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Characterization of [^{3}H]ucb 30889 binding to synaptic vesicle protein 2A in the rat spinal cord

Nathalie Lambeng [a,*], Michel Gillard [a], Pascale Vertongen [b], Bruno Fuks [a], Pierre Chatelain [a]

[a] *UCB S.A., CNS In Vitro Pharmacology, Building R4, Chemin du Foriest, B-1420 Braine-l'Alleud, Belgium*

[b] *Université Libre de Bruxelles, Faculté de Médecine, Department of Biochemistry and Nutrition, CP 611, 808 route de Lennik, B-1070 Bruxelles, Belgium*

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Abstract

The novel antiepileptic drug levetiracetam ((2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®) possesses a specific binding site in brain, which has very recently been identified as the synaptic vesicle protein SV2A. The aim of this study was to evaluate the presence of a levetiracetam binding site in the spinal cord and compare its properties to that in rat brain. We used [^{3}H]ucb 30889 ((2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide), a levetiracetam analogue, to perform binding assays, photoaffinity labelling and autoradiography experiments, and revealed the presence of SV2A by Western-blot analysis. [^{3}H]ucb 30889 binding kinetics at 4 °C were biphasic and saturation binding curves were compatible with the labelling of a homogenous population of binding sites with a K_d similar to that in brain. Competition curves with ligands known to interact with levetiracetam binding sites and photolabelling experiments indicated that [^{3}H]ucb 30889 labels the same 90 kDa protein in both spinal cord and brain. Levetiracetam binding site was localised in the grey matter of the spinal cord and its expression was not modified in a model of neuropathic pain. This study demonstrates the presence of a specific levetiracetam binding site in the rat spinal cord, which is similar to that found in rat brain.

Keywords: Levetiracetam; SV2A; Rat spinal cord; Binding

1. Introduction

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide, KEPPRA®) is a novel antiepileptic drug used as adjunctive therapy for the treatment of refractory partial epilepsy in adults (Hovinga, 2001; Nash and Sangha, 2001). Levetiracetam has a broad spectrum of activity in suppressing seizures as add-on treatment and monotherapy, and is safe and well-tolerated. Levetiracetam also has a favourable pharmacokinetic profile characterised by rapid and nearly complete absorption, very low potential for drug interactions and a prolonged pharmacodynamic effect that permits twice-daily dosing (for review, see Ben-Menachem, 2003). However, its mechanism of action is not fully elucidated. Previous studies revealed that levetiracetam binds saturably, reversibly and stereospecifically to an unidentified binding site in rat brain (Noyer et al., 1995; Gillard et al., 2003). Screening of a large number of known antiepileptic drugs and other neuroactive compounds failed to identify any with affinity for the levetiracetam binding site (Gillard et al., 2003), providing support for the novelty of this site. After identifying the site as a 90 kDa membrane protein enriched in synaptic vesicles (Fuks et al., 2003), the molecular nature of levetiracetam's binding site was recently discovered (Lynch et al., 2004). Levetiracetam and its related analogue ucb 30889 ((2*S*-(2-[4-(3-azidophenyl)-2oxopyrrolidin-1-yl]butanamide) bind specifically to the synaptic vesicle protein SV2A, which is totally different from the classical targets of established antiepileptic drugs ($GABA_A$ receptor, Na^+ channels and low-voltage activated Ca^{2+} currents).

Three SV2 isoforms, called SV2A, SV2B, and SV2C, have been identified and show a unique distribution in

* Corresponding author. Tel.: +32 2 386 23 32; fax: +32 2 386 33 97.
E-mail address: nathalie.lambeng@ucb-group.com (N. Lambeng).

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brain (Bajjalieh et al., 1992, 1993; Feany et al., 1992; Janz and Sudhof, 1999). These proteins are twelve transmembrane glycoproteins localised in synaptic vesicles and implicated in the regulation of synaptic vesicle exocytosis (Crowder et al., 1999; Janz et al., 1999). SV2A is the most abundant, being ubiquitous in the central nervous system and expressed in endocrine cells (Buckley and Kelly, 1985; Bajjalieh et al., 1994). Its function remains unknown. However, SV2A knockout (KO) mice, as well as double SV2A/SV2B knockouts, exhibit a severe seizure phenotype, and SV2A seems to have a crucial role in the regulation of vesicle function, but not in vesicle biogenesis or synaptic morphology (Crowder et al., 1999; Janz et al., 1999).

