Graded response to GABA by native extrasynaptic $GABA_A$ receptors.

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Footnotes:

Extrasynaptic $GABA_A$ receptor, GABARex; THDOC, (3α, 21-dihydroxy-5α-pregnan-20-one); TES, (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid)

Abstract

GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA in the brain is commonly associated with a fast, point-to-point form of signalling called synaptic transmission (phasic inhibition) but there is growing evidence that GABA participates in another, slower and more diffuse form of signalling often referred to as tonic inhibition. Unresolved questions regarding the tonic neuronal inhibition concern activation and functional properties of extrasynaptic $GABA_A$ receptors (GABARex) present on neurons. Extrasynaptic receptors are exposed to sub-micromolar GABA concentrations and may modulate the overall excitability of neurons and neuronal networks. Here we examined GABA-activated single-channel currents in dentate gyrus granule neurons in rat hippocampal slices. We activated three types (I, II, III) of GABARex channels by nanomolar GABA concentrations (EC_{50}; I: 27 ± 12; II: 4 ± 3; III: 43 ± 19 nM). The channels opened after a delay and the single-channel conductance was graded (γ_{max}; I: 61 ± 3; II: 85 ± 8, III: 40 ± 3 pS). The channels were differentially modulated by 1 μM diazepam, 200 nM zolpidem, 1 μM flumazenil and 50 nM THDOC (3α, 21-dihydroxy-5α-pregnan-20-one) consistent with the following minimal subunit composition of GABARex I $\alpha_1\beta\gamma_2$, GABARex II $\alpha_4\beta\gamma_2$ and GABARex III $\alpha\beta\delta$ channels.

Keywords: hippocampus, tonic inhibition, GABA channels, patch-clamp, single-channel current

Running title: GABARex channels generate tonic conductance

The most prominent inhibitory channels in the brain are the $GABA_A$ receptors. The receptors contain a chloride channel that is normally closed but can be opened by GABA. They are located at inhibitory synapses but are also outside of synapses (extrasynaptic or non-synaptic, GABARex channels) on the soma, the dendrites and on the axon (Somogyi *et al*., 1989; Kullmann *et al*., 2005). It is thought that the GABARex channels are the major generators of the tonic inhibition. Despite growing literature on tonic inhibition (Bai *et al*., 2001; Mody, 2004; Semyanov *et al*., 2004; Farrant & Nusser, 2005; Hemmings *et al*., 2005) relatively little is known about the functional and pharmacological properties of specific extrasynaptic $GABA_A$ receptors in intact neurons (Birnir *et al*., 1994; Eghbali *et al*., 1997; Brickley *et al*., 1999; Birnir *et al*., 2001; Lindquist *et al*., 2003). To investigate the effects of GABA on the GABARex channels in brain tissue we measured, in hippocampal slices, currents through a single receptor, the single-channel current. Previously we have shown that GABARex channels in the dentate gyrus granule neurons activated by low micromolar concentrations of GABA are blocked by the specific $GABA_A$ inhibitor bicuculline (Birnir *et al*., 1994). Here we explore the relationship between GABA and the channel conductance over a range of GABA concentrations.

Materials and methods.

Materials. All of the chemicals and drugs for the extracellular solutions were purchased from Sigma (St. Louis, MO) except flumazenil which was obtained from Tocris (Bristol, UK).

Hippocampal slice preparation. Experiments were approved by the Animal Care and Use Committee at Lund University. The animals were killed by decapitation. Experiments were carried out on neurons in the granule layer of the dentate gyrus region of hippocampal slices from Wistar rats. The methods used for preparation of the slices have been described previously (Birnir *et al*., 1994). Briefly, 17 - 21-day old rats were decapitated and the brain removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.3 $MgSO_4$, 2.5 NaH_2PO_4, 2.5 $CaCl_2$ and 20 glucose. The pH of this solution when equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 was 7.4. The hemi brain was glued to the cutting stage and immediately submerged in ice-cold ACSF. Slices about 400 μm thick were cut normal to the septo-temporal axis using a vibrating microslicer (Campden Instruments, UK). The hippocampus was gently separated from the surrounding brain tissue and incubated in ACSF at 37 °C for an hour. At the end of the incubation period, the chamber containing the slices was removed and stored at room temperature.

Recording solutions. The extracellular bath solution contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, TES (N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) 10 and the pipette solution (mM): CholineCl 140, NaCl 5, KCl 1, $MgCl_2$ 1, $CaCl_2$ 1.8, TES 10 and 0.0002 saclofen. In some experiments the choline was replaced with Na^+ in the pipette solution. The pH of the solutions was adjusted to 7.4 with NaOH. GABA was dissolved in the pipette solution. Diazepam, zolpidem, flumazenil and THDOC were dissolved in DMSO and then in the bath or pipette solution. The final concentration of DMSO was 190 μM. We have previously shown that at this concentration DMSO does not affect channel properties (Eghbali *et al*., 1997).

