Sequestration of $\gamma$-Aminobutyric Acid$_A$ Receptors on Clathrin-Coated Vesicles During Chronic Benzodiazepine Administration In Vivo

MOHAMMAD H. JALILIAN TEHRANI and EUGENE M. BARNES, Jr.
Verna and Mars McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas
Accepted for publication June 6, 1997

ABSTRACT
Chronic administration of benzodiazepine agonists produces use-dependent down-regulation of $\gamma$-aminobutyric acid$_A$ (GABA$_A$)/benzodiazepine receptors is a potential cellular mechanism. We previously identified GABA$_A$ receptors on clathrin-coated vesicles from rat brain, suggesting that surface receptors can be internalized via endocytosis. To examine a role for coated vesicles in GABA$_A$ receptor down-regulation in vivo, fractions were obtained from mouse brain microsomes through density centrifugation and treatment with 0.1% Triton X-100. This coated vesicle preparation was enriched in clathrin subunits and clathrin light-chain kinase and had twice the level of [H]flunitrazepam binding as did vesicles not exposed to Triton. Adult mice were treated with lorazepam (2 mg/kg/day) for 7 days via osmotic minipump, achieving a serum level of 103 ± 8.9 ng/ml. The level of flunitrazepam bound to coated vesicles from lorazepam-treated mice was increased by 83 ± 13% in the lorazepam-treated mice compared with vehicle-treated controls. The $B_{max}$ value for [H]flunitrazepam binding to synaptic membranes from lorazepam-treated animals was 33 ± 4% lower than that of controls. The amount of GABA$_A$ receptor alpha-1 subunits, as quantified by Western blotting, followed a similar pattern. Relative to controls, immunoreactivity for alpha-1 subunits in coated vesicles from lorazepam-treated mice was increased by 60.0 ± 10.3%, whereas that in synaptic membranes declined by 12 ± 6%. These results indicate that lorazepam-dependent GABA$_A$ receptor sequestration occurs in mouse brain. Furthermore, it is suggested that this sequestration may play a role in GABA$_A$ receptor down-regulation in vivo.

The benzodiazepines are a family of hypnotic, anxiolytic and anticonvulsant drugs that have major clinical significance. The development of tolerance is a common outcome of prolonged exposure to these compounds, a phenomenon that can be attributed to functional rather than pharmacokinetic accommodation (Greenblatt and Shader, 1986; Rosenberg and Chiu, 1985). The major site of benzodiazepine action in the central nervous system is on GABA$_A$ receptors. Benzodiazepines bind with high affinity to a major subgroup of GABA$_A$ receptors, potentiating the gating of chloride currents by GABA and thus enhancing postsynaptic inhibition (Macdonald and Olsen, 1994). Chronic administration of benzodiazepines to rodents produces a decline both in GABA$_A$ receptor-mediated currents (Gallager et al., 1984) and GABA-gated $^{36}$Cl$^-$/flux in certain brain regions that coincides with the onset of tolerance (Lewin et al., 1989; Marley and Gallager, 1989; Miller et al., 1988). However, the molecular events that underlie this loss of receptor function are not well defined. In benzodiazepine-treated animals, reductions have been detected in the number of binding sites (down-regulation) for GABA$_A$ receptor ligands (Miller et al., 1988; Tietz et al., 1986; Wu et al., 1994) or in the allosteric coupling between the benzodiazepine and GABA binding sites on receptors (Tietz et al., 1989; Xie and Tietz, 1992). Although reductions in GABA$_A$ receptor subunit mRNAs have also been documented in benzodiazepine-treated rodents (Heninger et al., 1990; Kang and Miller, 1991; O'Donovan et al., 1992; Zhao et al., 1995), it has been suggested that these changes may occur after the onset of tolerance and loss of receptor binding (Kang and Miller, 1991). Similarly, chronic administration of GABA agonists to chick embryos or to cortical neurons in vitro produces a down-regulation of GABA$_A$ receptor binding and receptor polypeptides that precedes detectable changes in receptor subunit mRNAs (Baumgartner et al., 1994; Calkin et al., 1994; Calkin and Barnes, 1994a, 1994b) or translational rates of alpha-1 subunits (Miranda and Barnes, 1997).

