0270-6474/85/0511-2882$02.00/0

The Journal of Neuroscience
Vol. 5, No. 11, pp. 2882-2888
November 1985

The Characterization of the Specific Binding of [^{3}H]-*N*-Acetylaspartylglutamate to Rat Brain Membranes[1]

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Abstract

[^{3}H]-*N*-Acetylaspartylglutamate (NAAG) bound saturably and reversibly to crude synaptosomal rat brain membranes. Optimal binding occurred in Tris-HCl buffer, pH 7.2, at 37°C using previously frozen, preincubated membranes. Saturation experiments revealed an apparent K_D of 383 ± 33 nM and a B_{max} of 31 ± 2 pmol/mg of protein. [^{3}H]NAAG specific binding was displaceable by serine-*o*-sulfate, quisqualate, ibotenate, and glutamate with K_i's in the nanomolar range, whereas the amino-phosphono analogues displaced [^{3}H] NAAG in the micromolar range (APB > APV > APH). No specific binding was found in peripheral tissues. Within the central nervous system, the thalamus exhibited the greatest amount of binding, whereas binding was lowest in cortex. Calcium ions enhanced the specific binding, whereas sodium ions caused a concentration-dependent inhibition. These results suggest that [^{3}H]NAAG labels an acidic amino acid receptor site designated "A-4," which recognizes the antagonist, 2-amino-4-phosphonobutyric acid, and that this receptor may mediate the neurophysiologic effects of endogenous NAAG.

N-Acetylaspartylglutamate (NAAG) is an acidic peptide found in brain that exhibits high affinity for a subpopulation of brain receptor sites labeled with [^{3}H]-L-glutamate (Zaczek et al., 1983; Koller and Coyle, 1984a). Ionophoretic application of NAAG onto pyramidal cells in stratum radiatum of the hippocampus (Bernstein et al., 1985) or on pyramidal cells in the prepyriform cortex receiving excitatory innervation from the lateral olfactory tract (LOT) results in excitation (ffrench-Mullen et al., 1985). Similar to the endogenous neurotransmitter released by the LOT, but in contrast to L-glutamate itself, the excitatory effects of NAAG are antagonized by D,L-2-amino-4-phosphonobutyric acid (APB). In addition, the excitatory effects of NAAG are not antagonized by 2-amino-5-phosphonovaleric acid (APV), which has relatively high specificity for blocking receptors activated by N-methyl-D-aspartic acid (NMDA) (J. M. H. ffrench-Mullen, K. J. Koller, R. Zaczek, J. T. Coyle, N. Hori, and D. O. Carpenter, unpublished observation).

On the basis of neurophysiologic, neuropharmacologic, and ligand-binding studies, Watkins and Evans (1981) and, more recently, Foster and Faag (1984) have distinguished several excitatory amino acid receptors; these include the NMDA receptor, the kainate receptor, and the quisqualate receptor. A fourth receptor has been described on the basis of the observation that L-APB selectively blocks synaptic transmission at sites that are not affected by NMDA receptor antagonists and at concentrations that do not affect the excitation induced by kainate or quisqualate. On the basis of ligand-binding studies, Foster and Fagg (1984) have designated this site as the "A-4" receptor, which binds [^{3}H]APB, exhibits chloride dependence, and is activated by calcium.

The results from displacement studies with NAAG for the specific binding of [^{3}H]-L-glutamate are consistent with NAAG interacting with an excitatory receptor subpopulation that is chloride dependent and activated by calcium (Koller and Coyle, 1984a). Furthermore, the neurophysiologic studies suggest that APB is a selective antagonist of NAAG-induced excitation (ffrench-Mullen et al., 1985). With the recent availability of [^{3}H]NAAG at high specific radioactivity, it is now possible to characterize directly the recognition sites with which it interacts. In a preliminary communication, we demonstrated that [^{3}H]NAAG binds with a high degree of specificity to a single population of noninteracting recognition sites in rat brain membranes (Koller and Coyle, 1984b). In the present paper, we have provided a detailed evaluation of the conditions for and kinetics of the specific binding of [^{3}H]NAAG and present evidence that this endogenous peptide may label the "A-4" excitatory receptor subtype.

Materials and Methods

Materials

Albino Sprague-Dawley rats (100 to 150 gm) were used in all experiments (Harlan-Sprague Dawley, Indianapolis, IN). Aspartylglutamate was a gift from K. Biziere of Clini-Midi, Montpellier, France, and cold NAAG was synthesized from this as previously described (Koller et al., 1984). The identity of NAAG was verified by amino acid analysis and by fast atom bombardment mass spectroscopy (Zaczek et al., 1983). [^{3}H]NAAG (46.6 Ci/mmol) was kindly donated by Dr. Y. P. Wan of the New England Nuclear Corp. (Boston, MA). The ligand was demonstrated to be pure (>95%) by high pressure liquid chromatography criteria (Koller et al., 1984). All other compounds were obtained from Sigma Chemical Co. (St. Louis, MO), Vega Biochemicals (La Jolla, CA), or Cambridge Research Biochemicals (Cambridge, United Kingdom).

