Structure–activity relationship study on senktide for development of novel potent neurokinin-3 receptor selective agonists†

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Neurokinin B (NKB) regulates the secretion of gonadotropin-releasing hormone (GnRH) in the hypothalamus via activation of the cognate neurokinin-3 receptor (NK3R). The stimulatory effect of NKB and the derivatives on gonadotropin secretion can potentially be used for development of novel regulatory and therapeutic agents for reproductive dysfunctions. Here, we report a comprehensive structure–activity relationship study on the NK3R-selective agonist peptide, senktide. Substitution of the N-terminal succinyl-Asp substructure in senktide with oxalyl-Glu, oxalyl-D-Glu or oxalyl-L-2-amino adipic acid (Aad) increased receptor binding and NK3R activation. Among these modifications, the oxalyl-D-Glu substructure prevented neutral endopeptidase (NEP) 24.11-mediated degradation, thus providing a novel NK3R agonist peptide with favourable biological and stability properties.

Introduction

Neurokinin-3 receptor (NK3R) belongs to the tachykinin receptor family and is known as a cognate receptor of neurokinin B (NKB, Table 1). Recently, the hypothalamic regulation of reproduction by the NKB–NK3R axis has received considerable attention.1,2 The genetic study of TAC3 and TACR3, which encode NKB and NK3R, respectively, highlighted the prevalence of hypogonadotropic hypogonadism by inactivating mutations of these genes.3,4 Exogenous administration of kisspeptin or gonadotropin-releasing hormone (GnRH) into patients with these genetic mutations restores the circulating level of luteinising hormone (LH),5 suggesting that NKB positively regulates the reproductive hormone cascade via GnRH neuron activation.6 The influence on LH secretion by central administration of an NK3R agonist into ovariectomised and estrogen-primed rats has also been reported previously.7 Because LH secretion from the pituitary gland is implicated in a number of gonadal functions including testosterone production in males, maturation of pre-ovulatory ovarian follicles, and ovulation in females,8,9 selective NK3R modulators have been expected to be a novel class of therapeutic agents for gonadal dysfunctions as well as reproductive disorders. The NK3R modulators would also be applicable to the improvement of the low reproductive efficiency of livestock such as cattle.10

In 2007, it was reported that NKB is colocalised with kisspeptin and dynorphin A (DynA) in a single subpopulation in the hypothalamic arcuate nucleus (ARC) in sheep.11 These peptides cooperatively regulate the secretion of GnRH: NKB and kisspeptin mediate induction, while DynA mediates inhibition of GnRH secretion. Cells containing these three peptides are currently recognised as KNDy (kisspeptin, NKB and DynA) neurons.12 Although NKB is expressed in many other brain regions, the colocalisation of these peptides is unique to ARC, which is conserved among mammalian species including human.13–15

Two NK3R-selective agonist peptides have been reported: [MePhe7]-NKB16 and senktide (Table 1).17 [MePhe7]-NKB was identified through a structure–activity relationship study on NKB(4–10), a short NKB analogue, which was designed based on the sequence of the neurokinin-1 receptor (NK1R)/NK3R agonist peptide, DiMeC7.16 Our previous structure–activity relationship study on naturally occurring tachykinin peptides revealed that substitution of Phe3 with an acidic amino acid (Asp5 or Glu5) and of Val7 with MePhe7 in NKB is indispensable for potent activity and selectivity of [MePhe7]-NKB for NK3R.18 On the basis of these data, a novel potent NK3R agonist with higher NK3R selectivity compared with

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4md00514g
Table 1  Sequences of NKB and senktide

| Peptide     | Sequence                                      |
|-------------|------------------------------------------------|
| Neurokinin B (NKB) | H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH2 |
| [MePhe7]-NKB | H-Asp-Met-His-Asp-Phe-MePhe-Gly-Leu-Met-NH2  |
| Senktide    | Succinyl-Asp-Phe-MePhe-Gly-Leu-Met-NH2        |

[MePhe7]-NKB was identified. Senktide is the most common NK3R agonist peptide, which was incidentally identified in an N-methylamino acid scanning study on substance P (SP)-related peptides for the development of NK1R-selective agonists. It has been used in a number of in vitro and in vivo experiments to investigate NK3R functions. For example, senktide-mediated NK3R stimulation evoked dopamine release from dopamine neurons in vitro. In addition, the function of the KNDy neurons in the pulsatile secretion of GnRH was demonstrated by in vivo administration of senktide. Although senktide is subjected to peptidase-mediated degradation, a novel peptidomimetic derivative of senktide exerts a prolonged effect on the GnRH pulse generator in ovariectomised (OVX) goats.

