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European Journal of Pharmacology 431 (2001) 305–310

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# Regional mapping of low-affinity kainate receptors in mouse brain using [$^3$H](2*S*,4*R*)-4-methylglutamate autoradiography

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Received 6 August 2001; received in revised form 5 October 2001; accepted 12 October 2001

**Abstract**

Recent data indicate that (2*S*,4*R*)-4-methylglutamate is a selective agonist for low affinity (GluR5 and GluR6) kainate receptor subunits. In the present study, we have employed [$^3$H](2*S*,4*R*)-4-methylglutamate to examine low affinity kainate receptor distribution in mouse brain. [$^3$H](2*S*,4*R*)-4-Methylglutamate labelled a single site in murine cerebrocortical membranes ($K_d = 9.9 \pm 2.7$ nM, $B_{max} = 296.3 \pm 27.1$ fmol mg protein$^{-1}$). The binding of 8 nM [$^3$H](2*S*,4*R*)-4-methylglutamate was displaced by several non-NMDA receptor ligands ($K_i \pm$ S.E.M.): domoate ($1.1 \pm 0.2$ nM) > kainate ($7.1 \pm 1.1$ nM) ≫ L-glutamate ($187.6 \pm 31.9$ nM) ≫ (*S*)-α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) (> 50 μM). [$^3$H](2*S*,4*R*)-4-Methylglutamate autoradiography revealed a widespread regional distribution of low affinity kainate receptors. Highest binding densities occurred within deep layers of the cerebral cortex, olfactory bulb, basolateral amygdala and hippocampal CA3 subregion. Moderate labelling was also evident in the nucleus accumbens, dentate gyrus, caudate putamen, hypothalamus and cerebellar granule cell layer. These data show that [$^3$H](2*S*,4*R*)-4-methylglutamate is a useful radioligand for selectively labelling low affinity kainate receptors. 

*Keywords:* Kainate receptor; AMPA receptor; Radioligand binding; BALB/c, mouse

## 1. Introduction

In the mammalian brain, L-glutamate is the principal excitatory amino acid neurotransmitter acting at both ligand-gated ion channels (ionotropic) and G-protein coupled (metabotropic) glutamate receptors. The ionotropic glutamate receptor family is divided into *N*-methyl-D-aspartate (NMDA), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) and kainate receptors. Kainate receptor subunits are subdivided further into two groups based on their relative affinity for [$^3$H]kainate, termed "low" affinity ($K_d \approx 60$ nM) GluR5–GluR7 subunits and "high" affinity ($K_d \approx 10$ nM) KA-1 and KA-2 subunits (Dingledine et al., 1999).

Previous studies have employed [$^3$H]kainate autoradiography to map kainate receptors in both the human (Jansen et al., 1990; Kunig et al., 1995) and rodent brain (Monaghan and Cotman, 1982; Garcia-Ladona and Gombos, 1993). However, data interpretation from these studies is hampered by the fact that the [$^3$H]kainate concentrations employed to examine "low" affinity kainate receptor subunits can label both high and low affinity kainate receptor subunits. In recent years, (2*S*,4*R*)-4-methylglutamate has been reported to be a potent agonist, selective for GluR5 and GluR6 kainate receptor subunits (Gu et al., 1995; Zhou et al., 1997; Donevan et al., 1998). We have recently synthesized and characterized [$^3$H](2*S*,4*R*)-4-methylglutamate binding to rabbit brain homogenates (Toms et al., 1997). We have now extended our studies to report here the mapping of low-affinity kainate receptors with [$^3$H](2*S*,4*R*)-4-methylglutamate in the murine brain using quantitative autoradiographic techniques.

## 2. Materials and methods

### 2.1. Homogenate binding

Preparation of plasma membranes and homogenate binding were performed as described previously by Toms et al. (1997). All experimental procedures were performed at 4 °C. Briefly, membranes were prepared from adult

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0014-2999/01/$ - see front matter 
PII: S0014-2999(01)01463-7

male BALB/c mice cerebral cortices. Membranes (75 μg protein) were incubated under equilibrium conditions for 60 min in buffer (50 mM Tris-acetate, pH 7.4) in the presence of either increasing concentrations of [$^3$H](2*S*,4*R*)-4-methylglutamate (saturation studies) or 8 nM (approx. $K_d$ value) [$^3$H](2*S*,4*R*)-4-methylglutamate (competition studies). Non-specific binding was determined using 1 mM L-glutamate. In all homogenate experiments, incubations were terminated by centrifugation (13,000 × *g*, 7 min) and the resultant pellets superficially washed twice with 1 ml of buffer. The pellet was dissolved overnight in 2% (w/v) sodium dodecyl sulphate and bound radioactivity determined by liquid scintillation spectrometry. Protein levels were determined by a Lowry assay using standards of bovine serum albumin.

