

QUANTITATIVE AUTORADIOGRAPHIC DISTRIBUTION OF L-[³H] GLUTAMATE-BINDING SITES IN RAT CENTRAL NERVOUS SYSTEM¹

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Abstract

Quantitative autoradiography was used to determine the distribution of L-[³H]glutamate-binding sites in the rat central nervous system. Autoradiography was carried out in the presence of Cl⁻ and Ca²⁺ ions. Scatchard plots and Hill coefficients of glutamate binding suggested that glutamate was interacting with a single population of sites having a K_D of about 300 nM and a capacity of 14.5 pmol/mg of protein. In displacement studies, ibotenate also appeared to bind to a single class of non-interacting sites with a K_I of 28 μ M. However, quisqualate displacement of [³H]glutamate binding revealed two well-resolved sites with K_I s of 12 nM and 114 μ M in striatum. These sites were unevenly distributed, representing different proportions of specific glutamate binding in different brain regions. The distribution of glutamate-binding sites correlated very well with the projection areas of putative glutamatergic pathways. This technique provides an extremely sensitive assay which can be used to gather detailed pharmacological and anatomical information about L-[³H]glutamate binding in the central nervous system.

Abundant electrophysiological and biochemical evidence supports the role of glutamate, or a glutamate-like substance, as a major excitatory transmitter in the central nervous system (for recent reviews, see Watkins, 1978; Davies et al., 1979; Cotman et al., 1981; Cotman and Nadler, 1981; Watkins and Evans, 1981; Fagg and Foster, 1983; Zaczek et al., 1983). Electrophysiologically, glutamate is almost universally excitatory, indicating the presence of glutamate receptors on most central nervous system neurons. With the development of various glutamate analogues, including antagonists of excitatory amino acids, physiologists have proposed numerous glutamatergic pathways (Watkins and Evans, 1981; Fagg and Foster, 1983), as well as multiple receptor types (Cotman et al., 1981; Watkins and Evans, 1981; McLennan, 1981). Biochemical evidence also strongly suggests a transmitter function for glutamate. Glutamate is selectively located in neurons and nerve terminals (Kuhar and Snyder, 1970; Storm-Mathisen et al., 1983) and is transported into synaptosomes by a high-affinity, sodium-dependent uptake system (Wofsey et al., 1971; Balcar and Johnston, 1972; Divac et al., 1977). Glutamate is released in a calcium-dependent fashion upon depolarization of the neuronal terminals of excitatory pathways (Potashner, 1978; Sandoval and Cotman, 1978; Pearce and Dutton, 1981), and it binds with high affinity to neuronal membranes in dendritic zones (Mi-

chaelis et al., 1974; Roberts, 1974; Foster and Roberts, 1978; Biziere et al., 1980; Baudry and Lynch, 1981a; Foster et al., 1981; Werling and Nadler, 1982; Greenamyre et al., 1983). By examining certain of these biochemical markers, such as high-affinity uptake and calcium-dependent release, in the projection areas of lesioned excitatory afferents, investigators have proposed glutamatergic pathways which are in good agreement with those suggested on physiological grounds (Watkins and Evans, 1981; Fagg and Foster, 1983).

The binding sites for L-glutamate have several characteristics suggesting that they are related to postsynaptic glutamate receptors (for reviews, see Baudry and Lynch, 1981b; Michaelis et al., 1981; Roberts and Sharif, 1981). L-[³H]Glutamate binding is saturable and reversible, and is highest in dendritic zones and in membrane fractions enriched in synaptic junctions. Binding is inhibited by a variety of glutamate analogues including antagonists of glutamate-induced depolarization. After lesions of projection areas of putative glutamatergic pathways, glutamate binding is decreased (Biziere et al., 1980; Roberts et al., 1982), suggesting a postsynaptic location of the binding sites.

Attempts to describe the regional distribution of [³H]glutamate-binding sites have been limited by available dissection methods and by the amount of tissue required for reliable binding assays and have yielded conflicting results (Biziere et al., 1980; Head et al., 1980; Baudry and Lynch, 1981a). Recently, *in vitro* autoradiographic techniques for examining the binding of [³H]glutamate to rat brain sections have been described (Greenamyre et al., 1983; Halpain et al., 1983; Monaghan et al., 1983). We report here a detailed description of the regional distribution of glutamate-binding sites determined in the presence of both chloride and calcium ions.

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Materials and Methods

Materials. L-[^3H]Glutamic acid (45 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Ibotenic acid was purchased from Regis Chemical Co. (Morton Grove, IL). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue preparation. Sprague-Dawley male rats (150 to 300 gm) were decapitated, and the brains were quickly removed and blocked, mounted on a microtome chuck with Lipshaw embedding matrix, and frozen under powdered dry ice. Twenty-micrometer sections were cut on a Lipshaw cryostat and thaw-mounted onto subbed slides. Sections were washed for 30 min at 20°C in 50 mM Tris-HCl (pH 7.2), containing 2.5 mM CaCl_2 , in order to remove endogenous glutamate, and then were blown dry with a stream of cool air (room temperature).

Autoradiography. In regional distribution studies, the tissue was incubated for 45 min at 2°C with 8 ml of 200 nM L-[^3H]glutamate (diluted with unlabeled glutamate to a "working" specific activity of 4.5 Ci/mmol) in 50 mM Tris-HCl containing 2.5 mM CaCl_2 . Under these conditions, "zone A" conditions are maintained and less than 5% of free ligand is bound. In competition studies unlabeled drugs were included in the assay mixture. Concentrations of glutamate between 20 nM and 2.5 μM were used in saturation experiments. Nonspecific binding was determined in the presence of 1 mM unlabeled glutamate and represented 5 to 15% of total binding at a [^3H]glutamate concentration of 200 nM.

After the incubation, sections were rinsed three times with cold buffer, then rinsed twice with 2 ml of cold 2.5% glutaraldehyde in acetone, in order to dry the slides rapidly and minimize uneven dissociation during drying. The total rinse time was approximately 10 sec. (Because glutamate has a rapid dissociation rate and a rinse in buffer alone results in uneven dissociation during drying, a number of different rinse procedures were examined to minimize this problem. It was found that a rinse with 2.5% glutaraldehyde in acetone markedly improved the reproducibility of our results, without altering the characteristics or regional distribution of binding. The time from first contact with this solution until the slides were dry was 2 to 3 sec. Thus, there is less chance for the tritium label to move or dissociate during drying.) Slides were blown dry with warm air, placed in x-ray cassettes with appropriate radioactive standards (Pan et al., 1983), and exposed to Ultrafilm ^3H (LKB).

