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The Separation of ^{3}H-Benzodiazepine Binding Sites in Brain and of Benzodiazepine Pharmacological Properties

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DUBNICK, B., A. S. LIPPA, C. A. KLEPNER, J. COUPET, E. N. GREENBLATT AND B. BEER. *The separation of ^{3}H-benzodiazepine binding sites in brain and of benzodiazepine pharmacological properties.* PHARMACOL. BIOCHEM. BEHAV. **18**(2) 311–318, 1983.—In addition to anxiolytic and anticonvulsant properties, benzodiazepines (BDZ) produce sedation, ataxia, and muscular relaxation. In general, it was difficult to separate these properties within this chemical class during the search for clinically useful anxiolytics; and when BDZ's were used to characterize ^{3}H-BDZ binding sites they indicated only a single homogenous class of receptors. A new chemical series was discovered, triazolopyridazines (TPZ, prototype CL 218,872), which showed anticonflict activity in rats and monkeys without sedation or ataxia and inhibited ^{3}H-BDZ binding in brain membranes with kinetic characteristics suggesting the presence of multiple BDZ receptors. High affinity and low affinity sites for the TPZ were demonstrated, the former designated at Type 1 and the latter as Type 2. Anatomical and *in vivo* studies have supported different distributions of each receptor in brain. Lately, the physical separation of discrete proteins which bind ^{3}H-BDZ has been reported. The multiple receptors and the variety of endogenous substances which have been proposed as modulators and ligands of the receptors might explain variability as well as selectivity in pharmacological properties in these drugs.

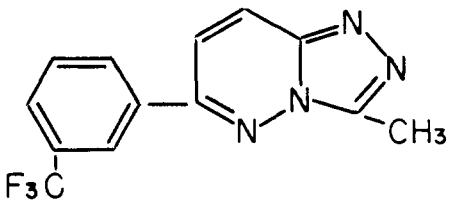
CL 218,872 Triazolopyridazine (TPZ) Benzodiazepine (BDZ) Pentylenetetrazol Brain receptors
Anticonvulsant Anticonflict Sedation Kinetic analysis Type 1 receptors Type 2 receptors
Gammaaminobutyric acid (GABA) Variability Anxiolytics

IN a review of benzodiazepine (BDZ) anxiolytics, Gschwend (1979) concluded that ". . . the anxiolytic activity with which we are concerned is almost always accompanied by a variety of other properties. The spectrum ranges from antianxiety effects, which are virtually always accompanied by slight sedation, through muscular relaxation, anticonvulsant properties, to hypnosis and ataxia" [10]. That is, as a group, the benzodiazepines are considered to be more alike than different from each other. Babbini *et al.* [1] studied a series of 12 BDZs in rats for separation of antianxiety (conflict behavior) from sedative (motor activity) effects. The separation of sedative to antianxiety ratios for these BDZs ranged from 0.2 (i.e. more sedative) and 3.8 (more anxiolytic) respectively, leading them to conclude that "substantial" separations can be obtained. However, the development of a nonsedating anxiolytic and the rationale for achieving that separation of properties was an "ultimate goal" which "still remains to be reached" [10].

Separation of Properties.

Apart from the therapeutic desirability of separating the several pharmacological properties associated with the anxiolytic activity in BDZs, certain lines of evidence suggested that this would be possible. Synder and Enna [48] argued that BDZs probably exert their sedative action by mechanisms similar to the other sedatives with which they share cross-tolerance, e.g. barbiturates and alcohol, drugs which have much weaker anti-punishment and muscle relaxant actions than the BDZs. Secondly, certain drugs can be shown to antagonize selectively only particular properties of benzodiazepines; picrotoxin and isoniazid, for example, antagonized the ataxia at doses which did not affect the anti-punishment effects of chlordiazepoxide [18,19]. Also, tolerance develops selectively to some of the actions of BDZs and not to others. Tolerance developed to the anti-bicuculline activity of diazepam, but not to the anti-pentylenetetrazole (PTZ) activity [15,20]. Based on the differential tolerance to the depressant but not the anti-punishment properties of the BDZ (e.g., [6,31]) it was proposed that anti-PTZ activity, like the anti-punishment activity, may reflect the anxiolytic properties of BDZs. A strategy for finding new types of nonsedating anxiolytics was suggested based on this selectivity of anticonvulsant activity [18].

