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GABA-Induced Uncoupling of GABA/Benzodiazepine Site Interactions Is Associated With Increased Phosphorylation of the $GABA_A$ Receptor

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The use-dependent regulation of the $GABA_A$ receptor occurs under physiological, pathological, and pharmacological conditions. Tolerance induced by prolonged administration of benzodiazepines is associated with changes in $GABA_A$ receptor function. Chronic exposure of neurons to GABA for 48 hr induces a downregulation of the $GABA_A$ receptor number and an uncoupling of the GABA/benzodiazepine site interactions. A single brief exposure ($t_{1/2} = 3$ min) of rat neocortical neurons to the neurotransmitter initiates a process that results in uncoupling hours later ($t_{1/2} = 12$ hr) without alterations in the number of $GABA_A$ receptors and provides a paradigm to study the uncoupling mechanism selectively. Here we report that uncoupling induced by a brief $GABA_A$ receptor activation is blocked by the coincubation with inhibitors of protein kinases A and C, indicating that the uncoupling is mediated by the activation of a phosphorylation cascade. GABA-induced uncoupling is accompanied by subunit-selective changes in the $GABA_A$ receptor mRNA levels. However, the GABA-induced downregulation of the $\alpha 3$ subunit mRNA level is not altered by the kinase inhibitors, suggesting that the uncoupling is the result of a posttranscriptional regulatory process. GABA exposure also produces an increase in the serine phosphorylation on the $GABA_A$ receptor $\gamma 2$ subunit. Taken together, our results suggest that the GABA-induced uncoupling is mediated by a posttranscriptional mechanism involving an increase in the phosphorylation of $GABA_A$ receptors. The uncoupling of the $GABA_A$ receptor may represent a compensatory mechanism to control GABAergic neurotransmission under conditions in which receptors are persistently activated.

Key words: $GABA_A$ receptors; GABA; posttranslational modification; plasticity

The use-dependent regulation of $GABA_A$ receptors by different ligands is a form of homeostatic plasticity that is relevant to physiological, pharmacological, and pathological conditions in which the receptors are persistently activated. There are physiological conditions, such as the menstrual cycle and pregnancy, in which $GABA_A$ receptors are chronically exposed to high concentrations of allopregnanolone, an endogenous positive allosteric receptor modulator. Under these conditions of chronic exposure, a tolerance mechanism, accompanied by subunit-selective alterations in $GABA_A$ receptor proteins, has been described (for review see Turkmen et al., 2010). Benzodiazepines are routinely prescribed to treat anxiety, insomnia, seizure disorders, etc. However, the chronic administration of benzodiazepines induces tolerance, limiting their usefulness. Chronic treatment of rats with benzodiazepines results in an allosteric uncoupling between the GABA and benzodiazepine sites and produces changes in the expression of several $GABA_A$ receptor subunits (for review see Bateson, 2002). The relationship between the uncoupling and the changes in the receptor subunit expression has not been established. The activation-induced regulation of the $GABA_A$ receptor is more evident under certain pathological conditions. For example, GABAergic neurons fire at a very high frequency (800 Hz) during cortical spike-wave electrographic seizures in cats (Timofeev et al., 2002). Alterations in $GABA_A$

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receptor subunit levels have been observed during epileptogenesis in animal models of epilepsy (Brooks-Kayal et al., 1998, 2009).

The receptor subunit composition is the major determinant of the pharmacological profile of the $GABA_A$ receptors. The expression of the different $GABA_A$ receptor subunits is controlled by complex transcriptional mechanisms that are not completely understood. For example, the transcription of the $GABA_A$ receptor $\alpha 1$ subunit is differentially regulated by protein kinases A and C (PKA, PKC), leading to changes in the receptor's cell surface expression (Hu et al., 2008). PKC activation induces an increase in the gene expression of the $\alpha 1$ subunit by the phosphorylation of the cAMP response element-binding protein (CREB). In contrast, PKA activation produces a decrease in the $\alpha 1$ subunit expression through the phosphorylation of CREB and the synthesis of the inducible cAMP early repressor.

The phosphorylation of neurotransmitter receptors is another potential mechanism for the plasticity of ion channels in the central nervous system. The main intracellular loops of the $GABA_A$ receptor β and γ subunits can be phosphorylated at serine, threonine, and tyrosine residues by a variety of kinases, such as PKA, PKC, the tyrosine kinase Src, and the Ca^{2+}/calmodulin type II-dependent kinase. The phosphorylation of the $GABA_A$ receptors regulates the channel function and cell surface trafficking; however, the signaling pathways that control this posttranslational modulation are not completely elucidated (Brandon et al., 2002; Kittler and Moss, 2003; Mody, 2005; Houston et al., 2007). Phosphorylation can stimulate or inhibit $GABA_A$ receptor function depending on the receptor subunit composition (McDonald et al., 1998).

