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$GABA_B$ receptor autoradiography in hippocampal sclerosis associated with human temporal lobe epilepsy

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1 Metabotropic γ-aminobutyric acid receptors ($GABA_B$) exist both pre- and postsynaptically throughout the brain, mediating the suppression of neurotransmitter release and late inhibitory postsynaptic potentials. Investigation of $GABA_B$ receptors in rodent models of temporal lobe epilepsy (TLE) suggests that expression or function of these receptors may be altered in the disorder.

2 The aim of the present study was to investigate the expression of $GABA_B$ receptors in samples of hippocampus surgically resected from patients with hippocampal sclerosis (HS) related intractable TLE, and compare this expression with samples of neurologically normal post-mortem (PM) control hippocampal tissue. Appropriate measures of neuronal loss associated with HS were investigated for comparison with receptor binding data.

3 Receptor autoradiography with [^{3}H]-GABA in the presence of isoguvacine, and quantitative densitometric analysis were used to investigate $GABA_B$ receptor expression (B_{max}) and affinity (K_D) in 11 HS samples and eight controls. A three-dimensional cell counting technique was used to assess neuronal density in both groups.

4 $GABA_B$ receptor density was significantly reduced in CA1, CA2, CA3, hilus and dentate gyrus, and increased in the subiculum, of HS cases as compared with PM controls. Neuronal loss was significant in all regions measured. When adjusted for neuronal loss, CA1 $GABA_B$ receptor expression appeared significantly upregulated ($P<0.05$).

5 In HS/TLE, $GABA_B$ receptor expression per remaining neurone appears increased in CA1. This finding, and increased [^{3}H]-GABA affinity at CA3 and hilar $GABA_B$ receptors, suggests altered $GABA_B$ receptor function may occur in human HS/TLE, possibly as a result of synaptic reorganization.

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Abbreviations: HS, hippocampal sclerosis; SGDG, stratum granulosum dentate gyrus; SMDG, stratum moleculare dentate gyrus; TLE, temporal lobe epilepsy

Introduction

The control of neuronal excitation in the CNS is regulated to a large extent by the major inhibitory neurotransmitter γ-aminobutyric acid (GABA). $GABA_B$ receptors, the metabotropic receptors for GABA, are G protein-coupled receptors (GPCR) which regulate neuronal excitability both pre- and postsynaptically. The action of GABA at presynaptic $GABA_B$ receptors is to reduce Ca^{2+} influx, and thus inhibit neurotransmitter release (Takahashi *et al*., 1998). These receptors may exist on GABAergic terminals (autoreceptors), or on terminals arising from cells containing other neurotransmitters, such as glutamate (heteroreceptors). Postsynaptically, $GABA_B$ receptors are responsible for the generation of the late inhibitory postsynaptic potential (IPSP), *via* the opening of K^+ channels, and inhibit adenylate cyclase (see Bettler *et al*., 1998). Abnormality in either of these functions could have consequences for the generation and/or prevention of epileptic seizures.

The first $GABA_B$ protein ($GABA_{B(1)}$) was discovered using expression cloning by Kaupmann *et al*. (1997). Functional expression of $GABA_{B(1)}$ in cell lines proved difficult (Couve *et al*., 1998), until a second protein $GABA_{B(2)}$ was discovered by database and yeast two-hybrid screens (Jones *et al*., 1998; Kaupmann *et al*., 1998; White *et al*., 1998). When co-expressed the two proteins appeared to form a functional $GABA_B$ receptor, as a heterodimer. The $GABA_{B(1)}$ gene has been mapped to a locus on chromosome 6, which is associated with some familial forms of epilepsy (Peters *et al*., 1998).

Electrophysiological studies of neurones from human hippocampal sclerosis (HS) specimens, have indicated a reduction in evoked IPSPs as compared with neurones in specimens from patients with structural lesions (Isokawa *et al*., 1991; Knowles *et al*., 1992). This suggests that GABA mediated inhibition may be compromised in HS patients.

