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Introduction of lipidization–cationization motifs affords systemically bioavailable neuropeptide Y and neurotensin analogs with anticonvulsant activities

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The neuropeptides galanin (GAL), neuropeptide Y (NPY) or neurotensin (NT) exhibit anticonvulsant activities mediated by their respective receptors in the brain. To transform these peptides into potential neurotherapeutics, their systemic bioavailability and metabolic stability must be improved. Our recent studies with GAL analogs suggested that an introduction of lipoamino acids in the context of oligo-Lys residues (lipidization–cationization motif) significantly increases their penetration into the brain, yielding potent antiepileptic compounds. Here, we describe an extension of this strategy to NPY and NT. Rationally designed analogs of NPY and NT containing the lipidization–cationization motif were chemically synthesized and their physicochemical and pharmacological properties were characterized. The analogs NPY-BBB2 and NT-BBB1 exhibited increased serum stability, possessed log *D* > 1.1, retained high affinities toward their native receptors and produced potent antiseizure activities in animal models of epilepsy following intraperitoneal administration. Our results suggest that the combination of lipidization and cationization may be an effective strategy for improving systemic bioavailability and metabolic stability of various neuroactive peptides. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: NPY; neurotensin; chemical modifications; bioavailability; epilepsy; blood–brain barrier

Introduction

Several endogenous neuropeptides, such as NPY (YPSKP-DNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2), NT (ZLY-ENKPRRPYIL), and GAL (GWTLNSAGYLLGPHAVGNHRSFS DKNGLTS-NH_2), possess both antiepileptic and antinociceptive properties mediated by their respective receptors in the CNS [1–3]. These neuropeptides show promise as potential first-in-class therapeutics for the treatment of epilepsy and/or pain. However, the lack of CNS bioavailability and marginal metabolic stability has slowed their preclinical and clinical development. The difficulty of delivering peptides, proteins, and other drugs across the BBB has long been challenged using a variety of strategies [4–6]. BBB-permeable analogs of NPY, NT, or GAL could provide useful pharmacological tools that could also validate their native receptors as drug targets in humans.

NPY is a 36-amino acid anticonvulsant peptide that is an endogenous ligand for five receptor subtypes (Y1–Y5) [7]. Among the five subtypes, the Y2 receptor was previously identified as the potential molecular target for the treatment of epilepsy, as it is implicated in modulating the neural excitability in the hippocampus [1,8]. Furthermore, over-expression of NPY in the rat brain using an adeno-associated virus has shown therapeutic promise as a treatment for epilepsy and is poised to enter clinical trials [9,10]. The NPY-based gene therapy strategy underscores the lack of systemically active NPY analogs that penetrate the BBB. Such analogs would provide lead compounds for developing NPY-based pharmacological therapies.

SAR studies of NPY identified critical amino acid residues for interactions with subtypes of NPY receptors [11,12]. Results from alanine-scan and truncated analog studies suggested that the *C*-terminal fragment of NPY was necessary for high affinity binding to Y2 receptors [13–15]. α-Helical character of the *C*-terminal fragment of NPY was found necessary for Y2-binding [11,16]. Replacement of naturally occurring amino acid residues with L-alanine perturbed helix formation and resulted in peptides with decreased binding affinities [11]. Based on the receptor selectivity of the *C*-terminal decapeptide and the stabilizing effects

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Abbreviations used: *BBB, blood–brain barrier; GAL, galanin; K_p, N^{ε}-palmitoyl-L-lysine; NPFF, neuropeptide FF; nH_2O, nanopure water; NT, neurotensin; NTR-1, neurotensin receptor subtype 1; Y2, neuropeptide Y receptor subtype 2; Z, pyroglutamic acid.*

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of *N*-terminal amino acids, the Beck-Sickinger group designed a series of centrally truncated NPY analogs. These analogs were compared for their binding affinities for both Y1 and Y2 receptor subtypes; analogs possessing the *C*-terminal fragment NPY (25–36) could readily discriminate between receptor subtypes with low nanomolar affinities for Y2 [11,12,16]. Taken together, it is possible to develop reduced-size peptide analogs that are potent agonists for the Y2 receptor.

NT mediates a number of physiological responses in the CNS [17,18]. This 13-residue peptide has been widely studied for its antinociceptive properties, shown to be mediated through two receptor subtypes, NTR-1 and NTR-2 [19,20]. More recently, Lee and coworkers demonstrated the ability of NT and its glycosylated analogs to suppress epileptic seizures in the brain with remarkable potency (for NT, $ED_{50} = 0.001$ nmol in the 6 Hz mouse model of epilepsy, intracerebroventricular injection) [2]. SAR studies have revealed that the *C*-terminal portion of the peptide, NT (8–13), is sufficient for receptor activation [21,22]. There have been many attempts to generate metabolically stable, BBB-permeable analogs of NT, including peptide minimization, cyclization, backbone modification, and amino acid substitution [18,23,24]. Work by our group has recently shown that conjugated polyamine motifs could be coupled to the NT active fragment, resulting in an analog with improved CNS bioavailability [25]. The polyamine-containing NT analog possessed subnanomolar binding affinity ($K_i = 0.25$ nM) and low nanomolar agonist potency toward NTR-1 ($EC_{50} = 1.4$ nM) [25]. Based on the large body of evidence, NT is not only a good model to study novel modifications for improving CNS bioavailability, but this neuropeptide also offers an attractive template for designing future drugs for pain, psychiatric diseases, and even perhaps for epilepsy.

Our recent research with the anticonvulsant neuropeptide, GAL, showed that combining lipidization (by addition of Lys-palmitoyl, (K_p)) and cationization (through incorporation of three Lys residues) significantly improved CNS bioavailability of the resulting GAL analogs. The modified GAL analogs exhibited potent antiepileptic activity that was also accompanied by increased log *D* values and improved metabolic stability [26–28]. Lipidization of peptides is known to improve BBB-penetration of peptides [6,29]. Similarly, increased cationic character of the peptides has been previously shown to improve their delivery across biological membranes, including the BBB [30,31]. Despite its effectiveness in generating systemically active GAL analogs with antiepileptic activity, the lipidization–cationization strategy has not been evaluated on any other anticonvulsant neuropeptides.

