The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam

Berkley A. Lynch*†, Nathalie Lambeng‡, Karl Nocka§, Patricia Kensel-Hammes¶, Sandra M. Bajjalieh¶, Alain Matagne‖, and Bruno Fuks‡

Departments of *Molecular and Cellular Biology and §Bioinformatics and Target Discovery, UCB Research Inc., 840 Memorial Drive, Cambridge, MA 02139; Departments of ‡In Vitro Pharmacology and ‖CNS Pharmacology, UCB S.A., Pharma Sector, Chemin du Foriest, B-1420 Braine L'Alleud, Belgium; and ¶Department of Pharmacology, University of Washington, D429 HSB, Box 357280, Seattle, WA 98195-7280

Edited by William A. Catterall, University of Washington School of Medicine, Seattle, WA, and approved May 17, 2004 (received for review December 10, 2003)

Here, we show that the synaptic vesicle protein SV2A is the brain binding site of levetiracetam (LEV), a new antiepileptic drug with a unique activity profile in animal models of seizure and epilepsy. The LEV-binding site is enriched in synaptic vesicles, and photoaffinity labeling of purified synaptic vesicles confirms that it has an apparent molecular mass of ≈90 kDa. Brain membranes and purified synaptic vesicles from mice lacking SV2A do not bind a tritiated LEV derivative, indicating that SV2A is necessary for LEV binding. LEV and related compounds bind to SV2A expressed in fibroblasts, indicating that SV2A is sufficient for LEV binding. No binding was observed to the related isoforms SV2B and SV2C. Furthermore, there is a high degree of correlation between binding affinities of a series of LEV derivatives to SV2A in fibroblasts and to the LEV-binding site in brain. Finally, there is a strong correlation between the affinity of a compound for SV2A and its ability to protect against seizures in an audiogenic mouse animal model of epilepsy. These experimental results suggest that SV2A is the binding site of LEV in the brain and that LEV acts by modulating the function of SV2A, supporting previous indications that LEV possesses a mechanism of action distinct from that of other antiepileptic drugs. Further, these results indicate that proteins involved in vesicle exocytosis, and SV2 in particular, are promising targets for the development of new CNS drug therapies.

Epilepsy, a group of diseases characterized by recurrent spontaneous seizures, is a prevalent chronic neurological disorder (1). An often devastating disease, epilepsy is frequently resistant to conventional antiepileptic drugs (AEDs), even when they are used in polytherapy (1). Traditional AEDs mainly target ion channels or postsynaptic receptors. Given that traditional AEDs do not control seizures in all patients, identification of alternative molecular pathways and targets for therapeutic intervention in epilepsy is warranted. Levetiracetam [LEV; (*S*)-α-ethyl-2-oxo-pyrrolidine acetamide; KEPPRA] is an antiepileptic drug approved by the Food and Drug Administration in the year 1999 as an adjunctive therapy for the treatment of refractory partial epilepsy in adults (2, 3). LEV possesses several properties that distinguish it from classical AEDs (4). It has a distinctive pharmacological profile in animal models of seizures and epilepsy, as demonstrated by its lack of activity in the acute seizure models traditionally used to screen for antiepileptic drugs (5). This lack of activity against acutely generated seizures contrasts LEV's potent seizure protection in animal models of chronic epilepsy, including genetic and kindling models (5). Compared with traditional AEDs, LEV has the ability to inhibit neuronal hypersynchronization when epileptiform activity is evoked in rat hippocampal slices (6, 7). Also exceptional among AEDs is LEV's ability to counteract the development of amygdala electrical kindling in rodents even after drug dosing is terminated (8).

LEV's pharmacological profile has been presumed to relate to a distinctive mechanism of action (9). Additionally, LEV does not seem to act by means of any of the three main mechanisms currently accepted for the antiseizure action of established AEDs: (*i*) γ-aminobutyratergic (GABAergic) facilitation, (*ii*) inhibition of Na^+ channels, or (*iii*) modulation of low-voltage activated Ca^{2+} currents (9, 10). Previous studies revealed that LEV binds saturably, reversibly, and stereospecifically to an unidentified binding site in rat brain (11). Screening of a large number of known AEDs and other neuroactive compounds failed to identify any with high affinity for the LEV-binding site (11), providing support for the novelty of the LEV-binding site. Testing a series of LEV analogs revealed a strong correlation between their affinities for the brain binding site and their antiseizure potencies in the audiogenic mouse model of epilepsy (11). This finding indicates a functional role for the unidentified brain binding site in the antiseizure actions of LEV.

Detection of a LEV-binding site in brain provided the rationale to search for the LEV-binding molecule. Further characterization of the binding site led to its classification as an integral membrane protein of widespread distribution in brain, localized in neurons and enriched in the synaptic vesicle membrane fraction (11, 12). SDS/PAGE on the LEV-binding protein from brain membranes cross-linked to a tritiated photoaffinity LEV derivative determined that the protein has an apparent molecular mass of ≈90 kDa (12). Among possible candidate proteins matching these characteristics is the synaptic vesicle protein 2 (SV2).

