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Autoradiographic visualization of group III metabotropic glutamate receptors using [^{3}H]-L-2-amino-4-phosphonobutyrate

Camilla Hudtloff & [1]Christian Thomsen

Novo Nordisk A/S, Department of Molecular Pharmacology, Novo Nordisk Park, Denmark

1 *In vitro* receptor autoradiography using [^{3}H]-L-2-amino-4-phosphonobutyrate ([^{3}H]-L-AP4) binding to sections of rat brain has been characterized and shown to most likely represent labelling of group III metabotropic glutamate receptors.

2 Specific [^{3}H]-L-AP4 binding to rat brain sections was observed at high densities in the molecular layer of the cerebellar cortex and the outer layer of the superior colliculus. Moderate levels were observed throughout the cerebral cortex, in the molecular layer of the hippocampal dentate gyrus, in thalamus, striatum, substantia nigra and in the medial geniculate nucleus. Low levels of [^{3}H]-L-AP4 binding were found in other regions of the hippocampal formation, in the entorhinal cortex and the granule cell layer of cerebellum.

3 Inhibitors of sodium- or calcium/chloride-dependent glutamate uptake did not displace [^{3}H]-L-AP4 binding to rat brain sections indicating that the observed binding does not represent [^{3}H]-L-AP4 uptake via these carriers. Furthermore, in contrast to [^{3}H]-L-AP4 uptake into cerebellar membranes, [^{3}H]-L-AP4 binding to brain sections was sensitive to guanosine-5′-O-(3-thio)trisphosphate-γ-S.

4 In the molecular layer of the cerebellar cortex, [^{3}H]-L-AP4 binding showed a maximal binding density (B_{max}) of 0.52 ± 0.06 pmol mg^{-1} tissue and an affinity (K_d) of 346 nM. The rank order of affinity for displacement of [^{3}H]-L-AP4 binding to rat brain sections was: L-AP4 > L-serine-O-phosphate > glutamate > (L)-2-aminomethyl-4-phosphonobutanoate > (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylate which is in agreement with a group III metabotropic glutamate receptor pharmacology.

Keywords: Metabotropic glutamate receptor; [^{3}H]-L-2-amino-4-phosphonobutyrate; L-AP4 receptor; receptor autoradiography; guanine nucleotide shift

Introduction

L-2-Amino-4-phosphonobutyrate (L-AP4) is a phosphonic analogue of the major excitatory amino acid, glutamate. Unlike L-glutamate, L-AP4 does generally not lead to excitation of glutamatergic pathways but is often associated with depression of synaptic transmission via a presynaptic mechanism (Evans *et al*., 1982; Lanthorn *et al*., 1984; Forsythe & Clements, 1990; Thomsen, 1997). The observed depression of synaptic transmission in hippocampal and retinal glutamatergic pathways leads to the proposal of a novel glutamate receptor, termed the L-AP4 receptor (Koerner & Cotman, 1981; Shiells *et al*., 1981). Furthermore, the existence of multiple L-AP4 receptor subtypes has been proposed since synaptic transmission via the lateral perforant path is depressed by low micromolar concentrations of L-AP4 while 20–50 fold higher concentrations of L-AP4 are needed to produce these effects in the medial perforant path (Koerner & Cotman, 1981). Previously, [^{3}H]-AP4 has been used in attempts to define this L-AP4 receptor in receptor binding experiments (Butcher *et al*., 1983; Monaghan *et al*., 1983; Robinson *et al*., 1985). However, no correlation between the depressant actions of a number of L-AP4-analogues in glutamatergic pathways and the affinity for [^{3}H]-AP4 binding sites in rat brain membranes was observed (Fagg & Lanthorn, 1985; Robinson *et al*., 1985). Indeed, it is currently believed that [^{3}H]-AP4 'binding' to rat membranes represents Ca^{2+}/Cl^--dependent sequestration of glutamate into synaptic vesicles rather than labelling of the L-AP4 receptor (Monaghan *et al*., 1983; Zaczek *et al*., 1987).

More recently, it has been shown that L-AP4 depress monosynaptic transmission via a GTP-binding protein in cultured olfactory bulb neurones, suggesting that the L-AP4 receptor is a G-protein coupled receptor (Thrombley & Westbrook, 1992). With the cloning of eight subtypes of metabotropic glutamate receptors (mGluRs), the molecular basis for a G-protein coupled receptor which is activated by L-AP4 has been provided (Suzdak *et al*., 1994; Pin & Duvoisin, 1995). Currently, L-AP4-sensitive mGluRs includes mGluR4, mGluR6, mGluR7 and mGluR8, which are placed in the same group (termed group III mGluRs) based on their amino acid sequence homology, agonist pharmacology and similar coupling to inhibition of adenylate cyclase when expressed in mammalian cell lines (Pin & Duvoisin, 1995). The localization of mRNA or receptor protein of group III mGluRs in rat brain has been determined by *in situ* hybridization techniques and immunocytochemistry experiments which revealed a heterogeneous and often complementary distribution of L-AP4-sensitive receptor subtypes (Nakajima *et al*., 1993; Kristensen *et al*., 1993; Kinzie *et al*., 1995; Bradley *et al*., 1996; Kinoshita *et al*., 1996; Saugstad *et al*., 1997; Shigemoto *et al*., 1997). However, the overall relative density of high affinity L-AP4 receptors is not easily compared from these studies. In addition, the actual affinities of L-AP4 for group III mGluRs expressed *in situ* are difficult to predict from functional studies in cloned cell lines since these may be dependent on the nature and the efficiency of the functional coupling. Thus, in order to determine the relative distribution of high affinity L-AP4 receptors in rat brain, a [^{3}H]-L-AP4 receptor autoradiography technique was developed based on a previously described [^{3}H]-

