Extrasynaptic $\alpha\beta$ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons

Martin Mortensen and Trevor G. Smart

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK

Extrasynaptic $GABA_A$ receptors that are tonically activated by ambient GABA are important for controlling neuronal excitability. In hippocampal pyramidal neurons, the subunit composition of these extrasynaptic receptors may include $\alpha5\beta\gamma$ and/or $\alpha4\beta\delta$ subunits. Our present studies reveal that a component of the tonic current in the hippocampus is highly sensitive to inhibition by Zn^{2+}. This component is probably not mediated by either $\alpha5\beta\gamma$ or $\alpha4\beta\delta$ receptors, but might be explained by the presence of $\alpha\beta$ isoforms. Using patch-clamp recording from pyramidal neurons, a small tonic current measured in the absence of exogenous GABA exhibited both high and low sensitivity to Zn^{2+} inhibition (IC_{50} values, 1.89 and 223 μM, respectively). Using low nanomolar and micromolar GABA concentrations to replicate tonic currents, we identified two components that are mediated by benzodiazepine-sensitive and -insensitive receptors. The latter indicated that extrasynaptic $GABA_A$ receptors exist that are devoid of $\gamma2$ subunits. To distinguish whether the benzodiazepine-insensitive receptors were $\alpha\beta$ or $\alpha\beta\delta$ isoforms, we used single-channel recording. Expressing recombinant $\alpha1\beta3\gamma2$, $\alpha5\beta3\gamma2$, $\alpha4\beta3\delta$ and $\alpha1\beta3$ receptors in human embryonic kidney (HEK) or mouse fibroblast (Ltk) cells, revealed similar openings with high main conductances (~25–28 pS) for $\gamma2$ or δ subunit-containing receptors whereas $\alpha\beta$ receptors were characterized by a lower main conductance state (~11 pS). Recording from pyramidal cell somata revealed a similar range of channel conductances, indicative of a mixture of $GABA_A$ receptors in the extrasynaptic membrane. The lowest conductance state (~11 pS) was the most sensitive to Zn^{2+} inhibition in accord with the presence of $\alpha\beta$ receptors. This receptor type is estimated to account for up to 10% of all extrasynaptic $GABA_A$ receptors on hippocampal pyramidal neurons.

(Resubmitted 26 July 2006; accepted after revision 4 October 2006; first published online 12 October 2006)
Corresponding author T. G. Smart: Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK. Email: t.smart@ucl.ac.uk

The distribution of specific $GABA_A$ receptor isoforms at synaptic and extrasynaptic locations will profoundly influence neuronal excitability. Currently, the archetypal synaptic $GABA_A$ receptor is likely to be composed of $\alpha\beta\gamma$ subunits (Moss & Smart, 2001; Farrant & Nusser, 2005), whereas extrasynaptic $GABA_A$ receptors will not only comprise these subtypes (Thomas *et al.* 2005), but also $\alpha\beta\delta$ subtypes (Farrant & Nusser, 2005; Mangan *et al.* 2005). In addition, some populations of extrasynaptic receptors will also contain specific α isoforms, such as $\alpha4$, $\alpha5$ and $\alpha6$ (Semyanov *et al.* 2004; Caraiscos *et al.* 2004; Farrant & Nusser, 2005). Thus expressing such a diverse spectrum of receptor subunits will confer quite distinctive pharmacological and physiological profiles on the extrasynaptic $GABA_A$ receptors. With regard to their physiology, low concentrations of ambient GABA can activate extrasynaptic receptors causing a small, but persistent, Cl^- current (Isaacson, 2000; Mody, 2001), which results in tonic inhibition thus enabling neuronal excitability to be regulated (Mitchell & Silver, 2003).

Previous studies of cerebellar (Brickley *et al.* 1999) and hippocampal neurons (Yeung *et al.* 2003) indicate that some of the $GABA_A$ receptors mediating tonic inhibition have a high sensitivity to GABA. Variations in GABA potency have been reported to depend on the α subunit present in recombinant $\alpha\beta\gamma$ receptors, with a relative order, based on GABA EC_{50} values determined from dose–response curves, of: $\alpha6 > \alpha1 > \alpha2 > \alpha4 > \alpha5 \approx \alpha3$ (Knoflach *et al.* 1996; Böhme *et al.* 2004; Feng & Macdonald, 2004). However, as $\alpha6$ subunit-containing receptors are found exclusively in cerebellar granule cells (Fritschy & Brünig, 2003) and the dorsal cochlear nucleus (Sieghart & Sperk, 2002), they cannot account for the high GABA sensitivity associated with tonic inhibition in hippocampal pyramidal cells. Alternatively, the high GABA potency might indicate the presence

 DOI: 10.1113/jphysiol.2006.117952

of δ subunit-containing receptors. Recombinant $\alpha4\beta3\delta$ receptors are highly sensitive to GABA (Brown *et al.* 2002) and $\alpha4\beta\delta$ isoforms have been proposed as extrasynaptic receptors on hippocampal pyramidal cells (Mangan *et al.* 2005). Furthermore, a comparison of GABA potency on recombinant $\alpha1\beta2/3\gamma2$ and $\alpha1\beta2/3$ receptors, revealed that receptors lacking $\gamma2$ subunits are at least 5-fold more sensitive to GABA (Verdoorn *et al.* 1990; Sigel *et al.* 1990; Fisher & Macdonald, 1997; Amato *et al.* 1999), raising the possibility that $\alpha\beta$ receptors may also contribute to the extrasynaptic receptor population. However, generally it is thought that $\alpha\beta$ receptors are unlikely to exist in neurons; however, immunocytochemistry (Sieghart & Sperk, 2002) and single-channel (Brickley *et al.* 1999) studies have both provided some evidence to the contrary. As the $\gamma2$ subunit is important in mediating the clustering of $GABA_A$ receptors near to the scaffold protein gephyrin at inhibitory synapses (Moss & Smart, 2001; Luscher & Keller, 2004), it appears that $\alpha\beta$ $GABA_A$ receptors are unlikely to reside in significant numbers at synapses.

The aim of this study was to investigate whether $\alpha\beta$ subunit $GABA_A$ receptors are expressed on the surface of hippocampal pyramidal cells. By using a combination of pharmacological and electrophysiological approaches, we show that δ and γ subunit-lacking $\alpha\beta$ $GABA_A$ receptors are likely to be expressed in low numbers in the extrasynaptic membranes of pyramidal neurons where they can contribute to the level of tonic inhibition.

Methods

Culturing of primary hippocampal neurons

Hippocampal neurons were cultured from embryonic day (E) 18 Wistar rat fetuses. Pooled hippocampi ($n = 6$), were incubated for 15 min in 1 mg ml^{-1} trypsin in Hank's balanced salt solution (HBSS) at 37°C (95% air–5% CO_2), followed by three 5 min washes in HBSS. Neurons were dissociated by mechanical trituration (three times) using polished Pasteur pipettes in minimum essential medium (MEM) with Earle's salts (Invitrogen) supplemented with 10% fetal calf serum (FCS), 0.06% (w/v) D-glucose and 50 units ml^{-1} penicillin-G and 50 μg ml^{-1} streptomycin. The final cell suspension was centrifuged for 10 min at 100 *g*. The cells were resuspended in supplemented MEM, prior to seeding onto poly-L-lysine-coated coverslips and incubation at 37°C (95% air–5%CO_2). After 3–5 h of incubation, the medium was replaced by Neurobasal media (Invitrogen) supplemented with 2% FCS, 0.36% w/v D-glucose, 115 units ml^{-1} penicillin-G and 115 μg ml^{-1} streptomycin, 0.5 mM glutamine and 0.02 arbitrary units (50-fold dilution) of the additive, B-27 (Invitrogen). Neurons were used for electrophysiological recordings after 7–10 days *in vitro*.

Cell lines and expression of recombinant $GABA_A$ receptors

Human embryonic kidney (HEK) cells were cultured as previously described (Wooltorton *et al.* 1997). HEK cells were plated onto poly-L-lysine-coated glass coverslips and transfected using a calcium phosphate protocol. cDNAs for the selected combination of human $\alpha1/5$, $\beta2/3$ and $\gamma2$ $GABA_A$ receptor subunits (Hadingham *et al.* 1993*a*,*b*) and enhanced green fluorescent protein (EGFP) were present in equal amounts (1 μg of each per culture dish). The DNA solutions were mixed with 340 mM $CaCl_2$ before the precipitate was formed by gentle addition of an equal volume of a double-strength HBSS containing (mM): NaCl 280, Na_2HPO_4 2.8, Hepes 50; pH 7.2 to the DNA–$CaCl_2$ solution. The DNA–calcium phosphate suspension was carefully added to the seeded HEK cells with the transfection proceeding overnight while incubating at 37°C. Cells were used for electrophysiological recording 18–72 h after transfection.

To examine the properties of $\alpha4\beta3\delta$ $GABA_A$ receptors, we promoted the stable expression of this receptor in Ltk cells (Brown *et al.* 2002). The cells were maintained in DMEM supplemented with 4.5 mg ml^{-1} glucose, 4 mM L-glutamine, 0.11 mg ml^{-1} sodium pyruvate, 10% FCS, 1 mg ml^{-1} geneticin and 0.2 mg ml^{-1} zeocin. After seeding the cells onto glass coverslips (same method as for HEK cells), the expression of the $GABA_A$ receptors was induced overnight in supplemented DMEM plus 0.5 μM dexamethasone. Electrophysiological recordings were performed within 48 h after the induction of receptor expression.

Patch-clamp electrophysiology

Whole-cell and single-channel GABA currents were recorded from hippocampal pyramidal neurons, transfected HEK cells or Ltk cells using an Axopatch 200B patch-clamp amplifier. Patch electrodes (4–6 MΩ for whole cell and 9–16 MΩ for single channels) were filled with an internal solution containing (mM): CsCl 120, $MgCl_2$ 1, EGTA 11, tetraethylammonium hydroxide 33, Hepes 10, $CaCl_2$ 1 and ATP 2; pH adjusted to 7.1 with HCl (approximately 8 mM). The cells were constantly perfused with a Krebs solution containing (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.52, glucose 11 and Hepes 5; pH 7.4. In whole-cell recordings, the Krebs solution was supplemented with 0.5 μM tetrodotoxin (TTX), 20 μM D-amino-5-phosphonopentanoic acid (AP5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), to block voltage-activated Na^+ channels and NMDA and non-NMDA receptor-mediated EPSCs, respectively. Membrane currents were filtered at 2.3–2.5 kHz (single-channel currents) or 5 kHz (whole-cell currents; −3 dB, 8-pole Bessel, 48 dB octave^{-1}), and digitized

using a Digidata 1320A (Axon Instruments) prior to being recorded directly onto a Dell PC Pentium IV, using Clamplex 8.2 (whole-cell recording). For single-channel experiments, currents were recorded onto a DTR-1201 digital tape-recorder prior to off-line A/D conversion and final analysis with the single channel analysis suite: SCAN and EKDIST (http://www.ucl.ac.uk/Pharmacology/dcpr95.html). Any change exceeding 10% in the membrane conductance and/or series resistance resulted in the termination of the recording.

Analysis of whole-cell current data from hippocampal pyramidal cells

The amplitudes of GABA induced membrane currents (I) were determined at a holding potential of −70 mV. The GABA concentration–response relationships were determined by normalizing the GABA currents to the response induced by a maximum saturating concentration of GABA in control Krebs solution (I_{max}) and subsequently fitted with the Hill equation:

$$I/I_{max} = (1/(1 + (EC_{50}/[A])^n),$$

where EC_{50} represents the concentration of the agonist ($[A]$) inducing 50% of the maximal current evoked by a saturating concentration of the agonist and n is the Hill coefficient.

