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Antagonist-Induced Reversal of Functional and Structural Measures of Hippocampal Benzodiazepine Tolerance[1]

ELIZABETH I. TIETZ, XU J. ZENG, SHANYI CHEN, SCOTT M. LILLY, HOWARD C. ROSENBERG, and PETER KOMETIANI

Department of Pharmacology, Medical College of Ohio, Toledo, Ohio

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ABSTRACT

One week oral flurazepam (FZP) administration in rats results in anticonvulsant tolerance in vivo, tolerance measured in vitro in hippocampal CA1 pyramidal cells, and regulation of hippocampal γ-aminobutyric acid$_A$-receptor subunit protein expression. A single injection (4 or 20 mg/kg i.p) of the benzodiazepine antagonist flumazenil (FLM) was given 1 day after FZP treatment, and tolerance and subunit protein expression were evaluated 1 day later. In vivo tolerance was measured by a reduced ability of the α_1-subunit-selective agonist zolpidem to suppress pentylenetetrazole-induced seizures. This tolerance was reversed by 20 but not 4 mg/kg FLM. In in vitro hippocampal slices, there was tolerance to the effect of zolpidem to prolong the decay of pyramidal cell miniature inhibitory postsynaptic currents, which was reversed by FLM (4 mg/kg) pretreatment. A reduction in miniature inhibitory postsynaptic current amplitude (~50%) was also restored by FLM injection. [^{3}H]Zolpidem binding measured 0, 2, and 7 days after FZP treatment was significantly decreased in the hippocampus and cortex at 0 days but not thereafter. Changes in α_1- and β_3-subunit protein expression were examined via quantitative immunohistochemical techniques. α_1-Subunit protein levels were down-regulated in the CA1 stratum oriens and β subunit levels were up-regulated in the stratum oriens and stratum radiatum of the CA3 region. Chronic FZP effects on α_1- and β_3-subunit protein levels were also reversed by prior FLM injection. FLM's effect on both functional and structural correlates of benzodiazepine tolerance suggests that each of these measures plays an interdependent role in mediating benzodiazepine tolerance.

Through their actions to potentiate fast, γ-aminobutyric acid (GABA)-mediated inhibitory neurotransmission benzodiazepines are potent anticonvulsants and useful adjuncts for intractable epilepsies. Nonetheless, the emergence of functional tolerance, i.e., a reduced anticonvulsant effect with prolonged use, is a significant drawback limiting their clinical value. Numerous animal studies have suggested that a dysfunction of the GABAergic system, and especially of the GABA$_A$ receptor (GABAR), underlies benzodiazepine anticonvulsant tolerance.

Mammalian GABARs are a heterogeneous population, composed of various combinations of five subunit proteins, derived from homologous families with multiple variants [α(1–6), β(1–3), γ(1–3), δ(1), and ϵ(1)] (Davies et al., 1990; Macdonald and Olsen, 1994, Rabow et al., 1995). Benzodiazepines bind to an allosteric site on GABARs, increasing the frequency of GABA-gated Cl^- channel opening (Macdonald and Olsen, 1994). The diversity of native GABAR subtypes in specific brain areas (cf. Rabow et al., 1995; McKernan and Whiting, 1996) is likely responsible for the functional heterogeneity of GABA and allosteric modulator responses in different brain regions such as the hippocampus (Kapur and Macdonald, 1996, Tietz et al., 1999b) and provides a basis for the differential sensitivity of GABARs to regulation by chronic benzodiazepine treatment.

Although impaired GABAR function appears central to benzodiazepine tolerance, the locus and nature of the dysfunction is still controversial. Several studies suggest that changes in subunit gene expression underlie a fundamental change in GABAR subunit composition, whereas others indicate that a posttranslational mechanism mediates a change in GABAR function. For example, localized changes in specific GABAR subunit mRNA and protein expression were detected after chronic benzodiazepine treatments that result in anticonvulsant tolerance (Heninger et al., 1990; Kang and Miller, 1991; Holt et al., 1996; Impagnatiello et al., 1996; Pesold et al., 1997; Chen et al., 1999; Tietz et al., 1999a) but not after treatment with novel benzodiazepines (imidazenil and abercarnil), which fail to induce tolerance (Holt et al., 1996; Impagnatiello et al., 1996; Pesold et al., 1997). On the

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GABAR, GABA$_A$ receptor; IPSC, inhibitory postsynaptic current; PTZ, pentylenetetrazole; FZP, flurazepam; FLM, flumazenil.

other hand, a consistent finding is a decreased allosteric coupling between the GABA and benzodiazepine binding site, measured by the reduced ability of GABA to increase benzodiazepine binding affinity (Klein et al., 1995; Rabow et al., 1995; Primus et al., 1996). Gallager and colleagues, and more recently other laboratories, have shown that, exposure to the benzodiazepine antagonist flumazenil (FLM) can rapidly reverse some of the functional and biochemical changes associated with chronic diazepam treatment (Gonsalves and Gallager, 1985, 1987; Klein et al., 1995; Primus et al., 1996). Based on the rapidity of reversal, the latter findings suggest that a conformational or other posttranslational change, e.g., protein phosphorylation/dephosphorylation, may play a key role in mediating tolerance.

Our laboratory uses a 1-week oral FZP treatment, which results in a reduction in diazepam, zolpidem, and $GABA_A$ agonist potency in the CA1 region of in vitro hippocampus (Xie and Tietz, 1992; Zeng and Tietz, 1999), and in anticonvulsant tolerance (Rosenberg, 1995) but not in dependence in vivo. Changes in GABAR structure and function associated with this treatment have suggested circumscribed effects on the GABAergic system in specific brain areas, namely, the hippocampus and cortex. Prolonged FZP treatment was associated with a localized decrease in GABA-mediated inhibitory function in CA1 pyramidal neurons (Xie and Tietz, 1991; Zeng and Tietz, 1997, 1999). Down-regulation of benzodiazepine binding sites, examined autoradiographically with [^{3}H]flunitrazepam, was short-lived in the hippocampus and cortex (Tietz et al., 1986, Chen et al., 1995). Regardless, the down-regulation of specific (α_1 and β_3) GABAR subunit mRNAs (Tietz et al., 1994, 1999) and proteins (Chen et al., 1999) in the same brain areas during oral FZP administration suggests that the variable expression of GABAR subunits contributes to changes in GABAR physiology and GABA and benzodiazepine pharmacology.

We evaluated the effect of FLM to reverse the behavioral and in vitro measures of tolerance, as well as GABAR subunit expression. In vivo tolerance was measured by the altered ability of the α_1-selective imidazopyridine zolpidem to suppress pentylenetetrazole (PTZ)-induced seizures (Rosenberg, 1995). In vitro tolerance was assessed by the shift in zolpidem's ability to prolong the decay of miniature inhibitory postsynaptic currents (mIPSCs) recorded in hippocampal CA1 pyramidal cells (Zeng and Tietz, 1999). Regional changes in [^{3}H]zolpidem binding, as a function of time after treatment, were also measured with autoradiographic techniques (Tietz et al., 1986). Localized changes in α_1- and β_3-subunit protein expression were examined with quantitative immunohistochemical techniques (Chen et al., 1999). Evaluating the differential effects of the benzodiazepine antagonist to affect both functional and structural measures of benzodiazepine tolerance might provide some insights into the role these measures play in mediating anticonvulsant tolerance.

Materials and Methods

In Vivo Drug Administration

Chronic FZP Treatment. After a 2-day vehicle-acclimation period, adult, male Sprague-Dawley rats (initial weight 180–225 g) were given FZP in 0.02% saccharin as their drinking water for 1 week. Matched control rats received saccharin water for the same length of time and were handled identically throughout all experiments. The goal was to achieve 100 mg/kg for 3 days and 150 mg/kg for 4 days. In practice, rats usually achieve a dose of ~75 to 95 mg/kg in the first 3 days and 125 to 145 mg/kg in the last 4 days. Thus, rats that did not meet the criterion dose, a weekly average of ≥100 mg/kg, were excluded. To determine whether tolerance had developed by the 3rd day of treatment, an additional group of rats was treated for 3 days to achieve 100 mg/kg p.o. or given 0.02% saccharin water. For rats treated for only 3 days, the criterion dose was an average of 75 mg/kg. After drug removal, FZP-treated rats were given saccharin water for 2 days until used for in vitro or in vivo experiments. At this time, 2 days after drug removal, residual benzodiazepine and metabolites are no longer detectable in the hippocampus (<3 ng FZP and metabolites/g hippocampus) and thus do not confound electrophysiological recordings (Xie and Tietz, 1991). Moreover, rats treated with FZP in this manner for 1 week are tolerant to the ability of the benzodiazepines to suppress PTZ seizures in vivo, up to 4 days after treatment is stopped (Rosenberg et al., 1985). Furthermore, neither PTZ nor bicuculline seizure threshold was altered per se as a result of 1-week FZP treatment, 2 days after drug removal (Rosenberg, 1995). Anticonvulsant tolerance in vivo is no longer evident 7 days after ending FZP treatment (Rosenberg et al., 1985).

