Block of Glutamate-Glutamine Cycle Between Astrocytes and Neurons Inhibits Epileptiform Activity in Hippocampus

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Bacci Alberto, Giulio Sancini, Claudia Verderio, Simona Armano, Elena Pravettoni, Riccardo Fesc, Silvana Franceschetti, and Michela Matteoli. Block of glutamate-glutamine cycle between astrocytes and neurons inhibits epileptiform activity in hippocampus. J Neurophysiol 88: 2302–2310, 2002; 10.1152/jn.00665.2001. Recurrent epileptiform activity occurs spontaneously in cultured CNS neurons and in brain slices in which GABA inhibition has been blocked. We demonstrate here that pharmacological treatments resulting in the block of glutaminase activity by astrocytes or the inhibition of glutamine uptake by neurons suppress or markedly decrease the frequency of spontaneous epileptiform discharges both in primary hippocampal cultures and in disinhibited hippocampal slices. These data point to an important role for the neuron-astrocyte metabolic interaction in sustaining episodes of intense rhythmic activity in the CNS, and thereby reveal a new potential target for antiepileptic treatments.

INTRODUCTION

Enhanced activity of excitatory synapses, produced by an imbalance of excitation and inhibition, has been considered as a possible mechanism for sustained epileptic hyperexcitability. In particular, evidence from experimental models of epilepsy and from patients indicates that glutamate and aspartate play an important role in the initiation, spread, and maintenance of epileptic discharges and that compounds that modify glutamate release alter seizure expression (Chapman 1998; Lee and Hablitz 1989; Loscher 1998; Malouf et al. 1990; McBain et al. 1988; Meldrum 1994). Whereas considerable attention has been focused on the involvement of glutamatergic neurotransmission in epilepsy, little is known about the mechanisms sustaining the spontaneous synaptic activities allowing recurrent seizures. In this scenario, one may expect that neurons, during epileptiform activity, require a strong metabolic support, in the form of precursors and proper energy substrates, to fill the rapid-turnover neurotransmitter pool and to continuously adjust the ionic gradients which tend to be altered by the increased neuronal activity.

In the CNS, neurons are metabolically coupled to astrocytes and the key-site of this neuron-glia interaction is the synapse (Bacci et al. 1999a; Haydon 2001; Pfrieger and Barres 1996; Tsacopoulos and Magistretti 1996), which is the most specialized structure responsible for transmitting and processing information between neurons. Among other functions, astrocytes are responsible for removing potassium ions (Cooper 1995) and glutamate (Bergles et al. 1999) accumulating in the synaptic microenvironment during synaptic activity. Gial cells also provide the presynaptic terminal with the glutamate precursor glutamine and with lactate, which is used by neuronal cells as a preferential energy substrate (for reviews see Bacci et al. 1999a; Deitmer 2001; Pfrieger and Barres 1996; Rothman et al. 1999; Tsacopoulos and Magistretti 1996). One might therefore hypothesize that astrocytes play a role in supporting an efficient neuronal functionality, via these multiple processes, during prolonged synaptic activity. In agreement with this possibility, it has been previously demonstrated that the glial-specific metabolic blocker fluoroacetate, which selectively inhibits glial cell function, decreases spontaneous and evoked synaptic transmission (Keyser and Pellmar 1994).

In a previous study, we reported that synchronous epileptiform activity in hippocampal cultures is dependent on the presence of adjacent glial cells (Verderio et al. 1999). Here we show that the maintenance of epileptiform activity in cultured hippocampal neurons and in hippocampal slices in which GABA-inhibition has been blocked crucially depends on the delivery of glutamine from astrocytes to neurons. These results reveal a novel function of glial cells in supporting the long term feeding of neuronal cells during enhanced neuronal activity.

METHODS

Hippocampal cell culture

Primary neuronal cultures were prepared from the hippocampi of 18-day-old fetal rats as previously described (Bartlett and Banker 1984; Verderio et al. 1999). In some experiments, to warrant the strict proximity with glial cells, neurons were plated on coverslips already containing monolayers of hippocampal astrocytes, prepared as described (Calegari et al. 1999).

