Amino Acid Neurotransmitter Candidates: Sodium-Dependent High-Affinity Uptake by Unique Synaptosomal Fractions

Abstract. *Glutamic and aspartic acids and glycine are accumulated by high-affinity uptake systems into synaptosomal preparations in central nervous tissue. Sodium is required by these high-affinity transports, but not by the low-affinity transports for these and other amino acids. The sodium-requiring amino acid uptake systems label unique synaptosomal fractions. Observations suggest that these amino acids serve specific synaptic functions, presumably as neurotransmitters.*

Several amino acids, especially glutamic and aspartic acids and glycine, have been suggested as neurotransmitter candidates in the central nervous system on the basis of microiontophoretic (*1*), regional (*2*), autoradiographic (*3*), and selective release (*4*) studies. We observed transport of glutamic and aspartic acids and glycine into synaptosomal preparations by unique high-affinity uptake systems (*5*). The selective localization of the glycine uptake system to the spinal cord but not the cerebral cortex paralleled the distribution of neurons that are specifically sensitive to glycine. Furthermore, we observed a unique synaptosomal fraction in the cerebral cortex which selectively accumulated glutamic and aspartic acids (*6*).

We report here that sodium is required for activity of the high-affinity uptake systems for glutamic and aspartic acids in the cerebral cortex and spinal cord and for glycine in the spinal cord; this requirement is not manifested by the low-affinity transport systems for these and other amino acids (*7*). Moreover, although glutamic acid is accumulated into a unique synaptosomal fraction in the presence of sodium (*6*), the subcellular localization of glutamic acid is indistinguishable from that of other amino acids if sodium is absent.

Male Sprague-Dawley rats (150 to 200 g) were decapitated; the cerebral cortex or lumbosacral spinal cord was rapidly dissected and homogenized in 20 volumes of 0.32*M* sucrose (4°C) in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle, and amino acid uptake into synaptosomal-rich pellets was examined (*5*). After centrifugation at 1000*g* for 10 minutes, the pellet was discarded. A 0.2-ml portion was added to 3.8 ml of Krebs-Ringer medium buffered with phosphate (*p*H 7.4) when normal, deficient, or excessive sodium concentrations were desired, or to Krebs-Ringer medium buffered with tris(hydroxymethyl)aminomethane (tris) (*p*H 7.4) when no sodium ion was desired. The standard sodium concentration employed, unless otherwise indicated, was 143 m*M*. In sodium-deficient media, isotonicity was maintained by equimolar amounts of 0.32*M* sucrose. The media also contained 11.1 m*M* glucose and varying concentrations of unlabeled amino acids. After 4 minutes of incubation at 37°C, samples were cooled to 4°C, and a uniform tracer amount of isotopically labeled amino acid (1 to 5 c/mmole; New England Nuclear) was added. The mixtures were then agitated another 4 minutes at 37°C, rapidly cooled to 4°C, and centrifuged at 27,000*g* for 10 minutes. The supernatant fluid was assayed for radioactivity, and the pellet was washed with 10 ml of 0.9 percent NaCl (4°C) and recentrifuged at 48,000*g* for 10 minutes at 4°C. Accumulated radioactivity was extracted into Triton X-100 : toluene phosphor and assayed by liquid scintillation spectrometry. Under these conditions, we had shown that amino acid uptake is linear with time at all concentrations employed, that accumulated amino acids are not metabolized to a significant extent, and that essentially all radioactivity accumulated in the pellet is localized in synaptosomal fractions isolated by isopycnic sucrose gradient centrifugation (*5*). For subcellular fractionation studies, the incubation procedures described above were followed, and the "crude synaptosomal" pellets were rinsed with 0.32*M* sucrose, layered on linear continuous (1.5 to 0.5*M*) sucrose gradients, centrifuged for 15 minutes at 100,000*g*, and fractionated as described (*6*). Ionic requirements for the high- and low-affinity uptakes were compared by the use of low ($10^{-5}M$) and high ($10^{-3}M$) concentrations of amino acids.

