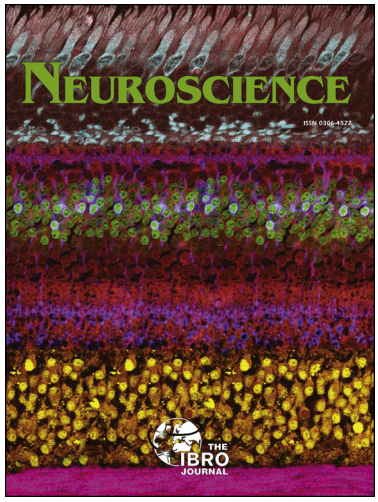

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List of abbreviations

γ-aminobutyric acid	(GABA)
voltage-gated calcium channel	(VGCC)
calmodulin-dependent protein kinase II	(CaMKII)
phosphate buffered saline	(PBS)
radioimmunoprecipitation assay	(RIPA)
tris buffered saline	(TBS)
horseradish peroxidase	(HRP)
phenylmethylsulfonyl fluoride	(PMSF)
ethylenediaminetetraacetic	(EDTA)
flunitrazepam	(FNZ)
bovine serum albumin	(BSA)
polymerase chain reaction	(PCR)
standard error of the mean	(SEM)

Abstract

Persistent activation of $GABA_A$ receptors triggers compensatory changes in receptor function that are relevant to physiological, pathological and pharmacological conditions. Chronic treatment of cultured neurons with GABA for 48 h has been shown to produce a down-regulation of receptor number and an uncoupling of GABA/benzodiazepine site interactions with a half-time of 24-25 h. Down-regulation is the result of a transcriptional repression of $GABA_A$ receptor subunit genes and depends on activation of L-type voltage-gated calcium channels. The mechanism of this uncoupling is currently unknown. We have previously demonstrated that a single brief exposure of primary neocortical cultures to GABA for 5-10 min ($t½$ = 3 min) initiates a process that results in uncoupling hours later ($t½$ = 12 h) without a change in receptor number. Uncoupling is contingent upon $GABA_A$ receptor activation and independent of voltage-gated calcium influx. This process is accompanied by a selective decrease in subunit mRNA levels. Here, we report that the brief GABA exposure induces a decrease in the percentage of α3-containing receptors, a receptor subtype that exhibits a high degree of coupling between GABA and benzodiazepine binding sites. Initiation of GABA-induced uncoupling is prevented by co-incubation of GABA with high concentrations of sucrose suggesting that is dependent on a receptor internalization step. Moreover, results from immunocytochemical and biochemical experiments indicate that GABA exposure causes an increase in $GABA_A$ receptor endocytosis. Together, these data suggest that the uncoupling mechanism involves an initial increase in receptor internalization followed by activation of a signaling cascade that leads to selective changes in receptor subunit levels. These changes might result in the assembly of receptors with altered subunit compositions that display a lower degree of coupling

between GABA and benzodiazepine sites. Uncoupling might represent a homeostatic mechanism that negatively regulates GABAergic transmission under physiological conditions in which synaptic $GABA_A$ receptors are transiently activated for several minutes.

Keywords: $GABA_A$ receptors, GABA, benzodiazepine, uncoupling, endocytosis.

1.1 Introduction

Brain function depends on a balance between excitatory and inhibitory neurotransmission. Alterations in the function of $GABA_A$ receptors, which mediate the main inhibitory transmission of the central nervous system, are relevant for neuropsychiatric disorders such as schizophrenia, anxiety, epilepsy and autism (for review see (Mohler, 2006, Charych et al., 2009, Hines et al., 2011)). The importance of $GABA_A$ receptors in normal brain function, in pathological conditions and as targets of clinically relevant drugs has attracted interest to the regulatory mechanisms that control the activity of these receptors.

Several lines of evidence suggest the existence of homeostatic mechanisms that control synaptic inhibition via changes in the number and/or function of post-synaptic $GABA_A$ receptors. Neuronal activity regulates $GABA_A$ receptor ubiquitylation and degradation by the ubiquitin-proteosome system in the endoplasmic reticulum, which leads to alterations in cell surface receptor expression. Chronically increased neuronal activity produces a decrease in the level of $GABA_A$ receptor ubiquitylation, whereas persistent inhibition of neuronal activity induces an increase in the level of receptor ubiquitylation (Saliba et al., 2007). Neuronal activity also alters the diffusion properties of $GABA_A$ receptors. Enhanced excitatory activity induces an increase in the receptor diffusion coefficient and a decrease in synaptic confinement, leading to a reduction in synaptic GABA responses (Bannai et al., 2009). On the other hand, a recent report indicates that rapid modulations of $GABA_A$ receptor insertion in the plasma membrane are induced by acute changes in neuronal activity (Saliba et al., 2012). This regulatory process is mediated by Ca^{2+} influx through L-type voltage-gated calcium channels (VGCC) and the phosphorylation of the $GABA_A$ receptor β3 subunit by CaMKII.

Different reports have demonstrated that chronic exposure of $GABA_A$ receptors to endogenous allopregnanolone during the estrous cycle and pregnancy induces tolerance that is accompanied by changes in receptor subunit expression (Turkmen et al., 2011). Chronic *in vivo* administration of $GABA_A$ receptor positive allosteric modulators in rodents has been reported to produce adaptive alterations in receptor function. Persistent ethanol consumption alters the functional properties of $GABA_A$ receptors via changes in receptor expression, subcellular and synaptic localization and post-translational modifications (for review see (Kumar et al., 2004)). Chronic administration of benzodiazepines induces tolerance and subunit-selective changes in $GABA_A$ receptor subunit levels (Chen et al., 1999, Tietz et al., 1999a, Tietz et al., 1999b).

In rats, a single dose of diazepam produces a decrease in GABA potentiation of benzodiazepine binding in the cortex and cerebellum 4 h later, and this process is referred to as uncoupling (Holt et al., 1999). These results suggest that uncoupling between GABA and benzodiazepine binding sites can occur *in vivo* under pharmacologically relevant conditions.

Several lines of evidence indicate that synaptic $GABA_A$ receptors can be persistently activated by GABA. GABA released as a consequence of a single action potential reaches concentrations of 1-5 mM in the synaptic cleft and is then cleared biphasically with time constants of 100 μs and 2 ms (Clements, 1996). However, GABAergic neurons are fast-spiking neurons that exhibit basal firing rates of 5-50 Hz (equivalent to one action potential every 200 and 20 ms, respectively) (Mountcastle et al., 1969, Hajos et al., 2004) in the neocortex that can last for many minutes (Czurko et al., 1999). Moreover, activation of $GABA_A$ receptors far outlasts the presence of free GABA because the GABA response decays with time constants of 50 and 171 ms

(Jones and Westbrook, 1995, Bianchi and Macdonald, 2001). In conclusion, the persistent activation of a substantial fraction (approximately 30 %) of post-synaptic $GABA_A$ receptors over several minutes by millimolar concentrations of GABA can occur *in vivo* under physiological conditions, which suggests the existence of homeostatic mechanisms that negatively regulate GABA responses.

Studies performed using cultured neurons have shown that prolonged activation of $GABA_A$ receptors induces changes in receptor function. Chronic exposure of brain neurons to GABA produces down-regulation of receptor number and uncoupling of GABA/benzodiazepine site interactions with a t½ of 24-25 h (Roca et al., 1990). GABA-induced $GABA_A$ receptor down-regulation and uncoupling are produced by activation of distinct signal transduction pathways. Down-regulation is mediated by transcriptional repression of $GABA_A$ receptor subunit genes and depends on the activation of L-type VGCC, whereas uncoupling is independent of channel activation (Lyons et al., 2000, Russek et al., 2000, Lyons et al., 2001). Exposure of neurons to benzodiazepines, barbiturates and neurosteroids causes uncoupling without altering receptor number, further supporting the hypothesis that down-regulation and uncoupling are produced by different mechanisms (Friedman et al., 1996). The mechanism of uncoupling has not yet been elucidated.

We have previously reported that a single brief exposure of neocortical neurons to 1 mM GABA for 5-10 minutes (t½ 3 min) initiates a process that results in the uncoupling of the allosteric site interactions 24-48 h later (t½ 12 h). This phenomenon occurs in the absence of receptor down-regulation, which provides a paradigm to selectively investigate the uncoupling mechanism (Gravielle et al., 2005). GABA induces uncoupling in a concentration-dependent manner with a half-maximal concentration of 460 μM. Initiation of uncoupling is prevented by co-incubation of

GABA with picrotoxin but is insensitive to nifedipine suggesting that uncoupling is dependent on $GABA_A$ receptor activation but independent of L-type VGCC activation. Additionally, uncoupling is accompanied by a selective decrease in steady state levels of $GABA_A$ receptor subunits. The aim of this work was to investigate the molecular mechanism underlying GABA-induced uncoupling. Here, we show that uncoupling is associated with a change in $GABA_A$ receptor subunit composition that is mediated by a mechanism that involves a receptor internalization step.

