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# Brain Specific Benzodiazepine Receptors

By CLAUS BRÆSTRUP and RICHARD F. SQUIRES

**SUMMARY Brain membranes from rat and human contain a single class of brain specific binding sites for pharmacologically and clinically active benzodiazepines. There is good correlation between the pharmacological effects of benzodiazepines and the affinity for the $^3$H-diazepam binding site.**

**Benzodiazepine binding sites are not present on glial cells. Selective neuronal degeneration experiments in rats indicate a neuronal localization. $^3$H-Flunitrazepam is a very suitable ligand for affinity binding and it binds to the same class of binding sites as $^3$H-diazepam.**

**Our results indicate that the *in vitro* $^3$H-diazepam and $^3$H-flunitrazepam binding sites are the receptors which *in vivo* mediate various pharmacological and clinical effects of benzodiazepines.**

## Introduction

High affinity binding techniques have been used for years to identify and study receptors for a large number of peptide hormones, and since 1973 also for neurotransmitters such as catecholamines, serotonin and acetylcholine.

The fundamental approach is to label a hormone or transmitter, or a drug having high affinity for a particular receptor, to high specific activity, preferably with tritium or iodine-131, and then measure whether a suitable tissue preparation will specifically bind the labelled 'ligand'.

A good affinity binding method should meet the following criteria:

(1) binding should be saturable, reflecting a definite number of binding sites in the tissue sample;

(2) the labelled ligand should bind to its receptor(s) with high affinity which greatly facilitates isolation of the receptor-ligand complex;

(3) binding should be specific, i.e. the ligand should bind to only one (or a few) well defined receptor(s) with as low non-specific binding as possible.

An additional criterion for establishing the physiological significance of a particular binding site is the demonstration of relatively high concentrations of these sites in expected target tissues.

The study of opiate receptors is outstanding compared to other receptor studies since no hormones or neurotransmitters or neuronal systems were known when the receptors were discovered (Terenius, 1973; Pert and Snyder, 1973; Simon, Hiller, and Edelman, 1973). The opiate receptor study was the first example of a drug-receptor leading to discoveries of new neuronal systems and putative neurotransmitters.

## Benzodiazepine Receptors

### *Brain specific $^3$H-diazepam binding site*

In the course of routine investigations of neurotransmitter receptors for pharmacological studies, we thought that the very potent and rather selective actions of the benzodiazepines might indicate that they acted through a receptor. We were fortunate to receive from Dr Willy Haefely at Roche a suitable labelled ligand, $^3$H-diazepam, with high specific activity (14.4 Ci/mmol). High specific activity increases



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the sensitivity of high affinity binding methods, and this is important for receptor studies where the total number of receptors is rather small.

The technique of high affinity binding is quite simple. The labelled ligand is incubated with a suitable tissue homogenate. We used the so-called $P_2$-fraction from the brain, which is a relatively crude membrane preparation containing synaptic plasma membranes as well as many other cellular membranes such as axonal membranes, glial membranes, Golgi apparatus, some mitochondria, etc. After incubation we separated the free and bound ligand by filtration through glass fiber filters. Biological membranes together with the bound ligand are retained on glass fibre filters, probably by adsorption. The amount of bound ligand is easily measured by conventional scintillation counting.

'Specific binding' is the amount of binding expected to occur at the receptor. 'Non-specific binding' is the remaining binding, i.e. adsorption of the ligand to glass filters, the amount of ligand dissolved in the biological membranes, and is usually defined as the amount of bound labelled ligand which cannot be displaced by excess of unlabelled ligand. Specific binding thus, is defined as total binding minus non-specific binding.

Figure 1 shows one of our very early experiments. We measured the amount of both specific and non-specific binding to rat brain membranes with increasing concentrations of $^3$H-diazepam in our *in vitro* assays. Several important features of the $^3$H-diazepam binding site are evident from this experiment, which we have now repeated many times. Specific binding is obviously saturable, indicated by the limiting value in binding which was reached at a $^3$H-diazepam concentration of ca. 50 nM. No increase in specific binding was observed even up to 900 nM $^3$H-diazepam (not shown). Further, there appears to be only one class of binding sites in the brain. This is indicated by a single straight line on the Scatchard plot. When two or more binding sites with different affinities for the ligand are present, the Scatchard plot will be curved or, possibly, resolve into several components. Third, Fig 1 shows that the binding occurs with high affinity, the affinity constant is about $3 \times 10^{-9}$M.

Physiologically meaningful receptors are more readily detected using labelled ligands with affinity constants below 10 nanomolar, while binding sites with affinity constants much above 10 nM are often artifactual, probably because many substances bind non-specifically to proteins and various surfaces at higher

