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Neuropharmacology 51 (2006) 121–128
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Selective changes in thalamic and cortical $GABA_A$ receptor subunits in a model of acquired absence epilepsy in the rat

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Received 22 September 2005; received in revised form 6 March 2006; accepted 7 March 2006

Abstract

Neonatal treatment of Long–Evans Hooded rats with the cholesterol synthesis inhibitor (CSI) AY9944 has been shown to increase occurrence of spike-waves in EEG recordings and decrease benzodiazepines sensitivity of $GABA_A$ receptor-mediated responses in neurons from the thalamic reticular nuclei (nRt, Wu et al., 2004). The present experiments were designed to investigate the changes in the $\gamma 2$ and $\alpha 1$ subunits of the $GABA_A$ receptor in CSI model rats as possible mechanisms of these changes. Western blot, immunohistochemistry and real-time PCR techniques were performed to measure the levels of $GABA_A$ receptor $\gamma 2$ and $\alpha 1$ subunit transcripts and protein in the nRt and ventrobasal (VB) relay nuclei of thalamus and in somatosensory cortex. In CSI model animals, Western blot results showed that $\gamma 2$ subunit expression significantly decreased in thalamus (control, $n = 6$: 0.17 ± 0.02 relative to actin vs. CSI model, $n = 6$: 0.11 ± 0.01, $P < 0.05$) but neither in cortex nor in hippocampal tissues. Conversely, $\alpha 1$ subunit expression decreased in CSI model somatosensory cortex, but not in nRt and VB. The present results demonstrate that neonatal block of cholesterol synthesis produces region- and subunit-specific decreases in $GABA_A$ receptor subunits in thalamus and cortex. Selective reductions in $GABA_A$ receptor subunits in thalamus may play a role in pathophysiology of absence epilepsy.

Keywords: Cholesterol synthesis inhibitor; $GABA_A$ receptor subunit; Thalamic reticular nucleus; Ventrobasal relay nucleus; Somatosensory cortex; Absence epilepsy

1. Introduction

Absence seizures, previously referred to as petit mal seizures, are important clinical entities in children and in some adults. Atypical absence seizures present with frequent episodes of lost awareness, may be associated with falls and injuries, and are especially difficult to treat (Dreifuss and Ogunyemi, 1992). Much work has elucidated the mechanisms of the genetically-transmitted absence epilepsies in animal model systems (Danober et al., 1998), and this work has highlighted the key role played by the reticular nucleus of thalamus (nRt) in generation of thalamocortical spike-waves. Until recently, no good animal models have existed for study of acquired absence epilepsy, which usually results from a cerebral insult early in a child's life. However, a model of acquired absence epilepsy now can be derived from inhibition of cholesterol synthesis in neonatal rats (CSI model). The inhibitor, AY9944 (*trans*-1,4-bis(2-chlorobenzylaminoethyl) cyclohexane 2HCl), was shown to produce long-standing absence epilepsy after a single injection, or a small series of injections in the first few weeks of life (Smith and Fisher, 1996). In distinction to the many drugs that can produce seizures, AY9944 can produce epilepsy (Cortez et al., 2001). Study of the CSI model might lead to insights about the mechanisms of epilepsy secondary to intrauterine or neonatal insults.

This paper focuses on the potential stoichiometric changes in heteromeric $GABA_A$ receptors in thalamus. The $\gamma 2$ subunit is of particular interest. Benzodiazepine and muscimol binding is

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doi:10.1016/j.neuropharm.2006.03.003

enhanced in various regions of brain after administration of neurosteroids or their precursors (Canonaco et al., 1989; Bertz et al., 1995; Nguyen et al., 1995), with a resulting increase in GABAergic inhibition. Type I benzodiazepine binding occurs in a cleft between the α1 and γ2 subunits (Sigel and Buhr, 1997; Sigel, 2002). We previously reported that neurons acutely dissociated from reticular nuclei (nRt) of Long–Evans Hooded rats subjected to early life block of cholesterol synthesis lose $GABA_A$ receptor sensitivity to benzodiazepines (BZs, Wu at al., 2004). This observation suggests that a reduction of $GABA_A$ receptor γ2 subunits may occur in the thalamus of CSI model animals. To test the hypothesis that alterations in thalamic $GABA_A$ receptor subunits may contribute to seizures in absence epilepsy, we investigated the levels of γ2 subunits of the $GABA_A$ receptor in the nRt and ventrobasal (VB) relay nuclei of thalamus, and also in somatosensory cortex, in animals previously given AY9944. To test the specificity of change in the γ2 subunit we also measured levels of the most common α subunit, α1.

2. Methods

2.1. Preparation of model animals

Experiments were carried out according to protocols approved by the Stanford Institutional Animal Care and Use Committee. Pregnant Long–Evans Hooded rats were purchased from Charles River. Newborn pups were given injections of AY9944, suspended in olive oil, intramuscularly at days 1, 5, 9, 13 and 17 of life, in doses totaling 7.5 mg/kg of weight. Pups were maintained on standard diets, including suckling with their mother. Each litter was split into those receiving AY9944 injections and those receiving olive oil control injections. Since handling of neonatal rats can influence $GABA_A$ receptor composition (Bolden et al., 1990; Caldji et al., 2000, 2003, 2004), we were careful to handle control and model animals in the same way.

EEGs from animals over 77 days of age were recorded by means of headplugs, cage rotary commutators and cables from Plastics One (Roanoke, VA). Animals had four bilateral bone screws, and a reference screw over the frontal sinus attached to the skull and connected to a Plastics One head plug, affixed in place with dental cement. A ground electrode was placed in the neck muscle. Implantation was performed under general anesthesia with ketamine (40 mg/kg), xylazine (2.5 mg/kg), and acepromazine maleate (0.8 mg/kg) by intramuscular injection in volumes of 0.2 ml. Animals were allowed to recover for at least 48 h before start of recording. EEG and concurrent video data were recorded on a Nicolet (Madison, WI) System 6000 recorder in digital form, and stored to CDs. Bandpass was 1–70 Hz (Anschel et al., 2004).

