Design, Synthesis, and Characterization of High-Affinity, Systemically-Active Galanin Analogues with Potent Anticonvulsant Activities

Grzegorz Bulaj,*[,†] Brad R. Green,[†] Hee-Kyoung Lee,[†] Charles R. Robertson,[†] Karen White,[†] Liuyin Zhang,[†] Marianna Sochanska,[†] Sean P. Flynn,[‡] Erika Adkins Scholl,[‡] Timothy H. Pruess,[‡] Misty D. Smith,[‡] and H. Steve White[‡]

Department of Medicinal Chemistry, Department of Pharmacology and Toxicology, College of Pharmacy, University of Utah, Salt Lake City, Utah 84108

Received September 2, 2008

Galanin is an endogenous neuropeptide that modulates seizures in the brain. Because this neuropeptide does not penetrate the blood−brain barrier, we designed truncated galanin analogues in which nonessential amino acid residues were replaced by cationic and/or lipoamino acid residues. The analogues prevented seizures in the 6 Hz mouse model of epilepsy following intraperitoneal administration. The most active analogue, Gal-B2 (NAX 5055), contained the -Lys-Lys-Lys(palmitoyl)-Lys-NH_2 motif and exhibited high affinity for galanin receptors (K_i = 3.5 nM and 51.5 nM for GalR1 and GalR2, respectively), logD = 1.24, minimal helical conformation and improved metabolic stability. Structure−activity-relationship analysis suggested that cationization combined with position-specific lipidization was critical for improving the systemic activity of the analogues. Because the anticonvulsant activity of galanin is mediated by the receptors located in hippocampus and other limbic brain structures, our data suggest that these analogues penetrate into the brain. Gal-B2 may lead to development of first-in-class antiepileptic drugs.

Introduction

Anticonvulsant neuropeptides are potent modulators of neuronal excitability.[1,2] This class of peptides include galanin,[3] neuropeptide Y (NPY[a]),[4,5] somatostatin,[6] or dynorphin A;[7] when endogenously expressed or delivered directly into the central nervous system (CNS), these peptides not only can suppress seizures in animal models of epilepsy but they also mediate antinociceptive effects. Furthermore, there is a growing body of evidence that some anticonvulsant neuropeptides may have disease-modifying properties, such as slowing, halting, or perhaps even preventing development of epileptic seizures.[8−10] Therefore, the anticonvulsant neuropeptides provide an opportunity to develop disease-modifying therapeutics for epilepsy.[6,8,11,12] If these peptides could be specifically targeted into the CNS, they may become first-in-class cures for many neurological diseases.

Galanin is an example of such a promising anticonvulsant peptide that exhibits disease-modification properties.[8,13,14] The acute administration of galanin receptor agonists or virus-mediated overexpression of galanin in the hippocampus has been found to inhibit limbic status epilepticus, pentylenetetrazol, and picrotoxin seizures in rats and mice.[13,15−19] Furthermore, the seizure threshold of galanin overexpressing transgenic animals is increased in status epilepticus and kindling models.[14,20,21] In vitro, galanin inhibits glutamate release from the hippocampus.[20,22] Galanin elicits multiple effects in the brain via three galanin receptors belonging to the G protein-coupled receptors (GPCRs).[23−25] Galanin receptor subtype 1 (GalR1) is present in many brain areas but displays the highest expression in the hippocampus.[26] The galanin receptor subtype 2 (GalR2) is expressed in the hypothalamus, the hippocampus, the amygdala, piriform cortex, basal forebrain (medial septum/diagonal band), the cerebellum, and the brainstem. Galanin receptor subtype 3 (GalR3) is most abundant in the hypothalamus but is absent from the hippocampus. Results from studies with the GalR1 knockout mice and rats treated with GalR2 peptide nucleic acid antisense suggests that galanin exerts its anticonvulsant effect through an action at both GalR1 and GalR2.[27,28] Furthermore, GalR2 is thought to play an important role in the neuroprotective effects of galanin in hippocampal neurons.[17,27,29,30] Results from a study conducted in a model of rapid kindling suggest that hippocampal GalR1 and GalR2 exert antiepileptogenic effects although via different signaling pathways.[8] Therefore, on the basis of the current literature, systemically active galanin receptor agonists that penetrate into the hippocampus might offer promising novel therapeutics that not only control epileptic seizures, but could also modify the progression of epilepsy.

Previous attempts to generate systemically active galanin agonists provided two compounds, galnon and galmic (Figure 1A, Table 1).[16,19,31] Galnon became a useful pharmacological tool to study the effects of galanin receptors in the CNS.[21,32,33] However, subsequent studies revealed that, at a concentration of 10 μM, both galnon and galmic interacted with a variety of nongalanin receptors including 5-HT-1A, 1B receptors, D2 dopamine receptors, ghrelin and melanocortin receptors, NPY receptors (galnon only), or μ-opioid receptors (galmic only), suggesting that some of the pharmacological properties of galnon or galmic might be mediated by nongalanin receptors.[34] Thus, despite these previous efforts, there is a continuous need for systemically active and selective galanin receptor ligands. Improved penetration of peptides into the CNS was successfully

* To whom correspondence should be addressed. Phone: (801) 581-4629. Fax: (801)581-7087. E-mail: bulaj@pharm.utah.edu. Address: Department of Medicinal Chemistry, College of Pharmacy, University of Utah, 421 Wakara Way, Suite 360, Salt Lake City, Utah 84108.

† Department of Medicinal Chemistry, College of Pharmacy, University of Utah.

‡ Department of Pharmacology and Toxicology, College of Pharmacy, University of Utah.

[a] Abbreviations: AEDs, antiepileptic drugs; AUC, area under the curve; BBB, blood−brain barrier; CD, circular dichroism; CMC, critical micelle concentration; CNS, central nervous system; Gal-B2, lead galanin compound described in this work; GalR1, galanin receptors subtype 1; GalR2, galanin receptors subtype 2; GPCRs, G-protein coupled receptors; ip, intraperitoneally; LAA, lipoamino acid; NPY, neuropeptide Y; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; TFE, 2,2,2-trifluoroethanol; TPE, time to peak effect.

10.1021/jm801088x CCC: $40.75
Published on Web 11/21/2008

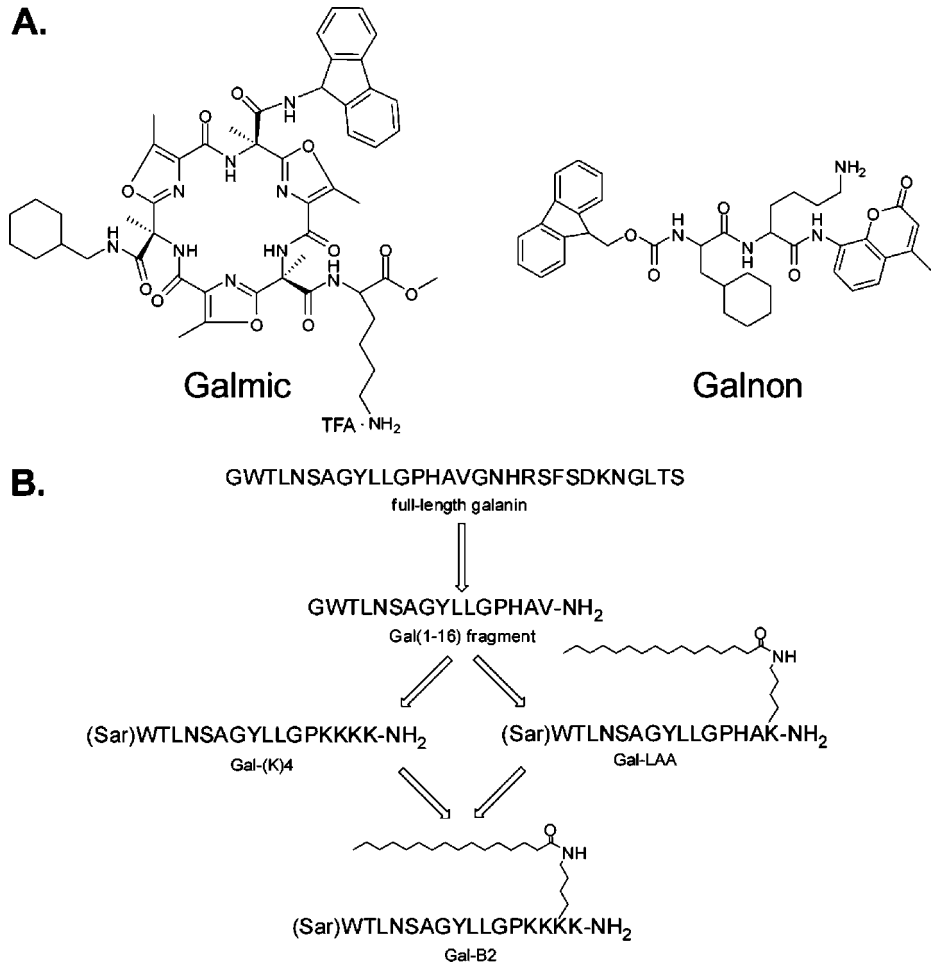

Figure 1. Developing systemically active galanin receptor ligands. (A) structures of two galanin agonists, galmic and galnon, discovered through combinatorial approaches. (B) A rational design of systemically active galanin analogues described in this work.

Table 1. Selected Galanin-Based Ligands and Their Affinities to the Galanin Receptors[a]

ligand	affinity[a], K_d [nM]		
	hGalR1	hGalR2	hGalR3
agonists			
hGAL(1−29)	0.4	2.3	69.0
pGAL(1−16)	3.2	5.0	315.0
GAL(2−11)	879	1.8	n.d.
agonists that cross blood−brain barrier			
galnon	12000	24000	n.d.
galmic	34000	>100000	n.d.
antagonists			
M35	0.1	2	15
M15 (galantide)	0.3	1	40

[a] K_d values are taken from the ref 25.

achieved by introducing such chemical modifications as polyamines (cationization), lipids, or glycosyl groups.[35−37] In this research, we investigated how selected chemical modifications may improve CNS bioavailability of galanin. Here, we report that the combination of C-terminal cationization/lipidization to the Gal(1−13) core provided metabolically stable and high-affinity galanin analogues that exert potent anticonvulsant activity following systemic administration.