Acute FLM Treatment. All FZP-treated and control rats were given a single i.p. injection of FLM (4 mg/kg) (Gonsalves and Gallager, 1985) or the FLM vehicle (1 ml/kg) 24 h after ending a week-long FZP treatment and 24 h before electrophysiological recording, immunohistochemical studies, or seizure threshold testing. An additional group of rats tested with PTZ received 20 mg/kg FLM or 2 ml/kg of the vehicle. The elimination half-life of 10 mg/kg FLM in the rat brain is 16 min and thus is no longer detectable within 90 min (Lister et al., 1984). This finding was confirmed by radioreceptor assay in rats administered 4 mg/kg i.p. after chronic diazepam treatment (Gonsalves and Gallager, 1985). The experimenters were not informed of the rats' treatment histories until the experiments were completed.

Whole-Cell Recording

Hippocampal Slice Preparation. Electrophysiological studies were carried out in in vitro hippocampal slices from FZP-treated and control rats. Rats were decapitated, and transverse dorsal hippocampal slices (500 μm) were cut on a vibratome (Pelco 101, Ted Pella, Inc., Redding, CA) in ice-cold pregassed ($95\%O_2/5\%CO_2$) buffer containing 120 mM NaCl, 5.0 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose; 288 mOsm, pH 7.2 to 7.4. Slices were stored at room temperature for ≥2 h but not >8 h in gassed buffer. mIPSCs were recorded at room temperature during constant perfusion (1.5 ml/min) with gassed buffer.

mIPSC Recording. $GABA_A$-mediated, action-potential-independent mIPSCs were isolated from CA1 pyramidal neurons in the presence 1 μM tetrodotoxin and the excitatory amino acid receptor antagonists, 50 μM DL-2-amino-5 phosphonovaleric acid and 10 μM 6,7-dinitroquinoxaline-2,3-done, with whole-cell voltage-clamp techniques as previously described (Zeng and Tietz, 1999). Briefly, patch pipettes (4–7 MΩ), pulled from borosilicate capillaries (nonfilamented, 1.5 mm *A*, Sutter Instruments Co., Novato, CA) on a Flaming-Brown electrode puller (P-97, Sutter), were filled with internal solution containing: 130 mM CsCl, 1.0 mM EGTA, 0.5 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM ATP, 10.0 mM HEPES, pH adjusted to 7.2. Cs^+ was included in the recording electrode to eliminate $GABA_B$-mediated events. *N*-Ethyl bromide quaternary salt (2 mM) was also included to block the spontaneous firing of CA1 pyramidal neurons (Zeng and Tietz, 1997, 1999). Neurons were voltage clamped (V_h = −70 mV) in continuous mode with an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA). The current output was low-pass filtered (10 KHz), offset, and amplified 10,000-fold, as previously described (Zeng and Tietz, 1999). The signal was continuously monitored on-line (PClamp 6.0 Software, Axon), digitized (Digidata 1200, Axon), and stored on VCR tape for later off-line analysis. Baseline

mIPSC activity was recorded for at least 5 min in each cell. Recorded events above the level of background noise (±2.0 pA) with a duration ≥3 ms were detected and averaged with Strathclyde CDR and SCAN software (J. Dempster, University of Strathclyde, Glasgow, Scotland). Peak mIPSC amplitude was measured from baseline. mIPSC decay kinetics were estimated with a single [$y(t) = a * \exp(-t/\tau)$] or biexponential [$y(t) = a1 * \exp(-t/\tau1) + a2 * \exp(-t/\tau2)$] function. Differences between amplitude (pA) and decay (τ) measures between groups were compared by Student's t test with a significance level of $p \leq .05$.

Zolpidem Effect on mIPSC Amplitude and Decay

In a subset of FZP-treated and control cells, mIPSC activity was recorded for 8 min in the presence of 1 μM zolpidem after the 5-min baseline recording. The final 3-min segment under basal conditions and in the presence of zolpidem was used for off-line analysis of mIPSC amplitude and decay kinetics. It was previously shown in a larger number of CA1 neurons that the proportion of control (65%) and FZP-treated (62%) neurons best fit with a biexponential versus monoexponential decay were similar (Zeng and Tietz, 1999). In this study, a comparison of residual variances (r^2) among cells within groups revealed a similar result. A biphasic decay was a better fit in 66% of control cells and 50% of FZP-treated cells. In the remaining FZP-treated cells, the fits of the decay function were indistinguishable. The degree of zolpidem potentiation of mIPSC decay was expressed as a fraction of the control monophasic decay constant, i.e., using the simpler of the two equations.

Tissue Preparation for Autoradiographic and Immunohistochemical Studies

Rats were used for autoradiographic experiments either immediately (0 days) or 2 or 7 days after ending FZP or saccharin treatment. These rats were intracardially perfused with 300 ml of ice-cold PBS (0.1 M, pH 7.4) after ketamine (80 mg/kg i.m.) anesthesia. Rats used for immunohistochemical experiments were anesthetized with ketamine 2 days after ending FZP treatment and then intracardially perfused with 300 ml ice-cold 0.9% NaCl. All brains were quickly removed and frozen for 15 s in isopentane, cooled in an acetone/dry ice bath (−70°C), and then placed into the cryostat (−14°C) for 1 h before sectioning. Parasagittal sections (autoradiography, 10 μm; immunohistochemistry, 20 μm) were thaw mounted onto 0.5% gelatin/0.05% chrome-alum-coated slides and stored at −70°C until used for binding or immunohistochemical studies. Brain sections were brought to room temperature under vacuum before use.

Quantitative [^{3}H]Zolpidem Autoradiography

[^{3}H]Zolpidem Binding. Brain sections from treated and control rats were handled in parallel throughout all procedures. Sections were prewashed 5 min in 50 mM K_2HPO_4, 50 mM NaH_2PO_4, and 200 mM NaCl (pH 7.4) before 3 × 10-min washes in the assay buffer: 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl (pH 7.4 at 4°C). Prewashed sections were then quickly air dried under a cool stream of air before ligand incubation. This washing procedure removes 94% of the GABA from tissue as determined by HPLC analysis of slide-mounted brain sections (E.I.T., D. Godfrey, W. F. Ferencak III, S.C., unpublished data). With similar autoradiographic techniques in FZP-treated rats, it was also demonstrated that the residual drug in the brain immediately after ending FZP treatment could be effectively removed by excessive prewashing of sections (Tietz et al., 1986). Residual drug is no longer detectable in the hippocampus or whole brain 2 days after the cessation of drug treatment (Xie and Tietz, 1992). Sections were incubated in slide mailers for 60 min in 5 nM [^{3}H]zolpidem (54.3 Ci/mmol; Amersham, Arlington Heights, IL) in ice-cold assay buffer. Nonspecific binding was determined in the presence of 2 μM unlabeled FLM. After incubation, slides were dipped in 300 ml assay buffer, then rinsed an additional three times 30 s in a similar volume of assay buffer to remove unbound ligand, then a final dip in dH_2O to remove salts. Slides were rapidly dried under a cool stream of air and fixed in a vacuum dessicator with paraformaldehyde vapor for 2 h at 80°C.

Quantitative Autoradiography. Brain sections were apposed to [^{3}H]Ultrofilm (Leica Instruments, Nussloch, Germany) for 6 days with 10-μm, slide-mounted ^{3}H standards, made by incorporating increasing concentrations of [^{3}H]thymidine (6.7 Ci/mmol; Amersham) into brain paste, as previously described (Tietz et al., 1986). One brain section per rat was labeled with [^{3}H]zolpidem and one brain section was used for nonspecific binding. Brain sections from FZP-treated rats and their matched control rats, i.e., sacrificed at the same time point, were exposed together in the same cassette. Autoradiographic films were developed 5 min in D-19 (Kodak, Rochester, NY), stopped in 3% acetic acid, fixed 4 min in Rapid Fixer (Kodak), and rinsed 30 min in dH_2O.