2.2. Total RNA isolation, reverse transcription and real-time polymerase chain reaction (PCR)

Adult Long–Evans rats (postnatal days 130–150) were anesthetized with halothane and decapitated. The brain quickly was removed and placed in cold (under 4 ° C) oxygenated artificial cerebrospinal fluid (ACSF), which contained (in mM): $NaCl_2$ 123, KCl 4, NaH_2PO_4 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 26, dextrose 10, and $CaCl_2$ 2.0, adjusted to pH 7.4. The somatosensory cortex and thalamic nRt and VB nuclei then were separately collected. Briefly, brains were cut horizontally by using a vibratome. Cortical sections containing somatosensory area (Fr1–2, HL Par1, Paxinos and Watson, 1997) and thalamic horizontal sections (Bregma ~ −5.3–6.8) containing nRt and VB nuclei were trimmed and collected. Samples were stored at −80 °C for later protein or RNA analysis.

We employed real-time PCR to detect changes in expression of $GABA_A$ subunit mRNA. The isolation of total RNA was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The purity and concentration were determined spectrophotometrically. cDNA was prepared from total RNA using oligo primers and Superscript reverse transcriptase according to the accompanying protocol (Invitrogen, Carlsbad, CA, USA). Genomic DNA was eliminated by DNase I. For real-time PCR, the reactions were conducted by placing 10 μl of material into 96-well plates, using the Quantitect SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA). Briefly, 5 μl of a mixture of 2× SYBR green and 0.4 μl of 10 μM $GABA_A$ subunit primers were loaded with 5 μl diluted cDNA template in each well. The PCR parameters were 94 °C for 5 min, then 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, repeating for 40 cycles. Melting curves of fluorescence versus temperature were performed to screen for primer dimers and other coamplification products. PCR sequencing was performed to confirm the PCR products. The amplification of synaptophysin RNA was used as an internal control. The data from real-time PCR experiments were analyzed by the comparative C_t method. The parameter C_t is derived for each cDNA sample and primer pair; C_t is an expression of amplification kinetics referring to the cycle at which log-phase amplification reaches a pre-determined threshold. For a given sample, C_t values for synaptophysin RNA were subtracted from the C_t of each $GABA_A$ subunit to arrive at a ΔC_t value. The mean ΔC_t from all control animal reactions was then subtracted from the ΔC_t for each control or treated sample to arrive at $\Delta\Delta C_t$. This parameter ($\Delta\Delta C_t$) reflects the fold-difference of over- or under-expression of $GABA_A$ subunits in CSI model rat tissue, relative to control according to the expression $2^{-\Delta\Delta C_t}$. In each experiment, samples were analyzed in triplicate for the indicated numbers of rats. Table 1 lists the sequence of primers used in the experiment.

2.3. Western blot analysis

We used specific antibodies directed against the $GABA_A$ receptor α1 and γ2 subunit protein in thalamic nRt and VB or somatosensory cortex. The brain tissue was sonicated in homogenization buffer containing: 12.5% 0.5 M Tris–HCl, 10% glycerol, 2% SDS and 0.7% β-mercaptoethanol. The homogenates were centrifuged at 14,000 × *g* for 10 min, and supernatant collected for Western blot analysis. All procedures were carried out at 0–4 °C. A sample of 40 μg protein from control and model brain tissue were separated by 10% Tris–HCl running gel and transferred to nitro-cellulose membranes. The membranes were then blocked with 5% nonfat milk in TBS buffer for 2 h at room temperature, and incubated with primary antibodies overnight at 4 °C. The dilution of antibodies was 1:1000 for rabbit polyclonal anti-α1 (Upstate, Lake Placid, NY) and rabbit polyclonal anti-γ2 subunits (Chemicon International Inc., Temecula, CA), and 1:5000 for mouse monoclonal anti-β-actin (Sigma). After several washes, the membranes were incubated with secondary antibodies at dilution of 1:10,000–20,000 for 1–2 h at room temperature. After further extensive washing, the immunoreactive bands were detected with ECL reagents (Amersham, UK). Quantification of optical absorbency of Western blots was performed by using Quantify One software (Bio-Rad, CA). The relative protein expression of $GABA_A$ receptor subunits was calculated as the OD value of $GABA_A$ receptor subunit/OD value of β-actin. Data were expressed as mean ± S.E.M. Statistical significance was compared between control and CSI model groups, using two-tailed Student t-tests at a $P < 0.05$ significance criteria.

2.4. Immunohistochemistry

Six rats (three controls and three CSI models, p130–150) were deeply anesthetized by halothane and then were perfused trans-cardiac with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, and post-fixed in the same fixative for 4–5 h and then transferred to 30% sucrose in PBS for cryoprotection at 4 °C. Sections of 40 μm

Table 1
Oligonucleotide primers used for real-time PCR

Gene	5′-primer ($n = 20$)	3′-primer ($n = 20$)	GenBank code
α1	aaggacccatgacagtgctc	ggctcccttgtccactcata	NM183326
γ2	gacgatgaccactctcagca	acagtccttgccatccaaac	NM183327
Synaptophysin	actactcctcgtcggctgaa	acagggtccctcagttcctt	NM012664

thickness were prepared on a Vibratome. Every fifth section was collected for immunostaining. Sections from control rats and CSI model rats were processed in the same well and viewed under the same light intensity. Negative controls were performed concurrently during the same experiment, with omission of the primary antibodies during processing. For DAB staining, sections were quenched with 3% hydrogen peroxide for 5 min. Non-specific binding was blocked by 10% normal goat serum (Jackson Immunoresearch Labs Inc.) and 0.5% Triton X-100 for 60 min, followed by primary antibodies, anti-$GABA_A$ receptor $\alpha 1$ or $\gamma 2$, the same as those used in performance of the Western blot, or use of the mouse anti-neuronal nuclei monoclonal antibody (NeuN, 1:1000 dilution, Chemicon International) overnight at 4 °C. Subsequently, the sections were incubated with a biotinylated secondary antibody (1:200, Vector Labs, Burlingame, CA) for 2 h at room temperature followed by 60 min in ABC reagent (Elite, Vector ABC). Finally, the sections were reacted with diaminobenzidine (Vector Labs) for 5–8 min. After several rinses, two sections (one from control and one from CSI model rats) were mounted on the same glass slides, dehydrated with graded alcohol and xylene, and protected with a coverslip. The results were examined under a light microscope. Images were taken from cortex, nRt and VB with a 10× (for cortex) or 40× (for nRt or VB) objective lens by using a digital camera and quantified blinded to experimental conditions. Relative $GABA_A$ receptor subunit abundance was determined by measuring mean immunoreactive intensity. NeuN staining was quantified by counting the stained cells. The expression of immunoreactive intensity was calculated by mean luminosity of the stained area subtracted from mean luminosity of the negative control sections obtained in the same experiment. Two images were taken from each section; two to three sections were taken from each animal. Data were expressed as mean ± S.E.M. Significance of differences was assessed by a two-tail Student t-test, according to a $P < 0.05$ criterion.

