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Amygdala levels of the GluA1 subunit of glutamate receptors and its phosphorylation state at serine 845 in the anterior hippocampus are biomarkers of ictal fear but not anxiety

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Running title: GluA1 phosphorylation states in the limbic system as biomarker of ictal fear

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Abstract: Fear is a conscious state caused by exposure to real or imagined threats that trigger stress responses that affect the body and brain, particularly limbic structures. A sub-group of patients with mesial temporal lobe epilepsy related to hippocampus sclerosis (MTLE-HS) have seizures with fear, which is called ictal fear (IF), due to epileptic activity within the brain defensive survival circuits structures. Synaptic transmission efficacy can be bi-directionally modified through potentiation (LTP, long-term potentiation) or depression (LTD, long-term depression) as well as the phosphorylation state of Ser831 and Ser845 sites at the GluA1 subunit of the glutamate AMPA receptors, which has been characterized as a critical event for this synaptic plasticity. In this study, GluA1 levels and the phosphorylation at Ser845 and Ser831 in the amygdala (AMY), anterior hippocampus (aHIP) and middle gyrus of temporal neocortex (CX) were determined with Western blots and compared between MTLE-HS patients who were showing (n = 06) or not showing (n = 25) IF. Patients with IF had an 11% decrease of AMY levels of the GluA1 subunit ($p = 0.05$) and a 21.5% decrease of aHIP levels of P-GluA1-Ser845 ($p = 0.009$) compared to patients not showing IF. The observed associations were not related to imbalances in the distribution of other concomitant types of aura, demographic, clinical or neurosurgical variables. The lower levels of P-GluA1-Ser845 in the aHIP of patients with IF were not related to changes in the levels of the serine/threonine-protein phosphatase PP1-alpha catalytic subunit or protein kinase A activation. Taken together, the GluA1 subunit levels in AMY and P-GluA1-Ser845 levels in the aHIP show an overall accuracy of 89.3% (specificity 95.5% and sensitivity 66.7%) to predict the presence of IF. AMY levels of the GluA1 subunit and aHIP levels of P-GluA1-Ser845 were not associated with the psychiatric diagnosis and symptoms of patients. This is the first report to address neuroplasticity features in human limbic structures connected to the defensive survival circuits, which has implications for the comprehension of highly prevalent psychiatric disorders and symptoms.

1. Introduction

Fear is a distinct and recognized human emotion that is considered to be a conscious state caused by exposure to real or imagined threats.[1,2] Defensive survival circuits detect and respond to threats, which initiates stress responses in the brain and body that indirectly contribute to conscious fear.[2] In predisposed individuals, acute and intense stress has been associated with post-traumatic stress disorder, and chronic and repetitive stress has been associated with depression and anxiety disorders.[3]

Epilepsies are characterized by recurrent spontaneous hyperexcitable and hypersynchronic brain activity[4,5] that occurs in approximately 0.5 to 1% of the world population. Thirty percent of all patients who have drug-resistant epilepsy are candidates for pre-surgical evaluation.[6] Mesial temporal lobe epilepsy related to hippocampus sclerosis (MTLE-HS) is the most common type of surgically treatable epilepsy.[7–9] In MTLE-HS, the hippocampus (HIP) is involved in seizure onset for 48.5% of cases, the amygdala (AMY) is involved in 26.7% of cases, and synchronous onset in the two structures occurs for the remaining 24.8% of cases.[10] Before consciousness is impaired, patients can become aware of their seizure symptoms in a phenomenon called epileptic aura.[11] The typical MTLE-HS aura includes olfactory, abdominal, autonomic, cephalic or psychic sensations, including déjà-vu, jamais-vu and fear.[14] The aura of fear, which is also termed ictal fear (IF), is characterized by a sudden, often short, conscious state of fear that occurs during the seizure and is unrelated to any real or imagined threats, including the fear of a seizure itself.[13–16] In MTLE-HS patients evaluated with stereotactic implanted depth electrodes (SEEG), the IF sensation and associated behaviour occurred when epileptic discharges involved or interfered with orbito-prefrontal, anterior cingulate, and temporal limbic cortices but did not occur if only the AMY was activated by the epileptic discharge. Interestingly, sensation of fear without associated behavioural changes can be evoked by electric stimulation of the AMY.[15]

Active synapses are bi-directionally modifiable in brain regions, such as the AMY, HIP and neocortex.[17,18] A long-lasting increase in synaptic transmission, called long-term potentiation (LTP), is usually induced by high-frequency neuronal stimulation.[17] Decreases in synaptic efficacy are caused by long-term depression (LTD) after low-frequency stimulation (LFS).[17] *In vivo* pharmacological evidence suggests there is an association between LTP and the fear associative memory task one-trial inhibitory avoidance,[19–22] which is thought to induce LTP in the HIP.[23] Fear conditioning, which is another fear associative memory task, can be inactivated by LTD and reactivated by LTP in the AMY, which supports a causal link between these synaptic processes and fear associative memory.[18] AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are heterotetrameric assemblies of GluA1-4 subunits, and the phosphorylation states of Ser831 and Ser845 of the GluA1 subunit are involved in LTP and LTD.[24–29] LTP induction increases the phosphorylation of both sites.[28,29]

Conversely, in naive synapses, LTD induction dephosphorylates Ser845, whereas in potentiated synapses, Ser831 is dephosphorylated by LTD induction. The level of GluA1 subunit phosphorylation on the Ser831 and Ser845 sites can be used as biomarkers of synaptic plasticity changes in human brain samples.[30]

Because the AMY is a part of a set of defensive survival circuits and its activation contributes to feelings of fear[2] and the anterior hippocampus (aHIP) is mostly connected to the AMY and associated with emotional encoding,[31] we investigated whether the occurrence of IF was differentially associated with the levels of the GluA1 subunit and its phosphorylation at the Ser831 and Ser845 sites in the AMY and aHIP of MTLE-HS patients. For comparison, we analysed samples resected from the middle temporal neocortex (CX). We also investigated if the IF and the levels of the GluA1 subunit and its phosphorylation in the AMY and aHIP were independently associated with the psychiatric diagnosis and symptoms found in our patients.

