Two classes of \(N\)-methyl-\(d\)-aspartate recognition sites: Differential distribution and differential regulation by glycine

\((N\)-methyl-\(d\)-aspartate receptors/excitatory amino acids/autoradiography\)

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ABSTRACT The \(N\)-methyl-\(d\)-aspartate (NMDA) receptor, a subtype of excitatory amino acid receptor, mediates synaptic responses in many regions of the central nervous system. This receptor plays a critical role in the mechanisms of both synaptic plasticity and excitotoxicity. Although these receptors were generally thought to be a single homogeneous receptor population, we report observations indicating that two anatomically distinct forms of the NMDA-receptor complex exist. (i) The distribution of NMDA receptors, as labeled by the NMDA agonist \(L\)-\([3H]\)glutamate, differs from that obtained with the radiolabeled antagonist \(d\)-\([3H]2-amino-5-phosphonopentanoate (\(d\)-\([3H]D-AP5\)). Relative to \(L\)-\([3H]\)glutamate, \(d\)-\([3H]D-AP5\) binding is low in the striatum and septum and high in the thalamus and inner cerebral cortex. (ii) NMDA antagonists are relatively more potent than agonists at displacing \(L\)-\([3H]\)glutamate binding in the thalamus and cerebral cortex; agonists are relatively more potent in the striatum and cerebellum. (iii) Glycine, which potentiates NMDA-receptor responses to glutamate, causes a greater percentage increase in \(L\)-\([3H]\)glutamate binding to NMDA receptors in the thalamus and cerebral cortex than in the striatum, septum, and cerebellum. Radiolabeled NMDA-antagonist binding, in contrast, is inhibited by glycine. Thus, as observed for \(\gamma\)-aminobutyric acid type A receptors, NMDA receptors have an agonist-prefering binding-site population and an antagonist-prefering binding site population. These may represent two distinct receptors and/or two interconverting forms. It could be of significant clinical importance if these two sites differ in their response to NMDA.

\(N\)-methyl-\(d\)-aspartate (NMDA) receptors mediate a potent excitatory response in many brain regions. Studies with selective NMDA antagonists have shown that this receptor appears capable of initiating certain forms of experience-dependent synaptic plasticity. Excessive NMDA receptor activation, such as that which occurs during seizures, ischemia, and hypoglycemia, results in cell death (1–7). Consequently, identification of multiple populations of NMDA receptors would be important for understanding basic mechanisms of synaptic function and clinical pharmacology. Structure–activity studies of the NMDA-receptor complex generally indicate that the NMDA-receptor population is homogeneous. However, some observations suggest heterogeneity (8–10).

Given that multiple populations of NMDA receptors are not likely to have identical distributions, we tested for possible receptor heterogeneity in three ways. (i) The distributions of NMDA receptors as labeled by \(L\)-\([3H]\)glutamate and \(\{propyl-1,2-\}[3H]-[\((\pm)2\)-carboxypiperazine-4-yl]propyl-1-phosphonic acid (\([3H]CPP\)) were compared. (ii) The relative potency of various glutamate analogues in displacing \(L\)-\([3H]\)glutamate binding to NMDA receptors was evaluated in various brain regions. (iii) The effects of NMDA-receptor allosteric modulators on NMDA receptor binding in different brain regions were determined.

MATERIALS AND METHODS

Chemicals and Animals. \(L\)-Glutamate (\(3,4\)-\([3H]\)), 50–52 Ci/mmol; \([3H]CPP\) (27 Ci/mmol) were obtained from New England Nuclear. \(d\)-\([3H]2-amino-5-phosphonopentanoate (\(d\)-\([3H]D-AP5\)) (26 Ci/mmol) was custom-made by Amersham. Glutamate analogues were obtained from either Tocris (Essex, U.K.) or Sigma. Other chemicals were obtained from Sigma. Male Sprague–Dawley rats (45–60 days old) were obtained from Simonsen Laboratories (Gilroy, CA). Male Wistar rats were home-bred (Bristol) and used in the \(d\)-\([3H]D-AP5\)-binding assays. Tritium-sensitive film and \(^3\)H microscale standards were obtained from Amersham.

