0026-895X/94/061056-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:1056–1062, 1994.



# Chronic Exposure to Benzodiazepine Receptor Ligands Uncouples the γ-Aminobutyric Acid Type A Receptor in WSS-1 Cells

GARRY WONG, TRACEY LYON, and PHIL SKOLNICK

*Laboratory of Neuroscience, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892*

Received March 28, 1994; Accepted September 1, 1994

**SUMMARY**

Chronic exposure to benzodiazepines can result in an "uncoupling" of γ-aminobutyric acid (GABA) receptors and benzodiazepine receptors (BzR) both in primary neuronal cell cultures and *in vivo*. The effect of chronic exposure to BzR ligands was examined in an engineered cell line (WSS-1) stably expressing "type I" $GABA_A$ receptors. Chronic exposure to flurazepam produced a concentration- ($EC_{50}$, ~1.1 μM after a 48-hr exposure) and time-dependent ($t_{½}$, ~3 hr at 100 μM) reduction in the efficacy ($E_{max}$) of GABA to enhance [$^3$H]flunitrazepam binding to BzR, a characteristic of uncoupling in native $GABA_A$ receptor isoforms. Uncoupling of $GABA_A$ receptors and BzR without concomitant changes in BzR density was also produced by chronic exposure to other, structurally diverse, BzR ligands, including Ro 15–1788 and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, but was not manifested after exposure to the 5-hydroxytryptamine reuptake blocker fluoxetine. Chronic (12–48-hr) exposure to flurazepam did not remarkably alter levels of α1 and γ2 mRNAs, which constitute $GABA_A$ receptors in this cell line. Based on these findings, it is hypothesized that uncoupling of $GABA_A$ receptors and BzR in this engineered cell line can proceed without the elaboration of additional novel subunits and could involve either post-translational modification of $GABA_A$ receptor proteins or changes in subunit stoichiometry.

The $GABA_A$ receptors are a heterogeneous group of multimeric, ligand-gated, chloride ion channels (1, 2). $GABA_A$ receptors that possess many of the pharmacological properties of native receptors (including responsiveness to BzR ligands) can be formed by the expression of at least two of three different (α, β, and γ) subunits (2–4). Expression of cloned $GABA_A$ receptor subunits indicates that the functional heterogeneity of $GABA_A$ receptors is produced by various combinations of the multiple α, β, and γ subunits (as well as several splice variants) that have so far been identified in the mammalian central nervous system (1, 5).

Tolerance to many of the pharmacological actions of Bz can be observed in both experimental animals and humans after chronic administration (reviewed in Ref. 6). Although the molecular mechanisms responsible for this phenomenon remain unknown, chronic exposure to Bz has been reported to decrease GABAergic function, and a disruption of the allosteric interactions between GABA receptors and BzR has been described both in primary neuronal cell cultures (7, 8) and *in vivo* (9–14). This latter effect has been termed "uncoupling" (8, 11) and can be demonstrated by both neurochemical (8, 11, 13, 14) and electrophysiological (7, 13) measures. The phenomenon of uncoupling has been proposed to underlie the clinical tolerance associated with chronic use of Bz (8, 11, 13, 14).

The WSS-1 cell line stably expresses "type I" $GABA_A$ receptors composed of α1 and γ2 subunits (3). The ability of Bz to augment GABA-gated chloride currents (an action blocked by Ro 15–1788) and of GABA to enhance [$^3$H]flunitrazepam binding indicates an appropriate allosteric coupling between GABA receptors and BzR in this and other cell lines expressing α1 and γ2 subunits (3, 4). The present study examined the effects of chronic exposure to BzR ligands on the allosteric interactions between GABA receptors and BzR in WSS-1 cells, to determine the suitability of this engineered cell line for examining the molecular mechanisms responsible for uncoupling of $GABA_A$ receptors.

## Materials and Methods

**Cell culture.** WSS-1 cells (CRL 2029; American Type Culture Collection, Rockville, MD) were cultured as described previously (3). Cells were grown in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) with 4.5 g/liter glucose, 2 mM gluta-

G.W. was supported by the Academy of Finland during some of the writing of this manuscript.

**ABBREVIATIONS:** GABA, γ-aminobutyric acid; Bz, benzodiazepine(s); BzR, benzodiazepine receptor(s); DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; ANOVA, analysis of variance.

mine, 100 μg/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). Cells were maintained at 37° in 5% $CO_2$ and were treated with drug or vehicle 24 hr after division. Drugs were dissolved in the minimum amount of water (flurazepam, GABA, and fluoxetine) or dimethylsulfoxide (diazepam, DMCM, Ro 15–1788, and Ro 16–6028). Control cultures were treated with the appropriate vehicle. No consistent differences in GABA-enhanced [$^3$H]flunitrazepam binding were observed between untreated cells and those treated with either water or dimethylsulfoxide (data not shown). In experiments to determine the reversibility of flurazepam-induced changes in GABA-enhanced [$^3$H]flunitrazepam binding, flurazepam-containing medium was removed from the cells, the culture dishes (10 cm, round; Falcon, Lincoln Park, NJ) were gently washed twice with 10 ml of serum-containing medium, and the original medium (minus flurazepam) was replaced.

**Cell membrane preparation.** Culture medium was removed from the dishes, and the cells were washed once with phosphate-buffered saline. Cells were scraped from the dishes, centrifuged at 1500 × *g* for 10 min, and resuspended in assay buffer (50 mM Tris-citrate buffer, pH 7.8) (~3 × $10^6$ cells/10-cm dish). Each experiment routinely contained cells from five or six plates washed in 7 ml of buffer. The cells were disrupted with a Brinkmann Polytron (setting 6, 10 sec), followed by centrifugation for 20 min at 20,000 × *g*. The buffer was decanted and the resulting pellet was resuspended in ~7 ml of assay buffer/15–18 × $10^6$ cells. This washing procedure was repeated four more times. Cell membranes were resuspended in assay buffer at an initial concentration of ~3 × $10^6$ cells/ml.