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Studies in animal models of TLE have shown varying degrees of impairment in $GABA_B$ receptor mediated neurotransmission. In the rat amygdala kindling model, a decrease in the sensitivity of presynaptic $GABA_B$ heteroreceptors was reported, indicated by a lack of paired pulse depression (Asprodini *et al*., 1992). A reduction in presynaptic $GABA_B$ receptor function was also reported in dentate granule cells of kindled rats (Buhl *et al*., 1996), and kainic acid treated rats (Haas *et al*., 1996), and in CA1 of partially kindled rats (Wu & Leung, 1997). Impairment of postsynaptic $GABA_B$ receptor function was not apparent in these studies. In contrast, Mangan & Lothman (1996), observed reductions in both pre- and postsynaptic $GABA_B$ receptor function in CA1 neurones in a rat hippocampal kindling model.

The $GABA_A$ receptor complex has been the focus of GABA receptor study in relation to human TLE (see Blümcke *et al*., 1999), but $GABA_B$ receptors may also play an important role in the control of neuronal excitability resulting in epileptic seizures. The aim of the present study was to investigate abnormalities in $GABA_B$ receptor binding characteristics in HS related TLE, using [^{3}H]-GABA saturation receptor autoradiographic analysis (under $GABA_B$ receptor preferring conditions), and compare this data with quantitative neuropathology from the same patients.

Methods

Subjects

Hippocampal tissue was obtained at surgery from 11 patients suffering from intractable HS associated TLE (median age 34 years, range 22–48 years), whose clinical details are summarized in Table 1. In all patients, HS had been confirmed by histological examination and magnetic resonance imaging (MRI), and all patients had been chronically prescribed antiepileptic medication (see Table 1). Frozen post-mortem control hippocampal tissue was obtained from The Parkinson's Disease Brain Bank, Institute of Neurology, (median age 56 years, range 32–77 years) the clinical details of which are summarized in Table 2. Control specimens were derived from individuals presenting no evidence of neurological disorder.

Table 1 Clinical data from 11 HS/TLE patients studied

Patient no	*Gender*	*Age* (age at onset) (years)	*CP seizure frequency* (per year)	*Secondary generalization*	*Medication*
1	M	38 (8)	56	Y	CBZ, LTG
2	F	28 (1)	144	Y	LTG, PHT
3	M	22 (5)	72	N	CLB, LTG
4	F	34 (2)	48	N	VPA, LTG
5	M	33 (2)	104	Y	LTG, CLB, VGB
6	M	42 (5)	48	Y	CBZ, LTG
7	F	36 (5)	70	Y	CBZ, LTG
8	F	40 (2)	120	Y	CBZ, TPM
9	F	23 (2)	36	Y	LTG, PHT
10	M	38 (23)	95	N	CBZ, LTG
11	F	48 (2)	48	N	CBZ, TPM

CP, complex partial; Y, presented with secondary generalized seizures (more than 1 per year, 10 in lifetime); N, no presentation with secondary generalized seizures (less than 1 per year, less than 10 in lifetime); CBZ, carbamazepine; CLB, clobazam; VGB, vigabatrin; LTG, lamotrigine; PHT, phenytoin; TPM, topiramate; VPA, sodium valproate.

Tissue preparation

The temporal lobe and hippocampal surgical resections were carried out according to the procedure described by Spencer *et al*. (1984). Approximately 2–2.5 cm of *en bloc* resection of the anterior mesial temporal structures including anterior hippocampus (head and body) was sliced at 0.5 cm intervals in a coronal plane and a single tissue block from the middle of this specimen was taken for receptor studies. Hippocampal tissue was resected and frozen within 20 min on dry ice, and subsequently stored at −80°C. Slices of control brain cut 1 cm thick were snap frozen between brass plates and hippocampi dissected out from appropriate slices. These were stored at −80°C until 10 μm cryostat sections were cut and thaw-mounted on charged microscope slides (Superfrost Plus, BDH), which were also stored at −80°C until assay.