### 2.2. Autoradiography

Autoradiography was performed as previously described by Kitchen et al. (1997). Briefly, adult male BALB/c mice were killed by cervical dislocation, intact brains removed and rapidly frozen in isopentane (−20 °C) and stored (−80 °C) for up to 1 month. Adjacent coronal sections (20 μm) were cut for receptor mapping at 300 μm intervals for determination of total and non-specific [$^3$H](2*S*,4*R*)-4-methylglutamate (10 nM) binding. In addition, sagittal sections were cut for qualitative assessment of binding from fore to hind brain. Non-specific binding was determined in the presence of 1 mM L-glutamate. Following pre-washing (3 × 5 min) in ice-cold 50 mM Tris-acetate buffer (pH 7.4), slides were then incubated (90 min, 4 °C) with 10 nM [$^3$H](2*S*,4*R*)-4-methylglutamate, a concentration to the approximate $K_d$ value. Following further slide washing (3 × 5 min), sections were dried and apposed to [$^3$H]-sensitive Hyperfilm together with [$^3$H]microscales (0.07–33.4 nCi mg$^{-1}$). After 5 weeks, film was developed and analysed by video-based computerised densitometry (MCID, Imaging Research).

### 2.3. Materials

[$^3$H](2*S*,4*R*)-4-methylglutamate (27 Ci mmol$^{-1}$), (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ((*S*)-AMPA), kainate, *N*-methyl-D-aspartate, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid, L-2-amino-4-phosphonobutanoic acid, L-*trans*-pyrrolidine-2,4-dicarboxylic acid and domoate were all obtained from Tocris Cookson, Bristol, UK. Tritium-sensitive Hyperfilm and [$^3$H]-microscales were purchased from Amersham Life Science, Little Chalfont, UK. All other chemicals were purchased from Sigma-Aldrich, Dorset, UK.

### 2.4. Data analysis

Films were analysed by video based computerised densitometry using an MCID image analyser (Imaging Research, Canada) and $^3$H-microscales (Amersham) used to determine fmol mg tissue$^{-1}$ equivalents after subtraction of non-specific binding images. Measurements were carried out as described in Kitchen et al. (1997) and structures were identified with reference to the mouse brain atlas of Franklin and Paxinos (1997). Data from the homogenate binding experiments were analysed with the iterative non-linear regression program GraphPad Prism (v.2.0). $K_i$ values were calculated from the $IC_{50}$ values using the Cheng–Prusoff equation. The *F*-test was used to assess whether data fitted a two-site model significantly better than the simpler one-site model.

## 3. Results

### 3.1. Homogenate binding

Between 1 and 64 nM [$^3$H](2*S*,4*R*)-4-methylglutamate displayed saturable binding (Fig. 1A). The saturation isotherm was best resolved (F-test) to one binding site

