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RAPID COMMUNICATION

Mu Opioid Receptor mRNA Expression, Binding, and Functional Coupling to G-Proteins in Human Epileptic Hippocampus

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ABSTRACT: Mu opioid receptors (MOR) are known to be involved in seizure activity. The main goal of the present study was to characterize the MOR mRNA expression, binding, as well as G protein activation mediated by these receptors in epileptic hippocampus of patients with pharmacoresistant mesial temporal lobe epilepsy (TLE). In contrast with autopsy samples, hippocampus obtained from patients with mesial TLE demonstrated enhanced MOR mRNA expression (116%). Saturation binding experiments revealed significantly higher (60%) B_{max} values for the mesial TLE group, whereas the K_d values were not statistically different. Although mesial TLE group demonstrated high levels of basal binding for the G proteins (136%), DAMGO-stimulated [^{35}S]GTPγS binding did not demonstrate significant alterations. In conclusion, our present data provide strong evidence that the epileptic hippocampus of patients with pharmacoresistant mesial TLE presents significant alterations in MOR. Such changes may represent adaptive mechanisms to compensate for other as yet unknown alterations. © **2010 Wiley Periodicals, Inc.**

KEY WORDS: mu opiod receptor; mRNA; G-protein; binding; hippocampus; mesial temporal lobe epilepsy

Opioid receptors are thought to be involved in epileptic activity. The activation of mu opioid receptors (MOR), which interact with Go, Gi (Chan et al., 1995), and Gs proteins (Chakrabarti et al., 2005), has been associated with anticonvulsant (Albertson et al., 1984) or proconvulsant effects (Tortella et al., 1987). Further studies support dual effects of MOR, i.e., they facilitate the epileptogenesis process, but increase refractoriness to subsequent seizures during the postictal period (Rocha et al., 1991). Patients with temporal lobe epilepsy (TLE), the most common form of epilepsy among adults characterized by chronic seizures originated in the hippocampal formation (Sloviter, 1994), are often resistant to medical therapy and become candidates to surgical resection of the epiletogenic zone. In these patients, positron emission tomography (PET) studies reveal an enhancement of the opioid receptor binding availability in the temporal neocortex ipsilateral to the epileptic focus during the postictal and interictal periods (Frost et al., 1988; Hammers et al., 2007). Although these observations lead to suggest an enhanced activity of the opioid system in the epileptic brain, a recent study from our group provides strong evidence that the signal transduction mechanisms downstream of MOR are decreased in the temporal neocortex ipsilateral to the epileptic focus of patients with mesial TLE (Rocha et al., 2009). The hippocampal formation is a brain region sensitive to the effects of opioid peptides. Activation of MOR in this brain area induces excitatory effects on the activity of pyramidal neurons via disinhibitory mechanisms involving GABAergic interneurons (Siggins and Zieglgansberger, 1981; Madison and Nicoll, 1988). In experimental models of epilepsy, the MOR changes in hippocampus depend on the type of seizure evaluated and the rate of neuronal excitability (Bausch and Chavkin, 1997, Skyers et al., 2003, Rocha et al., 1993). Concerning human brain, PET studies demonstrate decreased or no significant changes in opiod receptor binding availability in the epileptic hippocampus (Frost et al., 1988). However, there is no further data concerning signal transduction mechanisms downstream of the MOR in the human epileptic hippocampus of patients with mesial TLE. The main goal of the present study was to provide more information concerning MOR changes associated with epilepsy in human hippocampus. We characterized MOR mRNA expression, binding, and G protein activation in hippocampus of patients with pharmacoresistant mesial TLE who underwent epilepsy surgery. The evaluation of this tissue is an excellent opportunity to determine MOR receptor changes in the brain area that represents the epileptic focus in the mesial TLE (Sloviter, 1994). The protocol design was previously approved by the scientific committees of the institutions involved in the present

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TABLE 1.

