CHEMMEDCHEM

DOI: 10.1002/cmdc.200800421

Glycosylated Neurotensin Analogues Exhibit Sub-picomolar Anticonvulsant Potency in a Pharmacoresistant Model of Epilepsy

Hee-Kyoung Lee,[a] Liuyin Zhang,[a] Misty D. Smith,[b] H. Steve White,[b] and Grzegorz Bulaj*[a]

Neurotensin (NT) is an endogenous neuropeptide involved in a variety of central and peripheral neuromodulatory effects. Herein we show the effects of site-specific glycosylation on the in vitro and in vivo pharmacological properties of this neuropeptide. NT analogues containing O-linked disaccharides (β-melibiose and α-TF antigen) or β-lactose units linked by a PEG_3 spacer were designed and chemically synthesized using Fmoc chemistry. For the latter analogue, Fmoc-Glu-(β-Lac-PEG_3-amide) was prepared. Our results indicate that the addition of the disaccharides does not negatively affect the sub-nanomolar affinity or the low-nanomolar agonist potency for the neurotensin receptor subtype 1 (NTS1). Interestingly, three glycosylated analogues exhibited sub-picomolar potency in the 6 Hz limbic seizure mouse model of pharmacoresistant epilepsy following intracerebroventricular administration. Our results suggest for the first time that chemically modified NT analogues may lead to novel antiepileptic therapies.

Introduction

Neurotensin (NT) is a tridecapeptide (ZLYENKPRRPYIL, in which Z = pyroglutamate) involved in a variety of central and peripheral neuromodulatory effects.[1–4] NT and its analogues exhibit potent antinociceptive activity[5,6] and may also play a major role in the pathophysiology of several brain diseases.[7] As recently reviewed by Boules et al.,[8] metabolically stable NT analogues that penetrate the blood–brain barrier (BBB) could be used as effective therapeutics for the treatment of pain, schizophrenia, or other neurological and psychiatric diseases. Various approaches have been undertaken to improve the bioavailability and pharmacological properties of NT, including truncations,[9] cyclization,[10–12] backbone modification,[13–15] substitution of individual amino acids with unnatural residues,[16,17] and mimetics.[18] The efforts of several research groups, including those of Richelson[18–21] and Dix,[17,22–24] resulted in a number of BBB-permeable NT analogues. These analogues exhibit potent antinociceptive activity following systemic administration. Furthermore, they have been tested as potential antipsychotic drugs.[19–21]

Glycosylation of neuroactive peptides is a promising strategy in the development of new therapies for neurological and psychiatric disorders.[25] For example, glycosylation has proven useful for enhancing the bioavailability of opioid peptides. In this regard, improved analgesia has been reported for glycosylated deltorphin,[26] a cyclized Met-enkephalin analogue,[27] and linear Leu-enkephalin analogues.[28] A glycosylated NT analogue found in the venoms of cone snails, contulakin-G, was recently shown to posses potent analgesic activity.[29] When delivered intrathecally, contulakin-G produced pronounced antinociceptive effects in rat models of inflammatory pain.[30] Two additional examples of naturally occurring glycosylation of neuropeptides are vespulakinin (a bradykinin analogue) from wasps[31,32] and somatostatin from catfish.[33] Interestingly, the glycosylated derivatives of both neuropeptides exhibit equipotent or better bioactivity relative to carbohydrate-free analogues. In the present study, we investigated the effects of site-specific glycosylation on the pharmacological properties of NT. Our results suggest that the glycosylated analogues exhibit low-nanomolar affinities and agonist activities for neurotensin receptor subtype 1 (NTS1), and suppress seizures with sub-picomolar potency in the pharmacoresistant model of epilepsy following intracerebroventricular (icv) injections.

Results

Design of glycosylated neurotensin analogues

To investigate how glycosylation affects the pharmacological properties of NT, we designed several analogues containing O-linked sugar moieties (α-D-Gal-(1→6)-β-D-Glc (β-melibiose) and β-D-Gal-(1→3)-α-D-GalNAc (α-Thomsen–Freidenreich antigen, α-TF)) attached to position 7 of NT (Table 1). Mel-S is the β-melibiose–Ser conjugate, TF-T is the α-TF–Thr conjugate, and Lac-PEG_3-E is the Lac-PEG_3–Glu conjugate. The structures of glycoamino acids introduced onto NT analogues are shown in Figure 1. Position 7 was selected specifically because the naturally occurring glycosylated NT analogue, contulakin-G, has a β-D-Gal-(1→3)-α-D-GalNAc-(1→)(α-TF) attached to Thr 10 (boldface) in the following sequence: ZSEEGGSNA**T**KKPYIL.[30] Therefore, Thr 10 in contulakin-G is equivalent to Pro 7 in NT with re-

[a] *H.-K. Lee, L. Zhang, Prof. G. Bulaj*
Department of Medicinal Chemistry, University of Utah
421 Wakara Way, Suite 360, Salt Lake City, UT 84112 (USA)
Fax: (+1) 801-581-7081
E-mail: bulaj@pharm.utah.edu

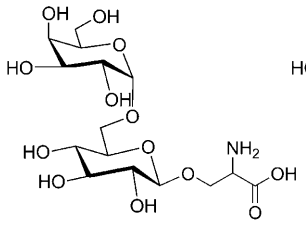
[b] *M. D. Smith, Prof. H. S. White*
Department of Pharmacology and Toxicology, University of Utah
417 Wakara Way, Suite 3211, Salt Lake City, UT 84112 (USA)

Table 1. Summary of MS and HPLC analysis of the NT analogues studied herein.