For quantification, films were placed on a light box under constant illumination (Northern Light, Imaging Research, Inc., St. Catherines, Ontario, Canada), and digitized images, acquired under identical conditions with a high-resolution CCD camera (Sierra Scientific, Inc., Sunnyvale, CA), were stored on disk for later analysis. With National Institutes of Health image software (v. 1.59), gray values, representing film density, were measured over ^{3}H standards to construct a standard curve relating film density to nanocuries ^{3}H per milligram protein (r^2 = 0.95–0.99). Total binding was determined from the film density over hippocampal subregions and other brain areas of interest. Hippocampal subregions measured included CA1 stratum (s.) oriens (SO), s. pyramidale (SP), s. radiatum (SR), and s. lacunosum (SL); CA2 (SP); CA3 (SO, SP); and dentate gyrus (DG) molecular (MOL), granule (GRN), and polymorph (PC) cell layers. Additional areas measured included the frontal, parietal, and occipital cortices, layers I to VI, and the molecular and granule cell layers of the cerebellum. Nonspecific binding densities were 25 to 65% of total binding in the various hippocampal subregions measured and 10 to 30% of total binding in cortical subregions. Nonspecific values were not significantly different between groups (Student's t test: 0 day, $p = .96$; 2 day, $p = .91$; 7 day, $p = .39$) and were subtracted from measures representing total binding to estimate specific binding (pmol of zolpidem per mg of protein) from the standard curve. The mean specific-binding density was compared between FZP-treated and control groups by ANOVA followed by pairwise comparisons with the method of Scheffé with a significance level of $p \leq .05$. For comparison of changes in specific binding between antibodies and among time points, the data are expressed as a percentage of control binding.

Quantitative Immunohistochemistry

Immunohistochemical Procedures. Specific, affinity-purified GABAR subunit antibodies raised in rabbits against specific, nondegenerate portions of the α_1- (1–9) and β_3- (345–408) subunits were obtained from W. Seighart. A complete description of antibody specificity is provided elsewhere (Buchstaller et al., 1991; Jechlinger et al., 1998; Chen et al., 1999). The quantitative immunohistochemical procedures were similar to those previously described (Chen et al., 1999). All brain sections were treated identically throughout all immunocytochemical procedures. Sections were warmed to room temperature for 15 min under vacuum, postfixed 8 min in 4% paraformaldehyde in 0.3 M Tris-HCl (pH 7.2 at 22°C), rinsed 3 × 5 min in Tris buffer, and blocked for 1 h in 10% normal goat serum plus 0.02% Triton X-100. The slides were then incubated overnight at 4°C with primary antibody (α_1, 3 μg/ml; β_3, 10 μg/ml) in blocking solution. After rinsing, the slides were incubated with biotinylated anti-rabbit IgG $F(ab')2$ fragment (1:250, v/v; Boehringer Mannheim, Mannheim, Germany) for 1 h at room temperature, rinsed well, and incubated for 1 h with avidin-biotin peroxidase complex (ABC; 1:100, v/v; Vector Laboratories, Inc., Burlingame, CA). After rinsing, immunostaining was visualized with 0.6% (w/v) diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO) plus 0.02% (v/v) hydrogen peroxide. The DAB reaction was stopped within 5 min. The slides

were dehydrated in ethanol (75, 95, and 100%; 5 min each), cleared in xylene 5 min, and covered with Permount (Fisher Scientific, Pittsburgh, PA).

Quantification of Immunostaining Density. The density of colorimetric DAB reaction on treated versus control brain sections was digitized as described above. This method of analysis, developed in our laboratory, results in reliable quantification of antibody staining and has been validated by Western blot analysis (Chen et al., 1999). Immunostaining density was measured in the following hippocampal subregions: CA1 s. oriens (SO), s. pyramidale (SP), s. radiatum (SR), and s. lacunosum (SL); CA2 (SO, SP, SR); CA3 (SO, SP, SR); and DG molecular, granule, and polymorph cell layers. The other brain areas measured included many of the same areas in which [^{3}H]zolpidem binding was evaluated, plus additional areas that included the inferior colliculus and the superficial gray, optic, and intermediate gray layers of the superior colliculus and the molecular and granule cell layers of the cerebellum. Background immunostaining density was measured over the corpus callosum immediately superior to the hippocampal CA1 region. The mean gray values ± S.E., reflecting specific immunostaining density, were compared between FZP-treated and control groups to assess FZP's effect on α_1- and β_3-GABAR subunit levels. The effect of prior benzodiazepine antagonist administration was compared between FZP-treated and control groups injected with FLM or the FLM vehicle 24 h before perfusion. Comparisons of gray-level values between the groups were made by ANOVA followed by pairwise comparisons with the method of Scheffé with a significance level of $p \leq .05$. For comparison of changes in levels of protein expression between antibodies and among groups, the data are expressed as a percentage of control expression.

Behavioral Measures of Zolpidem Effects

The acute effect of benzodiazepine agonists was evaluated by their effect to raise PTZ and bicuculline seizure threshold. This drug effect was measured in rats as an increase in the chemoconvulsant dose eliciting the motor manifestations of a seizure, i.e., myoclonic jerks and forelimb clonus (Rosenberg, 1995). In vivo cross-tolerance is reflected in an attenuation of the ability of zolpidem (or, e.g., diazepam) to suppress chemoconvulsant-induced seizures. This loss of drug effect can be measured as a reduction in the dose (milligrams per kilogram i.v.) of the chemoconvulsant required to induce forelimb clonic seizures in the presence of zolpidem. Comparisons among groups were by two-way ANOVA with chronic treatment and acute FLM or vehicle treatment as independent, grouping variables. Pairwise comparisons were made by the Tukey test with a significance level of $p \leq .05$.

Seizures were induced with either chemoconvulsant in separate groups of 1-week FZP-treated and control rats 2 days after FZP removal from the drinking water. Twenty-four hours before testing, FZP-treated and saccharin-treated rats were injected with FLM (4 mg/kg) or the FLM vehicle (1 ml/kg). An additional group of rats tested with PTZ received 20 mg/kg FLM or 2 ml/kg of the vehicle. To evaluate whether the length of benzodiazepine treatment affects FLM's ability to reverse anticonvulsant tolerance, another group of rats given FZP or 0.02% saccharin water for only 3 days was evaluated for the tolerance to diazepam's (5 mg/kg i.p.) ability to increase PTZ seizure threshold. The prototype benzodiazepine diazepam was used in these initial studies, because it was not known whether 3-day FZP treatment would produce tolerance. All FZP- and saccharin-treated control rats were tested in parallel between 9:00 and 11:00 AM. PTZ (20 mg/ml) or bicuculline (0.1 mg/ml) were infused i.v. (0.5 ml/min) via the rat's tail vein 30 min after zolpidem or diazepam administration (5 mg/kg i.p). The latency to the onset of forelimb clonus was defined as the endpoint. The volume of chemoconvulsant infused to achieve that endpoint was used to calculate the seizure threshold dose based on the rat's body weight. All rats were euthanized with sodium pentobarbital (120 mg/kg i.p.) immediately after seizure threshold determination.

Drug Solutions

FZP dihydrochloride (pH 6.4) was from Research Biochemicals International (Natick, MA). FLM, a gift of Hoffmann LaRoche, was dissolved in 0.2% carboxymethylcellulose and 0.1% Tween 80. Zolpidem was kindly provided by Synthélabo Recherché (Bagneux, France). For in vivo injection, zolpidem was made in a solution of 40% propylene glycol, 10% EtOH, 1.5% benzyl alcohol, 5% Na benzoate, 2.25% benzoic acid in dH_2O. PTZ was made fresh daily in 0.9% saline. Bicuculline was made immediately before use in 0.001 N HCl.

Drugs used for superfusion during whole-cell recording were dissolved at 100 times their final concentration and added to the perfusate with a syringe pump (Razel; World Precision Instruments, Inc., Sarasota, FL) at a rate of 26 to 78 μl/min to achieve their final concentration. Zolpidem was dissolved in dH_2O. DL-2-Amino-5 phosphonovaleric acid and 6,7-dinitroquinoxaline-2,3-dione were dissolved in ≤0.001% dimethyl sulfoxide. Tetrodotoxin, dissolved in dH_2O, and lidocaine, *N*-ethyl bromide quaternary salt, dissolved in the micropipette internal solution, were from Alamone Labs (Jerusalem, Israel). Buffer chemicals were from Sigma Chemical Co. or Fisher Scientific. To estimate nonspecific binding in receptor autoradiographic studies, a stock solution of the unlabeled benzodiazepine antagonist was made by dissolving FLM in ethanol to a final concentration of 1 mM.

