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In Vitro Binding Characteristics of a New Selective Group II Metabotropic Glutamate Receptor Radioligand, [^{3}H]LY354740, in Rat Brain

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ABSTRACT

The *in vitro* binding of [^{3}H]LY354740, the first high affinity group II-selective metabotropic glutamate (mGlu) receptor radioligand, was characterized in rat cortical, hippocampal, and thalamic membranes as well as in rat brain sections. [^{3}H]LY354740 binding was saturable in all regions investigated. Nonspecific binding (in the presence of 10 μM DCG-IV) was ≈8% of the total. Ionotropic glutamate receptor agonists, *N*-methyl-D-aspartate, (*R*,*S*)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid/kainate, a Na^+-dependent glutamate uptake blocker as well as a group I-selective mGlu receptor agonist (all up to 1 mM) did not inhibit [^{3}H]LY354740 binding to cortical membranes. However, several known metabotropic receptor ligands inhibited the binding with the following rank order of potency: LY354740 = LY341495 > (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine = (2*S*,1′*S*,2′*S*)-2-(2-carboxycyclopropyl)glycine > glutamate = (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid > (2*S*,1′*S*,2′*S*)-2-methyl-2-(2-carboxycyclopropyl)-glycine > quisqualate > ibotenate > L-2-amino-3-phosphonopropionic acid = (*S*)-α-methyl-4-carboxyphenylglycine > L-(+)-2-amino-4-phosphonobutyric acid. *N*-Acetyl-aspartyl-glutamate, (2*S*)-α-ethylglutamic acid, and (*R*,*S*)-α-methyl-4-phosphonophenylglycine inhibited [^{3}H]LY354740 binding in a biphasic manner. Guanosine-5′-*O*-(3-thiotriphosphate concentration-dependently and almost completely inhibited the binding. Finally, in parasagittal sections of rat brain, a high density of specific binding was observed in the accessory olfactory bulb, cortical regions (layers 1–3 > 4–6), caudate putamen, molecular layers of the hippocampus and dentate gyrus, presubiculum, retrosplenial cortex, anteroventral thalamic nuclei, and cerebellar granular layer, reflecting its preferential (perhaps not exclusive) affinity for presynaptic and postsynaptic mGlu2 receptors. Thus, the pharmacology, tissue distribution, and sensitivity to guanosine-5′-*O*-(3-thiotriphosphate show that [^{3}H]LY354740 binding probably occurs to group II mGlu receptors in rat brain.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and plays a role in integrative brain function, development of the nervous system, and neuronal cell survival and death (Choi and Rothman, 1990; Watkins *et al.*, 1990; Nakanishi, 1992, 1994). Two distinct families of mammalian glutamate receptors are known (Nakanishi, 1994): ionotropic glutamate receptors, which are glutamate-gated cation channels, and mGlu receptors, which are G protein-coupled receptors that modulate the production of intracellular second messengers (Pin and Duvoisin, 1995). Eight different mGlu receptors have been cloned, and they are classified into three groups on the basis of their sequence homology, signal transduction mechanism, and agonist selectivity (Nakanishi, 1992; Pin and Duvoisin, 1995). Group I (mGlu1 and mGlu5) stimulate phosphatidylinositol hydrolysis when expressed in mammalian cells and *Xenopus laevis* oocytes (Abe *et al.*, 1992; Aramori and Nakanishi, 1992) and are activated selectively by 3,5-dihydroxyphenylglycine (Ito *et al.*, 1992; Schoepp *et al.*, 1994). Group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8) receptors are negatively coupled to cAMP production when expressed in mammalian cells. Group III receptors are activated by L-AP4 (Tanabe *et al.*, 1993; Duvoisin *et al.*, 1995; Saugstad *et al.*, 1997), whereas group II receptors are insensitive to this compound (Thomsen *et al.*, 1992; Nakajima *et al.*, 1993; Tanabe *et al.*, 1993; Okamoto *et al.*, 1994; Saugstad *et al.*, 1994). *In situ* hybridization histochemical and immunohistochemical studies of rat brain (Tanabe *et al.*,

ABBREVIATIONS: mGlu, metabotropic glutamate; LCCG-I, (2*S*,1′*S*,2′*S*)-2-(2-carboxycyclopropyl)glycine; (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; MCPG, (*S*)-α-methyl-4-carboxyphenylglycine; EGLU, (2*S*)-α-ethylglutamic acid; L-AP4, L-(+)2-amino-4-phosphonobutyric acid; MPPG, (*R*,*S*)-α-methyl-4-phosphonophenylglycine; AMPA, (*R*,*S*)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; *S*-DHPG, (*S*)-3,5-dihydroxyphenylglycine; MCCG, (2*S*,1′*S*,2′*S*)-2-methyl-2-(2-carboxycyclo-propyl)-glycine; LTHA, L-(−)-*threo*-3-hydroxy-aspartic acid; NAAG, *N*-acetyl-aspartyl-glutamate; NMDA, *N*-methyl-D-aspartate; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate; DCG-IV, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine; L-AP3, L-2-amino-3-phosphonopropionic acid.

