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Reduction in Potency of Selective γ-Aminobutyric Acid$_A$ Agonists and Diazepam in CA1 Region of *in Vitro* Hippocampal Slices from Chronic Flurazepam-Treated Rats[1]

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ABSTRACT

The potency and efficacy of selective γ-aminobutyric acid$_A$ (GABA$_A$) agonists (GABA, muscimol, isoguvacine and 4,5,6,7-tetrahydroisoxazolo-[5,4-c]-pyridin-3-ol), the GABA$_B$ 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 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 μM, 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, 1989; 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.*, 1989). Decreased expression of the mRNAs for the α_1 (Heninger *et al.*, 1990; Kang and Miller, 1991) and γ_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 benzodiazepine-treated 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-

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ABBREVIATIONS: GABA, γ-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 of week 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 antipentylenetetrazol effects of the benzodiazepines (Rosenberg *et al.*, 1985, 1991), subsensitivity 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 × 3 days and 150 mg/kg × 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 [^{3}H]FNP 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 D-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 response 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 base line. 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 perfusate 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 to 99% 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.* 6.6%). The doses of each GABA agonist to be tested were chosen in other preliminary studies (MUS, 0.5–20 μM; ISO, 2.5–50 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-

cotic acid (200 μM). The IC_{50} for nipecotic acid blockade of GABA uptake in rat brain slices was reported to be 9 ± 1 μM (Krogsgaard-Larsen and Johnston, 1975). Nipecotic 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 $GABA_A$ 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 (EC_{50}). 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 (EC_{40}) in the absence, then presence, of DZP. The ability of the benzodiazepine to potentiate the ISO response was plotted as the −log dose ratio [EC_{50} (ISO + benzodiazepine)/EC_{50} (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, nipecotic 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 $GABA_A$ agonist, ISO, to inhibit the population spike in slices from control and FZP-treated rats is 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, nipecotic acid (200 μM), increased GABA potency 2.5-fold (fig. 2). Consistent with previous findings (Kemp *et al.*, 1986), BAC, a selective $GABA_B$ 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 (EC_{50}) and 1 μM (EC_{100}) were chosen for studies in slices from FZP-treated and control rats. Choosing concentrations on the linear (EC_{50}) and maximal (EC_{100}) 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 actions. GABA (fig. 2, $P = .047$) and the selective $GABA_A$ 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 (EC_{50} 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 $GABA_B$ agonist, BAC, between treated (EC_{50}, 7.2 μM) and control slices (EC_{50}, 6.5 μM) (fig. 2).

Effect of chronic FZP treatment on benzodiazepine agonist actions. DZP was used to evaluate the development of tolerance *in vitro* inasmuch as it is a full agonist at the benzodiazepine receptor in this preparation (Kemp *et al.*, 1987) and because anticonvulsant tolerance could be measured to 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 actions. 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.*, 1986).

Discussion

The principal findings of this study were a reduction in both $GABA_A$ and benzodiazepine agonist effects in CA1 region of *in vitro* hippocampal slices from rats 2 days after cessation of 1 week of FZPtreatment. There was a significant decrease in the actions of superfused $GABA_A$ agonists (GABA, ISO, MUS and

