Benzodiazepine Tolerance at GABAergic Synapses on Hippocampal CA1 Pyramidal Cells

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KEY WORDS flurazepam; dependence; interneuron; release; hippocampus; IPSCs

ABSTRACT Modulation of GABA function following 1 week oral administration of flurazepam (FZP) was investigated in chloride-loaded, rat hippocampal CA1 pyramidal neurons. Rats were sacrificed 2 or 7 days after ending drug treatment, when anticonvulsant tolerance was present or absent in vivo, respectively. Spontaneous (s)IPSCs and miniature (m)IPSCs were recorded using whole-cell voltage-clamp techniques. s/mIPSCs were bicuculline-sensitive, voltage-dependent, and reversed their polarity at 0 mV, the predicted ECl−. Comparisons of s/mIPSCs between FZP-treated and control groups were made at Vh = -90, -70, and -50 mV. The frequency of sIPSCs, but not mIPSCs, was significantly decreased in FZP-treated neurons 2 days, but not 7 days, after FZP treatment, suggesting a decrease in interneuron activity. These conclusions were supported by the negative findings of additional studies of [3H]GABA release from hippocampal slices and [3H]GABA uptake from hippocampal synaptosomes. The lack of change in the paired-pulse depression of GABAA-mediated IPSPs suggested that autoreceptor function was also not impaired following chronic FZP treatment. A large reduction in both sIPSC and mIPSC amplitude (60%) in FZP-treated neurons, the absence of mIPSCs in one-third of FZP-treated cells, and a measurable reduction in synaptic and unitary conductance confirmed that postsynaptic GABAA receptor function was profoundly impaired in FZP-treated CA1 neurons. Zolpidem, an α1-selective benzodiazepine receptor ligand, enhanced mIPSC amplitude and decay, but its ability to prolong mIPSC decay was reduced in FZP-treated neurons. Several pre- and postsynaptic changes at GABAergic synapses on CA1 pyramidal cells might be related to the decreased tonic GABA inhibition in FZP-treated CA1 neurons associated with the expression of benzodiazepine anticonvulsant tolerance. Synapse 31:263–277, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

Benzodiazepines exert their therapeutically useful anticonvulsant actions by potentiating inhibitory neurotransmission (Eghbali et al., 1997; Rogers et al., 1994; Study and Barker, 1981) at the GABAA receptor, a pentameric protein constituting a chloride (Cl−) channel formed from a combination of subunits with multiple variants (α1–6, β1–4, γ1–3, δ1 or ε1) (Davies et al., 1997; Delorey and Olsen, 1992; Macdonald and Olsen, 1994). Repeated benzodiazepine administration results in anticonvulsant tolerance (for review see Hutchinson et al., 1996). Findings of in vivo electrophysiological studies and [36Cl−] flux studies in tolerant animals showed reductions in GABA and benzodiazepine agonist actions. Studies measuring the affinity and number of GABA and benzodiazepine binding sites were inconclusive despite consistent reductions in allosteric coupling between the two binding sites (for reviews see Barnes, 1996; Gallager et al., 1991; Hutchinson et al., 1996). The modulation of expression of several GABAA receptor subunit mRNAs (Heninger et al., 1990; Holt et al., 1996; Impagnatiello et al., 1996; Kang and Miller, 1991; Tietz et al., 1994; Zhao et al., 1994) implied a change in the subunit composition of GABAA receptors. Notwithstanding such findings, the definitive changes that occur at GABAergic synapses during sustained allosteric activation of GABAA receptors and their functional consequences remain unknown.

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A series of electrophysiological studies in the CA1 region of in vitro hippocampal slices derived from FZP tolerant rats led us to hypothesize that presynaptic, as well as postsynaptic, mechanisms may contribute to reduced GABAergic inhibition (Xie and Tietz, 1991, 1992; Zeng and Tietz, 1994, 1997; Zeng et al., 1995). A major focus of the present experiments was to further investigate GABAergic inhibitory function in CA1 pyramidal cells in hippocampal slices derived from rats sacrificed at two time points after one week oral flurazepam administration when tolerance in vivo (Rosenberg et al., 1985) and in vitro (Xie and Tietz, 1992) is near maximal or absent, respectively. The possibility that interneuron excitability was altered was supported by the negative findings of additional biochemical studies of GABA uptake and action-potential independent Cl⁻-evoked GABA release and by studies of presynaptic, GABA<sub>A</sub> receptor-mediated, paired-pulse depression. To further evaluate postsynaptic changes at the GABA<sub>A</sub> receptor suggested by the reductions in action potential-dependent sIPSCs and action potential-independent mIPSCs and in evoked IPSC amplitude (De Koninck and Mody, 1992; Nusser et al., 1997), unitary Cl⁻-channel conductance was estimated from non-stationary fluctuation analyses of mIPSCs. In addition, since in situ hybridization and immunocytochemical studies had suggested that the α1 subunit mRNA and protein were selectively reduced in CA1 pyramidal cells (Chen et al., 1996; Huang et al., 1995; Tietz et al., 1994), in vitro tolerance was investigated in concentration-response studies of the α1-selective benzodiazepine receptor ligand, zolpidem, on mIPSC kinetics.

MATERIALS AND METHODS
Oral flurazepam administration

Male Sprague-Dawley rats (initial weight 185–225 g) were offered FZP in 0.02% saccharin solution as their only source of drinking water. The concentration of FZP was adjusted daily according to each rat's body weight and fluid consumption (100 mg/kg X 3 days and 150 mg/kg X 4 days). Only rats that consumed a criterion dose of an average ≥ 100 mg/kg/day were accepted for study. Saccharin water was offered 2 days prior to initiating drug treatment and for 2 or 7 days after ending treatment. Pair-handled control rats received saccharin water for the same length of time. The experimenter was not informed the rats' treatment histories until after the data analysis was completed.

