the higher frequency of seizures was associated with the higher protein expression of REST/NRSF in the hippocampus ($r = 0.658$, $p < 0.05$) (Table 1, Fig. 3). No significant correlations were found between the expression of REST/NRSF and other clinical variables. It is important to mention that the positive correlation between the protein expression of REST/NRSF and age that was found in autopsied subjects was not evident in patients with MTLE (Table 1, Fig. 1).

Clinical evaluation revealed that 13 patients with MTLE presented comorbid mood alterations: 3 patients had anxiety, 5 had depression, and 5 underwent anxiety plus depression. These patients with MTLE plus mood disorders exhibited an overexpression of REST/NRSF in mRNA (55% vs autopsies, $p > 0.05$) and protein (148% vs autopsies, $p < 0.05$). Fifteen patients had MTLE without psychiatric disorders. The hippocampus of these patients showed a more evident overexpression of REST/NRSF (mRNA, 171%, $p < 0.001$ vs autopsies; protein, 230%, $p < 0.001$ vs autopsies) (Fig. 4). No clinical conditions correlate with the overexpression of REST/NRSF in patients with and without mood disorders.

4. Discussion

The present study was focused to evaluate the expression of the transcription factor REST/NRSF in the hippocampus of autopsies and patients with pharmacoresistant MTLE. In autopsies, the protein expression of REST/NRSF was age-dependent, a condition not evident in the hippocampus of patients with MTLE. An important finding was that REST/NRSF was overexpressed in the epileptic hippocampus. The protein overexpression correlated with the seizure frequency. In addition, the higher expression of REST/NRSF was more evident in patients with MTLE without anxiety and depression.

For the present study, it was essential to verify the viability and quality of the autopsy samples in order to consider them as control condition. In this regard, the spectrophotometric and integrity assessments were consistent with the established criteria (see Materials and methods section) [32,33]. It is known that the integrity of mRNA can be affected by a prolonged PMI [34]. Under our experimental conditions, we did not find a significant correlation between the PMI (range of 10 to 18 h) of autopsies and their mRNA and protein expression. These findings support that the values obtained from the autopsies can be considered as a control condition.

During brain development, REST/NRSF activity is high because it regulates the differentiation of nonneuronal cells to neurons through the silencing of proneuronal genes [5]. In the mature brain, neurodevelopmental activity decreases, and the levels of REST/NRSF are low [6]. The results obtained from autopsies support the low expression of REST/NRSF in mature brain, a condition that can be regulated by epigenetic factors. Concerning this issue, it is known that the silencing of the *REST* gene results from the methylation of its promoter in three regions with high frequency of cytosines and guanines prone to be methylated (CpG islands). [35]. The expression of REST/NRSF is also repressed by miR-124 [19]. In spite of its low expression, REST/NRSF plays an important role in the mature brain. A previous study indicates that the expression of REST/NRSF in the human prefrontal cortex and hippocampus positively correlates with a good preservation of cognitive functions during aging, a condition that is lost in subjects with Alzheimer's disease [6]. An interesting finding from our experiments was that age correlated with the REST/NRSF protein, but not mRNA expression, in the hippocampus of autopsies. This group of evidence supports the notion that the age-dependent protein expression of REST/NRSF may modulate neuroprotective mechanisms against toxic insults that increase with aging [6]. The mismatch between the expression of mRNA and protein of REST/NRSF in autopsies can be due to the participation of many posttranscriptional mechanisms involved in translating

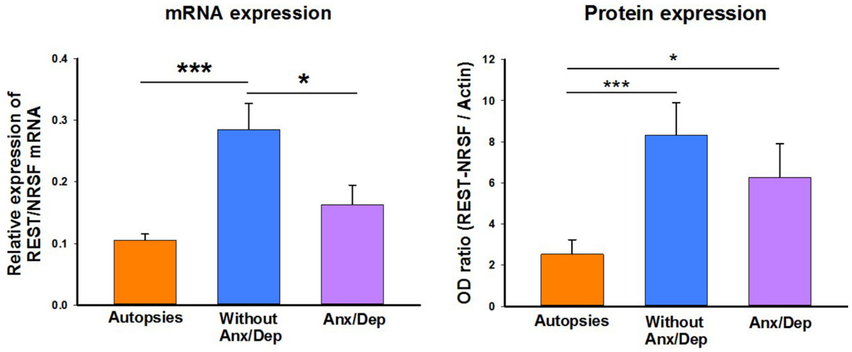

Fig. 4. REST/NRSF expression in the hippocampus of patients with drug-resistant MTLE with and without comorbid anxiety and depression. The graphs show the mRNA (left) and protein (right) expression of REST/NRSF in autopsies, patients with MTLE without mood disorders, and patients with MTLE and comorbid anxiety and depression. The results are expressed as mean ± SEM. ANOVA and Dunn–Sidak post hoc test, $^{*}p < 0.05$.

mRNA [36]. These mechanisms can modify the rate of transcription depending on the cellular necessities of protein expression. The above information suggests that under normal conditions, the transcription factor is translated according to cellular requirements, independently of mRNA levels.