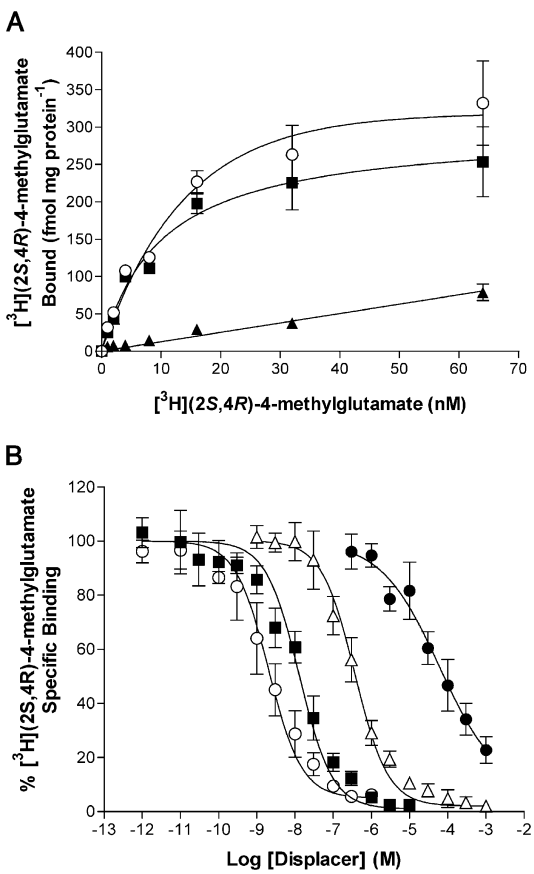


Fig. 1. (A) Saturation data for the total (○), non-specific (▲) and specific (■) binding of [$^3$H](2*S*,4*R*)-4-methylglutamate to mouse cerebrocortical membranes. Non-specific binding was determined in the presence of 1 mM L-glutamate. (B) Displacement of [$^3$H](2*S*,4*R*)-4-methylglutamate (8 nM) binding from mouse cerebrocortical membranes by domoate (○), kainate (■), L-glutamate (△) and (*S*)-AMPA (●). Non-specific binding was determined in the presence of 1 mM L-glutamate. Data represent the mean (±S.E.M.) of at least three independent experiments each performed in quadruplicate.

($K_d = 9.9 \pm 2.7$ nM, $B_{max} = 296.3 \pm 27.1$ fmol mg protein$^{-1}$, $n = 4$). Binding of 8 nM [$^3$H](2*S*,4*R*)-4-methylglutamate achieved equilibrium after 30 min (data not shown) and exhibited approximately 88% specific binding.

Displacement studies were performed, using 8 nM [$^3$H](2*S*,4*R*)-4-methylglutamate, to confirm the low affinity kainate receptor subunit selectivity of [$^3$H](2*S*,4*R*)-4-methylglutamate (Fig. 1B). Several selective ionotropic glutamate receptor ligands displaced specific [$^3$H](2*S*,4*R*)-4-methylglutamate binding ($K_i$ value ± S.E.M.): domoate ($1.1 \pm 0.2$ nM) > kainate ($7.1 \pm 1.1$ nM) ≫ L-glutamate ($187.6 \pm 31.9$ nM) ≫ (*S*)-AMPA (> 50 μM). All displacing agents fitted a one-site displacement model significantly better (*F*-test) than a two-site model. Neither, *N*-methyl-D-aspartate, the metabotropic glutamate receptor agonists (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid and L-2-amino-4-phosphonobutanoic acid, nor L-*trans*-pyrrolidine-2,4-dicarboxylic acid (an inhibitor of $Na^+$-dependent high-affinity glutamate transporters; Bridges et al., 1991) influenced [$^3$H](2*S*,4*R*)-4-methylglutamate binding when tested at 100 μM (data not shown).

### 3.2. Autoradiography

In the BALB/c mouse brain, [$^3$H](2*S*,4*R*)-4-methylglutamate (10 nM) binding exhibited a widespread regional distribution (Fig. 2, Table 1). Non-specific bindbing

