Pergamon

Pharmacology Biochemistry and Behavior, Vol. 48, No. 3, pp. 765–772, 1994

0091-3057/94 $6.00 + .00

0091-3057(94)E0053-K

Comparison of Anticonvulsant Tolerance, Crosstolerance, and Benzodiazepine Receptor Binding Following Chronic Treatment With Diazepam or Midazolam

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Received 16 February

RAMSEY-WILLIAMS, V. A., Y. WU AND H. C. ROSENBERG. *Comparison of anticonvulsant tolerance, crosstolerance, and benzodiazepine receptor binding following chronic treatment with diazepam or midazolam.* PHARMACOL BIOCHEM BEHAV **48**(3) 765–772, 1994.—In a previous study, rats treated chronically with flurazepam were tolerant to the anticonvulsant action of some benzodiazepines (BZs), but not others (34). To determine if this differential crosstolerance was unique to flurazepam, rats were treated chronically with diazepam or midazolam, and tested for tolerance to the anticonvulsant actions of diazepam, midazolam, clonazepam, and clobazam. Regional benzodiazepine receptor binding in brain was also studied. In contrast to previous findings with flurazepam, 1 week treatment with diazepam or with midazolam did not cause tolerance. Rats treated with diazepam for 3 weeks were tolerant to diazepam, clonazepam, clobazam, and midazolam. In contrast, rats treated 3 weeks with midazolam were tolerant to diazepam and midazolam, but not clobazam or clonazepam. Neither diazepam nor midazolam treatment for 3 weeks altered BZ binding in cerebral cortex, cerebellum, or hippocampus. The effects of chronic BZ treatment depended not only on the BZ given chronically, but also on the BZ used to evaluate these effects, suggesting drug-specific interactions of different BZs with their receptors.

Tolerance Crosstolerance Diazepam Midazolam Clonazepam Clobazam Benzodiazepine receptor Anticonvulsant

THE development of tolerance to benzodiazepines (BZs) is well documented. For example, tolerance develops to the sedative effects of alprazolam (23), clonazepam (12), lorazepam, and triazolam (6); to the motor-impairing effects of flurazepam (30,31) and lorazepam (22); and to the anticonvulsant effects of diazepam (7,11,15), flurazepam (34), and clonazepam (38). Comparison of the effects of different chronic BZ treatments suggests that all BZs do not have the same tolerance-inducing potentials, and that the detection and characteristics of tolerance depend upon which BZ is administered chronically. Differences in the ability to produce tolerance have been suggested between diazepam and CGS 9896 (1), diazepam and clorazepate (8), clonazepam and clorazepate (37), and between clobazam and clonazepam (14,33,47).

BZ crosstolerance studies have shown that, in addition to the choice of BZ for chronic treatment, the choice of BZ used to measure tolerance may influence the detection and characteristics of tolerance. In a study of the antipentylenetetrazol (PTZ) action of BZs, chronic treatment of rats with flurazepam resulted in tolerance to flurazepam, and crosstolerance to some, but not all BZs tested (34). Ruhland (35) found various patterns of tolerance to three actions of diazepam, bromazepam, clobazam, and metaclazepam (a 2-methoxy derivative) in separate groups of mice treated daily for 9 days with each of these BZs. In another study in dogs (8), in which PTZ seizure threshold elevation was used to measure BZ action, tolerance to diazepam was detected within a week. When dogs were treated with clorazepate (which is completely converted to desmethyl-diazepam during absorption), they were tolerant to diazepam, but not to desmethyl-diazepam.

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The differing patterns of tolerance and crosstolerance produced by BZs may indicate nonuniform interactions of BZs with their receptor sites both during chronic treatment and when the animal is challenged with a test drug.

Changes in BZ receptor binding provide another measure of chronic drug effects on the central nervous system (CNS), and may also be dependent on the drug used for chronic treatment. For example, treatments with lorazepam or alprazolam in mice resulted in different regional patterns of BZ receptor downregulation, as well as differential effects on type II (CL 218,872-resistant) BZ binding (23). Clonazepam treatment of mice resulted in a greater decrease in BZ binding site density than did chlordiazepoxide treatment (4). Differential chronic effects of BZs suggests distinctive receptor interactions among the BZs.