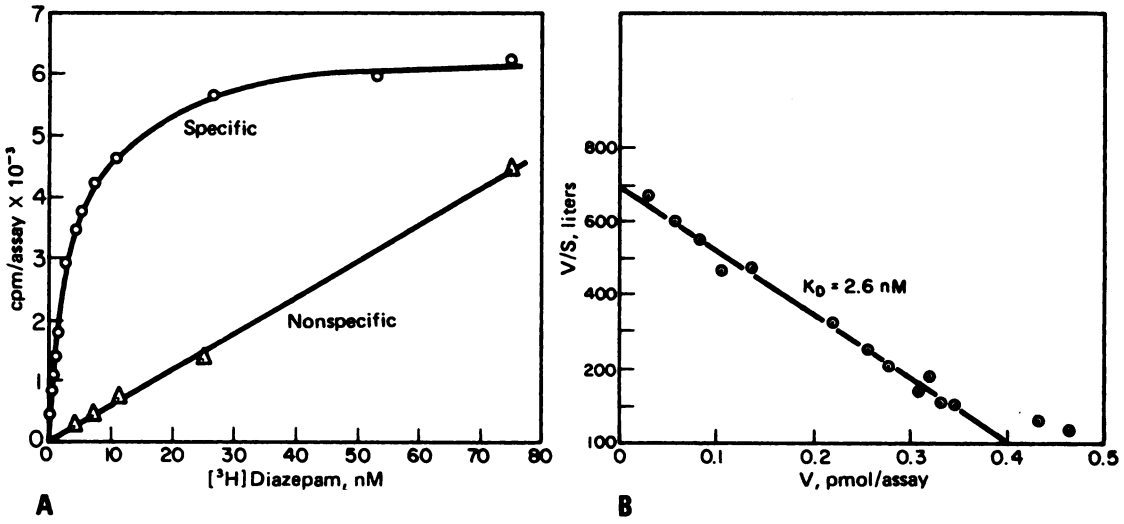



FIG 1.—Rat brain $P_2$-fractions in 50 mM, TRIS, HCL, pH 7.4 were incubated with $^3$H-diazepam in concentrations from 0.25–70 nM (A). Scatchard analysis (B) of the same data indicates only one binding site. Data from Braestrup and Squires (1977).

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concentrations. The above result as well as several other results in this section have been confirmed by Möhler and Okada (1977a, b) and Bosmann, Case and Stefano (1977).

We investigated the characteristics of the high affinity binding of $^3$H-diazepam to the rat brain membranes in some detail (Bræstrup and Squires, 1977) and found results consonant with the presence of a single, membrane bound, proteinaceous receptor, which is only found in brain tissue and which is relatively insensitive to cations.

The binding of $^3$H-diazepam is not a general characteristic of sedative, lipid soluble drugs. $^3$H-Hexobarbital ($^3$H(G), New England Nuclear) was tested under the same conditions as for $^3$H-diazepam (Bræstrup and Squires, 1977) and did not exhibit any specific binding (Nielsen and Bræstrup, unpublished).

The brain specific binding was not distributed evenly in the brain. In rats (Bræstrup and Squires, 1977) and humans (Bræstrup *et al*, 1977; see further details in Möhler, this volume) the highest levels were found in cortical areas, a result which suggests a predominantly cortical action of the benzodiazepines. No gross differences were found between the rat brain and human brain receptor (Bræstrup *et al*, 1977).

*Pharmacological characterization of the benzodiazepine receptors*

The binding site described above for $^3$H-diazepam exhibits many of the characteristics of a receptor: saturability, high affinity, organ specificity and membrane localization. The structural specificity of the receptor was investigated by adding increasing concentrations of clinically or pharmacologically active benzodiazepines to the $^3$H-diazepam-receptor complex. Fig 2 shows some typical displacement curves. Compounds with high affinity for the receptor will displace $^3$H-diazepam at low concentrations (see, e.g. clonazepam). Affinity can be expressed as the concentration of test substance required to displace 50 per cent of the specific $^3$H-diazepam binding ($IC_{50}$ values). However, since $IC_{50}$ values are dependent on the concentration of $^3$H-diazepam used, we have preferred to

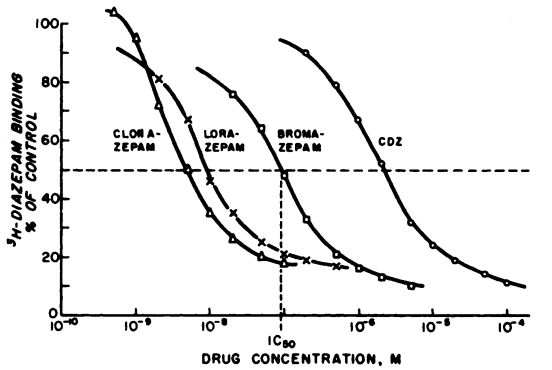



FIG 2.—Displacement of total $^3$H-diazepam binding in rat brain $P_2$-fractions by four benzodiazepines. The data are adapted from Bræstrup and Squires (1978) CDZ is chlordiazepoxide.

calculate the corresponding inhibitor constants, $K_i$ (see Table I).

A seven-membered ring with two aromatic rings attached is found in the benzodiazepines. In general, electronegative groups in position 7 of the A-ring (Cl, F, $NO_2$) are necessary for activity and further substitution in the 2′ position of the B ring enhances activity. Fig 3 shows some extremes of active configurations. The basic flurazepam and the acidic oxazepam hemisuccinate and chlorazepate rapidly loose their basic and acidic groups *in vivo*. Both $^3$H-diazepam displacement *in vitro* as well as pharmacological activity *in vivo* are stereospecific (Möhler and Okada, 1977b).