1992, 1993; Ohishi *et al.*, 1993a, 1993b; Catania *et al.*, 1994b; Ohishi *et al.*, 1994; Testa *et al.*, 1994; Neki *et al.*, 1996; Petralia *et al.*, 1996) have revealed that although mGlu2 receptors essentially are localized to the main and accessory olfactory bulb, cerebral cortices, striatum, molecular layers of the hippocampus and dentate gyrus, certain thalamic nuclei, and cerebellar Golgi neurons, mGlu3 receptors (albeit localized with an antibody recognizing both mGlu2 and mGlu3) apparently are distributed more widely in forebrain neurons and glia [e.g., in cerebral cortex (particularly layer 3)], striatum, reticular thalamic nucleus, dentate gyrus granule cells, and white matter (e.g., corpus callosum, fimbria, internal capsule fibers), where they are expressed in oligodendrocytes. There have been few meaningful studies with receptor radioautography (Catania *et al.*, 1994b) because [^{3}H]glutamate, a nonselective radioligand, has been used.

Recently, a new selective agonist, LY354740 [(+)-2-aminobicyclo-[3.1.0]hexane-2,6-dicarboxylate], was described to inhibit potently forskolin-stimulated cAMP formation in cells expressing human mGlu2 and mGlu3 or in cortical slices, without acting on group I and III mGlu receptors (Schoepp *et al.*, 1997).

In the current study, we characterized the binding of [^{3}H]LY354740 to cortical, hippocampal, and thalamic rat brain membranes as well as to rat brain sections.

Materials and Methods

Male SPF Füllinsdorf albino rats (weight, 120–200 g) were obtained from Biological Research Laboratories (Füllinsdorf, Switzerland). LCCG-I, (1*S*,3*R*)-ACPD, MCPG, EGLU, MPPG, AMPA, S-DHPG, L-AP4, MCCG, LTHA, NAAG, and kainate were obtained from Tocris Cookson (Bristol, UK). Quisqualate, L-AP3, glutamate, and ibotenate were purchased from RBI (Zurich, Switzerland). NMDA and GTPγS were from Sigma (Buchs, Switzerland). [^{3}H]LY354740 (specific activity, 35 Ci/mmol) was synthesized by Drs. H. Stadler and P. Huguenin (Chemical and Isotope Laboratories, F. Hoffmann-La Roche, Basel, Switzerland). LY354740, DCG-IV, and LY341495 [2-amino-2-(2-carboxycyclopropan-1-yl)-3-(dibenzopyran-4-yl)propanoic acid], as a mixture of four diastereoisomers, were synthesized by R. Jakob-Røtne, J. Wichmann, and T. Woltering (F. Hoffmann-La Roche), respectively.

[^{3}H]LY354740 Binding to Rat Brain Membranes

Membrane preparation. Halothane-anesthetized rats were killed by decapitation, and the cerebral cortex, hippocampus, and thalamus were dissected on ice. The different regions were homogenized in 25 volumes of 50 mM Tris·HCl (w/v), pH 7.1, with a polytron (14,000 rpm; Kinematica AG, Littau, Switzerland) for 20 sec. The homogenates were centrifuged at 48,000 × *g* for 10 min, the supernatant was discarded, and the pellet was homogenized under the same conditions as above. The final homogenate was incubated at 37° for 10 min and centrifuged as described above. The pellet was resuspended with the polytron, and the homogenate was frozen at −80°.

[^{3}H]LY354740 binding experiments. The membranes were thawed and washed three times in assay buffer containing 50 mM Tris·HCl and 2 mM $MgCl_2$, pH 7.4, by centrifugation at 48,000 × *g* and resuspended. After the last centrifugation, the pellet was resuspended in assay buffer with use of the polytron and added to a Beckman Instruments (Palo Alto, CA) plate containing [^{3}H]LY354740, buffer, and test compounds in a volume of 1 ml (final protein concentration, 0.25 mg/assay). The time course for association was estimated by the addition of [^{3}H]LY354740 (3 nM) to the membranes at different times 0–100 min before filtration. The time course of dissociation was measured by the addition of 10 μM LY354740 at different times before filtration to membranes previously incubated for 1 hr with 3 nM [^{3}H]LY354740. For saturation experiments, final concentrations of 0.3–300 nM [^{3}H]LY354740 were incubated with the membranes for 1 hr at room temperature. For competition experiments, 3 nM [^{3}H]LY354740 was incubated for 1 hr at room temperature in the presence of different concentrations of compounds. The plates were filtered onto glass-fiber filters (Unifilter-96 GF/B plate; Packard, Zürich, Switzerland) with a 96-well plate filtration unit (Filtermate 196; Packard). The filters were washed five times with cold assay buffer. Then, 40 μl of Microscint 40 (Packard) was added, and a Top Count counter (Packard) was used. Nonspecific binding was defined in presence of 10 μM DCG-IV. Protein concentration was measured according to the method of Pierce (Socochim, Lausanne, Switzerland) using bovine serum albumin as standard.