Clinical Data of Patients With Pharmacoresistant Mesial Temporal Lobe Epilepsy

Patient	Age (years)	Gender	Age of seizure onset (years)	Duration of epilepsy (years)	Frequency of seizures (per month)	Side of focus
HUM-114	29	M	2	27	4	Left
HUM-115	14	F	6	8	150	Left
HUM-116	29	F	6	23	8	Right
HUM-117	28	F	27	1	170	Right
HUM-118	24	M	1	23	20	Bilateral
HUM-119	24	M	5	19	6	Left
HUM-120	30	F	8	22	10	Right
HUM-121	36	F	2	34	252	Bilateral
HUM-122	52	F	13	39	3	Right
HUM-123	24	F	9	15	10	Right
HUM-124	33	F	3	30	5	Left
HUM-125	20	M	1	20	36	Left
HUM-129	38	M	33	5	15	Left
HUM-130	49	M	17	32	8	Right

M, male; F, female.

research. Epileptic hippocampal tissue was obtained from patients with intractable mesial TLE recruited from the Epilepsy Clinic Program of Hospital General de Mexico (Table 1). Each patient signed an informed consent. All the patients were on medication with one or two of the following antiepileptic drugs: carbamazepine, valproic acid, phenobarbital, and phenytoin. None of the patients involved in the present study demonstrated gross structural lesions other than hippocampal sclerosis. In 12 patients noninvasive studies with concordant results were enough to program a temporal lobectomy. In two patients a Phase II invasive protocol was mandatory. It includes either bilateral hippocampal electrodes or basolateral grid implantation with continuous video-EEG monitoring (Velasco et al., 2000). Epileptic patients had "en block" anterior lobectomy, ipsilateral to the epileptic focus at least 48 h after the last seizure. During the surgical procedure, hippocampal biopsies were collected immediately upon resection, quickly frozen in pulverized dry ice and stored at −70°C. The whole hippocampus obtained at autopsy from subjects with no evidence of neurological disease was used as control tissue since previous reports indicate that MOR mRNA, binding and agonist-stimulated [^{35}S]GTPγS binding are preserved for several hours after death (Platzer et al., 2000; González-Maeso et al., 2002; Escribá et al., 2004). Hippocampus was dissected at the time of autopsy, with a postmortem interval of 3–10 h, immediately stored at −70°C and then manipulated as described below no later than 1 week after they were obtained (Table 2). For evaluation of MOR and GAPDH mRNA expression, total RNA isolation and reverse transcription polymerase chain reaction for human MOR and GAPDH mRNAs were carried out according to Yalcin (2004). Human MOR (gene bank: NM_001145283) primers were used as described previously (Tripathi et al., 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (gene bank: NM_002046) primers were newly designed using Primer3 software (Rozen and Scaletsky, 2000). The forward primers for MOR and GAPDH genes were 5′-TCTGGCTCCAAAGAAAAGGA-3′ and 5′-GAGTCAACGGATTTGGTCGT-3′, respectively. The reverse primers for MOR and GAPDH were 5′-CAATGCAGAAGTGCCAAGAA-3′, 5′-TTGATTTTGGAGGGATCTCG-3′, respectively. Conditions for PCRs were optimized in a gradient cycler (Techne 512, UK) with regard to primers and various annealing temperatures. Optimized settings were transferred to real-time PCR protocols on a Stratagene Mx3000P real-time detection system (Stratagene, USA). Amplification of 1 μl RT mixture (cDNA diluted 1:5) was carried out using 1 μl (10 pmol) forward and reverse primers, 12.5 μl Brilliant SYBR® Green Q PCR 2× Master Mix (Stratagene, USA) and 9.5 μl nuclease-free water in a total volume of 25 μl. Cycling parameters were as follows: 5 min at 94°C, 45 s at 94°C followed by 40 cycles of 30 s at 60°C and 50 s at 72°C. An additional cycle for melting curve analyses was 1 min at 95°C, 30 s at 55°C. Both cDNA synthesis and PCR amplifications included negative control reactions, which were set up by excluding RNA and DNA templates, respectively. The melting temperatures (T_m) for MOR and GAPDH were 80.7 and 73.6°C, respectively. GAPDH gene was used as an endogenous control for normalization. The relative expressions of target genes were quantified according to ABI Prism 7700 Sequence Detection System User Bulletin No. 2 (Applied Biosystems, Foster City, CA) and Livak and Schmittengen (2001). The PCR products were analyzed using 2% agarose gel which contained ethidium bromide. For saturation binding and [^{35}S]GTPγS functional assays, crude membrane fraction from human hippocampus was prepared according to the method previously described (Benyhe et al., 1997). Protein levels were determined by the method of Lowry et al. (1951). Saturation binding assay was performed according with the procedure described previously (Gabilondo

TABLE 2.

