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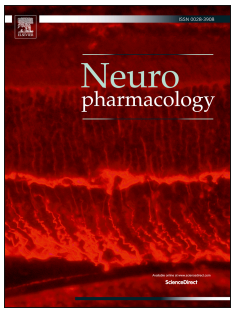

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QUANTITATIVE EXPRESSION AND LOCALIZATION OF $GABA_B$ RECEPTOR PROTEIN SUBUNITS IN HIPPOCAMPI FROM PATIENTS WITH REFRACTORY TEMPORAL LOBE EPILEPSY

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ABSTRACT

This study investigates $GABA_B$ protein expression and mRNA levels in three types of specimens. Two types of specimens from patients with temporal lobe epilepsy (TLE), secondary to hippocampal sclerosis, sclerotic hippocampal samples (TLE-HS), and tissue from the structurally preserved ~~healthy,~~ non-spiking ipsilateral superior temporal gyrus (TLE-STG) removed from the same patient during epilepsy surgery; and third specimen is hippocampal tissue ~~specimen~~ from individuals with no history of epilepsy (*post-mortem* controls, PMC).

mRNA expression of $GABA_B$ subunits was quantified in TLE-HS, TLE-STG and PMC specimens by qRT-PCR. Qualitative and quantitative Western blot (WB) and immunohistochemistry techniques were employed to quantify and localize $GABA_B$ proteins subunits.

qRT-PCR data demonstrated an overall decrease of both $GABA_{B1}$ isoforms in TLE-HS compared to TLE-STG. These results were mirrored by the WB findings. $GABA_{B2}$ mRNA and protein were significantly reduced in TLE-HS samples compared to TLE-STG; however they appeared to be upregulated in TLE-HS compared to the PMC samples. Immunohistochemistry (IHC) showed that $GABA_B$ proteins were widely distributed in PMC and TLE-HS hippocampal sections with regional differences in the intensity of the signal. The higher expression of mature $GABA_B$ protein in TLE-HS than PMC is in agreement with previous studies. However, these findings could be due to post-mortem changes in PMC specimens. The TLE-STG samples examined here represent a better 'control' tissue compared to TLE-HS samples characterized by lower than expected $GABA_B$ expression. This interpretation provides a better explanation for previous functional studies suggesting reduced inhibition in TLE-HS tissue due to attenuated $GABA_B$ currents.

KEYWORDS: human temporal lobe epilepsy, hippocampal sclerosis, $GABA_B$ qRT-PCR, quantitative Western blot, immunohistochemistry.

1. INTRODUCTION

The main inhibitory neurotransmitter in the mammalian central nervous system (CNS), γ-aminobutyric acid (GABA), plays important roles in regulating neuronal activity, plasticity, and pathophysiology. Its action is mediated through distinct receptor types: ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$). Both $GABA_A$ and $GABA_B$ receptors have been implicated in many important physiological functions and pathological conditions in the brain (Gassmannn and Bettler 2012, Castelli and Gessa, 2016), such as absence seizures (Stewart et al. 2009)

$GABA_B$ receptors have been demonstrated at both pre- and postsynaptic sites of both excitatory and inhibitory neurones (Chen et al. 2004). Presynaptic receptor stimulation reduces the evoked release of GABA and other neurotransmitters, whereas postsynaptic $GABA_B$ receptor activation increases neuronal K^+ conductance to generate long-lasting inhibitory postsynaptic potentials (IPSPs).

Along with other findings, previous pharmacological and physiological studies have suggested the existence of two distinct $GABA_{B1}$ receptor subtypes at pre- and postsynaptic sites and in different cells types and brain structures (Bowery 1997; Deisz et al.1997; Dutar and Nicoll 1988; Pitler and Alger 1994). The evidence for two different $GABA_{B1}$ receptor isoforms ($GABA_{B1a}$ and $GABA_{B1b}$) was first characterised by Kaupmann and colleagues (1997). A second subunit was subsequently characterised (Kaupmann et al. 1998; Jones et al. 1998; White et al. 1998).

The distribution of $GABA_{B1}$ receptors in human hippocampus has been demonstrated with receptor binding autoradiography (Princivalle et al. 2002). Expression of $GABA_{B1}$ mRNA in the rat CNS, human hippocampus and spinal cord has been established by radiolabelled riboprobes recognising the two $GABA_{B1}$ mRNA variants (Kaupmann et al. 1997; Benke et al. 1999; Liang et al. 2000; Towers, et al. 2000). The expression of $GABA_{B2}$ messengers has also been described widely expressed in rat brain (Clark et al. 2000; Calver et al. 2000). In addition $GABA_{B1}$ (a/b) and $GABA_{B2}$ immunoreactivity has been demonstrated in the rat CNS (Ige et al. 2000; Princivalle et al. 2000a; 2000b; 2001;

Charles et al. 2001). Nevertheless, it is still unclear how the two $GABA_{B1}$ variants and the $GABA_{B2}$ mature proteins are distributed in different neuronal regions and cell types in human brain tissue such as the hippocampus, or how the transcription of $GABA_{B1}$ and $GABA_{B2}$ may be affected by pathological states such as epilepsy.

Temporal lobe epilepsy (TLE) is the commonest and most researched drug-refractory focal epilepsy. Electrophysiological evidence has demonstrated that there is a lack of inhibition in TLE due to the abolished slow component of $GABA_B$ receptor-mediated IPSPs (Mangan and Lothman, 1996, Teichgräber et al. 2009). In addition, there is pharmacological and physiological evidence that $GABA_B$ receptor is impaired in animal models of TLE (Chandler et al. 2003; Furtinger et al. 2003a; Mares and Kubová 2015; Leung et al. 2016). However, the localization and quantitative expression of $GABA_B$ isoforms and subunits have not yet been elucidated in animal models or in human TLE.

Previous studies reported impaired $GABA_B$ receptor mediated currents in TLE (Straessle et al. 2003; Rocha et al. 2015). This study aimed to examine possible differences in $GABA_{B1a,}$ $GABA_{B1b}$ and $GABA_{B2}$ mRNA and protein expression. We investigated whether $GABA_B$ protein expression showed a reduction in the hippocampal tissue of patients with mesial temporal sclerosis (TLE-HS) compared to tissue taken from the same patients' superior temporal gyrus (TLE-STG) and post-mortem hippocampal control (PMC) tissue from individuals with no history of epilepsy.

2. MATERIALS AND METHODS

2.1. Patient tissue collection and clinical data

The majority of surgical samples were obtained from the Royal Hallamshire Hospital (R&D approval STH15210). The post-mortem immunohistochemistry samples were obtained from The National Hospital for Neurology and Neurosurgery. All samples were obtained with the understanding and the written consent of each patient. The sample collection procedure fully conformed with the Code of Ethics of the World Medical Association (Declaration of Helsinki), *British Medical Journal* (1964), and the Institute of

Neurology Joint Research Ethics Committee [Ethics Committee Protocol Pro-Forma (January 1998)]. The study was approved by the South Yorkshire Research Ethics Committee (08/H1310/49).

The surgical sclerotic human hippocampal tissue (TLE-HS) and non-sclerotic (TLE-STG) samples were obtained from the same patient with medically refractory TLE, undergoing surgical resection. Only patients with TLE secondary to unilateral hippocampal sclerosis were included. Clinical and demographic information about these patients is in Table 1. The excision of the samples was based on pre-surgical clinical evaluation including interictal and ictal EEG studies and magnetic resonance imaging (MRI) in all cases. Each sample was divided into two parts, one part was snap frozen (Kingsbury et al. 1996) and stored at -80°C until RNA and protein extraction were performed. All pre-operative diagnoses of HS were confirmed after surgery by histopathological examination based on established diagnostic criteria (Thom at al. 2002),. The second part of the sample was fixed as previously described (Thom at al. 2002): briefly they were post-fixed in 4% paraformaldehyde, then dehydrated through ethanol at increasing concentration, paraffin-embedded overnight, sliced by vibratome at 10 µm, mounted on slides, dried and stored at R.T. until use for histopathological analysis and immunohistochemistry experiments. The TLE-STG specimens were taken from the superior temporal gyri which looked structurally preserved on MRI, and had not been shown to generate ictal or inter-ictal epileptiform activity during pre-surgical electroencephalographic monitoring. If this kind of samples does not follow the above criteria they were not collected.

The flash-frozen post-mortem hippocampal samples were obtained from the UCL Brain Bank (08/H0718/54). They were from individuals with no previous medical history of neurological or psychiatric disease (Table 2). At autopsy the hippocampi were dissected, pH was checked to be between 6 and 7, and the samples were flash frozen and stored at -80°C.