Receptor autoradiography

Autoradiography was carried out essentially as described by Bowery *et al*. (1987). Briefly, on the day of assay, slides were allowed to equilibrate to room temperature and then washed in assay buffer (50 mM TRIS/HCl pH 7.4, 2.5 mM $CaCl_2$) at 23°C for 20 min, followed by a further 60 min in fresh assay buffer. Saturation binding was carried out with one of six concentrations (10–400 nM) of [^{3}H]-GABA (100 Ci mmol^{-1}, NEN) in the presence of 40 μM isoguvacine (RBI, U.K.) (to preclude binding at $GABA_A$ sites) in assay buffer for 20 min at 23°C. Non-specific binding was determined in the presence of 100 μM (−)-baclofen. Following incubation the slides were aspirated, rinsed in assay buffer (2 × 3 s), and dipped briefly in distilled water prior to drying under a stream of cool air. Slides were co-apposed to [^{3}H]-sensitive autoradiographic film (Hyperfilm, Amersham, U.K.) in lightproof cassettes with ^{3}H-impregnated plastic standards, for 21 days at room temperature.

Cell counting

Neuronal densities for hippocampal subregions were determined in 20–25 μm cresyl violet stained sections of paraffin embedded tissue from an adjacent part of the same samples, using a three-dimensional counting technique, as described previously (Williams & Rakic, 1988). Cell counts were not performed on the subiculum in this study.

Table 2 Clinical details of post-mortem control hippocampal tissue

Case no	*Gender*	*Age* (years)	*PMI* (hours)	*Cause of death*
1	M	67	5.5	Left ventricular failure
2	M	77	27.5	Cancer of pancreas
3	F	77	18	Unknown
4	M	43	15	Cardiac failure
5	F	63	34	Myocardial infarction
6	M	32	32	Pneumonia
7	M	50	34	Acute cardiac failure
8	M	49	44	Ruptured aortic aneurysm

Data analysis

[^{3}H]-GABA binding images were analysed by densitometry using an MCID M4 image analysis system (Imaging Research Inc., Ontario, Canada), and optical density converted to fmol mg^{-1} of bound ligand using the image generated by the [^{3}H]-impregnated plastic standard strips. Total binding was assessed in two to four sections per concentration of [^{3}H]-GABA. Regions to be analysed were identified by cresyl violet staining of sections used for autoradiography. The entire subregion under investigation was outlined, and a mean optical density across the region was obtained. Binding parameters (receptor density (B_{max}) and affinity (K_D)) were determined by use of a Langmuir equation in Prism PC software (GraphPad Software, San Diego, CA, U.S.A.).

Statistical analysis was performed with a Student's *t*-test (unpaired, 2-tailed) to compare mean values of neuronal density, B_{max} and K_D with $P<0.05$ as the significance level (Prism software). Values of *n* are quoted as a range where hippocampal subregions were missing from some resected tissue samples.

Results

Neuronal density

Significant reductions in neuronal density were found in regions CA1, CA2, CA3, dentate hilus and dentate gyrus granule cell layer (SGDG) of HS tissue as compared with post-mortem controls (Figure 1).

GABA$_B$ receptor density

A representative example of a saturation plot from CA1 of a single post-mortem control sample is shown in Figure 2. Specific binding appeared to be saturable within the concentration range used (10–400 nM), and non-specific binding increased in a linear fashion, a fact reflected in the continued increase in total binding after saturation of the specific component.

