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A Unique Synaptosomal Fraction, Which Accumulates Glutamic and Aspartic Acids, in Brain Tissue

(rat/rabbit/neurotransmitters/sucrose density gradients)

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ABSTRACT **Subcellular fractionation of rat cerebral cortical slices on sucrose density gradients provides evidence for the existence of a unique synaptosomal fraction (enriched in pinched-off nerve endings) that selectively accumulates glutamic and aspartic acids. The particles in this fraction sediment to a less dense portion of sucrose gradients than do particles that accumulate aromatic, basic, and neutral (large and small) amino acids. Particles that store gamma-aminobutyric acid are even less dense than those that contain exogenous glutamic and aspartic acids. The distribution of endogenous glutamic acid encompasses both that of exogenous glutamic acid and that of the neutral and basic amino acids. These findings provide neurochemical support for the suggestion that glutamic and/or aspartic acid has a specialized synaptic function, perhaps as a neurotransmitter, in the mammalian brain.**

Neurophysiological experiments have suggested that several amino acids, notably glutamic, aspartic, gamma-aminobutyric (γ-NH_2But), and glycine, may be neurotransmitters in the central nervous system (1–3). Subcellular fractionation of brain tissue can provide valuable neurochemical evidence to help identify possible transmitters. When brain tissue is homogenized in isotonic sucrose, nerve terminals pinch off to form membrane-bound sacs that contain cytoplasm and synaptic vesicles, called "synaptosomes" (4, 5), that can be isolated by a combination of differential and density gradient centrifugation (4, 5). The localization of a given chemical to synaptosomes provides suggestive evidence for a neurotransmitter role (4, 5). However, such conclusions must always be made with caution, since most synaptosomal preparations are contaminated by many unidentified particles.

Transport systems into nerve terminals have been described for several putative neurotransmitters, including norepinephrine (NE) (6–9), serotonin (10–13), and γ-NH_2But (14, 15). If certain amino acids are neurotransmitters, then perhaps analogous uptake systems might transport them into nerve terminals. If such is the case, the exogenous amino acid, which would label selectively a "neurotransmitter pool", should be more highly localized in synaptosomal fractions than would be the endogenous amino acids, which would include "metabolic" as well as "transmitter" pools. Recently, we (16) obtained evidence in support of this possibility. Brain slices were incubated with a large number of exogenous, radioactive amino acids; certain ones, including glutamic acid, were highly localized in synaptosomal fractions, even though endogenous glutamic acid was distributed no differently than was a general cytoplasmic marker.

Using the technique of "incomplete equilibrium sedimentation" in sucrose density gradients, we have been able to separate synaptosomal fractions storing different putative neurotransmitters (17, 18). We have applied this technique to the subcellular fractionation of brain tissue labeled with various radioactive amino acids. We report here that whereas most exogenous amino acids show the same uptake pattern in sucrose density gradients, glutamic and aspartic acids are localized to a unique synaptosomal fraction that can be distinguished from the particles that store the other amino acids.

METHODS AND MATERIALS

Adult male rats (Sprague-Dawley, 150–200 g) were killed by decapitation and their brains were quickly removed. Slices of cerebral cortex (30 mg) were excised according to the method of Glowinski and Iversen (19).

The cortical slices were incubated in 2 ml of modified Krebs–Henseleit bicarbonate medium with glucose and one-half the original concentration of calcium, containing labeled amino acids (5×10^{-7} M), NE (10^{-7} M), and γ-NH_2But (10^{-6} M). Brain slices accumulate NE (9), γ-NH_2But (15), and amino acids (16, 20, 21) by specific transport systems that appear to label the endogenous pools. Nialamide, a monoamine oxidase inhibitor, was present in the incubation medium (10^{-5} M) when tissue was incubated with NE to prevent degradation of NE. Amino-oxyacetic acid (10^{-5} M) was used to prevent metabolism of γ-NH_2But. All incubations were performed on a Dubnoff metabolic shaker in a 95% O_2–5% CO_2 atmosphere at 30°C. Incubations were performed at 30°C instead of 37°C in order to minimize the actions of catabolic enzymes, while maintaining transport systems. Under these conditions, 90–95% of the radioactive compounds were unmetabolized as determined by paper chromatography in three solvent systems. After incubation, the slices were removed from the medium, homogenized in 2.5 ml of fresh, ice-cold, 0.32 M sucrose (Mallinkrodt Analytic Reagent Sucrose) in a Potter–Elvehjem glass homogenizer fitted with a teflon pestle (0.10–0.15 mm clearance), and subjected to differential centrifugation. Crude mitochondrial pellets that contained synaptosomes were obtained, centrifuged for 15 min ($100,000 \times g$) in a linear continuous sucrose gradient (1.5–0.32 M), and fractionated as was described (17).

