Electrophysiological Properties of Mouse Horizontal Cell GABA<sub>A</sub> Receptors

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INTRODUCTION

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

GABA<sub>A</sub> receptors consist of various combinations of ≥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<sub>A</sub> receptors to synaptic sites has recently been suggested (Moss and Smart 2001).

Horizontal cells are a class of second-order interneurons that 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 establish the antagonistic receptive field structure of bipolar 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 of whether GABA is released by a vesicular or a nonvesicular 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 (Cuevas 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. Because 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<sub>A</sub> receptors expressed by solitary mouse horizontal cells, and we determine their single-channel characteristics.

METHODS

Dissociation of the retina and identification of horizontal cells

Two- to 4-mo-old C57/B mice were deeply anesthetized by intraperitoneal injection of a 0.1 ml solution containing equal parts of 5% peritoneal injection of a 0.1 ml solution containing equal parts of 5% pentobarbital and 3% chloral hydrate. The animals were then perfused transcardially with 0.9% saline followed by a fixative containing 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Horizontal cells were isolated after enzymatical 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<sub>A</sub> receptor antagonist bicuculline (IC<sub>50</sub> = 1.7 μM), indicating expression of GABA<sub>A</sub> but not GABA<sub>C</sub> receptors. Their affinity for GABA was moderate (EC<sub>50</sub> = 30 μM), and the Hill coefficient was 1.3, corresponding to two GABA binding sites. GABA responses were partially reduced by picrotoxin with differential effects on peak and steady-state current values. Zinc blocked the GABA response with an IC<sub>50</sub> value of 7.3 μM in a noncompetitive manner. Furthermore, GABA receptors of horizontal cells were modulated by extracellular application of diazepam, zolpidem, methyl 6,7-dimethoxy-4-ethyl-3-carboxylate, pentobarbital, and alphaxalone, thus showing typical pharmacological properties of CNS ligand-gated ion channels. GABA-evoked currents were characterized by a main conductance state of 29.8 pS and two subconductance states (20.2 and 10.8 pS, respectively). Kinetic analysis of single-channel events within bursts revealed similar mean open 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%.

Acknowledgments

Andreas Feigenspan, Andreas and Reto Weiler. Electrophysiological properties of mouse horizontal cell GABA<sub>A</sub> receptors. J Neurophysiol 92: 2789–2801, 2004. First published July 7, 2004; 10.1152/jn.00284.2004. 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<sub>A</sub> receptor antagonist bicuculline (IC<sub>50</sub> = 1.7 μM), indicating expression of GABA<sub>A</sub> but not GABA<sub>C</sub> receptors. Their affinity for GABA was moderate (EC<sub>50</sub> = 30 μM), and the Hill coefficient was 1.3, corresponding to two GABA binding sites. GABA responses were partially reduced by picrotoxin with differential effects on peak and steady-state current values. Zinc blocked the GABA response with an IC<sub>50</sub> value of 7.3 μM in a noncompetitive manner. Furthermore, GABA receptors of horizontal cells were modulated by extracellular application of diazepam, zolpidem, methyl 6,7-dimethoxy-4-ethyl-3-carboxylate, pentobarbital, and alphaxalone, thus showing typical pharmacological properties of CNS GABA<sub>A</sub> receptors. GABA-evoked single-channel currents were characterized by a main conductance state of 29.8 pS and two subconductance states (20.2 and 10.8 pS, respectively). Kinetic analysis of single-channel events within bursts revealed similar mean open 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%.

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coated with 1 mg/ml concanavalin A (Sigma). The cells were kept in
Those fractions containing horizontal cells were
decreasing open diameter, and after each trituration step, the cell
sequently, the retina was triturated with
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centrifuged at 1,000 rpm (5 min, 22
°
albumin (Sigma), and 100 U/ml DNase I (Sigma) in EBSS. The tissue
C). This solution contained
°
40–45 min digestion at 37°C, the retina was transferred to trituration
buffer to stop papain activity (5 min, 37°C). 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 1,000 rpm (5 min, 22°C), and the pellet was
resuspended in minimum essential medium (MEM; Sigma). Subse-
sequently, the retina was triturated with fire-polished Pasteur pipettes
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, 55% O₂ at 37°C. 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 h. 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 h. Binding of the primary antibody was visualized with a goat
anti-rabbit Alexa 568 (diluted 1:200; Molecular Probes, Eugene, OR)
secondary antibody (2 h). 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 horizon-
tal 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.