In the current study, we report the structure–activity relationships of senktide derivatives with modification of component amino acids or N-terminal functional group to identify a novel potent NK3R agonist. The stability under peptidase-mediated degradation conditions and the in vivo bioactivity of potent NK3R agonist derivatives are also discussed.

Results and discussion

Synthesis of senktide analogue peptides
All peptide chains were constructed by standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) on Rink-amide resin. Succinylation, carbamoylation, or hydroxycarbamoylation of the peptide’s N-terminus was carried out on resin as previously reported. Modification with oxalyl, aminocarbamoyl, methyl oxalyl, 2-amino-2-oxoacetyl, or sulfamoyl groups at the peptide N-terminus was conducted using appropriate reagents as described in the experimental procedures. The final deprotection and cleavage from the resin with the cocktail TFA/thioanisole/m-cresol/1,2-ethanedithiol/H2O (80:5:5:5:5), followed by RP-HPLC purification afforded the expected peptides as TFA salts. All peptides were identified by ESI-MS or MALDI-TOF-MS and the purity was determined to be more than 98% by analytical HPLC.

Structure–activity relationships of tachykinin consensus sequences in senktide
The C-terminal sequence, Phe-Xaa-Gly-Leu-Met-NH2, is common among mammalian tachykinin peptides including SP, neurokinin A (NKA) and NKB, which are endogenous agonists of NK1R, neurokinin-2 receptor (NK2R) and NK3R, respectively. In addition, tachykinin peptides share similar sequences among mammalian species, whereas the consensus sequence often contains several amino acid substitutions in other species. For example, insect-derived tachykinin-related peptides with the consensus Phe-Xaa-Gly/Ala-Yaa-Arg-NH2 sequence show potent agonistic activity towards the tachykinin peptide receptor, STKR, which has 41% homology with human NK3R. Octopus-derived tachykinin peptides have a variety of C-terminal sequences, including oct-TKRP I with Gly-Thr-Arg-NH2 and oct-TKRP-V with Pro-Pro-Lys-NH2. These oct-TKRP peptides show potent agonistic activity towards the oct-TKRP receptor. To optimise the amino acids of the consensus sequences in senktide for receptor binding and selectivity, we designed senktide derivatives based on these invertebrate tachykinin-related peptide sequences (see the ESI†). However, these modifications resulted in bioactivity loss, except for the peptide with Gly to Pro substitution, which exhibited weak NK3R binding. Substitution of Phe in the consensus sequence with an aromatic amino acid (Tyr, Trp or His) also led to significantly weak or no NK3R binding. These results suggest that the amino acids in the tachykinin consensus sequence, including the C-terminal Met-NH2, are indispensable for the biological activity of senktide towards NK3R.

MePhe is a characteristic amino acid that contributes to the high NK3R selectivity of [MePhe7]-NKB and senktide. We next designed several senktide derivatives with substitution at the MePhe position with other N-methylamino acids (Table 2). Among derivatives 1a–c with an aliphatic N-methylamino acid, the MeVal- (1a) and Melle-derivatives (1b) showed slightly lower biological activity than senktide (1a: IC50 = 104 nM, EC50 = 64 pM; 1b: IC50 = 91 nM, EC50 = 50 pM). In contrast, substitution with MeLeu resulted in loss of binding to NK3R. The MeTyrr- (1d) and MeTrp-substituted peptides (1e) also maintained the NK3R binding activity (1d: IC50 = 28 nM, EC50 = 41 pM; 1e: IC50 = 123 nM, EC50 = 33 pM). Substitution with D-MePhe resulted in loss of NK3R binding, suggesting that NK3R recognises the side chain orientation derived from the L-MePhe chiral centre in senktide. As the highly acidic succinyl-Asp substructure in senktide is likely to interact with the potential basic functional group(s) of NK3R, replacement of MePhe with MeAsp was also attempted; however, the MeAsp derivative (1g) showed no binding to NK3R. Taken together, some appropriate aliphatic or aromatic N-methylamino acids are acceptable at the MePhe position for potent NK3R agonists. Of note, the potent peptides 1a, 1b, 1d and 1e showed no binding affinity to NK1R and NK2R at 10 μM.