1.2 Experimental procedures

Cell Cultures

Primary cultures were prepared from 18 day-old rat embryos (Sprague-Dawley) as previously described (Gravielle et al., 2005). Briefly, whole brains were removed and cerebral cortices were dissected under a microscope and placed in ice-cold Hanks´ solution. Tissue was minced with a small pair of scissors, triturated with a serological pipette and centrifuged for 5 min at 500 *g*. The resulting pellet was resuspended in 5 ml of plating medium (Neurobasal™ medium plus 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine, Invitrogen) and triturated again with a serological pipette. The cell suspension was added to a final volume of plating medium and plated at a density of ¾ cortices per 100 mm culture dish coated with poly-L-lysine (0.1 mg/ml, Invitrogen). For immunocytochemical studies, neurons were plated onto 12 mm coverslips (Marienfeld GmbH) coated with poly-L-lysine at a density of 12,500 cells/cm^2. Cultures were

incubated at 37˚C in 5% CO_2, and after 1 h, the medium was aspirated and replaced with serum-free medium containing B27 serum-free supplement.

Drug treatments

On day 7, 100 mm dishes containing 14 ml of medium were treated as follows: 7 ml of medium (conditioned medium) was removed and kept in the incubator and 70 µl of concentrated GABA (final concentration of 1 mM), picrotoxin (final concentration of 100 µM), GABA plus picrotoxin or vehicle was added to each dish. GABA and picrotoxin stocks were prepared in Hanks´ solution. Dishes were returned to the incubator and incubated for 10 min. For experiments examining the effects of sucrose, neurons were preincubated for 10 min with sucrose (0.35 M) and then GABA (final concentration 1 mM) was added and co-incubated for 10 min. Cultures were washed twice with 5 ml of warm Hanks´ solution, and 7 ml of conditioned medium was re-added to the cultures. Dishes were kept in the incubator for 48 h. GABA treatments for the experiments measuring receptor internalization are described in the sections entitled *Immunocytochemistry to study receptor internalization* and *Biotinylation to study receptor internalization*.

Western blot assay to measure total subunit proteins

Cells were washed twice with 5 ml of ice-cold phosphate buffered saline (PBS), scraped from the dishes and centrifuged at 500 *g* for 5 min. The pellet was homogenized with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics) and incubated at 4˚C for 20

min with rotation. The lysates were centrifuged for 5 min at 2,000 *g*. Proteins from the supernatant (approximately 40 μg) were separated on 10% acrylamide gels and transferred to a nitrocellulose membrane. The blots were blocked for 2 h with 5% nonfat dry milk in 20 mMtris buffered saline (TBS) buffer containing 0.1% Tween-20. Blots were incubated with antibodies against α1 (1:1,000 dilution, Millipore), α3 (1:1,000 dilution, Millipore), γ2 (1:1,000 dilution, Alpha Diagnostic) or β2/3 subunits (1:1,000 dilution, Chemicon) overnight at 4˚C in blocking solution. The protein subunits were detected by incubation with secondary horseradish peroxidase (HRP)-conjugated antibodies (1:2,000 dilution, Santa Cruz) for 1.5 h at room temperature followed by enhanced chemiluminescence (ECL detection kit, Pierce). The blots were stripped and reprobed by incubation with an anti-actin antibody (1:500 dilution, Sigma Aldrich) overnight at 4°C, followed by incubation with a secondary HRP-conjugated antibody (1:2,000, Santa Cruz) for 1.5 h at room temperature. The subunits signals were normalized to that of the actin to control for loading amount variability. Densitometry was performed with the NIH Image J program.

Receptor immunoprecipitation

Cells were collected with 5 ml of ice-cold PBS as described above and centrifuged at 500 *g* for 5 min. To extract $GABA_A$ receptors, the pellet was homogenized as previously described (Jechlinger et al., 1998) in deoxycholate buffer containing 10 mMTris-HCl pH 8.5, 150 mMNaCl, 0.5% sodium deoxycholate, 0.05% phosphatidylcholine, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated in a rotator shaker for 20 min at 4˚C. The lysate was centrifuged at 27,000 *g* for 30 min and supernatants were diluted to 1 mg/ml protein with PBS buffer. The cell

lysate was pre-cleared by addition of 20 µl protein A agarose beads (Santa Cruz) to 500 µl of cell lysate and incubated at 4˚C for 20 min with rotation. The lysates were centrifuged for 5 min at 2,000 *g* and pellets were discarded. Aliquots of supernatants (total lysate) were collected to measure total receptor levels (protein input). The remaining supernatants were incubated with 2 µg rabbit anti-γ2 antibody (Alpha Diagnostic) overnight at 4˚C in a rotator shaker. Negative controls were carried out by incubating the reaction mixture with non-immune IgG. Immunocomplexes were captured by the addition of 20 µl of protein A agarose beads and incubation overnight at 4˚C in the shaker. The agarose beads were collected by centrifugation (5 min at 2,000 *g*) and pellets were washed 3 times by resuspension in ice-cold deoxycholate buffer followed by centrifugation. The final pellets were resuspended in 30 µl of denaturing sample buffer. Proteins were analyzed by western blot as described above. Blots were incubated with goat antibodies against the α1, α3 or α5 subunits (1:200 dilution, Santa Cruz) overnight at 4˚C. The protein subunits were detected by incubation with anti-goat HRP-conjugated antibody (1:2,000 dilution, Santa Cruz) for 1.5 h at room temperature. The blots were stripped and reprobed by incubation with an anti-rabbit HRP-conjugated antibody (1:2,000 dilution, Santa Cruz) for 1.5 h at room temperature to measure the amount of IgG; i.e., the antibody used to immunoprecipitate the $GABA_A$ receptors. The subunits signals were normalized to that of the rabbit IgG to control for loading amount variability. Protein input was measured by incubation with a rabbit antibody against γ2 subunit (1:1,000 dilution, Alpha Diagnostic) overnight at 4˚C and a secondary anti-rabbit HRP-conjugated antibody (1:2,000 dilution, Santa Cruz) for 1.5 h at room temperature.

Biotinylation assay to study cell surface receptors

Cells were place on ice and washed with 5 ml of ice-cold PBS containing 1.5 mM $MgCl_2$ and 0.2 mM $CaCl_2$ (PBS/Ca^{2+}/Mg^{2+}). Cells were then incubated for 20 min with 1 mg/ml of sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS/Ca^{2+}/Mg^{2+} with gentle shaking. Biotin solution was removed and cultures were washed twice for 15 min with 100 mM glycine in PBS/$Ca^{2+/}$ Mg^{2+} to quench the unbound biotin. After two washes with 5 ml of PBS/Ca^{2+}/Mg^{2+}, cells were lysed with ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics). Homogenates were incubated for 30 min at 4˚C and centrifuged for 30 min at 27,000 *g*. An aliquot of supernatant (total lysate) was collected to measure the levels of subunits and actin. Biotinylated proteins were captured from an aliquot of the remaining supernatant containing 500 µg of protein by incubation with 150 µl of Neutravidin agarose beads (Thermo Scientific) overnight at 4˚C in a rotator shaker. Beads were precipitated by centrifugation at 2,000 *g* for 5 min and washed three times with RIPA lysis buffer containing 500 mM of NaCl. The final pellet was resuspended in 35 µl of sample buffer. Immunoblot experiments were carried out as described above. Surface subunits were detected by incubation with antibodies anti α1 (1:1000 dilution, Millipore), α3 (1:1000 dilution, Millipore) or β2/3 subunits (1:1,000 dilution, Chemicon) overnight at 4°C. The subunit signals were detected by incubation with a secondary HRP-conjugated antibody (1:2,000 dilution, Santa Cruz). To confirm the absence of biotinylation of intracellular proteins, blots were stripped and reprobed by incubation with an anti-actin antibody (1:500 dilution, Sigma Aldrich) overnight at 4 °C followed by incubation with a secondary HRP-conjugated antibody (1:2,000 dilution, Santa Cruz) for 1.5 h at room

temperature. Surface subunit expression was normalized to the actin levels from the total lysates.

Immunocytochemistry to measure surface receptor number

Cultured neurons were fixed for 20 min at room temperature under nonpermeabilizing conditions (4% paraformaldehyde in PBS containing 4% sucrose). The cells were then washed with PBS, blocked for 2 h with 10% bovine serum albumin (BSA) in PBS. Cell surface receptors were labeled by incubation with a mouse monoclonal antibody against the extracellular region of the $GABA_A$ receptor β2/3 subunit (1:50 dilution in conditioned medium, Chemicon) for 60 min at room temperature. Control cultures for background measurement were not incubated with the primary antibody. Cultures were then washed with PBS and incubated for 60 min with a secondary Cy3-conjugated antibody (1:100 dilution in PBS blocking solution, Jackson). Images were captured with a Nikon microscope (ECLIPSE 50i) with a 40 X objective. All specimens were imaged under identical conditions. Analyses of images were performed with the NIH Image J program. Surface fluorescence was quantified after the determination of a threshold and the subtraction of the background. Signals were normalized to the total area of a selected cell. Data were analyzed from 15-18 fields for each condition (1-3 neurons/field). Three independent cultures were studied.

Binding assay

Cells were collected with PBS as described above and centrifuged at 500 *g* for 5 min. The pellet was homogenized in 1 mM EDTA/1 mM PMSF (1 ml per dish) with

12 strokes using a glass Dounce homogenizer and dialyzed against 4 X 4 l of potassium phosphate buffer (pH 7.4) overnight at 4°C.

