0026-895X/94/040657-07$03.00/0

MOLECULAR PHARMACOLOGY, 45:657-663

Reduced Expression of γ-Aminobutyric Acid Type A/ Benzodiazepine Receptor γ2 and α5 Subunit mRNAs in Brain Regions of Flurazepam-Treated Rats

TAI-JUN ZHAO, TED H. CHIU, and HOWARD C. ROSENBERG
Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699
Received August 23, 1993; Accepted January 21, 1994

SUMMARY

Previous studies showed that chronic benzodiazepine administration in rats affected the γ-aminobutyric acid $(GABA)_A$/benzodiazepine receptor. The present experiment investigated the effects of chronic flurazepam treatment on the mRNA levels for α1, α5, γ2, and γ2L (an alternatively spliced product of the γ2 gene) subunits of the $GABA_A$/benzodiazepine receptor in rat cerebral cortex, cerebellum, and hippocampus. Rats were treated with flurazepam for 2 or 4 weeks, and the mRNA levels were measured while rats were still receiving drug or 48 hr after 4-week flurazepam treatment had been stopped. The level of α5 mRNA was also measured in other rats 4 hr after a single injection of flurazepam or diazepam. The levels of mRNAs were analyzed by Northern blotting using digoxigenin-labeled oligonucleotide probes. Compared with the pair-handled controls, the levels of γ2 subunit mRNA in cortex and hippocampus were not changed after flurazepam treatment for 2 weeks. However, with rats treated with flurazepam for 4 weeks the levels of γ2 subunit mRNA were significantly reduced in cortex (31%) and hippocampus (39%) but not in cerebellum. The values returned to control levels by 48 hr after termination of the treatment. The regional distribution and time course of reduced γ2 levels matched the decrease in benzodiazepine binding produced by the same chronic flurazepam treatment. The amounts of α5 mRNA were reduced in cortex (23%) and hippocampus (18%) 4 hr after a single dose of flurazepam but not diazepam. The levels of α5 mRNA remained reduced in cerebral cortex and hippocampus (about 50%) after 2 weeks but returned to control after 4 weeks of chronic treatment with flurazepam. No change in α1 or γ2L subunit mRNAs was observed in any of the three brain regions examined after 4 weeks of flurazepam treatment. These results suggest that benzodiazepine receptor down-regulation after chronic benzodiazepine treatment may be related to the reduced expression of γ2 subunit mRNA, and they also suggest differential temporal and regional regulation of α5 and γ2 subunit mRNAs in rat brain.

Both clinical and experimental evidence indicates that prolonged benzodiazepine administration results in the development of functional tolerance, i.e., tolerance associated with a reduction in the sensitivity of the central nervous system to benzodiazepine action (1, 2). The effects of benzodiazepines are generally accepted to be mediated by potentiation of the action of GABA at $GABA_A$ receptors (3, 4). Therefore, identification of the changes that may occur in this receptor is of particular interest for understanding benzodiazepine tolerance. The effects of chronic benzodiazepine administration on $GABA_A$/benzodiazepine receptors have been studied at several levels of GABA and benzodiazepine interaction. After chronic treatment of animals or neuronal cultures with benzodiazepines for various periods of time, benzodiazepine binding has been found to be decreased (5–10) or not changed (11–16). Chronic exposure of animals or neuronal cultures to benzodiazepines has been shown to result in decreased GABA potentiation of benzodiazepine binding (13, 14, 16–18), a decrease in GABA- or muscimol-stimulated $^{36}Cl^-$ flux (10, 19, 20), and a reduced ability of benzodiazepines to enhance GABA-stimulated $^{36}Cl^-$ influx (21–24). The reduction in $GABA_A$ receptor function also has been demonstrated electrophysiologically (14, 25) and behaviorally (26).

The cloning of $GABA_A$ receptor subunits makes it possible to evaluate the molecular basis of altered $GABA_A$ receptor function after chronic benzodiazepine administration. $GABA_A$ receptors are hetero-oligomeric protein complexes consisting of several homologous membrane-spanning glycoprotein subunits (α, β, γ, δ, and ρ). Many of these exist as several isoforms, each encoded by a different gene (e.g., α1–6, β1–3, and γ1–3). Expression of recombinant receptors in mammalian cells revealed that inclusion of a γ subunit is required to demonstrate benzodiazepine sensitivity (27) and that the particular α subunit isoform present appears to be the major determinant of receptor subtype selectivity for benzodiazepine ligands (28, 29). These findings

This work was supported by National Institute on Drug Abuse Grant RO1-DA02194.

ABBREVIATIONS: GABA, γ-aminobutyric acid; SDS, sodium dodecyl sulfate.

suggest that changes in the expression of genes encoding the $GABA_A$ receptor subunits may underlie the functional changes at this receptor after chronic benzodiazepine treatment. Several groups have studied this hypothesis by estimating the brain levels of mRNA encoding $GABA_A$ receptor subunits after chronic benzodiazepine treatment. Decreased expression of the mRNA for $\alpha 1$ (30, 31) and $\gamma 2$ (31, 32) subunits has been reported. On the other hand, a decrease in $\alpha 5$ and increases in $\alpha 3$ and $\alpha 6$ but no changes in $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\beta 3$, or $\gamma 2$ mRNA levels have been demonstrated (33, 34). The relationship of these changes to tolerance or to receptor down-regulation is not yet clear.

