lys olivacea (*11*). Thus, this phenomenon appears to be widespread and has direct implication for conservation efforts that involve artificial rearing of sea turtle eggs. Attempts to incubate eggs in hatcheries aboveground or in central beach hatcheries (*1*) should only take place after temperature-dependent sex determination is defined for the species in question. To disregard temperature is to risk producing all male, all female, or even intersex hatchlings. A beach hatchery is an effective way to incubate eggs in order to produce natural sex ratios if care is taken to duplicate as closely as possible the depth, the amount of shading, and egg chamber dimensions of natural nests. A thermal transect of the beach from water's edge into vegetation, taken at nest depth, will indicate where to locate the hatchery so that temperature ranges will produce desired sex ratios among hatchlings. Finally, in the absence of data on temperature-dependent sex determination and a thermal transect of a beach, artificial hatcheries should not be used. Rather, efforts should be directed to marking natural nests as they are made and to enclose them in wire mesh fences as soon as possible. Under these circumstances, protecting these natural nests from human and natural predators offers the best opportunity of enhancing production of large numbers of hatchlings while maintaining a natural sex ratio (*12*).

STEPHEN J. MORREALE
Department of Biology,
State University College at Buffalo,
Buffalo, New York 14222
GEORGITA J. RUIZ
Facultad de Medicina Veterinaria y Zootécnia, Universidad Nacional Autónoma de México, Mexico City,
and *Rutgers University,*
New Brunswick, New Jersey 08903
JAMES R. SPOTILA
EDWARD A. STANDORA
Department of Biology,
State University College at Buffalo

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Micromolar Affinity Benzodiazepine Receptors: Identification and Characterization in Central Nervous System

Abstract. *Receptors that selectively bind micromolar concentrations of benzodiazepines are present in rat brain membrane. These micromolar receptors exhibit saturable, stereospecific binding, and the potency of benzodiazepine binding to these receptors is correlated with the ability of the benzodiazepines to inhibit maximum electric shock–induced convulsions. Benzodiazepine receptors with nanomolar affinity differ from the micromolar receptors in their binding, kinetic, and pharmacologic characteristics. The micromolar receptors also bind phenytoin, a non-benzodiazepine anticonvulsant. These results provide evidence for a distinct class of clinically relevant benzodiazepine receptors that may regulate neuronal excitability and anticonvulsant activity.*

Benzodiazepines are widely administered therapeutic drugs with diverse clinical applications (*1*). Several of the pharmacologic effects of the benzodiazepines have been attributed to a class of well-characterized, stereospecific receptors that selectively bind benzodiazepines in the nanomolar range (*2–5*). Although these receptors appear to mediate some of the clinical effects of the benzodiazepines, several studies show that they do not account for the complete range of benzodiazepine therapeutic actions (*4, 5*). In particular, the potency of benzodiazepine binding to these receptors is not significantly correlated with the ability of the benzodiazepines to inhibit maximum electric shock–induced convulsions in animals or their ability to alter the behavior of animals in conditioned avoidance tests (*4, 5*). These observations suggest that other benzodiazepine receptors are present in brain.

The existence of a distinct class of benzodiazepine receptors with binding affinities in the micromolar range is suggested by studies demonstrating that micromolar concentrations of benzodiazepines in the brain are pharmacologically active (*6*). Furthermore, micromolar concentrations of benzodiazepines bound to brain membrane inhibit Ca^{2+}-calmodulin protein kinase activity (*7*). We have now identified and characterized a stereospecific benzodiazepine receptor that has micromolar binding affin-

Table 1. Differences in binding and pharmacologic characteristics for the micromolar and the nanomolar benzodiazepine receptors in brain membrane.

Characteristic	Nanomolar receptor	Micromolar receptor
K_D (M)	3.1×10^{-9}	4.5×10^{-5}
B_{max} (pmole per milligram of protein)	0.893	360.4
Stereospecific binding	Yes	Yes
Dissociation rate constant, k_{-1} at 4°C (sec^{-1}) (*14*)	2.69×10^{-3}	5.50×10^{-3}
Association rate constant, k_{+1} at 4°C (M^{-1} sec^{-1}) (*14*)	1.13×10^{6}	1.07×10^{2}
Pharmacologic correlation (*17*)		
Maximum electric shock–induced seizures	No	Yes
Muscle relaxant activity	Yes	No
Mouse rotorod performance	Yes	No
Pentylenetetrazol-induced seizures	Yes	No
Conditioned avoidance tests	No	No
Relative affinity for Ro5-4864 (*16*)	> 10,000	5.7
Enhanced binding (*15*)		
$10^{-4}M$ GABA	Yes	No
$10^{-4}M$ muscimol	Yes	No

