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Distinct expression patterns of four GABARA subunits in human epileptic cortex and low-grade glioma

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Abstract

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain. Dysfunction in GABA receptor type A ($GABAR_A$) signalling has frequently been implicated in a number of different pathologies. The functionality of the ionotrophic $GABAR_A$ is dependent on its subunit composition. Whilst its involvement in epilepsy disorders is well reported, recent evidence suggests that it may also be involved in tumour development, due proliferative activity. This study investigated the expression of four $GABAR_A$ subunits in human epileptic cortex and low-grade gliomas. Tissue microarrays were prepared from 38 patients with a diagnosis of medically intractable focal epilepsy and 56 patients with a diagnosis of WHO grade II glioma. *In vivo* Immunohistochemical screening was carried out using antibodies for $GABAR_A$ subunits α1, θ, ρ2 and γ1 to determine their distribution in different lesion and glioma types. Subunits were expressed in tumour cells of gliomas, but were not expressed in the glial component of benign WHO grade I gliomas. High expression of θ and ρ2 subunits was found in gemistocytic glioma types, and the expression of these subunits was positively correlated ($R(56)=0.46,\ p<0.001$). These results suggest a role for $GABAR_A$ in glioma development, and implicate specific subunits in this process.

Introduction

The γ-aminobutyric acid receptor type A ($GABAR_A$) is a member of the cys-loop super-familly, and is an ionotrophic receptor, responding to GABA binding. It is normally composed of five subunits and is expressed throughout the central nervous system, functioning as one of the key mediators of cortical inhibition in the brain. So far there have been 19 different subunits identified – six α, three β, three γ, one δ, one ε, one π, one θ, and three ρ (Sieghart 2006). The receptor is activated by GABA binding to the extracellular domain at the interfaces between subunits α and β or θ (depending on subunit composition), causing the channel pore to open and permitting negative ions (primarily chloride) to cross the membrane (Kaila *et al.* 1993, Ernst *et al.* 2003). This is essential for controlling the membrane potential of cells, giving rise to phasic inhibition from synaptic $GABAR_A$, and tonic inhibition from extrasynaptic $GABAR_A$.

Certain subunits have been shown to be confined to specific regions of the brain, whilst others are expressed more uniformly (Pirker *et al.* 2000). Receptor subunit composition has also been shown to affect many features, such as allosteric binding sites for drugs, electrophysiological profile (Sigel *et al.* 1990, Fisher and Macdonald 1997), and sensitivity to GABA (Bohme *et al.* 2004, Minier and Siegel 2004). 17-20% of all neurones in the brain are GABAergic, so even rarer combinations of subunits will be expressed in a relatively large number of cells (Somogyi *et al.* 1998).

Considering the abundance of each $GABAR_A$ subtype in the brain it is remarkable how limited our understanding is of many of these different compositions.

The most common $GABAR_A$ in the brain is formed from two α, two β, and two γ subunits, of which the α1, β2, γ2 receptor occurs the most frequently. δ, ε and π subunits have been found to take the place of the γ subunit when forming functional channels, and the θ subunit has been found to take the place of β subunits (Sieghart 2006). ρ subunits form homomeric receptors previously known as $GABAR_C$ channels, though there are studies to suggest they can also form channels with other subunits (Ekema *et al.* 2002, Pan and Qian 2005).

Role of $GABAR_A$ in epilepsy

Epilepsy is a group of conditions characterised by an imbalance between excitatory and inhibitory transmission in the brain, whereby seizure activity, in the form of uncontrolled electrical firing, spreads throughout the brain or a region of the brain. These seizures are often classified as either generalized, in which the source of the seizure is distributed, or focal, in which it is localised. As the most common inhibitory neurotransmitter in the brain, GABA is essential for maintenance of inhibitory inputs. Extrasynaptic $GABAR_A$ is responsible for tonic inhibition, which helps maintain the overall level of excitability of local neurones. These channels respond to nM concentrations and mediate slow inhibitory post-synaptic currents due to continuous activation by ambient GABA (Semayanov *et al.* 2004, Belelli *et al.* 2009).

Dysfunction in this process is frequently postulated as being an underlying factor in epilepsy pathogenesis, due to loss of tonic inhibition, particularly from GABAergic interneurons (Olsen and Avoli 1997, Avoli *et al.* 2005). In focal epilepsy, this loss is commonly due to the cortical restructuring that occurs as a result of a lesion in the brain. Epileptiform activity originates in the perilesional cortex, where altered ion homeostasis and disrupted neurotransmitter transport alters the excitation/inhibition balance (Wolf *et al.* 1996, Shamji *et al.* 2009). Lesions that can lead to these sorts of changes can be due to a variety of changes, such as developmental anomalies, tumour development, scarring and sclerosis. However, more recent research suggests that $GABAR_A$ may not play such a straightforward role in these lesions, due to discoveries of its more complex roles in the cortex.

Treatment is normally possible with anti-epileptic drugs, such as benzodiazepines. However, in some cases, these drugs are not effective, and resection of the lesion may be an alternative treatment, particularly for those with focal seizures (Elger 2002, Gelziniene *et al.* 2008). $GABAR_A$ subunit composition may also be altered by the cortical restructuring that occurs, changing the pattern of tonic inhibition in the epileptogenic zone (Wolf *et al.* 1994). This can lead to expression of $GABAR_A$ with different allosteric binding sites that have decreased sensitivity to anti-epileptic drugs (Sata *et al.* 2002).

Role of $GABAR_A$ in low-grade gliomas

Of those cases diagnosed with medically refractory focal epilepsy, 12-51% are caused by low-grade gliomas, and in fact, pharmacoresistant epilepsy is commonly the first identifiable symptom of a low-grade glioma (Bartolomei *et al.* 1997). Gliomas are tumours of glial origin, and those that are considered “low-grade” are those classified as Grade I or II by the WHO, with lower proliferative activity and lower malignancy (Louis *et al.* 2007). As a result, the average survival after diagnosis can range from months to many years. Another means of classifying gliomas is by the type of glial cell they originate from. Some gliomas originate from only one cell type, such as oligodendrogliomas which originate from oligodendrocytes, and astrocytomas which originate from astrocytes. There are also gliomas of mixed glial cell origin, such as oligoastrocytomas which

originate from both oligodendrocytes and astrocytes. Some gliomas may contain swollen, reactive tumour cells with eccentric nuclei. These can be either gemistocytes or minigemistocytes depending on their precise morphology, and tumours containing a large proportion of these cells are known as gemistocytic astrocytomas or minigemistocytic oligoastrocytomas (Louis *et al.* 2007).