140

141 **2.2. Quantitative real-time polymerase chain reaction (qRT-PCR)**

142 ***2.2.1. RNA extraction:*** The total RNA was extracted from samples using SV Total RNA
143 Isolation System kit according to manufacturer's instructions (Promega). Briefly, the
144 hippocampal tissue lysates were prepared by adding 1 ml RNA lysis buffer to 342 mg of
145 tissue weight. The tissue lysates were diluted with SV RNA dilution buffer and RNA was
146 then adsorbed to a silica membrane-based column where it was purified by a spin
147 method. RNA was subjected to DNase treatment, washed and eluted with 100 µl of
148 Nuclease-free water. The RNA purity was checked by the NanoDrop-1000
149 spectrophotometer, and the RNA integrity was checked by 1% agarose gel
150 electrophoresis.

151 ***2.2.2. cDNA synthesis*:** Complementary DNA (cDNA) was synthesised by using the
152 Superscrpit III first strand synthesis system (Life Technologies, 18080-051) according to
153 the manufacturer's recommendation. Starting from 1µg of total RNA, cDNA was
154 synthesised by using 50 µM of oligo $(dT)_{20}$ primer, 40 U of RNaseOUT and 200 U of
155 Superscrpit III reverse transcriptase enzyme. The cDNA was then purified by using
156 QIAquick PCR purification kit (Qiagen, 28104) and quantified with the NanoDrop-1000
157 spectrophotometer.

158 ***2.2.3. qRT-PCR*:** The mRNA expression of $GABA_{B1}$ and $GABA_{B2}$ subunits was
159 investigated by qRT-PCR in 26 TLE-HS and 11 TLE-STG specimens (Table 1) and 10
160 post-mortem samples (Table 2). The qRT-PCR was performed on a StepOnePlus™ Real-
161 Time PCR System (Applied Biosystems) using TaqMan gene expression assays (Table
162 3). A 10 µl volume of PCR reaction mix was prepared by combining template cDNA
163 sample, TaqMan Universal PCR Master Mix (Applied Biosystems, 4352042) and TaqMan
164 gene expression assays (Life Technologies). Cyclophilin A (PPIA) and cyclin-dependent
165 kinase inhibitor 1B (CDKN1B) were selected as reference genes for our study as they
166 were among the most stably expressed genes in TLE (Wierschke et al. 2010). Results

were analysed using the $2^{-\Delta Ct}$ method and presented as relative gene expression normalised to the average threshold cycle of the two housekeeping genes.

2.3 Quantitative two colour Western blot (qWB)

2.3.1. ***Protein extraction and quantification:*** The hippocampi tissues were homogenised at 4°C in CelLytic™ (C3228, Sigma) and protease inhibitor cocktail (P8340, Sigma). The lysate was centrifuged twice at 500 XG for 15 minutes at 4°C. The supernatant was centrifuged at 20000 XG for 40 minutes at 4°C and pellet was suspended in 50mM TrisHCl buffer pH 7.5 (TBS). The total protein was then quantified by Bicinchoninic acid protein assay kit according to the manufacturer's protocol (BCA1, B9643, Sigma-Aldrich).

2.3.2. ***Quantitative WB:*** The $GABA_B$ receptor subunits were investigated by qWB in 9 TLE-HS, 6 TLE-STG, and 4 PMC samples (according to sample availability). 20 µg of protein was loaded on 8% sodium dodecyl sulphate-polyacrylamide gel for electrophoresis (SDS-PAGE). The separated proteins were electro-transferred onto a nitrocellulose membrane, which was washed briefly in phosphate buffered saline (PBS) for few minutes. The membranes were then blocked with 5% w/v non-fat dry milk (NFDM) in PBS and 0.1% Tween 20 (PBST) for 1 hour at room temperature (RT). Then they were incubated with primary diluted antibodies (Table 4) over night at 4°C with gentle shaking. A generous amount of 0.1% PBST buffer was used to wash the membranes 4 times for 5 minutes each. Then membranes were incubated with infrared-labelled secondary antibodies for 1 hour at RT followed by 4 washes with 1X PBS for 5 minutes each. The membranes were scanned on an Odyssey infrared imaging system (LI-COR, Biosciences, NE, U.S.A.). The 700nm and 800nm channel scanning intensities were set to 4 and 6 respectively. The images acquired were quantified on the Odyssey software (version 1.2) according to the software manual and Picariello et al. (2006). $GABA_{B1(a-b)}$, and $GABA_{B2}$ bands intensities were normalized to β-actin to eliminate any loading variation.

2.4 Immunohistochemistry (IHC)

***2.4.1. Brain sections preparation*:** Sections (10 µm) of paraffin-embedded human hippocampal tissue were cut by a microtome, mounted onto charged microscope slides (BDH Superfrost Plus) and stored with desiccant in plastic slide boxes at RT until required.

2.4.2. Tissue pre-treatment and application of antibodies

The immunohistochemistry antibodies sub-types specificity to human $GABA_{B1a}$, $GABA_{B1b}$ or $GABA_{B2}$ was previously tested (Calver et al. 2000). Immunohistochemistry (IHC) was conducted on 7 TLE-HS (Table 1) and 5 PMC specimens (Table 2) according to specimen availability. Following antigen retrieval, sections were rinsed in PBS, endogenous peroxidase activity blocked by incubation with hydrogen peroxide (0.3% in PBS) for 30 minutes, and followed by a rinse in fresh PBS. Sections were then incubated with normal goat serum (NGS) (1:10 in PBS) for 75 minutes, and subsequently overnight at 4°C with the primary antibodies (Table 4) respectively in PBS containing 1% NGS.

Following incubation with primary antibodies, the sections were washed with fresh PBS for 1 hour then incubated with secondary biotinylated antibodies (Table 4) for 75 minutes, rinsed for 1 hour in PBS and incubated with the avidin-biotin peroxidase complex (ABC; Vector) for 75 minutes. Peroxidase staining was performed by incubating the sections in 0.002% 3.3'diaminobenzidine and 0.002% H_2O_2 in 50mM Tris buffer, pH 7.6. The sections were dehydrated, and cover-slipped with diethyl-pyro carbonate (DPX).

2.4.3. Microscope visualization and quantitative IHC (qIHC)

Neuronal counting was performed as before using a stereological method as previously described (Princivalle et al. 2002; 2003). The number and intensity of $GABA_B$ receptor subunits were quantified in pyramidal and granular cells in TLE-HS and PMC IHC sections using the *Q-Capture Pro 7*™ (QCapture 10, 2010) connected to an *Olympus BX60* microscope.

In order to quantify the immunosignals of the $GABA_{B1}$ receptor isoforms and subunit, 13 sections from TLE patients and 5 from PMC were analysed. The microscope amplification used for quantification of each slide was 10 (ocular lens) x 20 (objective lens), giving a total amplification of 200x. For each slide 6 images of the area of interest (hippocampus) were captured. The raw relative optical density (ROD) of $GABA_B$ immunosignals was determined using the measuring tools of *Q-Capture Pro 7*™ software. The pyramidal cells were marked with a yellow triangle and granular cells with a blue square measuring tool. The ROD was normalized by subtracting the background (calculated by averaging 10 background spots in each slide). To correct for neuronal loss, ROD per neuron was calculated by dividing the total ROD on the number of $GABA_B$ immunopositive neurons and excluding glial cells.

2.4.4. Statistical analysis

The GraphPad Prism 6 software for Windows, version 6.05 was used for the statistical analysis (San Diego, CA, USA; www.graphpad.com). The Shapiro-Wilk W test was performed to test the normality of the data. The simple linear regression was used to do the correlation analysis. The Kruskal-Wallis with Conover-Inman *post hoc* analysis test was used, for any experiment, to identify significant differences between samples (* $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data presented as median and interquartile range values.

3. RESULTS

In this study we have investigated the expression of GABAB receptor subunit transcripts and proteins in human samples of TLE-HS, TLE-STG, and PMC. The median age of TLE-HS patients was 38 years (range 22-63). Patients had had epilepsy for a median of 23 years prior to surgery (range 2-53). Patients were taking a median of 3 antiepileptic drugs at the time of surgery. The patients had simple or complex partial seizures and 36% of them had also generalized tonic clonic seizures. 26% of patients had a history of febrile

seizures. Only 14 from 23 patients (60%) of patients were seizure free after 1 year of epileptic surgery.

3.1. qRT-PCR

The correlation between PMC mRNA samples versus age and post-mortem interval in demonstrates no correlation between the mRNA findings and these factors that could have influence the mRNA expression (supplementary material). The data from qRT-PCR, obtained from the whole resected hippocampi, show a very similar trend for both $GABA_{B1}$ and $GABA_{B2}$ subunits. The comparison of TLE-HS and the PMC samples reveals no difference in $GABA_{B1}$ subunit expression between the groups, but possibly an increased $GABA_{B2}$ expression in the TLE-HS tissue. In contrast, the comparison of TLE-HS with the TLE-STG samples showed a statistically significant lower level of expression of $GABA_{B2}$ in the TLE-HS tissue (see Figure 1).