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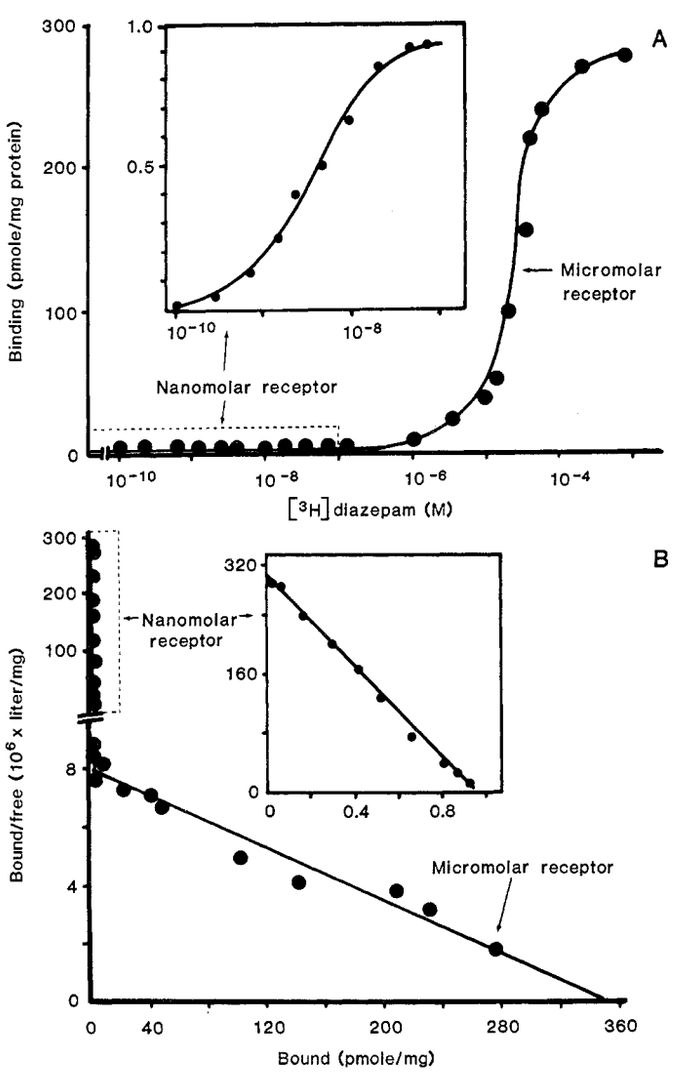
ities and that is biochemically and pharmacologically distinct from the receptors that bind nanomolar concentrations of benzodiazepines.

Benzodiazepine binding to synaptic membrane was studied with the use of membrane fractions prepared from rat brain as described (*8*). In the binding assays, nonspecific and total binding were determined by incubating membrane fractions with [^{3}H]diazepam (specific activity, 87.6 Ci/mmole) in the presence and absence of high concentrations of unlabeled diazepam (*9*). The assay was terminated in less than 3 seconds by filtration, and membrane-bound radioactivity on the filters was measured by liquid scintillation counting (*10*). The amount of specifically bound [^{3}H]diazepam was calculated by subtracting nonspecific binding from total binding.

Saturation studies over a wide range of [^{3}H]diazepam concentrations (0.10 n*M* to 800 μ*M*) revealed specific, saturable binding to two discrete benzodiazepine receptors (Fig. 1A). In addition to the receptors with nanomolar affinity, these studies demonstrated novel benzodiazepine receptors in the micromolar range. Nonspecific binding was nonsaturable and linear throughout the examined range of concentrations. Since therapeutic doses of diazepam produce plasma concentrations in the 0.1 to 50 μ*M* range and brain concentrations that are significantly larger (*6*), the brain levels of diazepam that exert clinical effects are adequate to significantly bind to this new class of micromolar-affinity benzodiazepine receptors.