This study compared the effects of chronic treatment with diazepam and midazolam on tolerance to the anti-PTZ action of four BZs, and on BZ receptor binding. Diazepam and midazolam were chosen because flurazepam-tolerant rats were tolerant to diazepam, but not midazolam (34), and because both have been used in studies of BZ tolerance in the rat (2,15,36). The tolerance and crosstolerance patterns produced by 1- and 3-week treatment with diazepam or midazolam were compared. In addition, the effects of 3-week exposure to diazepam or midazolam on BZ receptor binding were assayed. Diazepam and midazolam treatment intensities were evaluated using a radioreceptor assay to monitor drug concentrations in brain.

METHOD

Experimental subjects were adult male, Sprague–Dawley rats weighing 250–375 g at the time of testing or sacrifice. Different animals were used for anticonvulsant tolerance testing and binding assays. BZs used for tolerance testing were dissolved in a solvent of 60% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, 5% sodium benzoate, 2.25% benzoic acid, and distilled water. PTZ was purchased from Sigma. [^{3}H]Flunitrazepam (81–85 Ci/mmol) for use in BZ binding assays was purchased from Amersham. Diazepam, midazolam, and clonazepam were provided by Hoffmann-LaRoche, Inc. Clobazam was supplied by Hoechst-Roussel Pharmaceuticals.

Chronic Diazepam Treatment

Diazepam was continuously administered to rats via release from SC silastic reservoirs as described previously (11). This treatment has previously been shown to produce brain diazepam levels of approximately 250 ng/g, and to result in tolerance to the anti-PTZ effect of BZs (11,15). Silastic reservoirs containing 90 mg of recrystallized diazepam were prepared, and implanted in rats under ether anesthesia, as previously described (11). Control animals were handled identically, but received empty, sealed silastic tubes. Two tubes were initially implanted per rat, followed by a supplemental tube on day 10 in 3-week diazepam-treated rats. All tubes were removed, during ether anesthesia, 12 h before testing on day 7 or 21 of treatment.

Chronic Midazolam Treatment

Large doses of midazolam, which has appreciable water solubility, can be administered to rats in the drinking water (5). Midazolam was administered the same way as had been done for chronic treatment of rats with flurazepam (30). Rats were offered as their sole source of fluid either 0.02% saccharin solution (control) or saccharin solution containing midazolam. A 20 mg/ml stock solution of midazolam, dissolved in 0.1 N HCl, was used to prepare the drugged saccharin water. The maximal midazolam concentration was 0.5 mg/ml. Based on each rat's body weight and volume of fluid consumed, the drug concentrations were adjusted for the next day in efforts to achieve the target dose of 40 mg/kg/day. Midazolam treatment was continued for 1 or 3 weeks. Twelve hours before testing on day 7 or 21 of treatment, drugged water was replaced with saccharin solution.

Tolerance Testing

Rats were tested for tolerance 12 h after ending the chronic treatment. The observer was unaware of the chronic treatment each animal had received. BZ (either diazepam, clonazepam, midazolam, or clobazam) was injected IP, and PTZ (100 mg/kg, IP) was injected 10 min later. Doses of BZs were chosen based on the previous study of tolerance in flurazepam-treated rats (34), and are indicated in the tables. The 10-min interval was based on observations of the time course for ataxia produced by larger doses of these same BZs, and on the pharmacokinetics of IP diazepam in the rat (9).

Convulsions were observed and rated for 30 min following PTZ injection. A scale of 0–4 was used to assign a seizure rating to each rat according to the most severe seizure observed (34). The seizure ratings and their corresponding behavioral components were: 0 = no observable convulsive activity; 1 = myoclonic jerking; 2 = forelimb clonus; 3 = clonus of all four limbs or a nonlethal tonic–clonic seizure (tonic extension of the front legs and clonus of the back legs); 4 = lethal tonic–clonic seizure. The occurrence and time at onset of each seizure component was noted.

