Reduction in Potency of Selective $\gamma$-Aminobutyric Acid$_A$ Agonists and Diazepam in CA1 Region of in Vitro Hippocampal Slices from Chronic Flurazepam-Treated Rats

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ABSTRACT
The potency and efficacy of selective $\gamma$-aminobutyric acid$_A$ (GABA$_A$) agonists (GABA, muscimol, isoguvacine and 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol), the GABA$_A$ agonist, baclofen, and the benzodiazepine agonist, diazepam, were examined using extracellular recording techniques in in vitro hippocampal slices from rats sacrificed 2 days after 1 week of flurazepam treatment. Population spikes elicited by stimulation of Schaffer collaterals were recorded in the CA1 pyramidal cell region with NaCl containing glass micropipettes. GABA$_A$ agonists were superfused in increasing concentrations for 5 min. Drug responses, averaged over the last 2 min for each concentration, were compared to the predrug base line. GABA$_A$ agonists, but not baclofen, showed a significant, 2-fold, decrease in potency, but not efficacy, to reduce CA1-evoked responses in treated vs. control slices. The benzodiazepine effect was evaluated by the shift in the isoguvacine dose-response curve in the absence, then presence, of diazepam. A reduction in diazepam potency was demonstrated in vitro by a significantly reduced shift in the isoguvacine curve by 300 nM, but not 1 $\mu$M, diazepam after chronic but not acute in vivo pretreatment. The results indicated a selective GABA$_A$ agonist subsensitivity and diazepam tolerance in hippocampus after 1 week of flurazepam treatment and establish the hippocampal slice preparation as a valuable substrate for investigating synaptic mechanisms of benzodiazepine tolerance.

Prolonged administration of benzodiazepines to animals results in the development of functional tolerance, a reduction in the sensitivity of the central nervous system to benzodiazepine actions (cf. Rosenberg and Chiu, 1985). The pharmacological actions of benzodiazepines, which bind to an allosteric site on the central GABA$_A$ receptor, are mediated by the enhancement of a GABA-activated Cl$^-$ conductance (Haefely, 1985). Although the precise cellular and molecular mechanisms underlying benzodiazepine tolerance are not well understood, many changes at the GABA$_A$ receptor have been detected after chronic benzodiazepine treatment. These include downregulation of benzodiazepine binding sites (Tietz et al., 1986, 1988; Miller et al., 1988), upregulation of low-affinity GABA$_A$ receptors (Gallager et al., 1984b) and a reduced coupling between GABA and benzodiazepine binding sites (Gallager et al.; 1984a; Tietz et al., 1988). Decreased expression of the mRNAs for the $\alpha$ (Heninger et al., 1990; Kang and Miller, 1991) and $\gamma_2$ (Heninger et al., 1990) subunits of the GABA$_A$ receptor have also been reported. Changes in GABA function associated with these receptor-related modifications include a decrease in GABA agonist-mediated Cl$^-$ influx (Miller et al., 1988; Marley and Gallager, 1989) and a reduced ability of benzodiazepines to enhance GABA-stimulated Cl$^-$ influx in brain microsacs (Yu et al., 1988). Benzodiazepine tolerance and GABA agonist subsensitivity have also been observed in brains of chronic benzodiazepinetreated rats using in vivo electrophysiological (Gallager et al., 1985; Wilson and Gallager, 1988; Tyma et al., 1988) and behavioral methods (Tietz and Rosenberg, 1988; Ramsey et al., 1991). Alterations in the GABA$_A$ receptor with chronic benzodiazepine treatment and the accompanying changes in GABA function show a regional heterogeneity and have been localized to, among other brain areas, cerebral cortex, dorsal raphé, SNpr and hippocampus (Gallager et al., 1985; Tietz et al., 1986; Miller et al., 1988; Tyma et al., 1988; Wilson and Gallager, 1988).