Aliquots of homogenates (75-100 µg protein) were incubated at a final volume of 0.5 mL for 60 min at 0 °C with 0.5 nM [^{3}H]flunitrazepam ([^{3}H]FNZ) alone or in the presence of 1 mM GABA. Nonspecific binding was determined in the presence of 100 µM diazepam and subtracted from total binding to yield specific binding. The reaction was stopped by the addition of 5 ml ice-cold PBS and the aliquots were immediately vacuum filtered through glass fiber filters (Whatman GF/B). Filters were washed 3 times with 5 ml ice-cold PBS. Radioactivity retained on the filters was quantified with liquid scintillation spectrometry. Coupling represents the potentiation of [^{3}H]FNZ binding by GABA and was estimated as follows: (% potentiation $_{treated}$/% potentiation $_{control}$) X 100. Uncoupling was defined as the decrease in GABA-potentiated [^{3}H]FNZ binding and calculated as follows: [1-(% potentiation $_{treated}$/% potentiation $_{control}$)] X 100.

Immunocytochemistry to study receptor internalization

On day 7, the medium from the culture dishes was removed and kept in the incubator. Cell surface receptors were tagged in living cultured neurons by incubation with a mouse monoclonal antibody against the extracellular region of the $GABA_A$ receptor β2/3 subunit (1:50 dilution in conditioned medium, Chemicon) for 20 min at room temperature. Coverslips were washed with Hanks' solution, and conditioned medium was added. Cells were treated with 1 mM GABA or vehicle for 10 min. After incubation, cultures were washed twice with Hanks' solution, and conditioned medium was added. Cultures were returned to the incubator, and, after 20 min, the cells were fixed for 20 min at room temperature under nonpermeabilizing conditions (4%

paraformaldehyde in PBS containing 4% sucrose). Control cultures for background measurement were fixed immediately after the incubation with the primary antibody. The cells were then washed with PBS, blocked for 2 h with 10% bovine serum albumin (BSA) in PBS and incubated for 60 min with a secondary Cy5-conjugated antibody (1:250 dilution in PBS blocking solution, Jackson) for detection of cell surface $GABA_A$ receptors. Cells were permeabilized with 0.3% Triton X-100 for 15 min and incubated for 2 h with a secondary Alexa 488-conjugated antibody (1:250 dilution in PBS blocking solution, Molecular Probes) for detection of internalized $GABA_A$ receptors. After washing, coverslips were mounted in Mowiol mounting medium (AppliChem).

Images were captured with a confocal microscope (Olympus FV300) with a 60 X oil-immersion objective. All specimens were imaged under identical conditions. Analyses of images were performed with the NIH Image J program. Surface and internalized fluorescences were calculated as integrated densities after the determination of a threshold intensity and the subtraction of the background. To measure receptor internalization, the internalized signal was normalized to the total signal. Data were analyzed from 17-19 fields for each condition (1-3 neurons/field). Four independent cultures were studied.

Biotinylation assay to study receptor internalization

On day 7, the conditioned medium from cultures was removed and kept in the incubator. Dishes were placed on ice and washed with 5 ml of ice-cold PBS/Ca^{2+}/Mg^{2+}. Neurons were then incubated with 1 mg/ml of sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS/Ca^{2+}/Mg^{2+} for 20 min with gentle shaking. Cultures were washed twice with 100 mM glycine in PBS/Ca^{2+}/Mg^{2+} for 15 min to quench unbound biotin.

Control dishes used to measure background internalization were kept on ice. The rest of the dishes were incubated with conditioned medium containing 1 mM GABA, 100 μM picrotoxin, GABA plus picrotoxin or vehicle for 10 min in the presence of 100 μg/ml leupeptin (a lysosomal inhibitor) at 37°C. After incubation, cultures were washed twice with Hanks' solution, the conditioned medium was re-added and the dishes were returned to the incubator for 20 min. Cultures were placed on ice, and all dishes were incubated twice with glutathione buffer (50 mM glutathione, 75 mM NaOH, 75 mM NaCl, 1 mM EDTA and 1% BSA) for 15 min to cleave biotin from cell surface receptors. All samples were washed twice with PBS/Ca^{2+}/Mg^{2+} and lysed with ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics). Homogenates were incubated for 60 min at 4˚C with rotation and centrifuged for 30 min at 27,000 *g*. An aliquot of supernatant (total lysate) was collected to measure total receptor levels. Biotinylated proteins were captured from an aliquot of the remaining supernatant containing 500 μg of protein by incubation with 150 μl of Neutravidin agarose beads (Thermo Scientific) overnight at 4˚C in a rotator shaker. Beads were precipitated by centrifugation at 2,000 *g* for 5 min and washed three times with RIPA buffer containing 500 mM NaCl. The final pellets were resuspended in 35 μl of sample buffer. Immunoblot experiments were carried out as described above. Receptor subunits were detected by incubation with a mouse anti β2/3 antibody (1:500, Chemicon), or a rabbit anti α3 antibody (1:500, Millipore), overnight at 4°C and a secondary HRP-conjugated antibody (1:2,000 dilution, Santa Cruz) for 1.5 h at RT. The internalized subunit signals were normalized to the total receptor signal. To confirm the absence of biotinylation of intracellular proteins, blots were stripped and reprobed with an anti-actin antibody as described above.

1.3 Results

Chronic exposure of cerebral cultures to GABA for 48 h induces uncoupling of binding sites for GABA and benzodiazepines and down-regulation of $GABA_A$ receptor number (Roca et al., 1990). In a previous report (Gravielle et al., 2005), we demonstrated that a brief $GABA_A$ receptor activation (t½ 3.2 min) by GABA induces an uncoupling between GABA and benzodiazepine recognition sites hours later (t½ 12 h) without changing total receptor number.

Although the brief exposure to GABA does not induce a change in receptor number, previous results from real-time PCR experiments (Gravielle et al., 2005) have demonstrated a reduction in the mRNA levels of certain subunits (α1, α3 and β1-3) with no changes in the α2, α4, α5, γ1 or γ2 subunits. Because the degree of coupling between binding sites for GABA and benzodiazepines depends on the subtype of α subunit present in the $GABA_A$ receptor complex (α3> α1/ α5 (Puia et al., 1991, Wafford et al., 1993, Smith et al., 2001)), it is possible that short duration exposure of neurons to GABA produces alterations in the α subunit assembly of receptors. To test this hypothesis, we first verified that the reported reduction in the mRNA levels of the receptor subunits is associated with a concomitant decrease in protein levels (Fig. 1). Results from western blots assays indicated that a 10 min exposure of primary neocortical cultures to 1 mM GABA followed by a 48 h incubation period induced a significant decrease in the α1, α3, β2-3 subunit protein levels. These decreases were prevented by co-incubation with picrotoxin, suggesting that are mediated by $GABA_A$ receptor activation. Our results also indicated that GABA treatment failed to alter γ2 subunit levels.

To directly study GABA-induced changes in the percentage of receptors containing α1, α3 or α5 subunits, we performed immunoprecipitation experiments using an antibody against γ2, a subunit that is present in most of the receptors and is not down-regulated by GABA treatment, followed by western blot assays (Fig. 2). We observed a significant decrease in α3 and no changes in α1 or α5 immunoreactivities, suggesting that the percentage of α3γ2-containing receptors was reduced. These data suggest that uncoupling is accompanied by an alteration in the receptor subunit combination that changes coupling efficiency.

We next asked whether brief $GABA_A$ receptor activation induces a change in the subunit composition of receptors located in the plasma membrane. To this end, we studied the effect of GABA on the cell surface expression of α3 subunits. Cultures were treated with 1 mM GABA for 10 min, washed and incubated for 48 h. At the end of this treatment, surface proteins were labeled with biotin and precipitated with Neutravidin beads. Surface and total proteins were separated by gel electrophoresis and probed with an antibody against α3 subunits (Fig. 3A and B). In agreement with the reduction in the percentage of α3-containing receptors, results from these experiments showed that GABA induced a decrease in the surface levels of α3 subunits. In addition, we analyzed the effect of GABA treatment on the surface expression of α1 and β2/3 subunits. Results from these experiments (Fig. 3 A and B) indicated that GABA failed to induce a change in the surface levels of these subunits. The lack of alterations in the surface expression of β2/3 subunits, which are present at most of the $GABA_A$ receptors, suggest that GABA exposure did not induce a change in the number of cell surface receptors. These results were confirmed by immunocytochemical experiments (Fig. 3C y D) that showed that GABA did not produce changes in the surface expression of β2/3 subunits.

Several reports suggest that uncoupling is mediated by an increase in $GABA_A$ receptor endocytosis (Tehrani and Barnes, 1997, Ali and Olsen, 2001). To study whether the GABA-induced uncoupling process is initiated by an increase in $GABA_A$ receptor internalization, we studied the effect of inhibiting clathrin-mediated receptor endocytosis with highly hypertonic concentrations of sucrose (Heuser and Anderson, 1989) (Fig. 4). The uncoupling produced by a brief exposure to GABA was blocked by co-incubation with 350 mM sucrose suggesting that this regulatory process depends on a receptor endocytosis step.