Recently, CL 218,872 (Fig. 1), a novel triazolopyridazine (TPZ), was reported to have selective anti-punishment and anti-convulsant activity in animals without the attendant motor depression and ataxia associated with BDZs [21,22]. Like BDZ, CL 218,872 increased punished responding and



CL 218,872

3-methyl-6-[3-(trifluoromethyl)phenyl]-1,2,4-triazolo [4,3-b] pyridazine

FIG. 1. Chemical structure of CL 218,872.

protected against PTZ-induced convulsions in rats at doses comparable to diazepam. However, CL 218,872 was very weak (about 10-fold weaker relative to the benzodiazepines and about 100-fold weaker relative to its own potency in the anti-PTZ and anti-punishment tests) in producing motor depression and inclined screen deficits. It was also reported that CL 218,872 was a selective anti-convulsant, relatively weak in its ability to inhibit the convulsions produced by bicuculline, isoniazid and strychnine. This was interpreted as reflecting a lack of GABA-ergic and glycine-ergic properties in CL 218,872 and the unimportance of those neurotransmitter systems in mediating its anxiolytic actions [21,22].

Although an excellent correlation was found between convulsant potency and ability to inhibit the binding of ^{3}H-diazepam to brain receptors [42] in a series of eight tetrazole derivatives which differed more than 100 fold in potency as convulsants, it is uncertain whether one can generalize from neurotransmitter systems which might be involved in convulsant effects to the involvement of those same systems in anxiety or behavioral suppression in a conflict paradigm. In this regard, it is noteworthy that a new series of nonbenzodiazepine (quinoline), nonsedative anxiolytics has been described which seem to lack anti-convulsant properties but which displace ^{3}H-BDZ from binding sites in addition to having a potent (of the order of diazepam and chlordiazepoxide) anti-conflict effect in rats [17].

Receptors for Benzodiazepines

An important tool for studying the actions of BDZ at a molecular level became available with the discovery of brain-specific binding sites for ^{3}H-BDZ in several vertebrate species, including humans [4, 5, 35, 36, 49]. Characterization of these binding sites led to the conclusion that they are neuronally localized receptors on which BDZs act initially to produce their effects [5]. Early kinetic analyses of the binding indicated only a single homogeneous class of sites [29,36]. Figure 2, reproduced from Braestrup and Squires [4], shows the saturation isotherm for binding of ^{3}H-diazepam to rat brain membranes and Scatchard analysis of the same data indicating one binding site. Hill analyses of displacement curves for BDZ having varying affinities for the binding sites yielded slopes near one, i.e. no deviation from simple mass action for a bimolecular reaction, indicating a single, homogeneous binding site (Table 1). Furthermore, a series of BDZ showed similar Ki values in both frontal cortex and cerebellum of mammalian brain [5, 35, 49] areas of the brain where distinct differences could be demonstrated using TPZ (see below). This similarity among a series of 14 BDZ in human brain regions [5] is shown in Table 2.

Receptor Heterogeneity

The case for a single homogeneous BDZ receptor was based on (a) *monophasic heat inactivation* of the binding sites in Tris-HCl buffer; (b) *linear kinetic plots* when binding data were analyzed according to Scatchard; (c) *Hill coefficients that were near unity* for 15 BDZs that varied widely in their affinities for the receptor; (d) *Near linear dissociation curves* from brain binding sites for ^{3}H-diazepam and ^{3}H-flunitrazepam; and (e) *similar brain regional Ki values* for 14 BDZs in human frontal cortex and cerebellum. However, the TPZs with their selective pharmacological properties provided a rationale, a tool and an impetus to re-examine this conclusion.

(a) Whereas *heat inactivation* of BDZ binding sites in rat brain membranes suspended in 50 mM Tris. HCl, pH 7.4 showed a first order disappearance with a half-life of about 10 minutes at 60°C [50], when heat inactivation was carried out in 50 mM sodium phosphate buffer, pH 7.5, ^{3}H-flunitrazepam binding sites disappeared in an apparently biphasic fashion with half-lives of about 10 minutes and 70 minutes at 60°C (Fig. 3).

