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SUBCELLULAR DISTRIBUTION OF γ-HYDROXYBUTYRATE BINDING SITES IN RAT BRAIN PRINCIPAL LOCALIZATION IN THE SYNAPTOSOMAL FRACTION

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SUMMARY : γ-Hydroxybutyrate binding sites, first described on crude membranes from rat brain, have been further studied on subcellular fractions. The nerve ending fraction (fraction C) exhibits the maximal capacity for GHB binding. The two classes of binding sites (high and low affinities) described for the crude membrane preparation are enriched in this synaptosomal fraction. This result is further evidence in favor of a role for GHB as a neurotransmitter or neuromodulator in rat brain.

INTRODUCTION : γ-Hydroxybutyrate (GHB) in brain is a reductive catabolite of GABA. The existence of a specific succinic semialdehyde reductase involved in its synthesis (1), located in neurons (2), the neuropharmacological and neurophysiological effects of administered GHB (for review see 3) and its distribution in different brain areas (4) implicate the existence of receptor sites for GHB action in the CNS. Recently, high affinity binding sites for GHB have been demonstrated in rat brain (5).

The described characteristics of its synthesis and of the distribution of its binding sites are consistent with a neurotransmitter or neuromodulator role for GHB in brain. It was thus very important to demonstrate the localization of these binding sites on synaptic membranes. Here, we report on the distribution of γ-hydroxybutyrate binding sites in membranes prepared by subcellular fractionation of rat brain tissue and the predominance of GHB binding sites at the synaptic level.

MATERIALS AND METHODS : For subcellular fractionation of rat brain, the method of De Robertis et al. (6) was used. H_2O was added to all the fractions, including the nuclear and microsomal fraction, in order to obtain a concentration of 0.15 M sucrose before centrifuging for 30 min at 100,000 g. The pellets obtained were resuspended in 10 vol. (w/v) of 50 mM Tris-HCl, pH 7.2, homogenized for 30 sec with a polytron and then centrifuged at 50,000 g for 20 min. The pellets obtained were resuspended in Tris-HCl

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medium and stored at -20°C. The binding assays were performed exactly as described by Benavides et al. (5)- using $25 \cdot 10^{-9}$M (^{3}H) GHB. As previously shown (5) and in the experimental conditions used, the *in vitro* data are in favour of two classes of binding sites. In order to obtain an estimation of the extent of the binding to the two sites, the binding displaceable by 5 mM non radioactive GHB was investigated.

RESULTS : The subcellular distribution of γ-hydroxybutyrate binding is shown in Table 1. Results are expressed as relative binding (RB) and as fentomoles of [^{3}H]-GHB mg protein^{-1}. The richest compartment was membranes derived from fraction C of the sucrose gradient (between 1.0 and 1.2 M sucrose). Fraction C has been described by De Robertis et al. (6) to contain essentially nerve endings and is the richest source of acetylcholine and acetylcholinesterase. Nuclear, myelin and mitochondrial fractions are almost devoid of GHB binding sites. The soluble compartment (cytosol) was not tested.

We investigated some characteristics of GHB binding in the richest fraction (fraction C). GHB binding was saturable and the data for specific binding were plotted by the Scatchard method (Fig. 1). Two populations of binding sites (or a single class with negative cooperativity) were found as previously described for crude membranes of rat brain (5). A Gauss-

TABLE 1 SUBCELLULAR DISTRIBUTION OF GHB BINDING SITES (1)

Fraction	SPECIFICALLY BOUND (^{3}H) GHB	
	RB	fmoles [^{3}H] GHB per mg protein
Nuclear	0.52	105
Microsomal	1.23	658
Myelin (A)	0.80	150
Nerve endings (B)	1.83	254
Nerve endings (C)	4.25	975
Nerve endings + Mitochondria (D)	1.12	319
Mitochondria (E)	0.97	155
Cytosol	N.D.	N.D.

(1) Average of three experiments which varies less than 10 %

RB : relative binding :

$$\frac{\%\text{ binding displaceable by 5 mM non radioactive GHB}}{\%\text{ protein recovered}}$$

N.D. : binding not determined.

