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Regional distribution of endogenous and parenteral glutamate, aspartate and glutamine in rat brain

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Glutamic acid, a non-essential amino acid present in high concentrations in mammalian brains [1], is an important participant in numerous metabolic reactions [2, 3] and may also function as a neurotransmitter [4]. Observations that have been interpreted as supporting this latter role for glutamic acid include: (a) its decreased levels in the olfactory cortex after removal of the olfactory bulb [5], (b) its excitatory effects when applied iontophoretically to cortical neurons [6], (c) its release from cortical and caudate slices after electrical stimulation [7], (d) its autoradiographic association with nerve terminals after incubation with cortical synaptosomes [8], (e) the decrease in the efflux of glutamate from hippocampal slices prepared from brains of animals previously subjected to hippocampal deafferentation [9], and (f) the decrease in the concentration of glutamate in the cerebellums of animals afflicted with a viral disease that destroys granule cells [10].

It is difficult to distinguish the molecules of brain glutamate present in neurotransmitter pools from the presumably larger amounts present in metabolic pools. One potentially useful way of differentiating between these pools involves analyzing the glutamate concentrations in various brain regions [11]; regions containing large numbers of glutaminergic synapses might also have relatively high concentrations of the amino acid. Utilizing this approach, we affirm previous reports [11, 12] that glutamic acid levels do vary considerably among regions of the rat brain, and show that the distribution of glutamate differs from those of glutamine and aspartate.

Very large doses of monosodium glutamate (MSG) [13, 14], aspartic acid [15], and other acidic amino acids [16] are toxic to brains and retinas [17] of infant rodents and reportedly accumulate in certain small brain regions [18, 19]. This paper describes the effects of giving these amino acids, alone or in combination, on the concentrations of glutamate, glutamine, and aspartate in whole brain, various brain regions, and retinas of adult rats. It also shows that the effects of giving glutamate and aspartate are not additive to their combined concentration in rat brain or retina.

Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass.) of various sizes were exposed to light (Vita-Lite, Duro-Test Corp., North Bergen, N.J.) from 8:00 a.m. to 8:00 p.m. daily and given *ad lib.* access to water and to food (Big Red Rat Chow, Agway Co., Syracuse, N.Y.), except for 12 hr before an experiment. Drugs were administered intraperitoneally, except when specified. Animals were decapitated at 2-min intervals between 10:30 and noon, 1 hr after receiving drugs or amino acids. Brains were removed and placed on a chilled glass plate for dissection. Eyes were rapidly enucleated and hemisected, after which retinas were gently scraped away from the choroid layer and pooled for assay.

Immediately after dissection, tissues were frozen, weighed and homogenized in 10 vol. of 0.4 N perchloric acid. The homogenates were then centrifuged in a Sorvall centrifuge at 30,000 *g* for 20 min. After decanting, 100 μl of a solution containing 0.1 N KOH and 0.02 N KCl were added to precipitate the perchlorate ion, which interferes with the enzymes used in the aspartate and glutamine assays. The samples were then frozen at −20° until assayed.

Immediately before the assay, the samples were warmed to 0° and centrifuged under refrigeration at 2000 *g* to pack the potassium perchlorate into a dense pellet.

For the preparation of sera, blood collected from the cervical wound was kept in an ice bath until clotting occurred, and then centrifuged. Sera were mixed with equal volumes of 0.8 N perchloric acid and centrifuged at 30,000 *g*; the supernatant fluids were then separated, and their perchloric acid was precipitated by the addition of 2 vol. of a solution containing 0.4 N KOH and 0.1 N KCl. The samples were then frozen until assay. After thawing, the samples were immediately centrifuged to pack the potassium perchlorate pellet, and the supernatant fluids were separated for assay. Twenty μl of each were used for aspartate assay, and 10 μl of each were used for glutamate and glutamine assays.

The glutamic acid and glutamine assays were based on the conversion of NAD to NADH and subsequent measurement of the native fluorescence of the NADH. The aspartic acid assay was based on the conversion of NADH to NAD and treatment of the NAD with a strong base to convert it to a fluorescent product. These assays are minor modifications of procedures described previously [20, 21].

NADH, NAD, ADP and Trizma base were obtained from the Sigma Chemical Co., St. Louis, Mo.; malate dehydrogenase, glutamic-oxalacetic transaminase and glutamate dehydrogenase were obtained from Boehringer-Mannheim, Mannheim, Germany; and glutaminase was obtained from Worthington Biochemical Corp., Freehold, N.J. MSG, aspartic acid, α-ketoglutarate and glutamine (Sigma) were dissolved in water and brought to pH 7 (with 1 N NaOH) before injection.

Student's *t*-test was used for all statistical analyses.