(b) Kinetic analysis according to Hofstee of the ability of several TPZs to inhibit ^{3}H-BDZ binding to rat cerebral cortex yielded not linear, but curvilinear plots, which could be resolved into two components [23] (Fig. 4). CL 218,872, a member of the TPZ series, competitively displaces ^{3}H-BDZ from brain-specific binding sites with a potency comparable to the BDZs [21,22]. However, unlike flunitrazepam or diazepam, the potency of CL 218,872 as well as its calculated *Hill coefficient, varied as a function of brain region* [16,23]. Whereas the Hill coefficient in cerebellum was 0.9, the Hill coefficients in frontal cortex and dorsal hippocampus (0.7 and 0.6 respectively) were significantly less than unity. CL 218,872 was found to be most potent in cerebellar vermis, less potent in frontal cortex and least potent in dorsal hippocampus (Table 3). Hofstee analysis revealed that these differences were not due to any differences in the affinity of CL 218,872 for the two receptor types (i.e., Kd_1 and Kd_2 did not differ with respect to brain region) but rather to differences in the relative proportions of receptor types. Approximately 90% of the ^{3}H-BDZ binding sites in cerebellar vermis were Type 1, whereas only 40% of the ^{3}H-BDZ binding sites in dorsal hippocampus were Type 1 receptors [23]. The high affinity sites for each TPZ were designated as Type 1 receptors (along with their calculated Kd_1 and $Bmax_1$), while the low affinity sites were designated as Type 2 receptors (with their respective calculated Kd_2 and $Bmax_2$ values). The different TPZs varied with respect to their relative affinities for Type 1 and Type 2 receptors (Kd_1 and Kd_2). Despite these differences, and regardless of the particular TPZ studied, approximately 60% of the receptors in cerebral cortex were calculated to be Type 1 (23). Curvilinear dissociation curves calculated by displacing ^{3}H-BDZ binding with TPZ also could be resolved into both a high and low affinity component [50]. In cerebral cortex, the proportions of Type 1 and Type 2 receptors using this method closely approximated the 60% figure obtained from inhibition of equilibrium binding.

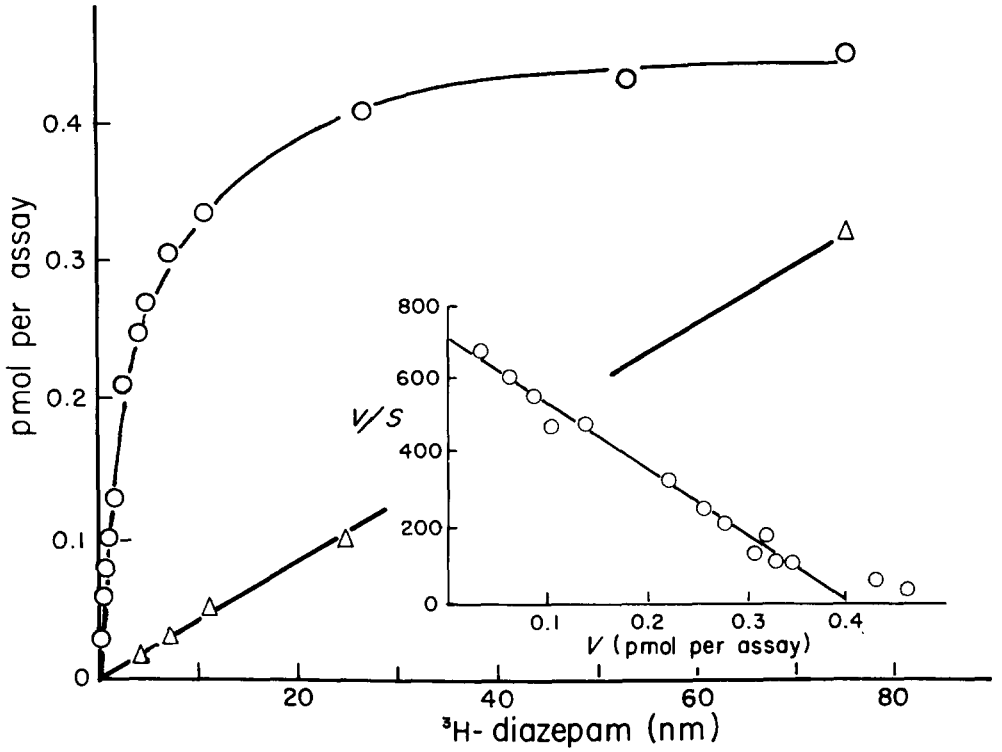


FIG. 2. Saturation isotherm and Scatchard analysis (inset) of ^{3}H-diazepam binding to rat brain P_2 suspensions indicating one binding site. From Braestrup and Squires [4] with permission.

These data demonstrate the existence of two pharmacologically distinct ^{3}H-BDZ binding sites. BDZ and TPZ have a high affinity for Type 1 receptors, while only BDZ have a high affinity for Type 2 receptors.

Recently, binding assays for ^{3}H-CL 218,872 have been developed [24, 38, 52]. Linear Scatchard plots were observed in cerebellum but curvilinear plots were observed in cortex and hippocampus. Quantification of binding parameters revealed values for Kd_1 and Kd_2 and $Bmax_1$ and $Bmax_2$ which were very similar to those values obtained from the Hofstee analyses of displacement studies cited above. Furthermore, BDZ competitively inhibited ^{3}H-CL 218,872 binding in all brain regions with Hill coefficients approximating unity.