Structure–activity relationships of N-terminal substructures in senktide
Senktide possesses two anionic carboxy groups at the N-terminus, which have been suggested to provide high NK3R selectivity. We next investigated the modification of the senktide N-terminal substructure with a number of potential bioisosteres.

Initially we substituted the N-terminal succinyl group in senktide with L-Asp or D-Asp derivatives (Table 3). As the succinyl group in senktide is derived from the side chain of L-Asp in NKB, regeneration of a chiral centre was expected to allow favourable orientation of negatively charged functional
The introduction of unmodified L-Asp (2a) resulted in a significant decrease in receptor binding and agonistic activity (2a: IC_{50} = 175 nM, EC_{50} = 63 pM). The acetyl capping of the Asp ε-amino group (3a) restored the potent bioactivity (3a: IC_{50} = 68 nM, EC_{50} = 31 pM), suggesting that a basic functional group at the N-terminal region is unfavourable for NK3R binding and activation. Among derivatives 3a–d with a modification at the second acidic amino acid, the L-Glu-substituted derivative 3c showed 5-fold more potent receptor binding and 3-fold more potent agonist activity compared with those of senktide (3c: IC_{50} = 7.7 nM, EC_{50} = 8.1 pM). A similar structure–activity relationship was obtained among the 4a–d series of peptides with an Ac–D-Asp modification at the N-terminus, in which the most potent L-Glu peptide 4c exhibited 3-fold more potent receptor binding and 2-fold stronger agonist activity compared with those of senktide (4c: IC_{50} = 13 nM, EC_{50} = 12 pM). The advantage of L-Glu for NK3R activation is consistent with the potent biological activity of PG-KII with an L-Asp–L-Glu substructure at this position, which was derived from the Australian frog, *Pseudophryne guentheri.*

Taking advantage of these key insights, further investigations were carried out for optimisation of the spatial arrangements of two N-terminal acidic functional groups in senktide (Table 4). Among the senktide derivatives in which L-Asp was substituted with other acidic amino acids including D-Asp, L-Glu, D-Glu or L-2-aminoadipic acid (L-Aad) (5b–e), substitution with D-Asp (5b), L-Glu (5c) or L-Aad (5e) resulted in 2- or 3-fold more potent NK3R agonist activity (5b: IC_{50} = 16 nM, EC_{50} = 29 pM; 5c: IC_{50} = 6.1 nM, EC_{50} = 30 pM; 5e: IC_{50} = 9.9 nM, EC_{50} = 19 pM) compared with that of senktide, whereas peptide 5d with D-Glu showed slightly less potent agonist activity (5d: IC_{50} = 24 nM, EC_{50} = 84 pM). Interestingly, significant improvement in bioactivity was observed among the N-terminally oxayl-modified derivatives, 6a–e. The derivatives with the L-Glu (6c), D-Glu (6d) or L-Aad (6e) substitution exhibited 7- to 9-fold more potent biological activity (6c: IC_{50} = 0.43 nM, EC_{50} = 9.1 pM; 6d: IC_{50} = 0.51 nM, EC_{50} = 14 pM; 6e: IC_{50} = 1.4 nM, EC_{50} = 7.4 pM) compared with that of senktide. Although the peptides with the L-Asp (6a) or D-Asp (6b) substitution showed 10-fold more potent binding inhibition, significant improvement in NK3R activation was not observed (6a: IC_{50} = 3.6 nM, EC_{50} = 83 pM; 6b: IC_{50} = 3.5 nM, EC_{50} = 22 pM). These results suggest that a combination of carboxy functional groups with a short tether at the N-terminus and with a long side chain at the L-Asp

