Synthesis and Biological Activity of Human Neuropeptide S Analogues Modified in Position 2

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Received September 25, 2007

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 Phe^2 which has been replaced with a series of coded and noncoded amino acids. Thirty-one human NPS analogues were synthesized and pharmacologically tested for intracellular calcium mobilization by using HEK293 cells stably expressing the mouse NPSR. The results of this study demonstrated the following NPS position 2 structure–activity features: (i) lipophilicity but not aromaticity is crucial, (ii) both the size of the chemical moiety and its distance from the peptide backbone are important for biological activity, and (iii) this position plays a role in both receptor binding and activation, since [4,4′-biphenyl-Ala^2]hNPS behaved as a partial agonist.

Introduction

Neuropeptide S (NPS[a])[1] is the last endogenous peptide identified via the reverse pharmacology approach.[2] The human form of NPS is a 20 residue peptide of the following primary sequence, SFRNGVGTGMKKTSFQRAKS, which is highly conserved among species. The serine (S) N-terminal residue of NPS is perfectly conserved across all species thus far examined.[3] After its pairing with NPS, the previously orphan G-protein-coupled receptor (GPCR) GPR154 was named the NPS receptor and abbreviated as NPSR.[1,4] NPSR shows low homology to other members of the GPCR family. In situ hybridization reveals that the 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 brain stem.[5] Cells stably expressing NPSR display a transient increase in the initial Ca^{2+} concentration levels in response to nanomole concentrations of NPS, thus suggesting that NPS behaves as an excitatory transmitter.[1] When injected supraspinally in rodents, NPS produces anxiolytic-like effects associated with stimulation of wakefulness.[1] The biological originality of this peptide−receptor system relies on its combined effects on anxiety and arousal, a behavioral profile that differs from those of all the known anxiolytic and arousal promoting agents.[6]

Future studies in the field of NPS and its receptor may substantially increase our knowledge about arousal and anxiety and possibly other brain functions. The development of selective ligands for NPSR is now mandatory to investigate the functions of this novel neurotransmitter system.

Previous structure–activity relationship (SAR) and conformational studies consistently demonstrated that the N-terminal part of the peptide is crucial for biological activity.[4,7–9] In particular, based on findings obtained from Ala- and D-amino acid-scan studies, Phe^2-Arg^3-Asn^4 were identified as the most important residues in the hNPS sequence.[7,8] In this paper we present results obtained both in a SAR study performed by replacing Phe^2 of hNPS with coded amino acids and in a more focused second round of synthesis performed by replacing Phe^2 with a series of noncoded Phe analogues whose structures are reported in Figure 1. These Phe analogues were used with the aim of examining in detail the effects on the hNPS biological activity of the following: (i) modifications of the aromaticity, length, size, and position of the amino acid side chain, (ii) conformational restrictions, and (iii) insertion of different chemical groups in the phenyl ring. By altering the electronic, steric, and lipo/hydrophilic features of this residue, these modifications may provide useful indications for the design of novel NPSR ligands.

Results and Discussion

Compounds **1**–**31** and the reference compound hNPS, whose chemicophysical properties are available as Supporting Information, were prepared with good yield by solid phase peptide synthesis. For the synthesis of compound **26**, the amino acid 2-aminotetralin-2-carboxylic acid (Atc) was employed as a racemic mixture, the corresponding [Atc^2]hNPS diastereomers were not fully separated in preparative high performance liquid chromatography (HPLC), and therefore they were evaluated pharmacologically as a diastereomeric mixture. All of the compounds were tested for intracellular calcium mobilization by using HEK293 cells stably expressing mouse NPSR in the fluorometric imaging plate reader FlexStation II.

In the first round of synthesis (Table 1) we explored the SAR requirements of hNPS position 2 by replacing Phe with some selected proteinogenic amino acids with aromatic, lipophilic, and hydrophilic side chains.

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[a] Abbreviations: DMF, *N*,*N*-dimethylformamide; GPCR, G-protein-coupled receptor; 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; NMM, 4-methylmorpholine; NPS, neuropeptide S; NPSR, NPS receptor; TFA, trifluoroacetic acid; SAR, structure–activity relationship.

