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Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator γ-hydroxybutyrate

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ABSTRACT

γ-Hydroxybutyrate (GHB) is an endogenous neuromodulator with therapeutical applications in anesthesia, sleep disorders, and drug addiction. We report the cloning of a GHB receptor from a rat hippocampal cDNA library. This receptor has a molecular mass of 56 kDa and belongs to the seven-transmembrane receptor family. The peptidic sequence has no significant homology with any known receptor, including $GABA_B$ receptors. Its mRNA is restricted to the brain and is particularly abundant in the hippocampus, cortex, striatum, thalamus, olfactory bulbs, and cerebellum, matching the distribution of GHB binding sites in rat brain. Southern blot revealed the presence of homologous sequences in several species including the human. Binding assays on transfected CHO cells showed a dissociation constant (K_d) of 426 nM for GHB and no affinity for GABA, baclofen, or glutamate. In patch-clamp experiments, transfected CHO cells revealed a functional G-protein-coupled receptor as demonstrated by GTP-γ-S-induced irreversible activation. Application of 0.1-15 μM GHB specifically induced an inward current at negative membrane potentials that was not reproduced by application of baclofen (10 μM). CGP-55845, a $GABA_B$ receptor antagonist, did not inhibit the GHB-induced response nor did the GHB receptor antagonist NCS-382, suggesting that the GHB receptor system includes several subtypes.

Key words: receptor for neuromodulator • drug of abuse • GHB • sleep regulation

The natural occurrence of γ-hydroxybutyrate (GHB) in the mammalian brain was demonstrated more than 30 years ago (1). GHB in the brain is a metabolite of GABA. When administered peripherally, GHB readily and rapidly crosses the blood brain barrier to potentiate and modify the endogenous GHB system (2, 3). Synthetic GHB has been used to induce anesthesia and long-term sedation as well as to treat ethanol addiction and withdrawal (2, 4). In July 2002, the U.S. Food and Drug Administration approved GHB for the treatment of narcolepsy (5, 6, 7). Among potential therapeutical applications currently under study are the treatments of fibromyalgia (8) and of age-related sleep disturbances. In animal studies, GHB was claimed to have a protective action against hypoxia at a cellular and whole organ level (9). However, in some experimental animals and particularly in rodents, GHB has been shown to produce seizure-like behavior and the compound is being used to produce an

absence-like seizure model (10). GHB has recently gained notoriety as a drug of abuse, initially among bodybuilders and subsequently among participants of "rave parties" and polydrug abusers. Anecdotal reports indicate that GHB may provide effects similar to those of ethanol without some of the perceived negative consequences. Numerous cases of GHB intoxication have been documented, and symptoms of tolerance, dependence, and withdrawal have been reported (11, 12, 13, 14).

Endogenous GHB has properties consistent with a role as a neurotransmitter/neuromodulator modulating GABAergic and dopaminergic activity. It is heterogeneously distributed in the brain of all species so far examined (rat, monkey, human, cat, guinea pig, and bovine; refs 15, 16, 17). GHB is the result of GABA transamination by GABA-T, which produces succinic semialdehyde (SSA), which is then reduced by a specific succinic semialdehyde reductase (SSR) into GHB (18, 19). SSR and GHB are localized in the neuronal compartment of the brain (20) from where GHB can be released via a depolarization-evoked, calcium-dependent mechanism (21). The role of GHB as a signaling molecule between neurons is also supported by the existence of high affinity GHB binding sites, both in the brain and in neuronal cell lines (22, 23). These binding sites appear to have specific kinetics, pharmacology, and ontogenesis (22, 24). In addition, they are heterogeneously distributed in the human and rat brain with the highest density in the cortex, hippocampus, striatum, thalamus, and olfactory tracts (25, 26).

We report here the molecular cloning and the characterization of the first member of a GHB receptor (GHB-R) family mainly expressed in the rat hippocampus. The pharmacological profile of this receptor and its electrophysiological properties are consistent with its involvement in the mediation of some of the effects of GHB in the rat brain.

METHODS

Materials

Reagents were purchased from SIGMA unless otherwise stated.

Purification and partial sequencing of GHB-R

Rat brain membranes were prepared from adult Wistar rats in the presence of EGTA, phenylmethylsulfonylfluoride, and a low concentration of KCl. Under these conditions, the presence of a high affinity binding site for [^{3}H]GHB can be demonstrated. For solubilization of GHB-R, a membrane pellet was homogenized with a polytron in potassium phosphate buffer containing 5% Triton X-100 and 3.5 M NaCl (solution A). Saturation curves using the solubilized receptors showed dissociation constant (K_d) values ranging from 5 to 15 µM for GHB and different GHB related structural analogs but no affinity for GABA (27).

The solubilized receptor was then purified by affinity chromatography, using activated agarose beads (activated CH sepharose 4B, Pharmacia Biotech, Sweden) linked to a GHB structural analog, NCS-400 {5-[4-(aminoprop-1-ynyl) phenyl]-4-hydroxypentanoïque, sodium salt}. The affinity of this compound for GHB-R was previously measured by competition with [^{3}H]GHB (100 Ci/mmol; CEA, Saclay, France) on rat brain membranes (two sites with $IC_{50\text{-}1}$=33 nM and $IC_{50\text{-}2}$=66 µM, nonlinear regression line, Graphpad Prism program, r=0.99). Compared with the population of [^{3}H]GHB binding sites present in the membrane extract, ~56% was retained by the

column. After being washed with solution A, proteins were specifically eluted with a pulse of the same medium containing 10 mM GHB. The eluate was collected and submitted to preparative electrophoresis on a 10% polyacrylamide/SDS gel (25 cm×1.5 mm). After being stained with Coomassie blue staining, two protein bands (one major band and one minor band) were observed, with a molecular mass of 54 and 47 kDa, respectively (Fig. 1). The two bands were isolated separately by cutting from the stained gel.

Protein hydrolysis and peptide sequencing

The putative GHB-R protein was digested in gel with modified pig trypsin (28). Seven peptides were isolated by HPLC (Vydac C_{18} 5m column, 250 mm×1.6 mm with an Applied Biosystem microbore apparatus) and sequenced (Applied Biosystem sequencer model 477A). The following sequences were obtained: GLGPGRLHA, LENEIQTYR, YENEVALR, LRENTFLLKF, SAQTELEH, FTTELCVW, and SEISELNR.

Rat hippocampal cDNA library construction

Adult Wistar rats were killed, and the hippocampus was rapidly extracted under sterile conditions and frozen in liquid nitrogen. Total RNA was isolated from the tissue by the guanidinium thiocyanate extraction method (29), and poly(A^+) RNA was prepared with an oligo(dT) affinity column (30). First-strand cDNA was synthesized by reverse transcription in the presence of a 50-base oligonucleotide primer constituted with a *Xho*I restriction site protected by a "GAGA" sequence from nucleases and an 18-base poly(dT) at the 3′ end and Moloney murine leukemia reverse transcriptase. The second strand was synthesized in the presence of RNase H and rat DNA polymerase I. The double-stranded cDNA was blunt-ended with *Pfu* DNA polymerase, and *Eco*RI adapters were ligated to. This construction was then inserted into the Zap Express vector in a sense orientation (*Eco*RI-*Xho*I) with respect to *lacZ* promoter. The library was finally packaged into lambda bacteriophage with the DNA in vitro packaging set (Stratagene Zap Express Kits: cDNA synthesis and Gigapack II).

PCR-amplified probes

Automatic mass excised plasmid cDNAs were prepared by inoculating Xl1-blue MRF' cell cultures with the amplified lambda bacteriophage library (10 times the primary library size) in the presence of ExAssist helper phage to serve as template for PCR reactions. Based on the above peptide sequences, the following degenerated oligonucleotides: GGNYTNGGNCCNGGNHGNYTNCAYGCN, CTBGARAAYGARATYCARACHTAYGG, TAYGARAAYGARGTSGCHCTBAGRC, YTNMGNGARAAYACNTTYYTNYTNAARTTY, ATYGAGATCWSYGAGCTGAAYMGG, and WSNGCNCARACNGARYTNGARCAY, TTYACNACNGARYTNTGYGTNTGG, and their reverse-complements were synthesized and used as primers in randomly paired combinations. PCR was performed in 25 µl reaction medium containing 2.5 µl 10x buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.5 µl dNTPs (5 mM each), 0.25 µl of each randomly paired primers (1 µg/µl each), 0.1 µl *Taq* polymerase (0.02 u/µl; HotGoldstar, Eurogentec), and 0.25 µg of template. PCR reactions were started with a first cycle at 94°C for 3 min, followed by 30 cycles of denaturation at 92°C for 45 s, annealing at 50°C for 1 min, polymerization at 72°C for 2 min, and termination by a 7 min elongation cycle at 72°C. We adopted a primer extension procedure consisting of coupling the

T3 and T7 RNA polymerase promoter sites at the 5′ ends of the PCR-amplified double-stranded cDNA for direct sequencing.

Screening of the cDNA library

Plaque-forming units (10^6) from the cDNA library were plated on NZY-agar plates at 50,000 per plate with 600 µl Xl1-Blue MRF' and 8 ml NZY top agarose and incubated overnight at 37°C. The plates were chilled at 4°C for 1 h, and phage plaques were transferred to Biodyne A nylon membranes for 5 min. The membranes were then successively incubated in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min, neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0), and washing buffer (0.2 M Tris-HCl, pH 7.5, 2x SSC). The membranes were heated at 80°C for at least 2 h before prehybridization in 5x SSC buffer containing 20 mM NaH_2PO_4, 0.4% SDS, 5x PAF (polyvinylpyrrolidone/BSA/Ficoll), and 500 µg/ml denatured salmon sperm DNA for 6 h at 60°C. The hybridization step was performed at 60°C overnight in the presence of the [^{32}P]-labeled PCR-amplified probe with gentle shaking. Putative positive plaques were isolated and re-screened at lower densities until homogeneity was reached. The purified clones were stored as bacteriophage suspensions in SM buffer at 4°C until further use.

