Structure-Activity Studies on Neuropeptide S
IDENTIFICATION OF THE AMINO ACID RESIDUES CRUCIAL FOR RECEPTOR ACTIVATION*

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Neuropeptide S (NPS) has been recently recognized as the endogenous ligand for the previous orphan G-protein-coupled receptor GPR154, now referred to as the NPS receptor (NPSR). The NPS-NPSR receptor system regulates important biological functions such as sleeping/wakening, locomotion, anxiety, and food intake. To collect information on the mechanisms of interaction between NPS and its receptor, a classical structure-activity relationship study was performed. Human (h) NPS derivatives obtained by Ala and D-scan and N- and C-terminal truncation were assessed for their ability to stimulate calcium release in HEK293 cells expressing the human recombinant NPSR. The results of this study indicate that (i) the effect of hNPS is mimicked by the fragment hNPS-(1–10); (ii) Phe^2, Arg^3, and Asn^4 are crucial for biological activity; (iii) the sequence Thr^8-Gly^9-Met^{10} is important for receptor activation, although with non-stringent chemical requirements; and (iv) the sequence Val^6-Gly^7 acts as a hinge region between the two above-mentioned domains. However, the stimulatory effect of hNPS given intracerebroventricularly on mouse locomotor activity was not fully mimicked by hNPS-(1–10), suggesting that the C-terminal region of the peptide maintains importance for in vivo activity. In conclusion, this study identified the amino acid residues of this peptide most important for receptor activation.

Many drugs produce therapeutic activities by interacting with G-protein-coupled receptors (GPCRs),[3] which represent, at present, the most important biological target for drug discovery (1, 2). This may remain true in the future, as at least 800 different genes coding for putative GPCRs have been identified in the human genome, ~360 of which encode transmitter GPCRs (3), yet the endogenous ligand is known for only ~240 receptors, whereas the others are still orphans (2). Novel drugs acting on orphan GPCRs are likely to provide innovative treatments for a variety of pathological conditions and diseases. The first step in understanding the function and the potential of an orphan receptor as drug target is the identification of its endogenous ligand. In the last 10 years, the reverse pharmacology technique (4), i.e. the use of a recombinant orphan GPCR as a target for identifying its endogenous ligand, has been validated as a successful approach for the identification of novel transmitter systems. In particular, several novel peptide receptor systems have been identified with such an approach, including nociceptin/orphanin FQ, prolactin-releasing peptide, orexins, apelin, ghrelin, urotensin II, metastatin, neuropeptide B, neuropeptide W, and neuropeptide FF and their receptors (for a recent review, see Ref. 2). These novel peptide receptor systems have been demonstrated to play important roles in regulating several biological functions, e.g. food intake, pain transmission, cardiovascular homeostasis, sleep behavior, stress responses, and drug reward (4). One of the latest neuropeptides identified by the reverse pharmacology approach is neuropeptide S (NPS) (5, 6). Human (h) NPS is a 20-residue peptide with the primary sequence SFRNGVGTGMKKTSFQRAKS, which is highly conserved across species. The N-terminal serine residue, which is present in all species analyzed so far, gave the name to this novel neuropeptide. As with other neuropeptides, hNPS is cleaved from a larger precursor protein containing a hydrophobic signal peptide and proteolytic processing sites (7). hNPS (as well as the rat and mouse isoforms of this peptide) selectively binds and activates a previous orphan GPCR known as GPR154 (GenBank™ accession number NM20127), which shows low homology to other members of the GPCR family (5). After its pairing with NPS, the GPR154 receptor was renamed the NPS receptor and abbreviated as NPSR by Civelli and co-workers (5). In this study, we have adopted the abbreviations NPS for the peptide and NPSR for its receptor; however, we would like to emphasize that the latter abbreviation has to be considered provisional because it is not in line with the recommendations of the International Union of Basic and Clinical Pharmacology for receptor nomenclature (8): the receptor name should not include the letter R as an abbreviation for receptor.

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[3] The abbreviations used are: GPCRs, G-protein-coupled receptors; NPS, neuropeptide S; h, human; NPSR, neuropeptide S receptor; HEK, human embryonic kidney; FLIPR, fluorescence imaging plate reader; WT, wild-type; Fmoc, N-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance.

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In situ hybridization studies revealed that NPSR mRNA is widely expressed throughout the nervous system. In contrast, NPS precursor protein mRNA is strongly expressed only in the locus coeruleus area and in few nuclei of the brainstem (5). hNPS binds with high affinity (K_d = 0.33 nM) to the hNPSR expressed in HEK293 cells, where it increases the intracellular calcium levels with high potency (EC_{50} = 9.4 nM) (5). *In vivo*, NPS injected intracerebroventricularly in mice at subnanomolar doses stimulates spontaneous locomotor activity and consistently induces anxiolytic-like effects in a battery of behavioral tests (open field, light-dark, elevated plus maze, marble burying) (5). These anxiolytic-like properties of NPS were recently confirmed in the mouse four-plate model and demonstrated to be mediated by a non-benzodiazepine mechanism (9). Moreover, NPS given intracerebroventricularly suppresses all stages of sleep in rats, indicating that this peptide may induce wakefulness (5). The profile of behavioral effects evoked by the intracerebroventricular administration of NPS (stimulation of arousal and wakefulness together with anxiolytic-like effects) appears to be quite unique because stimulants such as amphetamine and cocaine potently induce arousal but, at the same time, are anxiogenic. On the other hand, anxiolytics such as benzodiazepines produce sedative effects and are widely used as hypnotics. A variant form of the NPSR was recently identified as a candidate gene for asthma susceptibility (10); in addition, the NPSR was found to be significantly up-regulated in mouse lung after ovalbumin challenge in sensitized compared with non-sensitized mice (10). An N107I variant of the NPSR was recently characterized pharmacologically, and the mutation was demonstrated to cause a gain of function and to lead to an increase in agonist potency without affecting binding affinity (11). Finally, in line with NPSR expression in the hypothalamus, a key brain region for the regulation of food intake, a recent publication demonstrated an inhibitory effect of NPS given intracerebroventricularly on food intake in rats (12).

