Electrophysiological properties of mouse horizontal cell

GABA_A receptors

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GABA-induced currents have been characterized in isolated horizontal cells from lower vertebrates, but not in mammalian horizontal cells. Therefore, horizontal cells were isolated after enzymatical and mechanical dissociation of the adult mouse retina and visually identified. We recorded from horizontal cell bodies using the whole-cell and outside-out configuration of the patch-clamp technique. Extracellular application of GABA induced inward currents carried by chloride ions. GABA-evoked currents were completely and reversibly blocked by the competitive GABA\textsubscript{A} receptor antagonist bicuculline (IC\textsubscript{50} = 1.7 µM), indicating expression of GABA\textsubscript{A} but not GABA\textsubscript{C} receptors. Their affinity for GABA was moderate (EC\textsubscript{50} = 30 µM), and the Hill coefficient was 1.3, corresponding to two GABA binding sites. GABA responses were partially reduced by picrotoxinin with differential effects on peak and steady-state current values. Zinc blocked the GABA response with an IC\textsubscript{50} value of 7.3 µM in a non-competitive manner. Furthermore, GABA receptors of horizontal cells were modulated by extracellular application of diazepam, zolpidem, DMCM, pentobarbital, and alphaxalone, thus showing typical pharmacological properties of CNS GABA\textsubscript{A} receptors. GABA-evoked single-channel currents were characterized by a main conductance state of 29.8 pS and two subconductance states (20.2 pS and 10.8 pS, respectively). Kinetic analysis of single-channel events within bursts revealed similar mean open times and closed times for the main conductance and the 20.2-pS subconductance state, resulting in open probabilities of 44.6% and 42.7%, respectively. The ratio of open to closed times, however, was significantly different for the 10.8-pS subconductance state with an open probability of 57.2%.
INTRODUCTION

GABA acts mainly as an inhibitory neurotransmitter in the mammalian central nervous system (Bormann 1988; Sivilotti and Nistri 1991), although excitatory actions of GABA have been described, which seem especially important for developmental processes (Ben Ari 2002; Owens and Kriegstein 2002). GABA activates ionotropic GABA_A and GABA_C receptors by opening of an integral ion channel selectively permeable to chloride ions (Bormann et al. 1987; Bormann and Feigenspan 1995). Whereas bicuculline competitively blocks GABA_A receptors, it has no effect on GABA_C receptors (Feigenspan et al. 1993; Polenzani et al. 1991; Qian and Dowling 1993).

GABA_A receptors consist of various combinations of at least 14 different subunits, which determine their physiological and pharmacological properties (Barnard et al. 1998; Sieghart 1995). Although the multiplicity of subunits suggests a daunting number of possible combinations, it seems that only some of these combinations are realized. So, native receptors contain at least one α, one β, and one γ subunit, with the other subunits as possible substitutes for γ (McKernan and Whiting 1996). The importance of subunit composition for correct targeting of assembled GABA_A receptors to synaptic sites has recently been suggested (Moss and Smart 2001).

Horizontal cells are a class of second-order interneurons which modulate the signal transfer between photoreceptors and bipolar cells in the outer plexiform layer (OPL) of the vertebrate retina. They are extensively coupled via gap junctions, and thereby help establishing the antagonistic receptive field structure of bipolar cells and ganglion cells. Whereas most mammalian species possess two morphologically distinct types of horizontal cell, only one type has been described in the retina of the mouse (Jeon et al. 1998; Peichl and Gonzalez-Soriano 1994). The dendrites of this type synapse exclusively with cones, and the axon terminal system receives synaptic input exclusively from rods. The GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) has been localized to horizontal cells of the cat.
retina (Sarthy and Fu 1989), pinpointing GABA as a potential neurotransmitter of horizontal cells. The question whether GABA is released by a vesicular or by a non-vesicular mechanism has not been unequivocally answered yet. However, recent data demonstrate the presence of a vesicular GABA transporter in horizontal cells, suggesting that GABA is transported and stored into vesicles, although expression of the vesicular transporter in the plasma membrane cannot be excluded (Cueva et al. 2002).

The physiological properties of mammalian horizontal cells have so far been studied mainly by measuring their light responses using intracellular recording techniques. With the exception of glutamate receptors (Blanco and de la Villa 1999; Rivera et al. 2001), a detailed analysis of their equipment with ligand-gated ion channels is not available. Since the mouse has become increasingly important in the study of retinal neurobiology, we developed a preparation to investigate the physiological fingerprint of horizontal cells by using whole-cell and single-channel configurations of the patch-clamp technique. In this paper, we describe the physiological and pharmacological properties of GABA_A receptors expressed by solitary mouse horizontal cells, and we determine their single-channel characteristics.
METHODS

Dissociation of the retina and identification of horizontal cells

2-4 months old C57/B mice were deeply anesthetized by intraperitoneal injection of a 0.1 ml solution containing equal parts of 5% ketamine (Ceva, Düsseldorf, Germany) and 1% xylazine (Ceva) and subsequently killed by cervical dislocation. After removal of the cornea, lens, vitreous body, and sclera, the retina was transferred to 1 ml of digestion buffer containing 20 U/ml papain (Worthington Biochemical, Freehold, NJ) and 200 U/ml DNase I (Sigma Deisenhofen, Germany) in Earle’s Balanced Salt Solution (EBSS; Sigma). After 40-45 min digestion at 37ºC, the retina was transferred to trituration buffer to stop papain activity (5 min, 37ºC). This solution contained 1 mg/ml ovomucoid inhibitor (Worthington), 1 mg/ml bovine serum albumin (Sigma) and 100 U/ml DNase I (Sigma) in EBSS. The tissue was centrifuged at 1000 rpm (5 min, 22ºC), and the pellet was resuspended in minimum essential medium (MEM; Sigma). Subsequently, the retina was triturated with fire-polished Pasteur pipettes of decreasing open diameter, and after each trituration step, the cell suspension was carefully checked for the presence of horizontal cells. Those fractions containing horizontal cells were finally pooled, and the cell suspension was plated on glass coverslips, which had been coated with 1 mg/ml concanavalin A (Sigma). The cells were kept in an incubator in 5% CO₂ and 55% O₂ at 37ºC. After 15-20 min, 1% fetal calf serum (Sigma) was added to improve viability of the cells.

Immunocytochemistry

Horizontal cells plated on glass coverslips were immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min. After several rinses in PB, the coverslips were incubated in a solution containing 5% normal goat serum (NGS) and 0.3% Triton X-100 in PB for 1 hr. A polyclonal antibody against the calcium-binding protein calbindin D-28K (SWant, Bellinzona, Switzerland) was diluted 1:500 in a solution containing 3% NGS and
0.3% Triton X-100 in PB for 12-14 hr. Binding of the primary antibody was visualized with a goat anti-rabbit Alexa 568 (diluted 1:200; Molecular Probes, Eugene, OR) secondary antibody (2 hr). To prevent bleaching, the cells were embedded in VectaShield (Vector Laboratories, Burlingame, CA). Images were taken on a confocal laser scanning microscope (Leica, Nussloch, Germany) using the 568 line of a krypton-argon laser.

