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The novel antiepileptic drug levetiracetam (ucb L059) appears to act via a specific binding site in CNS membranes

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Abstract

Levetiracetam ((*S*)-α-ethyl-2-oxo-pyrrolidine acetamide, ucb L059) is a novel potential antiepileptic agent presently in clinical development with unknown mechanism of action. The finding that its anticonvulsant activity is highly stereoselective (Gower et al., 1992) led us to investigate the presence of specific binding sites for [^{3}H]levetiracetam in rat central nervous system (CNS). Binding assays, performed on crude membranes, revealed the existence of a reversible, saturable and stereoselective specific binding site. Results obtained in hippocampal membranes suggest that [^{3}H]levetiracetam labels a single class of binding sites ($n_H = 0.92 \pm 0.06$) with modest affinity ($K_d = 780 \pm 115$ nM) and with a high binding capacity ($B_{max} = 9.1 \pm 1.2$ pmol/mg protein). Similar K_d and B_{max} values were obtained in other brain regions (cortex, cerebellum and striatum). ucb L060, the (*R*) enantiomer of levetiracetam, displayed about 1000 times less affinity for these sites. The binding of [^{3}H]levetiracetam is confined to the synaptic plasma membranes in the central nervous system since no specific binding was observed in a range of peripheral tissues including heart, kidneys, spleen, pancreas, adrenals, lungs and liver. The commonly used antiepileptic drugs carbamazepine, phenytoin, valproate, phenobarbital and clonazepam, as well as the convulsant agent *t*-butylbicyclophosphorothionate (TBPS), picrotoxin and bicuculline did not displace [^{3}H]levetiracetam binding. However, ethosuximide ($pK_i = 3.5 \pm 0.1$), pentobarbital ($pK_i = 3.8 \pm 0.1$), pentylenetetrazole ($pK_i = 4.1 \pm 0.1$) and bemegride ($pK_i = 5.0 \pm 0.1$) competed with [^{3}H]levetiracetam with pK_i values comparable to active drug concentrations observed in vivo. Structurally related compounds, including piracetam and aniracetam, also displaced [^{3}H]levetiracetam binding. (*S*) Stereoisomer homologues of levetiracetam demonstrated a rank order of affinity for [^{3}H]levetiracetam binding in correlation with their anticonvulsant activity in the audiogenic mouse test ($r^2 = 0.84$, $n = 12$, $P < 0.0001$). These results support a possible role of this binding site in the anticonvulsant activity of levetiracetam and substantiate the singular pharmacological profile of this compound. This site remains however to be further characterised.

Keywords: Levetiracetam; ucb L059; Epilepsy; Anticonvulsant; Receptor binding

1. Introduction

Levetiracetam ((*S*)-α-ethyl-2-oxo-pyrrolidine acetamide, ucb L059) is a novel broad-spectrum anticonvulsant agent, with potential antiepileptogenic and antiabsence activity and a unique safety profile. This compound is presently in clinical development (phase II) in Europe and in the USA for the treatment of epileptic patients.

Gower et al. (1992) reported that levetiracetam exerts anticonvulsant effects within the range of 5.0–30.0 mg/kg, in a variety of generalised seizure models, including tonic and clonic audiogenic seizures in mice, tonic seizures in the maximum electroshock seizure test in mice and tonic seizures induced in rodents by a variety of chemoconvulsants including pentylenetetrazole, picrotoxin and *N*-methyl-D-aspartate (NMDA). The anticonvulsant activity of levetiracetam was most marked against submaximal rather than maximal doses of the γ-aminobutyric acid (GABA) related chemoconvulsants. Löscher and Hönack (1993) demonstrated that levetiracetam also exerts anticonvulsant effects in seizure threshold tests and markedly reduces the severity and duration of both focal and secondary generalized seizures elicited with suprathreshold stimuli in amygdala-kindled rats (Löscher and Hönack, 1993), a model of complex partial seizures with secondary generalisation (Löscher et al., 1986). The potency of leve-

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tiracetam in these tests was comparable with that of standard drugs, such as phenytoin and valproate.

In addition to an anticonvulsant action, levetiracetam was also demonstrated by Gower et al. (1992) to inhibit the development of pentylenetetrazole-induced kindling in mice (a model of epileptogenesis; McNamara, 1989) and against amygdala kindling in rats (Löscher, personal communication). Drugs such as phenytoin and carbamazepine are inactive against the development of kindling, whether chemical or electrical, although all of these drugs will prevent seizures elicited in fully kindled animals. In contrast, benzodiazepines and valproate are active both against kindling development and fully kindled seizures (Löscher and Schmidt, 1988). These data demonstrate that the anticonvulsant profile of levetiracetam is unique as it displays a broad spectrum of action, incorporating features in common with several different types of antiepileptic drugs. Most interestingly, these results also demonstrate that this compound clearly differs from all standard antiepileptic drugs since it displays a particularly high therapeutic index with, for example, a dose ratio of 50–100 times for sedation versus anticonvulsant properties. Moreover, in contrast to most clinically established antiepileptic drugs, levetiracetam appears to be free of adverse cognitive effect, and, in fact, demonstrated nootropic properties in a scopolamine-induced amnesia model in mice (Verloes et al., 1988).

