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[^{3}H]DIAZEPAM BINDING IN MAMMALIAN CENTRAL NERVOUS SYSTEM: A PHARMACOLOGICAL CHARACTERIZATION

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Abstract

Two types of benzodiazepine binding sites for [^{3}H]diazepam in mammalian central nervous tissue were identified using selective *in vitro* tissue culture and *in situ* kainic acid lesion techniques. These two binding sites were pharmacologically distinguished by differential displacement of the [^{3}H]diazepam radioligand using the centrally active benzodiazepine, clonazepam, and the centrally inactive benzodiazepine, RO5-4864. Clonazepam-displaceable binding sites were found to be located principally on neuronal membranes, while RO5-4864-displaceable binding sites were found to be located on non-neuronal elements. These pharmacological distinctions can be used to characterize the predominant cell types which bind benzodiazepines in nervous tissue. It is suggested that one quantitative measure of different cell populations is the ratio of clonazepam- to RO5-4864-displaceable [^{3}H]diazepam binding within a single neuronal tissue sample.

Binding sites for benzodiazepines in brain which have high affinity and show saturability and stereospecificity have been described (Squires and Braestrup, 1977; Möhler and Okada, 1977a). These binding sites appear to represent sites of pharmacological actions of benzodiazepines based upon the high degree of correlation between the ability of a series of benzodiazepines to displace binding of [^{3}H]diazepam from the high affinity sites in brain and their activity in a number of behavioral tests including conflict, muscle relaxant, and anticonvulsant tests (Tallman et al., 1980a). In such tests, one of the most potent compounds observed is clonazepam, 5-(*o*-chlorophenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one; in addition, clonazepam is a potent (K_i < 2 nM) displacer of [^{3}H]diazepam binding to brain specific sites (Braestrup et al., 1977; Möhler and Okada, 1977b). At the other end of the spectrum of potency is RO5-4864, 7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepin-2-one, which elicits none of the behavioral effects of the benzodiazepines and is relatively inactive (K_i > 100 μM; Braestrup et al., 1977) in displacing [^{3}H]-diazepam from the high affinity sites in brain. The lack of potency of RO5-4864 is particularly striking because it is quite similar to diazepam, differing only by possessing a *p*-chloro substituent.

In addition to brain, initial investigations of the binding of [^{3}H]diazepam indicated that specific high affinity binding of [^{3}H]diazepam could be obtained in several peripheral tissues including kidney (Braestrup et al., 1977). The sites on the kidney cells, although possessing a high affinity for [^{3}H]diazepam, showed an entirely different pharmacological spectrum from the brain site. In this spectrum, which does not correlate with the behavioral profile of the benzodiazepines, RO5-4864 is a rather potent (5 nM) displacer of [^{3}H]diazepam binding; in contrast, clonazepam is much weaker (>100 nM; Braestrup et al., 1977). Thus, although peripheral tissues possess high affinity sites for diazepam, those sites seem to be different from the central sites (Table I).

In addition to the kidney, the "peripheral sites" seem to be on cultured cells of various types, including those of presumptive neuronal origin (Syapin and Skolnick, 1979; Strittmatter et al., 1979; Guidotti et al., 1979; Tallman et al., 1980b). Both rat astrocytoma (Syapin and Skolnick, 1979; Tallman et al., 1980b) and neuroblastoma (Syapin and Skolnick, 1979; Guidotti et al., 1979; Tallman et al., 1980b) cells possess a large number of these peripheral (RO5-4864-displaceable) sites but binding is not displaced by clonazepam.

The present series of experiments was designed to investigate the cellular localization and possible significance of these pharmacologically distinct binding sites using *in vitro* tissue culture and *in vivo* lesioning techniques. Our studies indicate that both clonazepam and RO5-4864 displace [^{3}H]diazepam binding within the central nervous system (CNS). However, the amount of each type of binding depends upon the type of cellular element studied. Clonazepam-displaceable binding appears to occur only on neuronal elements. Destruction of neurons by lesioning *in vivo* with kainic acid or selective proliferation of non-neuronal elements in *in vitro* tissue cul-

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TABLE I

Properties of [^{3}H]diazepam binding on in vivo preparations

Binding of [^{3}H]diazepam was assayed in membrane fragments prepared from different tissues alone or in the presence of 10^{-7} M of the indicated displacing drug.

Concentrations of [^{3}H]Diazepam	[^{3}H]Diazepam Bound		
	Total	Clonazepam Displaceable	RO5-4864 Displaceable
	fmol/mg protein		
Adult rat brain (2 nM)	278 ± 4	270 ± 7	24 ± 2
Adult rat kidney (2 nM)	257 ± 4	34 ± 5	253 ± 9

tures increases the RO5-4864-displaceable binding and concomitantly decreases clonazepam-displaceable binding.

Thus, it is suggested that the ratio of clonazepam- to RO5-4864-displaceable binding from [^{3}H]diazepam binding sites may be used to determine cell type and may predict possible pharmacologically or physiologically induced alterations of cellular composition within the central nervous system.

Materials and Methods

Cerebral cortices from fetal rats (Sprague-Dawley/Taconic Farms) in the 14th or 15th day of gestation were removed and single cell suspensions were prepared by trituration of the pooled tissue from three to five pregnant rats (15 to 20 passes in a Pasteur pipette) in calcium-free Eagle's minimal essential medium (see below). The cell suspensions were filtered through HD 155 Nitex mesh (Nitex Co.) and cells were counted in a Coulter model ZBI counter.

