THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 22, Issue of June 3, pp. 20954–20960, 2005
Printed in U.S.A.
© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.

GABA Induces Activity Dependent Delayed-onset Uncoupling of GABA/Benzodiazepine Site Interactions in Neocortical Neurons*

Received for publication, January 5, 2005, and in revised form, March 31, 2005
Published, JBC Papers in Press, April 1, 2005, DOI 10.1074/jbc.M500131200

María C. Gravielle, Ramona Faris, Shelley J. Russek, and David H. Farb‡

From the Laboratory of Molecular Neurobiology, Department of Pharmacology, Boston University School of Medicine, Boston, Massachusetts 02118

Changes in the function of type A γ-aminobutyric acid receptors ($GABA_A$Rs) are associated with neuronal development and tolerance to the sedative-hypnotic effects of $GABA_A$R positive modulators. Persistent activation of $GABA_A$Rs by millimolar concentrations of GABA occurs under physiological conditions as GABAergic fast-spiking neurons in neocortex and cerebellum exhibit basal firing rates of 5 to 50 Hz and intermittent rates up to 250 Hz, leaving a substantial fraction of synaptic receptors occupied persistently by GABA. Persistent exposure of neurons to GABA has been shown to cause a down-regulation of receptor number and an uncoupling of GABA/benzodiazepine (BZD) site interactions with a half-life of ~24 h. Here, we report that a single brief exposure of neocortical neurons in primary culture to GABA for 5–10 min ($t_{½}$ = 3.2 ± 0.2 min) initiates a process that results in uncoupling hours later ($t_{½}$ = 12.1 ± 2.2 h). Initiation of delayed-onset uncoupling is blocked by co-incubation with picrotoxin or α-amanitin but is insensitive to nifedipine, indicating that uncoupling is contingent upon receptor activation and transcription but is not dependent on voltage-gated Ca^{2+} influx. Delayed-onset uncoupling occurs without a change in receptor number or a change in the proportion of α1 subunit pharmacology, as zolpidem binding affinity is unaltered. Such activity dependent latent modulation of $GABA_A$R function that manifests as delayed-onset uncoupling may be relevant to physiological, pathophysiological, and pharmacological conditions where synaptic receptors are transiently exposed to GABA agonists for several minutes.

Enhanced tolerance to the anticonvulsant and sedative/hypnotic *versus* anxiolytic effects of benzodiazepines (BZD)[1] is an important characteristic of their therapeutic effects (1–3). Attempts to uncover the molecular mechanism(s) that underlie tolerance to chronic *in vivo* administration of BZDs began 21 years ago with the discovery that a subsensitivity of allosteric interactions between the GABA and BZD recognition sites occurs after chronic *in vivo* administration of diazepam to rats (4). This was subsequently referred to as uncoupling of allosteric interactions based on experiments in primary cultures of chick brain (5).

Surprisingly, a single dose of diazepam results in subsensitivity after only 12 h (6). These results suggest that $GABA_A$R subsensitivity is produced via an interaction of diazepam with GABA-mediated synaptic transmission because BZDs potentiate the $GABA_A$R-mediated response (7).

In a similar fashion, a single convulsive dose of pentylenetetrazole causes a reduction in the GABA potentiation of BZD binding *in vivo* and this is accompanied by selective decreases in subunit-subtype mRNA levels without down-regulation of receptor number (8). These studies suggest that uncoupling between GABA and BZD binding sites can occur *in vivo* under pharmacologically relevant conditions and without alteration receptor number.

Chronic treatment with flurazepam induces tolerance and yields subunit-specific changes in the levels of region-specific $GABA_A$R subunit mRNAs and proteins (9–11), consistent with the hypothesis that persistent $GABA_A$R activation can regulate $GABA_A$R subunit gene expression. Whether such changes in gene expression underlie tolerance to drug administration remains unknown.

Insight into the potential molecular and cellular mechanisms underlying adaptive changes of receptor function can be derived from studies using primary neuronal cultures expressing native $GABA_A$Rs. Such results have demonstrated that persistent exposure of cultured neurons to positive modulators of the GABA response, such as BZDs, produces uncoupling of allosteric interactions (5, 12–14) that is not accompanied by changes in receptor number (5). Prolonged exposure of neurons in culture to steroids such as pregnanolone, as well as barbiturates, also leads to uncoupling (5, 15–17) in the absence of regulation of receptor number (5, 15, 16). In contrast to BZD treatment, occupancy of $GABA_A$Rs by GABA induces a down-regulation in receptor number (15, 18, 19), a reduction in the allosteric interactions between GABA and BZD binding sites (homologous uncoupling), and a reduction in allosteric interactions between BZD and barbiturate recognition sites (heterologous uncoupling (15, 18).

Because exposure to BZDs, steroids, and barbiturates can cause uncoupling of allosteric modulatory sites on the $GABA_A$R without producing down-regulation, it has been postulated that down-regulation and uncoupling are controlled by different mechanisms of action. Down-regulation is most likely the product of a transcriptional repression of $GABA_A$R subunit genes (20, 21) that depends on activation of L-type voltage-gated calcium channels (22).

Previous studies from our laboratory demonstrate that chronic exposure of neurons to GABA induces down-regulation of receptor number with a $t_{½}$ of 25 h and uncoupling occurs with a similar time course ($t_{½}$ = 24 h) (18). Uncoupling is unlike down-regulation in that it is independent of voltage-

* This work was supported by the National Institute on Alcohol Abuse and Alcoholism and NICHD, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Boston, MA 02118. Tel.: 617-638-4480; Fax: 617-638-4329; E-mail: dfarb@bu.edu.

[1] The abbreviations used are: BZD, benzodiazepine; $GABA_A$R, type A γ-aminobutyric acid receptor; FNZ, flunitrazepam; IPSC, inhibitory postsynaptic current.

This paper is available on line at http://www.jbc.org

gated calcium channel activation (22).

Our initial reports using neurons in primary culture revealed a $t_{1/2}$ of 18 h for BZD-induced uncoupling but no uncoupling was detected for exposures of 6 h or less (5). Contrary to this result in neurons, BZD-induced uncoupling in transfected cell lines occurs more rapidly and appears to be independent of transcription. The results from stably transfected Ltk^- fibroblast (uncoupling a $t_{1/2}$ = 32 min) and WSS-1 kidney (uncoupling $t_{1/2}$ = 3 h) cell lines expressing recombinant $GABA_A$Rs argue against a mechanism involving transcriptional regulation (23, 24). Similarly, the results obtained using the transiently transfected Sf9 insect cell line support internalization as a mechanism for uncoupling (25). These results appear to demonstrate that BZD-induced uncoupling is the result of a post-transcriptional regulatory mechanism.