Previous studies from our laboratory demonstrated that chronic treatment of rats with flurazepam for 4 weeks resulted in a brain region-specific reduction in benzodiazepine binding and tolerance to the benzodiazepine potentiation of GABA-mediated $^{36}Cl^-$ influx (5, 6, 21–23). The reduction in receptor density and tolerance to benzodiazepine potentiation returned to control 48 hr after treatment (5, 21). In the present report, the possible mechanisms for regulating the number and function of $GABA_A$ receptors were investigated by estimating the mRNA levels for $\alpha 1$, $\alpha 5$, $\gamma 2$, and $\gamma 2L$ receptor subunits in cortex, cerebellum, and hippocampus of rats treated with the same chronic flurazepam administration known to produce tolerance and benzodiazepine receptor down-regulation. We chose to investigate $\alpha 1$, $\alpha 5$, and $\gamma 2$ mRNAs not only because they are among the most abundant $GABA_A$ receptor subunit mRNAs (35) but also because they have been shown to be reduced after certain chronic benzodiazepine regimens (30–33). Our recent work indicated that zolpidem (a selective type I $GABA_A$/benzodiazepine receptor ligand) binding was decreased in cerebral cortex, cerebellum, and hippocampus after 4 weeks of flurazepam treatment (36), which suggests that $\alpha 1$ subunit mRNA may be decreased after chronic benzodiazepine administration. Down-regulation of benzodiazepine receptors found after chronic benzodiazepine treatment (5–10) suggests that $\gamma 2$ subunit mRNA may be altered, because the $\gamma 2$ subunit is necessary for benzodiazepine sensitivity (27). The $\gamma 2L$ mRNA was also studied, because it is an alternatively spliced $\gamma 2$ gene product (37) with eight additional amino acid residues, containing a consensus sequence for protein kinase C phosphorylation. In addition, rats were given a single dose of diazepam or flurazepam. The latter has been shown to reduce $\alpha 5$ mRNA expression in whole brain as early as 2 hr after a single dose (33).

Experimental Procedures

Materials. Oligonucleotide probes for the rat $\alpha 1$ subunit, encoding subunit residues 342–356 (5′-GGGGTCACCCCTGGCTAAGTTAGG GGTATAGCTGGTTGCTGTAGG-3′) (38), $\alpha 5$ subunit, encoding amino acid residues 355–369 (5′-ATTCCCAGTCCCGCCTGGAAGC TGCTCCTTTGGGATGTTTGGAGG-3′) (39), $\gamma 2$ subunit, encoding subunit residues 359–371 (5′-ATCCAAACACTCATAGCCATATTCT TCATCCCTCTCTTG-3′) (40), and $\gamma 2L$ subunit, encoding subunit residues 336–347 (5′-AGGGGCCTTGAAGGAAAACATCCGAAGAA GAGGGTT-3′) (37), were synthesized by Oligos Etc., Inc. (Wilsonville, OR). The $\gamma 2L$ probe consists of the alternatively spliced length of 24 nucleotides plus an additional six nucleotides on each end. The cDNA probe for β-actin was prepared by conventional methods from a human cDNA clone kindly provided by Dr. Craig Thompson (University of Michigan, Ann Arbor, MI). Oligonucleotide and cDNA probes were labeled with digoxigenin/dUTP by 3′-tailing and random primer techniques, respectively, according to the manufacturer's protocols (Boehringer Mannheim Corp., Indianapolis, IN). All other materials for mRNA isolation and hybridization were of the highest grade commercially available.

Animals and chronic treatment. The protocol for the treatment of rats and measurement of subunit mRNAs is shown in Table 1. Male Sprague-Dawley rats (initial weight, 150–174 g; weight at sacrifice, 250–300 g) were treated as described previously (5, 6). Flurazepam was dissolved in 0.02% saccharin solution, which was used as the only drinking water source. Based on the body weight and the volume consumed over the previous 24 hr, the drug concentration was adjusted to provide 100 mg/kg for 1 week, followed by 150 mg/kg for 1 or 3 weeks. Rats were sacrificed at 0 and 48 hr after flurazepam treatment was stopped. The drug-containing water was replaced with 0.02% saccharin solution during the drug-withdrawn period. Control rats received 0.02% saccharin solution and were handled the same way as the treated rats. This 4-week treatment protocol produces a brain flurazepam-like concentration of about 2 μM at 0 hr, which decreased to a negligible level 48 hr after the 4-week treatment was stopped (9). In another set of experiments, rats were administered 40 mg/kg flurazepam or 10 mg/kg diazepam intraperitoneally and were sacrificed 4 hr later. These acute flurazepam and diazepam injections caused profound ataxia, reduced muscle tone, and sedation in all rats given the intraperitoneal injections.

mRNA isolation and quantitation. Rats were sacrificed by decapitation between 8 and 10 a.m. Brain regions, including cortex, cerebellum, and hippocampus, were removed quickly, placed into 50-ml sterile polypropylene tubes, rapidly frozen with liquid nitrogen, and then stored at −70°. Poly(A)$^+$ mRNAs were extracted according to the modified proteinase K digestion method, as described previously (41). Each sample was homogenized in preheated (45°) lysis buffer (0.2 M NaCl, 2% SDS, 0.2 M Tris·HCl, pH 8.0 at room temperature) containing 1 mg/ml proteinase K, using a Polytron PT 10 homogenizer at a setting of 3 for 20 sec or until the tissues were completely disrupted. The lysates were incubated for 45 min at 45° and the concentration of NaCl was adjusted to 0.5 M. About 30 mg of oligo(dT)-cellulose were prepared by equilibration with buffer (0.01 M Tris·HCl, pH 7.5, 0.5 M NaCl) for 1 hr and then washing with TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0) one time. The equilibrated oligo(dT)-cellulose was added to the lysate and the mixture was incubated for 45 min at room temperature, on a rocking platform. After washing by inversion of the tube twice with 10 ml of a solution of 0.05 M Tris·HCl, pH 7.5, 0.5 M NaCl, and 0.1% SDS, the pellet was transferred with 500 μl of the same washing buffer to a spin column for additional washing until the A_{260} of the washing buffer was <0.05. Poly(A)$^+$ mRNA was eluted with 2 ×

TABLE 1
Chronic treatment of rats

Group	No. of rats[a]	Treatment	Sacrifice[b]	mRNA species measured
			hr	
1	8	Flurazepam for 4 weeks	0	$\gamma 2$, $\alpha 1$, $\alpha 5$
2	4	Flurazepam for 4 weeks	0	$\gamma 2$
3	8	Flurazepam for 4 weeks	48	$\gamma 2$
4	8	Flurazepam for 4 weeks	0	$\gamma 2L$
5	8	Flurazepam for 2 weeks	0	$\gamma 2$, $\alpha 1$, $\alpha 5$
6	6	One dose of flurazepam or diazepam	4	$\alpha 5$

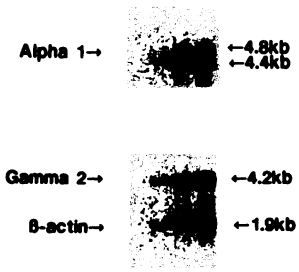
[a] For each treatment group except group 2, an equal number of control rats were given vehicle. For group 2, three control rats were used.
[b] Rats were sacrificed while still drinking drug-containing water (0 hr), 48 hr after drug treatment had been stopped, or 4 hr after a single intraperitoneal administration of 40 mg/kg flurazepam or 10 mg/kg diazepam.

