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Interaction between ghrelin and the ghrelin receptor (GHS-R1a), a NMR study using living cells

Manuel Martín-Pastor [a], Antonia De Capua [a,†], Carlos J. P. Álvarez [b,c,d], M. Dolores Díaz-Hernández [e], Jesús Jiménez-Barbero [e], Felipe F. Casanueva [c,d], Yolanda Pazos [b,c,*]

[a] *Unidade de Resonancia Magnética, RIAIDT, Universidad de Santiago de Compostela, Campus Sur. 15706 Santiago de Compostela, Spain*
[b] *Bioquímica Estructural de la Patología Endocrinológica, Instituto de Investigación Sanitaria, Lab. No 4, Complejo Hospitalario Universitario de Santiago de Compostela, A Choupana, s/n. 15706 Santiago de Compostela, Spain*
[c] *CIBER Fisiopatología de la Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Spain*
[d] *Laboratorio de Endocrinología Molecular y Celular, Instituto de Investigación Sanitaria, Lab. No 2, Complejo Hospitalario Universitario de Santiago de Compostela, A Choupana, s/n. 15706 Santiago de Compostela, Spain*
[e] *Centro de Investigaciones Biológicas, CIB-CSIC, 28040 Madrid, Spain*

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ABSTRACT

The study of the interaction of ghrelin (**1**), the endogenous ligand for the GH secretagogues receptor (GHS-R1a), and des-acyl ghrelin (**2**) with the GHS-R1a by NMR using living cells is presented, using GHS-R1a stably transfected cell lines (CHO and HEK 293) and wild type cells. Therefore, the interaction of **1** and **2** with the GHS-R1a receptor has been performed using quasi-physiological conditions. Ghrelin (**1**), showed a higher number of residues affected by chemical shift perturbation (CSP) or chemical shift exchange (CSE) effects: Ser3, Phe4, Leu5, Val12, Gln13/Gln14, Lys16/Lys19, Glu17 and Lys24 were much more affected in **1** than in des-acyl ghrelin (**2**). The chemical shift index CSI values indicated the presence of a possible α-helical region between Glu8 and Lys20 for ghrelin (**1**). After analysing the NMR data, two possible structures have arisen, which present different proline rotamers: the *EEZE* and the *EZEZ* conformers, at positions Pro7, Pro21, Pro22 and Pro27, respectively, keeping a left-handed α-helix from Glu8 to Lys20. These experimental evidences might imply that the GHS-R1a receptor is acting as a prolyl-*cis*/*trans* isomerase.

1. Introduction

Ghrelin (**1**), the endogenous ligand for the growth hormone secretagogue receptor,[1] is a 28-amino residue peptide with a post-translational octanoyl modification on Ser3 (Fig. 1), which was first discovered in rat and human stomach tissues.[2] This hormone is mainly synthesized in the stomach, but substantially lower amounts have been detected in other tissues.[3] Functionally, ghrelin (**1**) stimulates growth hormone (GH) secretion from pituitary somatotropes[2,4] and increases food intake and body weight.[5] Indeed, it has been proposed that **1** acts directly on the hypothalamic regulatory nuclei that control energy homeostasis acting as an orexigenic peptide.[6] On the other hand, des-acyl ghrelin (**2**), which presents the same structure with the exception of the *n*-octanoyl modification on Ser-3, does not show the same functionalities.[7] The receptor GHS-R1a transduces the information provided by ghrelin (**1**) and the group of growth hormone secretagogues (GHS), not structurally related to it. These striking properties have been explained on the basis of the existence of a common binding domain, as demonstrated, using GHS peptide and non-peptide agonists, by site-directed mutagenesis studies assisted by molecular modelling procedures.[8]

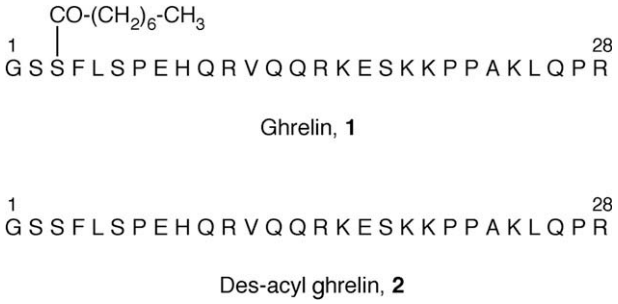

Figure 1. Primary structure of human ghrelin (**1**) and of its des-acylated related peptide, **2**.

* Corresponding author. Tel.: +34 981 95 50 72; fax: +34 981 95 61 89.
E-mail address: yolanda.pazos@usc.es (Y. Pazos).
† Present address: Interdisciplinary Research Centre on Biomaterials, Department of Materials and Production Engineering, University 'Federico II', Piazzale Tecchio, 80. 80125, Napoli, Italy.

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Since the discovery of ghrelin (**1**), there have been several studies aimed at determining which are the minimal structural requirements that permit to detect ghrelin receptor biological activity. Bednarek et al. reported the first ghrelin-based minimally active structure–activity studies, demonstrating that the minimum sequence necessary for GHS-R1a activation encompassed the first five residues, with the octanoyl modification on Ser-3. The employed protocol involved binding assays and activation of GHS-R1a by measuring intracellular calcium mobilisation, using HEK 293 cells transfected with GHS-R1a.[9] However, it was later demonstrated that this truncated analogue was not capable of stimulating GH secretion from somatotrope cells.[10] In principle, this discrepancy might be attributed to the fact that the rise in calcium obtained for the truncated analogues does not reflect the complete activation of the signal transduction systems as required, for example, to activate GH secretion. At the present moment, no data are available regarding the bioactive conformation of ghrelin (**1**) and its mode of interaction of GHS-R1a at the key binding site. In principle, given the presence of four Pro residues within the 28-amino acid primary sequence, as well as the *n*-octanoylation at Ser3, one could expect the presence of conformational heterogeneity and a fair amount of flexibility. To the best of our knowledge, three publications have reported approaches to determine the 3D structure of ghrelin (**1**) in solution. The ^{1}H NMR studies performed by Silva Elipe et al.[11] showed that ghrelin behaves as an unstructured and/or fast interconverting peptide at acidic pH. Later, Beevers and Kukol[12] reported a molecular dynamics (MD) simulation study at neutral pH in water and in the presence of a lipid bilayer, proposing the existence of stable secondary structural features for **1** in the latter case. In particular, the presence of a short α-helix from Pro7 to Glu13 and a hairpin structure with Glu17 to Lys20 in the bending region. Very recently, Dehlin et al. have reported on the CD study of ghrelin (**1**) and des-acyl ghrelin (**2**) in the presence of Tris pH 7.4 and of the α-helix stabilizing solvent, TFE. Although rather qualitative, it was described that the helical content in **1** and **2** was enhanced from 12% to 23% and 49%, respectively.[13]