Brain Benzodiazepine Determination by Radioreceptor Assay

The amount of BZ present in brain on days 7 and 21 of chronic diazepam treatment and at 12 h after removing the diazepam-filled reservoirs, was evaluated by the BZ radioreceptor assay. For midazolam-treated rats, radioreceptor assays were performed on days 3, 7, 14, and 21 of treatment. These rats were killed at 2200 h (during the period when the rats were most actively drinking) because no residual BZ could be detected in brain extracts from rats killed in the morning. This was probably due to the short half life (0.5 to 1 h) of midazolam in the rat (5,40).

The assay is based on the ability of an extract, prepared from the treated rat brain, to displace [^{3}H]flunitrazepam specifically bound to brain membranes from naive rats. Displacement of specifically bound [^{3}H]flunitrazepam is due to the total BZ-displacing activity in the brain extract, including that due to active metabolites, and will reflect the concentration of each drug species, as well as its binding affinity at the receptor. Rats were sacrificed by decapitation. Based on the method of Fujimoto and Okabayashi (10), whole rat brains were homogenized in 10 vol ice-cold ethanol and centrifuged at 13,000 $\times$ g for 20 min at 4°C. The supernatants were saved and 50 or 100 μl aliquots evaporated to complete dryness in a test tube. These extracts were resuspended in assay buffer (50 mM Tris-HCl$^-$, pH 7.4 at 4°C). For midazolam brain extracts, the dried residue was resuspended in 50 μl 0.001 N HCl. The same concentration of HCl was added to all tubes in midazolam assays. Radioreceptor assays were performed as previously described (32) using 2 nM [^{3}H]flunitrazepam bound to cerebral cortical membranes obtained from a drug naive rat. Non-

specific binding was determined in the presence of 2 μM clonazepam. The assay mixture was incubated for 1 h after adding brain homogenate and an additional 1 h after adding [^{3}H]flunitrazepam. The displacement of specific binding by the brain extract (in duplicate) was compared to a standard displacement curve constructed using known amounts of diazepam or midazolam (in triplicate). The amount of BZ-displacing activity in the extracts was calculated using a log-probit calculation program.

Benzodiazepine Binding Assay

The effect of chronic BZ treatment on brain BZ receptor binding was measured in brain homogenates from treated rats killed 12 h after removing diazepam-filled reservoirs, and in the morning at the end of the 3-week midazolam treatment. Rats were sacrificed by decapitation and the brains removed and dissected on ice. Regions assayed included cerebral cortex, hippocampus, and cerebellum. If not used immediately, each brain section was weighed, wrapped in foil, and placed in a closed vial, then quickly frozen in an acetone/dry ice bath and stored at −70°C. At the time of assay, brain sections were placed in ice cold 0.32 M sucrose and homogenized by 15 passes in a Teflon/glass homogenizer. The homogenates were centrifuged at 4°C for 10 min at 1000 × *g*. The supernatants were transferred to clean tubes and centrifuged for an additional 20 min at 20,000 × *g* at 4°C. The crude pellets were then washed three times by resuspending each pellet in 25 ml Tris-HCl$^-$ buffer (50 mM, pH 7.4 at 4°C) and centrifuging for 20 min at 20,000 × *g* at 4°C. Assay tubes each contained 50 μl buffer or unlabelled clonazepam (2 μM final concentration, to measure nonspecific binding), and 50 μl of the appropriate [^{3}H]flunitrazepam concentration (0.5–20 nM final concentration). Brain homogenate (400 μl) was added to initiate the binding reaction. Tubes were incubated on ice for 1 h and then filtered through glass fiber filters (Whatman GF/B) that had been soaked in ice-cold buffer. Filters were then washed three times with 5 ml ice cold buffer. Radioactivity remaining on the filters was counted in 5 ml Cytoscint cocktail (ICN Biomedicals). Scatchard analysis of all saturation binding data was performed. Protein concentrations were measured using the BCA (bicinchoninic acid) reagent kit (Pierce Chemical Co.).