In summary, the NPS-NPSR system seems to play an important role in regulating central and peripheral functions; the development of NPSR-selective ligands is now needed to determine the therapeutic potential of novel drugs interacting with this receptor. NPSR agonists might have clinical application in the treatment of obesity, hypersomnia/narcolepsy, and anxiety disorders without causing sedation.

In this work, we report the results of a classical structure-activity relationship study in which individual amino acids of the primary sequence were replaced with an alanine residue (Ala-scan) or the corresponding enantiomer (D-scan). Moreover, the N- and C-terminal portions of the peptide were progressively shortened (N- and C-terminal truncation) by single amino acid residue elimination. An HEK293 clonal cell line stably expressing the hNPSR was generated, and a functional assay was developed. All the NPS-related peptides were assayed for their ability to activate or inhibit using hNPSR-mediated Ca^{2+} mobilization. hNPS-(1–10) was identified as the minimum sequence required to activate the hNPSR *in vitro* with similar potency and efficacy as full-length hNPS. In addition, the *in vivo* effects of hNPS and its analog hNPS-(1–10) on locomotion were assessed after intracerebroventricular administration in mice.

EXPERIMENTAL PROCEDURES

Generation of a Stable HEK293 Clonal Cell Line Expressing Human GPR154—The open reading frame of the hNPSR (human GPR154, GenBank™ accession number NM_20127) was cloned into the expression vector pcDNA3.1 (Invitrogen). HEK293 cells were transfected by the FuGENE 6 method (Roche Applied Science, Basel, Switzerland) at a plasmid/reagent ratio of 1:3. On day 2 post-transfection, Geneticin (400 μg/ml) was added to the culture medium. After 6 days of selection, clonal foci of cells expressing the Geneticin resistance gene encoded by the pcDNA3.1-hNPSR plasmid were selected and expanded. Functional expression of the hNPSR was evaluated by measuring NPS-stimulated calcium response over several passages in 10 different clones. On the basis of the robustness of the calcium response to hNPS measured by the fluorescence imaging plate reader (FLIPR) assay, clone 6 of HEK293 cells expressing the hNPSR (referred to as HEK293/hNPSR cells) was chosen for all further studies.

Cell Culture—HEK293/hNPSR cells were maintained in minimal essential medium containing Earle's salts supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, and 400 μg/ml Geneticin. Wild-type (WT) HEK293 cells were maintained in the same medium in the absence of Geneticin. Both cell lines were cultured at 37 °C and 5% CO_2 in a humidified environment.

FLIPR Experiments—HEK293/hNPSR and WT HEK293 cells were seeded at a density of 50,000 cells/well into poly-D-lysine-coated, 96-well black wall, clear bottom plates (BioCoat, BD Biosciences). The day after, the medium was removed by aspiration, and labeling medium (100 μl/well; cell culture medium supplemented with 20 mM HEPES, 2.5 mM probenecid, 2 μM fluo-4 acetoxymethyl ester (Invitrogen), and 0.01% pluronic acid) was added. After 45 min at 37 °C, the labeling medium was aspirated, and assay buffer (100 μl/well; Hanks' balanced salt solution supplemented with 20 mM HEPES, 2.5 mM probenecid, and 500 μM brilliant black) was added. Peptides were dissolved in Hanks' balanced salt solution/HEPES (20 mM), and serial dilutions were carried out in Hanks' balanced salt solution/HEPES (20 mM) containing 0.02% (w/v) bovine serum albumin (fraction V) to prepare a master plate at a 3-fold concentration. Carbachol (10 μM final concentration), which stimulates muscarinic receptors endogenously expressed by HEK293 cells, was included in each FLIPR experiment for evaluating cell viability and responsiveness. Fluorescence changes were measured (excitation λ = 488 nm and emission λ = 510 nm) at room temperature. On-line additions were carried out in a volume of 50 μl/well. The maximum change in fluorescence over the base line was used to determine agonist response. The agonist activity of hNPS analogs was evaluated by generating concentration-response curves in the range of 1 pM to 1 μM. Antagonist activity was investigated by generating inhibition curves for hNPS analogs (1 pM to 1 μM concentration range, 10-min preincubation time) against the stimulatory effect elicited by 30 nM hNPS (which corresponds approximately to the EC_{80}) (see Fig. 1).