Here we have examined the relationship between $GABA_A$R activation and the subsequent establishment of uncoupling in neocortical neurons. Delayed-onset uncoupling was found to be a transcription-dependent form of receptor regulation that occurs through a two-step mechanism: a rapid initiation process taking several minutes and requiring $GABA_A$R activation, followed by a delayed-onset transduction process independent of the presence of agonist.

MATERIALS AND METHODS

Cell Cultures—Primary cultures were prepared from 18-day-old rat embryos (Sprague-Dawley). Whole brains were quickly removed and placed in ice-cold Ca^{2+}/Mg^{2+}-free buffer. Cerebral cortexes were dissected under a microscope and placed in 5 ml of ice-cold Ca^{2+}/Mg^{2+}-free buffer. Tissue was finely minced, 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 B27 serum-free supplement, Invitrogen; 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine) and triturated again with a serological pipette. This cell suspension was added to a final volume of plating medium and plated at a density of 3/4 cortex per 100-mm tissue culture dish coated with poly-L-lysine (0.1 mg/ml). Cultures were incubated at 37 °C in 5% CO_2, and after 1 h, the medium was aspirated and replaced with serum-free medium.

Drug Treatment—Cultures, 2–3 × 100-mm dishes/treatment group, containing 14 ml of medium were treated on day 7 as follow: 7 ml of medium (conditioned medium) was removed and kept in the incubator, a small volume (70 μl) of concentrated drug stock or vehicle was added. Drug stocks were prepared in medium, except for nifedipine, which was dissolved in Me_2SO. The final concentration of Me_2SO in the medium was 0.1%. Dishes were returned to the incubator, and after 1 to 20 min, the medium was aspirated. Cultures were washed twice with 7 ml of Neurobasal™ medium, and the 7 ml of the conditioned medium was added back. Picrotoxin (100 μM) was added to this conditioned medium in the experiments that are shown in Table II. After 0–48 h of incubation, cells were collected. For experiments studying α-amanitin effects, cultures were preincubated for 50 min with the transcriptional inhibitor alone (5 μg/ml), and then GABA was added and co-incubated for 10 min. The cells were washed twice and harvested 0–96 h later.

Binding Assays—Cultures were rinsed with 7 ml of ice-cold PBSS (123 mM NaCl, 5.4 mM KCl, 11 mM NaH_2PO_4, 0.4 mM $MgSO_4$, 0.9 mM $CaCl_2$, 22.2 mM glucose, pH 7.4), and the cells were scraped from the dishes with a rubber policeman and centrifuged at 500 × *g* for 5 min. For osmotic shock studies, the pellet was homogenized in 10 volumes of double-distilled H_2O by 12 strokes using a glass Dounce homogenizer and centrifuged at 100,000 × *g* for 60 min. The final pellet was homogenized in 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and dialyzed against 4× 4 liter of potassium phosphate buffer (pH 7.0) overnight at 4 °C. For the rest of the experiments, the first pellet (after centrifugation at 500 × *g*) was directly homogenized in EDTA/phenylmethylsulfonyl fluoride buffer before dialysis.

Aliquots of homogenates (75–100 μg of protein) were incubated in a final volume of 0.5 ml for 60 min at 4 °C with [^{3}H]flunitrazepam ([^{3}H]FNZ, 0.01–10 nM, PerkinElmer Life Sciences) or [^{3}H]muscimol (1 to 200 nM, PerkinElmer Life Sciences) in saturation binding experiments. To measure GABA potentiation of BZD binding, 0.5 nM [^{3}H]FNZ was used alone or in the presence of 1 mM GABA. Competition binding assays were performed using 0.5 nM [^{3}H]Ro15 1788 with 1 nM to 0.1 mM zolpidem. Nonspecific binding was determined in the presence of 100 μM flurazepam, muscimol, or Ro15 1788 in [^{3}H]FNZ, [^{3}H]muscimol, and [^{3}H]Ro15 1788 binding experiments, respectively, and subtracted from total binding to yield specific binding. The reaction was stopped by the addition of 5 ml of ice-cold PBSS, immediately filtered under vacuum through glass fiber filters (Whatman GF/B), and washed 3 times with 5 ml of ice-cold PBSS. Radioactivity retained on the filters was quantified by liquid scintillation spectrometry in 5 ml of Ecolite(+) scintillation fluid (ICN).

Real-time PCR Experiments—Total RNA was extracted from rat cortical neuronal cultures using a RNeasy® midi kit (Qiagen). Primers (Oligos Etc) were designed to amplify segments of rat $GABA_A$ receptor subunit cDNAs encoding amino acids located in the cytoplasmic loop between transmembrane domains M3 and M4 (an area with amino acid sequences divergent for the different subunits) using Primer Express software (Applied Biosystem). Rat $GABA_A$ receptor subunit probes were synthesized by Applied Biosystems (TaqMan probes). Ribosomal RNA probe and primers were purchased from Applied Biosystems. The sequence of primers and probes were: α1 primers, 5′-CCCCGGCTTGGCAACTA-3′ and 5′-CGGTTTTGTCTCAGGCTTGAC-3′; α1 probe, 5′-TGCTAAAAGTGCGACCATAGAACCGAAAGA-3′; α2 primers, 5′-GCATTACTGAAGTCTTCACCAACATC-3′ and 5′-CGAAAGAAAACATCTATTGTATATTCCATATC-3′; α2 probe, 5′-ATGTGACCAGTTTTGGCCCTGTCTCAGA-3′; α3 primers, 5′-CGCGACGGCCATGG-3′ and 5′-CAAATTCAATCAGTGCAGAAAATACAA-3′; α3 probe, 5′-CTGGTTCATGGCCGTCTGTTATGCC-3′; α4 primers, 5′-TGAAATCCTGAGGTTGAACAATATG-3′ and 5′-AGACAGATTTCTTTCCATTCCTGAAG-3′; α4 probe, 5′-TGGTCACCAAAGTTTGGACCCCTGAT-3′; α5 primers, 5′-GGCTCTTGGATGGCTATGACA-3′ and 5′-CACCTGCGTGATTCGCTCT-3′; α5 probe, 5′-CAGACTGCGGCCTGGGCTGG-3′; β1 primers, 5′-CCGGCAAGGGTCGCA-3′ and 5′-TCAGTCAAGTCAGGGATCTTCACT-3′; β1 probe, 5′-CCGCAGACGTGCCTCTCAGCTCA-3′; β2 primers, 5′-ACCCCAGGAGCACAATGC-3′ and 5′-AGGCAACCCAGCTTTCCG-3′; β2 probe, 5′-TGCCTATGATGCCTCCAGCATCCAGTA-3′; β3 primers, 5′-AGAGCATGCCCAAGGAAGG-3′ and 5′-AGGTGCGTCTTCTTGTGCG-3′; β3 probe, 5′-CTTCTGTCTCCCATGTACCGCCCATG-3′; γ1 primers, 5′-CCCAGGTCTCCATGCTGG-3′ and 5′-TTCCCCTTGAGGCATAGAAATG-3′; γ1 probe, 5′-TACTCTGATTCCCATGAACA-3′; γ2S primers, 5′-AAACCCTGCCCCTACCATTG-3′ and 5′-GGCATTGTTCATTTGGATCGT-3′; γ2S probe, 5′-TCCGTCCCAGATCAG-3′.