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GLUTAMATE-GLUTAMINE CYCLE IN EPILEPSY

Fura-2 videomicroscopy and patch-clamp recordings from hippocampal neurons in culture

Cultures were loaded for 40 min at 37°C with 2–4 mM Fura-2 pentacetoxy-methylester in Krebs-Ringer solution buffered with HEPES (KRH) with the following composition (in mM): 150 NaCl, 5 KCl, 1.2 MgSO4, 2 CaCl2, 10 glucose, and 10 HEPES/NaOH; pH 7.4) and transferred to the recording chamber of an inverted microscope (Axiovert 100; Zeiss) equipped with a calcium imaging unit. A modified CAM-230 dual wavelength microfluorimeter (Jasco, Tokyo, Japan) was used as a light source for the assays. The experiments were performed at room temperature (24–25°C) using an Axon Imaging Workbench 2.2 equipped with a PCO Super VGA SensiCam (Axon Instruments). Conventional whole cell patch clamping was employed using an Axopatch 200B amplifier (Axon Instruments) controlled by a PC computer. During acquisition and data analysis, pClamp (Axon Instruments) and Origin (Microcal) software was routinely used. The patch pipette (3–5 MΩm resistance) contained the following (in mM): 140 potassium gluconate, 2 MgCl2, 10 HEPES, 1 EGTA, and 2.5 MgATP. Miniature EPSCs (mEPSCs) were recorded in 1 µM tetrodotoxin (TTX) and 100 µM APV. All the data are reported as mean ± SE. Error bars represent SE. Statistical comparisons were done by using Student’s t-test. Differences were considered significant if P < 0.05.

Extracellular field potential recordings from hippocampal slices

Wistar rats (adult, both sexes) were deeply anesthetized by ether inhalation and decapitated. The brain was removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 3.5 KCl, 2 CaCl2, 2 MgSO4, 1.2 NaH2PO4, 26 NaHCO3, and 10 glucose (pH 7.3–7.4) and was bubbled with 95% O2–5% CO2. Transverse hippocampal slices 400–600 µm thick were cut from dorsal hippocampus using a vibratome, transferred to an interface chamber, and perfused with ACSF. Moreover, in some experiments, MeAIB 12.5 mM was freshly added to standard ACSF. Miniature sEPSCs (mEPSCs) were recorded in 1/4 normal strength ACSF. CNQX (10 µM), d-APV (10 µM), NBQX (10 µM), dissolved in 0.1% DMSO, and α-methylaspartic acid (MeAIB; 12.5 mM) were freshly added to standard ACSF. CNQX (10 µM), d-APV (10 µM), NBQX (10 µM), dissolved in 0.1% DMSO, and α-methylaspartic acid (MeAIB; 12.5 mM) were freshly added to standard ACSF.

Field potentials were recorded from CA1 region (pyramidal layer and/or stratum radiatum). Series of field responses were evoked by low frequency (0.2 Hz) electrical stimulation of the Schaffer collaterals. Signals were continuously monitored by means of chart recording, stored on a magnetic tape, and digitized off-line on a PC (sampling frequency of 2–4 kHz). The frequency of SEDs was calculated as the reciprocal of the mean inter-SED interval from recordings samples of 16 min. The slope of the descending portion of the population EPSP was extracted by a computer home made program using linear regression. Paired t-test or Wilcoxon test were used for statistical analysis.

RESULTS

Epileptiform activity in cultured hippocampal neurons relies on metabolically intact astrocytes

Cultured hippocampal neurons (12–20 days old) were loaded with the calcium indicator Fura-2 and recorded in physiological medium (KRH) by means of calcium imaging and whole cell current-clamp techniques. We have previously demonstrated that neurons undergo spontaneous [Ca2+]i oscil-
which use it as a precursor for the synthesis of glutamate (Bacci et al. 1999a; Pfrieger and Barres 1996; Rothman et al. 1999; Fig. 6). To directly test the functional significance of the astrocyte-dependent glutamate-glutamine pathway, cultures were exposed to 100 μM methionine sulfoximine (MSO), a selective inhibitor of the enzyme glutamine synthetase, which is specifically expressed by glial cells (Martinez-Hernandez et al. 1977). This experimental treatment has been previously found to result in a block of glutamine production in astrocytes (Derouiche and Rauen 1995; Laake et al. 1995; Ronzio et al. 1969). One hour of treatment with MSO was sufficient to produce either a complete block of epileptiform activity, leaving basal synaptic activity only (Fig. 2A), or a relevant reduction of the burst frequency (80.7 ± 4.2% reduction; Fig. 2B; n = 10). The MSO-induced block of oscillations was always prevented by the simultaneous exposure of the neuronal cultures to 100 μM glutamine (Fig. 2A; n = 7). Likewise, fluctuations of both membrane potential (Fig. 2B; n = 3) and [Ca^{2+}], (Fig. 2C; n = 11) in MSO-treated cells were partially rescued by the acute application of 100 μM glutamine. MSO-