Since the gliomas are composed primarily of electrically inert glial cells, the seizure activity in these cases is also assumed to originate in neuronal populations in the peritumoural region, where the infiltrating tumour cells disrupt the arrangement of glial cells and their connnexons (Chernov *et al.* 2005), and abnormal migration leads to neurones being found in the white matter (Goldring *et al.* 1986). Mass effect of the tumour and the resulting hypoxia due to decreased perfusion has been proposed as another mechanism of tumour epileptogenesis (Wick *et al.* 2005), although this does not account for the fact that more rapidly proliferating, high-grade tumours correlate with a lower incidence of epilepsy (Moots *et al.* 1995, Ruda *et al.* 2010).

The tumour cells themselves may also express transporters or receptors that further alter the normal signalling - Aronica *et al.* (2001) found that both ionotropic (iGluR) and metabotropic glutamate (mGluR) receptors were overexpressed in glioneuronal tumours of epilepsy patients, and other studies have supported this finding (Lee *et al.* 2006). It has even been suggested that mGluR activation could lead to down-regulation of $GABAR_A$ via second messengers (Rodriguez-Moreno *et al.* 1997). This would therefore be expected to disrupt the balance of excitation in the surrounding tissue.

However, some studies have shown decreased immunohistochemical (IH) staining in GABAergic neurones (Haglund *et al.* 1992), whilst others have found no change in the level staining between peritumoural cortex and normal cortex (Wolf *et al.* 1995). It appears now that the role of $GABAR_A$ in tumour-associated epilepsy (TAE) is more complex than previously thought, since in some cases its activation can lead to depolarization of cells. Chloride homeostasis can be disrupted in tumour pathology, allowing tumour cells to retain abnormally high intracellular concentrations of chloride ions $[Cl^-]_i$ via manipulation of chloride cotransporters (Sontheimer 2008). This increases the reversal potential for chloride ions, so that upon activation, $GABAR_A$ generates a depolarizing current, rather than the normal hyperpolarizing current, and this process can be seizure productive (Ransom *et al.* 2001, Aronica *et al.* 2007, Kahle *et al.* 2008, Conti *et al.* 2011). A number of studies have shown that $GABAR_A$ has much lower expression, or even a complete lack of expression, in high-grade gliomas, compared to low-grade gliomas (Jussofie *et al.* 1994, Labrakakis *et al.* 1998, Synowitz *et al.* 2001). This supports the theory of increased $[Cl^-]_i$, since lower expression of $GABAR_A$ in high-grade gliomas is not correlated with a higher incidence of epilepsy, as would be expected if $GABAR_A$ activity was inhibitory (Cascino 1990, Herman 2002, Ruda *et al.* 2010).

This depolarizing action of $GABAR_A$ has been proposed to play a further role in tumour pathology, since studies have reported its involvement it in proliferation (Fiszman *et al.* 1999, Liu *et al*. 2005, Goffin *et al.* 2008). High $[Cl^-]_i$ in the normal brain is therefore a unique feature of proliferating cell populations, and it is possible that $GABAR_A$ activity may have some involvement in driving trophic factors of proliferating gliomas. The differential expression of $GABAR_A$ between high and low grade gliomas also suggests a link to tumour growth.

Preliminary research by Z. Jin and B. Birnir (unpublished obervations) found differential expression of a number of $GABAR_A$ subunits in gliobastoma multiforme samples (high-grade glioma) and in grade II (low-grade) glioma samples, indicating that certain subunits were expressed to a significantly greater degree in the grade II glioma samples. The four subunits with the greatest difference in expression (α1, θ, ρ2 and γ1) were used as the focus of this experiment.

In this study, the pattern of expression of $GABAR_A$ subunits α1, θ, ρ2, and γ1 were investigated by quantitatively and qualitatively assessing immunohistochemical (IH) staining of tissue microarrays (TMAs) containing samples taken from different human epileptic cortex lesions and perilesional areas (*epilepsy* samples) and samples taken from different WHO grade II gliomas (*tumour* samples). It is the first reported use of the θ antibody for IH staining.

Methods

Tissue samples

1. *Epilepsy samples:* Lesion or perilesional tissue samples were taken from 38 patients who underwent neurosurgery at Uppsala University Hospital for medically refractory epilepsy between 1991 and 2005. Samples were used only after the patients' informed consent had been given. A final diagnosis was given after re-evaluation of the tissue samples by a neuropathologist (E. Aronica). The number of patients assigned to each diagnosis category is shown in Table 1. Gangliomas and dysembryoplastic neuroepithelial tumours are WHO Grade I gliomas. Astrocytomas and oligodendrogliomas are WHO Grade II gliomas.

Table 1: Neuropathological diagnosis of e*pilepsy* patients (n = 38)

Diagnosis	**Number of patients (%)**
Ganglioma (GG)	7 (18.4)
Dysembryoplastic Neuroepithelial Tumour (DNET)	2 (5.3)
Astrocytoma	2 (5.3)
Oligodendroglioma	1 (2.6)
Cortical Dysplasia	4 (10.5)
Gliosis	5 (13.2)
Hippocampal Sclerosis (HS)	10 (26.3)
Leptomeningeal Scarring (LS)	2 (5.3)
Malformations of Cortical Development (MCD)	1 (2.6)
Cavernous Hemangioma	4 (10.5)

2. *Tumour samples:* Tissue samples were taken from 111 patients who underwent neurosurgery at Uppsala University Hospital between 1982 and 1999 with a primary histopathological diagnosis of supratentorial WHO grade II glioma. Samples were used only after the patients' informed consent had been given. A final diagnosis was given after re-evaluation of the tissue samples by a neuropathologist (A. Orrego), leading to the exclusion of 44 patients who did not fit the criteria of tissue taken from the bulk of a supratentorial WHO grade II glioma. A further 11 patients were excluded after tissue preparation due to poor quality of the tissue samples. The number of patients assigned to each diagnosis category is shown in Table 2.

Table 2: Neuropathological diagnosis of *tumour* patients (n = 56)

Diagnosis	**Number of patients (%)**
Astroctyoma	22 (37.3)
Gemistocytic Astrocytoma	9 (15.3)
Minigemistocytic Oligoastrocytoma	3 (5.1)
Oligoastrocytoma	10 (16.0)
Oligodendroglioma	12 (20.0)

Clinical data was collected from patient records to include: date of operation, sex, age at symptoms onset (≤40 or >40), date of death, tumour size (≤6cm or >6cm), and epilepsy at diagonsis (yes or no). The clinical data collected from the patients is shown in Table 3.