Autoradiographic Procedures. As described (11), male Sprague–Dawley rat brain tissue sections on microscope slides were washed for 30 min in ice-cold 50 mM Tris acetate buffer, pH 7.0, preincubated at 30°C for 10 min, and then incubated for 10 min in ice-cold buffer containing 100 nM \(L\)-\([3H]\)glutamate. NMDA receptors were selectively labeled by the inclusion of 100 μM 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid, 5 μM amino-3-hydroxy-5-methylisoxazole-4-propionic acid, and 1 μM kainic acid, which displace all detectable non-NMDA-receptor binding. Under these conditions the specific binding is fully displaced by NMDA agonists and antagonists at concentrations appropriate for NMDA receptors (12–18).

Sections were then rinsed for 30 s, air dried, and placed against tritium-sensitive film along with tritium standards. Tissue sections in the experiments described in Fig. 2 were incubated for 20 min followed by a 20-s rinse. \([3H]CPP\) autoradiography was done as described (19). Briefly, after similar tissue preparation and preincubation, 100 nM \([3H]\)CPP was incubated in 50 mM Tris-HCl, pH 7.6, for 20 min at 0°C followed by a 15-s rinse. A similar autoradiographic pattern, of lower density, was obtained with 50 mM Tris acetate, pH 7.0, due to the lowered pH (9, 20). Quantitative analysis was performed with Spatial Data and Imaging Research Inc. digital image analysis systems.

Binding in Membrane Fractions. Membrane preparation and corresponding radioligand binding procedures for

Abbreviations: NMDA, \(N\)-methyl-\(d\)-aspartate; \(d\)-AP5, \(d\)-2-amino-5-phosphonopentanoate; \([3H]D-AP5\), \([3H]2-amino-5-phosphonopentanoate; \([3H]CPP\), \(L\)-\([3H]\)glutamate; \(\{propyl-1,2-\}[3H]-[\((\pm)2\)-carboxypiperazine-4-yl]propyl-1-phosphonic acid; PLSD, probable least square difference.
NMDA-sensitive L-[3H]glutamate and [3H]CPP binding are as described (14). Briefly, previously frozen crude synaptic membranes (P2 as defined in ref. 14) were lysed, incubated with 0.04% saponin at room temperature for 15 min, and washed in 200 μM Tris acetate, pH 7.2, buffer four times. Membranes (200 μg of protein) were incubated with 4 nM L-[3H]glutamate in 50 mM Tris acetate, pH 7.0, or 4 nM [3H]CPP in 50 mM Tris-HCl, pH 7.6, for 20 min at 0-4°C. Incubations were terminated by 100-fold dilution in ice-cold buffer, then vacuum filtered with Whatman GF/B filters after 10 s, and washed twice. The D-[3H]AP5-binding assay was done as described (20) with 1 mM L-glutamate used to define nonspecific binding. All experiments were repeated three or four times and are expressed as the mean ± SEM.

RESULTS

Comparison of L-[3H]Glutamate and [3H]CPP Binding Sites.

The distribution of NMDA-sensitive L-[3H]glutamate binding sites is similar to, but different from, that of [3H]CPP binding sites (Fig. 1). In the parietal cortex the density of [3H]CPP binding sites is greater than NMDA-sensitive L-[3H]glutamate binding sites in layers IV and V1, resulting in a distribution of more uniform density in [3H]CPP autoradiograms. The high level of [3H]CPP binding in layer IV accounts for the wider appearance of the superficial layer in [3H]CPP autoradiograms (Fig. 1A and B). Among other brain regions, NMDA-sensitive L-[3H]glutamate-binding site levels are relatively higher in the medial striatum and the septum; [3H]CPP-binding sites are relatively high in the hippocampus, cerebral cortex, and the lateral thalamic nuclei (Fig. 1). These results are also consistent with our previous observations of a high density of binding sites for the NMDA antagonist D-[3H]AP5 found in the thalamus and the reduced degree of lamination in the cerebral cortex (see figure 2B of ref. 21).

