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Immunoproteasome expression is induced in mesial temporal lobe epilepsy

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ABSTRACT

Immunoproteasome has been associated to neurodegenerative and autoimmune diseases as a marker and regulator of inflammatory mechanisms. Its expression in the brain may occur upon neuroinflammation in different cell types and affect a variety of homeostatic and inflammatory pathways including the oxidized protein clearance and the self-antigen presentation. In the present study we investigated the immunoproteasome expression in hippocampi and cortex of patients affected by different histopathological forms of pharmaco-resistent mesial temporal lobe epilepsy. We identified a pathology-specific pattern of immunoproteasome expression, which could provide insight into the complex neuroinflammatory pathogenic components of this disease.

1. Introduction

The multiple subunit 20S proteasome is the central catalytic unit of the ubiquitin proteasome system (UPS) and the catalytic core of the 26S proteasome that is built by the association of two 19S regulator complexes with the catalytic 20S core [19S-20S-19S]. With its N-terminal threonine residue as the single active site of the β-subunits (β1, β2, and β5), the 20S proteasome is a N-terminal nucleophilic hydrolase responsible for the degradation of the greater part of the cytoplasm proteins as well as for the generation of the vast majority of virus- or self-derived peptides presented by the MHC class I molecules [1,2]. This later function is generally aided by the interferon-γ (IFN-γ)-induced synthesis of the proteasome activator subunits PA28-α and PA28-β as well as of the alternative catalytic subunits β1i, β2i, β5i (also known as LMP2, MECL-1, and LMP7, respectively) with concomitant formation of immunoproteasome (i-proteasome) subtypes [3,4]. Very recently i-proteasomes, which possess altered proteolytic properties, showed to play also a pivotal role in both cytokine-mediated inflammation and in the clearance of oxidized proteins and aggresome-like induced structures upon INF-γ stimuli [5–7]. Although in young healthy human brain i-proteasomes are almost absent, they have been detected in brain areas from elderly subjects as well as from patients affected by Alzheimer or Huntington diseases [8,9] and Multiple Sclerosis (MS), in the latter with a concomitant expression of the PA28-αβ complex [10]. The induction of i-proteasome and PA28-αβ expression in brain (likely upon neuroinflammation) could have effects on different pathways because UPS regulates in neurons, for example, the synaptic plasticity and the protein turnover [11]. The role of neuroinflammation is emerging as a key element in another neurological disease such as the epilepsy, a brain disorder that affects about 50 million people worldwide and is characterized by an enduring predisposition to generate seizures as well as by emotional and cognitive dysfunctions. The most common form of partial-onset epilepsies is the Mesial Temporal Lobe Epilepsy (MTLE), which is characterized by electrophysiological and clinical evidence for seizure generation within the mesial temporal lobe and is often resistant to the antiepileptic drugs [12]. MTLE is a heterogeneous clinical entity with different aetiologies and clinical histories [13,14]. Pharmacoresistant MTLE is the most common type of epilepsy undergoing surgical treatment, with a postoperative

Abbreviations: Ab, antibody; FCD, focal cortex dysplasia; MTLE, mesial temporal lobe epilepsy; MTS, mesial temporal sclerosis; IFN-γ, interferon-γ; UPS, ubiquitin proteasome system.

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favourable outcome in about 70% of patients submitted to anterior temporal lobectomy along with uncus-amygdalo-hippocampectomy [15]. On histological examinations, the most frequently encountered alterations in such patients are represented by mesial temporal sclerosis (MTS) (in hippocampal specimens) and focal cortical dysplasia (FCD) (in temporal lobe cortex), alone or in association (double/dual pathology). MTS is characterized by neuronal loss, gliosis/sclerosis as well as different levels of granule cell dispersion and mossy fiber sprouting. In FCD, the temporal lobe cortex shows abnormal lamination of cortical layers, which may be (FCD type II) or not be (FCD type I) associated to the presence of dysmorphic neurons and balloon cells [16].

