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BRIEF COMMUNICATION

Absence of Tolerance to the Excitatory Effects of Benzodiazepines: Clonazepam-Evoked Shaking Behavior in the Rat

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PRANZATELLI, M. R., A. DAILEY, M. LEVY AND A. DOLLISON. *Absence of tolerance to the excitatory effects of benzodiazepines: Clonazepam-evoked shaking behavior in the rat.* PHARMACOL BIOCHEM BEHAV **39**(4) 1021–1024, 1991.—Select benzodiazepine (BDZ) agonists, such as clonazepam, evoke wet-dog shakes (WDS) in the rat, a behavior which may be influenced by serotonergic drugs. To further study the role of serotonin (5-HT) and BDZ receptors in BDZ-induced WDS, we injected adult rats daily for 21 days with clonazepam and measured WDS and 5-HT_1, 5-HT_2, and BDZ receptors. Clonazepam 5 mg/kg upregulated 5-HT_1 and 5-HT_2 receptors in frontal cortex, but not in brainstem or spinal cord, compared to vehicle controls, without a change in BDZ receptors. A 10 mg/kg dose of the same drugs, however, did not alter 5-HT receptors. Chronic treatment with clonazepam failed to decrease clonazepam-induced WDS, resulting instead in a significant increase. The increase was prevented by chronic cotreatment with the 5-HT_2 antagonist ketanserin, which significantly down-regulated 5-HT_2 and BDZ sites. In vitro, clonazepam did not inhibit radioligand binding at 5-HT_1 or 5-HT_2 receptors in frontal cortex, brainstem, or spinal cord. Lack of tolerance to WDS evoked by clonazepam suggests different mechanisms for the excitatory and inhibitory effects of BDZs. The dose-independent effects of chronic clonazepam administration on 5-HT receptors are not mediated by activity of clonazepam at 5-HT receptor recognition sites.

Clonazepam 5-HT receptors Tolerance Benzodiazepines Shaking behavior

THE mechanism by which benzodiazepine (BDZ) agonists, used clinically for their inhibitory properties, paradoxically evoke excitatory phenomena such as wet-dog shakes (WDS) in the rat is unknown. WDS, generated in limbic structures (19, 34, 39) by a variety of pharmacologic manipulations (1, 2, 9, 17, 18, 38), are rapid shakes involving head and trunk (15). No human correlate of WDS has been established because WDS may be associated experimentally with limbic seizures (29), but also resemble myoclonic jerks and tics. Some WDS are linked to activation of serotonin$_2$ (5-HT_2) receptors (8, 27, 36, 40), while others are not (12,35). We recently reported that BDZ agonists with an N-group on their A-ring, such as clonazepam and nitrazepam, induced dose-dependent WDS, which were inhibited by the 5-HT_{1A} agonist 8-OH-DPAT but not by 5-HT_2 receptor ligands or by the BDZ receptor antagonist Ro 15-1788 (27). A number of other experimental and clinical observations also suggest a link between BDZ and 5-HT mechanisms at some level (6, 7, 31, 33, 37).

To further study the role of 5-HT and BDZ receptors in BDZ-evoked WDS, we measured BDZ-induced WDS, 5-HT_1, 5-HT_2, and central BDZ receptors in the central nervous systems of rats chronically treated with clonazepam. Other rats were chronically cotreated with the 5-HT_2 receptor antagonist ketanserin, which down-regulates 5-HT_2 sites (13).

Animals

Male 150–200 g Sprague-Dawley rats (Charles River) were housed four to a cage at 23°C under a 12-hour light-dark cycle with free access to food and water.

Drugs

Clonazepam (Roche) and ketanserin tartrate (Janssen) were prepared each day by warming and sonication in 50% ethanol and 50% propylene glycol. Drug injection volume was 1 ml/kg.

Chronic Drug Injections

All rats received only one drug (or two-drug combination) at a single dose for 21 consecutive days. Naive rats were injected

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once daily (8–11 a.m.) with clonazepam 5 or 10 mg/kg or vehicle. A different group of rats was also coinjected with vehicle or ketanserin.

Behavioral Scoring

WDS were counted immediately following drug injection on the 21st day of dosing by an observer blind to drug status. Subtotals were recorded at 5-minute intervals. Rats were tested before and after chronic drug treatments.