(c) On careful examination *^{3}H-diazepam* can be considered to *dissociate from its binding sites* in rat brain membranes in a *bi-or polyphasic fashion*, an effect which can be amplified by carrying out the dissociation in the *presence of excess CL 218,872* ([50], Fig. 5). The two components of ^{3}H-diazepam binding (at 0°C and pH 7.5 in either 50 nM sodium phosphate or Tris:HCl) have half-lives of 2 and 9 minutes after addition of 1 μM nonradioactive diazepam or flunitrazepam. When CL 218,872 (1 μM) is used as the displacing substance, the fast component is unaffected while the half-time of the slow component increases from 9 to 27 minutes suggesting that CL 218,872 may have significantly lower affinity for the "slow receptor" than for the "fast receptor."

(d) The *in vivo differential affinity of CL 218,872 for Type 1 receptors* was demonstrated by treating groups of rats with equi-effective anti-conflict doses of diazepam, chlordiazepoxide or CL 218,872 followed after 30 minutes by a tracer dose of ^{3}H-diazepam. ^{3}H-diazepam binding was meas-

TABLE 1

INHIBITION OF SPECIFIC ^{3}H-DIAZEPAM (1.9 nM) BINDING TO RAT BRAIN MEMBRANES BY SEVERAL BENZODIAZEPINES§

No.	Compound	K_i (nM)*	a†	r^2‡
1	Clonazepam	1.9	1.5	0.997
2	U 39.219	2.1	1.2	0.996
3	U 35.005	2.2	1.4	0.998
4	Flunitrazepam	2.8	1.03	0.996
5	RO 5-3027	4.4	1.09	0.998
6	RO 5-3590	7.2	1.02	0.996
7	Estazolam	12	0.89	0.998
8	Nitrazepam	19	0.85	0.995
9	Bromazepam	30	1.01	0.998
10	U 31.957	60	1.03	0.999
11	RO 5-4528	274	0.91	0.999
12	Chlordiazepoxide	574	1.02	0.996
13	RO 5-3785	3,060	0.88	0.998
14	Medazepam	3,850	1.03	0.996
15	RO 5-3636	4,000	0.88	0.976

*$K_i = IC_{50}/1 + C/K_D$); where C = ^{3}H-diazepam concentration; K_D = affinity constant = 3.4 nM; IC_{50} = concentration causing 50% inhibition of ^{3}H-diazepam binding.

†a = Hill coefficient.

‡r = Linear regression coefficient for Hill plot.

§Data reproduced from Braestrup and Squires [4] with permission.

TABLE 2

INHIBITION OF SPECIFIC ^{3}H-DIAZEPAM BINDING (1.9 nM) TO HUMAN BRAIN MEMBRANES BY BENZODIAZEPINES*

No.	Compound	K_i (nM) Frontal Cortex	K_i (nM) Cerebellar Cortex
1	Lorazepam	1.5	1.9
2	Flunitrazepam	3.5	3.2
3	Clonazepam	4.3	3.4
4	Diazepam	11	7.1
5	Estazolam	11	13
6	Flurazepam	21	21
7	Nitrazepam	36	36
8	Bromazepam	37	43
9	Tranxene	71	56
10	Oxazepam	150	120
11	Chlordiazepoxide	980	590
12	Medazepam	1,780	840
13	RO 5-3636	1,910	1,410
14	RO 5-4864	24,500	13,200

Serial dilution of benzodiazepines in duplicate were added to the high-affinity binding assays from either frontal cortex or cerebellar cortex.

*Data reproduced from Braestrup and Squires [4], with permission.

ured in control and treated animals ([25], Table 4). All three compounds inhibited *in vivo* ^{3}H-diazepam binding in cerebellum by approximately 50 per cent. Diazepam and chlordiazepoxide also inhibited *in vivo* cortical binding to a similar extent. However, CL 218,872 inhibited cortical binding by less than 20 per cent. These brain regional variations in the *in vivo* potency of CL 218,872 parallelled those observed in *in vitro* and support a differential affinity of CL 218,872 for Type 1 receptors as well as an enrichment of this receptor subtype in the cerebellum.

(e) Recently, the *physical separation of Type 1 and Type 2 receptor sites* has been achieved [28] with rat, calf, guinea pig and human brain. The two distinct receptors were solubilized differentially by various detergents. Those that resisted solubilization (designated Type 1) were highly concentrated in cerebellum and showed a high affinity for CL 218,872, whereas several benzodiazepines did not differentiate between Type 1 and the more readily solubilized Type 2 receptors. The presence of multiple BDZ binding proteins in cortex and hippocampus (but only one in cerebellum) separable by electrophoresis on polyarylamide gel has also been reported [25,44].

