SYNAPSE 25:125–136 (1997)

Depression of Early and Late Monosynaptic Inhibitory Postsynaptic Potentials in Hippocampal CA1 Neurons Following Prolonged Benzodiazepine Administration: Role of a Reduction in Cl^- Driving Force

XU ZENG AND ELIZABETH I. TIETZ*
Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008

KEY WORDS gamma aminobutyric acid; $GABA_A$ receptor; $GABA_B$ receptor; flurazepam; tolerance

ABSTRACT GABAergic synaptic responses were studied by direct, monosynaptic activation of GABAergic interneurons in the CA1 region of in vitro hippocampal slices from rats made tolerant to the benzodiazepine, flurazepam. Monosynaptic IPSPs were elicited in CA1 pyramidal neurons, following 1 week oral flurazepam administration, by electrical stimulation at the stratum oriens/stratum pyramidale or stratum radiatum/stratum-lacanosum border $\leq$ 0.5 mm from the recording electrode plane. Excitatory input to pyramidal cells and interneurons was eliminated by prior superfusion of the glutamate receptor antagonists, APV (50 μM) and DNQX (10 μM). $GABA_A$ receptor-mediated early IPSPs were further isolated by perfusion of the $GABA_B$ antagonist, CGP 35348 (25 μM) or by diffusion of Cs^+ from the recording electrode. $GABA_B$ receptor-mediated late IPSPs were pharmacologically isolated by perfusion of the $GABA_A$ antagonist, picrotoxin (50 μM). There was a significant decrease in the amplitude of pharmacologically isolated early and late IPSPs in FZP-treated neurons without a change in passive membrane properties. A shift of the early IPSP, but not the late IPSP, reversal potential in FZP-treated neurons suggested that a change in the driving force for anions, presumably Cl^-, in CA1 neurons was one important factor related to the decreased early IPSP amplitude after prolonged activation of $GABA_A$ receptors by flurazepam. A decreased early IPSP amplitude accompanied by a decreased late IPSP amplitude suggested that presynaptic GABA release onto FZP-treated pyramidal cells may also be reduced. We conclude from these data that an impairment of GABAergic transmission in CA1 pyramidal neurons associated with the development of tolerance during chronic benzodiazepine treatment may be related to the regulation of both pre- and postsynaptic mechanisms at the GABA synapse. **Synapse 25:125–136, 1997.**

INTRODUCTION

Benzodiazepines bind to the benzodiazepine recognition site on the $GABA_A$ receptor and selectively potentiate fast inhibitory neurotransmission by increasing the frequency of chloride channel opening in response to GABA (Burt and Kamatchi, 1991; Macdonald and Angelotti, 1993). With repeated or prolonged administration, the anticonvulsant action of benzodiazepines and their clinical benefit are diminished. This reduced sensitivity to benzodiazepines actions, i.e., functional tolerance, has been well documented in several animal models and has been associated with decreased benzodiazepine and GABA responses at the postsynaptic $GABA_A$ receptor. GABA agonist-stimulated Cl^- influx into synaptosomes (Gallager et al., 1991; Miller et al., 1988), as well as the ability of benzodiazepines to potentiate

Contract grant sponsor: NIDA; Contract grant numbers RO1 = DA04075; KO2 = 00180.

*Correspondence to: Elizabeth I. Tietz, Ph.D., Department of Pharmacology, Medical College of Ohio, P.O. Box 10008, Toledo, OH 43699-0008.

Received 1 March 1996; Accepted 13 May 1996.

GABA-mediated Cl^- influx (Yu et al., 1988), was reduced following different chronic benzodiazepine treatments. A reduction in the ability of benzodiazepines and GABA actions to decrease neuronal firing rates was shown in several brain regions in in vivo electrophysiological studies following chronic flurazepam (FZP) or diazepam administration (Gallager et al., 1991; Tyma et al., 1988; Waterhouse et al., 1984).

The native $GABA_A$ receptor is a multimeric protein composed of five subunits (α_{1-6}, β_{1-4}, γ_{1-3} and δ) with an integral Cl^- channel and numerous allosteric regulatory sites, including a benzodiazepine binding site (Burt and Kamatchi, 1991; Macdonald and Angelotti, 1993). Changes in benzodiazepine and $GABA_A$ receptor affinity and number have been reported following those chronic benzodiazepine treatments associated with the development of functional tolerance (Gallager et al., 1991; Marangos and Crawley, 1982; Miller et al., 1988; Tietz et al., 1986, 1989), though a reduced allosteric coupling between GABA and benzodiazepine binding sites is a more consistent finding (Chen et al., 1995; Gallager et al., 1991; Tietz et al., 1989; Wong et al., 1994). Kinetic differences in $GABA_A$ receptor binding and changes in GABA and benzodiazepine binding site number have been related to modulation of $GABA_A$ receptor subunit mRNA expression (Gallager et al., 1991; Huang et al., 1994; Kang and Miller, 1991; O'Donovan et al., 1992; Tietz et al., 1994; Zhao et al., 1994). These findings suggested that chronic benzodiazepine treatment may regulate the gene expression of several $GABA_A$ receptor subunit proteins. Despite this large body of evidence documenting benzodiazepine tolerance and GABAergic subsensitivity in vivo and the accompanying changes at multiple regulatory sites on the postsynaptic $GABA_A$ receptor, no clear understanding of the locus and the nature of changes at the GABA synapse associated with prolonged benzodiazepine administration has emerged.

The well documented GABAergic local circuit in the CA1 region of the hippocampus has provided a useful substrate for a systematic study of benzodiazepine tolerance mechanisms. The hippocampus is dissected from rats sacrificed 2 days after the end of 1 week oral FZP administration when rats are tolerant to the antipentylenetetrazole effects of benzodiazepines in vivo (Rosenberg, 1995; Rosenberg et al., 1985). The CA1 region of the hippocampus has a high density of $GABA_A$ receptors, and several populations of GABAergic interneurons have been identified in this region using a combination of immunohistochemical and electrophysiological approaches (Buhl et al., 1994; Lacaille et al., 1989). In previous experiments in in vitro hippocampal slices from FZP-treated rats, a reduction in paired-pulse inhibition in the CA1 region, mediated by feedforward (Zeng and Tietz, 1994) and feedback (Xie and Tietz, 1991) GABA inhibitory circuits, was demonstrated. As with the decreased benzodiazepine and GABA sensitivity reported with other chronic benzodiazepine treatments (Gallager et al., 1991; Tyma et al., 1988; Waterhouse et al., 1984), a reduction in $GABA_A$ agonist and diazepam potency, but not efficacy, was demonstrated in the CA1 region of hippocampus following chronic FZP treatment (Xie and Tietz, 1992). The amplitude and duration of compound GABA-mediated inhibitory synaptic potentials (IPSPs) in CA1 pyramidal neurons, elicited by stimulation of the Schaffer collateral pathway, were reduced following the same oral FZP treatment (Zeng et al., 1995). These findings suggested that endogenous GABA neurotransmission in the CA1 region was fundamentally impaired following prolonged activation of the $GABA_A$ receptor with FZP.

Both pyramidal neurons and GABAergic interneurons in the CA1 region of the hippocampus bear excitatory amino acid receptors which can be activated by stimulation of the Schaffer collateral pathway. The glutamate receptor-mediated excitatory postsynaptic potentials (EPSPs) immediately precede and overlap the early, $GABA_A$ receptor-mediated hyperpolarization. The offset of the early IPSP and the onset of the late, $GABA_B$ receptor-mediated IPSP also overlap. Since EPSP amplitude was also shown to be increased following oral FZP administration (Zeng et al., 1995), the measurement of heterosynaptically-activated GABA-mediated hyperpolarizations were potentially confounded. Recent studies have shown that both early and late IPSPs can be evoked monosynaptically in the presence of glutamate receptor antagonists with a stimulating electrode placed near ($\leq$0.5 mm) the pyramidal cell soma or dendrites (Davies et al., 1990; Lupica et al., 1992). These pure, monosynaptic IPSPs were shown to be TTX sensitive and to result from direct stimulation of GABAergic interneurons and/or soma (Davies et al., 1990). The mixed GABA-mediated hyperpolarizations can be further distinguished using $GABA_A$ and $GABA_B$ receptor antagonists. The main objective of this study was to test the hypothesis that the amplitude of pure monosynaptic, $GABA_A$ receptor-mediated early IPSPs is reduced in the hippocampal CA1 region 2 days after the end of 1 week oral FZP treatment. A secondary goal was to establish whether $GABA_B$-mediated responses are decreased in the absence of an influence from $GABA_A$-mediated responses.

MATERIALS AND METHODS

Chronic benzodiazepine treatment

Rats administered oral FZP continuously for 1 week develop tolerance to the benzodiazepines ability to suppress pentylenetetrazole seizures (Rosenberg, 1995; Rosenberg et al., 1985). Tolerance can be measured up to 4 days, but not 7 days after ending 1 week FZP treatment (Rosenberg et al., 1985). During and following this treatment regimen, rats show no signs of intoxication or other overt behavioral effects and no signs of spontaneous withdrawal. Weight loss is not

seen upon FZP withdrawal (Rosenberg, 1995) and mild precipitated abstinence signs can only be elicited with very high doses of the benzodiazepine antagonist, flumazenil (25 mg/kg, iv). Thus, physical dependence is not likely to be present following 1 week oral FZP administration.

