Conformation−Activity Relationship of Neuropeptide S and Some Structural Mutants: Helicity Affects Their Interaction with the Receptor

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Neuropeptide S (NPS) is the endogenous ligand of the previously orphan G-protein coupled receptor now named NPSR. The NPS−NPSR receptor system regulates important biological functions such as sleep/waking, locomotion, anxiety and food intake. Recently, exhaustive Ala scan and D-amino acid scan studies, together with systematic N- and C-terminal truncation, led to the identification of key residues for biological activity. Because conformational preferences might also play an important role, we undertook a detailed conformational analysis of NPS and several analogues in solution. We show that helicity induced by substitution of three flexible residues in the 5−13 regulatory region abolishes biological activity. A parallel pharmacological and conformational study of single and multiple substitutions of glycines 5, 7, and 9 showed that helicity can be tolerated in the C-terminal part of the peptide but not around Gly[7]. The identification of hNPSR partial agonists heralds the possibility of designing pure NPS receptor antagonists.

Introduction

G-protein coupled receptors (GPCRs[a]) are ubiquitously present in the central and peripheral nervous system where they regulate a variety of biological functions. Therefore, it is not surprising that they are considered, at present, the most important biological target for drug discovery.[1,2] Although more than 800 genes coding for putative GPCR have been identified in the human genome,[3] including 350 olfactory receptors and 30 other chemosensory receptors, the corresponding endogenous ligands are only known for a fraction of them, approximately 240. GPCRs whose endogenous ligand has not yet been identified are called orphan GPCRs[2] and are actively investigated because their ligands may well hint at novel drugs, possibly providing innovative treatments for several diseases.

In the early 1990s, a novel strategy named reverse pharmacology was developed and validated: via this approach, several oGPCR were used in functional experiments aimed at the identification of their endogenous ligands.[2] One of the latest oGPCR ligands identified with these techniques is neuropeptide S (NPS).[4] In all species, NPS is a 20-residue peptide with the N-terminal sequence (seven residues) highly conserved across species, while the few differences are mainly located in the C-terminal part of the peptide. Human NPS, like many other neuropeptides, is proteolytically cleaved from a larger precursor polypeptide containing a hydrophobic signal peptide and proteolytic processing sites.[5] hNPS selectively binds and activates a previously orphan receptor known as GPR154,[6] GPRA,[7] or VRR1[8] and now referred to as NPSR. The NPSR is a class A G-protein coupled receptor that shows low homology to most members of the G-protein coupled receptor family.

As far as NPS biological actions are concerned, it has been reported that NPS given intra-cerebro-ventricularly stimulates locomotor activity and reduces sleep, indicating that this peptide may induce wakefulness.[4] This action is associated with anxiolytic-like effects, as indicated by results obtained in several different behavioral tests.[4] Thus, the profile of behavioral effects evoked by the icv administration of NPS (stimulation of arousal/wakefulness associated with anxiolytic-like effects) appears to be quite unique and candidates this peptide as the first activating anxiolytic signal.[9] The human NPSR, in particular its isoleucine 107 variant, characterized by an increase in agonist potency without modifications of ligand affinity[10] is also known as GPRA, with reference to asthma susceptibility. The link between the isoleucine 107 variant of human NPSR and asthma was recorded in several different populations.[7,11−13] However, a very detailed study performed on mice lacking the NPSR receptor gene failed to detect a clear phenotype of these animals in a large panel of models of airways pathologies.[14] Recent studies point to the involvement of the NPS−NPS receptor system in the regulation of food intake.[15−17] Certainly, the development of novel ligands for NPSR is mandatory to determine the role played by the NPS−NPS receptor system in regulating different

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[a] Abbreviations: In addition to the JUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1985**, *260*, 14−42), this paper uses the following additional symbols and abbreviations: GPCR, G-protein coupled receptor; Aib, α-aminoisobutyric acid; ANOVA, analysis of variance; Boc, *tert*-butyoxycarbonyl; DMF, *N*,*N*-dimethylformamide; Fmoc, fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high performance liquid chromatography; NPS, neuropeptide S; NPSR, neuropeptide S receptor; Pmc, 2,2,5,7,8-pentamethylchroman; tBu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl.

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biological functions and ultimately to predict the therapeutic potential of novel drugs interacting with this receptor.

Recently, by an exhaustive structure−activity relationship study, we were able to identify the main requirements for biological activity in the primary structure of NPS. Single residue replacement, either by an alanine residue (Ala-scan) or by the corresponding enantiomer (D-scan),[18] led to the identification of key residues for biological activity. Moreover, the N- and C-terminal portions of the peptide were progressively shortened (N- and C-terminal truncation) by single amino acid residue elimination. It was shown that residues Phe,[2] Arg,[3] and Asn[4] are essential for biological activity, whereas the sequence Thr[8]-Gly[9]-Met[10] is important for receptor activation, albeit without stringent requirements on the constitution of the side chains. The sequence Gly[5]-Val[6]-Gly[7] acts as a hinge region between the two above-mentioned domains. The major findings described by Roth et al.[18] were also reported by Bernier et al.[19] who independently performed a similar study and, in addition to the mentioned essential residues of the message domain, found that hNPS(1−6) is the shortest sequence required to activate hNPSR in vitro with potency and efficacy similar to those of the full-length peptide. In addition to the SAR study, Bernier et al., on the basis of a preliminary NMR and CD structural characterization, proposed a "nascent helix" in the region spanning residues 5−13 and hypothesized that such a helix may be important for proper interaction of NPS with its receptor.[19] Although based on diagnostically significant NH−NH effects, this result is somewhat surprising because, in our hands, an exploratory NMR investigation showed that the whole peptide is very flexible, as expected from the presence along its sequence of a high proportion of conformationally flexible residues. In addition to the three Gly, we considered also three Ser and two Thr. Strictly speaking, Ser and Thr cannot be classified as "flexible residues" in the same way as Gly because they do favor β structures. However, we included them among "flexible residues" for NPS on account of their low propensity to promote helicity and of the small encumbrance of the side chains.