Specificity of the antisera is supported by Western blot results obtained from membrane protein and immunoprecipitation experiments (not shown), which showed that the anti-$GABA_A$ receptor $\gamma 2$ antibody stained only one single band of size 45–47 kDa: the same as the vendor's results, and in correspondence to previously published immunohistochemical studies (Sergeeva et al., 2002; Ramoino et al., 2004).

3. Results

3.1. Seizures in the model animals

Skull plugs were implanted in six control animals and six AY9944-treated animals to record EEGs, and data were obtained, respectively, from six and five animals (the head plug came off in one). The mean time for recording EEGs was 106.4 ± 29.7 (range 77–147) days after the first AY9944 injection. None of the animals had tonic-clonic seizures, but several animals in the AY9944-treated group demonstrated apparent absence-seizure behaviors, with arrest of activity, staring and twitching of the vibrissae. Since these behaviors often were subtle, and not easily distinguished from normal rat behavior, we did not examine them systematically, but rather, used the presence of spike-waves (SWs) on the EEG to determine the degree of epileptogenicity. Fig. 1 illustrates a typical SW EEG pattern in an animal previously treated with AY9944.

Difference in SWs between controls and AY-9944 Long–Evans Hooded rats, reviewed blind to treatment group, were quantitative, not qualitative, since all animals had some SWs in at least one recording session. The difference in seizures and EEG SWs between groups of CSI model animals and control animals previously was reported by Cortez and colleagues (Cortez et al., 2001). In our experiments, the five AY9944-treated animals had SWs in every recording session (25/25 = 100%); whereas the six control animals had SWs in only 48% (15/31). We defined the SW score for each animal as the number of recording sessions (each 1 h long) showing SWs, divided by the total number of recording session. SW scores for the control animals were: 0.20, 0.80, 1.00, 0.20, 0.33 and 0.75. SW scores for AY9944-treated animals were 1.00, 1.00, 1.00, 1.00 and 1.00. A two-tailed *t*-test on independent means for the SW scores (no assumption of equal variance) showed $t = -3.21$, $df = 5$, $P = 0.024$, significantly different. Therefore, the treated animals were more likely to show SWs in a recording session, and in fact, the AY9944-treated animals showed SWs in every recording. In this paper, which was focused on receptor changes, we did not systematically evaluate the behavioral aspects of seizures.

3.2. Differential alterations in expression of $GABA_A$ subunits $\gamma 2$ and $\alpha 1$ protein in control and CSI model thalamus and cortex

We employed Western blot analysis of proteins to detect changes in expression of $GABA_A$ subunits $\gamma 2$ and $\alpha 1$ in control ($n = 6$) and CSI model ($n = 6$) thalamus and cortex. A significant decrease in relative $\gamma 2$ protein expression (compared to

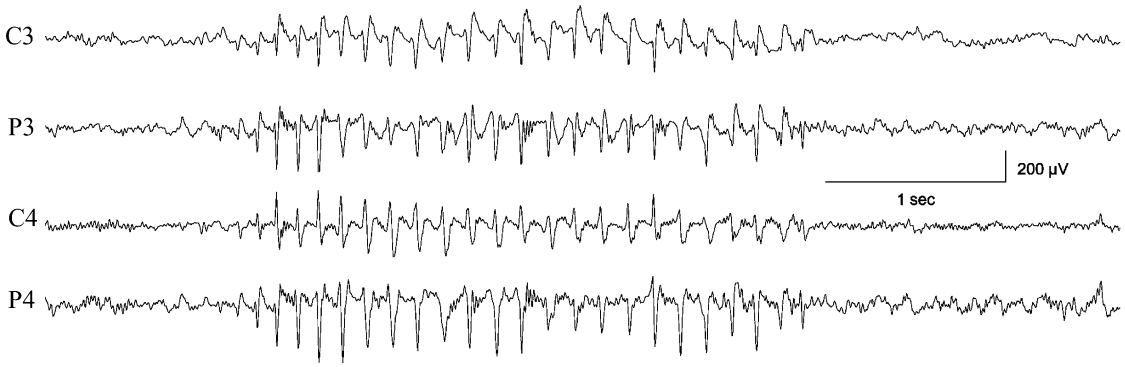

Fig. 1. EEG recording in a CSI model rat, demonstrating approximately 6 per second spike-waves. The reference electrode was a bone screw over the frontal sinus. C3, left central bone screw; C4, right central bone screw, P3, left parietal bone screw; P4, right parietal bone screw. Filters were set to low cutoff 1.0 Hz, high cutoff 70 Hz, 60-Hz notch filter on, and digitized at 200 Hz.

actin) in CSI model nRt and VB nuclei of thalamus was observed, with control of 0.17 ± 0.02 vs. CSI model of 0.11 ± 0.01 ($P < 0.05$, Fig. 2A1,B1). This difference in $\gamma 2$ protein expression was not seen in cortex, for which control values were 0.96 ± 0.15 vs. CSI model values of 0.92 ± 0.07 ($P = 0.8$, Fig. 2A2,B2). Similarly, hippocampal tissues from six control and six CSI model rats demonstrated no significant difference in $\gamma 2$ subunit protein levels (0.126 ± 0.01 $n = 6$, vs. 0.122 ± 0.01, $n = 6$, $P = 0.86$). Conversely, $GABA_A$ subunit $\alpha 1$ protein expression was lower in CSI model cortex. The level of $\alpha 1$ protein expression in cortex was less in model animals than in controls (1.32 ± 0.11 vs. CSI model cortex: 0.83 ± 0.12, $P < 0.05$), but not in thalamus (control thalamus: 0.5 ± 0.09 vs. CSI model thalamus: 0.38 ± 0.06, $P = 0.3$, Fig. 2).

