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# Chronic Benzodiazepine Agonist Treatment Produces Functional Uncoupling of the γ-Aminobutyric Acid-Benzodiazepine Receptor Ionophore Complex in Cortical Neurons

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**SUMMARY**

We have investigated the effect of chronic flurazepam HCl treatment on the γ-aminobutyric acid $(GABA)_A$ receptor complex in cultured mammalian cortical neurons. Chronic flurazepam (1–5 μM, for 1–10 days) treatment did not produce any changes in the morphological appearance or the cell protein content of cortical neurons. The basal binding of [$^3$H]flunitrazepam, [$^3$H]Ro15–1788, and [$^3$H]Ro15–4513 was also not altered after the chronic treatment. However, chronic flurazepam treatment produced uncoupling between GABA and pentobarbital sites and the [$^3$H]flunitrazepam binding site. The $EC_{50}$ values of GABA and pentobarbital were not significantly altered after chronic flurazepam treatment; however, their $E_{max}$ values were decreased by ~50%. The effect of chronic flurazepam treatment on the observed uncoupling was both time and concentration dependent. Furthermore, the binding of [$^3$H]GABA and *t*-butylbicyclophosphoro[$^{35}$S]thionate was also not altered by chronic flurazepam treatment. The effect of GABA on $^{36}Cl$ influx was not altered after chronic flurazepam treatment; however, treatment significantly attenuated the ability of diazepam to enhance GABA-induced $^{36}Cl$ influx. Chronic flurazepam-induced uncoupling and decreased diazepam efficacy were reversed by the concomitant presence of the benzodiazepine antagonist Ro15–1788, suggesting that these events are mediated via the benzodiazepine receptor site. Taken together, these results suggest that chronic benzodiazepine treatment produces uncoupling of GABA and pentobarbital sites from the benzodiazepine site and decreased coupling between the benzodiazepine site and GABA receptor-gated $Cl^-$ channels. The uncoupling and decreased efficacy may be due to an alteration in the levels of various α subunits and may be responsible for the tolerance associated with chronic benzodiazepine agonist treatment.

The benzodiazepines are widely used in clinical medicine as anxiolytic, sedative-hypnotic, muscle relaxant, and anticonvulsant agents. There is ample evidence from behavioral, electrophysiological, and neurochemical studies to suggest that benzodiazepines exert their pharmacological effects on the central nervous system by potentiating the inhibitory synaptic transmission mediated by GABA, acting at $GABA_A$ receptors, to produce an allosteric modulation of the complex that results in an increase in the frequency of bursting activity of the GABA-gated $Cl^-$ channels (1–4). It is well established that benzodiazepine agonists enhance and inverse agonists inhibit GABA-ergic transmission. Furthermore, the effects of benzodiazepine agonists and inverse agonists are blocked by Ro15–1788, suggesting modulation via the same receptor site (5). It is also known that chronic administration of benzodiazepines can produce tolerance to the effects of these drugs, which limits their clinical efficacy (6–9).

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The potential mechanisms involved in the development of tolerance to benzodiazepines have yet to be defined. There are controversial data in the literature regarding the consequences of chronic benzodiazepine treatment on the $GABA_A$-ergic system. Thus, chronic benzodiazepine exposure have been reported to cause no change (10–13), an increase (14), or a decrease (15–17) in the benzodiazepine receptor number. $GABA_A$ receptor function was also reported to be either unchanged (13, 18, 19) or decreased after chronic benzodiazepine treatment (15, 17, 20, 21). However, chronic benzodiazepine treatment has been reported to produce decreased coupling between GABA and benzodiazepine receptor sites (11, 21). The reason for these discrepancies is not clear but could be due to differences in methodology, drug choice, pharmacokinetics, or species used.

In view of these conflicting results, we investigated the effects of chronic flurazepam treatment on the GABA/benzodiazepine receptor ionophore complex in intact cultured cortical neurons, using both radioligand binding assays and $^{36}Cl$ influx assays. The experiments were performed under precisely controlled

**ABBREVIATIONS:** GABA, γ-aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBPS, *t*-butylbicyclophosphorothionate; MEM, minimal essential medium.

conditions, independently of pharmacokinetic variability, in well characterized cortical neurons. The purpose of this study was to explore whether chronic benzodiazepine receptor agonist exposure altered the benzodiazepine binding, caused an uncoupling, and/or altered $GABA_A$ receptor-gated $Cl^-$ channel function and to provide additional information on the GABA/benzodiazepine receptor complex associated with the $Cl^-$ channel after chronic flurazepam treatment.

## Experimental Procedures

**Materials.** Female and male C57BL/6CR mice (18–22 g) were purchased from Charles River (Boston, MA). $^{36}Cl$ (HCl) was purchased from ICN Radiochemicals (Irvine, CA). [$^3$H]Flunitrazepam, [$^3$H]GABA, [$^3$H]Ro15–1788, [$^3$H]Ro15–4513, and [$^{35}$S]TBPS were purchased from New England Nuclear (Boston, MA). GABA, (+)-nipecotic acid, sodium pentobarbital, picrotoxin, poly-L-lysine hydrobromide ($M_r$ >300,000), 5-fluoro-2-deoxyuridine, uridine, and flurazepam HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Diazepam and Ro15–1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo [1,5$\alpha$][1,4]benzodiazepine-3-carboxylate) were kindly supplied by Hoffmann-LaRoche (Nutley, NJ). Bicinchoninic acid protein assay reagents were purchased from Pierce Chemical Co. (Rockford, IL). MEM and serum were obtained from GIBCO (Santa Clara, CA). All other chemicals were purchased from commercially available sources.