Results

Whole-Cell Recordings

mIPSC Amplitude and Decay. With a symmetric Cl^- concentration inside and outside the neuron and in the presence of the excitatory amino acid antagonists and tetrodotoxin, mIPSCs were observed in CA1 neurons ($V_h = -70$ mV) (Fig. 1A) as previously reported (Zeng and Tietz, 1999). Averaged mIPSCs recorded in neurons from rats 2 days after FZP was removed from the drinking water were compared with those from control rats (Fig. 1 and Table 1). mIPSCs were recorded in 100% of 22 control neurons. Averaged mIPSC amplitude in control neurons, from rats injected with the FLM vehicle 24 h before recording, ranged from −7.1 to −37.5 pA with a mean of −21.1 pA. In neurons from FZP-treated rats injected with vehicle, events were detected less frequently (Fig. 1A). Twenty-eight percent (7 of 25) of cells from FZP-treated rats, which had been injected with vehicle, had no detectable mIPSC events, i.e., were "silent", similar to the previous report (32%; Zeng and Tietz, 1999). In rats with detectable events, the mIPSC amplitude, which ranged from −5.0 to −18.3 pA, was significantly reduced by ~50% (−10.8 pA, $p < .01$) in comparison to mIPSCs in control neurons (Fig. 1B and Table 1). There were no differences ($p = .65$) in the decay constant (τ) of mIPSCs between treated and control groups that had been injected with vehicle 24 h before (Table 1).

Prior FLM injection had no effect on the amplitude (−21.7 pA) of mIPSC responses in control neurons (Table 1; control FLM, range −9.6 to −40.5 pA). FLM injection after chronic FZP treatment restored mIPSC amplitude to near-basal levels (−18.8 pA, $p = .45$, range −7.7 to −36.6 pA). In contrast to FZP-treated control cells, none of the CA1 neurons (0 of 15 cells) from treated rats injected with FLM cells were silent. As with vehicle-injected rats, mIPSC decay was also not different between FZP-treated and control groups receiving a prior FLM injection.

Zolpidem Effects on mIPSC Amplitude and Decay. The ability of 1 μM zolpidem to enhance mIPSC amplitude

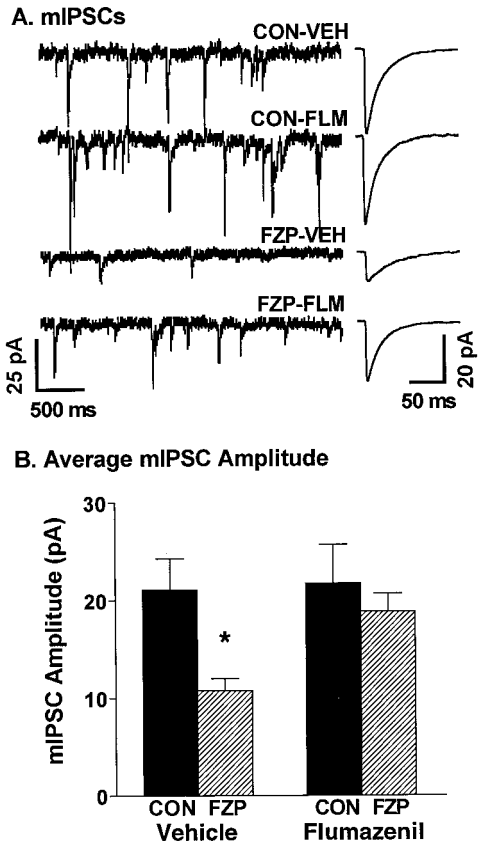

Fig. 1. Effects of FLM injection (4 mg/kg i.p.) on mIPSC amplitude in CA1 neurons recorded in in vitro rat hippocampal slices 2 days after ending FZP treatment. A, representative mIPSCs recorded in hippocampal CA1 neurons from control or FZP-treated rats injected with vehicle (VEH) or FLM 24 h before recording. The amplitude and frequency of mIPSCs recorded in FZP-VEH CA1 neurons was profoundly reduced, whereas that in FZP-FLM neurons was similar to that in control neurons. B, mIPSC amplitude, averaged from 5-min individual mIPSC recordings, was compared across control and FZP-treated groups. A decreased postsynaptic receptor-mediated response to GABA in FZP-treated CA1 neurons (n = 12) was reflected in a significant, 50% reduction in peak mIPSC amplitude in comparison to control neurons (n = 12). There was no significant difference in peak mIPSC amplitude between FZP-treated (n = 15) and control (n = 10) neurons from rats injected with FLM 24 h before recording as shown in Table 1.

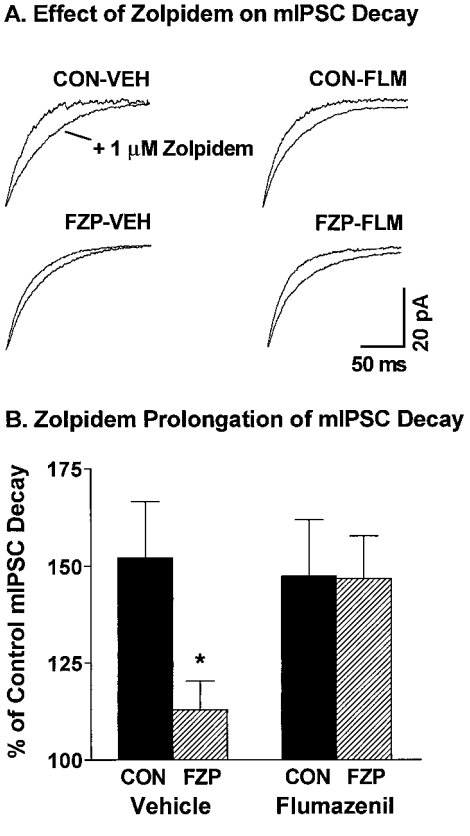

Fig. 2. Effects of FLM injection (4 mg/kg i.p.) on tolerance to zolpidem's ability to prolong mIPSC decay in CA1 neurons recorded in in vitro rat hippocampal slices 2 days after ending 1-week FZP treatment. A, representative traces of the decay phase of averaged mIPSCs, before and after superfusion of 1 μM zolpidem. CA1 neurons were recorded from control (CON) and FZP-treated rats injected with vehicle or FLM 24 h before recording. The peak amplitude of averaged mIPSCs from an FZP-treated neuron was normalized to the peak amplitude of control averaged mIPSCs for comparison. B, effects of 1 μM zolpidem to prolong mIPSC decay in CA1 neurons was expressed as a percentage of the baseline average mIPSC decay recorded in the absence of zolpidem. Zolpidem tolerance was measured as a significant reduction in the ability of zolpidem to enhance mIPSC decay in FZP-VEH neurons (n = 6) in comparison to CON-VEH neurons (n = 7). There was no significant difference in zolpidem's ability to prolong decay between neurons from FZP-treated (n = 6) and control (n = 7) rats injected with FLM 24 h before recording, as shown in Table 1.

and decay was evaluated in neurons from control and FZP-treated rats injected with vehicle or FLM 24 h before recording. Zolpidem (1 μM) increased the amplitude of mIPSCs in control and FZP-treated cells from rats injected with vehicle a similar amount (~120–130% of baseline; Table 1, p = .49). As expected from previous work, zolpidem increased the mIPSC decay constant (τ) by 52% in control neurons. In vitro tolerance was measured as a decrease in the ability of 1 μM zolpidem to enhance the mIPSC decay constant (Table 1 and Fig. 2A). That is, in FZP-treated rats previously injected with vehicle, zolpidem was significantly (p = .02) less effective, prolonging mIPSC decay by only 11%.

In control neurons, FLM injection tended to increase zolpidem's effect on mIPSC amplitude in comparison to neurons from vehicle-injected control rats, but this trend was not significant (Table 1, p = .11). In contrast to the zolpidem tolerance measured in neurons from FZP-treated rats after prior vehicle injection, there was no difference (p = .58) between zolpidem's ability to prolong decay in control (147% of baseline) and FZP-treated (159% of baseline) FLM-injected neurons; i.e., tolerance was no longer evident (Fig. 2B).

Time Course of Changes in [^{3}H]Zolpidem Binding

Binding of the α_1-selective ligand zolpidem was significantly reduced in the CA1 region (SO, −30%, p = .01; SR, −30%, p = .04; and SL, −33%; p < .03) of the hippocampus immediately (0 days) after oral FZP treatment was stopped (Fig. 3; Table 2). Down-regulation was also measured in CA3 SO (−34%, p = .01) immediately after treatment cessation. A similar, large reduction (−28.2%) in [^{3}H]zolpidem binding in the molecular layer of DG was not significant (p = .06). Although there was still a trend toward down-regulation in hippocampal subregions either 2 or 7 days after ending FZP treatment, there were no significant differences in [^{3}H]zolpidem binding between groups at these time points (Fig. 3; Table 2). Therefore, the effect of FLM injection on [^{3}H]zolpidem binding was not evaluated.