In any case, the proper structure–activity relationship study should be based on the knowledge of the bioactive conformation of **1** and **2** when bound to the receptor. NMR techniques are suitable to this end when working with isolated protein receptors,[14–16] and even recently, new NMR experiments using receptor-rich living cells have been reported.[17] Provided that the system holds the right kinetic features, these experiments, with living cells, could avoid the requirement for isolating the protein receptor. On this basis, the approach presented herein aims at the determination of the bioactive conformation of ghrelin (**1**) when interacting with its receptor, at neutral pH. Indeed, since the NMR study of ghrelin (**1**) is performed with living cells highly decorated with the GHS-R1a receptor, physiological conditions similar to those taking place in Nature are kept. Moreover, as the *n*-octanoyl modification of **1** has been shown to be essential for its physiological role, the des-acyl ghrelin analogue (**2**) has also been analysed using the same approach. Thus, the NMR analysis of the interaction of **1** and **2** with the ghrelin receptor (GHS-R1a) was performed in CHO and HEK 293 cell lines.

2. Results and discussion

2.1. Preliminary ^{1}H NMR experiments

To assure time stability of the samples, and therefore reliability and reproducibility of the NMR experiments, a series of 1D ^{1}H NMR spectra of the samples with living cells were collected at regular intervals during the course of one day. These spectra were recorded for ghrelin (**1**) and des-acyl ghrelin (**2**) in the presence of wild type and GHS-R1a-containing cells. The ^{1}H NMR spectra of the **1**:CHO-GHSR1a sample and of the **1**:CHO sample showed drastic changes for most of the exchangeable amide protons (data not shown). In fact, many signals gradually broadened and eventually disappeared within ca. 7 h after sample preparation. This was not the case for cell-free samples and, since buffer conditions (pH 7.0) were employed, the observed changes could, in principle, be attributed to the interaction of the peptides with the cells (probably through the receptor), thus affecting the water-exchange process for the amide protons. Nevertheless, the ^{1}H NMR spectra also showed significant changes for some of the non-exchangeable aliphatic resonances, especially for ghrelin (**1**) in the presence of transfected cells, even with the appearance of new sets of signals (Fig. 2).

After analyzing the ^{1}H NMR experiments taken at regular intervals of time for **1** and **2** in the presence of wild type cells and GHS-R1a transfected cells, it could be deduced that changes of the NMR signals only took place for ghrelin (**1**), and only when the transfected version of the cells was employed. No effect was observed for **2**. Additionally, no effects were observed for the wild-type cells. Even more important, the changes in the NMR spectra were shown to be

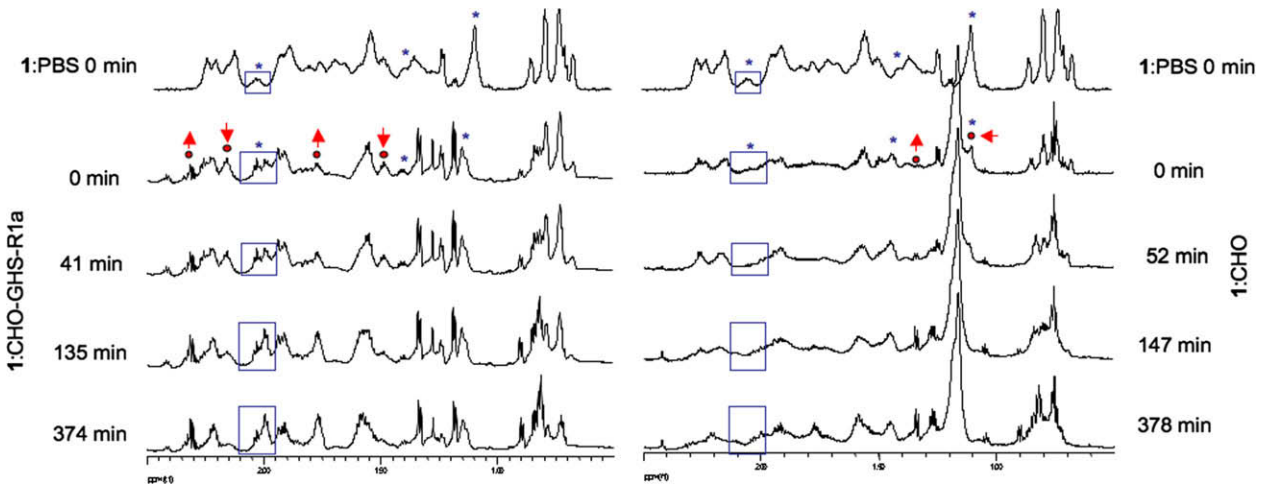

Figure 2. Time-course of the aliphatic section of the ^{1}H NMR spectra of **1**:CHO-GHS-R1a (left) and **1**:CHO (right) compared to the **1**:PBS data at time 0 min. The ^{1}H NMR spectra show important changes (red arrows) for some of the non-exchangeable aliphatic resonances and the appearance of new sets of signals for ghrelin (**1**) in the presence of the receptor (blue squares). The signals for the octyl group are marked with blue asterisks.

reversible. When the transfected cells were eliminated from the sample also containing **1**, the original chemical shifts for **1** in PBS buffer were recovered.

2.2. NMR cell titration experiment

Additional experiments were performed by increasing the number of cells in the NMR tube containing either **1** or **2**. The results of the cell titration study are shown in Figure 3. The signals of the amide protons disappeared gradually from the spectra and some of the non-exchangeable aromatic protons between 6.5 and 7.5 ppm (Fig. 3A–C) had a gradual change in their chemical shifts with the increase in the number of cells. Besides, new sets of signals were progressively appearing in the aliphatic region of the spectrum (Fig. 3D–F), with the increment in the number of cells in the NMR tube.

