Characterization of the Binding and Comparison of the Distribution of Benzodiazepine Receptors Labeled with [3H]Diazepam and [3H]Alprazolam

James K. Wamsley, Ph.D., Lisa L. Longlet, B.S., MaryAnne E. Hunt, Ph.D., Donald R. Mahan, and Mario E. Alburges, Ph.D.

The binding characteristics of [3H]diazepam and [3H]alprazolam were obtained by in vitro analysis of sections of rat brain. Dissociation, association, and saturation analyses were performed to optimize the conditions for obtaining selective labeling of benzodiazepine receptors with the two tritiated compounds. Both drugs approached equilibrium rapidly in vitro. Rosenthal analysis (Scatchard plot) of the saturation data indicated a similar finite number of receptors was being occupied by both ligands. Competition studies, using various ligands to inhibit both [3H]diazepam and [3H]alprazolam indicated that these two compounds bind to the tissue sections as typical benzodiazepine drugs and apparently do not overlap onto other subtypes of receptors. These experiments were performed by both binding assay in tissue sections and by light microscopic autoradiography. The major difference between the labeling of the two compounds is represented by the peripheral benzodiazepine sites, which are recognized by [3H]diazepam, but not occupied by [3H]alprazolam (at nanomolar concentrations). This difference was readily apparent in the autoradiograms. Other pharmacokinetic or pharmacodynamic properties must distinguish these two benzodiazepines.
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KEY WORDS: *Benzodiazepine receptors; Anxiolytics; Diazepam; Alprazolam; Autoradiography; Localization; Density; Binding characteristics*

Alprazolam is a triazolobenzodiazepine that is distinct from other compounds in its class due to its unique clinical profile (Fawcett and Kravitz 1982; Dawson et al. 1984; Ciraulo et al. 1986; Dunner et al. 1986). Alprazolam has been used as an anxiolytic, antidepressant, and in the treatment of panic disorder (Feighner et al. 1983; Pitts et al. 1983; Shehi and Patterson 1984; Rickels et al. 1985; Alexander and Alexander 1986; Leibowitz et al. 1986; Mendels and Schless 1986), whereas many other typical benzodiazepines are not as efficacious in these latter two areas, but are remarkably effective anxiolytics (others are used as sedative-hypnotics or anticonvulsants). Results from several studies have suggested alprazolam may be recognizing other receptor sites in addition to benzodiazepine receptors (Cernansky et al. 1982; Sethy and Hodges 1982; Charney and Heninger 1985; Eriksson et al. 1986; Kostowski et al. 1986; Söderpalm and Engel 1989), in the central nervous system (CNS). The in vitro analysis of [3H]alprazolam binding in synaptosomal preparations of rat brain has not been able to verify this as a direct effect (McCabe et al. 1990). To analyze the potential for over-

From the Neuropsychiatric Research Institute, Fargo; and the Departments of Pharmacology and Neuroscience, University of North Dakota, School of Medicine, Grand Forks and Fargo, North Dakota; and The Upjohn Company, Kalamazoo, Michigan.
Address reprint requests to: James K. Wamsley, Ph.D., Director of Basic Science Research, Neuropsychiatric Research Institute, 700 First Avenue South, Fargo, North Dakota 58103.
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lap of [^{3}H]alprazolam onto other sites, a receptor autoradiographic approach (Kuhar et al. 1985) was used in the present study. Previous investigations, aimed at analyzing the distribution of benzodiazepine receptors in the CNS, have principally involved the use of [^{3}H]flunitrazepam as the ligand of choice (Young and Kuhar 1979, 1980). Relatively little information is available on the binding of [^{3}H]diazepam after its initial characterization in membrane preparations (Braestrup and Squires 1977; Gallager et al. 1981). We sought to use a typical anxiolytic compound for comparison with alprazolam and to provide the binding conditions and characteristics for obtaining selective labeling of both [^{3}H]diazepam and [^{3}H]alprazolam to slide-mounted tissue sections for autoradiographic analysis.

MATERIALS AND METHODS

Male Long-Evan's rats (weighing between 180 and 200 gm) were deeply anesthetized with CO_2 and perfused intracardially with ice-cold isotonic saline. The brain tissues of the animals were rapidly dissected free from surrounding structures and frozen onto microtome chucks by slow immersion in isopentane at −70°C. Sections, 10 μ in thickness, either frontal or sagittal plane, were cut on a cryostat microtome and thaw-mounted onto subbed microscope slides. The brains of some animals were removed from the skull and gently homogenized in a glass tube with a Teflon-coated pestle. Frozen sections of this homogenate were cut in the cryostat as before. This procedure assured a uniform distribution of different cell types in each section so more consistent data could be obtained. All tissues were stored overnight in the presence of desiccant prior to being utilized in the incubation procedures.