3.2. Qualitative and Quantitative WB

Figure 2A shows a double-labelled Western blot image demonstrate a fairly consistent level of β-actin expression in the three study groups. However, there is a clear gradient of the expression of all three $GABA_B$ variants across the study groups. These proteins are expressed most strongly in TLE-STG, less strongly in TLE-HS and least strongly in PMC tissue. The data obtained by quantitative double-labelled analysis (Figure 2B) follows the same trend although differences between the TLE-HS and the TLE-STG comparisons were only significant for $GABA_{B2}$. Comparing TLE-HS to PMC, statistically significant up regulation ~~differences~~ was ~~are~~ observed for $GABA_{B1a}$, $GABA_{B1b,}$ and $GABA_{B2}$.

3.3. Distribution and comparison of $GABA_B$ receptor protein immunoreactivity in PMC and TLE-HS hippocampi

$GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ receptor proteins appeared to have a similar location in the TLE-HS and PMC hippocampal sections; furthermore, no evidence of single subunit

labelling was observed in the hippocampal subregions of either sample category (Figure 3A-F). In PMC cases $GABA_{B2}$ and $GABA_{B1b}$ exhibited the highest and the lowest immunoexpression respectively. All the three proteins displayed the highest expression in the dentate gyrus (DG) followed by the different *cornu ammonis* (CA) areas (all with comparable immunointensity), and the subiculum, which showed the lowest level of immunopositivity.

Figure 4A shows the total number of pyramidal and granular cells per mm^3 highlighting neuronal loss in the TLE-HS. 5B and 5C show the percentage of $GABA_B$ positive pyramidal and granular neurons respectively. Whereas immunopositivity to $GABA_{B1}$ was greater in pyramidal PMC than TLE-HS cells it was lower in granular PMC than TLE-HS cells. In contrast, $GABA_{B2}$ immunopositivity was more marked in TLE-HS than PMC in both types of neurons. Figures 4D and 4E show semi-quantitative immunosignal measurements demonstrating the intensity of immunopositivity per remaining neuron in PMC and TLE-HS. The $GABA_{B2}$ signal intensity is higher while $GABA_{B1a}$ is lower in TLE-HS patients compared to PMC in both pyramidal and granular cells. The comparison of $GABA_{B1b}$ intensity between TLE-HS and PMC cells on the other hand showed higher $GABA_{B1b}$ intensity in granular and lower intensity in pyramidal cells (resulting not only from the image shown but from the averaged analysis of 5 patients); however, these differences did not achieve significance in the small number of samples available for comparison.

Figure 5 and 6 show how representative pyramidal cells in CA areas and DG granular neurones reacted with the three antibodies for $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ at higher magnification. The immunosignal proved to be specific for all three antibodies. The left panel in Figure 5 represents pyramidal neurones in of CA1. The immunoreactivity was mainly expressed by the cell bodies and apical dendrites; there was no nuclear staining at all, either in PMC or in the TLE-HS sections. The main difference between PMC and TLE-HS CA1 was the intensity of immunoreactivity in most of the neuronal cells. $GABA_{B1a}$ and $GABA_{B2}$ immunoreactivity appeared stronger in a few neurones, whilst the $GABA_{B1b}$

immunosignal seemed fainter in the majority of TLE-HS compared to PMC neurons. Figure 5, right panel shows CA2 pyramidal neurones. The immunosignal, for all three antibodies, was confined to the cell bodies and apical dendrites in the control specimen. In the TLE-HS hippocampi there was neuronal loss. Furthermore the remaining neurones appeared smaller and contracted and the immunosignal seemed stronger in the cytoplasmic membrane. Figure 6, left panel displays pyramidal neurones in CA3. Immunopositivity was mainly confined to the neuronal bodies with almost no apical dendrites being immunolabelled with any of three antibodies in the PMC hippocampus. In TLE-HS neuronal loss was evident, the cells appeared to be smaller, and the immunoreactivity was present on the cytoplasmic membrane. There was also an apparent proliferation of glial cells as reported in literature (Charles et al. 2003; Kim et al. 1990; de Lanerolle 2012). The right panel of Figure 6 exhibits DG granular cells at higher magnification. In the PMC specimen the immunoreactivity with all three antibodies was present exclusively in the cell *somata*. In TLE-HS sections neuronal loss was evident, in addition the granule cells were smaller and more dispersed, and immunolabelling was more intense.

Most of the pyramidal neurons in CAs areas and granule cells in DG were immunopositive. In addition, supported by recent evidence (Huyghe et al. 2014), some interneurons and possibly some astrocytes appeared immunopositive to the $GABA_B$ antibodies. It would be appropriate in future to perform double fluorescent immunostaining to verify which subpopulation of neurons and glia express $GABA_B$ receptors.

4. DISCUSSION

Previous studies have indicated that changes in the $GABA_B$ receptors subunits could be implicated in the pathophysiology of pharmaco-resistant TLE associated with HS (Billinton et al. 2001; Fürtinger et al. 2003b; Princivalle et al. 2003). Therefore, studying $GABA_B$ receptor protein expression may provide an important contribution to our understanding of one of the most important mechanisms implicated in temporal lobe epilepsy.

The qRT-PCR results obtained in this study showed that there is no major difference in $GABA_B$ expression between TLE-HS and PMC samples. This is in agreement with previous data (Billinton et al. 2001). In contrast, the TLE-STG samples demonstrated a higher expression of both subunits compared to TLE-HS and PMC samples. The quantitative Western blot perfectly mirrored the trend of PCR data for $GABA_{B2}$, but not for $GABA_{B1.}$ Figure 1 and 2 clearly demonstrate that the $GABA_{B2}$ subunit expression is significantly lower in TLE-HS samples compared to the bioptic TLE-STG, and higher compared to the PMC as well as the IHC shows. It is difficult to compare qRT-PCR $GABA_{B2}$ mRNA to previous *in situ* hybridization data (Princivalle et al. 2003; Fürtinger et al. 2003b). However, overall both techniques indicate a higher expression of $GABA_{B2}$ mRNAs in the epileptic hippocampi compared to the PMC control.

The protein quantification obtained from qWB demonstrated that $GABA_{B1}$ and $GABA_{B2}$ expression mirror the mRNA level in TLE-HS and TLE-STG. Visual comparison of the three proteins by IHC between PMC control and TLE-HS patients displayed a wide distribution of $GABA_B$ isoforms and subunits in both types of specimen. However, as previously reported (Princivalle et al. 2003; Fürtinger et al. 2003b), the quantitative comparison showed that, despite neuronal loss in TLE-HS hippocampal samples, there was an increment of $GABA_{B1b}$ and $GABA_{B2}$ protein expression per remaining neuron in the CA areas and DG, compared to the PMC samples.

It may be argued that our findings are contradictory because the quantification of Western blot and IHC showed opposite trend for $GABA_{B1a}$. However, it is important to point out that WB data represents the total $GABA_{B1a}$ expression as we used homogenates of hippocampal tissue containing neurones, microglia and astrocytes rather than just the neuronal portion. In contrast, the quantitative IHC data represent $GABA_{B1a}$ expression per neurone. Comparing the mRNA and protein expression in Figures 1 and 2, it is evident that the trend of the receptor subunits is the same, demonstrating that $GABA_{B2}$

357 expression is very much lower in the hippocampi of pharmaco-resistant patients
358 compared to TLE-STG. Previous binding and present immunohistochemical data in
359 human hippocampal PMC control and epileptic specimens appear in reasonable
360 agreement (Princivalle et al. 2002).

361 In the IHC the higher expression of $GABA_{B1b}$ and $GABA_{B2}$ in the surviving neurones of the
362 DG reflects the mRNA per neurone levels reported elsewhere (Princivalle et al. 2003;
363 Furtinger et al. 2003b). In addition, the $GABA_B$ receptor autoradiography binding assays,
364 corrected for neuronal loss (Billinton et al. 2000; Princivalle et al. 2002), showed a
365 significant increase in receptor density per neurone in specific hippocampal subregions of
366 the TLE-HS compared to PMC samples.