It is postulated that neuropathic pain bears several similarities to epileptic seizures, including a therapeutic benefit from anticonvulsant drugs (Swerdlow, 1984). It was furthermore recently shown that levetiracetam induces an anti-hyperalgesic effect in chronic pain models (Ardid et al., 2003) and decreases neuropathic pain in patients (Price, 2004). It was therefore tempting to hypothesize that levetiracetam may act on a site expressed in the spinal cord. However, the presence of the levetiracetam binding site in the rat spinal cord has not been established. Only mRNA expression of SV2A (Bajjalieh et al., 1993, 1994) or immunostaining with a monoclonal antibody that does not distinguish between the three isoforms (Booj et al., 1989; Wang et al., 2000; Roosen et al., 2001; Brooke et al., 2004) have been described.

The synthesis of the new radioligand [^{3}H]ucb 30889 with a 20-fold higher affinity for the levetiracetam binding site than tritiated levetiracetam permitted further analysis of levetiracetam binding sites in the brain (Gillard et al., 2003), and in the spinal cord. By using [^{3}H]ucb 30889, we report here the existence of a levetiracetam binding site in the rat spinal cord. Binding and photoaffinity labelling experiments, autoradiography and Western-blot studies were performed to characterize it and compare its properties to the brain sites.

2. Materials and methods

2.1. Drugs and radioligands

Levetiracetam (2*S*-(2-oxo-1-pyrrolidinyl)butanamide), ucb L060 (2*R*-(2-oxo-1-pyrrolidinyl)butanamide), ucb 30889 (2*S*-(2-[4-(3-azidophenyl)-2oxopyrrolidin-1-yl]butanamide) and ucb 34714 (2*S*-(2-[4*R*-(2-oxo-4-propylpyrrolidin-1-yl]butanamide)) were synthesized at UCB (Braine-l'Alleud, Belgium). [^{3}H]ucb 30889 (32 Ci/mmol) was custom labelled by Amersham Biosciences (Roosendaal, The Netherlands). Bemegride, chlordiazepoxide, and pentobarbital were purchased from Sigma-Aldrich (Bornem, Belgium). Pentylenetetrazol was bought from Acros Organics (Pittsburgh, USA).

2.2. Spinal cord membranes preparation

Sprague–Dawley adult rats (200–300 g) from Iffa-Credo (Belgium) were sacrificed by decapitation. Spinal cords were quickly removed before homogenisation (10% w/v) in 20 mM Tris–HCl buffer (pH 7.4) containing 250 mM of sucrose (buffer A) at 4 °C. The homogenates were spun at 30,000 ×*g* at 4 °C for 15 min and the pellets resuspended in 50 mM Tris–HCl buffer (pH 7.4). 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 15 to 20 mg/ml and stored at −70 °C.

2.3. Binding studies

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

Experiments were performed essentially as described in Gillard et al. (2003). Membrane proteins (0.1 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 (1.12 nM) and increasing concentrations of unlabelled competing drugs. Nonspecific binding was defined as the residual binding observed in the presence of 1 mM 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. 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, [^{3}H]ucb 30889 binding was measured at the indicated times after addition of the membranes. For dissociation studies, membranes were first incubated for 120 min at 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 (0.1 mg of proteins) were incubated 120 min at 4 °C with concentrations of [^{3}H]ucb 30889 ranging from 1 to 120 nM (concentrations above 35 nM were obtained by isotopic dilutions).