Results

Design Strategy. Galanin is a 29−30 amino acid long peptide (Figure 1B),[38] where the first N-terminal 14 residues are highly conserved within different animal species.[39] The N-terminal core fragment comprises critical residues: Gly1, Trp2, Asn5, Tyr9, and Gly12, and it was previously shown to maintain high affinity toward galanin receptors.[40,41] Indeed, the truncated Gal(1−16)-NH_2 analogue is a low nanomolar agonist for both GalR1 and GalR2, slightly preferring GalR1. Alanine-walk analogues of Gal(1−16) indicated that individual replacements of amino acid residues within the (1−12) fragment affected binding properties to both galanin receptors, with GalR2 being more sensitive.[42,43] All analogues of Gal(1−13) with the ε-amino group of Lys14 conjugated to different groups varying in size retained high affinity, suggesting that the C-terminal portion of the truncated analogues may be amenable to the introduction of bulky modifications.[44] Additional SAR information utilized in our design was that the N-methylation of Gly1 did not affect receptor binding properties, whereas it improved metabolic stability of the analogue containing the N-terminal sarcosine residue.[45]

To develop systemically active galanin analogues, we first applied two common strategies: lipidization and cationization (Figure 1B). The introduction of a lipoamino acid residue at the C-terminus of the [Gly1Sar]Gal(1−16)-NH_2 could serve two functions: (1) to improve penetration of the analogue across the membranes by increasing lipophilicity and (2) to increase the metabolic stability, as was observed in other peptides.[46−49] The Lys-palmitoyl (Lys-P) residue was introduced at the C-terminus of the [Gly1Sar]Gal(1−16)-NH_2 analogue (analogue Gal-LAA). We also tested how the presence of positively charged residues at the C-terminus of [Gly1Sar]Gal(1−16)-NH_2 may improve anticonvulsant activity. Because the N-terminal

Table 2. Sequences, Mass Spectrometry Data, HPLC Retention Times and logD Values for the Galanin analogues[a]

analogue	structure	mass spec data (calc/exp)	HPLC retention time[b]	logD[c]
Gal(1−16)	GWTLNSAGYLLGPHAV-NH_2	1653.86/1654.67	19.32 ± 0.37	**0.69 ± 0.02**
Gal-LAA	(Sar)WTLNSAGYLLGP**HA(Lys-P)**	1936.30/1936.32	28.31 ± 0.04	**1.45 ± 0.02**
Gal-(K)4	(Sar)WTLNSAGYLLGP**KKKK**	1873.09/1873.14	15.13 ± 0.42	**0.34 ± 0.02**
Gal-B1	(Sar)WTLNSAGYLLGP**KKK(Lys-P)**	2112.60/2112.33	25.99 ± 0.16	**1.25 ± 0.02**
Gal-B2	(Sar)WTLNSAGYLLGP**KK(Lys-P)K**	2112.60/2112.33	25.84 ± 0.10	**1.24 ± 0.02**
Gal-B3	(Sar)WTLNSAGYLLGP**K(Lys-P)KK**	2112.60/2112.26	25.96 ± 0.29	**1.25 ± 0.03**
Gal-B4	(Sar)WTLNSAGYLLGP**(Lys-P)KKK**	2112.60/2112.29	25.88 ± 0.14	**1.24 ± 0.02**
Gal-B5	WTLNSAGYLLGP**KK(Lys-P)K**	2040.46/2040.13	25.62 ± 0.10	**1.22 ± 0.02**
Gal-B6	GWTLNSAGYLLGP**KK(Lys-P)K**	2098.60/2098.18	22.53 ± 3.20	**0.96 ± 0.24**
Gal-B7	(Ahx)WTLNSAGYLLGP**KK(Lys-P)K**	2154.70/2154.44	25.80 ± 0.12	**1.24 ± 0.02**
Gal-B8	(Sar)WTLNSAGYLLGP**RR(Lys-P)R**	2195.51/2195.43	26.36 ± 0.09	**1.28 ± 0.02**
Gal-B9	(Sar)WTLNSAGYLLG$P_D$$K_D$K(Lys-P)$_D$K	2112.60/2112.33	25.44 ± 0.12	**1.21 ± 0.02**
Gal-B10	(Sar)WTLNSAGYLL**KKKK(Lys-P)K**	2213.61/2213.29	24.00 ± 0.13	**1.08 ± 0.02**
Gal-B11	(Sar)WTLNSAGYLLG**KK(Lys-P)K**	2015.50/2015.30	25.67 ± 0.14	**1.23 ± 0.02**
Gal-B12	(Sar)WTLNSAGYLL**KK(Lys-P)K**	1957.42/1957.18	25.82 ± 0.10	**1.24 ± 0.02**

[a] Lys-P is Lysine-palmitoyl, Sar is sarcosine, Ahx is 6-aminohexanoic acid. [b] Linear gradient of H_2O/acetonitrile. [c] Calculated values based on HPLC retention times, as shown in Supplementing Information Table S1 and Figure S2.

galanin fragment is relatively hydrophobic and it lacks positively charged residues (known to improve penetration of peptides across biological membranes), several Lys residues were added to the C-terminus of the analogue (Gal-(K)4). The overall design strategy is summarized in Figure 1B. These two analogues were chemically synthesized and screened by the Anticonvulsant Screening Program at the NIH. Our initial screening results indicated that these two analogues failed to effectively protect mice from limbic seizures induced by 6 Hz corneal stimulation, although the observed activity of the analogue Gal-(K)4 provided a hint that cationization might in fact be an effective strategy to improve systemic bioavailability. However, because of apparent motor impairment toxicity of Gal-(K)4, we discontinued exploring increasing or replacing the positively charged residues at the C-terminus.

Next, we tested whether combining cationization and lipidization could be more effective in improving systemic activity of the analogues, as compared to each of the modifications alone. Therefore, a series of four analogues (Gal-B1, Gal-B2, Gal-B3, and Gal-B4) was designed, chemically synthesized, and screened; these analogues contained a Lys-palmitoyl residue in combinations with three Lys residues. Once we discovered that the combined presence of lipoamino acid at the position 16 and cationization was important for improving systemic activity of Gal-B2, subsequent subsets of SAR analogues were designed to address several questions: (1) Does the number and type of positively charged residues play a role in systemic bioavailability? (2) Does a further truncation of galanin analogues affect systemic bioavailability? (3) And is the N-terminal methylation important? Structures of the galanin analogues described in this work are summarized in Table 2

Synthesis and Physicochemical Characterization of the Galanin Analogues. Galanin analogues were synthesized on a solid support using automated peptide synthesizer and the Fmoc chemistry. After removal of the peptides from the resin, the analogues were purified using reversed-phase HPLC separations on a diphenyl preparative column. The peptides were quantified by measuring UV absorbance at 280 nm, aliquoted, and dried in a speed vac. Mass spectrometry analysis confirmed the chemical identity of all analogues. HPLC retention times are summarized in Table 2. The purity of all analogues is summarized in Supporting Information Figure S1 and Table S1, and the HPLC trace of the lead analogue, Gal-B2, showing 98.8% purity, is provided in Supporting Information Figure S2.

To characterize the lipophilicity of the analogues, we determined their water/octanol partitioning coefficients (logD) using previously established methods.[50,51] First, the shake-flask method was employed for the selected analogues to determine logD between *n*-octanol and aqueous phase (Supporting Information Table S1). The capacity factor (k') for the analogues were calculated by the formula $k' = (t_r - t_o)/t_o$, where t_r was the retention time of the test peptide and t_o is the solvent front. From the linear correlation (Supporting Information Figure S3), the capacity factors and subsequent logD's were determined for all the analogues.

As summarized in Table 2, unmodified Gal(1−16) analogue was found to have logD = 0.69. Presence of the N-terminal methyl and the C-terminal Lys-palmitoyl residue significantly increased logD to 1.45 (analogue Gal-LAA). Conversely, cationization by four C-terminal lysine residues, analogue Gal-(K)4, increased hydrophilicity, with accompanied shift in the logD to 0.34. For analogues containing Sar1, Lys-palmitoyl, and three Lys residues, logD was around 1.2. Conversion of the N-terminal glycine to sarcosine seen in analogue Gal-B2, reduced the positive charge, effectively increasing lipophilicity to a logD from 0.96 to 1.24 (comparing analogues Gal-B6 and Gal-B2). The shortening of the peptide did not affect logD but replacing Gly12-Pro13 with two Lys residues decreased logD from 1.25 to 1.08. Taken together, most galanin analogues in this series had a CNS-favorable logD greater or equal to 1.0.