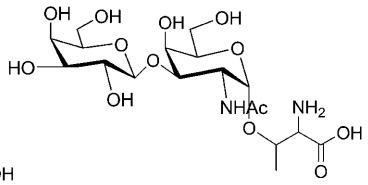
Analogue	Sequence	$Mass_{calcd}$ [Da]	$Mass_{exptl}$ [Da]	t_R [min][a]	Purity [%][a]
Neurotensin	ZLYENKPRRPYIL-OH	1671.91	1672.90	16.6	99.6
NT-Mel	ZLYENK(Mel-S)RRPYIL-OH	1985.99	1986.94	16.7	98.0
NT-TF	ZLYENK(TF-T)RRPYIL-OH	2041.04	2042.28	16.3	99.9
NT(8–13)	H-RRPYIL-OH	816.50	817.53	13.5	98.5
NT(8–13)-Mel	H-(Mel-S)RRPYIL-OH	1227.63	1228.71	14.8	98.0
NT(8–13)-PEG_3-Lac	H-(Lac-PEG_3-E)RRPYIL-OH	1400.74	1401.77	15.3	99.9

[a] HPLC was carried out with a linear gradient (5→65%) of buffer B (90% CH_3CN and 0.1% TFA in H_2O) for 30 min.

β-Melibiose-Ser (Mel-S)



α-Thomsen-Freidenreich antigen-Thr (TF-T)



β-Lactose-PEG_3-Glu (Lac-PEG_3-E)

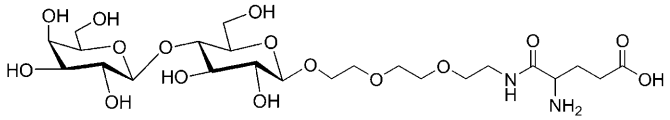
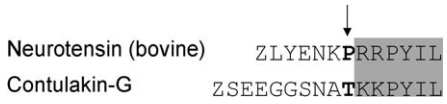


Figure 1. The structures of glycoamino acids introduced to NT and the C-terminal hexapeptide, NT(8–13).

spect to the active fragment -RRPYIL (shaded sequence), as shown below:

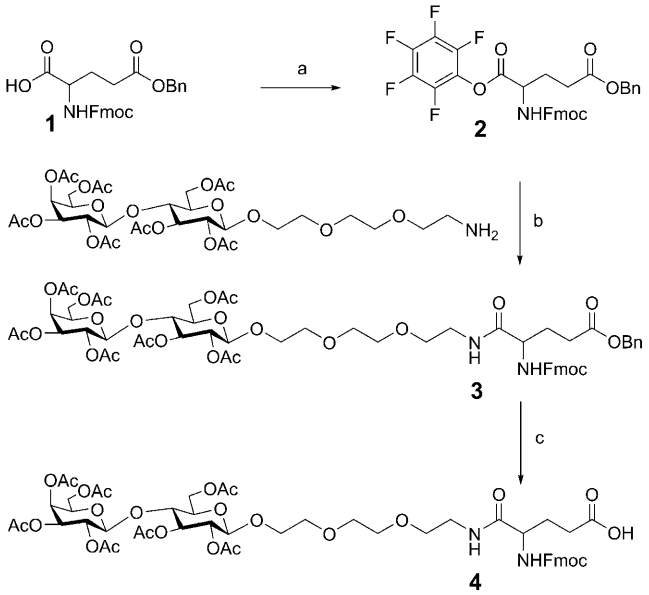


One of the sugar moieties, α-D-Gal-(1→6)-β-D-Glc (β-melibiose), was chosen based on extensive studies of glycosylated enkephalin analogues.[34] The enkephalin-based glycopeptides were shown to increase bioavailability to the central nervous system (CNS). When administered intravenously (iv), the glycosylated enkephalin containing melibiose was 20-fold more potent as an analgesic than the nonglycosylated parent peptide. The other sugar moiety, β-D-Gal-(1→3)-α-D-GalNAc (α-TF), was chosen based on the structure of contulakin-G.[30] The full-length NT analogues, NT-Mel and NT-TF, had identical amino acid sequences except for the different glycoamino acid residue (Table 1).

The C-terminal hexapeptide, NT(8–13), retained the biological activity of NT, including the affinity and agonist potency for NT receptors.[35,36] Therefore, we introduced the glycoamino acid Mel-S to the N terminus of NT(8–13). Once we discovered that all NT analogues containing 'natural' glycoamino acids were very potent in the receptor binding assay, we also explored how 'unnatural' glycosylation would affect the pharmacological properties of NT. To this end, we designed an analogue of NT(8–13) containing an 'extended' glycoamino acid: a β-lactose unit linked by a PEG_3 spacer (Figure 1). This particular glycoamino acid was chosen based on the commercial availability of the synthetic intermediate. The structures of all NT analogues studied herein are summarized in Table 1.

