0026-895X/90/010037-07$02.00/0

MOLECULAR PHARMACOLOGY, 37:37–43

Chronic Agonist Exposure Induces Down-regulation and Allosteric Uncoupling of the γ-Aminobutyric Acid/Benzodiazepine Receptor Complex

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Received July 14, 1989; Accepted October 16, 1989

SUMMARY

Using [^{3}H]flunitrazepam as a probe for the benzodiazepine-sensitive modulator site located on the γ-aminobutyric acid $(GABA)_A$ receptor complex, we have investigated the cellular regulation of the $GABA_A$ receptor in neuronal cultures derived from embryonic chick brain. Treatment of cultures with 1 mM GABA for 48 hr causes a reversible 35% decrease in the number of [^{3}H]flunitrazepam binding sites with no change in affinity. The EC_{50} for chronic GABA-induced down-regulation is 94 μM and the half-time is 25 hr. The effect of GABA is blocked by SR-95531, a $GABA_A$ receptor antagonist, and mimicked by muscimol but not baclofen. Consistent with the decrease in [^{3}H]flunitrazepam binding, chronic GABA exposure causes a 43% decrease in the binding of [^{35}S]*t*-butylbicyclophosphorothionate, a ligand for the receptor-associated chloride ionophore. In addition to chronic GABA-induced down-regulation, allosteric interactions between GABA and benzodiazepine recognition sites are uncoupled by 34%. The half-time and pharmacology for chronic GABA-induced uncoupling is indistinguishable from that for GABA-induced down-regulation, consistent with the hypothesis that the action of GABA at a common site induces both down-regulation and uncoupling.

Prolonged treatment with centrally acting drugs of various types leads, in many instances, to the development of tolerance to their effects. In certain cases this phenomenon may be ascribed to adaptive changes in the number and/or function of neurotransmitter receptors (1, 2).

Benzodiazepines are used extensively for their anticonvulsant, anxiolytic, and muscle relaxant properties. Unfortunately, the development of tolerance to these effects frequently limits their usefulness (3, 4). Benzodiazepines are thought to owe their therapeutically desirable properties to an ability to enhance those actions of the inhibitory neurotransmitter GABA that are mediated by the $GABA_A$ receptor (5–7). This receptor complex contains an integral transmembrane chloride channel, a GABA recognition site, and a number of allosteric regulatory binding sites through which several important pharmacological agents, including the benzodiazepines, act to alter GABA-mediated transmission (7–9).

Numerous studies, performed both *in vivo* and in culture, have focused on the effects of chronic benzodiazepine exposure on GABA/benzodiazepine-receptor number. These experiments have yielded conflicting results. Thus, chronic benzodiazepine exposure has been reported to cause no change (10), an increase (11), or a decrease (12–14) in receptor number and/or a change in the interaction between GABA and benzodiazepine binding sites (12–15).

Because it is likely that the effects of chronic benzodiazepine exposure reflect the prolonged potentiation of GABA action, we have sought to gain an insight into the fundamental mechanisms of GABA/benzodiazepine receptor regulation by examining the effects of prolonged GABA treatment directly. Specifically, we have studied the effects of chronic GABA exposure on embryonic chick brain neurons in primary dissociated culture. We report here that such treatment results in a reduction in the number of [^{3}H]flunitrazepam and [^{35}S]TBPS binding sites as well as a decrease in the allosteric coupling between remaining GABA and benzodiazepine recognition sites.

Experimental Procedures

Materials

[^{3}H]Flunitrazepam (85 Ci/mmol) and [^{35}S]TBPS (110.7 Ci/mmol) were purchased from Amersham; GABA and muscimol were from Sigma; baclofen and SR-95531 were from Research Biochemicals. The

Research support was provided by National Institutes of Health Grants NS-23140 and NS-22116.

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ABBREVIATIONS: GABA, γ-aminobutyric acid; PBSS, phosphate-buffered saline solution; TBPS, *t*-butylbicyclophosphorothionate; TCA, trichloroacetic acid.

benzodiazepine drugs were a gift of Hoffman-La Roche. Dialysis tubing was from Spectrapor and Liquiscint was obtained from National Diagnostics.

Methods

Cell cultures. Brains were removed from 7-day chick embryos (SPAFAS, Inc.) and placed in a Ca^{2+}- and Mg^{2+}-free saline solution (Puck's D_1G). The tissue was then minced into small fragments, incubated with trypsin (0.025%, 5 min, 25°), and centrifuged (800 rpm, 5 min). The pellet was resuspended in Eagle's Minimum essential medium [supplemented to a final concentration of 10% heat-inactivated horse serum (GIBCO), 5% chick embryo extract, 2.4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin], triturated with fire-polished Pastuer pipets, plated onto collagen-coated plastic culture dishes (approximately one brain per 100-mm dish or three 60-mm dishes), and maintained at 37° in 5% CO_2 and 100% humidity. Cytosine arabinoside (1 μM) was added after 1 day to control the proliferation of nonneuronal cells. One day later medium was replaced with fresh minimum essential medium that was supplemented as described above, with the exception of glucose (final concentration, 20 mM) and chick embryo extract (final concentration, 2.5%). Partial replacement of medium was carried out at intervals of 2 days thereafter. Cultures were generally used after 1 week.