Although the number and biochemical characteristics of BDZ binding sites are not yet firmly established (see discussion by Lippa, Meyerson and Beer [26]), a current view of sites designated as Type 1 and Type 2 is summarized in Table 5.

Anatomical Differentiation Based on Radioautography and Different Rates of Ontogeny

Autoradiographic studies of slide-mounted tissue sections which had been labelled by incubation with ^{3}H-flunitrazepam in the absence of, or in the presence of, concentrations of CL 218,872 [53] clearly demonstrated that ^{3}H-BDZ binding in some regions was substantially affected by CL 218,872 while

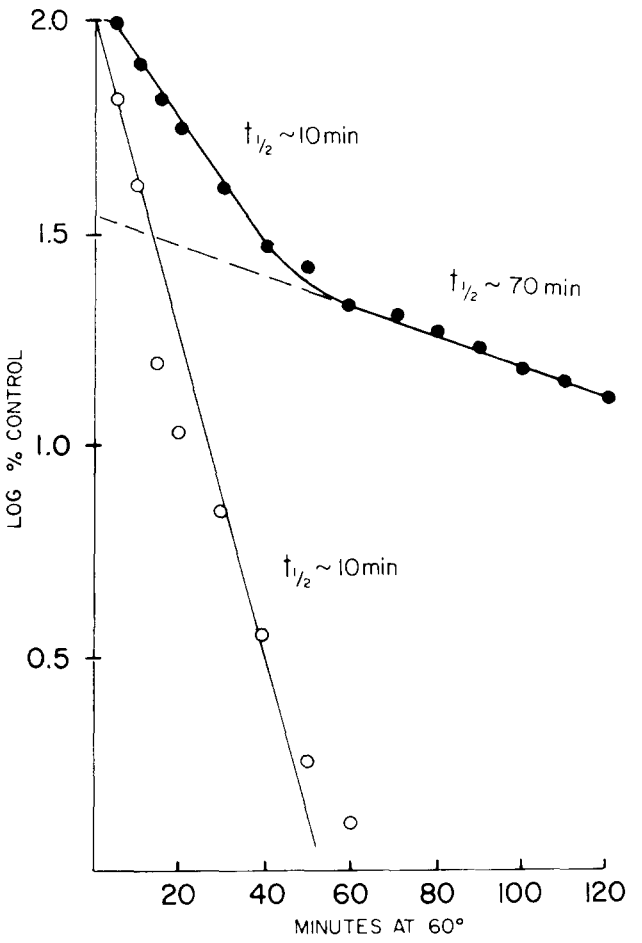


FIG. 3. Heat inactivation of ^{3}H-flunitrazepam binding sites in a P_2-synaptosomal fraction from rat brain. Tubes containing P_2 membrane protein in 50 mM sodium phosphate (●) or Tris·HCl buffer (○), pH 7.5, were kept at 60° in a water bath. After subsequent cooling, either buffer or clonazepam was added to each tube, followed by ^{3}H-flunitrazepam. From Squires *et al.* [50] with permission.

other regions were much less affected; clonazepam (a BDZ) displaced all of the ^{3}H-BDZ to the same extent in all of the brain regions. Biochemical studies on these same slide-mounted sections demonstrated calculated Hill slopes significantly less than unity for the displacement of bound ^{3}H-BDZ by CL 218,872. Autoradiographic visualization revealed a high degree of regional enrichment with either Type 1 or Type 2 receptors. A high proportion of Type 1 receptors was found in the cerebellum, globus pallidus and parts of the cerebral cortex. Regions with a high proportion of Type 2 receptors included the superficial layer of the superior colliculus and the caudate-putamen. Furthermore, even within a heterogeneous region such as the hippocampus, Type 1 and Type 2 receptors appeared to be separately concentrated in particular areas.

This differential neuroanatomical localization was paralleled by different rates of ontogeny for two receptor types in rat cerebral cortex demonstrated by the ability of CL 218,872 to inhibit ^{3}H-BDZ binding [27]. Most of the receptor population at birth was Type 2; the number of this receptor type

TABLE 3

POTENCY AND HILL COEFFICIENTS OF CL 218,872 AND FLUNITRAZEPAM IN FRONTAL CORTEX. DORSAL HIPPOCAMPUS AND CEREBELLAR VERMIS*

	CL 218,872		Flunitrazepam	
Brain Region	Hill Coefficient	IC_{50} (nM)	Hill Coefficient	IC_{50} (nM)
Cerebellar Vermis	0.9 ± 0.05	37.4 ± 2.1	0.9 ± 0.07	2.54 ± 0.96
Frontal Cortex	0.7 ± 0.02	142 ± 37	1.0 ± 0.05	2.90 ± 0.29
Dorsal Hippocampus	0.6 ± 0.03	330 ± 65	1.0 ± 0.05	2.32 ± 0.52

*Data are expressed as Mean ± SEM for four separate displacement experiments. Graded concentrations of flunitrazepam (0.2–20 nM) or CL 218,872 (1–1000 nM) were used to displace ^{3}H-flunitrazepam. Data reproduced from Lippa *et al.* [23] with permission.

doubled during the first postnatal week. The Type 1 receptors did not significantly increase in number until the second postnatal week.

