Further Studies on Nociceptin-Related Peptides: Discovery of a New Chemical Template with Antagonist Activity on the Nociceptin Receptor

Remo Guerrini, Girolamo Calo′,[†] Raffaella Bigoni,[†] Anna Rizzi,[†] Katia Varani,[†] Geza Toth,[‡] Stefania Gessi,[†] Eiji Hashiba,[#] Yoshio Hashimoto,[#] David G. Lambert,[#] Pier Andrea Borea,[†] Roberto Tomatis, Severo Salvadori, and Domenico Regoli*[,†]

Department of Pharmaceutical Sciences and Biotechnology Center and Department of Experimental and Clinical Medicine, Section of Pharmacology, University of Ferrara, Via Fossato di Mortara 19, 44100 Ferrara, Italy, Isotope Laboratory Institute of Biochemistry, Biological Research Centre, Szeged H-6701, Hungary, and University Department of Anaesthesia, Leicester Royal Infirmary, Leicester LE1 5WW, U.K.

Received March 17, 2000

Three series of nociceptin (NC)-related peptides were synthesized and their abilities (i) to bind to the NC sites expressed in mouse forebrain membranes, (ii) to inhibit the electrically evoked contraction of the mouse vas deferens, and (iii) to inhibit forskolin-stimulated cAMP accumulation in Chinese hamster ovary cells expressing the human recombinant NC receptor (CHO_{NCR}) were investigated. The compounds of the first series (**a** series) have an ordinary Xaa^1-Gly^2 bond, those of the second series (**b** series) have a $Xaa^1\Psi(CH_2\text{-}NH)Gly^2$ pseudopeptide bond, and those of the third series (**c** series) have a peptoid ($Nxaa^1$-Gly^2) structure. The affinity values measured in the binding assay and in the two functional assays with the compounds of the three series showed high levels of correlation. Thus, (I) the compounds of the **a** series in which Phe^1 was substituted with Tyr, Cha, or Leu acted as potent NC receptor agonists; (II) the **b** series compounds behaved as NC receptor antagonists in the mouse vas deferens and as full agonists in CHO_{NCR} cells with different potencies depending on the first amino acid residue, $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]NC(1-17)NH_2$ and $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]NC(1-13)NH_2$ being the most potent compounds; (III) the compounds of the third series were all inactive both as agonists and as antagonists with the exception of $[Nphe^1]NC(1-17)NH_2$ and $[Nphe^1]NC(1-13)NH_2$, which behaved as NC receptor antagonists both in the isolated tissue and in CHO_{NCR} cells (pK_B 6.1−6.4). In conclusion, this study demonstrates that chemical requirements for NC receptor agonists are different from those of antagonists. Moreover, modifications of the steric orientation of the aromatic residue Phe^1 in the NC sequence as obtained with the pseudopeptide bond between Phe^1 and Gly^2 or with the displacement of the benzyl side chain by one atom, as in $Nphe^1$, lead respectively to reduction or elimination of efficacy. Indeed, in contrast to $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]NC(1-13)NH_2$ which has been reported to exhibit agonist activity in several assays involving either central or recombinant NC receptors, $[Nphe^1]NC(1-13)NH_2$ antagonizes the effect of NC at human recombinant NC receptors and in the mouse tail withdrawal assay.

Introduction[1]

Nociceptin (NC)/orphanin FQ is the endogenous ligand of the ORL-1 (opioid receptor like 1) receptor,[2,3] the G-protein-coupled receptor which inhibits adenylyl cyclase,[2,3] activates potassium channels,[4] and inhibits calcium channels.[5] The NC receptor is expressed in different regions of the brain, in the spinal cord, and in peripheral organs such as intestine, vas deferens, liver, and spleen (see refs 6, 7 for a review). Sequentially, NC shows some structural homology with dynorphin A, the endogenous ligand of the κ opioid receptor. Despite structural similarities of receptors and ligands between the opioid and the NC systems, they are pharmacologically distinct, since NC does not bind opioid receptors and opioid ligands have no affinity for the NC receptor.