2.3. Comparison of the data for GHSR1a-containing cells with those for wild type cells with 2D TOCSY experiments

For each of the two peptides studied, **1** and **2**, 2D TOCSY spectra were acquired for different NMR samples and prepared under different conditions. Namely, spectra were recorded for **1** and **2** in PBS (without cells), and also in the presence of CHO cells or HEK cells. In each case, two types of cells were employed, either wild type or GHSR1a-transfected. Exclusive chemical shift perturbations (CSP) and slow conformational exchange (SCE) effects occurring in the two transfected cell samples were identified by comparison among TOCSY spectra: the two obtained for transfected and wild type cells of the same cell line, and the spectrum of the peptide in PBS, for which no CSP or SCE effects were obviously detected.

2.4. CSP and SCE effects in ghrelin (1)

Figure 4 shows part of the amide region 2D TOCSY spectra of **1** with CHO-GHSR1a cells (A) and CHO cells (B). Many of the correlations involving the amide protons have been drastically affected in the presence of the receptor-containing cells.

The evaluation of the spectra (using the spectrum acquired in absence of cells, **1**:PBS, as reference, Fig. S1) revealed that the following changes were exclusive for sample **1**:CHO-GHSR1a: (i) there are two sets of signals having SCE for protons Hβ and/or Hγ of Gln13 (and/or Gln14), in addition to a CSP of −0.08 ppm for the amide proton of Gln13 (and/or Gln14); (ii) there is a negative CSP effect for the amide proton of Leu5, which resonates ca. 0.07 ppm lower than in the **1**:CHO sample (Fig. 4B) or in the **1**:PBS sample (Fig. S1); and, (iii) the signals of the amide proton/s of Lys16 (and/or Lys19) are separated in two different sets. In the new set of signals for Lys16 (and/or Lys19), there was a CSP of −0.11, +0.01 and −0.04 ppm for Hα, Hβ1 and Hγ, respectively. The amide proton of Glu-17 had a positive CSP effect of +0.1 ppm in **1**:CHO-GHSR1a respect to **1**:PBS. A negative CSP effect of −0.16 ppm was measured for the aromatic signals of Phe4. The amide proton of Lys24 displayed a CSP of +0.03 ppm in **1**:CHO-GHSR1a.

The 2D TOCSY spectra shown in Figure 5 compare the aliphatic region for **1**:CHO-GHSR1a (A) and **1**:CHO (B) samples. Significant differences were observed for the **1**:CHO-GHSR1a spectrum, which did not occur for the **1**:CHO or for the **1**:PBS analogues: (i) two sets of signals generated by a SCE effect are observed for the *n*-octanoyl group attached to Ser3; (ii) the cross peak corresponding to protons Hβ and Hγ of Val12 presented a CSP effect of −0.10 and +0.05 ppm, respectively, with respect to the same peak in the **1**:CHO sample (Fig. 5B), or to the **1**:PBS sample (Fig. S2). These observed SCE or CSP effects matched the evolutions observed in the preliminary ^{1}H NMR experiments described above.

The authenticity of the conformational exchange previously found for ghrelin (**1**) was assessed by variable temperature 2D TOCSY experiments. The spectra were focussed in the double set of signals for the first methylene of the octyl group attached to Ser3. Four different temperatures of 8, 15, 25 and 35 °C were tested for **1** in the presence of HEK GHS-R1a enriched cells. These TOCSY spectra exhibited a modulation of the intensities for these methylene signals with the temperature. While at 8 °C the intensity of the new methylene signal was rather small, at 35 °C the two signals had practically the same intensity. As the temperature was raised, the intensity of the new methylene signal, which did not appear in cell-free ghrelin (**1**) sample, consistently increased at expenses of the former, indicating the presence of two conformers in the equilibrium (Fig. 6).

In order to check the consistency of the obtained results with the CHO cell line, the same study was performed with another cell

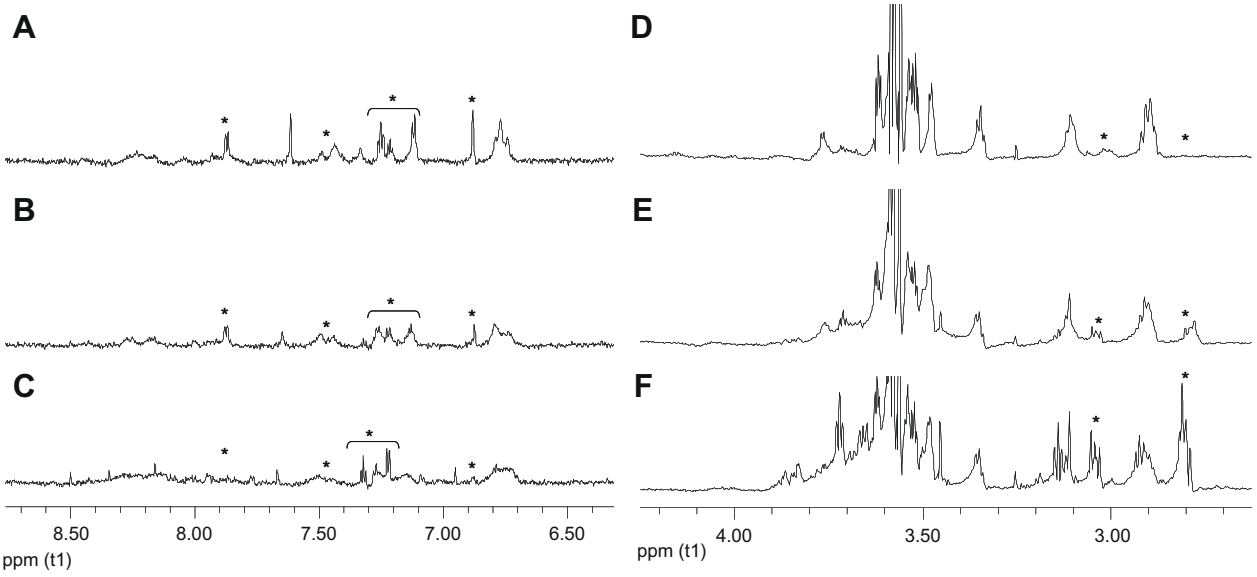

Figure 3. ^{1}H NMR titration study of **1**:CHO-GHSR1a. Spectra A, B and C correspond to the amide/aromatic region with 0, 2×10^6 and 4×10^6 cells, respectively. Spectra D, E and F correspond to the aliphatic region with 0, 2×10^6 and 4×10^6 cells, respectively. The evident and significant changes in some signals have been marked with an asterisk.