To further identify the regional distributions of the specific alterations in $\gamma 2$ and $\alpha 1$ $GABA_A$ receptor subunits in thalamus and cortex, we performed immunohistochemistry experiments. As shown in Fig. 3, both nRt and VB nuclei of thalamus expressed relatively less $\gamma 2$ subunit immunoreactivity, measured by immunostaining intensity, in tissue samples from CSI model rats compared to tissue from control rats (control nRt: 38 ± 4 vs. CSI model nRt: 26 ± 7, $P < 0.05$; control VB: 43 ± 4 vs. 31 ± 3, $P < 0.05$, Fig. 3A,B). However, the numbers of neurons in nRt and VB, as measured by treatment-blinded counts of cells marked by NeuN, were similar (control nRt: 103 ± 17 vs. CSI model nRt: 108 ± 21; control VB: 101 ± 17 vs. CSI model VB: 121 ± 20, Fig. 3A,C).

$\alpha 1$ immunoreactivity was very low in nRt (control nRt: 7 ± 7 vs. CSI model nRt: 8 ± 5). In contrast, relatively strong labeling is evident in VB, suggesting that the subunit mainly exists in VB (control VB: 74 ± 6 vs. CSI model VB: 64 ± 3), which is consistent with the observations of others (Wisden et al., 1992; Fritschy and Mohler, 1995; Browne et al., 2001).

The immunoreactivity of $\gamma 2$ and $\alpha 1$ subunits in cortex is shown in Fig. 4. A moderate decreased expression of $\alpha 1$ subunits was seen in CSI model cortex (control: 124 ± 7 vs. CSI model: 115 ± 7, $P < 0.05$, Fig. 4A,B1). The reduction of $\alpha 1$ immunoreactivity in cortex is relatively homogeneous, and no difference was evident as a function of cortical lamina. In cortex, no difference in $\gamma 2$ subunit immunoreactivity was observed (control: 88 ± 7 vs. 86 ± 7, $P = 0.58$). Numbers of neurons (control: 739 ± 24 vs. CSI model: 754 ± 18, $P = 0.9$) in control and CSI model cortex also did not differ (Fig. 4A,B2,B3).

3.3. Expression of $GABA_A$ subunits $\gamma 2$ and $\alpha 1$ mRNA in control and CSI model thalamus and cortex

To examine whether the regional decrease in $\gamma 2$ and $\alpha 1$ subunits was associated with altered levels of corresponding mRNAs, we employed real-time PCR to measure the expression of $GABA_A$ subunit mRNAs in control and CSI model thalamus and cortex. Expression of $\gamma 2$ and $\alpha 1$ subunit mRNAs did not differ for thalamic or cortical tissue from control and CSI model animals. Table 2 details the normalized fold-changes of $GABA_A$ receptor $\gamma 2$ and $\alpha 1$ subunit mRNA expression in control and CSI model thalamus and cortex.

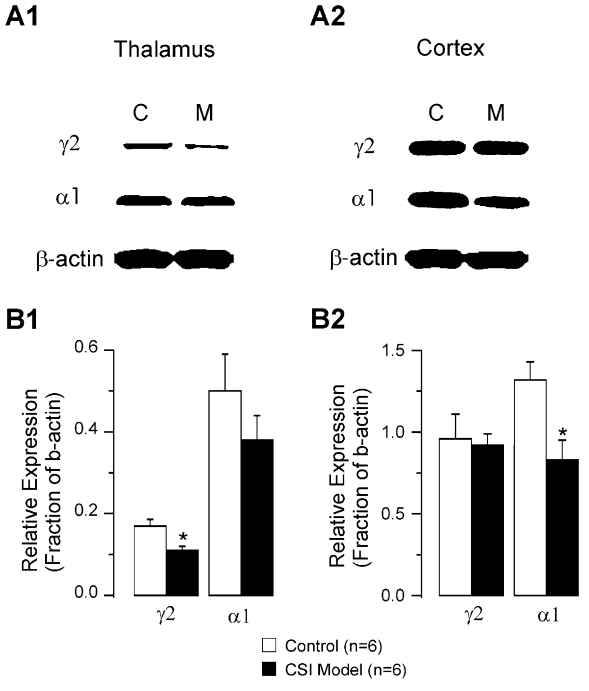

Fig. 2. Alterations in $GABA_A$ receptor $\gamma 2$ and $\alpha 1$ subunits in CSI model thalamus and cortex. (A1) Representative Western blot demonstrating $\gamma 2$ and $\alpha 1$ immunoreactivity in control (C) and CSI model (M) thalamus. (A2) Representative Western blot demonstrating $\gamma 2$ and $\alpha 1$ immunoreactivity in control (C) and CSI model (M) cortex. (B1) Histograms showing $\gamma 2$ and $\alpha 1$ immunoreactivity as a fraction of β-actin in control and CSI model thalamus. (B2) Histograms showing $\gamma 2$ and $\alpha 1$ immunoreactivity as a fraction of β-actin in control and CSI model cortex. Data are expressed as mean ± S.E.M. $^*P < 0.05$.

4. Discussion

Two main conclusions emerge from the present study. First, early-life block of cholesterol synthesis results in a significantly higher occurrence of EEG spike-waves, compared to those in control siblings, which is consistent with previous reports (Cortez et al., 2001; Smith and Fisher, 1996; Wu et al., 2004). Certain litters of Long–Evans rats have high baseline rates of EEG spike-waves on a genetic basis (Cortez et al., 2001), but imposition of early-life cholesterol blockade increases the prevalence of spike-waves essentially to 100%. Second, rats with early life block of cholesterol synthesis do not lose neurons in thalamus or cortex, but exhibit selective regional alterations in $GABA_A$ receptor subunits. The $\gamma 2$ subunit immunoreactivity decreases in both nRt and VB nuclei in thalamus, but not in cortex. In addition, the expression of the $\alpha 1$ subunit, as measured by Western blot and immunostaining, decreases in cortex, but not in VB of thalamus.