For the biochemical assays, slide-mounted tissue sections of homogenized brain were initially given a 3-minute preincubation period in distilled water to osmotically disrupt the cells and release any gamma aminobutyric acid (GABA) or other constituents that may interfere with the subsequent binding of the radioactive ligand (McCabe et al. 1988). This was followed by two 5-minute rinses, after which individual groups of sections were incubated for 60 minutes in the presence of the radioactive ligand in Tris HCl buffer (0.17 mol/L, pH 7.6) at between 0 and 4°C. Initially, a 2 nmol/L concentration of [^{3}H]alprazolam (15 to 45 Ci/mmol, supplied by the Upjohn Company, Kalamazoo, MI) or [^{3}H]diazepam (85.2 Ci/mmol, Dupont NEN, Boston, MA) was used to label the sections. The rinse time was varied and the tissue sections were wiped from the microscope slides with filter paper and the bound radioactivity determined by liquid scintillation counting. Next, the incubation time was varied, with the rinse time held constant. This was followed by the incubation of groups of sections in various concentrations of the radioactive ligands to establish saturation. Competition studies were performed by labeling sections with a 2-nmol/L concentration of the radiolabeled ligand using the parameters established in the previously outlined experiments, in the additional presence of 10^{-4} to 10^{-11} mol/L concentrations of the following compounds: clonidine, CL218,872, sulpiride, desipramine, imipramine, 4-OH alprazolam, or flurazepam. The IC_{50} values were obtained by plotting the percent of radioligand bound versus the percent of radioligand bound multiplied by the molar concentration of the competing ligand. In all cases, adjacent sections were incubated in the presence of 10^{-6} mol/L clonazepam to establish nonspecific binding.

For the autoradiographic studies, slide-mounted sections (20 μ in thickness) of whole brain were labeled using the optimum binding conditions and, instead of wiping the sections from the slide, the sections were dried by blowing cool dry air over the tissue surface. These sections were subsequently exposed to tritium-sensitive film (Amersham Hyperfilm, Arlington Heights, IL). After an appropriate exposure period, the films were developed and analyzed by computerized microdensitometry (Palacios et al. 1981) using an MCID system (Imaging Systems Corporation, St. Catherines, Ontario). Tritium standards (Amersham, Arlington Heights, IL) were included in the exposure of each film to quantitate femtomoles of ligand bound per milligram tissue. Another group of sections was incubated in a K_d concentration of the radioactive ligand (in an effort to occupy a major portion of the sites recognized by the radioactive compound) in the additional presence of increasing concentrations of the other benzodiazepine. Thus, incubations were performed with [^{3}H]diazepam in the presence of increasing concentrations of unlabeled alprazolam and [^{3}H]alprazolam was used to label sections in the presence of nonradioactive diazepam. These sections were analyzed by receptor autoradiography to determine if the radioactive compound occupied sites where the binding was not inhibited by the other benzodiazepine.

RESULTS

Comparison of the binding characteristics of [^{3}H]alprazolam and [^{3}H]diazepam to slide-mounted tissue sections of rat brain indicated many similarities. The initial osmotic shock of the slide-mounted tissue sections dramatically reduced the binding of [^{3}H]alprazolam. The magnitude of this reduction in binding was as high as 50% and affected only specific binding (nonspecific binding remained unchanged). Binding of [^{3}H]diazepam was also reduced by the osmotic shock, but not to the extent of the alprazolam binding. The labeling with both ligands was temperature dependent and all subsequent experiments were performed at ice-bath temperature. Analysis of the dissociation data (Fig. 1) was performed using a nonlinear regression program (Graph Pad), which resulted in a calculated K_{-1} (Bylund and Yamamura 1990) of 0.034 $minute^{-1}$ for alprazolam. From the association data it could be established that equilibrium was achieved with [3H]alprazolam (Fig. 1) after a 20-minute incubation period. The K_1 calculated for alprazolam was 0.0075 $min^{-1}/nmol/L^{-1}$ providing a kinetically derived estimate of the K_d equal to 4.5 nmol/L. Similar analyses were performed for [3H]diazepam (not shown). Analysis of saturation isotherms (using a rectangular hyperbola) best fit a one-site model with a K_d of 3.2 nmol/L for [3H]alprazolam and a 6.2 nmol/L for [3H]diazepam. These figures are slightly lower than those obtained from Scatchard analysis (Rosenthal plot) of the saturation curve data (see Fig. 1). A B_{MAX} of 189.8 and 195.7 fmol of receptor bound per milligram tissue was obtained for [3H]alprazolam and [3H]diazepam, respectively. Inhibition curves (Fig. 2) generated by incubating various compounds in the presence of the two radioactive ligands, again indicated similar sites were being recognized by both of these compounds. The inhibition curves for flurazepam, 4-hydroxyalprazolam, CL218,872, and diazepam against [3H]alprazolam are shown in Figure 2. The inhibition constant (K_i) for each substance is indicated in Table 1.