ABBREVIATIONS: GABA, $\gamma$-aminobutyric acid; CCV, clathrin-coated vesicle; DTT, dithiothreitol; TCW, Triton-extracted coated vesicle; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(\alpha-aminoethyl ether)-$N,N,N',N''$-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Acute treatment of cortical neurons in culture with GABA or clonazepam induces the internalization of GABA<sub>A</sub> receptor binding sites (Tehrani and Barnes, 1991), and comparable amounts of GABA<sub>A</sub> receptor polypeptides are also seques-
tered from the cell surface (Calkin and Barnes, 1994a, 1994b). A large fraction of the internalized GABA<sub>A</sub> receptor polypeptides appear to be degraded, providing a potential mechanism for use-dependent down-regulation (Calkin and Barnes, 1994a, 1994b; Miranda and Barnes, 1997). The ident-
ification of GABA<sub>A</sub> receptor ligand binding on highly puri-
ified CCVs from rat brain suggests that receptor sequestration also occurs in vivo (Tehrani and Barnes, 1993). To further examine this pathway of GABA<sub>A</sub> receptor regulation, loraz-
epam was chronically administered to mice using procedures that produce tolerance (Miller et al., 1988). Here we report that this benzodiazepine treatment leads to accumulation of GABA<sub>A</sub> receptors on coated vesicles, whereas receptors on synaptic membranes declined. Some of these data have been presented in a preliminary form (Tehrani and Barnes, 1994).

**Methods**

**Materials.** [3H]Flunitrazepam (84.3 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA), and [γ-
32P]ATP was from ICN (Costa Mesa, CA). Lorazepam was provided by Wyeth-Ayerst Research (Princeton, NJ), and clonazepam was provided by Hoffmann-LaRoche (Nutley, NJ). Osmotic pumps were purchased from Alza (Palo Alto, CA). Polyethylene glycol 400 was obtained from ICN (Costa Mesa, CA). Lorazepam was provided as a gift by Hoffmann-LaRoche (Nutley, NJ). Clonazepam was chronically administered to mice using procedures that produce tolerance (Miller et al., 1988). Here we report that this benzodiazepine treatment leads to accumulation of GABA<sub>A</sub> receptors on coated vesicles, whereas receptors on synaptic membranes declined. Some of these data have been presented in a preliminary form (Tehrani and Barnes, 1994).

**Methods**

**Materials.** [3H]Flunitrazepam (84.3 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA), and [γ-
32P]ATP was from ICN (Costa Mesa, CA). Lorazepam was provided by Wyeth-Ayerst Research (Princeton, NJ), and clonazepam was provided by Hoffmann-LaRoche (Nutley, NJ). Osmotic pumps were purchased from Alza (Palo Alto, CA). Polyethylene glycol 400 and poly-L-lysine (64 kDa) were from Sigma Chemical (St. Louis, MO).

**Lorazepam administration.** Adult (10–12 weeks old) C57BL/6J mice were obtained from a breeding colony at Baylor College of Medicine. The animals were maintained in a light- and temperature-controlled environment and fed laboratory chow and water ad libi-
tum. Lorazepam was administered as described by Miller et al. (1988). In brief, lorazepam was dissolved in polyethylene glycol 400 and placed in Alzet 2001 osmotic pumps to deliver 2 mg/kg/day. The mice were lightly anesthetized with methoxyflurane, and the pumps were implanted subcutaneously. Control animals were implanted with pumps containing only polyethylene glycol. The mice were treated for 7 days and then killed.

**Tissue preparations.** Tissue samples and brains were collected rapidly and chilled to 4°C. Coated vesicle fractions were isolated as described by Tehrani and Barnes (1993). The brains were homogenized individually in 3 volumes of isolation buffer [10 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine, 0.1 mg/ml bacitracin and 0.1 mg/ml soy-
bean trypsin inhibitor]. The homogenate was centrifuged at 20,000 g for 30 min. The supernatant was collected, and the pellet was resuspended with pumps containing only polyethylene glycol. The mice were treated for 7 days and then killed.

**Results**

CCV fractions from mouse brain were obtained essentially as previously described (Tehrani and Barnes, 1993) using modifications of the procedures of Bar-Zvi and Branton (1986). Microsomal fractions were centrifuged in 6.25% Ficoll 400/6.25% sucrose to produce a light vesicle supernatant greatly enriched in CCVs (crude CCVs). Further purification of coated vesicles was obtained by extracting smooth vesicle contaminants in 0.1% Triton X-100 and then centrifuging at 100,000 x g for 1 hr to obtain a supernatant (cytoplasmic fraction) and a microsomal pellet. This pellets were resuspended in 2 ml of isolation buffer, mixed with 2 ml of a solution containing 12.5% Ficoll 400 (w/v) and 12.5% sucrose (w/v) in isolation buffer and then centrifuged at 40,000 g for 40 min. The resulting supernatant was collected and mixed with 3 volumes of isolation buffer containing Triton X-100 (where indicated) to give a final concentration of 0.1%. The mixture was incubated for 30 min on ice and then centrifuged at 100,000 x g for 90 min. The resulting pellet (TVC) was resuspended in isolation buffer.