Changes in expression of $GABA_A$ receptor subunits have been studied extensively in animal models of temporal lobe epilepsy, but relatively few studies have been done in absence epilepsy models, including the GAERS (genetic absence epilepsy rats from Strasbourg) and the WAG/Rij (Wistar albino Glaxo from Rijswijk, see review by Crunelli and Leresche,

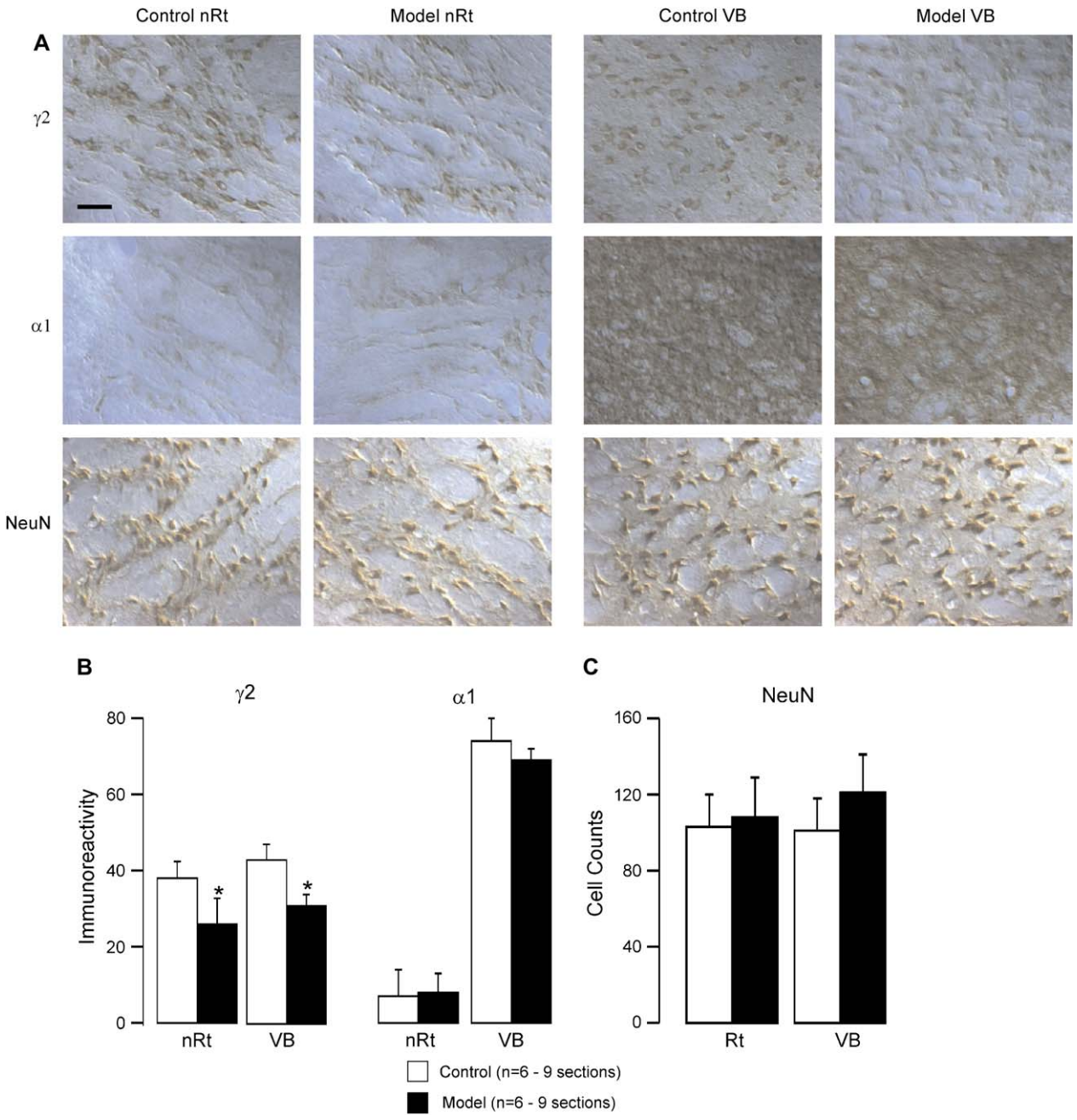

Fig. 3. Expression of $GABA_A$ γ2 subunit is low in CSI model thalamus. (A) Photomicrographs of nRt and VB showing γ2 (first row), α1 (middle row), and NeuN (bottom row) positive immunostainings in control nRt (left column), CSI model nRt (second left column), control VB (second right column) and CSI model VB (right column). (B) Histograms demonstrating mean immunoreactivity of γ2 and α1 subunits. Histogram shows similar numbers of NeuN positive neurons in control and CSI model thalamus. $*P < 0.05$. Scale bar = 50 μm and applies to all images.

2002). Two mutations in $GABA_A$ receptor subunits have been discovered in human absence epilepsy, involving the $GABA_A$ receptor γ2 subunit gene (Baulac et al., 2001; Wallace et al., 2001; Harkin et al., 2002); and $GABA_A$ α1 subunit gene (Cossette et al., 2002). To our knowledge, the selective changes in $GABA_A$ subunits documented in this paper are the first described in an acquired model of absence epilepsy. The model provides an example of how an early-life insult can result in lasting changes to GABA receptor subunits in brain, of a type that may predispose to absence seizures.