Table 3: Percentage of *tumour* patients in each variable group (n = 56)

Grouping Variable	**Number of patients (%)**
Male	35 (62.5)
Female	21 (37.5)
Age at onset ≤40	32 (57.1)
Age at onset >40	24 (42.9)
Tumour size ≤6cm	40 (71.4)
Tumour size >6cm	16 (28.6)
Epilepsy at onset	46 (82.1)
No epilepsy at onset	10 (17.9)

Tissue microarray preparation

After surgery, epilepsy samples were fixed in 4% paraformaldehyde for 24 h and then paraffin embedded. Tissue microarrays (TMAs) were prepared by the Swedish Proteome Resource Centre (HPR) facilities at the Rudbeck laboratory, Uppsala University Hospital. Samples were sectioned, hematoxylin-eosin stained and treated with immunohistochemical stains. Donor blocks were then prepared, from which was taken one to two 1.0mm diameter punches by an automated tissue arrayer (Beecher Instruments, Silver Spring, MD) and transferred to the appropriate TMA block. Digital recordings of each TMA were taken using a Luminescent Image Analyzer, LAS-3000 (Fuji Film, Japan).

1. *Epilepsy samples:* For each lesion, an average of six to seven representative areas were chosen for each donor block, from which the punches were then taken and transferred to the appropriate TMA block. For each immunohistochemical stain, four TMA blocks consisting of 260 pieces of tissue were prepared.

2. *Tumour samples:* For each tumour, punches were taken from one representative area and transferred to the appropriate TMA block. For each immunohistochemical stain, two TMA blocks consisting of 185 pieces of tissue were prepared.

Immunohistochemical staining

Immunohistochemical (IH) staining was carried out as described by Qu *et al.* (2009), though different stains were used, as described below. Tissue sections were deparaffinised, hydrated in grade alcohol and microwave treated. IH staining was performed by avidin-biotin peroxidise staining technique (Vector elite) using 3,3 diaminobenzidine as a substrate. The following commercial antibodies against the following antigens were used as diagnostic markers by the neuropathologists in their final diagnosis:

1. *Epilepsy samples*: GFAP (1:500; Rabbit polyclonal, DAKO, Denmark), vimentin (1:80; Mouse clone V9, Sigma), neuronal nuclear protein (1:1000; Mouse clone MAB377, Chemicon, Temecula, CA), microtubule-associated protein (1: 100; Mouse clone HM2, Sigma), HLA-DR (1:30; Mouse clone Tal1b5, DAKO), CD68 (1:100; Mouse monoclonal, DAKO), neurofilament (1:20; Mouse monoclonal, Immunostain), Ki67 (1:500; Rabbit polyclonal, DAKO), synaptophysin (1:200; Mouse monoclonal, Novocastra), CD31 (1:50; Mouse monoclonal, DAKO) and CD34 (1:100; Mouse monoclonal, DAKO).
2. *Tumour samples*: GFAP (1:500; Rabbit polyclonal, DAKO, Denmark), Ki67 (1:500; Rabbit polyclonal, DAKO), microtubule-associated protein (1: 100; Mouse clone HM2, Sigma)

Staining was carried out with antibodies against the $GABAR_A$ subunits, of which only the α1 subunit was a commercial antibody: $GABAR_A$ α1 subunit (1:50; Mouse monoclonal, MAB339, Chemicon), $GABAR_A$ θ subunit (1:100; Rabbit polyclonal, HPA002063, Atlas Antibodies), $GABAR_A$ ρ2 subunit (1:75; Rabbit polyclonal, HPA016467, Atlas Antibodies), $GABAR_A$ γ1 (1:30; Rabbit polyclonal, HPA035622, Atlas Antibodies).

Evaluation of immunostaining and statistics

1. *Epilepsy samples:* TMAs stained for the four $GABAR_A$ subunits were examined at the University of Amsterdam Hospital by a neuropathologist (E. Aronica) and an associate (H. Pedder) using a light microscope. At the cellular level, each tissue type and subcellular location was recorded as either immunopositive or immunonegative for the presence of the stain at each position on the TMAs, and the pattern of staining was qualitatively assessed. Cellular tissue types encountered were neurones, glial cells, tumour cells and neuropil. Subcellular staining was recorded either as nuclear, or as membrane and cytoplasmic.

2. *Tumour samples:* Digital recordings of the TMAs stained for the four $GABAR_A$ subunits were examined on a computer using ImageScope (ScanScope, version 11.0.2, Aperio Technologies) under varying magnifications at the Karolinska Institute, Stockholm, by a researcher (T. Elsir) and an associate (H. Pedder). For each position on the TMAs, the intensity of staining was recorded from 0 (no staining) to 4 (weakest staining). For samples where there had been two punches taken, the mean of the scores was calculated. These "intensity values" were also used to calculate "absolute values", by treating values greater than 1 as immunopositive and values equal to or below 1 as immunonegative for the presence of the stain, to take into account the possible presence of artefacts. Absolute values were used in certain statistical analysis methods. Statistical analysis was carried out using SPSS Statistics (version 17.0, IBM) for Windows.

Results

Epilepsy Results

The general pattern of staining showed that strong immunoreactivity (IR) was found for the α1 subunit in almost all samples (Fig. 1A). The staining was in neuropil and in neurone membranes and cytoplasm, although neuropil staining was slightly weaker in hippocampal sclerosis (HS) samples. Weaker IR was found for the θ subunit in most samples, although strong staining was visible in the

nuclei of neurones in some (Fig 1B). Besides the nuclear staining, in samples in which IR was found, it was moderate in both the neuropil and in neurone membranes and cytoplasm. Strong IR was found for the ρ2 subunit in neurone membranes and cytoplasm for most samples, with moderate staining also in the neuropil of most (Fig 1C). Moderate IR was found for the γ1 subunit in most samples, located in neurone membranes and cytoplasm, though neuropil staining was entirely absent in all the samples (Fig. 1D). There was no distinctive difference in IR for any of the stains between lesion and perilesional samples. There was no IR found in glial cells in any of the tissue samples.

The overall pattern of IR is shown in Table 4, showing the general distribution of $GABAR_A$ subunit expression in the lesions and perilesional space.

