Benzodiazepine Receptor: Demonstration in the Central Nervous System

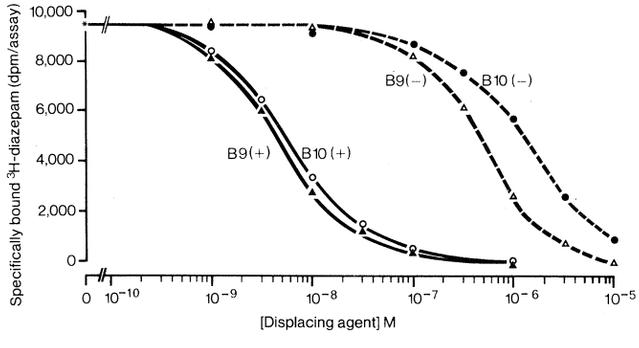
Abstract. *Diazepam, a potent minor tranquilizer, binds with high affinity to a specific benzodiazepine receptor that occurs exclusively in the central nervous system. The receptor is mainly localized in the synaptic membrane fraction. Binding to the receptor is stereospecific. Competition for the receptor by various benzodiazepines closely parallels their pharmacological potency.*

The benzodiazepines are a group of compounds with wide therapeutic application as anxiolytics, anticonvulsants, hypnotics, and muscle relaxants (*1*). Their main representative is diazepam. The site and mechanism of action of the benzodiazepines on the molecular level are yet unknown. We now describe the presence of a specific benzodiazepine receptor in the central nervous system (CNS). It was identified by high-affinity [^{3}H]diazepam binding and is mainly localized in the synaptic membrane fraction. The binding is stereospecific. A close parallel exists between the pharmacological potency of benzodiazepines and their affinity for the receptor.

Rat cerebral cortex was homogenized in 0.32*M* sucrose and a crude synaptosomal fraction (P_2) containing pinched-off nerve terminals and free mitochondria was prepared. Portions (1 mg of protein) were incubated for the binding assay in 2 ml of Krebs-Ringer-tris buffer, *p*H 7.4, containing 1.5 n*M* [^{3}H]diazepam (specific radioactivity, 14.2 c/mmole) (*2*) in the presence and absence of high concentrations of unlabeled diazepam or other pharmacologically potent benzodiazepines (1 μM) at 4°C for 15 minutes at which time equilibrium had been reached (*3*). The incubation was terminated by filtration, under vacuum, through Whatman GF/B glass fiber filters, which were washed twice with 5 ml of ice-cold Krebs-Ringer-tris buffer, *p*H 7.4. The synaptosomal material on the filter was solubilized in Protosol to count the radioactivity bound. [^{3}H]Diazepam bound in the presence of 1 μM diazepam (or other pharmacologically potent benzodiazepines) accounted for only 5 percent of that bound in their absence ("total binding") and was termed "nonspecific." Nonspecific binding was subtracted from total binding to obtain the [^{3}H]diazepam binding termed "specific." There was no degradation of free or bound [^{3}H]diazepam during the incubation (*4*). [^{3}H]Diazepam was radiochemically pure (> 98 percent) as judged with thin-layer chromatography (*3*).

Evidence for the localization of the benzodiazepine binding site comes from studies of the subcellular distribution of [^{3}H]diazepam binding. Specific binding in the synaptosomal fraction (P_2, rat cerebral cortex) was highest in both specific activity [9700 ± 300 disintegrations per minute (dpm) per milligram of protein] and amount (3.3 ± 0.5 × 10^6 dpm per entire fraction); the amount accounted for 60 percent of [^{3}H]diazepam specifically bound in the whole homogenate, the remainder being equally distributed between the nuclear (P_1) and microsomal fraction (P_3). The specific activity of [^{3}H]diazepam specific binding in these two fractions was low (4900 ± 800 and 6700 ± 900 dpm per milligram of protein, respectively). Thus, the highest density of diazepam specific binding sites occurs in the synaptosomal fraction.