2.3.2. [^{3}H]ucb 30889 photoaffinity labelling

For the investigation of irreversible binding to proteins, rat spinal cord membranes were resuspended in 50 mM Tris–HCl (pH 7.4), 2 mM EDTA containing protease inhibitors (Complete, Roche Diagnostics) and incubated for 120 min at 4 °C in the dark with 24 nM [^{3}H]ucb 30889 in the absence or presence of 1 mM levetiracetam. The samples were then washed three times with 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ at 30,000 ×*g* for 5 min at 4 °C. Aliquots of 2 mg were placed in 3-ml quartz cuvettes (1-cm light path) and irradiated with a Spectroline UV light (model CM-10) for 30 min at 254 nm at 4 °C in the dark. The membranes were washed five times in the same buffer and stored at −70 °C.

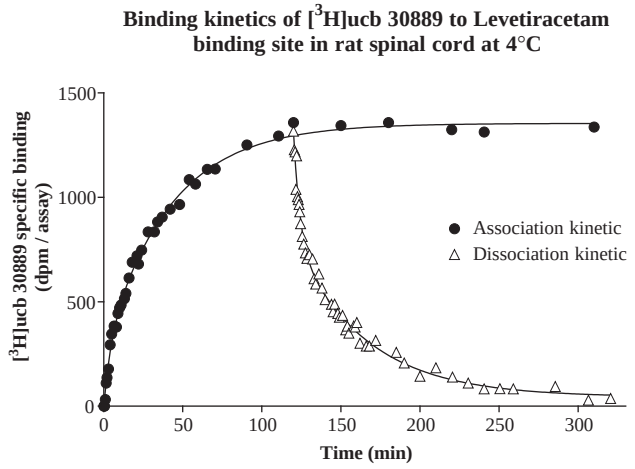

Fig. 1. Binding kinetics of [^{3}H]ucb 30889 in rat spinal cord. The binding kinetics were measured as described in Materials and methods at 4 °C. Data are representative of at least three independent experiments. The curves are the best fits obtained with equations describing the interaction of a ligand with two independent sites. Kinetic constants calculated are given in Table 1.

Samples were next subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels (Laemmli, 1970). For the analysis of the incorporated radioactivity, proteins were stained with Coomassie brilliant blue R-250 and gel slices were cut out and dissolved overnight in 3.5-fold diluted SOLUENE 350 (Packard Bioscience) followed by determination of the incorporated radioactivity by liquid scintillation counting.

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

Rat spinal cords were quickly dissected out and frozen in isopentane placed on dry-ice. Frontal slices were cut with a cryostat and mounted on microscope slides covered with gelatin. Slides were pre-incubated twice for 10 min at room temperature in a 50 mM Tris–HCl buffer (pH 7.4) with 0.5% bovine serum albumin (BSA). Then, incubation with 2 pmol/slide of [^{3}H]ucb 30889 was performed for 120 min at 4 °C in a 50 mM Tris–HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.05% bacitracin, 2 mM EGTA and 0.5% BSA. 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. Western-blot analysis of SV2A expression

Forty micrograms of total protein from spinal cord membranes was loaded on a Tris–glycine/8% polyacrylamide gel and developed. After transfer to a nitrocellulose membrane, the blots were probed with a polyclonal antibody specific for the SV2A isoform (sc-11936, Santa Cruz Biotechnology) and, when indicated, with an antibody directed against the synaptic vesicle protein synaptophysin (sc-7568, Santa Cruz Biotechnology).

2.5. Streptozotocin-induced rat neuropathic model

Sprague–Dawley adult rats (200–300 g) from Iffa-Credo (Belgium) were intraperitoneally injected with streptozotocin (60 mg/kg, Sigma) dissolved in citrate–saline solution (pH 4.5) or with the vehicle only. Diabetic phenotype was detected by blood glucose determination one week after injection and weight loss. All animals were sacrificed by decapitation three weeks later, and spinal cord collected before homogenization as described above.

2.6. Data analysis

Data analysis was performed by computerised nonlinear curve fitting methods using Graphpad Prism 4 (Graphpad Prism® software, San Diego, CA), according to equations describing several binding models (Molinoff et al., 1981). K_d values from saturation binding assays and IC_{50} values from the competition binding assays were determined with one site binding or one site competition curve fitting equations. IC_{50} values were corrected to K_i by applying the Cheng and Prusoff's (1973) equation. Autoradiography data were analysed and quantified after greyscale scanning using Scion Image software (Scion, Frederick, MD).