[1] Author for correspondence at: Novo Nordisk A/S, Department of Molecular Pharmacology, Novo Nordisk Park, DK-2760 Måløv, Denmark.

L-AP4 receptor binding assay to membranes from baby hamster kidney (BHK) cells expressing mGluR4a (Eriksen & Thomsen, 1995).

Methods

Autoradiographic procedure using [^{3}H]-L-AP4

The brains from male Wistar rats (weighing 200–250 g) were quickly removed and immediately frozen in crushed dry ice. The frozen brain was mounted on a specimen stage with Tissue-Tek embedding matrix and 20 μm sections were cut throughout the brain in a cryostat (1720 Digital, Wild Leitz) at -20°C. Tissue sections were thaw-mounted on gelatine/chomalun coated slides and dried for 10 min (30°C) before being stored at -20°C (for less then 3 days). For autoradiographic experiments, sections of rat brain were thawed and prewashed for 20 min in an assay buffer composed of 10 mM HEPES, pH 8.0, 110 mM NaCl, 1.2 mM $MgCl_2$ and 0.3 mM PMSF in order to remove endogenous ligand. This assay buffer composition and pH has previously been shown to be optimal for measuring [^{3}H]-L-AP4 binding to BHK cells expressing mGluR4a (Eriksen & Thomsen, 1995). The incubation was performed in assay buffer at 25°C for 30 min with 30 nM [^{3}H]-L-AP4. Non-specific binding was defined as binding in the presence of 0.1 mM L-serine-O-phosphate (L-SOP). Test compounds were dissolved in assay buffer and pH adjusted to 8.0. Following incubation with [^{3}H]-L-AP4 the sections were quickly rinsed with 3×7 ml of assay buffer, and rapidly dried under a stream of air. The duration of the washing procedure was less than 10 s per slide. The dry, labelled tissue sections were exposed to tritium sensitive Hyperfilm (Amersham, Buckinghamshire, U.K.) together with two standards (^{3}H microscales, 5.0 and 50.0 nCi, Amersham, Buckinghamshire, U.K.) for 14 days at 5°C. The film was developed in Kodak D-19.

Materials

[^{3}H]-L-AP4 was obtained from Tocris Cookson (Bristol, U.K.) with a specific activity of 50–54 Ci mmol^{-1}. The compounds used in this study were purchased from Tocris Cookson, Research Biochemicals Int. (Natick, MA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.).

Data analysis

The autoradiographic images were digitized on an Eagle Eye II scanner (Stratagene, CA, U.S.A.) and analysed by use of Inquiry software, (Loats Associates Inc., Westminster, MD, U.S.A.) for binding densities. IC_{50} values were calculated by a non-linear regression analysis using the GraphPad Prism programme (GraphPad Software, San Diego, CA, U.S.A.). Inhibitory constants (K_i) were calculated from the IC_{50} values using the equation: $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of [^{3}H]-L-AP4. Statistical analysis was performed were appropriate with the InStat programme (GraphPad Software, San Diego, CA, U.S.A.).

Results

Since specific [^{3}H]-L-AP4 binding to rat brain section was readily washed off the sections it was essential to minimize the time for the washing procedure, since no specific binding was observed if the sections were washed for 1 min (data not shown). The optimal incubation temperature for [^{3}H]-L-AP4 autoradiography was about 25°C when examined at 0°C, 25°C and 37°C using 60 min of incubation (specific binding was 26 ± 9 fmol mg^{-1} tissue, 58 ± 8 fmol mg^{-1} tissue and $32 \pm$