The mechanism of action of levetiracetam is unknown as yet. Its anticonvulsant activity, which appears to be mediated by the parent compound and not by its metabolites (Gower and Matagne, 1994), is highly stereoselective since ucb L060, its (*R*) enantiomer, lacks activity (Gower et al., 1992). The capacity of the drug to interact with a large variety of binding sites related to convulsions has been investigated, using labelled classical neurotransmitters as radioligands. As no significant affinity for known receptors was found in these experiments (Table 3), the presence of a specific binding site for levetiracetam was investigated in rat brain tissues using [^{3}H]levetiracetam as radioligand. This study reports the discovery of a specific binding site for [^{3}H]levetiracetam which could be involved in the anticonvulsant properties of this compound. Some of these data were communicated to the British Pharmacological Society meeting in April, 1994 (Gillard et al., 1994).

2. Materials and methods

2.1. Animals

Sprague-Dawley male rats (200–300 g), from Iffa-Credo, Belgium, killed by decapitation, were used for the binding experiments. Male or female DBA-derived mice aged 4–5 weeks (14–22 g) and genetically sound-sensitive were used for the audiogenic seizure tests.

2.2. Audiogenic seizures in mice

The mice were administered by the intraperitoneal route with the test substance and 60 min later placed in individual cages in a sound-attenuated cabinet. After 30 s, a 90-dB, 10-to 20-Hz acoustic stimulus was delivered for 30 s via loudspeakers positioned above each cage. The presence of tonic convulsions was noted for each mouse. Percent protection afforded by each dose of drug was calculated and the ED_{50} value, defined as the effective dose producing 50% protection, was computed as described by Gower et al. (1992).

2.3. Membrane preparation

Brain tissue

Brains were quickly removed and the cerebral tissues were dissected on ice. All subsequent operations were performed at 4°C. The tissues were homogenised in 20 mM Tris-HCl buffer (pH 7.4) containing 250 mM of sucrose (buffer A). The homogenates were spun at $30\,000 \times g$ for 15 min and the pellets resuspended in the same buffer. After incubation at 37°C for 15 min, the membranes were washed 3 times using the same centrifugation protocol. The final pellets were resuspended in buffer A at a protein concentration of 10–15 mg/ml and stored in liquid nitrogen.

Subcellular fractions from cortical tissue were isolated according to Jones and Matus (1974) with minor modifications. The tissue was homogenized in 9 vols. (w/w) of 5 mM Tris-HCl (pH 8.0), 0.32 M sucrose buffer. The homogenate was centrifuged at $1000 \times g$ for 10 min and the supernatants removed and centrifuged at $9000 \times g$ for 20 min. The resulting pellet was resuspended in hypotonic buffer (5 mM Tris, pH 8.0) and incubated for 60 min at 4°C followed by 6 strokes in a hand-operated glass-Teflon homogenizer. After centrifugation at $30\,000 \times g$ for 15 min, the pellet was resuspended in 1.2 M sucrose. The fractionation was achieved on a discontinuous sucrose gradient (0.6, 0.8, 1.0 and 1.2 M) with the sample being in the 1.2 M phase. Centrifugation was achieved at $60\,000 \times g$ for 120 min (4°C) in a swing-out rotor. The pellet and each of the interphases were kept for further characterisation by binding experiments with [^{3}H]NMS, [^{3}H]-RX821002 and [^{3}H]PK11195, to label synaptic plasma membranes and mitochondrial membranes (Antkiewicz-Michaluk et al., 1988). Protein was determined by the method of Lowry et al. (1951).

Peripheral tissues

Peripheral tssues were homogenised in buffer A and washed 3 times at $30\,000 \times g$ as for brain tissue. The

final pellet was resuspended in the same buffer at a protein concentration of 10–15 mg/ml and stored in liquid nitrogen.

2.4. Binding assays with [^{3}H]levetiracetam

Membrane proteins (0.2–0.3 mg/assay) were incubated 60 min at 25°C or 120 min at 4°C in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, [^{3}H]levetiracetam (8–10 nM) and increasing concentrations of unlabelled competing drugs. Non-specific binding was assessed by including 1 mM of unlabelled levetiracetam in the assay. Similar levels of non-specific binding were observed with structurally unrelated drugs in competition experiments. At the end of the incubation period, the membrane-bound radioligand was recovered by rapid filtration through GF/C glass fiber filters presoaked in 0.1% polyethyleneimine and 1 mM levetiracetam. The membranes were washed 4 times with 2 ml of ice-cold Tris buffer. The total filtration procedure did not exceed 10 s per sample. The filters were dried and the radioactivity was determined by liquid scintillation.

Saturation studies

Membranes were incubated with varying concentrations of [^{3}H]levetiracetam (concentrations above 20 nM were obtained by isotopic dilution) for 120 min at 4°C in 50 mM Tris buffer, pH 7.4, as described above. The specific binding obtained at each concentration was corrected for dilution using the following equation:

$$B_{TOT} = B_{OBS} \times \left(1 + \frac{[\text{non-radioactive levetiracetam}]}{[\text{radioactive levetiracetam}]}\right)$$

where: B_{TOT} = total levetiracetam specific binding and B_{OBS} = experimentally observed specific binding.

Kinetic studies

For association kinetics, specific [^{3}H]levetiracetam binding was measured at the indicated times after addition of the membranes. For dissociation studies, membranes were first incubated 60 min (25°C) or 120 min (4°C) with [^{3}H]levetiracetam 10 nM. Further association of the radioligand was then prevented by the addition of 1 mM levetiracetam and the samples were filtered thereafter at the times indicated.