**FIG. 1.** Fluoroacetate blocks neuronal epileptiform activity in cultured neurons. A: simultaneous current-clamp and calcium-imaging recordings from the same neuron. Long bursts of action potentials, resembling epileptiform activity, coincide with calcium transients. B and C: the gliotoxic drug fluoroacetate (FAc; 200 μM) gradually reduces the frequency of [Ca^{2+}], oscillations. B: plot of oscillation frequency in control and in FAc-treated neurons calculated in 10-min intervals and normalized to control (10 min before FAc application). C: temporal analysis of [Ca^{2+}], oscillations measured from a neuron in a fura-2-loaded culture. FAc reduces oscillations with a latency to onset approximately 10 min following addition (arrow), up to a complete block of the activity after approximately 40 min. The 2 traces are continuous in time. Application of 30 μM glutamate at the end of the experiment, induces a calcium response in the FAc-treated neuron. D: calcium responses elicited by 30 μM glutamate application before and after 30 min of FAc application in the same neuron. E: histogram quantifying the responses to glutamate before and after FAc application. F: current-clamp recordings from a neuron in control solution (top), from a neuron exposed for 1 h to 200 μM FAc (middle), and from a neuron exposed for 1 h to 200 μM FAc in the simultaneous presence of 100 μM glutamine (bottom). In FAc-treated cultures, spontaneous postsynaptic potentials are still present. Glutamine prevents the FAc-induced inhibition of the oscillatory activity. G: plot showing the percentage of oscillating cells in control conditions, after FAc intoxication and after FAc plus glutamine treatment.
treated neurons did not display any change in resting potential and input resistance (data not shown). Furthermore, MSO application did not change the receptor responsivity to the application of exogenous glutamate (data not shown).

To investigate whether the block of glutamine synthetase may play a direct role in glutamate release, mEPSCs were recorded, on MSO treatment, from hippocampal neurons growing in culture on top of astrocytes. A significant reduction of mEPSC frequency was recorded on MSO treatment (mEPSC frequency recorded 20–30 min after MSO application: 47.8 ± 11.8% compared with controls; 0–7 min of recording; n = 4; P < 0.05). No significant effect was produced by MSO, when applied in the concomitant presence of 100 μM glutamine. In the absence of glial cells, the frequency of mEPSC recorded 20–30 min after MSO application was 86.58 ± 5.05% compared with controls (0–7 min of recording; n = 4; P > 0.05) thus indicating that MSO affects miniature activity through an effect on glial cells.

Spontaneous epileptiform discharges induced in hippocampal slices by GABA-antagonists are reduced by block of glutamine synthesis in astrocytes

We then tested whether the glutamate-glutamine shuttle between neurons and glial cells is able to sustain the long-term maintenance of recurrent spontaneous epileptiform discharges (SEDS) in hippocampal slices, following induction of long-lasting hyperexcitability. The perfusion with 5–10 μM bicuculline methiodide or 25–50 μM picrotoxin added to ACSF consistently resulted in hyperexcitability phenomena, which occurred spontaneously. SED frequency reached a steady-state condition after about 30 min from the onset of GABA antagonist perfusion.

The effect of MSO on SED frequency was tested about 60 min following the perfusion of GABA A-receptor blockers to allow epileptiform discharges to reach a steady-state frequency. After 30 min of MSO (2 mM) perfusion, a decline in SED frequency could be observed (Fig. 3, A and B; 41.4 ± 0.1% of reduction; P < 0.01; n = 9), and this decay became more evident during the following 60–90 min of MSO perfusion (Fig. 3C; 66.1 ± 4.7% of reduction, P < 0.005, n = 9). The addition of 100–200 μM glutamine invariably recovered the frequency of SEDs to values close to those observed before MSO perfusion (82.7 ± 10.6%).