Many pharmacological data and some clinical data are available (Randall *et al*, 1974; Cook and Sepinwall, 1975; further references in Bræstrup and Squires, 1978) and we find good correlations between the drugs' affinities for the receptor ($K_i$) and their corresponding potencies in those pharmacological tests thought to be predictive of anxiolytic effects in man. Classical pharmacological tests are shown in Fig 4 (Bræstrup and Squires, 1978) and in Fig 5 we have compared *in vitro* affinities with 'anti-conflict' potencies *in vivo* (data from Cook and Sepinwall, 1975) and find a good correlation. When the clinical potencies of the drugs are expressed as recommended daily dosage, there is correlation to the $K_i$ values obtained from human brain (Bræstrup *et al*, 1977). Medazepam

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TABLE I

*Inhibition of specific $^3$H-diazepam (1.9 nM) binding or $^3$H-flunitrazepam (1.0 nM) binding to rat brain membranes by several kinds of benzodiazepines and a few other compounds (Braestrup and Squires (1978), by courtesy of* European Journal of Pharmacology). *Serial dilutions in duplicate were added to the binding assay to estimate $IC_{50}$ values (concentration causing 50 per cent inhibition of $^3$H-diazepam binding). $K_i = IC_{50}/(1 + C/K_D)$ where C = ligand concentration; $K_D$ = affinity constant = 3.4 nM for $^3$H-diazepam and 1.0 nM for $^3$H-flunitrazepam*

| No. | Compound | $K_i$ (nM) $^3$H-diazepam | α* $^3$H-diazepam | $K_i$ (nM)† $^3$H-flunitrazepam |
|---|---|---|---|---|
| 1 | KC-4-2846 | 1.3 | — | |
| 2 | Clonazepam | 1.9 | 1.5 | 1.3 |
| 3 | U 39.219 | 2.1 | 1.2 | |
| 4 | U 35.005 | 2.2 | 1.04 | |
| 5 | Flunitrazepam | 2.8 | 1.03 | 1.8 |
| 6 | Lorazepam | 3.3 | | 1.8 |
| 7 | Ro 5–3027 | 4.4 | 1.09 | |
| 8 | Ro 5–3590 | 7.2 | 1.02 | |
| 9 | Demethyl-diazepam | 8.8 | | |
| 10 | Diazepam | 8.9 | | 4.7 |
| 11 | Estazolam | 12 | 0.89 | 3.6 |
| 12 | Ro 5–2904 | 14 | | |
| 13 | Flurazepam | 16 | | |
| 14 | Nitrazepam | 19 | 0.85 | 10 |
| 15 | Bromazepam | 30 | 1.01 | 26 |
| 16 | Chlorazepate | 30 | | |
| 17 | Oxazepam | 49 | | |
| 18 | U 31.957 | 60 | 1.03 | |
| 19 | Ripazepam | 189 | | |
| 20 | Ro 5–4528 | 274 | 0.91 | |
| 21 | Chlorodiazepoxide | 574 | 1.02 | 412 |
| 22 | Ro 5–5807 | 2,600 | | |
| 23 | Ro 5–3785 | 3,060 | 0.88 | |
| 24 | Medazepam | 3,850 | 1.03 | |
| 25 | Ro 5–3636 | 4,000 | 0.88 | |
| 26 | Ro 5–4864 | 101,000 | | 23.000 |
| | Chlorpromazine | 86,700 | | |
| | Chlorzoxazone | 115,600 | | |

* α = Hill coefficient. Linear regression coefficients for Hill plots averaged, 997 ± .002.

† $K_i$-values for $^3$H-flunitrazepam (performed at 0°C) not previously published.

and estazolam appear to be more active clinically than predicted from their $K_i$-values. Medazepam is metabolized *in vivo* to desmethyldiazepam which accumulates and is clinically active.

Flurazepam appears to be less clinically active than predicted, which may be due to its rapid metabolism and elimination *in vivo*.

The above-mentioned correlations strongly support the contention that the benzodiazepine receptor characterized *in vitro* is the physiologically relevant receptor *in vivo*.

The benzodiazepine receptor is specific for benzodiazepines. No group of drugs other than benzodiazepines and benzodiazepine-like substances (i.e. ripazepam) are active on the receptor. More than 200 drugs from more than 22 different pharmacological classes have been tested including barbiturates, ethanol, meprobamate, and various convulsants and anticonvulsants. Drugs acting on the GABA receptors, such as GABA, muscimol, THIP, isoguvacine and bicuculline are inactive on the benzodiazepine receptor, confirming that the benzodiazepine receptor is not identical to the GABA receptor. Other known and putative neurotransmitters, such as noradrenaline, dopamine, serotonin, acetylcholine, glutamate,