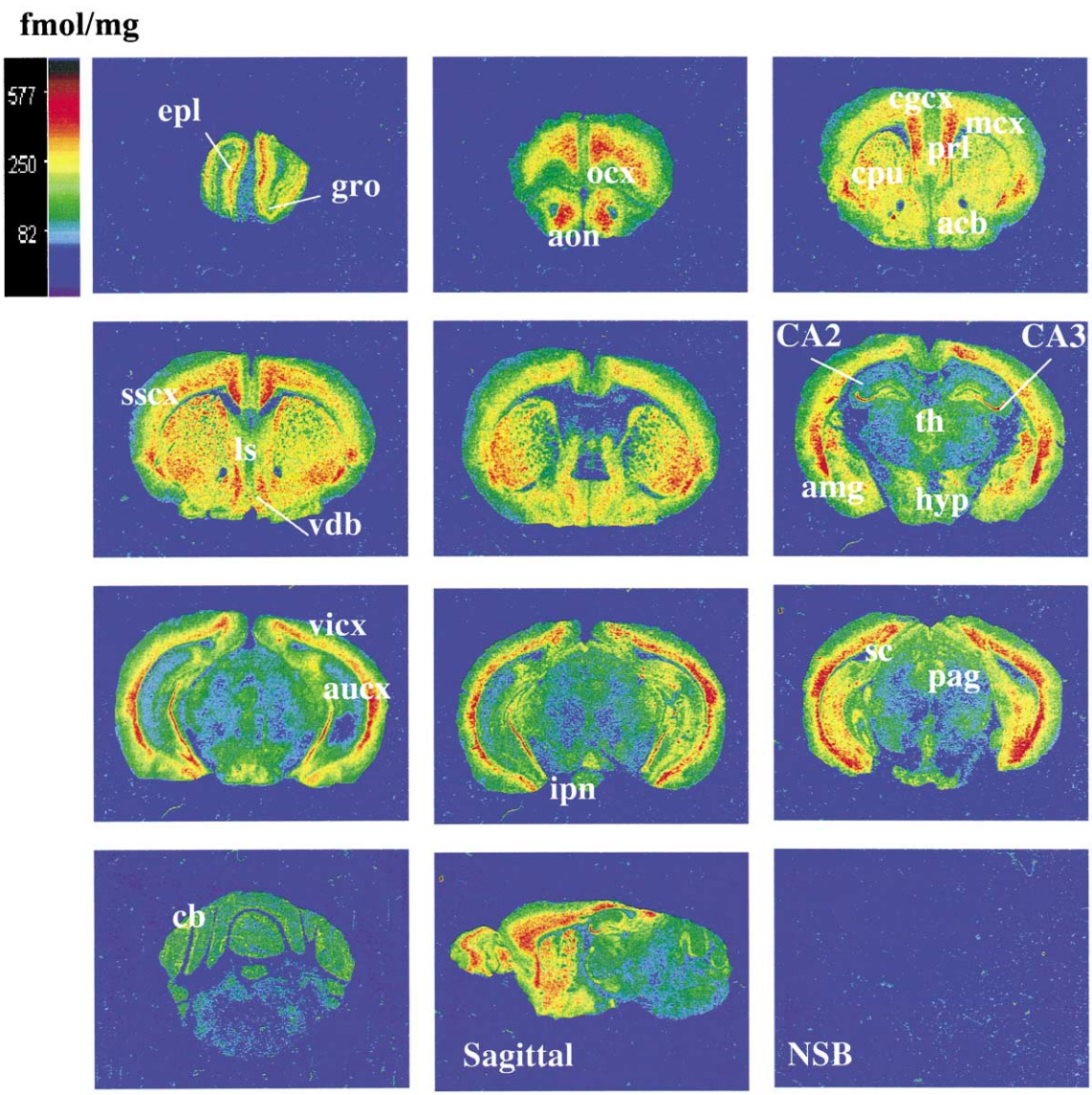


Fig. 2. Computer-enhanced colour autoradiograms of coronal (plus one sagittal) brain sections showing the regional distribution of [$^3$H](2*S*,4*R*)-4-methylglutamate (10 nM) binding in adult BALB/c mice. The colour bar represents a pseudo-colour interpretation of black and white film images in fmol mg tissue$^{-1}$ equivalents. Epl and gro: external plexiform and internal granular layers of the olfactory bulb; aon: anterior olfactory nucleus; cpu: caudate putamen; ocx, mcx,cgcx, sscx, vicx, aucx: orbital, motor, cingulate, somatosensory, visual and auditory cortex; ls: lateral septum; CA2, CA3: fields of hippocampus; th: thalamus; amg: amygdala; hyp: hypothalamus; ipn: interpeduncular nucleus; sc: superior colliculus; pag: periaqueductal gray; cb: cerebellum. NSB = non-specific binding.

Table 1
Quantitative autoradiography of mouse brain low affinity kainate receptor subunits labelled with [$^3$H](2*S*,4*R*)-4-methylglutamate (10 nM)

