Brain Research, 511 (1990) 310–318
Elsevier

BRES 15279

Sodium-dependent D-aspartate 'binding' is not a measure of presynaptic neuronal uptake sites in an autoradiographic assay

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(Accepted 8 August 1989)

Key words: D-Aspartate; Autoradiography; Excitatory amino acid; Glia; Glutamate; Neuron; Sodium; Uptake

The binding of D-[^{3}H]aspartate to sections of rat brain was examined in an autoradiographic assay. Binding was entirely dependent on the presence of sodium ions, but not chloride ions, and was optimal at 2 °C. D-Aspartate bound rapidly, reached equilibrium within 20 min and remained stable for 45 min. The rate of dissociation was relatively rapid with a $t_{1/2}$ of 56 s, but was not as fast as anticipated, perhaps because of some sequestration of ligand. Binding had a K_d of 6.8 ± 1.2 μM and a B_{max} of 49.4 ± 8.6 pmol/mg protein. The high B_{max} value may further indicate some sequestration of D-aspartate. L-Glutamate, unlabeled D-aspartate, and D,L-*threo*-hydroxyaspartate, a potent inhibitor of synaptosomal uptake, each competed for D-[^{3}H]aspartate binding with IC_{50}s of 7.0 ± 4.3 μM, 5.4 ± 1.5 μM, and 2.5 ± 1.0 μM, respectively. *N*-methyl-D-aspartate (NMDA), quisqualate, and kainate had no affinity for this site. The regional distribution of D-aspartate binding sites was unique and did not conform to the distribution of neuronal uptake sites described by others. Striatal D-aspartate binding was unaffected by unilateral decortication or striatal quinolinic acid lesions. In contrast, binding to NMDA, quisqualate, and kainate receptors was reduced by 80–90% by quinolinate lesions of the striatum. The results of D-aspartate binding after lesions strongly suggest that this site is not associated with either lesioned glutamatergic afferents or intrinsic neurons of the striatum; it may be associated with glia.

INTRODUCTION

It is generally believed that the synaptic actions of glutamate and aspartate are terminated when these excitatory amino acids are removed from the synaptic cleft by a high-affinity uptake system[18]. High-affinity uptake mechanisms for excitatory amino acids are found in both neurons and glia, but in crude homogenate preparations uptake is predominantly associated with neuronal synaptosomes[18]. In contrast, glial uptake of L-glutamate or D-aspartate may predominate in primary cell cultures or well-preserved slices of cerebellum[13,14,28,36].

Synaptosomal uptake of tritiated L-glutamate, L-aspartate or D-aspartate can be used as a marker of the relative density of glutamate or aspartate nerve terminals[17,37]. Similarly, in fresh slices of tissue from hippocampus or forebrain, neuronal uptake of labeled L-glutamate, L-aspartate, or D-aspartate can be demonstrated[16,31]. When putative glutamate or aspartate pathways are lesioned, a subequent decrease in uptake is seen in the terminal fields of those pathways in both synaptosomal and tissue slice preparations (For review see Fonnum[18]). In this way, uptake may be used to map putative aspartate or glutamate pathways and provides a way to assess the integrity of these pathways after lesions.

In recent years, there has been interest in examining the roles of excitatory amino acids in human neurological diseases[15,21]. Since high-affinity sodium-dependent uptake is one of the few relatively specific markers for excitatory amino acid neurons, there has been an effort to apply this technique to the study of human postmortem tissue. Unfortunately, studies in rat brain have indicated that freezing results in a 50–80% decrease in uptake activity compared to fresh tissue[22,29,30] and prolonged freezing results in a further decrement of uptake. Several laboratories have reported that sodium-dependent D-aspartate or L-glutamate binding has several characteristics consistent with an uptake site[2,9,32]. Subsequently, a number of investigators examined sodium-dependent D-aspartate or L-glutamate binding in postmortem human brains, since it was felt that this represented a more suitable and stable marker for the uptake site[5,8,9,24,27]. However, lesion studies have suggested that at least 40% of sodium-dependent L-glutamate binding is associated with intrinsic neurons[32], and not with lesioned glutamatergic afferent terminals. More recently, Danbolt and Storm-Mathisen[12], have demonstrated that sodium-dependent D-aspartate 'binding' in homogenates actually represents sequestration into membrane-bounded saccules and 'does not represent binding to the transport carrier . . .' It would therefore appear that sodium-

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dependent D-aspartate binding in homogenates is not a reliable marker of uptake sites.

The present study was undertaken to characterize sodium-dependent D-aspartate binding in an autoradiographic assay and to determine its suitability as a marker for presynaptic neuronal uptake sites.

MATERIALS AND METHODS

Materials

D-[^{3}H]Aspartic acid (sp. act. 26 Ci/mmol), L-[^{3}H]glutamic acid (59 Ci/mmol) and [^{3}H]kainic acid were obtained from Amersham (Arlington Heights, IL, U.S.A.). D,L-[^{3}H]-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was obtained from New England Nuclear (Boston, MA, U.S.A.). *N*-methyl-D-aspartate and quisqualate were obtained from Cambridge Research Biochemicals (Valley Stream, NY, U.S.A.). D,L-*threo*-β-hydroxyasparticacid was obtained from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were obtained from Sigma.

Lesions

Cortical ablations. Male Sprague–Dawley rats (175–250 g) were anesthetized with xylazine and ketamine, and their scalps were incised and retracted. Using a dental drill, a rectangular flap of skull from about 3 mm posterior to about 6 mm anterior to bregma and from 2 mm lateral to the mid-line to the lateral edge of the calvarium was removed. The underlying cortex was removed by aspiration, and cortex lateral to the opening in the skull was undercut with a suture needle. After a survival time of one week, the animals were killed by decapitation, and their brains quickly removed and processed for autoradiography.