Male, Sprague-Dawley rats (185–225 g) were adapted to a 0.02% saccharin water vehicle for 2 days. FZP in 0.02% saccharin was then offered as the sole source of drinking water for 1 week. The concentration of FZP, based on the volume consumed, was increased gradually (100 mg/Kg × 3 days; 150 mg/Kg × 4 days). Saccharin water was offered after ending treatment until the time of sacrifice. Only rats which reached the criterion dose of an average of 100 mg/kg/week were included in the study. Control rats were handled identically to FZP-treated rats and received saccharin water throughout the entire adaptation, treatment, and washout periods. Benzodiazepine tolerant rats were sacrificed 2 days after the end of treatment at a time when levels of FZP and its metabolites are no longer detectable in the hippocampus as determined by radioreceptor assay (Xie and Tietz, 1991). Electrophysiological data were therefore not confounded by the presence of residual drug. The experimenter was not informed of the rat's treatment history until after analysis of electrophysiological data was completed.

Hippocampal slice preparation

The experiments were conducted in the in vitro hippocampal slice preparation. The rats were killed by decapitation, the brain was rapidly removed, and the left hippocampus was carefully dissected from the temporal lobe. Five hundred micrometer-thick transverse slices were prepared from the dorsal hippocampus using a vibratome (Ted Pella, Inc., Redding, CA). The slices were transferred to a beaker containing artificial cerebrospinal fluid (ACSF) (in mM): 126 NaCl, 3 KCl, 25.9 $NaHCO_3$, 1.2 $NaHPO_4$, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 10 D-glucose (pH 7.4) saturated with 95% O_2/5% CO_2. Slices were maintained at room temperature for at least 1 h prior to electrophysiological recording. For intracellular recording, a single slice was moved to the recording chamber and held between two nylon nets. Slices were submerged in continuously gassed (95% O_2/5% CO_2) ACSF perfused at a constant flow rate of 1.5 ml/min. The temperature of the ACSF was maintained at 32°C.

Electrophysiological recording

Micropipettes for intracellular recordings from CA1 pyramidal cells were pulled on a Flaming-Brown micropipette puller (P97, Sutter Instruments Co., San Rafael, CA) from fiber-filled borosilicate glass micropipettes (1.2 mm O.D., thick wall, 60–90 MΩ, Sutter). Micropipettes contained 3 M potassium acetate (KAc) or 3 M cesium acetate (CsAc). In neurons studied with CsAc, IPSPs were activated by stimulation at the pyramidal cell/stratum oriens border (see below). Therefore, 200 mM QX314, a Na^+ channel blocker was also included in the micropipette to block antidromic activation of CA1 pyramidal cells. QX-314, like Cs^+, also blocks the $GABA_B$-activated postsynaptic K^+ conductance (Nathan et al., 1990).

Stimulating and recording electrodes were positioned visually. Placement was controlled using a micropositioner and the overlying net as guides. A concentric (75 μm O.D.), bipolar tungsten stimulating electrode was used to activate pyramidal cells by distal stimulation at the stratum radiatum/lacanosum border of the CA1 region, 0.3–0.4 mm from the pyramidal cell layer ≤ 0.5 mm from the vertical plane of the recording electrode or by proximal stimulation with an electrode placed at the border of the stratum oriens/stratum pyramidale layer in the CA1 region ≤ 0.5 mm from the recording electrode. Stratum oriens interneurons and basket cells are activated by the former placement whereas interneurons synapsing on distal dendrites, in particular those in the stratum lanconosum-moleculare are activated by the latter placement (Lupica et al., 1992). Synaptic potentials were elicited at ≥1 min intervals with a 0.1 ms, constant-current pulses using a Master-8 Stimulator (A.M.P.I., Jerusalem, Israel) coupled to an Iso-Flex constant current stimulus isolation unit (A.M.P.I.).

Intracellular signals were displayed on a storage oscilloscope (Tektronix, Beaverton, OR) amplified using an Axoclamp 2A amplifier (Axon Instruments, Inc., Foster City, CA). Membrane potential was altered by passing current through the recording electrode, balanced with an active bridge circuit. Signals were simultaneously displayed and digitized using pClamp 5.5 software and a PC-Labmate AD converter. Digitized records were stored on a PC computer hard disk and tape (Colorado Memory Systems, Loveland, CO) for later analysis.

Passive membrane characteristics of pyramidal neurons (resting potential, input resistance, time constant, and AP amplitude) were evaluated during ACSF superfusion. The input resistance of the cell membrane was obtained by calculating the slope of the current-voltage (I–V) relationship which resulted from applying a group of current pulses (range from +3 nA to −8 nA, 150 ms duration) to the cell membrane. The time constant was defined as the time required to reach 63% of the plateau value of the charge curve. Synaptic potentials were elicited at several stimulus intensities. *Just*-subthreshold stimulation referred to a stimulus intensity just below that intensity which elicited an action potential (AP) in the pyramidal cell on 50% of four trials. The threshold stimulating intensity was defined as the lowest stimulus intensity required to fire the pyramidal cell being recorded, although it may have been above or

below the threshold for neighboring cells. Measurements of all synaptic potentials were made at the peak of the maximal response. Following the determination of intrinsic characteristics, FZP-treated and control CA1 neurons were current clamped at −60 mV. Maximal IPSPs, which represent the average of four responses, were obtained by gradually increasing stimulus strength until no further increment in response was attained in the presence of glutamate and GABA receptor antagonists.

To isolate early and late IPSPs, slices were perfused for 10 min with the selective glutamate antagonists, 50 μM D-2-amino-5-phosphonopentanoate (APV) and 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX). The early IPSP was isolated in the presence of 25 μM p-(3-aminopropyl)-P-diethoxymethylphosphinic acid (CGP 35348) or using CsAc-filled micropipettes. The late IPSP was isolated in the presence of 50 μM picrotoxin. All drugs were dissolved in distilled water and added to the superfusate with a syringe pump (Razel, World Precision Instruments, Inc., Sarasota, FL).

To investigate the basis for the reduction in IPSP amplitudes, the reversal potentials of early and late IPSPs for each cell were measured under each condition at the peak of the response with the membrane potential current clamped from −55 mV to −90 mV. The relationship between the peak postsynaptic potential and membrane potential was determined from the mean of three trials at each membrane potential for each cell. The data were fit to a linear function (early and late IPSP) or a sigmoidal curve (early IPSP) using a four parameter logistic function (Graphpad Prism Software, San Diego, CA). The curve fitting algorithms minimized the sum of squares of the actual distance of the points from the curve. Since the degrees of freedom was invariant between linear and sigmoidal curve fits, a comparison of the sum of squares was used to determine the goodness of fit. The reversal potential for the early IPSP in each cell was estimated from the equation for the best-fit line. The reversal potential for the late IPSP was extrapolated from the equation for the best-fit line to a linear function.

FZP hydrochloride, APV, and DNQX were obtained from Research Biochemicals. CGP 35348 was a gift of Dr. M.F. Pozza (CIBA Geigy, Ltd., Basel, Switzerland). QX-314 was from Alamone Labs (Jerusalem, Israel). Picrotoxin was from Sigma Chemical Co. (St. Louis, MO). All other buffer chemicals were from Sigma or Fisher Scientific Co. (Pittsburgh, PA).

Data are presented as mean ± S.E.M. Data collected using each stimulus protocol were analyzed separately by multivariate analysis of variance. Pairwise comparison were made by orthogonal contrasts. Given four or fewer comparisons per cell, the *P*-value was set to $P \leq 0.05$ according to the method of Bonferroni.

TABLE I. Passive membrane properties of CA1 pyramidal neurons 2 days after oral flurazepam administration[1]

Membrane properties	Control (n = 23)	FZP-treated (n = 24)
Resting potential (mV)	−66.9 ± 0.7	−67.4 ± 0.6
Input resistance (MΩ)	51.3 ± 1.9	49.5 ± 2.3
Time constant (ms)	11.5 ± 1.0	11.5 ± 0.8
AP amplitude (mV)	69.5 ± 1.5	72.2 ± 1.9

[1]All values are means ± S.E.M.

RESULTS

Passive membrane properties

The electrophysiological data were based on recordings from 32 FZP-treated neurons (n = 15 rats) and 32 control CA1 pyramidal cells (n = 22 rats). Of those cells recorded in KAc (control 23/32; FZP-treated: 24/32), cells had resting membrane potentials (RMP) greater than −55 mV and action potential (AP) amplitudes larger than 60 mV. Most cells were stable from 1 to 5 h. As reported previously (Zeng et al., 1995), CA1 pyramidal neurons from FZP-treated rats, sacrificed 2 days after the end of 1 week oral FZP administration, showed no differences in comparison to control cells in their RMP, input resistance, time constant, or AP amplitude (Table I). The additional CA1 neurons (control: 9/32; FZP-treated: 8/32) were recorded with CsAc.

