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Glutamic Acid as a Transmitter Precursor and as a Transmitter

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Glutamate is one of the most active neuroexcitatory substances present in the CNS of vertebrates, where it may function as an important synaptic transmitter (12). In brain, glutamate also functions as the precursor for gamma-aminobutyric acid (GABA), a very important inhibitory transmitter. The quantity of glutamic acid decarboxylase (GAD), the enzyme that converts glutamate into GABA, does not appear to be rate limiting although it is mainly located in presynaptic GABAergic terminals. If the enzyme were ubiquitous, local applications of the excitatory transmitter glutamate would trigger an increased formation of GABA, and if the postsynaptic cells possessed specific GABAergic receptors to GABA, a biphasic response may ensue. This consideration brings up the question of whether GAD is a regulatory step for GABA and/or glutamate steady state. Recent reports have indicated (17) that GAD is only partially saturated by its cofactor, pyridoxal-5-phosphate (pyridoxal-P) in the intact brain and that there is a rapid postmortem increase in the degree of saturation of the enzyme by pyridoxal-P. It appears that in the absence of glutamate, pyridoxal-P is tightly bound to GAD, but glutamate, the substrate of GAD, promotes dissociation of the cofactor from the enzyme (16). This GAD inactivation promoted by glutamate appears to be very slow at low concentrations of glutamate and increases rapidly as glutamate reaches saturating concentrations. Pyridoxal-P is tightly bound to GAD in the absence of glutamate but this cofactor is loosely bound when this substrate is present. Thus, since GAD utilizes glutamate to form GABA, the pyridoxal-P will tend to stay on the enzyme; therefore, a continuous function of the enzyme in these conditions requires a constant supply of pyridoxal-P. The question then is: What are the regulatory biochemical events that maintain at steady state the glutamate pool that serves as a substrate for GAD? Is GAD regulation by the supply of pyridoxal-P the mechanism that nerve impulses utilize to increase the rate of GABA formation in the presence of an increased rate of GABAergic neuronal activity?

In cortex, hippocampus, cerebellum, and other brain nuclei, both glutamatergic (12) and GABAergic (7) neurons have been described. Lesion studies have shown that in striatum a degeneration of specific glutamatergic or GABAergic tracts causes little or no change in the tissue content of glutamate or GABA, respectively (5,13).

Thus, in certain brain nuclei, the amount of glutamate and GABA involved in neuronal transmission is not a preponderent part of the total brain content. Moreover, this finding suggests that a change in GABA and glutamate content cannot be a ubiquitous biochemical index of the changes in activity of glutamatergic and GABAergic neurons; probably, the turnover rate of GABA and glutamate are better indices. Biochemical measurements directed to ascertain the dynamic state of the glutamate functioning as a transmitter must differentiate this pool from the other neuronal pool where glutamate functions as a GABA precursor. Both pools must in turn be differentiated from the neuronal and glial cell pool where glutamate is a product of intermediary metabolism. If the precursors of glutamate were different in these various pools, one would have gained an important advantage in using glutamate turnover rate to estimate participation of glutamatergic neurons in brain function. Based on metabolic considerations, Quastel (24) suggested that glutamine might function in replenishing the transmitter pools of glutamate and GABA. Undoubtedly, blood glutamine is an excellent source of C and N for GABA or glutamate because intra- and extracellular levels of glutamine are high (32) and it can readily pass from blood to brain (20,22). In brain, glutamine could originate from glial cells (29) where CNS glutamine synthetase appears to be located (31). The glutamine taken up by axons could be metabolized by glutaminase (4) and by glutamate decarboxylase to generate GABA (26). Having none of the excitatory or inhibitory properties of glutamate and GABA, glutamine would be ideally suited for the function of precursor of glutamate and GABA. Thus, with glutamine present in the cerebral spinal fluid at concentrations around 500 μM, a considerable concentration gradient would drive glutamine into nerve terminals where it would be converted into glutamate and thereby either contribute to the energy metabolism or in particular neurons contribute to the glutamate pool functioning as a transmitter (4). The evidence that at least certain types of terminals convert glutamine into GABA was provided by studies with brain synaptosomes showing that ^{14}C-glutamine is rapidly metabolized to glutamate and GABA (4). According to current knowledge (33), there is a net flux of glutamate and GABA from neurons to glial cells caused by the impulse-mediated release of these amino acids from nerve terminals. Probably, glutamine formed in glial cells is taken back into the neurons for restoration of the transmitter pools of glutamate and GABA. This theoretical model has not yet been verified by *in vivo* studies; however, some *in vitro* experiments in which the brain structure was not disrupted were performed with hemisections of toad brain (28). These experiments supported a role of glutamine in replenishing neuronal pools of GABA and glutamate by showing increased uptake and conversion of radioactive glutamine to glutamate and GABA following K^+-induced depolarization (28). However, these results failed to indicate the extent to which glutamine contributes to maintain the steady state of the glutamate in the various brain areas where glutamate functions both as a transmitter and as a precursor of GABA. Unfortunately, experiments on glutamine uptake and synthesis using synaptosomes are not useful for extrapolations to "*in vivo*" conditions. One important drawback concerning the use of synaptosomes is their low content of glutamate and GABA