Autoradiographic analysis of the binding sites occupied by diazepam and alprazolam showed many regions of overlap (Fig. 3 and Table 2). The highest densities were found in the cerebral cortex, hippocampus, and molecular layer of the cerebellum and substantia nigra. Somewhat lower densities were found in many thalamic and hypothalamic regions as well as at the cau-

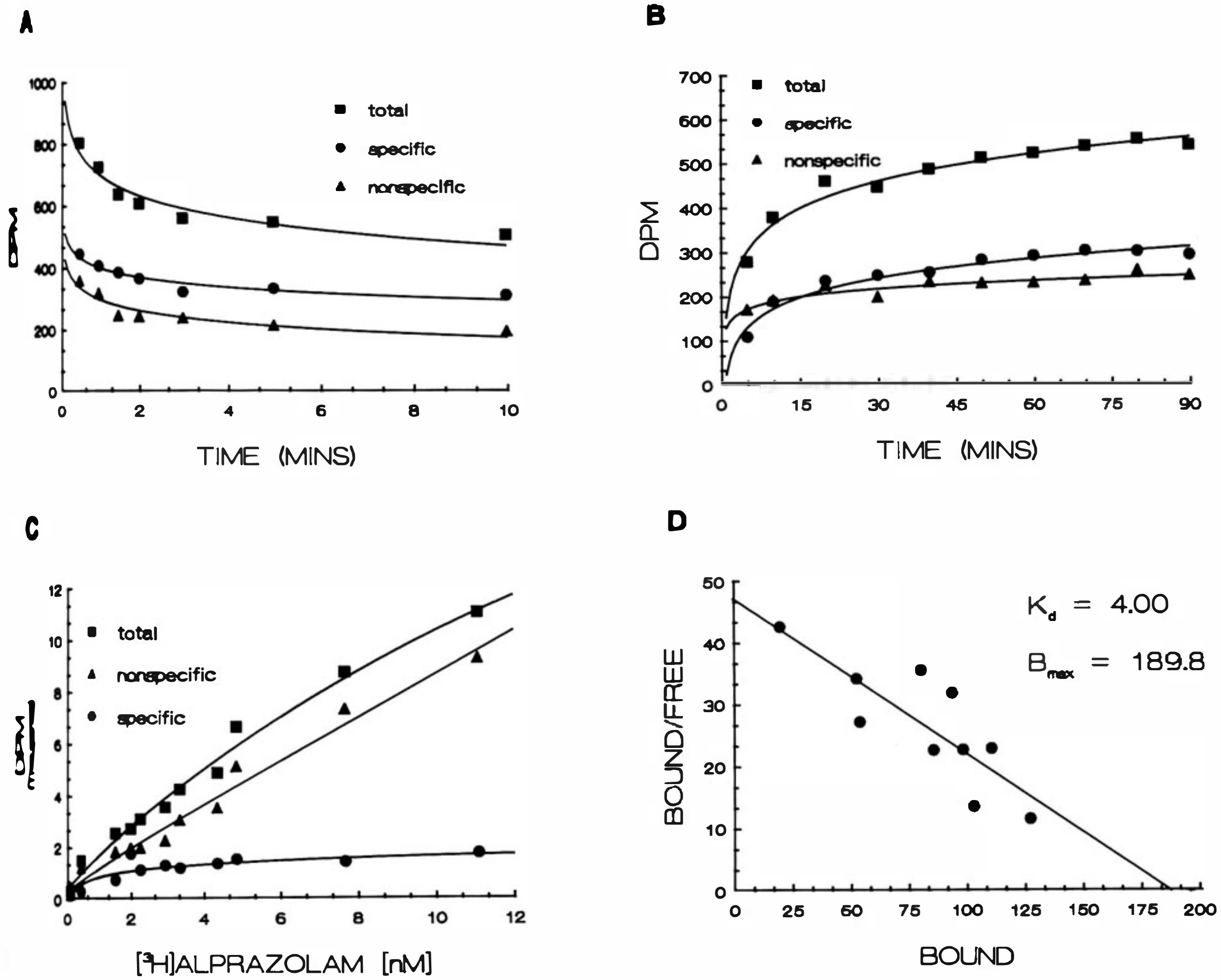

Figure 1. Graphs illustrating characterization of [3H]alprazolam binding. **A** = dissociation; **B** = association; **C** = saturation; and **D** = Rosenthal (Scatchard) plot of [3H]alprazolam binding to slide-mounted tissue sections of rat brain. The Rosenthal plot-derived K_d is 4.0 nmol with a B_{max} of 189.8 fmol of receptor bound/mg tissue. Similar analysis of [3H]diazepam showed a K_d of 7.4 nmol with a B_{max} of 195.7 fmoles/mg tissue.

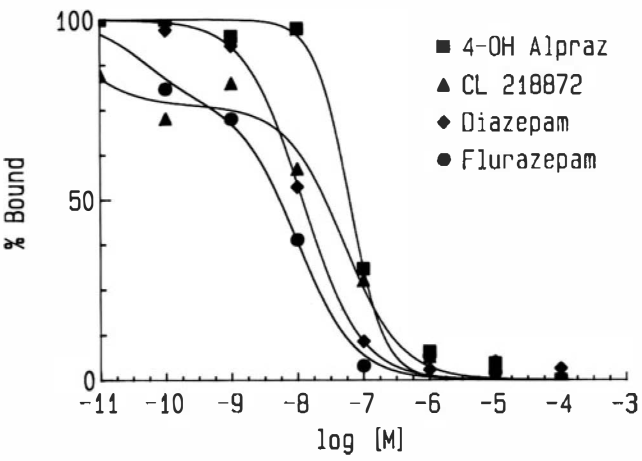

Figure 2. Graph illustrating selectivity of [^{3}H]alprazolam binding. The ability of several ligands to compete for the sites recognized by [^{3}H]alprazolam was analyzed in tissue sections. Ligands with reasonable affinity for the sites occupied by [^{3}H]alprazolam included 4-hydroxyalprazolam (an alprazolam metabolite), diazepam, CL218,872, and flurazepam. The BZ_1 receptor selective agent CL218,872 (a triazolopyridazine) inhibited the binding in a very shallow manner indicating the recognition of more than one site of the parent compound.

date putamen and nucleus accumbens. A similar distribution was found with both ligands except that the compounds differed in their ability to recognize the peripheral benzodiazepine sites (Figs. 3 and 4). Diazepam quite readily labeled these sites in the ependymal