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Binding of [^{3}H]CB 34, a selective ligand for peripheral benzodiazepine receptors, to rat brain membranes

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Abstract

The 2-phenyl-imidazo[1,2-*a*]pyridine derivative CB 34 is a ligand for peripheral benzodiazepine receptors. The binding of [^{3}H]CB 34 to rat cerebrocortical membranes was characterized. Specific binding was rapid, reversible, saturable and of high affinity. Kinetic analysis yielded association and dissociation rate constants of 0.2×10^8 M^{-1} min^{-1} and 0.29 min^{-1}, respectively. Saturation binding experiments revealed a single class of binding sites with a total binding capacity of 188 ± 8 fmol/mg protein and an apparent dissociation constant of 0.19 ± 0.02 nM. Specific [^{3}H]CB 34 binding was inhibited by ligands selective for peripheral benzodiazepine receptors, whereas, with the exception of flunitrazepam and diazepam, ligands for central benzodiazepine receptors were inactive. Of the brain regions examined, the density of the [^{3}H]CB 34-binding sites was greatest in the hypothalamus and lowest in the cerebral cortex. [^{3}H]CB 34 is thus a potent and selective ligand for peripheral benzodiazepine receptors and should be proven useful for studies of the roles of these receptors.

Keywords: CB 34; Benzodiazepine receptor, peripheral; Cerebral cortex, rat; Ligand binding, radiolabeled

1. Introduction

Classical benzodiazepine drugs exert their anxiolytic and other central effects by acting at high-affinity central benzodiazepine receptors located in the central nervous system (Braestrup and Squires, 1977). These receptors are actually binding sites associated with the $GABA_A$ receptor, a macromolecular complex that functions as a γ-aminobutyric acid- (GABA-)-activated Cl^- channel and which contains binding sites for several other modulators (Olsen and Homanics, 2000). Various benzodiazepines also bind to a different class of receptors known as peripheral benzodiazepine receptors that are located predominantly in peripheral tissues and glial cells in the brain (Papadopoulos, 1993). These latter receptors exhibit a high affinity for 4′ -chlordiazepam (Ro 5-4864) and a relatively high affinity for diazepam, but they only interact weakly with other potent ligands of central benzodiazepine receptors such as clonazepam and flumazenil (Marangos et al., 1982). Peripheral benzodiazepine receptors also bind with high affinity to imidazopyridine alpidem but exhibit low affinity for zolpidem (Benavides et al., 1983), an imidazopyridine that acts as a ligand for central benzodiazepine receptors. In addition, peripheral benzodiazepine receptors bind isoquinolines (PK 11195) (Le Fur et al., 1983), indoleacetamides ($FGIN_{1-27}$) (Romeo et al., 1992) and pyrrolobenzoxapines (Campiani et al., 1996) with high affinity.

Peripheral benzodiazepine receptors are localized predominantly in the mitochondria (Anholt et al., 1985), where they are restricted to the outer mitochondrial membrane (Anholt et al., 1986). They are composed of at least three subunits: an 18-kDa subunit containing the binding site

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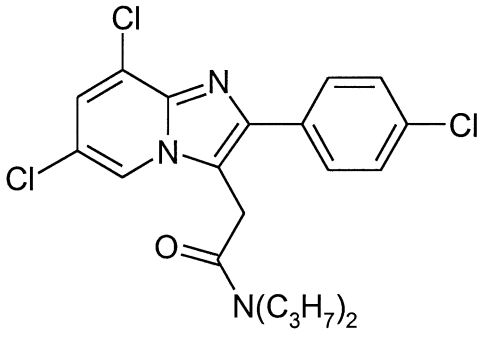
for isoquinolines, a 32-kDa subunit that functions as a voltage-dependent anion channel and binds benzodiazepines and a 30-kDa subunit that functions as an adenine nucleotide carrier and also binds benzodiazepines (McEnery et al., 1992).