2. Materials and Methods

2.1. Patients

Thirty-one adult patients who were surgically treated between May 2009 and December 2012 at Centro de Epilepsia de Santa Catarina were prospectively included in this study, which was approved by the Ethics Committee for Human Research of Universidade Federal de Santa Catarina (365-FR304969). Written informed consent was obtained from all participants. They had seizures impairing awareness at least once a month despite adequate treatment with antiepileptic drugs (AEDs).[32] The anamnesis, neurological examination, psychiatric and neuropsychological evaluation, surface video-EEG analysis, and magnetic resonance imaging (MRI, 1.5 Tesla) were consistent with unilateral MTLE-HS.[7–9,33–37] The analysed variables were gender, race, marital status, current work activity, history of initial precipitating injury (IPI), laterality of HS, AEDs, psychiatric diagnosis, age, level of education, disease duration, monthly frequency of seizures, and quality of life. Psychiatric diagnoses were determined by the Fourth Edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV)[38] and the identification of psychiatric conditions frequently associated with epilepsy.[34,39,40] Quality of life was evaluated using the Quality of Life in Epilepsy Inventory-31 (QOLIE-31).[7,8,41] Anxiety and depressive symptoms were assessed by the Hospital Anxiety and Depression Scale (HADS)[37,42] in the last 26 patients who were included in the study.

2.2. Characterization of IF and other epileptic auras

Patients were evaluated by a board-certified clinical neurophysiologist with expertise in epilepsy surgery and who were well familiar with auras, including IF. In all patients, the seizure semiology was essentially the same for several years. The reported auras included epigastric,

cephalic, fear, déjà vu, sternal, jamais-vu, dizziness, autonomic, olfactory, gustatory, sensations of ascending body chills, or poorly defined symptoms. Patients described only one type of aura or a sequence of two or three different auras. IF was assessed as previously described[13] using a standardized interview and confirmed only if (1) it was reported as being concomitant with an epileptic seizure; (2) it arose spontaneously out of context without any external or mental motivation; and (3) it could be clearly distinguished from fear of a seizure. IF perception was described by our using the words “fear”, “fear sensation”, “sensation of death”, “thoughts of dread”, “impending death” or “bad feeling of fear”. Care was taken to avoid suggestive questioning. Patients who could not remember any type of aura were classified in the group without aura (see the supplementary table 1). A careful VEEG analysis showed that all patients with IF (n=06) showed a horrified, tense or preoccupied facial expression during the seizure. Two of the twenty-five patients who did not report IF showed facial behaviour that suggested fear during their seizures. However, because they did not report ictal fear, they were classified in the group without IF. No patient had hypermotor behaviour that suggested frontal lobe semiology.

2.3. Anaesthesia protocol

The anesthetic protocol was the same for all patients,[30] starting between 7:30 to 8:30 a.m. with intravenous (i.v.) bolus of propofol (2 mg/kg), fentanyl (2 µg/kg) and rocuronium (0.9 mg/kg), followed by i.v. remiphentanil infusion (0.1-0.2 µg/kg/min) and isofluorane inhalation (0.5-0.6 MAC). A dexamethasone bolus (10 mg i.v.) was infused immediately after intubation as an adjunctive anti-inflammatory in 20 patients. Hydration was done with isotonic saline (1.2 ml/kg/h) plus the half volume of diuresis. Cephalotine (30 mg/kg) was given 30 min before the anesthesia. Oral AEDs were maintained until the day of surgery (6 a.m.). Patients received 20 mg/kg of phenytoin i.v. 12 hours before the surgery and those under phenytoin at home only received their oral dose at the day of surgery. All patients received a phenytoin bolus (5 mg/kg i.v.) after the brain samples were collected.

2.4. Surgery, intraoperative variables and brain tissue sampling

The analysed samples from brain tissue were removed by a standard anterior and temporal lobectomy[8,9] without thermo-coagulation following the recommended prospective collection model[43] as previously described.[30,44] A 1-cm^2 sample of middle temporal cortex (CX) localized 3 cm posterior to the temporal lobe pole was gently dissected from the white matter. After assessing the mesial temporal region, two-thirds of the AMY, including its basal and lateral nucleus, were resected. Finally, the HIP head and body were removed "en bloc", and the anterior hippocampus (aHIP) was quickly dissected on ice-refrigerated glass. Immediately after collection, the samples were transferred to an Eppendorf tube, frozen in liquid nitrogen

and stored in a -80°C freezer for later analysis. The anaesthesia duration for collecting the brain samples was controlled. Arterial blood gases, electrolytes, haematocrit/haemoglobin, pH, mean arterial pressure, heart and respiratory rate during the AMY/aHIP sampling were controlled. Haemodynamic and respiratory parameters remained stable during all procedures, and there were no surgical complications.

2.5. Biochemical analysis

All samples were homogenized by the same researcher on the same day and stored at -80ºC until the analysis. The phosphorylation levels and total amount of target proteins were determined in a blinded manner for all clinical data by western blot (WB) as previously described.[30,45–47] Briefly, the brain samples were mechanically homogenized in buffer solution containing 50 mM Tris, pH 7.0, 1 mM EDTA, 100 mM NaF, 0.1 mM PMSF, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail and centrifuged 10,000 x g at 4°C for 10 min. The supernatants were diluted in electrophoresis buffer. The protein content was estimated by the method described by Peterson (1977).[48] The proteins (60 µg per track) were electrophoresed in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Proteins were detected with specific antibodies [anti-phospho-GluA1-Ser831 (Sigma-Aldrich, A4352); anti-phospho-GluA1-Ser845 (Sigma-Aldrich, A4477); anti-total-GluA1 (Santa Cruz Biotechnology, sc-13152); anti-PP1 (Santa Cruz Biotechnology, sc-7482); anti-phospho-PKA substrates (Cell Signaling, #9624); anti-EAAT1 (Cell Signaling, #5684); anti-EAAT2 (Cell Signaling, #3838); anti-GFAP (Cell Signaling, #3670) in a 1:1000 dilution. The blots were developed by chemiluminescent reaction. For load control all membranes were incubated with anti-β-actin antibody (Santa Cruz Biotechnology, sc-47778, 1:2000). The phosphorylation level was determined as a ratio of the optic density (OD) of the phosphorylated band relative to the OD of the total band. The protein immunocontent was determined as a ratio of the OD of the protein band to the OD of the β-actin band.[30] Due to the lack of brain tissue samples from healthy controls, an internal control (IC) sample was applied as a reference in all electrophoresis. The reference sample was obtained from 3 pooled HIP prepared as all other samples. The OD ratio (phosphorylated/total or total/β-actin) for each target protein in the reference sample was considered 100% and the data were expressed as percentage variation from the reference sample.[30]

2.6. Statistical analysis

Continuous variables showed a normal distribution (Kolmogorov–Smirnov, $p<0.10$) and differences between patients with and without IF were analysed with the Student's t-test. Categorical variables were analysed by Fisher's exact test. Pearson's coefficient was used for

analysis of correlations. A univariate analysis was done to identify imbalances in the distribution of demographic, clinical, laboratorial and neurosurgical variables between patients with and without IF with a $p<0.20$. These variables were included in a multiple binary regression analysis to determine the independent association between IF and the target variables. Because we had a predetermined hypothesis and to avoid a type II error, no corrections for multiple comparisons were applied, and $p<0.05$ was considered statistically significant.

