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Autoradiographic Demonstration of the Selectivity of Two 1-N-Trifluoroethyl Benzodiazepines for the BZD-1 Receptors in the Rat Brain

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WAMSLEY, J. K., J. S. GOLDEN, H. I. YAMAMURA AND A. BARNETT. *Autoradiographic demonstration of the selectivity of two 1-N-trifluoroethyl benzodiazepines for the BZD-1 receptors in the rat brain.* PHARMACOL BIOCHEM BEHAV **23**(6) 973–978, 1985.—Receptor autoradiographic techniques have been used to demonstrate the selectivity of two trifluoroethyl-containing benzodiazepines for one of the subtypes of benzodiazepine receptor. Indirect localization of the binding sites for quazepam and halazepam was accomplished by using the ability of these compounds to displace [^{3}H]-flunitrazepam binding. The appropriate binding parameters were selected on the basis of initial studies aimed at identifying the binding characteristics of several benzodiazepine compounds in comparison with the triazolopyridine CL218,872. Autoradiographic analysis of the benzodiazepine sites displaceable with quazepam and halazepam revealed the two benzodiazepine compounds preferentially labeled receptor sites in regions of the brain dominated by the type 1 benzodiazepine receptor subtype. Thus, quazepam and halazepam preferentially bind to benzodiazepine type-1 receptors in lamina IV of the cerebral cortex, the zona incerta, substantia nigra and the cerebellum.

Benzodiazepine receptors BZ-1 and BZ-2 sites Autoradiography Receptor localization
Benzodiazepine receptor subtypes Selective benzodiazepines Quazepam

BINDING studies, performed in homogenate membrane preparations, have indicated the existence of two distinct receptors for the class of anxiolytic, sedative-hypnotic, muscle relaxant and anticonvulsant drugs known as the benzodiazepines [6, 12, 17, 19, 23]. The characterization of these two benzodiazepine receptor subtypes (termed BZD-1 and BZD-2 or Type-1 and Type-2) was based on the binding of a representative of a non-benzodiazepine class of compounds known as triazolopyridazines [6, 8, for review see 18]. The most frequently used drug of this class is CL218,872, a compound selective for the BZD-1 receptor subtype. Later studies have also demonstrated that a group of benzodiazepine receptor antagonists, the beta-carbolines, show selectivity for the BZD-1 receptor subtype as well [12]. The identification of these benzodiazepine receptor subtypes has raised the possibility that more selective sedative-hypnotic or anxiolytic compounds could be generated which do not produce the undesired ataxic side effects seen with the more classic benzodiazepines.

The study of the individual roles BZD-1 receptor vs. BZD-2 receptor stimulation plays in producing the actions of BZD compounds has been hampered by the lack of benzodiazepines themselves, which demonstrate selectivity for these receptor subtypes and has even called into question their actual existence [10]. Recent studies, performed with the new sedative-hypnotic quazepam, have indicated that this compound is capable of producing sedation with less ataxia and tolerance than other benzodiazepines [1,14]. Homogenate preparations of brain membranes have indicated that several of these trifluoroethyl substituted benzodiazepines show an apparent selectivity for the benzodiazepine receptors in the cerebellum, a tissue thought to contain only the BZD-1 receptor subtype [2, 4, 16]. In the present study, we sought to use autoradiographic techniques [15, 26, for review see 7 or 21] to demonstrate that two of the 1-N-trifluoroethyl benzodiazepines, quazepam and halazepam, show selectivity for the BZD-1 receptor subtype found in several discrete microscopic regions of the brain.

METHOD

Initial biochemical studies were performed to examine the binding characteristics of several benzodiazepine compounds by their ability to displace [^{3}H]-flunitrazepam ([^{3}H]-flu) binding on tissue slice preparations. Male Sprague-Dawley rats (200–250 g) were sacrificed by intracardial perfusion with an ice-cold saline solution while the animals were under chloroform anesthesia. The brain of each animal was rapidly dissected and frozen onto a microtome chuck with dry ice. Cryostat sections (10 microns in thickness) were