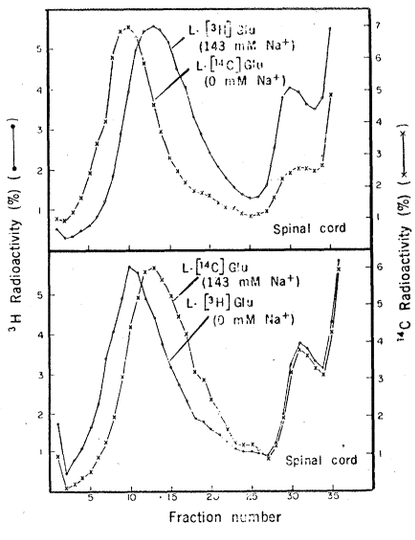

Fig. 1. Distribution patterns in linear, continuous sucrose density gradients of synaptosomal fractions that accumulate [^{3}H]- and [^{14}C]glutamic acid in the presence of normal sodium concentration (143 m*M*) or no added sodium. Homogenates from rat spinal cord were incubated with $10^{-5}M$ L-glutamic acid (L-[^{3}H]Glu or L-[^{14}C]Glu) in Krebs-Ringer-phosphate solution (143 m*M* sodium) or Krebs-Ringer-tris solution (0 m*M* sodium) and subjected to subcellular fractionation.

When low amino acid concentrations were used, glutamic and aspartic acid uptake in the cerebral cortex was markedly dependent on the presence of sodium (Table 1). With omission of sodium from the medium, glutamic and aspartic acid uptake fell by 93 to 96 percent; glycine, alanine, leucine, and arginine uptakes were much less affected. In the spinal cord, glutamic and aspartic acid transport showed a sodium requirement similar to that observed in the cerebral cortex. Glycine accumulation in the spinal cord required sodium, whereas uptake of this amino acid in the cerebral cortex was not markedly influenced by sodium. By contrast, none of the amino acids examined at high concentrations showed a marked sodium requirement in the cerebral cortex or spinal cord; and the partial sodium dependence for uptake of glutamic and aspartic acids and glycine may simply reflect amino acid still entering the high-affinity systems. The sodium requirement for the high-affinity transport systems for glutamic and aspartic acids and glycine could not be satisfied by equimolar concentrations of tris, choline, or lithium.

The sodium requirement for transport of 10^{-3} and $10^{-5}M$ glutamic and aspartic acids and glycine was further examined with ten different sodium concentrations between 0 and 200 m*M*. Uptake of $10^{-5}M$ glutamic and aspartic acids in cerebral cortex and spinal cord declined about 75 percent when sodium was reduced from 30 to 0 m*M*. The

Table 1. Effects of sodium omission on the uptake of various amino acids into synaptosomal fractions. Homogenates from rat cerebral cortex or lumbosacral spinal cord were incubated with 10^{-3} and $10^{-5}M$ amino acids in Krebs-Ringer-phosphate solutions for normal sodium concentration (143 mM) or Krebs-Ringer-tris solutions for deficient sodium concentration (0 mM). Isotonic sucrose (0.32M) was used to replace sodium in the sodium-deficient media. After centrifugation, the pellets were analyzed for accumulated [^{3}H]amino acids. Values for glutamic acid do not take into account the endogenous glutamic acid released into the medium by homogenization ($1.5 \times 10^{-5}M$). Data are means ± standard error (four separate determinations). The uptake ratio is 100 times the uptake obtained with 0 mM sodium (B or B′) divided by that obtained with 143 mM sodium (A or A′). Abbreviations: L-Glu, L-glutamic acid; L-Asp, L-aspartic acid; Gly, glycine; L-Ala, alanine; L-Leu, leucine; and L-Arg, L-arginine.