200 μl of TE buffer. The A_{260} of the combined eluate provided a measure of the recovery of mRNA. mRNA was precipitated overnight with 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate. The entire mRNA samples from each group were separated on one 1.2% denaturing formaldehyde-agarose gel. The separated mRNAs were transferred to Nytran membranes (Schleicher & Schuell, Keene, NH) and immobilized with UV irradiation (80,000 μJ/cm^2), and the membranes were baked at 80° for 2 hr.

Hybridization and detection of mRNA using the digoxigenin/dUTP-labeled probes were performed basically according to the protocols provided by the manufacturer (Boehringer Mannheim Corp.). Membranes were prehybridized at 42° for 4 hr in 5× standard saline citrate (1× = 0.15 M NaCl + 15 mM sodium citrate, pH 7.0), 50% formamide, 0.1% SDS, 200 μg/ml denatured salmon sperm DNA, 2% blocking reagent, for nucleic acid hybridization. The digoxigenin/dUTP-labeled γ2 (6 pmol/ml) and β-actin (0.5 pmol/ml) probes were added to the same solution and hybridization was carried out overnight (at least 16 hr) at 42°. The amounts of labeled probes added were such that similar intensities of signal on the autoradiograms, within the linear range, were obtained for both probes. After hybridization and posthybridization washes, the membranes were incubated with alkaline phosphatase-conjugated antidigoxigenin antibody. The bound antibody was detected with chemiluminescent substrate (Lumi-Phos 530). Autoradiograms were obtained at −70° by using Kodak XAR film with an intensifying screen. The intensities of autoradiograms were scanned with a Bio-Rad imaging densitometer (model GS-670) and areas were quantified. The amount of γ2 subunit mRNA for each sample was normalized to the corresponding β-actin mRNA (as an internal standard) measured on the same blots. Because repeated stripping of the membranes did not affect subsequent hybridizations (42), some of the blots were stripped two or three times (Table 1, groups 1 and 5), each for 2 × 30 min, by immersion in 0.1× standard saline citrate, 0.1% SDS, at 95° (42). They were rehybridized with α1 (10 pmol/ml) and β-actin (1 pmol/ml) probes or α5 (12.5 pmol/ml) probe alone, because the sizes of α5 and β-actin mRNAs are very similar. The intensity of each α1 or α5 mRNA signal was normalized to that of the corresponding β-actin mRNA. For γ2L mRNA measurement, the blot was incubated with 17.5 and 1.5 pmol/ml γ2L and β-actin probe, respectively. The relative intensities of the β-actin mRNAs in the stripped blots were not altered, which indicated that multiple stripping did not affect subsequent hybridizations, confirming the previous report (42). Barring contamination, the used probe solutions can be reused two or three times when supplemented with additional fresh probes. For the rats in group 1 (Table 1), the densities of the β-actin bands were measured in arbitrary units. This information was used to evaluate the supposition that benzodiazepine treatment had not altered β-actin mRNA levels. The relative intensity of each GABA$_A$ receptor subunit mRNA was determined as the ratio of the density of the band for that mRNA in each sample to the density of the corresponding β-actin band. The mean of the ratios for all controls was calculated and set at 100%. The result from each lane for control and treated rats was then expressed as a percentage of that value. Statistical comparisons of the data were made by Student's t test. Significant differences were accepted at $p < 0.05$.

Results

The recovery of mRNA from the three brain regions was not significantly different between control and treated animals (data not shown). As shown in Fig. 1, increasing the concentration of poly(A)$^+$ mRNA in the Northern blots caused a linear increase in the intensities of the signals for α1 and γ2 GABA$_A$ receptor subunit mRNAs and β-actin mRNAs (Fig. 1). This linear range of intensities was used for later Northern blot analysis. Fig. 1 also demonstrates that β-actin and γ2 probes each recognized a single species of mRNA. On the other hand, the α1 probe recognized two species of mRNA, as reported previously (38, 43–45). It is likely that both mRNA species represent α1 mRNAs, because probes of varying length and different sequences were used in these reports. Fig. 2 shows that the concentrations of constitutively expressed β-actin mRNA in cerebral cortex, cerebellum, and hippocampus of group 1 rats (Table 1) were not changed after 4 weeks of



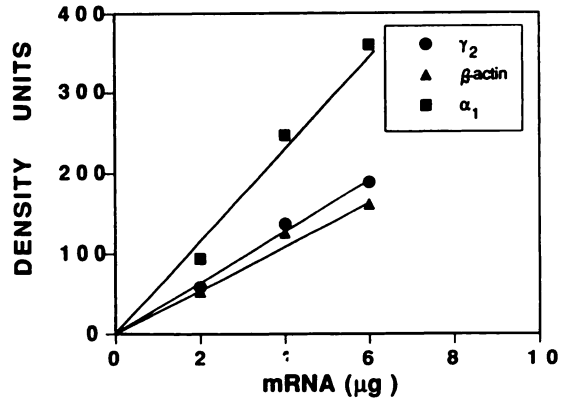


Fig. 1. Concentrations of mRNA versus intensities on autoradiograms for α1, γ2, and β-actin mRNAs. mRNA was extracted from a control whole brain. Increasing amounts (2, 4, and 6 μg) of mRNA were added to the three lanes. The Northern blot was hybridized with digoxigenin/dUTP-labeled probes for β-actin and γ2. The blot was stripped and later hybridized with a probe for α1. The autoradiograms were scanned with a densitometer. The two α species were combined for analysis. The sizes of the mRNAs (in kilobases) were estimated from a GIBCO BRL RNA standard (not shown).