Characterisation of subcellular fractions by radioligand binding

Aliquots of each fraction (150 μg of proteins for [^{3}H]levetiracetam, [^{3}H]RX821002 and [^{3}H]PK11195 binding or 20 μg of proteins for [^{3}H]NMS binding) were incubated for 120 min at 4°C with 10 nM [^{3}H]levetiracetam or for 60 min at 25°C with 1 nM [^{3}H]RX821002, 0.2 nM [^{3}H]PK11195 or 0.2 nM [^{3}H]NMS. Non-specific binding of [^{3}H]levetiracetam, [^{3}H]RX821002, [^{3}H]PK11195 and [^{3}H]NMS was determined by the inclusion in the assay of 1 mM levetiracetam, 10 μM phentolamine, 1 μM PK11195 and 1 μM atropine, respectively.

Pharmacological specificity

In order to characterise the nature of the [^{3}H]levetiracetam binding site, a range of compounds from different pharmacological classes were examined for their ability to inhibit [^{3}H]levetiracetam binding, as described above.

2.5. Receptor binding assay profile of levetiracetam

Competition binding assays with unlabelled levetiracetam were effected on various membrane receptors by the Company NovaScreen, Baltimore, MD, USA, using published protocols.

2.6. Drugs and radioligands

γ-Amino butyric acid (GABA), (±)-HA-966, phenytoin, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195), *tert*-butyl-bicyclo[2.2.2]phosphorothionate (TBPS), baclofen and nipecotic acid were purchased from RBI (Natick, MA, USA). Bemegride was purchased from Aldrich Chemicals (Bornem, Belgium). Bicuculline, carbamazepine, dimethadione, ethosuximide, pentylenetetrazole, picrotoxin, pronase, *l*-phenylalanine and valproate were bought from Sigma Chemicals (Bornem, Belgium). Trypsin was purchased from Gibco BRL (Gent, Belgium). Phenobarbital was purchased from VEL (Leuven, Belgium). Diazepam and clonazepam were obtained from Hoffmann-La Roche (Basle, Switzerland). Levetiracetam (ucb L059), its homologues and aniracetam were synthesised at UCB Pharmaceutical Sector, Belgium.

[*N*-methyl-^{3}H]PK11195 (85 Ci/mmol) was purchased from New England Nuclear (Brussels, Belgium). (1,4-[6,7(*n*)-^{3}H]benzodioxan-2-methoxy-2-yl)-2-imidazoline ([^{3}H]RX821002; 62 Ci/mmol) and *l*-[*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS; 84 Ci/mmol) were obtained from Amersham (Gent, Belgium). [^{3}H]Levetiracetam (36.6 Ci/mmol) was from Isotopchim (France).

3. Results

3.1. Saturation and kinetic studies for [^{3}H]levetiracetam binding

[^{3}H]Levetiracetam displayed complex association and dissociation kinetics. At 4°C, equilibrium was

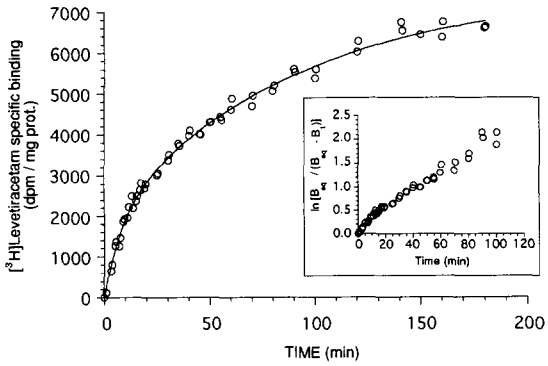


Fig. 1. Time course for the association of [^{3}H]levetiracetam binding to hippocampal membranes at 4°C. Specific binding of 10 nM [^{3}H]levetiracetam was determined at various time intervals. The curve is representative of three experiments each performed in duplicate. The inset shows transformed data where B_t is the specific binding at time t and B_{eq} is the specific binding measured at equilibrium.

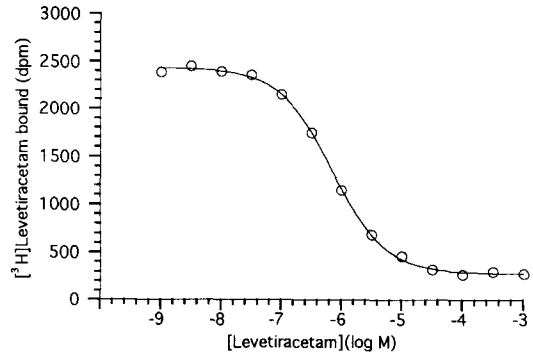


Fig. 3. Inhibition of [^{3}H]levetiracetam binding to hippocampal membranes. [^{3}H]Levetiracetam (10 nM) was incubated at 4°C in the presence of increasing concentrations of non-radioactive levetiracetam. The curves are representative of three separate experiments done in duplicate. pIC_{50} value was 6.1 ± 0.1.