Mouse Spontaneous Locomotor Activity—Male Swiss albino mice (Morini Farm, Reggio Emilia, Italy) weighing 30–35 g were used. They were housed in 425 × 266 × 155-mm cages
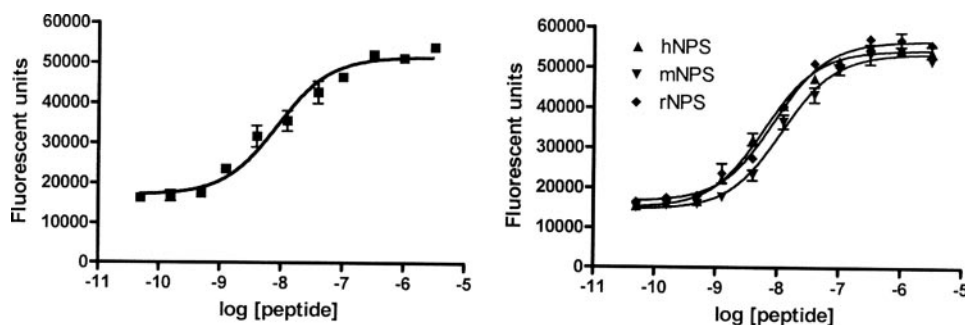
FIGURE 1. **FLIPR calcium assay.** Shown are the concentration-response curves of Ca^{2+} mobilization induced by human NPS synthesized in-house (*left panel*) compared side by side with the commercially available human, mouse (*m*), and rat (*r*) peptides (*right panel*) in an HEK293 clonal cell line expressing the hNPSR. The data are the means ± S.E. of a single experiment performed in triplicate.

(Tecniplast, Mantova, Italy), 9 animals/cage, under standard conditions (22 °C, 55% humidity, 12-h light-dark cycle, light on at 7:00 a.m.) with food (Mangime Italiano da Laboratorio, standard diet, Morini Farm) and water *ad libitum* for at least 7 days before experiments began. Mice were intracerebroventricularly injected (injection volume of 2 μl) under light ether anesthesia (*i.e.* just sufficient for losing the righting reflex) using the "free hand" technique described by Laursen and Belknap (13). In brief, a 27-gauge needle attached via a polyethylene tube to a 10-μl Hamilton syringe was used for the injection at an ~45° angle at 2 mm lateral to the bregma midline. Each mouse received only one intracerebroventricular injection. Experiments were carried out between 9:00 a.m. and 1:00 p.m. Dose-response curves for hNPS (dose range of 0.01–1 nmol) and hNPS-(1–10) (dose range of 0.1–10 nmol) were generated in mice acclimatized to the test chamber for 1 h prior to the injection. Locomotor activity was assessed using Basile activity cages as described previously (14). At least 12 mice were randomly assigned to each treatment.

Peptide Synthesis—hNPS and its analogs were synthesized according to published methods (15) using Fmoc/t-butyl chemistry with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten Germany). Preloaded Fmoc 4-benzyloxybenzyl ester resin (Fluka, Buchs, Switzerland) 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. Crude peptides were purified by preparative reversed-phase HPLC, and the purity grade was checked by analytical HPLC analyses and mass spectrometry using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker BioScience Corp., Billerica, MA) and an electrospray ionization Micromass ZMD-2000 mass spectrometer (Waters).

Drugs and Reagents—Mouse and rat NPS were purchased from Phoenix Pharmaceuticals, Inc. (Belmont, CA). hNPS was also purchased from the same supplier for comparison with the homemade peptide. Cell culture media and supplements were from Invitrogen. All other reagents were purchased from Sigma.

Data Analysis and Terminology—*In vitro* data were analyzed by nonlinear regression with GraphPad Prism software (Version 4.0) using the four-parameter logistic equation, allowing iterative fitting of the resultant responses. The data presented are the means of at least three independent experiments performed in triplicate. The pharmacological terminology adopted in this study is in line with the recommendations of the International Union of Basic and Clinical Pharmacology (17): the 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 maximum possible effect. For *in vivo* data, locomotor activity was expressed both as impulses/5 min over the time course of the experiment and as cumulative impulses over the first and second 30-min observation periods. Differences between groups were assessed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $p < 0.05$ *versus* saline (*in vivo*) or *versus* full-length hNPS (*in vitro*) was considered to be significantly different.

RESULTS

In Vitro Studies

HEK293/hNPSR Cell Line Characterization and Effects of Species-specific NPS Isoforms—A typical concentration-response curve for hNPS in this clonal hNPSR cell line is shown in Fig. 1. The in-house synthesized hNPS produced a concentration-dependent increase in intracellular calcium levels ($[Ca^{2+}]_i$), with a pEC_{50} of 8.39 ± 0.08 and an E_{max} corresponding to 3.7 ± 0.2-fold the basal fluorescence levels. Similar results in terms of both potency and maximum effects were obtained by testing the commercially available human NPS ($pEC_{50} = 8.57 \pm 0.09$), mouse NPS ($pEC_{50} = 8.66 \pm 0.16$), and rat NPS ($pEC_{50} = 8.83 \pm 0.17$) as shown in Fig. 1. None of the above-mentioned peptides was able to induce Ca^{2+}_i mobilization in WT HEK293 cells (data not shown), whereas HEK293/hNPSR and WT HEK293 cells were similarly sensitive to the stimulatory effect of 10 μM carbachol (4.4 ± 0.8- and 4.2 ± 0.2-fold over basal levels, respectively), acting on a muscarinic receptor endogenously expressed in HEK293 cells.

hNPS Ala-scan—Amino acid side chain contribution to the biological effect of hNPS was evaluated by systematic alanine replacement of each residue of the peptide primary sequence. Thus, 19 Ala-substituted hNPS analogs (Ala is present in the natural sequence at position 18) were synthesized and tested for their ability to stimulate Ca^{2+}_i mobilization in HEK293/hNPSR cells. The results of these experiments are summarized in Table 1. The Ala replacement of Ser[1] did not significantly modify either peptide potency or efficacy. In contrast, the substitution of Phe[2] with Ala generated an inactive analog. The substitution of Arg[3] with Ala produced a statistically significant decrease in peptide potency and efficacy. In fact, the maximum effect elicited by [Ala[3]]hNPS was approximately half that produced by the naturally occurring peptide. The N4A substitution produced a dramatic loss of potency (at

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TABLE 1

Ala-scan of hNPS

Data are the means ± S.E. of at least three separate experiments. E_{max} is expressed as a percentage of the maximum effect elicited by hNPS in the same experiment. Inactive, inactive up to 1 μM; CRC incomplete, concentration-response curve incomplete (the effect elicited by the compound at 1 μM is reported).