Table 4: General pattern of immunoreactivity in tissue from *epilepsy* patients

$GABAR_A$ Subunit	**Immunoreactivity**		
	Neurones		**Neuropil**
	Nucleus	Membrane/cytoplasm	
α1	negative	Strong	strong
θ	strong	Moderate	moderate
ρ2	negative	Strong	moderate
γ1	negative	Moderate	negative

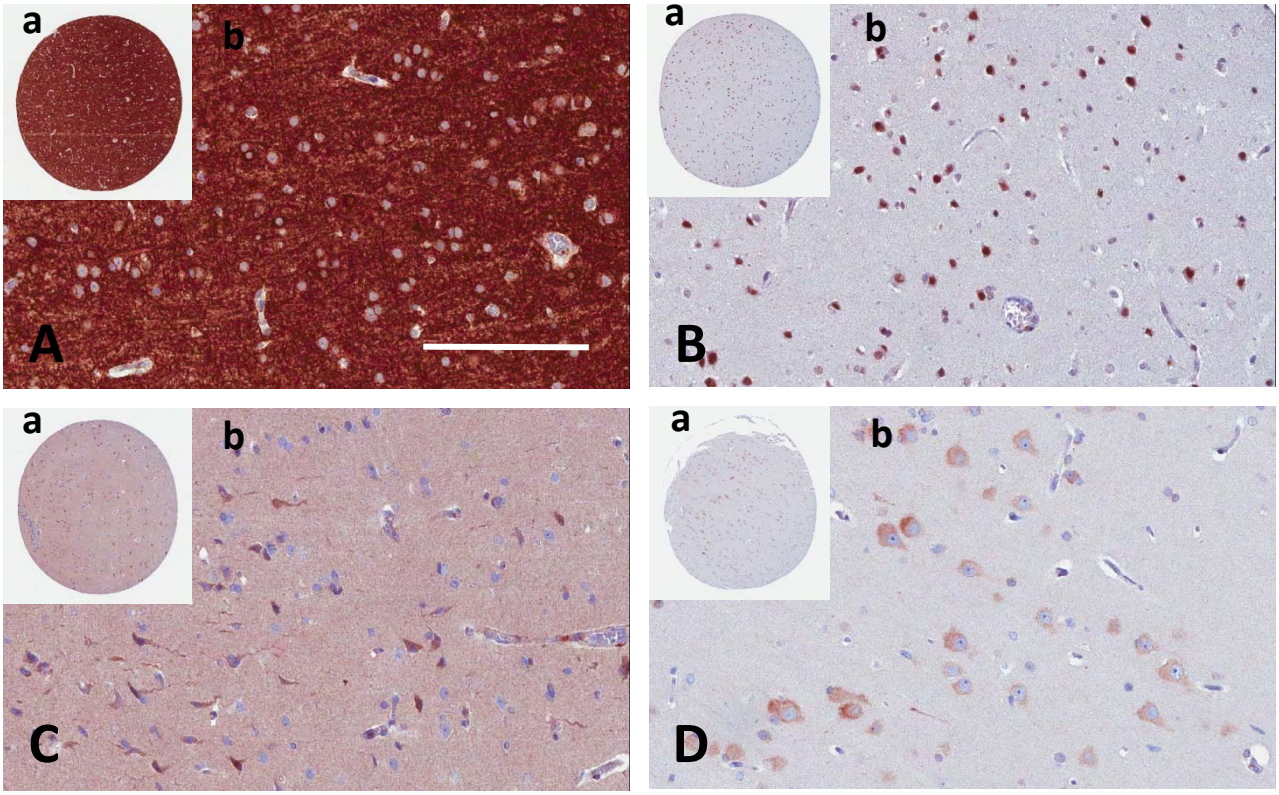

Figure 1: Photographs of tissue microarrays of tissue from within hippocampal sclerosis (HS), gliosis, and cavernous hemangioma (CH) lesions stained with antibodies for $GABAR_A$ subunits. Areas shown are representative of the general pattern of immunoreactivity for each stain. Sections are counterstained with hematoxylin. The white bar shown in A(b) represents 100µm. The diameter of the punches shown in (a) are 1000µm. **(A)** α1 subunit stain of tissue from a HS. Staining visible in neuropil and neuronal membranes and cytoplasm.**(B)** θ subunit stain of tissue from a gliosis. Staining visible in neuropil and neuronal membranes, cytoplasm and nucleus. **(C)** ρ2 subunit stain of tissue from a CH. Staining visible in neuropil and neuronal membranes and cytoplasm. **(D)** γ1 subunit stain of tissue from gliosis scarring. Staining visible in neurone membranes and cytoplasm.

IR for the ρ2 subunit was found in tumour cells in the astrocytoma samples, and IR for the γ1 subunit was found in tumour cells of oligodendroglioma samples, both of which are WHO Grade II gliomas. However, IR was not observed in glial cells of the WHO Grade I glioma samples despite being found in the neuronal component of the tumours, and was found in only one gliosis sample, despite being expressed in neurones.

Tumour results

The general IR for each subunit was similar to that found for the *epilepsy* samples, with α1 showing the strongest and γ1 the weakest. ρ and θ subunits showed similar levels of IR on the whole, and again, θ IR was primarily located in the nuclei.

Of the 56 samples, IR for at least one of the subunits was found in 50 samples. The relative expression of each subunit is shown for each tumour type in Table 5, indicating that the α1 subunit was expressed the most frequently, followed by the θ subunit, then the ρ2 subunit, and finally the γ1

subunit. In astrocytoma, oligoastrocytoma and oligodendroglioma, the α1 subunit was expressed considerably more often in samples than any of the other subunits, but in gemistocytic astrocytoma and minigemistocytic oligoastrocytoma the frequencies of both θ and ρ2 subunits were higher. This pattern was most visible in minigemistocytic oligoastrocytoma, where both θ and ρ subunit IR was positive in all samples, compared to α1 and γ1 subunit expression in only 33% of samples (Fig. 3). For the α1 subunit, this was much lower than in other tumour types, whilst for the γ1 subunit this was the tumour type in which the γ1 subunit was expressed most frequently, since no IR was visible in any gemistocytic astrocytoma or oligoastrocytoma samples. For the θ subunit the frequency of expression was lowest in oligoastrocytoma samples, where positive IR was found in only 10% of samples immunopositive for $GABAR_A$ subunits, considerably lower than in other tumour types. For the ρ2 subunit it was lowest for astrocytoma samples.