TABLE 1

[^{3}H]-FLUNITRAZEPAM DISPLACEMENT IN SLIDE-MOUNTED TISSUE SECTIONS

Compound	IC_{50} (nM)
Clonazepam	1.72
Triazolam	2.81
Alprazolam	5.61
Desmethyldiazepam	11.21
Flurazepam	21.63
Temazapam	22.91
Nitrazepam	31.60
Diazepam	
Forebrain	7.24
Cerebellum	12.3
Quazepam	
Forebrain	89.13
Cerebellum	18.19
Halazepam	
Forebrain	124.45
Cerebellum	20.65
CL218,872	
Forebrain	223.87
Cerebellum	32.4

Serial slide-mounted tissue sections of rat brain (coronal sections through the head of the caudate nucleus or through mid-cerebellar levels) were incubated in 1 nM [^{3}H]-flunitrazepam either alone or in the presence of various concentrations of the compounds listed above. The sections were rinsed and then wiped from the slides for radioactivity measurement by liquid scintillation counting. The IC_{50} value indicates the concentrations of unlabeled compound necessary to displace 50% of the specific [^{3}H]-flunitrazepam binding.

taken through both the rostral forebrain (including caudate, frontal cortex, nucleus accumbens, etc) and cerebellum. These sections were thaw-mounted onto cold, chrome-alum/gelatin coated microscope slides. The slide-mounted tissue sections were then incubated for 60 minutes at 0–4°C in 0.17 M Tris-HCl buffer (pH 7.4) containing 1 nM [^{3}H]-flu (84.3 Ci–mmol, New England Nuclear; Boston, MA) followed by a 2-minute rinse in fresh buffer. These sections represented total binding. Triplicate sets of tissue sections of either forebrain or cerebellum were incubated in buffered media containing the radioactively-labeled flunitrazepam plus various individual concentrations of the benzodiazepines clonazepam, diazepam, desmethyldiazepam, alprazolam, temazepam, flurazepam, quazepam, halazepam, nitrazepam, triazolam or the TPZ compound CL218,872 (concentrations ranged from 10^{-11}M to 10^{-3}M). After rinsing, the sections were wiped from the glass slides using microfiber glass filter discs, and the radioactivity remaining in the tissue sections were determined by conventional liquid scintillation counting techniques.

For autoradiography, slide-mounted tissue sections were obtained as outlined above, from areas throughout the brain, and incubated in the presence of [^{3}H]-flu alone (total binding) or in the added presence of 200 nM CL218,872, 100 nM quazepam, 100 nM halazepam, 20 nM flurazepam, 30 nM nitrazepam or 2 nM triazolam (these concentrations approximate the IC_{50} values, that is, the amount of drug able to displace 50% of the specific binding of [^{3}H]-flu from the tissue section), or in the presence of 1 micromolar clonazepam as a control. These sections were dried on the slides using a stream of cool, dry, filtered air and then apposed to sheets of LKB Ultrofilm (LKB Instruments; Rockville, MD) in X-ray cassettes (for review see [7,21]). Autoradiograms generated on the tritium-sensitive film were developed after a 2-week exposure period and examined using a Leitz Orthoplan (Leitz, West Germany) microscope. Density measurements of the autoradiographic grains appearing on the film were made using a DADS Model 560 microdensitometer (an Oki computer interfaced with an MPV Compact microphotometry system attached to the Orthoplan microscope). These readings were converted to femtomoles/mg tissue (wet weight) by comparison to the grain densities generated by tritium brain paste standards developed according to Unnerstall *et al.* [20].

All drugs were generous gifts from the respective pharmaceutical agencies (clonazepam, diazepam, desmethyldiazepam, nitrazepam and flurazepam were from Hoffmann LaRoche, Nutley, NJ; CL218,872 was from Lederle Labs., Pearl River, NY; alprazolam and triazolam were from Upjohn, Kalamazoo, MI; temazepam was from Sandoz, Inc., E. Hanover, NJ; and quazepam and halazepam were from Schering Corp., Bloomfield, NJ).