Electrophysiological recordings. Currents were recorded in cell-attached or inside-out patches using an Axopatch 200B (Molecular Devices) at depolarized 40 mV (pipette potential – 40 mV) relative to the chloride reversal potential, filtered at 2 or 5 kHz, digitized at 10 kHz using a DigiData 1322A analogue-to-digital converter interfaced with a computer and analyzed by pClamp software (Molecular Devices). Pipettes were made from borosilicate glass and fire polished. They generally had a resistance between 10 and 20 MΩ when filled with the pipette solution. Conventional patch-clamp techniques were used when establishing a gigaohm seal in slices. Recordings were mostly done in the cell-attached patch-clamp configuration since in this configuration channels are in contact with an unaltered intracellular environment in terms of ions, cellular/protein modulatory mechanisms and architecture. Experiments were done at room temperature (20 – 22 ºC).

Data analysis and statistics. Channel conductance was calculated by dividing the current amplitude by the difference between the pipette potential (Vp, generally – 40 mV) and the chloride reversal potential. The channel currents both in cell-attached and inside-out patch configurations reversed at Vp ~ 0 mV. This is to be expected not only for the excised patches where the chloride concentration across the patch is almost symmetrical but also for the cell-attached patches as the chloride reversal potential is close to the reversal membrane potential of the cells (Ben-Ari, 2002). In experiments where choline was the main cation in the pipette solution we did not correct for the small (-2.6 mV) liquid junction potential generated. Outward currents across the membrane (inward chloride movement) are shown as positive, upward currents and inward currents are shown as negative, downward currents. The amplitude of currents was measured either from all-points amplitude probability histograms with a bin width of 0.1 pA or from direct measurements of the amplitude of individual currents with similar outcome. The mean current was measured as the average of the deviations of all data points from zero (the midline of the baseline current). Concentration-conductance

data were fitted using a Hill-type equation: $\gamma = \gamma_{max} * [GABA]^h / [EC_{50}]^h + [GABA]^h$ where γ_{max} is the maximal conductance, EC_{50} is the concentration of GABA that evoked half-maximal conductance and h is the Hill coefficient. Results are presented as mean ± s.e.m. for three or more experiments. Data were statistically compared using Student's t test.

Results

GABA activates three types of GABARex channels.

We recorded GABARex single-channel currents in intact neurons using the cell-attached patch configuration. This ensures that factors and intracellular proteins that might normally interact with the receptors are present during our recordings (Birnir *et al*., 2001; Everitt *et al*., 2004). Three distinct types of channels (I, II and III) were activated as determined by maximal conductance at 1 to 100 μM GABA. Representative currents activated by 1 μM GABA are shown in Fig. 1A and 30 s all-points histograms in Fig. 1B. The maximal single-channel current amplitudes were 2.4 pA (60 pS) of GABARex I (Fig. 1Aa), 3.0 pA (75 pS) of GABARex II (Fig. 1Ab) and 1.7 pA (43 pS) of GABARex III (Fig. 1Ac). The average current-amplitudes determined from the all-points histograms (Fig. 1B) were similar, 2.4 pA (Ba), 3.0 pA (Bb) and 1.6 pA (Bc), respectively. The current-voltage (IV) relationship for the three channel types is shown in Fig. 1C. GABARex I (Ca) and II (Cb) show outward rectification whereas the IV relationship is more linear for GABARex III (Cc). When cell-attached (solid circles) patches were made inside-out (open circles, Ca and b), the IV relationship did not change indicating that the rectification is an inherent property of the GABARex I and II channels and not due to the low intracellular chloride concentration in the cell-attached recordings.

The GABA concentration sets the GABARex channel conductance.

The maximal current amplitude of the channels was related to the GABA concentration applied. We activated channels with 0.5 nM to 100 μM GABA at the pipette potential of depolarized 40 mV (Vp = - 40 mV). The single-channel conductance (γ) is the current amplitude divided by the difference between the pipette potential and the chloride reversal potential. The measured (a, b, c) and the average (d, e, f) single-channel conductance for GABARex I (a, d), II (b, e) and III (c, f) is plotted against the GABA