3. Results

3.1. Binding kinetics and saturation binding isotherms

In the rat spinal cord, [^{3}H]ucb 30889 binding is reversible (Fig. 1). Binding kinetics at 4 °C are biphasic with a fast

Table 1
Binding kinetics constants of [^{3}H]ucb 30889 in rat spinal cord

	Rat spinal cord
Dissociation	
k_{off} fast (min^{-1})	0.28±0.07
k_{off} slow (min^{-1})	0.018±0.006
% fast	43.3±6.5
$t_{1/2}$ fast (min)	2.6±0.6
$t_{1/2}$ slow (min)	42.6±14.4
Asssociation	
k_{obs} fast (min^{-1})	0.26±0.06
k_{obs} slow (min^{-1})	0.019±0.005
% fast	23.0±9.6
$t_{1/2}$ fast (min)	2.8±0.5
$t_{1/2}$ slow (min)	38.5±9.2

The binding kinetic constants of [^{3}H]ucb 30889 were calculated by nonlinear regression analysis of data experiments such as depicted in Fig. 1 and according to equations provided by Prism® software describing the interaction of a ligand with two independent binding sites (reported here as the slow and fast components). Results are the mean±S.D. from 3 independent 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 (1.12 nM in our experiments). $t_{1/2}$ is the half-time for dissociation or association.

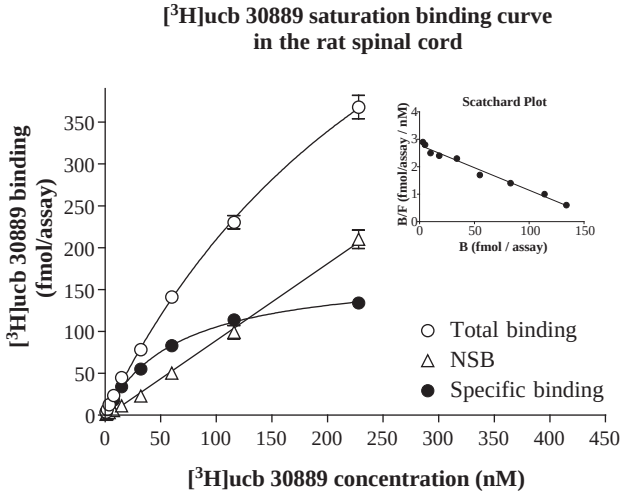

Fig. 2. Saturation isotherm of [^{3}H]ucb 30889 in rat spinal cord. Membranes were incubated with increasing concentrations of [^{3}H]ucb 30889 for 120 min at 4 °C. Nonspecific binding (NSB) was determined as the residual binding in the presence of 1 mM levetiracetam. The specific binding is obtained after substraction of the NSB from the total binding. Results are representative of four independent experiments. Inset: the Scatchard plot from the transformed data.

component having, respectively, half-times of association (at a radioligand concentration of 1.12 nM) and dissociation of 2.8±0.5 and 2.6±0.6 min and a slow component of 38.5±9.2 and 42.6±14.4 min (n=3). Binding kinetic constants are indicated in Table 1. Saturation binding curves of [^{3}H]ucb 30889 were compatible with the labelling of a homogeneous population of binding sites (Fig. 2). Affinity (K_d) was 52±14 nM (n=3) and maximum binding capacity (B_{max}) was 1633 ±126 fmol/mg protein (n=3).

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

	Rat spinal cord	Rat cortex (Gillard et al., 2003)
	pK_i±S.D.	pK_i±S.D.
Levetiracetam	5.9±0.3	5.8±0.2
ucb 30889	7.2±0.1	7.1±0.2
ucb 34714	7.3±0.1	7.0±0.1
ucb L060	<3	<3
Pentylenetetrazol	3.9±0.0	3.9±0.1
Bemegride	4.9±0.1	4.7±0.1
Pentobarbital	3.6±0.1	3.8±0.0
Chlordiazepoxide	5.6±0.2	5.3±0.1

Results are the mean±S.D. from 3 independent experiments. Data from competition curves such as depicted in Fig. 3 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.