Table 5: Relative expression of $GABAR_A$ subunits α1, θ, ρ2 and γ1 in different WHO grade II gliomas, calculated as the number of samples with positive immunoreactivity (IR) for each subunit divided by the total number of samples in which positive IR for any $GABAR_A$ subunit was found. (n = 50) *Calculations were done using absolute expression values.*

Glioma Type	**$GABAR_A$ Subunit**			
	α1	**Θ**	**ρ2**	**γ1**
Astrocytoma	86%	57%	10%	19%
Gemistocytic astrocytoma	50%	83%	67%	0%
Minigemistocytic oligoastrocytoma	33%	100%	100%	33%
Oligoastrocytoma	80%	10%	30%	0%
Oligodendroglioma	90%	50%	20%	20%
Total	78%	52%	28%	14%

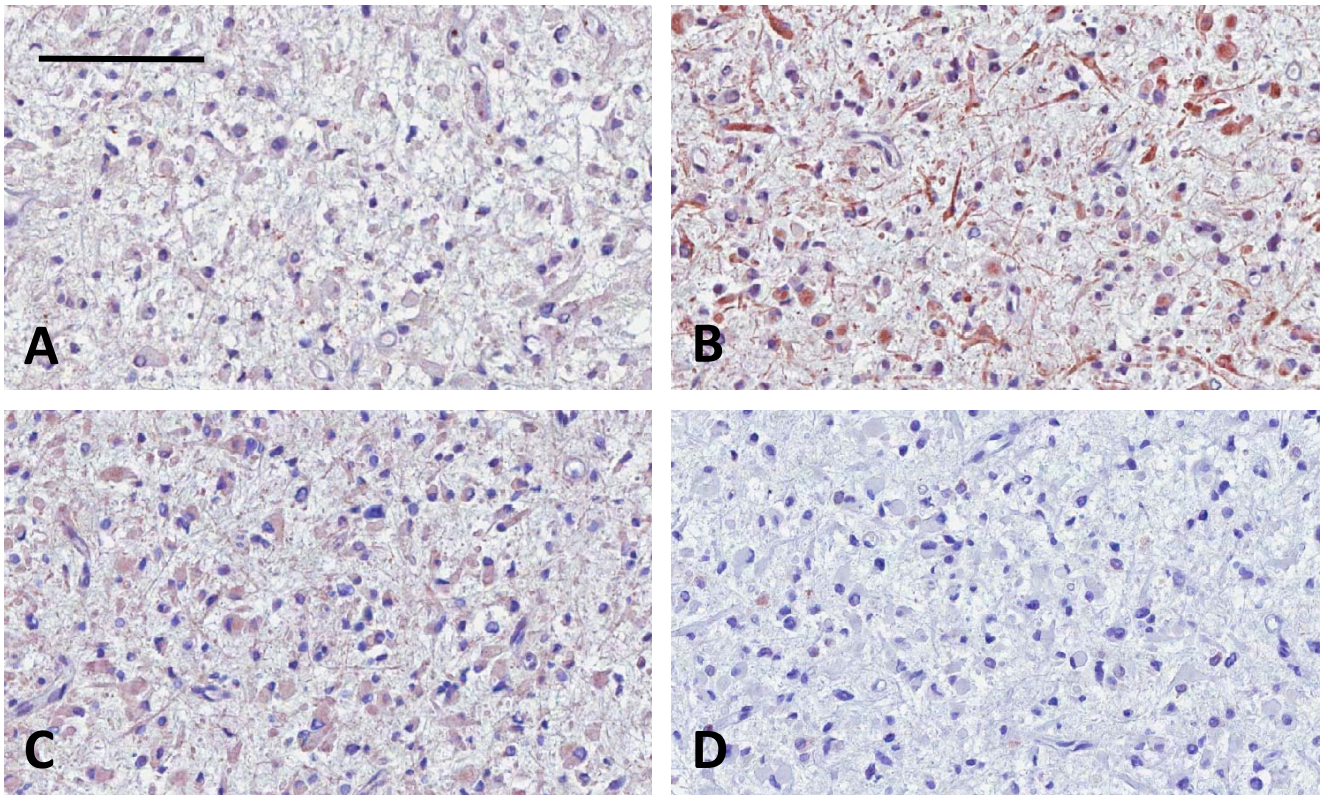

Figure 3: Photographs of tissue microarrays (TMAs) of tissue from within a gemistocytic astrocytoma stained with antibodies for $GABAR_A$ subunits. Sections are counterstained with hematoxylin. TMAs shown are all taken from the same glioma sample. The black bar shown in A represents 100µm. **(A)** α1 subunit staining. Positive immunoreactivity (IR) can be seen, though it is much weaker than in other α1 subunit stained samples. **(B)** θ subunit staining. Positive IR is clearly visible and is strong. **(C)** ρ2 subunit staining. Positive IR can be seen that is of moderate strength. **(D)** γ1 subunit staining. No IR is visible.

A correlation analysis was performed on the intensity expression values to see if there was any consistent variation in IR of the subunits. A highly significant positive correlation was found for the expression of the θ and ρ2 subunits (*R(56)=0.46, p<0.001*), though with a Pearson *R* value of 0.46 the correlation was only of moderate strength. No other two subunits were significantly correlated, even when investigating correlations in specific tumour types.

Mann Whitney-U tests were carried out using intensity expression values to compare the means between the two groups for each patient data variable (Table 4). No significant difference was found in subunit expression between groups in any of the four variables for which data were available.

Discussion

In this study, immunohistochemical (IH) staining of TMAs was used to assess the pattern of $GABAR_A$ subunit expression in epileptic cortex lesions and WHO grade II glioma tissue. The subunits investigated were α1, θ, ρ2 and γ1. This is the first reported study to assess the distribution of individual $GABAR_A$ subunit expression in human brain tumour tissue, and is also the first study to

report the use of the θ subunit antibody in IH staining. Although little was found to suggest differential subunit expression between lesions and perilesional areas, patterns of expression of subunits were found in different types of tumours, with some subunits being predominantly expressed in some tumour types more than in others. These results suggest a link between $GABAR_A$ expression and glioma morphology, providing direction for further research into $GABAR_A$ expression in brain tumours.

$GABAR_A$ and epilepsy

The subunits that form $GABAR_A$ give the channel different biochemical and biophysical properties, such as varying GABA sensitivity, chloride conductance, and rate of activation and desensitization (Fisher and Macdonald 1997, Lavoie *et al.* 1997, Brickley *et al.* 1999, Brown *et al.* 2002, Bohme *et al.* 2004). Localisation of the receptor also affects subunit composition, both within different brain regions and within different regions of cell membranes (Farrant and Nusser 2005). Disruption in GABA signalling is thought to play a role in epilepsy pathology, due to its importance as the major inhibitory signalling mechanism in the brain (Avoli *et al.* 2005). Studies have found the presence of seizure characteristics in a number of autosomal dominant mutations in $GABAR_A$ subunits (Baulac *et al.* 2001, Bowser *et al.* 2002, Dibbens *et al.* 2004, Audenaert *et al.* 2006, Maljevic *et al.* 2006, Lachance-Touchette *et al.* 2011). This lends support to the theory of GABAergic disruption in epilepsy, and the anti-seizure action of benzodiazepines via binding to $GABAR_A$ is further evidence for this. A novel area of research in GABA-associated epileptogenesis is the disruption in chloride homeostasis, which is thought to lead to the depolarizing activity of $GABAR_A$. It has also now been proposed that this could be a potential mechanism for tumour proliferation.

