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Brief Article

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Development of Novel Neurokinin 3 Receptor (NK3R) Selective Agonists with Resistance to Proteolytic Degradation

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Supporting Information

ABSTRACT: Neurokinin B (NKB) regulates the release of gonadotropin-releasing hormone (GnRH) via activation of the neurokinin-3 receptor (NK3R). We evaluated the biological stability of NK3R selective agonists to develop novel NK3R agonists to regulate reproductive functions. On the basis of degradation profiles, several peptidomimetic derivatives were designed. The modification of senktide with (*E*)-alkene dipeptide isostere generated a novel potent NK3R agonist with high stability and prolonged bioactivity.

INTRODUCTION

Neurokinin B (NKB) is one of the mammalian tachykinin peptides and an endogenous ligand for the neurokinin-3 receptor (NK3R). NKB regulates dopamine release as a neurotransmitter in the central nervous system[1,2] and induces contraction of the portal vein, venoconstriction of the mesenteric bed, and an increase in heart rate in the periphery.[3−5] Recently, it has been demonstrated that NKB-NK3R signaling is involved in the central control of reproduction.[6−8] A landmark discovery was the identification of missense mutations in either the *Tac3* or *Tacr3* genes (which encode NKB or NK3R, respectively) that cause severe gonadotropin deficiency in humans.[9] In kisspeptin/NKB/dynorphin A (KNDy) neurons in the hypothalamic arcuate nucleus (ARC), NKB, with kisspeptin and dynorphin A, cooperatively modulates the release of gonadotropin-releasing hormone (GnRH).[10]

The tonic mode of GnRH release is pulsatile, which is driven by the hypothalamic neural substrate, the so-called GnRH pulse generator. Because the GnRH pulse frequency controls baseline levels of circulating gonadotropins, this neural substrate has been thought to play a pivotal role in the hypothalamo-pituitary-gonadal (HPG) axis.[11] In goats, a method that monitors the electrophysiological manifestations of the GnRH pulse generator as periodic bursts of multiple-unit activity (MUA volleys) has been established.[8,12] Using this method, it has been demonstrated that intracerebroventricular (icv) administration of NKB[8] or intravenous injection of an NK3R-selective agonist, senktide,[13] immediately induced the MUA volley, suggesting involvement of NKB-NK3R signaling in GnRH pulse generation.[14]

NKB shares the C-terminal common sequence, −Phe-Xaa-Gly-Leu-Met-NH_2, with other tachykinin peptides including substance P (SP) and neurokinin A (NKA).[15−17] NK3R-selective agonists that have been reported so far include [MePhe[7]]-NKB[18] and senktide (Table 1). These peptides have been employed for a number of in vitro experiments using primary cultures or cell lines.[1,19,20] In vivo studies using NKB or senktide also provided insights regarding regulation of the HPG axis function. However, the reported actions of these NK3R agonists remain controversial. For example, icv treatment with NKB had no effect in male rats,[21] whereas icv administration of senktide to female rats resulted in decreased plasma LH levels.[22] In contrast, another study reported that senktide increased plasma LH levels in female rats.[23] These apparently inconsistent data may be attributable to enzymatic degradation following in vivo administration of the peptides. Indeed, it has been reported that NKB is degraded by neutral endopeptidase (NEP) 24.11 (also known as neprilysin or enkephalinase) at four peptide bonds (e.g., Asp[4]-Phe[5], Phe[5]-Phe[6], Phe[6]-Val[7] and Gly[8]-Leu[9], Table 1).[24] We envisioned that substitution of the degradable peptide bond(s) in NK3R selective agonists with appropriate noncleavable isosteres would confer resistance to enzymatic degradation while maintaining NK3R agonistic activity. This could potentially provide novel therapeutic agents, with prolonged effects, for reproductive disorders of human and domestic animals. This may also prove to be useful research tool(s) to investigate the role of NKB-NK3R pair in HPG axis regulation, addressing a major confounding influence of enzymatic degradation.