3. Results

Six patients (19.4%) had no aura, 25 (80.6%) had at least one aura type, and six (19.4 %) had IF alone or in combination with other auras (supplementary table 1).

Table 1 shows that patients with IF had lower levels of GluA1 (-11%) in the AMY ($p = 0.05$) but not in the aHIP ($p=0.82$) and CX ($p=0.38$) compared to patients without IF. Patients reporting IF also had 21.5% lower levels of P-GluA1-Ser845 in the aHIP ($p=0.009$) but not in the AMY ($p=0.28$) and CX ($p=0.20$). Patients with IF showed a non-significant trend ($p=0.14$) for lower levels of P-GluA1-Ser831 in the aHIP, but not in the AMY and CX, compared to patients without IF. The results remained unchanged when the two patients without IF that exhibited facial expressions of fear were excluded from the analysis ($p=0.03$ for the AMY levels of GluA1 subunit and $p=0.01$ for the a-HIP levels of P-GluA1-Ser845, data not shown).

Because IF could be related to imbalances in tissue gliosis, we compared the levels of glial fibrillary acidic protein (GFAP) between patients reporting or not reporting IF (Table 1). There were no differences in the GFAP levels in the AMY ($p=0.75$), aHIP ($p=0.52$) and CX ($p=0.84$) between patients with or without IF. There was also no correlation between the GluA1 subunit and the GFAP levels in the AMY ($r=0.03$, $p=0.88$, data not shown).

As a second marker of gliosis, and because glutamate transmission could be affected by changes in glutamate reuptake by astrocytes, the levels of excitatory amino acid transporter type 1 and 2 (EAAT1 and EAAT2) were determined in the same analysed samples (Table 1). No significant association ($p\geq0.64$) was observed between the occurrence of IF and the levels of EAAT1 and EAT2 in all of the analysed structures.

Multiple linear regressions were performed to investigate the independent association between the levels of the GluA1 subunit in the AMY or P-GluA1-845 in the aHIP and the IF (see Suppl. Table 2). After controlling for the distribution of other frequent auras, only IF was independently associated with aHIP levels of P-GluA1-Ser45 (supplementary table 2, final model 1) or the AMY levels of the GluA1 subunit (supplementary Table 2, final model 2).

The demographic and clinical variables are shown in table 2. The patients were mostly female (58.1%), had a mean age of 36.4 years, 6.6 years of education, 9 seizures impairing awareness per month and 24 years of disease duration. None of the investigated variables

were significantly associated with the occurrence of IF. There was a non-significant trend (p = 0.17) for lower prevalence of IF in patients taking benzodiazepines. Supplementary Table 3 shows that surgical and laboratory variables, e.g., storage time of samples and time since the last seizure before surgery, were not associated with IF. There was a non-significant trend for higher levels of arterial PO_2 pressure during surgery (p=0.13) and longer storage time (p=0.15) for the samples from patients with IF (supplementary Table 3).

Table 3 shows that after controlling for imbalances in the distribution of the arterial PO_2, benzodiazepine use, and time of sample storage with multiple binary regression, the presence of IF remains independently associated with aHIP levels of P-GluA1-Ser845 (adjusted OR 0.92, CI 95% 0.85-0.99, p=0.04) and shows a trend for association with GluA1 subunit levels in the AMY (adjusted OR 0.92, CI 95% 0.84-1.01, p=0.09). Considering the biological plausibility and the small sample size, we believe the observed trend (p=0.09) was a false negative result and both biomarkers were maintained in the final binary regression model (table 3). The aHIP levels of P-GluA1-Ser845 alone had an overall accuracy of 92.1% (specificity 95.5% and sensitivity 33.3%) to predict the occurrence of IF. The AMY levels of the GluA1 subunit alone had an overall accuracy of 87.2% (specificity 100% and sensitivity 33.3%) to predict the occurrence of IF. Together, the AMY levels of GluA1 and the aHIP levels of P-GluA1-Ser845 showed an overall accuracy of 89.3% (specificity 95.5% and sensitivity 66.7%) to predict the IF occurrence.

Because protein kinase A (PKA) phosphorylates and the serine/threonine-protein phosphatase PP1-alpha catalytic subunit (PP1) dephosphorylates GluA1-Ser845, we investigated the correlation between the aHIP levels of P-GluA1-Ser845 and PKA activation or PP1. There was a significant positive correlation between PKA activation and P-GluA1-Ser845 levels (figure 1A). The PP1 levels were not correlated with P-GluA1-Ser845 levels (figure 1B). However, the multiple linear regression analysis revealed that only IF, but not the levels of PKA activation or PP1 levels, were independently associated with the aHIP levels of P-GluA1-Ser845 (figure 1). The results indicate the association between IF and lower aHIP levels of P-GluA1-Ser845 was not related to changes in the levels of PKA activation or PP1.

Finally, IF was not associated with the psychiatric diagnosis (DSM criteria, p=0.78) or with anxiety (p=0.77) or depression (p=0.53) symptoms (Table 2). No significant correlations were observed between the AMY levels of the GluA1 subunit and HADS scores for anxiety (figure 2A, r=0.27, p=0.21) or depression (figure 2B, r=0.18, p=0.41). Finally, the aHIP levels of P-GluA1Ser-845 were also not associated with HADS scores for anxiety (figure 2C, r=0.08, p=0.71) or depression (figure 2D, r=0.04, p=0.85).

Representative Western blot results are shown in suppl. figure 1.

4. Discussion

Patients with unilateral drug-resistant MTLE-HS and IF had significantly lower levels of P-GluA1-Ser845 in the aHIP and GluA1 subunit in the AMY ipsilateral to the HS than patients without IF. The association between P-GluA1-Ser845 levels and IF was not related to changes in PKA activation or PP1 levels in the aHIP. The phosphorylation of GluA1-Ser845 also can be modulated by protein phosphatases 2A and 2B[27] as well as by protein kinase G.[49] Furthermore, GluA1-Ser-845 can also be modified by O-linked N-acetylglucosamine (O-GlcNAc),[50] a post-translational modification regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase, which are enzymes that were not analysed in this present study. It should be noted that this process could impair GluA1-Ser-845 phosphorylation and might be associated with hippocampal LTD.[50] Therefore, these mechanisms might affect GluA1-Ser845 phosphorylation and deserve further investigation.