Cells were cultured in Eagle's minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum; gentamicin sulfate was routinely added to a final concentration of 50 μg/ml. This medium is referred to as MEM. Incubation was at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

"Neuronal" and "non-neuronal" cultures. Cells were seeded in MEM on collagen-coated plastic Petri dishes at a cell density of 50,000 to 90,000 cells/cm^2. After 72 hr incubation, the medium was replaced with fresh MEM. In the case of the "neuronal" cultures, the medium was supplemented additionally with 20 μg/ml of cytosine arabinoside, which is reportedly selectively toxic for dividing cells (Furlong and Gresham, 1971). After an additional 24 hr incubation, the medium was aspirated, the dish was washed once with 10 ml of MEM, and fresh MEM was added. Cultures treated in this manner were harvested on the 5th and 11th day of incubation. Cultures not exposed to cytosine arabinoside, in which other cell types appeared to overgrow the neurons, were harvested on 11th day and are referred to as "non-neuronal" cultures.

"Neuronal enriched" cultures. Cells were seeded in MEM in collagen-coated plastic Petri dishes (100 mm diameter) at a cell density of 350,000 cells/cm^2. After 24 hr incubation, the MEM was replaced with Medium #1 as described by Sotelo et al. (1980) containing modified Dulbecco-Vogt medium with 10% fetal calf serum, 10% horse serum, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (instead of $NaHCO_3$), 80 units/liter of insulin, 50 μg/ml of gentamicin sulfate, and dextrose in a final concentration of 10 mg/ml. Final pH was 7.2 to 7.4. After 4 days in Medium #1, the culture medium was replaced with Medium #1 containing 4 μg/ml of cytosine arabinoside. On day 9 in culture, Medium #1 was replaced with Medium #2 as described by Sotelo et al. (1980). Medium #2 was similar to Medium #1 but no fetal calf serum was added, horse serum concentration was increased to 20%, and cytosine arabinoside was included in a concentration of 10 μg/ml. Medium #2 was replaced with fresh Medium #2 on day 15 in culture. Cells were harvested at 20 days in culture and are referred to as "neuronal enriched" cultures.

"Fibroblast" cultures. Fetal cortical cells were seeded on uncoated, 60-mm diameter, plastic Petri dishes in MEM at a cell density of 150,000 cells/cm^2. The medium was changed on the 3rd, 5th, and 9th days of incubation. On the 12th day, one Petri dish, which appeared confluent with a variety of cell types, was trypsinized by standard procedures and the cells were transferred to a single 75-cm^2 tissue culture flask. Thereafter, these cells, which appeared fibroblastoid in morphology, were maintained in culture by standard methods (i.e., the medium was replaced on Mondays and Wednesdays and the culture was split 1:5 after trypsinization each Friday). Benzodiazepine binding was measured on membranes prepared from these cells in the third passage.

C_6 cultures. Rat glioma line C_6 (CCl 107) was originally obtained from American Type Culture Collection, Rockville, MD. C_6 cells (41 to 68 passages) were grown in Dulbecco's minimal essential medium with 4.5 mg/ml of glucose, 5% fetal calf serum, and 4 mM L-glutamine as previously described (Strittmatter et al., 1979). Cultures were propagated in Falcon plastic culture flasks at 37°C in a humidified atmosphere of 5% CO_2, 95% air and assayed after they had become confluent, 4 to 5 days after seeding.

Preparation of membranes from cell cultures for binding assay. For binding studies, the medium was carefully aspirated from the culture plate and the cells were rinsed with 2 ml of ice cold phosphate-buffered saline (PBS; pH 7.4). Cells were removed from the culture plates with a rubber spatula and added to a centrifuge tube in 4 ml of PBS. The cells were pelleted by low speed centrifugation (3000 × *g*, 10 min) at 4°C and the supernatant was aspirated. Membrane fragments were prepared by a 30-sec sonication (Kontes Ultrasonic Cell Disrupter; 5.2 total watts) in 1.5 ml of cold Tris-HCl buffer. [^{3}H]Diazepam binding was measured in washed membrane fragments by diluting and sonicating membrane fragments in 5 ml of Tris-HCl buffer (0.05 M; pH 7.4 at 4°C). The suspension was centrifuged at 18,000 × *g* for 10 min. This washing procedure was repeated two additional times. The final tissue pellet was resuspended in 1.0 to 1.5 ml of Tris-HCl and a 100-μl aliquot (containing 0.1 to 0.3 mg of protein) was assayed for [^{3}H]diazepam binding as described under "Benzodiazepine binding assay."

Kainic acid lesions. A total of 17 male Sprague-Dawley rats (Taconic Farms/220 to 260 gm) were used in these experiments. One or 2 μg of kainic acid (Sigma, St. Louis, MO) was dissolved in 1 μl of phosphate-buffered

saline (PBS, pH 7.4) and injected over a 3-min period into the right striatum. Coordinates adapted from König and Klippel (1970) (AP 7.9, L 2.6, V 4.5) were employed. Control (sham) injections of 1 μl of PBS were made in the contralateral striatum of each animal. Fourteen days post-injection, rats were decapitated and their striata were rapidly dissected at 4°C. Each lesioned and control striatum was analyzed individually for both benzodiazepine binding and glutamic acid decarboxylase (GAD) activity. Striata weighing 47 ± 1 mg were homogenized in 50 vol (w/v) of 0.05 M Tris-HCl buffer, pH 7.5 at 4°C. Following homogenization, a 100-μl aliquot was removed for analysis of GAD activity as described below. The remaining fraction was used for benzodiazepine binding measurements.