**Reverse transcriptase-PCR**

For reverse transcriptase-PCR, visually identified isolated horizontal cells were harvested with a patch pipette. The intracellular solution contained 140 mM KCl, 0.5 mM EGTA, and 10 mM Hepes, pH 7.4. After seal formation, the cellular contents of individual horizontal cells were carefully aspirated into the pipette by applying negative pressure. The electrode was then lifted from the bath under constant visual control to avoid contamination with neighboring cells or debris. The tip of the pipette was finally broken into an Eppendorf tube containing 20 U RNase inhibitor (RNasin; Promega, Mannheim, Germany). After brief centrifugation, the tube was frozen on dry ice and stored at -80°C.

Contaminating genomic DNA was digested with DNase I (Amplification Grade, Invitrogen) according to the manufacturer's protocol. cDNA synthesis was carried out in a final volume of 25 µl. Each sample contained 1x first-strand buffer (Promega) 0.6 µM oligo(d)T primer (Promega), 0.6 µM random primer (Promega), 0.5 mM of each dNTP (Eppendorf, Hamburg, Germany) and 0.8 U/µl RNasin ribonuclease inhibitor. After primer annealing for 10 min at 72°C, the samples were briefly chilled on ice and incubated for 2 min at 42°C, before 0.3 U/µl AMV reverse transcriptase (Promega) were added. cDNA synthesis was carried out for 1 h at 42°C and stopped by incubating the samples for 5 min at 95°C. cDNAs were stored at -20°C. PCR reactions were carried out in a total volume of 25 µl. This included 6 µl horizontal cell cDNA, 1x reaction buffer (Promega), 1.25 mM MgCl$_2$, 0.2 mM of each dNTP (Eppendorf), 0.8 µM of each primer (MWG) and 1 unit Taq-polymerase (Promega). Reactions were
overlaid with 35 µl mineral oil (Sigma). The Taq-polymerase was added after incubating the
samples for 2 min at 95°C (hot start). The specific primer set for the detection of calbindin
included the forward primer (5'-GACGCTGATGGAAGTGGTTAC-3') and the reverse
primer (5'-ACGGTCTTGTTGGCTTTCTCT -3'), both of which were designed according to
the mouse calbindin coding sequence (GenBank accession number: NM031984). The
predicted size of the amplicon was 340 bp. Amplifications of calbindin transcripts were
carried out in a Stratagene Robocycler using the following protocol: 95°C for 2 min, 40 cycles
of 95°C for 1 min, 60°C for 1.5 min, 72°C for 3 min, and finally 72°C for 5 min. 20 µl of
each amplification product were analyzed on a 2% agarose gel and visualized on a
transilluminator after ethidium bromide staining.

Electrophysiological recordings

Cells were allowed to settle at least 30 min before commencement of recordings. Coverslips
with retinal neurons were placed in a recording chamber (Luigs and Neumann, Ratingen,
Germany) on the stage of an upright microscope (Leica). Horizontal cells were identified as
described below using 40x and 63x water immersion objectives equipped with Nomarski
optics (Leica). Whole-cell voltage-clamp and outside-out single-channel recordings were
performed with an EPC9 double patch-clamp amplifier (Heka, Lambrecht, Germany). Current
traces were monitored with a digital oscilloscope (Tektronix, Beaverton, OR) and directly
stored to the hard disk of a personal computer. Data were acquired with a sample frequency of
200 Hz in the whole-cell mode, and with a frequency of 10 kHz for single-channel
experiments. Patch pipettes were pulled from borosilicate glass (1.5 mm outer diameter, 1.275
mm inner diameter; Hilgenberg, Malsfeld, Germany) using a horizontal electrode puller
(Sutter, Novato, CA). Electrodes with a resistance ranging from 4 to 7 MΩ were connected to
the amplifier with an Ag/AgCl wire. The electrode holder combined with the headstage were
mounted on a mechanical, remote-controlled device attached to a three-dimensional
micromanipulator (Luigs and Neumann). In whole-cell experiments, the series resistance of the electrodes usually ranged between 8 and 15 MΩ and was not compensated for. However, the series resistance was carefully monitored in the time course of an experiment, and only those recordings with a stable series resistance value were considered for analysis. Drugs were applied to horizontal cells or outside-out membrane patches in the extracellular bath solution by the pressure-driven application system DAD-12 Superfusion System (ALA Scientific Instruments, Westbury, NY).

Isolated horizontal cells were continuously superfused (0.5 ml/min) at room temperature with an extracellular solution containing (in mM): 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Hepes, and 10 glucose (pH 7.4). The intracellular solution for recordings of whole-cell and single-channel currents contained (in mM): 120 CsCl, 20 TEA-Cl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 Hepes (pH 7.2). Diazepam, zolpidem, alphaxalone, methyl 6,7-dimethoxy-4-ethyl-β-carboxylate (DMCM) (all from Sigma) were prepared as 10-mM stock solutions in DMSO and stored at -20°C. The maximal final concentration of DMSO was 0.03%, which had no effect on GABA-induced currents. Bicuculline methiodide and picrotoxinin (both from Sigma) were freshly prepared and added to the GABA containing solution. ZnCl₂ (Fluka, Buchs, Switzerland). Pentobarbital (Sigma) was prepared as 10-mM stock solutions in extracellular solution and kept frozen at -20°C.

Data analysis

Current amplitudes were normalized and expressed as the ratio of the GABA-induced peak current in the presence of the drug relative to the control GABA response. For dose-response curves, current amplitudes were normalized to the maximum response, either obtained with saturating concentrations of GABA or, for studies of inhibitory effects, in the absence of antagonists. Data points of dose-response curves were fitted with a sigmoidal logistic function
using a Simplex algorithm: \( I / I_{\text{max}} = 1 / (1 + (c / k)^n) \), where \( c \) denotes the concentration of agonist or antagonist, \( k \) the half-maximally effective concentration, and \( n \) the Hill coefficient.