(25) caused by the lack of Na^+ during synaptosomal preparation. In the absence of Na^+, the amino acid carrier cannot function in maintaining amino acid steady state and transports glutamate and GABA out of the cell rather than keeping them inside.

In order to evaluate glutamatergic function, it appears necessary to resort to *in vivo* measurements of glutamate turnover; therefore, it is important to consider the neuroanatomy of the glutamatergic pathways and select one pathway that can be manipulated with facility and is located in a brain structure of known neurochemical characteristics.

NEUROANATOMY OF THE GLUTAMATERGIC SYSTEM

Glutamate is the most abundant amino acid in the adult CNS, with the highest concentration in n. accumbens, cerebral cortex, and cerebellum (Table 1). When the tissues are ranked according to the concentration of glutamate present, it appears evident that the amount of glutamate present bears no relationship with that of GABA (Table 1). Although GABA content may change from area to area by as much as sixfold (compare cerebellum and substantia nigra contents), the glutamate content changes by less than twofold and the glutamine content practically fails to change (Table 1). An interpretation of these data could be that in various brain areas the content of glutamine, a compound that readily crosses the blood-brain barrier, is in equilibrium with the blood; in contrast, glutamate and GABA contents vary in function of abundance of specific cells that store these amino acids. As a corollary of these considerations, we can conclude that it is difficult to assume that the glutamine content plays a regulatory role for the rate of glutamate synthesis because glutamate content in various brain areas appears to be independent from blood glutamine.

Support for the idea that glutamate may be an excitatory neurotransmitter in mammalian brain is provided by a specific Na^+-dependent high-affinity uptake system in brain synaptosomes (15) and slices (14). Using biochemical and elec-

TABLE 1. *GABA, glutamate, and glutamine content in various areas of the rat brain*

	nmoles/mg protein ± SEM		
	Glutamate	GABA	Glutamine
N. accumbens	125 ± 3.4	47 ± 2.2	—
Cortex	120 ± 6.0	15 ± 2.0	49 ± 4.9
Cerebellum	110 ± 3.0	15 ± 0.5	58 ± 6
Globus pallidus	95 ± 2.8	89 ± 5.5	—
Diencephalon	95 ± 5.0	22 ± 0.4	51 ± 7.2
Hippocampus	93 ± 4.0	21 ± 2.0	66 ± 2.7
N. caudatus	81 ± 4.0	20 ± 3.0	72 ± 7
Substantia nigra	79 ± 2.8	95 ± 4.4	—

Each value is the average of at least four assays. Sprague-Dawley rats weighing 150 g were microwaved and the brain processed for mass fragmentographic assay as described previously (2) or in the chapter by T. Giacometti (*this volume*).