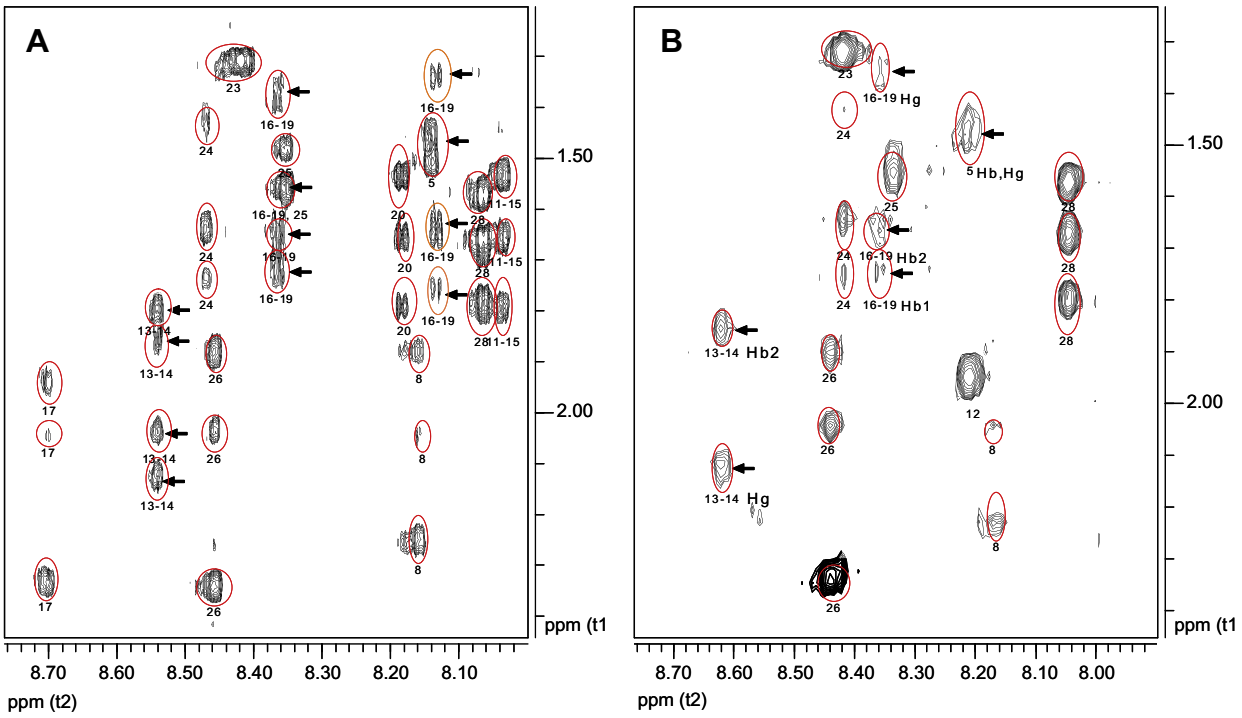

Figure 4. Amide/aromatic proton region in the 2D TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

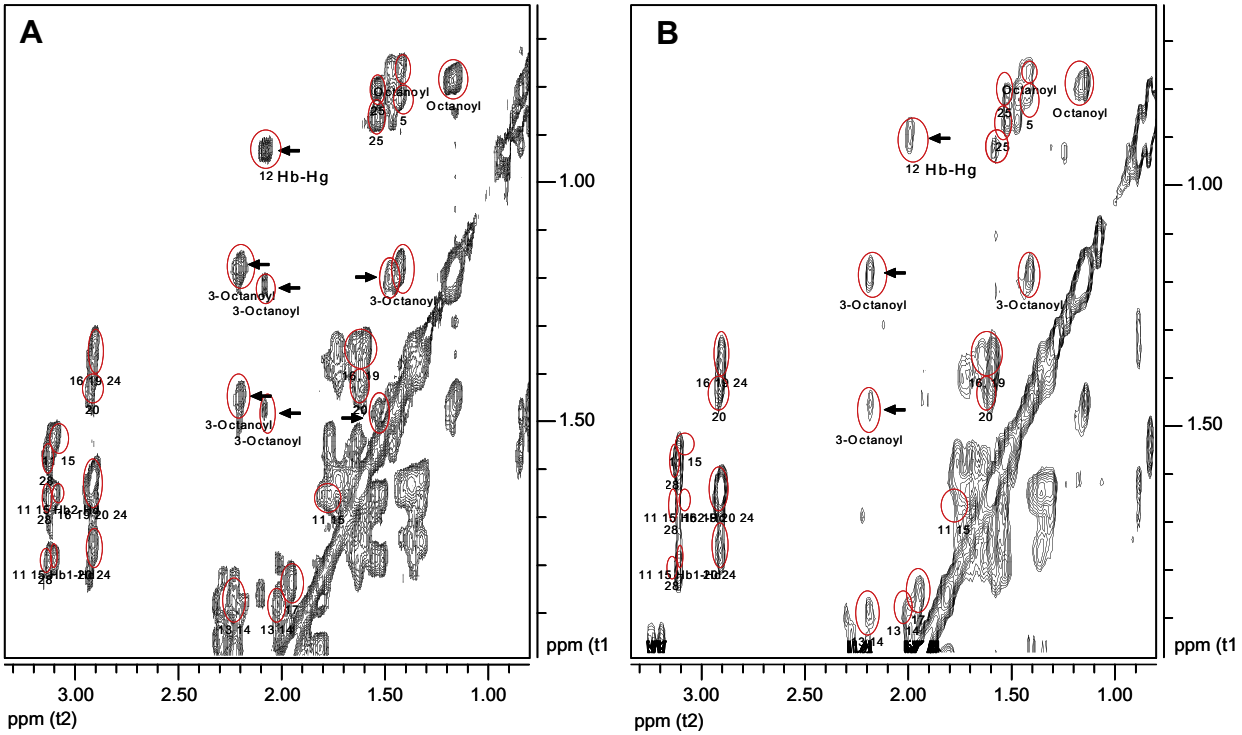

Figure 5. H-alpha/aliphatic proton region in the 2D TOCSY spectra of **1**:CHO-GHSR1a (A) and **1**:CHO (B). The numbering is indicated under the cross peaks. Differences between peaks are marked with an arrow.

line, HEK 293. Most of the aforementioned effects were reproduced again with this new HEK cell line. The unique difference observed between the two cell lines was the presence of two sets of signals for the Hβ and Hγ protons of Val12 due to a SCE effect in the **1**:HEK-GHSR1a version, instead of the CSP effect previously described for the **1**:CHO-GHSR1a sample (Fig. S3).

