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Comparison of the autoradiographic binding distribution of [^{3}H]-gabapentin with excitatory amino acid receptor and amino acid uptake site distributions in rat brain

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1 Gabapentin is a novel anticonvulsant with an unknown mechanism of action. Recent homogenate binding studies with [^{3}H]-gabapentin have suggested a structure-activity relationship similar to that shown for the amino acid transport system responsible for the uptake of large neutral amino acids (LNAA).

2 The autoradiographic binding distribution of [^{3}H]-gabapentin in rat brain was compared with the distributions for excitatory amino acid receptor subtypes and the uptake sites for excitatory and large neutral amino acids in consecutive rat brain sections.

3 Densitometric measurement of the autoradiographic images followed by normalisation with respect to the hippocampus CA1 stratum radiatum, was carried out before comparison of each binding distribution with that of [^{3}H]-gabapentin by linear regression analysis. The correlation coefficients observed showed no absolute correlation was observed between the binding distributions of [^{3}H]-gabapentin and those of the excitatory amino acid receptor subtypes. The acidic and large neutral amino acid uptake site distributions demonstrated a much closer correlation to the [^{3}H]-gabapentin binding site distribution. The correlation coefficients for D-[^{3}H]-aspartate, L-[^{3}H]-leucine and L-[^{3}H]-isoleucine binding site distributions were 0.76, 0.90 and 0.88 respectively.

4 Concentration-dependent inhibition by unlabelled gabapentin of autoradiographic binding of L-[^{3}H]-leucine and L-[^{3}H]-isoleucine was observed, with non-specific binding levels being reached at concentrations between 10 and 100 μM.

5 Excitotoxic quinolinic acid lesion studies in rat brain caudate putamen and autoradiography were carried out for the amino acid uptake sites mentioned above. The resulting glial infiltration of the lesioned areas was visualized by autoradiography using the peripheral benzodiazepine receptor specific ligand [^{3}H]-PK11195. A significant decrease in binding density in the lesioned area compared with sham-operated animals was observed for D-[^{3}H]-aspartate, L-[^{3}H]-leucine, L-[^{3}H]-isoleucine and [^{3}H]-gabapentin, whilst [^{3}H]-PK11195 showed a significant increase in binding density indicative of glial infiltration into the lesioned area. These results suggest that the gabapentin binding site and the acidic and LNAA uptake site may be present on cell bodies of a neuronal population of cells.

6 From these studies it appears that [^{3}H]-gabapentin, L-[^{3}H]-leucine and L-[^{3}H]-isoleucine bind to the same site in rat brain. The inhibition of [^{3}H]-gabapentin binding by the LNAA uptake system-specific ligand, BCH, suggests that [^{3}H]-gabapentin may label this uptake site, termed system-L. Conversely these ligands could be labelling a novel site that coincidentally has a similar structure-activity relationship to this uptake site. These results suggest a novel mechanistically relevant site of action for gabapentin and may enable further anti-epileptic agents of this type to be developed.

Keywords: Gabapentin; large neutral amino acid uptake site distribution; L-leucine; D-aspartate; rat brain; autoradiography

Introduction

Gabapentin is a novel anticonvulsant with an unknown mechanism of action which was initially synthesized as a GABA-mimetic capable of penetrating the blood-brain barrier. Gabapentin has since been shown to have no effect on $GABA_A$ or $GABA_B$ receptors, does not influence neural uptake of GABA and does not inhibit the GABA-degradative enzyme, GABA transaminase (E.C. 2.6.1.19) (Chadwick, 1992). Gabapentin has been shown to be active orally in various animal models of epilepsy, including maximal electroshock in rats and pentylenetetrazol- or audiogenically-induced seizures in mice (Bartoszyk *et al.*, 1986; Dooley *et al.*, 1986; Chadwick, 1992; Goa & Sorkin, 1993). Following the study of [^{3}H]-gabapentin binding to rat synaptic plasma membranes by Suman-Chauhan *et al.* (1993), a novel high affinity binding site for [^{3}H]-gabapentin was described. The same group described the autoradiographic localization of the gabapentin binding site in rat brain (Hill *et al.*, 1993). The binding site has been shown to be present in mouse and pig cerebral cortex synaptic plasma membranes with similar structure-activity relationships (Thurlow *et al.*, 1993). It was evident that the binding site appeared to be discretely localized in regions of the brain thought to be involved in the genesis of epileptiform activity.

Studies into excitatory amino acid NMDA receptor distribution and its association with the glycine modulatory site have been described extensively (Bristow *et al.*, 1986; Bowery *et al.*, 1988; Maragos *et al.*, 1988; McDonald *et al.*, 1990; Young & Fagg, 1991). Due to a possible link to excitatory amino acid receptors illustrated by animal and electrophysiological studies (Oles *et al.*, 1990; Sprosen, 1990), the distribution of [^{3}H]-gabapentin binding sites in rat brain was compared with the published distribution of [^{3}H]-glycine (McDonald *et al.*, 1990) and [^{3}H]-glutamate (Monaghan & Cotman, 1985). When the correlation coefficients between these three distributions were calculated it was clear from the low value obtained with the [^{3}H]-gabapentin binding site dis-

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tribution that there was no significant correlation with either the NMDA receptor or the glycine co-agonist site (Hill *et al.*, 1993). Following these studies it was suggested that the distribution of the gabapentin binding site was similar to that of AMPA since the distribution in the cerebellum appeared to correlate well with that described in the literature (Nielsen *et al.*, 1990). For this reason studies were performed to test the correlation of the binding site distribution for [^{3}H]-gabapentin with these and other excitatory amino acid receptor subtypes.

Further studies into the structure-activity relationships with amino acids and related compounds and gastrointestinal absorption studies using [^{3}H]-gabapentin, have indicated that the gabapentin binding site may use the large neutral amino acid uptake system, or system-L for uptake into cells (Thurlow *et al.*, 1993; Stewart *et al.*, 1993; Su *et al.*, 1995). Following these studies the relative distribution of the [^{3}H]-gabapentin binding site has been compared with that of L-[^{3}H]-leucine and L-[^{3}H]-isoleucine and also with a further excitatory amino acid uptake site as labelled by D-[^{3}H]-aspartate in the presence of NMDA, AMPA and kainate (100 μM each). The results of this study will be described here.