**Preparation of cell cultures.** Cerebral hemispheres were dissected from 15-day-old C57BL/6CR mouse embryos. The embryos, in their sacs, were removed and placed in a 60-mm culture dish containing ice-cold aerated (95% $O_2$/5% $CO_2$) Puck's buffer, pH 7.4 (100 ml of 10× Puck's saline, 10 ml of 1 M HEPES, and 50 ml of 12% glucose/30% sucrose solution, <320–330 mOsM), and cerebral hemispheres were dissected out under a microscope fitted with a light source. The cerebral hemispheres were minced with iridectomy scissors in an empty, sterile, 25-mm Petri dish. Tissue was then taken up in nutrient medium, pH 7.4, containing 80% Eagle's MEM, 33.3 mM glucose, 26.2 mM $NaHCO_3$, 10% heat-inactivated (56° for 30 min) horse serum, and 10% fetal bovine serum (MEM 10/10) and was transferred to a sterile tube. Tissue fragments were subjected to dissociation by trituration. This cycle of resuspension in MEM 10/10, followed by trituration, was repeated and supernatant volume of 2–3 ml/cortex was attained. Dissociated cells were plated on poly-L-lysine-coated, sterile, 25-mm, round coverslips, by addition of 0.5 ml of suspension to dishes containing 1 ml of MEM 10/10 that had been preincubated with 95% $O_2$/5% $CO_2$ for at least 1 hr at 37°.

The plated cultures were incubated for 24 hr, at which time 1 ml of growth medium was replaced with the same volume of medium containing 10% heat-inactivated horse serum only (MEM 10) and a mixture of sterile 5-fluoro-2′-deoxyuridine and uridine (2 mg/ml 5-fluoro-2′-deoxyuridine and 5 mg/ml uridine), at a final concentration of 10 $\mu$g/ml, was added to control the proliferation of non-neuronal cells; this treatment was given once each week. After 3 days of culture *in vitro*, chronic flurazepam HCl exposure studies were initiated, and all of the medium (MEM 10) with appropriate drugs was replaced every 24 hr; untreated cultured neurons prepared in parallel were used as controls. The experiments were performed on neurons after 2 weeks in culture.

**Radioligand binding studies.** Binding studies on intact primary cultured cortical neurons were carried out as described earlier (22). Briefly, coverslips attached with cultured cortical neurons were removed from the tissue culture medium and rinsed three times at room temperature in HEPES-buffered saline (136 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.0 mM $NaH_2PO_4$, 20 mM HEPES, adjusted to pH 7.4 with Tris base), for approximately 5 sec. After this, triplicate cultures were incubated with [$^3$H]flunitrazepam (2 nM), [$^3$H]Ro15–1788 (1 nM), or [$^3$H]Ro15–4513 (2 nM), with or without other drugs, for 30 min at room temperature in HEPES-buffered saline, pH 7.4. Nonspecific binding was determined in parallel in the presence of 10 $\mu$M Ro15–1788. After incubation, the coverslips with attached neurons were rapidly transferred to 1000 ml of ice-cold HEPES-buffered saline and then immersed for 7 sec in another beaker containing 1000 ml of ice-cold HEPES-buffered saline being stirred continuously, followed by a rapid rinse with 2 ml of HEPES-buffered saline solution in a 35-mm Petri dish. After this, each coverslip was drained on tissue paper and transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH. After at least 1 hr of digestion, one half of the mixture was removed for protein assay, and the balance was neutralized with 1.0 N HCl (200 $\mu$l), mixed with 10 ml of Hydrofluor, and counted by liquid scintillation counting.

For the $EC_{50}$ value determination, specific [$^3$H]flunitrazepam binding was determined in the presence of varying concentrations of the ligands. The ability of GABA or sodium pentobarbital to increase [$^3$H]flunitrazepam binding (percentage enhancement) was used as an indicator of the degree to which the GABA/pentobarbital and benzodiazepine sites are "allosterically coupled." Protein was estimated by the bicinchoninic acid protein assay (23).

Drugs that were insoluble in water were dissolved in dimethylsulfoxide and used at a final concentration of ≤0.1%. Control experiments showed that dimethylsulfoxide at up to 0.1% had no significant effect on radioligand binding or $^{36}Cl$ influx.

For [$^{35}$S]TBPS and [$^3$H]GABA binding studies, a mitochondrial and microsomal ($P_2$ plus $P_3$) fraction was prepared (24, 25). Briefly, cultures were scraped from flasks and homogenized in cold 0.32 M sucrose with a Teflon-glass homogenizer, followed by centrifugation at 1000 × *g* for 10 min. The supernatant was centrifuged at 140,000 × *g* for 30 min to obtain the mitochrondrial and microsomal ($P_2$ plus $P_3$) fraction. This fraction was resuspended in ice-cold double-distilled water and homogenized with a Brinkman Polytron at a setting of 6 for two 10-sec bursts, 15 sec apart. The suspension was centrifuged at 140,000 × *g* for 30 min and the pellet was washed three times, by homogenization and centrifugation, with buffer (for [$^{35}$S]TBPS binding, 200 mM KCl, 5 mM Tris·HCl, pH 7.4; for [$^3$H]GABA binding, 50 mM KCl, 50 mM Tris·HCl, pH 7.4) and was frozen at −70°. On the day of assay, the tissue was thawed, similarly centrifuged, washed two more times, and resuspended in buffer. Aliquots of membrane suspension were incubated with 5 nM [$^{35}$S]TBPS for 180 min or with 4 nM [$^3$H]GABA for 10 min, as described earlier (25). [$^3$H]GABA binding was measured by a centrifugation assay, whereas [$^{35}$S]TBPS binding was determined by a filtration assay. Nonspecific binding was determined in the presence of 10 $\mu$M GABA (for [$^3$H]GABA) or 10 $\mu$M picrotoxin (for [$^{35}$S]TBPS). All values for radioligand binding were expressed per milligram of cellular protein.