### Table 2 Structure-activity relationship of senktide derivatives with a modification at the MePhe position

| Peptide           | Xaa          | NK3R IC_{50} (nM) | NK3R EC_{50} (pM) | NK1R IC_{50} (nM) | NK1R EC_{50} (pM) | NK2R IC_{50} (nM) | NK2R EC_{50} (pM) |
|-------------------|--------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Senktide          | MePhe        | 45                | 17                | >10 000           | >10 000           | >10 000           | >10 000           |
| 1a MeVal          | 104          | 64                | 50                | >10 000           | >10 000           | >10 000           | >10 000           |
| 1b Melle          | 91           | 50                | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 1c MeLeu          | >10 000      | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 1d MeTyr          | 28           | 41                | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 1e MeTrp          | 123          | 33                | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 1f D-MePhe        | >10 000      | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 1g MeAsp          | >10 000      | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |

* IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of [[^{125}I]Hist]{2}–MePhe to NK3R, [[^{125}I]-BH-SP to NK1R, and [[^{125}I]-NKA to NK2R. * EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

### Table 3 Structure-activity relationship of senktide derivatives with a modification of the N-terminal acetyl-Asp substructure

| Peptide        | R            | Xaa  | NK3R IC_{50} (nM) | NK3R EC_{50} (pM) | NK1R IC_{50} (nM) | NK1R EC_{50} (pM) | NK2R IC_{50} (nM) | NK2R EC_{50} (pM) |
|----------------|--------------|------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Senktide       | Succinyl     | i-Asp| 40                | 23                | >10 000           | >10 000           | >10 000           | >10 000           |
| 2a H-ε-Asp     | 175          | 63   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 3a Ac-ε-Asp    | 68           | 31   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 3b Ac-d-Asp    | 83           | 24   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 3c Ac-ε-Asp    | 7.7          | 8.1  | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 3d Ac-d-Asp    | 37           | 18   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 4a Ac-d-Asp    | 73           | 18   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 4b Ac-d-Asp    | 55           | 17   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 4c Ac-d-Asp    | 13           | 12   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |
| 4d Ac-d-Asp    | 68           | 17   | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           | >10 000           |

* IC_{50} values indicate the concentration needed for 50% inhibition of receptor binding of [[^{125}I]Hist]{2}–MePhe to NK3R, [[^{125}I]-BH-SP to NK1R, and [[^{125}I]-NKA to NK2R. * EC_{50} values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.
The position of senktide would be appropriate for potent agonist activity towards NK3R.

Further modifications of the senktide N-terminus with iso-

stere functional groups were performed in an attempt to

improve the biological activity (7–12) (Table 5). All the deri-

vatives maintained the potent binding inhibition and agonist

activity toward NK3R (IC50 = 4.1–39 nM; EC50 = 2.5–43 pM).

Peptides 10 and 12, with 2-methoxy-2-oxoacetyl and amin- o-
sulfonl groups, respectively, showed slightly more potent

biological activity compared with that of senktide [10: IC50 =

4.1 nM, EC50 = 2.9 pM; 12: IC50 = 5.6 nM, EC50 = 2.5 pM],
suggesting that a charged anionic functional group is not
critical for the N-terminus of senktide. Using the potent pepti-
daes, 10 and 12, we further optimised the \( l \)-Asp position

using acidic amino acids (Table S3†). However, the improve-

ment in agonist activity towards NK3R by modifying the

\( l \)-Asp position was limited, which was inconsistent with the
case of the series of derivatives with an N-terminal oxalyl

group. Altogether, we concluded that the oxalyl group is the

best N-terminal functional group for NK3R agonists.

The highly potent senktide derivatives, 6c–e, were evalu-

ated for agonist activity towards NK3R derived from other

mammalian species including rat, goat and cattle (Table 6).

