The Interaction of Highly Helical Structural Mutants with the NOP Receptor Discloses the Role of the Address Domain of Nociceptin/Orphanin FQ

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Abstract: Nociceptin is a heptadecapeptide whose sequence is similar to that of Dynorphin A, sharing a message domain characterized by two glycines and two aromatic residues, and a highly basic C-terminal address domain but, in spite of these similarities, displays no opioid activity. Establishing the relative importance of the message and address domains of nociceptin has so far been hampered by its extreme conformational flexibility. Here we show that mutants of this peptide, designed to increase the helical content in the address domain, can be employed to explain the mode of interaction with the NOP receptor. Nociceptin analogues in which Ala residues are substituted with aminoisobutyric acid (Aib) show a substantial increment of activity in their interaction with the NOP receptor. The increment of biological activity was attributed to the well-documented ability of Aib to induce helicity. Here we have verified this working hypothesis by a conformational investigation extended to new analogues in which the role of Aib is taken up by Leu. The NMR conformational analysis confirms that all Ala/Aib peptides as well as [Leu^{7,11}]-N/OFQ-amide and [Leu^{11,15}]-N/OFQ-amide mutants (N/OFQ = nociceptin/orphanin FQ) have comparable helix content in helix-promoting media. We show that the helical address domain of nociceptin can place key basic residues at an optimal distance from complementary acidic groups of the EL_2 loop of the receptor. Our structural data are used to rationalize pharmacological data which show that although [Leu^{11,15}]-N/OFQ-amide has an activity comparable to those of Ala/Aib peptides, [Leu^{7,11}]-N/OFQ-amide is less active than N/OFQ-amide. We hypothesize that bulky residues cannot be hosted in or near the hinge region (Thr^5-Gly^6-Ala^7) without severe steric clash with the receptor. This hypothesis is also consistent with previous data on this hinge region obtained by systematic substitution of Thr, Gly, and Ala with Pro.

Keywords: conformation analysis · NMR spectroscopy · nociception · peptides · receptors · structure–activity relationships

Introduction

Nociceptin/orphanin FQ (N/OFQ)[1,2] is a 17-amino acid neuropeptide (H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH) that binds with a high affinity to the N/OFQ receptor (NOP, formerly known as ORL_1) such that the receptor binding aites are saturated. The NOP receptor is a member of the super-family of seven transmembrane G-protein-coupled receptors (GPCR's) that, in spite of a high degree of amino acid sequence homology

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with the cloned opioid receptor types (μMOP, δDOP, and κKOP), does not selectively bind opioid agonists or antagonists.[3–8] It has been demonstrated that N/OFQ, through NOP activation, modulates several biological functions, including pain threshold, morphine analgesia, food intake, anxiety, locomotor activity, memory processes, and cardiovascular, renal, respiratory, and gastrointestinal functions.[9,10]

Over the last few years, systematic structure–activity studies on N/OFQ, largely performed in our laboratory,[11,12] have pinpointed key residues in the N-terminal four residues (Phe-Gly-Gly-Phe, message domain), in the address domain (from Arg^{8} to Gln^{17}) and in the hinge region connecting the two domains (Thr-Gly-Ala). Initially we studied the activities of N/OFQ fragments to determine the minimal sequence (N/OFQ(1–13)-amide) that retains full agonist activity.[13] N/OFQ(1–13)-amide has since served as a template for the design of further compounds. Early attempts to find more active agonists and/or to change agonists into antagonists, focused on the message domain of N/OFQ. In order to protect the N terminus from degradation by amino peptidases, we prepared [$Phe^{1}\Psi(CH_2\text{-}NH)Gly^{2}$]-N/OFQ(1–13)-amide and noted that it behaves as a NOP antagonist in a variety of in vitro assays, while acting as a potent agonist in most in vivo assays.[14] This compound, which has been widely used in N/OFQ studies, is in fact a partial agonist whose intrinsic activity strongly depends on the efficiency of the stimulus/response coupling (for a detailed discussion of this topic see reference [10]). Finally, using a variety of different approaches we modified the N-terminal Phe of N/OFQ(1–13)-amide and identified [$NPhe^{1}$]-N/OFQ(1–13)-amide[15] as a pure and selective antagonist in vitro at recombinant human NOP receptors,[16] and at native peripheral[17–19] and central[20,21] NOP sites.

Overall, a series of structure–activity relationship (SAR) studies performed on N/OFQ[22–24,11–15] suggests that Phe^{1} and Phe^{4} represent the critical residues of the message domain of N/OFQ (Phe^{1}-Gly^{2}-Gly^{3}-Phe^{4}), which should be involved in receptor binding and activation, whereas the positively charged residues that are present in the address domain of the molecule ($Arg^{8,12}$, $Lys^{9,13}$) appear to be crucial for receptor occupation. As a result, the emphasis in SAR studies shifted somewhat to the address domain of N/OFQ. We have previously reported that the main conformational differences between N/OFQ and Dynorphin A (Dyn-A), the peptide ligand of κ-opioid receptor, seem confined to the address moieties.[25] According to a detailed conformational study on Dyn-A performed in our laboratory,[26,27] we found that the conformation of the address domain of Dyn-A is dominated by the presence of Pro^{10} that separates two groups of basic residues and limits its flexibility, whereas the corresponding domain of N/OFQ does not contain relevant constitutional constraints. Accordingly, the whole molecule is extremely flexible and the placement of key residues of the two domains inside the receptor is probably directed by a process of induced fit. In particular, the basic residues of the address domain can only make a productive interaction with acidic residues in the receptor if arranged in a precise conformation. Interestingly, Okada et al.[28] reported a series of N/OFQ analogues in which additional Arg-Lys dipeptides were strategically placed at positions 6–7, 10–11, or 14–15 adjacent to the parent Arg-Lys motifs (8–9 and 12–13) in lieu of existing residue pairs in the address domain of the peptide. As a result, [Arg^{14}, Lys^{15}]-N/OFQ, emerged as an analogue 17-fold more potent in the GTPγS functional assay compared to native N/OFQ. It is fair to hypothesize that the increase of positive charges can compensate for the lack of a regular conformation prior to receptor interaction.