Using MRI, Cendes et al.[14] showed that MTLE-HS patients reporting IF had a significant reduction in their AMY volume (16%) compared to patients without IF, and their post-operative histopathology correlated well with AMY atrophy.[14] We believe the 11% reduction in the AMY levels of the GluA1 subunit observed in our patients with IF may reflect the neurochemical aspects of the MRI results reported by Cendes at al. several years ago.[14]

In rodents, the HIP encodes contextual aspects of conditioned fear and has major projections to both the prefrontal cortex and the basolateral AMY.[51] Inhibitory avoidance learning promotes an increase in P-GluA1-Ser831 but not GluA1-Ser-845 in HIP synaptoneurosomes.[23,52] The phosphorylation pattern of these two sites of the GluA1 subunit in relation to fear memory resembles what occurs in the LTP induced in posterior hippocampal area CA1 by high-frequency stimulation.[23,53] In addition, phosphorylation of the GluA1 subunit at Ser845 by PKA has been implicated in the enhancement of AMPAR-mediated currents,[54,55] insertion of AMPARs into the postsynaptic membrane,[54,56] and LTP induction after prior LTD.[29] During LTD, the P-GluA1-Ser845 levels may be decreased[56,57] and associated with the removal of AMPARs from synapses, whereas LTP is associated with the delivery of AMPARs to synapses.[25,28,29] Our results may indicate an LTD-like neuroplasticity in the aHIP of patients showing IF compared to patients without IF. Moreover, in contextual fear conditioning, the increased HIP levels of P-GluA1-Ser831 seem to be specifically associated with learning rather than a non-specific effect of aversive stimuli (such as a foot shock or novel context exposure).[23,52,53,58] This outcome could mean that the slight, but not significant, decrease in Ser831 phosphorylation in the aHIP ($p = 0.14$) observed in this study is an indication that IF is distinct from fear conditioning.

The association between the lower aHIP levels of P-GluA1-Ser-845 and IF may be related to previous findings collected with magnetic resonance spectroscopy (MRS) that show a higher degree of neuronal dysfunction in the aHIP of MTLE-HS patients reporting IF.[13] Taken together, both results agree with the classical view of a functional role for aHIP within fear and

anxiety-related behaviours and the endocrine stress response.[31] In physiological conditions, fear caused by exposure to threats results in stress responses[1,59]. Increased levels of glucocorticoids released during chronic stress reduces dendritic branching and spine count in the rat hippocampus[60,61] and has been associated with HIP atrophy[62] and psychiatric illness, including anxiety and mood disorders.[3] However, we did not find any association between the psychiatric diagnoses or symptoms of depression or anxiety and the presence of IF as well the aHIP levels of P-GluA1-Ser-845 and the AMY levels of the GluA1 subunit.

The relationship between fear caused by exposure to real or imagined threats and the unmotivated aura of fear in temporal lobe epilepsy seizures is unknown. The differential diagnosis between panic attacks and IF can be challenging,[15,63] and several findings suggest that both disorders can be part of a continuum of abnormal hyperexcitability or involvement of defensive survival circuits.[63–65] We speculate that our findings in patients with MTLE-HS may have some implications for the role of neuroplasticity in panic attacks. Testing this idea will require some ingenuity since the occurrence of spontaneous fear cannot be investigated under experimental conditions. Temporal lobe epilepsy surgery is the only opportunity to obtain samples from defensive survival circuit structures under adequate conditions to investigate biomarkers of synaptic plasticity. However, our study design does not allow us to make a definitive conclusion as to whether IF is a cause, consequence, or an epiphenomenon of the lower levels of GluA1 in the AMY and P-GluA1-Ser-845 in the aHIP.

Variations in gliosis in aHIP and the AMY of MTLE-HS[66,67] patients could be a confounding bias in our study. Because histopathological analysis was not feasible in the samples used for WB analysis, determining the GFAP and astrocytic glutamate transporters levels were viable alternatives for controlling the gliosis distribution in our samples. The small sample size is a well-known limitation in WB studies, and false negative results are definitely possible. However, the significant associations that were found in a small sample strengthens the credibility of the results.

We would like to emphasize the positive aspects of our study: i) the hypothesis was established prior to the analysis; ii) the prospective study design had a blinded analysis; iii) use of the HADS questionnaire avoided reliance on identifying aspects of the somatic symptoms of psychiatric illness; iv) the extensive control applied to clinical variables and collection of the brain samples; and v) the multivariate analysis approach, which is rarely applied in studies using Western blot results of protein phosphorylation under clinical scenarios. Therefore, we do believe that our results provide reliable information concerning neuroplasticity in fear-related brain structures.

In conclusion, recurrent IF is associated with lower levels of P-GluA1-Ser-845 in the aHIP and the GluA1 subunit in the ipsilateral AMY of patients with unilateral MTLE-HS. This is the first report to address neuroplasticity features in human limbic structures connected to

the defensive circuit, which may have implications for understanding highly prevalent psychiatric disorders and symptoms.

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Table 1: Variation in the neurochemical parameters levels are expressed as a percentage of the reference sample in AMY, aHIP and CX according to the presence of IF.

Variables	All cases n = 31 Mean (SE)	No Ictal Fear n = 25 Mean (SE)	Ictal Fear n = 06 Mean (SE)	"p" value
Amygdala				
GluA1 subunit	97.5 (12.5)	99.6 (9.0)	88.6 (20.6)	***0.05*** [a]
P-GluA1-Ser845	108.1 (18.3)	109.7 (17.7)	99.8 (21.3)	0.28
P-GluA1-Ser831	109.3 (16.8)	109.8 (15.7)	107.1 (22.6)	0.73
GFAP	108.2 (10.6)	107.9 (11.3)	109.5 (8.3)	0.75
EAAT1	95.5 (23.0)	96.0 (24.3)	93.5 (18.2)	0.82
EAAT2	93.6 (15.2)	94.1 (16.8)	91.1 (15.1)	0.65
Anterior Hippocampus				
GluA1 subunit	96.7 (15.9)	97.5 (15.3)	94.0 (19.7)	0.82
P-GluA1-Ser845	104.2 (19.2)	108.8 (17.0)	87.3 (18.5)	***0.01*** [b]
P-GluA1-Ser831	97.0 (19.8)	99.8 (19.7)	88.7 (16.7)	0.14
GFAP	105.0 (7.4)	104.3 (7.8)	107.7 (5.7)	0.52
EAAT1	96.1 (22.6)	95.1 (23.8)	99.5 (19.4)	0.65
EAAT2	90.3 (20.6)	90.7 (19.8)	88.7 (25.4)	0.97
P-PKA substrates	97.5 (25.7)	101.1 (23.6)	83.9 (31.2)	0.15
PP1	95.6 (12.0)	96.6 (13.2)	92.0 (4.1)	0.36
Middle temporal neocortex				
GluA1 subunit	101.5 (8.1)	102.5 (7.0)	97.8 (11.5)	0.38
P-GluA1-Ser845	11.7 (17.2)	114.8 (15.6)	103.1 (16.2)	0.12
P-GluA1-Ser831	118.6 (17.2)	118.3 (17.5)	119.7 (17.9)	0.88
GFAP	112.4 (16.1)	110.3 (7.5)	116.7 (12.5)	0.84
EAAT1	108.6 (14.7)	109.9 (14.2)	104.8 (16.9)	0.64
EAAT2	106.5 (11.2)	106.5 (9.5)	106.2 (17.6)	0.87

