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The Benzodiazepine Receptor in Normal and Pathological Human Brain

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SUMMARY Benzodiazepines bind with high affinity to a specific benzodiazepine receptor, which occurs exclusively in the central nervous system. The affinity of various benzodiazepines to the receptor closely parallels their pharmacological and therapeutic potency. Binding to the receptor is stereospecific. The receptor is mainly localized in the synaptic membrane fraction and has its highest density in cortical areas of the brain. In Huntington's chorea a decrease in benzodiazepine receptor binding is found in caudate nucleus and putamen, which, at least in putamen, is due to a loss of benzodiazepine receptors apparently located on GABA neurones, which degenerate in Huntington's chorea. The loss of benzodiazepine receptors might explain why the ameliorative effects of benzodiazepines in the early stages of the disease are not sustained in the later stages.

Introduction

Despite the well-established clinical usefulness of benzodiazepines in the treatment of anxiety, insomnia, seizures and muscle spasms, the site and molecular mechanism of action of this group of compounds is not clear. This report describes the presence of a selective benzodiazepine receptor in human brain, which appears to be the specific brain structure to which benzodiazepines are bound in order to elicit central actions.

Using equilibrium binding techniques, which recently allowed the biochemical identification of neurotransmitter receptors (18), we incubated, as described by Möhler *et al* (9), the non-soluble fraction of homogenates from previously frozen human post mortem tissue (1 mg protein) with 1.5 nM ^{3}H-labelled diazepam (specific radioactivity 14.2 Ci/mmole) in Krebs-Tris-Ringer-buffer pH 7.1 at 4°C. An identical assay was run in parallel, containing in addition a high concentration (1 μM) of a pharmacologically potent benzodiazepine. After equilibrium between ligand and binding site was reached (15 min), the assay was terminated by filtration under vacuum. The material on the filter was washed twice and the radioactivity bound to the material was counted.

The difference in radioactivity bound to the tissue in the two parallel assays represents the amount of ^{3}H-diazepam displaceable by high concentrations of benzodiazepines and is termed 'specifically bound'. Non-displaceable ^{3}H-diazepam is called 'non-specifically bound'. For saturation experiments the incubation contained increasing concentrations of ^{3}H-diazepam (0.5–10 nM).

Postmortem brain tissue from control patients who died of various heart diseases with no disorder of the central nervous system (CNS), was obtained from the Institute of Pathology, Basle. Huntington's chorea brain tissue and the respective controls were kindly provided by Dr E. Bird, Addenbrooke's Hospital, Cambridge, England. Keeping the tissue stored frozen did not alter the binding characteristics of the benzodiazepine binding site (14). For the subcellular distribution studies, non-frozen tissue was used immediately after dissection of the brain.

In order to establish that specific binding of

^{3}H-diazepam represents an interaction with a pharmacologically meaningful brain structure (receptor), several criteria have to be fulfilled: (1) There should be only a limited number of specific binding sites, i.e. the specific binding sites should be saturable with increasing ligand concentration. (2) A high affinity binding site would be expected in view of the high potency of some benzodiazepines in vivo. (3) The affinity of different benzodiazepines, including stereoisomers, to the binding site should parallel their pharmacological and therapeutic potency *in vivo*. (4) Assuming that the specific binding site plays a role in synaptic transmission, one would expect, among different subcellular fractions, an enrichment of the binding site in the synaptic membrane fraction. (5) Due to the neuronal heterogeneity of the brain an uneven distribution of the binding site would be expected.

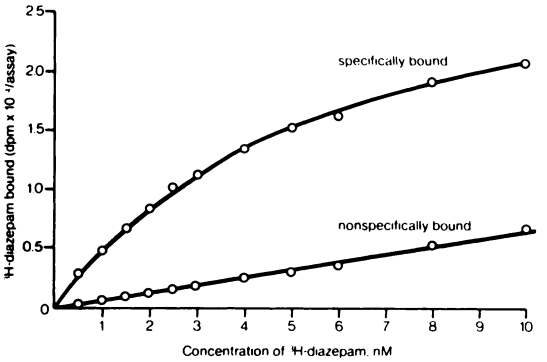

FIG 1A.—Saturation of ^{3}H-diazepam specific binding. Homogenates from human frontal cerebral cortex (1 mg protein) were incubated in triplicate at 4°C for 15 min in 2 ml Krebs-Ringer-Tris-buffer pH 7.4 containing 0.5 to 10 nM ^{3}H-diazepam in the absence and presence of high concentrations (1 μM) of unlabelled diazepam. The points are the means of triplicate determinations with SEM < 3 per cent.

