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NET UPTAKE OF L-GLUTAMATE AND GABA BY HIGH AFFINITY SYNAPTOSOMAL TRANSPORT SYSTEMS

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Abstract—Reuptake of neuroactive amino acids by high affinity transport systems in the CNS is thought to terminate the neurotransmitter activity of these substances. This notion has been challenged since the homoexchange of synaptosomal and exogenous L-glutamate and the corresponding homoexchange of synaptosomal and exogenous GABA has been demonstrated. We reported that depolarizing media (56 mM-KCl, 1 mM-$CaCl_2$) lowers the GABA content of synaptosomes. In such synaptosomes, net and apparent (radioactive) GABA uptake are similar. When rat cortical synaptosomes (1 mg protein/ml) are incubated with 10 μM-[^{14}C]L-glutamate, net and apparent (radioactive) uptake are similar. When the synaptosome levels are decreased to 0.5 mg protein/ml or less, then net uptake becomes a fraction of radioactive uptake (exchange ensues). Net L-glutamate uptake is Na^+-dependent and temperature-dependent. Furthermore, a 1 mM concentration of KCl or RbCl supports net L-glutamate and GABA uptake. LiCl, NH_4Cl, CsCl and choline chloride are ineffective. In addition, diaminobutyric acid (but not β-alanine) inhibits net and apparent GABA uptake. The demonstration of net uptake of L-glutamate and GABA by their respective high affinity systems is consonant with the idea that these systems may play a role in neurotransmitter inactivation in the synaptic region.

L-Glutamate and GABA are postulated excitatory and inhibitory neurotransmitters, respectively, in the vertebrate CNS (KRNJEVIĆ, 1970). Nerve terminal preparations (synaptosomes) sequester these radiolabeled substances by high affinity (low K_m) and low affinity (high K_m) transport systems (LOGAN & SNYDER, 1971; IVERSEN & JOHNSTON, 1971; WEINSTEIN *et al.*, 1965; LEVI & RAITERI, 1974; MARTIN, 1974). The high affinity transport systems are Na^+- and temperature-dependent. Each exhibits narrow specificity for its neuroactive substrate with a K_m of about 10^{-5} M. In contrast, the low affinity transport systems are associated with both neuroactive and inactive amino acids, exhibit broader specificity, are Na^+- and temperature-independent and exhibit a K_m of about 10^{-3} M (LOGAN & SNYDER, 1971). The high affinity uptake systems have been suggested to function in terminating neurotransmitter action by removing their specific substrates from the synaptic region (IVERSEN, 1971). Before this can be concluded for L-glutamate, GABA or other possible neurotransmitters, it must be demonstrated that the corresponding high affinity systems mediate net inward transport.

Levi and co-workers (LEVI & RAITERI, 1974; LEVI *et al.*, 1974) reported that the high affinity L-glutamate system mediates exchange of endogenous and external L-glutamate, but little or no net uptake. These experiments were performed with 0.25–0.5 mg/ml of synaptosomal protein. LEVI & RAITERI (1974) and Simon and co-workers (SIMON *et al.*, 1974) reported that analogous GABA exchange is Na^+- and temperature-dependent and is associated with the high affinity system. We confirmed the results of these two groups (RYAN & ROSKOSKI, 1977). Uptake was also measured in conventional synaptosomes and those treated with depolarizing media which lowers the synaptosomal content of GABA. We found that GABA-depleted synaptosomes exhibited Na^+- and temperature-dependent net GABA uptake in the absence of exchange. The present experiments were performed to determine whether synaptosomes will mediate net L-glutamate uptake. Therefore, the extent of L-glutamate uptake, and not rate of uptake, was measured. Following the methodology of LEVI *et al.* (1974) with 0.25 mg/ml of synaptosomal protein, appreciable exchange occurs. When the synaptosomal protein concentration is increased to 1 mg/ml, then net and apparent uptake become similar. A preliminary account of some of these findings has appeared (ROSKOSKI, 1977).