Sources of Variability in Physiological Response to BDZs

The framework for this discussion is variability among the clinically used BDZs and possible explanations for variability to be found among the biological properties of anxiolytics or in the experimental or clinical circumstances surrounding their use (see other contributors to this symposium). In this regard, no attempt has been made to deal with psychological or clinical variability which might result from varying diagnostic categories or semantics.

Our discussion concerning evidence for multiple receptors has been based largely on the involvement of our own laboratory in the new development of the TPZs; it has been related to "anxiety" as manifested in the conflict paradigm used for animal behavioral studies and on the anticonvulsant properties of anxiolytic drugs as a class, particularly anti-PTZ activity. Factors which can influence the response to an anti-anxiety drug and which can be related to

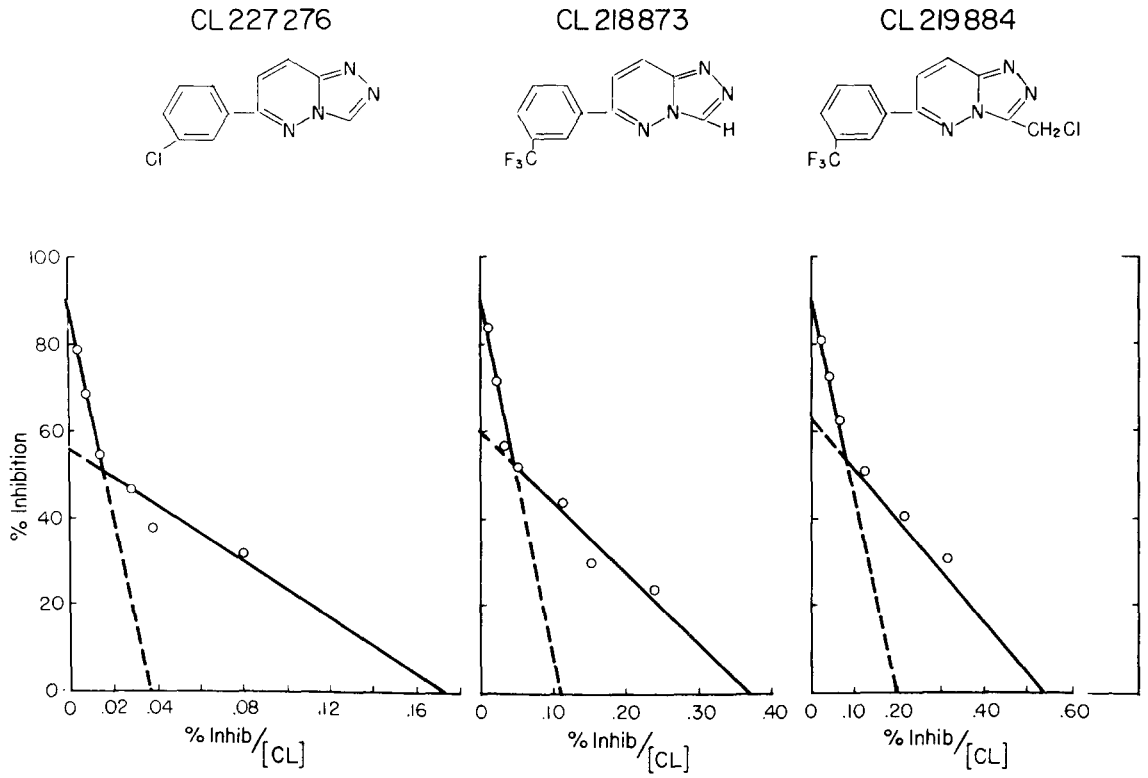


FIG. 4. The effects of triazolopyridazines on ^{3}H-diazepam binding in rat frontal cortex. Graded concentrations of CL 219,884 (0.1–4μM), CL 218,873 (0.1–4μM) and CL 227,276 (0.4–20μM) were incubated with 1.5 nM ^{3}H-diazepam at 0°C. Graphical analysis was performed according to the method of Hofstee. From Lippa *et al.* [23] with permission.