Because of the amphipathic character of the galanin analogues, we selected a representative analogue, Gal-B2, containing Lys-palmitoyl and three Lys residues to determine the critical micelle concentration (CMC). We employed a fluorescence-based method in 96-well microtiter plates. The surfactants, sodium dodecyl sulfate (SDS), and polyoxyethylene (20) sorbitan monolaurate, were used as controls. On the basis of changes in the fluorescence of fluorescein as a function of peptide concentration (Figure 2A), the CMC of Gal-B2 was calculated as 3.65 μM. This value is higher as compared to the reported CMC for human galanin 0.4 μM.[52] CMC value for polyoxyethylene (20) sorbitan monolaurate was 0.02 mM, whereas for SDS it was 0.43 mM (Supporting Information Figure S4), comparable to those previously reported.[53]

Structural Characterization of the Galanin Analogues. Normally unstructured galanin acquires α-helical conformation in complex with its receptor, as well as in 50% TFE.[54−56] As illustrated in Figure 2B, a presence of Lys-palmitoyl residue at the C-terminus would result in possible additional interactions with other hydrophobic residues that could favor helical conformation. To better understand structural consequences of introducing the lipoamino acid and cationization into the galanin analogues, their conformational properties were studied using circular dichroism (CD). The CD spectra were acquired in

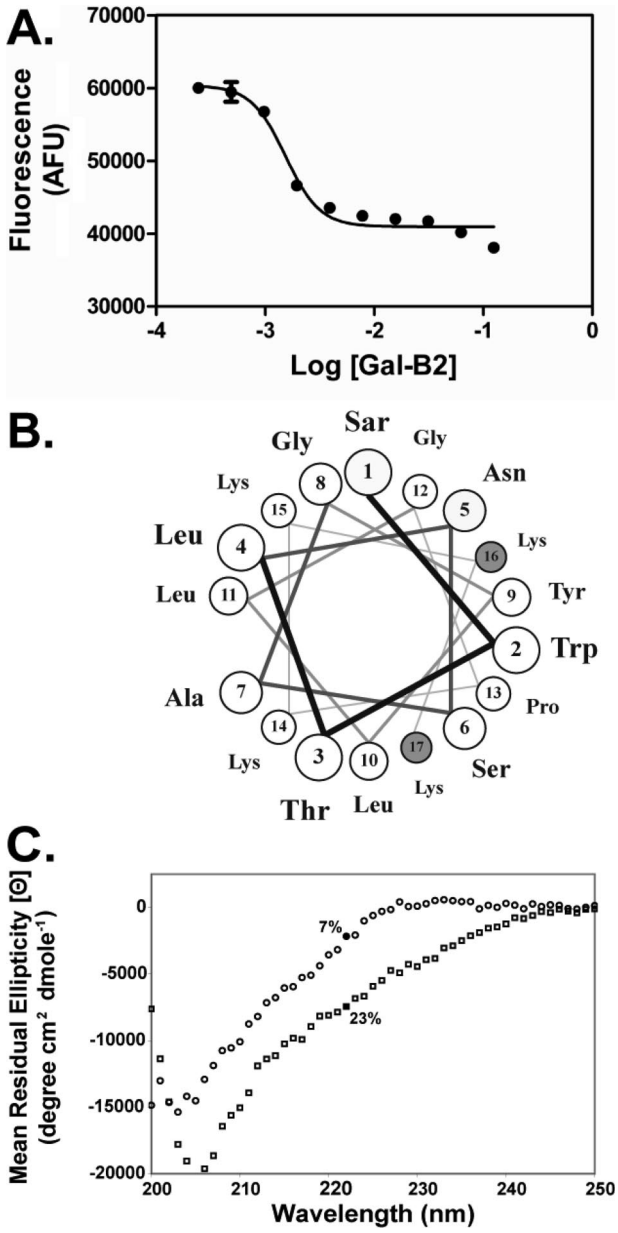

Figure 2. Characterization of the Gal-B2 analogue. (A) Determination of the critical micelle concentration using the fluorescence-based method. (B) Wheel representation of the Gal-B2 helical structure. (C) CD spectra of the Gal-B2 analogue in PBS (circles) and 50% TFE/PBS (squares).

aqueous 150 mM NaF phosphate buffer, pH 7.4 in the absence or presence of 50% (v/v) 2,2,2-trifluoroethanol (TFE). Figure 2C shows representative CD spectra and Table 3 summarizes amount of calculated α-helical structure in the galanin analogues. Conformation of Gal(1−16) in buffer was similar to published results for human galanin.[57] In the absence of TFE, Gal(1−16) gave a spectrum typical of random coil, with Θ_{222} showing ~1% helical structure. In 50% TFE, helical structure was stabilized and Gal(1−16) was calculated to have 22% helical structure, which coincides with literature results. For Gal(1−16), this equates to one helical turn (in TFE), however small distortions of the carbonyl chromophore from regular helical geometries in short peptides impair proper CD measurements and these values are considered to be the lower limit.

Some galanin analogues containing lipoamino acid showed increased tendency to form helical structure in aqueous solution (Table 3). The increase was generally dependent on the position

Table 3. Summary of the CD Spectrometry and the in Vitro Serum Stability Analyses

analogue	% α-helix (in water)	% α-helix (in 50% TFE)	half-life in 25% rat serum
Gal(1−16)	**1**	22	28 min
Gal-LAA	**0**	13	>10 h
Gal-(K)4	**0**	14	4.6 h
Gal-B1	**31**	16	>10 h
Gal-B2	**7**	23	9.4 h
Gal-B3	**2**	18	8.6 h
Gal-B4		20	>10 h
Gal-B5	**6**	17	>10 h
Gal-B6	**4**	16	>10 h
Gal-B7	**4**	19	>10 h
Gal-B8	**3**	16	4.8 h
Gal-B9	**0**	1	>10 h
Gal-B10	**10**	42	8.7 h
Gal-B11	**10**	36	5.6 h
Gal-B12	**20**	40	>10 h

of the Lys-palmitoyl residue. For example, the analogue Gal-B1 with the very terminal Lys-palmitoyl residue contained over 30% α-helicity, suggesting that the palmitoyl moiety may interact with specific side chains to stabilize the conformation (Figure 2B). Interestingly, this effect was diminished in the presence of 50% TFE. The Gly12-Pro13 fragment significantly affected the conformation of the analogues: their replacement or removal dramatically stabilized the helical conformation. Upon addition of TFE, the α-helical structure was further induced in most of the analogues. Interestingly, Gal-B1, that contained 31% α-helical conformation, was destabilized. The percentage of helical structure of analogue Gal-B2 was correlated to the position of Lys-palmitoyl residue. An α-helical wheel projection, shown in Figure 2B, shows that the lipoamino acid may stabilize the conformation through possible interactions with Tyr9, which is centered about a hydrophobic core (Ser6-Ala7-Gly8-Tyr9-Leu10-Leu11) where the helix resides.

Metabolic Stability of Galanin Analogues. To assess effects of the chemical modifications on the metabolic stability of the galanin analogues, their resistance to proteolytic degradation was determined in 25% rat serum incubated at 37 °C. Quantification of the analogues was determined by HPLC assay (Supporting Information Figure S5), and the recovery of the analogues at the initial time ($t = 0$ min) was higher than 80%. Representative time-courses for disappearance of the galanin analogues are illustrated in Figure 3 and calculated half-life are summarized in Table 3. Unmodified galanin analogue Gal(1−16) was very unstable with $t_{1/2} = 7.8$ min. All analogues exhibited significantly improved metabolic stability, with nearly half of them having $t_{1/2} > 10$ h.

Receptor Binding Studies. Affinities of the galanin analogues against human hGalR1 and hGalR2 were determined using time-resolved fluorescence based competitive binding assay. The assay was essentially performed in the same manner, as previously described.[58] Representative binding curves for Gal(1−16) and Gal-B2 analogues are shown in Figure 4; K_i values are summarized in Table 4. Consistent with the literature data, Gal(1−16) exhibited low nanomolar affinities toward both subtypes, with about 20-fold preference toward GalR1 subtype. The presence of lipoamino acid decreased the affinity of the analogues for both GalR1 and GalR2, but the effects were dependent on the sequence position of Lys-palmitoyl. The C-terminal cationization did not affect binding constants. The central truncations of Gal-B2 resulted in a decrease in the receptor affinities. The most potent, yet "nonselective" analogue was Gal-B7, displaying K_i values of 21.5 and 51 nM toward

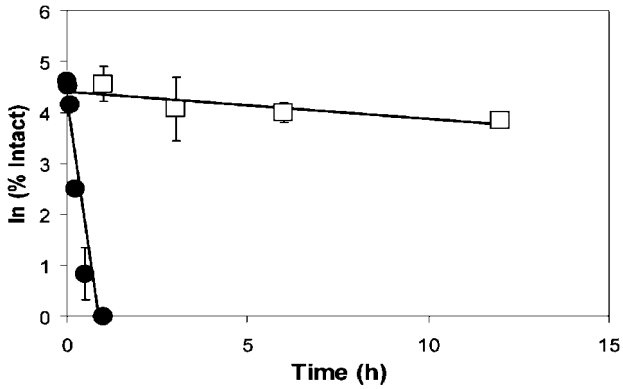

Figure 3. In vitro serum stability assay. Determination of the in vitro metabolic stability of the unmodified Gal(1−16) (closed circles) and the Gal-B2 analogue (open squares). Time-course of the disappearance of the intact peptides in the presence of 25% rat serum incubated at 37 °C is shown. The analogues were quantified by HPLC as shown in Supporting Information Figure S5. Half-lives for the Gal(1−16) and Gal-B2 analogues were calculated as 7.8 min and 9.4 h, respectively.

GalR1 and GalR2, respectively. In summary, most analogues exhibited nanomolar affinity toward the galanin receptors, and the presence of the C-terminal −Lys-Lys-Lys(palmitoyl)-Lys-NH_2 motif did not significantly affect binding affinities toward the galanin receptors.

Anticonvulsant Screening. Because all designed analogues maintained high affinities toward galanin receptors, their anticonvulsant activities were determined in the animal screening assay using the 6 Hz (32 mA) seizure model. The 6 Hz seizure test is a robust screening assay employed by the NIH-sponsored Anticonvulsant Screening Program to evaluate antiepileptic drug candidates. This acute seizure test is a model of pharmacoresistant epilepsy that displays a unique pharmacological profile. Moreover, seizures evoked by 6 Hz stimulation, unlike acute seizures evoked by maximal electroshock and/or pentylenetetrazol are blocked by levetiracetam. Prior to the anticonvulsant screening following systemic administration, we demonstrated that the Gal(1−16) fragment was very potent in suppressing seizures in this model of pharmacoresistant epilepsy following intracerebroventricular (icv) injection. ED_{50} was determined as 1.7 nmole (95% CI = 0.8 − 4.7). For the screening, each galanin analogue was administered intraperitoneally (ip) to 5 groups of mice at an identical dose of 4 mg/kg. At 15, 30, 60, 120, and 240 min after the drug administration, mice were challenged with a 6 Hz corneal stimulation. Mice not displaying characteristic limbic seizures were considered protected. Table 4 summarizes the results from this in vivo screening assay, and Figure 5 illustrates the area under the curve (AUC) for the time course of the anticonvulsant activities of the analogues.