In some experiments, MSO-resistant SEDs slightly increased in duration, showing a greater number of population spikes (Fig. 3A). In the presence of GABA blockers (bicuculline or picrotoxin) the number of population spikes was 4.9 ± 0.8, and remained substantially unchanged during the first 30–60 min of MSO perfusion (4.3 ± 0.8), whereas it increased to an average value of 7.9 ± 1.0 during the following 30–60
min \( n = 9 \). This phenomenon was however inconsistent and the difference did not reach a statistical significance.

The action of MSO was irreversible: the recovery of SED frequency to values close to those observed before MSO perfusion did not occur in case of MSO washout and was found to directly depend on the presence of glutamine in the perfusing medium (Fig. 3D). Glutamine perfusion recovered SED frequency to 50\% of control value in 15.9 \pm 1.6 min, reaching a steady state frequency after 33.9 \pm 1.6 min. A subsequent glutamine washout produced again a decrease of epileptiform discharge frequency (Fig. 3E; \( n = 4 \)). SED frequency was not affected when glutamine was added to the disinhibited slices not previously treated with MSO (Fig. 3F; \( n = 3 \)). CNQX quickly abolished SED recurrence (data not shown).

**Epileptiform activity in culture and in brain slices is inhibited by impairment of glutamine uptake in neurons**

The major carriers for glutamine uptake within the brain are represented by systems A, L, and ASC. System A, a sodium-dependent, unidirectional transporter that exhibits reduced activity at low pH, primarily mediates glutamine uptake in neurons. As system A family of transporters, which includes the recently cloned GlnT/SAT1 (Varoqui et al. 2000), represents a crucial link in the glutamate-glutamine cycle, playing a role as a possible gateway for the synthesis of glutamate in neuronal cells, we have investigated whether an impairment of its function could affect epileptiform activity in hippocampal neurons. A characteristic feature of system A, which differentiates it from systems L and ASC, is the ability to transport \( N \)-methylated substrates such as the non metabolizable amino acid analog \( \alpha \)-(methylamino)isobutaric acid (MeAIB), which competes with glutamine for uptake. MeAIB, used at concentrations previously shown to inhibit glutamine uptake (10–50 mM) (Varoqui et al. 2000), reduced or completely blocked the occurrence of spontaneous oscillatory activity in fura-2-loaded cultures of hippocampal neurons, in a reversible manner (Fig. 4A). MeAIB-treated neurons did not display any change in resting potential or input resistance (data not shown). Furthermore, MeAIB did not affect glutamate receptor responsivity (amplitude of the calcium response to 10 \( \mu \)M glutamate after MeAIB treatment: 97.2 \pm 4.8\% with respect to controls; \( n = 6 \); \( P > 0.05 \); Fig. 4, B and C).

We then tested the effect of MeAIB on epileptiform discharges in disinhibited hippocampal slices. MeAIB (12.5 mM) reduced SED frequency in few minutes (Fig. 5, A and B), reaching a frequency of 43.1 \pm 7.0\% of the control value (\( n = 8 \); \( P < 0.01 \)). MeAIB effect was completely reversible on wash (87.2 \pm 2.2\% of the control; Fig. 5C). The decrease of SED frequency was found to be more prominent when the experiments were performed by adding MeAIB to ACSF (\( n = 4 \), 33.0 \pm 4.6\% of the control frequency) compared with the experiments in which MeAIB was equimolarly substituted to NaCl (\( n = 4 \), 59.1 \pm 6.4\%). To exclude any possible osmotic effect on epileptiform discharge frequency, we performed similar experiments applying 12.5 mM glucose instead of MeAIB. Glucose, which is transported through cell membranes with similar kinetics as MeAIB (Chaudhry et al. 2002; Maher et al. 1996), was found to be ineffective on SED frequency (\( n = 3 \), 95.0 \pm 5.5\% of the control values). As the neuronal glutamine transporter is Na\(^+\)-dependent (Varoqui et al. 2000), these data suggest that the difference in MeAIB potency may be ac-
counted for by extracellular Na\(^+\) concentration. To assess the effect of MeAIB on excitatory neurotransmission, we tested the substance on the glutamate-mediated population EPSPs (Collingridge et al. 1983) evoked in slices perfused with standard ACSF (n = 4). As shown in Fig. 5D, the peak amplitude of population EPSPs recorded in stratum radiatum was reversibly depressed in the presence of MeAIB. The peak amplitude and the slope of population EPSP were respectively reduced by 27.8 ± 4.0% and 31.8 ± 6.9% with respect to the control values (P < 0.05).

