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QUANTITATIVE AUTORADIOGRAPHY OF L-[^{3}H]GLUTAMATE BINDING TO RAT BRAIN

JOHN T. GREENAMYRE, ANNE B. YOUNG* and JOHN B. PENNEY

University of Michigan, Neuroscience Lab. Building, 1103 East Huron, Ann Arbor, MI 48109 (U.S.A.)

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A technique has been developed to investigate sodium-independent L-[^{3}H]glutamate binding in rat brain sections using quantitative autoradiography and tritium-sensitive film. Binding is rapid (reaching equilibrium in 5 min) and reversible (having a $t_{1/2}$ of dissociation of 0.38 min). Glutamate apparently bound to a single population of sites with a K_d of about 1.0 μM. The pharmacology of this binding site is similar to that observed in homogenate studies. There is marked regional variation in the amount of glutamate bound. Of the areas analyzed in detail, the density of sites is greatest in stratum moleculare of hippocampus, followed by striatum and cortex.

Abundant electrophysiological and biochemical evidence supports the role of glutamate as a putative excitatory neurotransmitter in the mammalian central nervous system (CNS). The examination of biochemical markers for the neurotransmitter pool of glutamate, such as high-affinity uptake and Ca^{2+}-dependent release, in conjunction with lesion studies, has led investigators to propose numerous glutamatergic pathways including: corticostriate [5, 12]; corticothalamic and corticospinal [23]; perforant path [21]; hippocampal commissural [13]; and cerebellar granule cell [22] pathways. Recently, high affinity binding of L-[^{3}H]glutamate to membrane fractions of CNS has been demonstrated by several laboratories [1, 3, 6, 20]. L-[^{3}H]Glutamate binding is saturable and reversible, is highest in fractions enriched in synaptic junctions [4], is inhibited by various glutamate analogues and antagonists of glutamate-induced depolarization, and appears to be related to synaptic glutamate receptors. Attempts to define the anatomical localization of glutamate binding sites have until recently been limited by available dissection methods [7] and have yielded contradictory results [1, 3, 9]. With the development of quantitative receptor autoradiography, using either the emulsion technique or tritium-sensitive film, the precise regional distribution of various receptor types has been described [10, 14, 15, 17, 24]. It has been technically difficult to apply

* Author for correspondence.

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autoradiographic techniques to glutamate binding because glutamate binding sites typically have dissociation constants in the high nanomolar to low micromolar range and an extremely rapid dissociation rate. Recently, however, a brief, preliminary report indicated that autoradiography of glutamate binding sites may be feasible [8]. We report here the use of quantitative autoradiography to study kinetic, saturation and competition data of [^{3}H]glutamate binding in highly localized regions of rat brain.

Sprague–Dawley male rats (150–175 g) were decapitated, the brains quickly removed, blocked, placed in ice-cold buffered 0.32 M sucrose (pH 7.4) and allowed to cool to about 4°C. The tissue was then mounted on a microtome chuck with Lipshaw embedding matrix and frozen under powdered dry ice. Twenty μm coronal brain sections were cut on a Lipshaw cryostat and thaw-mounted onto subbed slides. Sections were washed for 30 min in ice-cold 50 mM Tris-HCl (pH 7.4), containing 2.5 mM $CaCl_2$, in order to remove endogenous glutamate and then were blown dry with a stream of cool air. The tissue was routinely incubated for 30 min