Although the functions of peripheral benzodiazepine receptors remain unknown, these receptors are implicated in the regulation of cell proliferation, Ca^{2+} flow, cellular respiration and cellular immunity (Cantor et al., 1984; Wang et al., 1984; Lenfant et al., 1986; Hirsch et al., 1989). In addition, they appear to play a role in the biological response to stress and in anxiety disorders: acute stress thus increases the density of these receptors, whereas receptor density is reduced in the platelets of individuals with generalized anxiety disorder, panic disorder or obsessive–compulsive disorder (Gavish et al., 1999; Veenman and Gavish, 2000). Moreover, compounds that activate peripheral benzodiazepine receptors also increase the steroidogenesis in both the periphery and the brain (Papadopoulos, 1993). These receptors are abundant in steroidogenic tissues, in which their activation promotes cholesterol transport and steroid synthesis (Papadopoulos, 1993). Given that certain ligands that potently and selectively activate peripheral benzodiazepine receptors exhibit a potential for the treatment of a variety of emotional and affective disorders (Gavish et al., 1999; Rupprecht et al., 2001), a search for new and more selective drugs that interact with these receptors and thereby stimulate brain and peripheral steroidogenesis with high efficacy has been initiated.

A new class of compounds, 2-phenyl-imidazo[1,2-*a*]pyridine derivatives, some of which exhibit high affinity for peripheral benzodiazepine receptors and stimulate brain and peripheral steroidogenesis, was recently prepared by the reaction of substituted 2-aminopyridines with bromoketoamides (Trapani et al., 1997, 1999; Serra et al., 1999). One of these compounds, CB 34 (Fig. 1), also exhibited a marked anticonflict effect in the Vogel test and selectively stimulated the synthesis of neuroactive steroids in the brain of rats with great efficacy (Serra et al., 1999). More recently, CB 34 has been labeled (Latrofa et al., 2001), and we now describe the characterization of the binding of [^{3}H]CB 34 to rat brain membranes.



Formula corretta del CB34

Fig. 1. Molecular structure of CB 34.

2. Materials and methods

Female Sprague–Dawley CD rats (Charles River, Como, Italy) were killed with a guillotine, and the brain was rapidly removed, dissected and stored at −80 °C until use. The tissue was subsequently thawed and then homogenized in 50 volumes of Dulbecco's phosphate-buffered saline (PBS, pH 7.4) at 4 °C with a Polytron PT 10 disrupter (setting 5 for 20 s). The homogenate was centrifuged at 40,000 × *g* for 30 min, and the resulting pellet was resuspended in 50 volumes of PBS and was recentrifuged. The new pellet was resuspended in 10 volumes of PBS and was used for the assay.

The binding of [^{3}H]CB 34 was measured in a final volume of 1000 μl, comprising 100 μl of membrane suspension (0.35–0.40 mg of protein), 100 μl of [^{3}H]CB 34 (127 Ci/mmol, Amersham Pharmacia Biotech; final assay concentration of 1 nM), 5 μl of drug solution or solvent and 795 μl of PBS. The binding reaction was performed at 4 or 25 °C for 90 or 120 min and was initiated by the addition of membranes. The incubation was terminated by rapid filtration through glass-fiber filter strips (Whatman GF/B) that had been presoaked with 0.3% polyethyleneimine and placed in a Cell Harvester filtration manifold (Brandel). The filters were washed five times with 4 ml of ice-cold PBS, after which filter-bound radioactivity was quantified by liquid scintillation spectrometry. Nonspecific binding was defined as the binding in the presence of 10 μM of unlabeled PK 11195. Specific binding was determined by subtracting the nonspecific from the total binding and was ~80% of the total binding. Diazepam was purchased from FIS (Vicenza, Italy), DMCM and FG 7142 were kindly provided by Schering (Berlin, Germany), and imidazenil and flumazenil were gifts from Fidia (Abano Terme, Italy) and Hoffmann-La Roche (Basil, Switzerland), respectively. PK 11195, $FGIN_{1-27}$, zopiclone, ethylβ-carboline-3-carboxilate and flunitrazepam were from RBI/Sigma (Sigma-Aldrich, Milano, Italy). CL 218,872, chlordesmethyldiazepam, lorazepam and all other reagents were from Sigma (Sigma-Aldrich).