Automatic excision of the recombinant plasmid

Two *E. coli* strains (Xl1-Blue MRF' and XLOLR) were grown separately overnight in NZY medium containing 0.2% maltose. In a sterile tube, 200 µl of the Xl1-Blue MRF' culture were inoculated with 250 µl of a positive phage suspension in the presence of ExAssist phage helper for 15 min at 37°C. Three millilters of NZY were added, and incubation was performed at 37°C for 3 h. The tube was then heated at 70°C for 20 min and centrifuged at 1,000 *g* for 15 min. An aliquot of the supernatant (10 µl) was added to 200 µl of the XLOLR culture and incubated at 37°C for 15 min. After addition of 300 µl of NZY, incubation was continued for 45 min at 37°C. Two-hundred microliters of the mixture were plated on LB-agar plates containing 50 µg/ml of Kanamycin and incubated overnight at 37°C. A distinct colony was picked up and grown overnight in LB-Kanamycin medium to prepare plasmid DNA for further analysis. This preparation was made according to the QIAGEN midiprep protocol.

Eukaryotic expression

CHO cells were transiently transfected with the plasmid DNA preparation using the FuGene6™ transfection reagent kit (ROCHE). Briefly, CHO cells were grown until 50-70% confluence was attained. Six microliters of FuGene6 were diluted into 97 µl serum-free DMEM and added to 2 µl of the pBK-CMV-inserted cDNA (1 µg/µl). This complex was distributed to cells, and incubation continued until complete confluence was attained (48 h). Cells were harvested and used for radioligand binding experiments.

Ligand binding experiments

The CHO cells were transiently transfected as described above. Cells were harvested and resuspended in cold phosphate buffer (100 mM KH_2PO_4, pH 6.0), containing 5 mM EDTA and centrifuged at 18,000 *g* for 10 min at 4°C. The pellet was resuspended in EDTA-free phosphate

buffer and centrifuged at 30,000 *g* for 20 min. The membrane preparation was resuspended in phosphate buffer and used immediately for binding experiments.

Saturation experiments were performed using 0.3-0.4 mg protein per assay. The incubation was carried out in phosphate buffer, pH 6.0, for 25 min at 0°C in the presence of various concentrations of [^{3}H]GHB (10^{-10}-2.10^{-6} M; 100 Ci/mmol, CEA, France). Nonspecific binding was determined in the presence of 5 mM unlabeled GHB. The binding reaction was terminated by the addition of ice-cold phosphate buffer, and bound was separated from free [^{3}H]GHB by rapid filtration through GF/B filters (Whatman). After two rinses with ice-cold phosphate buffer, membrane-associated [^{3}H]GHB on filters were determined by liquid scintillation counting in 5 ml Rotiszint® (Roth).

B_{max} and K_d were determined by fitting saturation binding curves by nonlinear regression (Graphpad prism software, San Diego, CA). Protein concentration was determined by the BCA protein assay (Pierce).

Competition experiments were performed at 0°C with 0.3-0.4 mg of protein per assay in 600 µl phosphate buffer, pH 6.0, for 25 min in the presence of 50 µM of either GHB or GHB structural analogs or of glutamate, GABA, or baclofen, with 200 nM [^{3}H]GHB. The separation of bound from free [^{3}H]GHB was performed as described above, and results were expressed in percentage of [^{3}H]GHB displaced by the various substances. IC_{50} was determined only for compounds exhibiting significant [^{3}H]GHB displacement.

Electrophysiological recordings

CHO cells were plated at low density (3.10^4 cells per dish) in 35 mm noncoated dishes (Nunk) and transiently transfected with the cloned cDNA using the FuGene6 transfection reagent as described above. After 48 h, the transfected cells were recorded either in the cell-attached or whole cell configuration of the patch-clamp technique (31).

For cell-attached recordings, the cell membrane potential was clamped close to 0 mV with a KCl bathing solution containing (in mM) 1 NaCl, 140 KCl, 10 HEPES, 1 $MgCl_2$, 0.5 $CaCl_2$, 1 EGTA, and 10 D-glucose with pH adjusted to 7.4 with KOH. The recording pipette had 3 to 4 MΩ resistance when filled up with the solution of the following composition (in mM): 140 NMDG, 15 TEA-Cl, 1 KCl, 5 EGTA, 10 $MgCl_2$, and 10 HEPES with pH adjusted to 7.4 with HCl. For whole-cell recordings, solutions had the following composition (in mM): 132 NaCl, 5 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, and 10 D-glucose for the bath and 122 K-gluconate, 3 NaCl, 3 KCl, 16.5 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 1 ATP for the pipette medium. The pH was adjusted to 7.4 and 7.2 with NaOH and KOH, respectively.

Drugs were diluted to the desired concentrations in the external solution and applied by gravity to the recorded cell through a horizontal multibarrel perfusion system. Each barrel has 300 µm inner diameter. The selected barrel was placed at ~20 µm away from the recorded cell. Solution changes were performed by manual switching, and the application was started by placing the corresponding tube in front of the cell.

Controls are nontransfected cells, and the data were recorded using an axopatch 200B amplifier (Axon Instruments, CA) and the clampex routine of the pClamp software package (Axon Instruments, CA). The signal was filtered (1 kHz) before acquisition (2 kHz sampling frequency) with the Digidata 1322A card interface (Axon Instruments, CA).

Northern blot

Ten micrograms of total RNA were submitted to electrophoresis on 1.0% agarose-formaldehyde gels and transferred to hybond membranes (Amersham Pharmacia Biotech, Orsay, France). Hybridization and washing conditions were carried out as described by Kalinyak and Perlman (32). A [^{32}P]-labeled GHB-R cDNA probe was used. Membranes were exposed to Kodak Biomax MR Film overnight.

Southern blot

Ten micrograms of genomic DNA from the brains of mouse, guinea pig, hamster, rat, rabbit, pig, monkey, and human were digested overnight in 400 µl total volume with 3 units of *Eco*RI per µg DNA at 37°C. Before precipitation at -80°C for 1 h, 0.1 volume of 3 M sodium acetate, and 2.5 volumes of ice-cold 100% ethanol were added. The digested DNA was pelleted by centrifugation at 20,000 *g* for 20 min. The dried supernatant was resuspended in 15 µl of 10 mM Tris, pH 7.5, and subjected to electrophoresis on a 0.8% agarose gel along with DNA marker I for genomic DNA analysis (MBI Fermentas) at 40 V, 35 mA for 6-16 h.

Southern transfer to nylon membrane

Just before the transfer, the Nylon membrane (Hybond-N, Amersham Pharmacia Biotech) was floated on top of distilled water to wet thoroughly and let stand in transfer buffer until use. The gel was denatured by soaking in at least two gel volumes of 1.5 M NaCl/0.5 N NaOH for 2 × 15 min. The transfer was then set up in a large electrophoresis tray filled with 10x SSC buffer into the two side reservoirs and run overnight. The membrane was then washed in 5x SSC buffer for 5 min, and DNA was immobilized.

Prehybridization

The membrane was placed in hybridization bottle with 100 µl/cm^2 of prehybridization buffer (50% deionized formamide, 6x SSPE, 5x Denhardt's reagent, 0.5% SDS, and denatured 100 µg/ml salmon testes DNA (Sigma D-9156) in distilled water) and incubated in hybridization oven at 42°C for 1-2 h.

Radioactive probe labeling

Twenty to fifty nanograms of cDNA-probe were labeled with [^{32}P]-α-dCTP using the Random Primer Labeling Kit (Gibco-BRL). We have used a mixture of two probes generated by PCR using the following couples of primers: AAAGCTGGTGGCCATTTGAG AAGTGGCTCA/AGTACAGCCCCAGTTCAGCCCCAGTCCA and GGCTGGACTG GGGCTGTTTACCACA/TTAATTGAGTCGCTCGTAGCCATCCTGCCA. The amplified cDNA fragments, respectively, 319 bp and 447 bp, are located at the 3′ region of the cloned GHB-R.

Hybridization

The probes were heat denatured and added to the prehybridization solution. Incubation was performed at 42°C overnight.

Washing

The washing steps are performed in a hybridization oven rotating at very low speed. The membrane was first soaked twice for 15 min each with 7x SSPE/0.5% SDS at room temperature and then washed twice for 15 min each with 1x SSPE/0.5% SDS at 37°C. Finally, the membrane was soaked for 1 h in 0.1x SSPE/1% SDS at 37°C.

Detection

After being washed, the membrane was exposed to X-ray film (Biomax-MR, Kodak) with an intensifying screen at –80°C for 1 wk.

In situ hybridization

Adult Wistar rats were intracardially perfused with 4% *p*-formaldehyde. The brains were excised and frozen in nitrogen liquid until cryostat sectioned (20 μm thick). Frozen slides were immediately immersed in an acetone bath for 3-5 min to remove lipids and incubated in a solution of 0.1 M triethanolamine (TEA), pH 8.0, and finally, in a TEA, 0.25% anhydride acetic acid bath. After two rinses with 2x SSC, slides were prehybridized and hybridized with the appropriate probe as described previously (33).

Prehybridization

Slides were incubated in a prewarmed 1x SSC solution containing 50% formamide at 60°C for 10 min and dehydrated successively with 50, 75, and 100% cold ethanol solutions. The slides were finally dried at room temperature.

Hybridization

An antisense [^{35}S]-labeled oligonucleotide designed from the GHB-R cDNA sequence was used as a probe. The probe (25,000 cpm/μl) was diluted into the following medium: 50% formamide, 0.6 M NaCl, 10 mM Tris buffer, pH 7.5, 10 mM Denhardt's solution, 1 mM EDTA, 500 μg/ml t-RNA, 1 mM DTT, 500 mg/ml dextran sulfate, and salmon sperm DNA and was incubated at 50°C for 2 h. Fifty microliters of this solution were added to each slide, and incubation was performed at 52°C overnight.

