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Relationship Between Benzodiazepine Receptors and Experimental Anxiety in Rats[1]

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LIPPA, A. S., C. A. KLEPNER, L. YUNGER, M. C. SANO, W. V. SMITH AND B. BEER. *Relationship between benzodiazepine receptors and experimental anxiety in rats.* PHARMAC. BIOCHEM. BEHAV. 9(6) 853–856, 1978.—The *in vitro* and *in vivo* ability of benzodiazepines to inhibit specific ^{3}H-diazepam binding correlated with their ability to increase punished responding in a conflict situation. Conflict and foot shock, the punishing stimulus used in most conflict procedures, also altered ^{3}H-diazepam binding. These data implicate ^{3}H-diazepam binding sites in mediating at least some of the anxiolytic properties of benzodiazepines and suggest the existence of some endogenous substance which might be involved in the etiology of anxiety.

Benzodiazepine receptors Conflict/punishment Endogenous ligands

AN understanding of the mechanisms by which the benzodiazepines produce their anxiolytic actions might provide valuable insights into identifying those neuronal substrates responsible for the production of anxiety. Recently, brain stereo-specific, high affinity binding sites for ^{3}H-diazepam have been reported in several species including humans [2, 12, 13, 16], and suggested to represent a substrate by which the benzodiazepines produce their pharmacological actions. This suggestion was supported by observations that the ability of a large number of benzodiazepines to inhibit ^{3}H-diazepam binding was significantly correlated with their pharmacological activity. In addition, pharmacologically inactive benzodiazepines, as well as pharmacologically inactive enantiomers of active benzodiazepines, did not affect ^{3}H-diazepam binding [2, 12, 13, 16].

Although this evidence indicates that ^{3}H-diazepam binding sites may somehow mediate the pharmacological actions of benzodiazepines, two points concerning the therapeutic and physiological relevance of these binding sites still remain unclear. First, benzodiazepines produce several pharmacological actions which may or may not relate to their anxiolytic actions [6, 14, 17]. The pharmacological property which best correlated with the ability of benzodiazepines to inhibit ^{3}H-diazepam binding was the cat muscle relaxant test [2, 12, 16], a procedure of dubious predictive value for anxiolytics [6]. There have been no reports correlating the ability of benzodiazepines to inhibit ^{3}H-diazepam binding with their ability to increase punished responding in a conflict situation, a procedure with high validity for predicting the anxiolytic effects of drugs [4,6]. Therefore, it is still uncertain whether the anxiolytic properties of the benzodiazepines are related to these ^{3}H-diazepam binding sites. Second, the physiological relevance of these ^{3}H-diazepam binding sites is also unknown. The possibility has recently been raised that these ^{3}H-diazepam binding sites may represent post-synaptic receptors for some as yet unidentified neurohumoral system in an analogous manner to that in which opiate binding sites represent post-synaptic receptors for opiate-like endogenous peptides [8, 9, 11, 13, 16].

To better understand the function of these ^{3}H-diazepam binding sites, we have investigated the relationship between ^{3}H-diazepam binding and a measure of situational anxiety as reflected by the ability of electric shock to suppress behavior in a conflict situation. Numerous studies in animals and humans have demonstrated that drugs used in the treatment of anxiety are able to selectively release behavior previously suppressed by a punishing stimulus (see [4,6] for review). For these reasons, it has been suggested that conflict procedures may serve as a model for situational anxiety [1,3]. We now report that: (1) the ability of benzodiazepines to increase punished responding in a conflict situation is significantly correlated with the ability of these drugs to inhibit *in vitro* ^{3}H-diazepam binding; (2) the *in vivo* administration of diazepam inhibits the subsequent *in vitro* binding of ^{3}H-diazepam in parallel with its ability to increase punished responding; and (3) exposure to aversive stimuli reduces the subsequent *in vitro* binding of ^{3}H-diazepam.