**Radioligand binding assays.** Saturation studies were performed in a total volume of 500–1000 μl, consisting of tissue (~200 μg of protein), 0.2 M NaCl, [$^3$H]flunitrazepam (~0.4–24 nM; specific activity, 74.1–85.8 Ci/mmol), and assay buffer to volume. Nonspecific binding was determined by the addition of 10 μM diazepam. Determination of total binding was performed in duplicate, with one nonspecific determination per data point. GABA enhancement of [$^3$H]flunitrazepam binding was assayed in a final volume of 500 μl, consisting of tissue (~200 μg of protein), 0.2 M NaCl, [$^3$H]flunitrazepam (~5 nM), GABA (10 nM to 1 mM or 50 μM, as specified), and assay buffer to volume. Nonspecific binding was determined by the addition of diazepam (10 μM) and was typically <20% of total binding. Determinations were performed in triplicate, with nonspecific binding measured in duplicate. Incubations (0–4°) were terminated after 60 min by rapid filtration through no. 32 glass filters (Schleicher and Schuell, Keene, NH) and two 5-ml assay buffer washes with a M24R cell harvester (Brandel, Gaithersburg, MD). The radioactivity retained on the filters was measured in a Beckman LS5801 liquid scintillation counter. Data were analyzed using GraphPAD Inplot 4.0 (Intuitive Software, San Diego, CA).

**Northern blot analysis.** After incubation with drugs or vehicle, cells were washed once in phosphate-buffered saline and the mRNA was extracted by guanidinium isothiocyanate denaturation followed by oligo(dT) affinity chromatography, using a commercially available kit (Invitrogen, San Diego, CA). mRNAs from five 10-cm dishes were pooled and 1 μg was electrophoresed on a 0.8% agarose/7% formaldehyde gel. The RNAs were transferred to Nytran (Scheicher and Schuell), cross-linked by UV irradiation, and hybridized to the respective probes as described (15). Full length, $^{32}$P-labeled, cDNA probes for rat GABA$_A$ receptor α1 and γ2 subunits and human β-actin were prepared by random priming (Strategene, San Diego, CA) to specific activities of >5 × $10^8$ cpm/μg. Radiolabeled cDNAs were hybridized to the Nytran filter in 0.5 M sodium phosphate, pH 7.0, 7% SDS, 0.5% bovine serum albumin, 2 mM EDTA, at 68° for 16–20 hr. The filters were washed once in 2× SSC (sodium chloride, 3 M; sodium citrate, 0.3 M)/0.1% SDS at 25° for 20 min and once in 0.1× SSC/0.1% SDS at 55° for 20 min. The filter was air dried and exposed to X-OMAT film (Kodak, Rochester, NY) for the indicated times (α1, 24 hr; γ2, 24 hr; β-actin, 48 hr). The γ2 probe was hybridized initially to the filter. After exposure, the filter was stripped by two washes for 10 min in 0.1× SSC/0.1% SDS at 95° and was rehybridized with α1 followed by β-actin radiolabeled probes. This experiment was repeated three times. Film densities were quantitated with an M4 image analyzer (Image Research, St. Catherines, Ontario, Canada), and values were standardized to β-actin.

**Statistics.** Data were analyzed by ANOVA with Dunnett's *post hoc* comparions. In experiments examining the effects of chronic drug exposure on GABA-enhanced [$^3$H]flunitrazepam binding (Table 1), comparisons were made with the percentage increase in [$^3$H]flunitrazepam binding produced by GABA, defined as (specific binding in the presence of GABA/specific binding in the absence of GABA) × 100.

**Materials.** Ro 15–1788, Ro 16–6028, Ro 5–4864, and other Bz were donated by Hoffmann-LaRoche (Nutley, NJ). DMCM was purchased from Research Biochemicals (Natick, MA). [$^3$H]Flunitrazepam was obtained from DuPont-NEN (Boston, MA) and [$^{32}$P]dCTP from Amersham (Arlington Heights, IL). Other materials were purchased from standard commerical sources.

## Results

Consistent with observations with native GABA$_A$ receptors, GABA enhanced [$^3$H]flunitrazepam binding to WSS-1 cells through a reduction in the apparent $K_d$, with no apparent effect on $B_{max}$ (Fig. 1). GABA enhanced [$^3$H]flunitrazepam binding to WSS-1 cell membranes in a concentration-dependent manner ($EC_{50}$, 0.30 ± 0.10 μM; $E_{max}$, 71 ± 17%; three experiments) (Fig. 2). Exposure of cells to 10 μM flurazepam for 48 hr markedly decreased maximal enhancement ($E_{max}$, 24 ± 8.4%) without significantly affecting the potency ($EC_{50}$, 0.56 ± 0.37 μM), whereas a higher concentration (100 μM) essentially abolished GABA enhancement of [$^3$H]flunitrazepam binding (Fig. 2). A 48-hr exposure to diazepam (100 μM) produced similar reductions in the $E_{max}$ of GABA (three experiments; data not shown).

Subsequent experiments were performed using a maximally effective concentration of GABA (50 μM), as determined from concentration-response curves (Fig. 2). The enhancement of [$^3$H]flunitrazepam binding by this concentration of GABA was 47 ± 5% (30 experiments). The potency of flurazepam to reduce GABA-enhanced [$^3$H]flunitrazepam binding was estimated after a 48-hr exposure. Under these conditions, the $EC_{50}$ value of flurazepam was 1.1 ± 0.2 μM (Fig. 3). The $EC_{50}$ for diazepam (1–200 μM) obtained under identical conditions was 12.4 μM (data pooled from six experiments; not shown). To determine the time required to effect an uncoupling of GABA receptors and BzR, WSS-1 cells were exposed to flurazepam for periods of up to 72 hr and GABA-enhanced [$^3$H]flunitrazepam binding was measured (Fig. 4A). A $t_{1/2}$ of ~3 hr was obtained with both 10 and 100 μM flurazepam. The reversibility of this effect was examined by treating cells with 100 μM flurazepam for 48 hr, followed by removal of this medium, superficial washing of the cells with flurazepam-free medium, and incubation with serum-containing Dulbecco's modified Eagle's medium for the indicated intervals (Fig. 4B). The $t_{1/2}$ for recovery of GABA-enhanced [$^3$H]flunitrazepam binding was ~2 hr. Under these conditions, recovery was only partial, with a maximal return to ~67% of pre-exposure values (Fig. 4B).