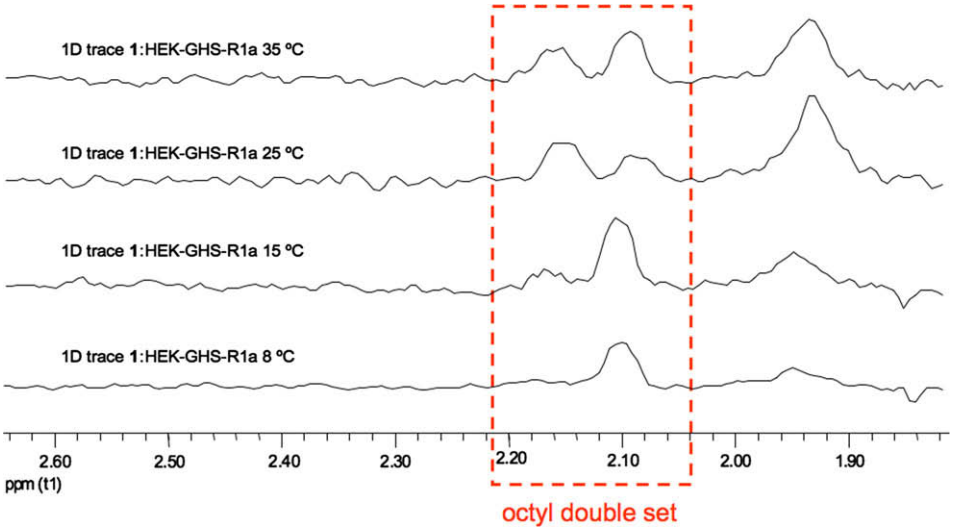

Figure 6. Traces from the variable temperature ^{1}H 2D TOCSY experiments performed in the sample **1**:HEK-GHSR1a. The experiments were not performed consecutively with the raising in temperature.

2.5. CSP and SCE effects in des-acyl ghrelin (2)

The amide protons region of **2**:HEK-GHSR1a (A) and **2**:HEK (B) samples is represented in Figure S4. Many amide protons have vanished or completely disappeared. The only clear difference exclusive to the spectrum of the **2**:HEK-GHSR1a sample (Fig. S4A) refers to the amide proton of Leu5, which has a large CSP effect of +0.19 ppm respect to that of **2**:HEK (Fig. S4B), or **2**:PBS (Fig. S6). There are other changes in the amide region (Fig. S4) that occur in both spectra with HEK-GHSR1a and HEK cells, which differ from those for the **2**:PBS sample (Fig. S6). These changes were identified as a double (or triple) set of signals for the amide proton of Ala23 and Glu17/Gln26 (Fig. S4A). For **2**:HEK (Fig. S4B) there was a double set of signals occurring in the amide proton of residue Val12 (as seen in the correlation 12NH/12Hγ). Nevertheless, this correlation could not be seen in **2**:HEK-GHSR1a due to a fast exchange of the Val12 amide proton (Fig. S4A).

Figure S5 gathers the comparison between samples **2**:HEK-GHSR1a (A) and **2**:HEK (B) in the Hα region. No exclusive modification for **2**:HEK-GHSR1a sample was found. There was one common variation to both spectra, the appearance of a double set of signals in SCE for the Hα of Ala23, which was not seen in the **2**:PBS spectrum (Fig. S7). There was also an exclusive SCE effect in the **2**:HEK spectrum (Fig. S5B), a double set of signals for the Hα of Val12, which did not occur in the **2**:HEK-GHSR1a (Fig. S5A) or in the **2**:PBS sample (Fig. S7).

Therefore, different effects are observed for both peptides, some being unique for ghrelin (**1**) in the transfected cell samples and others being observed for des-acyl ghrelin (**2**), but common to the wild type or transfected cells. The exclusive CSP and SCE effects detected for ghrelin (**1**) occurring *only* in the transfected cell samples are presumably due to the interaction of **1** with the GHS-R1a receptor.

In contrast, those effects occurring for **2** but for both wild type and transfected cells (especially in the HEK 293 cell line) could probably reflect the interaction of this peptide with other receptors at the cell membrane. Indeed, it has been previously described that this peptide exerts its biological actions through an unknown receptor, which is distinct from the GHS-R1a receptor,[18] and could be present in the HEK 293 cell line. The scheme of Figure 7 summarises all the SCE and CSP effects observed in the TOCSY spectra of **1**, which are exclusive for GHS-R1a transfected cells. The SCE effects

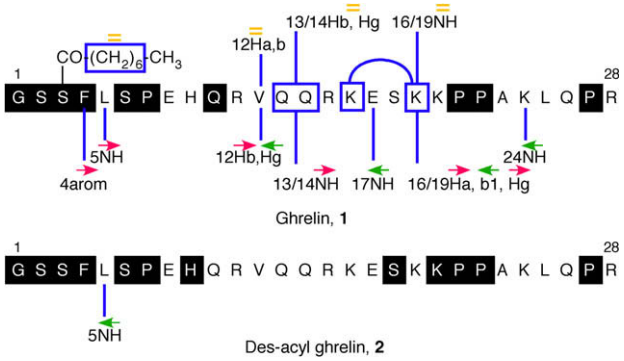

Figure 7. Description of the exclusive interactions of ghrelin (**1**) and des-acyl ghrelin (**2**) with the GHS-R1a receptor. Positive and negative CSP are indicated with arrows pointing to the left and right respectively. SCE effects are indicated with equal signs. No information is available for the NH amide protons of the residues shown in black background.

are possibly reflecting a change in the relative population of some of the conformers available to this flexible peptide. The large, both positive and negative, CSP effects detected enhance the signal dispersion of the amide protons of **1**. Both results could be related to the selection of a preferred conformation upon binding with the GHS-R1a receptor. The average lifetime of this preferred conformation should be stable within the chemical shift time scale to be observed in slow exchange in the NMR spectrum. The scheme of Figure 7 represents a reduced and conservative map of the changes observed for ghrelin (**1**) with the GHS-R1a receptor, because there were a number of residues for which no information could be obtained. The reasons for the absence of information are twofold: on one hand, the ambiguities introduced in the assignment of the lateral chains due to the extensive changes occurred in the presence of the cells; and, on the other hand, the fast NH amide exchange phenomenon that caused that many residues did not give correlations in the key amide region of the TOCSY spectrum. These residues have been represented in black background in the scheme of Figure 7. Nevertheless, the relative large number of SCE and CSP effects detected in the interaction of ghrelin (**1**) with the GHS-R1a receptor suggests that the presence of its receptor causes a significant conformational change in the peptide.