There is no uptake of diazepam into brain cells (cortical slices) at low concentrations (up to $10^{-7}M$) (*4*). This suggests a site of action for benzodiazepines on the cell surface, rather than within the cell. This argument could be supported by fractionation experiments. When the synaptosomal fraction, prior to the binding assay, was lysed by hypotonic shock, a mitochondria-myelin fraction and a fraction enriched in synaptic membranes could be isolated. The latter fraction accounted for most (70 percent) of the [^{3}H]diazepam bound to the intact synaptosomal fraction. The specific activity of



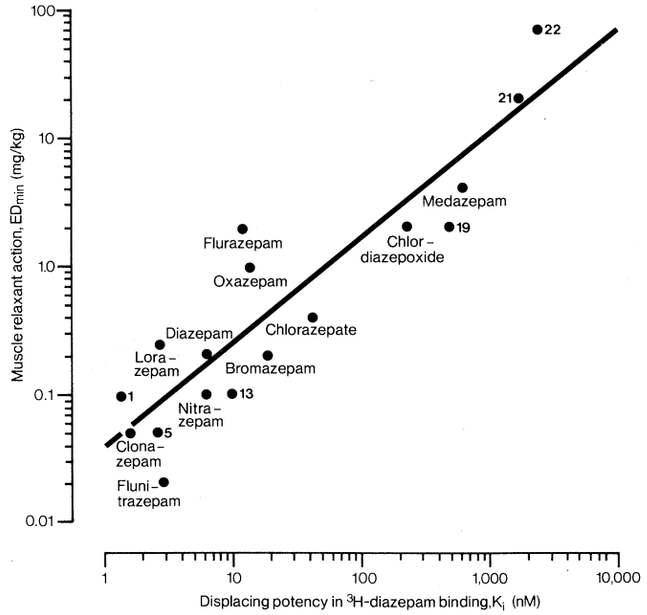


Fig. 1 (left). Stereospecificity of [^{3}H]diazepam specific binding. Synaptosomal preparations (1 mg of protein) from rat cerebral cortex were incubated at 4°C for 15 minutes in 2 ml of Krebs-Ringer-tris buffer, *p*H 7.4, containing 1.5 n*M* [^{3}H]diazepam and increasing concentrations of the (+) or (−) enantiomer of two benzodiazepines, B9 and B10 (Table 1), as described in the text. The points are the means of triplicate determinations with standard error of the mean < 3 percent. The K_i values obtained from three experiments are given in Table 1. Fig. 2 (right). Correlation between K_i values for the inhibition of specific [^{3}H]diazepam binding by various benzodiazepines and their pharmacological potency [cat muscle relaxant action, minimum effective dose (ED_{min}), milligrams per kilogram, orally]; correlation coefficient $r = .905$ ($P < .0001$). Benzodiazepines for which ED_{min} values of their cat muscle relaxant action were available (*1*) were used. K_i values were taken from Table 1. The numbers refer to the abbreviations of the benzodiazepines in Table 1. A possible contribution of active metabolites to the muscle relaxant action of the benzodiazepines would be of major significance only if a metabolite had a considerably higher muscle relaxant potency than the parent compound. Among the benzodiazepines, whose main metabolites have been tested for their muscle relaxant action—diazepam, chlordiazepoxide, flurazepam, medazepam, and nitrazepam (*1*)—only metabolites of medazepam are more potent than the parent compound. Thus the ED_{min} of "unmetabolized" medazepam would be somewhat higher than the value given.

specific binding to the synaptosomal membrane fraction (8300 ± 600 dpm per milligram of protein) was comparable to that of the intact synaptosomal fraction, in contrast to the low specific activity in the mitochondrial fraction (2800 ± 150 dpm per milligram of protein). The specific binding sites are thus mainly localized in the synaptic membrane fraction.

In view of the high pharmacological potency of diazepam in vivo, a high affinity of diazepam to the benzodiazepine receptor would be expected. Furthermore, the receptors should be saturable with increasing concentrations of diazepam. We have found that specific [^{3}H]diazepam binding to the synaptosomal preparation from rat cerebral cortex was saturable, involving a single population of specific binding sites, as shown by Scatchard plot analysis (*3*). The dissociation constant for diazepam was $K_d = 3.6 \pm 0.1$ nM. This value compares well with the K_i value for diazepam obtained in displacing [^{3}H]diazepam by increasing concentrations of unlabeled diazepam ($K_i = 6.3 \pm 0.3$ nM; Table 1). By contrast, nonspecific binding, which accounted for only 5 to 10 percent of total binding at 0.5 to 10 nM [^{3}H]diazepam concentration, was not saturable and increased linearly with increasing [^{3}H]diazepam concentration.

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 receptor, the (+) enantiomers should have a much higher affinity than the (−) enantiomers. We found that the pharmacologically potent (+) enantiomers of two benzodiazepines, B9(+) and B10(+) (for abbreviations see Table 1), had a 120- and 220-fold higher displacing potency than their respective, pharmacologically weak (−) enantiomers, as shown by their K_i values, in inhibiting specific [^{3}H]diazepam binding (Fig. 1 and Table 1).

