0026-895X/94/040626-11$03.00/0

MOLECULAR PHARMACOLOGY, 45:626–636

Metabotropic Glutamate Receptor Heterogeneity in Rat Brain

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Received September 20, 1993; Accepted January 6, 1994

SUMMARY

Metabotropic glutamate receptors (mGluRs) are G protein-linked receptors that operate through the formation of different second messengers. Utilizing quantitative autoradiographic techniques, we have characterized [^{3}H]glutamate binding to mGluRs in discrete regions of adult rat brain. [^{3}H]Glutamate binding, in the presence of high concentrations of α-amino-3-hydroxymethyl-4-isoxazolepropionic acid (10 μM), *N*-methyl-D-aspartate (100 μM), and 2.5 mM calcium chloride ($CaCl_2$), was saturable. Scatchard plots were linear in all regions examined and revealed similar affinity constants of about 500 nM. The largest number of sites was found in the outer cerebral cortical layers (10 pmol/mg of protein). [^{3}H]Glutamate binding was displaced by quisqualate, *trans*-1-amino-1,3-cyclopentane dicarboxylic acid (*t*-ACPD) (racemic mixture), and (1*S*,3*R*)-ACPD but not by (1*R*,3*S*)-ACPD. The guanine nucleotide analogue guanosine-5′-*O*-(3-thio) triphosphate (100 μM) reduced the binding by affecting the affinity but not the total number of sites, as predicted for G protein-coupled receptor sites. Quisqualate displacement curves were always biphasic and resolved two binding sites, with K_i values in the low nanomolar (15 nM) and micromolar (63 μM) ranges. (1*S*,3*R*)-ACPD displaced [^{3}H]glutamate binding both in the absence and in the presence of 2.5 μM quisqualate, suggesting that both high and low affinity quisqualate sites are linked to mGluRs. (1*S*,3*R*)-ACPD competition curves were broad (Hill coefficient = 0.73) but monophasic under both conditions, with K_i values in the micromolar range (14–116 μM), suggesting that (1*S*,3*R*)-ACPD acts on the two quisqualate sites with similar apparent affinities. The regional distributions of the two sites were different. The highest levels of the high affinity quisqualate binding site were found in the cerebellar molecular layer. The highest levels of the low affinity quisqualate binding sites were found in the outer cerebral cortex. The pharmacological profile and regional distribution suggest that the high and low affinity quisqualate-sensitive components of [^{3}H]glutamate binding sites might correspond to the mGluR1/mGluR5 and mGluR2/mGluR3 subgroups of cloned mGluRs, respectively.

EAA transmission is likely to be involved in mechanisms underlying synaptic plasticity during development and neurodegeneration (1). The functional versatility of the EAAs is due to the existence of different glutamate receptors. EAA receptors have been tentatively classified in two distinct groups, named "ionotropic" and "metabotropic" receptors and mGluRs (2). Ionotropic receptors consist of several distinct subunits assembled together to form glutamate-gated channels in the plasma membrane that are permeable to specific ions (3). Metabotropic receptors (mGluRs) are coupled, through G proteins, to enzymatic systems that generate second messengers (4, 5). The first mGluR to be described was that coupled to the phospholipase C-mediated hydrolysis of membrane PI and consequent formation of inositol-1,3,4-trisphosphate and diacylglycerol (4, 6, 7). Diacylglycerol activates protein kinase C in the presence of calcium and phosphatidylserine. Inositol-1,3,4-trisphosphate releases calcium from the endoplasmic reticulum through a receptor-mediated response (8). EAA-mediated PI hydrolysis is very high during the first weeks of postnatal life in several brain regions and progressively declines later in development (7, 9–12). mGluRs linked to PI hydrolysis have been shown also to be functionally activated under different experimental conditions in adult animal brains, such as ischemia (13), long term potentiation (14), spatial learning (15), and kindling (16–19). These results suggest a role for mGluRs in synaptogenesis and synaptic plasticity both in early development and in adult life.

mGluRs are activated by glutamate, QUIS, IBO, and *t*-ACPD but not by NMDA, kainate, or AMPA. Among these agonists, *t*-ACPD has been found to be the only specific compound for the mGluRs (20, 21). mGluR agonists have different rank orders of potency in different experimental preparations, suggesting the existence of several subtypes with different pharmacological profiles and localization in the central nervous system (22). Using molecular biological approaches, multiple mGluR genes have been isolated, some of which are linked to PI hydrolysis

This work was supported by United States Public Health Service Grants NS19613 and AG08671.

ABBREVIATIONS: EAA, excitatory amino acid; PI, phosphoinositides; mGluR, metabotropic glutamate receptor; QUIS, quisqualate; ACPD, 1-amino-1,3-cyclopentane dicarboxylic acid; AMPA, α-amino-3-hydroxymethyl-4-isoxazolepropionic acid; IBO, ibotenic acid; AP4, 2-amino-4-phosphonobutyric acid; HCSA, L-homocysteine sulfinic acid; *threo*-HA, DL-*threo*-β-hydroxyaspartic acid; AP3, 2-amino-3-phosphonopropionic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; NNKQ, non-*N*-methyl-D-aspartate-, non-kainate, non-QUIS-sensitive; NMDA, *N*-methyl-D-aspartate; ANOVA, analysis of variance.

and others of which are coupled to other second messengers (23–26). The mGluR1 and mGluR5 genes appear to be coupled to PI hydrolysis and the mGluR2, -3, -4, and -6 genes appear to be linked to inhibition of adenylate cyclase. Although the regional distributions of mGluR gene expression have been examined with *in situ* hybridization techniques (23–26), these data do not provide information about resultant protein localization. Antibodies to one subtype, mGluR1, have been used to investigate receptor localization (27); antibodies to the other subtypes are not yet available and immunocytochemical techniques do not provide quantitative data. Neither of these techniques can be used to directly examine the biochemical and pharmacological properties of these receptor subtypes in mammalian brain.

Ligand binding studies have predicted or confirmed the existence of different subtypes of EAA receptors proposed by electrophysiological and biochemical studies (28–30). In the case of mGluRs, the lack of available specific, high affinity ligands has made the development of binding protocols suitable for these receptor populations difficult. It has been shown, however, that, in the presence of saturable concentrations of NMDA (100 μM) and AMPA (10 μM), [^{3}H]glutamate binding displaceable by QUIS (2.5 μM) is likely to represent binding to mGluRs linked to PI hydrolysis (31). Another glutamate binding site, insensitive to NMDA (100–900 μM), kainate (1 μM), and QUIS (2.5 μM) (the NNKQ glutamate site), has been described in rat brain (32). The regional distribution of this latter binding site is very distinct, and the site has an unusual developmental profile (32). In the present study, we have examined the pharmacology and regional distribution of these two different [^{3}H]glutamate binding sites and related their properties to those of the cloned mGluRs and those of the recently identified [^{3}H]glutamate binding sites linked to mGluRs in bovine brain coated vesicles (33) and rodent forebrain (34, 35). The results suggest the existence of at least two pharmacologically distinct mGluR binding sites and indicate that each has a distinct distribution pattern in the central nervous system.

Experimental Procedures

Materials. Tris and L-glutamic acid were obtained from Fisher Biotech (Fair Lawn, NJ). Potassium thiocyanate was purchased from Mallinckrodt Inc. (Paris, KY). [^{3}H]Glutamate (45–56 Ci/mmol) was obtained from New England Nuclear (Boston, MA). NMDA, QUIS, AMPA, and *t*-ACPD were obtained from Research Biochemicals Inc. (Natick, MA). (1*S*,3*R*)-ACPD, (1*R*,3*S*)-ACPD, IBO, L-*trans*-pyrrolidine-2,4-dicarboxylic acid, L-AP4, L-HCSA, and (*RS*)-α-amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid were purchased from Tocris Neuramin (Buckhurst Hill, England). Kainic acid, dihydrokainic acid, *threo*-HA, L-cystine, DL-AP3, and GTPγS were purchased from Sigma Chemical Co. (St. Louis, MO).

