

[³H]LY341495 Binding to Group II Metabotropic Glutamate Receptors in Rat Brain

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ABSTRACT

[³H]LY341495 is a highly potent and selective antagonist for group II metabotropic glutamate (mGlu) receptors (mGlu2 and mGlu3), which has been used to label these receptors in cells expressing recombinant receptor subtypes. In this study, we characterized the kinetics, pharmacology, and distribution of [³H]LY341495 binding to mGlu receptors in rat brain tissue. Equilibrium experiments in the rat forebrain demonstrated binding to a single site that was saturable, reversible, and of high affinity (B_{max} , 3.9 ± 0.65 pmol/mg of protein, K_d , 0.84 ± 0.11 nM). The relative order of potencies for displacement of [³H]LY341495 by mGlu receptor ligands was LY341495 \gg L-glutamic acid $>$ LY354740 $>$ (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine $>$ 4-(2R,4R)-aminopyrrolidine-2,4-dicarboxylate $>$ (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid $>$ (R,S)- α -methyl-4-phosphonophenylglycine $>$ (R,S)3,5-dihydroxyphenylglycine $>$ L-(+)-2-amino-4-phosphonobutyric acid.

[³H]LY341495 was not displaced by the selective ionotropic glutamate receptor agonists *N*-methyl-D-aspartic acid, (R,S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or kainate at concentrations up to 1 mM. Comparison of [³H]LY341495 binding in rat brain with recombinant mGlu receptor subtypes demonstrated a very high correlation with mGlu3 receptor binding ($r^2 = 0.957$), a significant, but lower, correlation with mGlu2 receptor binding ($r^2 = 0.869$), but no significant correlation to mGlu8 receptor binding ($r^2 = 0.284$). Regional studies using autoradiography showed a similar distribution of [³H]LY341495 binding to that for group II mGlu receptors previously reported by others using immunocytochemical techniques. These studies indicate that [³H]LY341495 selectively labels group II (mGlu2/3) receptors, but under the conditions used, [³H]LY341495 may bind predominantly to mGlu3 receptor populations in the rat forebrain.

Metabotropic glutamate (mGlu) receptors are G-protein-coupled modulatory receptors in the glutamate receptor family. Cloning studies have identified eight different mGlu receptors, which are divided into three groups based on sequence homology, second messenger coupling, and pharmacology (Nakanishi, 1992; Pin and Duvoisin, 1995). Group I mGlu receptors (mGlu1 and mGlu5) are positively coupled to phosphoinositide hydrolysis; group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8) mGlu receptors are negatively coupled to adenylyl cyclase when expressed in nonneuronal mammalian cell lines.

Metabotropic glutamate receptors are highly expressed in the rat brain where they have overlapping, but distinct, distributions and functions. mGlu receptor functions include the modulation of excitatory and inhibitory neuronal transmission in many physiologically important synapses and pathways throughout the central nervous system (Anwyl,

1999). Metabotropic glutamate receptors also modulate the release of multiple neurotransmitters, including amino acids, such as glutamate and γ -aminobutyric acid, monoamines, such as serotonin and dopamine, purines, and neuropeptides (Cartmell and Schoepp, 2000). The availability of high-affinity ligands would be highly useful to study mGlu receptor pharmacology and regulation in physiological and pathological conditions involving these neurotransmitter systems. Nevertheless, to date there have been relatively few binding studies reported for mGlu receptors due to the lack of selective radioligands with high affinities (Schoepp et al., 1999; Mutel et al., 2000).

Our earlier studies using 1S,3R-ACPD-sensitive [³H]glutamate binding established conditions for binding to group II mGlu receptors in the rat brain (Schoepp and True, 1992; Wright et al., 1994). Using 1S,3R-ACPD-sensitive [³H]glutamate binding, LY341495 was identified as a highly potent

ABBREVIATIONS: mGlu, metabotropic glutamate; L-CCG-I, (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine; 1S,3R-ACPD, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid; L-AP3, L-(+)-2-amino-4-phosphonopropionic acid; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; DCG-IV, *n*-(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine; DHPG, (RS)- α -methyl-4-phosphonophenylglycine; MPPG, (R,S)3,5-dihydroxyphenylglycine; 2R,4R-APDC, 4-(2R,4R)-aminopyrrolidine-2,4-dicarboxylate; 1S,3S-ACPD, (1S,3S)-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, (R,S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartic acid; *t*-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; MPDC, L-*anti-endo*-3,4-methanopyrrolidinedicarboxylic acid; MCPG, (S)- α -methyl-4-carboxyphenyl glycine; NAAG, *N*-acetyl-L-aspartyl-L-glutamic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; (RS)PPG, (R,S)-4-phosphonophenylglycine.

and selective mGlu receptor ligand (Ornstein et al., 1998 b,c). Subsequent work showed that LY341495 is a low-nanomolar potent selective competitive antagonist for recombinant group II mGlu receptors and will also antagonize other mGlu receptor subtypes at higher concentrations (Kingston et al., 1998). LY341495 was radiolabeled subsequently, and [³H]LY341495 was proven to be quite useful for studying mGlu receptor binding in cell lines expressing cloned human group II (mGlu2 and mGlu3) receptors (Johnson et al., 1999). At higher ligand concentrations, [³H]LY341495 also labels certain group III mGlu receptors, such as mGlu6, mGlu7, and mGlu8 (Wright et al., 2000). Preliminary work in rat brain tissue also indicated that [³H]LY341495 may be useful in labeling mGlu receptors in rat tissue (Ornstein et al., 1998a).

In this study, we report on the kinetics, pharmacology, and distribution of [³H]LY341495 binding to metabotropic glutamate receptors in rat brain tissue using membrane preparations and receptor autoradiographic techniques. These studies indicate that [³H]LY341495 can be used to selectively label group II (mGlu2/3) receptors in the rat brain and thus, may be a useful new high-affinity antagonist radioligand tool for studying mGlu receptor pharmacology and regulation.