Abbreviations: γ-NH_2But, gamma-aminobutyric acid; NE, norepinephrine.

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To measure endogenous glutamic acid, gradient fractions in conical centrifuge tubes were immersed in boiling water for 15 min and centrifuged at 10,000 × *g* for 10 min. Endogenous glutamic acid was measured in aliquots of the supernatant fluid by the method of Graham and Aprison (22). Protein was determined by the method of Lowry *et al.* (23), with bovine serum albumin as a standard. Lactic acid dehydrogenase activity was measured as described (16). Radioactivity in gradient fractions was measured in Bray's phosphor (24) in a model 3375 Packard Tricarb liquid scintillation spectrometer.

All labeled compounds were obtained from New England Nuclear Corporation. Specific activities of the ^{3}H compounds were between 2 and 40 Ci/mmol, while specific activities of ^{14}C compounds varied from 20 to 250 Ci/mol.

RESULTS

To compare the subcellular localization of 17 exogenous amino acids, we incubated brain slices with two amino acids at a time, one labeled with ^{14}C, the other with ^{3}H, and synaptosomal fractions were obtained by incomplete equilibrium sedimentation. This procedure (17) resolves particles that store different neurotransmitters better than density equilibrium procedures (4, 5), and, by labeling one putative transmitter with tritium and another with carbon, subtle differences in sedimentation properties can be reliably detected. We examined representatives of each class of amino acids, including acidic, basic, neutral (large and small), and aromatic amino acids. Identical patterns of distribution in density gradients (Figs. 1, 4) were displayed by aromatic, basic, and neutral amino acids.

On the other hand, the acidic amino acids, glutamic and aspartic acids, were distributed in a slightly less dense region of the gradient and they could be separated reliably from nonacidic amino acids (Figs. 2, 3). To ensure that separations of different labeled amino acids in our gradients were not simply due to adventitious factors, we prepared a control gradient in each experiment in which a brain slice was incubated with carbon and tritiated isotopes of the same amino acid (Figs. 2, 3). In these cases, the two isotopes always showed identical patterns of distribution. Also, all separations of particles that stored different amino acids were confirmed in experiments in which the isotopes were reversed, e.g., an experiment contrasting [^{3}H]tyrosine and [^{14}C]glutamic acid was confirmed by contrasting [^{3}H]-glutamic acid and [^{14}C]tyrosine (Figs. 2, 3). Whereas particles accumulating labeled glutamic and aspartic acid could be separated from particles storing other amino acids, they could not be separated from each other (Fig. 3). In experiments with other brain regions, including hypothalamus and midbrain, similar separations of acidic and nonacidic aminoacid-accumulating particles were obtained.

Although particles that stored exogenous nonacidic acids were more dense than those containing labeled glutamic and aspartic acid, they proved to be less dense than those that stored radioactive NE (Fig. 3). On the other hand, particles that had accumulated labeled γ-NH_2But were localized to a portion of the gradients even less dense than