Amino acid	$10^{-3}M$ amino acid			$10^{-5}M$ amino acid		
	Uptake rate (nmole per gram per 4 minutes) with		Uptake ratio (100 B/A)	Uptake rate (nmole per gram per 4 minutes) with		Uptake ratio (100 B′/A′)
	143 mM Na^+ (A)	0 mM Na^+ (B)		143 mM Na^+ (A′)	0 mM Na^+ (B′)	
			Cerebral cortex			
L-Glu	804 ± 90	365 ± 31	45*	200 ± 15	8.4 ± 1.0	4.2*
L-Asp	652 ± 49	273 ± 28	42*	143 ± 12	8.9 ± 1.0	6.2*
Gly	703 ± 55	651 ± 36	93	30.4 ± 4	25 ± 3	82
L-Ala	776 ± 52	709 ± 50	91	39.6 ± 3	34 ± 2.6	86
L-Leu	690 ± 130	592 ± 73	86	25.4 ± 2	23.4 ± 1.0	92
L-Arg	110 ± 7	109 ± 10	99	12.0 ± 1.5	15.4 ± 1.7	131
			Spinal cord			
L-Glu	160 ± 18	63 ± 4	40*	7.2 ± 0.3	0.30 ± 0.05	4.0*
L-Asp	137 ± 18	97 ± 16	71	12 ± 1.3	0.6 ± 0.08	5.0*
Gly	220 ± 10	147 ± 11	67	20.0 ± 1.4	3.9 ± 0.3	19*
L-Ala	151 ± 8	105 ± 13	70	4.7 ± 0.6	3.4 ± 0.4	72
L-Leu	166 ± 11	163 ± 11	98	3.9 ± 0.1	3.9 ± 0.1	100
L-Arg	130 ± 35	122 ± 7	94	2.3 ± 0.4	3.4 ± 0.4	148

* $P < .001$ for difference between uptake rates with 0 and 143 mM Na^+.

optimal sodium concentration for accumulation of $10^{-5}M$ glutamic and aspartic acids (cerebral cortex and spinal cord) and of $10^{-5}M$ glycine (spinal cord) was 140 mM; uptake declined by about 50 percent when 200 mM sodium was used.

These results suggest that the unique high-affinity uptake systems for glycine and glutamic and aspartic acids (*5*) have a marked sodium dependence, while the low-affinity uptake systems for these and other amino acids do not require sodium. This conclusion was supported by double reciprocal kinetic analysis. When sodium was present, glutamic and aspartic acids (cerebral cortex and spinal cord) and glycine (spinal cord) were accumulated by both high-affinity [Michaelis constant (K_m) $\simeq 2 \times 10^{-5}M$] and low-affinity ($K_m \simeq 2 \times 10^{-4}M$) systems, confirming our earlier results (*5*). In the absence of sodium only the low-affinity uptake systems could be demonstrated.

We reported that low concentrations of exogenous glutamic and aspartic acids labeled a unique synaptosomal fraction in the cerebral cortex, while endogenous glutamic acid was uniformly distributed throughout the total synaptosomal population (*6*). If the low-affinity transport for glutamic acid represents accumulation by all synaptosomes homogeneously, and if the high-affinity, sodium-requiring glutamic acid transport labels a unique population of particles storing glutamic acid, it should be possible to separate the two populations of synaptosomes by including or omitting sodium in the incubation medium. Accordingly, homogenates from rat spinal cord were incubated with [^{3}H]- or [^{14}C]glutamic acid in the presence or absence of sodium (Fig. 1), and subjected to subcellular fractionation by incomplete equilibrium sedimentation (*8*). The particles accumulating glutamic acid in the presence of sodium sedimented in a less dense region of the gradient than did those from preparations incubated in the absence of sodium. The less dense particles thus showed sedimentation characteristics identical to those of the unique glutamic acid–accumulating synaptosomal fraction described (*6*). Experiments with reversed isotopes confirmed that the separations obtained were not artifacts of the isotope used or of the experimental technique. Moreover, when control homogenates were incubated with both isotopes in the presence of the same concentration of sodium, identical patterns of gradient distribution were obtained. The demonstration of unique synaptosomal fractions for aspartic acid in cerebral cortex and spinal cord and for glycine in the spinal cord (*9*) also depended on the presence of sodium in the incubation medium.

