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 ([3H]EBOB) binding to the GABA_A receptor complex was characterized autoradiographically in rat brain and then its binding in human brain was investigated. [3H]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 [3H]EBOB binding and saturation analysis indicated a competitive interaction. Isoguvacine inhibited [3H]EBOB binding with regionally different effects. Bicuculline increased [3H]EBOB binding only in the cerebellar granule cell layer. In human cerebellum, a high level of [3H]EBOB binding sites was seen in the granule cell layer. These results suggest that [3H]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 [3H]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 [3H]α-dihydropicrotoxinin (Ticku et al., 1978), [35S]l-butylbicycloguanylylphosphorothionate ([35S]TBPS) (Lawrence and Casida,
2.1. Subjects

Kd of [3H]EBOB is approximately 2 nM which is without neurologic diseases were obtained at necropsy, were cut in the horizontal plane on a Lipshaw cryostat. chunks, and frozen in Lipshaw embedding matrix sur-
mogenates, characterization of [3H]EBOB binding for (Palmer and Casida, 1991; Deng et al., 1991; Cole and
mographic binding experiments. A disadvantage of [35S]TBPS is greatly improved in specific binding and affinity, and has been used most commonly for both homogeneate and autoradiographic studies. Recently, a new tritiated ligand was introduced, [3H]4’-ethynyl-4-n-[2,3-3H2]propylbicycloorthobenzoate ([3H]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 Kd of [3H]EBOB is approximately 2 nM which is almost one tenth of that of [3H]TBOB (Cole and Casida, 1992). The high affinity, high specific binding, and high specific activity of [3H]EBOB predict that [3H]EBOB is an excellent ligand for autoradiographic studies. Although several investigators have used [3H]EBOB to study GABA_A receptors in brain homogenates, characterization of [3H]EBOB binding for quantitative autoradiography has not been reported.

In this study, we have characterized [3H]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 [3H]EBOB binding sites in the rat brain. In addition, we have investigated [3H]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 x 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 x subbed gelatin-coated slides and stored at -20°C until assay. Assays were run 24 h after sectioning.

2.2. [3H]EBOB Binding

Slide-mounted tissue sections were warmed to room temperature and prewashed for 3 x 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 [3H]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 opposing 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 14C 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 (lateral dorsal thalamic nucleus and lateral posterior thalamic nucleus), septum, superior colliculus, inferior colliculus, periaque ductal 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 x 10 min washed in 50 mM Tris-HCl (pH 7.4 at 4°C), 3 x 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 [3H]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 \(\mu M\) 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 \(\mu M\) picrotoxin, 1 nM to 100 \(\mu M\) isoguvacine, 1 \(\mu M\) 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 \([^{3}H]EBOB\) autoradiography 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-\(\pi\)-[2,3-\(^{3}H\)_2]propylbicycloorthobenzoate (\([^{3}H]EBOB\), specific activity = 50.5 Ci/mmoll) was purchased from Dupont New England Nuclear (Boston, MA, USA) and \([^{3}H]r\)-butylbicycloorthobenzoate (\([^{3}H]TBOB\), specific activity = 25.0 Ci/mmoll) from Amer-

sham (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.

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 $4.59 \pm 0.47$ nM and $B_{\text{max}}$ of $4.83 \pm 0.13$ pmol/mg protein (mean $\pm$ 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 $[^3H]$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 $1.40 \pm 0.04$ (mean $\pm$ 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 $[^3H]$EBOB binding in all areas examined, but there were regional differences in isoguvacine inhibition of $[^3H]$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, stratum and superior colliculus. Bicuculline produced a regionally specific increase in $[^3H]$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 $[^3H]$EBOB binding in the cerebellar granule cell layer and carefully analyzed the autoradiographs (Fig. 5). In the presence of 50 $\mu$M 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 $[^3H]$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, $[^3H]$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.

### 3.3. Regional distribution of $[^3H]$EBOB binding sites

The regional distribution of $[^3H]$EBOB binding sites was heterogeneous (Table 2). High levels of binding
were found in lamina IV of neocortex, the pre/para-subiculum 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 \([3H]TBOB\) binding sites. Correlation of the regional distribution of \([3H]EBOB\) binding sites with the regional distribution of \([3H]TBOB\) binding sites revealed significant correlation (Fig. 6).

3.4. Human cerebellar cortex

Specific \([3H]EBOB\) 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).

4. Discussion

4.1. \([3H]EBOB\) binding

\([3H]EBOB\) 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-wash.
Table 1
Inhibition of [3H]EBOB binding in various brain regions by isoguvacine

<table>
<thead>
<tr>
<th>Brain region</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>With high sensitivity to isoguvacine</strong></td>
<td></td>
</tr>
<tr>
<td>Neocortex</td>
<td></td>
</tr>
<tr>
<td>Laminae I-III</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>CA1 stratum radiatum</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Septum</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
</tr>
<tr>
<td>Molecular layer</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>Granule cell layer</td>
<td></td>
</tr>
<tr>
<td>Outer zone</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Inner zone</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td><strong>With intermediate sensitivity to isoguvacine</strong></td>
<td></td>
</tr>
<tr>
<td>Neocortex</td>
<td></td>
</tr>
<tr>
<td>Lamina IV</td>
<td>12.0 ± 1.9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>CA3 stratum radiatum</td>
<td>8.5 ± 1.0</td>
</tr>
<tr>
<td>Pre/para subiculum</td>
<td>8.2 ± 0.5</td>
</tr>
<tr>
<td>Thalamus</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>Periaqueductal gray matter</td>
<td>9.0 ± 1.1</td>
</tr>
<tr>
<td><strong>With low sensitivity to isoguvacine</strong></td>
<td></td>
</tr>
<tr>
<td>Neocortex</td>
<td></td>
</tr>
<tr>
<td>Laminae V-VI</td>
<td>17.6 ± 0.9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>CA1 stratum oriens</td>
<td>27.9 ± 4.6</td>
</tr>
<tr>
<td>CA3 stratum oriens</td>
<td>15.3 ± 2.0</td>
</tr>
<tr>
<td>Striatum</td>
<td>23.1 ± 2.7</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>24.0 ± 2.4</td>
</tr>
</tbody>
</table>