Therefore, we decided to examine the conformation−activity relationship of NPS in more detail, with the aim of finding a relationship between local conformational tendencies and biological activity. Here we present a conformational analysis of NPS and several analogues, based on NMR spectroscopy in solvent media that can promote ordered structures.

Results and Discussion

Biological Results. Helical Segments. The sequence of NPS is highly conserved across several species.[18,19] To test its conformational properties, we designed analogues with particular emphasis on residues with high intrinsic conformational flexibility along tract 5−13 and residues connecting the message domain and tract 5−13. Flexible residues in the 5−13 tract include glycines 5, 7, and 9; to encompass the whole tract and keeping in mind the features of Ser and Thr (vide supra), we included also Thr13.

There are several natural residues that can impart helicity, but because we did not wish to alter too much the global shape of the helix with respect to the virtual natural one, it is necessary to limit the choice to residues whose side chain has an encumbrance similar to those of Gly and Thr. Thus, to design possible peptides in which Gly/Thr residues are substituted by natural residues with higher helical propensity but minimal steric encumbrance of the side chain, Ala is the natural choice.

As a rough guideline for peptide design, we used AGADIR,[20] one of several available programs that can calculate helix probability for isolated chains. Although these predictions are not always easy to compare with experimental data, they yield a reliable comparative view of conformational tendencies. When AGADIR is used, the percentage of isolated helix in water at pH 7 is 0.28 for wild type NPS. It becomes 0.26, 0.49, 0.96, and 3.07 for [Ala5]-NPS, [Ala7]-NPS, [Ala9]-NPS, and [Ala7,9,13]-NPS, respectively. Even more important, if we want to locate the boundaries of the helical tract, the individual Ncap probability for residues preceding the substitutions is 0.19 at residue 5 for wild type NPS, 0.05 at residue 1 for [Ala5]-NPS, 1.02 at residue 5 for [Ala7]-NPS, 1.39 at residue 5 for [Ala9]-NPS (but also 0.97 at residue 8), and 5.29 at residue 5 for [Ala7,9,13]-NPS. Taken collectively, these predictions tell us that formation of a helix for wild type NPS and for [Ala5]-NPS is unlikely both from the intrinsic tendencies of individual residues and for the location of a possible Ncap. On the other hand, a helix including residue 7 is strongly favored by Ncap at 5 and it is also possible to have a helix in the C-terminal part starting from residue 9.

Table 1. Analytical Properties of the hNPS and Analogues

peptide	t_r[a] I	t_r[a] II	MH^+[b] calcd	MH^+[b] found
hNPS	9.86	14.02	2188.5	2188.6
[Ala[1]]hNPS	9.61	13.63	2172.5	2172.6
[Ala[5]]hNPS	9.76	14.36	2202.6	2202.8
[Ala[7]]hNPS	9.90	14.10	2202.6	2202.4
[Ala[9]]hNPS	9.72	14.09	2202.6	2202.4
[Ala[13]]hNPS	9.77	14.33	2158.5	2158.8
[Ala[7,9,13]]hNPS	10.06	14.34	2186.6	2187.0
[Aib[5]]hNPS	10.16	14.23	2216.6	2216.2
[Aib[7]]hNPS	9.84	13.90	2216.6	2216.4
[Aib[9]]hNPS	10.07	14.07	2216.6	2216.2
[His[4]]hNPS	9.18	13.36	2211.6	2212.2
[Pro[4]]hNPS	9.97	14.02	2171.5	2172.2
[D-Pro[4]]hNPS	10.02	14.07	2171.5	2172.2
[D-Ala[5]]hNPS	9.87	14.22	2202.6	2202.8
[D-Ala[7]]hNPS	9.82	14.12	2202.6	2203.2
[D-Ala[9]]hNPS	9.75	14.25	2202.6	2202.8
[Aib[1]]hNPS	9.88	13.94	2186.5	2186.4

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

Among powerful nonproteic residues that can induce helicity, the best choice, consistent with the above steric requirements, is α-aminoisobutyric acid (Aib).[21] Based on these considerations, to probe the conformational preferences of region 5−13, we designed the following peptides: [L/D-Ala[7]]hNPS, [L/D-Ala[9]]-hNPS, [Ala[13]]hNPS, [Ala[7,9,13]]hNPS, [Aib[7]]hNPS, and [Aib[9]]-hNPS.