For quantifying the suppression of the tonic GABA current, the shifts in the baseline current were normalized to the maximum change usually achieved with 1 mM Zn^{2+} or 30 μM bicuculline. All-point histograms were constructed for the tonic current taking data samples before and during drug application (10 or 20 s) and fitted with a single Gaussian distribution function of the form:

$$f(x) = A\frac{e^{-(x-\mu)^2/2\sigma_i^2}}{\sigma\sqrt{2\pi}} + C$$

The fit was constrained symmetrically around the peak frequency to avoid any bias caused by the presence of miniature IPSCs (mIPSCs; these are depicted in the histogram as the 'shoulder'). A defines the amplitude and C is a constant defining the pedestal of the histogram. This function provided the Gaussian mean baseline current (μ) and standard deviation (σ). Paired t test analysis was used to compare the effects of tricine, bicuculline and Zn^{2+} on tonic and phasic currents.

To determine the potency of Zn^{2+} where the inhibition–concentration relationship for the mean baseline current was monophasic, the data were fitted to the equation:

$$I/I_{max} = 1 - [1/(1 + (IC_{50}/B)^n)],$$

where the IC_{50} is the antagonist concentration (B) eliciting half-maximal inhibition of the tonic current. For those inhibition–concentration relationships which were clearly biphasic, the data were fitted to the equation:

$$I/I_{max} = [1 - (a/(1+ (IC'_{50}/B)^n)+(b/(1 + (IC''_{50}/B)^n)],$$

where a and b represent the relative proportions of each individual component described by IC_{50}' and IC_{50}'', respectively.

Analysis of single-channel records

Single GABA channel currents were recorded from excised outside-out membrane patches held at −70 mV. Patches showing channel current stacking, which indicated multiple channels in a patch, were only included in the analysis if the number of multiple channel openings never exceeded 2% of all detected openings (Macdonald *et al.* 1989; Smart, 1992). Further evaluation of channel numbers in each patch was performed as previously reported (Mortensen *et al.* 2004). Single-channel records were initially filtered at 10 kHz prior to storage on DAT. Records were then digitized at 20 kHz ensuring that additional filtering (∼2.5 kHz, 36 dB octave^{-1}) did not suppress the amplitude of very brief openings. This was important because the precise determination of various single-channel amplitude levels was critical for identifying the presence of particular $GABA_A$ receptor assemblies. Channel openings and closures were idealized using time course fitting using SCAN. SCAN automatically corrects for any baseline current drift that may occur. For the analysis in EKDIST, only openings longer than twice the rise time of the filter were considered. A minimum time-resolution was usually set at 100 μs for both open and shut times. The amplitude distributions were then fitted with multiple Gaussian components that defined the mean current levels, their standard deviations and the total areas of all components, by using a non-linear least-squares routine. The single-channel conductances were calculated from the mean current levels determined from the Gaussian curve fits, and the difference between the patch holding potential and GABA response reversal potential.

Drugs and solutions

Drugs and solutions were rapidly applied to the HEK cells using a modified Y-tube positioned approximately 300 μm from the recorded cell (Wooltorton *et al.* 1997). The 10–90% solution exchange times of the application system were within 18–25 ms as measured in open-tip recordings. All drugs were dissolved in the Krebs solution.

Results

GABA potencies on pyramidal neurons and recombinant $\alpha\beta\gamma$ $GABA_A$ receptors

An EC_{50} value for GABA, determined for a single neuron, will reflect the combined sensitivities to GABA of all the different $GABA_A$ receptors contributing to its response (synaptic and extrasynaptic), weighted according to the relative amounts of each isoform present on the cell surface. Whole-cell GABA concentration–response curves, generated using data obtained from hippocampal pyramidal neurons (Fig. 1*A*), revealed a mean GABA EC_{50} of $1.24 \pm 0.04\ \mu$M (slope, 1.20 ± 0.04, $n = 10$ cells; Fig. 1*B*). This is a relatively low value when compared to GABA EC_{50} values obtained from recombinant $\alpha\beta\gamma$ receptors, which normally range from 3 to 17 μM (Fisher & Macdonald, 1997; Mortensen *et al.* 2004; Böhme *et al.* 2004; Feng & Macdonald, 2004; Caraiscos *et al.* 2004). Although the reported variation in the EC_{50} will partly depend on the speed of GABA application, this higher sensitivity of pyramidal neurons to GABA could reflect the presence of mixed $GABA_A$ receptor populations with some possessing a higher affinity for GABA. Extrasynaptic $GABA_A$ receptors that underpin tonic inhibition have already been proposed to have a higher sensitivity to GABA than their synaptic counterparts (Stell & Mody, 2002). In this regard, previous studies have suggested that extrasynaptic $GABA_A$ receptors in pyramidal neurons could be composed of $\alpha5\beta\gamma$ (although these are not particularly sensitive to GABA compared to $\alpha1$ subunit-containing receptors; Caraiscos *et al.* 2004), as well as the higher sensitivity $\alpha4\beta\delta$ receptors (Brown *et al.* 2002; Mangan *et al.* 2005). Furthermore, in the cerebellum, higher affinity $\alpha\beta$ isoforms have also been proposed as extrasynaptic receptors (Brickley *et al.* 1999).

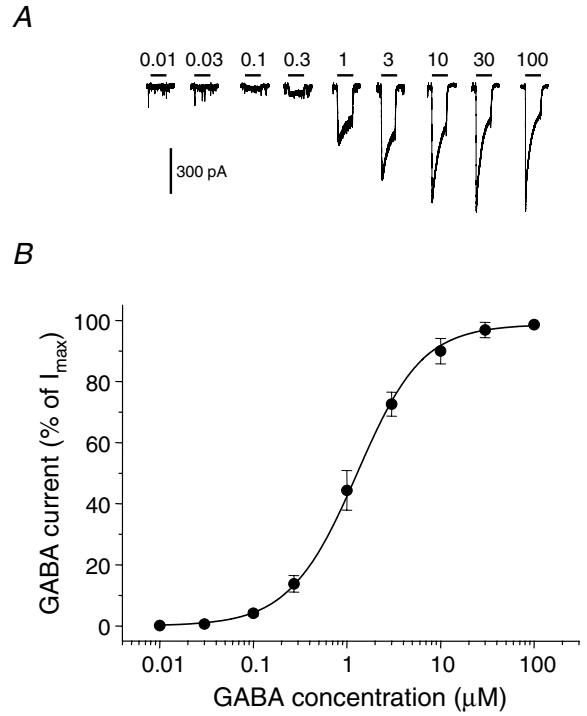

Figure 1. GABA sensitivity of hippocampal pyramidal neurons
A, membrane currents recorded from a single pyramidal neuron voltage clamped at −70 mV. The currents were activated by range of GABA concentrations applied for 4 s (at time shown). The occasional downward deflections represent miniature IPSCs. *B*, GABA concentration–response curve for peak GABA-activated currents. Responses have been normalized to the response induced by a saturating concentration of GABA. The data were accrued from cultured hippocampal pyramidal neurons after 10–14 days in vitro (DIV). Each data point represents mean ± S.E.M. ($n = 10$). The data are fitted with the Hill equation.

Modulation of the tonic conductance in cultured hippocampal neurons

Both the phasic and tonic GABA current components were isolated in whole-cell patch-clamp recordings from hippocampal pyramidal neurons in the presence of TTX, AP5 and CNQX (see Methods; Fig. 2*A*). We used Zn^{2+} as a pharmacological tool to separate $GABA_A$ receptors lacking γ subunits ($\alpha\beta$ and $\alpha\beta\delta$) from those containing γ subunits ($\alpha\beta\gamma$). This cation readily discriminates between $\alpha\beta$ and $\alpha4\beta\delta$ receptors with their high sensitivities to inhibition (IC_{50}, 88 nM and 6–16 μM, respectively) from the less sensitive $\alpha\beta\gamma$ $GABA_A$ receptors (IC_{50}, 300 μM; Krishek *et al.* 1998; Hosie *et al.* 2003). Benzodiazepines were also used to distinguish between $\alpha\beta/\alpha4\beta\delta$ and $\alpha\beta\gamma$ receptors as these ligands will not modulate $GABA_A$ receptors that lack the γ subunit (Pritchett *et al.* 1989; Sigel & Buhr, 1997; Klausberger *et al.* 2001). To first check that the ambient background levels of Zn^{2+} were not persistently occluding the activity of highly Zn^{2+}-sensitive extrasynaptic $GABA_A$ receptor isoforms, a high concentration (10 mM) of the Zn^{2+} chelator tricine was applied. This had only a minor effect (< 5% increase in holding current) in a few cells (3/14) while no effect was observed on the mean holding current in the majority of cells (11/14; Fig. 2*B*, $P > 0.05$). In addition, 10 mM tricine neither changed the mean mIPSC frequency (4.4 ± 1.4 Hz, $P > 0.05$) nor the mean amplitude (33 ± 7 pA, $P > 0.05$). This indicated that a very low ambient Zn^{2+} concentrations was likely to be present during perfusion with our Krebs solution and from previous titration studies, this concentration is likely to be less than 90 nM (Hosie *et al.* 2003). Therefore, any suppression by Zn^{2+} of high affinity $GABA_A$ receptor populations in the extrasynaptic compartment was assumed to be minimal (Fig. 2*B*). The GABAergic nature of the phasic and tonic currents was established using the competitive GABA antagonist bicuculline. At 30 μM, this antagonist

completely blocked the phasic $GABA_A$ receptor mIPSCs and the tonic inhibition (Fig. 2*C*). The application of Zn^{2+} also inhibited both the tonic and the phasic inhibitory $GABA_A$ receptor currents, but the tonic current was relatively more sensitive to inhibition than the phasic current at lower Zn^{2+} concentrations. The mean tonic current of -107 ± 12 pA appeared unaffected by 1 μM Zn^{2+} (-101 ± 0.6 pA). However, it was significantly reduced to -89 ± 2 pA in the presence of 10 μM Zn^{2+}, and maximally reduced to -65 ± 5 pA in the presence of 1 mM Zn^{2+} ($P < 0.05$), leaving only the residual holding current. The standard deviation of the tonic noise (6.2 ± 1.3 pA) was unaffected by 1 μM Zn^{2+} (5.9 ± 0.2 pA). However, it was reduced to 4.2 ± 0.4 pA in the presence of 10 μM Zn^{2+}, and to 3.9 ± 0.3 pA in 1 mM Zn^{2+} (Fig. 2*D–F*, $n = 9$, $P < 0.05$). These data predicted the IC_{50} for inhibition by Zn^{2+} of the tonic current to be approximately 5–30 μM. With regard to the phasic current, the mean mIPSC amplitude of 48 ± 3 pA was unaffected by 1 μM Zn^{2+}, decreased to 40 ± 0.2 pA in the presence of 10 μM Zn^{2+}, and to 17 ± 1.4 pA in 1 mM Zn^{2+} (Fig. 2*D–F*, $P < 0.05$). These data predicted an IC_{50} of Zn^{2+} for inhibition of the phasic current of approximately 300 μM. Taken together, these results implied that the $GABA_A$ receptor component involved in tonic inhibition possessed a relatively high sensitivity to Zn^{2+}.