Brain tissue. Adult male Sprague-Dawley rats (200–250 g; Charles River) were killed by decapitation. The entire brain was rapidly removed, completely covered by embedding matrix (Lipshaw), and frozen in powdered dry ice. Brains were stored at −70° until use (never more than 20 days). Before sectioning, brains were transferred to −20° for 3–6 hr. Horizontal sections (20 μm thick) were cut on a Lipshaw cryostat and thaw-mounted onto gelatin-coated slides. Slides were stored at −20° for no more than 24 hr. Before assay, sections were warmed to room temperature, prewashed for 30 min at 4° in a large volume (700–1000 ml) of 50 mM Tris·HCl buffer, pH 7.2, containing 2.5 mM $CaCl_2$ and 30 mM KSCN, and dried under a stream of warm air.

Receptor autoradiography. Autoradiography of mGluR distribution was performed as described previously (31). Briefly, slides were incubated with [^{3}H]glutamate (specific activity, 45–56 Ci/mmol; Amersham), in 50 mM Tris·HCl buffer, pH 7.2, containing 2.5 mM $CaCl_2$ and 30 mM KSCN, for 45 min at 4° in the presence of saturating concentrations of NMDA (100 μM) and AMPA (10 μM). In saturation experiments, the labeled ligand (above 100 nM) was diluted with unlabeled ligand (to a "working" specific activity of 2.5 Ci/mmol). In saturation experiments, specific binding was always determined in the presence of 1 mM unlabeled glutamate. Nonspecific binding represented 15–50% of total binding. Detailed competition studies were performed in the presence of 80–100 nM [^{3}H]glutamate (specific activity, 45–56 Ci/mmol), using QUIS, (1*S*,3*R*)-ACPD, and (1*R*,3*S*)-ACPD concentrations ranging from 1 nM to 2.5 mM. In some experiments, Tris-acetate buffer, pH 7.2, in the absence of KSCN, was used instead of Tris·HCl. Incubation was terminated by removal of slides from the ligand solution and rapid rinsing with four 4-ml squirts of 50 mM Tris·HCl buffer (at 4°) containing 2.5 mM $CaCl_2$, followed by two 2-ml squirts of 2.5% (v/v) glutaraldehyde in acetone. Slides were then dried under a stream of hot air and apposed to tritium-sensitive film (Hyperfilm ^{3}H; Amersham) in light-tight cassettes, along with standards containing known amounts of radioactivity (ARC Inc., St. Louis, MO). After 3–6 weeks of exposure at 4°, films were developed in Kodak D-19 developing solution.

Analysis of autoradiograms. Ligand binding was quantitated by computer-assisted densitometry using the MCID system (Imaging Research Inc., St. Catharine's, Ontario, Canada). Ten to 50 readings from each area were averaged. 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. Data from outer cerebral cortex, striatum, cerebellar molecular layer, and stratum radiatum of the CA1 region of the hippocampus (from two to four animals in at least two separate experiments) were analyzed to determine binding parameters. K_d and B_{max} values were determined graphically by Scatchard analysis. Hill coefficients were determined graphically by plotting log $(B/B_{max} - B)$ versus log free ligand (F), assuming that F is the ligand (L) concentration. K_i values for competitors were calculated by log-logit regression analysis using the equation of Cheng and Prusoff (36), $K_i = IC_{50}/(1 + L/K_d)$, for monophasic inhibition curves in situations in which the Hill plots constructed from competition curves gave Hill coefficients not statistically different from 0.8–1.2. Competition curves were analyzed for one or two sites using the iterative curve-fitting computer program LIGAND for determination of K_i values (37).

Results

Saturation experiments. Specific [^{3}H]glutamate binding to mGluRs (in the presence of 100 μM NMDA and 10 μM AMPA) was measured in the outer layers of cerebral cortex, striatum, cerebellar molecular layer, and stratum radiatum of the CA1 region of the hippocampus. Addition of higher concentrations of AMPA (100 μM) and NMDA (1 mM) resulted in identical data in experiments performed using a single concentration (100 nM) of [^{3}H]glutamate (data not shown). Saturation curves indicated that, under our experimental conditions, [^{3}H]glutamate binding was saturable in every region examined. Scatchard plots of [^{3}H]glutamate saturation curves were linear (Hill coefficient = 0.96 ± 0.02; correlation coefficient = 0.87 ± 0.07), indicating that glutamate binds one site or more sites with the same apparent affinity (Fig. 1). The glutamate affinity constants (K_d values) were similar in the areas examined, but the maximum number of sites (B_{max}) varied between regions (Table 1).

Competition experiments. QUIS, *trans*-ACPD (racemic mixture), and the two enantiomers (1*S*,3*R*)- and (1*R*,3*S*)-

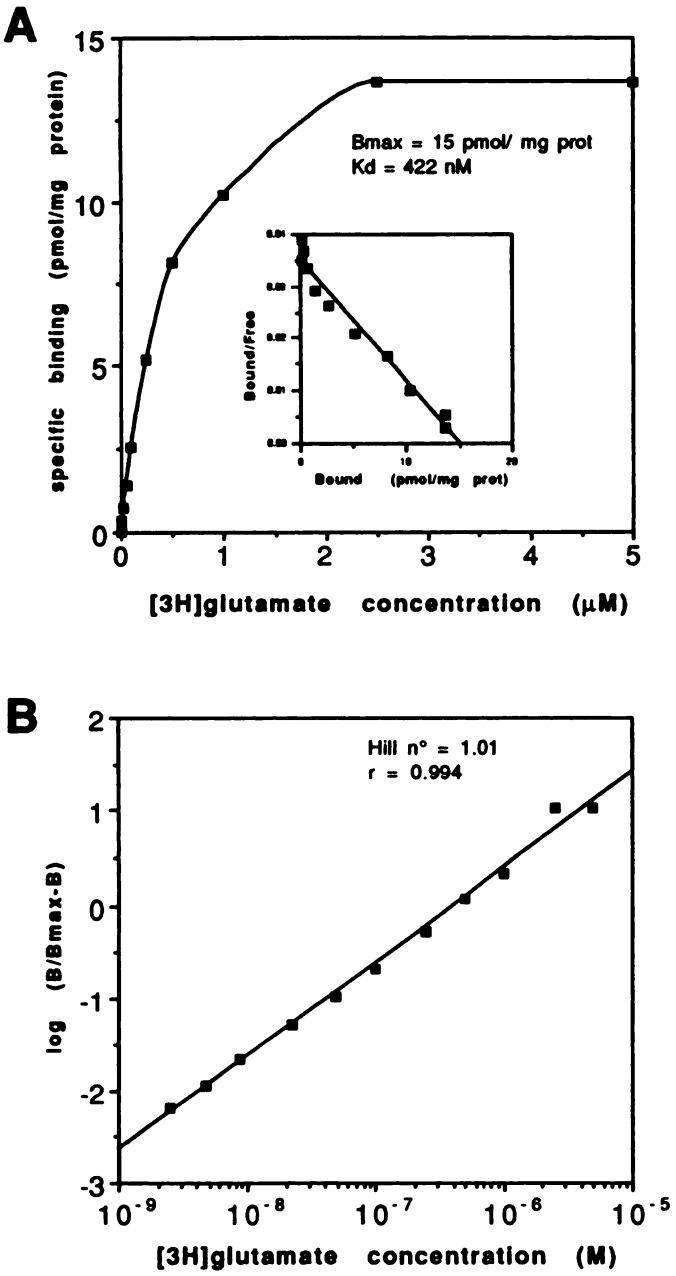

Fig. 1. Representative saturation curve for L-[^{3}H]glutamate binding in the outer cortex in the presence of 100 μM NMDA and 10 μM AMPA (A), with linear Scatchard (*inset*) and Hill (B) plots derived from the same saturation curve. L-[^{3}H]Glutamate concentrations used varied from 2.5 nM to 5 μM. The data fit best with a one-site model. Data represent points from one animal in one experiment, which was performed with four animals with identical results.