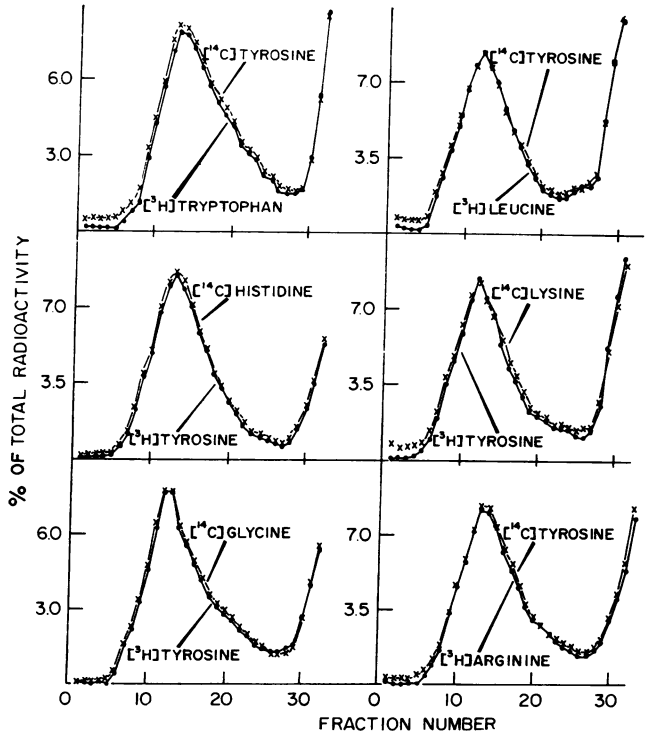

FIG. 1. Distribution patterns in linear, continuous sucrose density gradients of synaptosomal fractions that accumulate aromatic, basic, and neutral (large and small) amino acids. Particles were prepared from rat cerebral cortical slices and incubated with both ^{14}C- and ^{3}H-labeled amino acids. After centrifugation in a linear, continuous sucrose density gradient (100,000 × *g*, 15 min), we compared the distributions of the amino acids two at a time. Each experiment was repeated 3 to 6 times.

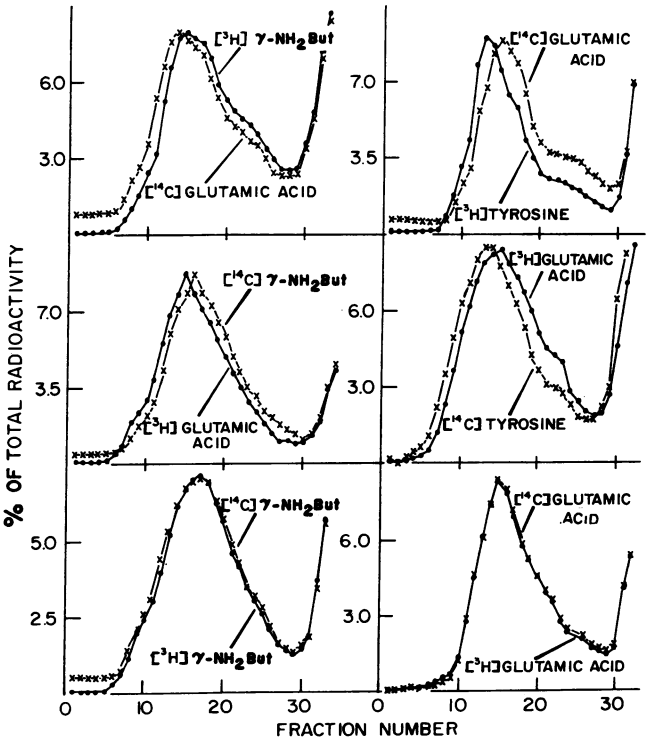

FIG. 2. A comparison of the distribution patterns, in linear, continuous sucrose density gradients, of synaptosomal fractions that contain labeled γ-NH_2But, glutamic acid, and tyrosine. Experimental details are as in Fig. 1.

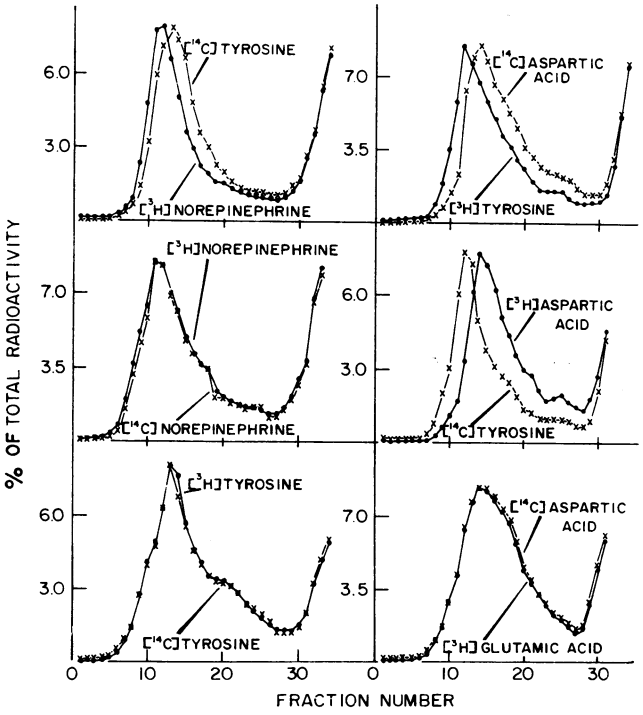

FIG. 3. A comparison of the distribution patterns of synaptosomal fractions that contain labeled norepinephrine, tyrosine, aspartic, and glutamic acids. Experimental details as in Fig. 1.