concentration in Fig. 2 (circles). The diamonds (green) represent the measured (a, b, c) and the average (d, e, f) single-channel conductance obtained in the presence of GABA plus 1 μM diazepam. The best fit of a Hill-type equation to the data is shown by the solid (GABA) or broken (GABA plus diazepam) lines. GABARex II has the highest apparent affinity (EC_{50}) for GABA (4 ± 3 nM, n = 56) and the largest maximal single-channel conductance (γ_{max}, 85 ± 8 pS). The EC_{50} and γ_{max} were lower for GABARex I (27 ± 12 nM, 61 ± 3 pS, n = 70) and GABARex III (43 ± 19 nM, 40 ± 3 pS, n = 42). The Hill coefficient for GABARex I, II and III were 0.5 ± 0.1, 0.4 ± 0.1 and 0.6 ± 0.2. When in the presence of 1 μM diazepam, GABARex I channel current was modulated but not those of GABARex II (n = 23) or GABARex III (n = 23). The EC_{50} of GABARex I for GABA was now 2 ± 0.4 nM (n = 27) comparable to that of GABARex II but the γ_{max} (59 ± 2 pS) and the Hill coefficient (0.5 ± 0.1) did not change.

The GABARex channels are activated after a delay from the GABA application. One of the features of the GABARex channels is the delay in activation upon GABA application. The channels did not open immediately after forming the patch but rather after a delay (latency) often lasting tens to hundreds of seconds. The concentration-dependent maximal conductance also often developed over additional tens of seconds and this is exemplified in Fig. 3A by activation of a GABARex II channel by 1 μM GABA. The initial conductance was 45 pS but within 30 s, it increased to 78 pS. The conductance did not increase further over the next 5 min the patch was held in the cell-attached configuration. We examined if the latency of activation of the concentration-dependent maximal channel conductance differed between GABARex I, II and III (Fig. 3B). Apart from channels activated by 2 nM GABA, GABARex I channels, on the average, were maximally conducting within the first 100 s from the GABA application whereas both GABARex II and III took longer time to develop the maximal conductance.

The three GABARex channels are differentially modulated by drugs. Most $GABA_A$ receptors in the CNS contain alpha (α_1 - α_6) and beta (β_1-β_3) subunits with the third subunit type often being the γ_2 or the δ subunit in the pentameric receptor (Barnard *et al*., 1998; Sigerhart & Sperk, 2002; Korpi & Sinkkonen, 2005). An extensive characterization of $GABA_A$ receptors of known subunit composition has been carried out using molecular pharmacological tools (Korpi *et al*., 2002). Diazepam enhancement of currents in mature animals requires the γ_2 together with the $\alpha_{1,2,3 \text{ or } 5}$ subunit (Barnard *et al*., 1998). To further probe the subunit composition of the GABARex channels we examined effects of drugs with a subunit-specific profile. Current enhancement by 200 nM zolpidem indicates α_1 with γ_2 subunit as the EC_{50} values for zolpidem potentiation of the GABA-activated current in α_2 (~500 nM, Sanna *et al.*, 2002) or α_3 (~ 800 nM, (Munakata et al., 1998; Wingrove *et al*., 2002) containing receptors are significantly higher concentrations than zolpidem EC_{50} values recorded in α_1 (~ 100 nM, Munakata *et al.*, 1998; Wingrove *et al*., 2002; Sanna *et al*., 2002) containing receptors. The α_5 subunit is not expressed in the dentate gyrus granule cells (Korpi *et al.*, 2002). One μM flumazenil only enhances currents in receptors containing α_4 (or α_6) with the γ_2 subunit (Whittemore *et al*., 1996) and receptors containing the δ rather than the γ subunit show stronger GABA-modulatory effect of neurosteroids (Belelli and Lambert, 2005). In dentate gyrus granule cells, Stell *et al.* (2005) showed that tonic currents from wild-type but not $\delta^{-/-}$ mice were modulated by 10 – 100 nM of the neurosteroid THDOC. We used 50 nM THDOC to examine if the δ subunit might be present in the receptors. GABARex channels were first activated by 20 nM GABA (Fig. 4). Channels were activated in cell-attached patches and after about 4 to 5 min when the channel had attained its maximal concentration-dependent conductance, the patch was made inside-out. We have shown previously that when lipid-soluble drugs are added to the intracellular side of the patch they do reach their site of action (Eghbali *et al*., 1997).

Since 20 nM GABA is a sub-saturating concentration, modulation of the channels should be recorded, if it takes place. Fig. 4 shows 20 nM GABA-activated currents after the patches had been made inside-out (A) and then after the addition of a drug (B, zolpidem (a), flumazenil (b) or THDOC (c)). The all-points histograms are from 30-s current traces from the patches shown. In Fig. 4C the average responses to the drugs are shown. The conductance is expressed relative to the 20 nM GABA-evoked maximal channel conductance in the cell-attached patch before it was ripped-off to obtain the inside-out configuration. Each of the three drugs had GABARex type-specific effects. 200 nM zolpidem increased the single-channel current amplitude of GABARex I by 1.6 (Fig. 4Aa, Ba, Ca) and 50 nM THDOC increased that of GABARex III by 2.4 (Fig. 4Ac, Bc, Cc) whereas 1 μM flumazenil had only a slight effect (1.2) on the current amplitude of GABARex II but appeared to affect the kinetics of the channel (Fig. 4Ab, Bb, Cb).