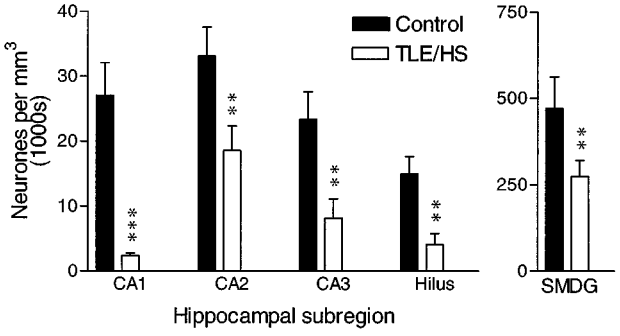

Figure 1 Mean changes in neuronal density in resected epileptic hippocampal tissue ($n=7-11$) as compared with post-mortem control hippocampal samples ($n=7-8$). Three-dimensional counts were performed on formalin fixed tissue sections (20–25 μm thickness). Data are represented as mean$\pm$s.e.mean. Statistical analysis used unpaired Student's *t*-test (2-tailed), where $^{**}P<0.01$ and $^{***}P<0.001$. SGDG, stratum granulosum dentate gyrus.

In post-mortem control tissue, the regions of highest GABA$_B$ receptor density were SMDG, CA1 and subiculum. CA2, CA3 and dentate hilus showed moderate binding density (Figure 3a).

GABA$_B$ receptor binding was found to be increased in subiculum, (to 125$\pm$9% of control, $P<0.05$) but reduced in all other regions of HS hippocampus as compared with post-mortem controls (Figure 4). The greatest deficit was observed in CA1, with a reduction in B_{max} to 19$\pm$3% of control ($P<0.001$), and SMDG showed the least decrease in binding density (to 74$\pm$5% of control, $P<0.05$).

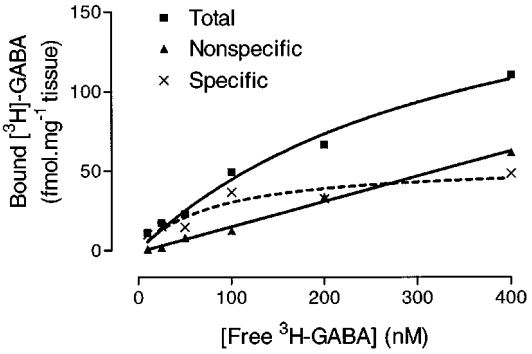

Figure 2 Representative plot of saturation binding of [^{3}H]-GABA in the presence of 40 μM isoguvacine in subregion CA1 of a control human hippocampus. Concentrations of [^{3}H]-GABA were in the range 10 to 400 nM. Specific binding (dashed line) is the difference between total and non-specific binding. Non-specific binding was determined in the presence of 40 μM isoguvacine and 100 μM (−)baclofen. Data shown are from a single tissue sample measured in duplicate.

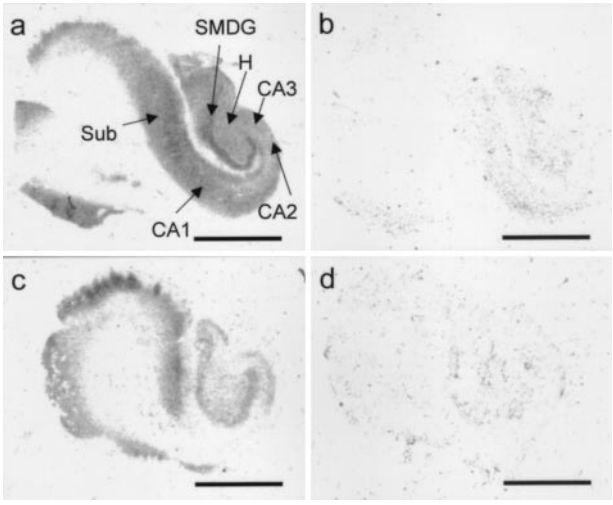

Figure 3 Autoradiographic images showing the total binding of 50 nM [^{3}H]-GABA (in the presence of 40 μM isoguvacine) to GABA$_B$ receptors in 10 μm frozen sections of human hippocampus from (a) a post-mortem control (neurologically normal) and (c) a TLE patient resection sample. An apparent loss of GABA$_B$ receptors can be seen throughout the 'epileptic' hippocampus (c) relative to control (a), with the most profound deficit being in region CA1. Non-specific binding in the presence of 40 μM isoguvacine and 100 μM (−)baclofen is shown in panels (b) and (d). H, hilus; SMDG, stratum moleculare dentate gyrus; Sub, subiculum. Scale bar represents 4 mm.