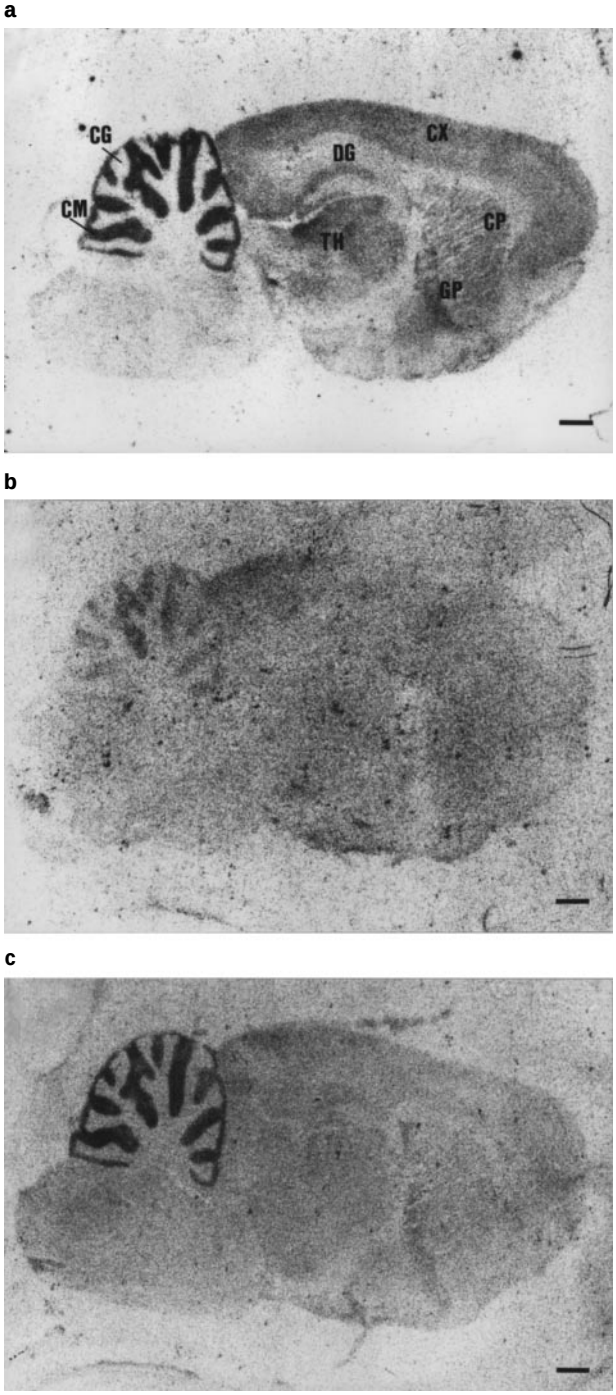

Figure 1 Autoradiographs of [^{3}H]-L-AP4 binding to parasagittal sections of rat brain. (a) Control, (b) in the presence of 0.1 mM L-SOP and (c) in the presence of 0.1 mM GTP-γ-S. L-SOP is used to define non-specific binding. At this concentration of [^{3}H]-L-AP4 (30 nM), very little specific binding was observed in the presence of 0.1 mM GTP-γ-S while only a 37% reduction in binding was observed in the molecular layer of the cerebellar cortex. See Figure 2 for a kinetic analysis of the GTP-γ-S sensitivity in the molecular layer of the cerebellar cortex. Abbreviations: CG (cerebellar cortex, granule cell layer); CM (cerebellar cortex, molecular layer); CP (caudate putamen); CX (cerebral cortex); DG (molecular layer of dentate gyrus); GP (globus pallidus); TH (thalamus). Calibration bar corresponds to 1.3 mm.

6 fmol mg^{-1} tissue, respectively in the molecular layer of the cerebellar cortex). Maximal specific [^{3}H]-L-AP4 binding was achieved after 30 min of incubation with the radioligand at 25°C (data not shown) and these conditions were used in subsequent experiments. As shown in Figure 1, [^{3}H]-L-AP4 binding to rat brain sections was effectively competed off by 0.1 mM L-SOP which displaced 50–65% of total binding. In the presence of 0.1 mM guanosine-5′-O-(3-thio)trisphosphate-γ-S (GTP-γ-S) [^{3}H]-L-AP4 binding was diminished, as shown in Figure 1c, suggesting that a G-protein coupled receptor is labelled. In most of the more weakly labelled brain regions very little specific binding was observed in the presence of GTP-γ-S but in the molecular layer of the cerebellar cortex 63 ± 3% specific [^{3}H]-L-AP4 binding remained in the presence of this nucleotide which allowed for a more detailed analysis. In the absence of GTP-γ-S, [^{3}H]-L-AP4 binding showed relatively low affinity a determined by saturation binding experiments (mean K_d was 346 nM and 95% confidence interval was 230–462 nM, $n=8$) and a limited number of an apparently single population of binding sites ($B_{max}=0.52\pm0.06$ pmol mg^{-1} tissue) in the molecular layer of the cerebellar cortex (Figure 2). In the presence of GTP-γ-S, B_{max} was significantly lower ($B_{max}=0.28\pm0.04$ pmol mg^{-1} tissue) when compared to binding in the absence of this GTP analogue ($P<0.05$, $n=5$, unpaired t test). The affinity of [^{3}H]-L-AP4 was not significantly altered in the presence of GTP-γ-S (mean K_d was 472 nM and 95% confidence interval was 297 ± 647 nM). When measuring [^{3}H]-L-AP4 binding to cerebellar membranes as described previously (Butcher *et al*., 1983; Monaghan *et al*., 1983) no effects of GTP-γ-S were observed ($K_d=4.3$ μM (2.6–6.0 μM); $B_{max}=109\pm31$ pmol mg^{-1} protein, $n=4$ and $K_d=4.0$ μM (2.4–5.6 μM); $B_{max}=100\pm26$ pmol mg^{-1} protein, $n=4$ in the absence and presence of 0.1 mM GTP-γ-S, respectively).

