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γ-Aminobutyric Acid$_A$ Receptor Regulation in Culture: Altered Allosteric Interactions Following Prolonged Exposure to Benzodiazepines, Barbiturates, and Methylxanthines

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SUMMARY

In previous reports, we have described the use of primary neuronal cultures derived from chick brain to study the regulation of the γ-aminobutyric acid$_A$ (GABA$_A$) receptor complex. Chronic exposure of cultures to GABA, benzodiazepines, or methylxanthines results in decreased enhancement of [^{3}H]flunitrazepam binding by GABA, consistent with an allosteric uncoupling of GABA and benzodiazepine recognition sites of the GABA$_A$ receptor. In the present communication, we extend our studies of the pharmacology of benzodiazepine- and methylxanthine-induced uncoupling of GABA/benzodiazepine recognition site interactions and present evidence to show that certain barbiturates (barbital and pentobarbital) also induce uncoupling. Chronic exposure to flurazepam (a high efficacy benzodiazepine) elicits no change in the number of benzodiazepine binding sites or the affinity of benzodiazepine binding in the absence of GABA. Whereas flurazepam and theophylline decrease coupling, Ro15-1788 (a low efficacy benzodiazepine) inhibits flurazepam-induced but not theophylline-induced uncoupling, suggesting that theophylline and flurazepam act through separate receptors. Flurazepam-induced uncoupling is not prevented by SR-95531 or picrotoxin (specific inhibitors of GABA action) and, therefore, is not an indirect effect mediated by endogenous GABA. The onset of flurazepam-induced uncoupling ($EC_{50} \approx 1$ μM) exhibits a $t_{1/2}$ of about 18 hr, in general agreement with the half-time for receptor turnover. Uncoupling is reversible following washout and recovery at 37°. These results are discussed in terms of mechanisms of GABA$_A$ receptor regulation in response to chronic exposure to functionally homologous or heterologous ligands.

Regulation of neurotransmitter receptor number is of central importance to nervous system function. In many receptor systems, chronic exposure to agonists induces a decrease in receptor number (down-regulation), whereas prolonged exposure to antagonists induces an increase in receptor number (up-regulation) (for review see Ref. 1). Such changes in response to agonist or antagonist exposure probably represent a mechanism for the regulation of overall cellular responsiveness to transmitter.

Benzodiazepines are used extensively for their anxiolytic, anticonvulsant, and muscle relaxant properties, whereas barbiturates are widely used for their anticonvulsant, sedative, and anesthetic properties. Both classes of compounds are thought to act in part by modulating the interaction of the inhibitory neurotransmitter GABA with the GABA$_A$ receptor. This receptor complex contains an integral transmembrane chloride channel, a GABA recognition site, and a variety of allosteric modulatory sites through which compounds such as the benzodiazepines and barbiturates can alter receptor function (2).

How does the central nervous system react to chronic exposure to neuromodulators such as the benzodiazepines and barbiturates? Although a number of studies of the effects of chronic benzodiazepine exposure have appeared, there has been no consensus as to the nature or direction of the changes in receptor binding that result. Following chronic treatment of animals with benzodiazepines for various periods of time, some groups have reported the binding of radiolabeled benzodiazepines to be increased (3–5) or decreased (5–8), but most investigators have found no change (9–14). Although less has been done, there has been similar disagreement as to whether GABA$_A$ receptor number in neuronal cultures is decreased (15) or unaffected (16, 17) by chronic exposure to benzodiazepines.

We find that chronic exposure of neuronal cultures to benzodiazepines results in decreased enhancement of [^{3}H]flunitra-

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ABBREVIATIONS: GABA, γ-aminobutyric acid; cpt-cAMP, chlorophenylthio-cAMP; TCA, trichloroacetic acid; PBSS, phosphate-buffered balanced salt solution.

zepam binding by GABA, consistent with an allosteric "uncoupling" of GABA and benzodiazepine recognition sites of the GABA$_A$ receptor, with no change in receptor number (18–20). In the present communication, we extend our studies of the pharmacology and dynamics of benzodiazepine-induced uncoupling of GABA/benzodiazepine recognition site interactions and present evidence to show that certain barbiturates (barbital and pentobarbital) also induce uncoupling. These results are discussed in terms of possible mechanisms of GABA$_A$ receptor regulation in response to chronic exposure to functionally homologous or heterologous ligands.

Experimental Procedures

Materials. [^{3}H]Flunitrazepam (85 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham. GABA, veratridine, tetrodotoxin, and barbiturates were purchased from Sigma. cpt-cAMP was obtained from Boehringer Mannheim Biochemicals. The benzodiazepine drugs were a gift of Hoffman-La Roche. Dialysis tubing (15,000 molecular weight cutoff) was from Spectrapor. Liquiscint was obtained from National Diagnostics.

Cell culture. 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 mg/ml streptomycin], triturated with fire-polished Pasteur pipets, plated onto collagen-coated plastic culture dishes (approximately one brain/100-mm dish), 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. Medium was replaced with fresh minimum essential medium [supplemented as described above, with the exception of glucose (final concentration, 0.2 M) and chick embryo extract (final concentration, 2.5%)] 1 day later and again as needed; 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 5–10 ml of growth medium. 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) unless otherwise indicated. Using light microscopy, the cells appeared to be unaffected by chronic drug treatment.

Cell homogenate. Cultures were washed once with ice-cold PBSS, scraped from culture dishes, and centrifuged (1000 rpm, 5 min), and the pellet was resuspended in 1 mM EDTA/1 mM phenylmethylsulfonyl fluoride, using a Dounce homogenizer (35–50 strokes) (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.* (21). 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.

[^{3}H]Flunitrazepam binding to intact cells. Primary brain cell cultures were grown in 24-well culture plates (16-mm diameter wells; Falcon). Binding experiments were carried out 1 week after plating. To remove treatment drugs, the medium was aspirated and the cultures were washed three times with ice-cold PBSS (pH 7.4, 45 min/wash). Solutions containing the appropriate combinations of drugs and [^{3}H]flunitrazepam were added (in PBSS, pH 7.4, 0–4°, 1 ml/well). After the appropriate period of incubation, the solution was aspirated and each well was washed for 10 sec with 1.5 ml of ice-cold PBSS (three changes). For some experiments involving large numbers of multiwell plates, an alternate method of washing was employed. Plates were inverted rapidly to drain the reactant solutions and then dipped sequentially into three 2-liter beakers containing ice-cold PBSS. This method yielded results identical to individual processing of each well. Dishes were air-dried overnight and 0.5 ml of 0.5% sodium deoxycholate in 1 N NaOH was added to each well to digest cells. Aliquots were taken for liquid scintillation counting and for protein determination.

[^{3}H]Flunitrazepam binding to cellular homogenates. Samples of cell homogenate (150 mg of protein) were incubated in PBSS (total volume, 1 ml) for 60 min at 4°, in the presence of 0.1–20 nM [^{3}H]flunitrazepam. The incubation was terminated by 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. 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 concentration is 7 times the K_D. For the measurement of GABA potentiation of benzodiazepine binding, 1 nM [^{3}H]flunitrazepam was used, except where otherwise noted. The extent to which the binding of [^{3}H]flunitrazepam (1 nM, unless otherwise indicated) was potentiated by GABA (50 μM, unless otherwise indicated) was used as an indicator of the degree to which GABA and benzodiazepine recognition sites are allosterically "coupled."

