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Binding characteristics of [^{3}H]ucb 30889 to levetiracetam binding sites in rat brain

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Abstract

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®), a novel antiepileptic drug, has been shown to bind to a specific binding site located in brain (levetiracetam binding site [Eur. J. Pharmacol. 286 (1995) 137]). However, [^{3}H]levetiracetam displayed only micromolar affinity for these sites making it an unsuitable probe for further characterization. The present study describes the binding properties of an analogue of levetiracetam: [^{3}H]ucb 30889, (2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide. [^{3}H]ucb 30889 binds reversibly to specific binding sites in rat brain. Kinetics at 4 °C were biphasic with half-times of association and dissociation of, respectively, 3 and 4 min for the fast component and 47 and 61 min for the slow component. [^{3}H]ucb 30889 saturation binding curves were compatible with the labelling of a homogenous population of binding sites having a B_{max} of 4496 ± 790 fmol/mg protein (mean ± S.D., $n = 5$) and a K_d of 62 ± 20 nM (mean ± S.D., $n = 5$), a 20-fold increase in affinity compared to [^{3}H]levetiracetam. Competition binding curves with ligands known to interact with levetiracetam binding sites and tissue distribution restricted to the brain indicated that [^{3}H]ucb 30889 and [^{3}H]levetiracetam bind to the same site. Although levetiracetam binding sites and $GABA_A$ (γ-aminobutyric acid) receptors share some ligands such as pentobarbital and pentylenetetrazol, experiments performed with [^{35}S]TBPS (*tert*-butyl-bicyclo[2.2.2]phosporothionate), a probe for the $GABA_A$ Cl^- channel do not support the hypothesis that levetiracetam binding sites are part of the $GABA_A$ receptor complex. Preliminary autoradiography studies in rat brain revealed that [^{3}H]ucb 30889 labels specific sites in all brain regions and that this binding is concentration-dependently displaced by levetiracetam.

Keywords: Antiepileptic; Binding; Brain; Levetiracetam

1. Introduction

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®) is a novel antiepileptic drug efficacious as adjunctive therapy in the treatment of partial-onset epileptic seizures in adults (Hovinga, 2001; Nash and Sangha, 2001). Compared to other antiepileptics, levetiracetam has favorable pharmacokinetics (Patsalos, 2000), and does not interact with cytochromes *P*450 (Nicolas et al., 1999). It displays a unique pharmacological profile as it is devoid of anticonvulsant activity in the acute maximal electroshock and maximal pentylenetetrazol tests yet shows potent protection in electrically and pentylenetetrazol kindled animals (Klitgaard, 2001; Klitgaard et al., 1998).

The biochemical target and the mechanism(s) of action of levetiracetam remain to be defined. There is little or no effects of levetiracetam on GABA (γ-aminobutyric acid), glutamate or glycine levels in brain nor on the activity of GABA transaminase or glutamic acid decarboxylase (Sills et al., 1997; Tong and Patsalos, 2001). Levetiracetam does not modulate voltage-gated Na^+ or T-type Ca^{2+} currents (Zona et al., 2001) but partially inhibits N-type Ca^{2+} current in hippocampal neurones (Niespodziany et al., 2001). Levetiracetam has also been reported to antagonise negative modulators (Zn^{2+} and β-carbolines) of the $GABA_A$ and glycine receptors, thereby increasing Cl^- fluxes (Rigo et al., 2002). Using tritiated levetiracetam we identified, several years ago, a specific binding site in rat brain that was named levetiracetam binding site (Noyer et al., 1995). Levetiracetam binding sites are unique and do not correspond to any known receptor or channel that might be involved in neuroexcit-

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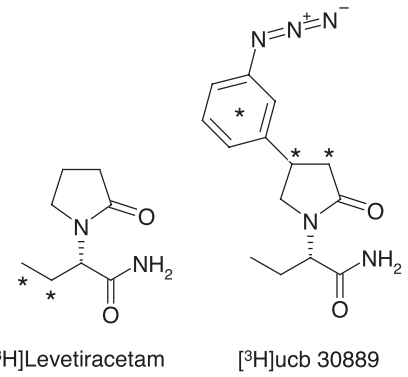

Fig. 1. Chemical structures of [^{3}H]ucb 30889 and [^{3}H]levetiracetam. * denotes the positions of the tritium atom.

ability. Only a few drugs, such as chlordiazepoxide, pentylenetetrazol, bemegride and pentobarbital competed with levetiracetam for its binding sites. Although these drugs are known to interact with the $GABA_A$ receptor complex, GABA, benzodiazepines and carbolines did not modulate levetiracetam binding.

There is a good correlation between the affinity of levetiracetam analogues for levetiracetam binding sites and their potency in suppressing tonic seizures in audiogenic sensitive mice. However, the rather low affinity of labelled levetiracetam for levetiracetam binding sites (close to 1 μM) has thwarted all efforts to further characterise this binding site.

In this paper, we describe the binding characteristics of [^{3}H]ucb 30889 ((2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide) (Fig. 1), a new radioligand with higher affinity for levetiracetam binding sites that circumvents the limitations encountered using tritiated levetiracetam. Furthermore, we demonstrate that this ligand can be used in autoradiography studies.