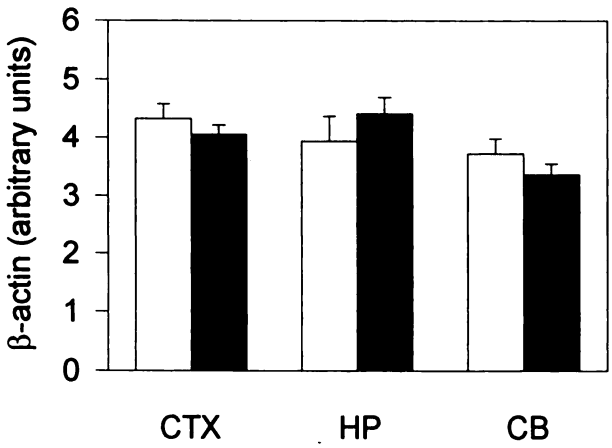


Fig. 2. Relative amounts of β-actin mRNA in cerebral cortex (*CTX*), hippocampus (*HP*), and cerebellum (*CB*) from control (□) and flurazepam-treated (4 weeks) (■) rats. Data are expressed in arbitrary density units (mean ± standard error, eight experiments).

flurazepam treatment, which indicated that chronic flurazepam administration did not adversely affect the survival of neurons.

To minimize experimental variations, the entire control and treated mRNA samples from each group were run together in the same Northern blot. Representative autoradiograms are shown in Fig. 3-6. Quantitation of these autoradiograms showed that the relative amount of γ2 mRNA in cortex and hippocampus was significantly reduced in both group 1 and group 2 rats at 0 hr for flurazepam-treated rats. The combined results are shown in Fig. 3. This was very similar to the results of a pilot study using γ2 probe labeled with [α-^{32}P]dATP, which showed a significant reduction (about 40%) of this mRNA in the cortex. No change in the level of γ2 mRNA was observed in cerebellum

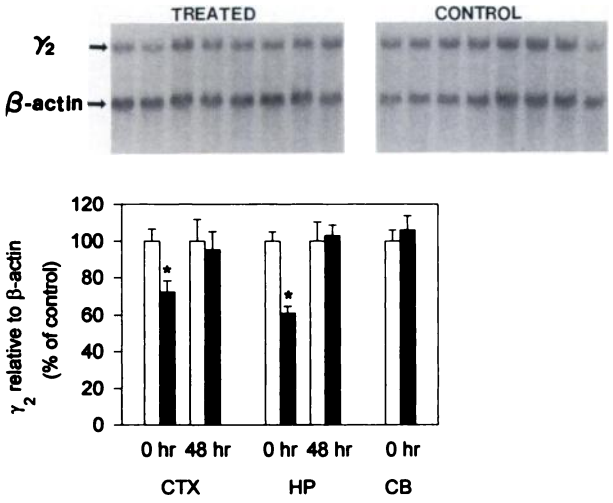


Fig. 3. *Top*, Northern blot of γ2 and β-actin mRNAs extracted from hippocampus of control and flurazepam-treated rats studied at the end of the 4-week treatment. *Bottom*, the relative intensities of the autoradiographic signals for the γ2 mRNAs from cerebral cortex (*CTX*), hippocampus (*HP*), and cerebellum (*CB*) of control (□) and treated (■) rats were normalized to those of the corresponding β-actin mRNA. Data from rats sacrificed immediately (0 hr) or 48 hr after the end of the 4-week treatment are expressed as percentage (mean ± standard error) of the corresponding control means (11 and 12 experiments for control and treated CTX at 0 hr and eight experiments for others). *, $p < 0.0005$.

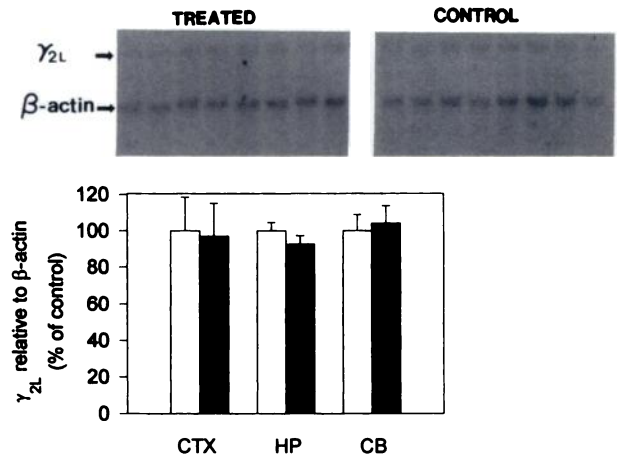


Fig. 4. *Top*, Northern blot of γ2L and β-actin mRNAs extracted from hippocampus of control and flurazepam-treated rats. *Bottom*, the relative intensities of the autoradiographic signals for the γ2L mRNAs from cerebral cortex (*CTX*), hippocampus (*HP*), and cerebellum (*CB*) of control (□) and treated (■) rats were estimated similarly to those in Fig. 3. Data are expressed as percentage (mean ± standard error) of the corresponding control means (eight experiments).