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	pEC$_{50}$	E_{max}
	Peptide sequence																				Agonist effect	
																						%
hNPS	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	7.94 ± 0.03	100
[Ala1]hNPS	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.79 ± 0.09	85 ± 4
[Ala2]hNPS	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Inactive	Inactive
[Ala3]hNPS	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6.86 ± 0.09[a]	49 ± 5[a]
[Ala4]hNPS	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CRC incomplete	25 ± 5
[Ala5]hNPS	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.51 ± 0.13	89 ± 9
[Ala6]hNPS	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.76 ± 0.15	98 ± 5
[Ala7]hNPS	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	CRC incomplete	31 ± 7
[Ala8]hNPS	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	8.02 ± 0.13	93 ± 11
[Ala9]hNPS	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	8.11 ± 0.12	96 ± 7
[Ala10]hNPS	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	8.09 ± 0.10	97 ± 5
[Ala11]hNPS	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	7.78 ± 0.10	96 ± 5
[Ala12]hNPS	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	–	7.95 ± 0.16	97 ± 5
[Ala13]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	–	8.01 ± 0.13	95 ± 9
[Ala14]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	–	7.92 ± 0.12	105 ± 12
[Ala15]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	7.97 ± 0.15	103 ± 18
[Ala16]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	7.99 ± 0.19	92 ± 4
[Ala17]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	7.90 ± 0.19	87 ± 4
[Ala19]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	8.03 ± 0.09	95 ± 5
[Ala20]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	7.98 ± 0.07	94 ± 9

[a] $p < 0.05$ versus hNPS (ANOVA followed by Dunnett's test).

TABLE 2

D-scan of hNPS

Data are the means ± S.E. of at least three separate experiments. E_{max} is expressed as a percentage of the maximum effect elicited by hNPS in the same experiment. Inactive, inactive up to 1 μM; CRC incomplete, concentration-response curve incomplete (the effect elicited by the compound at 1 μM is reported).

Compound	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	pEC$_{50}$	E_{max}
	Peptide sequence																				Agonist effect	
																						%
hNPS	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	8.10 ± 0.24	100
[D-Ser1]hNPS	s	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.81 ± 0.12	90 ± 17
[D-Phe2]hNPS	–	f	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6.51 ± 0.30[a]	109 ± 10
[D-Arg3]hNPS	–	–	r	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.03 ± 0.14[a]	106 ± 4
[D-Asn4]hNPS	–	–	–	n	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.12 ± 0.26[a]	101 ± 5
[D-Val6]hNPS	–	–	–	–	–	v	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CRC incomplete	40 ± 3
[D-Thr8]hNPS	–	–	–	–	–	–	–	t	–	–	–	–	–	–	–	–	–	–	–	–	7.92 ± 0.07	112 ± 7
[D-Met10]hNPS	–	–	–	–	–	–	–	–	–	m	–	–	–	–	–	–	–	–	–	–	7.80 ± 0.09	100 ± 6
[D-Lys11]hNPS	–	–	–	–	–	–	–	–	–	–	k	–	–	–	–	–	–	–	–	–	7.66 ± 0.14	105 ± 3
[D-Lys12]hNPS	–	–	–	–	–	–	–	–	–	–	–	k	–	–	–	–	–	–	–	–	7.85 ± 0.12	97 ± 8
[D-Thr13]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	t	–	–	–	–	–	–	–	7.75 ± 0.17	105 ± 3
[D-Ser14]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	s	–	–	–	–	–	–	7.90 ± 0.07	101 ± 6
[D-Phe15]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	f	–	–	–	–	–	7.87 ± 0.13	99 ± 8
[D-Gln16]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	q	–	–	–	–	7.80 ± 0.20	102 ± 8
[D-Arg17]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	r	–	–	–	7.79 ± 0.16	93 ± 4
[D-Ala18]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	a	–	–	7.83 ± 0.28	105 ± 7
[D-Lys19]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	k	–	7.78 ± 0.14	97 ± 6
[D-Ser20]hNPS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	s	7.89 ± 0.15	104 ± 11

[a] $p < 0.05$ versus hNPS (ANOVA followed by Dunnett's test).

least 100-fold) because [Ala4]hNPS produced a weak stimulatory effect (26% of that produced by the natural peptide) on Ca^{2+}_i mobilization only at the highest concentration tested. Interestingly, the substitution of Gly[7] with Ala generated a peptide with only a weak stimulatory effect (37% of that produced by hNPS) at the highest concentration tested. Ala substitutions from positions 8 to 20 did not significantly modify either the potency or efficacy of hNPS analogs.

hNPS D-scan—The contribution of the spatial orientation of the amino acid side chain to the biological effect of hNPS was evaluated by systematic D-amino acid replacement of each residue of the peptide primary sequence. Thus, 17 hNPS analogs (the achiral residue Gly is present in the natural sequence at positions 5, 7, and 9) were synthesized and tested for their ability to stimulate Ca^{2+}_i mobilization in HEK293/hNPSR cells.