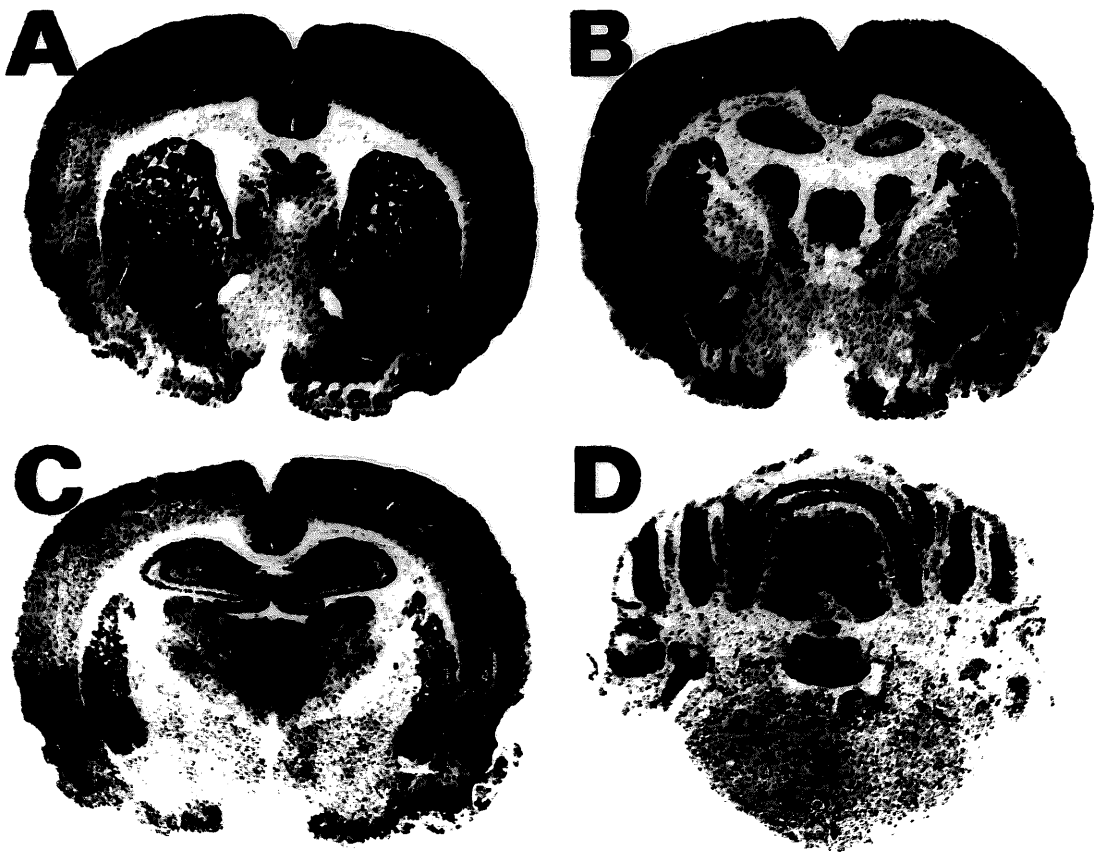

Fig. 1. Autoradiographs of L-[^{3}H]glutamate binding. A: rostral striatum. B: striatum, globus pallidus and rostral hippocampus. C: hippocampus, thalamus and hypothalamus. D: cerebellum and medulla. L-[^{3}H]Glutamate concentration was 97 nM.

at 37°C with various concentrations (50–1000 nM) of [^{3}H]glutamate (43 Ci/mmol, Amersham) in 50 mM Tris-HCl containing 2.5 mM $CaCl_2$. Sections were incubated for 0–30 min for kinetic studies. In competition studies unlabeled drugs were included in the assay mixture. Non-specific binding was determined in the presence of 1 mM unlabeled glutamate. Non-specific binding represented 25–35% of total binding at [^{3}H]glutamate concentrations of 50–1000 nM. However, at higher ligand concentrations non-specific binding represented a higher proportion of total binding. After the incubation, sections were rinsed 3 times with cold buffer, followed by a rinse with cold 5% glutaraldehyde in acetone in order to dry the slides rapidly and minimize uneven dissociation during drying. The total rinse time was approximately 10 sec. For dissociation studies the sections received 3 standard buffer rinses, followed by immersion in a large volume ('infinite dilution') of cold buffer for various times (15 sec to 10 min), and finally the glutaraldehyde/acetone rinse. Slides were blown dry with warm air, placed in X-ray cassettes with appropriate standards (16) and apposed to Ultrofilm ^{3}H (LKB).

