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Characterization of the pharmacology and regional distribution of (S)-[^{3}H]-5-fluorowillardiine binding in rat brain

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1 This study examined the binding of the new radioligand (S)-[^{3}H]-5-fluorowillardiine to rat brain synaptic membranes. Specific binding represented greater than 80% of the total binding and was increased by 10% in the presence of 100 mM potassium thiocyanate (KSCN).

2 In the absence of KSCN, (S)-[^{3}H]-5-fluorowillardiine identified two binding sites with $K_{D1} = 22.5$ nM, $B_{max1} = 1.4$ pmol mg^{-1} protein and $K_{D2} = 1.5$ μM, $B_{max2} = 10.8$ pmol mg^{-1} protein. In the presence of 100 mM KSCN the affinities of both the binding sites were increased, yielding values of $K_{D1} = 6.9$ nM and $K_{D2} = 0.4$ μM KSCN was without effect on the B_{max} values.

3 (S)-[^{3}H]-5-fluorowillardiine binding was displaced by non-NMDA receptor ligands with the rank order of potency: 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX) > domoate > (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ≈ L-glutamate > 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) > kainate > > (R)-5-fluorowillardiine. In contrast, both N-methyl-D-aspartate (NMDA) and the metabotropic glutamate receptor agonist, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) were inactive.

4 By use of quantitative autoradiography the regional distribution of (S)-[^{3}H]-5-fluorowillardiine binding in rat brain was assessed. The highest levels of binding were in the dentate gyrus and the CA1 region of the hippocampus. Lower levels of binding were detected in the cerebral cortex, olfactory system, lateral septum, caudate putamen and nucleus accumbens.

5 We conclude that the pharmacological profile and regional distribution of (S)-[^{3}H]-5-fluorowillardiine binding is consistent with its specific interaction with AMPA receptors. The advantages of high percentage specific binding and recognition of the two binding sites, in the absence of KSCN, suggest that (S)-[^{3}H]-5-fluorowillardiine may be the ligand of choice for the future study of AMPA receptors.

Keywords: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor; (S)-[^{3}H]-5-fluorowillardiine; rat brain synaptic membranes; receptor autoradiography

Introduction

L-Glutamate exerts its excitatory effects in the mammalian CNS by the activation of at least three classes of glutamate receptor, namely the N-methyl-D-aspartate (NMDA), non-NMDA and metabotropic receptors. The non-NMDA receptor class is subdivided into the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors on the basis of their distinct pharmacology and regional distributions (Monaghan & Cotman, 1982; Monaghan *et al.*, 1984). Molecular biology provides further evidence for the existence of separate AMPA and kainate receptors and to date, nine subunits of the non-NMDA receptors have been cloned, namely GluR1–7 and KA-1 and KA-2 (Hollmann *et al.*, 1989; Keinänen *et al.*, 1990). Homomeric expression of GluR1–4 reveals that all four form functional receptors which display high nanomolar affinity for [^{3}H]-AMPA (Keinänen *et al.*, 1990). The remaining non-NMDA receptor subunits GluR5–GluR7 form channels selectively activated by kainate (Bettler *et al.*, 1990; 1992; Egebjerg *et al.*, 1991) whilst KA-1 and KA-2 display high nanomolar affinities for [^{3}H]-kainate but not [^{3}H]-AMPA (Herb *et al.*, 1992; Werner *et al.*, 1991).

Study of the AMPA receptor subtype has been advanced by the discovery of the potent and selective agonist, AMPA (Krogsgaard-Larsen *et al.*, 1980; 1982). However, more recently a series of structural analogues halogenated in the 5-position of the amino acid willardiine, 1-(2-amino-2-carboxyethyl)pyrimidine-2,4-dione, has been described (Patneau *et al.*, 1992; Wong *et al.*, 1994). In rat cultured hippocampal neurones (Patneau *et al.*, 1992) and motoneurones of the neonatal rat (Jane *et al.*, 1991) the rank order of potencies of this series is the reverse of that seen at the kainate receptors found in both cultured dorsal root ganglion (DRG) cells (Wong *et al.*, 1994) and dorsal root fibres (Blake *et al.*, 1991). The most potent and selective for the AMPA receptor is (S)-5-fluorowillardiine (Figure 1). Here we describe firstly the characterization of the binding of the newly synthesized radiolabelled form of this compound, (S)-[^{3}H]-5-fluorowillardiine, to rat brain synaptic membranes and secondly, we provide receptor autoradiographic data for the regional distribution of its binding sites in rat brain. A preliminary report of some of these results has been previously presented in abstract form (Hawkins *et al.*, 1994).

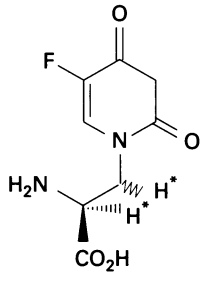

Figure 1 The structure of (S)-5-fluorowillardiine ((1'RS,2'S)-1-(2'-amino-2'-carboxyethyl)-5-fluoropyrimidine-2,4-dione). *Denotes the incorporation of ^{3}H at the 1' and 2' positions of the (S)-5-fluorowillardiine molecule.

