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Structure–activity study at positions 3 and 4 of human neuropeptide S

Valeria Camarda [a], Claudio Trapella [b], Girolamo Calo' [a], Remo Guerrini [b,*], Anna Rizzi [a], Chiara Ruzza [a], Stella Fiorini [b], Erika Marzola [b], Rainer K. Reinscheid [c], Domenico Regoli [a], Severo Salvadori [b]

[a] Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience Center, University of Ferrara, and National Institute of Neuroscience, Italy
[b] Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, Via Fossato di Mortara 19, 44100 Ferrara, Italy
[c] Department of Pharmaceutical Sciences, University of California Irvine, 360 Med Surge 2, Irvine, CA 92697, USA

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ABSTRACT

Neuropeptide S (NPS) has been identified as the endogenous ligand of a previously orphan receptor now named NPSR. Previous studies demonstrated that the N-terminal sequence Phe^2-Arg^3-Asn^4 of the peptide is crucial for biological activity. Here, we report on a focused structure–activity study of Arg^3 and Asn^4 that have been replaced with a series of coded and non-coded amino acids. Thirty-eight human NPS analogues were synthesized and pharmacologically tested for intracellular calcium mobilization using HEK293 cells stably expressing the mouse NPSR. The results of this study demonstrated the following NPS position 3 structure–activity features: (i) the guanidine moiety and its basic character are not crucial requirements, (ii) an aliphatic amino acid with a linear three carbon atom long side chain is sufficient to bind and fully activate NPSR, (iii) the receptor pocket allocating the position 3 side chain can accommodate slightly larger side chains at least to a certain degree [hArg, Arg(NO_2) or Arg($Me)_2$ but not Arg(Tos)]. Position 4 seems to be more sensitive to amino acids replacement compared to position 3; in fact, all the amino acid replacements investigated produced either an important decrease of biological activity or generated inactive derivatives suggesting a pivotal role of the Asn^4 side chain for NPS bioactivity.

1. Introduction

Neuropeptide S (NPS, human primary sequence SFRNGVGT GMKKTSFQRAKS) has been identified as the endogenous ligand of the G protein coupled receptor now named NPSR.[1] In cells expressing the recombinant NPSR, NPS produced concentration dependent increases of calcium levels and cAMP formation, suggesting transductional mechanisms involving Gq and Gs.[2] NPSR mRNA is widely expressed throughout the nervous system while the NPS precursor mRNA is strongly expressed only in the locus coeruleus area and in a few other nuclei of the brainstem.[1,3] The anatomical distribution of NPSR is consistent with the biological effects elicited by supraspinal administration of NPS in rodents, namely anxiolytic like effects,[1,4,5] arousal promoting action,[1,5] stimulation of locomotor activity,[1,5–8] and inhibition of food intake.[8–10] Up to now the involvement of NPSR in the in vivo actions of NPS has been demonstrated only for the locomotor stimulant action using the non-peptide NPSR antagonist SHA 68.[6] The development of selective ligands for NPSR is of paramount importance for investigating the functions and roles of this novel neurotransmitter system in central nervous system physiology and pathology. To this aim, we started a systematic investigation of NPS chemical and conformational requirements for NPSR binding and activation. First, in line with previous observations[2], we identified the N-terminal sequence Phe^2-Arg^3-Asn^4 as the most important structural component for biological activity[7], a finding confirmed by other researchers.[11] Second, we focussed on the chemical requirements of Phe^2 of NPS demonstrating that this position is important for both receptor binding and activation.[12] Third, we performed a conformation–activity relationship study[13] suggesting that helicity can be tolerated in the C-terminal part of NPS but not around Gly[7], a result which is only in part in line with the nascent helix spanning residues 5 through 13 proposed by Bernier et al.[11]

In this paper, we present results obtained from a SAR study performed by replacing Arg^3 and Asn^4 with several coded and non-coded amino acid residues. 38 novel NPS analogues were synthes-

Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; DMF, *N*,*N*-dimethylformamide; DMEM, Dulbecco's modified Eagles's medium; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HATU, [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; mRNA, messenger ribonucleic acid; Ndm, 3-(dimethylcarbamoyl)-2-aminopropanoic acid; NMM, 4-methylmorpholine; Nmm, 3-(methylcarbamoyl)-2-aminopropanoic acid; NPS, neuropeptide S; NPSR, NPS receptor; Ova, 2-amino-4-oxo-pentanoic acid; tBu, *tert*-butyl; TFA, trifluoroacetic acid; SAR, structure–activity relationship; SAH 68, 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-*a*]pyrazine-7-carboxylic acid 4-fluorobenzylamide.