METHOD

Animals

Male Wistar rats (Royalhart) were group housed 4–6 animals per cage with ad lib access to food and water. Prior to

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TABLE 1

CORRELATION OF ANTI-CONFLICT ACTIONS OF BENZODIAZEPINES WITH THEIR ABILITY TO INHIBIT ^{3}H-DIAZEPAM BINDING*

Treatment	Minimal Effective Anti-Conflict Dose†	Rank Order of Potency	Ki (nM)‡	Rank Order of Potency
Lorazepam	0.4	1	2.7	1
Diazepam	1.2	2	6.3	2
Nitrazepam	1.5	3	6.4	3
Chlordiazepoxide	4.5	4	220.0	6
Flurazepam	8.0	5	11.0	4
Oxazepam	35.0	6	14.0	5

*Pearson r = .83, $p<0.05$.
†Data expressed as mg/kg, IP.
‡Data from [12].

testing, rats were 48 hr water and 24 hr food deprived and weighed 160–240 g at time of testing.

Conflict Procedure

The unconditioned conflict procedure used in these studies has previously been described [6,7]. Briefly, food (24 hr) and water (48 hr) deprived naive male rats were placed into a black Plexiglas test chamber. A 10% dextrose-water solution was available through a stainless steel spout located on the back wall of the chamber. After locating the spout, rats were allowed 25 sec of free (no shock) drinking. Electric shock (200 μA) was then applied through the drinking spout on a 5 sec on–5 sec off schedule. The number of shocks received during a 5 min test session was recorded.

^{3}H-Diazepam Binding

Rats were sacrificed by decapitation and frontal cortex was removed by an oblique razor cut (approximately 30° from vertical) at the furthest anterior extent of the caudate nucleus. This tissue, minus olfactory bulbs and tubercles, was homogenized gently in 20 volumes of ice cold 0.32 M sucrose and centrifuged twice at 1000 G for 10 min. Pellets were discarded and supernatants were recentrifuged at 30,000 G for 20 min to produce a crude P_2-synaptosomal fraction. The P_2-fraction was resuspended, twice the original volume, in 50 mM Tris·HCl (pH 7.4). Three hundred μl of the P_2-fraction suspension, 100 μl of ^{3}H-diazepam (1.5 nM) and 100 μl of unlabeled diazepam (3 μM) or deionized water were added to cold tubes containing 1.5 ml of 50 mM Tris·HCl (pH 7.4). Incubation for 20 min at 0°C was terminated by filtration, under vacuum, through Whatman GF/C glass fiber filters. The filters were washed twice with 5 ml cold 50 mM Tris·HCl (pH 7.4) and placed in scintillation vials. After drying at 50–60°C for 30 min, 10 ml of Beckman Ready-Solve HP was added and radioactivity determined in a Beckman Scintillation Counter. All binding was expressed as specific binding, calculated as total binding minus binding in the presence of 3 μM diazepam.

Drugs

Drugs used in these studies were: lorazepam, 0.1–1.6 mg/kg, IP; diazepam, 0.3–5 mg/kg, IP, and 2.5–10 mg/kg, PO; nitrazepam, 0.75–6 mg/kg, IP; chlordiazepoxide 1–18 mg/kg, IP; flurazepam 2–16 mg/kg, IP; oxazepam 15–75 mg/kg, IP. ^{3}H-Diazepam (N-methyl-^{3}H; 39 Ci/mmole) was obtained from New England Nuclear.

RESULTS

In the first series of experiments, we attempted to determine if the *in vivo* potencies of several selected benzodiazepines in a conflict procedure correlated with their reported *in vitro* potencies for inhibiting ^{3}H-diazepam binding. Groups of 6–10 rats were intraperitoneally (IP) injected with graded doses of drugs or isovolumetric amounts of vehicle (2% starch suspension containing 5% polyetheylene glycol). After 30 minutes, all animals were placed into the drinking chamber and the number of shocks were recorded for each animal. All drugs produced increases in the number of shocks accepted. Minimally effective doses (MED) were defined as the lowest dose producing a statistically significant ($p<0.05$, Mann Whitney U Test) increase in shocks over vehicle treated controls and are presented in Table 1. As can be seen, potencies in the conflict procedure significantly correlated (Pearson r=.83, $p<0.05$) with the previously reported abilities of these drugs to inhibit ^{3}H-diazepam binding *in vitro*.