Data Analysis

Maximal seizure scores were analyzed by the Kruskal–Wallis test. Times to onset of convulsive activity (jerking) were analyzed by Student's *t*-test, using data only from those rats that had PTZ-induced jerking. BZ binding data were analyzed by *t*-test. Tolerance was indicated by a decrease in the acute BZ effect, which was measured as a decrease in the maximum seizure score, or by an increased latency to onset of convulsive activity.

RESULTS

Chronic Diazepam Treatment

The results of the radioreceptor assays of BZ-displacing activity in 7 and 21 day diazepam-treated rats (Table 1) were similar to those reported previously for rats receiving the same diazepam treatment (11). In brain extracts from rats that had been killed 12 h after ending the 3-week diazepam treatment, the amount of BZ-displacing activity (expressed in diazepam equivalents) was below the limits of detection (<15 ng/g). Diazepam-treated rats tended to gain less weight over the 3-week treatment period than empty tube-treated controls. For example, one group of diazepam-treated rats gained 60.8 ± 3.9 g, whereas the average weight gain for their corresponding controls was 84.0 ± 3.2 g. Body weights for control and treated rats before implantation were not different from each other.

One week diazepam treatment did not result in significant tolerance to the anti-PTZ effect of diazepam (5 mg/kg), or crosstolerance to clonazepam (0.5 mg/kg), clobazam (10 mg/kg), or midazolam (5 mg/kg) when maximal seizure severity was evaluated (Kruskal–Wallis test, $p > 0.05$; Table 2). The latency to onset of convulsive activity (jerking) also failed to show tolerance in rats treated 1 week with diazepam (Student's *t*-test, $p > 0.05$; data not shown).

In contrast to the shorter diazepam treatment, 3-week diazepam treatment produced tolerance to the anti-PTZ action of the BZs. Three-week diazepam-treated rats had more severe PTZ-induced convulsions than control rats (Table 3) after acute pretreatment with diazepam (5 mg/kg), clonazepam (0.5 mg/kg), or midazolam (5 mg/kg) (Kruskal–Wallis test, $p < 0.01$). There were no differences in maximum seizure scores between control and treated rats acutely pretreated with the other dose of clonazepam (1 mg/kg) or midazolam (2 mg/kg), nor in rats pretreated with either the 10 or 15 mg/kg dose of clobazam. When the latency to onset of convulsion was evaluated, it was noted that chronically treated rats had a shorter latency to onset of PTZ-induced convulsive activity than the controls (Fig 1A). This measure tended to be quite

TABLE 1

BZ-DISPLACING ACTIVITY IN RAT BRAIN EXTRACTS DURING CHRONIC TREATMENT WITH DIAZEPAM OR MIDAZOLAM, EXPRESSED AS DIAZEPAM EQUIVALENTS (ng/g BRAIN OR μM)

Chronic BZ	Treatment Duration	*n*	[Diazepam] (ng/g)	[Diazepam] (μM)
Diazepam	7 days	5	203.34 ± 31.81	0.71 ± 0.11
	21 days	6	273.13 ± 39.70	0.96 ± 0.14
Midazolam	3 days	6	82.92 ± 21.70	0.29 ± 0.08
	7 days	5	193.75 ± 58.98	0.68 ± 0.21
	14 days	6	120.81 ± 32.18	0.43 ± 0.11
	*21 days	5	45.05 ± 9.54	0.16 ± 0.03

*Significantly different from 1 week midazolam-treated rats ($p < 0.05$, Student's *t*-test).

TABLE 2

DISTRIBUTION OF MOST SEVERE SEIZURE COMPONENTS IN CONTROLS AND 1 WEEK BZ-TREATED RATS GIVEN THE ACUTE TEST DRUG 10 MIN BEFORE PTZ

Chronic Treatment	Test* Drug (mg/kg)	n	Group	Most Severe Seizure				
				No Seizure	Jerks	Front Leg Clonus	4-Leg Clonus	Tonic-Clonic
1 week diazepam	DZP	6	Control	3	–	2	1	–
	5	6	Treated	3	–	3	–	–
	CZP	6	Control	1	–	4	–	1
	0.5	6	Treated	–	1	5	–	–
	MDZ	6	Control	1	1	2	–	2
	5	6	Treated	1	–	4	–	1
	CBZ	10	Control	1	–	8	1	–
	10	9	Treated	–	–	7	1	1
1 week midazolam	DZP	8	Control	5	–	3	–	–
	5	7	Treated	2	–	4	–	1
	CZP	4	Control	–	–	3	–	1
	0.5	4	Treated	–	–	4	–	–
	MDZ	5	Control	3	1	1	–	–
	5	6	Treated	2	–	2	–	2

