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# Depression of Glutamate and GABA Release by Presynaptic $GABA_B$ Receptors in the Entorhinal Cortex in Normal and Chronically Epileptic Rats

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**Key Words**

$GABA_B$ receptors · Entorhinal cortex · Glutamate release · GABA release · Epilepsy

**Abstract**

Presynaptic $GABA_B$ receptors ($GABA_BR$) control glutamate and GABA release at many synapses in the nervous system. In the present study we used whole-cell patch-clamp recordings of spontaneous excitatory and inhibitory synaptic currents in the presence of TTX to monitor glutamate and GABA release from synapses in layer II and V of the rat entorhinal cortex (EC) in vitro. In both layers the release of both transmitters was reduced by application of $GABA_BR$ agonists. Quantitatively, the depression of GABA release in layer II and layer V, and of glutamate release in layer V was similar, but glutamate release in layer II was depressed to a greater extent. The data suggest that the same $GABA_BR$ may be present on both GABA and glutamate terminals in the EC, but that the heteroreceptor may show a greater level of expression in layer II. Studies with $GABA_BR$ antagonists suggested that neither the auto- nor the heteroreceptor was consistently tonically activated by ambient GABA in the presence of TTX. Studies in EC slices from rats made chronically epileptic using a pilocarpine model of temporal lobe epilepsy revealed a reduced effectiveness of both auto- and heteroreceptor function in both layers. This could suggest that enhanced glutamate and GABA release in the EC may be associated with the development of the epileptic condition.



## Introduction

Transmitter release at cortical synapses is controlled by an array of presynaptic receptors. These provide a sensitive means of dynamically regulating transmission in a synapse-delimited way and, consequently, at the network level. In this laboratory, we have been investigating the control of both glutamate and GABA release by presynaptic receptors in the entorhinal cortex (EC). This is an area of the temporal lobe thought to be pivotally involved in processes of learning and memory, but also a site of network disruption in epilepsy, schizophrenia and a number of other neurological disorders. We have been particularly interested in comparing modulation of synaptic activity by auto- and heteroreceptors in neurones of the superficial layers of the EC, the main origin of input to the adjacent hippocampus, to that in the deeper layers, which are the major relay of hippocampal output. For example, in slices of rat EC, we have shown that NMDA autoreceptors tonically facilitate glutamate release in both layer II and layer V [1–3], but NMDA heteroreceptors seem to be present at GABA terminals only in layer II [2, 4]. Group III metabotropic glutamate receptors, rather surprisingly, increase spontaneous glutamate release in layer V, but decrease it in layer II [5]. However, the same receptors decrease GABA release at inhibitory synapses in layer II, but not in layer V [6].

The level of spontaneous GABA release, assessed by whole-cell patch-clamp recordings of spontaneous inhibitory postsynaptic currents (sIPSCs), is considerably

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greater in layer II compared to layer V [7]. In a recent study we investigated whether this could be dependent on differences in modulation of release by presynaptic $GABA_B$ autoreceptors in the two layers [8]. Using the $GABA_BR$ agonist, baclofen, we found that these receptors depressed GABA release in the two layers to around the same extent. However, high concentrations of a $GABA_BR$ antagonist actually increased the release of GABA, suggesting a tonic feedback activation of the autoreceptor. The latter effect was restricted to layer V, but was of insufficient magnitude to fully explain the difference in frequency of sIPSCs recorded in the two layers.

In addition to their postsynaptic actions and their role as autoreceptors, $GABA_BR$ have long been known to act as heteroreceptors, controlling the release of a wide variety of neuroactive agents, including amines, peptides and amino acids. In the present study we have examined the heteroreceptor control of glutamate release by $GABA_BR$ in the EC. There has been some debate over whether different subtypes of $GABA_BR$ mediate auto- and heteroreceptor functions [9–14]. The development of more potent and specific $GABA_BR$ agonists and antagonists has allowed a more detailed examination of the possibility of receptor subtypes at distinct spatial locations. One agonist, CGP44533, has been suggested to be largely inactive at postsynaptic receptors [15], whilst displaying a greater potency at heteroreceptors compared to autoreceptors [14, 16]. We have used this agonist to assess whether there may be differences in hetero- and autoreceptors in the EC.

The EC has been strongly implicated in the generation and propagation of temporal lobe epilepsies [see 4], and we have been looking at changes in control of transmitter release in chronically epileptic animals [e.g. 3]. It is apparent that mice that lack functional $GABA_BR$, either by deletion of the $GABA_{B1}$ subunit or mutation of the $GABA_{B2}$ subunit, display a phenotype characterized by profound tonic-clonic seizure activity [17–19]. $GABA_B$ autoreceptor function has been shown to be decreased in the hippocampus [20, 21] after electrical kindling, and similar changes in heteroreceptors in both hippocampus and amygdala have also been shown [22–24]. Currently, there is little information on whether presynaptic $GABA_BR$ are altered in the EC, although Gloveli et al. [25] reported an enhancement of autoreceptor function in layer III in kindled animals. Thus, in the second part of the present study, we examined the question of whether $GABA_BR$ control of either glutamate or GABA release is altered in rats with chronic pilocarpine-induced spontaneous seizures. Some of the results in this paper have been published in abstract form [26].

## Methods

*Slice Preparation*

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document. Combined entorhinal-hippocampal slices were prepared from male Wistar rats, as previously described [27]. Rats were anaesthetised with an intramuscular injection of ketamine (120 mg/kg) plus xylazine (8 mg/kg) and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (ACSF) chilled to 4°C. Slices (400 μm) were cut using a Vibroslice, and stored in ACSF bubbled with 95% $O_2$/5% $CO_2$, at room temperature. Following recovery for at least 1 h, individual slices were transferred to a recording chamber mounted on the stage of an Olympus BX51 microscope. The chamber was perfused (2 ml/min) with oxygenated ACSF (pH 7.4) at 30–32°C. The ACSF contained (in mM): NaCl (126), KCl (2.5), $NaH_2PO_4$ (1.25), $NaHCO_3$ (24), $MgSO_4$ (2), $CaCl_2$ (2.5), and D-glucose (10). Neurones were visualized using differential interference contrast optics and an infrared video camera.

*Electrophysiological Recordings*

Patch pipettes (1–4 MΩ) were pulled from borosilicate glass on a Flaming/Brown microelectrode puller. For recording EPSCs, pipettes were filled with a solution containing (in mM): Cs-methanosulphonate (120), HEPES (10), QX-314 (5), EGTA (10), $CaCl_2$ (0.34), NaCl (4), $MgCl_2$ (1), ATP-Na (4), and GTP-Na (0.4). IPSCs were recorded using a patch pipette solution containing: CsCl (120), HEPES (10), QX-314 (5), EGTA (10), $CaCl_2$ (0.34), $MgCl_2$ (1), ATP (4), and GTP (0.4). Solutions were adjusted to 290 mosm, and to pH 7.25–7.3 with CsOH. Whole-cell voltage-clamp recordings (holding potential –60 mV) were made from neurones in layer V and layer II of the medial division of the EC, using an Axopatch 200B amplifier. Series resistance compensation was not employed, but access resistance (10–30 MΩ) was monitored at regular intervals throughout each recording and neurones were discarded from analysis if it changed by more than ±10%. Liquid junction potentials were estimated using the calculator of pClamp 8 software, and compensated for in the holding potentials.