The mean channel conductance is a function of the single-channel conductance and the open probability of the channel. It is therefore a good measure of a change in channel kinetics that may happen upon drug exposure. We measured the mean conductance in 10-s current traces before and after exposure to drugs (Fig. 4D). 200 nM zolpidem similarly increased the mean and the single-channel conductance of GABARex I (γ 1.6, γ_{mean} 1.6). For 1 μM flumazenil and 50 nM THDOC the effects were larger on the mean conductance as compared to the single-channel conductance in GABARex II (γ 1.2, γ_{mean} 1.5) and GABARex III (γ 2.4, γ_{mean} 5.2), respectively, demonstrating an increase in the open probability of the channel in addition to the effect on the channel conductance in the presence of the drugs.

Discussion

Although interest in tonic neuronal inhibition has increased in recent years we still know relatively little about the channels generating the tonic currents in various neuronal populations. Here we report that in the dentate gyrus granule neurons there are at least three types of GABARex channels that differ in their maximal conductance, latency of activation, sensitivity to drugs and apparent affinity for GABA. The very high apparent affinity for GABA (4 - 40 nM) and a saturated response at about 1 μM GABA makes these receptors sensitive to the extracellular GABA concentration within the relevant physiological range (Lerma *et al*., 1986; Tossmann *et al*., 1986). In this respect we have previously reported differences in response to GABA between receptors recorded in cell-attached patches and outside-out patches (see Birnir *et al*., 2001) and shown that co-expression of the intracellular protein GABARAP with $GABA_A$ receptor subunits changes the conductance properties of the receptors (Everitt *et al*., 2004). The nanomolar affinity of the receptors for GABA we measured in this study may be related to the receptors being recorded in intact neurons where they are in contact with intracellular structures, proteins and milieu required to sensitize the receptors to GABA.

The number of conductance levels recorded for GABARex I, II and III is greater than the normally accepted number of binding sites (2) for the $GABA_A$ receptors and the number of subunits (5) that make up the receptors (Barnard *et al*., 1998; Farrant & Nusser, 2005). An explanation for our results that does not require a large number of binding sites is related to Koshland's "induced-fit" hypothesis (Yankeelov & Koshland, 1965). In a pentameric receptor, occupation of a binding site by GABA might induce moulding of the receptor protein around the ligand. The moulding would be related to

the GABA concentration and governed by the occupancy of GABA at the ligand-binding site. The GABA concentration-dependent conformational changes at the binding site might then be transmitted through the protein and drive the progressive changes in the ion conduction pathway resulting in the recorded graded conduction. That a ligand-binding core can adopt a range of conformations that are related to discrete conductance states of the ion channel was recently shown for the ionotropic glutamate receptors (Jin *et al.*, 2003). Another possible explanation is e.g. that closely clustered channels may be able to open cooperatively. The "single-channel" currents would then contain contributions from a number of coupled pentameric channels (Eghbali et al., 1997; Everitt et al., 2004).

The GABARex channels did not open immediately when GABA was applied. In the light of the different roles the tonic (extrasynaptic) and the phasic (synaptic) GABAergic inhibition are proposed to have in neuronal and network function (Semyanov *et al*., 2004) the latency of activation of GABARex channels is perhaps not as surprising as it initially appears to be. It ensures that these receptors are not activated in a random fashion since the local, extracellular GABA concentration has to be maintained for a relatively long time (e.g., tens to hundreds of seconds compared to the ms time-scale of synaptic transmission) in order to evoke the extrasynaptic, tonic conductance. What determines the local extracellular GABA concentration is not clear but release from astrocytes, reverse co-transport and spill-over from synapses have all been indicated in contributing to the GABA concentration in the extracellular space (Brickley *et al*., 1996; Liu *et al*., 2000; Wu *et al*., 2003).

The pharmacological profile of the GABARex channels is consistent with the following minimal subunit composition of GABARex I $\alpha_1\beta\gamma_2$, GABARex II $\alpha_4\beta\gamma_2$ and

GABARex III αβδ (Fig. 2 and 4) but does not rule out that other subunit compositions might have these properties. Data from tonic current measurements or immunogold labelling have located α_4- and δ-containing receptors extrasynaptically in dentate gyrus granule neurons (Mody, 2004) whereas α_1-containing receptors have been located extrasynaptically both in hippocampal interneurons (Semyanov *et al*., 2004) and dentate gyrus granule cells (Nusser *et al*., 1995). Benzodiazepine-sensitive tonic and single-channel currents indicate extrasynaptic γ_2-containing receptors and have been reported both in the hippocampal interneurons and the CA1 pyramidal neurons (Lindquist *et al*., 2003; Semyanov *et al*., 2004, Caraiscos *et al*., 2004).