2. Materials and methods

2.1. Drugs and radioligands

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide), piracetam (2-(2-oxo-1-pyrrolidinyl)acetamide), ucb 30889 ((2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide) and analogues were synthesised at UCB (Braine-l'Alleud, Belgium). Bemegride, bicuculline, carbamazepine, chlordiazepoxide, ethosuximide, felbamate, gabapentin, pentobarbital, phenytoin, picrotoxin, vigabatrin and zonisamide were purchased from Sigma-Aldrich (Bornem, Belgium). Pentylentetrazol was bought from Acros Organics (Pittsburgh, USA). Diazepam was obtained from Hoffmann-La Roche (Basle, Switzerland). Tiagabine was a gift from Novo Nordisk (Denmark). [^{3}H]Levetiracetam (36 Ci/mmol) and [^{3}H]ucb 30889 (32 Ci/mmol) were custom labelled by Isotopchim (France) and Amersham Biosciences (Roosendaal, The Netherlands), respectively. [^{35}S]TBPS (*tert*-butyl-bicyclo[2.2.2]phosporothionate, 200 Ci/mmol) was purchased from Perkin Elmer Life Sciences (Zaventem, Belgium).

2.2. Animals and membrane preparation

2.2.1. Brain tissue

Sprague–Dawley male rats (200–300 g) from Iffa-Credo (Belgium) were sacrificed by decapitation. Brains were quickly removed and the cerebral tissues were dissected on ice. All subsequent operations were performed at 4 °C. The tissues were homogenised (10% w/v) in 20 mM Tris–HCl buffer (pH 7.4) containing 250 mM of sucrose (buffer A). The homogenates were spun at 30,000 × *g* at 4 °C for 15 min and the pellets resuspended in the same buffer. After incubation at 37 °C for 15 min, the membranes were washed three times using the same centrifugation protocol. The final pellets were resuspended in buffer A at a protein concentration of 10 to 15 mg/ml and stored in liquid nitrogen.

For [^{35}S]TBPS binding experiments, membranes were prepared as described by Hulme and Birdsall (1992) with slight modifications. Briefly, membranes from cerebral cortex obtained as described above were thawed and centrifuged at 30,000 × *g* for 15 min (4 °C). The pellet was resuspended in deionized water, homogenised (Ultra-turrax, 10 s at 20,000 rpm) and centrifuged at 55,000 × *g* for 30 min (4 °C). This step was repeated once. The pellet was resuspended in a 10 mM K^+ phosphate buffer (pH 7.4) containing 100 mM KCl and the centrifugation step repeated. The final membrane pellet was resuspended in the same buffer at a protein concentration of 5 mg/ml and used immediately in binding studies.

2.2.2. Peripheral tissues

Peripheral rat tissues were homogenised (10% w/v) in buffer A and washed three times at 30,000 × *g* as for brain tissue. The final pellets were resuspended in the same buffer at a protein concentration of 10–15 mg/ml and stored in liquid nitrogen.

2.3. Binding studies

2.3.1. [^{3}H]levetiracetam and [^{3}H]ucb 30889 binding

Experiments were performed essentially as described in Noyer et al. (1995) for [^{3}H]levetiracetam.

Membrane proteins (0.2–0.3 mg/assay) were incubated 120 min at 4 °C in 0.5 ml of a 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, [^{3}H]ucb 30889 (1 to 2 nM) or [^{3}H]levetiracetam (8 to 10 nM) and increasing concentrations of unlabelled competing drugs. Nonspecific binding was defined as the residual binding observed in the presence of 1 mM of unlabelled levetiracetam. At the end of the incubation period, the membrane-bound radioligand was recovered by rapid filtration through GF/C glass fibre filters pre-soaked in 0.1% polyethyleneimine and 1 mM levetir-

acetam. The membranes were washed with 8 ml of ice-cold Tris buffer (pH 7.4). The total filtration procedure did not exceed 10 s per sample. The filters were dried and the radioactivity determined by liquid scintillation.

For association kinetics, specific [^{3}H]ucb 30889 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]ucb 30889. Further association of the radioligand was then prevented by the addition of 1 mM levetiracetam and the samples were filtered at increasing intervals of time thereafter.

For saturation binding studies, membranes (1 mg of proteins) were incubated 240 min at 4 °C with concentrations of [^{3}H]ucb 30889 ranging from 1 to 500 nM (concentrations above 35 nM were obtained by isotopic dilution).

2.3.2. [^{35}S]TBPS binding

Membranes (200 μg of proteins) were incubated at 25 °C in 0.5 ml of a 10 mM K^+ phosphate buffer (pH 7.4) containing 100 mM KCl and 1 nM [^{35}S]TBPS. For equilibrium experiments, competing drugs were preincubated for 30 min with the membranes before the addition of the radioligand. After 2 h incubation, membrane bound and free radioligand were separated as described above. To measure the effect of drugs on the dissociation rate of [^{35}S]TBPS, binding of the radioligand was allowed to proceed for 2 h before the addition of drugs; 40 min later samples were filtered as described above. Nonspecific binding was defined as the residual binding observed in the presence of 10 μM picrotoxin.

2.3.3. [^{3}H]ucb 30889 autoradiography

Rat brains were quickly removed and frozen in isopentane placed on dry-ice. Frontal slices (25-μm-thick) were cut with a cryostat and mounted on microscope slides covered with gelatin. Slides were preincubated twice for 10 min at room temperature in a 50 mM Tris–HCl buffer (pH 7.4) with 0.5% bovine serum albumin. The incubation was performed at 4 °C for 2 h in a 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.05% bacitracin, 2 mM EGTA, 0.5% bovine serum albumin and 1 pmol/slide of [^{3}H]ucb 30889. Subsequently, slides were washed twice for 10 min in ice-cold preincubation buffer and dipped once in ice-cold water before being dried and exposed to ^{3}H-hyperfilm (Amersham Biosciences) for 3 weeks at −20 °C. Nonspecific binding was defined in the presence of 1 mM levetiracetam.