Chronic treatment. Cultures were treated on day 7 by adding 100 μl of a concentrated drug stock to each dish, which contained 5 or 10 ml of growth medium (depending on dish size). For GABA, 1 day after this first treatment half the medium was removed and replaced with fresh medium containing the final concentration of drug desired. This treatment paradigm yielded reproducible results; more frequent supplementation did not increase the effects of chronic GABA exposure. All drugs were made up in PBSS (in mM: NaCl, 123; KCl, 5.4; NaH_2PO_4, 11; $MgSO_4$, 0.4; $CaCl_2$, 0.9; glucose, 22.2; pH 7.4), except veratridine, which was made up in ethanol (final concentration, 0.05%). Control cultures were treated with PBSS or the appropriate vehicle. In no case did drug treatment alter the morphological appearance of the cultures, as judged by phase-contrast microscopy (100× and 200× magnification).

Cell homogenate. At the end of the treatment period, cultures were washed once with ice-cold PBSS, scraped from culture dishes, and centrifuged (1000 rpm, 5 min), and the resulting pellet was resuspended in 1 mM EDTA/1 mM phenylmethylsulfonyl fluoride, 0.5 ml/100-mm culture dish (final protein concentration, approximately 6 mg/ml). To remove endogenous GABA and drugs added during treatment, the homogenate was then dialyzed against 4 liters of 25 mM potassium phosphate buffer (pH 7.0) for 24 hr at 4°, with four changes of buffer. Following dialysis, the homogenate was diluted with PBSS to a final concentration of 1.5 mg of protein/ml and used immediately. Protein concentrations were determined by the method of Lowry *et al.* (16). Membranes used for studies of enhancement of [^{3}H]flunitrazepam binding with GABA were not frozen, because we have found that freezing tends to reduce enhancement.

Reversible binding. Samples of cell homogenate (150 μg protein) were incubated in PBSS (total volume, 0.5 or 1 ml) for 60 min at 4° in the presence of 0.1–15 nM [^{3}H]flunitrazepam. The incubation was terminated upon the addition of 5 ml of ice-cold PBSS, rapidly followed by filtration over a Whatman GF/B glass fiber filter, which was then washed three times with a total of 15 ml of PBSS. Unless indicated otherwise, nonspecific binding (generally 10–20% of total) was determined in the presence of 100 μM flurazepam and was subtracted from total binding to yield specific binding (saturation experiments were performed in triplicate; all other determinations were the result of quintuplicates). Radioactivity retained on filters was determined by liquid scintillation counting in 5 ml of Liquiscint (National Diagnostics).

In experiments investigating possible changes in benzodiazepine binding site number, 15 nM [^{3}H]flunitrazepam was used. In our hands, this represents a concentration that is 7 times the K_D. The decrease in [^{3}H]flunitrazepam binding is expressed as a percentage of control, calculated as follows:

$$(1 - B_{treated}/B_{control}) \times 100$$

where $B_{treated}$ represents binding in cell homogenate derived from treated cultures and $B_{control}$ represents binding in cell homogenate derived from control cultures.

For the measurement of GABA potentiation of benzodiazepine binding, 1 nM [^{3}H]flunitrazepam was used. The ability of GABA (50 μM, unless stated otherwise) to potentiate 1 nM (unless stated otherwise) [^{3}H]flunitrazepam binding (percentage of potentiation) was used as an indicator of the degree to which the GABA and benzodiazepine sites are "coupled."

Data for enhancement of benzodiazepine ligand binding by GABA is expressed as percentage of potentiation of [^{3}H]flunitrazepam binding, defined as follows:

$$\% \text{ Potentiation} = \left(\frac{\text{specific binding in presence of GABA}}{\text{specific binding in absence of GABA}} - 1\right) \times 100$$

In some cases, change in enhancement of [^{3}H]flunitrazepam binding by GABA after chronic treatment is expressed as percentage of uncoupling, defined as:

$$\% \text{ Uncoupling} = \left(1 - \frac{(\% \text{ potentiation})_{treated}}{(\% \text{ potentiation})_{control}}\right) \times 100$$

[^{35}S]TBPS binding. [^{35}S]TBPS binding was performed in a manner similar to that described by Squires and Saederup (17). Cell homogenates were dialyzed as described earlier. The last change of dialysate, however, was 5 mM Tris·HCl in place of 25 mM potassium phosphate buffer. Samples of 400 μg of protein were incubated in 50 mM Tris·HCl/200 mM KBr (total volume, 0.5 ml) for 120 min at room temperature in the presence of 2 or 25 nM [^{35}S]TBPS. The 25 nM [^{35}S]TBPS was supplemented with 25 nM TBPS to give a final concentration of 50 nM TBPS. TBPS was made up in ethanol (final concentration, 0.005%). The incubation was terminated upon the addition of 5 ml of ice-cold 50 mM Tris·HCl, followed by filtration over Whatman GF/B glass fiber filters, which were then washed two times with a total of 10 ml of Tris/HCl. Nonspecific binding (approximately 15% of total) was determined in the presence of 50 μM picrotoxinin and subtracted from total binding to yield specific binding. All determinations were performed in quintuplicate. Radioactivity retained on filters was counted as described above.

