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GAMMA-HYDROXYBUTYRIC ACID BINDING SITES IN RAT AND HUMAN BRAIN SYNAPTOSOMAL MEMBRANES

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Abstract—The binding of γ-hydroxy[2,3-^{3}H]butyric acid (GHB) was characterized in rat and human brain synaptosomal membranes. Binding was shown to be saturable, pH dependent, and linear with protein concentration. There was a distinct regional distribution of binding sites in both rat and human brain, with the hippocampus being the richest and the cerebellum the poorest, in density of [^{3}H]GHB binding sites. Competition and saturation experiments revealed two different populations of binding sites, a high-affinity site with a K_{D_1} of 580 nM and a B_{max_1} of 1.8 pmoles/mg protein and a low-affinity site with a K_{D_2} of 2.3 μM and a B_{max_2} of 11.3 pmoles/mg protein. [^{3}H]GHB binding was not inhibited by γ-aminobutyric acid (GABA), GABA receptor agonists, opiate antagonists or anticonvulsant drugs. These data suggest that GHB may play a role as a neurotransmitter or neuromodulator in brain independent of GABA.

Gamma-hydroxybutyric acid (GHB) is a naturally occurring substance [1, 2] which has diverse neuropharmacologic and neurophysiological properties [3]. Because of the structural similarity of GHB to γ-aminobutyric acid (GABA) and its derivation from GABA in brain [4, 5], many of the protean neurobiological effects of this compound have been attributed to GABAergic mechanisms [6–8]. However, the thesis that GHB is a direct GABA agonist is difficult to reconcile with data indicating that GHB has no affinity for GABA receptor binding sites in brain [9, 10]. An alternative mechanistic explanation for the biologic effects of GHB is that this compound functions as a neurotransmitter or neuromodulator that is independent of GABA [11]. Such a role would presumably require the presence of binding sites in brain specific for GHB. Indeed, there are recent data to support such a site [12].

The object of the following experiments, therefore, was to further identify and characterize GHB binding sites in rat and human brain.

MATERIALS AND METHODS

Chemicals and drugs. γ-Hydroxy{2,3-^{3}H}butyric acid, ammonium salt (sp. act. 20.2 Ci/mmole), was synthesized by the Amersham Corp. (Arlington Heights, IL). Purity of the radiochemical was assessed before and at the end of the assay procedure by thin-layer chromatography (TLC) in three different solvent systems [ethanol–ammonium hydroxide (0.88 M): 6/2; ethanol–water: 50/50; and ethanol–sodium hydroxide (2M)–water: 75/16/9]. Channeled LK6DF TLC plates (Whatman, Clifton, NJ) were spotted with radioisotope and developed to 10 cm in the above solvent systems; 1-cm sections were then cut and counted.

Naloxone, ethosuximide, valproic acid, and trimethadione were donated by Endo Laboratories (Wilmington, DE), Warner Lambert Laboratories (Ann Arbor, MI), and Abbot Laboratories (North Chicago, IL) respectively. All other drugs and chemicals were obtained from commercial sources.

Tissue source. Rat brains were obtained from adult male Sprague–Dawley rats [Crl: CS(SD)BR, Charles River] weighing 200–350 g. All animals were fed and watered *ad lib.* and maintained on a 12-hr light–dark cycle. Animals were killed by decapitation and the brains were rapidly removed, dissected, and subjected to the binding procedure outlined below.

Post-mortem human brain tissue was obtained from patients ranging in age from 32 to 68 years (mean = 43.7), who had died of nonneurologic disease and were autopsied within 12 hr of death (mean = 4.23 hr). All brains were dissected where possible into frontal, temporal, parietal, and occipital cortex, caudate, putamen, globus pallidus, hippocampus, hypothalamus, thalamus, cerebellum, pons, and medulla. The brain tissue samples were then frozen at −76° until the binding procedures were done.