2.4. Data analysis

Data analysis was performed by computerised nonlinear curve fitting methods (Graphpad Prism® software, San Diego, CA), according to equations describing several binding models (Molinoff et al., 1981). IC_{50} values were corrected to K_i by applying the Cheng and Prusoff (1973) equation. Autoradiography data were analysed and quantified after greyscale scanning using Scion Image software (Scion, Frederick, MD).

3. Results

3.1. Binding kinetics

[^{3}H]ucb 30889 (Fig. 1) binding is reversible with binding kinetics that are temperature dependent. In rat cerebral cortex, at 4 °C, kinetics are biphasic (Fig. 2) with a fast component having, respectively, half-times of association (at a radioligand concentration of 1 nM) and dissociation of 3 ± 2 and 4 ± 1 min and a slow component having half-times of association and dissociation of 47 ± 13 and 61 ± 15 min. At 25 °C, only one fast component with a half-time of 2 ± 2 min remains (Fig. 2) and the total number of sites labelled is decreased by more than 40% (data not shown). Since increasing the temperature accelerates the binding kinetics, the fast component seen at 4 °C is probably lost at 25 °C due to experimental limitations (e.g. filtration time). We therefore conducted all subsequent experiments at 4 °C. A comparison of the binding kinetics of [^{3}H]ucb 30889 in hippocampus and cerebral cortex did not reveal any significant differences (Table 1).

3.2. Saturation binding isotherms

Because of the slow binding kinetics observed at 4 °C, samples were incubated for 4 h to allow the lowest concentrations of [^{3}H]ucb 30889 to reach binding equilibrium. The

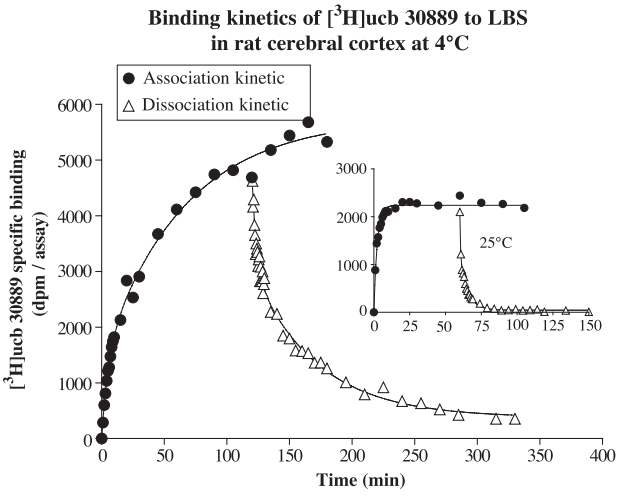

Fig. 2. Binding kinetics of [^{3}H]ucb 30889 in rat cerebral cortex. The binding kinetics were measured as described in Materials and methods either at 4 °C or at 25 °C (inset). Data presented are representative of three separate experiments. The curves are the best fits obtained with equations describing the interaction of a ligand with two independent sites at 4 °C and one site at 25 °C. Kinetic constants calculated at 4 °C are given in Table 1.

Table 1
Binding kinetics constants of [^{3}H]ucb 30889

	Cortex	Hippocampus
Dissociation		
k_{off} fast (min^{-1})	0.16 ± 0.03	0.34 ± 0.11
k_{off} slow (min^{-1})	0.012 ± 0.003	0.012 ± 0.006
% fast	51 ± 9	47 ± 1
$t_{1/2}$ fast (min)	4 ± 1	2 ± 2
$t_{1/2}$ slow (min)	61 ± 15	77 ± 39
Association		
k_{obs} fast (min^{-1})	0.25 ± 0.11	0.50 ± 0.14
k_{obs} slow (min^{-1})	0.016 ± 0.004	0.036 ± 0.019
% fast	22 ± 3	24 ± 2
$t_{1/2}$ fast (min)	3 ± 2	2 ± 1
$t_{1/2}$ slow (min)	47 ± 13	24 ± 12

The binding kinetic constants of [^{3}H]ucb 30889 were calculated by nonlinear regression analysis of data from experiments such as depicted in Fig. 2 and according to equations provided by Prism® software describing the interactions of a ligand with two independent sites (reported here as the slow and fast components). The results are the mean ± S.D. from three separate experiments performed at 4 °C. k_{off} is the dissociation kinetic constant and $k_{obs} = k_{on}L + k_{off}$ where k_{on} is the association kinetic constant and L is the concentration of radioligand (2 nM in our experiments). $t_{1/2}$ is the half time for dissociation or association.

results showed that in rat cerebral cortex, [^{3}H]ucb 30889 binds with high affinity ($K_d = 62 \pm 20$ nM, $n = 5$) to an homogenous population of binding sites (Fig. 3). Under identical experimental conditions, the comparison of saturation isotherms showed that [^{3}H]ucb 30889 has 20-fold higher affinity than [^{3}H]levetiracetam while the total number of sites labelled is comparable (B_{max} of [^{3}H]ucb 30889 = 4496 ± 790 fmol/mg protein, $n = 5$, versus 4391 ± 367 for [^{3}H]levetiracetam, $n = 3$, data not shown). The signal to noise ratio for [^{3}H]ucb 30889 defined as the ratio between total binding and nonspecific binding was excellent at concentrations up to 30 nM where this ratio was above 30.

3.3. Tissue distribution

A panel of rat tissues was tested for the presence of levetiracetam binding sites. Specific binding of [^{3}H]ucb 30889 was only detected in the central nervous system where similar levels of binding sites were observed in cerebral cortex, hippocampus and cerebellum (Fig. 4).