GABA$_B$ receptor affinity

The affinity of [^{3}H]-GABA at GABA$_B$ receptors increased in CA3 and hilus of HS tissue, as compared with post-mortem controls with significant decreases in K_D value to 51±9% ($P<0.01$) and 64±10% ($P<0.05$) of control respectively (Figure 5).

Relationship between GABA$_B$ receptor density and neuronal density

Data for GABA$_B$ receptor density and neuronal density were used to give an assessment of [^{3}H]-GABA binding to GABA$_B$ receptors per remaining neurone (Figure 6). This analysis indicated that there was a significant upregulation of GABA$_B$ receptor binding per neurone in hippocampal subregion CA1 of HS cases as compared with post-mortem controls (to 209±38% of control, $P<0.05$). GABA$_B$ receptor binding was not significantly altered in other hippocampal regions (Figure 6).

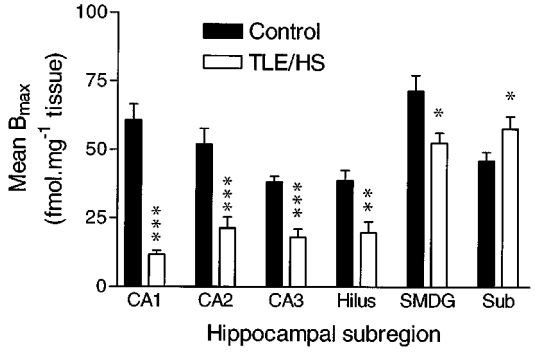

Figure 4 Mean changes in receptor population (B_{max}) in resected epileptic hippocampal tissue ($n=9-11$) as compared with post-mortem control hippocampal samples ($n=8$). Data are represented as mean±s.e.mean. Statistical analysis used unpaired Student's *t*-test (2-tailed), where $*P<0.05$; $**P<0.01$ and $***P<0.001$. SMDG, stratum moleculare dentate gyrus; Sub, subiculum.

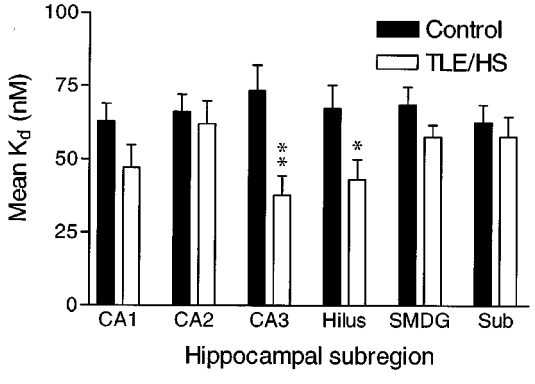

Figure 5 Mean changes in receptor affinity (K_D) in resected epileptic hippocampal tissue ($n=9-11$) as compared with post-mortem control hippocampal samples ($n=8$). Data are represented as mean±s.e.mean. Statistical analysis used unpaired Student's *t*-test (2-tailed), where $*P<0.05$. $**P<0.01$. SMDG, stratum moleculare dentate gyrus; Sub, subiculum.

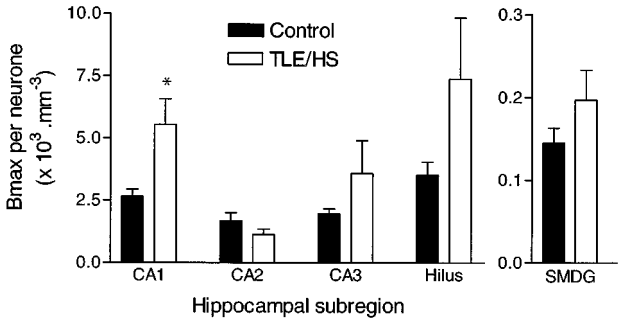

Figure 6 Receptor population expressed as a ratio of neuronal density in resected epileptic hippocampal tissue ($n=5-10$) as compared with post-mortem control hippocampal samples ($n=7-8$). Data are represented as mean±s.e.mean. Statistical analysis used unpaired Student's *t*-test (2-tailed), where $*P<0.05$. SMDG, stratum moleculare dentate gyrus.