* Corresponding author. Tel.: +39 0532 455 988; fax: +39 0532 455953.
E-mail address: r.guerrini@unife.it (R. Guerrini).

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ised and pharmacologically evaluated in a calcium mobilization assay using HEK293 cells stably expressing mouse NPSR ($HEK293_{mNPSR}$) and the fluorometric imaging plate reader FlexStation II.

2. Results and discussion

Compounds **1–38** and the reference compound human NPS were prepared with good yield by solid phase peptide synthesis. For the synthesis of compounds **35**, **37** and **38**, the commercially unavailable amino acids Nmm and Ndm were synthesized in good yield by amidation of the Fmoc-Asp-*Ot*Bu side chain with mono or dimethyl amine followed by TFA deprotection of the C^{α} carboxylic function. The non-coded amino acid Ova was synthesized and Fmoc protected[14] following procedures reported in the literature.[15] The chemical formulae of the non-coded amino acids employed in this study are displayed in Figure 1.

Tables 1 and 2 summarize data obtained investigating the SAR requirements of NPS positions 3 and 4, respectively. In line with previous results[2,12], in the calcium mobilization assay NPS increased intracellular calcium levels in a concentration dependent manner showing high potency (pEC_{50} ≈8) and efficacy (E_{max} ≈ 2.5-fold over the basal values).

For investigating the chemical features required in positions 3 and 4 of NPS for NPSR interaction, we adopted a classical approach in which the original amino acid residues were replaced with coded and non-coded amino acids. The substitution of Arg^3 with aromatic residues (compounds **1**–**3**) produced a drastic loss of biological activity; the best compound ([His^3]NPS) being more than 100-fold less potent than the natural peptide. Similar results were obtained with non-aromatic lipophilic residues (compounds **4**–**5**). The introduction in position 3 of residues with hydrophilic neutral (compounds **6**–**11**), basic (compound **12**) or acidic (compounds **13–14**) character produced low potency or inactive NPS analogues. Among these, only [Lys^3]NPS displayed a potency value similar to