*DZP, diazepam; CZP, clonazepam; MDZ, midazolam; CBZ, clobazam.

variable, and many control rats were completely protected, and so could not be used for this analysis. Even so, the shorter latency to onset of PTZ-induced convulsions was statistically significant in diazepam treated rats that had been acutely pretreated with diazepam or with clobazam (Fig. 1A).

[^{3}H]Flunitrazepam binding in rat brain regions taken 12 h after ending the 3-week diazepam treatment revealed no changes in B_{max} or K_d in cerebral cortex, hippocampus, or cerebellum (Table 4). The protein concentrations in hippocampi and cerebella were not different in treated and control rats after 3 weeks of diazepam treatment; however, cortical protein concentration was greater in treated rats than in controls (23.54 ± 1.80 mg/g tissue vs. 17.90 ± 1.01 mg/g tissue).

Chronic Midazolam Treatment

The average daily dose of midazolam consumed by rats in the 1- and 3-week midazolam-treated groups was 39.01 ± 0.37 mg/kg/day. There were no differences in midazolam doses consumed by rats in different acute BZ pretreatment groups ($p > 0.05$, ANOVA). Brain BZ-displacing activity, expressed in diazepam equivalents, is shown in Table 1. BZ-displacing activity in midazolam-treated rat brains was significantly reduced at the 3-week time point (Student's t-test, $p < 0.05$), as compared with BZ activity at 1 week.

One-week midazolam treatment did not result in significant tolerance to the anti-PTZ effect of midazolam (5 mg/kg), or crosstolerance to clonazepam (0.5 mg/kg) or diazepam (5 mg/kg) when maximal seizure severity (Kruskal–Wallis test, $p > 0.05$; Table 2), or jerking latency was tested (t-test, $p > 0.05$; data not shown). Three-week midazolam treatment induced crosstolerance to the anti-PTZ effect of diazepam, as indicated by the more severe seizures in midazolam-treated rats as compared to controls (Kruskal–Wallis test, $p < 0.01$; Table 3). There was no difference in maximal seizure severity ($p > 0.05$) between control and chronic midazolam-treated rats tested after acute pretreatment with midazolam, clonazepam, or clobazam (Table 3). However, examining the latency to onset of jerking revealed a tendency toward shorter latencies in 3-week midazolam treated rats after most pretreatments (Fig. 1B). However, this was statistically significant only in the rats pretreated with 5 mg/kg midazolam.

Following 3-week midazolam treatment, there was no change in BZ binding density (B_{max}) or affinity (K_d) in the cerebral cortex, cerebellum, or hippocampus of treated rats (Table 4). Protein concentrations did not differ in any brain region examined after chronic midazolam treatment. Extracts of brain homogenates taken from midazolam treated rats showed no measurable [^{3}H]flunitrazepam-displacing activity in the radioreceptor assay.

DISCUSSION

This study evaluated the effects of chronic diazepam and midazolam treatments measured behaviorally, by anticonvulsant tolerance and crosstolerance, and biochemically, by receptor binding density and affinity. These results, along with those of a previous study (34), showed that the effects of chronic BZ treatment depended on which BZ was given chronically, and the choice of drug used to test the effects of BZ treatment.