Experimental Procedures

Materials. [³H]-2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)propionic acid ([³H]LY341495) (17.5 Ci/mmol) was radiolabeled by Chemsyn (Lenexa, KA) (Ornstein et al., 1998a). LY341495, LY354740, LY366563, LY307452, 4-(2R,4R)-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APDC), L-(+)-2-amino-4-phosphonopropionic acid (L-AP3), and (R,S)3,5-dihydroxyphenylglycine (DHPG) were synthesized at Eli Lilly and Company (Indianapolis, IN). L-Quisqualate, (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I), (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD), (1S,3S)-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3S-ACPD), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), (R,S)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), kainate, *n*-(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), (R,S)- α -methyl-4-phosphonophenylglycine (MPPG), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*t*-PDC), L-*anti-endo*-3,4-methanopyrrolidinedicarboxylic acid (MPDC), (R,S)-4-phosphonophenylglycine [(R,S)PPG], and (S)- α -methyl-4-carboxyphenylglycine (MCPG) were obtained from Tocris Cookson (Ballwin, MO). L-Glutamic acid and L-aspartic acid were obtained from Sigma (St. Louis, MO). N-Acetyl-L-aspartyl-L-glutamic acid (NAAG) was obtained from Bachem California (Torrance, CA). ScintiSafe liquid scintillation cocktail was obtained from Fisher Scientific (Pittsburgh, PA). Coomassie protein assay reagent was obtained from Pierce Chemical (Rockford, IL).

[³H]LY341495 Binding to Rat Brain Membranes. Brain tissue was obtained by decapitating adult male Sprague-Dawley rats (150–250 g, Harlan Bioproducts for Science, Indianapolis, IN) in accordance with the Eli Lilly and Company animal care and use policies and prepared as described previously (Wright et al., 1994). For most studies, the forebrain (cortex, striatum, and hippocampus) was used. For brain region studies, the brain was dissected into seven regions using the method of Glowinski and Iversen (1966). The brain tissue was homogenized in 30 mM Tris-HCl + 2.5 mM CaCl₂ buffer (pH 7.6 at 5°C) and washed three times by centrifugation, incubated for 30 min at 37°C followed by three more washes, and then resuspended in 10 volumes of buffer and frozen at –20°C.

Frozen pellets of rat brain homogenate were thawed on the day of assay and washed three times with ice-cold assay buffer (10 mM potassium phosphate + 100 mM potassium bromide, pH 7.6). For ion experiments, 10 mM potassium phosphate, pH 7.6, was used as the base buffer, to which was added the respective potassium salts for

the anions and bromide salts for the cations. To start the reaction, tissue (0.02–0.06 mg of protein) was added to deep-well polypropylene microtiter plates, which contained [³H]LY341495 (1 nM) and appropriate concentrations of test compounds in assay buffer. Final assay volume was 0.5 ml. Nonspecific binding was defined with 1 mM L-glutamate. Assay plates were incubated on ice for 30 min, and bound and free radioligands were separated by rapid filtration with 5 × 1 ml of ice-cold assay buffer using Whatman GF-B filters (Brandel Inc., Gaithersburg, MD). Protein concentration was determined using the Pierce Coomassie micro assay.

Data analysis was performed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA) and Ludson Software (Ludson Software, Inc., Chagrin Falls, OH). K_i values for the displacers were calculated from IC₅₀ values using the equation of Cheng and Prusoff (1973). The observed on and off rates were calculated from association-dissociation curves using the one-phase exponential association and decay equations in the Prism program. K_{on} was calculated by subtracting the off rate from the observed on rate and dividing by the radioligand concentration. K_d was calculated two ways, from the saturation curves using the Ludson1 program (Ludson Software, Inc.) and from the on and off rates: $K_d = K_{off}/K_{on}$.

[³H]LY341495 Autoradiography. Whole brains were obtained from adult male Sprague-Dawley rats (approximately 200 g), rapidly frozen in powdered dry-ice, and mounted on cryostat chucks. Horizontal sections (20 μ m) were cut and thaw-mounted on gelatin-coated slides. Sections were stored at –20°C before the experiment. Tissue sections were preincubated in ice-cold 10 mM potassium phosphate buffer with 100 mM potassium bromide (phosphate/bromide buffer), pH 7.6, for 30 min to remove endogenous receptor ligands, then rapidly dried under a stream of cool air. The sections were incubated for 90 min in phosphate/bromide buffer with 5 nM [³H]LY341495. Nonspecific binding was determined using adjacent sections with 1 mM L-glutamate in the buffer solution. Following incubation, the sections were rinsed by immersing in ice-cold phosphate/bromide buffer for 30 s, followed by a another 30-s fresh buffer rinse and a final 30-s rinse in ice-cold purified water, then quickly dried under a stream of warm air.

Tissue sections were apposed to tritium-sensitive film (Hyperfilm-³H, Amersham Pharmacia Biotech, Piscataway, NJ) for 2 weeks. Radioactive standards, calibrated with known amounts of tritium ([³H] micro-scales, Amersham RPA 510), were coexposed with each film. Quantitative autoradiogram analysis was performed densitometrically using a computer-assisted image analyzer (Imaging Research Inc., St. Catherines, ON, Canada). Optical density values were converted to femtomoles per milligram of protein using a computer generated regression analysis that compared film densities produced by tissue sections and radioactive standards. Three density readings were obtained from each area per animal and averaged for each determination. Separate measurements were obtained from five individual animals.

Results

Analysis of [³H]LY341495 binding under equilibrium conditions revealed a single binding site that was saturable ($B_{max} = 3.9 \pm 0.65$ pmol/mg of protein, $n = 3$) and of high affinity ($K_d = 0.84 \pm 0.11$ nM, $n = 3$). Figure 1 shows the results of a typical saturation experiment. As shown in Fig. 2, the binding of [³H]LY341495 was linear between 5 and 60 μ g of protein, and specific binding, defined using 1 mM L-glutamate as displacer, was greater than 95% of the total binding with 1 nM [³H]LY341495.