Striatal lesions. Male Sprague–Dawley rats (200–230 g) were anesthetized with nembutal (50 mg/kg) and were positioned in a Kopf stereotaxic frame. Unilateral striatal lesions were made with quinolinate at a dose of 60 nmol at uniform stereotaxic coordinates (1 mm anterior to bregma, 2.5 mm lateral to bregma, 5 mm deep from the cortical surface). Quinolinate was dissolved in 0.1 M phosphate buffer adjusted to pH 7.4 and injected in a small volume (0.5–1.0 μl) via a Hamilton syringe over a period of 8 min. The syringe was left in position for another 5 min prior to removal. After 3 months, the animals were killed and the brains removed and processed for autoradiography.

Tissue preparation

After decapitation, brains were quickly removed, mounted on cryotome pedestals with Lipshaw embedding matrix, and were frozen under powdered dry ice. Twenty-μm sections were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides, then allowed to dry at room temperature. Sections were either used immediately or stored for less than 24 h at –20 °C. Prior to assay, sections were pre-washed for 30 min at 2 °C in 50 mM Tris-acetate buffer (pH 7.20) in order to remove endogenous free excitatory amino acids. This procedure does not completely remove excitatory amino acids, but harsher treatments damage the mounted tissue. Sections were blown dry under a stream of room temperature air.

Autoradiography

D-Aspartate. In the 'standard' D-aspartate autoradiographic assay, slide-mounted tissue sections were incubated for 45 min at 2 °C in 50 mM Tris-acetate buffer (pH 7.40) containing 150 mM sodium acetate (NaAc) in the presence of 90–500 nM D-[^{3}H]aspartate. In saturation studies, the concentration of D-aspartate ranged from 100 nM to 100 μM. In studies examining the association and dissociation of binding, the D-aspartate concentration was 100 nM; in competition studies and studies examining the effect of sodium on binding, the D-aspartate concentration was 500 nM. In experiments examining the sodium-dependence of binding, sodium was added as either the chloride or acetate salt. To control for the effects of increased osmolality in the presence of sodium salts, binding was also performed in 50 mM Tris-acetate buffer containing 300 mM sucrose or 150 mM choline chloride or 150 mM potassium acetate. Non-specific binding was defined by 500 μM D,L-*threo*-hydroxyaspartic acid. Non-specific binding represented approx. 30% of total binding at a D-aspartate concentration of 100 nM.

'Sodium-dependent' glutamate binding. L-[^{3}H]Glutamate binding was determined in 50 mM Tris-acetate buffer containing 150 mM NaCl or 150 mM NaAc. In the case of glutamate binding in the presence of NaCl, no other drugs were included in the incubation, and the 'blank' was determined with 1 mM unlabeled glutamate. When glutamate binding was measured in the presence of NaAc, 100 μM NMDA, 1 μM quisqualic acid, and 1 μM kainic acid were included in the incubation. Under these conditions, 500 μM D,L-*threo*-hydroxyaspartic acid served as a blank. These assays were otherwise identical to the sodium-dependent D-aspartate binding assay.

Receptor binding. Glutamate binding to the NMDA receptor was determined in 50 mM Tris-acetate buffer (pH 7.2) in the presence of 1 μM quisqualic acid and 1 μM kainic acid. Non-specific binding was defined as binding in the presence of 1 mM NMDA. Quisqualate receptors were measured with [^{3}H]AMPA binding in 50 mM Tris-HCl buffer containing 2.5 mM $CaCl_2$ and 100 mM potassium thiocyanate (KSCN). AMPA binding in the presence of 1 mM unlabeled glutamate defined non-specific binding. Kainate receptors were assayed with [^{3}H]-α-kainate in 50 mM Tris-acetate buffer (pH 7.20) using 1 mM unlabeled glutamate to define non-specific binding. For all receptor assays, binding was performed at 2 °C with an incubation period of 45 min.

For all assays, after the incubation, sections were rinsed quickly 3 times with cold buffer, and then rinsed with cold 2.5% (v/v) glutaraldehyde in acetone. Sections were then blown-dry with warm air. The rinse and drying procedure took no more than 10 s. Dried sections were placed in X-ray cassettes with appropriate radioactive standards and apposed to Hyperfilm (Amersham). The film was exposed to the tissue sections for 14–21 days at 4 °C and then developed, fixed, and dried. Autoradiograms were analyzed using an MCID image processing system (Imaging Research, St. Catharine's, Ont., Canada). All data presented were analyzed from autoradiographic images.

IC_{50} values were determined by logit transformation. Statistical analysis of lesion-induced changes in binding were determined by paired *t*-tests.

RESULTS

Binding characteristics of D-aspartate

The binding of 500 nM D-aspartate was entirely dependent on the presence of sodium ions (Fig. 1a). No significant specific binding was found in the absence of sodium. Osmolality alone could not explain the effects of sodium since under sodium-free conditions, there was minimal specific binding in 300 mM sucrose, 150 mM choline chloride or 150 mM potassium acetate. A double-reciprocal (Lineweaver-Burke) plot of D-aspartate binding versus sodium concentration yielded a straight line with a correlation coefficient of 0.99 (Fig. 1b). Results were identical when sodium was added as either the acetate salt or the chloride salt. This suggests that a single sodium ion is bound for each D-aspartate molecule.

