Supporting Information

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SI Materials and Methods

Antibodies and Expression Constructs. Polyclonal rabbit anti-α4 antibodies were provided by Verena Tretter and Werner Sieghart (Medical University Vienna, Vienna). The peroxidase-conjugated IgG secondary antibody was from Jackson ImmunoResearch Laboratories. Fluorescently labeled α-bungarotoxin (α-Bgt) was purchased from Invitrogen. α1, α4, β3, and δ cDNAs were cloned into the mammalian cytomegalovirus promoter vector PRK5 for transgene expression (1). dsRed monomer fluorescent protein (RFP) was introduced after the fourth amino acid of the mature α4 subunit followed by the bungarotoxin (Bgt) binding site sequence (WRYYESSLEPYPD). Likewise, pHluorin was introduced between amino acids 4 and 5 of the α1 subunit, and all mutations were verified by DNA sequencing. Plasmids were transfected into monkey kidney (COS-7) or human embryonic kidney (HEK) 293 cells using electroporation (Bio-Rad) with 2 μg of plasmid DNA per construct. Nuclofection (AMAXA Systems) was used to introduce the respective plasmids into freshly dissociated hippocampal neurons that were used at 10–14 days in vitro (Div).

Cell Culture, Metabolic Labeling, and Immunoprecipitation. Cultures and slices were labeled with 0.5 mCi/mL [^{32}P]orthophosphoric acid for 1–4 h in phosphate-free DMEM before lysis. The α4 subunit was isolated using immunoprecipitation with α4 antibodies, after correction for protein content and the specific activity of labeling. Results were attained by SDS/PAGE followed by autoradiography (1).

Biotinylation. Cultures were washed once with ice-cold PBS and then incubated in 2 mL of ice-cold PBS containing 1 mg/mL NHS-SS-biotin (Pierce) for 20 min to label surface proteins with biotin. After labeling, the biotin was quenched by incubating cells in PBS containing 25 mM glycine and 10 mg/mL BSA. Cells were then lysed in lysis buffer and sonicated. For hippocampal slice experiments, slices were incubated in artificial cerebrospinal fluid (ACSF) described above at 30 °C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/mL NHS-SS-biotin. Excess biotin was removed by washing slices three times in ice-cold ACSF and lysed. For both COS-7 cells and hippocampal slices, insoluble material was removed by centrifugation. The supernatant lysates were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 °C. Bound material was eluted with sample buffer and subjected to SDS/PAGE and then immunoblotted with the indicated antibodies. Blots were then quantified using the CCD-based FujiFilm LAS 300 system.

Patch-Clamp Electrophysiology. Cells were superfused, at a rate of 2 mL/min at 32–33 °C, with an extracellular solution containing (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 Hepes, and 11 glucose, and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2–5 MΩ) will contain (in mM) 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 Hepes, and 2 ATP (Mg^{2+} salt) and adjusted to pH 7.4 with KOH. Experiments were started 3–5 min after achieving the whole-cell configuration. GABA was applied once every 120 s via a fast-step perfusion system (Warner Instruments). All experiments were carried out at 32 °C using recording chamber and in-line perfusion heaters (Warner Instruments). Cells were voltage-clamped at –60 mV. Tetrahydrodeoxycorticosterone (THDOC) was applied to the cell either internally via passive dialysis from the pipette solution or superfused into the recording chamber. $PKC_{19–36}$ inhibitory peptide (Promega) and botulinum neurotoxin type A (BotA) (Sigma–Aldrich and List Biological Laboratories, Inc.) were added to the internal solution.

Data Acquisition and Analysis. For biochemical and immunofluorescent experiments, data are presented as means ± SEM. Statistical analysis was performed by using Student t test, where $P < 0.05$ is considered significant. For electrophysiological experiments, currents were recorded with an Axopatch 200B amplifier (Molecular Devices), filtered at 2 kHz, and digitized at 20 kHz with a Digidata 1320A data acquisition system (Molecular Devices), and analyzed using either Clampfit (pClamp, Molecular Devices) or GraphPad Prism version 4 software (GraphPad Software, Inc.).