Synthesis of glycosylated neurotensin analogues

Fmoc-Ser(β-Mel-Ac_7)-OH and Fmoc-Thr(α-TF-Ac_6)-OH were obtained from a commercial source for the preparation of NT-Mel, NT(8–13)-Mel, and NT-TF. For the preparation of NT(8–13)-PEG_3-Lac, Fmoc-Glu-(β-Lac-PEG_3-amide) **4** was synthesized as shown in Scheme 1. First, Fmoc-Glu(OBn)-OH **1** was activated



Scheme 1. Synthesis of Fmoc-Glu-(β-Lac-PEG_3-amide) **4**, the intermediate compound for the preparation of NT(8–13)-PEG_3-Lac. a) PFP, DCC, CH_2Cl_2, room temperature, 72%; b) DIPEA, CH_2Cl_2, 57%; c) H_2, Pd/C (10%), EtOAc, 70%.

with pentafluorophenol (PFP) in the presence of 1,3-dicyclohexylcarbodiimide (DCC) to generate Fmoc-Glu(OBn)-OPFP, **2**. The acylation reaction between **2** and β-Lac-PEG_3-amine in dichloromethane and *N*,*N*-diisopropylethylamine (DIPEA) generated Fmoc-Glu(OBn)-(β-Lac-PEG_3-amide), **3**. Debenzylation of **3** with 10% palladium on carbon in ethyl acetate under hydrogen (1 atm) produced the final product, Fmoc-Glu-(β-Lac-PEG_3-

amide) **4**. Degradation of the Fmoc group was found to be minimal under the hydrogenation conditions, and the minor impurity was removed easily in a single chromatographic purification step. The overall yield (70%) of hydrogenation was sufficient to get the final product, **4**.

For all NT analogues, excess Fmoc-protected and peracetylated glycoamino acids (~25–50 μmol) were incorporated manually. The peptides were synthesized by using preloaded Fmoc-Wang resin, and PyBop™ was used as a coupling reagent in the presence of DIPEA. The Fmoc group was removed in the final synthesis step. After peptide synthesis, NT analogs were cleaved from the resin by incubation with reagent K (trifluoroacetic acid/water/ethanedithiol/phenol/thioanisole) for 2 h, precipitated with methyl *tert*-butyl ether (MTBE) at −20 °C for 30 min, and purified by reversed-phase (RP) HPLC. After the purification of peracetylated NT analogues was complete, deacetylation reactions were performed in a solution of sodium methoxide (50 mM in methanol) for 2 h. The progress of deacetylation was monitored by RP-HPLC. The change in HPLC retention time for the representative NT analogue, NT-TF, is shown in Figure 2. The chemical identity of the final products was confirmed by MALDI-TOF mass spectrometry (Table 1).

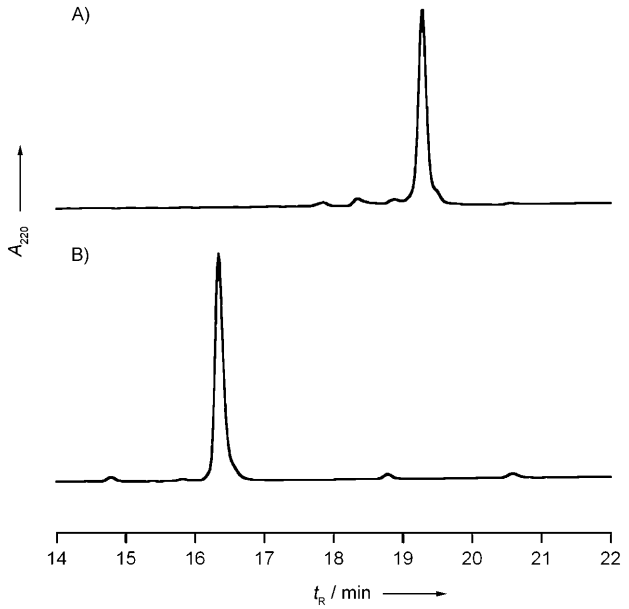


Figure 2. HPLC profile of NT-TF A) before deacetylation and B) after deacetylation. The retention time (t_R) changed from 19.5 to 16.3 min during the deacetylation reaction. The analogues were purified by RP-HPLC using a semi-preparative C_{18} column in a linear gradient (5→65%) of buffer B (90% CH_3CN, 0.1% TFA in H_2O). The molecular weights of the analogues were confirmed by MALDI-TOF mass spectrometry.

Receptor binding and agonist activities

The binding affinity of NT analogues for the human recombinant NTS1 receptor was tested with europium-labeled NT (Eu-NT) using membrane preparations derived from HEK-293T cells. The displacement of Eu-NT fluorescence was detected as a function of the concentration of NT analogues (Figure 3A). Competition binding curves were analyzed using the sigmoidal dose–response (variable slope) classical equation for nonlinear

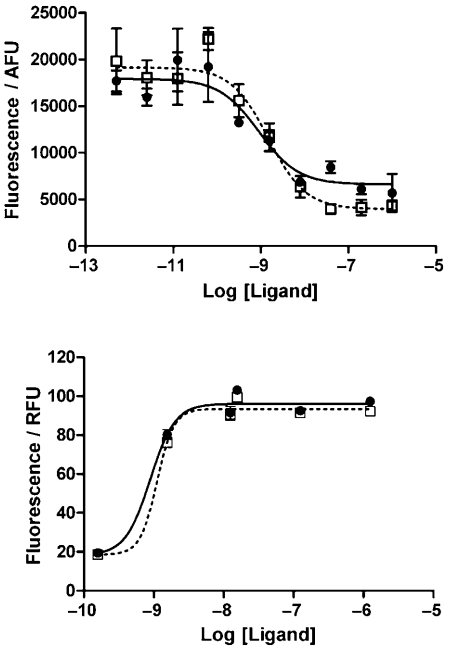


Figure 3. A) Receptor binding curve for NT analogues and B) the agonist activity from functional assays. The binding affinity of NT (-----□-----) and NT-TF (——●——) for NTS1 remains the same: 0.5 nM. The EC_{50} values determined by intracellular Ca^{2+} mobilization assays for NT and NT-TF are 1.1 and 0.9 nM, respectively.

regression analysis, yielding K_i values reported in Table 2. Neither glycosylation nor truncation affected the binding affinity for NTS1. All analogues studied retained high affinity for the receptor, similar to that measured for unmodified NT. Even the truncated NT analogue containing the unnatural glycoamino acid (NT(8–13)-PEG_3-Lac) displayed a sub-nanomolar K_i value, further underscoring the critical role of the C-terminal hexapeptide motif in receptor recognition.