The hippocampus is an important site of benzodiazepine actions (Haefely, 1985), in particular anticonvulsant actions (Rock and Taylor, 1986). Recently, by using an vitro hippocampal slice preparation, proven to be a useful substrate for the study of GABA/benzodiazepine pharmacology (Kemp et al., 1986; Kemp et al., 1987), we demonstrated a significant reduc-

ABBREVIATIONS: GABA, $\gamma$-aminobutyric acid; SNpr, substantia nigra pars reticulata; FZP, flurazepam; EPSP, excitatory postsynaptic potential; DZP, diazepam; ISO, isoguvacine; MUS, muscimol; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol; BAC, baclofen.
tion in paired-pulse inhibition in CA1 region of hippocampus of rats sacrificed 2 days, but not 7 days, after 1 week of FZP treatment. Moreover, there was a significant prolongation of the field EPSP half-decay time 2 days after FZP treatment without a change in its initial slope or maximal amplitude (Xie and Tietz, 1991). These findings are consistent with an impairment in endogenous GABA function in hippocampus of chronic benzodiazepine-treated rats which coincides with benzodiazepine anticonvulsant tolerance in the whole animal (Rosenberg et al., 1985, 1991).

One purpose of this study was to directly test the hypothesis that GABA_A receptor function is impaired in in vitro hippocampal slices prepared from chronic benzodiazepine-treated rats by evaluating the ability of exogenous GABA_A and GABA_B agonists to inhibit CA1 pyramidal cell-evoked responses. Because behavioral studies from our laboratory had shown that the regulation of benzodiazepine and GABA_A agonist effects were dissimilar in SNpr after chronic benzodiazepine treatment (Tietz and Rosenberg, 1988; Ramsey et al., 1991), experiments were also designed to investigate the possibility of differential regulation in hippocampus by measuring the ability of several GABA agonists to inhibit CA1-evoked responses after chronic benzodiazepine treatment. The electrophysiological actions of benzodiazepines can be measured in the hippocampal slice preparation by their ability to potentiate the effect of GABA agonists (Kemp et al., 1987). To establish whether benzodiazepine tolerance could be measured locally in hippocampus after chronic benzodiazepine treatment, the effect of DZP was tested in hippocampal slices prepared from chronic benzodiazepine-treated rats by its capacity to shift the dose-response curve of the GABA_A agonist, ISO.

**Materials and Methods**

**Chronic and acute benzodiazepine treatment.** The 1-week FZP treatment used in this study has been shown to result in tolerance to the antipentyleneetrazol effects of the benzodiazepines (Rosenberg et al., 1985, 1991), sub-sensitivity to the behavioral actions of benzodiazepine and GABA agonists microinjected into SNpr (Tietz and Rosenberg, 1988), suppression of spontaneous firing of SNpr neurons (Tyma et al., 1988) and downregulation of benzodiazepine receptors in several brain regions including SNpr and hippocampus (Tietz et al., 1986). This chronic treatment does not produce any overt behavioral effects, e.g., ataxia, or does it result in spontaneous or precipitated withdrawal effects (Tietz and Rosenberg, 1988). Details of the chronic treatment regimen have been provided previously (Rosenberg et al., 1985, 1990; Tietz et al., 1986; Tietz and Rosenberg, 1988).

After a 2-day adaptation period during which rats received a 0.02% saccharin water vehicle, male Sprague-Dawley rats (initial weight, 175–200 g) were offered FZP for 7 days (100 mg/kg x 3 days and 150 mg/kg x 4 days) in their drinking water. Only rats that consumed an average of 100 mg/kg/day for the 1-week treatment period were included in the study (Tietz and Rosenberg, 1988; Ramsey et al., 1991; Rosenberg et al., 1991). After the FZP treatment, rats received saccharin vehicle for 2 days until sacrifice. Paired-handled controls received saccharin water for the entire treatment period.