ACPD were used as competitors of [^{3}H]glutamate binding. The QUIS competition curve was clearly biphasic in all four regions examined (Fig. 2). Computer analysis of the data from QUIS competition curves revealed two binding sites, with K_d values in the nanomolar and micromolar range. The relative distribution of the two sites was uneven, as indicated by the B_{max} ratio in the different regions (Tables 2 and 3). *t*-ACPD (racemic mixture) competition curves were broader than expected for a single uniform site in all the regions examined (Hill coefficient = 0.73 ± 0.1), with K_d values of 51 μM (outer cortex), 82 μM (striatum), 28 μM (cerebellar molecular layer), and 54 μM

TABLE 1

Data from Scatchard analysis of [^{3}H]glutamate (2.5 nM to 5 μM) binding in the presence of 100 μM NMDA and 10 μM AMPA in different regions

Results are mean ± standard error values of eight saturation curves with eight animals from four separate experiments.

Regions[a]	B_{max}	K_d	Hill coefficient
	pmol/mg of protein	*nM*	
OUT CTX	12.8 ± 0.7[b]	450 ± 28	0.97 ± 0.04
STR	9.2 ± 0.8	478 ± 46	0.94 ± 0.04
CML	9.5 ± 0.8	494 ± 63	0.99 ± 0.05
CA1 SR	10.0 ± 0.7	528 ± 57	0.95 ± 0.05

[a] OUT CTX, outer cortex (external third of the entire cerebral cortex); STR, striatum; CML, cerebellar molecular layer; CA1 SR, stratum radiatum of the CA1 region of the hippocampus.
[b] Different from other regions ($p < 0.01$ by ANOVA with *post hoc* Fisher test).

(stratum radiatum of the CA1 region of hippocampus). Analysis of these curves for multiple sites by LIGAND (37), however, consistently revealed a best fit for only one site. The two enantiomers (1*S*,3*R*)- and (1*R*,3*S*)-ACPD were then tested as competitors of [^{3}H]glutamate binding in the presence of 100 μM NMDA and 10 μM AMPA, without or with 2.5 μM QUIS to block the high affinity QUIS sites. (1*S*,3*R*)-ACPD displaced [^{3}H]glutamate binding both in the absence and in the presence of 2.5 μM QUIS (Fig. 3; Table 4). (1*S*,3*R*)-ACPD displayed the same apparent K_i values in the outer cortex and striatum regardless of the presence of QUIS. The K_i of (1*S*,3*R*)-ACPD was greater in the cerebellar molecular layer and stratum radiatum of the hippocampus when the competition experiment was performed in the presence of 2.5 μM QUIS (Table 4). Interestingly, the Hill coefficients for competitive inhibition with (1*S*,3*R*)-ACPD were closer to unity than were those for racemic *t*-ACPD. (1*R*,3*S*)-ACPD never displaced more than 20% of binding in any region examined under either condition; it is possible that the inactive isomer affected the inhibition of binding in the racemic mixture (Fig. 3).

GTPγS effect on [^{3}H]glutamate binding. Inclusion of 100 μM GTPγS in the incubation mixture resulted in an overall reduction of [^{3}H]glutamate binding by 13% ($p = 0.0001$, two-way ANOVA) in all of the regions examined. This effect was due to a decrease in the affinity, because Scatchard analysis of saturation data from experiments in the presence of GTPγS showed an overall 29% increase ($p = 0.002$, two-way ANOVA) in the K_d values in the regions examined, without any change in the number of receptor sites, compared with binding in the absence of GTPγS (Fig. 4). GTPγS treatment did not alter the linearity of Scatchard plots (Hill coefficient = 0.95 ± 0.02; correlation coefficient = 0.85 ± 0.03).

Characterization of the low affinity QUIS site. Inclusion of 2.5 μM QUIS in the incubation mixture, together with 100 μM NMDA and 10 μM AMPA, blocked [^{3}H]glutamate binding to the high affinity QUIS site and allowed us to study only the low affinity QUIS component of [^{3}H]glutamate binding. Calcium chloride allowed the expression of this binding site, which was not detectable in the absence of these ions (Fig. 5). Kainate (100 μM) had no effect on binding to the low affinity QUIS site (Fig. 5). Binding remaining in the presence of high concentrations of QUIS was not further displaced by *t*-ACPD.

It has been proposed that [^{3}H]glutamate binding in the presence of $CaCl_2$ represents a chloride-dependent glutamate uptake site, rather than a receptor site (38, 39). We tested the effect of various glutamate uptake inhibitors on the low affinity

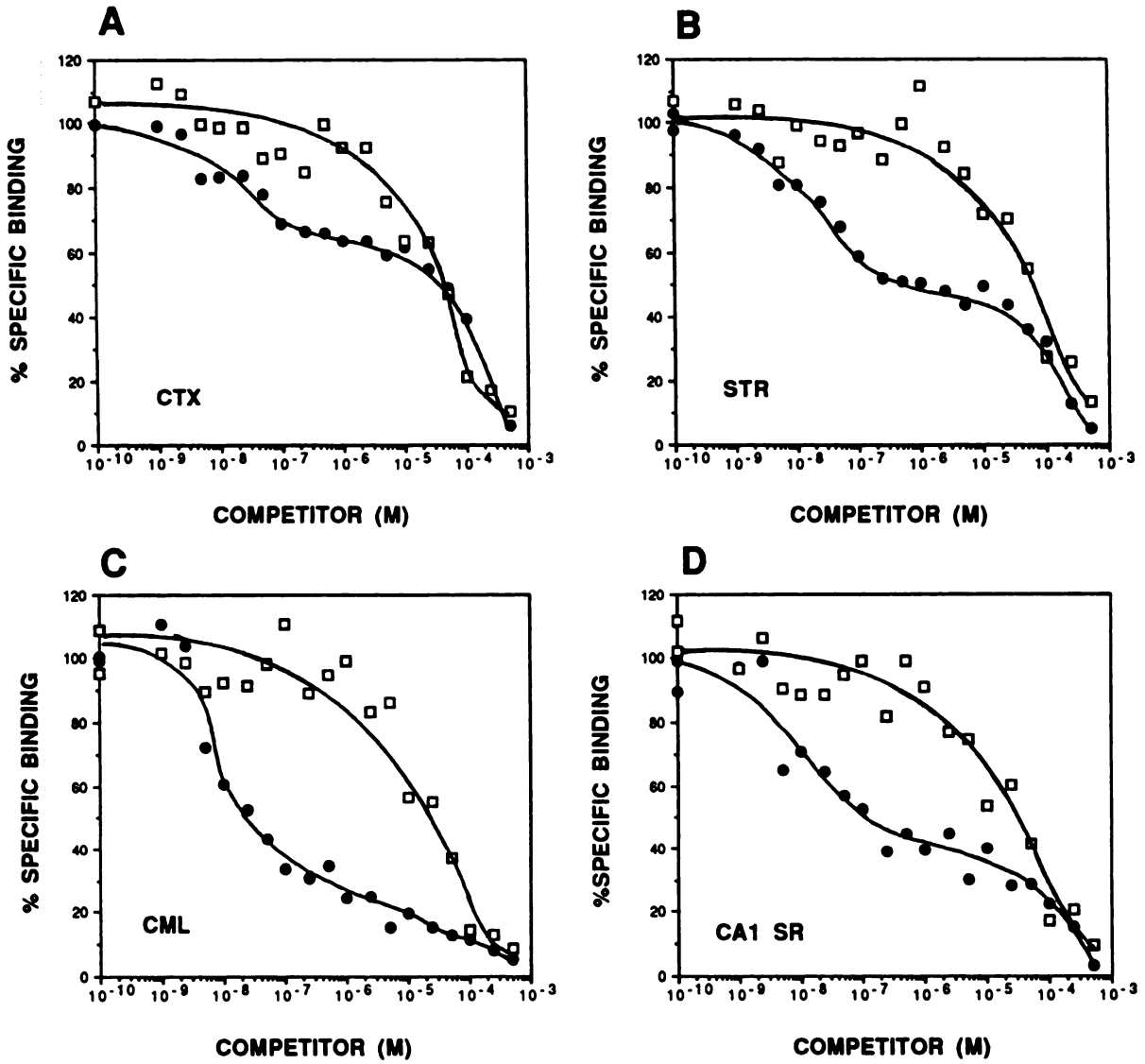

Fig. 2. QUIS (●) and *t*-ACPD (□) competition curves for L-[^{3}H]glutamate binding in the presence of AMPA (10 μM) and NMDA (100 μM) in the outer cortex (A), striatum (B), cerebellar molecular layer (C), and stratum radiatum of the hippocampus (D). The L-[^{3}H]glutamate concentration was 100 nM. The blanks were considered 1 mM QUIS and 2.5 mM *t*-ACPD, respectively. Each *point* represents the mean of determinations in four animals. Standard deviations were always less than 10%. *CTX*, outer cortex (external third of the entire cortex, corresponding to layers 1, 2, and 3); *STR*, striatum; *CML*, cerebellar molecular layer; *CA1 SR*, stratum radiatum of the CA1 region of the hippocampus.