Endogenous glutamate or other endogenous ligands are unlikely to account for these two distributions because of the

![Fig. 1. Differing distributions of NMDA receptors in a horizontal plane of the rat brain as determined with the radioligands L-[3H]glutamate (A) and [3H]CPP (B). Digital images of binding site density are color coded with high-to-low densities represented from red-yellow to green-blue. (C) Quantitative values from these experiments. To compare ligands each regional binding value was divided by the average binding level from the eight regions shown. Average L-[3H]glutamate binding was 0.91 ± 0.13 pmol/mg of protein; average [3H]CPP binding was 0.59 ± 0.13. (D) The mean [3H]CPP/L-[3H]glutamate normalized binding ratios ± SEM (among animals; n = 4). HC, hippocampus; MS, medial striatum; LS, lateral striatum; S, septum; CB, cerebellar granule cell layer; TH, thalamus; OC, outer (I-III) parietal cortex; IC, inner (IV-VI) parietal cortex; and G, granule cell layer.]
following: (i) Endogenous competitors would alter both distributions in the same manner. (ii) Binding density and distributions were not affected by incubating the tissue sections in 0.1% saponin (10 min at 30°C) and 1 mM EDTA (10 min at 30°C) before the standard preincubation. (iii) Binding densities and distributions were also unaffected by preincubating sections in 100 μM L-glutamate, D-AP5, or glycine for 10 min and two 10-min washes before ligand binding.

**Heterogeneity of L-[³H]Glutamate Binding Sites.** Quantitative autoradiography was used to determine whether the pharmacological properties of NMDA-sensitive L-[³H]glutamate binding sites exhibit regional variations. Sliding-mounted tissue sections were incubated with L-[³H]glutamate in the presence of IC₅₀ concentrations [determined from our previously reported (14)] Kᵣ values] of the NMDA agonists and NMDA antagonists. Alternate sections were used for agonists and antagonists. In addition to reducing the overall binding levels by an average of 58 ± 5%, these compounds displayed two different patterns of displacement corresponding to the presence of an agonist or an antagonist.

Regional variations in drug potency were quantified (Fig. 2) by determining the binding density in each given region and dividing that value by the average binding found in the other regions in the same autoradiogram. This analysis is thus sensitive to preferential displacement of binding in a given region and avoids interautoradiogram error when making comparisons between autoradiograms. Relative to other brain regions, antagonists were less effective displacers of L-[³H]glutamate binding in the cerebellar granule cell layer (CB) and medial striatum (Fig. 2). In contrast, in the cerebral cortex and lateral thalamus antagonists displayed greater effectiveness at displacing L-[³H]glutamate binding than did agonists. Differential agonist and antagonist activity is indicated by statistical analysis of differences between compounds within brain regions; 32 comparisons are significant (P < 0.05; Fisher PLSD), 31 of which represent differences between an agonist and an antagonist (L-Asp potency differed from quinolinate in the inner parietal cortex).

For further analysis, the data were pooled into agonists and antagonists groups (Fig. 2B). These values were then expressed relative to binding obtained in the absence of added drugs by dividing the normalized binding value in the presence of a drug by the normalized binding value obtained in the absence of drug (this value is further described in the figure legend). This analysis reveals that both agonists and antagonists exhibit a regional variation in their potency as inhibitors of L-[³H]glutamate binding and that the cerebellum and striatum are significantly (P < 0.005) more sensitive to agonists, whereas the thalamus and cortex are significantly more sensitive to antagonists. Thus, although L-[³H]glutamate predominately labels an agonist-preferring population, some of its binding appears to be to an antagonist-preferring population.

