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Quantitative autoradiography of 4′-ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate binding to the $GABA_A$ receptor complex

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Abstract

4′-Ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate ([^{3}H]EBOB) binding to the $GABA_A$ receptor complex was characterized autoradiographically in rat brain and then its binding in human brain was investigated. [^{3}H]EBOB binding was saturable, specific and identified a single population of binding sites. The K_d obtained from saturation studies was 4.59 nM. Picrotoxin produced dose-dependent inhibition of [^{3}H]EBOB binding and saturation analysis indicated a competitive interaction. Isoguvacine inhibited [^{3}H]EBOB binding with regionally different effects. Bicuculline increased [^{3}H]EBOB binding only in the cerebellar granule cell layer. In human cerebellum, a high level of [^{3}H]EBOB binding sites was seen in the granule cell layer. These results suggest that [^{3}H]EBOB binds to the picrotoxin binding site associated with the $GABA_A$ receptor complex, that regional differences in $GABA_A$ agonist and antagonist modulation of [^{3}H]EBOB binding reflect underlying regional differences in $GABA_A$ receptor subunit composition, and that there is a species difference in $GABA_A$ receptor distribution between human and rat cerebellum.

Keywords: EBOB (4′-ethynyl-4-*n*-propylbicycloorthobenzoate); $GABA_A$ receptor complex; Picrotoxin; Brain, human; Autoradiography

1. Introduction

The $GABA_A$ receptor is the major mediator of synaptic inhibition in the central nervous system and structurally is a hetero-oligomeric protein comprised of five (or fewer) subunits per active complex (Burt and Kamatchi, 1991). Molecular cloning studies have provided evidence for the existence of a number of different subunits forming the $GABA_A$ receptor and each of these subunits has a distinct regional distribution in the brain (Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Lüddens and Wisden, 1991; Doble and Martin, 1992; Wisden et al., 1992). Functionally, the $GABA_A$ receptor incorporates a chloride ionophore as well as binding sites for several classes of ligands which interact allosterically to either potentiate or inhibit receptor function (Burt and Kamatchi, 1991; Sieghart, 1992).

Among the several binding sites of the $GABA_A$ receptor complex, a site of particular interest is the picrotoxin site. In binding studies, picrotoxin ligand binding is regulated by the activation of other sites on the $GABA_A$ receptor complex. $GABA_A$ agonists and zinc inhibit picrotoxin ligand binding (Squires et al., 1983; Lawrence et al., 1985; Edgar and Schwartz, 1990; Kume et al., 1994; Sakurai et al., 1994). Benzodiazepines and neurosteroids have complex effects (Gee et al., 1986, 1988, 1989; Lloyd et al., 1990; Im and Blakeman, 1991; Concas et al., 1990; Sakurai et al., 1994). The regulation of picrotoxin ligand binding by other sites on the $GABA_A$ receptor complex allows picrotoxin ligand binding assays to be used as functional probes of $GABA_A$ receptor function and pharmacology.

The picrotoxin site on the $GABA_A$ receptor complex has been characterized with [^{3}H]α-dihydropicrotoxinin (Ticku et al., 1978), [^{35}S]*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) (Lawrence and Casida,

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1983; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Edgar and Schwartz, 1990) and [^{3}H]*t*-butylbicycloorthobenzoate ([^{3}H]TBOB) (Lawrence et al., 1985; Van Rijn et al., 1990; Sakurai et al., 1994). [^{3}H]α-Dihydropicrotoxinin suffered from low specific binding and low affinity and is no longer used. [^{35}S]TBPS is greatly improved in specific binding and affinity, and has been used most commonly for both homogenate and autoradiographic binding experiments. A disadvantage of [^{35}S]TBPS is the short half-life of the radiolabel and a poorer degree of resolution than tritiated ligands when used for film autoradiography. [^{3}H]TBOB is a less costly alternative to [^{35}S]TBPS and has been used in homogenate and autoradiographic studies. Recently, a new tritiated ligand was introduced, [^{3}H]4′-ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate ([^{3}H]EBOB) (Palmer and Casida, 1991; Deng et al., 1991; Cole and Casida, 1992; Hawkinson and Casida, 1992). EBOB is structurally related to TBOB. The specific binding is approximately 90% of total binding and the reported K_d of [^{3}H]EBOB is approximately 2 nM which is almost one tenth of that of [^{3}H]TBOB (Cole and Casida, 1992). The high affinity, high specific binding, and high specific activity of [^{3}H]EBOB predict that [^{3}H]EBOB is an excellent ligand for autoradiographic studies. Although several investigators have used [^{3}H]EBOB to study $GABA_A$ receptors in brain homogenates, characterization of [^{3}H]EBOB binding for quantitative autoradiography has not been reported.

In this study, we have characterized [^{3}H]EBOB binding to rat brain slices for quantitative autoradiography, determined the effect of compounds acting at the $GABA_A$ receptor complex, and determined the distribution of [^{3}H]EBOB binding sites in the rat brain. In addition, we have investigated [^{3}H]EBOB autoradiography in human brain.

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats (Harlan Labs, Indianapolis, IN, USA; weight, 175–199 g) were decapitated, their brains rapidly removed, mounted on cryotome chunks, and frozen in Lipshaw embedding matrix surrounded by powdered dry ice. 20 μm-thick sections were cut in the horizontal plane on a Lipshaw cryostat. Sections were obtained at the level including the caudo-putamen, and thaw-mounted onto 2 × subbed gelatin-coated slides on a warming plate and stored at −20°C until the time of assay. All assays were performed 24 h after decapitation. Each experiment was run in triplicate.

Six human cerebellar hemispheres from individuals without neurologic diseases were obtained at necropsy, sectioned in a sagittal plane into 1 cm slabs, frozen in crushed dry ice, placed in sealed plastic bags, and stored at −70°C. The average postmortem delay to storage was 17.2 h (range, 7–23 h), and the average age at death was 49.3 years (range, 24–80 years). At the time of assay, slabs were warmed overnight to −20°C and blocks containing the cerebellar cortex were cut out of the slabs. Blocks were mounted on cryotome chunks, and 20 μm-thick sections were cut sagittally on a Lipshow cryostat. Sections were thaw-mounted onto 2 × subbed gelatin-coated slides and stored at −20°C until assay. Assays were run 24 h after sectioning.