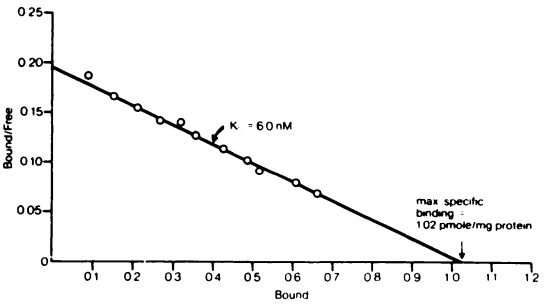

FIG 1B.—Scatchard plot of ^{3}H-diazepam specific binding. The data of Fig 1a were plotted according to Scatchard. Bound = pmole specifically bound ^{3}H-diazepam per mg protein; free = concentration of unbound ^{3}H-diazepam in the incubation medium (nM). The apparent dissociation constant obtained using frontal cerebral cortex from four different brains was K_D = 7.0 ± 0.8 nM, the maximal specific binding 1.2 ± 0.2 pmole/mg protein.

High affinity binding to a specific CNS receptor site

Provided benzodiazepines are bound to specific binding sites in the central nervous system, these sites should be saturable with increasing ligand concentration. In homogenates of human cerebral cortex specific binding of ^{3}H-diazepam was found to be saturable with increasing ^{3}H-diazepam concentration (Fig 1a), involving a single population of binding sites with an apparent dissociation constant for diazepam K_D = 7.0 ± 0.8 nM, as shown by Scatchard plot analysis (Fig 1b). All benzodiazepines appear to be bound to that site, since various benzodiazepines—clonazepam, triazolam, flurazepam, chlorazepate, chlordiazepoxide, diazepam—were found to inhibit specific binding of ^{3}H-diazepam to the binding site in a competitive fashion (14), as illustrated for clonazepam in Fig 2.

The high apparent affinity of diazepam to the binding site appears to be in the range expected, in view of the high *in vivo* potency of the drug, as judged from animal studies. In the cat, in which the regional brain concentration of diazepam is known, the minimal effective dose of diazepam enhancing the presynaptic inhibition in spinal cord (0.1 mg/kg i.v.) results in an initial (1 min), maximal brain concentration of about 1–2 μM of diazepam. However, in analogy to the diazepam distribution in blood (1), possibly only about 1 per cent of the diazepam present in the brain represents the pool of free drug, which would amount to 10–20 nM diazepam. This concentration is in the range expected, assuming that its action is mediated via a binding site with an apparent affinity for diazepam of 7 nM. This argument is strengthened by the fact that, at least in man and rat, the apparent affinity of diazepam to the

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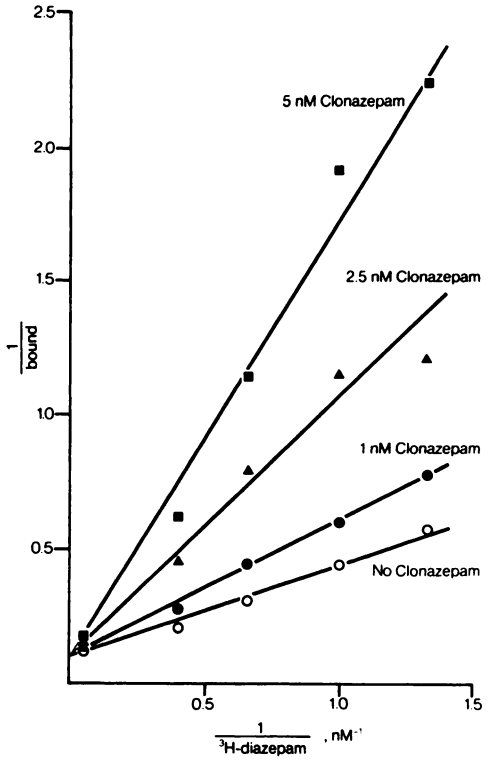