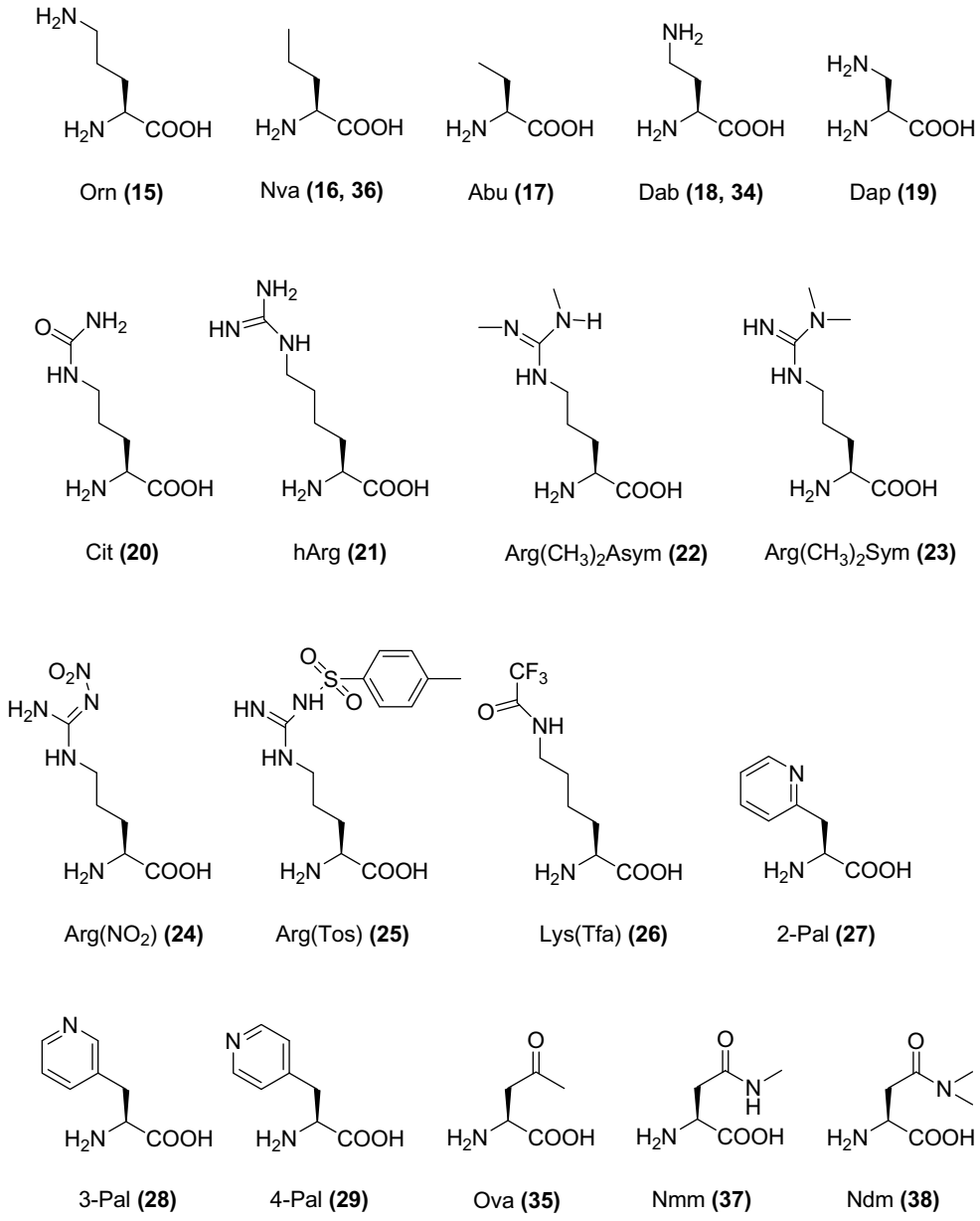

Figure 1. Chemical formula of the non-coded amino acids employed in this study.

Table 1
Effects of NPS and [X^3]NPS analogues in HEK293 cells expressing the mNPSR

No.	Compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM
	NPS	7.92 (7.83–8.01)	267 ± 10%
1	[Phe^3]NPS	Crc incomplete: at 10 μM 46 ± 6%	
2	[Tyr^3]NPS	Crc incomplete: at 10 μM: 73 ± 4%	
3	[His^3]NPS	5.81 (5.20–6.42)	273 ± 18%
4	[Leu^3]NPS	Crc incomplete: at 10 μM 36 ± 9%	
5	[Val^3]NPS	Crc incomplete: at 10 μM 129 ± 30%	
6	[Thr^3]NPS	6.06 (5.97–6.15)	272 ± 20%
7	[Ser^3]NPS	Crc incomplete: at 10 μM 175 ± 19%	
8	[Cys^3]NPS	6.43 (5.97–6.89)	256 ± 26%
9	[Gly^3]NPS	5.90 (5.71–6.09)	264 ± 7%
10	[Asn^3]NPS	Crc incomplete: at 10 μM 161 ± 33%	
11	[Gln^3]NPS	6.89 (6.76–7.02)	295 ± 21%
12	[Lys^3]NPS	7.74 (7.70–7.78)	290 ± 17%
13	[Asp^3]NPS	Crc incomplete: at 10 μM 73 ± 24%	
14	[Glu^3]NPS	Crc incomplete: at 10 μM 89 ± 3%	
15	[Orn^3]NPS	7.50 (7.24–7.76)	318 ± 11%
16	[Nva^3]NPS	6.87 (5.95–7.79)	207 ± 23%
17	[Abu^3]NPS	6.29 (5.43–7.15)	218 ± 17%
18	[Dab^3]NPS	6.69 (6.12–7.26)	207 ± 32%
19	[Dap^3]NPS	6.62 (6.32–6.87)	196 ± 17%
20	[Cit^3]NPS	6.78 (6.45–7.11)	285 ± 11%
21	[hArg^3]NPS	7.97 (7.70–8.24)	288 ± 16%
22	[Arg(Me)$_2$^{3}(Asym)]NPS	7.65 (7.52–7.78)	281 ± 15%
23	[Arg(Me)$_2$^{3}(Sym)]NPS	7.50 (7.40–7.60)	272 ± 10%
24	[Arg(NO$_2$)^3]NPS	7.66 (7.49–7.83)	288 ± 17%
25	[Arg(Tos)^3]NPS	Crc incomplete: at 10 μM 104 ± 16%	
26	[Lys(Tfa)^3]NPS	7.07 (6.60–7.54)	231 ± 18%
27	[2'-Pal^3]NPS	Crc incomplete: at 10 μM 19 ± 4%	
28	[3'-Pal^3]NPS	Crc incomplete: at 10 μM 42 ± 11%	
29	[4'-Pal^3]NPS	Crc incomplete: at 10 μM 154 ± 17%	

pEC_{50}: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect.
$CL_{95\%}$: 95% confidence limits.
E_{max}: the maximal effect elicited by the agonist expressed as % over the baseline.
SEM: standard error of the mean.
crc: concentration response curve.