Rats receiving 1 week oral FZP treatment showed a loss in the ability of benzodiazepines to protect against pentylentetrazol-induced seizures up to 4, but not 7, days after ending drug treatment (Rosenberg et al., 1985; Rosenberg, 1995). Therefore, FZP-treated and paired-handled control rats were sacrificed 2 or 7 days after 1 week FZP-treatment when benzodiazepine functional tolerance is near-maximal or absent, respectively. Moreover, 1 week FZP-treated rats are tolerant but not dependent (Tietz and Rosenberg, 1988). The initial brain levels of benzodiazepine metabolites are equivalent to or less than those using other treatment protocols (Gallager et al., 1991; Hutchinson et al., 1996). In addition, FZP and its active metabolites were not detected in the hippocampus 2 days after 1 week FZP administration (Xie and Tietz, 1991). Thus, electrophysiological measurements were not affected by residual benzodiazepine metabolites.

Hippocampal slice preparation

Experiments were carried out in 500 μm hippocampal slices from FZP-treated and control rats as previously described (Zeng et al., 1995; Zeng and Tietz, 1997). Briefly, rats were decapitated and transverse dorsal hippocampal slices were prepared on a vibratome (Ted Pella, Inc., Redding, CA) in ice-cold pre-gassed (95%O<sub>2</sub>/5%CO<sub>2</sub>) ACSF containing (in mM) NaCl 120, KCl 5.0, MgSO<sub>4</sub> 1.3, NaH<sub>2</sub>PO<sub>4</sub> 1.2 CaCl<sub>2</sub> 2.4, NaHCO<sub>3</sub> 26, D-glucose 10; 288 mOsm, pH 7.3. Slices were stored at room temperature for ≈ 1 hr in gassed ACSF. During recording individual slices were continuously perfused at a constant rate of 1.5 ml/minute with pre-gassed ACSF at room temperature.

Electrophysiological studies
Whole-cell recordings

Tight-seal, whole-cell voltage-clamp recordings were made from CA1 pyramidal neurons. Patch pipettes were pulled from borosilicate capillaries (nonfilamented, 1.5 mm O.D., Sutter Instruments Co., Novato, CA) on a Flaming-Brown electrode puller (P-97, Sutter Instruments Co.) using a two-stage pull to a tip diameter of about 1 µm (4–7 MΩ). To isolate GABA<sub>A</sub>-mediated IPSCs, pipettes were filled with a filtered, Cs<sup>+</sup> containing internal solution containing (in mM): CsCl 130, EGTA 1.0, CaCl<sub>2</sub> 0.5, MgCl<sub>2</sub> 2.0, ATP 2.0, HEPES 10.0, pH adjusted to 7.2. Cells were Cl⁻-loaded to minimize the possible contribution of intracellular Cl⁻ accumulation to the reduction of GABA-mediated inhibition suggested by a shift in the E<sub>IPSP</sub> in previous experiments in FZP-treated neurons (Zeng and Tietz, 1997). Chloride-loading was demonstrated to reverse the use-dependent shift in E<sub>Cl</sub> due to prolonged GABA activation (Ling and Bernardo, 1995). QX-314 (2 mM), an intracellular Na<sup>+</sup> channel blocker, was also included to block the spontaneous firing of CA1 pyramidal neurons (Lupica, 1995; Zeng and Tietz, 1997).

s/mIPSC recordings. CA1 pyramidal cells, patched using ‘blind’ techniques, were voltage-clamped in continuous mode (cSEVC) using an Axoclamp 2A amplifier (Axon Instruments Inc., Foster City, CA). The current output was low-pass filtered at 10 KHz and amplified 100X. The amplified DC current was offset and further amplified 100X (FL4, 4 pole Bessel Filter, Dagan Corp., Minneapolis, MN). The signal was monitored continuously on-line using PClamp 6.0 Software interfaced
with a Digidata 1200 A/D converter (Axon Instruments Inc.) and stored on VCR tape with a VR100 Digital Recorder (Instrutech Corp., Mineola, NY) for off-line analysis. If series resistance varied by > 80%, the recording was abandoned. In most cells, the amplitude of s/mIPSCs was constant for > 2 hours. To ensure that the relatively smaller events in FZP-treated neurons were reliably detected and to avoid voltage-clamp errors, sIPSCs and mIPSCs were recorded in each cell at three different holding potentials (V_h = -50, -70, and -90 mV). At these negative membrane potentials, inward s/mIPSCs were detected in the presence of the glutamate antagonists APV (50 µM) and DNQX (10 µM). sIPSCs were recorded in one group of control (n = 9) and FZP-treated (n = 10) neurons. In another group of cells, mIPSCs were recorded in the presence of 1 µM TTX, after a 5-minute stable sIPSC recording was obtained.

s/mIPSCs analysis. For signal detection, the data on VCR tape was played back, amplified 10 X and low-pass filtered at 10 kHz. Off-line analysis was performed on an IBM compatible computer using Strathclyde CDR and SCAN software (J. Dempster, University of Strathclyde, Glasgow, Scotland). Non-stationary fluctuation analysis was carried out using NSAN software (Y. De Koninck, McGill University, Montreal, Canada). Spontaneous and miniature synaptic currents were analyzed from 2-minute segments of data acquired from each neuron under each recording condition. Detection threshold for individual sIPSC and mIPSC events was set just above the maximum recording system background noise. Data from individual neurons was omitted if the background noise exceeded 3 pA. Events with a duration shorter than 3 ms were also excluded (Lupica, 1995). For statistical comparisons of s/mIPSC amplitude and frequency, relative cumulative frequency distribution for the population of event amplitudes and inter-event intervals was compared between experimental and control groups using the Kolmogorov-Smirnov (K-S) test (Lupica, 1995; Otis and Mody, 1992). Curve fitting to estimate mIPSC decay kinetics was using a single exponential function [y(t) = a * exp (-t/t_1)] or a bi-exponential function [y(t) = a1 * exp (-t/t_1) + a2 * exp (-t/t_2)] (J. Dempster, University of Strathclyde, Glasgow, Scotland). Non-stationary fluctuation analysis was carried out on mIPSCs as described by De Koninck and Mody (1992). Unitary current (i) and channel number (N) were estimated from the variance analysis using the equation \sigma^2 = I_{M} - I_{M}/N where \sigma = unitary current; I_{M} = mean current and N = channel number. Unitary conductance (\gamma) was derived from the unitary current estimate.