The allosteric interaction between GABA receptors and BzR was also examined after a 48-hr exposure to other BzR ligands exhibiting a range of actions along the described pharmacological continuum for these compounds (16). Reductions in GABA (50 μM)-enhanced [$^3$H]flunitrazepam binding comparable to or greater than those produced by 10 μM flurazepam were also

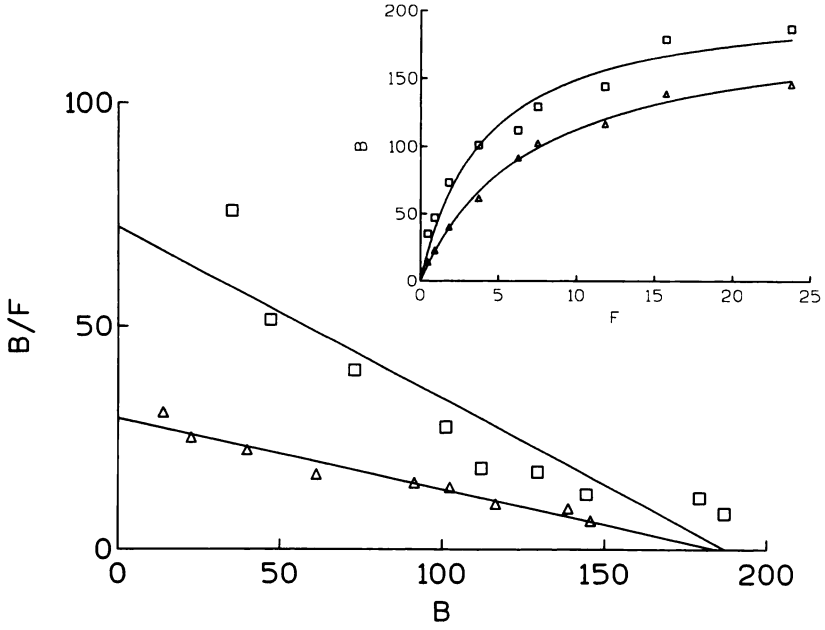


**Fig. 1.** Effect of GABA on [$^3$H]flunitrazepam binding to WSS-1 cells. Membranes were prepared and incubated with ~0.4–24 nM [$^3$H]flunitrazepam in the absence (△) or presence (□) of GABA (50 μM). In this representative experiment, the $K_d$ of [$^3$H]flunitrazepam was reduced from 7.1 to 4.0 nM; the corresponding $B_{max}$ values were 193.3 and 209.8 fmol/mg of protein. *Inset*, Saturation isotherm. *B*, bound (fmol/mg of protein); *F*, free radioligand (nM). This experiment was repeated, with similar results.

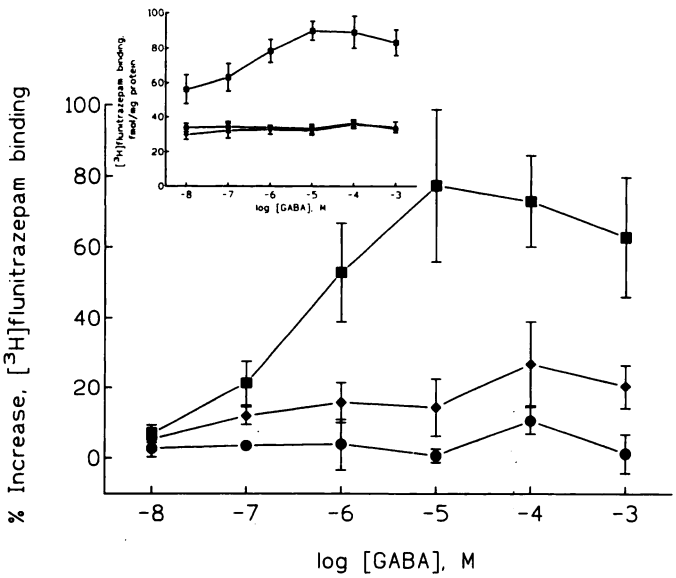


**Fig. 2.** Effects of chronic flurazepam exposure on GABA-enhanced [$^3$H]flunitrazepam binding. Membranes were prepared from WSS-1 cells treated for 48 hr with vehicle (■), 10 μM flurazepam (◆), or 100 μM flurazepam (●). Membranes were extensively washed and incubated with [$^3$H]flunitrazepam (~5 nM) and GABA (0.01 μM to 1 mM) as described in Materials and Methods. Values represent the mean ± standard error of three experiments. The maximal enhancement ($E_{max}$) of [$^3$H]flunitrazepam binding by GABA was 71 ± 17% in vehicle-treated cells; the $EC_{50}$ of GABA was 0.30 ± 0.10 μM. *Inset*, data replotted as GABA-enhanced [$^3$H]flunitrazepam binding (fmol/mg of protein). In these experiments, basal [$^3$H]flunitrazepam binding was 53 ± 9, 29 ± 3, and 33 ± 3 fmol/mg of protein for vehicle-, 10 μM flurazepam-, and 100 μM flurazepam-treated cells, respectively.

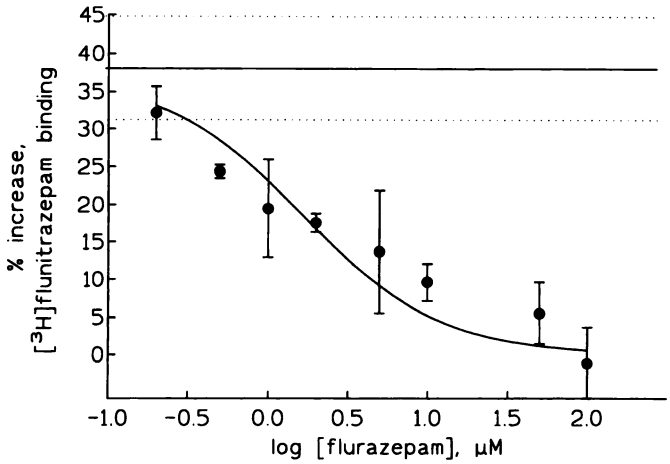


**Fig. 3.** Concentration-effect curve for uncoupling of $GABA_A$ receptors by flurazepam. WSS-1 cells were treated with flurazepam (0–100 μM) for 48 hr. Membranes were prepared and incubated with [$^3$H]flunitrazepam (~5 nM) in the presence of GABA (50 μM), as described in Materials and Methods. The $E_{max}$ of GABA in vehicle-treated cells was 37 ± 7% (*solid line*, mean; *dashed lines*, standard error; nine experiments) and the basal binding was 55 ± 3 fmol/mg of protein. The values at each concentration of flurazepam represent the mean ± standard error of three experiments. The $EC_{50}$ of flurazepam was 1.1 ± 0.2 μM.