Characteristics of Control Subjects

Subject	Gender	Age (years)	Cause of death	Postmortem delay (h)
C1	M	66	Gastrointestinal bleeding	4
C2	M	50	Hepatitis	6
C3	F	71	Diabetes	5
C4	F	77	Hypovolemic shock	3
C5	F	39	Ovarian cancer	3
C6	F	20	Chronic renal failure	4
C7	M	29	Hypovolemic shock	5
C8	M	37	Hypovolemic shock	10

M, male; F, female.

et al., 1995). The data obtained were first fit to the rectangular hyperbolic function followed by linear transformation (Scatchard, bound/free vs. bound) to determine equilibrium dissociation constant (K_D) and receptor density (B_{max}) using the program Prism (GraphPad Software). [^{35}S]GTPγS functional assay was used to evaluate receptor-mediated G-protein activation as described previously (Rocha et al., 2009). G-protein activation was given as percent of the specific [^{35}S]GTPγS binding observed in absence of receptor ligands (basal activity). [^{35}S]GTPγS binding experiments were performed in triplicates. Data were subjected to nonlinear regression analysis of concentration effect curves performed by Prism (GraphPad Software) to determine Log EC_{50} and E_{max} values. Results from all experiments were examined statistically by Student's t test and a value of $P < 0.05$ was considered statistically significant. Epileptic hippocampi were obtained from 14 patients (six male and eight female) ranged in age from 14 to 52 yrs (30.7 ± 2.7 yrs old) with intractable mesial TLE history. Their clinical data were as follows: 21.2 ± 2.9 yrs of epilepsy duration; age of seizure onset at 9.5 ± 2.6 yrs old; and 49.7 ± 21.3 seizures per month (Table 1). Autopsy samples were acquired from 8 subjects (4 male and 4 female) ranging in age from 20 to 77 yrs (44.5 ± 7.3 yrs), who died by different causes and without history of neurological disease (Table 2). Total RNA contents from mesial TLE group (1.61±0.59) were similar to those found for autopsy samples (1.47±0.68) (Fig. 1A). The expected PCR products for MOR and GAPDH from autopsies and epileptic patients were detected at 172 and 238 bp, respectively (Fig. 1B). We found relative gene expression levels of MOR significantly higher in hippocampus of patients with mesial TLE (2.71 ± 0.38, min 0.28, max 4.78, $P < 0.05$) in contrast with autopsy samples (1.25 ± 0.50, min 0.37, max 2.54) (Fig. 1C). The specific MOR binding obtained from the equilibrium binding experiments was found to be saturable in hippocampus of both, autopsy and mesial TLE groups. [^{3}H]DAMGO binding from autopsy samples correlated positively with age at death ($r = 0.899$, $P < 0.05$) and supports data obtained from previous studies (Gabilondo et al., 1995). Even though autopsy samples were obtained from older subjects, the B_{max} value from the mesial TLE group was signifi-