SV2, an integral membrane protein present on all synaptic vesicles, is a small gene family consisting of three isoforms, designated SV2A, SV2B, and SV2C. SV2A is the most widely distributed isoform, being nearly ubiquitous in the CNS, as well as being present in endocrine cells (13, 14). SV2B is brain specific, with a wide but not ubiquitous distribution, and SV2C is a minor isoform in brain. The brain distribution of the LEV-binding site, as revealed by autoradiography, matches the equivalent distribution of SV2A as determined by immunocytochemistry (14, 15). Both $SV2A^{-/-}$ and $SV2B^{-/-}$ homozygous knockout (KO) mice have been reported, as well as double A/B KOs (16, 17). SV2A and SV2A/B KOs exhibit a severe seizure phenotype whereas the SV2B KOs do not. Studies of the SV2 KOs indicate that SV2 has a crucial role in the regulation of vesicle function, although not in vesicle biogenesis or synaptic morphology (16, 17).

Here, we report studies designed to test the hypothesis that SV2 is the LEV-binding site. We demonstrate that the protein SV2A is the LEV-binding site in brain and that there is an excellent correlation between the binding affinity of LEV and derivatives in brain and to heterologously expressed human

PHARMACOLOGY

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AED, antiepileptic drug; LEV, levetiracetam; SV2, synaptic vesicle protein 2; hSV2A, human SV2A; KO, knockout; β-gal, β-galactosidase; DM, *n*-dodecyl-β-D-maltoside; [^{3}H]ucb 30889, (2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide; pIC_{50}, $-\log IC_{50}$.

†To whom correspondence should be addressed. E-mail: berkley.lynch@ucb-group.com.

SV2A in fibroblasts. These data have implications for the mechanism of action of LEV as an antiepileptic drug, and potentially for future research into the contribution of presynaptic mechanisms to seizure initiation and propagation in the brain.

Materials and Methods

Reagents. LEV and derivatives were synthesized at UCB Pharma. [^{3}H]ucb 30889, (2*S*)-2-[4-(3-azidophenyl)-2-oxopyrrolidin-1-yl]butanamide (32 Ci/mmol; 1 Ci = 37 GBq), was custom labeled by Amersham Pharmacia.

WT and SV2 KO Animals. All animal experiments were approved by the local ethics committee for animal experimentation, according to U.S. and Belgian law. SV2A KO mice have been reported (16). SV2B KOs were bred with animals heterozygous for the SV2A gene disruption to produce SV2A$^{+/-}$SV2B$^{-/-}$ breeders, which were used to generate SV2A/B KOs. WT C57BL/6 and SV2 KO mouse brain membranes for Western blot and binding analyses were prepared as follows. Frozen whole brains were homogenized (10% wt/vol) in 20 mM Tris·HCl buffer (pH 7.4) containing 250 mM sucrose (buffer A). The homogenates were spun at 30,000 × *g* at 4°C for 15 min, and the pellets were resuspended in the same buffer. After incubation at 37°C for 15 min, the membranes were washed two times, resuspended in buffer A, and frozen.

Photoaffinity Labeling Experiments. Purification of crude synaptosomal (P2) and synaptic vesicle (LP2) fractions from rat brain was performed as reported (12), except that the LP2 fraction was washed at 100,000 × *g* for 60 min. Photoaffinity labeling was performed as described (12), except for using synaptic vesicle fractions instead of brain membranes. The inclusion of 1 mM LEV in the reaction was shown to prevent the photolabeling. For size analysis of the labeled binding site, membranes were extracted and run on SDS/PAGE. The developed gel was sliced, and the slices were solubilized and counted as described (12).

Synaptic Vesicle Purification from WT and KO Mice. Synaptic vesicles were purified by using the technique of Huttner *et al*. (18) as described (19), except that the final two centrifugation steps were done by using a SW 50 rotor.

Cloning and Expression of SV2 Isoforms. Human SV2A (hSV2A) was PCR-amplified from a human fetal brain cDNA with added GATEWAY (Invitrogen) attB1 and attB2 flanking sequences. The resultant product was cloned by recombination into a pDONR201 vector (Invitrogen). hSV2B and hSV2C were PCR-amplified from first-strand cDNA synthesized from human adult brain total RNA (Ambion, Austin, TX) and recombined by TA cloning into a GATEWAY pENTR/SD/D vector (Invitrogen). All three isoforms were recombined into pd40 expression vectors (Invitrogen), supporting expression of the native, nonfused protein from a cytomegalovirus immediate-early promoter. For transfections, one of four vectors was used, either a control vector containing the β-galactosidase (β-gal) gene, or hSV2A, hSV2B, or hSV2C in pd40 vectors. Transient transfections in COS-7 cells were performed on preconfluent cells by using either the reagent Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche), with equivalent results. For confluent cell assays, cells were grown, transfected, and assayed in 24-well plates. For suspension cell assays, cells were grown in, transfected in, and harvested from 10-cm plates (and stored at −80°C for later use). Cells were used 48 h posttransfection.