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Methods

Synaptic membrane preparation

Male Wistar rats (250–300 g) were stunned and decapitated, whole brains minus cerebella were rapidly removed and homogenized in 10 ml of buffered 0.32 M sucrose (5 mM Tris-HCl pH 7.4 at 4°C) using 10 passes at 6000 r.p.m. of a tight fitting Teflon-glass homogenizer. All procedures were performed at 4°C unless otherwise indicated. The homogenate was centrifuged at 1000 *g* for 10 min and the pellet discarded. After centrifugation of the supernatant (17000 *g* for 20 min) the resulting pellet was resuspended in 5 mM Tris-HCl (pH 7.4 at 4°C) and lysed on ice for 15 min and spun at 17000 *g* for a further 20 min. The 'buffy coat layer' was collected and washed twice in 5 mM Tris-HCl (17000 *g* for 20 min) and finally washed a further four times in 50 mM Tris-HCl (pH 7.4 at 4°C) at 50,000 *g* for 10 min. The resulting pellet was resuspended in 20 ml of 50 mM Tris-HCl and stored at −70°C until used. Prior to the binding assays, membranes were thawed and resuspended in 20 ml of 50 mM Tris-HCl and centrifuged at 50,000 *g* for 10 min. This wash step was repeated twice. The final pellet was resuspended in assay buffer (50 mM Tris-HCl/100 mM KCl (pH 7.4 at 4°C)) and used immediately. In experiments to investigate binding under chaotropic conditions 100 mM KCl was replaced by 100 mM potassium thiocyanate (KSCN).

Saturation studies

The method used was that described by Morgan *et al.* (1991). Briefly, aliquots (300 µl) of the membrane suspension (100–150 µg protein) were incubated in the presence of 1 to 3000 nM (S)-[^{3}H]-5-fluorowillardiine for 40 min at 4°C. Concentrations of radioligand between 1 and 50 nM were achieved by increasing the concentration of radioligand, while those above 50 nM were produced by adding increasing concentrations of unlabelled ligand to a fixed concentration of radioligand (50 nM). Non-specific binding was defined in the presence of 1 mM L-glutamate. The experiment was terminated by centrifugation for 7 min at 13,000 *g* in a Heraeus Biofuge 13. The supernatant was removed by vacuum aspiration and the pellet superficially washed with two 1 ml aliquots of ice-cold assay buffer. The tip of the microfuge tube was removed and placed in 0.5 ml of 2% sodium dodecyl sulphate (SDS) solution overnight to solubilize the pellet. After the addition of 3 ml of scintillation fluid the radioactivity in each sample was determined in a Wallac 1409 scintillation counter. Protein concentrations were determined by a modified Lowry assay with bovine serum albumin as the standard.

Displacement studies

The ability of various compounds to displace (S)-[^{3}H]-5-fluorowillardiine binding (10 nM) was assessed by use of at least ten concentrations ranging between 0.01 nM to 1 mM. All concentration points were determined in quadruplicate. For [^{3}H]-kainate binding, the membranes were incubated for 60 min with 10 nM [^{3}H]-kainate in 50 mM Tris acetate buffer (pH 7.1 at 4°C). Non-specific binding was defined in the presence of 1 mM L-glutamate.

Receptor autoradiography

Male Wistar rats (250–300 g) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}) and perfused (intracardiac) with 280 ml phosphate buffered saline. The brains were removed and frozen in isopentane chilled with dry ice. Sections of the frozen brain (10 µm) were cut at −15°C and thaw-mounted onto gelatin-coated slides and stored at −70°C until used. All sections were thawed at room temperature prior to a 30 min preincubation in 50 mM Tris-HCl/100 mM KCl buffer (pH 7.4 at 4°C). Sections were dried under a stream of cool air. The sections were incubated in 200 µl of 50 mM Tris-HCl/100 mM KCl buffer containing either 10 nM (S)-[^{3}H]-5-fluorowillardiine or (S)-[^{3}H]-AMPA for 40 min at 4°C. Non-specific binding was defined in the presence of 1 mM L-glutamate. The ability of various compounds to displace (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding was assessed by use of at least five concentrations, each performed in triplicate. Unbound radioactivity was removed by three washes in ice cold buffer and finally in ice cold water; the total wash time was 8 s. Sections were then dried under a stream of cool air. The sections were exposed to ^{3}H-Hyperfilm (Amersham) for 4 weeks, alongside ^{3}H microscales (Amersham). The films were developed and the signal quantified by computer-assisted densitometry (Cambridge 970 System). For each region analysed, between two and four determinations were made from each section.