Saturation binding data were analyzed by Scatchard plot, and the K_d (dissociation constant) and B_{max} (maximal number of binding sites) were calculated. In inhibition experiments, each median inhibitory concentration (IC_{50}) was converted into the inhibition constant (K_i) with the equation $K_i = IC_{50}/1+([L]/K_d)$ (Cheng and Prusunoff, 1973), where [*L*] is the free concentration of the radio-

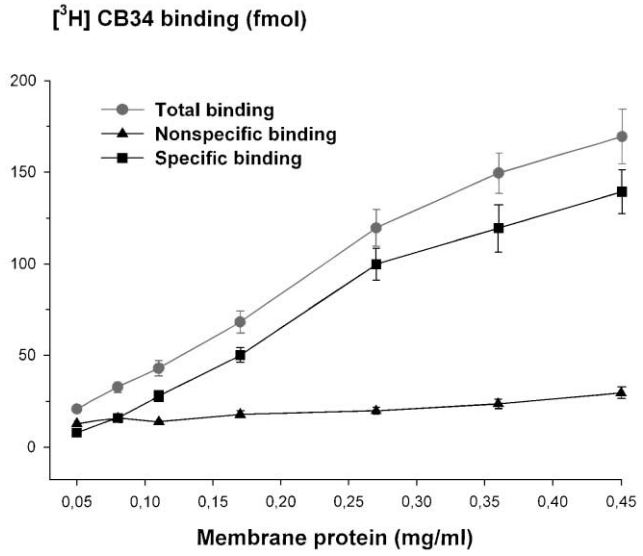


Fig. 2. Dependence of [^{3}H]CB 34 binding to rat cerebral cortical membranes on membrane protein concentration. Membranes at the indicated concentrations were incubated for 90 min at 25 °C with 1 nM [^{3}H]CB 34, after which the extents of total, nonspecific and specific binding were determined. The data are means of triplicates from an experiment that was repeated for a total of three times with similar results.

ligand. The association and dissociation rate constants were calculated from second- and first-order equations, respectively.

3. Results

Specific binding of 1 nM [^{3}H]CB 34 to rat cerebral cortical membranes was proportional to the concentration of the membrane protein in the reaction mixture with up to a value of at least 0.27 mg/ml (Fig. 2). The binding achieved equilibrium after incubation for ~ 90 min at 25 °C (Fig. 3A), and this level of binding was maintained for at least 240 min (data not shown). When the incubation was performed at 4 °C, specific [^{3}H]CB 34 binding had not reached a plateau by 120 min (data not shown). The addition of 1 μM of unlabeled CB 34 to the reaction mixture after binding equilibrium had been achieved at 25 °C that resulted in the rapid dissociation of specifically bound [^{3}H]CB 34 from the cerebrocortical membranes with a half-time of 20 min (Fig. 3B). The rate constants of association (k_{+1}) and dissociation (k_{-1}) were 0.2×10^8 M^{-1} min^{-1} and 0.29 min^{-1}, respectively, for the experiment shown in Fig. 3. The kinetically derived dissociation constant ($K_d = k_{-1}/k_{+1}$) was therefore 14 nM.

A saturation isotherm of specific [^{3}H]CB 34 binding to rat cerebrocortical membranes is shown in Fig. 4. Scatchard analysis of these data (Fig. 4, inset) indicated that [^{3}H]CB 34 binds to a single population of high-affinity binding sites, with K_d and B_{max} values calculated as 0.19 ± 0.02 nM and 188 ± 8 fmol/mg protein, respectively.