The only change observed for des-acyl ghrelin (**2**) in the GHS-R1a transfected cells corresponded to the CSP effect of the amide proton of Leu5, which is shown in the scheme of Figure 7. As for ghrelin (**1**), the residues for which it was not possible to obtain information are shown in black background. However, the large number of differences found between both schemes is evident and, very probably, indicates that the interactions of des-acyl ghrelin (**2**) with GHS-R1a are not as extended or specific as those taking place for ghrelin (**1**).

The analysis of the Chemical Shift Index (CSI), a semi empirical protocol to determine the secondary structure of a peptide or protein was then employed.[19] This approach compares the Hα chemical shifts of every aminoacid within the peptide of interest with those described for the corresponding residue in an average of random coil structures. Although the method has its drawbacks, it accurately matches the data determined by X-ray crystallography, even for small proteins. Thus, in the absence of extensive NOE data, we decided to apply this protocol to the data obtained for the different ghrelin (**1**) samples to verify whether the different CSI suggested, or not, the presence of α-helix between Pro7 and Pro21. Indeed, residues showing α-helix preference, with the exception of Val12 (CSI = 0) that is bulky and awkward due to branched beta carbon, basically compose that region of the molecule. The CSI values for the **1**:H_2O sample showed the characteristics of an unstructured peptide (data not shown), while those for the **1**:PBS analogue showed a high-field shift for all of the Hα of the previously described region, with the related CSI's suggesting the presence of certain population of α-helix. This discrepancy between the two samples could be explained by the presence of the phosphate ion, a known α-helix stabilizing osmolyte.[20] The **1**:CHO sample showed approximately the same chemical shifts as the PBS sample, while those measured for the **1**:CHO-GHSR1a sample were even more shifted to high field, which could indicate further stabilisation of the putative α-helix. These data are in agreement with the idea that the loop from Ser18 to Lys20 described by Kukol could be incorporated in a longer α-helix.[21] The NMR studies performed might indicate that ghrelin (**1**) in PBS is only partially structured, with a low percentage of α-helix from Glu8 to Lys20. This secondary structure is further stabilized in the presence of the GHS-R1a. In a parallel manner, the experiments performed in the presence of the receptor showed the development of new sets of ^{1}H NMR signals in several residues. Since this was shown to be a reversible process (see above), this fact could be explained by the isomerization of any of the prolines present in the ghrelin (**1**) skeleton: Pro7, Pro21, Pro22, or/and Pro27. Although the work of Schubert et al has shown that Δ^{13}C is an accurate indicator of the proline *N*-terminal peptide bond isomerization state,[22] it was impractical herein due to the nature of our samples. As an alternative, simulated annealing calculations were performed by restricting the torsion angles for adopting a α-helix structure from Glu8 to Lys20. When a α-helix was forced in this peptide, there was only one possibility to adopt this conformation between these residues, namely a left-handed α-helix. Nevertheless, although the prolines could adopt two possible conformations *E* ($\omega = 180°$) and *Z* ($\omega = 0°$), the results showed two structures with left-handed α-helix, with the prolines adopting the following conformations: *EEZE* and *EZEZ*, for Pro7, Pro21, Pro22, and Pro27, respectively, as the best among the 32 possible structures (Fig. 8).

Peptides usually adopt several conformations in solution. Therefore, in most of the cases, the assessment of one single 3D structure is unrealistic and, indeed, NMR may easily generate virtual conformations, when ensemble averages are not properly taken into account. A good strategy to decrease conformational exchange is the use of low temperatures before the freezing point (0–8 °C). It has been speculated that these temperatures may induce the formation of structure that is similar to the conformation of the peptide in the receptor bound state.[23–25] Although in all the experiments described herein, the temperature was set at 5 °C, we could only infer a low population of α-helix in the PBS sample, and not in the H_2O sample. Additionally, the (*reversible*) appearance of new sets of signals, indicative of the possible proline *cis*/*trans* isomerizations, exclusively arose in the presence of the receptor. Indeed, additional NMR experiments carried out under experimental conditions mimicking membrane-like environments (SDS-micelles) did not show the presence of additional NMR signals, but just suggested, on the basis of chemical shifts and NOEs (data not shown), the existence of an additional percentage, although still small, of helical structure. These pieces of information might suggest that the GHS-R1a receptor is acting as a prolyl-*cis*/*trans* isomerase and that, prior to bind to its receptor, ghrelin (**1**) needs to adopt a specific conformation.

2.6. Conclusion

The NMR study described above has shown that ghrelin (**1**) displays a large number of residues affected by CSP or SCE effects by interaction with the GHS-R1a receptor. In contrast, des-acyl ghrelin (**2**) only presents one residue with a marked CSP effect, a result that is consistent with the known higher affinity of **1**.[2,11,26] Moreover, the *n*-octanoic group at Ser3 of **1** was seen to be clearly involved and necessary for the interaction with the GHS-R1a receptor, a fact coherent with previous studies of ghrelin analogues that claimed that this pendant chain plays a defining role in its bioactivity.[2,11] The lack of CSP or SCE effects observed for des-acyl

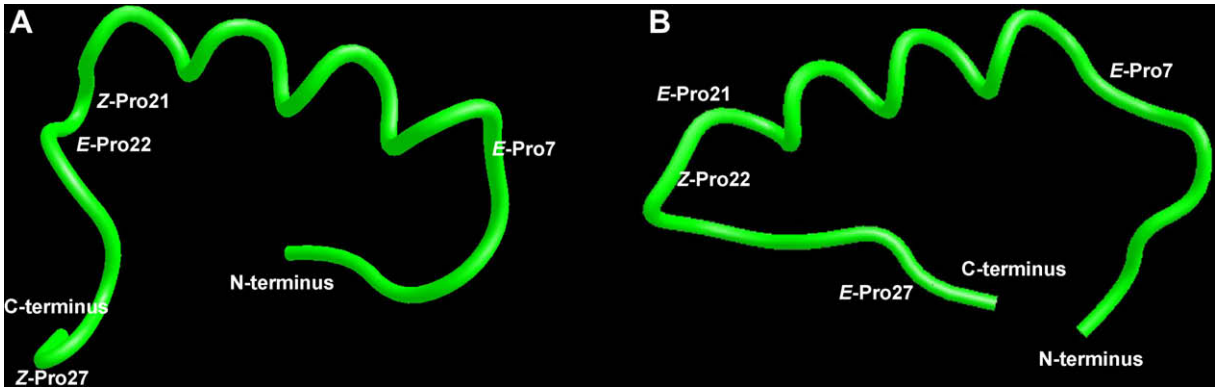

Figure 8. Possible conformations of ghrelin (**1**) in the presence of its receptor GHS-R1a. The most stable conformations were *EEZE* (A) and *EZEZ* (B) with a pre-defined left-handed α-helix from Glu8 to Lys20.

ghrelin (**2**) with the GHS-R1a receptor in the NMR experiments corroborated this piece of information. This result also supports the conclusion that NMR data using living cells accurately report on the functional interaction of these peptides.