Based on these considerations, we asked whether i-proteasomes and PA28-αβ could be involved in different forms of MTLE and whether their expression might be induced by IFN-γ stimulus. Accordingly, we here report the presence of i-proteasome subunits (LMP2 and LMP7) and PA28-β, not accompanied by the presence of IFN-γ, in hippocampal and cortical specimens of MTS, FCD, MTS + FCD, assessed by Western blot assays. Subsequently, we revealed by immunohistochemistry (IHC) which cell-types were responsible for i-proteasome expression, focusing particularly on hippocampus in consideration of the complexity of its structure (Hammon Horn, Dentate gyrus, pyramidal neurons, granular cells) and its role in epileptogenesis and neuropsychological alterations with implications in memory functions and learning capabilities [17].

2. Materials and methods

2.1. Patients and controls

Specimens obtained from fifteen patients (Table 1), who underwent epilepsy surgery (tailored anterior temporal resection along with uncus-amygdalo-hippocampectomy) for pharmacoresistant MTLE, were retrieved from the files of the Section of Pathology of the Department of Haematology and Oncology of the University of Bologna at Bellaria Hospital (Bologna). An informed written consent to participate in this study was obtained from all patients. Blocks were serially cut, stained with H & E and immunostained with anti NeuN (Chemicon, Temecula, CA, USA, clone A60, dilution 1:1000) antiserum in order to assess the presence of MTS, according to classification proposed by Blümcke et al. [14], and of FCD, following the recently published indications proposed by ILAE Commission [18]. MTLE specimens were compared to specimens removed during autopsy from donors not affected by epilepsy or other neurological disorders (Table 1). Autopsies were carried out after 24 h from the death and almost all brain was included for histological examination and diagnostic definition of the disease.

2.2. Western blot assay

Twenty milli grams of frozen tissue from human hippocampus and cortex of control adult ($n = 2$) and MTLE subjects ($n = 2$) were lysed in TEAD buffer (Tris–HCl 20 mM pH 7.5, EDTA 1 mM, NaN_3 1 mM, DTT 1 mM), homogenized using a motor-driven homogenizer and centrifuged at 25000g for 1 h at 4 °C. The supernatant containing the total protein extract was quantified by Bradford's method. Western blot analysis on total protein extract (30 μg) was performed as previously described [19]. The antibodies (Abs) used in Western blot assays are reported in Table 2 and were previously tested on human brain and liver [8,10,20].

2.3. Immunohistochemistry (IHC)

Immunostaining was performed as previously described [10] by using mouse monoclonal anti-LMP2 and -LMP7 Abs, which are specific for i-proteasomes, mouse monoclonal anti-β1, which is specific for constitutive proteasomes (c-proteasomes) and mouse monoclonal anti-α4 Ab, which marks any human 20S proteasome (Table 2). IHC was performed on hippocampi of MTS ($n = 6$), FCD ($n = 5$), MTS + FCD ($n = 4$) and control ($n = 3$) donors (Table 1).

3. Results

3.1. i-proteasome and PA28- subunit expressions are induced in MTLE hippocampus and cortex

The total proteins extracted from hippocampi of patients affected by FCD, MTS + FCD and of controls not affected by any MTLE or other neurological diseases were stained for different subunits of the 20S proteasome core and of the regulatory complex PA28-αβ. Although the Western blot assay provided information about the presence of the target proteins, the small number of samples did not allowed quantification with adequate statistical power. The two catalytic subunits β1 and β5, which are distinctive of c-proteasomes, and the α4 subunit, which is incorporated in any

Table 1
Relevant clinical data on donors used in this study. Age at tissue collection was significantly higher in autopsy controls (donors # 16-18). Surgery is a tailored polar anterior temporal resection along with uncus-amygdalo-hippocampectomy. F, female; M, male; RTL and LTL, right and left temporal lobe, respectively; MTS, mesial temporal sclerosis; FCD, focal cortical dysplasia; GG, ganglioglioma.