Receptor Binding Studies

Twenty-four hours after the last drug injection, brainstem (midbrain, pons, medulla), spinal cord, and cortex were dissected on a glass petri dish over ice, frozen on dry ice, and stored at −80°C. Tissue was homogenized (polytron setting 6 × 10 seconds) in 50 volumes of buffer (50 mM Tris-HCl, pH 7.4 at 37°C). The homogenate was preincubated for 15 minutes at 37°C before it was centrifuged at 49,000 × g for 20 minutes at 2–4°C. The pellet was resuspended (polytron setting 2 × 10 seconds) in Tris buffer (50 vol.) containing 4 mM $CaCl_2$, 10 mM pargyline, and 0.1% (wt./vol.) ascorbic acid.

In the 5-HT_1 binding assay (3), 100 μl of [^{3}H]5-HT (spec.act. 29.5 Ci/mmol, NEN) (20 to 0.5 nM for Scatchard studies) was added last to tubes containing 100 μl cold 5-HT (10^{-5} M) or blank and 800 μl of tissue homogenate. In displacement studies, a single isotope concentration of 5 nM was used. After a 10-minute incubation at 37°C, the reaction was terminated by rapid filtration using a Brandel Harvester and #32 Schleicher & Schuell glass fiber filters. Filters were washed three times (5 ml each) with ice-cold Tris buffer and counted by liquid scintillation spectroscopy (45% counting efficiency).

The 5-HT_2 assay (20) differed in that the tissue homogenate was not preincubated and the assay buffer was plain Tris HCl 7.4 at 37°C. Fifty μl [^{3}H]ketanserin (spec.act. 76.5 Ci/mmol, NEN) (10 to 0.1 nM for Scatchard studies), 50 μl methysergide (10^{-5} M), and 400 μl homogenate were incubated at 37°C for 20 minutes. In displacement studies, a single isotope concentration of 1 nM was used.

In the BDZ receptor binding assay (30), tissue homogenates were prepared as described above with the following exceptions. The washing procedure was repeated three times using Tris citrate (pH 7.1 at 25°C) and there was no preincubation. In the assay, 50 μl [^{3}H]flunitrazepam (spec.act. 81.8 Ci/mmol, NEN) (10 to 0.3 nM) was added last to tubes containing 400 μl tissue homogenate and 50 μl clonazepam (10^{-6} M) or blank. Clonazepam was dissolved in ethanol to give a final ethanol concentration of 0.1%, a concentration which did not significantly inhibit [^{3}H]flunitrazepam binding. The assay was incubated at 0–4°C for 30 minutes.

Statistics

B_{max} and K_d were obtained by Scatchard analysis. The Hill coefficient (n_H) was obtained by Hill plot analysis. Correlation coefficients (r) were obtained by linear regression of both Scatchard and Hill plot data. Comparison between multiple groups for B_{max} or K_d were made using analysis of variance (ANOVA). For significant main effects, multiple comparisons between drug treatments were then analysed using the Student-Newman-Keuls test. Comparisons between two groups were made using the *t*-test.

RESULTS

Behavioral Studies

BDZ agonists which induced WDS acutely after a single injection continued to do so with chronic injection without attenuation (Table 1). Clonazepam treatment significantly increased clonazepam-evoked WDS ($p<0.05$). Ketanserin given chronically as cotreatment with clonazepam prevented clonazepam-induced increases in WDS.

TABLE 1

EFFECT ON CLONAZEPAM-EVOKED WDS OF CHRONIC TREATMENT WITH CLONAZEPAM WITH OR WITHOUT KETANSERIN

	WDS/30 Minutes	
	Acute	Chronic
Clonazepam + Vehicle	38 ± 8	*70 ± 10
Clonazepam + Ketanserin	22 ± 5	32 ± 11

Ten mg/kg of drugs singly or in combination.
*$p<0.05$, chronic compared to acute.
Data are means ± S.E.M. (n = 4–6 rats).