As shown in Table 4, neither Gal(1−16) nor the lipoamino acid containing analogue, Gal-LAA, produced any anticonvulsant activity following ip administration. In contrast, Gal-(K)4 exhibited some degree of protection against seizures with a time to peak effect (TPE) of 30 min. Strikingly, one out of four analogues containing combined lipoamino acid and Lys residues was very active in this test, providing 100% protection against seizures from 30 min to 2 h following drug administration. As mentioned earlier, Gal-B2 was selected as a lead compound to further advance the SAR analysis. The analogues with the modifications at the N-terminus (Gal-B5, Gal-B6, and Gal-B7) all displayed pronounced antiepileptic activity. The presence of additional Lys residues or replacing Lys with Arg or D-Lys did not significantly modify the anticonvulsant activity, although the D-Lys containing analogue was, as predicted, longer-lasting when compared to Gal-B2. Removal of Pro13 significantly decreased anticonvulsant activity, an effect consistent with changing the relative position of Lys-palmitoyl, as observed in the analogue Gal-B3. The GalR2-preferring analogue, Gal-B5, was less active as compared to GalR1-preferring Gal-B2 (both analogues are identical except Gal-B5 lacks N-terminal sarcosine). The most active analogue, Gal-B2, yielded an ED_{50} of 0.8 mg/kg (95% CI = 0.43 − 1.56) determined at the time-to-peak effect (TPE) of 1 h. Even at the highest efficacious doses tested, no behavioral toxicity was observed. The rotarod test was employed to evaluate the effect of higher doses of Gal-B2 on motor function. In this study, the median toxic dose, or TD_{50}, was determined to be 21 mg/kg (Figure 6). Gal-B2 exhibits the protective index (PI = TD_{50}/ED_{50}) above 25.

Discussion

This work describes our ongoing research efforts on developing systemically active galanin analogues. To the best of our knowledge, this is the first report that describes applying chemical modifications to galanin to improve its CNS activity. Previous attempts to produce systemically active galanin analogues, such as galnon or galmic, included combinatorial libraries based on peptidomimetic scaffolds.[16,19,31,59] Chemical modifications, such as glycosylation, lipidization, or cationization, have been extensively studied for opioid peptides, neurotensin or somatostatin, but were never a subject of a rational design using galanin.[60] On the basis of available SAR data, we designed a series of truncated galanin analogues in which the N-terminal Gly1 was replaced by sarcosine, and the C-terminal residues were replaced by a combination of lipoamino acid and Lys residues. These modifications did not significantly affect binding affinity toward galanin receptors but dramatically improved systemic bioavailability, as determined by high anticonvulsant potency following systemic administration. There are two major outcomes of this research: (1) combining chemical modifications improves bioavailability of peptides, and (2) the Gal-B2 analogue provides a new pharmacological tool to study the role of galanin receptors in the nervous system.

Chemical modifications such as lipidization or cationization are commonly used to improve bioavailability of peptides.[37,61,62] Lipoamino acids have been introduced to a number of neuropeptide analogues including somatostatin,[46,63] conotoxins,[47] or opioid peptides.[64] A reversible lipidization strategy was successfully applied to improve bioavailability of opioid peptides[65] or octreotide.[49] Furthermore, increased lipophilicity of peptides has been well-established factor to improve their BBB penetration.[66] However, lipidization of the [Gly1Sar]Gal(1−16)-NH_2 analogue did not improve its systemic bioactivity despite significantly increasing its logD value and the in vitro metabolic stability. Although introduction of the lysine-palmitoyl moiety noticeably decreased the affinities toward both GalR1 and GalR2, a lack of the antiepileptic activity of Gal-LAA could not be easily accounted for by a 1−2 order of magnitude decrease in the receptor affinities (analogues Gal-B10 and Gal-B12 had comparable affinities to that of Gal-LAA, but both analogues were significantly more active than Gal-LAA following systemic administration, as shown in Table 4). Thus, the presence of a lipoamino acid alone did not improve systemic bioavailability. Similarly, cationization is well-known to improve both penetration across biological membranes[67,68] and penetration into the CNS via adsorptive-mediated endocytosis.[69,70] Indeed, introduction of four Lys residues (analogue Gal-(K)4) resulted in an increase of the anticonvulsant activity following

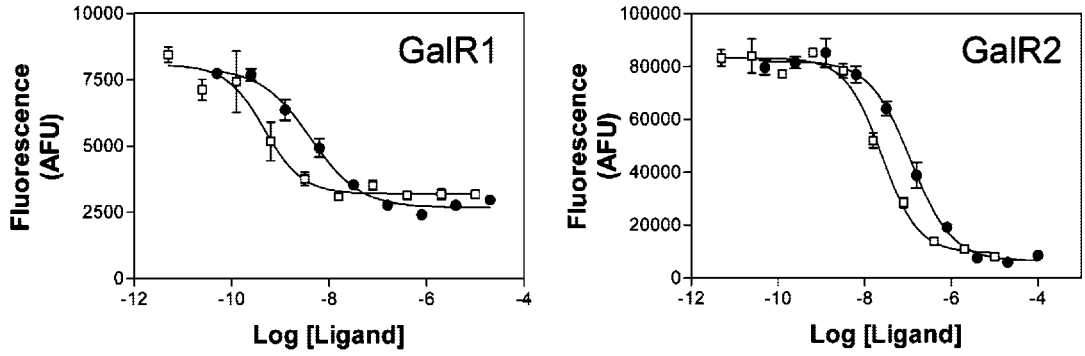

Figure 4. Galanin receptor binding studies. A representative graph from competitive receptor binding studies for GalR1 (left) and GalR2 (right). Binding assay was performed with GalR1 and GalR2 membrane preparations for Gal-B2 (closed circle) and Gal(1−16) (open square). Each data point represents the average of four assay points. Europium-labeled galanin was used as a ligand for the assay. K_i values for the analogues are summarized in Table 4.

Table 4. In Vitro and in Vivo Pharmacological Properties of the Galanin Analogues

	in vitro assay (receptor binding)		in vivo assay (anticonvulsant activity)					
			6 Hz, 32 mA, 4 mg/kg					
analogue	GalR1 K_i [nM]	GalR2 K_i [nM]	15′	30′	60′	120′	240′	AUC [a]
Gal(1−16)	0.5 ± 0.2	13.0 ± 5.4	0/4	0/4	0/4	0/4	0/4	0
Gal-LAA	85.0 ± 58.0	160.0 ± 9.9	0/4	0/4	0/4	0/4	0/4	0
Gal-(K)4	0.4 ± 0.1	24.0 ± 9.9	0/4	3/4	1/3	0/3	0/4	3186
Gal-B1	1.9 ± 0.6	22.0 ± 9.2	1/4	1/4	2/4	0/4	0/4	3000
Gal-B2	3.5 ± 1.0	51.5 ± 34.4	3/4	4/4	4/4	4/4	0/4	16313
Gal-B3	6.0 ± 1.4	28.0 ± 4.9	1/4	3/4	0/4	0/4	0/4	1875
Gal-B4	25.5 ± 6.4	108.0 ± 15.6	0/4	1/4	1/4	0/4	1/4	3188
Gal-B5	387.0 ± 123.0	48.0 ± 11.3	2/4	1/4	2/4	2/4	0/4	8250
Gal-B6	5.3 ± 3.0	22.5 ± 3.5	1/4	3/4	3/4	0/4	0/4	5250
Gal-B7	21.5 ± 12.0	51.0 ± 11.3	4/4	3/4	3/4	1/4	1/4	9563
Gal-B8	3.3 ± 0.3	65.5 ± 0.7	2/4	3/4	4/4	3/4	0/4	13313
Gal-B9	0.9 ± 0.2	15.0 ± 8.5	2/4	3/4	4/4	2/4	3/4	15563
Gal-B10	41.5 ± 12.0	214.5 ± 24.7	2/4	2/4	3/4	3/3	2/3	17871
Gal-B11	25.5 ± 4.9	75.0 ± 10.6	1/4	2/4	2/4	0/4	0/4	3563
Gal-B12	306.5 ± 12.0	1,323.5 ± 27.6	1/4	4/4	4/4	1/4	0/4	9188

[a] area under the curve values summarize the anticonvulsant time−response curves. The percent of animals protected at each time-point was plotted against time, and the AUC values were calculated as described in the Methods Section.

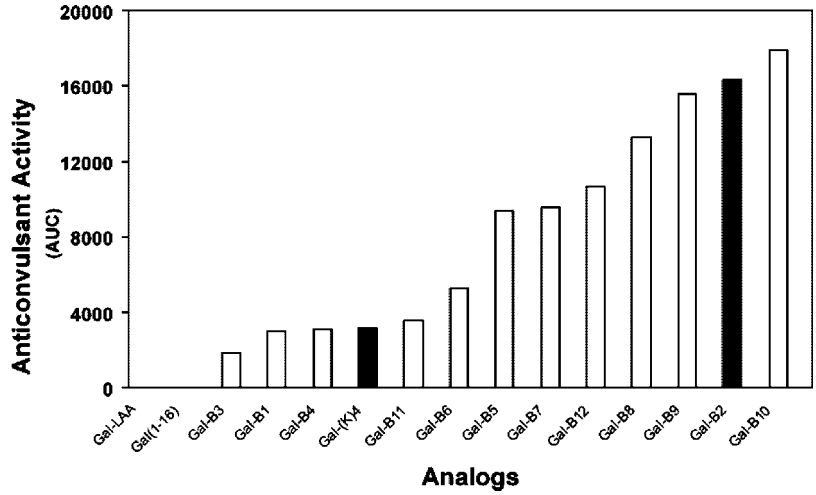

Figure 5. Anticonvulsant screening of the galanin analogues. Bar graphs represent area under the curve values for each of the analogue calculated from a time−response study for a single bolus dose of 4 mg/kg (ip).

systemic administration. However, the relatively modest antiepileptic activity with a slow onset and a short duration of action was accompanied by an apparent toxic effect thereby precluding further attempts to optimize this scaffold. To our surprise, the combination of lipoamino acid and lipidization appeared superior in producing very active antiepileptic compounds that voided behavioral neurotoxicity at the efficacious doses.