High concentrations of Zn^{2+} are known to affect the release of neurotransmitters and to modulate the function of voltage-activated ion channels (Xie & Smart, 1991; Xie *et al.* 1994; Smart *et al.* 1994; Harrison & Gibbons, 1994). To ensure that the observed effects of Zn^{2+} on the holding current in our study derived mainly from the inhibition of $GABA_A$ receptors, we first blocked these receptors with a supersaturating concentration (100 μM) of bicuculline (Ueno *et al.* 1997) and then co-applied our highest concentration of Zn^{2+} (1 mM). Under these conditions, only a small additional outward current was observed (3.5 ± 1.1% change of holding current, $P > 0.05$) suggesting that bicuculline and Zn^{2+} were both mainly targeting $GABA_A$ receptors (Fig. 2*G*). The very small additional inhibition by Zn^{2+} may reflect inhibition of a non-GABAergic conductance. The block resulting from the higher concentrations of Zn^{2+} agreed with with the level of block caused by the $GABA_A$ receptor antagonist picrotoxin (10 μM), which was also assumed to reflect a complete block of the tonic current (data not shown).

A rebound current response was often observed after the application of only higher concentrations of Zn^{2+} (Fig. 2*F*). This might be explained by the inhibited or shut $GABA_A$ receptors rapidly entering one or more open channel states directly after Zn^{2+} unbinding. Alternatively,

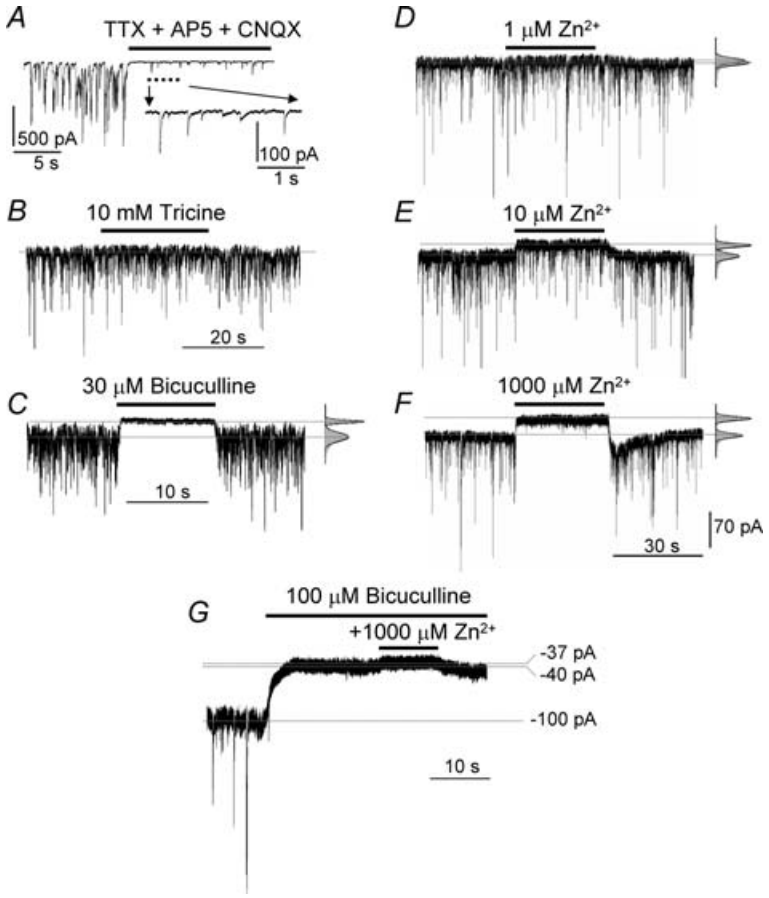

Figure 2. Functional profile of phasic and tonic inhibitory membrane currents from pyramidal neurons
A, spontaneous and miniature synaptic currents recorded from a cultured hippocampal pyramidal neuron held at −70 mV before and after the application of 0.5 μM TTX, 20 μM D-amino-5-phosphonopentanoic acid (AP5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to the Krebs solution. A selected portion of the record is shown below at high time resolution. GABA-mediated miniature IPSCs (mIPSCs) and the tonic (baseline) current were recorded in the presence of TTX, AP5 and CNQX, before and after recovery from the application of 10 mM tricine (*B*), 30 μM bicuculline (*C*), 1 μM Zn^{2+} (*D*), 10 μM Zn^{2+} (*E*) and 1 mM Zn^{2+} (*F*). $GABA_A$ receptors are almost solely responsible for the tonic current, as 1 mM Zn^{2+} only has a negligible inhibitory effect after $GABA_A$ receptors have been completely blocked with 100 μM bicuculline (*G*). The tonic current before (lower) and after (upper) ligand exposure are shown as dotted lines. The Gaussian curves depict the mean current and standard deviation of the noise neglecting the distortion caused by the mIPSCs (see Methods).

it could simply reflect the 'collection' of many $GABA_A$ receptors in one or more Zn^{2+}-blocked states (Gingrich & Burkat, 1998) and, following the removal of Zn^{2+}, the tonic current transiently over-recovers following the reactivation of the 'collected' $GABA_A$ receptors before resuming the steady-state tonic current level. Many studies have indicated that the inhibition caused by Zn^{2+} is seemingly not dependent on the open/shut state(s) of the GABA receptor but largely a function of the subunit composition (Legendre & Westbrook, 1991; Smart, 1992; Berger *et al.* 1998). Generally, for $\alpha\beta$ receptors, Zn^{2+} inhibition is non-competitive and largely independent of the liganded state of the receptor (Smart & Constanti, 1990; Draguhn *et al.* 1990). However, for $\alpha\beta\gamma$ isoforms, Zn^{2+} inhibition is dependent on the state of the receptor whereby ligand-exposed $GABA_A$ receptors are preferentially blocked (Gingrich & Burkat, 1998). This type of block was best described by using the generic mechanism of mixed inhibition (Smart & Constanti, 1986; Gingrich & Burkat, 1998). Notably, the major difference in Zn^{2+} sensitivity between $\alpha\beta$ and $\alpha\beta\gamma$ receptors is maintained in all the above studies, ranging from 50-fold (Gingrich & Burkat, 1998) to 3000-fold (Hosie *et al.* 2003), which is why we used Zn^{2+} as a tool to detect the presence of $\alpha\beta$ isoforms.

Analysis of the Zn^{2+} inhibition–concentration relationship for the tonic current revealed a clear biphasic curve (Fig. 3), indicative of a mixed population of highly Zn^{2+}-sensitive (IC_{50}', $1.89 \pm 0.3\ \mu$M, $n = 8$) and less Zn^{2+}-sensitive $GABA_A$ receptors (IC_{50}'', $223 \pm 7\ \mu$M $n = 8$) being involved in tonic inhibition on hippocampal pyramidal neurons. The highly Zn^{2+}-sensitive receptors represented the smallest component ($35 \pm 5\%$ of the total) compared to the low sensitivity receptors ($65 \pm 6\%$).

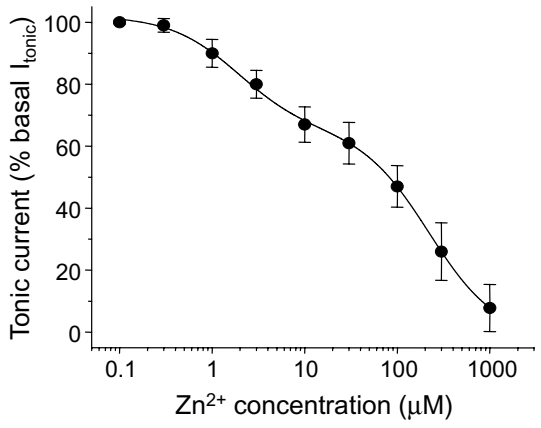

Figure 3. Zn^{2+} concentration–response curve for the inhibition of the tonic current in pyramidal neurons
The tonic current prior to application of Zn^{2+} was defined as 100% and used to normalize the level of inhibition. Each data point represents mean ± S.E.M. ($n = 8$). The biphasic curve fit was achieved using the inhibition function (see Methods). This yielded two IC_{50} values: $1.89 \pm 0.3\ \mu$M ($35 \pm 5\%$ of the population) and $223 \pm 7\ \mu$M ($65 \pm 6\%$).

Recruitment of different $GABA_A$ receptor populations using low ambient GABA concentrations

Although dissociated hippocampal cultures exhibited some degree of tonic $GABA_A$ receptor activation, the level of inhibition by Zn^{2+} appeared variable. This variation was assumed to reflect differences in the ambient concentration of GABA. In order to compensate and thus ensure stable levels of tonic inhibition, low concentrations of GABA were added to the external solution (Fig. 4*A*). After titration, 10 nM GABA was found to have little effect on the level of tonic inhibition, whereas 100 nM GABA was an appropriate compensating concentration because it consistently increased the level of tonic inhibition without affecting the frequency, amplitude or the decay of mIPSCs (data not shown). By contrast, the higher concentrations of 300–1000 nM significantly activated the cell surface $GABA_A$ receptors and caused inhibition of the mIPSC amplitudes (Fig. 4*A*).

In the presence of 100 nM ambient GABA, Zn^{2+} displayed consistent inhibition of the tonic baseline current (Fig. 4*B*). This resulted in a monophasic Zn^{2+} inhibition curve with an IC_{50} of $9.5 \pm 1.1\ \mu$M (Fig. 5*A*, $n = 9$) indicating that the tonic GABA concentration was sufficient to activate highly Zn^{2+} sensitive $GABA_A$ receptors, possibly those receptors that would be lacking a $\gamma 2$ subunit. Nevertheless, if a significant component of this tonic current was also mediated by $\alpha\beta\gamma$ receptors (i.e. activated by 100 nM GABA), then the Zn^{2+} inhibition curve should be laterally displaced to higher concentrations of Zn^{2+} if the activity of such receptors was increased, because they would become the dominant component of the inhibition curve and they exhibit lower sensitivity to Zn^{2+} inhibition. However, the application of 200 nM diazepam did not significantly shift the Zn^{2+} inhibition curve in the presence of 100 nM GABA as would have been expected if many $\alpha\beta\gamma$ receptors had been active (IC_{50}, $12.6 \pm 1.2\ \mu$M; Fig. 5*A*).