We have shown that extrasynaptic $GABA_A$ receptors can effectively sense and respond to the low GABA concentrations present in the extracellular fluid of the brain. The response is graded and develops over seconds. The high nanomolar affinity for GABA and the dynamic channel conductance make the GABARex channels ideal components of an efficient tonic inhibitory system.

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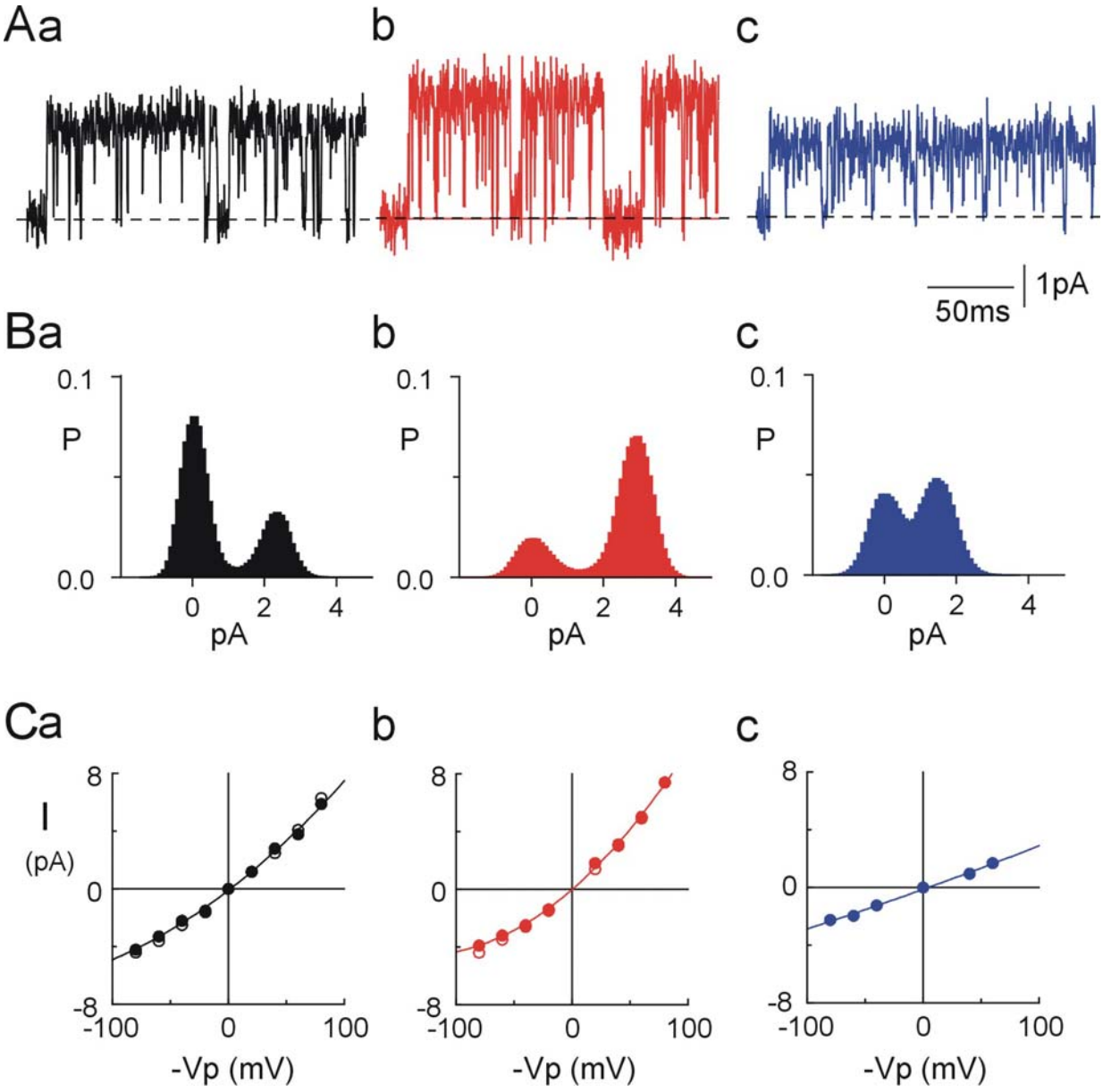

Figure 1.

Three types of GABARex channels in dentate gyrus granule neurons.

