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AN AUTORADIOGRAPHICAL SATURATION KINETIC STUDY OF THE DIFFERENT BENZODIAZEPINE BINDING SITES IN RAT BRAIN BY USING [^{3}H] FLUNITRAZEPAM AS A RADIOLIGAND

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Abstract—The present study reports on the equilibrium association constant (K_D) and receptor density (B_{max}) values of a number of brain areas from the mesencephalon, cerebral cortex, hippocampus, and cerebellum for the overall benzodiazepine (BZ) binding sites as well as for benzodiazepine binding site subtype 1 (BZ1) and subtype 2 (BZ2), determined by autoradiographical procedures using [^{3}H] flunitrazepam. The differences between BZ1 and BZ2 binding sites were analyzed using the specific BZ1 agonist zolpidem as inhibitor of the radioligand. Statistically significant differences in the affinities of BZ2 with respect to BZ and BZ1 binding sites were mainly found in cortical layers when pK_D (negative logarithms of K_D values) values were compared (ANOVA-SNK test). The distribution of B_{max}, as well as the percentages of BZ1 and BZ2 and Hill coefficients which, surprisingly, are always close to 1 (>0.9) for all the saturation kinetics analyzed, are also described. The possibility of heterogeneity related to anatomical distribution in the different subtypes is discussed.

Key words: GABA; benzodiazepine receptors; BZ1; BZ2; zolpidem; binding

Two pharmacologically different types of central benzodiazepine binding sites, BZ1[‖] and BZ2, have been described by using specific ligands for BZ1 such as the BZ1-selective imidazopyridine hypnotic drug zolpidem [1, 2]. In recent years, a new picture of the GABAR has emerged due to reports of an increasingly higher number of subunits and their different subtypes [3, 4]. On the basis of sequence similarities, 5 different GABAR subunit families (α, β, γ, δ, and ρ) have been identified, most of them having multiple subtypes (α1–6, β1–4, γ1–3, δ, and ρ1–2) [3]. Current understanding of the molecular structure of the GABA-ion channel complex suggests that it is a heteropentameric glycoprotein composed of combinations of multiple polypeptide subunits. Depending on their combination, different affinities of the benzodiazepines for the GABA receptor have been described in recombinant expressed receptors [5, 6]. It should be mentioned, however, that in these receptors flunitrazepam binds α1 = α2 = α3 = α5 [5], which implies that complexes including α6 subunits found exclusively in cerebellum [7] are not labelled with the radioligand used here. *In situ* hybridization studies show that different combinations of GABAR subunit subtypes are present in different neuronal populations throughout the brain [8] and it has become necessary to use a more accurate resolution to distinguish whether differences in affinity of this receptor exist in different brain areas. These data can be obtained by performing autoradiographic saturation studies as reported here, rather than mapping with a single concentration of radioligand.

Only a few saturation studies on tissue sections mounted on slides have been performed with benzodiazepines as radioligands. In most of them, tissue sections are wiped off with filter paper which is then evaluated in a scintillation counter [9, 10]. As far as we know, only one autoradiographic saturation study on [^{3}H] flunitrazepam has been carried out using an image analyzer [11], although a detailed study of the K_D and B_{max} of the different structures, as well as differences between BZ1 and BZ2, were not described therein. However, the autoradiographic distributions of BZ1 and BZ2 are well documented in rodents [9, 12–14], humans and primates [15], mapping at a single concentration with different benzodiazepine radioligands. Thus, to our knowledge, no studies have yet been carried out characterizing [^{3}H] flunitrazepam K_D and B_{max} saturation parameters to total benzodiazepine binding sites, as well as to BZ1 and BZ2 binding sites, in a wide number of brain structures, by means of autoradiography.

METHODS

One group of 10 male Wistar rats aged 28 days (125–130 g) were kept in a room under standard conditions of temperature (20–22°C), light (12-hr light/dark cycle), and feeding [16].

Brains were removed immediately after decapitation and rapidly frozen in liquid nitrogen. Transverse sections 10 μm thick were cut with a cryostat, mounted onto gelatin-coated slides, and stored in a freezer (–20°C) until use.