GABA-induced single-channel events in outside-out patches were analyzed after low-pass filtering at 1 kHz (-3 dB, 4-pole Bessel filter). Bursts were constructed by choosing 5 ms as the intraburst interval. Time frames containing bursts of single-channel openings were selected, converted into all-point histograms, and subsequently fitted with multiple Gaussian distributions. Open times, closed times, and open probabilities were determined by half-amplitude threshold analysis. For open time distributions, number of events per burst were binned in 2ms-intervals, and the data were fitted with a first-order exponential probability density function: \( f(t) = a \exp(-t/\tau) \), where \( \tau \) denotes the mean of the distribution. Desensitizing and deactivating current traces were also fitted with a first-order exponential function. All data analysis was performed with the Pulsefit (Heka), MatLab (MathWorks, Natick, MA) and Origin software packages (Microcal, Natick, MA).
RESULTS

Identification of horizontal cells

It has been shown previously that only one type of horizontal cell is present in the retina of mice and rats (Jeon et al. 1998; Peichl and Gonzalez-Soriano 1994). This type of horizontal cell typically displays a multipolar morphology, with a long, thin axon, extending within the outer plexiform layer and ramifying in an elaborate axon terminal system. Thus, these cells belong to the axon-bearing type or B-type of horizontal cells, as described in other mammalian species like cats and primates (Boycott et al. 1978; Kolb et al. 1980). In the mammalian retina, antibodies to the calcium-binding protein calbindin D-28K have been effectively used to label horizontal cells in the rabbit (Röhrenbeck et al. 1987; Röhrenbeck et al. 1989) and the mouse (Haverkamp and Wässle 2000).

Horizontal cells were obtained from the mouse retina after enzymatical and mechanical dissociation. Because their thin axons are likely to be ruptured, the dissociation process resulted in horizontal cell bodies and axon terminals as separate entities. Horizontal cell somata were characterized by a polygonal-shaped perikaryon that measured 14 µm on average and gave rise to 5–8 primary dendrites (Fig. 1A,B). To avoid confusion, it should be noted that we recorded exclusively from horizontal cell bodies, which will simply be referred to as horizontal cells in the remainder of the text. Although the axon and probably the fine distal dendrites were lost during the dissociation procedure, horizontal cells were viable and readily accessible for patch-clamp electrodes. To confirm their identity, we harvested the cytoplasm of five visually identified horizontal cells after obtaining their electrophysiological fingerprint, and subsequently we performed single-cell reverse transcriptase-PCR with primers specific for calbindin D-28K. In all cases, we obtained a signal at the expected size of 340 bp (Fig. 1B, inset). In addition, immunocytochemistry with polyclonal antibodies directed against calbindin D-28K was carried out on paraformaldehyde-fixed isolated cells. All cells previously identified as horizontal cells showed calbindin-like immunoreactivity (Fig. 1B),
thus confirming our PCR results and strongly suggesting that the cells chosen for recordings were horizontal cells. In addition, we performed control experiments on dissociated cells with bipolar morphology, which do not contain calbindin D-28K. Both reverse transcriptase-PCR and immunocytochemistry were negative for these cells (data not shown).

Finally, horizontal cells of the mouse retina were characterized electrophysiologically by the presence of non-inactivating calcium currents. With voltage-dependent potassium channels blocked, stepwise depolarization of the membrane from a holding potential of −70 mV to 0 mV induced calcium-mediated, small-amplitude inward currents with properties reminiscent of L-type channels (Fig. 1C). This inward current was observed in all horizontal cells tested. Since voltage-dependent ion channels are subject of a different study, they were not investigated further. In summary, we used (1) morphological criteria, (2) the presence of calbindin D-28K, and (3) the presence of L-type-like calcium channels to identify horizontal cells in a preparation of acutely dissociated retinal cells.

**GABA concentration-response relationships**

Morphologically identified horizontal cells were voltage-clamped at a holding potential of −70 mV with equal concentrations of chloride on the intra- and extracellular sides of the membrane. In the whole-cell mode of the patch-clamp technique, extracellular application of GABA induced chloride-mediated inward currents in all horizontal cells tested. We obtained successful recordings from a total of 174 horizontal cells. Stable seals with resistances between 2 and 10 GΩ could be established in more than 90% of the recordings, indicating that the dissociation procedure did not impair the overall structure of the cell membrane. Because GABA concentrations less or equal 1 µM consistently failed to induce measurable currents, GABA was applied at concentrations ranging from 3 to 1000 µM. Desensitization of GABA receptor-mediated currents was dose-dependent and became apparent at GABA concentrations of 30 µM and above (Fig. 2A). The maximal current measured at a saturating
concentration of GABA (1 mM) was variable and ranged in amplitude from –241 to –1210 pA with a mean value of –633 ± 100 pA (mean ± SE, n = 11).

For each horizontal cell, inward currents induced by increasing concentrations of GABA were normalized to the maximal value obtained with 1 mM GABA. When compared between cells, responses appeared most variable in the linearly rising part of the dose-response plot, with a maximal standard deviation of 0.16 measured at 30 µM (Fig. 2B,C). EC$_{50}$ values for individual cells ranged from 16.7 to 53.0 µM with a mean value of 32.6 µM. For each GABA concentration, the normalized mean of all horizontal cells was plotted and fitted to a sigmoidal logistic function (Fig. 2C). The EC$_{50}$ value obtained from the fit was 30.1 ± 1.9 µM with a Hill coefficient of 1.3 ± 0.1 corresponding to two GABA binding sites on each GABA receptor. The maximal amplitude of GABA-induced currents derived by the best fit to the logistic equation was –662 ± 25 pA, as compared to the mean peak current value of –633 pA measured in 11 horizontal cells. The insignificant difference between the two values indicated that peak current amplitudes were not affected by desensitization or redistribution of chloride ions even at saturating concentrations of GABA.

Current-voltage (I-V) relationships of GABA-induced currents were obtained by ramping the membrane potential from -70 mV to 70 mV (100 mV s$^{-1}$) in the presence of 100 µM GABA (Fig. 2D). Non-specific leak conductances were determined by performing the same ramping protocol in the absence of GABA and eventually subtracting the two current traces. The I-V-curve was linear with no sign of rectification, as indicated by fitting the data points with a linear regression function. The current reversed sign close to 0 mV, which is the expected equilibrium potential given symmetrical chloride concentrations on both sides of the membrane.

In addition, we determined the kinetics of activation, desensitization, and deactivation of mouse horizontal cell GABA$_{A}$ receptors. The average rate of activation was measured as the 10–90% rise time of currents elicited by 1 mM GABA. The 10–90% rise time to peak
current amplitudes was 89 ± 9 ms (n = 19). 3-s pulses of GABA (1 mM) were used to determine desensitization kinetics. The decline of currents was well fitted using a first-order exponential function with a time constant of 1142 ± 50 ms (n = 19; Fig. 2E). Similarly, the deactivation properties of GABA receptor channels were measured by fitting a first-order exponential function to the current trace immediately after a 3-s application of GABA (50 µM). Currents decayed slowly with a time constant of 490 ± 24 ms (n = 19; Fig. 7F).