Donor	Gender	Age at surgery	Age onset epilepsy	Duration epilepsy	Specimen type	Brain area	Histological diagnosis	Clinical diagnosis
1	F	21	7	14	Biopsy	LTL	MTS	TLE
2	F	30	23	7	Biopsy	RTL	FCD-II	TLE
3	M	36	29	7	Biopsy	LTL	MTS + FCD-IIIa	TLE
4	F	38	12	26	Biopsy	RTL	MTS + FCD-IIIa	TLE
5	M	17	15	2	Biopsy	RTL	MTS + FCD-IIIa	TLE
6	M	49	9	40	Biopsy	LTL	MTS	TLE
7	M	53	29	24	Biopsy	RTL	MTS	TLE
8	F	29	16	13	Biopsy	RTL	MTS	TLE
9	F	46	17	29	Biopsy	LTL	MTS + FCD-IIIa	TLE
10	F	31	8	23	Biopsy	LTL	GG + FCD-IIIb	TLE
11	M	30	14	16	Biopsy	RTL	FCD-I	TLE
12	F	45	13	32	Biopsy	RTL	MTS	TLE
13	F	36	19	17	Biopsy	LTL	MTS	TLE
14	M	36	33	3	Biopsy	LTL	FCD-I	TLE
15	M	24	17	7	Biopsy	LTL	FCD-I	TLE
16	F	61	/	/	Autopsy	Hippocampus + cortex	Normal	Cerebral ischemia
17	M	44	/	/	Autopsy	Hippocampus + cortex	Normal	Small cell lung cancer
18	M	57	/	/	Autopsy	Hippocampus + cortex	Normal	Pulmonary edema

Table 2
Technical details of the antibodies used for IHC and Western blot analysis.

Antibody	Clone	Manufacturer	Dilution IHC	Dilution WB
β1	PW8140	Enzo Life Sciences, Plymouth meeting, PA, USA	1:300	1:1500
β5	PW8895	Enzo Life Sciences, Plymouth meeting, PA, USA	–	1:2000
LMP2	PW8840, LMP2-13	Enzo Life Sciences, Plymouth meeting, PA, USA	1:95	1:750
LMP7	PW8845, LMP7-1	Enzo Life Sciences, Plymouth meeting, PA, USA	1:90	1:750
PA28-β	CST#2409	Cell Signalling Technology, Beverly, MA, USA	–	1:1000
α4	PW8120	Enzo Life Sciences, Plymouth meeting, PA, USA	1:90	1:2000
IFN-γ	AF285NA	R and D Systems Inc, Minneapolis, MN, USA	–	1:750

proteasome isoform, were detected in all samples (Fig. 1). In contrast, although the i-proteasome catalytic subunits LMP2 and LMP7 as well as PA28-β were virtually absent in control hippocampi, they were detected in hippocampi of MTS + FCD and FCD (in a lower amount) patients. Accordingly, LMP2, LMP7 and PA28-β were not identified in cortex of controls whereas they were expressed in the cortex of the patient affected by MTS + FCD (Fig. 1). It is worthy to note that by Western blot assay and based on their molecular weight, we identified the processed forms of both LMP2 and LMP7, suggesting that both the subunits were incorporated in the assembled i-proteasomes. Generally, the staining of LMP2 was less pronounced and showed a very weak signal in control hippocampi and MTS + FCD cortex, whereas control cortex was negative. Because i-proteasomes are usually induced upon IFN-γ stimulus, we stained the MTLE and control specimens by anti-IFN-γ Abs but we failed to detect this cytokine both in cortex and hippocampi.

3.2. Which cells did express LMP2 in MTLE hippocampi?

In order to understand which cell types express i-proteasome subunits in MTLE, hippocampus of controls as well as of patients affected by different forms of MTLE were stained with anti-LMP2 Ab. In accord with our previous observations [8,10] and Western blot's results (heading 3.1), LMP2 was not detected in neurons (neither in granular nor in pyramidal neurons), whereas endothelial and few glia cells were positive to the staining in the hippocampus of control donors (Fig. 2A). Conversely, in MTS specimens, although granular neurons remained negative to the LMP2 staining (Fig. 2B), pyramidal neurons and glia cells were clearly marked in addition to luminal endothelial cells (Fig. 2C). Intriguingly, not only the latter three cell types but also granular neurons were intensely stained by anti-LMP2 Ab both in FCD (Fig. 2D) and in MTS + FCD (Fig. 2E) hippocampi.