To test the hypothesis that the combination of lipidization and cationization could improve systemic activity of structurally diverse anticonvulsant neuropeptides, this chemical modification strategy was applied to NPY and NT. Herein we report the design, chemical synthesis, and characterization of several BBB-penetrating analogs of both NPY and NT. Our results suggest that the lipidization–cationization strategy might be more broadly applicable across diverse neuropeptides toward improving metabolic stability and CNS bioactivity.

Materials and Methods

Chemical Synthesis

Synthesis of all analogs was carried out on solid phase using pre-loaded Rink Amide (NPY) or Wang (NT) resins. Syntheses were carried out at 100 μmol scale using Fmoc-Tyr(tBu)-Rink Amide-MBHA resin (subs. 0.41 meq/g) or Fmoc-Leu-Wang resin (subs. 0.57 meq/g). Three couplings (30, 30, and 40 min) were performed using 0.2 M PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), 0.4 M DIPEA (*N*,*N*-diisopropylethylamine), and 200 mM of the appropriate amino acid. Fmoc-protecting groups were removed prior to the addition of the next amino acid by 30-min treatment with 20% piperidine. Protected amino acids, including Fmoc-Lys(palmitoyl) and Fmoc-6-aminohexanoic acid, were obtained from Chem-Impex International. Peptides were cleaved from resin by treatment with reagent K (82.5% TFA, 5% water, 5% ethanedithiol, 2.5% thioanisole v/v, 75 mg/ml phenol) followed by vacuum filtration to separate peptide from resin, and finally precipitated with chilled methyl-*tert*-butyl ether. Crude peptides were then purified by preparative reversed-phase HPLC over a linear gradient of Buffer B (90% acetonitrile in 0.1% TFA) ranging from 5 to 95% in 45 min. Purities of HPLC separations were assessed by analytical reversed-phase HPLC using a linear gradient ranging from 5 to 95% Buffer B in 30 min. HPLC fractions with peptide purities greater than 95% were pooled and quantified by measuring UV absorbance at 274.6 nm. Molecular masses of all analogs were confirmed by MALDI-TOF MS.

Partitioning Coefficient, log *D*

As an *in vitro* measure of lipophilicity of the analogs, log *D* values were calculated using the shake-flask method. Water-saturated *n*-octanol was prepared by mixing equal volumes of *n*-octanol and water for 24 h at room temperature. Lyophilized peptides were then reconstituted in 1 ml phosphate-buffered saline (PBS), followed by addition of an equal volume of water-saturated *n*-octanol. Samples were then allowed to mix on a rotary mixer for 24 h. Aqueous phases were carefully removed and analyzed by analytical HPLC over a gradient of Buffer B ranging from 5 to 95% in 30 min. The amount of peptide in the aqueous phase was determined by HPLC using a standard curve constructed for each peptide. Log *D* values for each analog were then calculated using the following equation [26,32]:

$$\log D = \log\left(\frac{[\text{Peptide}_{\text{Octanol}}]}{[\text{Peptide}_{\text{Aqueous}}]}\right) \quad (1)$$

Serum Stability Assay

The stability of peptides in the presence of 25% rat blood serum was evaluated for each analog by incubation at 37 °C for 0 min, 30 min, 1 h, 2 h, and 4 h. Samples were prepared by adding 10 μg linear peptide, resuspended in nH_2O, to pre-heated tubes containing 25% rat blood serum and 0.1 M Tris–HCl, pH 7.5 to yield a final peptide concentration of 20 μM. At the appropriate time points, reactions were quenched by precipitation of serum proteins through addition of 100 μl isopropanol/water/trichloroacetic acid (45%: 40%: 15% by volume). Samples were then incubated at −20 °C for 20 min, followed by centrifugation at 10 000 rpm for 3 min to remove serum proteins. Supernatants were then analyzed by analytical HPLC equipped with a Waters YMC ODS-A 5 μm 120 Å column using a gradient ranging from 5 to 95% Buffer B in 45 min including a 15-min pre-equilibration. Metabolic stability was assessed by determining a time-course of the disappearance of an intact peptide. Half-lives ($t_{1/2}$) for each peptide were determined from at least three independent time-course experiments using

the equation below (where m is the slope of the line and b is the y-intercept [26]):

$$t_{1/2}(\text{h}) = \frac{[\ln(50) - b]}{m} \quad (2)$$

Circular Dichroism

The α-helical content of NPY analogs was determined by CD. A measured quantity of 0.1 mg peptide was reconstituted in 1.0 ml 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 combined with 0.5 ml TFE. After 1 h, 250 µl of each sample was loaded into a 0.1-cm quartz cuvette and analyzed using an Aviv 410 CD spectropolarimeter set to 25 °C. Scans were collected over the range of 250–200 nm in 1.0 nm steps with 1 s dwell time. Data were averaged from three scans and processed using Eqn (3), where M_r is the molecular weight, c is concentration (mg/ml), d is path length (cm), and n is the number of peptide bonds. Percent helicity was determined using Eqns (4) and (5) [26,33]:

$$[\Theta] = \frac{\Theta \times 100 \times M_r}{c \times d \times n} \quad (3)$$

$$[\Theta]_{max} = -39\,500\left[1 - \left(\frac{2.57}{(n)}\right)\right] \quad (4)$$

$$\%\,\alpha\text{-helix} = \frac{[\Theta_{222}]}{[\Theta_{max}]} \times 100 \quad (5)$$

CD spectra for NT analogs were not collected as it has been shown previously that this peptide lacks secondary structure in both aqueous and sodium dodecyl sulfate ($C_{12}H_{25}OSO_3Na$)-containing environments [34].