Data analysis

The data were analysed with the iterative non-linear regression programme GraphPAD Prism. All experiments were analysed

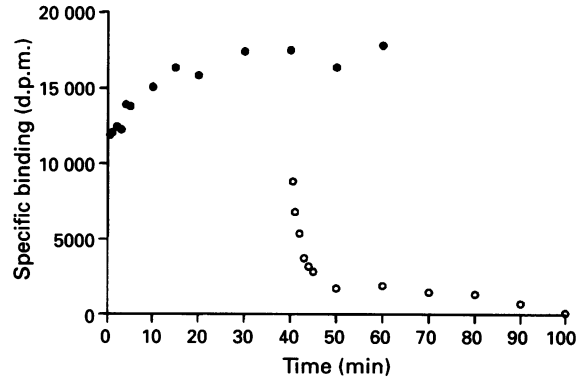

Figure 2 Time course of association (●) and dissociation (○) of the specific binding of (S)-[^{3}H]-5-fluorowillardiine (10 nM) to rat brain synaptic membranes. Binding shows rapid association and is readily reversible. Dissociation was initiated by the addition of 1 mM L-glutamate after 40 min. In the dissociation experiments non-specific binding represented the binding remaining after 120 min incubation in the presence of 1 mM L-glutamate. The data points represent the mean of quadruplicate determinations from a single experiment. The experiment was repeated twice with similar results.

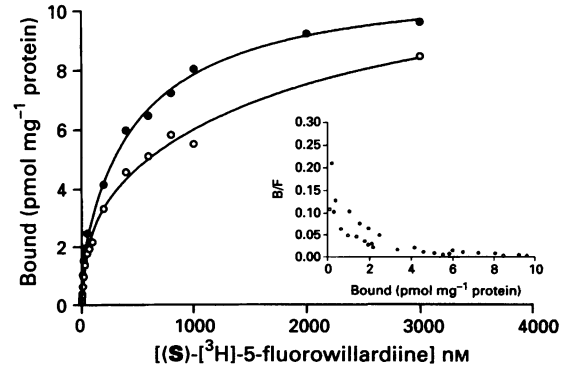

Figure 3 Saturation data for (S)-[^{3}H]-5-fluorowillardiine binding, to rat brain synaptic membranes, measured in either the presence (●) or absence (○) of 100 mM potassium thiocyanate. These data are from a single experiment which was repeated twice with similar results. The inset represents the Scatchard transformation of these data. Analysis of the saturation data by non-linear regression (GraphPAD Prism) identified two binding sites, the K_D and B_{max} values are shown in Table 1.

Table 1 The binding constants for (S)-[^{3}H]-5-fluorowillardiine binding to rat brain synaptic membranes in both the presence and absence of the chaotropic agent potassium thiocyanate

	K_D (nM)	B_{max} (pmol mg^{-1})	K_D (nM)	B_{max} (pmol mg^{-1})
Presence KSCN	6.97 ± 0.45*	1.19 ± 0.24	450 ± 20.0*	10.72 ± 3.04
Absence KSCN	22.56 ± 4.33	1.45 ± 0.40	1590 ± 290	10.86 ± 2.52

The results represent the mean ± s.e.mean of three experiments performed in quadruplicate. The values were then analysed using the Mann-Whitney U-test. *P = 0.05 compared to the value in the absence of KSCN.

independently, the IC_{50} values described representing the mean of at least three such experiments. For the one-site model the IC_{50} values were calculated from the following equation Y = A + (B-A)/1 + $10^{x-logIC_{50}}$ where A and B are the minimum and maximum percentage specific binding respectively. Similarly, for the two site model Y = A + (B-A)(Fraction 1/1 + $10^{x-logIC_{501}}$) + (1-Fraction 1/1 + $10^{x-logIC_{502}}$). The K_i values were calculated from the IC_{50} values using the Cheng-Prusoff equation. The F-test was used to assess whether the more complex two-site model was a significantly better ($P < 0.05$) fit than the simpler one-site model. When statistics were performed the Mann-Whitney U-Test was used with significance taken at the 5% level.

Materials

[^{3}H]-kainate (58 Ci mmol^{-1}) was obtained from Du Pont-NEN. (S)-[1′,2′-^{3}H]-5-fluorowillardiine (specific activity 44–46 Ci mmol^{-1}), (S)-[^{3}H]-AMPA (specific activity 40–45 Ci mmol^{-1}), (S)-AMPA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), domoate, (S)-5-fluorowillardiine, (R)-5-fluorowillardiine, 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2-3-dione (NBQX), N-methyl-D-aspartic acid (NMDA) and (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) were obtained from Tocris Cookson (Bristol, U.K.). L-Glutamate and kainate were from Sigma (U.K.). All compounds were dissolved directly in assay buffer, with the exception of NBQX and 5-fluorowillardiine which were first dissolved in a 10% excess of sodium hydroxide solution, and CNQX in dimethyl sulphoxide (DMSO), before diluting into buffer.