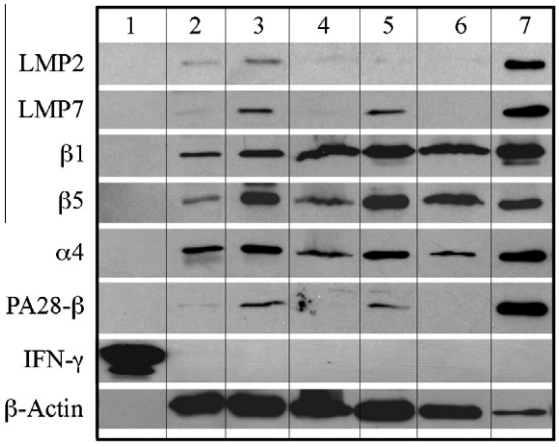

Fig. 1. Western blot assays performed on a representative set of MTLE and non-MTLE total protein extract specimens. Loading order: 1. Purified IFN-γ protein; 2. Hippocampus of FCD patient; 3. Hippocampus of patient affected by MTS + FCD; 4. Hippocampus of not-MTLE; 5. Cortex of patient affected by MTS and FCD; 6. Cortex of non-MTLE control; 7. Human liver (positive control [20]).

3.3. Which cells did express LMP7 in MTLE hippocampi?

Because LMP2 and LMP7 subunits could be incorporated independently in i-proteasomes [21] and LMP2 and LMP7 showed to differentially impinge upon inflammation [7,22], we performed, on the same specimens described above, the LMP7 IHC staining. The findings substantially overlapped the LMP2 expression pattern for neuronal populations. Indeed, the control hippocampi showed no LMP7 expression but in luminal endothelial cells (Fig. 3A). In contrast, pyramidal neurons in hippocampi of MTS (Fig. 3B and C), FCD (Fig. 3D) and MTS + FCD (Fig. 3E) were positive to LMP7 staining. Similarly to LMP2, also LMP7 seemed to be expressed in granular cells in FCD and MTS + FCD (Fig 3D and E) but not in MTS (Fig. 3B). Differently to LMP2, glia cells were negative or only weakly positive to LMP7 in MTS and FCD patients, while they were steeply positive in MTS + FCD cases (Fig. 3E).

As expected and as previously observed [8,10], IHC on hippocampi specimens of MTLE and controls performed by Abs specific for both β1 and α4 proteasome subunits stained all cell types (data not shown).

4. Discussion

We have shown by Western blot and IHC assays that i-proteasomes and PA28-β expression is induced in MTLE hippocampi and cortex in different cell types, including pyramidal and granular neurons, apparently in an IFN-γ-independent manner. The physiological role of i-proteasomes upon inflammation and their involvement in diseases with a neuroinflammatory component is an expanding area and represents a hot topic in biomedical research. In last years, breaking-down results, produced by exploiting LMP2 and LMP7 knock out mice, suggested that i-proteasomes regulate anti-viral humoral and innate immunity, cytokine production as well as clearance of oxidized proteins and aggresome-like induced structures [5,22,23]. Intriguingly, Seifert and colleagues showed that a deficit of LMP7 subunit can also affect the brain, worsening the onset and severity of experimental autoimmune encephalitis, an animal model of MS [5]. In MS, the i-proteasomes and PA28-α subunit are expressed in different cell types (including neurons) and LMP2 polymorphism may have a protective effect by altering a specific self-antigen presentation [10]. In addition, i-proteasomes were detected in human brain from old subjects and from patients affected by Huntington and Alzheimer diseases [8,9].

Although i-proteasome subunits are usually synthesised upon IFN-γ stimulus, how neurons are induced to express i-proteasome is still a matter of debate. A neuronal induction of i-proteasome expression upon different stimuli has been described both *in vitro* and *in vivo* in mice, even if the literature is contradictory about the basal expression of the i-proteasome subunits in neurons [24–27]. We did not observed, by Western blot assay, IFN-γ expression in MTLE hippocampi suggesting that, at least in this disease, neuronal i-proteasome expression was induced by stimuli other than this cytokine. The i-proteasome synthesis that we observed in hippocampal neurons of MTLE patients might be induced