By combining lipidization and cationization, we have synthesized a number of galanin analogues that are highly active anticonvulsants following systemic administration. Importantly, the combined chemical modifications did not affect affinity toward galanin receptors. In contrast, the modifications increased lipophilicity, metabolic stability, and in some instances, an amount of α-helical structure in aqueous solution and in 50%

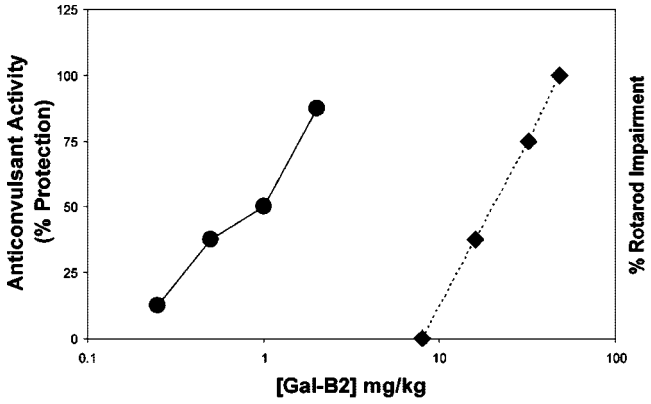

Figure 6. Separation of the potency and toxicity of the Gal-B2 analogue in the 6 Hz model (32 mA) of pharmacoresistant epilepsy in mice. Bolus injections of Gal-B2 were administered intraperitoneally, and the dose−response data were generated for the 1 h time point (time-to-peak effect). On the basis of these results, ED_{50} and TD_{50} were calculated as 0.8 and 21 mg/kg, respectively, yielding the protective index (PI) of 26.

TFE. It is well-known logD and metabolic stability are two important factors that should be considered when developing systemically active peptide analogues that target the CNS. The optimal range of logD for CNS drugs is between 1.5 and 2.7,[71] thus the Gal-B2 analogues display a favorable logD value. The increased lipophilic character of the analogues was not reflected in lower water solubility, in fact, the Gal-B2 analogue could be easily dissolved in aqueous solutions (water, phosphate buffered saline (PBS)) at concentrations as high as 10−20 mg/mL. The amphipathic properties of the combined cationization/lipidization were also reflected by their ability to form micelles at a CMC value of 3.65 μM. The presence of the lipoamino acid/cationization moieties not only increased lipophilic and amphipathic properties of the galanin analogues studied here, but they also improved their metabolic stability, as determined by extended half-life in the in vitro serum stability test. Our structure−stability relationship data suggest that each of the modifications (cationization and lipidization) played a significant role to the increased resistance to a proteolytic degradation; this effect might be in part accounted for by increased binding to serum albumin. For example, acetylation of insulin with fatty acids is known to increased their binding properties to serum albumin,[72,73] and similarly cationic peptides can also bind to albumin.[74,75] It is, therefore, conceivable that the increased protein binding may extend half-lives of the galanin analogues in serum solutions, allowing a better penetration into the brain.

The mechanism of penetration of the analogues into the brain remains unclear and can be only speculated at this time. Furthermore, a major limitation of this work is the lack of sensitive bioanalytical methods to reliably determine the brain levels of the Gal-B2 (although this is a work in progress). Apparently, the efficacious levels of the analogues reach the limbic brain structures and the galanin receptors responsible for suppressing seizures.[8,76] Our current hypothesis is that a passive diffusion (lipidization) and an adsorptive-mediated endocytosis (cationization) may both contribute to the improved penetration across the BBB.[37] Given the number of the transport mechanisms that exist at the BBB,[61] we cannot rule out additional mechanisms. Recently, Nelson and co-workers showed that the "−(Lys-myristoyl)-Lys-Lys-Lys-NH_2" motif was able to transport a fluorescently labeled peptide into living cells,[77] suggesting that such cationization/lipidization modifications are capable of transporting a cargo similarly to the cell-penetrating peptides.[78] A cell-permeable analogue of galanin, namely transportan, has been described;[79] however, it is not known whether this galanin analogue exhibits the anticonvulsant activity following systemic administration. Transportan was, however, effective in delivering peptide-nucleic acids to the nervous system.[80] A role of helical conformation in improving systemic bioavailability of galanin analogues is less apparent, although many antimicrobial peptides that penetrate membranes have helical conformations.[81] Differences between the amount of the helical structure and the anticonvulsant activity of two analogues: Gal-B2 (very active, 7% helix in buffer) and Gal-B1 (poor anticonvulsant, 31% helix in buffer) suggest that there is no such relationship (these two analogues exhibit comparable logD values, metabolic stabilities, and receptor binding properties). Our current studies are focused on quantifying the brain penetration by the Gal-B2 analogue, as well as the mechanism of transport of the analogues across the cell-based biological membranes.

Perhaps the most important discovery described in this work is the finding that the Gal-B2 analogue exhibits potent antiepileptic activity following systemic administration. This compound has nanomolar affinity toward both GalR1 and GalR2 receptors. In this respect, it displays a selectivity profile comparable to that of Gal(1−16). The Gal-B2 analogue is a useful pharmacological tool to study a role of galanin receptors in the nervous system, including the CNS. Moreover, because galanin also produces analgesic effects,[82−84] the systemically active galanin analogues are likely to provide new pain therapeutics as well. Indeed, the Gal-B2 analogue appeared very active in several animal pain models (E. Adkins Scholl, et al., manuscript in preparation).

Methods

Chemical Synthesis. All analogues were synthesized in an automated peptide synthesizer using Rink Amide resin (25 or 50 μmole scales) and standard Fmoc-based coupling protocols. Fmoc-Lys(Palmitoyl)-OH was obtained from Chem-Impex International Inc. The peptide analogues were removed from the resin by a 3 h treatment with the reagent K (90/5/2.5/7.5/5 by volume; trifluoroacetic acid/water/ethanedithiol/phenol/thioanisole) and then precipitated out in cold methyl-*tert*-butyl ether. Crude peptides were purified by reversed-phase HPLC using preparative HPLC column (Vydac diphenyl, 219TP1011522) and eluted with a linear gradient of acetonitrile (0.1% TFA). The flow rate was 10 mL/min, and the elution was monitored by UV detection at 220 nm. The purities of peptides were determined by analytical reversed-phase HPLC separations (Vydac diphenyl column): buffer A (0.1% TFA in water) and buffer B (0.1% TFA, v/v, in 90% aqueous acetonitrile) were used to produce a linear gradient from 20 to 100% of buffer B over 40 min. The flow rate was 1 mL/min, and the elution was monitored by measuring absorbance at 220 nm. Purified analogues were quantified by measuring UV absorbance at 279.8 nm (molar absorbance coefficient $\varepsilon = 7000$). The molecular masses of peptides were determined by MALDI-TOF MS at the University of Utah Core Facility.

Partitioning Coefficient, logD. Shake-Flask Method. Water saturated *n*-octanol was made by shaking equal volumes of *n*-octanol and water for 24 h at room temperature. Lyophilized peptides (0.4 mg) were reconstituted in 1 mL of PBS, pH 7.4, for 20 min; peptides with low aqueous solubility were reconstituted in 1 mL water saturated octanol. To the peptide solutions, 1 mL of water saturated octanol was added (1 mL PBS to peptides in organic phase), and the biphasic solution was placed on a rotary mixer for 24 h at room temperature. The samples were allowed to settle for 30 min, and the organic phase was removed (950 μL) without disturbing the meniscus. The aqueous layer was sonicated in a bath sonicator for 5−10 s. Peptide concentration in a 50 μL aliquot of the aqueous phase was quantified by HPLC. By mass balance, the concentration of peptide in the organic phase was determined to

be total peptide concentration minus the aqueous peptide concentration. For each analogue, four independent sample preparations and logD measurements were carried out. LogD was calculated:

$$\log D = \log\left(\frac{[\text{octanol}_{\text{peptide}}]}{[\text{aqueous}_{\text{peptide}}]}\right)$$

HPLC Capacity Factor (k') Method. For the logD determination, the HPLC buffers were buffer A (1 L H_2O + 1 mL TFA) and buffer B (900 mL acetonitrile + 100 mL H_2O + 1 mL TFA). A 5.0 μg standard (run in triplicate) was injected onto a Vydac diphenyl column using a linear gradient starting at 80/20 buffer A:buffer B and ending at 10/90 buffer A:Buffer B in 15 min before immediate return to initial conditions. The retention times are the average of 3 runs. The capacity factors (k') of the peptides were calculated using the formula below; t_o is the solvent front, t_r is the retention time of the peptide. The logD's obtained from the shake-flask method were plotted against their peptides respective k' values giving a linear plot. LogD's for all other peptides were calculated of this standard curve.

$$k' = \frac{t_r - t_o}{t_o}$$

Determination of Critical Micelle Concentration. CMC was determined by measuring changes in fluorescence of fluorescein as a function of peptide concentration. The Gal-B2, sodium dodecyl sulfate or polyoxyethylene (20) sorbitan monolaurate were dissolved in PBS and serial dilutions were prepared in the Costar 96-well clear plate. Next, 60 μL of 50 nM fluorescein in PBS solution was added to every well containing 200 μL of the test compound. The plate was subjected to a gentle shaking for 24 h, followed by recording the fluorescence at 485/535 nm using VICTOR^3 spectrofluorometer. The data were analyzed using GraphPad Prism and the sigmoidal dose−response (variable slope) equation.