A crucial question, as yet partially unanswered, is the relative spatial disposition of the two domains. A combined conformational and pharmacological study on analogues of N/OFQ(1–13)-amide has shown that if one rigidifies the hinge region (Thr-Gly-Ala) by systematically substituting each residue with Pro there is a dramatic drop in activity. The decrease is most pronounced for [Pro^{5}]-N/OFQ(1–13)-amide and [Pro^{7}]-N/OFQ(1–13)-amide, whereas [Pro^{6}]-N/OFQ(1–13)-amide retains an activity comparable to that of other active analogues.[29] This result was interpreted as an indication that the angle between the two domains ought to be similar to that imposed by a β-turn centered on Gly^{6}-Ala^{7}. This hypothesis is of limited validity because both domains are very flexible from a conformational point of view. However, while it is well known that the N-terminal domain is intrinsically flexible, like that of opioids,[25,29] the address domain does have conformational tendencies that can be enhanced by environmental conditions and/or specific substitutions. Accordingly, we sought the best conditions to reveal its intrinsic conformational tendencies.

Zhang et al.[30] designed a series of conformationally constrained analogues of N/OFQ and N/OFQ-amide by replacing Ala residues in the address domain of the peptide with H-Me-Ala (or Aib; Aib = aminoisobutyric acid) residues. These authors prepared a series of N/OFQ and N/OFQ-amide analogues containing Aib as a replacement for Ala^{7}, Ala^{11}, or Ala^{15}. It was anticipated that if the bioactive conformation of N/OFQ is characterized by an amphipathic helix within the address portion of the sequence, the Aib-containing peptides would have binding affinities and functional potencies similar to, or higher than that of N/OFQ. In fact, their results show that the adoption of an amphipathic helix within the "address" segment of N/OFQ in the receptor-bound state leads to binding affinities and potencies superior to those of N/OFQ itself.

Since Aib is known to favor 3_{10} helices,[31] and also considering that it is not a genetically encoded, that is, "natural", residue, we decided to investigate the importance of α helices, using natural amino acid residues to modify the helical propensity of N/OFQ sequences. Here we present a conformational study of new N/OFQ analogues, containing Leu residues in place of Ala: [$Leu^{7,11}$]-, [$Leu^{11,15}$]- and [$Leu^{11,15}$,Glu^{16}]-N/OFQ-amide. For comparison purposes we have also studied the parent peptides N/OFQ, N/OFQ-amide, the peptides originally proposed by Zhang et al.[30] [Aib7]-, [Aib^{11}]-, [$Aib^{7,11}$]-N/OFQ-amide, [Glu^{16}]-N/OFQ-

amide, and [Pro^6]-, [Pro^7]- and [Pro^{11}]-N/OFQ-amide, as a negative control, exploiting the helix-breaking property of Pro.

All the peptides were tested on the mouse vas deferens (MVD), a pharmacological preparation sensitive to N/OFQ and the tendency of the peptides to adopt a preferred conformation was investigated experimentally by NMR spectroscopy. The pharmacological profiles of the new peptides show that helicity does indeed play a key role. However, a combination of conformational studies in solution and docking to a reliable receptor model show that it is necessary to take into account the precise features of residues connecting the message and address domains.

Results

Peptide design and synthesis: To design possible peptides in which Ala residues are substituted by natural residues with helical propensity higher than that of Ala, we used two popular programs, Agadir[32] and PSIPRED.[33] Agadir, an algorithm based on the helix–coil transition theory, predicts the helical propensity of isolated peptides. Accordingly, although not written to predict a possible interaction with the receptor, Agadir is ideal for an appraisal of relative conformational tendencies. PSIPRED, a new accurate secondary structure prediction method based on neural networks,[33] yielded comparable results. There are several natural residues that can impart helicity, but if one wants to keep the essential distinctive features of the address domain of N/OFQ, for example, its predominantly basic character, it is evident that Glu, a powerful helix-inducer, should be excluded. Actually, we used mutations involving Glu only as a negative control (vide infra). By the same token, we did not want to use aromatic residues that could compete with the message domain. After several trials among possible candidates, we chose Leu as the best natural residue in place of Aib to substitute Ala residues of the original N/OFQ sequence. Both prediction methods yielded low helicity values for single substitutions, but higher values for double substitutions. Based on these predictions, we designed the following peptides: [Leu^{7,11}]-, [Leu^{11,15}]- and [Leu^{11,15},Glu^{16}]-N/OFQ-amide. The first two have mutations consistent with those described by Zhang et al.[30] The last one was synthesized to gauge the balance between increased helicity and the (likely) detrimental effect on binding caused by the presence of an acidic residue.

Pharmacological data were also acquired for the parent peptides N/OFQ and N/OFQ-amide, for all Aib-containing peptides originally proposed by Zhang et al.,[30] that is, [Aib^7]-, [Aib^{11}]-, [Aib^{7,11}]-N/OFQ-amide, for [Glu^{16}]-N/OFQ-amide, and finally for [Pro^6]-, [Pro^7]- and [Pro^{11}]-N/OFQ-amide. Pro was chosen since it is a widely used helix-breaker, but [Pro^6]- and [Pro^7]-N/OFQ-amide, as well as representing a negative control for helicity, play a crucial role in connection to the issue of the flexible hinge connecting the two domains of N/OFQ.[29]

Table 1 shows the ten analogues of N/OFQ, synthesized according to published methods by using standard solid-phase synthesis techniques[34] with a Milligen 9050 synthesizer as described in detail in the Experimental Section.

Table 1. Analytical properties of N/OFQ analogues.

	t_r[a]		$[M+H]^+$[b]	
Abbreviated names	I	II	calcd	found
[Aib^7]-N/OFQ-amide	13.12	21.17	1823.1	1823.1
[Aib^{11}]-N/OFQ-amide	13.88	21.51	1823.1	1822.8
[Aib^{7,11}]-N/OFQ-amide	14.07	22.17	1837.1	1837.3
[Leu^{7,11}]-N/OFQ-amide	14.88	23.07	1893.2	1893.1
[Leu^{11,15}]-N/OFQ-amide	15.12	23.15	1893.2	1893.2
[Leu^{11,15}, Glu^{16}]-N/OFQ-amide	15.76	23.81	1908.2	1907.9
[Glu^{16}]-N/OFQ-amide	12.76	18.08	1824.1	1824.3
[Pro^6]-N/OFQ-amide	13.81	21.51	1849.1	1849.5
[Pro^7]-N/OFQ-amide	13.34	21.76	1835.1	1835.3
[Pro^{11}]-N/OFQ-amide	14.22	22.66	1835.1	1835.1

[a] t_r is the retention time determined by analytical HPLC. [b] The mass ion $[M+H]^+$ was obtained by MALDI-TOF mass spectrometry.