Quantitative one-step real-time PCR was performed in an ABI prism Applied Biosystems 7900HT Sequence Detection System using a QuantiTect™ Probe RT-PCR kit (Qiagen). Standard curves for relative quantification were generated with 1 to 500 ng of total RNA isolated from control cultures (treated with vehicle). Total RNA (10–40 ng) from cultures treated with GABA was tested. Reactions were performed in triplicate in a total volume of 50 μl containing QuantiTect RT mixture, QuantiTect Probe RT-PCR master mixture, 250 μM receptor probe, 900 nM receptor primers, 1 μM rRNA probe, and 250 nM rRNA primers. Two aliquots of 20 μl/reaction were loaded in a 384-well plate. Incubation conditions were 48 °C for 30 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. The relative amount of each $GABA_A$ receptor subunit RNA was normalized to the relative amount of rRNA (internal control).

Data Analysis—Coupling represents the potentiation of [^{3}H]FNZ binding by GABA and was estimated as: (% $potentiation_{treated}$/% $potentiation_{control}$) × 100. Uncoupling was defined as the decrease in GABA-potentiated [^{3}H]FNZ binding and calculated as: [1 − (% $potentiation_{treated}$/% $potentiation_{control}$)] × 100. Concentration effect, time course, and saturation binding data were analyzed by computer-aided nonlinear regression.

RESULTS

We have previously reported that 48-h exposure of primary neuronal cultures to 1 mM GABA results in down-regulation of the number of BZD binding sites ($t_{1/2}$ = 25 h) and an uncoupling between binding sites for GABA and BZDs ($t_{1/2}$ = 24 h (15, 18, 19)). To investigate whether the continuous presence of GABA during the 48-h period is required to induce $GABA_A$R regulation, we studied the effect of brief incubations of rat cerebral cortical neurons with GABA. Neurons were exposed to 1 mM GABA for 0–20 min, whereupon GABA was removed, cells returned to the incubator for 48 h and then harvested. Such brief exposures to GABA ($t_{1/2}$ = 3.2 min) are sufficient to induce a decrease in the potentiation of [^{3}H]FNZ binding by GABA (46 ± 6% maximal uncoupling, Fig. 1) without producing a

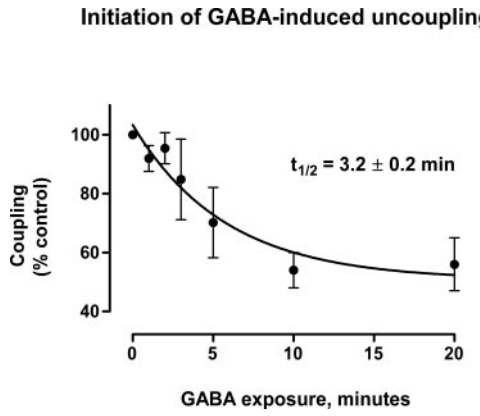

FIG. 1. **Initiation of delayed-onset GABA-induced uncoupling.** Cultures were incubated with vehicle or 1 mM GABA for the indicated times, washed twice, returned to the incubator, and cells were harvested 48 h later. The *points* represent the mean ± S.E. of four independent determinations.

change in the affinity and number of [^{3}H]muscimol and [^{3}H]FNZ binding sites (Table I).

Blockade of uncoupling by inclusion of picrotoxin (100 μM) during incubation demonstrates that uncoupling depends on GABA$_A$R activation (Fig. 2). Brief exposure of neurons to GABA induces uncoupling in a concentration-dependent manner with a half-maximal concentration of 460 ± 40 μM (Fig. 3).

To investigate whether the incubation period after exposure to GABA is required to produce uncoupling, cultures were incubated with 1 mM GABA for 10 min, washed, and then incubated for different times (0 to 96 h) in the absence of GABA (Fig. 4). We did not detect uncoupling when cells were harvested immediately after GABA exposure; however, uncoupling increased with incubation time, reaching a maximum at 24 to 48 h. These results suggest that an incubation period of at least 24 h is necessary to produce uncoupling. A reversion phase followed the peak of uncoupling, and uncoupling was lost after a 72-h incubation period. In contrast, uncoupling produced by continuous exposure to GABA persisted for at least 84 h in GABA (18).

To ensure that activation of the GABA$_A$R did not occur from residual GABA that may not have been removed by the washing procedure, we added picrotoxin to the medium after brief GABA exposure (Table II) and uncoupling (38 ± 5%) was not significantly different from control ($p < 0.05$). This demonstrates that initiation of delayed-onset uncoupling is because of the presence of the agonist exclusively during the initial 10 min of incubation.

We have previously demonstrated that GABA induces Ca^{2+} influx in embryonic brain neurons kept in culture. Moreover, blockade of L-type voltage-gated Ca^{2+} channels by nifedipine inhibits down-regulation of GABA$_A$R number (22). However, nifedipine does not prevent uncoupling induced by the 48-h treatment with GABA (22). To ask whether Ca^{2+} influx mediates fast GABA-induced initiation of delayed-onset uncoupling, we co-incubated cultures with nifedipine during the initiation phase (Fig. 2). Application of nifedipine failed to inhibit delayed-onset uncoupling, suggesting that the uncoupling mechanism induced by either a pulse application of GABA or persistent exposure to GABA is independent of L-type Ca^{2+} channel activation.

Results from Ali and Olsen (25) performed in an insect cell line suggest that uncoupling involves a receptor internalization process induced by the acidic environment inside intracellular compartments. To investigate whether an internalization mechanism is responsible for GABA-induced uncoupling in neurons, a similar procedure was performed as described by Ali and Olsen (25) where intracellular compartments that persisted in membrane homogenates were lysed by osmotic shock. Results from these experiments indicate that such treatment does not inhibit uncoupling (Table III), suggesting that a receptor internalization process seen in insect cell lines may not be related to uncoupling in neurons.

Studies from cell lines suggest that uncoupling is the result of a post-transcriptional regulatory process (23–25). To ask whether a transcriptional event is part of the uncoupling mechanism in neurons, we studied the effect of α-amanitin, a potent inhibitor of transcription, on the initiation phase of GABA exposure. Delayed-onset uncoupling was completely prevented when α-amanitin was added to the cultures 50 min before the addition of GABA and left in the medium during the 10-min GABA exposure period (Table III). Incubation of neurons with α-amanitin alone did not produce a significant effect on the potentiation of [^{3}H]FNZ binding by GABA. These results show that uncoupling is contingent upon transcription.