Zolpidem effects on mIPSCs. Since \alpha1 mRNA (Huang et al., 1994; Tietz et al., 1994) and protein (Chen et al., 1996) expression were selectively reduced in the CA1 region following 1 week FZP treatment, the ability of the \alpha1-selective imidazopyridine, zolpidem (30 nM–100 µM), to enhance CA1 pyramidal cell mIPSCs was compared in control and FZP-treated cells. After 3 minutes baseline mIPSC activity was obtained zolpidem was added to the perfusate in increasing concentrations (4–10 concentrations/cell) for 8 minutes each. The final 3-minute segment of data under each concentration was used for off-line analysis of mIPSCs amplitude and decay kinetics and for non-stationary fluctuation analysis. The degree of zolpidem potentiation at each concentration was expressed as a fraction of the control response.

Evoked IPSCs. In other groups of rats sacrificed 2 days after FZP treatment was stopped, synaptic conductance was estimated from the current-voltage response to maximal stimulation of GABAergic interneurons. Voltage-clamp recordings (-70 to +70 mV) were made with micropipettes (6–10 MΩ) filled with the same internal solution as described above. IPSCs were evoked, monosynthetically, in the presence 50 µM APV and 10 µM DNQX, with 0.1 ms constant-current pulses from a Master-8 stimulator (A.M.P.I., Jerusalem, Israel) coupled to an Iso-Flex constant current stimulus isolation unit (A.M.P.I.). Current was delivered through a concentric (75 mm O.D.), bipolar tungsten stimulating electrode placed at the str. oriens/str. pyramidale (SP) border ± 0.5 from recording electrode. The mean synaptic conductance (\gamma), derived from the slope of the current-voltage relationship in each cell, was compared between FZP-treated and control groups by Student’s t-test.

Intracellular recordings

GABA_A autoreceptor function. GABA_A autoreceptor function was measured by the degree of paired-pulse depression of CA1 pyramidal cell responses in slices from rats sacrificed 2 days after ending FZP treatment. Since postsynaptic GABA_A receptor function was shown to be profoundly impaired in FZP-treated neurons, GABA_A-mediated IPSPs, rather than GABA_A-mediated IPSPs, were used to evaluate paired-pulse depression (c.f. Davies et al. 1990; Mott and Lewis, 1994). Though the concentration-response of the selective GABA_A receptor agonist, baclofen, to inhibit CA1 population spikes was unchanged in FZP-treated slices (Xie and Tietz, 1991), a reduction in the amplitude and duration of the GABA_A receptor-mediated slow IPSP amplitude and duration was also detected (Zeng et al., 1995; Zeng and Tietz, 1997). Thus, to determine whether the postsynaptic GABA_A response was altered by FZP-treatment, the effect of the GABA_A receptor agonist, baclofen, to hyperpolarize CA1 neurons (Beck et al., 1995) was first evaluated between groups.

Baclofen-induced hyperpolarization. Intracellular recordings were made with filaments glass micropipettes (1.2 mm O.D., 60–90 MΩ, Sutter Instruments, San Rafael, CA) filled with 3 M potassium acetate. At
minimum 2 minute baseline (V_H = -65 mV; Axoclamp 2A, Axon Instruments, Foster City, CA) was recorded in the presence of 1 µM TTX prior to baclofen (100 nM to 100 µM) superfusion. Membrane potential was allowed to return to baseline during drug wash-out, prior to incrementing each baclofen concentration. Only one cell per slice was used. Concentration-response curves were constructed for each cell from the peak amplitude of the membrane hyperpolarization at each baclofen concentration and fit to a sigmoid curve using a four parameter logistic function (GraphPad, PRISM Software, San Diego, CA). The log EC50s and the maximal responses for individual cells were compared by Student’s t-test.

Paired-pulse depression of GABA_A-mediated IPSPs. Monosynaptic IPSPs were evoked in CA1 neurons using the same electrode placement and stimulus parameters as described above. IPSPs were blocked with 50 µM APV and 10 µM DNQX. The GABA_A receptor antagonist, picrotoxin (50 µM) was used to isolate the GABA_A receptor-mediated slow IPSP. Membrane potential was set to -60 mV. To activate the GABA_B autoreceptor, the amplitude of conditioning IPSPs was adjusted to 4.0–4.5 mV across control and FZP-treated neurons. Paired-pulse depression was induced by stimulating interneurons, twice with stimuli of equivalent intensity, at intervals from 250 ms to 1,250 ms and was expressed as the ratio of the peak amplitudes of conditioning and test responses. Paired-pulse depression curves at each concentration between groups was analyzed by ANOVA. Post-hoc analyses of individual data points were made using orthogonal contrasts (P ≤ .05).