Cultured neurons chronically exposed to GABA for 48 hr display a downregulation of the $GABA_A$ receptor number and the uncoupling of the GABA/benzodiazepine site interactions with a time constant of 24–25 hr (Roca et al., 1990a). The decrease in the $GABA_A$ receptor number is mediated by the transcriptional repression of the receptor subunit genes via the activation of the L-type voltage-gated calcium channels (VGCC; Lyons et al., 2000, 2001; Russek et al., 2000). We previously demonstrated that a single brief exposure (5–10 min) of GABA to neocortical neurons induces the uncoupling of the $GABA_A$ receptors 24–48 hr later without any alteration in the receptor number (Gravielle et al., 2005). This uncoupling is dependent upon receptor activation but is independent of calcium influx through the L-type VGCC. The objective of this study was to investigate the molecular basis of GABA-induced uncoupling of GABA/benzodiazepine site interactions and test the hypothesis that activation of a protein quinase cascade mediates the uncoupling produced by a brief $GABA_A$ receptor activation. The results suggest that the GABA-induced uncoupling of the receptor is mediated by PKA and PKC activation and occurs as a result of an increase in the phosphorylation state of the $GABA_A$ receptor $\gamma 2$ subunit.

MATERIALS AND METHODS

Cell Cultures

Primary cultures were prepared from 18-day-old rat embryos (Sprague-Dawley) as previously described by Gravielle et al. (2005). Briefly, whole brains were removed and cerebral cortices were dissected under a microscope and placed in ice-cold Hanks' solution. The 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 plating medium (Neurobasal medium plus 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine; Invitrogen, Caralsbad, CA) 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 0.75 cortices per 100-mm culture dish coated with poly-L-lysine (0.1 mg/ml; Invitrogen). The cultures were incubated at 37°C in 5% CO_2, and after 1 hr the medium was aspirated and replaced with serum-free medium containing the B27 serum-free supplement.

Drug Treatments

After 7 days in culture, 100-mm dishes containing 14 ml medium were treated as follows. Seven milliliters of medium (conditioned medium) was removed and remained in the incubator, and a small volume (70 μl) of concentrated drug stock or vehicle was added. Cells were incubated for 10 min at 37°C. For the experiments examining the effects of the kinase inhibitors, neurons were preincubated for 10 min at 37°C with H-89 (Sigma, St. Louis, MO), chelerythrine (Sigma), or vehicle; then GABA (Sigma) or the vehicle was added and coincubated for 10 min at 37°C. Cultures were washed twice with 5 ml warm Hanks' solution, and 7 ml conditioned medium was added to the cultures. The culture dishes were maintained in the incubator for 48 hr, and at the end of this incubation the medium was aspirated. The GABA solution (final concentration 1 mM) was prepared in Hanks' solution, and the H-89 (final concentration 1 μM), chelerythrine (final concentration 5 μM), nifedipine (Sigma; final concentration 10 μM), and saclofen (Sigma; final concentration 200 μM) solutions were prepared in Me_2SO. The final concentration of the Me_2SO in the culture medium was 0.1%.

Binding Assay

Cells were washed twice with 5 ml ice-cold phosphate buffered saline (PBS), scraped from the dishes, and centrifuged at 500*g* for 5 min. The resulting pellet was homogenized in 1 ml of 1 mM EDTA/1 mM phenylmethylsulfonil fluoride (PMSF) with 12 strokes of a glass Dounce homogenizer and dialyzed against 4 × 4 liters of potassium PB (pH 7.4) overnight at 4°C.

The homogenate aliquots (75–100 μg protein) were incubated in 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 the total binding to yield the 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 then washed three times with 5 ml ice-cold PBS. Radioactivity retained on the filters was quantified with liquid scintillation spectrometry. The coupling represents the GABA-potentiated [^{3}H]FNZ binding and was estimated as (% potentiation $_{treated}$/% potentiation$_{control}$) × 100. The uncoupling was defined as the decrease in the GABA-potentiated [^{3}H]FNZ binding and was calculated as [1 − (% potentiation $_{treated}$/% potentiation $_{control}$)] × 100.