Structural data: To assess conformational tendencies on an experimental basis we undertook a systematic NMR study in several media that are known to favor helicity. The number and intensity of NOE's and, to some extent, even the conformation of short linear peptides in solution can be influenced by the use of cryoprotective mixtures,[35–37] for example, solvent mixtures of viscosity higher than that of pure water but comparable to that of cytoplasm.[38,39] Notwithstanding, the NOESY spectra of N/OFQ in water, dimethyl sulfoxide (DMSO), in mixtures of water with DMSO or in several hydroalcoholic mixtures show a limited spread of the NH resonances corresponding to little ordering of the peptide.[29] The same behavior was shown by analogues containing Pro in key positions,[29] including those studied in the present paper, namely [Pro^6]-N/OFQ-amide, [Pro^7]-N/OFQ-amide and [Pro^{11}]-N/OFQ- amide (data not shown) consistently with the low helix-inducing property of this residue.

In contrast, analogues containing Aib or Leu residues in place of Ala show rich NOESY spectra consistent with a high helical content in all media that are known to favor helical conformations. Alcohols, either neat or mixed with water are the most popular media used to induce helicity in peptides.[40–43] We ran spectra of nocistatin in mixtures of water with 2,2,2-trifluoroethanol (TFE) and hexafluoroacetone trihydrate (HFA). This last mixture has been shown to behave like TFE/water mixtures, but with a much higher helix-inducing propensity.[44] It was also shown that the structuring effect of HFA/water does not overrule conformational preferences encoded in residue types, but rather reflects intrinsic residues tendencies faithfully: β-endorphin, another long-chain opioid, in HFA/water assumes a regular helical structure only in the C-terminal address domain sequence, leaving the first 12 residues completely disordered.[45]

Figure 1 shows the comparison of partial 500 MHz NOESY spectra of [Aib^{7,11}]- and [Leu^{7,11}]-N/OFQ-amide in

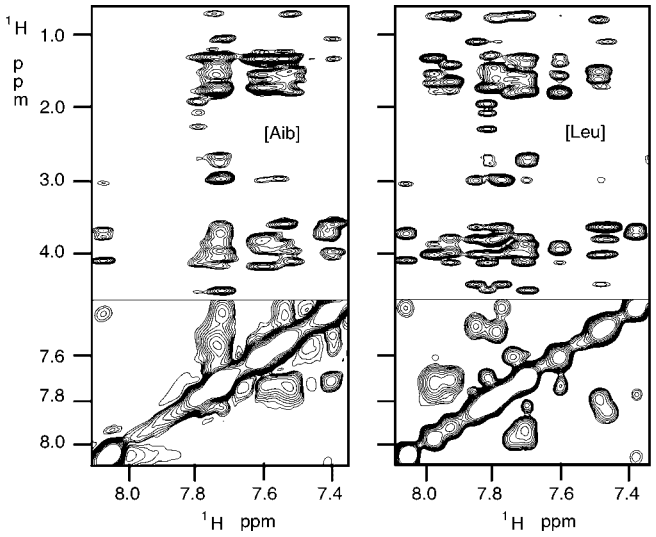

Figure 1. Comparison of partial 500 MHz ^{1}H NOESY spectra of [Aib^{7,11}]- and [Leu^{7,11}]-N/OFQ-amide in HFA/water (1:1, v:v) at 300 K. The spectra were recorded with a mixing time of 200 ms. Water suppression was achieved either by presaturation or by using the WATERGATE pulse sequence.[57]

HFA/water (1:1, v:v) at 300 K. Both spectra show NOEs between protons of residues spaced by three or four positions along the sequence, consistent with a high helical content. Figure 2A shows the bar diagrams of the two peptides, summarizing the main diagnostic effects derived from NMR spectra. Figure 2B shows the corresponding distribution of NOE-derived constraints along the sequence. The higher number of constraints that can be measured for [Leu^{7,11}]-N/OFQ-amide allows a full structure determination. Introduction of restraints derived from intraresidue, sequential and medium range NOEs in DYANA[46] generated 20 structures of [Leu^{7,11}]-N/OFQ-amide with good values of the usual target function[46] out of 30 randomly 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. The whole sequence from Thr^5 to Ala^{15} is a fairly regular α helix. Small deviations from a canonical α-helical structure may originate from an insufficient number of constraints in the refinement procedure. Figure 3 shows the bundle of the ten best structures of [Leu^{7,11}]-N/OFQ-amide calculated by DYANA and refined by means of restrained simulated annealing (left) and the ribbon representation of the mean structure (right).

All peptides containing Aib and those with two Leu residues in lieu of two Ala yield NMR spectra typical of peptides with a high helical content. For all peptides other than [Leu^{7,11}]-N/OFQ-amide, instead of reverting to the standard (but time-consuming) structure determination employed for this peptide, we built models that were subsequently refined by restrained energy minimization. In order to build good