We next sought to determine if the *in vivo* actions of diazepam on the benzodiazepine receptor were related to the ability of diazepam to increase punished responses in the conflict test. Various groups of food and water deprived rats were injected with diazepam (2.5, 5 and 10 mg/kg, IP; 3, 6, 12 and 18 mg/kg, PO) or isovolumetric amounts of the vehicle solution. Thirty minutes after IP administration and 60 minutes after oral administration, approximately half of these rats were placed into drinking chambers, and the number of punished responses (shocks) were recorded. The remaining naive animals were decapitated and frontal cortex was removed for the *in vitro* determination of ^{3}H-diazepam binding. As can be seen in Fig. 1, both IP and PO administration of diazepam produced a dose-related inhibition of the subsequently measured specific ^{3}H-diazepam binding, with the first statistically significant ($p<0.05$, *t*-test) effects observed at 2.5 mg/kg, IP, and 6 mg/kg, PO. Diazepam administration also produced the expected increase in punished responding with the minimally effective anti-conflict doses (2.5 mg/kg, IP, and 6 mg/kg, PO) paralleling the minimally effective doses inhibiting ^{3}H-diazepam binding (see Fig. 1). These data demonstrate that *in vivo* administered diazepam can affect

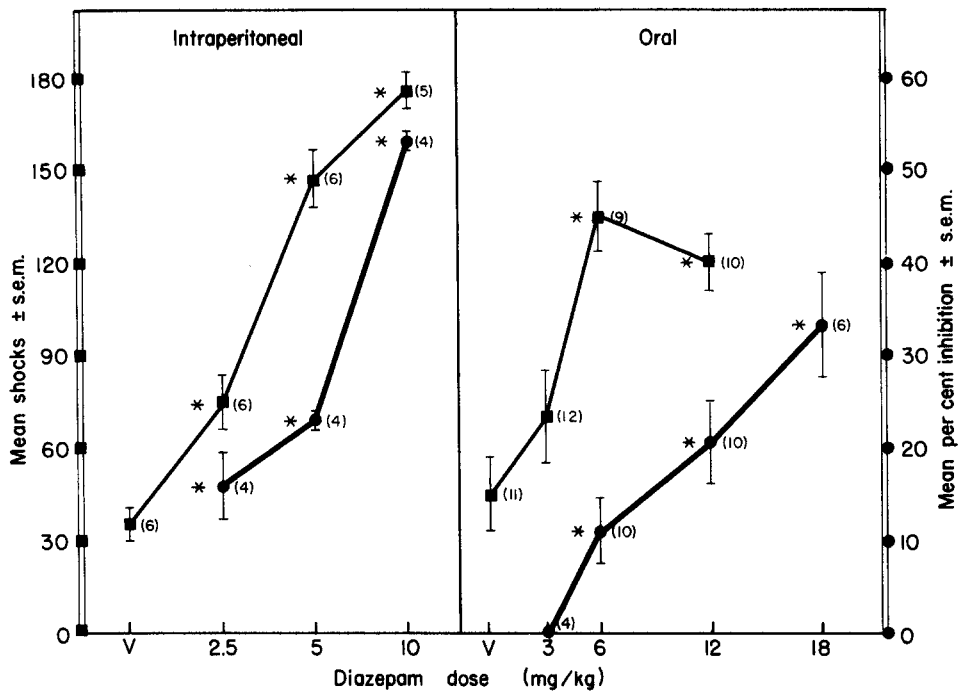

FIG. 1. Effects of *in vivo* administration of diazepam on conflict responding and *in vitro* ^{3}H-diazepam binding. Solid squares represent the mean shocks received during a 5 min conflict test. Solid circles represent the mean percent inhibition of specific ^{3}H-diazepam binding. Percent inhibition was determined by the following formula:

$$\text{Percent inhibition} = \frac{(\rho\text{moles } ^3\text{H-diazepam/mg protein in vehicle controls}) - (\rho\text{moles } ^3\text{H-diazepam/mg protein in drugged animals})}{\rho\text{moles } ^3\text{H-diazepam in vehicle controls}} \times 100$$

Mean ^{3}H-diazepam binding in IP treated controls was 0.504 ± 0.017 ρmoles/mg protein, n=4, and in PO treated controls was 0.436 ± 0.011 ρmoles/mg protein, n=20. Numbers in parenthesis represent the number of animals per group. *$p<0.05$, t-test.

benzodiazepine receptor activity in parallel with its ability to overcome the effects of punishment.