Real-Time PCR

Total RNA was extracted using the RNeasy midi kit (Qiagen, Hilden, Germany). The primers (Tecnolab)

and the probe (TaqMan; Applied Biosystems, Foster City, CA) were designed in Primer Express software (Applied Biosystems). The sequences of the α3 primers were 5′-CACCA TGACCACCTTGAGTATCA-3′ and 5′-CCGTCGCGTAT GCCACTT-3′. The sequence of the α3 probe was 5′-TGCC AGAAACTCTTTAC-3′. These sequences were designed to amplify a region in the cytoplasmic loop between transmembrane domains M3 and M4 (an area with amino acid sequences divergent among the different subunits).The ribosomal RNA probe and primers were purchased from Applied Biosystems. Quantitative one-step real-time PCR was performed in an Applied Biosystems 7500 real-time PCR system with an AgPath ID one-step RT PCR kit (Ambion, Grand Island, NY). The standard curves for relative quantification were generated with 2.5–100 ng total RNA isolated from control cultures (treated with vehicle). The PCR reactions were performed in triplicate in a total volume of 25 μl containing AgPath ID master mixture, 250 nM of the α3 subunit probe, 900 nM of the α3 subunit primers, 50 nM of the 18S rRNA probe, and 50 nM of the 18S rRNA primers. The reaction conditions were 45°C for 10 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 45 sec. The relative amount of the α3 subunit mRNA was normalized to the relative amount of the 18S rRNA (internal control).

Immunoprecipitation and Western Blot

The cells were collected in 5 ml ice-cold PBS as described above and centrifuged at 500*g* for 5 min. The pellet was homogenized in ice-cold lysis buffer containing 25 mM HEPES pH 7.5, 0.3 N NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM β-glycerophosphate, 0.1 mM sodium metavanadate, 1 mM PMSF, 0.83 mM 1,4-dithiothreitol and incubated on a rotator shaker for 20 min at 4°C. The lysates were centrifuged at 27,000*g* for 30 min, and the supernatants were diluted to a protein concentration of 1 mg/ml in PBS buffer. The lysates were then boiled for 5 min and precleared by the addition of 20 μl protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) to 500 μl of the cell lysate. Lysates were incubated at 4°C for 20 min with rotation and centrifuged for 5 min at 2,000*g*. The resulting supernatants were incubated with 2 μg of rabbit anti-γ2 or anti-β2 antibodies (Alpha Diagnostic, San Antonio, TX) overnight at 4°C in a rotator shaker. Negative controls were produced by incubating the reaction mixture without the specific antibody. Immunocomplexes were captured by the addition of 20 μl protein A agarose beads and incubating overnight at 4°C with gentle shaking. The agarose beads were collected by centrifugation (5 min at 2,000*g*), and the pellets were washed three times by resuspension in ice-cold lysis buffer followed by centrifugation. The final pellets were resuspended in 30 μl denaturing sample buffer. Proteins were separated on 10% acrylamide gels and transferred to a nitrocellulose membrane. The blots were blocked for 2 hr with 5% nonfat dry milk in 20 mM of Tris-buffered saline (TBS) buffer containing 0.1% Tween-20. The blots were incubated with mouse antiphosphotyrosine or antiphosphoserine antibodies (1:500 dilution; Millipore, Billerica, MA) overnight at 4°C. The phosphorylated proteins were detected by incubating with an anti-mouse HRP-conjugated antibody (1:2,000 dilution; Santa Cruz) for 1.5 hr at room temperature. The blots were stripped and reprobed with an anti-rabbit HRP-conjugated antibody (1:2,000 dilution; Santa Cruz Biotechnology) for 1.5 hr at room temperature to measure the amount of rabbit IgGs, i.e., the antibodies used to immunoprecipitate the $GABA_A$ subunits. The subunit signals were normalized to the signals of the rabbit IgGs to control for the amount of loading variability.

RESULTS

We previously demonstrated that brief GABA-induced activation of the $GABA_A$ receptors by GABA for 5–10 min ($t_{1/2}$ = 3 min) leads to a delayed-onset uncoupling of the GABA/benzodiazepine site interactions, which takes 24–48 hr to unfold ($t_{1/2}$ = 12 hr; Gravielle et al., 2005). Because this use-dependent regulation of the $GABA_A$ receptors occurs in the absence of changes to the receptor number, we used this paradigm to selectively study the uncoupling mechanism. Although the number of $GABA_A$ receptors remains constant, the GABA-induced uncoupling is accompanied by a decrease in the mRNA levels of the α1, α3, β1, β2, and β3 $GABA_A$ receptor subunits. However, the mRNA levels of some receptor subunits (α2, α4, α5, γ1, and γ2) do not change (Gravielle et al., 2005). We recently demonstrated that the GABA-induced uncoupling is associated with a decrease in the proportion of $GABA_A$ receptors containing α3 subunits (Gutiérrez et al., 2014), a receptor subtype that exhibits the highest coupling strength between the GABA and benzodiazepine sites (Puia et al., 1991; Wafford et al., 1993; Smith et al., 2001).