The results of these experiments are summarized in Table 2. Whereas inversion of the chirality of Ser[1] did not affect the potency and efficacy of the peptide, the chirality of Phe[2], Arg[3], and Asn[4] seemed to be of high importance. Substitution of these amino acids with their respective enantiomers caused a statistically significant loss of potency (in the range of 10–30-fold). [D-Val6]hNPS produced a weak stimulatory effect (40% of that produced by the natural peptide) on Ca^{2+}_i mobilization only at the highest concentration tested. D-Amino acid substitution from positions 8 to 20 did not modify either the potency or efficacy of the peptides, producing hNPS analogs that behaved as potent full agonists.

hNPS N- and C-terminal Truncation—The contribution of the N- and C-terminal domains of the peptide to the biological effects of hNPS were investigated by progressive deletion of 10

TABLE 3

N- and C-terminal truncation of hNPS

Data are the means ± S.E. of at least three separate experiments. E_{max} is expressed as a percentage of the maximum effect elicited by hNPS in the same experiment. Inactive, inactive up to 1 μM; CRC incomplete, concentration-response curve incomplete (the effect elicited by the compound at 1 μM is reported).

Compound	Peptide sequence																				Agonist effect	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	pEC$_{50}$	E_{max}
																						%
hNPS	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	8.54 ± 0.03	100
hNPS-(2–20)		F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	7.80 ± 0.11[a]	107 ± 4
hNPS-(3–20)			R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(4–20)				N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(5–20)					G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(6–20)						V	G	T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(7–20)							G	T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(8–20)								T	G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(9–20)									G	M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(10–20)										M	K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(11–20)											K	K	T	S	F	Q	R	A	K	S	Inactive	Inactive
hNPS-(1–19)	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A	K		8.52 ± 0.06	102 ± 3
hNPS-(1–18)	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R	A			8.52 ± 0.34	103 ± 4
hNPS-(1–17)	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q	R				8.30 ± 0.31	105 ± 5
hNPS-(1–16)	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F	Q					8.64 ± 0.11	98 ± 2
hNPS-(1–15)	S	F	R	N	G	V	G	T	G	M	K	K	T	S	F						8.29 ± 0.15	102 ± 2
hNPS-(1–14)	S	F	R	N	G	V	G	T	G	M	K	K	T	S							8.59 ± 0.03	110 ± 5
hNPS-(1–13)	S	F	R	N	G	V	G	T	G	M	K	K	T								8.45 ± 0.07	109 ± 5
hNPS-(1–12)	S	F	R	N	G	V	G	T	G	M	K	K									8.39 ± 0.25	103 ± 7
hNPS-(1–11)	S	F	R	N	G	V	G	T	G	M	K										8.31 ± 0.41	92 ± 3
hNPS-(1–10)	S	F	R	N	G	V	G	T	G	M											8.32 ± 0.06	106 ± 2

[a] $p < 0.05$ *versus* hNPS (ANOVA followed by Dunnett's test).

TABLE 4

N- and C-terminal truncation of hNPS-(1–10)

Data are the means ± S.E. of at least three separate experiments. E_{max} is expressed as a percentage of the maximum effect elicited by hNPS in the same experiment. Inactive, inactive up to 1 μM; CRC incomplete, concentration-response curve incomplete (the effect elicited by the compound at 1 μM is reported).

Compound	Peptide sequence										Agonist effect	
	1	2	3	4	5	6	7	8	9	10	pEC$_{50}$	E_{max}
												%
hNPS-(1–10)	S	F	R	N	G	V	G	T	G	M	8.03 ± 0.06	107 ± 2
hNPS-(2–10)		F	R	N	G	V	G	T	G	M	CRC incomplete	39 ± 15
hNPS-(1–9)	S	F	R	N	G	V	G	T	G		7.77 ± 0.18	103 ± 7
hNPS-(1–8)	S	F	R	N	G	V	G	T			7.33 ± 0.16[a]	101 ± 4
hNPS-(1–7)	S	F	R	N	G	V	G				CRC incomplete	67 ± 7
hNPS-(1–6)	S	F	R	N	G	V					CRC incomplete	21 ± 4
hNPS-(1–5)	S	F	R	N	G						Inactive	Inactive
hNPS-(1–4)	S	F	R	N							Inactive	Inactive

[a] $p < 0.05$ *versus* hNPS (ANOVA followed by Dunnett's test).

residues from both sides. The results of these experiments are summarized in Table 3. The deletion of Ser[1] produced a statistically significant reduction in the potency of the peptide without modifying its efficacy. Additional truncations of the N terminus eliminating Phe[2] caused a dramatic loss of potency, rendering peptides inactive at the highest concentration tested (1 μM).

The progressive deletion of single amino acids from the C terminus of the molecule from Ser[20] to Lys[11] did not affect either peptide potency or efficacy. In fact, hNPS-(1–10) produced a similar maximum effect and potency as the reference peptide hNPS-(1–20).

N- and C-terminal Truncation of hNPS-(1–10)—As hNPS-(1–10) maintained the same biological activity of hNPS, we performed a second round of N- and C-terminal truncated peptide synthesis with hNPS-(1–10) aimed at the identification of the minimum sequence required for hNPSR activation. The results of these additional truncations are summarized in Table 4. A dramatic loss of potency was observed when Ser[1] was deleted (hNPS-(2–10)). The elimination of Met[10] and of Gly[9]-Met[10] produced only slight reductions of potency of ~3- and 5-fold,

respectively. In contrast, the additional deletion of Thr[8] caused a >100-fold loss of potency with hNPS-(1–7), displaying a pEC$_{50}$ of <6. Similar results were obtained with the compounds in which the C terminus was further shortened.

hNPS-(1–10) Analogs—The previous results suggested that hNPS-(1–10) is the shortest hNPS sequence that maintains the same potency and efficacy as the natural ligand. To confirm and extend this proposal and to compare the chemical requirements for the biological activity of this fragment with those of the natural sequence, hNPS-(1–10) was resynthesized together with a short series of hNPS-(1–10) analogs. The results of these experiments are summarized in Table 5. The replacement of Val[6] with the corresponding enantiomer and the substitution of Gly[7] with Ala generated inactive analogs. The substitution with Ala at positions 8 and 10 alone or in combination produced only a small reduction of the potency of the reference peptide hNPS-(1–10).

