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Unraveling the identity of benzodiazepine binding sites in rat hipppocampus and olfactory bulb

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Abstract

The goals of the work reported here were (i) to identify distinct $GABA_A$/benzodiazepine receptors in the rat hippocampus and olfactory bulb using receptor binding assays, and (ii) to determine the affinities and selectivities of benzodiazepine receptor ligands from structurally diverse chemical families at each site identified. These studies were aided by the use of software AFFINITY ANALYSIS SYSTEM, developed in our laboratory for analysis of receptor binding data that allows the determination of receptor heterogeneity using non-selective radioligands. Saturation binding assays using [^{3}H]RO15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate) revealed two binding sites in each of these two tissues. The higher affinity site corresponds to α_5 subunit-containing $GABA_A$ receptor and the lower affinity site to a combination of α_1, α_2, and α_3 subunit-containing receptors. These results should be useful in the challenging task of identifying the various functional $GABA_A$ receptors in the central nervous system, and in providing a link between receptor affinities and in vivo activities of the $GABA_A$/benzodiazepine receptor ligands studied.

Keywords: $GABA_A$/benzodiazepine receptor; Hippocampus; Olfactory bulb; Ligand affinity; Receptor heterogeneity; α subunit

1. Introduction

Benzodiazepines are therapeutic agents that have hyperphagic, anxiolytic, sedative, hyperthermic, anticonvulsive and cognitive effects (Braestrup et al., 1982; Burt and Kamatchi, 1991). These drugs exert their effect by binding to allosteric sites on the hetero-pentameric γ-aminobutyric acid ($GABA_A$) chloride ion channel receptors and modulating the ability of $GABA_A$ to open the chloride channel (Olsen and Tobin, 1990). Thus, to further elucidate the molecular basis for their diverse pharmacological effects, it is important to identify the nature and extent of $GABA_A$/benzodiazepine receptor heterogeneity in the brain. Both the number and the uncertainty of subunit composition of the $GABA_A$ receptors in the central nervous system contribute to the difficulty of identifying those that are associated with a specific behavioral activity.

Cloning and sequencing have demonstrated the existence of 21 different isoforms of the five $GABA_A$ receptor subunits, including α_1–α_6, β_1–β_4, γ_1–γ_4, δ, ρ_1–ρ_3, π, ε, and θ (Bonnert et al., 1999; Burt and Kamatchi, 1991; Olsen and DeLorey, 1999). In principle, these subunits can combine to form a vast number of functional $GABA_A$ receptors and the study of cloned receptor subunits in transfected cells cannot by itself lead to the identification of the particular combinations present in the central nervous system receptors. Other experimental approaches can aid in this process. Specifically, immunohistochemistry and in situ hybridization studies have identified different subunits that are expressed in various regions of the brain (Thompson et al., 1992; Wisden et al., 1988; Zimprich et al., 1991). Furthermore, co-immunoprecipitation studies have identified the subunits that possibly form the functional receptors (Khan et al., 1994; McKernan and Whiting, 1996; Quirk et al., 1994).

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These diverse studies provide evidence for at least 13 distinct functional combinations in vivo. In addition, several studies indicate that functional $GABA_A$ receptors include α, β and γ subunits (Prichett et al., 1989a; Siegel et al., 1990; Smith and Olsen, 1995). It is also now clear that there is more than one type of functional $GABA_A$ receptor in virtually every region of the brain (Laurie et al., 1992; McKernan and Whiting, 1996; Wisden and Seeburg, 1992).

The nature of the α subunit expressed in each specific pentameric receptor is emerging as the main determinant of receptor affinity for $GABA_A$/benzodiazepine receptor (Ebert et al., 1997; Prichett et al., 1989b; Wong and Skolnick, 1992). Thus, one useful indicator of the different possible $GABA_A$/benzodiazepine receptor types in a given brain region is the number of different α subunits expressed in that region. For example in the cerebellum the major $GABA_A$ α subunits expressed are α_1 and α_6 (Laurie et al., 1992; Thompson et al., 1992). Thus, in binding studies two major receptor sites are expected to be present, as observed previously (Lameh et al., 2000; Uusi-Oukari, 1992; Wisden et al., 1996). In both hippocampus and olfactory bulb, all the major $GABA_A$ α subunits are expressed with the exception of α_6 subunit (Fritschy and Möhler, 1995; Laurie et al., 1992; Sperk et al., 1997; Sur et al., 1999; Thompson et al., 1992). Thus, several receptor types can potentially be expressed in these tissues.

The goals of the present study were two fold: first to identify the number of distinct $GABA_A$/benzodiazepine receptors in rat hippocampus and olfactory bulb, and second to measure the affinities of several benzodiazepine ligands for each of the identified receptor type. Hippocampus and olfactory bulb were chosen because $GABA_A$ receptors are expressed at very high levels and have important functions in these tissues. In the olfactory bulb, the $GABA_A$ receptors, together with the glutamate receptors are involved in olfactory coding (Wellis and Kauer, 1993) which in turn, at least in rodents, are involved in learning and conditioning (Okutani et al., 1999; Trombley and Shepherd, 1992). In the hippocampus, the $GABA_A$ receptors are involved in memory processing (Frankland et al., 1998; Milanovic et al., 1998; Phillips and LeDeux, 1992).