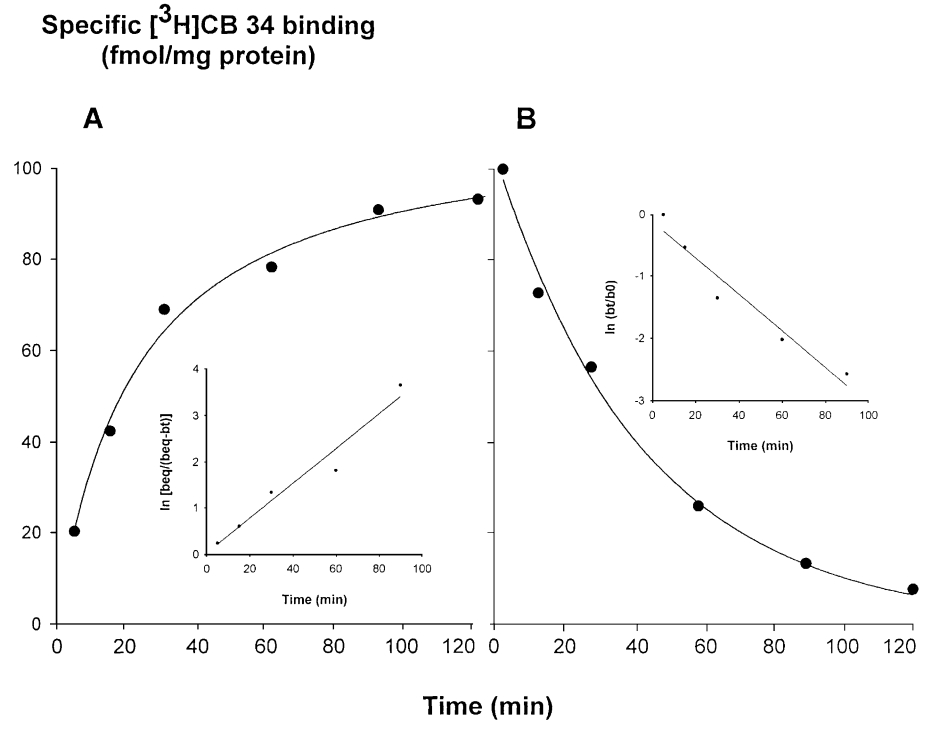


Fig. 3. Association (A) and dissociation (B) kinetics for the specific binding of [^{3}H]CB 34 to rat cerebrocortical membranes. Membranes were incubated with 1 nM [^{3}H]CB 34 for 120 min at 25 °C, after which unlabeled CB 34 was added to the reaction mixture to a final concentration of 1 μM. Specific binding of [^{3}H]CB 34 was determined at the indicated times. The data are means of triplicates from an experiment that was repeated for a total of three times with similar results. The insets show the respective representations of the data plotted as linear relations of $\{\ln[B_{eq}/(B_{eq} - B_t)]$ versus time and of $\ln(B_t/B_0)$ versus time, where B_{eq} is the extent of binding at equilibrium, B_t is the extent of binding at time t, and B_0 is the extent of binding at time zero.

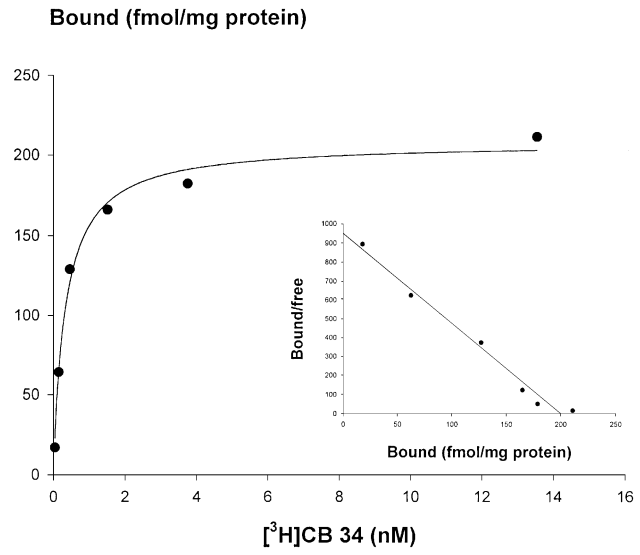


Fig. 4. Saturation isotherm and Scatchard analysis (inset) of [^{3}H]CB 34 binding to rat cerebrocortical membranes. Membranes were incubated with various concentrations of [^{3}H]CB 34 for 120 min at 25 °C, after which the extent of specific binding was determined. The data are means of triplicates from an experiment that was repeated for a total of four times with similar results.

Table 1
Inhibition constant values for the effects of various drugs on [^{3}H]CB 34 binding to rat cerebrocortical membranes

Compound	K_i (M)
Nonbenzodiazepines	
CB 34	$0.41 \pm 0.15 \times 10^{-9}$
PK 11195	$3.7 \pm 0.05 \times 10^{-9}$
$FGIN_{1-27}$	$1.3 \pm 0.09 \times 10^{-8}$
Zopiclone	$>1.9 \times 10^{-6}$
DMCM	$>1.9 \times 10^{-6}$
CL 218,872	$>1.9 \times 10^{-6}$
Ethyl β-carboline-3-carboxylate	$>1.9 \times 10^{-6}$
FG 7142	$>1.9 \times 10^{-6}$
Benzodiazepines	
4'-Chlordiazepam	$4.2 \pm 0.3 \times 10^{-8}$
Diazepam	$5.7 \pm 0.4 \times 10^{-7}$
Flunitrazepam	$1 \pm 0.04 \times 10^{-8}$
Chlordesmethyldiazepam	$>1.9 \times 10^{-6}$
Lorazepam	$>1.9 \times 10^{-6}$
Imidazenil	$>1.9 \times 10^{-6}$
Flumazenil	$>1.9 \times 10^{-6}$

The inhibition of [^{3}H]CB 34 binding was determined as described in the legend of Fig. 5. Data are means ± S.E.M. of values from three independent experiments.