SV2A Solubilization and Immunoprecipitation. Rat brain membranes were diluted in a solubilization buffer [20 mM Tris·HCl (pH 7.4), 0.25 M sucrose, and protease inhibitors (Complete, Roche)] containing 15 mM *n*-dodecyl-β-D-maltoside (DM), incubated for 2 h at 4°C and centrifuged at 4°C for 1 h at 100,000 × *g*. Four micrograms of anti-SV2A antibodies (sc-11936, Santa Cruz Biotechnology) or normal goat IgG (sc-2028, Santa Cruz Biotechnology) was added to 1.5 mg of supernatant for overnight incubation at 4°C, and then incubated with protein A-Sepharose beads for 1 h at 4°C. After several washes, the immune pellets were collected by centrifugation and boiled for 5 min in SDS sample buffer containing 2-mercaptoethanol. Proteins were separated by SDS/PAGE and transferred to nitrocellulose for immunoblotting with anti-SV2A antibodies (see above). Twenty micrograms of rat brain membranes without immunoprecipitation was immunoblotted as a control.

Western Blot Analysis of Protein Expression. Approximately 10 μg of total protein from WT and KO brain membranes was loaded on a Tris·glycine/4–12% polyacrylamide gel and developed. After transfer to a nitrocellulose membrane, the blots were probed with either a monoclonal cross-reactive to all SV2 proteins (13) (obtained from the Developmental Studies Hybridoma Bank, supported by the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa, Iowa City) or with a polyclonal antibody specific for the SV2A isoform (sc-11936, Santa Cruz Biotechnology, as above). For the synaptic vesicle purification experiment, 2 μg of each fraction was analyzed by immunoblot for SV2A, by using an SV2A-specific polyclonal antibody (14), and for the synaptic vesicle protein synaptophysin, by using an anti-synaptophysin monoclonal antibody from Chemicon. Analysis of the expression of SV2 isoforms in COS-7 cells was performed by immunoblot against 13 μg (SV2A, SV2B, β-gal), or 39 μg of total protein (SV2C), and probing with the SV2 monoclonal as described above.

Binding of [^{3}H]ucb 30889 to Brain Membranes, Heterologous SV2 Proteins, and Immunoprecipitated SV2A. WT and KO membrane and synaptic vesicle preparation binding assays were performed as described (15), either by using 100 μg of protein (brain membranes) and 1.8 nM or by using 20 μg of protein (synaptic vesicle fractions) and 3.6 nM [^{3}H]ucb 30889 per assay. Binding assays with solubilized and immunoprecipitated SV2A used similar conditions (1.8 nM [^{3}H]ucb 30889). For binding experiments on the different SV2 isoforms, transfected cells were incubated for 2 h at 4°C in PBS with [^{3}H]ucb 30889 (1.8 nM), in the presence or absence of 1 mM LEV. The assay was terminated by harvesting in a 24-well GF/B filter plate (PerkinElmer) with rapid washing using 4°C PBS. IC_{50} curves of LEV, ucb 30889, and ucb L060 against hSV2A were determined by using confluent cells in 24-well plates and binding conditions as above, followed by rinsing the cells three times rapidly with 4°C PBS. After a final aspiration, 200 μl of 0.1 N NaOH was added to lyse the cells, and the samples were counted. For binding experiments on the larger series of LEV derivatives to hSV2A, aliquots of previously frozen transfected COS-7 cells, containing 2–3 × 10^4 cells, were incubated for 120 min at 4°C in 0.2 ml of a RPMI medium 1640-Hepes 25 mM solution containing [^{3}H]ucb 30889 (1.8 nM) and increasing concentrations of unlabeled competing drugs. The termination of the binding reaction by filtration and radioactivity counting was performed as described above. The −log IC_{50}s (pIC_{50}s) were determined by nonlinear curve fitting.

Audiogenic Seizure Mouse Model. Antiseizure activity of LEV and analogues was assessed in sound-susceptible mice (20) by exposing the mice to acoustic stimuli of 90 db, 10–20 kHz for 30 s, 60 min after i.p. pretreatment. The reported ED_{50} values were obtained from testing of four to eight groups (n = 10) administered different doses and reflect the potency of the compounds for inhibiting clonic convulsions.

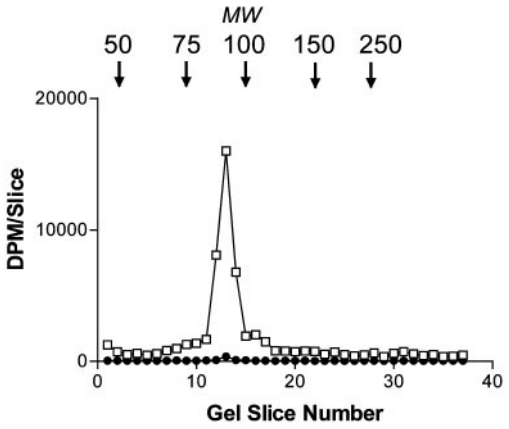

Fig. 1. Photolabeling of the LEV-binding site in synaptic vesicles from rat brain. After photoaffinity labeling with [^{3}H]ucb 30889, SDS-solubilized proteins were run on an SDS/PAGE gel, and then the gel was cut into thin slices and counted for ^{3}H content. Photoaffinity labeling of crude synaptosomal (P2) (●) and synaptic vesicle (LP2) (□) fractions by using [^{3}H]ucb 30889 is shown. Photoaffinity labeling identifies a protein of ≈90 kDa enriched in the synaptic vesicle fraction.