Table 1. Sequences of NKB, [MePhe[7]]-NKB, and Senktide

peptide	sequence
neurokinin B (NKB)	H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
[MePhe[7]]-NKB	H-Asp-Met-His-Asp-Phe-Phe-MePhe-Gly-Leu-Met-NH_2
senktide	succinyl-Asp-Phe-MePhe-Gly-Leu-Met-NH_2

In this study, we investigated proteolytic digestion of NKB-selective agonist peptides in animal serum, hypothalamic

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extracts, and by recombinant NEP 24.11. In addition, on the basis of degradation of these peptides, novel peptidomimetic senktide analogues with resistance against proteinase-mediated degradation were designed and synthesized using a series of dipeptide isosteres. In vivo biological activity of the analogues was assessed using the MUA recording method in conscious goats.

RESULTS AND DISCUSSION

Proteolytic Degradation of NK3R-Selective Agonists. Initially, we investigated the proteolytic degradation of [MePhe^7]-NKB in rat, pig, goat, and cattle serum. Treatment of [MePhe^7]-NKB with pig serum provided the fragment peptides **1**−**5** by N-terminus degradation (Figure 1B). Peptides **1**−**5** were similarly produced by treatment with sera derived from the other animal species, although the degradation patterns were different (Supporting Information). Peptides **2**, **3**, and **5** were mainly obtained by serum derived from cattle, pig, and goat, respectively. Fragment **6** was also observed following 24 h treatment with rat serum. The proteolytic degradation was terminated at the Phe-MePhe peptide bond in sera of all animals, and the resistance could be attributed to the *N*-methyl group of MePhe. We also analyzed the proteolytic degradation of [MePhe^7]-NKB in pig hypothalamic extracts, which is the site of action of NKB in the central nervous system (Figure 1C). [MePhe^7]-NKB was immediately degraded by treatment with hypothalamic extract to generate peptides **5**, **6**, and the C-terminal carboxylate **7**. Under these conditions, fragments **1**−**4** were not observed, probably owing to the rapid degradation of [MePhe^7]-NKB.

Next, we investigated the biological activities of fragment peptides **1**−**7**, which were synthesized separately by Fmoc-based solid-phase peptide synthesis (Table 2). Receptor binding was evaluated by competitive binding assay using [^{125}I]-BH-SP for human NK1R, [^{125}I]-NKA for human NK2R, and ([^{125}I]His^3, MePhe^7)-NKB for human NK3R, respectively. The shorter fragments among peptides **1**−**5** exhibited less inhibitory activity for NK3R binding, whereas peptides **6** and **7** did not inhibit NK3R binding. In contrast, slightly improved NK1R binding inhibition was observed for shorter peptide fragments **1**−**5** and moderate inhibitory activity for NK2R. NK3R agonistic activity was assessed by monitoring intracellular Ca^{2+} flux induced by NK3R activation, with peptides **1**−**5** exhibiting similar activity (EC_{50} = 0.091−0.35 nM), which is apparently inconsistent with the structure−activity relationship data obtained by binding assays. This may be because of the heterologous binding competition, in which the IC_{50} values do not always truly reflect competitor binding, in the binding assay.[25−27]

The proteolytic degradation of senktide in serum and pig hypothalamic extracts was investigated (Supporting Information). Senktide was stable in animal sera and pig hypothalamic extracts over 24 h, indicating that N-terminal succinyl capping on senktide prevents degradation by exopeptidases in serum and hypothalamus. We also analyzed senktide degradation by NEP 24.11. In contrast to treatment in serum and hypothalamic extracts, more than 80% of senktide was digested by NEP 24.11 within 24 h (Figure 2). Enzymatic hydrolysis occurred at the Gly^8-Leu^9 bond, which corresponds to the cleavage site of NKB by NEP 24.11.[24] The resulting senktide N-terminal fragment **8** and C-terminal fragment **9** did not exhibit agonistic activity at 100 nM (data not shown), suggesting that NEP 24.11 is able to rapidly deactivate the senktide administered in vivo.