The pellets from the 20,000 x g centrifugation were used to prepare crude synaptic membranes with a freeze-thaw/wash proce-
dure (Tehrani and Barnes, 1986). Serum samples were prepared by incubating trunk blood at 37°C for 30 min and then overnight at 4°C, followed by centrifugation at 8000 x g for 10 min. The protein content of the fractions was determined according to the method of Lowry et al. (1951).

**[3H]Flunitrazepam binding assay.** Crude synaptic membrane (60–100 µg of protein) or coated vesicle fractions (30–60 µg of protein) were added to an assay mixture containing 10 mM sodium phosphate, pH 7.4, 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl<sub>2</sub> and 10 nM [3H]Flunitrazepam in a final volume of 400 µl. For Scatchard analysis, concentrations of [3H]Flunitrazepam from 0.25 to 40 nM were used. Nonspecific binding was defined with 1 µM clonazepam. Incubations were carried out for 30 min at 4°C and then terminated by the addition of 3.5 ml of ice-cold phosphate buffer and filtration through glass-fiber filters (no. 32; Schleicher & Schuell, Keene, NH) that had been treated with 0.3% polyethyleneimine. The filters were washed twice with 3.5 ml of ice-cold assay buffer, dried and counted by liquid scintillation.

**Clathrin light-chain kinase assay.** Clathrin kinase activity in crude microsomal and CCV fractions was determined as previously described (Tehrani and Barnes, 1993). In brief, 35 µg of protein from tissue fractions was added to an assay mixture containing 25 mM HEPES, pH 7.4, 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and, where indicated, 5 µg of poly-L-lysine in a final volume of 50 µl. The mixtures were incubated for 5 min at 30°C, followed by the addition of [γ-32P]ATP (20 µM final concentration, 5 Ci/mmol). After 3 additional min, the assay was terminated by the addition of 25 µl of stop solution (1% SDS, 20% glycerol, 0.2% phenol red, 0.1 M DTT). Aliquots were electrophoresed on 10% polyacrylamide-SDS gels (Garfin, 1990) that were dried and exposed to Kodak X-Omat AR film.

**Quantitative immunoblotting.** GABA<sub>A</sub> receptor alpha-1 subunit immunoreactivity was determined by Western blotting using an antibody (RP4) against an alpha-1(1331–381) fusion protein described by Miranda et al. (1997). Clathrin heavy chains were detected on Western blots using monoclonal antibody F21–35C (provided by Dr. E. M. Lafer) and ECL enhanced chemiluminescent detection kits (Amersham, Arlington Heights, IL). Signal intensities on x-ray film were quantified using a laser scanner and QuantiScan software ( Biosoft, Ferguson, MO). Western blots with a series of membrane protein concentrations were used to determine the linear range of analysis for GABA<sub>A</sub> receptor alpha-1 subunit immunoreactivity (Miranda and Barnes, 1997). All of the alpha-1 subunit quantifica-
tion was carried out within this linear range.
centration of lorazepam, a competitive binding assay was used. Unlabeled lorazepam displaced the specific binding of $[^{3}H]$flunitrazepam in a dose-dependent manner with an IC$_{50}$ value of 25 nM (fig. 2A). The displacement plot was used as a standard curve to estimate the lorazepam content of serum. Specific $[^{3}H]$flunitrazepam binding to synaptic membranes was displaced by sera from lorazepam-treated animals in a volume-dependent manner (fig. 2B). Serum from vehicle-treated animals was ineffective. The amount of lorazepam estimated from the standard curve was proportional to the amount of serum up to 20 µl (fig. 2C). At the end of the 7-day treatment with lorazepam (2 mg/kg/day), the average serum level in the experimental group was 103.3 ± 8.9 ng/ml (fig. 2D). Using the same treatment protocol, Miller et al. (1988) found a lorazepam level of 80 ng/ml through chromatographic analysis. Thus, the regimen used in our study appears to be sufficient to produce tolerance to lorazepam as defined by Miller et al. (1988).