Brief exposure to GABA does not produce a change in the number of BZD binding sites (Table I) consistent with the fact that subunit mRNA levels specific to the γ2 subunit do not change. Uncoupling in the absence of down-regulation, however, could be because of altered expression of GABA$_A$Rs that display different strengths in the coupling between their GABA and BZD modulatory binding sites ($\alpha3 > \alpha1$ (26–28)). As a first step to determine whether brief exposure to GABA produces a change in the expression of receptors with different coupling efficiencies, we used a ligand binding assay to detect whether there was an increase in the proportion of zolpidem-sensitive receptors, based on the fact that zolpidem shows selectivity for GABA$_A$Rs containing the α1 subunit. Competition binding experiments were performed using [^{3}H]Ro15 1788 as radioligand in the presence of different concentrations of zolpidem (Fig. 5). The IC_{50} for zolpidem did not change significantly after delayed-onset uncoupling, strongly indicating that an increase in the proportion of α1-containing receptors is not responsible for uncoupling in neurons. This result is consistent with the fact that α1 mRNA levels do not increase in response to delayed-onset uncoupling. In contrast, although the receptor number remains unchanged there is a selective decrease ($p < 0.05$) in α1 (−29.6 ± 2.38%), α3 (−32.5 ± 6.4%), β1 (−24.5 ± 3.9%), β2 (−27.5 ± 6.6%), and β3 (−18.3 ± 3.4%) mRNA levels with no change in α2 (−2.3 ± 2.0%), α4 (−8.2 ± 4.5%), α5 (3.3 ± 5.7%), γ1 (−7.4 ± 1.9%), and γ2 (2.0 ± 6.5%) mRNAs. It remains to be determined whether decreased expression of α3 subunit mRNAs contributes functionally to delayed-onset uncoupling or whether altered transcription of regulatory factors such as protein kinases, phosphatases, or GABA$_A$R-associated proteins play a major role in the uncoupling process.

DISCUSSION

GABA$_A$R activation-dependent plasticity of GABAergic synapses has been described both in neonatal and adult brain and has been suggested to play a role in the development and function of neuronal networks (29–31). Evidence that GABA$_A$R activation alters GABA$_A$R subunit gene expression also comes from studies of neurons in culture (20, 21) and cultures treated with the GABA$_A$R positive endogenous modulator allopregnanolone at different stages of development (32).

Changes in the number and function of GABA$_A$Rs have been associated with different neuropsychiatric disorders such as anxiety, epilepsy, and schizophrenia. For example, in pseudopregnant rats, an anxiogenic syndrome induced by withdrawal from progesterone is accompanied by a decrease in basal and BZD potentiated GABA responses, and an increase in α4 containing GABA$_A$Rs (33). Augmentation of postsynaptic GABA responses accompanied by an increase in

TABLE I

Exposure of cortical neurons to 1 mM GABA for 10 min has no effect on [^{3}H]muscimol and [^{3}H]FNZ binding

Cultures were exposed for 10 min to 1 mM GABA (GABA) or vehicle (mock), washed twice, and incubated for 48 h in the absence of agonist. Data represent the mean ± S.E. of three independent experiments performed by triplicate.

	[^{3}H]Muscimol binding		[^{3}H]FNZ binding	
	K_d	B_{max}	K_d	B_{max}
	nM	*pmol/mg protein*	*nM*	*pmol/mg protein*
Mock	0.37 ± 0.04	37.51 ± 3.88	1.42 ± 0.10	0.38 ± 0.03
GABA	0.55 ± 0.07	48.10 ± 4.66	1.62 ± 0.08	0.37 ± 0.03

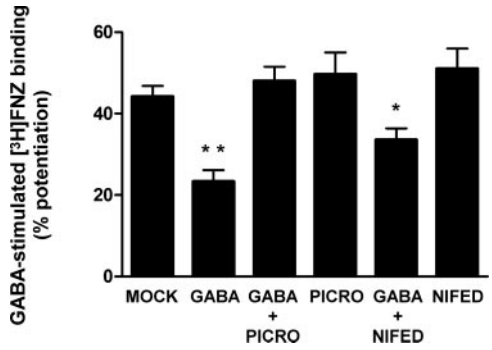

FIG. 2. **Initiation of delayed-onset GABA-induced uncoupling is mediated by GABA$_A$R activation.** Cells were incubated for 10 min with vehicle (*MOCK*), 1 mM GABA (*GABA*), 1 mM GABA plus 100 μM picrotoxin (*GABA + PICRO*), 100 μM picrotoxin (*PICRO*), 1 mM GABA plus 10 μM nifedipine (*GABA + NIFED*), or 10 μM nifedipine (*NIFED*), washed twice, and harvested 48 h later. Data represent the mean ± S.E. of 3–11 independent determinations. Significant differences: **, GABA *versus* MOCK ($p < 0.001$); *, GABA + NIFED *versus* NIFED ($p < 0.05$), one-way analysis of variance and Tukey's post-hoc test.

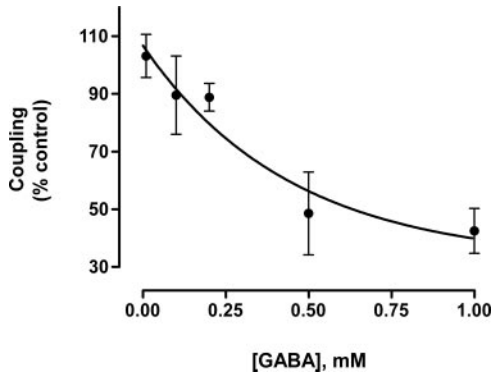

FIG. 3. **Concentration dependence for initiation of delayed-onset GABA-induced uncoupling.** Cultures were treated for 10 min with the indicated concentrations of GABA, washed twice, and cells were harvested 48 h later. The *points* represent the mean ± S.E. of three independent determinations.