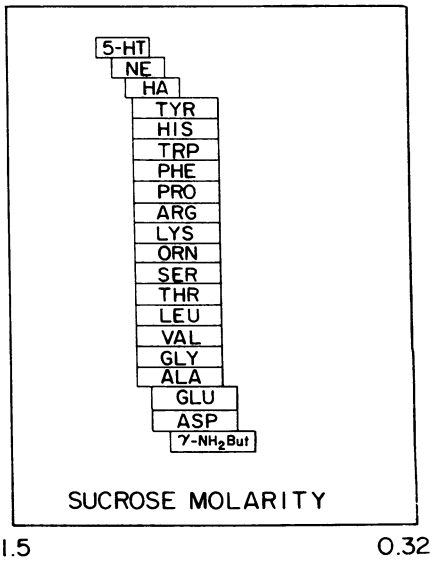

FIG. 4. A schematic diagram showing the patterns of distribution of seventeen amino acids, serotonin (5-HT), norepinephrine (NE), and histamine (HA) in linear, continuous sucrose density gradients. Serotonin, norepinephrine, and histamine appear in denser regions of the gradients, and in narrower profiles, than do the amino acids. γ-NH_2But appeared in the least-dense part of the gradient, whereas glutamic and aspartic acids appeared in less-dense regions than the other amino acids. Experimental details as in Fig. 1. Except for histamine, all profiles reflect the distribution of exogenous compounds. Under these centrifugal conditions, endogenous and exogenous serotonin show identical profiles (18).

those that stored glutamic and aspartic acids. In other studies, we found that particles containing endogenous and exogenous serotonin sediment to a denser portion of these gradients than those that contain NE (18), whereas particles that contain endogenous histamine are similar in their sedimentation characteristics, although slightly less dense (25), to those that accumulate labeled NE. Thus, using our techniques of incomplete equilibrium sedimentation, we can rank the synaptosomal fractions that store compounds we have examined in the following order of decreasing density: serotonin > norepinephrine > histamine > nonacidic amino acids > acidic amino acids > γ-NH_2But (Fig. 4). Fig. 4 also illustrates that the acidic amino acids and the other amino acids show broader patterns of distribution than do amine-storing particles.

Transport systems for all amino acids exist in most mammalian tissues, presumably subserving general metabolic functions of these compounds. By raising the concentration of exogenous amino acids in the incubation mixture, one might overload a small, high-affinity "neurotransmitter-specific" pool and force amino acids into more generalized transport systems. In line with such reasoning, we incubated brain slices with glutamic acid concentration of 1×10^{-3} M, 6×10^{-5} M, and 5×10^{-7} M, and found in three replicate experiments that the peaks of glutamic acid and tyrosine in our gradients were separated by two fractions at 5×10^{-7} M and 6×10^{-5} M, but by only 1 fraction at 10^{-3} M.

Since synaptosomes are osmotically-sensitive particles (4), amino acids should be released by osmotic shock if present unbound in the cytoplasm of synaptosomal fractions. When the crude mitochondrial pellets were subjected to osmotic shock by resuspension in 0.05 M sucrose, and then centrifuged, 95% of the radioactivity from all of the [^{3}H]aminoacids examined, including glutamic and aspartic acids, γ-NH_2But, glycine, leucine, and tyrosine, was released into the supernatant fluid.