All the peptides showed more potent agonist activity towards

mammalian NK3R compared with that of senktide. The most

potent peptide, 6e, with oxalyl-\( L \)-Asp at the N-terminus

exhibited 3-, 2- and 4-fold higher potency than senktide in

activation of rat, goat and cattle NK3R, respectively.38

Table 4 Structure–activity relationship of N-terminal succinyl and oxalyl senktide derivatives

| Peptide | R         | Xaa    | NK3R IC50a (nM) | NK1R IC50a (nM) | NK2R IC50a (nM) |
|---------|-----------|--------|-----------------|-----------------|-----------------|
| Senktide| Succinyl  | \( l \)-Asp | 43              | >10000          | >10000          |
| 5b      | Succinyl  | \( d \)-Asp | 16              | >10000          | >10000          |
| 5c      | Succinyl  | \( l \)-Glu | 6.1             | >10000          | >10000          |
| 5d      | Succinyl  | \( d \)-Glu | 24              | >10000          | >10000          |
| 5e      | Succinyl  | \( l \)-Aad | 9.9             | >10000          | >10000          |
| 6a      | Oxalyl    | \( l \)-Asp | 3.6             | >10000          | >10000          |
| 6b      | Oxalyl    | \( d \)-Asp | 3.5             | >10000          | >10000          |
| 6c      | Oxalyl    | \( l \)-Glu | 0.43            | >10000          | >10000          |
| 6d      | Oxalyl    | \( d \)-Glu | 0.51            | >10000          | >10000          |
| 6e      | Oxalyl    | \( l \)-Aad | 1.4             | >10000          | >10000          |

\( a \) IC50 values indicate the concentration needed for 50% inhibition of receptor binding of \([^{125}\text{I}]\text{His}^3, \text{MePhe}^7\)-NKB to NK3R, \([^{125}\text{I}]\)-BH-SP to

NK1R, and \([^{125}\text{I}]\)-NKA to NK2R. \( b \) EC50 values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.

Table 5 Optimisation of the N-terminal accessory substructure

| Peptide | R         | NK3R IC50a (nM) | NK1R IC50a (nM) | NK2R IC50a (nM) |
|---------|-----------|-----------------|-----------------|-----------------|
| Senktide|            | 29              | 3.4             | >10000          |
| 7       |            | 39              | 13              | >10000          |
| 8       |            | 10              | 43              | >10000          |
| 9       |            | 34              | 6.0             | >10000          |
| 10      |            | 4.1             | 2.9             | >10000          |
| 11      |            | 7.9             | 11              | >10000          |
| 12      |            | 5.6             | 2.5             | >10000          |

\( a \) IC50 values indicate the concentration needed for 50% inhibition of receptor binding of \([^{125}\text{I}]\text{His}^3, \text{MePhe}^7\)-NKB to NK3R, \([^{125}\text{I}]\)-BH-SP to

NK1R, and \([^{125}\text{I}]\)-NKA to NK2R. \( b \) EC50 values are the concentration needed for 50% of the full agonist activity induced by 100 nM senktide.
Proteolytic degradation of potent NK3R agonist peptides in serum and by endopeptidase treatment

NKβ is degraded and inactivated by cleavage at the Gly-Leu peptide bond by membrane-anchored neutral endopeptidase 24.11 (NEP 24.11; also known as nephrisin or enkephalinase). Senktide is also inactivated by NEP 24.11-mediated cleavage at the same site. To identify potent NK3R agonists with resistance to NEP 24.11-mediated degradation, we assessed the stability of the potent derivatives obtained in the above structure–activity relationship experiments.

Peptides 6c and 6d were stable in rat, pig, goat and cattle serum for 24 h similarly to senktide (Fig. S1†), suggesting that the N-terminal oxalyl capping also prevents the possible degradation by peptidases in the serum, which was observed in the case of [MePhe]-NKβ. Next, the resistance of peptides 6c–e to NEP 24.11-mediated degradation was investigated (Fig. 1). More than 70% of 6c and 6e were digested at the Gly-Leu dipeptide bond by 24 h of incubation with NEP 24.11. In contrast, little degradation of 6d by NEP 24.11 was observed after 24 h. These results suggest that substitution of t-Asp in senktide with d-Glu is effective for resistance against NEP 24.11-mediated proteolytic degradation at the distal Gly-Leu cleavage site. Thus, peptide 6d with oxalyl-d-Glu at the N-terminus is a potent NK3R agonist with high biological stability.