trophysiological indices, the presence of glutamatergic neurons was identified: (a) in the lateral olfactory tract (4,10); (b) in the afferents from the entorhinal cortex to the hippocampal formation via the fibers of the perforant pathway (3,21,33a); (c) in the pathways connecting the frontal cortex with striatum (13); (d) in the cerebellar parallel fibers originating from the granule cells (27); and (e) in the septum, the septal-hippocampal cholinergic pathway is regulated by a glutamatergic feed back loop that links the hippocampus to the septum (J. T. Coyle, *personal communication;* and this laboratory, *unpublished*).

In selecting an appropriate model from these five pathways to study biochemically the regulation of the turnover of transmitter glutamate, we gave certain weight to the availability of additional knowledge on transmitter neurochemistry and ordinary neuroanatomical information of the selected brain structure. Thus, we have selected the striatum because in this area we can separate extrinsic afferent pathways

TABLE 2. *Effect of striatal injections of kainic acid and cortical ablation on the neurotransmitter profile of rat striatum*

	Cortical ablation	Kainic acid
Neurochemical parameters	Percent controls	
GABAergic neurons		
GAD	99	42[a]
GABA content	100	35[a]
GABA turnover	45[a]	—
^{3}H-GABA binding	70[a]	—
GABA uptake	94	46[a]
Cholinergic neurons[b]		
CAT	83	49[a]
ACh content	95	30[a]
ACh turnover	64[a]	30[a]
Dopaminergic neurons		
TH	108	97
DA content	102	98
HVA	105	110
^{3}H-halop-binding	68[a]	64[a]
DA-sensitive adenylcyclase	95	5[a]
Glutamatergic neurons		
Glutamate content	87[a]	97
Glutamate uptake	61[a]	98
Enkephalinergic neurons		
Enkephalin content	92	45[a]
^{3}H-naloxone binding	70[a]	60[a]

The different parameters were determined 21 days after cortical ablation or 15 days after intrastriatal injection of 1 μg of kainic acid, according to the following methods: GABA, glutamate content, and GABA turnover rate (18), GABA binding (8), GABA and glutamine uptake (11), ACh (acetylcholine) content and turnover (35), TH (36), DA and HVA (6), enkephalin (34), and naloxone binding (23). The other data were from Schwarcz et al. (30).

[a] The difference from controls was significant, $p < 0.05$.

[b] From Moroni et al., ref. 19.

from intrinsic interneurons by comparing neurochemical parameters in rats with cortical deafferentation and with striatal lesion caused by local injection of kainic acid (5). The results of Table 2 show that striatal deafferentation reduces GABA and ACh turnover but fails to change CAT, GABA uptake, or the content of ACh and GABA, suggesting that the reduction in the turnover of ACh and GABA is due to a decrease of an afferent stimulation caused by decortication. Probably, decortication eliminates glutamatergic afferents among a number of other corticostriatal connections. This suggestion is confirmed by the data in Table 2, which shows that cortical deafferentation reduces glutamate content and uptake. Both parameters fail to change following kainic acid treatment, which destroys striatal interneurons (5) but not afferent axons (Table 2). Cortical ablation fails to change HVA, TH, or DA content, adding significance to the changes in the parameters that are indices of glutamergic activity that were changed by decortication (Table 2). In contrast, the data of Table 2 show that kainic acid injections that destroy a large number of intrinsic striatal neurons reduce the indices of enkephalinergic, GABAergic, and cholinergic activities, indicating that these three types of neurons reside within the striatum and, presumably, receive the corticostriatal innervation. Finally, the data of Table 2 also indicate that the corticostriatal afferent fibers that include glutamatergic axons may contain in their membranes GABA, enkephalin, and opiate receptors. The neuroanatomical inferences that can be drawn from the data of Table 2 and from other data from this laboratory are illustrated in the working model of Fig. 1.