To further investigate whether the mechanism of GABA-induced uncoupling is initiated by a receptor internalization process, we used two independent approaches. The first approach was based on immunofluorescent labeling of receptors (Fig. 5A and B). Live cultures were incubated with an antibody against an extracellular epitope of β2/3 subunits because these subunits are present in most of $GABA_A$ receptors and interact with the clathrin/activator protein 2 (AP2) (Kittler et al., 2005). Cells were washed and exposed to vehicle or 1 mM GABA for 10 min at 37°C. After this incubation, cultures were washed and returned to the incubator for 20 min to allow maximal internalization to occur (Kittler et al., 2004). $GABA_A$ receptors that remained on the cell surface were visualized after fixation with a fluorescent secondary antibody, and internalized receptors were subsequently detected after membrane permeabilization using a secondary antibody coupled to a different fluorochrome. Receptor internalization was quantified as the fluorescence intensity of the internalized receptors normalized to that of the total receptor population. Internalized $GABA_A$ receptor immunoreactivity was almost undetectable in untreated cultures. The brief GABA treatment induced a significant stimulation of receptor endocytosis (an approximately 2-

fold increase). The immunoreactivity from $GABA_A$ receptors remaining on the cell surface did not significantly change ($p<0.05$).

The second approach with which we investigated the effect of GABA exposure on receptor internalization employed biotinylation assays followed by western blots (Fig. 5C and D). Surface proteins were biotinylated, and cultures were exposed to vehicle (control) or 1 mM GABA for 10 min at 37°C, washed and incubated for another 20 min at 37°C to achieve maximal receptor internalization. The biotin on the cell surface proteins was cleaved with reduced glutathione, and the remaining biotinylated proteins were precipitated with Neutravidin beads. Western blots were performed on total and biotinylated proteins with antibodies against β2/3 and α3 subunits. GABA induced a significant increase in receptor endocytosis compared to control conditions. Taken together, the results of both approaches suggest that stimulation of receptor internalization is part of the uncoupling mechanism.

1.4 Discussion

Long-term potentiation and long-term depression of GABAergic synapses have been described both in the neonatal and adult brain (Gaiarsa et al., 2002). Experiments using early postnatal cortical neurons in culture indicate that exposure to allopregnanolone, a positive endogenous modulator of $GABA_A$ receptors, augments the rate of the developmental switchover of $GABA_A$ receptor subunits (Poulter et al., 1997). These forms of activity-dependent plasticity might have a role in the development and function of neuronal networks (Ben-Ari et al., 2007, Farrant and Kaila, 2007).

Chronic treatment of primary neuronal cultures with GABA induces down-regulation of receptor number and decreases in the potentiation of benzodiazepine

binding by GABA in a process termed uncoupling (Roca et al., 1990). We have previously reported that brief $GABA_A$ receptor activation by GABA for 5-10 min ($t½$ = 3.2 min) is sufficient to produce uncoupling 24-48 h later ($t½$ = 12 h) (Gravielle et al., 2005). This uncoupling process occurs in the absence of alterations in total receptor number, which provides a paradigm that allowed us to study the uncoupling mechanism exclusively. As already mentioned, persistent activation of $GABA_A$ receptors by high GABA concentrations occurs under physiological conditions as GABAergic neurons in neocortex exhibit high spiking rates (5-50 Hz) (Mountcastle et al., 1969, Hajos et al., 2004) and GABA currents have a relatively slow decay (time constants of 50 and 171 ms) (Jones and Westbrook, 1995). However, it is important to point out that our experimental conditions, 10 min exposure of cultured neurons to 1 mM GABA, do not completely reproduce the physiological situation since only a fraction of $GABA_A$ receptors at synapses (approximately 30 %) is continuously activated (Jones and Westbrook, 1995). Thus, uncoupling may be relevant for this fraction of receptors that remains persistently bound to the neurotransmitter.

We previously demonstrated that brief GABA exposure produces a decrease in the mRNA levels of certain receptor subunits. The present results indicate that these reductions are accompanied by corresponding changes in α1, α3 and β2-3 subunit protein levels, but γ2 subunit levels are not altered. These changes are blocked by picrotoxin, a non-competitive $GABA_A$ receptor antagonist, suggesting that are dependent on receptor activation. Our receptor immunoprecipitation experiments suggest that uncoupling is associated with a decrease in the percentage of α3-containing receptors. This observation is consistent with reports showing the strengths of allosteric interactions between the GABA and benzodiazepine binding sites of $GABA_A$ receptors vary based on the subtypes of α subunit in the following rank order: α3> α1/5 (Puia et

al., 1991, Wafford et al., 1993, Smith et al., 2001). Therefore, uncoupling might be the consequence of alterations in receptor subunit composition that results in receptors with lower degrees of coupling. The decrease in the proportion of α3-containing $GABA_A$ receptors was further confirmed by the GABA-induced reduction in cell surface expression of α3, which suggests that uncoupling is accompanied by changes in the subunit compositions of the receptors located at the plasma membrane. We failed to detect changes in the surface expression of α1 or β2/3 subunits (Fig 3) supporting our hypothesis that GABA treatment specifically induces a reduction in the percentage of α3-containing receptors.

The absence of alterations in the number of receptors containing α1 subunits is consistent with our previous reports that show that uncoupling is not accompanied by a change in zolpidem affinity; zolpidem is a compound that exhibits selectivity for α1-containing receptors (Gravielle et al., 2005). The lack of an exact correspondence between changes in subunit levels and alterations in receptor subunit compositions could be due to the synthesis of a vast excess of subunits. It has been reported that only 20% of subunits are assembled into $GABA_A$ receptors (Baumgartner et al., 1994, Miranda and Barnes, 1997). Indeed, Mehta and Ticku (Mehta and Ticku, 1999) used immunoprecipitation and binding assays to demonstrate that the ethanol-induced decrease in α1 subunit expression in the rat cerebellum and cerebral cortex is not associated with a reduction in the number of $GABA_A$ receptors containing this subunit.

Although brief GABA treatments do not induce changes in <u>total</u> receptor number, we failed to detect increases in any receptor subpopulation that would counterbalance the reduction in the percentage of α3-containing receptors. It is possible that this decrease is compensated for by small undetectable changes in the amount of varied alternative receptor subtypes. The results presented in Figure 2 support this

possibility by showing a small increase in the number of α5-containing receptors that was not statistically significant.

Results from biotinlylation and immunocytochemical experiments (Fig 3) indicate that GABA exposure does not induce alterations in the cell surface expression of β2/3 subunits, present in most of the $GABA_A$ receptors, suggesting that uncoupling is not accompanied by a change in the number of surface receptors.

Several reports suggest that $GABA_A$ receptor uncoupling is mediated by receptor sequestration in intracellular vesicles. Chronic *in vivo* treatment of mice with benzodiazepines has been shown to induce increases in the maximal binding of benzodiazepines in clathrin-coated vesicle fractions (Tehrani and Barnes, 1997). In addition, experiments using an insect cell line suggest that the uncoupling mechanism induced by benzodiazepine exposure involves a receptor internalization process (Ali and Olsen, 2001). It has been hypothesized that internalized receptors may provide the signal for subsequent regulation of $GABA_A$ receptor subunit expression (Barnes, 2000, Bateson, 2002). As a first step to investigate the intracellular signaling pathway that mediates GABA-induced uncoupling in neurons, we asked whether uncoupling depends on an initial increase in receptor internalization. To do this, we studied the effect of high sucrose concentrations that inhibit receptor endocytosis (Heuser and Anderson, 1989). We found that uncoupling was prevented by the co-incubation of GABA with the sucrose solution, suggesting that this process is initiated by a receptor internalization step.

We next studied the effect of $GABA_A$ receptor activation on receptor endocytosis with immunocytochemical and biotinylation assays. Results from these experiments showed that a 10 min exposure to GABA induced an increase in the percentage of internalized receptors, which suggests that the first step of the uncoupling

mechanism involves the stimulation of receptor endocytosis. The GABA-induced receptor internalization is prevented in the presence of picrotoxin, suggesting that this process is mediated by $GABA_A$ receptor activation. A previous report indicated that the surface expression of $GABA_A$ receptors in the hippocampus is not regulated by GABA binding (Goodkin et al., 2008); however, that study analyzed a lower neurotransmitter concentration (100 μM). Therefore, these differences in GABA concentrations and the brain area studied may explain this discrepancy in the results.

1.5 Conclusion

In summary, we demonstrated that uncoupling induced by a brief exposure of cortical neurons to GABA is associated with a decrease in the percentage of α3-containing $GABA_A$ receptors and is dependent on a receptor internalization step. It is possible that brief $GABA_A$ receptor activation induces an increase in receptor endocytosis followed by activation of a signaling cascade that leads to selective changes in receptor subunit transcription. These changes might result in the assembly of receptors with altered subunit compositions that display lower degrees of coupling between the GABA and benzodiazepine sites.

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Disclosures:

There are no known conflicts of interest.

References

Ali NJ, Olsen RW (2001) Chronic benzodiazepine treatment of cells expressing recombinant GABA(A) receptors uncouples allosteric binding: studies on possible mechanisms. J Neurochem 79:1100-1108.