The $GABA_A$ receptor is assembled as five subunits from 19 possible subunits, most importantly, variants of alpha, beta, gamma and delta subunit moieties (Hevers and Luddens, 2002). The $GABA_A$ receptor usually is comprised of one gamma, two alpha and two beta subunits (Sarto et al., 2002), in a pentamer. Benzodiazepine binding occurs in a cleft between the α and γ2 subunits (Sigel, 2002). The γ2 subunit normally is present in both nRt and VB cells (Browne et al., 2001) and is an important element in determining positive modulation of $GABA_A$ receptors by benzodiazepines

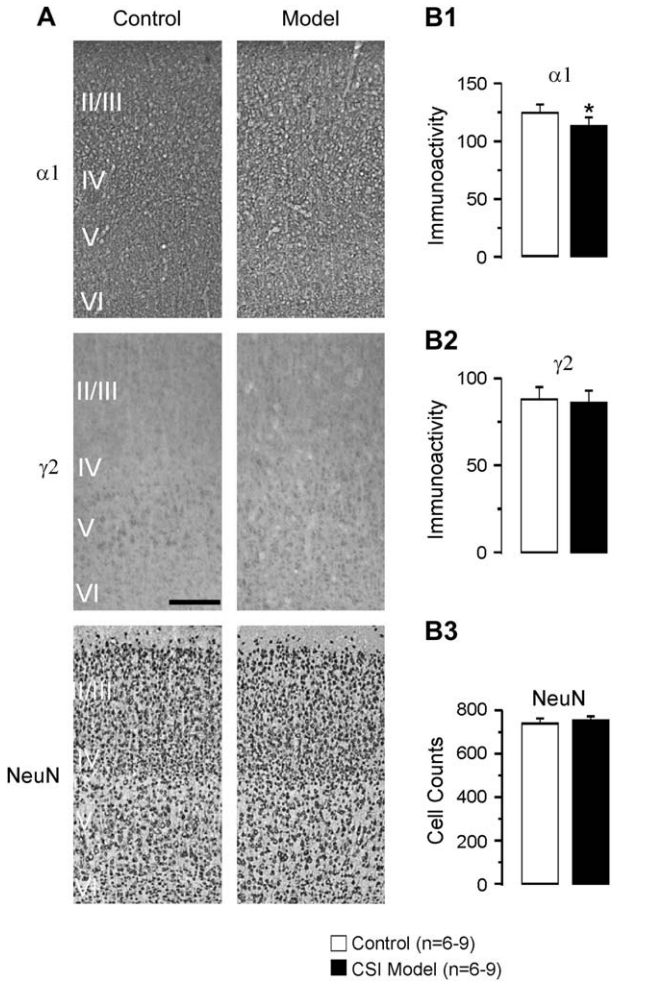

Fig. 4. CSI model cortex expresses lower $GABA_A$ receptor α1 subunits. (A) Photomicrograph of cortex showing α1 (first row), γ2 (middle row), and NeuN (bottom row) positive immunostainings in control cortex (left column) and CSI model cortex (right column). (B1) Histogram demonstrating that the mean immunoreactivity of α1 subunits in CSI model cortex is slight but significantly lower compared to control cortex. (B2) Histogram showing no significant difference in mean immunoreactivity of γ2 subunits in control and CSI model cortex. (B3) Histogram showing similar numbers of NeuN positive neurons in control and CSI cortex. $*P < 0.05$. Scale bar in control γ2 image = 200 μm and is applied to all images.

(Pritchett et al., 1989). γ2 subunit expression is positively correlated with benzodiazepine sensitivity and inversely correlated with Zn^{2+} blockade (Sieghart, 1995; Dominguez-Perrot et al., 1996; Zhu et al., 1996; Barnard, 1998; Alsbo et al., 2001).

$GABA_A$ receptor α1 and γ2 subunits are developmentally regulated and substantially increase with brain maturation (Laurie et al., 1992; Poulter et al., 1992; Golshani et al., 1997). The γ2 subunit protein reduction in CSI model nRt and VB of thalamus and α1 subunit decrease in somatosensory cortex are not accompanied by changes in the corresponding mRNAs. The discrepancy between the unchanged mRNA levels by RT-PCR and the protein changes suggests that the observed changes in γ2 and α1 proteins are not through a transcriptional regulation, but a post-translational modification (Jones et al., 1997; Petrie et al., 2001).

Table 2
$GABA_A$ receptor subunit expression in control and CSI model thalamus and cortex

	Thalamus		Cortex	
	Control ($n = 6$)	Model ($n = 6$)	Control ($n = 6$)	Model ($n = 6$)
α1	1.03 ± 0.11	1.22 ± 0.17	1.03 ± 0.06	1.06 ± 0.12
γ2	1.06 ± 0.15	1.03 ± 0.12	1.02 ± 0.10	0.98 ± 0.13

We did not detect a significant decrease of α1 subunit in thalamus; although, there was a trend toward reduced expression. The possibility of a non-genomic change in the $GABA_A$ α1 subunit, such as receptor internalization (Naylor et al., 2005), cannot be excluded by our experiments.

$GABA_A$ receptor α1 subunits are required for the assembly of most αβ2/3γ2 receptors in brain. Benzodiazepine binding sites, located at the cleft of α and γ2 subunits are severely affected by deletion of the α1 subunit. Previous studies suggest that up to 70% of benzodiazepine binding sites (type I) are α1 subunit containing receptors (Braestrup and Nielsen, 1981). The present study demonstrates that thalamic nRt has a low level of expression of α1 subunits, consistent with previous literature (Wisden et al., 1992; Fritschy and Mohler, 1995; Browne et al., 2001). The lack of α1 subunits in nRt also suggests that nRt may predominantly exhibit α2 or α3 containing (Type II) benzodiazepine binding sites (Pritchett et al., 1989). The early life block of cholesterol synthesis selectively decreases the α1 subunit in cortex but not in thalamic VB relay nuclei. This observation raises the possibility that the affected type of benzodiazepine binding sites in CSI model thalamus may be different from those in cortex, which may lead to differential changes in $GABA_A$ receptor pharmacology and function in the thalamocortical circuit.

The mechanisms by which cholesterol synthesis inhibition in newborn rat brain leads to alteration in brain physiology is unknown. Future possible explanations will have to consider, not only reductions in cholesterol itself, but in reduction of compounds, such as neurosteroids derived from cholesterol, which also are decreased in this model (Cortez et al., 2002). Neurosteroids, for example, alphaxalone, are positive modulators of the $GABA_A$ receptor (Majewska, 1992). Reduction of neurosteroids can alter $GABA_A$ receptor subunits during development (Follesa et al., 2002), such that inhibition in the nucleus reticularis of thalamus might become deficient, and lead to enhanced thalamically-generated spike-waves (Huntsman et al., 1999). Accumulation of cholesterol precursors in animals treated with cholesterol synthesis inhibitors also could play a role in altered physiology.