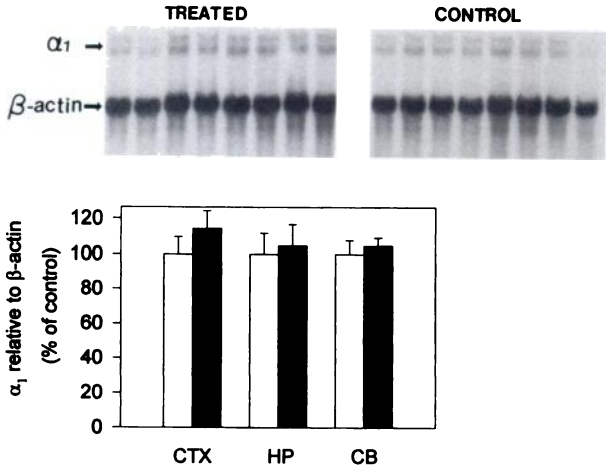


Fig. 5. *Top*, Northern blot of α1 and β-actin mRNAs extracted from hippocampus of control and flurazepam-treated rats. *Bottom*, the relative intensities of the autoradiographic signals for the α1 mRNAs from cerebral cortex (*CTX*), hippocampus (*HP*), and cerebellum (*CB*) of control (□) and treated (■) rats were measured similarly to those in Fig. 3. Data are expressed as percentage (mean ± standard error) of the corresponding control means (eight experiments).

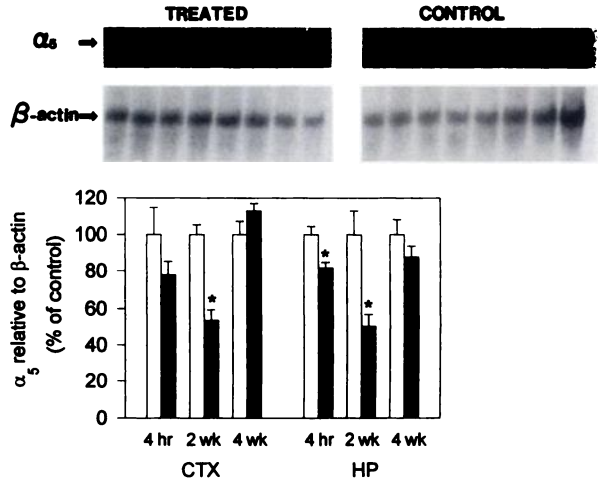


Fig. 6. *Top*, Northern blot of α5 and β-actin mRNAs extracted from cerebral cortex of control and flurazepam-treated rats. *Bottom*, the relative intensities of the autoradiographic signals for the α5 mRNAs from cerebral cortex (*CTX*) and hippocampus (*HP*) of control (□) and treated (■) rats were measured similarly to those in Fig. 3. Data are expressed as percentage (mean ± standard error) of the corresponding control means (six experiments for the rats sacrificed 4 hr after a single flurazepam injection and eight experiments for the rats sacrificed immediately after completing 2 or 4 weeks of flurazepam treatment). *, $p <$ 0.005.

(Fig. 3). The decrease in γ2 mRNA detected in cortex and hippocampus returned to control levels 48 hr after the flurazepam treatment was stopped (Fig. 3). Flurazepam treatment of shorter duration (2 weeks) did not affect the expression of γ2 mRNA in cortex and hippocampus (95 ± 9% of control for cortex and 102 ± 6% of control for hippocampus, eight experiments).

The γ2L probe recognized a single species of mRNA (Fig. 4). Its signal was very weak in all three brain regions examined, compared with that of γ2 mRNA (Fig. 3). The relative concentration of γ2L mRNA, an alternatively spliced transcriptional product of the γ2 gene (37), was not changed in cerebral cortex, cerebellum, or hippocampus after 4 weeks of flurazepam ad-

ministration (Fig. 4). Similarly, the relative amounts of each of the two species of α1 mRNA were not altered in the three brain regions examined after chronic administration of flurazepam for 4 weeks. The intensities of both species were combined for analysis as shown in Fig. 5. Flurazepam treatment of shorter duration (2 weeks) also did not affect the expression of α1 mRNA in cortex and hippocampus (99 ± 7% of control for cortex and 106 ± 8% of control for hippocampus, eight experiments).

No α5 mRNA could be detected in cerebellum of either control or flurazepam-treated rats. In cerebral cortex and hippocampus, the α5 probe hybridized to a single species of mRNA (Fig. 6). The relative amount of α5 mRNA in cerebral cortex and hippocampus was decreased 23% and 18%, respectively, 4 hr after a single dose (40 mg/kg) of flurazepam (Fig. 6). In contrast, acute administration of an equipotent dose of diazepam (10 mg/kg) did not affect the level of α5 mRNA in cortex and hippocampus (128 ± 21% of control for cortex and 108 ± 5% of control for hippocampus, six experiments). The expression of α5 mRNA in cerebral cortex and hippocampus was further reduced to about 50% of control after chronic flurazepam administration for 2 weeks (Fig. 6). However, it returned to control after 4 weeks of flurazepam treatment (Fig. 6).

Discussion

The data show that mRNA levels for the γ2 subunit of the GABA$_A$/benzodiazepine receptor in cortex and hippocampus, but not in cerebellum, were significantly decreased by 30–40% in nonwithdrawn rats after chronic flurazepam treatment for 4 weeks. Because the expression of α1 subunit mRNA was not altered in these regions in these same rats, the reduction in γ2 mRNA was a specific drug effect. The levels of γ2 mRNA returned to control 48 hr after termination of flurazepam administration. No change in the abundance of γ2 subunit mRNA was found after only 2 weeks of flurazepam treatment. The time courses for reduction of γ2 subunit mRNA levels and for recovery, as well as regional selectivity, were consistent with our previous observations using the same chronic treatment protocol. In rats given the same flurazepam treatment, a 14–18% reduction of benzodiazepine receptors was found in cerebral cortex and hippocampus, but not in cerebellum (6). This down-regulation of benzodiazepine receptors, as well as tolerance to the gross ataxia produced by large doses of flurazepam, was present after 4 weeks but not after only 2 weeks of treatment and was reversed 48 hr after the end of the 4-week treatment (5). Similarly, benzodiazepine potentiation of GABA-mediated $^{36}Cl^-$ uptake in cortical plus hippocampal vesicles was attenuated by 70–100% at 0 hr but disappeared 48 hr after drug treatment (21). Because study of recombinant receptors expressed in mammalian cells using different subunit mRNAs revealed that the γ2 subunit is necessary for the assembled receptors to demonstrate benzodiazepine sensitivity (27), it is hypothesized that reduced benzodiazepine binding, reduced benzodiazepine enhancement of GABA-mediated Cl^- flux, and tolerance to motor impairment during chronic flurazepam administration may be due to the reduced expression of γ2 subunit mRNA, resulting in decreased production of γ2 subunit protein and decreased assembly of receptors containing γ2 subunits. It should be noted that changes in mRNA abundance may not reflect changes in the steady state level of protein, because the latter is also influenced by factors such as post-translational modification, efficiency of subunit assembly, and stability of subunit interaction. Direct assessment of changes in the level of subunit protein could be accomplished by using subunit-specific antibodies.