Isolation of inhibitory postsynaptic potentials with glutamate antagonists

Postsynaptic potentials were activated in CA1 pyramidal neurons bathed in ACSF at stimulus intensities *just*-subthreshold and at threshold for an AP by direct activation of excitatory and inhibitory terminals in the vicinity of the stimulating electrode positioned near the soma or distal dendrites. Figure 1A shows a typical response of a CA1 hippocampal neuron to *just*-subthreshold intensity stimulation. This stimulation resulted in an EPSP and a biphasic hyperpolarization. The first hyperpolarizing component represents the $GABA_A$ receptor-mediated, early IPSP, whereas the second component represents the $GABA_B$ receptor-mediated, late IPSP. Exposure of the same cell to the selective excitatory amino acid (EAA) blockers APV (50 μM) and DNQX (10 μM) for 10 min resulted in a complete inhibition of the EPSP. Complete blockade of the EPSP was seen in every cell tested. A pure IPSP which consisted of early and late components was retained as shown in the control cell in Figure 1A. In comparison, responses from an FZP-treated cell are shown in Figure 1B. The amplitude of the early and the late IPSP was significantly reduced in the FZP-treated neuron (Fig. 1B). Table II shows a summary of postsynaptic potential data recorded in neurons from FZP-treated and control groups in response to *just*-subthreshold and threshold stimulation during ACSF superfusion and to maximal stimulation during superfusion of

glutamate antagonists. As reported following heterosynaptic activation of synaptic potentials (Zeng et al., 1995), the amplitude of both early and late IPSPs recorded in CA1 neurons from the FZP-treated group (n = 24) was significantly ($P < .05$) smaller than in the control group (n = 23) under all conditions. As reported previously (Zeng et al., 1995), there was a significant increase in EPSP amplitude in FZP-treated neurons relative to control cells ($P < .05$) prior to APV and DNQX superfusion.

Isolation of early IPSP with CGP 35348

Since $GABA_A$-mediated early IPSPs and $GABA_B$-mediated late IPSPs appear in close temporal sequence (Davies et al., 1990), a separate analysis of isolated IPSPs was necessary to compare the relative peak amplitudes between FZP-treated and control groups. CGP 35348, a $GABA_B$ receptor antagonist (Olpe et al., 1990), was used to pharmacologically isolate the $GABA_A$-mediated component following direct stimulation of interneurons in the proximity of the stimulating electrode placed near the distal apical dendrite. The response of the control cell is shown in the presence of EAA antagonists and after 10 min perfusion of 25 μM CGP 35348 (Fig. 1A, lower trace). The late IPSP disappeared in the presence of the $GABA_B$ antagonist. Both the compound IPSP and the isolated early IPSP were reduced in amplitude in FZP-treated neurons (Fig. 1B, lower trace). There was a significant decrease in the amplitude of early IPSPs recorded in the FZP-treated group (n = 13) when compared to the control group (n = 13) (Table III).

Isolation of the early IPSP with intracellular Cs^+

At least three populations of GABAergic interneurons have been identified in CA1 region of hippocampus based on their anatomical location and physiological characterization (Buhl et al., 1994; Lacaille et al., 1989). IPSPs elicited by stimulation of stratum pyramidale in the presence of EAA antagonists are probably due to the direct activation of basket cells and stratum oriens interneurons or their axons in or near the cell layer (Lupica et al., 1992). Recordings following stimulation at the pyramidal cell layer border were obtained with electrodes filled with 3M CsAc instead of 3M KAc. Cs^+ diffuses from the recording electrode into the pyramidal cell cytoplasm and blocks the $GABA_B$ receptor-activated postsynaptic K^+ conductance (Alger, 1984). The use of cesium offers the advantage over perfusion of $GABA_B$ receptor antagonists in that presynaptic $GABA_B$-mediated actions remain intact. QX-314 (200 mM), a sodium channel blocker (Nathan et al., 1990), was also included in the recording electrode to block antidromic activation of CA1 pyramidal neurons. The rate at which QX-314 blocked APs appeared depen-

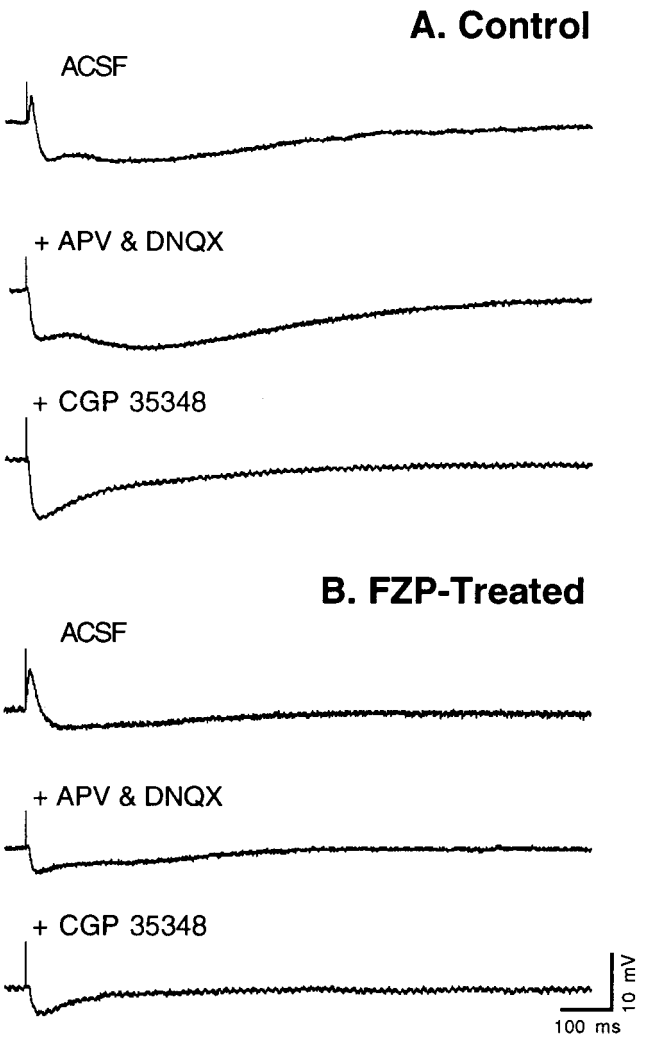

Fig. 1. Isolation of monosynaptic, $GABA_A$ receptor-mediated early IPSPs. A stimulating electrode was placed at the stratum radiatum/stratum lacanosum border ≤ 0.5 mm from the recording electrode to evoke IPSPs in (**A**) a control and (**B**) a FZP-treated CA1 neuron in the absence and presence of glutamate antagonists. The *just*-subthreshold stimulating intensity evoked an EPSP followed by an early and a late IPSP (upper traces). Peak EPSP amplitude was greater in the FZP-treated cell (control; 4.0 mV; FZP-trated 6.8 mV) whereas the peak amplitude of the early (control: 6.5 mV; FZP-treated: 3.4 mV) and late (control: 6.6 mV; FZP-treated: 3.3 mV) IPSP was reduced in the FZP-treated neuron when compared to the control cell. Following 10 min superfusion of 50 μM APV and 10 μM DNQX, the EPSP and AP were eliminated (middle traces). The peak early (control: 8.5 mV; FZP-treated: 4.3 mV) and late (control: 10.0 mV; FZP-treated: 2.6 mV) monosynaptic IPSP amplitudes attained with maximal stimulation were reduced in the FZP-treated cell vs. the control neuron. While maximal responses were triggered in the presence of excitatory amino acid antagonists, the late IPSP was eliminated as GCP 35348 (25 μM), a $GABA_B$ antagonist, was added to the ASCF for 5 min (lower traces). In the FZP-treated neuron, the peak early IPSP amplitude (4.3 mV) was reduced in comparison to the control cell (10 mV). Stimulus artifacts were truncated. (RMP: control, −72 mV; FZP-treated, −70 mV; membrane potential was held at −60 mV, average of four responses/cell).

TABLE II. Postsynaptic potentials recorded ± glutamate antagonists 2 days after oral flurazepam administration[1]

	ACSF		
	EPSP (mV)	Early IPSP (mV)	Late IPSP (mV)
Subthreshold intensity			
Control (n = 23)	4.5 ± 0.3	6.3 ± 0.6	6.0 ± 0.6
FZP-treated (n = 24)	5.7 ± 0.5*	2.3 ± 0.4*	2.6 ± 0.4*
Threshold intensity			
Control (n = 23)	—	6.4 ± 0.6	6.1 ± 0.5
FZP-treated (n = 24)	—	3.0 ± 0.5*	3.5 ± 0.4*

	50 µM APV + 10 µM DNQX	
	Early IPSP (mV)	Late IPSP (mV)
Maximal intensity		
Control (n = 23)	7.5 ± 0.5	6.7 ± 0.5
FZP-treated (n = 24)	3.3 ± 0.5*	3.8 ± 0.3*

[1]All values are means ± S.E.M.
*Denotes significance at P ≤ .05.