Inhibitory effects of bicuculline and picrotoxinin

The plant alkaloid bicuculline has been described as a competitive and reversible blocker of GABA<sub>A</sub> receptor-mediated currents. Concentrations of bicuculline ranging from 0.1 to 100 µM were co-applied with 50 µM GABA. As shown in Fig. 2C, this GABA concentration induced about 66% of the maximal inward current. Because the application of seven different concentrations took a considerable amount of time, it was crucial to continuously monitor experimental conditions. Therefore, control applications of 50 µM GABA without bicuculline were frequently performed in the course of an experiment. A final application of GABA served to indicate that the observed inhibition was indeed caused by the drug and not by rundown of currents or deterioration of the recording conditions. Only those horizontal cells displaying stable GABA-induced inward currents were considered for analysis.

Fig. 3A shows a consecutive series of current traces obtained from co-application of 50 µM GABA with increasing concentrations of bicuculline. GABA-induced inward currents were reduced by bicuculline in a dose-dependent manner. The effect of the drug was fully reversible as indicated by the current amplitude under washout conditions. Complete dose-response curves were recorded from six horizontal cells. Low concentrations of bicuculline (1 µM) inhibited GABA-induced currents to 0.64 on average, whereas the current was completely eliminated by 100 µM (Fig. 3A). Low concentrations of bicuculline had a stronger effect on peak amplitudes than on steady-state values, whereas the overall response became
non-desensitizing with concentrations above 3 µM bicuculline (Fig. 3A). The IC$_{50}$ values for individual cells ranged from 1.2 to 3.5 µM with a median value of 1.6 µM. Because the inhibitory effect of bicuculline appeared homogeneous across horizontal cells, we calculated the mean of all responses obtained at a given concentration of bicuculline and fitted the data points with a sigmoidal logistic function (Fig. 3B). The IC$_{50}$ value obtained from the best fit to the data points was 1.7 ± 0.1 µM with a Hill coefficient of 1.1 ± 0.1. These results indicate that horizontal cell GABA responses are sensitive to the inhibitory action of bicuculline.

Furthermore, GABA receptor currents are mediated exclusively by GABA$_A$ receptors, but not by GABA$_C$ receptors, as indicated by the complete block of GABA-induced currents with high concentrations of bicuculline.

In addition, we confirmed the competitive mode of inhibition exerted by bicuculline. When a dose-response curve for GABA was measured in the presence of 3 µM bicuculline, responses induced by low concentrations of GABA were more strongly affected by the antagonist than those evoked by higher concentrations (Fig. 3C). The inhibitory action of bicuculline could be entirely abolished by a 1000-fold excess of GABA. Bicuculline induced a rightward shift in the dose-response curve towards higher GABA concentrations, with EC$_{50}$ values changing from 38.5 ± 5.7 µM (n = 7) under control conditions to 201.7 ± 10.0 µM (n = 7) in the presence of bicuculline. These results are consistent with a competitive block of GABA$_A$ receptors by bicuculline.

Picrotoxinin has been described as an open channel blocker selective for GABA- and glycine-gated chloride channels. To determine its use-dependent mode of inhibition, picrotoxinin was applied twice at a given concentration and then thoroughly washed out before the next concentration was tested (Fig. 4A). Consecutive applications of picrotoxinin exerted a differential effect on peak and steady-state current values. At 10 µM picrotoxinin, 0.60 of the control peak current remained during the first application, whereas the current was reduced to 0.38 during the second application (Fig. 4A,B). A similar effect was observed at 100 µM
concentration with 0.28 and 0.15 of control current values remaining after the first and second application of picrotoxinin, respectively. When the steady-state values of the currents were compared, however, two applications of picrotoxinin blocked very similar fractions of the GABA-induced current. The fractions measured for the two applications at 10 µM (0.45 and 0.46) and 100 µM picrotoxinin (0.13 and 0.16) did not show a significant difference (p < 0.01, t-test). The mean current amplitudes reflecting these findings are summarized in Fig. 4B. This effect was observed in all eight horizontal cells tested. Additional applications of picrotoxinin did not further reduce GABA-evoked currents. Interestingly, we consistently found that GABA responses of mouse horizontal cells were not entirely abolished even at high concentrations of picrotoxinin. The mean amplitudes of peak and steady-state control responses before and after washout of the drug did not show a statistically significant difference (p < 0.01, t-test), indicating that the inhibitory effect of picrotoxinin on GABA receptor-mediated chloride currents was almost fully reversible (Fig. 4B).

**Inhibition of GABA receptor currents by Zn^{2+}**

The divalent transition metal cation Zn^{2+} has been shown to non-competitively antagonize GABA_{A} receptor-mediated currents (Hosie et al. 2003; Legendre and Westbrook 1991; Westbrook and Mayer 1987). Zn^{2+} ranging in concentrations from 1 to 1,000 µM was co-applied with 50 µM GABA to eight isolated horizontal cells. Fig. 5A shows the inhibitory effect of increasing concentrations of Zn^{2+}. Block of GABA-evoked currents by Zn^{2+} was fully reversible after complete removal of the blocker from the extracellular solution. It appears that the presence of Zn^{2+} slowed the onset of the GABA-induced inward currents (Fig. 5A). This effect was consistently observed in all horizontal cells tested.

Horizontal cell GABA receptors showed high sensitivity towards the blocking effects of Zn^{2+}. Concentrations as low as 1 µM Zn^{2+} blocked on average 0.26 of the control inward current, which was completely eliminated by 1 mM Zn^{2+}. We measured the dose-response curve by
calculating the normalized mean residual current at each concentration of Zn\(^{2+}\) and subsequently fitting the data points to a sigmoidal logistic equation (Fig. 5B). The IC\(_{50}\) value of Zn\(^{2+}\) inhibition obtained from the fit was 7.3 ± 1.9 µM with a Hill coefficient of 0.7 ± 0.1.

When eight different dose-response curves were generated for every single horizontal cell, the median IC\(_{50}\) value was 7.5 µM with a Hill coefficient of 0.7. These results are very similar to the numbers obtained from the best fit to the mean values of all cells, indicating homogeneous inhibition of mouse horizontal cell GABA\(_A\) receptors by Zn\(^{2+}\).

Furthermore, we determined the mode of horizontal cell GABA\(_A\) receptor inhibition by Zn\(^{2+}\). Dose-response curves for GABA were measured under control conditions and in the presence of 5 µM Zn\(^{2+}\) (Fig. 5C). EC\(_{50}\) values calculated from fitting the dose-response curves with a sigmoidal logistic equation were 32.7 ± 3.6 µM (n = 9) for control conditions, and 34.3 ± 2.5 µM (n = 9) in the presence of 5 µM Zn\(^{2+}\). These values were not significantly different (p < 0.01, t-test). In addition, the ratio of GABA-induced currents with and without Zn\(^{2+}\), respectively, could be well fitted with a linear regression line displaying a slope of 0.02 ± 0.04, suggesting that the amount of block exerted by Zn\(^{2+}\) does not depend on the GABA concentration (Fig. 5D). These results indicate that Zn\(^{2+}\) blocks GABA\(_A\) receptors of mouse horizontal cells in a non-competitive manner.