Our data suggest that particles that contain exogenous glutamic and aspartic acids differ in sedimentation properties from those that contain nonacidic amino acids. However, it is conceivable that there is no difference in the sedimentation properties of the particles that store acidic and nonacidic amino acids, but that leakage of labeled aspartic and glutamic acids from particles during their passage through the gradient results in a pattern of distribution for the acidic amino acids that appears less dense than that of the nonacidic amino acids. In this case, one might expect the profile of total radioactivity from glutamic or aspartic acids to differ from the profile for the particulate-bound form of these compounds. Accordingly, in some experiments, gradient fractions were diluted to a final concentration of 0.45 M sucrose and centrifuged for 30 min at 50,000 $\times$ *g*, and ^{3}H and ^{14}C in the pellets were assayed. In these experiments, we obtained the same separation of acidic and nonacidic amino acids in particulate matter alone as we did when we monitored the total radioactivity in the gradient fractions.

To determine whether the localization of different synaptosomal fractions could be generalized to different animal species, we compared the sedimentation properties of particles that accumulated radioactive acidic and nonacidic amino acids in the rabbit. In rabbit cerebral cortical tissue, as in the rat, acidic amino acid-storing particles proved less dense than particles that contained nonacidic amino acids.

Lactate dehydrogenase and protein content have been

used as markers for the total synaptosomal fraction of density gradients (4). To determine how the distribution of amino acids related to the total population of synaptosomal particles, we measured the protein concentration and activity of lactate dehydrogenase (EC 1.1.1.27) in density gradients, as well as the concentrations of labeled glutamic acid and tyrosine (Fig. 5). Protein and lactate dehydrogenase activity distributed similarly to [^{14}C]tyrosine, which was used as a marker for the nonacidic amino acids. The profile of [^{3}H]-glutamic acid, however, differed from that of the other components, being in a less dense region of the gradient. These results suggest that the nonacidic amino acids label all nerve terminals in the brain fairly homogeneously, whereas a significant proportion of labeled glutamic acid and aspartic acid enter a unique synaptosomal fraction. The pattern of distribution of endogenous glutamic acid, however, encompassed the profiles of both acidic *and* nonacidic amino acids, which suggests that glutamic acid is associated with the total synaptosomal fraction, as well as with the unique fraction labeled by exogenous glutamic acid.

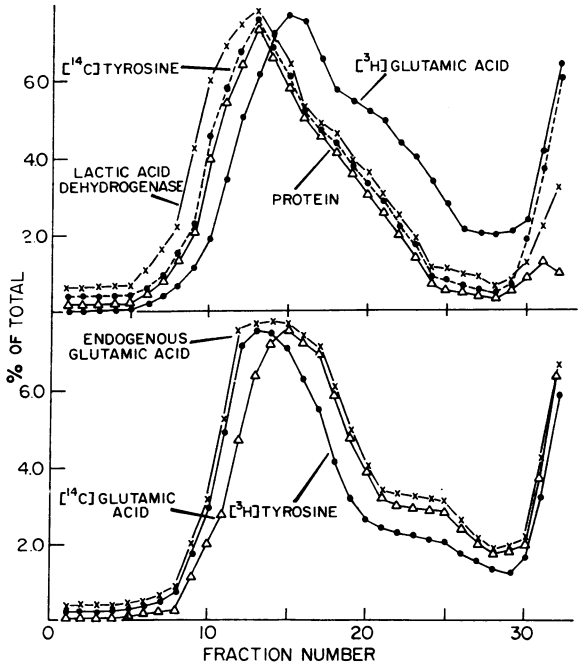

FIG. 5. A comparison of the distribution patterns of synaptosomes that contain [^{14}C]tyrosine, [^{3}H]glutamic acid, endogenous glutamic acid, protein, and lactate dehydrogenase activity. Assays are described in *Methods*. Other experimental details are the same as in Fig. 1.

DISCUSSION

The major finding of the present study is that the profile of the synaptosomal fractions that store exogenous glutamic and aspartic acids can be reliably differentiated from that of all nonacidic aminoacids examined. What factors might account for this separation?

a. Since microsomal fragments occur predominantly in less dense portions of the gradient, perhaps the acidic amino acids are associated with ribosomal components of the microsomes for incorporation into peptide chains more frequently than other amino acids, producing a shift in the profile of the acidic aminoacid-storing synaptosomal fraction. If such an association contributed significantly to the profile, then other amino acids, especially leucine, should occur in less dense areas of the gradient than the acidic amino acids, since under our conditions of incubation of brain slices, the incorporation into protein of labeled leucine is ten times that of glutamic acid (16).