Table 1 shows the analytical data for all peptides, synthesized according to published methods using standard solid-phase synthesis techniques with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany), as described in detail in the Experimental Section. It should be noted that the synthesis of analogs with single Ala substitutions has already been described in Roth et al.[18]

Pharmacological data were acquired by testing all analogues on mNPSR, stably expressed in HEK293 cells and measuring the calcium mobilization response. Table 2 summarizes the pharmacological actions of hNPS and of all analogues tested. hNPS produced a concentration-dependent increase in $[Ca^{2+}]$ levels showing nanomolar potency (pEC_{50} 8.04) and maximal effects corresponding to 275% of the basal values. Substitutions of Gly/Thr with Ala in positions 5, 7, 9, and 13 or in combination 9−13 and 7−9−13 show that while the presence of Ala in positions 1, 5, 9, or 13 is well-tolerated, substitution in 7 is highly detrimental for biological activity. Such a behavior

Table 2. Pharmacological Profile of hNPS and Analogues Tested against mNPSR Stably Expressed in HEK293 Cells

	pEC_{50}[a] ($CL_{95\%}$[b])	E_{max}[c] ± sem[d] (%)
hNPS	8.04 (7.76−8.32)	175 ± 18
[Ala[1]]hNPS	7.58 (7.27−7.89)	182 ± 24
[Ala[5]]hNPS	7.61 (7.31−7.91)	178 ± 16
[Ala[7]]hNPS	Crc incomplete; at 10 μM	129 ± 15
[Ala[9]]hNPS	7.63 (7.06−8.20)	164 ± 14
[Ala[13]]hNPS	7.72 (7.21−8.23)	172 ± 13
[Ala[9,13]]hNPS	7.82 (7.32−8.32)	191 ± 22
[Ala[7,9,13]]hNPS	Crc incomplete; at 10 μM	69 ± 24
[D-Ala[5]]hNPS	7.55 (7.03−8.07)	90 ± 15[e]
[D-Ala[7]]hNPS	7.63 (7.08−8.19)	164 ± 24
[D-Ala[9]]hNPS	7.67 (7.14−8.20)	172 ± 26
[His[4]]hNPS	Crc incomplete; at 10 μM	147 ± 38
[Pro[4]]hNPS	6.80 (6.48−7.12)	167 ± 19
[D-Pro[4]]hNPS	Crc incomplete; at 10 μM	66 ± 48
[Aib[1]]hNPS	8.17 (7.67−8.67)	184 ± 32
[Aib[5]]hNPS	6.72 (6.43−7.00)	65 ± 9%[e]
[Aib[7]]hNPS	Crc incomplete; at 10 μM	14 ± 4
[Aib[9]]hNPS	7.99 (7.58−8.40)	190 ± 19

[a] pEC_{50}: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. [b] $CL_{95\%}$: 95% confidence limits. [c] E_{max}: the maximal effect elicited by the agonist expressed as % over the baseline. [d] sem: standard error of the mean. [e] $p < 0.05$ vs NPS, according to Anova, followed by the Dunnet test.

is paralleled by the observation that substitution in 5 does not induce helicity, whereas substitutions in 7 or 9 do favor helicity (vide infra). On the contrary, replacement of Gly with D-Ala in positions 5, 7, and 9 of the peptide is well-tolerated and yields analogues with potencies comparable to that of NPS. Such a behavior is consistent with the fact that obviously D-Ala[7] cannot promote a right-handed helix, consistent with the chirality of the other (L-)residues. Substitution of residues in positions 5, 7, and 9 with Aib showed that, similarly to substitutions with Ala, only [Aib[7]]NPS has a dramatic decrease of activity. To correlate these findings with conformational tendencies, we undertook an exhaustive NMR study in several solvent systems.

Message Domain. The N-terminal domain Ser[1]-Phe[2]-Arg[3]-Asn[4]-Gly[5] has not been investigated from a conformational point of view. We explored possible ways to make the N-terminal region more rigid, with the hope of enhancing the biological activity of the whole peptide. The presence of Asn[4]-Gly[5] in the N-terminal region hints at the possibility for this domain to assume, in its bioactive conformation, a regular turn, because these residues are found with high frequency in positions $i+1$ and $i+2$, respectively, of many turns of globular proteins.[22] A simple way to compare the likeliness of different turns may be to rate each turn on the basis of the occurrence of its residues in proteins. Thus, if one calculates the sum for individual residues of the potentials of Hutchinson and Thornton,[22] for a turn of sequence Arg^{i}-Asn^{i+1}-Gly^{i+2}-Val^{i+3}, corresponding to residues 3−6 of NPS, the highest scores are obtained for type I′ and type II turns. If the role of Asn in hNPS is mainly structural, that is if its side chain is not involved in a specific interaction with the receptor, it ought to be possible to substitute it with other residues compatible with regular β-turns and, at the same time, have useful indications about the precise type of turn. Thus, we designed three peptides in which Asn[4] was substituted with His, Pro or D-Pro and two in which Gly[5] was substituted with L/D-Ala or Aib. According to the frequencies of residues observed in proteins,[22] Pro could be a good substitution for a type II turn, whereas His could be a good candidate for a type I′ turn, as well as for more frequent type I. D-Pro has been suggested specifically as a substitute for Asn in Asn-Gly turns (both type I′ and type II′) in β-hairpin nucleation.[23] D-Ala is often chosen in lieu of Gly to take into account the lack of chiral preferences of this residue. Aib[5] on the other hand has no chirality like Gly but, in addition, is able to influence profoundly the local conformation. All these substitutions did not induce substantial changes in the conformational tendencies of the whole peptide that remained essentially disordered in all media (vide infra).