Under the above recording conditions, EPSCs in both layer II and V are mediated by the spontaneous release of glutamate acting at both AMPA and NMDA receptors [28]. No attempt was made to distinguish between events mediated by the two receptors, although pure NMDA receptor-mediated events are infrequent and most EPSCs reflect the activation of postsynaptic AMPA receptors. We did not pharmacologically block $GABA_A$ receptors in these recordings. However, using the internal and external solutions detailed above, all inward currents at the holding potential of –60 mV could be blocked by a combination of NBQX and 2-AP5 [29]. When recording IPSCs, the ACSF routinely contained NBQX (6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione di-sodium; 20 μM) and 2-AP5 (D,L-2-amino-5-phosphonovalerate; 50 μM) to block AMPA and NMDA receptors, respectively. Under these recording conditions, i.e. with similar concentrations of $Cl^-$ intra- and extracellularly and $V_h$ at –60 mV, IPSCs are recorded as inward currents, mediated by GABA acting at $GABA_A$ receptors [7, 8]. The inclusion of CsCl and QX-314 in the patch pipette solutions ensured that in all studies, postsynaptic $GABA_BR$ were blocked in the recorded neurones. Spon-

taneous release at both glutamate and GABA synapses is composed of events driven by action potentials invading presynaptic terminals, and quantal events, which are activity-independent, miniature EPSCs (mEPSCs) and mIPSCs. To avoid global network effects of bath-applied drugs, we restricted our studies to activity-independent events, and all studies were performed in the presence of TTX (1 μM).

Data were recorded to computer hard disk using Axoscope software. Minianalysis (Synaptosoft, USA) was used for analysis of inter-event interval (IEI), amplitude, rise time (10–90%) and decay time (to 50%) of mPSCs off-line. Frequencies of events were determined as reciprocal of IEI. mPSCs were detected automatically using a threshold-crossing algorithm. Threshold varied from neurone to neurone but was always maintained at a constant level in any given recording. At least 200 events were sampled during a continuous recording period for each neurone under each condition. To compare pooled data under control and drug conditions, we determined mean values of parameters of mPSCs in each cell and calculated the fractional changes before and after drug applications (e.g. the mean frequency in the presence of CGP44533 as a fraction of that in TTX alone). All error values stated in the text refer to standard error of the mean. We also assessed the significance of shifts in cumulative probability distributions of pooled data using the non-parametric Kolmogorov-Smirnov test. Student's t test and one-way ANOVA were used to assess differences in fractional changes before and after drug application.

*Chronically Epileptic Animals*

Initial experiments to determine the effects of $GABA_B$ agonists and antagonists were performed in slices taken from juvenile animals (4–6 weeks). In studies comparing chronically epileptic rats with age-matched controls, the animals were aged 4–6 months at the time of electrophysiological experiments.

Rats were rendered chronically epileptic using a modified version of the pilocarpine model described by Glien et al. [30]. They were pretreated with LiCl (200–300 mg/kg i.p.). Twenty-four hours later they received α-methyl scopolamine (1 mg/kg i.p.) to block the peripheral cholinergic effects of pilocarpine, and 15 min later received pilocarpine, administered in very low doses (5–10 mg/kg i.p.) repeated as necessary (up to a maximum of 40 mg/kg) at 30-min intervals until an acute status epilepticus (SE) was induced. After induction of SE, animals were administered the central muscle relaxant, xylazine (4 mg/kg i.m.), which reduced the severity of clonic contractions without preventing electrical seizure activity. After 1.5 h in SE, seizures were terminated with diazepam (10 mg/kg i.p.). When necessary they were orally administered glucose in normal saline for rehydration on the day of the SE and on the subsequent 2–3 days. Using this modified approach, we achieved a mortality rate of around 5–10%, and development of spontaneous recurrent seizures in 100% of animals. Age-matched control animals received the initial injection of LiCl, but nothing else.

Following the acute SE, animals developed evidence of epileptic seizure activity during a latent period of 6–10 weeks. This included the appearance of occasional head twitches, wet dog shakes, forepaw clonus, oro-facial movements and whole body jerks. Eventually, all animals began to display spontaneous recurrent seizures, which were characterized by rearing plus loss of balance, and accompanied by generalized clonic seizures. The licensing of our chronic experiments required that the animals be used for electrophysiology as soon as possible after developing chronic seizures. In practice this meant that most animals were used within 2 weeks after the first seizure was noted, up to a maximum of 4 weeks. Because animals were used as soon as possible, it was not possible to correlate adaptive changes with baseline seizure frequency.

*Materials*

Salts used in preparation of ACSF were 'Analar' grade and purchased from Merck/BDH (UK). All drugs were applied by bath perfusion. NBQX and 2-AP5 were obtained from Tocris (UK), $GABA_BR$ agonists (CGP44533 and CGP35024) and antagonists (CGP55845 and CGP64213) were a kind gift from Novartis Pharma (Basel, Switzerland), and TTX was supplied by Alamone Laboratories (Jerusalem, Israel). Concentration-response curves to agonists were constructed cumulatively, with each concentration being applied for a 10-min period. When the effects of antagonists were tested, after establishing control responses to agonists, the antagonists were perfused for at least 15 min, before re-testing of agonists.

## Results

Preliminary experiments determined that activation of $GABA_BR$ with either baclofen (3 μM) or CGP44533 (3 μM) reduced the frequency of sEPSCs in both layer II and layer V neurones without changing amplitude, indicative of a presynaptic depression of glutamate release [M.O. Cunningham, PhD thesis, unpubl. results]. All subsequent studies, described below, were made in the presence of TTX to isolate activity-independent release and rule out network effects of $GABA_BR$ activation. The experiments described were conducted on 67 neurones in layer II and 74 neurones in layer V of the medial EC. In a representative sample of neurones in the two layers the mean IEI was 296.3 ± 47.5 ms in layer V (n = 27) and 151.1 ± 20.5 ms in layer II (n = 30), reflecting average frequencies of around 3.5 and 6.6 Hz, respectively. Corresponding mean amplitudes were 9.7 ± 0.9 and 9.0 ± 0.8 pA.

*Effects of $GABA_BR$ Agonists on mEPSCs*

The effect of $GABA_BR$ agonist, CGP44533, was tested at concentrations from 10 nM to 25 μM on a total of 37 neurones in layer II and 44 in layer V. The drug caused a robust and reversible decrease in frequency of mEPSCs in both layers. Figure 1a illustrates this effect in one neurone recorded in layer II. The mean IEI in the control situation was 176 ± 4 ms. During perfusion with CGP44533 (0.5 μM) this increased to 259 ± 6.8 ms. Subsequent addition of the agonist at 1, 5 and 10 μM resulted

