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Localization and Characterization of ^{35}S-t-Butylbicyclophosphorothionate Binding in Rat Brain: An Autoradiographic Study

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^{35}S-t-butylbicyclophosphorothionate (TBPS) binding to slide-mounted rat brain sections was characterized for subsequent autoradiographic analysis. Cortical brain mash slices, preincubated with EDTA to remove endogenous GABA, were used for biochemical characterization. Steady state for ^{35}S-TBPS binding was reached by 3 hr of incubation at 22°C. The association rate constant (K_1) and dissociation rate constant (K_2) were 0.377 min^{-1}μM^{-1} and 0.011 min^{-1}, respectively. Dissociation was monophasic and slow ($t_{1/2}$ = 80 min). The kinetically derived K_D was 29.4 nM. Scatchard analysis indicated a single population of binding sites with a K_D of 21.0 ± 2.2 nM and a B_{max} of 1.59 ± 0.13 pmol/mg protein. Both picrotoxin and muscimol inhibited ^{35}S-TBPS binding completely with IC_{50}s of 251 ± 13 nM and 203 ± 41 nM and n_Hs of 0.98 and 1.4, respectively. The distribution of ^{35}S-TBPS binding sites in the rat brain resembles that of other ligands that bind to GABA$_A$ receptor complex with some regionally specific differences. Regions with a high degree of ^{35}S-TBPS binding included the inferior colliculus, medial septal nucleus, central and paracentral nuclei of the thalamus, olfactory tubercle, zona incerta, dentate gyrus, and substantia nigra. ^{35}S-TBPS preferentially bound to the molecular vs granular layer of the cerebellum. Omission of the preincubation markedly but variably decreased ^{35}S-TBPS binding. The greatest regional decreases occurred in areas with a high degree of GABA synthesis. In addition, ^{35}S-TBPS binding was inhibited to different degrees in the cell layers of the cerebellum. The addition of 1 μM GABA to the incubation medium of preincubated slices also produced variable decreases in ^{35}S-TBPS binding to cerebellar layers. These findings support previous studies that demonstrate GABA$_A$ receptor heterogeneity. Our study confirms the suitability of ^{35}S-TBPS for use as a ligand in autoradiography and demonstrates that the distribution of ^{35}S-TBPS binding sites is significantly influenced by the preincubation–incubation conditions used.

GABA, a major inhibitory neurotransmitter in brain, binds to the GABA$_A$ receptor and activates membrane chloride permeability to produce neuronal inhibition. The GABA$_A$ receptor is a multimeric protein complex containing a chloride channel that is opened when GABA occupies the GABA recognition site. In addition, the GABA$_A$ receptor complex contains recognition sites for benzodiazepines, barbiturates, and cage convulsants, such as picrotoxin and t-butylbicyclophosphorothionate (TBPS; for review, see Schwartz, 1988). Binding studies in brain membrane homogenates have used ^{3}H-ligands for the GABA$_A$ and benzodiazepine recognition sites, and these ligands have also been used to localize GABA$_A$ receptors in brain using receptor autoradiography (Unnerstall et al., 1981; Wamsley et al., 1983; Olsen et al., 1984; Richards et al., 1988). The ^{35}S-labeled cage convulsant, TBPS, has been reported to be a suitable probe for the GABA-gated chloride channel in brain membrane homogenates (Lawrence and Casida, 1983; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984). Since very short film exposure times can be used for the ^{35}S-labeled brain slices, ^{35}S-TBPS is also an attractive ligand for autoradiographic studies of the GABA-gated chloride channel. Quantitative *in vitro* receptor autoradiography is a highly useful technique for determining the location and density of binding sites in brain (Young and Kuhar, 1979). Thus, several investigators have used ^{35}S-TBPS to label GABA$_A$ receptors in brain slices for receptor autoradiography (Wamsley et al., 1983; Concas et al., 1986; McCabe and Wamsley, 1986), although a characterization of ^{35}S-TBPS binding to slide-mounted sections has not been reported. In addition, there is limited information on the distribution and quantitation of ^{35}S-TBPS sites in brain using computerized densitometry. In this study, we have characterized ^{35}S-TBPS binding to rat brain slices for autoradiographic analysis and determined the effect of removing endogenous GABA. In addition, we have provided quantitative data for the distribution of ^{35}S-TBPS sites throughout the rat brain.

Materials and Methods

Animal preparation. Male Sprague-Dawley rats weighing 150–200 gm were housed under standard laboratory conditions and given access to food and water *ad libitum*. After decapitation, brains were rapidly removed for the preparation of cerebral cortical brain mash slices or slide-mounted brain sections.

Cortical brain mash slice preparation. Brain mash slices were prepared according to the method of Rothman et al. (1983) for use in the biochemical experiments. Cortex was dissected free of white matter on ice and finely minced with a razor blade. Minced cortex was placed in a 12 × 75 mm polypropylene tube, centrifuged at 3000 rpm (4°C, 30 sec) and frozen in isopentane at −30°C for 10 min. The tube was placed in water at 22°C for 1 min, and the resulting "sausage" was removed and stored on dry ice until slicing. After mounting the mash preparation on a cryostat chuck, 20 μm slices were cut at −14°C and thaw-mounted on gelatin-coated glass slides. Slides were placed in a vacuum desiccator overnight at 4°C and then stored at −70°C until the date of assay.

Biochemical characterization of ^{35}S-TBPS binding. Before assay, cortical mash slices were thawed and preincubated (10 min, 22°C) in buffer containing 1 mM EDTA to remove endogenous GABA (Squires et al.,

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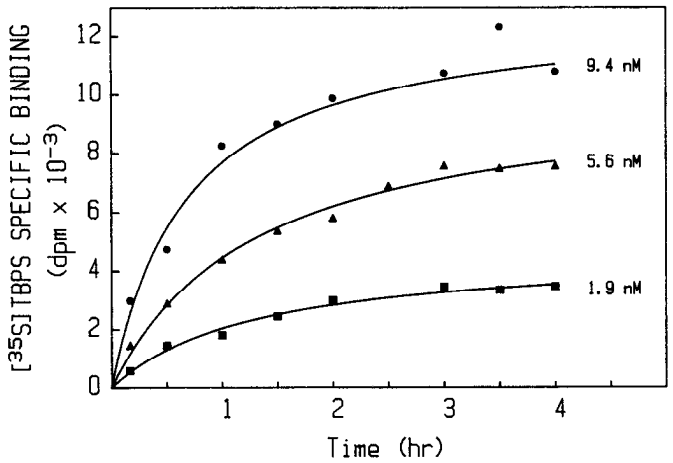

Figure 1. Association rates of ^{35}S-TBPS binding to slide-mounted cortical brain mash slices. After a 10-min preincubation in buffer containing 1 mM EDTA, slices were incubated in three concentrations of ^{35}S-TBPS for up to 4 hr at 22°C. Slices were washed, dried, and scraped for liquid scintillation counting as described in Materials and Methods. Each data point represents the mean dpm from 3 slices, and each curve represents a typical experiment.