Interestingly, cerebellar NMDA receptors, which are unique in their low affinity for tiemylenphencyclidine at the NMDA ion channel (22), are also anomalous in being relatively insensitive to both agonists and antagonists (Fig. 2). Lateral septal binding sites are relatively low in [³H]CPP binding; yet, the septal L-[³H]glutamate sites appear to be equally sensitive to agonists and antagonists.

**Heterogeneity of Glycine Modulation.** Glycine is known to allosterically potentiate glutamate responses at the NMDA receptor (23). Because preliminary experiments indicated that glycine enhances the binding of L-[³H]glutamate in autoradiographic preparations, we evaluated the regional distribution of glycine stimulation of L-[³H]glutamate binding to determine whether NMDA receptors are uniform in their regulation by glycine. As shown in Fig. 3A, NMDA receptors in different brain regions show a different sensitivity to glycine. In regions such as the striatum, there is only a 20% stimulation by 1 and 10 μM glycine. In contrast, the cerebral cortex and lateral thalamus show a 50% stimulation. This regional variation suggests that there might be a quantitative relationship between glycine stimulation and the agonist/antagonist-preferring properties of NMDA receptors. As indicated in Fig. 3B, there is a good correlation between regions with a high density of antagonist-preferring sites relative to agonist-preferring sites and regions exhibiting a greater glycine stimulation of glutamate binding.

Because glycine causes a greater enhancement of agonist binding (L-[³H]glutamate) in brain regions with a high density of antagonist-preferring binding sites, glycine is suggested to have different actions on the antagonist-preferring binding sites and the agonist-preferring binding sites. This hypothesis was directly tested by examining the effect of glycine on radiolabeled agonist and radiolabeled antagonist binding in synaptic membrane fractions. As shown in Fig. 4A, glycine oppositely affects agonist and antagonist binding. Higher concentrations of glycine enhanced NMDA-sensitive L-[³H]glutamate binding to membrane fractions and tissue sections followed by a lack of stimulation at even higher glycine concentrations. In contrast, D-[³H]AP5 binding is reduced by glycine, with only ~50% of the binding inhibited at maximal glycine concentrations. The effects of glycine upon both agonist and antagonist binding occur mostly in the 0.1–1 μM range, concentrations similar to that found for glycine enhancement of NMDA-induced responses (23). D-Serine, which also acts at the physiological glycine site,
also enhances L-[3H]glutamate binding and inhibits antagonist binding as labeled by D-[3H]AP5 and [3H]CPP (Fig. 4B).

**DISCUSSION**

We obtained three lines of evidence indicating that NMDA radioligands bind to at least two anatomically distinct populations of binding sites, which we term agonist-preferring and antagonist-preferring. (i) NMDA receptors labeled by the NMDA agonist L-[3H]glutamate have a differing distribution than those labeled by the NMDA antagonist [3H]CPP. (ii) L-[3H]Glutamate itself exhibits a regional variation in its sensitivity to displacement by agonists and a different regional variation in antagonist sensitivity. (iii) Glycine stimulation of L-[3H]glutamate binding is heterogeneous; greater binding stimulation is seen in brain regions with a relatively high proportion of antagonist-preferring sites. Consistent with this observation was the finding that radiolabeled agonists and radiolabeled antagonists are oppositely affected by glycine (and d-serine). Agonist binding is enhanced, whereas antagonist binding is reduced by glycine.

Each of these three independent lines of evidence indicate heterogeneous distributions with similar patterns. Antagonists show a greater displacement of L-[3H]glutamate binding in brain regions where radiolabeled antagonist binding is high relative to agonist binding. These brain regions also correspond to those regions showing a greater stimulation of L-[3H]glutamate binding by glycine; the agonist-preferring class has the highest relative levels in the medial striatum, and the antagonist-preferring population has the highest relative levels in the lateral thalamus and cerebral cortex.

Together these results indicate that there are multiple NMDA receptor sites and/or states with different distributions, different affinities, and different responses to glycine and d-serine.