FIG 2.—Competitive inhibition of ^{3}H-diazepam specific binding by clonazepam. Homogenates of human frontal cerebral cortex (1 mg protein) were incubated at 4°C for 15 min with ^{3}H-diazepam (0.5 to 10 nM) and a fixed concentration of clonazepam in the absence and presence of 1 μM diazepam. The points are the means of triplicate determinations with SEM < 3 per cent. The experiment was replicated three times.

binding site is rather similar throughout the brain, and there appears to be no species difference in the affinity to the binding site (Table I) (2, 9).

Main localization of the receptor

There is no uptake of diazepam into cells (rat cortical slices) at low concentrations (up to 10^{-7} M), suggesting a site of action for benzodiazepines on the cell surface rather than within the cell. This argument is supported by the subcellular distribution of ^{3}H-diazepam specific binding in human frontal cerebral cortex (14). Subcellular fractions were obtained by differential centrifugation of the tissue homogenate according to Zukin *et al* (20). The

TABLE I

Benzodiazepines: Comparison of their affinity to the benzodiazepine binding site in human cerebral cortex with their therapeutic and pharmacological potencies

Benzodiazepine action (n = 12–16)	Correlation with the displacing potency in ^{3}H-diazepam binding in man*	
	Correlation coefficient r	Significance p
Displacing potency in ^{3}H-diazepam binding in rat cerebral cortex, K_i	0.99	<0.0001
Muscle relaxant action in cat, ED_{min}	0.92	<0.0001
Antagonism of pentetrazol induced convulsions in mice, ED_{50}	0.90	<0.0001
Average therapeutic dose**, including medazepam, μmol/d	0.79	<0.005
Average therapeutic dose** without medazepam, μmol/d	0.83	<0.005

n = number of benzodiazepines used in the correlations.

* The data are taken from Möhler *et al* (11), where the potency values for individual benzodiazepines are given.

** The dose recommended by the manufacturers for the use of benzodiazepines as anxiolytics and/or hypnotics, was used except clonazepam which is exclusively used as anticonvulsant. For details, see ref. 9.

amount of ^{3}H-diazepam specifically bound was highest (166 ± 17 fmole/mg protein) in the crude synaptosomal fraction (P_2) containing pinched off nerve terminals and mitochondria. When the synaptosomal fraction is lysed by hypoosmotic shock, a crude synaptic membrane fraction can be obtained by differential centrifugation, which shows an equally high amount of specifically bound ^{3}H-diazepam (157 ± 22 fmole/mg protein). Thus, the benzodiazepine binding site in human frontal cerebral cortex appears to be mainly localized in the synaptic membrane fraction. The crude nuclear fraction (P_1) and the crude microsomal fraction (P_3)

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contained a much smaller amount of specifically bound ^{3}H-diazepam (81 ± 16 and 99 ± 18 fmole/mg protein).

Correlation between receptor affinity and therapeutic potency

On the assumption that the therapeutic and pharmacological potency of benzodiazepines parallels their affinity to the benzodiazepine receptor site, the pharmacologically potent benzodiazepines should have a much higher affinity than the weaker ones. As a measure of the affinity, we determined in homogenates of human frontal cerebral cortex the dose of various benzodiazepines resulting in half-maximal displacement of specifically bound ^{3}H-diazepam from the binding site (Fig 3).

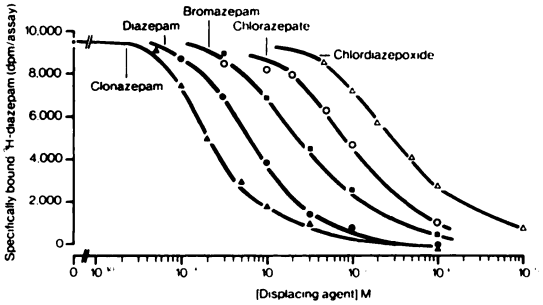

FIG 3.—Potency of different benzodiazepines in displacing ^{3}H-diazepam specifically bound. Homogenates of human frontal cerebral cortex were incubated with 1.5 nM ^{3}H-diazepam and increasing concentrations of various benzodiazepines. The points are the means of triplicate determinations with SEM < 3 per cent. The K_i-values from three different experiments are given in Fig 4.