The regional distributions of glutamate, aspartate and glutamine in rat brain are described in Table 1. Glutamate concentrations varied over almost a 2-fold range among regions of rat brain. The lowest concentrations were noted in the brain stem, and the highest levels were found in the cortex, the caudate-putamen and the amygdala. In the brain regions studied, the concentrations of aspartate were lower than those of glutamate, varied over a smaller range, and generally failed to parallel those of glutamate. The lowest aspartate levels were found in the cerebellum, caudate-putamen and septum; the highest levels were measured in the medulla and pons. Glutamine concentrations varied even more than those of glutamate and exhibited still another pattern of distribution: they were lowest in the cortex and pons and highest in the hypothalamus. Calculations of the glutamate-to-aspartate ratios in various brain regions confirmed that these amino acids are distributed very differently in rat brain (Table 1); the same was true for the glutamate-to-glutamine ratios.

The administration of α-ketoglutarate (2 g/kg) caused no significant changes in whole brain glutamate, glutamine or aspartate levels, or in the serum concentrations of glutamate or glutamine (Table 2). Glutamine administration (2 g/kg) markedly elevated whole brain and serum glutamine concentrations but failed to affect glutamate or aspartate levels. Treatment with MSG (2 g/kg) elevated the serum glutamate concentration by 25- to 30-fold, but failed in most experiments to increase the glutamate level in

Table 1. Regional distribution of glutamate, glutamine and aspartate*

Region	Glutamate (μmoles/g)	Aspartate (μmoles/g)	Glutamine (μmoles/g)	Glu/Asp†	Glu/GluNH$_3$‡
Cortex	13.0 ± 0.6 (8)§	3.8 ± 0.1 (7)	2.6 ± 0.8 (3)	3.4	4.4
Caudate-putamen	12.1 ± 0.4 (10)	2.9 ± 0.2 (7)	5.0 ± 0.2 (3)	4.2	2.5
Olfactory bulb	10.7 ± 0.3 (10)	3.8 ± 0.2 (7)	4.8 ± 0.2 (3)	2.8	2.3
Septum	11.7 ± 0.7 (11)	2.9 ± 0.2 (8)	5.3 ± 0.9 (3)	4.0	2.8
Amygdala	14.1 ± 0.7 (7)	3.7 ± 0.3 (7)		3.8	
Hippocampus	12.6 ± 0.3 (11)	3.2 ± 0.2 (8)	4.6 ± 0.3 (3)	3.9	3.0
Thalamus	12.9 ± 0.6 (10)	3.9 ± 0.2 (6)	3.6 ± 0.6 (3)	3.2	3.7
Hypothalamus	11.1 ± 0.5 (11)	3.9 ± 0.1 (6)	6.4 ± 0.2 (3)	2.8	2.1
Mesencephalon	9.5 ± 0.3 (10)	3.7 ± 0.2 (7)	3.3 ± 0.3 (3)	2.6	3.0
Cerebellum	11.7 ± 0.2 (9)	3.0 ± 0.2 (6)	3.8 ± 0.4 (3)	3.9	3.1
Pons	8.8 ± 0.3 (10)	4.0 ± 0.3 (7)	2.6 ± 0.2 (3)	2.2	3.9
Medulla	8.2 ± 0.2 (10)	4.0 ± 0.2 (7)	3.3 ± 0.1 (3)	2.1	2.7

* Each determination was done on one brain. The rats weighed 250–280 g.
† Glu/Asp = the ratio of the glutamate concentration to the aspartate concentration.
‡ Glu/GluNH$_3$ = the ratio of the glutamate concentration to the glutamine concentration.
§ Number of determinations for each group is given in parentheses.

whole rat brain (Table 2). (In one of five experiments, this dose did produce a significant 10 per cent increase in brain glutamate concentration.) Aspartic acid (2 g/kg) administration elevated serum and brain aspartate concentrations. The joint administration of aspartic acid and MSG (2 g/kg of each) elevated serum [glutamate plus aspartate] levels by more than twice as much as the administration of only one of the amino acids, but still either failed to affect brain glutamate or aspartate levels (Table 2) or had the same effect as 2 g/kg of glutamate alone. The effects of exogenous MSG (2 g/kg) on glutamate concentrations were examined in eleven brain regions. One hr after receiving this dose of MSG, no region exhibited any significant elevation in its glutamate concentration.

The concentration of glutamate in rat retina (4.99 μmoles/g) was lower than that observed in any brain region (Table 1), as was that of aspartate (2.07 μmoles/g). Doses of glutamate or aspartate [2 g/kg, i.p. (Table 3) or by stomach tube] that caused 50-fold or greater increases in serum glutamate or aspartate concentrations failed to affect the levels of these amino acids in the retina. The combined administration of both amino acids (4 g/kg) by either route was also without effect on the retinas.