*Effects of benzodiazepines, barbiturates and steroids*

GABA\(_A\) receptors are known to be modulated by benzodiazepines like diazepam or flunitrazepam, which potentiate GABA-induced currents by increasing the frequency of channel opening (Study and Barker 1981). Furthermore, it has been shown that the \(\gamma2\) subunit is required to confer benzodiazepine sensitivity to GABA\(_A\) receptors (Pritchett et al. 1989). To study the effects of benzodiazepines on horizontal cell GABA\(_A\) receptors, we applied diazepam in concentrations ranging from 1 to 10 µM together with 50 µM GABA. Diazepam potentiated the peak current in all eight horizontal cells tested, with a mean enhancement of
2.51 ± 0.16 at the highest concentration of 10 µM (Fig. 6A). A dose-response curve was obtained by calculating the normalized mean responses of all cells and fitting the data points with a logistic function (Fig. 6B). The EC_{50} value for diazepam was 6.6 µM with a Hill coefficient of 0.8. The potentiating effects diazepam were fully reversible after washout of the drug.

To determine the pharmacology of the benzodiazepine binding site, we applied the BZ1-selective imidazopyridine zolpidem (Macdonald and Olsen 1994; Pritchett et al. 1989) in the concentration range of 1 to 10 µM. Like diazepam, zolpidem augmented the inward currents induced by 50 µM GABA with a maximal enhancement of 2.48 ± 0.13 at 10 µM (Fig. 6C). Again, a dose-response curve was generated by normalizing the current amplitudes to the control response to 50 µM GABA and plotting the normalized values against the concentration of zolpidem (Fig. 6D). The EC_{50} value was 85 nM with a Hill coefficient of 0.7, indicating high affinity of horizontal cell GABA_{A} receptors for the BZ1-selective drug zolpidem.

In addition, GABA-induced currents were uniformly inhibited by the inverse benzodiazepine agonist DMCM. At a concentration of 1 µM, currents were reduced to 0.67 ± 0.02 of control values. Barbiturates have been described to modulate GABA_{A} receptor function by increasing the mean open duration time of the chloride channels (Macdonald et al. 1989), whereas the synthetic neuroactive steroid alphaxalone acts by increasing the average open time and opening frequency (Twyman and Macdonald 1992). Both compounds enhanced GABA_{A} receptor-mediated currents of horizontal cells. Pentobarbital (50 µM) increased current amplitudes to 2.68 of control values (n = 11), and alphaxalone (1 µM) augmented the GABA response to 5.1 (n = 10). The pharmacological properties of mouse horizontal cell GABA_{A} receptors are summarized in Table 1.
Single-channel recordings

We determined the single-channel conductance of GABA<sub>A</sub> receptors by recording from outside-out patches pulled from the cell bodies of isolated horizontal cells. Channel openings induced by 10 µM GABA (10 s) were recorded at a holding potential of -70 mV. Because of the low density of GABA<sub>A</sub> receptors on the surface of horizontal cell bodies, single channel events could be recorded in less than 50% of all membrane patches. In those patches containing GABA<sub>A</sub> receptors, extracellular application of GABA induced single channel openings to multiple conductance levels (Fig. 7A).

The amplitude distribution of the main and two subconductance states was determined in five patches recorded at -70 mV holding potential. When all-point histograms were fitted with Gaussian distributions, we obtained mean current values of -2.15 ± 0.02 pA for the main level and -1.45 ± 0.01 pA and -0.73 ± 0.01 pA for the two sublevels, respectively (Fig. 7B-D).

In addition, we measured the main conductance and both subconductance states by recording GABA-induced single channel currents at holding potentials ranging from -70 mV to 70 mV. The resulting single-channel current amplitudes were plotted against voltage and fitted with a linear regression line. The conductances measured from the slope of the I-V relationships were 29.8 ± 0.7 pS for the main level, and 20.2 ± 0.3 pS and 10.8 ± 0.1 pS for the two subconductance states, respectively (Fig. 7E).

Finally, we determined the open time distribution of GABA-induced single-channel events to the main conductance level (Fig. 7F). The majority of openings took place within 1-3 ms, whereas open times longer than 10 ms occurred very infrequently. The distribution was fitted by an exponential probability density function with a mean of 2.43 ± 0.04 ms. The mean open time, the mean closed time, and the open probability (P<sub>o</sub>) for the main conductance and the two subconductance states are summarized in Figure 7G. The main conductance (M) and the second subconductance state (S2) showed similar open times (2.23 ± 0.15 ms for M, 2.14 ±
0.32 ms for S2), and closed times (2.43 ± 0.23 ms for M, 2.30 ± 0.25 ms for S2). Thus, open probabilities for M and S2 were not significantly different (44.6 ± 1.9% for M, 42.4 ± 3.2% for S2; p < 0.01, t-test). In contrast, the first subconductance state (S1) was characterized by a significantly increased value for $P_o$ (Fig. 7G). Although open times are slightly smaller compared to M and S2, closed times are even more reduced (Fig. 7G). The single channel properties of mouse horizontal cell GABA_A receptors are summarized in Table 2.
DISCUSSION

To study the electrophysiology of mouse horizontal cells, we developed a preparation in which individual neurons were separated from each other by enzymatical and mechanical dissociation of the retina. The overall morphology of horizontal cells was surprisingly well conserved in this preparation, and it was used as an unambiguous criterion for cell identification. In addition, the physiological properties of the cells chosen for recordings were remarkably similar, both with respect to voltage-gated channels and to the response to extracellular application of GABA. We consistently failed to observe TTX-sensitive action potentials corresponding to the apparent lack of large-amplitude voltage-dependent sodium currents. However, we cannot exclude low expression of sodium channels, which might be partially masked by simultaneous activation of calcium channels after depolarizing voltage steps. In the cat retina, sodium currents have been demonstrated in 50% of A-type horizontal cells, but not in B-type cells (Ueda et al. 1992). In contrast, a high percentage of immature rabbit retinal cells grown in monolayer culture express TTX-sensitive sodium channels (Löhrke and Hofmann 1994). Thus, the presence of voltage-dependent sodium channels appears to differ between species, cell types, and preparations.

It has been shown previously that exposure to proteases like papain does not affect the properties of GABA-gated chloride channels of dopaminergic amacrine cells of the mouse retina (Gustincich et al. 1997). Similarly, other studies have also reported no alterations of the GABA response when comparing digested tissue and cultured neurons (Kapur and Macdonald 1996).