In order to assure that the effects observed in hippocampal slices were specific to chronic benzodiazepine treatment, an acute dose of the FZP active metabolite, desalkyl-FZP, was given to another group of rats. The acute desalkyl-FZP dose to be administered was determined in preliminary radioreceptor assay studies carried out as described previously (Xie and Tietz, 1991). Briefly, ethanol extracts of whole brain from rats given several different doses of desalkyl-FZP (1–10 mg/kg p.o.) or 1 week of chronic FZP treatment were compared for their ability to displace 2 nM [3H]JNPN binding. The level of benzodiazepine activity in the brain immediately after the end of treatment was estimated from a standard DZP displacement curve and expressed in DZP equivalents, nanograms of DZP per/gram of brain. A single dose of desalkyl-FZP (2.5 mg/kg p.o.) resulted in a level of benzodiazepine activity in brain (162.4 ng/g of DZP; 0.57 μM, n = 2) equivalent to that found in the brains of rats after 1 week of chronic FZP treatment (161.1 ± 35.9 ng/g DZP; 0.57 μM, n = 6). Twelve hours before intubation, the food was removed from the cage. Two days before sacrifice, 2.5 mg/kg of desalkyl-FZP was administered by gastric intubation in an emulsion of peanut oil, water and acacia (4:2:1). Control rats received the same volume of the emulsion vehicle by gastric intubation.

**Hippocampal slice preparation.** Hippocampal slices (400 μm) from treated or control rats were cut on a tissue chopper (Stoelting, Wood Dale, IL) from the left dorsal hippocampus. Slices were placed in ice-cold pregassed (95% O_2-5% CO_2) buffer (mM): NaCl, 120; KCl, 5.0; MgSO_4, 1.3; NaH_2PO_4, 1.2; CaCl_2, 2.4; NaHCO_3, 26.0; and glucose, 10.0; 288 mOsm, pH 7.6), then transferred to a recording chamber maintained at 33–34°C. After slices were allowed to equilibrate for at least 1 hr at an interface with buffer and humidified gas, the slices were superfused with warm, gassed buffer for 30 min.

**Extracellular CA1-evoked responses.** CA1 population spikes (fig. 1) elicited by stimulation of the Schaffer collateral pathway were recorded in the stratum pyramidale of CA1 with 2 M NaCl-filled glass microelectrodes (2–5 megohms). A concentric, bipolar, tungsten-stimulating electrode was placed into the stratum radiatum near the CA1/CA2 border and a monophasic, 0.1-msec square-wave pulse (3–15 V) was delivered at 30-sec intervals with an S44 stimulator (Grass Instrument Co., Quincy, MA) connected to a stimulus isolation unit. The amplified (1000-fold), filtered (10 Hz, 3 kHz) population spikes, monitored on an audio monitor, were digitized using a MacLab analog-to-digital converter unit (World Precision Instruments, Inc., Sarasota, FL) interfaced with a MAC II computer, and were displayed using MacLAB software which emulated a digital oscilloscope. Evoked responses data was stored on computer disk for subsequent analysis.

A stimulus-response curve [population spike (millivolts) vs. stimulus intensity (volts)] was constructed for each electrode placement by increasing stimulus intensity manually in 0.5- to 1-V increments from threshold (the stimulus intensity required to elicit a 1-mV potential) until the maximum population spike amplitude was achieved. Half-maximum (1/2 Max), the stimulus intensity required to elicit a population spike 50% of maximum, was used as the standard stimulus intensity. Healthy responses were selected for analysis based on the magnitude of the maximum evoked response (>2.7 mV) and the absence of multiple population spikes before and after drug application. After a stable response was obtained, the last four responses were averaged to serve as the predrug baseline. Only one slice was used from each treated or control rat.

**GABA agonist effects.** GABA agonists were dissolved in distilled water, at a concentration 100 to 1000 times the desired final concentration and were delivered to the hippocampal slice with a calibrated syringe pump (Razel, Stamford, CT) at a rate of 10 to 100 μl/min. Each concentration was perfused for 5 min to ensure that a maximal response was achieved and the last four responses at each concentration averaged to minimize inter-response variability. Drug doses were added cumulatively and the dose-response curve was constructed by plotting drug concentration against the percentage reduction of the baseline population spike amplitude. Drugs were washed out with buffer for 20 to 25 min until the postdrug response returned to 95% of the predrug response.