All inactive peptides were also tested for putative antagonist activity for the hNPSR by generating inhibition curves (from 1 pM to 1 μM) against the stimulatory effect elicited by 30 nM hNPS. None of these peptides was able to inhibit the hNPS-mediated response (data not shown). Finally, all peptides were

TABLE 5

hNPS-(1–10) analogs

Data are the means ± S.E. of three separate experiments. E_{max} is expressed as a percentage of the maximum effect elicited by hNPS in the same experiment. Inactive, inactive up to 1 μM; CRC incomplete, concentration-response curve incomplete (the effect elicited by the compound at 1 μM is reported).

Compound	Peptide sequence										Agonist effect	
	1	2	3	4	5	6	7	8	9	10	pEC_{50}	E_{max}
												%
hNPS-(1–10)	S	F	R	N	G	V	G	T	G	M	8.32 ± 0.06	104 ± 3
[D-Val[6]]hNPS-(1–10)	–	–	–	–	–	v	–	–	–	–	Inactive	Inactive
[Ala[7]]hNPS-(1–10)	–	–	–	–	–	–	A	–	–	–	Inactive	Inactive
[Ala[8]]hNPS-(1–10)	–	–	–	–	–	–	–	A	–	–	7.63 ± 0.12[a]	97 ± 1
[Ala[10]]hNPS-(1–10)	–	–	–	–	–	–	–	–	–	A	8.00 ± 0.41	101 ± 2
[Ala[8], Ala[10]]hNPS-(1–10)	–	–	–	–	–	–	–	A	–	A	7.65 ± 0.05[a]	106 ± 9

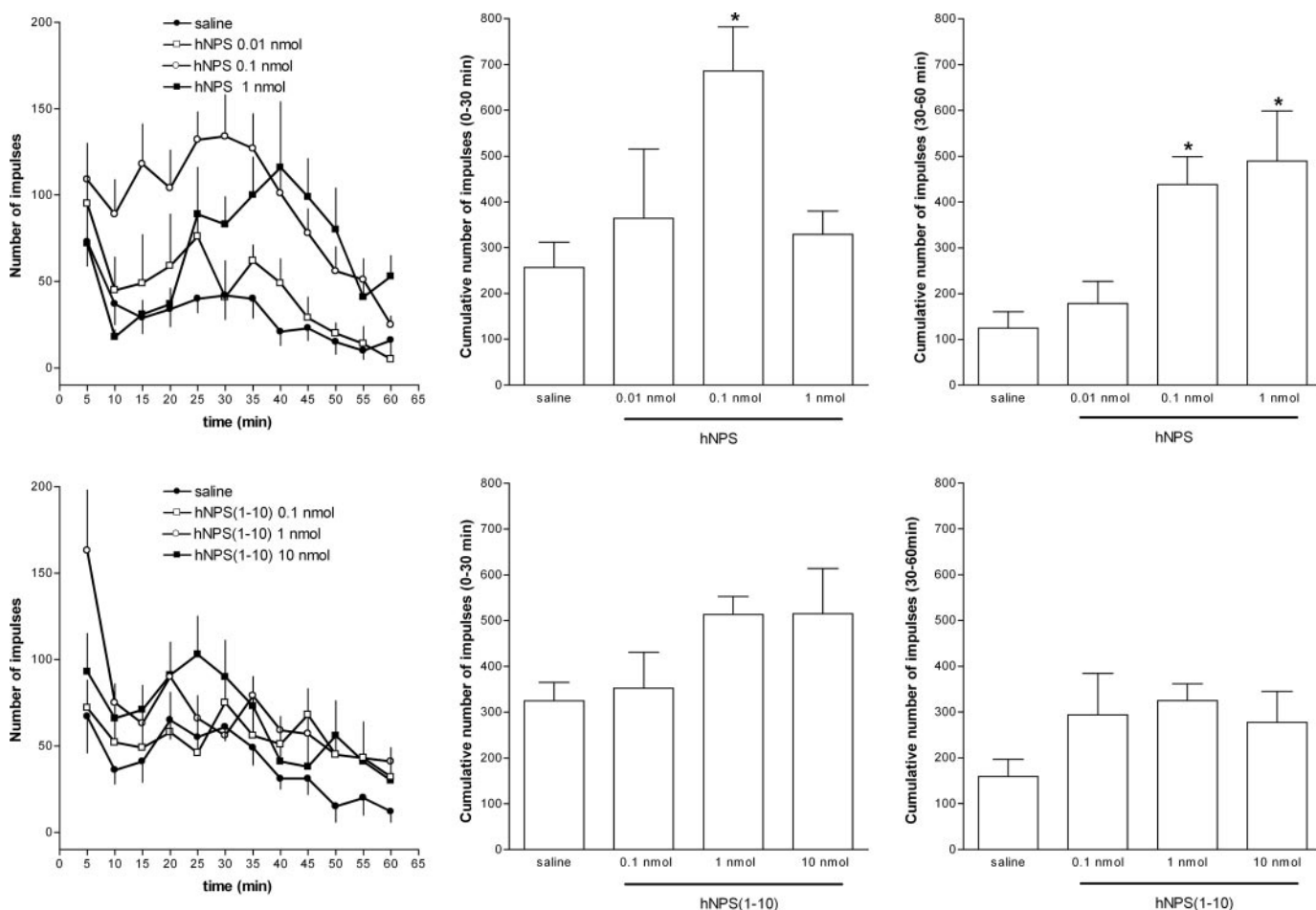[a] $p < 0.05$ *versus* hNPS (ANOVA followed by Dunnett's test).