Benzodiazepine receptors were labelled with concentrations ranging from 0.08 nM to 10 nM of [^{3}H] fluni-

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‖ Abbreviations: BZ, benzodiazepine; BZ1, benzodiazepine binding site subtype 1; BZ2, benzodiazepine binding site subtype 2; CNS, central nervous system; Curio, Ci; GABA, γ-aminobutyric acid; GABAR, GABA/BZ receptors; K_D, equilibrium association constant; pK_D, negative logarithms of K_D values; B_{max}, receptor density.

trazepam (83 Ci/mmol, New England Nuclear, U.K.) in 0.17 M Tris HCl, pH 7.4 at 4°C for 40 min, followed by washing in the same buffer for 2 min and drying at 4°C as previously described [17]. Characterization of benzodiazepine BZ2 binding sites was carried out directly by co-incubating [^{3}H] flunitrazepam with 1 μM zolpidem (RBI Natick, U.S.A.), which specifically binds to benzodiazepine BZ1 binding sites, and the BZ1 binding sites described herein are the differences between [^{3}H] flunitrazepam specific binding and [^{3}H] flunitrazepam specific binding to BZ2 binding sites. To determine the zolpidem concentration required to inhibit [^{3}H] flunitrazepam binding to BZ1 binding sites, incubation of sections in [^{3}H] flunitrazepam either in the absence or presence of increased zolpidem concentrations (0.1 nM to 1 μM) was performed. Nonspecific binding was determined in the presence of 1 μM clonazepam (ROCHE, Basel, Switzerland).

Autoradiograms were generated by exposing the labelled tissue sections to tritium sensitive film (Hyperfilm, Amersham, Little Chalfont, U.K.) for periods ranging from 8 to 240 days.

Optical densities of autoradiographic labelling in the areas studied were determined from autoradiograms using a computerized image analyzer system (VIDAS, Carl Zeiss). Optical density values were converted to nCi bound per mg tissue using suitable autoradiographic standards (Amersham). These values were converted to fmol of [^{3}H] flunitrazepam bound per mg tissue using nonlinear regression between radioactivity standards and their respective optical density values, with the data fitted to a polynomial second-order equation. Consecutive sections were stained with cresyl violet to identify the anatomical areas corresponding approximately to Fig. 26 from the Paxinos and Watson rat brain atlas [18] as well as the cerebellum level.

ANOVA-SNK tests to determine statistical significances among affinities by using pK_D values (negative logarithms of K_D values), the Shapiro-Wilk's W tests for normality, and the *t*-tests were performed using STATISTICA for Windows R 4.5 (StatSoft Inc, 1993). Saturation parameters were determined using the LIGAND computer program (Elsevier-Biosoft). Graphics and curve fittings were made with the SIGMAPLOT V 1.02 program for Windows (Jandel Scientific Corporation, Erkrath, Germany).

RESULTS

The affinities of [^{3}H] flunitrazepam recognition sites in each of the brain structures studied are illustrated in Table 1 and studied areas are shown in Fig. 1. Figure 2 shows the profile of inhibition of [^{3}H] flunitrazepam binding by increased concentrations of either zolpidem or clonazepam in both a BZ1-rich area (cerebellum) and a BZ2-rich area (dentate gyrus). It may be observed that zolpidem concentrations higher than 0.1 μM do not enhance inhibition. Saturation kinetic studies of each area revealed a single affinity for [^{3}H] flunitrazepam. Scatchard plots for BZ, BZ1, and BZ2 binding sites, as well as the Hill plot for BZ binding sites of this ligand in layer L3 of the visual cortex, are shown as examples in Fig. 3 (a–d). In the structures studied, K_D values ranged between 1.17 nM (dentate gyrus) and 3.4 nM (substantia nigra). K_D values ranged between 2.04 nM (deep grey layer of the superior colliculus) and 4.79 nM (layer L1 of the auditory cortex) when zolpidem 1 μM was added to the incubation media. The K_D values for displaceable zolpidem binding sites ranged between 1.12 nM (dentate gyrus) and 2.98 nM (substantia nigra).

The B_{max} values for the [^{3}H] flunitrazepam recognition sites in the different areas studied are shown in Table 1. The highest B_{max} values were obtained in layer L3 of the auditory cortex (340 fmol/mg tissue). Layer L1 of the enthorinal cortex revealed the lowest B_{max} values (219 fmol/mg tissue). After inhibition of radioligand binding with 1 μM zolpidem, the B_{max} values obtained in the different structures ranged between 32 fmol/mg tissue and 174 fmol/mg tissue (substantia nigra and dorsal dentate gyrus, respectively), with the exception of cerebellum and the red nucleus which lacked [^{3}H] flunitrazepam binding under these conditions. The B_{max} values for displaceable zolpidem binding sites ranged between 159 and 308 fmol/mg tissue (layer L1 of the entorhinal cortex and substantia nigra, respectively).