During the study by Hill and coworkers (1993), excitotoxic lesions were performed in the caudate putamen, a brain region showing medium density of [^{3}H]-gabapentin binding. These quinolinic acid lesions in the striatum demonstrated a decrease in specific [^{3}H]-gabapentin binding. This is consistent with the distribution in the hippocampus which suggests the binding site is present on a neuronal population, probably on the dendritic tree of granule or pyramidal neurones, as the resulting glial infiltration into the lesioned area did not illustrate an increase in binding (Hill *et al.*, 1993).

The distributions of L-[^{3}H]-leucine and L-[^{3}H]-isoleucine binding in brain have not been described in the literature to date. In order to investigate the distribution with a possible link to system-L, the specific ligand, 2-(−)-endoamino-bicycloheptane-2-carboxylic acid (BCH), and the corresponding unlabelled ligand were used to describe non-specific binding for each of these ligands. Further to the correlations of these binding distributions with [^{3}H]-gabapentin binding site distribution, quinolinic acid lesion studies, as described by Hill *et al.* (1993), were performed to distinguish whether the cellular localizations of these distributions differed.

The glial infiltration component for this type of lesion has been illustrated by the subsequent increase in the level of binding of [^{3}H]-PK11195 to the omega-3 benzodiazepine site (Dubois *et al.*, 1988). This technique has been included in the lesion studies described in this paper and the results of this study will be discussed.

Methods

[^{3}H]-gabapentin autoradiography

Rats (approximately 250 g) were stunned, decapitated and the brains removed. These were rapidly frozen with isopentane at −35°C and sections (10 μm) were cut using a cryostat. These sections were mounted onto gelatin-subbed slides, thawed, dried and stored at −20°C until required. On the day of the experiment, sections were thawed and dried and then preincubated in 10 mM HEPES buffer (pH 7.4 at 22°C, sodium-free; pH achieved with KOH) at room temperature for 30 min to remove any endogenous inhibitors of [^{3}H]-gabapentin binding. Adjacent sections were incubated in 10 mM HEPES buffer containing 20 nM [^{3}H]-gabapentin at 20°C in the presence and absence of 10 μM (S)-(+)-3-isobutyl GABA to define non-specific binding. After a 30 min incubation, the radiolabelled sections were subjected to a series of four sequential washes of 1 min each in ice-cold 100 mM NaCl, dipped in deionised water to remove excess salt and dried under a stream of cold air. The dried sections were exposed to tritium-sensitive film (Amersham) for 3 weeks together with [^{3}H]-microscales (Amersham). Quantitative analysis of autoradiographs was performed using the Microcomputer Imaging Device (MCID - Imaging Inc.). Incubations were performed in the presence and absence of L-leucine, 2-(−)-endoamino-bicycloheptane-2-carboxylic acid (BCH) and methylaminoisobutyric acid (MeAIB) at concentrations of 100 μM.

[^{3}H]-glycine autoradiography

Autoradiography of rat brain sections was performed as described by McDonald *et al.* (1990). Briefly, sections were brought to room temperature, dried and preincubated in 50 mM Tris-citrate (pH 7.4 at 20°C) for 45 min to remove any endogenous inhibitors of [^{3}H]-glycine binding. The sections were incubated in 50 mM Tris-citrate (pH 7.4) containing 100 nM [^{3}H]-glycine at 4°C for 20 min. Non-specific binding was determined by inclusion of 1 mM glycine in the incubation buffer. In order to determine the distribution of any strychnine-sensitive sites in the brain and their autoradiographical distribution, a third incubation was performed using consecutive sections in the presence of 10 μM strychnine. The incubation was terminated by a single rapid wash in 50 mM Tris-citrate buffer (pH 7.4 at 4°C) followed by a 1 min wash in fresh buffer at 4°C. The sections were then rapidly dip-washed in deionised water at 4°C and dried in a stream of cold air. The sections were then exposed to tritium-sensitive film for 3 to 4 weeks.

[^{3}H]-AMPA autoradiography

Sections were brought to room temperature and washed in 50 mM Tris-HCl buffer (pH 7.2) at 2°C for 30 min to allow dissociation of possible endogenous inhibitors of AMPA binding. Sections were then incubated in 50 mM Tris-HCl buffer (pH 7.2) at 2°C containing 40 nM [^{3}H]-AMPA for 45 min. Non-specific binding was determined by the inclusion of 100 μM unlabelled AMPA in the incubation buffer. Incubations were performed in the presence and absence of 100 mM potassium thiocyanate, as this has been shown to enhance the binding of [^{3}H]-AMPA in brain sections (Nielsen *et al.*, 1988). The incubations were terminated by three very rapid rinses in 50 mM Tris-HCl buffer (pH 7.2) at 2°C followed by warm air drying. The rinse and drying time took no more than 10 s for each slide and they were then exposed to tritium-sensitive film for 2 to 3 weeks.

[^{3}H]-MK-801 autoradiography

The method proposed by Bowery *et al.* (1988) was used to investigate the distribution of MK-801 binding sites within rat brain for comparison and correlation with the distribution obtained with [^{3}H]-gabapentin. Briefly, slide-mouthed sections were brought to room temperature and rinsed in 50 mM Tris-HCl (pH 7.4) at 23°C and allowed to dry in air. These slides were then incubated at 23°C in the presence of 30 nM [^{3}H]-MK-801 for 20 min. After incubation, the sections were rinsed twice for 40 s duration in 250 ml of fresh Tris-HCl buffer (pH 7.4) at 23°C. Non-specific binding was determined by including 100 μM MK-801 in the incubation buffer. Sections were then exposed to tritium-sensitive film for 2 to 3 weeks.

L-[^{3}H]-glutamate autoradiography

Sections were brought to room temperature and washed in 50 mM Tris-acetate for 45 min at 2°C (pH 7.2) to allow dissociation of possible endogenous inhibitors of L-[^{3}H]-glutamate binding. Sections were then transferred to fresh 50 mM Tris-acetate buffer at 30°C (pH 7.2) for 10 min as this has been shown to increase specific binding (Monaghan & Cotman, 1985). Following this preincubation, sections were transferred to fresh Tris-acetate buffer (pH 7.2) at 2°C containing 100 nM [^{3}H]-L-glutamate for 45 min. Non-specific binding was defined by use of 1 mM glutamate or 100 μM NMDA or 100 μM AMPA for consecutive sections. The incubation was termi-

nated by three rapid washes in 50 mM Tris-acetate (pH 7.2) at 2°C; sections were dried in a stream of cold air and exposed to tritium-sensitive film for 3 to 4 weeks.