Protein synthesis. Synthesis of general cellular protein was measured as previously described (18). Briefly, cultures were incubated for 1 hr at 37° with [^{35}S]methionine and then washed thoroughly to remove unincorporated radioactivity. Cells were solubilized with 0.2% sodium dodecyl sulfate/2% Nonidet P40/8 M urea (2 ml/dish), aliquots were removed, and ice-cold TCA was added to a final concentration of 10%. TCA-precipitable radioactivity was determined by filtration through Whatman GF/B filters, which were washed at 0°, under vacuum, with PBSS/10% TCA. Radioactivity retained on the filters was determined by liquid scintillation counting.

Data analysis. Data were analyzed by computer using a nonlinear regression program written by Dr. Terrell Gibbs, (Dept. of Anatomy and Cell Biology, SUNY Health Science Center at Brooklyn), based on the "patternsearch" algorithm of Hooke and Jeeves (19). Data were weighted according to the standard error. Results are presented as the mean ± standard error. Significance was determined using Student's *t* test.

Results

Exposure of 7-day embryonic chick brain cells to 0.5 mM GABA for 48 hr resulted in a decrease in the B_{max} of [^{3}H] flunitrazepam binding (Fig. 1). The pooled results from four independent experiments yielded a K_D of 1.75 ± 0.2 nM and a B_{max} of 120 ± 20 fmol/mg of protein for control, whereas cell

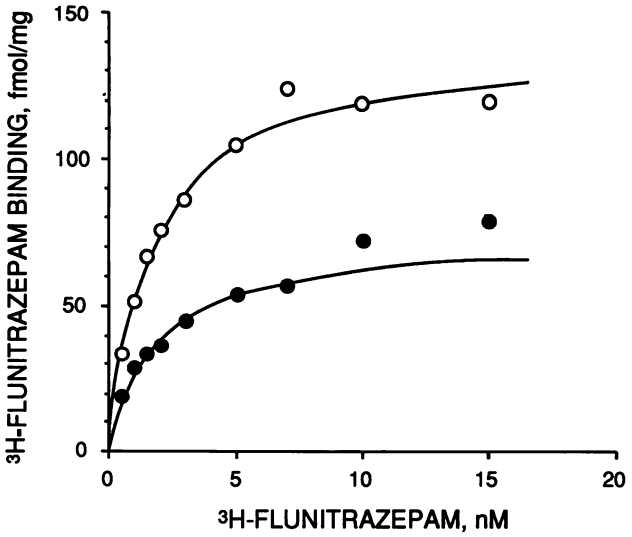

Fig. 1. Chronic GABA exposure induces a decrease in the number of benzodiazepine recognition sites. Seven-day chick embryo brain cultures were treated for 48 hr with 0.5 mM GABA (ten 100-mm dishes/group). Nonspecific binding was defined using 10 μM flurazepam plus 2.5 μM Ro5-4864. The *points* are the mean of three to five replicates. The standard errors are contained within the *symbols*. This figure shows a typical experiment. The *lines* represent computer fits to the data using the Michaelis-Menten equation. For this experiment B_{max} was 138 fmol/mg for control and 73 fmol/mg for GABA treated. The K_D was 1.7 and 1.6 nM for control and treated cultures, respectively. Similar results were seen in a total of four experiments. ○, Control; ●, GABA-treated.

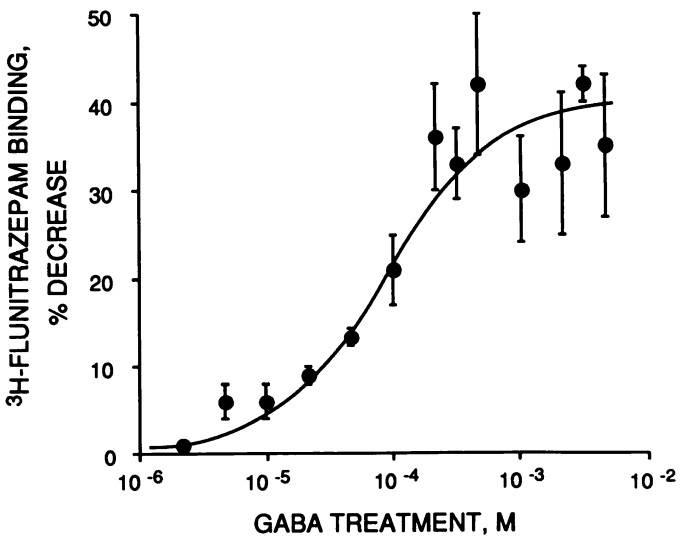

Fig. 2. Down-regulation of the GABA/benzodiazepine receptor obeys Michaelis-Menten kinetics. In each experiment five, six, or seven 100-mm dishes were treated for 48 hr with the indicated concentrations of GABA. The binding of 15 nM [^{3}H]flunitrazepam was measured as described in Experimental Procedures. Each *point* represents the mean ± standard error of three to eight experiments. The *line* drawn is a computer fit to the data using the logistic equation. The EC_{50} = 94 μM, n = 0.96, and the maximum decrease in [^{3}H]flunitrazepam binding is 42.2%.

homogenates derived from treated cells yielded a K_D of 1.8 ± 0.04 nM and a B_{max} of 78 ± 18 fmol/ml of protein. This represents a 35% decrease in the number of binding sites ($p <$ 0.05, paired Student's t test) with no change in affinity. Homogenates derived from cells treated chronically with 1 mM GABA for 48 hr also showed a decrease in the binding of [^{35}S]TBPS, a noncompetitive GABA antagonist (9) that binds at a site closely associated with the GABA-gated chloride ionophore (20, 21). The binding of 2 nM [^{35}S]TBPS decreased 42.8 ± 4%, whereas the binding of 50 nM [^{35}S]TBPS decreased 42.7 ± 3% (three experiments).