Increasing the ambient GABA concentration to $2\ \mu$M resulted in a biphasic Zn^{2+} inhibition curve (IC_{50} values, $2.4 \pm 0.5\ \mu$M, 43%; and $130 \pm 25\ \mu$M, 57%; Fig. 5*B*), suggesting that at least two $GABA_A$ receptor populations were active. In the presence of 200 nM diazepam, the component with low Zn^{2+} sensitivity became dominant resulting in a monophasic inhibition curve. This indicates that at this higher GABA concentration, a component of the GABA tonic current was presumably supported by $\gamma 2$ subunit-containing $GABA_A$ receptors (IC_{50}, $94 \pm 14\ \mu$M; Fig. 5*B*). Although the pharmacological analyses with Zn^{2+} and benzodiazepines indicated the likelihood of at least two populations of receptors expressed with and

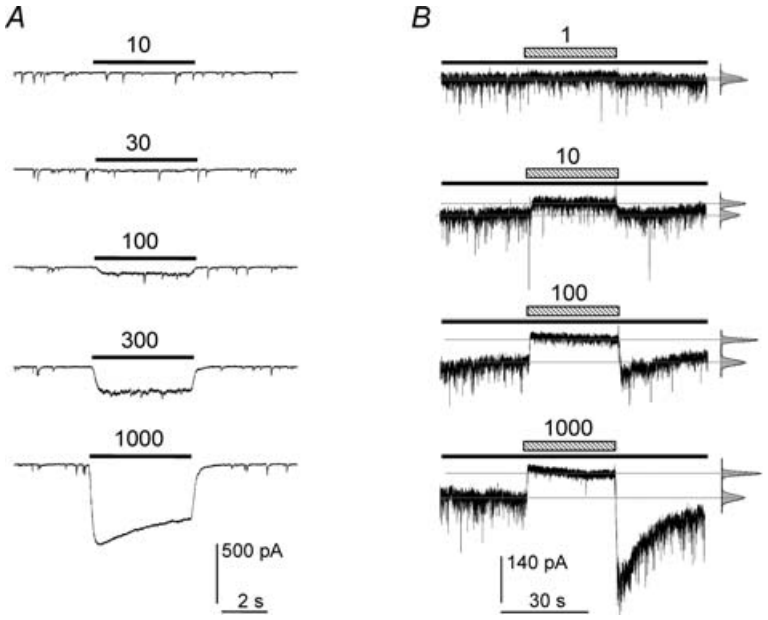

Figure 4. Titration of exogenous GABA concentrations required to reproduce the tonic current in pyramidal neurons
A, application of low GABA concentrations (10, 30, 100, 300 and 1000 nM) and their effect on the tonic and phasic currents in the presence of 0.5 μM TTX, 20 μM D-amino-5-phosphonopentanoic acid (AP5) and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). *B,* in the presence of a background 100 nM concentration of GABA (continuous lines), the tonic and phasic currents were exposed to 1, 10, 100 and 1000 μM Zn^{2+} (hatched bars). The dotted lines reveal the shifts in the tonic current.

without the $\gamma 2$ subunit, single-channel recording was used to provide corroborating evidence. This was important because identifying $\alpha\beta$ from $\alpha\beta\delta$ $GABA_A$ receptors by Zn^{2+} sensitivity alone is quite difficult given that Zn^{2+} potency differs by only 30-fold between these two receptor isoforms.

Single-channel conductance levels of recombinant $\alpha\beta$, $\alpha\beta\gamma$ and $\alpha\beta\delta$ $GABA_A$ receptors

Previous studies using light and electron microscopic immunofluorescence and immunogold methods have previously reported the presence of $\alpha 1$–5, $\beta 1$–3, $\gamma 2$ and δ subunits in hippocampal neurons (Fritschy *et al.* 1998; Pirker *et al.* 2000; Brünig *et al.* 2002; Fritschy & Brünig, 2003). By monitoring Zn^{2+} inhibition in these cells, our results indicated a prevalence of $\gamma 2$ subunit-containing $GABA_A$ receptors; however, these receptors are unlikely to account for the differential sensitivity to Zn^{2+} given that such receptors containing $\alpha 1/2/3$ subunits are considered the dominant synaptic and perisynaptic forms of the $GABA_A$ receptor (Craig *et al.* 1994; Somogyi *et al.* 1996) and $\alpha 5$ subunit-containing receptors are likely to be the dominant type in extrasynaptic compartments (Caraiscos *et al.* 2004). The pharmacological data implied that some extrasynaptic $GABA_A$ receptors must lack the $\gamma 2$ subunit. If this is so, then these receptors may be detectable using single-channel recording to reveal their unique properties (Moss *et al.* 1990; Smart, 1992; Angelotti & Macdonald, 1993).

In order to obtain precise identifiable profiles for some of the $GABA_A$ receptors likely to be expressed in the synaptic and extrasynaptic membranes of hippocampal pyramidal neurons, we first analysed single GABA channel currents recorded from the following four recombinant $GABA_A$ receptors: $\alpha 1\beta 3\gamma 2$ (Fig. 6*Aa*), $\alpha 5\beta 3\gamma 2$ (Fig. 6*Ba*), $\alpha 4\beta 3\delta$ (Fig. 6*Ca*) and $\alpha 1\beta 3$ (Fig. 6*Da*). Our primary focus was on the single-channel conductance levels that could

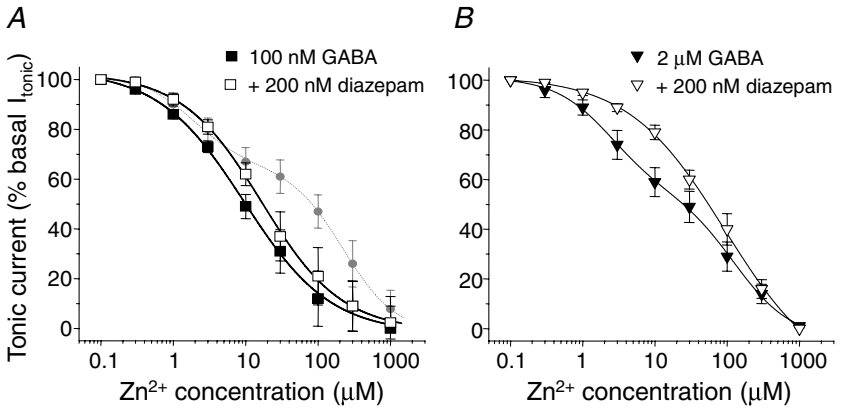

Figure 5. Two components characterize the Zn^{2+} inhibition of the tonic current induced by GABA application
Zn^{2+} inhibition concentration–response curves for the tonic current of cultured pyramidal neurons induced by 100 nM GABA (*A*) or 2 μM GABA (*B*), in the absence (filled symbols) or presence (open symbols) of 200 nM diazepam. The currents are normalized to the control tonic current in the absence of Zn^{2+}. The dotted line in (*A*) represents the biphasic Zn^{2+} inhibition curve taken from Fig. 3 for comparison. Each data point represents mean ± S.E.M. ($n = 8$–9).

be used to characterize each receptor. To define these levels clearly, we applied near maximal concentrations of GABA (selected from dose–response curves: $\alpha\beta\gamma$, 100 μM; $\alpha4\beta3\delta$, 30 μM; $\alpha\beta$, 10 μM) to activate bursts and clusters of channel openings that could be identified as the activations of single ion channels. This is a particularly useful diagnostic indicator for identifying $\alpha\beta$ receptors given their lower conductance state compared to $\alpha\beta\gamma$ and $\alpha\beta\delta$ subunit-containing receptors (Moss *et al.* 1990; Angelotti *et al.* 1993; Fisher & Macdonald, 1997).

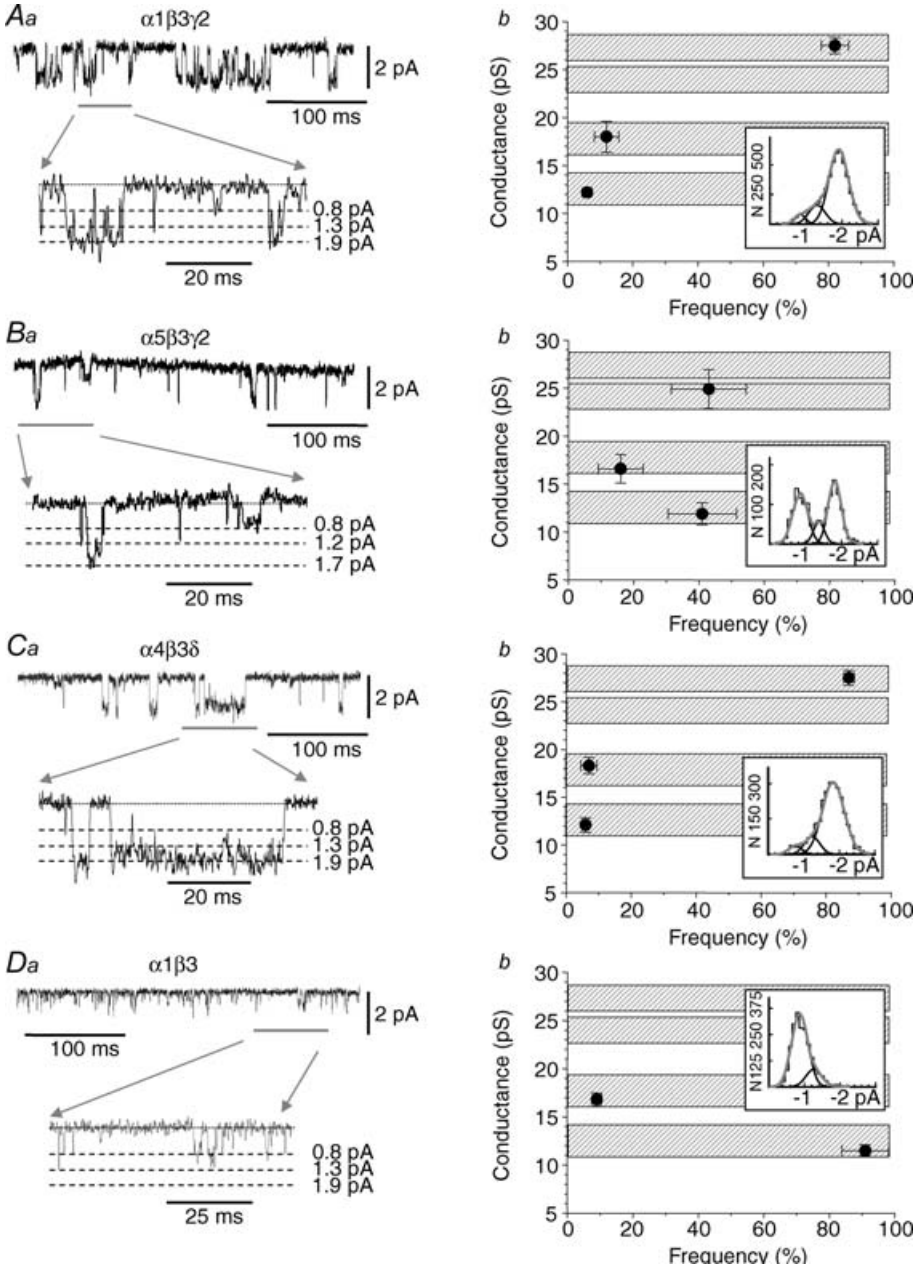

Figure 6. Single-channel conductances associated with recombinant $GABA_A$ receptors
The lefthand column (*Aa–Da*) shows GABA-activated single channel currents recorded from outside-out patches taken from HEK (*Aa*, *Ba* and *Da*) or Ltk (*Ca*) cells expressing $\alpha1\beta3\gamma2$ (*Aa*; 100 μM GABA), $\alpha5\beta3\gamma2$ (*Ba*; 100 μM GABA), $\alpha4\beta3\delta$ (*Ca*; 30 μM GABA) and $\alpha1\beta3$ (*Da*; 10 μM GABA). Selected portions of the single-channel current recordings (grey line) are shown at high time resolution (lower traces) together with the associated zero current level (dotted line) and various current amplitude levels (dashed lines). The righthand column shows the frequencies of the various conductance levels obtained by analysis of the single channel records for $\alpha1\beta3\gamma2$ receptors (*Ab*), $\alpha5\beta3\gamma2$ (*Bb*), $\alpha4\beta3\delta$ (*Cb*) and $\alpha1\beta3$ receptors (*Db*). Data points are means ± S.E.M. from $n = 4$–8 patches. The hatched bars indicate the approximate range of conductance levels that are common to all four receptors. The insets show the frequency distributions for the various conductance levels.