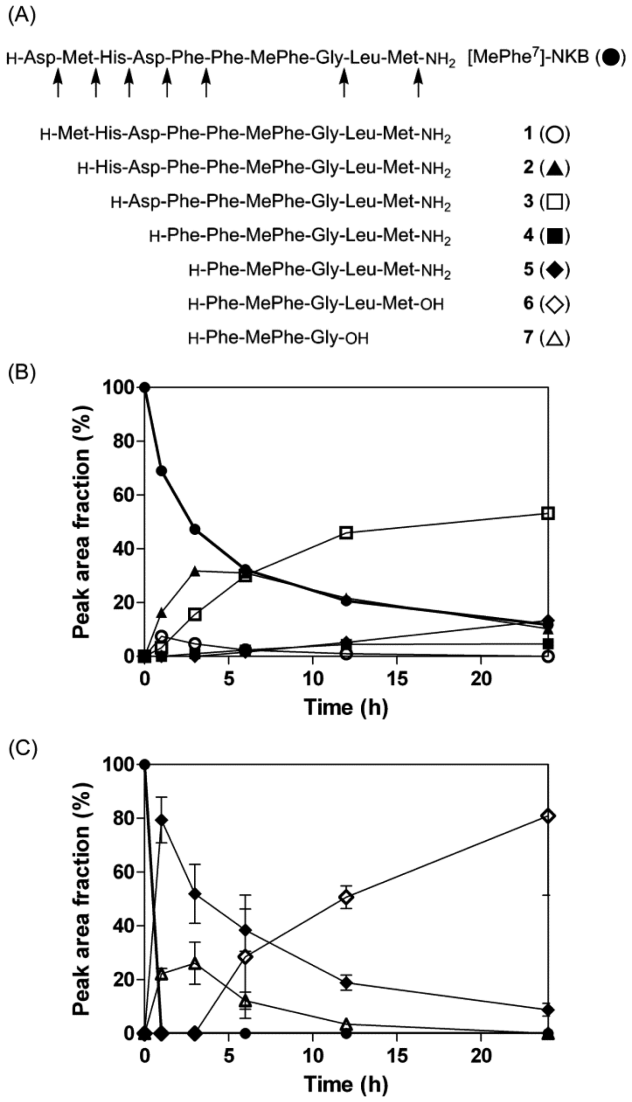

Figure 1. Proteolytic degradation of [MePhe^7]-NKB in serum or hypothalamic extracts. (A) Cleavage sites of [MePhe^7]-NKB in animal sera or pig hypothalamic extracts and the sequences of the digested peptides. The degradation profiles of [MePhe^7]-NKB following treatment with (B) pig serum, (C) pig hypothalamic extracts [MePhe^7]-NKB (closed circles), **1** (opened circles), **2** (closed triangles), **3** (opened squares), **4** (closed squares), **5** (closed diamonds), **6** (opened diamonds), and **7** (opened triangles). [MePhe^7]-NKB was incubated in serum or hypothalamic extracts at 37 °C. The sample was analyzed by HPLC (detection at 220 nm). Data represent the mean ± SD (n = 3).