Methods

Tissue preparation. Rat forebrain was removed from adult male albino Sprague-Dawley rats and homogenized in 10 vol of ice-cold 0.32 M sucrose. After centrifugation at 1,000 × *g* for 10 min to remove nuclei, the supernatant was centrifuged at 18,000 × *g* for 20 min to isolate a crude synaptosomal preparation (P_2). The P_2 fraction was resuspended in 10 vol of distilled H_2O with a Polytron (setting 6*g*, 30 sec) and centrifuged at 7,500 × *g* for 20 min.

Received November 26, 1984; Revised March 26, 1985;
Accepted May 3, 1985

[1] We thank Alice Trawinski for her excellent secretarial assistance. This work was supported by United States Public Health Service Grant NS-13584 and Research Scientist Development Award MH 00125.

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The supernatant and buffy coat were removed and diluted with 5 ml of 50 mM Tris-HCl, pH 7.2, and the suspension was centrifuged at 40,000 × *g* for 10 min. The supernatant fluid was discarded and the pellet was frozen at −80°C for future use. The frozen pellet was sonicated in 15 vol of 50 mM Tris-HCl, pH 7.2, and preincubated for 30 min at 37°C. Following centrifugation at 40,000 × *g* for 10 min, the pellet was washed two more times in 15 vol of buffer each time. The final pellet was resuspended in buffer such that 0.2 ml contained 100 to 140 μg of protein. Protein was measured by the method of Lowry et al. (1951).

For developmental studies, four pups were sacrificed at each time point (days of age: 2, 8, 14, 21, 28, and 35) from four different litters, and the whole brain, including olfactory bulbs, was prepared as above. The regional study was carried out on tissue from adult male Sprague-Dawley rats dissected by the method of Glowinski and Iversen (1966).

Assay conditions. To measure total binding of [^{3}H]NAAG, each tube contained 100 to 140 μg of protein and 2 pmol of [^{3}H]NAAG in 1 ml of 50 mM Tris HCl, pH 7.2, at 37°C. Nonspecific binding was determined by addition of 5 nmol of L-glutamate or 1 nmol of nonradioactive NAAG, each of which gave equivalent blank values. Saturation isotherms were generated by diluting 2 nM [^{3}H]NAAG with increasing concentrations of nonradioactive NAAG with correction for the final specific radioactivity of the NAAG.

The reaction mixture was incubated at 37°C for 45 min in glass 12 × 75 mm tubes, and the assay was terminated by filtration over Whatman GF/B glass-fiber filters in a modified Brandel Cell harvester. Each filter was washed with 15 ml of 50 mM Tris-HCl, pH 7.2, at 37°C, and radioactivity was measured by scintillation spectrometry in a Beckman LS 2800 scintillation counter after solubilizing the filters in 10 ml of Formula 947 (New England Nuclear).

Results

Effects of assay conditions on the specific binding of [^{3}H]NAAG. In the initial experiments, incubation conditions and buffer concentrations previously demonstrated to be associated with optimal specific binding of [^{3}H]-L-glutamate that is displaceable by NAAG were used to characterize the specific binding of [^{3}H]NAAG. In these initial assays, 2 nM [^{3}H]NAAG was incubated with 100 μg of membrane protein prepared from lysed synaptosomes for 45 min at 37°C in Tris-HCl buffer at pH 7.2. The ligand-receptor complex was harvested by filtration over glass fiber filters. As shown in Table I, in the absence of synaptosomal homogenate, binding to the filter represented only 52 cpm. The addition of 0.5 mM L-glutamate or 0.1 mM unlabeled NAAG in the presence of buffy coat membranes resulted in binding only 2-fold greater than in the absence of tissue.

TABLE I

Effect of various tissue treatments on total [^{3}H]NAAG binding

Crude synaptosomal membranes from rat forebrain were prepared as described under "Materials and Methods." Tissue was used either fresh or frozen at −80°C for at least 24 hr. Standard conditions refer to tissue (100 μg of protein) incubated for 45 min at 37°C in Tris-HCl buffer, pH 7.2, in the presence of 2 nM [^{3}H]NAAG with or without 0.1 mM unlabeled NAAG or 0.5 mM glutamate. Values are the mean ± SEM of three experiments performed in duplicate.

Condition	Total [^{3}H]NAAG Bound (cpm)
No tissue	52 ± 4
Fresh tissue	1116 ± 18
Fresh tissue + 500 μM L-Glu	120 ± 9
Fresh tissue + 100 μM NAAG	118 ± 4
Fresh; preincubated (37°C, 30 min)	1982 ± 55
Fresh; preincubated (37°C, 30 min) + 500 μM L-Glu	67 ± 9
Fresh, heat treated (85°C, 15 min)	232 ± 10
Fresh, heat treated (85°C, 15 min) + 500 μM L-Glu	213 ± 12
Frozen	1471 ± 23
Frozen + 500 μM L-Glu	79 ± 4
Frozen, preincubated (37°C, 30 min)	2165 ± 45
Frozen, preincubated (37°C, 30 min) + 500 μM L-Glu	61 ± 3

Optimal specific binding was observed with buffy coat membranes that had been frozen at −80°C and then preincubated for 30 min at 37°C prior to the addition of [^{3}H]NAAG. Freshly prepared membranes preincubated at 37°C for 30 min or membranes that had been frozen at −80°C but not preincubated yielded significantly lower levels of specific binding. Heat denaturation of the buffy coat membranes by incubation at 85°C for 15 min resulted in nearly complete loss of specific binding.