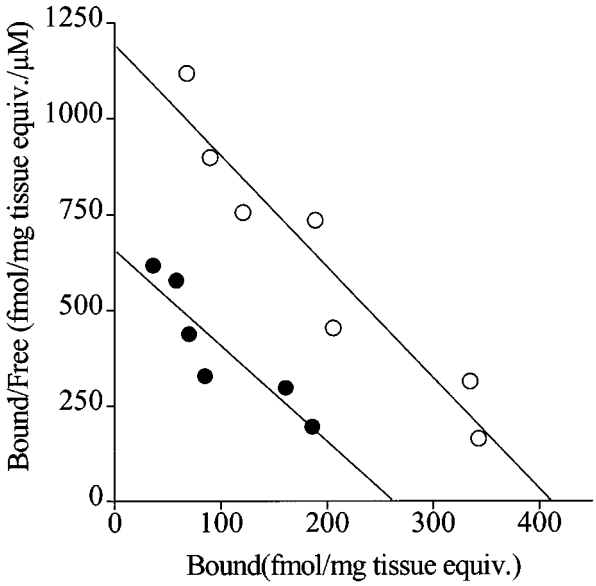

Figure 2 Scatchard transformations of saturation binding experiments with [^{3}H]-L-AP4 to brain sections from rat brain as measured in the molecular layer of the cerebellar cortex. The experiments were performed in the absence (open circles) or presence (solid circles) of 100 μM GTP-γ-S. [^{3}H]-L-AP4 (30 nM) was diluted with unlabelled L-AP4 at 7 concentrations ranging from 0.01 μM to 10 μM, and the experiments ($n=5-8$) were performed in triplicate as described in Methods. Non-specific binding was defined as the binding in the presence of 0.1 mM L-SOP.

In order to distinguish further [^{3}H]-L-AP4 binding to rat brain sections from the abundant [^{3}H]-L-AP4 uptake site present in membrane preparations, the pharmacology of [^{3}H]-L-AP4 autoradiography was characterized with compounds showing selectivity for group III mGluRs and for [^{3}H]-L-AP4 uptake, respectively. As shown in Table 1, ligands of group III mGluRs displaced [^{3}H]-L-AP4 binding to the molecular layer of the cerebellar cortex while no effects of inhibitors of Ca^{2+}/Cl^--dependent L-glutamate uptake (L-α-aminoadipate, 4-acetamido-4-isothiocyanatostilbene-2,2-disulphonic acid (SITS) and L-cystine) or Na^+-dependent glutamate uptake (L-(trans)-pyrrolidine-2,4-dicarboxylate) were observed. Furthermore, quisqualate is a potent inhibitor of [^{3}H]-L-AP4 uptake ($IC_{50}=0.5$ μM) (Butcher *et al*., 1983; Sheardown & Thomsen, 1996) and its activity at displacing [^{3}H]-L-AP4 binding to rat brain sections (38% at 300 μM) is therefore more in agreement with labelling of group III mGluRs ($EC_{50}=129$ μM) (Kristensen *et al*., 1993) than inhibition of [^{3}H]-L-AP4 uptake. Agonists of ionotropic glutamate receptors (AMPA, kainate and NMDA) and antagonists of group I and II mGluRs ((**S**)-4-carboxy-3-hydroxyphenylglycine and (+)-α-methyl-4-carboxyphenylglycine) were also ineffective displacers of [^{3}H]-L-AP4 binding (Table 1). The data in Table 1 represent displacement of [^{3}H]-L-AP4 binding from the molecular layer of the cerebellar cortex. However, the effects of the compounds listed in Table 1 on binding densities were also measured in the cerebral cortex, striatum, hippocampus dentate gyrus, nucleus accumbens and thalamus but no overt pharmacological differences were observed between these regions (data not shown). In the molecular layer of the cerebellar cortex, the rank order of potency for group III

Table 1 Pharmacology of [^{3}H]-L–AP4 binding to rat brain sections

	Inhibition of [^{3}H]-L-AP4 binding (%)	*Affinity for [^{3}H]-L-AP4 binding* ($-\log K_i$)
Group III mGluR agonists		
L-AP4 (0.1 mM)	93±3	6.52±0.10
L-SOP (0.1 mM)	100	5.74±0.06
Glutamate (0.3 mM)	81±8	5.15±0.13
(1**S**, 3**R**)-ACPD (1 mM)	62±5	3.64±0.04
mGluR antagonists		
L-MAP4 (1 mM)	81±4	4.30±0.15
(**S**)-4-Carboxy-3-hydroxyphenyl-glycine (1 mM)	11±7	
(**S**)-α-Methyl-4-carboxyphenyl-glycine (1 mM)	25±11	
Other glutamate receptor agonists		
AMPA (0.1 mM)	4±4	
Kainate (0.1 mM)	1±1	
NMDA (0.3 mM)	0±0	
Quisqualate (0.3 mM)	38±8	
Inhibitors of glutamate uptake		
L-α-Aminoadipate (0.1 mM)	10±1	
SITS (0.1 mM)	2±3	
L-Cystine (1 mM)	6±5	
L-(trans)-Pyrrolidine-2,4-dicarboxylate (1 mM)	0±0	