After a 14- to 21-day exposure at 4°C, the film was developed in D-19 for 3 min at 20°C, fixed, and dried. The film was placed in a

photographic enlarger, and the optical densities of areas of film were determined with a computer-assisted microdensitometer located at the center of the enlarger's image plane. Sixteen readings from each area were averaged, and the radioactivity was determined by a computer-generated polynomial regression analysis, which compared film densities produced by the tissue sections with those produced by the radioactive standards.

Following exposure and development of the autoradiographs, the unfixed tissue sections were stained for Nissl substance with thionin. Photographs of the autoradiographs and the Nissl-stained sections were made in mirror-image relation to each other. The Nissl-autoradiograph composites represent the *same one-half* of a single tissue section.

Results

Kinetics of L-[^3H]glutamate binding. In saturation studies, using glutamate concentrations between 20 nM and 2.5 μM , glutamate apparently bound to a single population of sites with a K_D of 285 ± 52 nM as indicated by linear Scatchard plots (Fig. 1). The maximum number of binding sites was 14.5 ± 1.2 pmol/mg of protein. Hill plots of these data yielded a value of 0.92 ± 0.03 , again suggesting that glutamate is binding to a single class of sites. Inclusion of ibotenate (100 nM to 1000 μM) in the reaction mixture inhibited the binding of glutamate to the tissue sections with a K_I of 27.7 ± 0.3 μM and a Hill coefficient of 0.88 ± 0.08 (Fig. 2). Quisqualate (1 nM to 1000 μM) resolved two distinct binding sites in competition studies (Fig. 2). The K_I s of these sites were determined by an iterative curve-fitting computer program (Munson and Rodbard, 1980) to be 12 ± 5 nM and 114 ± 3 μM . In striatum, approximately 43% of the sites were of the high-affinity type and the remainder had low affinity for quisqualate. The proportion of high affinity sites was about 78% in the molecular layer of cerebellum and about 36% in the granule cell layer (Fig. 3). It thus appears that L-[^3H]glutamate is labeling at least two distinct sites, which are indistinguishable in saturation binding studies but which can be resolved by quisqualate competition. The sites appear to have different distributions.

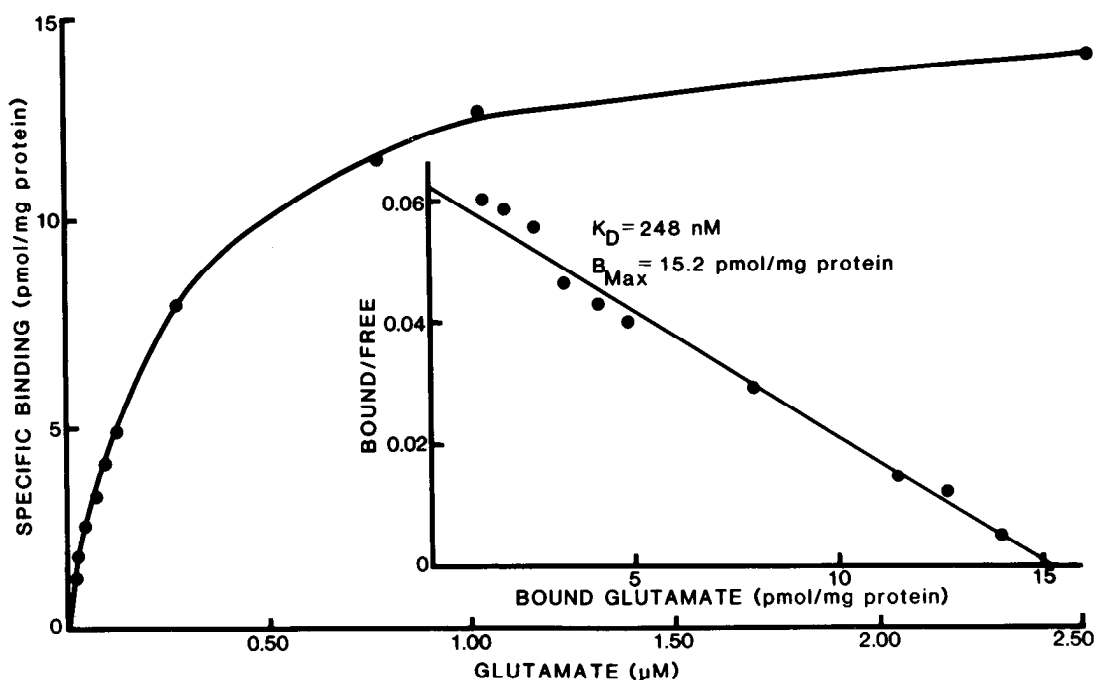


Figure 1. Representative saturation and Scatchard plots of L-[^3H]glutamate binding in striatum. Ligand concentrations between 20 nM and 2.5 μM were used in saturation studies. Autoradiography was performed as described in the text. Each point represents specific binding (the average of 16 readings in an area of interest minus readings from an adjacent section incubated in the presence of 1 mM glutamate).