Studies of structurally diverse benzodiazepine receptor ligands in transfected cells have revealed little or no selective binding to reconstituted $GABA_A$ receptors with varying α subunits in combination with β_2, γ_2 subunits. A major impediment to binding studies as a tool for characterization of native receptors has been the lack of radioligands selective for each of the different types of receptors. The commercial software programs currently available for analysis of receptor binding data require the use of a radioligand capable of distinguishing between different binding sites in order to detect multiple binding affinities at these sites for a non-radioactive ligand. This obstacle was overcome in the present study by use of AFFINITY ANALYSIS SYSTEM developed in our laboratory, which is comprised of two independent analysis programs. The first program, SINGLE LIGAND, performs analysis of saturation and self-competition data. The other program, COMPETE, allows the determination of the affinities and densities of competing cold ligands for multiple binding sites using a non-selective radioligand that binds to all the sites with similar affinity.

Eight different chemical families were represented in the ligands chosen for the present study. These were 1,4-benzodiazepines (flunitrazepam), imidazobenzodiazepines (RO15-1788, RO15-4513, RO16-6028, RO23-0364, RO41-7812 and RO42-8773), imidazopyridines (Zolpidem and AHR 14749), β-carbolines (Abecarnil), pyrazoloquinolines (CGS 8216, CGS 9895 and CGS 9896), pyrroloquinazolinone (AHR 11797), quinoxalinone (U78875) and quinoline (RO23-1590). Systematic binding studies were carried out with these ligands to elucidate the heterogeneity of the $GABA_A$/benzodiazepine receptors in the olfactory bulb and hippocampus and to determine their affinities at each site identified.

2. Materials and methods

2.1. Materials

The following compounds were received as generous gifts: zolpidem ([*N*,*N*,6-trimethy-1-2-(4-methylphenyl)imidazo[1,2-a]pyridine-3-acetamide hemitartrate]) (Synthelabo Recherche, Bagneux, France), AHR-11797 (5,6-dihydro-6-methyl-1-phenyl-3*H*-pyrrolo[3,2,1-ij]quinazolin-3-one), AHR 14749 (1-*N*, *N*-dimethyl-amido-imidazo[2,3*a*]-2-chloropyridine) (A.H. Robins, Richmond, VA, USA), RO15-1788 (8-fluoro-3-carboxy-5,6-dihydro-5-methyl-6-oxo-414-imidazo[1,5-*a*]1,4 benzodiazepine), RO15-4513 (ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate), RO16-6028 (*t*-butyl(s)-8-bromo-11,12,13,13*a*-tetrahydro-9-oxo-9*H*-imidazo[1,5-*a*][1,4]benzodiazepine-1-carboxylate), RO23-0364 (6-[2-chlorophenyl]-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxamide), RO23-1590 (2-(*p*-chloro phenyl)-4-(4-*N*-ethylamide piperazinyl) quinoline), RO41-7812 (7-chloro-4,5-dihydro-3-(3-hydroxy-1-propynyl)-5-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepin-6-one) and RO42-8773 (7-chloro-3-[3-(cyclopropylmethoxy)1-propynyl]-4,5-dihydro-5-methyl-6*H*-imidazo[1,5*a*][1,4]benzodiazepine-6-one) (Hoffmann-LaRoche, Nutley, NJ), abecarnil (isopropyl 6-benzyloxy-4-methoxymethyl-beta-carboline-3-carboxylate)(Schering, Berlin, Germany), U78875 (3-(5-cyclopropyl-1,2,4-oxadiazol-3-yl)-5-(1-methylethyl) imidazo[1,5-*a*]-quinoxalin-4(5*H*)-one) (Upjohn, Kalamazoo, MI, USA), CGS 8216 (2-Phenylpyrazo-lo[4,3-c]quinolin-3(5*H*)-one, 2-*p*-chlorophenylpyrazolo[4,3-*c*]quinolin-3(5*H*)-one) CGS 9896 (2-*p*-chlorophenylpyrazolo[4,3-*c*]quinolin-3(5*H*)-one) and

CGS 9895 (2-(4-methoxy-phenyl)-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one) (Ciba-Geigy, Summit, NJ, USA). [^{3}H]RO-15-4513 and [^{3}H]RO15-1788 were purchased from New England Nuclear (Boston, MA, USA), flunitrazepam (5-[2-fluorophenyl]-1-methyl-7-nitro-3*H*-1,4-benzodiazepin-2[1*H*]one) from Sigma (St. Louis, MO, USA). All other chemicals were from standard commercial sources. All drugs were made as 10 or 5 mM stock in 100% ethanol. The dilutions of the drugs were made in reaction buffer. The final ethanol concentration in the assay tube was less than 0.1%.