3.4. Competition experiments

The affinity of ucb 30889 as determined in a competition binding experiment with [^{3}H]levetiracetam (100 nM) or [^{3}H]ucb 30889 (80 nM) agrees well with the K_d of [^{3}H]ucb 30889 determined by direct saturation binding experiments (62 nM). In order to further ascertain that [^{3}H]ucb 30889 and [^{3}H]levetiracetam are indeed labelling the same binding sites, we compared the affinity of a variety of ligands and levetiracetam analogues in competition binding studies using both radioligands. Results are shown in Fig. 5 and compiled in Table 2. The correlation obtained was excellent ($r^2 = 0.99$) for a wide range of pIC_{50} values (4.0 to 7.5). Because the concentrations of radioligand used in these experiments (1 and 3 nM for [^{3}H]ucb 30889 and [^{3}H]levetiracetam) are still far below their respective K_d values (60 nM and 1 μM), there is no significant correction to be made between the pIC_{50} and the pK_i values. ucb 30889 and ucb 30890 are two diasterioisomers and their affinity for levetiracetam binding sites differ by a factor of 3. None of the known antiepileptics including carbamazepine, diaze-

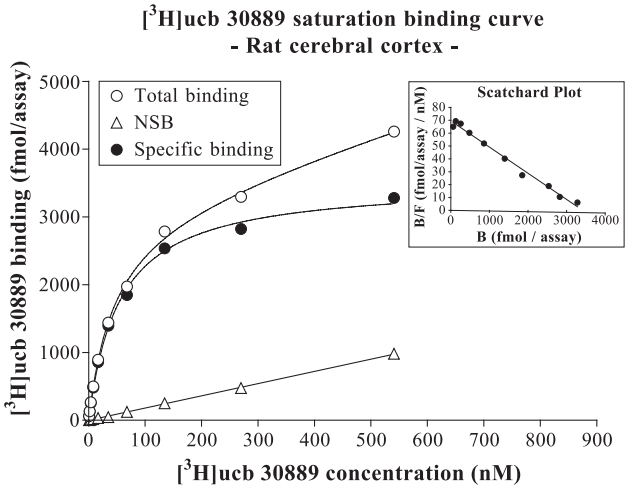

Fig. 3. Saturation isotherm of [^{3}H]ucb 30889 in rat cerebral cortex. Membranes were incubated with increasing concentrations of [^{3}H]ucb 30889 for 240 min at 4 °C. Nonspecific binding (NSB) was determined as the residual binding observed in the presence of 1 mM levetiracetam. The specific binding is obtained after subtraction of the NSB from the total binding. Results are representative of four experiments. Inset: the Scatchard plot from the transformed data.

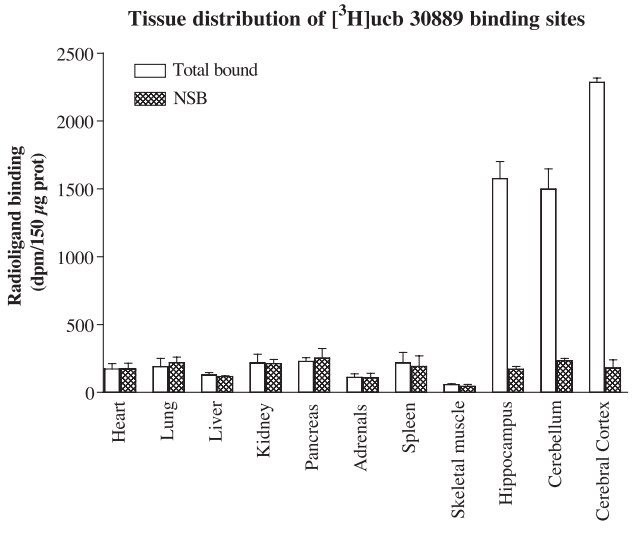

Fig. 4. Selective distribution of [^{3}H]ucb 30889 binding in the central nervous system. Total and nonspecific binding of 2 nM [^{3}H]ucb 30889 were measured in a variety of tissues (150 μg of proteins were used for all tissues). Results are given as the mean ± S.D. of triplicates and are representative of two experiments.