After a 14–21-day exposure at 4°C, the film was developed in D-19 for 4 min at 20°C, fixed and dried. The film was placed in a photographic enlarger and the optical densities of areas of film determined with a computer-assisted microdensitometer, located at the center of the enlarger's image plane. Sixteen readings from each region were averaged and the radioactivity was determined by a computer-generated polynomial regression analysis, which compared film densities produced by the sections with those produced by the standards, as previously described [16].

The binding of L-[^{3}H]glutamate to tissue sections showed marked regional heterogeneity (Fig. 1). Cortex, striatum, hippocampus and the molecular layer of the cerebellum all are thought to receive extensive glutamatergic input, and by this technique show the highest densities of binding sites. It is of interest that in hippocampus and cerebellum the density of sites is heaviest in stratum moleculare and the molecular layer, respectively, areas sparse in neuronal cell bodies where dendritic arborization and putative glutamatergic synaptic activity are extensive [18]. This is consistent with findings from hippocampus [2] and cerebellum [19] that the ontogeny of glutamate binding sites closely parallels the development of the hippocampal molecular layers and the parallel fiber system, respectively, and further suggests that these sites are related to postsynaptic glutamate receptors.

Scatchard analysis of glutamate binding reveals an apparent single homogeneous population of binding sites with a K_d of about 1 μM (Fig. 2A). Data analyzed from 3 regions of forebrain indicate that the density of binding sites is greatest in the stratum moleculare of the hippocampus, (K_d = 960 ± 35 nM, B_{max} = 1.99 ± 0.08 pmol/mg tissue) followed by striatum (K_d = 891 ± 96 nM, B_{max} 1.17 ± 0.09 pmol/mg tissue) and cortex, layers 5 and 6 (K_d = 957 ± 46 nM, B_{max} = 1.08 ± 0.18 pmol/mg tissue). Hill coefficients of 1.0 were calculated from these saturation data, suggesting an apparent single class of binding sites. However, it should be noted that the saturation curves have not as yet been carried to very high ligand con-