Association-dissociation experiments demonstrated that [³H]LY341495 binding (1 nM) reaches equilibrium within 30 min when incubated on ice and is displaced completely within approximately 45 min after 1 μ M cold LY341495 is

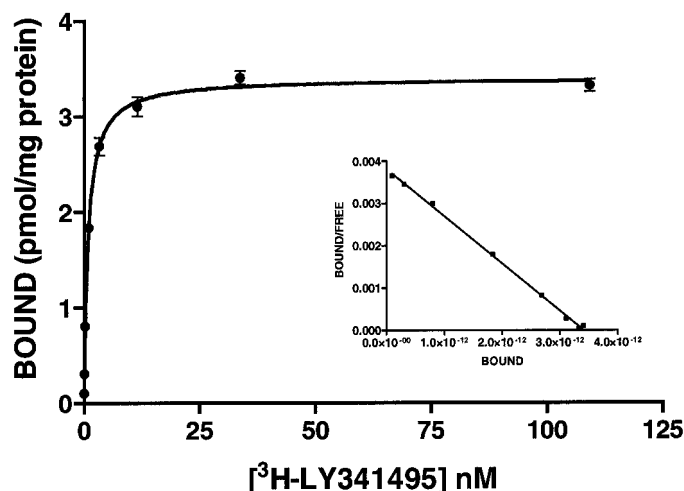


Fig. 1. Representative saturation analysis and Scatchard plots of [³H]LY341495 binding in rat forebrain homogenate. Tissue was incubated 30 min on ice with increasing concentrations of [³H]LY341495 in 10 mM potassium phosphate buffer (pH 7.6 at 5°C) with 100 mM potassium bromide. Nonspecific binding was defined with 1 mM L-glutamate. Assay was terminated by rapid filtration with ice-cold buffer. Each data point was determined in triplicate for each experiment. Data were analyzed using the Lunden Software program.

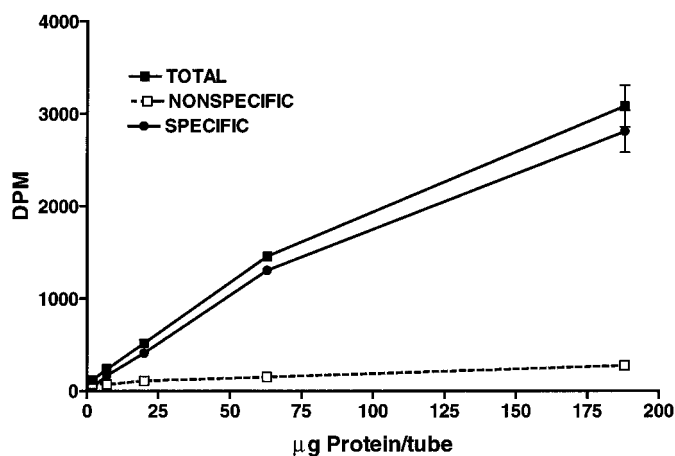


Fig. 2. Protein linearity experiment showing specific versus nonspecific and total binding. Tissue was incubated 30 min on ice with [³H]LY341495 (1 nM) in 10 mM potassium phosphate buffer (pH 7.6 at 5°C) with 100 mM potassium bromide. Nonspecific binding was defined with 1 mM L-glutamate. Assay was terminated by rapid filtration with ice-cold buffer. Total and nonspecific bound were determined with increasing amounts of rat forebrain homogenate. Protein concentrations were determined using the Pierce Coomassie micro assay. Data represent the mean and standard error of triplicates in a representative experiment.

added to the reaction tubes. Analysis of the association-dissociation curves resulted in on and off rates of 0.160 nM⁻¹ min⁻¹ and 0.053 min⁻¹, respectively. The K_d calculated from these data (K_{off}/K_{on}) was 0.33 nM (Fig. 3, A and B).

The effects of different ions on binding are shown in Fig. 4. Relatively high levels of specific binding were obtained in the presence of bromide, chloride, or nitrate (>1000 fmol/mg of protein), and binding was moderate in the presence of acetate, iodide, and thiocyanate (between 300 and 800 fmol/mg of protein) and very low in the presence of fluoride and potassium phosphate buffer alone (<200 fmol/mg of protein) (Fig. 4A). Specific binding in the presence of bromide was slightly higher than that in the presence of chloride (about 1200 versus 1000 fmol/mg of protein). In the presence of

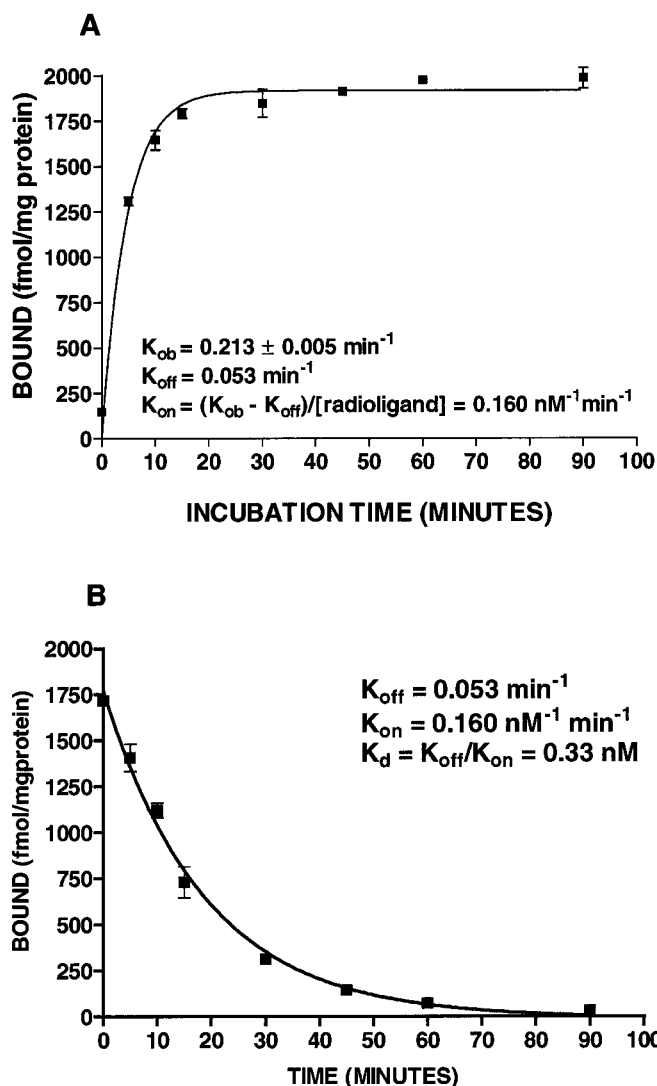


Fig. 3. Association and dissociation time course curves for [³H]LY341495 binding in rat forebrain. Assay was performed as described in the legend to Fig. 2 and under *Experimental Procedures*. For the association data (A), assay components were incubated for the length of time indicated, then terminated by rapid filtration. For the dissociation data (B), assay components were incubated for 30 min, then an excess of cold LY341495 (1 µM) was added, followed by rapid filtration at the times indicated for each data point. Data represent mean and standard error of triplicates from a representative experiment.

increasing concentrations of bromide ion, the level of specific binding plateaued at 100 mM (Fig. 4B). The presence of bromide salts of different mono- or divalent cations (potassium versus sodium versus magnesium versus calcium) did not have much effect at up to 100 mM on the specific binding of [³H]LY341495 in the rat forebrain under normal assay conditions (Fig. 4B).