Washing steps

The slides were washed twice with 1x SSC containing 50% formamide at 55°C for 1 h followed by two rinses at room temperature for 5 min. Then, they were incubated in a 1x NTE solution in the presence of 3 μg/ml of RNase at 37°C for 30 min. Finally, the slides were washed twice with 2x SSC, 50% formamide at 55°C for 1 h, and 0.1x SSC at 55°C for 15 min and dehydrated with successively 50 and 75% and absolute cold ethanol solution. Once the slides were dry, they were

autoradiographed at room temperature for 3-7 days. Autoradiographic microscales were used for calibration of autoradiographs.

RESULTS

Cloning of GHB-R and sequence analyses

The PCR-amplified cDNA with some homology to known G-protein coupled receptors was used as a probe to screen a brain hippocampal cDNA library. This method resulted in isolating a full-length cDNA sequence of 1567 bp. An ATG codon, located at nucleotide 22 is followed by an open reading frame of 1535 bp and terminated by a stop codon at nucleotide 1558 (Fig. 2). The open reading frame encodes for a protein of 512 amino acid residues with a calculated molecular weight of 56,972 Da (Fig. 2). The peptides GLGPGRLHA, LRENTFLLKF, SAQTELEH, and FTTELCVW were found at positions 7 to 15, 124 to 133, 251 to 258, and 377 to 384 of the protein sequence, respectively. On the other hand, the peptides LENEIQTYR, YENEVALR, and SEISELNR were not present. This is probably due to the fact that the band A (Fig. 1) was a mixture of proteins with similar molecular weights. A search for secondary structure patterns using PredictProtein programs (34) revealed the presence of seven putative transmembrane regions as observed for G-protein-coupled receptors. The deduced amino acid sequence shows a number of putative phosphorylation sites (34) for protein kinase C and casein kinase II. Comparison of the amino acid sequences of $GABA_B$-R1, $GABA_B$-R2, and GHB-R by multiple sequence alignment analysis revealed that only 2.47% of the residues were found identical and shared between the three proteins. These residues are located in the extracellular domain and in the first and third transmembrane domains of $GABA_B$ receptors. Obviously, there is no significant identity between $GABA_B$ receptors and GHB-R.

Expression of GHB-R mRNA in rat brain tissue

The expression of GHB-R mRNA was examined in several brain regions by in situ hybridization in order to verify the regional brain distribution of GHB-R expression and to try to correlate the distribution of mRNA with the distribution of [^{3}H]GHB binding. These experiments confirmed that the GHB-R gene is expressed in specific brain regions (Fig. 3). With the use of radioactive microscales and quantitative image analysis of the autoradiographs, the following distribution of GHB-R mRNA was found (nCi/g tissue equivalent±SE): crus ansiform cerebellar lobules = 1019 ± 200; hippocampus (dentate gyrus) = 924 ± 205; olfactory bulbs = 912 ± 223; hippocampus (CA_2 and CA_3) = 281 ± 44; piriform cortex = 254 ± 21; cingular cortex = 206 ± 45; nucleus accumbens (shell) = 190 ± 32; frontal cortex = 146 ± 59; parietal cortex = 142 ± 16; caudate putamen = 139 ± 24; thalamic nuclei = 125 ± 19; and amygdala = 87 ± 9. Most other brain regions showed very low basal level GHB-R gene expression. Although this distribution probably does not reflect the expression of the entire family of GHB-R, it nevertheless corresponds to the distribution of GHB binding sites in the rat brain (Table 1), as measured by saturation binding experiments with [^{3}H]GHB in dissected brain regions (35).

Expression of GHB-R gene in peripheral organs

To verify the tissue-specific pattern of GHB-R expression, the total RNA of heart, lung, liver, testis, and kidney was extracted and submitted to Northern blot analysis. All the peripheral

organs tested were devoid of GHB-R mRNA. Controls carried out with total RNA extracted from brain showed at least three distinct bands. This result suggests the existence of a family of GHB-R in the brain (Fig. 4).

Southern blot of brain genomic DNA from different species

The GHB-R specific probes found their complements in the genomes of different species including human. Figure 5 shows that the major spot in the rat, located around 5500 bp, was present in all brain tissues. This result is of major importance because it demonstrates the presence of the GHB-R in all the species studied and suggests that the GHB-R gene contains probably more than one exon.

Pharmacology of GHB-R expressed in CHO cells

The GHB-R expression construct was transiently transfected into CHO cells. Membranes prepared from these cells exhibited concentration-dependent binding of [^{3}H]GHB and identified a single binding site with a K_d of 426 ± 150 nM and a B_{max} of 4.8 ± 1.5 pmol/mg protein (Fig. 6). This affinity for GHB is of the order of the affinity measured for ex vivo brain membranes (see ref 3 and Table 1). Competition experiments carried out with 50 and 200 μM of the tested substance revealed no affinity of the cloned receptor for GABA, baclofen, or glutamate. Various GHB analogs that exhibit affinity for the GHB binding site(s) ex vivo have been screened for their ability to displace GHB binding at 50 μM concentration (Table 2). Under these conditions, γ-phenyl-GHB possesses a similar potency than GHB itself, in agreement with results obtained on rat brain membranes (36). For some more potent compounds, IC_{50} values have been determined with reference to GHB itself. Trans-4-hydroxycrotonate (T-HCA) and its derivatives (γ-*p*-nitrophenyl-T-HCA and γ-*p*-chlorophenyl-T-HCA) were more potent than GHB, with IC_{50} of 297 ± 102, 597 ± 150, and 706 ± 159 nM, respectively (Fig. 7). These results appear to indicate that the cloned GHB-R belongs to a subclass of GHB-R with a particular affinity for T-HCA and its derivatives. However, NCS-382, which possesses antagonistic properties for some of the described effects of GHB in vivo and in vitro (37), did not bind to the cloned GHB-R, even at 200 μM concentration.

Functional expression of the GHB-R

The cloned cDNA was transiently expressed in CHO cells. After 48 h of incubation the cells were recorded either in the cell-attached or whole cell mode of the patch-clamp technique. Application of GHB at concentrations up to 20 μM to control cells (n=20) did not elicit any significant current (Fig. 8). In contrast, 75.4% of the transfected cells responded to GHB when applied at concentrations ranging from 0.5 to 20 μM (n=65 cells). Figure 8 illustrates the current response obtained in the cell-attached recording mode. The fact that current activation can be observed in the physically bath isolated recorded patch of membrane while the drug was applied to the rest of the cell demonstrates that the link between the two sites is a cytoplasmic diffusible factor. In the cell-attached mode, this should contribute at least in part to the delay observed between the onset of drug application and the response (Fig. 8).

At negative membrane potentials, the current response was directed inwardly and lasted for ~1-10 min (see Figs. 8, 10, and 11). In the whole cell mode, the I-V relation of the steady-state

current amplitude recorded in the absence of GHB was linear and reversed at a potential of -24.0 mV (Fig. 9). The GHB-induced current was also linear over the potential range tested (-50 to 30 mV) and reversed at a potential of –2.5 mV in the case illustrated in Fig. 9 as estimated by linear regression. The mean calculated reversal potential value was –2.1 ± 0.1 mV (n=5 cells). This value corresponded to the equilibrium potential of the monovalent cations (E_{cat}=-1.7 mV) in our whole cell recording conditions. This demonstrates that the ion species that carry the GHB induced current here were mainly sodium and potassium ions.

The GHB induced response was dose dependent. Figure 10*A* shows the mean normalized dose-response curve obtained from seven different cells recorded in the whole cell mode. The distribution of the data points was sigmoidal and well described by the Hill equation. Interestingly, a value close to 2 was obtained for the Hill coefficient parameter (mean value=2.2±0.8). This indicates allosteric interaction of at least two binding sites for receptor activation. The maximal response varied strongly from cell to cell, and extreme values of 0.1 and 4 nA were obtained with a mean at 1244 ± 1860 pA. The mean EC_{50} calculated from individual dose responses had a value of 4.0 ± 0.3 μM.

Figure 10*B* illustrates some pharmacological properties of the cloned GHB-R expressed in CHO cells. CGP-55845, a $GABA_B$ antagonist, used at a concentration corresponding to three times its apparent K_d on the $GABA_B$ receptor (38), i.e., 0.1 μM, was unable to block the response induced by 1 or 5 μM GHB. In the case of Fig. 10*B*, the amplitude of the response was -5.5 and -5.7 pA in the absence or presence of CGP, respectively. The response was not antagonized with even 10 time higher concentration of CGP-55845 (not shown). Moreover, application of baclofen to transfected cells, at concentrations up to 10 μM, was not able to elicit any detectable current under our conditions (5 transfected and 5 nontransfected cells tested, not shown). These results are in agreement with the binding data (see above) and strongly suggest that the transfected cDNA expressed a receptor that did not recognize the specific $GABA_B$ receptor ligands. Surprisingly, in our experiments the well-documented GHB-R antagonist NCS-382 did not block the response elicited in the presence of GHB. Indeed, there was no significant difference in the amplitude response to 1 μM GHB when applied in the absence or presence of 5 or 50 μM NCS-382. Responses of 6.9, 7.3, and 6.2 pA amplitude were obtained, respectively, as shown in Fig. 10*B*.