Other studies have also attempted to determine the effects of chronic benzodiazepine treatment on GABA$_A$ receptor α1 and γ2 subunit mRNA levels and the possible role of such changes in tolerance. One such study found that mice treated with lorazepam for 14 days but not 10 days had reduced amounts of these mRNAs in cerebral cortex but not in hippocampus or cerebellum (31). The same treatment was found to cause tolerance as well as benzodiazepine receptor down-regulation in both cerebral cortex and hippocampus as early as day 7 of treatment (10). Therefore, although changes in α1 and γ2 mRNA might be related to the behavioral and biochemical changes found (10), they did not correlate with the time course of tolerance or with the time course and regional distribution of benzodiazepine receptor down-regulation. In other work, rats treated with diazepam were found to have reduced amounts of α1 and γ2 subunit mRNA in cerebral cortex but not in hippocampus or cerebellum (30, 32). In spite of these changes, the same diazepam treatment was found to cause no change in the binding of several ligands to the benzodiazepine receptor (46). These studies and the present results all show that chronic benzodiazepine administration is associated with changes in brain levels of GABA$_A$ receptor subunit mRNA. They also demonstrate regional differences in this effect, with cerebellum appearing to be the most resistant. Such regional differences may be related to the molecular diversity of receptor subunit composition among brain regions (29). The differences among the studies in results with the α1 subunit and the differing results with the γ2 subunit in hippocampus suggest that differences in the chronic treatment protocols (e.g., dose, duration, species, and benzodiazepines used) may determine the pattern of changes in GABA$_A$ receptor subunit mRNA. This could explain observations such as the report that virtually identical treatment with two different benzodiazepines produced regionally different changes in benzodiazepine binding (47).

The γ2L mRNA is an alternatively spliced γ2 gene product that contains an additional eight amino acid residues located between amino acid residues 337 and 338 of γ2 mRNA (37). These additional amino acid residues were found to include a consensus sequence for protein kinase C phosphorylation (37). Because GABA$_A$/benzodiazepine receptor subunits are substrates for phosphorylation/dephosphorylation, which plays an important role in receptor regulation, we investigated the expression of γ2L mRNA after chronic flurazepam administration. No alteration of γ2L mRNA was found after chronic flurazepam administration for 4 weeks. In the present study, the probe used for the detection of γ2 mRNA also hybridizes to γ2L mRNA. Thus, the reduction of γ2 mRNA observed in cerebral cortex and hippocampus after 4 weeks of flurazepam treatment should be greater than that shown in Fig. 3. However, the adjustment would be rather small, because the expression of γ2L mRNA was much less than that of γ2 mRNA.

As in the present study, O'Donovan *et al.* (33, 34) studied GABA$_A$ receptor mRNAs in rats treated with flurazepam. However, regional comparisons are not possible because those authors used whole brain. Large, time-dependent changes in α3, α5, and α6 mRNAs were found, but no change in α1 was observed (33). Interestingly, we found a similar time-dependent

reduction of $\alpha 5$ mRNA in cerebral cortex and hippocampus after acute and chronic flurazepam treatment. However, acute diazepam administration did not affect the expression of $\alpha 5$ mRNA. The reasons for this apparent ligand-specific regulation of $\alpha 5$ subunit mRNA are not clear. In contrast, those authors did not find changes in $\gamma 2$ subunit mRNA (33). It is important to keep in mind that the treatment used by O'Donovan *et al.* (33, 34), i.e., once-daily intraperitoneal injection of 40 mg/kg flurazepam, was quite different from that used in the present study and so might not be expected to have the same effects on $GABA_A$ receptors. For example, in rats, 40 mg/kg flurazepam injected intraperitoneally causes obvious ataxia and other evidence of intoxication (48), whereas rats treated with flurazepam in the drinking water would not be expected to achieve as high peak brain levels and, in fact, do not display motor impairment during chronic treatment (5). Furthermore, once-daily treatment does not produce continuous drug exposure because rats metabolize benzodiazepines very rapidly, so that flurazepam and its active metabolites disappear with half-lives of <2 hr (49, 50).

The time course and regional distribution of changes in the expression of $\gamma 2$ mRNA match those of benzodiazepine receptor down-regulation measured in brain homogenates of rats given the same chronic flurazepam treatment. However, these data must be interpreted with caution. A quantitative autoradiographic study showed localized down-regulation of benzodiazepine binding, including parts of cerebral cortex and CA1 and CA3 of hippocampus, after 1 week of treatment (9). In addition, tolerance to the benzodiazepine potentiation of GABA-mediated $^{36}Cl^-$ flux was also observed after a 1-week treatment (22, 23). In the present experiment, we did not find any change in the mRNA levels for $\alpha 1$ or $\gamma 2$ subunits after only 2 weeks of flurazepam administration. However, we cannot exclude the possibility that, just as localized down-regulation of benzodiazepine binding can be present after shorter treatment periods, localized changes in the mRNA levels for some receptor subunits may occur after benzodiazepine administration of shorter duration.