The ability of various glutamate receptor and transporter ligands to displace [³H]LY341495 binding in adult rat forebrain membranes is shown in Fig. 5 and Table 1. Compounds that are selective for group II mGlu receptors displaced binding with a large range of potencies, which is generally consistent with their potencies at group II recombinant receptors (Schoepp et al., 1999). For example, LY341495 was by far the most potent compound, with a K_i value of 0.8 nM. The group II agonist LY379268 was also highly potent, with a K_i

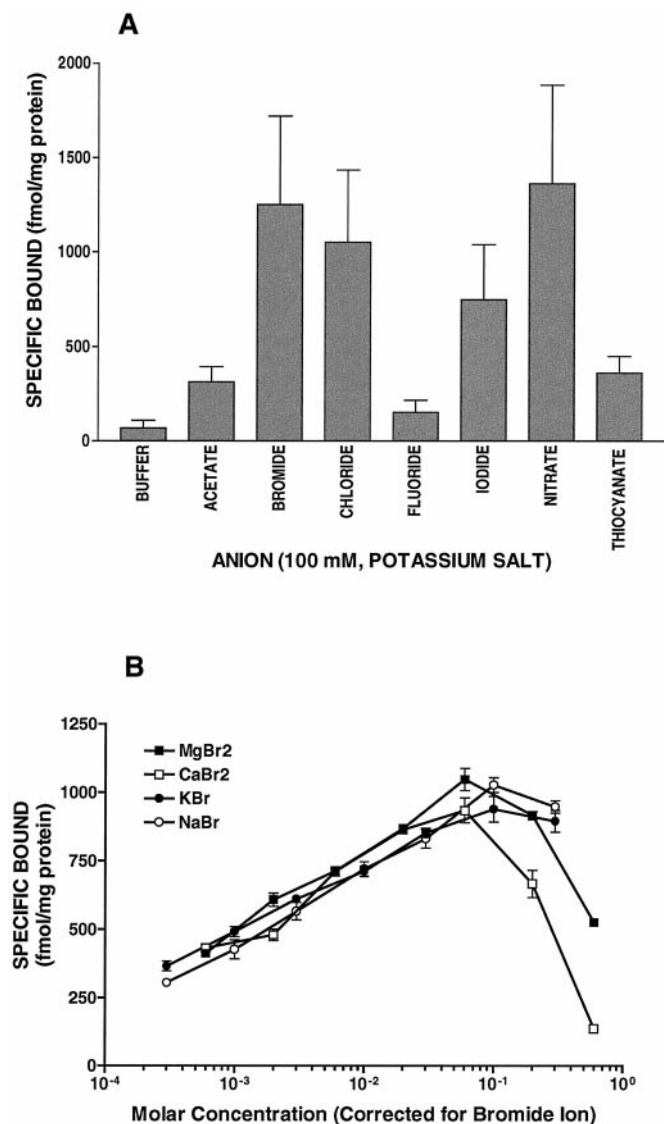


Fig. 4. Results of experiments performed to determine the effects of ions on [³H]LY341495 binding in rat forebrain. Assays were performed as described in Fig. 2 legend and under *Experimental Procedures*. Anion experiments (A) were performed using 10 mM potassium phosphate buffer as the base buffer, then adding the potassium salt of each anion tested to a concentration of 100 μM. Cation experiments (B) were performed using 10 mM potassium phosphate buffer, then adding the bromide salt of each cation tested at increasing concentrations. Data are shown corrected for bromide concentration.

value of 6.5 nM. DCG-IV and LY354740 displaced the binding at nanomolar concentrations (in the range of 50–100 nM). L-CCG-1 was slightly less potent with affinity in the 300 nM range. The compounds LY307452, 1S,3S-ACPD, and 2R,4R-APDC displaced at low micromolar concentrations. The non-selective endogenous ligand L-glutamate potently displaced [³H]LY341495 ($K_i = 90$ nM).

The ionotropic receptor agonists NMDA, AMPA, and kainic acid did not appreciably displace [³H]LY341495 binding at concentrations up to 1 mM, whereas the endogenous NMDA agonist, L-aspartate, displaced the binding at high-micromolar concentrations. The group I mGlu receptor-selective agonists DHPG and quisqualate displaced specific [³H]LY341495 binding but with micromolar potencies. The group III mGlu receptor-selective agonist, L-AP4, also displaced the binding at high

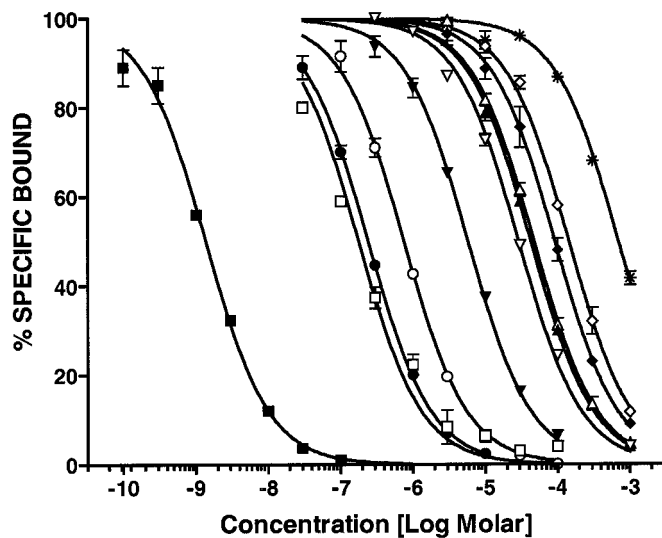


Fig. 5. Displacement of [³H]LY341495 binding in rat forebrain by glutamate receptor ligands with varying selectivity and potency. Assay was performed as described in the legend to Fig. 2 and under *Experimental Procedures*. Data points represent percentage of total specific bound in the absence of a displacer (mean and standard error of at least three separate experiments performed in duplicate). Nonspecific binding, defined by the amount bound in the presence of 1 mM L-glutamate, was subtracted from all values. ■, LY341495; □, L-glutamate; ●, LY354740; ○, L-CCG-I; ▼, APDC; ▽, 1S,3S-ACPD; ▲, quisqualate; △, LY366563; ◆, MPPG; ◇, DHPG; *, L-AP4.