Previous binding data on the native GHB-R had shown that this receptor belongs to the G-protein linked family. To verify whether the expressed receptor-mediated responses involved G-protein, we used here GTP-γ-S, a nonhydrolyzable GTP analog. The expected effect of this compound when introduced into the cell is to block the receptor activated G-protein in its active state. Consequently, the response is no longer reversible. Indeed, when GTP-γ-S was added to the pipette solution at concentrations of 0.5 or 1 mM, in six out of seven transfected cells tested, the response to GHB 1-5 μM was not reversible during the recording time (1-2 h) whereas no detectable current was induced when GHB was applied to nontransfected cells (Fig. 11; 8 cells tested). These results strongly suggest that the expressed GHB-R was linked to a G-protein.

DISCUSSION

GHB is emerging as an important neuromodulator of the central nervous system, playing a fundamental role in the regulation of GABAergic and dopaminergic activities. These properties

are the basis for its use as a therapeutic and/or as a recreative substance. The cloning of GHB-R is of fundamental importance to delineate the pathways of GHB neurotransmission in the brain and to define and characterize the subtypes of receptors involved in the pharmacological effects of GHB. Our findings provide several lines of evidence supporting the existence of a family of GHB-R. The present report describes the isolation of the first member of this family and provides several lines of evidence suggest that the cloned GHB-R is a G-protein linked receptor.

Analysis of the 512 amino acid sequence of the cloned receptor revealed the presence of seven putative transmembrane domains and a number of phosphorylation sites for PKC and casein kinase II. These phosphorylations could occur on a serine residue at position 294 and threonine residues at positions 231, 293, and 300 and participate in the regulation of the receptor activity. Our results showed that some rigid structural analogs of GHB, related to T-HCA and its derivatives, were more effective than GHB itself on the activity of the cloned receptor. Such a pharmacological discrimination has already been predicted from comparative binding studies of [^{3}H]-T-HCA and [^{3}H]GHB on rat brain membranes (39). T-HCA is an endogenous substance in the rat brain (3, 40), and this substance and its derivatives have pharmacological effects closely related to those of GHB, especially on GHB-mediated modulation of dopaminergic activity (41). Thus, it can be proposed that the cloned GHB-R belongs more specifically to a subclass of GHB-R involved in the control of dopaminergic activity in the rat brain.

Although several studies have proposed that high levels of GHB in brain (i.e., those found after peripheral administration of GHB) could interfere with $GABA_B$ receptors (42), our results confirm the existence of a GHB-specific receptor that is different from the $GABA_B$ receptor. Competition experiments carried out on membrane preparations from transfected CHO cells indeed revealed that neither GABA nor baclofen or glutamate showed any affinity to the receptor, highlighting the specificity of the cloned receptor for GHB. Thus, under physiological conditions when micromolar GHB concentrations are present in the brain, it seems clear that a series of specific GHB-R are responsible for the effects of GHB. This substance is structurally related to GABA, but these two compounds are pharmacologically distinct in many of their actions, even though they can be converted to each other in the brain through a common metabolite, succinic semialdehyde (3).

The electrophysiological response elicited by GHB in our conditions was selectively present in the CHO cells transfected with the GHB-R coding cDNA. As expected from the binding experiments described above, the observed pharmacological profile supports the concept that the cloned cDNA codes for a relatively high affinity GHB-R. Interestingly, the fact that the GHB-induced response was insensitive to the classically reported GHB-R antagonist NCS-382 may suggest that the cloned receptor is part of a family of GHB-R in the brain, with some of them having no affinity for NCS-382. The existence of GHB-R diversity was already suggested by the diversity of pharmacological effects reported for GHB (43, 44, 45).

The fact that GHB responses could be recorded in the cell-attached mode and the sensitivity of the response to GTP-γ-S suggests that the expressed GHB-R is a member of the G protein-coupled receptors superfamily (46). This is in agreement with the presence in the deduced amino acid sequence of the GHB-R of a homologous sequence to a consensus G-protein binding domain. However, the GHB response observed here corresponded to an activation of a cationic ion conductance and it cannot be excluded that the involvement of this conductance may be a

consequence of the host cell protein machineries, as was the case for the expression of the 5HT-2 in *Xenopus* oocyte (47). Thus, the naturally occurring effector coupling with this cloned GHB-R will need to be identified in native cells to definitely conclude that the expressed GHB-R is a G-protein linked receptor.

We have investigated the brain regional expression of the cloned receptor by in situ hybridization, and we found some labeling in the cerebellum despite the fact that previous reports did not find high affinity GHB binding sites in this region. Taking into account the fact that the K_d for GHB binding in cerebellum is somewhat higher than in other brain regions (Table 1), it is probable that this binding is washed out from brain slices used for autoradiography (25). However, Snead and Liu (26) reported some GHB binding in the cerebellum, consistent with the results shown in Table 1. In the majority of the brain regions investigated, the K_d values for GHB binding are in the range of 0.8 to 1.3 μM. These values correspond to previous results reported for GHB binding in total rat brain (35) or in differentiated NCB-20 cell lines (23). The lower value of GHB binding measured in CHO cells might be due to receptor expression in heterologous cells.

Our search for the human counterpart of the cloned rat GHB-R, using FASTA programs that provides sequence similarity and homology searching against nucleotide and protein databases, has so far been unsuccessful. Indeed, the best score was a 65% homology in 175 amino acids overlapping with a member of the four transmembrane superfamily (4TMSF) tetraspanins (48, 49, 50). Tetraspanins may play a role in the regulation of axon growth as a link between β1-integrin and various intracellular signaling molecules including phosphatidylinositol 4-kinase and PKC (51). However, the similarity is restricted to the 175 amino acids located at the N terminus of the GHB-R sequence. Moreover, in contrast to the wide tissue distribution of 4TMSF, the cloned GHB-R is expressed only in brain tissue. Despite the failure to identify the human GHB-R in the database search, results from Southern blot analyses clearly demonstrate the presence of this receptor in the genomic DNA from various species. As shown in the southern blot (Fig. 5), a major band is found in almost all species (including human). The presence of three to four bands could represent the existence of three to four exons for the GHB-R gene. Therefore, approaches that are more labor intensive than the standard database search may be necessary to identify the human counterpart of the rat GHB-R described in the present report as well as to isolate and clone the other members of the GHB-R family.

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Table 1

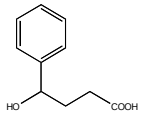
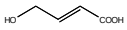
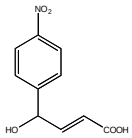
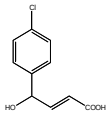
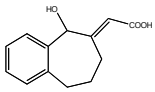
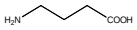
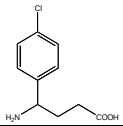
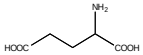
Regional distribution of GHB binding sites in the rat brain

	$K_d \pm$ SD (nM)	$B_{max} \pm$ SD (pmol/mg)	R_2
Hippocampus	821 ± 72	26.4 ± 0.80	0.99
Cortex	466 ± 54	22.7 ± 0.87	0.98
Striatum	550 ± 36	15.2 ± 0.33	0.99
Olfactory bulbs	819 ± 71	13.7 ± 0.45	0.98
Thalamus	1256 ± 163	7.1 ± 0.39	0.98
Cerebellum	1309 ± 198	1.6 ± 0.10	0.98

K_d and B_{max} of [^{3}H]GHB binding determined on membranes obtained from various rat brain regions. Saturation curves experiments were calculated by nonlinear fitting with the Graphpad-Prism program. Each experimental point is means ± SD of triplicate.

Table 2

Effects of GHB and analogs on [^{3}H]GHB binding

Abbreviations	Structures	Compounds	[^{3}H]GHB Displacement ± SD (Arbitrary Units)
GHB		γ-hydroxybutyrate	100
pph-GHB		γ-p-phenyl-hydroxybutyrate	106 ± 29
T-HCA		*trans*-4-hydroxycrotonic acid	229 ± 18
p-NO_2ph- T-HCA		γ-p-nitrophenyl-t-hydroxycrotonic acid	288 ± 28
NCS–356		γ-p-chloro-phenyl-THCA	427 ± 30
NCS-382		6,7,8,9-tetrahydro-5-[H]-benzocycloheptene-5-ol-4-ylidene acetic acid	0
GABA		γ-aminobutyric acid	16 ±10
Baclofen		γ-p-chloro-phenyl-GABA	0
Glu		Glutamate	0

Competition experiments were carried out with 200 nM [^{3}H]GHB in the presence of 50 µM GHB or GHB analogs. Specific displacements induced by 50 µM GHB have been arbitrarily set to 100. Under these conditions, several derivatives of T-HCA provoked a more potent displacement.