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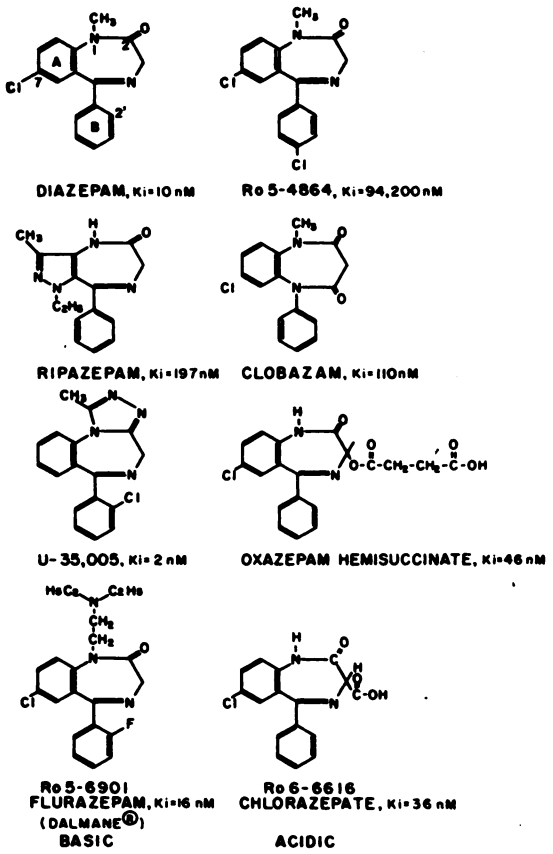



FIG 3.—Benzodiazepine structure-activity relations.

glycine, metenkephalin, substance P, somatostatin and VIP are all inactive (Squires and Bræstrup, 1977; Bræstrup and Squires, 1978).

*Ontogenetic and phylogenetic development of the benzodiazepine receptors*

The ontogenesis of the benzodiazepine receptor in the rat is characterized by a steep increase in binding just after birth in contrast to several neurotransmitter receptors which develop later (Bræstrup and Nielsen, 1978). Phylogenetically, the benzodiazepine receptors exhibit a remarkably late evolutionary appearance (Nielsen *et al*, 1978). Among 18 vertebrate and 5 invertebrate species the highest levels were found in mammals, especially small mammals which have high neuron/glia ratios, followed by birds, reptilia and amphibia. No differences in affinity constants were evident among these four classes. Higher bony fishes had low receptor levels and polyphasic Scatchard plots, while primitive fishes (Agnatha) as well as all the invertebrate species studied were devoid of benzodiazepine receptors.

Lobsters which possess a well developed GABA-ergic neuro-transmission system, were completely devoid of benzodiazepine receptors in their head ganglia.

*Temperature effects on binding*

$^3$H-diazepam binding to the benzodiazepine receptor exhibits a marked and unexpected temperature-dependence. Binding is maximal at 0°C while very little binding occurs at 37°C (Bræstrup and Squires, 1977). Does this mean that the receptor only functions at 0°C and not at physiological conditions? Chang and Snyder (1978) have shown that the very potent benzodiazepine $^3$H-flunitrazepam, when given intravenously to mice, will bind specifically *in vivo* to a particulate brain fraction. This specific binding was inhibited *in vivo* by administration of pharmacologically active benzodiazepines in small intraperitoneal doses. These findings (Chang and Snyder, 1978) indicated that $^3$H-flunitrazepam binds to specific receptors at 37°C and we therefore compared $^3$H-flunitrazepam and $^3$H-diazepam binding, first, to be sure that they acted on the same receptor and secondly to investigate whether $^3$H-flunitrazepam binding was less temperature dependent than $^3$H-diazepam binding.

Figure 6 shows that $^3$H-flunitrazepam and $^3$H-diazepam bind to about the same number of receptors at 0°C in the same membrane preparation. Flunitrazepam has higher affinity than diazepam ($K_D$ = 1 nM compared to 3.8 nM, at 0°C) and both ligands exhibit a single straight line on Scatchard plots indicating only one binding site. $^3$H-flunitrazepam is an excellent ligand for receptor studies, high specific activity can be obtained (87 Ci/mmol, NEN) giving high sensitivity, and low blank values (binding in the presence of surplus unlabelled benzodiazepine is only 3–4 per cent of total binding).

The final proof that $^3$H-diazepam and $^3$H-flunitrazepam bind to the same receptors is

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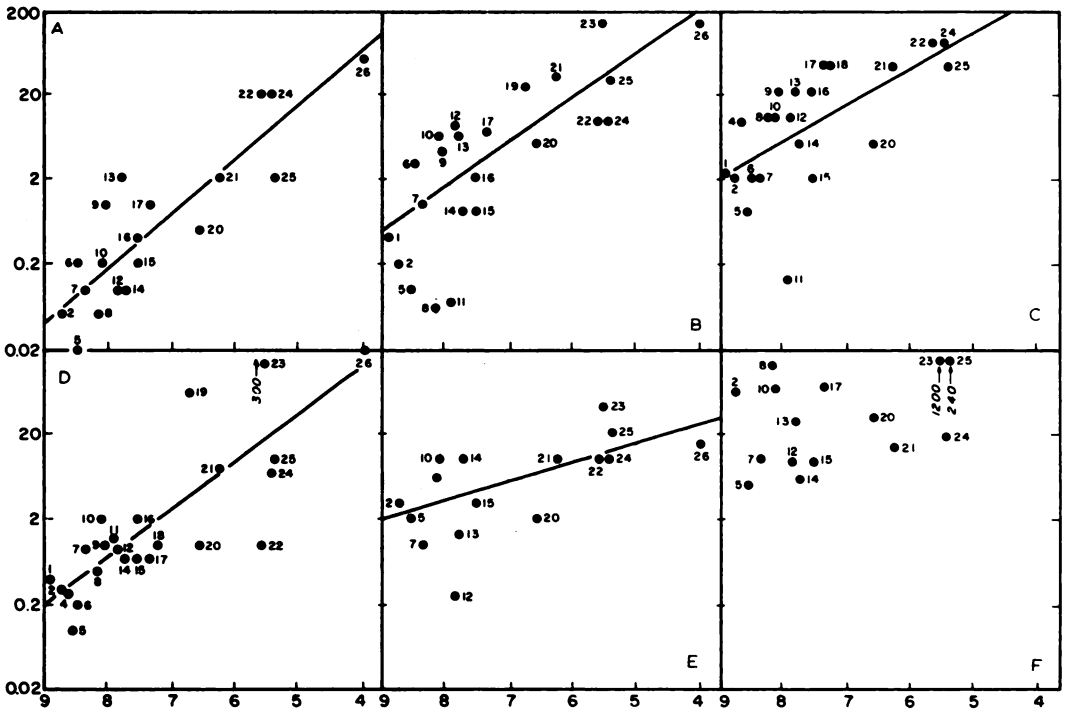