Roth et al.[18] have demonstrated that Phe[2], Arg[3], and Asn[4] are crucial for biological activity, whereas Asn[4] can be important both for potential interactions of its side chain with groups of the receptor and for the structural influence on the potential turn centered around Asn[4]-Gly.[5] Thus, the only residue that might influence the conformation of the N-terminal message domain without affecting the (necessary) flexibility at the hinge region is the first one. Accordingly, we designed and probed also [Aib[1]]hNPS, an analogue that might induce some additional conformational rigidity in the first four residues of the N-terminal domain without affecting too much the conformational flexibility between Gly[5] and Gly.[7] To probe the conformational preferences of the message domain, we synthesized the following peptides: [His[4]]hNPS, [Pro[4]]hNPS, [D-Pro[4]]hNPS, [L/D-Ala[5]]-hNPS, [Aib[5]]hNPS, [Ala[1]]hNPS, and [Aib[1]]hNPS.

Among the changes of residues of the N-terminal domain, [Aib[1]]hNPS was designed with the hope of inducing some conformational rigidity in the first four residues domain without affecting too much the conformational flexibility between Gly[5] and Gly[7]. Although this substitution did not induce a significant change in the structure of the whole peptide with respect to the parent peptide (data not shown), the biological activity of [Aib[1]]-hNPS is slightly higher than that of the parent peptide. This finding indicates that, while Aib[1] is not able to change significantly the spectral properties of hNPS, it may favor a moderate local ordering of the first residues. This result is fully consistent with the previous Ala substitution and, together with the decrease of biological activity when the first residue is deleted,[18] hints at the possibility that whatever structure of the message domain is attained in the interaction with the receptor, it does not place any special requirement on the side chain of the first residue. In other words, Ser[1] is not required for the features of its side chain.

The most interesting results on the conformation−activity relationship within the N-terminal domain are obtained with changes involving the Asn[4]Gly[5] dipeptide. The fact that substitutions of Gly[5] with either Ala or D-Ala are equally well tolerated speaks in favor of either I′ or II′ turns centered on Asn[4]Gly.[5] However, it is difficult to ascertain the true nature of the putative turn because substitutions in position 4 with His, Pro, and D-Pro do not lead to active analogues. These results do not rule out the possibility of a different type of turn centered on Asn[4]Gly[5] because the substitutions we tried are not exhaustive. Another possible interpretation of these results is that the side chain of Asn[4] is involved in a very specific interaction with the receptor. It is difficult, at this stage, to discriminate between these alternatives. However, it is very interesting to note that the substitution of Gly[5] with D-Ala[5] and expecially with Aib[5] reduced not only peptide potencies (3- and 10-fold, respectively) but also their efficacies.

To fully characterize their pharmacological profiles, we compared the receptor activation curves of these two analogues with respect to wild type NPS. Figure 1 A and B show the comparison of the response (in terms of fluorescent intensity units) of the NPSR to [Aib[5]]hNPS and [D-Ala[5]]hNPS, respectively. In $HEK293_{mNPSR}$ [Aib[5]]hNPS and [D-Ala[5]]hNPS mimicked hNPS effects showing, however, reduced potency (pEC_{50}

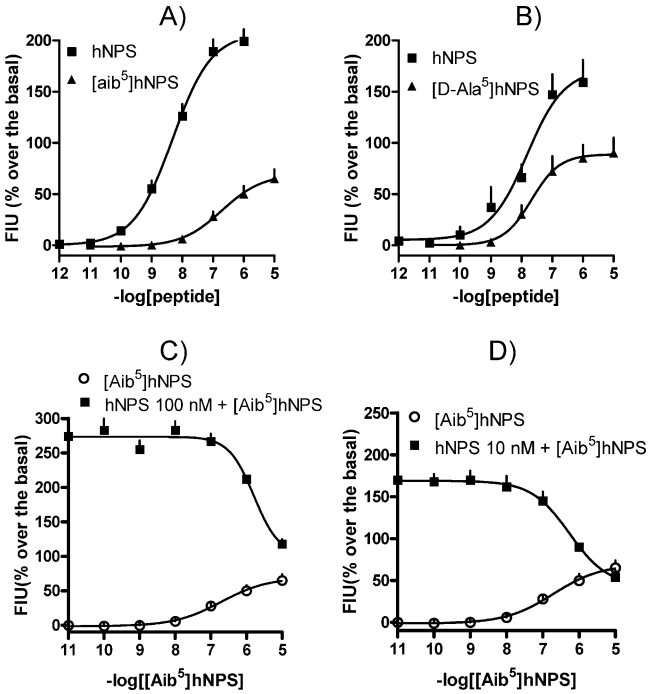

Figure 1. Response of HEK293$_{mNPSR}$, in fluorescence intensity units, versus peptide concentration. (A) hNPS vs [Aib^5]hNPS; (B) hNPS vs [D-Ala^5]hNPS. When tested against a concentration of 100 (C) or 10 (D) nM hNPS, [Aib^5]hNPS is able to inhibit in a concentration-dependent manner the stimulatory effect of hNPS (see Methods).

6.72 and 7.55, respectively) and maximal effects ($\alpha \sim 0.3$ and 0.6, respectively). As shown in Figure 1C,D, when tested against fixed concentrations of hNPS (10 or 100 nM), [Aib^5]hNPS was able to inhibit, in a concentration-dependent manner, the stimulatory effect of hNPS with pIC_{50} values of 6.27 and 5.79, respectively (see Methods).

The data illustrated in Figure 1C,D describe typical pharmacological profiles of partial agonists. The change from full agonist to partial agonist is generally taken as an initial indication for the design of an antagonist. In the present experiment, this indication emerges from a residue (Aib) whose side chain is expected to not be directly involved in the interaction with the active receptor site; the replacement of Gly^5 with Aib is expected to induce a change of conformation that could be favorable for antagonism. Substitution of Gly^5 with Aib^5 does not produce a significant increase in helicity (vide infra) but has certainly a profound influence on the conformation of the hinge region. The lack of helicity can be explained by the lack of an efficient Ncap among preceding residues (*vide supra*)[20] or by the fact that residues following Aib^5 do not favor helicity or propagation of a helix.