TABLE 2

Data from computer analysis of competition by QUIS for [^{3}H] glutamate binding in the presence of NMDA (100 μM) and AMPA (10 μM)

The concentration of glutamate was 92 nM. The blank was considered 1 mM QUIS. Data are mean ± standard error of results from determinations in eight animals in four separate experiments (two animals/experiment). Abbreviations are as in Table 1. Data are from Ref. 35).

Regions	$B_{max_{high}}$	K_{high}	$B_{max_{low}}$	K_{low}	B_{max} ratio, high/low
	pmol/mg of protein	*nM*	*pmol/mg of protein*	*μM*	
OUT CTX	3.6 ± 1.0	10.4 ± 4.4	8.5 ± 1.5[a]	55.3 ± 4.7	0.4
STR	3.3 ± 0.7	21.7 ± 5.8	4.9 ± 0.6[b]	57.9 ± 8.0	0.6
CML	5.3 ± 0.5	12.1 ± 2.9	1.6 ± 0.1[a,b,c]	72.0 ± 13	3.3
CA1 SR	3.8 ± 1.0	13.6 ± 4.1	2.9 ± 0.6[b]	66.3 ± 11	1.3

[a] Different from $B_{max_{high}}$ for some regions ($p < 0.01$ by paired *t* test).
[b] Different from outer cortex ($p < 0.05$ by ANOVA with *post hoc* Fisher test).
[c] Different from striatum ($p < 0.05$ by ANOVA with *post hoc* Fisher test).

QUIS site, in comparison with the effects of putative agonists and antagonists of mGluRs (Fig. 6). The same concentration (100 μM) was used for all of the tested compounds. Cystine and L-*trans*-pyrrolidin-2,4-dicarboxylic acid inhibited about 40% of binding, whereas dihydrokainate and DL-*threo*-HA had no effect in any region examined. Among the mGluR agonists, *t*-ACPD and IBO were the most potent inhibitors, displacing about 70% of binding. L-HCSA inhibited about 50% of binding in cortex and striatum and 70% of binding in the cerebellar molecular layer. L-AP4 and DL-AP3 inhibited 50% and 60%, respectively, of binding in all regions examined. No strong regional effect was observed except for the more effective displacement of HCSA in the cerebellar molecular layer.

Regional distribution of the high and low affinity QUIS mGluRs. A study of the regional distribution of the two [^{3}H]glutamate binding sites identified here was based on the assumption that the glutamate affinity constant does not

TABLE 3

Regional distribution of metabotropic binding sites

Data are mean ± standard error values of determinations in six animals (met 1) or four animals (met 2), from two experiments. The [^{3}H]glutamate concentration was 100 nM. Binding values were determined from adjacent sections, as described in the text. Anatomy was defined by comparison with Nissl-stained adjacent sections.

Regions	Binding		Ratio of met 1/met 2
	Met 1[a]	Met 2	
	pmol/mg of protein		
Frontal cortex			
Layer 1	0.26 ± 0.06	1.49 ± 0.05	0.18
Layer 2	0.33 ± 0.07	1.54 ± 0.05	0.21
Layer 3	0.45 ± 0.29	1.42 ± 0.11	0.31
Layer 4	0.42 ± 0.03	1.13 ± 0.05	0.38
Layer 5	0.44 ± 0.03	0.98 ± 0.10	0.45
Layer 6	0.35 ± 0.08	0.76 ± 0.07	0.46
Parietal cortex			
Layer 1	0.31 ± 0.08	1.41 ± 0.05	0.22
Layer 2	0.45 ± 0.05	1.47 ± 0.08	0.30
Layer 3	0.43 ± 0.07	1.44 ± 0.18	0.30
Layer 4	0.42 ± 0.08	1.04 ± 0.16	0.40
Layer 5	0.39 ± 0.06	0.99 ± 0.14	0.40
Layer 6	0.29 ± 0.06	0.59 ± 0.08	0.49
Entorhinal cortex			
Layer 1	0.61 ± 0.12	1.08 ± 0.13	0.56
Layer 2	0.45 ± 0.07	0.79 ± 0.12	0.57
Layer 3	0.45 ± 0.07	0.85 ± 0.14	0.53
Layer 4	0.62 ± 0.03	0.53 ± 0.11	1.19
Layer 5	0.47 ± 0.08	0.42 ± 0.10	1.12
Septum	0.66 ± 0.04	0.63 ± 0.13	1.11
Striatum	0.55 ± 0.04	0.96 ± 0.13	0.59
Thalamus			
Anterior nucleus	0.55 ± 0.07	0.94 ± 0.09	0.59
Reticular nucleus	0.22 ± 0.06	0.40 ± 0.08	0.55
Ventral posterior	0.44 ± 0.06	0.69 ± 0.14	0.64
Lateral geniculate	0.45 ± 0.08	0.82 ± 0.13	0.54
Medial geniculate	0.41 ± 0.09	0.73 ± 0.12	0.56
Medial nuclei	0.41 ± 0.05	0.62 ± 0.05	0.66
Hippocampus			
Dentate gyrus			
Molecular layer	0.70 ± 0.10	0.80 ± 0.13	0.78
Granule layer	0.38 ± 0.06	0.35 ± 0.06	1.08
Polymorphic cell layer	0.53 ± 0.08	0.22 ± 0.09	1.33
CA3			
Stratum oriens	0.52 ± 0.05	0.36 ± 0.09	1.45
Stratum pyramidale	0.29 ± 0.02	0.29 ± 0.07	1.02
Stratum lucidum	0.51 ± 0.04	0.33 ± 0.11	1.52
Stratum radiatum	0.41 ± 0.04	0.38 ± 0.07	1.07
Stratum moleculare	0.54 ± 0.07	0.77 ± 0.12	0.70
CA1			
Stratum oriens	0.67 ± 0.08	0.51 ± 0.12	1.32
Stratum pyramidale	0.29 ± 0.07	0.45 ± 0.08	0.65
Proximal stratum radiatum	0.56 ± 0.07	0.49 ± 0.1	1.13
Distal stratum radiatum	0.47 ± 0.06	0.52 ± 0.11	0.89
Stratum moleculare	0.48 ± 0.08	1.01 ± 0.14	0.48
Cerebellum			
Molecular layer	0.91 ± 0.13	0.29 ± 0.09	3.10
Granular layer	0.36 ± 0.09	0.18 ± 0.08	1.96

[a] Met 1, high affinity QUIS site; met 2, low affinity QUIS site.

change in the different regions of the brain nor do the inhibition constants (K_i) of QUIS and (1*S*,3*R*)-ACPD. The high affinity QUIS binding site (metabotropic type 1 binding site) was studied by assessing binding in the presence of 2.5 μM QUIS as blank. Specific binding to the low affinity QUIS site (metabotropic type 2 binding site) was calculated by subtracting binding in the presence of 2.5 mM (1*S*,3*R*)-ACPD from binding in the presence of 2.5 μM QUIS (Fig. 7). The relative distribution of the two [^{3}H]glutamate binding sites is shown in Table 3. The highest levels of high affinity QUIS sites (met 1) were found in the cerebellar molecular layer, septum, stratum moleculare of