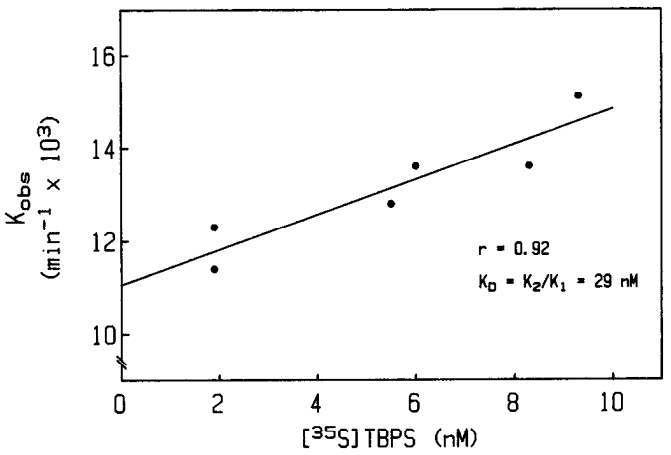

Figure 2. Determination of the ^{35}S-TBPS equilibrium dissociation constant (K_D) from observed association rates (K_{obs}) in slide-mounted cortical brain mash tissue. The K_{obs} value for each set of association data was obtained as the slope of the ln-linear transformation $\ln([*DR]_{eq}/([*DR]_{eq}-[*DR]))$/time (not shown). Each data point represents the K_{obs} value obtained from one experiment. The association rate constant (K_1) is 0.377 $min^{-1}\mu M^{-1}$ (determined from the slope value), and the dissociation rate constant (K_2) is 0.011 min^{-1} (determined from the y-intercept value).

1983). In certain experiments, the preincubation step was omitted. The buffer composition was 50 mM K_2HPO_4/NaH_2PO_4 and 200 mM NaCl, pH 7.4. Slices were then incubated in polyethylene mailers containing buffer (no EDTA) and 2 nM ^{35}S-TBPS (65–100 Ci/mmol, New England Nuclear, Boston, MA) for 3 hr (22°C) unless otherwise noted. Nonspecific binding was determined in the presence of 100 μM picrotoxin. In biochemical experiments, nonspecific binding was 2–4% of total binding, whereas in autoradiographic experiments nonspecific binding was not detectable. The incubation was terminated with two 15-min washes in buffer (22°C) and one dip in distilled water. After being dried under a stream of cool air, individual slices were scraped from the slide and placed in scintillation cocktail (toluene, triton X-100, PPO, and POPOP) for counting by liquid scintillation spectroscopy (Beckman LS 3801; 80% efficiency). The protein content of brain mash slices was determined by the method of Lowry et al. (1951).

Autoradiography. Sagittal and coronal slide-mounted brain sections were prepared as previously described by Herkenham and Pert (1982). After removal, the brain was immersed in isopentane (−40°C, 20 sec) and placed in dry ice until mounting on the cryostat chuck. Slices (20 μm) were cut, mounted, and stored as described for cortical brain mash slices.

Autoradiographic sections were assayed as described for biochemical experiments. Dried sections were then apposed to Hyperfilm-Betamax (Amersham, Arlington Heights, IL) for 2–3 days at 22°C unless otherwise noted. Autoradiograms were developed using Kodak developer D-19 and Kodak fixer. The corresponding slices were stained with cresyl violet for histological examination.

Data analysis. Autoradiographic images were digitized and analyzed using an Amersham RAS 1000 computer-assisted image analysis system. Standards were prepared by incubating cortical brain mash slices with various concentrations of ^{35}S-TBPS. After washing and drying, the standards were either scraped for scintillation counting or apposed to film along with brain sections for autoradiography. The standard curve relating the fmol/mg protein of ^{35}S-TBPS bound to the optical density was generated by the Loats image analysis program and was described by a power function. The fmol ^{35}S-TBPS bound/mg protein in each brain region was divided by the fmol ^{35}S-TBPS bound/mg protein in white matter of the same brain slice in order to control for variations across the film or between cassettes (Schwartz, 1986).

Results

Biochemical characterization of ^{35}S-TBPS binding

The kinetic and steady-state characteristics of ^{35}S-TBPS binding were determined using slide-mounted slices of cerebral cortical mashes. After a 10-min preincubation, specific ^{35}S-TBPS binding increased with increasing incubation times. Steady state was reached by 3 hr of incubation at 22°C (Fig. 1). Specific binding was stable at incubation times of up to 4 hr but declined variably after longer incubation times, apparently due to degeneration of the slide-mounted tissue. Therefore, slices were incubated with ^{35}S-TBPS for 3 hr in equilibrium-binding experiments. Association experiments were carried out using three ^{35}S-TBPS concentrations (Fig. 1). The K_{obs} value for each set of association data was determined as the slope of the ln-linear transformation $\ln([*DR]_{eq}/([*DR]_{eq} - [*DR]))$/time (not shown). The K_{obs} value obtained at each ^{35}S-TBPS concentration was then plotted against the ^{35}S-TBPS concentration to determine the values for the association (K_1) and dissociation (K_2) rate constants (Fig. 2). From the graph, K_1 = 0.377 $min^{-1}\mu M^{-1}$ and K_2 = 0.011 min^{-1}. The kinetically derived equilibrium dissociation constant (K_D) was 29.4 nM.

The K_2 value also was obtained from dissociation experiments. Slices were incubated with ^{35}S-TBPS (1.9 nM), and after 3 hr, dissociation was initiated by adding 100 μM picrotoxin. The K_2 values were obtained as the slope of the plot $\ln([B]/[B]_0)$ vs time (Fig. 3). Dissociation was slow and monophasic. The mean K_2 determined from three experiments was 0.008 ± 0.001 min^{-1}, corresponding to a dissociation half-time ($t_{1/2}$) of 88 min.

Saturation experiments performed at equilibrium indicated that nearly all ^{35}S-TBPS binding sites were occupied with 110 nM ^{35}S-TBPS (Fig. 4*A*). Nonspecific binding (determined in the presence of 100 μM picrotoxin) was linear over the range of ^{35}S-TBPS concentrations used and was 2–4% of the total binding at 2 nM. Scatchard analysis of the saturation data (Fig. 4*B*) indicated a single population of binding sites with a K_D of 21.0 ± 2.2 nM (n = 3). This agrees with the value calculated from the kinetic data. Scatchard analysis also revealed the density of ^{35}S-TBPS binding sites (B_{max}) in cortical tissue to be 1.59 ± 0.13 pmol/mg protein.