Since the first two experiments implicated benzodiazepine receptors in mediating the anti-anxiety actions of the benzodiazepines, we next sought to determine whether exposure to anxiety-provoking situations would alter binding at these receptors. In the third experiment, food (24 hour) and water (48 hour) deprived rats (n=6) were placed into the conflict procedure for a standard 5 minute test. These animals received an average of 20 shocks. Immediately after testing, animals were sacrificed by decapitation and frontal cortex was removed to determine ^{3}H-diazepam binding. An equal number of deprived animals, which were not exposed to the conflict procedure, were used as controls. Exposure to the conflict procedure produced an approximately 25% decrease ($p<0.01$, t-test) in ^{3}H-diazepam specifically bound (Table 2).

In the final experiment, deprived rats were placed into a small test chamber (9 × 8 × 8 in.) with a stainless steel grid floor. The experimental group (n=8) was placed into the test chamber and electric foot shock (100 msec of 300 μA scrambled sine wave current) was applied every 15 sec for 5 min, giving a total of 20 shocks. The control group (n=8) was also placed into the chamber for 5 min, but electric shock was not administered. At the end of the 5 minute period, all rats were decapitated and frontal cortex removed for the determination of ^{3}H-diazepam binding. The application of electric foot shock produced a smaller (relative to the conflict procedure) but still significant ($p<0.05$, t-test) decrease in specifically bound ^{3}H-diazepam (Table 2).

TABLE 2

EFFECTS OF AVERSIVE STIMULI ON ^{3}H-DIAZEPAM BINDING

Treatment	N	^{3}H-Diazepam Binding (pmoles/mg protein)*
Deprived Controls	6	0.535 ± 0.060
Conflict	6	0.408 ± 0.036‡
Deprived Controls	8	0.580 ± 0.035
Foot Shock	8	0.501 ± 0.031†

*Data expressed as mean ± SEM.
†$p<0.05$, t-test.
‡$p<0.01$, t-test.

DISCUSSION

Recent reports of brain-specific receptors for benzodiazepines [2, 12, 13, 16] raise the possibility that these sites represent a neuronal substrate upon which the benzodiazepines act to produce their pharmacological properties. The present studies demonstrate that the *in vitro* ability of benzodiazepines to inhibit ^{3}H-diazepam binding correlates with their ability to increase punished responding in a conflict situation, a procedure with high validity for predicting the anxiolytic effects of drugs [1, 3, 4, 6]. However, this *in vitro* measure of benzodiazepine receptor activity should be viewed cautiously, since it ignores the drug metabolism and absorption which take place in the experimental animal, as well as in man. For this reason, it is significant that the *in vivo* actions of diazepam on the benzodiazepine receptor were related to the ability of diazepam to increase punished responses in the conflict test. These data demonstrate that *in vivo* administered diazepam can affect benzodiazepine receptor activity in parallel with its ability to overcome the effects of punishment and support the hypothesis that these receptor sites may be involved in the anti-anxiety actions of this drug.

The importance of these findings is perhaps best illustrated by highlighting recent parallel developments in research on the mechanism of action of narcotic analgesics. In this area, it has been shown that those sites which bind radioactively labelled opiate analgesics may serve as receptor sites for endogenous peptides, which are released in response to painful stimuli, and which produce analgesia when administered centrally (see [5, 10, 15] for review). In an analogous manner, we hypothesize that ^{3}H-diazepam binding sites may serve as neuronal receptors for some as yet unidentified endogenous ligand. This hypothesis is supported by reports of endogenous, competitive inhibitors of benzodiazepine binding [11], as well as the recent demonstration of a neuronal localization of benzodiazepine receptors [8,9]. We further hypothesize that the endogenous ligand is normally released when an organism is exposed to anxiety-provoking stimuli and interacts at those same receptor sites which selectively bind benzodiazepines. According to this model, the presently observed decrease in ^{3}H-diazepam binding after exposure to anxiety-provoking stimuli (conflict and electric shock) may reflect the increased release and occupation of receptor sites by the endogenous ligand.