Biochemical studies

GABA release. To provide an additional measure of action-potential independent GABA release, the fraction of [3H]GABA released from pre-loaded hippocampal slices was evaluated following K+ depolarization. Four 200 µm dorsal hippocampal slices, cut on a tissue chopper (Stoelting), were placed on a stainless steel grid in a wire mesh basket and were pre-incubated 10 minutes in 1.5 ml of pre-warmed (37°C) buffer under continuous oxygenation (95%O2/5%CO2). Slices were then incubated in 1.5 ml .23 µM [3H]GABA (Amersham, Arlington Heights, IL, 59 Ci/mmol) plus 50 µM aminooxyacetic acid for 30 minutes at 37°C. EDTA (30 µM) was added in a few experiments to block Ca²⁺-mediated terminal release. Slices were washed 30 seconds in 1.5 ml buffer to remove excess [3H]GABA and transferred to a 1 ml filtration chamber (Millipore). To prevent continued uptake of [3H]GABA during spontaneous and KCl-evoked release, buffer containing 200 µM nipeptic acid was superfused at a constant rate of 1 ml/minute. Fractions of the perfusate were collected at 30-second intervals over 25 minutes. When the rate of release had reached steady-state (15 minutes), 1M KCl was injected for 2 minutes via a septum (20 ml/minutes) with a calibrated syringe pump (Razel World Precision Instr. Inc., Sarasota, FL) (final concentration 25 mM). Fractions were counted 5 minutes in 5 ml CytoScint (ICN Biochemicals, Costa Mesa, CA). Slices were solubilized with 0.5 ml 0.5 N NaOH, sonicated, and the total [3H]GABA remaining was counted. After this, 1 µM TTX was added in a few experiments to block Ca²⁺-mediated release. Membrane potential was then allowed to return to baseline during drug wash-out, prior to incrementing each baclofen concentration. Only one cell per slice was used. Concentration-response curves were constructed for each cell from the peak amplitude of the membrane hyperpolarization at each baclofen concentration and fit to a sigmoid curve using a four parameter logistic function (GraphPad, PRISM Software, San Diego, CA). The log EC50s and the maximal responses for individual cells were compared by Student’s t-test. 

GABA uptake into hippocampal synaptosomes. Methods for [3H]GABA uptake assays were modified from Abita et al. (1977). The hippocampus was isolated from FZP-treated rats sacrificed 2 days after ending treatment (n = 6) and matched control rats (n = 5). Each hippocampus was suspended by homogenization (10 strokes at 700 rpm) in 10 volumes of .32 M sucrose in 5 mM Tris HCl Buffer (pH 7.4, 0°C) and centrifuged at 1,200g for 5 minutes at 4°C (Beckman J 21, Beckman, Palo Alto, CA). The supernatant was centrifuged at 23,000g at 20 minutes at 4°C. The P2 pellet was resuspended in 1 ml ice-cold 0.3 M Sucrose in buffer and layered on a discontinuous sucrose gradient (0.8 M, 1.0 M, 1.2 M; 83,000g for 60 minutes at 4°C, Beckman SW27). Synaptosomes were collected at the 1.0/1.2 M sucrose interface and stored at 4°C until used. Synaptosomes were resuspended in 1 ml incubation buffer (in mM: 140 NaCl, 5 KCl 2.8 CaCl2, 1.3 MgSO4, 20 mM Tris-Cl, pH 7.4) plus 50 ml 0.6 M sodium valproate. The reaction was initiated with addition of 10 µl of [3H]GABA + unlabeled GABA to a final concentration of 10 or 50 mM per ml of synaptosomes and proceeded for 15 minutes. The time-course and concentrations for maximal GABA uptake were determined in preliminary assays. Non-specific uptake was in the presence of 500 mM nipeptic acid. The incubation mixture was filtered over 0.45 mm Millipore filters, pre-soaked 2 hours in 1% BSA. Filters were washed 2X with buffer, air-dried, and counted 5 minutes in 5 ml CytoScint. Protein concentration was determined by the method of Lowry et al. (1951).

Drug application and drug solutions

All drug stock solutions were made at 100X the final concentration and added to the perfusate with a syringe pump (Razel, World Precision Instruments, Inc., Sarasota, FL) at a rate of 25–75 µl/minute to achieve their final concentration. Drugs were dissolved in distilled water or as indicated. FZP (flurazepam dihydrochloride) (pH 6.4); APV (DL-2-amino-5 phosphonovaleric acid), DNQX (6,7-dinitroquinoxaline-2,3-dione) in DMSO (to a final concentration of ≤ .001%), TTX (tetraodo-
toxin), (+)-bicuculline methiodide, and (±)baclofen were purchased from Research Biochemicals International (Natick, MA). Picrotoxin was from Sigma Chemical Co. (St. Louis, MO). QX-314 was from Alamone Labs (Jerusalem, Israel). Zolpidem was kindly provided by Synthélabo Recherche (Bagneux, France). Buffer chemicals were from Sigma or Fisher Scientific Co. (Pittsburgh, PA).

RESULTS

Characteristics of sIPSCs and mIPSCs

Whole-cell patch-clamp recordings were made from FZP-treated and control CA1 neurons in hippocampal slices from young adult rats (240–260 g). Spontaneous inward s/mIPSCs were observed in neurons voltage-clamped at negative membrane potentials (Figs. 1A and 2A). Since APV (50 µM) and DNQX (10 µM) were perfused throughout the entire recording period, any mixed s/mEPSCs were eliminated. When bicuculline methiodide (BMI, 5 µM), a GABA_\text{A} receptor competitive antagonist, was superfused, sIPSCs were completely blocked. sIPSCs reappeared after BMI washout (n = 4, data not shown). After TTX (1 µM) was introduced to the bath for 5 minutes, most events of larger amplitude disappeared (Fig. 2A), indicating that the occurrence of sIPSCs was dependent on the spontaneous firing of interneurons. The remaining events were action potential-independent mIPSCs and had a lower amplitude, and a lower frequency of appearance as compared to sIPSCs. The averaged amplitude of sIPSCs after TTX perfusion (mIPSCs) was significantly (P < 0.01) smaller than before TTX perfusion (sIPSCs). The relative cumulative frequency distributions for event amplitudes were significantly (P < 0.001) shifted toward smaller amplitude events, indicating a decrease in event amplitude after TTX superfusion. With a symmetric Cl^- concentration inside and outside the neuronal membrane, mIPSCs reversed their polarity at about 0 mV, as predicted by the Nernst equation (Fig. 2B, n = 4). The voltage-dependency (V_h = -90 to +90 mV) of mIPSCs is also shown in the current-voltage (I-V) response of the same control cell in Figure 2B. Similar observations were found during sIPSC recordings (n = 3, data not shown).