Receptor Binding

Determination of binding affinity (K_i) for NPY analogs was provided by the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH PDSP). Binding affinities for the NPY-Y2 subtype were obtained using a nonlinear regression of radioligand competition binding isotherms. K_i values were calculated from best fit IC_{50} values using the Cheng–Prusoff equation [35]. Competitive binding of NT analogs toward the NTR-1 receptor subtype was assessed using a fluorescence-based assay. The assays were conducted in quadruplicate in a 96-well format on AcroWell filter plates using NTR-1 membrane preparations and Eu-labeled NT (Perkin-Elmer). Samples were incubated at room temperature followed by repeated rinsing with wash buffer (50 mM Tris–HCl, pH 7.5, and 5 mM $MgCl_2$) using a vacuum manifold. Prior to analysis, samples were incubated in 200 µl enhancement solution for 30 min at room temperature. Plates were read using a Perkin-Elmer VICTOR^3 spectrofluorometer. Competitive binding curves were generated by GraphPad Prism software using a nonlinear regression, sigmoidal dose–response (variable slope) curve with no weights or constraints [27].

Anticonvulsant Activity

The anticonvulsant activity of the analogs was studied in the 6 Hz (32 mA) model of pharmacoresistant epilepsy following a bolus i.p. administration of a dose of 4 mg/kg to five groups of CF-1 mice ($n = 4$). At discrete time points (15, 30, 60, 120, and 240 min) following the drug administration, mice were challenged with a 6 Hz corneal stimulation (32 mA for 3 s delivered via corneal electrodes). Mice which did not exhibit classical limbic seizure behavior (i.e. jaw chomping, vibrissae twitching, forelimb clonus, or Straub tail) were classified as protected. In addition to the 6 Hz screening, dose–response studies were carried out for the NPY-BBB2 and NT-BBB1 analogs. Briefly, peptide analogs were administered i.p., and at 1 h (time-to-peak effect), mice were then challenged with a 32-mA corneal stimulation. Based on the numbers of protected animals at each dosage, ED_{50} values were determined for each peptide using Probit software.

Results

Design Strategy

The lipidization–cationization strategy, previously applied to the truncated GAL analogs such as Gal-B2 (NAX-5055), was applied to the bioactive fragments of NPY and NT. To design NPY-derived compounds, two key SAR results were taken into account: (i) the *C*-terminal fragment of NPY, NPY(25–36), retains the agonist activity toward the Y2 receptor and (ii) bulky substituents can be added to the *N*-terminus of NPY(25–36) [12,15,36]. As illustrated in Figure 1, the Y2-targeting analogs were based on previously characterized [Ahx$^{5-24}$]NPY, which contains the *C*-terminal fragment (NPY 25–36) [12,36]. To introduce the lipidization–cationization motif, a positively charged core was created near the *N*-terminus by replacement of the Pro-Ser sequence with Lys-Lys, whereas, Lys4 in [Ahx$^{5-24}$]NPY was replaced with a N^{ε}-palmitoyl-L-lysine (K_p)

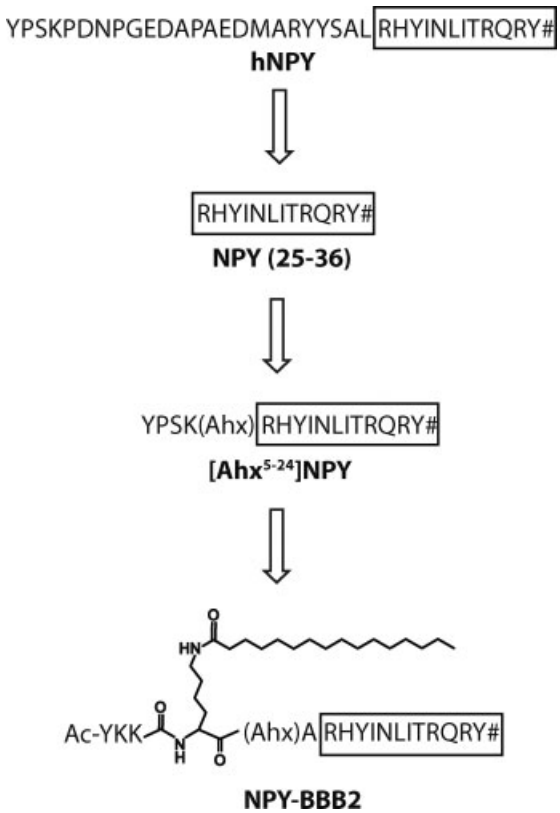

Figure 1. Rational design of systemically active NPY analogs with improved penetration across the BBB. Analogs were designed based on the Y2 receptor subtype selectivity of the NPY pharmacophore NPY (25–36) and the stabilizing effects of *N*-terminal residues observed in the previously described analog [Ahx$^{5-24}$] NPY [12]. NPY-BBB2 combines the strategies of cationization and lipidization into centrally truncated NPY. (#) denotes *C*-terminal amidation of peptides, (Ahx) is 6-aminohexanoic acid.

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Table 1. Summary of sequences, mass spectrometry data, HPLC retention times, and log *D* values for NT and NPY analogs[a]

Peptide analog	Structure	Molecular mass (calc/exp)	HPLC retention time[b]	Log *D*[c]
NPY				
NPY-BBB0	Ac-**(Ahx)A**RHYINLITRQRY$^{\#}$	1857.05/1856.81	16.8 ± 0.08	−2.13 ± 0.68
NPY-BBB1(K3)	Ac-Y**KKK(Ahx)**RHYINLITRQRY$^{\#}$	2333.36/2333.55	15.5 ± 0.01	−1.38 ± 0.12
NPY-BBB2(K3)	Ac-Y**KKK(Ahx)A**RHYINLITRQRY$^{\#}$	2404.4/2404.46	15.8 ± 0.07	−1.11 ± 0.31
NPY-BBB1	Ac-Y**KK(K_p)(Ahx)**RHYINLITRQRY$^{\#}$	2571.59/2572.4	21.5 ± 0.03	2.82 ± 0.74
NPY-BBB2	Ac-Y**KK(K_p)(Ahx)A**RHYINLITRQRY$^{\#}$	2642.63/2642.7	21.8 ± 0.01	1.76 ± 0.01
NT				
NT-BBB0	RRPYIL$^{\wedge}$	816.5/816.5	14.0 ± 0.14	−1.31 ± 0.35
NT-BBB1(K4)	Ac-**KKKK**PYIL$^{\wedge}$	1058.69/1058.70	13.1 ± 0.03	0.48 ± 0.01
NT-BBB1	Ac-**K(K_p)KK**PYIL$^{\wedge}$	1296.91/1296.9	22.7 ± 0.01	1.19 ± 0.12