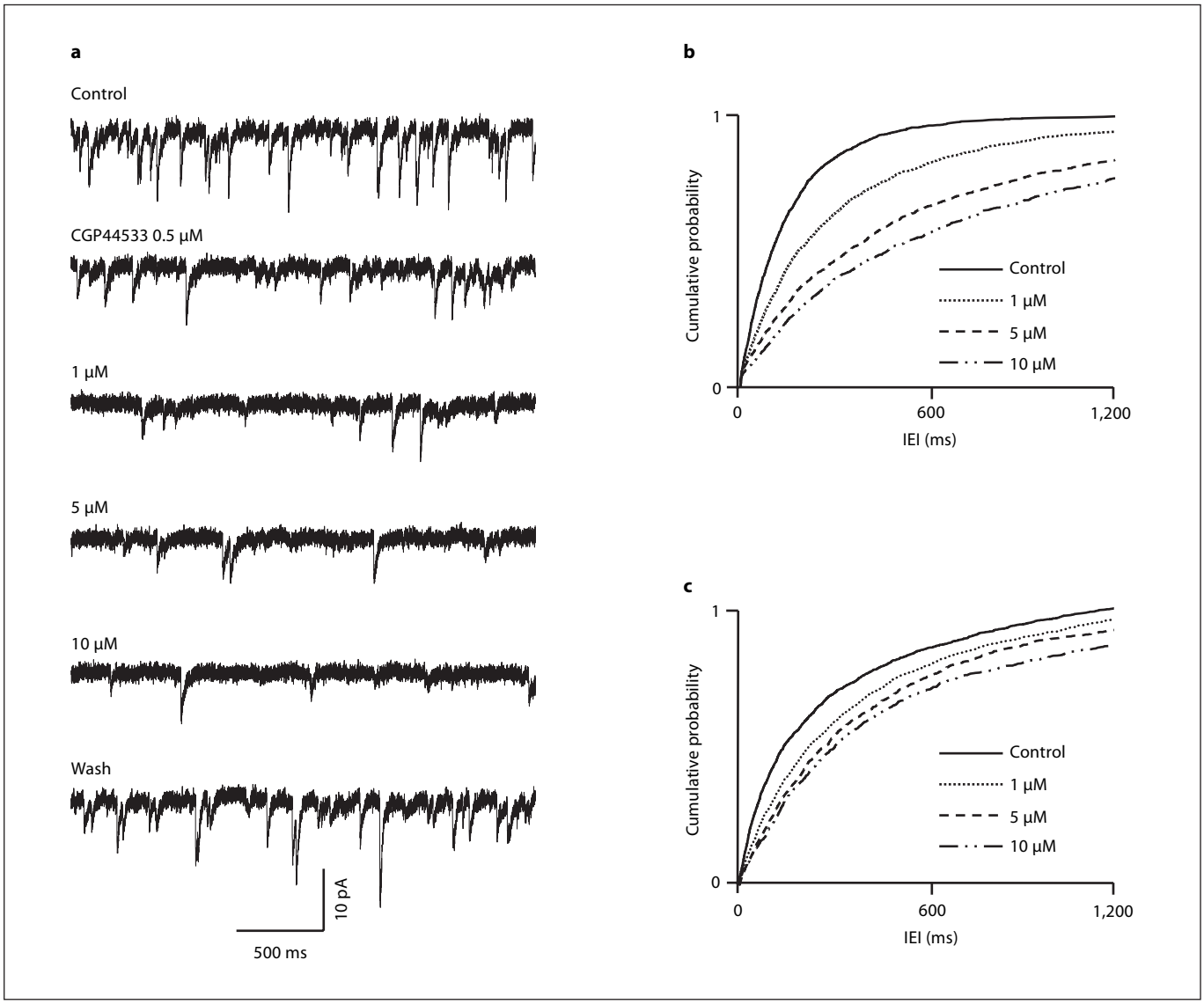


**Fig. 1.** Presynaptic $GABA_BR$ reduce glutamate release. **a** Whole-cell voltage-clamp recordings from a layer II neurone. The control trace shows mEPSCs recorded in the presence of TTX. The records below show the reduced frequency of mEPSCs in the presence of cumulative applications of CGP44533 at increasing concentrations, followed by recovery on washing. **b** The graphs show cumulative probability relationships of pooled data from neurones in layer II (n = 5) for inter-event interval (IEI) at three concentrations of CGP44533. The progressive shifts to the right illustrate the prolongations of IEI reflecting the reduced frequencies of events. **c** The cumulative probability data distributions for layer V neurones (n = 5) show less shift to the right, illustrating the reduced effectiveness of the agonist in this layer.

in increments in IEI to 389 ± 16, 897 ± 41 and 1,040 ± 52 ms, respectively. These changes reflect a concentration-dependent decrease in average instantaneous frequency from 5.7 Hz to 3.9, 2.6, 1.1 and 0.9 Hz over the range of concentrations tested. Figure 1b shows cumulative probability distributions of pooled IEI data for 1 (n = 12), 5 (n = 12) and 10 µM (n = 15) CGP44533. The substantial shift of these distributions to the right reflects the decreasing frequency of events and the change was highly significant at all concentrations (Kolmogorov-Smirnov test, $p > 0.001$). There was no change in amplitude of events at any concentration tested. In the presence of

TTX alone the mean amplitude of events was 9.5 ± 0.3 pA, and with the subsequent application of increasing concentrations of CGP44533, the corresponding mean values were 9.5 ± 0.3, 9.5 ± 0.3, 10.4 ± 0.4 and 9.4 ± 0.3 pA. Likewise, neither rise nor decay times of events were altered by the agonist. The mean control rise time was 3.3 ± 0.5 ms and the respective values in the presence of the four agonist concentrations were 3.6 ± 0.6, 3.1 ± 0.2, 3.7 ± 0.6 and 3.7 ± 0.3 ms. Corresponding values for decay times were 5.2 ± 0.7 ms (control), 6.4 ± 0.8 (0.5 μM), 5.7 ± 0.7 (1 μM), 6.1 ± 0.5 (5 μM) and 6.0 ± 0.4 (10 μM).

CGP44533 also reduced the frequency of mEPSCs without affecting the amplitude in layer V neurones. Cumulative probability curves for pooled IEI data are shown for 1, 5 and 10 μM concentrations of the agonist in figure 1c. The mean control IEI was 247 ± 8 ms. CGP44533 (0.5 μM) increased this to 289 ± 10 ms. At 1, 5 and 10 μM the agonist resulted in increments in IEI to 339 ± 25, 640 ± 63 and 739 ± 88 ms, respectively. These changes reflect a decrease in instantaneous frequency from 4.1 Hz to 3.5, 2.9, 1.6 and 1.4 Hz over the concentrations tested and indicate that the drug may be less effective at reducing mEPSC frequency in layer V compared to the layer II. However, as in layer II, the agonist had no effect on amplitude (table 1), rise or decay time of events (not shown).

A more detailed comparison of the effect of the agonist in the two layers is illustrated by the pooled data from all cells shown in figure 2. When the fractional reduction in mEPSC frequency is plotted against concentration (fig. 2a), the curve for layer V is shifted to the right of that for layer II, suggesting that the activation of the $GABA_B$ heteroreceptor is more effective at reducing glutamate release at synapses in the superficial layer. The divergence between the effect in the two layers increases with increasing concentration. The difference was significant at 5, 10 and 25 μM (t test, $p < 0.01$) but not at lower concentrations. Thus, the depression of mEPSC frequency was maximal with 10–25 μM CGP44533 in both layers, but the maximal depression was greater in layer II than in layer V. Figure 2b shows the concentration-response data normalized to the maximum depression of frequency. $EC_{50}$ values determined from these data were virtually the same (0.79 μM in layer II and 0.91 μM in layer V). The fractional frequencies and amplitudes for the data illustrated in figure 2b are shown in table 1.