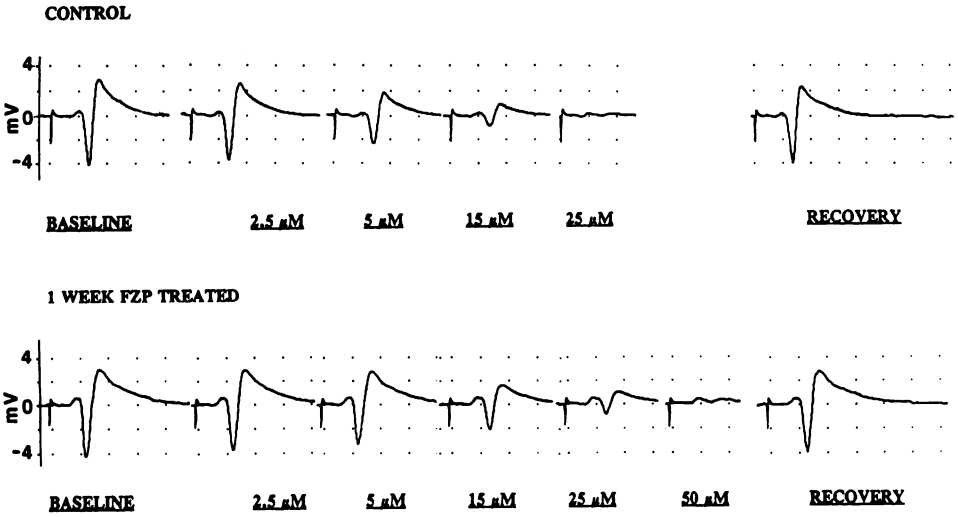

Fig. 1. Representative extracellular-evoked potentials (population spikes) recorded in CA1 pyramidal cell layer in control and treated hippocampal slices during superfusion of ISO (2.5–50 μM). Horizontal distance between dots = 5 msec; vertical distance between dots = 2 mV.

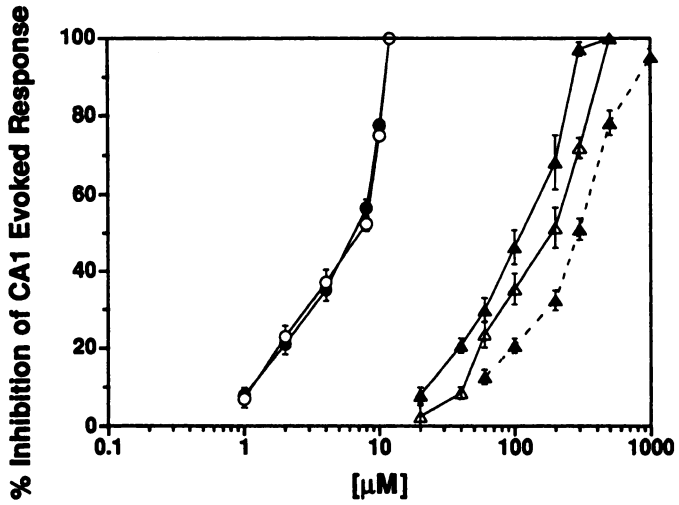

Fig. 2. Cumulative BAC (●, ○) and GABA (▲, △; plus 200 μM nipecotic acid) concentration-response curves in hippocampal slices from control rats (▲, ●) and rats sacrificed 48 hr after 1 week of FZP treatment (△, ○). Drugs (10–100 μl) were added to the superfusate (2 ml/min), incrementing the concentration every 5 min. Drug responses, averaged over the last 2 min, were calculated as a fraction of the predrug baseline. GABA showed a significant decrease in potency in treated (EC_{50}, 186.4 μM) *vs.* control slices (EC_{50}, 112.8 μM). There was no significant difference in the response to BAC between treated (EC_{50}, 7.2 μM) and control slices (EC_{50}, 6.5 μM). Each point represents the mean ± S.E.M. for each concentration. Each curve was generated from six slices taken from six rats/group. The response to GABA alone (EC_{50}, 294.7 μM; in the absence of nipecotic acid) is also shown (– – –, three slices/three rats).