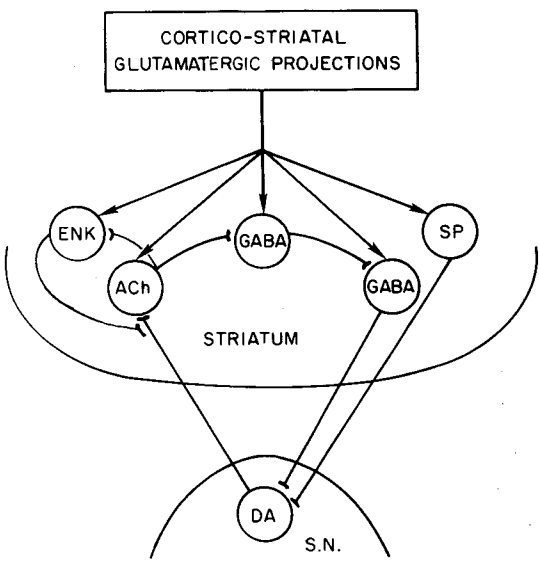

FIG. 1. Schematic representation of interaction of various striatal transmitters as revealed by the data of Table 2. S.N., substantia nigra; DA, dopamine; S.P., substance P; ENK, enkephalin; ACh, acetylcholine.

MASS FRAGMENTOGRAPHIC STUDIES OF THE *IN VIVO* RELATIONSHIPS BETWEEN GLUTAMINE AND GLUTAMATE

In order to ascertain whether or not glutamate biosynthesis depends on a conversion of glutamine into glutamate, male Sprague-Dawley rats were infused intraventricularly with 400 nmoles of 1,2,3,3,D_4-glutamine/min in a volume of 2 μl/min for various time periods. The rats (approximately 140 g in weight) were killed by exposing their heads for 2.5 sec to a focused high-intensity microwave beam as described by Guidotti et al. (9). The brains were removed from the skull and the parietal cortex, cerebellum, caudate, hippocampus, and diencephalon were carefully dissected and frozen on Dry Ice. Each brain area was subsequently homogenized in 1 ml of 1 N-acetic acid containing 300 nmoles of glutamine-D_4, 200 nmoles of glutamate-D_5, and 50 nmoles of N-aminoisovaleric acid (AVA), which were used as internal standards for glutamine, glutamate, and GABA, respectively. The whole homogenate was centrifuged and the supernatant was loaded onto a small column containing 0.5-ml Dowex 50 × 8, 200–400 mesh in the acid form. The columns were washed previously with 1 ml 2N-hydrochloric acid, which was followed by water rinses until the eluate had a neutral pH.

The amino acids were eluted from the resin with 1 ml of 3N NH_4OH and aliquots (100 to 200 μl) of the eluate were transferred to glass vials and evaporated to dryness with a stream of nitrogen. To the dry residue, 50 μl of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Pierce Co., Rockford, Ill.) and 100 μl pentafluoropropionic anhydride (PFPA) (Pierce Co.) were added. The vials were sealed, heated for 1 hr at 60° C and stored at 4° C overnight. Just before the mass fragmentographic analysis, the reaction mixture was evaporated to dryness. The residue was dissolved in 15 to 50 μl of ethylacetate and aliquots of 1 to 3 μl were injected into the gas chromatograph-mass spectrometer (LKB 9000). The separation was made on a 2 m × 3 mm i.d. silanized glass column packed with 3% OVI on Gas chrom Q 100–200 mesh (Applied Science Lab., State College, Pa.), maintained at a temperature of 150° C. The temperature of the flash heater was 200° C and the ion source was kept at 250° C. The electron energy and the trap current were set at 70 eV and 60 A, respectively. The following ions were recorded with the m.i.d.: 230 and 234 for glutamine and glutamine-D_4; 230 and 235 for glutamate and glutamate-D_5; 399 for GABA and 413 for AVA.