3.2. Competition experiments

To ensure that [^{3}H]ucb 30889 labels the same binding site in the rat spinal cord as in the rat cortex, we performed competitive binding assays with various ligands and levetiracetam analogues (Fig. 3). Calculated pK_i are shown in Table 2. In these experiments, no significant correction between the pIC$_{50}$ and the pK_i was needed, since the concentration of [^{3}H]ucb 30889 (1.12 nM) is far below its K_d value (50 nM). For levetiracetam and its analogues (ucb 30889, ucb 34714), the pK_i were equivalent in both tissues. As in the cortex, the binding of [^{3}H]ucb 30889 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. The affinity of ucb 30889 as determined in a competition binding

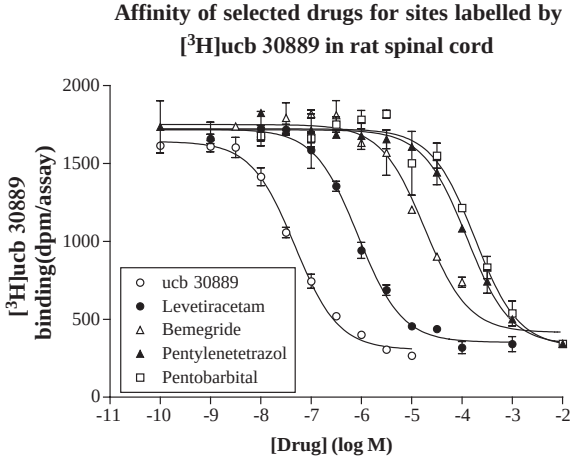

Fig. 3. Competition curves of selected drugs against [^{3}H]ucb 30889 as radioligand. Compounds were incubated at increasing concentrations with 1.12 nM of [^{3}H]ucb 30889 for 120 min at 4 °C as described in Materials and methods. Data were analysed by nonlinear regression with Prism® according to a model with variable slope. The results are representative of three independent experiments. pK_i values are shown in Table 2.

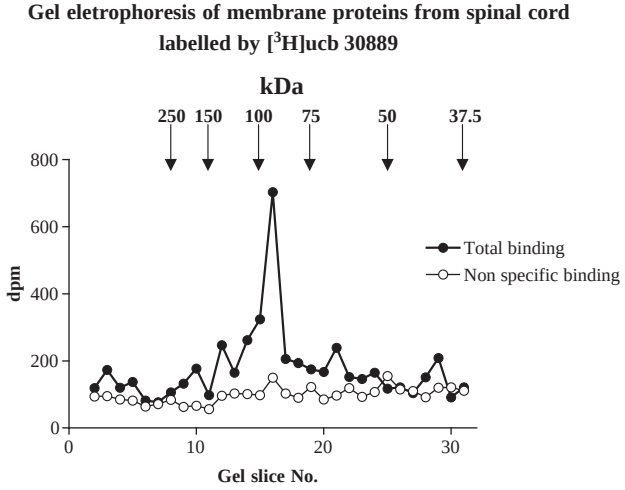

Fig. 4. Gel electrophoresis of membrane proteins labelled by [^{3}H]ucb 30889. Photoaffinity labelling was performed in the absence (closed circles) or in the presence (open circles) of 1 mM levetiracetam. The arrows represent the position of molecular weight markers and the numbers above indicate their molecular sizes.

experiment with [^{3}H]ucb 30889 (63 nM) agreed well with the K_d of [^{3}H]ucb 30889 determined by saturation binding experiments (Fig. 2, 52 nM). All other tested compounds that are known to displace the binding of [^{3}H]ucb 30889 in the rat cerebral cortex (Gillard et al., 2003) inhibited the binding of the radioligand in the rat spinal cord with a similar pK_i (Table 2).