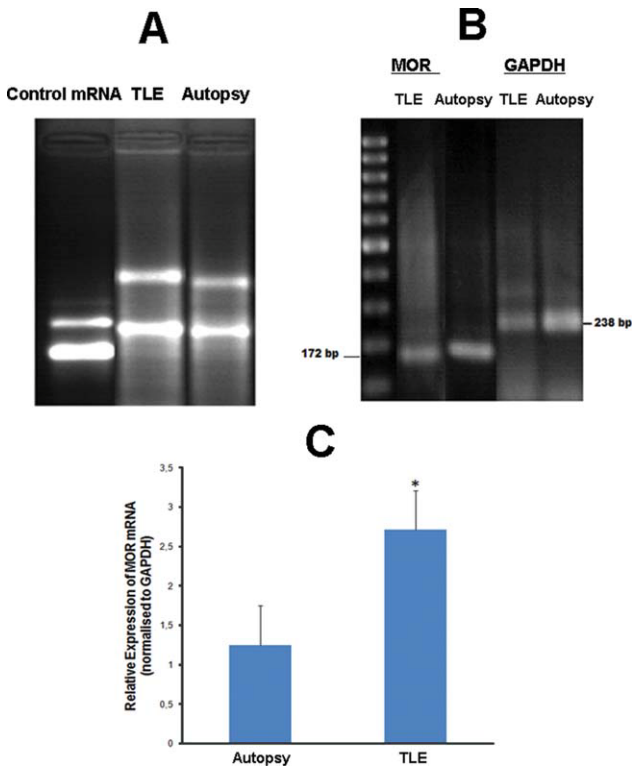

FIGURE 1. RT-PCR analysis showing the presence of MOR in human hippocampus. mRNA from patients with mesial temporal lobe epilepsy (MTLE) was compared with mRNA isolated from autopsy (control) samples. (A) Total RNA integrity analysis. Lane 1: Control RNA sample; Lane 2: total RNA isolated from a sample of a patient with MTLE; Lane 3: total RNA isolated from a control sample. (B) Agarose gel analysis of MOR and GAPDH genes in one control and one MTLE samples. The expected PCR products for MOR and GAPDH were 172 and 238 bp, respectively. Lane 1: Marker DNA 100bp; Lane 2: MOR expression in the hippocampus of a patient with MTLE; Lane 3: MOR expression in a control tissue; Lane 4: GAPDH expression in the hippocampus of a patient with MTLE; Lane 5: GAPDH expression in a control sample. (C) Relative gene expression levels of MOR in control ($n = 4$) and MTLE ($n = 7$) groups. Data represent mean ± S.D of three independent real-time polymerase chain reaction experiments. *$P < 0.05$ vs. autopsy samples. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

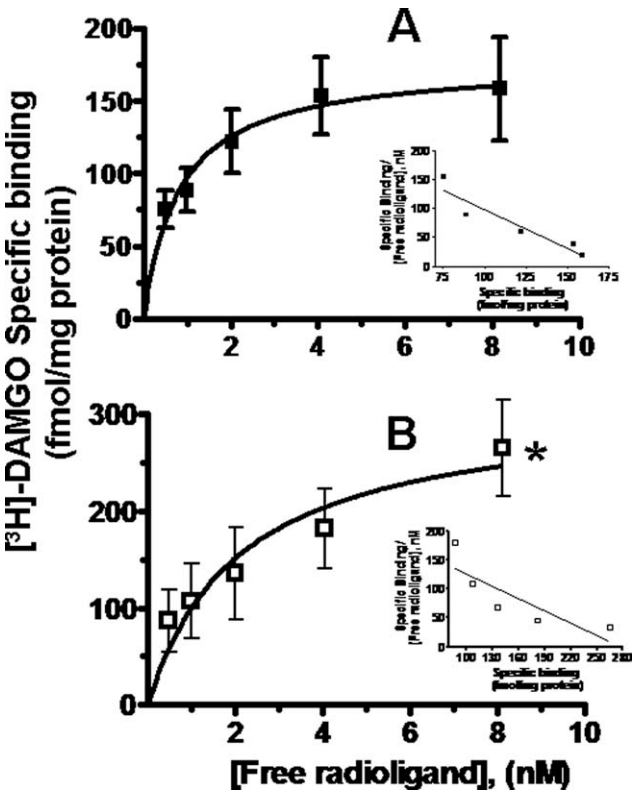

FIGURE 2. Saturation analysis for [^{3}H]-DAMGO binding in hippocampus membranes of autopsy samples (n = 8, ■) and epileptic patients (n = 14, □). Membranes were incubated with increasing concentrations of [^{3}H]-DAMGO in the absence (total binding) or presence of Naloxone (10 μM, nonspecific binding). Points represent mean ± S.E.M of the experiments performed in triplicate. The corresponding Scatchard plot is given as inset. Note that the receptor density (B_{max}) value for the epilepsy group was higher with respect to the autopsy samples. *$P < 0.05$ vs. autopsy group.