Tissue homogenization and preparation for binding assay. Brain tissue samples were prepared by sonication for 30 sec at 5.2 total watts using a Kontes Ultrasonic Cell Disrupter in ice cold 0.05 M Tris-HCl buffer (pH 7.4 at 4°C). Tissues were diluted to 5 mg of tissue/100 μl. After 100 μl of homogenate was removed for GAD activity analysis, the sample was resuspended in 50 vol of Tris buffer, rehomogenized, and spun at 18,000 × *g* for 10 min. The membrane pellet then was resuspended in 50 vol (w/v) of Tris (0.05 M, pH 7.5) and centrifuged at 18,000 × *g* four additional times to remove endogenous GABA (γ-aminobutyric acid) (Gallager et al., 1980b). Washed membranes were suspended in cold 0.05 M Tris to a final concentration of 0.2 to 0.5 mg of protein/100 μl. Benzodiazepine binding was assayed as described under "Benzodiazepine binding assay." Proteins were determined for each sample according to Lowry et al. (1951) and data is expressed per milligram of protein.

Analysis of GAD activity. To 100 μl of fresh or frozen (−70°C), thawed homogenate, 50 μl of a solution containing 50 mM potassium phosphate buffer (KPB; pH 6.9), 0.2 mM pyridoxal phosphate (PP), 1 mM *S*-(2-aminoethyl)isothiuronium bromide hydrobromide (AET), 0.1 mM EDTA (ethylenediaminetetraacetate), and 0.25% (w/v) Triton X-100 was added. Samples were rehomogenized in a glass-glass homogenizer on ice and centrifuged for 30 min at 18,000 × *g*. Forty microliters of the resulting supernatant was added to 10 μl of a mixture containing 20 mM L-monopotassium glutamate, 200 mM KPB (pH 6.9), 1 mM AET, and 0.2 mM PP with 0.5 μCi of L-[1-^{14}C]glutamic acid. The assay was run in duplicate and carried out according to the method of Tappez et al. (1976) with an incubation time of 60 min at a temperature of 37°C. Proteins were determined by the method of Lowry et al. (1951) and the assay was linear at various protein concentrations up to 60 min.

Benzodiazepine binding assay. Benzodiazepine binding to membranes from brain or cell culture tissues was measured using an incubation volume of 500 μl consisting of: 100 μl of homogenate, 350 μl of Tris-HCl buffer or drug dissolved in Tris buffer, and 50 μl of [^{3}H]diazepam (specific activity, 83.5 Ci/mmol, New England Nuclear, Boston) in a concentration of 5 nM. When [^{3}H]flunitrazepam was used as the benzodiazepine radioligand, (specific activity, 88 Ci/mmol, New England Nuclear, Boston), it too was added in a concentration of 5 nM. Incubations were carried out at 4°C for 30 min and terminated by rapid filtration through Whatman GF/B filters with two 7-ml washes of ice cold Tris-HCl buffer. Filters were suspended in 10 ml of Aquassure (New England Nuclear, Boston) and radioactivity was measured with a Beckman liquid scintillation spectrometer. Total [^{3}H]diazepam binding was assayed in triplicate. Concentrations of 10^{-10} to 10^{-6} M clonazepam or 10^{-9} to 10^{-5} M RO5-4864 were added as indicated to displace [^{3}H]diazepam binding. Ratio of clonazepam-displaceable to RO5-4864-displaceable [^{3}H]diazepam binding was determined following the addition of either 10^{-7} M clonazepam or 10^{-7} M RO5-4864 to successive homogenate samples. Duplicate displacement assays were run; more replications were obtained when sufficient homogenate was available.

Results

In vitro studies

Primary cell culture. In longitudinal studies starting from the same dissection, primary cortical "neuronal" cultures grown in Eagle's minimal essential medium had measurable total diazepam binding which increased with age in culture from 214 fmol/mg of protein on day 5 to 447 fmol/mg of protein on day 11 (Fig. 1, *neuronal*). In these cultures which had been treated with the antimitotic agent, cytosine arabinoside (Furlong and Gresham, 1971), a diminution of non-neuronal cells occurred, evidenced in light microscopic examination (R. Henneberry, unpublished observations). [^{3}H]Diazepam binding to membranes in these neuronal cultures was maximally displaced by 10^{-7} M clonazepam ($K_i = 5 \times 10^{-9}$) while RO5-4864 had little potency at this concentration ($K_i > 10^{-5}$ M) (Fig. 2, *neuronal*). This is similar to pharmacology observed in adult rat cortex (Table I). The ratio of clonazepam (10^{-7} M)-displaceable [^{3}H]diazepam binding to RO5-4864 (10^{-7} M)-displaceable [^{3}H]diazepam binding was 2.2 at both 5 and 11 days in culture. In contrast, cultures started from the same dissection as the "neuronal" cultures but not treated with an antimitotic agent showed an apparent overgrowth of non-neuronal elements and were designated "non-neuronal" cultures. However, no attempt was made to remove possible remaining neurons from these "non-neuronal" cultures. These "non-neuronal" cultures had less total [^{3}H]diazepam binding (294 fmol/mg of protein) at 11 days in culture than the "neuronal" cultures (Fig. 1, *non-neuronal*). In addition, RO5-4864 was more potent in displacing [^{3}H]diazepam than was clonazepam (Fig. 2, *non-neuronal*). In these "non-neuronal" cultures, the ratio of clonazepam (10^{-7} M)-displaceable to RO5-4864 (10^{-7} M)-displaceable binding was 0.88.