Examination of the effects of temperature on D-aspartate binding did not reveal any temperature depen-

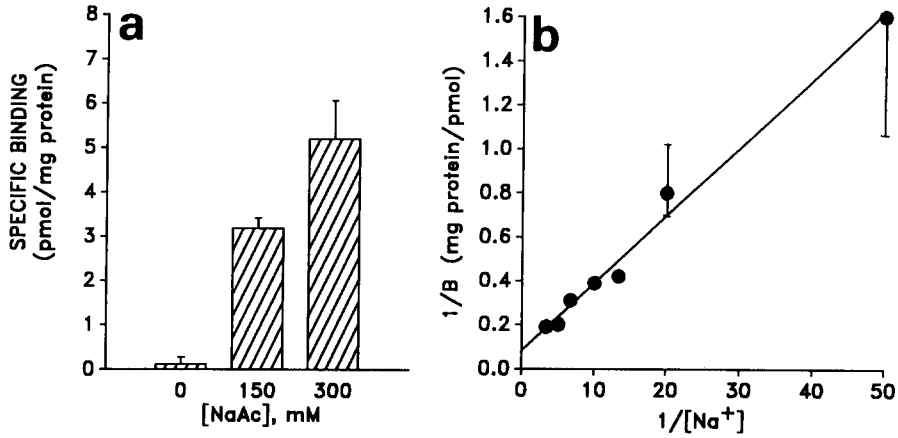

Fig. 1. Panel a: histograms demonstrating sodium-dependence of D-aspartate binding. Bars represent specific binding ± S.E.M. (n = 3). Sodium was added as sodium acetate salt. No specific binding was detected in the absence of sodium ions. D-[^{3}H]Aspartate concentration was 500 nM. Panel b: double-reciprocal plot of D-aspartate binding vs sodium concentration. Sodium was added as acetate salt, but identical results were obtained with NaCl. Points represent mean ± S.E.M. of specific binding from 3 animals. The S.E.M. was smaller than the size of the point in cases where no error bars are seen. The plot demonstrates a linear relationship between 1/B and 1/[Na] (r = 0.99).

dence (data not shown). However, at 2 °C, binding was much more reproducible; binding at higher temperatures revealed a patchy, smeared binding artifact.

Kinetic experiments indicated that D-aspartate bound to tissue sections rapidly, reaching equilibrium within 20 min and remaining stable for at least 45 min (Fig. 2). After a 45-min incubation, when individual tissue sections at equilibrium were subjected to 'infinite dilution' by placement in 800 ml of ice-cold 50 mM Tris-acetate buffer, binding dissociated rapidly, with a $t_{1/2}$ of 56 s. A plot of ln (B/B_0) versus time yielded a biphasic curve with an initial, more rapid rate of dissociation followed by a slowing of the dissociation rate (data not shown). An estimate of k_{-1} was made by measuring the slope of the initial portion of this plot and yielded a k_{-1} = 0.75 min^{-1}. Using this value, k_{+1} was determined to be 3.68 × 10^6 M^{-1} min^{-1}. The ratio k_{-1}/k_{+1} = 0.20 μM.

In saturation experiments, using D-aspartate concentrations from 100 nM to 100 μM, a K_d of 6.8 ± 1.2 μM was determined in striatum. Scatchard plots yielded a B_{max} of 49.4 ± 8.6 pmol/mg protein (Fig. 3). The Hill coefficient was 0.79 ± 0.07, and was not significantly less than unity.

Because of previous reports that D-aspartate 'binding' in homogenate preparations was disrupted by freezing and thawing prior to assay[12], this was examined in the autoradiographic assay. Sections prepared in standard fashion were assayed in parallel with sections which had received 4 additional freeze-thaw cycles. Binding to sections prepared in the standard manner (0.56 ± 0.05 pmol/mg protein) was not significantly different than binding in tissue sections which had undergone repeated

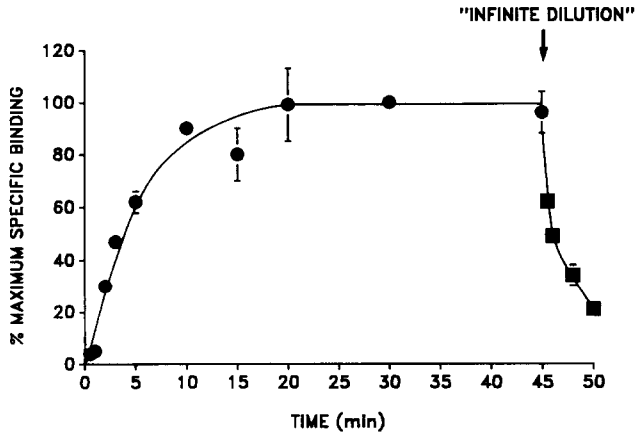

Fig. 2. Association and dissociation of D-aspartate binding. Points represent mean ± S.E.M. of specific binding (n = 3). The S.E.M. was smaller than the size of the point in cases where no error bars are seen. D-[^{3}H]Aspartate concentration was 100 nM. D-Aspartate binds rapidly and reaches equilibrium within 20 min. When tissue sections at equilibrium were placed in 800 ml of cold buffer ('infinite dilution') binding dissociated with a $t_{1/2}$ of 56 s.