($^{\#}$) denotes an amidated *C*-terminus. ($^{\wedge}$) represents carboxylation at the *C*-terminus.
[a] K_p is N^{ε}-palmitoyl-L-lysine, Ahx is 6-aminohexanoic acid.
[b] Linear gradient of H_2O/acetonitrile.
[c] Log *D* values calculated by the shake-flask method.

residue (Figure 1). No additional positively charged residues were introduced, as the presence of the Arg-His sequence provided additional positively charged residues located adjacent to the KKK_p(Ahx) motif (NPY-BBB1). Based on our previous SAR study with Gal-B2 [26], we designed an additional NPY analog, NPY-BBB2, in which the lipidization–cationization motif, 'KKK_p(Ahx)', was further separated from the active fragment by an additional Ala residue (Table 1, Figure 1). As the spacing between the active neuropeptide fragment and the lipidized-cationic motif was important for improving bioavailability of the GAL analogs, as determined by truncation of Gal-B2, we chose to insert an additional alanine into NPY-BBB1 (resulting in NPY-BBB2) rather than further truncate NPY-BBB1. In addition to the modified peptides, three additional NPY analogs were designed as controls (Table 1). All NPY-derived analogs were acetylated at the *N*-terminus and amidated at the *C*-terminus.

As summarized in Figure 2, a similar design strategy was applied to engineer NT analogs. The *C*-terminal hexapeptide sequences of NT (RRPYIL) and Contulakin-G (KKPYIL) (Contulakin-G is a venom-derived glycosylated conopeptide) are the active NT fragments that bind specifically to the NT receptors [37]. Noteworthy, the naturally occurring 'Lys-Lys' sequence in Contulakin-G provided part of the lipidization–cationization motif, whereas the lipoamino acid, Lys-palmitoyl, was introduced proximal to the Lys-Lys sequence. The position of Lys-palmitoyl was based on our previous finding that this position could accommodate a Lys-spermine residue without affecting receptor binding properties toward NTR-1 subtype of the NT receptors [25]. One additional Lys residue was added to the *N*-terminus, resulting in the KK_pKK motif. All NT analogs were acetylated at the *N*-terminus and contained a free carboxyl group at the *C*-terminus.

Chemical Synthesis

All analogs were synthesized on solid support using an automated peptide synthesizer and standard Fmoc protocols. Peptides were cleaved from resin by treatment with reagent K and were purified by preparative reversed-phase HPLC. Peptides were quantified by UV absorbance at 274.6 nm, aliquoted, and lyophilized. Masses of all compounds were confirmed by MALDI-TOF MS. Final yields of purified peptide analogs ranged between 2% for the modified peptides and 8% for the unmodified active fragments. Overall yields were calculated from the averages of multiple syntheses with respect to the amount of resin used in each 100 µmole synthesis. Retention times were calculated from the average of three independent analyses of each analog and are summarized in Table 1.

The partitioning coefficient (log *D*) for each analog was determined using the shake-flask method. Calculated values for partitioning are summarized in Table 1. The unmodified active fragments NT-BBB0 and NPY-BBB0 were hydrophilic in nature indicated by negative log *D* values of −1.31 and −2.13, respectively. Cationization through conjugation of the oligo-Lys motif to the *N*-terminus of the bioactive fragments showed slight improvement in partitioning with values of 0.48 for NT-BBB1 (K4), −1.38 for NPY-BBB1(K3), and −1.11 for NPY-BBB2(K3). The analogs possessing the combination of cationization and lipidization showed dramatic improvement over both the peptide active fragments and the cationic analogs. NT-BBB1 had a log *D* of 1.19, NPY-BBB1 a log *D* of 2.82, and NPY-BBB2 a log *D* of 1.76 (Figure 3).

Structural Characterization of NT/NPY Analogs

To evaluate effects of the chemical modifications on helical content of the resulting NPY analogs, CD experiments were carried out (Figure 4). Recordings were performed in aqueous 150 mM NaF phosphate buffer, pH 7.4 both in the absence and in the presence of 50% (v/v) TFE. Helical content (Θ_{222}) for full-length NPY in the presence of TFE was calculated as 21.4%. The NPY-BBB0 analog exhibited comparable helicity at 18.1%. Increased cationization of the active fragment in the BBB1(K3) and BBB2(K3) analogs disrupted the secondary structure and resulted in helical contents of 9.7 and 2.2%, respectively. For NPY analogs containing the lipoamino acid and oligo-Lys components, helicity was determined as 12.6 and 15.0% for BBB1 and BBB2, respectively (Figure 4). We did not characterize NT analogs using CD, as the previous work has shown NT lacks secondary structure [33,34.]