In preliminary studies in slices from control rats, the effect of superfusion time on the magnitude of the evoked response was evaluated in the presence of buffer alone or 2.5 μM ISO. There was a small increase in the size of the population spike over 60 min during both buffer and ISO perfusion (7.5% vs. 6.8%). The doses of each GABA agonist to be tested were chosen in other preliminary studies (MUS, 0.5–20 μM; ISO, 2.5–60 μM; THIP, 25–500 μM; GABA, 60 μM-1 mM; and BAC, 1–12 μM). The effects of GABA in treated and control slices were determined in the presence of the GABA uptake inhibitor, nipe.
nicotinic acid (200 μM). The IC₅₀ for nicotinic acid blockade of GABA uptake in rat brain slices was reported to be 2.0 ± 1 μM (Krogsgaard-Larsen and Johnston, 1975). Nicotinic acid alone (200 μM) had no effect on the population spike whereas 500 μM depressed baseline-evoked responses. MUS, ISO, THIP (Brehm et al., 1979) and BAC (Bowery et al., 1983) are not substrates for GABA uptake sites.

**Benzodiazepine agonist effects.** Because the benzodiazepines act by potentiation of GABA function, benzodiazepines would be expected to potentiate the inhibitory effects of GABA on the CA1 population spike in a dose-dependent manner as shown by Kemp et al. (1986). The action of DZP was tested in slices from saccharin-treated control rats by its ability to shift the GABA agonist, ISO, dose-response curve (fig. 4). After superfusion with ISO (2.5–20 μM), DZP (100 nM–1 μM, dissolved in 1% ethanol) was added to the superfusate for 20 min (20 μl/min) before a 2nd ISO dose-effect curve was determined in the presence of DZP. A single slice was used for each DZP concentration tested. The experiment was repeated 3 to 8 times per DZP concentration. The results of these studies served as the basis for the DZP concentrations chosen for study in FZP-treated and control slices.

**Data analysis.** The population spike was measured from the midpoint of the tangent to the field EPSP onset and offset to the maximum negative point of the population spike (fig. 1) (Adamec et al., 1981; Tuff et al., 1983; Xie and Tietz, 1991). GABA agonist potency was measured by the concentration of agonist which produced 50% inhibition of the population spike (EC₅₀). Because the concentrations of ISO and DZP chosen for studies in hippocampal slices derived from treated and control rats did not always result in 50% inhibition, the shift of ISO response by DZP was measured as the dose of ISO to produce 40% inhibition of the population spike (EC₄₀). The ability of the benzodiazepine to potentiate the ISO response was plotted as the log dose ratio [EC₅₀ (ISO + benzodiazepine)/EC₅₀ (ISO alone)] vs. benzodiazepine concentration (nanomolar) (fig. 4) (Kemp et al., 1987). The differences in the concentration-response curves among groups were analyzed by a two-way repeated measures analysis of variance. Post-hoc comparisons were made by Bonferroni t test between control and corresponding treatment groups. Changes were deemed significant if P < .05.

**Drugs.** Drugs were obtained from the following sources: DZP and desalkyl-FZP (Hoffmann-La Roche Inc., Nutley, NJ); GABA, nicotinic acid and BAC (Sigma Chemical Co., St. Louis, MO); MUS (Research Organics, Cleveland); ISO hydrochloride and THIP hydrochloride (Research Biochemicals, Inc., Wayland, MA).

**Results**

**Effect of GABA and benzodiazepine agonists on the CA1-evoked response.** Electrical stimulation of Schaffer collaterals elicited a synchronous firing of the CA1 pyramidal cells that could be measured extracellularly. An example of extracellular recordings and the effect of the selective GABAₐ agonist, ISO, to inhibit the population spike in slices from acute desalkyl-FZP vs. emulsion-treated rats was shown in figure 1. GABA agonists inhibited the CA1 population spike in *in vitro* rat hippocampus in a dose-related manner with relative potencies (MUS > BAC > ISO > THIP > GABA) similar to that reported by Kemp et al. (1986) (figs. 2 and 3). The specific GABA uptake blocker, nicotinic acid (200 μM), increased GABA potency 2.5-fold (fig. 2). Consistent with previous findings (Kemp et al., 1986), BAC, a selective GABAₐ agonist, produced 100% inhibition of the CA1 population spike.