Our results revealed that the epileptic hippocampus of patients with MTLE showed an overexpression of REST/NRSF. In contrast to autopsies, the REST/NRSF expression in patients with MTLE was not age-dependent. It is possible that in brain disorders such as epilepsy, the REST/NRSF expression depends on abnormal insults such as excessive oxidative stress. This situation leads to the silencing of target genes that are essential to prevent neuronal death [7].

The overexpression of REST/NRSF in epileptic tissue may result from the activation of the canonical Wingless/Integrated (WNT) pathway. In this pathway, β-catenin directly regulates the *REST* gene and other genes related to the control of progenitor cells [37]. The overexpression of β-catenin and other effectors of the WNT pathway is associated with aberrant hippocampal neurogenesis in MTLE [38]. In addition, Wnt/β-catenin signaling is involved in seizure-induced neurogenesis, seizure susceptibility, and epileptogenesis [39]. Other studies indicate that the activation of WNT pathway is a mechanism that reduces the high extracellular levels of glutamate by the expression of Excitatory Amino Acid Transporter 2 (EAAT2) [40]. Further studies are necessary to determine how the REST/NRSF expression regulated by β-catenin modifies the epileptic activity.

It is known that the *REST* gene is positively regulated by cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein [35]. The activation of CREB results from the increase in extracellular levels of glutamate [41], a condition found in the hippocampus of patients with drug-resistant MTLE [42]. In addition, the enhanced expression of CREB is a consequence of the decreased expression of miR-124 in epileptic tissue [43]. The chronic activation of CREB induced in the epileptic hippocampus [44] represents other possible trigger factor for the REST/NSRF overexpression found in the present study.

The results obtained revealed that subjects with the highest frequency of epileptic seizures exhibited the highest REST/NRSF protein expression. It is known that the induction of an ictal event produces an increase in REST/NRSF expression, which returns to baseline 24 h later [18]. Because the release of glutamate augments with each epileptic seizure [45], it is possible that a high frequency of ictal events maintains the elevated expression of REST/NRSF by increasing CREB and/or β-catenin. It is important to notice that both the overexpression of β-catenin and excess in glutamate augment the expression of P-glycoprotein in brain endothelial cells [46]. The overexpression of this multidrug transporter in the blood–brain barrier is a condition that facilitates the pharmacoresistant phenotype in epilepsy [47,48]. In the future, it will be important to determine the role of the overexpression of REST/NRSF in pharmacoresistant epilepsy.

One important finding from our experiments was that patients with MTLE without psychiatric comorbidities showed a more evident overexpression of the REST/NRSF. This result supports a previous study indicating that the increase in REST/NRSF is associated with the reduction of psychiatry symptoms associated with stress [11]. The overexpression of REST/NRSF related with the absence of psychiatric comorbidities also supports a regulation mediated by miR-124 and CREB in the manifestation of mood disorders. The hippocampus of subjects with depression presents a high expression of miR-124 [49], a condition associated with low expression of CREB, in both its native and phosphorylated forms [50–52]. On the other hand, the transcriptional induction of CREB in the hippocampus of mice has an antidepressant and anxiolytic effect [53], and treatment with antidepressants leads to an increase in CREB expression [54,55]. In addition, the chronic antidepressant administration modifies hippocampal expression of multiple components of the Wnt/β-catenin cascade, including an increase of WNT2, a subtype of Wnts [56]. In contrast to patients with MTLE without comorbid disorders, patients with MTLE plus anxiety and depression showed a less significant overexpression of REST/NRSF. Future studies should be carried out to determine the mechanism by which the REST/NRSF-induced silencing of genes avoids psychiatric disorders in MTLE.

The AEDs received by patients during the presurgical period are another factor that can explain the changes in REST/NRSF expression. Topiramate and oxcarbazepine positively regulate CREB and activate the WNT pathway through Glycogen Synthase Kinase 3 (GSK3) [57–59]. In contrast, drugs such as levetiracetam and valproic acid negatively regulate CREB and inhibit GSK3β [60–62]. Antiepileptic drugs such as clobazam and lamotrigine do not induce changes in regulatory molecules of the WNT pathway [62]. In the present study, it was not possible to identify the influence of each AED administered during the presurgical period because all patients received polytherapy. Future studies in experimental models should be designed to identify the role of AEDs in the overexpression of REST/NRSF.

5. Conclusions

The findings obtained in the present study indicate that REST/NRSF is overexpressed in the hippocampus of patients with pharmacoresistant MTLE and that its protein expression is dependent on the frequency of epileptic seizures. In addition, our results support the notion that the increased expression of REST/NRSF is associated with lower comorbid mood disorders. Finally, the overexpression of this repressor transcription factor may be important in the regulation of genes that actively participate in the excitatory or inhibitory processes that lead to the development of MTLE and its comorbidities.

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Conflict of interest

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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