The percentages of displaceable and nondisplaceable zolpidem binding sites are shown in Table 1. The greatest differences among the structures studied appeared in motor function-related areas. Roughly, displaceable zolpidem binding sites appear in high quantities in mesencephalic areas, in a variable range in cortical layers, and at a similar displaceable:nondisplaceable binding site ratio in the hippocampus and superficial grey layer of the superior colliculus.

Because different [^{3}H] flunitrazepam affinities for BZ1 and BZ2 binding sites were observed throughout the brain areas studied, statistical comparisons between [^{3}H] flunitrazepam affinity values for BZ1, BZ2, and total BZ binding sites were carried out using pK_D values. Tests for normality showed that for BZ pK_D values the hypothesis of a Gaussian distribution was rejected ($P < 0.05$). ANOVA analysis followed by the SNK test of the pK_D values were performed in such a way that each structure was first considered as a group before 10 pK_D values per group were considered. From these tests, the only significant differences ($P < 0.05$) found were those between pK_D values of the substantia nigra and geniculate lateral nucleus with respect to almost all the groups. BZ2 as well as BZ1 pK_D values fit Gaussian distributions when tested for normality ($P < 0.05$). ANOVA-SNK analysis carried out by comparing BZ2 pK_D values showed that only the geniculate lateral nucleus presented significant differences ($P < 0.05$) with most areas, with no significant differences found for the remaining structures. ANOVA-SNK analysis comparing BZ1 pK_D values only revealed significant differences ($P < 0.05$) between the superficial grey layer of the superior colliculus and the inner layer of the superior colliculus and the periaqueductal grey matter (ventral area). From *t*-tests, statistically significant differences ($P < 0.05$) were obtained between the pK_D mean values of BZ2 and BZ1 and the pK_D mean values of BZ2 and BZ, whereas no differences were found for pK_D mean values of BZ and BZ1.

When the ANOVA-SNK test was performed by including BZ and BZ2 pK_D values, statistically significant decreases in affinity were found in most cortical areas studied as well as in the dentate gyrus and periaqueductal grey matter (medial area), as shown in Table 1. The ANOVA-SNK test was also performed to compare BZ2 and BZ1 pK_D values and found that only a few cortical structures showed significant differences as shown in

Table 1. [^{3}H]flunitrazepam K_D (nM) and B_{max} (fmol/mg tissue) values for BZ, BZ1, and BZ2 binding sites in areas of cortex, hippocampus, mesencephalon, and lateral geniculate nucleus. Values are indicates as mean ± SEM (standard error mean). $n = 10$.