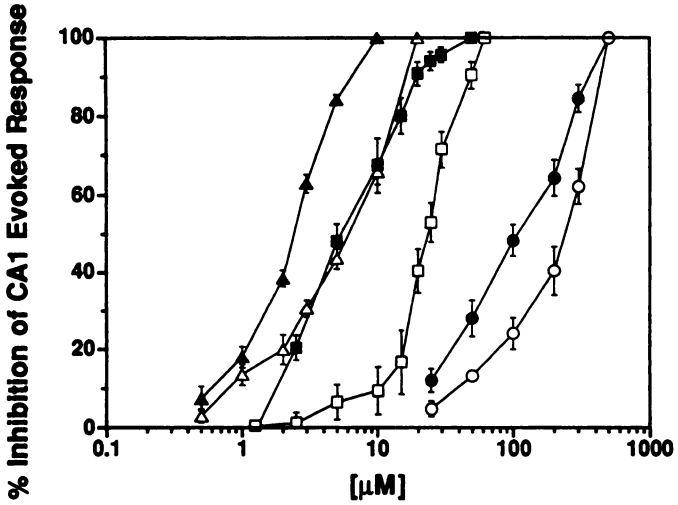

Fig. 3. MUS (▲, △), ISO (■, □) and THIP (●, ○) concentration-response curves in hippocampal slices from control rats (▲, ■, ●) and rats sacrificed 48 hr after 1 week of FZP treatment (△, □, ○). Drugs (10–100 μl) were added to the superfusate (2 ml/min), incrementing the concentration every 5 min. Drug responses, averaged over the last 2 min, were calculated as a fraction of the predrug baseline. Each point represents the mean ± S.E.M. for each concentration. Each curve was generated from six slices taken from six rats/group per drug tested. There was a significant decrease in potency between treated and control slices for each drug (EC_{50} in micromolar: MUS, 6.1 *vs.* 2.4; ISO, 23.6 *vs.* 5.2; and THIP, 238.3 *vs.* 106.7).

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 $GABA_B$ 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

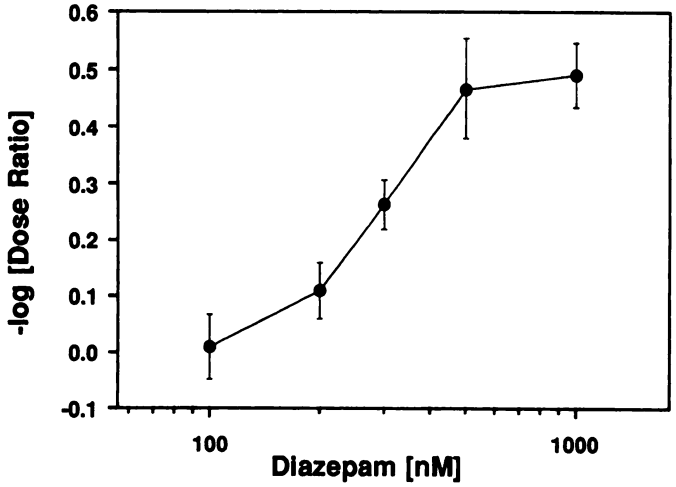

Fig. 4. Concentration-response curve for the ability of DZP to increase the potency of ISO in hippocampal slices from control rats. DZP concentration was plotted against the −log [Dose Ratio]. [Dose Ratio] = EC_{50} (ISO + DZP)/EC_{50} (ISO). Each point is the mean ± S.E.M. of three to eight determinations.

GABA (Kemp *et al.*, 1986) and benzodiazepine (Kemp *et al.*, 1987) actions in hippocampal slices was highly correlated with their ability to displace [^{3}H]GABA and [^{3}H]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 the finding that the allosteric interaction between GABA and benzodiazepine receptor sites was not altered in hippocampal 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 $GABA_A$ 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 $GABA_A$ presynaptic receptors cannot be ruled out (Waldmeir and Baumann, 1990). The significant correlation between $GABA_A$ agonist potency and the average duration of Cl^- channel opening in spinal cord cultures (Barker and Mathers, 1981) would suggest that the reduction in $GABA_A$ 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.