10.1021/jm701204n CCC: $40.75 © 2008 American Chemical Society
Published on Web 01/09/2008

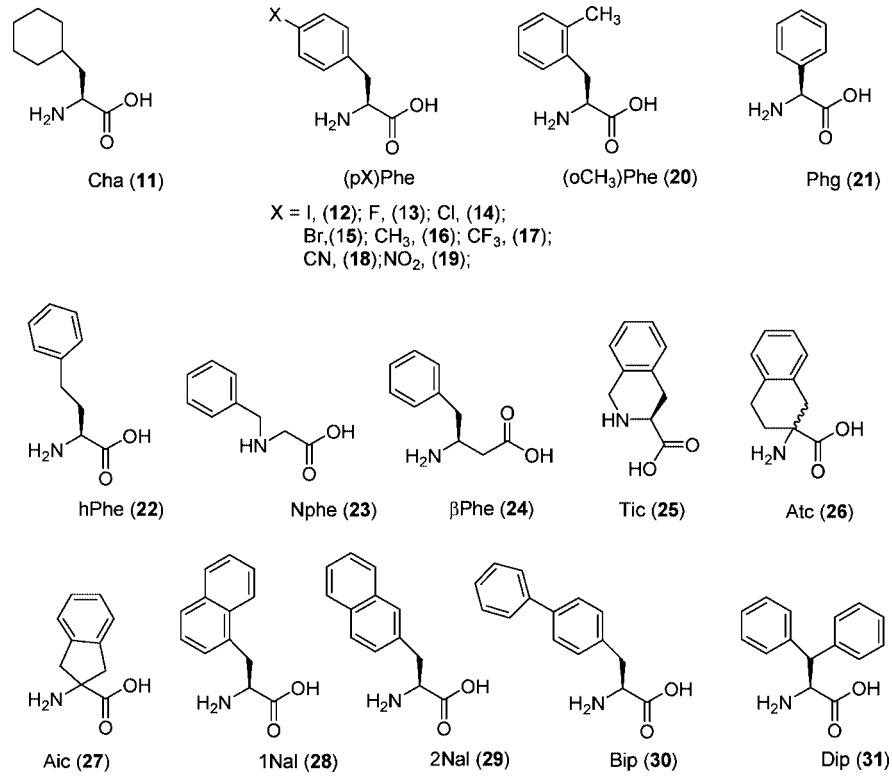

Figure 1. Chemical formulas of the noncoded amino acids employed in this study.

Table 1. Effects of hNPS and [X^2]hNPS Analogues Substituted with Coded Residues in HEK293 Cells Expressing the mNPSR[a]

no.	compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM
	hNPS	8.96 (8.81–9.11)	270 ± 12%
1	[His^2]hNPS	6.88 (6.79–6.97)	288 ± 5%
2	[Tyr^2]hNPS	8.63 (8.38–8.88)	312 ± 5%
3	[Trp^2]hNPS	8.65 (8.45–8.85)	323 ± 4%
4	[Leu^2]hNPS	8.08 (7.92–8.24)	282 ± 15%
5	[Lys^2]hNPS	crc incomplete: at 10 μmol 103 ± 15%	
6	[Asp^2]hNPS	crc incomplete: at 10 μmol 39 ± 3%	
7	[Glu^2]hNPS	crc incomplete: at 10 μmol 170 ± 20%	
8	[Asn^2]hNPS	crc incomplete: at 10 μmol 49 ± 2%	
9	[Gln^2]hNPS	crc incomplete: at 10 μmol 54 ± 6%	
10	[Thr^2]hNPS	crc incomplete: at 10 μmol 114 ± 5%	

[a] pEC_{50}: 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}: Maximal effect elicited by the agonist expressed as percent over the baseline. SEM: Standard error of the mean. crc: Concentration response curve.