Basal specific zolpidem binding density was one- to three-

TABLE 1
Effect of FLM on CA1 pyramidal cell mIPSCs

Group	mIPSCs		Group	Zolpidem Potentiation	
	Amplitude	τ		Amplitude	τ
	pA	*ms*		%	
CON-VEH (n = 12/9)	−21.1 ± 3.2	23.4 ± 1.9	CON-VEH (n = 7/6)	121.1 ± 10.1	152.1 ± 14.5
CON-FLM (n = 10/6)	−21.7 ± 4.8	22.2 ± 1.1	CON-FLM (n = 7/5)	168.4 ± 28.3	147.4 ± 14.6
p value	.89	.59		.11	.81
FZP-VEH (n = 10/7)	−10.8 ± 1.9	27.6 ± 1.1	FZP-VEH (n = 6/5)	129.1 ± 7.5	111.3 ± 6.2
p value vs. CON-VEH	.01*	.11		.48	.02*
FZP-FLM (n = 15/9)	−18.8 ± 1.9	22.9 ± 1.8	FZP-FLM (n = 6/5)	117.5 ± 14.0	158.8 ± 15.7
p value vs. CON-FLM	.45	.65		.12	.57

Values are means ± S.E. n = cells/rats. FLM: i.p. injection of FLM; VEH: i.p. injection of FLM vehicle. Zolpidem potentiation (%) is expressed as a percentage of the baseline value in the absence of zolpidem. *, significant difference ($p \leq .05$) between 1-week FZP- and saccharin-treated control (CON) groups. p values are versus control of respective group.

fold greater in the frontal, parietal and occipital cortical layers (~120–410 fmol/mg of protein) than in the various hippocampal subregions (~15–120 fmol/mg of protein). The specific binding density of the molecular layer of the cerebellum was in a similar range to the cortex (~300 fmol/mg of protein) (Fig. 3A). Compared with controls, specific cortical cell layers also showed significant decreases (−30%) in [^{3}H]zolpidem binding 0 days after oral FZP administration was stopped (frontal VI, 204.4 ± 32.7 versus 294.6 ± 22.9 fmol/mg of protein, p = .02; parietal IV, 273.8 ± 32.7 versus 412.3 ± 31.6 fmol/mg of protein, p = .02; parietal V–VI, 174.1 ± 25.5 versus 260.3 ± 20.9 fmol/mg of protein, p = .02). A similar trend toward down-regulation in layers V–VI was seen in the occipital cortex (occipital V–VI, 174.1 ± 25.5 versus 260.3 ± 20.9 fmol/mg of protein, p = .07). [^{3}H]Zolpidem binding was also decreased by 33% in the molecular layer of the cerebellum of the FZP-treated group (195.5 ± 28.3 versus 293.0 ± 31.4 fmol/mg of protein, p = .03). These effects persisted in the frontal cortex, layer VI, 2 days after ending FZP treatment (134.6 ± 19.8 versus 188.9 ± 18.1 fmol/mg of protein, p = .02). A similar trend was seen in layer V (134.6 ± 19.8 versus 188.9 ± 18.1 fmol/mg of protein, p = .06). Seven days after ending treatment, a significant 30% reduction in [^{3}H]zolpidem binding was still detected in layer IV of the parietal (178.0 ± 22.5 versus 257.6 ± 30.6 fmol/mg of protein, p = .04) and occipital (129.1 ± 10.1 versus 179.5 ± 23.1 fmol/mg of protein, p = .05) cortices.

Quantitative Immunohistochemistry

α_1-Subunit Protein. With similar quantitative immunohistochemical techniques in previous studies, α_1- and β_3-subunit protein expression in the hippocampus and cortex were shown to be down-regulated immediately after 1-week oral FZP administration (Chen et al., 1999). Two days after ending chronic FZP treatment, down-regulation (−13.6%) of $GABA_A$ receptor α_1-subunit protein immunostaining persisted in the SO region of CA1 (Table 3; p = .04). There was also still a trend (p = .06) toward a decrease in the SL. This region had shown among the largest degrees of down-regulation (−35%) immediately after ending FZP administration (Chen et al., 1999). All other hippocampal areas still showed a trend toward α_1-subunit down-regulation 2 days after the end of treatment, but no area showed a significant difference (Table 3). Significant changes in α_1-subunit immunostaining were no longer present in any of the other cortical, collicular, or cerebellar areas measured 2 days after ending 1-week FZP treatment.

Acute exposure to FLM did not effect α_1-subunit protein levels in the hippocampus of control rats (Table 3). As noted above, significant down-regulation of the α_1-subunit protein was detected in several hippocampal subregions immediately after ending FZP treatment and persisted in the CA1 SO area 2 days after chronic treatment was stopped. In FZP-treated rats, an acute injection of FLM reversed these changes so that α_1-subunit protein levels in the CA1 SO (−8.4%) were no longer significantly different (p = .33) in comparison to the FZP-treated group injected with vehicle (Fig. 4 and Table 3).

β_3-Subunit Protein. Chronic FZP treatment modulated GABAR β_3 subunit protein levels in specific subregions of hippocampus (Fig. 5 and Table 3). In contrast to the down-regulation of the β_3-subunit protein immediately at the end of the 1-week treatment (Chen et al., 1999), β_3-subunit protein immunostaining density in hippocampal CA3 was significantly increased in the dendritic regions (SO, +10.3%, p = .02, and SR, +8.7%, p = .05) but not the cellular region (SP, +3.4%, p = .55) (Fig. 5 and Table 3).

As with the α_1 subunit protein, prior injection of control rats with FLM had no effect on β_3-immunostaining. In contrast, FLM injection restored the elevated β_3-subunit levels in the CA3 region of FZP-treated rats to basal levels (SO, +2.9%, p = .38; SP, +1.5%, p = .50; SR, +3.7%, p = .45) (Fig. 5 and Table 3).

Behavioral Measures of Zolpidem Effects

Chemoconvulsant Seizure Threshold. In vivo cross-tolerance to zolpidem was first assessed in a group of FZP and saccharin-treated rats (n = 4/group) 2 days after ending FZP treatment. These groups were given no prior FLM or vehicle injection before PTZ seizure-threshold testing. Zolpidem tolerance was measured by a 28% difference in the dose of PTZ required to induce forelimb clonus after zolpidem pretreatment (control, 51.1 ± 3.3 mg/kg i.v., n = 4; FZP treated, 36.8 ± 4.5 mg/kg i.v., n = 4; p = .01; Fig. 6). FZP-treated rats injected with vehicle 24 h before PTZ seizures were induced required a similar 26% lower dose of PTZ to

elicit clonus, indicative of zolpidem cross-tolerance (control, 56.9 ± 4.6 mg/kg i.v., $n = 15$; FZP treated, 41.9 ± 4.0 mg/kg i.v., $n = 15$; $p < .01$; Fig. 6). The same dose of FLM (4 mg/kg i.p.) that reversed the effects of prolonged FZP treatment, with measures of hippocampal GABA-mediated function (Figs. 1 and 2 and Table 1) and measures of the regulation of GABAR subunit protein (Figs. 4 and 5 and Table 3), appeared to have a slight effect to reverse in vivo zolpidem tolerance. However, tolerance was still evident; i.e., the PTZ dose required to elicit clonus was still significantly lower, by 20%, in FZP-treated rats (control, 42.5 ± 3.6 mg/kg i.v., $n = 15$; FZP treated, 34.0 ± 2.4 mg/kg i.v., $n = 15$; $p = .03$; Fig. 6). Reversal of in vivo zolpidem tolerance by a larger dose of FLM (20 mg/kg i.p.) was indicated by the absence of a significant difference in PTZ threshold dose after either zolpidem or vehicle pretreatment (control, 52.3 ± 3.3 mg/kg i.v., $n = 8$; FZP treated, 48.4 ± 4.3 mg/kg i.v., $n = 7$; $p = .21$; Fig. 6).

Rats treated with FZP for 3 days were not tolerant to the anticonvulsant effect of diazepam (5 mg/kg i.p.) to suppress PTZ-induced forelimb clonus (FZP treated, 90.6 ± 9.7 mg/kg i.v., $n = 5$; control, 87.8 ± 12.1 mg/kg i.v., $n = 6$; $p = .85$). Although the average daily FZP dose consumed in the 3-day treatment group was lower than in the 7-day treatment, it was expected that such treatment, if extended for 7 days, would result in a reduction in CA1 neuron mIPSC amplitude (Poisbeau et al., 1997). Because in vivo tolerance was not observed, additional studies of tolerance to zolpidem's anticonvulsant effect, or of FLM's ability to reverse tolerance, were not carried out in 3-day FZP-treated rats.