Data analysis. The inhibition curves were fitted with a four-parameter logistic equation that provided IC_{50} values and Hill coefficients through the use of Deltagraph (Deltapoint, Monterey, CA). Saturation experiments were analyzed with iterative nonlinear curve-fitting software (EBDA-LIGAND, G. A. McPherson, Elsevier-Biosoft, Cambridge, UK). The analysis of the NAAG, EGLU, and MPPG inhibition curves was carried out using Grafit (Erithacus Software, Staines, UK). The statistical significance of the difference between a one- and a two-site curve-fitting model was evaluated using a *F* test. The experiments were all repeated three times in triplicate.

[^{3}H]LY354740 Binding to Tissue Sections

Tissue preparation. Rat brain was dissected rapidly from halothane-anesthetized albino Füllinsdorf SPF rats (weight, ≈120 g) and frozen immediately in dry ice. Parasagittal cryostat sections (≈20 μm thick) were mounted on precleaned slides and stored at −20° until use.

***In vitro* binding assay.** Equilibrium was reached within 60 min at 4°, and the optimal rinse time (producing the maximal relative specific binding) was twice for 30 sec plus 1 min at 4°. In all binding experiments, four tissue sections per parameter were used.

For regional distribution studies, sections were preincubated at room temperature (22°; twice for 10 min) in 50 mM Tris·HCl buffer, pH 7.0, 2 mM $MgCl_2$, and 2 mM $CaCl_2$ (final volume, 130 ml) and then incubated with 50 nM [^{3}H]LY354740 in the same volume of buffer (containing $MgCl_2$ and $CaCl_2$) for 60 min at 22°. This was followed by three washes at 4° (twice for 30 sec plus 1 min) in 130 ml of buffer alone; nonspecific binding was determined in the presence of 10 μM DCG-IV. Sections were exposed, with tritium microscales, to Tritium Ultrofilm (Amersham International, Buckinghamshire, UK) for 1 week. Quantitative radioautography was carried out densitometrically using an MCID M2 image analyzer (Imaging Research, St. Catherine's, Ontario, Canada).

Results

Specific [^{3}H]LY354740 binding to rat brain membranes was linear with a tissue concentration of 0.025–0.25 mg of protein (data not shown). Treatment of the membranes with EDTA before the experiment, in absence of added ions, totally prevented [^{3}H]LY354740 specific binding. $CaCl_2$ or $MgCl_2$, both at 2 mM, induced an enhancement of specific [^{3}H]LY354740 binding to cortical membranes not treated with EDTA by ≈2- and ≈3-fold, respectively. The combination of $CaCl_2$ and $MgCl_2$ did not increase further the specific binding, and NaCl (120 mM) did not affect the [^{3}H]LY354740 specific binding. Finally, the stimulations obtained with 2 mM $MgSO_4$ and $MgCl_2$ were equal.

Specific [^{3}H]LY354740 binding was the highest at 25°.

Slightly less specific binding was observed at 4°, and a decrease of ≤50% was observed at 37° in all areas. Finally, specific [^{3}H]LY354740 binding did not differ at pH 6–8; however, it was reduced by 80% at pH 10.

With 3 nM [^{3}H]LY354740, 20% of the maximal binding at equilibrium was bound to cortical membranes within <1 min, and the equilibrium was achieved within ≈60 min (Fig. 1). The dissociation was almost complete by 20 min after the addition of an excess of LY354740.

Saturation analysis was carried out with cortical, hippocampal, and thalamic membranes. The different curves were fitted with a one- or two-site model using the computer program LIGAND. In thalamus and hippocampus, a one-site model statistically fitted the curves better than a two-site model (three and five experiments for thalamus and hippocampus, respectively). The calculated K_D and B_{max} values were 8 ± 1.3 nM and 500 ± 100 fmol/mg of protein, respectively, in hippocampus, and 10 ± 1 nM and 250 ± 89 fmol/mg of protein in thalamus. In cortex, 7 of 10 saturations were better fitted statistically with a two-site than with a one-site model. For these 7 saturations, the mean K_D values of the high and low affinity sites were equal to 5 ± 0.7 and 60 ± 27 nM with B_{max} values of 659 ± 60 and 550 ± 100 fmol/mg of protein, respectively. Fig. 2 shows a saturation isotherm obtained in cortical membranes. With 3 nM [^{3}H]LY354740, in the presence of 10 μM DCG-IV the nonspecific binding did not exceed 8 ± 1% of total.