2.2. [^{3}H]EBOB binding

Slide-mounted tissue sections were warmed to room temperature and prewashed for 3 × 10 min in buffer (50 mM Tris-HCl + 1 mM EDTA pH 7.4 at 4°C) and dried under a stream of cool air. Binding of [^{3}H]EBOB (2 nM) was carried out in buffer (50 mM Tris-HCl + 120 mM NaCl pH 7.4 at room temperature) for 120 min at room temperature. The incubation was terminated by two 60 min rinses in buffer (50 mM Tris-HCl pH 7.4 at 4°C) at 4°C followed by a brief dip in distilled water. Each slide was then dried under a stream of hot air. Non-specific binding was assessed in the presence of 20 μM picrotoxin. Autoradiograms were generated by apposing the slides to tritium-sensitive film (Hyperfilm, Amersham) in light-tight cassettes along with standards containing known amounts of radioactivity. After 3 weeks, films were developed in Kodak D-19. Ligand binding was quantitated with computer-assisted densitometry using the MCID system (Imaging Research, St. Catherines, Ontario, Canada). To quantify ligand binding density, the optical density of co-exposed standards was determined and a standard curve generated by fitting standard values with a fourth-degree polynomial regression equation. Standards were commercial ^{14}C plastic standards (ARC, St. Louis, MO, USA). Use of the standards and derived standard curve allows conversion of areal optical density to pmol/mg protein values. Areas read included the cortical laminae I-III, lamina IV and laminae V-VI, hippocampal CA1 stratum oriens, CA1 stratum radiatum, CA3 stratum oriens, CA3 stratum radiatum and dentate gyrus, striatum, thalamus (laterodorsal thalamic nucleus and lateral posterior thalamic nucleus), septum, superior colliculus, inferior colliculus, periaqueductal gray matter, and cerebellar molecular and granule cell layers. Ten to twenty readings per area from triplicate sections were averaged.

Optimal prewashing was determined by using 2 different prewashing regimens: 3 × 10 min washed in 50 mM Tris-HCl (pH 7.4 at 4°C), 3 × 10 min washes in 50 mM Tris-HCl + 1 mM EDTA (pH 7.4 at 4°C). Sections were then processed as described above using 2 nM [^{3}H]EBOB.

For association experiments, sections were incubated in assay buffer containing 2 nM [^{3}H]EBOB for 12 time points between 2 and 360 min, and rinsed as described above.

For saturation studies, sections were incubated in 10 concentrations of [^{3}H]EBOB ranging from 10 pM to 10 nM. Non-specific binding was assessed with addition of 200 μM picrotoxin. Films were analyzed as described above and data further analyzed with Scatchard plots.

2.3. Effects of picrotoxin, isoguvacine and bicuculline

To determine the regulation of [^{3}H]EBOB binding by $GABA_A$ ligands, unlabelled drugs were included in the incubation mixture with 2 nM [^{3}H]EBOB. Drugs evaluated included: 1 nM to 10 μM picrotoxin, 1 nM to 100 μM isoguvacine, 1 μM to 1 mM bicuculline methobromide. IC_{50} values for competitors were calculated by log-logit analysis. The interaction of picrotoxin with [^{3}H]EBOB was further examined by performing saturation analysis as described above in the presence of 600 nM picrotoxin.

2.4. Regional distribution of [^{3}H]EBOB binding sites

The regional distribution of [^{3}H]EBOB binding sites was established using horizontal sections cut at the level including the caudo-putamen. Sections were processed for [^{3}H]EBOB autoradiography as described above using 2 nM [^{3}H]EBOB. We compared the regional distribution of [^{3}H]EBOB with that of [^{3}H]-TBOB. [^{3}H]TBOB autoradiography was performed as described previously (Kume et al., 1994; Sakurai et al., 1994) using 20 nM [^{3}H]TBOB. Correlation of the regional distribution of [^{3}H]EBOB binding sites with the regional distribution of [^{3}H]TBOB was determined with linear regression.

2.5. Human cerebellar cortex

Human cerebellar sections were processed for [^{3}H]EBOB autoradigraphy as described above using 2 nM [^{3}H]EBOB. To compare [^{3}H]EBOB binding with [^{3}H]TBOB binding, [^{3}H]TBOB autoradiography was performed as described previously (Kume et al., 1994; Sakurai et al., 1994) using 20 nM [^{3}H]TBOB. In generating autoradiograms, sections were exposed to tritium-sensitive film for 5 weeks.

2.6. Materials

4'-Ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate ([^{3}H]EBOB, specific activity = 50.5 Ci/mmol) was purchased from Dupont New England Nuclear (Boston, MA, USA) and [^{3}H]*t*-butylbicycloorthobenzoate ([^{3}H]-TBOB, specific activity = 25.0 Ci/mmol) from Amersham (Arlington Heights, IL, USA). Isoguvacine was purchased from Cambridge Research Biochemicals (Cambridge, UK). Bicuculline and picrotoxin were purchased from Research Biochemicals (Natick, MA, USA). The remaining reagents were purchased from Sigma Chemicals (St. Louis, MO, USA) and were of the highest possible purity.

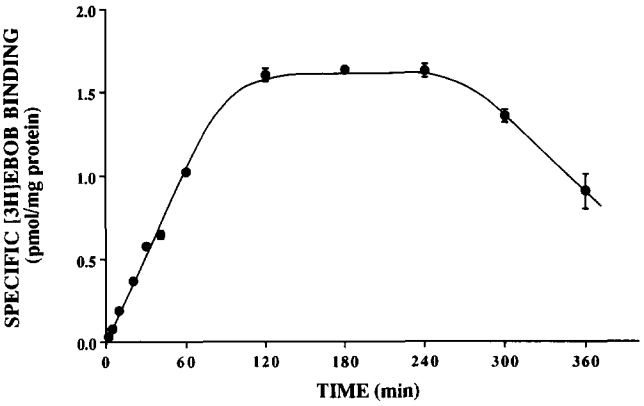

Fig. 1. Association curve for specific [^{3}H]EBOB binding to cortical lamina IV. Rat brain sections were incubated in 2 nM [^{3}H]EBOB for 12 time points and then rinsed for 2×60 min in buffer. Data represent mean ± S.E.M. of 3 animals.