Through Scatchard analysis, the parameters for $[^{3}H]$flunitrazepam binding to synaptic membranes were determined for the vehicle- and lorazepam-treated animals. K$_{d}$ values for the control and drug-treated groups, 1.55 ± 0.04 nM and 1.86 ± 0.52 nM (mean ± S.E.M., n = 6 preparations), respectively, did not differ significantly, whereas the B$_{max}$ value for the mice receiving lorazepam showed a 33% reduction relative to controls (fig. 3, top). Because of the limited amounts of coated vesicles obtainable from mouse brain (~100 µg/animal; table 1), saturation studies of $[^{3}H]$flunitrazepam binding were carried out only on tissue from untreated animals. For the coated vesicle fraction, a K$_{v}$ value of 2.08 ± 0.14 nM and a B$_{max}$ value of 220 ± 7 fmol/mg were obtained. A 10 nM concentration of $[^{3}H]$flunitrazepam was used to estimate the level of receptors in TCVs from treated mice. As shown in fig. 3 (bottom), the level of binding to coated vesicles from the group exposed to lorazepam was increased by 82.9 ± 12.8% compared with controls. Assays of microsomal fractions did not reveal any significant differences in binding for the vehicle- vs. drug-treated mice (not shown).

Quantitative Western blotting was used to determine the amounts of clathrin heavy chain and of GABA$_{A}$ receptor alpha-1 subunits in the membrane fractions. As indicated in fig. 4 (left), immunoreactivity of the 170-kDa clathrin heavy chain in TCVs showed little difference in the lorazepam-treated animals compared with controls. Quantification of similar immunoblots of three independent TCV preparations from each treatment group (not shown) revealed that the clathrin level from the drug-treated mice represented 98.7 ± 12.8% in the absence of lorazepam.
3.9% (mean ± S.E.M.) of that of the vehicle-treated controls. This demonstrates that lorazepam administration had no gross effects on the yield of coated vesicles. On the other hand, the level of the GABAA receptor 51-kDa alpha-1 subunit in TCVs from treated mice showed an increase compared with controls (fig. 4, right). The smaller amount of immunoreactivity at ~100 kDa appears to be nonspecific. Preabsorption of the antibody with the GABA_A receptor alpha-1(331–381) fusion protein used as the antigen (Miranda et al., 1997) eliminated the immunoreactive 51-kDa band but had little effect on the 100-kDa band (not shown). The relative amounts of the 51-kDa alpha-1 subunit on similar Western blots of three TCV preparations from each experimental group were determined with a previously validated method (Miranda and Barnes, 1997); this revealed that the content of GABAA receptor alpha-1 subunits in TCVs from lorazepam-treated animals was increased by 58.5 ± 13.5% (mean ± S.E.M.) relative to the controls. When clathrin heavy-chain immunoreactivity was used as an internal control on each Western blot to normalize the amounts of GABAA receptor alpha-1 subunits, similar results were obtained. This analysis showed that TCVs from the mice exposed to lorazepam had a 60.0 ± 10.3% (n = 3) elevation of alpha-1 subunits compared with those from the vehicle-treated animals. Consistent with fig. 4, quantification of immunoblots of crude synaptic membranes showed only an 11.5 ± 60.1% decline in alpha-1 subunits from the drug-treated mice compared with controls.

**Discussion**

The number of neurotransmitter receptors on the synaptic plasma membrane is regulated by the traffic of intracellular vesicles. Golgi-derived vesicles provide newly synthesized receptors to the cell surface, whereas CCVs are the initial vehicles for sequestration of surface receptors, which are ultimately degraded or recycled. Muscarinic acetylcholine receptors and beta-adrenergic receptors, which are subject to agonist-induced sequestration and down-regulation (Benovic et al., 1988; Thompson and Fisher, 1990), are found on CCVs (Chuang et al., 1986; Silva et al., 1986).