Our results support the hypothesis that absence seizures acquired by early-life block of cholesterol synthesis are associated with reduced γ2 subunits of the $GABA_A$ receptor in the nRt and VB nuclei of thalamus. Such a reduction is relatively specific, since no significant α1 subunit reduction is seen in VB, and the decrease in γ2 subunits is not due to cell loss. Although changes in $GABA_A$ receptor subunits can result from handling in the neonatal period, the difference between model and control animals, which were handled the same, argue

that handling is not the main cause of the difference. The number of neurons measured by NeuN staining was similar in control and CSI model nRt and VB of thalamus. The reduction in the γ2 subunit is regionally specific, since γ2 subunits are not decreased in CSI model cortex. These data are not meant to be a comprehensive survey of $GABA_A$ receptor subunits, but rather to focus on the γ2 subunits, with measurement of α1 subunits as an index of specificity for the γ2 changes. The decrease in levels of the CSI model thalamus γ2 subunit may account for the electrophysiological and benzodiazepine pharmacological changes observed in our previous studies (Wu et al., 2004). Selective reduction in thalamic γ2 subunits could contribute to the increased seizure susceptibility in CSI model rats, by reducing inhibition in nRt, and thereby increasing firing in thalamic relay nuclei (for review see Huguenard, 1999). Documenting these assertions in the CSI model will require further experimentation.

Acknowledgements

R.S.F. was supported by the Maslah Saul MD Chair and the James and Carrie Anderson Fund for Epilepsy Research. H.L. was supported by the Susan Horngren Fund and the Epilepsy Foundation. J.R.H. was supported by NIH RO1 NS34774. We gratefully acknowledge Wyeth-Ayerst for supplying the AY-9944 drug for the study and Dr. Mark Kay for the loan of laboratory equipment.

References

Alsbo, C.W., Kristiansen, U., Møller, F., Hansen, S.L., Johansen, F.F., 2001. $GABA_A$ receptor subunit interactions important for benzodiazepine and zinc modulation: a patch-clamp and single cell RT-PCR study. Eur. J. Neurosci. 13, 1673–1682.

Anschel, D.A., Ortega, E.L., Kraus, A.C., Fisher, R.S., 2004. Focally injected adenosine prevents seizures in the rat. Exp. Neurol. 190, 544–547.

Barnard, E.A., 1998. Subtypes of gamma-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. International Union of Pharmacology. XV. Pharmacol. Rev. 50, 291–313.

Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J.F., Baulac, M., Brice, A., Bruzzone, R., LeGuern, E., 2001. First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat. Genet. 28, 46–48.

Bertz, R.J., Reynolds, I.J., Kroboth, P.D., 1995. Effect of neuroactive steroids on [^{3}H]flumazenil binding to the $GABA_A$ receptor complex in vitro. Neuropharmacology 34, 1169–1175.

Bolden, S.W., Hambley, J.W., Johnston, G.A., Rogers, L.J., 1990. Neonatal stress and long-term modulation of GABA receptors in rat brain. Neurosci. Lett. 111 (3), 258–262.

Braestrup, C., Nielsen, M., 1981. [^{3}H]Propyl beta-carboline-3-carboxylate as a selective radioligand for the BZ1 benzodiazepine receptor subclass. J. Neurochem. 37, 333–341.

Browne, S.H., Kang, J., Akk, G., Chiang, L.W., Schulman, H., Huguenard, J.R., Prince, D.A., 2001. Kinetic and pharmacological properties of GABA-A receptors in single thalamic neurons and GABA-A subunit expression. J. Neurophysiol. 86, 2312–2322.

Caldji, C., Francis, D., Sharma, S., Plotsky, P.M., Meaney, M.J., 2000. The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. Neuropsychopharmacology 22 (3), 219–229.

Caldji, C., Diorio, J., Meaney, M.J., 2003. Variations in maternal care alter GABA(A) receptor subunit expression in brain regions associated with fear. Neuropsychopharmacology 28 (11), 1950–1959.

Caldji, C., Diorio, J., Anisman, H., Meaney, M.J., 2004. Maternal behavior regulates benzodiazepine/GABAA receptor subunit expression in brain regions associated with fear in BALB/c and C57BL/6 mice. Neuropsychopharmacology 29 (7), 1344–1352.

Canonaco, M., O'Connor, L.H., Pfaff, D.W., McEwen, B.S., 1989. Longer term progesterone treatment induces changes of $GABA_A$ receptor levels in forebrain sites in the female hamster: quantitative autoradiography study. Exp. Brain Res. 77, 407–411.

Cortez, M.A., McKerlie, C., Snead, O.C., 2001. A model of atypical absence seizures: EEG, pharmacology, and developmental characterization. Neurology 56, 341–349.

Cortez, M.A., Cunnane, S.C., Snead III, O.C., 2002. Brain sterols in the AY-9944 rat model of atypical absence seizures. Epilepsia 43, 3–8.

Cossette, P., Liu, L., Brisebois, K., Dong, H., Lortie, A., Vanasse, M., Saint-Hilaire, J.M., Carmant, L., Verner, A., Lu, W.Y., Wang, Y.T., Rouleau, G.A., 2002. Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy. Nat. Genet. 31, 184–189.

Crunelli, V., Leresche, N., 2002. Childhood absence epilepsy: genes, channels, neurons and networks. Nat. Rev. Neurosci. 3, 371–382.

Danober, L., Deransart, C., Depaulis, A., Vergnes, M., Marescaux, C., 1998. Pathophysiological mechanisms of genetic absence epilepsy in the rat. Prog. Neurobiol. 55, 27–57.

Dominguez-Perrot, C., Feltz, P., Poulter, M.O., 1996. Recombinant $GABA_A$ receptor desensitization: the role of the gamma 2 subunit and its physiological significance. J. Physiol. 497, 145–159.

Dreifuss, F.E., Ogunyemi, A.O., 1992. Classification of epileptic seizures and the epilepsies: an overview. Epilepsy Res. Suppl. 6, 3–11.

Fritschy, J.M., Mohler, H., 1995. $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J. Comp. Neurol. 359, 154–194.