Table 2. Receptor binding (K_i) and agonist activity (EC_{50}) toward NTS1, and anticonvulsant activity (ED_{50}) of NT and NT analogues.

Analogue	K_i [nM]	EC_{50} [nM][a]	ED_{50} [nmol] (icv)[b]
Neurotensin	0.5 ± 0.2	1.1	0.0010
NT-Mel	1.0 ± 0.3	ND	0.0004
NT-TF	0.5 ± 0.1	0.9	0.0004
NT(8–13)	0.2 ± 0.1	ND	0.0011
NT(8–13)-Mel	0.4 ± 0.1	ND	0.0004
NT(8–13)-PEG_3-Lac	0.3 ± 0.1	ND	< 0.0010

[a] ND = not determined. [b] In a 6 Hz mouse model of epilepsy.

The ability of a selected NT analogue to stimulate intracellular Ca^{2+} mobilization was tested in HEK-293T cells expressing human NTS1 (Figure 3B). The NT-TF analogue retained full po-

tency as an NTS1 agonist, indicating that the presence of glycoamino acids does not affect the binding mechanism of the peptide to the receptors (Table 2). The activation of intracellular Ca^{2+} mobilization was concentration dependent, with EC_{50} values of 1.1 and 0.9 nM for NT and NT-TF, respectively. The EC_{50} values for stimulation of intracellular Ca^{2+} release confirmed the similar binding affinities of NT and NT-TF.

Anticonvulsant activity

To investigate the in vivo activity of the NT analogues, we tested their ability to suppress seizures in an animal model of epilepsy. This assay was chosen based on a preliminary report that the glycosylated NT analogue contulakin-G possesses potent anticonvulsant activity in the Frings mouse model of epilepsy (USPTO; patent 6,696,408). All analogues were evaluated in the mouse 6 Hz partial psychomotor seizure model following icv injections (Figure 4). Animals were considered protected if they did not display a motor seizure characterized by vibrissae twitching, jaw chomping, or forelimb clonus. The median effective dose (ED_{50}) calculated from dose–response curves are reported in Table 2. The ED_{50} values for all the analogues were found to be in the picomolar range. Notably, three analogues, NT-Mel, NT-TF, and NT(8–13)-Mel, exhibited sub-picomolar potency. The glycosylated NT analogues were not found to be anticonvulsant following systemic administration (intraperitoneally, ip) at doses as high as 10 mg kg^{-1}, suggesting that the glycosylation does not improve CNS bioavailability for these peptides.

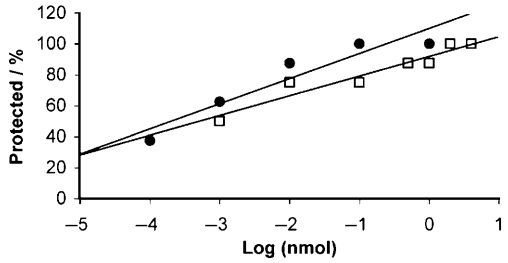


Figure 4. Dose–response curves of NT (□) and a representative glycosylated NT analogue, NT-TF (●), in the 6 Hz (32 mA) anticonvulsant assay in mice following icv administration. The log values of dose were plotted against the percentage of mice protected from seizures. The ED_{50} values for NT and NT-TF were 1.0 pmol and 0.4 pmol, respectively.

Discussion

Herein we describe how 'nature-inspired' glycosylation of NT analogues affects their pharmacological properties. We present a novel design strategy, solid-phase chemical synthesis of several glycopeptide analogues, including synthesis of an unnatural glycoamino acid, as well as an in vitro pharmacological characterization of the resulting analogues (receptor binding and agonist activities), and in vivo pharmacology in a mouse model of pharmacoresistant epilepsy. Our design strategy of the glycosylated analogues is unique, as it is based on the study of peptide-based marine natural products and available SAR data on NT analogues. Despite numerous studies on the engineering of glycosylated neuropeptides,[25,37] to the best of our knowledge, this is the first comprehensive study of applying natural-product-inspired posttranslational modifications to bioactive fragments of neuropeptides.

NT analogues containing O-linked sugar moieties (α-D-Gal-(1→6)-β-D-Glc and β-D-Gal-(1→3)-α-D-GalNAc) or β-lactose units linked to the peptide by a PEG_3 spacer had comparable receptor binding properties, suggesting that the relatively bulky modification does not interfere with peptide–receptor interactions. Even the presence of an unnatural glycoamino acid, β-lactose linked through a short PEG spacer, does not change receptor binding affinity. Furthermore, glycosylation does not affect the agonist activity of the analogues. Our findings are also different from those reported for contulakin-G and its deglycosylated analogue, [Thr 10]contulakin-G,[30] as the disaccharide-free analogue has >40-fold higher affinity, as well as >30-fold higher agonist potency, for human NTS1. The apparent differences of how glycosylation affects the receptor affinities and agonist potencies of NT (this work) and contulakin-G[30] suggest that these two peptides may have different binding mechanisms at NT receptors; perhaps the shorter length of NT can better accommodate the sugar moiety in complex with the target receptor. These results are encouraging for further explorations of neo-glycopeptide NT analogues as a strategy to improve bioavailability.