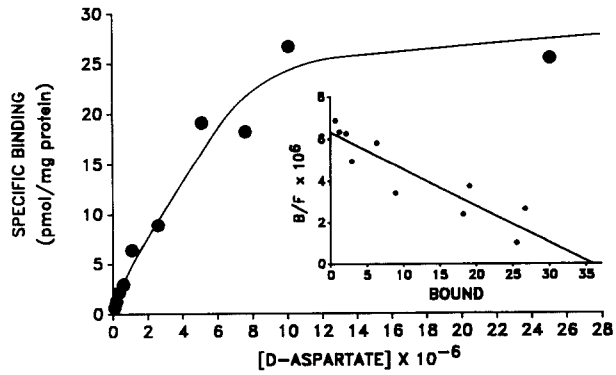

Fig. 3. Representative saturation curve of D-[^{3}H]aspartate binding in striatum. Points represent specific binding. Inset: Scatchard plot of saturation data. Mean K_d = 6.8 ± 1.2 μM and B_{max} = 49.4 ± 8.6 pmol/mg protein (n = 3).

freeze-thaw cycles (0.56 ± 0.07 pmol/mg protein).

Pharmacological specificity of D-aspartate binding

Of the compounds tested, the high-affinity uptake blocker D,L-*threo*-hydroxyaspartic acid[1] was the most potent competitor for D-aspartate binding sites, with an IC_{50} of 2.5 ± 1.0 μM (Fig. 4). The Hill coefficient of this compound, 0.66 ± 0.08, was significantly less than 1 ($P < 0.05$). L-Glutamate competed for D-aspartate binding sites with an IC_{50} of 7.0 ± 4.3 μM and a Hill coefficient of 0.88 ± 0.11. Unlabeled D-aspartate competition for D-[^{3}H]aspartate binding sites gave an IC_{50} of 5.4 ± 1.5 μM and a Hill coefficient of 0.79 ± 0.07. The Hill coefficients of unlabeled D-aspartate and L-glutamate did not significantly differ from unity.

The receptor-specific compounds, NMDA (300 μM), quisqualate (300 μM) and kainate (100 μM), did not significantly displace D-aspartate binding. The anion blocker 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), at a concentration of 100 μM, did not compete for D-aspartate binding. Similarly, 10 μM L-cystine, which has been reported to inhibit chloride-dependent sodium-independent glutamate 'binding'[23], had no effect on D-aspartate binding.

Regional distribution of D-aspartate binding

Sodium-dependent D-aspartate binding had a unique distribution in the rat brain (Fig. 5) which was unlike binding to NMDA, quisqualate, or kainate receptors[20,25]. The highest levels of binding were found in the cerebellar molecular layer, followed by lateral septum, entorhinal cortex, and dentate gyrus of the hippocampal formation. Overall, binding in forebrain was rather uniform, with relatively little regional variation, as compared with the distribution of excitatory amino acid receptors. Within the neocortex, lowest levels of binding were found in layer 1. There was relatively little lamination of binding in the hippocampus (Fig. 6), in contrast to binding to NMDA, quisqualate, and kainate receptors[20,25]. In the basal ganglia, there was less binding in globus pallidus than in caudate–putamen, but this difference was less than that seen with receptor binding.

The binding of L-[^{3}H]glutamate in Tris-buffer containing 150 mM NaAc, 100 μM NMDA, 1 μM quisqualate, and 1 μM kainate, produced very similar regional distribution to that seen with D-aspartate binding (Fig. 5). Binding of L-glutamate in the presence of 150 mM NaCl, in the absence of receptor-specific compounds, resulted in a distinct regional distribution which was similar to the combined distributions of NMDA, quisqualate, and kainate receptors, as well as D-aspartate sites.

Effects of lesions on binding

In an effort to determine the synaptic localization of D-aspartate binding sites, binding was examined after unilateral decortications or striatal quinolinate lesions (Fig. 7). Following unilateral frontoparietal lesions, striatal D-aspartate binding was examined ipsilateral and contralateral to the lesion by means of saturation studies. Striatal binding ipsilateral to the lesion (K_d = 6.82 ± 0.86 μM; B_{max} = 50.2 ± 7.0) did not differ from binding contralateral to the lesion (K_d = 6.77 ± 1.19 μM; B_{max} = 49.4 ± 8.6). Similarly, binding to NMDA, quisqualate, and kainate receptors was not significantly changed by unilateral decortication (Table I). Glutamate binding in the presence of NaCl (without receptor-specific drugs) and glutamate binding in the presence of NaAc (containing NMDA, quisqualate, and kainate) were unchanged by decortication.

After striatal quinolinate lesions, D-aspartate binding was unchanged and equal to that contralateral to the lesion (Table I). Similarly, L-glutamate binding in the presence of NaAc and receptor-specific drugs was unaffected by the quinolinate lesions (data not shown). In contrast, binding to NMDA, quisqualate, and kainate receptors was reduced by 80–90% in the region of the lesion. Binding of L-glutamate in the presence of NaCl (and in the absence of receptor-specific drugs) was

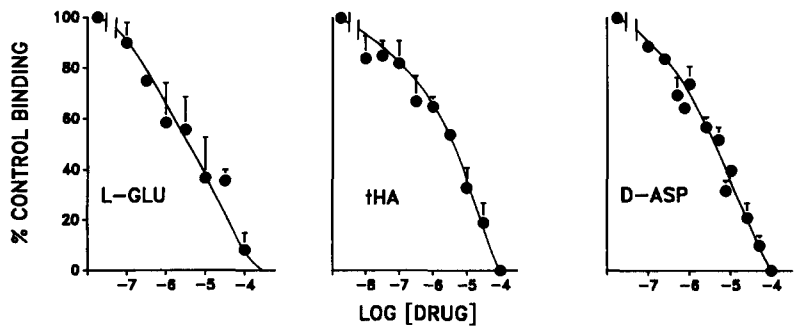

Fig. 4. Competition curves of L-glutamate (L-GLU), D,L-*threo*-hydroxyaspartic acid (tHA) and unlabeled D-aspartate (D-ASP). Points represent mean ± S.E.M. of percent control binding in absence of competing drugs (n = 3). The S.E.M. was smaller than the size of the point in cases where no error bars are seen. The Hill coefficient of tHA was significantly less than 1 (0.66 ± 0.08).