Data for enhancement of [^{3}H]flunitrazepam binding by GABA are 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, changes in enhancement of [^{3}H]flunitrazepam binding by GABA after chronic treatment are expressed as percentage of uncoupling, defined as:

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

Protein synthesis. Synthesis of general cellular protein was measured as described previously (22). Briefly, cultures were incubated 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 P-40/8 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 with PBSS/10% TCA at 0° under vacuum. Radioactivity retained on the filters was determined by liquid scintillation counting.

Protein degradation. To examine degradation of general cellular protein, cultures were labeled with [^{35}S]methionine (36 hr, 37°), washed with complete medium containing 2 mM L-methionine (to prevent further incorporation of label), and incubated at 37°. At each time point, a small sample of medium was removed and replaced with fresh medium. Ice-cold TCA was added (final concentration, 10%) to the sample and radioactivity in the supernatant was determined after centrifugation for 5 min in a Beckman microfuge at 4°. At the end of the experiment, cell-associated radioactivity was determined in the washed cells after solubilization with 0.2% sodium dodecyl sulfate/2% Nonidet P-40/8 M urea. Radioactivity released was expressed as a percentage of total radioactivity, according to the equation, % Release = (cumulative [^{35}S]methionine released) × 100/(total specific incorporation), where total incorporation equals cumulative release plus radioactivity remaining at the end of the experiment.

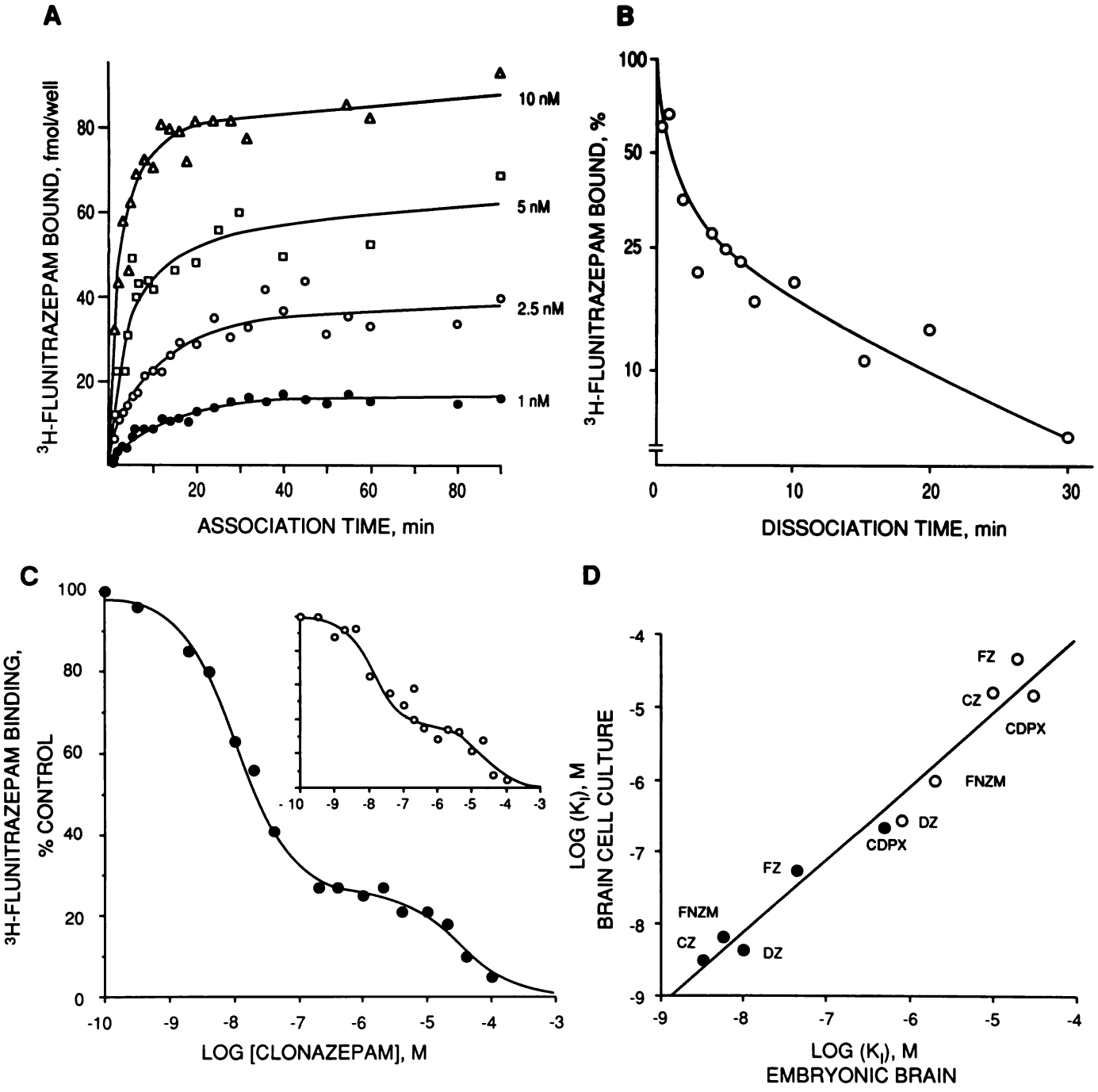

Fig. 1. [^{3}H]Flunitrazepam binding to cell culture homogenate is representative of binding to intact culture neurons and embryonic brain homogenates. A, Time course of binding of [^{3}H]flunitrazepam to intact neuronal cultures. Binding to intact cultures was carried out as described in Experimental Procedures. Cultures (grown in 24-well multiwell plates) were exposed to 1 (●), 2.5 (○), 5 (□), or 10 nM (△) [^{3}H]flunitrazepam at 4° for the indicated period of time, washed, and solubilized for scintillation counting, as described in Experimental Procedures. Nonspecific binding, determined at each time point in the presence of 10 μM flurazepam, has been subtracted. B, Dissociation of [^{3}H]flunitrazepam from intact cultures. Cultures were incubated 1 hr with 5 nM [^{3}H]flunitrazepam and then dissociation was initiated by addition of 1 μM nonradioactive flunitrazepam. After the indicated period of time, cultures were washed and solubilized for scintillation counting, as described in Experimental Procedures. Nonspecific binding, determined from cultures incubated with 5 nM [^{3}H]flunitrazepam plus 1 μM nonradioactive flunitrazepam, has been subtracted. C, High and low affinity benzodiazepine binding sites in cellular homogenates and intact cells are similar. Cellular homogenates or intact cells (*inset*) were incubated 1 hr in the presence of 20 nM [^{3}H]flunitrazepam and the indicated concentration of clonazepam. Binding was determined as described in Experimental Procedures. Nonspecific binding was determined in the presence of 1 mM flurazepam. Clonazepam was used as competitor, because it exhibits the greatest selectivity for high affinity benzodiazepine binding sites. The *points* represent the means of triplicate determinants. The *lines* drawn are the best two-site fits to the data. For cell homogenate K_i = 11.6 nM for the high affinity site and 33 μM for the low affinity site. For intact cells the corresponding K_i values are 13 nM and 24 μM, respectively. Similar results were obtained using flunitrazepam as competitor. D, Binding affinities determined in culture homogenate correlate with binding to embryonic brain membranes. K_i values were determined for high (●) and low affinity (○) benzodiazepine binding sites by competition with [^{3}H]flunitrazepam, using either culture homogenate or P_2 membrane homogenate prepared from 7-day embryonic chicken brain. The *line* drawn represents a linear regression of the data (r^2 = 0.966). *FNZM*, flunitrazepam; *CZ*, clonazepam; *DZ*, diazepam; *FZ*, flurazepam; CDPX, chlordiazepoxide.