FIGURE 2. **Mouse locomotor activity assay.** Shown are the dose-response curves for hNPS (0.01–1 nmol; *upper panels*) and hNPS-(1–10) (0.1–10 nmol; *lower panels*) injected intracerebroventricularly. The locomotor activity of the mice over the 60-min time course of the experiment is displayed in the *left panels* and as cumulative impulses over the first 30-min period in the *middle panels* and the second 30-min period in the *right panels*. *Data points* indicate the means, and *vertical lines/error bars* indicated the S.E. of at least four separate experiments (12 mice/group). *, $p < 0.05$ *versus* saline (ANOVA followed by Dunnett's test).

also evaluated in WT HEK293 cells, in which they failed to promote Ca^{2+}_i mobilization (data not shown).

In Vivo Studies

In habituated mice, intracerebroventricular injection of hNPS in the range of 0.01–1 nmol caused a dose-dependent increase in spontaneous locomotor activity compared with saline-injected mice. In particular, in the first 30-min observation period, only 0.1 nmol produced a statistically significant stimulatory effect on locomotor activity, whereas in the second 30-min period, both 0.1 and 1 nmol produced a similar and

statistically significant stimulatory effect (Fig. 2, *upper panels*). Under the same experimental conditions, hNPS-(1–10) at 0.1–10 nmol failed to evoked statistically significant effects. However, it should be noted that a clear trend for a stimulatory effect of 1 and 10 nmol was evident in the first 30-min observation period, whereas this was no longer observed in the second period of observation (Fig. 2, *lower panels*).

DISCUSSION

In this work , we report the first systematic structure-activity relationship study on the human neuropeptide NPS. By applying

the classical peptide structure-activity relationship approach, we were able to obtain relevant information on the chemical requirements needed for NPSR activation.

The first step of this study was to develop an *in vitro* assay suitable for screening the pharmacological activity of hNPS peptide analogs. As reported previously (5), the hNPSR mediates intracellular Ca^{2+} release; therefore, a FLIPR readout was chosen for monitoring peptide activity for the hNPSR. hNPS activated the human receptor with high potency similar to that reported by Xu *et al.* (5) under similar experimental conditions. In addition, the rat and mouse isoforms of NPS displayed similar effects as the human peptide. All these peptides failed to modify calcium levels in WT HEK293 cells. These results nicely confirm previous findings (5) and demonstrate that (i) the NPSR is likely coupled to G-proteins of the G_q type, and (ii) the human receptor does not discriminate between human and rodent NPS.

The Ala scan of hNPS clearly demonstrated that (i) the benzyl ring of Phe[2] is crucial for NPS binding to its receptor; (ii) a similar important role in receptor binding is played by the amide function of the Asn[4] side chain; and (iii) the guanidine moiety of Arg[3] contributes to the receptor binding properties of the peptide, but is also important for receptor activation because [Ala[3]]hNPS displayed reduced efficacy, behaving as a partial agonist. Finally, the G7A substitution produced a detrimental effect with a loss of potency of >100-fold. This substitution had a double effect: it increased the steric hindrance of the amino acid and generated an additional chiral carbon in the peptide backbone. Ala substitution at positions 1, 5, 6, and 8–20 did not affect the activation properties of the peptide.

The indications obtained with the Ala-scan approach were confirmed and extended by the D-scan. In fact, the inversion of chirality of Phe[2], Arg[3], and Asn[4] caused a significant loss of potency of at least 10-fold; this confirms the crucial role played by the hNPS sequence Phe-Arg-Asn in biological activity and suggests a "message" role for this domain of the peptide. This is in line with recent findings by Reinscheid *et al.* (11) demonstrating that the peptide hNPS-(4–20) is inactive on both the human and mouse NPSRs. Interestingly, the inversion of chirality of Val[6] (which can be replaced by Ala without affecting activity) produced a dramatic loss of potency. This finding, together with the fact that introduction of chirality at position 7 (replacement of Gly with Ala) also had a detrimental effect on receptor interaction, strongly suggests that the sequence Val[6]-Gly[7] makes an important contribution to maintaining/favoring the bioactive conformation of hNPS, possibly acting as a hinge region. Finally, the inversion of chirality at positions 1 and 8–20 did not produce a change in NPS activity.

The N-terminal truncation of hNPS demonstrated that deletion of Ser[1] produced a significant loss of potency. This indicates that an amino acid residue (or most likely just a chemical moiety) must occupy position 1; however, neither the chemical function of its side chain nor its spatial orientation is crucial for biological activity. The additional deletion of position 2 produced a drastic loss of potency, again suggesting a crucial role for Phe[2] in receptor interaction. Because hNPS-(3–20) is virtually inactive, the contribution of the other N-terminal amino acids to biological activity could not be evaluated.
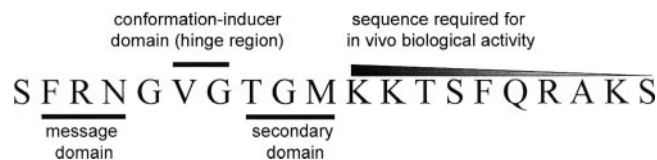

FIGURE 3. **Schematic representation of the suggested pharmacophoric domains of hNPS.**

In line with indications emerging from Ala- and D-scan studies, the deletion of C-terminal amino acids from positions 20 to 11 did not affect hNPS activity. Collectively, these results demonstrated a negligible role for the *in vitro* activity of hNPS-(11–20).