Conformational Studies. α-Helical conformation of each analogue was studied using circular dichroism. Lyophilized peptides (0.1 mg) were reconstituted in 1.0 mL of NaF/phosphate buffer (50 mg KH_2PO_4, 54 mg Na_2HPO_4, 1.55 g NaF, 250 mL H_2O, pH 7.4 with Na_2HPO_4) or in 0.5 mL NaF/phosphate buffer and 0.5 mL 2,2,2-trifluoroethanol. After 1 h of reconstitution, 250 μL of peptide solution were loaded into a 0.1 cm quartz cuvette and placed into an Aviv 62DS CD spectropolarimeter, at room temperature. Scans were collected from 250 to 200 nm every 1.0 nm with 1 s dwell time. Data were averaged from 5 scans and processed by eq 1, where M_r is molecular weight, c is concentration (mg/mL), d is path length (cm), and n is the number of peptide bonds. Percent helical content are calculated using eqs 2 and 3.

$$[\Theta] = \frac{\theta \times 100 \times M_r}{c \times d \times n} \quad (1)$$

$$[\Theta_{\max}] = -39500\left[1 - \left(\frac{2.57}{n}\right)\right] \quad (2)$$

$$\%\alpha \text{ helix} = \frac{[\Theta_{222}]}{[\Theta_{\max}]} \times 100\% \quad (3)$$

Metabolic Stability Assay. Peptide stability was assessed in a rat serum assay. One mL of 25% rat serum was incubated at 37 °C for 10 min prior to addition of the analogues. Reactions were prepared by adding each analogue, dissolved in nanopure H_2O, to a solution containing 25% rat blood serum and 0.1 M Tris-HCl, pH 7.5 to a final peptide concentration of 20 μM. At appropriate time intervals (ranging up to 8 h), 200 μL aliquots were withdrawn and added to 100 μL "quenching solution" (15% trichloroacetic acid in 40% isopropanol). Isopropanol (40%, aqueous solution) was added to quenching mixture (this step improved recovery of the Gal-B2 and other analogues). Upon precipitation with the quenching mixture, the samples were incubated at −20 °C for 15 min and centrifuged at 12000 rpm. The supernatant was analyzed using HPLC separation with an YMC ODS-A 5 μm 120 Å column (Waters, cat. no.: AA12S052503WT). In cases where analogue peaks overlapped with peaks observed in the "serum-only" control samples, the gradient was optimized by changing the composition of the mobile phases, column temperature, or HPLC column (for example C_8 rather than diphenyl column). Recovery of the analogues was assessed by spiking "serum-only" control samples after the trichloroacetic acid precipitation with known amounts of the analogue. Metabolic stability was assessed by monitoring the disappearance of the analogues over a period of 8 h. This was accomplished by comparison the area under the curve for the peak corresponding to the intact analogue at each time point. Half-life, $t_{1/2}$, for each analogue was calculated using the average of three independent experiments for each time point. Results were plotted on a log-scale plot using the Kaleidagraph software. Linear curve-fit analysis was used to fit the time-course of the degradation of the analogues according to the following formula: $t_{1/2}$ (h) = ln(50) − b)/(m), where "m" represents the slope of the line and "b" is the y-intercept.

Receptor Binding Assay. The fluorescence-based assay with europium-labeled galanin was employed to determine binding affinities for GalR1 and GalR2 receptors. Competitive binding assay was performed on AcroWell filter plates using receptor membrane preparations and Eu-galanin (Perkin-Elmer), and the samples were tested in quadruplicate. Both GalR1 (expressed in HEK-293 EBNA) and GalR2 (expressed in CHO-K1) membrane preparations were purchased from Perkin-Elmer. Binding assay was carried out with 6 μg of membrane protein (1.4 pmol/mg protein) and 2 nM of Eu-galanin in a volume of 100 μL of the binding buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 μM EDTA, and 0.2% BSA). Samples were incubated at room temperature for 90 min, followed by washing four times with wash buffer (50 mM Tris-HCl pH 7.5 and 5 mM $MgCl_2$) using a vacuum manifold. Enhancement solution (200 μL) was added, and the plates were incubated at room temperature for 30 min. The plates were read on a VICTOR^3 spectrofluorometer using a standard time-resolved fluorescence measurement for europium-based compounds (excitation at 340 nm, delay for 400 μs, and emission at 615 nm). Competition binding curves were analyzed using GraphPad Prism software. The sigmoidal dose−response (variable slope) equation for nonlinear regression analysis was used to calculate the EC_{50}, and K_i values were calculated using the formula: $K_i = EC_{50}/(1 + [\text{Eu-Gal}]/K_d)$, where K_d (4.3 nM) was provided by Perkin-Elmer.

Anticonvulsant Activity. Each galanin analogue was administered intraperitoneally to five groups of CF-1 mice ($n = 4$ mice) at a dose of 4 mg/kg. At various times (i.e., 15, 30, 60, 120, and 240 min) after ip administration, mice were challenged with a 6 Hz corneal stimulation (32 mA for 3 s delivered via corneal electrodes). Mice not displaying a characteristic limbic seizure (jaw chomping, vibrissae twitching, forelimb clonus, Straub tail) were considered protected. The percent of animals protected at each time-point was plotted against time, and the AUC values were calculated with GraphPad Prism software suite using the trapezium rule and the formula below:

$$\sum\left(\frac{\%\text{protected}}{2}\right) \times \Delta\text{time}$$

Acknowledgment. This work was supported in part by the Epilepsy Therapy Grants Program from the Epilepsy Research Foundation, the University of Utah Startup Funds, and the University of Utah Research Foundation. Pharmacology of Gal-B2 was presented at the IX EILAT Conference on New Antiepileptic Drugs. We thank the Anticonvulsant Screening Program (ASP) at the NIH/NINDS, and in particular the ASP Director, James Stables, and his dedicated ASP staff for their support with screening galanin analogues. Invaluable support and help with data analysis, preparation of figures, and laboratory management efforts from Dan McDougle is also greatly appreciated. We thank Professors Andrey Mazarati and Tony Yaksh for numerous discussions on the pharmacology of

galanin. We are also indebted to Professor Bob Schackmann and Scott Endicott from the DNA/Peptide Synthesis Core Facility at the University of Utah for their help with the peptide synthesis. G.B. and H.S.W. are scientific cofounders of NeuroAdjuvants, Inc.