Data are expressed as the mean (SD) level of the neurochemical parameter expressed as percentage of the reference sample which was considered 100%;

[a] Significant decrease of 11 % in AMY levels of GluA1 subunit in patients with IF;

[b] Significant decrease of 21.5 % in HIP levels of P-GluA1-Ser845 in patients with IF.

Table 2: Clinical, demographic, neuroradiological, neurophysiological, and surgical variables of patients with MTLE-HS according to the presence of IF.

Variables	All cases n = 31	Ictal Fear		"p" value
		No n = 25 (80.6)	Yes n = 06 (19.4)	
Gender				
Female	18 (58.1)	15 (60.0)	03 (50.0)	
Male	13 (41.9)	10 (40.0)	03 (50.0)	0.67
Race				
Caucasian	27 (87.1)	22 (88.0)	05 (83.3)	
Others	04 (12.9)	03 (12.0)	01 (16.7)	1.0
Marital status				
Single	17 (54.8)	14 (56.0)	03 (50.0)	
Married	10 (32.3)	09 (36.0)	01 (16.7)	
Divorced or Widower	04 (12.9)	02 (8.0)	02 (33.3)	0.22
Current work activity				
Working	11 (35.5)	08 (32.0)	03 (50.0)	
House wife	06 (19.4)	04 (16.0)	02 (33.3)	
Health Insurance	04 (12.9)	04 (16.0)	0	
Not working	10 (32.3)	09 (36.0)	01 (16.7)	0.48
History of initial precipitant injury				
No	07 (22.6)	05 (20.0)	02 (33.3)	
Yes	24 (77.4)	20 (80.0)	04 (66.7)	0.60
MRI side of HS				
Right side	16 (51.6)	13 (52.0)	03 (50.0)	
Left side	15 (48.4)	12 (48.0)	03 (50.0)	1.0
Antiepileptic drugs regimen[c]				
Monotherapy	09 (29.0)	06 (24.0)	03 (50.0)	
Two or more drugs	22 (71.0)	19 (76.0)	03 (50.0)	0.32
Benzodiazepines				
No	16 (51.6)	11 (44.0)	05 (83.3)	
Yes	15 (48.4)	14 (56.0)	01 (16.7)	***0.17***
Carbamazepine				
No	06 (19.4)	05 (20.0)	01 (16.7)	
Yes	25 (80.6)	20 (80.0)	05 (83.3)	1.0
Phenobarbital				
No	19 (61.3)	15 (60.0)	04 (67.7)	
Yes	12 (38.7)	10 (40.0)	02 (33.3)	1.0
Diphenilhydantoin				
No	28 (90.3)	22 (88.0)	06 (100.0)	
Yes	03 (9.7)	03 (12.0)	0	1.0

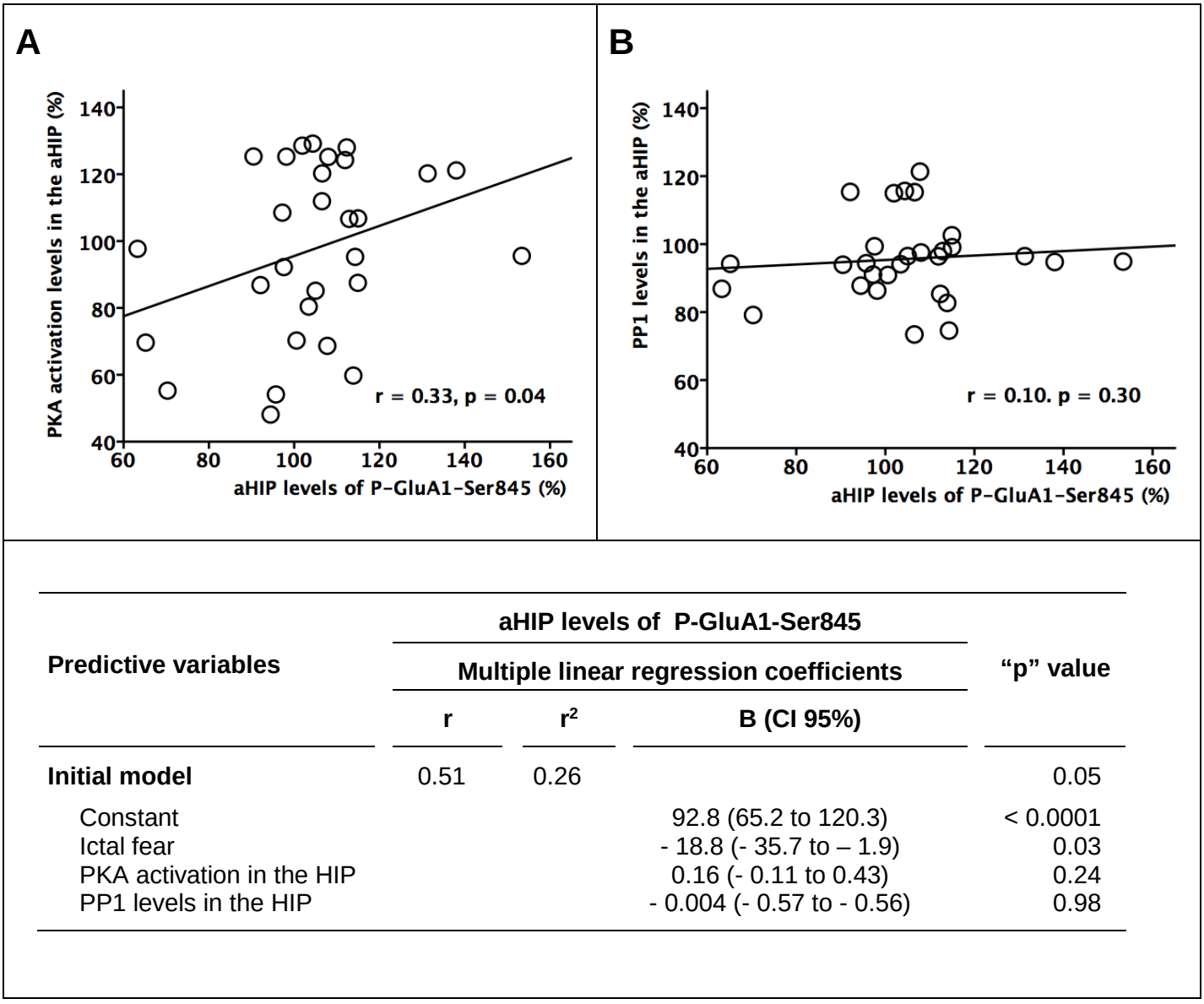
Valproic acid				
No	27 (87.1)	21 (84.0)	06 (100.0)	
Yes	04 (12.9)	04 (16.0)	0	
				0.56
Lamotrigine				
No	27 (87.1)	21 (84.0)	06 (100.0)	
Yes	04 (12.9)	04 (16.0)	0	0.56
Topiramate				
No	29 (93.5)	23 (92.0)	06 (100.0)	
Yes	02 (6.5)	02 (8.0)	0	1.00
Hand dominance				
Right	27 (87.1)	21 (84.0)	06 (100.0)	
Non-right	04 (12.9)	04 (16.0)	0	0.56
Psychiatric comorbidities				
No diagnosis	15 (48.4)	13 (52.0)	02 (33.3)	
Depressive disorder	08 (25.8)	06 (24.0)	02 (33.3)	
Anxiety disorder [a]	03 (9.7)	02 (8.0)	01 (16.7)	
Interictal dysphoric disorder	03 (9.7)	02 (8.0)	01 (16.7)	
Ictal psychosis	02 (6.4)	02 (8.0)	0	0.78
HADS anxiety scores [b]	8.3 (3.4)	8.5 (3.2)	8.0 (4.3)	0.77
HADS depression scores [b]	7.0 (3.9)	7.3 (4.0)	6.0 (3.9)	0.53
Age (years)	36.4 (12.1)	36.7 (12.1)	34.8 (13.1)	0.75
Education (years)	6.6 (3.0)	6.8 (2.9)	5.7 (3.5)	0.41
Disease duration (years)	24.3 (11.7)	24.6 (11.1)	23.0 (11.9)	0.77
Monthly seizures frequency [c]	7.5 (4.9)	7.1 (4.68)	9.6 (6.5)	0.32
QOLIE-31 overall score [d]	35.2 (15.3)	34.6 (14.6)	37.7 (19.6)	0.66