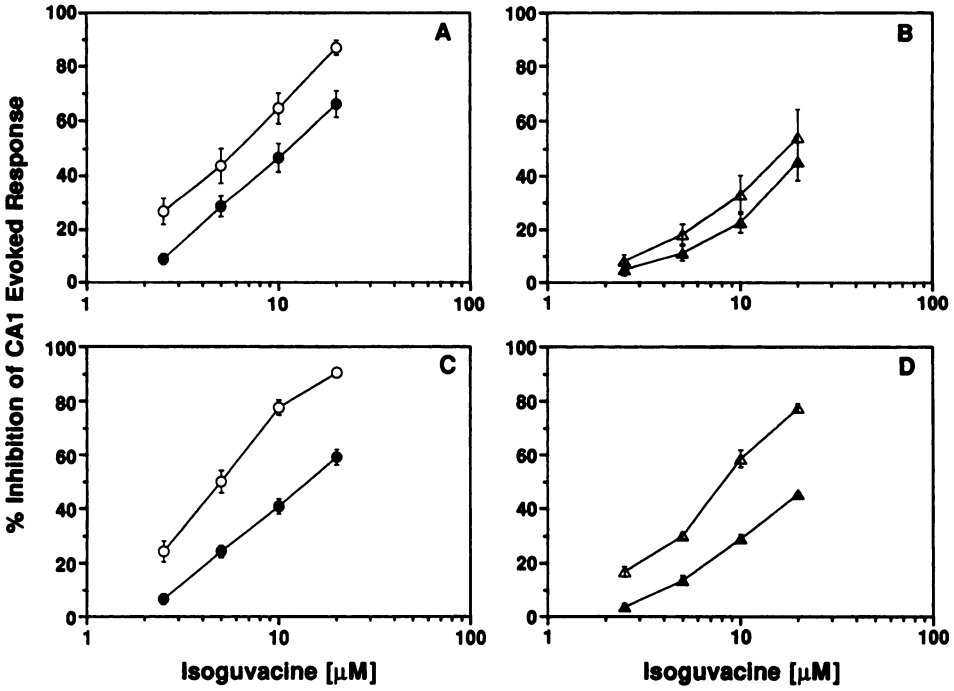

Fig. 5. DZP was tested in slices 2 days after 1 week of FZP treatment by its ability to shift the ISO dose-response curve. After superfusion of ISO (2.5–20 μM), DZP (300 nM or 1 μM) was superfused for 20 min before a second ISO concentration-effect curve was determined in the presence of DZP. In control slices (A and C), 300 nM DZP (A; n = 8 slices/8 rats) and 1 μM DZP (C; n = 6 slices/6 rats) significantly shifted the ISO curve (ISO alone, ●; ISO + DZP, ○). In treated slices (B and D), the ability of DZP to shift the ISO concentration-response curve (ISO alone, ▲; ISO + DZP = △) was significantly decreased with 300 nM DZP (B; n = 8 slices/8 rats) but not 1 μM DZP (D; n = 6 slices/6 rats). Each point represents the mean ± S.E.M. for each concentration.

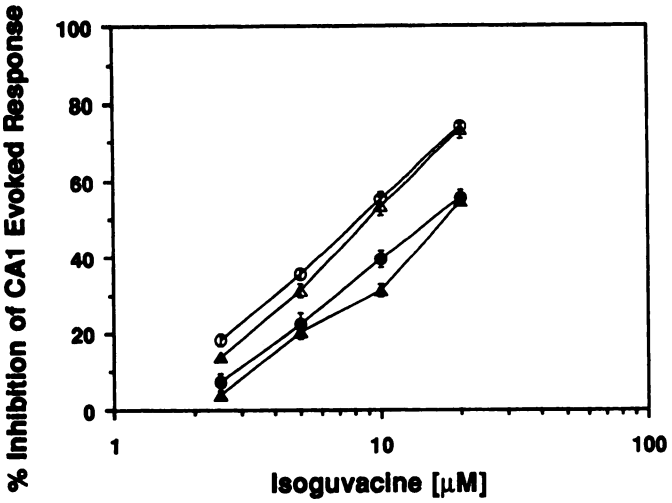

Fig. 6. The ability of DZP (300 nM) to shift the ISO concentration-response curve (2.5–20 μM) was tested *in vitro* 2 days after a single, acute pretreatment with desalkyl-FZP (2.5 mg/kg p.o.). There was no significant difference between the ISO concentration-response curve generated in treated (▲; EC_{40} = 13.7 μM) *vs.* control slices (●; EC_{40} = 10.2 μM) or in the action of 300 nM DZP to shift the ISO curve (ISO + DZP: treated, △, EC_{40} = 7.0 μM *vs.* control; ○, EC_{40} = 6.1 μM). Each point represents the mean ± S.E.M. for each concentration. Each curve was generated from four slices taken from four rats/group per drug tested.