Bannai H, Levi S, Schweizer C, Inoue T, Launey T, Racine V, Sibarita JB, Mikoshiba K, Triller A (2009) Activity-dependent tuning of inhibitory neurotransmission based on GABAAR diffusion dynamics. Neuron 62:670-682.

Barnes J, Eugene M. (2000) Intracellular trafficking of GABAA receptors. Life Sciences 66:1063-1070.

Bateson AN (2002) Basic Pharmacologic Mechanisms Involved in Benzodiazepine Tolerance and Withdrawal. Curr Pharmacol Des 8:5-21.

Baumgartner BJ, Harvey RJ, Darlison MG, Barnes EM, Jr. (1994) Developmental up-regulation and agonist-dependent down-regulation of GABAA receptor subunit mRNAs in chick cortical neurons. Brain Res Mol Brain Res 26:9-17.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215-1284.

Bianchi MT, Macdonald RL (2001) Agonist Trapping by GABAA Receptor Channels. J Neurosci 21:9083-9091.

Clements JD (1996) Transmitter timecourse in the synaptic cleft: its role in central synaptic function. Trends Neurosci 19:163-171.

Czurko A, Hirase H, Csicsvari J, Buzsaki G (1999) Sustained activation of hippocampal pyramidal cells by 'space clamping' in a running wheel. Eur J Neurosci 11:344-352.

Charych EI, Liu F, Moss SJ, Brandon NJ (2009) GABA(A) receptors and their associated proteins: implications in the etiology and treatment of schizophrenia and related disorders. Neuropharmacology 57:481-495.

Chen S, Huang X, Zeng XJ, Sieghart W, Tietz EI (1999) Benzodiazepine-mediated regulation of alpha1, alpha2, beta1-3 and gamma2 GABA(A) receptor subunit proteins in the rat brain hippocampus and cortex. Neuroscience 93:33-44.

Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of GABA(A) receptor signalling. Prog Brain Res 160:59-87.

Friedman LK, Gibbs TT, Farb DH (1996) gamma-aminobutyric acidA receptor regulation: heterologous uncoupling of modulatory site interactions induced by chronic steroid, barbiturate, benzodiazepine, or GABA treatment in culture. Brain Res 707:100-109.

Gaiarsa JL, Caillard O, Ben-Ari Y (2002) Long-term plasticity at GABAergic and glycinergic synapses: mechanisms and functional significance. Trends Neurosci 25:564-570.

Goodkin HP, Joshi S, Mtchedlishvili Z, Brar J, Kapur J (2008) Subunit-specific trafficking of GABA(A) receptors during status epilepticus. In: J Neurosci, vol. 28, pp 2527-2538 United States.

Gravielle MC, Faris R, Russek SJ, Farb DH (2005) GABA Induces Activity Dependent Delayed-onset Uncoupling of GABA/Benzodiazepine Site Interactions in Neocortical Neurons. J Biol Chem 280:20954-20960.

Hajos N, Palhalmi J, Mann EO, Nemeth B, Paulsen O, Freund TF (2004) Spike timing of distinct types of GABAergic interneuron during hippocampal gamma oscillations in vitro. J Neurosci 24:9127-9137.

Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. The Journal of Cell Biology 108:389-400.

Hines RM, Davies PA, Moss SJ, Maguire J (2011) Functional regulation of GABA(A) receptors in nervous system pathologies. Curr Opin Neurobiol.

Holt RA, Bateson AN, Martin IL (1999) Decreased GABA enhancement of benzodiazepine binding after a single dose of diazepam. J Neurochem 72:2219-2222.

Jechlinger M, Pelz R, Tretter V, Klausberger T, Sieghart W (1998) Subunit composition and quantitative importance of hetero-oligomeric receptors: GABAA receptors containing alpha6 subunits. J Neurosci 18:2449-2457.

Jones MV, Westbrook GL (1995) Desensitized states prolong GABAA channel responses to brief agonist pulses. Neuron 15:181-191.

Kittler JT, Chen G, Honing S, Bogdanov Y, McAinsh K, Arancibia-Carcamo IL, Jovanovic JN, Pangalos MN, Haucke V, Yan Z, Moss SJ (2005) Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. Proc Natl Acad Sci U S A 102:14871-14876.

Kittler JT, Thomas P, Tretter V, Bogdanov YD, Haucke V, Smart TG, Moss SJ (2004) Huntingtin-associated protein 1 regulates inhibitory synaptic transmission by modulating gamma-aminobutyric acid type A receptor membrane trafficking. Proc Natl Acad Sci U S A 101:12736-12741.

Kumar S, Fleming RL, Morrow AL (2004) Ethanol regulation of gamma-aminobutyric acid A receptors: genomic and nongenomic mechanisms. Pharmacol Ther 101:211-226.

Lyons HR, Gibbs TT, Farb DH (2000) Turnover and down-regulation of GABA(A) receptor alpha1, beta2S, and gamma1 subunit mRNAs by neurons in culture. J Neurochem 74:1041-1048.

Lyons HR, Land MB, Gibbs TT, Farb DH (2001) Distinct signal transduction pathways for GABA-induced GABA(A) receptor down-regulation and uncoupling in neuronal culture: a role for voltage-gated calcium channels. J Neurochem 78:1114-1126.

Mehta AK, Ticku MK (1999) Prevalence of the GABAA receptor assemblies containing alpha1-subunit in the rat cerebellum and cerebral cortex as determined by immunoprecipitation: lack of modulation by chronic ethanol administration. Brain Res Mol Brain Res 67:194-199.

Miranda JD, Barnes EM, Jr. (1997) Repression of gamma-aminobutyric acid type A receptor alpha1 polypeptide biosynthesis requires chronic agonist exposure. J Biol Chem 272:16288-16294.

Mohler H (2006) GABAA receptors in central nervous system disease: anxiety, epilepsy, and insomnia. J Recept Signal Transduct Res 26:731-740.

Mountcastle VB, Talbot WH, Sakata H, Hyvarinen J (1969) Cortical neuronal mechanisms in flutter-vibration studied in unanesthetized monkeys. Neuronal periodicity and frequency discrimination. J Neurophysiol 32:452-484.

Poulter MO, Ohannesian L, Larmet Y, Feltz P (1997) Evidence that GABAA receptor subunit mRNA expression during development is regulated by GABAA receptor stimulation. J Neurochem 68:631-639.

Puia G, Vicini S, Seeburg PH, Costa E (1991) Influence of recombinant gamma-aminobutyric acid-A receptor subunit composition on the action of allosteric modulators of gamma-aminobutyric acid-gated Cl- currents. Mol Pharmacol 39:691-696.

Roca D, Rozenberg I, Farrant M, Farb D (1990) Chronic agonist exposure induces down-regulation and allosteric uncoupling of the gamma-aminobutyric acid/benzodiazepine receptor complex. Mol Pharmacol 37:37-43.

Russek SJ, Bandyopadhyay S, Farb DH (2000) An initiator element mediates autologous downregulation of the human type A gamma -aminobutyric acid receptor beta 1 subunit gene. Proc Natl Acad Sci U S A 97:8600-8605.

Saliba RS, Kretschmannova K, Moss SJ (2012) Activity-dependent phosphorylation of GABA(A) receptors regulates receptor insertion and tonic current. Embo J 31:2937-2951.

Saliba RS, Michels G, Jacob TC, Pangalos MN, Moss SJ (2007) Activity-dependent ubiquitination of GABA(A) receptors regulates their accumulation at synaptic sites. J Neurosci 27:13341-13351.

Smith AJ, Alder L, Silk J, Adkins C, Fletcher AE, Scales T, Kerby J, Marshall G, Wafford KA, McKernan RM, Atack JR (2001) Effect of alpha subunit on allosteric modulation of ion channel function in stably expressed human recombinant gamma-aminobutyric acid(A) receptors determined using (36)Cl ion flux. Mol Pharmacol 59:1108-1118.

Tehrani MH, Barnes EM, Jr. (1997) Sequestration of gamma-aminobutyric acidA receptors on clathrin-coated vesicles during chronic benzodiazepine administration in vivo. J Pharmacol Exp Ther 283:384-390.

Tietz EI, Huang X, Chen S, Ferencak WF, 3rd (1999a) Temporal and regional regulation of alpha1, beta2 and beta3, but not alpha2, alpha4, alpha5, alpha6, beta1 or gamma2 GABA(A) receptor subunit messenger RNAs following one-week oral flurazepam administration. Neuroscience 91:327-341.

Tietz EI, Zeng XJ, Chen S, Lilly SM, Rosenberg HC, Kometiani P (1999b) Antagonist-induced reversal of functional and structural measures of hippocampal benzodiazepine tolerance. J Pharmacol Exp Ther 291:932-942.

Turkmen S, Backstrom T, Wahlstrom G, Andreen L, Johansson IM (2011) Tolerance to allopregnanolone with focus on the GABA-A receptor. Br J Pharmacol.

Wafford KA, Whiting PJ, Kemp JA (1993) Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant gamma-aminobutyric acidA receptor subtypes. Mol Pharmacol 43:240-244.

Figure legends:

Figure 1.GABA exposure induces decreases in specific $GABA_A$ receptor subunits.