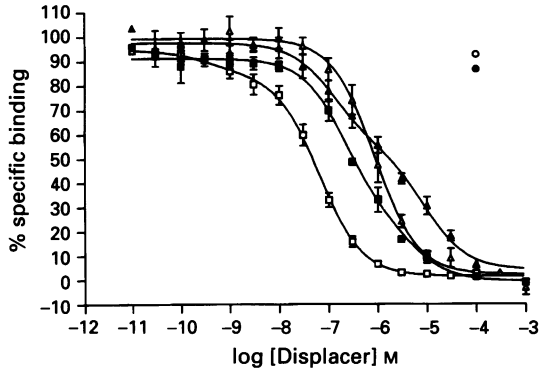

Figure 4 Displacement of (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding to rat brain synaptic membranes, in the absence of KSCN, by the non-NMDA receptor ligands NBQX (□), (S)-AMPA (■), CNQX (△) and domoate (▲). At concentrations up to 100 μM both NMDA (●) and (1S,3R)-ACPD (○) were inactive as displacers of (S)-[^{3}H]-5-fluorowillardiine binding. The data points represent the mean ± s.e.mean of three experiments each performed in quadruplicate. The K_i values were calculated using the Cheng-Prusoff equation (for details see data analysis) and are summarized in Table 2. For all curves, with the exception of CNQX, the two-site model provided a significantly better fit (F-test $P < 0.05$).

Results

(S)[^{3}H]-5-fluorowillardiine binding studies

From preliminary studies the optimal conditions for (S)-[^{3}H]-5-fluorowillardiine binding were established at 4°C and pH 7.4 (data not shown). Consequently, these conditions were used throughout this study. Previous studies have demonstrated that in the absence of KSCN [^{3}H]-AMPA binding is maximal on the addition of 100 mM KCl to the assay buffer (Morgan *et al.*, 1991). Consequently similar conditions were used in this study; however, the removal of 100 mM KCl from the assay buffer had no effect on (S)-[^{3}H]-5-fluorowillardiine binding (data not shown). The binding of (S)-[^{3}H]-5-fluorowillardiine (10 nM) in the absence of KSCN was rapid, equilibrium was reached within 30 min and remained stable for at least 1 h

Table 3 Summary of the IC_{50} values of the non-NMDA receptor ligands displacing [^{3}H]-kainate (10 nM) binding

Compound	*Site 1 IC_{50}* (nM)	*Site 2 IC_{50}* (nM)
Domoate	1.5 ± 0.4 (38)	55.0 ± 20.9
(S)-5-fluorowillardiine	275.6 ± 123.1 (43)	34,200 ± 6,900
(S)-AMPA	18,400 ± 1500	/

The values represent the mean ± s.e.mean from at least three experiments analyzed independently. The values in the parentheses represent the proportion of binding represented by Site 1.

Table 2 Summary of the K_i values of the non-NMDA receptor ligands displacing (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding in the absence of KSCN

Compound	*Site 1* K_i (nM)	*Site 2* (K_i) (μM)
NBQX	0.2 ± 0.06 (18)	0.06 ± 0.04
(S)-5-Fluorowillardiine	22* ± 4	1.5* ± 0.2
Domoate	98 ± 17 (38)	12 ± 2
(S)-AMPA	142 ± 66 (25)	4.3 ± 0.5
L-Glutamate	375 ± 270 (25)	3.2 ± 0.2
CNQX	479 ± 49 (100)	/
Kainate	970 ± 277 (45)	88 ± 34
(R)-5-Fluorowillardiine	> 50,000	/
NMDA	> 100,000	/
(1S,3R)-ACPD	> 100,000	/

The values represent the mean ± s.e.mean from at least three experiments analyzed independently. The values in the parentheses represent the proportion of binding represented by Site 1. *K_D values obtained from the saturation analysis.

(Figure 2). Consequently an incubation time of 40 min was chosen for future experiments. On the addition of 1 mM L-glutamate, the binding was rapidly and fully reversed (Figure 2). Specific (S)-[^{3}H]-5-fluorowillardiine binding, under non-chaotropic conditions represented 80% of the total binding. The inclusion of 100 mM KSCN in the assay buffer increased the percentage specific binding to 90%.

At concentrations ranging between 1 nM and 3 μM, (S)-[^{3}H]-5-fluorowillardiine displayed saturable binding in both the presence and absence of KSCN (Figure 3). In the absence of KSCN the saturation data were best resolved into two binding sites (F-test; $P<0.05$) with $K_{D1}=22.5$ nM and $K_{D2}=1.5$ μM and B_{max} values of 1.4 and 10.8 pmol mg^{-1} protein respectively. The inclusion of 100 mM KSCN in the assay buffer significantly (Mann-Whitney U-test; $P=0.05$) increased the affinity of the high and low affinity binding sites to $K_{D1}=6.9$ nM and $K_{D2}=0.4$ μM with no effect on the B_{max} values (Table 1).