Whereas specific binding of [^{3}H]NAAG appeared linearly related to protein concentration up to 150 μg of protein ($r = 0.97$, $N = 6$), deviation from linearity became apparent with concentrations of 200 μg protein or greater. Since buffer conditions play a critical role in the characteristics of receptor site labeled by [^{3}H]-L-glutamate (Foster and Fagg, 1984), the influence of different buffers on the specific binding of [^{3}H]NAAG was examined. The pH curve generated with Tris-HCl buffer revealed optimal binding between pH 7 and pH 7.5 with striking reductions in the amount specifically bound at a greater or lesser pH (Fig. 1). With pH held constant at 7.2, the effects of buffer type and temperature of incubation on the specific binding of [^{3}H]NAAG were examined (Table II). Optimal binding was observed with Tris-HCl buffer, whereas negligible binding was detected with Tris-citrate or $NaKPO_4$ buffer. That chloride ion played an important role in specific binding is suggested by the observation that MOPS (3-(*N*-morpholino)propanesultonic acid) buffer plus 10 mM NH_4Cl resulted in specific binding of approximately 50% of that seen with Tris-HCl. Specific binding was temperature dependent, with the greatest amount observed at 37°C, and binding was reduced by 60 to 70% at 0°C in the presence of chloride-containing buffer.

Kinetics of the specific binding of [^{3}H]NAAG. Saturation isotherm for the specific binding of [^{3}H]NAAG (2 nM) was carried out by sequential dilution with unlabeled NAAG in which the final ligand concentration was corrected for specific radioactivity. As shown in Figure 2, the specific binding of [^{3}H]NAAG is saturable. Hill transformation of these data (Bylund, 1980) revealed a Hill coefficient of 0.98 and an apparent K_D of 327 nM. Using Scatchard analysis (Scatchard, 1949), the K_D is 383 ± 33 nM and the B_{max} is 31 ± 2 pmol/mg of protein ($N = 4$). With endogenous NAAG purified to homogeneity as previously described (Zaczek et al., 1983), the Hill transformation of the displacement curves indicated an apparent K_D of 320 nM with a Hill coefficient of 0.99. Thus, endogenous NAAG and synthetic NAAG exhibited identical kinetic interactions with the binding site. With 2 nM [^{3}H]NAAG, binding achieved equilibrium between 30 and 45 min (Fig. 3). This binding was shown to be

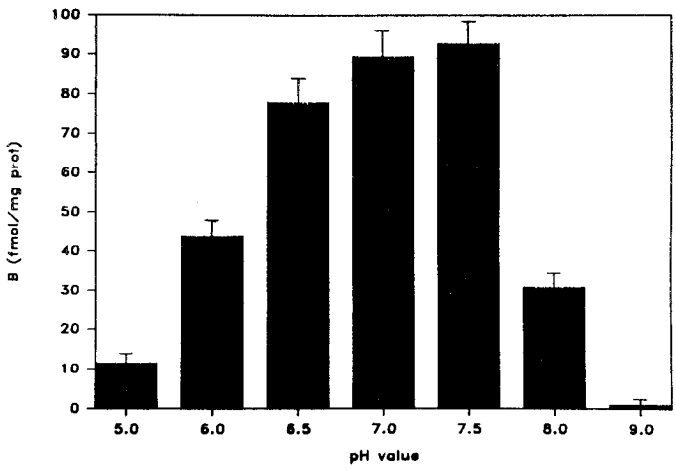

Figure 1. The effect of pH on specific binding of [^{3}H]NAAG. Crude synaptosomal membranes were prepared in Tris buffer adjusted to the appropriate pH with concentrated HCl. One hundred micrograms of protein for each pH value were incubated for 45 min at 37°C in the presence of 2 nM [^{3}H]NAAG with or without 0.5 mM L-glutamate. Each value represents the mean of two separate experiments conducted in triplicate.

TABLE II

Effects of various assay conditions on [^{3}H]NAAG binding

Crude synaptosomal membranes were prepared as described under "Materials and Methods." Fresh tissue was preincubated at 37°C for 30 min before use. For standard conditions, 100 μg of protein were incubated for 45 min at 37°C in the designated buffer in the presence of 2 nM [^{3}H]NAAG with or without 0.5 mM glutamate. Values are the mean ± SEM of three separate experiments performed in duplicate.

Condition		Specifically Bound [^{3}H]NAAG (fmol/mg of protein)
Buffer	Temperature	
Tris-HCl, pH 7.2	0°C	35.6 ± 1.2
	25°C	83.9 ± 3.3
	37°C	113.4 ± 6.4
Tris-citrate, pH 7.2	0°C	1.1 ± 0.9
	25°C	1.8 ± 0.9
	37°C	2.0 ± 0.7
$NaKPO_4$, pH 7.2	0°C	1.4 ± 0.5
	25°C	0.55 ± 0.2
	37°C	UD[a]
MOPS + 10 mM	0°C	16.7 ± 2.0
NH_4Cl, pH 7.2	25°C	37.2 ± 3.5
	37°C	65.4 ± 4.6

[a] UD, undetectable.