Inhibition studies using the $GABA_A$ agonist muscimol and the antagonist picrotoxin showed that both compounds were able to completely inhibit ^{35}S-TBPS (1.9 nM) binding in the slide-

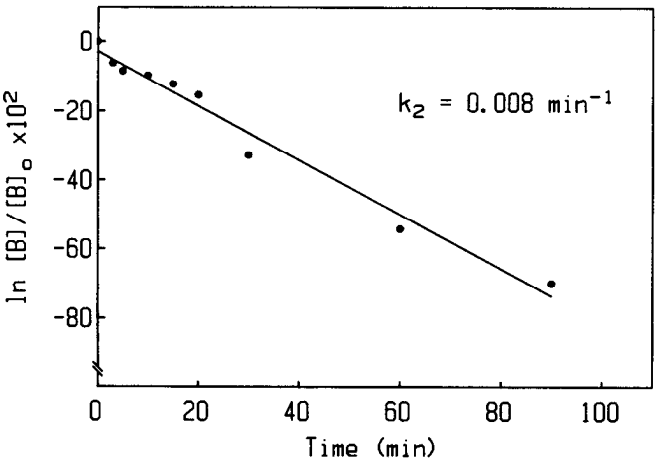

Figure 3. Rate of ^{35}S-TBPS dissociation from cortical brain mash slices. After slices had been incubated with 1.9 nM ^{35}S-TBPS (3 hr, 22°C), dissociation was initiated by the addition of 100 μM picrotoxin. Slices were washed, dried, and counted as described in Materials and Methods. B_0 represents the ^{35}S-TBPS bound immediately before the addition of picrotoxin. B represents the ^{35}S-TBPS bound at each time point after the addition of picrotoxin. The dissociation constant (K_2) was determined from the graph using the equation K_2 = slope/−2.303. The graph shows the results of a typical experiment, which was replicated twice.

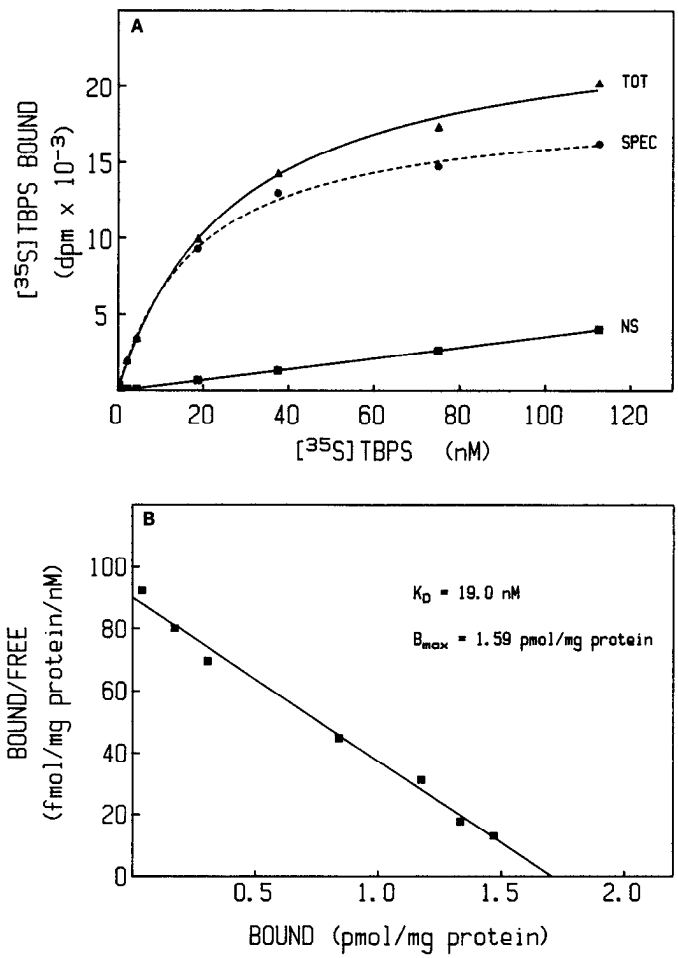

Figure 4. *A*, Saturation analysis of ^{35}S-TBPS binding to slide-mounted cortical brain mash slices. Preincubated slices were incubated with ^{35}S-TBPS (1–110 nM) for 3 hr at 22°C. Nonspecific binding was determined in the presence of 100 μM picrotoxin. Slices were washed, dried, scraped, and counted as described in Materials and Methods. Nonspecific dpm were subtracted from total dpm to determine specific dpm. The graph shows results of a typical experiment, which was replicated twice. *B*, Scatchard analysis of the specific binding data in *A*. The mean ± SEM of 3 experiments indicated a K_D of 21.0 ± 2.2 nM and a B_{max} of 1.59 ± 0.13 pmol/mg protein.

mounted sections (Fig. 5). Muscimol inhibited ^{35}S-TBPS binding with an IC_{50} of 203 ± 41 nM and a Hill coefficient (n_H) of 1.4. Picrotoxin inhibited ^{35}S-TBPS binding with an IC_{50} of 251 ± 13 nM and a n_H of 0.98.

Because GABA has been shown to inhibit the binding of ^{35}S-TBPS in homogenized membrane preparations (Squires et al., 1983), our standard conditions included a 10-min preincubation of the slices in buffer containing 1 mM EDTA to remove endogenous GABA. ^{35}S-TBPS binding was maximal after preincubation times of 5–10 min in both cortical mash slices and cerebellar slices. Seventy-one to 98% of maximal binding was achieved at a preincubation time of 2 min (data not shown). In some cases, preincubations of 20 min or longer resulted in a slight decrease from the maximal binding. Nonspecific binding was not affected by the preincubation time. Removal of endogenous GABA was also evident from the ln-linear dissociation rate plot (Fig. 3). When the preincubation was omitted, the ^{35}S-TBPS binding in cortical brain mash slices was markedly decreased (>60%) (Fig. 6). Note that nonpreincubated slices were assayed at a ^{35}S-TBPS concentration 2.5 times higher than preincubated slices (Fig. 6). In preincubated slices, 1 μM GABA decreased ^{35}S-TBPS binding by 50%. ^{35}S-TBPS binding in slices receiving no preincubation was further decreased (50%) by the addition of 1 μM GABA.

Autoradiographic localization of ^{35}S-TBPS binding

The distribution of ^{35}S-TBPS binding in several brain regions from coronal and parasagittal tissue sections is shown in Table 1 and Figure 7. ^{35}S-TBPS binding was greatest in the cortex of the inferior colliculus. Very dense binding was observed also in the medial septal nucleus and the nucleus of the vertical limb of the diagonal band. Areas of moderately high binding included the islands of Calleja of the olfactory tubercle, zona incerta, amygdaloid nuclei, strata radiatum and moleculare of the hippocampus, the dentate gyrus molecular layer, substantia nigra, and layer IV of the cerebral cortex. Lower levels of binding were found in the globus pallidus, nucleus accumbens, superior colliculus, striatum, entorhinal cortex, lateral septal nucleus, and cerebellum. Within the cerebellum, ^{35}S-TBPS preferentially labeled the molecular cell layer compared to the granule cell layer.