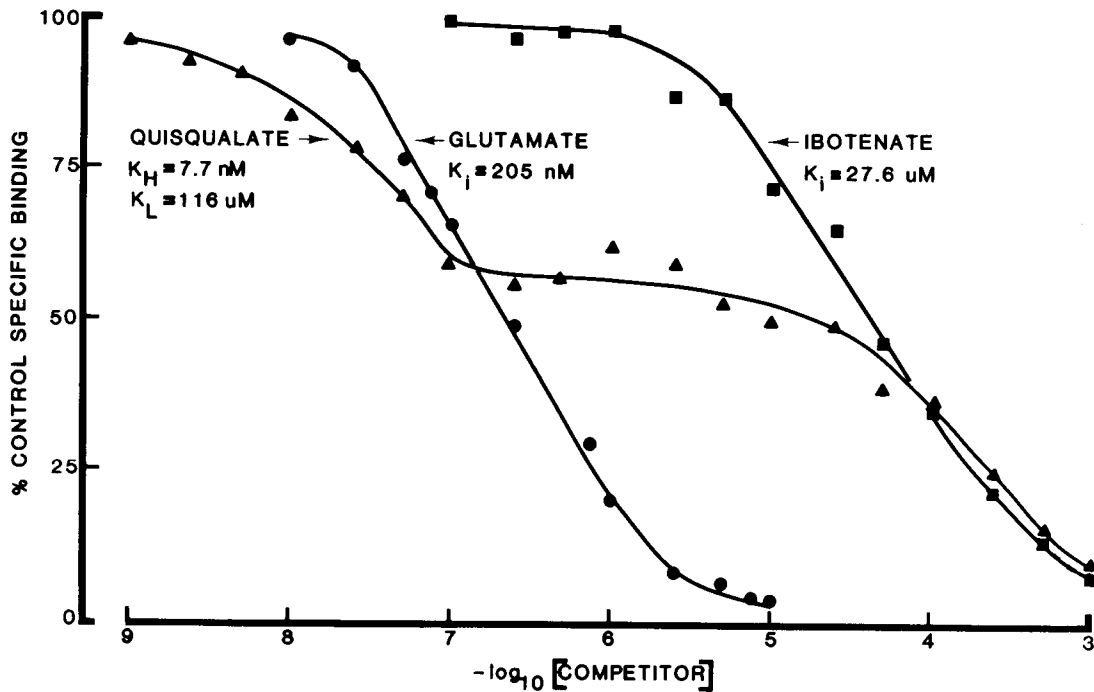


Figure 2. Representative plots of inhibition of striatal $[^3\text{H}]$ glutamate binding by quisqualate (1 nM to 1000 μM), unlabeled glutamate (10 nM to 1000 μM), and ibotenate (100 nM to 1000 μM). Each point represents specific binding. The concentration of $[^3\text{H}]$ glutamate was 200 nM. Hill coefficients near 1 were calculated for ibotenate and unlabeled glutamate, and inhibition constants (K_i) for these compounds were determined by log-logit regression analysis using the formula: $K_{1/2} = \text{IC}_{50} / (1 + L/K_D)$. The $K_{1/2}$ s for quisqualate displacement were determined by an iterative curve-fitting computer program (Munson and Rodbard, 1980).

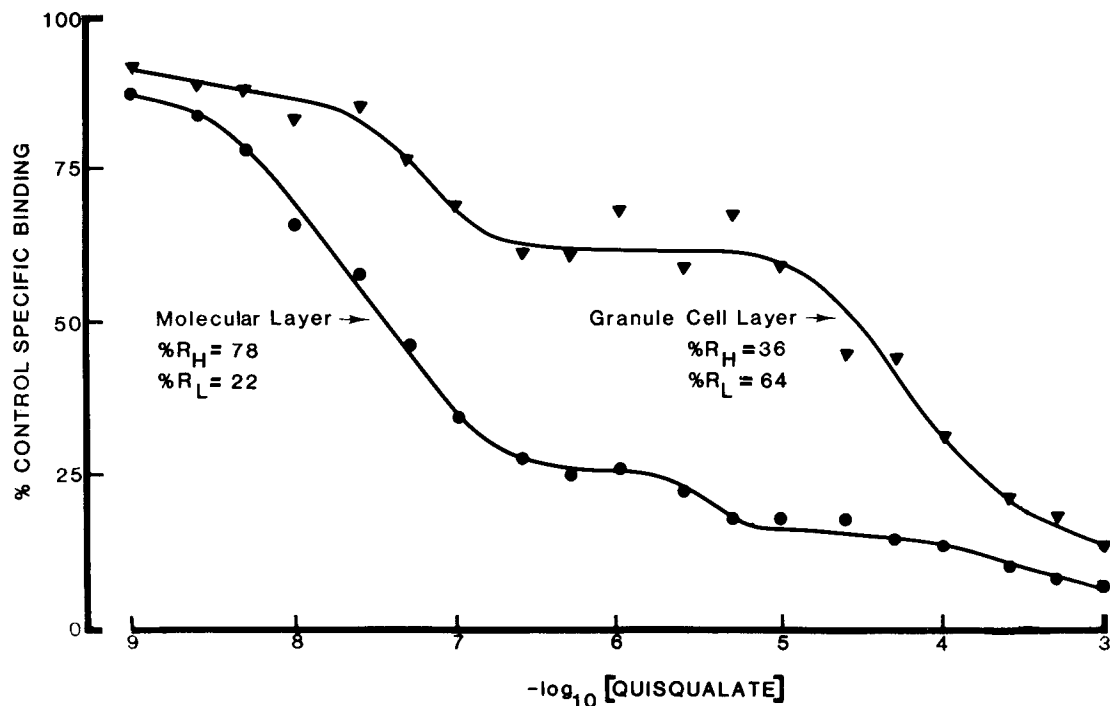


Figure 3. Inhibition of $[^3\text{H}]$ glutamate binding by quisqualate in cerebellar molecular and granule cell layers. Points represent the average specific binding from four determinations (16 readings per determination) in two separate experiments. The concentration of $[^3\text{H}]$ glutamate was 200 nM. The proportion of sites in the molecular layer having a high affinity for quisqualate ($\%R_H$) was 78. In the granule cell layer $\%R_H$ was 36.

Regional distribution of L- $[^3\text{H}]$ glutamate-binding sites. The binding of glutamate to rat brain sections exhibited a marked regional heterogeneity, with the highest levels found in the stratum moleculare of the dentate gyrus. The distribution of glutamate-binding sites is summarized in Table I, both in terms

of the absolute amount of bound glutamate and the amount of binding relative to that in stratum moleculare. Representative autoradiographs and Nissl-stained sections are shown in Figure 4; the composites represent the same side of the same section, as described under "Materials and Methods."

TABLE I

Regional distribution of glutamate-binding sites in rat brain

Values represent the mean \pm SEM of three experiments (3 animals). L-[³H]Glutamate concentration was 200 nM. Autoradiography and quantitation of autoradiographs were performed as described in the text.