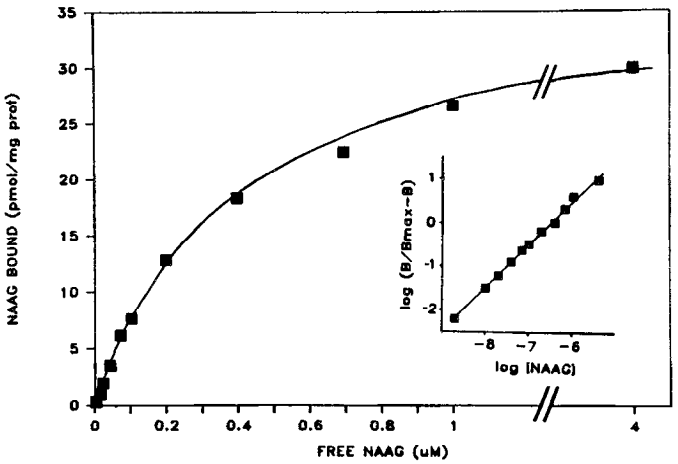

Figure 2. Saturation isotherm for [^{3}H]NAAG specific binding in rat forebrain membranes. Buffy coat membranes prepared from rat forebrain were preincubated for 30 min at 37°C, isolated by centrifugation, and resuspended in 50 mM Tris-HCl buffer, pH 7.2. Aliquots containing 100 μg of protein were incubated for 30 min in the presence of varying concentrations of [^{3}H]NAAG; nonspecific binding was measured with 100 μM L-glutamate. Membrane-ligand complex was isolated by aspiration over Whatman GF/B glass-fiber filters. The *inset* presents the data transformed into a Hill plot. The Hill coefficient is 0.98 and K_D is 327 nM.

reversible since the addition of an excess of L-glutamate after equilibrium was reached resulted in a rapid decrease in [^{3}H]NAAG specifically bound.

Displacement curves of the specific binding of 2 nM [^{3}H]NAAG were generated for a variety of substances reputed to act as agonists or antagonists at glutamate receptors or analogues of NAAG. As shown in Figure 4, unlabeled NAAG, L-glutamate, and APB exhibited parallel displacement curves with NAAG having approximately 3-fold greater affinity than L-glutamate and APB being approximately 10 times less potent than L-glutamate. All three displacers exhibited Hill coefficients of approximately 1.0, consistent with their interaction at a single population of noninteracting sites.

Pharmacology. Displacement of [^{3}H]NAAG by various compounds was studied and their K_i values are listed in Table III. Notably, the site labeled by [^{3}H]NAAG exhibited stereospecificity since D-Glu was nearly 10 times less potent than L-glutamate and, although L-aspartate exhibited a K_i of 3 μM, D-aspartate was ineffective at the site. Of the peptides studied (all L-isomers with α-peptide bonds), those enriched in acidic amino acids were active at the site. Specifically, Glu-Glu and Asp-Glu were very potent, whereas Glu-Asp, Asp-Asp, *N*-acetyl-Glu-Asp and Val-Gly-Asp-Glu, a chemotactic substance, all interacted with the site to a much lower degree. In addition, a large number of sulfur-containing compounds were recognized by this site. Notably, serine-*o*-sulfate was the most potent compound studied. L-Cysteine, D-homocysteic acid, L-cysteic acid, and L-cysteinesulfonic acid also displace [^{3}H]NAAG. Both quisqualate and ibotenate were potent in their interaction with K_i's in the nanomolar range. Finally, the amino-phosphono analogues were also active at

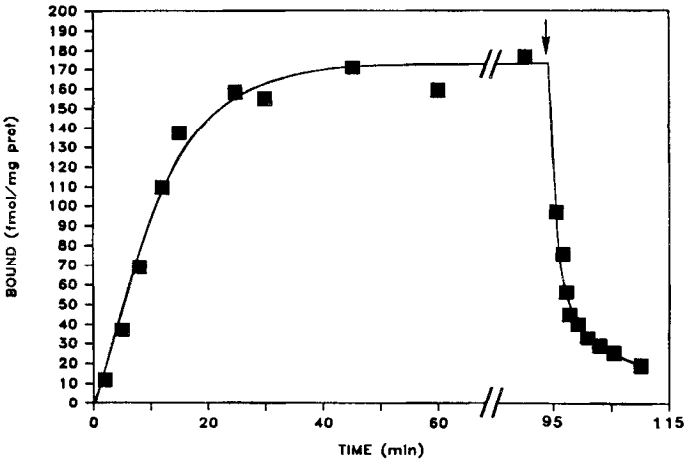

Figure 3. Reversibility of [^{3}H]NAAG specific binding. Crude synaptosomal membranes of rat forebrain were prepared as described under "Materials and Methods" and were incubated at 37°C in the presence of 2 nM [^{3}H]NAAG with or without 0.5 mM L-glutamate. The assay was terminated at various times and the total amount bound was determined. After equilibrium, a large excess of L-glutamate was added (at *arrow*), and at various times, the assay was terminated and the amount of [^{3}H]NAAG specifically bound was determined. The results are the mean of two experiments with triplicate determinations.