In 20-day cortical cultures from a separate dissection, media conditions were altered to enhance selectively neuronal growth and differentiation according to the method of Sotelo et al. (1980). In this "neuronal enriched" culture, total benzodiazepine binding (529 fmol/mg of protein) was approximately the same as previously observed for "neuronal" cultures grown in the less selective media (Eagle's minimal essential medium, Fig. 1) at one-half this age in culture. However, benzodiazepine binding was primarily displaceable by clonazepam (10^{-7} M) while RO5-4864 (10^{-7} M) had little potency at this dose (ratio: clonazepam-displaceable to RO5-4864-dis-

placeable [^{3}H]diazepam binding = 4.4, Fig. 1, *neuronal enriched*).

Continuous cell cultures. Membranes obtained from transformed cell cultures of C_6 glioma and a tertiary cell culture obtained from the selective growth of fibroblasts derived from embryonic rat cortex also exhibited [^{3}H]-diazepam binding (Fig. 1, *fibroblasts*, *C_6 glioma*). However, this benzodiazepine binding was displaced maximally by 10^{-7} M RO5-4864 (apparent $K_i = 5 \times 10^{-9}$ M) and only slightly displaced by 10^{-7} M clonazepam (apparent $K_i = 2 \times 10^{-6}$ M) (Fig. 2, *fibroblasts*). Ratio of 10^{-7} M clonazepam-displaceable to 10^{-7} M RO5-4864-displaceable [^{3}H]diazepam binding in these continuous cell cultures was 0.13 (*fibroblasts*, Fig. 1) and 0.03 (*C_6 glioma*, Fig. 1).

These data from both primary cell cultures and continuous cell cultures suggest that clonazepam displaces primarily neuronal [^{3}H]diazepam binding while RO5-4864 displaces primarily non-neuronal binding. However, it is not certain whether binding sites in cultured tissues are identical to those observed in the intact mammalian CNS. Thus, a second series of experiments explored the pharmacological characterization of binding sites within the CNS with intact and lesioned neurons.

In vivo studies

Kainic acid lesions. Intrastriatal injection of kainic acid (KA) has been reported to produce striatal neuronal degeneration (Olney et al., 1974; Schwarcz and Coyle, 1977) and gliosis (Wuerthele et al., 1978; Nicklas et al., 1979). If pharmacological distinctions can be made between binding in neurons and non-neuronal elements, then one could postulate that binding characteristics should be significantly altered by KA lesioning. In these experiments, 14 days following intrastriatal KA injections, lesioned and sham-lesioned tissue from the contralateral striatum were analyzed in each injected animal for [^{3}H]diazepam and [^{3}H]flunitrazepam (Speth et al., 1978) binding and GAD activity. As previously reported by others (Biggio et al., 1979; Chang et al., 1980), no marked reductions in total [^{3}H]diazepam or [^{3}H]flunitrazepam binding were observed following a 1-μg KA injection at 14 days post-lesion (Table II). Only a 2-μg dose of KA was able to cause a significant increase ($p < 0.05$) in [^{3}H]diazepam binding in the lesioned side (Table III). However, a marked change in the pharmacology of [^{3}H]diazepam binding was observed following both the 1- and 2-μg doses of KA (Tables II and III). In both cases, the amount of RO5-4864-displaceable binding was significantly increased in KA-lesioned striatal tissue. In addition, the ratio of clonazepam (10^{-7} M)-displaceable to RO5-4864 (10^{-7} M)-displaceable binding was dramatically decreased in the KA-lesioned tissue. A highly significant correlation was observed ($r = 0.78$; $p < 0.01$) between the