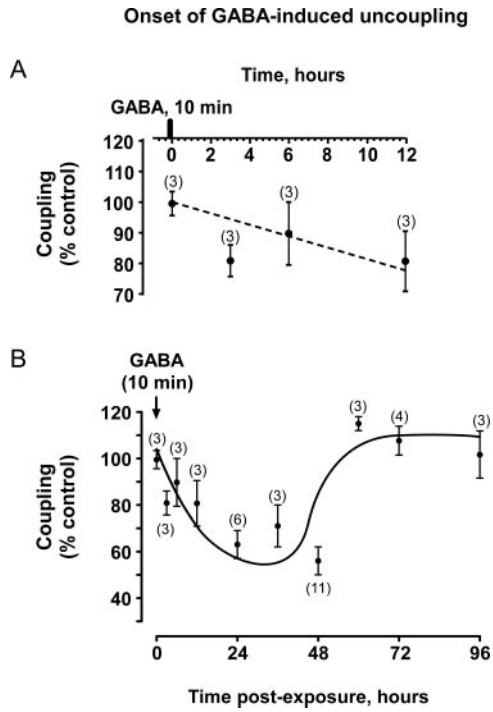

FIG. 4. **Time course for GABA-induced delayed-onset uncoupling.** Cells were incubated with vehicle or 1 mM GABA for 10 min, washed twice, and harvested after the indicated times. The *points* represent the mean ± S.E., where the number in parentheses indicates the number of independent experiments. *A*, initial phase of uncoupling onset. Data were fit using linear regression analysis (slope is significantly different from 0, $p = 0.05$). *B*, time course of uncoupling onset and reversion.

TABLE II

Delayed-onset uncoupling is not a consequence of residual GABA

Cells were exposed for 10 min to 1 mM GABA (GABA) or vehicle (mock), washed twice, incubated for 48 h with 100 μM picrotoxin, and harvested for the binding assay. Data represent the mean ± S.E. of three independent experiments performed by triplicate.

Treatment	GABA stimulated [^{3}H]FNZ binding	
	Potentiation	Coupling
	%	
Mock, PICRO	45 ± 4	100
GABA, PICRO	27 ± 1[a]	62 ± 5

[a] Significantly different from mock: $p < 0.02$ (Student's t test).

the number of synaptic GABA$_A$Rs in the hippocampus (34) has been reported in the kindling model of temporal-lobe epilepsy and an enhancement in GABA$_A$R binding is seen in certain areas of the cerebral cortex of the postmortem schizophrenic brain (35). Moreover, increases in extracellular GABA concentrations as a result of cerebral ischemia, through reversal of the GABA transporter (36), or through extrasynaptic release of GABA during development (37) suggest that the release of GABA may be increased during both physiological and pathological conditions.

We have previously shown that chronic treatment of primary neuronal cultures with GABA for 48 h induces down-regulation of receptor number and a decrease in the potentiation of BZD binding by GABA, a phenomenon we termed "uncoupling" (38). To attempt to separate GABA$_A$R activation from the subsequent cellular process resulting in uncoupling, we asked whether GABA-induced regulation of GABA$_A$R function depends upon the continuous presence of GABA during the 48-h incubation period. We found that brief exposure of rat cerebral cortical cultures to GABA for 5–10 min ($t_{½}$ = 3.2 min) induces a decrease in the potentiation of [^{3}H]FNZ binding by GABA (uncoupling) 48 h later. This delayed-onset GABA-induced uncoupling is prevented by co-incubating GABA during the 10-min initiation period with the non-competitive antagonist, picrotoxin. However, delayed-onset uncoupling is not blocked by addition of picrotoxin during the 48-h of incubation following

TABLE III
Effects of osmotic shock and inhibition of transcription on delayed-onset GABA-induced uncoupling

Cultures were treated as follow: 10 min with vehicle (mock); 10 min with 1 mM GABA (GABA); 50 min with α-amanitin alone and then 10 min with α-amanitin plus vehicle (α-amanitin); 50 min with α-amanitin alone and then 10 min with α-amanitin plus 1 mM GABA (GABA + α-amanitin). Cells were washed twice and harvested 48 h later. An osmotic shock treatment was performed in some of the membrane preparations as indicated. Data represent the mean ± S.E. of three to four independent experiments performed by triplicate.

Treatment	GABA stimulated [^{3}H]FNZ binding	
	Potentiation	Coupling
	%	
Mock	44 ± 3	100
GABA	23 ± 3[a]	53 ± 6
Mock, osmotic shock	39 ± 6	88 ± 10
GABA, osmotic shock	17 ± 4[a]	39 ± 6
α-Amanitin	36 ± 6	82 ± 9
GABA + α-amanitin	50 ± 5	112 ± 7

[a] Significantly different from mock: $p < 0.05$ (Student's t test).

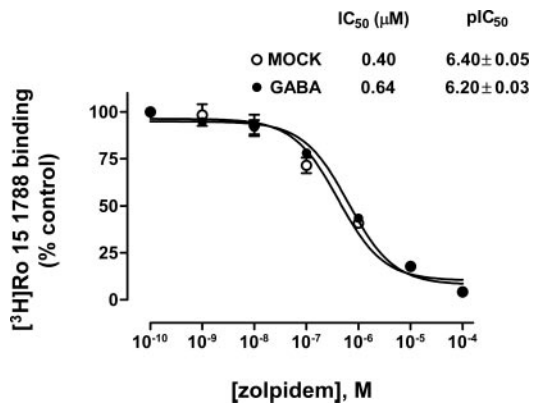

FIG. 5. **Effect of GABA-induced delayed-onset uncoupling on the pharmacology of GABA$_A$Rs.** Cells were incubated with vehicle (*MOCK*) or 1 mM GABA (*GABA*) for 10 min, washed twice, and harvested after 48 h. Competition binding experiments were performed using 0.5 nM [^{3}H]Ro15 1788 as a radioligand in the presence of different concentrations (1 nM-0.1 mM) of zolpidem. The *points* represent the mean ± S.E. of three independent determinations. IC_{50} values are the mean of the three independent curve fits, averaged as negative logarithms (pIC_{50}) to estimate the S.E.

GABA exposure. This observation indicates that receptor activation by GABA is required only for a brief time, and that delayed onset of uncoupling can occur without the continued presence of neurotransmitter action. Uncoupling of modulatory interactions by brief GABA exposure also occurs in the absence of any change in the number of GABA or BZD binding sites that had been detected earlier using chronic (48 h) GABA exposure. Because brief exposure to GABA induces delayed-onset uncoupling without down-regulation, these findings demonstrate that uncoupling and down-regulation are mediated by independent signaling pathways and provide a paradigm to selectively isolate the mechanism of uncoupling.

Previous results from our laboratory suggest that down-regulation and uncoupling are mediated by the activation of two distinct signal transduction pathways (22). Down-regulation is mediated by an elevation of intracellular Ca^{2+} concentrations through voltage-gated Ca^{2+} channels (22) and seems to involve transcriptional repression of GABA$_A$R subunit genes (20, 21), whereas uncoupling is independent of channel activation. In agreement with these results, we now report that uncoupling induced by a brief exposure to GABA is also resistant to nifedipine, a specific L-type voltage-gated Ca^{2+} channel blocker (Fig. 6).