Follesa, P., Porcu, P., Sogliano, C., Cinus, M., Biggio, F., Mancuso, L., Mostallino, M.C., Paoletti, A.M., Purdy, R.H., Biggio, G., Concas, A., 2002. Changes in $GABA_A$ receptor gamma 2 subunit gene expression induced by long-term administration of oral contraceptives in rats. Neuropharmacology 42, 325–336.

Golshani, P., Truong, H., Jones, E.G., 1997. Developmental expression of GABA(A) receptor subunit and GAD genes in mouse somatosensory barrel cortex. J. Comp. Neurol. 383, 199–219.

Harkin, L.A., Bowser, D.N., Dibbens, L.M., Singh, R., Phillips, F., Wallace, R.H., Richards, M.C., Williams, D.A., Mulley, J.C., Berkovic, S.F., Scheffer, I.E., Petrou, S., 2002. Truncation of the GABA(A)-receptor gamma2 subunit in a family with generalized epilepsy with febrile seizures plus. Am. J. Hum. Genet. 70, 530–536.

Hevers, W., Luddens, H., 2002. Pharmacological heterogeneity of gamma-aminobutyric acid receptors during development suggests distinct classes of rat cerebellar granule cells in situ. Neuropharmacology 42, 34–47.

Huguenard, J., 1999. Neuronal circuitry of thalamocortical epilepsy and mechanisms of antiabsence drug action. Adv. Neurol. 79, 991–999.

Huntsman, M.M., Porcello, D.M., Homanics, G.E., DeLorey, T.M., Huguenard, J.R., 1999. Reciprocal inhibitory connections and network synchrony in the mammalian thalamus. Science 283 (5401), 541–543.

Jones, A., Korpi, E.R., McKernan, R.M., Pelz, R., Nusser, Z., Makela, R., Mellor, J.R., Pollard, S., Bahn, S., Stephenson, F.A., Randall, A.D., Sieghart, W., Somogyi, P., Smith, A.J.H., Wisden, W., 1997. Ligand-gated ion channel subunit partnership: $GABA_A$ receptor alpha6 subunit gene inactivation inhibits delta subunit expression. J. Neurosci. 17, 1350–1362.

Laurie, D.J., Wisden, W., Seeburg, P.H., 1992. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J. Neurosci. 12, 4151–4172.

Majewska, M.D., 1992. Neurosteroids: endogenous bimodal modulators of the GABA-A receptor. Mechanism of action and physiological significance. Prog. Neurobiol. 38, 379–395.

Naylor, D.E., Liu, H., Wasterlain, C.G., 2005. Trafficking of GABA(A) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. J. Neurosci. 25, 7724–7733.

Nguyen, Q., Sapp, D.W., Van Ness, P.C., Olsen, R.W., 1995. Modulation of $GABA_A$ receptor binding in human brain by neuroactive steroids: species and brain regional differences. Synapse 2, 77–87.

Paxinos, G., Watson, C., 1997. The Rat Brain in Stereotaxic Coordinates, Edition 3. Academic Press, San Diego.

Petrie, J., Sapp, D.W., Tyndale, R.F., Park, M.K., Fanselow, M., Olsen, R.W., 2001. Altered GABA-A receptor subunit and splice variant expression in rats treated with chronic intermittent ethanol. Alcohol Clin. Exp. Res. 25, 819–828.

Poulter, M.O., Barker, J.L., O'Carroll, A.M., Lolait, S.J., Mahan, L.C., 1992. Differential and transient expression of $GABA_A$ receptor alpha-subunit mRNAs in the developing rat CNS. J. Neurosci. 12, 2888–2900.

Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H., Schofield, P.R., Seeburg, P.H., 1989. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338, 582–585.

Ramoino, P., Scaglione, S., Diaspro, A., Beltrame, F., Fato, M., Usai, C., 2004. $GABA_A$ receptor subunits identified in Paramecium by immunofluorescence confocal microscopy. FEMS Microbiol. Lett. 238, 449–453.

Sarto, I., Klausberger, T., Ehya, N., Mayer, B., Fuchs, K., Sieghart, W., 2002. A novel site on gamma 3 subunits important for assembly of GABA-A receptors. J. Biol. Chem. 277, 30656–30664.

Sergeeva, O.A., Krister, S., Eriksson, K.S., Sharonova, I.N., Vorobjv, V.S., Haas, H.L., 2002. GABA(A) receptor heterogeneity in histaminergic neurons. Eur. J. Neurosci. 16, 1472–1482.

Sigel, E., 2002. Mapping of the benzodiazepine recognition site on GABA(A) receptors. Curr. Top. Med. Chem. 2, 833–839.

Sigel, E., Buhr, R., 1997. The benzodiazepine binding site of $GABA_A$ receptors. Trends Pharmacol. Sci. 18, 425–429.

Sieghart, W., 1995. Structure and pharmacology of gamma-aminobutyric $acid_A$ receptor subtypes. Pharmacol. Rev. 47, 181–234.

Smith, K., Fisher, R.S., 1996. The selective GABAB antagonist CGP-35348 blocks spike-wave bursts in the cholesterol synthesis rat absence epilepsy model. Brain Res. 729, 147–150.

Wallace, R.H., Marini, C., Petrou, S., Harkin, L.A., Bowser, D.N., Panchal, R.G., Williams, D.A., Sutherland, G.R., Mulley, J.C., Scheffer, I.E., Berkovic, S.F., 2001. Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. Nat. Genet. 28, 49–52.

Wisden, W., Laurie, D.J., Monyer, H., Seeburg, P.H., 1992. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. 12, 1040–1062.

Wu, J., Ellsworth, K., Ellsworth, M., Schroeder, K.M., Smith, K., Fisher, R.S., 2004. Abnormal benzodiazepine and zinc modulation of GABA(A) receptors in an acquired absence epilepsy model. Brain Res. 1013, 230–240.

Zhu, W.J., Wang, J.F., Vicini, S., Grayson, D.R., 1996. Alpha 6 and gamma 2 subunit antisense oligodeoxynucleotides alter gamma- aminobutyric acid receptor pharmacology in cerebellar granule neurons. Mol. Pharmacol. 50, 23–33.