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## WIDESPREAD OCCURRENCE OF SPECIFIC, HIGH AFFINITY BINDING SITES FOR AMINO ACIDS

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The high affinity binding of several amino acids to various membrane and protein preparations has been measured. Binding of radioactive amino acids suspected of being neurotransmitters and also of leucine and tyrosine to brain, liver and heart muscle membranes was saturable, reversible and stereospecific. Similar characteristics were found using chloroform-methanol extracted brain tissue and heat denatured albumin. Compounds thought to act as blockers of postsynaptic binding such as strychnine, bicuculline and kainic acid did not inhibit binding. Thus, specific high affinity interactions between amino acids and proteins are widespread and largely unrelated to neurotransmission.

## INTRODUCTION

The affinity binding of several amino acids such as glutamic acid, glycine and gamma-aminobutyric acid has been reported in membrane preparations of brain (1-4). This binding has been used as a measure of postsynaptic sites specific for a particular amino acid neurotransmitter. Criteria used to establish the identity of these sites include: 1) specificity of the interaction and also stereospecific selectivity in several cases; 2) saturable, reversible binding kinetics with a high affinity between membrane and ligand; 3) independence of binding on the presence of the sodium ion (5); 4) no energy dependence of the interaction, temperature should not have a major effect on binding velocity; 5) physiological relevance and appropriate inhibition by known antagonists.

We are here reporting that several of these characteristics can be found in interactions between brain membranes and amino acids not thought to be neurotransmitters and also in interactions between putative amino acid transmitters and non-neural protein preparations. Such binding can be of high affinity, stereospecific, reversible and saturable. However, specific neurotransmitter antagonists have little inhibitory effect on the binding. It is likely that most specific high affinity binding of amino acids by nervous tissue is not related to synaptic function.

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### MATERIALS AND METHODS

Preparation of membranes - Optic lobe, liver and heart tissue was dissected out from embryonic or new hatched chicks, weighed and frozen at  $-80^{\circ}\text{C}$ . Frozen regions were homogenized in 20 volumes of 0.32M sucrose and the suspension centrifuged (ten minutes, 40,000 g). Precipitates were resuspended in 20 volumes cold distilled water and recentrifuged (ten minutes, 40,000 g). The final pellet was then frozen for one hour at  $-20^{\circ}\text{C}$ , thawed and suspended in 20 volumes cold distilled water. Another cycle of centrifuging and refreezing was then carried out. The final pellet was taken up in 20 mM Tris-HCl (pH 7.1) to make a concentration of around 10 mg original tissue/ml. A 0.1 ml aliquot of this was used in the binding study.

In some instances the final suspension was extracted with an equal volume of chloroform-methanol (2:1 v/v) and after centrifugation, the protein residue was washed with 10 ml Tris Buffer, centrifuged and taken up in Tris buffer for incubation.

Heat-denatured albumin and globulin preparations were prepared by heating solutions in distilled water (20 mg/ml) to  $72^{\circ}\text{C}$  for three minutes. The washed precipitate was taken up in Tris buffer (1 mg/ml) for affinity binding assay.

Binding - The standard binding assay was carried out in a volume of 1 ml 50 mM Tris-HCl buffer at  $30^{\circ}\text{C}$  for 15 minutes. The reaction mixture consisted of 0.1 ml of a membrane preparation, and 0.1 to 1  $\mu\text{Ci}$  of a labelled ligand. Non-radioactive ligand or inhibitors were also included in some experiments. Specific activities of the tritiated ligands used were 2- $^3\text{H}$ -glycine (9.39 Ci/mmole), L-6- $^3\text{H}$ -glutamic acid (25 Ci/mmole), L-4-5- $^3\text{H}$  leucine (58 Ci/mmole),  $^3\text{H}$ -GABA (gaseously labelled 12.6 Ci/mmole), L-3-5- $^3\text{H}$ -tyrosine (53 Ci/mmole), ethyl-2- $^3\text{H}$  (n) dopamine (8.4 Ci/mmole), D-4-5- $^3\text{H}$ -leucine (1 Ci/mmole).

The final concentration of labelled compounds was around  $4 \times 10^{-8}\text{M}$ . At the end of incubation, tubes were centrifuged (ten minutes, 40,000 g). Supernatants were collected to determine unbound radioactivity. Pellets were washed once in 4 ml Tris buffer and recentrifuged. These pellets were then dissolved in 0.5 ml of a tissue solubilizer (NCS, Amersham Searle, Inc.) at  $45^{\circ}\text{C}$ . This solution was then mixed with 5 ml of scintillation fluid and radioactivity counted at an efficiency of 31.5%.

### RESULTS

#### Binding of amino acids to brain membranes

Many of the amino acids studied bound to cerebral membranes with high affinity. This binding was stereospecific, saturable and reversible. Scatchard plots were used to determine  $K_D$  values and these were around  $10^{-7}\text{M}$  (Table 1). In our studies, receptor concentration was limiting and was considerably below the  $K_D$  values. Thus, the affinity constants found are not likely to have been underestimated (6).