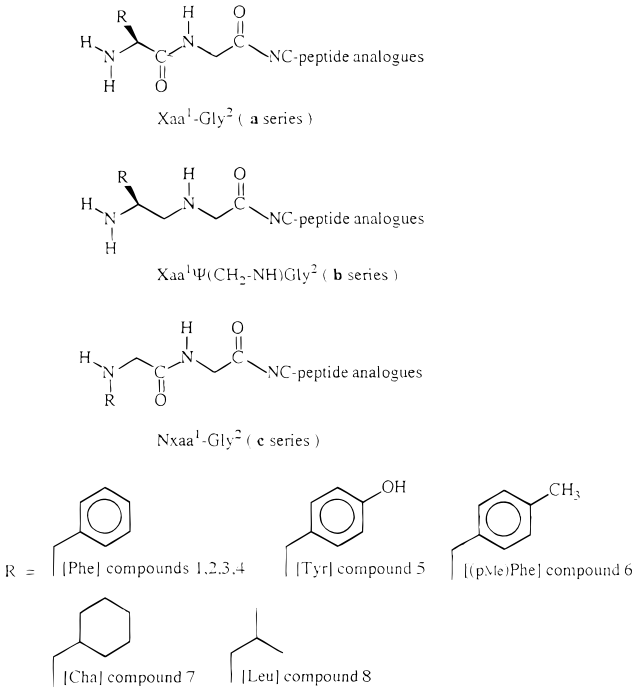
NC exerts several biological effects in the central nervous system (see refs 6, 7 for a review) and inhibits the release of neurotransmitters such as dopamine,[8] glutamate,[9] acetylcholine,[10] noradrenaline,[11] and serotonin.[12,13] Similarly, in the periphery, NC inhibits the release of neurotransmitters from sympathetic,[14−16] parasympathetic,[16,17] and peptidergic nerves[18−21] and causes hypotension, bradycardia,[22−24] and diuresis.[25]

Structure−activity studies on NC have been performed over the last 2 years: First, we studied the activities of NC fragments to determine the minimal sequence ($NC(1-13)NH_2$) that maintains full agonistic activity and is protected from degradation at the C-terminus.[26] This tridecapeptide has served as a template for the identification of further compounds. We were also able to determine that the active group(s) of NC are in the N-terminus tetrapeptide Phe-Gly-Gly-Phe (message), while the other nine residues (5−13) are required for binding (address) to the NC receptor.[27] Second, in an attempt to protect the N-terminus from degradation by aminopeptidases, we discovered that $[Phe^1\Psi(CH_2\text{-}NH)Gly^2]NC(1-13)NH_2$ is an antagonist of NC receptors expressed in the mouse vas deferens,[28] in a variety of in vitro peripheral preparations,[18,29−32] and

* To whom correspondence should be addressed. Tel: +39-0532-291227. Fax: +39-0532-291205. E-mail: d.regoli@unife.it.
[†] Department of Experimental and Clinical Medicine, University of Ferrara.
[‡] Biological Research Centre.
[#] Leicester Royal Infirmary.

10.1021/jm990075h CCC: $19.00
Published on Web 07/07/2000

Chart 1. N-Terminus Dipeptide Structures of the **a**−**c** Series Compounds



also in vivo in the cardiovascular system of the mouse.[22] However, this compound maintains agonistic activities, mimicking the actions of NC especially when tested in central nervous system preparations[11,12,33−37] (see the review article[38] for a detailed analysis of the pharmacological profile of [Phe$^1\Psi$(CH$_2$-NH)Gly^2]NC(1−13)NH$_2$).

In the present investigation, we have further modified the N-terminus Phe and identified new antagonists, which, although of low potency, show an interesting pharmacological profile.