cantly higher (60%, $P < 0.05$), whereas the K_d value from both groups (0.75 ± 0.08 nM, autopsy; 1.4 ± 0.61 nM, epilepsy) was not statistically different (Fig. 2). These results suggest that the hippocampus of patients with mesial TLE present an increased density of MOR, whereas their equilibrium dissociation constant is not modified. MOR-mediated G-protein activation was assayed by the radiolabel [^{35}S]GTPγS bound to the G protein. Basal activity from mesial TLE group was significantly higher (136%, $P < 0.05$) when compared with autopsy samples (Fig. 3A). DAMGO-stimulated [^{35}S]GTPγS binding was concentration-dependent and saturable. In autopsy samples, [^{35}S]GTPγS binding stimulation by DAMGO showed a positive correlation for net stimulation values in relation to age at death (r = 0.870, $P < 0.01$) (data not shown), supporting the influence of aging on functional [^{35}S]GTPγS binding (González-Maeso et al., 2002). [^{35}S]GTPγS binding assays including the analysis of all autopsy samples revealed a maximal stimulation (E_{max}) of 42.8% and a potency (EC_{50}) value within the micromolar range (−6.48 ± 0.16). To obtain a clear interpretation of the specific role of transduction mechanisms in epilepsy, values obtained from mesial TLE group were compared with those obtained from autopsies of subjects in a range of age from 20 to 50 yrs old. According to this comparison, both groups demonstrated similar Emax (27.8 and 20.8%, autopsy and mesial TLE groups, respectively) and EC_{50} (−7.0± 0.2 and −6.82 ± 0.2, autopsy and mesial TLE groups, respectively) values (Fig. 3B). These results indicated that, although the epileptic hippocampus presents higher density of MOR (see saturation binding results), it does not show significant changes in the efficacy (E_{max}) and potency (EC_{50}) of DAMGO for the stimulation of the G protein. To our knowledge, this is the first time that alterations in MOR mRNA expression, binding and induced G-protein activation in hippocampus of patients with pharmacoresistance mesial TLE are described. We found an enhancement in MOR gene expression that was consistent with significant increases in receptor density. Surprisingly, there were not significant alterations in DAMGO stimulated [^{35}S]GTPγS binding. According to previous studies, it is important to consider that the autopsy samples were collected at postmortem intervals that allow the preservation of their physical conditions (González-Maeso et al., 2002; Escribá et al., 2004). Then, values obtained from autopsy samples under our experimental conditions can be considered as control situation. Other important factor to be considered is that the density as well as MOR-mediated G-protein stimulation are directly correlated with age (Gabilondo et al., 1995; González-Maeso et al., 2002), a notion that was supported by our results obtained from autopsy samples. Gene expression is modulated by proteins acting at DNA regulatory sequence. Regulation by transcription activators and repressors could be involved in the enhanced MOR mRNA expression found in the epileptic hippocampus. In this context, it has been shown that the overexpression of poly(ADPribose) polymerase-1 (PARP-1), a 116-kDa nuclear protein known to have DNA binding activity and enzymatic activity of ADP-ribosylation (Soldatenkov et al., 2002), upregulates MOR gene transcription (Ono et al., 2009). Similarly, interleukin-6 strongly induces MOR mRNA in the human neuroblastoma cell line SH SY5Y, an effect dependent on the transcription factors 1 (STAT1) and STAT3 (Börner et al., 2004). PARP-1 activation has been proposed to occur following status epilepticus (Fujikawa, 2005), whereas interleukin-6 blood concentrations are chronically increased in patients with TLE (Liimatainen et al., 2009). In the future, more detailed studies are needed to assess the mechanisms involved in the augmentation of MOR mRNA expression in the human epileptic hippocampus. In spite of their known neuronal localization, MOR are also expressed in glia (Murphy and Pearce, 1987). It is possible to suggest that the enhanced gliosis found in hippocampus of patients with mesial TLE may account for the high [^{3}H]DAMGO binding detected in the present study. Although no direct evidence exists in the human brain, experiments carried out in rats support a reduced opioid peptides release in hippocampus during the interictal period (Rocha et al., 1997). The enhanced density in MOR found in the epileptic hippocampus could be a homeostatic reaction caused by a decreased release of endogenous opioid peptides in this brain area. Despite of the high MOR mRNA and density