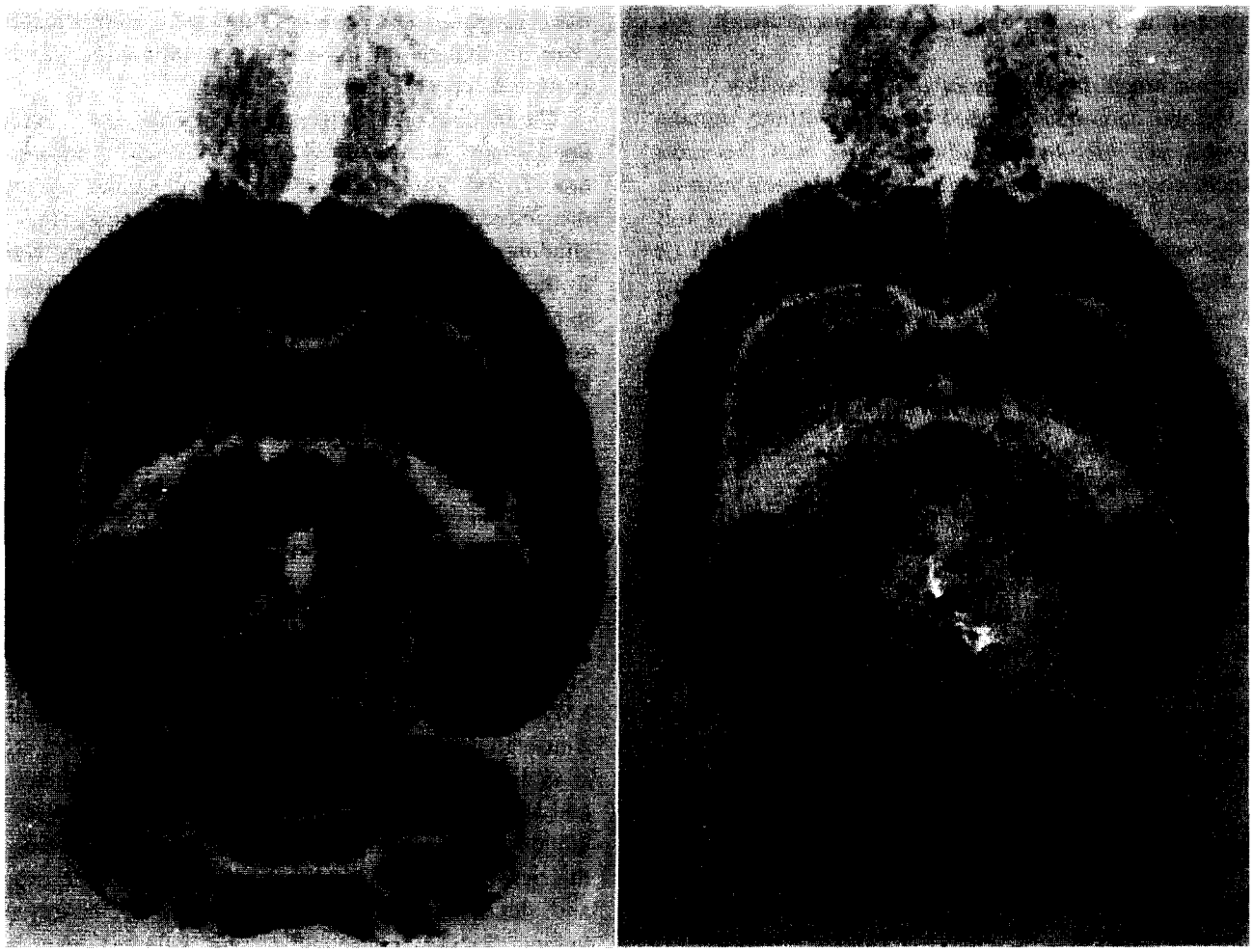

Fig. 5. Left panel: the regional distribution of D-aspartate binding in a horizontal section of rat brain. Note the high concentration of binding sites in the cerebellar molecular layer, and the lower level and relatively uniform distribution of binding sites in forebrain. There is very little binding in layer I of cortex. Right panel: regional distribution of L-glutamate binding sites assayed in the presence of 150 mM Na-acetate, 100 μM NMDA, 1 μM quisqualate, and 1 μM kainate. The distribution of L-glutamate binding sites under these conditions is similar to that of D-aspartate sites.

reduced by an average of 61% in the region of the lesion, intermediate between the loss of receptors and lack of change in the aspartate binding.