A. Representative maximal single-channel currents activated by 1 μM GABA in cell-attached patches. GABARex **a**. I, 2.4 pA. **b**. II, 3.0 pA. **c**. III, 1.7 pA. **B**. Histograms of 30-s current recordings, including the corresponding traces shown in A. GABARex **a**. I **b**. II **c**. III. **C**. Current-voltage relations for currents activated by 1 μM GABA in cell-attached (solid circles) patches and then after

the same patches had been made inside-out (open circles). GABARex **a**. I **b**. II **c**. III.

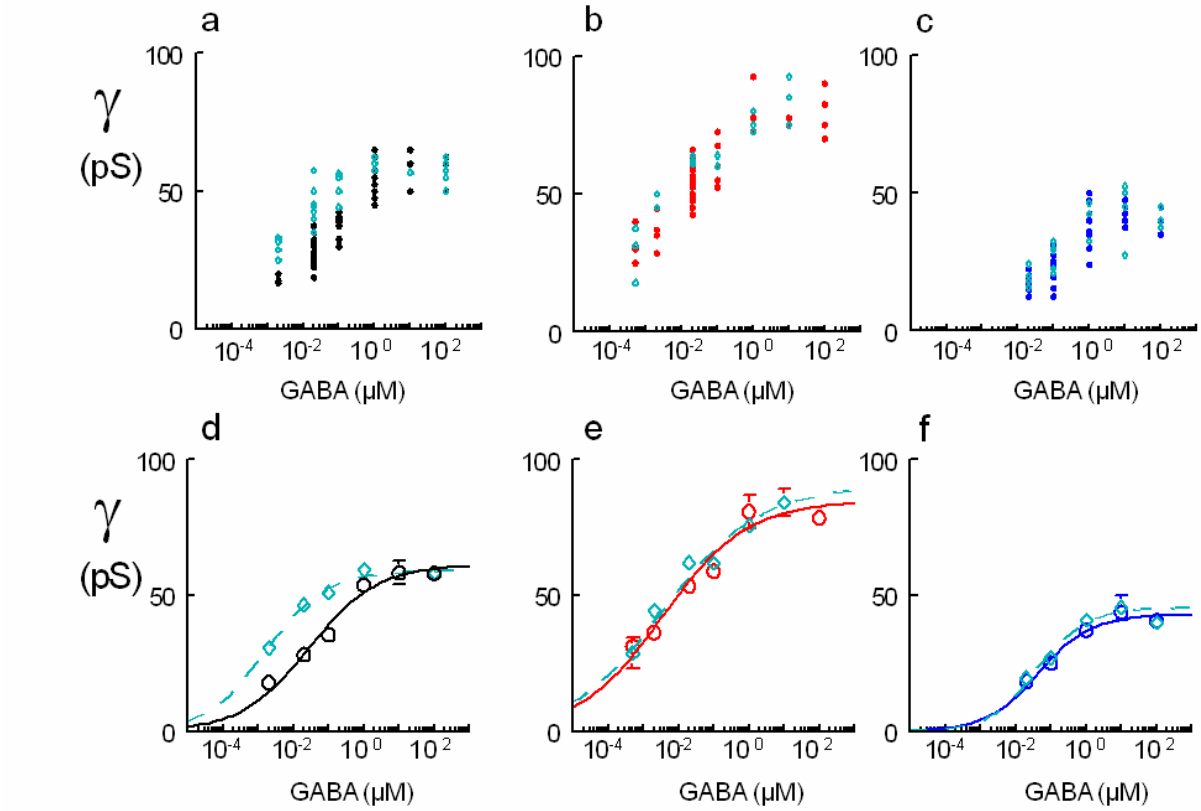

Figure 2.

The maximal single-channel conductance is set by the GABA concentration.

Maximal single-channel conductance (γ_{max}) in cell-attached patches plotted against the GABA concentration. In a, b and c individual experiments are plotted whereas in d, e and f the average conductance is shown. **a & d** GABARex I: GABA alone (n = 70), GABA plus 1 μM diazepam (n = 27) **b & e**. GABARex II: GABA alone (n = 56), GABA plus 1 μM diazepam (n = 23) **c & f**. GABARex III: GABA alone (n = 42), GABA plus 1 μM diazepam (n = 23). The lines are a fit of a Hill-type equation to the data, GABA alone (circles, solid line), GABA + 1 μM diazepam (diamonds, broken line). Concentration-dependent average conductance ± s.e.m. with $3 \leq n \leq 33$.