Preparation of lysed synaptosomal membranes. Synaptosomal plasma membranes were prepared by a modification of the method of Enna and Snyder [13]. Briefly, brain tissue was homogenized in 8 vol. of ice-cold 0.32 M sucrose, pH 7.5. After an initial centrifugation at 900 *g* for 10 min, the supernatant fraction was centrifuged at 26,000 *g* for 20 min to obtain a crude synaptosomal pellet which was washed by resuspension and centrifugation first with ice-cold deionized water and then with 50 mM Tris, pH 7.2. The final pellet was then frozen in 50 mM Tris, pH 7.2, for 18 hr at −60°, thawed, and washed again with ice-cold deionized water.

Protein was determined by the method of Lowry *et al.* [14].

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GHB receptor binding procedure. Specific {[3H]GHB binding was measured by modification of the procedure of Benavides *et al.* [12]. In brief, aliquots of resuspended pellet (protein concentration 2–3 mg/ml) were incubated in triplicate at 0° for 30 min in a 1 ml total volume of buffer made up of 50 mM Pipes (1,4-piperazine-diethanesulfonic acid), pH 6.5, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 12 mM Cl^-, 50 mM K^+, and [3H]GHB as indicated in Results. Tubes containing 5 mM unlabeled GHB plus all other components were used to account for non-specific binding of [3H]GHB. The binding reaction was terminated at the end of the incubation period by centrifugation at 48,000 *g*; the membranes were washed twice with ice-cold deionized water and solubilized with Protosol (New England Nuclear, Boston, MA); and the radioactivity present was assayed by liquid scintillation spectrometry.

Competition curves were constructed by addition of concentrations of unlabeled GHB ranging from 10 to 100,000 nM to incubation mixtures containing 50 nM [3H]GHB. Saturation curves were generated by varying the concentration of [3H]GHB in the incubation mixture from 50 to 500 nM using blank mixtures as described above.

[3H]GHB displacement by other ligands was assessed by adding membranes to incubation mixtures that contained 50 nM [3H]GHB and concentrations of the competing drug that ranged from 10 μM to 1 mM. Human hippocampus was used for these studies.

The variability of binding with pH and protein concentration was determined by incubating 10 nM [3H]GHB in incubation mixtures which contained protein concentrations that ranged from 1 to 5 mg/ml; the pH values of the mixture varied from 5 to 8. The presence of nonneuronal binding sites was sought in kidney and liver.

Data analysis. K_D and B_{max} were calculated from the competition experiments by the method of Akera and Cheng [15] and by logit analysis and from the saturation experiments by Scatchard and Hill plots [16]. Results of inhibition of [3H]GHB by other drugs are expressed as percent inhibition of binding displaced by 5 mM nonradioactive GHB [12].

Table 1. Density and regional distribution of 3H-binding sites in rat brain*

Region	Specific 3H-binding density (fmoles/mg protein ± S.E.M.)
Cerebellum	31.75 ± 4.3
Pons	ND†
Medulla	26.1 ± 1.3
Striatum	108.0 ± 9.3
Thalamus	52.9 ± 1.2
Hypothalamus	11.0 ± 0.73
Hippocampus	215.0 ± 24.1
Cortex	133.0 ± 11.4
Whole brain	150.0 ± 13.3

* Each determination is the mean of three experiments performed in triplicate which varied less than 5%.
† ND = no detectable binding.

RESULTS

[3H]GHB was isographic in the three solvent systems used. Ligand purity was confirmed in these systems at the end of the assay procedure. The number of cpm added in the standard binding assay was 250,000; the number of total counts bound was 13,500; and the number of counts displaced by a saturating concentration of GHB was 6000. The [3H]GHB binding was saturable, pH dependent, and linear with tissue concentration (Fig. 1). Binding was destroyed by boiling and was not found in liver or kidney.