Competition experiments were performed to evaluate the pharmacology of the binding sites labelled by (S)-[^{3}H]-5-fluorowillardiine. The non-NMDA receptor ligands which recognised two binding sites displaced the binding from the higher affinity binding site with the following rank order of potency: NBQX > domoate > (S)-AMPA ≈ L-glutamate > kainate (Figure 4; Table 2). With the exception of domoate, a similar rank order of potency was observed in the displacement of the lower affinity binding site labelled by (S)-[^{3}H]-5-fluorowillardiine (Table 2). The non-NMDA receptor antagonist, CNQX, displaced (S)-[^{3}H]-5-fluorowillardiine binding with a Hill coefficient close to unity indicating the recognition of a single binding site (Figure 4). At concentrations up to 100 μM both NMDA and the metabotropic receptor agonist (1S,3R)-ACPD were inactive. Similarly (R)-5-fluorowillardiine, was more than 3000 times less active than the (S)-enantiomer, with a K_i value of 53 μM; this weak activity may represent contamination (1%) by the (S)-enantiomer.

[^{3}H]-kainate binding was performed to evaluate the selectivity of (S)-5-fluorowillardiine for AMPA and kainate receptors. Domoate was the most potent displacer of [^{3}H]-kainate binding recognising two binding sites with differing affinities (Table 3). At the concentrations tested (S)-5-fluorowillardiine and (S)-AMPA could displace up to 80% of the kainate binding with (S)-5-fluorowillardiine being more potent than (S)-AMPA.

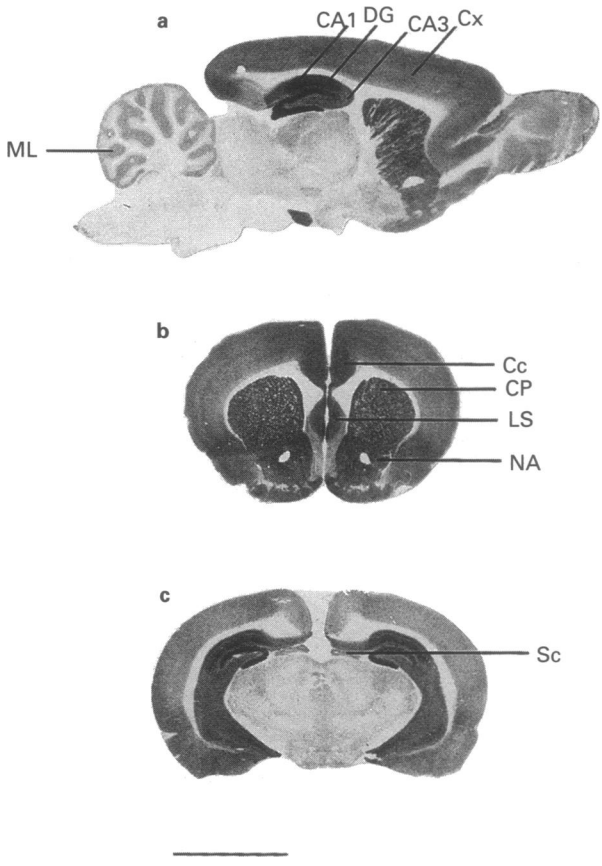

Figure 5 Autoradiograms of (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding, in the absence KSCN, to rat brain sections, printed directly from the tritium-sensitive film. Dark regions correspond to areas of high binding density. Non-specific binding represented less than 10% of the total binding and was indistinguishable from background. Planes of sections; (a) parasaggital; (b) and (c), coronal. Abbreviations: Cx, cortex; CA1 and CA3, the CA1 and CA3 regions of the hippocampus respectively; DG, dentate gyrus; ML, molecular layer of the cerebellum; Cc, cingulate cortex; CP, caudate putamen; LS, lateral septum; NA, nucleus accumbens and Sc, superior colliculus. Bar = 5 mm.

Regional distribution of (S)-[^{3}H]-5-fluorowillardiine binding

Densitometric analysis of the autoradiograms from sections obtained from at least three rats indicated that the highest

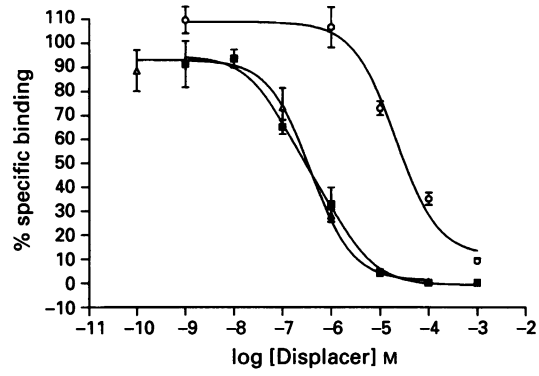

Figure 6 Displacement of (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding to rat brain cortex by (■) S-AMPA, (○) kainate and (△) CNQX as determined by receptor autoradiography. The results are representative of the other brain regions investigated. The IC_{50} values obtained by non-linear regression (for details see data analysis) for all brain regions considered are summarized in Table 4. The data points represent the mean ± s.e.mean of three experiments performed in triplicate. For all the curves the one-site model provided a significantly better fit (F-test $P>0.05$).