**$^{36}Cl$ influx studies.** $^{36}Cl$ influx was measured as described earlier (5). Briefly, coverslips with attached cultures were removed from tissue culture medium, rinsed quickly three times at room temperature with HEPES-buffered saline solution, pH 7.4, and drained rapidly on tissue paper, followed by immediate transfer to 2 ml of HEPES-buffered saline containing $^{36}Cl^-$ (2.0 $\mu$Ci/ml), in the absence or presence of various drugs. GABA-mediated $^{36}Cl$ influx was measured in the presence of the uptake blocker nipecotic acid (100 $\mu$M). Influx was terminated after 5 sec by rapid transfer of the coverslips with attached cultures to 1000 ml of ice-cold stop solution, pH 7.4 (150 mM NaCl, 5.4 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.0 mM $NaH_2PO_4$, 5.0 mM HEPES, adjusted to pH 7.4 with Tris base), and then coverslips were immersed for 7 sec in another beaker containing 1000 ml of ice-cold stop solution being stirred continuously. After that, each coverslip was drained on tissue paper, transferred to a scintillation vial containing 1.5 ml of 0.2 N NaOH, and processed as described for binding studies. All values for $^{36}Cl$ influx were expressed per milligram of cellular protein.

Statistical analysis was performed using Student's *t* test; *p* values of <0.05 was considered statistically significant.

## Results

**Effects of chronic flurazepam HCl treatment on cultured cortical neurons.** The protein content determined for coverslips that had been treated with 5 $\mu$M flurazepam for 10

days was similar to that of controls in a large number of experiments. Results from five representative experiments are presented in Table 1 and indicate that chronic flurazepam treatment did not affect the cellular protein content of cortical neurons. Furthermore, chronic flurazepam treatment did not affect the morphological characteristics of cortical neurons, as visualized by phase-contrast microscopy (Fig. 1).

The cultured cortical neurons used in the present study contain all of the components of the $GABA_A$/benzodiazepine receptor ionophore complex (26). In our initial studies, we examined the effect of chronic flurazepam treatment on the basal binding of various benzodiazepine ligands to the benzodiazepine receptor site. Table 2 shows that chronic flurazepam (5 $\mu$M, 10 days) treatment did not alter the basal binding of [$^3$H]flunitrazepam (agonist), [$^3$H]Ro15–1788 (antagonist), or [$^3$H]Ro15–4513 (inverse agonist) to intact cortical neurons. The results in Table 2 also indicate that the protocols used in our study are effective in removing flurazepam and there is no residual drug in our chronically treated cultures before the measurement of the binding.

TABLE 1

**Effects of chronic flurazepam HCl (5 $\mu$M, 10 days) treatment on total cellular protein content in cultured cortical neurons**

Results are from five representative experiments. The values are mean ± standard deviation of triplicate determinations.

| Expt. | Protein | |
|---|---|---|
| | Control group | Treated group |
| | *μg/coverslip* | |
| 1 | 135 ± 22 | 137 ± 34 |
| 2 | 163 ± 19 | 161 ± 14 |
| 3 | 148 ± 32 | 146 ± 17 |
| 4 | 174 ± 10 | 174 ± 13 |
| 5 | 159 ± 11 | 157 ± 14 |

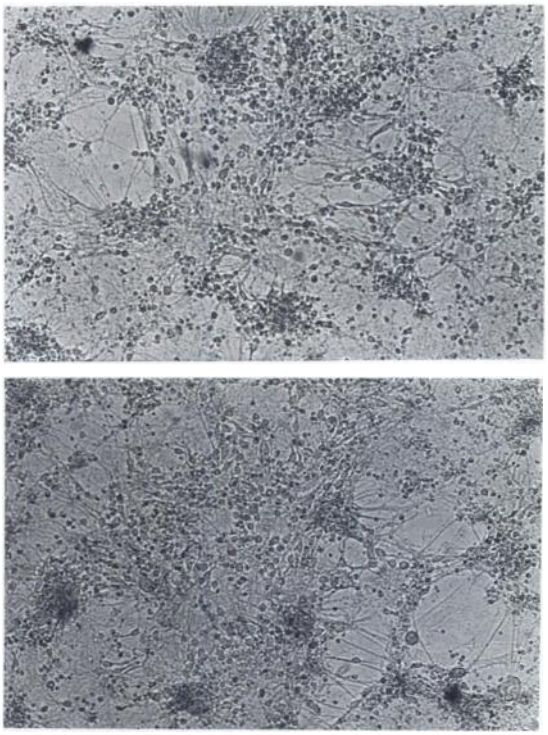

**Fig. 1.** Phase-contrast micrographs (× 50) of control (*top*) and chronically flurazepam HCl (5 $\mu$M, 10 days)-treated (*bottom*) cultured primary cerebral cortical neurons.