Three very similar channel conductance levels were identified for $\alpha1\beta3\gamma2$, $\alpha5\beta3\gamma2$ and $\alpha4\beta3\delta$ receptors, designated as high, medium and low, with values of 25–28, 17–19 and 12–13 pS, respectively (Fig. 6*Ab*, *Bb* and *Cb*). The frequencies of openings to these conductance levels were quite similar for $\alpha1\beta3\gamma2$ and $\alpha4\beta3\delta$ receptors. Openings to the high conductance state (27–28 pS) dominated the frequency distributions for both $\alpha1\beta3\gamma2$ and $\alpha4\beta3\delta$ receptors ($82 \pm 4\%$ and $87 \pm 2\%$, respectively, Fig. 6*Ab* and *Cb*). By contrast, for $\alpha5\beta3\gamma2$ receptors, both high (24.9 ± 2.0 pS) and low (11.9 ± 1.1 pS) conductances appeared with equal frequency ($43 \pm 12\%$ and $41 \pm 11\%$, Fig. 6*Bb*). For $\alpha1\beta3$ receptors, although channel openings with high conductance (25–28 pS) were absent as previously reported, two conductance states could still be discerned which were comparable to the medium and low conductance states of the $\alpha\beta\gamma$ and $\alpha4\beta\delta$ receptors. For the $\alpha1\beta3$ receptors, channel openings to the medium conductance level (16.8 ± 0.7 pS) were quite infrequent ($9 \pm 1.4\%$; Fig. 6*Db*) with the majority ($91 \pm 7\%$) of openings occurring to the low conductance level (11.5 ± 0.6 pS). As a result of the overlapping conductance levels between the $\alpha\beta$ and $\alpha\beta\gamma$ and $\alpha4\beta\delta$ receptors, the possiblity cannot be excluded that the openings to the lower conductance levels observed for $\alpha1\beta3\gamma2$, $\alpha5\beta3\gamma2$ and $\alpha4\beta3\delta$ receptors do not reflect incompletely assembled γ or δ subunit-lacking $GABA_A$ receptors (see below).

Single-channel conductance levels for $GABA_A$ receptors in hippocampal neurons

We used the single-channel conductance profiles for the selected recombinant $GABA_A$ receptors to facilitate our interpretation of GABA single-channel conductance levels in hippocampal neurons. Using outside-out patches, the application of 10 μM GABA revealed channel openings to four different conductance levels (Fig. 7*A* and *B*). We used 10 μM GABA as this exceeds the EC_{50} for activation of $GABA_A$ receptors with both high and low sensitivity to GABA. Two quite close high conductance levels at 24 ± 0.6 ($28 \pm 7\%$) and 27.7 ± 0.5 pS ($39 \pm 6\%$) were identified. These conductances are very similar to those measured for the recombinant $\alpha1\beta3\gamma2$, $\alpha4\beta3\delta$ and $\alpha5\beta3\gamma2$ receptors (27.5 pS for $\alpha1\beta3\gamma2/\alpha4\beta3\delta$ and 24.9 pS for $\alpha5\beta3\gamma2$), indicating that the native $GABA_A$ receptor population probably contains mixtures of $\alpha1\beta\gamma$, $\alpha4\beta\delta$ and $\alpha5\beta\gamma$ receptors. The lower frequencies of openings to the higher conductance levels in neurons, compared with those observed for recombinant $\alpha1/5\beta3\gamma2$ and $\alpha4\beta3\delta$ receptors, is another indication of the expected heterogeneity in the $GABA_A$ receptor population in pyramidal cells. Although channel openings to higher conductance levels are believed to mainly originate from $\alpha1/\alpha5\beta\gamma$ and $\alpha4\beta\delta$ receptors, we cannot exclude the possibility that some openings may stem from other γ subunit-containing $GABA_A$ receptors (e.g. $\alpha2$–$4\beta\gamma$ receptors). Two further conductance states were also identified: a medium conductance level

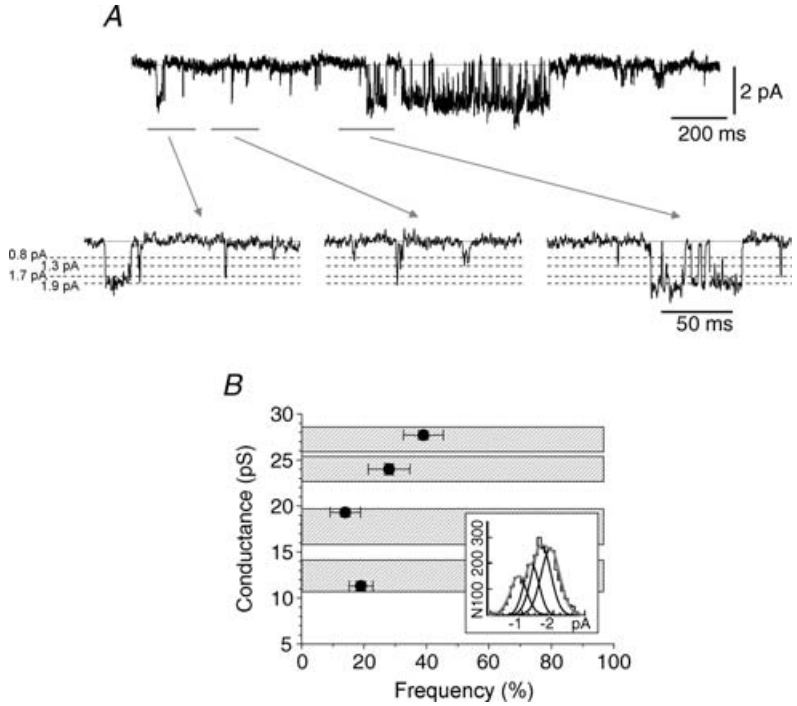

Figure 7. Multiple single GABA channel conductances are present in hippocampal pyramidal neurons
A, single-channel currents activated by applying 10 μM GABA to an outside-out patch taken from a hippocampal pyramidal neuron. Three segments (grey lines) are shown at high time resolution (lower panel) and the relevant conductance levels are shown by dashed lines. *B*, relationship between the four conductance levels and their relative frequencies are shown. All the data points are mean ± S.E.M. from $n = 9$ patches. Hatched bars indicate the conductance level ranges found in the analyses of the recombinant $GABA_A$ receptors taken from Fig. 6 for comparison.

(19.3 ± 0.5 pS, 14 ± 5%) and a low conductance level (11.3 ± 0.5 pS, 19 ± 3.5%) that correspond to the medium and low conductance states measured with the selected recombinant $GABA_A$ receptors (Fig. 7*B*). The latter conductance state could be indicative of the low conductance states of $\alpha\beta\gamma$ or $\alpha\beta\delta$ receptors, but equally it might also represent the existence of $\alpha\beta$ receptor isoforms in neurons, especially when considering the relative frequency of openings to this low conductance level.

Identification of a Zn^{2+}-sensitive GABA channel conductance level in hippocampal pyramidal neurons

To identify any $\alpha\beta$ subunit-containing $GABA_A$ receptors in pyramidal neurons, single GABA-activated channel currents were evoked by 10 μM GABA prior to the addition of 10 μM Zn^{2+}. This concentration of Zn^{2+} is predicted to inhibit the activity of virtually all $\alpha\beta$ receptors (by > 98%; IC_{50}, 88 nM; Hosie *et al.* 2003) and substantially reduce $\alpha\beta\delta$ receptor activity (by approximately 37–65%; IC_{50}, 6–16 μM; Krishek *et al.* 1998; Nagaya & Macdonald, 2001; Brown *et al.* 2002) without causing any significant antagonism at $\alpha\beta\gamma$ $GABA_A$ receptors (< 3%, IC_{50}, 300 μM). Under these conditions, single-channel current analyses revealed that only the frequency of occurrence of the low conductance level (11–12 pS; Fig. 8*A*) in hippocampal neurons was significantly reduced by 10 μM Zn^{2+} (from 19 ± 3.5% to 9 ± 1.7%; Fig. 8*B* and *C*, $n = 9$, $P < 0.05$). The incomplete reduction in activity of this conductance state by Zn^{2+} most probably reflects the presence of similar-sized low conductance states originating from the less Zn^{2+}-sensitive $\alpha\beta\gamma$ and $\alpha4\beta\delta$ receptors, as demonstrated previously with recombinant $GABA_A$ receptors.

To control for the presence of low conductance openings which originate from $\alpha\beta\gamma$ receptors, the $\alpha1\beta3\gamma2$ receptor subunit combination was expressed in HEK cells. Outside-out patches revealed three conductance states in the presence of 0.1 μM GABA as observed previously; however, the frequency of the low conductance state was relatively high compared to the frequencies of the medium and high conductance states (Fig. 9*Aa*). The application of 10 μM Zn^{2+} did not affect these low conductance states (Fig. 9*Aa* and *Ab*) indicating that the sensitivity to Zn^{2+} is determined by the receptor isoform as expected (Draguhn *et al.* 1990; Smart *et al.* 1991; Hosie *et al.* 2003), rather than by the conductance level.

Another factor that may confound our identification of those receptors that underlie the Zn^{2+}-sensitive, low-conductance states is that $\alpha5\beta3\gamma2$ receptors are reported to have a higher sensitivity to Zn^{2+} than other $\gamma2$ subunit-containing receptors (IC_{50}, 20 μM; Burgard

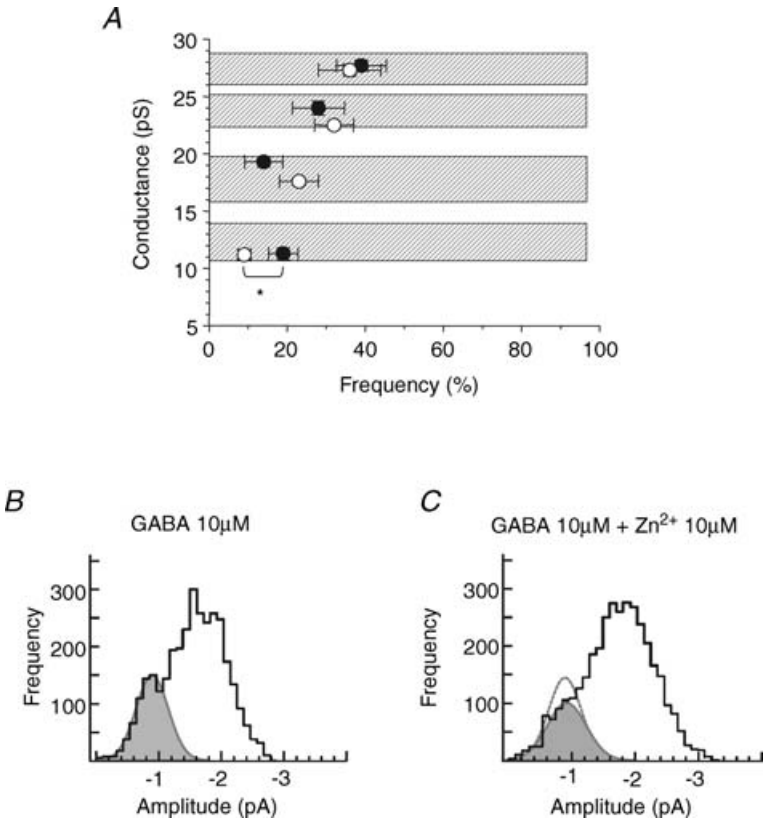

Figure 8. Zn^{2+} inhibition of the GABA channel low conductance level in pyramidal neurons
A, relationship between the openings of the GABA channel to various conductance levels, following activation by 10 μM GABA and their relative frequency before (●, taken from Fig. 7*B*) and then after the co-application of GABA with 10 μM Zn^{2+} (○). Data were recorded from nine outside-out patches at −70 mV. The asterisk indicates that only the lowest conductance state was significantly inhibited by Zn^{2+} ($P < 0.05$). *B* and *C*, GABA single-channel current amplitude histograms compiled following activation of the channels by 10 μM GABA in the absence (*B*) and presence (*C*) of 10 μM Zn^{2+}. A single Gaussian (grey filled area) has been fitted to both distributions to highlight the low conductance level (~0.8 pA; 11–12 pS). The area of this Gaussian decreases from 19% in control (*B*) to 9% in the presence of Zn^{2+} (*C*). The control Gaussian is superimposed on *C* as a dotted line.