observed with diazepam (20 μM) and DMCM (10 μM) (Table 1). Ro 15–1788 (1, 5, and 100 μM) produced a concentration-dependent reduction in GABA-enhanced [$^3$H]flunitrazepam binding, whereas a combination of Ro 15–1788 (5 μM) and diazepam (20 μM) produced a reduction comparable to that produced by diazepam (Table 1). More modest, nonsignificant reductions in GABA-enhanced [$^3$H]flunitrazepam binding were observed after incubation with Ro 5–4864 (10 μM) and GABA (100 μM). In contrast, the 5-hydroxytryptamine reuptake blocker fluoxetine (10 μM) (17) was without effect (Table 1).

The effects of 48-hr exposure to a group of structurally diverse BzR ligands on the apparent affinity of [$^3$H]flunitrazepam and BzR density were also examined. No statistically significant changes in BzR density were elicited by flunitrazepam (10 μM), diazepam (100 μM), DMCM (10 μM), or Ro 16–6028 (10 μM), as determined by ANOVA (Table 2). Only Ro 16–6028 produced a modest, albeit statistically significant, increase in the apparent $K_d$ of [$^3$H]flunitrazepam.

Northern blot analyses demonstrated no statistically significant change in the levels of α1 and γ2 mRNAs, relative to β-actin, after a 12-, 24-, or 48-hr exposure to flurazepam (Fig.

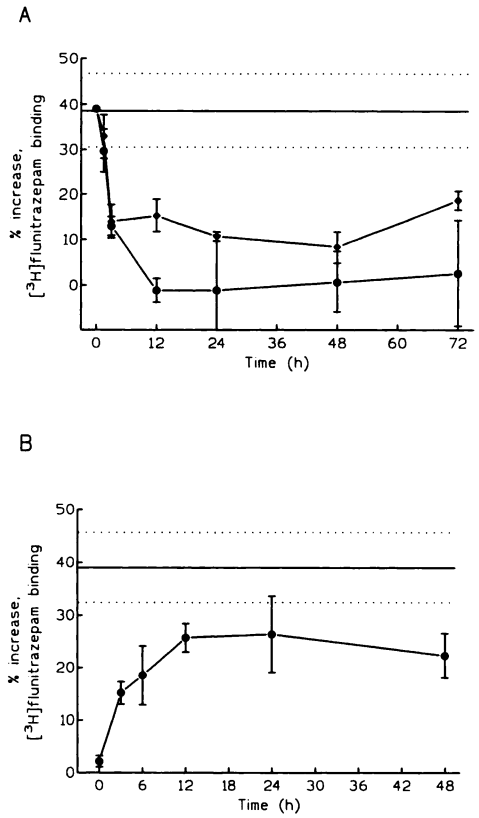


**Fig. 4.** Time course (A) and reversibility (B) of uncoupling of GABA$_A$ receptors by flurazepam. A, WSS-1 cells were treated with 10 μM (♦) or 100 μM (●) flurazepam for the indicated times. Cell membranes were prepared and incubated with [$^3$H]flunitrazepam (~5 nM) in the presence of GABA (50 μM). *Ordinate*, percentage increase in [$^3$H]flunitrazepam binding produced by GABA. In control cultures, GABA increased [$^3$H] flunitrazepam binding by 39 ± 7.0% (*solid line*, mean; *dashed lines*, standard error; 12 experiments); basal binding was 50 ± 4 fmol/mg of protein. *Abcissa*, time of incubation with flurazepam. Each *point* represents the mean ± standard error of three experiments. B, Cells were treated with 10 μM flurazepam for 48 hr. Flurazepam-containing medium was aspirated from the cultures and replaced by flurazepam-free medium for the indicated times (*abcissa*). Note that this superficial washing of cells did not completely remove flurazepam. Assay of the supernant from cells after removal of drug-containing medium indicated the presence of ~0.8 μM flurazepam (or an active metabolite) (data not shown). After removal of drug-containing medium, cells were assayed for coupling at the indicated post-treatment times, as described above.

5). β-Actin mRNA, used as a standard for comparison of the relative densities of GABA$_A$ receptor mRNAs, was also unaffected by exposure to flurazepam (Fig. 5, legend). A representative Northern blot is shown in Fig. 6. Similar results were obtained with a slot blot (data not shown).

## Discussion

A reduction in GABAergic function has been reported after chronic regimens of Bz, which result in the development of tolerance to these drugs in a variety of behavioral measures. Bz-induced changes in GABAergic function are manifested at both the cellular and subcellular levels and include a subsensitivity to GABA in electrophysiological studies (9, 12, 13) and a reduced augmentation by GABA of [$^3$H]Bz binding (11, 13, 14). Reductions in BzR density have also been observed by some (18–20) but not all (13, 14) investigators after chronic Bz administration. Similar alterations in the allosteric interaction

TABLE 1

**Effects of Chronic exposure to BzR ligands on GABA-enhanced [$^3$H]flunitrazepam binding**

WSS-1 cells were treated with the indicated substances for 48 hr. GABA (50 μM) enhancement of [$^3$H]flunitrazepam binding to well washed cell membranes was determined as described in Materials and Methods. The enhancement of [$^3$H]flunitrazepam binding by GABA was 69 ± 11% in control cells (nine experiments). Values represent the mean ± standard error of at least three determinations for drug-treatment cells. The basal [$^3$H]flunitrzepam binding in control cells was 31 ± 3 fmol/mg of protein.