^{35}S-TBPS binding in preincubated and nonpreincubated sections

A preincubation time of 10 min removed sufficient endogenous GABA to obtain maximal ^{35}S-TBPS binding (see above). However, by omitting the preincubation, we were able to study the distribution of ^{35}S-TBPS binding in the presence of endogenous GABA. An overall decrease in ^{35}S-TBPS binding was observed in sections that were not preincubated when compared to preincubated sections. Nonpreincubated sections also exhibited a high degree of variability in ^{35}S-TBPS binding. The variability apparently was related to the position of the sections on the slide in the incubation medium, as demonstrated in Figure 8. Two slides, each holding two brain sections arranged vertically, were incubated in a vertical position as shown. (Note that nonprein-

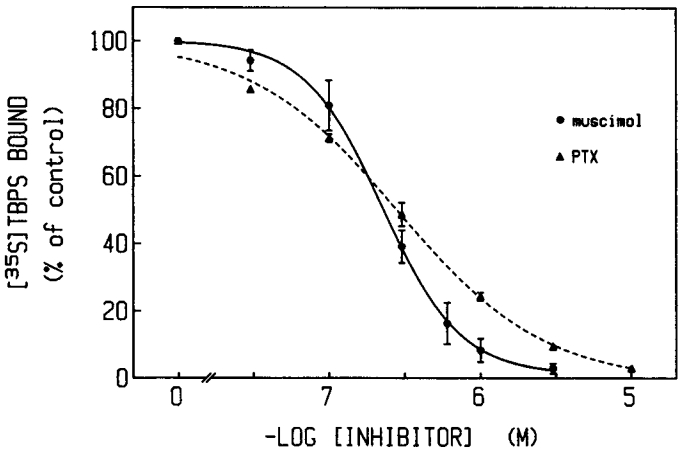

Figure 5. Inhibition of ^{35}S-TBPS binding to cortical brain mash slices by muscimol and picrotoxin. Preincubated slices were incubated with ^{35}S-TBPS (1.9 nM) and various concentrations of each inhibitor (3 hr, 22°C). The values are the mean ± SEM from three experiments performed in triplicate. For muscimol, IC_{50} = 203 ± 41 nM and n_H = 1.4. For picrotoxin, IC_{50} = 251 ± 13 nM and n_H = 0.98 (n = 3).

cubated sections were incubated at a higher ^{35}S-TBPS concentration, 5 nM, than preincubated sections, 2 nM, in order to obtain clearly visible autoradiographic images of the nonpreincubated sections.) Preincubated sections (*A, B*) showed identical binding patterns independent of their positions on the slide. However, in nonpreincubated slides (*C, D*), ^{35}S-TBPS binding was much greater in the upper sections than in the lower ones. We believe this was due to a release of GABA from the upper section, which accumulated in the lower section, attaining concentrations high enough to inhibit ^{35}S-TBPS binding. In addition, the relative distribution of ^{35}S-TBPS binding in nonpreincubated sections differed from that in preincubated sections. In the nonpreincubated section (Fig. 8*C*), ^{35}S-TBPS binding became more prominent in layer I of the cerebral cortex and less prominent in layers II and III relative to the other cerebral cortical layers. ^{35}S-TBPS binding in the external plexiform layer

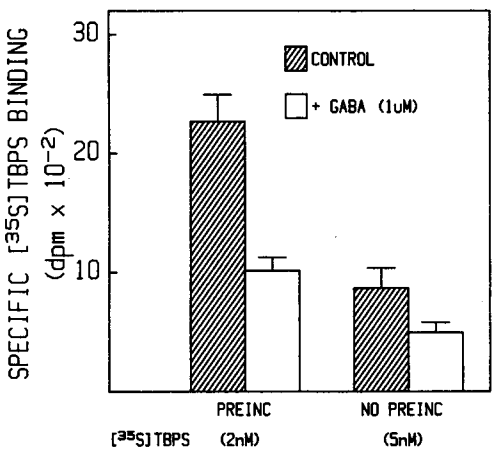

Figure 6. Inhibition of ^{35}S-TBPS binding to preincubated and nonpreincubated cortical brain mash slices by GABA. Preincubated slices were incubated with 2 nM ^{35}S-TBPS ± GABA, and nonpreincubated slices were incubated with 5 nM ^{35}S-TBPS ± GABA. Slices were washed, dried, scraped, and counted as described in Materials and Methods. Data are the mean ± SEM of three experiments performed in triplicate.

Table 1. Distribution of ^{35}S-TBPS binding in rat brain

Brain region	Specific ^{35}S-TBPS binding ratio: fmol/mg protein brain region / fmol/mg protein white matter
Inferior colliculus (Cortex)[a]	8.93 ± 1.96
Septal nuclei	
Medial/vertical	
Limb of diagonal	
Band	7.45 ± 0.35
Lateral	1.56 ± 0.30
Thalamus	
Centrolateral, paracentral, and centromedial nuclei	6.66 ± 0.14
Olfactory tubercle	4.90 ± 1.5
Zona incerta	4.89 ± 1.5
Amygdaloid nuclei	4.80 ± 0.99
Hippocampus	
Strata radiatum and moleculare	4.48 ± 0.97
Stratum oriens	2.45 ± 0.68
Dentate gyrus molecular layer	4.05 ± 0.88
Substantia nigra	4.06 ± 1.5
Frontoparietal cortex	
Layer IV	4.00 ± 0.47
Layers V and VI	3.66 ± 0.18
Layer I	2.56 ± 0.33
Layers II and III	2.21 ± 0.30
Globus pallidus	3.55 ± 0.69
Cerebellum	
Molecular layer	3.40 ± 0.25
Granular layer	
Internal	2.82 ± 0.18
External	2.30 ± 0.17
Nucleus accumbens	2.65 ± 0.50
Superior Colliculus	
Superficial grey	2.61 ± 0.10
Striatum	2.34 ± 0.20
Entorhinal cortex	2.17 ± 0.19

Sections were assayed using 2 nM ^{35}S-TBPS as described in Materials and Methods. Data are expressed as the mean ± SEM from 3–5 rats, 8 sections/rat. Values for ^{35}S-TBPS binding in white matter areas ranged from 4 to 195 fmol/mg protein.

[a] Described by Meininger et al. (1986).

of the olfactory bulb was decreased dramatically in the nonpreincubated section when compared to the preincubated section despite the higher concentration of ^{35}S-TBPS in the incubation medium of the nonpreincubated slide (Fig. 8, *C, D*).