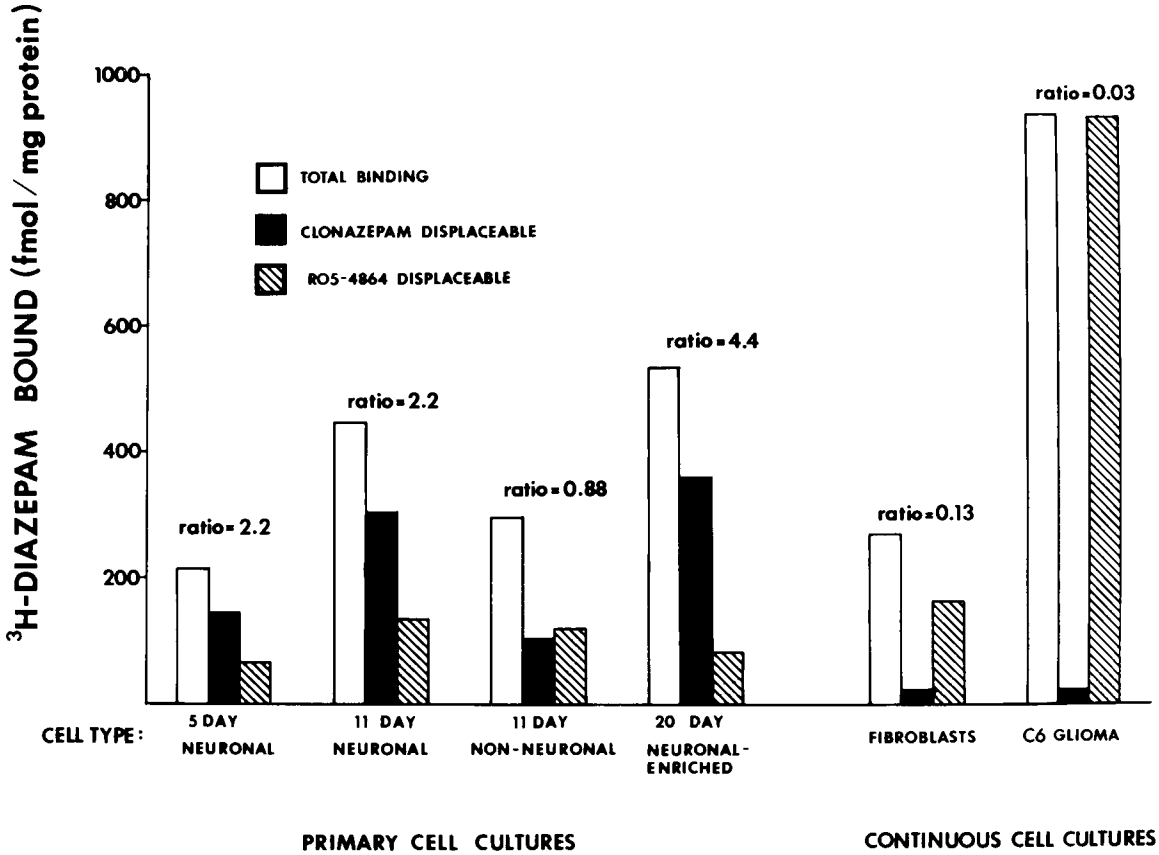

Figure 1. Histograms indicate the amount (in femtomoles per milligram of protein) of total [^{3}H]diazepam bound (*open bar*), amount of bound [^{3}H]diazepam displaced by 10^{-7} M clonazepam (*solid bar*), and amount of bound [^{3}H]diazepam displaced by 10^{-7} M RO5-4864 (*crosshatched bar*) with 5 nM [^{3}H]diazepam added to the final membrane assay mixture from representative cultures. Various culture conditions and preparation of cell membranes for benzodiazepine binding are described under "Materials and Methods." Ratio indicates proportion of clonazepam-displaceable to RO5-4864-displaceable binding.