To investigate the signaling pathway that is involved in the uncoupling mechanism, we first studied the effect of specific PKA and PKC inhibitors. The results from these experiments (Fig. 1) indicate that the addition of the specific PKA (H-89) or PKC (chelerythrine) inhibitors during the 10-min GABA exposure blocked the uncoupling between the GABA and benzodiazepine binding sites. These results suggest that the uncoupling is mediated by the PKA and PKC signaling cascades.

Because $GABA_B$ receptor activation can modulate PKA and PKC activities (for review see Gassmann and Bettler, 2007), we asked whether GABA-induced uncoupling is mediated by $GABA_B$ receptors. Results from binding experiments (Fig. 2) showed that saclofen, a

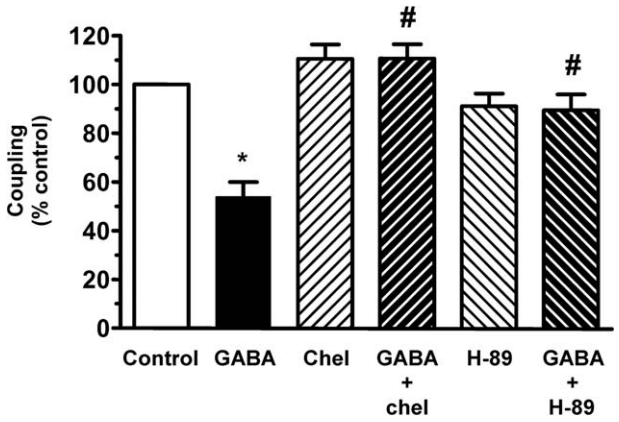

Fig. 1. GABA-induced uncoupling is mediated by PKA and PKC activation. Cells were treated as follows: 20 min with vehicle (100% coupling, control); 10 min with vehicle and then 10 min with 1 mM GABA (GABA); 20 min with 5 μM chelerythrine (Chel); 10 min with chel and then 10 min with GABA plus chel (GABA + chel); 20 min with 1 μM H-89 (H-89); 10 min with H-89 and then 10 min with GABA plus H-89 (GABA + H-89). At the end of treatments, cultures were washed and incubated for 48 hr before harvesting. The coupling between the GABA and benzodiazepine binding sites was measured as the potentiation of the [^{3}H]FNZ binding by GABA and expressed as a percentage of the control. Data represent the mean ± SEM of five to eight independent determinations. $\star P < 0.05$ vs. 100, one-sample Student's *t*-test. $^{\#}P < 0.05$ vs. GABA, one-way analysis of variance with Tukey's post hoc test.

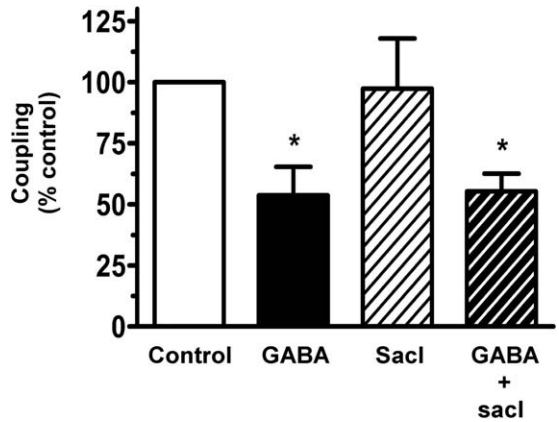

Fig. 2. GABA-induced uncoupling is independent of $GABA_B$ receptor activation. Cells were exposed to 1 mM GABA (GABA), 200 μM saclofen (Sacl), GABA plus sacl (GABA + sacl), or vehicle (control, 100%) for 10 min and then washed and incubated for 48 hr. At the end of treatments, the cultures were washed and incubated for 48 hr before harvesting. The coupling between the GABA and benzodiazepine binding sites was measured as the potentiation of the [^{3}H]FNZ binding by GABA and expressed as a percentage of the control. Data represent the mean ± SEM of three independent determinations. $\star P < 0.05$ vs. 100, one-sample Student's *t*-test.