TABLE 2

**Effects of chronic flurazepam HCl (5 $\mu$M, 10 days) treatment on the specific binding of [$^3$H]flunitrazepam, [$^3$H]Ro15-1788, and [$^3$H]Ro15-4513 in intact cultured cortical neurons**

The values are mean ± standard deviation from three or four experiments, each performed in triplicate.

| Radioligand | Specific binding | |
|---|---|---|
| | Control group | Treated group |
| | *fmol/mg* | |
| [$^3$H]Flunitrazepam (2 nM) | 230 ± 21 | 228 ± 24 |
| [$^3$H]Ro15-1788 (1 nM) | 218 ± 12 | 218 ± 13 |
| [$^3$H]Ro15-4513 (2 nM) | 418 ± 7 | 419 ± 14 |

**Effects of chronic flurazepam treatment on coupling/uncoupling of the $GABA_A$ receptor complex.** After a 10-day exposure to flurazepam (5 $\mu$M), although the [$^3$H]flunitrazepam specific binding was not altered, there was a significant decrease in the enhancement of [$^3$H]flunitrazepam binding by ligands that bind to other sites of the $GABA_A$ receptor complex. Fig. 2 shows the concentration-dependent enhancement of [$^3$H]flunitrazepam binding by GABA (Fig. 2A) and pentobarbital (Fig. 2B) in control and chronically (5 $\mu$M, 10 days) treated neurons. Chronic flurazepam treatment decreased the $E_{max}$ values for GABA (Fig. 2A) and pentobarbital (Fig. 2B) without altering their $EC_{50}$ values (Table 3). Fig. 3 shows that the effect of chronic flurazepam treatment on the uncoupling of GABA and pentobarbital was time dependent. The half-maximal uncoupling occurred at 3 days of treatment, with maximal uncoupling occurring at 5–10 days.

Fig. 4 shows that the effect of chronic flurazepam treatment on GABA (Fig. 4a) and pentobarbital (Fig. 4b) enhancement was concentration dependent, with half-maximal effect occurring at 1–2 $\mu$M and maximal effect occurring at 5 $\mu$M.

**Effects of chronic flurazepam treatment on [$^3$H]GABA and [$^{35}$S]TBPS binding.** Table 4 compares the effect of chronic flurazepam (5 $\mu$M, 10 days) treatment on specific [$^3$H]GABA and [$^{35}$S]TBPS binding. Chronic flurazepam treatment did not alter the binding of GABA or TBPS to their respective binding sites.

**Effects of chronic flurazepam treatment on GABA-induced $^{36}$Cl influx.** To investigate the effect of chronic flurazepam (5 $\mu$M, 10 days) treatment on the functional aspects of GABA-ergic transmission, we measured the GABA-mediated $^{36}$Cl influx in these neurons. GABA produced a concentration-dependent increase in $^{36}$Cl influx, with an $EC_{50}$ value of 13.5 ± 2 $\mu$M and maximal enhancement of 89 ± 10% in control neurons (Fig. 5; Table 5). GABA produced a similar concentration-dependent enhancement of $^{36}$Cl influx in chronically treated neurons (Fig. 5). The $E_{max}$ and $EC_{50}$ values of GABA were not altered after the chronic treatment (Table 5). We next examined the effect of chronic flurazepam treatment on diazepam enhancement of GABA-induced $^{36}$Cl influx. Table 6 shows the effect of chronic flurazepam treatment on diazepam enhancement of GABA-induced $^{36}$Cl influx. Chronic flurazepam treatment decreased the enhancing effect of diazepam on GABA-mediated $^{36}$Cl influx. This effect was reversed by withdrawal of the drug for 72 hr, suggesting that the observed effect is reversible (data not shown).

**Reversal by Ro15–1788 of chronic flurazepam-induced uncoupling and decreased diazepam efficacy.** To

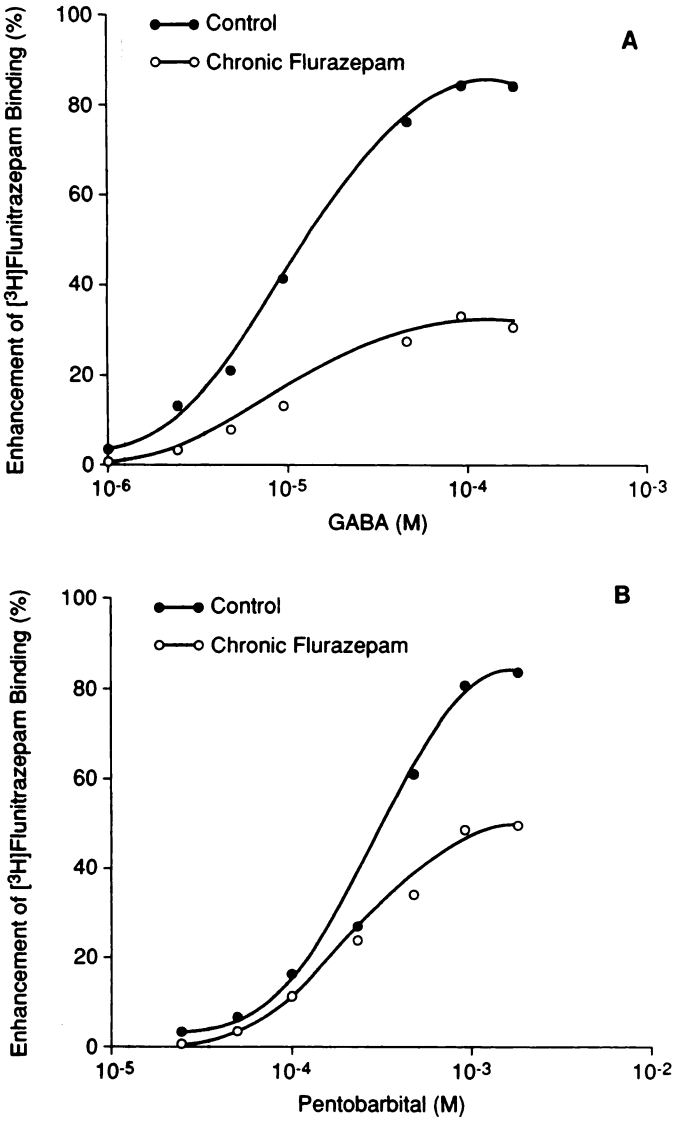


**Fig. 2.** Concentration-dependent enhancement of the specific [$^3$H][flunitrazepam (2 nM) binding by GABA (A) and pentobarbital (B) in control and chronically flurazepam HCl (5 $\mu$M, 10 days)-treated cultured cortical neurons. $EC_{50}$ and $E_{max}$ values are summarized in Table 3. Data are representative of four separate experiments performed in triplicate.