Table 2
Effects of hNPS and [X^4]hNPS analogues in HEK293 cells expressing the mNPSR

No.	Compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM
	NPS	7.99 (7.65–8.33)	234 ± 12%
30	[Asp^4]NPS	Crc incomplete: at 10 μM 146 ± 6%	
31	[Phe^4]NPS	Crc incomplete: at 10 μM 118 ± 19%	
32	[Thr^4]NPS	6.99 (6.86–7.12)	253 ± 10%
33	[Gln^4]NPS	6.25 (5.65–6.85)	210 ± 19%
34	[Dab^4]NPS	Crc incomplete: at 10 μM 169 ± 18%	
35	[Ova^4]NPS	Crc incomplete: at 10 μM 136 ± 17%	
36	[Nva^4]NPS	Crc incomplete: at 10 μM 240 ± 39%	
37	[Nmm^4]NPS	Crc incomplete: at 10 μM 188 ± 18%	
38	[Ndm^4]NPS	Crc incomplete: at 10 μM 89 ± 12%	

pEC_{50}: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect.
$CL_{95\%}$: 95% confidence limits.
E_{max}: the maximal effect elicited by the agonist expressed as % over the baseline.
SEM: standard error of the mean.
crc: concentration response curve.

that of native NPS. These results, obtained by exchanging Arg^3 with coded amino acids, suggest that position 3 does not tolerate substitution with aromatic and aliphatic branched residues. The Arg^3 replacement with hydrophilic residues with short side chain produced in general low potency peptides (compounds **6-10**). Interestingly, while the substitution of Arg^3 with Gln (compound **11**) reduced peptide potency only by 10-fold, exchanges to Asn or Glu lead to inactive analogs. These results, together with the inactivity of [Val^3]NPS, indicate that a linear 3-carbon atom moiety of the amino acid side chain, which is similar in both Arg and Gln, but not in Val or Ala,[7,11] is important for binding the NPSR pocket that harbors the Arg^3 side chain. However, this same pocked does not tolerate an acidic side chain as demonstrated by the lack of activity of [Glu^3]NPS. Moreover, the data obtained by the replacement Arg/Gln suggested that basicity in position 3 is not crucial for bioactivity.

The shortening of the Lys side chain by one carbon atom (compound **15**) appears to be well tolerated producing a peptide only 2-fold less potent. In addition, the elimination of the primary amino function (compound **16**) produced a modest reduction of biological activity corroborating the hypothesis that basicity in position 3 is not a stringent requirement for NPSR interaction. Further shortening of the side chain (compound **17**) reduced peptide potency while the addition on the ethyl moiety of an amino function, as in Dab (compound **18**), produced an NPS analog with similar potency as compound **16**. Finally, the elimination of a methylene group from the side chain of **18** (compound **19**) produced negligible changes in biological activity. Collectively, these data indicate that the length of the side chain of the amino acid in position 3 and its linear shape seem to be more important for biological activity than its basic character. Surprisingly, the transformation of the guanidine function into urea (compound **20**) generated a peptide 10-fold less potent than NPS. Since compound **20** is also less potent than compound **12** or **15**, it is probable that the reduction of NPSR binding is due to the urea carbonyl (C=O), rather than to its non-basic character.

An increase in length of the side chain of Arg^3 (compound **21**) or its methylation (compounds **22** and **23**) are completely tolerated. The elimination of the basic character of the Arg^3 side chain obtained with compound **24** was tolerated while that obtained with compound **25** was not. Collectively these results corroborate the hypothesis that the guanidine moiety and its basicity are not particularly relevant for biological activity. In addition, the steric hindrance of the guanidine moiety does not seem to represent a particularly stringent chemical requirement since it can be enlarged (as in compounds **22**–**24**) without loss of potency. However, this does not apply for moieties as bulky as tosyl since compound **25** was found inactive.