Physiological properties of horizontal cell GABA\(_A\) receptors

In summary, GABA responses were characterized by (1) high threshold doses of at least 3 µM of the agonist, (2) variable but generally small amplitudes even at saturating concentrations of GABA, and (3) rather low affinities. Although current amplitudes differed within half an
order of magnitude, values normalized to the maximum current were remarkably similar. The $EC_{50}$ value measured for mouse horizontal cells was 30.1 µM, which is in about the same range than that of acutely dissociated pyramidal neurons, adult cortical neurons, and thalamic neurons (Celentano and Wong 1994; Oh et al. 1995). Individual $EC_{50}$ values were evenly distributed around the mean. These results suggest expression by horizontal cells of a variable number of GABA$_A$ receptors, but very similar affinities of these receptor molecules for GABA.

Pharmacological properties and receptor subunit composition

It has been a common observation that GABA-induced currents of all horizontal cells tested were affected by the various modulators and inhibitory substances in a very similar manner. Thus, given the physiological similarity described above, it is tempting to speculate that all horizontal cells express the same or a highly related GABA$_A$ receptor subunit composition. The effect of subunit composition on the affinity of GABA$_A$ receptors for GABA has been studied in detail using heterologous expression systems (Ebert et al. 1994; Saxena and Macdonald 1996).

GABA-induced currents were completely and reversibly blocked by the competitive GABA$_A$ receptor antagonist bicuculline, indicating that mouse horizontal cells express exclusively GABA$_A$ receptors. In contrast, horizontal cells of teleost fish have been shown to contain both GABA$_A$ and GABA$_C$ receptors (Qian and Dowling 1993). The measured $IC_{50}$ value is very similar to that obtained for acutely isolated dopaminergic amacrine cells of the mouse retina and cultured amacrine cells of the rat retina (Feigenspan et al. 2000; Feigenspan and Bormann 1998).

Benzodiazepine receptors are usually subdivided into two different pharmacological types, BZ1 and BZ2. Whereas BZ1 sites show high affinity for CL-218872, zolpidem, and $\beta$-carbolines, BZ2 sites are characterized by a lower affinity for these substances and a high
affinity for flunitrazepam. GABA responses of all horizontal cells were modulated by the BZ1-preferring agonist zolpidem, as well as the benzodiazepine agonist diazepam and the inverse agonist DMCM. The EC\textsubscript{50} value for zolpidem was 85 nM, whereas the affinity of the GABA receptors for diazepam was about an order of magnitude lower (6.6 µM), indicating BZ1-like pharmacological properties. However, maximal enhancement of GABA-induced currents was very similar (2.51 for diazepam and 2.48 for zolpidem). Studies in heterologous expression systems have suggested that sensitivity to benzodiazepines is conferred by the presence of the \(\gamma_2\) subunit (Pritchett et al. 1989). The augmentation by diazepam in all horizontal cells tested could therefore be explained by the expression of the \(\gamma_2\) subunit. In addition, the characteristics of a BZ1 binding site are consistent with the presence of the \(\alpha_1\) subunit. Since the presence of the \(\alpha_4\) subunit in combination with one \(\beta\) and the \(\gamma_2\) subunit confers insensitivity to benzodiazepines (Wisden et al. 1991), we exclude the possibility of \(\alpha_4\) expression in horizontal cell GABA\textsubscript{A} receptors.

With an IC\textsubscript{50} value of 7.3 µM, GABA receptor currents of horizontal cells displayed a moderate to high sensitivity to the divalent metal cation Zn\textsuperscript{2+}. The effects of Zn\textsuperscript{2+} on GABA-induced currents are determined by the receptor isoforms (Draguhn et al. 1990; Smart et al. 1991). The presence of \(\alpha_1\) or \(\beta_x\) (\(x = 1-3\)) confers high sensitivity to Zn\textsuperscript{2+}, whereas on addition of a \(\gamma\) subunit, the inhibitory effect of Zn\textsuperscript{2+} is lost. However, it is reasonable to assume that the presence of a \(\gamma\) subunit causes a decrease in the susceptibility to Zn\textsuperscript{2+} rather than a total loss (Saxena and Macdonald 1996). Therefore, the observed sensitivity of horizontal GABA\textsubscript{A} receptors to the inhibitory action of Zn\textsuperscript{2+} is compatible with the presence of the \(\gamma_2\) subunit in all receptors.

Even high concentrations of the open channel blocker picrotoxinin did not entirely block GABA-mediated inward currents. Picrotoxinin has been shown to directly activate chloride
channels by interacting with the β1 subunit (Sigel et al. 1989), suggesting the presence of this subunit in mouse horizontal cells.

It has been shown that the neuromodulatory effects of steroids are at least partially determined by subunit composition (Gee and Lan 1991; Korpi and Luddens 1993; Lan et al. 1991; Puia et al. 1990; Puia et al. 1993). A large potentiating effect has been associated with expression of the α3 subunit (Lambert et al. 1995; Lan et al. 1991), whereas the presence of the δ subunit inhibits neurosteroid modulation (Zhu et al. 1996). In addition, recombinant receptors containing α1 are more sensitive to neurosteroids than those containing α6, but the identity of the β subunit apparently does not play a crucial role (Zhu et al. 1996). In our hands, the neuroactive steroid alphaxalone caused a large potentiation of GABA-induced currents. The magnitude of this effect is very similar to the augmentation of GABA<sub>Α</sub> receptor currents observed in dopaminergic amacrine cells (Feigenspan et al. 2000), and it is in good agreement with a GABA receptor containing α1, but lacking the δ subunit.

The kinetic properties of mouse horizontal cell GABA<sub>Α</sub> receptors are similar to those of heterologously expressed combinations of α1β3γ2L (Haas and Macdonald 1999). The slow time constants obtained by fitting the current traces with first-order exponential functions range within the same order of magnitude.

Finally, preliminary evidence indicates that GABA-induced currents of horizontal cells are modulated by extracellular dopamine. This effect is most likely mediated by activation of cAMP-dependent protein kinase A (Feigenspan and Bormann, 1994), and biochemical work has identified the β3 subunit as the main target for PKA (Browning et al. 1993).

In summary, the physiological and pharmacological data indicate that GABA<sub>Α</sub> receptors expressed by mouse horizontal cells are comprised of the α1, β1, β3, and γ2 subunits.