3. Results

3.1. [^{3}H]EBOB binding

Prewashing in buffer containing 1 mM EDTA provided specific [^{3}H]EBOB binding of 1.13 ± 0.077 pmol/mg protein (mean ± S.E.M.) in cortical lamina IV, which is 1.5 times as high as that prewashed in buffer without EDTA. Therefore, in all experiments slices were prewashed in buffer containing 1 mM EDTA. Specific binding of [^{3}H]EBOB was 99% of total binding when sections received 2 × 60 min rinses in 50 mM Tris-HCl (pH 7.4 at 4°C).

In association experiments, specific [^{3}H]EBOB binding increased with increasing incubation time and steady state was reached by 2 h of incubation at room temperature (Fig. 1). Specific binding was stable at incubation times up to 4 h but declined variably after longer incubation times, apparently due to degeneration of the slide-mounted tissue. Therefore, slices were incubated with [^{3}H]EBOB for 2 h in equilibrium-binding experiments.

Saturation experiments showed that specific [^{3}H]-EBOB binding sites were occupied with 7 nM [^{3}H]-EBOB (Fig. 2A). Non-specific binding was linear over the range of [^{3}H]EBOB concentrations used and the percentage of non-specific binding was 3–6% of total binding at 2 nM. Scatchard plot was well fitted to a simple linear regression ($r = -0.923$, $P < 0.001$) and

n_H approximated 1 (Fig. 2B), indicating a single population of binding sites. The binding parameters from experiments with 3 animals yielded a K_d of 4.59 ± 0.47 nM and B_{max} of 4.83 ± 0.13 pmol/mg protein (mean ± S.E.M.) in cortical lamina IV.

3.2. Effects of picrotoxin, isoguvacine and bicuculline

Compounds which modulate the activity of the $GABA_A$ receptor complex modulated binding of [^{3}H]EBOB. Picrotoxin produced dose-dependent inhibition with an IC_{50} of 600 nM and saturation analysis performed in the presence of 600 nM picrotoxin indicated a competitive interaction (Figs. 2B, 3, and 4). The n_H for picrotoxin was 1.40 ± 0.04 (mean ± S.E.M) and significantly greater than 1 ($P < 0.0005$ on t-test). The $GABA_A$ agonist, isoguvacine, also produced dose-dependent inhibition of [^{3}H]EBOB binding in all areas examined, but there were regional differences in isoguvacine inhibition of [^{3}H]EBOB binding (Figs. 3 and 4). IC_{50} values for isoguvacine were significantly different among brain regions ($P < 0.0001$ on ANOVA, Table 1) with IC_{50} values lowest in cortical laminae I-III, hippocampal CA1 stratum radiatum and dentate gyrus, septum and the cerebellar molecular and granule cell layers. Relatively low IC_{50} values were observed also in cortical laminae IV, thalamus, inferior colliculus and periaqueductal gray matter. Relatively high IC_{50} values were found in cortical laminae V-VI, hippocampal CA1 stratum oriens and CA3 stratum oriens, striatum and superior colliculus. Bicuculline produced a regionally specific increase in [^{3}H]EBOB binding that was restricted to the cerebellar granule cell layer. The enhancement of binding by bicuculline was greater in the inner zone of the granule cell layer than in the outer zone of the granule cell layer. In this experiment, we paid particular attention to the regional heterogeneity of allosteric modulation of [^{3}H]EBOB binding in the cerebellar granule cell layer and carefully analyzed the autoradiographs (Fig. 5). In the presence of 50 μM bicuculline, the enhancement of binding was 90% in the outer zone and 244% in the inner zone of the granule cell layer. Significant difference in the enhancement by bicuculline was noted between the two zones ($P < 0.05$ on t-test). Such zonally different modulation of [^{3}H]EBOB binding in the granule cell layer was found to some extent in experiments prewashed in buffer without EDTA, but not in inhibition experiments with picrotoxin. In sections prewashed in buffer without EDTA, [^{3}H]EBOB binding in the molecular layer and outer granule cell layer was reduced relative to binding in the granule cell layer bordering the white matter.

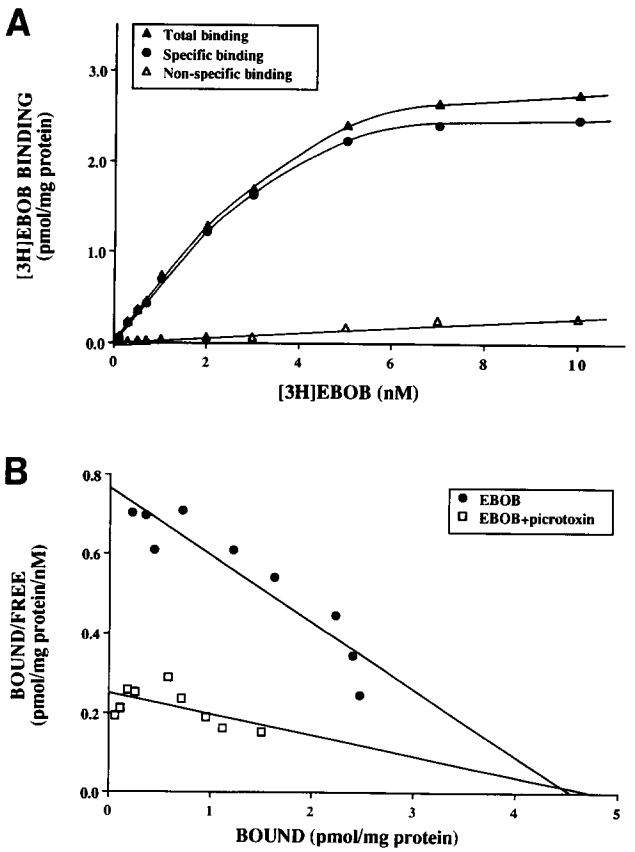

Fig. 2. A: Saturation curves for [^{3}H]EBOB binding to cortical laminae V-VI. Sections were incubated in concentrations of [^{3}H]EBOB ranging from 10 pM to 10 nM for 120 min at room temperature and then rinsed for 2×60 min in buffer at 4°C. Non-specific binding was determined in the presence of 200 μM picrotoxin. The graph shows the result of a typical experiment. B: Scatchard plots of the specific binding data in (A). For the graph, $K_d = 5.90$ nM, $B_{max} = 4.51$ pmol/mg protein and $n_H = 1.01$. In separate experiments, 600 nM picrotoxin was included in the incubation mixture of saturation experiments.