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 $GABA_A$, 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 actions (Blaxter and Carlen, 1985).

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 (Haefely, 1985). Therefore, chronic benzodiazepine 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 [^{3}H]GABA release (Hitchcott *et al.*, 1990). However, recent data from our laboratory has shown that KCl-evoked [^{3}H]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. 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 Krogsgaard-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 ($EC_{40} \approx 20$ 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 (α_{1-6}, β_{1-3}, γ_2 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

and benzodiazepine agonists. Chronic benzodiazepine treatment has been reported to decrease the expression of α_1 (Heninger *et al.*, 1990; Kang and Miller, 1991) and γ_2 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 $GABA_A$ subunit mRNAs are expressed in the hippocampus, including the CA1 region (Möhler *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 $GABA_A$ 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 $GABA_A$ 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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References

ADAMEC, R. E., MCNAUGHTON, B., RACINE, R. AND LIVINGSTON, K. E.: Effects of diazepam on hippocampal excitability in the rat: Action in the dentate area. Epilepsia (Basel) **22:** 205–215, 1981.

ALGER, B. E. AND NICOLL, R. A.: Pharmacological evidence for two kinds of GABA receptors on rat hippocampal pyramidal cells studied *in vitro*. J. Physiol. (Lond.) **328:** 125–141, 1982.

ANDRADE, R., MALENKA, R. C. AND NICOLL, R. A.: A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science (Wash. DC) **234:** 1261–1265, 1986.

BARKER, J. L. AND MATHERS, D. A.: GABA analogues activate channels of different duration on cultured mouse spinal neurons. Science (Wash. DC) **212:** 358–360, 1981.

BLAXTER, T. J. AND CARLEN, P. L.: Pre- and postsynaptic effects of baclofen in the rat hippocampal slice. Brain Res. **341:** 195–199, 1985.

BOWERY, N. G., HILL, D. R. AND HUDSON, A. L.: Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. Br. J. Pharmacol. **78:** 191–206, 1983.

BREHM, L., KROGSGAARD-LARSEN, P. AND JACOBSEN, P.: GABA uptake inhibitors and structurally related "pro-drugs." *In* GABA Neurotransmitters: Pharmacological, Biochemical and Pharmacological Aspects, ed. by P. Krogsgaard-Larsen, J. Scheel-Kruger and H. Kofod, pp. 247–262, Academic Press, New York, 1979.

CHU, D. C. M., ALBIN, R. L., YOUNG, A. B. AND PENNEY, J. B.: Distribution and kinetics of $GABA_B$ binding sites in rat central nervous system: A quantitative autoradiographic study. Neuroscience **34:** 341–357, 1990.

FALCH, E. AND KROGSGAARD-LARSEN, P.: The binding of the GABA agonist [^{3}H]THIP to rat brain synaptic membranes. J. Neurochem. **38:** 1123–1129, 1982.

GALLAGER, D. W., LAKOSKI, J. M., GONSALVES, S. F. AND RAUCH, S. L.: Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity. Nature (Lond.) **308:** 74–76, 1984a.

GALLAGER, D. W., MALCOLM, A. B., ANDERSON, S. A. AND GONSALVES, S. F.: Continuous release of diazepam: Electrophysiological, biochemical and behavioral consequences. Brain Res. **342:** 26–36, 1985.

GALLAGER, D. W., RAUCH, S. L. AND MALCOLM, A. B.: Alterations in a low affinity GABA recognition site following chronic benzodiazepine treatment. Eur. J. Pharmacol. **98:** 159–160, 1984b.

GIBBS, T. T., CHAN, C. Y., CZAJKOWSKI, C. M. AND FARB, D. H.: Benzodiazepine receptor photoaffinity labeling: Correlation of function with binding. Eur. J. Pharmacol. **110:** 171–180, 1985.

HAEFELY, W.: The biological basis of benzodiazepine action. *In* The Benzodiazepines: Current Standards for Medical Practice, ed. by D. E. Smith and D. R. Wesson, pp. 7–41, MTP Press, Lancaster, 1985.