reached after 120 min incubation, and the half-lives of [^{3}H]levetiracetam dissociation were 1.2 ± 0.8 min ($k_{off} = 0.8 \pm 0.6$ min^{-1}) calculated from the fast component ($31 \pm 9\%$ of the binding sites) and 37 ± 5 min ($k_{off} = 0.019 \pm 0.003$ min^{-1}) from the slow component (Figs. 1–3). At 25°C, binding steady state was achieved within 10–15 min (not shown) and the off-rates were very fast with $66 \pm 6\%$ of [^{3}H]levetiracetam dissociating with a $t_{1/2}$ of 0.5 ± 0.2 min ($k_{off} = 1.5 \pm 0.8$ min^{-1}) and the remaining 34% with a $t_{1/2}$ of 5 ± 2 min ($k_{off} = 0.14 \pm 0.05$ min^{-1}) (Fig. 2). At the radioligand concentration used (8–10 nM), the specific binding was much higher at 4°C than at 25°C (88% versus 70% of total binding), giving a much better ratio of total to non-specific binding (not shown). This is a consequence of the 2-fold higher affinity of [^{3}H]levetiracetam for its binding sites at 4°C ($pIC_{50} = 6.11 \pm 0.06$) compared to the affinity at 25°C ($pIC_{50} = 5.85 \pm 0.07$). The slower kinetics and the sligthly higher affinity of [^{3}H]levetiracetam at 4°C prompted us to perform most of the binding experiments at this temperature.

On account of the relatively low affinity of [^{3}H]levetiracetam for its binding sites, it was necessary to obtain saturation curves by isotopic dilution of the radioligand with unlabelled levetiracetam, although this method causes some imprecision in the determination of the maximun number of binding sites. A representative experiment is shown in Fig. 4, where the data are

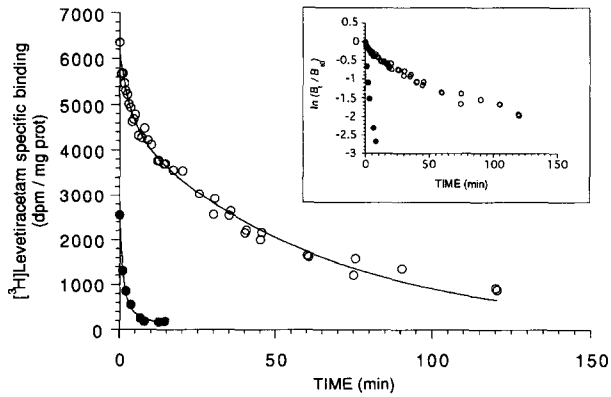


Fig. 2. Time course of the dissociation of [^{3}H]levetiracetam binding from hippocampal membranes. [^{3}H]Levetiracetam (10 nM) was incubated during 120 min at 4°C (open circles) or during 60 min at 25°C (closed circles). Tracer dissociation was induced by the addition of 1 mM unlabelled levetiracetam and the amount of specifically bound radioligand was determined at various time intervals. The curves are representative of three separate experiments each performed in duplicate. The inset shows the same data transformed as a semilog plot. The curvilinear nature of the curve at 4°C suggests dissociation from more than one site.

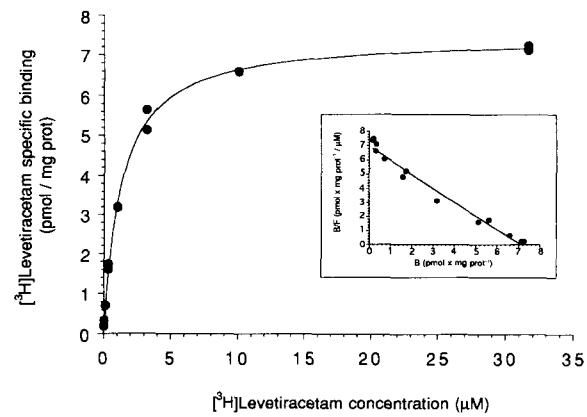


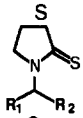
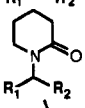
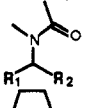
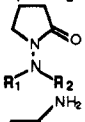
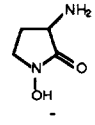
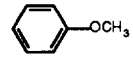
Fig. 4. Saturation of [^{3}H]levetiracetam binding to hippocampal membranes. High tracer concentrations were obtained by isotopic dilution with unlabelled levetiracetam and specific binding was calculated as explained in Materials and methods. The graph shows a saturation curve from a single experiment in which each point was determined in duplicate. The inset shows a Scatchard plot of the same data, suggesting binding to a single population of binding sites. Data from five separate experiments yielded a mean K_D of 0.8 ± 0.2 μM and a maximum binding capacity (B_{max}) of 9.1 ± 1.2 pmol/mg protein.

transformed according to the equation given in Materials and methods. It appeared that [^{3}H]levetiracetam recognised a homogeneous population of binding sites in rat hippocampus with a K_d of 780 ± 115 nM, a Hill coefficient of 0.92 ± 0.06 and a B_{max} of 9.1 ± 1.2 pmol/mg protein.

The binding of [^{3}H]levetiracetam is highly stereoselective since ucb L060, the D-stereoisomer of levetiracetam, was 1000-fold less potent than levetiracetam in inhibiting the binding of the radioligand (40–60% inhibition at 1 mM for ucb L060 compared to a pIC_{50} of 6.1 for levetiracetam).