Fig. 1

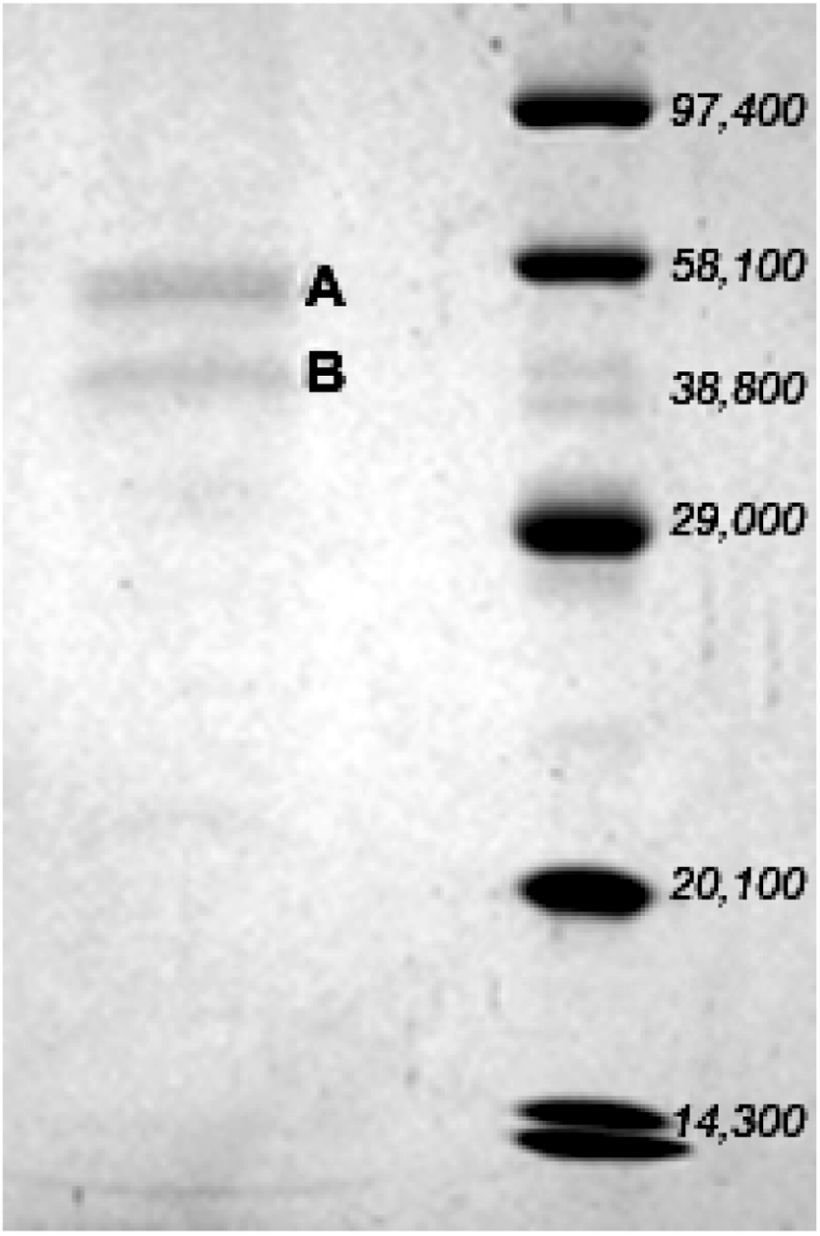


Figure 1. Preparative gel electrophoresis of the proteins eluted from the affinity column by a pulse of 10 mM GHB. The major band (***A***) is a ~54 kDa protein that was partially hydrolyzed to give peptides the sequences of which were used to design PCR primers. The quantity of protein in the second band (***B***) was too low to be sequenced. Molecular weight markers: 1 = 97,400 (phosphorylase b subunit); 2 = 58,100 (catalase subunit); 3 = 38,800 (alcohol dehydrogenase subunit); 4 = 29,000 (carbonic anhydrase); 5 = 20,100 (trypsin inhibitor); 6 = 14,300 (lysozyme).

Fig. 2

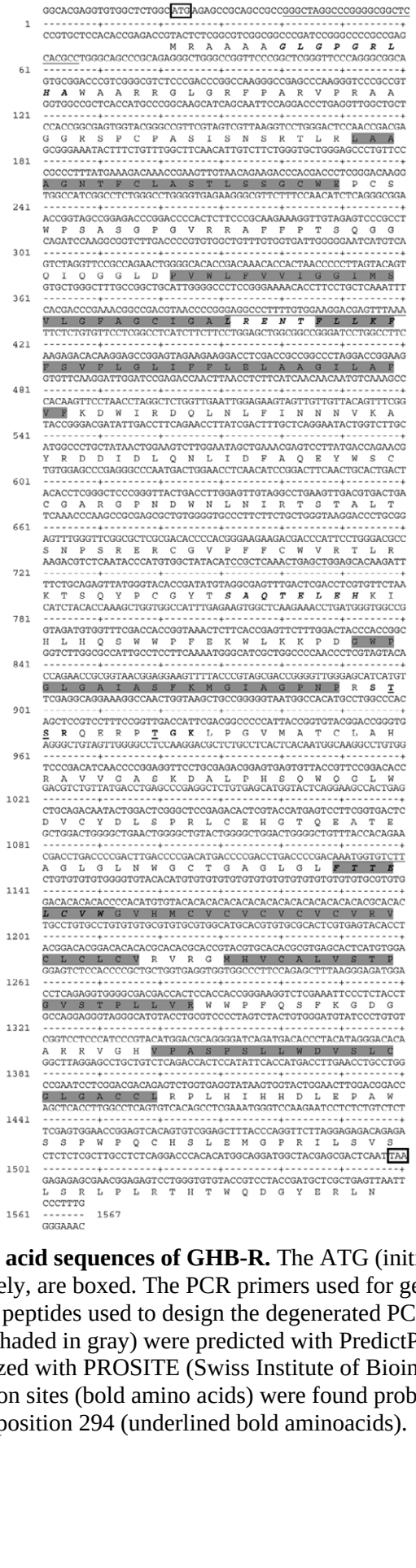


Figure 2. Nucleotide and amino acid sequences of GHB-R. The ATG (initiation codon) and TAA (stop codon) at position +22 and +1558, respectively, are boxed. The PCR primers used for generating the 344 bp cDNA probe are underlined, and the corresponding peptides used to design the degenerated PCR primers are in bold italic letters. The 7 putative transmembrane regions (shaded in gray) were predicted with PredictProtein, a secondary structure prediction software. The sequence was analyzed with PROSITE (Swiss Institute of Bioinformatics; 47), and putative protein kinase C and casein kinase II phosphorylation sites (bold amino acids) were found probably involving threonine residues at positions 293 and 300 and serine residue at position 294 (underlined bold aminoacids).

Fig. 3

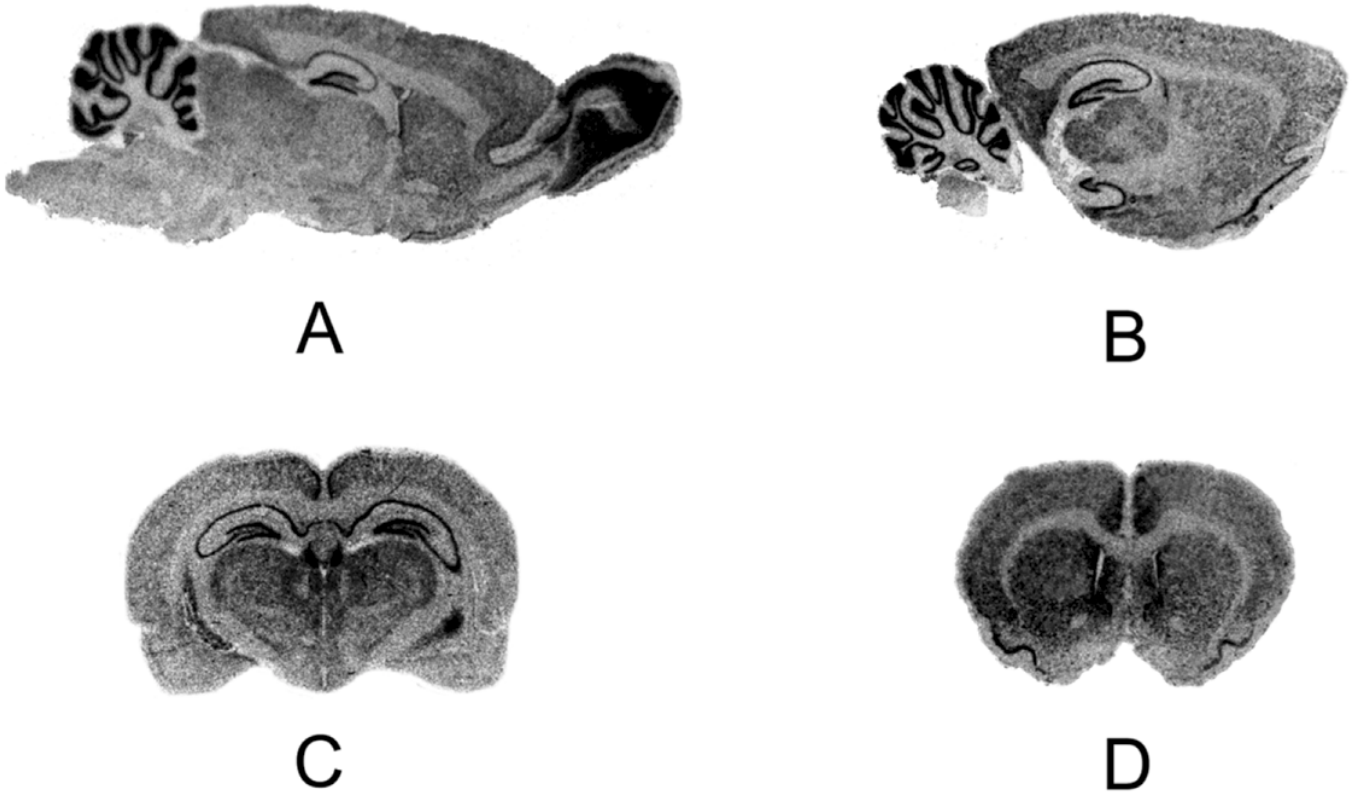


Figure 3. Brain regional distribution of GHB-R mRNA. In situ hybridization was performed on brain sections of rats using as probe an antisense ^{35}S-labeled oligonucleotide designed from the GHB-R cDNA sequence. The figure shows two sagittal (***A*** and ***B***) and frontal (***C*** and ***D***) slices of rat brain. Hippocampus, olfactory bulbs, cortex, striatum, and thalamus are heavily labeled. The cerebellar cortex is also labeled, but caudal parts of the brain contain no or very low levels of GHB-R receptors (pons-medulla and hypothalamus).