EXPERIMENTAL PROCEDURES

Synaptosome preparation. Partially purified rat cortical synaptosomes were prepared by differential centrifugation as described by COTMAN *et al.* (1976). GABA and L-glutamate-depleted synaptosomes were prepared by 56 mM-KCl–1 mM-$CaCl_2$ treatment as previously described (RYAN & ROSKOSKI, 1977). GABA uptake and measurement (fluorometric and radioactive) were also performed as previously documented (RYAN & ROSKOSKI, 1977).

The composition of Ringer solution, unless otherwise noted, was 10 mM-glucose, 150 mM-NaCl, 1.0 mM-KCl, 1.2 mM-$MgSO_4$, 1.2 mM-Na_2HPO_4, 10 mM-Tris-(hydroxymethyl)-aminoethane–HCl (pH 7.4). After preparing the synaptosomes they were harvested by centrifugation, suspended in Ringer solution (with additions where specified),

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incubated 10 min (25°) and centrifuged (3000 *g*, 5 min, 0°). They were resuspended in Ringer by vortexing and recentrifuged. Finally, the synaptosomes were suspended again in Ringer (15 ml/g of cortex) giving about 1 mg/ml protein except when otherwise noted.

Glutamate transport experiments. For each determination ten individual synaptosome fractions (about 0.38 mg protein in 0.38 ml) were pre-incubated for 5 min at 25°C, then 20 μl of [^{14}C]L-glutamate or [^{14}C] or [^{3}H]GABA (100 Ci/mol uniformly labeled) as specified was added to five fractions to give a final exogenous concentration of 10 μM and 20 μl of Ringer solution was added to the others and incubations were continued an additional 10 min (25°). The samples were then centrifuged in a Brinkman microfuge (1 min, 13,000 *g*). The supernatants were aspirated and 1.00 ml of distilled water was added. The pellet was suspended by sonication (Kontes Micro-Ultrasonic Cell Disrupter). The suspensions were then treated at 90°C for 3 min to denature metabolizing enzymes. The suspensions were centrifuged 15 min (13,000 *g*) and 20 μl samples were taken for radioactivity measurements and suitable aliquots were taken for the fluorometric determination of L-glutamate using the general methodology of GRAHAM & APRISON (1966). Strip scanner analysis of the supernatant or extracts of the pellet using the methodology previously described (RYAN & ROSKOSKI, 1975) showed that 96% or more of the radioactivity co-migrated with authentic L-glutamate.

As previously noted for GABA (RYAN & ROSKOSKI, 1977), there is a small amount of efflux of this substance into the medium during the 5 min preincubation to give a concentration of 1 μM or less. There is also some efflux of L-glutamate to give a concentration between 1 and 2 μM. For each experiment, the initial GABA and L-glutamate concentrations were measured in five parallel samples after the 5 min preincubation and this was used to correct for the initial exogenous concentration in the calculation of the apparent radioactive uptake. The chemical content of these substances was measured in the synaptosomal pellet after the incubation and no correction is required.

The native fluorescence of NADH formed during the enzymatic reaction of L-glutamate and NAD^+ was used to measure this amino acid in the samples. Suitable dilution of the water extracts of the synaptosome pellet were added to 0.45 ml of glycine–hydrazine buffer (previously treated with Norit (GRAHAM & APRISON, 1966)). After addition of 20 μl of 3 mg/ml NAD^+, then 10 μl of 5 mg/ml L-glutamate dehydrogenase was added. After 30 min at ambient temperature, 0.6 ml of 0.1 M-EDTA (pH 10) was added and fluorescence of NADH formed was measured (350 nm excitation, 450 nm emission, 25°C). Internal standards were routinely employed and the coefficient of variation was less than 1.5% (GRAHAM & APRISON, 1966). Radioactive substances were purchased from New England Nuclear Co. and enzymes and other compounds from Sigma Chemical Co.