The decrease in 15 nM [^{3}H]flunitrazepam binding caused by chronic GABA exposure was dose dependent (Fig. 2). The GABA dose-response curve exhibited an EC_{50} of 94 μM, a Hill slope of 0.96, and a maximum decrease in [^{3}H]flunitrazepam binding of 42%. Fig. 3 demonstrates that the chronic GABA-induced decrease in [^{3}H]flunitrazepam binding was also time dependent. Data were fit to a single exponential with $t_{½}$ = 25 hr and a maximum down-regulation of 40%.

To determine whether the GABA-induced decrease in [^{3}H]flunitrazepam binding was reversible, cells were treated for 48 hr with 1 mM GABA, washed, and then allowed to recover for 36 hr (Fig. 4). The GABA-induced reduction in binding recovered from 33 ± 2% to 9 ± 6%, representing a recovery of approximately 70%. This suggests that the decrease in binding was not due to cell death or some other irreversible mechanism.

When the incorporation of [^{35}S]methionine was measured, as an indicator of general cell protein synthesis, no difference was observed between untreated and GABA-treated cells. In cells treated with 1 mM GABA for 48 hr, [^{35}S]methionine incorporation was 95 ± 7% of control (four experiments). Therefore, GABA-induced down-regulation was not a reflection of a nonspecific decrease in protein synthesis.

In initial experiments, bicuculline (100 μM) partially blocked

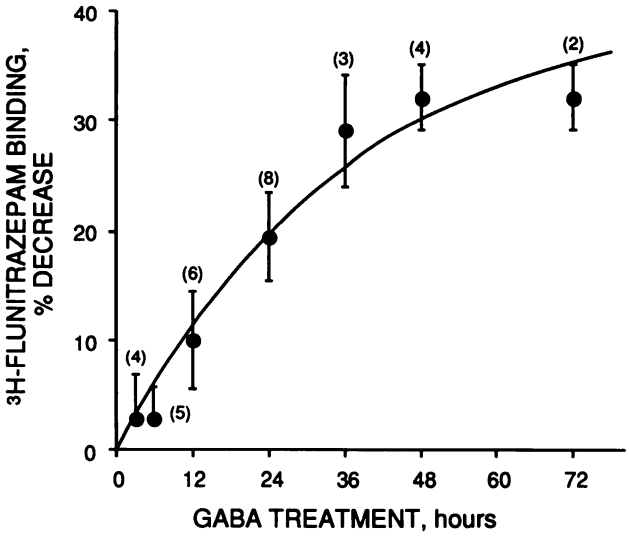

Fig. 3. GABA-induced receptor down-regulation obeys apparent first-order kinetics. In each experiment six 100-mm dishes were treated for the indicated times with 0.5 mM GABA. The binding of 15 nM [^{3}H]flunitrazepam was measured as described in Experimental Procedures. The *points* represent the mean ± standard error of the number of experiments shown in parentheses. The *line* represents a computer-generated single-exponential fit to the data. The $t_{½}$ = 25 hr and the maximum decrease in [^{3}H]flunitrazepam binding is 40.5%.

the ability of chronic GABA to decrease [^{3}H]flunitrazepam binding. However, we observed that when cells were treated with bicuculline the pH of the growth medium decreased from 7.0 to 6.1. For this reason, in subsequent experiments we used the compound SR-95531, a potent water-soluble GABA$_A$ receptor antagonist (9, 22, 23), which did not alter the pH of the growth medium. As shown in Fig. 5B, exposure of cells to 0.5 mM GABA for 48 hr caused a 37.1 ± 1% decrease in 15 nM [^{3}H]flunitrazepam binding. When cells were cotreated with 0.5 mM GABA and 100 μM SR-95531, only a 7.6 ± 2% decrease

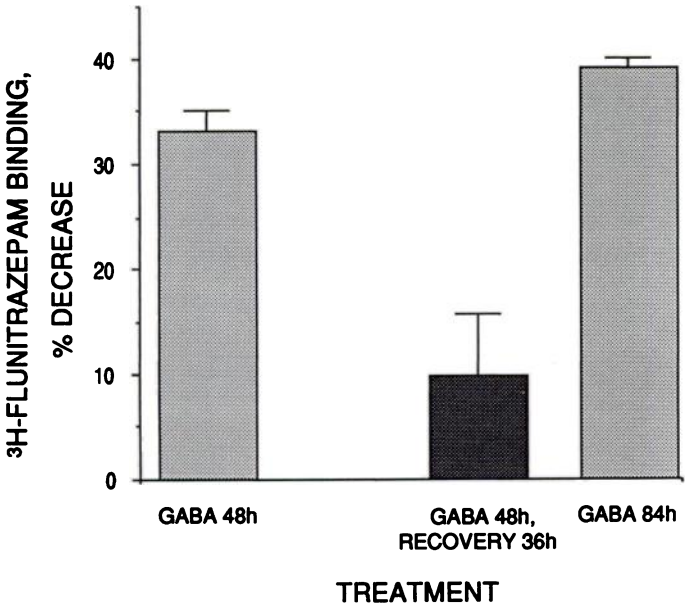

Fig. 4. The GABA-induced decrease in [^{3}H]flunitrazepam binding is reversible. Cultures were treated for 48 hr with 1 mM GABA. At this time, one group of dishes was assayed for 15 nM [^{3}H]flunitrazepam binding. Two further groups were rinsed twice with conditioned medium. One of these groups was then treated with 1 mM GABA for an additional 36 hr (total treatment time, 84 hr). The other group was allowed to recover for 36 hr. The numbers represent the mean ± standard error of three independent experiments (seven dishes/group). Both the 48- and 84-hr time points are significantly different from the recovered group ($p < 0.05$; paired Student's *t* test).