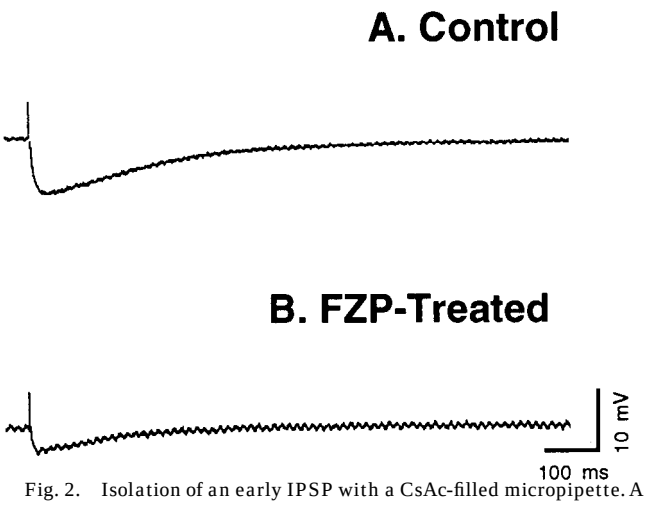

Fig. 2. Isolation of an early IPSP with a CsAc-filled micropipette. A stimulating electrode was placed at the pyramidal cell layer/stratum oriens border ≤ 0.5 mm from the recording electrode in (**A**) a control and (**B**) a FZP-treated neuron. APV (50 µM) and DNQX (10 µM) were superfused to block the EPSP. Late IPSPs were suppressed by intracellular diffusion of Cs^+. QX-314 (200 mM), an intracellular sodium channel blocker, was included in the micropipette to block action potentials generated by antidromic activation of the pyramidal cell. There was a reduction in the peak early IPSP amplitude to maximal stimulation in the FZP-treated cell (4.6 mV) when compared to the control neuron (9.9 mV). Stimulus artifacts were truncated. (RMP: control, −52 mV; FZP-treated, −54 mV; membrane potential was held at −60 mV, average of four responses/cell.)

dent upon the size of the electrode tip through this relationship was not systematically studied. QX-314 typically required 30 min to reach its maximum effect, but at times required up to 2 h. During recording, some cells gradually depolarized probably due to the depolarizing effect of both cesium and QX-314 (Nathan et al., 1990) requiring further injection of hyperpolarizing current to maintain the membrane potential at −60 mV. AP blockade by QX-314 was followed by APV and DNQX perfusion until only the early IPSP remained (Fig. 2A). The amplitude of early IPSP in the FZP-treated cell was smaller than in control neurons (Fig. 2B). The mean amplitude of early IPSPs isolated using CsAc-filled electrodes was significantly reduced in FZP-treated neurons as compared to control cells (Table III).

Isolation of the late IPSP with picrotoxin

GABA released from the presynaptic terminal can act at two main subtypes of postsynaptic receptors, the $GABA_A$ and $GABA_B$ receptor. Previous studies had shown that the amplitude of the late $GABA_B$-mediated IPSP amplitude was decreased in FZP-treated relative to control cells (Zeng et al., 1995). However since the offset of the early IPSP and the onset of the late IPSP overlap, the amplitude of the late IPSP may have been underestimated in FZP-treated neurons. We directly tested the possibility that the $GABA_B$-mediated response was decreased by isolating the late IPSP with the non-competitive $GABA_A$ receptor antagonist, picrotoxin. Blockade of the early IPSP component is shown in a control cell after 10 min addition of 50 µM picrotoxin to the medium in presence of APV and DNQX (Fig. 3A). The maximal amplitude of the late IPSP in the FZP-treated cell was decreased in comparison to the control response (Fig. 3B). In comparison to the group of control cells tested in the presence of picrotoxin (n = 11), FZP-treated cells (n = 10) showed a significant reduction in their late IPSP amplitude (Table III).

Reversal potential of the early and late IPSP

$GABA_A$ receptors activate a Cl^- conductance, whereas $GABA_B$ receptors activate a postsynaptic K^+ conductance. To indirectly evaluate Cl^- and K^+ distribution across the membrane, the reversal potentials for the early and late IPSPs were recorded at membrane potentials ranging from −55 mV to −90 mV, both during EAA superfusion and GABA antagonist superfusion. The voltage-dependence of the early IPSP amplitude recorded using a CsAc-filled electrode is shown in Figure 4. Figure 4A,B shows examples of early IPSPs recorded in a control and a FZP-treated neuron at different membrane potentials. These data and the early IPSP reversal potential data from individual fits of all control and FZP-treated cells under each condition were best-fit to a sigmoidal curve as shown in Figure 4C. This fit is consistent with the report of Lupica (1995) who demonstrated a similar non-linear I–V relationship for $GABA_A$-mediated IPSCs as a function of membrane potential, i.e., IPSP amplitude appeared to plateau with larger amplitude responses at more positive and more negative membrane potentials. This deviation of the I–V curve from a linear fit is probably due to the interaction of several factors which

TABLE III. Isolation of inhibitory postsynaptic potentials 2 days after oral flurazepam administration[1]

Isolation of $GABA_A$-mediated IPSP—distal stimulation			
	50 μM APV + 10 μM DNQX		+25 μM CGP 35348
	Early IPSP (mV)	Late IPSP (mV)	Early IPSP (mV)
Control (n = 13)	6.8 ± 0.7	5.8 ± 0.6	7.1 ± 0.9
FZP-treated (n = 13)	4.1 ± 0.8*	3.1 ± 0.5*	3.6 ± 0.8*
Isolation of $GABA_B$-mediated IPSP—proximal stimulation			
			3M CsAc; 200 μM QX314
			Early IPSP (mV)
Control (n = 9)			6.9 ± 0.6
FZP-treated (n = 8)			3.8 ± 0.5*
Isolation of $GABA_B$-mediated IPSP—distal stimulation			
	50 μM APV + 10 μM DNQX		+50 μM picrotoxin
	Early IPSP (mV)	Late IPSP (mV)	Late IPSP (mV)
Control (n = 10)	7.9 ± 0.7	8.0 ± 0.6	7.8 ± 0.8
FZP-treated (n = 11)	3.3 ± 0.8*	4.2 ± 0.5*	4.5 ± .05*

[1]All values are means ± S.E.M.
*Denotes significance at $P \leq .05$.

have been described in hippocampal pyramidal cells (Fatima-Shad and Barry, 1992; Gray and Johnston, 1985). Outward rectification was more pronounced at hyperpolarized membrane potentials, consistent with reports in cultured hippocampal cells (Fatima-Shad and Barry, 1992) and adult hippocampal CA1 neurons (Gray and Johnston, 1985). In addition, less $GABA_A$ receptor desensitization was reported at depolarized vs. hyperpolarized membrane potentials in hippocampal cells (Yoon, 1994). The decreased IPSP amplitude at the extremes of the curve may represent an increase in the inability to resolve the rapid rise to peak of larger amplitude responses. The equation for the best-fit line was used to derive the reversal potential for each cell. As shown in Figure 4C, there was a depolarizing shift in the reversal potential of the early IPSP in the FZP-treated cell relative to the control neuron. Based on estimates derived from each cell under all isolation conditions, there was a significant shift in the mean reversal potential for the early IPSP (Table IV). A similar shift in the IPSP reversal potential was found when the E_{IPSP} was estimated from the linear regression of the I–V curve (data not shown). The late IPSP reversal was extrapolated from the best-fit of the data to a linear function. As reported previously (Zeng et al., 1995), there was no change in the mean reversal potential for the late IPSP (Table IV).

DISCUSSION

Several populations of interneurons mediating GABA inhibition have been localized to the CA1 region of hippocampus (Buhl et al., 1994; Lacaille et al., 1989). Excitation of these local circuit GABAergic interneurons mediates feedforward and feedback inhibition in the CA1 region by activation of $GABA_A$ and $GABA_B$ receptors located on CA1 pyramidal cells. GABA activates early and late IPSPs, respectively, which hyperpolarize the neuron. Findings of extracellular electrophysiological studies had demonstrated that both recurrent and feedforward inhibition were reduced in the CA1 region of hippocampal slices from rats sacrificed after 1 week of oral FZP administration (Xie and Tietz, 1991; Zeng and Tietz, 1994). In studies designed to characterize the intracellular electrophysiological characteristics of CA1 pyramidal cells from chronic FZP-treated rats, a decrease in the amplitude of heterosynaptically-activated, compound early and late IPSPs was noted in the absence of a change in intrinsic membrane characteristics (Zeng et al., 1995). These findings suggested that endogenous GABAergic transmission was impaired in the CA1 region of hippocampus after chronic benzodiazepine treatment. A decrease in potency, but not efficacy, of exogenously applied $GABA_A$ and benzodiazepine agonists to inhibit CA1 evoked responses was also detected (Xie and Tietz, 1992). Further, the transient reduction in GABA function was temporally correlated with the presence of benzodiazepine anticonvulsant tolerance in the whole animal (Rosenberg, 1995; Rosenberg et al., 1985).