Both the pharmacologic activity of the benzodiazepines and their binding to the nanomolar receptor is stereospecific (*2–5*). The stereospecificity of the binding to the micromolar receptor was demonstrated by displacement studies which revealed that the pharmacologically active benzodiazepine B10(+) displaced [^{3}H]diazepam from the micromolar receptor, whereas the pharmacologically inactive enantiomer B10(−) showed no



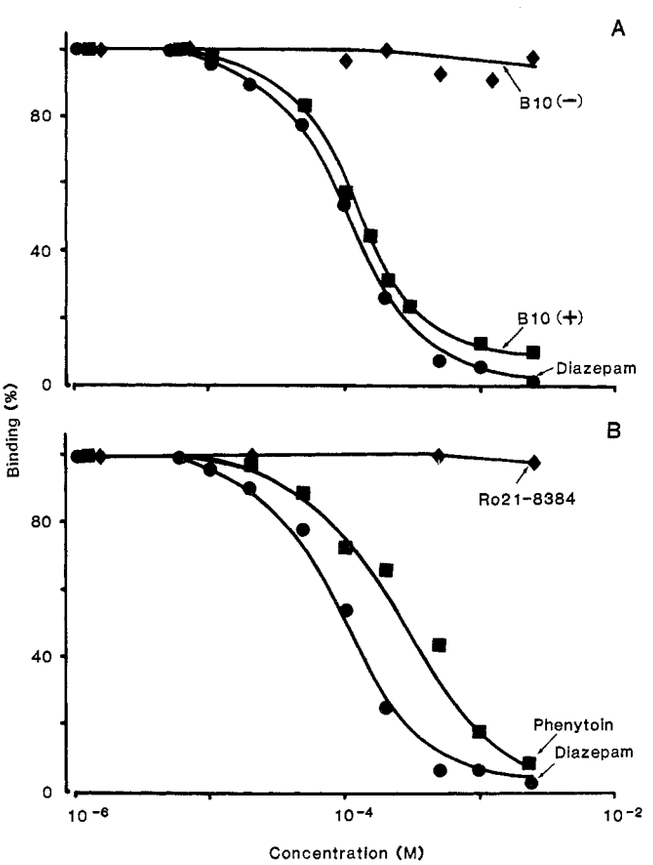


Fig. 1 (left). (A) Saturation curve demonstrating the presence of two distinct classes of saturable benzodiazepine receptors. Binding assays were performed with [^{3}H]diazepam concentrations in the 100 p*M* to 800 μ*M* range. For the nanomolar receptor, our binding assay yielded results similar to those obtained in (*2*). Three hundred micrograms of protein were used for each measurement, and each point is representative of at least ten separate determinations. (B) Scatchard transformation of the saturation data showing different binding parameters for the two receptors. Statistical analysis of the data in the Scatchard plot indicated a K_D of 45.0 μ*M* and a B_{max} of 360.4 pmole per milligram of protein, and r = .969 for the micromolar receptor; and a K_D of 3.1 n*M*, a B_{max} of 0.893 pmole per milligram of protein, and r = .997 for the nanomolar receptor. Fig. 2 (right). (A) Stereospecificity of binding is demonstrated by the displacement of [^{3}H]diazepam by the pharmacologically active B10(+) in comparison to the negligible displacement exhibited by the pharmacologically inactive B10(−). Binding assays were performed under standard conditions with 10 μ*M* [^{3}H]diazepam and various concentrations of displacing agents. The data are representative of at least six separate determinations. (B) Specific affinity of the micromolar receptor for anticonvulsant agents as demonstrated by displacement studies with diazepam, phenytoin, and Ro21-8384. Diazepam yielded a K_i of 85 μ*M* and phenytoin, a nonbenzodiazepine anticonvulsant, exhibited a K_i of 155 μ*M*. Ro21-8384, a benzodiazepine which is ineffective as an anticonvulsant (*18*), did not exhibit significant displacement of [^{3}H]diazepam up to concentrations of 2.50 m*M*. The [^{3}H]diazepam was used at a concentration of 10 μ*M*. The data are representative of at least six separate determinations.

significant displacement (Fig. 2A). To show that binding to the micromolar receptor is a general property of the benzodiazepines, we performed displacement experiments with several different benzodiazepines and obtained the following inhibition constants (K_i, expressed as micromolar values) (*11, 12*): Ro5-5345, 39; flunitrazepam, 42; Ro5-5807, 55; Ro5-2180, 73; diazepam, 85; B10(+), 102; medazepam, 121; clonazepam, 182; oxazepam, 243; nitrazepam, 369; bromazepam, 372; chlordiazepoxide, 393; Ro5-4864, 491; flurazepam, 1120; B10(−), > 2000.

