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 two specimens from patients with TLE, secondary to hippocampal sclerosis, removed during epilepsy surgery (sclerotic hippocampal samples) TLE-HS, and tissue from the healthy, non-spiking ipsilateral superior temporal gyrus (TLE-STG); and hippocampal tissue specimen from individuals with no history of epilepsy (*post-mortem* controls, PMC).

mRNA expression of $GABA_B$ subunits was quantified in PMC, TLE-STG and TLE-HS 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. 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 co-localisation of $GABA_{B1(a-b)}$ and $GABA_{B2}$ seemed to support the idea that the receptor is heterodimeric. 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. Possibly the TLE-STG samples examined here represent a better 'control' tissue. If so, TLE-HS samples would be characterised by lower than expected $GABA_B$ expression. This interpretation would provide a better explanation for 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 pathogenesis. 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 (Mott and Lewis 1994; Misgeld et al. 1995, Bowery 1997; Deisz et al.1997; Bettler et al. 1997).

$GABA_B$ 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 in rat brain (Kaupmann et al. 1998; Jones et al. 1998). 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 best 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 $GABA_B$ 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.

This study aimed to examine how possible differences in $GABA_{B1a,}$ $GABA_{B1b}$ and $GABA_{B2}$ mRNA and protein expression may contribute to the impaired $GABA_B$ mediated currents reported in TLE (Straessle et al. 2003; Rocha et al. 2015). 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 patients 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. In summary, the median age of 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. 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. The second part of the sample was fixed for histopathological analysis and all pre-operative diagnoses of HS were confirmed after surgery by histopathological examination based on established diagnostic criteria (Thom at al. 2002), and immunohistochemistry experiments. The TLE-STG specimens were taken from the superior temporal gyri which looked structurally healthy 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.

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

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

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

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

2.2.4. Data analysis: 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. 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 Kruskal-Wallis with Conover-Inman *post hoc* analysis test was used to identify significant differences between samples ($P < 0.05$).

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).

2.3.3. *Data analysis:* $GABA_{B1(a-b)}$, and $GABA_{B2}$ bands intensities were normalized to β-actin to eliminate any loading variation. The GraphPad Prism 6 software version 6.01 for Windows was used for all the statistical analysis (San Diego, CA, USA). The Shapiro-Wilk W test was performed to test the normality of the data. The Kruskal-Wallis with Conover-Inman *post hoc* analysis test was used to identify significant differences between samples ($P < 0.05$).

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 - 0.08% nickel ammonium sulphate 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 (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.

3. RESULTS

3.1. qRT-PCR

The correlation between PMC mRNA samples versus age and post-mortem interval in figure 1 demonstrates no correlation between the mRNA findings and these factors which could have influence the mRNA expression. 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 an increased $GABA_{B2}$ expression in the TLE-HS tissue. In contrast, the comparison of TLE-HS with the TLE-STG samples showed a lower level of expression of both $GABA_B$ in the TLE-HS tissue (see Figure2).

3.2. Qualitative and Quantitative WB

Figure 3A 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 3B) follows the same trend although differences between the TLE-HS and the TLE-STG comparisons were only significant for $GABA_{B2}$.

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 4 A-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 5A 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 5D and 5E 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 6 and 7 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 6 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 6, 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 7, 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 7 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, 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 2 and 3 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 2 and 3, it is evident that the trend of the receptor subunits is the same, demonstrating that $GABA_{B2}$ expression is very much lower in the hippocampi of pharmaco-resistant patients compared to TLE-STG. Previous binding and present immunohistochemical data in human hippocampal PMC control and epileptic specimens appear in reasonable agreement (Princivalle et al. 2002).

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

The lower expression of both $GABA_B$ receptor subunits in TLE-HS compared to TLE-STG, could indicate a decline in $GABA_B$ receptors which would provide an explanation for the compromised $GABA_B$ functionality previously reported in pharmacological and electrophysiological studies in animal model and in human TLE (Billinton et al. 2000; Princivalle et al. 2002; Fürtinger et al. 2003b; Mareš and Kubová 2015; Leung et al. 2016; Rocha et al. 2015). This may be affecting the formation of fully functional $GABA_B$ receptors: since the heterodimerisation of $GABA_{B1}$ and $GABA_{B2}$ in 1:1 stoichiometry is essential for

receptor trafficking and G-protein activation, the $GABA_{B2}$ subunit could be a potential target for the development of new agonists or activating transcription factors drugs, which may have a major clinical impact on the treatment of pharmaco-resistant TLE-HS patients. However, there are other factors which could explain the reduced GABAergic inhibition (Gill et al. 2010; Armstrong et al. 2016), and there is a strong possibility of co-causation.