The elimination of the Lys side chain basicity (compound **26**) produced a 3-fold decrease of activity compared to **12**; this could be due, at least in part, to the increase of the side chain steric hindrance, similar to what observed with compound **25**. Finally we inserted in position 3 amino acids with a pyridine nucleus as side chain, which combines in the same moiety aromatic character and a nitrogen atom able to accept hydrogen bond (compounds **27**–**29**). In line with previous findings (see compounds **1**–**3**) this chemical substitution was not tolerated, corroborating the evidence that aromaticity in position 3 is detrimental for NPSR binding.

Data obtained substituting Asn^4 in NPS with a limited series of coded and non-coded amino acids are summarized in Table 2. The replacement of position 4 with amino acids with acidic (compound **30**) or aromatic (compound **31**) side chains produced inactive derivatives. Previous findings[13] indicated that aromaticity in position 4 is detrimental for biological activity since [His^4]NPS was found more than 100 fold less potent than NPS. A 10-fold reduction of potency has been obtained by replacing Asn with Thr (compound **32**) while the Asn related amino acid Gln (compound **33**) produced a larger decrease of biological activity (55-fold). To investigate in details the contribution of the primary amide function of Asn^4 to biological activity, we substituted either alone (compound **34** and **35**) or in combination (compound **36**) the C=O and $-NH_2$ groups with $-CH_2$ and $-CH_3$, respectively. A profound loss of biological activity (>1000-fold) was observed with all these analogs suggesting a pivotal role of the amide function of the Asn^4 side chain for biological activity. In compounds **37** and **38** this moiety was mono- or dimethylated generating, also in this case, inactive

peptides. These latter modifications reduced or eliminated the hydrogen bond donor properties of the amide function and at the same time progressively increased the steric hindrance of position 4. The relative importance of these factors in the loss of NPSR binding can not be clearly unraveled. Collectively, these results, together with previous Ala- and D-amino acid scan indications[7,11], underline the importance of Asn[4] for the activity of NPS and suggest strict chemical requirements of the agonist binding pocket in NPSR allocating the side chain of this residue.

3. Conclusions

In the present SAR study, we investigated the chemical requirements of NPS position 3 (Arg) and 4 (Asn) for NPSR binding. Results related to position 3 suggest that (i) the guanidine moiety and its basic character are not crucial requirements, (ii) an aliphatic amino acid with a linear three carbon atom long side chain is sufficient to bind and fully activate NPSR, (iii) the receptor pocket allocating the side chain of position 3 can accommodate slightly larger side chains at least to a certain degree [hArg, Arg(NO_2) or Arg($Me)_2$ but not Arg(Tos)]. Position 4 seems to be more sensitive to amino acids replacement compared to position 3; in fact, all the amino acid replacements investigated produced an important decrease of biological activity or generated inactive derivatives suggesting a pivotal role of the Asn[4] side chain for NPS bioactivity.

4. Experimental

4.1. General information

Amino acids, protected amino acids, resins for solid phase synthesis and chemicals were purchased from Bachem, Novabiochem, Fluka or Chem-Impex International. Stock solutions (1 mM) of peptides were made in distilled water and kept at −20 °C until use. All other reagents were from Sigma (Poole, UK) or Merck (Darmstadt, Germany) and were of the highest purity available.

4.2. General procedures for the solid phase peptide synthesis

All peptides were synthesised following the procedures previously reported in details[12] using Fmoc/*t*-butyl chemistry with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten Germany). Fmoc-Ser(*t*Bu)-4-Benzyloxybenzyl Alcohol resin was used for the synthesis of all peptides. The peptides were cleaved from the resin using reagent B[16]; after filtration of the exhausted resin, the solvent was concentrated in vacuo, and the residue was triturated with ether.

4.3. Peptide purification and analytical determinations

Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with a Jupiter column C_{18} (250 × 30 mm, 300 A, 15 μm spherical particle size). The column was perfused at a flow rate of 25 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0% to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity and retention time (t_R) of the peptides were determined using two different HPLC conditions.