Studies performed in cell lines expressing recombinant GABA$_A$Rs suggest that uncoupling is the result of post-transcriptional regulatory mechanisms (23–25). Ali and Olsen (25) have reported that uncoupling in an insect cell line is prevented by applying an osmotic shock treatment to membrane preparations, and produced when binding assays are performed at low pH. It is suggested that exposure of GABA$_A$Rs to an acidic environment in intracellular compartments, as a result of an internalization mechanism, induces uncoupling. However, using the same experimental procedure to lyse internal vesicles of cortical neurons, uncoupling is not prevented. This suggests that uncoupling in mammalian neurons may occur via a different process than in insect cell lines.

In contrast to studies using cell lines, we also show that uncoupling is blocked by incubation with the transcriptional inhibitor, α-amanitin, indicating that uncoupling in neurons is dependent upon a transcriptional event. The results of our studies are consistent with evidence demonstrating that stimulation of neurons with neurotransmitters can rapidly induce gene transcription (39).

Several mechanisms that involve transcriptional processes may play a role in uncoupling (40). Uncoupling may be the direct result of an alteration in the transcription of GABA$_A$R subunit genes. This could occur through GABA$_A$R stimulation of an intracellular signal transduction pathway that triggers specific transcription factor activation, or a change in the transcription of particular factors that regulate subunit-specific gene expression. It has been reported that the allosteric coupling between GABA and BZD binding sites depends on the subtype of α subunit present in the GABA$_A$R ($\alpha3 > \alpha1/\alpha2$) (26–28).

As an initial step to address the possibility that uncoupling is the result of a change in receptor subunit composition we examined whether increased expression of the α1 subunit, present in one of the most abundant BZD-sensitive GABA$_A$R, could account for the decrease in coupling between BZD/GABA binding sites. However, the zolpidem binding affinity remains unchanged indicating that the percentage of α1-containing receptors also remains constant. The results do not exclude the possibility that a decrease in the proportion of α3 containing receptors may account for delayed-onset uncoupling.

In the simple case where GABA$_A$Rs are composed of only one α subunit isoform a decrease in α3 gene expression might be compensated by an equal increase in gene expression for an alternative α subunit to maintain a fixed number of receptors. However, the results show that delayed-onset uncoupling is accompanied by significant decreases in several subunit mRNA levels (α1, α3, β1, β2, and β3) although some subunit mRNAs (α2, α4, α5, γ1, and γ2) do not change. This suggests that uncoupling does not occur because of a change in the proportion of $\alpha3\beta\gamma$ *versus* $\alpha1\beta\gamma$ containing receptors. Alternatively, in the case of receptors containing more than one α subunit isoform (referred to as heterologous α subunit receptor subtypes (41)) a decrease in α3 gene expression could occur in the absence of an increase in the gene expression of an alternative α subunit if that α subunit is in vast excess, such as has been described for α1 (42–44). In this instance, for example, $\alpha3\alpha3\beta2\gamma2$ and/or $\alpha1\alpha3\beta2\gamma2$ receptors could convert into $\alpha1\alpha1\beta2\gamma2$ receptors.

Delayed-onset uncoupling could also be the consequence of a mechanism in which brief exposure of GABA$_A$Rs to GABA triggers an intracellular cascade that modifies the transcription of a receptor regulatory factor. This regulatory factor might be a protein kinase or phosphatase that alters the function of GABA$_A$Rs by a phosphorylation or dephosphorylation reaction. Phosphorylation has been shown to regulate the function of the GABA$_A$R (45), and although there is no evidence to

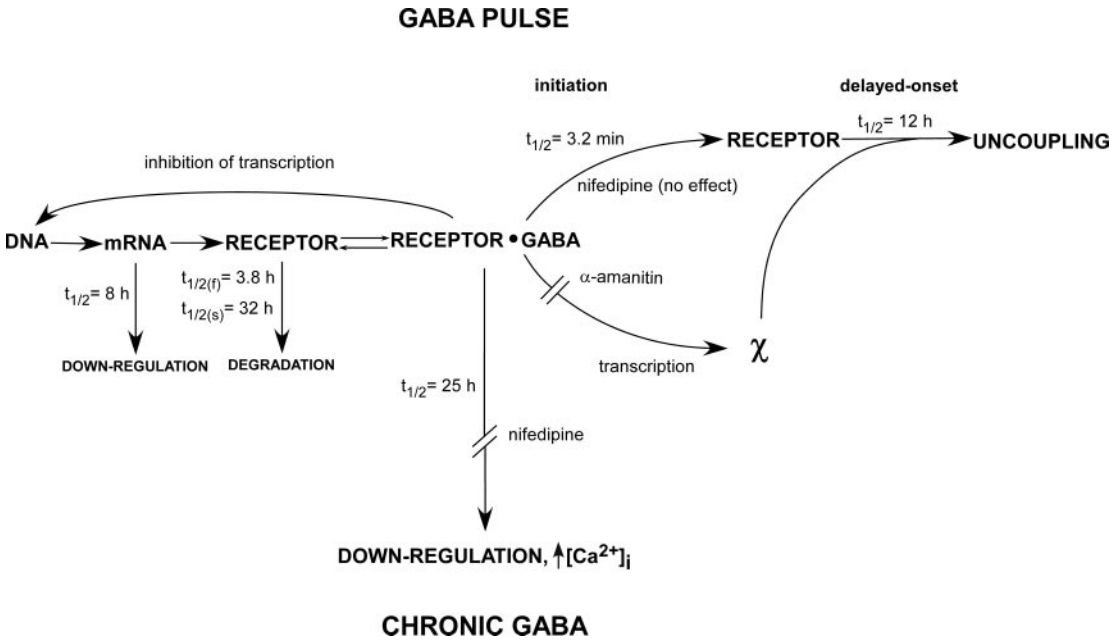

FIG. 6. **Diagrammatic representation of the cellular mechanisms involved in GABA-induced GABA$_A$R uncoupling and down-regulation.** Brief exposure of neocortical neurons to GABA for a few minutes leads to uncoupling of allosteric interactions between BZD and GABA recognition sites many hours later without an accompanying down-regulation of receptor number. Delayed-onset uncoupling is blocked by picrotoxin during the initial exposure period but not thereafter, indicating that initiation of uncoupling requires receptor activation. This phenomenon therefore comprises two steps: a rapid initiation ($t_{½}$ = 3.2 min) phase and a slow onset ($t_{½}$ = 12 h) phase. Delayed-onset uncoupling is blocked by α-amanitin, indicating that it involves transcriptional activation of an unknown gene(s) (χ). The GABA pulse also produces a delayed decrease in the levels of specific GABA$_A$R subunit gene mRNA levels, presumably because of this initial inhibition of transcription. Delayed-onset uncoupling is unlike receptor down-regulation induced by persistent exposure to GABA in that it is not blocked by nifedipine. The time constants for receptor and mRNA down-regulation, and receptor degradation were previously reported (20, 21, 64, 65).

indicate that this covalent modification can affect allosteric interactions between GABA and BZD binding sites, run-down of GABA$_A$R mediated modulation by positive modulators such as BZDs, barbiturates, and steroids is contingent on the loss of phosphorylation factors (46). Alternatively, the regulatory factor modified by exposure to GABA might be a GABA$_A$R-associated protein that controls the function, assembly, or trafficking of the GABA$_A$R (47). For example, the importance of the protein p130 in controlling receptor function has been revealed in electrophysiological studies performed in the hippocampus of p130 knock-out mice that show impairment in the modulation of GABA-induced currents by diazepam (48).