Effect of peripheral administration of peptide 6d on the induction of periodic burst of multiple-unit activity (MUA) volleys in goat

It has been reported that pulsatile GnRH secretion is synchronized with the induction of periodic burst of multiple-unit activity (MUA) volleys. To assess the in vivo effect on pulsatile secretion of GnRH, we monitored the induction of MUA volleys after intravenous administration of 6d. MUA volleys with intervals less than 80% of the average spontaneous intervolley intervals (T) were defined as ligand-induced, whereas those with intervals higher than 80% of T were spontaneous. The action duration (R) was the period from the injection until the onset of the following spontaneous MUA volley, and the number of MUA volleys occurring in this period (V) was counted. Peptide 6d (200 nmol) immediately induced several MUA volleys. The R and V values of 6d were similar to those of senktide (Table 7 and Fig. S2†). This is inconsistent with the results in our recent study, in which the novel biostable NK3R-selective peptidomimetic agonist 13 [succinyl-Asp-Phe-MePhe-Gly-ψ[(E)-CH==CH]-Leu-Met-NH2] showed prolonged in vivo bioactivity in goat compared with that of senktide. These results may suggest that the clearance of the peptide from the circulation should be further optimised to develop NK3R agonists with prolonged in vivo bioactivity.

Conclusions

In this study, we investigated the structure–activity relationships of senktide derivatives in relation to agonist activity towards NK3R. The consensus sequence of tachykinin peptides in senktide is important for NK3R binding and activation, while MePhe can be substituted with several hydrophobic N-methyllamino acids. Optimisation of the N-terminal structures resulted in identification of novel potent NK3R agonists (6c–e) with an oxalyl group at the peptide N-terminus. Although further improvement to prevent the possible rapid clearance of senktide and the derivatives is needed, peptide 6d with its enhanced metabolic stability could be an appropriate lead peptide for designing novel NK3R agonists with prolonged in vivo bioactivity.

Experimental procedures

General method for peptide synthesis

The protected linear peptides were constructed by Fmoc-based solid-phase synthesis on Rink-amide resin (0.66 mmol g⁻¹,
45.5 mg, 0.025 mmol). Fmoc-protected amino acids (0.075 mmol) were coupled by N,N’-diisopropylcarbodiimide (DIC, 0.0116 cm³, 0.075 mmol) and HOBr·H₂O (11.5 mg, 0.075 mmol) in DMF. Coupling of amino acid (0.075 mmol) to N-methylamino acid was carried out with HATU (28.5 mg, 0.075 mmol) and (iPr)₂NET (DMPEA, 0.013 cm³, 0.075 mmol). Completion of each coupling reaction was ascertained using the Kaiser ninhydrin test. The Fmoc-protecting group was removed by treating the peptide resin with TFA/thioanisole/pyridine (45.5 mg, 0.025 mmol) in DMF (1.0 cm³) for 1.5 h to produce hydroxycarbamoyl-modified peptide resin for peptides 1a-d and 3a–e.

**Sucyclination.** The peptide resin (0.025 mmol) was treated with succinic anhydride (12.5 mg, 0.125 mmol) and DIPEA (0.0436 cm³, 0.125 mmol) in DMF (1.0 cm³) for 1.5 h to produce succinyl-modified peptide resin for peptides 1a–g, 5b–e and 3a–r.

**Oxalylation.** The peptide resin (0.025 mmol) was treated with tert-butyl chloro(oxo)acetate (20.6 mg, 0.125 mmol) and DIPEA (0.0436 cm³, 0.25 mmol) in CH₂Cl₂ (1.0 cm³) for 1.5 h to produce oxalyl-modified peptide resin for peptides 6a–e.

**Carbamoylation.** The peptide resin (0.025 mmol) was treated with chlorosulfonyl isocyanate (0.0109 cm³, 0.125 mmol) and pyridine (0.0202 cm³, 0.25 mmol) in CH₂Cl₂ (1.0 cm³) for 16 h. Subsequently, the resin was treated with H₂O (0.050 cm³) for 48 h to produce carbamoyl-modified peptide resin for peptide 7.