Due to the many different subunits and subtypes of $GABAR_A$ that can be formed, a consistently altered state of the receptors in specific pathologies is difficult to ascertain. Of the four subunits studied in this experiment, the α1 subunit was expressed most frequently, as has already been reported to be the case in most areas of the normal brain (Laurie et al. 1992, Wisden et al. 1992, Pávai et al. 2010). In *epilepsy* samples, no changes were found between lesion and perilesion expression of the subunit, contrary to what might have been expected, since previous research describes a general decrease in α1 subunit immunoreactivity (IR) in the perilesion compared to normal tissue (Wolf *et al.* 1996). However, since the comparison in this study was a comparison to lesion tissue, rather than to normal tissue, it is possible that the changes in IR described by Wolf *et al.* occur in both the lesion and the perilesion, thus explaining the similar pattern of expression found here, and there is no previous data on lesion and perilesional differences in expression of the other three subunits we investigated.

A slight reduction in α1 subunit IR was also found in the neuropil of hippocampal sclerosis (HS) samples. This is similar to results from studies of hippocampal specimens, which found highly significant reductions in α1 subunit IR, though this reduction was closely related to neurone loss, suggesting that it may be a secondary cause of this loss, rather than a mechanism for epileptogenesis (Wolf *et al.* 1994, Fritschy *et al.* 1998, Loup *et al.* 2000). Although it is not clear from these studies if loss of neurones was selective for those expressing the α1 subunit compared to other α subunits, Brooks-Kayal *et al.* (1999) did find a marked decrease in α1 subunit expression relative to other α subunits, suggesting that α1-expressing neurones may die more rapidly than others. Strong evidence for the importance of the α1 subunit in inhibition is shown by Raol *et al.* (2006), who found that enhanced α1 subunit expression in the dentate gyrus reduced the number of rats developing epilepsy after pilocarpine induced seizures. Mutations in the α1 subunit are also a common cause of familial epilepsy (Maljevic *et al.* 2006, Lachance-Touchette *et al.* 2011).

However, other studies in both humans and in seizure-induced animal models have reported no change in overall α1 subunit expression in the hippocampus (Poulter *et al.* 1999, Loup *et al.* 2006). Loup *et al.* (2009) reported a disorganized network of α1-positive white matter neurones

in a number of patients with temporal lobe epilepsy (TLE), a form of focal epilepsy, suggesting that the disruption to the GABA system in the hippocampus may be more in the organisation of α1-expressing neurones, partly via death of α1-expressing interneurones, resulting in disruption of the inhibitory networks themselves, rather than disruption of $GABAR_A$ functionality.

Decreases in α1 subunit expression would also potentially be an important factor in medically refractory epilepsy, due to its role in providing benzodiazepine binding sites with relatively high binding affinity. Studies have shown that receptor complexes formed from α1 subunits have a higher binding affinity for zolpiderm than other α subunits, with such a strong difference that receptors containing α subunits have been classified as type I or type II $GABAR_A$-benzodiazepine receptors (Pritchett *et al.* 1989). Thus, α1 subunit expression reduction *relative* to the expression of other α subunits, as found by Brooks-Kaval *et al.* (1998), could help provide an explanation for forms of pharamcoresistant epilepsy, particularly since decreased sensitivity to benzodiazepine-binding site 1 (BZ1) agonists has been correlated to the decrease in α1 subunit expression (Brooks-Kayal *et al.* 1999).

Whilst the α1 subunit has frequently been the focus of studies, due to its abundance in the normal brain and its reliability of measurement (Laurie et al. 1992, Wisden et al. 1992), there has been much considerably less research on θ, ρ2 and γ1 subunit expression. For all *epilepsy* samples except for astrocytoma, dysembryoplastic neuroepithelial tumour (DNET) and ganglioma (GG) in this study there was no pattern in the differences in θ, ρ2 and γ1 subunit expression between different lesion types, or between lesion and perilesion tissue. Studies have reported that during epileptogenesis, γ2 subunit expression changes from synaptic to extrasynaptic to replace δ subunit expression (Zhang *et al.* 2007), and the resulting receptor has decreased sensitivity to GABA, reducing its capacity for inhibition (Saxena and Macdonald 1994). This change could be considered to be a key mechanism in disruption of GABAergic signalling. However, there have been no studies suggesting that γ1 subunit expression is altered by a similar means.

Relative γ1 subunit expression was found to be unchanged in dentate granule neurones of epileptic rats compared with controls (Brooks-Kayal *et al.* 1998) and, after kainite-induced seizures in rats, Tsunashima *et al.* (1997) found no difference in γ1 subunit expression in the stratum of hippocampi. One study found high levels of α1 and γ1 subunit expression in paediatric TLE patients relative to other subunits measured, also reporting very low, yet quantifiable, relative expression of the θ subunit (Porter *et al.* 2005). Whilst the same, strong α1 subunit expression was found in our study, γ1 expression was found to be the weakest of the four subunits that were studied, with θ subunit expression stronger in all subcellular locations. This finding may also be considered to be unusual, since θ subunit expression has previously been reported as being fairly low (Bonnert *et al.* 1999).

In the normal brain, functional $GABAR_A$ channels containing a θ subunit are formed from α, β and γ subunits, though exact possible combinations are not fully understood (Bonnert *et al.* 1999). It has a similar genetic sequence to the β subunit, yet studies show that it cannot fully provide the same functionality, due to the benzodiazepine insensitivity of θ-containing receptors (Ranna *et al.* 2006). Due to the limited investigations into this subunit, it was unexpected to find such high expression in this study. In *tumour* patients θ subunit expression was found in 52% of samples in which $GABAR_A$ was expressed, a much higher value than reported previously by Bonnert *et al.* (1999) in healthy patients, where it was expressed in only 20% of receptors. As this study was the first reported use of the θ subunit stain for IH, it is difficult to find sufficient data to which to compare results, and it is therefore possible that much of the positive staining found in this study was due to artefacts. However, the Swedish Human Protein Atlas Project (2011) also found strong nuclear staining when investigating θ subunit expression in various other tissues, though the stain was given an "uncertain" rating for reliability in tissue staining due to the lack of supportive data.