	K_D			B_{max}			Percentage	
	BZ	BZ2	BZ1	BZ	BZ2	BZ1	%BZ2	%BZ1
1 Cerebellum	1.6 ± 0.4	—	—	266 ± 32	—		0	100
2 Cingulum (OUT. L.)	1.6 ± 0.1	2.9 ± 0.3	1.9 ± 0.2	254 ± 8	72 ± 16	231 ± 16	29	91
3 Cingulum (IN. L.)	1.6 ± 0.2	3.5 ± 0.5**	2.0 ± 0.3	274 ± 12	90 ± 8	239 ± 17	33	87
4 Vis. cortex L1	1.7 ± 0.2	4.3 ± 0.5**	1.7 ± 0.2**	267 ± 14	96 ± 8	202 ± 17	36	76
5 Vis. cortex L2	1.5 ± 0.1	3.9 ± 0.1**	1.4 ± 0.1**	327 ± 11	103 ± 7	265 ± 8	31	81
6 Vis. cortex L3	1.6 ± 0.2	2.4 ± 0.2	1.9 ± 0.3	318 ± 12	85 ± 6	269 ± 21	27	85
7 Aud. cortex L1	1.6 ± 0.2	4.8 ± 0.8**	1.9 ± 0.4**	235 ± 11	104 ± 16	192 ± 21	44	82
8 Aud. cortex L2	1.5 ± 0.1	4.8 ± 0.7**	1.4 ± 0.0**	326 ± 13	135 ± 15	244 ± 7	42	75
9 Aud. Cortex L3	1.6 ± 0.1	4.6 ± 0.6**	1.6 ± 0.1**	340 ± 9	150 ± 20	257 ± 9	44	76
10 Aud. Cortex L4	1.6 ± 0.1	3.8 ± 0.7**	1.6 ± 0.1*	310 ± 14	127 ± 15	233 ± 11	41	75
11 Enth. cortex L1	1.6 ± 0.1	3.8 ± 0.6*	2.0 ± 0.2	219 ± 8	82 ± 13	159 ± 22	38	73
12 Enth. cortex L2	1.9 ± 0.2	4.1 ± 0.5*	1.8 ± 0.2*	285 ± 21	117 ± 12	192 ± 34	41	67
13 Enth. cortex L3	1.8 ± 0.1	4.6 ± 1.0**	1.7 ± 0.1**	313 ± 12	146 ± 17	214 ± 13	47	68
14 Enth. cortex L4	1.7 ± 0.1	3.7 ± 0.4**	1.7 ± 0.1	328 ± 22	151 ± 11	209 ± 24	46	64
15 Subiculum	1.4 ± 0.4	2.6 ± 0.4	2.6 ± 1.1	225 ± 44	107 ± 15	167 ± 66	47	74
16 Alveus (CA1)	1.5 ± 0.2	2.3 ± 0.7	2.2 ± 0.8	235 ± 14	86 ± 23	174 ± 41	47	74
17 Pyramidal (CA1)	1.3 ± 0.0	2.5 ± 0.3	1.5 ± 0.2	248 ± 12	107 ± 11	172 ± 6	43	69
18 Dent. gyr. (D.A.)	1.2 ± 0.0	3.4 ± 0.2**	1.2 ± 0.2	318 ± 9	174 ± 10	222 ± 4	55	70
19 Dent. gyr. (M.A.)	1.2 ± 0.0	2.8 ± 0.6*	1.1 ± 0.2	301 ± 9	154 ± 17	197 ± 5	51	66
20 Dent. gyr. (V.A.)	1.2 ± 0.2	2.8 ± 0.6*	1.1 ± 0.3	286 ± 25	154 ± 17	178 ± 26	54	62
21 Sup. col. (S.G.L.)	1.2 ± 0.1	2.4 ± 1.1	1.2 ± 0.1	303 ± 7	158 ± 28	208 ± 9	52	69
22 Sup. col. (OPT. L.)	1.6 ± 0.1	2.7 ± 1.1	2.2 ± 0.6	276 ± 7	76 ± 21	245 ± 3	28	89
23 Sup. col. (IN. L.)	1.9 ± 0.1	2.0 ± 0.5	2.6 ± 0.5	273 ± 7	69 ± 11	248 ± 18	25	91
24 Per. grey N. (D.A.)	1.9 ± 0.2	3.5 ± 0.3**	2.2 ± 0.3	322 ± 10	73 ± 10	280 ± 14	23	87
25 Per. grey N. (M.A.)	2.4 ± 0.2	2.4 ± 0.6	2.3 ± 0.7	268 ± 9	54 ± 10	210 ± 42	20	78
26 Per. grey M. (V.A.)	2.3 ± 0.5	2.1 ± 0.6	2.7 ± 0.3	267 ± 31	60 ± 14	255 ± 19	22	96
27 Red nucleus	2.6 ± 0.3	—	—	231 ± 34	—	—	0	100
28 Substantia nigra	3.4 ± 0.7	2.7 ± 0.3	3.0 ± 0.9	308 ± 13	32 ± 2	308 ± 12	11	100
29 Lat. gen. nuc.	3.0 ± 0.5	1.6 ± 0.4	2.7 ± 0.6	253 ± 26	38 ± 8	253 ± 48	15	100

The symbol — means that because nonspecific binding was found, no saturation studies could be done. Column BZ2 displays the statistically significant differences (*$P > 0.05$, **$P < 0.01$) when BZ-BZ2 pK_D values are compared following the ANOVA-SNK test. Column BZ1 displays significant differences when BZ2-BZ1 pK_D values are compared using the ANOVA-SNK test. %BZ2 column shows the percentages of BZ2 binding sites obtained directly from zolpidem inhibition data with respect to BZ binding sites. %BZ1 are B_{max} percentages obtained from B_{max} calculated from BZ1 specific binding (indirect data obtained by subtracting BZ and BZ2 binding sites) with respect to BZ binding sites. AUD.: auditory. D.A.: Dorsal area. Dent. gyr.: dentate gyrus. Enth.: entorhinal. IN. L.: Inner layers. L1–L4: Cortical layers corresponding to different autoradiography density bands. Lat. gen. nuc.: Lateral geniculate nucleus. M.A.: Medial area. OPT. L.: Optical layer. OUT. L.: Outer layers. P. G. M.: Periaqueductal grey matter. Sup. col.: Superior colliculus. S.G.L.: Superficial grey layer. V.A.: Ventral area. Vis.: Visual.

Table 1. When the ANOVA-SNK test was performed by including BZ and BZ1 pK_D values, no statistically significant differences were found in any of the structures.