Specific binding increased linearly with protein concentrations up to 2 mg/ml (not shown). Heating the tissue at 65°C for 20 min or treating the tissue with trypsin (0.01%) or pronase (0.01%) for 20 min at 37°C before incubation with [^{3}H]levetiracetam abolished about 60–80% of the specific binding. This binding was

Table 1
Affinity of levetiracetam analogues for the [^{3}H]levetiracetam binding site in rat CNS membranes. Comparison with their anticonvulsant activity in the audiogenic mouse test

Compound	Structure			Binding	Audiogenic mouse	
	R1		R2	pKi	ED_{50} mmol/kg	Range
Piracetam	H	-	CONH2	4.5±0.1	2.1	1.3-7.6
Levetiracetam	C2H5	S	CONH2	6.1±0.1	0.05	0.04-0.07
ucb L060	C2H5	R	CONH2	<3.0	>1.0	ND
3	CH3	S	CONH2	5.5±0.1	0.10	0.04-0.19
13	CH3	R	CONH2	<3.0	#3.0	
4	C3H7	S	CONH2	5.3±0.0	0.13	0.03-0.22
14	cycloC3H5	-	CONH2	#4.0	#1.0	
5	isoC3H7	S	CONH2	4.7±0.1	0.66	ND
15	C4H9	R	CONH2	<3.0	0.41	0.37-0.47
6	C4H9	S	CONH2	5.0±0.0	0.18	0.13-0.32
16	isoC4H9	S	CONH2	<4.0	#1.0	
7	C6H13	S	CONH2	5.3±0.1	0.24	0.20-0.29
17	C6H13	R	CONH2	4.3	0.13	ND
18	C2H4CONH2	d l	CONH2	#4.0	#1.0	
8	C2H5	S	CONHCH3	4.9	0.4	0.25-0.61
19	C2H5	d l		#4.0	#1.0	
9	C2H5	S	CSNH2	5.0	0.46	ND
20	C2H5	S	CONHCH2Φ	#4.0	#0.56	
21	C2H5	S	COOH	#4.0	#3.0	
10	C2H5		CONH2	6.3	0.043	0.022-0.060
11	C2H5		CONH2	5.2±0.1	0.42	0.18-1.45
12	C2H5		CONH2	4.8	0.92	0.64-1.45
22	C2H5		CONH2	#4.0	#1.0	
HA 966	-		-	#3.0	0.073[a)]	0.048-0.096
Aniracetam	0	-		3.0±0.2	>3.0	ND

pK_i values are means ± S.D. from at least three separate experiments using 8 nM [^{3}H]levetiracetam and rat hippocampal membranes at 4°C. The ED_{50} values for protection against the tonic convulsions are means with 95% confidence limits. # signifies inactivity at the dose indicated (maximum dose tested). [a)] Compound was administered 30 min before accoustic stimulus.

also dependent upon pH with optimal levels of specific binding at pH 7.4–8.0 (not shown). These data support a protein nature of the levetiracetam binding site.

3.2. Tissue distribution of [^{3}H]levetiracetam binding sites

In order to determine whether the [^{3}H]levetiracetam binding site was unique to the brain, [^{3}H]-levetiracetam was incubated with crude membranes prepared from a variety of peripheral tissues (Fig. 5). Strikingly, specific binding was only detected in the brain structures tested (hippocampus, cortex and cerebellum) and none could be measured in any of the peripheral tissues investigated (liver, lung, kidney, spleen, pancreas, heart, adrenals). [^{3}H]Levetiracetam binding characteristics, with respect to the affinity and the B_{max}, were quite similar in all brain regions investigated (data not shown).

3.3. Subcellular distribution of [^{3}H]levetiracetam binding

A subcellular fractionation of a cortex homogenate was carried out according to Jones and Matus (1974), using [^{3}H]NMS, [^{3}H]RX821002 and [^{3}H]PK11195 as markers for synaptic plasma membranes and mitochondrial membranes, and the binding of [^{3}H]levetiracetam was determined among the different cellular substructures. As illustrated in Fig. 6, the majority of the [^{3}H]levetiracetam binding was detected in the same fractions as the binding of [^{3}H]NMS and [^{3}H]-RX821002, i.e. in the synaptic plasma membranes. As for [^{3}H]NMS and [^{3}H]RX821002, only a small amount of [^{3}H]levetiracetam binding was located in the mitochondrial membrane fractions (sucrose 1.2 M) labelled with [^{3}H]PK11195 and no binding was found in the myelin containing fraction (sucrose 0.6 M).

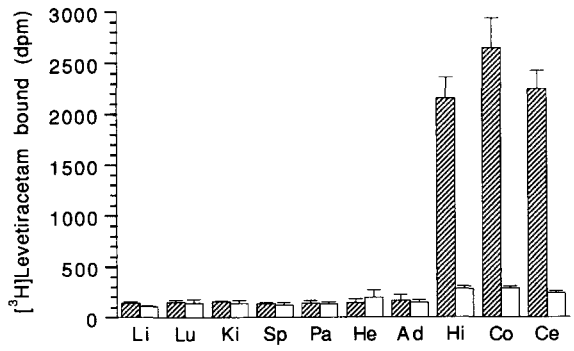


Fig. 5. Comparison of [^{3}H]levetiracetam binding in rat brain and in a variety of peripheral tissues. 300 μg of proteins from each tissue were incubated 120 min at 4°C with [^{3}H]Levetiracetam (10 nM) in the absence (total binding: hatched bars) or in the presence of 1 mM unlabelled levetiracetam (non-specific binding: open bars). Samples were done in triplicate. Li, liver; Lu, lung; Ki, kidney; Sp, pleen; Pa, pancreas; He, heart; Ad, adrenal; Hi, hippocampus; Ce, cerebellum; Co, cortex.