TABLE 1

Potencies of various excitatory amino acid analogs in displacement of [³H]LY341495 binding in rat forebrain membranes

Data are mean + S.E., $n = 3$.

Compound	K_i μM	Hill Coefficient
LY341495	0.0008 ± 0.0002	1.01 ± 0.01
LY379268	0.0065 ± 0.0004	0.98 ± 0.01
DCG-IV	0.061 ± 0.001	0.90 ± 0.02
L-Glutamate	0.090 ± 0.018	0.75 ± 0.08
LY354740	0.111 ± 0.006	0.99 ± 0.04
L-CCG-I	0.344 ± 0.023	1.03 ± 0.04
LY307452	2.01 ± 0.49	1.03 ± 0.04
2R,4R-APDC	2.73 ± 0.27	0.96 ± 0.03
1S,3S-ACPD	8.69 ± 0.58	0.85 ± 0.01
1S,3R-ACPD	15.69 ± 1.15	0.91 ± 0.02
Quisqualate	19.83 ± 1.38	0.99 ± 0.07
LY366563	19.83 ± 0.52	1.02 ± 0.04
NAAG	37.60 ± 1.11	0.80 ± 0.04
MPPG	43.66 ± 2.58	0.96 ± 0.06
DHPG	63.51 ± 3.66	1.05 ± 0.04
(RS)PPG	61.33 ± 4.42	0.81 ± 0.07
t-PDC	110.02 ± 8.24	0.90 ± 0.12
L-AP3	117.23 ± 25.97	0.86 ± 0.11
MCPG	247.47 ± 15.81	0.90 ± 0.09
L-AP4	341.43 ± 13.56	0.93 ± 0.04
L-Aspartate	375.40 ± 61.89	0.85 ± 0.07
D-Glutamate	509.53 ± 41.62	0.75 ± 0.05
Anti-endo-MPDC	837.43 ± 188.36	0.81 ± 0.06
AMPA	% displacement at 1 mM	19 ± 2%
Kainate	% displacement at 1 mM	5 ± 3%
NMDA	% displacement at 1 mM	5 ± 2%
SITS	% displacement at 1 mM	11 ± 4%

micromolar concentrations. (RS)PPG, which is a nanomolar potent and selective mGlu8 receptor agonist, only displaced binding at high micromolar concentrations. The neuropeptide NAAG, which is reported to be selective for mGlu3 receptors, displaced binding with a K_i value of 37 μM. Among the glutamate transport inhibitors, t-PDC displaced binding at high mi-

cromolar concentrations, however, near millimolar concentrations of anti-*endo*-MPDC were required. The chloride-dependent glutamate uptake blocker SITS did not displace the binding appreciably at up to 1 mM.

Stereoselectivity of [³H]LY341495 binding was demonstrated by the difference in potencies of L-glutamate and LY354740 and their stereoisomers, D-glutamate and LY366563. These isomers differed in potency by factors of about 10,000 and 100, respectively. For all compounds that displaced [³H]LY341495 binding, the Hill coefficients were between 0.75 and 1.05.

In Fig. 6, we compared the K_i values for displacement of [³H]LY341495 binding to the rat forebrain with values we reported previously using membranes from human mGlu2, mGlu3, and mGlu8 receptor-expressing cells (Johnson et al., 1999; Wright et al., 2000). Compared with rat brain tissue, a very high correlation with mGlu3 receptor binding ($r^2 = 0.957$, $p < 0.0001$) was observed. A highly significant ($p < 0.0001$), but lower ($r^2 = 0.869$), correlation with mGlu2 receptor binding was noted. When rat brain binding was compared with mGlu8 receptors, no significant correlation was found ($p > 0.05$, $r^2 = 0.284$). In Table 2, we compared the affinity and kinetics of [³H]LY341495 binding to rat forebrain membranes with our previously reported values for recombinant mGlu subtypes. The K_d value for rat brain tissue was not significantly different from the K_d value for mGlu3 binding, but was about half the K_d value for mGlu2 binding. As shown, [³H]LY341495 has a much lower affinity for mGlu8 receptors when compared with these other tissues. Likewise, the off and on rates and pharmacological correlations for rat brain [³H]LY341495 binding were most like mGlu3 receptors when compared with these other tissues. The on and off rates for the mGlu8 receptor are not shown, as association and dissociation binding to this tissue was too rapid to be measured accurately (Wright et al., 2000).

Figure 7 illustrates the distribution of [³H]LY341495 (1 nM) binding in grossly dissected adult rat brain regions. The regions with the highest levels of specific binding were in the forebrain: the cortex, striatum, and hippocampus (2477 ± 84 , 2126 ± 81 , and 1940 ± 23 fmol/mg of protein, respectively). Moderate levels of specific binding were found in the hypothalamus (midbrain), olfactory bulb, and cerebellum (1142 ± 26 , 936 ± 18 , and 815 ± 21 fmol/mg of protein, respectively). The lowest level of binding occurred in the region of the pons and medulla (375 ± 2 fmol/mg of protein).

Figure 8 and Table 3 show results for quantitative autoradiographic studies on [³H]LY341495 (5 nM) distributions in sagittal sections of the rat brain. Here, nonspecific binding was minimal when compared with total binding [Fig. 8, compare upper panel (total binding) and lower panel (nonspecific binding)]. Highest specific binding (>1000 fmol/mg of protein) was found in many regions of the cerebral cortex (frontal, occipital, retrosplenial) (particularly layers I and II), dentate gyrus, caudate putamen, nucleus accumbens, and olfactory tubercle. Regions with moderate to high specific binding (500–1000 fmol/mg of protein) included the amygdala, lateral geniculate nucleus, anterior olfactory bulb, subiculum, hippocampus (stratum oriens and radiatum, pyramidal layer, granular layer of dentate), thalamus, substantia nigra, superior colliculus, and the granular layer of the cerebellum. Areas with measurable, but relatively lower binding (<500 fmol/mg of protein), included the globus pallidus, in-