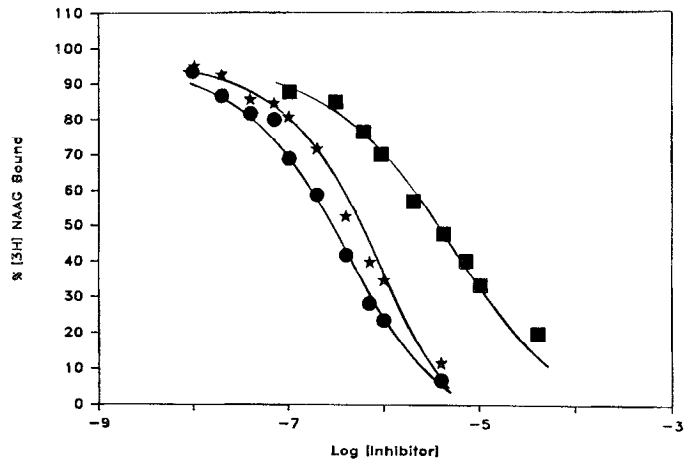

Figure 4. Inhibition of the specific binding of [^{3}H]NAAG by unlabeled NAAG, glutamate, and APB. Crude synaptosomal membranes from rat forebrain were prepared as described under "Materials and Methods" and incubated (45 min, 37°C) in the presence of [^{3}H]NAAG (2 nM) with at least 10 concentrations of unlabeled NAAG (●—●), L-glutamate (★—★), and APB (■—■). Nonspecific binding was determined by the addition of 0.5 mM L-glutamate, and the reaction was terminated by filtration. The results are the mean of two experiments with triplicate determinations.

this site. APB was the most potent (K_i = 3.86 μM), and both APV and D,L-2-amino-7-phosphonoheptanoic acid (APH) displaced with K_is of 6 and 11.4 μM, respectively.

Table IV lists the compounds tested with K_i values greater than 0.1 mM. Among these substances are amino acids such as GABA, glycine, *N*-acetylaspartate, and glutamate diethylester. A variety of aspartyl-peptides were inactive, as was the neuronal tripeptide, thyrotropin-releasing hormone. Of the excitatory amino acid analogues tested, NMDA, kainate, and quinolinic acid were also ineffective. In addition, 2-amino-3-phosphonopropionate was the only amino-phosphono analogue tested that was ineffective.

Regional and peripheral distribution of specifically bound [^{3}H]NAAG. The regional distribution of [^{3}H]NAAG binding was examined in seven regions of the brain as well as in spinal cord (Table V). Thalamus exhibited the greatest amount of binding at 2 nM (210 ± 25 fmol/mg of protein), and spinal cord had the next highest amount (203 ± 13 fmol/mg of protein). Cortex had substantially lower specific binding (132 ± 14 fmol/mg of protein), being only 63% of the level in thalamus. No significant binding of NAAG was seen in the peripheral tissues studied (Table VI). The amount of specific binding in heart, liver, kidney, spleen, and lung ranged from −0.8% to 1.4% of the amount seen in the thalamus. Notably, no specific binding was seen in the intestine, an area where many other neuronal peptides are active.

Ion effects. As demonstrated in Table II, the binding of [^{3}H]NAAG

TABLE III

K_i values for displacers of [^{3}H]NAAG

The compounds indicated were incubated with crude synaptosomal membranes (100 μg of protein) prepared from rat forebrain in the presence of [^{3}H]NAAG (2 nM) as described under "Materials and Methods." Each value is the mean of three separate experiments consisting of five concentrations of the competing agent. Each drug concentration was assayed in duplicate. The K_i values were calculated according to the method of Bylund (1980). Nonspecific binding was determined with 0.5 mM L-glutamate.

Compound	K_i (μM)	Compound	K_i (μM)
Amino Acids		*Excitatory Amino Acid Analogues*	
L-Cysteine	0.331	Serine-*o*-sulfate	0.115
L-Glu	0.460	Quisqualate	0.407
L-Asp	3.00	Ibotenate	0.525
D-Glu	5.00	α-Aminosuberic acid	1.33
N-Acetyl-Glu	19.0	D-Homocysteic acid	1.34
D-Asp	>100	L-Cysteinesulfonic acid	2.37
Peptides		L-Cysteic acid	2.50
Glu-Glu	0.275	D,L-2-Amino-4-phosphonobutyric acid	3.86
Asp-Glu	0.400	D(−)-2-Amino-5-phosphonovaleric acid	6.00
Glu-Asp	5.00	D,L-2-Amino-7-phosphonoheptanoic acid	11.4
Glutathione (reduced)	12.5		
Asp-Asp	13.8		
Val-Gly-Asp-Glu	23.6		
N-Acetyl-Glu-Asp	74.5		

TABLE V

Regional distribution of [^{3}H]NAAG binding

Various regions of the rat CNS were dissected and prepared as described under "Materials and Methods." Two-tenths milliliter of the washed sonicate (100 μg of protein) was added to tubes containing 2 nM [^{3}H]NAAG with or without 0.5 mM L-glutamate. Following a 45-min incubation at 37°C, the reaction was terminated by filtration, and the radioactivity was determined by liquid scintillation spectrophotometry. Values represent the mean ± SEM of five separate experiments each done in duplicate.