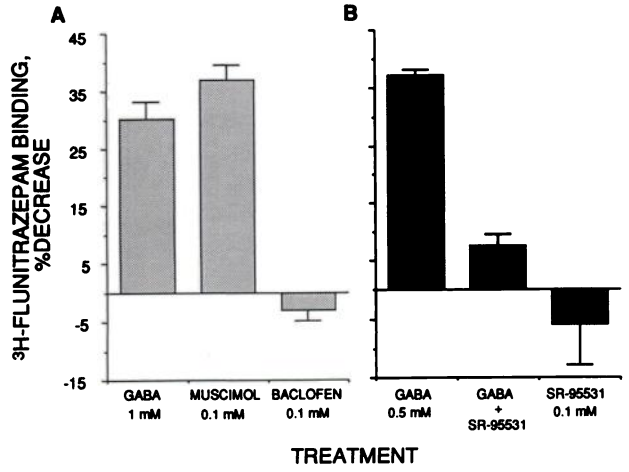

Fig. 5. The GABA-induced decrease in [^{3}H]flunitrazepam binding is mediated through the $GABA_A$ recognition site. A, In each experiment, five 100-mm or six 60-mm dishes were treated for 48 hr with either 1 mM GABA, 100 μM muscimol, or 100 μM baclofen. The binding of 15 nM [^{3}H]flunitrazepam was measured as described in Experimental Procedures. The *bars* represent the mean ± standard error of four or five independent experiments. Both GABA and muscimol treatment resulted in significant decreases in [^{3}H]flunitrazepam binding, as compared with control ($p < 0.01$; paired Student's *t* test). B, In each experiment, five or six dishes were treated for 48 hr with 0.5 mM GABA and/or 100 μM SR-95531. The *bars* represent the mean standard errors derived from three independent experiments. SR-95531 significantly reduced the GABA-induced decrease in [^{3}H]flunitrazepam binding ($p < 0.01$; paired Student's *t* test).

was observed. Treatment with SR-95531 alone had no significant effect.

To further determine whether the decrease in [^{3}H]flunitrazepam binding caused by chronic GABA exposure was exerted through the $GABA_A$ recognition site, cells were treated with 100 μM muscimol (a $GABA_A$ receptor agonist) or 100 μM baclofen (a $GABA_B$ receptor agonist). Chronic muscimol treatment caused a 37 ± 2% decrease in 15 nM [^{3}H]flunitrazepam binding, whereas baclofen had no significant effect on benzodiazepine binding (Fig. 5A). Together, these results suggest that the GABA-induced decrease in [^{3}H]flunitrazepam binding requires $GABA_A$ receptor occupancy.

It has previously been demonstrated that chronic GABA exposure results in a marked decrease in synaptic activity in neuronal cultures (24). When cells were treated for 36 hr with 100 nM tetrodotoxin (a concentration of the voltage-gated sodium channel blocker sufficient to inhibit all synaptic activity among chick spinal cord neurons, as measured by intracellular recording)[2] or 10 μM veratridine (a sodium channel activator), the binding of 1 nM [^{3}H]flunitrazepam remained unchanged (97 ± 5% and 94 ± 5% of control respectively; six experiments), suggesting that down-regulation is not simply the result of an overall change in neuronal activity.

To determine whether chronic GABA exposure has any effects on GABA/benzodiazepine binding site interactions, the acute enhancement of 1 nM [^{3}H]flunitrazepam binding by GABA was measured. The pooled results from six independent GABA dose-response experiments demonstrated a 34% decrease in the maximum GABA potentiation of 1 nM [^{3}H] flunitrazepam binding following chronic GABA treatment (Fig. 6). We refer to this decrease in the ability of GABA to potentiate benzodiazepine binding as "uncoupling" (see also Ref. 15).