To amplify 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 1× 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 ribo-
nuclease 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) was 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, 1× reaction buffer (Promega),
1.25 mM MgCl₂, 0.2 mM of each dNTP (Eppendorf), 0.8 µM of each
primer (MWG), and 1 U 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’-GACGCTGATGGAACTGGTAC-3’) and the reverse primer
(5’-ACGGTCTTGGTCTTCTCTTC-3’), both of which were designed according to the mouse calbindin coding sequence
GenBank Accession No. NM031984). The predicted size of the
amplicon was 340 bp. Amplifications of calbindin transcripts were
 carr for out 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 1 min. The final cycle was followed by 10 min at 72°C. The
product were analyzed on a 2% agarose gel and visualized on a
transilluminator after ethidium bromide staining.

Electrophysiological recordings
Cells were allowed to settle ≥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 de-
scribed in the following text using ×40 and ×63 water-immersion
 objectives equipped with Nomarski optics (Leica). Whole cell volt-
age-clamp and outside-out single-channel recordings were performed
with an EPC9 double patch-clamp amplifier (Heka, Lambrecht, Ger-
many). 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 borosili-
cate glass (1.5 mm OD, 1.275 mm ID; 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 mechan-
ic, remote-controlled device attached to a three-dimensional
micromanipulator (Luigs and Neumann). In whole cell experi-
ments, the series resistance of the electrodes usually ranged be-
tween 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 resis-
tance value were considered for analysis. Drugs were applied to
horizontal cells or outside-out membrane patches in the extracel-
lular bath solution by the pressure-driven application system
DAD-12 Superfusion System (ALA Scientific Instruments, West-
bury, 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, and methyl 6,7-dimethoxy-4-ethyl-β-carbo-
ylate (DMCM) (all from Sigma) were prepared as 10-mM stock
solutions in DMSO and stored at −20°C. The maximal final concen-
dration of DMSO was 0.03%, which had no effect on GABA-induced
currents. Bicuculline methiodide and picrotoxin (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 am-
plitudes were normalized to the maximum response, obtained either
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: 
\[ \text{I} \text{nt}_{\text{max}} = \frac{1}{1 + \left(\frac{c}{(c \text{M})^n}\right)} \]
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, numbers of events per burst were binned in 2-ms 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, 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 five to eight primary dendrites (Fig. 1, A and 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 noninactivating calcium currents. With voltage-dependent potassium channels blocked, stepwise depolarization of the membrane from a holding potential of –70 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. Because voltage-dependent ion channels are subject of a different study, they were not investigated further. In summary, we used morphological criteria, the presence of calbindin D-28K, and 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 >90% of the recordings, indicating that the dissociation procedure did not impair the overall structure of the cell membrane. Because GABA concentrations ≤1 μM consistently failed to induce measurable currents, GABA was applied at concentrations ranging from 3 to 1,000 μM. Desensitization of GABA receptor-mediated currents was dose-dependent and became apparent at GABA concentrations of ≥30 μM (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 (SE) pA (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 SD of 0.16 measured at 30 μM (Fig. 2B). 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 with 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 to 70 mV (100 mV/s) in the presence of 100 μM GABA
Nonspecific 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 ($n = 19$). Three-second 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 1,142 ± 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. 2F).

**Inhibitory effects of bicuculline and picrotoxin**

The plant alkaloid bicuculline has been described as a competitive and reversible blocker of GABA$_A$ 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 ~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.

Figure 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 (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 (1 μM) inhibited GABA-induced currents to 0.64 on average, whereas the current was completely eliminated by 100 μM (Fig. 3A).
bicuculline had a stronger effect on peak amplitudes than on steady-state values, whereas the overall response became non-desensitizing with concentrations >3 μM bicuculline (Fig. 3A). The IC₅₀ 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 IC₅₀ value obtained from 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 1,210 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).

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application of picrotoxin, respectively. When the steady-state values of the currents were compared, however, two applications of picrotoxin 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 picrotoxin (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 picrotoxin 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 picrotoxin. The mean amplitudes of peak and steady-state currents, which are plotted as fractions of the peak currents (C), Steady-state currents were measured at the end of the 5-s application. Each column represents the mean ± SE of 8 horizontal cells.