[a] Anxiety disorders: generalized anxiety disorder (two patients in the group without fear), social phobia (one patient in IF group);
[b] HADS anxiety and depression were applied only in 26 patients (5 had IF);
[c] Seizures impairing awareness;
[d] QOLIE-31 = Quality of Life in Epilepsy Inventory-31 overall score.

Table 3: Independent association between IF and aHIP levels of P-GluA1-Ser-845 and AMY levels of GluA1 after controlling for imbalances in the distribution of potential confounding variables.

Predictive variables	Crude OR (CI 95%)	"p" level	Adjusted OR (CI 95%)	"p" levels
Initial model				
HIP levels of P-GluA1-Ser845	0.92 (0.86 to 0.99)	0.03	0.88 (0.74 to 1.05)	0.17
AMY levels of GluA1 subunit	0.93 (0.97 to 1.00)	0.07	0.91 (0.80 to 1.04)	0.16
PO_2 pressure during surgery (mmHg)	0.99 (0.98 to 1.00)	0.20	0.98 (0.95 to 1.00)	0.16
Storage time of samples (months)	1.08 (0.97 to 1.20)	0.15	0.96 (0.73 to 1.26)	0.76
Benzodiazepines use	0.16 (0.02 to 1.55)	0.11	6.6 (0.18 to 238.7)	0.30
Final model [a]				
aHIP levels of P-GluA1-Ser845	0.92 (0.86 to 0.99)	0.03	0.92 (0.85 to 0.99)	0.04
AMY levels of GluA1 subunit	0.93 (0.97 to 1.00)	0.07	0.92 (0.84 to 1.01)	0.09

[a] Overall accuracy 89.3% (specificity 95.5% and sensitivity 66.7%) to predict the occurrence of IF (Nagelkerke $R^2 = 0.50$);
HIP levels of P-GluA1Ser845 alone has an overall accuracy of 92.1% (specificity 95.5% and sensitivity 33.3%) to predict the occurrence of IF (Nagelkerke $R^2 = 0.34$);
AMY levels of GluA1 subunit alone has an overall accuracy of 87.2%% (specificity 100% and sensitivity 33.3%) to predict the occurrence of IF (Nagelkerke $R^2 = 0.17$).



Predictive variables	aHIP levels of P-GluA1-Ser845			"p" value
	Multiple linear regression coefficients			
	r	r^2	B (CI 95%)	
Initial model	0.51	0.26		0.05
Constant			92.8 (65.2 to 120.3)	< 0.0001
Ictal fear			- 18.8 (- 35.7 to – 1.9)	0.03
PKA activation in the HIP			0.16 (- 0.11 to 0.43)	0.24
PP1 levels in the HIP			- 0.004 (- 0.57 to - 0.56)	0.98

Figure 1: Correlations between the variation in the level of PKA activation (A) and PP1 (B) in aHIP and the variation in the P-GluA1-Ser845 levels in the aHIP. Data are expressed as the level of the neurochemical parameter determined as percentage of the reference sample which was considered 100%. PKA activation was determined using an antibody against phospho-PKA substrates (indirect measure of PKA activation) which detects peptides and proteins containing a phospho-serine/threonine residue with arginine at the -3 and -2 positions, which is a consensus sequence that undergoes PKA-dependent phosphorylation. There was a significant positive correlation between the levels PKA activation and the P-GluA1-Ser845 (r = 0.33, p = 0.04). No association was observed between the P-GluA1-Ser845 and the PP1 levels (r = 0.10, p = 0.30). Statistical analysis done by Pearson correlation (1-tailed). After the multiple linear regression analysis (bottom of Figure 1) only the presence of the IF, but not the levels of PKA activation and PP1, remain independently and negatively associated with the P-GluA1Ser845 variation in the aHIP. The validity of the model was confirmed by many aspects: there were no outliers, the data points were independent, the distribution of residuals satisfied the normality assumptions, the variance was constant and there was no multicollinearity.