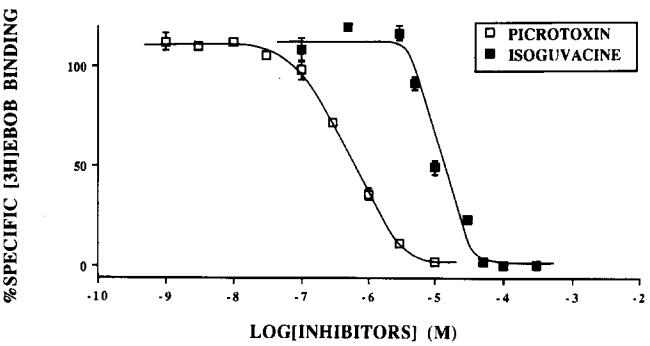

Fig. 3. Inhibition of [^{3}H]EBOB binding to cortical lamina IV by picrotoxin and isoguvacine. Sections were incubated in 2 nM [^{3}H]EBOB in the presence of increasing concentrations of picrotoxin and isoguvacine. For picrotoxin, $IC_{50} = 588 \pm 37$ nM and $n_H = 1.40 \pm 0.04$. For isoguvacine, $IC_{50} = 12.3 \pm 0.97$ μM. Data represent mean ± S.E.M. of 3 animals.

3.3. Regional distribution of [^{3}H]EBOB binding sites

The regional distribution of [^{3}H]EBOB binding sites was heterogeneous (Table 2). High levels of binding

were found in lamina IV of neocortex, the pre/parasubiculum of the hippocampal formation; intermediate levels of binding were found in laminae I-III of neocortex, laminae V-VI of neocortex, most areas of hippocampal formation, superior colliculus, inferior colliculus, periaqueductal gray matter, the cerebellar molecular layer; and low levels of binding in septum, striatum, thalamus, the cerebellar granule cell layer. This distribution pattern was virtually identical with that of [^{3}H]TBOB binding sites. Correlation of the regional distribution of [^{3}H]EBOB binding sites with the regional distribution of [^{3}H]TBOB binding sites revealed significant correlation (Fig. 6).

3.4. Human cerebellar cortex

Specific [^{3}H]EBOB binding sites were found within the human cerebellar cortex (Fig. 7). The distribution of binding sites was heterogeneous, with higher levels of binding in the granule cell layer (mean 0.44 ± 0.026 (S.E.M.) pmol/mg protein) and lower levels in the molecular layer (0.060 ± 0.0086 pmol/mg protein). Highest levels of binding were seen in the granule cell layer bordering the white matter (0.62 ± 0.044 pmol/mg protein) and specific binding was 94% of total binding. The distribution of [^{3}H]EBOB binding in human cerebellar cortex was different from that found in rat cerebellar cortex, where [^{3}H]EBOB binding was always higher in the molecular layer than in the granule cell layer. While [^{3}H]EBOB demonstrated a high specific binding in human brain tissue, specific [^{3}H]TBOB binding to the human cerebellar cortex was quite low and sometimes indistinguishable from its non-specific binding (data not shown).

4. Discussion

4.1. [^{3}H]EBOB binding

[^{3}H]EBOB is a suitable radioligand for quantitative autoradiography of picrotoxin binding sites. The EDTA prewash has been shown to be more efficient in removing endogeneous tissue GABA than conventional pre-