| Region | Bregma co-ordinates (mm) | [$^3$H](2*S*,4*R*)-4-Methylglutamate specific binding (fmol mg tissue$^{-1}$) |
|---|---|---|
| Olfactory bulb | 3.56 | |
| External plexiform layer | | 295 ± 27 |
| Internal granular layer | | 168 ± 20 |
| Anterior olfactory nucleus | 2.46 | 367 ± 13 |
| Cortex | | |
| Orbital | 2.46 | |
| Deep layers | | 231 ± 27 |
| Agranular insular | 1.42 | |
| Superficial layers | | 104 ± 10 |
| Intermediate layers | | 222 ± 21 |
| Deep layers | | 335 ± 17 |
| Prelimbic | 1.42 | |
| Deep layers | | 339 ± 13 |
| Motor | 1.42 | |
| Superficial layers | | 93 ± 9.5 |
| Intermediate layers | | 156 ± 8 |
| Deep layers | | 269 ± 13 |
| Cingulate | 1.10 | |
| Superficial layers | | 124 ± 10 |
| Intermediate layers | | 190 ± 10 |
| Deep layers | | 332 ± 18 |
| Primary somatosensory | 1.10 | |
| Superficial layers | | 83.0 ± 9.3 |
| Intermediate layers | | 157 ± 9 |
| Deep layers | | 260 ± 14 |
| Auditory and visual | −2.70 | |
| Superficial layers | | 101 ± 6 |
| Intermediate layers | | 176 ± 12 |
| Deep layers | | 268 ± 12 |
| Nucleus accumbens | 1.18 | 211 ± 28 |
| Caudate putamen | 1.10 | 233 ± 15 |
| Septum | 0.74 | 199 ± 18 |
| Vertical limb of diagonal band | 0.74 | 184 ± 16 |
| Globus pallidus | −0.22 | 51.5 ± 8.5 |
| Thalamus | −1.46 | 124 ± 10 |
| Basolateral amygdala | −1.46 | 283 ± 29 |
| Hypothalamus | −1.46 | 147 ± 9 |
| Ventral medial hypothalamic nuclei | −1.46 | 144 ± 22 |
| Hippocampus | −1.46 | |
| CA2 | | 63.1 ± 7.7 |
| CA3 | | 290 ± 50 |
| Dentate gyrus | | 148 ± 13 |
| Periaqueductal gray | −3.40 | 131 ± 8 |
| Superficial grey layer of superior colliculus | −3.40 | 200 ± 12 |
| Cerebellum | −6.48 | |
| Granular layer | | 168 ± 9 |
| Molecular layer | | 101 ± 3 |

The data represent mean (± S.E.M.) specific binding in fmol mg tissue$^{-1}$ in four brains. Bregma co-ordinates taken from the mouse brain atlas of Franklin and Paxinos (1997).

(1 mM L-glutamate) was close to background. In the olfactory bulb, the external plexiform layer displayed dense [$^3$H](2*S*,4*R*)-4-methylglutamate binding with the adjacent granule layer possessing intermediate binding levels. Within the cerebral cortex, [$^3$H](2*S*,4*R*)-4-methylglutamate binding showed a distinct laminar distribution (deep layers > intermediate layers > superficial layers). Highest binding densities were observed in deep layers of the agranular insular, prelimbic, cingulate, motor, auditory and visual, orbital and primary somatosensory cortices. Marked [$^3$H](2*S*,4*R*)-4-methylglutamate binding was also observed in the nucleus accumbens, caudate putamen, hypothalamus, vertical limb of diagonal band, amygdala and septum. In the hippocampus, the CA3 region displayed intense [$^3$H](2*S*,4*R*)-4-methylglutamate binding with moderate expression in the dentate gyrus. In the cerebellum, the granule cell layer displayed high [$^3$H](2*S*,4*R*)-4-methylglutamate binding with moderate binding in the adjacent molecular layer.

## 4. Discussion

The present study has revealed that [$^3$H](2*S*,4*R*)-4-methylglutamate represents a useful ligand for low affinity kainate receptors in the murine brain. Saturation analysis revealed that [$^3$H](2*S*,4*R*)-4-methylglutamate occupies a single high affinity site in murine brain membranes. High affinity single site [$^3$H](2*S*,4*R*)-4-methylglutamate binding has also recently been reported to occur in (a) *Macaca fascicularis* monkey brain (Carroll et al., 1998) and (b) our previous study (using rabbit brain homogenate) with concentrations of [$^3$H](2*S*,4*R*)-4-methylglutamate below 60 nM (Toms et al., 1997).

The rank order of potency of displacing ligands was comparable with that previously reported for [$^3$H]kainate binding to GluR5 and GluR6 kainate receptor subunits (Bettler et al., 1992; Lomeli et al., 1992; Sommer et al., 1992). Indeed, since domoate was found to be a more potent displacer than kainate, [$^3$H](2*S*,4*R*)-4-methylglutamate binding to high affinity (KA-1 and KA-2) kainate receptor subunits is unlikely (Werner et al., 1991; Herb et al., 1992). We have reported a similar rank order of displacing agent potency in rabbit brain (Toms et al., 1997) and this was confirmed by Carroll et al. (1998) in *M. fascicularis* monkey brain. Recent studies report (2*S*,4*R*)-4-methylglutamate to possess relative low affinities (> 3 μM) for cloned (EAAT1 And EAAT2) $Na^+$-dependent glutamate transporters (Vandenberg et al., 1997; Lieb et al., 2000). In our hands, employing low nM [$^3$H](2*S*,4*R*)-4-methylglutamate concentrations, no discernible [$^3$H](2*S*,4*R*)-4-methylglutamate interaction with glutamate transporters could be detected.