Cultures were incubated for 10 min with vehicle (control), 1 mM GABA, 100 μM picrotoxin (PTX) or GABA plus PTX (GABA + PTX), washed and incubated for 48 h. **A**. Representative western blot of protein homogenates from cultures. **B**. Bar graph of the densitometry analyses of subunit levels normalized to actin expression. The results are expressed as percentage changes relative to control values (defined as 0). Data represent the mean ± the S.E.M. of 3-4 independent experiments. Significant differences: * GABA *versus* zero ($p<0.05$, one sample Student's *t* test); # GABA *versus* GABA + PTX ($p<0.05$, one-way ANOVA and Tukey´s post-hoc test).

Figure 2.Effect of GABA exposure on $GABA_A$ receptor subunit composition.

Cells were treated for 10 min with vehicle (control) or 1 mM GABA, washed and incubated for 48 h. Receptor immunoprecipitation experiments were carried out with a rabbit antibody against the γ2 subunit. **A**. Representative western blot of the immunoprecipitated material. In control experiments (negative controls), immunoprecipitation was performed in the absence of the specific antibody. The abundance of α subunits was measured with specific goat antibodies. **B**. Densitometry analyses of subunit levels normalized to the rabbit IgG signal to control for loading variability. Data are expressed as percentages of control values (defined as 100) and represent the mean ± the S.E.M. of 5-7 independent experiments. * Significantly different from one hundred ($p<0.05$, one sample Student's *t* test).

Figure 3. GABA-induced decrease in cell surface expression of α3 subunit.

Cultures were treated for 10 min with vehicle (control) or 1 mM GABA, washed and incubated for 48 h. **A**. Biotinylated (surface) proteins were visualized by western blot with antibodies against α1, α3 or β2/3 subunits. Actin levels from total cell lysates were measured to control for loading variability. **B.** Densitometry analyses of the blots. Cell surface levels of subunits were normalized to the actin signal. The data are expressed as percentages of control values (defined as 100) and represent the mean ± the S.E.M. of 4 independent experiments. * Significantly different from one hundred ($p<0.05$, one sample Student's *t* test). **C.** Images from epifluorescence microscopy of surface β2/3-containing $GABA_A$ receptors (red signal). Scale bar: 10 µm. **D**. Quantitative analysis of immunocytochemical experiments. Fluorescence was normalized to the total area of a selected cell. The data represent the mean ± the S.E.M. of 15-18 optic fields (1-3 neurons per field) of 3 independent cultures. * Significantly different from control values ($p<0.05$, unpaired Student's *t* test).

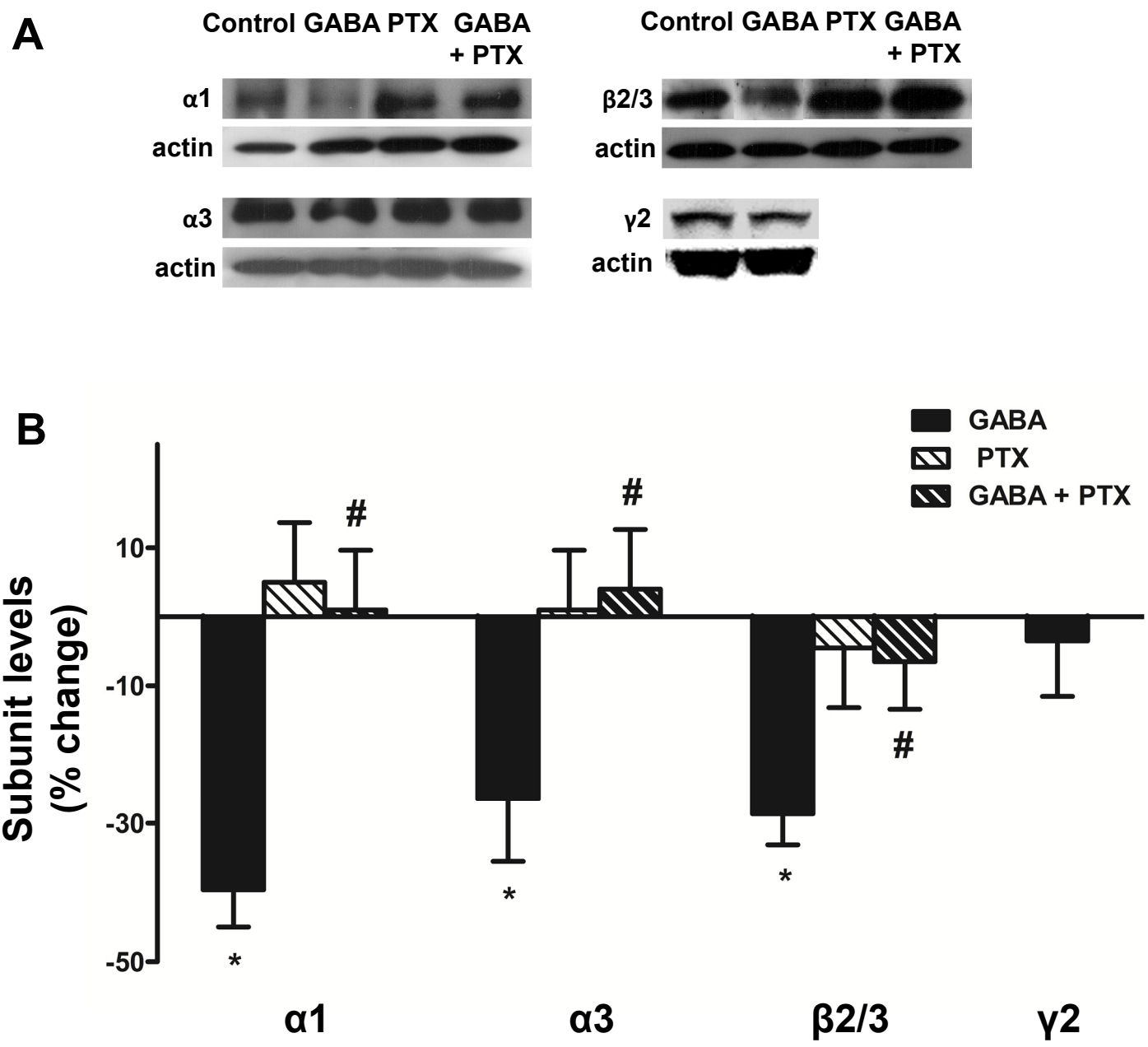
Figure 4. Initiation of GABA-induced uncoupling is prevented by co-incubation with high concentrations of sucrose.

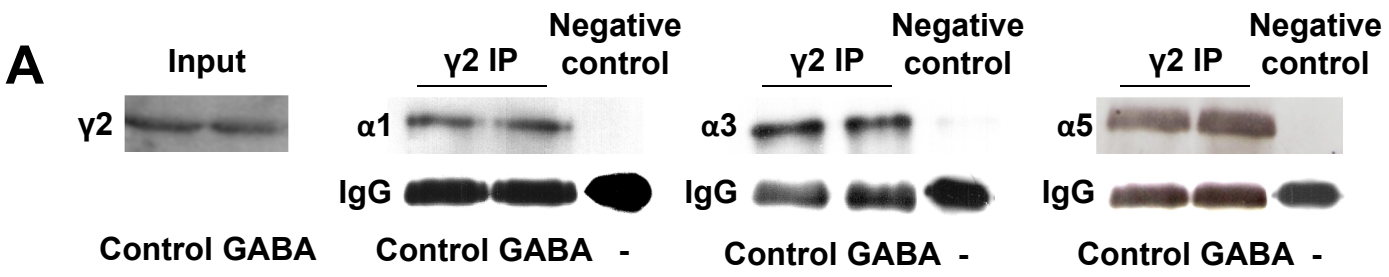
Cultures were pre-incubated for 10 min with 350 mM sucrose or vehicle and then treated for 10 min with 1 mM GABA or vehicle. Cells were washed twice and incubated for 48 h. Coupling between the GABA and benzodiazepine binding sites was measured as the potentiation of [^{3}H]flunitrazepam binding by GABA. The data are expressed as percentages of control values (defined as 100) and represent the mean ± the S.E.M. of 3 independent experiments. Significant differences: * GABA *versus*