As shown in figure 4, DZP dose-dependently potentiated the inhibitory effect of ISO on the CA1 population spike. Higher concentrations of DZP (>1 μM) had direct, presumably non-specific, inhibitory effects on the amplitude of the CA1 population spike (data not shown) (Kemp et al., 1987). The 1% ethanol vehicle alone had no effect on the base-line response. During perfusion of slices with 1% ethanol over 60 min, the size of the population spike increased by 6.9%, similar to the change in the evoked response over time during perfusion with buffer alone. Based on the results of DZP dose-response studies (fig. 4), DZP at concentrations of 300 nM (EC₅₀) and 1 μM (EC₁₀₀) were chosen for studies in slices from FZP-treated and control rats. Choosing concentrations on the linear (EC₅₀) and maximal (EC₁₀₀) portions of the DZP concentration-response curve allowed for detection of changes in DZP potency and/or efficacy.

**Effects of chronic FZP treatment on GABA agonist effects.** GABA (fig. 2, P = .047) and the selective GABAₐ agonists, MUS, ISO and THIP (fig. 3), showed significant (P < .001) decreases in potency in hippocampal slices derived from rats sacrificed 2 days after ending 1 week of FZP treatment. This was indicated by a rightward shift in the dose-response curves for their ability to inhibit the CA1 population spike in treated compared to control slices (EC₅₀ in micromolar: GABA, 186.4 vs. 112.8; MUS, 6.1 vs. 2.4; ISO, 23.6 vs. 5.2; and THIP, 238.3 vs. 106.7; n = 6/group). However, there was no significant difference (P = .56) in the CA1 response to the selective GABAₐ agonist, BAC, between treated (EC₅₀, 7.2 μM) and control slices (EC₅₀, 6.5 μM) (fig. 2).

**Effect of chronic FZP treatment on benzodiazepine agonist effects.** DZP was used to evaluate the development of tolerance in *vitro* inasmuch as it is a full agonist at the benzodiazepine receptor. A single dose (1 μM) of DZP in *vivo* after chronic FZP treatment (Rosenberg et al., 1991). Two days after 1 week of FZP treatment, there was a significant decrease in the ability of DZP to shift the ISO dose-response curve (2.5–20 μM) in treated (1.3-fold shift) vs. control (1.8-fold shift) slices (fig. 5). The decreased ability of DZP to shift the ISO response was observed at 300 nM DZP (n = 8/group, P = .02), but not 1 μM DZP (n = 6/group, P = .49; treated: 2.5-fold shift; control: 2.4-fold shift), indicating a decrease in DZP potency, but not efficacy in CA1 region after chronic FZP treatment.

**Effect of acute desalkyl-FZP treatment on GABA and benzodiazepine agonist effects.** Two days after a single dose of desalkyl-FZP (2.5 mg/kg p.o.), slices from both acutely treated and control rats were tested. There was no significant difference (P = .73) in the ability of ISO to decrease the CA1-evoked response in slices from acute desalkyl-FZP vs. emulsion intubated rats (fig. 6). There was also no significant difference (P = .40) between groups in the ability of 300 nM DZP to shift the ISO dose-response curve (fig. 6; 2.0-fold vs. 1.7-fold shift; n = 4/group). There was no change in the effect of ISO or of DZP in hippocampal slices from rats sacrificed 2 days after pretreatment with a single dose of DZP (10 mg/kg p.o. in 0.5% TWEEN 80, n = 3/group) vs. control slices (data not shown). This acute treatment also results in benzodiazepine brain levels equivalent to that found after 1 week of FZP treatment (Tietz et al., 1991).