lining of the ventricles, the choroid plexus, and other regions where the blood-brain barrier is permeable. Alprazolam, however, did not label these sites at the concentrations used in this study. This was quite remarkable on the autoradiograms generated by inhibiting the binding of one compound with the other (Fig. 4). In those sections labeled with [^{3}H]diazepam in the presence of alprazolam, high concentrations of the latter compound inhibited all of the diazepam binding except that in regions of the peripheral sites. Under the opposite condition, all of the sites occupied by alprazolam were inhibited by diazepam. No other regions of labeling could be shown to be statistically different.

Table 1. Competition of [^{3}H]Alprazolam Binding

Ligand	Ki [nM][a]
Clonidine[b]	>60,000
CL218,872	31.29
Sulpiride[b]	>60,000
Desipramine[b]	>60,000
Imipramine[b]	>60,000
Diazepam	7.46
4-OH Alprazolam	38.62
Flurazepam	6.12

[a] The K_i values were computed by calculation of the IC_{50} from the inhibition curves, using Graph Pad software (Bylund and Yamamura, 1990). The experiments were performed in triplicate and each sample was repeated three times; the results are expressed as the mean of these experiments. Tissue sections were selected from four different animals and the data pooled. The sections were wiped from the slides and the residual radioactivity remaining bound to the tissue was determined by ligand scintillation counting.

[b] The antidepressant imipramine (selective for 5-HT uptake), clonidine (an α_2-adrenergic receptor agonist), sulpiride (a DA_2 receptor antagonist), and desipramine (an antidepressant selective for norepinephrine uptake) did not compete for [^{3}H]alprazolam binding.