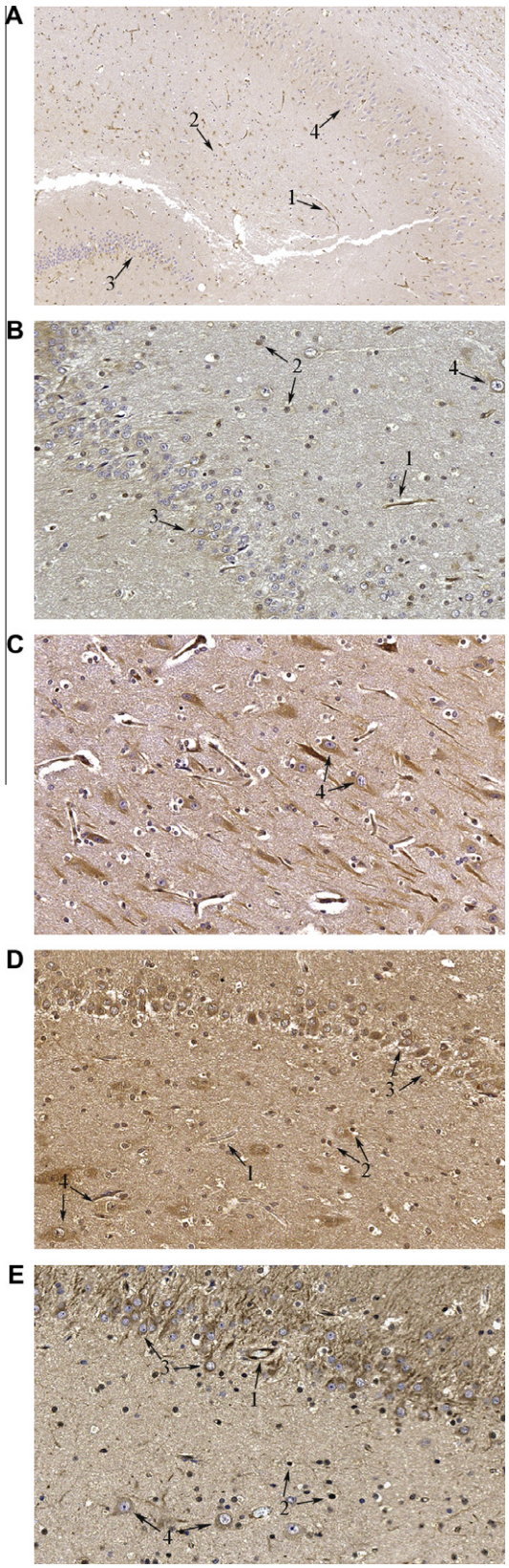

Fig. 2. Distribution of i-proteasome LMP2 subunit in hippocampi of controls as well as MTS, FCD and MTS + FCD patients. IHC staining by anti-LMP2 Abs in representative hippocampus of control (A), MTS (B and C), FCD (D) and MTS + FCD (E) donors. Representative cell types are marked as following: 1, luminal endothelial cells; 2, glia (oligodendrocytes or microglia); 3, granular neurons; 4, pyramidal neurons. Magnification: A, 40×; B, 200×; C, 200×; D, 200×; E, 200×.