HENINGER, C., SAITO, N., TALLMAN, J. F., GARRETT, K. M., VITEK, M. P., DUMAN, R. S. AND GALLAGER, D. W.: Effects of continuous diazepam administration on $GABA_A$ subunit mRNA in rat brain. J. Mol. Neurosci. **2:** 101–107, 1990.

HITCHCOTT, P. K., FILE, S. E., EKWURU, M. AND NEAL, M. J.: Chronic diazepam treatment in rats causes long-lasting changes in central [^{3}H]-5-hydroxytryptamine and [^{14}C]-γ-aminobutyric acid release. Br. J. Pharmacol. **99:** 11–12, 1990.

KANG, I. AND MILLER, L. G.: Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. Br. J. Pharmacol. **103:** 1283–1287, 1991.

KEMP, J. A., MARSHALL, G. R., WONG, E. H. F. AND WOODRUFF, G. N.: The affinities, potencies and efficacies of some benzodiazepine-receptor agonists, antagonists and inverse-agonists at rat hippocampal $GABA_A$-receptors. Br. J. Pharmacol. **91:** 607–608, 1987.

KEMP, J. A., MARSHALL, G. R. AND WOODRUFF, G. N.: Quantitative evaluation of the potencies of GABA-receptor agonists and antagonists using the rat hippocampal slice preparation. Br. J. Pharmacol. **87:** 677–684, 1986.

KROGSGAARD-LARSEN, P. AND JOHNSTON, G. A. R.: Inhibition of GABA uptake in rat brain slices by nipecotic acid, various isoxazoles and related compounds. J. Neurochem. **25:** 792–802, 1975.

LISTA, A., BLIER, P. AND MONTIGNY, C. D.: Benzodiazepine receptor modulating serotonergic neurotransmission in rat hippocampus do not desensitize after long-term diazepam treatment. Brain Res. **526:** 161–164, 1990.

MARLEY, R. J. AND GALLAGER, D. W.: Chronic diazepam treatment produces regionally specific changes in GABA-stimulated chloride influx. Eur. J. Pharmacol. **159:** 217–223, 1989.

MILLER, L. G., GREENBLATT, D. J., BARNHILL, J. G. AND SHADER, R. I.: 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, 1988.

MÖHLER, H., MALHERBE, P., DRAGUHN, A. AND RICHARDS, J. G.: $GABA_A$-receptors: Structural requirements and sites of gene expression in mammalian brain. Neurochem. Res. **15:** 199–207, 1990.

NEWBERRY, N. R. AND NICOLL, R. A.: Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells. Nature (Lond.) **308:** 450–451, 1984.

RAMSEY, V. A., TIETZ, E. I. AND ROSENBERG, H. C.: Chronic flurazepam differentially regulates a behavioral effect of GABA agonists. Pharmacol. Biochem. Behav. **38:** 659–663, 1991.

REYNOLDS, J. N., WU, P. H., KHANNA, J. M. AND CARLEN, P. L.: Ethanol tolerance in hippocampal neurons: Adaptive changes in cellular responses to ethanol measured *in vitro*. J. Pharmacol. Exp. Ther. **252:** 265–271, 1990.

ROCK, D. M. AND TAYLOR, C. P.: Effects of diazepam, pentobarbital, phenytoin and pentylenetetrazol on hippocampal paired-pulse inhibition *in vivo*. Neurosci. Lett. **65:** 265–270, 1986.

ROSENBERG, H. C. AND CHIU, T. H.: Regional specificity of benzodiazepine receptor down-regulation during chronic treatment of rats with flurazepam. Neurosci. Lett. **24:** 49–52, 1981.

ROSENBERG, H. C. AND CHIU, T. H.: Time course for development of benzodiazepine tolerance and physical dependence. Neurosci. Biobehav. Rev. **9:** 123–131, 1985.

ROSENBERG, H. C., TIETZ, E. I. AND CHIU, T. H.: Tolerance to the anticonvulsant action of benzodiazepines: Relationship to decreased receptor density. Neuropharmacology **24:** 639–644, 1985.