Region	[^{3}H]NAAG Specifically Bound (fmol/mg of protein)	Percentage of Thalamus
Thalamus	210 ± 25	100
Spinal cord	203 ± 13	97
Hippocampus	202 ± 17	96
Cerebellum	191 ± 26	91
Corpus striatum	171 ± 16	81
Midbrain	154 ± 8	73
Brainstem	147 ± 22	70
Cortex	132 ± 14	63

TABLE VI

[^{3}H]NAAG binding to peripheral tissues

The indicated tissue was dissected, frozen immediately on dry ice, and stored at −80°C for at least 24 hr. Prior to assay, the tissue was sonified in 20 vol of 50 mM Tris-HCl buffer, preincubated at 37°C for 30 min, and washed three times by centrifugation. Two-tenths-milliliter portions of the tissue representing 100 μg of protein were incubated for 45 min at 37°C in the presence of 2 nM [^{3}H]NAAG with or without 0.5 mM L-glutamate. Values are the mean ± SEM of three separate experiments each done in triplicate.

Region	[^{3}H]NAAG Bound (fmol/mg of protein)		Specifically Bound % of Thalamus
	Total	Nonspecific	
Heart	6.4 ± 3.0	3.9 ± 1.4	1.2
Liver	6.5 ± 1.7	8.2 ± 4.3	−0.8
Kidney	4.0 ± 0.3	3.2 ± 0.2	0.4
Intestine	7.5 ± 1.6	7.9 ± 1.2	−0.2
Spleen	6.7 ± 1.8	3.8 ± 0.8	1.4
Lung	7.1 ± 1.2	6.2 ± 1.0	0.4

TABLE IV

Compounds with a K_i value greater than 0.1 mM

The compounds indicated were incubated with 100 μg of protein from rat forebrain crude synaptosomal membranes as described under "Materials and Methods." Compounds were tested at 0.1 mM and inhibited the specific binding of [^{3}H]NAAG (2 nM) by less than 50%. Nonspecific binding was determined with 0.5 mM L-glutamate.

Amino Acids	Peptides	Excitatory Amino Acid Analogues
γ-Aminobutyric acid	L-Aspartyl-L-phenylalanine	Kainate
β-Alanine	L-Aspartyl-L-lysine	Dihydrokainate
Leucine	L-Aspartyl-L-alanine	Quinolinic Acid
Glycine	L-Aspartyl-L-valine	AMPA
L-Pyroglutamate	β-Aspartyl-histidine	Cystathione
Taurine	L-Aspartyl-L-leucine	2-Amino-3-phosphonopropionic acid
N-Acetylaspartate	L-Aspartyl-L-glycine	*cis*-2,3-Piperidine dicarboxylic acid
D-Aspartate	γ-D-Glutamylglycine	*cis*-2,4-Piperidine dicarboxylic acid
Glutamate diethylester	Thyrotropin-releasing hormone	*cis*-2,5-Piperidine dicarboxylic acid
N-Methyl-D,L-Aspartate	(pGlu-His-Pro-NH_2)	*cis*-2,6-Piperidine dicarboxylic acid

was dependent on the presence of chloride ions in the buffer. In addition to chloride, however, the specific binding of [^{3}H]NAAG in forebrain membranes was enhanced by calcium (Fig. 5). Calcium acetate had no effect on binding at 10 μM, but at 100 μM, binding was enhanced 57%. A peak of enhancement occurred at 1 mM (+119%), whereas at 100 mM, specific binding fell to 10% of control values. Sodium ion caused a concentration-dependent decrease in specific binding of [^{3}H]NAAG (Fig. 5). No significant change was seen at 10 μM, but binding decreased 12% at 0.1 mM and was 75% less than control at 100 mM.

The effect of nucleotides on [^{3}H]NAAG specific binding. Table VII illustrates the effect of various nucleotides at displacing [^{3}H]NAAG. 5′-Guanylylimidodiphosphate (GppNHp), the nonhydrolyzable analogue of GTP, was the most potent among the guanyl nucleotides. Both GTP and GDP were effective at displacing, whereas GMP, cGMP, and guanosine were ineffective. In addition to the guanyl nucleotides, however, adenyl, cytodyl, and uradyl nucleotides displaced [^{3}H]NAAG. The triphosphates were the most potent, followed by the diphosphates. AMP, cAMP, and adenosine were also ineffective. The interaction of GppNHp with the receptor appears to be competitive since saturation isotherms of [^{3}H]NAAG with and without 10 μM GppNHp resulted in a K_D shift from 354 nM in control to 634 nM with GppNHp. No significant change in B_{max} was observed (33.2 versus 31.2 pmol/mg of protein; data not shown).