In the present study, GABA-mediated inhibition in the CA1 region of the hippocampus was investigated following monosynaptic stimulation of proximal and distal inhibitory synaptic inputs, i.e., in the absence of an influence from excitatory afferents onto pyramidal neurons or interneurons. Pharmacologically isolated $GABA_A$ and $GABA_B$ receptor-mediated IPSPs, activated by distal stimulation, were significantly reduced in amplitude in CA1 neurons recorded in in vitro hippocampal slices from rats sacrificed 2 days after 1 week oral FZP administration. In recordings with Cs^+

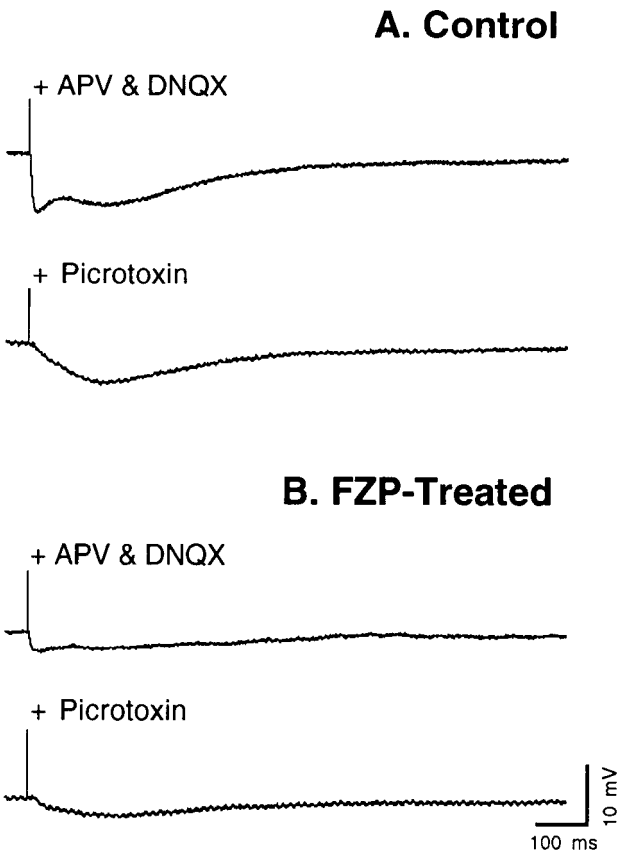

Fig. 3. $GABA_B$ receptor-mediated late IPSPs. Maximal, late IPSPs were elicited in (**A**) a control and (**B**) a FZP-treated neuron in the presence of excitatory amino acid antagonists (50 µM APV and 10 µM DNQX) and the non-competitive, $GABA_A$ receptor antagonist, picrotoxin (50 µM). The stimulating electrode was placed at the stratum radiatum/stratum lacanosum, ≤0.5 mm from the recording pipette. Prior to picrotoxin superfusion, both early (control: 10.5 mV; FZP-treated: 4.0 mV) and late (control: 9.3 mV; FZP-treated: 34 mV) IPSPs were significantly reduced in the FZP-treated cell in comparison to the control cell. After exposure to 50 µM picrotoxin, the early IPSP was absent. The late IPSP amplitude was reduced in the FZP-treated cell (3.6 mV) when compared to the control neuron (7.3 mV). Stimulus artifacts were truncated. (RMP: control, −64 mV; FZP-treated, −62 mV; membrane potential was held at −60 mV; average of four responses/cell).

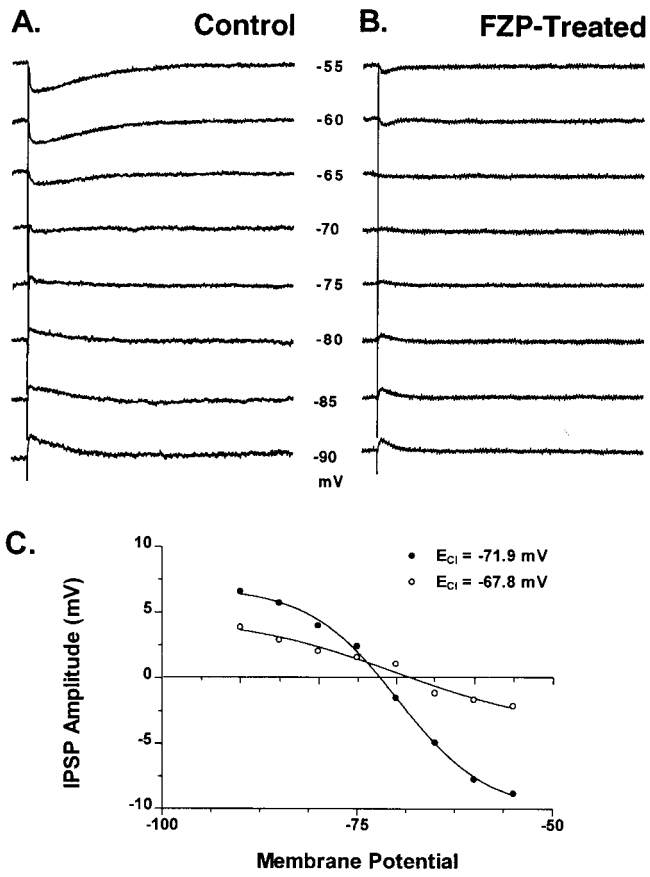

Fig. 4. I–V curve generated with a CsAc-filled micropipette. Early IPSPs recorded at membrane potentials from −55 mV and −90 mV in representative (**A**) control and (**B**) FZP-treated neurons. **C:** The amplitude of the control and FZP-treated responses, measured at peak, were plotted against membrane potential. A sigmoidal curve best-fit the data points generated in both the control and FZP-treated cell. There was a positive shift of the early IPSP reversed potential direction in the FZP-treated cell (−68 mV), whereas the early IPSP in the control cell (−72 mV) reversed at the Cl^- reversal potential. Stimulus artifacts were truncated.

in the micropipette, which blocks only the postsynaptic $GABA_B$ receptor-mediated late IPSP, the early IPSP amplitude was also significantly depressed (50%) in FZP-treated neurons following direct stimulation of interneurons in the vicinity of the pyramidal cell soma. The reduction in amplitude of the $GABA_A$ receptor-mediated early IPSP was accompanied by a somewhat smaller decrease (40%) in the $GABA_B$ receptor-mediated late IPSP in FZP-treated neurons. A reduction in IPSP amplitude in the hippocampus following chronic benzodiazepine treatment could arise from a decrease in presynaptic GABA release or a postsynaptic change in Cl^- conductance. With respect to the $GABA_A$ receptor, the latter could arise from a shift in the Cl^- equilibrium potential or more fundamental changes in

TABLE IV. Reversal potentials of isolated inhibitory potentials 2 days after oral flurazepam administration[1]

	50 µM APV + 10 µM DNQX			
	ACSF	CGP 35348	CsAc;QX314	Picrotoxin
Early IPSP				
Control	−71.5 ± 1.1 (n = 23)	−72.7 ± 2.1 (n = 13)	−75.2 ± 1.7 (n = 9)	—
FZP-treated	−67.9 ± 0.9* (n = 24)	−65.4 ± 2.1* (n = 13)	−70.3 ± 2.5* (n = 8)	—
Late IPSP				
Control	−90.8 ± 1.2 (n = 23)	—	—	−96.7 ± 1.7 (n = 11)
FZP-treated	−93.2 ± 2.1 (n = 23)	—	—	−96.5 ± 1.9 (n = 10)

[1]All values are means ± S.E.M.
*Denotes significance at P ≤.05.

the function of the Cl^- channel in response to GABA, related to an alteration in the postsynaptic $GABA_A$ receptor. In conjunction with the findings of previous electrophysiological, biochemical, and molecular studies in rats treated for 1 week with FZP, the findings of the present study suggest that changes in presynaptic and postsynaptic mechanisms may each contribute to the reduction in IPSP amplitude in CA1 neurons and thus the reduction in GABA-mediated inhibition in the benzodiazepine tolerant rat hippocampus.