Polyethylene cannulae were implanted into the lateral ventricle of rats and D_4-glutamine was infused for various periods of time. The brains from animals infused with glutamine-D_4 were homogenized in a solution containing only AVA as an internal standard. The next steps of the analytical procedure were identical to those used for the measurements of the levels of amino acids, but when the samples were injected into the gas chromatograph-mass spectrometer in order to monitor the percent of incorporation of the deuterium into the endogenous glutamine, glutamate, and GABA pools, the following fragments were recorded: 230 and 234 for glutamine and glutamine-D_4; 250 and 254 for glutamate and glutamate-D_4; 399 and 403 for GABA and GABA-D_4, respectively.

The data reported in Table 3 show the enrichment of deuterated glutamine at

TABLE 3. *Relative enrichment of the D_4-variant into glutamine and glutamate during various periods of intraventricular infusion[a] with glutamine-D_4*

	Glutamate-D_4 (nmoles/mg prot.)	Glutamate-D_4/Glutamate-D_0 · 100			Glutamine-D_4/Glutamine D_0 · 100			Glutamine-D_0 (nmoles/mg prot.)
		8′	10′	15′	8′	10′	15′	
Caudate	81 ± 4.0	0.26 ± 0.09	0.58 ± 0.15	0.59 ± 0.19	12 ± 4.5	12 ± 8	21 ± 6.6	72 ± 7
Hippocampus	93 ± 4.0	0.34 ± 0.22	1.23 ± 0.29	2.0 ± 0.5	24 ± 6.5	60 ± 13	88 ± 19	66 ± 2.7
Cerebellum	110 ± 6.0	0	0	0	6.9 ± 2.1	4.2 ± 2	7.9 ± 28	58 ± 6
Cortex	120 ± 6.0	0.65 ± 22	1.2 ± 0.34	1.1 ± 0.33	37 ± 9	54 ± 14	55 ± 5	49 ± 4.9

[a] 400 nmoles/min at 2 μl/min.

various times during the intraventricular infusion of this deuterated compound. It appears that cortex and hippocampus contain a greater amount of deuterated glutamine than caudate and cerebellum. Since these areas contain approximately the same amount of glutamine, this difference can be explained by the pattern of the location of the uptake mechanism in various brain regions or by differences in the turnover rate of glutamine in various brain areas. Since, as discussed earlier, there is no blood-brain barrier for glutamine, regional differences in the uptake mechanism may not be operative in explaining the distribution pattern of D_4-glutamine of Table 3. We have therefore invoked a faster turnover rate in cerebellum and caudate than in hippocampus and cortex to explain the lower amount of D_4-glutamine in this area. We are, however, aware that other factors may be operative. Our interest in developing a method to measure glutamate turnover rate from the conversion of glutamine into glutamate was reduced by the data shown in Table 3. Glutamine in hippocampus and cortex has a specific activity higher than that in caudate and cerebellum. Also, the specific activity of the glutamate formed in the hippocampus and cortex is several fold greater than that formed in caudate or cerebellum. Thus, it appears that not only is the conversion of glutamine to glutamate minimal but it follows first-order kinetics. On the basis of these results, we should conclude that the role of glutamine in the formation of glutamate should be minimal. However, it could be argued that this conversion, which we consider small, reflects almost exclusively the turnover of that pool of glutamate that functions as a transmitter.

To test this possibility we plan to compare, in rats, the conversion of deuterated glutamine in glutamate in the striatum ipsilateral to that contralateral to a monolateral ablation of cortex. Since we failed to detect labeling in the GABA pool of striatum, hippocampus, and cortex, we are also interested in pursuing further the possibility that blood-borne glutamine may be a suitable precursor to use in measuring the turnover of glutamate in the glutamate pool that functions as a transmitter.