Among the many different compounds tested, including known and putative neurotransmitters and various peptides, only benzodiazepines were competitors for [^{3}H]diazepam specific binding sites (Table 1). Their displacing potencies, represented by their K_i values, correlated significantly with their relative pharmacological potencies in tests that are considered to be a measure of their anxiolytic, anticonvulsant, or sedative properties (*1*): the cat muscle relaxant action (correlation coefficient $r = .905$, $P < .0001$; Fig. 2), the inhibition of electric shock–induced fighting of mice ($r = .779$, $P < .001$), the antagonism of pentetrazol-induced convulsions in mice ($r = .722$, $P < .001$), the performance of squirrel monkeys in a conditioned avoidance test (shock increase, $r = .786$, $P < .01$), and the impairment of mouse rotarod performance ($r = .691$, $P < .01$). The potencies of benzodiazepines in other test systems—the taming action in cynomolgus monkeys, the inhibition of electric shock–induced convulsions of mice, and the performance of rats in conditioned avoidance tasks (shock increase or escape failure)—show poor or no correlation to either the K_i values (Table 1) or the potencies of benzodiazepines in the formerly mentioned test systems (*5*). It is noteworthy that a pharmacologically inactive structural analog of diazepam, B21, is also inactive as competitor for specific [^{3}H]diazepam binding (Table 1).

The density of [^{3}H]diazepam specific binding sites varies in different rat CNS regions. The highest density was found in cerebral cortex (305 ± 10 fmole per milligram of protein) followed by hypothalamus (290 ± 22), cerebellum (270 ± 21), midbrain (269 ± 15), hippocampus (252 ± 13), striatum (180 ± 13), medulla oblongata-pons (162 ± 22), and spinal cord (90 ± 22). A similarity of this rank order of the density of neurotransmitter receptors exists to some extent only to that of the γ-aminobutyric acid (GABA) receptor (*6*), supporting the important role attributed to GABA neurons in the action of benzodiazepines (*7*). The GABA receptor itself, however, does not appear to be the diazepam specific binding site. GABA agonists and antagonists fail to influence diazepam binding (Table 1), and benzodiazepines fail to influence GABA receptor binding (*6*, *8*). Likewise, ligands to other neurotransmitter receptors do not affect diazepam binding. The suggestion that the glycine receptor is a site of action of benzodiazepines (*9*) is, in addition, at variance with electrophysiological evidence (*10*). Barbiturates and meprobamate, despite similarities in pharmacological profile with benzodiazepines, do not influence diazepam binding (Table 1).

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, the characteristics of binding are fundamentally different from those in the CNS: the most potent benzodiazepines

Table 1. Inhibition of specific [^{3}H]diazepam binding to synaptosomal preparations from rat cerebral cortex by various benzodiazepines. Specific [^{3}H]diazepam binding to the crude synaptosomal preparation from rat cerebral cortex was assayed as described in the text. Inhibition of specific binding by six to ten concentrations of various compounds was determined by calculating the 50 percent inhibitory concentration (IC_{50}) by log logit analysis. Since the inhibition of binding was competitive (*4*), the IC_{50} values were converted to K_i according to the equation $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of the radioactive ligand and K_d its dissociation constant. The K_i values are the means of three experiments ± S.E.M. Other compounds without inhibitory effect at least up to $10^{-6}M$ include, for example, acetylcholine, noradrenaline, dopamine, serotonin, GABA, glycine, L-glutamate, L-aspartate, taurine, physostigmine, phenoxybenzamine, propranolol, strychnine, (+) bicuculline, picrotoxin, clozapine, methysergide, meprobamate, phenobarbital, naloxone, morphine, haloperidol, diphenylhydantoin, theophylline, L-tryptophan and 21 other L-amino acids, met-enkephalin and 20 other brain and gastrointestinal peptides, and prostaglandins (*4*). Water-insoluble benzodiazepines were dissolved (3 to 10 mM) in methanol and diluted with Krebs-tris buffer, pH 7.4; the maximum concentration of methanol in the binding assay was 0.1 percent (by volume). Methanol alone, up to at least 0.5 percent, had no effect on the amount of total or specific [^{3}H]diazepam binding. Likewise, the affinity of the specific binding site seemed unaffected by methanol, since the K_i of chlordiazepoxide, a water-soluble benzodiazepine, was unchanged by the presence of 0.5 percent methanol in the binding assay (*4*).