DISCUSSION

The data presented here using [^{3}H] flunitrazepam as a radioligand without inhibition by the BZ1 binding agonist zolpidem point to a single affinity (Hill coefficient > 0.9). Differences in affinity have been reported [10] in some areas of the human CNS (cerebellum, striatum, and frontal cortex). Our data show higher K_D values of [^{3}H] flunitrazepam for BZ binding sites in the mesencephalon, although statistically significant differences were not found among the different brain areas studied, except for the substantia nigra and lateral geniculate nucleus in the thalamus (which differ with respect to almost all the remaining structures). In spite of the discrepancy between these two structures, the findings described here agree with a number of reports on the affinity of this radioligand based on membrane binding saturation kinetic studies [9, 15, 19–21]. The two main differences between autoradiographic techniques and membrane binding are that the former do not use the extraction procedures employed for membrane binding and the quantification procedures are different. The agreement of our results with those obtained with membrane-binding techniques confirms that the results of autoradiographic saturation studies with this radioligand are comparable to those using membrane binding, despite the different procedures. This offers the advantage of greater anatomical accuracy.

To determine the zolpidem concentrations required to inhibit [^{3}H] flunitrazepam binding to BZ1 binding sites, we performed inhibition assays in which similar [^{3}H] flunitrazepam binding values were observed when zolpidem concentrations higher than 0.1 μM were used in BZ2-rich areas, such as the dentate gyrus. In the same way, similar binding values were observed in cerebellum, a BZ1-rich area, when zolpidem concentrations higher than 30 nM were used. When the inhibition profiles of zolpidem found in areas such as the dentate gyrus or superficial grey layer of the superior colliculus were compared to those obtained with clonazepam, which inhibits [^{3}H] flunitrazepam binding to both BZ1 and BZ2 binding sites, it was observed that zolpidem was not able

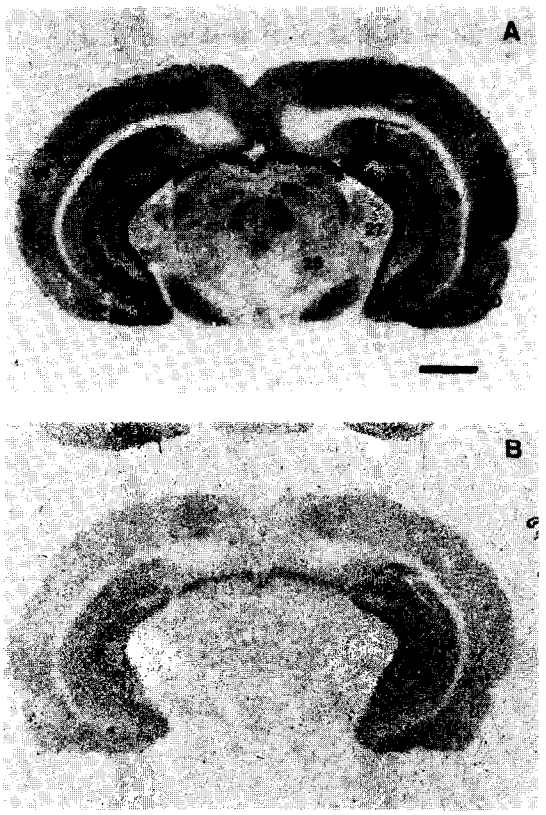

Fig. 1. Autoradiogram obtained using 2 nM [^{3}H] flunitrazepam as radioligand (A) and the specific BZ1 binding site agonist 1 μM zolpidem as inhibitor (B). Transversal level corresponding to Fig. 26 of the Paxinos-Watson Atlas [18]. 1 and 2: Outer and inner layers of the cingulum, respectively; 3, 4, and 5: L1, L2, and L3 visual cortical layers, respectively; 6, 7, 8, and 9: L1, L2, L3, and L4 auditory cortical layers, respectively; 10, 11, and 12: L1, L2, and L3 entorhinal cortical layers, respectively; 13: Subiculum; 14: Alveus in CA1; 15: Piramidal layer in CA4; 16, 17, and 18: Dorsal, medial and ventral areas, respectively, of the dentate gyrus; 19, 20, and 21: Superficial grey, optical, and internal layers, respectively, of the superior colliculus; 22, 23, and 24: Dorsal, medial and ventral areas of the periaqueductal grey matter, respectively; 25: Red nucleus; 26: Substantia nigra; 27: Lateral geniculate nucleus. Bar 1 mm.

to inhibit part of the radioligand binding in these areas but clonazepam was. Nevertheless, in areas such as the cerebellum, a similar degree of binding inhibition could be observed with high zolpidem or clonazepam concentrations (>30 nM). These facts confirm previous results attesting to the presence and distribution of some BZ2-rich areas using these or different ligands [9, 21]. The lack of an enhanced binding inhibition for zolpidem concentrations higher than 0.1 μM in BZ2-rich areas shows that the whole of BZ1 binding is inhibited with this concentration. Because 1 μM zolpidem does not increase the inhibition of [^{3}H] flunitrazepam binding, it seems to be an appropriate concentration to fully inhibit the binding of [^{3}H] flunitrazepam to these receptors.