Metabolic Stability of NT/NPY Analogs

To determine how the lipidization–cationization changed the *in vitro* metabolic stability of the NPY and NT analogs, the selected peptides were incubated in 25% rat blood serum at

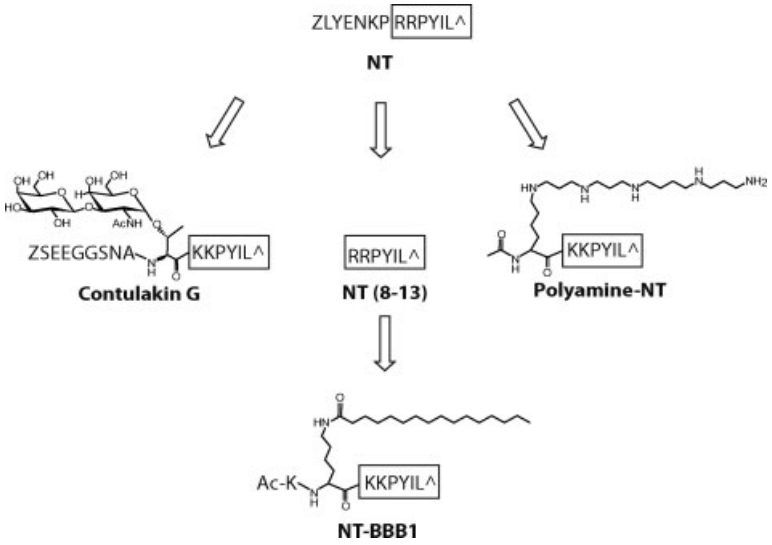

Figure 2. Rational design of systemically active NT-based analogs with improved penetration across the BBB. The Contulakin-G isolated from cone snail venom [37], the NT active fragment RRPYIL [21], and polyamine-NT [25] are all potent ligands for the NT receptor. In NT-BBB1, the lipoamino acid was introduced in the position proximal to the *C*-terminal active fragment. The cationization part was achieved by adding the *N*-terminal Lys residue to two already existing Lys residues that comprised the NT active fragment. (Z) denotes pyroglutamic acid and (^) represents carboxylated *C*-terminus.

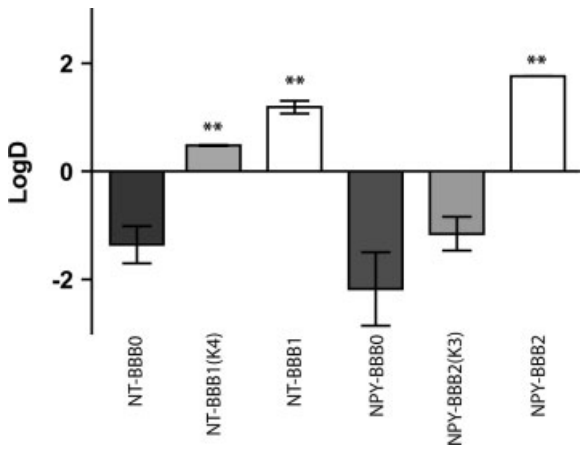

Figure 3. Summary of log *D* values of NT and NPY analogs. Log *D* values were obtained from the average of three independent experiments via the shake-flask method using a 50 : 50 ratio of *n*-octanol/PBS, pH 7.4 at 25 °C. The amount of peptide in the aqueous phase was determined by peak area using analytical HPLC over a gradient ranging from 5 to 95% Buffer B in 30 min. Results were obtained from the slopes of at least three independent experiments. Statistical comparisons between unmodified and modified analogs were performed using the two-tailed *t*-test function of GraphPad software. Statistical significance was noted as: (**) *P*-value <0.01.

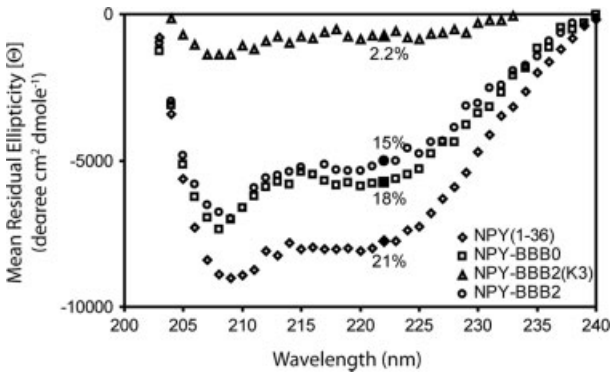

Figure 4. Helical properties of NPY-BBB2. CD spectra showing the relative α-helical content of NPY analogs: Full-length NPY (◇), NPY-BBB0 (□), NPY-BBB2 (○), and NPY-BBB2(K3) (△). Percent helicity was calculated at 222 nm and is noted by filled data points. Data were obtained in aqueous 150 mM NaF phosphate buffer, pH 7.4 both in the absence and in the presence of 50% (v/v) TFE.

37 °C and degradation was monitored by HPLC. Aliquots were withdrawn after 30 min, 1 h, 2 h, and 4 h and quenched with TCA followed by HPLC separations [26,38]. The time courses of the disappearance of the intact peaks were plotted and half-lives were calculated.

As summarized in Figure 5, *in vitro* metabolic stability was significantly increased using the strategy described here. For unmodified NPY and NT analogs, the $t_{1/2}$ values were 40 and 4.7 min, whereas the NPY-BBB1, NPY-BBB2, and NT-BBB1 analogs had $t_{1/2}$ values of 3.2, 3.0, and 2.7 h, respectively. Noteworthy, the *in vitro* metabolic stability was also increased in the analogs containing only oligo-Lys motifs, such as NT-BBB1(K4), NPY-BBB1(K3), and NPY-BBB2(K3). It was previously shown that short cationic peptides exhibit increased binding to serum albumin [39,40]. Furthermore, improved metabolic stability was observed for the GAL analogs: conjugation of the oligo-Lys motif to the GAL active fragment ($t_{1/2}$ = 28 min) increased $t_{1/2}$ by nearly tenfold ($t_{1/2}$ = 4.6 h) [26]. It is therefore possible that increased cationization of NPY, NT, and GAL analogs could result in extended $t_{1/2}$ values through increased peptide binding to albumin in serum-containing solutions.