DISCUSSION

The benzodiazepines represent a diverse set of compounds all with many similar characteristics (Haefely 1989; Williams and Olsen 1989). In fact, it has been pointed out that since many of these compounds share the same long-acting metabolites, they may indeed be similar in their pharmacodynamics (Arendt et al. 1987). The triazolobenzodiazepine, alprazolam, appears to be unique on the basis of its clinical profile since it, as opposed to other benzodiazepines, appears to be useful in the treatment of depression and panic disorder (Dawson et al. 1984). The binding of [^{3}H]alprazolam however, looks very similar to [^{3}H]diazepam (McCabe et al. 1990). Both label a finite receptor population that shows the characteristics of benzodiazepine receptors. The kinetics of the binding with the two ligands are similar and subject to modulation by GABA since osmotic shock removed some of the specific binding in each case. Competition of one compound against the other also supports the conclusion that the binding is taking place at the same sites.

Several studies have indicated an apparent overlap of alprazolam's effects onto other neurotransmitter systems including β-adrenergic, α-adrenergic, and dopaminergic receptors. The analysis of [^{3}H]alprazolam binding in synaptosomal preparations provided no indication of a direct interaction between the benzodiazepine and these adrenergic receptor subtypes (McCabe et al. 1990). Testing of many other compounds against the binding of [^{3}H]alprazolam also indicated there was not any overlap onto sites outside of the benzodiazepine receptors.

Pharmacologic studies have indicated the presence of at least three subtypes of benzodiazepine receptors in the CNS (Klepner et al. 1979; Nielsen and Braestrup 1980; Sieghart and Karobath 1980; Braestrup and Nielsen 1981; Hirsch et al. 1982; Squires 1983). These are the BZ_1, BZ_2, and peripheral benzodiazepine sites that are also known as ω_{1-3}, respectively (Langer and Arbilla 1988). The so-called peripheral benzodiazepine sites have been discovered in the brain (Benavides et al. 1983b; Gehlert et al. 1983, 1985; Schoemaker et al. 1983; Anholt et al. 1984) and these sites appear to have