Results

Identification and characterization of benzodiazepine binding sites *in situ*. The kinetics of [^{3}H]flunitrazepam association (Fig. 1A) and dissociation (Fig. 1B) determined using intact primary cultures of chick brain neurons were consistent with the kinetics of [^{3}H]flunitrazepam binding to culture homogenates[3] or to membrane homogenates prepared from brain (23, 24). Association and dissociation kinetics were biphasic, and equilibrium binding was achieved in 30–60 min (1–10 nM). In equilibrium binding experiments with cell culture homogenates, clonazepam competed with 20 nM [^{3}H]flunitrazepam in a complex manner (Fig. 1C), suggesting the presence of multiple binding sites. Similar results were obtained when binding experiments were carried out on intact cells in culture (Fig. 1C, *inset*), confirming that binding to homogenates accurately reflects the binding properties of the receptor *in situ*. Nonlinear regression analysis indicates that the results of competition binding experiments are consistent with a two-site model. These results are in good agreement with previous results reported for binding to membrane homogenates prepared from embryonic brain (Fig. 1D) (25). Because of an unfavorable ratio of specific to nonspecific binding in the high concentration range, the number of low affinity [^{3}H]flunitrazepam binding sites could not be determined accurately; however, in view of the low affinity of flunitrazepam for this site (26), it is evident that for neuronal cells in culture, as in early embryonic chick brain, low affinity [^{3}H]flunitrazepam binding sites are considerably more abundant than high affinity sites (26). Because the low affinity site is not linked with the GABA$_A$ receptor (25), subsequent experiments focused on the high affinity component of [^{3}H]flunitrazepam binding.

Allosteric uncoupling by benzodiazepines. Binding sites for GABA and benzodiazepines are allosterically coupled, as demonstrated by the ability of GABA to enhance the binding of [^{3}H]flunitrazepam. That is, GABA decreases the K_D (increases the affinity) of [^{3}H]flunitrazepam binding, but does not affect B_{max} (20). Enhancement of [^{3}H]flunitrazepam binding by GABA is inhibited by bicuculline (Fig. 2), which competes for the GABA recognition site on the GABA$_A$ receptor, with an IC$_{50}$ of about 1 μM.

Chronic exposure of cells to flurazepam did not affect either the K_D or the B_{max} of [^{3}H]flunitrazepam binding to dialyzed cellular homogenates in the absence of GABA (Fig. 3). In the presence of GABA, however, homogenates of flurazepam-treated cells bind less [^{3}H]flunitrazepam than do homogenates of untreated sister cells (Fig. 4) (19, 20), indicating a decrease in the capacity of GABA to enhance [^{3}H]flunitrazepam binding. In other words, chronic flurazepam treatment leads to an allosteric uncoupling of GABA/benzodiazepine recognition site interactions. Similar results were observed when cultures were treated with 10 μM diazepam for 36 hr (two experiments).

After removal of flurazepam, enhancement of [^{3}H]flunitrazepam binding by GABA recovered almost to control levels. In homogenates derived from cells that were treated for 25 hr with 10 μM flurazepam, GABA potentiation of 1 nM [^{3}H]flunitrazepam binding was 67% of control; 16 hr after drug removal, GABA potentiation returned to 98% of control. In sister cultures treated with 100 μM flurazepam, the corresponding results were 50% and 82%. These results indicate that flurazepam-induced uncoupling is reversible. As previously reported, potentiation of [^{3}H]flunitrazepam binding by GABA was unaffected when cells were first homogenized and the homogenates were subjected to chronic flurazepam exposure, indicating that the uncoupling process requires intact cells (20). The results are consistent with a model in which cellular metabolism mediates the regulation of receptor coupling.

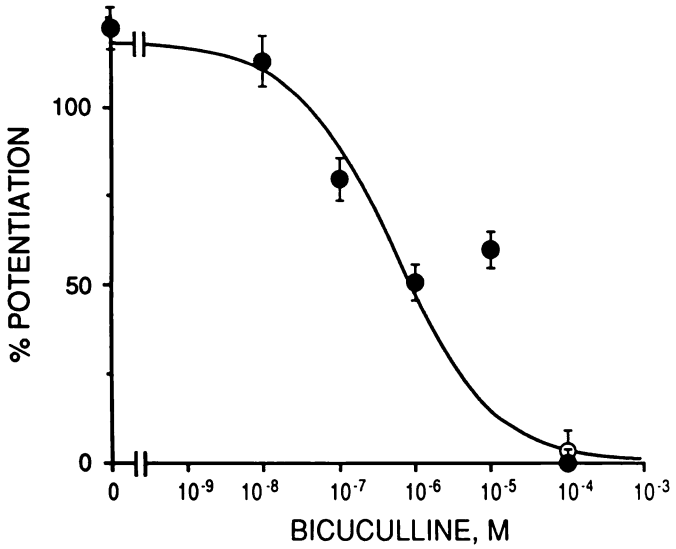

Fig. 2. Enhancement of [^{3}H]flunitrazepam binding by GABA is inhibited by bicuculline. Intact cultures were incubated with 1 nM [^{3}H]flunitrazepam in the presence (●) or absence (○) of 10 μM GABA and the indicated concentration of bicuculline. Values are percentages of enhancement of [^{3}H]flunitrazepam binding over binding in the absence of GABA (mean ± standard error of triplicate determinations).

The degree of uncoupling was dependent upon the concentration and duration of exposure to flurazepam, with an apparent EC$_{50}$ of 1 μM (Fig. 5) and a $t_{1/2}$ of about 18 hr (Fig. 6). Ro15-1788, a low efficacy benzodiazepine (27) was less effective than flurazepam in inducing uncoupling (Fig. 7). Moreover, Ro15-1788 antagonized flurazepam-induced uncoupling, arguing that Ro15-1788 and flurazepam act via a common site, the benzodiazepine-sensitive modulatory site of the GABA$_A$ receptor. Uncoupling of GABA/benzodiazepine recognition site interactions was also observed following chronic exposure to theophylline (20), but theophylline-induced uncoupling was unaffected by Ro15-1788 (Fig. 7). Conversely, flurazepam-induced uncoupling was unaffected by 2-chloroadenosine, which antagonizes theophylline-induced uncoupling (20), consistent with the view that flurazepam and theophylline act at different sites.