We have tested the effect of a second agonist, CGP35024 (0.1–10 μM), on a total of 16 neurones in layer II and 14 in layer V. A similar picture emerged, with the drug being more effective in reducing mEPSC frequency in the superficial compared to the deep layer. Additionally, the concentration-response relationships in each layer were shifted a little to the left with respect to CGP44533. In layer II, fractional mEPSC frequencies for CGP35024 at 1 and 5 μM were 0.48 ± 0.04 (n = 8) and 0.20 ± 0.05 (n = 4) compared to 0.53 ± 0.05 (n = 12) and 0.25 ± 0.3 (n = 12) for CGP44533. Corresponding values in layer V were 0.58 ± 0.06 (n = 5) and 0.31 ± 0.07 (n = 3) for CGP35028, and 0.63 ± 0.05 (n = 14) and 0.38 ± 0.04 (n = 13) for CGP44533. Estimated $EC_{50}$ values for CGP35028 in layer II and layer V were 0.64 and 0.73 μM,

**Table 1.** Concentration-dependent effect of CGP44533 on mEPSCs in EC

| CGP44533 μM | Layer II mEPSCs | | | Layer V mEPSCs | | |
|---|---|---|---|---|---|---|
| | fractional frequency | fractional amplitude | n | fractional frequency | fractional amplitude | n |
| 0.01 | 0.89 ± 0.08 | 1.07 ± 0.07 | 4 | 0.91 ± 0.01 | 0.96 ± 0.04 | 9 |
| 0.1 | 0.76 ± 0.07 | 0.91 ± 0.10 | 3 | 0.83 ± 0.01 | 0.99 ± 0.06 | 7 |
| 0.5 | 0.67 ± 0.05 | 0.99 ± 0.09 | 14 | 0.76 ± 0.05 | 0.97 ± 0.05 | 14 |
| 1 | 0.53 ± 0.05 | 0.99 ± 0.48 | 12 | 0.63 ± 0.05 | 1.01 ± 0.02 | 14 |
| 2 | 0.39 ± 0.05 | 1.01 ± 0.02 | 9 | 0.48 ± 0.06 | 0.99 ± 0.07 | 10 |
| 5 | 0.25 ± 0.03 | 1.11 ± 0.10 | 12 | 0.38 ± 0.04 | 0.98 ± 0.05 | 13 |
| 10 | 0.17 ± 0.03 | 0.94 ± 0.04 | 15 | 0.29 ± 0.04 | 0.99 ± 0.08 | 15 |
| 25 | 0.17 ± 0.02 | 1.00 ± 0.06 | 8 | 0.29 ± 0.05 | 0.95 ± 0.09 | 11 |

Frequencies of events were calculated from IEI and fractional frequencies at each concentration represent the frequency in the presence of the drug as a fraction of that in the presence of TTX alone.

respectively. Again, CGP35028 had no significant effect on amplitude of mEPSCs in either layer (data not shown). In layer V the mean control amplitude was 8.2 ±1.2 pA, 9.6 ± 2.5 pA at 1 μM, and 8.4 ± 2.3 pA at 5 μM. In layer II, the fractional amplitude of events in the presence of the agonist were 0.91 ± 0.4 and 1.06 ± 0.03 at 1 and 5 μM, respectively, and 1.06 ± 0.12 and 1.03 ± 0.11 in layer V.

*Effects of $GABA_BR$ Antagonists on mEPSCs*

The reduction in frequency of mEPSCs by CGP44533 (10 μM) was completely blocked by pre-perfusion with the $GABA_BR$ antagonist, CGP64213 (10 μM) in both layer V (n = 3) and layer II (n = 3). In layer II, the fractional frequency of mEPSCs was 0.18 ± 0.03 in the presence of the agonist alone and 1.04 ± 0.02 when the antagonist was present. In layer V, the corresponding values were 0.30 ± 0.03 and 0.98 ± 0.06. Another $GABA_BR$ antagonist (CGP55845, 5 μM) also abolished the effects of CGP44533 (not shown). In the presence of the antagonists alone, occasional neurones showed a tendency towards an increase in frequency of mEPSCs, but overall there was no significant effect on either frequency or amplitude in either layer. The results of these studies are summarized in table 2.

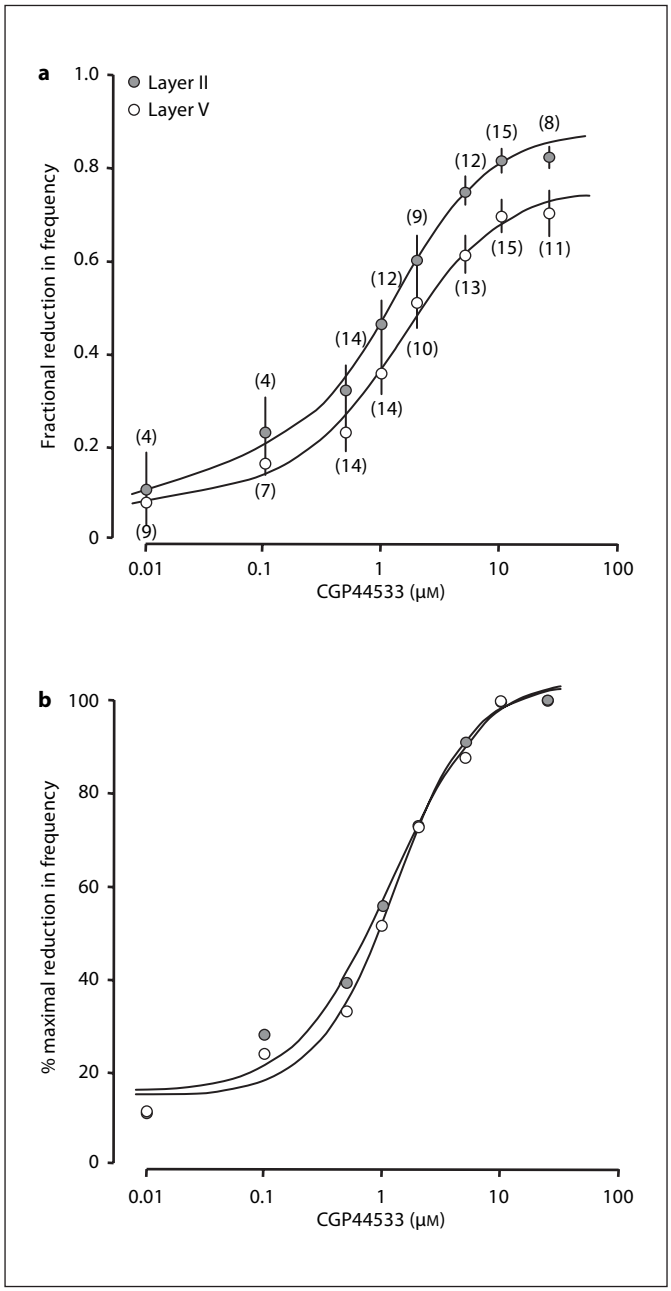


**Fig. 2.** Concentration-response data for the reduction in mEPSC frequency by CGP44533 in layer V and layer II neurones. **a** Data plotted as fractional reduction in frequency against concentration show that the $GABA_BR$ agonist was consistently less effective at reducing mEPSC frequency in layer V compared to layer II. The difference was significant from 10–25 μM, where it is clear that there is a reduced maximal effect in the deep layer. Figures in parentheses are the numbers of neurones tested at each concentration. **b** When the data are normalised to the maximal depression in frequency, the curves are closely superimposed.