2.2. Membrane preparation

Frozen (Pel Freeze, Rogers, AR, USA) rat brains were used for dissection of the hippocampus and the olfactory bulbs. The dissected tissues were homogenized with a polytron homogenizer in 40 volumes of 50 mM Tris–HCl, pH 7.7 at 4°C and centrifuged at 20,000 × *g* for 10 min at 4°C. The supernatant was discarded and the pellet homogenized and centrifuged two more times as above. The pellet was resuspended in minimal volume of buffer and frozen at −86°C overnight. After thawing, the pellet was resuspended in the same volume of buffer and washed two more times by centrifugation and homogenization. The final membrane pellet was resuspended to a tissue concentration of 100 mg wet weight/ml of buffer and stored in aliquots at −86°C until use (Maguire et al., 1992).

2.3. Single ligand binding assay

These studies were performed as saturation assays. Increasing concentrations of [^{3}H]RO15-4513 from 40 pM to 1 nM were used followed by displacing from 1 nM with unlabeled RO15-4513 concentrations of 0.058 nM to 10 μM. The range of the total ligand concentration was from 40 pM to 10 μM. The assay was carried out in 1 ml total reaction volume with 30–50 μg of membrane protein per assay tube. Incubation was at 0°C for 90 min. The assay was terminated by rapid filtration through Whatman GF/B filters using a FilterMate cell harvester (Packard Instruments) followed by three washes, 4 ml each with ice cold buffer. Radioactivity retained on the filters was measured using Microscint 0 in a TopCount liquid scintillation counter (Packard Instruments). All assays were carried out in triplicate.

Single ligand binding assays were also carried out for [^{3}H]RO15-1788 as described above for [^{3}H]RO-15-4513.

2.4. Competition binding assay

Membranes (30–50 μg/tube) were incubated with 0.5–1.0 nM [^{3}H]RO15-4513 and increasing concentrations (24–32) of unlabeled ligand in a total of 1 ml reaction volume. Incubation and filtration conditions were as described above for the single ligand assays. All assays were carried out in triplicate.

2.5. Data analysis

2.5.1. Single ligand binding: saturation and self-competition

Ligand binding is controlled by the equation

$$b = \sum_{j=1}^{n} \frac{d_j f}{a_j + f} + Nf, \quad (1)$$

where f is the concentration of free ligand, b is the concentration of bound ligand, n is the number of sites, d_j is the density of site j in the substrate, a_j is the (dissociative) affinity for site j, and N is the non-specific binding factor (Klotz, 1997). Free ligand is measured as the difference between total ligand T and bound ligand b: $f = T - b$.

Self-competition experiments mix radioligand with unlabeled ligand. Let Tr be the total concentration of radioligand and Tu be the total concentration of unlabeled ligand. Also, let br be the concentration of bound radioligand, and let bu be the concentration of bound unlabeled ligand. Since labeled and unlabeled ligand bind equally,{br}/{bu} = {Tr}/{Tu} The quantities Tr, Tu, and br are the only ones that can be measured directly, but f can be derived from the equation $f = (\mathrm{Tr} + \mathrm{Tu}) - (\mathrm{br} + \mathrm{bu})$.

Typically one starts with 24 data triples (Tr, Tu, br) where the concentration of ligand Tr + Tu range from nearly zero to levels that saturate the binding sites. The curve-fitting problem is to determine the binding parameters n, d_j, a_j, and N from these data.

2.5.2. Competition binding

When two ligands compete for binding sites, the binding is determined by the equation

$$b = \sum_{j=1}^{n} \frac{d_j \dfrac{f}{a_j}}{1 + \dfrac{f}{a_j} + \dfrac{g}{c_j}} + Nf, \quad (2)$$

where b is the concentration of first bound ligand, f is the concentration of free first ligand, g is the concentration of free second ligand, n is the number of sites recognized by the second ligand, d_j is the density of site i, a_j is the (dissociative) affinity of the first ligand for site j, c_I is the affinity of the second ligand for site j, and N is the non-specific binding factor for the first ligand (Cheng and Prusoff, 1973). In practice, Eq. (2) is used when the first ligand is a radioligand whose parameters n, d_j, a_j,and N have been determined for a particular substrate by single ligand methods.

The problem for competition experiments involving a radiolabeled and a cold ligand is to determine the number of sites, n, the density of each site, d_i, and the affinity at each site, j, for both the radiolabeled ligand, a_j, and the competing cold ligand, c_j. At first glance it might seem that the number of sites and the density of each site has

already been determined through the analysis of the saturation data for the radiolabeled ligand. However, the methods used do not assume that the competing cold ligands recognize the same sites as the radiolabeled ligand. It is possible that a cold ligand distinguishes between two sites that the hot ligand bound with equal affinities. Such a pair of sites would appear, in the saturation analysis, as a single site. It is also possible that a cold ligand fails to bind at a site bound by the hot ligand. The methods used can resolve these situations, although they are not able to determine if a cold ligand is binding at a site unrecognized by the hot ligand.