We turned our attention to development of novel senktide derivatives with an increased enzymatic stability. An approach that has been explored to provide resistance against enzymatic degradation while maintaining NK3R agonistic activity includes the use of an appropriate dipeptide isostere at the cleavable Gly-Leu dipeptide moiety. Because the introduction of a dipeptide isostere structure to bioactive peptides might lead to loss of the original bioactivity, we designed several senktide derivatives containing a variety of dipeptide isostere substructures. The Gly-Leu dipeptide surrogates for the standard Fmoc-based solid-phase peptide synthesis were synthesized according to the facile preparation approach that we previously reported.[28] The amidoxime- and amidine-containing peptides **10d,e** were also obtained using aldoxime resin.[29,30]

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Table 2. Biological Activities of [MePhe^7]-NKB Degradation Products

peptide	IC_{50} (μM)[a]	EC_{50} (nM)[b]	binding inhibition (%)[c] NK1R	NK2R
[MePhe^7]-NKB	0.012 ± 0.004	0.064 ± 0.016	<10	57 ± 1.3
1	0.022 ± 0.008	0.33 ± 0.014	22 ± 1.3	78 ± 1.9
2	0.043 ± 0.012	0.28 ± 0.041	18 ± 1.6	74 ± 1.3
3	0.059 ± 0.022	0.091 ± 0.021	15 ± 2.6	73 ± 0.98
4	2.9 ± 1.8	0.24 ± 0.054	49 ± 0.81	84 ± 1.6
5	2.3 ± 0.34	0.35 ± 0.093	70 ± 0.55	77 ± 0.53
6	>10000	>100	–[d]	–[d]
7	>10000	>100	–[d]	–[d]

[a]IC_{50} values are the concentrations for 50% inhibition of ([^{125}I]His^3, MePhe^7)-NKB (0.1 nM) binding to human NK3R (n = 3). [b]EC_{50} values are the concentrations needed for 50% induction of Ca^{2+} influx in human NK3R expressing CHO cells (n = 3). [c]Binding inhibition (%) was calculated by binding inhibition assay using a radioactive ligand (0.1 nM) and each peptide (10 μM). 100% binding inhibition was calculated based on background signal, obtained by measurement of the wells without receptor membrane. [d]Not evaluated.

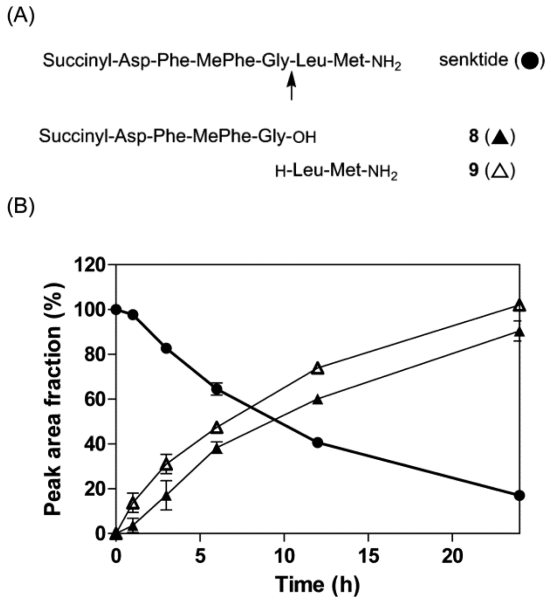
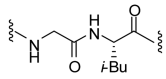
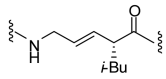
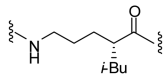
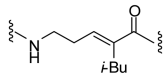
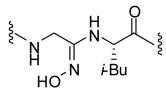
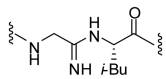


Figure 2. Proteolytic degradation of senktide by NEP 24.11. (A) Cleavage site of senktide and the sequences of the digested peptides. (B) The degradation profile of senktide following treatment with NEP 24.11: senktide (closed circles), **8** (closed triangles), **9** (open triangles). Senktide was incubated in NEP 24.11 solution at 37 °C and analyzed by HPLC (detection at 220 nm). Data represent the mean ± SD (n = 3).