**Table 2.** Effect of $GABA_BR$ antagonists on mEPSCs

| Antagonist μM | Layer II mEPSCs | | | Layer V mEPSCs | | |
|---|---|---|---|---|---|---|
| | fractional frequency | fractional amplitude | n | fractional frequency | fractional amplitude | n |
| CGP55845 (5) | 1.03 ± 0.06 | 1.03 ± 0.14 | 5 | 1.11 ± 0.25 | 0.96 ± 0.12 | 3 |
| CGP64213 (10) | 1.01 ± 0.01 | 0.98 ± 0.19 | 16 | 1.00 ± 0.03 | 0.99 ± 0.06 | 13 |

Neither drug had any effect on frequency or amplitude of events.

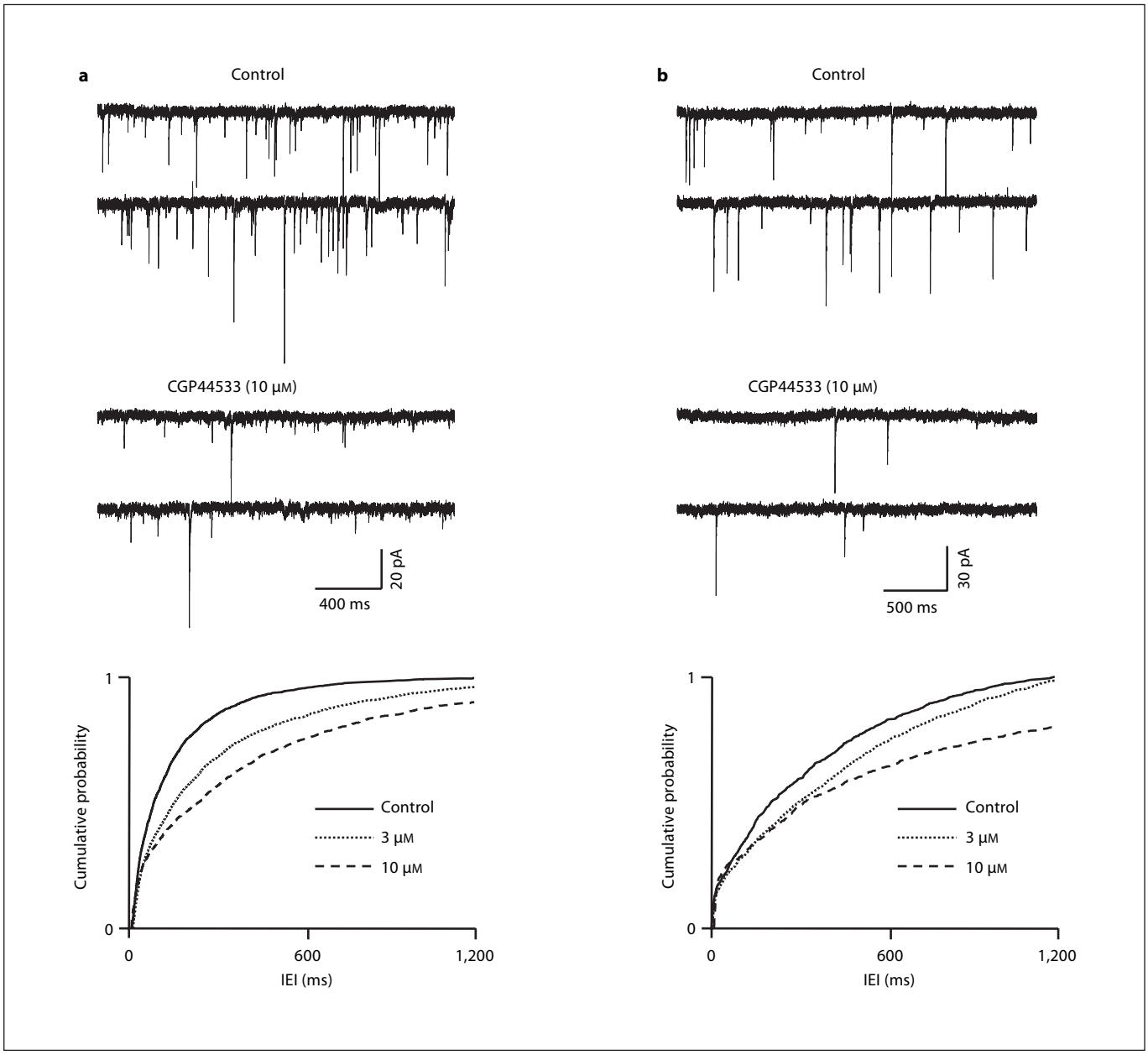


**Fig. 3.** Presynaptic $GABA_BR$ reduce GABA release. **a** Voltage-clamp recordings of mIPSCs in a layer II neurone (TTX present throughout). CGP44533 substantially reduced the frequency of events. The cumulative probability curves show pooled data from 15 neurones with two concentrations of the agonist. The shifts of the curves to the right illustrate the concentration dependent increase in IEI. **b** Similar data for layer V neurones (n = 15).

*Effect of CGP44533 on mIPSCs*

We have previously described depression of mIPSC frequency by baclofen in the EC [8]. In the present studies we have compared the effect of CGP44533 on mIPSCs in layer II and layer V to those described above for mEPSCs. In a typical sample of layer V neurones (n = 10) mIPSCs had an IEI of 325 ± 46 ms and amplitude of 18.8 ± 2.4 pA. Rise and decay times were 2.8 ± 0.2 and 6.9 ± 0.9 ms, respectively. Corresponding mean values in layer II neurones (n = 10) were 29 ± 4 ms (IEI), 19.2 ± 1.4 pA (amplitude), 2.5 ± 0.1 ms (rise) and 7.5 ± 0.4 ms (decay). The only difference lay in the frequency of events, which

**Table 3.** Effect of CGP44533 on mIPSCs

| CGP44533 μM | Layer II mIPSCs | | | Layer V mIPSCs | | |
|---|---|---|---|---|---|---|
| | fractional frequency | fractional amplitude | n | fractional frequency | fractional amplitude | n |
| 3 | 0.54 ± 0.06 | 1.04 ± 0.06 | 15 | 0.56 ± 0.06 | 0.97 ± 0.06 | 15 |
| 10 | 0.35 ± 0.05 | 0.94 ± 0.08 | 15 | 0.40 ± 0.05 | 0.98 ± 0.04 | 15 |

Neither drug had any effect on frequency or amplitude of events. Frequencies of events were decreased to about the same extent in the two layers, and amplitudes were unaltered.

was much higher in layer II (approx. 34 Hz) than in layer V (3 Hz), as we previously described [7]. For the purposes of comparison of the effects of CGP44533, we used concentrations that were intermediate (3 μM) and essentially maximal (10 μM) for effects on mEPSCs.

The records in figure 3a show a strong reduction in mIPSC frequency by CGP44533 (10 μM) in an individual layer II neurone and the cumulative probability curves represent pooled data at the two concentrations tested (n = 15 in each case). The shift to the right of the IEI distributions in the presence of the agonist reflects a concentration-dependent reduction in mIPSC frequency. Figure 3b shows data from layer V neurones (n = 15 at each concentration), and illustrates that the agonist had very similar effects to that seen in layer II. In layer V the mean IEI was increased from 569 ± 59 to 1,146 ± 184 ms in the presence of 5 μM CGP44533, and further to 1,863 ± 366 ms when 10 μM was added. In layer II, the control IEI was 140 ± 35 ms and this increased to 289 ± 58 and 536 ± 87 ms in the presence of the two agonist concentrations. The mean fractional mIPSC frequencies in the two layers reflect these changes (table 3). Amplitudes of mIPSCs were unaffected by the agonist in either layer (table 3) and likewise no changes in rise or decay times were observed (not shown).