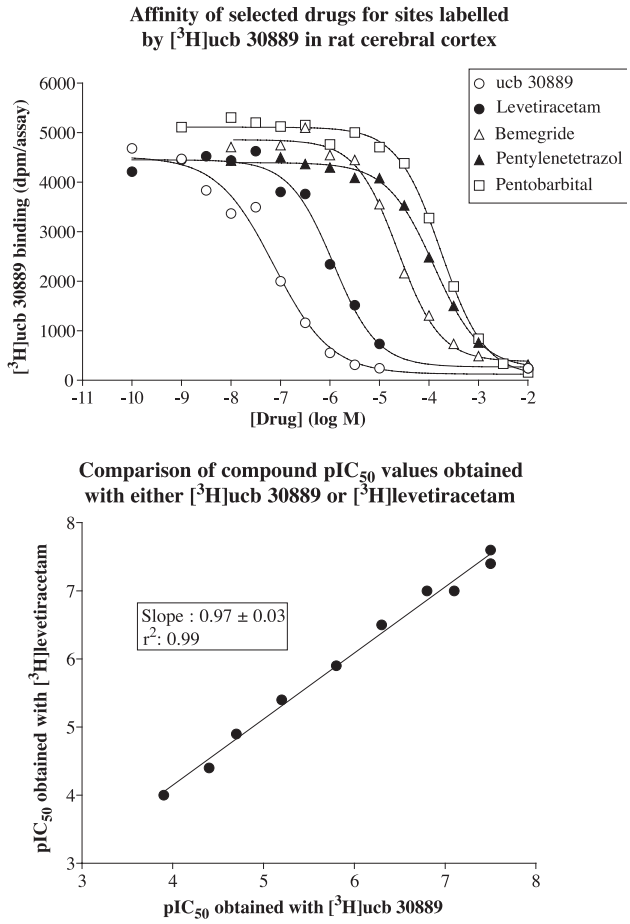

Fig. 5. Correlation in the affinity of selected drugs for levetiracetam binding sites labelled by either [^{3}H]ucb 30889 or [^{3}H]levetiracetam in rat cerebral cortex. Top: Competition curves of selected drugs against [^{3}H]ucb 30889 as radioligand. Compounds were incubated at increasing concentrations with 1 nM of [^{3}H]ucb 30889 for 120 min at 4 °C as described in Materials and methods. Data were analyzed by nonlinear regression with Prism® according to a model with variable slope. The results are representative of three experiments. Bottom: Drugs presented in the top part and several levetiracetam analogues were tested in competition experiments using either [^{3}H]ucb 30889 or [^{3}H]levetiracetam as radioligand. pIC_{50} values (see Table 2) from the two sets of curves (each repeated three times) were plotted against each other and a linear regression was applied to calculate the correlation.

pam, ethosuximide, felbamate, gabapentin, phenytoin, tiagabine, vigabatrin and zonisamide competed with [^{3}H]ucb 30889 when tested at 10 μM, confirming and extending the results of the previous study (Noyer et al., 1995).

3.5. [^{35}S]TBPS binding experiments

Besides analogues of levetiracetam, most drugs that are able to compete for binding to levetiracetam binding sites are also known to interact with the $GABA_A$ receptor complex either at the channel level (pentylenetetrazol, bemegride) or at allosteric sites (pentobarbital) (Table 2). We therefore investigated the possible interaction of levetiracetam and ucb 30889 with these same sites using [^{35}S]TBPS, alone or

Table 2
Affinity of selected compounds for levetiracetam binding sites labelled by [^{3}H]ucb 30889 or [^{3}H]ucb levetiracetam

	[^{3}H]ucb 30889	[^{3}H]Levetiracetam
	$pK_i \pm$ S.D.	$pK_i \pm$ S.D.
Pentylenetetrazol	3.9 ± 0.1	4.1 ± 0.1
Pentobarbital	3.8 ± 0.0	3.8 ± 0.0
Bemegride	4.7 ± 0.1	4.8 ± 0.1
Chlordiazepoxide	5.3 ± 0.1	5.2 ± 0.1
Levetiracetam	5.8 ± 0.2	6.1 ± 0.1
ucb 30889	7.1 ± 0.2	7.0 ± 0.1
ucb 30890	6.6 ± 0.1	6.6 ± 0.1
ucb L060	< 3.0	< 3.0

Results are the mean ± S.D. from three experiments. Data from competition curves such as depicted in the upper part of Fig. 5 were analysed by nonlinear regression and pIC_{50} were corrected to pK_i as explained in Materials and methods. Hill coefficients were not different from unity.

in combination with the above mentioned compounds. Since the buffer and experimental conditions used to measure [^{35}S]TBPS binding are quite different from the ones used

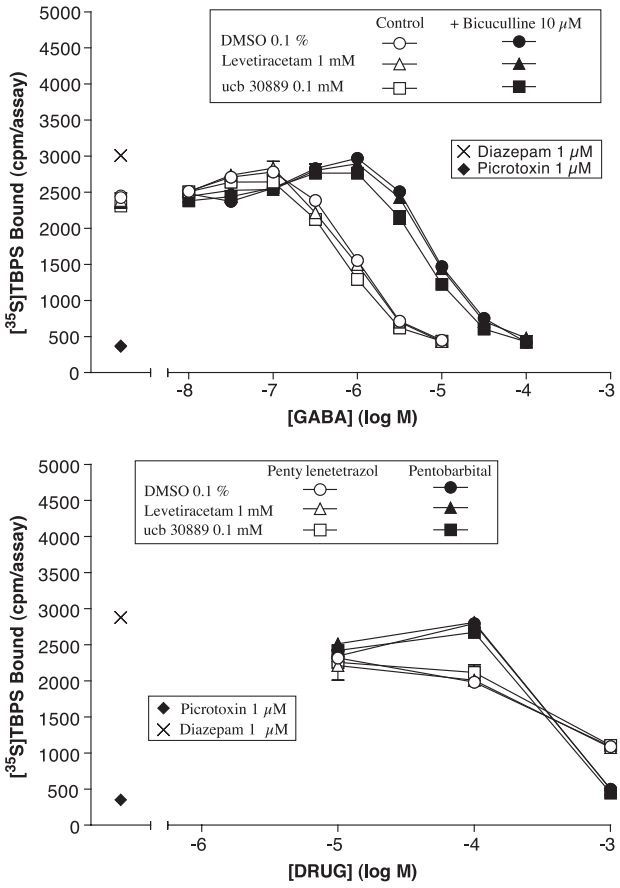

Fig. 6. Levetiracetam and ucb 30889 do not modulate the binding properties of GABA, bicuculline, pentylenetetrazol or pentobarbital to $GABA_A$ receptors. The inhibition of equilibrium [^{35}S]TBPS binding to the Cl^- channel by GABA ± bicuculline (top) or pentylenetetrazol and pentobarbital (bottom) was measured in the presence or absence of levetiracetam or ucb 30889 as explained in Materials and methods. Results presented are representative of three experiments. Diazepam was used as a positive modulator of [^{35}S]TBPS to validate the membrane batch and picrotoxin was used to define the nonspecific binding.