Area	Glutamate Bound <i>pmol/mg of protein</i>	Percentage Binding Relative to Stratum Moleculare
Olfactory bulb		
External plexiform layer (EPI)	4.0 \pm 0.7	58.7
Anterior olfactory nucleus, lateral (AOL)	4.3 \pm 0.6	62.7
Internal granular layer (IGr)	2.7 \pm 0.3	40.2
Anterior olfactory nuclei (AON)	4.1 \pm 0.8	61.1
Cortex		
Frontal, layers 1 & 2 (Fr)	6.0 \pm 0.7	88.5
Frontal, layers 5 & 6 (Fr)	3.7 \pm 0.5	54.2
Anterior cingulate (ACg)	5.3 \pm 0.7	78.6
Posterior cingulate (PCg)	3.1 \pm 0.1	45.4
Piriform, pyramidal cell layer (Pfc)	2.7 \pm 0.4	39.7
Visual cortex (Str)	5.0 \pm 0.6	74.1
Septal area		
Nucleus accumbens (Acb)	4.3 \pm 0.5	63.7
Lateral septal nuclei (LS)	4.6 \pm 0.6	67.8
Medial septum (MS)	1.4 \pm 0.2	20.9
Diagonal band, vertical (VDB)	1.5 \pm 0.3	22.8
Hippocampus		
Stratum moleculare (SMo)	6.8 \pm 0.2	100.0
Stratum oriens (Or)	5.1 \pm 0.2	75.1
Stratum radiatum (Rad)	5.9 \pm 0.2	87.7
Granular layer, dentate gyrus (GrDG)	2.8 \pm 0.3	41.6
Subiculum dorsal (Sd)	3.1 \pm 0.1	46.4
Thalamus		
Lateral dorsal (LD)	4.3 \pm 0.1	63.6
Medial dorsal (MD)	3.6 \pm 0.1	52.4
Anteroventrolateral/medial (AVL/M)	2.6 \pm 0.3	38.1
Reticular (RT)	1.5 \pm 0.1	21.5
Lateral habenular (LHb)	1.6 \pm 0.4	24.2
Ventral posterior lateral/medial (VPL/M)	3.3 \pm 0.3	48.3
Posterior (Po)	2.0 \pm 0.2	29.2
Dorsal lateral geniculate (DLG)	3.6 \pm 0.1	52.7
Ventral lateral geniculate (VLG)	1.6 \pm 0.1	24.1
Medial geniculate (MG)	3.8 \pm 0.2	56.3
Basal ganglia		
Caudate-putamen (CPu)	4.6 \pm 0.4	67.4
Globus pallidus (GP)	1.3 \pm 0.1	19.1
Amygdala		
Lateral nucleus (La)	3.7 \pm 0.2	54.8
Basolateral nucleus (BL)	4.1 \pm 0.2	60.0
Forebrain, other		
Subthalamic nucleus (STN)	1.7 \pm 0.1	23.9
Zona incerta (ZI)	1.1 \pm 0.1	15.8
Olfactory tubercle (Tu)	3.8 \pm 0.2	55.8
Nucleus lateral olfactory tr. (LOT)	3.6 \pm 0.3	53.3
Lateral hypothalamus (LH)	1.2 \pm 0.1	17.3
Bed nucleus stria terminalis (BST)	2.5 \pm 0.1	36.6
Corpus callosum (CC)	0.7 \pm 0.1	9.7
Brainstem		
Superior colliculus (SC)	3.5 \pm 0.1	52.0
Periaqueductal gray (PAG)	2.0 \pm 0.1	29.0
Substantia nigra reticulata (SNr)	2.0 \pm 0.1	29.0
Substantia nigra compacta (SNc)	1.3 \pm 0.1	19.2
Medial mammillary nucleus, posterior (MP)	2.2 \pm 0.1	31.9
Interpeduncular nucleus, central (IPC)	1.9 \pm 0.3	28.5
Deep mesencephalic nuclei (DpMe)	0.8 \pm 0.1	12.6
Inferior colliculus, dorsal (ICD)	1.9 \pm 0.1	28.2
Pontine nuclei (Pn)	1.2 ^a	17.3

^a Mean of two experiments.

TABLE 1—continued

Area	Glutamate bound <i>pmol/mg of protein</i>	Percentage Binding Relative to Stratum Moleculare
Dorsal cochlear nucleus (DCo)	1.7 ± 0.3	24.9
Nucleus solitarius, medial (SolM)	1.7 ± 0.1	25.4
Hypoglossal nucleus (12)	0.7 ± 0.1	10.6
Inferior olivary nuclei (IO)	1.5 ± 0.2	22.6
Spinal nucleus of V, caudal (SP5C)	1.4 ± 0.3	21.2
Cerebellum		
Molecular layer (Mol)	3.3 ± 0.2	49.0
Granule cell layer (Gr)	2.1 ± 0.2	26.3
Spinal cord		
Substantia gelatinosa, cervical (CSG)	2.6 ± 0.4	38.0
Substantia gelatinosa, thoracic (TSG)	2.1 ± 0.4	30.4
Substantia gelatinosa, lumbar (LSG)	2.8 ± 0.4	40.8
Ventral horn, cervical (CVH)	0.7 ± 0.2	10.4
Ventral horn, thoracic (TVH)	0.9 ± 0.2	13.2
Ventral horn, lumbar (LVH)	0.8 ± 0.2	11.8

Forebrain. As can be seen in Figures 4 and 5, the amount of glutamate binding varies widely in the forebrain. The olfactory bulb nuclei and molecular layers exhibited relatively high levels of binding, whereas the glomerular layer did not bind glutamate above background levels. Cortical areas differed more than 2-fold in glutamate binding. The highest cortical binding was found in superficial frontal cortex, the lowest in the pyramidal cell layer of piriform cortex. It was a consistent finding that more glutamate bound to superficial layers of cortex than to deep layers, and to anterior cingulate cortex more than to posterior cingulate cortex.

The basal ganglia showed marked differences in the levels of bound glutamate, with the caudate-putamen having many more binding sites than the globus pallidus. Similarly, in the septal area there were large differences in glutamate binding in adjacent structures. The lateral septal nuclei and the nucleus accumbens bound approximately 3 times as much glutamate as the medial septum or vertical limb of the diagonal band.

In the hippocampus, glutamate binds in a distinctive laminar pattern (Fig. 6), with highest levels in the stratum moleculare and much less in the granule cell layer and subiculum. Stratum radiatum and stratum oriens both showed a high density of sites. Subnuclei of the amygdala bound different amounts of glutamate, as can be seen in Figure 4F. The lateral and basolateral nuclei bind more glutamate than do other amygdaloid nuclei.

Various thalamic nuclei were readily distinguished by the amount of bound glutamate. The lateral dorsal nucleus bound the most glutamate, and the reticular and lateral habenular nuclei had relatively little glutamate binding. The ventral posterior thalamus (lateral and medial portions) stands out in sharp contrast to other posterior thalamic nuclei because of its high density of binding sites (Fig. 4G). Heavy glutamate binding was also found in medial and dorsal lateral geniculate bodies; the ventral lateral geniculate showed much less binding.

In other forebrain regions, the subthalamus could be distinguished from zona incerta by its higher level of binding. Both the olfactory tubercle and nucleus of the lateral olfactory tract had relatively high levels of binding sites. The bed nucleus of the stria terminalis bound an intermediate amount of glutamate, and the lowest levels of glutamate binding in the forebrain were found in the hypothalamus and in the white matter of the corpus callosum.