3.3. Photoaffinity labelling

The radioligand [^{3}H]ucb 30889 was designed with an azidophenyl motif capable of forming a covalent complex with the protein after UV light irradiation. This capability to bind covalently to the levetiracetam binding site was used to determine the molecular weight of this protein in the rat spinal cord. The optimal photolabelling conditions were determined in a previous study (Fuks et al., 2003). After loading photolabelled samples onto SDS-PAGE, it was found that the radioactivity was irreversibly incorporated into a protein with a molecular weight of approximately 90 kDa (Fig. 4). The amount of radioactivity incorporated into the 90-kDa band was quantified by measuring the radioactivity in individual gel slices by liquid scintillation counting. Addition of 1 mM of levetiracetam in the binding assay inhibited the photoincorporation of radioactivity into this protein, indicating that this photolabelling was specific.

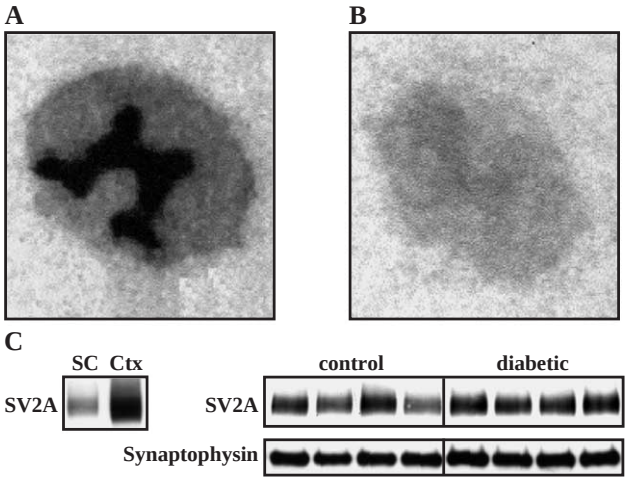

Fig. 5. Localization of levetiracetam binding site in rat spinal cord by [^{3}H] ucb 30889 autoradiography and analysis of SV2A expression. Spinal cord slices mounted on microscope slides were incubated with [^{3}H]ucb 30889 for 120 min at 4 °C in the absence (panel A) or in the presence of 1 mM levetiracetam (panel B) as described in Materials and methods . Western-blot analysis of SV2A expression in rat spinal cord (SC) and cerebral cortex (Ctx) (C, left panel), and in spinal cord of rats treated or not with the streptozotocin toxin to induce a diabetic-linked neuropathic pain (C, right panel, $n=4$ for each group). In this latter case, expression of synaptophysin was used as a control of synaptic vesicle proteins quantity. Results are representative of two to three independent experiments.

3.4. Binding of [^{3}H]ucb 30889 on spinal cord slices and SV2A expression

Autoradiography from slices of spinal cord is shown in Fig. 5. The levetiracetam binding site was distributed throughout the grey matter (Fig. 5A). Binding is homogeneous within this structure and unchanged throughout the spinal cord from the brain stem down to the sacral region (not shown). No [^{3}H]ucb 30889 binding was observed in the white matter (Fig. 5A) or in the presence of 1 mM levetiracetam (Fig. 5B). Western-blot analysis revealed that SV2A is expressed two- to three-fold less in rat spinal cord than in cerebral cortex (Fig. 5C).

As levetiracetam had previously shown anti-hyperalgesic effects in a rat model of neuropathic pain (Ardid et al., 2003), the streptozotocin-induced diabetic rat model (Courteix et al., 1993), we examined whether SV2A expression was altered in this hyperalgesic model. However, despite the establishment of a diabetic phenotype (glycemia above 4.75 g/l), we did not observe any significant change in B_{max} (1558±252 and 1783±422 fmol/mg protein for control and diabetic rats ($n=6$), respectively) or in SV2A expression level (Fig. 5C). Expression of the synaptic vesicle synaptophysin was also analysed on the same Western-blot to exclude any artifactual modification of SV2A expression.