2.5.3. Computational methodology

Calculations of affinities and site densities were performed by the AFFINITY ANALYSIS SYSTEM developed in our laboratory, consisting of two complementary analysis programs, SINGLE LIGAND that analyzes saturation and self-competition data and COMPETE that analyzes competition data. Both programs were written in the Delphi environment (Imprise) and run as stand-alone executables under Microsoft Windows 95 and 98 and Windows NT 4.0. They are available from the Molecular Research Institute's web site: http://www.molres.org.

Both programs follow the same general protocol. They use a method based on linear programming (Tobler and Engel, 1983) to approximate the densities and affinities, then use a genetic algorithm for curve fitting (Storn and Price, 1995) to refine these parameters. Genetic algorithms are optimization algorithms that model Darwinian evolution. They start with between 10 and 50 approximate solutions and modify them to find the optimal solution. The modifications are based on a biological model. Individual solutions undergo random modifications (mutation); parts of different solutions are combined (crossover); and at each stage the best solutions are retained while less accurate ones are discarded (natural selection) (Bäck and Schwefel, 1993). The program SINGLE LIGAND also uses Marquardt minimization and the bootstrap method (Press, 1992) to estimate the covariance matrix for the estimated densities and affinities.

To apply the linear programming method to self-competition data, the self-competition Eq. (1) must first be recast into the form:

$$\frac{\mathrm{br}}{\mathrm{Tr}-\mathrm{br}} = \sum_{j=1}^{n} \frac{d_j}{a_j + f} + N \tag{3}$$

The linear programming method yields estimates for the number of sites n and the site densities d_j and affinities a_j. It also estimates the non-specific binding factor N. Once these factors are estimated, the genetic algorithm adjusts them to fit the equation to the data. The goodness of fit criterion is χ^2, the sum over all data of $(\{\text{data} - \text{estimate}\}/\{\text{data}\})^2$.

Similarly, the competition Eq. (2) can be recast into the required form:

$$b - Nf = \sum_{j=1}^{n} \frac{d_j \dfrac{f}{a_j + f}}{1 + \left(\dfrac{a_j}{a_j + f}\right)\dfrac{g}{c_j}} \tag{4}$$

In the competition calculation, the linear programming method determines not affinities and densities directly but the related factors $m_j = d_j\{f\}/\{a_j + f\}$ and $e_j = \{d_j\}/\{c_j(a_j + f)\}$. One such pair of values is produced for each site recognized by the both the cold ligand and the hot ligand. In the simplest situation the hot and cold ligands bind to the same sites, so all that remains is to match the pairs of cold ligand parameters (m_j, e_j) to the pairs of hot ligand parameters (d_j, a_j). Once this is done the cold ligand affinities c_i can be calculated. To determine the correct matching, the software tries all possible permutations, uses the resulting estimated cold ligand affinities as a starting point for fitting Eq. (4) to the data, and selects the match that gives the best fit based on the χ^2 criterion.

The simplest situation is not always encountered. It is possible that the radiolabeled ligand binds to two sites with equal affinity, but that these present themselves with different affinities to the cold ligand (Lameh et al., 2000). The software developed can recognize this situation. It does not assume that the pairs of factors produced by the linear programming method must correspond to the sites found by the hot ligand. Rather, the software assumes only that each site recognized by the cold ligand is a subsite of a site recognized by the hot ligand. Thus, if the site were not recognized by the hot ligand, then there is no way the displacement measurements used here could detect it. Once a cold ligand site is matched to a hot ligand site, the affinity a_j of the hot ligand for the site is known and the density d_j and cold ligand affinity c_j can be determined from the factors m_j and e_j. An assignment of cold ligand sites to hot ligand sites is considered plausible if the sum of the estimated densities for the cold ligand subsites at each hot ligand site approximately equals the known density of the site. Plausible assignments are then used as starting points for fitting the data to Eq. (4), and the best fit is used to determine the final estimates of affinities and densities of sites for the cold ligand.

One simplifying assumption is made when applying Eq. (4). It is that the concentrations of free first and second ligand f and g are assumed equal to the total concentrations. The equations are not sensitive to small changes in the factors for free hot ligand (f) and cold ligand (g), and simulation experiments confirm that replacing free ligand values with total ligand values changes the estimates for affinities and densities by no more than the relative change in the data. For this work the ratio of bound ligand to free ligand never surpassed 4%. Thus, we can be confident that

the simplifying assumption did not significantly affect our results.

Before using it to analyze binding data, the software was evaluated by use of different sets of simulated data that included up to 5% random error. For each test set of data, the software consistently found the correct number of receptor sites and accurately estimated receptor densities and affinities at each site.

In addition to providing support for the accuracy of the results, use of simulated data allowed the determination of the conditions under which multiple binding sites can be identified. Sites with affinities differing by at least a factor of four can be distinguished provided the densities are within a factor of two of each other. If the densities are vastly different, then the ability to distinguish sites is diminished. Furthermore, the reliability of the analysis is dependent on the accuracy of the experimental data. Use of a large number of data points for each experiment (at least 24) and errors less than 5% among repeated measurements are required to assure reliable computational results.