It is difficult to know whether or not BZ binding sites behave as a population with a single affinity when labelled with this radioligand. Some evidence suggests that BZ binding sites do not constitute such a population. In this regard, we observed that the pK_D values do not fit a Gaussian distribution and that some areas (substantia nigra and lateral geniculate nucleus) show significant differences with respect to almost all the structures studied. From the literature, lower Hill coefficients have been reported in some areas such as the hippocampus when this radioligand is used ($n = 0.6$–0.7) [22, 23], and biphasic association and dissociation plots have been described that correspond to more rapid association and dissociation kinetics of BZ1 and BZ2 receptors [24]. However, as mentioned above, the Hill coefficients close to 1, the linearity of our Scatchard plots, and the lack of differences in affinity in most structures point to a single population with a single affinity. BZ binding sites would then act as an average of BZ1 and BZ2 binding sites and the significant differences in the means of affinities of this radioligand for BZ2 with respect to those of BZ and BZ1, as well as the existence of significant differences in [^{3}H] flunitrazepam affinity for BZ2, BZ1, or BZ binding sites in a number of structures, appear to corroborate this (in spite of the statistical significance observed in some areas, the differences in affinities are so slight that caution should be exercised in their interpretation). If BZ2 binding sites have less affinity than BZ1 for this radioligand, the affinity for BZ should depend on the BZ1/BZ2 density ratio, having more affinity for BZ when BZ1 density was higher. However, this is not so in many structures (see Table 1). A possible explanation for this discrepancy could be the existence of populations of both BZ1 and BZ2 binding sites with differences in affinities to [^{3}H] flunitrazepam, as well as in their relative densities in each structure. However, we were not able to confirm this from our statistical analysis. From our data, it appears that differences in affinity are related to anatomical areas or functional systems, although more specific studies should be carried out to fully elucidate this aspect.

In recent years, the heterogeneous distribution of the GABAR subunits along the CNS as well as the way in which these subunits interact with certain specific ligands have attracted much attention [3, 5, 8]. Site-directed mutagenesis has been used to locate the amino acid responsible for [^{3}H] flunitrazepam binding in the α1, α2, α3, and α5 subunits [25]. Molecular data are in agreement with the anatomical study of affinities reported here. In general terms, we observed lower affinities for BZ2 binding sites in cortical areas than in the hippocampus and mesencephalon and, by contrast, higher affinities for BZ1 (it is to be noted that affinities for BZ1 are calculated indirectly). This would agree with a higher expression of the α1 subunit in the cortex and cerebellum. This subunit has been described as being responsible for BZ1 binding sites when expressed with β and γ subunits in recombinant GABARs [6]. Moreover, zolpidem has a relatively higher affinity for recombinant GABARs containing α1 subtypes, but varies in affinity for GABARs containing other α subtypes when expressed in recombinant GABARs [6]. The way in which different possible configurations of GABAR subunits appear along the CNS is as yet unknown although the described heterogeneous distribution of α subunits [8] could be responsible for the different affinities discussed above. We should also point out that there is an exclusive presence of α6 in cerebellum [7] and that [^{3}H] flunitrazepam does not label this subunit [5]. Additionally, it should be taken into account that BZ1 receptors of cerebellar granular cells are not labelled by [^{3}H] flunitrazepam. Our data suggest that a more accurate pharmacological study together with the emerging molecular