Although only a few rats ($n = 5$ or 6/group) were used to look for tolerance to zolpidem's antibicuculline seizure effect, there was a nonsignificant trend ($p = .08$) toward tolerance to zolpidem's ability to suppress bicuculline-induced seizures. A 17.6% lower dose of bicuculline was needed in FZP-treated rats to elicit clonus (control, 0.51 ± 0.06, $n = 5$; FZP treated, 0.42 ± 0.06 mg/kg i.v.). Prior FLM treatment with the lower dose of FLM did not alter this trend (control, 0.50 ± 0.04 mg/kg i.v.; FZP treated, 0.43 ± 0.02 mg/kg i.v.; $p = .08$)

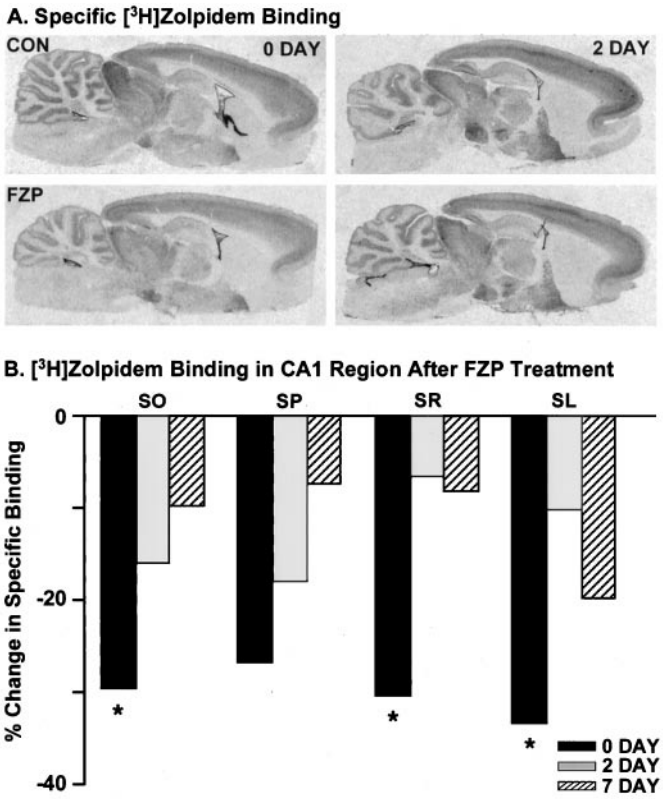

Fig. 3. Time course of [^{3}H]zolpidem binding in hippocampal CA1 subregions, 0, 2, and 7 days after ending FZP treatment. A, specific 5 nM [^{3}H]zolpidem binding in representative 10-μm brain sections from control and FZP-treated rats sacrificed immediately (0 days) or 2 days after ending FZP treatment. B, rats sacrificed 0 days (black column) after ending treatment showed a significant reduction in [^{3}H]zolpidem binding in CA1 dendritic subregions (SO, SR, SL). There was a trend toward reduced binding 2 days (gray column) after ending treatment, and values approached baseline after 7 days (hatched column). Because [^{3}H]zolpidem binding was not significantly reduced 2 days after ending treatment, FLM reversal was not investigated. Measures are shown as percent change from the mean control specific-binding density. *$p \leq .05$, significant differences between specific binding values (mean ± S.E.) in control and FZP-treated rat brains, as shown in Table 2.

Discussion

The goal of this study was to establish whether FLM could reverse the functional and structural characteristics of FZP-induced tolerance in the hippocampus and anticonvulsant tolerance in the whole animal. FLM reversal was previously reported in other in vivo (Gonsalves and Gallager, 1985, 1987) and in vitro (Klein et al., 1995; Primus and Gallager, 1996) models. Whereas the latter studies in recombinant systems are invaluable for their ability to isolate important experimental variables, key in vivo elements critical for understanding tolerance mechanisms, i.e., neural connectivity and endogenous neurotransmitter activity, may be absent. In our study, it was hypothesized that tolerance, evident in vivo (PTZ seizure threshold) and in vitro (zolpidem prolongation of mIPSC decay) and the reduced sensitivity of CA1 pyramidal cells to tonically released GABA (mIPSC amplitude) would be reversed by a single FLM injection. Because structural changes in hippocampal GABARs (Tietz et al., 1994, 1999a; Chen et al., 1999) may be related to these functional alterations (Xie and Tietz, 1992; Zeng and Tietz, 1997, 1999), the effect of FLM to regulate GABAR subunit protein levels was also evaluated. Without exception, FLM (4 or 20 mg/kg i.p.) was able to restore each correlate of chronic FZP administration to near-basal values.

As shown previously (Poisbeau et al., 1997; Zeng and Tietz, 1999), 1-week of FZP administration significantly reduced (~50%) GABA-mediated mIPSC amplitude (Table 1 and Fig. 1). Furthermore, the percentage (~30%) of silent CA1 neurons, without measurable mIPSCs, was also similar to previous reports. However, chronic benzodiazepine treatment did not alter GABAR-mediated responses in other hippocampal principal cell types (Poncer et al., 1996; Poisbeau et al., 1997). For example, dentate granule cell mIPSC amplitude was unaltered after a similar 1-week oral FZP treatment (Poisbeau et al., 1997). These findings suggest a unique susceptibility of CA1 pyramidal cell GABARs during chronic benzodiazepine exposure. In this study, 4 mg/kg of the antagonist restored mIPSC to 87% of control, and silent neurons were no longer detected. Similarly, Gonsalves and Gallager (1985, 1987) reported that FLM could reverse GABA subsensitivity after chronic diazepam treatment in dorsal raphé. Note that, in these studies, FLM was administered at a time when residual benzodiazepine is no longer present, and manifestations of tolerance were measured at a time

TABLE 2
Time course of specific [^{3}H]zolpidem binding in hippocampus 0, 2, and 7 days after 1-week flurazepam treatment

Group	CA1				CA3		DG		
	SO	SP	SR	SL	SO	SP	MOL	GRN	PC
	fmol/mg protein								
0 Day									
CON	97.0 ± 6.2	59.4 ± 4.9	80.3 ± 6.0	117.3 ± 8.4	43.1 ± 2.7	16.5 ± 3.3	72.8 ± 5.5	31.6 ± 3.8	19.1 ± 4.3
FZP	68.3 ± 8.7	43.6 ± 7.8	55.9 ± 9.8	78.2 ± 14.5	28.6 ± 4.5	13.8 ± 3.2	52.3 ± 9.2	21.2 ± 5.7	18.1 ± 4.9
p value	.01*	.09	.04*	.03*	.01*	.54	.06	.12	.86
2 Day									
CON	63.8 ± 6.6	38.5 ± 4.5	50.6 ± 5.4	78.6 ± 9.3	37.5 ± 5.2	17.0 ± 2.5	48.5 ± 7.0	24.3 ± 3.2	19.1 ± 3.5
FZP	53.6 ± 4.5	31.6 ± 1.9	47.3 ± 5.4	70.5 ± 7.7	29.3 ± 3.3	13.4 ± 2.7	40.4 ± 3.0	18.2 ± 2.3	13.2 ± 1.2
p value	.04*	.20	.15	.06	.18	.25	.28	.13	.12
7 Day									
CON	60.3 ± 7.2	40.5 ± 5.6	55.3 ± 7.1	85.0 ± 13.5	37.1 ± 4.8	19.4 ± 3.9	59.6 ± 6.1	26.3 ± 3.8	18.1 ± 3.2
FZP	54.4 ± 5.8	37.6 ± 5.7	50.8 ± 5.4	68.2 ± 7.3	29.9 ± 3.1	19.2 ± 2.5	52.5 ± 6.4	28.0 ± 4.8	19.4 ± 4.1
p value	.51	.70	.60	.26	.20	.96	.41	.76	.78

Values are mean ± S.E. specific binding.
*, significant difference ($p \leq .05$) between 0.02% saccharin-treated control (CON) and FZP-treated groups; n = eight sections/group.