Figure 4 shows a quantitative comparison of the effects of CGP44533 on mEPSCs and mIPSCs in the two layers. At both 3 and 10 μM, the agonist induced a similar depression of mIPSC frequency in layer II and layer V. The depression of mEPSC frequency was quantitatively similar to that of mIPSCs in layer V, but that induced in layer II was significantly greater ($p < 0.05$) at both agonist concentrations. Thus, there was a differential effect of CGP44533 at excitatory terminals in layer V compared to layer II and compared to inhibitory terminals in both layers.

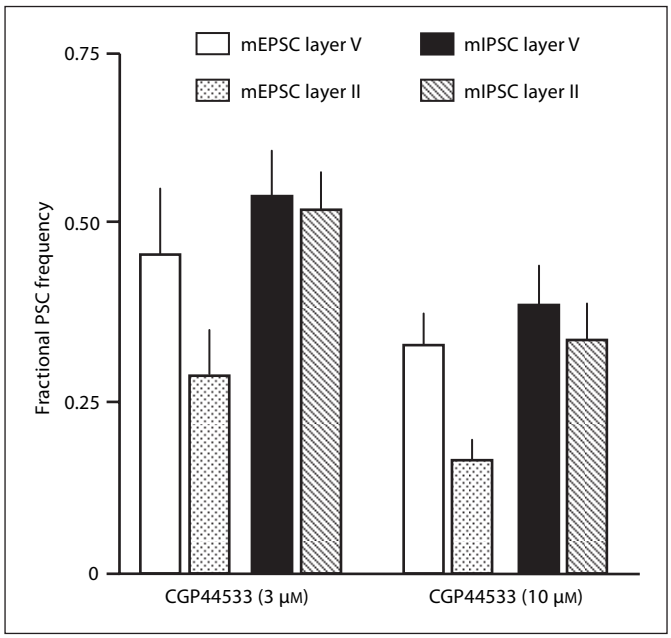


**Fig. 4.** Summary data for the effects of CGP44533 on mPSC frequency. The columns represent the mean fractional frequencies of events in 3 μM (left) and 10 μM of the agonist and the errors are standard error of the mean. Quantitatively, the only difference was a significantly greater reduction in mEPSC frequency in layer II, which was seen at both concentrations. The degree of reduction of mEPSCs in layer V and mIPSCs in both layers was similar. n = 15 in all cases except for mEPSCs in layer II (n = 13) and layer V (n = 10).

*Effects of Antagonists on mIPSCs*

As with mEPSCs, the reduction in frequency of mIPSCs by CGP44533 was abolished by a $GABA_BR$ antagonist. In layer II the fractional frequency in the presence of the CGP44533 alone (10 μM) was 0.35 ± 0.05, but when applied together with CGP55845 (5 μM) it was 1.06

± 0.06 (n = 3). Likewise, in three neurones in layer V the corresponding values were 0.40 ± 0.04 and 0.97 ± 0.04, respectively.

CGP55845 alone had no significant effect on mIPSC frequency, amplitude, rise or decay times. Fractional frequencies in the presence of CGP55845 were 1.02 ± 0.06 in layer II (n = 5) and 1.11 ± 0.25 in layer V (n = 6). As was the case with mEPSCs, occasional neurones did show quite a clear and consistent increase in frequency when CGP55845 was applied, particularly in layer V, but most showed no effect, or minor increases or decreases in frequency. Also, CGP64213 (10 μM) had no effect on mIPSCs in either layer II (1.01 ± 0.01, n = 16) or layer V (1.0 ± 0.03, n = 10).

*$GABA_BR$ Function in Epileptic Animals*

There are some changes in the frequency and characteristics of mEPSCs and mIPSCs associated with the development of the chronic epileptic condition. Some of these have been described briefly elsewhere [31–33]. Detailed accounts of these changes are beyond the scope of the current paper, and are currently in preparation [G. Ayman, G.L. Woodhall and R.S.G. Jones, unpubl. results]. Generally, data in a large population of neurones indicate a slightly increased frequency of mEPSCs in both layers, accompanied by a small increase in amplitude and a slowing of both rise and decay times, but it should be stressed that none of the differences are pronounced. mIPSCs, in contrast to mEPSCs, show a decreased frequency in epileptic animals, but amplitudes were also concurrently increased. Rise times of mIPSCs are generally unaltered but decay times, in both layers, are prolonged. These data indicate that there may be pre- and postsynaptic changes in both excitation and inhibition in the epileptic EC, but that these changes, at least assessed by means of spontaneous synaptic currents, are not dramatic and there is considerable overlap and variation. Thus, in the smaller samples of cells studied below, the differences noted in our larger population of data are not necessarily reflected.

*Effects of CGP44533 on mEPSCs in Epileptic Animals*

Perfusion with CGP44533, produced a similar depression of mEPSC frequency in older age-matched (AM) control animals to that seen in juvenile animals (see above). However, when tested on mEPSCs in slices from epileptic animals, the drug was considerably less effective. For example, in 5 layer II neurones in AM control slices a mean IEI of 143 ± 20 ms was increased to 462 ± 84 ms in the presence of CGP44533 at 10 μM, equating to a mean fractional frequency of 0.33 ± 0.07. In corresponding studies in epileptic slices, the IEI was increased from 169 ± 45 to 333 ± 71 ms by CGP44533 at 10 μM, giving a fractional frequency of 0.66 ± 0.11 (n = 7). The mean amplitude of events in AM control slices, as in juvenile slices, was unaltered by CGP44533 (12.4 ± 0.9 vs. 11.9 ± 0.7 pA), and this was the same in epileptic slices (13.1 ± 1.8 vs. 12.0 ± 1.7 pA). Likewise, rise times (AM control 1.6 ± 0.2 vs. 1.5 ± 0.1 ms; epileptic 2.8 ± 0.6 vs. 3.0 ± 0.7 ms) and decay times (AM control 2.2 ± 0.2 vs. 2.0 ± 0.1 ms; epileptic 3.2 ± 0.3 vs. 3.1 ± 0.4 ms) of events were unaltered by the agonist in either group. These results were mirrored by those with the lower concentration (3 μM) of CGP44533 in layer II. The fractional frequency of mEPSCs in these studies was 0.63 ± 0.11 (n = 4) in AM control slices but only 0.81 ± 0.17 (n = 6) in layer II neurones recorded in slices from epileptic animals. Thus, these data reflect a considerable reduction in the effectiveness of the $GABA_BR$ agonist at depressing mEPSC frequency. Very similar results were seen in layer V neurones and the results of changes in frequency for both layer II and layer V are summarized by the bar graphs in figure 5a. Again, as in layer II, the amplitudes rise and decay times of mEPSCs in layer V were unaffected by CGP44533 in slices from AM control or epileptic animals.