367 The lower expression of both $GABA_B$ receptor subunits in TLE-HS compared to TLE-
368 STG, could indicate a decline in $GABA_B$ receptors which would provide an explanation for
369 the compromised $GABA_B$ functionality previously reported in pharmacological and
370 electrophysiological studies in animal model and in human TLE (Billinton et al. 2000;
371 Princivalle et al. 2002; Fürtinger et al. 2003b; Mareš and Kubová 2015; Leung et al. 2016;
372 Rocha et al. 2015). This may be affecting the formation of fully functional $GABA_B$
373 receptors: since the heterodimerisation of $GABA_{B1}$ and $GABA_{B2}$ in 1:1 stoichiometry is
374 essential for receptor trafficking and G-protein activation, the $GABA_{B2}$ subunit could be a
375 potential target for the development of new agonists or activating transcription factors
376 drugs, which may have a major clinical impact on the treatment of pharmaco-resistant
377 TLE-HS patients. However, there are other factors which could explain the reduced
378 GABAergic inhibition (Gill et al. 2010; Armstrong et al. 2016), and there is a strong
379 possibility of co-causation.
380
381 The findings of this study could be interpreted in two different ways: $GABA_B$ protein
382 expression in epileptogenic hippocampal tissue could be down-regulated (because of the
383 higher expression in TLE-STG tissue) or it could be up-regulated (because of the lower

384 expression in PMC tissue). The decision which explanation is more likely depends on the
385 relative merits of the two non-epileptogenic “control”-tissues. Unfortunately, neither PMC
386 nor non-spiking TLE-STG is a perfect match for the TLE-HS samples of interest for the
387 kind of experiments conducted here. However, there is no real alternative in human
388 studies and it is not the first time that neocortex (STG) has been used in studies on TLE
389 (Teichgräber et al. 2009; Rocha et al. 2015). Even human non-epileptogenic hippocampi
390 removed for other reasons (such as temporal lobe tumours) cannot be considered an
391 ideal control tissue for TLE-HS samples (Kovács et al. 2012). Son et al. (2015)
392 demonstrated that tissue surrounding or adjacent to a tumour is physiologically and
393 molecularly perturbed by the tumour itself or by previous irradiation.
394 In view of these difficulties, many studies investigating TLE pathophysiology have recently
395 compared their results obtained in epileptogenic TLE specimens to other surgically
396 resected samples such as neocortex. The strength of this approach includes the fact that
397 both sample types contain the same DNA (reducing the risk of intersubjective variability
398 caused by gene-gene or gene-environment interactions) and that both samples were
399 obtained and processed in the same way. This approach also avoids the difficulties
400 associated with comparing TLE-HS tissue removed during epilepsy surgery with PMC-HS
401 tissue possibly affected by an agonal state and *post-mortem* changes (Preece et al. 2003;
402 Tomita 2004; Teichgräber et al., 2009; Rocha et al. 2015).

403 In this study, the expression of mature $GABA_B$ receptor proteins was investigated for the
404 first time in TLE-HS, and in both types of ~~potential~~ “control” tissues, surgically resected
405 non-spiking TLE-STG and PMC specimens.

406

407 **CONCLUSIONS**

408 In agreement with older studies, we found a statistically significant increase in overall
409 expression of $GABA_B$ receptor protein in TLE-HS versus PMC. This finding suggests that
410 the previously reported reduction in slow IPSPs in TLE-HS cannot be explained by a

411 decreased protein expression of the $GABA_B$ receptor subunit. Instead this
412 neurophysiological observation could be due to other causes including post-translational
413 modification of the $GABA_B$ protein. On the other hand, this study shows a statistically
414 significant lower expression of $GABA_{B2}$ in TLE-HS samples than in non-epileptogenic
415 TLE-STG from the same patients. Considering that the PMC values were affected by
416 agonal or post-mortem changes (or due to undetected differences in clinical or
417 demographic factors between TLE-HS and PMC subjects) the TLE-STG samples may
418 represent a more appropriate "control" tissue. Therefore, the downregulation of $GABA_{B2}$
419 transcription and $GABA_{B2}$ mature protein subunit in TLE-HS could represent one of the
420 reasons for the impaired GABAergic inhibition reported in epileptogenic hippocampal
421 tissue in the literature.
422
423 **LIST OF ABBREVIATIONS**

424	CA	*cornu ammonis*
425	GABA	γ-aminobutyric acid
426	$GABA_B$	γ-aminobutyric acid receptor B
427	DG	dentate gyrus
428	HS	hippocampal sclerosis
429	IHC	immunohistochemistry
430	IR	immunoreactivity
431	PMC	post-mortem control
432	PMI	post-mortem interval
433	TLE	temporal lobe epilepsy
434	DG	dentate gyrus
435	ROD	relative optical density
436	STG	superior temporal gyrus

437

This paper is dedicated to the memory of Professor Norman G. Bowery (1944-2016)

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Disclosure of Conflict of Interest

The authors do not have any competing interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issue involved in ethical publication and affirm that this report is consistent with those guidelines.

Authors' contributions

MAS made substantial contributions in production, acquisition of data, analysis of WB, qRT-PCR, and interpretation of all data.

DB resected and collected the human specimen, and clinical data.

LC made significant contributions in acquisition of data and analysis of IHC.

MR, DB, and JD, have been involved in revising manuscript critically for important intellectual content.

APP made substantial contributions to conception and design of the project, analysis, drafting and revising the manuscript.

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FIGURE LEGENDS

Figure 1 Quantitative real time PCR of $GABA_B$ mRNA receptor subunits.

qRT-PCR mRNA expression of $GABA_{B1}$ and $GABA_{B2}$ in 26 TLE-HS, 11 TLE-STG and 10 PM control using TaqMan gene expression Assays and Comparative delta Ct analysis ($2^{-\Delta CT}$) method. Statistical analysis: Kruskal-Wallis with Conover-Inman post hoc analysis test was used to identify significant differences between (* $P \leq 0.05$, ~~** P < 0.01, *** P< 0.001~~). Data presented as median and interquartile range values.

Figure 2 Qualitative and quantitative Western blot

(A) Qualitative WB of $GABA_{B1a}$, $GABA_{B1b}$, $GABA_{B2}$ and β-Actin, revealed by double labelling with IRDye 680 and IRDye 800 secondary antibodies. **(B)** Quantitative expression of $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ relative to β-Actin. Bands quantification was done on Odyssey infrared imaging system and Image Studio lite 4.0 software. Statistical analysis: Kruskal-Wallis with Conover-Inman post hoc analysis test was used to identify significant differences between (* $P \leq 0.05$, ** P < 0.01, ~~*** P< 0.001~~). Data presented as median and interquartile range values.

Figure 3 Qualitative immunohistochemistry

Distribution of $GABA_{B1a,}$ $GABA_{B1b}$ and $GABA_{B2}$ in PMC and TLE-HS hippocampi. Photomicrographs show $GABA_{B1a}$ **(A, D)**, $GABA_{B1b}$ **(B, E)** and $GABA_{B2}$ **(C, F)** IR in three adjacent sections from a post-mortem control and TLE-HS specimen. $GABA_{B2}$ show the highest immunosignal, $GABA_{B1a}$ demonstrated a lower immunoreactivity and $GABA_{B1b}$ displays the lowest immunopositivity. Scale bars represent 4mm in A, B, C and 8 mm in D, E, F (magnification 5X).

Figure 4 Quantitative immunohistochemistry

(A) Neuronal densities obtained by adjacent section of both TLE-HS (n=6-11) and PMC (n=5) stained with Cresyl Violet/Luxol Fast blue and. **(B, C)** graphs illustrate the

679 percentage of $GABA_B$ positive pyramidal and granular neurons respectively compared to
680 PMC. **(D, E)** graphs show semi-quantitative expression in pyramidal and granular cells of
681 $GABA_B$ subunits in 6 TLE-HS and 2 PMC. Semi-quantitative analysis obtained is
682 expression of GABA subunits in ROD per neurones. Statistical analysis: Kruskal-Wallis
683 with Conover-Inman post hoc analysis test was used to identify significant differences
684 between (* $P \leq 0.05$, ** $P < 0.01$, *** $P< 0.001$). Data presented as median and
685 interquartile range values.
686
687 **Figure 5 Brightfield photomicrograph displaying immunoreactivity in PMC and TLE-**
688 **HS CA1 and CA2**
689 Photomicrographs showing the distribution of $GABA_{B1a}$, $GABA_{B1b}$, and $GABA_{B2}$ in human
690 PMC and TLE-HS patients in the pyramidal cells of the CA1 (panel **A**); CA2 (panel **B**); red
691 harrows show glial cells. Scale bars: 120 µm.
692
693 **Figure 6 Brightfield photomicrograph displaying immunoreactivity in PMC and TLE-**
694 **HS CA3 and DG**
695 Photomicrographs showing the distribution of $GABA_{B1a}$, $GABA_{B1b}$, and $GABA_{B2}$ in human
696 PMC and TLE-HS patients in the pyramidal cells of the CA3 (panel **A**); DG (panel **B**); red
697 harrows show glial cells. Scale bars: 120 µm.