In the calcium mobilization assay hNPS increased the initial Ca^{2+} concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 8.96 and 270% over the basal ones, respectively. The replacement of Phe^2 with His (compound **1**) produced a 100-fold reduction in potency while that with Tyr (compound **2**) and Trp (compound **3**) generated only a slight decrease in biological activity. The nonaromatic lipophilic residue Leu produced a peptide (compound **4**) about 10-fold less potent than hNPS. The series of hydrophilic residues with basic (compound **5**), acidic (compounds **6** and **7**), and neutral (compounds **8–10**) side chains produced a drastic (>3 log unit) loss of potency. These results suggested that the lipophilicity of the side chain of the residue in position 2 is indeed a crucial requirement for the NPSR interaction. However, steric hindrance is also important since Ala/Phe^2 substitution has been consistently reported[7,8] to be highly detrimental for biological activity. Although aromaticity is not strictly required, none of the investigated hNPS analogues displayed higher potency than the natural sequence, thus indicating that the benzyl moiety of Phe^2 is the most effective chemical structure in promoting receptor binding.

On the aforementioned bases, a second round of synthesis was planned to investigate in details the SAR requirements of the Phe^2 benzyl moiety. The biological results of this study are summarized in Table 2. The elimination of aromaticity (compound **11**) is well tolerated and generates an hNPS analogue equipotent to the natural ligand. Data obtained with compound **11** confirmed those relative to compound **4** indicating that aromaticity in position 2 is not crucial for the NPSR interaction. However, cyclic lipophilicity seems to be favored, since [Cha^2]hNPS is equipotent to the reference peptide while 6-fold more potent than [Leu^2]hNPS.

The introduction, into the para position of the phenyl ring, of an atom of the halogen series (compounds **12–15**) as well as of chemical groups with electron donor (compound **16**) or withdrawing (compounds **17–19**) properties seemed to be well tolerated, and it generated hNPS analogues with similar potency to that of the natural sequence. Similarly, the introduction of a methyl group into the ortho position of the phenyl ring (compound **20**) did not affect the biological activity at NPSR. Collectively, these data indicate that the modulation of the

Table 2. Effects of hNPS and [X^2]hNPS Analogues Substituted with Noncoded Residues in HEK293 Cells Expressing the mNPSR[a]

no.	compound	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM
	hNPS	8.93 (8.86–9.00)	260 ± 12%
11	[Cha^2]hNPS	8.87 (8.29–9.45)	221 ± 16%
12	[(pI)Phe^2]hNPS	8.75 (8.54–8.96)	243 ± 25%
13	[(pF)Phe^2]hNPS	8.96 (8.72–9.20)	291 ± 21%
14	[(pCl)Phe^2]hNPS	9.15 (8.81–9.49)	298 ± 27%
15	[(pBr)Phe^2]hNPS	9.19 (8.93–9.45)	309 ± 26%
16	[(pCH_3)Phe^2]hNPS	9.17 (8.77–9.57)	317 ± 18%
17	[(pCF_3)Phe^2]hNPS	8.59 (8.34–8.84)	286 ± 12%
18	[(pCN)Phe^2]hNPS	8.48 (8.19–8.77)	296 ± 14%
19	[(pNO_2)Phe^2]hNPS	8.44 (8.24–8.64)	290 ± 10%
20	[(oCH_3)Phe^2]hNPS	8.73 (7.89–9.57)	222 ± 40%
21	[Phg^2]hNPS	crc incomplete: at 10 μmol 141 ± 24%	
22	[hPhe^2]hNPS	8.38 (7.43–9.33)	216 ± 23%
23	[Nphe^2]hNPS	7.81 (7.25–8.37)	246 ± 13%
24	[βPhe^2]hNPS	crc incomplete: at 10 μmol 177 ± 24%	
25	[Tic^2]hNPS	8.09 (8.01–8.17)	244 ± 19%
26	[Atc^2]hNPS	7.95 (7.30–8.60)	257 ± 27%
27	[Aic^2]hNPS	7.47 (6.84–8.10)	211 ± 34%
28	[1Nal^2]hNPS	8.71 (8.24–9.18)	232 ± 6%
29	[2Nal^2]hNPS	8.66 (8.44–8.88)	213 ± 14%
30	[Bip^2]hNPS	7.70 (6.98–8.42)	170 ± 19%[b]
31	[Dip^2]hNPS	crc incomplete: at 10 μmol 130 ± 31%	

[a] pEC_{50}: 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}: Maximal effect elicited by the agonist expressed as percent over the baseline. SEM: Standard error of the mean. crc: Concentration response curve. [b] $p < 0.05$ vs NPS according to one-way analysis of variance followed by the Dunnett test.

electronic asset of the benzyl side chain of Phe^2 is not important for the NPSR interaction.