Affinities were calculated from competition binding experiments ($n=4$) as described in Methods and expressed as mean±s.e.mean of the $-\log_{10}$ value of the K_i. % inhibition of [^{3}H]-L-AP4 binding is expressed relative to the inhibition by 0.1 mM L-SOP at the concentrations indicated. In separate experiments full dose-response curves were generated for active compounds (see Figure 3).

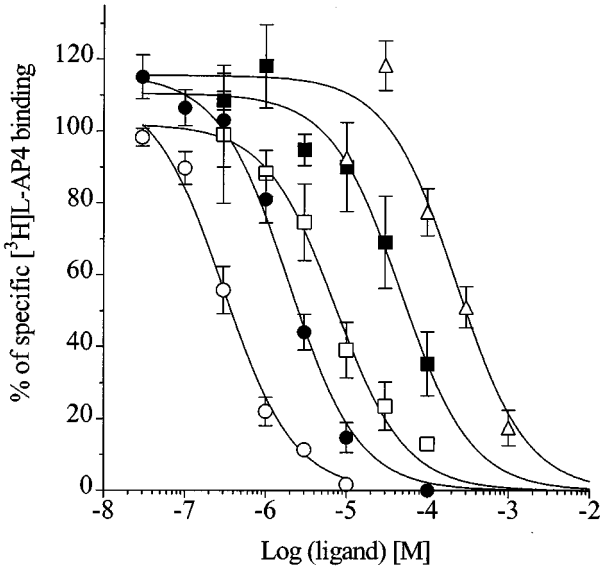

Figure 3 Displacement of [^{3}H]-L-AP4 binding to rat brain sections by L-AP4 (open circles), L-SOP (solid circles), glutamate (open squares), L-MAP4 (solid squares) and (1**S**,3**R**)-ACPD (open triangles). The data are expressed as % of specific [^{3}H]-L-AP4 binding as measured in the molecular cell layer of the cerebellar cortex and are mean of 4 experiments which were performed in duplicate; vertical lines show s.e.mean. Non-specific binding was defined in the presence of 0.1 mM L-SOP and accounted for 50–60% of total binding in these experiments. For further information see Table 2.

ligands at displacing [^{3}H]-L-AP4 binding was [K_i in parentheses): L-AP4 (0.3 μM) > L-SOP > (1.8 μM) glutamate > (L)-2-aminomethyl-4-phosphonobutanoic acid (L-MAP4) (50 μM) > (1**S**,3**R**)-1-aminocyclopentane-1,3-dicarboxylic acid ((1**S**,3**R**)-ACPD) (230 μM) > quisqualate (> 1 mM) in agreement with a group III mGluR pharmacology (Figure 3).

Since these data suggested that [^{3}H]-L-AP4 indeed label group III mGluRs, the distribution of [^{3}H]-L-AP4 binding was determined in more detail by using coronal sections cut throughout the rat brain (Figure 4). The densities of [^{3}H]-L-AP4 binding in selected brain regions are shown in Table 2. A moderate labelling (15–25 fmol mg^{-1} tissue) of most of the cerebral cortex was observed (Figure 4) but low levels (< 10 fmol mg^{-1} tissue) were found in the entorhinal cortex (Table 2). In caudate putamen, globus pallidus and olfactory tubercle there was a moderate degree of [^{3}H]-L-AP4 binding (Figure 1a and Table 2). High levels of [^{3}H]-L-AP4 binding were observed in the outer layer of the superior colliculus and moderate to high levels were found in the medial geniculate nucleus and substantia nigra (Figure 4). In the hippocampal formation a moderate level of [^{3}H]-L-AP4 binding was observed in the molecular layer of the dentate gyrus, whereas other regions in the hippocampus showed very little bound radioactivity (Figure 4). The highest levels of [^{3}H]-L-AP4 binding were observed in the molecular layer of the cerebellar cortex with a clear separation from the granule cell layer (Figure 4).