Results

Photoaffinity Labeling of LEV-Binding Site in Synaptic Vesicles Labels a 90-kDa Protein. [^{3}H]ucb 30889 is a photoactivable derivative of LEV that shows a higher affinity for the LEV-binding site in brain but otherwise behaves as a surrogate for LEV (15). This photoaffinity ligand was used to label the LEV-binding site in a synaptic vesicle fraction purified from rat brain membranes by using density centrifugation (Fig. 1). Gel electrophoresis (SDS/PAGE) of the photo-cross-linked sample revealed that the radioligand labeled a protein with an approximate molecular mass of 90 kDa, consistent with results previously seen in crude brain membranes, where it was demonstrated that labeling occurred to an ≈90-kDa integral membrane protein (12). The synaptic vesicle localization of the binding site, along with the measured size and an integral membrane character led us to consider SV2 proteins as the primary candidate for the binding site.

[^{3}H]ucb 30889 Binds Only to Animal Brain Membranes and Synaptic Vesicles Containing the SV2A Isoform. To determine whether SV2 is necessary for LEV and related compound binding, we measured [^{3}H]ucb 30889 binding to brain membranes from WT and SV2 KO mice. We found that [^{3}H]ucb 30889 binds only to membranes from animals expressing SV2A (Fig. 2 *A* and *B*). Western analysis of brain membranes from WT and SV2 KOs, using a monoclonal antibody cross-reactive to all isoforms (SV2A, SV2B, and SV2C), or using a polyclonal specific to SV2A, confirmed the expected genotype, with no SV2A immunoreactivity in the SV2A or SV2A/B KOs (Fig. 2*A*). There was no binding of [^{3}H]ucb 30889 to brain membranes from animals lacking SV2A and no significant binding in the SV2A/B double homozygote, which still expresses SV2C (Fig. 2*B*). Membranes from animals lacking only SV2B show binding to [^{3}H]ucb 30889 that is roughly equivalent to that seen in WT (Fig. 2*B*). However, we do measure a statistically significant difference between total binding to the WT and SV2B KO membranes (at $P < 0.05$ by two-tailed t test). We have confirmed this observation by Scatchard analysis of binding to the WT and SV2B KO (data not shown), but no difference was observed between the affinities of [^{3}H]ucb 30889 for the brain membranes from the WT or SV2B KO animals. Taken together, these results indicate that the observed binding in WT mouse brain is completely dependent on the presence of SV2A. However, the results raise the possibility that the presence of SV2B might affect the binding to SV2A. If there is a residual binding to SV2C in brain, it is so low that it cannot be responsible for the observed binding site in WT

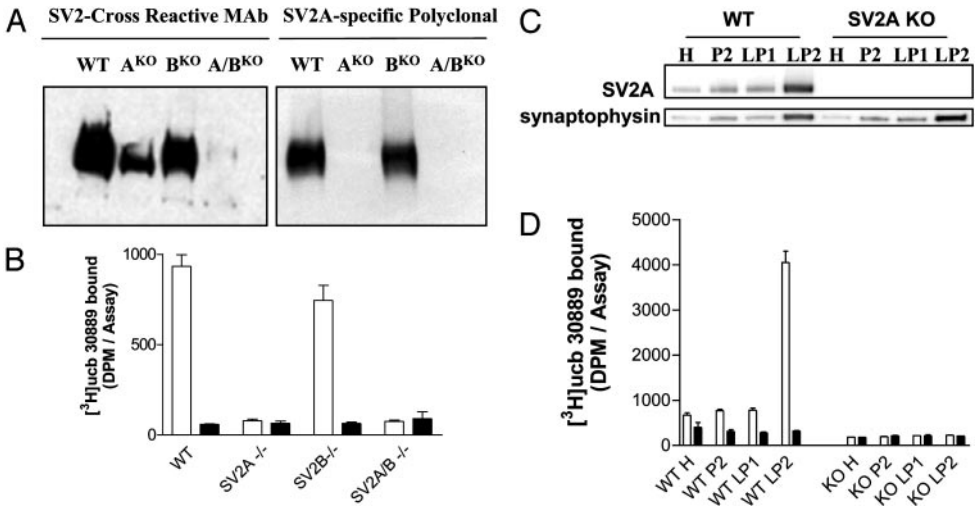

Fig. 2. Binding of [^{3}H]ucb 30889 to WT and KO brain membranes and synaptic vesicles. (*A*) Western blot of brain membranes from WT and homozygous KO mice probed with an anti-SV2 monoclonal antibody (cross-reactive to all isoforms) or with an anti-SV2A-specific polyclonal antibody. Lanes 1, WT; lanes 2, SV2A$^{-/-}$ KO; lanes 3, SV2B$^{-/-}$ KO; lanes 4, SV2A$^{-/-}$/B$^{-/-}$ double KO. (*B*) Binding of [^{3}H]ucb 30889 to brain membranes from WT, SV2A$^{-/-}$, SV2B$^{-/-}$, and SV2A$^{-/-}$/SV2B$^{-/-}$ KO mice. Binding is observed only to membranes from animals expressing SV2A. □, [^{3}H]ucb 30889 alone; ■, [^{3}H]ucb 30889 plus 1 mM LEV . Error bars are the SD of experiments performed with five WT brains and four KO brains, with three replicates within each experiment. (*C*) Purification of synaptic vesicles enriches for the synaptic vesicle proteins and LEV binding. Shown are blots of mouse brain homogenate (H), crude synaptosomes (P2), plasma and heavy membranes (LP1), and synaptic vesicles (LP2) (2 μg of each fraction) that were probed for the synaptic vesicle proteins SV2A (*Upper*) and synaptophysin (*Lower*). The synaptic vesicle fraction from WT animals displays enrichment of both synaptic vesicle proteins and LEV-binding proteins, whereas material from SV2A KOs shows enrichment of synaptophysin only. (*D*) Binding to the different fractions using [^{3}H]ucb 30889 shows significant binding only to the WT LP2 fraction, containing SV2A-rich synaptic vesicles. Shown are [^{3}H]ucb 30889 alone (open bars) and [^{3}H]ucb 30889 plus 1 mM LEV (filled bars). Shown are representative examples of two experiments. Error bars are the SD of two replicates.