Total Internal Reflection Fluorescence Microscopy. HEK cells or hippocampal neurons expressing fluorescent $GABA_A$ receptor ($GABA_AR$) subunits were subject to live total internal reflection fluorescence (TIRF) microscopy imaging using a Nikon Eclipse Ti Inverted TIRF Microscope (Nikon Instruments) at 32 °C. Cells were allowed to equilibrate in the stage top incubator for 10 min before conducting experiments. Samples were viewed through a Plan Apo TIRF 60× high-resolution (1.45 N.A.) oil immersion objective. Fluorescent images were collected with an iXion EMCCD camera (Andor Technology) interfaced to a PC running Nikon NIS-Elements software. Images were collected with a 200 ms exposure over a time course of 20 min before and immediately after addition of THDOC. For BotA (BotA-Sigma) experiments, 50 μM of activated toxin was added 10 min before THDOC addition. Total insertion events per minute were marked and taken as insertion frequency (2). GraphPad Prism Software was used for data analysis.

1. Abramian AM, et al. (2010) Protein kinase C phosphorylation regulates membrane insertion of GABAA receptor subtypes that mediate tonic inhibition. *J Biol Chem* 285(53):41795–41805.

2. Lin CY, et al. (2010) Surface plasmon-enhanced and quenched two-photon excited fluorescence. *Opt Express* 18(12):12807–12817.

Fig. S1. The effects of THDOC on α4 subunit insertion are dependent on vesicular-dependent membrane trafficking. HEK-293 cells expressing RFPα4β3 subunits were subject to TIRF before and after exposure to BotA (50 μM) and THDOC (100 nM). (Scale bar, 10 μM.) α4 insertion was reduced from 6.9 ± 1.4 to 4.6 ± 0.9 events per minute in BotA/THDOC ($P = 0.035$; $n = 6$).

Fig. S2. THDOC has no effect on α4 subunit endocytosis. Cells expressing RFPα4β3 were labeled with 50 nM Alexa–Bgt for 20 min. Cells were then incubated at 37 °C over a 20-min time course under control conditions or in the presence of 100 nM THDOC and imaged via confocal microscopy. (Scale bar, 5 μm.) The ratio of Alexa/RFP fluorescence was determined and normalized to that evident at t = 0 (100%) under control conditions (blue line) or in the presence of 100 nM THDOC (red line).

Fig. S3. The lack of effects of internal THDOC on α1β3 receptor-mediated current rundown. (*A*) GABA-activated currents recorded from α1β3 receptors at 0 and 20 min after the start of the experiment either in the absence (control, upper currents) or presence of internal 100 nM THDOC (lower currents). (*B*) Time dependence relationship for ~EC_{50} GABA-activated currents recorded in the presence of either internally applied DMSO control (white square) or 100 nM THDOC (black square) is shown.

Fig. S4. The effects of THDOC on receptors composed of α4(Q241L)β3. (*A*) Time dependence relationship for ~EC_{50} GABA-activated currents recorded from cells expressing α4(Q241L)β3 subunits in the presence of internally applied control (DMSO) (white square) or 100 nM THDOC (black square). Data were normalized to I_{GABA} at t = 0. Under control conditions, I_{GABA} was reduced to 30 ± 14% at 20 min, whereas it was reduced to 70 ± 8% of the initial response in the presence of internal THDOC (*n* = 3–4; *P* = 0.026). (*B*) Cells expressing α4(Q241L)β3 subunits were subject to biotinylation, followed by immunoblotting, and data were normalized to control (100%; dotted line). Cell surface levels of the α4(Q241L) subunit were increased to 145.4 ± 17% of control by THDOC (*P* = 0.021; *n* = 4).

Fig. S5. Imaging α4 subunit and gephyrin expression in hippocampal neurons. The 21–29 Div hippocampal neurons were permeabilized and stained with antibodies against the inhibitory scaffold protein gephyrin and the $GABA_AR$ α4 subunit followed by confocal microscopy. The images in the three right panels are enlargements of the box area in the left panel. (Scale bar, 10 μm.)