Pharmacological Characterization

NPY and NT analogs were characterized both in the receptor binding assays and in a mouse model of epilepsy. Binding affinities were determined for the Y2 receptor through a competition assay against [^{125}I] Peptide YY, whereas for the NTR-1 receptor, a time-resolved fluorescence-based competitive binding assay

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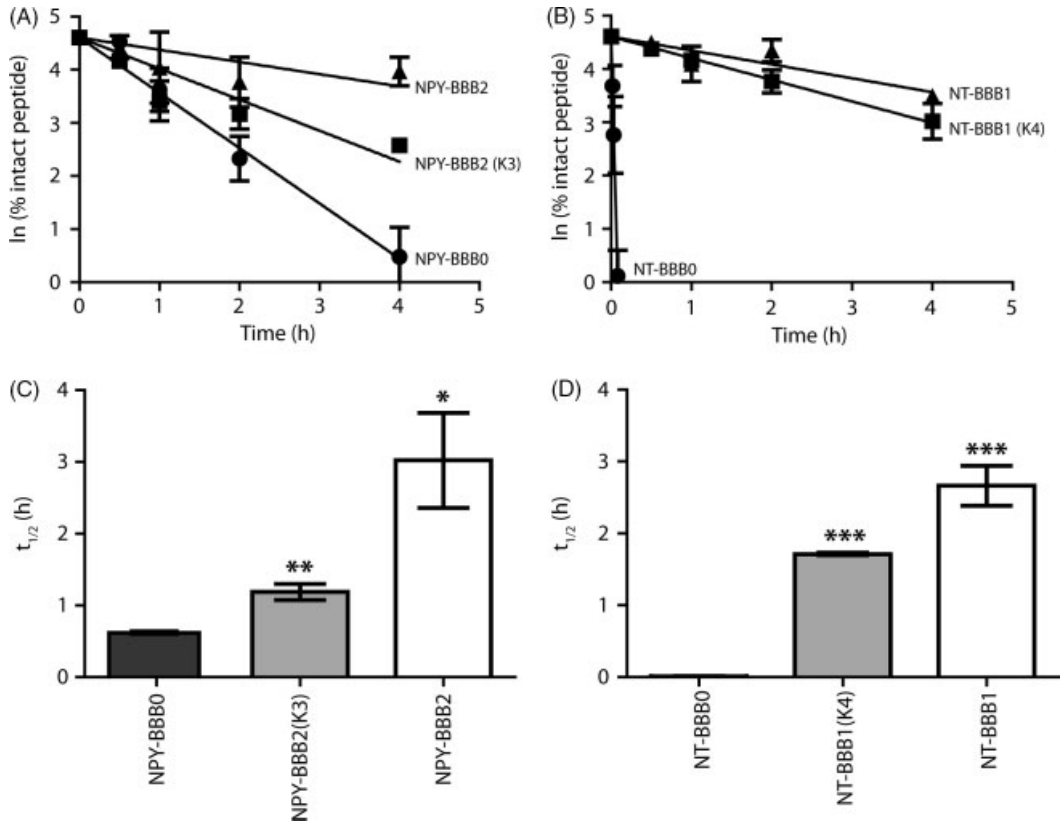

Figure 5. Metabolic stability of the NT and NPY analogs determined in the *in vitro* serum stability assay. (A) Time-course of disappearance of NPY-based analogs. (B) Time-course of disappearance of NT-based analogs. (C) Half-lives for NPY-BBB0, NPY-BBB2(K3), and NPY-BBB2 were calculated as 0.67, 1.2, and 3.0 h, respectively. (D) $t_{1/2}$ for NT-BBB0, NT-BBB1(K4), and NT-BBB1 were calculated as 0.01, 1.7, and 2.7 h, respectively. Results were obtained from the slopes of at least three independent experiments. Statistical comparisons of unmodified and modified analogs were performed using the two-tailed *t*-test function of GraphPad software. Statistical significance was noted as: (*) *P*-value <0.05, (**) *P*-value <0.01, and (***) *P*-value <0.0001.

was employed. The antiepileptic activity of the analogs was assessed in the 6 Hz model of epilepsy in mice following i.p. administration. All pharmacological data are summarized in Table 2.

All modified analogs exhibited low nanomolar affinities toward their target receptors (Table 2 and Figure 6), suggesting that the chemical modifications did not affect interactions with the target receptors. The K_i value for the unmodified NPY-BBB0 was determined as 60.6 nM. Noteworthy, NPY-BBB1(K3) and NPY-BBB1 actually exhibited tighter binding toward the Y2 receptor as compared to the active fragment by itself. For NT analogs, the addition of two Lys residues did not affect the affinity of the NT active fragment toward NTR-1; however, incorporation of the lipoamino acid decreased the affinity by 1 order of magnitude (K_i for NT-BBB1 was 4.0 n m).

Next, the analogs were characterized for their ability to suppress seizures in mice following systemic administration. The time–response curves were determined by measuring the number of mice protected from seizures (in groups of four mice) at the following time points: 15, 30, 60, 120, and 240 min. The unmodified and oligo-Lys containing analogs of NPY did not exhibit significant anticonvulsant activity at 4 mg/kg, i.p. (this dose was previously used for *in vivo* screening of anticonvulsant analogs of GAL). The dose of 4 mg/kg of NPY-BBB1 produced, at most, a very modest anticonvulsant activity at 30 and 60 min post-drug administration, whereas at the same dose, NPY-BBB2 yielded a significant anticonvulsant effect with time-to-peak effect between 30 and 60 min. For NT analogs, the unmodified active fragment (NT-BBB0) lacked antiepileptic activity. The lipoamino acid containing NT-BBB1 was very active in the epilepsy test, producing a full protection from seizures at 60 min. The two most active analogs (NPY-BBB2 and NT-BBB1) were further evaluated in a dose–response study. Figure 6 illustrates the dose–response curves, and the calculated ED_{50} data are shown in Table 3. ED_{50} values for NT-BBB1 and NPY-BBB2 were calculated as 2.86 mg/kg (95% CI = 1.44–4.36 mg/kg) and 1.07 mg/kg (95% CI = 0.41–1.80 mg/kg), respectively.