3. Results

Saturation and competition binding assays were carried out to determine the binding characteristics in the hippocampus and olfactory bulb of 16 benzodiazepine ligands with diverse chemical structures. The binding data were analyzed with new saturation (self-competition) and competition software programs (SINGLE LIGAND and COMPETE, respectively). The competition software (COMPETE) allows characterization of the binding profile of an unlabelled ligand with different binding affinities to sites to which a labeled ligand binds with similar affinities.

In the rat hippocampus, self-competition binding assays with [^{3}H]RO15-4513 identified two distinct binding sites with affinities of 2.7 ± 0.2 nM and 0.25 ± 0.17 nM (Table 1). Comparisons with results for this ligand in transfected cells (Hadingham et al., 1993; Liu et al., 1995; Quirk et al., 1996) allowed the identification of the lower affinity site as a composite of $GABA_A$ receptors containing α_1, α_2 and α_3 subunits and of the higher affinity site as one corresponding to α_5 subunit-containing receptors. According to previous studies (Liu et al., 1995) RO15-4513 has similar affinities for α_1, α_2 and α_3 subunit-containing receptors and thus cannot distinguish between these three types of receptors.

No evidence for either α_6 or α_4 subunit-containing $GABA_A$/benzodiazepine receptors was found in hippocampus, as seen by the identification of only one binding site in self-competition assay with RO15-1788. The failure to detect α_6 subunit-containing $GABA_A$/benzodiazepine receptors in hippocampus is consistent with lack of detection of this subunit in this brain region. However, in spite of some reports indicating the presence of the α_4 subunit in the hippocampus (Benke et al., 1997; Sperk et al., 1997), the studies reported here were not able to detect any α_4 subunit-containing receptors using either [^{3}H]RO15-4513 or [^{3}H]RO15-1788 as the radioligand.

As shown in Table 1, in addition to RO15-4513, nine of the benzodiazepine receptor ligands studied had differential affinities at the composite α_1, α_2 and α_3 subunit-containing receptors compared to the α_5 subunit-containing receptors. All these ligands except AHR 14749 bound to the α_5 subunit-containing receptors with affinities greater than 3 nM. The drug with the highest affinity for the α_5 subunit-containing receptors was CGS 8216 with an affin-

Table 1
Affinities of several benzodiazepines to the rat hippocampus receptors

Ligand	Affinity α_1, α_2, α_3 (nM)	% total binding α_1, α_2, α_3	Affinity α_5 (nM)	% total binding α_5
RO15-4513[a]	2.7 ± 0.2	68 ± 39	0.25 ± 0.17	32 ± 39
RO16-6028	0.5 ± 0.2	95 ± 6	0.32 ± 0.2	5 ± 6
RO23-0364	9.1	74	1.45	26
RO23-1590	101 ± 26	90 ± 2	0.94 ± 0.68	10 ± 2
AHR 11797	214 ± 7	92 ± 1	2.8 ± 1	8 ± 1
AHR 14749	223 ± 53	74 ± 12	2758 ± 2015	26 ± 12
CGS 8216	0.8 ± 0.9	83 ± 15	0.09 ± 0.11	17 ± 15
CGS 9895	0.2 ± 0.1		ND	
CGS 9896	1.3 ± 0.9		ND	
ZOLPIDEM[b]	11.4 ± 4.5 (α_1)	39 ± 7 (α_1)		
	442 ± 34 (α_2, α_3)	61 ± 7 (α_2, α_3)		
ABECARNIL	0.43 ± 0.52		ND	
RO41-7812	4.5 ± 1.1	84 ± 6.8	0.18 ± 0.27	16 ± 6.8
RO42-8773	0.8 ± 0.2	86 ± 7	0.40 ± 0.5	14 ± 7
U78875	38 ± 41	91 ± 2.0	0.25 ± 0.3	9 ± 2.0

Competition binding assays were carried out against the [^{3}H]RO15-4513 as described under Section 2.
The values in the table are average values $\pm$ S.D. from two or more experiments each carried out in triplicate.
ND: None detected.
[a] Values from self-competition experiments.
[b] Competition was carried out against [^{3}H]Ro15-1788.

ity of 0.09 ± 0.11 nM. By contrast, four compounds, CGS 9895, CGS 9896, zolpidem and abecarnil did not bind to α_5 subunit-containing receptors in hippocampus with detectable affinities (Table 1).

The affinities of the ligands studied also varied for the combination of α_{1-3} subunit-containing receptors, ranging from subnanomolar, for example, for CGS 8216 and RO16-6028 to 100–200 nM for RO23-1590, AHR 11797 and AHR 14749.