Fig. 4

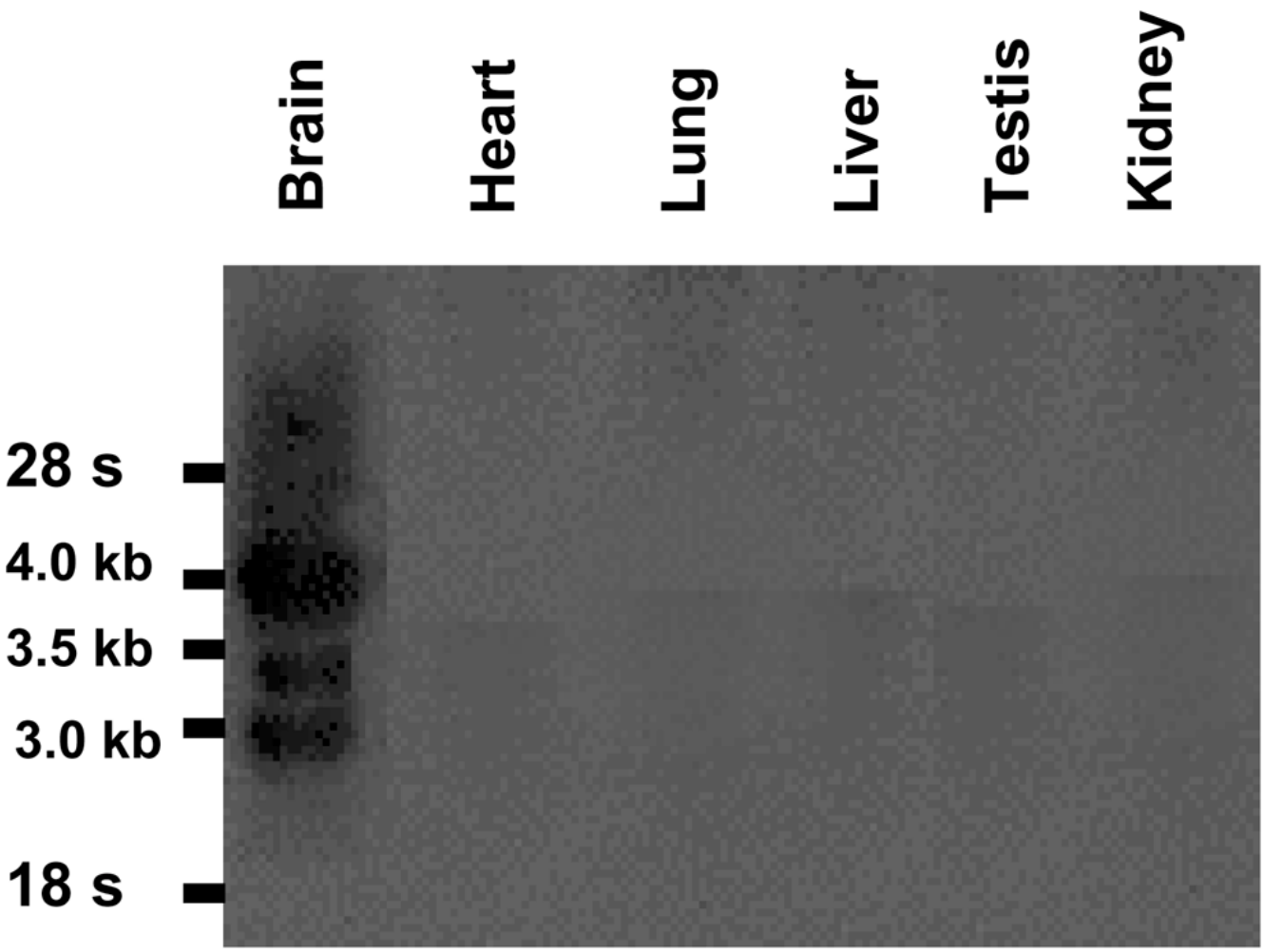


Figure 4. Northern blot analysis of 10 µg total RNA from rat brain, heart, liver, and lung. The blot was probed with ^{32}P-α-dCTP-labeled GHB-R cDNA; 28S (4.9 kb) and 18S (1.9 kb) rRNA were used as size markers. The probe recognized only 3 RNA species from rat brain samples. The peripheral tissues analyzed showed no bands, suggesting that GHB-R was specific to brain tissue. These three RNA bands also suggest the existence of different GHB-R isoforms.

Fig. 5

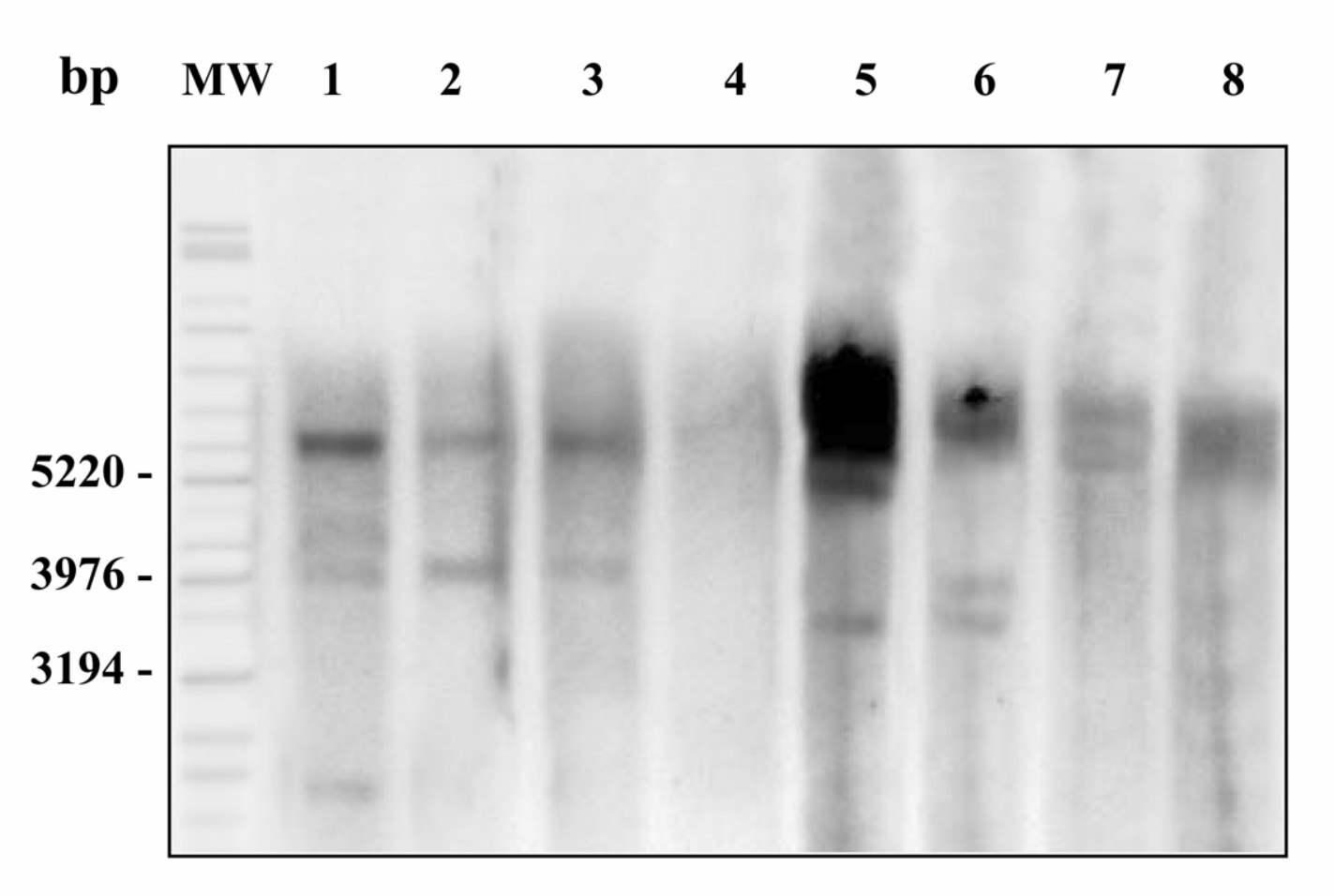


Figure 5. Southern blot analysis of brain genomic DNA. Ten micrograms of genomic DNA from the brains of mouse (lane 8), guinea pig (lane 7), hamster (lane 6), rat (lane 5), rabbit (lane 4), pig (lane 3), monkey (lane 2), and human (lane 1) were digested with *Eco*RI and subjected to electrophoresis on a 0.8% agarose gel along with DNA marker I (MBI Fermentas). Two PCR-amplified fragments were ^{32}P-labeled and used as hybridization probes.