$GABA_B$ receptor antagonist, failed to prevent uncoupling between GABA and benzodiazepine binding sites produced by GABA exposure. This suggests that GABA-induced uncoupling is independent of $GABA_B$ receptor activation.

We next examined whether PKA or PKC activation leads to transcriptional repression of the α3 subunit. To answer this question, we studied the effect of the protein kinase inhibitors on the GABA-induced downregulation of the α3 subunit mRNA levels. Quantitative real-time PCR (Fig. 3) demonstrated that the GABA-induced reduction of the α3 mRNA levels was not prevented by the addition of the kinase inhibitors, suggesting that the decrease of the α3 mRNA levels is not related to the GABA-induced uncoupling. Chelerythrine alone induced a small decrease in the α3 mRNA levels; however, this effect was not statistically significant.

It has been established that the mechanism of the reduction of the $GABA_A$ receptor number induced by a chronic exposure of brain neurons to GABA for 48 hr is the result of transcriptional repression of the receptor subunit genes through L-type VGCC activation (Lyons et al., 2001). Conversely, the receptor uncoupling induced in neurons that have been briefly or chronically exposed to GABA is independent of calcium channel activation (Lyons et al., 2001; Gravielle et al., 2005). To analyze further the relationship between the receptor uncoupling and the regulation of receptor subunit mRNA levels, we investigated whether the decrease in

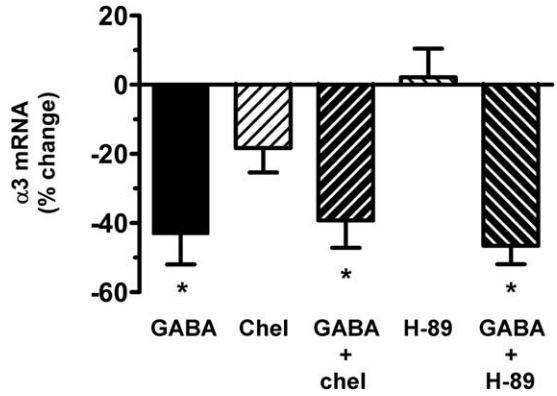

Fig. 3. GABA-induced downregulation of the $GABA_A$ receptor α3 subunit mRNA levels is independent of PKA or PKC activation. Cells were treated as follows: 20 min with vehicle (control, 0%); 10 min with vehicle and then 10 min with 1 mM GABA (GABA); 20 min with 5 μM chelerythrine (Chel); 10 min with chel and then 10 min with GABA plus chel (GABA + chel); 20 min with 1 μM H-89 (H-89); 10 min with H-89 and then 10 min with GABA plus H-89 (GABA + H-89). At the end of treatments, the cultures were washed and incubated for 48 hr before harvesting. Quantitative real-time PCR was performed using total RNA. 18S RNA was used as an internal control to normalize the results. The results are expressed as a percentage of change compared with control experiments and represent the mean ± SEM of four independent experiments. $\star P < 0.05$ vs. 0, one-sample Student's *t*-test.

the α3 mRNA levels induced by a brief activation of $GABA_A$ receptors is associated with receptor uncoupling or constitutes part of the receptor downregulation mechanism that would require a longer receptor activation to

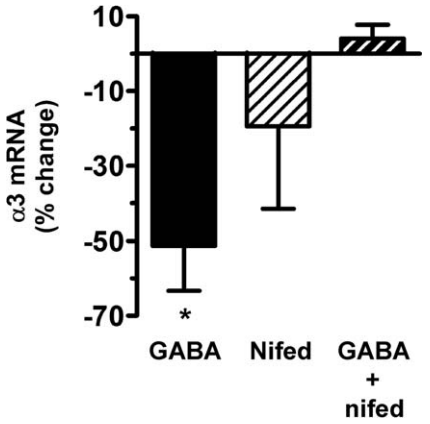

Fig. 4. GABA-induced downregulation of the $GABA_A$ receptor α3 subunit mRNA levels is prevented by coincubation with nifedipine. Cells were exposed to 1 mM GABA (GABA), 10 μM nifedipine (Nifed), GABA plus nifed (GABA + nifed), or vehicle (control, 0%) for 10 min; then washed, and incubated for 48 hr. Quantitative real-time PCR was performed using total RNA. 18S RNA was used as an internal control to normalize the results. Values are expressed as a percentage of change compared with control experiments and represent the mean ± SEM of four independent experiments. $^{\star}P < 0.05$ vs. 0, one-sample Student's *t*-test.

complete. To answer this question, we studied the effect of nifedipine, a compound that blocks receptor downregulation but not its uncoupling (Lyons et al., 2001; Gravielle et al., 2005), on the downregulation of the α3 subunit mRNA induced by a 10-min exposure to GABA (Fig. 4). The findings indicate that nifedipine prevented the reduction of the α3 mRNA levels, suggesting that this alteration might be associated with the reduction in the receptor number rather than the uncoupling of the receptor. Nifedipine alone induced a small decrease in the α3 subunit mRNA levels, but this effect was not statistically significant.