**Discussion**

The principal findings of this study were a reduction in both GABAₐ and benzodiazepine agonist effects in CA1 region of *in vitro* hippocampal slices from rats 2 days after cessation of 1 week of FZP treatment. There was a significant decrease in the actions of superfused GABAₐ agonists (GABA, ISO, MUS and
THIP) to inhibit population spikes recorded extracellularly from CA1 pyramidal cells (figs. 2 and 3). GABA_A agonist concentration-response curves were shifted 2-fold to the right without a change in maximal effect, indicating a decrease in GABA_A agonist potency, but not efficacy. The reduction in the postsynaptic actions of GABA in hippocampal slices was selective for the GABA_A receptor because there was no change in the action of the GABA_A agonist, BAC, to inhibit CA1-evoked potentials after chronic FZP treatment (fig. 2). In addition, the ability of the benzodiazepine agonist, DZP, to shift the ISO dose-response curve was reduced in chronically treated as compared to control slices. The tolerance to DZP measured in hippocampal slices was produced by chronic FZP administration, but not by a single desalkyl-FZP pretreatment (fig. 6), and was only observed after superfusion with a DZP concentration of 300 nM (EC_{50}), not 1 μM (EC_{100}), indicating a decrease in DZP potency, not efficacy (fig. 5).

This is the first report of a reduction in both GABA_A and benzodiazepine agonist potency, rather than efficacy, after a 1-week chronic benzodiazepine treatment. The presence of residual FZP or its active metabolites in the slice, which would result in an apparent increase in GABA and benzodiazepine agonist potency, could not have affected these observations because residual benzodiazepines were not detectable in hippocampus 2 days after withdrawal from 1-week FZP treatment (Xie and Tietz, 1991). These data suggest that there is an alteration in the affinity of both GABA and benzodiazepine binding sites during chronic benzodiazepine treatment. This conclusion is supported by the finding that the rank order of potency of
the finding that the allosteric interaction between GABA and benzodiazepine receptor sites was not altered in hipppocampal membranes after chronic FZP administration (Tietz et al., 1989).

The current results extend our previous findings of a reduction in paired-pulse inhibition and a prolongation of the EPSP half-decay time in CA1 region of hippocampus 2 days after the end of 1-week chronic FZP treatment (Xie and Tietz, 1991). The subsensitivity to exogenous GABA and benzodiazepine agonists in the CA1 region supports the hypothesis that the reduction in paired-pulse inhibition in the hippocampus was due to a decrease in endogenous GABA-mediated inhibition and suggests that the decreased inhibition is due, at least in part, to a decreased postsynaptic response to GABA, although a change in GABA, presynaptic receptors cannot be ruled out (Waldmeir and Baumann, 1990). The significant correlation between GABA, agonist potency and the average duration of Cl channel opening in spinal cord cultures (Barker and Mathers, 1981) would suggest that the reduction in GABA, agonist actions in the CA1 pyramidal cell region of the hippocampus after chronic FZP treatment may be the result of an alteration in Cl channel kinetics rather than a decrease in conductance. With respect to benzodiazepine actions, lowering benzodiazepine binding affinity by photoinactivation of the benzodiazepine receptor reduced the ability of chlordiazepoxide to increase GABA-induced Cl conductance (Gibbs et al., 1985). Chronic treatment of spinal cord and cortical cell cultures with clonazepam resulted in a decrease in benzodiazepine affinity for the receptor (Sher et al., 1983; Sher, 1986) and was associated with a reduced ability of DZP to facilitate GABA-induced conductance (Sher et al., 1983). The reduced potency of DZP in hippocampal slices from FZP-treated rats might result in a similar change. Intracellular current and voltage clamp recordings from CA1 pyramidal cells in slices from benzodiazepine-treated rats should further elucidate the synaptic mechanisms underlying the impairment in GABA-mediated inhibition in the hippocampus of benzodiazepine-tolerant rats.