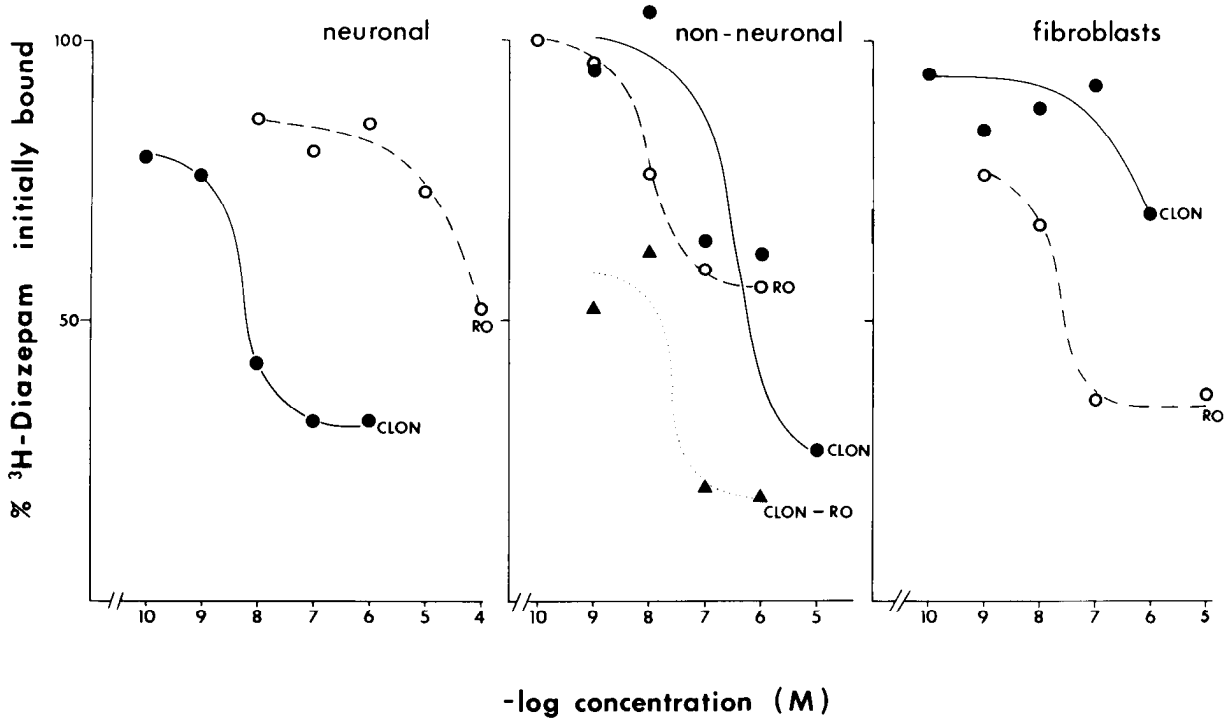

Figure 2. Dose-displacement curves for inhibition of 5 nM [^{3}H]diazepam by clonazepam (●——●, *CLON*) and RO5-4864 (○- - -○, *RO*) in representative neuronal, non-neuronal, and fibroblast cultures as described under "Materials and Methods." Data is plotted as the percentage of total [^{3}H]diazepam (447 fmol/mg of protein in neuronal, 294 fmol/mg of protein in non-neuronal, and 977 fmol/mg of protein in fibroblast cultures) versus the log of the concentration of the displacing benzodiazepine. ▲·····▲ (*CLON – RO*), clonazepam minus RO5-4864.

TABLE II

[^{3}H]Benzodiazepine binding following striatal kainic acid lesions

One microgram of kainic acid was injected into the striatum in a volume of 1 μl of PBS and the contralateral striatum was injected with 1 μl of PBS as above.

	[^{3}H]Diazepam Binding		[^{3}H]Flunitrazepam Binding	
	Lesion (N = 11)	Sham (N = 11)	Lesion (N = 11)	Sham (N = 11)
	fmol/mg protein		*fmol/mg protein*	
Total binding	422 ± 49	435 ± 51	642 ± 80	732 ± 24
10^{-7} M clonazepam-displaceable binding	274 ± 30	331 ± 49	512 ± 64	552 ± 20
10^{-7} M RO5-4864-displaceable binding	122 ± 28[a]	53 ± 11	56 ± 20	24 ± 12
	pmol CO_2/hr/μg protein			
GAD activity	224.8 ± 14.8[b]	323.5 ± 27.1		

[a] $p < 0.02$ paired t test.
[b] $p < 0.02$ t test.

TABLE III

[^{3}H]Diazepam binding following striatal kainic acid lesions

Two micrograms of kainic acid were injected into the striatum in a volume of 1 μl of phosphate-buffered saline (PBS) over a 3-min injection period. One microliter of PBS was injected into the contralateral striatum.

	[^{3}H]Diazepam Binding		Paired t Test
	Lesion (N = 6)	Sham (N = 6)	
	fmol/mg protein		
Total binding	573 ± 39	484 ± 30	$p < 0.05$
10^{-7} M clonazepam-displaceable binding	279 ± 37	342 ± 28	N.S.[a]
10^{-7} M RO5-4864-displaceable binding	245 ± 42	59 ± 24	$p < 0.02$
Ratio: clonazepam:RO5-4864-displaceable binding	1.3 ± 0.2	7.2 ± 1.1	$p < 0.01$

[a] N.S., not significant.

lesion-induced decrease in GAD activity (ratio of GAD activity in lesioned striatum versus GAD activity in sham-lesioned striatum) and the ratio of clonazepam-displaceable (10^{-7} M)/RO5-4864-displaceable (10^{-7} M) [^{3}H]diazepam binding in lesioned striatum (R_L) to the ratio of clonazepam-displaceable (10^{-7} M)/RO5-4864-displaceable (10^{-7} M) [^{3}H]diazepam binding in sham-lesioned striatum (R_S) (Fig. 3).

These *in vivo* data further reinforce the view that neuronal and non-neuronal [^{3}H]diazepam binding sites within the central nervous system are pharmacologically distinguishable. As previously observed (Speth et al., 1978; Braestrup et al., 1979) and apparent from the data in Table II, [^{3}H]flunitrazepam appears to bind with greater sensitivity than [^{3}H]diazepam to CNS tissue. However, a direct comparison of these benzodiazepine radioligands in the same tissue homogenates indicates that [^{3}H]flunitrazepam does not bind to any significant degree (at the concentration used) to the RO5-4864-displaceable site (Table II) in contrast to binding with [^{3}H]diazepam.