Effects of chronic FZP treatment on CA1 pyramidal cells GABA_\text{A} receptor-mediated currents

sIPSCs recorded 2 days after 1 week FZP treatment

One group of paired-handled FZP-treated and control rats, which were sacrificed 2 days after 1 week FZP treatment, were used for recording sIPSCs. Figure 1A

### MECHANISMS OF BENZODIAZEPINE TOLERANCE

### RESULTS

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One group of paired-handled FZP-treated and control rats, which were sacrificed 2 days after 1 week FZP treatment, were used for recording sIPSCs. Figure 1A
shows representative sIPSCs traces recorded from a control and a FZP-treated neuron. Each neuron was sequentially voltage-clamped at \( V_h = -90, -70, \) and \(-50 \) mV. At each membrane holding potential, averaged sIPSCs, recorded in neurons from rats 2 days after one week FZP treatment, were significantly reduced (58–64\%) in amplitude \(-50 \) mV: Control (\( n = 11 \), -43.1 ± 3.2 pA; FZP-Treated (\( n = 8 \), -17.7 ± 1.6 pA; -70 mV: Control, -58.7 ± 4.6 pA; FZP-Treated, -24.4 ± 4.4 pA; -90 mV: Control, -73.1 ± 7.3 pA; FZP-Treated, -30.0 ± 4.4 pA) and appeared less frequently when compared to those detected in control neurons. The frequency distribution of mean ± S.E.M. sIPSCs amplitudes at \( V_h = -70 \) mV showed a loss of both small and large amplitude sIPSCs in FZP-treated neurons (data not shown). Relative cumulative frequency distributions of event amplitude (Fig. 1C) and inter-event interval (Fig. 1D) were constructed between control (\( n = 9 \)) and FZP-treated (\( n = 10 \)) neurons (\( V_h = -70 \) mV). Compared to control neurons, there was a significant shift in the sIPSC amplitude distribution toward smaller amplitude events at \( V_h = -90 \) mV (\( P < 0.001 \)), -70 mV (\( P < 0.001 \)), and -50 mV (\( P < 0.001 \)) in FZP-treated neurons as compared to control neurons.

mIPSCs recorded 2 days after 1 week FZP treatment. mIPSCs were recorded in another group of FZP-treated (\( n = 9 \)) and control (\( n = 9 \)) neurons (Fig. 2A). mIPSC event amplitudes in FZP-treated neurons were significantly smaller than control mIPSC event amplitudes indicated by the leftward shift of the relative cumulative frequency distribution of mIPSC amplitudes (Fig. 2C, \( V_h = -70 \) mV). Averaged mIPSC amplitudes in control neurons (\( n = 26 \)) ranged from -5.5 pA to -60.7 pA (Mean -25.7 ± 2.9 pA; Median -23.3 pA). In a large sample of FZP neurons recorded, 32\% of cells (21/64) had no detectable mIPSC events, as reported by Poisbeau et al. (1997). In FZP-treated neurons in which events were detected, event amplitude ranged from -6.3 to -21.2 pA (Mean: -11.9 ± 0.7 pA; Median -11.1 pA). Analysis of the relative cumulative frequency distributions of event amplitudes recorded in hippocampal CA1 neurons after 1 week FZP administration indicated a significant decrease in event amplitudes at \( V_h = -90 \) mV (\( P < 0.001 \)), -70 mV (\( P < 0.001 \)), and -50 mV (\( P < 0.001 \)). There were no significant differences between control and FZP-treated neuron mIPSC inter-event interval distributions (Fig. 2D, \( V_h = -70 \) mV) at any holding potential recorded (\( V_h = -90 \) mV (\( P > 0.01 \)), -70 mV (\( P > 0.01 \)).
TABLE I. mIPSC kinetics and unitary conductance in CA1 pyramidal cells

**Monophasic Fit**

<table>
<thead>
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<th>Group (#cells)</th>
<th>Amp. (pA)</th>
<th>( \tau ) (msec)</th>
</tr>
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<tr>
<td>Control (n = 9)</td>
<td>-25.6 ± 5.3</td>
<td>25.1 ± 1.7</td>
</tr>
<tr>
<td>FZP-Treated (n = 13)</td>
<td>-10.4 ± 2.1</td>
<td>26.9 ± 4.3</td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.01*</td>
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**Biphasic Fit**

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<th>Group (#cells)</th>
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<th>( \tau_1 ) (msec)</th>
<th>( A_2 ) (pA)</th>
<th>( \tau_2 ) (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 17)</td>
<td>-12.2 ± 2.4</td>
<td>10.2 ± 1.0</td>
<td>-14.4 ± 2.1</td>
<td>33.0 ± 2.0</td>
</tr>
<tr>
<td>FZP-Treated (n = 20)</td>
<td>-5.0 ± 0.6</td>
<td>11.1 ± 1.1</td>
<td>-8.4 ± 0.9</td>
<td>34.1 ± 2.3</td>
</tr>
<tr>
<td>p value</td>
<td>p &lt; 0.02*</td>
<td>p = 0.64</td>
<td>p = 0.07</td>
<td>p = 0.65</td>
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</tbody>
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Mean (± S.E.M.); asterisks denote significant difference at \( p \leq 0.05 \).
SCs in voltage-clamped ($V_h = -70$ to $+70$ mV) CA1 pyramidal cells. The IPSCs evoked in cells from rats killed 2 days after ending oral FZP administration were compared to those in cells from matched control rats (Fig. 6A). The reversal potential for the $E_{IPSC}$ was 0 mV in both groups. Outward currents recorded at positive holding potentials were greater in magnitude than at comparable negative potentials indicating outward rectification (Fig. 6B). The slope of the I-V relationship was used as an estimate of synaptic conductance and was significantly lower in FZP-treated vs. control cells [$2.9 \pm 1.0$ (n = 6) vs. $10.8 \pm 2.2$ (n = 6); $P = .008$].