RESULTS

Competition curves generated by displacing [^{3}H]-flunitrazepam binding, in sections of the forebrain, with clonazepam, diazepam, flurazepam, alprazolam, temazepam, desmethyldiazepam, triazolam and nitrazepam followed the law of mass action giving IC_{50} values of 1.72 nM, 7.24 nM, 21.63 nM, 5.61 nM, 22.91 nM, 11.21 nM, 2.81 nM and 31.60 nM, respectively (Table 1). Unlike these compounds, however, CL218,872, quazepam and halazepam showed somewhat shallow displacement curves which indicated a deviation from simple Michaelis-Menten kinetics. These compounds showed IC_{50} values of 223.87 nM (CL218,872), 89.13 nM (quazepam), and 124.45 nM (halazepam). Hill plots of the data obtained from sections of forebrain showed Hill coefficients significantly less than 1 ($p<0.05$) for these latter three compounds while all of the other benzodiazepines showed Hill coefficients of 1 in these same tissues. All of the compounds had Hill coefficients which approached unity when tested using sections of cerebellar tissues.

Autoradiographic localization of [^{3}H]-flu binding in sections through the mesencephalon showed the presence of high concentrations of benzodiazepine receptors in the superficial lamina of the superior colliculus, molecular layer of the dentate gyrus, retrosplenial cortex, periaqueductal gray matter, mammillary bodies and substantia nigra. Addition of 200 nM concentrations of CL218,872 to the incubation medium greatly attenuated the binding in the substantia nigra while producing little perturbation of the binding in the superior colliculus, dentate gyrus, retrosplenial cortex or mammillary bodies (Table 2; Fig. 1). The binding in the periaqueductal gray matter was partially reduced. Using an approximate IC_{50} value concentration of quazepam or halazepam to displace [^{3}H]-flu binding produced a quantifiable pattern of labeling similar to that of CL218,872 (Table 2). Selective displacement of [^{3}H]-flu binding by these three compounds was also seen in such regions as: lamina IV of the cerebral cortex (Fig. 2), the cingulate gyrus, the globus pallidus, several thalamic nuclei, the zona incerta and the

TABLE 2

REGIONAL COMPARISON OF CL218,872, QUAZEPAM AND HALAZEPAM DISPLACEABLE [^{3}H]-FLUNITRAZEPAM BINDING

Area	Specific [^{3}H]-Flu	CL218,872	% Displaced†	Quazepam	% Displaced†	Halazepam	% Displaced†
Basal forebrain	61.2 ± 2.1	25.6 ± 0.8	58	26.0 ± 1.4	58	25.3 ± 1.7	59
Dentate gyrus molecular layer	63.7 ± 2.4	53.0 ± 1.2	17	56.5 ± 1.5	11	65.2 ± 1.1	−2
Zona incerta	36.2 ± 1.2	15.7 ± 0.9	57	15.0 ± 1.1	59	12.1 ± 0.6	67
Substantia nigra Pars reticulata	53.0 ± 1.0	10.7 ± 0.3	80	12.5 ± 0.3	16	14.9 ± 1.1	72
Mammillary body	53.0 ± 1.5	54.0 ± 2.4	−2	51.8 ± 2.7	2	55.5 ± 2.0	−5
Superior colliculus Superficial lamina	70.6 ± 1.4	51.2 ± 0.7	27	51.5 ± 0.8	27	64.7 ± 1.3	8
Periaqueductal gray matter	48.9 ± 1.6	20.2 ± 0.7	59	18.8 ± 2.2	62	23.2 ± 0.9	53

*The values listed indicate the femtomoles of [^{3}H]-flu bound per mg tissue plus or minus the standard error of the mean. These measurements were obtained using a DADS Model 560 computerized microdensitometry system to quantitate the autoradiographic grain densities associated with each indicated region. The readings (% transmission) were converted to femtomoles by comparison with tritium standards.

†Percent of specific [^{3}H]-flu binding displaced by an IC_{50} value concentration of the indicated compound.