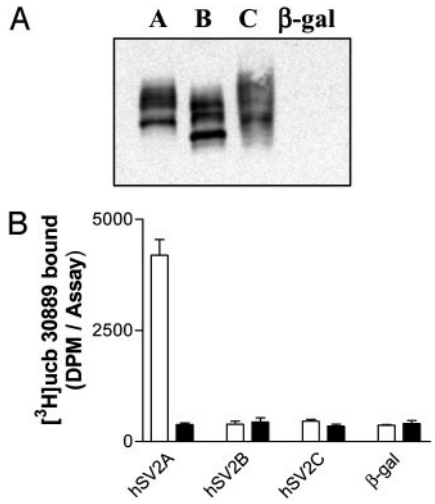

Fig. 3. Binding of [^{3}H]ucb 30889 to heterologously expressed SV2 isoforms. (*A*) Western blot with cross-reactive anti-SV2 monoclonal showing roughly equivalent amounts of hSV2A (lane A), hSV2B (lane B), and hSV2C (lane C). There is no detectable SV2 immunoreactivity in the β-gal-transfected cells (β-gal, fourth lane). In the case of hSV2C, three times as much total protein was loaded as used for the other samples. (*B*) Binding of [^{3}H]ucb 30889 to hSV2A, hSV2B, or hSV2C (three times the number of cells as other samples) transiently expressed in COS-7 cells. Significant binding is observed only to hSV2A, not to hSV2B or hSV2C. Shown are [^{3}H]ucb 30889 alone (open bars) and [^{3}H]ucb 30889 plus 1 mM LEV (filled bars).

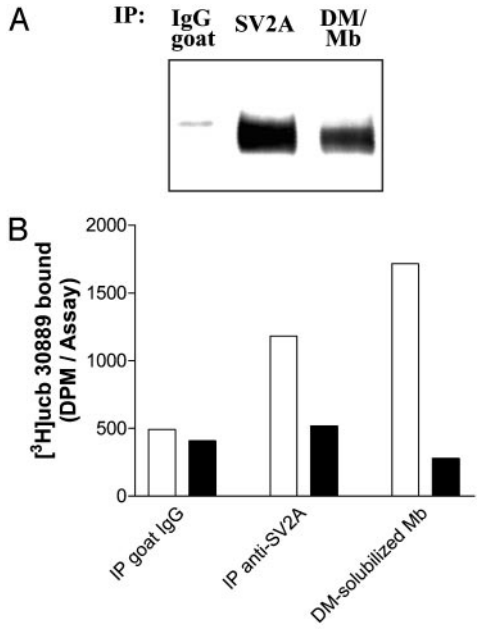

Fig. 4. Binding of [^{3}H]ucb 30889 to immunoprecipitated SV2A. (*A*) Western blot of immunoprecipitated SV2A. Immunoprecipitation was performed with a goat antibody against SV2A, or normal goat IgG. Western blotting with the SV2A antibody revealed the presence of the protein in the former condition only. Mb, membrane; IP, immunoprecipitate. (*B*) Binding of [^{3}H]ucb 30889 to SV2A purified by IP from a detergent-soluble fraction of rat cortical membranes. Binding occurs only to the DM-solubilized membrane or anti-SV2A immunoprecipitate, not to the control IgG immunoprecipitate. Shown are [^{3}H]ucb 30889 alone (open bars) and [^{3}H]ucb 30889 plus 1 mM LEV (filled bars). Shown is a representative example of three experiments.

animals based on the binding capacity in total mouse brain membranes.

To determine whether LEV binds synaptic vesicle-associated SV2, we compared [^{3}H]ucb 30889 binding across synaptic vesicle preparations from WT and SV2A KO mice (Fig. 2 *C* and *D*). Western blot analysis of the fractions obtained across the purification confirms the coenrichment of the synaptic vesicle proteins synaptophysin and SV2A with [^{3}H]ucb 30889 binding. In contrast, the equivalent SV2A KO samples show enrichment of synaptophysin in the synaptic vesicle-enriched fraction LP2, but an absence of SV2A expression and [^{3}H]ucb 30889 binding throughout all samples. The results confirm the synaptic vesicle localization of the LEV-binding site (12) and support the identity of the LEV-binding site as SV2A.