Table 1: Patient clinical data

Patients	Gender	age at sugery (yrs)	Samples	age at onset epilepsy	History of febrile seizures	Seizures type	Current AED	ILAE surgery outcome *	Method
Pt. 01	F	36	TLE-HS	4	Yes	Simple or complex partial/ Secondary generalised tonic clonic	PHT, VPA	NA	RT-PCR
Pt. 02	M	42	TLE-HS	2	Yes	Simple or complex partial/ Secondary generalised tonic clonic	CBZ, LCS	1	RT-PCR
Pt. 03	M	54	TLE-HS	NA	NA	Simple or complex partial	CBZ, LCS, PHT	2	RT-PCR
Pt. 04	F	24	TLE-HS	1	NA	Simple or complex partial/ Secondary generalised tonic clonic	LCS, LEV	2	RT-PCR
Pt. 05	F	45	TLE-HS	29	No	Simple or complex partial/ Secondary generalised tonic clonic	None	1	RT-PCR
Pt. 06	M	33	TLE-HS	9	NA	Simple or complex partial	None	1	RT-PCR
Pt. 07	M	33	TLE-HS	2	No	Simple or complex partial/ Secondary generalised tonic clonic	LMT, LEV	NA	RT-PCR
Pt. 08	F	22	TLE-HS	NA	Yes	Simple or complex partial/ Secondary generalised tonic clonic	LMT, CBZ	NA	RT-PCR
Pt. 09	F	39	TLE-HS	2	No	Simple or complex partial	None	NA	RT-PCR
Pt. 10	F	29	TLE-HS	1	Yes	Simple or complex partial/ Secondary generalised tonic clonic	CBZ, LMT	NA	RT-PCR
Pt. 11	M	23	TLE-HS	7	No	Simple or complex partial/ Secondary generalised tonic clonic	LMT	NA	RT-PCR
Pt. 12	F	27	TLE-HS	18	No	Simple or complex partial	CBZ, LEV	1	RT-PCR

Table 1: Patient clinical data (continued)

Patients	Gender	age at sugery (yrs)	Samples	age at onset epilepsy	History of febrile seizures	Seizures type	Current AED	ILAE surgery outcome *	Method
Pt. 13	M	31	TLE-HS	11	No	Simple or complex partial	LEV, CBZ	NA	RT-PCR
Pt. 14	M	48	TLE-HS	7	Yes	Simple or complex partial	None	1	RT-PCR
Pt. 15	M	48	TLE-HS	NA	NA	NA	VPA, LEV	NA	RT-PCR
Pt. 16	F	44	TLE-HS	NA	NA	Simple or complex partial	PGB, VPA	NA	RT-PCR
Pt. 17	M	63	TLE-HS	17	Yes	Simple or complex partial	LEV,CLB	1	RT-PCR
Pt. 18	M	38	TLE-HS	NA	NA	Simple or complex partial/ Secondary generalised tonic clonic	LMT, CBZ, GBP	5	RT-PCR /WB
Pt. 19	M	25	TLE-HS	2	Yes	Simple or complex partial/ Secondary generalised tonic clonic	ZNS, TPM	1	RT-PCR/ WB
Pt. 20	M	30	TLE-HS	28	No	Simple or complex partial	LEV, TMP, LCS	NA	WB
Pt. 21	M	44	TLE-HS	6	Yes	Simple or complex partial	CLB,CBZ,TPM	NA	IHC
Pt. 22	F	38	TLE-HS	NA	NA	Simple or complex partial	NA	NA	IHC
Pt. 23	M	30	TLE-HS	19	No	Simple or complex partial/ Secondary generalised tonic clonic	CBZ , PGB, CLB	NA	IHC
Pt. 24	M	27	TLE-HS	15	Yes	Simple or complex partial	LMT, VPA	NA	IHC
Pt. 25	F	24	TLE-HS	12	Yes	Simple or complex partial	LEV, PGB	NA	IHC
Pt. 26	F	40	TLE-HS	NA	NA	NA	NA	NA	IHC
Pt. 27	F	39	TLE-HS	22	No	Simple or complex partial/ Secondary generalised tonic clonic	LMT, CBZ, TPM	NA	IHC

Table 1: Patient clinical data (continued)

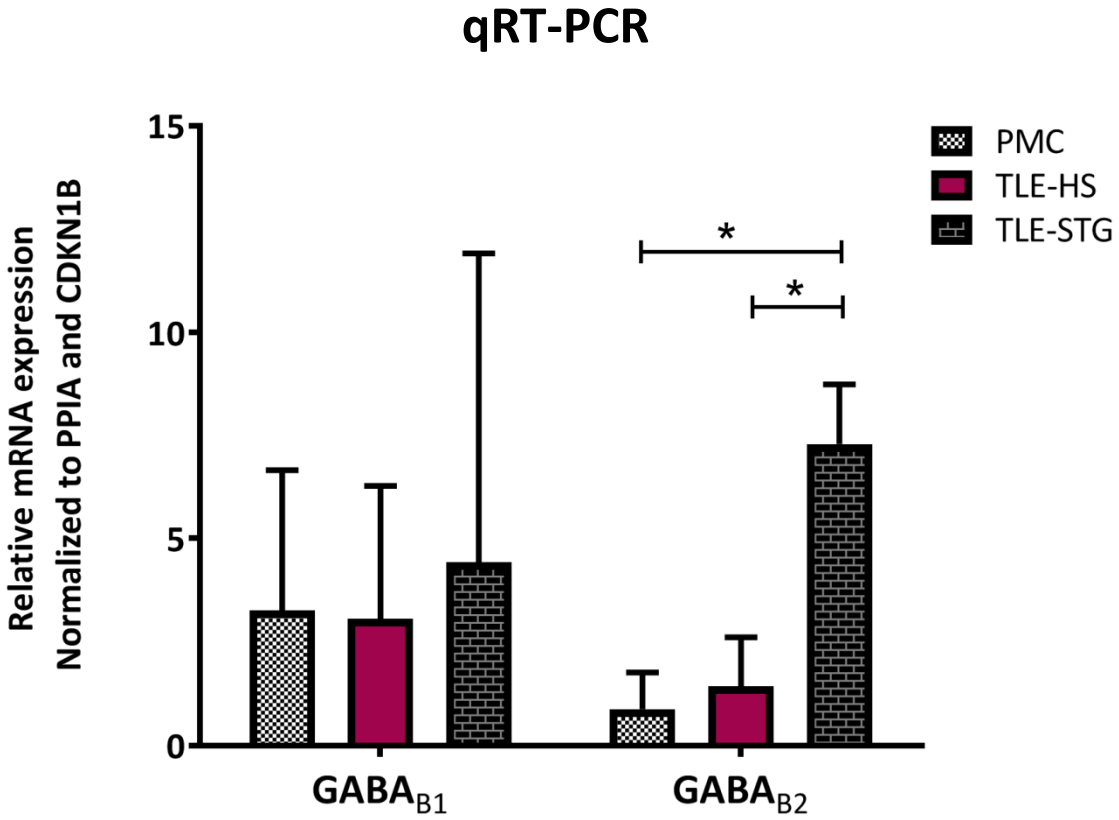
Patients	Gender	age at sugery (yrs)	Samples	age at onset epilepsy	History of febrile seizures	Seizures type	Current AED	ILAE surgery outcome *	Method
Pt. 28	M	42	TLE-HS/TLE-STG	NA	Yes	Simple or complex partial	CBZ, LCS, CLB	4	RT-PCR
Pt. 29	F	32	TLE-HS/TLE-STG	19	No	Simple or complex partial/ Secondary generalised tonic clonic	LMT, CBZ, GBP	4	RT-PCR
Pt. 30	F	54	TLE-HS/TLE-STG	1	Yes	Simple or complex partial	LMT,PGB	1	RT-PCR
Pt. 31	M	41	TLE-HS/TLE-STG	12	No	Simple or complex partial	CBZ , LMT	1	RT-PCR
Pt. 32	M	61	TLE-HS/TLE-STG	NA	NA	Simple or complex partial	LMT, CBZ, GBP	3	WB
Pt. 33	F	31	TLE-HS/TLE-STG	29	NA	Simple or complex partial	PB	1	WB
Pt. 34	M	35	TLE-HS/TLE-STG	17	No	Simple or complex partial/ Secondary generalised tonic clonic	CBZ, LEV	1	WB
Pt. 35	F	44	TLE-HS/TLE-STG	16	Yes	Simple or complex partial	TPM	1	RT-PCR
Pt. 36	F	34	TLE-HS/TLE-STG	0.5	NA	Secondary generalised tonic clonic	LMT, LEV	1	RT-PCR
Pt. 37	M	51	TLE-HS/TLE-STG	45	No	Simple or complex partial	NA	NA	RT-PCR
Pt. 38	F	22	TLE-HS/TLE-STG	9	No	Simple or complex partial/ Secondary generalised tonic clonic	LCS, LMT, TPM, PB	5	RT-PCR
Pt. 39	M	48	TLE-HS/TLE-STG	1	No	Simple or complex partial	CBZ, PER	4	RT-PCR/WB
Pt. 40	F	51	TLE-HS/TLE-STG	40	Yes	Simple or complex partial/ Secondary generalised tonic clonic	LCS, LEV	1	RT-PCR/WB
Pt. 41	F	25	TLE-HS/TLE-STG	19	No	Simple or complex partial/Secondary generalised tonic clonic	LEV, PB, LCS	4	RT-PCR/WB