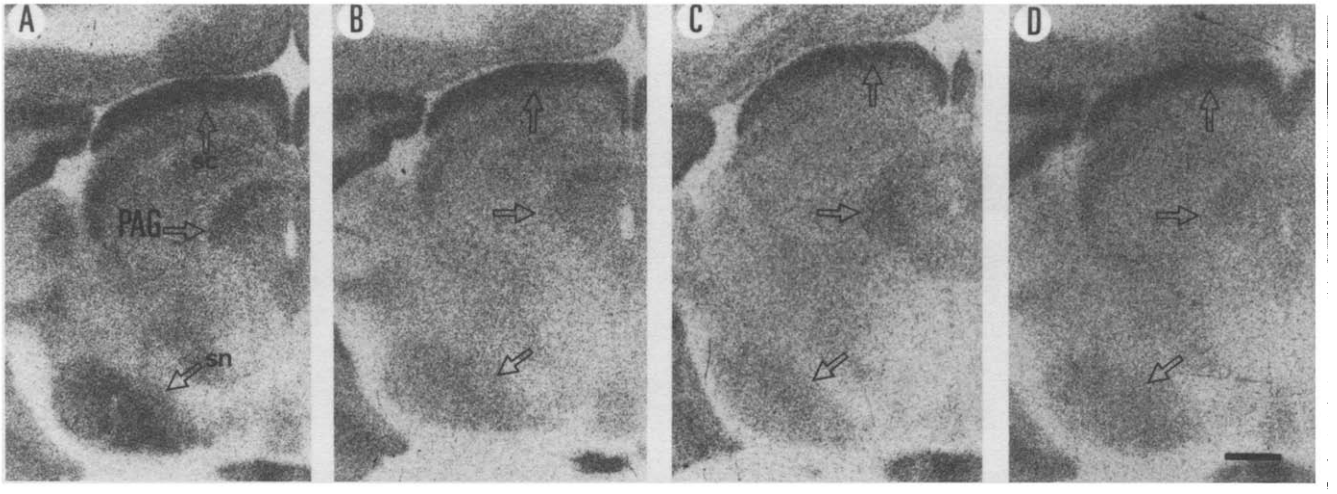

FIG. 1. A. The autoradiogram shown in this photomicrograph shows the total density of [^{3}H]-flu binding in a section of rat brainstem at the level of the substantia nigra (sn). B. The section used to generate this autoradiogram was adjacent to the one shown in A. It was labeled with [^{3}H]-flu in the presence of 200 nM CL218,872. Note the reduction in labeling of the substantia nigra and periaqueductal gray matter (PAG). Labeling in the superior colliculus (sc), as well as other structures in the field (molecular layer of the dentate gyrus and the mammillary body), do not change appreciably in the presence of the selective BZD-1 displacer. C. This section was labeled with [^{3}H]-flu in the presence of an IC_{50} concentration of halazepam. D. The section which was used to generate this autoradiogram was incubated in the presence of 100 nM quazepam. Note again the reduction in grain density appearing in the substantia nigra and periaqueductal gray matter in relation to that present in A. Bar=500 microns.

cerebellum (Fig. 3). None of the other compounds tested showed any preferential displacement in these areas (Table 3).

DISCUSSION

Analysis of the competition curves showed that CL218,872, quazepam and halazepam were less potent displacers of [^{3}H]-flu binding in the forebrain than were the other benzodiazepines tested. Hill plots of this data demonstrated that only CL218,872, quazepam and halazepam had Hill coefficients significantly less than 1 in forebrain sections, whereas all of the drugs showed Hill coefficients which approximated unity in sections of cerebellum. These observations are in accordance with previous biochemical studies performed in tissue homogenate preparations and indicate that quazepam and halazepam [4,16], like CL218,872 [6, 8, 11], preferentially bind to the BZD-1 subtype of benzodiazepine receptor.

Localization of the BZD receptors labeled with [^{3}H]-flu, using autoradiographic techniques, allowed the demonstration of high densities of these sites in many brain regions. The distribution of BZD sites found in the present study was