RESULTS

Time course of L-*glutamate and GABA uptake*

The synaptosome fractions were incubated with 10 μM each [^{14}C]L-glutamate and [^{3}H]GABA for the time indicated. The suspension was centrifuged and the radioactivity (apparent) uptake and total L-glutamate (fluorometric) uptake into the pellet was measured. The rate of uptake of radioactive and labeled GABA was linear for 5 min and then leveled off at 10 min as previously described (RYAN & ROSKOSKI, 1975; 1977). The rate of L-glutamate transport is linear for 2–3 min and levels off by 5 min (Fig. 1). In most of the following experiments, a 10 min incubation was performed so that the total and not the rate of L-glutamate uptake was determined.

Net and apparent L-*glutamate uptake as a function of* L-*glutamate content*

We previously reported that synaptosomes depleted of GABA by treatment with 56 mM-KCl–1 mM-$CaCl_2$ mediated net and apparent uptake of this substance. Appreciable exchange, however, occurred in the conventional and GABA treated synaptosomes. Using a similar methodology, rat cortical synaptosomes were incubated with 50 μM-L-glutamate, 56 mM-KCl–1 mM-$CaCl_2$ or Ringer solution. These treatments increased and decreased, respectively, the L-glutamate content (Table 1). Net uptake was about 95% that of apparent uptake in the L-glutamate depleted and also in the conventional synaptosomes. In the glutamate-loaded synaptosomes, however, net uptake was significantly less than apparent uptake (Table 1). LEVI *et al.* (1974) reported that synaptosome fractions, comparable to the control system, were incapable of mediating net L-glutamate uptake. Therefore, a systematic investigation of the variables which may be responsible for this difference was undertaken.

Net and apparent uptake were measured as a function of synaptosomal protein concentration. Using protein concentrations of 0.25–0.5 mg/ml, similar to

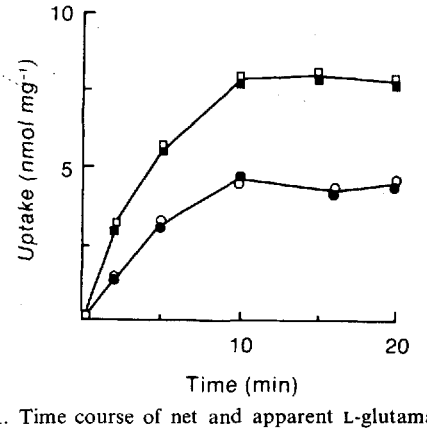

FIG. 1. Time course of net and apparent L-glutamate (□) and GABA (○) uptake into synaptosomes. Synaptosomes were treated with 56 mM-KCl, 1 mM-$CaCl_2$ as previously described (RYAN & ROSKOSKI, 1977). Then uptake was measured at the specified times as described in Table 1 except that the zero time was preincubated at 0° (5 min) instead of at 25°. Net (chemical) and apparent (radioactive) uptake is represented by the hollow and solid symbols, respectively. L-Glutamate and GABA were measured fluorometrically as described in the Experimental Procedures and as previously documented (RYAN & ROSKOSKI, 1977), respectively. The standard error of the mean for each assay is less than 0.5 nmol/mg of protein.

TABLE 1. SYNAPTOSOMAL GLUTAMATE CONTENT AND GLUTAMATE UPTAKE*

Pretre	Synaptosomal glutamate content (nmol/mg)	Apparent uptake (nmol/mg)	Net uptake (nmol/mg)
1. Control	52.3	8.1	8.0‡
2. 50 μM-L-glutamate	59.1†	6.4	4.2§
3. 56 mM-KCl, 1 mM-$CaCl_2$	41.5†	8.7	8.4‡