Discussion

The present study shows a loss of GABA$_B$ receptor binding in all hippocampal subregions examined except for the subiculcum. The decreases in B_{max} seen were mirrored by the loss of neurones in these regions. However, in CA1 there appeared to be a significant upregulation of GABA$_B$ receptors when adjusted for neuronal loss. An increase in the affinity of [^{3}H]-GABA for GABA$_B$ receptors in CA3 and hilus was also observed.

The distribution of GABA$_B$ receptors in the human hippocampal formation confirms previous autoradiographic findings with [^{3}H]-GABA (Chu *et al*., 1987). Despite large increases in glial cell populations associated with HS, a similar increase in GABA$_B$ receptor binding was not observed in the present study. This suggests that GABA$_B$ receptors are not be glial, as previously reported (Kaupmann *et al*., 1997), though cultured astrocytes have demonstrated GABA$_B$ binding (Hosli & Hosli, 1990). However, an increase in GABA$_B$ receptor population was seen in the subiculum, though cell counts were not taken in this region. The study of Glass *et al*. (1996), reported neurones were not depleted in the subiculum of HS patients. This implies an increase in GABA$_B$ receptors possibly at both pre- and/or postsynaptic locations, in the subiculum in the present study, provided that tissue shrinkage is limited in this region. This could be part of a mechanism to reduce secondary generalization of the seizure from the hippocampus, and increased presynaptic receptors would contribute to the control of this mechanism. This increase in receptor protein is supported by an increase in GABA$_{B(1)}$ mRNA in the subiculum of the same patients and controls (Billinton *et al*., 2000). The decrease in binding observed in the stratum molecular of the dentate gyrus, could result from a combination of granule cell loss, and a widening of the stratum moleculare, effectively dispersing the binding present.

There are caveats associated with the use of PM human tissue, which should be considered alongside the interpretation of data arising from such studies. Factors such as agonal state and post-mortem interval (PMI) may affect binding parameters. Sample storage times of 4–10 years have been reported to influence GABA$_B$ stimulated GTPase activity, which may affect agonist binding (Odagaki *et al*., 1998). All

PM samples used in the present study were stored for less than 4 years. However, [^{3}H]-GABA binding in human tissue has been shown to be independent of age, PMI, sex or storage time (Lloyd & Dreksler, 1979). The possibility of this effect could be avoided by the use of an antagonist radioligand for GABA$_B$ receptors, but this does not give an indication of receptor functionality, as agonist binding does. Further studies of HS/TLE and control tissue using a GABA$_B$ receptor antagonist radioligand are planned. The difference in median ages of the two groups is difficult to control, and this has to be considered when comparing groups with different median ages. GABA$_B$ receptor binding in rat brain is reported to reduce over the first few months of life, but then remain fairly constant throughout adulthood (Turgeon & Albin, 1994). Several groups report altered GABA$_B$ receptor function with age, such as decreased sensitivity of rat hippocampal GABA$_B$ receptors to pertussis toxin (Knott *et al*., 1993), and decreased GABA$_B$ mediated late IPSP in rat hippocampal neurones (Billard *et al*., 1995). This last observation may relate to the functioning of specific subsets of hippocampal interneurones with ageing, but this has yet to be clarified.