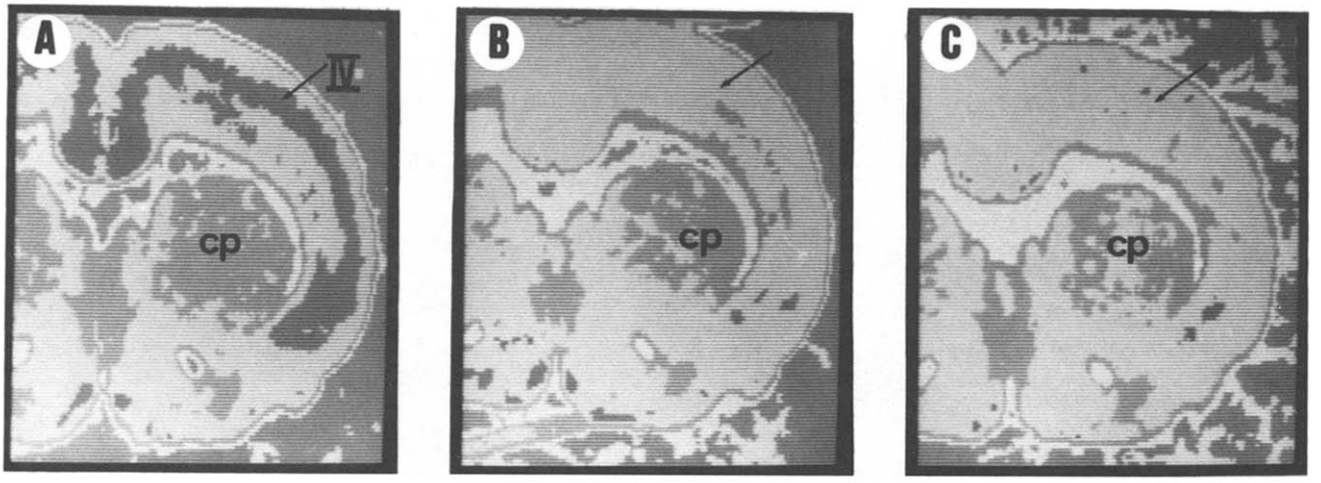

FIG. 2. A. Computer reconstructed scan of the grain densities appearing over a section of rat forebrain labeled with [^{3}H]-flunitrazepam. Heavy labeling is indicated by the dark color seen in lamina IV of the cerebral cortex (IV) extending medially into the cingulate cortex. A lower grain density (indicating a lower BZD receptor density) is indicated by the higher shade of gray apparent in the caudate-putamen (cp). B. A tissue section adjacent to the one shown in A was labeled and scanned in the same fashion. This section, however, was incubated in the presence of 200 nM CL218,872. Note the receptor labeling is reduced in lamina IV (arrow), but remains essentially unchanged in the caudate-putamen (cp). C. Another serial section was incubated in the presence of a 100 nM concentration of quazepam. The scan of this section appears similar to that shown in B indicating preferential displacement by quazepam of the BZD-1 sites present in the cortex.