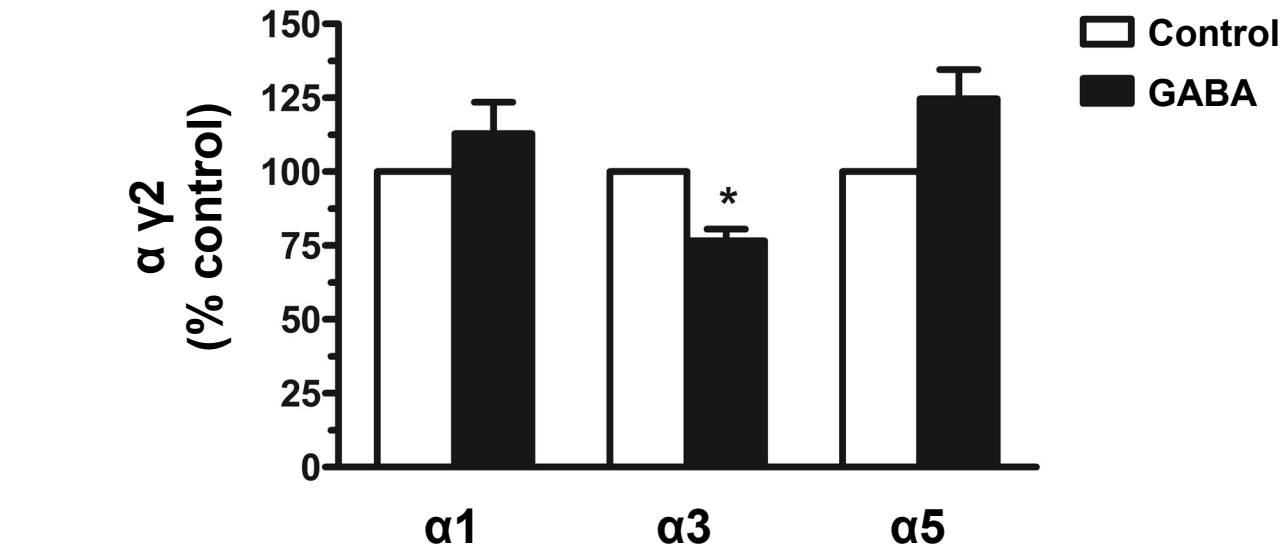
control, defined as 100 ($p<0.05$, one sample Student's *t* test); # GABA *versus* GABA + sucrose ($p<0.05$, one-way ANOVA and Tukey´s post-hoc test).

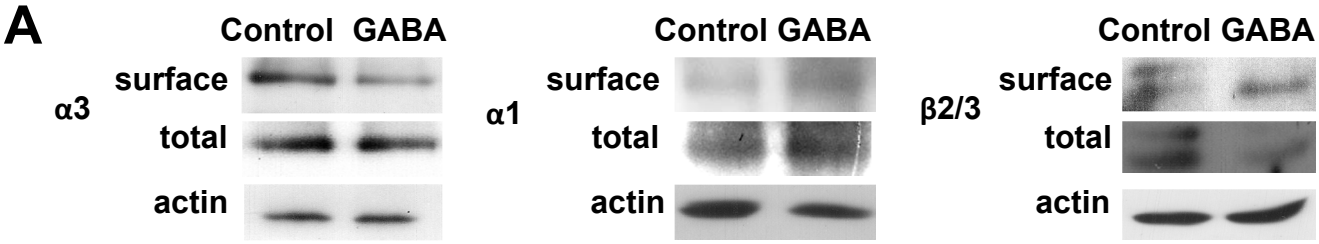
Figure 5.GABA-induced $GABA_A$ receptor internalization.

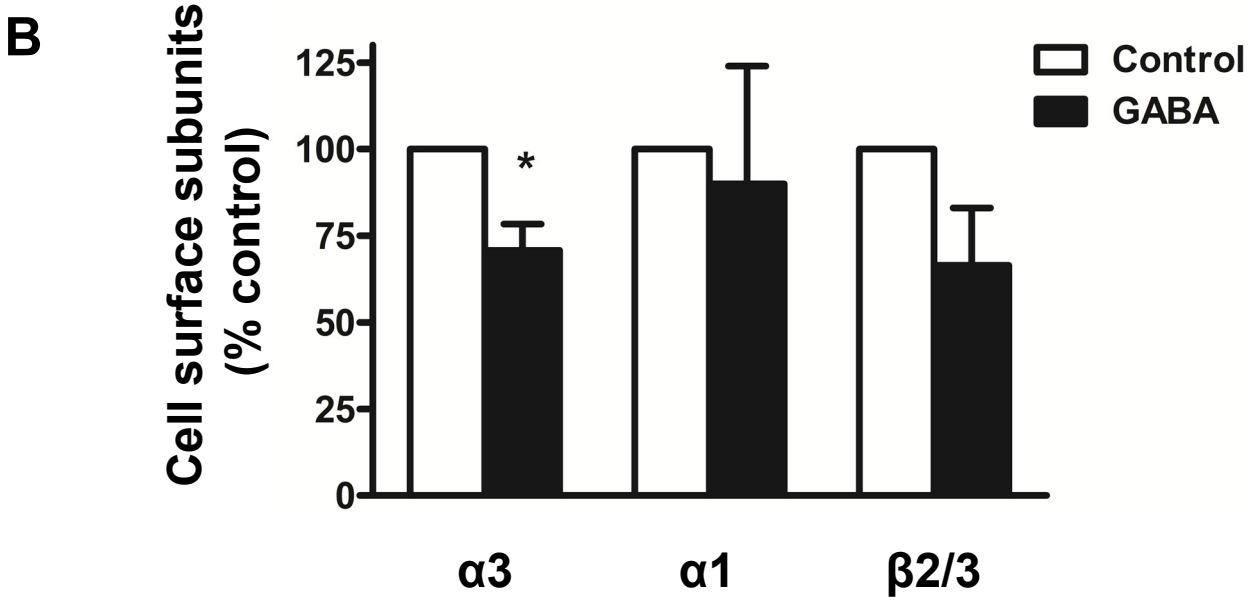
Cultures were exposed for 10 min to vehicle (control), 1 mM GABA, 100 μM picrotoxin (PTX) or GABA plus PTX (GABA + PTX) at 37°C, washed and incubated for 20 min at 37°C in the absence of neurotransmitter. **A**. Images from epifluorescence microscopy of surface (red signal) and internalized (green signal) β2/3-containing $GABA_A$ receptors. Scale bar: 10 μm. **B**. Quantitative analysis of immunocytochemical experiments. Receptor internalization was measured as the percentage of internalized fluorescence relative to total fluorescence. The data represent the mean ± the S.E.M. of 17-19 optic fields (1-3 neurons per field) of 4 independent cultures. * Significantly different from control values ($p<0.05$, unpaired Student's *t* test). **C.** Biotinylated (internalized) proteins were visualized by western blot with antibodies against α3 or β2/3 subunits. The signal of the internalized receptors (Int) was normalized to total receptor signal. **D**. Densitometry analyses of the blots. The data are expressed as percentages of control values (defined as 100) and represent the mean ± the S.E.M. of 4 independent experiments. Significant differences: * GABA *versus* control, defined as 100 ($p<0.05$, one sample Student's *t* test); # GABA *versus* GABA + PTX ($p<0.05$, one-way ANOVA and Tukey´s post-hoc test).