IC50 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 IC50 values among regions is noted on one-way ANOVA (P < 0.0001).

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

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

The affinity of [3H]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 [3H]-

Fig. 5. [3H]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 [3H]EBOB and then rinsed for 2×60 min in buffer. In control sections prewashed with buffer containing 1 mM EDTA, density of [3H]EBOB binding was homogeneous through the granule cell layer (A). However, in sections co-incubated with bicuculline and sections prewashed with buffer without EDTA, [3H]EBOB binding differed within the granule cell layer. Co-incubation with 50 μM bicuculline enhanced [3H]EBOB binding to the granule cell layer and significantly more so in the inner zone (B). In sections prewashed with buffer without EDTA, [3H]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

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

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).

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 economical than \([^{3}H]TBBOB\) 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-

Fig. 6. Correlation of \([^{3}H]EBOB\) binding sites with \([^{3}H]TBBOB\) 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.
port the conclusion that \[^3\text{H}\]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 \[^3\text{H}\]EBOB binding. Isoguvacine, a GABA\(_A\) agonist, inhibited \[^3\text{H}\]EBOB binding in all brain regions but there was a regional difference in inhibitory effect by isoguvacine. Bicuculline, a GABA\(_A\) antagonist, enhanced \[^3\text{H}\]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 \[^35\text{S}\]TBPS and \[^3\text{H}\]TBOB (Gee et al., 1983; 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 \(\alpha6\) 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 \[^35\text{S}\]TBPS binding in the cerebellar granule cells is inherent to \(\alpha6\) subunit-containing receptors. In this study, we observed bicuculline enhancement of \[^3\text{H}\]EBOB binding only in the cerebellar granule cell layer. This property, a differential coupling of bicuculline sites and \[^3\text{H}\]EBOB binding sites in cerebellar granule cells, is probably determined by the expression of \(\alpha6\) 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 \[^3\text{H}\]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 \[^3\text{H}\]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 \[^35\text{S}\]TBPS binding was relatively less potent in the inner zone of the cerebellar granule cell layer. We observed the same phenomenon with \[^3\text{H}\]EBOB binding. Bicuculline enhanced \[^3\text{H}\]-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 \[^3\text{H}\]EBOB binding, \[^3\text{H}\]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 \[^3\text{H}\]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 (\(\alpha1, \alpha6, \beta2, \beta3, \gamma2, \delta\)) 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 (\(\alpha\alpha\beta\beta\gamma\delta\), \(\alpha\alpha\beta\beta\delta\delta\) – 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 \[^3\text{H}\]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; Gambara 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 \[^3\text{H}\]EBOB binding sites

Reported regional distributions of picrotoxin ligand binding sites, such as \[^35\text{S}\]TBPS and \[^3\text{H}\]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 \[^3\text{H}\]EBOB binding sites. The regional distribution pattern of \[^3\text{H}\]EBOB binding sites was virtually identical with that of \[^3\text{H}\]TBOB binding sites. This suggests that \[^3\text{H}\]EBOB binds exactly to the \[^3\text{H}\]TBOB binding site and that determinants of \[^3\text{H}\]EBOB binding are iden-
tical with those of [3H]TBOB binding. In prior studies, correlation of regional distribution of [3H]TBOB binding sites with those of [3H]zolpidem (Sakurai et al., 1994) and [3H]2-oxo-quazepam (Olsen et al., 1990) suggested that regional expression of benzodiazepine type I binding and [3H]TBOB binding have common underlying determinants. Sakurai et al. (1994) found a number of similarities between regional distribution of [3H]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 [3H]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 [3H]TBOB binding sites was somewhat different from that of [35S]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 [3H]EBOB autoradiography and previously reported [35S]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 [35S]TBPS, [3H]TBOB and [3H]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 absence of suitable radioligands for autoradiographic study. The commonly used radioligand, [3H]TBOB, did not make suitable autoradiographs in this experiment. In contrast, [3H]EBOB binding displayed excellent results with a high specific binding in human cerebellar cortex. A prior homogenate binding study revealed that [3H]EBOB has a specific binding for human brain tissue as high or higher than that for rodent brain tissue and the affinity of [3H]EBOB is more than 10-fold higher than that of [3H]TBOB (Cole and Casida, 1992). Those results are consistent with our data. The high affinity and high specific binding of [3H]EBOB make it possible to perform autoradiographic studies of picrotoxin binding sites in human brain.

We observed a species difference in distribution of [3H]EBOB binding sites. In rat cerebellum, there is a high density of [3H]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 [3H]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 [3H]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 [3H]EBOB binding site in human cerebellar granule cells.

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