et al. 1996). To address this point we compared the Zn^{2+} sensitivity of single-channel openings, induced by 10 μM GABA, from recombinant $\alpha1\beta3$ and $\alpha5\beta3\gamma2$ receptors (Fig. 9*B* and *C*). Single-channel openings by $\alpha1\beta3$ receptors were almost completely blocked by 10 μM Zn^{2+} (Fig. 9*Ba* and *Bb*) with only a residual level of 2–3% of openings observed during Zn^{2+} application (Fig. 9*Bc*). By contrast, single-channel openings for $\alpha5\beta3\gamma2$, exhibited only very low sensitivity to 10 μM Zn^{2+} (Fig. 9*Ca* and *Cb*), where 86–93% of openings remained during Zn^{2+} application (Fig. 9*Cc*). An analysis of the open times for $\alpha5\beta3\gamma2$ receptors in the absence and presence of Zn^{2+} was performed to address whether Zn^{2+} had affected the open state kinetics. The dwell times and their areas resolved in the presence of 10 μM GABA (τ_1, 0.38 ± 0.05 ms, $91 \pm 9\%$; τ_2, 3.41 ± 0.16 ms, $9 \pm 4\%$) were not statistically different from those resolved in the presence of 10 μM GABA plus 10 μM Zn^{2+} (τ_1, 0.43 ± 0.02 ms, $92 \pm 7\%$; τ_2, 3.55 ± 0.23 ms, $8 \pm 3\%$; $n = 4$).

These results suggest that in hippocampal neurons, the low conductance states that remain in the presence of Zn^{2+} (Fig. 8) probably reflect the activation of $\alpha5\beta\gamma$ and $\alpha4\beta\delta$ receptors. The frequency of openings to the low conductance state for a 'pure' population of recombinant $\alpha4\beta\delta$ receptors is only around 5% of the total openings (Fig. 6*Cb*). Given that Zn^{2+} will inhibit such openings for $\alpha4\beta\delta$ receptors by approximately 50%, the largest inhibition of low conductance states that could be expected from these receptors alone would only be approximately 2.5%. Similarly, the relatively frequent openings to the low conductance state for a 'pure' recombinant $\alpha5\beta3\gamma2$ receptor population (~40%, Fig. 6*Bb*) will be inhibited by Zn^{2+} by approximately 10% yielding a maximal inhibition of only 4%. Of course, the proportions of low conductance openings from $\alpha5\beta\gamma$ and $\alpha\beta\delta$ receptors in the mixed populations of extrasynaptic $GABA_A$ receptors on hippocampal pyramidal cells that remain in the presence of Zn^{2+} are likely to be considerably lower

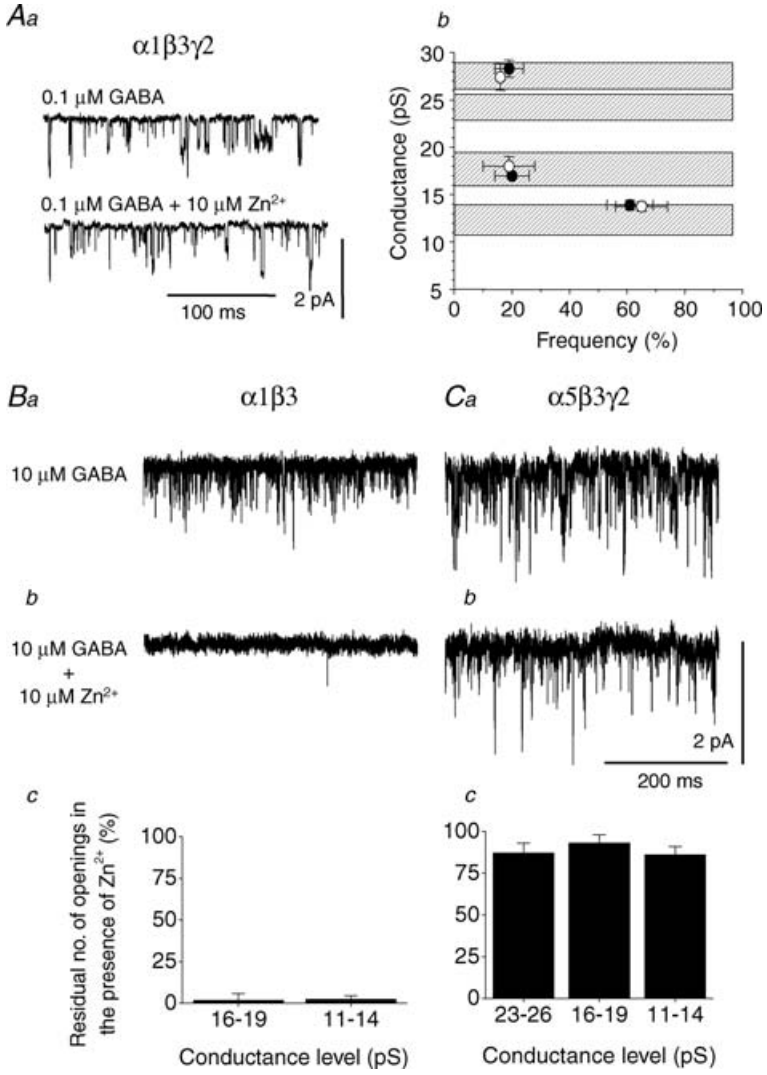

Figure 9. Zn^{2+} inhibition of GABA channel conductance states for $\alpha1\beta3\gamma2$, $\alpha1\beta3$ and $\alpha5\beta3\gamma2$ $GABA_A$ receptors expressed in HEK cells
Aa, single GABA channel currents activated by 0.1 μM GABA for $\alpha1\beta3\gamma2$ receptors in the absence or presence of 10 μM Zn^{2+}. *Ab*, relationship between the openings of the $\alpha1\beta3\gamma2$ GABA channel to various conductance levels and their relative frequency before (●) and after co-application of 0.1 μM GABA and 10 μM Zn^{2+} (○; $n = 7$ patches). The concentration of GABA was titrated to 0.1 μM to promote the opening frequency of GABA channels to the lowest conductance state. *Ba* and *b*, single-channel openings by $\alpha1\beta3$ $GABA_A$ receptors induced by 10 μM GABA in the absence (*a*) and presence (*b*) of 10 μM Zn^{2+}. *Bc*, bargraph of the number of residual $\alpha1\beta3$ channel openings to the medium and low conductance levels in the presence of Zn^{2+} ($n = 3$). *Ca*, single-channel openings for $\alpha5\beta3\gamma2$ $GABA_A$ receptors induced by 10 μM GABA in the absence (*a*) and presence (*b*) of 10 μM Zn^{2+}. *Cc*, bargraph of GABA channel openings for $\alpha5\beta3\gamma2$ receptors in the presence of Zn^{2+} ($n = 4$). All currents are recorded from outside-out patches at −70 mV.

than in the pure recombinant receptor populations. Thus, allowing for their relative sensitivities to Zn^{2+} inhibition, the $\alpha4$ and $\alpha5$ subunit-containing receptors cannot account for the 10% inhibition in the low conductance states observed in Fig. 8. Based on these considerations, we suggest that up to 10% of extrasynaptic $GABA_A$ receptors on hippocampal pyramidal cells are likely to be formed from the highly Zn^{2+}-sensitive $\alpha\beta$ isoform.

Discussion

A growing body of evidence suggests that a continuous activation of extrasynaptic $GABA_A$ receptors by low basal concentrations of GABA results in a tonic inhibition of neurons (Mody, 2001; Semyanov *et al.* 2004; Farrant & Nusser, 2005). Several factors will influence the extent to which extrasynaptic $GABA_A$ receptors are tonically active. These include the $GABA_A$ receptor isoforms that are present and their affinities for GABA, the basal GABA concentration around the extrasynaptic domains resulting from spillover from nearby GABAergic synapses, and the activity of GABA transporters that will tightly regulate basal GABA concentrations.

The innate level of tonic inhibition in our cultured hippocampal neurons is in close agreement with previous findings using similar preparations (Bai *et al.* 2001; Caraiscos *et al.* 2004), but variations in the level of inhibition are also evident. Although Bai *et al.* (2001) reproduced their results in brain slices, others have had to pretreat their slices with vigabatrin, an inhibitor of GABA transaminase, to achieve a resolvable inhibition of the tonic current with gabazine (Overstreet & Westbrook, 2001). Semyanov *et al.* (2003) in another brain slice study, reported a tonic current in stratum radiatum interneurons, but no tonic current in pyramidal cells. However, by also using hippocampal slices, Stell & Mody (2002) observed a tonic current in CA1 pyramidal neurons. It is quite probable that the different levels of tonic inhibition reflect varying ambient GABA concentrations in different preparations under different experimental conditions. It was for this reason that we choose to normalize the GABA concentration in our cultures to provide a consistent level of tonic inhibition.

Estimates of ambient GABA concentrations from *in vivo* microdialysis range from tens of nanomolar to a few micromolar (Lerma *et al.* 1986; Tossman *et al.* 1986; Xi *et al.* 2003); however, it is likely that this method will fail to accurately detect variations in GABA concentrations near inhibitory synapses. In our study, the small and variable tonic current in hippocampal neurons, which could be inhibited by bicuculline and Zn^{2+} in the absence of exogenous GABA, suggested that very low GABA concentrations were in close proximity to the neurons. This was supported by the induction of small current responses to low (30–50 nM) GABA concentrations which indicated that the ambient GABA concentration was probably lower than 30 nM. However, this estimate is a mean value and probably endogenous GABA concentrations have a highly non-uniform distribution between the synaptic and extrasynaptic zones (see below).