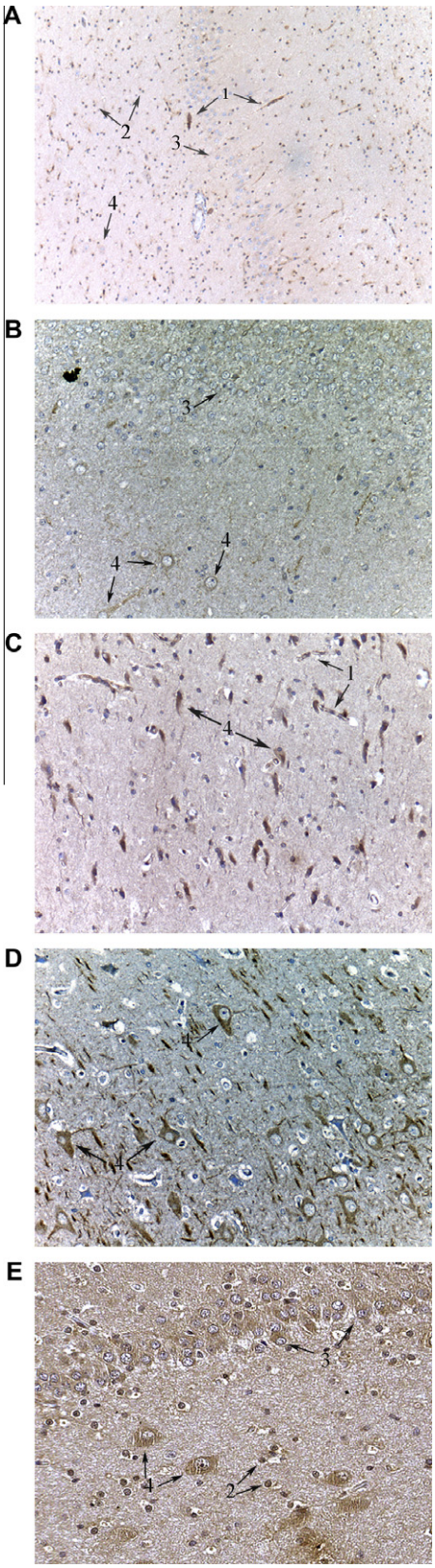

Fig. 3. Distribution of i-proteasome LMP7 subunit in hippocampi of controls as well as MTS, FCD and MTS + FCD patients. IHC staining by anti-LMP7 Abs in representative hippocampus of control (A), MTS (B and C), FCD (D) and MTS + FCD (E) donors. Representative cell types are marked as in Fig. 2. Magnification: A, 100×; B, 100×; C, 200×; D, 200×; E, 200×.

by both neuroinflammation through IFN-γ-independent pathways, *e.g.* TNF-α or by activating TLR-4, or directly by seizures. Indeed, in MTLE as well as in other forms of epilepsy, a vicious circle between neuroinflammation and seizures may occur [12]. In this complex interplay i-proteasome might play a role, taking into account the relevance of proteasomes in neuronal function. Indeed, proteasomes regulate pre- and post-synaptic axonal density and have a pivotal role in NF-κB activation (through IκB-α degradation and p105 and p100 cleavage), which is a common transducer of the main pathways identified in epilepsy's neuroinflammation such as TLR-4 and IL-1β [11,12]. Such an hypothesis raises the question whether i-proteasome presence in neurons has protective or deleterious implications. For other neuroinflammatory conditions and pathologies such as ageing and AD progression we hypothesized that neuronal expression of i-proteasomes in hippocampus might be an extreme attempt of neurons to cope with the increasing accumulation of aggregates and oxidised proteins, hypothesis supported by other indirect evidences [5,8,28,29]. Intriguingly, even in neurons of AD patients, i-proteasome subunit expression seems to be finely regulated because in cerebellum, at variance with other neurons, Purkinje cells were i-proteasome-free. Similarly, in MTS, but not in FCD or MTS + FCD, i-proteasome subunits were detected only in pyramidal neurons whereas granular cells were i-proteasome negative. Whether those neurons that express i-proteasomes have a healthier phenotype than the i-proteasome-negative neuronal cells is still an open question. The comparison between different forms of MTLE suggest that neuronal loss as well as the local seizure generation are likely not the factors leading to the i-proteasome expression in surrounding neurons, as neuronal i-proteasomes were observed in both MTS and FCD hippocampi. Likely, neuroinflammatory stimuli, common to different forms of MTLE [13] and maybe partially regulated by cortical seizures (because of the similar i-proteasome expression in different neurons in FCD and MTS + FCD hippocampi), can be the trigger of the neuronal i-proteasome expression with effects on neuronal viability that are still unknown.

In summary, we demonstrated that, in contrast to controls, i-proteasome and PA28-β subunits were present in MTLE hippocampi and cortex parenchyma apparently in an IFN-γ-independent manner. In hippocampus, i-proteasomes were detected both in neurons and glia cells with a different neuronal expression pattern between MTS and FCD or MTS + FCD. Whether i-proteasomes formation in neurons represents a pro-survival or a deleterious event and which role it plays in the complex neuroinflammatory scenario of MTLE, as well as other neurological diseases, remains an open question that will be worthwhile to properly address.

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