Unitary conductance and channel number were estimated in control (n = 12) and FZP-treated (n = 11) CA1 neurons 2 days after the end of 1 week FZP treatment by the variance analysis of mIPSC decay (Table I). Unitary conductance ($\gamma$) was derived from the unitary current estimate. In control cells the conductance esti-
mates ranged from 7 pS to 54 pS whereas in FZP-treated cells conductance estimates ranged from 2 to 9 pS. There was no significant difference in the mean channel number (N) (Table I) between control and FZP-treated cells.

Zolpidem potentiation of GABA-mediated currents. Zolpidem (30 nM–100 µM) concentration-dependently prolonged the decay of mIPSCs in CA1 pyramidal cells (Fig. 7A and C). In control neurons (n = 7) the concentration-effect of zolpidem to prolong mIPSC decay showed two plateaus. However, the data could not be fit using a two-site equation. The effect of zolpidem to enhance mIPSC decay was significantly reduced in FZP-treated neurons (n = 7) (Fig. 7C). Zolpidem (100 µM) enhancement of mIPSC amplitude in control cells indicated an average maximal potentiation of 156.7 ± 14.7% (Fig. 7C). In one control cell, with a low estimated unitary conductance (g = 7) the maximal zolpidem (10 µM) potentiation was 212%. Since the baseline mIPSC amplitude in CA1 pyramidal cells from FZP-treated rats was ~ 40% of that recorded in control cells, the FZP-treated response was normalized to the control response for comparison (Fig. 7D).

DISCUSSION
These in vitro studies in hippocampal CA1 pyramidal cells suggested that both GABAergic pre- and postsynaptic...
aptic mechanisms may contribute to benzodiazepine anticonvulsant tolerance in vivo. Taken together, the results of whole-cell, intracellular, and biochemical studies suggested that reduced presynaptic GABAergic function in the hippocampus was related to decreased interneuron activity. Confirming our preliminary studies of m/sIPSCs in this model (Zeng et al., 1995), profound changes in the function of the postsynaptic GABAA receptor were indicated by the 60% reduction in s/mIPSC amplitude and the absence of mIPSCs in one-third of cells recorded from FZP-treated rats. A reduction in postsynaptic GABAA receptor function was further supported by the decreased synaptic and unitary conductance measured in CA1 neurons. Moreover, tolerance was demonstrated in CA1 pyramidal cells in vitro by the reduced capacity of the GABAA receptor α1 subtype-selective ligand, zolpidem, to prolong mPSC decay, paralleling the discretely localized reductions in CA1 pyramidal cell α1 subtype mRNA and protein expression in this animal model (Chen et al., 1996; Huang et al., 1994; Tietz et al., 1994).

Accumulating evidence suggests that GABA released from a diverse array of GABAergic interneurons (c.f., Freund and Buszaki, 1996) interacts with heterogeneous populations of GABAA receptors (Fritschy and Möhler, 1995; Spyer et al., 1997; Wisden et al., 1992) located on multiple, subcellular compartments of hippocampal pyramidal cells (Freund and Buszaki, 1996; Freund and Gulyás, 1997; Miles et al., 1996; Nusser et al., 1996), resulting in functionally heterogeneous GABA responses in the hippocampus (Alger and Nicoll, 1982; Kapur and Macdonald, 1996; Miles et al., 1996; Pearce et al., 1995; Schönrock and Bormann, 1993). The amount of GABA released is related to the level of presynaptic GABAergic interneuron network activity (Edwards and Sakmann, 1990; Hájos and Mody, 1997; Otis and Mody, 1992; Ropert et al., 1990) as well as random, i.e., tonic GABA release. Whereas the frequency of sIPSCs is a sum of the interneuron network activity, the latter process is reflected in the mIPSP frequency and was not altered in FZP-treated CA1 neurons (Fig. 2). KCl-evoked release was a sensitive measure of increased GABA release from hippocampal slices following chronic diazepam treatment (Hitchcott et al., 1990). However, neither KCl-evoked release, due to direct terminal depolarization, nor [3H]GABA uptake, were altered by FZP-treatment (Fig. 4). Presynaptic GABA receptors autoreceptor function, which plays a role in feedback regulation of GABA release, and can be reflected in the degree of paired-pulse depression (Isaacson et al., 1993; Mott and Lewis, 1994; Pearce et al., 1995), was also unchanged by chronic FZP treatment (Fig. 5). Taken together, these negative findings suggested that an alteration in GABAergic terminal release processes may not be central to benzodiazepine tolerance.

The change in sIPSC frequency in FZP-treated cells may relate to a regulation of interneuron network activity in the hippocampus, i.e., the change in excitability in those interneurons targeting CA1 pyramidal cells. The excitability of these interneurons may be
regulated by a change in their membrane properties or by ionotropic and metabotropic receptor-mediated, excitatory inputs, and GABAA receptor-mediated inhibitory inputs (Freund and Gulyás, 1997; Lacaille et al., 1989; Sah et al., 1990; Samulack et al., 1993; Whittington et al., 1995). Since sIPSC frequency was not altered in the presence of ionotropic glutamate antagonists (Otis and Mody, 1992) and was superfused in the present experiments, these receptors were not candidates for regulation of sIPSC frequency in FZP-treated rats. Recent electrophysiological, morphological, and immunohistochemical studies have established GABAA receptor-mediated inhibitory connections among hippocampal interneurons. For example, sIPSCs can be recorded in interneurons (Hájos and Mody, 1997). The positive a1, b2, and g2 GABAA receptor subtype immunoreactivity