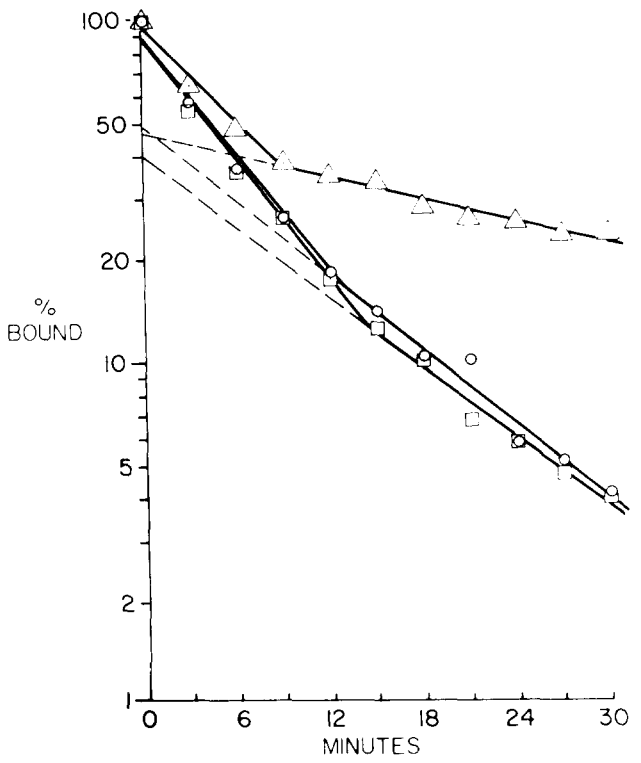


FIG. 5. Dissociation of ^{3}H-diazepam from binding sites in rat brain membranes in presence of 1.0μM each of unlabeled diazepam (○), flunitrazepam (□) and CL 218,872 (△). Equilibrium binding of ^{3}H-diazepam was established before adding the unlabeled displacer. From Squires *et al.* [21] with permission.

TABLE 4

EFFECTS OF CL 218,872, DIAZEPAM AND CHLORDIAZEPOXIDE ON IN VIVO ^{3}H-DIAZEPAM BINDING*

Drug	Dose (mg/kg)	Percent Inhibition Cortex	Cerebellum
CL 218,872	1.5	11 ± 9	53 ± 9
Diazepam	1.5	58 ± 8	59 ± 8
Chlordiazepoxide	3.0	49 ± 7	52 ± 5

*Data expressed as mean ± SEM. All drugs were injected intraperitoneally 30 minutes prior to intravenous injection of 50 μCi ^{3}H-diazepam. Values for vehicle treated controls were 51 ± 15, and 31 ± 11 pmoles/g protein in cortex and cerebellum, respectively.

TABLE 5

DISTINGUISHING PROPERTIES OF TYPE 1 AND TYPE 2 BDZ RECEPTORS (SEE TEXT)

TYPE 1	TYPE 2
High affinity for BDZ AND TPZ	High affinity only for BDZ, *NOT* TPZ
Competitively inhibited by CL 218,872	Non-competitively inhibited by CL 218,872
Relatively thermolabile	Relatively thermostable; protected by GABA
Resists solubilization by detergent	Readily solubilized by detergent
May be related to anxiolytic effects	May be related to sedative effects
Absent from rat cortex at birth	Major population in rat cortex at birth
Regional enrichment in rat brain: cerebellum, globus pallidus	Regional enrichment in rat brain: dentate gyrus, superior colliculus
Identified with electrophoretic band at 49K	Identified with electrophoretic bands at 51K, 55K, 59K

intersubject variability such as differences of species, age, gender and the development of tolerance to some properties are more relevant to other discussions. However, some additional factors are perhaps worthy of notice here since they are germane to our main subject, multiple receptors. These are (a) speculations concerning endogenous ligands, (b) endogenous modulators, (c) agonist and antagonist substances including the possibility that a single molecule may have mixed properties, and (d) the complex nature of the receptor sites which can influence interactions among various ligands, modulators and ions.

(a) Endogenous ligands. The discovery of BDZ receptors initiated a search for an endogenous ligand which may be involved in controlling manifestations of anxiety. Naturally occurring substances shown to bind to the BDZ receptor include the purines, inosine and hypoxanthine [46], β-carbolines [40], nicotinamide [37], tryptophan derivatives [30] and several high and low molecular weight peptide factors [14,32]. The presence of endogenous ligands implies endogenous occupancy of receptors which can affect responses to drugs acting on those receptors.

Endogenous modulators. Evidence for the relationship of neurotransmitters to anxiety, either as mediators or as modulators, has been reviewed [12]. A role for substances such as dopamine, histamine, acetylcholine, purines, amino acids and peptides can be suggested and, at least minimally, supported. However, a role for serotonin (5-HT) has a stronger basis and the relationship of gamma-aminobutyric acid (GABA) to BDZ actions and to BDZ receptors seems firm. The question whether all BDZ receptors are coupled to GABA receptors is unresolved [23].