Brainstem and cerebellum. In general, there was less binding

in brainstem than in forebrain. The superficial gray of the superior colliculus had the most glutamate binding of any midbrain structure. There was a differential distribution of sites within the substantia nigra, with approximately 50% more binding in pars reticulata than in pars compacta. Intermediate levels of binding were found in periaqueductal gray matter, the mammillary body, the interpeduncular nucleus, and the inferior colliculus. Very little binding was found in deep mesencephalic nuclei.

Of the other brainstem nuclei where binding was measured, the most glutamate was bound in the medial nucleus of the solitary tract, followed closely by the dorsal cochlear nucleus, the inferior olivary nuclei, and the spinal nucleus of the trigeminal nerve, pars caudalis. Slightly less binding was found in the pontine nuclei and even less glutamate bound to the hypoglossal nucleus. There was very little binding in pontine or medullary tegmentum.

The molecular layer of cerebellum bound about one-half the amount of glutamate that was found in stratum moleculare in hippocampus. Forty percent less binding was found in the granule cell layer.

Spinal cord. Relatively high levels of glutamate were bound to substantia gelatinosa of the spinal cord at all levels. Uniformly little binding was seen in the ventral horn of the spinal cord.

Discussion

This report confirms earlier findings that quantitative autoradiography may be used to examine the pharmacological specificity and regional distribution of glutamate binding in central nervous system tissue sections (Greenamyre et al., 1983; Halpain et al., 1983; Monaghan et al., 1983). As previously reported (Greenamyre et al., 1983), the binding of glutamate to tissue sections is saturable and reversible, having a $t_{1/2}$ of dissociation of 0.38 min. In the present study, glutamate bound to tissue with an affinity similar to that found in other autoradiographic studies as well as homogenate studies (Roberts and Sharif, 1981; Slevin and Coyle, 1981). Our earlier studies were carried out at 37°C (Greenamyre et al., 1983). Subsequently, it was found that the same pattern of binding was obtained at 2°C. At this temperature, total binding was at least as great as at 37°C, and nonspecific binding was reduced and more uniform. Saturation studies yielded linear Scatchard plots, and in com-

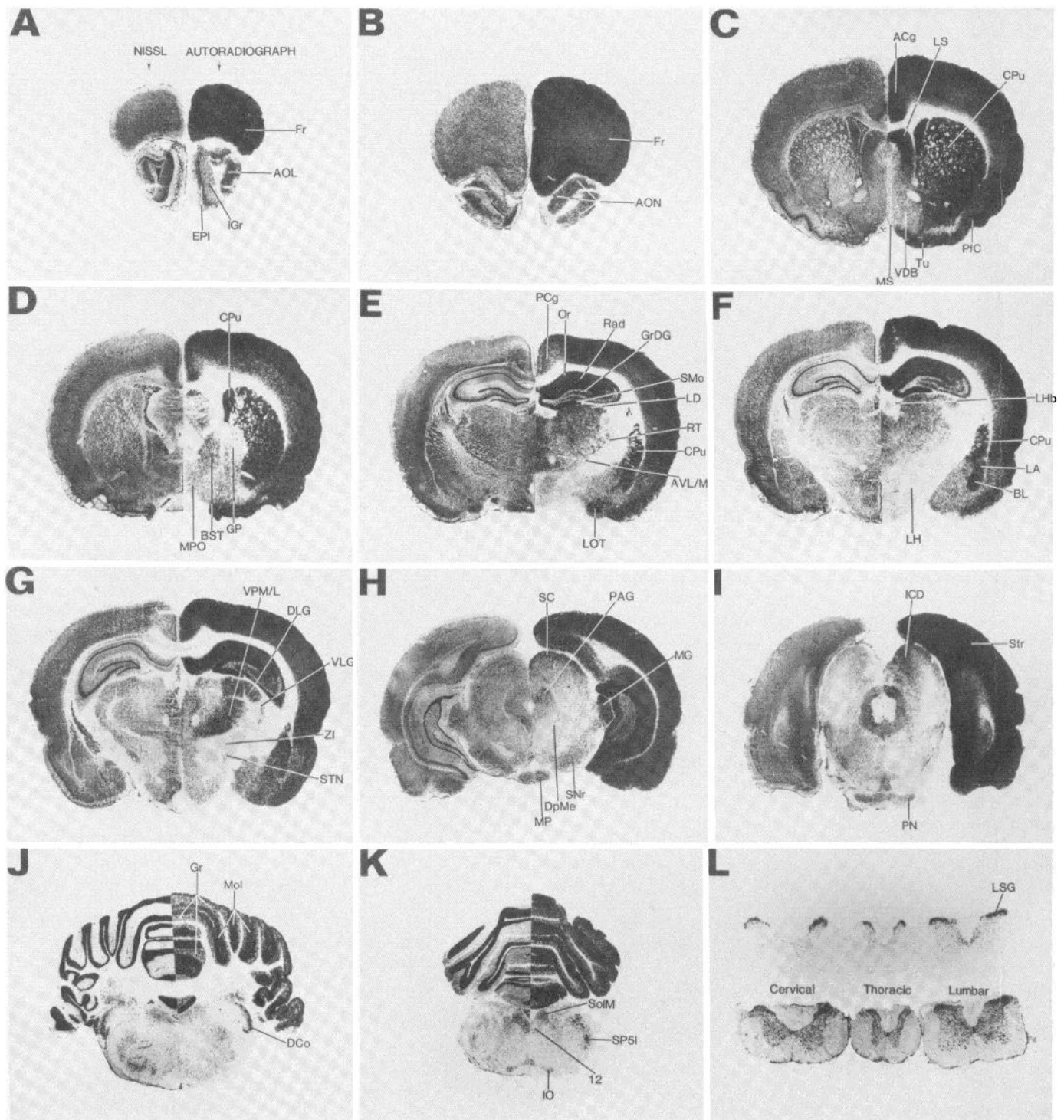


Figure 4. Representative autoradiograph-Nissl stain composites. Autoradiography and quantitation were performed as described in the text. Photographs of autoradiographs and Nissl-stained sections were prepared in mirror-image relation to each other so that a composite represents the same one-half of a single tissue section. Each photograph and composite was printed individually; therefore, quantitative comparisons between photographs are not possible. Abbreviations are defined in Table I.

petition studies with unlabeled glutamate, Hill coefficients near unity were calculated. These results suggested that glutamate was binding to a single class of sites. Ibotenate also appeared to interact with a single site in this system, with an affinity lower than that of glutamate, as others have reported (Honore et al., 1981; Fagg et al., 1983a). When quisqualate (1 nM to 1000 μ M) was added to the assay in competition studies, two well-resolved sites became apparent. Biphasic displacement of

glutamate binding by quisqualate has recently been described by others (Slevin et al., 1982; Werling and Nadler, 1982; Fagg et al., 1983a; Werling et al., 1983).