A large amount of experimental evidence suggests that modifications of the postsynaptic $GABA_A$ receptor are likely to contribute to the reduction of the early IPSP amplitude following prolonged benzodiazepine administration. Changes in $GABA_A$ receptors in the hippocampus among other brain regions, have been reported following various chronic benzodiazepine treatments (Chen et al., 1995; Gallager et al., 1991; Miller et al., 1988; Tietz et al., 1986). Consistent with these findings, changes in the affinity, the number, and the allosteric coupling of benzodiazepine and GABA binding sites have been reported following several chronic benzodiazepine treatments (Gallager et al., 1991; Marangos and Crawley, 1982; Miller et al., 1988) including chronic FZP treatment (Chen et al., 1995; Tietz et al., 1986, 1989; Wong et al., 1994). However, 2 days following 1 week FZP treatment, only changes in the functional coupling between GABA and benzodiazepine binding sites persists in the CA1 region of the hippocampus (Chen et al., 1995). Further, using Northern blot techniques, the expression of mRNAs encoding various-$GABA_A$ receptor subunits ($\alpha_{1,5}$, $\beta_{2,3}$, and γ_2) was shown to vary with brain region following different chronic benzodiazepine treatments (Gallager et al., 1991; Kang and Miller, 1991; O'Donovan et al., 1992; Zhao et al., 1994). Using in situ hybridization techniques, the α_1 and β_3 subunit mRNA expression was decreased, whereas β_2 subunit mRNA expression was increased, in the hippocampal CA1 region immediately and 2 days, but not 7 days, after ending 1 week oral FZP treatment (Huang et al., 1994; Tietz et al., 1994). Preliminary findings suggest that these changes in $GABA_A$ receptor subunit mRNAs are accompanied by a decrease in α_1 and β_3 subunit proteins in apical and basal dendritic layers of the CA1 pyramidal cell region (Huang et al., 1995). Studies of recombinant $GABA_A$ receptors suggest that GABA affinity is influenced by subunit composition. For example, point mutations of the α_1 subunit of the $GABA_A$ receptor can decrease GABA agonist and antagonist affinity (Smith and Olsen, 1995). Recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes from α_{1-3} subunits showed differential GABA sensitivity (Levitan et al., 1988). Further, HEK 293 cells expressing α_1 subunits combined with β_3 subunits showed a three-fold greater GABA sensitivity than those combined with β_1 or β_2 subunits (Ducić et al., 1995; Hadingham et al., 1993). Therefore, regulation of $GABA_A$ receptors in pyramidal cell dendrites could represent a shift in $GABA_A$ receptor subunit composition and relate to the decreased GABA agonist potency (Xie and Tietz, 1992) and early IPSP amplitude following FZP treatment.

A significant new finding of the present study was a positive shift in the E_{IPSP} of FZP-treated neurons, detected under all recording conditions, in the presence of glutamate antagonists (Table IV). In the former study, there was no shift in the IPSP reversal potential in FZP-treated neurons recorded in the absence of EAA antagonists (Zeng et al., 1995). Since fast inhibitory and excitatory conductances overlap (Dingledine et al., 1987), these conflicting findings in FZP-treated neurons can likely be explained by the presence of the EPSP in the previous study (Zeng et al., 1995) which was significantly increased in amplitude relative to control cells, as in the present study prior to EAA superfusion (Table II). Repeated low frequency activation of inhibitory synapses has been reported to reduce early IPSP amplitude and to shift E_{IPSP} as a result of a accumulation of intracellular Cl^- (Thompson and Gähwiler, 1989). Due to the relatively low concentration of Cl^- in the cytoplasm, a small increase in $[Cl^-]_i$ could significantly decrease the early IPSP amplitude. Using the Nernst equation, the 5 mV change in the reversal potential in a FZP-treated neuron due to Cl^- flux alone (though other anions also permeate the $GABA_A$ receptor channel; Fatima-Shad and Barry, 1993; see below) would be expected to result in a ~25% increment in $[Cl^-]_i$ relative to a control cell. Therefore, the shift in E_{IPSP} in FZP-treated neurons is probably related to a change in the driving force for Cl^-, perhaps as a result of sustained potentiation of $GABA_A$ receptor-activated Cl^- conductance with prolonged benzodiazepine administration.

If intracellular Cl^- accumulation plays a significant role in the reduction of GABA inhibition after prolonged benzodiazepine treatment, then certain predictions can be made regarding the electrophysiological response of chronic FZP-treated neurons. For example, repeated or intense activation of the $GABA_A$ receptor in the CA1 region also results in $GABA_A$ receptor-mediated depolarizing potentials in distal dendrites (Grover et al., 1993; Huguenard and Alger, 1986; Lambert et al., 1991; Staley et al., 1995; Thompson and Gähwiler, 1989). Several hypotheses have been proposed to account for this phenomenon including the existence of "extrasynaptic" $GABA_A$ receptors (Alger and Nicoll, 1982) and regional differences in Cl^-/cation co-transport mechanisms (Misgeld et al., 1986). Since $GABA_A$ receptor channels carry a mixed Cl^-/HCO_3^- current, Staley et al. (1995) proposed that $GABA_A$ receptor-mediated depolarizations are the result of differential localized shifts in anion gradients and a net positive shift in E_{GABA}. Consistent with the latter model, the shift in E_{IPSP} found in the present experiment would underlie

the appearance of the depolarizing event detected previously in FZP-treated, but not control, neurons (Zeng et al., 1995). The appearance of depolarizing GABA responses is also one factor which might have contributed to the increased EPSP amplitude in FZP-treated neurons (Grover et al., 1993). Thus, as with the barbiturates (Alger and Nicoll, 1982), experimental manipulations which increase mean Cl^- channel open time can induce $GABA_A$ receptor-mediated depolarizations (cf. Staley et al., 1995). However, it is important to note that the benzodiazepine effect described, i.e., the positive shift in E_{IPSP} (Fig. 4), is the functional consequence of a sustained, not acute, increase in the frequency of Cl^- channel openings since residual FZP or other benzodiazepine metabolites are not detected in the hippocampus at the time of the recording (Xie and Tietz, 1991).

Nonetheless, the concept of a subpopulation of postsynaptic $GABA_A$ receptors, perhaps extrasynaptic (Alger and Nicoll, 1982), mediating shifts in anion gradients in pyramidal cell dendrites cannot be excluded by the model of Staley et al. (1995). The heterogeneity of native $GABA_A$ receptors (Burt and Kamatchi, 1991; Macdonald and Angelotti, 1993) is supported by studies in the hippocampal CA1 region. For example, GABA and allosteric modulators of native $GABA_A$ receptors in the hippocampus, including diazepam and Zn^{2+}, have multiphasic effects to modulate GABA whole-cell currents in acutely dissociated CA1 pyramidal cells suggesting that multiple $GABA_A$ receptor isoforms may exist on different CA1 pyramidal neurons (Tietz et al., 1995). In addition, Grover et al. (1993) noted that $GABA_A$-mediated hyperpolarizing responses were less sensitive to perfusion with HCO_3^- free medium than depolarizing responses and suggested that these biphasic GABA-mediated responses might show differential sensitivity to changes in pH. Interestingly, a preliminary report by Kaila and colleagues suggests the co-existence of $GABA_A$ receptors populations in acutely dissociated CA1 pyramidal cells, recorded in HCO_3^- free medium, with different GABA affinities which are differentially sensitive to extracellular pH and Zn^{2+} (Pasternack et al., 1995). Further, since hydration forces can significantly alter ion entry into the $GABA_A$ receptor channel and anions can interact within the channel (Fatima-Shad and Barry, 1993) differences in channel properties related to regional differences in $GABA_A$ receptor isoforms could modulate anionic fluxes in different portions of the neuron. Thus, changes in $GABA_A$ receptor subunit composition during chronic benzodiazepine treatment could consequently modulate shifts in anion gradients.

Sustained activation of GABA activity during prolonged FZP administration could also result in a decreased early IPSP amplitude related to changes in Cl^- channel conductance. Though results are somewhat indeterminate, subunit composition can influence the main conductance state and kinetics of the $GABA_A$ receptor (Macdonald and Angelotti, 1993). GABA agonist potency has also been correlated with the duration of Cl^- channel opening (Segal and Barker, 1984) suggesting that a change in channel kinetics could also underlie the decreased early IPSP amplitude. This point will require further analysis in benzodiazepine tolerant hippocampal neurons under conditions in which the concentration and speed of GABA application can be carefully controlled. Single-channel studies in FZP-treated cells will also be necessary to address these hypotheses. Changes in post-transductional mechanisms associated with chronic benzodiazepine treatment, e.g., phosphorylation state of the $GABA_A$ receptor (Macdonald and Angelotti, 1993) or the regulation of other Ca^{2+}-dependent processes, can also not be ruled out. These possibilities are intriguing since the $GABA_A$ receptor-mediated depolarization can activate NMDA-receptor mediated Ca^{2+} influx (Staley et al., 1995). Ca^{2+}-dependent protein kinases and phosphatases have been shown to regulate native and recombinant $GABA_A$ receptors (for reviews, see Macdonald and Angelotti, 1993; Stelzer, 1992). Accordingly, increasing $[Ca^{2+}]_i$ causes run-down of GABA-mediated currents in hippocampal CA1 neurons whereas buffering $[Ca^{2+}]_i$ stabilizes GABA responses (however, see Soltesz and Mody, 1995). Modulation of $[Ca^{2+}]_i$ was also shown to modify GABA and diazepam affinity (Akaike et al., 1989).