Variations in regional concentrations of compounds have historically been cited as evidence that brain constituents might function as neurotransmitters. Regions characterized as having relatively high concentrations of a given compound were presumed to contain neurotransmitter pools of the compound, especially if they could be localized within nerve terminals. In confirmation of other reports [11, 12], our examination of the regional distribution of glutamate in the brain showed that glutamate concentrations in parts of the cerebrum were almost twice those in the brain stem (Table 1). The regional distribution of aspartate was completely different from that of glutamate; the lowest concentrations of aspartate were found in the caudate-putamen and septum, while the highest concentrations were in the brain stem (Table 1). Glutamine levels also varied widely, but according to a different pattern from those of glutamate or aspartate. We hoped it would be possible to obtain a better delineation of regions containing neurotransmitter glutamate pools by examining regional glutamate levels in animals given large doses of MSG. None of the eleven regions chosen for examination exhibited significant changes in glutamate concentration 1 hr after animals received 2 g/kg of the amino acid intra-

Table 2. Effect of various glutamate precursors on brain and serum levels of glutamate, glutamine and aspartate*

	Brain (μmoles/g)			Serum (μmoles/ml)		
Treatment	Glutamate	Glutamine	Aspartate	Glutamate	Glutamine	Aspartate
Saline	10.6 ± 0.48 (8)†	3.9 ± 0.22 (8)	2.5 ± 0.25 (8)	0.15 ± 0.01 (7)	0.56 ± 0.02 (3)	0.10 ± 0.01 (4)
α-Keto-glutarate	10.8 ± 0.14 (8)	3.9 ± 0.27 (8)	2.8 ± 0.12 (8)	0.16 ± 0.03 (3)	0.52 ± 0.04 (3)	
Glutamine	9.9 ± 0.27 (8)	6.2 ± 0.42‡ (8)	2.5 ± 0.17 (8)	0.31 ± 0.12 (3)	2.2 ± 0.12§ (3)	
MSG	10.3 ± 0.16 (8)	3.5 ± 0.21 (8)	2.2 ± 0.11 (8)	4.42 ± 0.43‡ (7)	1.1 ± 0.31 (3)	0.61 ± 0.07 (4)
MSG + aspartate	11.0 ± 0.51 (8)	4.1 ± 0.32 (6)	2.8 ± 0.16 (6)	8.91 ± 1.7‡ (7)	1.8 ± 0.19§ (3)	8.49 ± 0.12 (3)
Aspartate	9.55 ± 0.24 (8)	3.9 ± 0.23 (8)	3.2 ± 0.21§ (8)	1.33 ± 0.20 (7)	0.70 ± 0.02 (3)	6.89 ± 0.57 (4)

* Rats (90–120 g) were injected intraperitoneally with 2 g/kg of all drugs except [MSG + aspartate]; rats in this group received 2 g/kg of each drug. All drugs were given 1 hr before sacrifice.
† Number of determinations is given in parentheses.
‡ $P < 0.01$, differs from control group.
§ $P < 0.05$, differs from control group.

Table 3. Effect of giving MSG or aspartate on their concentrations in retina and serum*

Treatment	Retina (μmoles/g)		Serum (μmoles/ml)	
	Glutamate	Aspartate	Glutamate	Aspartate
Saline	4.99 ± 0.24	2.07 ± 0.05	0.21 ± 0.01	0.10 ± 0.01
MSG	4.98 ± 0.25	1.78 ± 0.13	5.15 ± 0.38†	0.61 ± 0.07†
Aspartate	4.29 ± 0.27	2.18 ± 0.18	2.03 ± 0.16†	6.89 ± 0.57†
MSG + aspartate	4.92 ± 0.24	2.44 ± 0.13	10.44 ± 2.22†	8.49 ± 0.12†

* Groups of eight rats (185–190 g) received MSG and/or aspartate (2 g/kg of each) intraperitoneally 1 hr before sacrifice. Two retinas were pooled for each assay.
† $P < 0.001$ differs from saline-treated group.

peritoneally, nor were changes noted in the retinas of animals given the acidic amino acid intraperitoneally (Table 3) or by stomach tube. This lack of response is somewhat surprising in view of the evidence that radioactively labeled glutamate is taken up into brain [22], and the reports of others [18, 19] that selective, many-fold increases occur in regional glutamate levels after the administration of neurotoxic doses of the amino acid. The possibility remains that brain regions smaller than those examined do selectively accumulate exogenous glutamate. In one study, glutamate levels in the arcuate nucleus reportedly rose 2-fold 1 hr after 4-day-old mice received 12 m-moles/g of MSG [18]. We are now examining the glutamate concentrations of individual hypothalamic nuclei from control rats and animals given exogenous glutamate.