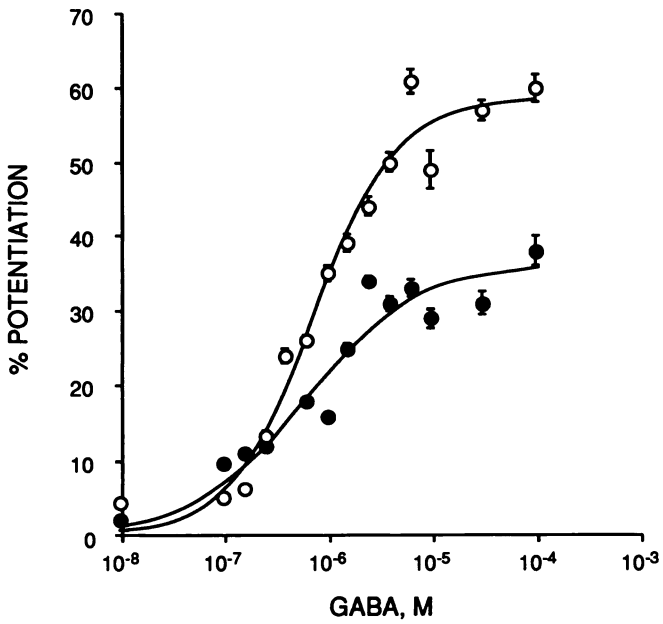

Fig. 6. Chronic GABA exposure decreases GABA enhancement of [^{3}H] flunitrazepam binding. Ten culture dishes were treated for 48 hr with 1 mM GABA and the potentiation of 1 nM [^{3}H]flunitrazepam binding was measured as described in Experimental Procedures. ○, Data from control cells; ●, data from treated cells. The results shown are from a single experiment. The *lines* drawn are the computer-generated best fits to the data using the logistic equation. Pooled results from six independent experiments gave the following values: for control, maximum potentiation = 49.0 ± 4.0%, n = 1.5 ± 0.3, and EC_{50} = 856 ± 146 nM; following GABA treatment, maximum potentiation = 32.3 ± 1.2%, n = 1.4 ± 0.3, and EC_{50} = 706 ± 105 nM. Chronic GABA exposure significantly reduced the maximum potentiation ($p < 0.01$; paired Student's *t* test).

[2] D. Mierlak, personal communication.

Chronic GABA-induced uncoupling was dose dependent (Table 1) and time dependent, with an approximate $t_{1/2}$ of 24 hr (Fig. 7). The result shown in Fig. 7 also demonstrates that uncoupling was reversible. Cells were treated for 48 hr with 1 mM GABA, washed, and then allowed to recover for 36 hr. Uncoupling was 39.3 ± 5% and 42.6 ± 1% in homogenates derived from cells treated for 48 and 84 hr, respectively. In homogenates derived from cells allowed to recover, uncoupling was 13 ± 3% (i.e., 68% recovery).

The GABA antagonist SR-95531 reduced the uncoupling caused by chronic GABA exposure (Fig. 8A). Uncoupling was 51.7 ± 4% in homogenates from 1 mM GABA-treated cells and 16 ± 6% in homogenates from cells co-treated with 200 μM SR-95531. Treatment with SR-95531 alone had no significant effect. This suggests that, like GABA-induced down-regulation, GABA-induced uncoupling requires binding to the GABA$_A$ receptor. But, when cells were co-treated with GABA and SR-95531, some of the chronic GABA-induced uncoupling persisted. To determine whether GABA-induced uncoupling was mediated through the GABA$_A$ receptor, cells were treated with the specific GABA$_A$ receptor agonist muscimol. Chronic 100 μM muscimol treatment caused uncoupling similar to that following chronic GABA exposure (Fig. 8B), suggesting that chronic GABA-induced uncoupling is mediated through the GABA$_A$ receptor.

TABLE 1

Chronic exposure to GABA receptor agonists uncouples the allosteric interactions between GABA and benzodiazepine binding sites

Embryonic brain cell cultures were treated with the indicated drugs for 48 hr. Uncoupling was measured as described in Experimental Procedures.

Chronic treatment	No. of experiments	Uncoupling
		%
GABA		
100 μM	8	18 ± 5[a]
500 μM	8	33 ± 4[b]
1 mM	21	39 ± 3[b]
Muscimol, 100 μM	3	40 ± 5[b]

[a] Significantly different from zero, $p < 0.05$, unpaired Student's t test.
[b] Significantly different from zero, $p < 0.01$, unpaired Student's t test.

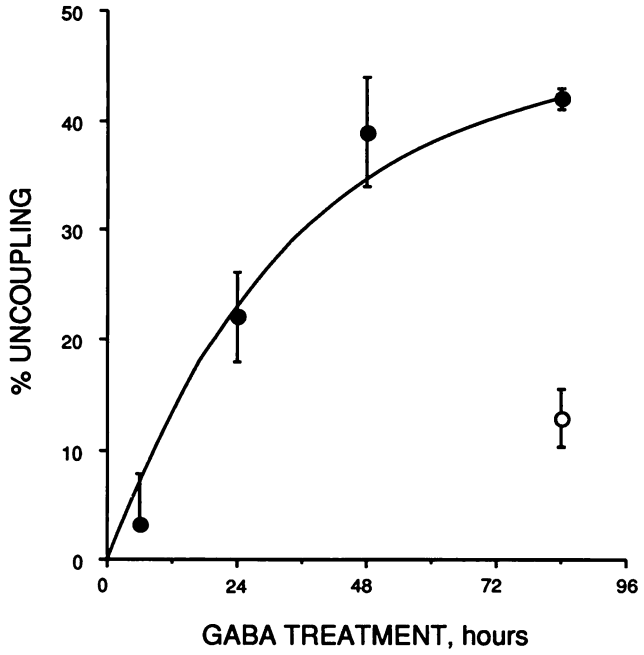

Fig. 7. Chronic GABA-induced uncoupling is time dependent and reversible. Cells were treated with 1 mM GABA for the indicated times. To examine reversibility, at 48 hr one group of culture dishes was assayed for 1 nM [^{3}H]flunitrazepam binding. At the same time two other groups were rinsed twice with conditioned medium. One of the groups was treated with GABA for an additional 36 hr and is represented by the 84-hr time point. The other treated cultures were returned to the incubator and allowed to recover for 36 hr before being assayed for 1 nM [^{3}H] flunitrazepam binding (○, at the 84-hr time point). The *points* represent the mean ± standard error of three independent experiments, except for the 6- and 24-hr time points, which are from two and four experiments, respectively. In each experiment, values were the result of quintuplicate determinations. All points except the 6-hr time point are significantly different from zero ($p < 0.01$; Student's t test). Both the 48- and 84-hr time point are significantly different from the recovered group ($p < 0.01$; Student's t test). The *line* drawn represents a computer-generated single-exponential fit to the data. The $t_{1/2}$ is 24 hr and the maximum uncoupling is 46%.