* Synaptosomes were pretreated with Ringer solution, Ringer solution containing 50 μM unlabeled L-glutamate, or Ringer solution containing 56 mM-KCl and 1 mM-$CaCl_2$ according to previously outlined procedures (RYAN & ROSKOSKI, 1977). After incubation (10 min, 25°), synaptosomes were collected by centrifugation (3000 *g*, 5 min), washed once, and resuspended in Ringer solution. Synaptosomal L-glutamate was measured fluorometrically as described in Methods. To measure net uptake and apparent uptake, synaptosomes (400 μg in 380 μl Ringer) were preincubated at 25° for 5 min. Then 20 μl of 200 μM-[^{14}C] L-glutamate was added for an additional 10 min. After centrifugation at 13,000 *g* (60 s, ambient temperature), the total glutamate was measured in the pellet by the enzymatic fluorometric method by measuring NADH fluorescence and radioactive [^{14}C] L-glutamate was measured in a 20 μl portion by liquid scintillation spectroscopy as documented in Methods. The values are the means of six determinations; the standard errors are less than 3% of the values given.

† Significantly different from the control ($P < 0.05$).

‡ Net uptake not significantly different from apparent uptake.

§ Net uptake significantly less than apparent uptake ($P < 0.01$).

those used by LEVI *et al.* (1974), net uptake is about 50% of apparent uptake indicating that appreciable exchange occurs (Fig. 2). When the synaptosome concentration is increased to 1 mg/ml, about 8 nmol of L-glutamate are taken up which corresponds to 80% of that in the incubation medium. Net and apparent uptake are similar. The chief difference between the present work and those of Levi and Raiteri therefore seems to be related to the synaptosomal protein concentration. The mechanism for the exchange exhibited at low concentrations and not higher concentrations requires further investigation.

Na^+- *and Temperature-dependence of* L-*glutamate uptake*

In addition to a K_m in the micromolar range, the high affinity uptake systems are Na^+ and temperature-dependent (LOGAN & SNYDER, 1971). When choline chloride (150 mM) or sucrose (300 mM) is substituted for NaCl in the Ringer solution, net uptake is negligible (not shown). The uptake at 4° is only 20% that observed at 25° (Fig. 2). The net and apparent uptake were similar at 25° and 37°. Since 80% of the total exogenous L-glutamate uptake occurs at 25° (10 min), it is therefore not surprising that net uptake is not significantly greater at 37°C.

Effect of monovalent cations on net and apparent L-*glutamate and GABA uptake*

Net GABA uptake was previously shown to be dependent on exogenous K^+ (RYAN & ROSKOSKI, 1977). BENNETT *et al.* (1973) also reported that K^+ is required for L-glutamate uptake. Other monovalent cations were substituted for KCl to ascertain if they might mediate net uptake. RbCl (1 mM), but not LiCl, CsCl, NH_4Cl or choline chloride (each 1 mM) are unable to mediate net L-glutamate or GABA uptake

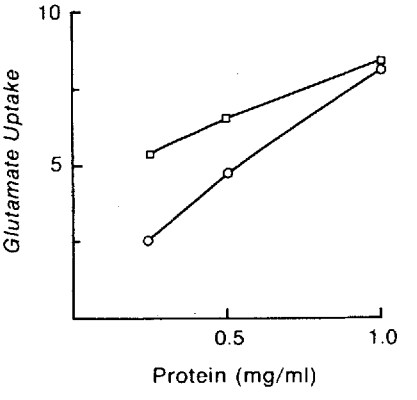

FIG. 2. Variation of net and apparent L-glutamate uptake with synaptosomal protein concentration. Ten samples of synaptosomes containing the specified quantity of protein were incubated for 5 min at 25°. Then 10 μl of [^{14}C]-L-glutamate was added to give a final concentration of 10 μM to five samples. After 10 additional minutes, the samples were centrifuged and the pellets were collected for fluorometric determination of L-glutamate. The initial L-glutamate content was 13.1 ± 0.2 nmol/0.25 mg, 26.3 ± 0.4 nmol/0.5 mg and 53.2 nmol/1 mg. (○--○) represents the final minus initial glutamate content. (□--□) is the radioactive uptake. The standard error of the mean for each assay is less than 0.5 nmol/mg of protein.