Structure–activity studies of NT showed that its C-terminal hexapeptide NT(8–13) is equipotent to, or even more potent than, native NT in binding to NT receptor (membrane preparations from human frontal cortex) in radioligand binding assays, while maintaining the same biological and pharmacological properties.[9] Our results obtained from the fluorescence-based binding assays are consistent with previous studies, with K_i values of 0.5 ± 0.2 and 0.2 ± 0.1 nM for NT and NT(8–13), respectively. Both NT and NT(8–13) display equipotent anticonvulsant ED_{50} values (i.e., 0.0010 nmol, icv). As the glycosylated C-terminal hexapeptide also maintains the same activity as NT in vitro and in vivo, this finding supports the critical role of the RRPYIL motif. The apparent correlation between receptor affinity, agonist potency, and anticonvulsant activity suggests that the suppression of seizures might be explained by targeting NT receptors.

Perhaps the most important finding from our study is that NT and the glycosylated analogues exhibit picomolar, or even sub-picomolar, anticonvulsant potencies in the 6 Hz model of epilepsy. Although previous studies reported correlations between NT and seizures,[38,39] there is only one reported example of the anticonvulsant activity of an NT analogue (contulakin-G) determined in the Frings mouse model of epilepsy (USPTO; patent 6,696,408). Although the hypothermic activity of NT may partially account for some of its anticonvulsant activity, this neuropeptide was recently shown to potentiate GABAergic activity in the rat hippocampus CA1 region.[40] Because NT is known to exert hypothermia, the anticonvulsant activity observed in the present study may contribute to the anticonvulsant action of the modified NT analogues described herein;

however, we did not observe a direct correlation between changes in body temperature and the percentage of animals protected in the seizure test. The mechanism of NT-mediated anticonvulsant activity remains unknown, and requires further pharmacological studies.

The immediate impact of the discovery that NT and its glycosylated analogues exhibit high potencies in suppressing seizures is a new research direction for exploring BBB-permeable NT analogues as potential first-in-class antiepileptic drugs. The unexpected anticonvulsant role of NT in the brain will trigger more mechanistic studies to be carried out by neuropharmacologists. Furthermore, many research groups in both the academic and industrial spheres have been working for two decades on NT analogues with improved bioavailability.[1–21] As a result, a number of CNS-active lead compounds derived from NT analogue libraries have been identified.[8,16] We hope that this work will encourage these research groups to test their BBB-permeable NT agonists in various models of epilepsy (for example, by using the NIH-sponsored Anticonvulsant Screening Program).

Experimental Section

General: Chemicals were obtained from Aldrich Chemical and were used without prior purification. β-Lac-PEG_3-amine, Fmoc-Ser(β-Mel-Ac_7)-OH, and Fmoc-Thr(α-TF-Ac_6)-OH were purchased from Sussex Research Laboratories Inc. Fmoc-protected amino acids and preloaded Fmoc-Leu-Wang resins were obtained from Chem-Impex International Inc. Reactions were performed under N_2 atmosphere, unless otherwise indicated. Chromatography refers to flash chromatography on silica gel (Whatman 230–400 mesh ASTM silica gel). Analytical thin-layer chromatography (TLC) was performed using Whatman glass plates coated with silica gel (0.25 mm thickness) containing PF_{254} indicator. NMR spectra were recorded at 400 MHz (^{1}H), 100 MHz (^{13}C), and 376 MHz (^{19}F) at 25 °C. Proton and carbon chemical shifts are given in ppm relative to tetramethylsilane (TMS) as the internal standard; external standards were used for ^{19}F ($CFCl_3$, $\delta = 0.00$ ppm). MALDI-TOF MS data were collected at the University of Utah Core Facility. Optical rotations were measured on a polarimeter (PerkinElmer, model 343) using a quartz cell with a path length of 10 cm.

Fmoc-Glu(OBn)-OPFP, 2: DCC (0.246 g, 1.19 mmol) was added to a solution of Fmoc-Glu(OBn)-OH (0.459 g, 1 mmol) and PFP (0.190 g, 1.03 mmol) in CH_2Cl_2 (5 mL) at 0 °C and then stirred at room temperature for 16 h, filtered, washed with $NaHCO_3$ solution, and purified by chromatography (hexane/EtOAc 3:1) to afford **2** (0.45 g, 72%) as white solid: $R_f = 0.25$ (CH_2Cl_2/CH_3OH, 7:1); mp: 144.0–146.0 °C; $[\alpha]_D^{20} = -13.0$ ($c = 3.8$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.66$ (d, $J = 7.2$ Hz, 2H), 7.49 (m, 2H), 7.18–7.30 (m, 9H), 5.51 (d, $J = 8.4$ Hz, 1H), 5.06 (s, 2H), 4.70 (m, 1H), 4.35 (m, 2H), 4.12 (t, $J = 6.8$ Hz, 2H), 2.46 (m, 2H), 2.34(m, 1H), 2.10 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.60$, 168.62, 156.126, 143.95, 143.77, 141.56, 135.75, 128.86, 128.67, 128.57, 128.02, 127.34, 125.26, 120.26, 67.54, 67.08, 53.55, 47.32, 30.31, 27.27 ppm; ^{19}F NMR (376 MHz, $CDCl_3$): $\delta = -152.55$ (d, $J = 15.6$ Hz, 2F), -157.33 (t, $J = 24.4$, 22.8 Hz, 1F), -162.06 ppm (t, $J = 24.4$, 22.8 Hz, 2F); HRMS (MALDI) m/z $[M+Na]^+$ calcd for $C_{33}H_{24}F_5NO_6Na$: 648.1416, found: 648.1422.