The relative binding indicates the capacity of the subcellular compartment as a percentage of the total capacity. The amount of radioactive GHB specifically bound per mg of protein represents only the density of binding sites in the different subcellular compartments.

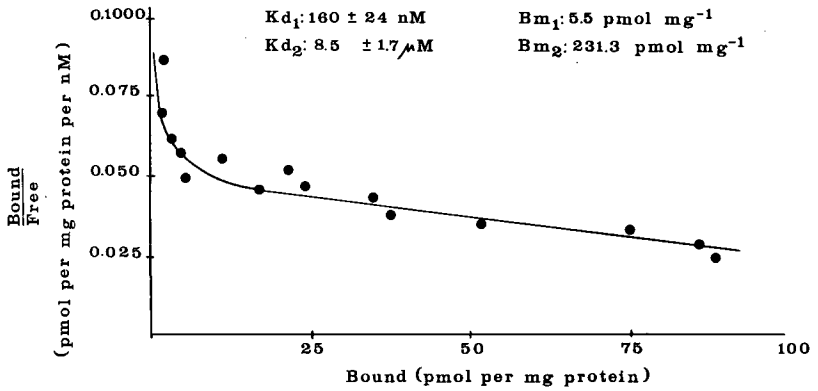

Figure 1.: Analysis by Scatchard plot of (^{3}H) γ-hydroxybutyrate binding to rat brain synaptosomal membranes (fraction C). The data is derived from two separate experiments performed in triplicate at each concentration (variation < 10 %).

Newton fitting of the data (7) indicates that the high affinity binding has a Kd_1 of 160 ± 25 10^{-9}M and a $Bmax_1$ of 5.5 ± 1.5 pmole per mg protein whereas the lower affinity site has a Kd_2 of 8.5 ± 1.7 10^{-6}M and a $Bmax_2$ of 231 ± 35 pmole/mg (Table 2).

DISCUSSION : The present results show that γ-hydroxybutyrate binding sites previously described on a crude preparation of membrane from rat brain are essentially located in the subcellular fraction containing nerve endings.

Compared to the data published previously concerning GHB binding sites on crude membranes prepared from the P_2 fraction (5), the purified synap-

TABLE 2 COMPARISON OF THE BINDING PARAMETERS OF (^{3}H) GHB IN CRUDE MEMBRANES AND IN PURIFIED MEMBRANES OF SYNAPTIC ORIGIN

	HIGH AFFINITY BINDING SITE	LOW AFFINITY BINDING SITE
CRUDE MEMBRANES (P_2 FRACTION)	Bmax = 0.560 ± 0.035 pmole/mg Kd = 93.5 ± 12.4 10^{-9}M	Bmax = 47.9 ± 2.0 pmole/mg Kd = 16.7 ± 1.4 10^{-6}M
MEMBRANES PREPARED FROM NERVE ENDINGS (FRACTION C)	Bmax = 5.5 ± 1.5 pmole/mg Kd = 160 ± 25 10^{-9}M	Bmax = 231 ± 35 pmoles/mg Kd = 8.5 ± 1.7 10^{-6}M
ENRICHMENT FACTOR IN BINDING SITES	x 9.8	x 4.8

tosomal membrane (fraction C) apparently exhibits the same two classes of binding sites (one of high affinity and another of lower affinity). However, compared to a crude membrane fraction, the number of binding sites increases about 9.8 fold for the high affinity system and about 4.8 fold for the low affinity system in the purified synaptosomal fraction (see Table 2). This result also indicates the predominant distribution of GHB binding sites in the synaptosomal membranes (fraction C). In fact, the capacity (relative binding) of the nerve ending fraction (fraction C) appears to be the largest compared to the other subcellular compartments. The density of GHB binding sites (fmoles GHB per mg protein) is also maximal in fraction C. It is highly probable that the apparent high density of binding sites in the microsomal fraction is due to the well known contamination of this fraction by synaptic and plasma membrane.

These data are in favour of the existence of receptor sites for GHB at the synaptic level. This is additional evidence for a neurotransmitter or neuromodulator role of GHB, since the existence of a specific enzyme that synthesises GHB is present in neurons and nerve terminals and a high affinity uptake system for GHB has been demonstrated in membranes vesicles from synaptic origin (8).

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