Discussion

Our previous work on engineering systemic bioavailability in GAL analogs yielded very potent antiepileptic compounds [26–28,41]. In the present work, we successfully applied the same strategy, namely combining a lipoamino acid with several adjacent Lys residues (lipidization–cationization motif) to two other anticonvulsant neuropeptides, NPY and NT. The apparent similarities of physicochemical and pharmacological properties of all three analogs, GAL-B2, NPY-BBB2, and NT-BBB1, are summarized in Table 3. Two analogs described in this work, namely NPY-BBB2 and NT-BBB1, appeared as potent antiepileptic compounds following systemic delivery while exhibiting improved *in vitro* serum stability, suggesting that this strategy might be

Table 2. Pharmacological properties of NT and NPY analogs

Peptide analog	Receptor binding assay K_i [nM]	*In vivo* anticonvulsant activity 6 Hz, 32 mA, 4 mg/kg 15 min	30 min	60 min	120 min	240 min
NPY						
NPY-BBB0	60.6 ± 4.6			n.d.		
NPY-BBB1(K3)	3.6 ± 0.4***	1/4	0/4	0/4	0/4	0/4
NPY-BBB2(K3)	n.d.	1/4	0/4	0/4	0/4	0/4
NPY-BBB1	14.2 ± 0.7***	0/4	1/4	1/4	0/4	0/4
NPY-BBB2	26.1 ± 1.7***	1/4	3/4	3/4	0/4	0/4
NT						
NT-BBB0	0.2 ± 0.1	0/4	0/4	0/4	0/4	0/4
NT-BBB1(K4)	0.5 ± 0.1*	0/4	0/4	0/4	0/4	0/4
NT-BBB1	4.0 ± 0.2***	3/4	2/4	4/4	3/4	1/4

K_i values were calculated as the average of three independent binding experiments. Statistical significance between unmodified and modified analogs, calculated using the two-tailed *t*-test function GraphPad software, was noted as: (*) *P*-value <0.05, (**) *P*-value <0.01, and (***) *P*-value <0.0001. 6 Hz data obtained from five independent groups of four mice each. Data represent the number of mice within each group that did not exhibit classical seizure activity at each time point.

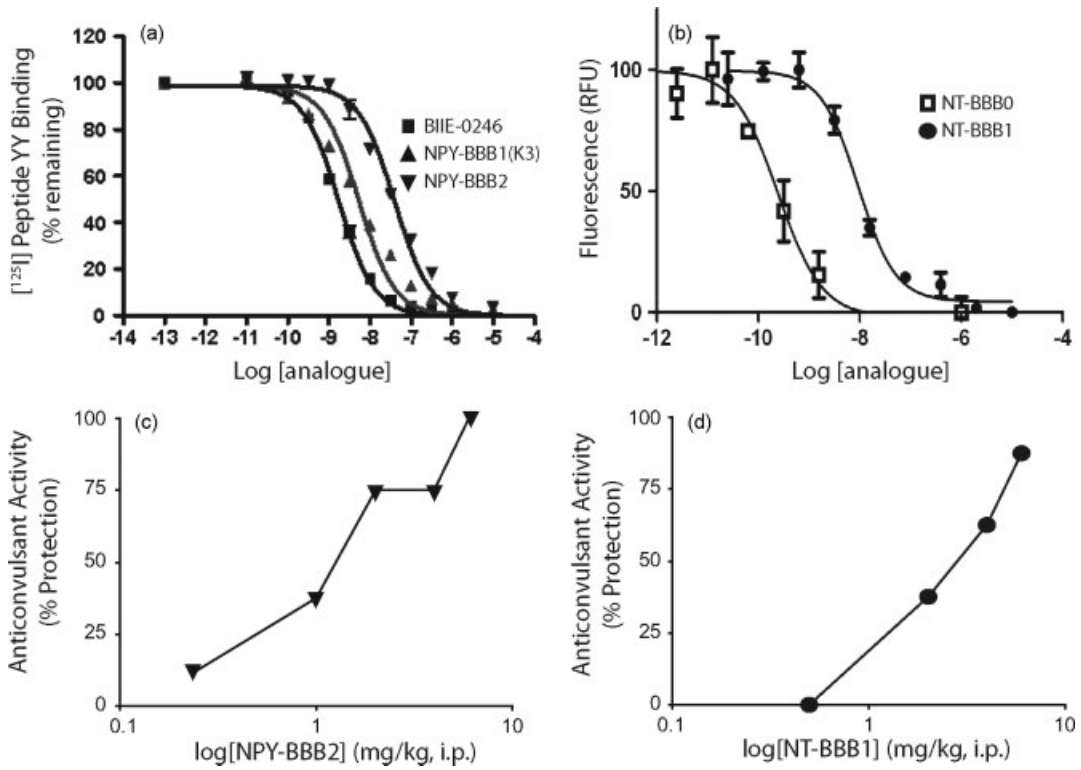

Figure 6. Pharmacological characterization of NPY and NT analogs. (A) NPY analogs were screened in a competitive binding assay against ^{125}I-labeled NPY. (A) The binding curves for NPY-BBB1 (K3) (K_i = 3.6 nM) and NPY-BBB2 (K_i = 26 nM) and the BIIE-0246 reference standard (K_i = 1.2 nM). (B) Binding affinities of NT analogs toward the NTR-1 receptor were determined using fluorescence-based competitive binding assay. Shown here are the binding curves for NT-BBB0 and NT-BBB1 (K_i = 0.2 and 4.0 nM, respectively). (C) Bolus injections of NPY-BBB2 and NT-BBB1 (shown in panel D) were administered i.p. and dose–response curves were generated at the 1 h time point. Based on the dose–response data, the calculated ED50 values for NPY-BBB2 and NT-BBB1 were calculated as 1.07 and 2.86 mg/kg, respectively, using Probit software.

more generally applicable to neuropeptides to improve their penetration across the BBB.