*Effects of CGP44533 on mIPSCs in Ipileptic Animals*

mIPSCs showed a similar change in sensitivity to CGP44533 as mEPSCs in neurones recorded in slices from epileptic animals. Thus, the depression in frequency of mIPSCs by the $GABA_BR$ agonist was less marked compared to that seen in control slices, in both layer V and layer II. The data for layer V neurones with CGP44533 at 10 μM are representative. In AM control slices, the agonist increased mean IEI to 400 ± 49 from 133 ± 19 ms, representing a fractional frequency of 0.36 ± 0.03 in the presence of the drug. In contrast, in neurones (n = 5) in epileptic slices the mean IEI was 103 ± 35 ms before the drug was applied and increased to 159 ± 88 ms in its presence, a fractional frequency of 0.63 ± 0.12. Amplitudes of mIPSCs were unaltered in either AM control (13.9 ± 2.8 vs. 15.1 ± 3.1 pA) or epileptic slices (21.9 ± 4.3 vs. 20.1 ± 2.9 pA). As with mEPSCs, neither rise times (AM control 2.5 ± 0.3 vs. 2.2 ± 0.3 ms: epileptic 2.4 ± 0.4 vs. 2.8 ± 0.5 ms) nor decay times (AM control 5.3 ± 1.5 vs. 5.4 ± 1.4 ms; epileptic 7.7 ± 2.5 vs. 6.9 ± 3.4 ms) were altered by the agonist. The data on frequency changes together with those for a lower dose of the agonist, and also for layer II neurones, are summarized in figure 5b.

*Effects of $GABA_BR$ Antagonists in Epileptic Animals*

Finally, we examined the effects of a $GABA_BR$ antagonist alone on mEPSCs and mIPSCs in AM control and epileptic rats. CGP55845 (1 and 5 μM) had little discernible effect on the frequency or amplitude of either current, in either layer and in either experimental group. Thus, in layer II neurones fractional mEPSC frequencies in the presence of the antagonist (at 5 μM) were 0.99 ± 0.08 in control neurones (n = 5) and 0.94 ± 0.12 in epileptics (n = 6). In layer V the corresponding values were 1.12 ± 0.04 (n = 4) and 0.97 ± 0.04 (n = 4). Effects on mIPSCs were more variable, but there were no significant differences detected. In layer II, the fractional frequency was 1.04 ± 0.06 in controls (n = 4) and 1.24 ± 0.29 in epileptics (n = 6), and in layer V the respective values were 1.12 ± 0.07 (n = 3) and 0.95 ± 0.10 (n = 5).

## Discussion

Using the $GABA_BR$ agonist, baclofen, we have shown previously that spontaneous release of GABA at synapses in both deep and superficial layers of the EC can be depressed via the activation of a $GABA_B$ autoreceptor [8] with baclofen. We have now confirmed these observations with a second agonist, CGP44533, which induced a similar depression of GABA release. In terms of the percentage reduction in mIPSC frequency, this was similar in layer V and layer II. This suggests that there is probably a single $GABA_BR$ responsible for control of GABA release in the deep and superficial layers, or that CGP44533 cannot discriminate between different receptors present in the two layers. The mechanism of the autoreceptor effect remains unknown. We have shown that the depression of release applies not only to activity driven release [8] but, like others [34–36], to quantal release occurring in the absence of spontaneous action potentials also. The latter effect is unlikely to be related to the established action of $GABA_BR$ on GIRKs [37, 38] or voltage-gated calcium channels [39, 40]. Others have suggested that the action of $GABA_B$ autoreceptors may involve G-protein interaction with protein kinase C [37, 41], protein kinase A [42], or direct effects on the exocytotic machinery [43], but we have no information on such possible mechanisms at present.

We now demonstrate that, in common with many other brain regions [9], glutamate terminals in both deep and superficial layers of the EC bear inhibitory $GABA_B$ heteroreceptors, and that these receptors can suppress quantal, activity-independent glutamate release. We have

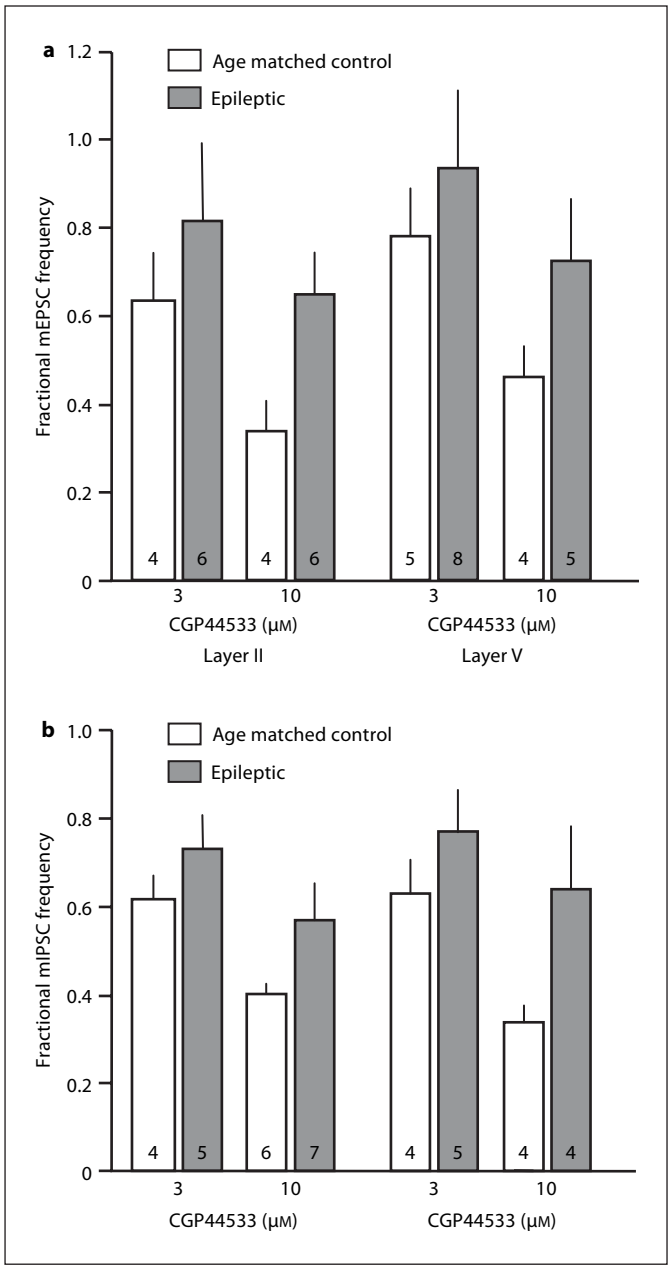


**Fig. 5.** Summary of the effects of presynaptic $GABA_BR$ activation on glutamate and GABA release in slices from epileptic rats. **a** The reduction in frequency of mEPSCs was less in epileptic animals. This was the case at both concentrations and in both layer II and layer V, although only the difference at 10 μM reached significance. **b** A very similar picture was seen with mIPSCs, with a reduced effect in both layers. Again, only the difference at 10 μM was significant. Numbers of cells recorded are shown at the foot of the bars.

previously found that baclofen can substantially depress both sEPSC and mEPSC frequency in layer V neurones [M.O. Cunningham, PhD thesis, unpubl. results]. In the current study we found that two other potent $GABA_BR$ agonists, CGP44533 and CGP35024, also robustly decreased mEPSC frequency, with the latter drug being slightly more potent. $GABA_BR$ antagonists abolished the effects of CGP44533, and neither agonist had any effect on amplitude or kinetics of mEPSCs, so the results clearly indicate that they were acting at presynaptic $GABA_BR$ to reduce glutamate release. As with the autoreceptor, this heteroreceptor control of glutamate release could be exerted on activity-independent quantal events, but the precise mechanism requires further investigation.