Analysis of competitive binding studies of [^{3}H]RO15-4513 with zolpidem led to further separation of this composite site into α_1 and α_2/α_3 subunit-containing sites with distinguishable affinities of zolpidem to α_1 (11.4 ± 4.5 nM) vs. α_2/α_3 (442 ± 34 nM) subunit-containing receptors (Table 1). In addition, the results from competitive binding studies of zolpidem showed that the $GABA_A$/benzodiazepine receptors containing the α_1 subunit are expressed at lower levels (39% of zolpidem binding sites) than those containing the α_2 and α_3 subunits (61% of zolpidem binding sites).

These combined results indicate that rat hippocampus contains at least three populations of benzodiazepine binding sites. These correspond to α_1 subunit-containing, α_2/α_3 subunit-containing and α_5 subunit-containing $GABA_A$ receptors. Moreover, 20% of the $GABA_A$/benzodiazepine receptors could be attributed to those containing the α_5 subunit, 30% to those containing α_1 and the remaining 50% to receptors containing α_2 and α_3 subunits. This observation supports the fact that the distribution pattern of functional $GABA_A$/benzodiazepine receptor is consistent with the expression pattern of $GABA_A$ receptor subunits (Fritschy and Möhler, 1995).

As shown in Table 2, self-competition binding assays carried out in the olfactory bulb with [^{3}H]RO15-4513 identified two sites with affinities of 2.2 ± 0.5 nM for to the combination of α_1, α_2 and α_3 subunit-containing receptors and 0.051 ± 0.001 nM for α_5 subunit-containing receptors. Once again, the identification of the specific subunit-containing receptors in this brain region was based on values previously reported from studies in transfected cell lines and the expression pattern of various subunits in the olfactory bulb. The affinities for RO15-4513 determined in this study agree with published values for these receptors in transfected cells (Hadingham et al., 1993; Liu et al., 1995; Quirk et al., 1996).

While the affinities of [^{3}H]RO15-4513 at the composite α_1, α_2 and α_3 subunit-containing site and at α_5 subunit-containing site are consistent with those found for these two sites in hippocampus, the relative densities were significantly different. In hippocampus, the ratio of densities of the two sites was 68%:32% (Table 1), while in olfactory bulb it was 94%:6%.(Table 2). Thus, there is a much lower density of the α_5 subunit-containing receptor in the olfactory bulb. As a consequence, despite the rigor of the data analysis using the COMPETE program, characterization of the affinities of the cold ligands at the α_5 subunit-containing receptor in this brain region was much less robust. Thus, while most of the ligands studied that bound the α_5 subunit-containing receptors in the hippocampus, also bound these receptors in the olfactory bulb, no appreciable binding to this receptor was detected for CGS 8216, RO16-6028 and RO42-8773, as evidenced by only one binding site in competition against [^{3}H]RO15-4513 in the olfactory bulb. This problem was exacerbated for these ligands because the affinities of both the labeled and unlabeled ligands to α_5 site are very high. Under these circumstances, the competition software is unable to measure the affinity to this very high-affinity, low-density site. The reason is that the decrease in the overall labeled ligand binding due to the high affinity interaction of the unlabeled ligand to the low-density site is very small.

As in the hippocampus expression, of α_4 subunit protein has been previously demonstrated in the olfactory bulb. However, like the hippocampus, as shown in Table 3, self-competition experiments with [^{3}H]RO15-1788 detected only one binding site for this ligand. Binding studies

Table 2
Affinities of several benzodiazepines to the rat olfactory bulb receptors

Ligand	Affinity α_1, α_2, α_3 (nM)	% total binding α_1, α_2, α_3	Affinity α_5 (nM)	% total binding α_5
RO15-4513[a]	2.2 ± 0.5	94 ± 3	0.051 ± 0.001[b]	6 ± 2
RO16-6028	0.38 ± 0.11			
RO23-0364	5.5 ± 2	93 ± 2	0.2 ± 0.2	7 ± 2
RO23-1590	102 ± 49	89 ± 5	1.2 ± 0.5	11 ± 5
AHR 11797	50 ± 6	83 ± 12	26 ± 5	17 ± 12
CGS 8216	0.28 ± 0.14		ND	
RO41-7812	1.5 ± 1.7	76 ± 18	1.1 ± 0.8[b]	23 ± 18
RO42-8773	0.4 ± 0.02			
U78875	21 ± 17	79 ± 3	0.069 ± 0.055	21 ± 3

Competition binding assays were carried out against the [^{3}H]RO15-4513 as described under Section 2.
The values in the table are average values ± S.D. from two or more experiments each carried out in triplicate.
ND: None detected.
[a]Values from self-competition experiments.
[b]A high affinity, low density site may exist for α_5.