Fmoc-Glu(OBn)-(β-Lac-PEG_3-amide), 3: DIPEA (89.5 μL, 0.514 mmol) was added to a stirred solution of **2** (0.236 g, 0.514 mmol) and β-Lac-PEG_3-amine (0.329 g, 0.428 mmol) in CH_2Cl_2 (10 mL). After stirring for 16 h, the reaction mixture was concentrated and purified by chromatography (hexane/EtOAc 1:1) to afford **3** (0.296 g, 57%) as a white amorphous solid: $R_f = 0.41$ (EtOAc); $[\alpha]_D^{20} = -4.6$ ($c = 0.5$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.70$ (d, $J = 7.2$ Hz, 2H), 7.53 (d, $J = 6.8$ Hz, 2H), 7.24–7.34 (m, 9H), 6.67 (m, 1H), 5.64 (d, $J = 7.2$ Hz, 1H), 5.24 (d, 2H), 5.13–5.00 (m, 4H), 4.85 (m, 2H), 4.46–4.12 (m, 6H), 4.01 (m, 4H), 3.85 (m, 1H), 3.72 (m, 2H), 3.60 (m, 1H), 3.52–3.30 (m, 10H), 2.49–2.35 (m, 2H), 1.82–2.07 ppm (m, 23H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 173.24, 171.34, 170.66, 170.55, 170.40, 170.30, 170.06, 169.35, 158.91, 143.95, 141.49, 138.97, 135.39, 128.82, 128.55, 128.47, 127.99, 126.73, 125.34, 120.26, 101.34, 100.89, 76.51, 72.92, 71.83, 71.19, 70.82, 70.72, 70.54, 70.36, 69.75, 69.47, 69.29, 67.27, 66.74, 64.60, 62.27, 60.96, 54.26, 47.33, 39.61, 30.85, 30.52, 29.93, 28.73, 21.08, 21.02, 20.97, 20.85, 20.75, 19.34 ppm; HRMS (MALDI) m/z $[M+Na]^+$ calcd for $C_{59}H_{72}N_2O_{25}Na$: 1231.4316, found: 1231.4284.

Fmoc-Glu-(β-Lac-PEG_3-amide), 4: A solution of **3** (0.270 g, 0.24 mmol) and 10% Pd/C (54 mg) in EtOAc (10 mL) was stirred under H_2 (1 atm) for 48 h. The mixture was then filtered through Celite, and the solvent was evaporated under reduced pressure. Chromatography (EtOAc/MeOH 3:1) gave **4** (0.202 g, 70%) as a clear oil: $R_f = 0.52$ (EtOAc/MeOH 3:1); $[\alpha]_D^{20} = -3.6$ ($c = 1.0$ in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.68$ (d, $J = 7.2$ Hz, 2H), 7.50 (m, 2H), 7.29–7.33 (t, $J = 6.8$ Hz, 2H), 7.22 (d, $J = 4.4$ Hz, 2H), 6.99 (br, 1H), 5.99 (br, 1H), 5.24 (s, 1H), 5.11 (t, $J = 9.2$ Hz, 1H), 5.01 (t, $J = 8.4$, 9.6 Hz, 1H), 4.80–4.89 (m, 2H), 4.45–4.37 (m, 3H), 4.28 (m, 3H), 4.12 (m, 1H), 4.05–3.94 (m, 3H), 3.81 (m, 1H), 3.76–3.73 (m, 2H), 3.64–3.34 (m, 12H), 2.37 (m, 2H), 1.89–2.07 ppm (m, 23H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.79$, 170.64, 170.39, 170.32, 170.20, 170.12, 169.39, 144.04, 143.95, 141.44, 127.98, 127.35, 125.37, 120.22, 101.26, 100.84, 76.43, 72.89, 71.83, 71.18, 70.77, 70.69, 70.31, 69.67, 69.40, 69.32, 67.35, 66.82, 64.58, 62.22, 60.98, 54.26, 47.25, 39.56, 30.83, 29.89, 21.23, 21.07, 21.00, 20.94, 20.83, 20.73, 19.34 ppm; HRMS (MALDI) m/z $[M+Na]^+$: calcd for $C_{52}H_{66}N_2O_{25}Na$: 1141.3847, found: 1141.3832.

Peptide synthesis, purification, and characterization: Peptides were synthesized on a Symphony automatic peptide synthesizer (Protein Technologies Inc.) using Fmoc SPPS methods on a 25 μmol scale. Preloaded Fmoc-Leu-Wang resin was used, with PyBop™ as a coupling reagent and piperidine (20%) as a deprotection reagent. Unnatural amino acids were coupled manually, and the Fmoc group was removed during the final step. After synthesis, peptides were cleaved from the resin by incubating in reagent K (TFA/H_2O/ethanedithiol/phenol/thioanisole, 90:5:2.5:7.5:5 *v/v*) for 2 h, then precipitated with MTBE (6 mL) at −20 °C for 30 min and purified by RP-HPLC. After the purification of peracetylated NT analogues was complete, they were hydrolyzed in NaOMe (50 mM in MeOH) for 2 h to remove the acetyl group, and purified by RP-HPLC using a C_{18} semipreparative column (Vydac, 4.6 mm × 250 mm) under a linear gradient (5→65%) of buffer B (90% CH_3CN and 0.1% TFA in H_2O). The flow rate was 5 mL min^{-1}, and elution was monitored by UV detection at 220 nm. Prepared peptides were quantified by measuring UV absorbance at 274.6 nm ($\varepsilon =$ 1420.2 M^{-1} cm^{-1}). The molar masses of peptides were determined by MALDI-TOF mass spectrometry.