hSV2A Is Sufficient for [^{3}H]ucb 30889 Binding. To determine whether SV2A is solely responsible for the brain binding of LEV, we analyzed binding to SV2A expressed heterologously in nonneural cells (Fig. 3). hSV2A, hSV2B, and hSV2C were transiently expressed in the fibroblast cell line COS-7, and the expression was verified by Western blot analysis (Fig. 3*A*). Note that the heterogeneous pattern of staining of all three isoforms in the Western blot, which differs somewhat from that observed in brain (Fig. 2*A*), has been reported in the literature and attributed to heterogeneity in the SV2 protein's glycosylation (21). We generally observe that hSV2A and hSV2B have similar levels of expression in the COS-7 system, but that hSV2C expresses at a lower level. We thus corrected for the lower expression of hSV2C in the binding assay by increasing the amount of material added (3×) to yield approximately equivalent amounts of immunoreactive protein in all three isoforms (as shown in Fig. 3*B*). There are significant levels of binding of [^{3}H]ucb 30889 in cells expressing hSV2A, and this binding is displaced by excess LEV, indicating that it is specific. No statistically significant binding was observed under identical conditions to COS-7 cells transfected with hSV2B or hSV2C, or to COS-7 cells transfected with a vector encoding β-gal (Fig. 3*B*), consistent with the interpretation that SV2A is the sole LEV-binding SV2 isoform.

[^{3}H]ucb 30889 Binds to Immunoprecipitated SV2A. We next determined whether [^{3}H]ucb 30889 binds to native SV2A immunoprecipitated from membrane extracts. Rat cortical membranes were solubilized with DM and SV2A immunoprecipitated with a selective goat polyclonal antibody. Immunoprecipitation was confirmed by a Western blot analysis, which showed the presence of large amounts of SV2 immunoreactivity in the starting extract, and in the immunoprecipitated sample, but not in the IgG control sample (Fig. 4*A*). Binding to [^{3}H]ucb 30889 was observed in the anti-SV2A immunoprecipitated sample, but not in the negative control (Fig. 4*B*). This result suggests a direct interaction between [^{3}H]ucb 30889 and the immunopurified SV2A protein.

Compound Affinities to SV2A Correlate with Affinities to the LEV-Binding Site in Brain and to Antiseizure Potencies. To characterize in more detail the nature of the binding interaction of LEV and derivatives to the SV2A protein, we measured their relative affinities for hSV2A. In experiments testing the ability of unlabeled compounds to displace [^{3}H]ucb 30889 from hSV2A expressed in COS-7 cells, the affinities (pIC_{50s}) of ucb 30889, LEV, and LEV's enantiomer, ucb L060 (7.2, 5.7, and 3.6, respectively), show the same rank order and similar values (Fig. 5*A*) to those previously reported in studies of LEV binding to rat brain membranes (11, 15). Critically, ucb L060 binds with significantly less affinity to hSV2A than does LEV. The stereoselectivity for LEV over its opposite stereoisomer, ucb L060, is a key characteristic of the binding site in brain and was confirmed by these studies.

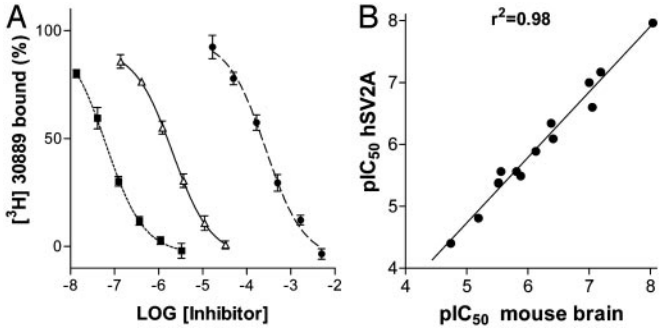

Fig. 5. Binding affinities of selected LEV derivatives for human SV2A. (*A*) IC_{50} curves of LEV, ucb L060, and ucb 30889 against hSV2A transiently expressed in COS-7, using [^{3}H]ucb 30889. Shown are LEV (△), ucb 30889 (■), and ucb L060 (●). Error bars are SEM, $n = 3$. (*B*) Correlation of binding of a series of LEV compounds to mouse brain and to hSV2A. Shown are pIC_{50}s measured by using [^{3}H]ucb 30889. There is a high degree of correlation between binding affinity of these compounds to hSV2A and to mouse brain membranes. pIC_{50} values are the mean of two independent experiments, where each determination lies within 0.2 log units of the mean.

Testing the binding of LEV and additional analogs to hSV2A expressed in COS-7 cells revealed that pIC_{50}s are highly correlated with the values obtained in mouse brain ($r^2 = 0.98$) (Fig. 5*B*) and rat brain membranes (data not shown). There was also a clear correlation between the affinities of these compounds for hSV2A in COS-7 and the potency of their antiseizure protection in the mouse audiogenic model of epilepsy ($r^2 = 0.84$) (Fig. 6). These data are consistent with the previous report of a correlation between binding of LEV analogs in rat brain and antiseizure potency (11). We also investigated the binding of other AEDs, including valproate, carbamazepine, phenytoin, ethosuximide, felbamate, gabapentin, tiagabine, vigabatrin, and zonisamide. None of these AEDs, at concentrations up to 100 μM, competed with [^{3}H]ucb 30889 for binding to SV2A (data not shown), consistent with previous studies testing AEDs against the LEV-binding site in rat brain (11, 15).