In summary, D-aspartate binding was unaffected by either decortication or quinolinate lesions. L-Glutamate binding in the presence of NaAc behaves identically. Binding to receptors was unchanged by decortication and was severely decreased by striatal lesions. Glutamate

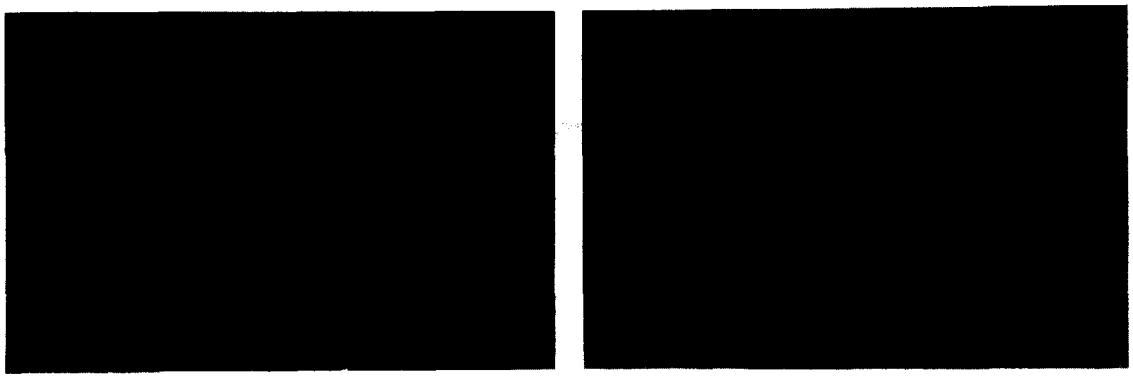

Fig. 6. Left panel: D-aspartate binding to hippocampus. Right panel: glutamate binding to NMDA receptors in the presence of quisqualate and kainate. The distribution of D-aspartate binding sites is much more uniform than that of NMDA receptors.

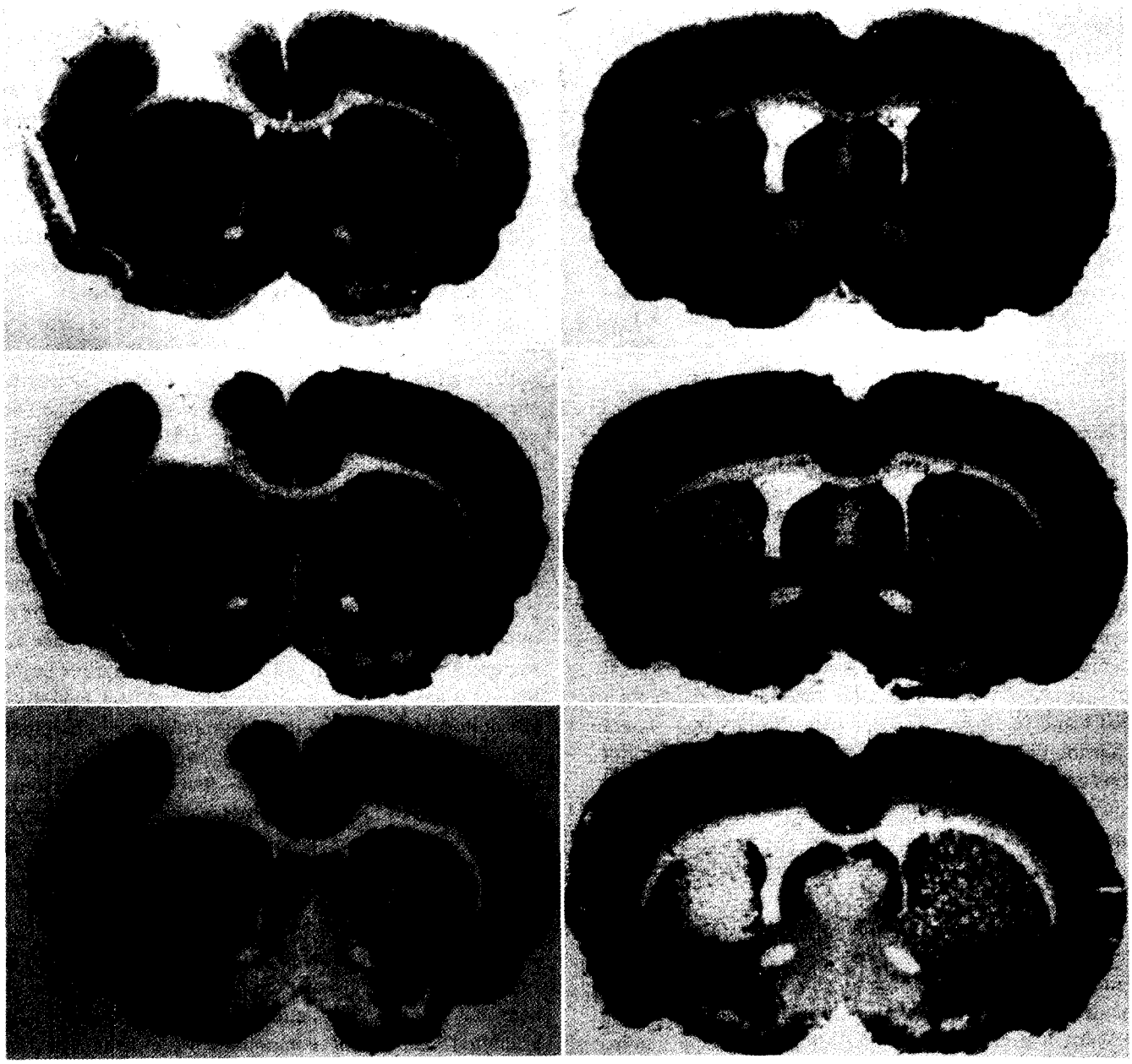

Fig. 7. Autoradiograms of D-[^{3}H]aspartate binding (top), L-glutamate binding in 150 mM NaCl without NMDA, quisqualate, or kainate to block receptor binding (center) and L-glutamate binding to NMDA receptors (bottom) in rats which had received unilateral decortications (left) or unilateral striatal quinolinic acid lesions (right). D-Aspartate binding was unaffected by decortication or QA lesion (Table I). Binding to NMDA receptors was reduced by 92 ± 1% after QA lesions. The reduction in L-glutamate binding in NaCl in the QA lesion was intermediate between the profound loss of NMDA receptors and the unchanged D-aspartate binding (see text).