Receptor binding assay: Competitive binding assays were performed on membrane preparations using Eu-NT (both from PerkinElmer), and the samples were tested in quadruplicate. Membrane preparations, Eu-NT, and ligands were diluted in binding buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 μM EDTA, 0.2% BSA). Samples were incubated at room temperature for 90 min in a total

volume of 200 μL. Following incubation, samples were washed four times with wash buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$). Enhancement solution (200 μL) was added, and the plates were incubated at room temperature for 30 min. The plates were read on a Wallace VICTOR^3 instrument using the standard Eu-TRF measurement (λ_{ex} = 340 nm, 400 μs decay, and emission collection (λ_{em} = 615 nm) for 400 μs). Competition curves were analyzed with GraphPad Prism using the sigmoidal dose–response (variable slope) classical equation for nonlinear regression analysis.

Agonist activity: HEK-293T cells transiently co-transfected with NTS1 were plated in 96-well plates, and grown to confluence. After incubation with Fluo-3/Am, cells were washed with HBS (10 mM HEPES pH 7.4, 150 mM NaCl) and equilibrated for 20 min. The fluorescence emission due to intracellular Ca^{2+} mobilization elicited by agonists of the expressed receptor was determined with a fluorescence imaging plate reader (FLIPR^{TM}, Molecular Devices Corporation). The results were analyzed using SOFTmax Pro and GraphPad Prism software. This assay was performed by Millipore Inc.

Anticonvulsant assay: Analogues were tested in the 6 Hz partial psychomotor seizure model following icv administration (total volume, 5 μL) to CF-1 male adult mice (Charles River). Mice were dosed with the analogues (icv), and 30 min later, animals were challenged with corneal stimulation (6 Hz, 32 mA, 3 s). Animals were considered protected if they did not display a motor seizure characterized by vibrissae twitching, jaw chomping, or forelimb clonus. The fitted curves were analyzed with GraphPad Prism using the dose–response classical equation for nonlinear regression analysis. ED_{50} values were determined based on 50% protection. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Acknowledgements

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 Anticonvulsant Screening Program (ASP) at the NIH/NINDS. G.B. also acknowledges financial support from NIH grants R21 NS055845 and Program Project GM 48677. We thank the ASP Director, Mr. James Stables, for numerous discussions. G.B. and H.S.W. are scientific co-founders of NeuroAdjuvants, Inc.