Differential binding patterns in the preincubated vs nonpreincubated sections were especially apparent in cerebellum. In preincubated tissue, ^{35}S-TBPS bound preferentially to the molecular layer of the cerebellum, with less binding in the granular layer of the brainstem (Fig. 9*A*). In nonpreincubated sections, ^{35}S-TBPS binding in the layers of the cerebellar cortex decreased overall. This made it necessary to appose slices to film for a longer time in order to generate a clearly visible autoradiographic image (Fig. 9*B*). In the nonpreincubated cerebellar sections, ^{35}S-TBPS binding in the molecular layer and the external granular layer was reduced relative to binding in the granular layer bordering the white matter (internal granular layer) and in the

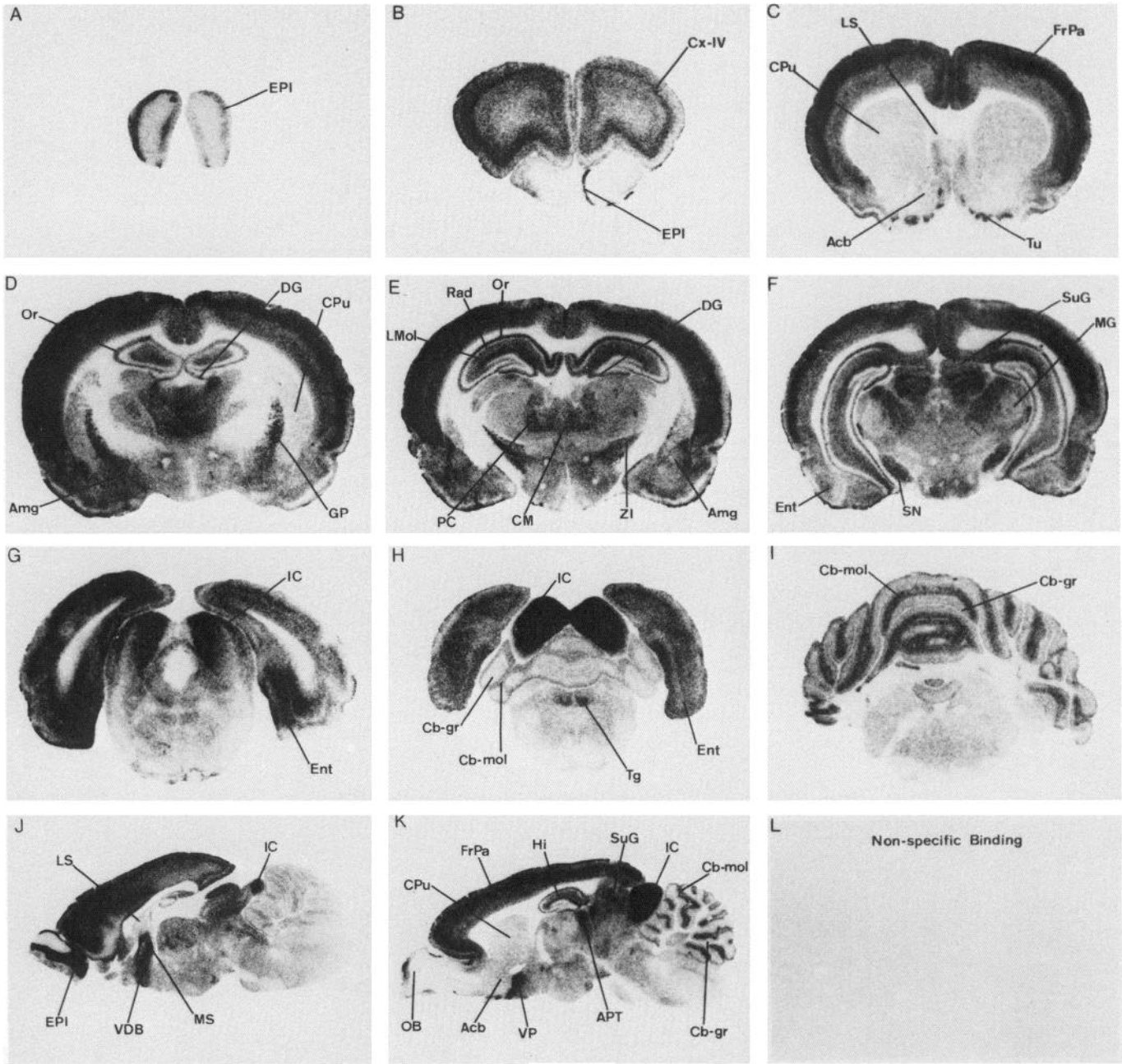

Figure 7. Autoradiographic images showing the distribution of ^{35}S-TBPS binding in the rat brain. All slices (16 μm) were preincubated for 10 min and incubated with ^{35}S-TBPS (2 nM) for 3 hr as described in Materials and Methods. Nonspecific binding was measured in the presence of 100 μM PTX (*L*). Acb, nucleus accumbens; Amg, amygdaloid nuclei; APT, anterior pretectal area; Cb-gr, granular layer of the cerebellum; Cb-mol, molecular layer of the cerebellum; CM, centromedial nucleus of the thalamus; CPu, caudate putamen (striatum); Cx, cerebral cortex; DG, dentate gyrus; Ent, entorhinal cortex; EPl, external plexiform layer of the olfactory bulb; FrPa, frontoparietal cerebral cortex; GP, globus pallidus; Hi, hippocampus; IC, inferior colliculus; LMol, lacunosum moleculare of the hippocampus; LS, lateral septal nucleus; MG, medial geniculate nucleus; MS, medial septal nucleus; OB, olfactory bulb; Or, stratum oriens of the hippocampus; PC, paracentral nucleus of the thalamus; Rad, stratum radiatum of the hippocampus; SN, substantia nigra; SuG, superficial grey of the superior colliculus; Tg, tegmental nuclei; Tu, olfactory tubercle (islands of Calleja); VDB, nucleus of the vertical limb of the diagonal band; VP, ventral pallidus; ZI, zona incerta.

brain stem (Fig. 9*B*). Quantitation of ^{35}S-TBPS binding in nonpreincubated sections was not performed because of variability of ^{35}S-TBPS binding within sections and between sections. However, it was possible to mimic the pattern of ^{35}S-TBPS binding in nonpreincubated sections by adding 1 μM GABA to the incubation medium of preincubated sections (Fig. 9*C*). ^{35}S-TBPS binding in such sections was reproducible and quantifiable and could be compared directly to preincubated sections. Two-way analysis of variance indicated that there was a regionally selective effect of GABA in cerebellum. In the presence of GABA, ^{35}S-TBPS binding decreased moderately in the molecular layer (32%) and most dramatically in the external part of the granular layer (55%) when compared to preincubated sections without added GABA (Table 2). ^{35}S-TBPS binding in the internal granular layer decreased only slightly (11%) with the addition of 1 μM GABA (Table 2).