Binding Studies

Chronic injection of clonazepam 5 mg/kg (Fig. 1) had a significant effect on B_{max} but not K_d for 5-HT_1, $F(2,9)=4.6$, $p<0.04$, and 5-HT_2 sites, $F(2,12)=5.15$, $p<0.02$. Clonazepam significantly upregulated 5-HT_1 receptors (+13%) in cortex. There were no significant increases in B_{max} of 5-HT_1 sites in brainstem or spinal cord. Clonazepam (+41%) upregulated cortical 5-HT_2 sites. There were no significant changes in corresponding BDZ receptors. However, clonazepam 10 mg/kg did not significantly alter any of the receptors studied (Table 2).

For drug combinations at 10 mg/kg (Table 2), there was a highly significant drug main effect on B_{max} of 5-HT_2 binding, $F(7,28)=3.72$, $p=0.006$, and on K_d, $F(7,28)=8.05$, $p=0.001$ (ANOVA). Significant differences in B_{max} were due to down-regulation of 5-HT_2 sites by ketanserin (−35%) and by the combination of ketanserin + clonazepam (−19%), $p<0.05$ (SNK test), compared to vehicle-treated controls and to other drug treatments. The B_{max} for rats treated with ketanserin alone

TABLE 2

EFFECTS ON CORTICAL 5-HT AND BDZ RECEPTORS OF CHRONICALLY ADMINISTERED DRUG COMBINATIONS AT 10 mg/kg IN NAIVE RATS

	B_{max} (pmol/g)	K_d (nM)
	5-HT_1	
Vehicle	10.86 ± 0.70	3.44 ± 0.36
Clonazepam	10.69 ± 0.52	3.73 ± 0.29
	5-HT_2	
Vehicle	14.36 ± 0.82	0.64 ± 0.04
Clonazepam	12.91 ± 0.84	0.56 ± 0.04
Ketanserin + Vehicle	*9.29 ± 0.97	*1.64 ± 0.20
Ketanserin + Clonazepam	*11.60 ± 1.13	*1.63 ± 0.39
	BDZ	
Vehicle	64.25 ± 6.37	2.65 ± 0.52
Clonazepam	57.05 ± 4.53	2.22 ± 0.21
Ketanserin + Vehicle	58.81 ± 4.55	3.02 ± 0.65
Ketanserin + Clonazepam	*44.73 ± 3.87	3.40 ± 1.11

Data are means ± S.E.M. (n = 4 rats).
*$p<0.05$, compared to vehicle, SNK test.

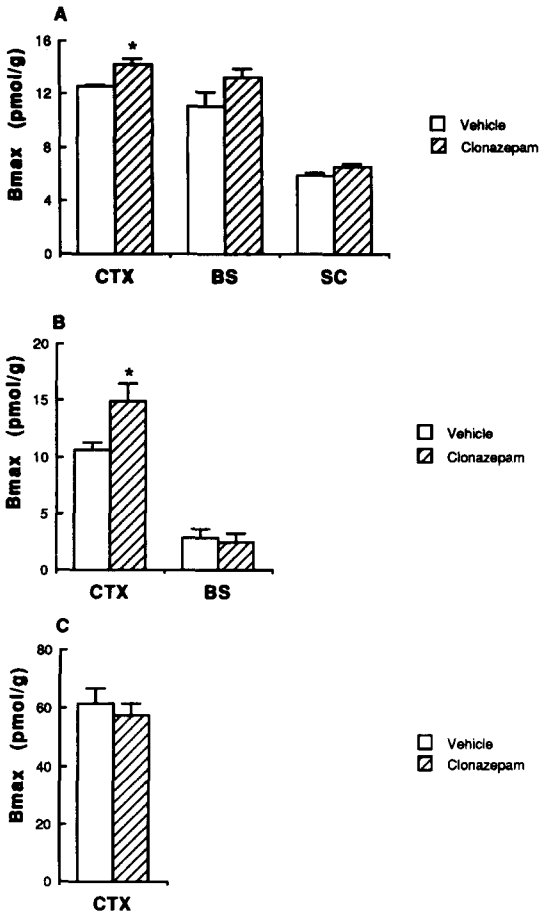

FIG. 1. Effect on 5-HT_1 (A), 5-HT_2 (B), and BDZ (C) receptor density in rat cortex (CTX), brainstem (BS), or spinal cord (SC) of chronic treatment with 5 mg/kg clonazepam. There were no significant changes in K_d. Data are means ± S.E.M. (n = 5). *$p<0.05$ compared to vehicle.

or in combination with clonazepam did not differ significantly. The K_d was significantly increased in the ketanserin and ketanserin + clonazepam groups, which did not differ significantly from each other. Mean Hill coefficients were between .98 and 1.06 for all drug groups and did not differ from unity. The combination of clonazepam with ketanserin at 10 mg/kg did not prevent the significant ketanserin-induced down-regulation of cortical 5-HT_2 sites, but did significantly reduce B_{max} of BDZ sites (−24%) (SNK test, $p<0.05$).