TABLE 3

**Effect of chronic flurazepam treatment on the $EC_{50}$ and $E_{max}$ values for GABA and pentobarbital enhancement of [$^3$H]flunitrazepam binding in cortical neurons**

Values are mean ± standard deviation of three or four experiments, each done in triplicate. Values in parentheses represent percentage uncoupling.

| | [$^3$H]Flunitrazepam binding | | | |
|---|---|---|---|---|
| | $EC_{50}$ | | $E_{max}$ | |
| | Control group | Treated group | Control group | Treated group |
| | $\mu$M | | % | |
| GABA | 11.7 ± 0.6 | 11.5 ± 2.4 | 92 ± 6 | 32 ± 2 (−65%)* |
| Pentobarbital | 357 ± 51 | 250 ± 37 | 78 ± 6 | 38 ± 9 (−51%)* |

*$p < 0.001$, compared with the control group.

examine the possible involvement of the benzodiazepine binding sites in uncoupling, we examined the effect of concomitant exposure of cortical neurons to flurazepam and the benzodiazepine antagonist Ro15–1788. Fig. 6 shows that chronic flurazepam-induced uncoupling of GABA and pentobarbital from the benzodiazepine receptor sites was blocked by concomitant exposure of the neurons to the benzodiazepine receptor antagonist Ro15–1788 (3 $\mu$M). Control cortical neurons treated with Ro15–1788 (3 $\mu$M) did not show alterations in basal [$^3$H]flunitrazepam binding (Fig. 6). Fig. 7 shows that the concomitant presence of Ro15–1788 also reversed the decreased efficacy of diazepam on GABA-induced $^{36}$Cl influx. In contrast, the GABA$_A$ receptor antagonist R5135 (1 $\mu$M) and the channel antagonist picrotoxin (10 $\mu$M) did not reverse uncoupling or the decreased efficacy of diazepam (data not shown). These results suggest that the observed events are mediated via the benzodiazepine receptor site.

## Discussion

Benzodiazepines are some of the safest pharmacological agents used to treat a variety of neurological disorders. Based on several lines of behavioral, biochemical, and electrophysiological evidence, it is clear that most of their pharmacological effects are mediated by facilitation of GABA$_A$-ergic transmission in the central nervous system. It is also clear that benzodiazepines have very selective and subtle effects on GABA receptor-gated responses, because they affect only the frequency of channel opening, without affecting the duration of channel opening (27).

Chronic benzodiazepine administration produces tolerance to the various pharmacological effects of these drugs (see the introduction). The present study was aimed at defining the potential mechanism that may be involved in the development of tolerance to the benzodiazepines. These studies were conducted in mammalian cultures from cerebral cortex, a region that is important in manifesting the clinical effects of the benzodiazepines. Our aim was to determine whether tolerance to the benzodiazepines may be mediated via a specific effect on the GABA$_A$ receptors, a phenomenon that could be easily studied in well characterized mammalian cortical neurons. These neurons contain all of the components of the GABA$_A$ receptor complex (26).

Chronic flurazepam treatment did not alter the morphological appearance of the neurons or the total cellular protein content, results consistent with previous reports (for example, see Ref. 15). Furthermore, chronic treatment altered neither the specific binding of a benzodiazepine agonist (flunitrazepam), antagonist (Ro15–1788), and inverse agonist (Ro15–4513) to the benzodiazepine recognition site nor the binding of [$^3$H]GABA and [$^{35}$S]TBPS to the GABA and picrotoxin sites, respectively. Thus, chronic benzodiazepine treatment does not produce down-regulation of any of the binding sites associated with the oligomeric GABA$_A$ receptor complex, as demonstrated in cortical neurons. Our results showing no change in benzodiazepine binding are similar to those reported in chick and mammalian neurons (11, 13) but differ from the membrane homogenate studies, where changes have been reported (10, 14, 16).

It is well known that various sites of the oligomeric GABA$_A$ receptor complex are allosterically coupled. We have previously demonstrated coupling of GABA and barbiturate sites with the benzodiazepine sites in intact cultured cortical neurons (26). A