To identify the minimum hNPS fragment able to activate the hNPSR *in vitro*, a second round of N- and C-terminal truncation was performed on hNPS-(1–10). The deletion of Ser[1] produced a dramatic decrease in potency, thus supporting the idea that a chemical moiety must be present at the N terminus of Phe[2], although its chemical requirements are not stringent. The elimination of Met[10] produced a slight decrease in peptide potency, which reached statistical significance when Gly[9] was deleted. The additional deletion of Thr[8] generated an inactive compound. Interestingly enough, the amino acids in Thr[8]-Gly[9]-Met[10] could be substituted with Ala or their corresponding enantiomers without loss of activity, whereas their deletion generated an inactive peptide. These results indicate that this sequence is indeed important for receptor activation, although without displaying stringent chemical requirements. This is also corroborated by the fact that residues 8 and 10 are the only ones within sequence 1–10 that show variability among species-specific forms of NPS (5).

To further validate the notion that hNPS-(1–10) and the full-length sequence peptide recognizes the NPSR similarly, a short series of hNPS-(1–10) analogs were synthesized and evaluated. The results of these studies confirmed the crucial role of the hinge sequence Val[6]-Gly[7] as well as the lack of stringent chemical requirements for the C-terminal tripeptide sequence Thr[8]-Gly[9]-Met[10].

The presented structure-activity relationship data allowed us to identify sequence 1–10 as the smallest fragment able to activate the hNPSR with similar potencies and efficacies as full-length hNPS. This peptide sequence contains three major portions: (i) the Phe[2]-Arg[3]-Asn[4] sequence, which likely represents the message domain of this peptide; (ii) the sequence Thr[8]-Gly[9]-Met[10], which is also important for receptor binding, although with non-stringent chemical requirements; and (iii) the sequence Val[6]-Gly[7], which probably acts as a hinge region, determining the correct spatial arrangement of the two above-mentioned domains and thus stabilizing/favoring the active conformation of the peptide. A schematic representation summarizing this hypothesis is shown in Fig. 3.

The biological activity of hNPS and hNPS-(1–10) was assessed *in vivo* after intracerebroventricular administration, measuring locomotor activity in mice habituated to the test cage. hNPS displayed a robust and dose-dependent stimulatory effect on locomotor activity, which was similar in terms of potency, duration, and overall effect to that reported previously by Xu *et al.* (5). The only difference between the present data

and those of Xu *et al.* is the delayed effect of 1 nmol of hNPS. This different result is difficult to interpret because both sets of data were obtained under very similar experimental conditions. The only issue worthy of mention is related to the strain of mice used: Swiss *versus* C57BL/6 in our experiments and those of Xu *et al.*, respectively. Despite this minor difference, our results confirmed and extended to another strain of mice the potent hyperlocomotor action of NPS. This effect is probably related to the powerful arousal-promoting action of NPS that is associated with a robust anxiolytic-like effect (5), making the behavioral profile of this novel neuropeptide, *i.e.* an activating anxiolytic, quite unique (18). Under identical experimental conditions, only a slight, short-lasting, and not statistically significant effect was obtained by testing hNPS-(1–10), even using doses (10 nmol/mouse) 100-fold higher than the first effective dose of hNPS (0.1 nmol/mouse). Thus, hNPS-(1–10) does fully mimic the *in vitro* effects of the natural ligand in the FLIPR assay, whereas this does not apply to its *in vivo* biological activity, where the peptide fragment produced only weak and short-lasting effects. The reason(s) for these *in vitro versus in vivo* discrepant results are at present unknown; however, the following two issues should be considered. (i) Peptidergic signals are terminated by the action of specific peptidases that efficiently process endogenous peptides into inactive fragments. It is possible that hNPS and hNPS-(1–10) substantially differ in their susceptibility to peptidases. Because the peptidase activity is likely to play a crucial role in the brain (*in vivo* studies), whereas its biological relevance is probably negligible in recombinant systems (*in vitro* studies), the metabolism issue may explain (at least in part) the discrepant results obtained with hNPS-(1–10). (ii) *In vitro* studies were performed on cells expressing the hNPSR protein, whereas *in vivo* studies were carried out in mice. It is therefore possible that peptide fragment 1–10 represents the minimum active sequence for the hNPSR, but not for the mouse NPSR ortholog. However, this possibility seems to be unlikely because, in general, natural peptides do not discriminate between species-specific receptor orthologs, whereas this feature is quite characteristic of non-peptide ligands (19). Future studies are required to identify the reasons underlying the different actions of hNPS-(1–10) *in vitro* and *in vivo*. Independent of these considerations, the data obtained with hNPS-(1–10) measuring locomotor activity in mice clearly demonstrated that the C-terminal sequence of hNPS maintains importance in determining the *in vivo* biological activity of this peptide. This has been also illustrated in Fig. 3.

In conclusion, this study investigated the structure-activity relationships of the novel peptide NPS and identified the amino acid residues of this peptide most important for receptor activation. This information will be used in future studies to design NPS analogs acting as selective NPSR ligands with improved potency (agonists) and reduced efficacy (partial agonists and possibly pure antagonists). The availability of such ligands will allow the thorough investigation of the biological functions regulated by this novel peptide receptor system.

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Structure-Activity Studies on Neuropeptide S: IDENTIFICATION OF THE AMINO ACID RESIDUES CRUCIAL FOR RECEPTOR ACTIVATION

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