Fig. 7. Effect of 1 week FZP administration on zolpidem potentiation of mIPSC decay and amplitude. A: Representative traces of the decay phase of averaged mIPSCs recorded from a control (bottom traces) and a FZP-treated (top traces) neuron during superfusion of increasing concentrations of zolpidem (300 nM–10 µM). At each zolpidem concentration, the average mIPSC was normalized to the average mIPSC amplitude of the control neuron. The best fit line to the decay, derived from the double exponential equation: \( y(t) = a1 \cdot \exp\left(-\frac{t}{t1}\right) + a2 \cdot \exp\left(-\frac{t}{t2}\right) \), is superimposed over each trace. B: Representative averaged mIPSCs in a control (top traces) and FZP-treated (bottom traces) cell during zolpidem superfusion (30 nM–100 µM). C: Concentration-response curve of the effect of zolpidem to prolong mIPSC decay. Data are expressed as a percent of the baseline average mIPSC decay recorded in the absence of zolpidem. Two plateaus were evident in the zolpidem concentration-response curve constructed from control cells (n = 7). There was a significant reduction in the ability of zolpidem to enhance mIPSC decay in FZP-treated cells (n = 7). D: The concentration-response curve of the effect of zolpidem to potentiate average mIPSC amplitude in the same control and FZP-treated CA1 pyramidal cells. Since mIPSC amplitude was significantly reduced in FZP-treated cells, the response in FZP-treated cells was normalized to the mean average mIPSC amplitude in the 7 control cells in the absence of zolpidem. The non-normalized response in FZP-treated cells is also shown (dashed line).
of subclasses of hippocampal interneurones (Gao and Fritschy, 1994; Moreno et al., 1992; Sperk et al., 1997) provides a possible molecular substrate for the regulation of interneuron excitability by chronic benzodiazepine administration, i.e., an increase in GABAergic inhibitory input to specific interneuron classes may result in a depression of their activity. In fact, α1 mRNA expression was downregulated on one subclass of interneurons at the str. pyramidale/str. oriens border after FZP-treatment (Tietz et al., in press). The importance of this subclass to CA1 pyramidal cell inhibition is not yet known. Calretin (CR)-positive interneuron-selective (IS) GABAergic interneurons, immunoreactive for the α1-subtype antibody, which may mediate gamma (~10–40 Hz) rhythms in the hippocampus (compare Freund and Buszaki, 1997; Jeffreys et al., 1996) are possible candidates. Other classes of interneurons receive GABAergic inputs from afferent pathways extrinsic to the hippocampus (Freund and Buszaki, 1996; Lacaille et al., 1989) and may mediate theta rhythms in CA1 neurons (Soltesz and Deschênes, 1993). Interestingly, FZP-treatment was shown to modulate power spectra in the theta frequency in vivo (Poisbeau et al., 1997). Additionally, GABA-mediated depolarizing responses were recorded in α1β2-2-immunopositive interneurons (Gao and Fritschy, 1994; Moreno et al., 1994; Guijarro et al., 1994; Sperk et al., 1997) in the hilus, which synchronized GABAergic responses recorded in dentate granule cells. Similar interneuron subclasses may exist in the CA1 region (Michelson and Wong, 1991). Thus, both electrophysiological and immunocytochemical evidence supports the possibility that altered sIPSC frequency in FZP-treated CA1 neurons may be related to a change in interneuron activity due to regulation of inhibitory inputs onto interneurons, resulting in a decrease in tonic GABAergic function.

Studies of maximally-evoked IPSCs (Fig. 6) indicated a reduction in synaptic conductance in FZP-treated rats. A change in synaptic conductance could reflect a decreased GABA concentration at the synaptic cleft or a decreased Cl− channel conductance. The lack of change in mIPSC frequency and the lack of modulation of GABA release or uptake made the first possibility unlikely. The mean unitary conductance estimated in control CA1 pyramidal cells (27 pS) was similar to that reported in single-channel studies in hippocampal culture (8 to 54 pS, Eghbali et al., 1997) and to that found under similar conditions in dentate granule cells (20 pS, De Koninck and Mody, 1992) and CA3 pyramidal cells (31 pS, Poncer et al., 1996). Unitary conductance was significantly greater in control CA1 cells in comparison to FZP-treated cells (6 pS). The ~75% reduction in the unitary conductance estimate (Table 1) strongly suggested that GABA_A receptor channel conductance was reduced following FZP treatment. Since conductance levels may reflect variations in GABA_A receptors subunit composition (Sigel et al., 1990; Verdoorn et al., 1990), in particular among hippocampal principle cell types (Fritschy and Mähler, 1995; Sperk et al., 1997; Wisden et al., 1992), the large reduction in unitary conductance in FZP-treated CA1 pyramidal cells could be explained by a switch in GABA_A receptor subunit composition. Nonetheless, a test of this hypothesis will require more direct measures of native GABA_A receptor subunit composition in individual pyramidal neurons in relation to changes in single channel conductance.