The synaptosomal uptake of the putative neurotransmitters—catecholamines (*10*), serotonin (*10*), and γ-aminobutyric acid (*11*)—are also markedly sodium-dependent. Although equilibrium accumulation of most amino acids is only partially dependent on sodium (*12*), a marked sodium dependence was reported for initial uptake rates of proline and aspartic acid in cerebral cortical synaptosomes (*13*). We have confirmed the sodium dependence of proline uptake and also observed a high-affinity synaptosomal transport system for proline (*14*).

Perhaps the unique synaptosomal fractions subserving the high-affinity, sodium-dependent uptake of glutamic and aspartic acids are made up of nerve terminals in which these amino acids serve a specific synaptic function, perhaps as neurotransmitters. However, it should be emphasized that all synaptosomal preparations are contaminated by unidentified particles.

As proposed for catecholamines, γ-aminobutyric acid, and serotonin (*15*), the uptake systems for glutamic and aspartic acids and glycine might serve to inactivate released amino acid. The marked sodium requirement may be a useful probe in elucidating the synaptic function of amino acids.

JAMES P. BENNETT, JR.*
WILLIAM J. LOGAN
SOLOMON H. SNYDER
Departments of Pharmacology and Experimental Therapeutics and *Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

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* Fourth-year medical student.

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Calcitonin Receptors of Kidney and Bone

Abstract. *Receptors for calcitonin, determined by activation of adenylate cyclase, were found in a distribution among zones of the kidney distinct from that of receptors for parathyroid hormone or vasopressin. Competitive binding studies showed that the receptors for calcitonin are similar in kidney and bone and that their high apparent affinity for salmon calcitonin accounts in part for the high biological potency in vivo of salmon calcitonin.*

Calcitonin, the polypeptide hormone discovered as a factor causing hypocalcemia in rats, has been identified throughout the vertebrate phylum (*1*).

In mammals, the hormone inhibits skeletal resorption (*1*) and influences renal function as well, causing natriuria, phosphaturia, and calciuria (*2*). We have compared the renal and skeletal receptors for the hormone by determining hormone-activated adenylate cyclase and binding of salmon calcitonin labeled with ^{125}I with cell membranes prepared from kidneys and bones of rats. The receptors for calcitonin were found to be similar in the two tissues. Salmon calcitonin, of all the species of the hormone tested, showed the highest apparent affinity for the receptors in both target tissues. This high affinity must account in part for the high potency of salmon calcitonin in vivo relative to the activity of mammalian species of the hormone.