Scatchard analysis of the saturation binding studies shows that the two classes of benzodiazepine receptors possess different properties (Fig. 1B). The apparent dissociation constant (K_D) of [^{3}H]diazepam for the micromolar receptor is 45.0 μM, whereas the K_D value for the nanomolar receptor is 3.1 nM (*2, 3*) (Fig. 1B). Furthermore, the density of the micromolar receptor in brain membranes, as reflected in the maximum binding capacity (B_{max}), is higher than that of the nanomolar receptor (Fig. 1B). Recent studies have indicated that there are two subclasses of the nanomolar receptor in rat brain (*13*). The linear Scatchard plot for the micromolar receptor (Fig. 1B) suggests the presence of a single, homogeneous population of binding sites in this concentration range. However, these results do not eliminate the possibility of more than one micromolar benzodiazepine receptor with very similar binding affinities.

The kinetics of benzodiazepine binding were examined by performing the binding assay with [^{3}H]diazepam after various incubation times and at different temperatures according to established procedures (*14*). Kinetic constants computed for the micromolar receptor, when compared with the kinetics for the nanomolar receptor (*14*), further distinguish these two receptors (Table 1). With a $t_{1/2}$ of 126 seconds for dissociation of the micromolar receptor at 4°C, the 3-second washing period in our filtration assay was sufficiently rapid to enable us to measure binding before significant dissociation from the receptor could occur (*14*). The K_D value for the micromolar receptor computed from the rate constants ($K_D = k_{-1}/k_{+1}$) is 51.4 μM, which is in good agreement with the K_D value determined by the Scatchard plot (Fig. 1B). The binding affinity of the micromolar receptor was not significantly enhanced by the addition of γ-aminobutyric acid (GABA) or muscimol, a GABA agonist (Table 1), to the assay mixture, whereas these compounds did have a potentiating effect on the benzodiazepine binding affinity of the nanomolar receptor (*15*).

Another distinguishing characteristic of the micromolar receptor is its relative affinity for Ro5-4864 (*16*). This benzodiazepine has a low relative affinity for the nanomolar receptor, but is as potent as many of the other benzodiazepines in displacing [^{3}H]diazepam from the micromolar receptor (Table 1). Although Ro5-4864 is inactive in most pharmacologic test systems, it is a relatively potent inhibitor of maximum electric shock–induced convulsions (*7*). A peripheral type of benzodiazepine receptor has been described which also binds Ro5-4864 (*2, 3*). However, the peripheral receptor is different from the micromolar receptor in many other respects: the K_D value of [^{3}H]diazepam for the peripheral receptor is approximately 40 nM (*2, 3*), and the peripheral receptor, in contrast to the micromolar receptor, has a characteristically low relative affinity for clonazepam (*2, 3*). Proteolytic digestion of membrane preparations with *Staphylococcus aureus* protease (*2, 3*) completely abolished the specific binding of [^{3}H]diazepam to the micromolar receptor, indicating that binding of the benzodiazepines to the micromolar receptor is dependent on the integrity of membrane proteins.

Previous studies have shown a significant correlation between the affinity of the nanomolar receptor and the ability of the benzodiazepines to act as muscle relaxants, impair mouse rotorod performance, and inhibit pentylenetetrazol-induced seizures in mice (*4, 5*). However, the potency of binding to the nanomolar receptor does not correlate with the potency of benzodiazepine inhibition of maximum electric shock–induced seizures in mice (*4, 5*). The highly significant correlation of micromolar receptor binding with benzodiazepine inhibition of maximum electric shock–induced seizures ($r = 0.9534$, $P < .001$) and the insignificant correlation of micromolar receptor binding with the other benzodiazepine pharmacologic test systems (*17*), demonstrate that the micromolar and the nanomolar receptors mediate different clinical effects. The correlation of micromolar receptor binding with inhibition of maximum electric shock–induced convulsions is further supported by the fact that Ro21-8384, a benzodiazepine which is ineffective in inhibiting maximum electric shock–induced convulsions (*18*), does not displace [^{3}H]diazepam from the micromolar receptor (Fig. 2B). The benzodiazepines appear to have two different anticonvulsant effects mediated by two distinct classes of receptors, the micromolar receptor (maximum electric shock) and the nanomolar receptor (pentylenetetrazol).

The anticonvulsant phenytoin is structurally similar to diazepam (*19*) and, like diazepam, phenytoin inhibits Ca^{2+}-calmodulin kinase activity (*20*) and is one of the best anticonvulsants for controlling maximum electric shock–induced seizures and status epilepticus (*21*). Phenytoin in therapeutic concentrations (*22*) displaced specifically bound [^{3}H]diazepam from the micromolar receptor with a K_i of 155 μM, suggesting that the micromolar receptor is also a phenytoin receptor. Conversely, we have shown (*23*) that micromolar concentrations of [^{3}H]phenytoin exhibit specific, saturable membrane binding, and that this binding can be displaced by diazepam and phenytoin in micromolar concentrations. Since diazepam and phenytoin represent two distinct classes of anticonvulsant drugs which exhibit similar binding affinities for the micromolar receptor, our results indicate that the micromolar benzodiazepine receptor may represent a generalized "anticonvulsant receptor" in brain membrane.