Discussion

We have identified SV2A as the binding site for the antiepileptic drug LEV and as a potential target for CNS therapy. Intriguingly, SV2A KO mice seem normal at birth, but develop an unusually strong seizure phenotype by 1.5 weeks of age, and usually die within 3 weeks after birth (16). SV2B KOs, on the other hand, do not display seizures or other gross phenotypes (17) (SV2C KO mice have not been reported). The severe disability of the homozygous SV2A KO mice has prevented us from performing certain desirable experiments, such as testing the effects of LEV on seizures in these animals, or indeed, fully characterizing the seizures by using implantable electrodes. Both inhibitory (16) and excitatory (V. Lopantsev and S.M.B., unpublished results) neurotransmission is reduced in the absence of SV2A. Likewise, studies in adrenal chromaffin cells from SV2A KO mice revealed reduced calcium-stimulated exocytosis, suggesting that the release probability of synaptic vesicles is reduced in the absence of SV2A (22). Also, hippocampal neurons cultured from mice lacking both SV2A and SV2B demonstrate altered activity-dependent synaptic depression (17). These examples of altered neurotransmission were all observed in the absence of changes in either synapse or synaptic vesicle density or morphology (16, 17, 22). Together these observations suggest that SV2A acts as a modulator of vesicle fusion although it is possible that SV2A has additional functions at the presynaptic terminal.

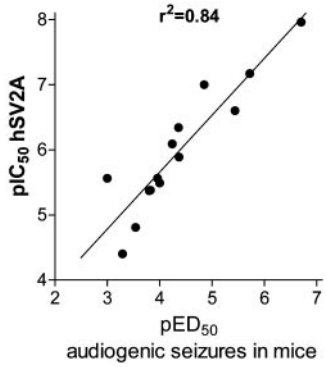

Fig. 6. Correlation between binding affinity and antiseizure potency of LEV derivatives. Correlation of binding of a series of LEV-related compounds to hSV2A assayed in transiently transfected COS-7 cells (pIC_{50}s measured by using [^{3}H]ucb 30889), and of antiseizure potencies shown as the $-\log ED_{50}$s (pED_{50}s) in the mouse audiogenic seizure model. There is a good correlation between antiseizure potency in audiogenic mice and affinity to hSV2A.

The molecular action of SV2A is unknown. The SV2s are twelve transmembrane integral membrane glycoproteins with a significant homology to the major facilitator superfamily (MFS) of transporters found in both bacteria and eukaryotes (23). Given their universal presence in synaptic vesicles, it has been proposed that the SV2s might transport a common constituent of the vesicles, such as calcium or ATP (14). However, no transport function has yet been found to rely on an SV2 protein. SV2A interacts with the presynaptic protein synaptotagmin, considered the primary calcium sensor for regulating calcium-dependent exocytosis of synaptic vesicles, and may affect synaptotagmin's function (19, 24). Also, SV2 proteins seem to contribute the majority of sugar side chains to the lumen of synaptic vesicles and perhaps are the source of a neurotransmitter-binding matrix hypothesized to exist in the synaptic vesicle interior (25). Given the growing recognition that many, if not most, proteins have multiple functions (26), it is possible that SV2A has several functions, including those discussed above, but perhaps others as well. The availability of a compound that acts through SV2A should provide a powerful tool to probe the structure and function of this protein and may contribute to the understanding of the regulation of neurotransmitter release.

The identification of the synaptic vesicle protein SV2A as the binding site in brain for LEV has important implications not only for the antiepileptic mechanism of action of LEV, but also for future drug discovery in epilepsy and other neurological conditions. The correlation between antiseizure properties of LEV derivatives and their affinity for SV2A strongly suggests a mechanistic link between the two. There are reports of other effects of LEV, including the partial inhibition of N-type voltage-gated Ca^{2+} channels and the reduction of inhibition of γ-aminobutyric acid (GABA)- and glycine-gated currents, induced by Zn^{2+} and β-carbolines (9). Currently, it is unclear whether these effects are mediated by the observed interaction with SV2A, or by alternate mechanisms. In either case, we believe that the correlation between SV2A binding and drug potency suggests that LEV is modulating one or more of the functions of SV2A, and correspondingly contributing to its efficacy in treating epilepsy.

Determining the effect of LEV on SV2A function is complicated by both the absence of proven SV2A functions, and also by the unusual pharmacology of LEV, which lacks effects on the electrophysiology of normal brain tissue and neurons (4, 27), and on standard amino acid neurotransmitter release as studied by microdialysis in normal brain (28). The apparent lack of effect on normal electrophysiology has implications for any hypothesis of LEV's effects on the function(s) of SV2A. We do not anticipate LEV to affect SV2A functions that are critical to

normal physiology of the neuron but rather think LEV might modulate a function of SV2A present only under pathophysiological conditions. It is thus unlikely that LEV alters synaptic release in normal brain and neurons, and indeed, there is no evidence for such an effect.

The strong seizure phenotype observed in the SV2A KO animals supports the interpretation that SV2 can influence mechanisms of seizure generation or propagation. The fact that the SV2A KO mice exhibit seizures, while LEV inhibits seizures, suggests that LEV is not acting simply as an antagonist of SV2A function (or LEV would presumably act as a proconvulsant instead of an anticonvulsant). It is possible that LEV binding enhances a function of SV2A that inhibits abnormal bursting in epileptic circuits, a function whose loss in the SV2A KOs results in seizures. Alternately, the seizure phenotype observed in SV2A KOs may arise indirectly from pathophysiological developmental changes in synaptic function caused by the SV2A deletion, changes that are not directly related to the antiseizure functions of SV2A that LEV presumably enhances.