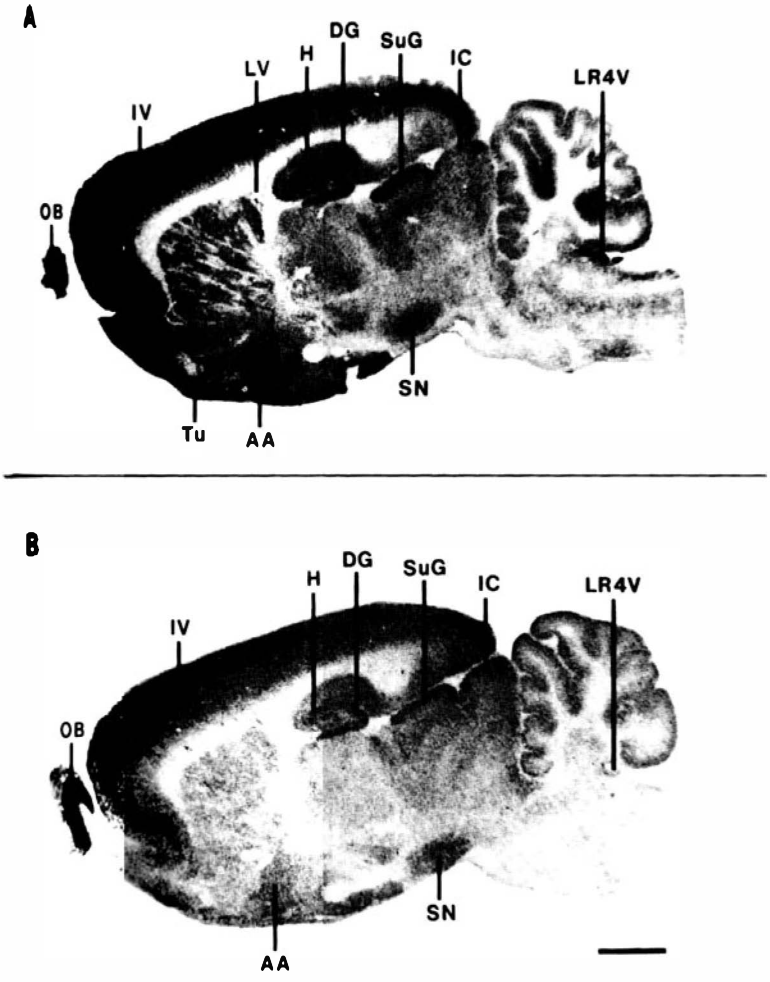

Figure 3. Autoradiograms of diazepam and alprazolam binding. Photomicrographs of the actual autoradiograms of sagittal sections of rat brain labeled with [^{3}H]diazepam (**A**) and [^{3}H]alprazolam (**B**) binding. Note the similarity in the regions showing the presence of autoradiographic grains (black against the white background). Although alprazolam binding is much lighter, the distribution is the same as that for diazepam except in the area of the lateral ventricle (choroid plexus). The exposure period for [^{3}H]alprazolam was already 4 months. Doubling this time would be expected to generate an autoradiogram similar to **A**. This can be performed by computer enhancement to compare the relative densities as seen in Figure 4. Abbreviations: OB = olfactory bulb; IV = lamina IV of the cerebral cortex; LV = lateral ventricle; H = hippocampus; DG = dentate gyrus; SuG = superficial grey layer of the superior colliculus; IC = inferior colliculus; Tu = olfactory tubercle; AA = amygdaloid area; SN = substantia nigra.

a unique subcellular distribution (Anholt et al. 1986; Basile and Skolnick 1986). Some of the centrally acting benzodiazepines recognize these sites whereas others do not. Nanomolar concentrations of agents such as diazepine and flunitrazepam will bind detectably to the peripheral sites whereas clonazepam, alprazolam, triazolam, fluraze pidem, do not. Most benzodiazepines recognize the other two subtypes of benzodiazepine receptor (BZ_1 and BZ_2) with equal high affinity. However, the benzodiazepine, quazepam, and the β-carbolines as well as several nonbenzodiazepine compounds (CL218,872, zolpidem, and CGC91164) preferentially bind the BZ_1 receptor (Klepner et al. 1979; Lippa et al. 1979; Nielsen and Braestrup 1980; Iorio et al. 1984; Wamsley et al. 1985; Billard et al. 1987). This receptor subtype also shows a unique distribution in various nuclei of the CNS (Young et al. 1981; Wamsley et al. 1985; Niddam et al. 1987; Yezuita et al. 1988). Both receptor subtypes appear to be allosterically bound to GABA receptors and they show a distribution similar to that of the high-affinity $GABA_A$ sites (Unnerstall et al. 1981; Wamsley and Palacios 1984; McCabe and Wamsley 1986).

The results of the present study support the conclusion that [^{3}H]alprazolam recognizes those sites labeled with [^{3}H]diazepam. Computerized microdensitometric analysis of the autoradiographic films generated by the two ligands show only relative differences in the amount of labeling in individual regions of the brain where benzodiazepine receptors exist. By standardizing these measurements to a central area, no statistically different amount of labeling could be obtained by one ligand versus the other, even though the labeling of diazepam is relatively higher in each individual area. Thus, it would appear that the receptor sites recognized by [^{3}H]alprazolam and [^{3}H]diazepam are the typical benzodiazepine receptors described in classic studies.

Using conditions that provide optimum signal-to-noise binding ratios, it was possible to create competition between one of the radioactive ligands against the other. As higher and higher concentrations were reached, it should have been possible to demonstrate

Table 2. Regional Distribution of [^{3}H]Diazepam and [^{3}H]Alprazolam Binding Site in Rat Brain[a]

	Bound (fmol/mg Tissue ± SEM)	
Brain Area	**[^{3}H]Diazepam**	**[^{3}H]Alprazolam**
Frontoparietal Cortex		
Laminae I–III	193.70 ± 7.51	165.17 ± 8.25
Laminae IV	217.51 ± 5.44	206.38 ± 5.91
Laminae V–VI	159.64 ± 4.33	156.90 ± 5.18
Caudate Putamen	84.66 ± 2.43	76.92 ± 3.20
Globus Pallidus	57.56 ± 2.77	50.25 ± 5.28
Ventral Pallidum	95.87 ± 5.77	87.82 ± 8.32
Field CA_1 of Ammon's Horn	155.77 ± 2.46	167.21 ± 4.47
Dentate Gyrus	182.67 ± 10.26	189.10 ± 6.30
Ventral Posterolateral/ Ventral Posteromedial Thalamic Nuclei	71.97 ± 7.96	58.25 ± 4.16
Zona Incerta, dorsal/ventral	90.41 ± 7.96	94.92 ± 3.82
Substantia Nigra	113.66 ± 6.26	114.77 ± 6.08
Superior Gray Layer of the Superior Colliculus	218.53 ± 16.33	182.22 ± 5.61
External Cortex of the Inferior Colliculus	152.36 ± 22.87	100.68 ± 7.26
Medial Geniculate Nuclei dorsal/ventral	90.72 ± 6.11	91.29 ± 10.17
Entorhinal Cortex	201.99 ± 14.22	161.13 ± 37.50
Microcellular Tegmental Nucleus	171.21 ± 23.86	150.56 ± 16.12
Molecular Layer of the Cerebellum	120.70 ± 3.02	122.21 ± 7.95
Granular Layer of the Cerebellum	29.30 ± 4.32	44.88 ± 8.86
Accumbens Nucleus	137.65 ± 9.50	121.70 ± 6.05