In summary, the present study shows that chronic treatment of rats with flurazepam decreased the mRNA levels for $GABA_A$/benzodiazepine receptor $\gamma 2$ and $\alpha 5$ subunits in cortex and hippocampus but not in cerebellum. The mRNA levels for $\alpha 1$ and $\gamma 2L$ subunits in the three regions examined were not altered. The down-regulation of benzodiazepine binding and the decrease of function after chronic flurazepam treatment may be explained by the reduced expression of mRNA for the $\gamma 2$ subunit. These results also suggest that distinct transcriptional mechanisms may be involved in the regulation of $\gamma 2$ and $\alpha 5$ subunit mRNAs in different brain regions.

References

1. Greenblatt, D. J., and R. I. Shader. Long-term administration of benzodiazepines: pharmacokinetic versus pharmacodynamic tolerance. *Psychopharmacol. Bull.* **22**:416–423 (1986).
2. Rosenberg, H. C., and T. H. Chiu. Time course for development of benzodiazepine tolerance and physical dependence. *Neurosci. Biobehav. Rev.* **9**:123–131 (1985).
3. Haefely, W., and P. Polc. Physiology of GABA enhancement by benzodiazepines and barbiturates, in *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties* (R. W. Olsen and J. C. Venter, eds.). Alan R. Liss, Inc., New York, 97–133 (1986).
4. Macdonald, R. L., and J. L. Barker. Benzodiazepines specifically modulate GABA mediated postsynaptic inhibition in cultured mammalian neurons. *Nature (Lond.)* **271**:563–564 (1978).
5. Rosenberg, H. C., and T. H. Chiu. Tolerance during chronic benzodiazepine treatment associated with decreased receptor binding. *Eur. J. Pharmacol.* **70**:453–460 (1981).
6. Rosenberg, H. C., and T. H. Chiu. Regional specificity of benzodiazepine receptor down-regulation during chronic treatment of rats with flurazepam. *Neurosci. Lett.* **24**:49–52 (1981).
7. Crawley, J. N., P. J. Marangos, J. Stivers, and F. K. Goodwin. Chronic clonazepam administration induces benzodiazepine receptor subsensitivity. *Neuropharmacology* **21**:85–89 (1982).
8. Sher, P. K., R. E. Study, J. Mazzetta, J. L. Barker, and P. G. Nelson. Depression of benzodiazepine binding and diazepam potentiation of GABA-mediated inhibition after chronic exposure of spinal cord cultures to diazepam. *Brain Res.* **268**:171–176 (1983).
9. Tietz, E. I., H. C. Rosenberg, and T. H. Chiu. Autoradiographic localization of benzodiazepine receptor downregulation. *J. Pharmacol. Exp. Ther.* **236**:284–292 (1986).
10. Miller, L. G., D. J. Greenblatt, J. G. Barnhill, and R. I. Shader. Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric $acid_A$ receptor function. *J. Pharmacol. Exp. Ther.* **246**:170–176 (1988).
11. Braestrup, C., M. Nielsen, and R. F. Squires. No changes in rat benzodiazepine receptors after withdrawal from continuous treatment with lorazepam and diazepam. *Life Sci.* **24**:347–350 (1979).
12. Shibla, D. B., M. A. Gardell, and J. H. Neale. The insensitivity of developing benzodiazepine receptors to chronic treatment with diazepam. *Brain Res.* **210**:471–474 (1981).
13. Farb, D. H., L. A. Borden, C. Y. Chan, C. M. Czajkowski, T. T. Gibbs, and G. D. Schiller. Modulation of neuronal function through benzodiazepine receptors: biochemical and electrophysiological studies of neurons in primary monolayer cell culture. *Ann. N. Y. Acad. Sci.* **435**:1–31 (1984).
14. Gallager, D. W., J. M. Lakowski, S. F. Gonsalves, and S. L. Rauch. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. *Nature (Lond.)* **308**:74–77 (1984).
15. Stephens, D. N., and H. H. Schneider. Tolerance to the benzodiazepine diazepam in an animal model of anxiolytic activity. *Psychopharmacology* **87**:322–327 (1985).
16. Schiller, G. D., and D. H. Farb. Enhancement of benzodiazepine binding by GABA is reduced rapidly during chronic exposure to flurazepam. *Ann. N. Y. Acad. Sci.* **463**:221–223 (1986).
17. Tietz, E. I., T. H. Chiu, and H. C. Rosenberg. Regional GABA/benzodiazepine receptor/chloride channel coupling after acute and chronic benzodiazepine treatment. *Eur. J. Pharmacol.* **167**:57–65 (1989).
18. Roca, D. J., G. D. Schiller, L. Friedman, I. Rozenberg, T. T. Gibbs, and D. H. Farb. γ-Aminobutyric $acid_A$ receptor regulation in culture: altered allosteric interactions following prolonged exposure to benzodiazepines, barbiturates, and methylxanthines. *Mol. Pharmacol.* **37**:710–719 (1990).
19. Lewin, E., J. Peris, V. Bleck, N. R. Zahniser, and R. A. Harris. Diazepam sensitizes mice to FG 7142 and reduces muscimol-stimulated $^{36}Cl^-$ flux. *Pharmacol. Biochem. Behav.* **33**:465–468 (1989).
20. Marley, R. J., and D. W. Gallager. Chronic diazepam treatment produces regionally specific changes in GABA-stimulated chloride influx. *Eur. J. Pharmacol.* **159**:217–223 (1989).
21. Yu, O., T. H. Chiu, and H. C. Rosenberg. Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. *J. Pharmacol. Exp. Ther.* **246**:107–113 (1988).
22. Ngur, D. O., H. C. Rosenberg, and T. H. Chiu. Modulation of GABA-stimulated Cl^- flux by a benzodiazepine agonist and an 'inverse agonist' after chronic flurazepam treatment. *Eur. J. Pharmacol.* **176**:351–356 (1990).
23. Li, M., H. C. Rosenberg, and T. H. Chiu. Tolerance to the effects of diazepam, clonazepam and bretazenil on GABA-stimulated chloride influx in flurazepam tolerant rats. *Eur. J. Pharmacol.* **247**:313–318 (1993).
24. Allan, A. M., L. D. Baier, and X. Zhang. Effects of lorazepam tolerance and withdrawal on $GABA_A$ receptor-operated chloride channels. *J. Pharmacol. Exp. Ther.* **261**:395–402 (1992).
25. Xie, X.-H., and E. I. Tietz. Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of *in vitro* hippocampus. *Brain Res.* **561**:69–76 (1991).
26. Tietz, E. I., and H. C. Rosenberg. Behavioral measurement of benzodiazepine tolerance and GABAergic subsensitivity in substantia nigra pars reticulata. *Brain Res.* **438**:41–51 (1988).
27. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
28. Pritchett, D. B., H. Luddens, and P. H. Seeburg. Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
29. Olsen, R. W., and A. J. Tobin. Molecular biology of $GABA_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
30. Heninger, C., N. Saito, J. F. Tallman, K. M. Garrett, M. P. Vitek, R. S. Duman, and D. W. Gallager. Effects of continuous diazepam administration on $GABA_A$ subunit mRNA in rat brain. *J. Mol. Neurosci.* **2**:101–107 (1990).
31. Kang, I., and L. G. Miller. Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. *Br. J. Pharmacol.* **103**:1285–1287 (1991).