A variation in the subunit composition of extrasynaptic $GABA_A$ receptors may also affect the tonic current. Some receptors may include the $\alpha5$ subunit particularly as it is prominently expressed in the hippocampus (Sieghart, 1995; Sur *et al.* 1998; 1999; Pirker *et al.* 2000; Brünig *et al.* 2002) and shows a diffuse extrasynaptic distribution (Brünig *et al.* 2002; Crestani *et al.* 2002), and this would agree with recent evidence of the importance of $\alpha5\beta\gamma$ receptors in tonic inhibition of pyramidal neurons (Caraiscos *et al.* 2004). Similarly, other studies have also shown that $\alpha4\beta\delta$ $GABA_A$ receptors may be important extrasynaptic receptors on pyramidal neurons (Mangan *et al.* 2005). However, this does not exclude the possibility that other $GABA_A$ receptors contribute to tonic inhibition in these cells. Our GABA concentration–response relationships for pyramidal neurons indicated a higher sensitivity to GABA than would be expected if only $\alpha\beta\gamma$ receptors were present extrasynaptically. Of course, whole-cell applications of GABA will unavoidably activate both synaptic and extrasynaptic receptors, but even so the reported EC_{50} values for GABA activating recombinant $\alpha1\beta2/3\gamma2$ receptors (3–17 μM) (Fisher & Macdonald, 1997; Mortensen *et al.* 2004; Böhme *et al.* 2004; Feng & Macdonald, 2004; Caraiscos *et al.* 2004) and $\alpha5\beta3\gamma2$ receptors (11–19 μM) (Böhme *et al.* 2004; Caraiscos *et al.* 2004) are not easily reconciled with values obtained for native hippocampal receptors. Therefore, other $GABA_A$ receptors that exhibit higher sensitivities to GABA, such as $\alpha1\beta1/3$ (EC_{50}, 1.0–2.7 μM (Angelotti *et al.* 1993; Fisher & Macdonald, 1997; Amato *et al.* 1999; Wilkins & Smart, 2002; Hosie *et al.* 2003) and $\alpha4\beta\delta$ receptors (EC_{50}, 0.5 μM) (Mangan *et al.* 2005), probably complement the extrasynaptic receptor population. Our proposition that not only $\alpha4\beta\delta$ receptors but also $\alpha\beta$ receptors are partly responsible for the tonic inhibition in these neurons was based initially on the sensitivity of the tonic current to Zn^{2+} inhibition. The Zn^{2+} inhibition curves obtained with sufficient GABA to differentially activate $\alpha\beta/\alpha4\beta\delta$ and $\alpha\beta\gamma$ receptors, displayed two components with high and low sensitivity to Zn^{2+}. These components correlated well with the different sensitivities to Zn^{2+} inhibition of recombinant $\alpha\beta/\alpha4\beta\delta$ and $\alpha\beta\gamma$ receptors, demonstrating that this ion is a useful tool to identify and separate $GABA_A$ receptor populations that differ in their incorporation of the $\gamma2$ subunit.

The biphasic inhibition curve determined from our cultured neurons (in the absence of co-applied agonist) could be explained by the selective activation of different

$GABA_A$ receptor populations by endogenous GABA. We propose that higher concentrations of endogenously released GABA (much higher than 30 nM) are likely to activate synaptic (most probably $\alpha\beta\gamma$) receptors with some spillover, and consequent dilution to lower concentrations, into the perisynaptic zone, where $\alpha\beta\gamma$ and other $GABA_A$ receptor isoforms (e.g. $\alpha\beta$ and $\alpha\beta\delta$) may reside. However, endogenous GABA reaching the extensive extrasynaptic zone is predicted to be so dilute (< 30 nM) that most extrasynaptic $GABA_A$ receptors (including $\alpha\beta$ and $\alpha\beta\delta$ receptors) would not be activated. This non-uniform GABA concentration gradient from the inhibitory synapses to the perisynaptic zones would be effectively abolished by applying a uniform low exogenous GABA concentration (100 nM), which increased the tonic current by mostly activating the more GABA-sensitive, extrasynaptic receptors (e.g. $\alpha\beta/\alpha4\beta\delta$ receptors) to such an extent that $\alpha\beta\gamma$ receptor activation (from synaptic and perisynaptic regions) was no longer resolved in the Zn^{2+} inhibition experiments. The resulting monophasic Zn^{2+} inhibition curve was also unaffected by diazepam, as expected if the majority of activated $GABA_A$ receptors lacked the $\gamma2$ subunit. When the ambient GABA concentration was further increased to low micromolar levels, both extrasynaptic $\alpha\beta/\alpha4\beta\delta$ and $\alpha\beta\gamma$ receptors were activated, resulting once more in a biphasic Zn^{2+} inhibition curve. Under these conditions, diazepam potentiated the activation of the $\gamma2$ subunit-containing receptors to such a degree that they dominated the GABA response thereby transforming the Zn^{2+} inhibition relationship into another monophasic curve. However, despite separating $\alpha\beta/\alpha\beta\delta$ from $\alpha\beta\gamma$ receptors in the extrasynaptic compartment based on their differential sensitivities to Zn^{2+} inhibition, using this criterion alone to separate $\alpha\beta$ from $\alpha\beta\delta$ receptors was not definitive. For this reason we relied on the acquisition of single-channel conductance 'fingerprints' to propose the existence of $\alpha\beta$ receptors on hippocampal neurons.

Single-channel currents for recombinant $\gamma2$ or δ subunit-containing receptors such as $\alpha1\beta3\gamma2$, $\alpha5\beta3\gamma2$ and $\alpha4\beta3\delta$, were characterized by three to four similar conductance levels which were dominated by the higher conductance states (25–28 pS). By contrast, recombinant $\alpha1\beta3$ receptors shared only the two lower conductance levels with $\gamma2$ or δ subunit-containing receptors, with the low conductance 11.5 pS state dominating. These data corresponded well with previous reports for the main conductance levels for $\alpha1\beta1/3\gamma2$S/l (27.1–32 pS) and $\alpha1\beta1/3$ (11–15.3 pS) receptors (Verdoorn *et al.* 1990; Angelotti & Macdonald, 1993; Fisher & Macdonald, 1997). For $\alpha1\beta3\delta$ receptors, a single-channel conductance state of 26.7 pS has been reported (Fisher & Macdonald, 1997). Our single-channel recordings from pyramidal neurons revealed two high conductance states in the 25–28 pS range which were comparable to values for recombinant $\alpha1\beta3\gamma2/\alpha4\beta3\delta$ and $\alpha5\beta3\gamma2$ receptors. In addition, two additional low conductance levels ($\sim$19 and $\sim$11 pS) were observed in pyramidal neurons. It was significant that the lowest level, which corresponded closely with the main conductance level for recombinant $\alpha\beta$ receptors, was relatively abundant (19% of all openings).

Further evidence of extrasynaptic $\alpha\beta$ receptors came from the sensitivity to Zn^{2+} inhibition of the low (11 pS) conductance state. However, not all $GABA_A$ receptors exhibiting this low conductance level can be simply classified as $\alpha\beta$ because Yeung *et al.* (2003) reported an increase in channel open probability by midazolam of all the three conductance levels that they resolved (including the 11 pS state) indicating the presence of $\gamma2$ subunits in these receptors. The most likely reason for this is that $\alpha\beta$, $\alpha\beta\delta$ and $\alpha\beta\gamma$ receptors can all induce openings to the lowest conductance state, although in our study these states were quite infrequent for $\alpha\beta\delta$ and $\alpha\beta\gamma$ receptors. With regard to their pharmacology, Zn^{2+} will inhibit the low conductance states arising from $\alpha\beta$ receptor activation, whereas midazolam will potentiate openings to similar conductance states that are induced by $\alpha\beta\gamma$ receptors. Although $\alpha5\beta3\gamma2$ receptors are reported to have a higher sensitivity to Zn^{2+} inhibition compared to other $\alpha\beta\gamma$ receptors, their IC_{50} is reported to be 20 μM, which is within the sensitivity range exhibited by $\alpha\beta\delta$ receptors (Burgard *et al.* 1996) and much lower than that observed with $\alpha\beta$ receptors. It is interesting that we did not observe such a high sensitivity to Zn^{2+} in our single-channel experiments on $\alpha5\beta3\gamma2$ receptors.

The number of $\alpha\beta$ receptors present in extrasynaptic membranes is probably quite low. From the single-channel conductance distributions the relative proportion can be estimated to be up to 10%. Their relatively rapid desensitization kinetics (Krampfl *et al.* 2000) might reduce their activity in the continued presence of GABA which could argue against a role in tonic inhibition. However, our single-channel results clearly show continued activity of $\alpha\beta$ receptors, even when applying exogenous GABA concentrations much higher than the ambient levels of GABA we believe exist around neurons. This clearly indicates that $\alpha\beta$ receptors do have the potential to support tonic inhibition *in vivo*. The existence of extrasynaptic $\alpha\beta$ receptors in cerebellar granule neurons has previously been postulated based on the observation of low conductance single-channel currents (Brickley *et al.* 1999), giving rise to the prospect that $\alpha\beta$ receptors may be more widely distributed on central neurons than previously assumed. Immunocytochemical data also support the notion of $\alpha\beta$ receptors in the CNS. It is worth noting that up to 50% of $\alpha4$ receptors in the forebrain are purported not to associate with $\gamma1$–3 subunits or δ subunits (Bencsits *et al.* 1999). In addition, the δ subunit knockout mouse revealed cerebellar $GABA_A$ receptors that lacked $\gamma1$–3

subunits (Tretter *et al.* 2001). Lastly, although under somewhat unusual conditions, the $\gamma 2$ knockout mouse displayed $GABA_A$ receptors with low single-channel conductances in accord with $\alpha\beta$ assemblies, suggesting that native neurons can indeed support the expression of such $GABA_A$ receptors (Gunther *et al.* 1995). The presence of $\alpha\beta$ receptors in the extrasynaptic membrane will offer neurons the capability of detecting low concentrations of ambient GABA while being highly sensitive to Zn^{2+} inhibition. Their low conductance and generally short mean open time will provide only limited opportunity for charge transfer across the cell membrane and thus a modest contribution to tonic inhibition. Nevertheless, such receptors will add further diversity to those $GABA_A$ receptors that underpin tonic inhibition.

References

Amato A, Connolly CN, Moss SJ & Smart TG (1999). Modulation of neuronal and recombinant $GABA_A$ receptors by redox reagents. *J Physiol* **517**, 35–50.

Angelotti TP & Macdonald RL (1993). Assembly of $GABA_A$ receptor subunits: $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunits produce unique ion channels with dissimilar single-channel properties. *J Neurosci* **13**, 1429–1440.

Angelotti TP, Uhler MD & Macdonald RL (1993). Assembly of $GABA_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J Neurosci* **13**, 1418–1428.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF & Orser BA (2001). Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. *Mol Pharmacol* **59**, 814–824.

Bencsits E, Ebert V, Tretter V & Sieghart W (1999). A significant part of native γ-aminobutyric $acid_A$ receptors containing $\alpha 4$ subunits do not contain γ or δ subunits. *J Biol Chem* **274**, 19613–19616.

Berger T, Schwarz C, Kraushaar U & Monyer H (1998). Dentate gyrus basket cell $GABA_A$ receptors are blocked by Zn^{2+} via changes in their desensitization kinetics: an in situ patch-clamp and single-cell PCR study. *J Neurosci* **18**, 2437–2448.

Böhme I, Rabe H & Lüddens H (2004). Four amino acids in the α subunits determine the γ-aminobutyric acid sensitivities of $GABA_A$ receptor subtypes. *J Biol Chem* **279**, 35193–35200.

Brickley SG, Cull-Candy SG & Farrant M (1999). Single-channel properties of synaptic and extrasynaptic $GABA_A$ receptors suggest differential targeting of receptor subtypes. *J Neurosci* **19**, 2960–2973.

Brown N, Kerby J, Bonnert TP, Whiting PJ & Wafford KA (2002). Pharmacological characterization of a novel cell line expressing human $\alpha 4\beta 3\delta$ $GABA_A$ receptors. *Br J Pharmacol* **136**, 965–974.

Brünig I, Scotti E, Sidler C & Fritschy JM (2002). Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. *J Comp Neurol* **443**, 43–55.

Burgard EC, Tietz EI, Neelands TR & Macdonald RL (1996). Properties of recombinant γ-aminobutyric $acid_A$ receptor isoforms containing the $\alpha 5$ subunit subtype. *Mol Pharmacol* **50**, 119–127.