Our data confirm previous reports that MSG administration can greatly elevate serum glutamate concentrations [23]. Indeed, the rise in plasma glutamate might explain most or all of the measured increase in brain glutamate that follows MSG administration. Assuming that each g of rat brain contains 20 μl of blood (an estimate lower than some in the literature, e.g. Ref. 24), one can calculate that 0.3 μmole of "brain" glutamate really is present in the plasma 1 hr after rats received 2 g/kg of glutamate intraperitoneally (Table 2). If this figure were used to correct all the brain glutamate concentrations that we measured after MSG administration, no significant differences would persist in glutamate (or glutamate plus aspartate) concentrations in any of our experiments. (As shown previously [25], brain glutamine levels do rise significantly after glutamine administration, even if a similar correction is made.) This lack of response even to very high doses of glutamate contrasts sharply with the effects on the brain concentrations of neutral amino acids that occur when animals are given much smaller doses of those compounds [26, 27].

When MSG and aspartic acid are administered together, the combined increase in the serum concentrations of the two amino acids is enormous; however, the changes in brain or retinal glutamate concentrations are no greater than those observed in animals receiving glutamate or aspartate alone (i.e. half as much total acidic amino acid). This observation is compatible with evidence *in vitro* [28] and *in vivo* [29] that acidic amino acids, like neutral amino acids, compete for a common mechanism for uptake into the brain. These data provide no basis for concern that the effects of consuming the two amino acids together would amplify risks of neuronal or retinal toxicity.

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Inhibition *in vitro* of norepinephrine *N*-methyltransferase by 2-aminotetralins, analogs of phenylethylamines with rigid conformation

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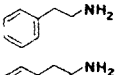
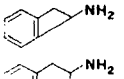
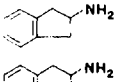
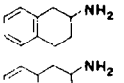
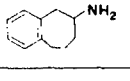
Norepinephrine *N*-methyltransferase (EC 2.1.1.28) is the terminal enzyme in epinephrine biosynthesis. This enzyme, which was referred to as phenylethanolamine *N*-methyltransferase (PNMT) prior to the 1972 recommendations of the International Union of Biochemistry [1], catalyzes the transfer of the methyl group from *S*-adenosylmethionine to norepinephrine. Although a variety of other amines [2–4] are methylated by the enzyme *in vitro*, conversion of norepinephrine to epinephrine may be its only physiological function. For several years inhibitors of norepinephrine *N*-methyltransferase have been sought as pharmacologic tools for interrupting epinephrine biosynthesis without altering the biosynthesis of dopamine and norepinephrine. Known inhibitors of the enzyme include various phenylethylamines and amphetamines [5–7], which are not substrates because they lack the requisite β-hydroxyl group (or similar functional group such as the β-amino); although these amines cannot accept a methyl group they nonetheless combine with and, therefore, inhibit the enzyme. We earlier studied the effect of ring substitution on norepinephrine *N*-methyltransferase inhibition by amphetamines (α-methyl-phenylethylamines) and found that 3, 4-dichloroamphetamine was the most active inhibitor among 33 derivatives that were compared [7]. This paper deals with studies *in vitro* on some phenylethylamine amphetamine analogs having rigid conformation which provides a greater degree of norepinephrine *N*-methyltransferase inhibition.

Norepinephrine *N*-methyltransferase from rabbit adrenal glands was prepared by ammonium sulfate fractionation of the supernatant fluid obtained by high speed centrifugation of tissue homogenates as described previously [8]. Enzyme activity was assayed with 40 μM l-norepinephrine bitartrate (Winthrop) as substrate in the assay method we previously devised using reineckate to precipitate unreacted *S*-adenosylmethionine-[methyl-^{14}C] (New England Nuclear) after incubation [8]. Inhibitors were tested at four to six concentrations, and pI_{50} values (negative logarithm of the molar concentration required for 50 per cent inhibition) were determined by interpolation between points on both sides of 50 per cent inhibition. All of the inhibitors were synthesized in the Lilly Research Laboratories, and their identity and purity were verified by appropriate physicochemical methods.

We started by studying several rigid conformational derivatives of phenylethylamine that had the amino-bearing carbon of the side chain connected to the ortho position of the ring (Table 1). The compound (II) having the carbons directly connected was slightly more active as an inhibitor than was phenylethylamine (I). The derivative (III) with an added methylene unit connecting the carbons had markedly increased inhibitory activity, and a second methylene unit increased the activity still more (IV). Further expansion of the ring then sharply reduced inhibitor activity (V). Thus, the optimum size for the second ring structure was six carbons, inhibitor activity decreasing mark-

Table 1. Inhibition of norepinephrine *N*-methyltransferase by phenylethylamine and related bicyclic compounds

	Structure	Per cent inhibition, Micromolar concentration 3	10	32	100	317	1000	3170	pI_{50}
I						22	40	69	2.83
II				0	5	35	74	95	3.31
III		9	33	66	87	95			4.74
IV		18	47	76	91	96			4.95
V						5	31	70	2.76