Ionotropic glutamate receptor agonists NMDA, AMPA, and kainate; the Na^+-dependent glutamate transporter blocker LTHA; and the selective group I mGlu receptor agonist S-DHPG displaced <25% of [^{3}H]LY354740 binding at 1 mM. However, several metabotropic receptor ligands concentration-dependently inhibited the binding to cortical membranes with the following rank order of potency: LY354740 = LY341495 > DCG-IV = LCCG-I > glutamate = (1*S*,3*R*)-ACPD > MCCG > quisqualate > ibotenate > L-AP3 = MCPG > L-AP4 (Fig. 3 and Table 1). Maximal inhibition (≈92%) was obtained with all these compounds; IC_{50} values were between 9 nM and 350 μM, and Hill coefficients were 0.8–1.15 (Table 1). In contrast, the inhibition curves of NAAG, EGLU, and MPPG were biphasic with 40% of high affinity sites. For these three compounds, a two-site model fitted the curve statistically better than a one-site model ($p < 0.05$, F test), and the calculated IC_{50} values obtained were 0.34 ± 0.05 and 245 ± 9 μM for NAAG, 0.06 ± 0.01 and 42 ± 10 μM for EGLU, and 0.3 ± 0.1 and 45 ± 10 μM for MPPG for the high and low affinity components, respectively. In addition, the NAAG inhibition curve was biphasic in the hip-

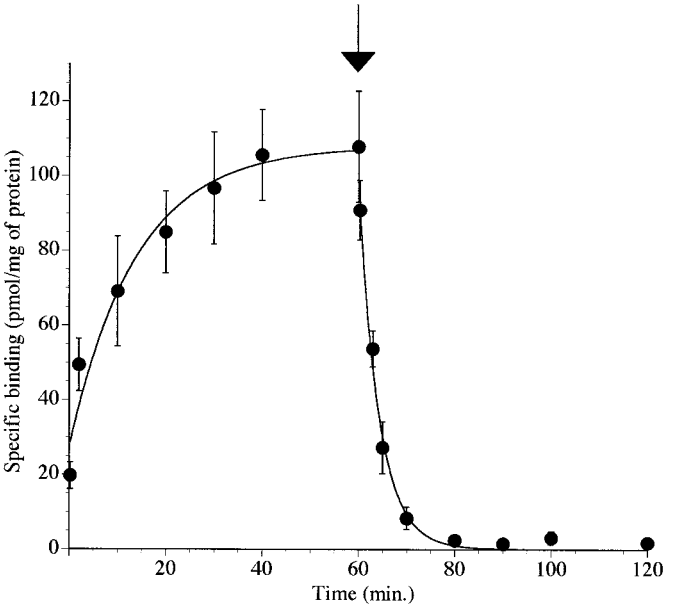

Fig. 1. Time course of association and dissociation of 3 nM [^{3}H]LY354740 to rat cortical membranes. Association was initiated by the addition of [^{3}H]LY354740 to membranes at different time before filtration. *Arrow*, dissociation experiments were performed by the addition of 10 μM DCG-IV at different times before filtration to membranes preincubated for 1 hr with 3 nM [^{3}H]LY354740. Results are shown as specific binding and are the mean ± standard deviation of three experiments performed in triplicate.

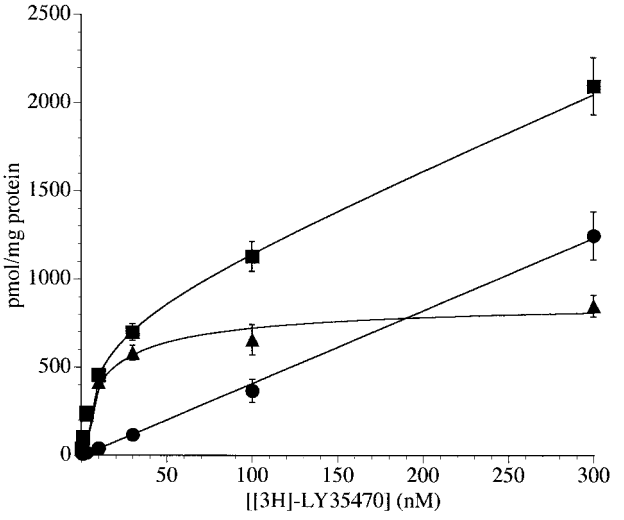

Fig. 2. Saturation isotherm of [^{3}H]LY354740 binding to rat cortical membranes. Specific binding (▲) was obtained by subtracting, at each point, the nonspecific binding (●) from the total binding (■). Each *point* represents the mean ± standard deviation of three separate determinations performed in triplicate.