GABA (Kemp et al., 1986) and benzodiazepine (Kemp et al., 1987) actions in hippocampal slices was highly correlated with their ability to displace [3H]GABA and [3H]RO15–1788 binding, respectively, to hippocampal membranes. The lack of change in DZP efficacy is consistent with the reversal of benzodiazepine receptor downregulation in hippocampal pyramidal cell regions within 2 days after the end of chronic FZP treatment (Tietz et al., 1986) and suggest that benzodiazepine receptor downregulation (Rosenberg and Chiu, 1981; Tietz et al., 1986; Miller et al., 1988) may not be necessary for the maintenance of benzodiazepine tolerance in hippocampus. Furthermore, the absence of a decrease in benzodiazepine efficacy, which has been shown to be related to the ability of GABA to alter the affinity of benzodiazepines for their receptor ("GABA-shift") in hippocampus (Kemp et al., 1987) is consistent with...
Isoproterenol (ISO) agonists may be exhibited after chronic benzodiazepine treatment. These studies had demonstrated the differential regulation of the circling response to several GABA_A agonists and FZP microinjected into SNpr (Tietz and Rosenberg, 1988; Ramsey et al., 1991). The time course for the development and the reversal of tolerance to FZP and subsensitivity to MUS were dissimilar (Tietz and Rosenberg, 1988) and subsensitivity was demonstrated to GABA, but not to THIP (Ramsey et al., 1991). A possible basis for this hypothesis may be found in other studies in the hippocampus (Alger and Nicoll, 1982; Woodson et al., 1989). A recent quantitative immunocytochemical study showed a heterogeneous population of GABAergic cells in the rat hippocampal formation. Two different types of GABA immunoreactive neurons were identified in the CA1/CA2 interface region (Woodson et al., 1989). The existence of two distinct, i.e., synaptic and extrasynaptic, GABA_A receptors have been proposed to mediate GABA-induced hyperpolarization and depolarization, respectively (Alger and Nicoll, 1982). THIP preferentially induced hyperpolarization (Alger and Nicoll, 1982) and has been found to label a subset of GABA receptors (Falch and Krosgaard-Larsen, 1982). In hippocampal slices from chronic FZP-treated rats, the potency of each GABA_A agonist was reduced to a similar extent (2-fold) 2 days after chronic benzodiazepine treatment. Although the shift in ISO potency was initially 4-fold (fig. 3), this was reduced to 2-fold when the number of test doses used was decreased from 8 to 4 (figs. 5 and 6). This was due to the increase in potency of ISO in control slices when the greater number of doses was used, perhaps due to a cumulative drug effect. The shift in the ISO curve in treated slices was maximal (EC_50 ~ 20 μM) using both paradigms. Both subsensitivity to GABA_A agonists and tolerance to DZP were detected concurrently in the hippocampus, suggesting that regulation of their actions may not follow different time courses as they did in SNpr. Studies at different time points after chronic treatment would be necessary to reject the hypothesis that responses to GABA_A agonists and benzodiazepines are regulated differentially in the hippocampus. Intracellular recording methods and localized pressure ejection techniques would be a logical approach to further investigate the possibility of differential regulation in the hippocampus after chronic benzodiazepine treatment.

Numerous experiments have demonstrated regional variations in the function of the GABA_A receptor complex after chronic benzodiazepine treatment. For example, subsensitivity to iontophoretically applied GABA was demonstrated in dorsal raphé (Gallager et al., 1984; Wilson and Gallager, 1988) but not in SNpr neurons (Wilson and Gallager, 1987). Furthermore, no changes were found in the GABA iontophoretic currents required to inhibit CA3 pyramidal cell firing in the hippocampus of chronic DZP-treated rats (Lista et al., 1990). The exact mechanisms mediating benzodiazepine tolerance still remain unknown. Regional variation in benzodiazepine and GABA receptor binding and changes in their allosteric interaction have also been associated with chronic benzodiazepine treatment (Gallager et al., 1984a, 1985; Tietz et al., 1986; Miller et al., 1988; Tyma et al., 1988; Wilson and Gallager, 1988; Tietz et al., 1989). The regional variations in changes in the GABA complex during chronic benzodiazepine treatment may reflect the differential distribution of GABA_A receptor subunit (α1-4, β1-3, γ2 and δ) mRNAs in the brain, specifically in hippocampal laminae (Möhler et al., 1990). Variable expression of the genes encoding for GABA_A receptor subunits could lead to the synthesis of a GABA_A receptor with an altered affinity for GABA.