In summary, we have shown that brief exposure of cortical neurons to GABA induces delayed-onset uncoupling of allosteric interactions between benzodiazepine and GABA recognition sites through a receptor activation-dependent process. Uncoupling induced by exposure to GABA for a few minutes takes a day to unfold. Uncoupling does not involve a change in total GABA$_A$R number, but is dependent upon transcription and not explicable in terms of an increase in α1 subunit composition (Fig. 6).

These results may be relevant both to fast-spiking depolarizing neurons in the adult brain as well as embryonic brain. Although depolarizing GABA actions have been initially described in immature brain (49), the evidence indicates that depolarizing GABA-induced responses also occur in adult brain (50). In the latter report, the theta activity in hippocampal CA1 pyramidal cells of adult rats is mediated by GABAergic postsynaptic depolarization. In addition, under pathological conditions that involve intense GABA$_A$R activation, such as during epileptic seizures, GABA responses also become depolarizing in the mature brain, and depolarizing GABA responses occur in slices of hippocampus from patients with temporal lobe epilepsy (51–53). These results indicate that the uncoupling mediated by depolarizing GABA actions is relevant to both immature and adult brain.

The following evidence supports the conclusion that persistent activation of synaptic GABA$_A$R by millimolar concentrations of GABA can occur for minutes. A single action potential leads to the release of millimolar (1 to 5 mM) concentrations of neurotransmitter that are then cleared from the synaptic cleft biphasically with time constants of 100 μs and 2 ms (54). However, neurons exhibit a repetitive firing behavior with average frequencies ranging from 5 to 50 Hz (55–57) that can persist for many minutes (58). In particular, fast-spiking neurons in neocortex are GABAergic (59) and Purkinje cells, which are GABAergic and provide the main output of the cerebellum, exhibit a basal firing rate of 50 Hz but can fire intermittently at 250 Hz (60, 61).

Moreover, activation of GABA$_A$Rs long outlasts the presence of free GABA (62). This prolonged postsynaptic response to GABA release is produced by neurotransmitter "trapping" on the GABA$_A$R (63). After an inhibitory postsynaptic current (IPSC) the GABA response decays biphasically with time constants of approximately τ = 50 and 171 ms (62), indicating that when the firing frequency is 5.8 Hz (equivalent to one action potential every 171 ms) a substantial fraction (about 30%) of synaptic GABA$_A$Rs will be continuously occupied. The implications of this are even more significant when it is considered that ongoing Purkinje cell-mediated inhibition is occurring at a rate of one IPSC every 20 ms and up to one IPSC every 4 ms. These results lead to the surprising conclusion that in the absence of an adaptive mechanism GABA$_A$Rs would be tonically activated all of the time. This would clearly be impossible from a systems standpoint as the information contained within the frequency of IPSC activity would be lost, leading us to propose the existence of a negative regulatory mechanism.

Under certain pathological conditions, the GABA$_A$R regulation produced by exposure to GABA on a time scale of minutes can be even more evident. For example, fast-spiking GABAergic neurons in cat neocortex fire at a very high frequency (800 Hz or one IPSC per 1.25 ms) during electro-

graphic seizures (52). In addition, extracellular concentrations of GABA increase 600-fold in ischemic human brain (36). It will, therefore, be important to determine the extent to which delayed-onset uncoupling of GABA/BZD site interactions may alter physiological and pathophysiological aspects of nervous system function.