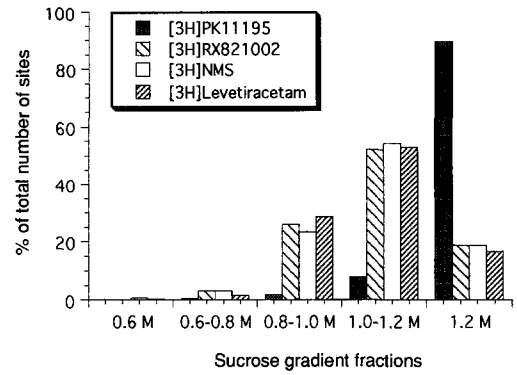


Fig. 6. Subcellular distribution of [^{3}H]levetiracetam binding in rat CNS. A P2 pellet from rat brain cortex was fractionated on a discontinuous sucrose gradient at 60000 × g according to Jones and Matus (1974). The pellet and each interphase were characterised by binding experiments with [^{3}H]NMS and [^{3}H]RX821002 to label synaptic plasma membranes and with [^{3}H]PK11195 to label mitochondrial membranes. Data are representative of two experiments and are expressed as percentage of the total specific binding found in pellet P2.

3.4. Pharmacological specificity of [^{3}H]levetiracetam binding

Levetiracetam homologues

The α-alkyl-2-oxo-pyrrolidine acetamide homologues of levetiracetam sharing a (R) absolute configuration (ucb L060 and compounds 13, 15 and 17) displayed much less affinity for the [^{3}H]levetiracetam binding sites than their respective (S) stereoisomers (levetiracetam and compounds 3, 6 and 7), with stereospecificity index (ratios of K_i values) comprised between 1000 and 10 (Table 1). The stereospecificity index between the optical isomers appeared to decrease with the length of the alkyl chain. Moreover, a relation appeared between the K_i values and the length of the alkyl chain for the (S)-α-alkyl-2-oxo-pyrrolidine acetamide homologues of levetiracetam. Piracetam was the least active ($pK_i = 4.5 \pm 0.1$) and levetiracetam the most active compound ($pK_i = 6.1 \pm 0.1$) among the drugs tested. The methyl homologue (compound 3) and the homologues with longer alkyl chain displayed intermediate affinities. Modifications of the acetamide moiety (compounds 8, 21 and 20ucb 29903), as well as the pyrolidinone ring (compounds 11 and 12) produced profound reduction of the affinity. However, the 2-thio-pyrrolidine derivative was equiactive to levetiracetam. HA 966 was inactive and aniracetam displayed a low affinity ($pK_i = 3.0 \pm 0.2$).

Relation to the anticonvulsant properties of levetiracetam homologues

The levetiracetam analogues mentioned above were also tested for their anticonvulsant activity in the audiogenic mouse model of epilepsy. As for the binding,

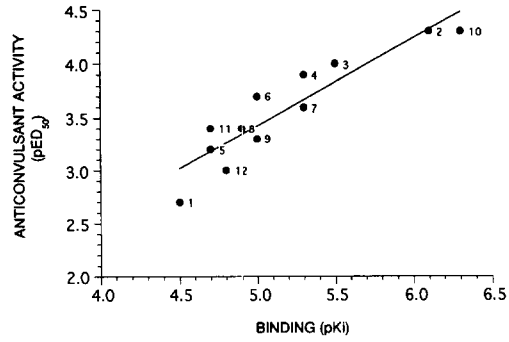


Fig. 7. Comparison between the affinity of (*S*) homologues of levetiracetam at the [^{3}H]levetiracetam binding site with the anticonvulsant activity of these compounds in the audiogenic mouse test. $r^2 = 0.84$, $P < 0.0001$, $n = 12$. 1 = piracetam; 2 = levetiracetam.

the α-alkyl-2-oxo-pyrrolidine acetamide homologues with a (*R*) absolute configuration were less effective in protecting the mice against sound-induced convulsions (Table 1). Moreover, a fairly good correlation ($r^2 = 0.84$; $n = 12$; $P < 0.0001$) was observed between the affinity and the anticonvulsant activity of the (*S*)-α-alkyl-2-oxo-pyrrolidine acetamide homologues of levetiracetam (Fig. 7).

Antiepileptic drugs

The results in Table 2 show that neither phenytoin, carbamazepine, sodium valproate, phenobarbital, dimethadione nor benzodiazepines (diazepam and clonazepam) display any relevant affinity for the levetiracetam binding site in comparison with pharmacological active doses, with IC_{50} values higher than 0.1–1.0 mM. The anti-absence drug, ethosuximide, was an exception with a pK_i of 3.5 ± 0.1, a value seemingly compatible with effective plasma levels in patients (0.4–0.8 mM), as reported by Meldrum and Porter (1986).

Table 2
Affinities for the [^{3}H]levetiracetam binding site of antiepileptic drugs and some GABA-related substances

Compounds	pK_i	Compounds	pK_i
Antiepileptics		*GABA-related*	
Phenobarbital	#3.0	GABA	#3.0
Phenytoin	#4.0	Nipecotic acid	#4.0
Carbamazepine	#4.0	Baclofen	< 3.0
Valproate	#3.0	Bicuculline	#4.0
Ethosuximide	3.5 ± 0.1	PK11195	≤ 4.0
Dimethadione [1]	≤ 2.0	Picrotoxin	#3.0
Diazepam	≤ 4.0	TBPS [2]	#5.0
Clonazepam	≤ 4.0	Pentylenetetrazole (PTZ)	4.1 ± 0.1
		Bemegride	5.0 ± 0.1
Miscellaneous			
l-Phenylalanine	< 4.0		

pK_i values are means ± S.D. from at least three separate experiments using 8 nM [^{3}H]levetiracetam and rat hippocampal membranes at 4°C. [1] Brain cortex membranes; [2] *t*-butylbicyclophosphorothionate, binding assay also done in presence of KCl 100 mM. Standard deviations are indicated when $n \geq 3$. # indicates less than 20% inhibition at the concentration indicated (concentration maximum tested).