**Hydroxycarbamoylation.** The peptide resin (0.025 mmol) was treated with p-nitrophenyl chloroformate (186.4 mg, 0.925 mmol) and DIPEA (0.161 cm³, 0.925 mmol) in THF/CH₂Cl₂ (1:1, 1.0 cm³) for 30 min. After removal of the above reagents, a solution of hydroxylamine hydrochloride (64.3 mg, 0.925 mmol) and DIPEA (0.322 cm³, 1.85 mmol) in DMF (1.0 cm³) was added. The suspension was stirred for 15 min to produce hydroxycarbamoyl-modified peptide resin for peptide 8.

**Aminocarbamoylation.** A solution of p-nitrophenyl chloroformate (100.8 mg, 0.5 mmol) in dry THF (1.0 cm³) was added to a solution of tert-butyl carbazate (66.1 mg, 0.5 mmol) and N-methylmorpholine (0.055 cm³, 0.5 mmol) in dry THF (4.0 cm³). After stirring for 40 h, the reaction mixture was concentrated to give Boc-NHNH·CO₂C₆H₄(4-NO₂). This reagent in dry THF (1.0 cm³) and TEA (0.069 cm³, 0.5 mmol) was added to the peptide resin (0.025 mmol). The suspension was agitated for 42 h to produce aminocarbamoyl-modified peptide resin for peptide 9.

**2-Methyl-2-oxoacetylation.** The peptide resin (0.025 mmol) was treated with methyl chloroglyoxalate (0.0114 cm³, 0.125 mmol) and pyridine (0.0201 cm³, 0.25 mmol) in CH₂Cl₂ (1.0 cm³) for 2 h to produce methylglyoxyl-modified peptide resin for peptides 10 and S2a–c.

**Amino-2-oxoacetylation.** The peptide resin (0.025 mmol) was treated with oxamic acid (11.1 mg, 0.125 mmol), DIC (0.0194 cm³, 0.125 mmol), and HOBr·H₂O (19.1 mg, 0.125 mmol) in CH₂Cl₂ (1.0 cm³) for 3 h to produce 2-amino-2-oxoacylated-modified peptide resin for peptide 11.

**Sulfamoylation.** tert-Butyl alcohol (0.0237 cm³, 0.25 mmol) in dry THF (0.1 cm³) was added to chlorosulfonyl isocyanate (0.0218 cm³, 0.25 mmol) in dry THF (0.15 cm³) and the mixture was stirred for 15 min at 0 °C. The reaction mixture and TEA (0.0693 cm³, 0.5 mmol) in dry CH₂Cl₂ (0.25 cm³) were added to the peptide resin (0.025 mmol), and the suspension was agitated for 3 h to produce sulfamoyl-modified peptide resin for peptides 12 and S3a–c.

### Abbreviations

- **Aad**: L-2-Aminoadipic acid
- **ARC**: Arcuate nucleus
- **BH-SP**: Bolton–Hunter labelled substance P
- **DIC**: N,N’-Diisopropylcarbodiimide
- **DynA**: Dynorphin A
- **GnRH**: Gonadotropin-releasing hormone
- **HOAt**: 4-Hydroxy-7-azabenzotriazole
- **LH**: Luteinising hormone
- **MUA**: Multiple-unit activity
- **NKA**: Neurokinin A
- **NKB**: Neurokinin B
- **NK1R**: Neurokinin-1 receptor
- **NK2R**: Neurokinin-2 receptor
- **NK3R**: Neurokinin-3 receptor
- **MePhe**: N-Methylphenylalanine
- **OVX**: Ovariectomised
- **SP**: Substance P
- **SPPS**: Solid-phase peptide synthesis

### Acknowledgements

We are grateful to Dr. Akira Hirasawa (Kyoto University) for his generous support in the experiments. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development, REP-2001, REP-2003 and REP-2005), by Grants-in-Aid for Scientific Research from JSPS, Japan, and by Platform for Drug Discovery, Informatics, and Structural Life Sciences.
Science from MEXT, Japan. R.M. and T.N. are grateful for JSPS Research Fellowships for Young Scientists.

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