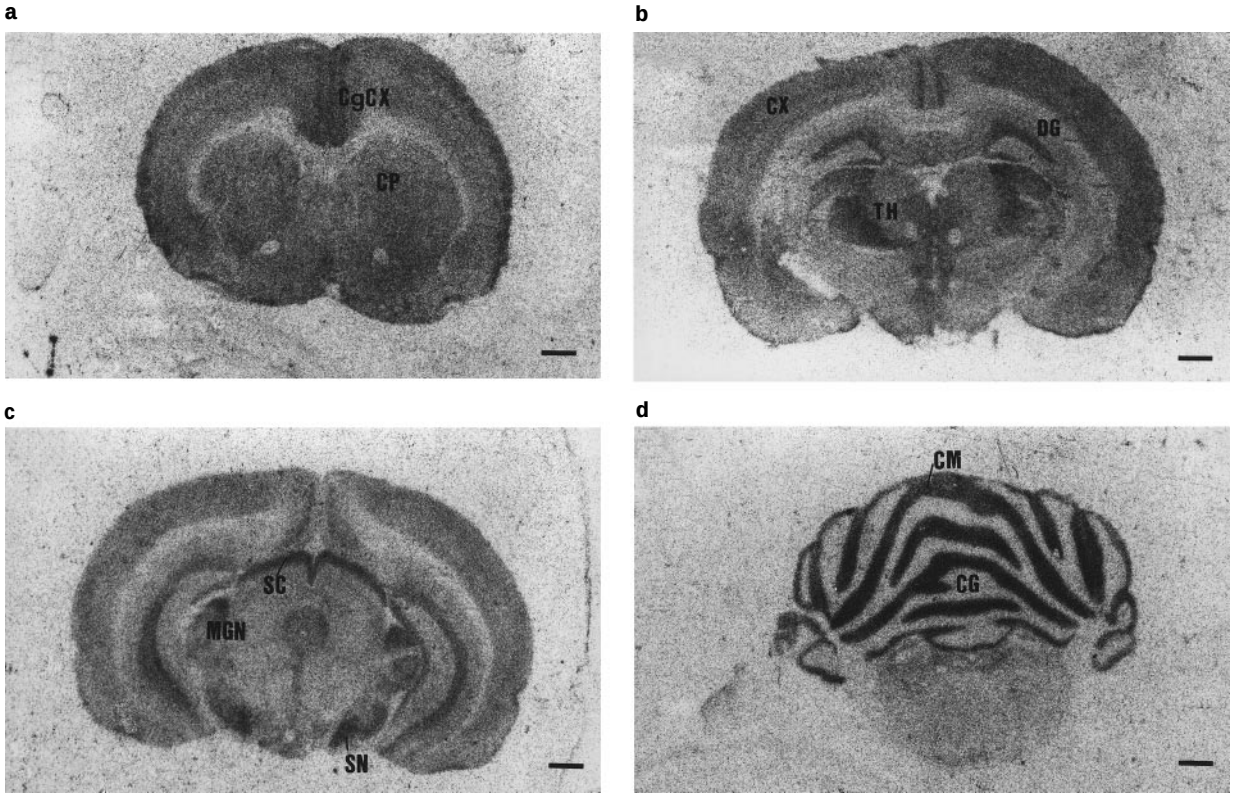

Figure 4 Autoradiographs of [^{3}H]-L-AP4 binding to coronal sections of rat brain. (a) Coronal section showing labelling in cingulate cortex (CgCX) and throughout caudate putamen (CP). (b) Coronal section showing labelling in the molecular layer of dentate gyrus (DG) of the hippocampal formation, throughout the cerebral cortex (CX) and in the thalamus (TH). (c) Coronal section showing labelling in the superior colliculus (SC), the medial geniculate nucleus (MGN) and in the substantia nigra (SN). (d) Coronal section showing labelling in the molecular layer of the cerebellar cortex (CM) but not in the granule cell layer (CG). Calibration bar corresponds to 1.3 mm.

Discussion

In order to label group III mGluRs in rat brain with [^{3}H]-L-AP4 it is important to ensure that the observed binding is not due to accumulation of isotope through the highly efficient uptake sites for glutamate and related analogues such as L-AP4, which are present in the brain (Lester *et al*., 1994). In contrast to [^{3}H]-L-AP4 uptake into rat brain membranes, which is absent at 0°C and optimal at 37°C (Butcher *et al*., 1983; Monaghan *et al*., 1983), specific [^{3}H]-L-AP4 binding to rat brain sections was measurable at 0°C and optimal at 25°C. Moreover, [^{3}H]-L-AP4 uptake sites were most abundant in membranes from the striatum, hippocampus and cerebral cortex and less prominent in cerebellar synaptosomes (Butcher *et al*., 1983), while [^{3}H]-L-AP4 binding to rat brain sections was predominant in the cerebellar cortex. However, the most obvious difference between the previously observed uptake site for L-AP4 and the autoradiographic representation of [^{3}H]-L-AP4 binding sites is with respect to their pharmacology. The rank order of potency for inhibition of L-AP4 uptake into rat brain membranes is: quisqualate = L-cystine > L-glutamate > L-AP4 > L-α-aminoadipate (Butcher *et al*., 1983; Kessler *et al*., 1987). In the present study, similar compounds showed a rank order of affinity of: L-AP4 > L-glutamate > > quisqualate > > L-cystine/L-α-aminoadipate. Furthermore, (**S**)-4-carboxy-3-hydroxyphenylglycine has been shown to be a very potent inhibitor of [^{3}H]-L-AP4-uptake into rat cortical membranes (Sheardown & Thomsen, 1996), but did not displace [^{3}H]-L-AP4 binding to rat brain sections in concentrations up to 1 mM. In contrast, the rank order of affinity of mGluR ligands for [^{3}H]-L-AP4 binding to rat brain sections was identical to that of [^{3}H]-L-AP4 binding to membranes from BHK cells expressing mGluR4a (L-AP4 > L-SOP > L-glutamate > L-MAP4 > (1**S**,3**R**)-ACPD > quisqualate) (Eriksen & Thomsen, 1995; Thomsen *et al*., 1997). Another distinction between [^{3}H]-L-AP4 uptake into brain membranes and [^{3}H]-L-AP4 autoradiography is that GTP-γ-S decreased binding to brain sections (Figure 2), but did not affect uptake kinetics (data not shown). Thus, the characteristics of [^{3}H]-L-AP4 binding as measured by *in vitro* receptor autoradiography is clearly different from the previously described uptake site measured in preparations of synaptosomes (Butcher *et al*., 1983; Monaghan *et al*., 1983; Fagg & Lanthorn, 1985; Robinson *et al*., 1985; Zaczek *et al*., 1987).