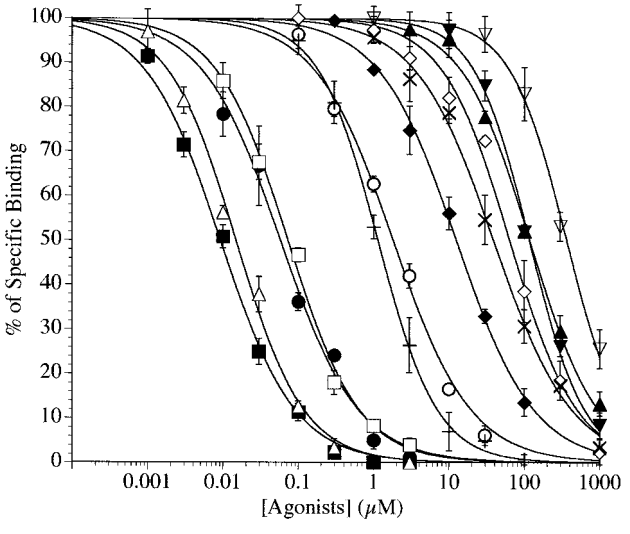

Fig. 3. Inhibition of [^{3}H]LY354740 binding to rat cortical membranes by varying concentrations of LY354740 (■), LY341495 (△), DCG-IV (●), LCCG-I (□), glutamate (+), (1*S*,3*R*)-ACPD (○), MCCG (♦), quisqualate (×), Ibotenate (◇), L-AP3 (▼), MCPG (▲), and L-AP4 (▽). Values are presented as percent of specific binding and represent mean ± standard deviation of three independent experiments performed in triplicate.

TABLE 1

Affinities of various ligand on adult rat cortical membranes

Binding assay was performed as described in the text. IC_{50} and Hill values (mean ± standard error, three experiments) were determined from inhibition curves with [3H]LY354740.

Compound	IC_{50}	Hill number
	μM	
LY354740	0.009 ± 0.0002	0.88 ± 0.02
LY341495	0.01 ± 0.002	0.98 ± 0.05
DCG-IV	0.06 ± 0.007	0.85 ± 0.1
LCCG-I	0.07 ± 0.003	0.94 ± 0.03
Glutamate	1.1 ± 0.1	1 ± 0.07
(1*S*,3*R*)-ACPD	2 ± 0.16	0.85 ± 0.02
MCCG	12 ± 0.5	0.85 ± 0.05
Quisqualate	40 ± 3	0.83 ± 0.01
Ibotenate	65 ± 5	0.96 ± 0.03
L-AP3	120 ± 1.5	1.13 ± 0.06
MCPG	120 ± 13	0.95 ± 0.03
L-AP4	350 ± 10	1.05 ± 0.12

pocampal and thalamic membranes ($p < 0.05$, *F* test), but the proportion of high affinity sites, which was similar in cortical (Fig. 4) and hippocampal membranes (40–50%) (not shown), was higher in thalamic membranes (70%). The IC_{50} values for the high and low affinity components were 0.2 ± 0.04 and 100 ± 2 μM for thalamus (Fig. 4) and 0.2 ± 0.04 and 240 ± 30 μM for hippocampus (not shown). Finally, the nonhydrolyzable analogue of GTP, GTPγS, inhibited [3H]LY354740 binding concentration-dependently to a maximum of 80% in brain membranes (see Fig. 4). The IC_{50} values calculated were 50 ± 9, 70 ± 7, and 40 ± 6 nM in cortex, hippocampus, and thalamus, respectively.

In brain sections (Fig. 5a and Table 2), radioautography and image analysis indicated a high density of specific binding in the accessory olfactory bulb, cerebral cortex (layers 1–3 > 4–6, with extremely high levels of binding in the retrosplenial cortex), caudate putamen, dorsomedial anteroventral thalamic nuclei, lacunosum moleculare layer of the hippocampus and molecular layer of the dentate gyrus, subiculum, presubiculum, and cerebellar granular layer. In these regions, binding occurred to cell processes (dendrites and axons) rather than to perikarya. No detectable binding to white matter areas (corpus callosum, internal capsule fibers, fimbria) was observed. Nonspecific binding (determined in the presence of 10 μM DCG-IV; Fig. 5b) was ≈10% of total.