FIG 4.—Correlation of $K_i$ for $^3$H-diazepam displacement on the one hand (abcissa, $-\log K_i$, see Table II) and: A, cat muscle relaxant effect (minimum effective dose mg/kg p.o.).
(B) mice rotarod performance ($ED_{50}$, mg/kg p.o.); (C) inhibition of electric foot shock-induced fighting in mice ($ED_{50}$, mg/kg p.o.); (D) inhibition of pentazol convulsions in mice ($ED_{50}$, mg/kg p.o.); (E) bioassay for human antianxiety (minimum effective dose, mg/day p.o.); (F) inhibition of conditioned avoidance (escape failure in rats (minimum effective dose, mg/kg i.p.).
Significant correlations are denoted by regression lines. Data from Bræstrup and Squires (1978).

shown in Fig 6B. Rat brain membranes were incubated with increasing concentrations of *both* $^3$H-diazepam and $^3$H-flunitrazepam (in the fixed concentration ratio 3.8:1). There were no additive effects in the number of receptors (intercept on the abcissa) compared to $^3$H-flunitrazepam alone. Further, as expected, the $K_i$ values for 9 clinically active benzodiazepines were similar for both ligands (Table I).

We then investigated the effects of temperature on the apparent $K_D$ and apparent number of receptors. With increasing temperature the apparent affinity constants increased for both ligands and, at the same time, the apparent number of receptors decreased. $^3$H-diazepam binding is clearly more temperature dependent than $^3$H-flunitrazepam binding (Table II).

We hypothesized that the extremely low $^3$H-diazepam binding at 37°C was an artifact of the filtration assay, due to washing specific $^3$H-diazepam binding away during the filtration procedure. Determinations of the receptor complex's dissociation rates showing increasingly rapid dissociation of $^3$H-diazepam from its receptors at increasing temperatures (Fig 7), is consistant with this assumption. Further, centrifugation assays of $^3$H-diazepam binding are less temperature sensitive than filtration assays (Bræstrup, unpublished).

In conclusion, we found that $^3$H-flunitrazepam and $^3$H-diazepam bind to the same

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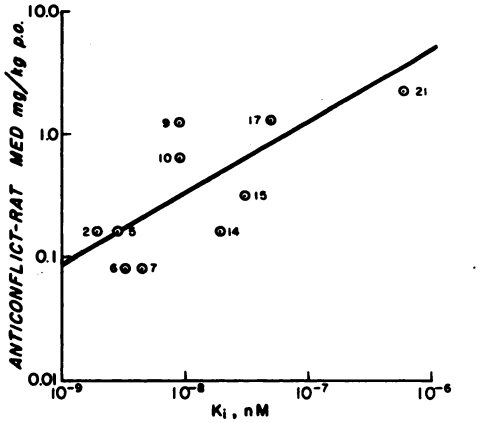



FIG 5.—Correlation between $K_i$ for $^3$H-diazepam displacement (Table I) and rat anticonflict activity (Cook and Sepinwall, 1975) (minimum effective doses, p.o.). (correlation coefficient, r = 0.743, P < 0.025).

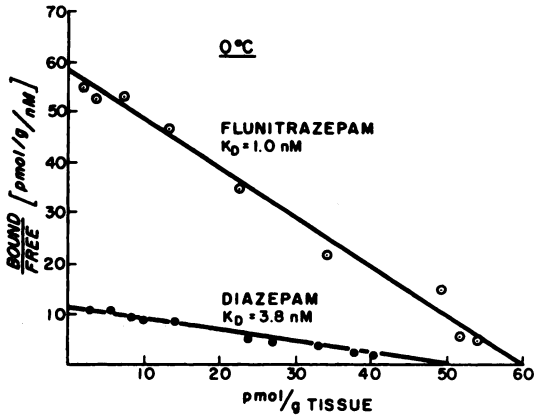



FIG 6A.—Specific binding at 0°C of $^3$H-flunitrazepam (specific activity 87 Ci/mmol, NEN) (0 07—8 nM), and $^3$H-diazepam (0.5—38 nM), assayed on rat brain $P_2$-fractions suspended in 50 vol. of buffer by the filtration technique described in Bræstrup and Squires (1977). Data are plotted according to Scatchard and the affinity constants, $K_D$, are shown on the figure.