L-[3 H]Glutamate-binding sites are regulated by a variety of ions including Na^+ , Ca^{2+} , and Cl^- (Baudry and Lynch, 1979, 1981a; Baudry et al., 1983b; Fagg et al., 1982, 1983a; Werling and Nadler, 1983). The Na^+ -dependent component appears to represent binding to the high-affinity uptake site (Vincent and

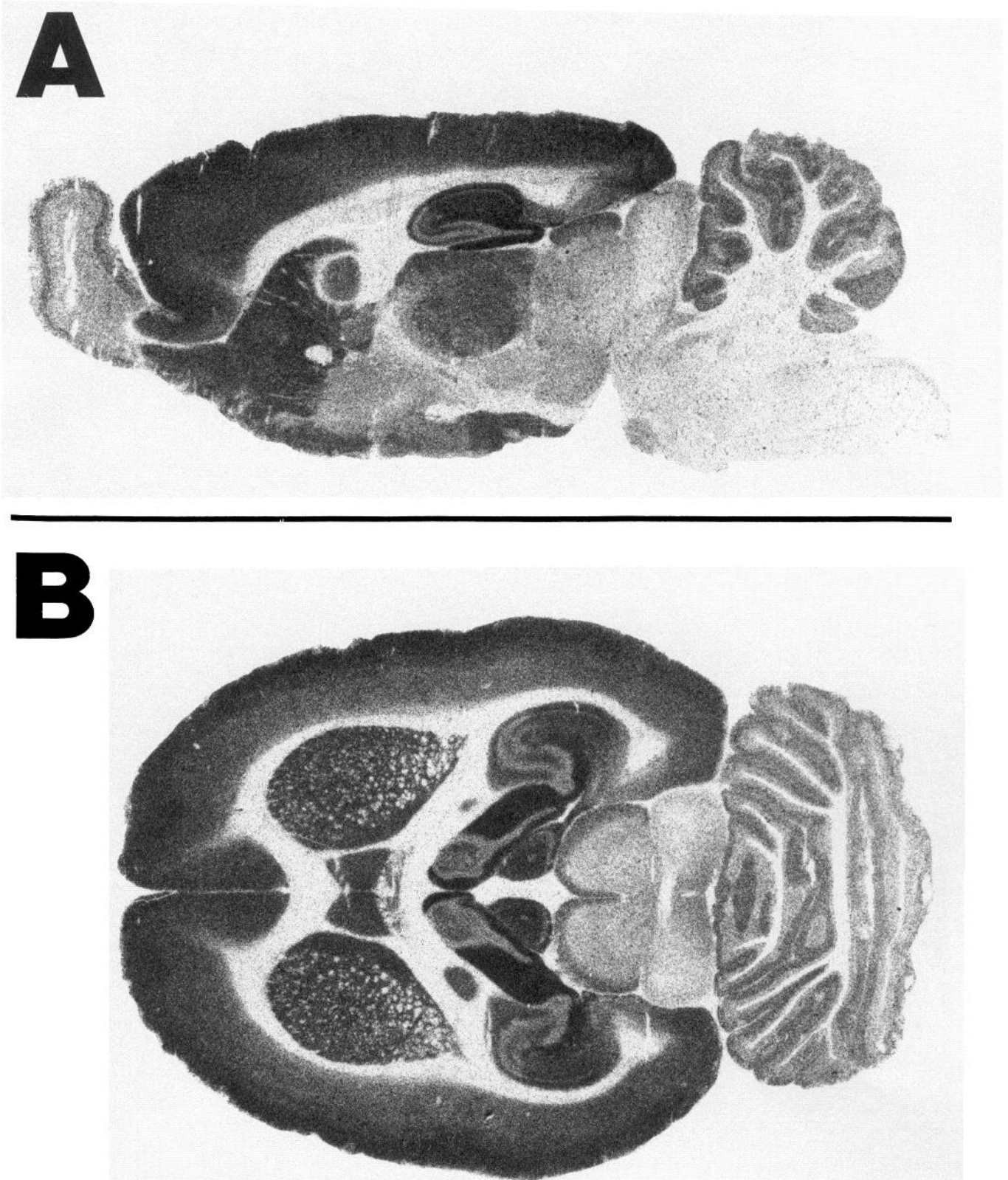


Figure 5. Autoradiographs of a sagittal (A) and a horizontal (B) section of rat brain. Many of the structures labeled in Figure 4 are readily discernible in these sections. Direct comparisons of density between rostral and caudal structures are possible in these photographs.

McGeer, 1980; Baudry and Lynch, 1981a). Na^+ -independent binding, on the other hand, either in the absence or presence of Ca^{2+} and/or Cl^- ions appears to be related to postsynaptic receptors. In the absence of sodium, Cl^- ions reportedly unmask, and Ca^{2+} ions enhance, a unique site that displays a high

affinity for both 2-amino-4-phosphonobutyric acid and quisqualic acid (Fagg et al., 1983a; Werling and Nadler, 1983); this site is labile to freezing in membrane preparations (Fagg et al., 1983b; Werling et al., 1983). The effects of Ca^{2+} , or $\text{Cl}^-/\text{Ca}^{2+}$, have been claimed to be both irreversible (Baudry and Lynch,