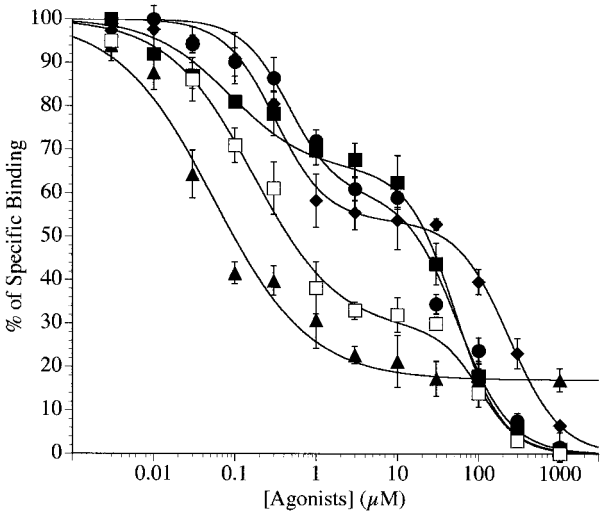

Fig. 4. Inhibition of [3H]LY354740 binding to rat brain cortex membranes by varying concentrations of NAAG (♦), MPPG (●), EGLU (■), and GTPγS (▲) and to rat brain thalamus membranes by NAAG (□). Values are presented as percent of specific binding and represent mean ± standard deviation of three independent experiments performed in triplicate.

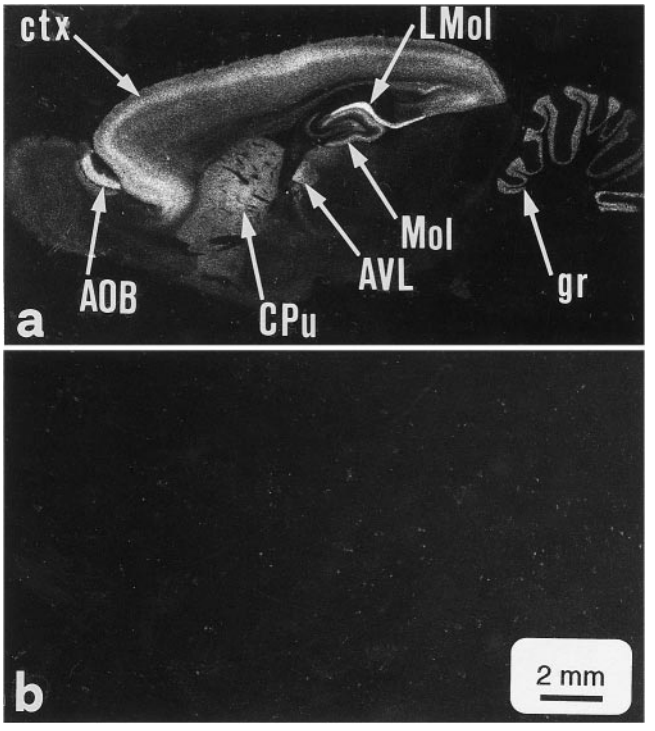

Fig. 5. *In vitro* binding of [3H]LY354740 to adjacent rat brain parasagittal sections (a, total binding; b, nonspecific binding in the presence of 10 μM DCG-IV). Note the high density of binding in cerebral cortex (*ctx*), accessory olfactory bulb (*AOB*), caudate putamen (*Cpu*), molecular layer of dentate gyrus (*Mol*), lacunosum moleculare of hippocampus (*LMol*), dorsomedial anteroventral thalamic nucleus (*AVL*), presubiculum, retrosplenial cortex, and cerebellar granular layer (*gr*) but the virtual absence of binding to white matter regions. *Scale bar*, 2 μm.

TABLE 2

Total *in vitro* binding values (~90% specific) for [3H]LY354740 (50 nM) to several brain regions measured densitometrically by quantitative radioautography and image analysis

Values are mean ± standard deviation.

Brain regions	Total binding
	fmol/mg of protein
Accessory olfactory bulb	3541 ± 574
Cerebral cortex layer I	2123 ± 270
Cerebral cortex layer II	2814 ± 350
Cerebral cortex layer III	3848 ± 373
Cerebral cortex layers IV–VI	2249 ± 255
Retrosplenial cortex	3183 ± 656
Subiculum	3688 ± 204
Presubiculum	3686 ± 384
Lacunosum moleculare hippocampus	5235 ± 200
Dentate gyrus granular layer	3008 ± 325
Caudate putamen	3180 ± 347
Anterodorsal thalamic nucleus	1824 ± 287
Other thalamic nuclei	598 ± 47
Cerebellar granular layer	2226 ± 250

Discussion

The only available radioligands for the metabotropic receptors have been of low affinity, selectivity, or both (e.g., [3H]glutamate, [3H]*trans*-ACPD, [3H]L-AP4). Nevertheless, some of them have been used to characterize cloned and/or

native mGlu receptors (Nicoletti *et al.*, 1988; Schoepp *et al.*, 1992; Catania *et al.*, 1994a; Eriksen and Thomsen, 1995; Laurie *et al.*, 1995). Because LY354740 was shown recently to be a highly selective and potent group II mGlu receptor agonist (Schoepp *et al.*, 1997), this molecule was radiolabeled and its binding properties were characterized in rat brain tissue.