Nuclear staining for a $GABAR_A$ subunit was unexpected, due to functional $GABAR_A$ channels operating only within the plasma membrane. However, it is possible that the θ expression found in this study does not produce a functional receptor, but plays a different role. The α1 subunit has

been shown to be expressed independently of functional $GABAR_A$, where it promotes apoptosis (Vaknin and Hann 2006), and it may be possible that other subunits have similar independent functions. Future studies must be carried out using this stain to determine its efficacy, as well as to further understand the role of the θ subunit, before any key conclusions can be drawn from these results.

ρ2 subunit expression was found in a large number of samples in this study. Whilst previous studies indicate that the ρ1 subunit is predominantly expressed in the retina and visual pathways, ρ2 expression has also been found in other areas of the CNS, such as the hippocampus and amygdala (Enz *et al.* 1995, Wegelius *et al.* 1998, Liu *et al.* 2004, Alakuijala *et al.* 2005, Rosas-Arellano *et al.* 2011). ρ subunits generally form heteromeric $GABAR_A$-ρ (previously known as $GABAR_C$ channels) together *in vivo*, though there has been some evidence suggesting that they may also be able to form homomeric receptors, due to expression studies and homomeric interaction signal findings (Bormann and Feigenspan 1995, Hackam *et al.* 1997, Enz and Cutting 1999, Pan *et al.* 2006). Due to the absence of other $GABAR_A$ subunits in these receptors, they have unusual functional properties, such as slow desensitization and insensitivity to many $GABAR_A$ ligands (Feigenspan *et al.* 1993). Whilst most studies have shown that they cannot form functional $GABAR_A$ channels with other subunits, there has been evidence for coassembly of ρ1 with α1 subunits in brainstem neurones (Milligan *et al.* 2004) and of ρ1 and ρ2 subunits with γ2 subunits after co-expression in *Xenopus* oocytes (Ekema *et al.* 2002, Pan and Qian 2005).

$GABAR_A$ in gemistocytic tumour types

A relatively high expression of θ and ρ2 subunits was found in gemistocytic astrocytomas and minigemistocytic oligoastrocytomas of *tumour* samples, in which α1 subunit expression was found to be lower than in other tumour types. Gemistocytic astrocytomas are classified as those containing at least 20% gemistocytic astrocytes. These are reactive, oval-shaped astrocytes with abundant cytoplasm and an eccentric nucleus (Louis *et al.* 2007). They are fairly uncommon, and are found in only 5-10% of patients with astrocytomas (Tatter *et al.* 1996, Elsievich 1999). Minigemistocytes are similar in morphology, yet smaller, and with slightly different patterns of GFAP staining (Tihan *et al.* 2006). Though there has been no previous research on $GABAR_A$ subunit expression as showing a characteristic pattern in gemistocytic gliomas, frequent mutations in TP53 are typical (Watanabe *et al.* 1998), and antibody Pm43 specifically reacts with these two cell types, suggesting a relationship between the cell types (Kros *et al.* 1991). In this study, a similarity in $GABAR_A$ subunit expression between these two gliomas is reported, as well as a striking difference from other tumours. This supports a relationship between the two cell types as well as a difference in regulation from other glioma types.

The presence of gemistocytes in a tumour has been correlated with worse prognosis (Shaw *et al.* 1989, Okamoto *et al.* 2004), although the proportion of gemistocytes in the tumour does not seem to have an effect (Yang *et al.* 2003). The reasons for development of gemistocytes in tumours are still poorly understood. Hoshino *et al.* (1975) found that they multiplied slowly, irrespective of tumour malignancy, and that neighbouring neoplastic cells showed higher proliferation, and as a result suggested that they may develop due to losing the competition for substrates with adjacent cells. Studies finding that fibroblast growth factors released from gemistocytes promote proliferation of neighbouring cells support this theory (Paulus *et al.* 1990, Stefanik *et al.* 1991), although it does not fully explain the development of pure gemistocytic tumours (Kros *et al.* 1991). If bFGF does increase α1-expressing cells, as reported previously (Antonopoulos *et al.* 1997), this theory also does not explain the decreased expression of the α1 subunit found in gemistocytic tumour types.

Expression of the θ and ρ2 subunits in normal tissue is relatively uncommon, and there has been no evidence to suggest that they could form a functional channel together, yet this study found

a very significant positive correlation in their expression. The correlation was not strong, suggesting that they were not co-expressed in all instances, though it is possible that the ρ2 subunit could have formed homomeric receptors, or that channels were formed with other $GABAR_A$ subunits for which we did not stain (Bormann and Feigenspan 1995, Enz and Cutting 1999). It is also possible that they could have been expressed without forming functional channels, particularly since normal transcription regulation in tumour cells is disrupted, and so may not follow the normal "rules" for $GABAR_A$ subunit expression.

Although these results are from a relatively small sample of only 12 patients assigned these diagnoses, the pattern of expression of these relatively rare subunits further strengthens the relationship between $GABAR_A$ and tumour pathology, and warrants further investigation into both these two glioma types and these two subunits. Due to the wide expression of $GABAR_A$ throughout the CNS, these rarer subunits could be important for the proper functioning of a number of different important systems.

$GABAR_A$ and proliferation

The expression of ρ2 and γ1 subunits in reactive tumour cells of WHO Grade II glioma *epilepsy* samples was supported by similar expression found in the larger number of *tumour* samples. This finding contrasted the apparent lack of $GABAR_A$ subunit expression in the glial component of WHO Grade I glioma samples, or in the reactive glial cells of gliosis. The difference in expression may reflect the different rates of proliferation that are found between these different disease states, and complicates findings that decreased $GABAR_A$ expression is associated with increased proliferation, as in high-grade gliomas.

However, the glioma type-specific expression of certain $GABAR_A$ subunits implicates the receptor involvement during certain stages of tumour development, suggesting that there may be a difference in post-operative survival between patients expressing certain subunits. For example, high expression of θ and ρ2 subunits may correlate with lower survival, since their expression was high in gemistocytic tumour types, which have been associated with poorer prognosis (Shaw *et al.* 1989, Okamoto *et al.* 2004). Future studies using a larger number of samples could investigate this. Since a higher rate of tumour growth is associated with lower survival, subunits whose expression was associated with poorer prognosis could be important for proliferation,

There appear to be two mechanisms by which $GABAR_A$ may affect proliferation. The first is through changes in chloride concentrations inside the tumour cells. The hyperpolarizing effect of activation of $GABAR_A$ is due to the flow of chloride ions into the cell from the interstitial fluid. This flow is due to the concentration gradient that exists across the plasma membrane, which is maintained by chloride transporters such as NKCC and KCC co-transporters (Payne *et al.* 2003). An imbalance in the expression of these co-transporters can lead to an increase in intracellular chloride concentration ($[Cl]_i$). When $[Cl]_i$ exceeds $[Cl]_o$, the membrane potential reverses and the opening of $GABAR_A$ will result in chloride flowing out of the cell, generating a depolarizing current (Ransom *et al.* 2001, Kahle *et al.* 2008). In neurones this can result in seizure activity, and is therefore a possible explanation for epileptogenesis, particularly in cases of medically refractory epilepsy, where pharmacological targeting of $GABAR_A$ provides limited therapy (Aronica *et al.* 2007, Huberfeld *et al.* 2007).