Table 4 Summary of the IC_{50} (μM) values for the displacement of (S)-[^{3}H]-5-fluorowillardiine (10 nM) binding to four regions of the rat brain as determined by receptor autoradiography

	Cortex	*Striatum*	*Dentate gyrus*	*CA1*
CNQX	0.4 ± 0.04	0.4 ± 0.02	0.3 ± 0.03	0.3 ± 0.04
(S)-AMPA	0.5 ± 0.2	0.4 ± 0.04	1.5 ± 0.7*	2.6 ± 0.9*
Kainate	21.7 ± 4.8	22.9 ± 5.5	16.5 ± 6.6	20.9 ± 4.9

The values represent mean ± s.e.mean from three experiments performed in triplicate. The values were analysed by the Mann-Whitney U-Test. *$P=0.05$ compared to the IC_{50} values for (S)-AMPA in the cortex and striatum.

density of (S)-[^{3}H]-5-fluorowillardiine binding was in the hippocampal formation, particularly in CA1 and the dentate gyrus. Other regions of the forebrain such as the cerebral cortex, anterior olfactory nucleus, caudate putamen, nucleus accumbens and the lateral septum displayed intermediate levels of binding (100–300 fmol mg^{-1} wet weight). Low levels of binding (< 100 fmol mg^{-1} wet weight) were also detected in the thalamus, inferior colliculus and the olfactory bulb. Within the cerebellum, higher densities of binding were observed in the molecular layer compared with the granule cell layer (Figure 5). The binding of (S)-[^{3}H]-5-fluorowillardiine to rat brain sections could be displaced by non-NMDA receptor ligands in all regions of rat brain investigated (Figure 6). Kainate and CNQX displayed similar IC_{50} values in the cortex, striatum, dentate gyrus and CA1 region of the hippocampus (Table 4). However, in both the CA1 region of the hippocampus and dentate gyrus the IC_{50} values for (S)-AMPA were significantly (Mann-Whitney U-Test; $P=0.05$) higher than those observed in the cortex and striatum (Table 4).

The regional distribution of (S)-[^{3}H]-5-fluorowillardiine binding in rat brain is similar to that observed for (S)-[^{3}H]-AMPA binding (Figure 7). However, in the absence of KSCN, (S)-[^{3}H]-AMPA displayed much lower levels of binding than (S)-[^{3}H]-5-fluorowillardiine (Table 5).

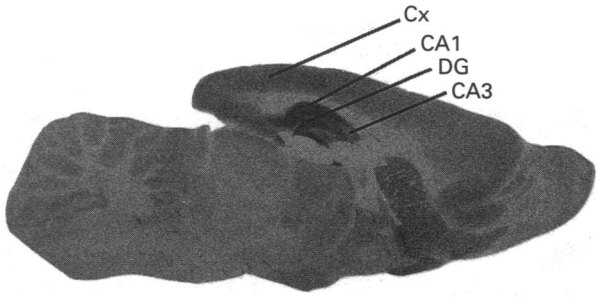

Figure 7 Autoradiogram of (S)-[^{3}H]-AMPA (10 nM) binding, in the absence of KSCN, to rat brain, sections showing the similarity in the regional distribution of the binding to that of (S)-[^{3}H]-5-fluorowillardiine. Non-specific binding was indistinguishable from background. These studies were performed on serial sections and under identical conditions to those described for (S)-[^{3}H]-5-fluorowillardiine. In the absence of KSCN (S)-[^{3}H]-AMPA displayed a much lower density of binding than (S)-[^{3}H]-5-fluorowillardiine (Table 5). Abbreviations: Cx, cortex; CA1 and CA3, the CA1 and CA3 regions of the hippocampus; DG, dentate gyrus. Bar = 5 mm.

Discussion

In electrophysiological studies the halogenated analogues of the amino acid, willardiine display reverse orders of potency for the AMPA and kainate receptors respectively (Wong *et al.*, 1994). The most potent and selective agonist for AMPA receptors within this series is (S)-5-fluorowillardiine. Subsequently (S)-5-fluorowillardiine has been shown to displace (S)-[^{3}H]-AMPA binding and to recognise both a high and low affinity binding site (Hawkins *et al.*, 1995). The high potency and the thousand fold difference in the affinity of (S)-5-fluorowillardiine for the two binding sites recognised by (S)-[^{3}H]-AMPA, together with the functional selectivity, indicate its potential as a useful tool to investigate further the AMPA receptor. In this study we have described the binding of the radiolabelled form of (S)-5-fluorowillardiine.