Table 3
Affinities of several benzodiazepines to the rat olfactory bulb receptors

Ligand	Affinity (NM)	% total binding
RO15-1788[a]	1.6 ± 1.8	
RO23-0364	15.9 ± 15.6	
RO23-1590	179 ± 218 (α_1, α_2, α_3)	79 ± 13 (α_1, α_2, α_3)
	4.3 ± 5.9 (α_5)	21 ± 13 (α_5)
AHR 11797[b]	407 ± 397	
AHR 14749	97 ± 13 (α_1, α_2, α_3)	68 ± 20 (α_1, α_2, α_3)
	4539 ± 1253 (α_5)	32 ± 20 (α_5)
CGS 8216	0.76 ± 0.4 (α_1, α_2, α_3)	33 ± 19 (α_1, α_2, α_3)
	0.02 ± 0.01 (α_5)	67 ± 19 (α_5)
CGS 9896	0.16 ± 0.03	
Zolpidem	15 ± 17 (α_1)	45 ± 53 (α_1)
	776 ± 963 (α_2, α_3)	55 ± 53 (α_2, α_3)
Flunitrazepam	0.16 ± 0.16 (α_2, α_5)	22 ± 11 (α_2, α_5)
	4.55 ± 1.4 (α_1, α_3)	78 ± 11 (α_1, α_3)
Abecarnil	5.9 ± 0.8	
RO41-7812	6.2 ± 3 (α_1, α_2, α_3)	59 ± 49 (α_1, α_2, α_3)
	0.30 ± 0.6 (α_5)	41 ± 49 (α_5)
RO42-8773[c]	0.41 ± 0.17	

Competition binding assays were carried out against the [^{3}H]RO15-1788 as described under Section 2.
The values in the table are average values ± S.D. from two or more experiments each carried out in triplicate.
[a]Values are from self-competition experiments.
[b]Another experiment showed two sites with affinities of 4.1 and 167 nM.
[c]Another experiment showed two sites with affinities of 0.078 and 0.29 nM.

carried out in transfected cell lines expressing alpha subunits together with gamma subunits have shown that affinity of RO15-1788 to $GABA_A$ receptors containing the α_1, α_2, α_3 and α_5 subunits is more than 100-fold higher than its affinity to α_4 and α_6 subunit-containing receptors (Liu et al., 1995; Scholze et al., 1996; Wisden et al., 1991). Thus, in the present studies only the $GABA_A$ receptors containing the α_1, α_2, α_3 and α_5 subunits were detected in the olfactory bulb.

Competition binding studies in the olfactory bulb were performed with selected cold ligands against [^{3}H]RO15-1788. Since RO15-1788 is non-selective at α_1, α_2, α_3 and α_5 subunit-containing receptors (Liu et al., 1995), an attempt was made to split the single binding site of the labeled ligand into multiple sites for the unlabeled ligand. The results are summarized in Table 3. As seen in this table, further characterization of receptor heterogeneity in the olfactory bulb was obtained from these competitive binding studies. Specifically, competition binding studies with RO23-1590, AHR 14749, CGS 8216 and RO41-7812 all led to the separate identification of the one composite non-selective binding site of [^{3}H]RO15-1788 into two sites corresponding to the α_{1-3} and α_5 subunit-containing receptors. By contrast, zolpidem and flunitrazepam split the single composite RO15-1788 binding site into two different combinations of sites corresponding to α_1 and α_2/α_3 or α_2/α_5 and α_1/α_3 subunit-containing receptors, respectively. In the competition binding assays against [^{3}H]RO15-1788, affinities determined for the various ligands were similar to those determined by competition with [^{3}H]RO15-4513.

These combined results indicate that, as in the rat hippocampus, the rat olfactory bulb contains at least three populations of benzodiazepine binding sites in similar order of relative densities. These correspond to α_1 subunit-containing, α_2/α_3 subunit-containing and α_5 subunit-containing $GABA_A$ receptors. In the olfactory bulb, 10% of the $GABA_A$/benzodiazepine receptor could be attributed to those containing the α_5 subunit, 40% to those containing α_1 and the remaining 50% to receptors containing α_2 and α_3 subunits. Thus, in both brain regions, the density of α_5 subunit containing receptors is the lowest, and the density α_1 subunit-containing receptors is somewhat less that that of α_2/α_3 subunit-containing receptors.

4. Discussion

Heterogeneity of benzodiazepine receptors in the hippocampus and olfactory bulb were characterized by a combination of saturation and competitive binding studies and the use of AFFINITY ANALYSIS SYSTEM (SINGLE LIGAND and COMPETE) for data analysis. The competition software (COMPETE) allows the determination of the affinities and densities of competing cold ligands for multiple binding sites using non-selective radioactive ligands that bind to all the sites with similar affinities. In these studies, the radioligands RO15-4513 and RO15-1788 were used to identify the different benzodiazepine binding sites in hippocampus and olfactory bulb and the affinities of sixteen benzodiazepine ligands for these binding sites determined.