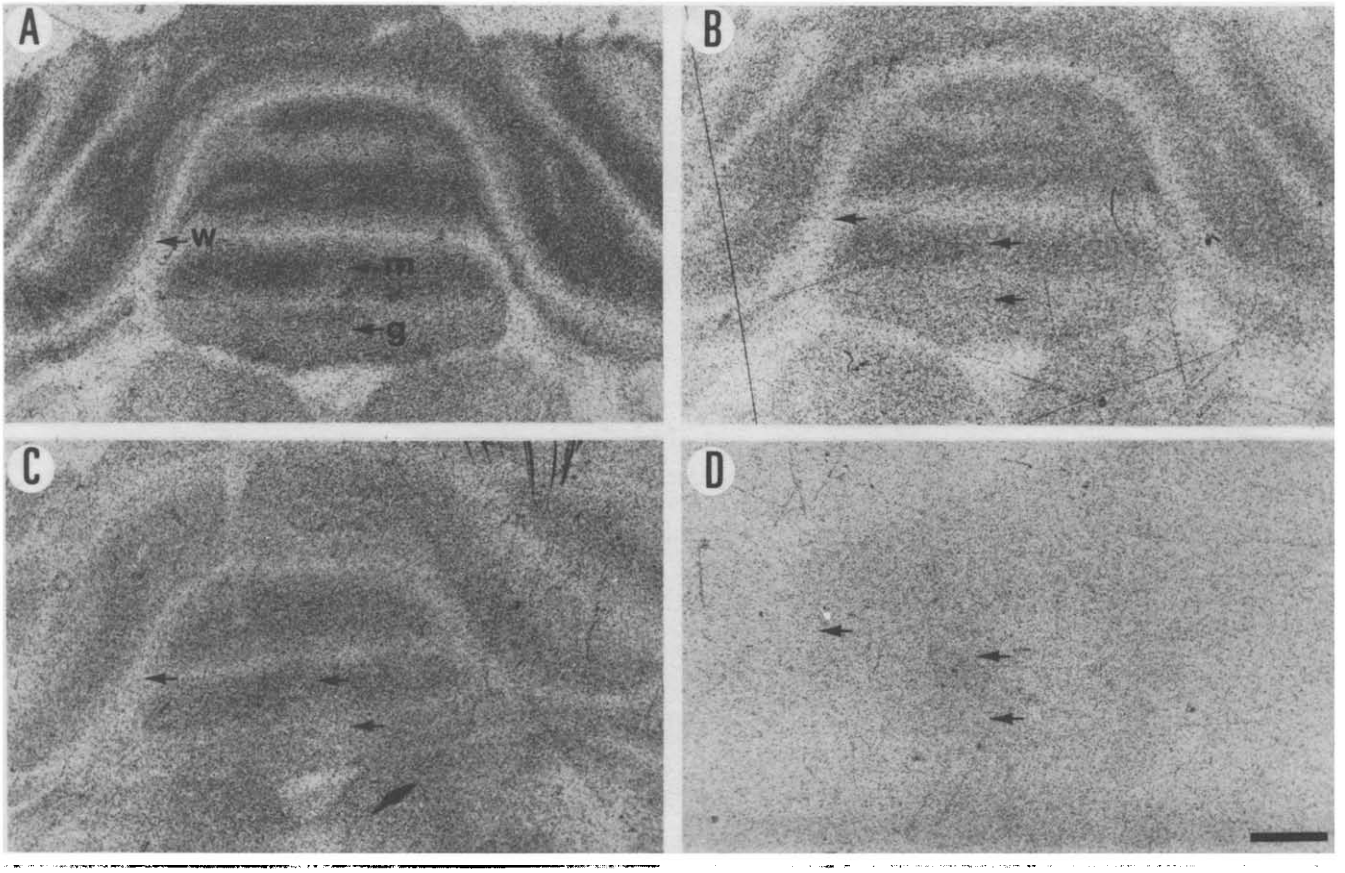

FIG. 3. A. A section through the rat cerebellum was labeled with [^{3}H]-flu and used to generate the autoradiogram shown in this photomicrograph. The BZD receptor density is highest in the molecular layer (m). B. A section adjacent to the one shown in A was incubated in the presence of CL218,872 and used to produce the autoradiographic grain distribution depicted on photomicrograph of the tritium-sensitive film. Note the overall reduction [^{3}H]-flu binding which takes place since the cerebellum is a BZD-1 tissue exclusively. C. This autoradiogram shows the BZD receptor binding remaining in a section incubated in the presence of 100 nM halazepam. The distribution and density of grains is similar to that shown in B for CL218,872 indicating halazepam is selectively displacing these sites in the same manner. D. In each case, adjacent sections labeled in the presence of excess clonazepam to indicate regions of nonspecific binding, show uniformly distributed low grain densities similar to those levels seen associated with the white matter pathways in these figures. An example is shown in this photomicrograph. Bar=500 microns.

TABLE 3

COMPARISON OF CL218,872 AND QUAZEPAM DISPLACEABLE [^{3}H]-FLUNITRAZEPAM BINDING WITH THAT OF OTHER SEDATIVE-HYNOTICS*

Area	Specific [^{3}H]-Flu	CL218,872	%	Quazepam	%	Flurazepam	%	Nitrazepam	%	Triazolam	%
Cortex											
Laminae I-III	35.1 ± 1.4	15.2 ± 1.3	57	12.3 ± 0.8	65	19.4 ± 0.6	45	15.4 ± 1.0	56	18.6 ± 1.1	47
†Lamina IV	116.8 ± 2.9	25.5 ± 1.6	78	24.8 ± 1.1	79	54.2 ± 1.4	54	65.1 ± 1.6	44	57.6 ± 2.1	51
Lamina V	32.6 ± 2.7	14.9 ± 1.7	54	11.7 ± 0.5	64	13.2 ± 0.5	60	15.9 ± 0.3	51	17.2 ± 1.8	47
†Lamina VI	41.5 ± 2.5	13.2 ± 2.7	68	13.2 ± 0.8	68	21.7 ± 0.6	48	19.1 ± 0.5	54	18.3 ± 4.6	56
Caudate-putamen	17.4 ± 1.1	15.8 ± 0.9	9	18.0 ± 1.0	−3	16.7 ± 1.1	4	15.8 ± 0.4	9	15.0 ± 0.5	14
Cerebellum											
†molecular layer	24.3 ± 3.9	2.6 ± 0.2	89	1.8 ± 0.1	93	7.5 ± 0.5	69	11.2 ± 1.3	53	12.7 ± 1.1	47
†granule cell layer	7.6 ± 1.1	1.3 ± 0.2	83	1.0 ± 0.1	87	3.6 ± 0.3	53	4.7 ± 0.2	38	4.3 ± 0.7	43
white matter	0.8 ± 0.4	0.7 ± 0.2		0.3 ± 0.3		0.7 ± 0.2		1.0 ± 0.4		1.1 ± 0.5	
Nucleus reticularis											
gigantocellularis	11.5 ± 0.9	4.1 ± 0.8	64	3.0 ± 0.8	74	5.6 ± 0.2	51	8.8 ± 0.5	23	6.4 ± 1.0	44
parvocellularis	9.6 ± 1.5	3.1 ± 0.2	68	2.5 ± 0.1	74	4.1 ± 0.4	57	5.5 ± 0.3	43	5.5 ± 0.8	43