Fig. 6

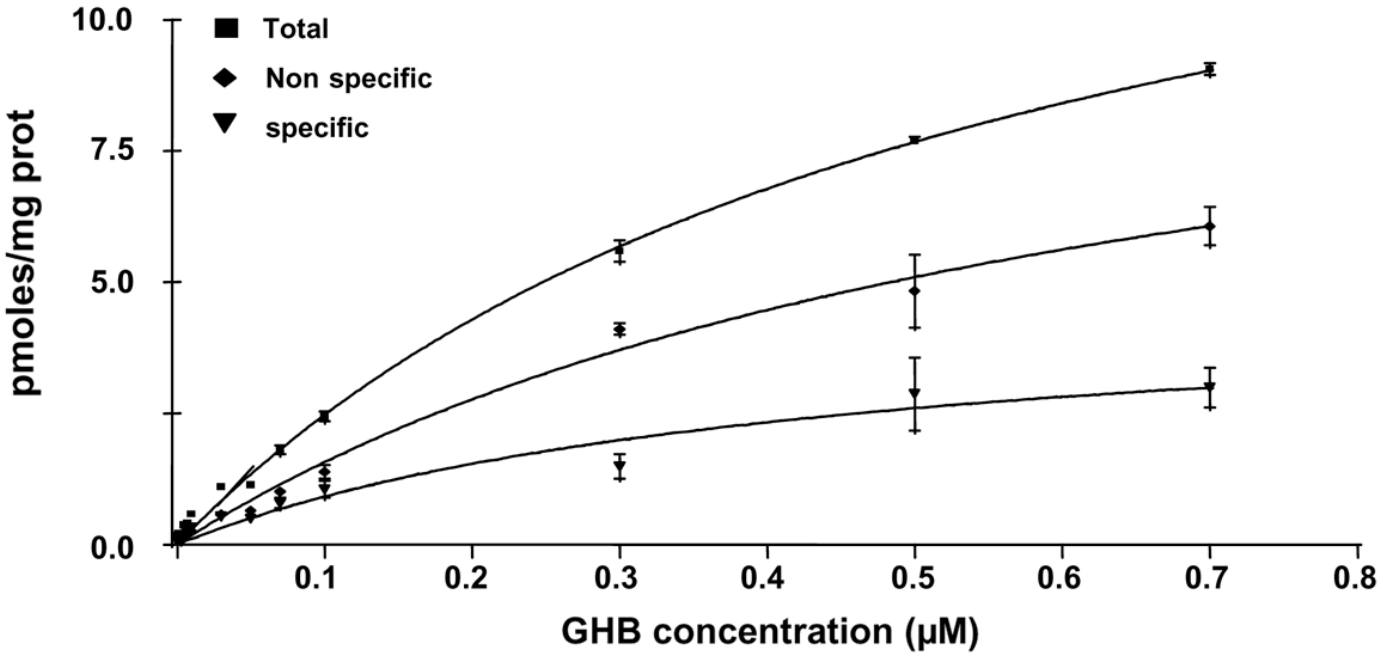


Figure 6. Saturation [^{3}H]GHB binding experiments (nonlinear regression line) with membranes of transfected CHO cells. K_d and B_{max} values are 425 ± 150 nM and 4.8 ± 1.5 pmol/mg protein, respectively. Results are means ± SD of 3 independent values, nonlinear fitting by the GraphPad-Prism program (San Diego, CA).

Fig. 7

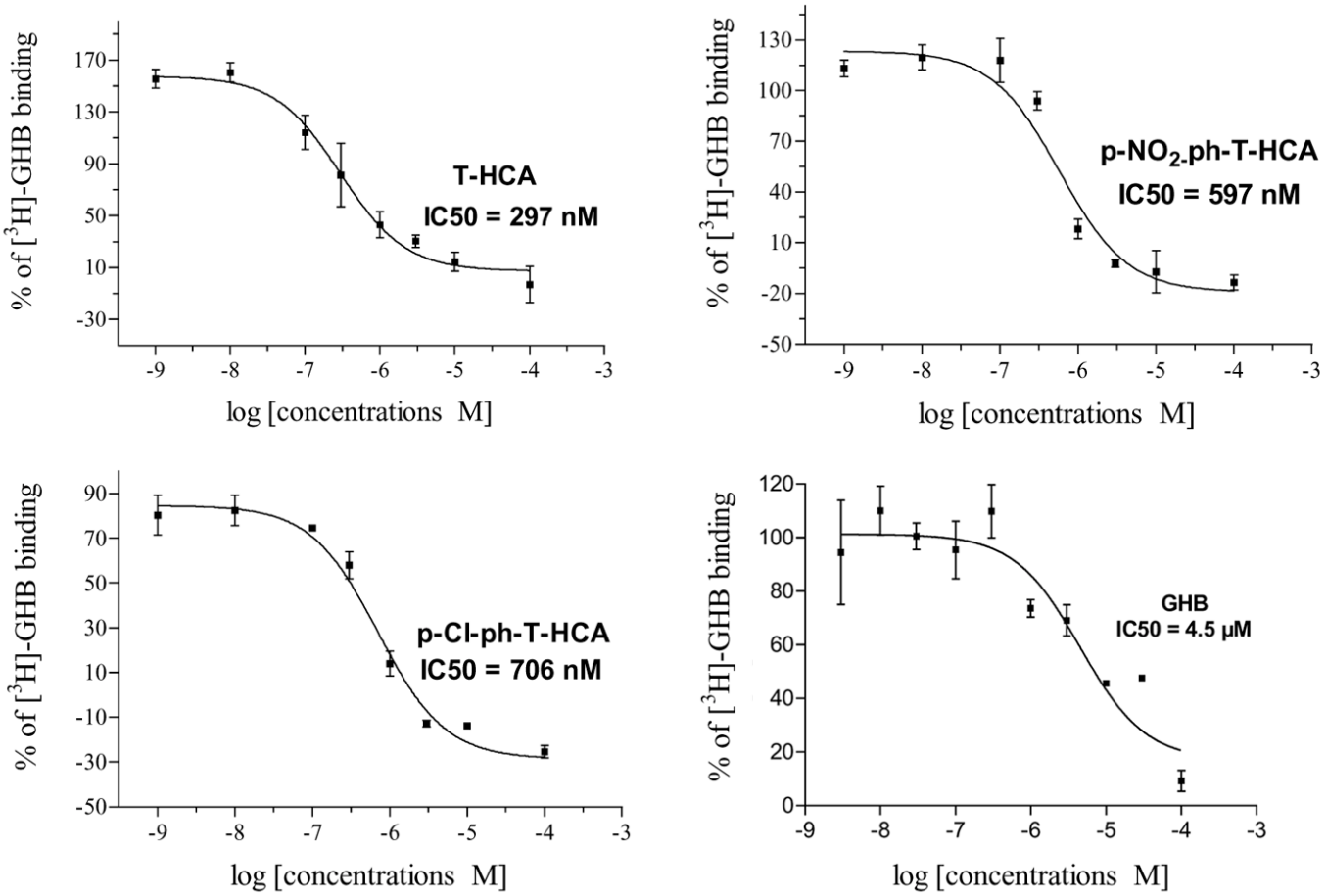


Figure 7. Competition curves for the displacement of [^{3}H]GHB from binding sites of transfected CHO cells in the presence of increasing concentrations of GHB structural analogs. Results are means ± SD of 3 independent values for each experimental point. Nonlinear fitting was performed using GraphPad-Prism program.

Fig. 8

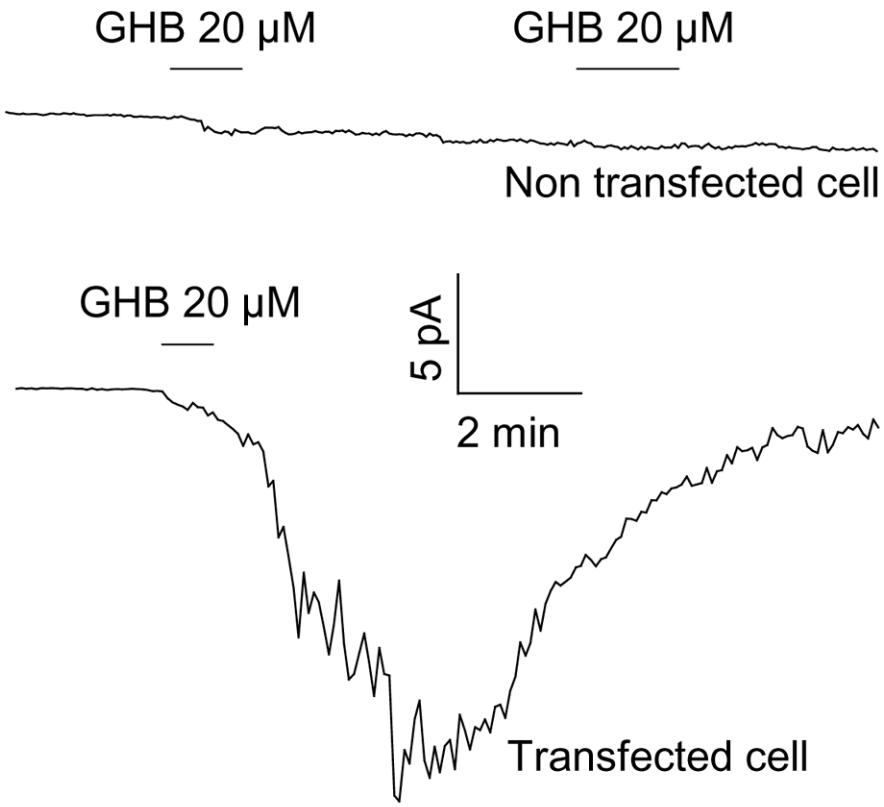


Figure 8. Functional expression of the cloned GHB-R in CHO cells. Typical current traces recorded in the cell-attached configuration of the patch-clamp technique from control (upper trace) and transfected cells (lower trace). The membrane potential was held to –80 mV. Bars indicate the periods of drug application.