Design and Biological Evaluations of Senktide Derivatives with Gly-Leu Dipeptide Isostere. All designed peptides (**10a**–**e**) worked as full NK3R agonists, exhibiting a good correlation between binding affinity and agonistic activity (Table 3). Among peptidomimetics **10a**–**e**, (*E*)-alkene dipeptide isostere-containing analogue **10a** was most potent (IC_{50} = 0.056 ± 0.012 μM; EC_{50} = 0.016 ± 0.002 nM), comparable to the parent senktide (IC_{50} = 0.056 ± 0.003 μM; EC_{50} = 0.011 ± 0.004 nM).[31] The alkene substructure, which mimics a planar amide bond may contribute to the high affinity and potent agonistic activity, which are in contrast to the lower bioactivities of a flexible ethylene-type derivative **10b** (IC_{50} = 19 ± 0.88 μM; EC_{50} = 0.92 ± 0.13 nM) and α,β-unsaturated derivative **10c** (IC_{50} = 13 ± 3.9 μM; EC_{50} = 1.9 ± 0.86 nM). Amidoxime **10d** and amidine derivative **10e** showed 34- and 9-times lower bioactivity compared with senktide, respectively (IC_{50}(**10d**) = 1.1 ± 0.082 μM; EC_{50}(**10d**) = 0.37 ± 0.13 nM; IC_{50}(**10e**) = 0.36 ± 0.091 μM; EC_{50}(**10e**) = 0.10 ± 0.027 nM).

Table 3. Biological Activities of Senktide Derivatives for NK3R

Peptide	GlyΨLeu	IC_{50} (μM)[a]	EC_{50} (nM)[b]
senktide		0.056 ± 0.003	0.011 ± 0.004
10a		0.056 ± 0.012	0.016 ± 0.002
10b		19 ± 0.88	0.92 ± 0.13
10c		13 ± 3.9	1.9 ± 0.86
10d		1.1 ± 0.082	0.37 ± 0.13
10e		0.36 ± 0.091	0.10 ± 0.027

[a]IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of ([^{125}I]His^3, MePhe^7)-NKB to human NK3R. [b]EC_{50} values indicate the concentration needed for 50% of the full agonistic activity for human NK3R induced by 100 nM senktide.

It is worth noting in terms of the receptor selectivity of peptides **10a**–**e** that binding to NK1R and NK2R was not observed at 30 μM, indicating that these peptides are NK3R-selective (Supporting Information).

To estimate the biological activity of **10a** in animals, we evaluated its agonistic activity against rat, cattle, and goat NK3R (Table 4). Peptide **10a** showed potency similar to senktide toward rat NK3R (EC_{50}(senktide) = 0.013 ± 0.004 nM; EC_{50}(**10a**) = 0.019 ± 0.004 nM), cattle NK3R (EC_{50}(senktide) = 0.34 ± 0.12 nM; EC_{50}(**10a**) = 0.36 ± 0.031 nM), and goat NK3R (EC_{50}(senktide) = 0.012 ± 0.004 nM; EC_{50}(**10a**) = 0.011 ± 0.004 nM).

The enzymatic stability of potent peptides **10a** and **10e** against NEP 24.11-mediated digestion was also evaluated (Figure 3). No significant cleavage of the Gly-Leu pseudopeptide bond in **10a** was observed, while peptide **10e** was

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Table 4. Biological Activities of Senktide and Peptide 10a

	senktide	10a
NK3R activation: EC_{50} (nM)[a]		
human	0.011 ± 0.004	0.016 ± 0.002
rat	0.013 ± 0.004	0.019 ± 0.004
cattle	0.34 ± 0.12	0.36 ± 0.031
goat	0.012 ± 0.004	0.011 ± 0.004
MUA volley[b]		
duration of the effect (R)	60.6 ± 9.9	92.1 ± 18.7[c]
the number of ligand-induced MUA volleys	3.4 ± 1.3	5.6 ± 2.3[c]

[a]EC_{50} values indicate the concentration needed for 50% of the full agonistic activity for human, rat, bovine, and goat NK3R induced by 100 nM senktide. [b]MUA volley induction in OVX goats by intravenous injection of NK3R ligands (200 nmol, n = 5). Values are represented as the mean ± SEM. [c]$P < 0.05$ (paired t-test).