It is well known that the phosphorylation of $GABA_A$ receptors alters receptor function. To determine whether the direct phosphorylation of $GABA_A$ receptors mediates receptor uncoupling, we tested whether brief GABA applications altered the phosphorylation state of the receptor subunits. We performed immunoprecipitation studies using antibodies raised against the β2 and γ2 subunits, followed by Western blotting with antiphosphoserine or antiphosphotyrosine antibodies (Fig. 5). The results revealed an increased phosphorylation state of the γ2 subunit at serine residues. We did not detect any phosphorylation state changes of the serine residues of the β2 subunit or the tyrosine residues of the γ2 subunit. Because serine residues on γ2 subunits can be phosphorylated by PKC (Song and Messing, 2005), we next investigated the effect of chelerythrine on GABA-induced phosphorylation of these residues (Fig. 5). Results indicated that chelerythrine prevented the phosphorylation of $GABA_A$ receptors at serine residues. These Western blot results suggest that a change in the phosphorylation state of $GABA_A$ receptors underlies the uncoupling mechanism.

DISCUSSION

The plasticity of the $GABA_A$ receptors is important during development; in the normal functioning of adult brain; in pathological conditions such as epilepsy, anxiety, schizophrenia, etc. (Gaiarsa et al., 2002; Fritschy and Brunig, 2003; Mohler, 2006); and in pharmacological situations of prolonged administration of benzodiazepines (Bateson, 2002; Vinkers and Olivier, 2012). Persistent occupancy of the $GABA_A$ receptors by GABA or positive allosteric modulators leads to alterations in the number and function of $GABA_A$ receptors (Roca et al., 1990a,b; Friedman et al., 1996). We previously reported that a single brief activation of the $GABA_A$ receptors by GABA produces the uncoupling of the GABA/benzodiazepine sites without changing the receptor number (Gravielle et al., 2005).

GABA-induced uncoupling is accompanied by a subunit-selective decrease in the mRNA levels of the $GABA_A$ receptor (Gravielle et al., 2005). Our recent results suggest that the receptor uncoupling is associated

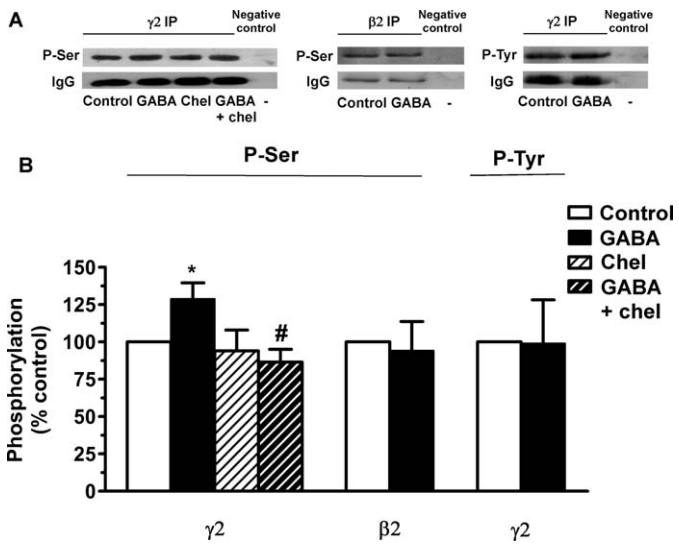

Fig. 5. Effect of GABA exposure on the the phosphorylation state of the $GABA_A$ receptor β2 and γ2 subunits. Cells were treated as follows: 20 min with vehicle (control, 100%); 10 min with vehicle and then 10 min with 1 mM GABA (GABA); 20 min with 5 μM chelerythrine (Chel); 10 min with chel and then 10 min with GABA plus chel (GABA + chel). At the end of treatments, the cultures were washed and incubated for 48 hr before harvesting. Immunoprecipitation was performed with rabbit antibodies raised against the β2 or γ2 subunits, followed by Western blotting using mouse antiphosphoserine or antiphosphotyrosine antibodies. **A:** Representative Western blot of the immunoprecipitated proteins. In control experiments (negative controls), the immunoprecipitation was performed in the absence of the specific antibody. **B:** Densitometry analyses of the phosphorylated subunits normalized to the rabbit IgG signals (loading control). Data are expressed as percentages of control values (defined as 100%) and represent the mean ± SEM of four to six independent experiments. IP, immunoprecipitation; P-Ser, antiphosphoserine; P-Tyr, ant-phosphotyrosine. $^{\star}P < 0.05$ vs. 100%, one-sample Student's *t*-test. $^{\#}P < 0.05$ vs. GABA, one-way analysis of variance and Tukey's post hoc test.