Besides, the CSI obtained could indicate the presence of certain population of α-helix for the region between Glu8 and Lys20 in ghrelin (**1**) in PBS solution, which is further stabilized in the presence of the GHS-R1a receptor. After analyzing the possible conformations with modelling calculations, two possible structures have arisen: the *EEZE* and the *EZEZ* conformers with a left-handed α-helix from Glu8 to Lys20.

3. Experimental section

3.1. Reagents

Ghrelin (**1**) was purchased from Global Peptides (Fort Collins, Co, USA). Des-acyl ghrelin (**2**) was obtained from Bachem AG (Bubendorf, CH). F-12 Ham was purchased from Sigma Chemical Co (St. Louis, MO, USA). DMEM was purchased from Cambrex Bio Science (Walkersville, MD, USA). D_2O was purchased from Spectra Stable Isotopes (Columbia, MD, USA).

3.2. Cell cultures

HEK 293 and CHO cell lines were cultured as described by the supplier (ECACC, Wiltshire, UK). Briefly, cells were seeded in 100-mm dishes and cultured in DMEM or F-12 Ham medium, respectively, supplemented with 10% foetal bovine serum (FBS), 100U/mL penicillin G, 100 μg/mL streptomycin sulfate and 2.5 μM L-glutamine with 5% CO_2 and 37 °C. Subculture routine was as follows: split sub-confluent cultures (70–80%) 1:4 seeding at 2–5 × 10,000 cells/cm^2 using 0.25% trypsin, 0.05% EDTA. Stably transfected cell lines HEK 293-GHS-R1a and CHO-GHS-R1a were cultured as described for the parent wild type cell line and selected on the basis of resistance to geneticin sulfate G-418 (500 μg/mL).[26,27]

3.3. NMR experiments

NMR spectra were acquired on a Varian INOVA spectrometer operating at 750 MHz and processed with MestRe-C v3.x software[28] using a standard inverse detection triple-resonance and triple-axis gradient probe. Temperature was set at 5 °C in all experiments to avoid internalisation of the ligand–receptor complex in these cells. It has been described that the complete desensitization–resensitization process takes for about 6 h.[26] A soft-watergate solvent suppression scheme was used to suppress the H_2O solvent signal in all the experiments.[29] All the spectra were acquired with an external reference of 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP, 0.0 ppm).

3.4. NMR sample preparation

NMR samples of the pure peptides **1** or **2** were prepared by dissolving 400 μg of each peptide in 0.5 mL of a mixture PBS buffer (pH 7.2):D_2O (95:5, v/v). These samples are referred in the text as **1**:PBS and **2**:PBS, respectively. To perform the NMR experiments with living cells, 4 × 10^6 cells were counted, washed, and dissolved in phosphate-buffered saline (PBS, pH 7.2). Samples were prepared by placing 400 μg of each peptide in 0.5 mL of a suspension of the cells in a mixture PBS (pH 7.2):D_2O (95:5, v/v). Different samples of peptides **1** and **2** were prepared as a suspension with the following cell lines: HEK 293, HEK 293-GHS-R1a, CHO, and CHO-GHS-R1a; and they are referred in the text as **1**:HEK, **1**:HEK-GHSR1a, **1**:CHO, **1**:CHO-GHSR1a, **2**:HEK and **2**:HEK-GHSR1a. Parallel experiments (Tripan Blue Dye) probed that, under the NMR experimental conditions, cells viability was about 80% at 24 h. The exact amount of receptors on the cell surface is unknowable. However, we estimate the binding-sites (BS) concentration present in the samples[26] and, therefore, the BS:**1** ratio. Ghrelin (**1**) concentration in the samples is 2.12 × 10^{-4} M, which means a BS:**1** ratio of 1:100.

3.5. 1D ^{1}H NMR experiments

Fifteen 1D ^{1}H NMR spectra were acquired for each NMR sample for 24 h, at regular time intervals, after sample preparation. Each 1D ^{1}H NMR spectrum was acquired with 256 scans (6 min).

3.5.1. NMR titration

A NMR titration study with **1**:CHO-GHSR1a was performed. Seventeen 1D ^{1}H NMR spectra were acquired for samples prepared with a crescent number of cells (0–4 × 10^6 cells) in 0.25 × 10^6 cells steps. The complete titration study was performed in a relative short time (ca. 4 h) to avoid cell sedimentation. Each 1D ^{1}H NMR spectrum was acquired with 256 scans (6 min).

3.6. 2D ^{1}H TOCSY experiments

2D ^{1}H TOCSY was acquired for each NMR sample. NMR samples were prepared as previously described for each peptide using 4 × 10^6 cells. For each sample, three 2D ^{1}H TOCSY experiments were acquired at different times after preparation to check sample stability. The experiments were acquired at time 0, 7 and 24 h after sample preparation. Each spectrum was acquired in ca. 2.5 h with 24 scans and 128 complex points in the t1 dimension using the states phase sensitive mode. The spectra were processed with a 90° shifted sinebell apodization function and Fourier transformed to 2048 × 512 real points in dimensions F2 and F1, respectively.

3.6.1. Variable temperature 2D ^{1}H TOCSY experiments

Variable temperature 2D ^{1}H TOCSY experiments were acquired with the same conditions described above with a sample of 400 μg ghrelin (**1**) and 4 × 10^6 cells transfected cells in 0.5 mL PBS (pH 7.2):D_2O (95:5, v/v). The experiments were acquired at the following temperatures: 8, 15, 25 and 35 °C.

