Supporting Information

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SI Materials and Methods

Materials. Bicuculline, diazepam, etomidate, muscimol, tracazolate, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP or gaboxadol), and valerenic acid were purchased from Tocris Bioscience.

Methods. ***Photolinking, proteolysis, and denaturing gel electrophoresis.*** Rat membrane preparations (30 μg protein) were incubated with 100 nM [^{125}I]4-hydroxy-4-[4-(2-azido-5-iodobenzyloxy)phenyl]butanoate γ-hydroxybutyric acid ([^{125}I]azido-BnOPh-GHB) in the absence or presence of 100 μM 4-hydroxy-4-[4-(2-iodobenzyloxy) phenyl]butanoate (BnOPh-GHB) (1) for 1 h (room temperature) in incubation buffer 50 mM KH_2PO_4 buffer (pH 6.0). The reaction was terminated by centrifugation at 4 °C, and the pellet was resuspended, washed, and UV-irradiated for 2 min (302 nm, 25 W). After two additional washing steps, the pellet was resuspended and incubated in proteinase K buffer [20 mM Tris buffer (pH 8.0) containing 0.5% SDS, 1.0% Triton-X 100, 50 mM DTT, proteinase K (5 ng/μL)] for the indicated time points at 37 °C. Proteolysis was terminated by the addition of 5 mM phenylmethylsulfonylfluoride (Sigma) and 4 μL Nupage LDS buffer sample buffer (Invitrogen). Proteins were separated on a 4–12% gradient gel (Invitrogen) along with ^{14}C-methylated molecular mass markers (CFA.626; GE Healthcare). The dried gel was exposed to a BAS-2040 imaging plate (Science Imaging Scandinavia AB) for 2–5 h and scanned by phosphorimaging in a BAS-2500 bioimaging analyzer (Fujifilm). Radioactive bands were excised for liquid chromatography (LC)–MS analysis.

Sample preparation and LC-MS analysis. The isolated gel bands were excised, destained, and subjected to in-gel digestion by trypsin (sequencing grade; Promega) as previously described (2). The resulting peptides were extracted in 0.5% acetic acid, concentrated on reversed-phase C18 tips, and analyzed by nanoscale LC-MS/MS on an LTQ-Orbitrap XL (Thermo Scientific) mass spectrometer using automatic lock mass recalibration (3). The peptides were separated by a linear gradient of increasing acetonitrile in 0.5% acetic acid (8–30% vol/vol) for 90 min on an Easy-nLC (Proxeon Biosystems), and the eluting peptides were directly electrosprayed into the orifice of the MS instrument. The mass spectrometer was operated in data-dependent acquisition mode to automatically switch between full-scan MS and collision-induced dissociation MS/MS. Full scans were recorded in the orbitrap mass analyzer at a resolution of 60,000 at m/z 400, whereas up to 10 MS/MS spectra were analyzed by the LTQ detector system in each scan cycle.

Target values for full scans were set to 10^6 and 10^4 for collision-induced dissociation MS/MS. The spray voltage was set to 2.2 kV, the source temperature was 200 °C, normalized collision energy (CE) was 35%, and a lock mass from ambient air (m/z = 445.120024) was used to recalibrate all full scans.

All raw LC-MS/MS files were processed with the MaxQuant software suite (4). Peptides were identified by searching the peak lists against a forward and reversed version of the rat and mouse IPI v.3.37 databases using Mascot (www.matrixscience.com), allowing the following variable modifications: oxidized methionine, N-terminal protein acetylation, and *N*-pyro-glutamine. The initial mass tolerance was set to 7 ppm for MS and 0.5 Da for MS/MS. Identified peptide hits were filtered to an estimated false discovery rate (FDR) < 0.01 and quantified using by the label-free quantitation algorithm and spectral MS/MS counts.

1. Wellendorph P, et al. (2010) Novel radioiodinated γ-hydroxybutyric acid analogues for radiolabeling and Photolinking of high-affinity γ-hydroxybutyric acid binding sites. *J Pharmacol Exp Ther* 335:458–464.
2. Lundby A, Olsen JV (2011) GeLCMS for in-depth protein characterization and advanced analysis of proteomes. *Methods Mol Biol* 753:143–155.
3. Olsen JV, et al. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 4:2010–2021.
4. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26:1367–1372.

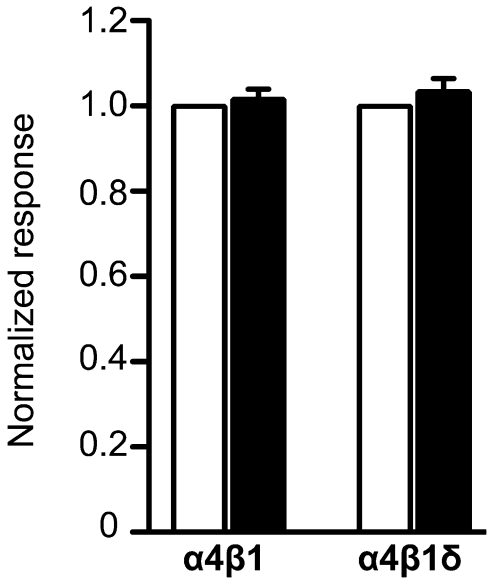


Fig. S1. Inability of GHB to antagonize the GABA response at α4β1- and α4β1δ-expressing oocytes. Normalized response of 1 mM GHB and 100 nM GABA (filled bars) and 100 nM GABA (open bars) compared with 100 nM GABA of oocytes injected with α4 and β1 or α4, β1, and δ $GABA_A$ receptor RNA. Based on reported GABA concentration response curves (1), 100 nM GABA corresponds to approximately 15% and 85% of the maximum responses for α4β1 and α4β1δ, respectively. The normalized responses (mean ± SEM) ($I_{GHB + GABA}/I_{GABA}$) were 1.02 ± 0.02 (α4β1; $n = 7$, $P = 0.54$, Z test) and 1.03 ± 0.03 (α4β1δ; $n = 7$, $P = 0.28$, Z test).