In vitro, clonazepam did not significantly inhibit (>20% displacement) the binding of serotonergic radioligands at concentrations as high as 100 μM.

DISCUSSION

The main finding of this study was that clonazepam-evoked WDS did not attenuate with chronic clonazepam treatment. Tolerance was expected since it develops to the inhibitory effects of BDZ agonists, such as anticonvulsant activity and sedation, with chronic use in humans (5,21). Since behavioral dose-response studies were not performed, too much attention to the increased behavioral responses we found would not be justified except to confirm the absence of tolerance. It is unlikely that the drug dose or dose regimen was insufficient to induce tolerance. These data suggest a different mechanism of BDZ-evoked excitatory and inhibitory behaviors.

Another finding was that lack of tolerance was not associated with dose-dependent changes in 5-HT or BDZ receptors. Receptors changed in unexpected ways. Reduction in BDZ and 5-HT_2 sites with chronic coadministration of ketanserin and clonazepam corresponded to prevention of clonazepam-induced increase in WDS rather than a decrease of drug-evoked WDS in naive rats. The increase in WDS with chronic treatment of 10 mg/kg clonazepam was not associated with a corresponding increase in 5-HT or BDZ receptors. While several studies support involvement of 5-HT_2 but not 5-HT_1 receptors in WDS evoked by serotonergic drugs in the rat (14, 22, 27) and by BDZs (head twitches) in mice (24,25), 5 mg/kg clonazepam upregulated both 5-HT_1 and 5-HT_2 sites. A similar, apparent paradox occurs in mice, in which BDZs evoked head twitches which are blocked by 5-HT_2 antagonists (24,25), but a single injection of the 5-HT_2 antagonist mianserin (or the 5-HT_{1A} agonist buspirone) acutely enhanced [^{3}H]flunitrazepam specific binding to mouse whole brain (16,26) without activity at BDZ sites in vitro.

Upregulation of cortical 5-HT_2 receptors by 5 mg/kg clonazepam but not diazepam also has been reported using [^{3}H]spiperone (37), but other brain regions were not studied, BDZ receptors were not measured, and no behavioral data were reported. The mechanism of upregulation does not appear to involve a direct effect of clonazepam at 5-HT receptor recognition sites since clonazepam was not active in vitro at 5-HT receptors. The restriction of 5-HT upregulation to one high clonazepam dose detracts from the argument that such receptor changes explain the clinical superiority if clonazepam over diazepam in clinical treatment of myoclonic seizures.

There have been several conflicting reports of the effects of chronic BDZ agonist administration in the rat on BDZ receptors, including down-regulation of [^{3}H]flunitrazepam sites by flurazepam (30), and no changes (4,23) or increases by diazepam (11). In vitro, reduction in BDZ specific binding attributed to a high affinity site has been reported in cerebral cortical cell cultures from fetal mice (33). Our data do not indicate a significant change, differing from a previous clonazepam study (10) which used an increasing rather than fixed dose, an earlier sampling time, and mice rather than rats, which respond differently to serotonergic drugs (15).

This study supports the existence of a yet undefined interaction between 5-HT and BDZ receptors. One limitation, however, is that only very high doses of clonazepam were used because lower doses are less effective in evoking WDS (28). These doses exceed the doses needed to stimulate BDZ receptors to exert anxiolytic and anticonvulsant effects, which were not measured in our rats, and may instead exert effects unrelated to BDZ receptors. Although control groups were used, the ethanol vehicle may have affected neurophysiological functions since ethanol is known to exert effects at the GABA/BDZ receptor complex, resulting in an interaction with clonazepam. The relative contribution of stimulation of type I vs type II central BDZ receptors in the effects we report also requires further study.

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