GABA-related compounds

GABA and baclofen, its β-chlorophenyl derivative, an agonist with greater specificity for $GABA_B$ receptors, did not demonstrate affinity (Table 2). This is in agreement with the data obtained with [^{3}H]GABA and unlabelled levetiracetam (Table 3). The phthalide isoquinoline (+)-bicuculline, a $GABA_A$ receptor antagonist, and nipecotic acid, a GABA uptake inhibitor, were also inactive.

Like the 'central-type' benzodiazepines (diazepam and clonazepam), the 'peripheral-type' benzodiazepine isoquinoline carboxamide (PK11195), characterised by a low affinity for the brain-type sites linked to the $GABA_A$ receptor, did not display any relevant affinity for the levetiracetam binding site ($pK_i \leq 0.1$ mM).

Table 3
Receptor profile of levetiracetam

Amino acid-related		*Adenosine*	
Quisqualate (1)	[^{3}H]AMPA	Adenosine 1 (6)	[^{3}H]CPX
Kainate (1)	[^{3}H]Kainic acid	Adenosine 2 (7)	[^{3}H]NECA
MK-801 (1)	[^{3}H]MK-801		
NMDA (1)	[^{3}H]CGS19755	*Channel proteins*	
Phencyclidine (1)	[^{3}H]TCP	Calcium, T and L (6)	[^{3}H]Nitrendipine
Sigma (2)	[^{3}H]DTG	Calcium, N (1)	[^{125}I]ω-Conotoxin
Glycine (3)	[^{3}H]Strychnine	Chloride (6)	[^{3}H]TBOB
Glycine (6)	[^{3}H]Glycine	Potassium (1)	[^{125}I]Apamin
$GABA_A$ (4)	[^{3}H]GABA		
$GABA_B$ (1)	[^{3}H]GABA		
Benzodiazepine (5)	[^{3}H]Flunitrazepam		

Levetiracetam did not displace any of the above radioligands at a concentration up to 10 μM. Origin of membranes as follows: (1) rat forebrain, (2) guinea-pig whole brain, (3) rat spinal cord, (4) bovine cerebellum, (5) bovine cortex, (6) rat cortex, (7) rat striatum. Abbreviations: CPX, cyclopentyl-1,3-dipropylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; AMPA, amino-3-hydroxy-5-methylisoxazole-4-propionic acid; TCP, *N*-(1-[2-thienyl]cyclohexyl) 3,4-piperidine; DTG, 1,3-di-(*o*-tolyl)guanidine; TBOB, *t*-butylbicyclo-orthobenzoate.

$GABA_A$ receptor ionophore antagonists were also investigated. Picrotoxin and the cage convulsant TBPS were both inactive (Table 2). The inactivity of TBPS is in agreement with the results obtained for competition binding of [^{3}H]TBOB, a parent compound of TBPS, and unlabelled levetiracetam (Table 3). Pentylenetetrazole and bemegride are two convulsivants reported to act at the picrotoxin site of the GABA complex. They display similar pharmacological profile, both in electrophysiology (Scholfield, 1982) and ligand binding studies (Ramanjaneyulu and Ticku, 1984). These two compounds both inhibited [^{3}H]levetiracetam binding with pK_i values of 4.1 ± 0.1 and 5.0 ± 0.1, respectively. The pK_i value measured for pentylenetetrazole is compatible with the effective concentrations inhibiting the GABA responses on neuronal cell cultures (De Deyn and Macdonald, 1989) and with the doses effective in vivo. The pK_i value measured for bemegride is also compatible with the dose of levetiracetam (100 μmol/kg) inhibiting bemegride (260 μmol/kg)-induced tonic convulsions in mice (Matagne and Gower, unpublished data).

3.5. Miscellaneous

Gabapentin is a new anticonvulsant which as been described to label a specific binding site in the rat CNS. This binding site may be a system-L-like neutral amino acid carrier in rat brain membranes, as [^{3}H]gabapentin binding is antagonised by neutral amino acids such as *l*-phenylalanine ($IC_{50} = 0.38$ μM) (Thurlow et al., 1993). In order to investigate possible similarity between the levetiracetam binding site and the gabapentin binding site, the affinity of *l*-phenylalanine for the [^{3}H]levetiracetam binding was measured. As shown in Table 2, the IC_{50} of *l*-phenylalanine for levetiracetam binding was higher than 100 μM.

3.6. Receptor profile

The affinity of levetiracetam was also evaluated at a variety of binding sites related to convulsions, such as amino acid-related receptors, adenosine receptors and ion channels. The results showed that levetiracetam was inactive at all the binding sites tested, up to the concentration of 10 μM (Table 3).

4. Discussion

The data reported demonstrate that [^{3}H]levetiracetam labels a homogeneous population of binding sites in the rat brain. This binding is characterised by a high stereoselectivity and an affinity in the micromolar range that is compatible with dose levels required to produce anticonvulsant properties of the compound. Levetiracetam binding appears to be brain specific, with a relatively high capacity, in the 10 pmol/mg protein range. Indeed, using experimental conditions identical to those that permit the labelling of brain membranes, no specific [^{3}H]levetiracetam binding could be detected in a variety of peripheral tissues. However, because of the rather low affinity of the radioligand, the presence of a small population of binding sites in the periphery cannot be excluded, but in any case, their concentration would be one order of magnitude smaller than in the central nervous system. Such an observation suggests that the binding site may not be associated with any general aspect of cellular physiology, which is in agreement with the pharmacological profile of levetiracetam. Moreover, the binding sites seem to be equally distributed in all the brain regions investigated.