Fig. 9

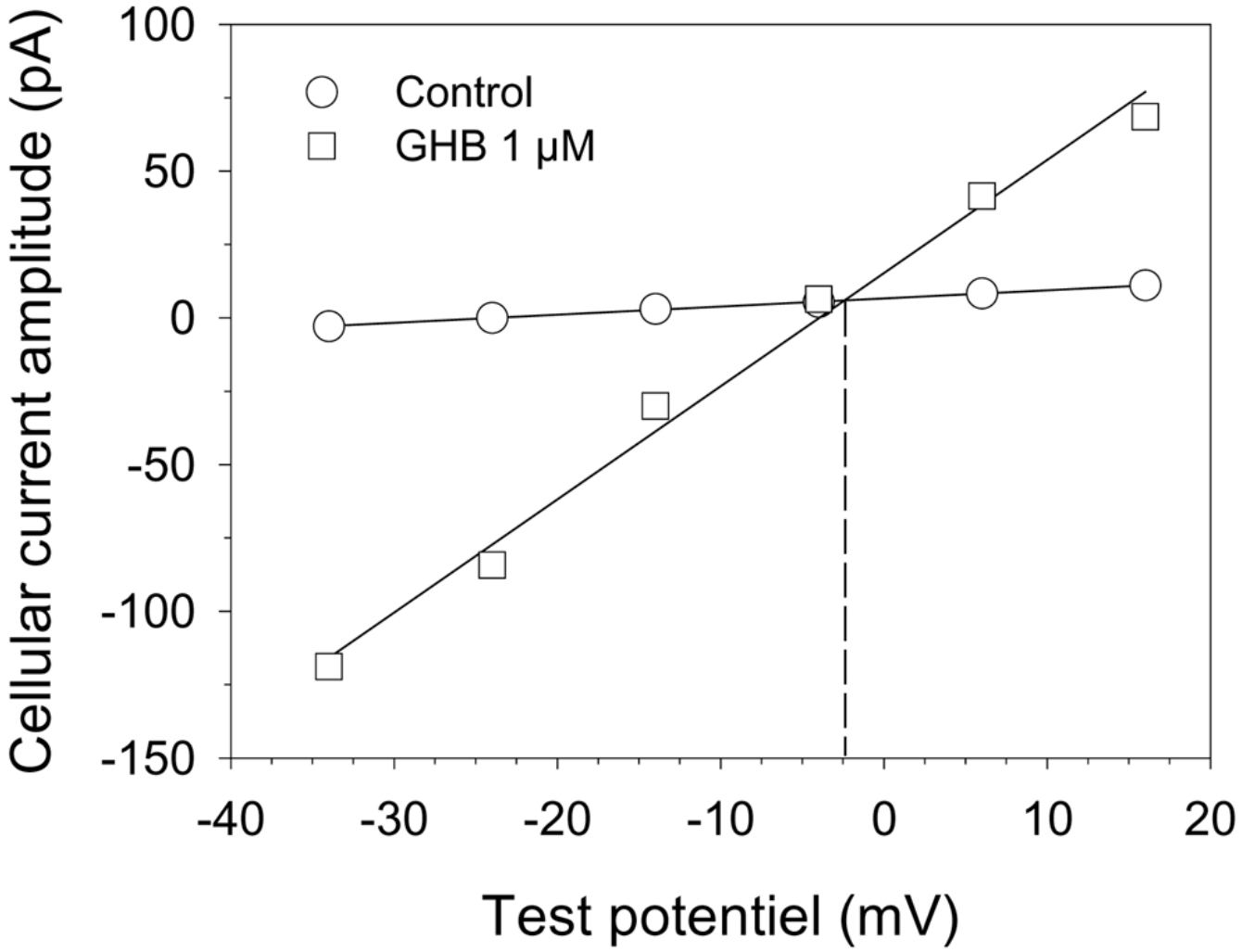


Figure 9. Typical current-voltage relationship of the steady-state current of a CHO cell in absence or presence of 1 µM GHB as indicated in the figure. Straight lines are linear regression fit to data points. The dashed line is the zero current potential of the GHB response. Test potential values were corrected for junction potential (14 mV), which was calculated using a clampex routine, according to the ionic composition of the bath and pipette solutions used in whole cell recording.

Fig. 10

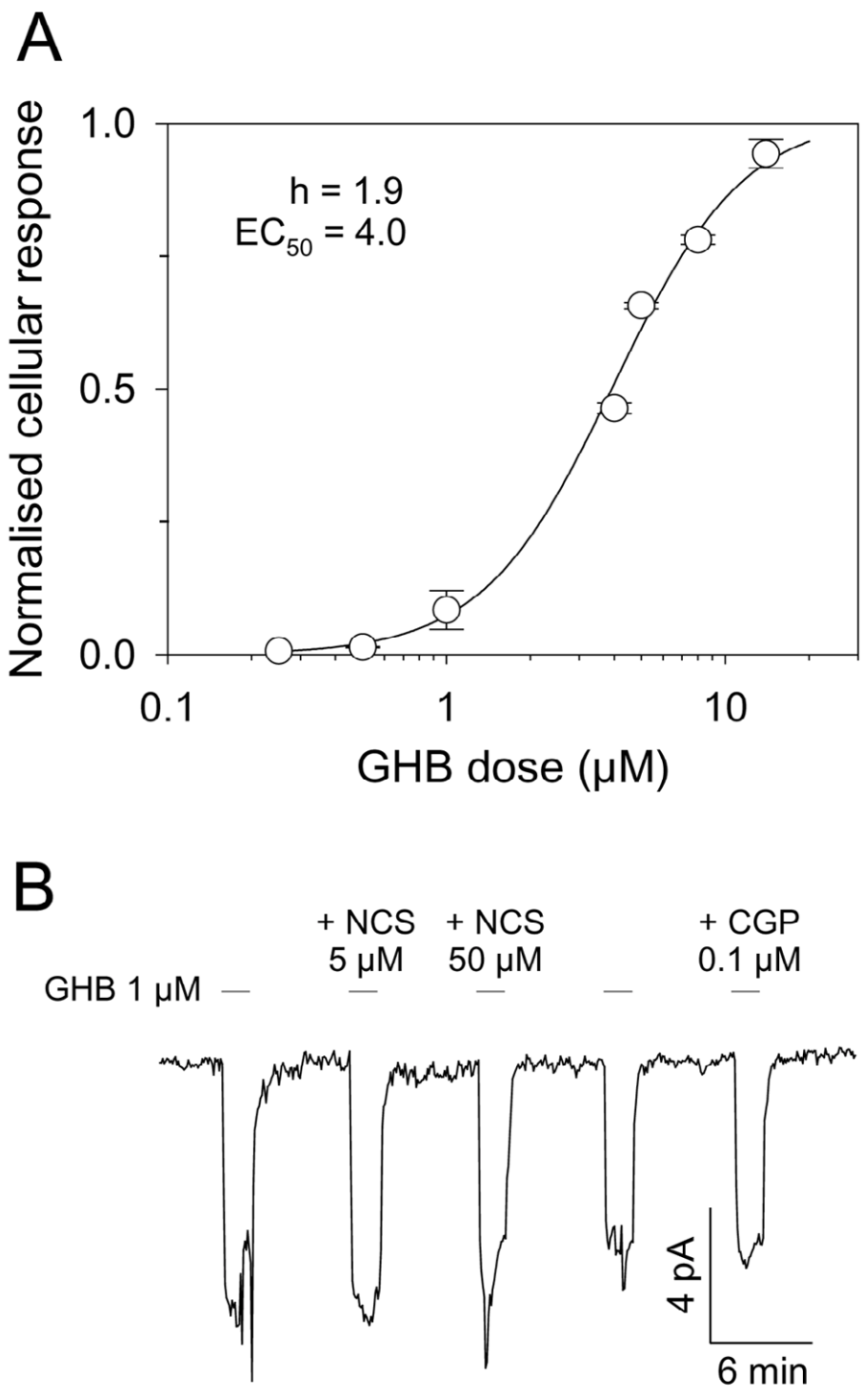


Figure 10. **Dose-response and pharmacological properties of GHB-induced response in whole cell. *A*)** Normalized mean dose-response curve obtained from 7 transfected cells with GHB-R. The normalization was done with respect to the calculated maximal response for each cell. The continuous curve corresponds to the Hill equation fit to data points. The calculated parameters are indicated in the figure. The membrane potential was –80 mV. ***B*)** Typical traces of responses to GHB (1 μM; horizontal bars) obtained in absence or presence (+) of the $GABA_B$ selective antagonist CGP-55845 (0.1 μM) or NCS-382 (5–50 μM) as indicated.

Fig. 11

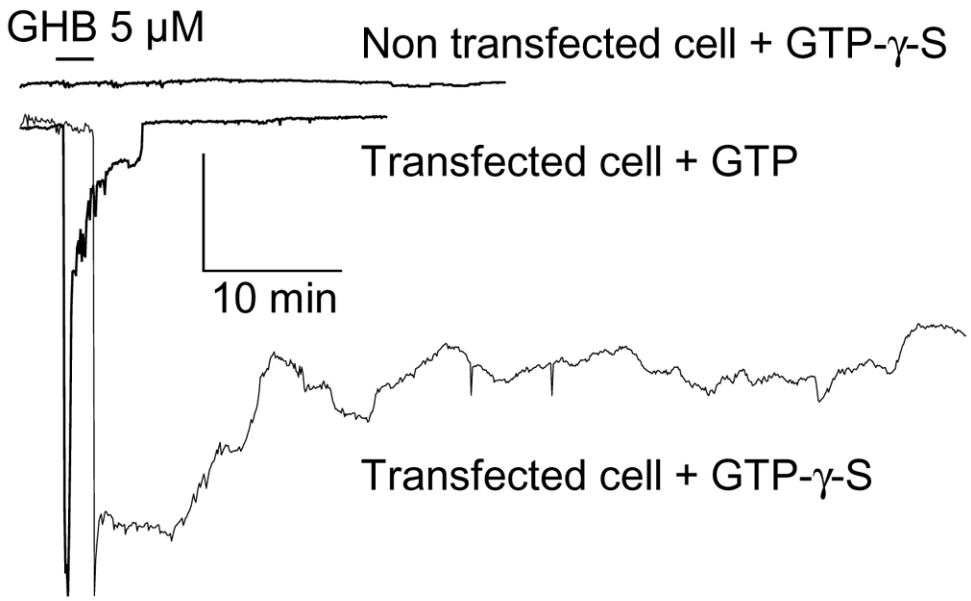


Figure 11. Sensitivity of the GHB response to GTP-γ-S. Superposed (with respect to the GHB application period, horizontal bar) traces of the current recorded in the presence of 5 µM GHB from control and transfected cells. GTP-γ-S was diluted in the pipette medium at a final concentration of 0.5 mM. Note that this nonhydrolyzable GTP analog specifically blocks the reversion of the GHB response. Vertical scale bar corresponds to 106 and 321 pA for GTP and GTP-γ-S transfected cells, respectively. The membrane potential was –80 mV.