Although the combination α1β2γ2 is the most abundant in the brain (McKernan and Whiting 1996) and considered to represent the BZ1 subtype, the nature of the β subunit does not
appear to be relevant for determining benzodiazepine pharmacology (Benke et al. 1994; Hadingham et al. 1993). Since 19% of the GABA_A receptors in the rat cerebral cortex contain both β1 and β3 subunits (Li and De Blas 1997), α1β1β3γ2 also seems a likely combination for horizontal cell GABA_A receptors. Concerning the insensitivity to picrotoxinin, it is also reasonable to assume two different types of GABA_A receptor, with those lacking the β1 subunit being resistant to the antagonist.

**GABA transporters**

We currently do not know whether or not mouse horizontal cells express GABA transporters as described in lower vertebrates (Schwartz 1982; Schwartz 1987). Immunocytochemical studies have demonstrated the presence of the GABA transporters GAT-1, GAT-2, and GAT-3 in amacrine cells, displaced amacrine cells, interplexiform cells, pigment epithelium, and Müller cells of the rat retina. Surprisingly, however, horizontal cells in the same preparation appeared negative for the known GABA transporters (Brecha and Weigmann 1994; Johnson et al. 1996). In contrast, a vesicular GABA transporter is expressed in horizontal cells (Cueva et al. 2002), indicating the possibility of vesicular GABA release in the mammalian retina, or expression of a vesicular transporter in the plasma membrane.

We found no evidence for the presence of a GABA transporter current in mouse horizontal cells. The GABA-induced response was completely blocked by the selective GABA_A receptor antagonist bicuculline, indicating that the current is entirely mediated by GABA_A receptors. The picrotoxinin-resistant fractional current, which served as evidence for a GABA transporter in a lower vertebrate retinal preparation (Dong et al. 1994), is most likely caused by a differential subunit composition of GABA_A receptors.

To our knowledge, there is no ultrastructural evidence for the existence of GABAergic synapses between horizontal cells. Thus, it is tempting to speculate that GABA receptors of horizontal cells are extrasynaptic, as described for GABA_A receptors elsewhere in the nervous system.
system (Fritschy et al. 1992; Nusser et al. 1995; Nusser et al. 1996). A possible function of these receptors would then be the continuous monitoring of the GABA concentration in the outer retina, which in turn affects the signal processing properties on the level of horizontal cells.
FIGURE LEGENDS

Figure 1
Identification of isolated horizontal cells. A: After enzymatical and mechanical dissociation, living solitary horizontal cells were identified with Nomarski optics because of their characteristic morphology. B: Confocal micrograph of a horizontal cell labeled with a polyclonal antibody directed against calbindin D-28K. Inset: Agarose gel electrophoresis of the cDNA fragments obtained after single-cell reverse transcriptase-PCR for calbindin D-28K transcript. The 340 bp product specific for calbindin D-28K mRNA was amplified from five different horizontal cells which are numbered 1–5. C: A horizontal cell was recorded in voltage-clamp mode at a holding potential of –70 mV. A depolarizing voltage step to 0 mV (100 ms duration) induced an inward current mediated by voltage-gated calcium channels. Scale bars, 20 µm.

Figure 2
GABA dose-response relationship of horizontal cells. A: GABA-induced whole-cell currents measured at a holding potential of –70 mV. The concentrations of GABA are indicated above the current traces. The bars delineate beginning and duration of GABA applications (3 s). B: Scatter plot showing the GABA concentration-response relationship of 11 horizontal cells. The data were normalized to the maximal current obtained with 1 mM GABA. C: The mean (± SE) of the cells in B were plotted versus the GABA concentration. The line represents the best fit of the data to a sigmoidal function. The EC50 value and Hill coefficient derived as free parameters from the equation of the sigmoidal function were 30.1 µM and 1.3, respectively. D: Current-voltage relation of GABA-induced currents. The membrane potential was continuously changed from -70 to 70 mV in the presence of 100 µM GABA. The straight line represents the best fit obtained by linear regression (R = 0.99967, P < 0.0001). E: The desensitizing response to GABA (1 mM) was fitted with an exponential function (straight
line) characterized by a time constant of 1210 ms. F: The kinetic properties of deactivation were determined by fitting an exponential function to the current trace after application of 50 µM GABA. The straight line represents the best fit to the data (τ = 753 ms). Bars indicate application of GABA (3 s).

**Figure 3**

Inhibition of GABA-induced currents by bicuculline. A: Consecutive series of inward currents evoked by application of 50 µM GABA together with increasing concentrations of bicuculline (BIC). Control and washout responses without bicuculline were measured at the beginning and end of each experiment, respectively. Concentrations of bicuculline are depicted above the bars, which reflect the application period (3 s). All current traces were recorded from the same horizontal cell. The plus sign (+) indicates that bicuculline was co-applied with 50 µM GABA. B: Response of all horizontal cells (n = 6) plotted against the concentration of bicuculline. The data points represent the mean (± SE) of current amplitudes normalized to the control response obtained with 50 µM GABA. The IC50 value (1.7 µM) and Hill coefficient (1.1) were derived from the equation of the sigmoidal function giving the best fit to the data. C: Dose-response curve of GABA measured under control conditions and in the presence of 3 µM bicuculline (n = 7). The data points represent the mean (± SE) of current amplitudes normalized to the response obtained with a saturating GABA concentration. Bicuculline induced a shift in the dose-response curve towards higher GABA concentrations and thus a decrease in apparent affinity, indicative of a competitive mode of action.

**Figure 4**

Inhibition of GABA-induced currents by picrotoxinin. A: Consecutive series of current traces reflecting the course of an experiment. After a control application of 50 µM GABA, picrotoxinin (PTX) was applied twice at a concentration of 10 µM. The amplitude of the control response returned to pre-drug levels, indicating that picrotoxinin was completely
removed from the extracellular solution. Following the same protocol, picrotoxinin was then applied at 100 µM. Concentrations of picrotoxinin are depicted above the bars, which reflect the application period (5 s). The plus sign (+) indicates that picrotoxinin was co-applied with 50 µM GABA. B: Summary of the inhibitory effect of picrotoxinin on absolute GABA-induced currents. Grey bars represent steady-state currents, which are plotted as fractions of the peak currents (white bars). Steady-state currents were measured at the end of the 5 s-application. Each column represents the mean (± SE) of 8 horizontal cells.

**Figure 5**

Inhibition of GABA-induced currents by the divalent metal ion Zn\(^{2+}\). A: Inward currents induced by extracellular application of 50 µM GABA (Control) were blocked by increasing concentrations of Zn\(^{2+}\). Numbers next to each current trace indicate concentrations of Zn\(^{2+}\) (in µM), and the horizontal bar represents the application period (3 s). All current traces were recorded from the same horizontal cell. B: Dose-response curve for Zn\(^{2+}\) obtained from 8 horizontal cells. Current amplitudes at each concentration of Zn\(^{2+}\) were normalized to control values measured in the absence of Zn\(^{2+}\). Individual data points represent mean (± SE). The IC\(_{50}\) value (7.3 µM) and Hill coefficient (0.7) were derived from the equation of the sigmoidal function which gave the best fit to the data. C: Dose-response curve in the absence (Control) and presence of 5 µM Zn\(^{2+}\). Zn\(^{2+}\) did not change the EC\(_{50}\) value, but significantly reduced the maximum GABA-induced current. D: The ratio of currents (I\(_{Zn}\)/I\(_{Control}\)) was calculated and plotted against the respective GABA concentration. Data points were fitted by a linear regression line with a slope close to 0, indicating non-competitive inhibition of GABA\(_{A}\) receptors by Zn\(^{2+}\).