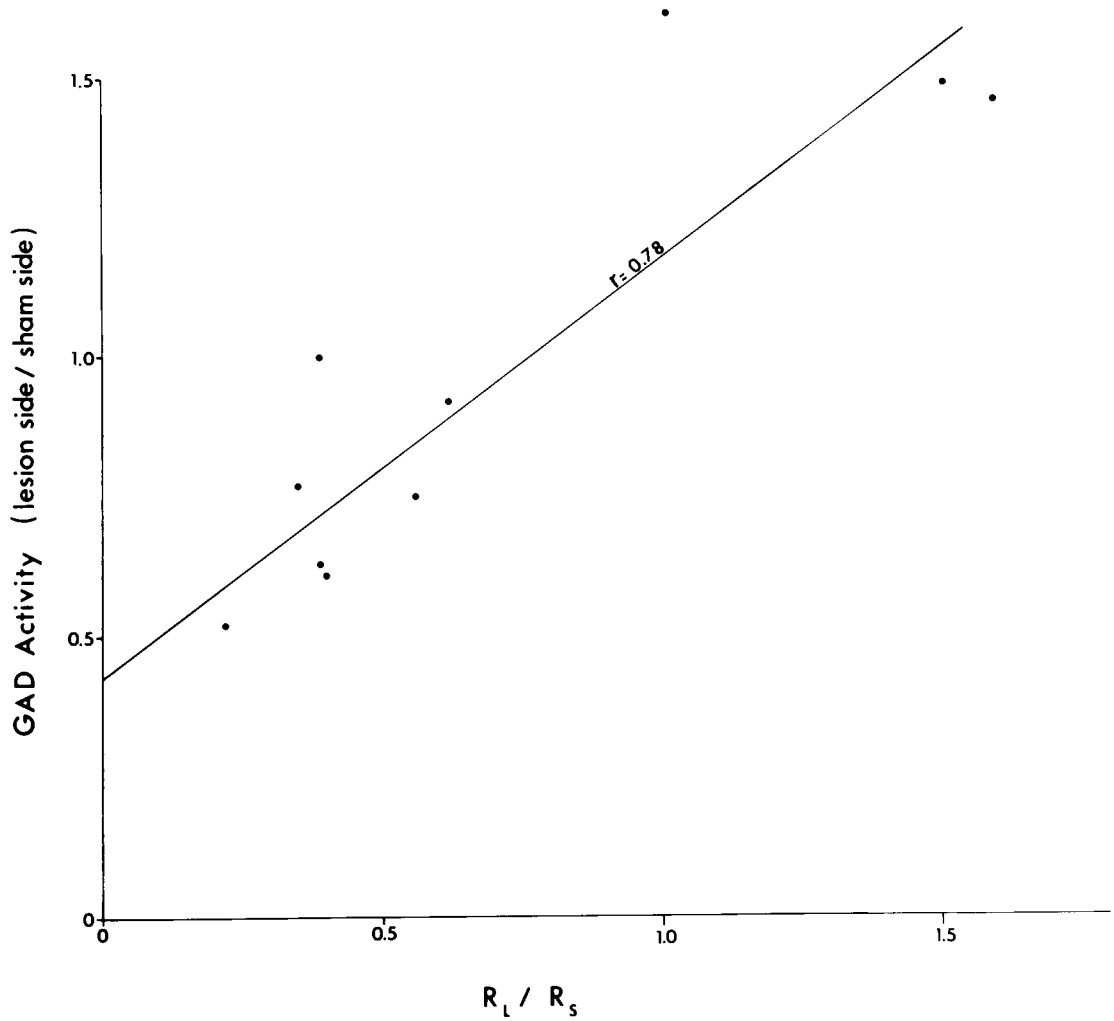

Figure 3. Effect of kainic acid lesions on GAD activity and pharmacology of [^{3}H]diazepam binding. Animals received a single 1- or 2-μg injection of kainic acid intrastriatally and a similar volume of PBS in the contralateral striatum. GAD activity was determined for each animal and plotted on the ordinate as a ratio of GAD activity remaining in the lesioned side to GAD activity in the sham-lesioned side for each animal. The ratio (R) of clonazepam (10^{-7} M)- to RO5-4864 (10^{-7} M)-displaceable [^{3}H]diazepam binding was measured in the lesioned and contralateral sham-lesioned striatum for each animal and plotted on the abscissa as the ratio of displaceable binding in the lesioned (R_L) to the sham-lesioned (R_S) [^{3}H]diazepam binding in striatal tissue. Thus, the relative change in GAD activity is compared to the relative changes in the displacement ratio of [^{3}H]diazepam binding within each animal. A significant correlation ($p < 0.01$) between lesioned-induced changes in GAD activity and lesion-induced changes in the pharmacology of [^{3}H]diazepam binding was observed.

Discussion

The results presented in this paper demonstrate that mammalian central nervous tissue contains at least two pharmacologically distinct benzodiazepine binding sites. Both *in vitro* cell cultures and *in vivo* lesion preparations have been utilized to determine the cellular localization of these two binding sites.

Cell culture techniques have been shown to be important for investigating various aspects of neuron and glial cell growth and differentiation. Primary neuronal cultures from rodent cortical cultures exhibit some of the biochemical (Raff et al., 1979; Hansson et al., 1980; Snodgrass et al., 1980; Sotelo et al., 1980) and physiological (Barker and Ransom, 1978; Dichter, 1978; MacDonald and Barker, 1978) properties characteristic of the central nervous system.

Benzodiazepine binding in primary cortical cultures has also been described by several laboratories (Dudai et al., 1979; Harden and McCarthy, 1979; Hertz and Mukerji, 1980; Huang et al., 1980). In these previous studies, benzodiazepine binding is most potently displaced by centrally active benzodiazepines, analogous to the pharmacological profile observed in cerebral tissue from adult (Braestrup et al., 1977; Möhler and Okada, 1977a, b; Tallman et al., 1980b) and postnatal animals (Braestrup and Nielson, 1978; Candy and Martin, 1979; Mallorga et al., 1980). However, the present studies have also demonstrated a significant amount of displacement of membrane-bound [^{3}H]diazepam by the centrally "inactive" benzodiazepine, RO5-4864. Displacement of membrane-bound [^{3}H]diazepam by RO5-4864 has been observed previously to be significant in transformed neuronal and glial cell cultures (Braestrup et al., 1978; Chang et al., 1978; Guidotti et al., 1979; Hertz and Mukerji, 1980; Strittmatter et al., 1979; Syapin and Skolnick, 1979).