Autoradiography of excitatory amino acid uptake sites

Anderson and co-workers (1990) showed that it is possible to label glutamate and aspartate uptake sites in rat brain using D-[^{3}H]-aspartate. Briefly, slides were warmed to room temperature prior to incubation. The sections were then preincubated for 10 min in 50 mM Tris-HCl buffer containing 300 mM NaCl, pH 7.4. Following preincubation, the sections were incubated in 100 nM D-[^{3}H]-aspartate for 10 min at 4°C. In addition, to enhance the selective visualization of the chloride-dependent binding site, NMDA, AMPA and kainate (100 μM each) were included in the incubation buffer. Non-specific binding was determined in the presence of 100 μM D,L-threo-β-hydroxy-aspartate. Following incubation, unbound radioactivity was removed by dipping slides in a series of four Coplin jars with ice-cold buffer for a total time of 30 s. The sections were then rapidly dried under a cold air stream. The sections were exposed to tritium-sensitive film for 4 to 5 weeks with methylacrylate standards (Microscales, Amersham).

Autoradiographic distribution of the system-L amino acid uptake site

Preliminary studies suggested that the assay developed for [^{3}H]-gabapentin provided 70 to 80% specific binding for L-[^{3}H]-leucine and L-[^{3}H]-isoleucine following a 30 min incubation in the presence and absence of the corresponding unlabelled ligand or the system-L specific ligand, BCH. Here, the sections were treated in the same way but the incubations were performed with 20 nM L-[^{3}H]-leucine or 20 nM L-[^{3}H]-isoleucine and non-specific binding was determined with the inclusion of 100 μM BCH or each of the corresponding unlabelled compound for the radioligands.

[^{3}H]-PK11195 autoradiography

This radiolabelled compound has been shown to be an effective marker for neuronal damage in rat brain following lesion studies (Price *et al.*, 1990) as it labels the peripheral-type benzodiazepine receptor shown to be present on glial cells, following gliosis of lesioned rat brain regions (Benavides *et al.*, 1989). The method of Benavides and coworkers (1990) was used for autoradiographical studies of lesioned rat brains following the procedures below. Coronal sections (10 μm) mounted on gelatin-coated slides were incubated with 1 nM [^{3}H]-PK11195 at 25°C in 0.17 M Tris-HCl, pH 7.4, for 30 min. The slides were washed twice for 5 min in the same buffer at 4°C and then rinsed quickly in deionised water before being dried in a cold air stream. Non-specific binding was achieved by including 1 μM Ro5-4864 in the incubation. Once dry the sections were apposed to tritium-sensitive film (Amersham) for 3 to 4 weeks. Contra- and ipsi-lateral regions of the resulting autoradiographs were then analysed densitometrically for specific [^{3}H]-PK11195 binding using MCID.

Quinolinic acid lesion studies

Male rats were anaesthetized with Hypnorm (fentanyl-fluanisone 0.3 ml kg^{-1}; fentanyl base 0.2 mg ml^{-1} and fluanisone 10 mg ml^{-1}) and diazepam (2.5 mg kg^{-1}) and placed in a Kopf stereotaxic apparatus. Unilateral lesions of the striatum were made by injecting 200 nmol of quinolinic acid dissolved in 1 μl of saline at a rate 0.5 μl min^{-1} (coordinates: anterior 0.5 mm, lateral 2.6 mm and ventral 4.4 mm, with bregma and the brain surface as reference points). Sham-lesioned animals received the same surgical procedures but saline was infused rather than the excitotoxic amino acid. This treatment has been shown to produce extensive loss of cell bodies in the injected region (Coyle & Schwarcz, 1976). Fourteen days after surgery the animals were killed and the brains removed and rapidly frozen.

Coronal sections were cut from brains of both lesioned and sham-lesioned animals and treated as described above for autoradiographical distribution studies for the ligands: [^{3}H]-gabapentin, L-[^{3}H]-leucine, L-[^{3}H]-isoleucine, D-[^{3}H]-aspartate, and [^{3}H]-PK11195.

Materials

Radiolabelled compounds were obtained from the following companies: [^{3}H]-gabapentin, specific activity 104 Ci mmol^{-1} (Cambridge Research Biochemicals, ICI, Macclesfield; custom synthesis); [^{3}H]-glycine, specific activity 48 Ci mmol^{-1} (Amersham); [^{3}H]-AMPA, specific activity 57 Ci mmol^{-1} (NEN Du Pont); [^{3}H]-MK-801, specific activity 22 Ci mmol^{-1} (NEN Du Pont); L-[^{3}H]-glutamate, specific activity 54 Ci mmol^{-1} (NEN Du Pont); D-[^{3}H]-aspartate, specific activity 25 Ci mmol^{-1} (Amersham); L-[^{3}H]-leucine, specific activity 143 Ci mmol^{-1} (Amersham); L-[^{3}H]-isoleucine, specific activity 93 Ci mmol^{-1} (Amersham); [^{3}H]-PK11195, specific activity 86 Ci mmol^{-1} (NEN Du Pont).

Other compounds were from Sigma except for Ro 05-4864 (7-chloro-5-(4-chlorophenyl)-N-methyl-N(1-methylpropyl)-3-isoquinoline-carboxamide) and MK-801((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; dizocilpine) which were purchased from Research Biochemicals Inc.

Results

Distribution of [^{3}H]-gabapentin binding sites in rat brain

The autoradiographical localization of [^{3}H]-gabapentin binding to a horizontal section of rat brain is illustrated in Figure 1. Binding of this radioligand is unevenly distributed in the brain with highest levels of binding (192 $\pm$ 28 fmol mg^{-1} tissue equivalent) being found in the outer layers of the frontal, parietal, occipital and entorhinal cortex. Non-specific binding levels were 40–50% of total binding when defined with either 100 μM gabapentin or 10 μM (S)-(+)-3-isobutyl GABA. The lowest levels of binding were observed in the white matter. It can be clearly seen from Figure 1 that within the cerebral cortex the highest degree of labelling was particularly pronounced in layers I and II of the cerebral cortex.