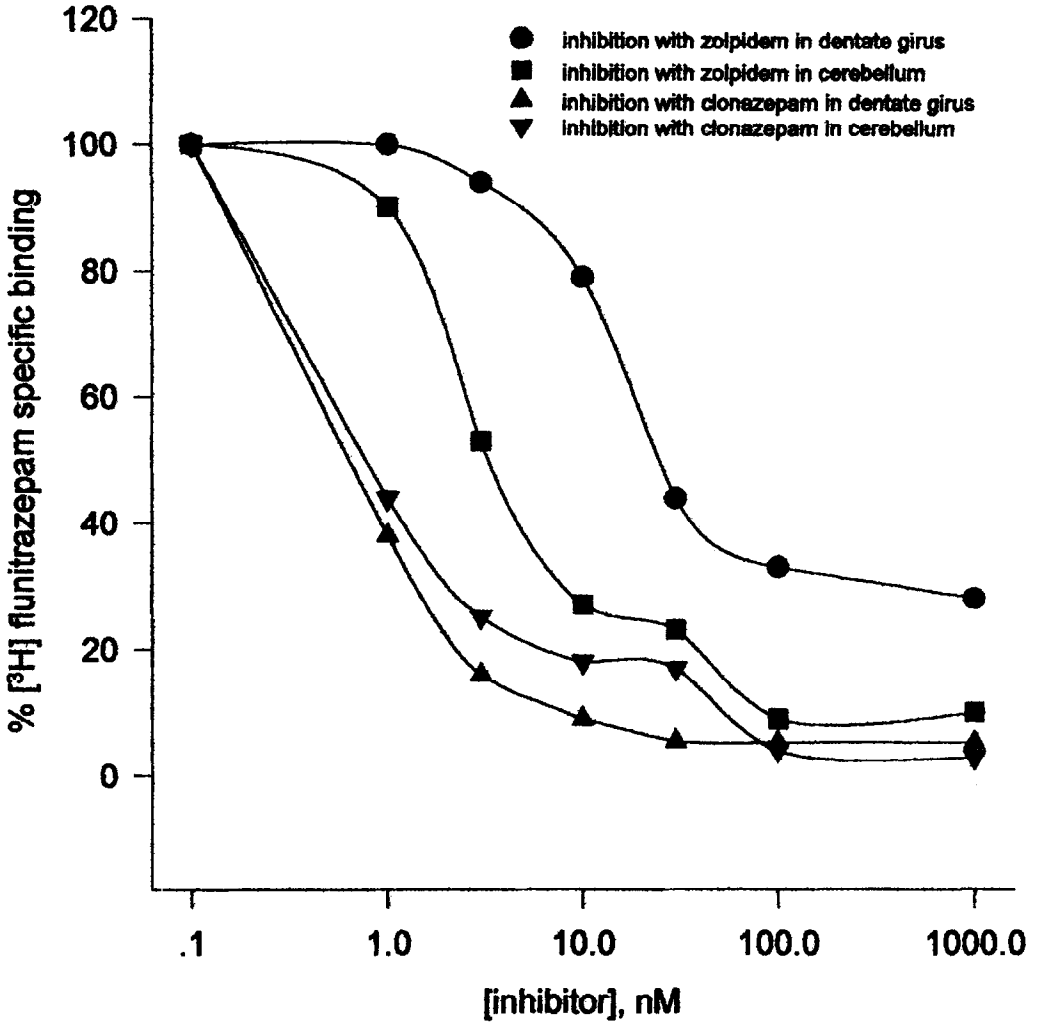

Fig. 2. Competition binding profiles by inhibiting 2 nM [^{3}H] flunitrazepam binding with concentrations ranging from 0.1 nM to 1 μM of the BZ1 specific agonist zolpidem as well as the agonist clonazepam, which inhibits radioligand binding to both BZ1 and BZ2 binding sites. The profile was made in cerebellum, where only BZ1 binding sites are detected, and dentate gyrus, where a higher number of BZ2 binding sites is observed. The plot shows that 1 μM zolpidem inhibits radioligand binding in the cerebellum and BZ2 binding sites of the dentate gyrus remain labelled.

data could contribute to clarifying the distribution of the different GABAR complexes throughout the brain, as well as their putative pharmacological relevance.

It should be stressed that because BZ1 binding sites are calculated indirectly, the data do not match perfectly. Thus, the result obtained from adding the BZ2 and the calculated BZ1 sites is higher than that for the overall BZ binding sites. This is a common problem when a radioligand labels more than one binding site and an inhibitor must be used to discriminate its different binding sites. The inhibitor, usually employed at high concentrations, might affect more than one binding site or might incompletely inhibit the radioligand binding to its binding site when used at low concentrations. Obviously, if this effect were the same for all structures, the affinities for a single binding site should be equally affected and differences in affinities such as those reported here suggest binding site heterogeneity.