[a] The data are expressed as the mean ± SEM in femtomoles of ligand bound per milligram of tissue. These values were obtained from digitized images of the autoradiograms and standards on the MCDI imaging system. Only relative differences can be seen. These values are not statistically significant from each other. $p < 0.01$.

labeling of the radioactive compound outside of the realm of sites recognized by the unlabeled compound, if such nonselective binding were occurring. Indeed this was demonstrated by the inability of alprazolam to inhibit [^{3}H]diazepam binding from the peripheral binding sites in the choroid plexus and the ependymal lining of the ventricles. This appeared to be the only difference between the two ligands that we could ascertain in our microscopic analysis. The relative importance of the latter observation in reference to the unique clinical profile of alprazolam is unknown. This could be meaningful, however, since there are many hypotheses regarding the potential roles the peripheral sites may play in biologic function coupled with the widespread appearance of these receptors in many tissues (Benavides et al. 1983a; Le Fur et al. 1983; Quirion 1984; Wang et al. 1984a,b; De Souza et al. 1985; Mestre et al. 1986; Starosta-Rubinstein et al. 1987; Verma et al. 1987).

There are several other possible explanations for alprazolam's effects that involve pharmacodynamic differences with other benzodiazepine compounds (Fawcett and Kravitz 1982; Sethy and Harris 1982a; Sethy 1983). For instance, low doses of alprazolam apparently result in an increase of benzodiazepine receptor numbers, rather than a decrease (Miller et al. 1987) as seen with higher doses (Sethy and Harris 1982b). Interestingly, the peripheral benzodiazepine binding sites have been associated with various effects including production of steroid hormones, renal hypertension and an interaction with anesthetic binding sites (Wang et al. 1984a,b; Eriksson et al. 1986; Mestre et al. 1986; Clark and Post 1990; Massotti et al. 1990; Papadopoulos et al. 1990). How these effects relate to the central mechanisms involved in the potential actions of compounds acting at these sites remains to be determined. Likewise, potential species differences in benzodiazepine receptors may preclude our ability to extrapolate our findings across phylogeny.

It appears likely that alprazolam's effects on other systems occur "downstream" rather than as a direct receptor-mediated phenomenon. There is a potential, however, for diazepam to affect the peripheral sites