Specific binding was also discretely localized in the molecular layer of the dentate gyrus and cerebellum as well as in the CA1 region of the hippocampus. The hippocampal distribution of [^{3}H]-gabapentin binding is not uniform; the highest levels were found in the CA1 region and the molecular layer of the dentate gyrus. These densely labelled regions could be clearly distinguished from the pyramidal cell layer in the hippocampus and the granule cell layer in the dentate gyrus. A similar distribution pattern of [^{3}H]-gabapentin binding was observed in the cerebellum. Here, the highest level of binding was observed in the molecular layer; the granule cell layer showed intermediate levels of binding, while binding was absent in the white matter. Other regions associated with [^{3}H]-gabapentin binding at intermediate levels were the caudate putamen, globus pallidus, central grey and the geniculate nucleus formation. Evidence for a structure-activity relationship for high affinity binding between different regions of rat brain was beyond the scope of this study.

Excitatory amino acid receptor ligand binding site distributions in rat brain

The distribution of some of the ligands tested are summarized in Table 1. The [^{3}H]-glycine binding site distribution showed high levels of binding in the CA1 region of the hippocampus, molecular layer of the dentate gyrus and frontal areas of the

cortex. Intermediate levels of binding were observed in the parietal cortex, caudate putamen, the granule cell layer of the dentate gyrus, dorsal geniculate nucleus and the granular layer of the cerebellum. Inclusion of strychnine in the incubation produced very little difference in the distribution in the forebrain but did demonstrate some displacement of [^{3}H]-glycine binding in the brain stem (data not shown).

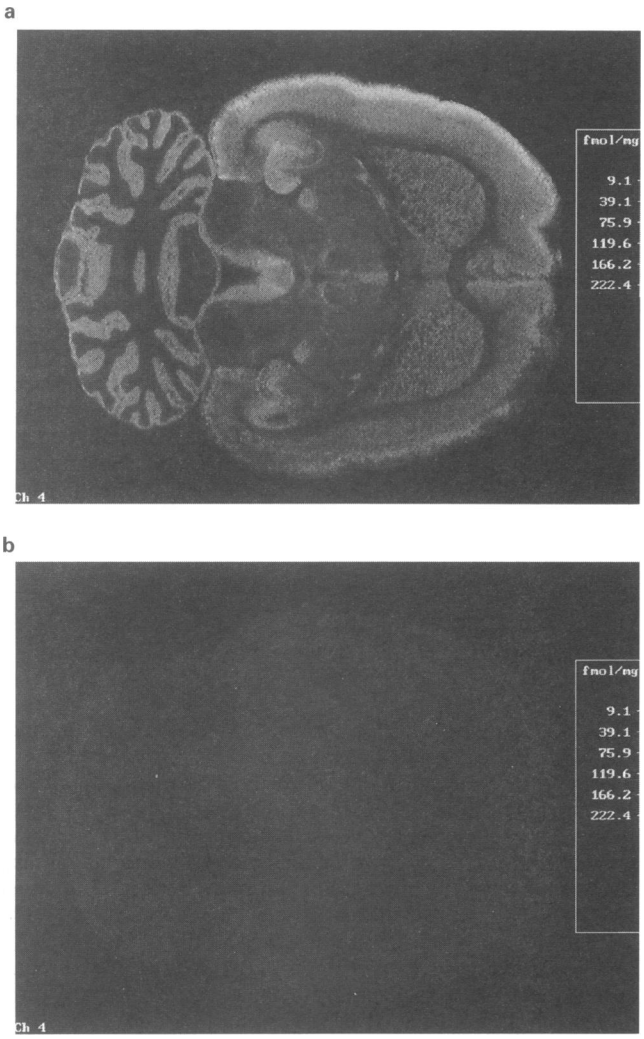

Figure 1 The autoradiographic distribution of the [^{3}H]-gabapentin binding site in rat brain. Binding distribution is shown in horizontal sections: (a) represents total binding distribution; (b) represents non-specific binding distribution as defined by the inclusion of 10 μM (S)-(+)-3-isobutyl GABA in the incubation.

The distribution of [^{3}H]-glutamate binding in the presence of NMDA is also summarized in Table 1. The highest levels for this binding site were found in the hippocampus and the molecular layer of the dentate gyrus. Intermediate levels were observed in the outer layers of the cerebral cortex, the granular cell layer of the dentate gyrus, the geniculate nucleus complex, cerebellum and caudate putamen. The distributions sensitive to glutamate and AMPA agree well with the literature but due to overlap with some of the binding densities of other radioligands described here, the data are not shown.

The density of the [^{3}H]-MK-801 binding sites was highest in the forebrain with the lowest levels of binding in the cerebellum. No differentiation between the molecular and granule cell layers in the cerebellum was observed. Highest levels of binding were detected in the hippocampus, dentate gyrus and the outer layers of the cortex. Intermediate levels were observed in the geniculate nucleus complex and the caudate putamen.

[^{3}H]-AMPA binding site density was highest in the hippocampus, dentate gyrus, outer layers of the cortex and the molecular layer of the cerebellum. Lowest levels of binding were detected in the geniculate nucleus complex, the granular cell layer of the cerebellum and the inner layers of the parietal cortex (Table 1). Inclusion of potassium thiocyanate in the incubation buffer led to a uniform increase in [^{3}H]-AMPA binding in all regions (data not shown).

Comparison of the binding distributions of excitatory amino acid receptor ligands with [^{3}H]-gabapentin binding in rat brain

The correlation of these binding distributions with the [^{3}H]-gabapentin binding site distribution is shown in Table 2. It can be clearly seen that there is a poor correlation between [^{3}H]-gabapentin binding sites and the binding sites for excitatory amino acid receptors. The best correlation was obtained with [^{3}H]-MK-801 ($r = 0.71$). However, the distribution for MK-801 binding in the cerebellum is completely different from that of [^{3}H]-gabapentin, with an equal binding density in both the granule cell and molecular layers for the former, whereas with [^{3}H]-gabapentin the distribution is high in the molecular layer but low in the granule cell layer.

Amino acid uptake site ligand binding site distribution in rat brain

The binding distributions of the amino acid uptake sites is shown in Figure 2(a), (b) and (c) and summarized in Table 3.