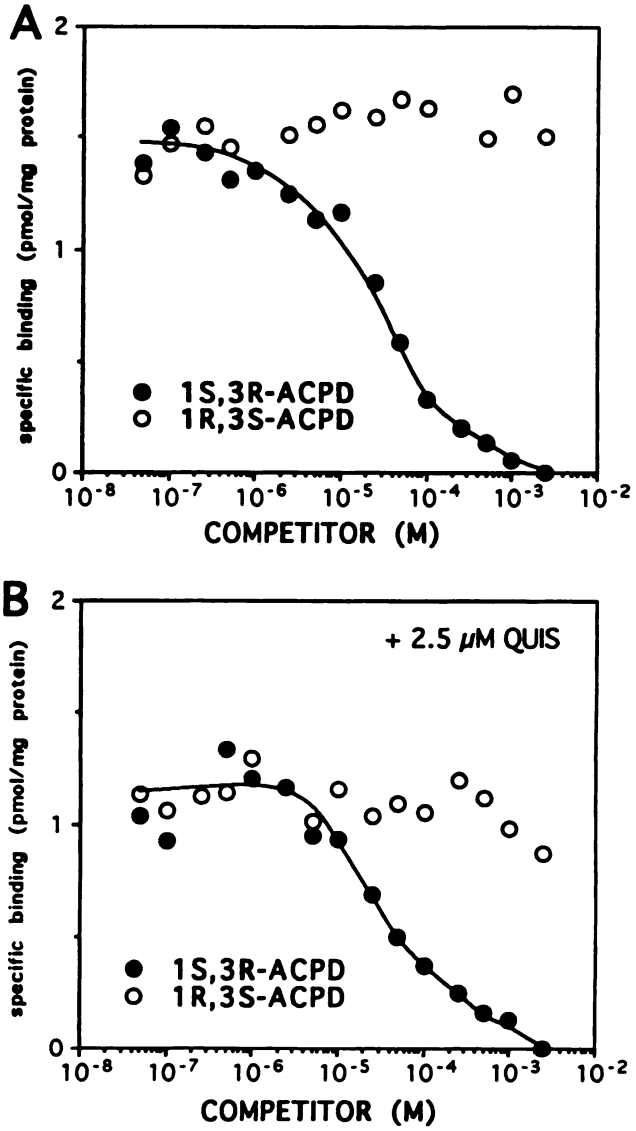

Fig. 3. Comparison of the L-[^{3}H]glutamate binding displacement by the enantiomers (1*S*,3*R*)-ACPD and (1*R*,3*S*)-ACPD in the absence (A) and presence (B) of 2.5 μM QUIS. Figure shows representative binding competition curves for the striatum from an experiment performed with four animals. The variability between animals was <10%. An identical experiment (three animals) gave similar results. Identical results were obtained in the other regions examined. The L-[^{3}H]glutamate concentration was 100 nM. The blank was considered 2.5 mM (1*S*,3*R*)-ACPD.

the dentate gyrus, and stratum oriens of the CA1 region of the hippocampus, as reported previously (31) (Fig. 7B). Entorhinal cortex also had a high density of high affinity QUIS sites. Intermediate levels were present in the striatum and thalamus except the reticular nucleus, which had only negligible levels of high affinity QUIS sites. Low levels of high affinity QUIS sites were found in the stratum pyramidale of the hippocampus. The outer cortex (layers 1, 2, and 3) exhibited the highest levels of low affinity QUIS binding sites (met 2). Intermediate levels of binding were found in the striatum, septum, thalamus, and stratum moleculare of the hippocampus. The lowest levels of low affinity QUIS binding sites were found in the cerebellum (Fig. 7C). Each region expressed different levels of high and low affinity QUIS binding sites. In particular, the cortex (with the exception of the inner entorhinal cortex), thalamus, stria-

TABLE 4

Data from analysis of competition by (1S,3R)-ACPD for [^{3}H] glutamate binding in the presence of 100 μM NMDA and 10 μM AMPA, with or without 2.5 μm QUIS

K_i values were calculated by log-logit regression analysis using the formula; $K_i = IC_{50}/(1 + L/K_d)$. The glutamate K_d was obtained from saturation data.

Regions	High + low affinity QUIS sites (−QUIS)		Low affinity QUIS sites (+2.5 μM QUIS)	
	(1S,3R)-ACPD K_i	Hill coefficient	(1S,3R)-ACPD K_i	Hill coefficient
	μM		μM	
OUT CTX	26.1 ± 4.3	0.88 ± 0.06	33.5 ± 5.0	0.87 ± 0.05
STR	30.6 ± 4.9	0.86 ± 0.05	43.8 ± 4.0	1.02 ± 0.07
CML	14.2 ± 2.0	0.71 ± 0.07	111.1 ± 31.2[a]	0.88 ± 0.17
CA1 SR	28.2 ± 4.6	0.84 ± 0.04	116.3 ± 13.3[a]	1.02 ± 0.20

[a] Different from outer cortex and striatum by ANOVA with *post hoc* Fisher test.

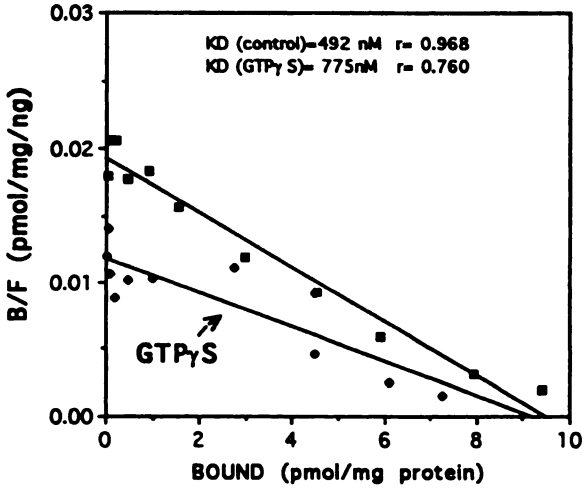

Fig. 4. Scatchard plot of L-[^{3}H]glutamate saturation curves in the absence and presence of 100 μM GTPγS. Binding was performed as described in Experimental Procedures. Figure is representative of determinations on one of four animals in the same experiment.

tum, stratum moleculare of the dentate gyrus, and CA1 and CA3 of the hippocampus expressed more low than high affinity QUIS binding sites. Layers 4 and 5 of the entorhinal cortex, septum, and stratum radiatum of CA1 and CA3 of the hippocampus expressed about the same amounts of both metabotropic binding sites. The cerebellar molecular layer is unique among the regions because it expressed the highest levels of high affinity QUIS binding sites of the brain and very low levels of low affinity binding sites.

In a separate experiment, parasaggital sections through the basal ganglia were assayed to assess the detailed localization in these nuclei (Table 5). High affinity QUIS sites varied 3–4-fold across the structures, with the most dense binding in striatum (ventral > dorsal) and pallidum (ventral > dorsal). The lowest binding was observed in entopeduncular nucleus, with intermediate levels in subthalamic nucleus and substantia nigra pars reticulata. The low affinity QUIS site was present in all basal ganglia nuclei, with striatum demonstrating about 3 times the density of pallidum and substantia nigra pars reticulata. Subthalamic nucleus showed the lowest levels of low affinity QUIS sites in basal ganglia.