Changes in BZ receptor binding and in BZ anticonvulsant effects are results of neural plasticity, which often involves regulation of receptor concentration (20). Chronic flurazepam treatment of rats caused BZ receptor downregulation in cerebral cortex and hippocampus, but not cerebellum (29,30,43). In the present study, neither diazepam nor midazolam treatment produced significant changes in BZ binding, though both treatments did cause anticonvulsant tolerance. A previous study (16) reported no change in binding of 0.5 nM [^{3}H]flunitrazepam to cerebral cortical homogenates after 3-week treatment with diazepam. The results of the saturation binding assays in cortex, hippocampus, and cerebellum confirmed and extended that finding, demonstrating that chronic diazepam did not alter BZ receptor density or affinity mea-

TABLE 3

DISTRIBUTION OF MOST SEVERE SEIZURE COMPONENTS IN 3 WEEK BZ-TREATED RATS GIVEN THE ACUTE TEST DRUG (mg/kg) 10 MIN BEFORE PTZ

Chronic Treatment	Test Drug (mg/kg)	n	Group	Most Severe Seizure				
				No Seizure	Jerks	Front Leg Clonus	4-Limb Clonus	Tonic-Clonic
3 week diazepam	*DZP	9	Control	6	2	1	–	–
	5	8	Treated	–	3	4	–	1
	*CZP	12	Control	4	3	4	–	1
	0.5	12	Treated	–	–	9	–	3
	CZP	8	Control	6	1	1	–	–
	1	8	Treated	5	–	1	1	1
	CBZ	11	Control	–	1	10	–	–
	10	12	Treated	–	1	8	–	3
	CBZ	7	Control	1	1	5	–	–
	15	8	Treated	1	1	5	–	1
	MDZ	8	Control	3	2	3	–	-
	2	8	Treated	1	–	6	–	1
	*MDZ	8	Control	7	–	1	–	–
	5	7	Treated	–	–	4		3
3 week midazolam	*DZP	9	Control	6	2	1	–	–
	5	6	Treated	–	–	2	–	4
	CZP	6	Control	1	–	5	–	–
	0.25	5	Treated	–	1	3	–	1
	CZP	10	Control	2	5	2	–	1
	0.5	10	Treated	2	4	3	–	1
	CZP	8	Control	6	1	1	–	–
	1	8	Treated	7	–	1	–	–
	CBZ	8	Control	1	1	6	–	–
	15	8	Treated	–	1	7	–	–
	MDZ	8	Control	2	3	2	1	–
	2	7	Treated	1	–	4	1	1
	MDZ	8	Control	5	1	2	–	–
	5	8	Treated	3	2	3	–	–

*$p < 0.05$, Kruskal-Wallis test.

sured in tissue homogenates from these three brain regions. It is possible that localized changes in BZ binding, not easily detected in homogenates, may have been present, as was found with quantitative receptor autoradiography in rats treated 1 week with flurazepam (43). If chronic treatment specifically affected one BZ receptor subtype, or if different subtypes were changed in opposing directions, as was reported in mice receiving lorazepam (13), changes in binding might be

TABLE 4

REGIONAL [^{3}H]FLUNITRAZEPAM BINDING FOLLOWING 3 WEEK BZ TREATMENTS

Chronic Treatment	Region	Group	n	B_{max} (pmol/mg)	K_d (nM)
Diazepam	Cortex	Control	5	2.37 ± 0.31	1.53 ± 0.13
		Treated	6	2.06 ± 0.25	1.46 ± 0.08
	Cerebellum	Control	5	1.90 ± 0.13	1.88 ± 0.21
		Treated	6	1.84 ± 0.07	1.64 ± 0.09
	Hippocampus	Control	4	2.37 ± 0.04	1.63 ± 0.30
		Treated	3	2.40 ± 0.07	1.54 ± 0.29
Midazolam	Cortex	Control	9	2.31 ± 0.10	1.69 ± 0.22
		Treated	9	2.18 ± 0.08	1.48 ± 0.11
	Cerebellum	Control	4	1.77 ± 0.03	1.55 ± 0.15
		Treated	4	1.73 ± 0.01	1.53 ± 0.14
	Hippocampus	Control	3	2.08 ± 0.25	1.24 ± 0.02
		Treated	3	1.96 ± 0.10	1.07 ± 0.08