As expected for the binding of an agonist to a G protein-coupled receptor, the specific binding of [^{3}H]LY354740 was absolutely dependent on the addition of divalent cations (Gilman, 1987). Chloride ions did not influence the specific binding, showing that unlike the $CaCl_2$-dependent [^{3}H]glutamate binding, [^{3}H]LY354740 binding did not involve the Cl^--dependent glutamate uptake (Pin *et al.*, 1984). A potent Na^+-dependent glutamate uptake blocker, LTHA (Robinson *et al.*, 1993), at a concentration of 1 mM, was unable to inhibit the binding. This, together with the lack of effect of NaCl on the binding, showed that in contrast to [^{3}H]glutamate, [^{3}H]LY354740 did not interact with Cl^-- and Na^+-dependent uptake sites (Baudry and Lynch, 1981; Fagg *et al.*, 1983; Pin *et al.*, 1984). The association curve showed that [^{3}H]LY354740 binding to rat brain cortex membranes was rapid, and equilibrium was achieved in <1 hr. The dissociation experiment showed a complete displacement of [^{3}H]LY354740 within ≈20 min The saturation analysis revealed the presence of single binding sites in thalamus and hippocampus with a similar K_D value of ≈10 nM. In the cortex, however, the compound apparently bound with equal proportion to two sites: one with a K_D value of 5 nM and one with a lower affinity (60 nM). This compares relatively well with the EC_{50} values reported for LY354740 for inhibition of forskolin-stimulated cAMP production in mGlu2- and mGlu3-transfected cells (5 and 24 nM, respectively) (Schoepp *et al.*, 1997), although the difficulty to separate accurately these two sites with close affinities may account for the increased variability of the K_D and B_{max} values for the low affinity site. The differences in B_{max} values observed between the different areas (cortex > hippocampus ≫ thalamus) may reflect their relative immunoreactivities with the use of a mGlu2/3 receptor antibody (Petralia *et al.*, 1996).

The specificity of the [^{3}H]LY354740 binding was assessed using the glutamate ionotropic receptor agonists NMDA, AMPA, and kainate, all of which were devoid of affinity up to 1 mM. LY354740 has been reported to be inactive on transfected group I mGlu receptors (Schoepp *et al.*, 1997). In agreement, S-DHPG, a selective group I agonist (Schoepp *et al.*, 1994), was unable to inhibit [^{3}H]LY354740 binding up to a concentration of 1 mM. The most potent inhibitors of the binding were LY354740 (Schoepp *et al.*, 1997) itself and the antagonist LY341495 (Ornstein *et al.*, 1996), and their binding affinities correspond well with their reported activities in functional assays performed on cloned mGlu receptors. L-AP4, which has been reported to be inactive on group II cloned receptors (Pin and Duvoisin, 1995), inhibited the binding, although with a low potency.

The inhibition induced by NAAG (Wroblewska *et al.*, 1997), EGLU (Jane *et al.*, 1996), and MPPG (Jane *et al.*, 1995) in the cortical membranes indicates again that [^{3}H]LY354740 bound to two populations of receptors and that these three compounds had differential affinities for these populations; the use of these compounds may help to characterize the individual populations. Interestingly, NAAG has been claimed to be a selective agonist for mGlu3, in comparison with mGlu2, receptors expressed in Chinese hamster ovary cells (Wroblewska *et al.*, 1997), but the selectivity of EGLU and MPPG has not been described. In our model, NAAG inhibited [^{3}H]LY354740 binding biphasically with a high affinity component, probably reflecting binding to mGlu3 receptors, and a lower affinity component, possibly relating to interaction with another receptor, such as mGlu2. This would reflect a preferential affinity of NAAG for mGlu3 but not an absolute selectivity. Quisqualate, which was described to be inactive on cloned mGlu2 and to be an agonist on mGlu3 receptors, with an EC_{50} value of 40 μM (Pin and Duvoisin, 1995), did not seem to inhibit partially the [^{3}H]LY354740 binding.