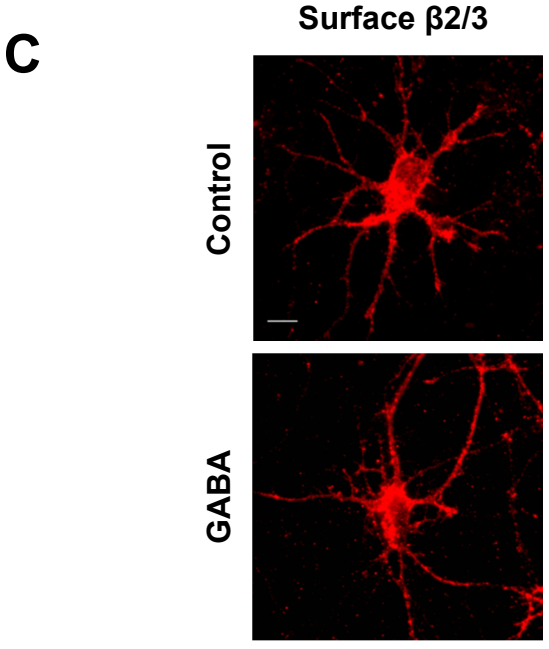


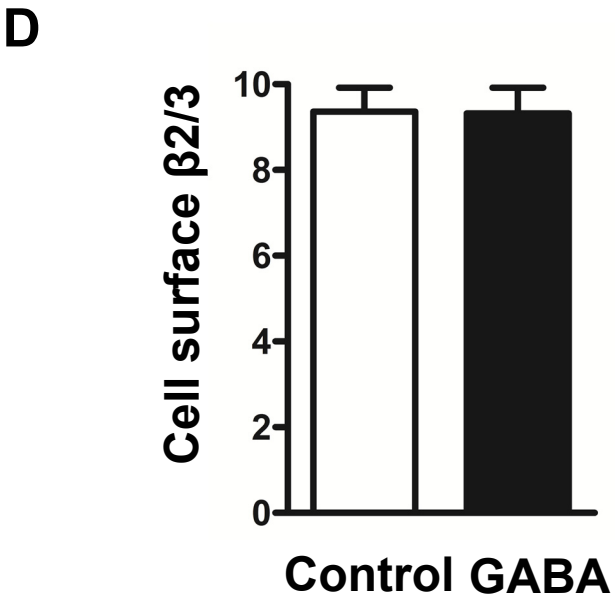


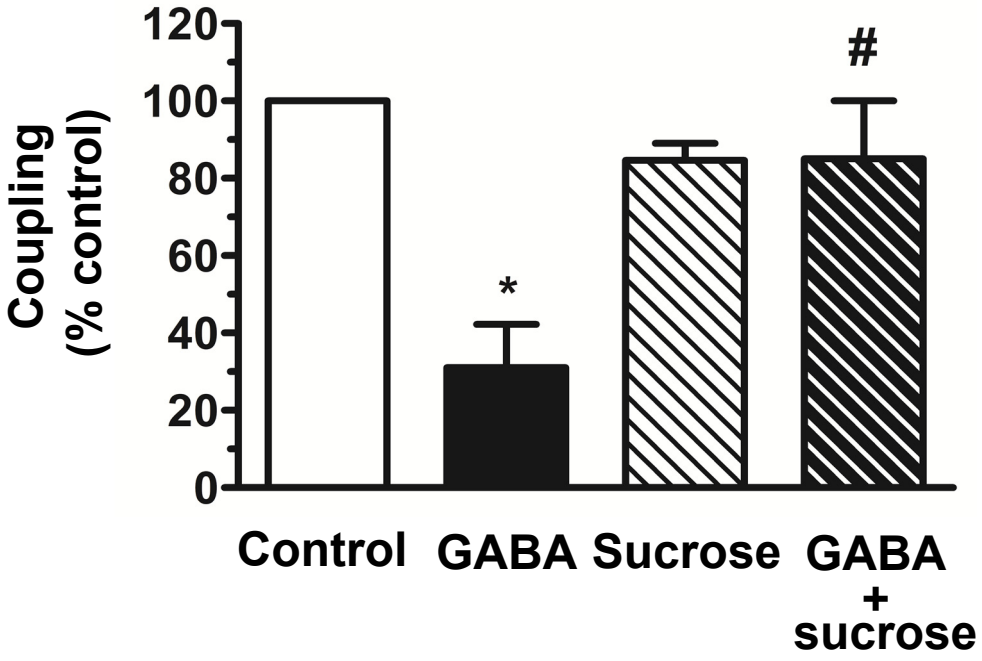


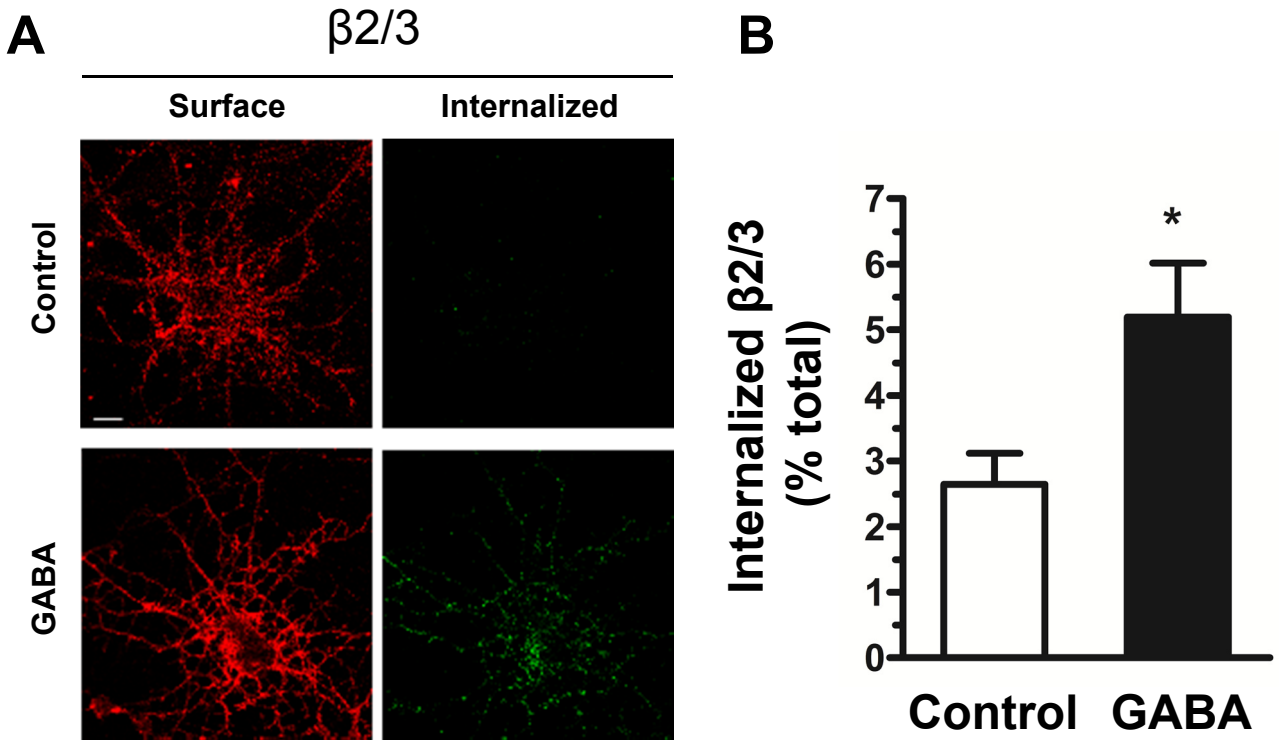








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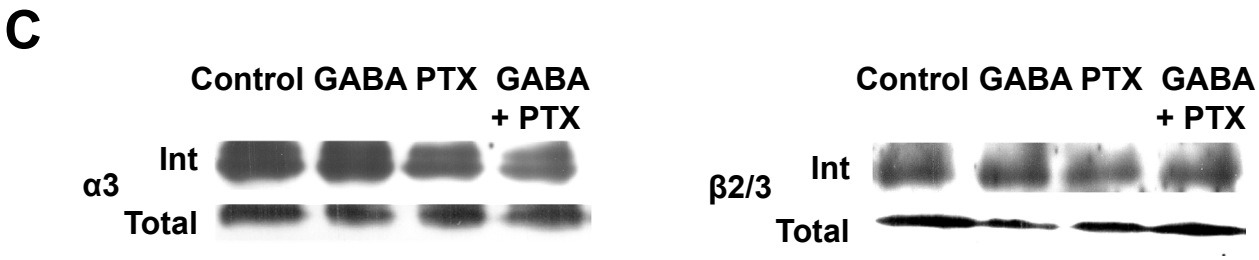
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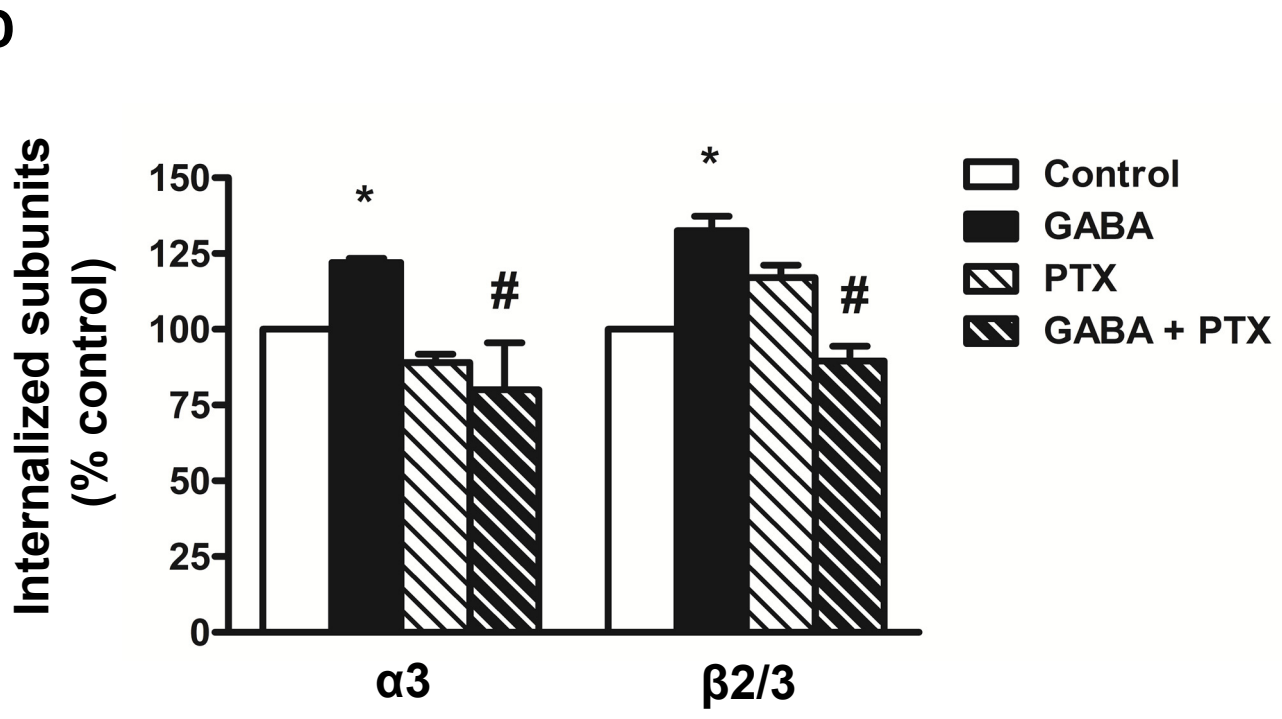
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GABA + PTX

Highlights

-A single brief GABA exposure induces uncoupling between GABA and benzodiazepine sites.

-Uncoupling is accompanied by a decrease in the proportion of α3-contaning receptors.

-Uncoupling mechanism is initiated by an increased $GABA_A$ receptor internalization.