4. Discussion

The results presented here demonstrate that the levetiracetam binding site is abundant in the rat spinal cord. This site which has never been described in the literature before exhibits similar characteristics to that present in the cerebral cortex. The affinity of various drugs and levetiracetam analogues was equivalent in both tissues showing that [^{3}H] ucb 30889 was indeed labelling the same site. Radioligand affinity found in rat spinal cord (52±14 nM) was comparable to that obtained in rat cerebral cortex (62±20 nM, Gillard et al., 2003), and photoaffinity labelling experiments indicated that [^{3}H]ucb 30889 binds to a protein with a molecular weight in the 90 kDa range as observed in rat cerebral cortex (Fuks et al., 2003). However, Scatchard analysis revealed that the number of binding sites (B_{max}) in spinal cord (1633 ±126 fmol/mg protein) is two to three times lower than in cerebral cortex (4496±790 fmol/mg protein, Gillard et al., 2003). This result was confirmed by Western-blot analysis of the protein SV2A. This difference may be attributed to the specific distribution of the levetiracetam binding site within the spinal cord. Indeed, autoradiography studies revealed that it is localized exclusively in the grey matter, suggesting that the levetiracetam binding site is diluted in the whole homogenate. Estimation of the relative amount of grey matter in spinal cord suggests that the number of sites is equivalent to that in cerebral cortex.

Autoradiographic mapping also revealed that the levetiracetam binding site is homogeneously distributed in the grey matter from the brain stem down to the sacral region. However, given the low resolution of the signal obtained, we could not detect small variations of levetiracetam binding site expression that may exist throughout the spinal cord. Histological studies with specific antibody for SV2A and quantitative analysis should be performed to confirm our results. As demonstrated in the brain (Crowder et al., 1999; Janz et al., 1999; Fuks et al., 2003), SV2A in the spinal cord is probably localized in the synaptic vesicles of neurons present in the grey matter. Indeed, using anti-SV2 monoclonal antibody that recognizes the three SV2 isoforms, an immunoreactive staining was observed in synaptic endings of sympathetic preganglionic neurons (Roosen et al., 2001) and at the neuromuscular synapse (Noakes et al., 1999; Wang et al., 2000). Immunohistological studies aimed at distinguishing the localization of the three SV2 isoforms in the grey matter are ongoing.

The kinetics of [^{3}H]ucb 30889 binding to rat spinal cord membranes are biphasic, as observed in rat brain (Gillard et al., 2003). We have previously showed that SV2A is the unique binding site of this radioligand in the brain (Lynch et al., 2004): it does not bind to SV2B or SV2C isoforms expressed in COS cells and no specific binding is detected in the brain of SV2A knockout mice. Therefore, this biphasic kinetics may only be due to an isomerization, to a negative cooperativity process or reflect the presence of multiple binding sites due to various conformations of SV2A. This protein is indeed glycosylated (Scranton et al., 1993; Janz et al., 1998) and phosphorylated (Gross et al., 1995; Pyle et al., 2000), and different binding properties of [^{3}H]ucb 30889 to each form cannot be ruled out. SV2A interaction with protein partners like synaptotagmin I (Schivell et al., 1996, 2005) may also modulate the binding of [^{3}H]ucb 30889. Additional experiments are needed to elucidate this point.

As mentioned in the Introduction, antinociceptive effects of anticonvulsant drugs have been reported in numerous preclinical studies. Moreover, levetiracetam induces an anti-hyperalgesic effect in a model mimicking human neuropathic pain, the streptozotocin-induced diabetic rat (Ardid et al., 2003), and may exert its anti-hyperalgesic action through its binding site expressed in the spinal cord. We therefore investigated if the levetiracetam binding site is modulated in this animal model, like, for example, for the vanilloid receptor 1 whose expression is increased under such pathological conditions (Rashid et al., 2003). Western-blot and binding studies showed no change of SV2A expression, suggesting there is no apparent dysregulation of SV2A during the development of this type of neuropathic pain. This does not, however, rule out the possibility of altered glycosylation or phosphorylation states or modified function of SV2A in animal models of other neurological disorders.

In conclusion, our data demonstrate the expression of SV2A in the rat spinal cord, with binding properties similar to the brain site, whose level of expression is not modified in an animal model of diabetic-induced neuropathic pain.

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