Table 1 The specific binding of [^{3}H]-gabapentin and various excitatory amino acid ligands in several rat brain regions

Brain region	*[^{3}H]-gabapentin* (fmol mg^{-1})	*[^{3}H]-glycine (strychnine-insensitive site)* (fmol mg^{-1})	*[^{3}H]-glutamate (NMDA-sensitive site)* (fmol mg^{-1})	*[^{3}H]-MK-801* (fmol mg^{-1})	*[^{3}H]-AMPA (+ thiocyanate)* (fmol mg^{-1})
Frontal cortex	127±15	82±7	21±0.2	372±35	330±20
Frontal parietal cortex inner	90±12	51±14	17±2	255±36	274±11
Frontal parietal cortex outer	192±28	59±21	25±0.2	389±33	311±24
Caudate putamen	65±10	53±4	18±0.1	230±37	331±18
Globus pallidus	57±13	24±7	5.5±0.1	94±16	75±9
Hippocampus CA1 radiatum	127±24	90±19	32±2	509±46	448±3
Hippocampus CA1 oriens	123±27	92±27	28±5	437±39	441±10
Dentate gyrus molecular layer	138±26	81±13	43±3	484±29	443±1
Dentate gyrus granular layer	117±25	55±14	13±3	346±60	388±16
Medial geniculate nucleus	67±14	21±2	6.5±2	215±31	121±29
Dorsal geniculate nucleus	55±9	50±6	13±0.1	248±51	151±5
Cerebellum granular layer	34±6	58±7	12±2	133±17	114±4
Cerebellum molecular layer	118±24	14±3	13±1	133±17	317±2

Data represent the arithmetic mean of measurements from sections of at least 3 different rat brains.

Table 2 The specific binding of [^{3}H]-gabapentin and amino acid uptake site specific ligands in several rat brain regions

Brain region	*[^{3}H]-gabapentin* (fmol mg^{-1})	*L-[^{3}H]-leucine (BCH-sensitive site)* (fmol mg^{-1})	*L-[^{3}H]-isoleucine (BCH-sensitive site)* (fmol mg^{-1})	*D-[^{3}H]-aspartate (glutamate uptale site)* (fmol mg^{-1})
Frontal cortex	127±15	53±1	56±5	160±14
Frontal parietal cortex inner	90±12	54±4	48±3	130±10
Frontal parietal cortex outer	192±28	101±7	82±4	198±31
Caudate putamen	65±10	43±3	41±3	113±11
Globus pallidus	57±13	10±1	5±1	19±8
Hippocampus CA1 radiatum	127±24	71±1	61±2	167±9
Hippocampus CA1 oriens	123±27	66±2	60±2	154±12
Dentate gyrus molecular layer	138±26	76±4	68±3	169±16
Dentate gyrus granular layer	117±25	75±1	63±3	149±14
Medial geniculate nucleus	67±14	38±1	34±2	47±6
Dorsal geniculate nucleus	55±9	57±5	50±4	85±8
Cerebellum granular layer	34±6	21±1	20±1	86±15
Cerebellum molecular layer	118±24	74±4	92±4	284±33

Data represent the arithmetic mean of measurements from sections of at least 5 different rat brains.

The system-L preferring ligand L-[^{3}H]-leucine had a distribution which appeared to be almost superimposable with that of [^{3}H]-gabapentin. In fact, unlabelled gabapentin displaced L-[^{3}H]-leucine and L-[^{3}H]-isoleucine binding to rat brain sections, in a concentration-dependent manner to the non-specific levels as defined by BCH at concentrations between 10 and 100 μM (data not shown). In the L-[^{3}H]-leucine binding site distribution, the highest levels of binding occurred in those regions of the brain which showed high levels of [^{3}H]-gabapentin binding, namely the hippocampus, dentate gyrus, outer layers of the frontal, parietal, occipital and entorhinal cortex and the molecular layer of the cerebellum. Conversely, the granule cell layer of the cerebellum had a low binding site density for L-[^{3}H]-leucine along with the medial geniculate nucleus. Intermediate levels of binding sites were found in the dorsal geniculate nucleus, caudate putamen and inner layers of the cerebral cortex. The specific binding distribution was identical whether non-specific binding was defined with 100 μM BCH or unlabelled L-leucine.

The binding distribution for L-[^{3}H]-isoleucine was also found to be almost identical to that of [^{3}H]-gabapentin and L-[^{3}H]-leucine. Once again specific binding was unaffected whether non-specific binding was determined by use of 100 μM BCH or unlabelled L-isoleucine.

The distribution of D-[^{3}H]-aspartate binding under conditions allowing exclusive labelling of the glutamate/aspartate uptake site, is shown in Figure 2(e) and (f). It can be seen that a pattern of binding similar to that of [^{3}H]-gabapentin is evident in the cerebellum. High levels of binding were seen in the molecular layer of the cerebellum, while intermediate levels were found in the hippocampus, dentate gyrus, outer layers of the cerebral cortex and caudate putamen. Lower levels were seen in the central grey and in the medial and dorsal geniculate nuclei.

Comparison of the distribution of the amino acid uptake sites with [^{3}H]-gabapentin binding sites

The similarity of these three distributions to that of [^{3}H]-gabapentin is shown in Table 3 and Figure 3. Correlation of the data normalized with respect to the hippocampus CA1 stratum radiatum yielded correlation coefficients from linear regression analysis of 0.90 and 0.88 for L-[^{3}H]-leucine and L-[^{3}H]-isoleucine respectively, while for D-[^{3}H]-aspartate the correlation coefficient was 0.76. Thus the correlation of gabapentin binding sites with that for glutamate/aspartate uptake sites is slightly lower than that for the system-L amino acid uptake site. The reason for this appears to be due to the relatively high values of binding for D-[^{3}H]-aspartate in the molecular layer of the cerebellum when compared to the stratum radiatum of the CA1 region of the hippocampus (Table 2). Direct comparison of the distributions can be seen in Figure 3.

Quinolinic acid lesion studies in the caudate putamen

The localization of the cellular site to which gabapentin binds was determined by unilateral quinolinic acid lesions to the caudate putamen. Data from four separate animals is represented in Table 4. Specific binding in the lesioned animals decreased in the lesioned, right-hand side (RHS) when compared to the unlesioned, left-hand side (LHS), or to sections from the sham-lesioned animals, for [^{3}H]-gabapentin, L-[^{3}H]-leucine, [^{3}H]-isoleucine and D-[^{3}H]-aspartate. [^{3}H]-PK11195 binding, however, increased from an undetectable level of specific binding in sham-lesioned animals to nearly 300 fmol mg^{-1} tissue equivalent specific bound.