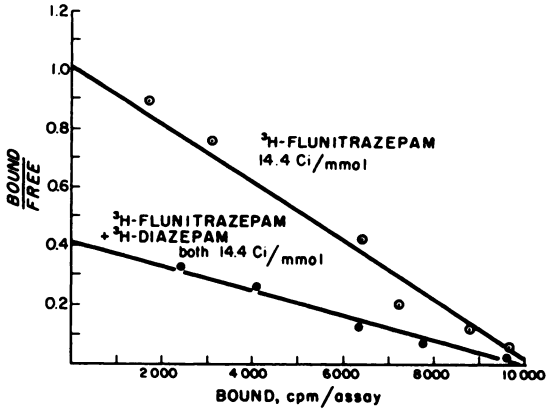



FIG 6B.—Scatchard plot for $^3$H-flunitrazepam alone (diluted with unlabelled flunitrazepam to a specific activity of 14.4 Ci/mmol) (0.5—24 nM) and a mixture of $^3$H-flunitrazepam (0.25—12 nM) plus $^3$H-diazepam (0.95—45.6 nM) in a fixed concentration ratio of 1:3.8. The almost identical intercept of the abcissa indicates that binding of $^3$H-flunitrazepam and $^3$H-diazepam are not additive.

TABLE II

| Temp. °C | $^3$H-diazepam | | $^3$H-flunitrazepam | |
|---|---|---|---|---|
| | $K_D$, nM | B max pmol/g tissue | $K_D$, nM | B max pmol/g tissue |
| 0° | 3.8 | 46 | 1.0 | 64 |
| 20° | 9.3 | 30 | 2.6 | 47 |
| 37° | 24 | 6.9 | 6.6 | 29 |

Temperature dependence of the apparent affinity constants, $K_D$, and apparent number of receptors (B max.) for $^3$H-diazepam and $^3$H-flunitrazepam in rat brain $P_2$ suspensions: each value the mean of two independent Scatchard analyses performed at 5 ligand concentrations in triplicate. For details of the method, see Bræstrup and Squires (1977).

receptors and that the apparent lack of $^3$H-diazepam binding at physiological temperature is probably a technical artifact due to high dissociation rates and the use of filtration assays.

## Neuronal Localization

A possible relation between glial cells and the effects of antipsychotic drugs was recently proposed because a large portion of brain dopamine and haloperidol binding sites seemed to be present on bulk isolated glial cells (Henn, Anderson and Sellström, 1977). We would not expect a physiological relevant benzodiazepine receptor to be located on glial cells. Primary cultures of mouse cerebral astrocytes appear to be the preparation of choice for investigation of

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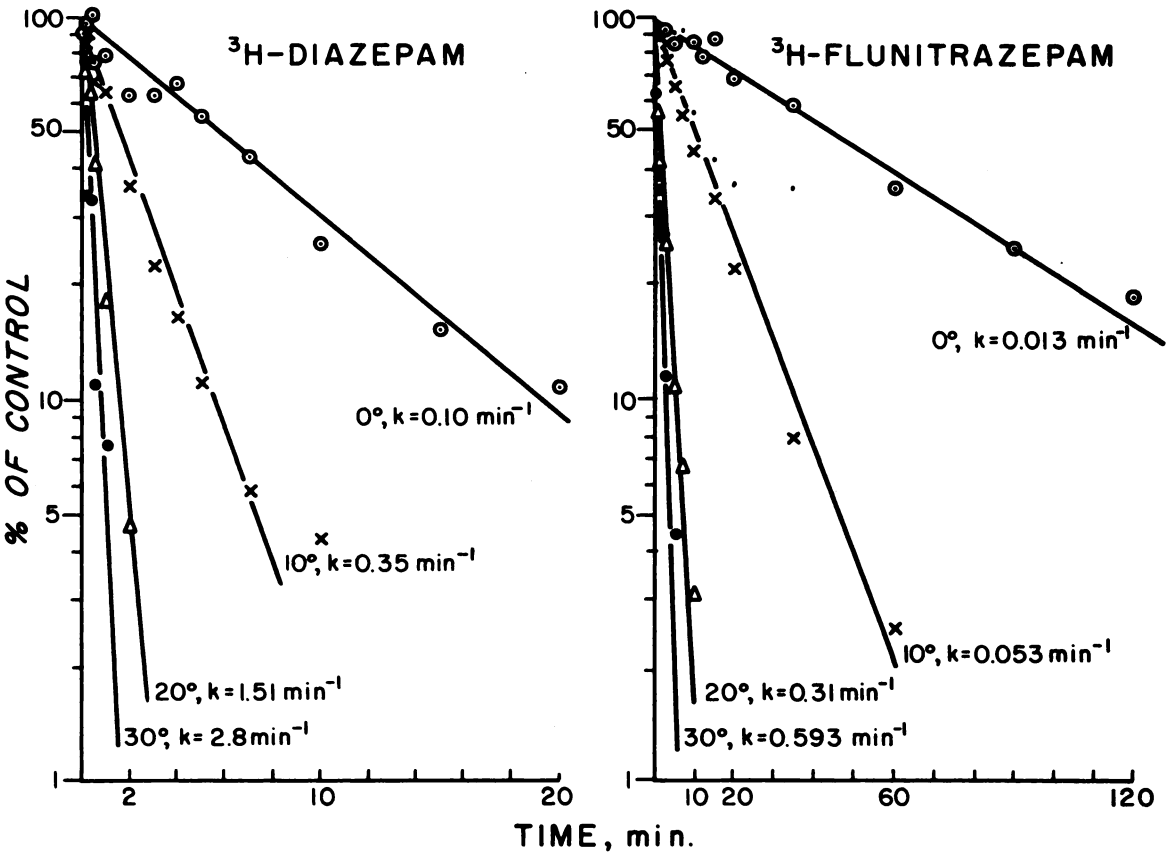