The displacement of [^{3}H]CB 34 from its binding sites in cerebrocortical membranes by various benzodiazepine receptor ligands was examined in order to assess the pharmacological specificity of these sites (Fig. 5, Table 1).

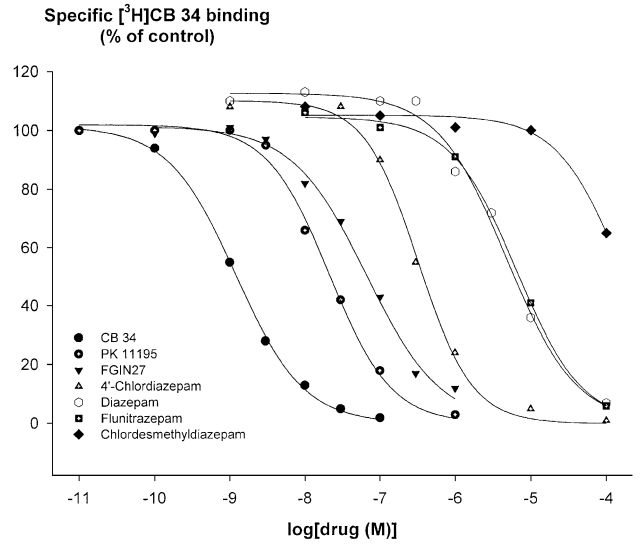


Fig. 5. Inhibition of [^{3}H]CB 34 binding to rat cerebrocortical membranes by various drugs. Membranes were incubated with 1 nM [^{3}H]CB 34 and indicated concentrations of drugs for 90 min at 25 °C, after which the extent of specific binding was determined. The data are means of triplicates from a representative experiment and are expressed as a percentage of specific [^{3}H]CB 34 binding apparent in the absence of drug. Nonlinear least-squares regression analysis gave the following Hill coefficients: CB 34 = 0.95; PK 11195 = 1.11; FGIN27 = 0.96; 4′-chlordiazepam = 1.29; diazepam = 0.97; and flunitrazepam = 0.99.

The binding of [^{3}H]CB 34 to cortical membranes was potently inhibited by unlabeled CB 34 and PK 11195 at concentrations in the nanomolar range (K_i values of 0.41 ± 0.15 and 3.7 ± 0.05 nM, respectively). The other two peripheral benzodiazepine receptor ligands tested, $FGIN_{1-27}$ and 4′-chlordiazepam, also competed with [^{3}H]CB 34 for binding but with a lower potency. With the exception of flunitrazepam and diazepam (K_i values of 1×10^{-8} and 5.7×10^{-7} M, respectively), all other ligands of the central benzodiazepine receptors tested were virtually inactive with regard to the inhibition of [^{3}H]CB 34 binding with K_i values of >1 μM.

The specific binding of [^{3}H]CB 34 varied in extent among membranes prepared from different brain regions. Of those regions examined, it was greatest in the hypothalamus and smallest in the cerebral cortex (Table 2). Equilibrium isotherms indicated that the regional differences in

Table 2
Regional distribution of specific [^{3}H]CB 34 binding sites in rat brain

Brain region	B_{max} (fmol/mg protein)	K_d (nM)
Hypothalamus	760 ± 33	0.17 ± 0.02
Olfactory bulb	514 ± 22	0.14 ± 0.01
Hippocampus	278 ± 12	0.12 ± 0.01
Cerebral cortex	188 ± 8	0.19 ± 0.02

Membranes prepared from the indicated brain regions were incubated with 0.5–16 nM [^{3}H]CB 34 as described in the legend of Fig. 4, and the B_{max} and K_d values for specific [^{3}H]CB 34 binding were determined by Scatchard analysis. Data are means ± S.E.M. of values from three independent experiments.

the [^{3}H]CB 34 binding are attributable primarily to the variation in receptor density, with the affinity of the recognition sites for [^{3}H]CB 34 being about the same in all brain regions studied (Table 2).