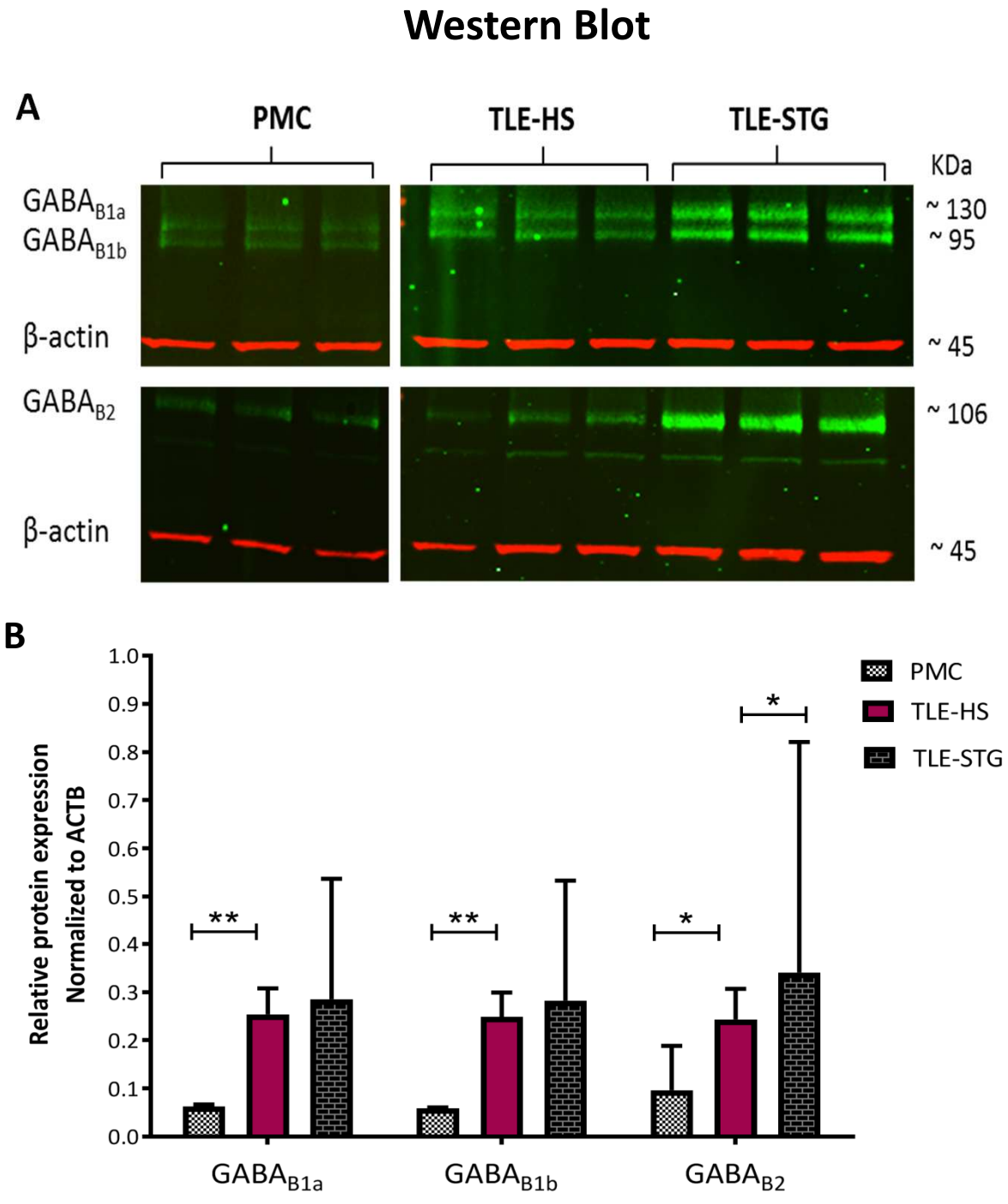
Table 1 reports the relevant clinical data of patients used in this study. **Samples:** Patients (1-27) sclerotic hippocampi from Temporal lobe epilepsy patients (**TLE-HS**). Patients (28-41): Superior temporal gyrus (**STG**) from TLE-HS patients. **Antiepileptic drugs (AEDs):** CBZ, Carbamazepine; CLB, Clobazam; CNP, Clonazepam; GB, Vigabatrin; LCS, Lacosamide; LEV, Levetiracetam; LMT,Lamotrigine; OXC, Oxcabazepine; PB, Phenobarbital; PER: Perampanel; PGB, Pregabalin, PHT, Phenytoin; TPM, Topiramate; VPA, Valproate. **(NA):** not available. *** ILAE Classification of Surgical outcome: 1)** completely seizure free, No auras; **2)** only auras,no other seizures; **3)** 1-3 seizure days per year, ± auras; **4)** 4 seizure days per year to 50% reduction of baseline seizure days, ± auras; **5)** less than 50% reduction of baseline seizure days, ± auras. **Methods**: quantitative real-time polymerase chain reaction (qRT-PCR), quantitative Western blot (qWB) and Immunohistochemistry (IHC), not available (NA).

Table 2: Post-*mortem* samples:

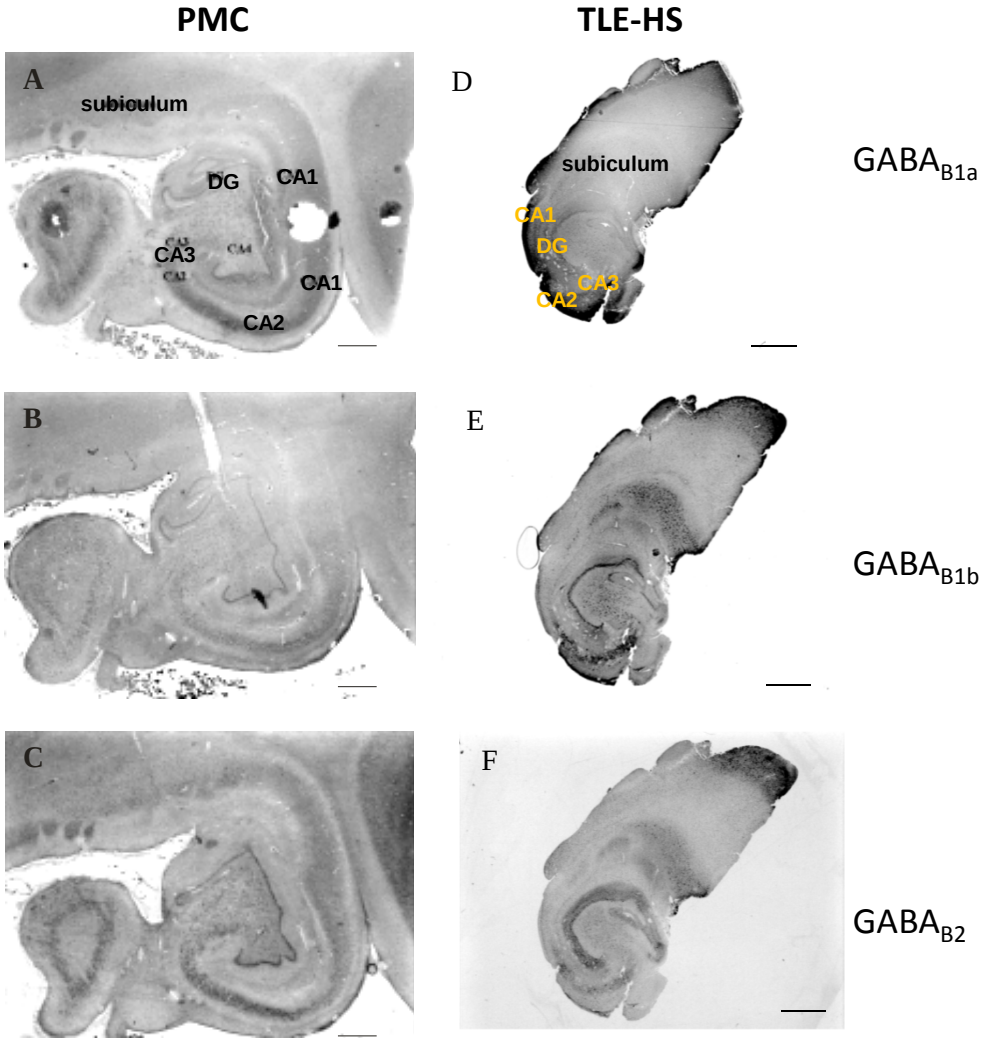
Sample	Gender	Age (yrs)	PMI (hrs)
PMC 01	M	71	38.5
PMC 02	M	38	80.35
PMC 03	M	63	42
PMC 04	M	43	15
PMC 05	F	53	29.5
PMC 06	F	78	23.3
PMC 07	F	80	49.1
PMC 08	M	85	51.3
PMC 09	F	78	51.3
PMC 10	F	64	79
PMC 11	M	91	48
PMC 12	F	83	20
PMC 13	F	88	49.25