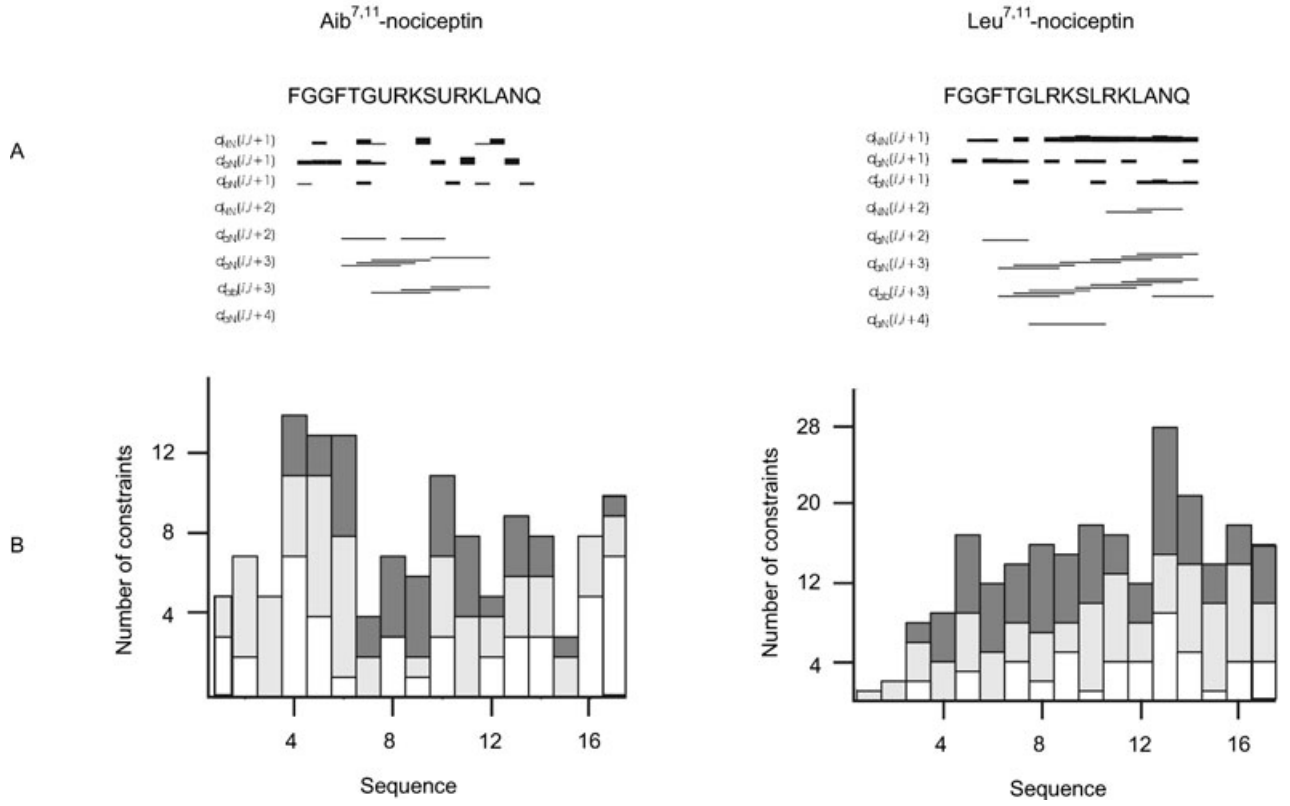

Figure 2. A) Bar diagrams summarizing the main diagnostic effects involving backbone NH and H$^\alpha$ and H$^\beta$ atoms derived from NMR spectra of [Aib^{7,11}]- (left) and [Leu^{7,11}]-N/OFQ-amide (right). B) Distribution of NOE derived constraints along the sequence for the same peptides.

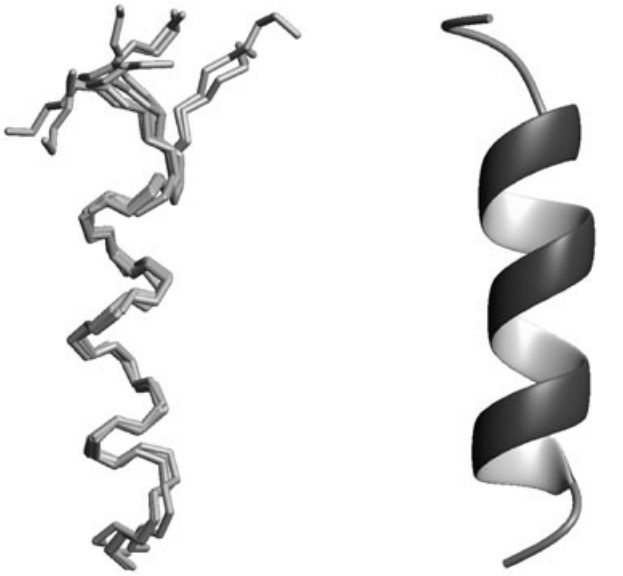

Figure 3. Bundle of the ten best structures of [Leu^{7,11}]-N/OFQ-amide calculated by DYANA and refined by means of restrained simulated annealing (left) and the ribbon representation of the mean structure (right).

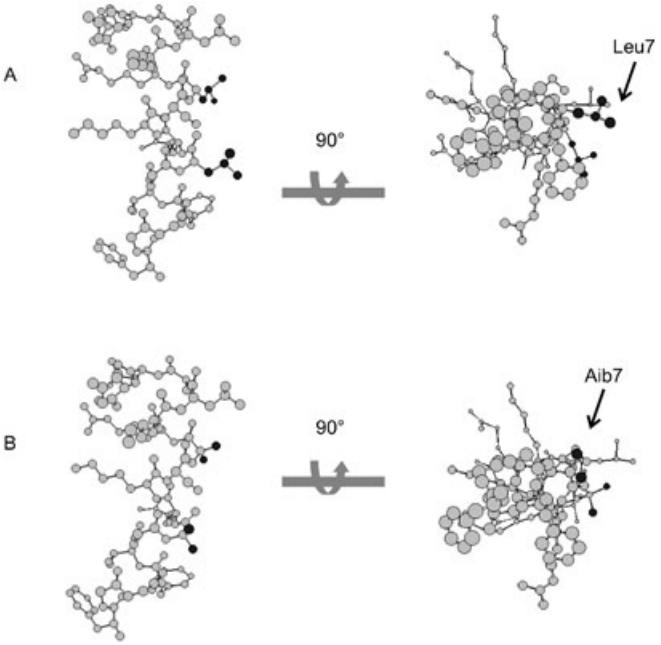

Figure 4. Comparison of the molecular models of [Leu^{7,11}]-N/OFQ-amide (A) and [Aib^{7,11}]-N/OFQ-amide (B). Both models are shown as stick-and-ball representations. Side chain atoms of Aib and Leu residues are in darker gray. The views on the right, seen along the axes of the helices, show that the side chain of Leu^7 protrudes beyond the mean encumbrance of the helix.

starting conformations, we adopted ϕ angles close to those of a regular α helix for the Leu peptides and close to a 3_{10} helix for the Aib peptides, with small manual adjustments to take into account experimental distance restraints. Each peptide was energy minimized by using a simple MM2 force-field in vacuo and using NOE-derived interatomic distances as the only restraints. After several cycles of restrained minimization, unrestrained energy minimization led to a final conformation that was checked for consistency against all NMR parameters. It was reassuring to note that the model of [Leu^{7,11}]-N/OFQ-amide is very similar to the structure calculated by using the standard procedure.