The inhibition of specific binding of  $5 \times 10^{-8}\text{M}$  amino acids by incubation together with various compounds at  $10^{-4}\text{M}$  is shown in Table 2. Specific binding was taken as that displaced by  $10^{-4}\text{M}$  glutamate and constituted around 80% of all bound  $^3\text{H}$ -glutamate. The only other amino acid that competed strongly for l-glutamate binding sites was l-aspartate. D-

TABLE 1

Density and Dissociation Constants of Specific Receptors  
From Chicken Optic Lobe Membranes

	Site Density (p moles/10 mg Tissue)	$K_D$ ( $\mu$ Molar)
Glutamate	7.2	0.57
Glycine	3.3	0.26
GABA	0.48	0.08
Tyrosine	12.7	0.30
Leucine	15.1	0.14

glutamate and several other amino acids had little effect on the binding of l-glutamate. If  $10^{-4}M$  l-glutamate was added after an initial incubation of membranes with  $5 \times 10^{-8}M$   $^3H$ -l-glutamate, 77% of the specifically bound counts could be displaced. Several amino acids that are not thought to be neurotransmitters also showed high affinity, stereospecific binding to brain membranes. Table 2 shows the extent of cross-competition between various compounds. Leucine binding was blocked by valine but not alanine; B-alanine inhibited glycine binding. No inhibition of glutamate binding by GABA was observed. A similar specificity has been found by Fiszer de Plazas and DeRobertis (7), but inhibition of glutamate binding by GABA has also been reported (3). The interactions we observed possessed a high degree of specificity. The biological relevance of such binding was suggested by the complete failure of two unnatural amino acids (d-leucine and alpha-aminoisobutyric acid) to act as ligands for specific binding.

These events were thought to be true binding and not high affinity transport phenomena for several reasons:

- 1) No dependence on ATP or other energy sources was found.
- 2) No major temperature dependence was detected.
- 3) Frozen-thawed, lysed membrane preparations were used successfully.
- 4) Alpha-aminoisobutyrate, a potent competitor in low affinity uptake systems for neutral amino acids (8) did not block binding.

TABLE 2

Inhibition of Specific Binding of  $5 \times 10^{-8}M$   $^3H$ -Amino Acids to Membranes from Optic Lobes, in the Presence of  $10^{-4}$  Non-Radioactive Amino Acids

Unlabelled Amino Acid	% Inhibition of $^3H$ -Amino Acid Binding				
	GLU	GABA	GLY	LEU	TYR
L-GLU	100	0	30	0	
L-ASP	100				
D-GLU	22				
GABA	0	100	15		
GLY	17	28	100		
L-LEU	20			100	
D-LEU				23	
L-VAL				100	
L-ALA				100	
B-ALA		0	74	0	
L-TYR	8				100
D-TYR					26
AIBA	3	11	31	28	22

- 5) Binding to brain membranes from 11 day old chick embryos was very similar to binding to brain membranes of new-hatched chicks. Thus, no developmental course was apparent.

It is possible that some of the binding measured is to the carrier proteins related to the transport process.

#### Effect of Postsynaptic Inhibitors

Several blockers of postsynaptic binding had little effect on the interaction between brain membranes and putative neurotransmitter amino acids (Table 3). Thus,  $10^{-4}M$  kainate or *n*-methyl aspartate did not block glutamate binding to a great extent,  $10^{-6}M$  strychnine did not interfere with glycine binding and GABA binding was not greatly inhibited in the presence of  $10^{-4}$  imidazole acetic acid, bicuculline or bicuculline methiodide, a derivative that is less subject to hydrolysis. The effect of  $10^{-4}M$  of the unlabelled amino acids on total binding (specific and nonspecific) is also given in order to illustrate the specific nature of most of the binding.

TABLE 3

Inhibition of Total Binding of  $5 \times 10^{-8}M$   $^3H$ -Amino Acids to Optic Lobe Membranes, by Unlabelled Amino Acids and by Pharmacological Agents

$^3H$ -Amino Acid	Addition	% Inhibition of Binding
a) L-GLU	L-GLU ( $10^{-4}M$ )	81
	Kainic acid ( $10^{-4}M$ )	22
	n-Methylaspartate ( $10^{-4}M$ )	14
b) GLY	GLY ( $10^{-4}M$ )	87
	Strychnine ( $10^{-6}M$ )	0
c) GABA	GABA ( $10^{-4}M$ )	66
	Bicuculline ( $10^{-5}M$ )	18
	Imidazoleacetic acid ( $10^{-4}M$ )	0

#### Binding to Non-Neural Membranes and to Non-Membranous Preparations

All compounds that bound to brain membranes also bound with similar characteristics to membranes prepared from liver or heart muscle (Table 4). The specificity, affinity and site-density of these interactions were suggestive of high affinity, specific binding. D-leucine and alpha-amino-isobutyrate did not bind to these membranes.