with a reduction in the percentage of α3-contaning $GABA_A$ receptors (Gutiérrez et al., 2014). On the other hand, it has been shown that PKA and PKC activations differentially regulate the transcription of the $GABA_A$ receptor α1 subunit in neocortical neurons (Hu et al., 2008). To determine whether the activation of a protein kinase cascade mediates the uncoupling induced by a brief $GABA_A$ receptor activation, we studied the effect of specific PKA and PKC inhibitors on the GABA-induced uncoupling (Fig. 1). The results suggest that the GABA-induced uncoupling is mediated by the activation of the PKA and PKC signaling cascades.

The slow inhibition via $GABA_B$ receptors plays an important role in the cerebral cortex, regulating network activity (Kohl and Paulsen, 2010). Because activation of $GABA_B$ receptors can modulate the activity of PKA and PKC (Gassmann and Bettler, 2007), we tested whether GABA-induced uncoupling is mediated by binding of GABA to these receptors. Our results (Fig. 2) indicate that uncoupling is independent of $GABA_B$ receptor activation. On the other hand, we previously demonstrated that uncoupling is prevented by coincubation of GABA with picrotoxin (Gravielle et al., 2005), further indicating that this regulatory process is mediated by activation of $GABA_A$ receptors.

The mechanism of uncoupling may involve transcriptional repression of the α3 subunit induced by the activation of PKA and/or PKC or the direct phosphorylation of $GABA_A$ receptors by these kinases. To test the first hypothesis, we investigated the effect of the protein kinase inhibitors on the downregulation of α3 mRNA levels (Fig. 3). Quantitative real-time PCR showed that PKA or PKC inhibition failed to prevent the GABA-induced decrease in the steady-state levels of α3 mRNA. These data suggest that the GABA receptor uncoupling is not produced by changes in the transcription of the genes for the $GABA_A$ receptor subunits and/or the stability of the receptor subunit mRNAs.

It has been shown that neocortical neurons exhibit excitatory GABA responses in our culture conditions (Kato-Negishi et al., 2004). GABA-induced downregulation of the $GABA_A$ receptor number is the result of transcriptional repression of receptor genes (Lyons et al., 2000; Russek et al., 2000) and is mediated by activation of L-type VGCC (Lyons et al., 2001). In contrast, uncoupling induced by chronic or brief exposure to GABA is independent of L-type VGCC activation (Lyons et al., 2001; Gravielle et al., 2005).

The GABA-induced reduction of the $GABA_A$ receptor α3 subunit mRNA levels may alternatively represent the first step of a receptor downregulation process that would require prolonged receptor activation to progress. To investigate further whether the receptor uncoupling is mediated by alterations in the mRNA levels of the $GABA_A$ receptor subunits, we studied the effect of nifedipine, a compound that blocks the reduction in receptor number but not its uncoupling (Lyons et al., 2001; Gravielle et al., 2005), on the downregulation of the α3 subunit mRNA levels. The results (Fig. 4) show that nifedipine prevents the decrease in the α3 subunit mRNA levels, suggesting that this process is not related to the receptor uncoupling and most likely mediates a reduction in receptor number.

We finally investigated the hypothesis that the receptor uncoupling is induced by the direct phosphorylation of the $GABA_A$ receptor subunits. The function and trafficking of $GABA_A$ receptors are regulated by receptor subunit phosphorylation of the intracellular domains between transmembrane domains 3 and 4. We analyzed the phosphorylation states of the serine residues on the β2 and γ2 subunits and the tyrosine residues on the γ2 subunits because they are the major residues phosphorylated by protein kinases on $GABA_A$ receptors (Fig. 5). The immunoprecipitation results indicate that the GABA treatment induces an increase in the phosphorylation state of the γ2 subunits at serine residues. The S327 and S348 residues of the γ2 (S and L) subunit and the S343 residue of the γ2L can be phosphorylated by PKC (Song and Messing, 2005), suggesting that the receptor uncoupling is mediated by serine phosphorylation on the $GABA_A$ receptor γ2 subunits by PKC. These results were confirmed by experiments showing that a PKC inhibitor inhibits GABA-induced phosphorylation of $GABA_A$ receptor γ2 subunits on serine residues (Fig. 5).