As reported previously for the compound IPSP (Zeng et al., 1995), the size of the pharmacologically isolated $GABA_B$ receptor-mediated late IPSP was significantly decreased in FZP-treated neurons (Table III). Since benzodiazepine binding sites are not coupled to $GABA_B$ receptors, they would not be expected to be directly influenced by chronic benzodiazepine treatment. This was shown in previous extracellular concentration-response studies in in vitro hippocampal slices in which neither the potency nor efficacy of superfused baclofen was altered following chronic FZP treatment (Xie and Tietz, 1992). The late IPSP results from a $GABA_B$ receptor-mediated postsynaptic K^+ conductance in response to synaptically released GABA (Alger, 1984). An alteration in intracellular K^+ concentration seems unlikely to be involved in the decreased late IPSP amplitude in FZP-treated cells since the reversal potential of isolated late IPSPs was not changed as the result of chronic FZP treatment (Table IV). Ling and Bernardo (1994) have demonstrated a decrease in the amplitude, but not the decay kinetics, of the late $GABA_B$-mediated IPSC in CA1 pyramidal cells following repetitive, low-frequency monosynaptic stimulation suggesting that a decrease in GABA release, not an increase in GABA uptake, may lead to use-dependent GABAergic depression. In addition, the finding that picrotoxin could suppress late IPSCs, led these investigators to propose that $GABA_A$-mediated interneurons may also mediate a feedforward recruitment of $GABA_B$-mediated activity.

These findings suggest that at least two presynaptic factors, a decrease in GABA release or a reduction in $GABA_A$-mediated responses in interneurons, may have contributed to the decrease in the $GABA_B$-mediated IPSP following prolonged FZP treatment.

Presynaptic regulation of GABA release following 1 week FZP treatment could occur on at least three different levels: 1) a decrease in the number of GABAergic interneurons; 2) a change in the function of the autoreceptor on GABAergic terminals; or 3) a change in the excitability of GABAergic interneurons. A decrease in the number of GABAergic interneurons is an unlikely possibility in view of previous findings, i.e., the restoration of GABA-mediated inhibition, 7 days after the end of 1 week FZP treatment (Xie and Tietz, 1991; Zeng and Tietz, 1994) when tolerance to anticonvulsant actions of benzodiazepines has reversed (Rosenberg et al., 1985). Although GABA release in some brain areas can be modulated by $GABA_A$ autoreceptors (Ennis and Minchin, 1988), use-dependent depression of GABA-mediated inhibition in the hippocampus has been demonstrated to be dependent on GABA release via $GABA_B$ autoreceptor activation (Dutar and Nicoll, 1988). Since $GABA_B$ receptor function is not modified by benzodiazepines, a direct effect of the benzodiazepines to alter $GABA_B$ autoreceptor function seems unlikely, however, a secondary effect on $GABA_B$ autoreceptor remains a possibility and will require further study. To evaluate the third possibility, recordings from different interneuron populations mediating both $GABA_A$ and $GABA_B$ IPSCs (Lacaille et al., 1989) are necessary to directly assess whether differences in interneuron excitability or activity between experimental groups exist. Nevertheless, evaluating spontaneous IPSPs, elicited by the spontaneous action potential firing of presynaptic interneurons offers an indirect method for assessing the excitability of GABAergic interneurons in the CA1 region (Alger and Nicoll, 1980; Lupica, 1995). Preliminary findings of whole-cell recordings made in the CA1 region of the hippocampus have indicated a reduction in the frequency and the amplitude of $GABA_A$ receptor-mediated spontaneous IPSCs in FZP-treated neurons recorded in symmetrical Cl^- solutions (Zeng and Tietz, 1995). A decrease in the frequency of spontaneous IPSPs suggests a depression of spontaneous GABAergic interneuron activity following oral FZP administration. On the other hand, only the amplitude of miniature IPSPs recorded in the presence APV, DNQX, and TTX was suppressed (Zeng and Tietz, 1995), which suggests that only action-potential dependent GABA release was affected by chronic benzodiazepine treatment.

In conclusion, these experiments demonstrated a significant reduction in the isolated $GABA_A$ receptor-mediated early IPSP amplitude in the CA1 region of hippocampus 2 days after 1 week FZP treatment when rats are tolerant to benzodiazepine anticonvulsant actions. Although a large body of evidence supports the possibility that a change in the postsynaptic $GABA_A$ receptor is partly responsible for the decreased GABAergic inhibition in the hippocampus, an increase in intracellular Cl^- concentration in the pyramidal neuron, thus a shift in Cl^- equilibrium potential is also likely to play a major role in the impaired GABA response following chronic FZP treatment. The concomitant decrease in the isolated late IPSP suggested that the decrease in early IPSP amplitude might also have resulted from a decreased GABA release. Further experiments will be required to understand the bases for the presynaptic and postsynaptic changes occurring during chronic FZP treatment which are responsible for the decreased GABAergic inhibition in the hippocampus of rats made tolerant to benzodiazepines.

ACKNOWLEDGMENTS

This study was supported by research grant RO1-DA04075 and Research Scientist Development Award (KO2-00180) to E.I.T. from the National Institute on Drug Abuse (NIDA). We thank William C. Ferencak III for technical assistance and Dr. Carl R. Lupica for critically reading the final manuscript.

REFERENCES

Akaike, N., Oyama, Y., and Yakushiji, T. (1989) Influences of external Ca^{++} on the GABA-induced chloride current and the efficacy of diazepam in internally perfused frog sensory neurons. Brain Res., 504:293–296.

Alger, B.E. (1984) Characteristics of a slow hyperpolarizing synaptic potential in rat hippocampal pyramidal cells in vitro. J. Neurophysiol., 52:892–910.

Alger, B.E., and Nicoll, R.A. (1980) Spontaneous inhibitory postsynaptic potentials in hippocampus: mechanism for tonic inhibition. Brain Res., 200:195–200.

Alger, B.E., and Nicoll, R.A. (1982) Feed-forward dendritic inhibition in rat hippocampal pyramidal cells studied in vitro. J. Physiol. (Lond.), 328:105–123.

Buhl, E.H., Halasy, K., and Somogyi, P. (1994) Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. Nature, 368:823–828.

Burt, D.R., and Kamatchi, G.L. (1991) $GABA_A$ receptor subtypes: from pharmacology to molecular biology. FASEB J., 5:2916–2922.

Chen, S., Cox, A., and Tietz, E.I. (1995) Temporal regulation of $GABA_A$ receptor (GABAR) binding and coupling in flurazepam (FZP) tolerant rats. Soc. Neurosci. Abstr., 21:1590.

Davies, C.H., Davies, S.N., and Collingridge, G.L. (1990) Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic rat responses in rat hippocampus. J. Physiol. (Lond.), 424:513–531.

Dingledine, R., Roth, A.A., and King, G.L. (1987) Synaptic control of pyramidal cell activation in the hippocampal slice preparation in the rat. Neuroscience, 22:553–561.

Ducic, I., Caruncho, H.J., Zhu, W.J., Vicini, S., and Costa, E. (1995) γ-Aminobutyric acid gating of Cl^- channels in recombinant $GABA_A$ receptors. J. Pharmacol. Exp. Ther., 272:434–445.

Dutar, P., and Nicoll, R.A. (1988) A physiological role for $GABA_B$ receptors in the central nervous system. Nature, 332:156–157.

Ennis, C., and Minchin, M.C.W. (1988) Modulation of the GABA auto-receptor by benzodiazepine receptor ligands. Neuropharmacology, 27:1003–1006.

Fatima-Shad, K., and Barry, P.H. (1992) A patch-clamp study of GABA- and strychnine-sensitive glycine-activated currents in postnatal tissue cultured hippocampal neurons. Proc. R. Soc. Lond. [Biol], 250:99–105.

Fatima-Shad, K., and Barry, P.H. (1993) Anion permeation in GABA- and glycine-gated channels of mammalian cultured hippocampal neurons. Proc. R. Soc. Lond. [Biol], 253:69–75.

Gallager, D.W., Marley, R.J., and Hernandez, T.D. (1991) Biochemical and electrophysiological mechanisms underlying benzodiazepine tolerance and dependence. In: The Biological Bases of Drug Tolerance and Dependence. J. Pratt, ed. Academic Press, New York, pp. 49–70.

Gray, R., and Johnston, D. (1985) Rectification of single GABA-gated chloride channels in adult hippocampal neurons. J. Neurophysiol., 54:134–142.

Grover, L.M., Lambert, N.A., Schwartzkroin, P.A., and Teyler, T.J. (1993) Role of $HCO3^-$ ions in depolarizing $GABA_A$ receptor-mediated responses in pyramidal cells of rat hippocampus. J. Neurophysiol., 69:1541–1555.

Hadingham, K.L., Wingrove, P.B., Wafford, K.A., Bain, C., Kemp, J.A., Palmer, K.J., Wilson, A.W., Wilcox, A.S., Sikela, J.M., Ragan, C.I., and Whiting, J.P. (1993) Role of β subunit in determining the pharmacology of human γ-aminobytyric acid type A receptor. Mol. Pharmacol., 44:1211–1218.

Huang, X. Ferencak, W.F., Chen, S., and Tietz, E.I. (1994) Temporal regulation of $GABA_A$ receptor α and β subunit mRNAs *in situ* after chronic flurazepam administration. Soc. Neurosci. Abstr., 20:499.