Changes of the distance between the Phe^2 phenyl ring and the peptide backbone obtained by eliminating (compound **21**) or adding (compound **22**) a methylene group produced very different results. In fact, compound **21** was almost inactive up to 10 μM while compound **22** was only 3-fold less potent than hNPS. The shift of the Phe^2 benzyl moiety with respect to the peptide backbone produced a 10-fold less active derivative in the case of N-shift (compound **23**) while substituting Phe with β-Phe (compound **24**) generated an inactive derivative. These results together with the detrimental effect obtained by changing the chirality of Phe^{27} demonstrated that the spatial disposition of the phenyl moiety relative to the peptide backbone was indeed very important for biological activity. This was further corroborated by the results obtained with the introduction in position 2 of constrained Phe analogues. In fact, the cyclization of the benzyl side chain on the nitrogen (compound **25**) or on the Phe chiral carbon (compounds **26** and **27**) produced NPS derivatives approximately 10-fold less potent than hNPS. The addition of a further phenyl ring on the Phe^2 side chain was well tolerated in the case of Nal isomers (compounds **28** and **29**) but not in the case of [Bip^2]hNPS (compound **30**) or [Dip^2]hNPS (compound **31**), which were >10- and >1000-fold less potent than the parent peptide, respectively. Collectively, these results suggest that the NPSR ligand-binding pocket allocating the side chain of Phe^2 does not show particularly stringent hindrance requirements. In fact, among the enlarged aromatic side chain analogues tested, only compound **31** completely lost biological activity.

Interestingly, compound **30** displays a statistically significant reduction of efficacy, with its maximal effect being only 62% of those elicited by the natural peptide (Figure 2). This suggests that the correct position of the phenyl ring of Phe^2 into the NPSR ligand-binding pocket is important not only for binding but also for receptor activation. Other NPSR ligands with reduced efficacy have been described by substituting Arg^3 with Ala^7 and Gly^5 with 2-aminoisobutyric acid (Aib) or D-Ala.[9] Collectively, these findings corroborate the proposal[7,8] that the N-terminal part of hNPS represents the message domain of this peptide.

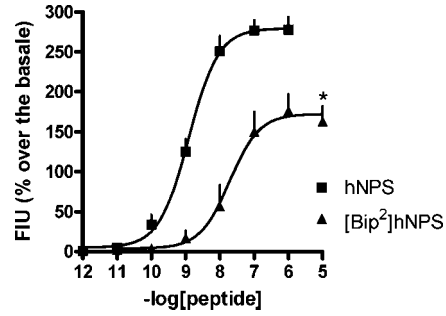

Figure 2. Concentration−response curves to hNPS and [Bip^2]hNPS obtained in the same plates of HEK293 cells stably expressing the mouse NPSR. Data are mean ± SEM of four experiments made in duplicate. *$p < 0.05$ vs hNPS according to the Student t test for unpaired data.

In conclusion, the present SAR studies performed on position 2 of hNPS demonstrated the following: (i) Lipophilicity but not aromaticity is crucial, and a cyclic lipophilic side chain seems to be favored. (ii) Both the size of the chemical moiety and its distance or position from the peptide backbone are important for biological activity. In particular, the size can be enlarged up to a naphthyl or reduced down to an isobutyl moiety while the distance can only be increased by one carbon atom without major changes of biological activity. (iii) Finally, position 2 plays a role in both receptor binding and activation, as demonstrated by the reduction in efficacy displayed by [Bip^2]hNPS.

Experimental Section

Materials. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka, or Chem-Impex International. All other reagents were from Sigma Chemical Co. (Poole, U.K.) or E. Merck (Darmstadt, Germany) and were of the highest purity available.