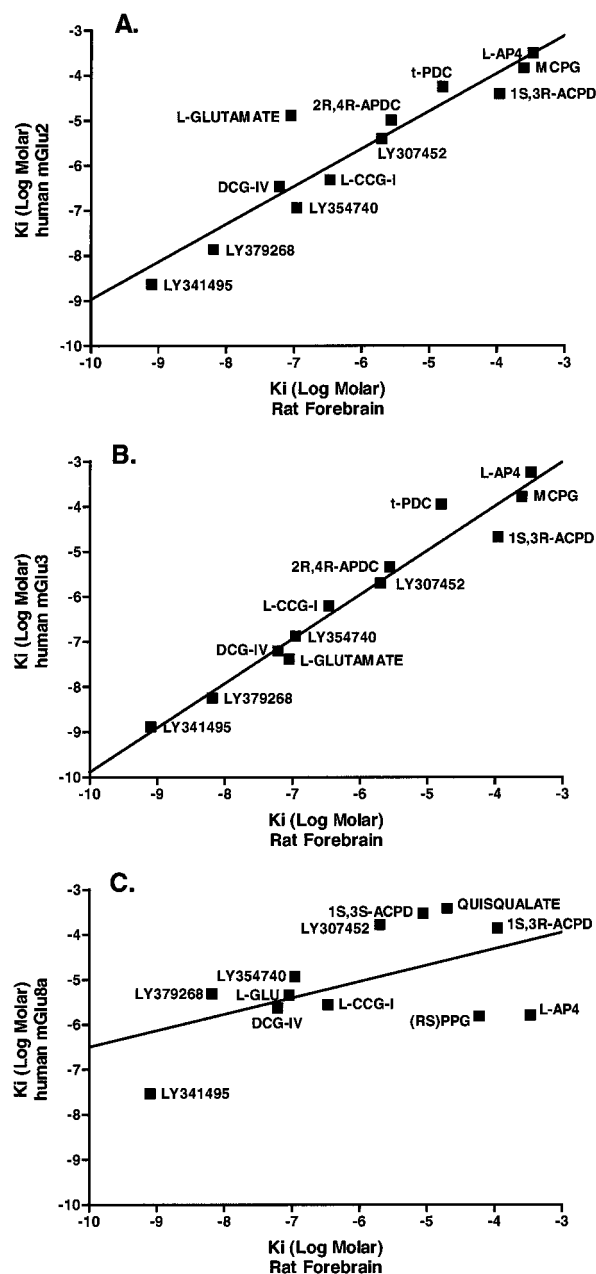


Fig. 6. Correlations of compound displacement of [³H]LY341495 binding to rat forebrain versus human recombinant mGlu2, mGlu3, and mGlu8 receptor subtypes. Data for rat forebrain were taken from Table 1. Data for human mGlu2 and mGlu3 receptors were from Johnson et al. (1999). Data for human mGlu8 receptors were from Wright et al. (2000).

ferior colliculus, mesencephalic nucleus, and cerebellum (molecular layer and interposed nuclei).

Discussion

Recently, considerable progress has been made in the discovery and characterization of potent agonists and antagonists for metabotropic glutamate receptor subtypes (Schoepp et al., 1999). Among these, LY341495 has been shown to be a competitive mGlu receptor antagonist with particularly high affinity (low nanomolar potency) for group II mGlu receptor subtypes. The high affinity for mGlu2 and mGlu3 receptors exhibited by LY341495 was demonstrated in assays showing

TABLE 2
Comparison of [³H]LY341495 binding in rat brain with binding in cells expression recombinant mGlu receptor subtypes

Binding Parameter	Tissue Preparation			
	Rat Forebrain	Human mGlu2 ^a	Human mGlu3 ^a	Human mGlu8 ^b
Affinity				
K_d at equilibrium	0.84	1.67	0.75	14.0
Off rate				
K_{off} (min ⁻¹)	0.053	0.246	0.069	
$t_{1/2}$ (min)	13.1	2.8	10.0	
On rate				
K_{on} (nM ⁻¹ min ⁻¹)	0.160	0.46	0.092	
Pharmacology				
Correlation vs. rat brain		$r^2 = 0.869$ ($p < 0.0001$)	$r^2 = 0.957$ ($p < 0.0001$)	$r^2 = 0.284$ ($p > 0.05$)

^a Data are from Johnson et al. (1999).

^b Data are from Wright et al. (2000).

reversal of functional responses (agonist suppressions of cAMP) in cell lines expressing these recombinant subtypes (Kingston et al., 1998). This was further verified by binding studies in mGlu2- and mGlu3-expressing cells using [³H]LY341495 (Johnson et al., 1999). In particular, the low nanomolar K_d values we obtained with [³H]LY341495 in mGlu2 and mGlu3 preparations made this compound a very good filtration assay ligand.

Initial [³H]LY341495 studies were in cell lines expressing a mGlu2 or mGlu3 receptor subtypes. However, LY341495 will also block other mGlu receptor subtypes at higher concentrations than those that block group II receptors. Using functional assays (Kingston et al., 1998), we found that the relative order of LY341495 potency in blocking mGlu receptor subtypes was mGlu3 \geq mGlu2 > mGlu8 > mGlu6 = mGlu7 > mGlu1 = mGlu5 > mGlu4. The range of concentrations for these activities differed by greater than 3 orders of magnitude (from IC₅₀ = 14 nM for mGlu3 receptors to 22 μ M for mGlu4). Nevertheless, LY341495 can be used to block all eight mGlu receptors subtypes by adding a sufficient concentration (~100 μ M). For example, in the work of Fitzjohn et al. (1998), we used this feature of LY341495 to study the role of mGlu receptors per se in hippocampal synaptic plasticity.

As LY341495 was active at <1 μ M against functional agonist responses in certain group III receptor-expressing cell lines, we have also recently characterized [³H]LY341495 binding to cells expressing certain group III mGlu receptors (Wright et al., 2000). Here we found that [³H]LY341495 could be used to label mGlu6, mGlu7, and mGlu8 receptor subtypes. However, we noted that the affinities of [³H]LY341495 for these sites was 10- to 100-fold lower than what we observed for mGlu2 and mGlu3 receptors. Nevertheless, [³H]LY341495 was a useful ligand for studying the pharmacology of these recombinant receptors in cell lines. Thus, in the rat brain, [³H]LY341495 may label these other receptors in part. The present study in rat brain tissue, which presumably expresses all mGlu subtypes to various degrees, was designed to investigate the mGlu receptor population(s), which [³H]LY341495 might label. The data presented are consistent with the notion that, at least under the conditions used in this study, [³H]LY341495 labels group II (mGlu2 and/or mGlu3 receptors). This conclusion is based on observations discussed below.