Although two distinct NMDA binding sites have not been previously identified, their existence accounts for differences in pharmacological properties obtained with various NMDA receptor radioligands. Antagonists generally display greater displacement potencies in [3H]antagonist-binding assays than in [3H]agonist assays; agonists are more potent in [3H]antagonist assays (9, 12–22, 24, 25). Some of the quantitative differences between agonist and antagonist binding could correspond to differences between one of the multiple points of agonist- and antagonist-receptor attachment, as we and others have suggested (26, 27). However, such differences do not explain the observed regional variations in receptor properties described in this report. Although agonists often display multiple affinities for a single receptor, this property in itself would not explain the different anatomical distributions seen for the two sites.

Glycine reduces antagonist binding and enhances agonist binding. Thus, glycine could be converting some of the antagonist-preferring sites into agonist-preferring sites, or, alternatively, inhibiting antagonist sites while independently enhancing agonist sites. Because glycine preferentially enhances L-[3H]glutamate binding in those regions rich in antagonist-preferring binding sites, the anatomic data suggest that the glycine-enhanced L-[3H]glutamate binding may be derived from the antagonist-preferring binding population. Thus, glycine might be converting some of the antagonist-preferring sites into agonist-preferring sites.
Fig. 5. The different potencies of D-AP5 displacement of agonist (L-[^3]H)glutamate) and antagonist (D-[^3]H)AP5 binding (16, 17, 21) are compared with D-AP5 potency at blocking NMDA-induced focal depolarizations and NMDA-receptor-mediated long-term potentiation (28). All measurements were made in the stratum radiatum of CA1 hippocampus, ▲, Long-term potentiation; ■, NMDA depolarization; ●, L-[^3]H]glutamate binding; and □, D-[^3]H]AP5.

The agonist- and antagonist-prefering sites may have differing functional properties. Quantitative comparison of the antagonist potency of D-AP5 against NMDA-induced responses in the rat hippocampus (Fig. 5) and spinal cord (29) shows a closer correspondence to the affinity of these compounds for the agonist-prefering L-[^3]H]glutamate binding site than for the antagonist-prefering [^3]H]AP5-binding site. Consequently, it is possible that the antagonist-prefering binding site corresponds to a physiologically less responsive (densensitized?) state of the receptor and it is the antagonist’s affinity for the agonist-prefering site that determines antagonist potency. This possibility could also account for the apparent paradox that CPP is three to five times more potent than D-AP5 as an NMDA antagonist and as a displacer of NMDA-sensitive L-[^3]H]glutamate binding, while being equipotent to D-AP5 as an inhibitor of D-[^3]H]AP5 binding (29, 30). Thus, taken together with the evidence that glycine might be interconverting antagonist-prefering sites into agonist-prefering sites, one can propose the hypothesis that glycine causes a conversion of NMDA receptors from a state that is relatively unresponsive to glutamate (agonist-prefering conformation) to a conformation highly responsive (agonist-prefering conformation).

The existence and differential distribution of the agonist- and antagonist-prefering NMDA receptors raises several important questions. Are the two sites expressed by different mRNAs and/or are the two sites differentiated by covalent modifications such as phosphorylation? What factor(s) maintain the proportions of the two populations of NMDA sites in the absence of glycine? Does the parallel between agonist- and antagonist-prefering y-aminobutyric acid type A receptors (31, 32) and NMDA receptors reflect a general receptor plan of genetically related receptors (33)? Do the two sites differ in their functional properties? If the agonist-prefering sites are more responsive to NMDA, then the striatum would be exceptionally sensitive to the excitotoxic actions of NMDA receptors. Exceptional striatal sensitivity has been reported for the NMDA agonist quinolinate (10, 34) and has been suggested to be occurring in Huntington disease (35, 36). The hypothesis of a receptor form exhibiting greater responsiveness could also account for observations of altered NMDA receptor activity occurring in development (37) and following kindling (38). Thus, resolution of these issues may have significant implications for the role of NMDA receptors in development, learning, and pathology.

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