Saturation experiments in rat brain indicated a high-affinity site with a K_{D_1} of 580 nM and a B_{max_1} of 1.8 pmoles/mg protein (Fig. 2). Competition experiments in rat brain indicated a low-affinity binding site with a K_{D_1} of 2.3 μM and a B_{max_2} of 11.3 pmoles/mg protein.

Regional analysis of rat brain (Table 1) showed that the highest densities of binding sites were in hippocampus and cortex with the lowest specific binding found in cerebellum, brain stem and hypothalamus.

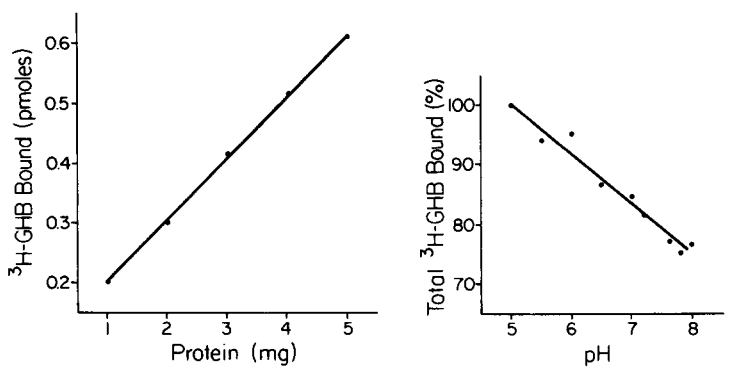

Fig. 1. Experiments showing pH dependence of [3H]-binding and linearity of binding with protein concentration. Each point represents the mean of three experiments performed in triplicate with a standard error of less than 5%.

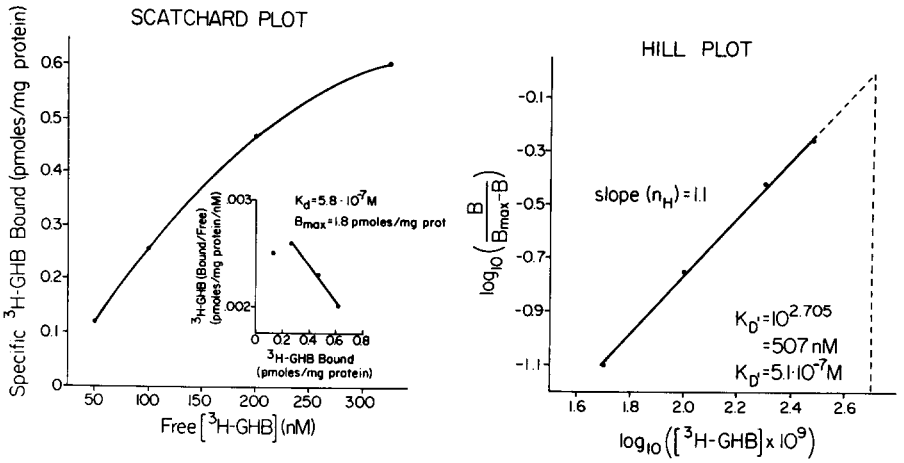

Fig. 2. Scatchard analysis and Hill plot of data generated from saturation experiments showing a K_{D_1} of 580 nM and a B_{max_1} of 1.8 pmoles/mg protein. Each point represents the mean of four experiments performed in triplicate with a standard error of less than 5%.

Binding studies done in human brain (Fig. 3) indicated that the highest specific binding occurred in hippocampus and pons with the lowest in cerebellum. Cortex and caudate were intermediate in binding density in human. Regional binding difference in both rat and human brain was due to a difference in B_{max}.

None of the other ligands tested produced significant inhibition of [³H]GHB binding (Table 2).