With the exception of the molecular layer of the cerebellar cortex virtually no specific binding was observed in most brain regions in the presence of GTP-γ-S, suggesting that the affinity of [^{3}H]-L-AP4 for its binding site was markedly decreased when uncoupled from the G-protein. This observation may suggest that the receptor labelled by [^{3}H]-L-AP4 in the molecular layer of the cerebellar cortex is of a different type from that labelled in other brain regions where the GTP-γ-S sensitivity is apparently higher. However, with the limited selectivity between individual mGluR subtypes of the group III mGluR ligands currently available, it is difficult to address the latter hypothesis. Thus, no obvious pharmacological difference were observed when [^{3}H]-L-AP4 binding was measured in other selected brain regions as compared to the molecular layer of the cerebellar cortex. The ability of GTP-γ-S to uncouple G-proteins from the receptors is believed to result in a shift of agonists to a lower affinity state, whereas the total number of receptors remains unchanged. Thus, in receptor binding experiments with a radiolabelled agonist to a G-protein coupled receptor GTP-γ-S will generally increase the dissociation constant (K_d) while leaving the B_{max} constant unchanged (Poyner, 1990). However, in the present study a significant decrease in B_{max} was observed, while K_d was not significantly increased in the presence of GTP-γ-S. The most parsimonious explanation for this observation may be related to methodological aspects rather than a reflection of complex receptor/G-protein interactions. Given the relatively low binding affinity of [^{3}H]-L-AP4 binding to rat brain sections ($K_d = 0.35$ μM) it was perhaps not unexpected that the isotope appeared to

Table 2 Regional distribution of [^{3}H]-L-AP4 binding to rat brain sections

Brain region	*Specific [^{3}H]L-AP4 binding* (fmol/mg^{-1} tissue)
Cerebral cortex	
Motor cortex	18±2
Sensory cortex	20±1
Pyriform cortex	17±2
Parietal cortex	16±2
Auditory cortex	17±1
Striate cortex 17	16±1
Striate cortex 18	15±2
Entorhinal cortex	8±2
Retrospenial cortex	14±1
Temporal cortex	16±1
Cingulate cortex	23±2
Limbic system	
Mammillary body	6±1
Nucleus accumbens	18±2
Olfactory tubercle	19±2
Hippocampus dentate, granule layer	12±2
Hippicampus dentate, mol. layer	20±3
Hippocampus CA1, pyramidal layer	5±2
Hippocampus CA3, pyramidal layer	6±2
Hippocampus CA4, pyramidal layer	6±2
Hippocampus, dendritic layer	8±3
Medial amygdala	10±2
Lateral amygdala	15±3
Caudate putamen	14±1
Globus pallidus	18±2
Thalamus	
Lateral habenula	9±3
Medial habenula	8±2
Medial geniculate	20±2
Lateral geniculate	13±2
Subthalamic nucleus	5±1
Monoaminergic nuclei	
Substantia nigra, pars reticular	18±2
Substantia nigra, pars compacta	9±2
Diencephalic structures	
Medial septum	10±1
Diagonal band of Broca	21±2
Corpus callosum	2±1
Lateral septum	4±2
Lateral hypothalamus	10±2
Medial hypothalamus	6±3
Superior colliculus, inner layer	16±2
Superior colliculus, outer layer	35±3
Brain stem	
Interpendicular nucleus	8±1
Ventral tegmental area	9±1
Vestibular nucleus	12±2
Cochelear nucleus	1±1
Cerebellum	
Granule cell layer	3±1
Molecular layer	54±6

The results are means±s.e.mean, $n = 6$, and represent binding densities (fmol mg^{-1} tissue equivilent) in different brain regions as determined by quantitative [^{3}H]-L-AP4 receptor autoradiography.

dissociate rapidly from its binding site (dissociation was too rapid to allow for actual kinetic experiments to be performed). Thus, it may be speculated that if GTP-γ-S indeed further decreased the affinity of [^{3}H]-L-AP4 for its recognition site (as suggested by the observed trend towards and increase in K_d) this population of receptors may not be detected with the current technique. If this is the case, the resulting saturation binding experiments would only reveal the population of receptors which remained in the high affinity G-protein coupled state and, thus, leading to an apparent decrease in B_{max}. Similar observations have been made with other G-protein coupled receptors that are negatively coupled to adenylate cyclase (e.g., the nociceptin receptor) (Fawzi *et al*., 1997).