These results imply a mechanism of action of LEV that is distinct from that of other antiepileptic drugs and suggests that other vesicle proteins in the presynaptic terminal may also represent promising targets for the discovery of drugs for treating epilepsy and other neurological disorders.

We thank Jacqueline Van Geffel and Michel Legrand for their technical expertise and Dr. Henrik Klitgaard for critically reading the manuscript. We thank Dina Anderson and Alex Golden for their work on cloning hSV2B and hSV2C, respectively. We also thank Dr. Jeffrey Noebels for many helpful discussions. Parts of this work were supported by National Institutes of Health Grant R01 MH59842 (to S.M.B.).

1. Scheuer, M. L. & Pedley, T. A. (1990) *N. Engl. J. Med.* **323,** 1468–1474.
2. Hovinga, C. A. (2001) *Pharmacotherapy* **21,** 1375–1388.
3. Nash, E. M. & Sangha, K. S. (2001) *Am. J. Health Syst. Pharm.* **58,** 1195–1199.
4. Klitgaard, H. (2001) *Epilepsia* **42,** Suppl. 4, 13–18.
5. Klitgaard, H., Matagne, A., Gobert, J. & Wulfert, E. (1998) *Eur. J. Pharmacol.* **353,** 191–206.
6. Margineanu, D. G. & Klitgaard, H. (2000) *Pharmacol. Res.* **42,** 281–285.
7. Niespodziany, I., Klitgaard, H. & Margineanu, D. G. (2003) *NeuroReport* **14,** 1273–1276.
8. Löscher, W., Hönack, D. & Rundfeldt, C. (1998) *J. Pharmacol. Exp. Ther.* **284,** 474–479.
9. Margineanu, D. G. & Klitgaard, H. (2002) in *Antiepileptic Drugs*, eds. Levy, R. H., Mattson, R. H., Meldrum, B. S. & Perucca, E. (Lippincott Williams & Wilkins, Philadelphia), 5th Ed., pp. 419–427.
10. Rigo, J. M., Hans, G., Nguyen, L., Rocher, V., Belachew, S., Malgrange, B., Leprince, P., Moonen, G., Selak, I., Matagne, A. & Klitgaard, H. (2002) *Br. J. Pharmacol.* **136,** 659–672.
11. Noyer, M., Gillard, M., Matagne, A., Hénichart, J.-P. & Wülfert, E. (1995) *Eur. J. Pharmacol.* **286,** 137–146.
12. Fuks, B., Gillard, M., Michel, P., Lynch, B. A., Vertongen, P., Leprince, P., Klitgaard, H. & Chatelain, P. (2003) *Eur. J. Pharmacol.* **478,** 11–19.
13. Buckley, K. & Kelly, R. B. (1985) *J. Cell Biol.* **100,** 1284–1294.
14. Bajjalieh, S. M., Frantz, G. D., Weimann, J. M., McConnell, S. K. & Scheller, R. H. (1994) *J. Neurosci.* **14,** 5223–5235.
15. Gillard, M., Fuks, B., Michel, P., Vertongen, P., Massingham, R. & Chatelain, P. (2003) *Eur. J. Pharmacol.* **478,** 1–9.
16. Crowder, K. M., Gunther, J. M., Jones, T. A., Hale, B. D., Zhang, H. Z., Peterson, M. R., Scheller, R. H., Chavkin, C. & Bajjalieh, S. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 15268–15273.
17. Janz, R., Goda, Y., Geppert, M., Missler, M. & Sudhof, T. C. (1999) *Neuron* **24,** 1003–1016.
18. Huttner, W. B., Schiebler, W., Greengard, P. & De Camilli, P. (1983) *J. Cell Biol.* **96,** 1374–1388.
19. Pyle, R. A., Schivell, A. E., Hidaka, H. & Bajjalieh, S. M. (2000) *J. Biol. Chem.* **275,** 17195–17200.
20. Chapman, A. G., Croucher, M. J. & Meldrum, B. S. (1984) *Arzneimittelforschung* **34,** 1261–1264.
21. Feany, M. B., Lee, S., Edwards, R. H. & Buckley, K. M. (1992) *Cell* **70,** 861–867.
22. Xu, T. & Bajjalieh, S. M. (2001) *Nat. Cell Biol.* **3,** 691–698.
23. Bajjalieh, S. M., Peterson, K., Shinghal, R. & Scheller, R. H. (1992) *Science* **257,** 1271–1273.
24. Schivell, A. E., Batchelor, R. H. & Bajjalieh, S. M. (1996) *J. Biol. Chem.* **271,** 27770–27775.
25. Reigada, D., Diez-Perez, I., Gorostiza, P., Verdaguer, A., Gomez de Aranda, I., Pineda, O., Vilarrasa, J., Marsal, J., Blasi, J., Aleu, J. & Solsona, C. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 3485–3490.
26. Jeffery, C. J. (2003) *Trends Genet.* **19,** 415–417.
27. Birnstiel, S., Wulfert, E. & Beck, S. G. (1997) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **356,** 611–618.
28. Tong, X. & Patsalos, P. N. (2001) *Br. J. Pharmacol.* **133,** 867–874.