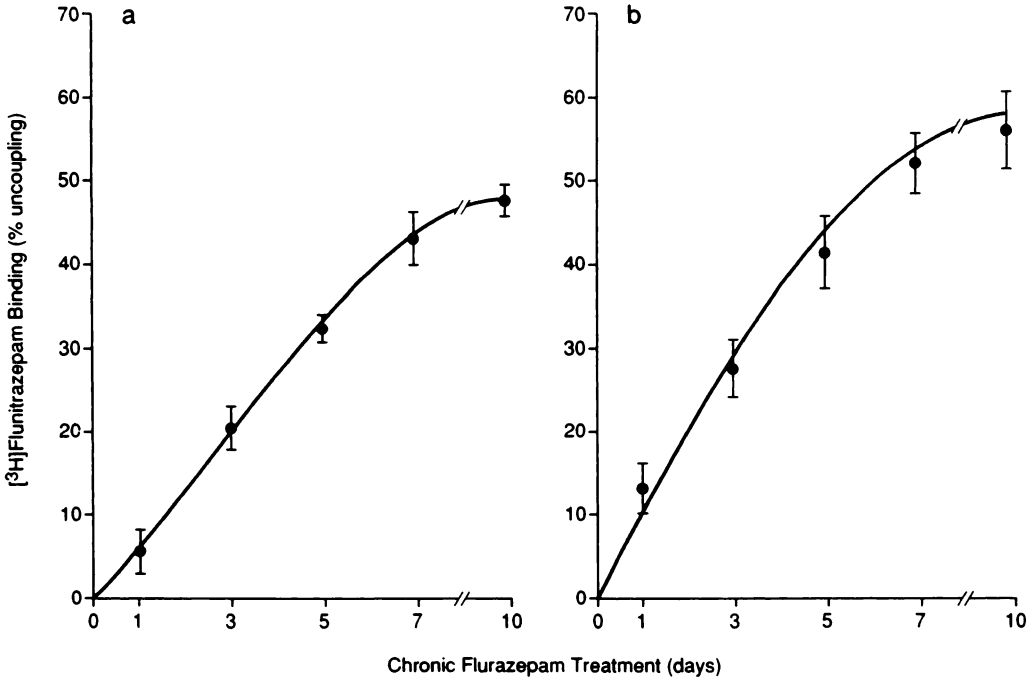


**Fig. 3.** Time course of the uncoupling produced by chronic flurazepam HCl (5 $\mu$M) treatment on GABA (5 × $10^{-4}$ M) (a) and sodium pentobarbital (5 × $10^{-4}$ M) (b) enhancement of [$^3$H]flunitrazepam (2 nM) binding. Cultured cortical neurons were incubated with 5 $\mu$M flurazepam HCl for 1–10 days before the measurement of binding. % *uncoupling*, decrease in the percentage of enhancement observed, relative to control neurons. Values are mean ± standard deviation of three or four experiments, each performed in triplicate.

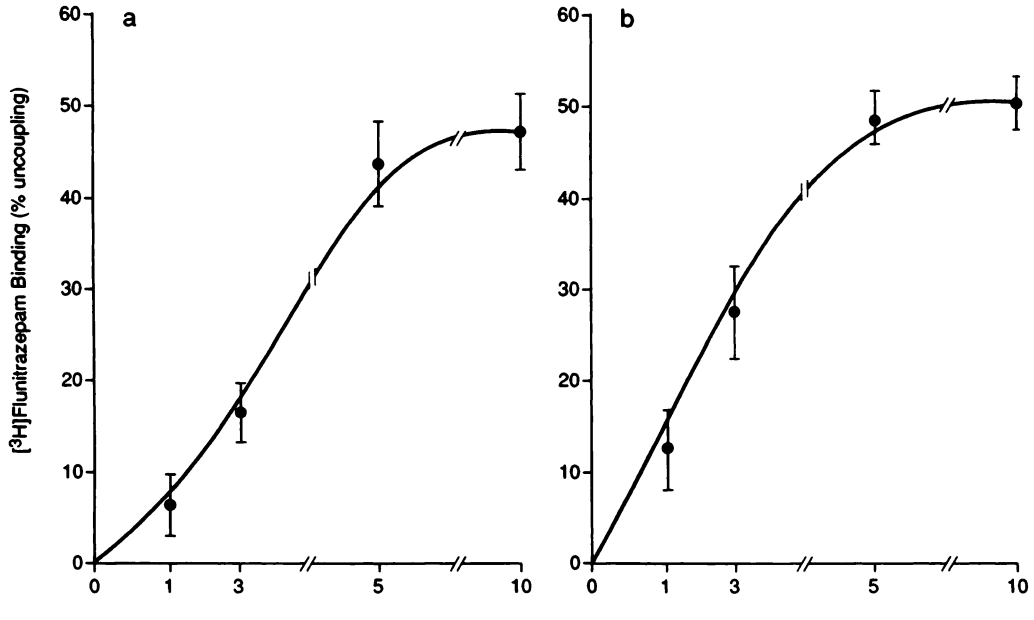


**Fig. 4.** Concentration-dependent effect of chronic flurazepam HCl treatment on uncoupling of GABA (a) and pentobarbital (b) enhancement of [$^3$H] flunitrazepam binding. Cultured cortical neurons were incubated for 10 days in the presence of different concentrations of flurazepam HCl, before the measurement of binding. Values represent mean ± standard deviation of three or four experiments, each performed in triplicate.

second approach in our study was to examine the effect of chronic flurazepam treatment on the coupling of GABA and pentobarbital to [$^3$H]flunitrazepam binding in intact cortical neurons. Chronic flurazepam treatment decreased the coupling of GABA and pentobarbital to the benzodiazepine receptor sites by ~50%. This decreased coupling or uncoupling was concentration and time dependent, with maximal effects occurring at 5 $\mu$M and with 5–10-day treatment. There was no effect on the $EC_{50}$ values for GABA and pentobarbital; however, their $E_{max}$ values were decreased. The uncoupling was susceptible to concomitant exposure of the neurons to the benzodiazepine receptor antagonist Ro15–1788, suggesting that the uncoupling was mediated selectively via the benzodiazepine recognition site. A previous study reported a similar uncoupling between GABA and benzodiazepine receptor sites in cultured brain neurons (11).

TABLE 4

**Effect of chronic flurazepam HCl (5 μM, 10 days) treatment on [$^3$H] GABA and [$^{35}$S]TBPS specific binding**

Cultured cortical neurons were treated with flurazepam (5 μM) for 10 days, and [$^3$H]GABA (4 nM) and [$^{35}$S]TBPS (5 nM) binding was measured as described in Experimental Procedures. Values are mean ± standard deviation of three separate experiments, each performed in triplicate.

| Radioligand | Specific binding | |
|---|---|---|
| | Control group | Treated group |
| | *fmol/mg of protein* | |
| [$^3$H]GABA | 76 ± 9.8 | 73 ± 5.7 |
| [$^{35}$S]TBPS | 73 ± 5.5 | 73 ± 0.4 |

Because the functional significance of coupling as measured by the binding sites is not clear, we measured the effect of chronic flurazepam treatment in the functional assay, i.e., GABA-induced $^{36}$Cl influx. Chronic flurazepam treatment did not alter the GABA-mediated $^{36}$Cl influx in cortical neurons. This observation is consistent with there being no apparent change in GABA receptor binding after chronic treatment. This contrasts with several previous studies that have reported a decrease in GABA receptor function after chronic benzodiazepine treatment (15, 21, 28). However, in our study diazepam enhancement of GABA-mediated $^{36}$Cl influx was greatly attenuated after chronic benzodiazepine treatment. These results also suggest that the uncoupling observed in the binding studies may have functional relevance. The observed effects could occur in both neurons and glial cells.