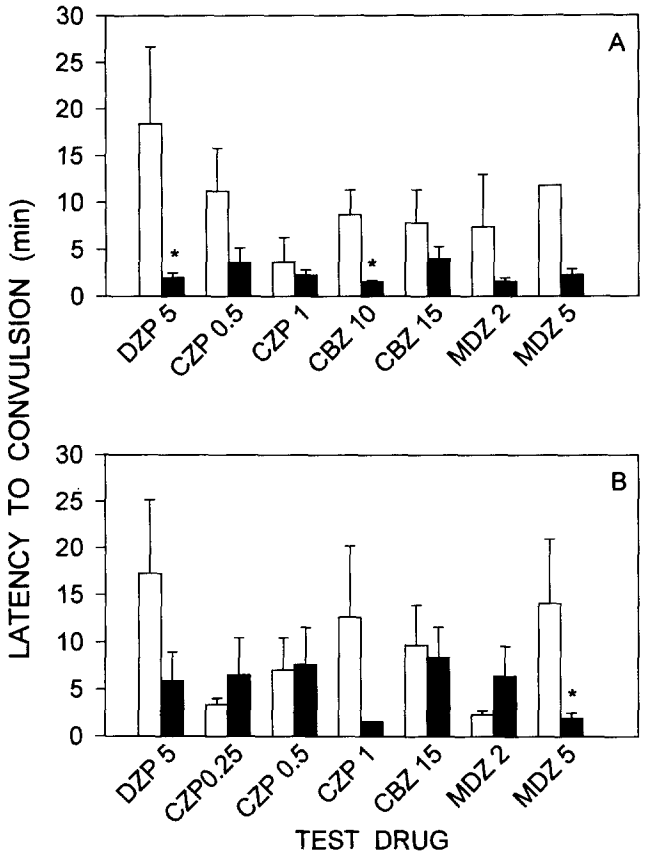

FIG. 1. Mean latency to first convulsive activity after pentylenetetrazol injection in control rats (open bars) and in rats treated for 3 weeks with benzodiazepine (solid bars). Vertical lines show one SEM. (A) Rats treated 3 weeks with diazepam. (B) Rats treated 3 weeks with midazolam. Rats were injected with benzodiazepine shown (abbreviations defined in Table 2) 10 min before pentylenetetrazol. Rats that were completely protected from convulsive activity are not included. Number of animals in each group can be obtained from Table 3. *$p < 0.05$, t-test.

more readily detected with a selective ligand rather than a nonselective BZ ligand such as flunitrazepam. Studies in rats treated with flurazepam for 4 weeks have shown just such a result, with [^{3}H]zolpidem binding revealing a more extensive BZ receptor downregulation, including in cerebellum, than could be detected previously (45). Similar studies will reveal if BZ receptor subtype-selective changes may be taking place during diazepam or midazolam treatments.

Comparing the patterns of tolerance and crosstolerance produced in 3-week BZ-treated rats, it was evident that the effects of diazepam and midazolam treatments are dissimilar. For example, 3-week diazepam treatment resulted in tolerance to the anticonvulsant action of diazepam and crosstolerance to clonazepam, midazolam, and clobazam. Three-week midazolam treatment resulted in tolerance to the anticonvulsant action of midazolam and crosstolerance to diazepam, but did not induce crosstolerance to clobazam or clonazepam. From these results, it was not clear if crosstolerance to clobazam and clonazepam might require a longer or even higher dose midazolam treatment, or if the adaptations that occur in response to chronic midazolam treatment do not interfere with the acute actions of these BZs. Whatever mechanism may underlie this differential crosstolerance, it does suggest that differences among the BZs in their interactions with BZ receptors can determine whether or not tolerance will be observed. It has been suggested that the efficacy of the BZ might determine the degree or rate of tolerance development (1,14,47). This could not, however, explain the differential crosstolerance found in this study. Evidence has been presented that flurazepam may be a partial agonist, with lower efficacy than diazepam to enhance GABA-induced conductance in spinal cord neurons (3). However, flurazepam was shown to induce tolerance after only 1 week of exposure (34), whereas 1 week of treatment with diazepam or midazolam failed to result in measurable tolerance to the anticonvulsant effect of BZs.