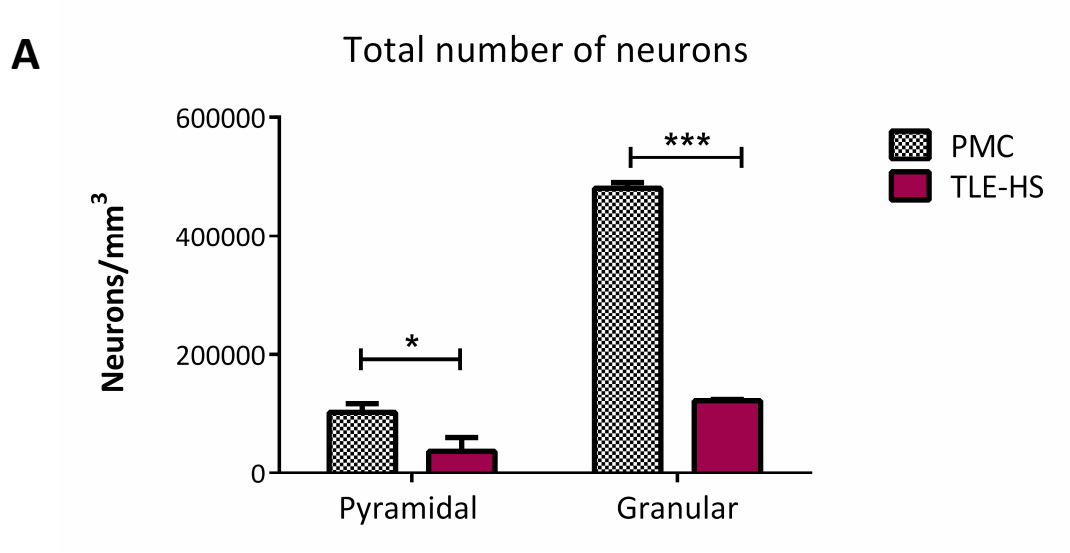
Table 2 shows post-mortem samples' features

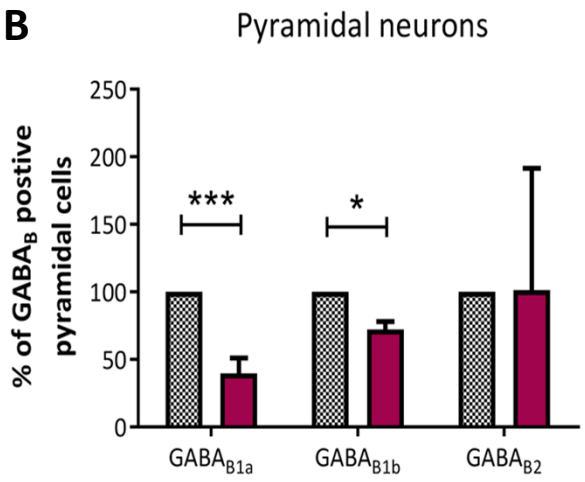

qRT-PCR
Relative mRNA expression
Normalized to PPIA and CDKN1B
15
10
5
0
PMC
TLE-HS
TLE-STG
*
*
GABA$_{B1}$
GABA$_{B2}$

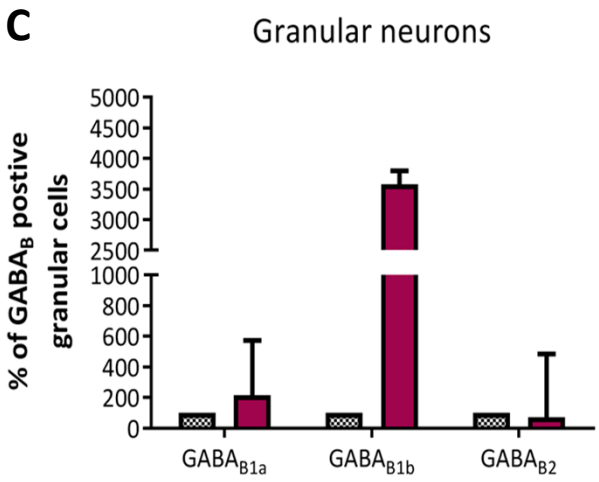

Western Blot
A
PMC
TLE-HS
TLE-STG
KDa
GABA$_{B1a}$
GABA$_{B1b}$
β-actin
~ 130
~ 95
~ 45
GABA$_{B2}$
β-actin
~ 106
~ 45
B
Relative protein expression
Normalized to ACTB
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
PMC
TLE-HS
TLE-STG
**
**
*
*
GABA$_{B1a}$
GABA$_{B1b}$
GABA$_{B2}$

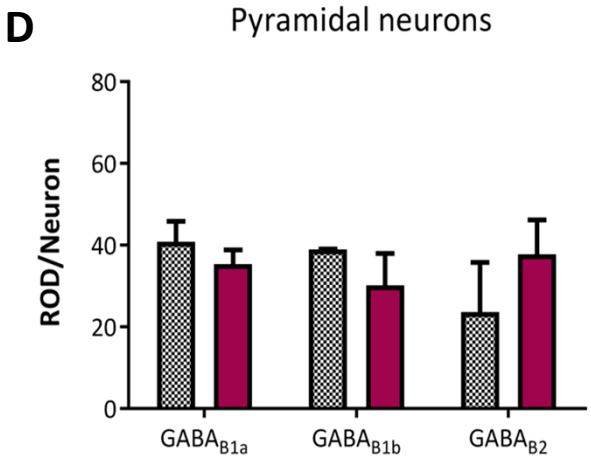
Immunohistochemistry

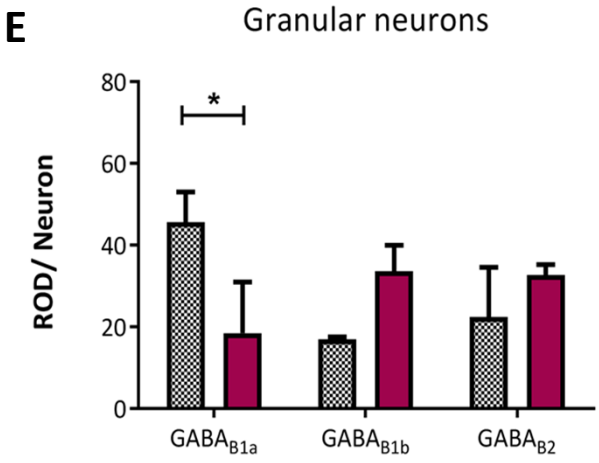


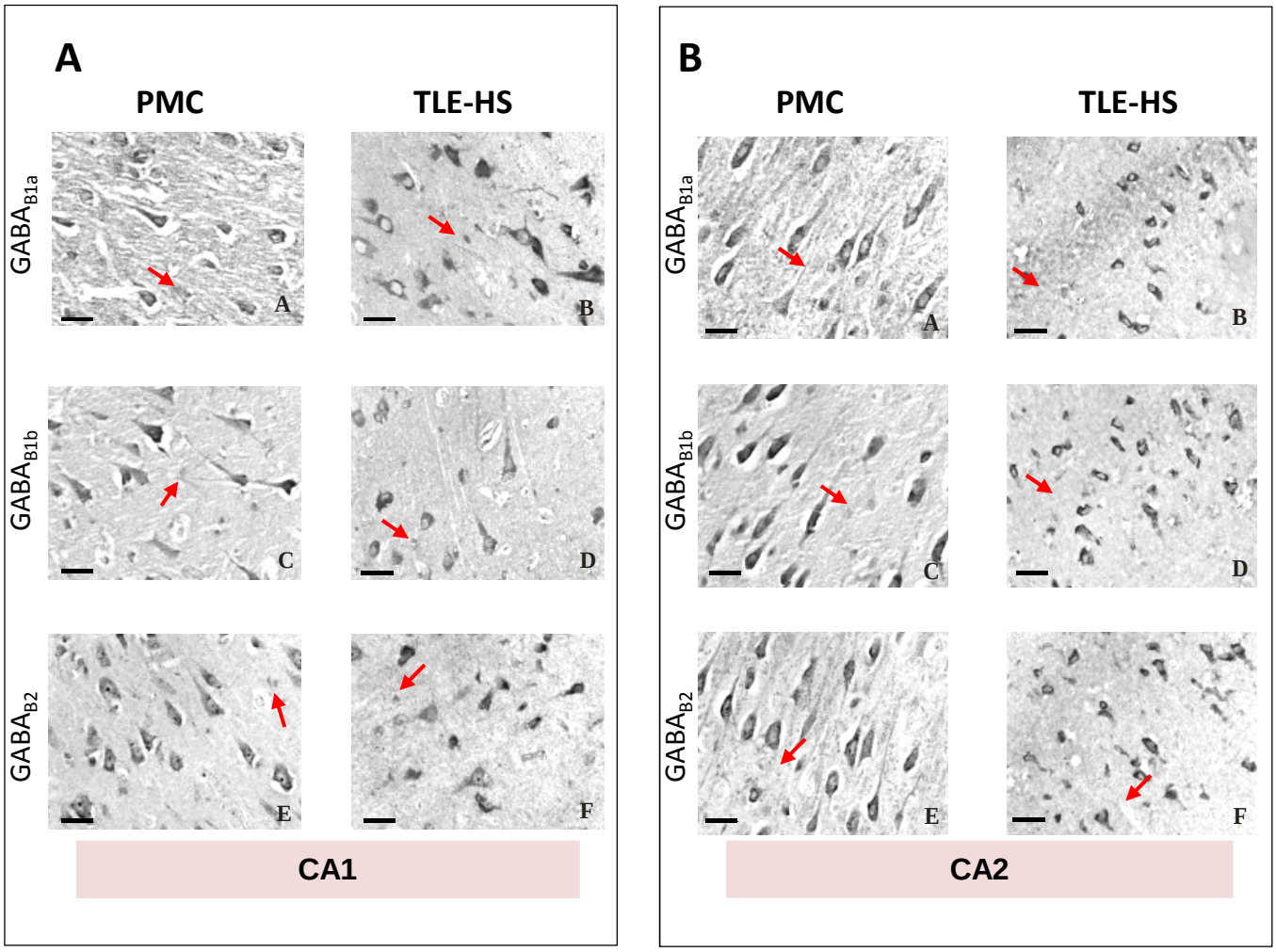

A
Total number of neurons
Neurons/mm3
600000
400000
200000
0
*
***
PMC
TLE-HS
Pyramidal
Granular