On the other hand the change of biological activity, with respect to the parent peptide, may be interpreted in terms of the ability of Aib residues to support more rigid conformations with respect to Gly residues and thus reduce the optimally interaction required from receptor activation. In other words, Gly and Aib are both achiral residues and possess conformational preferences possibly consistent with several kinds of turn, but Aib is conformationally less flexible than Gly and, accordingly, may force the local conformation into an incorrect bent structure. The formation of a slightly distorted turn, with respect to the optimal bioactive conformation, may be sufficient to occupy the active site but, at the same time, to suppress crucial interactions with receptor residues, for example, the interaction of the Asn side chain, thus reducing peptide efficacy.

Structural Data. NMR spectra of NPS in water hint at a completely disordered conformation. As pointed out by Bernier et al.,[19] it is possible to observe seven sequential NH−NH cross-peaks for residues 5−13 in the NOESY spectrum of NPS at pH 5.0. This observation led them to postulated the presence of a nascent helix but, in the absence of other diagnostic short or medium range effects, these are not sufficient to define accurately the secondary structure. The very concept of "nascent helix", as described by Dyson et al.,[24] requires that the observation of N−N cross-peaks in water be validated by stabilization into a true helix in water/trifluorethanol mixtures. In our hands, all attempts to observe a stable helix in water/trifluorethanol mixtures failed. Accordingly, we tried to slightly enhance the intrinsic helicity by combining the use of water/trifluorethanol mixtures with a systematic change of the "flexible" residues. A further advantage of this approach is that it is possible to discriminate among the "flexible" residues with respect to their relative importance for helicity and activity. To assess conformational tendencies on an experimental basis, we undertook a systematic NMR study in several media that favor ordered structures in linear peptides, in particular, media that favor helicity. We obtained spectra of hNPS in H_2O (pH 3.45 and 300 K), DMSO-d_6 at 300 K, DMSO-d_6−H_2O 90/10 at 278 K, 100 mM SDS_{d25} at 300 K, and finally, in mixtures of water with 2,2,2-trifluoro ethanol (9:1 TFE/water; at 300 K) and hexafluoro acetone trihydrate (HFA·3H_2O/water 1:1 v:v, corresponding to a mole fraction of HFA, $f = 0.085$, at 300 K). Mixtures of water and organic solvents like DMSO and alcohols are often referred to as cryoprotective mixtures.[25] The number and intensity of NOEs and, to some extent, even the conformational preferences of short linear peptides in solution can be influenced by the use of cryoprotective mixtures[26,27] possibly because these mixtures have viscosities higher than that of pure water but comparable to that of cytoplasm.[28,29] Alcohols, particularly fluorinated ones, either neat or mixed with water, are the most popular media used to induce helicity in peptides.[30−33]

Nevertheless, the NOESY spectra of hNPS in dimethyl sulfoxide (DMSO), in mixtures of water with DMSO or in several hydroalcoholic mixtures, do not show any increase of diagnostic NOEs with respect to neat water solutions, but they exhibited a limited spread of the NH resonances, corresponding to little ordering of the peptide. The same behavior was observed for all analogues containing changes only in the message N-terminal domain (data not shown). In particular, the lack of any tendency to assume helical conformations can be probably ascribed to the very high number of either intrinsically flexible residues (Gly) or of β-promoting residues like the three Ser and the two Thr out of a total of 20 residues. On the contrary, all Ala analogs designed to test the conformation−activity relationship in the tract 5−13 show some tendency to assume a helical conformation in the C-terminal moiety when their NMR spectra are run in hydroalcoholic mixtures. This tendency is enhanced, as expected, by Aib substitutions, but we focused our structural investigation on analogs containing Ala in lieu of Gly/Thr to restrict the analysis to proteic residues.

To put these observations on a more quantitative basis, we undertook a full structural characterization of [Ala^{7,9,13}]hNPS. NMR spectra of [Ala^{7,9,13}]hNPS were obtained in DMSO-d_6−H_2O 90/10 at 278 K, neat TFE at 300 K, and 9:1 TFE/water at 300 K. The high number of constraints that can be measured for [Ala^{7,9,13}]hNPS in 9:1 TFE/water at 300 K allows a detailed structure determination. Figure 2 shows the number of NOE-derived constraints for different ranges (panel A) and their