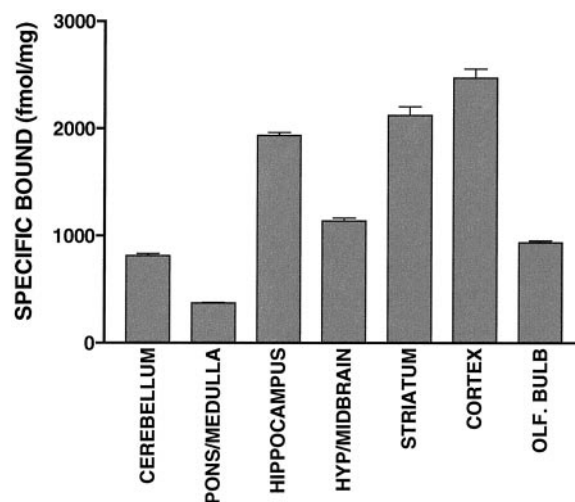


Fig. 7. Regional distribution of [³H]LY341495 binding in adult rat brain. Brains from three different rats were dissected into seven major regions by the method of Glowinski and Iverson (1966) and processed separately as described under *Experimental Procedures*. Assays were performed as described in the legend to Fig. 2 and under *Experimental Procedures*. Data represent mean and standard error of three different rat brains. Each data point was determined in triplicate. OLF. BULB, olfactory bulb.

[³H]LY341495 labeled a single population of sites with a very high affinity, comparable to mGlu2 and mGlu3 (although more like mGlu3, see below for additional discussion on this point). Using a range of structurally different mGlu receptor active agents (agonists and antagonists), a highly significant correlation was obtained between rat tissue and mGlu2 or mGlu3 receptor sites. In contrast, binding displacements in rat tissue by these agents clearly did not predict binding to mGlu8 receptors. Compounds of note were (RS)PPG and L-AP4, which are both agonists with nanomolar affinity for the mGlu8 receptor, but required high concentrations (micromolar to millimolar) to displace [³H]LY341495 binding to rat brain tissue. Hill coefficients, obtained with a wide range of pharmacological agents, including these two compounds, were ~0.8 to 1.0 (Table 1) and gave no indication for the displacement of multiple receptor populations.

Previously, we have characterized quisqualate-insensitive 1S,3R-ACPD-sensitive [³H]glutamate binding to rat brain tissue and have concluded that our conditions for this assay selectively label group II mGlu receptors (Schoepp and True, 1992; Wright et al., 1994). In fact, this was the assay where LY341495 was first noted as an extremely potent mGlu active agent (Ornstein et al., 1998b,c). We show here that the ionic requirements, expression level, regional distribution, and pharmacology of [³H]LY341495 binding to rat brain tissue very closely resembles that of 1S,3R-ACPD-sensitive [³H]glutamate binding (Wright et al., 1994). For example, like 1S,3R-ACPD-sensitive [³H]glutamate binding, [³H]LY341495 binding to rat brain was enhanced by bromide, chloride, and nitrate more than iodide, thiocyanate, acetate, and fluoride. Use of potassium phosphate buffer (10 mM) alone, resulted in very little binding for both ligands. The gross regional distribution of [³H]LY341495 binding agrees well with that of the previous studies using 1S,3R-ACPD-sensitive [³H]glutamate binding (Wright et al., 1994) with the higher levels of binding in the cerebral cortex, hippocampus, and striatum. As expected, the affinity of [³H]LY341495 in rat brain shown here is much