![FIG. 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). +, bicuculline was co-applied with 50 μM GABA. B: summary of the inhibitory effect of picrotoxin on absolute GABA-induced currents. □, steady-state currents, which are plotted as fractions of the peak currents (C). Steady-state currents were measured at the end of the 5-s application. Each column represents the mean ± SE of 8 horizontal cells.](http://jn.physiology.org/)

![FIG. 4. Inhibition of GABA-induced currents by picrotoxin. A: consecutive series of current traces reflecting the course of an experiment. After a control application of 50 μM GABA, picrotoxin (PTX) was applied twice at a concentration of 10 μM. The amplitude of the control response returned to predrug levels, indicating that picrotoxin was completely removed from the extracellular solution. Following the same protocol, picrotoxin was then applied at 100 μM. Concentrations of picrotoxin are depicted above the bars, which reflect the application period (5 s). +, picrotoxin was co-applied with 50 μM GABA. B: summary of the inhibitory effect of picrotoxin on absolute GABA-induced currents. ○, steady-state currents, which are plotted as fractions of the peak currents (C). Steady-state currents were measured at the end of the 5-s application. Each column represents the mean ± SE of 8 horizontal cells.](http://jn.physiology.org/)
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 picrotoxin on GABA receptor-mediated chloride currents was almost fully reversible (Fig. 4B).

**Inhibition of GABA receptor currents by Zn²⁺**

The divalent transition metal cation Zn²⁺ has been shown to noncompetitively antagonize GABAᵦ receptors, receptor-mediated currents (Hosie et al. 2003; Legendre and Westbrook 1991; Westbrook and Mayer 1987). Zn²⁺ ranging in concentrations from 1 to 1,000 μM was co-applied with 50 μM GABA to eight isolated horizontal cells. Figure 5A shows the inhibitory effect of increasing concentrations of Zn²⁺. Block of GABA-evoked currents by Zn²⁺ was fully reversible after complete removal of the blocker from the extracellular solution. It appears that the presence of Zn²⁺ 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 toward the blocking effects of Zn²⁺. Concentrations as low as 1 μM Zn²⁺ blocked on average 0.26 of the control inward current, which was completely eliminated by 1 mM Zn²⁺. We measured the dose-response curve by calculating the normalized mean residual current at each concentration of Zn²⁺ and subsequently fitting the data points to a sigmoidal logistic equation (Fig. 5B). The IC₅₀ value of Zn²⁺ 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 IC₅₀ 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 GABAᵦ receptors by Zn²⁺.

Furthermore, we determined the mode of horizontal cell GABAᵦ receptor inhibition by Zn²⁺. Dose-response curves for GABA were measured under control conditions and in the presence of 5 μM Zn²⁺ (Fig. 5C). EC₅₀ 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 Zn²⁺. These values were not significantly different (P < 0.01, t-test). In addition, the ratio of GABA-induced currents with and without Zn²⁺, 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 Zn²⁺ does not depend on the GABA concentration (Fig. 5D). These results indicate that Zn²⁺ blocks GABAᵦ receptors of mouse horizontal cells in a noncompetitive manner.

**Effects of benzodiazepines, barbiturates, and steroids**

GABAᵦ 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 γ2 subunit is required to confer benzodiazepine sensitivity to GABAᵦ receptors (Pritchett et al. 1989). To study the effects of benzodiazepines on horizontal cell GABAᵦ 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 EC₅₀ 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

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**FIG. 5.** Inhibition of GABA-induced currents by the divalent metal ion Zn²⁺. A: inward currents induced by extracellular application of 50 μM GABA (control) were blocked by increasing concentrations of Zn²⁺. Numbers next to each current trace indicate concentrations of Zn²⁺ (in μM), and the horizontal bar represents the application period (3 s). All current amplitudes were recorded from the same horizontal cell. B: dose-response curve for Zn²⁺ obtained from 8 horizontal cells. Current amplitudes at each concentration of Zn²⁺ were normalized to control values measured in the absence of Zn²⁺. Individual data points represent means ± SE. The IC₅₀ 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 Zn²⁺. Zn²⁺, did not change the EC₅₀ value but significantly reduced the maximum GABA-induced current. D: the ratio of currents (Iₓ/I₀) 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 noncompetitive inhibition of GABAᵦ receptors by Zn²⁺.
amplitudes were normalized to the values obtained with 50 nM GABA.

Current potentiated by co-application of increasing concentrations of zolpidem. Again, a dose-response curve was generated by normalizing the current amplitudes to the control response to 50 nM GABA and plotting the normalized values against the concentration of zolpidem (Fig. 6C). The EC50 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_A 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_A receptors on the surface of horizontal cell bodies, single-channel events could be recorded in <50% of all membrane patches. In those patches containing GABA_A 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 and −0.73 ± 0.01 pA for the two sublevels, respectively (Fig. 7, B–D).