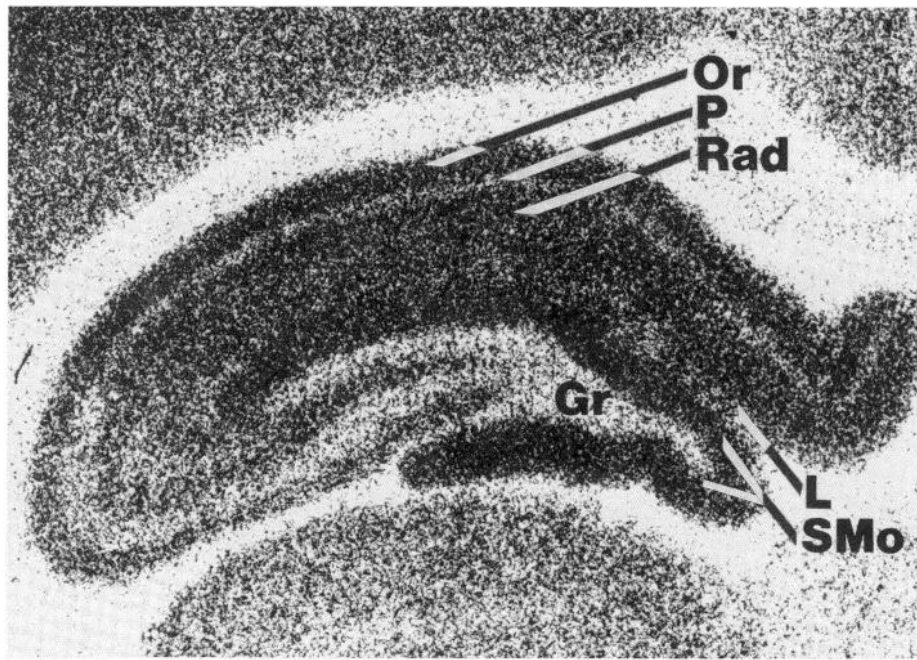


Figure 6. Detail of the laminar pattern of L-[³H]glutamate binding to hippocampus at the level of and under the same conditions as Figure 4F. Absolute and relative values of hippocampal glutamate binding may be found in Table I. *Or*, stratum oriens; *P*, pyramidal cell layer; *Rad*, stratum radiatum; *L*, stratum lacunosum-moleculare; *Gr*, granule cell layer of the dentate gyrus (GrDG).

1979; Baudry et al., 1983a) and fully reversible (Mena et al., 1982). The complex relationship between these ions remains to be defined unambiguously and was not addressed in the present study.

Recently, Monaghan et al. (1983) described autoradiographic evidence for four different glutamate-binding sites in rat hippocampus. Glutamate binding in the absence of Ca²⁺ was found to be sensitive to kainate in stratum lucidum of CA3, and in other areas binding was sensitive to *N*-methyl-D-aspartate (NMDA). A site partially displaced by 1 μM quisqualate or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid was also demonstrated. In the presence of Ca²⁺ and Cl⁻ an additional site was observed that was distributed in a manner consistent with the location of proposed glutamatergic pathways. Our study focuses on the distribution of binding in the presence of Ca²⁺ and Cl⁻ ions. The putative kainate receptor would be unlikely to represent a large proportion of binding in our system because Ca²⁺ is known to be an inhibitor of kainate binding (Beaumont et al., 1979). Furthermore, in the presence of Ca²⁺/Cl⁻, NMDA had little effect on the binding we observed (Greenamyre et al., 1983). [³H]Glutamate binding, in our studies, shows regional variation in its sensitivity to quisqualate.

In general, the distribution of glutamate-binding sites reported here (regardless of receptor type) correlates extremely well with the projection sites of putative glutamatergic pathways that have been proposed on physiological and biochemical bases. Because [³H]glutamate probably labels multiple receptors, this technique provides an assay for structures receiving excitatory input from afferents using acidic amino acids, or related compounds (Zaczek et al., 1983), as transmitters.

Glutamate, aspartate, and, very recently, *N*-acetylaspartyl-glutamate have all been proposed as the neurotransmitter of the lateral olfactory tract (Harvey et al., 1975; Bradford and Richards, 1976; Yamamoto and Matsui, 1976; Collins, 1979; Collins and Probett, 1981; French-Mullen et al., 1983). This tract is composed largely of the axons of the olfactory mitral cells. These cells make synaptic connections with periglome-

ular and granule cells in the external plexiform layer and internal granule cell layer, and also with cells of the anterior olfactory nucleus, olfactory tubercle, and piriform cortex, as well as the amygdala and entorhinal cortex (Shepherd, 1979). All of these regions have high levels of glutamate binding and each of the three proposed transmitters demonstrates high affinity for glutamate-binding sites (Greenamyre et al., 1983; Zaczek et al., 1983). Interestingly, the pattern of binding we observed in olfactory bulb correlates well with the levels of glutamic acid found in the dog olfactory bulb, but it is distinct from the pattern of kainate binding described by others (Nadi et al., 1980; Unnerstall and Wamsley, 1983).

There is evidence that glutamate may serve as a transmitter in the cerebral cortex (Jaspar and Koyama, 1969; Abdul-Ghani et al., 1981; Baughman and Gilbert, 1981). Consistent with this hypothesis, there is dense labeling of cortex by [³H]glutamate. We have found that superficial cortical layers have more binding sites than deeper layers have. There was about 75% more binding in anterior cingulate cortex than in posterior cingulate cortex; this has been confirmed by D. T. Monaghan (personal communication).

Much attention has been focused on defining transmitters used by various corticofugal pathways. Evidence supports glutamatergic projections from cortex to amygdala (Walker and Fonnum, 1983), cuneate nucleus (Stone, 1976, 1979; Rustioni and Cuenod, 1982), dentate gyrus (Storm-Mathisen, 1977; White et al., 1977; Wheal and Miller, 1980; Nadler and Smith, 1981), nucleus accumbens (Walaas, 1981), olfactory tubercle (Fonnum et al., 1981), pontine nuclei (Young et al., 1981, 1983; Thangnipon et al., 1983), spinal cord (Young et al., 1981, 1983), striatum (Spencer, 1976; Divac et al., 1977; McGeer et al., 1977; Reubi and Cuenod, 1979), substantia nigra (Fonnum et al., 1981; Carter, 1982), superior colliculus (Kvale et al., 1983), and thalamus (Bromberg et al., 1981; Fonnum et al., 1981; Rustioni et al., 1982), including the lateral geniculate body (Lund-Karlsen and Fonnum, 1978; Baughman and Gilbert, 1981). Again, each of these areas is readily apparent in autoradi-

ographs of glutamate binding. Equally important is the fact that nearby structures which receive little or no cortical input, such as the globus pallidus, show little glutamate binding. In this regard, it is noteworthy that the dorsal lateral geniculate body, which receives putative glutamatergic innervation from visual cortex, shows a high density of glutamate-binding sites. The ventral lateral geniculate body apparently does not receive glutamatergic cortical input and has little binding. The medial geniculate body shows dense labeling by [³H]glutamate and is known to receive a cortical projection (Shepherd, 1979), but there is not yet evidence to suggest that this input is glutamatergic. Similarly, the subthalamic nucleus, which receives a neurochemically undefined cortical input (Künzle, 1978), has more glutamate binding than does the surrounding zona incerta. Studies which examined the glutamatergic innervation of the substantia nigra (Fonnum et al., 1981; Carter, 1982) did not differentiate pars compacta from pars reticulata. We found higher levels of binding in pars reticulata than in pars compacta.