Finally, in agreement with the results found for many agonists binding to G protein-coupled receptors (Gilman, 1987), GTPγS inhibited in a concentration-dependent manner [^{3}H]LY354740 binding in all three areas with high potency. This result is in contrast with the moderate or lack of effect of GTPγS described by several authors for the binding of [^{3}H]glutamate on rat brain as well as mGlu3-transfected cell membranes (Catania *et al.*, 1994a, Laurie *et al.*, 1995).

The expression of mGlu2 and mGlu3 receptors has been described in studies using antibodies recognizing mGlu2 (Neki *et al.*, 1996) or mGlu2/3 (Ohishi *et al.*, 1994; Petralia *et al.*, 1996). Unequivocal evidence for the selective distribution of mGlu3 receptors is lacking in the literature. The distribution of binding sites for [^{3}H]LY354740 in parasagittal sections of rat brain generally correlated with that of mGlu2 transcripts and protein studied with the use of hybridization histochemistry (Tanabe *et al.*, 1992; Ohishi *et al.*, 1993a; Catania *et al.*, 1994b; Testa *et al.*, 1994) and immunohistochemistry (Ohishi *et al.*, 1994; Neki *et al.*, 1996; Petralia *et al.*, 1996) (as well as with recent unpublished findings [Richards JG] on the regional stimulation of GTPγ^{35}S binding by LY354740, revealed by radioautography).

For example, in the olfactory regions, the high density of binding sites corresponds with the reported strong hybridization signal and immunoreactivity for mGlu2 receptors in mitral and granule cells of this region. mGlu3 receptors generally are considered to be weakly expressed in the main and accessory olfactory bulb. In cortical regions, the laminar distribution of high density binding in most cortical regions (1–3 > 4–6) correlates as well with the distribution of mGlu2 receptor transcripts and protein. mGlu3 transcripts (Ohishi *et al.*, 1993b) have a much broader distribution, probably reflecting a high proportion of glial expression as revealed by mGlu2/3 antibodies (Petralia *et al.*, 1996). By far the highest density of binding in cortical regions was found in the retrosplenial cortex, which corresponds with the reported strong hybridization signal for mGlu2 receptors in entorhinal cortices (Catania *et al.*, 1994b). The lacunosum moleculare of the hippocampus, as well as the subiculum and presubiculum, also contained high densities of binding sites corresponding to immunohistochemical evidence for presynaptic mGlu2 receptors on axons of the perforant path projecting from the entorhinal cortex (i.e., their sites of synthesis). Finally, in the cerebellum, the Golgi cells of the granular layer have been shown to be the sites of both synthesis and expression of mGlu2 receptors. Therefore, the presence of a high density of binding sites in the granular layer seems to correspond with

the location of mGlu2 receptors on cell bodies, dendrites, and axon terminals of Golgi neurons.

The strong labeling of the caudate putamen matches its mGlu2 and mGlu2/3 immunoreactivity. The absence of mGlu2 mRNA in this brain region suggests that the binding may occur to presynaptic mGlu2 receptors as well as to postsynaptic and glial mGlu3 receptors. Although we cannot exclude the binding of [^{3}H]LY354740 to mGlu3 receptors in tissue sections of other brain regions, the radioautographic evidence suggests its affinity for mGlu2 receptors. The lack of binding to white matter regions (e.g., corpus callosum, internal capsule fibers, fimbria), despite evidence for the abundance of mGlu3 transcripts (and virtual lack of mGlu2 transcripts) in these regions, indicates a low level of mGlu3 protein beyond the limits of detection by radioautography. This, together with the reported lower affinity of this ligand for the mGlu3 receptor, may contribute to the overall lack of detectable signal. It also is possible to that under the conditions used for the radioautography, this ligand does not recognize mGlu3 receptors.

In conclusion, we have shown that in rat brain membranes, [^{3}H]LY354740 binds in a saturable manner, with a high affinity and specificity, to G protein-coupled receptors with a pharmacology of group II mGlu receptors. NAAG, EGLU, and MPPG inhibited the binding in a biphasic manner that indicates [^{3}H]LY354740 binds to at least two populations of receptors that may correspond to mGlu2 and mGlu3 on the basis of the reported affinity of LY354740 for these cloned receptors (Schoepp *et al.*, 1997).

The distribution of [^{3}H]LY354740 binding observed radioautographically indicates clearly its affinity for at least presynaptic and postsynaptic mGlu2 receptors. However, no definitive conclusions regarding the labeling of mGlu3 receptors can be drawn, and additional experiments are needed to clarify this issue.

Despite this, due to its high affinity and selectivity, [^{3}H]LY354740 seems to be the most suitable ligand currently available for the study of group II receptors in rat brain.

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