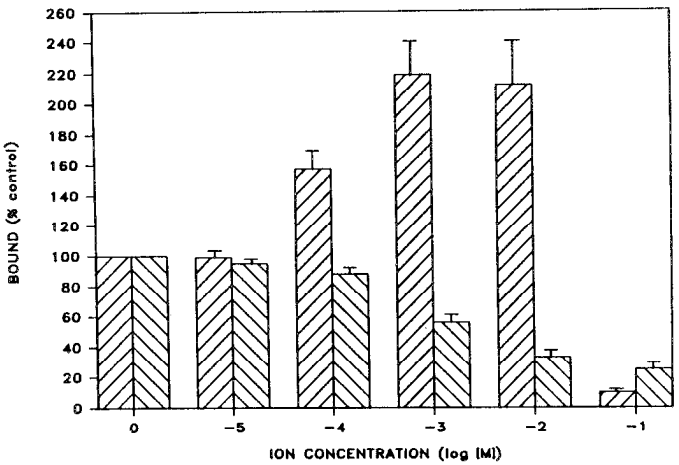

Figure 5. The effect of addition of ions on the specific binding of [^{3}H]NAAG. Crude synaptosomal membranes were prepared as described under "Materials and Methods." Tissue (100 μg of protein) was incubated (45 min, 37°C) in the presence of 2 nM [^{3}H]NAAG in 50 mM Tris-HCl, pH 7.2, with five concentrations of calcium acetate (▨) and sodium acetate (▧). Nonspecific binding was determined by the addition of 0.5 mM L-glutamate. Each value is the mean of four separate experiments. *Error bars* represent SEM.

TABLE VII

Effect of nucleotides on [^{3}H]NAAG binding

The compounds indicated were incubated with crude synaptosomal membranes (100 μg of protein) prepared from rat forebrain in the presence of 2 nM [^{3}H]NAAG as described under "Materials and Methods." Each value is the mean of three separate experiments consisting of 5 to 10 concentrations of the competing agent assayed in duplicate. K_I values were determined by the method of Bylund (1980).

Nucleotide	K_I (μM)
GppNHp	10.7
GTP	18.3
GDP	21.8
GMP	>400
CGMP	>400
Guanosine	>400
CTP	10.1
UTP	15.2
ATP	22.5
UDP	29.9
ADP	30.5
CDP	42.0
AMP	>400
cAMP	>400
Adenosine	>400

Development. The development of [^{3}H]NAAG binding was studied in rat whole brain. Saturation isotherms were performed at each age (N = 4), and the K_D and B_{max} were approximated by Scatchard (1949) analysis (Table VIII). Whereas the K_D at the ages studied increased slightly less than 2.5 times (from 197 nM to 472 nM), the B_{max} increased linearly more than 20-fold until day 35 of age, which was comparable to the adult density. Notably, the developmental profile differs from that observed for the postnatal increases in endogenous NAAG concentration in brain (Koller and Coyle, 1984c), although it is consistent with the developmental increases in other neurotransmitter receptors (Lanier et al., 1976; Coyle, 1977).

Discussion

As previously reported for synaptic membranes (Slevin et al., 1982) and supported by *in vitro* autoradiographic techniques (Monaghan et al., 1983a), [^{3}H]glutamate binds to recognition sites in brain that can be further subdivided on the basis of differential displacement by analogues and antagonists. The endogenous brain peptide, NAAG, distinguishes in displacement studies a subpopulation of these [^{3}H]glutamate recognition sites (Zaczek et al., 1983; Koller and Coyle, 1984a). The site to which [^{3}H]NAAG binds exhibits K_I values for L-glutamate and for quisqualate that are comparable to the values observed at the specific binding site for [^{3}H]-L-glutamate. These agents apparently bind with relatively high affinity to that portion of the [^{3}H]NAAG site that recognizes the glutamate moiety. Other acidic excitatory amino acids such as L-cysteine and serine-o-sulfate also probably interact with this portion of the recognition site. In contrast, the *N*-acetylated acidic amino acids, *N*-acetylglutamate and *N*-acetylaspartate, are quite weak at competing for the specific binding of [^{3}H]NAAG, which suggests that the *N*-acetyl moiety itself is not a major determinant of receptor affinity. The importance of a terminal glutamate is further reinforced by the fact that both Glu-Glu and Asp-Glu have substantial affinities for the NAAG recognition site, whereas Glu-Asp and Asp-Asp have at least a magnitude lower affinity. Notably, the receptor-specific agonists, NMDA and kainic acid, have negligible affinity for the [^{3}H]NAAG binding site.

These results suggest that this subpopulation of [^{3}H]glutamate

TABLE VIII

Development of [^{3}H]NAAG in rat whole brain

Rat pups were sacrificed at the ages indicated and the whole brain was removed. Crude synaptosomal membranes were prepared as under "Materials and Methods" and kept frozen at −80°C until use. Saturation isotherm experiments were conducted diluting 2 nM [^{3}H]NAAG with unlabeled NAAG to cover the concentration range of 2 nM to 4000 nM. Scatchard analysis was performed to estimate the K_D and B_{max} values. Each value is the mean ± SEM of four separate animals each taken from a distinct litter.