Discussion

[^{3}H]Glutamate binding, in the presence of ionotropic inhibitors, was saturable and Scatchard plots were linear, indicating

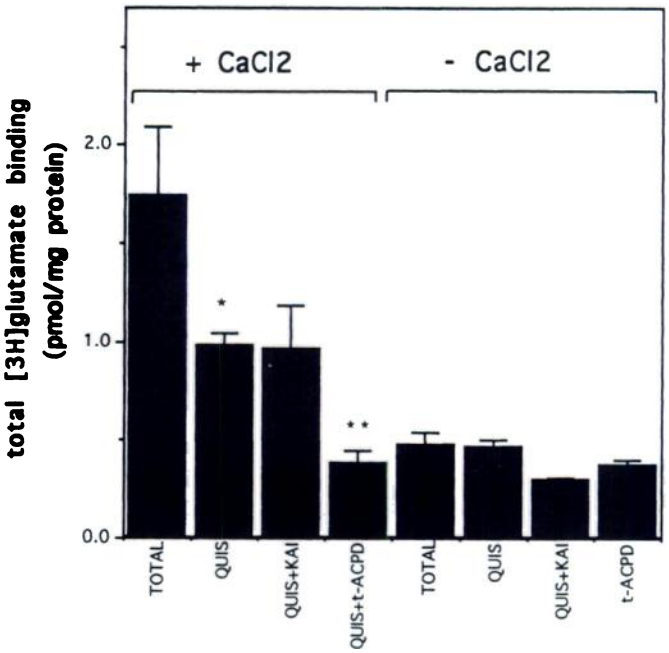

Fig. 5. L-[^{3}H]Glutamate binding to high and low affinity QUIS binding sites, measured in the presence and absence of $CaCl_2$ (2.5 mM), in stratum radiatum of area CA1 of the hippocampus. Binding was performed in the presence of 100 μM NMDA and 10 μM AMPA. In the presence of $CaCl_2$, QUIS displaced much of the total binding, revealing the low affinity QUIS site that was displaceable by *t*-ACPD. Kainate did not affect the low affinity QUIS site. In the absence of $CaCl_2$, neither QUIS nor *t*-ACPD displaced significant amounts of the total binding. Data represent the mean ± standard error from four animals. Similar results were obtained for all other regions. *, Different from total (in the presence of $CaCl_2$) by Scheffe *F* test ($p < 0.001$ by one-way ANOVA). **, Different from 2.5 μM (in the presence of $CaCl_2$) by Scheffe *F* test ($p < 0.001$ by one-way ANOVA). QUIS at 2.5 μM, kainate (*KAI*) at 100 μM, and *t*-ACPD at 1 mM were used.

that glutamate binds to a single population of sites or, alternatively, to different sites with the same apparent affinity. Although the binding data presented here could indeed represent binding to a single population of receptors with complex binding properties and negative cooperativity, the recent cloning of different receptors with distinct pharmacological properties suggests that the binding data represent binding to multiple sites. QUIS, in competition studies, resolved two distinct sites, one with a K_i in the low nanomolar range and the other with a K_i in the micromolar range. Interestingly, these two sites had a defined pattern of distribution in the brain; the high affinity QUIS site was more uniformally distributed than the low affinity QUIS site. Levels of the high affinity binding sites were not statistically different in the regions examined but tended to be higher in the cerebellar molecular layer. In contrast, the low affinity QUIS sites were highly differentially distributed, with maximal levels of binding in the external cortex and intermediate levels in the striatum and stratum radiatum of the hippocampus.

It was shown previously that a subpopulation of QUIS-sensitive, AMPA-insensitive, NMDA-insensitive [^{3}H]glutamate binding sites may represent the recognition site for mGluRs (31). That binding site was characterized by its high affinity for QUIS, IBO, and *t*-ACPD, whereas aspartate, kainate, and NMDA were weak or inactive. That binding site, defined by the binding displaced by 2.5 μM QUIS, appears to be identical to the high affinity QUIS site described in our

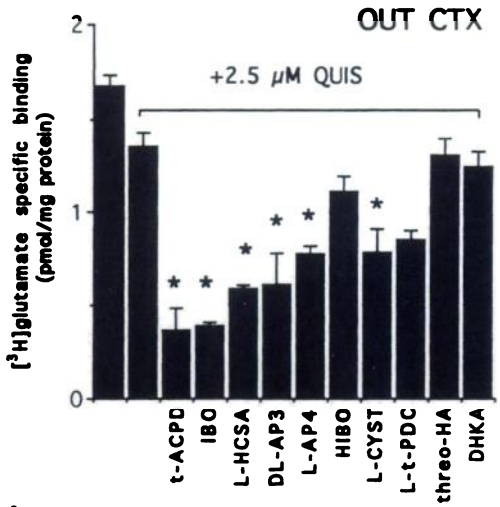

Fig. 6. L-[^{3}H]Glutamate binding to the low affinity QUIS binding site in the presence of various competitors in outer cortex. Binding was maximally reduced by *t*-ACPD (−73%) and IBO (−71.5%). Among different glutamate transport inhibitors, only L-cystine (−43%) and L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-t-PDC) (−42%) affected the low affinity QUIS site. All drugs were tested at 100 μM. The L-[^{3}H]glutamate concentration was 80 nM. The blank was considered to be binding in the presence of 1 mM *t*-ACPD. Data are mean ± standard error of determinations from four animals. $p < 0.001$ by one-way ANOVA. *, 0.05 by Scheffe *F* test. *HIBO*, (*RS*)-α-Amino-4-bromo-3-hydroxy-5-isoxazolepropionic acid; *L-CYST*, L-cystine; *L-t-PDC*, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; *DHKA*, dihydrokainic acid.

study, because 1) it is defined by the same QUIS concentration under the same experimental conditions (see Experimental Procedures) and 2) it has a similar regional distribution, with high levels of binding in the cerebellar molecular layer and lower levels in the striatum, hippocampus, and external cortex (31) (Tables 2 and 3).

We propose that the low affinity QUIS component of the AMPA- and NMDA-insensitive [^{3}H]glutamate binding found in the present study is also linked to mGluRs and that the high and low affinity QUIS sites represent pharmacologically distinct and differentially distributed subpopulations of mGluRs. *t*-ACPD displaced [^{3}H]glutamate binding monophasically, suggesting that this specific mGluR agonist is active at both sites with the same apparent affinity. A different sensitivity of mGluR-mediated responses to the two enantiomers of *t*-ACPD has been reported recently. When tested as competitors of [^{3}H]glutamate binding only, (1*S*,3*R*)-ACPD, but not (1*R*,3*S*)-ACPD, displaced binding both in the absence and in the presence of 2.5 μM QUIS, a concentration corresponding to the plateau of the QUIS competition curve. Interestingly, the (1*S*,3*R*)-ACPD inhibition constants were greater in the cerebellar molecular layer and hippocampus than in the other brain areas. These data have two possible explanations, i.e., 1) the specific number of low affinity QUIS binding sites is lower in the cerebellar molecular layer and CA1 region of the hippocampus than in the outer cortex and striatum, making it more difficult to obtain reproducible results from competition curves, or 2) the low affinity QUIS sites in these areas are pharmacologically different from those expressed in the cortex and striatum. The first possibility appears plausible for the molecular layer of the cerebellum, where there were very few low affinity QUIS sites. It is also possible that a third binding site, with low affinity for both QUIS and (1*S*,3*R*)-ACPD, exists in these areas. However, it remains to be determined whether, considering its low affinity for both agonists, this latter site could be identified as a mGluR. Our data are in accord with previous reports showing that (1*S*,3*R*)-ACPD is more potent than (1*R*,3*S*)-ACPD (40, 41), and they strongly support the hypothesis that the binding studied here is linked to mGluRs. It was shown, however, that in striatal and cerebellar cultured neurons (1*S*,3*R*)-ACPD was the only active compound after 2–3 days *in vitro* but both enantiomers were active at a later stage, with (1*R*,3*S*)-ACPD being more potent than (1*S*,3*R*)-ACPD (42). It is possible that binding to the (1*R*,3*S*)-ACPD-sensitive receptors cannot be detected under our experimental conditions or that these receptors represent a very small subpopulation of