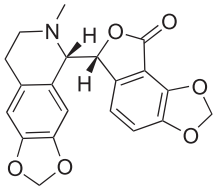
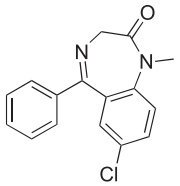
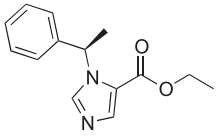
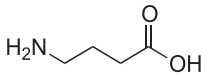
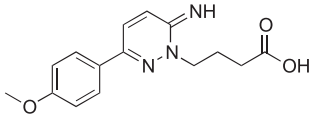
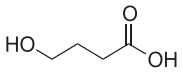
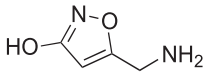
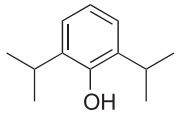
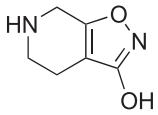
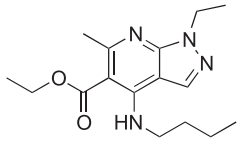
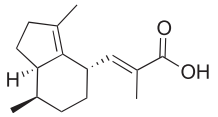
1. Karim N, et al. (2012) Low nanomolar GABA effects at extrasynaptic α4β1/β3δ $GABA_A$ receptor subtypes indicate a different binding mode for GABA at these receptors. *Biochem Pharmacol*, 10.1016/j.bcp.2012.05.017.

Table S1. List of proteins identified from radiophotoaffinity-labeled GHB binding sites (related to Fig. 1 and further described in Dataset S1)

Protein names	Gene names	Molecular mass (kDa)	MS/MS counts				
			18 kDa	21 kDa	28 kDa	50 kDa	Upper and lower gel bands
Brain-specific angiogenesis inhibitor 1-associated protein 2	Baiap2	59.65	4	3	5	10	28
Calcium/calmodulin-dependent protein kinase type II α-chain	Camk2a	55.35	64	46	55	47	41
2′,3′-cyclic-nucleotide 3′-phosphodiesterase	Cnp	47.27	42	28	40	23	58
GABA receptor subunit α-1	Gabra1	51.75	5	2	7	7	9
GABA receptor subunit β-1 and -2	Gabrb1; Gabrb2	54.63	1	1	3	6	9
Glial fibrillary acidic protein	Gfap	49.96	19	9	37	43	49
Guanine nucleotide binding protein G(i), α2-subunit	Gnai2	40.47	8	3	9	5	8
Guanine nucleotide binding protein G(o) subunit α	Gnao1	40.07	29	9	27	15	34
Neuroplastin	Nptn	43.93	3	3	4	10	15
Septin-5	Sept5	43.92	5	6	9	4	13
Excitatory amino acid transporter 1	Slc1a3	59.62	22	8	30	28	27
4F2 cell-surface antigen heavy chain	Slc3a2	58.07	6	3	2	2	8
Synaptotagmin-1	Syt1	47.42	21	7	24	16	17

Proteins identified in all gel bands before and after limited proteolysis by proteinase K treatment.

Table S2. Inhibitory affinity constants (K_i values) for $GABA_A$ receptor ligands in the [^{3}H] (*E,RS*)-(6,7,8,9-tetrahydro-5-hydroxy-5*H*-benzocyclohept-6-ylidene)acetic acid [^{3}H]NCS-382 binding assay

Compound	Concentration (μM)	K_i ($pK_i \pm$ SEM)	Structure
Bicuculline	100	*	
Diazepam	100	*	
Etomidate	100	*	
GABA	100	2.1 mM (2.7 ± 0.021)†	
Gabazine	100	15.6 μM (4.7 ± 0.107)	
GHB	100	4.3 μM (5.4 ± 0.041)‡	
Muscimol	100	*	
Propofol	100	*	
THIP/Gaboxadol	100	*	
Tracazolate	30	*	
Valerenic acid	100	*	

*Data from two independent experiments performed in triplicate showed no significant difference from control (one-way ANOVA; posttest Dunnett).
†Concentration inhibition curve was fitted to the value of nonspecific binding (1 mM GHB).
‡Modified from ref. 1.

1. Wellendorph P, Høg S, Skonberg C, Bräuner-Osborne H (2009) Phenylacetic acids and the structurally related non-steroidal anti-inflammatory drug diclofenac bind to specific γ-hydroxybutyric acid sites in rat brain. *Fundam Clin Pharmacol* 23:207–213.

Other Supporting Information Files

Dataset S1 (XLSX)