The displacing potencies of various benzodiazepines, represented by their K_i-values correlate highly significantly not only with their pharmacological potency in several animal test systems predictive of clinical effectiveness, but also with their therapeutic potency in man (Table I) (2, 9, 10, 11, 14, 19). There is a very close correlation between the affinity of benzodiazepines to the binding site and their muscle relaxant action (cat) (Table I). Likewise, the anticonvulsant potency of benzodiazepines (anti-pentetrazol, mice) correlates highly significantly with the affinity of the benzodiazepines to the binding site (Table I). Most remarkably, there is a good correlation between the affinity of benzodiazepines to the binding site and their therapeutic potency as anxiolytics and/or hypnotics in man (Table I, Fig 4). There is a less good correlation between the binding affinity and the potency of benzodiazepines in animal tests considered to reflect the sedative properties of benzodiazepines, such as the inhibition of electric shock induced fighting of mice (correlation coefficient $r = 0.75$, $p < 0.005$) and the impairment of mouse rotarod performance ($r = 0.59$, $p < 0.05$). There is no significant correlation with the inhibition of electric shock-induced convulsions in mice, the taming action in vicious monkeys and the performance of rats and squirrel monkeys in conditioned avoidance tasks.

The comparison of *in vitro* with *in vivo* potencies of benzodiazepines seems to be justified. A dominant contribution from metabolites to the *in vivo* potency in man would be expected only in the case of medazepam, the only benzodiazepine known whose main metabolite in man is more potent than the parent compound itself. The hypothetical *in vivo* potency of unmetabolized medazepam would be less than that shown in Fig 4, bringing the point closer to the regression line. Not surprisingly, without medazepam the correlation between the affinity

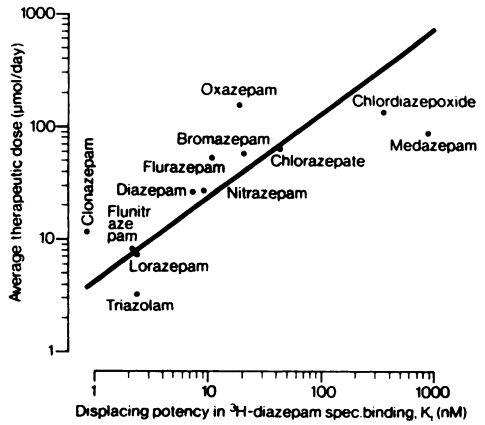

FIG 4.—Correlation between K_i-values for the inhibition of ^{3}H-diazepam specific binding by various benzodiazepines and their average therapeutic dose (the dose recommended by the manufacturers for their use as anxiolytics or hypnotics was used). Correlation coefficient $r = 0.79$ ($P < 0.005$).

of the benzodiazepines to the binding site and their therapeutic potency is improved (correlation coefficient r = 0.83 as compared with r = 0.79 when medazepam is included). Thus, even the exception proves the rule. The absorption of different benzodiazepines from the gastrointestinal tract is expected to be similar for this chemically rather homogeneous group of compounds. A correlation of binding affinity to the potency in different species is not surprizing, since the affinity of various benzodiazepines to the benzodiazepine binding site was found to be similar at least in human, rat and calf brain (Table I) (10, 11, 19).

The pharmacological activity of benzodiazepines containing an asymmetric carbon atom (C–3), is highly stereospecific, with most of the activity residing in the (+) enantiomers. Accordingly, on the assumption that the pharmacological potency parallels the affinity for binding to the benzodiazepine binding site, the (+) enantiomers should have a much higher affinity than the (−) enantiomers. We found (9) that the (+) enantiomer of a benzodiazepine (Ro 11–6896) had a 700 fold higher displacing potency than the corresponding, pharmacologically weak (−) enantiomer (Ro 11–6893), as shown by their K_i-values in inhibiting specific ^{3}H-diazepam binding (Fig 5).