Discussion

The understanding of the manner by which ligands may influence the activity of neuronal systems has been aided by autoradiographic studies which reveal the anatomical localization of ligand binding sites. For example, the correlation of NMDA-sensitive glutamate binding sites and [^{3}H]-glycine binding sites highlights the dependence for the activation of the NMDA receptor complex on glycine (Bristow *et al.*, 1986; Johnson & Ascher, 1987; Bowery *et al.*, 1988; Maragos *et al.*, 1988; McDonald *et al.*, 1990; Young & Fagg, 1991). The uneven distribution of excitatory amino acid receptors is in good agreement with the projection areas of the major excitatory amino acid pathways (Young & Fagg, 1991).

The distribution of the gabapentin binding site is discretely localized to brain regions which are highly associated with projection areas of major excitatory amino acid pathways, and areas where epileptiform activity occurs, such as the hippocampus, dentate gyrus and outer layers of the cerebral cortex. In fact, apart from the outer layers of the cortex, the highest levels of gabapentin binding density were found within the hippocampus and dentate gyrus, an area believed to be associated with seizure activity due to its low threshold to stimulation.

When the autoradiographic binding distribution of [^{3}H]-gabapentin in the hippocampus was inspected more closely it was evident that the binding paralleled the terminal fields of the excitatory circuitry. This circuitry was described by Walaas (1983), and it can be seen that binding was highest in the area dentata where a major excitatory pathway, arising in the entorhinal cortex (the perforant path), terminates on the dentrites of granule cells. The projections of these granule cells extend the excitatory pathway to the CA3 region of the hippocampus and the CA3 cells in turn feed forward to terminate in the CA1 region where binding was again pronounced. From this distribution it would be reasonable to assume that gabapentin may influence the excitatory input to this structure. However, in intracellular recordings from the CA1 region of hippocampal slices, gaba-

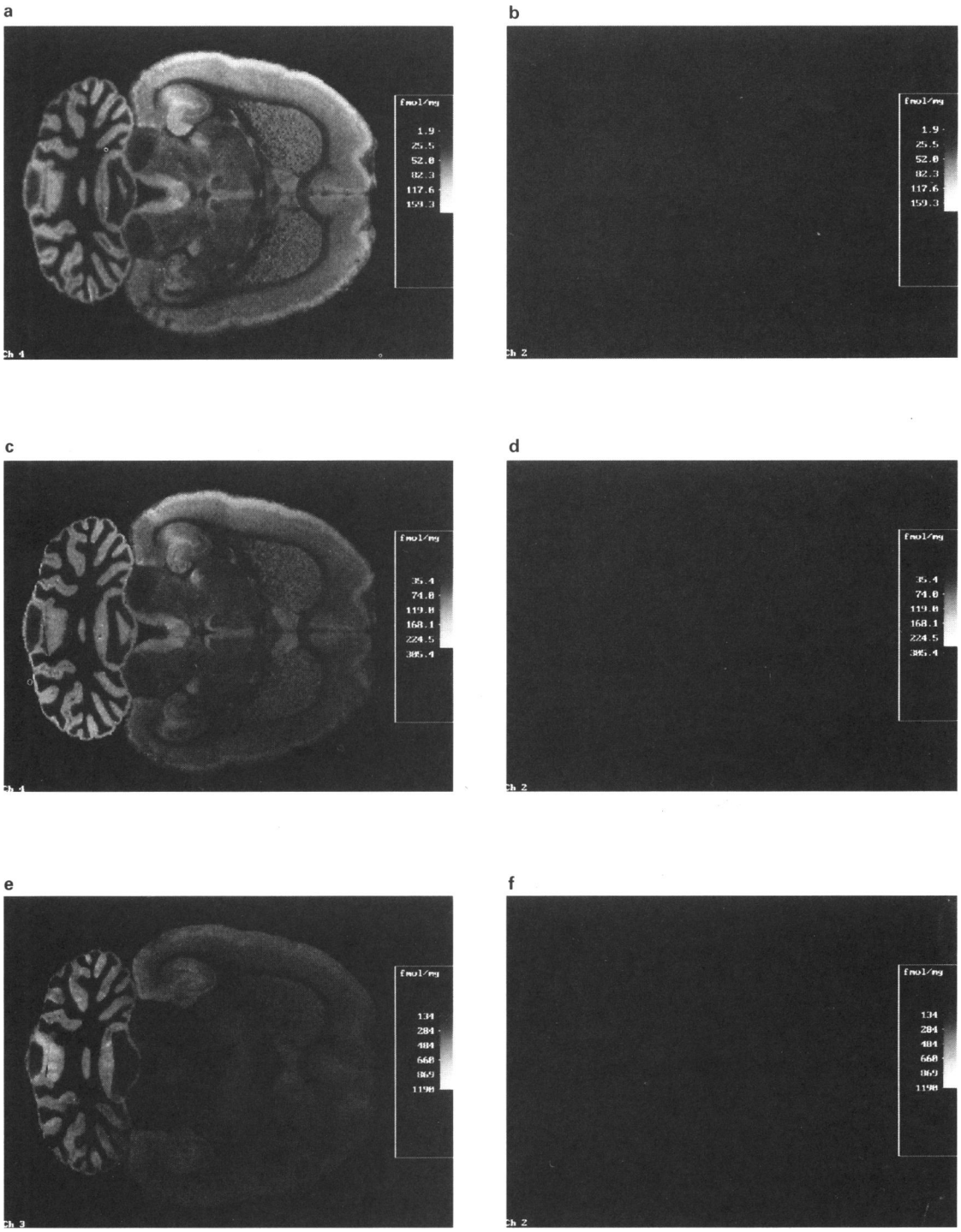

Figure 2 The autoradiographic distribution of binding in consecutive rat brain sections of: 20 nM L-[^{3}H]-leucine in the absence (a) and presence of 100 μM BCH (b); 20 nM L-[^{3}H]-isoleucine in the absence (c) and presence of 100 μM BCH (d); 100 nM D-[^{3}H]-aspartate in the absence (e) and presence of 100 μM D,L-threo-β-hydroxyaspartate (f).