FIG 7.—Temperature dependence of the apparent dissociation rates of the $^3$H-diazepam- and the $^3$H-flunitrazepam-receptor complexes. Rat brain $P_2$-fractions were prepared (Bræstrup and Squires, 1977), and each ligand was incubated for 30 min. at the indicated temperature and at a concentration of 1.9 nM and 1 0 nM, respectively. Excess of unlabelled respective ligands ($3 \times 10^{-6}$ M, final concentration) was added at t = 0. The samples were then filtered after t min. and washed with $2 \times 5$ ml icecold buffer before scintillation counting. Double incubations were performed at each time and temperature.

astroglial cells and we grew enough of these cultures to measure binding of $^3$H-diazepam, $^3$H-haloperidol as well as $^3$H-QNB (muscarinic) and $^3$H-naloxone (opiate).

There were no brain specific benzodiazepine receptors, nor muscarinic or opiate receptors on the astroglial cells. $^3$H-haloperidol exhibited appreciable specific binding, which however was different from dopamine or 'neuroleptic' receptors since the specific haloperidol binding was neither stereospecific towards α- and β-flupenthixol nor was it displaced by dopamine or apomorphine (Bræstrup, Nissen, Squires and Schousboe, 1978). Oligodendroglial cells are most abundant in white matter, areas with low concentrations of benzodiazepine receptors. These results, by exclusion, suggest a neuronal localization, even though other brain elements might be considered, i.e. microvessels.

Preliminary results with a detailed subcellular distribution (Bræstrup and Bock, in prep.) indicate that the benzodiazepine receptor follows the enzyme $Na^+$-$K^+$-ATPase which is a marker of outer cell membranes and not intracellular membranes. These results indicate that the benzodiazepine receptors are confined to neuronal cell membranes.

We then tried to localize the benzodiazepine

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receptors to well-known neuronal pathways in rat brains by making specific neuronal lesions. Our first series of experiments (Table III) indicated that benzodiazepine receptors are not concentrated on dopamine or noradrenaline terminals, on GABA-ergic or cholinergic neurons in the striatum, on granular cells in the hippocampus nor on climbing fibres in the cerebellum (Bræstrup, Nielsen, Squires and Laurberg, 1978). Further, injections of the neurotoxic agent kainic acid into cerebellum, which caused a reduction in glutamic acid dehydrogenase (GAD) (Bræstrup, unpublished) and cortical ablation, which decreases $^3$H-haloperidol receptors and glutamate uptake in striatum, did not decrease the benzodiazepine receptor concentrations (Snyder, unpublished).

Kainic acid injections in rat substantia nigra, however, partially depleted benzodiazepine receptors (Table IV). The presumed selectivity of kainic acid (Olney, Rhee and Ho, 1974; see also Coyle *et al*, 1977) might indicate that benzodiazepine receptors were located on glutamate-sensitive neurons in substantia nigra. The observed degeneration of caudate dopamine-terminals (reduced dopamine uptake) and the degeneration of nigral GAD-containing neurons or terminals calls for caution, however, in concluding which neurons are affected by the lesion.

*Benzodiazepine—GABA interaction?*

The comprehensive literature showing GABA-like effects of benzodiazepines is reviewed by W. Haefely and by E. Costa (this symposium). Is the benzodiazepine receptor connected in any way to the GABA-receptor? Obviously the GABA and the benzodiazepine receptors are not identical; neither do benzodiazepines act on presynaptic receptors releasing GABA to the synaptic cleft. Is the benzodiazepine receptor then an allosteric site at or near GABA-receptors? This question cannot be answered at present since several points still need clarification.

Barbiturates, which do not act on the benzodiazepine receptor, potentiate GABA in several electrophysiological studies (see e.g.

TABLE III

*Brain lesions without effects on the benzodiazepine receptor in rats*

| No. | Lesion | Region examined | Specific $^3$H-diazepam binding[e] per cent of controls |
|---|---|---|---|
| I | Hemisection just anterior to substantia nigra[a] | Striatum | 89 ± 6 (10) |
| | | Frontal cortex | 86 ± 5 (6) |
| | | Substantia nigra | 103 ± 4 (4) |
| II | 6-hydroxydopamine in ascending noradrenaline neurons[b] | Occipital cortex | 123 ± 10 (5) |
| III | 2 μg kainic acid in corpus striatum[c] | Striatum | 106 ± 8 (10) |
| IV | 60 mg/kg i.p. 3-acetylpyridine | Striatum | 100 ± 7 (5) |
| | | Cerebellum | 93 ± 3 (5) |
| V | X-irradiation of hippocampus[d] | Hippocampus | 106 ± 3 (5) |

[a] A complete hemisection at anterior 4.4 mm and lateral 0.0–3.5 mm.