32. Primus, R. J., and D. W. Gallager. GABA$_A$ receptor subunit mRNA levels are differentially influenced by chronic FG 7142 and diazepam exposure. *Eur. J. Pharmacol.* **226**:21–28 (1992).
33. O'Donovan, M. C., P. R. Buckland, G. Spurlock, and P. McGuffin. Bi-directional changes in the levels of messenger RNAs encoding γ-aminobutyric acid$_A$ receptor α subunits after flurazepam treatment. *Eur. J. Pharmacol.* **226**:335–341 (1992).
34. O'Donovan, M. C., P. R. Buckland, and P. McGuffin. Levels of GABA$_A$ receptor subunit mRNA in rat brain following flurazepam treatment. *J. Psychopharmacol.* **6**:364–369 (1992).
35. Wisden, W., D. J. Laurie, H. Monyer, and P. H. Seeburg. The distribution of 13 GABA$_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.* **12**:1040–1062 (1992).
36. Wu, Y., H. C. Rosenberg, and T. H. Chiu. Regional changes in [^{3}H]zolpidem binding to brain benzodiazepine receptors in flurazepam tolerant rat: comparison with changes in [^{3}H]flunitrazepam binding. *J. Pharmacol. Exp. Ther.*, **268**:675–682 (1994).
37. Whiting, P., R. M. McKernan, and L. L. Iversen. Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. USA* **87**:9966–9970 (1990).
38. Khrestchatisky, M., A. J. Maclennan, M.-Y. Chiang, W. Xu, M. B. Jackson, N. Brecha, C. Sternini, R. W. Olsen, and A. J. Tobin. A novel α subunit in rat brain GABA$_A$ receptors. *Neuron* **3**:745–753 (1989).
39. Malherbe, P., E. Siegel, R. Baur, E. Pershon, J. G. Richards, and H. Mohler. Functional expression sites of gene transcription of a novel α subunit of the GABA$_A$ receptor in rat brain. *FEBS Lett.* **260**:261–265 (1990).
40. Shivers, B. D., I. Killisch, R. Sprengel, H. Sontheimer, M. Kohler, P. R. Schofield, and P. H. Seeburg. Two novel GABA$_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**:327–337 (1989).
41. Lai, C.-C., T. H. Chiu, H. C. Rosenberg, and W.-H. Huang. Improved proteinase K digestion for the rapid isolation of mRNA from mammalian tissues. *BioTechniques* **15**:620–626 (1993).
42. Noppinger, K., G. Duncan, D. Ferraro, S. Watson, and J. Ban. Evaluation of DNA probe removal from nylon membrane. *BioTechniques* **13**:572–575 (1992).
43. Garrett, K. M., R. S. Duman, N. Saito, A. J. Blume, M. P. Vitek, and J. F. Tallman. Isolation of a cDNA clone for the *alpha* subunit of the human GABA-A receptor. *Biochem. Biophys. Res. Commun.* **156**:1039–1045 (1988).
44. Lolait, S. J., A.-M. O'Carroll, K. Kusano, J.-M. Muller, M. J. Brownstein, and L. C. Mahan. Cloning and expression of a novel rat GABA$_A$ receptor. *FEBS Lett.* **246**:145–148 (1989).
45. Montpied, P., A. L. Morrow, J. W. Karanian, E. I. Ginns, B. M. Martin, and S. M. Paul. Prolonged ethanol inhalation decreases γ-aminobutyric acid$_A$ receptor α subunit mRNAs in the rat cerebral cortex. *Mol. Pharmacol.* **39**:157–163 (1990).
46. Heninger, C., and D. W. Gallager. Altered γ-aminobutyric acid/benzodiazepine interaction after chronic diazepam exposure. *Neuropharmacology* **27**:1073–1076 (1988).
47. Galpern, W. R., L. G. Miller, D. J. Greenblatt, and R. I. Shader. Differential effects of chronic lorazepam and alprazolam on benzodiazepine binding and GABA$_A$-receptor function. *Br. J. Pharmacol.* **101**:839–842 (1990).
48. Rosenberg, H. C. Central excitatory actions of flurazepam. *Pharmacol. Biochem. Behav.* **13**:415–420 (1980).
49. Lau, C. E., J. L. Falk, S. Dolan, and M. Tang. Simultaneous determination of flurazepam and five metabolites in serum by high- performance liquid chromatography and its application to pharmacokinetic studies in rats. *J. Chromatogr.* **423**:251–259 (1987).
50. Sethy, V. H., J. W. Francis, and G. Elfring. Onset and duration of action of benzodiazepines as determined by inhibition of [^{3}H]-flunitrazepam binding. *Drug Dev. Res.* **10**:117–121 (1987).

Send reprint requests to: Ted H. Chiu, Department of Pharmacology, Medical College of Ohio, P.O. Box 10008, Toledo, OH 43699–0008.