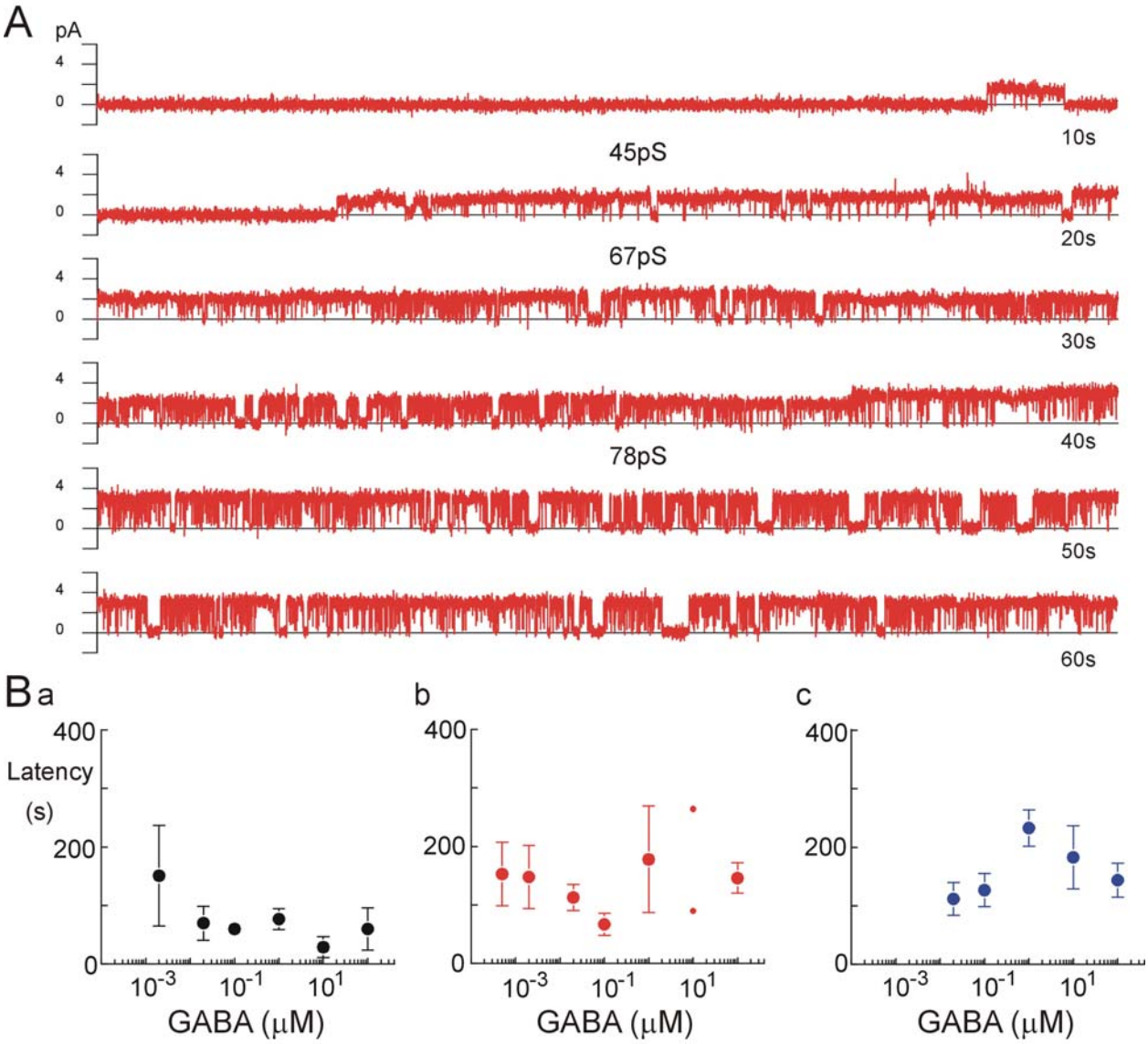

Figure 3.

The maximal single-channel conductance is evoked after a delay from the GABA application.

A. GABARex II currents activated in a cell-attached patch by 1 μM GABA increased with time from 45 pS to 78 pS. **B**. The delay (latency) between the GABA application and the appearance of the maximal conducting channel plotted against the GABA concentration. Concentration dependent average latency ± s.e.m with $3 \leq n \leq 24$ from the same data-set as used in Fig. 2. Small symbols represent individual experiments. GABARex **a**. I (n = 56) **b**. II (n = 56) **c**. III (n = 34).