Keywords: agonists · anticonvulsants · binding · glycosylation · neurotensin

[1] J.-P. Vincent, J. Mazella, P. Kitabgi, *Trends Pharmacol. Sci.* **1999**, *20*, 302–309.
[2] G. Bissette, C. B. Nemeroff, P. T. Loosen, J. A. J. Prange, M. A. Lipton, *Nature* **1976**, *262*, 607–609.
[3] P. Kitabgi, F. De Nadai, C. Labbe-Jullie, I. Dubuc, D. Nouel, C. J. , Y. Masuo, W. Rostène, J. Woulfe, L. Lafortune, *Clin. Neuropharmacol.* **1992**, *15*, 313 A-314 A.
[4] C. B. Nemeroff, G. Bissette, P. J. Manberg, A. J. Osbahr III, G. R. Breese, A. J. J. Prange, *Brain Res.* **1980**, *195*, 69–84.
[5] N. R. F. Al-Rodhan, E. Richelson, J. A. Gilbert, D. J. McCormick, K. S. Kanba, M. A. Pfenning, A. Nelson, E. W. Larson, T. L. Yaksh, *Brain Res.* **1991**, *557*, 227–235.
[6] P. R. Dobner, *Peptides* **2006**, *27*, 2405–2414.
[7] W. Rostène, A. Brouard, C. Dana, Y. Masuo, F. Agid, M. Vial, A. M. Lhiaubet, D. Pelaprat, *Ann. NY Acad. Sci. USA* **1992**, *668*, 217–231.
[8] M. Boules, P. Fredrickson, E. Richelson, *Peptides* **2006**, *27*, 2523–2533.
[9] K. S. Kanba, S. Kanba, A. Nelson, H. Okazaki, E. Richelson, *J. Neurochem.* **1988**, *50*, 131–137.
[10] P. Bredeloux, F. Cavelier, I. Dubuc, B. Vivet, J. Costentin, J. Martinez, *J. Med. Chem.* **2008**, *51*, 1610–1616.
[11] A. M. Sefler, J. X. He, T. K. Sawyer, K. E. Holub, D. O. Omecinsky, M. D. Reily, V. Thanabal, H. C. Akunne, W. L. Cody, *J. Med. Chem.* **1995**, *38*, 249–257.
[12] H. Bittermann, J. Einsiedel, H. Hübner, P. Gmeiner, *J. Med. Chem.* **2004**, *47*, 5587–5590.
[13] E. García-Garayoa, L. Allemann-Tannahill, P. Bläuenstein, M. Willmann, N. Carrel-Rémy, D. Tourwé, K. Iterbeke, P. Conrath, P. A. Schubiger, *Nucl. Med. Biol.* **2001**, *28*, 75–84.
[14] J. Einsiedel, H. Hübner, M. Hervet, S. Härterich, S. Koschatzky, P. Gmeiner, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 2013–2018.
[15] J. Couder, D. Tourwé, G. Van Binst, J. Schuurkens, J. E. Leysen, *Int. J. Pept. Protein Res.* **1993**, *41*, 181–184.
[16] K. P. Kokko, M. K. Hadden, K. L. Price, K. S. Orwig, R. E. See, T. A. Dix, *Neuropharmacology* **2005**, *48*, 417–425.
[17] J. T. Lundquist IV, T. A. Dix, *J. Med. Chem.* **1999**, *42*, 4914–4918.
[18] F. Hong, J. Zaidi, B. Cusack, E. Richelson, *Bioorg. Med. Chem.* **2002**, *10*, 3849–3858.
[19] B. M. Tyler-McMahon, J. A. Stewart, F. Farinas, D. J. McCormick, E. Richelson, *Eur. J. Pharmacol.* **2000**, *390*, 107–111.
[20] B. Cusack, M. Boules, B. M. Tyler, A. Fauq, D. J. McCormick, E. Richelson, *Brain Res.* **2000**, *856*, 48–54.
[21] B. M. Tyler, C. L. Douglas, A. Fauq, Y. P. Pang, J. A. Stewart, B. Cusack, D. J. McCormick, E. Richelson, *Neuropharmacology* **1999**, *38*, 1027–1034.
[22] K. P. Kokko, M. K. Hadden, K. S. Orwig, J. Mazella, T. A. Dix, *J. Med. Chem.* **2003**, *46*, 4141–4148.
[23] J. T. Lundquist IV, E. E. Büllesbach, T. A. Dix, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 453–455.
[24] J. T. Lundquist IV, T. A. Dix, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2579–2582.
[25] R. Polt, M. Dhanasekaran, C. M. Keyari, *Med. Res. Rev.* **2005**, *25*, 557–585.
[26] L. Negri, R. Lattanzi, F. Tabacco, L. Orrù, C. Severini, B. Scolaro, R. Rocchi, *J. Med. Chem.* **1999**, *42*, 400–404.
[27] R. D. Egleton, S. A. Mitchell, J. D. Huber, J. Janders, D. Stropova, R. Polt, H. I. Yamamura, V. J. Hruby, T. P. Davis, *Brain Res.* **2000**, *881*, 37–46.
[28] R. D. Egleton, S. A. Mitchell, J. D. Huber, M. M. Palian, R. Polt, T. P. Davis, *J. Pharmacol. Exp. Ther.* **2001**, *299*, 967–972.
[29] J. W. Allen, K. Hofer, D. McCumber, J. D. Wagstaff, R. T. Layer, R. T. McCabe, T. L. Yaksh, *Anesth. Analg.* **2007**, *104*, 1505–1513.
[30] A. G. Craig, T. Norberg, D. Griffin, C. Hoeger, M. Akhtar, K. Schmidt, W. Low, J. Dykert, E. Richelson, V. Navarro, J. Mazella, M. Watkins, D. Hillyard, J. Imperial, L. J. Cruz, B. M. Olivera, *J. Biol. Chem.* **1999**, *274*, 13 752–13 759.
[31] H. Yoshida, R. G. Geller, J. J. Pisano, *Biochemistry* **1976**, *15*, 61–64.
[32] M. Gobbo, L. Biondi, F. Filira, B. Scolaro, R. Rocchi, T. Piek, *Int. J. Pept. Protein Res.* **1992**, *40*, 54–61.
[33] L. Chen, K. J. Jensen, J. Tejbrant, J. E. Taylor, B. A. Morgan, G. Barany, *J. Pept. Res.* **2000**, *55*, 81–91.
[34] N. O. Elmagbari, R. D. Egleton, M. M. Palian, J. J. Lowery, W. R. Schmid, P. Davis, E. Navratilova, M. Dhanasekaran, C. M. Keyari, H. I. Yamamura, F. Porreca, V. J. Hruby, R. Polt, E. J. Bilsky, *J. Pharmacol. Exp. Ther.* **2004**, *311*, 290–297.
[35] R. Carraway, S. E. Leeman, in *Peptides: Chemistry, Structure and Biology*, Ann Arbor Science, Ann Arbor, **1975**, pp. 679–685.
[36] P. Kitabgi, C. Poustis, C. Granier, J. Van Rietschoten, J. Rivier, J. L. Morgat, P. Freychet, *Mol. Pharmacol.* **1980**, *18*, 11–19.
[37] S. Elliott, T. Lorenzini, S. Asher, K. Aoki, D. Brankow, L. Buck, L. Busse, D. Chang, J. Fuller, J. Grant, N. Hernday, M. Hokum, S. Hu, A. Knudten, N. Levin, R. Komorowski, F. Martin, R. Navarro, T. Osslund, G. Rogers, N. Rogers, G. Trail, J. Egrie, *Nat. Biotechnol.* **2003**, *21*, 414–421.
[38] G. Sperk, R. Wieser, R. Widmann, E. A. Singer, *Neuroscience* **1986**, *17*, 1117–1126.
[39] A. Shulkes, Q. L. Harris, S. J. Lewis, J. E. Vajda, B. Jarrott, *Neuropeptides* **1988**, *11*, 77–81.
[40] S. Li, J. D. Geiger, S. Lei, *J. Neurophysiol.* **2008**, *99*, 2134–2143.

Received: December 8, 2008
Published online on January 27, 2009