It has been demonstrated in recombinant expression systems that phorbol esters produce a reduction in the current amplitude of the $GABA_A$ receptors. This regulation of receptor function is mediated by PKC phosphorylating the serine residues on the β and γ2 subunits (Kellenberger et al., 1992; Krishek et al., 1994). In contrast, brain-derived neurotrophic factor transiently stimulates $GABA_A$ receptor function in hippocampal neurons, and this effect is temporally correlated with the phosphorylation of serine residues of the β3 subunit by PKC (Jovanovic et al., 2004).

Phosphorylation by PKC can also alter the actions of allosteric modulators on the $GABA_A$ receptors. Some studies suggest that PKC activation increases the potentiation of the GABA currents by benzodiazepines, barbiturates, and neurosteroids in *Xenopus* oocytes (Leidenheimer et al., 1993; Leidenheimer and Chapell, 1997). Conversely, other studies suggest that PKC activation induces a decreased coupling among the allosteric sites on the $GABA_A$ receptors. Experiments performed in neuron-like NT2-N cells demonstrated that PKC activation produced a decrease in the potency of benzodiazepines' ability to stimulate GABA currents (Gao and Greenfield, 2005). Mutant mice lacking PKCε are more sensitive to the acute behavioral effects of barbiturates, benzodiazepines, and neurosteroids. This supersensitivity is accompanied by an increase in the potentiation of muscimol-induced chloride influx by neurosteroids (Hodge et al., 1999, 2002). These observations suggest that PKCε inhibits $GABA_A$ receptor modulation by positive allosteric modulators.

It has been hypothesized that continuous exposure to GABA triggers a chain of events that alters the number and function of $GABA_A$ receptors (Barnes, 1996; Bateson,

2002). Initial activation of $GABA_A$ receptors is rapidly followed by desensitization. The rate and extent of desensitization depend on the receptor subunit composition. For example, $\alpha1\beta3\gamma2L$ $GABA_A$ receptors desensitize extensively (~90%) at fast ($\tau \sim 10$ msec), intermediate ($\tau \sim 150$ msec), and slow ($\tau \sim 1{,}500$ msec) rates (Bianchi and Macdonald, 2002). The molecular mechanism of desensitization remains unclear. Prolonged desensitization could provide the signal for uncoupling. Uncoupling occurs through a two-step mechanism: a rapid initiation process ($t_{1/2} = 3$ min) that requires $GABA_A$ receptor activation, followed by a delayed-onset process ($t_{1/2} = 12$ hr) that is independent of the presence of neurotransmitter (Gravielle et al., 2005). Longer activation of $GABA_A$ receptors would finally produce transcriptional repression of $GABA_A$ receptor subunit genes that would result in a decrease in the number of receptors (Lyons et al., 2000; Russek et al., 2000).

In summary, the results presented here indicate that the uncoupling of the GABA/benzodiazepine sites, induced by briefly exposing neocortical neurons to GABA, is mediated by a posttranscriptional mechanism involving the activation of the PKA and PKC cascades. This receptor uncoupling is accompanied by an increase in the phosphorylation state of the $GABA_A$ receptor $\gamma2$ subunits at serine residues by PKC. These results suggest that GABA-induced uncoupling is the result of a change in the phosphorylation state of $GABA_A$ receptors. The role of PKA activation on the uncoupling mechanism is unknown. PKA may phosphorylate a protein other than the $GABA_A$ receptor, such as a receptor-associated protein, which can contribute to the development of the receptor uncoupling. The receptor uncoupling may represent a homeostatic mechanism to downregulate $GABA_A$ receptor function under physiological, pathological, and pharmacological conditions in which the receptors are persistently activated. This negative regulatory mechanism may be important in controling both phasic inhibition mediated by synaptic $GABA_A$ receptors and tonic inhibition via extrasynaptic receptors. Results from behavioral experiments in knockin mice have suggested that $\alpha5$-containing $GABA_A$ receptors, a receptor population that can be found at extrasynaptic sites, are required for the development of tolerance to the sedative actions of diazepam (van Rijnsoever et al., 2004). These results might suggest the involvement of extrasynaptic $GABA_A$ receptors in the adaptive mechanism induced by prolonged exposure to benzodiazepines.

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