Increases in affinity of [^{3}H]-GABA for GABA$_B$ receptors in CA3 and hilus of HS/TLE cases as compared with controls could be explained by decreases in affinity associated with the older control group. Decreases in affinity of [^{3}H]-baclofen with age have been reported in mouse cerebellum (Ishige, 1995), as has a decrease in [^{3}H]-GABA affinity with age for rat brain GABA$_B$ receptors (Turgeon & Albin, 1994). More concentrations on the saturation curve would provide a more accurate estimate of affinity. Also, a shift in the ratio of pre- and postsynaptic GABA$_B$ receptors in HS patients could influence measures of affinity, particularly as the affinity of GABA differs at pre- and postsynaptic GABA$_B$ receptors (Pitler & Alger, 1994; Pozza *et al*., 1999). The patients whose tissue was used in the present study were all undergoing chronic anti-epileptic drug therapy, which have not been reported to interact directly with GABA$_B$ receptors. However, the indirect effect of such compounds on GABA$_B$ receptor binding is unknown. It has been shown, however, that chronic carbemazepine does not effect GABA$_A$ or benzodiazepine binding in rat brain sections (Clark *et al*., 1994). It is not possible to obtain tissue from epilepsy patients not chronically taking anti-epileptic therapy.

The apparent upregulation of GABA$_B$ receptors associated with remaining neurones in CA1 of the HS/TLE group is in contrast to the findings of Hand *et al*. (1997) which showed flumazenil binding to benzodiazepine receptors to be reduced over and above the loss of neurones in CA1 of HS/TLE cases. This may be explained by the existence of interneurones selectively expressing GABA$_A$ or GABA$_B$ receptors (Lacaille *et al*., 1992; Mody *et al*., 1994; Nurse & Lacaille, 1997). GABAergic interneurones are reported to be preserved in region of the sclerotic hippocampus (Babb *et al*., 1989), and therefore selective preservation of specific interneurones could account for apparent changes in binding. This increase in binding is mirrored by an increase in GABA$_{B(1)}$ mRNA level in CA1 of the same patients and controls (Billinton *et al*., 2000). This still does not preclude the possibility of increased receptor expression on afferent terminals, or as a result of neuronal sprouting. Animal models of TLE report reductions in pre- and postsynaptic GABA$_B$ receptor mediated effects (see Introduction). The present study is not predictive of specific impairments, as pre- and postsynaptic specific GABA$_B$ receptor ligands are not currently available. Changes in these receptor populations would have different functional consequences for neuronal excitability. Increases in presynaptic GABA$_B$ receptor populations would affect the release of GABA and could impair inhibitory responses, whereas increases in postsynaptic GABA$_B$ receptors could prolong the inhibitory effects of GABA on postsynaptic neurones. Also, signal transduction mechanisms beyond the level of the receptor may be compromised, rather than impairments being at the level of the receptor. For example, the Ca^{2+} binding protein calbindin-D$_{28k}$ shows reduced expression in the granule cells of TLE cases (Magloczky *et al*., 1997).

GABA$_B$ receptors function as a heterodimer of GABA$_{B(1)}$ and GABA$_{B(2)}$ proteins (Kaupmann *et al*., 1998; Jones *et al*., 1998; White *et al*., 1998), and GABA binding is only reported to occur at GABA$_{B(1)}$. Thus modulation of the expression or association of the components of the heterodimer could influence the GABA$_B$ receptor population, or may account for increases in affinity observed in the HS/TLE group. Further investigations into these possibilities are currently being undertaken to clarify these issues.

In summary, the present study has shown an overall loss of GABA$_B$ receptor binding in the HS/TLE hippocampus when compared with neurologically normal PM controls. However, when accounting for the pattern of cell loss associated with HS, the density of GABA$_B$ receptors associated with remaining neurones is increased in CA1. This may be the result of the selective preservation of GABA$_B$ receptor specific expressing interneurones. Increases in affinity of [^{3}H]-GABA for GABA$_B$ receptors, and increases in receptor population may be influenced by the association of GABA$_{B(1)}$ and GABA$_{B(2)}$ proteins to form a heterodimer. Investigation of these possibilities should provide important information relating to the pathogenesis of HS/TLE.

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