The lack of change in the actions of BAC in treated slices suggests that the impairment in GABA-mediated inhibition in the hippocampus is a selective dysfunction mediated by GABA_A receptors, rather than GABA_B receptors. GABA_B receptors which have been localized to the CA1 area of the rat brain (Chu et al., 1990) mediate both pre- and postsynaptic GABA_A actions (Blaxter and Carlen, 1986).

Activation of postsynaptic GABA_B receptors results in the hyperpolarization of hippocampal CA1 pyramidal cells (Newberry and Nicoll, 1984; Reynolds et al., 1990) through an increase in K⁺ conductance (Newberry and Nicoll, 1984; Andrade et al., 1986). Thus, the primary action of superfused BAC, as found in this study (fig. 2), was inhibition of CA1-evoked responses (Kemp et al., 1986). GABA_B receptors are not, like GABA_A receptors, coupled to the benzodiazepine receptor-linked Cl⁻ channel (Hafely, 1985). Therefore, chronic benzo diazepine treatment would not be predicted to modulate postsynaptic GABA_B receptor function. To our knowledge, this is the first demonstration of the presumed selectivity of the effect of chronic benzodiazepine treatment on postsynaptic GABA_A receptor function. Nonetheless, changes in the presynaptic actions of BAC as the result of chronic benzodiazepine treatment cannot be ruled out. BAC has been reported to decrease endogenous GABA release (Waldmeier et al., 1988), possibly as a result of presynaptic calcium channel blockade (Blaxter and Carlen, 1985). Changes in the presynaptic actions of BAC might be predicted from the report that chronic DZP treatment can modify [³H]GABA release (Hitchcott et al., 1990). However, recent data from our laboratory has shown KCl-evoked [³H]GABA release is unchanged in in vitro hippocampal slices 2 days after withdrawal from chronic FZP treatment (Tietz and Xie, 1991).

Behavioral studies from our laboratory in chronic FZP-treated rats led us to test the hypothesis that differential subsensitivity to the actions of GABA_A and benzodiazepine agonists may be exhibited after chronic benzodiazepine treatment.
and benzodiazepine agonists. Chronic benzodiazepine treatment has been reported to decrease the expression of α1 (Henninger et al., 1990; Kang and Miller, 1991) and γ2 mRNAs (Kang and Miller, 1991) in cerebral cortex although not in cerebellum, or interestingly, in the hippocampus. The latter findings may suggest the lack of involvement of an alteration in gene expression in the induction and maintenance of benzodiazepine tolerance in the hippocampus or that localized changes in the hippocampus, a very heterogeneous brain region, may be difficult to detect using molecular techniques in homogenized tissue. Because most GABA<sub>A</sub> subunit mRNAs are expressed in the hippocampus, including the CA1 region (Mohler et al., 1990), it is also possible that the various subunit mRNAs which have been analyzed thus far in the hippocampus are not those whose variable expression are associated with benzodiazepine tolerance phenomenon.

The current findings, i.e., the reduced potency of GABA<sub>A</sub> and benzodiazepine agonists in the CA1 region of in vitro hippocampus from chronic FZP-treated rats, establish the hippocampal slice preparation as a valuable substrate for investigating benzodiazepine tolerance mechanisms. The functional tolerance measured in the hippocampus correlates with the time course of the tolerance measured to the antipentylenetetrazol actions of the benzodiazepines in vivo (Rosenberg et al., 1985, 1991). By using intracellular electrophysiological techniques in combination with biochemical methods, i.e., autoradiographic binding and in situ hybridization techniques, this preparation will allow us to further investigate the changes in the GABA<sub>A</sub> receptor and its functions which are associated with chronic benzodiazepine treatment to gain a better understanding of the synaptic mechanisms underlying benzodiazepine tolerance.

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