Acute exposure of cortical neurons in vitro to GABA or clonazepam induces sequestration of GABAA ligand binding sites (Calkin and Barnes, 1994a, 1994b; Tehrani and Barnes, 1991). Consistent with operation of such a mechanism in vivo, we previously identified central benzodiazepine receptors on highly purified CCV fractions from rat brain (Tehrani and Barnes, 1993). However, in vivo studies have been hampered by difficulties in purifying CCV fractions from limited amounts of tissue. To facilitate such experiments, we changed the final purification step, eliminating gel filtration of crude CCVs and substituting an extraction with Triton.
X-100. Contamination by smooth vesicles, which account for 
50% of the protein in the crude coated vesicle fraction, was 
reduced through Triton extraction, a treatment that spares 
CCVs (Pearse, 1982; Weidenmann et al., 1985). We found 
earlier that 
70% of the [3H]flunitrazepam binding sites on 
column-purified CCVs could be recovered after Triton extrac-
tion (Tehrani and Barnes, 1993). In the present study, the 
purification of coated vesicles was monitored by enrichment 
for the clathrin heavy chain (Bar-Zvi and Branton, 1986) and 
clathrin light-chain kinase (Schook and Puszkin, 1985). By 
these measures, the degree of enrichment of coated vesicles 
obtained here was similar to that reported earlier (Tehrani 
and Barnes, 1993). The level of [3H]flunitrazepam binding 
obtained under standard conditions (10 nM radioligand) was 
also similar: 59 fmol/mg for column-purified CCVs (Tehrani 
and Barnes, 1993) and 74 fmol/mg for TCVs (table 1).

To examine the possibility that agonist-dependent seques-
tration of GABA A receptors occurs in vivo, we used proce-
dures described by Miller et al. (1988) for the administration 
of lorazepam to mice. In that study, lorazepam treatment (2 
mg/kg/day) for a 7-day period produced tolerance, a serum 
lorazepam level of 60 to 80 ng/ml and a 25% reduction in the 
B max value for [3H]flunitrazepam binding to crude synaptic 
membranes (P 2 fraction) from the cerebral cortex. Using an 
identical protocol for drug administration, we found a serum 
lorazepam level of 103±8.9 ng/ml and a 33±4% reduction 
in the B max value for [3H]flunitrazepam binding to synaptic 
membranes from whole brain. Thus, agreement between 
these two series of experiments seems to be excellent. As an 
alternative explanation of our results, the possibility that 
residual lorazepam could produce an artifactual displace-
ment of [3H]flunitrazepam binding was considered. However, 
this appears unlikely because the washing procedures used 
during isolation of TCVs or treatment of crude synaptic mem-
branes (see Methods) removes contaminating benzodiaz-
epines (Miller et al., 1988; Wu et al., 1994).

We found that the level of [3H]flunitrazepam binding to 
coated vesicles (TCVs) from the lorazepam-treated animals...
was nearly twice that from the vehicle-treated controls. This result is highly significant (P < 0.025) and indicates that use-dependent sequestration of GABA<sub>A</sub> receptors can occur in vivo. We considered three other explanations for our results. First, synthesis of some receptor subunits could have undergone an up-regulation as a result of the drug treatment (Galpern et al., 1990). Newly synthesized receptors are presumably present on smooth vesicles derived from the endoplasmic reticulum or Golgi complex, which could contaminate the coated vesicle fraction. Although such smooth vesicles should be present in even higher numbers in the microsomal fraction, we found that lorazepam administration had no significant effect on the microsomal binding of [3H]flunitrazepam. Second, the drug treatment could have produced an altered expression of GABA<sub>A</sub> receptor subunits (Wu et al., 1994), which increased the affinity of [3H]flunitrazepam in TCVs. However, nearly saturating levels of [3H]flunitrazepam were used in the TCV assays. Furthermore, lorazepam administration did not significantly alter the K<sub>d</sub> values for synaptic membranes. Finally, we considered the possibility that [3H]flunitrazepam binding to TCVs from the treated mice could have been stimulated allosterically by endogenous GABA. To remain after such extensive washing of membranes, any residual GABA would probably be trapped within vesicles, but intravesicular GABA should have been released by the Triton extraction. Furthermore, the GABA antagonist SR95531 has no effect on [3H]flunitrazepam binding to coated vesicles, showing that endogenous GABA does not compromise the assays (Tehrani and Barnes, 1995). Thus, we suggest that a lorazepam-dependent increase in receptor number on coated vesicles is the best explanation for the data. However, limitations in the amount of coated vesicles (table 1) that can be obtained from drug-treated mice prevented us from carrying out a complete saturation analysis of equilibrium [3H]flunitrazepam binding.

To show more directly that the drug treatment produced an increase in GABA<sub>A</sub> receptor number in coated vesicles, we used quantitative immunoblotting of the alpha-1 subunit. This polypeptide is the major site for [3H]flunitrazepam photolabeling in rat brain (Macdonald and Olsen, 1994). The TCV fractions from treated mice showed nearly a 60% rise in alpha-1 subunit immunoreactivity relative to controls, whereas the synaptic membrane fraction experienced a very small decline (12%). This redistribution of GABA<sub>A</sub> receptor alpha-1 subunits in the lorazepam-treated mice is similar to that observed for [3H]flunitrazepam binding. Thus, our data provide good support for the occurrence in vivo of lorazepam-dependent GABA<sub>A</sub> receptor sequestration.