In a previous study, we and others reported that chronic GABA treatment produced down-regulation of various binding sites, uncoupling, and decreased efficacy of GABA-mediated $^{36}$Cl flux in cortical neurons (26, 29, 30). In contrast, chronic benzodiazepine treatment of the same neurons produced only uncoupling and decreased efficacy of benzodiazepine potentiation of GABA-mediated $^{36}$Cl flux. Thus, the $GABA_A$ receptor and its functions are regulated differently by chronic GABA and flurazepam treatments. The ability of chronic benzodiazepine treatment only to affect coupling and to decrease the efficacy of benzodiazepine agonist potentiation of GABA responses further attests to the ability of benzodiazepines to modulate $GABA_A$-ergic transmission in a subtle and selective manner. Because benzodiazepines enhance GABA-ergic function, it is possible that the GABA recognition site on the receptor may mediate the observed effects. However, the ability of Ro15–1788 (but not R5135 or picrotoxin) to reverse both uncoupling and the decreased efficacy of diazepam suggests an involvement of the benzodiazepine recogniton site and rules out a role for the $GABA_A$ receptors in these events.

The molecular basis of uncoupling and decreased efficacy has yet to be established. Recent preliminary studies have measured the changes in $GABA_A$ receptor gene expression after chronic benzodiazepine treatment. These changes include decreases in the α1 and γ2 subunit mRNA levels (31, 32) and increases in the levels of α3 and α6 subunit mRNAs in whole rat brains (33). The findings of the latter study must be viewed with caution, because the study used intermittent drug treatment and examined the mRNA levels in whole brain. It is very likely that chronic benzodiazepine treatment modulates $GABA_A$ receptor function in a region-specific manner. Furthermore, the type of subunits and their levels vary from region to region (for example, see Ref. 34), and whole-brain measurements cannot explain the regional effects. From the cloning and expression studies, it is clear that benzodiazepine action requires the γ2 subunit in combination with the α and β variants. Furthermore, α variants are crucial in determining the degree of coupling between GABA and benzodiazepine sites and benzodiazepine potentiation of GABA responses in transfected cells. These studies have demonstrated that the α3 subunit gives maximal enhancement of benzodiazepine agonist binding by GABA and maximal efficacy of benzodiazepine agonists in enhancing GABA-ergic responses (35, 36). We postulate that chronic flurazepam treatment-induced uncoupling and decreased efficacy of diazepam, as observed in our study, may be due to an alteration in the levels of the α variants, which are crucial for generating functional assembled $GABA_A$ receptors. We speculate that chronic flurazepam treatment may decrease the levels of the α3 variant and/or increase the levels of the α2/α1 subunits in cortical neurons. However, we cannot rule out a role for other $GABA_A$ receptor subunits. Such an alteration

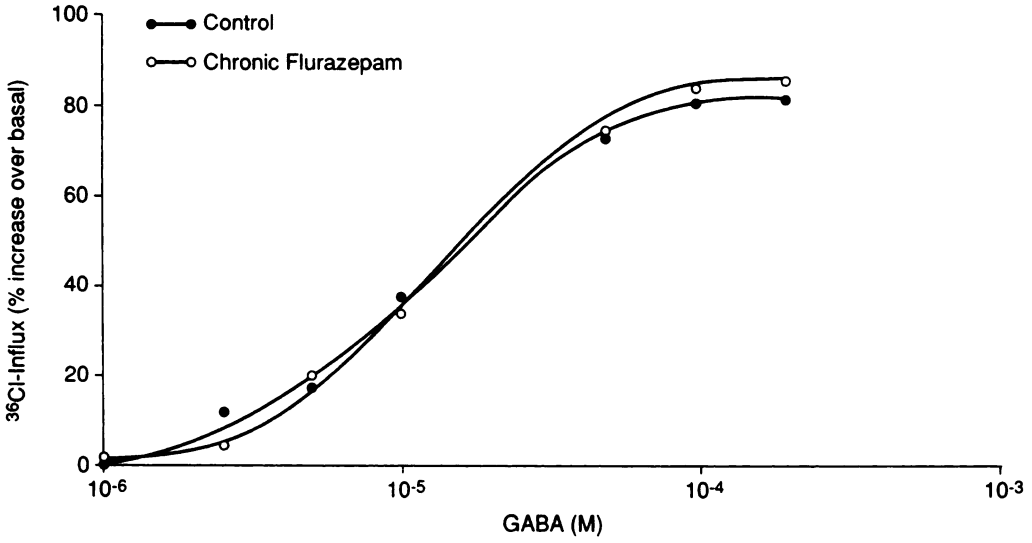


**Fig. 5.** Concentration-dependent enhancement of GABA-induced $^{36}$Cl influx in control and chronic flurazepam HCl (5 μM, 10 days)-treated cultured cortical neurons. Similar results were replicated three times. $EC_{50}$ and $E_{max}$ values are summarized in Table 5. Data are representative of three separate experiments performed in triplicate.