Huang, X., Chen, S., Sieghart, W. and Tietz, E.I. (1995) Regional changes in $GABA_A$ receptor (GABAR) α_1 subunit protein in flurazepam (FZP) tolerant rats: Immunocytochemical detection using computer-assisted image analysis. Soc. Neurosci. Abstr., 21:1590.

Huguenard, J.R., and Alger, B.E. (1986) Whole-cell voltage-clamp study of the fading of GABA-activated currents in acutely dissociated hippocampal neurons. J. Neurophysiol., 56:1–18.

Kang, L., and Miller, L.G. (1991) Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. Br. J. Pharmacol., 103:1283–1287.

Lacaille, J.-C., Kunkel, D.D., and Schwartzkroin, P.A. (1989) Electrophysiological and morphological characterization of hippocampal interneurons. In: The Hippocampus: New Vistas. V. Chan-Palay, and C. Kohler, eds. Alan R. Liss, New York, pp. 287–305.

Lambert, N.A., Borroni, A.M., Grover, L.M., and Teyler, T.J. (1991) Hyperpolarizing and depolarizing $GABA_A$ receptor-mediated dendritic inhibition in area CA1 of rat hippocampus. J. Neurophysiol., 66:1538–1548.

Levitan, E.S., Schofield, P.R., Burt, D.R., Rhee, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H.F., Stephenson, A., Darlison, M.G., Barnard, E.A., and Seeburg, P.H. (1988) Structural and functional basis for $GABA_A$ receptor heterogeneity. Nature, 335: 76–79.

Ling, D.S.F., and Bernardo, L.S. (1994) Properties of isolated $GABA_B$-mediated inhibitory postsynaptic currents in hippocampal pyramidal cells. Neuroscience, 63:937–944.

Lupica, C.R. (1995) μ And δ enkephalins inhibit spontaneous GABA-medicated IPSCs via a cyclic Amp-independent mechanism in the rat hippocampus. J. Neurosci., 15:737–749.

Lupica, C.R., Proctor, W.R., and Dunwiddle, T.V. (1992) Dissociation of μ and δ opioid receptor-mediated reductions in evoked and spontaneous synaptic inhibition in the rat hippocampus in vitro. Brain Res., 539:226–238.

Macdonald, R.L., and Angelotti, T.P. (1993) Native and recombinant $GABA_A$ receptor channels. Cell Physiol. Biochem., 3:352–373.

Marangos, P.J., and Crawley, J.N. (1982) Chronic benzodiazepine treatment increases [^{3}H]muscimol in mouse brain. Neuropharmacology, 21:81–84.

Miller, L.G., Greenblatt, D.J., Barnhill, J.G., and Shader, R.I. (1988) Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric acid A receptor function. J. Pharmacol. Exp. Ther., 246:170–176.

Misgeld, U., Deisz, R.A., Dodt, T.U., and Lux, H.D. (1986) The role of chloride transport in postsynaptic inhibition of hippocampal neurons. Science, 232:1413–1415.

Nathan, T., Jensen, M.S., and Lambert, J.D.C. (1990) The slow inhibitory postsynaptic potential in rat hippocampal CA1 neurones is blocked by intracellular injection of QX-314. Neurosci. Lett., 10:309–313.

O'Donovan, M.C., Buckland, P.R., Spurlock, G., and McGuffin, P. (1982) Bi-directional changes in the levels of messenger RNAs encoding γ-aminobutyric acid A receptor α subunits after flurazepam treatment. Eur. J. Pharmacol., 226:335–341.

Olpe, H.F., Karlsson, K., Pozza, M.F., Brugger, F., Steinmann, M., Riezen, H.V., Fagg, G., Hall, R.G., Froestl, G., and Bittiger, F. (1990) CGP 35348: a centrally active blocker of $GABA_B$ receptors. Eur. J. Pharmacol., 187:27–38.

Pasternack, M., Smirnov, S., and Kaila, K. (1995) Dural modulation of $GABA_A$ receptors by external H^+ ions in acutely isolated rat hippocampus neurones. Soc. Neurosci. Abstr., 21:2047.

Rosenberg, H.C., Tietz, E.I., and Chiu, T.H. (1985) Tolerance to the anticonvulsant action of benzodiazepines: relationship to decreased receptor density. Neuropharmacology, 24:639–644.

Rosenberg, H.R. (1995) Differential expression of benzodiazepine anticonvulsant cross-tolerance according to time following flurazepam or diazepam treatment. Pharmacol. Biochem. Behav., 51:363–368.

Segal, M., and Barker, J.L. (1984) Rat hippocampal neurons in culture voltage-clamp analysis of inhibitory synaptic connections. J. Neurophysiol., 52:469–487.

Smith, G.B., and Olsen, R.W. (1995) Functional domains of $GABA_A$ receptors. Trends Pharmacol. Sci., 16:162–168.

Soltesz, I., and Mody, I. (1995) Ca^{++}-dependent plasticity of miniature inhibitory postsynaptic currents after amputation of dendrites in central neurons. J. Neurophysiol., 73:1763–1773.

Staley, K.J., Soldo, B.L., and Proctor, W.R. (1995) Ionic mechanisms of neuronal excitation by inhibitory $GABA_A$ receptors. Science, 269:977–990.

Stelzer, A. (1992) Intracellular regulation of $GABA_A$ receptors function. In: Ion Channels. T. Narahashi, ed. Plenum Press, New York, pp. 83–136.

Thompson, S.M., and Gähwiler, B.H. (1989) Activity-dependent disinhibition. I. Repetitive stimulation reduces IPSP driving force and conductance in the hippocampus in vitro. J. Neurophysiol., 61:501–511.

Tietz, E.I., Rosenberg, H.C., and Chiu, T.H. (1986) Autoradiographic localization of benzodiazepine receptor downregulation. J. Pharmacol. Exp. Ther., 236:284–292.

Tietz, E.I., Chiu, T.H., and Rosenberg, H.C. (1989) Regional GABA/benzodiazepine receptor/chloride channel coupling after acute and chronic benzodiazepine treatment. Eur. J. Pharmacol., 167:57–65.

Tietz, E.I., Kapur, J., and Macdonald, R.L. (1995) Mutiphasic effects of allosteric modulators on GABA whole-cell currents in acutely dissociated hippocampal CA1 pyramidal cells. Soc. Neurosci. Abstr., 21:1346.

Tietz, E.L., Huang, X., Weng, X., Rosenberg, H.C., and Chiu, T.H. (1994) Expression of α_1, α_5, and γ_2 $GABA_A$ receptor subunit mRNAs measured in situ in rat hippocampus and cortex following chronic flurazepam administration. J. Mol. Neurosci., 4:277–292.

Tyma, J.L., Rosenberg, H.C., Tietz, E.I., and Chiu, T.H. (1988) Effects of chronic flurazepam treatment on firing rate of rat substantia nigra pars reticulata neurons. Brain Res., 453:344–348.

Waterhouse, B.D., Moises, H.C., Yeh, H.H., Geller, H.M., and Woodward, D.J. (1984) Comparison of norepinephrine- and benzodiazepine-induced augmentation of purkinje cell responses by γ-aminobutyric acid (GABA). J Pharmacol. Exp. Ther., 288:257–267.

Wong, G., Lyon, T., and Skolnick, P. (1994) Chronic exposure to benzodiazepine receptor ligands uncouples the γ-aminobutyric acid type A receptor in WSS-1 cells. J. Mol. Pharmacol., 46:1056–1062.

Xie, X.-H., and Tietz, E.I. (1991) Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of in vitro hippocampal slices. Brain Res., 561:69–76.

Xie, X.-H., and Tietz, E.I. (1992) Reduction in potency of selective $GABA_A$ agonists and diazepam in CA1 region of in vitro hippocampal slices from chronic flurazepam treated rats. J. Pharmacol. Exp. Ther., 262:204–210.

Yoon, K.W. (1994) Voltage-dependent modulation of $GABA_A$ receptor channel desensitization in rat hippocampal neurons. J. Neurophysiol., 71:2151–2160.

Yu, O., Chiu, T.H., and Rosenberg, H.C. (1988) Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. J. Pharmacol. Exp. Ther., 246:107–113.

Zeng, X., and Tietz, E.I. (1994) Impairment of feedforward inhibition in CA1 region of hippocampus after chronic benzodiazepine treatment. Neurosci. Lett., 173:40–44.

Zeng, X., and Tietz, E.I. (1995) Decreased amplitude and frequency of spontaneous IPSCs recorded in hippocampal CA1 pyramidal neurons after chronic flurazepam (FZP) treatment. Soc. Neurosci. Abstr., 21:1589.

Zeng, X., Xie. X.-H., and Tietz, E.I. (1995) Reduction of GABA-mediated IPSPs in hippocampal CA1 pyramidal neurons following oral flurazepam administration. Neuroscience, 66:87–99.

Zhao, T.J., Chiu, T.H., and Rosenberg, H.C. (1994) Decreased expression of γ-aminobytyric Acid type A/benzodiazepine receptor beta subunit mRNAs in brain of flurazepam tolerant rats. J. Mol. Neurosci., 5:181–192.