4. Discussion

We have shown that [^{3}H]CB 34 binds with high affinity to peripheral benzodiazepine receptors in rat brain. The binding of [^{3}H]CB 34 was time-dependent, reaching a plateau at 90 min at 25 °C. Specific [^{3}H]CB 34 binding was saturable, and Scatchard analysis revealed only a single population of high-affinity binding sites with a K_d of 0.19 nM. This K_d value, however, was 20 times lower than that derived from the kinetic experiments (14 nM). This difference is not due to a failure to achieve binding equilibrium in the saturation binding experiments. A discrepancy between the K_d values obtained from saturation and kinetic studies has previously been described for the binding of [^{3}H]Ro 5-4864 and [^{3}H]PK 14105 in various tissues (Doble et al., 1987; Benavides et al., 1989; Mak and Barnes, 1990) and was attributed to the heterogeneity of peripheral benzodiazepine receptors or to the complex association and dissociation kinetics (Benavides et al., 1989). Accordingly, the semilog plot of dissociation (depicted in Fig. 3B) may be nonlinear. A straightforward interpretation of such data is that there exist two different binding sites. However, another possible interpretation is that cooperativity exists between the sites such that the higher binding of the remaining complex occurs as fewer sites are occupied.

The compound [^{3}H]*N*-(2,5-dimethoxybenzyl)-*N*-(5-fluoro-2-phenoxyphenyl) acetamide ([^{3}H]DAA1106) was recently described as a ligand of peripheral benzodiazepine receptors with the highest affinity hitherto identified (Chaki et al., 1999). Although the tissue preparation used in this previous study (crude mitochondrial fraction) differed from that used in the present study (whole homogenate), the affinity of [^{3}H]CB 34 for peripheral benzodiazepine receptors appears to be equal to that of [^{3}H]DAA1106 (0.19 versus 0.12 nM, respectively).

The specificity of the binding of [^{3}H]CB 34 to peripheral benzodiazepine receptors was demonstrated by the selectivity of the displacement of this ligand by various drugs. With the exception of diazepam and flunitrazepam, both of which bind both central and peripheral benzodiazepine receptors (Marangos et al., 1982), all ligands of central benzodiazepine receptors tested failed to inhibit [^{3}H]CB 34 binding. Among the peripheral benzodiazepine receptor ligands examined, PK 11195 was the most potent in inhibiting [^{3}H]CB 34 binding to rat cerebrocortical membranes, with both 4′-chlordiazepam and $FGIN_{1-27}$ exhibiting lower affinities. This difference in the affinities of PK 11195 and 4′-chlordiazepam for the binding site labeled by [^{3}H]CB 34 further supports the idea that these two ligands bind to two distinct binding domains or to two different conformational states of peripheral benzodiazepine receptors (Papadopoulos, 1993).

Of the rat brain regions examined, the density of the [^{3}H]CB 34-binding sites was greatest in the hypothalamus. In contrast, the density of the binding sites labeled with [^{3}H]PK 11195, [^{3}H]Ro 5-4864 or [^{3}H]DAA1106 was highest in the olfactory bulb (Benavides et al., 1983; Schoemaker et al., 1983; Chaki et al., 1999). Given that the chemical structure of CB 34 differs from those of the benzodiazepine 4′-chlordiazepam and the isoquinoline PK 11195, the possibility that [^{3}H]CB 34 binds to a site on peripheral benzodiazepine receptors distinct from those bound by these other two drugs cannot be ruled out. Indeed, this notion is supported by the observation that the concomitant administration of PK 11195 and CB 34 to rats resulted in a reciprocal inhibition of their stimulatory effects on the steroid biosynthesis in the brain (Serra et al., 1999).

In conclusion, [^{3}H]CB 34 was shown to bind with high affinity to rat cerebrocortical membranes. Specific binding was saturable and was inhibited by nanomolar concentrations of ligands selective for peripheral benzodiazepine receptors. Further studies with this ligand may provide insight into the pharmacological and physiological significance of these receptors.

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