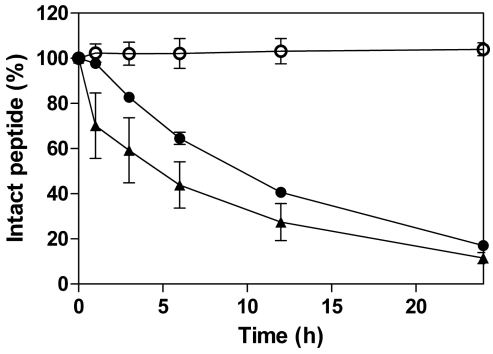

Figure 3. NEP 24.11-mediated degradation of senktide and derivatives: senktide (closed circles), **10a** (open circles), and **10e** (closed triangles). Senktide, **10a** and **10e** were incubated in NEP 24.11 solution at 37 °C and analyzed by HPLC (detection at 220 nm). Data represent the mean ± SD (n = 3).

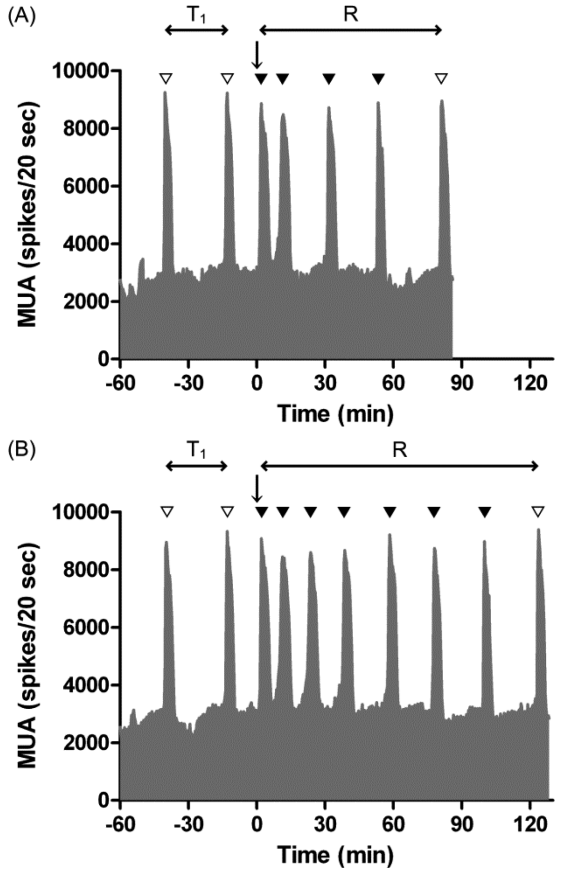

Figure 4. Effect of senktide and peptide **10a** on MUA volley in OVX goats. Representative profiles of MUA in OVX goats that received intravenous administration of senktide (200 nmol, A) and peptide **10a** (200 nmol, B) are shown. The arrow indicates timing of injection of NK3R agonist. The open and closed triangles indicate spontaneous and ligand-induced MUA volleys, respectively.

degraded. Interestingly, **10a** was partially degraded in rat serum to provide a product ($t_{1/2}$ >24 h), in which the C-terminal amide was hydrolyzed (Supporting Information). These indicate that introduction of (E)-alkene dipeptide isostere increased the stability of senktide against proteolytic cleavage. As such, the senktide derivative **10a** with favorable enzyme stability may be a promising NK3R agonist for regulating reproduction systems.