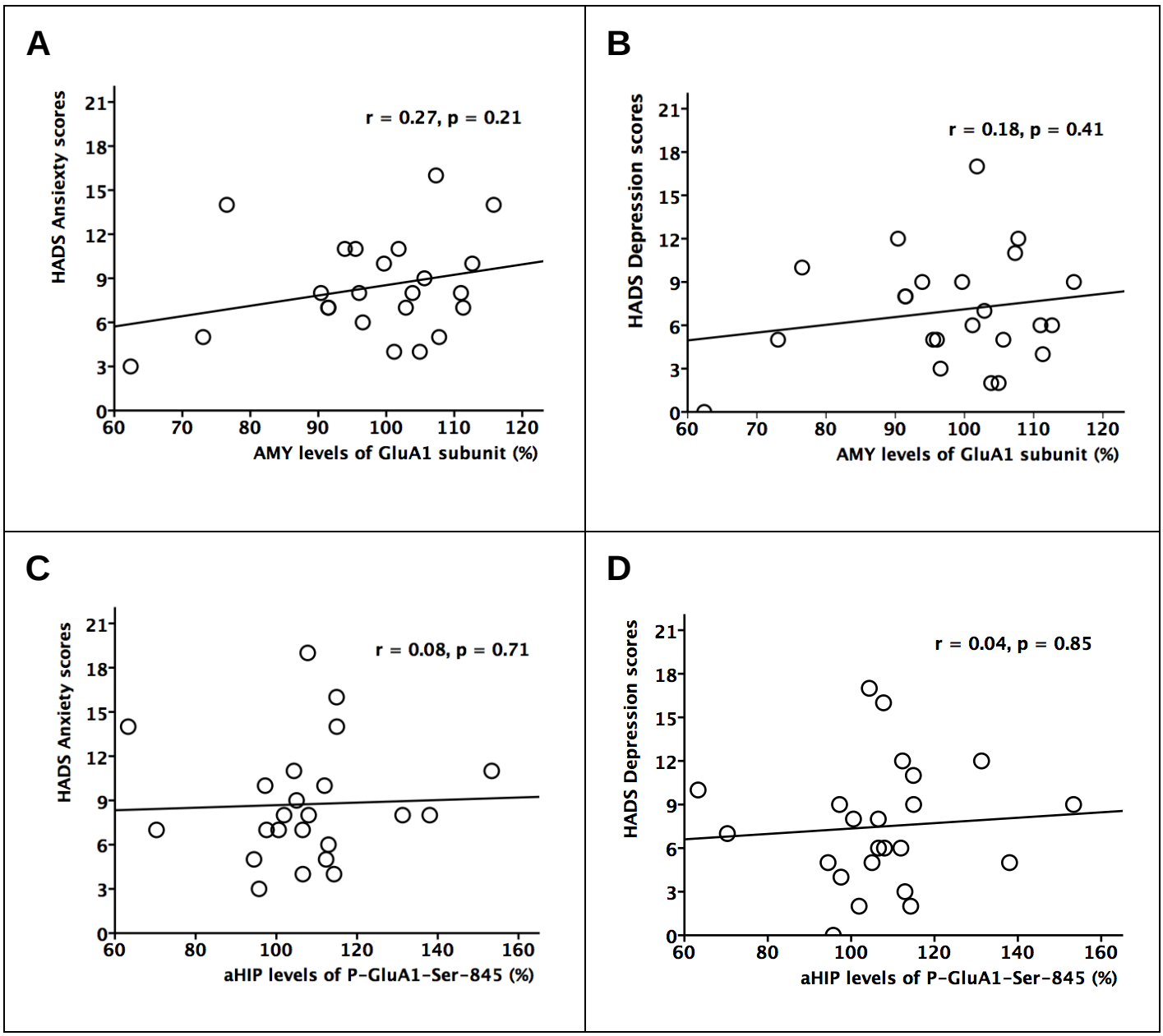


Figure 2: Pearson's correlation between the neurochemical changes in AMY and aHIP and the psychiatric symptoms of MTLE-HS patients (n = 26). A) AMY levels of GluA1 subunit and anxiety symptoms (HADS Anxiety); B) AMY levels of GluA1 subunit and depression symptoms (HADS Depression); C) aHIP levels of P-GluA1-Ser845 and anxiety symptoms (HADS Anxiety); D) aHIP levels of P-GluA1-Ser845 and depressive symptoms (HADS Depression).

SI Table 1: Frequency of different of auras reported by MTLE-HS patients.

Epileptic auras, n (%)	**All Cases n = 31 (%)**
None	06 (19.4)
Any type of aura [a]	25 (80.6)
Fear	06 (19.4)
Abdominal sensation	06 (19.4)
Chest sensation	05 (16.1)
Poorly defined symptoms	04 (12.9)
Cephalic sensation	03 (9.7)
Déjà-vu	02 (6.5)
Dizziness	02 (6.5)
Jamais-vu	01 (3.2)
Tachycardia	01 (3.2)
Olfactory	01 (3.2)
Body ascending chill	01 (3.2)

[a] Patients described only one type of aura or a sequence of two or three different auras.

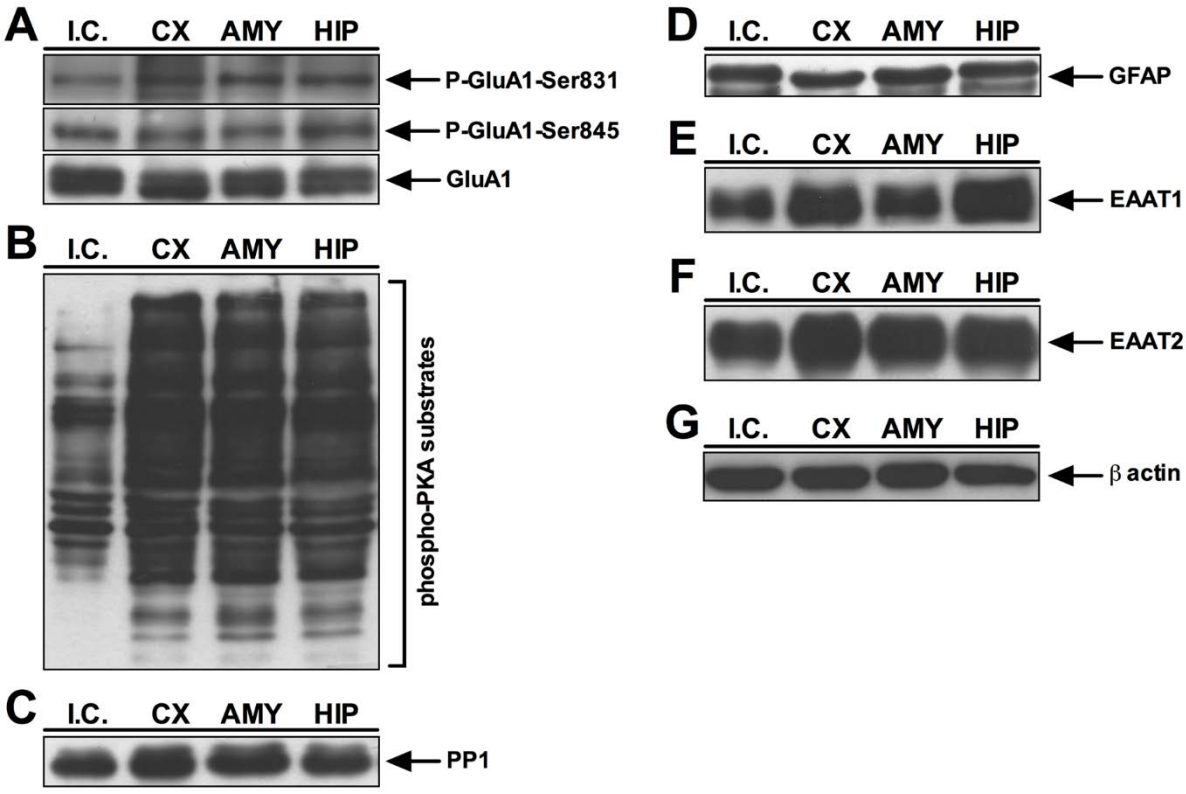
SI Table 2: Independent association between aura of fear and aHIP levels of P-GluA1-Ser45 and AMY levels of GluA1 after controlling for imbalances in the distribution of other types of aura.