REFERENCES

1. Swift, C. G., Swift, M. R., Hamley, J., Stevenson, I. H., and Crooks, J. (1984) *Age Ageing* **13,** 335–343
2. Seidel, W. F., Cohen, S. A., Wilson, L., and Dement, W. C. (1985) *Psychopharmacology* (*Berl.*) **87,** 194–197
3. Haigh, J. R., and Feely, M. (1988) *Trends Pharmacol. Sci.* **9,** 361–366
4. Gallager, D. W., Lakoski, J. M., Gonsalves, S. F., and Rauch, S. L. (1984) *Nature* **308,** 74–77
5. Roca, D. J., Schiller, G. D., Friedman, L., Rozenberg, I., Gibbs, T. T., and Farb, D. H. (1990) *Mol. Pharmacol.* **37,** 710–719
6. Holt, R. A., Bateson, A. N., and Martin, I. L. (1999) *J. Neurochem.* **72,** 2219–2222
7. Choi, D. W., Farb, D. H., and Fischbach, G. D. (1977) *Nature* **269,** 342–344
8. Walsh, L. A., Li, M., Zhao, T. J., Chiu, T. H., and Rosenberg, H. C. (1999) *J. Pharmacol. Exp. Ther.* **289,** 1626–1633
9. Tietz, E. I., Zeng, X. J., Chen, S., Lilly, S. M., Rosenberg, H. C., and Kometiani, P. (1999) *J. Pharmacol. Exp. Ther.* **291,** 932–942
10. Tietz, E. I., Huang, X., Chen, S., and Ferencak, W. F., 3rd (1999) *Neuroscience* **91,** 327–341
11. Chen, S., Huang, X., Zeng, X. J., Sieghart, W., and Tietz, E. I. (1999) *Neuroscience* **93,** 33–44
12. Prasad, A., and Reynolds, J. N. (1992) *Brain Res.* **591,** 327–331
13. Hu, X. J., and Ticku, M. K. (1994) *Mol. Pharmacol.* **45,** 618–625
14. Hu, X. J., and Ticku, M. K. (1994) *J. Pharmacol. Exp. Ther.* **270,** 485–490
15. Friedman, L. K., Gibbs, T. T., and Farb, D. H. (1996) *Brain Res.* **707,** 100–109
16. Friedman, L., Gibbs, T. T., and Farb, D. H. (1993) *Mol. Pharmacol.* **44,** 191–197
17. Follesa, P., Serra, M., Cagetti, E., Pisu, M. G., Porta, S., Floris, S., Massa, F., Sanna, E., and Biggio, G. (2000) *Mol. Pharmacol.* **57,** 1262–1270
18. Roca, D. J., Rozenberg, I., Farrant, M., and Farb, D. H. (1990) *Mol. Pharmacol.* **37,** 37–43
19. Montpied, P., Ginns, E. I., Martin, B. M., Roca, D., Farb, D. H., and Paul, S. M. (1991) *J. Biol. Chem.* **266,** 6011–6014
20. Lyons, H. R., Gibbs, T. T., and Farb, D. H. (2000) *J. Neurochem.* **74,** 1041–1048
21. Russek, S. J., Bandyopadhyay, S., and Farb, D. H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8600–8605
22. Lyons, H. R., Land, M. B., Gibbs, T. T., and Farb, D. H. (2001) *J. Neurochem.* **78,** 1114–1126
23. Klein, R. L., Whiting, P. J., and Harris, R. A. (1994) *J. Neurochem.* **63,** 2349–2352
24. Wong, G., Lyon, T., and Skolnick, P. (1994) *Mol. Pharmacol.* **46,** 1056–1062
25. Ali, N. J., and Olsen, R. W. (2001) *J. Neurochem.* **79,** 1100–1108
26. Puia, G., Vicini, S., Seeburg, P. H., and Costa, E. (1991) *Mol. Pharmacol.* **39,** 691–696
27. Wafford, K. A., Whiting, P. J., and Kemp, J. A. (1993) *Mol. Pharmacol.* **43,** 240–244
28. Smith, A. J., Alder, L., Silk, J., Adkins, C., Fletcher, A. E., Scales, T., Kerby, J., Marshall, G., Wafford, K. A., McKernan, R. M., and Atack, J. R. (2001) *Mol. Pharmacol.* **59,** 1108–1118
29. Caillard, O., Ben-Ari, Y., and Gaiarsa, J. L. (1999) *J. Physiol.* **518**, 109–119
30. Caillard, O., Ben-Ari, Y., and Gaiarsa, J. L. (1999) *J. Neurosci.* **19,** 7568–7577
31. Gaiarsa, J. L., Caillard, O., and Ben-Ari, Y. (2002) *Trends Neurosci.* **25,** 564–570
32. Poulter, M. O., Ohannesian, L., Larmet, Y., and Feltz, P. (1997) *J. Neurochem.* **68,** 631–639
33. Smith, S. S., Gong, Q. H., Li, X., Moran, M. H., Bitran, D., Frye, C. A., and Hsu, F. C. (1998) *J. Neurosci.* **18,** 5275–5284
34. Nusser, Z., Hajos, N., Somogyi, P., and Mody, I. (1998) *Nature* **395,** 172–177
35. Benes, F. M., Vincent, S. L., Marie, A., and Khan, Y. (1996) *Neuroscience* **75,** 1021–1031
36. Hutchinson, P. J., O'Connell, M. T., Al-Rawi, P. G., Kett-White, C. R., Gupta, A. K., Maskell, L. B., Pickard, J. D., and Kirkpatrick, P. J. (2002) *J. Neurol. Neurosurg. Psychiatry* **72,** 99–105
37. Takayama, C., and Inoue, Y. (2004) *Neurosci. Res.* **50,** 447–458
38. Roca, D. J., Schiller, G. D., and Farb, D. H. (1988) *Mol. Pharmacol.* **33,** 481–485
39. West, A. E., Griffith, E. C., and Greenberg, M. E. (2002) *Nat. Rev. Neurosci.* **3,** 921–931
40. Rabow, L. E., Russek, S. J., and Farb, D. H. (1995) *Synapse* **21,** 189–274
41. Benke, D., Fakitsas, P., Roggenmoser, C., Michel, C., Rudolph, U., and Mohler, H. (2004) *J. Biol. Chem.* **279,** 43654–43660
42. Baumgartner, B. J., Harvey, R. J., Darlison, M. G., and Barnes, E. M., Jr. (1994) *Brain Res. Mol. Brain Res.* **26,** 9–17
43. Miranda, J. D., and Barnes, E. M., Jr. (1997) *J. Biol. Chem.* **272,** 16288–16294
44. Mehta, A. K., and Ticku, M. K. (1999) *Brain Res. Mol. Brain Res.* **67,** 194–199
45. Brandon, N., Jovanovic, J., and Moss, S. (2002) *Pharmacol. Ther.* **94,** 113–122
46. Gyenes, M., Wang, Q., Gibbs, T. T., and Farb, D. H. (1994) *Mol. Pharmacol.* **46,** 542–549
47. Luscher, B., and Keller, C. A. (2004) *Pharmacol. Ther.* **102,** 195–221
48. Kanematsu, T., Jang, I. S., Yamaguchi, T., Nagahama, H., Yoshimura, K., Hidaka, K., Matsuda, M., Takeuchi, H., Misumi, Y., Nakayama, K., Yamamoto, T., Akaike, N., and Hirata, M. (2002) *EMBO J.* **21,** 1004–1011
49. Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J. L. (1989) *J. Physiol.* **416,** 303–325
50. Sun, M. K., Zhao, W. Q., Nelson, T. J., and Alkon, D. L. (2001) *J. Neurophysiol.* **85,** 269–279
51. Cohen, I., Navarro, V., Clemenceau, S., Baulac, M., and Miles, R. (2002) *Science* **298,** 1418–1421
52. Timofeev, I., Grenier, F., and Steriade, M. (2002) *Neuroscience* **114,** 1115–1132
53. Fujiwara-Tsukamoto, Y., Isomura, Y., Kaneda, K., and Takada, M. (2004) *J. Physiol.* **557,** 961–979
54. Clements, J. D. (1996) *Trends Neurosci.* **19,** 163–171
55. Mountcastle, V. B., Talbot, W. H., Sakata, H., and Hyvarinen, J. (1969) *J. Neurophysiol.* **32,** 452–484
56. Ranck, J. B., Jr. (1973) *Exp. Neurol.* **41,** 461–531
57. Hajos, N., Palhalmi, J., Mann, E. O., Nemeth, B., Paulsen, O., and Freund, T. F. (2004) *J. Neurosci.* **24,** 9127–9137
58. Czurko, A., Hirase, H., Csicsvari, J., and Buzsaki, G. (1999) *Eur. J. Neurosci.* **11,** 344–352
59. Connors, B. W., and Gutnick, M. J. (1990) *Trends Neurosci.* **13,** 99–104
60. Cheron, G., Gall, D., Servais, L., Dan, B., Maex, R., and Schiffmann, S. N. (2004) *J. Neurosci.* **24,** 434–441
61. Khaliq, Z. M., and Raman, I. M. (2005) *J. Neurosci.* **25,** 454–463
62. Jones, M. V., and Westbrook, G. L. (1995) *Neuron* **15,** 181–191
63. Bianchi, M. T., and Macdonald, R. L. (2001) *J. Neurosci.* **21,** 9083–9091
64. Borden, L. A., Czajkowski, C., Chan, C. Y., and Farb, D. H. (1984) *Science* **226,** 857–860
65. Borden, L. A., and Farb, D. H. (1988) *Mol. Pharmacol.* **34,** 354–362