General Procedures for the Solid Phase Peptide Synthesis. As an illustrative example, the synthesis of hNPS is described. Fmoc-Ser(tBu)-4-benzyloxybenzyl alcohol resin (Fmoc-Ser(tBu)-Wang resin) (0.62 mmol/g, 0.2 g) was treated with 20% piperine/*N*,*N*-dimethylformamide (DMF) and linked with Fmoc-Lys

(Boc)-OH by using [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) as the coupling reagent. The following N^{α}-Fmoc amino acids were sequentially coupled to the growing peptide chain: Fmoc-Ala-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Asn(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Phe-OH, and Fmoc-Ser(tBu)-OH. All of the N^{α}-Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using HATU (4 equiv) in DMF in the presence of an equimolar concentration of 4-methylmorpholine (NMM); the coupling reaction time was 1 h. To improve the analytical profile of the crude peptide, capping with acetic anhydride (0.5 M/DMF) in the presence of NMM (0.25 M/DMF) (3:1 v/v; 2 mL/0.2 g of resin) was performed at any step. To remove the Fmoc group at every step, 20% piperidine/DMF was used. The peptide resin was washed with methanol and dried in vacuo to yield the protected hNPS resin. The other peptides **1**–**31** were synthesized in a similar manner. The protected peptide resin was treated with reagent B[10] (trifluoroacetic acid (TFA)/H_20/phenol/triisopropylsilane 88:5:5:2; v/v; 10 mL/0.2 g of resin) for 1.5 h at room temperature. After filtration of the resin, the solvent was concentrated in vacuo, and the residue was triturated with ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

Peptide Purification and Analytical Determinations. Crude peptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Jupiter column C_{18} (250 mm × 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 at 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. The analytical purity and retention time (t_R) of the peptides were determined using two different HPLC conditions.

Retention time I was obtained using a Nucleodur C_{18} column (4.6 mm × 100 mm, 2 μm particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 0.6 mL/min using a linear gradient from 0% to 70% B over 25 min. Retention time II was obtained using a Hypersil BDS C_{18} column (4.6 mm × 150 mm, 5 μm particle size) with solvent A (35 mM NaH_2PO_4 at pH 2.1) and solvent B (59 mM NaH_2PO_4 at pH 2.1 and 60:40 v/v acetonitrile). The column was perfused at a flow rate of 1 mL/min with a linear gradient from 5% to 65% B over 25 min. The amino acid Atc was used, for the synthesis of the corresponding hNPS analogue **26**, as a racemic mixture. The preparative HPLC separation of the two diastereomers was not base peak resolved, and consequently this compound was purified and tested as a diastereomeric mixture. The retention time reported in the table available in the Supporting Information is the mean of the retention time of the two peaks. All analogues showed >95% purity when monitored at 220 nm. Molecular weights of compounds were determined with a mass spectrometer ESI Micromass ZMD-2000; values are expressed as MH^+. The analytical properties of the hNPS analogues are available in the Supporting Information.

Calcium Mobilization Experiments. Cell culture HEK293 cells stably expressing the mouse recombinant NPSR ($HEK293_{mNPSR}$) were generated as previously described,[4] maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and Hygromycin (100 mg/L)and, cultured at 37 °C in 5% CO_2 humidified air.

$HEK293_{mNPSR}$ was seeded at a density of 50 000 cells/well into poly D-lysine coated, 96-well, black, clear-bottom plates. The following day, the cells were incubated with medium supplemented with 2.5 mmol of probenecid, 3 μmol of the calcium sensitive fluorescent dye Fluo-4 AM, and 0.01% pluronic acid for 30 min at 37 °C. After that time, the loading solution was aspirated, and 100 μL/well of assay buffer (Hank's balanced salt solution; HBSS) supplemented with 20 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 mmol of probenecid, and 500 μmol of Brilliant Black (Aldrich) were added. Concentrated solutions (1 mmol) of hNPS and related peptides were made in bidistilled water and kept at −20 °C. Serial dilutions were carried out in HBSS/HEPES (20 mmol) buffer (containing 0.02% BSA fraction V) in order to prepare a master plate at 3× concentration. 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.

Data Analysis and Terminology. The data were expressed as mean ± SEM of at least four independent experiments made in duplicate. The maximum change in fluorescence, expressed in percent of baseline fluorescence, was used to determine the agonist response. Nonlinear 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 test for multiple comparisons or the Student *t* test for unpaired data, as specified in the table and figure legends.

Acknowledgment. This work was supported by funds from the Italian Ministry of the University (Cofin 2006 grant to S.S. and G.C.)

Supporting Information Available: Table reporting the retention time determined by analytical HPLC analyses using two different chromatographic systems and the calculated and found molecular weight. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM701204N