It is widely accepted that glutamate or a similar substance serves as a neurotransmitter of both hippocampal afferents and efferents. The stratum moleculare of the dentate gyrus receives putative glutamatergic input from the entorhinal cortex via the perforant path (Storm-Mathisen, 1977; White et al., 1977; Wheal and Miller, 1980; Nadler and Smith, 1981) and from hippocampal CA4 cells (Cotman and Nadler, 1981; Storm-Mathisen, 1981). In our autoradiographic assay, this structure had the highest level of glutamate-binding sites of any brain region. In keeping with the proposed glutamatergic and/or aspartergic innervation of stratum oriens and stratum radiatum from commissural and Schaffer collateral fibers (Cotman and Nadler, 1981; Storm-Mathisen, 1981; Corradetti et al., 1983), these dendritic zones also show high levels of binding. The CA3 pyramidal cells project bilaterally through the fimbria and fornix to the lateral but not the medial septal nuclei (Swanson and Cowan, 1977). Consistent with the proposal by Zaczek et al. (1979) that this projection is glutamatergic, we found that the lateral septum is selectively and heavily labeled by glutamate. Walaas and Fonnum (1980) have proposed that projections from subiculum to the nucleus of the diagonal band, bed nucleus of the stria terminalis, nucleus accumbens, mediobasal hypothalamus, and mammillary body may all use glutamate as a transmitter. All of these areas showed significant glutamate binding, although the levels of these sites were unevenly distributed. Nucleus accumbens was labeled most heavily, followed by the bed nucleus of the stria terminalis, mammillary body, diagonal band nucleus, and hypothalamus. This order, based on density of binding sites, matches that of Walaas and Fonnum (1980) for absolute levels of glutamate uptake in these areas, suggesting a correlation between the density of presynaptic terminals (uptake sites) and postsynaptic receptors.

In the brainstem gray matter there was a 5-fold difference between the highest and lowest densities of binding sites. In general, binding was lower than in forebrain. Several brainstem nuclei have already been mentioned with respect to glutamatergic innervation and glutamate binding and will not be discussed further. It is interesting to note that some nuclei, such as the periaqueductal gray matter, interpeduncular nucleus, inferior colliculus, and the inferior olivary nuclei, show substantial glutamate binding but, to date, lack any defined glutamatergic input. However, all of these nuclei, with the possible exception of the interpeduncular nucleus, receive afferents from cortex (Brodal, 1981). It has been suggested that glutamate may serve as the transmitter of certain primary afferents to brainstem nuclei. In particular, there is substantial evidence implicating glutamatergic function in the cochlear nucleus (Godfrey et al., 1977; Potashner, 1983; Altschuler et al., 1984; for a review, see Wenthold, 1981), in the nucleus of the tractus solitarius (Dietrich et al., 1981; Granata and Reis, 1983), and

in the spinal nucleus of the trigeminal nerve, pars caudalis (Salt and Hill, 1980, 1982). Subdivisions of the cochlear nucleus were not specifically examined for relative density of glutamate-binding sites, but it was readily apparent that there was substantial binding in the dorsal cochlear nucleus. There was also moderate binding in the nucleus solitarius and the caudal trigeminal nucleus (20 to 25% of the maximal binding found in the stratum moleculare). Of brainstem nuclei measured, the least binding was found in the deep mesencephalic nuclei and the hypoglossal nucleus. (Although there was even less binding in other brainstem regions, we were unable to define these areas anatomically in autoradiographs. Only measurements from positively identified areas were included in this report.)

One of the best characterized putative glutamatergic pathways is the granule cell/parallel fiber system of the cerebellum (Young et al., 1974; Hudson et al., 1976; Sandoval and Cotman, 1978). Granule cell axons project to the molecular layer of the cerebellum and, here, form the parallel fibers, which make putative glutamatergic synaptic connections with dendritic spines of Purkinje, basket, stellate, and Golgi cells. Furthermore, it has been proposed that the quisqualate receptor is the major excitatory amino acid receptor type present in this region (Foster and Roberts, 1981; Crepel et al., 1982). Autoradiographs reveal that the molecular layer binds about 60% more glutamate than does the granule cell layer. Approximately 80% of the molecular layer-binding sites are of the high-affinity quisqualate type and 20% are the low-affinity type. Binding sites in the granule cell layer may be postsynaptic to climbing fiber terminals, which are also believed to use an excitatory amino acid as their transmitter (Rea et al., 1980; Wiklund et al., 1982; Foster and Roberts, 1983; Toggenburger et al., 1983). Alternatively, they may be postsynaptic to mossy fibers, which also may use excitatory amino acids as transmitters (Freeman et al., 1983). It is interesting to note that the granule cell layer apparently displays a pharmacological profile which differs from that in the molecular layer. Less than 40% of the binding sites in this layer have a high affinity for quisqualate.

In the spinal cord, most of the glutamate binding is confined to the substantia gelatinosa. It has been proposed, but remains controversial, that glutamate may serve as a transmitter of some primary afferents in the spinal cord (Graham et al., 1967; Roberts and Keen, 1973, 1974; Zieglansberger and Puil, 1973; Johnson, 1977). Our findings are consistent with this hypothesis, but until further studies are carried out it cannot be stated with any certainty that these sites are postsynaptic to primary afferents. For example, it is known that intrinsic cells of the substantia gelatinosa and of lamina IV to VI are retrogradely labeled after D-[³H]aspartate injections into cervical dorsal horn (Rustioni and Cuenod, 1982). It is also thought that excitatory amino acids may be neurotransmitters of spinal cord interneurons (Davidoff et al., 1967; Watkins and Evans, 1981; Watkins et al., 1981). We find that there is a significant but relatively low level of binding in the remaining lamina of the spinal cord.

White matter has very low levels of glutamate binding. However, because white matter has a greater self-absorption (attenuation) of β particles than gray matter has (Alexander et al., 1981), we may have underestimated binding in those structures with high white matter content. In areas of pure white matter, this underestimation would be at most 30%. Thus, the value of 0.7 pmol/mg of protein (10% of the binding found in stratum moleculare) obtained for corpus callosum may be as high as 1.0 pmol/mg of protein (15% of the binding found in stratum moleculare).

Autoradiography of L-[³H]glutamate binding provides a sensitive assay which can be used to obtain detailed pharmacological data from highly circumscribed, well defined brain regions. It permits assays that are beyond the resolution of the microdissection methods and tissue requirements of conventional

binding assays. The results of the present study demonstrate a striking correlation between glutamate-binding sites and the terminal fields of previously proposed glutamatergic pathways. Structures adjacent to those suspected of receiving glutamatergic input have much less binding. This suggests that autoradiography may provide an assay for projection areas of pathways that use excitatory amino acids as neurotransmitters. This technique should help to better define the roles of excitatory amino acids in the central nervous system.

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