The potency of L-AP4 to induce functional responses via mGluRs expressed in mammalian cell lines is: 0.5–1.2 μM (mGluR4), 0.9 μM (mGluR6), 160 μM (mGluR7), 0.7 μM (mGluR8) and in excess of 1 mM for mGluR1, mGluR2, mGluR3 and mGluR5 (Thomsen *et al*., 1992; Nakajima *et al*., 1993; Tanabe *et al*., 1993; Okamoto *et al*., 1994; Suzdak *et al., 1994; Saugstad et al*., 1997). Thus, among mGluRs subtypes mGluR4 and mGluR8 are most likely to contribute to [^{3}H]-L-AP4 binding in rat brain, since mGluR6 is believed to be located mainly in the retina (Nakajima *et al*., 1993). Though, it should be kept in mind that the affinity of L-AP4 for mGluRs may be somewhat different when the receptor is expressed in its natural environment (e.g., rat brain). Although somewhat unlikely, the possibility that mGluR7 may also be labelled by [^{3}H]-L-AP4 in the present set of experiments cannot be totally excluded. However, the most striking similarity between the localization of putative L-AP4 sensitive mGluRs as revealed by [^{3}H]-L-AP4 autoradiography and the distribution pattern of L-AP4-sensitive mGluR subtypes was found with mGluR4a. A predominant expression of mGluR4a mRNA has been observed in cerebellar granule cells (Kristensen *et al*., 1993; Tanabe *et al*., 1993) which projects to the molecular layer where high levels of [^{3}H]-L-AP4 binding were found. Moreover, a mGluR4a specific antibody showed intense staining in this rat brain region (Kinoshita *et al*., 1996). Thus, the high levels of [^{3}H]-L-AP4 binding observed in the molecular layer of the cerebellar cortex is very likely to represent expression of mGluR4. Furthermore, the moderate expression of mGluR4a mRNA in the thalamus, caudate putamen and medial geniculate nuclear (Kristensen *et al*., 1993) is in agreement with the observed distribution of [^{3}H]-L-AP4 binding. In the hippocampus, immunoreactivity for mGluR4a has been shown to be restricted to a modest labelling of the dentate gyrus (Shigemoto *et al*., 1997), which also corresponds well to the observed pattern of [^{3}H]-L-AP4 binding in the hippocampus. However, subtype mGluR8 is also expressed in the dentate gyrus but not in other hippocampal regions (Shigemoto *et al*., 1997). Thus, the observed distribution of [^{3}H]-L-AP4 binding primarily in the dentate gyrus of the hippocampus is in accordance with labelling of mGluR4a and mGluR8 but not of mGluR7, since this subtype is expressed throughout the hippocampus (Bradley *et al*., 1996; Shigemoto *et al*., 1997). On the other hand, mGluR7 mRNA (but not mGluR4a, mGluR6 or mGluR8 mRNA) is concentrated in the superior colliculus and globus pallidus (Okamoto *et al*., 1994; Kinzie *et al*., 1995) which may suggest that this subtype is also labelled by [^{3}H]-L-AP4. However, the possibility that alternative spliced variants of mGluR4 (i.e., mGluR4a and mGluR4b) are labelled with equally high affinity by [^{3}H]-L-AP4 (Thomsen *et al*., 1997) should be borne in mind, but the regional distribution of mGluR4b has not been characterized in the rat. For example, alternative spliced variants of other group III mGluRs have been shown to differ markedly in their distribution in the hippocampus (Shigemoto *et al*., 1997) and it is therefore not clear whether mGluR4b may be expressed at high levels in the superior colliculus and/or globus pallidus. Keeping in mind the fact that the potency of L-AP4 at mGluR7 is quite low (Okamoto *et al*., 1997), significant expression of mGluR4b is perhaps a more likely explanation for the high levels of [^{3}H]-L-AP4 binding in these regions.

In conclusion, high affinity L-AP4 binding sites have been demonstrated in brain regions such as the molecular layer of the dentate gyrus, olfactory tubercle, cerebellar cortex and thalamus in which L-AP4 blocks synaptic transmission via presynaptic mechanisms (for a review see Thomsen, 1997). The pharmacology of the L-AP4 binding site corresponds well to that of group III mGluRs, lending further support to the hypothesis that the L-AP4 receptor, as defined in electrophysiological experiments, is contained within the family of mGluRs.

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