Recently, Conti *et al.* (2011) have found evidence for anomalous expression of these co-transporters in gliomas of epileptic patients, and other research has shown that tumour cells maintain high $[Cl]_i$, at concentrations of around 100mM (Ransom *et al.* 2001, Ernest *et al.* 2005, Habela *et al.* 2009). This depolarizing activity of $GABAR_A$ closely resembles that found in early postnatal life. Here, activation of $GABAR_A$ leads to an influx of calcium ions (Labrakakis *et al.* 1998, Ben-Ari 2002, Nguyen *et al.* 2003). However, there is opposing evidence regarding the effects of this influx as either pro- or anti-proliferative. An anti-proliferative effect of $GABAR_A$ activation has been

reported in GFAP-expressing progenitor cells (Liu *et al.* 2005), as well as other cells (LoTurco *et al.* 1995, Goffin *et al.* 2008), yet a pro-proliferative effect in cerebellar granule cells has also been shown (Fiszman *et al.* 1999). In gliomas, the higher expression of $GABAR_A$ in low-grade compared to high-grade tumours is supportive of anti-proliferative activity. Though it is not entirely clear what these effects of $GABAR_A$ activation might be, its role in proliferation is important when considering its expression in gliomas. $GABAR_A$ mediated calcium ion influx has also been shown to the production of brain-derived neurotrophic factor (BDNF), a promoter of cell survival (Porcher *et al.* 2011). Upregulation of BDNF has been found in glioma tissue (Yan *et al.* 2009), which could be in response to the depolarizing activity of $GABAR_A$.

This activity is a means by which $GABAR_A$ function could be altered independently of its subtype. Whilst this evidence suggests that changes in the $GABAR_A$ channel itself is not a cause for the depolarizing shift in chloride homeostasis, it is possible that particular subtypes of the receptor may be expressed more frequently in cells in which the chloride concentrations are reversed, perhaps via synthesis of specific subunits. Studies have reported a high expression of the γ1 subunit in neural precursor cells, which is shown to decrease with age (Goffin *et al.* 2008, Fillman *et al.* 2010, Muth-Köhne *et al.* 2010). The α1 subunit follows different pattern however, and is upregulated to replace the neonatally predominant α2 subunit expression as the brain develops (Fritschy *et al.* 1994). This suggests that the $GABAR_A$ subtypes on cells with high $[Cl^-]_I$ are not necessarily similar compositions, since the pattern of expression in this study found a high expression of the α1 subunit similar to those found in normal adult brain tissue, and relatively very low γ1 subunit expression, more closely resembling $GABAR_A$ subunit composition found commonly in the adult brain than in the neonate.

The second mechanism by which $GABAR_A$ might affect proliferation is through its interaction with basic fibroblast growth factor (bFGF). Autocrine loop mechanisms of growth have been suggested in tumours, whereby growth factors released from one tumour cell promote the growth of other tumour cells, which in turn produce their own growth factors, leading to a positive feedback mechanism of tumour development (Tang *et al.* 1997, Katoh 2008). Inhibition of FGFs could help impede this uncontrolled growth.

In *tumour* samples, α1 subunit expression was found in a very high proportion of samples. Previous research has shown that activation of $GABAR_A$ inhibits the proliferative action of bFGF. It has been hypothesised that this might be specific to cells expressing the α1 subunit, due to the finding that bFGF treatment increases the relative number of cotical progenitor cells expressing the α1 subunit, thus forming a negative feedback loop to maintain control of proliferation (Antonopoulos *et al.* 1997, Goffin *et al. 2008*). bFGF is thought to play a role in glioma growth and proliferation via angiogenesis (Morrison *et al.* 1994, Wang *et al.* 2011) and so the strong expression of the α1 subunit found in this study may be a reflection of the increased expression and activity of bFGF, acting as a negative feedback component in which $GABAR_A$ upregulation and subsequent activity inhibits the proliferative action of bFGF. This could account for the loss of $GABAR_A$ found in high-grade gliomas (Jussofie *et al.* 1994, Labrakakis *et al.* 1998, Synowitz *et al.* 2001), in which higher levels of bFGF, and increased expression of bFGF receptors, is found (Takahashi *et al.* 1991, Saxena and Ali 1992, Ueba *et al.* 1994). In these cases the increased rate of proliferation may be due to loss of GABA-ergic inhibition of bFGF-mediated proliferation. Alterations in FGF signal transduction pathways have been shown to develop as gliomas progress from a benign to a malignant phenotype (Morrison *et al.* 1994). The α1 subunit has also been found to have pro-apoptotic activity even when expressed independently of $GABAR_A$, another response which may counteract tumour growth (Vaknin and Hann 2006).

Due to the origin of gliomas, it also might be expected that $GABAR_A$ channels in glioma cells similar in structure and physiology to those found in glia. However, Labrakakis *et al.* (1998) found that their binding properties most resembled receptors found in neurones, with two binding sites for GABA which have strong allosteric interaction. Benzodiazepine binding sites have been found on glioma cell membranes, suggesting that their activity could be modulated by specific

pharmacological agents (Frattola *et al.* 1985, Jussofie *et al.* 1994). Gandolfo *et al*. (1999) showed that the actions of $GABAR_A$ could be altered by peptides acting at the benzodiazepine-binding site to modulate the proliferation of astrocytes. This opens the possibility for new tumour therapies targeting $GABAR_A$ to prevent tumour growth. However, due to the abundant expression of the receptor in the brain, it would be important to develop therapies with high-specificity. Further studies in this area could be carried out to assess the impact of benzodiazepine treatment in epileptic patients on tumour development.

The range of different forms and functions of $GABAR_A$ is a rapidly increasing area of research it seems, though considering the importance of this receptor our knowledge is surprisingly limited. Focussing on particular patterns of subunit expression without considering the other proteins and transporters involved in the regulation of $GABAR_A$ may limit the knowledge that can be drawn from such studies. Conversely, focussing too closely on the regulation of factors such as chloride concentrations without considering the receptor subtype may lead to important results being overlooked. It will be vital to keep a clear view of the bigger picture in future research.

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