The mechanism of benzodiazepine tolerance is not understood. There is evidence that GABA<sub>A</sub> receptor functions are compromised in some, but not all, brain regions of tolerant rodents (Marley and Gallagher, 1989; Miller et al., 1988; Ramsey et al., 1991; Wilson and Gallagher, 1988; Xie and Tietz, 1992). However, no clear picture of underlying alterations in receptor molecules has emerged. Some investigators have reported a down-regulation of GABA<sub>A</sub> receptor ligand binding sites (Crawley et al., 1982; Miller et al., 1988; Tietz et al., 1986; Wu et al., 1994), which is in accord with our current study of [3H]flunitrazepam binding to synaptic membranes. However, others found no change in ligand binding to tissues from tolerant rats (Gallager et al., 1984; Impagnatiello et al., 1996). For GABA<sub>A</sub> receptor subunit mRNAs, a complex pattern of drug-induced regional and down variations was reported, but this pattern differs from that for the corresponding subunit polypeptides (O'Donovan et al., 1992; Impagnatiello et al., 1996). Our data suggest that some of these inconsistencies may be due to a subcellular redistribution of GABA<sub>A</sub> receptors in the tolerant animals. For example, receptor sequestration on coated vesicles could reduce GABA-gated currents without producing changes detectable by ligand binding autoradiography or subunit immunoreactivity at a multicellular level. At a gross tissue level, the amount of GABA<sub>A</sub> receptors on coated vesicles represents only a small fraction of the total. However, sequestration, like down-regulation (Tietz et al., 1988; Impagnatiello et al., 1996; Wu et al., 1994), may be anatomically regionalized.

The significance of benzodiazepine-induced sequestration of GABA<sub>A</sub> receptors, either in vitro (Tehrani and Barnes, 1991) or in vivo, has not been clearly established. It has been shown that a fraction of sequestered GABA<sub>A</sub> receptors are rapidly degraded, providing a pathway for use-dependent down-regulation (Calkin and Barnes, 1994a, 1994b). Miranda and Barnes (1997) also suggested that sequestration could provide intracellular signals for the regulation of GABA<sub>A</sub> receptor expression. Alternatively, internalized receptors could be recycled to the neuronal surface (Barnes, 1996). Under appropriate conditions, such as drug withdrawal, this recycling could replenish the surface with GABA<sub>A</sub> receptors, returning membrane currents to the normal state. Based on our current data, we advance the hypothesis that GABA<sub>A</sub> receptor sequestration may contribute to the establishment of tolerance to benzodiazepines. Additional studies of coated vesicle components and their trafficking during benzodiazepine administration will be necessary to more definitively examine this hypothesis.

Acknowledgments
The technical assistance of Lee M. Savelle is gratefully acknowledged. We thank Dr. E. M. Lafer (University of Texas Health Science Center at San Antonio) for a monoclonal antibody against clathrin.

References
Gallager, D. W., Laskesi, J. M., Gonsalves, S. F. and Rauch, S. L.: Chronic


Miranda, J. D. and Barnes, E. M., Jr.: Depression of \( \gamma \)-aminobutyric acid type A receptor \( \alpha_1 \) polypeptide biosynthesis requires chronic agonist exposure. J. Biol. Chem. 272: 16282–16294, 1997.


O’Donovan, M. C., Buckland, P. R., Spurlock, G. and McGuffin, P.: Bidirectional changes in the levels of messenger RNAs encoding \( \gamma \)-aminobutyric acid \( \alpha \) receptor \( \alpha_1 \) subunits after flurazepam treatment. Eur. J. Pharmacol. 226: 335–341, 1992.


Wu, Y., Rosenberg, H. C., Chu, T. H. and Ramsey-Williams, V.: Regional changes in \( ^{1} \text{H} \)Hippolpid binding to brain benzodiazepine receptors in flurazepam tolerant rat: comparison with changes in \( ^{3} \text{H} \)Flunitrazepam binding. J. Pharmacol. Exp. Ther. 268: 675–682, 1994.


Send reprint requests to: Dr. Eugene M. Barnes, Biochemistry Department, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.