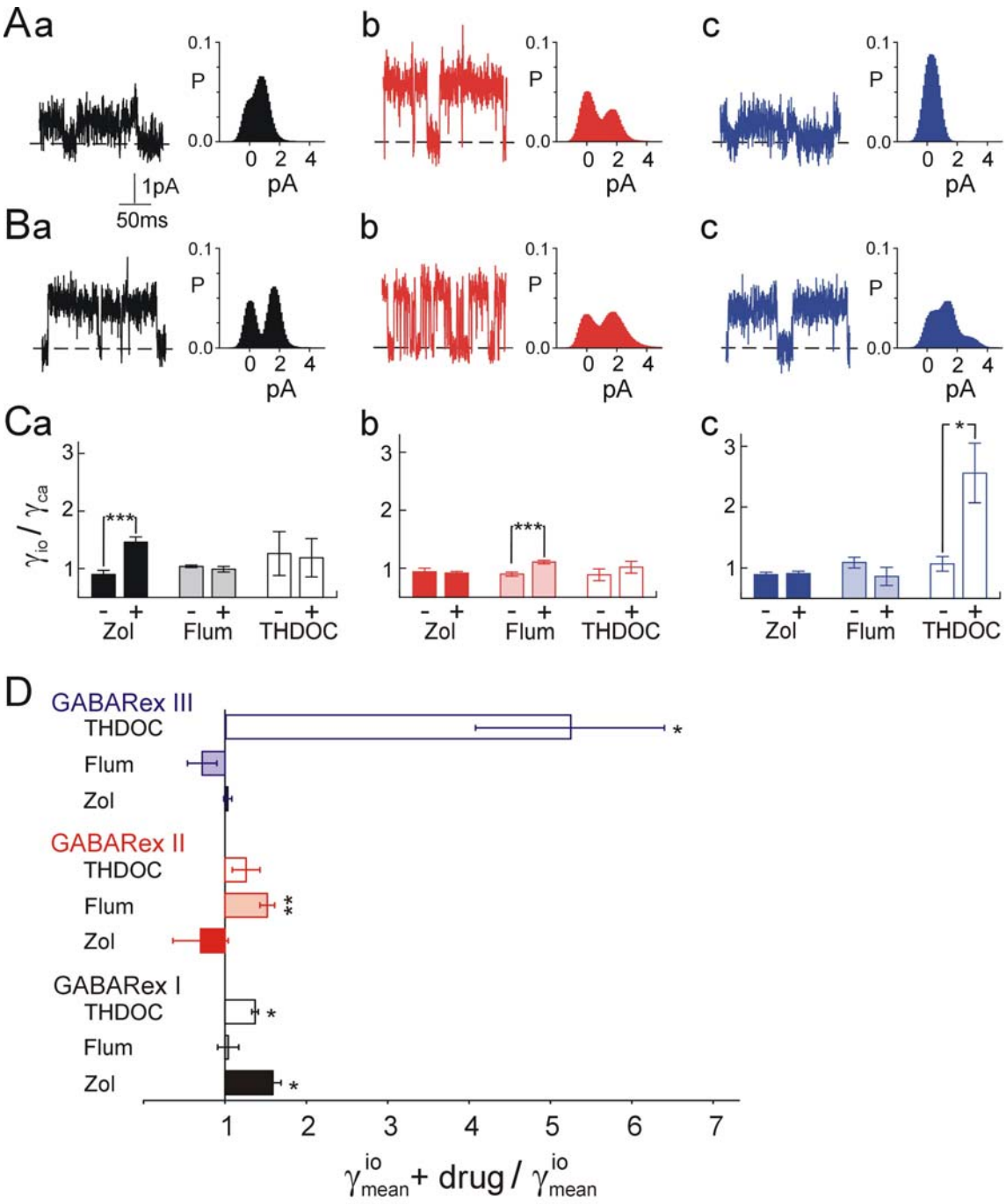

Figure 4.

The GABARex channels are differentially modulated by drugs.

Channels were activated by 20 nM GABA in cell-attached patches and then the patches were made inside-out. **A** Channels before and **B.** after the addition of a drug to the same

inside-out patch. The histograms are from 30-s current recordings and include the corresponding current trace. GABARex I (**a**), II (**b**) and III (**c**) in the absence (**A**) and presence **(B**) of 200 nM zolpidem (Zol, Ba), 1 μM flumazenil (Flum, Bb) or 50 nM THDOC (Bc), respectively. **C**. Average single-channel conductance from inside-out (io) patches normalized to the conductance recorded in the same cell-attached (ca) patches. GABARex (**a**) I, Zol (n = 6), Flu (4), THDOC (3) (**b**) II, Zol (2), Flum (8), THDOC (3) and (**c**) III, Zol (3), Flum (3), THDOC (8). Values are averages ± s.e.m. **D**. Mean conductance from 10-s current traces recorded in inside-out patches in the presence of drugs and shown as a fraction of the mean conductance in the absence of the drug. GABARex: I, Zol (n = 4), Flum (4), THDOC (3), II, Zol (2), Flum (8), THDOC (4) and III, Zol (3), Flum (3), THDOC (4). Values are averages ± s.e.m. $*$ $p < 0.05$; $**$ $p < 0.01$; $***$ $p < 0.001$, students t-test.