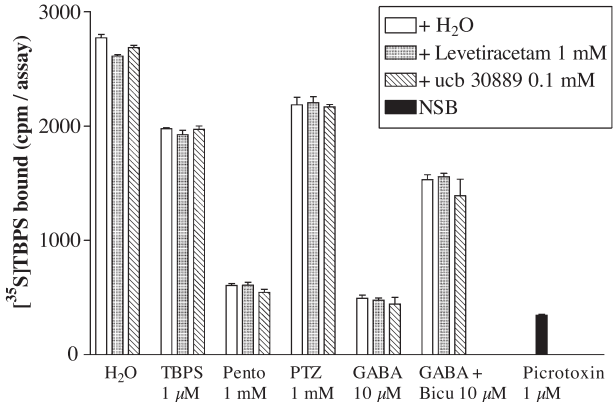

Fig. 7. Levetiracetam and ucb 30889 do not alter the modulator properties of GABA, bicuculline, pentylenetetrazol or pentobarbital on [^{35}S]TBPS dissociation kinetics. After 2-h incubation, [^{35}S]TBPS dissociation was induced by the addition of water (no dissociation), TBPS (control dissociation), GABA, GABA + bicuculline, pentylenetetrazol or pentobarbital in the absence or presence of levetiracetam or ucb 30889, both at a concentration high enough to saturate all levetiracetam binding sites. Samples were filtered and counted 40 min thereafter. Results are representative of three experiments.

for [^{3}H]ucb 30889 binding, we first verified the binding of ucb 30889 and levetiracetam to levetiracetam binding sites under these new conditions. We found a fall of about 0.5 log in affinity for levetiracetam binding sites with both compounds (data not shown). The first series of experiments were done under steady state conditions (Fig. 6). As previously shown (Ticku and Ramanjaneyulu, 1984), diazepam was able to increase the binding of [^{35}S]TBPS by 20 to 30% and was used as a control in all further experiments. GABA, at submicromolar concentrations increased [^{35}S]TBPS binding to the same extent as diazepam. At higher concentrations, GABA completely inhibited [^{35}S]TBPS binding with a pIC_{50} of 6.0. The addition of bicuculline, a GABA receptor antagonist, induced a parallel shift to the right of the GABA competition curve. Pentylenetetrazol and pentobarbital also inhibited [^{35}S]TBPS binding albeit at higher concentrations (>100 μM). As diazepam and GABA, pentobarbital first increased [^{35}S]TBPS binding. Neither ucb 30889 nor levetiracetam, at concentrations high enough to saturate all levetiracetam binding sites (0.1 and 1 mM, respectively), had a direct effect on [^{35}S]TBPS binding. Likewise, they did not modify the binding properties of GABA, bicuculline, pentylenetetrazol or pentobarbital. In an attempt to detect possible small changes, we also examined the effects of compounds on [^{35}S]TBPS dissociation kinetics. As shown in Fig. 7, TBPS, pentobarbital, pentylenetetrazol and GABA were all able to induce dissociation of [^{35}S]TBPS. Compared to TBPS, GABA and pentobarbital markedly accelerated the dissociation of [^{35}S]TBPS whereas bicuculline antagonised the effect of GABA. Again, neither ucb 30889 nor levetiracetam, alone or in combination with the other drugs, had any significant effect on [^{35}S]TBPS dissociation.

3.6. Binding of [^{3}H]ucb 30889 on brain slices

Having characterised the binding properties of [^{3}H]ucb 30889 in membrane preparations and given the affinity and good signal to noise ratio of this ligand, we then performed preliminary binding experiments on brain slices. Because even at 4 °C a portion of [^{3}H]ucb 30889 binding dissociates with fast kinetics, it was verified that no loss of selective binding occurred during the two 10-min washing steps.