REFERENCES

1. Beer, B. and B. Migler. Effects of diazepam on galvanic skin response and conflict in monkeys and humans. In: *Predictability in Psychopharmacology: Preclinical and Clinical Correlations*, edited by A. Sudilovsky, S. Gershon and B. Beer. New York: Raven Press, 1975, pp. 143–158.
2. Braestrup, C. and R. F. Squires. Pharmacological characterization of benzodiazepine receptors in the brain. *Eur. J. Pharmac.* **48:** 263–270, 1978.
3. Blackwell, B. and W. Whitehead. Behavioral evaluation of anti-anxiety drugs. In: *Predictability in Psychopharmacology: Preclinical and Clinical Correlation*, edited by A. Sudilovsky, S. Gershon and B. Beer. New York: Raven Press, 1975, pp. 121–142.
4. Cook, L. and A. B. Davidson. Effects of behaviorally active drugs in a conflict punishment procedure in rats. In: *The Benzodiazepines*, edited by S. Garattini, E. Mussini and L. O. Randall. New York: Raven Press, 1973, pp. 327–347.
5. Costa, E. and M. Trabucchi (Eds). *The Endorphins, Advances in Biochemical Psychopharmacology, Vol. 18.* New York: Raven Press, 1978.
6. Lippa, A. S., E. N. Greenblatt and P. A. Nash. Pre-clinical neuropsychopharmacological testing procedures for anxiolytic drugs. In: *The Anxiolytics, Industrial Pharmacology, Vol. 3*, edited by S. Fielding and H. Lal. New York: Futura Publishing Company, in press.
7. Lippa, A. S., E. N. Greenblatt and R. W. Pelham. The use of animal models for delineating the mechanisms of action of anxiolytic agents. In: *Animal Models in Psychiatry and Neurology*, edited by I. Hanin and E. Usdin. Oxford: Pergamon Press, 1977, pp. 279–291.
8. Lippa, A. S., M. C. Sano, J. Coupet, C. Klepner and B. Beer. Evidence that benzodiazepine receptors reside on cerebellar Purkinje cells: studies with "nervous" mutant mice. *Life Sci.* **23:** 2213–2218, 1978.
9. Lippa, A. S., M. C. Sano, J. Coupet, C. Klepner and B. Beer. Benzodiazepine receptors: cellular and behavioral characteristics. *Pharmac. Biochem. Behav.* in press.
10. Lord, J. A. H., A. A. Waterfield, J. Hughes and H. W. Kosterlitz (Eds.). *Opiates and Endogenous Opioid Peptides.* Amsterdam: Elsevier, 1976.
11. Marangos, P. J., S. M. Paul, P. Greenlaw, F. K. Goodwin and P. Skolnick. Demonstration of an endogenous, competitive inhibitor of ^{3}H-diazepam binding in bovine brain. *Life Sci.* **22:** 1893–1901, 1978.
12. Mohler, H. and T. Okada. Properties of ^{3}H-diazepam binding to benzodiazepine receptors in rat cerebral cortes. *Life Sci.* **20:** 2101–2110, 1977.
13. Mohler, H. and T. Okada. Benzodiazepine receptor: demonstration in the central nervous system. *Science* **198:** 849–851, 1977.
14. Randall, L. O. and B. Kappell. Pharmacological activity of some benzodiazepines and their metabolites. In: *The Benzodiazepines*, edited by S. Garattini, E. Mussini and L. O. Randall. New York: Raven Press, 1973, pp. 27–52.
15. Snyder, S. and S. Matthysse (Eds.). *Opiate Receptor Mechanisms.* Cambridge, Massachusetts: M.I.T. Press, 1975.
16. Squires, R. F. and C. Braestrup. Benzodiazepine receptors in rat brain. *Nature* **266:** 732–734, 1977.
17. Zbinden, G. and L. O. Randall. Laboratory and clinical correlations. In: *Pharmacology of Benzodiazepines: Advances in Pharmacology, Vol. 5*, edited by S. Garattini and P. A. Shore. New York: Academic Press, 1971, pp. 213–291.