The species of the hormone tested were: synthetic salmon calcitonin; synthetic human calcitonin M, the sulfoxide analog, and the (11-32)-amide fragment; purified porcine calcitonin [96 Medical Research Council units (MRCU) per milligram]; and bovine (60 MRCU/mg) and ovine (70 MRCU/mg) calcitonins, which were purified by published techniques (*3*). Renal plasma membranes were isolated from male Sprague-Dawley rats weighing 150 to 200 g by a method that has been described (*4*). Renal tissue was homogenized in a solution of 0.25*M* sucrose, 0.01*M* tris(hydroxymethyl)-aminomethane (tris), and 0.001*M* ethylenediaminetetraacetic acid (EDTA), at 4°C and *p*H 7.5, by using a loosely fitting Dounce homogenizer. A crude membrane fraction was obtained by differential centrifugation at 2200*g* in the same buffer and layered onto a continuous gradient of 32 to 42 percent sucrose (by weight) in 0.01*M* tris and 0.001*M* EDTA at *p*H 7.5. After centrifugation for 90 minutes at 100,000*g* at 4°C, the band appearing at a sucrose concentration of 38 percent was aspirated; aliquots were centrifuged and the pellets were stored under liquid nitrogen. Membranes from fetal rat calvaria were obtained by a modification of the method used to prepare skeletal adenylate cyclase (*5*). The tissue was powdered, while frozen, in a stainless steel mortar. The preparation was then homogenized in a solution containing 0.25*M* sucrose, 0.05*M* tris, and 0.02*M* EDTA at *p*H 7.5 by using an electrically driven rotating Teflon pestle. The homogenate was filtered through glass wool and centrifuged for 15 minutes at 2200*g* at 4°C. This crude membrane fraction was resuspended in 0.25*M* sucrose, 0.01*M* tris, and 0.001*M* EDTA at *p*H 7.5, centrifuged in aliquots, and stored as pellets under liquid nitrogen. Adenylate cyclase was assayed under the conditions given in the legend of Fig. 1. Synthetic calcitonin (salmon) was labeled with ^{125}I by a modification of the method of Hunter and Greenwood (*6*) to specific activities of 300 to 600 $\mu c/\mu g$. Binding studies were carried out for 4 hours at 22°C in 0.05*M* tris at *p*H 7.5; the solution contained 2 percent heat-inactivated albumin. Separation of the membrane-bound hormone was accomplished by

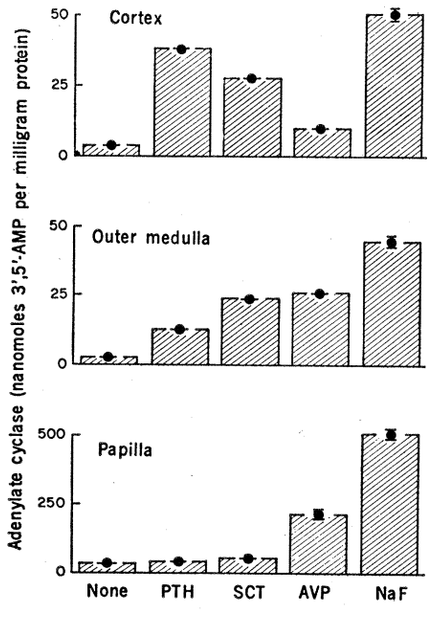

Fig. 1. Adenylate cyclase in membranes from three zones of the rat kidney. The kidneys were dissected into cortical, red (outer) medullary, and white medullary (papilla) zones and fractionated as described in the text. Adenylate cyclase was assayed at 22°C for 30 minutes in 50 m*M* tris, 0.013 percent bovine serum albumin, 30 m*M* KCl, 4.5 m*M* $MgCl_2$, 1.1 m*M* ATP, 4 m*M* creatinine phosphate, 10 μg creatine phosphokinase, and 9 m*M* theophylline, at *p*H 7.5; the total volume was of 70 μl. The ^{32}P-labeled cyclic 3',5'-adenosine monophosphate (AMP) that was formed was determined as described previously (*16*). The additives and their final concentrations were: parathyroid hormone (PTH) (1500 U.S. Pharmacopeia units per milligram), 20 μg/ml; salmon calcitonin (SCT) (4200 MRCU/mg), 200 ng/ml; arginine vasopressin (AVP) (60 international units per milligram), 800 ng/ml; sodium fluoride, 7 m*M*. The points represent the mean ± 1 S.E. of three determinations for the cortex and outer medulla, or the average and range of two determinations for the papilla. All three hormones and fluoride caused significant ($P < .001$) stimulation of the enzyme prepared from the cortex or outer medulla. In membranes from the outer medulla SCT caused greater activation ($P < .001$) than PTH, whereas the latter caused greater hormonal activation in the cortical preparation ($P < .001$).