**TABLE 1. Pharmacology of horizontal cell GABA_A receptors**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, µM</th>
<th>EC50, nM</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 Ic/Ic controls GABA-induced peak currents measured in the presence of the drug (Ic) and under control conditions (Ic). Values are expressed as means ± SE (n cells). DMCM, methyl 6,7-dimethoxy-4-ethyl-carboxylate.
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 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 >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_o) for the main conductance and the two subconductance states are summarized in Fig. 7G. The main conductance (M) and the second

**Fig. 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. Top: openings to the main conductance state (M), with an occasional opening of a 2nd GABA receptor channel (2M). Bottom: the 2 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 2 GABA_A receptor channels is reflected in an additional peak around −4 pA (B). E: current-voltage relation of the main and 2 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 (R^2 = 0.99932). G: mean open times, mean closed times, and open probabilities of the main conductance and 2 subconductance states. Bars represent means ± 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 with either the main conductance or the 2nd subconductance state S2 (*P < 0.05; **P < 0.01, t-test).
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 P0 (Fig. 7G). Although open times are slightly smaller compared with 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).

**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 in the preceding text, 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 and Bormann 1998; Feigenspan et al. 2000).

Benzodiazepine receptors are usually subdivided into two different pharmacological types, BZ1 and BZ2. Whereas BZ1 sites show high affinity for CL-218872, zolpidem, and β-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-prefering agonist zolpidem as well as the benzodiazepine agonist diazepam and the inverse agonist DMCM. The EC_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 γ2 subunit (Pritchett et al. 1989). The augmentation by diazepam in all horizontal cells tested could therefore be explained by the expression of the γ2 subunit. In addition, the characteristics of a BZ1 binding site are consistent with the presence of the α1 subunit. Because the presence of the α4 subunit in combination with one β and the γ2 subunit confers insensitivity to benzodiazepines (Wisden et al. 1991), we exclude the possibility of α4 expression in horizontal cell GABA_A receptors.

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**TABLE 2. GABA_A receptor single-channel properties**

<table>
<thead>
<tr>
<th></th>
<th>Main</th>
<th>Sub1</th>
<th>Sub2</th>
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<tbody>
<tr>
<td>Amplitude, pS</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>Conductance, pS</td>
<td>30.3 ± 0.6 (60)</td>
<td>11.2 ± 0.2 (14)</td>
<td>30.6 ± 0.4 (15)</td>
</tr>
<tr>
<td>Mean open time, ms</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>Mean closed time, ms</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>Open probability, %</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. Sub1 and Sub2, subconductance states 1 and 2, respectively.
HORIZONTAL CELL GABAₐ RECEPTORS

With an IC₅₀ value of 7.3 µM, GABA receptor currents of horizontal cells displayed a moderate to high sensitivity to the divalent metal cation Zn²⁺. The effects of Zn²⁺ on GABA-induced currents are determined by the receptor isoforms (Draguhn et al. 1990; Smart et al. 1991). The presence of α1 or βx (x = 1–3) confers high sensitivity to Zn²⁺, whereas on addition of a γ subunit, the inhibitory effect of Zn²⁺ is lost. However, it is reasonable to assume that the presence of a γ subunit causes a decrease in the susceptibility to Zn²⁺ rather than a total loss (Saxena and Macdonald 1996). Therefore, the observed sensitivity of horizontal GABAₐ receptors to the inhibitory action of Zn²⁺ is compatible with the presence of the γ2 subunit in all receptors.

Even high concentrations of the open channel blocker picrotoxin did not entirely block GABA-mediated inward currents. Picrotoxin 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; Korpri and Luddens 1993; Lan et al. 1991; Paia et al. 1990, 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 GABAₐ 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 GABAₐ 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 (PKA) (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 GABAₐ receptors expressed by mouse horizontal cells are composed 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). Because 19% of the GABAₐ 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 GABAₐ receptors. Concerning the insensitivity to picrotoxin, it is also reasonable to assume two different types of GABAₐ 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, 1987). Immunochemical studies have demonstrated the presence of the GABA transporters GAT-1, -2, and -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 and 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 GABAₐ receptor antagonist bicuculline, indicating that the current is entirely mediated by GABAₐ receptors. The picrotoxin-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 GABAₐ 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 GABAₐ receptors elsewhere in the nervous system (Fritschy et al. 1992; Nusser et al. 1995, 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.

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