Table 3 Comparison of the specific binding distributions of excitatory amino acid ligands and amino uptake sites with the specific binding distribution of [^{3}H]-gabapentin

[^{3}H]-gabapentin binding site distribution (n = 8) versus:	n	*Correlation coefficient*
Strychnine-insensitive [^{3}H]-glycine binding site distribution	3	0.38
NMDA-sensitive [^{3}H]-glutamate binding site distribution	3	0.66
[^{3}H]-MK-801 binding site distribution	5	0.71
[^{3}H]-AMPA (+SCN−) binding site distribution	6	0.20
BCH-sensitive L-[^{3}H]-leucine binding site distribution	5	0.90
BCH-sensitive L-[^{3}H]-isoleucine binding site distribution	5	0.88
D-[^{3}H]-aspartate (+ NMDA, + AMPA, + Kainate) binding site distribution	5	0.76

The binding distributions were normalised with respect to hippocampal CA1 stratum radiatum binding levels and correlations between the distributions were determined by linear regression (Grafit).

pentin did not influence the development of long-term potentiation (Taylor *et al.*, 1988) which is believed to be due to the activation of NMDA receptors (Harris *et al.*, 1984), and reductions in synaptic transmission in the hippocampus were observed only at high (1 mM) concentrations of gabapentin (Haas & Weiser, 1986).

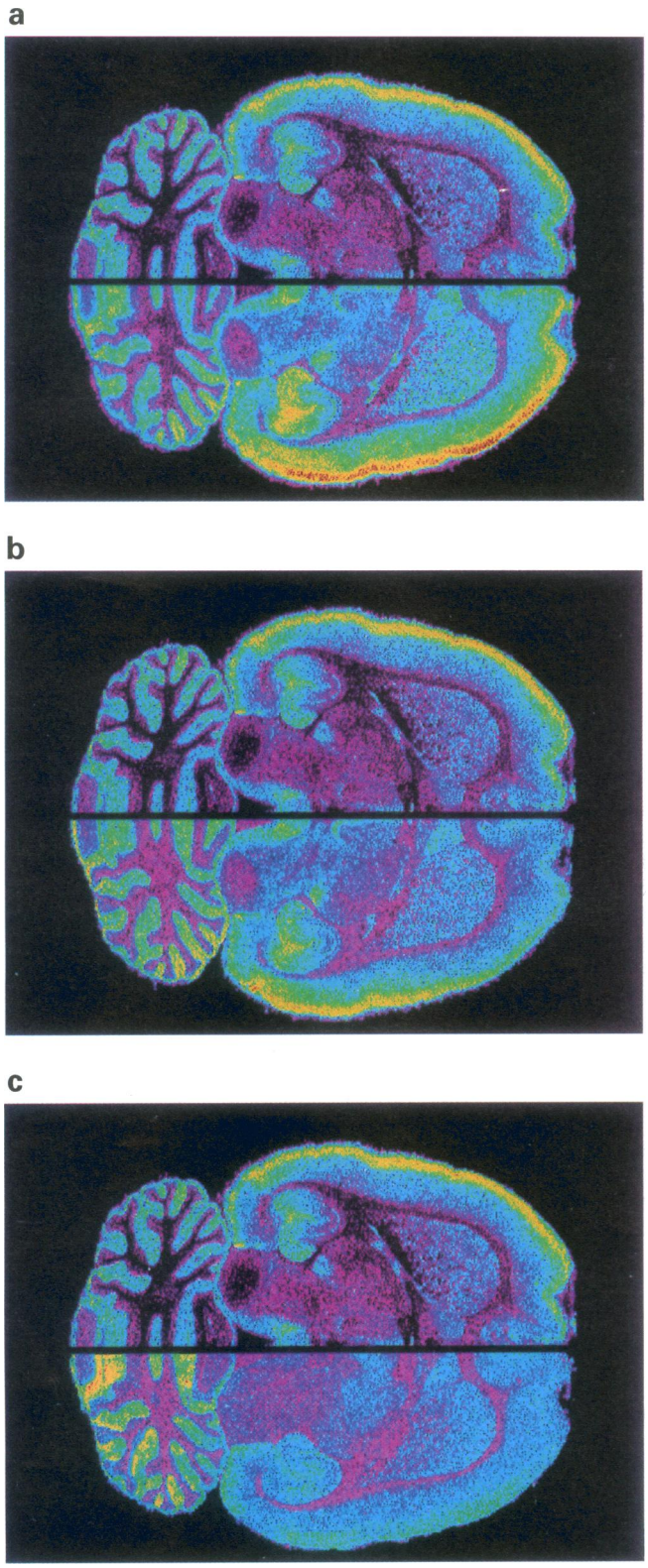

Figure 3 Comparison of the distributions of the binding sites for 20 nM [^{3}H]-gabapentin (top half of images) with: (a) 20 nM L-[^{3}H]-leucine (bottom half of image); (b) 20 nM L-[^{3}H]-isoleucine (bottom half of image); (c) 100 nM D-[^{3}H]-aspartate (bottom half of image) in rat brain. Specific binding images derived from total and non-specific images using MCID.

A comparison of the relative quantitative distribution of the NMDA/glycine receptor complex and that for the gabapentin suggests that these two distributions are different. The low correlation coefficients observed in this study and others (Hill *et al.*, 1993) suggest that the gabapentin binding site is not part of the NMDA receptor complex but may mirror the excitatory amino acid inputs as described above. Proportionally, the gabapentin binding site has highest levels of binding in the outer layers of the cortex while the highest levels of binding of the NMDA/glycine complex are in the CA1 region of the hippocampus.

Within the cerebellum the distribution of [^{3}H]-gabapentin binding was the reverse of that found for [^{3}H]-glycine. The correlation of these two distributions was in fact so low that, although they are qualitatively similar, the two sites were not related. It is this brain region which also decreases the correlation between the relative distributions of [^{3}H]-gabapentin and [^{3}H]-MK-801 binding.

From the study by Hill *et al.* (1993), the cerebellar distribution of gabapentin binding was believed to be close to that of [^{3}H]-AMPA and further study into this distribution was necessary. The results presented here indicate that despite the similarity of the distributions in this brain region, the overall relative brain distributions correlate very poorly. Indeed, maximal levels of binding occur in the hippocampus for [^{3}H]-AMPA, while for [^{3}H]-gabapentin they occur in the outer layers of the cerebral cortex.

The LNAA binding site distribution appears, from Figures 2(a), (b), (c) and (d), to show marked similarity to that of gabapentin. Throughout the brain the distribution appears to be almost the mirror image of the gabapentin binding site, a fact which is illustrated by Figure 3. Indeed the correlates are close to unity and in accordance with past research by Bowery and co-workers (1988) this degree of correlation is indicative of labelling of a shared or the same binding site. The non-specific binding levels as defined by BCH or L-leucine for L-[^{3}H]-leucine were identical and the same was true for the non-specific binding levels for L-[^{3}H]-isoleucine binding. Non-specific levels of binding could also be achieved for [^{3}H]-gabapentin autoradiography with the inclusion of 100 μM BCH in the incubation (data not shown).