| Treatment | Increase in [$^3$H]flunitrazepam binding |
|---|---|
| | % |
| Control | 69 ± 11 |
| Flurazepam, 10 μM | 18 ± 5[a] |
| Diazepam, 20 μM | 9 ± 2[b] |
| Ro 15-1788, 1 μM | 36 ± 3 |
| Ro 15-1788, 5 μM | 18 ± 4[a] |
| Ro 15-1788, 5 μM, + diazepam, 20 μM | 6 ± 3[b] |
| Ro 15-1788, 100 μM | 11 ± 2[b] |
| DMCM, 10 μM | −5 ± 3[b] |
| GABA, 100 μM | 44 ± 8 |
| Ro 5-4864, 10 μM | 39 ± 6 |
| Fluoxetine, 10 μM | 64 ± 7 |

[a] $p < 0.05$ versus control, Dunnett's *post hoc* comparisons.
[b] $p < 0.01$.

TABLE 2

**Effects of chronic exposure to BzR ligands on [$^3$H]flunitrazepam binding**

WSS-1 cells were treated for 48 hr and the membranes were assayed as described in Materials and Methods. Parameter values were estimated using GraphPAD Inplot 4.0 ($r^2 > 0.95$). Data are mean ± standard error of three experiments.

| Treatment | $K_d$ | $B_{max}$ |
|---|---|---|
| | nM | fmol/mg of protein |
| Control | 8.5 ± 1.1 | 218 ± 27 |
| Flurazepam, 10 μM | 6.0 ± 0.6 | 145 ± 23 |
| Diazepam, 100 μM | 12.8 ± 2.4 | 182 ± 14 |
| DMCM, 10 μM | 9.2 ± 1.6 | 141 ± 25 |
| Ro 16-6028, 10 μM | 15.4 ± 2.1[a] | 260 ± 63 |

[a] $p < 0.05$, Dunnett's *post hoc* test. All other $K_d$ and $B_{max}$ values were not significantly different from controls.

between GABA receptors and BzR have also been observed after exposure of primary neuronal cell cultures to Bz (7, 8, 21), but the present report is, to our knowledge, the first attempt to study the effects of long term exposure to Bz in a cell line stably expressing GABA$_A$ receptors.

In our initial studies, chronic exposure to flurazepam reduced the efficacy of GABA to enhance [$^3$H]flunitrazepam binding (Fig. 2; Table 1). This phenomenon (termed uncoupling or "functional uncoupling") (8, 11) has been reported both in primary cultures of chick brain (8, 21) and in animals chronically treated with Bz (11, 13). The extent of uncoupling in WSS-1 cells is much greater than observed in rat cortical membranes after chronic Bz treatment. For example, Gallager *et al.* (13) reported an ~50% reduction in the efficacy of GABA to enhance [$^3$H]flunitrazepam binding to rat cortical membranes after 21 days of parenterally administered diazepam (5 mg/kg). A more modest (~30%) but significant reduction in the efficacy of GABA was observed after 28 days of flurazepam administered to rats in their drinking water (11). Although the use of homogeneous versus heterogeneous populations of GABA$_A$ receptors could be invoked to explain this difference in extent of uncoupling, a complete uncoupling of GABA receptors and BzR was also achieved in primary neuronal cultures