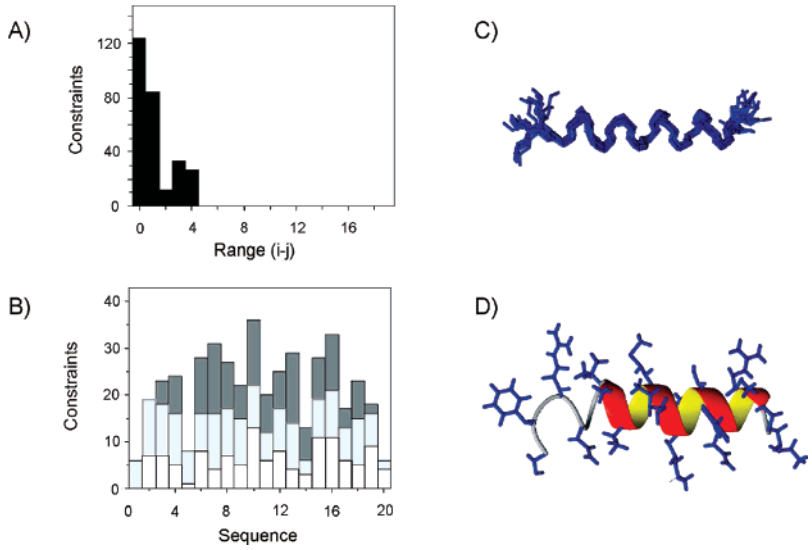

Figure 2. NOE constraints and the corresponding final structure for [Ala^{7,9,13}]hNPS in 9:1 TFE/water at 300 K. (A) Number of NOE-derived constraints for different ranges of interactions (sequential, short, and medium distance); (B) Distribution of NOE-derived constraints along the sequence; (C) Bundle of the 10 best structures of [Ala^{7,9,13}]hNPS calculated by DYANA and refined by means of restrained simulated annealing; and (D) Ribbon representation of the mean structure, with side chains shown as a neon representation. All molecular models were calculated by MolMol.[32]

distribution along the sequence (panel B). Introduction of all restraints derived from intraresidue, sequential, and medium range NOEs in DYANA[34] generated 20 structures of [Ala^{7,9,13}]-hNPS with good values of the usual target function[34] out of 30 random generated initial conformers. All 20 structures have similar values of the backbone torsion angles for the C-terminal part, but diverge in the N-terminal region.

Figure 2C shows the bundle of the 10 best structures of [Ala^{7,9,13}]hNPS calculated by DYANA and refined by means of restrained simulated annealing. The corresponding ribbon representation of the mean structure, as calculated by MolMol,[35] can be seen in Figure 2D.

The ribbon was superimposed by MolMol only to residues 7−18 because, strictly speaking, only these have canonical values of backbone angles for an α-helix. However, as shown by the Ramachandran plot for the average structure (Figure 1SI of Supporting Information), apart from the first two and the last two residues, the whole structure is essentially helical, although some pairs of ϕ/ψ angles, notably those of residues 3 and 6, are at the margin of the helical region. Small deviations from a canonical α-helical structure may originate from an insufficient number of constraints in the refinement procedure.

The sequence from Ala^7 to Ala^{18} is not only a fairly regular α helix, but is also endowed with remarkable stability for a linear peptide. Figure 3 shows the comparison of natural abundance 600 MHz ^{1}H−^{15}N HSQC correlation spectrum of [Ala^{7,9,13}]hNPS in neat TFE-d_3, recorded 24 h after dissolution (left panel), with the corresponding spectrum in 9:1 TFE-d_2/H_2O at 300 K (right panel). It can be appreciated that all residues comprised in the helical structure from Ala^7 to Ala^{18} do not exchange during this time.

A better understanding of the influence on biological activity of helicity in the 5−13 region can be achieved by examining separately single Ala or Aib analogues. [Ala^5]hNPS behaves as hNPS, that is it has no propensity to assume a helical conformation even in 9:1 TFE/water at 300 K. Interestingly, substitution of Gly^5, with the stronger helix inducer Aib, also failed to induce any helicity. Peptides [Ala^7]hNPS, [Aib^7]hNPS, [Ala^9]hNPS, and [Ala^{13}]hNPS, when examined in 9:1 TFE/water at 300 K, showed that only substitution of Gly^7, either with

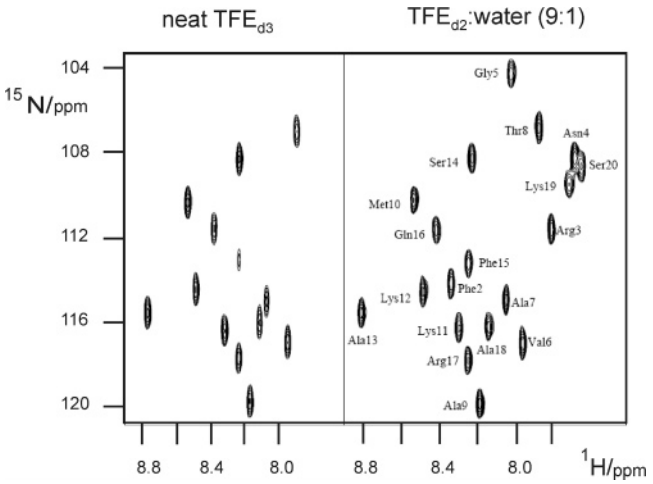

Figure 3. Comparison of natural abundance 600 MHz ^{1}H−^{15}N HSQC correlation spectrum of [Ala^{7,9,13}]hNPS in neat TFE-d_3, recorded 24 h after dissolution (left panel) with the corresponding spectrum in 9:1 TFE-d_2/H_2O at 300 K (right panel).

Ala or Aib, is able to induce substantial helical structure, whereas [Ala^9]hNPS and [Ala^{13}]hNPS show just a few effects hinting at some degree of helicity. A comparison of the amide regions of [Ala^5]hNPS, [Ala^7]hNPS, and [Ala^{7,9,13}]hNPS is reported in Figure 2SI of Supporting Information. Relevant NOEs are reported in detail in Methods (vide infra), but the main diagnostic NOEs are summarized in Figure 4.

Qualitative evaluation of these NOEs points to helical tracts approximately centered around the residue bearing the Ala substitution, but the data are insufficient for a more precise conformational characterization. Nonetheless, this conformational behavior together with the pharmacological data suggests a critical negative role for a helical conformation centered around residue 7.