Variables and models	**Linear regression coefficients**			**"p" value**
HIP levels of P-GluA1-Ser845	**r**	**r^2**	**B (CI 95%)**	
Initial Model 1	0.51	0.26		0.18
Constant			109.5 (98.4 to 120.6)	< 0.0001
Ictal fear (n = 06)			-21.1 (-38.0 to -3.6)	0.02
Abdominal sensation (n = 06)			-2.9 (-21.4 to 15.6)	0.75
Chest sensation (n = 05)			-3.6 (-23.5 to 16.2)	0.71
Cephalic sensation (n = 04)			-0.5 (-29.9 to 18.3)	0.62
Poorly defined symptoms (n = 03)			-7.5 (-14.7 to 29.8)	0.49
Final Model 1	0.47	0.22		
Constant			108.8 (101.5 to 116.0)	< 0.0001
Ictal Fear			-21.5 (-37.4 to -5.6)	0.01
AMY levels of GluA1	**r**	**r^2**	**B (CI 95%)**	
Initial Model 2	0.46	0.21		0.27
Constant			99.7 (92.9 to 106.6)	< 0.0001
Ictal fear (n = 06)			-9.9 (-21.7 to 1.7)	0.09
Abdominal sensation (n = 06)			-1.0 (-13.2 to 11.7)	0.87
Chest sensation (n = 05)			-6.0 (-19.2 to 7.2)	0.36
Cephalic sensation (n = 04)			12.3 (-4.0 to 28.6)	0.13
Poorly defined symptoms (n = 03)			-0.3 (-17.7 to 11.7)	0.68
Final Model 2	0.35	0.12		
Constant			99.6 (94.7 to 104.4)	< 0.0001
Ictal fear			- 11.0 (- 22.0 to 0.06)	0.05

SI Table 3: Surgical and laboratorial variables, storage time of samples and time since the last seizure before the epilepsy surgery according to the presence of IF.

Variables	All cases n = 31	Ictal Fear		"p" value
		No n = 25 (80.6)	Yes n = 06 (19.4)	
Mean arterial pressure (mmHg)	67.5 (9.6)	68.2 (10.4)	64.8 (5.3)	0.38
Heart rate (per minute)	73.7 (11.9)	73.9 (11.9)	72.8 (13.4)	0.98
Respiratory rate (per minute)	11.6 (1.7)	11.7 (1.8)	11.2 (1.3)	0.70
Biochemical analysis of blood [a]				
pH	7.41 (0.4)	7.41 (0.04)	7.43 (0.04)	0.52
Arterial PCO_2 pressure (mmHg)	28.6 (4.3)	28.6 (4.7)	29.0 (3.2)	0.94
Arterial PO_2 pressure (mmHg)	229.6 (61.5)	236.6 (53.1)	200.4 (88.7)	***0.13***
Hematocrit (%)	35.0 (3.8)	34.6 (3.7)	37.0 (3.9	0.21
Glucose (mg/dL)	116.3 (24.6)	118.7 (25.8)	104 (6.3)	0.28
Sodium (mEq/L)	138.2 (3.5)	138.0 (3.7)	139.0 (2.0)	0.63
Potassium (mEq/L)	4.1 (0.4)	4.1 (0.4)	4.2 (0.1)	0.21
Ionic calcium (mg/dL)	4.2 90.8)	4.2 (0.9)	4.5 (0.1)	0.51
Lactic acid (mg/dL)	2.1 (1.1)	2.1 (1.1)	2.0 (1.0)	0.88
Storage time of samples (months) [b]	24.0 (8.9)	22.8 (8.7)	28.7 (9.2)	***0.15***
Time since last seizure (hours) [c]	225 (418)	216 (330)	264 (455)	0.82
Time for CX sampling (min) [d]	188 (39)	192 (40)	173 (34)	0.30
Time for AMY/HIP sampling (min) [e]	260 (54)	262 (57)	246 (43)	0.62
Time of HIP manipulation (min) [f]	11.2 (4.9)	11.6 (4.8)	9.8 (5.7)	0.42
Dexamethasone, n (%) [g]				
No	11 (35.5)	08 (32.0)	03 (50.0)	
Yes	20 (64.5)	17 (68.0)	03 (50.0)	0.64

[a] Biochemical analysis was done in the arterial blood collected during surgery when AMY and HIP were resected;
[b] Time course since brain tissue sampling and storage until the neurochemical analysis;
[c] Time course since the last seizure attack occurrence and brain tissue sampling;
[d] Time course since anesthesia induction until CX tissue sampling;
[e] Time course since anesthesia induction until AMY/HIP tissue sampling;
[f] Time course since HIP vessels thermo-coagulation started until the complete resection of the HIP;
[g] Dexamethasone administered during anesthetic induction (single i.v. dose of 10 mg).



Sl Figure 1: Representative western blots of GluA1 subunit of AMPA receptor (A), PKA (B), PP1 catalytic subunit (C), GFAP (D) EAAT1 (E), EAAT2 (F) and β actin (G) in the middle temporal neocortex (CX), amygdala (AMY) and anterior hippocampus (HIP) of patients and the internal control sample (I.C.). The images are illustrative and represent the pattern detection of targets of interest.