Figure 4 shows the comparison of the molecular models of [Leu^{7,11}]- and [Aib^{7,11}]-N/OFQ-amide.

Pharmacology: The ten analogues of N/OFQ, reported in Table 1, were tested for their ability to inhibit electrically evoked contractions (twitch response) of the mouse vas deferens (MVD), an N/OFQ sensitive pharmacological preparation.[10] Results of this biological assay are presented in Table 2 as a pEC_{50} to describe agonist potency. The replacement of Ala^7, Ala^{11}, and Ala^{7,11} with Aib in the address sequence of N/OFQ improves activity in the MVD assay; [Aib^7]-, [Aib^{11}]-, [Aib^{7,11}]-N/OFQ-amide are approximately two- and threefold more potent than N/OFQ-amide and N/OFQ, respectively. These results are in agreement with the data reported by Zhang et al.[30] determined as a [^{35}S]GTPγS functional assay. To determine if NOP agonist activities of [Aib^7]-, [Aib^{11}]-N/OFQ-amide were mediated by the NOP receptor, the pA_2 for UFP-101, a selective NOP receptor antagonist,[18] and naloxone were determined in comparison with N/OFQ. While naloxone does not modify the NOP agonist activity up to 10 μM, the pA_2 values obtained for UFP-101 were comparable for all NOP agonists, confirming that in the MVD assay agonist activity of Aib peptides was mediated by the NOP receptor (Table 3). As reported in the paper of Zhang et al.,[30] these results may be interpreted on the basis of helicity, imparted by the Aib substitution to the address domain of the N/OFQ peptide. Amino acids such as Ala, Asp, Glu, Ile, Leu, and Met favor the formation of α helices, whereas, Gly and Pro favor disruption of the helix. This is particularly true for Pro, since it is a pyrrolidine-

Table 2. Effects of N/OFQ and N/OFQ related peptides in the electrically stimulated mouse vas deferens.

Peptide	pEC_{50} ($CL_{95\%}$)[a]	relative potency	E_{max}[b] [%]
N/OFQ	7.82 (7.64–8.00)	0.7	93 ± 1
N/OFQ-amide	7.97 (7.93–8.01)	**1**	90 ± 1
[Aib^7]-N/OFQ-amide	8.35 (8.24–8.46)	2.4	91 ± 1
[Aib^{11}]-N/OFQ-amide	8.38 (8.07–8.29)	2.6	91 ± 1
[Aib^{7,11}]-N/OFQ-amide	8.18 (8.07–8.29)	1.6	90 ± 2
[Leu^{7,11}]-N/OFQ-amide	7.60 (7.55–7.65)	0.4	84 ± 3
[Leu^{11,15}]-N/OFQ-amide	8.13 (8.08–8.18)	1.4	88 ± 1
[Leu^{11,15}, Glu^{16}]-N/OFQ-amide	7.64 (7.59–7.69)	0.5	84 ± 2
[Glu^{16}]-N/OFQ-amide	7.44 (7.20–7.68)	0.3	87 ± 2
[Pro^6]-N/OFQ-amide	6.60 (6.23–6.97)	0.04	92 ± 5
[Pro^7]-N/OFQ-amide	6.32 (5.82–6.82)	0.02	99 ± 1
[Pro^{11}]-N/OFQ-amide	6.06 (5.77–6.35)	0.01	90 ± 8

[a] pEC_{50} is the negative logarithm to base ten of the molar concentration of agonist that produces 50% of the maximal possible effect. $CL_{95\%}$: 95% confidence limits. [b] E_{max} is the maximal effect induced by an agonist expressed as percent inhibition of electrically induced twitches.

Table 3. Effects of naloxone and UFP-101 against the actions of N/OFQ-amide, [Aib^7]-, and [Aib^{11}]-N/OFQ-amide in the electrically stimulated mouse vas deferens.[a]

	pK_B values ($CL_{95\%}$)	
	naloxone	UFP-101
N/OFQ-amide	<6	7.04 (6.73–7.35)
[Aib^7]-N/OFQ-amide	<6	7.35 (7.11–7.59)
[Aib^{11}]-N/OFQ-amide	<6	7.37 (7.21–7.53)

[a] Antagonist potencies were evaluated using the Gaddum Schild equation: $pA_2 = \log((CR-1)/[\text{antagonist}])$ assuming a slope value equal to unity. Data are the mean ± s.e.m. of at least five separate experiments. For pK_B values, the 95% confidence limits are given in brackets.

based imino acid (HN=) whose structure significantly restricts movement about the peptide bond in which it is present, thereby interfering with extension of the helix. We replaced Ala^{7,11} and Ala^{11,15} with Leu and Gly^6, and Ala^7 or Ala^{11} with Pro, to see if natural amino acids such as Leu or Pro can confirm or disrupt the tendency of the C-terminal sequence of the peptide to adopt an ordered conformation (amphipathic helix) in the receptor interaction.