**Figure 6**

Benzodiazepine pharmacology of horizontal cell GABA\(_{A}\) receptors. A: GABA-evoked currents of horizontal cells were augmented by extracellular co-application of diazepam in a
concentration-dependent manner. B: Dose-response curve for diazepam obtained from eight horizontal cells. Data points representing mean (± SE) were derived from current amplitudes normalized to control currents induced by 50 µM GABA. The EC50 value and Hill coefficient calculated from the best fit of a logistic function to the data points were 6.6 µM and 0.8, respectively. C: Inward currents induced by 50 µM GABA were potentiated by co-application of increasing concentrations of zolpidem. D: Dose-response curve for zolpidem obtained from eight horizontal cells. Current amplitudes were normalized to the values obtained with 50 µM GABA and plotted as mean (± SE). The EC50 value obtained from fitting the data points with a logistic function was 85 nM with a Hill coefficient of 0.7.

Figure 7
Single-channel properties of horizontal cell GABA_A receptors. A: Single-channel events were induced by extracellular application of 10 µM GABA at −70 mV holding potential. The upper panel shows openings to the main conductance state (M), with an occasional opening of a second GABA receptor channel (2M). The lower panel shows the two subconductance states S1 and S2. B-D: All-point histograms of single-channel openings to the main and both subconductance states. The lines indicate the best Gaussian fit to the respective amplitude distributions. The Gaussian peak at 0 pA represents the closed state between channel openings. The simultaneous opening of two GABA_A receptor channels is reflected in an additional peak around -4 pA (B). E: Current-voltage relation of the main and two subconductance levels. Each data point represents the mean (± SE) of 10-15 measurements. The data were fit with linear regression lines (R=0.99883, P<0.0001 for Main; R=0.99991, P<0.0001 for Sub1; R=0.99961, P<0.0001 for Sub2). F: Open time distribution for the main conductance state. The solid line represents the best fit to an exponential probability density function. The time constant of the decay calculated from the fit was 2.43 ± 0.04 (R² = 0.99932). G: Mean open times, mean closed times, and open probabilities of the main
conductance and two subconductance states. Bars represent mean (± SE) of 14 to 60 bursts, each comprised of various numbers of single-channel events. Open and closed times are represented by the left y-axis, open probabilities by the right y-axis. The values for the subconductance state S1 show statistically significant differences when compared to either the main conductance or the second subconductance state S2 (*, P<0.05; **, P<0.01, t-test).
Table 1. Pharmacology of horizontal cell GABA<sub>A</sub> receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>Conc. (µM)</th>
<th>I/I&lt;sub&gt;c&lt;/sub&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>1</td>
<td>1.41 ± 0.07</td>
<td>9</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3</td>
<td>1.86 ± 0.17</td>
<td>9</td>
</tr>
<tr>
<td>Diazepam</td>
<td>10</td>
<td>2.51 ± 0.16</td>
<td>9</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>1</td>
<td>1.77 ± 0.07</td>
<td>9</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>3</td>
<td>2.17 ± 0.11</td>
<td>9</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>10</td>
<td>2.48 ± 0.13</td>
<td>9</td>
</tr>
<tr>
<td>DMCM</td>
<td>1</td>
<td>0.67 ± 0.02</td>
<td>8</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>50</td>
<td>2.68 ± 0.17</td>
<td>11</td>
</tr>
<tr>
<td>Alphaxalone</td>
<td>1</td>
<td>5.10 ± 0.54</td>
<td>10</td>
</tr>
<tr>
<td>Picrotoxinin</td>
<td>10</td>
<td>0.36 ± 0.02</td>
<td>8</td>
</tr>
<tr>
<td>Picrotoxinin</td>
<td>100</td>
<td>0.13 ± 0.01</td>
<td>8</td>
</tr>
</tbody>
</table>

The ratio I/I<sub>c</sub> represents GABA-induced peak currents measured in the presence of the drug (I) and under control conditions (I<sub>c</sub>). Values are expressed as mean ± SE (n cells).
Table 2. GABA<sub>A</sub> receptor single channel properties

<table>
<thead>
<tr>
<th></th>
<th>Main</th>
<th>Sub1</th>
<th>Sub2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amplitude (pA)</strong></td>
<td>-2.15 ± 0.02 (60)</td>
<td>-0.73 ± 0.01 (14)</td>
<td>-1.45 ± 0.01 (15)</td>
</tr>
<tr>
<td><strong>Conductance (pS)</strong></td>
<td>29.8 ± 0.7 (60)</td>
<td>10.8 ± 0.1 (14)</td>
<td>20.2 ± 0.3 (15)</td>
</tr>
<tr>
<td><strong>Mean open time (ms)</strong></td>
<td>2.23 ± 0.15 (60)</td>
<td>1.45 ± 0.12 (14)</td>
<td>2.14 ± 0.32 (15)</td>
</tr>
<tr>
<td><strong>Mean closed time (ms)</strong></td>
<td>2.43 ± 0.23 (60)</td>
<td>0.78 ± 0.11 (14)</td>
<td>2.30 ± 0.25 (15)</td>
</tr>
<tr>
<td><strong>Open probability (%)</strong></td>
<td>44.58 ± 1.85 (60)</td>
<td>57.15 ± 2.22 (14)</td>
<td>42.38 ± 3.24 (15)</td>
</tr>
</tbody>
</table>

Values in parenthesis indicate number of bursts considered for analysis.
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Figure 2

A

3 μM GABA
10 μM GABA
30 μM GABA
133 μM GABA
533 μM GABA
1 mM GABA

B

Normalized current vs. [GABA] (μM)

C

Normalized current vs. [GABA] (μM)

D

I (pA) vs. V (mV)

E

F

100 pA

50 pA
Figure 3
Figure 4

A

50 μM GABA

1. Appl. + 10 μM PTX

2. Appl. + 10 μM PTX

50 μM GABA

1. Appl. + 100 μM PTX

2. Appl. + 100 μM PTX

100 pA

2 s

B

Current (pA)

Peak

Steady-state


10 μM PTX

100 μM PTX
Figure 5
Figure 6
Figure 7