TABLE I

Effects of lesions on Na^+-dependent D-[^{3}H]aspartate binding, L-[^{3}H]glutamate binding in the presence of NaCl and L-[^{3}H]glutamate binding to NMDA receptors in striatum

Binding assays were performed as described in text. D-[^{3}H]Aspartate concentration was 96 nM and L-[^{3}H]glutamate concentration was 28 nM. Values represent mean ± S.E.M. (n = 3) and are expressed in pmol/mg protein (ipsi: ipsilateral; contra: contralateral).

Lesion	*D-[^{3}H]aspartate*		*L-[^{3}H]glutamate/NaCl*		*L-[^{3}H]glutamate/NMDA*	
	ipsi	*contra*	*ipsi*	*contra*	*ipsi*	*contra*
Decortication	0.84 ± 0.08	0.84 ± 0.10	0.94 ± 0.04	0.86 ± 0.09	0.28 ± 0.02	0.32 ± 0.02
Striatal	0.67 ± 0.05	0.65 ± 0.06	0.25 ± 0.02**	0.65 ± 0.01	0.02 ± 0.002*	0.23 ± 0.04

*$P < 0.05$, **$P < 0.02$ by paired t-test, compared to contralateral striatum.

binding in the presence of NaCl was unaffected by decortication and reduced by an intermediate amount in quinolinate-lesioned striata.

DISCUSSION

Quantitative autoradiography of sodium-dependent D-aspartate binding has been described previously[26], and has recently been applied to the study of human postmortem tissue[4,7,10]. However, detailed characterization of this autoradiographic binding site has not been reported, and evidence that it represents the neuronal uptake carrier is lacking.

Characteristics of D-aspartate binding

In our autoradiographic assay, D-aspartate binding was entirely dependent on the presence of sodium ions and a double reciprocal plot of binding versus sodium ion concentration suggested that one sodium ion was bound with each D-aspartate molecule. This finding is consistent with previous kinetic modeling of uptake systems[3,6]. In contrast, Danbolt and Storm-Mathisen[11] provided evidence that in homogenates, the ratio of sodium ions to D-aspartate was 2. The fact that binding was the same in NaCl and in NaAc, together with the fact that choline chloride did not increase binding, suggests that chloride ions exert no effect on binding. The enhanced binding in the presence of sodium salts was not simply due to increased osmolality since binding was not enhanced by increasing the osmolality with sucrose, choline chloride or potassium acetate.

Sodium-independent binding of D-aspartate has been reported previously, and has been used to measure binding to NMDA receptor sites[19]. Sodium-independent binding was not detected in our assay and 300 μM NMDA had no effect on the sodium-dependent binding of D-aspartate.

Kinetic experiments indicated that D-aspartate bound to tissue sections rapidly, reached equilibrium within 20 min and remained stable for at least 45 min. The dissociation of D-aspartate from its binding site appeared to be biphasic and slower than expected. Because of this, the ratio k_{-1}/k_{+1} gave a K_d of 0.20 μM, 20-fold lower than the K_d determined from saturation studies. The reason for this relatively slow rate of dissociation is unclear, but it may be related to sequestration of D-aspartate as seen in homogenates[12]. The discrepancy between the K_d determined kinetically and by saturation studies with Scatchard analysis could also be explained by multiple binding sites or non-equilibrium conditions. However, this latter explanation appears unlikely since association studies (Fig. 2) indicate that binding was at equilibrium.

Unlike membrane preparations in which the sequestration process was disrupted by freezing and thawing[12], autoradiographic binding of D-aspartate was unaffected by this treatment. Furthermore, unlike the membrane translocation scheme proposed by Danbolt and Storm-Mathisen[12], D-aspartate binding was not temperature-dependent. Considering the differences in sodium-dependence, temperature-dependence, and sensitivity to freezing and thawing, it is likely that sodium-dependent D-aspartate binding in homogenates and in autoradiographic assays represents two distinct processes.

Saturation analysis of sodium-dependent D-aspartate binding to tissue sections yielded a linear Scatchard plot with an affinity in the lower micromolar range, similar to the affinity of D-aspartate for the uptake site[1,6]. The Hill coefficient of D-aspartate binding was not significantly different from unity, suggesting that D-aspartate was interacting with a single class of sites. The B_{max} (50 pmol/mg protein) in the current study was approx. 5-fold lower than the B_{max} reported for sodium-dependent L-glutamate and D-aspartate 'binding' in homogenate preparations[12,32], consistent with the hypothesis that 'binding' in homogenate preparations is largely due to sequestration. Although the B_{max} reported here is lower than in some homogenate binding studies, it is nevertheless quite high relative to receptor numbers[20,25]. Together with the relatively slow, biphasic nature of D-aspartate dissociation, this may indicate that there was some sequestration of ligand in our assay. Thus, the apparent B_{max} in our studies may have been an over-estimate of the actual number of binding sites.