Lipidization–cationization was successfully applied to the KKPYIL motif (NT-BBB0). The *N*-terminal addition of Lys and Lys-palmitoyl residues resulted in an increased log *D* value and improved half-life in the serum stability assay, whereas the receptor binding retained high nanomolar affinity. The improvements of log *D* and metabolic stability were correlated with pronounced anticonvulsant activity in the 6 Hz mouse model of epilepsy after i.p. administration. Noteworthy, the lipidization–cationization alone did not produce antiseizure activity [28] suggesting that the anticonvulsant activities of the modified neuropeptides are mediated by their target receptors expressed in the brain. Although this work adds one more example of BBB-permeable NT-based analogs

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Table 3. Summary of physicochemical and pharmacological properties of three lead anticonvulsant neuropeptides containing the lipidization–cationization motif

Analog	Log *D*	$t_{1/2}$ (h)	ED_{50} (mg/kg)[a]	K_i [nM][b]
Gal-B2[a]	1.24	9.4	0.8 (0.4–1.6)	3.5
NPY-BBB2	1.76	3.0	1.1 (0.4–1.8)	26.1
NT-BBB1	1.19	2.7	2.9 (1.4–4.4)	4.0

Partitioning coefficients were calculated using the shake-flask method of log *D* determination. All modified analogs were shown to possess increased lipophilicity compared to the unmodified peptides. Modified GAL, NPY, and NT analogs showed increased resistance to proteolytic degradation in an *in vitro* serum stability assay as evidenced by increased serum half-lives ($t_{1/2}$).
[a] ED_{50} values were calculated at a time-to-peak effect of 1 h.
[b] K_i values for analogs were calculated against receptors implicated in seizure activity (i.e. hGalR1, hNTR-1, and hNPY-Y2, respectively).

(such as NT69L [42], JMV2012 [23], or KK13 [43]), it is the first report of the systemically active NT analog that has antiepileptic activity.

Systemically active NPY analogs were rationally designed based on Y2-selective analogs developed by the group of Beck-Sickinger [12]. NPY-BBB2 contained the lipidization–cationization motif and exhibited increased log *D*, improved metabolic stability and systemic bioavailability, while maintaining nanomolar affinity toward NPY-Y2 receptor. The *C*-terminal residues of NPY (NPY 25–36) are required for Y2 receptor subtype specificity [12,15] and NPY-BBB2 indeed maintained similar Y2-binding affinity to that described by Rist and colleagues for the Ac-NPY 25–36 analog (K_i = 26.1 nM vs 21 nM) [15]. We hypothesize that the anticonvulsant activity of NPY-BBB2 is mediated by targeting Y2 receptor subtypes expressed in hippocampus [1], but more *in vivo* pharmacological studies are needed to prove this hypothesis. In addition, we acknowledge that further receptor binding and functional studies are required to evaluate the subtype selectivity and agonist properties of NPY-BBB2. Without more detailed receptor studies, it is also difficult to reconcile the differences in anticonvulsant activity between NPY-BBB1 and NPY-BBB2. Our work encourages further engineering of systemically active NPY analogs not only as antiepileptic drugs, but also as analgesics and as drug leads for other indications. We are currently evaluating NPY analogs in analgesic models. It is possible to modify NPY analogs toward subtype selectivity for Y1 receptor by either point mutations, such as Q34P [12] or lactamization [15]. Such modifications may result in peptide-based leads for regulating food uptake, blood pressure, or anxiety [12,44].

Our efforts to generate systemically active analogs of GAL, NPY, and NT peptide analogs with anticonvulsant properties encourages the extension of the lipidization–cationization strategy for delivery of other neuropeptides into the brain and/or spinal cord. Table 4 summarizes examples of neuropeptides that are attractive targets for improving their BBB-penetration. Available SAR results facilitate the rational design of such BBB-permeable analogs by choosing optimal positions outside the active fragments for the introduction of the lipidization–cationization motif. Typically, either the *N*- or *C*-terminus in a given neuropeptide has been observed to be most amenable for modification. Based on the previous SAR studies, *N*-terminal modifications of NPFF or bombesin with oligo-Lys and lipoamino acids may improve their systemic bioavailabilities [45,46], whereas these modifications may be more effective at the *C*-terminus of orphanin and ghrelin [47–49]. Although our strategy of improving bioavailability of anticonvulsant neuropeptides (GAL, NT, and NPY) described here and in our previous studies requires further validation, nonetheless it offers an attractive alternative to the challenging development of systemically bioavailable non-peptidic agonists that are receptor-specific and subtype-selective [50]. Such BBB-permeable analogs could not only be useful pharmacological tools but perhaps could even offer first-in-class compounds to validate therapeutic targets in human clinical trials.

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Table 4. Selected examples of neuroactive peptides that may be modified with either *N*- or *C*-terminal lipidization–cationization motif for improved metabolic stability and bioavailability

Peptide	Structure	Target receptor(s)	Potential application	Literature reference
NPFF	FLFQPQRF#	Neuropeptide FF receptor (NPFFR1) agonist	Analgesia	45
Bombesin	ZQRLGNQWAVGHLM#	Bombesin receptor subtype 3 (BRS-3) agonist	Regulation of food intake; anxiolytic drug	46
Orphanin	FGGFTGARKSARKLANQ^	Nociceptin (NOP) receptor agonist	Regulation of food intake: anxiolytic drug; nociception	49
Ghrelin	GSS* FLSPEHQRVQQRKESKKPPAKLQPR^	Growth hormone secretagog receptor (GHS-R) agonist	Metabolic disorders	48

Selected neuroactive peptides that exert agonist activities through their respective receptors in the CNS. Underlined amino acid residues denote the biologically active fragment of each compound. Modified peptide analogs would retain this fragment while conjugating oligo-lysine and lipoamino acid motifs. (Z) represents pyroglutamic acid, (S*) denotes Ser(*O*-CO-C_7H_{15}) residue, (#) denotes an amidated *C*-terminus, and (^) represents carboxylation at the *C*-terminus.

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