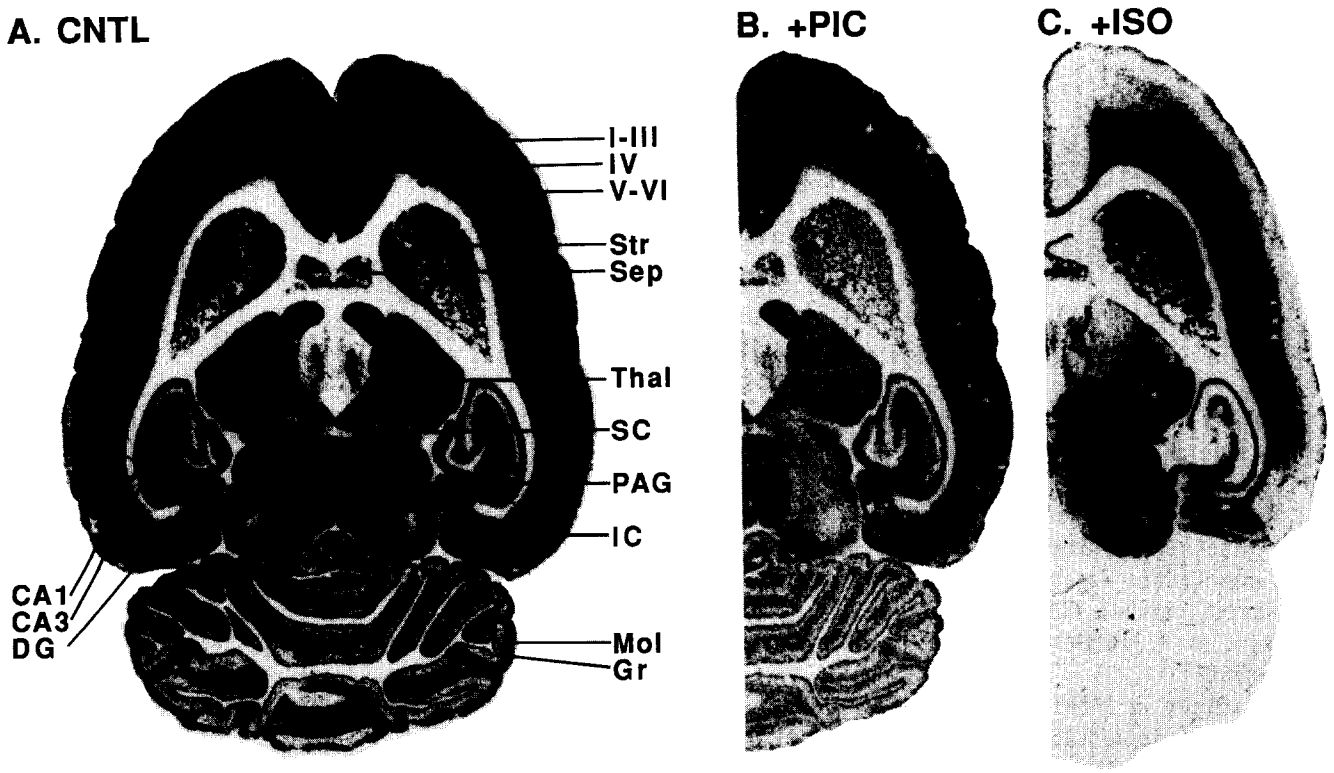

Fig. 4. Autoradiographs of [^{3}H]EBOB binding to horizontal section of rat brain without modulation (A), in the presence of 600 nM picrotoxin (B) and in the presence of 10 μM isoguvacine (C). Sections were incubated in 2 nM [^{3}H]EBOB. Co-incubation with 600 nM picrotoxin decreased [^{3}H]EBOB binding to the cortical laminae I-III, laminae V-VI, hipocampal CA1 stratum oriens, inferior colliculus and cerebellar molecular layer by 51, 55, 55, 64, 64%, respectively. Co-incubation with 10 μM isoguvacine decreased the binding by 96, 35, 16, 43, 100%, respectively. Picrotoxin displaced to a similar extent all of the [^{3}H]EBOB bound in all of the brain regions, but there were regional differences in isoguvacine inhibition of [^{3}H]EBOB binding. I-III: cortical laminae I-III, IV: cortical lamina IV, V-VI: cortical laminae V-VI, CA1: CA1 stratum oriens, CA3: CA3 stratum oriens, CNTL: control, DG: dentate gyrus, Gr: cerebellar granule cell layer, IC: inferior colliculus, ISO: isoguvacine, Mol: cerebellar molecular layer, PAG: periaqueductal gray matter, PIC: picrotoxin, SC: superior colliculus, Sep: septum, Str: striatum, Thal: thalamus.

Table 1
Inhibition of [^{3}H]EBOB binding in various brain regions by isoguvacine

Brain region	IC_{50} (μM)
With high sensitivity to isoguvacine	
Neocortex	
Laminae I-III	4.1±0.7
Hippocampus	
CA1 stratum radiatum	3.4±0.7
Dentate gyrus	4.6±0.5
Septum	1.1±0.3
Cerebellum	
Molecular layer	1.7±0.5
Granule cell layer	
Outer zone	0.8±0.1
Inner zone	0.6±0.2
With intermediate sensitivity to isoguvacine	
Neocortex	
Lamina IV	12.0±1.9
Hippocampus	
CA3 stratum radiatum	8.5±1.0
Pre/para subiculum	8.2±0.5
Thalamus	7.8±1.2
Inferior colliculus	12.7±1.2
Periaqueductal gray matter	9.0±1.1
With low sensitivity to isoguvacine	
Neocortex	
Laminae V-VI	17.6±0.9
Hippocampus	
CA1 stratum oriens	27.9±4.6
CA3 stratum oriens	15.3±2.0
Striatum	23.1±2.7
Superior colliculus	24.0±2.4

IC_{50} values were derived from inhibition curves with isoguvacine. Data are mean±S.E.M. values from experiments with $n = 3$ animals. A significant difference of the IC_{50} values among regions is noted on one-way ANOVA ($P < 0.0001$).

washes and has been shown previously to be necessary for [^{35}S]TBPS autoradiography (Edgar and Schwartz, 1990; Sapp et al., 1992) and [^{3}H]TBOB autoradiography (Sakurai et al., 1994). Likewise, EDTA prewash is necessary for optimal [^{3}H]EBOB autoradiography.

In homogenate studies, investigators have used incubation times ranging from 60 to 90 min, but the time to reach equilibrium was not indicated. Our association experiments indicate that an incubation time of 2 h is necessary for [^{3}H]EBOB binding to slices to reach equilibrium.

The affinity of [^{3}H]EBOB for slide-mounted sections was determined according to saturation analysis; $K_d = 4.59 \pm 0.47$ nM in cortical lamina IV. Our observed K_d values are somewhat higher than that found in the homogenate binding using animal and human brains (Cole and Casida, 1992; Hawkinson and Casida, 1992). Differences in K_d values may reflect differences in binding condition between studies.

As compared with reported K_d values of other picrotoxin ligands, our observed K_d values of [^{3}H]-EBOB is 4- to 5-fold lower than those of [^{35}S]TBPS (Squires et al., 1983; Edgar and Schwartz, 1990), 2- to 10-fold lower than those of [^{3}H]TBOB (Lawrence et al., 1985; Van Rijn et al., 1990) and virtually 6-fold lower than those of [^{3}H]TBOB obtained previously in our laboratory (Kume et al., 1994; Sakurai et al., 1994). Use of [^{3}H]EBOB confers some advantages for quanti-