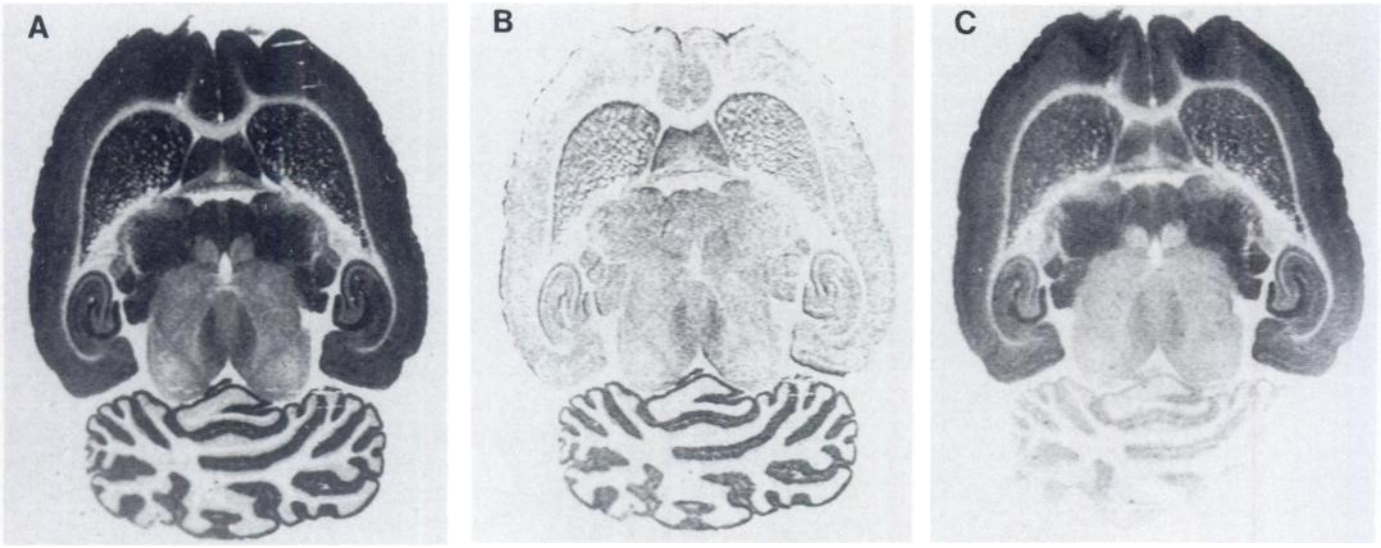

Fig. 7. Autoradiographic imaging of total binding of L-[^{3}H]glutamate in the presence of 100 μM NMDA and 10 μM AMPA (A), specific binding to the high affinity QUIS site (site 1) (B), and total binding in the presence of 100 μM NMDA, 10 μM AMPA, and 2.5 μM QUIS (C). Binding in the presence of 100 μM NMDA, 10 μM AMPA, 2.5 μM QUIS, and 2.5 mM (1*S*,3*R*)-ACPD was negligible. Image B was generated by subtraction of image C from image A, using the MCID program.

TABLE 5

Distribution of metabotropic binding sites in basal ganglia

Data are mean ± standard error values of determinations in six animals. The [^{3}H] glutamate concentration was 100 nM. Binding values were determined from adjacent sections, as described in the text. Anatomy was defined by comparison with Nissl-stained adjacent sections.

Regions	Binding		Ratio of met 1/ met 2
	Met 1[a]	Met 2	
	pmol/mg of protein		
Striatum	0.70 ± 0.05	1.18 ± 0.19	0.59
Ventral striatum	0.80 ± 0.09	1.22 ± 0.15	0.65
Globus pallidus	0.43 ± 0.06	0.31 ± 0.08	1.40
Ventral pallidum	0.53 ± 0.06	0.51 ± 0.10	1.04
Subthalamic nucleus	0.33 ± 0.06	0.19 ± 0.05	1.77
Entopeduncular nucleus	0.19 ± 0.05	0.22 ± 0.06	0.86
Substantia nigra pars reticulata	0.37 ± 0.05	0.34 ± 0.06	1.10

[a] Met 1, high affinity QUIS site; met 2, low affinity QUIS site.

receptors *in vivo*, in comparison with the (1*S*,3*R*)-ACPD-sensitive sites.

GTPγS treatment reduced AMPA- and NMDA-insensitive [^{3}H]glutamate binding. In this experiment specific binding was defined with 1 mM GLU and, thus, included both high and low affinity binding sites. GTPγS is a nonhydrolyzable GTP analogue and activates G proteins, irreversibly binding to them. The model of G protein interactions with a receptor-agonist ligand complex proposed by Gilman (43) postulates that the receptor is in a low affinity state when associated with a G protein in the presence of GTP. Our data are consistent with this model, because inclusion of GTPγS in the incubation mixture seemed to affect the affinity of the receptors for the ligand, without changing the number of binding sites.

[^{3}H]Glutamate bound to mGluRs only in the presence of $CaCl_2$. In fact, in Tris-acetate buffer alone total binding was consistently reduced and *t*-ACPD did not displace any specific binding. Kainate was initially omitted from the incubation mixture on the assumption that calcium ions would block the kainate binding sites (28, 30). In addition, it was shown previously that kainate does not displace the high affinity QUIS metabotropic site (31). We found in the present study that kainate did not have any effect on the low affinity QUIS metabotropic sites. We thus conclude that kainate sites are not labeled under our experimental conditions.

It has been proposed that $CaCl_2$-dependent [^{3}H]glutamate binding may represent binding to glutamate uptake sites rather than receptors sites (38, 39). However, whereas it has been shown that low temperature (0–4°) minimizes uptake systems and putative [^{3}H]glutamate binding to uptake sites is not sensitive to the increasing effect of $CaCl_2$ at low temperature (38), under our experimental conditions $CaCl_2$ dramatically enhanced binding at 0°. Furthermore, whereas L-cystine, the most potent inhibitor of chloride-dependent glutamate sequestration, reduced binding to the low affinity QUIS site, the L-cystine concentration used in our study was far above its K_i value for inhibition of glutamate uptake (0.2 μM) (39). Thus, the L-cystine concentration (100 μM) used should have completely blocked all of the glutamate uptake sites. Even if we cannot rule out the possibility that at least some of the binding measured in our study is to the uptake system, it is also possible that, at 100 μM, L-cystine is partially active at mGluRs. An action of uptake inhibitors on metabotropic sites would not be surprising, because L-aspartate-β-hydroxamate, another potent glutamate uptake inhibitor, has been shown to be a noncompetitive inhibitor of EAA-mediated PI hydrolysis in rat brain (44), probably through allosteric mechanisms at the receptor protein (45). In the same study, L-cysteine sulfinate and L-cysteate, compounds structurally similar to L-cysteine, were also inhibitors of EAA-induced PI hydrolysis, with IC_{50} values of 98 and 198 μM, respectively (44). The pharmacological profile described in our study for the low affinity QUIS-sensitive [^{3}H] glutamate binding site is more consistent with that of the mGluR recognition sites, because the inhibiting effects displayed by *t*-ACPD and IBO (−70%) were higher, at the same concentrations, than that exhibited by L-cystine (−40%), whereas we would expect the opposite for an uptake site. In addition, it has been reported recently that the external glutamate concentration in synaptosomes incubated with (1*S*,3*R*)-ACPD is unchanged, excluding a possible action of ACPD on the glutamate transporter (46). Thus, although one could theoretically include an uptake inhibitor in the assay for the metabotropic sites, the fact that many of the inhibitors may actually affect the metabotropic sites directly makes this strategy problematic.