DISCUSSION

Our experiments corroborate those studies that demonstrated the presence of a specific binding site for GHB [12] and extend those findings into human brain. We found that the binding of [³H]GHB to synaptic plasma membranes was saturable, pH dependent, and linear with protein concentration. We also demonstrated high- and low-affinity sites, as have others [12]. The high-affinity site had a K_{D_1} of 580 nM and a B_{max_1} of 1.8 pmoles/mg protein while the low-affinity site had a K_{D_2} of 2.3 μM and a B_{max_2} of 11.3 pmoles/mg protein. Since the pH optimum, the K_D and the B_{max} in our studies are all quite different from those reported for the GABA receptor [13], and since GABA did not compete for binding in our experiments, the GHB binding site appears to be independent of the GABA receptor. Similarly, the fact that γ-butyrolactone (GBL), a compound often used as a prodrug for GHB in neurophysiologic and neuropharmacologic experiments [17], had no affinity for the GHB binding site substantiates the thesis that this drug owes all its neurobiologic properties to its conversion *in vivo* to GHB [18, 19].

We examined a number of anticonvulsants used in petit mal epilepsy, as well as naloxone, in the inhibition experiments because of the unique abilities

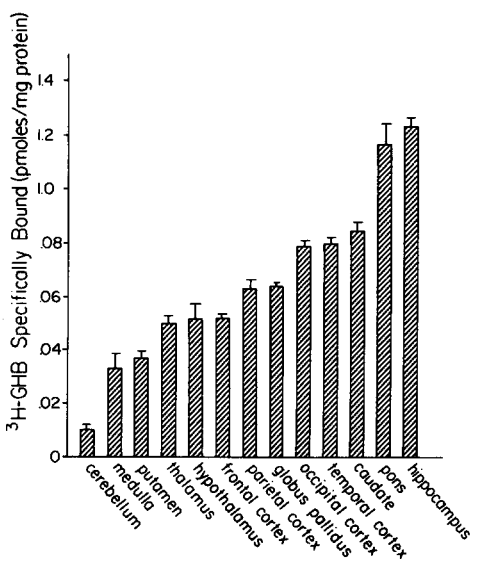

Fig. 3. Regional analysis of binding of [³H]GHB to synaptic membranes prepared from post-mortem human brain. Each value represents the mean ± S.E.M. of three determinations done in triplicate.

Table 2. Ligand inhibition of [³H]GHB binding*

Drugs	Binding of 50 nM [³H]GHB (% inhibition ± S.E.M.)
GHB	55 ± 1.73
γ-Butyrolactone	ND†
GABA	ND
Picrotoxin	ND
Muscimol	ND
Bicuculline	ND
Valproic acid	ND
Trimethadione	ND
Ethosuxmide	ND
Naloxone	ND
Kainic acid	ND

* Drug concentrations tested were 10 μM–1 μM. There were four rats in each group.

† ND = no detectable inhibition.

of these compounds to overcome the petit-mal-like epileptiform discharges which GHB is capable of inducing in experimental animals [20–21]. The fact that these compounds did not demonstrably compete with [^{3}H]GHB for binding in either rat or human brain suggests that their anti-petit-mal properties in the GHB model of petit mal epilepsy are not due to an action at the GHB binding site.

The regional analysis of specific binding in rat brain is in agreement with previous studies [12] showing the highest density of binding sites to be in hippocampus. This was also found to be the case in human brain. Human hippocampus was the richest source of GHB binding sites that we found while the cerebellum was the lowest in both species. This distribution may have some functional significance for, whereas cerebellum is relatively rich in endogenous GHB [22], the hippocampus appears to be quite sensitive to its electrophysiologic effects [23]. This inconstant relation between anatomic distribution of GHB concentration and GHB binding sites raises the possibility that in some areas of brain i.e. cerebellum, GHB occurs as an incidental GABA metabolite while in other areas, e.g. cortex, striatum, and hippocampus, GHB may have a functional role in neuromodulation or neurotransmission [11].

In summary these data, which demonstrate the existence of GHB binding sites in rat and human brain, may provide support for the existence of a specific mechanism by which GHB exerts its diverse effects in brain. The presence of a specific binding site for GHB provides further support for the hypothesis that GHB has a biologic role in brain independent of GABA.

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