*These data were obtained and represented in a fashion similar to that described for Table 2.
†Indicates regions where the BZD-1 receptor subtypes predominates.

in accordance with that originally reported by Young and Kuhar [25,27] using similar conditions. Likewise, sites in which the binding of [^{3}H]-flu was selectively displaced by CL218,872 (and, thus, regions where the BZD-1 receptor subtype predominates) overlapped with those originally described by Young *et al.* [28], again using similar conditions (for review see [22]). The quantitated values of BZD-1 and BZD-2 binding are close to those originally reported by Unnerstall *et al.* [20] in many brain regions. The novel finding in the present study is the demonstration that two trifluoroethyl-containing benzodiazepines show the same selectivity for BZD-1 receptors as CL218,872 in slide-mounted tissue sections, and this selectivity is manifest in microscopic regions of the brain where the BZD-1 site predominates. Thus, quazepam and halazepam competed for sites occupied by [^{3}H]-flu in lamina IV of the cerebral cortex, the zona incerta, substantia nigra and the cerebellum. Other brain regions shown in this study to have BZD-1 sites include the cingulate gyrus, retrosplenial cortex, caudal pontine reticular formation and the apparant mixture of BZD-1 and BZD-2 receptors which exists in the periaqueductal gray matter. The same concentration of quazepam and halazepam did not interfere with much of the binding in regions including the caudate-putamen, molecular layer of the dentate gyrus, superior colliculus, or the mammillary body; all regions where the BZD-2 receptors predominate.

The functional significance of stimulation of the BZD-1 receptor vs. the BZD-2 receptor subtype is unknown. Some behavioral meaning has been purported to result from selective activation of the BZ-1 receptor subtype [6, 8, 9], but the results remain controversial [13]. The anatomical location of the BZD-1 sites in regions of the brain involved in the control of movement (the globus pallidus, substantia nigra and the cerebellum) could indicate areas responsible for the affects of BZD-1 sites on influencing motor control. Localization of BZD-1 receptors in lamina IV of the cerebral cortex (where the specific thalamic afferents terminate), the thalamus, and the zona incerta (a rostral extension of the reticular system) could also be involved in the control of movement by playing a role in sensory ataxia. Interestingly, both animal and clinical studies of the sedative-hypnotic quazepam and the anxiolytic halazepam have indicated these benzodiazepines are free of some of the ataxic-producing side effects and dependence liability of other benzodiazepines [1, 5, 24]. Thus, it would appear that quazepam and halazepam interaction with the BZD-1 receptor to "inhibit" or "suppress" mechanisms involved in producing ataxia which originate from these regions. Alternatively, one could hypothesize that either quazepam or halazepam is only a partial agonist and its antagonistic activity is manifest at the BZD-1 site. Recent reports also indicate that these two trifluorethyl substituted benzodiazepines differ in their ability to compete for the "peripheral-type" benzodiazepine receptor labeled with RO5-4864 [3]. Fortunately, we now have these two benzodiazepine compounds to use as tools to study the significance of these benzodiazepine receptor subtypes.

Neurochemical or neurophysiological monitoring of the effects of benzodiazepine receptor binding in regions where BZD-1 receptors, BZD-2 or the "pheripheral-type" receptors predominate, will undoubtedly aid us in our attempts to understand the action of BZD drugs at these sites. Novel clinical studies on these compounds will allow determination of whether the BZD-1 receptor selectivity results in a clinically superior benzodiazepine.

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