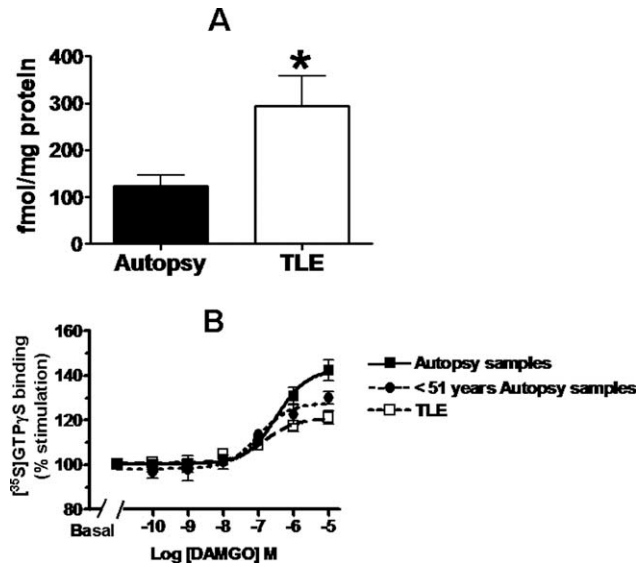

FIGURE 3. (A) Representation of absolute basal values obtained from autopsies (n = 8) and epileptic patients (n = 14). Values are represented as mean ± S.E.M. of fmol/mg of protein. (B) Specific [^{35}S]GTPγS binding to membranes of samples from all autopsies (-■-), from autopsies of subjects of less than 51 yrs old (n = 5, ···●···) and epileptic patients (-□-) as a function of increasing concentration of the mu receptor agonist DAMGO. Each point represents the mean ± S.E.M of the individual percentage stimulation over basal values. Note that in epileptic patients, the [^{35}S]-GTPγS binding percentage stimulation by DAMGO was no significantly different from autopsy samples of subjects of less than 51 yrs old. *P < 0.05 vs. autopsy group.

levels detected in the hippocampus of patients with mesial TLE, [^{35}S]GTPγS binding experiments indicated that MOR signaling is not altered. Receptor activation of G proteins is a vital step in the signal transduction pathways determining agonist efficacy (Kenakin, 1993), so the fact that the activity of the MOR signaling is not modified in the epileptic hippocampus can be considered as an intracellular counteradaptation that occur in the epileptic focus preventing increased MOR binding to result in altered intracellular signaling. An explanation for this situation is that high levels of receptor density may tend to produce saturation of stimulus-response mechanisms, leading to a limit in the maximal ordinate response measured. Other explanation could be that the increased [^{3}H]-DAMGO binding could reflect an increase in the density of receptors that comprises some with different intrinsic ability to activate G proteins. Indeed, since MOR couple to several different types of Gα subunits (Chakrabarti et al., 2005), epilepsy may have different effects on specific subtypes of Gα subunits with different affinities for GTP. An interesting result from our functional experiments was that the basal binding was increased in the epileptic hippocampus. It is possible that a substantial portion of the basal activity found in the present study represents preactivated G proteins, i.e., an spontaneous interaction established between receptors and G protein in the cell membrane, situation that results in a tonic level of GTPase stimulation (Scheer and Cotecchia, 1997). Previous studies indicate that part of the apparent preactivation of basal activity may result from an opioid receptor-dependent mechanism, in as much as small but significant reduction of basal activity is also observed following opioid-mediated downregulation (Costa et al., 1988). In addition, high levels of receptor density, as the one we found in the present study, are anticipated to increase basal binding as well as the precoupled or constitutively active forms of the receptors (Costa et al., 1990). The nonsignificant increase in DAMGO-induced G protein activation in proportion to the high MOR density in the epileptic hippocampus can be explained because the occupation of the constitutively active receptors by agonists may not increase [^{35}S]GTPγS binding much above basal levels (Costa et al., 1990). Since the increase of the constitutive activity of G protein-coupled receptors can be induced by mutations or they occur spontaneously in human diseases (Scheer and Cotecchia, 1997), we cannot exclude that it plays a significant role in the pathophysiology of the pharmacoresistant epilepsy. Finally, the present findings provide direct information on the functional status of MOR in the hippocampus of patients with pharmacoresistant mesial TLE that will probably aid in the design of better antiepileptic medication for this disorder. Further studies are necessary to elucidate the role of intracellular changes associated with MOR in the epileptic hippocampus during the epilepsy process.

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