Caraiscos VB, Elliott EM, You T, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF & Orser BA (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by $\alpha 5$ subunit-containing γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* **101**, 3662–3667.

Craig AM, Blackstone CD, Huganir RL & Banker G (1994). Selective clustering of glutamate and γ-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc Natl Acad Sci U S A* **91**, 12373–12377.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Möhler H & Rudolph U (2002). Trace fear conditioning involves hippocampal $\alpha 5$ $GABA_A$ receptors. *Proc Natl Acad Sci U S A* **99**, 8980–8985.

Draguhn A, Verdorn TA, Ewert M, Seeburg PH & Sakmann B (1990). Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by Zn^{2+}. *Neuron* **5**, 781–788.

Farrant M & Nusser Z (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci* **6**, 215–229.

Feng HJ & Macdonald RL (2004). Multiple actions of propofol on $\alpha\beta\gamma$ and $\alpha\beta\delta$ $GABA_A$ receptors. *Mol Pharmacol* **66**, 1517–1524.

Fisher JL & Macdonald RL (1997). Single channel properties of recombinant $GABA_A$ receptors containing $\gamma 2$ or δ subtypes expressed with $\alpha 1$ and $\beta 3$ subtypes in mouse L929 cells. *J Physiol* **505**, 283–297.

Fritschy JM & Brünig I (2003). Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol Ther* **98**, 299–323.

Fritschy JM, Johnson DK, Möhler H & Rudolph U (1998). Independent assembly and subcellular targeting of $GABA_A$-receptor subtypes demonstrated in mouse hippocampal and olfactory neurons in vivo. *Neurosci Lett* **249**, 99–102.

Gingrich KJ & Burkat PM (1998). Zn^{2+} inhibition of recombinant $GABA_A$ receptors: an allosteric, state-dependent mechanism determined by the γ-subunit. *J Physiol* **506**, 609–625.

Gunther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F *et al.* (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the $\gamma 2$ subunit gene of γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* **92**, 7749–7753.

Hadingham KL, Wingrove P, Le Bourdelles B, Palmer KJ, Ragan CI & Whiting PJ (1993*a*). Cloning of cDNA sequences encoding human $\alpha 2$ and $\alpha 3$ γ-aminobutyric $acid_A$ receptor subunits and characterization of the benzodiazepine pharmacology of recombinant $\alpha 1$-, $\alpha 2$-, $\alpha 3$-, and $\alpha 5$-containing human γ-aminobutyric $acid_A$ receptors. *Mol Pharmacol* **43**, 970–975.

Hadingham KL, Wingrove PB, Wafford KA, Bain C, Kemp JA, Palmer KJ, Wilson AW, Wilcox AS, Sikela JM & Ragan CI (1993*b*). Role of the β subunit in determining the pharmacology of human γ- aminobutyric acid type A receptors. *Mol Pharmacol* **44**, 1211–1218.

Harrison NL & Gibbons SJ (1994). Zn^{2+}: an endogenous modulator of ligand- and voltage-gated ion channels. *Neuropharmacology* **33**, 935–952.

Hosie AM, Dunne EL, Harvey RJ & Smart TG (2003). Zinc-mediated inhibition of $GABA_A$ receptors: descrete binding sites underlie subtype specificity. *Nat Neurosci* **6**, 362–369.

Isaacson JS (2000). Spillover in the spotlight. *Curr Biol* **10**, R475–R477.

Klausberger T, Sarto I, Ehya N, Fuchs K, Furtmüller R, Mayer B, Huck S & Sieghart W (2001). Alternate use of distinct intersubunit contacts controls $GABA_A$ receptor assembly and stoichiometry. *J Neurosci* **21**, 9124–9133.

Knoflach F, Benke D, Wang Y, Scheurer L, Lüddens H, Hamilton BJ, Carter DB, Möhler H & Benson JA (1996). Pharmacological modulation of the diazepam-insensitive recombinant γ-aminobutyric $acid_A$ receptors $\alpha_4\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$. *Mol Pharmacol* **50**, 1253–1261.

Krampfl K, Bufler J, Lepier A, Dudel J & Adelsberger H (2000). Desensitization characteristics of rat recombinant $GABA_A$ receptors consisting of $\alpha1\beta2\gamma2$S and $\alpha1\beta2$ subunits expressed in HEK293 cells. *Neurosci Lett* **278**, 21–24.

Krishek BJ, Moss SJ & Smart TG (1998). Interaction of H^+ and Zn^{2+} on recombinant and native rat neuronal $GABA_A$ receptors. *J Physiol* **507**, 639–652.

Legendre P & Westbrook GL (1991). Noncompetitive inhibition of γ-aminobutyric $acid_A$ channels by Zn. *Mol Pharmacol* **39**, 267–274.

Lerma J, Herranz AS, Herreras O, Abraira V & Martin del Rio R (1986). In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Res* **384**, 145–155.

Luscher B & Keller CA (2004). Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther* **102**, 195–221.

Macdonald RL, Rogers CJ & Twyman RE (1989). Kinetic properties of the $GABA_A$ receptor main conductance state of mouse spinal cord neurones in culture. *J Physiol* **410**, 479–499.

Mangan PS, Sun C, Carpenter M, Goodkin HP, Sieghart W & Kapur J (2005). Cultured hippocampal pyramidal neurons express two kinds of $GABA_A$ receptors. *Mol Pharmacol* **67**, 775–788.

Mitchell SJ & Silver RA (2003). Shunting inhibition modulates neuronal gain during synaptic excitation. *Neuron* **38**, 433–445.

Mody I (2001). Distinguishing between $GABA_A$ receptors responsible for tonic and phasic conductances. *Neurochem Res* **26**, 907–913.

Mortensen M, Kristiansen U, Ebert B, Frølund B, Krogsgaard-Larsen P & Smart TG (2004). Activation of single heteromeric $GABA_A$ receptor ion channels by full and partial agonists. *J Physiol* **557**, 389–413.

Moss SJ & Smart TG (2001). Constructing inhibitory synapses. *Nat Rev Neurosci* **2**, 240–250.

Moss SJ, Smart TG, Porter NM, Nayeem N, Devine J, Stephenson FA, Macdonald RL & Barnard EA (1990). Cloned GABA receptors are maintained in a stable cell line: allosteric and channel properties. *Eur J Pharmacol* **189**, 77–88.

Nagaya N & Macdonald RL (2001). Two γ2L subunit domains confer low Zn^{2+} sensitivity to ternary $GABA_A$ receptors. *J Physiol* **532**, 17–30.

Overstreet LS & Westbrook GL (2001). Paradoxical reduction of synaptic inhibition by vigabatrin. *J Neurophysiol* **86**, 596–603.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W & Sperk G (2000). $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* **101**, 815–850.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR & Seeburg PH (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* **338**, 582–585.

Semyanov A, Walker MC & Kullmann DM (2003). GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. *Nat Neurosci* **6**, 484–490.

Semyanov A, Walker MC, Kullmann DM & Silver RA (2004). Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27**, 262–269.

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

Sieghart W & Sperk G (2002). Subunit composition, distribution and function of $GABA_A$ receptor subtypes. *Curr Top Med Chem* **2**, 795–816.

Sigel E, Baur R, Trube G, Mohler H & Malherbe P (1990). The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron* **5**, 703–711.

Sigel E & Buhr A (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol Sci* **18**, 425–429.

Smart TG (1992). A novel modulatory binding site for zinc on the $GABA_A$ receptor complex in cultured rat neurones. *J Physiol* **447**, 587–625.

Smart TG & Constanti A (1986). Studies on the mechanism of action of picrotoxinin and other convulsants at the crustacean muscle GABA receptor. *Proc R Soc Lond B Biol Sci* **227**, 191–216.

Smart TG, Constanti A (1990). Differential effects of zinc on the vertebrate $GABA_A$ receptor complex. *Br J Pharmacol* **99**, 643–654.

Smart TG, Moss SJ, Xie X & Huganir RL (1991). $GABA_A$ receptors are differentially sensitive to zinc: dependence on subunit composition. *Br J Pharmacol* **103**, 1837–1839.

Smart TG, Xie X & Krishek BJ (1994). Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc. *Prog Neurobiol* **42**, 393–341.

Somogyi P, Fritschy JM, Benke D, Roberts JD & Sieghart W (1996). The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacology* **35**, 1425–1444.

Stell BM & Mody I (2002). Receptors with different affinities mediate phasic and tonic $GABA_A$ conductances in hippocampal neurons. *J Neurosci* **22**, RC223.

Sur C, Fresu L, Howell O, McKernan RM & Atack JR (1999). Autoradiographic localization of $\alpha 5$ subunit-containing $GABA_A$ receptors in rat brain. *Brain Res* **822**, 265–270.

Sur C, Quirk K, Dewar D, Atack J & McKernan R (1998). Rat and human hippocampal $\alpha 5$ subunit-containing γ-aminobutyric $acid_A$ receptors have $\alpha 5\beta 3\gamma 2$ pharmacological characteristics. *Mol Pharmacol* **54**, 928–933.

Thomas P, Mortensen M, Hosie AM & Smart TG (2005). Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. *Nat Neurosci* **8**, 889–897.

Tossman U, Jonsson G & Ungerstedt U (1986). Regional distribution and extracellular levels of amino acids in rat central nervous system. *Acta Physiol Scand* **127**, 533–545.

Tretter V, Hauer B, Nusser Z, Mihalek RM, Hoger H, Homanics GE, Somogyi P & Sieghart W (2001). Targeted disruption of the $GABA_A$ receptor δ subunit gene leads to an up-regulation of $\gamma 2$ subunit-containing receptors in cerebellar granule cells. *J Biol Chem* **276**, 10532–10538.

Ueno S, Bracamontes J, Zorumski C, Weiss DS & Steinbach JH (1997). Bicuculline and gabazine are allosteric inhibitors of channel opening of the $GABA_A$ receptor. *J Neurosci* **17**, 625–635.

Verdoorn TA, Draguhn A, Ymer S, Seeburg PH & Sakmann B (1990). Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron* **4**, 919–928.

Wilkins ME & Smart TG (2002). Redox modulation of $GABA_A$ receptors obscured by Zn^{2+} complexation. *Neuropharmacology* **43**, 938–944.

Wooltorton JR, Moss SJ & Smart TG (1997). Pharmacological and physiological characterization of murine homomeric $\beta 3$ $GABA_A$ receptors. *Eur J Neurosci* **9**, 2225–2235.

Xi ZX, Ramamoorthy S, Shen H, Lake R, Samuvel DJ & Kalivas PW (2003). GABA transmission in the nucleus accumbens is altered after withdrawal from repeated cocaine. *J Neurosci* **23**, 3498–3505.

Xie X, Hider RC & Smart TG (1994). Modulation of GABA-mediated synaptic transmission by endogenous zinc in the immature rat hippocampus in vitro. *J Physiol* **478**, 75–86.

Xie XM & Smart TG (1991). A physiological role for endogenous zinc in rat hippocampal synaptic neurotransmission. *Nature* **349**, 521–524.

Yeung JY, Canning KJ, Zhu G, Pennefather P, MacDonald JF & Orser BA (2003). Tonically activated $GABA_A$ receptors in hippocampal neurons are high-affinity, low-conductance sensors for extracellular GABA. *Mol Pharmacol* **63**, 2–8.

Acknowledgements

This work was supported by the MRC and Wellcome Trust. We thank Philip Thomas for his comments on the manuscript.