TABLE 3
Effect of FLM on α_1- and β_3-subunit antibody immunostaining in hippocampus after 1-week FZP treatment

Group	CA1				CA3			DG		
	SO	SP	SR	SL	SO	SP	SR	MOL	GRN	PC
	relative gray value									
α1-Subunit										
CON-VEH	31.7 ± 2.0	27.7 ± 2.1	25.7 ± 1.9	36.1 ± 2.9	22.6 ± 2.2	15.6 ± 2.4	23.0 ± 2.0	25.8 ± 1.5	19.6 ± 1.7	14.0 ± 1.2
FZP-VEH	27.4 ± 1.1	24.4 ± 1.0	23.2 ± 0.8	33.2 ± 1.1	21.2 ± 1.3	14.7 ± 1.1	21.2 ± 1.2	25.3 ± 1.1	17.7 ± 1.1	14.0 ± 1.3
p value	.04*	.12	.15	.23	.55	.69	.39	.76	.31	.98
CON-FLM	32.3 ± 1.8	27.9 ± 1.7	26.6 ± 1.4	37.9 ± 1.3	24.4 ± 1.1	16.9 ± 0.9	24.4 ± 1.2	28.3 ± 1.6	20.0 ± 1.6	16.0 ± 1.0
FZP-FLM	29.6 ± 1.9	26.9 ± 1.7	25.1 ± 2.0	36.2 ± 2.2	24.0 ± 1.6	16.8 ± 1.3	22.9 ± 1.6	27.9 ± 1.5	19.2 ± 1.3	15.0 ± 1.2
p value	.33	.71	.54	.49	.85	.92	.44	.87	.71	.49
	CA1				CA3			DG		
	SO	SP	SR	SL	SO	SP	SR	MOL	GRN	PC
	relative gray value									
β3-Subunit										
CON-VEH	69.5 ± 1.9	59.8 ± 5.2	64.5 ± 1.2	60.6 ± 2.2	65.5 ± 2.1	51.3 ± 2.8	66.5 ± 2.2	64.4 ± 2.1	49.0 ± 2.1	56.5 ± 1.6
FZP-VEH	72.4 ± 0.9	58.3 ± 2.0	67.0 ± 1.3	63.2 ± 1.9	72.2 ± 1.8	53.1 ± 1.7	72.3 ± 1.9	67.9 ± 1.9	49.7 ± 1.7	60.2 ± 1.7
p value	.76	.54	.24	.36	.02*	.55	.05*	.21	.79	.12
CON-FLM	70.1 ± 2.0	60.0 ± 2.2	66.2 ± 1.9	61.9 ± 1.9	72.6 ± 2.7	51.8 ± 2.1	70.6 ± 1.6	64.9 ± 5.2	50.8 ± 2.0	56.7 ± 2.1
FZP-FLM	71.5 ± 1.3	59.2 ± 1.8	66.4 ± 1.4	60.8 ± 2.0	70.2 ± 3.4	52.1 ± 2.2	69.7 ± 2.6	64.8 ± 2.2	50.1 ± 8.2	58.7 ± 2.0
p value	.82	.76	.92	.65	.57	.90	.76	.96	.82	.47

Values are mean ± S.E. relative gray level.
FLM: i.p. injection of flumazenil; VEH: i.p. injection of flumazenil vehicle α1. CON-VEH, n = 5; FZP-VEH, n = 7; CON-FLM, n = 6; FZP-FLM, n = 6; β_3, CON-VEH, n = 6; FZP-VEH, n = 8; CON-FLM, n = 7; FZP-FLM, n = 8.
*, significant difference ($p \leq .05$) between 1-week FZP and saccharin-treated control (CON) groups.

when FLM was also eliminated from brain (Lister et al., 1984; Gonsalves and Gallager, 1985; Xie and Tietz, 1992). Although the molecular mechanism for antagonist reversal of tolerance remains obscure, the findings of this study support the idea that, rather than directly antagonizing agonist actions, acute antagonist exposure "resets" the GABAR to its naive, pretreatment state.

Significant cross-tolerance was noted in zolpidem's ability to prolong mIPSC decay (Table 1 and Fig. 2; Zeng and Tietz, 1999), consistent with the decreased potency of zolpidem to potentiate GABA currents in dissociated CA1 pyramidal cells of chronic diazepam-treated rats (Itier et al., 1996). Similar to its actions to reverse GABA subsensitivity, FLM also reversed in vitro zolpidem cross-tolerance (Table 1 and Fig. 2). There was some apparent tolerance to zolpidem's effect on mIPSC amplitude (Table 1 and Fig. 2), which, unlike the effect on mIPSC decay, was not significant.

Findings of benzodiazepine-receptor regulation after various chronic treatments are inconsistent. Down-regulation of [^{3}H]FZP and [^{3}H]zolpidem binding sites was noted in hippocampus and cortex after some (Tietz et al., 1986; Miller et al., 1988; Wu et al., 1994) but not after other (Gallager et al., 1984; Wu et al., 1994; cf. Rabow et al., 1995; Impagnatiello et al., 1996) chronic treatments. Zolpidem binds to three hippocampal receptor populations (K_Ds = 15 nM, 225 nM, and 6 μM), likely corresponding to BZI (α_1-subunit-containing), BZIIA (α_2- or α_3-subunit-containing) and BZIIB (α_5-subunit-containing) receptors (Sieghart, 1995). The first two sites, more prominent in CA1 than in CA3 or DG, showed a high degree of GABA-mediated allosteric coupling (Ruano et al., 1992, 1993). This may be one factor rendering CA1 neurons particularly susceptible to regulation during chronic benzodiazepine treatment. In hippocampus, [^{3}H]zolpidem binding was significantly reduced at the end of FZP treatment and, although there was still a trend toward continued down-regulation (Table 2 and Fig. 3), it was not significant. How-

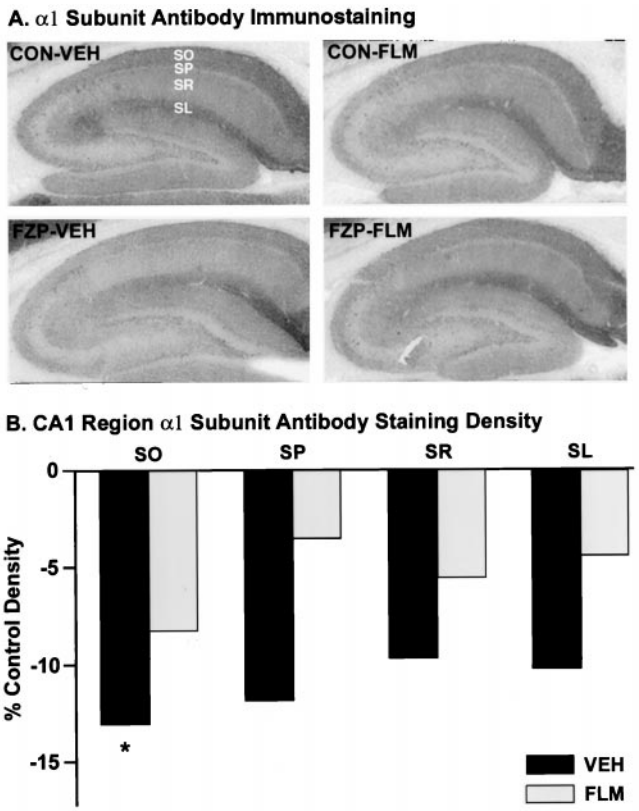

Fig. 4. Effect of FLM on FZP-induced modulation of $GABA_A$ receptor α_1-subunit protein in discrete hippocampal CA1 subregions, 2 days after ending FZP treatment. A, representative 20-μm brain sections from control and FZP-treated rats injected with vehicle or FLM 24 h before perfusion. B, rats injected with vehicle (black column) 24 h before perfusion showed a reduction in α_1 subunit protein immunostaining density in CA1 subregions, significant in the SO region, 2 days after ending chronic FZP treatment. There was no change in α_1 subunit antibody immunostaining density in rats that had received FLM injection (gray column) 24 h before perfusion. Measures are shown as percent change from mean control densities. $^*p \leq .05$, significant differences between relative gray values in control and FZP-treated brains (mean ± S.E.), as shown in Table 3.

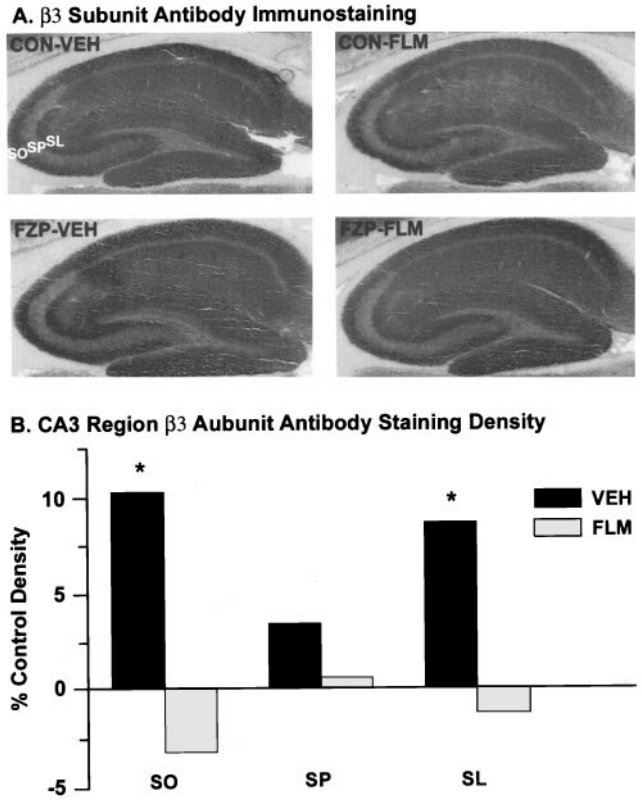

Fig. 5. Effect of FLM on FZP-induced modulation of $GABA_A$ receptor β_3-subunit protein in discrete hippocampal CA3 subregions, 2 days after ending FZP treatment. A, representative 20-μm brain sections from control and FZP-treated rats injected with vehicle or FLM 24 h before perfusion. B, rats injected with vehicle (black column) 24 h before perfusion showed a significant increase in β_3 subunit antibody immunostaining density in CA3 subregions (SO and SR) 2 days after ending chronic FZP treatment. There was no change in β_3 subunit antibody immunostaining density in rats that had received FLM injection (gray column) 24 h before perfusion. Measures are shown as percent change from mean control densities. $^*p \leq .05$, significant differences between relative gray values in control and FZP-treated brains (mean ± S.E.), as shown in Table 3.