**DISCUSSION**

The occurrence of spontaneous synchronous discharges is one of the hallmarks of epileptiform activity. A potentially useful approach to study the mechanisms underlying the seizure-like activity is represented by hippocampal neurons in primary culture, due to their relatively simplified circuitry. In this experimental model, long bursts of action potentials, coincident with calcium oscillations, commonly occur either spontaneously (Bacci et al. 1999b; Verderio et al. 1999) or on removal of magnesium ions from the external medium (Abele et al. 1990). This activity, which has been considered as an “in vitro” model of epilepsy (De Lorenzo et al. 1998; Furshpan and Potter 1989; Segal 1994; Segal and Furshpan 1990), relies on glutamatergic neurotransmission (Abele et al. 1990; Bacci et al. 1999b; Lawrie et al. 1993; Murphy et al. 1992; Nunez et al. 1996) and requires the removal of glutamate from the synaptic environment by adjacent astrocytes (Verderio et al. 1999). Once taken up by glial transporters, glutamate is converted by

![Image](https://example.com/image.png)
the enzyme glutamine synthetase into glutamine, which is then sent back to neurons. Glutamine is thought to efflux from astrocytes via specific carriers belonging to the System N family, whose expression is restricted to astrocytes (Chaudhry et al. 1999) and to be accumulated by neurons via specific carriers belonging to the MeAIB-sensitive System A family of neutral amino acid transporters (reviewed in Bode 2001). Glutamine is then used as the major precursor for the synthesis of glutamate (Bacci et al. 1999a; Pfrieger and Barres 1996; Rothman et al. 1999; Tsacopoulos and Magistretti 1996; Fig. 6). Therefore by removing glutamate from the synaptic cleft, astrocytes might also replenish the neuronal pool of neurotransmitter (Laake et al. 1995). In this study, we demonstrate that the functional integrity of the glutamate-glutamine cycle is indeed required for supporting epileptiform activity in hippocampus.

As a first step, we used the gliotoxic drug FAc, which is selectively taken up by astrocytes in the CNS (Hassel et al. 1992; Waniek and Martin 1998) and has specific effects on astrocyte metabolism. FAc has been previously used to demonstrate that glial cells play an integral role in hippocampal synaptic transmission, because FAc treatment of astrocytes was found to reduce both spontaneous and evoked synaptic transmission (Keyser and Pellmar 1994). After being converted to fluorocitrate, FAc inhibits aconitase in the tricarboxylic acid (TCA) cycle (Clarke et al. 1970; Hassel et al. 1992; Muir et al. 1986; Paulsen et al. 1987; Waniek and Martin 1998). In intoxicated astrocytes use glutamine as an alternative energy source, converting it into 2-ketoglutarate, which enters the TCA cycle, rescuing in part cell metabolism, and maintaining ATP levels near normal in the first hours after intoxication (Hassel et al. 1997; Swanson and Graham 1994; Fig. 6). Our finding that glutamine rescues the block of oscillations produced by FAc supported the hypothesis that the astrocyte-dependent glutamine supply to neurons could in fact be relevant in sustaining epileptiform activity.

To more specifically address this point, we pharmacologically interfered with the glutamate-glutamine cycle at two different levels (Fig. 6), either blocking the glutamine synthetase-mediated production of glutamine in astrocytes or inhibiting the GlnT/SAT1-operated glutamine uptake by neurons. Although the pharmacological approach may have some intrinsic limitations, due to the complex mechanisms sustaining and regulating recurrent epileptic discharges, the finding that both treatments resulted in a strong reduction of the frequency of SEDs occurring in primary cultures of hippocampal neurons, strongly pointed to a crucial role of the glutamate-glutamine cycle between astrocytes and neurons in supporting the enhanced synaptic activity. To then investigate whether the supply of glutamine from astrocytes might also contribute to maintain epileptic discharges in brain structures preserving their physiological connectivity, we blocked the glutamate-glutamine cycle in hippocampal slices perfused with GABA antagonists. Blockade of GABA-dependent synaptic inhibition is an extensively applied method to induce “in vitro” epileptiform discharges, which substantially depend on the unbalanced glutamatergic neurotransmission (Dingledine et al. 1986; Lee and Hablitz 1989; Traub and Wong 1983). The synchronous occurrence of SEDs in a large neuronal population give rise to complex field potentials composed by multiple population spikes occurring with a periodic time course (Traub and Wong 1983). Either MSO or MeAIB perfusion were capable of significantly decreasing the frequency of SEDs.