One possibility for the different effects of diazepam and midazolam treatments was that the two treatments were not equieffective. This was assessed with radioreceptor assays of the amount of BZ in brain during chronic treatment. The brain levels of BZ-displacing activity after 1 and 3 weeks of diazepam treatment were very similar to previously reported results (11). During midazolam treatment, no measurable BZ-displacing activity could be found in brain extracts 12 h after stopping treatment. That was in keeping with the data of Falk and Tang (5), who reported little or no midazolam in rat serum immediately following a 3 h period of scheduled-induced drinking of a midazolam solution, and almost no metabolites within 3 h after the end of the drug ingestion. After 1 week of midazolam treatment, brain BZ-displacing activity was similar to that in 1 week diazepam-treated rats. However, by the end of the 3 week midazolam treatment, brain BZ-displacing activity was less. The data suggested that chronic midazolam may have induced its own biotransformation, as has been reported during high-dose chlordiazepoxide exposure (18). Increasing the dose of chronic midazolam would result in greater brain levels of midazolam, and might produce crosstolerance to all the BZs tested, as did diazepam. Even if midazolam did induce biotransformation, it can not explain why tolerance was present for diazepam and midazolam, but not clonazepam or clobazam after 3 weeks of treatment. The brain concentration of BZ-like activity in 1 week flurazepam-treated rats was equivalent to 0.57 μM diazepam (46), similar to that found after 1 week of treatment with diazepam or midazolam (Table 1). However, in contrast to 1 week flurazepam treatment (34), tolerance or crosstolerance to the anticonvulsant effect of BZs was not detected after 1 week of treatment with diazepam or midazolam. Thus, BZ treatments of similar intensity and duration did not have the same ability to produce tolerance.

No obvious difference in the structural or pharmacokinetic properties of BZs used in this or in a previous study (34) for chronic treatment or for tolerance testing can explain why the results depended on the particular BZ studied. One possibility is that the results were determined by differences among the BZs in their interactions with $GABA_A$/BZ receptors both during chronic treatment and at the time of anticonvulsant tolerance testing. These drug-receptor interactions would be affected by any changes in the receptor produced by chronic BZ treatment. The $GABA_A$ receptor is a ligand-gated ion channel composed of homologous proteins (39). Genes encoding multiple isoforms of $GABA_A$ receptor subunits have been cloned and the characteristics of expressed $GABA_A$ receptors were found to depend on the combinations of $GABA_A$ receptor subunit isoforms (27,41,44). Differences in tolerance, crosstolerance and receptor binding after different chronic BZ treatments may be due to differential regulation of subunit

expression by each BZ treatment, and differences among the drugs in their receptor interactions at the time of testing. Regulation of $GABA_A$ receptor subunit mRNA expression by chronic BZ treatment has been demonstrated by decreases in the mRNA levels for the α_1 and γ_2 subunits after chronic diazepam or lorazepam treatments (17,19,26), and by a decrease in mRNA for α_5, increases in mRNA for α_3 and α_6, but no change in α_1 or γ_2 during flurazepam treatment (24,25). Plasticity of $GABA_A$ receptor subunit composition might also determine differing effects of chronic BZ treatment on responses to $GABA_A$ agonists, such as those noted in previous studies (28,42). Similarly, $GABA_A$ receptor subunit composition could determine the effects of chronic BZ treatment on BZ binding. Downregulation of receptor number would depend on the subunit composition of $GABA_A$ receptors in the region, and the effects of the particular chronic BZ on expression of those subunits. For example, exchanging α subunits in expressed $GABA_A$ receptors can alter the receptor sensitivity to GABA (21), but would not affect the number of BZ binding sites. Future experiments may reveal a relationship between changes in $GABA_A$ receptor subunit expression and patterns of BZ tolerance.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. Ted H. Chiu and Elizabeth I. Tietz for their contributions to this research, which included laboratory space and insightful discussion. We also wish to thank Dr. Peter F. Sorter of Hoffmann-LaRoche Inc., Nutley, NJ, for the gifts of midazolam, diazepam, and clonazepam used in these studies. Clobazam was a gift from Val K. Wagner, Hoechst-Roussel Pharmaceuticals, Somerville, NJ. This work was supported by research grant RO1-DA02194 from the National Institute on Drug Abuse.

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