Benzodiazepine (abbreviation or generic name)*	K_i (nM)
B1	1.3 ± 0.1
Clonazepam	1.5 ± 0.1
Thienobenzodiazepine	1.9 ± 0.2
B4	2.5 ± 0.4
B5	2.6 ± 0.2
Lorazepam	2.7 ± 0.2
Flunitrazepam	2.8 ± 0.6
B8	3.2 ± 0.6
B9(+)	3.6 ± 0.7
B10(+)	4.8 ± 0.5
Diazepam	6.3 ± 0.3
Nitrazepam	6.4 ± 0.3
B13	9.5 ± 0.6
Flurazepam	11 ± 1
Bromazepam	12 ± 4
Oxazepam	14 ± 1
Chlorazepate	41 ± 8
Chlordiazepoxide	220 ± 20
B9(−)	430 ± 90
B19	590 ± 100
Medazepam	600 ± 50
B10(−)	1040 ± 130
B21	1400 ± 200
B22	2800 ± 400
B23	†

*B1, Ro 5—3027; B4, Ro 21—8384; B5, Ro 5—3590; B8, Ro 21—3981; B9(+), Ro 11—3129; B9(−), Ro 11—3625; B10(+), Ro 11—6896; B10(−), Ro 11—6893; B13, Ro 5—2904; B19, Ro 5—3636; B21, Ro 5—5807; B22, Ro 5—4933; and B23, Ro 5—2181; for the structures of these benzodiazepines see (*1*); (+) and (−) indicate corresponding enantiomers. †No displacement at 1 μM.

have binding affinities to serum albumin 1/10,000th of that in the CNS; in addition, benzodiazepines are displaceable from albumin by L-tryptophan (*11*), in contrast to the binding site in cortex (Table 1). Benzodiazepine binding in rat kidney, liver, and lung also differs fundamentally from that in cortex (*4*). No diazepam binding to erythrocytes (*11*) or skeletal muscle has been observed (*4*). Pig CNS and calf CNS also contain [^{3}H]diazepam specific binding sites (*4*).

Note added in proof: The benzodiazepine receptor in human brain corresponds to that of rat brain in affinity stereospecificity and regional distribution (*4*).

The identification of the site of action of the benzodiazepines may provide new insight into their mechanism of action.

H. MÖHLER
T. OKADA*
Pharmaceutical Research Department, F. Hoffmann-La Roche, 4002 Basle, Switzerland

References and Notes

1. G. Zbinden and L. O. Randall, *Adv. Pharmacol.* **5**, 213 (1967); L. O. Randall, W. Schallek, L. H. Sternbach, R. Y. Ning, in *Psychopharmacological Agents*, M. Gordon, Ed. (Academic Press, New York, 1974), vol. 3, p. 175.
2. R. Schwob and J. Würsch, *J. Labelled Compd.*, in press.
3. H. Möhler and T. Okada, *Life Sci.* **20**, 2101 (1977)..
4. T. Okada and H. Möhler, in preparation.
5. Some of these results were confirmed by R. F. Squires and C. Braestrup, *Nature (London)* **266**, 732 (1977).
6. H. Möhler and T. Okada, *ibid.* **267**, 65 (1977).
7. W. Haefely, A. Kulcsar, H. Möhler, L. Pieri, P. Polc, R. Schaffner, in *Mechanism of Action of Benzodiazepines*, E. Costa and P. Greengard, Eds. (Raven, New York, 1975), p. 131; G. Biggio, B. B. Brodie, E. Costa, A. Guidotti, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3592 (1977).
8. S. R. Zukin, A. B. Young, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4802 (1974).
9. A. B. Young, S. R. Zukin, S. H. Snyder, *ibid.*, p. 2246.
10. D. R. Curtis, C. J. A. Game, D. Lodge, *Br. J. Pharmacol.* **56**, 307 (1976).
11. W. E. Müller and U. Wollert, *Biochem. Pharmacol.* **25**, 141 (1976); *Naunyn-Schmiedebergs Arch. Pharmacol.* **288**, 17 (1975).
12. We thank W. Haefely and A. Pletscher for critical reading of the manuscript and R. Schwob and J. Würsch for synthezising [^{3}H]diazepam.
* Permanent address: Biochemistry Department, Nippon-Roche Research Centre, 200 Kaiiwara, Kamakura-City, Japan.