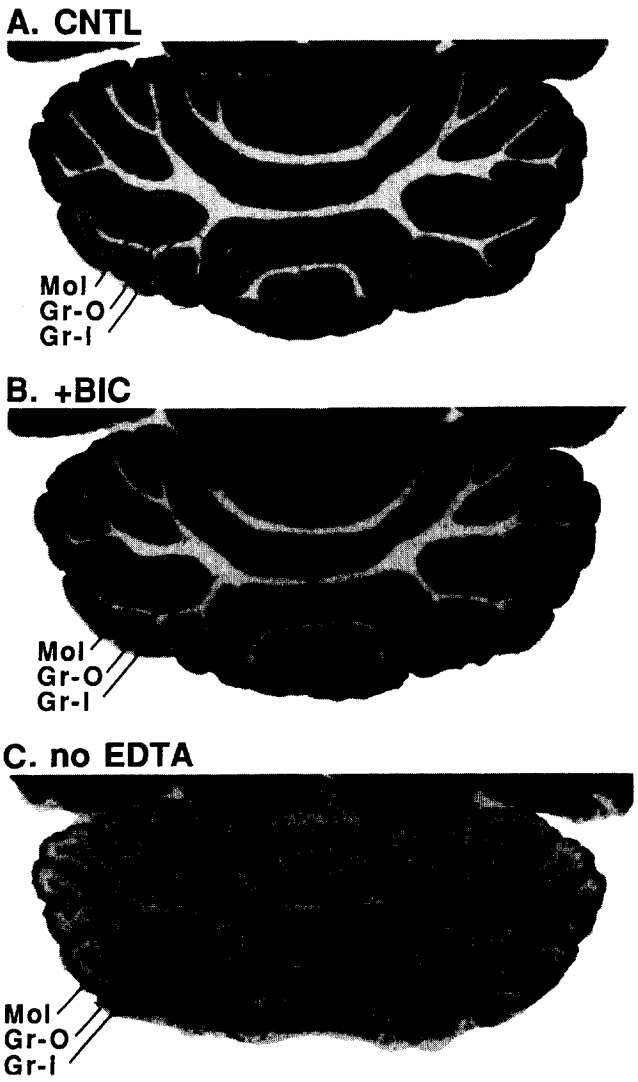

Fig. 5. [^{3}H]EBOB binding to the rat cerebellum without modulation (A), in the presence of 50 μM bicuculline (B), and prewashed with buffer without EDTA (C). All sections were incubated in 2 nM [^{3}H]EBOB and then rinsed for 2×60 min in buffer. In control sections prewashed with buffer containing 1 mM EDTA, density of [^{3}H]EBOB binding was homogeneous through the granule cell layer (A). However, in sections co-incubated with bicuculline and sections prewashed with buffer without EDTA, [^{3}H]EBOB binding differed within the granule cell layer. Co-incubation with 50 μM bicuculline enhanced [^{3}H]EBOB binding to the granule cell layer and significantly more so in the inner zone (B). In sections prewashed with buffer without EDTA, [^{3}H]EBOB binding in the outer zone of the granule cell layer was reduced relative to the binding in the inner zone bordering the white matter (C). BIC: bicuculline, CNTL: control, Gr-I: inner zone of the granule cell layer, Gr-O: outer zone of the granule cell layer, Mol: molecular layer.

Table 2
Regional distribution of [^{3}H]EBOB binding to rat brain sections

Brain region	Specific [^{3}H]EBOB binding (pmol/mg protein)		
	Mean ($n = 3$)	S.E.M.	Relative to cortical lamina IV (%)
Neocortex			
Laminae I-III	1.23	0.012	86
Lamina IV	1.42	0.058	100
Laminae V-VI	1.12	0.042	79
Hippocampus			
CA1 str. oriens	0.91	0.012	64
CA1 str. radiatum	0.68	0.033	48
CA3 str. oriens	0.91	0.019	64
CA3 str. radiarum	0.84	0.022	59
Dentate gyrus	1.00	0.024	70
Pre/para subiculum	1.38	0.015	97
Septum	0.52	0.040	37
Striatum	0.48	0.029	34
Thalamus			
Laterodorsal thal. ncl.	0.61	0.044	43
Latral post. thal. ncl.	0.61	0.039	43
Superior colliculus	0.96	0.046	68
Inferior colliculus	1.17	0.091	82
Periaqueductal gray matter	1.07	0.033	75
Cerebellum			
Molecular layer	0.85	0.040	60
Granular cell layer			
Outer zone	0.23	0.011	16
Inner zone	0.27	0.026	19

Data represent mean ± S.E.M. of 3 animals. Sections were incubated in 2 nM [^{3}H]EBOB for 120 min at room temperature and then rinsed for 2×60 min in 50 mM Tris-HCl buffer (pH 7.4 at 4°C). Quantification of autoradiograms was performed as described in the text. A significant difference of the specific [^{3}H]EBOB binding among regions is noted on one-way ANOVA ($P < 0.0001$).

tative autoradiography. First, a proper rinse provides an optimal autoradiographic image with a high contrast to the background because specific, high-affinity binding dissociates little while most of non-specific binding

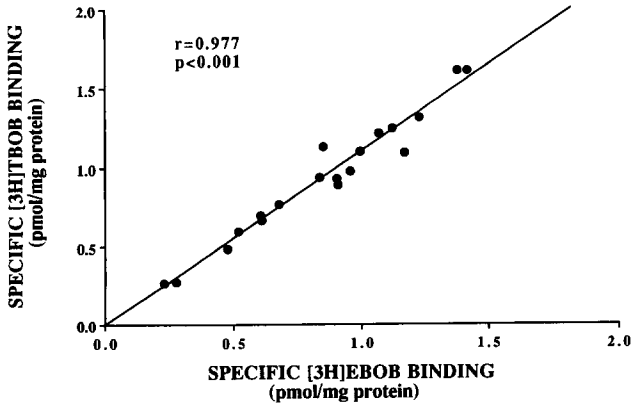

Fig. 6. Correlation of [^{3}H]EBOB binding sites with [^{3}H]TBOB was determined with linear regression.

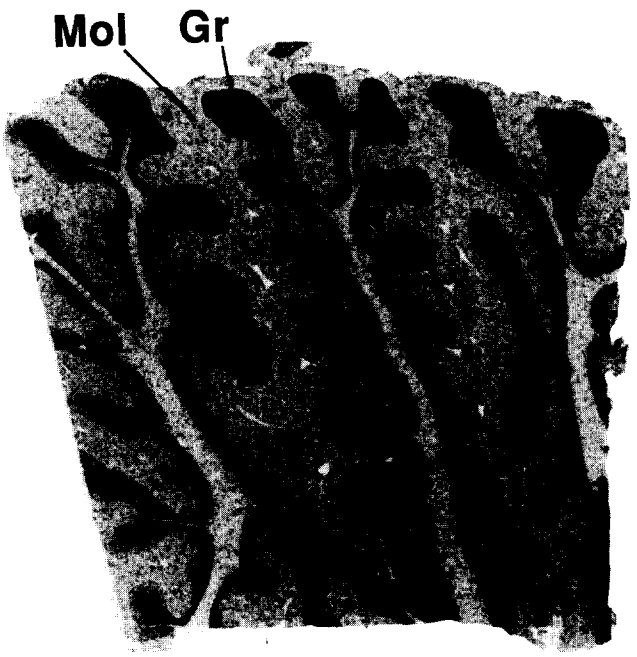

Fig. 7. Autoradiograph of [^{3}H]EBOB binding to human cerebellar cortex. Sections were incubated in 2 nM [^{3}H]EBOB and then rinsed for 2×60 min. The human sections were apposed to film for 5 weeks in order to obtain a clear visible image. There was a high density of [^{3}H]EBOB binding sites in the granule cell layer, and the highest density was seen in the inner zone bordering the white matter. Gr: granule cell layer, Mol: molecular layer.

dissociates rapidly. The high quality autoradiograph makes it easier to analyze small brain structures and to measure subtle changes in binding levels. Second, the high-affinity, high-specific-activity radioligand reduces the amount necessary for each experiment. Therefore, [^{3}H]EBOB is more ecconomical than [^{3}H]TBOB and [^{35}S]TBPS, particularly in a large series of pharmacological experiments and saturation studies. Third, [^{3}H]EBOB showed a high specific binding to human brain slices as well as rat brain slices (discussed below). [^{3}H]EBOB makes it possible to study human brain picrotoxin binding sites.