The role of 5-HT as a modifier of conflict in animal paradigms is supported by studies which show either an anti-conflict effect of the 5-HT synthesis inhibitor, parachlorophenylalanine [9,43] or its ability to alter chlordiazepoxide's behavioral effects [6], 5-HT antagonists such as cinanserin and methysergide also show anti-conflict activity (references in [6]).

BDZ receptors are functionally linked to the GABA recognition sites and from a GABA-BDZ-chloride ionophore complex [11,41]. GABA and certain GABA agonists enhance BDZ binding by altering the affinity of BDZs for the receptor [51]. Certain non-benzodiazepine pyrazolopyridine compounds have been found to enhance BDZ binding and GABA binding (additively); the enhancement is potentiated by chloride ion [2,33]. These compounds have anti-conflict activity in animals. Therefore, response to BDZs, no doubt,

can be regulated by endogenous GABA related to the receptor.

c) Several compounds have been recognized recently as having properties of BDZ antagonists. In addition to the BDZs, TPZs and quinolines [17] which bind to the BDZ recognition site and have anxiolytic properties, other chemical types, including β-carbolines [40], the pyrazoloquinolinone CGS 8216 [3,7] and imidazobenzodiazepines such as Ro-15-1788 [13] bind but antagonize the anxiolytic, anti-convulsant, muscle relaxant and sedative actions of BDZs in animals and man. A fundamental difference between the "agonist" and "antagonist" group of ligands on modulation of receptor sites *in vitro* is that the binding of the latter group is not altered (i.e., not enhanced) by GABA, chloride, barbiturates and pyrazolopyridines [47]. This may be a general distinction which suggests an additional complexity in the BDZ receptor unit as well as the possibility that compounds may be developed or are already in use which have mixed ("agonist-antagonist") properties.

SUMMARY

CL 218,872 (3-methyl-6-[3-trifluormethyl)phenyl]-1,2,4-triazolo- [4,3-b]pyridazine, TPZ) selectively increases punished responding and protects against pentylenetetrazol-induced convulsions at doses comparable to diazepam without the attendant motor depression and ataxia associated with the BDZ. The clear separation of anti-punishment activity in the TPZ from motor depression and inclined screen deficits (about 10-fold greater than with BDZ) provided a tool and an impetus to reexamine the early conclusion that brain receptors for BDZ's are uniform.

The case for a single homogeneous BDZ receptor was based, *in vitro*, on (a) monphasic heat inactivation of the binding sites, (b) linear Scatchard plots, (c) Hill coefficients near unity for a variety of BDZ's, (d) near linear dissociation curves from brain binding sites for ^{3}H-BDZ's and, (e) similar inhibitory potency in different brain regions (frontal cortex and cerebellum) for a variety of BDZ's. Upon reexamination and using the TPZ to displace ^{3}H-BDZ (or, recently, ^{3}H-TPZ as a ligand), all of the above *in vitro* criteria provided evidence for heterogeneity, i.e., non-linear heat inactivation, non-linear Scatchard and dissociation curves, brain regional differences and Hill coefficients less than one. High affinity and low affinity sites for the TPZ were demonstrated, the former designated as Type 1 and the latter as Type 2. Anatomical and *in vivo* studies have supported different distributions of each receptor in the brain. Autoradiography of slide-mounted tissue sections labelled with ^{3}H-BDZ in the absence or presence of CL 218,872 clearly demonstrated differential regional enrichment of Type 1 and Type 2 receptors. Different rats of ontogeny for two receptor sites in rat cerebral cortex were also demonstrated by the ability of CL 218,872 to inhibit ^{3}H-BDZ binding selectively at different ages. The *in vivo* selective affinity of CL 218,872 for Type 1 receptors was demonstrated by treating rats with equieffective anti-conflict doses of diazepam, chlordiazepoxide or CL 218,872 followed by a tracer dose of ^{3}H-diazepam. Whereas diazepam and chlordiazepoxide inhibited ^{3}H-diazepam binding equally in cerebellum and cortex, CL 218,872 was clearly more effective in cerebellum. Finally, recent reports have described the differential solubilization and physical separation of Type 1 (high affinity for BDZ and TPZ) and Type 2 (high affinity for BDZ, low affinity for TPZ) receptors.

Clearly, one explanation for variability among individuals responding to BDZ's could lie in the heterogeneity of the receptor. In addition, the current work on receptors has introduced speculations concerning endogenous ligands, endogenous modulators (such as GABA) and BDZ antagonist substances, all factors which could contribute to the variability of clinical response.

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