Conclusion

Although the structure−activity relationship of each residue of NPS has been thoroughly characterized by exhaustive Ala scan and D-Ala scan studies as well N- and C-terminal

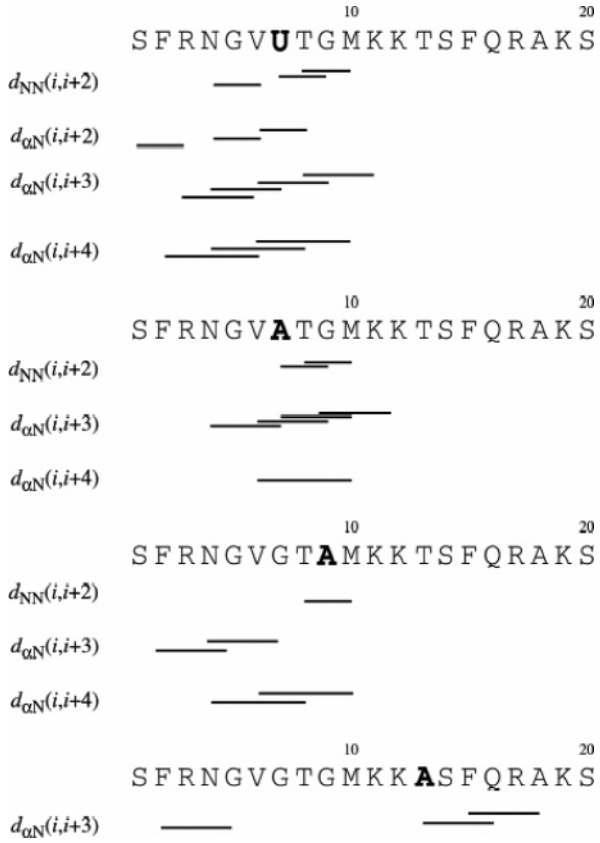

Figure 4. Bar diagram of diagnostic NOEs for peptides [Aib[7]]hNPS, [Ala[7]]hNPS, [Ala[9]]hNPS, and [Ala[13]]hNPS in 9:1 TFE-d_2/H_2O at 300 K. The single-letter code used for Aib is U.

truncation,[18,19] conformational aspects of the interaction of NPS with its receptor have not been thoroughly investigated. In the present paper we described a detailed conformational analysis of NPS and of several analogues in solution, based on NMR spectroscopy. The evaluation of conformational preferences was paralleled in each case by the corresponding biological activity. The main efforts were focused on the behavior of the 5−13 regulatory region because it had been suggested[19] that a helical conformation of this region could be instrumental for a regulatory role of the biological activity. NPS has a sequence rich with conformationally flexible residues; therefore, it was not surprising to find that this peptide has an essentially random conformation in water and that even in strong helix-inducing media it is not structured. Conversely, substitution of three flexible residues in the 5−13 region leads to a very stable helix spanning virtually all the sequence. However, triple substitution of Gly[7], Gly[9], and Thr[13] with Ala residues deprives the peptide of all its biological activity. To pinpoint the regions most sensitive to the presence of a rigid structure, conformation−activity relationship investigations were extended to singly substituted analogues. The analysis of [Aib[1]]hNPS, [Ala[5]]hNPS, [D-Ala[5]]hNPS, [Aib[5]]hNPS, [Ala[7]]hNPS, [Aib[7]]hNPS, [Ala[9]]-hNPS, and [Ala[13]]hNPS showed that the only substitutions that induced substantial structuring of the peptide were those of Gly[7], with either Ala or Aib. Because both analogues also have very low activity, it seems fair to conclude that the region for which helicity is detrimental for biological activity is that around residue 7. It is particularly striking that substitution of Gly[5] with either Ala or Aib did not lead to any structuring of the peptide. We can conclude that conformational changes along the sequence G[5]VGTG[9] are really critical, hinting at a key role of the (hinge) region adjacent to the N-terminal message domain. Even slight modifications can lead to complete loss of biological activity ([Ala[7]]hNPS, [Aib[7]]hNPS) or result in reduced efficacy ([Aib[5]]hNPS and [D-Ala[5]]hNPS).

The conformational analysis of the hinge region that led to a partial agonist heralds the design of pure antagonists in the near future. Because the Asn[4]Gly[5] dipeptide is a clear signature for residues $i+1$ and $i+2$ of several β-turns, we tried to stabilize it with the double goal of obtaining powerful agonists and of validating the type of turn. Substitutions of Asn[4] with His, Pro, or D-Pro, aimed at stabilizing I, II, or II′ beta turns centered on Asn[4]Gly[5], lead to a drastic reduction of activity. On the contrary, substitutions at Gly[5] with Ala, D-Ala, or Aib are well-tolerated, hinting at the possible presence of a different turn.

The lack of success with the substitutions we tried can be due either to the fact that the mutants' side chains interact badly with the receptor or more likely that the turn is a different one. For instance, other described canonical turns are type I, typeVIII, type VIa1, type VIa2, type VIb, and type IV (see PROMOTIF[37]). Our search could not possibly be exhaustive either in terms of turn types or in terms of residues. In addition to canonical turns, it is also possible to have noncanonical ones.[38]

It seems reasonable to expect that further investigations on the nature of the putative turn can lead to powerful and stable NPSR ligands.