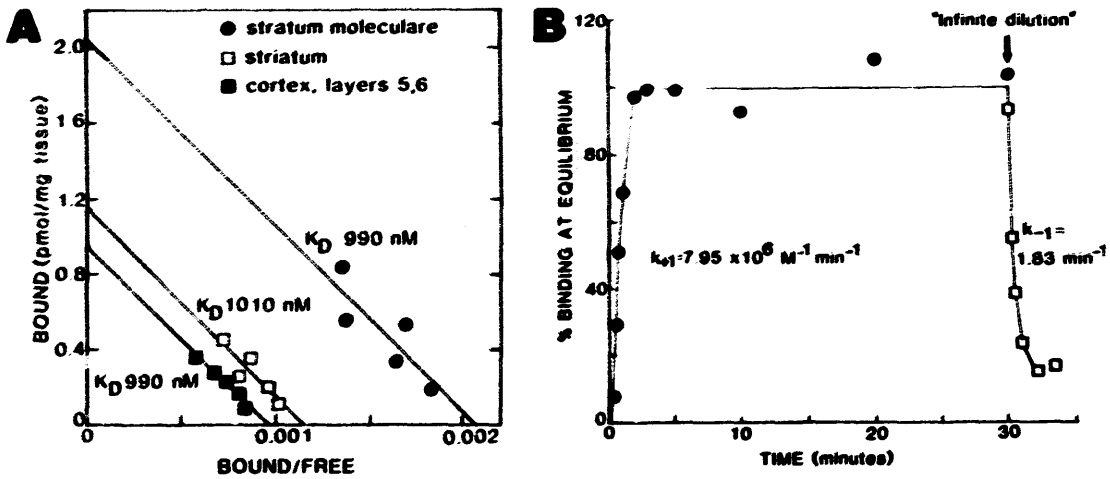

Fig. 2.A: representative Scatchard plots of L-[^{3}H]glutamate binding in stratum moleculare of hippocampus (B_{max} = 2.05 pmol/mg tissue, r = 0.86); striatum (B_{max} = 1.16 pmol/mg tissue, r = 0.89); and cortex, layers 5 and 6 (B_{max} = 0.95 pmol/mg tissue, r = 0.98). Ligand concentrations between 100 and 600 nM were used in this experiment. Autoradiography was performed as described in text. Each point represents specific binding (the average of 16 readings in an area of interest minus readings from adjacent sections incubated in presence of 1 mM glutamate). The experiment has been replicated 3 times. B: association and dissociation of L-[^{3}H]glutamate binding. Data were taken from striatum. The concentration of [^{3}H]glutamate was 97 nM. Points represent specific binding. Tissue was incubated for 0–30 min for association studies (closed circles) and additional sections were rinsed as described in text for dissociation studies (open squares).

centrations for technical reasons and thus it is possible that additional lower affinity sites exist [11]. This possibility is currently under investigation.

The binding of [^{3}H]glutamate to sections was rapid and reached equilibrium within 5 min (Figure 2B). Binding was stable for at least 30 min. The association rate constant (k_1) was determined to be 8.47 ± 0.72 × 10^6 M^{-1} min^{-1}. After 30

TABLE I

INHIBITION OF SPECIFIC BINDING OF L-[^{3}H]GLUTAMATE BY VARIOUS GLUTAMATE ANALOGUES.

Analogues were tested at 100 μM, 1 mM and 5 mM. The concentration of L-[^{3}H]glutamate was 47 nM. Values represent averages of 2 or 3 experiments. Data were taken from striatum.

Drug	% Displacement		
	5 mM	1 mM	100 μM
Quisqualic acid	79	81	49
Ibotenic acid	74[a]	73	32
L-Aspartic acid	64	44	23
D,L-Homocysteic acid	71	46	18
D,L-α-Aminoadipic acid	55	37	4
D,L-2-Amino-4-phosphonobutyric acid	39	17	0
Kainic acid	26	21	9
N-Methyl-D,L-aspartic acid	12	8	11

[a] 2.5 mM

min of incubation, when sections at equilibrium were placed in 300 ml of cold buffer ('infinite dilution'), the bound glutamate quickly dissociated from the binding site with a half time of 0.38 min. This half time corresponds to a dissociation rate constant (k_{-1}) of 1.83 ± 0.01 min^{-1}. The ratio, k_{-1}/k_1, is an estimate of the equilibrium dissociation constant of binding and gives a K_d of 216 nM. This K_d value is roughly within the range of that found from equilibrium saturation studies, the difference probably reflecting the difficulties inherent in measuring such rapid rates of association and dissociation.

In displacement studies unlabelled glutamate was the most potent inhibitor of [^{3}H]glutamate binding. Other compounds were tested at concentrations of 100 μM, 1 mM and 5 mM for their ability to inhibit binding in striatum (Table I). The cyclic glutamate analogues, quisqualate and ibotenate, were the next most potent diplacers, followed by D,L-homocysteic acid and L-aspartate. The putative antagonists, D,L-α-aminoadipic acid and D,L-2-amino-4-phosphonobutyric acid, were of intermediate potency. Kainic acid and *N*-methyl-D,L-aspartate were almost inactive. This rank order of potencies of the displacers is virtually identical to that of Foster and Roberts [6] using a cerebellar membrane preparation. It must be kept in mind, however, that glutamate binding sites in different areas of brain could exhibit distinct pharmacological specificities.

This report indicates that detailed kinetic, saturation and competition data on L-[^{3}H]glutamate binding can be obtained autoradiographically from highly circumscribed regions of brain. The data presented are consistent with reports from other laboratories using homogenate techniques and suggest that the glutamate binding site is related to postsynaptic glutamate receptors. Further support for this hypothesis is the fact that regions of brain suspected to receive extensive glutamatergic input show the greatest density of binding sites. In particular, autoradiography provides direct evidence that relatively acellular regions that are rich in dendritic processes and putative glutamatergic synapses show a high density of binding sites.

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