The putative mGluR antagonists L-AP4 and DL-AP3 consistently inhibited [^{3}H]glutamate binding at a concentration of 100 μM, in accord with their potency in inhibiting *t*-ACPD-mediated responses (IC_{50} = 100–300 μM) (22) in brain slices. Although a noncompetitive mechanism has been proposed for its antagonism of PI hydrolysis, L-AP3 probably acts on a mGluR protein, because it has no effect on adrenergic or muscarinic receptor-mediated PI hydrolysis (47). AP3 inhibits *t*-ACPD-induced, Ca^{2+}-dependent, long lasting chloride currents in *Xenopus* oocytes injected with rat brain mRNA (48) and mGluR1 mRNA (49). However, no AP3-sensitive effect has been detected in mammalian cells transfected with any of the cloned mGluR mRNAs (25, 49). In striatal neurons L-AP3 competitively inhibits QUIS-induced PI hydrolysis (50) but is ineffective on *t*-ACPD-induced inhibition of forskolin-stimulated cAMP formation in the same culture preparation (51). AP3 antagonizes the increase of intracellular Ca^{2+} levels in cultured granule cells (40) but not in cultured Purkinje cells (52). AP3 fails to block the mGluR-mediated postsynaptic electrophysiological effects in hippocampal neurons (53, 54) and cerebellar Purkinjie cells (55) but prevents the (1*S*,3*R*)-ACPD-mediated potentiation of glutamate release in cerebrocortical synaptosomes (46). It is possible that [^{3}H]glutamate binding under our experimental conditions recognizes both AP3-sensitive and -insensitive metabotropic recognition sites. AP4 is an agonist at mGluR4 (26) and its effects on binding could potentially reflect this action. The mGluR4 gene is most intensely expressed in cerebellar granule cells, however, and thus one would expect potentially high levels of the low affinity site in this region and a preferential effect of AP4 in this location as well. Unfortunately, the low affinity site is present at low levels in the cerebellar granule cell layer and AP4 has no differential effects there. Thus, our binding conditions may not allow binding to the mGluR4 protein.

The low affinity QUIS site described in our study presents strong similarities to a previously described [^{3}H]glutamate binding site defined as the NNKQ site (32, 56). The NNKQ site is dependent upon chloride ions, is enhanced by calcium ions, and has the same distribution as our low affinity QUIS site, with very high levels in the superficial layers of the frontal

cortex (1.94 ± 0.7 pmol/mg of protein) and intermediate levels in the stratum radiatum of the CA1 region of the hippocampus (0.44 ± 0.01 pmol/mg of protein) (32). Although it was defined as non-QUIS sensitive, the NNKQ site has been characterized in the presence of a QUIS concentration of 2.5 μM and not a higher concentration. A detailed description of the assay methods and distribution of the NNKQ site within the basal ganglia of the rat has been recently published (57). The basal ganglion distribution of the low affinity QUIS sites reported here and that of NNKQ sites are very similar, and thus the previous data are very comparable (57). The NNKQ site is transiently expressed in the neonatal rat brain and has been found to be reduced in hippocampal formation (58) and insular cortex (59) of patients with Alzheimer's disease, whereas the high affinity QUIS site was unchanged. The definition of the NNKQ site as a subtype of mGluR now offers a novel interpretation of these previous results, as well as a new methodological tool for the investigation of the selective role of mGluR subtypes in development and in neurological diseases.

Correlation between cloned receptors and binding sites. The existence of subtypes of EAA mGluRs, suggested by pharmacological studies in different experimental paradigms, has been confirmed by the cloning of different cDNAs coding for pharmacologically distinct subpopulations of mGluRs. Three groups have been identified, with different agonist selectivities and different sites of expression (26). mGluRs belonging to the first group (mGluR1 and mGluR5) are more sensitive to QUIS than *t*-ACPD, are coupled to PI hydrolysis, and are highly expressed in the cerebellum. mGluRs belonging to the second group (mGluR2 and mGluR3) are more sensitive to *t*-ACPD than QUIS, inhibit adenylate cyclase, and are expressed in different areas of the brain. mGluRs of the third group have low sensitivity to *t*-ACPD (26). We have identified two pharmacologically and regionally distinct sites of [^{3}H]glutamate binding to mGluRs. They are both sensitive to the enantiomer (1*S*,3*R*)-ACPD, which is active at mGluRs, and insensitive to the inactive (1*R*,3*S*)-ACPD, but they display different sensitivities to QUIS. We predict that the high affinity QUIS [^{3}H] glutamate binding sites are on receptors expressed by the first group of mGluR genes, whereas the low affinity QUIS [^{3}H] glutamate binding sites are mainly on mGluRs that belong to the second group. A precise correlation between the binding results and *in situ* hybridization data are not currently possible due to the lack of knowledge regarding the actual pre- or postsynaptic location of the receptor proteins and the absolute amounts of mRNA for each receptor subtype expressed in the different regions. However, the comparison between mGluR binding and *in situ* data for each region examined led us to some tentative conclusions.

In the cerebellar molecular layer most of the binding is to the high affinity QUIS site (metabotropic type 1 binding site), which has the same pharmacology as the cloned mGluR1 and mGluR5. The level of mGluR1 mRNA is very high in Purkinje cells (60), and immunoreactivity with specific antibodies that recognize mGluR1 is very high in the cerebellar molecular layer and Purkinje cells (61, 62). In contrast, no mGluR5 hybridization signal is detectable in the cerebellum, with the exception of a small population of Golgi cells (63). The high affinity QUIS sites (QUIS-sensitive, AMPA-insensitive, NMDA-insensitive [^{3}H]glutamate binding sites) are present at very low levels in Purkinje-defective mutant mice (31). We conclude that, in the cerebellar molecular layer of adult rat brain, most of the high affinity sites recognize receptors of the mGluR1 subtype that are located postsynaptically.

In the stratum radiatum of the CA1 region of the hippocampus, both high and low affinity QUIS sites are present. The mGluR1 mRNA is present at very low levels in the pyramidal cells of this region (24). Immunoreactivity with mGluR1 is high in the stratum oriens but virtually absent in the stratum radiatum (33, 61). In contrast, the levels of mGluR5 are very high in the CA1 pyramidal cells of the hippocampus, suggesting that the high affinity QUIS-sensitive [^{3}H]glutamate binding recognizes this specific mGluR subtype. Although no immunohistochemical study using specific antibodies for mGluR5 has yet been reported, we predict a postsynaptic location for this receptor, because kainate lesions of the CA3 region of the hippocampus did not alter [^{3}H]glutamate binding to the high affinity QUIS site in the stratum radiatum of the CA1 region.[1]

In the striatum high levels of mGluR5 mRNA have been detected (63). Although mGluR1 mRNA and mGluR1 immunoreactivity levels are low in the striatum, high levels of message for this receptor subtype have been detected in regions of the thalamus (but not the cerebral cortex) that project to the striatum. An immunohistochemical study performed with an antibody against mGluR1 has provided evidence for a presynaptic location of this receptor subtype in the striatum (61). It is thus possible that the high affinity QUIS sites recognize, in this structure, both mGluR1 and mGluR5, located pre- and postsynaptically, respectively. The outer cerebral cortex contains low levels of the high affinity QUIS site and these sites probably account principally for recognition sites located on mGluR5, because the message for this receptor subtype is present at relatively high levels in the outer cortex and mGluR1 immunoreactivity is low in this region.

Low affinity QUIS sites, in the striatum and outer cortex, exhibit a pharmacology [(1*S*,3*R*)-ACPD > QUIS] similar to but not identical to that of the mGluR2 and mGluR3 clones (*t*-ACPD ≫ QUIS). In the outer cortex, high levels of mGluR2 message have been found (25), which could account for the high levels of low affinity QUIS sites in this region. mGluR2 has been demonstrated to be coupled to the inhibition of adenylate cyclase that is present in the outer cortex (64). It is more difficult, however, to explain the high levels of low affinity QUIS sites in the striatum, where the levels of mGluR2 and mGluR3 are low, compared with other regions (25, 65). One explanation is that part of this binding may be located on presynaptic sites. In particular, mGluR2 levels are high in the thalamus and cortex. On the other hand, the existence of other unknown mGluRs linked to other mechanisms of signal transduction cannot be excluded. Interestingly, a new G protein-linked receptor, negatively coupled to cAMP formation and pharmacologically distinct from any of the cloned mGluRs, has been recently described in striatal culture (51). This receptor is sensitive to (1*S*,3*R*)-ACPD but not to (1*R*,3*S*)-ACPD and exhibits an EC_{50} of 45 μM for QUIS. It is, however, less sensitive to (1*S*,3*R*)-ACPD (70 μM) than is the binding site described in our study. It is possible that the binding to the low affinity QUIS sites is heterogeneous, despite the Hill coefficient for (1*S*,3*R*)-ACPD competition close to the unity. In the future, new selective, subtype-specific drugs may be developed to help

[1] M. V. Catania, unpublished observations.

distinguish these possibilities. These mGluR binding assays may prove useful for screening potential compounds.

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