Results and Discussion

Three series of eight compounds were synthesized and tested. As shown in Chart 1, the compounds of the first series (**a** series) have an ordinary Xaa^1-Gly^2 bond, those of the second series (**b** series) have a Xaa$^1\Psi$(CH$_2$-NH)-Gly^2 pseudopeptide bond, and those of the third series (**c** series) have a peptoid (Nxaa^1-Gly^2) structure.

Peptides **1**−**8** of the **a** and **c** series were prepared by solid-phase peptide synthesis performed on a Milligen 9050 synthesizer using a Fmoc-PAL-PEG-PS-resin (0.2 mmol/g, 0.2 g in all syntheses). Boc-protected amino aldehydes were prepared by the reduction of the Weinreb amide.[39] Peptides were assembled using Fmoc-protected amino acid (4 equiv), DIPCDI (4 equiv), and HOBt (4 equiv) as coupling agents for 1 h for each coupling. Side chain protecting groups used were Pmc for Arg, Trt for Asn and Gln, Boc for Lys, and tBu for Ser and Thr. Peptides **1b**−**8b** containing modification of the CO−NH bond were obtained by condensing Boc-Xaa-CHO with the NC fragments [NC(2−17)-, NC(2−13)-, NC(2−12)-, and NC(2−9)]-PAL-PEG-PS-resin and reducing the intermediate imine derivative in situ with NaBH$_3$CN in order to minimize racemisation.[28] As reported by Ho et al.,[40] the formation of diastereomeric peptides due to apparent racemization at the α-carbon of the Boc-amino aldehyde was observed in several cases. Protected amino aldehydes were used directly without further purification to avoid possible epimerization. To test the formation, during the synthesis of diastereomers of the **b** series compounds, we adopted different HPLC strategies. For the compound **2b** (the best analogue of this series), we prepared the relative diastereomer with a D-Phe residue in position 1, [D-Phe$^1\Psi$(CH$_2$-NH)Gly^2]NC(1−13)NH$_2$, [28] and we compared the HPLC behavior of the two peptides, under the analytical conditions reported in the Experimental Section. Under these conditions, the two peptides showed the same retention time. We also adopted an alternative HPLC solvent including a different ion-pairing system, e.g. triethylammonium phosphate (pH 6.5) and CH$_3$CN with various gradients to separate the two diastereomeric peptides. Unfortunately, we did not obtain any separation of the two peptides under these experimental conditions. In addition, we synthesized two diastereomeric tetrapeptides Boc-L- and -D-PheΨ(CH$_2$-NH)Gly-Gly-Phe-OH, and we compared their HPLC retention time under the latter HPLC conditions. Once again, the two diastereomer tetrapeptides were not distinguishable by HPLC analysis. Finally, we removed the Boc protection from these tetrapeptides to obtain H-L- and -D-PheΨ(CH$_2$-NH)Gly-Gly-Phe-OH, and we performed a new HPLC analysis under the following experimental conditions: chiral column Daicel Crownpak CR for RP-HPLC (15 × 0.4 cm, 5 μm) eluted with a solution of HClO$_4$ (pH 3.1) containing KCl (1 mM) and 10% MeOH with a flow rate of 0.65 mL/min at 5 °C temperature. Under these experimental conditions, we obtained two peaks, not completely baseline resolved, with retention times of 44.3 and 46.8 min corresponding to H-D- and -L-PheΨ(CH$_2$-NH)Gly-Gly-Phe-OH, respectively. We therefore conclude that the application of several HPLC methods did not result in complete separation of these diastereoisomeric tridecapeptides and N-terminus tetrapeptides. However, on the basis of results obtained with similar peptides by Ho et al.[40] and on the basis of our biological assay results ([D-Phe$^1\Psi$(CH$_2$-NH)Gly^2]NC(1−13)NH$_2$ is inactive both as NC receptor agonist and as antagonist[28]) we feel that isomerization (if it occurs) may be negligible.