[b] 4×8 μg/2 μl 6-hydroxydopamine were injected bilaterally at: A −0.4 mm; L ± 1.3 mm; V −1.9 and −2.9 mm (Bræstrup, 1977).

[c] (Coyle and Schwartz, 1976).

[d] Rats received 200 r on day 0, 1, 3 and 5 after birth (Laurberg and Hjorth-Simonsen, 1977), and they were killed on day 14.

[e] All results are the means ± S.E.M. of (n) pairs of values.
(Data from Bræstrup, Nielsen, Squires and Laurberg, 1978.)

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TABLE IV

*Reduced level of benzodiazepine receptors in rat substantia nigra after kainic acid*

| Nigral kainic acid, μg | Days | Percentage of control, mean ± S.E.M. Specific binding | GAD | Dopamine uptake in striatum |
|---|---|---|---|---|
| 1 | 8 | 71 ± 6** (6) | 57 ± 6** (6) | 21 ± 6*** (6) |
| 0.75 | 25 | 59 ± 5*** (5) | 83 ± 8 (5) | 57 ± 6*** (5) |
| 1 | 50 | 57 ± 8*** (5) | 59 ± 9** (5) | — |

** $p < 0.01$, *** $p < 0.001$, 8-day experiments with $^3$H-flunitrazepam, others with $^3$H-diazepam.

Male Wistar rats (numbers in brackets) received kainic acid in 1 μl saline, pH 6.5–7, into the left substantia nigra, zona reticulata. By macroscopic comparison with the König and Klippel-atlas, the coordinates were estimated to, A, 2.2. mm; L, 2.0 mm and 2.6 mm below zero. Rats received no injection in the right substantia nigra except for the experiments with 0.75 μg, where vehicle was applied. Glutamic acid decarboxylase (GAD) was assayed with 1-$C^{14}$-glutamic acid as substrate and dopamine uptake in striatum was estimated on a crude synaptosomal preparation (Randrup and Bræstrup, 1977).

Nicoll, 1978). Barbiturates can even antagonize effects of the GABA antagonist bicuculline (Bowery and Dray, 1976).

The regional distribution of GABA-receptors with $^3$H-GABA and $^3$H-muscimol as ligands is reminiscent but still *clearly* different from the benzodiazepine receptor distribution (Young *et al*, 1976; Beaumont *et al*, 1978).

*In vitro*, diazepam completely occupies the benzodiazepine receptors at a concentration of about 50 nM but much higher concentrations are needed to increase the apparent affinity for $^3$H-GABA as well as decrease the number of $^3$H-GABA receptors (Guidotti *et al*, 1978).

Benzodiazepine receptors are not present in head ganglia of Crustacae (i.e. lobsters) which have been useful preparations for GABA receptor studies.

The ontogenetic development of the benzodiazepine receptor preceds the development of GABA-receptors (Coyle and Enna, 1976).

Finally potentiation of GABA-ergic transmission has not shown any 'anxiolytic' activity. The GABA-increasing drug amino-oxyacetic acid did not exhibit any anticonflict activity in animal tests, neither alone nor in combination with diazepam (Cook and Sepinwall, 1975). Further, sedation but no anticonflict activity was observed after increasing peroral doses of muscimol (Cook, L., personal communication), the most potent and selective GABA agonist available. The lack of effect of muscimol is probably not a consequence of poor penetration into the brain after p.o. administration since sedation occurs at high doses and central effects of muscimol p.o. can be demonstrated in other pharmacological tests (J. Scheel-Krüger, unpublished).

## The Missing Endogenous Ligand

We have extracted brain tissue at different pH, temperatures and with different solvents to find a presumed endogenous ligand for the benzodiazepine receptor. These studies have been inconclusive until now. Brain tissue contains a water-soluble factor which inhibits $^3$H-diazepam binding to the brain membranes but this factor turned out to be a protein with a molecular weight of about 70,000, probably too high to be considered an endogenous ligand (Bræstrup *et al*, unpublished).

Cerebrospinal fluid in general does not exhibit inhibitory activity. Four out of 15 miscellaneous psychiatric patients without a history of benzodiazepine intake, however, exhibited weak inhibition in the benzodiazepine receptor assay (Nielsen and Bræstrup, unpublished). Human urine exhibits appreciable inhibitory activity which we have not been able to purify to high specific activity (Squires and Nielsen, unpublished).

## Conclusion

Brain tissue contains a single class of well-defined and well-characterized binding sites for pharmacologically active benzodiazepines. This receptor is probably the physiological target for the benzodiazepines. The most important tasks for future research seem to be (1) identification

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of a putative endogenous ligand, (2) identification of the neurons carrying benzodiazepine receptors, i.e. by autoradiographic techniques with irreversible ligands or with refined lesion techniques, (3) demonstration of changes in receptor affinity, number or characteristics in patological or experimental situations.

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