Pharmacological specificity

In our autoradiographic assay, the pharmacological specificity of D-aspartate binding was similar to that of neuronal uptake sites[1,6], although it should be noted that a limited number of compounds were tested in the current study. Similar to the uptake site, the D-aspartate binding site had a high-affinity for L-glutamate[1,6]. D,L-*threo*-hydroxyaspartic acid is a potent inhibitor of neuronal excitatory amino acid uptake[1,6] and it inhibited the binding of D-aspartate with high affinity, although it produced a somewhat shallow competition curve and a Hill coefficient of 0.66. The low Hill coefficient may indicate that D,L-*threo*-hydroxyaspartic acid is interacting with multiple sites, or that there is some degree of negative cooperativity in this system. However, a more likely explanation is that the D- and L-isomers of this compound have different affinities for the D-aspartate binding site. NMDA, quisqualate, and kainate had little affinity for this binding site, even at high concentrations. The anion blocker SITS inhibits sodium-independent, chloride-dependent glial uptake of glutamate[35], but had no effect in our assay. L-Cystine can be used to

distinguish sodium-indepenent, chloride-dependent glutamate sequestration from glutamate binding[23]. This compound had no effect on D-aspartate binding. Thus, it is unlikely that D-aspartate binding is associated with chloride-dependent glial uptake or chloride-dependent sequestration.

Regional distribution

Autoradiography of D-aspartate binding revealed a unique regional distribution. Binding in cerebellar molecular layer was 2- to 3-fold higher than in any region of forebrain. Within the forebrain, the distribution of binding sites was rather uniform. This pattern of binding is markedly different from the regional distribution of neuronal uptake sites determined in homogenates or fresh slices of forebrain[16,29–31,38]. In contrast to the very high levels of D-aspartate binding found in the cerebellum in our autoradiographic assay, homogenate studies in rat, cat, and human brains have indicated that high-affinity neuronal uptake is higher in several regions of forebrain than in cerebellum[29,30,38]. The high levels of D-aspartate binding in cerebellum reported here are more reminiscent of the glial uptake of D-aspartate reported in fresh slices of cerebellum[13,36]. Furthermore, the distinct laminar pattern of hippocampal neuronal uptake seen in fresh tissue slices[31] is different from the pattern of hippocampal D-aspartate binding reported here. In fresh slices of forebrain, the highest levels of cortical D-aspartate uptake are found in layer 1[16]. This is the cortical layer with the least D-aspartate binding. Thus, the anatomical pattern of D-aspartate binding sites is not consistent with the distribution of neuronal uptake sites described in several other studies. When glutamate binding is assayed in the presence of NaAc (with NMDA, quisqualate, and kainate to block binding to receptors), an identical pattern is produced.

Synaptic localization

Cortical afferents to striatum are believed to be glutamatergic. The corticostriatal pathway is predominantly ipsilateral with a small crossed component. One week after unilateral frontoparietal decortications, several laboratories have demonstrated a marked decrease in striatal synaptosomal L-glutamate or D-aspartate uptake ipsilateral to the lesion[17,33,34]. Following identical lesions, we were unable to demonstrate any change in the affinity or number of D-aspartate binding sites in striatum ipsilateral to the lesion. Because of the small crossed corticostriatal projection, our use of the contralateral striatum as a control after decortication may have resulted in a slight underestimation of any subsequent changes in binding. However, similar use of the contralateral striatum as a control still results in a large decrease in synaptosomal uptake ipsilateral to the lesion[33,34]. The fact that D-aspartate binding was not reduced ipsilateral to the lesion suggests that the sites are not located on lesioned corticostriatal glutamatergic terminals. Alternatively, uptake carrier proteins capable of binding D-aspartate might persist in striatum, even after degeneration of afferent terminals. In similar fashion, binding to NMDA, quisqualate and kainate receptors, as well as to glutamate binding sites in the presence of NaCl or NaAc was also unchanged by decortication.

After a unilateral quinolinate lesion of the intrinsic neurons of the striatum, NMDA, quisqualate and kainate receptor binding was reduced by 80–90%, consistent with a postsynaptic localization of these receptors. In contrast, D-aspartate binding was not decreased in the region of the lesion in adjacent tissue sections. L-Glutamate binding in the presence of NaAc, NMDA, quisqualate, and kainate also showed no changes in the lesion. L-Glutamate binding in the presence of NaCl, without receptor-specific blocking agents, was reduced by an intermediate amount (60%). These results strongly suggest that sodium-dependent D-aspartate binding sites are not located on intrinsic neurons of the striatum.

If these binding sites are not localized to afferent terminals or intrinsic neurons in the striatum, it must tentatively be concluded that D-aspartate binding sites are located on glia. The intermediate reduction in glutamate binding when assayed in the presence of NaCl, without agents to block binding to receptors, suggests that under these conditions, glutamate is binding to both receptors and the glial site. This is consistent with the report by Vincent and McGeer[32] that about half of the sodium-dependent glutamate binding sites in striatal membrane preparations are not associated with lesioned glutamatergic afferent terminals.

Relationship of D-aspartate binding to neuronal uptake

The results of this study suggest that sodium-dependent D-aspartate binding has several characteristics expected of presynaptic neuronal uptake. Binding is entirely sodium-dependent, it is inhibited by glutamate and D,L-*threo*-hydroxyaspartic acid, and it has an affinity consistent with the neuronal uptake site. However, the regional distribution of this binding site is not consistent with previously described neuronal uptake sites. Furthermore, the effects of lesions indicate that this site is not localized on pre- or postsynaptic neuronal elements. Together with evidence from homogenate experiments that a large portion of sodium-dependent L-glutamate binding is postsynaptic and that D-aspartate binding represents sequestration, the current autoradiographic study suggests that sodium-dependent D-aspartate binding is a poor measure of presynaptic neuronal uptake

sites. Previous studies using homogenate or autoradiographic preparations should be interpreted with extreme caution and their conclusions reassessed.

Acknowledgements. This work was supported by USPHS Grant AG 06155 and the Arbogast Foundation.

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