Autoradiographic mapping revealed that [$^3$H](2*S*,4*R*)-4-methylglutamate displayed widespread regional binding throughout the murine brain. Intense [$^3$H](2*S*,4*R*)-4-methylglutamate binding was observed in the hippocampal CA3 region with less so in the dentate gyrus (molecular layer).

Interestingly, little [$^3$H](2*S*,4*R*)-4-methylglutamate binding was observed in the murine CA2 region, in contrast to monkey hippocampus which is reported to display intense CA2/3 regional [$^3$H](2*S*,4*R*)-4-methylglutamate binding (Carroll et al., 1998). Previous studies employing [$^3$H] kainate (at concentrations expected to label both high and low kainate receptor subunits) have reported intense binding in rat CA2–CA4 (Monaghan and Cotman, 1982) and mouse CA3/CA4 (Garcia-Ladona and Gombos, 1993) hippocampal regions. Indeed, dense expression levels of Glu6 (and moderate Glu5 and Glu7) kainate receptor subunits have been reported to occur in rat hippocampal CA3 and dentate gyrus subregions (Petralia et al., 1994; Porter et al., 1997). Taken together, the data suggest species differences in the regional expression of the low affinity kainate receptor subunits within hippocampal structures.

Emerging data suggest that kainate receptors play a complex role in hippocampal CA3 subregion neurophysiology. Presynaptic kainate receptors (GluR5 and/or GluR6 receptor subunits) are reported to depress both excitatory mossy fibre and associational–commissural (Vignes et al., 1998; Contractor et al., 2000; Kamiya and Ozawa, 2000) inputs, and also to enhance perforant path (Contractor et al., 2000) inputs, to hippocampal CA3 pyramidal neurones. Additionally, postsynpatic GluR5 kainate receptor subunits have also been suggested to be expressed in hippocampal CA3 pyramidal neurones (Vignes et al., 1997).

In addition to the hippocampus, the mouse cerebral cortex possessed discrete layers of high [$^3$H](2*S*,4*R*)-4-methylglutamate binding, similar to that recently described in the monkey cortex (Carroll et al., 1998). Furthermore, the trilaminar cortical regional distribution is comparable with previously described [$^3$H]kainate binding loci in both the rat (Monaghan and Cotman, 1982) and mouse (Garcia-Ladona and Gombos, 1993). Moderate levels of [$^3$H](2*S*,4*R*)-4-methylglutamate binding occurred in the caudate putamen with very low levels in the globus pallidus. These data are comparable with previous reports describing [$^3$H](2*S*,4*R*)-4-methylglutamate binding to monkey (Carroll et al., 1998) and [$^3$H]kainate binding to rodent (Monaghan and Cotman, 1982; Garcia-Ladona and Gombos, 1993) basal ganglia.

In the cerebellum, [$^3$H](2*S*,4*R*)-4-methylglutamate binding was highest in the granule cell layer with weaker binding observed in the molecular layer. This corresponds with reported [$^3$H](2*S*,4*R*)-4-methylglutamate binding in monkey cerebellum (Carroll et al., 1998) and [$^3$H]kainate binding in the rodent cerebellum (Monaghan and Cotman, 1982; Garcia-Ladona and Gombos, 1993). Furthermore, mouse cerebellar [$^3$H](2*S*,4*R*)-4-methylglutamate binding correlates with the reported expression profiles of GluR6 in the granular and molecular layers of the rat cerebellum (Petralia et al., 1994; Porter et al., 1997).

In summary, the distribution of mouse brain low affinity kainate receptor subunits compares favourably with previous studies employing high [$^3$H]kainate concentrations. However, limited differences of low affinity kainate receptor expression profiles exist between mouse, rat and monkey brain. Therefore, [$^3$H](2*S*,4*R*)-4-methylglutamate represents a useful high specific binding radioligand which demonstrates selectivity for low affinity kainate receptor subunits.

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