Supporting Information Available: Tables S1 and S2 summarize properties of the galanin analogues related to logD and purity, as determined by HPLC. Figures S1−S5 show: the HPLC traces of the galanin analogues, integrated HPLC trace for the lead compound, Gal-B2, the plot with a linear correlation between the capacity factor and logD, fluorescence-based determination of CMC values for control compounds, polyoxyethylene (20) sorbitan monolaurate (Polysorbate 20) and sodium dodecyl sulfate, and HPLC traces for Gal-B2 from the in vitro serum stability experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hokfelt, T.; Broberger, C.; Xu, Z. Q.; Sergeyev, V.; Ubink, R.; Diez, M. Neuropeptides—an overview. *Neuropharmacology* **2000**, *39*, 1337–1356.
(2) Baraban, S. C.; Tallent, M. K. Interneuron Diversity series: Interneuronal neuropeptides—endogenous regulators of neuronal excitability. *Trends Neurosci.* **2004**, *27*, 135–142.
(3) Lerner, J. T.; Sankar, R.; Mazarati, A. M. Galanin and epilepsy. *Cell. Mol. Life Sci.* **2008**, *65*, 1864–1871.
(4) Baraban, S. C. Neuropeptide Y and epilepsy: recent progress, prospects and controversies. *Neuropeptides* **2004**, *38*, 261–265.
(5) Vezzani, A.; Sperk, G.; Colmers, W. F. Neuropeptide Y: emerging evidence for a functional role in seizure modulation. *Trends Neurosci.* **1999**, *22*, 25–30.
(6) Vezzani, A.; Hoyer, D. Brain somatostatin: a candidate inhibitory role in seizures and epileptogenesis. *Eur. J. Neurosci.* **1999**, *11*, 3767–3776.
(7) Solbrig, M. V.; Koob, G. F. Epilepsy, CNS viral injury and dynorphin. *Trends Pharmacol. Sci.* **2004**, *25*, 98–104.
(8) Mazarati, A.; Lundstrom, L.; Sollenberg, U.; Shin, D.; Langel, U.; Sankar, R. Regulation of kindling epileptogenesis by hippocampal galanin type 1 and type 2 receptors: The effects of subtype-selective agonists and the role of G-protein-mediated signaling. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 700–708.
(9) Tallent, M. K.; Qiu, C. Somatostatin: An endogenous antiepileptic. *Mol. Cell. Endocrinol.* **2008**, *286*, 96–103.
(10) Richichi, C.; Lin, E. J.; Stefanin, D.; Colella, D.; Ravizza, T.; Grignaschi, G.; Veglianese, P.; Sperk, G.; During, M. J.; Vezzani, A. Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. *J. Neurosci.* **2004**, *24*, 3051–3059.
(11) Noe, F.; Nissinen, J.; Pitkanen, A.; Gobbi, M.; Sperk, G.; During, M.; Vezzani, A. Gene therapy in epilepsy: the focus on NPY. *Peptides* **2007**, *28*, 377–383.
(12) Noe, F.; Pool, A. H.; Nissinen, J.; Gobbi, M.; Bland, R.; Rizzi, M.; Balducci, C.; Ferraguti, F.; Sperk, G.; During, M. J.; Pitkanen, A.; Vezzani, A. Neuropeptide Y gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy. *Brain* **2008**, *131*, 1506–1515.
(13) Mazarati, A. M.; Halaszi, E.; Telegdy, G. Anticonvulsive effects of galanin administered into the central nervous system upon the picrotoxin-kindled seizure syndrome in rats. *Brain Res.* **1992**, *589*, 164–166.
(14) Kokaia, M.; Holmberg, K.; Nanobashvili, A.; Xu, Z. Q.; Kokaia, Z.; Lendahl, U.; Hilke, S.; Theodorsson, E.; Kahl, U.; Bartfai, T.; Lindvall, O.; Hokfelt, T. Suppressed kindling epileptogenesis in mice with ectopic overexpression of galanin. *Proc. Natl. Acad. Sci. U.S.A* **2001**, *98*, 14006–14011.
(15) Mazarati, A. M.; Liu, H.; Soomets, U.; Sankar, R.; Shin, D.; Katsumori, H.; Langel, U.; Wasterlain, C. G. Galanin modulation of seizures and seizure modulation of hippocampal galanin in animal models of status epilepticus. *J. Neurosci.* **1998**, *18*, 10070–10077.
(16) Saar, K.; Mazarati, A. M.; Mahlapuu, R.; Hallnemo, G.; Soomets, U.; Kilk, K.; Hellberg, S.; Pooga, M.; Tolf, B. R.; Shi, T. S.; Hokfelt, T.; Wasterlain, C.; Bartfai, T.; Langel, U. Anticonvulsant activity of a nonpeptide galanin receptor agonist. *Proc. Natl. Acad. Sci. U.S.A* **2002**, *99*, 7136–7141.
(17) Haberman, R. P.; Samulski, R. J.; McCown, T. J. Attenuation of seizures and neuronal death by adeno-associated virus vector galanin expression and secretion. *Nat. Med.* **2003**, *9*, 1076–1080.
(18) Lin, E. J.; Richichi, C.; Young, D.; Baer, K.; Vezzani, A.; During, M. J. Recombinant AAV-mediated expression of galanin in rat hippocampus suppresses seizure development. *Eur. J. Neurosci.* **2003**, *18*, 2087–2092.
(19) Bartfai, T.; Lu, X.; Badie-Mahdavi, H.; Barr, A. M.; Mazarati, A.; Hua, X. Y.; Yaksh, T.; Haberhauer, G.; Ceide, S. C.; Trembleau, L.; Somogyi, L.; Krock, L.; Rebek, J., Jr. Galmic, a nonpeptide galanin receptor agonist, affects behaviors in seizure, pain, and forced-swim tests. *Proc. Natl. Acad. Sci. U.S.A* **2004**, *101*, 10470–10475.
(20) Mazarati, A. M.; Hohmann, J. G.; Bacon, A.; Liu, H.; Sankar, R.; Steiner, R. A.; Wynick, D.; Wasterlain, C. G. Modulation of hippocampal excitability and seizures by galanin. *J. Neurosci.* **2000**, *20*, 6276–6281.
(21) Schlifke, I.; Kuteeva, E.; Hokfelt, T.; Kokaia, M. Galanin expressed in the excitatory fibers attenuates synaptic strength and generalized seizures in the piriform cortex of mice. *Exp. Neurol.* **2006**, *200*, 398–406.
(22) Zini, S.; Roisin, M. P.; Langel, U.; Bartfai, T.; Ben-Ari, Y. Galanin reduces release of endogenous excitatory amino acids in the rat hippocampus. *Eur. J. Pharmacol.* **1993**, *245*, 1–7.
(23) Hokfelt, T.; Xu, Z. Q.; Shi, T. J.; Holmberg, K.; Zhang, X. Galanin in ascending systems. Focus on coexistence with 5-hydroxytryptamine and noradrenaline. *Ann. N.Y. Acad. Sci.* **1998**, *863*, 252–263.
(24) Branchek, T. A.; Smith, K. E.; Gerald, C.; Walker, M. W. Galanin receptor subtypes. *Trends Pharmacol. Sci.* **2000**, *21*, 109–117.
(25) Lundstrom, L.; Elmquist, A.; Bartfai, T.; Langel, U. Galanin and its receptors in neurological disorders. *Neuromol. Med* **2005**, *7*, 157–180.
(26) Burgevin, M. C.; Loquet, I.; Quarteronet, D.; Habert-Ortoli, E. Cloning, pharmacological characterization, and anatomical distribution of a rat cDNA encoding for a galanin receptor. *J. Mol. Neurosci.* **1995**, *6*, 33–41.
(27) Mazarati, A.; Lu, X.; Kilk, K.; Langel, U.; Wasterlain, C.; Bartfai, T. Galanin type 2 receptors regulate neuronal survival, susceptibility to seizures and seizure-induced neurogenesis in the dentate gyrus. *Eur. J. Neurosci.* **2004**, *19*, 3235–3244.
(28) Mazarati, A.; Lu, X.; Shinmei, S.; Badie-Mahdavi, H.; Bartfai, T. Patterns of seizures, hippocampal injury and neurogenesis in three models of status epilepticus in galanin receptor type 1 (GalR1) knockout mice. *Neuroscience* **2004**, *128*, 431–441.
(29) Pirondi, S.; Fernandez, M.; Schmidt, R.; Hokfelt, T.; Giardino, L.; Calza, L. The galanin-R2 agonist AR-M1896 reduces glutamate toxicity in primary neural hippocampal cells. *J. Neurochem.* **2005**, *95*, 821–833.
(30) Hwang, I. K.; Yoo, K. Y.; Kim, D. S.; Do, S. G.; Oh, Y. S.; Kang, T. C.; Han, B. H.; Kim, J. S.; Won, M. H. Expression and changes of galanin in neurons and microglia in the hippocampus after transient forebrain ischemia in gerbils. *Brain Res.* **2004**, *1023*, 193–199.
(31) Ceide, S. C.; Trembleau, L.; Haberhauer, G.; Somogyi, L.; Lu, X.; Bartfai, T.; Rebek, J., Jr. Synthesis of galmic: a nonpeptide galanin receptor agonist. *Proc. Natl. Acad. Sci. U.S.A* **2004**, *101*, 16727–16732.
(32) Lu, X.; Barr, A. M.; Kinney, J. W.; Sanna, P.; Conti, B.; Behrens, M. M.; Bartfai, T. A role for galanin in antidepressant actions with a focus on the dorsal raphe nucleus. *Proc. Natl. Acad. Sci. U.S.A* **2005**, *102*, 874–879.
(33) Badie-Mahdavi, H.; Behrens, M. M.; Rebek, J.; Bartfai, T. Effect of galnon on induction of long-term potentiation in dentate gyrus of C57BL/6 mice. *Neuropeptides* **2005**, *39*, 249–251.
(34) Lu, X.; Lundstrom, L.; Langel, U.; Bartfai, T. Galanin receptor ligands. *Neuropeptides* **2005**, *39*, 143–146.
(35) Poduslo, J. F.; Curran, G. L. Glycation increases the permeability of proteins across the blood−nerve and blood−brain barriers. *Brain Res. Mol. Brain Res.* **1994**, *23*, 157–162.
(36) Poduslo, J. F.; Curran, G. L. Polyamine modification increases the permeability of proteins at the blood−nerve and blood−brain barriers. *J. Neurochem.* **1996**, *66*, 1599–1609.
(37) Witt, K. A.; Gillespie, T. J.; Huber, J. D.; Egleton, R. D.; Davis, T. P. Peptide drug modifications to enhance bioavailability and blood−brain barrier permeability. *Peptides* **2001**, *22*, 2329–2343.
(38) Tatemoto, K.; Rokaeus, A.; Jornvall, H.; McDonald, T. J.; Mutt, V. Galanin—a novel biologically active peptide from porcine intestine. *FEBS Lett.* **1983**, *164*, 124–128.
(39) Langel, U.; Bartfai, T. Chemistry and molecular biology of galanin receptor ligands. *Ann. N.Y. Acad. Sci.* **1998**, *863*, 86–93.
(40) Land, T.; Langel, U.; Low, M.; Berthold, M.; Unden, A.; Bartfai, T. Linear and cyclic N-terminal galanin fragments and analogs as ligands at the hypothalamic galanin receptor. *Int. J. Pept. Protein Res.* **1991**, *38*, 267–272.
(41) Fisone, G.; Berthold, M.; Bedecs, K.; Unden, A.; Bartfai, T.; Bertorelli, R.; Consolo, S.; Crawley, J.; Martin, B.; Nilsson, S.; et al. N-terminal galanin-(1−16) fragment is an agonist at the hippocampal galanin receptor. *Proc. Natl. Acad. Sci. U.S.A* **1989**, *86*, 9588–9591.

(42) Carpenter, K. A.; Schmidt, R.; Yue, S. Y.; Hodzic, L.; Pou, C.; Payza, K.; Godbout, C.; Brown, W.; Roberts, E. The glycine residue in cyclic lactam analogues of galanin(1−16)-NH_2 is important for stabilizing an N-terminal helix. *Biochemistry* **1999**, *38*, 15295–15304.

(43) Jureus, A.; Langel, U.; Bartfai, T. L-Ala-substituted rat galanin analogs distinguish between hypothalamic and jejunal galanin receptor subtypes. *J. Pept. Res.* **1997**, *49*, 195–200.

(44) Pooga, M.; Jureus, A.; Razaei, K.; Hasanvan, H.; Saar, K.; Kask, K.; Kjellen, P.; Land, T.; Halonen, J.; Maeorg, U.; Uri, A.; Solyom, S.; Bartfai, T.; Langel, U. Novel galanin receptor ligands. *J. Pept. Res.* **1998**, *51*, 65–74.