[Leu^{7,11}]- and [Leu^{11,15}]-N/OFQ-amide maintain good agonist activity in the MVD assay although the first analogue is 2.5-fold less potent than N/OFQ-amide, while the second one is 1.4-fold more potent than reference peptide. Replacement of Asn^{16} with an acidic residue, as in the analogue [Leu^{11,15}, Glu^{16}]-N/OFQ-amide, does not improve the potency of the analogue. In fact, [Leu^{11,15}, Glu^{16}]-N/OFQ-amide is twofold less potent than N/OFQ-amide, in spite of the great helix-inducing propensity of Glu. On the other hand, the only substitution of Asn^{16} with Glu, as in [Glu^{16}]-N/OFQ-amide, is more detrimental for the activity: threefold less compared to reference N/OFQ-amide. It has been suggested that positively charged residues (Arg and Lys), in the C-terminal address domain of the peptide, are important for the interaction with NOP receptor.[22,23,11] Such interactions probably occur with the second extracellular loop (EL_2) of the NOP receptor that is rich in acidic residues (Asp and Glu). It is likely that the negative charge of Glu^{16} can interfere with acidic residues of the second loop of the receptor or reduce the interactions of basic residues present in the address domain of the N/OFQ. Considering that [Leu^{11,15}, Glu^{16}]-N/OFQ-amide is 1.6-fold more potent than [Glu^{16}]-N/OFQ-amide, we might speculate that Leu residue in the address domain of the peptide has a positive contribution in the bioactive conformation. These results are consistent with the high activity of [Leu^{11,15}]-N/OFQ-amide that is 3.1-fold more potent than [Leu^{11,15}, Glu^{16}]-N/OFQ-amide and fivefold more potent than [Glu^{16}]-N/OFQ-amide, an analogue without Leu residue in the address domain of the peptide.

The Pro-containing peptides, although still agonists, are far less potent than N/OFQ-amide, and the corresponding Aib- and Leu-containing peptides. In particular, the [Pro^{11}]-, is 210-fold less potent, in the MVD assay, than [Aib^{11}]- and 120-fold if compared with [Leu^{11,15}]-N/OFQ-amide. These data confirm that since Pro favors disruption of the helix conformation in the address domain of the N/OFQ, helicity itself plays an important role. The other two Pro analogues, that is, [Pro^6]-N/OFQ-amide and [Pro^7]-N/OFQ-amide, while still consistent with the role of a helical conformation in the address domain, shed light on the importance of the conformation of the hinge region. As discussed at length in a previous paper,[29] only a β-turn centered on Gly^6-Ala^7 is consistent with NOP activity. This observation is particularly relevant for the relative orientation of the two domains inside the receptor (vide infra).

Receptor binding in silico: The interaction of N/OFQ with the NOP receptor, similarly to that of Dyn-A with the κ-opioid receptor,[27,29] is thought to be mediated by EL_2, a very acidic external loop of the receptor. It is not known whether the function of this loop is to "capture" the agonist or to contribute also to the binding together with the active site hosting the message domain. The relevance of helicity for the interaction with the receptor, suggested by the working hypothesis of Zhang et al.[30] and confirmed by the data of the present work, hints at the possibility that a key interaction inside the receptor is the helix–helix interaction of the peptide with one or more of the TM helices, as proposed by Saviano et al.,[45] in the case of β-endorphin. If this were the case, the agonist would eventually find its optimal placement inside the receptor without further involvement of the loop.

To check this, we explored possible orientations of the NMR structure of [Leu^{11,15}]-N/OFQ-amide inside the receptor. The NOP receptor has been modeled by Topham et al.[47a] and more recently by Bröer et al.[47b] We chose the coordinates (courtesy of Dr. Bröer) of this last model for our docking. One major difficulty in this in silico complexation is that the conformations of the peptide and of the receptor are likely to undergo mutual reorganization. However, if we limit ourselves to simple topological and electrostatic considerations, it is possible to reach an unambiguous result. It is highly probable that the two aromatic rings of the message domain, that is, Phe^1 and Phe^4, should be placed inside the apolar cavity that is able to bind apolar nonpeptidic agonists.[47b] It is also imperative that the very basic C-terminal address domain of [Leu^{11,15}]-N/OFQ-amide can interact with acidic residues of the receptor.

As shown by Figure 5A, if one places the message domain inside the apolar cavity, the helix–helix interactions occur in parts of the receptor almost completely devoid of acidic residues. In contrast, if one rotates the model of the peptide by 180°, the basic residues can interact with the very acidic EL_2 loop (Figure 5B).

As shown in Figure 5C, manual docking of the peptide can easily optimize specific interactions between basic residues of the peptide and corresponding acidic residues of the receptor: Arg^8-Glu^{203}, Lys^9-Glu^{199}, Arg^{12}-Asp^{195}, and Lys^{13}-Glu^{197}. It is interesting to note that the relative orientation of the two domains in the the NMR structure of [Leu^{11,15}]-N/OFQ-amide is consistent with the β-turn centered on Gly^6-Ala^7.