22 April 1977; revised 12 July 1977

Cyclic Nucleotides Injected Intracellularly into Rat Superior Cervical Ganglion Cells

Abstract. *Intracellular iontophoresis of either adenosine 3′,5′-monophosphate or guanosine 3′,5′-monophosphate produces a membrane depolarization and an increased membrane conductance in sympathetic ganglion cells of the rat superior cervical ganglion. Since adenosine 3′,5′-monophosphate did not cause a membrane hyperpolarization, it is difficult to assign it a second messenger role in the mediation of the slow inhibitory postsynaptic potential. However, these results do not rule out the possibility that the cyclic nucleotides, at the intracellular concentrations attained in these experiments, participate in cellular processes that contribute to conductance changes which result in depolarization of the ganglion cell membrane.*

Reports (*1*–*3*) indicate that the concentrations of cyclic nucleotides, adenosine 3′,5′-monophosphate (cyclic AMP) and guanosine 3′,5′-monophosphate (cyclic GMP), determined by biochemical techniques, are increased in whole ganglia or slices thereof that have been treated with drugs such as dopamine, norepinephrine, or methylxanthines, or after orthodromic electrical stimulation. Thus a hypothesis has been proposed (*1*, *2*) that cyclic AMP mediates the slow hyperpolarizing response, the catecholamine-induced (*4*) inhibitory postsynaptic potential (IPSP), in sympathetic ganglia; and that cyclic GMP may mediate (*1*, *2*) the slow depolarizing response, the acetylcholine-induced (*5*) slow excitatory postsynaptic potential (slow EPSP).

According to the above hypothesis, a slow, hyperpolarizing response should be recorded from a ganglion cell when the intracellular concentration of cyclic AMP is increased. Electrophysiological investigations of this hypothesis have demonstrated that cyclic AMP or its dibutyryl derivative produce a hyperpolarization (*1*), no effect (*6*, *7*), or a depolarization (*6*, *8*). Because of the variability of results, interpretation has been difficult (*9*).

The hypothesis further suggests that if cyclic GMP is the mediator of the slow EPSP, then increasing the cyclic GMP within the cell should result in a slow depolarizing response. The results of studies with extracellular applications of cyclic GMP or its dibutyryl derivative have been consistent in that only a depolarization has been reported (*1*, *2*, *6*).

The variability in these results may be due to the fact that investigators have used only extracellular applications of cyclic AMP, cyclic GMP, their dibutyryl derivatives, or drugs that act by way of an adenyl cyclase mechanism. Thus, the biochemical and electrophysiological data are not in agreement as to the role of cyclic nucleotides in ganglionic transmission.

We have attempted to alter directly the intracellular concentrations of cyclic nucleotides in principal rat sympathetic ganglion cells by microiontophoretic injection of specific nucleotides into cells while recording the resultant effects on the effective membrane resistance (R_0), resting membrane potential (RMP), and orthodromic action potential. The rat superior cervical ganglion was used in this study because it produces an IPSP upon orthodromic stimulation (*10*), and because it possesses the highest density of small intensely fluorescent (SIF) cells in ganglia of commonly investigated mammals (*11*). The SIF interneurons are important because they are interposed between preganglionic axons and principal ganglionic neurons (*12*), and might be capable of modulating ganglionic transmission by releasing a postsynaptic inhibitory transmitter.

Rat superior cervical ganglia were maintained at 37°C in vitro, with Krebs solution aerated with 95 percent O_2 and 5 percent CO_2 (*13*). Double-barreled microelectrodes were used. The recording barrel (40 to 60 megohms) was filled with 3*M* KCl. The iontophoretic barrel (60 to 100 megohms) was filled with the acid of cyclic AMP (5 m*M*) or 5′-AMP (5 m*M*), or the sodium salt of cyclic GMP (0.1 m*M*) or 5′-GMP (0.1 m*M*) at *p*H 6.6 to 6.8. Only those cells in which an orthodromic action potential could be elicited were used in this study. Impaled cells were allowed to stabilize for a minimum of 10 minutes prior to nucleotide treatment. A bridge circuit was used to pass current and record the resultant electrotonic potentials simultaneously in order to obtain an estimate of membrane resistance. A cathodal holding current prevented leakage from the ejection barrel so that the nucleotide was only released during a 1-minute continuous anodal current of 2 to 50 na. The transfer number for cyclic AMP was determined by a technique similar to that described by Shoemaker *et al.* (*14*).

The tracings in Fig. 1 depict the effect of intracellularly injected cyclic AMP on R_0 and RMP of one cell and the effect of intracellularly injected cyclic GMP on RMP and the orthodromic action potential of another cell. Both nucleotides decreased the membrane resistance (an increased conductance) and depolarized the membrane. This at times resulted in blockade of the orthodromic action potential. In each case, these effects were