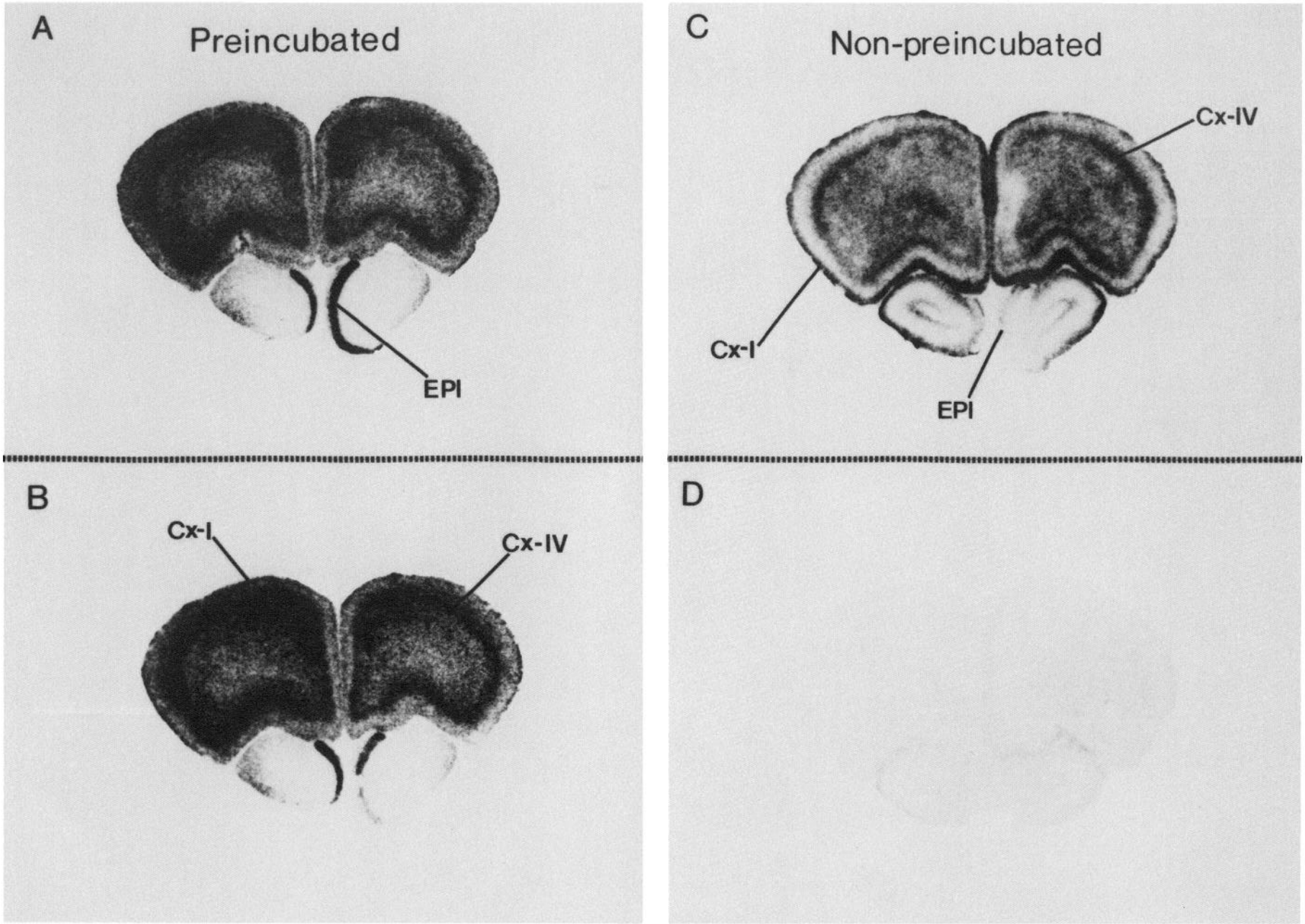

Figure 8. ^{35}S-TBPS binding to preincubated and nonpreincubated rat brain sections. Preincubated upper (*A*) and lower (*B*) sections from the same slide incubated in a vertical position with ^{35}S-TBPS (2 nM) for 3 hr. Nonpreincubated upper (*C*) and lower (*D*) sections on the same slide incubated in a vertical position with ^{35}S-TBPS (5 nM) for 3 hr. After washing and drying, all sections were apposed to film for 2 d. Cx-I, layer I of cerebral cortex; Cx-IV, layer IV of cerebral cortex; EPl, external plexiform layer of the olfactory bulb.

Discussion

The studies presented here indicate that ^{35}S-TBPS binds specifically, reversibly, and with high affinity to a site associated with the $GABA_A$ receptor in slide-mounted rat brain sections. The ^{35}S-TBPS binding distribution in the rat brain resembles that of other ligands that bind to the GABA-gated receptor complex, with a few regionally specific differences (discussed below). However, the degree and distribution of ^{35}S-TBPS binding can be highly dependent on the preincubation–incubation conditions used and on the presence or absence of GABA.

Biochemical characterization

Although ^{35}S-TBPS binding in homogenized brain preparations has been characterized (Squires et al., 1983; Wong et al., 1984; Casida and Lawrence, 1985), ^{35}S-TBPS binding to slide-mounted brain sections has not been fully characterized. Our initial experiments for determining association rates indicated that an incubation time of 3 hr was necessary for ^{35}S-TBPS binding to slices to reach equilibrium. Reported times to reach equilibrium in brain homogenates range from 90 min to 3.5 hr (Squires et al., 1983; Wong et al., 1984; Maksay and Ticku, 1985). In autoradiography studies, investigators have used an incubation time of 90 min, although the time to reach equilibrium was not indicated (Wamsley et al., 1983; Concas et al., 1986; McCabe and Wamsley, 1986).

We found ^{35}S-TBPS dissociation from slide-mounted tissue to be slow and monophasic, contrary to the findings of Squires et al. (1983), who observed a more rapid and biphasic dissociation in EDTA treated, water dialyzed membrane homogenates. However, the dissociation rate we observed was very similar to that observed by Maksay and Ticku (1985) in freeze-thawed membranes. These investigators also reported that dissociation became polyphasic and more rapid when either GABA was added or KBr was substituted for KCl in the buffer. Therefore, it is likely that differences in endogenous GABA levels and buffer anion content may account for observed variations in the dissociation rate.