Since high affinity binding was not confined either to nerve tissue or to amino acids suspected of being neurotransmitters, we investigated the possibility that intact membranes were also not essential for this phenomenon. We have used two non-membranous preparations, both free of lipid, heat denatured albumin and chloroform-methanol treated tissue (Table 5). Specific binding for a variety of amino acids was found in the chloroform-methanol insoluble residue of extracted brains, and this was often very stereospecific. Lesser binding was found in the case of heat denatured albumin, but in both preparations the bulk of binding was specific. A stereospecific low affinity binding site ( $K_D$   $1.6 \times 10^{-4}M$ ) for L-tryptophan has been reported for albumin (9).

TABLE 4

Specific Binding of  $5 \times 10^{-8}M$   $^3H$ -Amino Acids to Membranes  
 Prepared from Various Tissues of the Chick.  
 Values represent bound substances, displaceable by incubation  
 together with the homologous unlabelled amino acid at  $10^{-4}M$ .

	<u>p Moles Bound/10 mg Tissue</u>		
	<u>Brain</u>	<u>Liver</u>	<u>Heart Muscle</u>
L-GLU	7.2	2.3	7.0
GABA	0.48	0.33	0.39
GLY	3.3	1.1	2.8
L-LEU	15.1	2.0	14.9
L-TYR	12.7	7.8	8.9

#### DISCUSSION

The existence of widespread, high affinity binding sites for biological amino acids has not been previously described in higher organisms. However, such sites are known in bacteria where amino acid binding proteins have been purified and appear to be involved in amino acid transport systems (10,11). The specificity that we have found in chick tissue is as great as that reported in bacteria and the corresponding  $K_D$  values are of similar magnitude. Most vertebrate tissues have relatively low affinity transport mechanisms for amino acids with affinity constants around  $10^{-4}M$ . These systems are few in number and are relatively non-specific. The relation between the binding we are reporting and amino acid transport is not clear. It may be that a highly selective binding precedes the less discriminating transport of amino acids across the cell membrane. Amino acid concentrations within the cell membrane are not known and high affinity binding may be compatible with subsequent low affinity transport.

Since postulated blockers of amino acid binding to postsynaptic sites have little effect on the interactions between amino acids and membranes, the bulk of binding sites that we have assayed must be more numerous and dissimilar to postsynaptic binding sites. Such receptors may be resistant to pharmacological anta-

TABLE 5

Total Binding of  $5 \times 10^{-8}M$   $^3H$ -Amino Acids to Chloroform-Methanol Extracted Residual Protein of Chick Brain and to Heat Denatured Albumin

$^3H$ -Amino Acid	Unlabelled Additions ( $10^{-4}M$ )	p Moles Bound/mg Protein	
		CHCl <sub>3</sub> -MeOH Residue	Denatured Albumin
a) L-GLU	-----	1.46	1.38
	L-GLU	0.30	0.30
	D-GLU	1.44	1.37
b) GLY	-----	0.68	0.61
	GLY	0.13	0.10
c) L-LEU	-----	2.56	----
	L-LEU	0.50	----
	D-LEU	2.51	----
d) L-TYR	-----	1.26	0.61
	L-TYR	0.60	0.12
	D-TYR	1.30	0.58

gonists of synaptic binding because of their narrower range of acceptable ligands. Comparisons of density of receptor sites are difficult since other investigators have used a variety of species and brain regions. However, the density of glutamate receptors we found in chick optic lobes (7.2 pmoles/10 mg wet tissue) is considerably greater than that reported for rat brain using  $^3H$ -kainic acid (12) - 1.03 pmoles/mg protein. These authors found that kainic acid did not block glutamate binding and concluded the density of glutamate sites to be 8-10 times greater than the density of kainate sites. In addition, they reported specific glutamate binding in several non-neural tissues. Failure of bicuculline or imidazole acetic acid to severely block GABA binding has also been previously reported (13,14).

Several studies exist where the binding of labelled amino acids in the presence or absence of excess unlabelled amino acid, has been used to identify the properties of postsynaptic receptor sites. These include reports on GABA, glycine and glutamate (1-5, 14). It is possible that non-synaptic binding



could have contributed to the specific binding observed in these studies. Labelled pharmacological antagonists may be more likely than amino acids to have a specific affinity for synaptic binding sites. Another approach to the isolation of binding sites that are related to nerve function may be the chromatographic fractionation of proteins. While we have found that proteins insoluble in organic solvents are able to bind several amino acids stereospecifically, the postsynaptic binding sites for amino acids thought to act as transmitters may be hydrophobic proteolipids (7, 15). The separation of synaptic binding sites from a large excess of non-synaptic sites may be achieved by fractionation techniques.

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