Since benzodiazepines are centrally active drugs with negligible action in the periphery, a restriction of the benzodiazepine receptor to the CNS would be expected. Although benzodiazepines are bound to some structures outside the CNS, e.g. albumin, the characteristics of binding are fundamentally different from those in the CNS: the most potent benzodiazepines have binding affinities to serum albumin 1/10,000th of that in the CNS; in addition, benzodiazepines are displaceable from albumin by L-tryptophan (13), in contrast to the binding site in cortex. Benzodiazepine binding in rat kidney, liver and lung also differs fundamentally from that in rat cortex (3). No diazepam binding binding to erythrocytes (13) or skeletal muscle has been observed (3).

Thus, the close correlation between the affinity of benzodiazepines to the cerebral binding site and their therapeutic potency as anxiolytics and hypnotics, or their pharmacological potency as muscle relaxants and anticonvulsants, presented in this section, suggests that the benzodiazepine binding site identified in *in vitro* binding studies in the brain represents the site of central action of benzodiazepines, which seems to be involved in mediating the anxiolytic, hypnotic and anticonvulsant action of benzodiazepines. This conception is supported by displacement studies *in vivo*, which also show a parallelism between pharmacological potency and binding affinity of benzodiazepines in the brain (4).

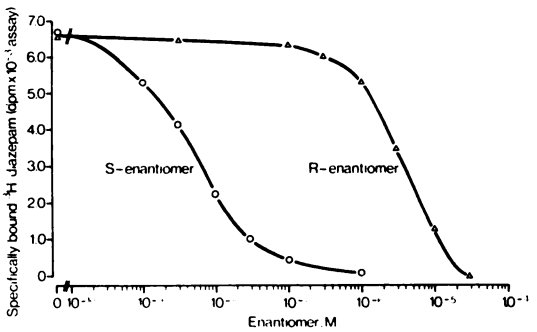

FIG 5.—Stereospecificity of ^{3}H-diazepam specific binding. Homogenates from human frontal cerebral cortex (1 mg protein) were incubated at 4°C for 15 min in 2 ml Krebs-Ringer-Tris-buffer pH 7.4 containing 1.5 nM ^{3}H-diazepam and increasing concentrations of the S- or R-enantiomer of a benzodiazepine (5-(0-fluorophenyl)-1,3-dihydro-1,3-dimethyl-7-nitro-2H-1,4-benzodiazepine-2-one). The points are the means of triplicate determinations with SEM < 3 per cent. The K_i-values obtained from three different brains are $K_i = 4.3 \pm 0.2$ nM for the (+) enantiomer (S-enantiomer) and $K_i = 2800 \pm 30$ nM for the (−) enantiomer (R-enantiomer). For details, see ref. (11).

Regional distribution of the benzodiazepine receptor

In order to investigate whether the benzodiazepine binding site has different binding properties in different brain regions, the density of these sites and their apparent affinity constant (K_D) for diazepam were determined in various regions of human brain (Table II). The apparent affinity of diazepam was rather similar in all brain areas tested, suggesting that the affinities of benzodiazepines to the benzodiazepine binding site throughout the brain are similar to those determined in frontal

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cerebral cortex (9). However, there is a 24-fold variation in the density of the benzodiazepine binding site in human brain with the highest values in cerebral and cerebellar cortex, followed by areas of the limbic system, the basal ganglia and the brain stem (Table II). Thus, the impact of benzodiazepine treatment on neuronal events might be most pronounced in cortical areas, assuming similar concentrations of free drug in all brain areas.

TABLE II

Maximal specific binding and apparent dissociation constant of diazepam in various regions of human brain