Differences in tissue preparation may also account for discrepancies in the reported K_D values for ^{35}S-TBPS. We obtained K_D values of 21.0 nM and 29.4 nM from saturation and kinetic data, respectively. Although K_D values have not been reported for slide-mounted sections, these values are consistent with the findings of other investigators who employed some method of GABA removal in homogenate preparations (Wong et al., 1984; Maksay and Ticku, 1985). However, K_D values obtained from membrane preparations that were not treated for the removal of endogenous GABA ranged from 56 nM to 84 nM (Lawrence and Casida, 1983; Supavilai and Karobath, 1984). The reason for this difference is not clear, since GABA has been reported to alter only the B_{max} and not the K_D value for ^{35}S-TBPS (Ticku

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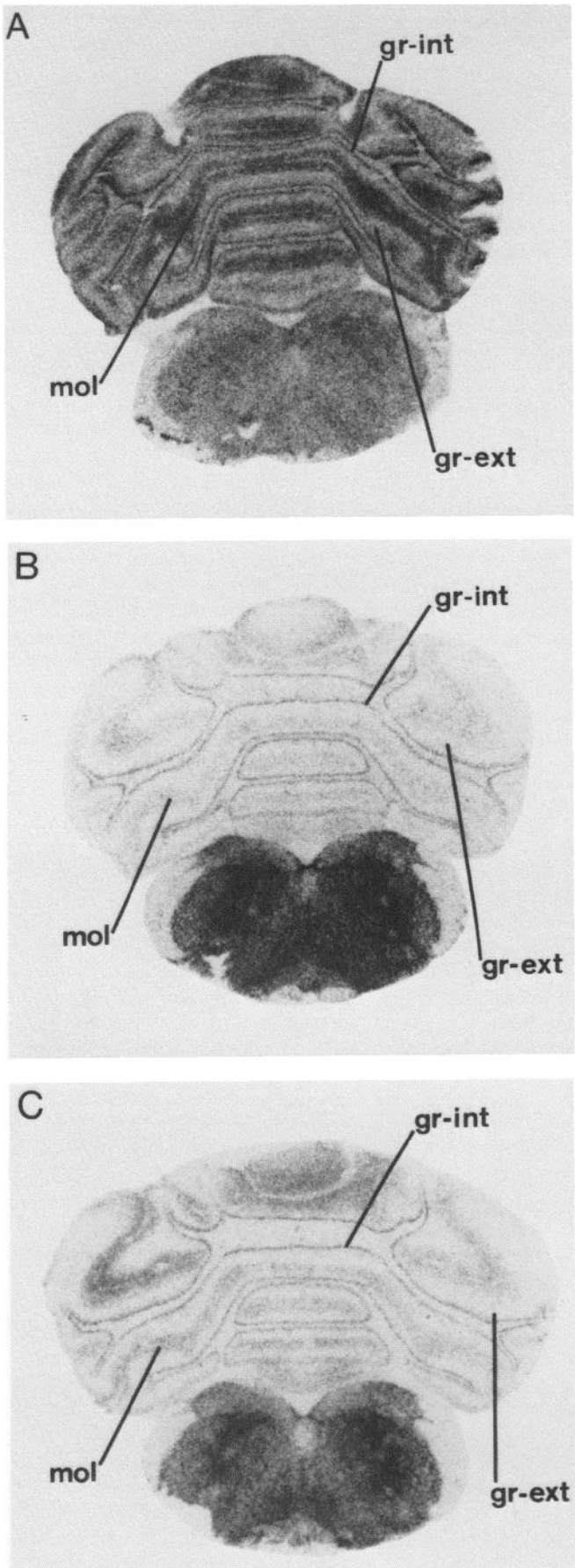

Figure 9. ^{35}S-TBPS binding to the cerebellum of a preincubated section (*A*), a nonpreincubated section (*B*), and a preincubated section assayed in the presence of 1 μM GABA (*C*). All sections were incubated with 2 nM ^{35}S-TBPS. After washing and drying, sections in *A* and *C* were apposed to film for 3.5 d. The section in *B* was apposed to film for 7 d in order to obtain a clearly visible image. gr-ext, external granular layer; gr-int, internal granular layer; mol, molecular layer.

Table 2. Inhibition of ^{35}S-TBPS binding in cerebellar cell layers by GABA

Cerebellar cell layer	Specific ^{35}S-TBPS binding ratio fmol/mg protein brain region / fmol/mg protein white matter	
	Control	1 μM GABA
Molecular layer	5.51 ± 1.0	3.74 ± 0.74 (−32%)
Granular layer		
Internal	4.14 ± 0.53	3.68 ± 0.38 (−11%)
External	3.63 ± 0.54	1.63 ± 0.29 (−55%)*

Preincubated slices were assayed with or without 1 μM GABA as described in Materials and Methods. Data are expressed as the mean ± SEM of 4 rats. Numbers in parentheses indicate percent decrease in ^{35}S-TBPS binding caused by GABA. Two-way analysis of variance indicated a regionally selective effect of GABA ($p < 0.05$).

* $p < 0.02$ compared to preincubated control (Student's t test).

and Ramanjaneyulu, 1984; Wong et al., 1984). Possibly, procedures used to remove endogenous GABA are also removing some other regulatory substance at the GABA receptor.

^{35}S-TBPS binding to slide-mounted sections was altered by other ligands of the GABA receptor complex in a predictable manner. Both muscimol and picrotoxin inhibited ^{35}S-TBPS binding to cortical brain mash tissue in a concentration-dependent manner. A n_H of 1.4 was obtained from inhibition studies with muscimol. This finding is consistent with a noncompetitive interaction between the ligands that occupy the $GABA_A$ and TBPS recognition sites (Wong et al., 1984; Akaike et al., 1985; Schwartz and Mindlin, 1988). The competitive inhibition of ^{35}S-TBPS binding by picrotoxin ($n_H = 0.98$) agrees with previous reports that these two convulsants bind to the same site (Wong et al., 1984; Maksay and Ticku, 1985).

Specific ^{35}S-TBPS binding is poorly detectable in rat brain membranes that have not been treated for the removal of endogenous GABA (Squires et al., 1983). Investigators have used EDTA–water dialysis, repetitive freeze-thaw techniques, and osmotic shock to remove GABA from homogenized membrane preparations (Squires et al., 1983; Maksay and Ticku, 1985; McCabe et al., 1988). After a 10-min preincubation in EDTA–buffer, we observed consistently high specific binding of ^{35}S-TBPS to slide-mounted brain tissue. Omission of the preincubation resulted in a substantial but highly variable decrease in ^{35}S-TBPS binding. Both regionally specific and nonspecific variations in ^{35}S-TBPS binding were observed within and between sections. These variations were evident in autoradiograms. For example, in Figure 8, regions known to have a high degree of GABA synthesis (high glutamate decarboxylase activity), such as the external plexiform layer (EPl) of the olfactory bulb (Ribak et al., 1977), exhibited dramatic decreases in ^{35}S-TBPS binding when the preincubation was omitted. In preincubated sections, ^{35}S-TBPS binding was high in the EPl, indicating that the endogenous GABA had been removed in the preincubation.