In the case of MSO, the effect was irreversible, persisted after long-lasting washout, but was regularly reverted by glutamine addition to the superfusing medium. The slow and progressive effect of MSO on SED frequency, well agrees with a gradual reduction of the available glial glutamine. This is in agreement with recent data indicating that the recurrent excitatory bursts, which occur in brain stem and sustain the respiratory rhythm, substantially depend from glutamine provided by glial cells, are suppressed by either FAc or MSO and recovered in the presence of exogenous glutamine (Hulsmann et al. 2000). MSO is known to exert a convulsant action “in vivo” (Cloix and Hevor 1998; Sellinger et al. 1968), through a mechanism which is still debated. This property might account for our observation of an increased duration of individual SEDs remaining during late MSO perfusion. This effect, which we found to occur in a delayed time window with respect to the early decline of SED frequency, is better accounted for by a...
late influence on cell excitability mediated by a slow impairment of ammonium or carbohydrate metabolism (Cloix and Hovor 1998; Sellinger et al. 1968) rather than by a direct effect of the drug on glutamate receptors (Shaw et al. 1999). On the other hand, no late “excitatory side effect” could be detected during perfusion with MeAIB, a competitive inhibitor of the system A family of neutral amino acid transporters, not active on systems L, ASC, and N (Bode et al. 2001; Varoqui et al. 2000). As expected by a pharmacological treatment not involving an enzymatic step, the inhibitory effect of MeAIB on SED frequency was very fast compared with MSO. MeAIB is actively transported through the neuronal membrane (Chaudhry et al. 2002; Su et al. 1997; Varoqui et al. 2000), so that, at least at the applied concentrations, it is not expected to exert any significant osmotic effect. In agreement, we did not find any change by applying equimolar concentrations of glucose, which is transported through the cell membrane with a Michaelis-Menten kinetics comparable to that of MeAIB (Maher et al. 1996). The greater efficiency of MeAIB in decreasing SED frequency when applied in the presence of physiological concentrations of extracellular Na+ well agrees with the Na+ dependence of the efficiency of GluT/SAT1 (Varoqui et al. 2000).

Our data also indicate that impairment of the glutamate-glutamine shuttle between astrocytes and neurons affects mEPSC activity of neuronal cells. Indeed, MSO was found to significantly reduce mEPSC frequency in neurons growing on top of glial cells, without having effects in neurons without astrocytes. As a dramatic decrease of mEPSC frequency occurs in rat hippocampal slices and cultures following treatment with bafilomycin A1, a potent blocker of the vacuolar-type ATPase, which results in a reduced synaptic vesicle filling (Zhou et al. 2000), our data open the possibility that the glutamate-glutamine cycle plays a role in glutamate replenishment of synaptic vesicles. In further support of this possibility, MeAIB has been recently found to affect mEPSC frequency and amplitude, selectively in neurons growing on top of glial cells (Armano et al. 2002), thus suggesting the possibility that the glutamate-glutamine shuttle between astrocytes and neurons plays a direct role in glutamate replenishment of synaptic vesicles.

In conclusion, our data support the view that the neuron-astrocyte coupling, through the restoration of the presynaptic glutamate pool, could mediate a plastic form of bidirectional communication, eventually leading to an enhancement of synaptic transmission in the long term range. This aspect of the glia-neuron coupling may have significative physiopathological implications. Glial glutamine could indeed contribute to the persistence of periodic fragmentary EEG and clinical epileptic activities that are known to characterize several forms of severe symptomatic epilepsy in humans (Chatrian et al. 1964; Westmoreland et al. 1986), which often persist for a long time despite antiepileptic treatments.

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