Brain region	Max. spec. binding (fmol/mg protein)	K_D (nM)
Cerebral cortex		
frontal	1 200 ± 200	7.0 ±0.8
precentral	1 200 ± 200	7.5 ± 1.9
postcentral	1 110 ± 60	7.0 ± 1.0
Cerebellum		
cortex	730 ± 70	5.8 ± 0.8
vermis	720 ± 90	8.0 ± 2.9
Amygdala	720 ± 100	6.7 ± 1.5
Hippocampus	610 ± 50	6.5 ± 1.4
Hypothalamus	520 ± 40	8.2 ± 0.6
Nucleus accumbens	430 ± 40	6.5 ± 0.8
Thalamus	410 ± 50	8.7 ± 1.5
Nucleus caudatus	380 ± 60	8.5 ± 1.3
Putamen	360 ± 60	6.8 ± 0.9
Globus pallidus	300 ± 10	6.2 ± 0.8
Substantia nigra	290 ± 50	10.6 ± 4.1
Tegmentum	180 ± 40	10.1 ± 3.3
Dentate nucleus	160 ± 30	8.7 ± 4.3
Olive	160 ± 30	14.1 ± 2.9
Pons	160 ± 10	14.2 ± 3.0
Medulla oblongata	150 ± 50	21.9 ± 9.1
Corpus callosum	50 ± 10	6.4 ± 1.9

Homogenates of different regions from human brain were incubated as described in the text. Specific binding of ^{3}H-diazepam was plotted according to Scatchard in order to obtain the apparent dissociation constant K_D and the value of maximal specific binding. The data are the means ± SEM from four different brains.

Loss of benzodiazepine receptors in Huntington's chorea

In an attempt to determine on which types of neurons benzodiazepine receptors may be localized, binding studies were performed in different regions of post-mortem brain of patients who died with the neurodegenerative disease of Huntington's chorea. The most characteristic neuropathological change of this disorder is a diffuse loss of neurons, most notably in the striatum (21). It was found that ^{3}H-diazepam specific binding was markedly reduced in Huntington's chorea brains in putamen and caudate nucleus as compared to control brain. In those regions of Huntington's chorea brains which are not known to be appreciably affected with neuronal cell loss, like frontal cerebral cortex, cerebellar cortex, thalamus and dentate nucleus, there was no significant change in benzodiazepine receptor binding, as compared with respective control regions (15). Thus the loss of benzodiazepine receptor binding in putamen is most likely due to a degeneration of neurones carrying benzodiazepine receptors. In putamen a loss of GABAergic and—in some patients—of cholinergic neurones is known to occur, as shown by the decrease in the respective marker enzyme activities, glutamic acid decarboxylase (GAD) and choline acetyl transferase (CAT) (21). A comparison between benzodiazepine receptor binding and GAD- and CAT activity suggests a localization of the benzodiazepine receptor on GABA neurons (15).

The reduction in benzodiazepine receptors binding in putamen and caudate nucleus of Huntington's chorea patients may explain why the ameliorative effect of benzodiazepine treatment in the early stages of the disease is not sustained in later stages (17).

Are benzodiazepine receptors involved in neuronal transmission?

There is good electrophysiological evidence for the influence of benzodiazepines on GABA mediated neuronal events (5, 7, 16). Although *in vitro* binding studies and electrophysiological studies suggest that benzodiazepines are not bound to the GABA receptor site (6, 10, 12, 19, 20), an enhancement of GABA-ergic synaptic transmission by benzodiazepines or a blockade of the GABA-antagonistic action of bicuculline by benzodiazepines have been found, depending on the brain area (8). Biochemically, there is evidence (see above), that, at least

in putamen, GABA neurons carry benzodiazepine receptors.

It remains to be demonstrated however that benzodiazepine receptors are associated with GABA synapses either pre- or postsynaptically. Furthermore, it has to be clarified whether benzodiazepine receptors are restricted to GABAergic synapses or show a wider pattern of distribution. The exact autoradiographic localization of the benzodiazepine receptor may help to clarify these points.

It cannot be excluded at present that, apart from benzodiazepines, a yet unknown brain constituent acts as physiological ligand of the benzodiazepine receptor. However so far, apart from benzodiazepines no other ligand has been found for the benzodiazepine receptor among known and putative neurotransmitters, drugs and other known brain constitutents (2, 10, 11, 14, 19). In any case, a function for the benzodiazepine receptor in neuronal transmission would be in accordance with its enrichment in the synaptic membrane fraction (see above) and its virtual absence in white matter (Table II). Thus the elucidation of the function of the benzodiazepine receptor may help our understanding of disorders ameliorated by benzodiazepine treatment.

It is remarkable that meprobamate and barbiturates, despite pharmacological similarities with benzodiazepines, are not bound to the benzodiazepine receptor.

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