Retention time I was obtained using a Luna C_{18} column (4.6 × 100 mm, 3 μm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 0.5 mL/min using a linear gradient from 0% to 65% B over 25 min. Retention time II was obtained using a Hypersil BDS C_{18} column (4.6 × 250 mm, 5 μm particle size) with solvent A: 35 mM NaH_2PO_4 (pH 2.1) and solvent B: 59 mM NaH_2PO_4 (pH 2.1)-acetonitrile (60:40 v/v). The column was perfused at a flow rate of 1 mL/min with a linear gradient from 5% to 60% B over 25 min. All analogues showed >95% purity when monitored at 220 nm. Molecular weights of compounds were determined by a mass spectrometer ESI Micromass ZMD-2000, values are expressed as MH^+. The analytical properties of NPS analogues are reported in Table 3.

4.4. Calcium mobilization experiments

$HEK293_{mNPSR}$ cells were generated as previously described[2] and maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, Hygromycin (100 mg/L), and cultured at 37 °C in 5% CO_2 humidified air. $HEK293_{mNPSR}$ cells were prepared for calcium mobilization experiments as previously described.[12] Concentrated solutions (1 mM) of NPS and related peptides were made in bidistilled water and kept at −20 °C. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and master plate) into the fluorometric imaging plate reader FlexStation II (Molecular Devices, Sunnyvale, CA), fluorescence changes were measured at room temperature (≈22 °C). On-line additions were carried out in a volume of 50 μL/well.

Table 3
Analytical properties of the NPS analogues

No.	Abbreviated names	[a]t_R		[b]MH^+	
		I	II	Calculated	Found
	hNPS	11.88	19.42	2188.5	2188.2
1	[Phe^3]NPS	11.32	19.95	2179.5	2178.9
2	[Tyr^3]NPS	10.67	18.86	2195.5	2194.3
3	[His^3]NPS	10.82	19.07	2169.5	2168.7
4	[Leu^3]NPS	11.40	20.15	2145.5	2144.7
5	[Val^3]NPS	11.44	19.11	2131.5	2131.0
6	[Thr^3]NPS	10.76	19.06	2133.4	2133.2
7	[Ser^3]NPS	11.14	18.87	2119.4	2118.9
8	[Cys^3]NPS	11.83	19.27	2134.4	2135.0
9	[Gly^3]NPS	11.12	19.55	2089.4	2087.9
10	[Asn^3]NPS	10.42	18.38	2146.4	2145.1
11	[Gln^3]NPS	10.98	19.42	2160.5	2159.5
12	[Lys^3]NPS	10.86	19.04	2160.5	2158.9
13	[Asp^3]NPS	10.84	18.99	2147.4	2146.8
14	[Glu^3]NPS	11.04	19.52	2161.4	2160.6
15	[Orn^3]NPS	10.06	17.94	2146.5	2147.1
16	[Nva^3]NPS	11.77	19.38	2131.5	2131.4
17	[Abu^3]NPS	11.33	19.12	2117.4	2116.4
18	[Dab^3]NPS	10.87	19.06	2132.5	2132.0
19	[Dap^3]NPS	10.08	18.00	2118.5	2117.6
20	[Cit^3]NPS	10.15	18.03	2189.5	2190.1
21	[hArg^3]NPS	10.20	18.11	2202.5	2201.8
22	[Arg(Me)$_2$^{3}(Asym)]NPS	10.60	18.86	2215.5	2216.0
23	[Arg(Me)$_2$^{3}(Sym)]NPS	11.00	19.28	2215.5	2216.2
24	[Arg(NO$_2$)^3]NPS	10.11	18.07	2233.5	2188.9
25	[Arg(Tos)^3]NPS	13.62	22.02	2342.7	2342.4
26	[Lys(Tfa)^3]NPS	13.18	21.96	2256.5	2256.4
27	[2'-Pal^3]NPS	11.30	19.46	2180.5	2180.2
28	[3'-Pal^3]NPS	11.90	19.35	2180.5	2180.2
29	[4'-Pal^3]NPS	10.89	19.39	2180.5	2180.2
30	[Asp^4]NPS	9.63	11.79	2189.5	2189.2
31	[Phe^4]NPS	11.07	13.88	2221.6	2222.0
32	[Thr^4]NPS	10.87	13.32	2175.5	2175.6
33	[Gln^4]NPS	11.18	13.79	2202.6	2203.2
34	[Dab^4]NPS	9.51	11.64	2174.5	2175.0
35	[Ova^4]NPS	10.91	13.28	2186.5	2186.2
36	[Nva^4]NPS	8.64	10.78	2173.6	2173.8
37	[Nmm^4]NPS	9.79	12.01	2202.5	2202.2
38	[Ndm^4]NPS	9.65	11.82	2216.6	2216.8

[a] t_R is the retention time determined by analytical HPLC.
[b] The mass ion (MH^+) was obtained by electro spray mass spectrometry.