TABLE 2. EFFECT OF MONOVALENT CATIONS ON NET AND APPARENT GABA AND GLUTAMATE UPTAKE*

Medium	GABA		L-Glutamate	
	Net	Apparent	Net	Apparent
	(nmol mg^{-1} protein)			
No KCl	0.2	3.5	0.2	5.9
1 mM-KCl	3.4	3.8	8.3	8.4
1 mM-RbCl	3.2	2.9	8.5	8.7
1 mM-LiCl	0	2.7	−0.4	4.4
1 mM-CsCl	0	1.4	−0.2	6.9
1 mM-Chloine chloride	0	2.5	−0.8	5.1
1 mM-NH_4Cl	−0.4	2.9	−0.8	4.8

* Depolarized synaptosomes depleted of GABA and glutamate by the methodology previously given (RYAN & ROSKOSKI, 1977), were resuspended in K^+-free Ringer solution. To 380 μl portions (420 μg protein) were added 5 μl of salt solution to give the 1 mM final concentration. After incubation for 5 min (25°), then 20 μl portions of [^{14}C]L-glutamate or [^{14}C] GABA were added for 10 min additional incubation. Each suspension was centrifuged (45 s, 13,000 *g*) and net and apparent uptake of GABA (RYAN & ROSKOSKI, 1977) and L-glutamate were measured as described in Methods.

(Table 2). Variable uptake of GABA or L-glutamate in the absence of exogenous K^+ may be related to leakage from the preparation. An appreciable amount of radioactive uptake occurs with the latter monovalent cations in the absence of net uptake (Table 2). At first it was hypothesized that the ability to mediate net transport might be related to the ability to serve as the external substrate for the Na^+–K^+ ATPase. That this is an unsatisfactory explanation, however, is indicated by the ability of NH_4^+ to substitute for K^+ in thee ATPase reaction (SKOU, 1960), but its inability to mediate net uptake (Table 2).

Effect of diaminobutyric acid and β-alanine on net and apparent GABA uptake

In the previous study (RYAN & ROSKOSKI, 1977), net and apparent uptake was greater in the synaptosome fraction than in the mitochondrial or myelin fraction. The synaptosome fraction, however, contains other membranous structures including glial fragments which might sequester GABA. Since diaminobutyrate preferentially inhibits GABA transport into neurons and β-alanine preferentially inhibits uptake into glia (MARTIN, 1976) the action of these compounds on GABA uptake into the synaptosome fraction was determined. With 1 mM-diaminobutyric acid, net and apparent GABA uptake (10 μM) was completely abolished (Table 3). β-alanine, however, failed to alter net or apparent GABA uptake. These studies provide supportive evidence that GABA transport into neuronal elements occurs in the present system.

TABLE 3. EFFECT OF DIAMINOBUTYRATE AND β-ALANINE ON NET AND APPARENT GABA UPTAKE*

Addition	GABA Uptake	
	Net	Apparent
None	4.1	4.2
1 mM-DABA	0.3	0.3
1 mM-β-alanine	3.8	3.9

* GABA-depleted synaptosomes (6.8 nmol mg^{-1} protein) were prepared as previously described (RYAN & ROSKOSKI, 1977). The suspensions (380 μl, 410 μg protein) were incubated 5 min (25°C) prior to the simultaneous addition of diaminobutyrate, or β-alanine and [^{14}C]GABA. Net and apparent uptake into the pellet was measured (in triplicate) as described in Table 1. The experiment was performed with three different synaptosome preparations and similar results were obtained.

DISCUSSION

To mediate GABA and L-glutamate inactivation, the respective uptake system must transport net amounts of the substance from the synaptic region into the cell. With high affinity (10 μM) and low affinity (1 mM) uptake systems, and their experimentally determined maximal velocities, it can be calculated that about 85% of the uptake at 10 μM substrate is mediated by the high affinity transport system (MARTIN, 1976). Net L-glutamate uptake is Na^+- and temperature-dependent and these are properties characteristic of the high affinity systems. Previous experiments demonstrated that net GABA uptake exhibits these characteristics. Furthermore, both transport systems require low concentrations (1–4 mM) of K^+ or Rb^+.