BIOCHEMICAL STUDIES OF GLUTAMATE AS A GABA PRECURSOR

The development of a very sensitive method for the simultaneous quantitation of glutamic acid and GABA content by mass fragmentography (1) has made it possible to measure the turnover rate of GABA in small brain structures. With this method, the carboxylic groups of glutamate and GABA are esterified with HFIP, whereas the amino groups are acylated with PFPA. To obtain an estimation of the GABA utilization, the changes with time in the ^{13}C enrichment of GABA and glutamate during constant rate of infusion of ^{13}C-glucose are monitored (2). Similar to the methods to measure the turnover rate of catecholamines (6) or acetylcholine (35) also the method to measure GABA turnover is based on a certain number of assumptions and it involves a number of approximations concerning the precursor pool. The method tacitly implies that the conversion of glucose into the various glutamate pools present in the brain areas where we measure GABA turnover proceeds at the same rate constant. Naturally, this is never completely true because

glutamate functions not only as a metabolite of intermediary metabolism, but also as a precursor of GABA and a transmitter in its own right. We know that the rate of transmitter biosynthesis is never constant and depends on transsynaptic regulation. Hence, in certain brain areas such as the cerebellum and hippocampus, where various glutamate pathways exist, it has been proved impossible to measure GABA turnover using ^{13}C-glucose, because the specific activity of GABA fails to relate to that of glutamate as it is known for a product and precursor relationship. In practice, if one minimizes the error due to the lack of linearity caused by feedback, one can measure the turnover rate of GABA in globus pallidus, n. caudatus, n. accumbens, and substantia nigra but not in hippocampus, cerebellar cortex, and deep cerebellar nuclei (2).

In the brain nuclei shown in Table 4, the value of the turnover rate of GABA is completely unrelated to the GABA content or to the glutamate content but relates to the efflux rate of ^{13}C-GABA (k_{GABA}), which is a function of the rate in which the specific activity of glutamate and GABA reach equilibrium. It is important to note from Tabie 3 that the lack of glutamatergic fibers reaching the striatum (Fig. 1) is associated with a decrease of GABA and ACh turnover rate (Table 2).

CONCLUSIONS

Evidence was reviewed suggesting that glutamine may serve as a precursor of toad brain glutamate, and probably as a precursor of that pool of glutamate that in this preparation functions as a transmitter. However, it is difficult to extrapolate from these experiments to *in vivo* experiments in the mammalian brain. A number of appropriate brain models were discussed to study the regulation of glutamate pool functioning as a transmitter. The validity of caudate as such a model was documented by a number of neurochemical data available. These experiments show that glutamatergic afferents to striatum stimulate the GABA and ACh metabolism presumably because glutamate functions as an excitatory transmitter. Using deuterated glutamine injected intraventricularly it is possible to label glutamate in hippocampus, cortex, and caudatus, but this labeling does not appear to have the

TABLE 4. *GABA turnover rate in various brain nuclei*

Nucleus	GABA (nmoles/mg prot.)	Glutamate (nmoles/mg prot.)	k_{GABA}/hr	TR_{GABA} (nmoles/mg prot/hr)
Substantia nigra	95 ± 4.4	79 ± 2.8	3.1 ± 0.50	290
Globus pallidus	89 ± 5.5	95 ± 2.8	4.0 ± 0.30	360
N. caudatus	19 ± 0.86	81 ± 4	18 ± 1.5	340
N. accumbens	47 ± 2.2	125 ± 3.4	5.1 ± 0.70	240

From Bertilsson et al., ref. 2, and Miller et al., ref. 16, with permission.

properties typical for a precursor–product relationship between glutamine and glutamate. However, interestingly enough, we could not detect labeling of GABA. Since the striatal GABA that is not labeled with D_4-glutamine is labeled with ^{13}C-glucose, which also labels glutamate, it is concluded that ^{13}C-glucose (and not D_4-glutamine) is a better precursor to study glutamate as a precursor, whereas D_4-glutamine warrants further study as a possible precursor of glutamate that functions as a transmitter in cortex, hippocampus, and caudatus.

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