Table 5 Regional distribution of (S)-[^{3}H]-5-fluorowillardiine and (S)-[^{3}H]AMPA binding, in the absence of KSCN, in rat brain as determined by quantitative autoradiography

Region	*Specific binding* (fmol mg^{-1} wet weight) *(S)-[^{3}H]-5-Fluorowillardiine*	*(S)-[^{3}H]-AMPA*
Cerebral cortex		
Layers I-III	192.5 ± 16.4 (44)	49.9 ± 2.3 (40)
Layers IV-VI	156.0 ± 13.2 (35)	51.5 ± 5.8 (41)
Cingulate	201.0 ± 45.5 (46)	60.8 ± 4.1 (49)
Hippocampal formation		
Dentate gyrus	434.9 ± 25.8 (99)	115.2 ± 7.2 (93)
CA1	436.4 ± 33.8 (100)	123.1 ± 9.8 (100)
CA3	240.7 ± 8.9 (55)	62.5 ± 0.4 (50)
Striatum		
Caudate putamen	157.5 ± 12.8 (36)	47.5 ± 1.1 (38)
Nucleus accumbens	235.0 ± 28.2 (53)	68.0 ± 1.5 (55)
Septum		
Dorsal/lateral	279.3 ± 56.1 (64)	90.2 ± 5.2 (73)
Thalamus	59.9 ± 20.9 (13)	25.4 ± 2.4 (20)
Superior colliculus		
Superficial grey layer	124.5 ± 16.4 (28)	28.8 ± 2.7 (23)
Inferior colliculus		
External cortex	119.9 ± 39.3 (27)	22.0 ± 4.5 (17)
Central nucleus	54.9 ± 19.7 (12)	
Cerebellum		
Molecular layer	109.98 ± 9.8 (25)	22.5 ± 1.8 (18)
Granule cell layer	65.2 ± 11.5 (14)	14.9 ± 3.5 (12)
Olfactory system		
Olfactory bulb	75.8 ± 11.9 (17)	27.4 ± 3.7 (22)
Anterior olfactory nucleus	205.5 ± 41.6 (47)	63.9 ± 8.8 (51)

Optical densities were converted to fmol mg^{-1} wet weight using the tritium microscales. Non-specific binding was defined with 1 mM L-glutamate, and was subtracted from all readings. Values are the mean ± s.e.mean of readings from two consecutive sections from at least three rats. Values in parentheses represent binding expressed as a percentage of the binding of the respective ligand in the CA1 region of the hippocampus.

(S)-[^{3}H]-5-fluorowillardiine binding to rat brain synaptic membranes is both saturable and reversible. In the absence of chaotropic agents specific binding represents greater than 80% of the total binding, while addition of 100 mM KSCN results in a small enhancement of specific binding. This is in contrast to the very large stimulatory effect of thiocyanate ions on [^{3}H]-AMPA binding (Honoré & Nielsen, 1985). (S)-[^{3}H]-5-fluorowillardiine recognises two binding sites $K_{D1} = 22.5$ nM and $K_{D2} = 1.5$ μM. The addition of 100 mM KSCN results in the affinities of both binding sites being increased whilst the binding site densities are unaltered. In both binding and autoradiographic studies, it is clear that in the absence of KSCN, [^{3}H]-AMPA appears to bind to only a single site. However in the presence of KSCN two binding sites are commonly identified (Honoré & Drejer, 1988; Nielsen *et al.*, 1990). Evidence to date suggests that KSCN increases the affinity of [^{3}H]-AMPA binding, although the exact mechanism by which this is effected is unknown. It was originally suggested that KSCN might increase the affinity of [^{3}H]-AMPA binding by influencing the equilibrium between high and low affinity binding states (Honoré & Drejer, 1988). However, more recently it has been reported that KSCN increases the affinity of both the high and low affinity binding sites (Hall *et al.*, 1992). (S)-[^{3}H]-5-fluorowillardiine recognises two binding sites in both the absence and presence of KSCN reinforcing the latter conclusion. The apparent absence of the second [^{3}H]-AMPA binding site in the absence of KSCN would thus be explicable in terms of its very low affinity and fast off-rate. In addition the failure of [^{3}H]-AMPA to recognise the low affinity binding site in the absence of KSCN explains why the B_{max} value observed for (S)-[^{3}H]-AMPA binding (Hawkins *et al.*, 1995) is lower than that observed for (S)-[^{3}H]-5-fluorowillardiine binding, even though both these radioligands recognise the same receptor.