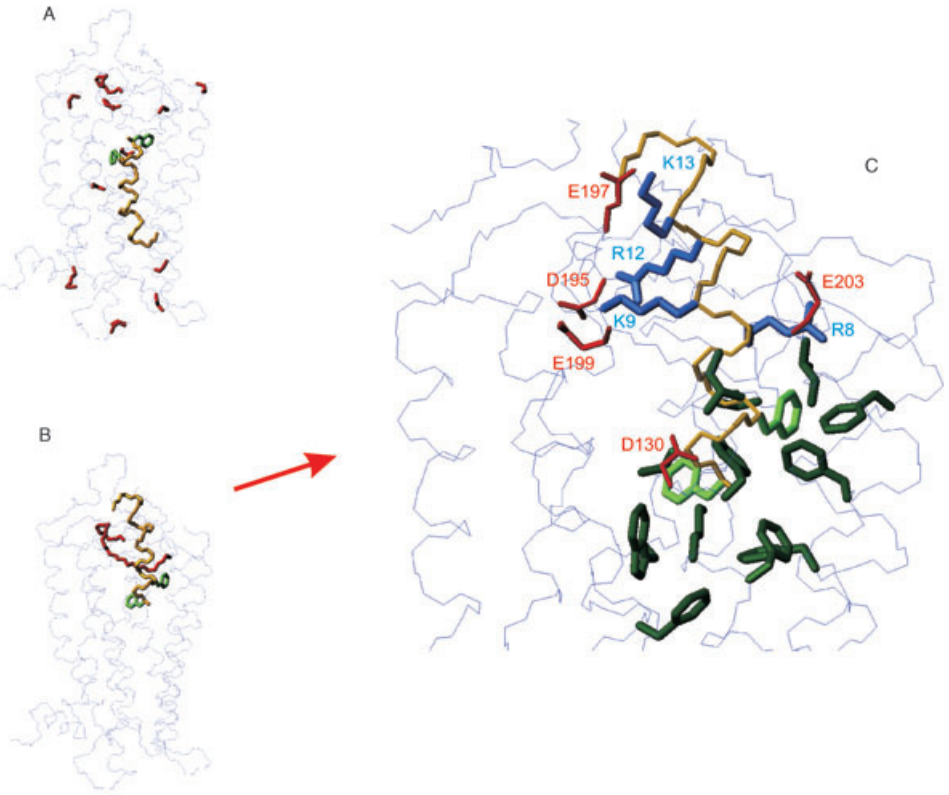

Figure 5. Two possible orientations of the model of [Leu^{11,15}]-N/OFQ-amide inside the receptor. The model of [Leu^{11,15}]-N/OFQ-amide is shown in a neon rendering with the backbone in gold and aromatic side chains in light green. The model of the receptor is shown as a line rendering of the backbone (blue). A) Message domain inside the apolar cavity and address domain among the TM helices. Backbone atoms of acidic residues are in neon and highlighted in red. B) Message domain inside the apolar cavity and the address domain in contact with the EL_2 loop (backbone as red neon). C) Interactions between basic residues of the peptide and corresponding acidic residues of the receptor: Arg^8-Glu^{203}, Lys^9-Glu^{199}, Arg^{12}-Asp^{195}, and Lys^{13}-Glu^{197}. The model of [Leu^{11,15}]-N/OFQ-amide and selected side chains of the receptor are shown in a neon rendering with the backbone in gold, basic side chains in blue, and aromatic side chains in light green. The acidic side chains of the EL_2 loop are shown in red, and the hydrophobic side chains lining the walls of the active site are represented in dark green.

Discussion

Helicity as a key factor: The combination of results from the conformational analysis and the pharmacological assays shows conclusively that helicity of the address domain of nociceptin plays a key role in the interaction with the NOP receptor. This result is of particular importance, since the parent peptide (N/OFQ) has very little tendency to assume a helical conformation in a variety of media,[29] although there have been claims to the contrary.[48] In full agreement with the work of Zhang et al.,[30] we have confirmed that single or multiple substitutions of Ala residues with Aib in the address domain of N/OFQ invariably lead to an increment of activity with respect to NOP interaction. The increment of biological activity was attributed by Zhang et al.[30] to the well-documented ability of Aib to induce helicity.[31] However, since these authors relied entirely on literature data to evaluate the helix-inducing ability of Aib, and did not support their hypothesis with an experimental conformational study, we chose to undertake a comparative structural and pharmacological analysis.

We have verified the hypothesis of Zhang et al.[30] experimentally, extending the study to new analogues in which the role of Aib is taken up by a natural amino acid, that is, Leu, a residue that is known to increment helicity. Predictions of secondary structure showed that single Ala/Leu substitutions are not sufficient to increase helicity considerably, but double substitutions, such as [Leu^{7,11}]- and [Leu^{11,15}]-N/OFQ-amide, do give encouraging helicity predictions.

The conformational analysis, based on NMR spectroscopy, confirms that all singly substituted Ala/Aib peptides as well as the doubly substituted [Aib^{7,11}]-N/OFQ-amide, which was studied to compare its conformational features to those of [Leu^{7,11}]-N/OFQ-amide, are highly helical. The same holds true also for [Leu^{7,11}]- and [Leu^{11,15}]-N/OFQ-amide. In fact, in helix-promoting media, the helix content of [Leu^{7,11}]- is even higher than that of [Aib^{7,11}]-N/OFQ-amide.

Helicity is not the only factor: [Leu^{11,15}]-N/OFQ-amide has an activity comparable to those of singly substituted Ala/Aib peptides and identical to that of [Aib^{7,11}]-N/OFQ-amide. Helicity is evidently not the only factor affecting the interaction with the receptor. We know that alteration of the basic nature of the message domain is detrimental for the interaction, as shown by numerous comparisons between N/OFQ peptides that contain only natural residues: those with a C-terminal carboxyl group are invariably less active than the corresponding C-terminal amidated analogues.[11] Confirmation also came from the Aib-containing sequences of Zhang et al.[30] that, when in the form of peptide amides, are more active than the corresponding C-terminal free acid derivatives. As an internal control, we studied also [Leu^{11,15},Glu^{16}]-N/OFQ-amide: the inclusion of Glu^{16} enhances the helical propensity, both owing to the intrinsic properties of Glu and for the favorable electrostatic interaction with Lys^{12}. Yet, the biological activity is only half that of the parent peptide (N/OFQ-amide). The interpretation of this result is straightforward, since it is well known that negative charges in the

address domain decrease binding dramatically. In this case, the presence of the negative charge of Glu^{16} more than offsets the gain in binding obtained through the stabilization of the helix, as indicated by the similar pEC_{50} values of [$Leu^{11,15}$, Glu^{16}]-N/OFQ-amide and [Glu^{16}]-N/OFQ-amide.

An important difference among Aib and Leu mutants is that the biological response of Leu mutations depends sharply on the position along the sequence. A less predictable outcome of our study is the fact that [$Leu^{7,11}$]-N/OFQ-amide is even less active than [$Leu^{11,15}$, Glu^{16}]-N/OFQ-amide. Since this peptide is as helical as any of the Aib/Ala peptides and has no significant change in the electrostatic properties, the only possible explanation is that bulky residues cannot be hosted in the hinge region (Thr-Gly-Ala) without severe steric clash with the receptor.

This finding is consistent with previous data on this hinge region obtained by systematic substitution of Gly and Ala with Pro. This study[29] showed that the angle between the two domains is very important for a productive interaction with the receptor. It is clear that a bulky residue at the end of the hinge can both change the angle and clash with receptor residues. It is interesting to see from Figure 4 that the only significant difference between the models of [$Aib^{7,11}$]- and [$Leu^{7,11}$]-N/OFQ-amide is the evident encumbrance of the side chain of Leu^7 with respect to that of Aib^7.