4.2. Effects of picrotoxin, isoguvacine and bicuculline

Previous binding studies have indicated that unlabeled EBOB inhibits [^{35}S]TBPS binding to the picrotoxin site (Hawkinson and Casida, 1992) and [^{3}H]EBOB binding is inhibited by a number of unlabeled compounds acting at the picrotoxin site, such as picrotoxin, lindane, TBPS and dithianes (Deng et al., 1991; Cole and Casida, 1992). Scatchard analysis demonstrated that lindane and picrotoxin are competitive inhibitors of [^{3}H]EBOB binding in the insect brain (Deng et al., 1991). In the present study, we observed that [^{3}H]-EBOB binding to the rat brain slices is inhibited by picrotoxin in a competitive manner. Our results sup-

port the conclusion that [^{3}H]EBOB binds to the picrotoxin site associated with the $GABA_A$ receptor complex.

We observed regional differences in allosteric effect by $GABA_A$ compounds on [^{3}H]EBOB binding. Isoguvacine, a $GABA_A$ agonist, inhibited [^{3}H]EBOB binding in all brain regions but there was a regional difference in inhibitory effect by isoguvacine. Bicuculline, a $GABA_A$ antagonist, enchanced [^{3}H]EBOB binding only to the cerebellar granule cell layer while picrotoxin uniformly inhibited its binding. Regionally different effects of allosteric modulators on picrotoxin ligand binding have been reported with [^{35}S]TBPS and [^{3}H]-TBOB (Gee et al., 1988; Sapp et al., 1992; Korpi et al., 1992; Korpi and Lüddens, 1993; Kume et al., 1994; Sakurai et al., 1994). The pharmacological and molecular biological evidence for regional heterogeneity of the $GABA_A$ receptor complex has been widely reported and regional/cellular differences in the expression of $GABA_A$ receptor subunit isoforms probably underlie, at least in part, regional/cellular differences in receptor function and pharmacology (Olsen and Tobin, 1990; Burt and Kamatchi, 1991). For example, the cerebellar granule cells have a unique collection of the $GABA_A$ receptor subunits (Shivers et al., 1989; Lüddens et al., 1990) and they are the single type of neuron expressing the $\alpha 6$ subunit in adult rat brain (Laurie et al., 1992a, b; Thompson et al., 1992). Korpi and Lüddens (1993) correlated the $GABA_A$ pharmacology in rat brain sections with the properties of recombinant receptors expressed from various subunit cDNAs selected on the basis of their regional expression, and provided evidence that a greater GABA sensitivity of [^{35}S]TBPS binding in the cerebellar granule cells is inherent to $\alpha 6$ subunit-containing receptors. In this study, we observed bicuculline enchancement of [^{3}H]EBOB binding only in the cerebellar granule cell layer. This property, a differential coupling of bicuculline sites and [^{3}H]EBOB binding sites in cerebellar granule cells, is probably determined by the expression of $\alpha 6$ subunit conferring exceptional sensitivity to the effects of the small amount of GABA remaining in sections after prewashes (Korpi and Lüddens, 1993). Thus, the regional difference in $GABA_A$ agonist/antagonist effects on [^{3}H]EBOB binding may well reflect the regional heterogeneity of $GABA_A$ isoforms.

It is interesting that the cerebellar granule cell layer showed regional heterogeneity of $GABA_A$ agonist/antagonist modulation of [^{3}H]EBOB binding despite its anatomically homogeneous appearance. This was first noted by Edgar and Schwartz (1990) and confirmed by Korpi and Lüddens (1993). They observed that GABA inhibition of [^{35}S]TBPS binding was relatively less potent in the inner zone of the cerebellar granule cell layer. We observed the same phenomenon with [^{3}H]EBOB binding. Bicuculline enhanced [^{3}H]-EBOB binding to the inner zone twice as much as to the outer zone. In sections prewashed with buffers without EDTA, where endogeneous GABA remains and decreases [^{3}H]EBOB binding, [^{3}H]EBOB binding in the outer zone of the granule cell layer was reduced relative to binding in the inner zone. These findings suggest that GABA and $GABA_A$ antagonist regulation on [^{3}H]EBOB binding sites differs with location within the granule cell layer, although all granule cells are histologically similar. A recent in situ hybridization study indicated the presence of six subunit mRNAs ($\alpha 1$, $\alpha 6$, $\beta 2$, $\beta 3$, $\gamma 2$, δ) in the rat cerebellar granule cells and suggested the existence of at least two subtypes of pentameric $GABA_A$ receptors on the granule cells or subtypes of granule cells expressing different $GABA_A$ receptors (Laurie et al., 1992a). A recent immunocytochemical study with antibodies for several $GABA_A$ receptor subunits on cultured cerebellar granule cells predict two subtypes of the $GABA_A$ receptor with heteropentameric structure ($\alpha\alpha\beta\beta\gamma 2$, $\alpha\alpha\beta\beta\delta$ – Caruncho et al., 1993). The existence of 2 different subtypes of $GABA_A$ receptor in granule cells may bring about differential GABA and GABA antagonist effects on [^{3}H]EBOB binding in the granule cell layer.