Zolpidem had the predicted effect to prolong mIPSC decay in CA1 pyramidal cells (De Koninck and Mody, 1992; Otis and Mody, 1992; Poisbeau et al., 1997; Poncer et al., 1996). Concentration-response studies also revealed a multiphasic effect of zolpidem on mIPSC decay, which may relate its differential affinity for GABA_A receptor a subunit variants (α1≫α2,α3≫α5; compare Sieghart, 1995) and the detection of multiple [3H]zolpidem binding sites in the hippocampus (Ruano et al., 1992). The decreased potency of zolpidem to potentiate GABA-induced currents in acutely dissociated CA1 pyramidal cells of chronic diazepam-treated rats (Itier et al., 1996) and the decreased ability of zolpidem to prolong mIPSC decay (Fig. 7C) may be explained by the localized decrease in α1 mRNA and protein expression in this model. The smaller degree of potentiation at lower concentrations may reflect zolpidem’s decreased relative potency at an altered α1 subunit. Zolpidem’s capacity to increase mIPSC amplitude is controversial (Fig. 7D). Benzodiazepines were shown to have no effect on mIPSC amplitude (De Koninck and Mody, 1992; Mellor and Randall, 1997; Otis and Mody, 1992; Poncer et al., 1996), suggesting that GABA saturates the synaptic cleft (Macacoche et al., 1994). Conversely, Perrais and Ropert (1997) reported that zolpidem (100 nM–100 µM) potentiated mIPSC amplitude (~140% of control) in cortical pyramidal cells, cerebellar Purkinje cells, and dentate granule cells recorded at 22°C, consistent with the idea that intrinsic variation in transmitter concentration and variance in stochastic channel properties can contribute to the variance in mIPSC amplitude (Bekkers et al., 1990; Freerking et al., 1995). In control CA1 pyramidal cells, zolpidem enhanced mIPSC amplitude to a maximum of 156% (Fig. 7D), suggesting that GABA concentration may not be saturating at CA1 pyramidal cells synapses in the present study or that, similar to diazepam’s effect on GABA-gated currents in hippocampal culture, zolpidem might increase Cl− channel conductance (Eghbali et al., 1997). For example, one cell in which conductance was initially low (8 pS) was potentiated to 212% of control by 10 µM zolpidem. The ability of zolpidem to enhance IPSCs suppressed by chronic FZP treatment suggested that either GABA_A receptor functionality could be partially restored or that another GABA_A receptor isofrom, presumably α2-containing, was preferentially activated.
A reduction in s/mPSCs amplitude could reflect a change in GABA_A receptor number or in channel conductance (DeKoninck and Mody, 1992; Nusser et al., 1997). However, estimates of GABA_A receptor channel number (N) derived from non-stationary fluctuation analysis (Table I) indicated no decrease in GABA_A receptor number following chronic benzodiazepine treatment. This finding was consistent with the absence of a change in [3H]GABA binding in the CA1 region of the hippocampus following the same FZP treatment (Chen et al., 1995). Alternatively, the functional changes detected in FZP-treated CA1 pyramidal cells may have their basis in a change in postsynaptic GABA_A receptor subunit composition. Investigations aimed at establishing whether such a switch occurs have demonstrated a variety of changes in subtype mRNA expression following different chronic benzodiazepine treatments, primarily in cerebral cortex (Heninger et al., 1990; Holt et al., 1996; Impagnatiello et al., 1996; Kang and Miller, 1991; O’Donovan et al., 1992; Zhao et al., 1994). A localized decrease in α1 and β3 subtype mRNA and protein expression was first detected in the CA1 pyramidal region after 1 week FZP treatment (Chen et al., 1996; Tietz et al., 1994, in press). The decreased α1 subunit mRNA content was replicated by RT-PCR analysis of the hippocampus from diazepam-treated rats (Impagnatiello et al., 1996). The functional consequences of such a switch in subunit composition have been investigated in heterologous expression systems. Kinetic analyses of GABA-gated currents in recombinant receptors have suggested that the structural determinants for the molecular transitions associated with ligand binding, resulting in channel activation and gating, may be localized to the α subunit (Gingrich et al., 1995). The β subunit, in addition to its role as a target for GABA binding (compare Smith and Olsen, 1995), plays a critical role in subcellular targeting of assembled receptors to regulate receptor localization or clustering (Connolly et al., 1996; Perez-Velazquez and Angelides 1993) and may also modulate GABA/benzodiazepine pharmacology (Ducic et al., 1995; Sieghart, 1995; Sigel et al., 1990). Chronic benzodiazepine treatment may remodel the GABA_A receptor by altering subunit composition from one with a higher affinity for GABA and benzodiazepines to one with a lower affinity and could account for the decreased GABA_A agonist and benzodiazepine potency in the CA1 region of the hippocampus of FZP-tolerant rats (Fig. 7C; Xie and Tietz, 1992). Such a switch could also account for the allosteric uncoupling of GABA/benzodiazepine binding sites associated with chronic benzodiazepine treatment (cf., Barnes, 1996) but does not preclude the possibility that altered post-translational processes, e.g., protein phosphorylation (Brown and Bristow, 1996; Klein et al., 1994; Primus et al., 1996; Tietz et al., in press), may contribute to the molecular transitions at the GABA_A receptor associated with decreased GABAergic function. Tolerance to the action of benzodiazepines to inhibit pentylentetrazol-induced seizures in vivo was measured up to 4 days after 1 week FZP treatment and had disappeared when measured at 7 days (Rosenberg, 1995; Rosenberg et al., 1985) when functional changes in CA1 neurons were reversed (Fig. 3). Poisbeau et al. (1997), using a slight modification of this FZP treatment regimen, evaluated changes in unitary current in CA1 neurons and dentate granule cells at several time-points after benzodiazepine administration was stopped. Reductions in unitary current in CA1 pyramidal cells were augmented on days 3–5 after the drug was removed and returned to control values by the 6th day. Since spontaneous or precipitated abstinence signs were not evident following FZP withdrawal (Tietz and Rosenberg, 1988), changes in conductance may not be related to benzodiazepine dependence. There was no change in unitary current in dentate granule cells exposed to FZP in vivo (Poisbeau et al., 1997). Moreover, exposure of hippocampal slice cultures to midazolam had no effect on CA3 pyramidal cell unitary conductance or on the ability of midazolam to prolong mPSC decay. However, the inability to demonstrate midazolam tolerance in vitro may account for the unchanged unitary conductance and may not be surprising since midazolam tolerance is difficult to induce in vivo (Ramsay-Williams et al., 1994). Nonetheless, α1 mRNA expression was reduced in all three principle cell groups while rats were still consuming FZP, yet the reduction in mRNA expression persisted only in CA1 pyramidal cells, the sole hippocampal cell group in which a change in function due to chronic benzodiazepine exposure has been detected.

ACKNOWLEDGMENTS


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