Discussion

A decrease in receptor number (down-regulation) is a recognized cellular response to chronic agonist exposure. Whereas the cellular dynamics of regulation of hormone receptors and/or neurotransmitter receptors coupled to second messengers are well established (1), comparatively little is known about the long term regulation of ligand-gated ion channels in the central nervous system. The GABA$_A$ receptor complex contains an integral chloride channel that is gated by GABA binding. Moreover, a number of modulatory sites that are apparently located on the receptor complex subserve key roles in the acute control of GABA receptor function. Examples of neuromodulators that act through the GABA receptor include benzodiazepines, barbiturates, cage convulsants, and certain steroids (7–9).

The results of the present investigation demonstrate that chronic exposure of brain neurons to GABA causes (a) a reduction in the number of both flunitrazepam and TBPS binding sites, indicative of a down-regulation of the GABA$_A$ receptor complex, and (b) a decrease in the allosteric interactions between GABA and benzodiazepine sites. These findings confirm

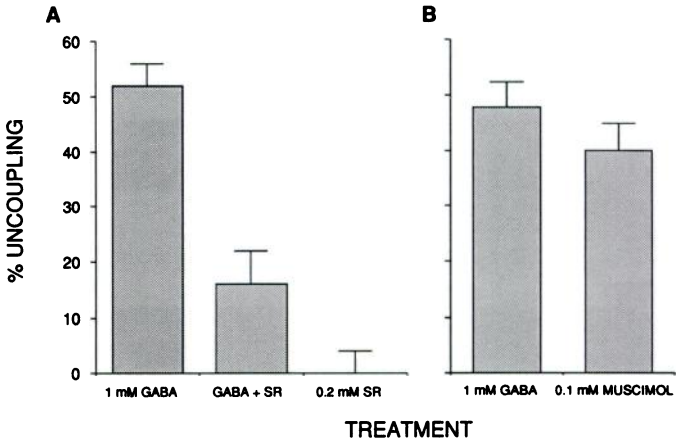

Fig. 8. GABA induces uncoupling through a GABA$_A$ recognition site. A, SR-95531 (*SR*) reduces chronic GABA-induced uncoupling. Six culture dishes were treated with 1 mM GABA and/or 200 μM SR-95531 for 48 hr. The *bars* represent the mean ± standard error of four independent experiments. In the presence of SR-95531, GABA-induced uncoupling was significantly reduced ($p < 0.05$; paired Student's t test). B, Chronic muscimol treatment causes uncoupling. Six culture dishes were treated for 48 hr with 1 mM GABA and 100 μM muscimol. The *bars* represent the mean ± standard error of three independent experiments. Both groups are significantly different from zero ($p < 0.01$; Student's t test).

and extend those of Maloteaux *et al.* (25) and Tehrani and Barnes (26), who reported chronic GABA-induced down-regulation of flunitrazepam binding sites in cultured rat and chick neurons. Results from earlier *in vivo* studies in which elevation of cerebral GABA levels was achieved following chronic treatment with inhibitors of GABA catabolism (27–29) are contradictory with regard to changes in [^{3}H]GABA or [^{3}H]muscimol binding. Our demonstration of allosteric uncoupling in response to chronic $GABA_A$ agonist exposure represents an important novel finding, because this form of regulation has previously only been observed following prolonged benzodiazepine or methylxanthine treatment (13, 15, 30–32).

Saturation binding demonstrates that chronic treatment of cultures with 0.5 mM GABA for 48 hr decreases the B_{max} of [^{3}H]flunitrazepam binding by 35%, with no change in affinity. The time course of down-regulation exhibits psuedo-first-order kinetics, with a half-time of 25 hr and a maximum reduction of receptor number of 41%. This half-time is similar to the slow phase of $GABA_A$ receptor degradation (32 hr), which we have measured previously by photoaffinity labeling (33). A fast component comparable to that observed during the initial phase of receptor degradation (3.8 hr) could not be identified, demonstrating that the fast phase of receptor degradation cannot be the rate-determining step in GABA-induced down-regulation of receptor.

The dependence of down-regulation upon GABA concentration is consistent with the Michaelis-Menten equation (EC_{50} = 94 μM; maximum down-regulation = 42%; n = 0.96). The EC_{50} for down-regulation is higher than that of 17 μM for $GABA_A$ responses measured electrophysiologically (34). However, due to GABA uptake (35), it is possible that the former is an overestimation and, hence, that this discrepancy may be exaggerated. Down-regulation was blocked by the specific $GABA_A$ receptor antagonist SR-95531, and an action of GABA at the $GABA_A$ receptor was confirmed by the fact that down-regulation was seen with chronic muscimol (a $GABA_A$ receptor agonist) but not with baclofen (a $GABA_B$ receptor agonist).