Although a slight inhibition of protein synthesis (23% inhibition; Table 1) was evident in cells chronically exposed to 100 μM flurazepam, protein synthesis was not affected by 10 μM flurazepam or by 100 μM theophylline, nor was there any effect of either flurazepam or theophylline on the kinetics of protein degradation of cells in culture (Fig. 8). Thus, uncoupling is not a consequence of a generalized cytotoxic effect on the cells in culture.

There was considerable variability between platings with respect to the degree of uncoupling induced by flurazepam or theophylline. There were occasional experiments in which little or no uncoupling was observed or, rarely, in which enhancement of [^{3}H]flunitrazepam binding increased after chronic treatment. Conversely, there were experiments in which uncoupling was considerably greater than average, and in some cases enhance-

[3] G. D. Schiller, unpublished data.

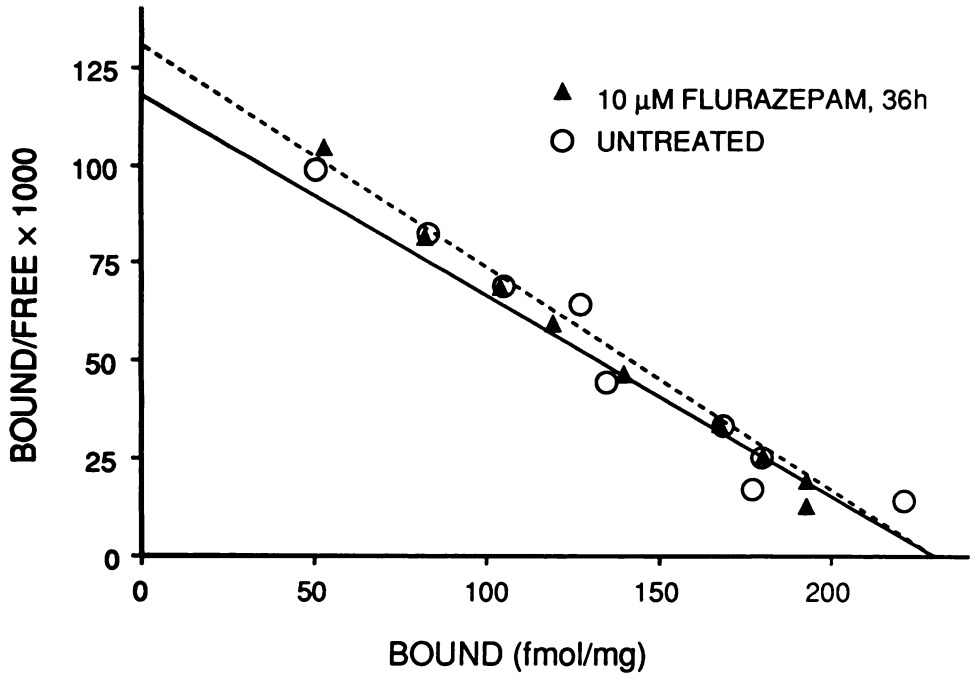

Fig. 3. Chronic flurazepam exposure does not alter [^{3}H]flunitrazepam binding in the absence of GABA. Ten culture dishes were incubated for 36 hr in the presence (▲) or absence (○) of 10 μM flurazepam. Binding of 0.5–15 nM [^{3}H] flunitrazepam to dialyzed culture homogenate was determined by filtration. Nonspecific binding, determined in the presence of 10 μM flurazepam, was subtracted. To prevent interference from low affinity sites, both total and nonspecific binding were determined in the presence of 2.5 μM Ro5-4864. K_D and B_{max} were determined by nonlinear regression, using untransformed data [control (——), K_D = 1.95 nM, B_{max} = 0.23 pmol/mg of protein; flurazepam-treated (– – –), K_D = 1.76 nM, B_{max} = 0.23 pmol/mg of protein]. Similar results were seen in six other experiments. In each experiment, values were the result of triplicate determinations.

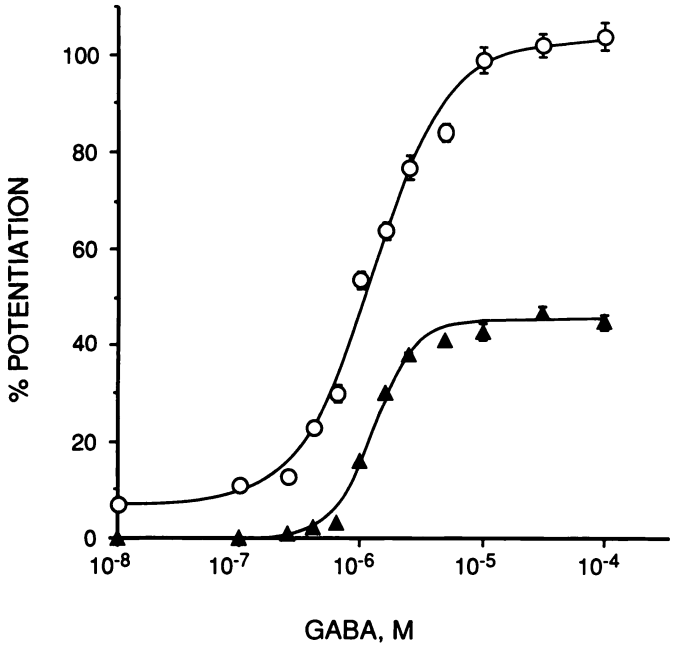

Fig. 4. Chronic exposure to flurazepam reduces enhancement of [^{3}H] flunitrazepam binding by GABA. GABA potentiation of [^{3}H]flunitrazepam binding to dialyzed homogenates of control cultures (○) or cultures exposed for 36 hr to 100 μM flurazepam (▲) was determined by filtration. Each *point* is the mean ± standard error of triplicate determinations. Redrawn with permission from Ref. 3.