Both hippocampus and olfactory bulb express all the $GABA_A$ receptor α subunits with the exception of α_6. Since the α subunit present in the $GABA_A$ receptor is believed to be a major determinant of the affinity of various ligands to the receptor (Ebert et al., 1997; Prichett et al., 1989b; Wong and Skolnick, 1992), at least five benzodiazepine receptor types could be expressed in each of these tissues. However, most of the radioligands currently available can only distinguish among some of the α subunits. For example, self-competition studies with RO15-1788 could only identify α_{1-3}, α_5 subunit-containing receptors from those containing the $\alpha_{4,6}$ (Scholze et al., 1996). Self-competition studies with RO15-4513 could only distinguish between α_{1-4}, α_6 subunit-containing receptors and α_5 subunit-containing receptors (Liu et al., 1996). Subsequently, binding affinities of fourteen other structurally diverse benzodiazepine receptor ligands at each of these binding sites were measured in these tissues. While most ligands cannot distinguish between α_1, α_2 and α_3 subunit-containing receptors, one ligand used in com-

petition studies, zolpidem, had measurably higher affinity at the α_1, vs. the α_2/α_3 subunit-containing sites. Thus, the combined results of self-competition and competition studies allowed the identification of three different benzodiazepine binding sites in the hippocampus and olfactory bulb corresponding to α_1 subunit-containing, α_2/α_3 subunit-containing and α_5 subunit-containing $GABA_A$ receptors.

While the α_4 subunit of $GABA_A$ receptors is present in both hippocampus (Benke et al., 1997; Sperk et al., 1997) and olfactory bulb (Benke et al., 1997; Laurie et al., 1992), no ligand binding to this receptor was detected in the present study. Specifically, RO15-1788 bound only to one site in self-competition studies and in competition studies against RO15-4513. The lack of RO15-1788 binding to α_4 subunit-containing receptors in these brain regions has two plausible explanations. The first is that the α_4 subunits present in the hippocampus and olfactory bulb do not form any benzodiazepine sensitive $GABA_A$ receptors. The second explanation is that although α_4 subunit is expressed in these tissues, it does not assemble to form functional $GABA_A$ receptors. There is substantial evidence for the first explanation. Based on previous reports, the distribution of α_4 subunits parallels that of the δ subunit in the hippocampus and olfactory bulb (Benke et al., 1997; Sperk et al., 1997). In addition, the δ subunit-containing $GABA_A$ receptors have been shown to be benzodiazepine insensitive (Saxena and Macdonald, 1994; Shivers et al., 1989). Furthermore, the immunoprecipitated α_4 subunit-containing $GABA_A$ receptors show very little RO15-4513 binding (Khan et al., 1996). These results indicate that α_4 subunits assemble with δ subunits, thus forming benzodiazepine insensitive $GABA_A$ receptors.

Receptors containing α_5 subunit were present in both hippocampus and olfactory bulb. In addition, α_{1-3} subunit-containing receptors were found to be present in both of these tissues. Overall, about 20% of all hippocampus benzodiazepine receptors expressed the α_5 subunit, while 30% expressed the α_1 subunit and 50% the α_2/α_3 subunits. This result is in agreement with previous reports that in the hippocampus, more α_2 subunit than α_1 subunit is expressed (Fritschy and Möhler, 1995). In the olfactory bulb, only about 10% of the $GABA_A$ receptors appeared to contain the α_5 subunit while 40% contained the α_1 subunit and 50% contained the α_2/α_3 subunits. In the olfactory bulb, as in the hippocampus, the distribution found provides direct evidence that the pattern of functional $GABA_A$/benzodiazepine receptor is consistent with the expression pattern of $GABA_A$ receptor subunits in this brain region (Fritschy et al., 1992; Fritschy and Möhler, 1995; Sur et al., 1999).

Among the benzodiazepine ligands characterized, zolpidem preferentially bound α_1 subunit-containing receptors (9 ± 6 nM in olfactory bulb and 11.4 ± 4.5 nM in hippocampus) over α_2/α_3 subunit-containing receptors (321 ± 343 nM in olfactory bulb and 442 ± 34 nM in hippocampus) as expected (Fritschy and Möhler, 1995). All other ligands did not distinguish between these three binding sites. These results are consistent with those obtained for zolpidem in transfected cell studies (Hadingham et al., 1993; Liu et al., 1995).

Most of the ligands bound to α_5 subunit-containing receptors with affinities 2–100-fold higher than affinities for the α_{1-3} subunit-containing receptors in both tissues. The only exception was the ligand AHR 14749, which bound to the α_5 subunit-containing receptor with affinities of 10–100-fold lower than that for the α_{1-3} subunit-containing receptors. Overall, the affinities of various ligands for the receptors containing the same subunit were similar in the two tissues.

In summary, the benzodiazepine binding sites present in two rat tissues rich in $GABA_A$ receptors, namely hippocampus and olfactory bulb, were identified using a novel data analysis software. Furthermore, the affinities of various benzodiazepine ligands with diverse structures to these binding sites were determined. These results should be useful in two important ways by aiding in the challenging task of identifying the various functional $GABA_A$ receptors in the central nervous system, and by providing a link between receptor affinities and in vivo activities of the $GABA_A$/benzodiazepine receptor ligands studied. Moreover, the data analysis software, AFFINITY ANALYSIS SYSTEM, will provide a useful tool for further characterization of currently available benzodiazepine ligands and new ligands under development. This software can be successfully used in testing the selectivity of benzodiazepine ligands to the various benzodiazepine binding sites in the brain, thus facilitating the identification of novel therapeutic agents.

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