b. The acid groups of glutamic and aspartic acids might bind nonspecifically to positive charges on microsomal particles in less dense portions of the gradient. Osmotic shock would not be expected to disrupt such ionic linkages, yet almost 100% of the particulate acidic amino acids was released into the supernatant fluid upon hypo-osmotic treatment. Moreover, γ-NH_2But, a nonacidic amino acid, which would not bind to positively-charged particles as well as glutamic and aspartic acids, was located in an even less dense region of the gradient than were the acidic amino acids.

c. Some portions of the labeled glutamic and aspartic acids might be selectively incorporated into hitherto unidentified particles, conceivably glial in origin, that appear in less dense portions of the gradient. However, Whittaker (4) failed to find any significant contamination of synaptosomal preparations by glia. Our own electron microscopic examination of fractions from gradients prepared in exactly the same way as those in the present study also showed only minimal contamination (17, 26). Although synaptosomal fractions prepared by incomplete equilibrium sedimentation contain free mitochondria, as well as synaptosomes, these occur in the denser area of the synaptosomal distribution (17, 26) and, hence, could not account for the profiles of the acidic amino acids that preponderate in the less dense areas.

d. The interpretation most compatible with our data is that a significant proportion of glutamic and aspartic acids enters a unique population of less dense synaptosomes. This conclusion is in accord with the large body of evidence that of all the amino acids examined in cerebral cortical tissue, glutamic and aspartic acids and γ-NH_2But best satisfy neurophysiological criteria as transmitter substances. Our results provide neurochemical evidence to support this view. However, it is important to bear in mind that synaptosomal preparations are contaminated by tissue constituents other than nerve endings, and that contamination by free mitochondria is greater with the technique of incomplete equilibrium sedimentation than when gradients are centrifuged to apparent density equilibrium. Moreover, there are numerous other unidentifiable particles in these fractions. Accordingly, it is possible that the accumulation of [^{3}H]glutamic and [^{3}H]aspartic acids by nonsynaptosomal particles might also account for the separation of these amino acids from other [^{3}H]aminoacids on our gradients.

Glutamic acid is the major metabolic precursor of γ-NH_2But (27, 28). It is unlikely that our results reflect glutamic acid entering γ-NH_2But neurons, since synaptosomes storing γ-NH_2But and glutamic acid could be reliably distinguished on density gradients. Moreover, kinetic studies have shown that glutamic acid has negligible affinity for the γ-NH_2But-transport system (15).

In our experiments, labeled glutamic acid and aspartic acid showed identical profiles in density gradients. Our methods cannot discriminate whether these two compounds enter the same particles or different ones with the same sedimentation characteristics.

Transport systems for all amino acids exist in most mammalian tissues (20, 21). Our results imply that unique transport systems in brain nerve terminals exist to preferentially accumulate glutamic and aspartic acids. Recently, kinetic evidence has been obtained for a unique, high-affinity transport system for glutamic acid in brain synaptosomal fractions (W. M. Logan and S. H. Snyder, in preparation). Synaptosomal fractions can accumulate various amino acids by transport systems with relatively low affinities (K_m about 10^{-3} M) (20, 21). Glutamic acid is accumulated into brain synaptosomal fractions by a similar system and, in addition, by a unique high-affinity system (K_m about 4×10^{-5} M). Accordingly, one might predict that low concentrations of glutamic acid would enter the synaptosomal fractions specific for acidic amino acids, whereas high concentrations of glutamic acid should enter all synaptosomal populations homogeneously. This prediction was borne out by our experiments in which particles prepared from brain slices incubated with 10^{-3} M glutamic acid proved less separable from the particles storing nonacidic amino acids than synaptosomes labeled with low concentrations of glutamic acid.

Uptake systems for compounds such as norepinephrine and serotonin, whose sole presumed function is as a neurotransmitter, appear to label the endogenous pools homogeneously (18, 29, 30). Even if glutamic and aspartic acids function as neurotransmitters, it is likely that only a small portion of the endogenous concentrations of these compounds serve in this role. Presumably, the high-affinity uptake system labels the pool of glutamic acid that has a special synaptic function, whereas the distribution of endogenous glutamic acid on our gradient reflects the sum of the synaptic, as well as the general metabolic pool, glutamic acid.

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