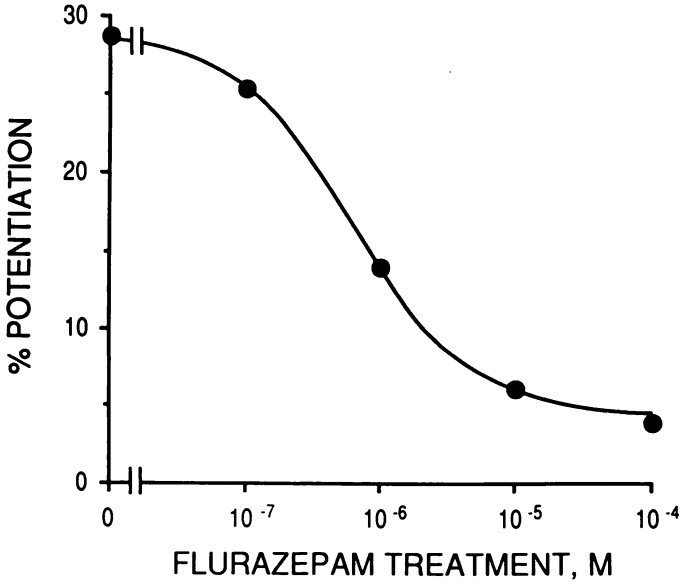

Fig. 5. Chronic flurazepam decreases GABA potentiation in a concentration-dependent manner. Cultures were incubated with the indicated concentration of flurazepam for 64 hr. Cell homogenates were dialyzed and potentiation of 1.5 nM [^{3}H]flunitrazepam was calculated as described in Experimental Procedures. The *points* are the means of triplicate determinations. This experiment was repeated with similar results.

ment of [^{3}H]flunitrazepam binding by GABA was virtually abolished. In cells treated for 36 hr with 100 μM flurazepam, 32 of 37 experiments showed 10% or greater uncoupling; in cells treated with 10 μM flurazepam for the same period of time, 23 of 39 experiments showed 10% or greater uncoupling. In cells that were treated with 1 μM theophylline, 32 of 47 experiments showed 10% or greater uncoupling. The source of this variability is unclear; however, it did not correlate with plating density or with the batch of horse serum used to prepare medium.

Barbiturate-induced uncoupling. Changes in allosteric coupling of binding sites associated with the benzodiazepine binding site were also observed following chronic exposure to the barbiturates barbital (1 mM) and pentobarbital (200 μM). In control cells, these barbiturates enhanced binding of 1 nM [^{3}H]flunitrazepam by 20–30%. After chronic barbiturate exposure, barbiturate enhancement of [^{3}H]flunitrazepam binding was virtually eliminated (Fig. 9, A and B). Interestingly, chronic exposure to barbiturates also reduced enhancement of [^{3}H] flunitrazepam binding by GABA (Fig. 9C). There was no change in maximal [^{3}H]flunitrazepam binding after chronic barbiturate exposure. The binding of 20 or 30 nM [^{3}H]flunitrazepam

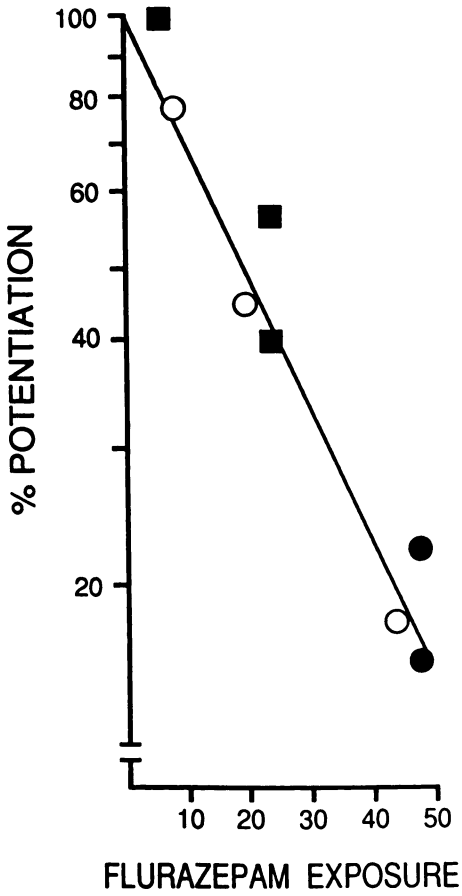

Fig. 6. Time-dependent loss of GABA enhancement of [^{3}H]flunitrazepam binding in cultures chronically incubated with flurazepam. Cultures were exposed to 100 μM flurazepam for the indicated number of hours and binding was determined in the presence and absence of 10 μM GABA. Binding was to culture homogenates (*squares*) or to intact cells (*circles*). The regression line corresponds to a $t_{1/2}$ of 18 hr. *Open circles* are all from the same experiment; *closed symbols* are from independent experiments.

to homogenates derived from cells treated for 48 hr with either 0.5 M pentobarbital or 1 mM barbital showed 95 ± 1% and 103 ± 7% of control binding, respectively (four experiments).

Mechanisms of uncoupling. In an effort to gain insight into the mechanism of uncoupling, we examined the effects of chronic cAMP exposure on cell cultures. Cells were treated with 200 μM cpt-cAMP (a membrane-permeant cAMP analog) for 36 hr and uncoupling was measured. Pooled results from eight experiments showed no significant effects (Table 2). To determine whether uncoupling was a reflection of an alteration in synaptic activity or membrane potential, cells were chronically treated with 100 nM tetrodotoxin (a blocker of the voltage-gated sodium channel) or 10 μM veratridine (a sodium channel activator) for 36 hr. Neither treatment resulted in any measurable effects (Table 2). The concentration of tetrodotoxin used was sufficient to inhibit all synaptic activity measured by intracellular electrode recording in spinal cord cells.[4] Flurazepam-induced uncoupling persisted when chronic treatment was carried out in the presence of the competitive GABA antagonist SR-95531 (Table 3) at a concentration (100 μM) that inhibits GABA-induced down-regulation of [^{3}H]flunitrazepam binding (28). Similarly, the noncompetitive GABA inhibitor picrotoxin (100 μM) failed to inhibit uncoupling induced by chronic exposure to flurazepam or sodium barbital.[5]

Discussion

The regulation of neuromodulation sites in the brain is an area of cellular neuroscience that is relatively unexplored. The GABA$_A$ receptor complex presents a unique opportunity to study sites whose function involves modulation rather than direct signal transduction and, as such, may be regarded as a model for other neuromodulatory receptors.

The characteristics of [^{3}H]flunitrazepam binding to intact neuronal cultures and cellular homogenates derived from culture are virtually identical to those obtained previously with P_2 membrane homogenates from embryonic brain (25). In particular, we find that GABA enhancement of [^{3}H]flunitrazepam binding to homogenates derived from neuronal cultures is similar to that previously determined for brain homogenates (26). Potentiation of [^{3}H]flunitrazepam binding by GABA is blocked by bicuculline, an antagonist selective for the GABA$_A$ receptor (29, 30). Thus, the high affinity binding of [^{3}H]flunitrazepam is due to the "neuronal" benzodiazepine binding site, which is allosterically coupled to the GABA$_A$ receptor. As in embryonic chick brain, neuronal cultures contain both high affinity and low affinity binding sites for [^{3}H]flunitrazepam. We have shown previously (26) that the low affinity component reflects a population of sites with high affinity for Ro5-4864 that do not contribute to modulation of GABA$_A$ receptor function (25), suggesting that these sites may be related to the "peripheral" benzodiazepine binding sites that are present in both brain and peripheral tissues (31). Due to their low affinity for [^{3}H]flunitrazepam, these sites do not contribute significantly to binding of [^{3}H]flunitrazepam over the range of concentrations employed in the present study.