Conclusion

In summary, our study confirms the importance of a predominantly helical conformation for the address domain of N/OFQ as a key factor for its interaction with the EL_2 loop of the receptor. As previously mentioned, N/OFQ shows very little tendency to assume ordered conformations, including helical ones, in in vitro studies in a variety of media.[29] The results of our conformational studies suggest that the helical conformation of the address domain may be attained, in vivo, through the induced fit with the receptor. The easy docking of the NMR structure of [$Leu^{11,15}$]-N/OFQ-amide inside the receptor lends validity to the relative location and orientation of the two domains. The message domain is hosted by the cavity that can bind apolar nonpeptidic agonists,[47b] whereas the acidic residues of the EL_2 loop are placed at ideal interacting positions to corresponding basic residues of [$Leu^{11,15}$]-N/OFQ-amide by the helical conformation of the address domain and by the angle between the two domains. The response to substitutions in the hinge region between the message and address domains hints at a precise spatial requirement for the relative orientation of the two domains and at a threshold in the bulkiness of residue number seven.

Experimental Section

Materials: Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka (Switzerland) or Chem-Impex International (USA). The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid]polyethyleneglycol/polystyrene support (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). Naloxone was from Tocris Cookson (Bristol, UK). Stock solutions (1 mM) of peptides were made up in distilled water and kept at −20 °C until use. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) was of the following composition (in mM): NaCl (118.5), KCl (4.7), KH_2PO_4 (1.2), $NaHCO_3$ (25), $CaCl_2$ (2.5), and glucose (10). All other reagents were from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany) and were of the highest purity available.

General procedures for the solid phase synthesis: As an illustrative example the synthesis of [Aib^7]-N/OFQ-amide is described. Fmoc-PAL-PEG-PS resin, (0.19 mmol g^{-1}, 0.5 g) was treated with piperine 20% DMF and linked with: Fmoc-Gln(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ala-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Aib-OH, Fmoc-Gly-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, and Fmoc-Phe-OH (4 equiv) by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (known as HATU)[49] (4 equiv) as coupling reagent. Double coupling was required in the acylation step of the Aib residue. The coupling reaction time was 1 h and piperidine (20%)/DMF was used to remove the Fmoc group in every step. The peptide resin was washed with methanol and dried in vacuo to yield the protected [Aib^7]-N/OFQ-resin. The other peptides were synthesized in a similar manner. The protected peptide resin was treated with reagent K[50] (TFA/$H_2$0/phenol/ethanedithiol/thioanisole 82.5:5:5:2.5:5; v/v; 10 mL per 0.5 g of resin) for 1 h at room temperature. After filtration of the exhausted resin, the solvent was concentrated in vacuo and the residue triturated with diethyl ether. The crude peptide was purified by preparative reverse-phase HPLC to yield a white powder after lyophilization.

Peptide purification and analytical determinations: Crude peptides were purified by preparative reverse-phase HPLC by using a Waters Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 Å, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 mL min^{-1} with solvent A (water, 0.1% TFA), and a linear gradient from 0 to 35% of solvent B (acetonitrile, 0.1% TFA) over 30 min was adopted for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 125 liquid chromatograph fitted with a Nucleodur C_{18} column (4.6 × 150 mm, 5 μm particle size) and equipped with a Beckman 168 diode array detector. Analytical purity and retention time (t_R) of each peptide were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 mL min^{-1} using a linear gradient: I) from 5% to 60% B over 25 min and II) from 0% to 40% B over 25 min. All analogues showed >97% purity when monitored at 220 nm. Molecular weights of compounds were determined by a MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) analysis using a Hewlett Packard G2025 A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix. Values are expressed as $[M+H]^+$.

The analytical properties of N/OFQ analogues are reported in Table 1.

Model building: Model building was performed by means of the program Chem3D (Cambridge Soft). Starting models were built from scratch by using only sequence information and choosing qualitatively the helical regions from the NMR parameters. The resulting conformer was energy minimized by using a simple MM2 force-field, in vacuo, with a dielectric constant of 15, and NOE-derived interatomic distances as the only restraints. Restrained minimization was continued until low values of the gradient (<0.001) were reached. Unrestrained energy minimization led to a final conformation consistent with all NMR parameters.

NMR measurements: Proton NMR spectra were run on Bruker DRX-500 and on Bruker DRX-600 spectrometers. A conventional set of 2D spectra, according to the scheme of sequential assignment described by Wuethrich[51] was recorded: DQF-COSY,[52] TOCSY,[53] and NOESY.[54] TOCSY spectra were collected with mixing times in the range 50 to 75 ms, using the clean MLEV-17 mixing scheme.[55] The NOESY spectra were recorded with mixing times of 50, 100 and 200 ms. Time-proportional phase Incrementation (TPPI) was applied to achieve quadrature detec-

tion in the virtual dimension.[56] Water suppression was achieved either by presaturation or by using the WATERGATE pulse sequence.[57]

Data processing was performed with standard Bruker software XwinNMR. Spectral analysis was performed with SPARKY.[58]

Structure calculation: The input data for the structure calculation with the program DYANA[46] were generated from the peak volumes obtained from SPARKY.[58] Figure 3 summarizes all measured NOEs, classified as intraresidue, sequential and medium range ($1<|i-j|<5$).

Based on the peak volumes observed on the NOESY spectra, the upper distance limits were generated with the program CALIBA.[46] 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 root mean square deviations (rmsd) values were carried out with the program MOLMOL.[59]

Bioassay studies: Male Swiss mice weighing 25–30 g were used. The bioassay experiments were performed as previously described.[14] The mouse vas deferens tissues were suspended in 5 mL organ baths containing Mg^{2+} free Krebs solution at 33 °C. The tissues were stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. The resting tension was maintained at 0.3 g. The electrically evoked contractions were measured isotonically by means of a Basile strain gauge transducer and recorded with a PC-based acquisition system (Autotrace, RCS, Florence, Italy). After an equilibration period of about 1 h the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration-response curves to N/OFQ and related peptides were performed (0.5 log unit steps). When required, antagonists (naloxone and UFP-101) were added to the Krebs solution 15 min before performing the concentration-response curve to agonists.

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