During development, granule cells are generated in the external granular layer of the cerebellum and migrate to their adult position where they will then elaborate dendrites which will become postsynaptic to the axon of the Golgi cells (Altman, 1972a,b; Rakic, 1971). Golgi cells form inhibitory GABAergic synapses with granule cells in synaptic glomeruli (Palay and Chan-Palay, 1974). As granule cells enter the granule cell layer, they form an outside-to-inside gradient with the early generated cells settling superficial to the newly arriving cells. The developing granule cells express $GABA_A$ receptor subunits only after they have achieved their adult position and elaborated dendrites (Meinecke and Rakic, 1990; Gambarana et al., 1990; Laurie et al., 1992b; Zheng et al., 1993). Therefore, in granule cells there probably exists some zonal difference of GABAergic synapse formation and expression of the $GABA_A$ receptor complex.

4.3. Regional distribution of [^{3}H]EBOB binding sites

Reported regional distributions of picrotoxin ligand binding sites, such as [^{35}S]TBPS and [^{3}H]TBOB binding sites (Gee et al., 1983; Lawrence et al., 1985; Edgar and Schwartz, 1990; Olsen et al., 1990; Sakurai et al., 1994), are heterogeneous. Our quantitative autoradiography demonstrated also regional heterogeneity of [^{3}H]EBOB binding sites. The regional distribution pattern of [^{3}H]EBOB binding sites was virtually identical with that of [^{3}H]TBOB binding sites. This suggests that [^{3}H]EBOB binds exactly to the [^{3}H]TBOB binding site and that determinants of [^{3}H]EBOB binding are iden-

tical with those of [^{3}H]TBOB binding. In prior studies, correlation of regional distribution of [^{3}H]TBOB binding sites with those of [^{3}H]zolpidem (Sakurai et al., 1994) and [^{3}H]2-oxo-quazepam (Olsen et al., 1990) suggested that regional expression of benzodiazepine type I binding and [^{3}H]TBOB binding have common underlying determinants. Sakurai et al. (1994) found a number of similarities between regional distribution of [^{3}H]TBOB binding sites and that of α1 subunit mRNA determined by in situ hybridization, and suggested that the presence of α1 subunit is an important determinant of [^{3}H]TBOB binding.

Although homogenate binding experiments have indicated that TBPS and TBOB act at the same population of binding sites (Lawrence et al., 1985), autoradiographic studies revealed that the regional distribution of [^{3}H]TBOB binding sites was somewhat different from that of [^{35}S]TBPS binding sites (Olsen et al., 1990; Sakurai et al., 1994). In this experiment, we confirmed a similar discord of the distribution of ligand binding sites between [^{3}H]EBOB autoradiography and previously reported [^{35}S]TBPS autoradiography (Edgar and Schwartz, 1990; Kume et al., unpublished data). TBPS, TBOB and EBOB are insecticides with a high toxicity and structurally consist of trioxabicyclooctane and X-substituents (Palmer and Casida, 1991; Casida, 1993; Hawkinson and Casida, 1993). The structure-toxicity relationships of trioxabicyclooctanes has revealed a discontinuous size-activity correlation for the X-substituents, i.e., high toxicity is conferred by small and large substituents and low toxicity by those of intermediate size (Milbrath et al., 1979; Palmer and Casida, 1988), and two distinct binding sites were therefore proposed: one for compounds with small X-substituents such as TBPS and one for trioxabicyclooctanes with large X-substituents such as TBOB and EBOB. The proposal of two subtypes of binding sites for trioxabicyclooctanes is consistent with our results of correlation analysis among [^{35}S]TBPS, [^{3}H]TBOB and [^{3}H]EBOB binding sites. The hypothesis of subtypes of picrotoxin binding sites is also consistent with our picrotoxin competition data. The n_H of the picrotoxin displacement curve was significantly greater than 1, which is consistent with the existence of multiple picrotoxin binding sites, although positive cooperativity between identical sites is also possible.

4.4. Human cerebellar cortex

Autoradiographic studies of the $GABA_A$ receptor complex in human cerebellum have reported localization of GABA and benzodiazepine binding sites in normal and diseased conditions (Young and Kuhar, 1979; Whitehouse et al., 1986; Albin and Gilman, 1990; Price et al., 1993). However, localization of picrotoxin binding sites in human cerebellum is not yet published, probably because of the abscence of suitable radioligands for autoradiographic study. The commonly used radioligand, [^{3}H]TBOB, did not make suitable autoradiographs in this experiment. In contrast, [^{3}H]EBOB binding displayed excellent results with a high specific binding in human cerebellar cortex. A prior homogenate binding study revealed that [^{3}H]EBOB has a specific binding for human brain tissue as high as or higher than that for rodent brain tissue and the affinity of [^{3}H]EBOB is more than 10-fold higher than that of [^{3}H]TBOB (Cole and Casida, 1992). Those results are consistent with our data. The high affinity and high specific binding of [^{3}H]EBOB make it possible to perform autoradiographic studies of picrotoxin binding sites in human brain.

We observed a species difference in distribution of [^{3}H]EBOB binding sites. In rat cerebellum, there is a high density of [^{3}H]EBOB binding sites in the molecular layer and a relatively low but significant density in the granule cell layer. In the human cerebellum, there was a high density of [^{3}H]EBOB binding sites in the granule cell layer, but there was a markedly reduced level of binding sites in the molecular layer. Similar species difference in receptor distribution in the cerebellum is reported for the benzodiazepine site of the $GABA_A$ receptor complex (Young and Kuhar, 1979; Albin and Gilman, 1990), where human brain shows a high density of benzodiazepine site in association with both the molecular layer and the granule cell layer while in the rat cerebellum there is a high density in the molecular layer but a markedly reduced level in the granule cell layer. Species difference in receptor distribution may reflect species differences in $GABA_A$ receptors in the cerebellar cortex.

In addition, we observed a zonally arranged difference in the level of [^{3}H]EBOB binding sites in the human cerebellar granule cell layer. The highest density was seen in the inner zone bordering the white matter. This finding is similar to that found in the rat cerebellar granule cell layer. As described above, we suppose some zonal difference in GABAergic synapse formation and expression of the $GABA_A$ receptor complex in the rat cerebellar granule cell layer. Like rat cerebellar granule cells, human granule cells are generated in the external granular layer of the cerebellum, migrate to their adult position and then mature (Rakic and Sidman, 1970). A similar zonal difference of GABAergic synapse formation and expression of the $GABA_A$ receptor complex may underlie the zonally arranged difference in density of [^{3}H]EBOB binding site in human cerebellar granule cells.

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