Uncoupling of GABA/benzodiazepine recognition site interactions by chronic benzodiazepine treatment. The question of whether benzodiazepines induce GABA$_A$ receptor down-regulation has become somewhat controversial (see Introduction). Our own results are qualitatively similar to those obtained *in vivo* by Gallager and co-workers (12, 32), who found that long term (3 weeks or more) treatment of mice with diazepam leads to decreased coupling between GABA and benzodiazepine recognition sites but does not alter the total number of receptors. There are at least three possible confounding situations that could account for the apparent discrepancies between the results obtained by different researchers:

The first is the presence of residual benzodiazepine. In investigating the effects of chronic benzodiazepine exposure on [^{3}H]flunitrazepam binding, it is crucial to verify that the results obtained are not due to competition by residual unlabeled drug, which would produce an apparent decrease in binding. Benzodiazepines are lipophilic and are difficult to remove from biological membranes (33). In our experiments, residual flurazepam was adequately removed from cell homogenates by exhaustive dialysis, as shown by the fact that the binding of 1 nM [^{3}H]flunitrazepam (in the absence of GABA) was the same in membranes from control (0.102 ± 0.01 pmol/mg) and flurazepam-treated cells (0.106 ± 0.01; 58 experiments). As an additional control, we verified that there was no change in either total [^{3}H]flunitrazepam binding or enhancement by GABA

[4] D. Mierlak, personal communication.

[5] L. Friedman, unpublished results.

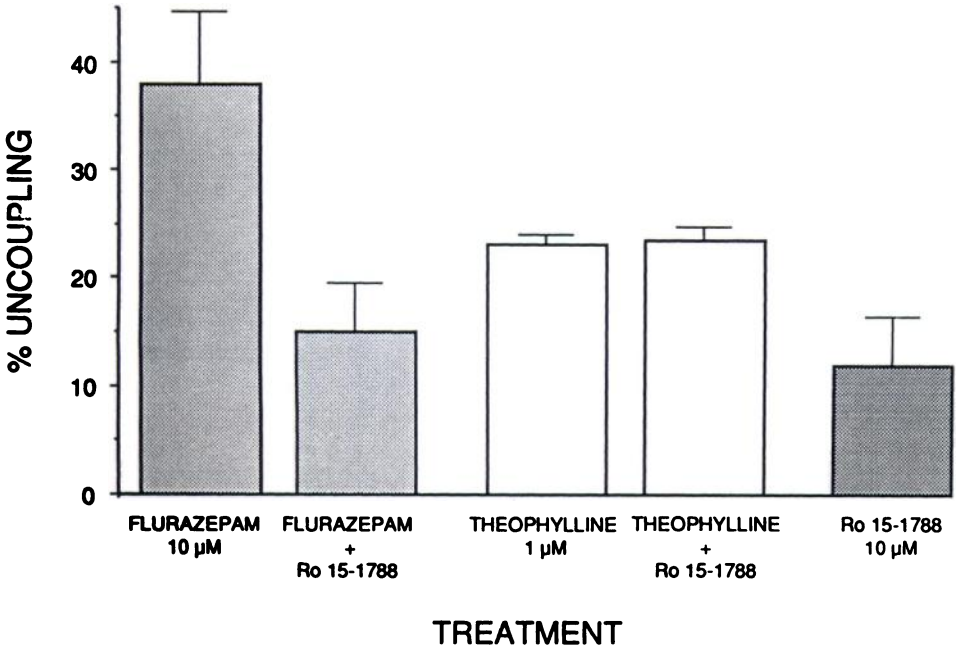

Fig. 7. Flurazepam- but not theophylline-induced uncoupling is reduced by Ro15-1788. Five or six culture dishes were exposed for 36 hr to flurazepam (10 μM) or theophylline (1 μM) alone or in combination with Ro15-1788 (10 μM), then homogenized, dialyzed, and assayed for enhancement of [^{3}H]flunitrazepam binding by GABA. Results are means ± standard errors of four experiments. In each experiment, values were the means of triplicate determinations. Flurazepam-induced uncoupling was significantly reduced by Ro15-1788 ($p < 0.001$, Student's paired t test), whereas theophylline-induced uncoupling was unaffected. The uncoupling induced by Ro15-1788 alone was not statistically significant.

TABLE 1

Uncoupling is not the result of a decrease in protein synthesis

Cultures were exposed to the indicated drug for 36 hr incubated (1 hr, 37°) with 5 nM [^{3}H]methionine and washed, and TCA-precipitable radioactivity was determined, as described in Experimental Procedures. Numbers in parentheses indicate number of independent experiments. Values shown are mean ± standard error.

Chronic treatment	Protein synthesis
	% of control
Flurazepam	
10 μM	111 ± 16 (3)
100 μM	79 ± 5 (5)[a]
Theophylline	
1 μM	108 ± 15 (3)[b]
100 μM	124 ± 18 (2)

[a] Significantly different from control, $p < 0.01$, paired Student's t test.
[b] From Roca *et al.* (20).

when cell homogenates, rather than living cells, were chronically exposed to flurazepam, indicating that a passive mechanism such as retention of drug cannot account for the observed results.

The second is the presence of endogenous GABA during the assay period. We find that the number of [^{3}H]flunitrazepam binding sites is unchanged after chronic benzodiazepine treatment. The fact that the allosteric coupling between GABA and benzodiazepine recognition sites is reduced, however, means that the potentiating effect of endogenous GABA would be expected to decrease following chronic treatment. Thus, if endogenous GABA were present during the assay, chronically treated cells would exhibit a decrease in [^{3}H]flunitrazepam binding that could be mistaken for down-regulation.

The third is the presence of endogenous GABA during the chronic exposure period. Because chronic GABA exposure induces $GABA_A$ receptor down-regulation (17, 28), it is reasonable to speculate that, if sufficient endogenous GABA is present during the chronic exposure period, then benzodiazepines may indirectly promote $GABA_A$ receptor down-regulation by enhancing the binding of GABA. Thus, either uncoupling or down-regulation could occur, depending upon whether endogenous GABA levels are low or high.

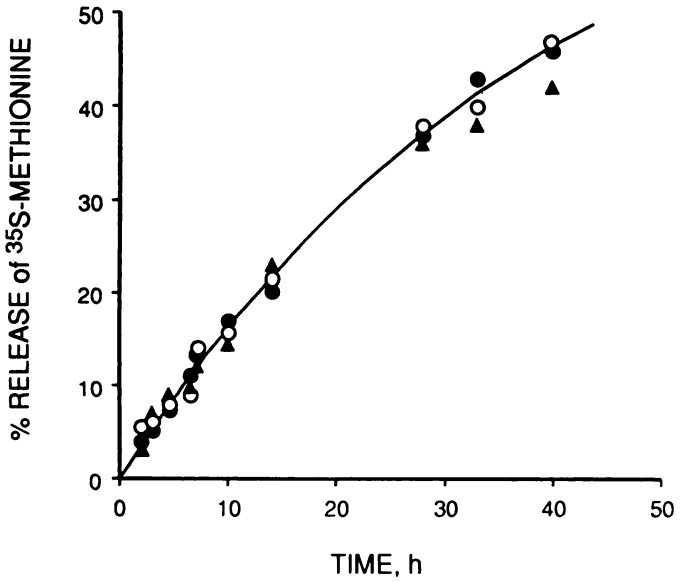

Fig. 8. Protein degradation is unaffected by chronic exposure to flurazepam or theophylline. Six 35-mm culture dishes were incubated with 4 nM [^{35}S]methionine for 36 hr at 37°, in the absence (○) or presence of 100 μM flurazepam (▲) or 100 μM theophylline (●), at which time ($t = 0$) the cultures were washed three times and the medium was replaced with fresh medium containing the same concentration of flurazepam or theophylline plus 2 mM nonradioactive methionine. The medium was periodically sampled and release of TCA-soluble radioactivity was determined as a function of time. Results shown are the combination of two experiments. Each point is the mean of six determinations ± standard error.