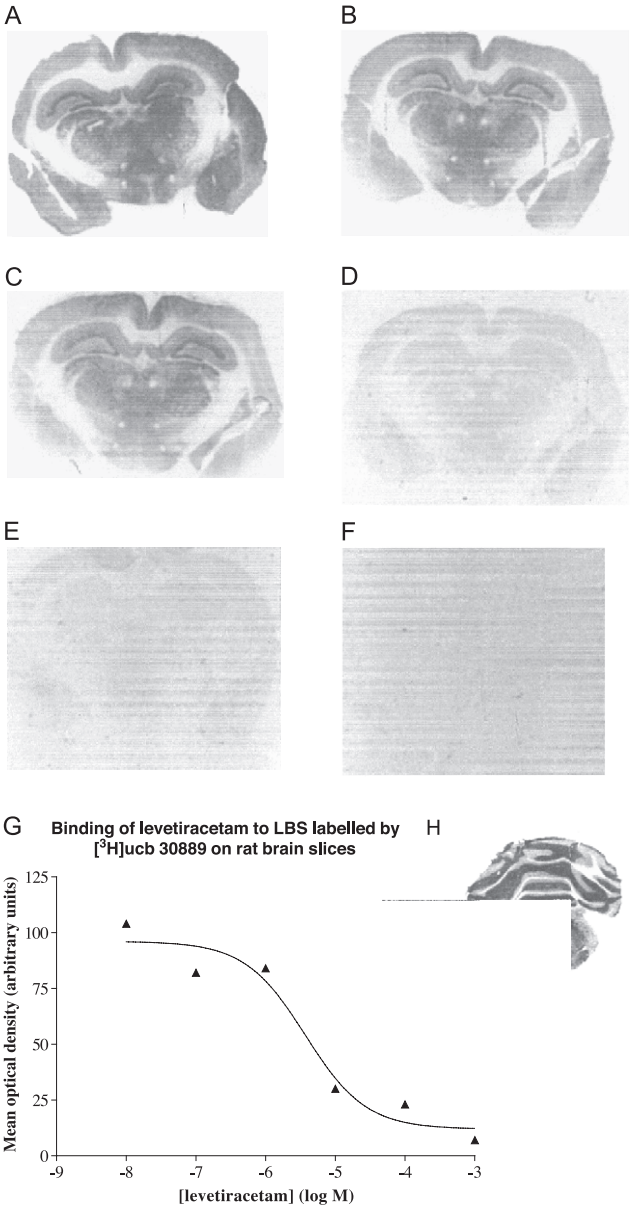

Fig. 8. Localisation of levetiracetam binding sites in rat brain by [^{3}H]ucb 30889 autoradiography. Consecutive brain slices mounted on microscope slides were incubated with [^{3}H]ucb 30889 for 2 h at 4 °C in the absence (panel A) or in the presence of increasing concentrations of levetiracetam (0.1, 1, 10, 100 and 1000 μM in panels B, C, D, E and F, respectively) as described in Materials and methods. Panel G shows the analysis of the labelling in the hippocampus based on pixel quantification using Scion software. Panel H shows [^{3}H]ucb 30889 binding in the cerebellum.

Autoradiographies from slices obtained using our standard procedure were compared with those from slices that had been washed by two 1-s dipping steps. Reducing the washing time led to a slightly higher background with no differences in the relative intensities of the brain structures being labelled (data not shown). A better signal to noise ratio was favoured and our original conditions were kept for further experiments. As depicted in Fig. 8, [^{3}H]ucb 30889 binds specifically to brain structures. The dentate gyrus is particularly well labelled and there was no detectable binding to white matter (Fig. 8A). Remarkably, in the cerebellum (Fig. 8H), [^{3}H]ucb 30889 labelling was intense in the molecular layer. The binding of [^{3}H]ucb 30889 is concentration dependently inhibited by levetiracetam. Quantification of the data led to a pIC_{50} value of 5.4 for levetiracetam, a value which, considering the differences in experimental conditions, is similar to that obtained from binding curves performed in brain homogenates ($pIC_{50} = 6.1 \pm 0.1$) (Table 2).

4. Discussion

This article describes a new radioligand with higher affinity for levetiracetam binding sites than [^{3}H]levetiracetam and its use to further characterise these binding sites. Previous results showed that the chiral carbon of levetiracetam (S-stereoisomer) is responsible for a marked 1000-fold stereoselectivity in binding affinity (ucb L060, the R-enantiomer virtually does not bind to the levetiracetam binding sites; Noyer et al., 1995). Based on these observations and confirmed by other pairs of enantiomers belonging to the same structural family, only S-stereoisomer compounds were synthesised. Substitutions in position 4 of the pyrrolidone, such as in ucb 30889, not only increased the affinity for levetiracetam binding sites but also introduced a second chiral centre. However, the resulting stereoselectivity was less significant with only a 3-fold change in affinity between ucb 30889 and ucb 30890 (Table 1).

Initial experiments were aimed at showing that both radioligands were indeed labelling the same sites. The results obtained with [^{3}H]ucb 30889 in comparison with [^{3}H]levetiracetam verified this hypothesis. B_{max} are comparable, affinities determined for a series of drugs and levetiracetam analogues spanning a large range of pIC_{50} values (over 4 log units) are independent of the radioligand used and both ucb 30889 and levetiracetam bind to the same population of sites labelled with either radioligand. Furthermore, the tissue distribution of the binding sites is identical for both radioligands, confirming the initial data obtained with [^{3}H]levetiracetam (Noyer et al., 1995).

Compared to [^{3}H]levetiracetam, [^{3}H]ucb 30889 displays 20-fold higher affinity for levetiracetam binding sites. Its binding is reversible with quite fast kinetics even at 4 °C. The kinetics are biphasic as was already observed with [^{3}H]levetiracetam (Noyer et al., 1995). This may have several explanations including isomerization, negative cooperativity or indicate heterogeneity of the binding sites. This latter possibility is not supported by our current data. At the concentration of [^{3}H]ucb 30889 used in these experiments and given the kinetic constants, one would label approximately equal amounts of these two hypothetical sites; however, all competing drugs tested thus far displaced [^{3}H]ucb 30889 with slopes not different from unity as would be expected for an homogenous population of binding sites. In addition, saturation isotherms obtained with [^{3}H]ucb 30889 are also best described by a single population of binding sites. Besides, proportions of fast and slow components do not vary significantly between the cerebral cortex and the hippocampus. We might have expected differences if we were in the presence of two different sites, as often observed with G-protein coupled receptor subtypes for example. Additional experiments are needed to further elucidate this point.

As with [^{3}H]levetiracetam, we showed that the distribution of the binding sites labelled by [^{3}H]ucb 30889 are essentially restricted to the central nervous system. Given the amount of proteins, the concentration and the K_d of [^{3}H]ucb 30889, we estimate that our lower limit of detection was a B_{max} of 150 fmol/mg protein. Since no specific binding was observed in any of the peripheral tissues examined (Fig. 4), we conclude that levetiracetam binding sites are at least 25 times more abundant in the central nervous system compared to the periphery.

Because of the enhanced binding affinity of ucb 30889, we have performed preliminary autoradiography studies in rat brain. As depicted in Fig. 8, [^{3}H]ucb 30889 is particularly well suited for these experiments as its nonspecific binding in tissue slices is virtually nil. The total binding was completely and concentration-dependently inhibited by levetiracetam. These first results indicate that levetiracetam binding sites are largely distributed in the brain gray matter although some structures such as the dentate gyrus and the molecular layer of the cerebellum are predominantly labelled. A more detailed analysis of levetiracetam binding sites distribution in the brain substructures is presented in the accompanying paper by Fuks et al. (2003, in press).