Age	K_D (nM)	B_{max} (fmol/mg of wet weight)
Day 2	197 ± 45	50 ± 24
Day 8	242 ± 22	137 ± 12
Day 14	268 ± 8	326 ± 57
Day 21	321 ± 28	790 ± 171
Day 28	366 ± 10	881 ± 103
Day 35	472 ± 20	1080 ± 177

recognition sites has a domain sufficiently large to bind the dipeptides NAAG, Glu-Glu, and Asp-Glu. In this regard, physiologic studies have indicated the excitatory effects of both NAAG and Asp-Glu, which exhibit physiologic and neuropharmacologic effects in slice studies in prepyriform cortex and hippocampus that are more consistent with an endogenous neurotransmitter role than glutamate itself (Bernstein et al., 1985; ffrench-Mullen et al., 1985). Whether NAAG and Asp-Glu are interacting at the same receptors, as suggested by the ligand-binding studies, or at different receptors remains to be determined.

These studies have revealed important regulatory properties for the subpopulation of the glutamate receptors labeled by [^{3}H]NAAG. The [^{3}H]NAAG binding site is highly dependent upon the presence of chloride consistent with a similar anionic dependency for a major population of the receptors labeled by [^{3}H]-L-glutamate (Fagg et al., 1982). Calcium ions have also been demonstrated to augment the specific binding of specific chloride-dependent binding of [^{3}H]-L-glutamate in a regionally specific fashion which is due to the increase in the B_{max} of the receptors for the ligand (Fagg et al., 1982). Similar activation of specific binding of [^{3}H]NAAG has been demonstrated with a greater than 2-fold increase in the specific binding in the presence of 1 mM Ca^{2+}. In contrast, sodium ion decreases the specific binding of [^{3}H]glutamate to the chloride-dependent site due to a reduction in the B_{max} (Foster and Fagg, 1984; Malouf et al., 1984); a similar dose-response relationship of inhibition with sodium ion has been demonstrated with the [^{3}H]NAAG-binding site. Taken together, these similarities in pharmacology, kinetics of inhibition, and ionic effects provide compelling evidence that [^{3}H]NAAG binds to a discrete subpopulation of chloride-dependent receptors labeled in a homogenous fashion by [^{3}H]glutamate.

The glutamate receptor has been reported to be linked to a regulatory subunit that utilizes guanine nucleotides (Foster and Roberts, 1980; Sharif and Roberts, 1980a, b). Accordingly, we examined the effects of various nucleotides on the specific binding of [^{3}H]NAAG. Although GTP and GppNHp both inhibited the specific binding of [^{3}H]NAAG, consistent with this hypothesis, ATP, ADP, CTP, CDP, UTP, and UDP also acted as inhibitors. In a kinetic analysis with GppNHp, the reduction in specific binding of [^{3}H]NAAG was shown to be due to a decrease in affinity, consistent with a competitive type of inhibition. The general displacement of [^{3}H]NAAG by tri- and diphosphates (and the inactivity of monophosphate, cyclic nucleotides, or the bases alone) suggests an ionic interaction rather than a specific guanine nucleotide-regulatory site interaction (Rodbell, 1980).

Recently, Foster and Fagg (1984) have classified the excitatory amino acid-binding sites into four receptors (A1 to A4) and one uptake site. This classification was based upon the pharmacology of the binding sites with different ligands and the effects of ions on this binding. With this classification scheme, the results obtained in the present studies suggest that [^{3}H]NAAG may bind to the acidic amino acid "A4" site. Both of these sites are dependent on chloride for binding, and binding is enhanced by Ca^{2+} and inhibited by Na^+. In addition to the ionic interactions, the pharmacology of these sites is quite comparable. Quisqualate, ibotenate, and serine-*o*-sulfate all display K_i's in the nanomolar range, whereas APB, APV, and APH are effective at micromolar concentrations. Notably, the sites seem to be relatively insensitive to NMDA and kainate. One discrepancy that does exist concerns the effects of freeze-thawing. Although the "A4" site is apparently abolished by freeze-thawing, the B_{max} of the sites labeled by [^{3}H]NAAG is not significantly different with fresh or frozen tissue, whereas the K_D decreases by 2-fold after freezing of the tissue (data not shown).

Aside from the discrepancy with regard to fresh versus frozen tissue, the site labeled by [^{3}H]NAAG and the "A4" site described by Foster and Fagg (1984) are remarkably similar. Notably, APB and glutamate are ligands that have been used previously to describe this site (Fagg et al., 1982; Butcher et al., 1983; Monaghan et al., 1983b). In the LOT, which has been considered an aspartergic/glutamatergic pathway (Harvey et al., 1975; Bradford and Richards, 1976; Collins, 1978), only the effects of NAAG and the endogenous neurotransmitter are blocked by the antagonist, APB, whereas the excitatory effect of glutamate is insensitive to this concentration of APB (ffrench-Mullen et al., 1985). Thus, the binding site labeled by [^{3}H]NAAG exhibits a pharmacology *in vitro* consistent with what has been described in the neurophysiologic studies. These results indicate that [^{3}H]NAAG is a useful ligand for studying this subpopulation of acidic amino acid receptors and will be of assistance in further characterizing the receptors that mediate its neurophysiologic effects in brain. They lend additional support to the hypothesis that small, acidic peptides in brain may act as the neurotransmitters at reputed glutamatergic synapses.

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