3.7. 2D gradient ^{1}H COSY magnitude experiments

2D gradient ^{1}H COSY magnitude experiments were acquired for the PBS samples. NMR samples were prepared as previously described for each peptide. Each spectrum was acquired in 1.25 h with 24 scans and 128 points in the t1 dimension. The spectra were processed with a cosinebell apodization function and Fourier transformed to 2048 × 512 real points in dimensions F2 and F1, respectively. 2D TOCSY and 2D COSY experiments were used for the signal assignment of the peptides **1** and **2**. This assignment was based in the assignment of the pure peptides in the PBS buffer at pH 7.2. The assignment of these peptides in H_2O at pH 3.0 had been previously described.[11]

3.8. CYANA calculations

Simulated annealing calculations were performed with CYANA v2.1.[30] Ghrelin (**1**) was built with the appropriated configuration for the prolines and the acylated serine at position 3. Simulated annealing calculations were performed starting from 100 different random structures that were submitted to 10,000 simulated annealing iterations. During the annealing, the torsion angles ϕ/ψ of residues 8–20 were restrained around the typical angles either of the right-handed α-helix (−60°/−50°) or of the left-handed α-

helix (60°/50°). The ω torsion angles of prolines 7, 21, 22, and 27 were restrained to either *E* ($\omega = 180°$) or *Z* ($\omega = 0°$). Each of the 16 possible combinations of the prolines was tested in different CYANA calculations (Tables S1 and S2).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.004.

References and notes

1. Pazos, Y.; Casanueva, F. F.; Camiña, J. P. *Vitam. Horm.* **2008**, *77*, 89.
2. Kojima, M.; Hosoda, H.; Date, Y.; Nakazato, M.; Matsuo, H.; Kangawa, K. *Nature* **1999**, *402*, 656.
3. van der Lely, A. J.; Tschop, M.; Heiman, M. L.; Ghigo, E. *Endocr. Rev.* **2004**, *25*, 426.
4. Takaya, K.; Ariyasu, H.; Kanamoto, N.; Iwakura, H.; Yoshimoto, A.; Harada, M.; Mori, K.; Komatsu, Y.; Usui, T.; Shimatsu, A.; Ogawa, Y.; Hosoda, K.; Akamizu, T.; Kojima, M.; Kangawa, K.; Nakao, K. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 4908.
5. Tshop, M.; Smiley, D. L.; Heiman, M. L. *Nature* **2000**, *407*, 908.
6. Nakazato, M.; Murakami, N.; Date, Y.; Kojima, M.; Matsuo, H.; Kangawa, K.; Matsukura, S. *Nature* **2001**, *409*, 194.
7. Pemberton, C. J.; Richards, A. M. *Vitam. Horm.* **2008**, *77*, 13.
8. Feighner, S. D.; Howard, A. D.; Prendergast, K.; Palyha, O. C.; Hreniuk, D. L.; Nargund, R.; Underwood, D.; Tata, J. R.; Dean, D. C.; Tan, C. P.; McKee, K. K.; Woods, J. W.; Patchett, A. A.; Smith, R. G.; van der Ploeg, L. H. T. *Mol. Endocrinol.* **1998**, *12*, 137.
9. Bednarek, M. A.; Feighner, S. D.; Pong, S.-S.; McKee, K. K.; Hreniuk, D. L.; Silva, M. V.; Warren, V. A.; Howard, A. D.; van der Ploeg, L. H. Y.; Heck, J. V. *J. Med. Chem.* **2000**, *43*, 4370.
10. Torsello, A.; Ghe', C.; Bresciani, E.; Catapano, F.; Ghigo, E.; Deghenghi, R.; Locatelli, V.; Muccioli, G. *Endocrinology* **2002**, *143*, 1968.
11. Silva Elipe, M. V.; Bednarek, M. A.; Gao, Y.-D. *Biopolymers* **2001**, *59*, 489.
12. Beevers, A. J.; Kukol, A. *J. Biomol. Struct. Dynam.* **2006**, *23*, 357.
13. Dehlin, E.; Liu, J.; Yun, S. H.; Fox, E.; Snyder, S.; Gineste, C.; Willingham, L.; Geysen, M.; Gaylinn, B. D.; Sando, J. J. *Peptides* **2008**, *29*, 904.
14. Meyer, B.; Peters, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 864.
15. Lepre, C. A.; Moore, J.; Peng, J. W. *Chem. Rev.* **2004**, *104*, 3641.
16. Zartler, E. R.; Yan, J.; Mo, H.; Kline, A. D.; Shapiro, M. J. *Curr. Top. Med. Chem.* **2003**, *3*, 25.
17. Mari, S.; Serrano-Gómez, D.; Cañada, F. J.; Corbí, A. L.; Jiménez-Barbero, J. *Angew. Chem., Int. Ed.* **2005**, *44*, 296.
18. Zhang, W.; Chai, B.; Li, J.-Y.; Wang, H.; Mulholland, M. W. *Endocrinology* **2008**, *149*, 4710.
19. Wishart, D. S.; Sykes, B. D. *Methods Enzymol.* **1994**, *239*, 363.
20. Celinski, S. A.; Scholtz, J. M. *Protein Sci.* **2002**, *11*, 2048.
21. The further stabilisation of the helical region could be indeed due to the presence of the receptor. Indeed, recent NMR data (including extensive NOE analysis) gathered in our labs demonstrates that both **1** and **2** do not show a significant α-helix population when included in membrane-like environments, such as SDS micelles (in preparation).
22. Schubert, M.; Labudde, D.; Oschkinat, H.; Schmieder, P. *J. Biomol. NMR* **2002**, *24*, 149.
23. Slupsky, C. M.; Sykes, D. B.; Gay, G. L.; Sykes, B. *Protein Sci.* **2001**, *10*, 1244.
24. Booth, V.; Slupsky, C. M.; Clark-Lewis, I.; Sykes, B. D. *J. Mol. Biol.* **2003**, *327*, 329.
25. Langelaan, D. N.; Bebbington, E. M.; Reddy, T.; Rainey, J. K. *Biochemistry* **2009**, *48*, 537.
26. Camiña, J. P.; Carreira, M. C.; El Messar, S.; Llorens-Cortes, C.; Smith, R. G.; Casanueva, F. F. *Endocrinology* **2004**, *145*, 930.
27. Casanueva, F. F.; Camiña, J. P.; Carreira, M. C.; Pazos, Y.; Varga, J. L.; Schally, A. V. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 20452.
28. Cobas, J. C.; Sardina, F. J. *Concepts Magn. Reson. Part A* **2003**, *19A*, 80.
29. Liu, M.; Mao, X.; Ye, C.; Huang, H.; Nicholson, J. K.; Lindon, J. C. *J. Magn. Reson.* **1998**, *132*, 125.
30. Güntert, P. *Methods Mol. Biol.* **2004**, *278*, 353.