Although ^{35}S-TBPS binding in the cerebellum decreased overall in the nonpreincubated sections as compared to the preincubated sections, the extent of the decrease in each cell layer varied. Generally, in nonpreincubated sections ^{35}S-TBPS labeling of the internal aspect of the granular layer (next to white matter) was not reduced relative to the other layers. This was also observed when the nonpreincubated condition was "mimicked" by adding 1 μM GABA to preincubated cerebellar sections. Since variability was not a problem in the latter case, quantitative autoradiography was performed in the cerebellar layers. This revealed an uneven inhibition of ^{35}S-TBPS binding in cerebellar cell layers (Table 2). These findings may indicate that GABA regulation of ^{35}S-TBPS binding sites differs in the cerebellar layers due to a heterogeneity of sites. In this regard, a recent study has demonstrated that reconstituted, cloned $GABA_A$ receptors are differentially sensitive to GABA depending on which α subunit (α-1, 2, or 3) accompanies the β subunit (Levitan et al., 1988).

Omission of the preincubation also revealed an unanticipated binding artifact that resulted from the positioning of brain sections on the slide. Because slides were incubated vertically for 3 hr, endogenous GABA from the uppermost section on the slide apparently was released and accumulated in the lower section on the same slide. Therefore, ^{35}S-TBPS binding in the lower section was inhibited to a much greater degree than binding in the upper section, making analysis of nonpreincubated sections virtually impossible.

Regional distribution

Regions showing the greatest specific ^{35}S-TBPS binding under standard (preincubated) assay conditions were the inferior colliculus, medial septal nucleus and vertical limb of the diagonal band, central and paracentral thalamic nuclei, islands of Calleja in the olfactory tubercle, external plexiform layer of the olfactory bulb, zona incerta, substantia nigra, and globus pallidus. Regions with relatively little ^{35}S-TBPS binding included the lateral septal nucleus, striatum, entorhinal cortex, nucleus accumbens, and the superficial grey of the superior colliculus. ^{35}S-TBPS binding in the frontoparietal cortex was most prominent in layer IV and also heavy in layers V and VI.

Several of the regions reported in our study were reported also to be regions of high ^{35}S-TBPS binding in other autoradiography studies (Gee et al., 1983; Wamsley et al., 1983; Concas et al., 1986). However, the ^{35}S-TBPS binding distribution we observed more closely parallels the binding distributions of (1) the benzodiazepine ligands, ^{3}H-flumazenil (RO15-1788) and ^{3}H-flunitrazepam (Wamsley et al., 1983; Richards et al., 1988), (2) the ligand for the low-affinity $GABA_A$ site, ^{3}H-bicuculline methochloride (Olsen et al., 1984), and (3) ^{3}H-t-butylbicycloorthobenzoate (TBOB) (O'Connor and McEwen, 1986; R. W. Olsen, personal communication), another ligand that labels the GABA-gated chloride channel (Lawrence et al., 1985). This was especially true in the case of the cerebellum. As with the benzodiazepine ligands, ^{3}H-bicuculline methochloride, and ^{3}H-TBOB, ^{35}S-TBPS binding was present in both layers of the cerebellum but was more prominent in the molecular layer than in the granule cell layer. In this respect, our findings differ from those of Wamsley et al. (1983) and Gee et al. (1983), who reported that ^{35}S-TBPS binds predominantly to the granule cell layer. We did confirm their observation of a gradient of binding within the granule cell layer; ^{35}S-TBPS binding was more dense in the internal aspect of the granule cell layer lining the white matter.

In contrast, the binding distribution of ^{35}S-TBPS was different from the distribution of high affinity $GABA_A$ sites labeled with ^{3}H-muscimol in the rat brain (Palacios et al., 1980; Unnerstall et al., 1981; McCabe and Wamsley, 1986). Low levels of ^{3}H-muscimol binding were observed in such regions as zona incerta and globus pallidus, 2 of the areas exhibiting dense ^{35}S-TBPS binding. In cerebellum, ^{3}H-muscimol bound almost exclusively to the granular as compared to the molecular layer.

The ^{35}S-TBPS binding distribution in cerebellum also differed from the distribution of $GABA_A$ receptor α and β subunits in rat brain. *In situ* hybridization (Montpied et al., 1988; Richards et al., 1988; Sequier et al., 1988) and immunohistochemical studies (Schoch et al., 1985; Richards et al., 1988) indicate that both the α and β subunits are more densely localized in the cerebellar granular layer than in the molecular layer. However, other regions of dense ^{35}S-TBPS binding, such as inferior colliculus, globus pallidus, and substantia nigra, hybridized with both α and β probes, although more strongly with the α than the β (Richards et al., 1988).

The differences in localization of the various recognition sites and subunits associated with the $GABA_A$ receptor complex may be due to $GABA_A$ receptor heterogeneity. Structural and functional heterogeneity of $GABA_A$ receptors has been demonstrated in several studies (Yasui et al., 1985; Levitan et al., 1988; Moffett et al., 1989). In addition, heterogeneity of central benzodiazepine binding sites has been documented (Klepner et al., 1979; Squires et al., 1979; Sweetnam and Tallman, 1986; Medina et al., 1989). Localization of benzodiazepine site subtypes (Chisolm et al., 1983; Sweetnam and Tallman, 1986; Medina et al., 1989) and various subunits of the $GABA_A$ receptor in brain (de Blas et al., 1988; Montpied et al., 1988; Schmitz et al., 1988; Sequier et al., 1988) indicates that $GABA_A$ receptor subtypes are unevenly distributed throughout brain regions, particularly in the cerebellum (Schmitz et al., 1988). Further studies are needed to establish the relationship between $GABA_A$ and benzodiazepine recognition site heterogeneities and subtypes.

The results of the present study indicate that ^{35}S-TBPS is useful for localizing the $GABA_A$ receptor-associated chloride ionophore in autoradiographic studies. The advantages of a 2–3 day film exposure time for ^{35}S as compared to 2–10 weeks for ^{3}H-ligands are obvious. Although the binding distribution of ^{35}S-TBPS correlates well with the distribution of low-affinity $GABA_A$ recognition sites and benzodiazepine binding sites in brain, there are some regionally specific differences in the distribution of ^{35}S-TBPS sites and (1) high-affinity $GABA_A$ recognition sites and (2) immunohistochemical and *in situ* hybridization probes for the $GABA_A$ receptor. However, it is clear that changes in either endogenous or exogenous GABA may alter the distribution pattern of ^{35}S-TBPS binding sites. Differences in endogenous GABA levels could also alter the binding patterns at other sites associated with $GABA_A$ receptor. These findings may be explained by the existence of heterogeneous populations of the $GABA_A$ receptor complex in brain.

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