4.5. Data analysis and terminology

The data were expressed as mean ± SEM of at least four independent experiments made in duplicate. Maximum change in fluorescence, expressed in percent of baseline fluorescence, was used to determine agonist responses. Non-linear regression analysis using GraphPad Prism software (v.4.0) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonist potencies are given as pEC_{50} (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect). Differences in maximal effects between ligands were statistically analyzed via one-way analysis of variance followed by the Dunnett's test for multiple comparisons.

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References and notes

1. Xu, Y. L.; Reinscheid, R. K.; Huitron-Resendiz, S.; Clark, S. D.; Wang, Z.; Lin, S. H.; Brucher, F. A.; Zeng, J.; Ly, N. K.; Henriksen, S. J.; de Lecea, L.; Civelli, O. *Neuron* **2004**, *43*, 487.
2. Reinscheid, R. K.; Xu, Y. L.; Okamura, N.; Zeng, J.; Chung, S.; Pai, R.; Wang, Z.; Civelli, O. *J. Pharmacol. Exp. Ther.* **2005**, *315*, 1338.
3. Xu, Y. L.; Gall, C. M.; Jackson, V. R.; Civelli, O.; Reinscheid, R. K. *J. Comp. Neurol.* **2007**, *500*, 84.
4. Leonard, S. K.; Dwyer, J. M.; Sukoff Rizzo, S. J.; Platt, B.; Logue, S. F.; Neal, S. J.; Malberg, J. E.; Beyer, C. E.; Schechter, L. E.; Rosenzweig-Lipson, S.; Ring, R. H. *Psychopharmacology (Berl.)* **2008**, *197*, 601.
5. Rizzi, A.; Vergura, R.; Marzola, G.; Ruzza, C.; Guerrini, R.; Salvadori, S.; Regoli, D.; Calo, G. *Br. J. Pharmacol.* **2008**, *154*, 471.
6. Okamura, N.; Habay, S. A.; Zeng, J.; Chamberlin, A. R.; Reinscheid, R. K. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 893.
7. Roth, A. L.; Marzola, E.; Rizzi, A.; Arduin, M.; Trapella, C.; Corti, C.; Vergura, R.; Martinelli, P.; Salvadori, S.; Regoli, D.; Corsi, M.; Cavanni, P.; Calo, G.; Guerrini, R. *J. Biol. Chem.* **2006**, *281*, 20809.
8. Smith, K. L.; Patterson, M.; Dhillo, W. S.; Patel, S. R.; Semjonous, N. M.; Gardiner, J. V.; Ghatei, M. A.; Bloom, S. R. *Endocrinology* **2006**, *30*, 30.
9. Beck, B.; Fernette, B.; Stricker-Krongrad, A. *Biochem. Biophys. Res. Commun.* **2005**, *332*, 859.
10. Cline, M. A.; Godlove, D. C.; Nandar, W.; Bowden, C. N.; Prall, B. C. *Comp. Biochem. Physio.l A Mol. Integr. Physiol.* **2007**, *148*, 657.
11. Bernier, V.; Stocco, R.; Bogusky, M. J.; Joyce, J. G.; Parachoniak, C.; Grenier, K.; Arget, M.; Mathieu, M. C.; O'Neill, G. P.; Slipetz, D.; Crackower, M. A.; Tan, C. M.; Therien, A. G. *J. Biol. Chem.* **2006**, *281*, 24704.
12. Camarda, V.; Trapella, C.; Calo, G.; Guerrini, R.; Rizzi, A.; Ruzza, C.; Fiorini, S.; Marzola, E.; Reinscheid, R. K.; Regoli, D.; Salvadori, S. *J. Med. Chem.* **2008**, *51*, 655.
13. Tancredi, T.; Guerrini, R.; Marzola, E.; Trapella, C.; Calo, G.; Regoli, D.; Reinscheid, R. K.; Camarda, V.; Salvadori, S.; Temussi, P. A. *J. Med. Chem.* **2007**, *50*, 4501.
14. Carpino, L. A.; Han, G. A. *J. Org. Chem.* **1972**, *37*, 3404.
15. Marcaurelle, L. A.; Bertozzi, C. A. *Tetrahedron Lett.* **1998**, *39*, 7279.
16. Sole', N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399.