Since our primary cortical cultures contained mixed populations of neurons, glia, and other non-neuronal elements, we wished to determine whether the pharmacological characteristics of binding could be correlated with cell type. We chose appropriate parameters for culturing which would result in the preferential survival of either neuronal or non-neuronal cell types. These studies confirmed that the ability of clonazepam and RO5-4864 to displace [^{3}H]diazepam binding is selectively affected by the predominant cell type.

Typically in peripheral tissues and cell cultures where RO5-4864-displaceable benzodiazepine binding sites have been observed, a lower affinity for [^{3}H]diazepam has been measured than for tissues with primarily clonazepam-displaceable binding sites (Braestrup et al., 1978). In the present experiments, displacement curves for [^{3}H]diazepam were obtained using a relatively high concentration (5 nM) of the radioligand in order for both sites to be measured. One study reported observing no differences in benzodiazepine binding affinities between selectively cultured populations of neurons and glia (Dudai et al., 1979), but possible differences may have been masked by the use of low concentration [^{3}H]flunitrazepam as the radioligand (see discussion below). The most selective discrimination between sites was found to be at a concentration of 10^{-7} M for both clonazepam and RO5-4864. When cultures were principally neuronal, [^{3}H]diazepam was maximally displaced by 10^{-7} M clonazepam but not significantly affected by 10^{-7} M RO5-4864. In contrast, when non-neuronal cell types predominated (as seen in both non-antimitotic agent-treated primary cortical cultures and continuous fibroblast and C_6 glioma cultures; Strittmatter et al., 1979; Tallman et al., 1980b), membrane-bound [^{3}H]diazepam was potently displaced by 10^{-7} M RO5-4864 and less affected by 10^{-7} M clonazepam. These data indicate that the ratio of clonazepam-displaceable to RO5-4864-displaceable [^{3}H]diazepam binding can be used to distinguish between neuronal and non-neuronal elements in *in vitro* culture.

In order to determine whether these pharmacological characterizations could be generalized to the mammalian central nervous system *in vivo*, characterization of benzodiazepine binding sites in adult rat brain was done. As previously observed, [^{3}H]diazepam binding to membranes of adult rat cerebral cortex were almost exclusively displaceable by clonazepam. However, a small amount of RO5-4864-displaceable [^{3}H]diazepam binding was measured at high ligand concentrations (5 nM). Selective neuronal lesions using kainic acid did not markedly alter total [^{3}H]diazepam (or [^{3}H]flunitrazepam) binding as previously reported (Biggio et al., 1979; Chang et al., 1980). However, these lesions did dramatically increase the amount of RO5-4864-displaceable [^{3}H]diazepam binding and significantly altered the ratio of clonazepam- to RO5-4864-displaceable binding. Such an effect could explain the decrease in binding affinity for [^{3}H]-diazepam following kainic acid lesions observed by Biggio et al. (1979).

To determine the extent of neuronal damage, GAD, the biosynthetic enzyme for GABA, activity was determined. A significant correlation between the ratio of these two types of displaceable binding and GAD activity within the same tissue homogenate has also been shown. These experiments demonstrate that the extent of neuronal destruction is related to the ratio of clonazepam- to RO5-4864-displaceable [^{3}H]diazepam binding. Although an intimate interaction between GABA and benzodiazepine binding has been reported previously (Tallman et al., 1980a), GAD activity is used in these studies as a measure of neuronal damage (McGeer and McGeer, 1978). It cannot be determined from our studies whether benzodiazepine binding sites are located principally on GABAergic neurons. However, several reports now suggest such a correlation (Huang et al., 1980; Möhler et al., 1980).

Interestingly, when [^{3}H]flunitrazepam was used as the radioligand, no significant alterations in either the total binding or the ratio of clonazepam- to RO5-4864-displaceable [^{3}H]flunitrazepam binding was observed. At this concentration of [^{3}H]flunitrazepam, the major portion of binding is clonazepam displaceable, consistent with the suggestion that [^{3}H]flunitrazepam binds preferentially to a "neuronal" site (Braestrup et al., 1979; Harden and McCarthy, 1979). However, in this same homogenate, RO5-4864 potently displaces [^{3}H]diazepam. These data suggest that [^{3}H]diazepam and [^{3}H]flunitrazepam may *not* be interchangeable as benzodiazepine radioligands. Despite its lower affinity, [^{3}H]diazepam may be the preferred radioligand in studies where pharmacological or physiological alterations may result in differential survival of cell populations.

In conclusion, these experiments demonstrate that mammalian central nervous tissue contains at least two pharmacologically distinct types of benzodiazepine binding sites. These binding sites are located on different cell types. Clonazepam-displaceable binding sites are located on neuronal membranes while RO5-4864-displaceable binding sites are located on non-neuronal elements. These pharmacological distinctions may be used to characterize the predominant cell types which bind benzodiazepines in nervous tissue. One quantitative measure of different cell populations is the ratio of clonazepam- to RO5-4864-displaceable binding within a single tissue sample. Such measures can potentially be used to demonstrate physiological (KA and surgical lesions) and pharmacological (Gallager et al., 1980a) manipulations which alter cell survival and/or proliferation within the mammalian nervous system.

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