TABLE 5

**Effect of chronic flurazepam (5 μM, 10 days) treatment on GABA-induced $^{36}Cl$ influx in cortical neurons**

GABA (1–200 μM)-induced $^{36}Cl$ influx was measured in control and chronically treated neurons, as described in the text. The data were plotted as described for Fig. 5, and $EC_{50}$ and $E_{max}$ values were obtained by graphical analysis. Values represent mean ± standard deviation of three experiments, each done in triplicate.

| | $^{36}Cl$ influx | | | |
|---|---|---|---|---|
| | Control group | | Treated group | |
| | $EC_{50}$ | $E_{max}$ | $EC_{50}$ | $E_{max}$ |
| | μM | % | μM | % |
| GABA | 13.5 ± 2 | 89 ± 10 | 16.5 ± 3 | 92 ± 7 |

TABLE 6

**Effects of chronic flurazepam HCl (5 μM, 10 days) treatment on GABA-induced $^{36}Cl$ influx in cultured cortical neurons**

Cultured cortical neurons were treated with flurazepam and GABA-induced $^{36}Cl$ influx was measured in the absence and presence of diazepam. Values are mean ± standard deviation of three separate experiments, each performed in triplicate. Values in parentheses represent percentage increase over the basal values.

| | $^{36}Cl$ influx | |
|---|---|---|
| | Control group | Treated group |
| | nmol/mg of protein | |
| Basal | 2.203 ± 0.177 | 2.198 ± 0.192 |
| GABA (10 μM) | 3.216 ± 0.136 (+46%) | 3.185 ± 0.283 (+45%) |
| GABA (10 μM) + diazepam (1 μM) | 4.459 ± 0.193 (+102%) | 3.719 ± 0.367 (+69%)* |

*$p < 0.05$, compared with control group.

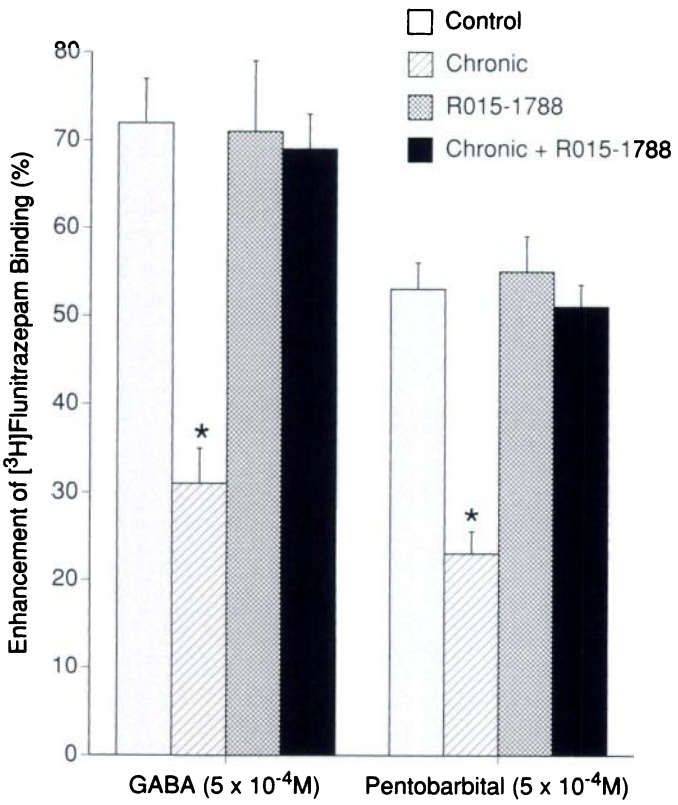


**Fig. 6.** Effect of chronic flurazepam (5 μM, 10 days) treatment and its reversal by concomitant exposure of cortical neurons to Ro15–1788 (3 μM, 10 days) on GABA and pentobarbital enhancement of [$^3$H]flunitrazepam (2 nM) binding. Each value is mean ± standard deviation of three or four experiments, each done in triplicate. *, $p < 0.01$, compared with control group.

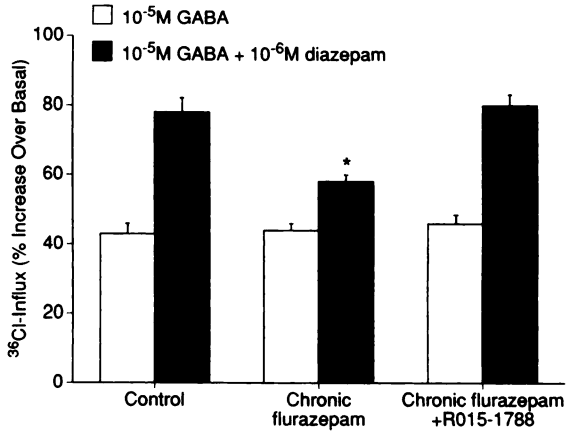


**Fig. 7.** Ro15–1788 (3 μM, 10 days) reversal of the effect of chronic flurazepam (5 μM, 10 days) treatment on diazepam enhancement of GABA-induced $^{36}Cl$ influx. Results are mean ± standard deviation of three experiments, each done in triplicate. *, $p < 0.001$, compared with control group.

could result in decreased uncoupling and decreased efficacy of benzodiazepine agonists and may form a molecular basis for benzodiazepine tolerance. This type of alteration in the case of receptors that exist in multiple isoforms may result in decreased response to the modulatory drug (e.g., diazepam), with no apparent change in the response to the endogenous neurotransmitter (i.e., GABA). Support for this hypothesis is provided by the observations that chronic flurazepam treatment altered neither GABA binding nor GABA-induced $^{36}Cl$ influx but only the facilitatory effect of diazepam. This hypothesis is currently being investigated in our laboratory by using subunit-specific antibodies and cDNA clones of various subunits to measure the levels of $GABA_A$ receptor subunit polypeptides and mRNA.

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