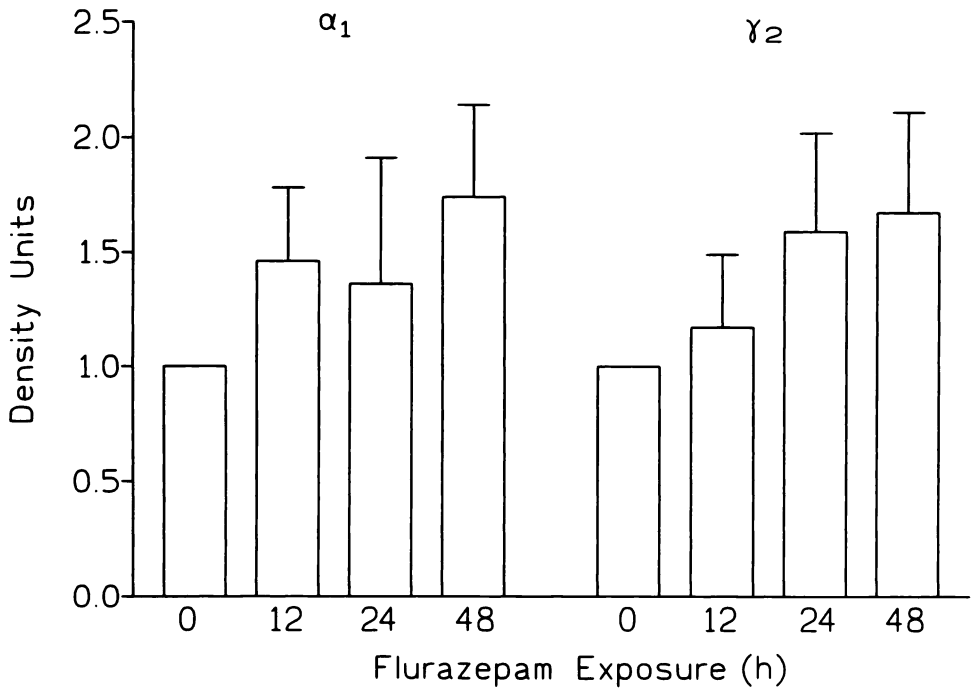


**Fig. 5.** Effects of chronic flurazepam exposure on $\alpha 1$ and $\gamma 2$ mRNA. Cells were treated with flurazepam (100 $\mu$M) for the indicated times. mRNA was extracted and probed with $\alpha 1$, $\gamma 2$, and $\beta$-actin cDNAs as described in Materials and Methods. Values represent the mean ± standard error of three independent experiments, representing analysis of Northern blots by densitometry using an M4 image analyzer, as described in Materials and Methods. The absorbance of $\beta$-actin (see Fig. 6) at time 0 was set at unity. Flurazepam exposure did not alter $\beta$-actin absorbance; the ratios of $\beta$-actin absorbance at 12, 24, and 48 hr to $\beta$-actin absorbance at time 0 were 0.88 ± 0.21, 1.09 ± 0.24, and 1.17 ± 0.20, respectively. Density units are defined as absorbance, [$\alpha 1$ (or $\gamma 2$)/$\beta$-actin] at 12, 14, or 48 hr/[$\alpha 1$ (or $\gamma 2$)/$\beta$-actin] at time 0. No statistically signicant (ANOVA) differences in absorbance were obtained for $\alpha 1$, $\gamma 2$, or $\beta$-actin as a function of time. A representative autoradiogram is illustrated in Fig. 6. In an independent series of two experiments, DMCM (100 $\mu$M) exposure did not alter $\beta$-actin absorbance; the ratios of $\beta$-actin absorbance at 12, 24, and 48 hr to $\beta$-actin absorbance at time 0 were 0.79, 1.14, and 1.14, respectively. The corresponding density units for $\alpha 1$ were 0.72, 1.09, and 1.15 and for $\gamma 2$ were 0.79, 1.59, and 1.30.

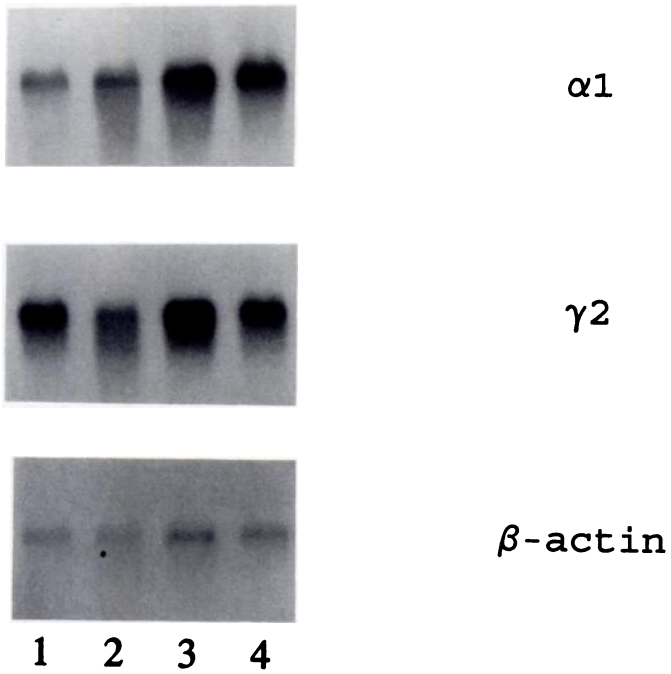


**Fig. 6.** Effects of chronic flurazepam exposure (100 $\mu$M) on levels of $\alpha 1$ and $\gamma 2$ mRNA. A Northern blot of mRNA from WSS-1 cells exposed to flurazepam is shown. *Lanes 1-4*, exposure for 0, 12, 24, and 48 hr, respectively. Approximately 1 $\mu$g of mRNA was electrophoresed and blotted to Nytran filters as described in Materials and Methods. Radiolabeled, full length, cDNA probes used were $GABA_A$ receptor rat $\alpha 1$ subunit, $GABA_A$ receptor rat $\gamma 2$ subunit, and human $\beta$-actin, as indicated. Films were exposed for 24 hr ($\alpha 1$ and $\gamma 2$) or 48 hr ($\beta$-actin) at −70°.

(8). The potency of flurazepam in this latter study ($EC_{50}$, ~1 $\mu$M after a 64-hr exposure) was similar to that observed in WSS-1 cells ($EC_{50}$, ~1.1 $\mu$M after a 48-hr exposure). Based on the time- and concentration-dependent uncoupling observed in both primary neuronal cultures (8) and WSS-1 cells (Figs. 3 and 4), delivery of sustained higher concentrations of Bz *in vivo* might produce a more extensive uncoupling than reported previously.

These findings demonstrate that the elements necessary for uncoupling of $GABA_A$ receptors are constitutively contained in cells of non-neuronal origin, because WSS-1 cells were derived from a human kidney cell line. WSS-1 cells were engineered with cDNAs encoding the $\alpha 1$ and $\gamma 2$ subunits, indicating that uncoupling can proceed in the absence of appropriately oriented, transcriptional control elements that are likely associated with the expression of these subunits in primary neuronal cultures and intact neurons (22). One potential explanation for uncoupling in WSS-1 cells is a redistribution of $GABA_A$ receptors between the cell surface and subcellular organelles. In view of the methods used to prepare cell membranes for radioligand binding assays, it would be predicted that such a shift would also be accompanied by changes in receptor density. However, the uncoupling of $GABA_A$ receptors in WSS-1 cells produced by flurazepam and other BzR ligands (see below) was not accompanied by consistent changes in BzR density (Table 2), indicating that this does not readily explain uncoupling. Uncoupling of $GABA_A$ receptors that is not accompanied by changes in receptor density has been reported in neuronal cell cultures after exposure to flurazepam (8) and in some (but not all) *in vivo* studies (12, 13, 19, 20). The reported differences among *in vivo* studies may be related to both the doses of Bz and the duration of treatment used.

Several studies have investigated changes in GABA$_A$ receptor subunit expression after chronic treatment with Bz (14, 23–25). Those studies indicate that chronic treatment with Bz leads to a region-specific decrease in levels of mRNAs encoding the $\alpha$1 and $\gamma$2 subunits. Moreover, O'Donovan *et al.* (25) recently reported that chronic treatment with flurazepam results in bidirectional changes in whole-brain levels of other $\alpha$ subunit mRNAs (a decrease in $\alpha$3 and an increase in $\alpha$5 mRNA). Because it appears that native GABA$_A$ receptors can be constituted with multiple $\alpha$ subunit isoforms (26) and transient transfection of cells with different $\alpha$ subunits affects the allosteric interactions between GABA receptors and BzR (27), substitution or switching of subunits represents a plausible mechanism for GABA$_A$ receptor uncoupling (25). Because only the $\alpha$1 and $\gamma$2 subunits are expressed in WSS-1 cells and levels of mRNAs encoding these subunits do not appear to change in a consistent, statistically significant fashion during flurazepam exposure (Figs. 5 and 6), uncoupling may proceed without the elaboration of additional GABA$_A$ receptor subunits. Consistent with this notion, in an *in vivo* model of tolerance to Bz decreases in $\alpha$1 and $\gamma$2 mRNA levels in cerebral cortex were not apparent until 14 days after treatment, whereas development of receptor down-regulation was noted after 7 days (23). Alternatively, uncoupling in WSS-1 cells could be explained by chronic Bz treatment inducing the expression of endogenous subunits in WSS-1 cells. Although the appearance of GABA$_A$ receptor mRNAs encoding other subunits was not examined after flurazepam treatment, the speed at which uncoupling occurs in WSS-1 cells ($t_{1/2}$, ~3 hr) (Fig. 4A) makes this explanation unlikely. Nonetheless, a change in subunit stoichiometry (e.g., assuming a pentameric configuration, a change from $3\alpha/2\gamma$ to $2\alpha/3\gamma$) remains a potential mechanism to explain uncoupling in this cell line.