Effect of Peripheral Administration of Peptide 10a on MUA Volley in Goats. In vivo biological activity was evaluated by single intravenous administration of senktide and peptide **10a** in ovariectomized (OVX) goats, using induction of the MUA volley as the index of GnRH pulse generator activation.[8,12] The injection of NK3R analogues immediately induced several MUA volleys with shorter intervolley intervals than the predetermined average value, which were followed by MUA volleys with the regular interval (Figure 4). It is thought that those MUA volleys with an interval less than 80% of the average spontaneous intervolley intervals (T) are ligand-induced, whereas ones with an interval not less than 80% of T were spontaneous. The duration of the analogue effect (R) was obtained during a period from injection until the onset of the following spontaneous MUA volley, where the number of MUA volleys occurring during this period was counted. Statistical analysis revealed that the duration is significantly longer ($p < 0.05$) and the number of ligand-induced MUA volleys is significantly larger ($p < 0.05$) in peptide **10a** than with senktide injections (Table 4). This result demonstrates that the novel NK3R agonist **10a**, with an (E)-alkene dipeptide isostere, exhibits biological action with longer duration and more potent activity than senktide via peripheral administration in goats.

■ CONCLUSIONS

In this study, we investigated the proteolytic degradation of NK3R-selective agonists, [MePhe^7]-NKB and senktide. [MePhe^7]-NKB was degraded in serum and hypothalamic extracts, whereas senktide was stable under the same conditions. Senktide was digested at the Gly-Leu peptide bond by NEP 24.11, an inactivating enzyme of NKB. On the basis of the proteolytic degradation profiles, we designed Gly-Leu dipeptide isostere-containing peptides. Among these, peptide **10a**, an (E)-alkene dipeptide isostere-containing derivative, maintained the highly potent bioactivity and selectivity of senktide for NK3R. Peptide **10a** showed prolonged effects on the GnRH pulse generator by single intravenous administration into OVX goats. As such, modification at the Gly-Leu substructure of senktide resulted in the improved metabolic stability accompanied by potent NK3R-selective activity.

■ EXPERIMENTAL SECTION

Chemistry. The peptides were synthesized by Fmoc-based solid-phase synthesis as described previously.[28,29,32] The peptides were

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purified by HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 mm × 250 mm). All peptides were characterized by ESI-MS, and the purity was verified as >98% by HPLC on a Cosmosil 5C18-ARII analytical column (Nacalai Tesque, 4.6 mm × 250 mm) (see the Supporting Information for details).

Evaluation of Peptide Stability in Serum and Pig Hypothalamic Extracts. First, 4 μL of peptide solution (10 mM in DMSO containing 0.1% of *m*-cresol as an internal standard) was dissolved in rat, pig, goat, and cattle serum or pig hypothalamic extracts (196 μL). After incubation at 37 °C, a 30 μL aliquot was sampled at intervals (indicated time). After addition of MeCN (90 μL), the sample solution was centrifuged (4 °C, 13000*g*, 10 min), and then the supernatant was analyzed by HPLC with a linear gradient of MeCN (5−50% over 45 min or 30−50% over 20 min; detection at 220 nm). The compound ratio was determined by the peak areas.

Evaluation of Peptide Stability in the Presence of NEP 24.11. First, 8 μL of peptide solution (10 mM in DMSO containing 0.1% of *m*-cresol) was dissolved in Tris·HCl (pH 7.5) containing 0.05% of Brij-35 (WAKO) (192 μL). After addition of NEP 24.11 (R&D Systems) (200 μL, 0.1 μg/mL in Tris·HCl (pH 7.5)), solutions were incubated at 37 °C. After incubation, a 30 μL aliquot was sampled at the indicated intervals and quenched by addition of MeCN (30 μL). The sample solution was analyzed by HPLC with a linear gradient of MeCN (5−50% over 45 min or 30−50% over 20 min; detection at 220 nm). The compound ratio was determined by the peak areas.