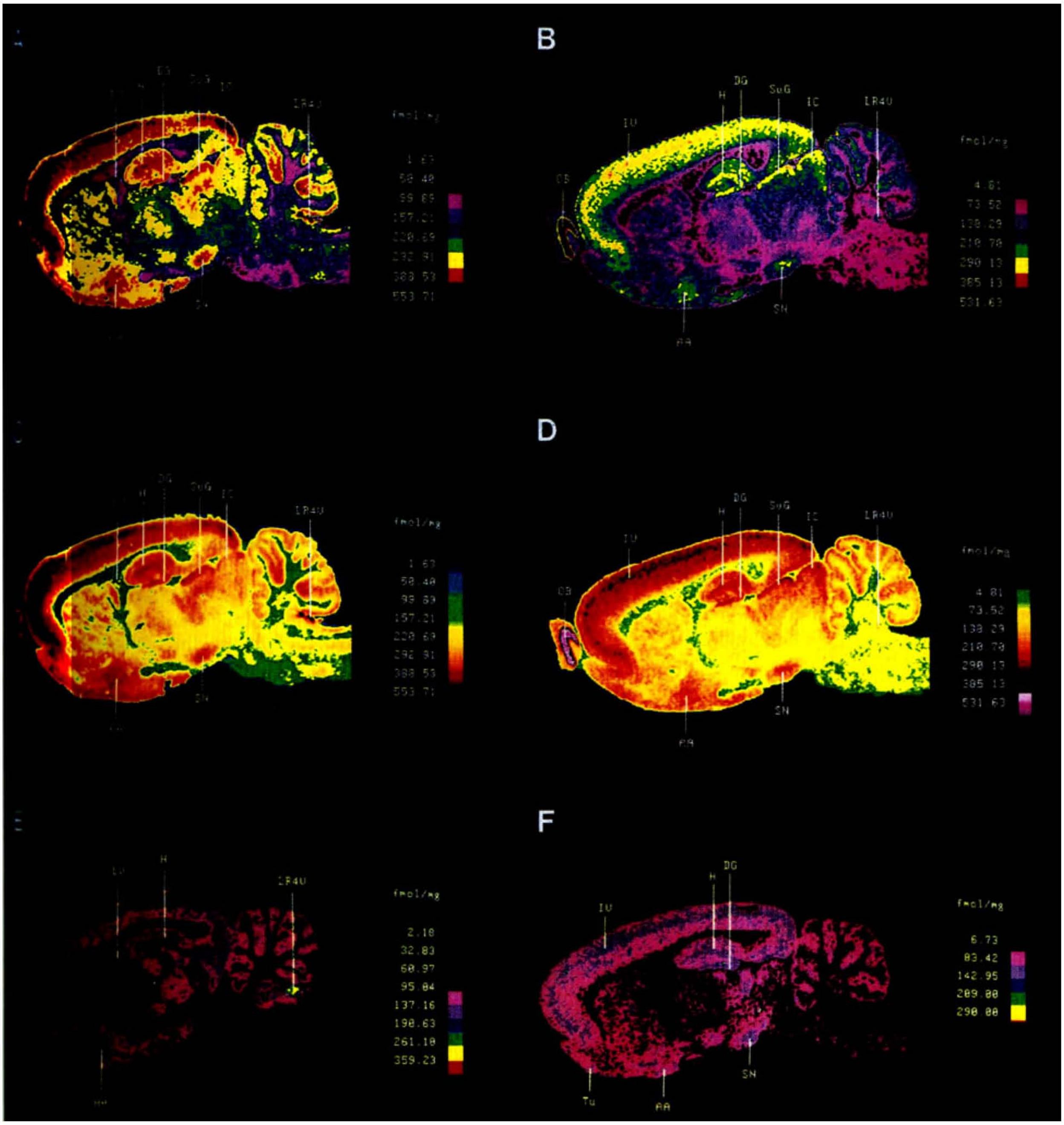

Figure 4. Comparison of [^{3}H]alprazolam and [^{3}H]diazepam receptor autoradiography. (A) Computer-generated images of autoradiograms produced by labeling sagittal sections of rat brain with (A) [^{3}H]diazepam or (B) [^{3}H]alprazolam. The color scale was held constant to show the level of binding in each case. The specific activity of the two ligands, however, makes a direct comparison difficult. In (B) and (C), the color scale was adjusted to artificially simulate the colors across the two sections. The relative differences between colors on the same section was not changed, so the binding can now be compared. Note the similarity between the sites recognized by each ligand. The section shown in (E) was labeled with [^{3}H]diazepam in the presence of 10^{-8} mol alprazolam. In (F), the section was labeled with [^{3}H]alprazolam in the presence of diazepam (10^{-8} mol). Note the similarities in the ability of each ligand to compete for the areas occupied by the other compound. One exception is readily noted in the fourth ventricle (LR4V). This area contains the peripheral sites recognized by diazepam, but not by low concentrations of alprazolam. Thus, these sites are bound by the radioactive drug, but not inhibited in (E). They are unrecognized by the radioactive compound in (F). Abbreviations: OB = olfactory bulb; IV = lamina IV of the cerebral cortex; LV = lateral ventricle; H = hippocampus; DG = dentate gyrus; SuG = superficial grey layer of the superior colliculus; IC = inferior colliculus; Tu = olfactory tubercle; AA = amygdaloid area; SN = substantia nigra.

directly, as well as BZ_1 and BZ_2 receptors, which could distinguish it from alprazolam, but not presumably other benzodiazepines like clonazepam. Molecular biologic studies indicate the presence of more than just three benzodiazepine receptor subtypes due to the identification of several distinct subunits of the GABA receptor (Lüddens and Wisden 1991). Drugs previously classified as BZ_1 selective have been shown to interact differently with the α-$GABA_A$ receptor subunit and other variants, providing a new level of differentiation of benzodiazepine compounds. Alprazolam and diazepam may have unique profiles in relation to these subunits that could account for their unique properties. Future experimentation in this area will be required to resolve these issues.

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