ROSENBERG, H. C., TIETZ, E. I. AND CHIU, T. H.: Differential tolerance to the anti-pentylenetetrazol activity of benzodiazepines in flurazepam-treated rats. Pharmacol. Biochem. Behav. **39:** 711–716, 1991.

ROSENBERG, H. C., TIETZ, E. I., ZHANG, H. AND CHIU, T. H.: Tolerance to positive and negative modulators of GABA function measured in substantia nigra of benzodiazepine tolerant rats. Life Sci. **46:** 519–525, 1990.

SHER, P. K.: Long-term exposure of cortical cell cultures to clonazepam reduces benzodiazepine receptor binding. Exp. Neurol. **92:** 369–368, 1986.

SHER, P. K., STUDY, R. E., MAZZETTA, J., BARKER, J. L. AND NELSON, G. P.: Depression of benzodiazepine binding and diazepam potentiation of GABA-mediated inhibition after chronic exposure of spinal cord cultures to diazepam. Brain Res. **268:** 171–176, 1983.

TIETZ, E. I., CHIU, T. H. AND ROSENBERG, H. C.: Regional GABA/benzodiazepine receptor/chloride channel coupling after acute and chronic benzodiazepine treatment. Eur. J. Pharmacol. **167:** 57–65, 1989.

TIETZ, E. I. AND ROSENBERG, H. C.: Behavioral measurement of benzodiazepine tolerance and GABAergic subsensitivity in the substantia nigra pars reticulata. Brain Res. **438:** 41–51, 1988.

TIETZ, E. I., ROSENBERG, H. C. AND CHIU, T. H.: Autoradiographic localization of benzodiazepine receptor downregulation. J. Pharmacol. Exp. Ther. **236:** 284–292, 1986.

TIETZ, E. I. AND XIE, X-H.: KCl-evoked GABA release unchanged in *in vitro*

hippocampus after chronic flurazepam treatment. Soc. Neurosci. Abstr. **17:** 1601, 1991.

TUFF, L. P., RACINE, R. J. AND ADAMEC, R.: The effects of kindling on GABA-mediated inhibition in the dentate gyrus of the rat. I. Paired-pulse depression. Brain Res. **277:** 79–90, 1983.

TYMA, J. L., ROSENBERG, H. C., TIETZ, E. I. AND CHIU, T. H.: Effects of chronic flurazepam treatment on firing rate of rat substantia nigra pars reticulata neurons. Brain Res. **453:** 344–348, 1988.

WALDMEIER, P. C. AND BAUMANN, P. A.: Presynaptic GABA receptors. Ann. N.Y. Acad. Sci. **604:** 136–151, 1990.

WALDMEIER, P. C., WICKI, P., FELDTRAUER, J.-J. AND BAUMANN, P. A.: Potential involvement of a baclofen-sensitive autoreceptor in the modulation of the release of endogenous GABA from rat brain slices *in vitro*. Naunyn-Schmiedeberg's Arch. Pharmacol. **337:** 289–285, 1988.

WILSON, M. A. AND GALLAGER, D. W.: Effects of chronic diazepam exposure on GABA sensitivity and on benzodiazepine potentiation of GABA-mediated responses of substantia nigra pars reticulata neurons of rats. Eur. J. Pharmacol. **136:** 333–343, 1987.

WILSON, M. A. AND GALLAGER, D. W.: GABAergic subsensitivity of dorsal raphe neurons *in vitro* after chronic benzodiazepine treatment *in vivo*. Brain Res. **473:** 198–202, 1988.

WOODSON, W., NITECKA, L. AND BEN-ARI, Y.: Organization of the GABAergic system in the rat hippocampal formation: A quantitative immunocytochemical study. J. Comp. Neurol. **280:** 254–271, 1989.

XIE, X.-H. AND TIETZ, E. I.: Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of *in vitro* hippocampus. Brain Res. **561:** 69–76, 1991.

YU, O., CHIU, T. H. AND ROSENBERG, H. C.: Modulation of GABA-gated chloride ion flux in rat brain by acute and chronic benzodiazepine administration. J. Pharmacol. Exp. Ther. **246:** 107–113, 1988.

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