B
Pyramidal neurons
% of GABAB postive pyramidal cells
250
200
150
100
50
0
***
*
GABAB1a
GABAB1b
GABAB2


C
Granular neurons
% of GABAB postive granular cells
5000
4500
4000
3500
3000
2500
1000
800
600
400
200
0
GABAB1a
GABAB1b
GABAB2


D
Pyramidal neurons
ROD/Neuron
80
60
40
20
0
GABAB1a
GABAB1b
GABAB2


E
Granular neurons
ROD/ Neuron
80
60
40
20
0
*
GABAB1a
GABAB1b
GABAB2


A
PMC
TLE-HS
GABA$_{B1a}$
GABA$_{B1b}$
GABA$_{B2}$
A
B
C
D
E
F
CA1
B
PMC
TLE-HS
GABA$_{B1a}$
GABA$_{B1b}$
GABA$_{B2}$
A
B
C
D
E
F
CA2

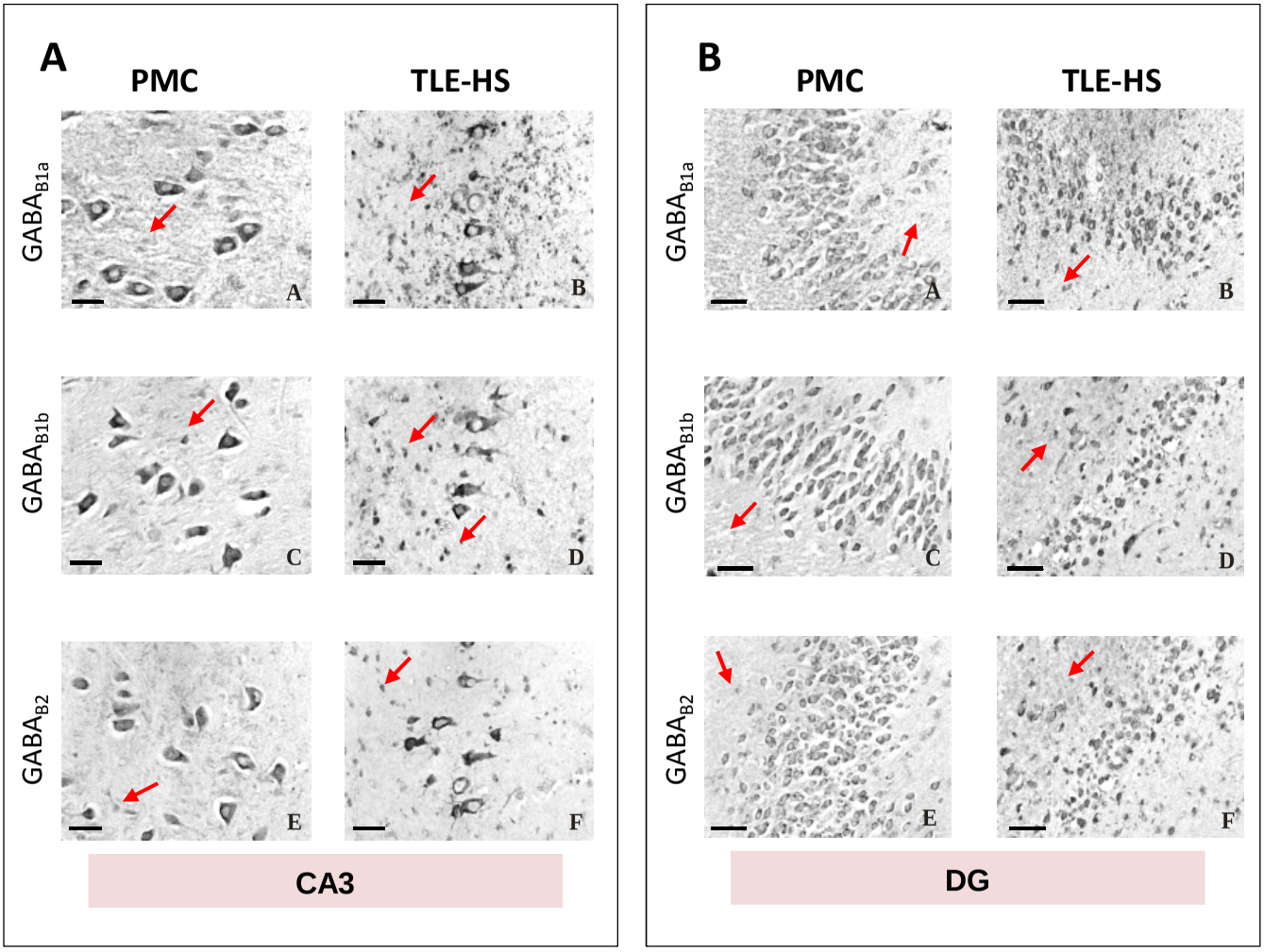


Table 3: TaqMan gene expression assays

Gene Symbol	Name	cellular function	Task	TaqMan assay ID	amplicons' length bp	R^2	Efficiency
GABBR 1 (a,b)	gamma-aminobutyric acid (GABA) B receptor, 1	synaptic transmission, GABA signalling pathway	Target gene	Hs00559488_m1	68	0.95	98.03
GABBR 2	gamma-aminobutyric acid (GABA) B receptor, 2	synaptic transmission, GABA signalling pathway	Target gene	Hs01554998_m1	158	0.98	96.45
PPIA	peptidylprolyl isomerase A	protein metabolism and folding	reference gene	Hs04194521_s1	97	0.97	96.84
CDKN1 B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	cell growth and division	reference gene	Hs01597588_m1	151	0.97	99.66

Table 4. Antibodies and their concentration used for WB & IHC

Quantitative western blot				
Proteins	**Primary antibody**	**Primary antibody dilution**	**Secondary antibody**	**secondary antibody dilution**
GABBR1 (a,b)	Rabbit GABBR1 (Cell Signaling Technology®, S3835)	1:500	IRDye® 800CW goat anti-rabbit IgG (926-32211,LI-COR® Bioscience)	1:5000
GABBR2	Rabbit GABBR2 (ab75838, abcam®)	1:500	IRDye® 800CW goat anti-rabbit IgG (926-32211,LI-COR® Bioscience)	1:5000
β-actin	Mouse β-actin	1:1000	IRDye® 680LT goat anti-mouse IgG (926-68020, LI-COR® Bioscience)	1:10000
IHC				
Proteins	**Primary antibody and sequence**	**Primary antibody dilution**	**Secondary antibody**	**secondary antibody dilution**
GABBR1a	Rabbit polyclonal $GABBR1_{1a}$ NH2-CHPPWEGGIRYRGLTRDQVK-COOH residues 33-51	1:500	biotinylated goat anti-rabbit	1:200
GABBR1b	Rabbit polyclonal $GABBR1_{1b}$ NH_2-HSPHLPRPHPRVPPHPS-COOH residues 30–47	1:500	biotinylated goat anti-rabbit	1:200
GABBR2	α glutathione *S*-transferase (GST) fusion protein was generated against the intracellular C-terminus amino acids 745–941	1:100	biotinylated goat anti-rabbit	1:200

All antibodies were diluted with 0.1% PBST buffer.

HIGHLIGHTS

- This study investigates $GABA_B$ in three types of human specimens: two types from patients with temporal lobe epilepsy with sclerotic hippocampal samples (TLE-HS), non-spiking ipsilateral superior temporal gyrus (TLE-STG) and third is hippocampal tissue from (p*ost-mortem* controls (PMC).
- This study investigates $GABA_B$ by using three different quantitative techniques: RT-PCR, Western blot, and immunohistochemistry in human specimens
- The higher expression of mature $GABA_B$ protein in TLE-HS than PMC is in agreement with previous studies
- On the other hand, this study shows a statistically significant lower expression of $GABA_{B2}$ in TLE-HS samples than in non-epileptogenic
- Therefore, the downregulation of $GABA_{B2}$ transcription and $GABA_{B2}$ mature protein subunit in TLE-HS could represent one of the reasons for the impaired GABAergic inhibition reported in epileptogenic hippocampal tissue