The pharmacology of (S)-[^{3}H]-5-fluorowillardiine binding is consistent with that of an AMPA receptor. Both NMDA and (1S,3R)-ACPD failed to displace (S)-[^{3}H]-5-fluorowillardiine binding, confirming the selectivity of (S)-[^{3}H]-5-fluorowillardiine for the non-NMDA ionotropic glutamate receptor class. (S)-[^{3}H]-5-fluorowillardiine does not label glutamate uptake sites as the binding does not display chloride dependence as the removal of 100 mM KCl from the assay buffer has no effect on the binding. In addition, D-aspartate, at concentrations up to 100 μM, failed to affect the binding (data not shown). The non-NMDA receptor agonists, (S)-AMPA, L-glutamate, kainate and the antagonist NBQX display the same rank order of potency for both the high and low affinity binding sites labelled by (S)-[^{3}H]-5-fluorowillardiine. The rank order is also similar to that observed when using either [^{3}H]-AMPA or [^{3}H]-CNQX to label the AMPA receptor (Monaghan *et al.*, 1984; Nielsen *et al.*, 1990). As mentioned above, results from binding studies using [^{3}H]-AMPA indicate the presence of two AMPA binding sites of different affinities. However, the non-NMDA receptor antagonist CNQX has been shown to have equal affinity for the two binding sites (Honoré *et al.*, 1989). Similarly in this study CNQX displaced (S)-[^{3}H]-5-fluorowillardiine binding with a Hill coefficient close to unity suggesting that CNQX also has the same affinity for the two binding sites identified by (S)-[^{3}H]-5-fluorowillardiine.

(S)-5-fluorowillardiine displaced [^{3}H]-kainate binding with IC_{50} values at least 10 times higher than the K_D values from the saturation studies, reinforcing the conclusion from electrophysiological studies (Wong *et al.*, 1994) that (S)-5-fluorowillardiine displays a selectivity for the AMPA receptor compared to the kainate receptor. The ability of both domoate and kainate completely to displace the binding of (S)-[^{3}H]-5-fluorowillardiine is in agreement with previous reports that whilst both these ligands display high affinity for kainate receptors they will also interact with the AMPA receptor subtype (Hampson *et al.*, 1992; Keinänen *et al.*, 1990).

The distribution of (S)-[^{3}H]-5-fluorowillardiine binding in rat brain parallels that observed using either [^{3}H]-AMPA or [^{3}H]-CNQX (Monaghan *et al.*, 1984; Nielsen *et al.*, 1990) and contrasts strongly with that of [^{3}H]-kainate (Monaghan & Cotman, 1982). Similarly the immunohistochemical localization of the AMPA receptor subunits, GluR1–3 exhibited greatest abundance in the anterior olfactory regions, cerebral cortex, hippocampus and striatum, while GluR4 immunoreactivity was restricted to the cerebellum (Martin *et al.*, 1993). (S)-[^{3}H]-5-fluorowillardiine binding was consistent with this distribution, and was quite distinct from that observed for the kainate subunits, which are particularly abundant in the CA3 region of the hippocampus (Werner *et al.*, 1991; Herb *et al.*, 1992). Furthermore in this study there was a good correlation between the regional distribution of (S)-[^{3}H]-AMPA and (S)-[^{3}H]-5-fluorowillardiine binding. (S)-[^{3}H]-AMPA displayed lower levels of binding than (S)-[^{3}H]-5-fluorowillardiine throughout rat brain as would be expected from the difference in the K_D values obtained for the two ligands from the homogenate binding studies. In addition, assuming the wash conditions employed in the autoradiographic studies were sufficient to remove low affinity binding, (S)-[^{3}H]-AMPA would be expected to recognise only a single binding site in the absence of KSCN (Hawkins *et al.*, 1995).

By use of receptor autoradiography the pharmacological specificity of (S)-[^{3}H]-5-fluorowillardiine binding sites in different brain regions could be compared. Saturation experiments using receptor autoradiography have not been performed and therefore the presence of the two binding sites in this preparation has yet to be confirmed. However, from displacement studies similar IC_{50} values for CNQX were observed in both the autoradiographic and homogenate binding studies. This is as expected, irrespective of the presence of one or two binding sites, as CNQX has equal affinity for the two AMPA binding sites. However for kainate the IC_{50} values in the receptor autoradiography are intermediate to the two sites identified in the homogenate binding studies suggesting the presence of the two sites. The failure to resolve the two components can be attributed to the restricted number of individual drug concentrations employed in the autoradiographic studies. (S)-AMPA displayed a significantly lower potency in displacing (S)-[^{3}H]-5-fluorowillardiine binding in the hippocampal formation compared to the cortex and striatum. From the IC_{50} values obtained in the autoradiographic studies it is tempting to conclude that the two AMPA binding sites recognised by (S)-AMPA in the homogenate studies have distinct regional locations within the rat forebrain, but such conclusions are limited by the absence of saturation data. However, previous reports have indicated regional variations in the pharmacology of [^{3}H]-AMPA binding with differences observed between the binding in the cerebellum compared with the forebrain (Porter & Greenamyre, 1994). In this study the pharmacology of (S)-[^{3}H]-5-fluorowillardiine binding in the cerebellum was not assessed.

In summary, the anatomical distribution and pharmacological characteristics of (S)-[^{3}H]-5-fluorowillardiine binding indicate recognition of the AMPA receptor. The high specific binding and ability to discriminate two binding sites of different affinities, under more physiological conditions, suggest that this is a useful radioligand for the future research of the AMPA receptor.

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