As demonstrated by subcellular fractionation of brain cortex tissue, the levetiracetam binding site appears to be preferentially localised on synaptic plasma membranes. Accordingly, it would be anticipated that the levetiracetam binding sites could modulate neuronal functions, provided binding to cortical membranes is representative of binding in other brain regions. The drug could affect neurons directly, by an action on neurone plasma membranes or indirectly via glial cell plasma membranes, as the localisation of the levetiracetam binding sites on glial elements cannot presently be ruled out. The determination of its precise anatomical localisation by appropriate experiments will probably help to understand the functional role of this binding site. In this regard, autoradiographic studies are now in progress.

Binding to the levetiracetam binding site is most sensitive to levetiracetam itself and to close homologues. Unsubstituted acetamide and (*S*)-alkyl pyrolidinine moieties are two structural requirements for a significant affinity at the [^{3}H]levetiracetam binding site. These structural features are more important than the chain length of the alkyl substituant at the α carbon. For the levetiracetam homologues, the absolute (*S*) configuration is also required for good anticonvulsant properties in the audiogenic mice model. Although not a proof, the correlation observed between the affinity of these compounds for the [^{3}H]levetiracetam binding site and their anticonvulsant properties in this model of epilepsy suggests that this binding site is pharmacologically relevant. More functional correlates are needed however to definitively assess a physiological role for this binding site.

Piracetam, the unsubstituted-2-oxo-pyrrolidine acetamide which is widely used to treat cognitive deficits in the elderly (Rosenberg et al., 1990; Sarter, 1991), also recognises the levetiracetam binding site, although with modest affinity. Piracetam is likewise much less active than levetiracetam in standard animal models of

epilepsy. However, this compound potentiates the antiepileptic action of carbamazepine in chronic experimental limbic epilepsy (Hawkins and Mellanby, 1986). It is also pertinent to note that piracetam is able to improve seizure protection in epileptic patients receiving carbamazepine (Chaudhry et al., 1991) and is highly effective as an adjunct therapy in human myoclonia (Brown et al., 1992).

The data obtained with the convulsant agents pentylenetetrazole and bemegride support the hypothesis that some interactions could exist between the levetiracetam binding site and the $GABA_A$ receptor complex. Pentylenetetrazole and bemegride are reported to act at the same site (Ticku and Maksay, 1983). Although a variety of actions have been reported for pentylenetetrazole, there is much evidence to suggest that it may cause convulsions by reducing GABA-mediated inhibition through an action at the picrotoxin site of the $GABA_A$ receptor-linked chloride channel. The results suggest that interactions are possible between levetiracetam and a yet undefined binding site on the $GABA_A$ complex related to the chloride ionophore. However, they do not support a direct action of the compound at this receptor complex.

Benzodiazepines do not recognise the levetiracetam binding site. This is in agreement with data demonstrating that the anticonvulsant action of levetiracetam is not antagonized by flumazenil, a benzodiazepine antagonist (Gower et al., 1994), and with the safety and the pharmacological profile of levetiracetam, particularly its potential cognition-enhancing activity, which is quite different to benzodiazepines.

In addition to benzodiazepines, a variety of clinically effective antiepileptics from different classes do not interact with the [^{3}H]levetiracetam binding sites. It is unlikely therefore that levetiracetam labels a known 'anticonvulsant' binding site, but the possibility cannot be excluded that levetiracetam labels a site involved in the action of a particular type of anticonvulsant. In this respect, the pK_i value obtained for the anti-absence drug ethosuximide indicates that levetiracetam and ethosuximide share a common binding site. Both compounds also share common anticonvulsant properties in animals, but they differ greatly in their pharmacological profile: levetiracetam displays a broader spectrum of anticonvulsant activity and a much higher safety margin. In contrast to levetiracetam, ethosuximide is only active in models representative of 'petit mal', such as the pentylenetetrazole threshold test for clonic seizures and spontaneous absence seizures in rats (Marescaux et al., 1984), and is not active in a test that predicts drugs effective in generalized tonic-clonic seizures ('grand mal'), such as the maximal electroshock seizure test in mice and rats (Löscher and Schmidt, 1988). The results with ethosuximide were confirmed independently by Sacaan and Lloyd (1994), who reported an $IC_{50} = 0.07$ mM in displacing [^{3}H]levetiracetam. These authors also reported displacement of [^{3}H]levetiracetam binding by amiloride, a T-type Ca^{2+} antagonist, whereas the N-type Ca^{2+} antagonist ω-conotoxin and the Na^+ channel antagonist saxitoxin were without effect.

In conclusion, the data presented in this report establish the lack of affinity of the novel anticonvulsant levetiracetam for a variety of binding sites related to convulsions. These results are in agreement with the unique pharmacological profile of levetiracetam, and particularly with the lack of its neurotoxic effect at high doses of the drug. This report also describes the presence in the rat brain of a specific binding site for levetiracetam. This binding site, which is highly stereoselective and present in high concentrations in synaptic plasma membranes, has not yet been characterised.

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