(45) Rivera Baeza, C.; Kask, K.; Langel, U.; Bartfai, T.; Unden, A. Analogs of galanin (1−16) modified in positions 1−3 as ligands to rat hypothalamic galanin receptors. *Acta Chem. Scand.* **1994**, *48*, 434–438.

(46) Dasgupta, P.; Singh, A.; Mukherjee, R. N-terminal acylation of somatostatin analog with long chain fatty acids enhances its stability and antiproliferative activity in human breast adenocarcinoma cells. *Biol. Pharm. Bull.* **2002**, *25*, 29–36.

(47) Blanchfield, J. T.; Dutton, J. L.; Hogg, R. C.; Gallagher, O. P.; Craik, D. J.; Jones, A.; Adams, D. J.; Lewis, R. J.; Alewood, P. F.; Toth, I. Synthesis, structure elucidation, in vitro biological activity, toxicity, and Caco-2 cell permeability of lipophilic analogues of a-conotoxin MII. *J. Med. Chem.* **2003**, *46*, 1266–1272.

(48) Blanchfield, J. T.; Lew, R. A.; Smith, A. I.; Toth, I. The stability of lipidic analogues of GnRH in plasma and kidney preparations: the stereoselective release of the parent peptide. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1609–1612.

(49) Yuan, L.; Wang, J.; Shen, W. C. Reversible lipidization prolongs the pharmacological effect, plasma duration, and liver retention of octreotide. *Pharm. Res.* **2005**, *22*, 220–227.

(50) Wang, J.; Wu, D.; Shen, W. C. Structure−activity relationship of reversibly lipidized peptides: studies of fatty acid−desmopressin conjugates. *Pharm. Res.* **2002**, *19*, 609–614.

(51) van de Waterbeemd, H.; Lennernas, H.; Artursson, P. *Drug Bioavailability: Estimation of Solubility, Permeability, Adsorption and Bioavailability*; Wiley-VCH Verlag GmbH & Co: Germany, 2003.

(52) Dagar, S.; Onyuksel, H.; Akhter, S.; Krishnadas, A.; Rubinstein, I. Human galanin expresses amphipathic properties that modulate its vasoreactivity in vivo. *Peptides* **2003**, *24*, 1373–1380.

(53) Cai, L. F.; Gochin, M. Colloidal aggregate detection by rapid fluorescence measurement of liquid surface curvature changes in multiwell plates. *J. Biomol. Screening* **2007**, *12*, 966–971.

(54) Dyson, H. J.; Rance, M.; Houghten, R. A.; Wright, P. E.; Lerner, R. A. Folding of immunogenic peptide fragments of proteins in water solution. II. The nascent helix. *J. Mol. Biol.* **1988**, *201*, 201–217.

(55) Dyson, H. J.; Merutka, G.; Waltho, J. P.; Lerner, R. A.; Wright, P. E. Folding of peptide fragments comprising the complete sequence of proteins. Models for initiation of protein folding. I. Myohemerythrin. *J. Mol. Biol.* **1992**, *226*, 795–817.

(56) Shin, H. C.; Merutka, G.; Waltho, J. P.; Wright, P. E.; Dyson, H. J. Peptide models of protein folding initiation sites. 2. The G-H turn region of myoglobin acts as a helix stop signal. *Biochemistry* **1993**, *32*, 6348–6355.

(57) Morris, M. B.; Ralston, G. B.; Biden, T. J.; Browne, C. L.; King, G. F.; Iismaa, T. P. Structural and biochemical studies of human galanin: NMR evidence for nascent helical structures in aqueous solution. *Biochemistry* **1995**, *34*, 4538–4545.

(58) Valenzano, K. J.; Miller, W.; Kravitz, J. N.; Samama, P.; Fitzpatrick, D.; Seeley, K. Development of a fluorescent ligand-binding assay using the AcroWell filter plate. *J. Biomol. Screening* **2000**, *5*, 455–461.

(59) Sollenberg, U.; Bartfai, T.; Langel, U. Galnon−a low-molecular-weight ligand of the galanin receptors. *Neuropeptides* **2005**, *39*, 161–163.

(60) Boules, M.; Fredrickson, P.; Richelson, E. Bioactive analogs of neurotensin: Focus on CNS effects. *Peptides* **2006**, *27*, 2523–2533.

(61) Egleton, R. D.; Davis, T. P. Bioavailability and transport of peptides and peptide drugs into the brain. *Peptides* **1997**, *18*, 1431–1439.

(62) Egleton, R. D.; Davis, T. P. Development of neuropeptide drugs that cross the blood−brain barrier. *NeuroRx* **2005**, *2*, 44–53.

(63) Dasgupta, P.; Mukherjee, R. Lipophilization of somatostatin analog RC-160 with long chain fatty acid improves its antiproliferative and antiangiogenic activity in vitro. *Br. J. Pharmacol.* **2000**, *129*, 101–109.

(64) Uchiyama, T.; Kotani, A.; Tatsumi, H.; Kishida, T.; Okamoto, A.; Okada, N.; Murakami, M.; Fujita, T.; Fujiwara, Y.; Kiso, Y.; Muranishi, S.; Yamamoto, A. Development of novel lipophilic derivatives of DADLE (leucine enkephalin analogue): intestinal permeability characateristics of DADLE derivatives in rats. *Pharm. Res.* **2000**, *17*, 1461–1467.

(65) Wang, J.; Hogenkamp, D. J.; Tran, M.; Li, W. Y.; Yoshimura, R. F.; Johnstone, T. B.; Shen, W. C.; Gee, K. W. Reversible lipidization for the oral delivery of leu-enkephalin. *J. Drug Targeting* **2006**, *14*, 127–136.

(66) Banks, W. A.; Kastin, A. J. Peptides and the blood−brain barrier: lipophilicity as a predictor of permeability. *Brain Res. Bull.* **1985**, *15*, 287–292.

(67) Fuchs, S. M.; Raines, R. T. Internalization of cationic peptides: the road less (or more?) traveled. *Cell. Mol. Life Sci.* **2006**, *63*, 1819–1822.

(68) Drin, G.; Cottin, S.; Blanc, E.; Rees, A. R.; Temsamani, J. Studies on the internalization mechanism of cationic cell-penetrating peptides. *J. Biol. Chem.* **2003**, *278*, 31192–31201.

(69) Tamai, I.; Sai, Y.; Kobayashi, H.; Kamata, M.; Wakamiya, T.; Tsuji, A. Structure−internalization relationship for adsorptive-mediated endocytosis of basic peptides at the blood−brain barrier. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 410–415.

(70) Drin, G.; Rousselle, C.; Scherrmann, J. M.; Rees, A. R.; Temsamani, J. Peptide delivery to the brain via adsorptive-mediated endocytosis: advances with SynB vectors. *AAPS PharmSci* **2002**, *4*, E26.

(71) Pajouhesh, H.; Lenz, G. R. Medicinal chemical properties of successful central nervous system drugs. *NeuroRx* **2005**, *2*, 541–553.

(72) Kurtzhals, P.; Havelund, S.; Jonassen, I.; Kiehr, B.; Larsen, U. D.; Ribel, U.; Markussen, J. Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem. J.* **1995**, *312* (Pt 3), 725–731.

(73) Havelund, S.; Plum, A.; Ribel, U.; Jonassen, I.; Volund, A.; Markussen, J.; Kurtzhals, P. The mechanism of protraction of insulin detemir, a long-acting, acylated analog of human insulin. *Pharm. Res.* **2004**, *21*, 1498–1504.

(74) Menezo, Y.; Khatchadourian, C. Peptides bound to albumin. *Life Sci* **1986**, *39*, 1751–1753.

(75) Svenson, J.; Brandsdal, B. O.; Stensen, W.; Svendsen, J. S. Albumin binding of short cationic antimicrobial micropeptides and its influence on the in vitro bactericidal effect. *J. Med. Chem.* **2007**, *50*, 3334–3349.

(76) Mazarati, A. M. Galanin and galanin receptors in epilepsy. *Neuropeptides* **2004**, *38*, 331–343.

(77) Nelson, A. R.; Borland, L.; Allbritton, N. L.; Sims, C. E. Myristoyl-based transport of peptides into living cells. *Biochemistry* **2007**, *46*, 14771–14781.

(78) Zorko, M.; Langel, U. Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv. Drug Delivery Rev.* **2005**, *57*, 529–545.

(79) Pooga, M.; Hallbrink, M.; Zorko, M.; Langel, U. Cell penetration by transportan. *FASEB J.* **1998**, *12*, 67–77.

(80) Pooga, M.; Soomets, U.; Hallbrink, M.; Valkna, A.; Saar, K.; Rezaei, K.; Kahl, U.; Hao, J. X.; Xu, X. J.; Wiesenfeld-Hallin, Z.; Hokfelt, T.; Bartfai, T.; Langel, U. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat. Biotechnol.* **1998**, *16*, 857–861.

(81) Tossi, A.; Sandri, L.; Giangaspero, A. Amphipathic, α-helical antimicrobial peptides. *Biopolymers* **2000**, *55*, 4–30.

(82) Hua, X. Y.; Hayes, C. S.; Hofer, A.; Fitzsimmons, B.; Kilk, K.; Langel, U.; Bartfai, T.; Yaksh, T. L. Galanin acts at GalR1 receptors in spinal antinociception: synergy with morphine and AP-5. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 574–582.

(83) Hua, X. Y.; Salgado, K. F.; Gu, G.; Fitzsimmons, B.; Kondo, I.; Bartfai, T.; Yaksh, T. L. Mechanisms of antinociception of spinal galanin: how does galanin inhibit spinal sensitization. *Neuropeptides* **2005**, *39*, 211–216.

(84) Liu, H. X.; Brumovsky, P.; Schmidt, R.; Brown, W.; Payza, K.; Hodzic, L.; Pou, C.; Godbout, C.; Hokfelt, T. Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors. *Proc. Natl. Acad. Sci. U.S.A* **2001**, *98*, 9960–9964.

JM801088X