The distribution of the excitatory amino acid high affinity uptake site, labelled by D-[^{3}H]-aspartate, can be seen to have a distribution similar to that of gabapentin, suggesting a close correlation to that of the excitatory input pathways in the brain. It is unlikely that gabapentin and the excitatory amino acid uptake site are the same molecular entity due to the relatively higher binding observed in the molecular layer of the cerebellum for [^{3}H]-gabapentin, while the density in the outer layers of the cortex for the same ligand was somewhat lower.

The cellular location of the gabapentin binding site has been suggested from previous quinolinic acid lesion studies in the caudate putamen to be on neuronal cell bodies, as illustrated by a decrease in the specific binding of [^{3}H]-gabapentin in the lesioned area (Hill *et al.*, 1993). As can be seen from Table 4, specific binding of L-[^{3}H]-leucine, L-[^{3}H]-isoleucine and D-[^{3}H]-aspartate decreased along with [^{3}H]-gabapentin binding in the area of the lesion. The specific binding of the peripheral benzodiazepine receptor ligand, [^{3}H]-PK11195, increased significantly in the lesioned area, which is indicative of glial infiltration into the region of damage (Dubois *et al.*, 1988).

It is interesting that in this study the level of D-[^{3}H]-aspartate binding decreased in the lesioned area, while in another study (Monaghan & Cotman, 1985) a transient increase in D-[^{3}H]-aspartate binding was observed in the hippocampus following an entorhinal cortex lesion. It is believed that this increase is due to increased amounts of uptake sites present on the glia which infiltrate the area of damage following the lesion. This leads to an increased capacity for the uptake of glutamate and aspartate, and so curtails the possible damage caused by a bolus release of excitatory amino acids following an insult such as ischaemic damage. The decrease described in this study is suggestive of a loss of excitatory amino acid uptake sites from neuronal cell bodies and not an increase in

Table 4 Binding of (A) 20 nM [^{3}H]-gabapentin, (B) 20 nM L-[^{3}H]-leucine, (C) 20 nM L-[^{3}H]-isoleucine, (D) 100 nM D-[^{3}H]-aspartate and (E) 1 nM [^{3}H]-PK11195 in rat brain following lesions to the caudate putamen with quinolinic acid

A				
[^{3}H]-gabapentin	*Amount bound* (fmol mg^{-1} tissue equivalent)			
	LHS		*RHS*	
	Total	*Non-specific*	*Total*	*Non-specific*
Sham	82.3±5.1	20.4±1.4	82.3±3.9	21.3±0.9
Lesioned	82.8±5.4	22.1±0.8	60.3±2.4*	18.9±0.9
B				
L-[^{3}H]-leucine	*Amount bound* (fmol mg^{-1} tissue equivalent)			
	LHS		*RHS*	
	Total	*Non-specific*	*Total*	*Non-specific*
Sham	66.4±4.4	12.2±0.6	71.6±4.0	10.0±0.9
Lesioned	60.9±7.1	7.1±0.9	41.5±6.1*	4.5±0.6
C				
L-[^{3}H]-isoleucine	*Amount bound* (fmol mg^{-1} tissue equivalent)			
	LHS		*RHS*	
	Total	*Non-specific*	*Total*	*Non-specific*
Sham	38.5±3.5	10.1±1.0	43.0±3.8	9.7±1.2
Lesioned	36.0±1.9	5.6±1.4	23.9±1.3*	6.0±1.3
D				
D-[^{3}H]-aspartate	*Amount bound* (fmol mg^{-1} tissue equivalent)			
	LHS		*RHS*	
	Total	*Non-specific*	*Total*	*Non-specific*
Sham	195.3±15.8	51.6±5.2	221.6±19.4	65.6±3.2
Lesioned	201.2±18.8	37.8±5.4	119.5±14.7*	46.5±5.9
E				
[^{3}H]-PK11195	*Amount bound* (fmol mg^{-1} tissue equivalent)			
	LHS		*RHS*	
	Total	*Non-specific*	*Total*	*Non-specific*
Sham	81.0±7.5	90.1±7.9	87.9±7.2	94.5±7.05
Lesioned	100.5±2.2	100.5±3.0	413.9±13.6*	118.9±5.4

Results represent mean of sections taken from 4 separate animals from sham-lesioned and lesioned animals; ±s.e.mean values are shown. *$P<0.05$; two tailed Mann-Whitney test; InStat V2.02.

the glial component for uptake. The reasons for the differences between these two studies are unclear at present and further work in this area is required.

In conclusion, [^{3}H]-gabapentin binds to rat brain with a discrete localization, somewhat similar to the terminals of excitatory amino acid input pathways. Correlations of the [^{3}H]-gabapentin binding site distribution with the binding distributions of excitatory amino acid receptors and uptake sites show no clear co-localization. The binding distributions of the LNAA uptake site-specific ligands L-[^{3}H]-leucine and L-[^{3}H]-isoleucine defined by the non-specific ligand BCH, exhibit marked similarity to the gabapentin binding site distribution. Lesion studies suggest that these sites and the excitatory amino acid uptake site are present on neuronal cell bodies. From these results it may be possible to conclude that [^{3}H]-gabapentin labels the LNAA uptake site, system-L.

Recent [^{3}H]-gabapentin and ^{3}H-large neutral amino acid uptake studies in a crude cerebral cortex synaptosomal preparation have demonstrated that gabapentin and amino acids such as L-leucine share a common uptake site namely system-L (Thurlow *et al.*, 1996). Upon further investigation it is apparent that the structure activity relationship at this uptake site for [^{3}H]-gabapentin is different from that observed in homogenate binding studies as (S)-(+)-3-isobutyl GABA has no significant effect on [^{3}H]-gabapentin uptake at 100 μM (Thurlow *et al.*, 1996) whilst in homogenate binding studies this concentration inhibits [^{3}H]-gabapentin binding to non-specific levels (Thurlow *et al.*, 1993). In a personal communication, Gee and co-workers have identified the solubilized and purified [^{3}H]-gabapentin binding site to be the $\alpha_2\delta$-subunit of a calcium channel. From this further information it appears that [^{3}H]-gabapentin and L-[^{3}H]-leucine indeed label a novel binding site and due to the low affinity of the uptake site (Thurlow *et al.*, 1996), it would appear that in this study the distributions described may be that of the $\alpha_2\delta$-subunit of a calcium channel.

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