The B_{max} values obtained in this study are difficult to compare with those described in the literature because different animal species, radioligands, or techniques have been used. The B_{max} values of [^{3}H] flunitrazepam binding obtained from whole brain membrane homogenates of rat [9] agree with our data in the cerebral cortex and hippocampus. The previous autoradiographic mapping describing BZ1 and BZ2 distribution [15] is roughly confirmed with the B_{max} reported here, in which there are striking differences in the BZ1:BZ2 ratio in different areas of the cortex and the superior colliculus as well as a scarce presence of BZ2 in motor function-related areas such as cerebellum, red nucleus, and substantia nigra. From the BZ2:BZ1 ratio, the BZ2 binding sites seem to play a role in the whole hippocampus, not only in the dentate gyrus. These data confirm the existence of different populations of BZ1 and BZ2 related to different functions or anatomical distributions, supporting the idea of a different role for the two hitherto distinguished pharmacological BZ populations.

As pointed out above, perhaps more than one BZ1 population could be recognized if studies taking into

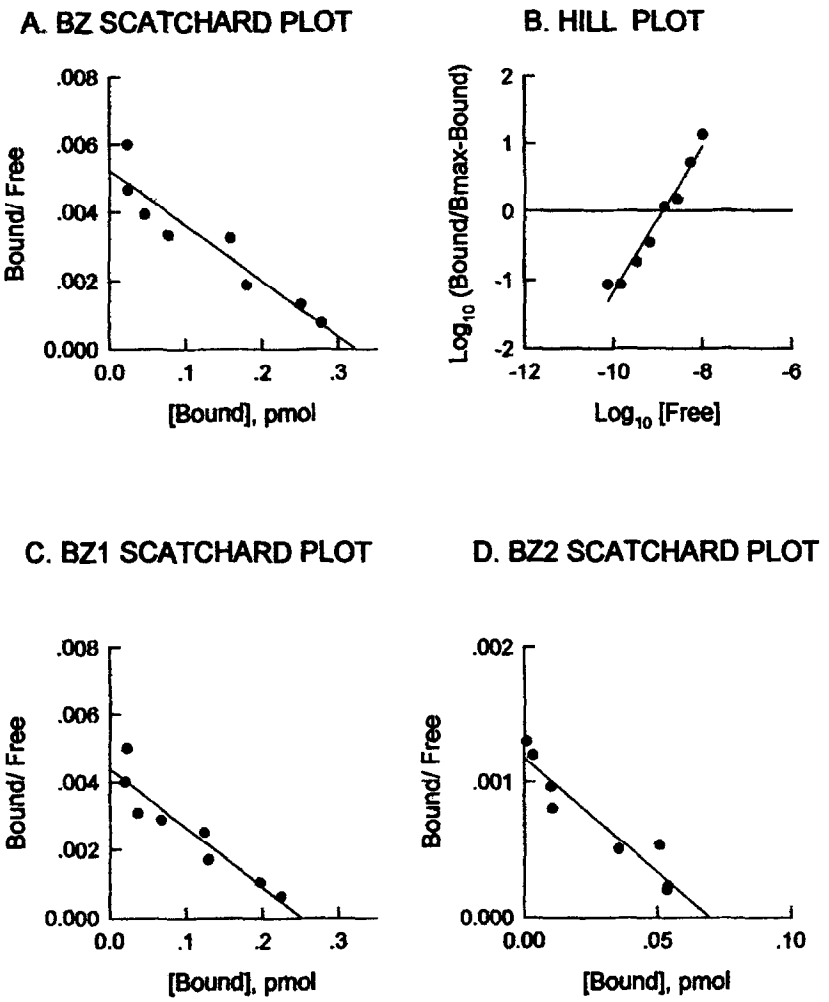

Fig. 3. Scatchard plots for BZ (A), BZ1 (C), and BZ2 (D) binding sites in Layer 3 of the visual cortex; Fig. 3 B displays a Hill plot of the same structure.

account regional differences were performed. For example, in the motor function-related areas described here, the presence of BZ2 is null or very low. The substantia nigra and red nucleus have K_D values similar to those described in the caudate nucleus [21]. However, the cerebellum displayed a somewhat significantly higher affinity than these areas (data not shown), suggesting differences in the BZ1 population. Because BZ1 were studied indirectly, a further study using a specific BZ1 radioligand together with a more accurate distribution of the GABAR subunits would be necessary to determine whether motor function-related areas display differences in the subunit composition of the GABAR complex, as suggested by our data.

Interspecific differences are difficult to analyze owing to the different techniques and areas studied. However, low receptor densities have been described in human and monkey substantia nigra [10, 15, 26], in contrast to those already described in rat [9, 27], and further confirmed in this study. This supports the idea that interspecific differences in the densities of this receptor have existed throughout evolution. Should this hypothesis be confirmed, an interesting field concerning the importance of BZ binding sites in the different brain functional systems throughout evolution would be opened.

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