Evaluation of Binding Affinity of Tachykinin Peptides to NK1R, NK2R, and NK3R. Binding inhibition assays were performed using membranes from NK1R-, NK2R-, or NK3R-expressing CHO cells as described previously.[32] Briefly, membranes were incubated with 50 μL of peptide, 25 μL of radioactive ligand ([^{125}I]-BH-SP for NK1R, [^{125}I]-NKA for NK2R, and ([^{125}I]His^{3}, MePhe^{7})-NKB for NK3R, respectively, 0.4 nM, PerkinElmer Life Sciences) and 25 μL of membrane solution in assay buffer (50 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% BSA). Reaction mixtures were filtered through GF/B filters, pretreated with 0.3% polyethylenimine. Filters were washed with (50 mM HEPES (pH 7.4), 500 mM NaCl, 0.1% BSA) and dried at 55 °C. Bound radioactivity was measured by TopCount (PerkinElmer Life Sciences) in the presence of MicroScint-O (30 μL) (PerkinElmer Life Sciences).

Evaluation of NK3R Agonistic Activity. NK3R agonistic activity of each peptide was evaluated by $[Ca^{2+}]_i$ flux assay as described previously.[32] NK3R expressing CHO cells (4.0×10^4 cells/50 μL/well) were inoculated in 10% FBS/Ham's F-12 onto a 96-well black clear-bottom plate (Greiner), followed by incubation at 37 °C overnight in 5% CO_2. After medium removal, 100 μL of pigment mixture (Calcium 4 assay kit, Molecular Devices) was dispensed into each well, followed by incubation at 37 °C for 1 h. Then 10 μM peptide in DMSO was diluted with HANKS/HEPES containing 2.5 mM probenecid, and the dilution was transferred to a 96-well sample plate (V-bottom plate, Coster). The cell and sample plates were set in FlexStation (Molecular Devices) and 25 μL of sample solution automatically transferred to the cell plate.

Evaluation of Effect of NK3R Agonists on MUA Volley in OVX Goats. All goat experiments were approved by the Committee on the Care and Use of Experimental Animals at the National Institute of Agrobiological Sciences (H23-002). Adult female Shiba goats (*Capra hircus*, 4−8 years old, weighing 23.0−32.0 kg, $n = 5$) were bilaterally ovariectomized under inhalation anesthesia. With an interval of more than one month, they were implanted with an array of bilateral recording electrodes consisting of six Teflon-insulated platinum−iridium wires at the posterior region of the ARC as described previously.[8,12] After recovery, the goats were kept in a condition-controlled room (12*L*/12*D*, 23 °C, and 50% relative humidity) and loosely held in an individual stanchion. Animals were maintained with a standard pellet diet and dry hay and had free access to water and supplemental minerals. MUA was monitored in conscious goats. Signals were passed through a buffer amplifier and integrated circuit directly plugged into an electrode assembly. After additional amplification and amplitude discrimination, MUA signal was stored as counts per 20 s on a personal computer.

In OVX goats, the intervolley interval of spontaneously occurring MUA volleys differed slightly among individuals ranging from 20 to 35 min but was relatively constant within an individual in which the interval variation was usually ±2 min, allowing for a timed treatment between experiments. On each experimental day, the average value of three successive intervolley intervals (T) was calculated for each OVX goat and sample injections made at $1/2T$ after the preceding MUA volley.

NK3R agonists were initially dissolved in DMSO at 10 mM and further diluted with saline to make a working concentration of 100 μM. All five goats received a bolus injection of senktide and peptide **10a** once through a jugular catheter. MUA was recorded throughout the experimental period. Analogue treatments were separated by at least 1 day.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, characterization data, degradation profile, and NMR spectra of peptides **10a−e**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ARC, arcuate nucleus; BH-SP, Bolton−Hunter labeled substance P; GnRH, gonadotropin-releasing hormone; MePhe, *N*-methylphenylalanine; MUA, multiple-unit activity; NKA, neurokinin A; NKB, neurokinin B; NK1R, neurokinin-1 receptor; NK2R, neurokinin-2 receptor; NK3R, neurokinin-3 receptor; HPG, hypothalamo-pituitary-gonadal; OVX, ovariectomized; SP, substance P

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