ever, the pattern appeared to differ from that of [^{3}H]FZP binding (Chen et al., 1995), which had recovered to control levels by 2 days after ending treatment and showed a trend toward up-regulation by day 7. Because there was no longer a significant decrease in [^{3}H]zolpidem binding 2 days after ending FZP treatment, studies of FLM reversal were not performed.

Several laboratories have proposed that a switch in GABAR subunit composition may underlie benzodiazepine tolerance (Heninger et al., 1990; Tietz et al., 1994, 1999a; Holt et al., 1996; Impagnatiello et al., 1996; Pesold et al., 1997; Chen et al., 1999). After 1 week of chronic FZP treatment, localized changes in the expression of α_1- and β_3-GABAR subunit mRNAs in specific regions of the hippocampus and cortex (Tietz et al., 1994, 1999a) were mirrored by changes in their respective proteins (Chen et al., 1999). A decrease in α_1-mRNA, paralleled by decreased α_1-subunit immunogold labeling, was also found in the cortex and hippocampus of chronic diazepam-treated rats (Impagnatiello et al., 1996). Decreased α_1-subunit levels were accompanied by increased α_5-subunit mRNA levels in the frontoparietal cortex, perhaps accounting for the lack of change in [^{3}H]FLM binding in that study. Interestingly, the α_5-subunit mRNA showed a trend toward up-regulation 7 days after ending 1-week FZP treatment (Tietz et al., 1999a). Moreover, rats given protracted treatment with the novel benzodiazepine imidazenil did not exhibit anticonvulsant tolerance or GABAR mRNA or protein changes. In another study, chronic diazepam, but not chronic abercarnil, which did not induce tolerance, resulted in GABAR subunit mRNA changes (Holt et al., 1996). In our study, in vitro cross-tolerance to zolpidem (Table 1 and Fig. 2) might be explained by the localized regulation of the α_1-protein in the hippocampal CA1 region (Chen et al., 1999), because FLM injection was capable of reversing both in vitro tolerance and α_1-subunit protein expression (Table 3 and Fig. 4). The role for the β_3-subunit in mediating tolerance may be more likely related to its ability to signal GABAR sequestration (Barnes, 1996) or to target receptors to the membrane (Connolly et al., 1996). Nonetheless, the FLM-mediated reversal of β_3 subunit up-regulation in the CA3 region (Table 3 and Fig. 5) in conjunction with the reversal of α_1-subunit down-regulation in the CA1 region strengthens the idea that regulation of both of these subunits is important for modifying GABAR function in the tolerant hippocampus.

Receptor down-regulation and changes in subunit gene

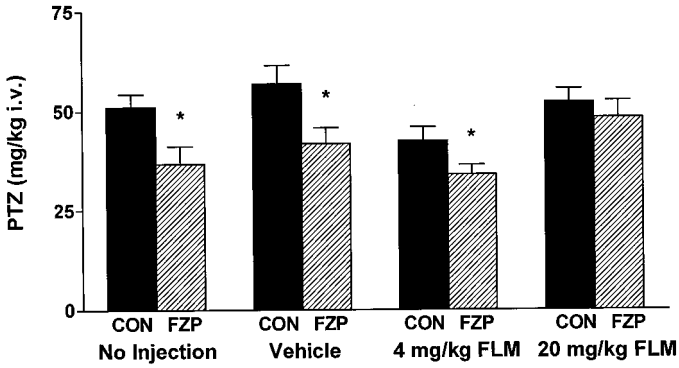

Fig. 6. FLM reversal of in vivo tolerance to zolpidem's ability to increase the threshold dose of PTZ to induce forelimb clonus 2 days after ending 1-week FZP treatment. Control and 1-week FZP-treated rats were injected with zolpidem (5 mg/kg i.p.) 30 min before an infusion of PTZ (20 mg/kg i.v.) via the tail vein. Zolpidem raises PTZ seizure threshold in control rats, which received 0.02% saccharin. Tolerance, indicated by a reduced ability of zolpidem to raise seizure threshold, was evident in FZP-treated rats that received no prior injection (n = 4/group) and in rats that received an i.p. injection of the FLM vehicle (n = 13/group) or FLM (4 mg/kg, n = 9/group) 24 h before PTZ testing. Tolerance was no longer present in groups of rats that received a single, five-fold greater dose of FLM (20 mg/kg i.p., n = 4/group) the day before testing.

expression are not always associated with long-term benzodiazepine-agonist exposure. On the other hand, allosteric uncoupling of GABA and benzodiazepine binding sites is consistently associated with GABA and benzodiazepine-agonist receptor occupation (cf. Rabow et al., 1995). Allosteric uncoupling in a baculovirus recombinant expression system was dependent on ligand efficacy, was subunit specific (e.g., zolpidem exposure decreased coupling in $\alpha_1\beta_2\gamma_2$- but not $\alpha_5\beta_2\gamma_2$-recombinant receptors), and was reversed by a brief exposure to FLM (Primus et al., 1996). In another study (Klein et al., 1995), mouse PA3 cells stably transfected with $\alpha_1\beta_1\gamma_{2L}$-receptors showed attenuated coupling after FZP exposure without any change in subunit mRNA or protein expression. The rapid time course of antagonist reversal (0.5 h) in these systems provided additional support for the role of a conformational change or posttranslational mechanism, e.g., receptor phosphorylation/dephosphorylation (Smart, 1997), in mediating the functional effects of chronic drug exposure. Interestingly, Poisbeau et al. (1999) provided evidence to suggest that mIPSCs recorded in CA1 pyramidal cells and in dentate granule cells were differentially affected by protein kinase A and protein kinase C, which may provide one basis for the differential susceptibility of GABARs on these two hippocampal cell types to chronic benzodiazepine treatment.

In addition to its specific actions in the hippocampus, FLM reversed anticonvulsant cross-tolerance to zolpidem in vivo. Nonetheless, a larger dose of FLM was required. One possible reason for the lower potency of FLM to reverse in vivo tolerance is the contribution of multiple GABAergic mechanisms. For example, a presynaptic mechanism, i.e., decreased GABAergic interneuron activity, was also demonstrated in the benzodiazepine-tolerant hippocampus (Zeng and Tietz, 1999). Another possibility is that non-GABA-mediated compensatory mechanisms were invoked by prolonged FZP treatment. It is also likely that other brain regions are important for tolerance to zoplidem's anti-PTZ effects. For example, the cerebral cortex, particularly the frontoparietal and parietooccipital regions, consistently show decreased subunit mRNA and protein expression (Heninger et al., 1990; Kang and Miller, 1991; Holt et al., 1996; Impagnatiello et al., 1996; Pesold et al., 1997; Chen et al., 1999). In this regard, the functional anatomy of the circuit(s) responsible for PTZ seizure expression may involve brain areas with different complements of GABAR subtypes requiring a larger dose of FLM to reset their receptors.

In summary, FLM was able to reverse each measure of benzodiazepine tolerance associated with chronic FZP administration, including in vivo and in vitro tolerance. The ability of the antagonist to normalize GABAR subunit protein expression suggested that a change in GABAR subunit composition may play a role in mediating tolerance. Moreover, findings in various systems suggest that the effects of chronic benzodiazepine treatment on transcriptional, translational, and posttranslational mechanisms are interdependent and may each contribute to the establishment and maintenance of tolerance. It will be important to determine the sequence and nature of the events from ligand binding to changes in GABAR physiology and pharmacology that contribute to the phenomenon of tolerance.

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Send reprint requests to: Elizabeth I. Tietz, Ph.D., Department of Pharmacology, Medical College of Ohio, Block Health Science Building, 3035 Arlington Ave., Toledo, OH 43614-5804. E-mail: etietz@mco.edu