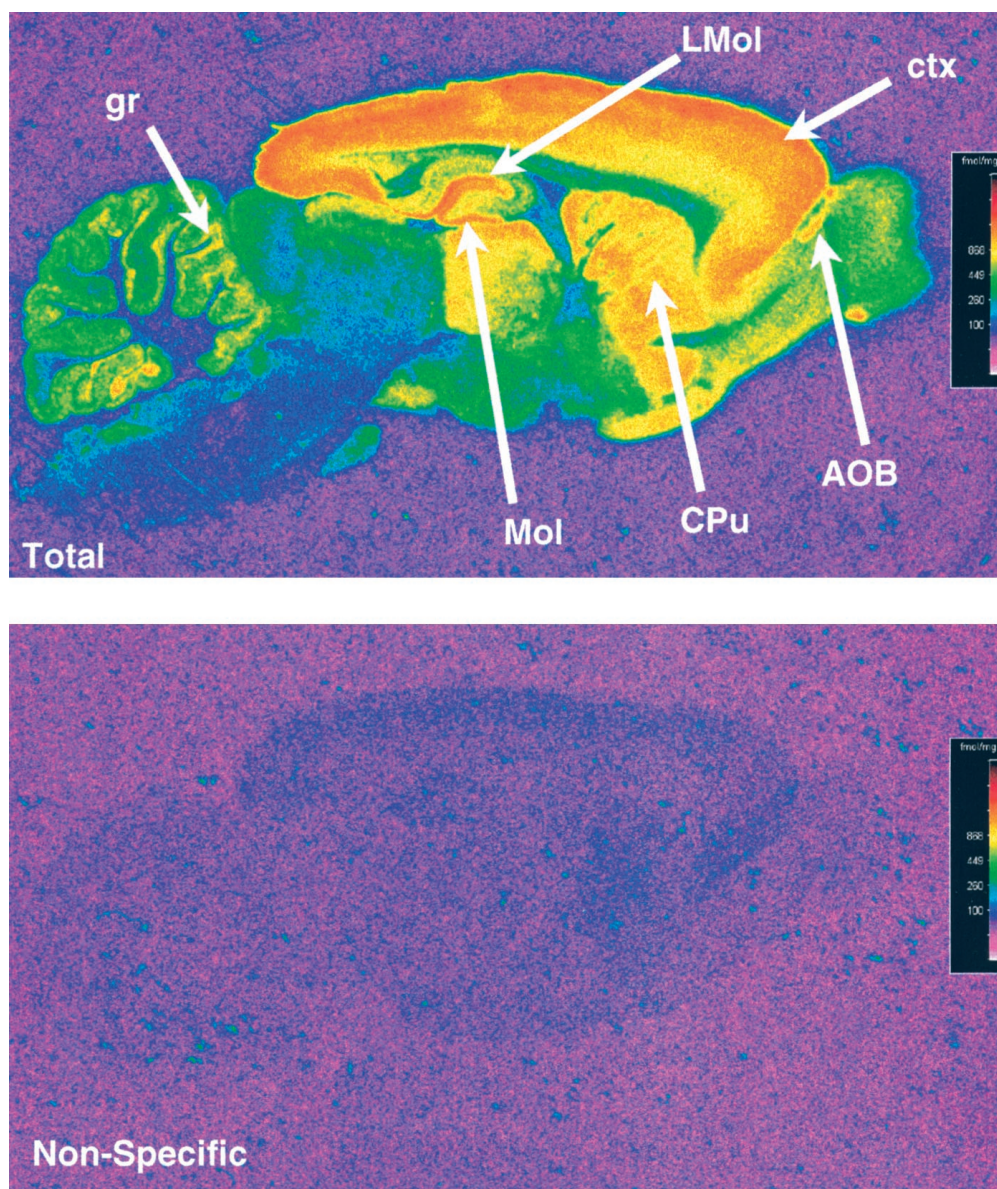


Fig. 8. Distribution of [³H]LY341495 binding to rat brain using autoradiography. Upper panel is a representative rat showing total binding with 5 nM [³H]LY341495. Lower panel is a representative rat showing nonspecific binding with 5 nM [³H]LY341495 in an adjacent section in the presence of 1 mM L-glutamate. Regions denoted for reference are Frontal cortex (ctx), lacunosum moleculare of the hippocampus (LMol), molecular layer of the dentate gyrus (Mol), caudate/putamen (CPu), accessory olfactory bulb (AOB), and granule cell layer of the cerebellum (gr).

higher than that observed for group II binding with [³H]glutamate ($K_d = 187$ nM). However, the apparent level of expression of the receptors in the adult rat forebrain membranes by the two methods were similar ($B_{max} = 3.9 \pm 0.65$ pmol/mg and 2.5 ± 0.27 pmol/mg for [³H]glutamate and [³H]LY341495, respectively).

Using quantitative autoradiography, we examined in further detail [³H]LY341495 binding distributions in the rat brain. A very distinct pattern of binding was noted, which compares very well with immunocytochemical labeling studies using antibodies against mGlu2/3 receptors (Petralia et al., 1996; Shigemoto et al., 1997) and for the binding of the mGlu2/3 agonist [³H]LY354740 (Schaffhauser et al., 1998). For example, within the hippocampal formation, we noted intense [³H]LY341495 binding to the lacunosum moleculare and the molecular layer of the dentate gyrus. The cerebral cortex, particularly layers I and II, also expressed high levels of [³H]LY341495 binding, along with the caudate-putamen and accessory olfactory bulb.

Interestingly, in the case of [³H]LY354740, the authors

hypothesized that this ligand may be selectively labeling mGlu2 (over mGlu3) receptors in the rat brain (Schaffhauser et al., 1998). This was based on the observation that [³H]LY354740 did not label white matter, despite evidence for mGlu3 transcript there. Furthermore, the neuropeptide NAAG has been reported to have selectivity for mGlu3 over mGlu2 in functional assays using cloned rat mGlu receptors (Wroblewska et al., 1997), and this compound exhibited a bi-phasic displacement of [³H]LY354740 binding. Lastly, LY354740 is somewhat more potent in functional assays for mGlu2 versus mGlu3, and thus it might be labeling mGlu2 preferentially (Schoepp et al., 1997). In contrast to these observations for [³H]LY354740, our data suggest that the antagonist radioligand [³H]LY341495 labels both mGlu2 and mGlu3 sites, but that mGlu3 receptor sites may predominate in forebrain homogenate membranes. This is based on the observation of highest pharmacological correlation with [³H]LY341495 rat brain binding to mGlu3 binding, kinetic constants (K_i , on rate, off rate) being most similar to mGlu3, and displacement of a single site with the compound NAAG.

TABLE 3
Specific binding of [³H]LY341495 to multiple regions of the rat brain using quantitative autoradiography

Rat Brain Region	Mean ± S.E. (n = Five Rats)
	<i>fmol/mg</i>
Amygdala	761.5 ± 6.3
Globus pallidus	279.3 ± 30.2
Lateral geniculate nuclei	706.1 ± 24.4
Access. olfactory bulb	1254.1 ± 45.9
Anterior olfactory nucleus	785.8 ± 29.4
Olfactory tubercle	1027.4 ± 39.8
Frontal ctx, layers I&II	1456.2 ± 37.3
Frontal ctx, layer III	1331.6 ± 69.1
Frontal ctx, layers IV&V	1029.9 ± 78.3
Occipital ctx, layers I&II	1532.6 ± 41.2
Occipital ctx, layer III	1269.4 ± 69.2
Occipital ctx, layers IV&V	1011.2 ± 38.1
Retrosplenial ctx	1498.9 ± 62.8
Presubiculum	1225.4 ± 39.5
Subiculum	664.1 ± 37.9
Lacunus moleculare hippo.	1345.4 ± 78.4
Oriens, hippocampus	590.8 ± 25.4
Pyramidal layer, hippocampus	509.2 ± 32.7
Stratum radiatum, hippocampus	564.7 ± 22.6
Dentate gyrus, granular	613.4 ± 14.3
Dentate gyrus, molecular	1223.6 ± 54.5
Caudate/putamen	1025.6 ± 34.0
Nucleus accumbens	1078.0 ± 22.1
Lateral dorsal/lateral posterior thalamus nuclei	860.4 ± 50.5
Ventral thalamus nuclei	521.0 ± 15.2
Substantia nigra	573.6 ± 39.9
Inferior colliculus	297.7 ± 16.6
Superior colliculus	601.7 ± 28.4
Deep mesencephalic nuclei	212.1 ± 39.0
Cerebellar granular layer	759.0 ± 17.8
Cerebellar molecular layer	438.0 ± 20.9
Interposed cerebellar nuclei	53.1 ± 0.0

Displacement of NAAG with an affinity ($K_i = 38 \mu\text{M}$) is consistent with its reported activity at mGlu3 receptors in functional assays ($\text{EC}_{50} = 65 \mu\text{M}$) (Wroblewska et al., 1997). As [³H]LY341495 also has a very high affinity for mGlu2 (as well as mGlu3), we cannot rule out binding to mGlu2 in certain brain areas and subregions. Future studies with newer agents and possibly transgenic animals may be useful in addressing this question. Also, it might be possible to find conditions for the labeling of endogenous group III receptors with this compound. In any case, [³H]LY341495 binding under the conditions described here appears useful for the study of endogenous group II mGlu receptors in the rat brain.

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