There are a number of differences between the two transport systems. First, net L-glutamate transport is readily demonstrable in conventional or non-depolarized fractions. On the other hand, net GABA transport is readily demonstrable in K^+–Ca^{2+} treated synaptosomes which are GABA-depleted (RYAN & ROSKOSKI, 1977) but not conventional synaptosomes. Synaptosomal GABA content may be elevated secondary to post-mortem elevation of brain GABA levels (BALCOM *et al.*, 1975) and depolarization may

normalize them. L-Glutamate elevation, on the other hand, has not been reported. The relationship of these preparations *in vitro* to those *in vivo*, however, can only be inferred. Two other major differences include the higher L-glutamate capacity and the greater rate of L-glutamate uptake. Although the absolute values vary among preparations, the glutamate content is 4–5-fold greater than that of GABA. The rate of L-glutamate uptake is linear for about 2 min (25°) and levels off at 5 min. For GABA, on the other hand, uptake is linear for 5 min and continues until 10 min. Some of these differences may be related to the general role of L-glutamate in the metabolism of all neuronal and glial cells whereas that of GABA is thought to be associated more with specialized neurotransmitter function.

The phenomenon of GABA and L-glutamate homoexchange, as well as the normal mechanism of amino acid uptake, is incompletely understood. The role of monovalent cations in net uptake by the high affinity system also remains to be determined. Experimentally, however, 1–4 mM-KCl or RbCl is required for net uptake.

The purpose of the reported experiments was to determine whether or not synaptosome preparations are capable of mediating net uptake of L-glutamate and GABA by the high affinity systems. The original protocol of LEVI & RAITERI (1974) measures extent and not rate of uptake. They incubated synaptosomes (0.25 mg protein/ml) in 10 μM-^{14}C-L-glutamate.

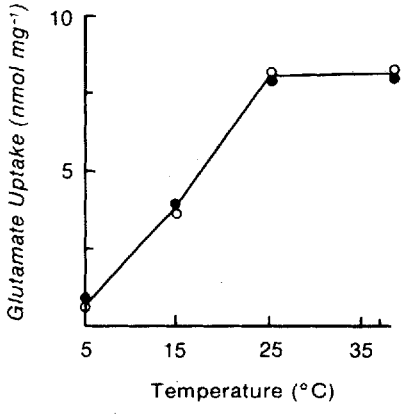

FIG. 3. Temperature-dependence of net and apparent L-glutamate uptake. After synaptosome suspensions (380 μl, 365 μg protein) were incubated for 5 min at the specified temperature, then 10 μl of [^{14}C]-L-glutamate was added and the incubations were incubated an additional 10 min. The suspensions were centrifuged 45 s (4°C) at 13,000 ***g***. Net and apparent uptake was measured as described in Table 1. The transport experiments were replicated five times as described in Methods and the standard error of the mean was less than 3% of the numbers given. The initial L-glutamate content was 51.3 $\pm$ 1.3 nmol mg^{-1}. (●), apparent uptake; (○), net uptake. The standard error of the mean for each assay is less than 0.5 nmol/mg of protein.

Although the synaptosomes took up radioisotope, the medium L-glutamate concentration remained unchanged. The authors postulated a homoexchange of synaptosomal and medium L-glutamate to account for the results. When the synaptosomal protein concentration is raised to 1 mg protein/ml radioisotope and L-glutamate uptake are equivalent (Table 1; Fig. 2). The mechanism of this effect is unknown. The process is Na^+- and temperature-dependent and concentrations of L-glutamate and GABA are in the range expected of the high affinity uptake systems. Since diaminobutyrate inhibits GABA uptake, this suggests that neuronal and not glial uptake is occurring. It is not yet possible, however, to ascertain the proportion of L-glutamate uptake into neuronal or glial elements in the present experiments. Nevertheless, nervous tissue preparations mediate net L-glutamate and GABA uptake at concentrations in the range of the high affinity transport system and this may therefore play a role in the inactivation of these neuroactive substances.

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