GABA$_A$ receptor uncoupling can be elicited in native receptors by GABA-positive BzR ligands (28) such as diazepam (13) and flurazepam (8, 11). This effect can be blocked by the GABA-neutral ligand Ro 15–1788 (8, 13). In contrast, uncoupling of GABA$_A$ receptors in WSS-1 cells was observed after exposure to GABA-positive, -neutral, and -negative BzR ligands. Because a range of pharmacological responses can be obtained from the appropriate assembly of recombinant GABA$_A$ receptor subunits that differ from those typically observed in wild-type receptors (29), it could be argued that DMCM and Ro 15–1788 act as GABA-positive ligands in WSS-1 cells. However, because Bz (e.g., midazolam and diazepam) augmentation of GABA-gated chloride currents is blocked by Ro 15–1788 in WSS-1 cells (and other cells expressing $\alpha$1$\gamma$2 subunits) (3, 4), it is concluded that occupation of BzR may be sufficient to uncouple GABA$_A$ receptors in this cell line. The inability of fluoxetine to uncouple GABA$_A$ receptors provides some indication that this action is not shared by non-BzR ligands (Table 1), whereas the modest nonsignificant uncoupling produced by 10 $\mu$M Ro 5–4864 may reflect the low affinity (30) of this 1,4-Bz for GABA$_A$ receptor-linked BzR. Preliminary experiments (Table 1) also demonstrated that exposure to GABA (100 $\mu$M, for 48 hr) effects a modest nonsignificant uncoupling of GABA$_A$ receptors, compared with BzR ligands. However, the concentrations of GABA used in those experiments were 5–10-fold lower than those effective in uncoupling GABA$_A$ receptors in native isoforms (31–33). Additional studies will be required to determine similarities between engineered and native cells with respect to compounds acting at other loci on the GABA$_A$ receptor complex. Flurazepam induced an uncoupling of GABA$_A$ receptors in WSS-1 cells far more rapidly ($t_{1/2}$, ~3 hr) than previously reported for primary cultures of chick brain ($t_{1/2}$, ~18 hr); the latter value is consistent with $t_{1/2}$ values reported for receptor turnover (8). This difference may be related to a fundamentally different mechanism for GABA$_A$ receptor uncoupling in native versus recombinant receptors or may be related to the expression of GABA$_A$ receptors in a non-neuronal environment.

Although the effects of flurazepam on GABA$_A$ receptor uncoupling appear to be only partially reversible (restored to a maximum of 67%) (Fig. 4B), the removal of flurazepam by superficial washing of the cells was incomplete. Thus, significant concentrations of flurazepam or its active metabolites (0.8 $\mu$M from 100 $\mu$M flurazepam-treated cells) were detected by radioreceptor assay in the supernatant of cell membranes harvested immediately after flurazepam removal (data not shown). Nonetheless, this partial restoration of GABA$_A$ receptor coupling is rapid and appears temporally symmetrical with uncoupling, which is consistent with the possibility that these processes are effected through a common mechanism. Moreover, the lack of a significant difference in the $K_d$ of [$^3$H]flunitrazepam after treatment of WSS-1 cells with flurazepam (Table 2) indicates that the extensive washing of cell membranes removes all of the drug before assay.

These findings suggest that BzR occupation is sufficient to produce a concentration- and time-dependent uncoupling of GABA$_A$ receptors in WSS-1 cells, perhaps by initiating processes leading to changes in either receptor stoichiometry or post-translational modifications of receptors (e.g., phosphorylation) (34). Although the uncoupling of BzR and GABA receptors in WSS-1 cells is not identical to the process observed after exposure to BzR ligands either in primary neuronal cultures or *in vivo*, this cell line represents a novel model that may provide insights into the molecular mechanisms of tolerance to Bz.

**Acknowledgments**

The authors thank Drs. Anthony Basile, Yoshitatsu Sei, and Yoshua Gozes for technical help and advice.

**References**

1. Burt, D. R., and G. L. Kamatchi. GABA$_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
2. Lüddens, H., and W. Wisden. Function and pharmacology of multiple GABA receptor subunits. *Trends Pharmacol. Sci.* **12**:49–52 (1991).
3. Wong, G., Y. Sei, and P. Skolnick. Stable expression of type I $\gamma$-aminobutyric acid$_A$/benzodiazepine receptors in a transfected cell line. *Mol. Pharmacol.* **42**:996–1003 (1992).
4. Im, H., W. B. Im, B. J. Hamilton, D. B. Carter, and P. F. Von Voiglander. Potentiation of $\gamma$-aminobutyric acid-induced chloride currents by various benzodiazepine site agonists with the $\alpha$1$\gamma$2, $\beta$2$\gamma$2, and $\alpha$1$\beta$2$\gamma$2 subtypes of cloned $\gamma$-aminobutyric acid type A receptors. *Mol. Pharmacol.* **44**:866–870 (1993).
5. Olsen, R., and A. Tobin. Molecular biology of GABA$_A$ receptors. *FASEB J.* **4**:1469–1480 (1990).
6. File, S. E. The biology of benzodiazepine dependence, in *Benzodiazepine Dependence* (C. Hallström, ed.). Oxford University Press, Oxford, UK, 95–118 (1993).
7. Prasad, A., and J. N. Reynolds. Uncoupling of GABA-benzodiazepine receptors in chick cerebral cortical neurons requires co-activation of both receptor sites. *Brain Res.* **591**:327–331 (1992).
8. Roca, D. J., G. D. Schiller, L. Friedman, I. Rozenberg, T. T. Gibbs, and D. H. Farb. $\gamma$-Aminobutyric acid$_A$ receptor regulation in culture: altered allosteric interactions following prolonged exposure to benzodiazepines, barbiturates, and methylxanthines. *Mol. Pharmacol.* **37**:710–719 (1990).
9. 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).