The mechanisms that could potentially lead to receptor down-regulation include receptor internalization, chemical modification of receptor, an increase in the rate of receptor degradation, or a decrease in receptor synthesis rate. Recovery from GABA down-regulation following the removal of GABA eliminates the possibility of an irreversible process such as neuronal death.

We have shown previously that 20% of the total number of $GABA_A$ receptors are sequestered or intracellular (36). Internalization of surface receptors cannot explain the phenomenon of down-regulation, because flunitrazepam is lipophilic, gaining access to both internal and external receptors. Moreover, the selective loss of intracellular receptors could not by itself account for $GABA_A$ receptor down-regulation because this could only give a maximum reduction of 20%, even if all of the intracellular sites were selectively degraded.

The β-adrenergic receptor has been shown to be internalized and transferred into a light vesicle fraction following chronic agonist exposure (37, 38). This could appear as a down-regulation if precautions are not taken to prevent the loss of such a light vesicle fraction in the supernatant after centrifugation. This is an unlikely explanation for our results, because at no time during the preparation of cell homogenate, in which we employed dialysis rather than centrifugation to remove residual drug, could a protein with a molecular weight of greater than 15,000 be lost. Reversible chemical modification is improbable, because it has been reported that protein synthesis inhibitors block recovery from chronic GABA-induced GABA receptor down-regulation in rat brain neurons in culture (25). Thus, it is unlikely that internalization, translocation, or chemical modification can explain chronic GABA-induced down-regulation. Taken together, these considerations leave us with only two possibilities, an increase in receptor degradation or a decrease in receptor synthesis. Recently, we have found that a reduction of $GABA_A$ receptor α-subunit mRNA appears to correlate with the reduction of receptor number.[3] This result favors a change in receptor synthesis; however, a change in both synthesis and degradation is not precluded.

Chronic exposure of cultured rat brain neurons to GABA has been shown to result in $GABA_A$ receptor down-regulation but not uncoupling (25). The reason for the discrepancy between this result and our own is not clear. The use of different experimental paradigms may be important. We found a 34% decrease in the ability of GABA to enhance 1 nM [^{3}H]flunitrazepam binding when complete GABA dose-response curves were analyzed (Fig. 6). It may have been difficult for Maloteaux *et al.* (25) to detect, with sufficient accuracy, a shift of this magnitude in the K_D for [^{3}H]flunitrazepam using a saturation binding protocol.

The uncoupling of GABA/benzodiazepine binding site interactions does not appear to be the result of a change in the potency of GABA but rather a change in its efficacy. This is suggested by the lack of effect of chronic GABA treatment on the EC_{50} of GABA potentiation of [^{3}H]flunitrazepam binding. The same is true for chronic flurazepam- and theophylline-induced uncoupling (15).

The observation that the $t_{1/2}$ for uncoupling is similar to the average $t_{1/2}$ for benzodiazepine turnover (18 hr) (18, 33) prompts the speculation that uncoupling might involve the synthesis of a new modified receptor population. It may be significant that, whereas GABA potentiates the binding of [^{3}H]flunitrazepam to cell surface receptors, the internal sites appear to be allosterically uncoupled (39). Internal and total cellular receptors exhibit identical turnover kinetics (40), and it seems possible that if surface receptors were down-regulated selectively then GABA-induced uncoupling might result from a reduction in the ratio of surface to internal receptors.

What kind of signal transduction mechanism might result in down-regulation and/or allosteric uncoupling? For the nicotinic acetylcholine receptor of skeletal muscle, membrane potential is thought to play a key regulatory role; treatment of chick myotubes with veratridine decreases acetylcholine receptor-specific mRNA and receptor number, whereas tetrodotoxin produces opposite effects (41). In contrast, chronic veratridine or tetrodotoxin treatment is without effect upon $GABA_A$ receptor number (or GABA/benzodiazepine binding site interactions),[4] suggesting that, although both are members of a superfamily of ligand-gated ion channels, the number of $GABA_A$ and nicotinic acetylcholine receptors may be regulated through different mechanisms. One possibility is that the GABA receptor is linked to an as yet uncharacterized second messenger that can activate a cascade that regulates transcription or mRNA stability. For example, it has been suggested that the indirect increase in intracellular calcium levels and the conse-

[3] Montepied *et al.*, manuscript in preparation.
[4] Roca *et al.*, manuscript in preparation.

quent decrease in cAMP levels following activation of voltage-gated sodium channels both contribute to the regulation of sodium channel α-subunit mRNA, possibly by altering transcription rates (42).

In summary, we have demonstrated that neurons derived from embryonic chick brain and maintained in primary monolayer cell culture respond to chronic GABA exposure with a down-regulation of the GABA$_A$ receptor complex. Moreover, chronic GABA exposure also reduces the allosteric interactions between the GABA and benzodiazepine recognition sites present on this complex. This is distinct from the regulation we observe following chronic benzodiazepine (15) or barbiturate[4] treatment, suggesting that the characteristics of GABA$_A$ receptor regulation are site dependent. The results are consistent with a sequence of events in which GABA binding, chloride channel activation, and desensitization occur rapidly (on the time scale of milliseconds to minutes), whereas persistent occupancy of the GABA recognition site induces a slow (hours to days) down-regulation of receptor number that is accompanied by a partial uncoupling of allosteric interactions.

Acknowledgments

We would like to thank Dr. Terrell Gibbs for helpful discussions, Dr. Fong-Sen Wu for critical comments on the manuscript, and Lalitha Reddy for expert technical assistance.

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