Uncoupling of GABA/benzodiazepine recognition sites by methylxanthines. Surprisingly, uncoupling of $GABA_A$/benzodiazepine receptor interactions is also observed following chronic exposure to methylxanthines (20). Although some methylxanthines bind to the benzodiazepine recognition site, they do so with low affinity (IC_{50} approximately 400 μM) (34), whereas methylxanthines are highly potent in inducing uncoupling (20). Moreover, methylxanthine-induced uncoupling is not affected by Ro15-1788, which inhibits flurazepam-induced

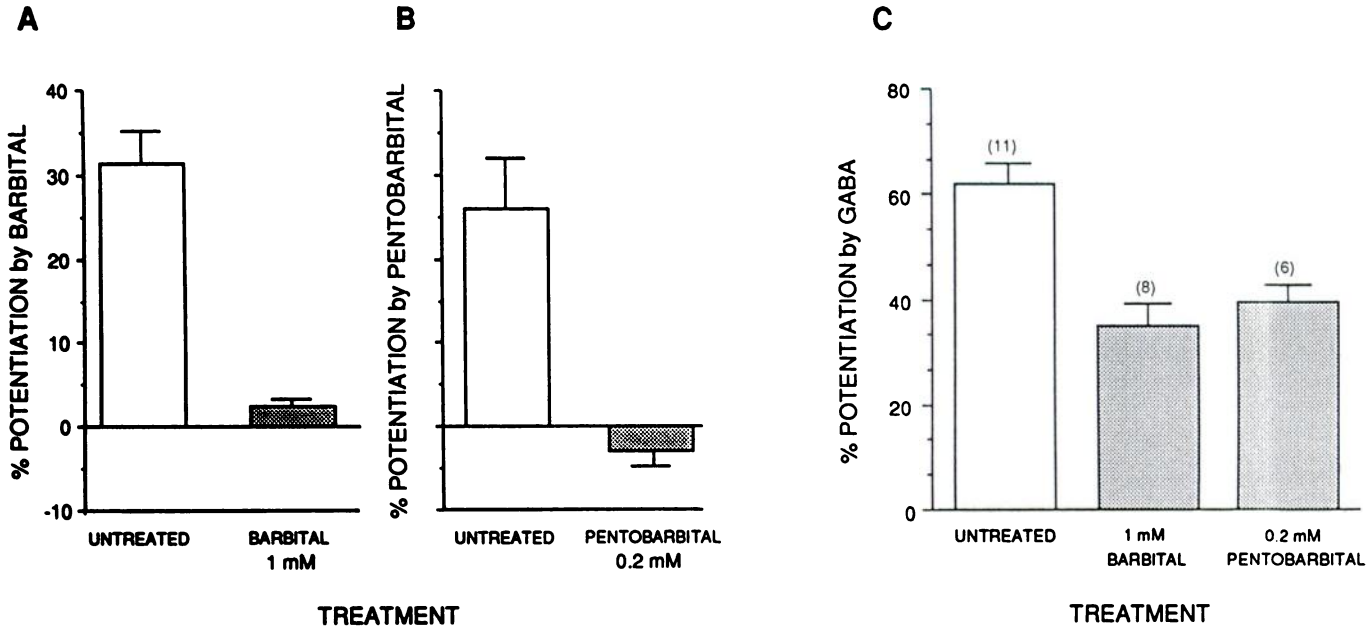

Fig. 9. Chronic barbital or pentobarbital treatment reduces the ability of barbiturates or GABA to potentiate [^{3}H]flunitrazepam binding. A and B, Direct enhancement of flunitrazepam binding by barbiturates is eliminated after chronic barbiturate treatment. Six 60-mm culture dishes were treated for 48 hr in the absence or presence of 1 mM barbital (A) or 0.2 mM pentobarbital (B). Potentiation of 1 nM [^{3}H]flunitrazepam binding by 1 mM barbital (A) or 0.2 mM pentobarbital (B) was then determined in quadruplicate samples of dialyzed cell homogenates. C, Chronic barbiturate treatment reduces GABA potentiation of [^{3}H]flunitrazepam binding. Six 60-mm culture dishes were incubated for 48 hr in the absence or presence of barbital or pentobarbital. In A, B, and C, results are expressed as percentages of potentiation of [^{3}H]flunitrazepam binding. Values represent means ± standard errors of five independent experiments in A and B or as indicated in the parentheses in C. Treated groups were significantly different from controls ($p < 0.01$, Student's paired t test).

uncoupling, but is inhibited by 2-chloroadenosine, an adenosine receptor agonist. Thus, it seems more likely that methylxanthine-induced uncoupling is mediated through a separate receptor, possibly a novel adenosine receptor.

Uncoupling by barbiturates. Like benzodiazepines, barbiturates at low concentrations potentiate the GABA response; however, barbiturates do not bind to the benzodiazepine recognition site, as shown by the fact that barbiturates enhance, rather than inhibit, the binding of radiolabeled benzodiazepines (35). We now report the finding that the enhancement of [^{3}H] flunitrazepam binding by barbiturates is decreased following chronic exposure to barbiturates, indicating a virtually complete elimination of allosteric coupling between the barbiturate and benzodiazepine recognition sites. Notably, chronic exposure to barbiturates also reduces enhancement of [^{3}H]flunitrazepam binding by GABA, suggesting that the uncoupling of benzodiazepine and barbiturate recognition sites from the GABA recognition site may be governed by a common phenomenon.

Barbiturates differ from benzodiazepines in that barbiturates have the capacity to open the GABA$_A$ receptor chloride channel even in the absence of GABA (36). Because chronic exposure to GABA results in GABA$_A$ receptor down-regulation (17, 28), it might be expected that barbiturates would produce the same effect, but we found no change in the [^{3}H]flunitrazepam B_{max} after barbiturate treatment. It should be noted, however, that barbiturates are substantially more potent in enhancing the GABA response than in opening the channel directly. In particular, the concentration of pentobarbital used in the present study (200 μM) produces only a small direct effect but a large enhancement of the GABA response (37).[6] It is possible that chronic exposure to greater barbiturate concentrations would produce down-regulation as well as uncoupling.

Mechanism of uncoupling. Although flurazepam-, barbiturate-, and theophylline-induced uncoupling are almost certainly mediated by different binding sites, a common modification of the receptor may be involved in the control of allosteric site interactions. Although GABA alone can also induce uncoupling (Table 4), it does not seem likely that uncoupling induced by flurazepam and barbiturates is a secondary consequence of enhancement of the action of endogenous GABA, because inhibitors of GABA-mediated synaptic transmission did not block uncoupling by flurazepam or barbiturates. Moreover, none of these compounds produced down-regulation, such as is seen following chronic exposure to GABA (17, 28).