10. Hernandez, T. D., C. Heninger, M. A. Wilson, and D. W. Gallager. Relationship of agonist efficacy to changes in GABA sensitivity and anticonvulsant tolerance following chronic ligand exposure. *Eur. J. Pharmacol.* **170**:145–155 (1989).
11. 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).
12. 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 $\gamma$-aminobutyric acid$_A$ receptor function. *J. Pharmacol. Exp. Ther.* **246**:170–176 (1988).
13. Gallager, D. W., J. M. Lakoski, S. F. Gonsalves, and S. L. Rauch. Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. *Nature (Lond.)* **308**:74–77 (1984).
14. Gallager, D. W., A. A. Jacobs, J. S. Crais, M. J. During, and T. D. Hernandez. Chronic treatments that produce reversible and irreversible changes in *gamma*-aminobutyric acid sensitivity, in *Transmitter Amino Acid Receptors: Structures, Transduction and Models for Drug Development* (E. A. Barnard and E. Costa, eds.), Vol. 6. Thieme Medical Publishing, New York, 113–128 (1991).
15. Boje, K. M., G. Wong, and P. Skolnick. Desensitization of the NMDA receptor complex by glycinergic ligands in cerebellar granule cell cultures. *Brain Res.* **603**:207–214 (1993).
16. Gardner, C. Functional *in vivo* correlates of the benzodiazepine agonist-antagonist continuum. *Prog. Neurobiol.* **31**:425–476 (1988).
17. Wong, D. T., F. P. Bymaster, L. R. Reid, and P. G. Threlkeld. Fluoxetine and two other serotonin reuptake blockers without affinity for neuronal receptors. *Biochem. Pharmacol.* **32**:1287–1293 (1983).
18. Crawley, J. N., P. J. Marangos, J. Stivers, and F. K. Goodwin. Chronic clonazepam administration induces benzodiazepine receptor subsensitivity. *Neuropharmacology* **21**:85–89 (1982).
19. 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).
20. Chiu, T. H., and H. C. Rosenberg. Reduced diazepam binding after chronic benzodiazepine treatment. *Life Sci.* **23**:1153–1158 (1978).
21. Schiller, G. D., and D. H. Farb. Enhancement of benzodiazepine binding by GABA is rapidly reduced during chronic exposure to flurazepam. *Ann. N. Y. Acad. Sci.* **435**:221–223 (1986).
22. Kirkness, E. F., and C. M. Fraser. A strong promoter element is located between alternative exons of a gene encoding the human $\gamma$-aminobutyric acid-type A receptor $\beta$3 subunit (GABARB3). *J. Biol. Chem.* **268**:4420–4428 (1993).
23. Kang, I., and L. G. Miller. Decreased GABA$_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. *Br. J. Pharmacol.* **103**:1285–1287 (1991).
24. 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).
25. O'Donovan, M. C., P. R. Buckland, G. Spurlock, and P. McGuffin. Bidirectional changes in the levels of messenger RNAs encoding $\gamma$-aminobutyric acid$_A$ receptor $\alpha$ subunits after flurazepam treatment. *Eur. J. Pharmacol.* **226**:335–341 (1992).
26. Fritschy, J. M., D. Benke, S. Mertens, W. H. Oerten, T. Bachi, and H. Möhler. Five subtypes of type A $\gamma$-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc. Natl. Acad. Sci. USA* **89**:6726–6730 (1992).
27. Pritchett, D., H. Lüddens, and P. Seeburg. Type I and type II GABA$_A$-benzodiazepine receptors produced in transfected cells. *Science (Washington D. C.)* **245**:1389–1392 (1989).
28. Squires, R., ed. *GABA and Benzodiazepine Receptors*. CRC Press, Boca Raton, FL (1988).
29. von Blankenfeld, G., S. Ymer, D. Pritchett, H. Sontheimer, M. Ewert, P. Seeburg, and H. Kettenmann. Differential benzodiazepine pharmacology of recombinant GABA$_A$ receptors. *Neurosci. Lett.* **115**:269–273 (1990).
30. Braestrup, C., R. Albrechtsen, and R. Squires. High densities of benzodiazepine receptors in human cortical areas. *Nature (Lond.)* **269**:702–704 (1978).
31. Montpied, P., E. I. Ginns, B. M. Martin, D. Roca, D. H. Farb, and S. M. Paul. $\gamma$-Aminobutyric acid (GABA) induces a receptor-mediated reduction in GABA$_A$ receptor $\alpha$ subunit messenger RNAs in embryonic chick neurons in culture. *J. Biol. Chem.* **266**:6011–6014 (1991).
32. Roca, D. J., I. Rozenberg, M. Farrant, and D. H. Farb. Chronic agonist exposure induces down-regulation and allosteric uncoupling of the $\gamma$-aminobutyric acid/benzodiazepine receptor complex. *Mol. Pharmacol.* **37**:37–43 (1990).
33. Mehta, A. K., and M. K. Ticku. Chronic GABA exposure down-regulates GABA-benzodiazepine receptor-ionophore complex in cultured cerebral cortical neurons. *Mol. Brain Res.* **16**:29–36 (1992).
34. Moss, S. J., T. G. Smart, C. D. Blackstone, and R. L. Huganir. Functional modulation of GABA$_A$ receptors by cAMP-dependent protein phosphorylation. *Science (Washington D. C.)* **257**:661–665 (1992).

**Send reprint requests to:** Phil Skolnick, NIH/NIDDK-LN, Building 8, Room 111, Bethesda, MD 20892–0008.