Specific binding sites have been described for other antiepileptic drugs. Gabapentin labels a specific binding site that was initially limited to the brain and that had similar levels of expression with levetiracetam binding sites (Hill et al., 1993). However, this site was later identified as the $\alpha_2\delta$ subunit of the L-type Ca^{2+} channel (Gee et al., 1996) and high levels of expression were detected in skeletal muscle. We found that gabapentin did not compete with either [^{3}H]levetiracetam or [^{3}H]ucb 30889 and furthermore, we did not detect specific binding of [^{3}H]ucb 30889 in skeletal muscle. SB-204269 is another anticonvulsant for which specific binding sites have been reported but the level of expression in rat forebrain is about 10 times less than for levetiracetam binding sites and levetiracetam did not show any affinity for these sites (Herdon et al., 1997).

Pentobarbital, bemegride, pentylenetetrazol and chlordiazepoxide are amongst the few drugs that compete with [^{3}H]levetiracetam or [^{3}H]ucb 30889 for levetiracetam binding sites. In addition, the B_{max} values found for levetiracetam binding sites are comparable to those reported for [^{35}S]TBPS or [^{3}H]GABA (Sousa and Ticku, 1997). This suggested some possible interactions of levetiracetam and ucb 30889 at the $GABA_A$ receptor complex level, although GABA, bicuculline and benzodiazepines, other than chlordiazepoxide, did not inhibit the binding of either radioligand. On the other hand, levetiracetam is known to potentiate the anticonvulsant effects of phenobarbital in amygdala-kindled rats (Klitgaard and Matagne, 2002) and in audiogenic seizure susceptible mice (Matagne et al., 2001). Levetiracetam was also shown to inhibit bicuculline induced hyperexcitability in rat hyppocampus (Margineanu and Wülfert, 1995) but this was later dismissed as a non-$GABA_A$ mediated effect (Margineanu and Wülfert, 1997) on the basis that levetiracetam antagonised bicuculline but not gabazine a selective $GABA_A$ receptor antagonist. To further resolve this issue, we performed a series of binding experiments with [^{35}S]TBPS. It is well known that GABA, pentobarbital, pentylenetetrazol and benzodiazepines are able to compete and to modulate, through allosteric interactions, the binding and kinetics of [^{35}S]TBPS or its analogue [^{35}S]TBPT to the Cl^- channel of $GABA_A$ receptors (Ticku and Ramanjaneyulu, 1984; Maksay, 1996). Accordingly, we observed that diazepam increased the binding at equilibrium. Likewise, GABA and pentobarbital at low concentrations increased [^{35}S]TBPS binding to the same extent as diazepam. These compounds have been shown to induce an increase of [^{35}S]TBPS affinity in membranes devoid of endogenous GABA by extensive washing steps (Liljequist and Tabakoff, 1993).

At higher concentrations, GABA, pentobarbital and pentylenetetrazol inhibited [^{35}S]TBPS binding. Bicuculline was shown to antagonise the effects of GABA (seen as a rightward shift of the GABA competition curve). GABA and pentobarbital also increased the dissociation rate of [^{35}S]TBPS compared to TBPS-induced dissociation. Neither levetiracetam nor ucb 30889 alone were able to inhibit [^{35}S]TBPS binding or to modify the kinetics. Similarly, co-incubation of levetiracetam or ucb 30889 with the other drugs did not affect their binding properties or interactions with [^{35}S]TBPS. The concentrations of levetiracetam and ucb 30889 used in these experiments were high enough to occupy all the levetiracetam binding sites on one hand and to prevent the binding of pentobarbital and pentylenetetrazol to levetiracetam binding sites on the other. Altogether, it seems unlikely that levetiracetam binding sites are part of, or interact with, the $GABA_A$ receptor complex. In addition, the distribution of levetiracetam binding sites in rat brain substructures (Fig. 8 and Fuks et al., 2003, in press) does not correspond to the mapping of $GABA_A$ receptors as revealed by [^{35}S]TBPS (Korpi and Lüddens, 1997), [^{3}H]flunitrazepam (Li et al., 2001) or [^{3}H]muscimol (Walsh et al., 1999) binding. In the cerebellum, [^{3}H]muscimol labels essentially the granular layer, [^{3}H]flunitrazepam and [^{3}H]ucb 30889 label preferentially the molecular layer while [^{35}S]TBPS labels equally the two layers. However, [^{3}H]flunitrazepam shows high binding in the paraventricular nucleus compared to the other thalamic nuclei whereas the opposite holds true for [^{3}H]ucb 30889. [^{3}H]flunitrazepam also equally labels the hippocampal formation while [^{3}H]ucb 30889 displays a significantly higher density of binding sites in the dentate gyrus.

Finally, as disclosed in the companion paper (Fuks et al., 2003, in press) the size of the protein labelled by [^{3}H]ucb 30889 (90 kDa) does not match with any of the subunits forming the $GABA_A$ receptor complex.

In conclusion, levetiracetam is a novel antiepileptic drug that binds to a specific binding site in the brain. In this paper, we showed that [^{3}H]ucb 30889 is a new radioligand with excellent binding properties, being more potent than [^{3}H]levetiracetam to label levetiracetam binding site and very well suited to further explore the biochemical and pharmacological properties of these sites. Moreover, [^{3}H]ucb 30889 contains an azido phenyl moiety enabling the formation of a covalent bond upon photo-activation with UV light. This was verified and proved useful to purify the levetiracetam binding site (Fuks et al., 2003, in press). Finally, although some $GABA_A$ receptor ligands such as pentobarbital, pentylenetetrazol or chlordiazepoxide also bind to levetiracetam binding sites with affinities similar to those displayed towards the $GABA_A$ receptor complex, our results altogether strongly suggest that there is no functional overlap between these sites.

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