The ability of benzodiazepines to inhibit Ca^{2+}-calmodulin–dependent membrane kinase activity is significantly correlated with their ability to inhibit maximum electric shock–induced convulsions (*7*). Micromolar concentrations of benzodiazepines also inhibit, in addition to membrane kinase activity (*7*), Ca^{2+}-calmodulin–dependent neurotransmitter release from both synaptic vesicle and intact synaptosome preparations (*24*). Although the full functional significance of micromolar benzodiazepine receptors remains to be elucidated, the present results suggest that these receptors may regulate specific neuronal enzyme systems and play an important role in modulating neuronal excitability and anticonvulsant activity.

ALLEN C. BOWLING
ROBERT J. DELORENZO
Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510

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9. Membrane preparations (130 μl) were first incubated with 5 μl of ethanol (total binding) or 5 μl of unlabeled excess drug suspended in ethanol (nonspecific binding) for 1 hour at 4°C, and then incubated with 5 μl of labeled drug for an additional 2 hours at 4°C. Nonspecific binding to the nanomolar receptor and micromolar receptor was studied by using unlabeled excess diazepam at final concentrations of 1.0 μ*M* and 2.5 m*M*, respectively. The wide range of [^{3}H]diazepam concentrations was obtained by either diluting the stock solution of [^{3}H]diazepam with ethanol or concentrating it with unlabeled diazepam.
10. For each binding condition in a single experiment, three identical assay mixtures were prepared. After the final incubation, three samples were taken from each assay mixture and placed on three separate Whatman GF/B glass fiber filters, thus providing triplicate values for each of the three identical mixtures. Filters were washed twice with 5 ml of buffer and each wash was conducted in less than 1.5 seconds. Each filter was placed in 10 ml of Aquasol and left at 4°C for at least 12 hours prior to liquid scintillation counting. The total quantity of [^{3}H]diazepam bound at each concentration was less than 10 percent of the initial amount of free [^{3}H]diazepam added to the assay mixture.
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16. The relative affinity of the receptors for Ro5-4864 is the ratio of the K_i of Ro5-4864 to the K_i of diazepam. This value was computed for the nanomolar receptor by using the data of C. Braestrup and R. F. Squires (*5*). The relative affinity of the micromolar receptor was calculated using the data from our displacement studies.
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Efferent Fibers to *Limulus* Eyes Synthesize and Release Octopamine

Abstract. *Octopamine synthesized in vitro from tyramine by* Limulus *lateral and ventral eyes was located by light microscopic and electron microscopic autoradiography in efferent fibers which innervate ventral photoreceptors and lateral eye ommatidia. Newly synthesized octopamine was released from efferent fibers in response to depolarization in high concentrations of potassium. We propose that octopamine is a neurotransmitter of efferent fibers that may modulate basic retinal processes such as photoreceptor sensitivity, photomechanical movements, and photoreceptive membrane turnover.*

Efferent innervation to retinas seems to be a feature common to the visual systems of many species (*1*) including that of the horseshoe crab *Limulus polyphemus* (*2*). In the *Limulus* visual system, a preparation that has been central to our understanding of basic mechanisms of vision (*3*), efferent fibers project from a circadian clock in the brain (*4*) and innervate all three types of eyes:

Fig. 1. Stereograph of a three-dimensional reconstruction of octopamine-synthesizing efferent fibers of the *Limulus* ventral eye. Regions of intense label seen in individual sections are part of a branched efferent innervation of photoreceptor cell bodies. Ventral eyes were incubated overnight in $6 \times 10^{-8}M$ [^{3}H]tyramine (New England Nuclear; 9.14 Ci/mmole), and then fixed (*10*), dehydrated, and embedded in Araldite 502. Thick sections (1 μm) mounted on glass slides were dipped in Kodak NTB-2 Nuclear Trak Emulsion, exposed for 7 to 14 days at 4°C, and developed with Dektol. Sections were stained with toluidine blue. Serial light autoradiographs were digitized and displayed by computer. Dotted lines show outlines of two photoreceptor cell bodies.

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