The findings of this study could be interpreted in two different ways: $GABA_B$ protein expression in epileptogenic hippocampal tissue could be down-regulated (because of the higher expression in TLE-STG tissue) or it could be up-regulated (because of the lower expression in PMC tissue). The decision which explanation is more likely depends on the relative merits of the two non-epileptogenic "control"-tissues. Unfortunately, neither PMC nor non-spiking TLE-STG is a perfect match for the TLE-HS samples of interest for the kind of experiments conducted here. However, there is no real alternative in human studies and it is not the first time that neocortex (STG) has been used in studies on TLE (Teichgräber et al. 2009; Rocha et al. 2015). Even human non-epileptogenic hippocampi removed for other reasons (such as temporal lobe tumours) cannot be considered an ideal control tissue for TLE-HS samples (Kovács et al. 2012). Son et al. (2015) demonstrated that tissue surrounding or adjacent to a tumour is physiologically and molecularly perturbed by the tumour itself or by previous irradiation.

In view of these difficulties, many studies investigating TLE pathophysiology have recently compared their results obtained in epileptogenic TLE specimens to other surgically resected samples such as neocortex. The strength of this approach includes the fact that both sample types contain the same DNA (reducing the risk of intersubject variability caused by gene-gene or gene-environment interactions) and that both samples were obtained and processed in the same way. This approach also avoids the difficulties associated with comparing TLE-HS tissue removed during epilepsy surgery with PMC-HS tissue possibly affected by an agonal state and *post-mortem* changes (Preece et al. 2003; Tomita 2004; Teichgräber et al., 2009; Rocha et al. 2015).

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

CONCLUSIONS

In agreement with older studies, we found the overall expression of $GABA_B$ increased in TLE-HS versus PMC. If correct, this finding suggests that the previously reported reduction in slow IPSPs in TLE-HS cannot be explained by a decreased protein expression of the $GABA_B$ receptor subunit. Instead this neurophysiological observation could be due to other causes including post-translational modification of the $GABA_B$ protein. On the other hand, this study shows a lower expression of $GABA_{B2}$ in TLE-HS samples than in non-epileptogenic TLE-STG from the same patients. Considering that the PMC values were affected by agonal or post-mortem changes (or due to undetected differences in clinical or demographic factors between TLE-HS and PMC subjects) the TLE-STG samples may represent a more appropriate "control" tissue. If so, 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 in the literature.

LIST OF ABBREVIATIONS

CA	*cornu ammonis*
GABA	γ-Aminobutyric acid
$GABA_B$	γ-Aminobutyric acid receptor B
DG	dentate gyrus
HS	hippocampal sclerosis
IHC	immunohistochemistry
IR	immunoreactivity

PMC	post-mortem control
PMI	post-mortem interval
TLE	temporal lobe epilepsy
DG	dentate gyrus
ROD	relative optical density
STG	superior temporal gyrus

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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 Graphical representation of linear regression

(A) shows the correlation between mRNA from PMC samples and age; **(B)** illustrates the correlation between mRNA from PMC samples and PMI.

Figure 2 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 Mean ± S.D.

Figure 3 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 Mean ± S.D.

Figure 4 Qualitative immunohistochemistry

Distribution of $GABA_{B1a,}$ $GABA_{B1b}$ and $GABA_{B2}$ in PMC and TLE-HS hippocampi. Photomicrographs showing $GABA_{B1a}$ (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 5 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 percentage of $GABA_B$ positive pyramidal and granular neurons respectively compared to PMC. (D, E) graphs show semi-quantitative expression in pyramidal and granular cells of $GABA_B$ subunits in 6 TLE-HS and 2 PMC. Semi-quantitative analysis obtained is expression of GABA subunits in ROD per neurones. Data presented as Mean ± S.D. The statistical test used was Kruskal-Wallis: for pairwise comparisons (Conover-Inman).

Figure 6 Brightfield photomicrograph displaying immunoreactivity in PMC and TLE-HS CA1 and CA2

Photomicrographs showing the distribution of $GABA_{B1a}$, $GABA_{B1b}$, and $GABA_{B2}$ in human PMC and TLE-HS patients in the pyramidal cells of the CA1 (panel A); CA2 (panel B); red harrows show glial cells. Scale bars: 120 µm.

Figure 7 Brightfield photomicrograph displaying immunoreactivity in PMC and TLE-HS CA3 and DG

Photomicrographs showing the distribution of $GABA_{B1a}$, $GABA_{B1b}$, and $GABA_{B2}$ in human PMC and TLE-HS patients in the pyramidal cells of the CA3 (panel A); DG (panel B); red harrows show glial cells. Scale bars: 120 µm.