There are at least four potential mechanisms whereby uncoupling could occur, 1) a lasting conformational change in the receptor, such as might occur during desensitization, 2) the production (or loss) of a regulatory factor that binds to the GABA$_A$ receptor and modifies its function, 3) a covalent modification of the GABA$_A$ receptor, such as by phosphorylation, or 4) a change in the subunit composition of the receptor.

A simple conformational change seems the least likely, in light of the slow onset of uncoupling, the absence of uncoupling after chronic incubation of homogenates with flurazepam, and the persistence of uncoupling through the lengthy period of dialysis. In particular, uncoupling does not appear to reflect desensitization of the GABA$_A$ receptor. The observation that the time course of the appearance of uncoupling has a $t_{1/2}$ of approximately 18 hr rather than several minutes indicates that this process does not conform to the generally accepted criteria for desensitization. The $t_{1/2}$ for desensitization of GABA$_A$ receptor by 10 μM GABA is approximately 10 sec (38).

[6] J. J. Celentano, unpublished results.

TABLE 2

Chronic exposure to $GABA_A$ receptor agonists, positive allosteric modulators, and adenosine receptor antagonists uncouples the allosteric interactions between the GABA and benzodiazepine binding sites

Embryonic brain cell cultures were treated with the indicated drugs for 36 hr (48 hr for barbiturate treatments). Uncoupling of GABA/benzodiazepine recognition site interactions was measured as described in Experimental Procedures. For the compounds that produced uncoupling [flurazepam, barbital, pentobarbital, theophylline, caffeine, and isobutylmethylxanthine (IBMX)], means and standard errors reflect all experiments carried out, regardless of the extent of uncoupling observed. The remaining compounds never produced significant uncoupling; however, because of concerns related to variability of uncoupling (see Results), means for these compounds are calculated using only those experiments in which sister cultures chronically treated with flurazepam showed uncoupling of at least 10%.

Chronic treatment	No. of expt.	Uncoupling
		%
Flurazepam 10 μM	39	18 ± 3[a]
100 μM	37	44 ± 4[a]
R 015-1788, 10 μM	4	12 ± 5
Barbital 1 mM	8	49 ± 12[b]
Pentobarbital 200 μM	8	44 ± 6[a]
Theophylline 1 μM	47	16 ± 2[a]
Caffeine[c] 1 μM	3	23 ± 6[d]
IBMX[c] 100 μM	9	22 ± 6[d]
2-Chloroadenosine[c] 10 nM	4	3 ± 4
10 μM	4	1 ± 2
cpt-cAMP 250 μM	8	3 ± 11
Tetrodotoxin 100 nM	6	6 ± 7
Veratridine 10 μM	6	3 ± 2
Carbachol[c] 100 μM	4	7 ± 6

[a] Significantly different than zero, $p < 0.001$, unpaired Student's t test.
[b] $p < 0.005$.
[c] From Roca *et al.* (20).
[d] $p < 0.05$

TABLE 3

Flurazepam-induced uncoupling is not antagonized by SR-95531

Embryonic brain cell cultures were treated with the indicated drugs for 48 hr. Uncoupling of GABA/benzodiazepine recognition site interactions was measured as described in Experimental Procedures. Values shown are mean ± standard error of four experiments.

Treatment	Uncoupling
	%
Flurazepam (50 μM)	54.7 ± 10.4
Flurazepam (50 μM) + SR-95531 (100 μM)	60.8 ± 14.4
SR-95531 (100 μM)	3 ± 1.3

The fact that uncoupling persists following exhaustive dialysis also argues against a small soluble regulatory factor, although the possibility of a large or membrane-bound regulatory factor cannot be excluded. Phosphorylation of the $GABA_A$ receptor complex has been shown to take place in membrane homogenates and in partially purified receptor preparations (39, 40). Moreover, there is electrophysiological evidence that phosphorylation may be important in maintaining $GABA_A$ receptor function in internally perfused neurons (41, 42). The lack of effect of chronic cpt-cAMP treatment fails to support the hypothesis that a cAMP-dependent protein kinase mediates uncoupling; however, the possibility that uncoupling may be mediated by a cAMP-independent phosphorylation mechanism has not been excluded.

Finally, the observation that the $t_{1/2}$ for uncoupling is similar to the average $t_{1/2}$ for benzodiazepine receptor turnover [18 hr (22, 43)] prompts the speculation that uncoupling may involve the synthesis of new receptors. Interestingly, transient expression studies using cloned $GABA_A$/benzodiazepine receptor subunits indicate that receptors constructed with the α_3 variant of the α subunit exhibit substantially greater potentiation of [^{3}H] flunitrazepam binding by GABA than do receptors constructed with the α_1 or α_2 variants (44). Thus, a change in receptor subunit composition could reasonably account for uncoupling.

TABLE 4

Regulation at the GABA recognition site is distinct from regulation at positive modulatory sites

	Down-regulation	Uncoupling
$GABA_A$ receptor agonists[a]	Yes	Yes
$GABA_A$ receptor positive modulators[b]	No	Yes
Adenosine receptor antagonists[c]	No	Yes

[a] GABA, muscimol (Roca *et al.*, *Mol. Pharmacol.* **37**:37–43 (1990)).
[b] Benzodiazepines (flurazepam, diazepam), barbiturates (pentobarbital, barbital).
[c] Methylxanthines (theophylline, caffeine, isobutylmethylxanthine) (20).

Physiological role of $GABA_A$ receptor regulation. The phenomenon of uncoupling is not unique to neurons in tissue culture, inasmuch as similar results have been reported in certain brain regions following chronic benzodiazepine treatment *in vivo* (12). The observation of uncoupling in dissociated cell culture argues that this form of regulation occurs on a cellular level and is not contingent upon the presence of intact neural pathways or cytoarchitecture of the central nervous system.

Although there is little doubt that the barbiturate- and benzodiazepine-sensitive modulatory sites located on the $GABA_A$ receptor mediate many of the pharmacological effects of these drugs, it is unknown whether these sites play any role in the normal functioning of the nervous system or whether they are only drug acceptor sites. The notion that an endogenous benzodiazepine-like or barbiturate-like substance may control levels of inhibition in the central nervous system remains an intriguing possibility (45). If these sites do indeed play a physiological role, it would make sense for there to be a mechanism for regulating modulator interactions independently of receptor number. The present results, which indicate that the strength of the allosteric interaction between the benzodiazepine and GABA recognition sites is regulated in response to chronic drug exposure, suggest that the interaction of benzodiazepines with the $GABA_A$ receptor is not fortuitous and they support the view that these modulatory sites may play a role in control of GABA-mediated inhibition in the central nervous system. It is possible that benzodiazepine- and barbiturate-induced uncoupling of modulator interactions with the $GABA_A$ receptor is an early step in the process by which clinical tolerance to these compounds develops.

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