There is an increasing body of evidence to indicate heterogeneity of $GABA_B$ auto- and heteroreceptors [10–14]. The reason for selecting CGP44533 as an agonist for use in our studies was because it has been suggested to be largely inactive at postsynaptic receptors [15] but to be selective for heteroreceptors over autoreceptors [14, 16]. Our studies do not directly support such a conclusion, although the situation was not straightforward. In terms of percentage change, the drug had a very similar effect at autoreceptors in layer II and layer V and also at heteroreceptors in layer V. However, CGP44533 was consistently more effective in layer II, and induced a greater maximum depression of mEPSC frequency in this layer. This could suggest that the heteroreceptor in layer II is different to that in layer V, and indeed to the autoreceptor in either layer. However, the $EC_{50}$ values for depression of mEPSC frequency were virtually identical in layer II and V, which more likely indicates that the receptor is the same in both layers, but that glutamate terminals in the superficial layer express a greater density of the receptor. It is also possible that the receptor is expressed at the same density, but is less efficiently coupled to the intracellular effector mechanism in layer V, or even coupled to a different effector. However, whilst the difference between layer II and V is interesting, it should be stressed that it is not dramatic, and our data do not strongly support heterogeneity of receptors on glutamate and/or GABA terminals in the EC.

In a previous investigation [8] we reported that a $GABA_BR$ antagonist, CGP55845, increased the frequency of mIPSCs in layer V (but not layer II) and we suggested that this resulted from blockade of tonic feedback activation of $GABA_BR$, similar to that seen in other brain areas [36, 44]. However, mIPSC frequency in either layer was not significantly affected by $GABA_BR$ antagonists in the present study, although there were occasions where a clear increase was seen. It should be noted that the increase in mIPSC frequency in layer V reported previously [8] occurred with very high concentrations of the antagonist (10–50 μM). In the present study, lower concentrations of the antagonists failed to consistently alter mIPSC frequency, despite the fact that the same concentrations were able to abolish the decrease in frequency induced by $GABA_BR$ agonists. Thus, at present we are unsure if the effects of the high concentrations of CGP55845 [8] are the result of blockade of tonic feedback activation of the autoreceptors, or result from another, as yet undetermined, effect of the drug.

In the case of mEPSCs, again the antagonists had no significant effect on mEPSC frequency at concentrations where the effects of bath-applied agonists were abolished, suggesting that in our slices, $GABA_B$ heteroreceptors were not normally tonically activated. Again, there were occasional instances where mEPSC frequency was clearly increased, which could indicate that under some circumstances there is a tonic spillover of GABA to heteroreceptors on glutamate terminals. However, in other studies [M.O. Cunningham and R.S.G. Jones, unpubl. results] we found that high concentrations of CGP55845 actually decreased mEPSC frequency and amplitude in layer V, so again there may be other effects of the antagonists to consider that may not be related to $GABA_BR$ blockade. It should be remembered that our experiments were all conducted in TTX where ambient GABA release would be reduced. Also, in contrast to slices, a much higher baseline release of GABA probably occurs in vivo, so tonic physiological activation of both auto- and heteroreceptors may well occur.

The role of the EC in temporal lobe epilepsy has received increasing attention in recent years, and there is strong clinical and experimental evidence to suggest a role in the initiation and propagation of seizures of temporal lobe-limbic system origin [see 4]. Here, we demonstrate that in a model of chronic temporal lobe seizures there is a decline in the depressant effects of presynaptic $GABA_BR$ on both glutamate and GABA release. The effect was further generalized in that both deep and superficial layers of the EC were affected. A decline in $GABA_B$ autoreceptor function in CA1 and the dentate gyrus has previously been reported in electrical kindling models of temporal lobe epilepsy [20, 21]. Likewise, decreased heteroreceptor function has been described in the amygdala and hippocampus in kindling or pilocarpine-induced chronic seizure animals [22–24], and there is an indication that both auto- and heteroreceptor function may be reduced in human neocortical tissue isolated from pa-

tients with temporal lobe epilepsy [45]. However, it should be noted that Gloveli et al. [25] have reported an *increased* $GABA_B$ autoreceptor function in layer III of the EC in electrically kindled rats. This was accompanied by an apparent increase in immunocytochemical labeling of $GABA_BR$ across all layers of the EC, although this labeling does not distinguish between pre- and postsynaptic receptors. We have not yet studied either auto- or heteroreceptor function in layer III in our pilocarpine-treated animals. It should be noted that layer III differs from the other layers of the EC to some extent, in that it shows pronounced cell loss in both human epileptic patients and in animal models [46, 47]. Thus, we should not assume that the decrease in presynaptic $GABA_BR$ function we find in layers II and V would be mirrored by a similar effect in layer III, and this requires further study.

The question arises as to the significance of our results for the genesis and maintenance of the chronic seizures. It is difficult to relate the results of other studies that have looked at the expression of $GABA_BR$ in animal models and human epilepsy tissue to the functional studies discussed above and reported here. To start with, expression and immunocytochemical labeling studies do not distinguish between pre- and postsynaptic receptors. Examination of the literature reveals a bewildering array of changes, including both up- and down-regulation, which vary markedly depending on the model used, the area examined, the receptor subunits studied and the time point in the process of chronic seizure generation [25, 48–53]. However, what is clear is that transgenic mice lacking functional $GABA_BR$ present epileptic phenotypes [17–19, 54], and although the specific contribution of loss of post- or presynaptic auto/heteroreceptor function in these phenotypes is unclear, it seems certain that $GABA_BR$ control helps to prevent hypersynchrony in cortical networks.

Clearly, both excitation and inhibition are subject to such control in the EC. It could be argued that the effects we observe on mPSCs may be less important than effects on action potential driven release in terms of epileptogenesis. However, there is no reason to presume a priori that quantal release of glutamate and GABA are unimportant in control of network excitability. In fact, the majority of spontaneous release of both transmitters in the EC is TTX-insensitive [1, 4, 7]. In any case, the reduced $GABA_BR$-mediated control of quantal release that we see mirrors the changes reported elsewhere for control of action potential-dependent release [20–25]. Thus, whatever the effector mechanisms involved, impaired $GABA_BR$ function would be felt by both forms of release in the intact epileptic brain. The loss of inhibitory control of glutamate release could result in enhanced excitatory transmission and increased network excitability leading to epileptogenesis. It is more difficult to see how the reduced autoreceptor control of GABA release may fit in. One possibility is that this is an adaptive change occurring in response to the epileptogenic state, designed to increase inhibition across the network and reduce the propensity to synchronization. However, GABA interneurones are increasingly being viewed as drivers of network synchrony [55–57], and that pathological changes in interneurone driven synchronization of principal neurones [58] and excitatory actions of GABA [59] could be underlying factors in epilepsy. Thus, a loss of feedback autoreceptor control of inhibition could be a destabilizing factor in network excitability, exacerbated by the concurrent loss of inhibitory control of glutamate release. If $GABA_A$ receptor-mediated depolarizing events contribute to epileptogenesis, then increased GABA release consequent upon decreased $GABA_B$ autoreceptor function could exacerbate this effect [59–61]. However, at present we do not know whether the reduced effectiveness of presynaptic $GABA_BR$ is a causal factor in chronic epilepsy or casually related to it. An important next step will be to determine the time course of changes in these presynaptic receptors in relationship to the development of the chronic recurring seizures.


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