Methods

Peptide Synthesis. hNPS and its analogues were synthesized according to published methods[38] using Fmoc/*t*-butyl chemistry with a Syro XP multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany). As an illustrative example, the synthesis of hNPS is described. Fmoc-Ser(tBu)-4-benzyloxybenzyl-alcohol resin (Fmoc-Ser(tBu)-Wang resin; 0.65 mmol/g, 0.2 g) was treated with 20% piperine/DMF and linked with Fmoc-Lys(Boc)-OH by using 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, Fmoc-Ser(tBu)-OH, and Fmoc-Aib-OH. All 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, and the coupling reaction time was 1 h. Capping with acetic anhydride (0.5 M) with the presence of NMM (0.25 M; 3:1 v/v; 2 mL/0.2 g of resin) was performed at any step. A 20% piperidine/DMF mixture was used to remove the Fmoc group at every step. The peptide resin was washed with methanol and dried in vacuo to yield the protected hNPS-resin.The other peptides were synthesized in a similar manner. The protected peptide−resin was treated with reagent B[39] (TFA/$H_2$0/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 exhausted resin, the solvent was concentrated in vacuo, and the residue was triturated with ether.

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 × 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 Nucleodur C_{18} column (4.6 × 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 × 150 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 65% 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, and values are expressed as MH^+.

The analytical properties of hNPS analogues are reported in Table 1.

Pharmacology. Cell Culture. HEK293 cells stably expressing the mouse recombinant NPSR ($HEK293_{mNPSR}$) were generated as previously described[10] 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.

Calcium Mobilization Experiments. $HEK293_{mNPSR}$ were 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 mM probenecid, 3 μM 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 mM HEPES, 2.5 mM probenecid, and 500 μM Brilliant Black (Aldrich) was added. Concentrated solutions (1 mM) of hNPS 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) 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). Online additions were carried out in a volume of 50 μL/well.

[Aib[5]]hNPS, tested against fixed concentrations of hNPS (10 or 100 nM), was able to inhibit the stimulatory effect of NPS with pIC_{50} values of 6.27 and 5.79, respectively. A pK_B of 7.16 was derived from the following equation:

$$KB = IC_{50}/([2 + ([A]/EC_{50})^n]^{1/n} - 1])$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [*A*] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response, and *n* is the Hill coefficient of the CRC to the agonist.[40]

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 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 ANOVA, followed by the Dunnett test for multiple comparisons.

NMR Spectroscopy. NMR samples were prepared by dissolving appropriate amounts of peptide in 0.5 mL of solvents to make approximately 1 mM solutions.

All spectra were run on Bruker Avance DRX-600 spectrometer equipped with cryoprobe, operating at 600 MHz for ^1H. A conventional set of 2D spectra, according to the scheme of sequential assignment described by Wuethrich[41] was recorded: DQF-COSY,[42] TOCSY,[43] and NOESY.[44] Natural abundance $^1H-^{15}N$ HSQC correlation experiments[45] were used to identify amide protons with slowed exchange, after dissolving freeze-dried peptide samples in 99.9% TFE-d_3 and in TFE-d_2.

Total correlated spectroscopy (TOCSY) spectra were collected with mixing times in the range 50 to 75 ms, using the clean MLEV-17 mixing scheme.[46] The nuclear Overhauser enhancement spectroscopy (NOESY) spectra were recorded with mixing times of 200 and 300 ms. TPPI was applied to achieve quadrature detection in the virtual dimension[47] Water suppression was achieved either by presaturation or by using the WATERGATE pulse sequence.[48]

Data processing was performed with standard Bruker software XwinNMR. Spectral analysis was performed with SPARKY.[49]

The most relevant NOESY cross-peaks measured for analogues [Ala[7]]hNPS, [Aib[7]]hNPS, [Ala[9]]hNPS, and [Ala[13]]hNPS, when examined in 9:1 TFE/water at 300 K, are: CHα4 → NH7, CHα6 → NH9, CHα6 → NH10, CHα7 → NH10, CHα9 → NH12; NH7 → NH9, and NH8 → NH10 for [Ala[7]]hNPS; CHα1 → NH3, CHα4 → NH6, CHα6 → NH8; CHα3 → NH6, CHα4 → NH7, CHα6 → NH9, CHα8 → NH11; CHα4 → NH8, CHα6 → NH10; NH4 → NH6, NH7 → NH9, and NH8 → NH10 for [Aib[7]]hNPS; CHα2 → NH5, CHα4 → NH7, CHα4 → NH8, CHα6 → NH10, and NH8 → NH10 for [Ala[9]]hNPS; and CHα2 → NH5, CHα13 → NH16, CHα15 → NH18 for [Ala[13]]hNPS, respectively.

Structure Calculation. The input data for the structure calculation with the program DYANA[34] were generated from the peak volumes obtained from SPARKY.[49] Figure 2A summarizes all measured NOEs, classified as intraresidue, sequential and medium range ($1 < |i - j| \leq 5$). Based on the peak volumes observed on the NOESY spectra, the upper distance limits were generated with the program CALIBA.[34] Computations were performed on SGI O2 computers. During the DYANA calculation using the simulated annealing protocol in torsion angle space, we introduced all available restraints.

To display the final structures, calculations of the mean coordinates of the ensemble structures and their rmsd values were carried out with the program MOLMOL.[35]

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Supporting Information Available: Ramachandran plot of the average structure of [Ala[7,9,13]]hNPS. Amide regions of [Ala[5]]hNPS, [Ala[7]]hNPS, and [Ala[7,9,13]]hNPS. This material is available free of charge via the Internet at http://pubs.acs.org.

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