Peptoids **1c**−**8c** with the Xaa^1 side chain N-shift were obtained by condensing Boc-Nxaa-OH with the same NC fragments, obtained by the solid-phase methods reported above (**b** series), in the presence of DIPCDI and HOBt in the final acylation step of peptide synthesis. The syntheses of the peptoid residues, Nxaa-OEt, were achieved by alkylation of an amine with ethyl bromoacetate or by reductive amination of the H-Gly-OEt amino function in the presence of NaBH$_3$CN. Nxaa-OEt was protected at the amino function with the Boc group and the ester function removed by basic hydrolysis to give Boc-Nxaa-OH.

The chemical features of the new compounds are presented in Table 1. *K′* was determined in two (I and II) solvent systems to assess the purity of each compound. Mass ion data are also reported to characterize the synthesized peptides.

The peptides of the three series were tested for their ability to inhibit the electrically evoked contraction (twitch response) of the mouse vas deferens (mVD), a pharmacological preparation shown to be sensitive to NC.[16] When they were found inactive as agonists, the

Table 1. Abbreviated Names and Analytical Properties of NC Analogues

		K' [a]		MH^+ [b]	
no.	abbreviated names	I	II	calcd	found
5a	[Tyr^1]NC(1−13)NH_2	2.41	3.78	1398.6	1399.0
6a	[(pMe)Phe^1]NC(1−13)NH_2	2.73	4.06	1396.5	1396.5
7a	[Cha^1]NC(1−13)NH_2	3.56	4.87	1388.6	1388.7
1b	[Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1−17)NH_2	3.86	4.48	1795.0	1794.8
3b	[Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1−12)NH_2	3.52	4.61	1240.4	1240.1
4b	[Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1−9)NH_2	3.81	4.38	926.0	926.3
5b	[Tyr$^1\Psi$(CH_2-NH)Gly^2]NC(1−13)NH_2	3.65	4.32	1384.6	1384.5
6b	[(pMe)Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1−13)NH_2	3.91	4.62	1382.5	1381.9
7b	[Cha$^1\Psi$(CH_2-NH)Gly^2]NC(1−13)NH_2	4.15	4.81	1374.6	1374.6
8b	[Leu$^1\Psi$(CH_2-NH)Gly^2]NC(1−13)NH_2	3.17	3.88	1334.5	1334.3
1c	[Nphe^1]NC(1−17)NH_2	3.88	4.81	1809.0	1808.7
2c	[Nphe^1]NC(1−13)HN_2	2.14	3.72	1382.5	1382.6
3c	[Nphe^1]NC(1−12)NH_2	2.28	3.91	1254.4	1255.0
4c	[Nphe^1]NC(1−9)NH_2	2.66	4.17	940.0	940.7
5c	[Ntyr^1]NC(1−13)NH_2	2.37	3.83	1398.6	1398.2
6c	[N(pMe)phe^1]NC(1−13)NH_2	2.79	4.31	1396.5	1396.2
7c	[Ncha^1]NC(1−13)NH_2	3.71	4.83	1388.6	1388.1
8c	[Nleu^1]NC(1−13)NH_2	2.32	3.61	1348.5	1348.0

[a] K' is the capacity factor determined by analytical HPLC. [b] The mass ion (MH^+) was obtained by MALDI-TOF mass spectrometry

peptides were assayed as antagonists against the reference agonist, NC(1−13)NH_2, in the same preparation. Results of biological assays are presented in Table 2 in terms of pEC_{50} to describe their agonist potency and in terms of pK_B to indicate their potency as antagonists. The same compounds were also evaluated in a recently described NC receptor binding assay performed on mouse forebrain membranes using [^{3}H]NC-NH_2 as a radioligand.[41] Results of binding experiments, expressed in terms of pK_i, are also presented in Table 2. The compounds of the three series were also tested in CHO_{NCR} cells for their ability to inhibit forskolin-stimulated cAMP accumulation. The results of these experiments are summarized in Table 3.

As reported by us previously,[27] position 1 of the ligand NC(1−13)NH_2 can be modulated in different ways, by replacing Phe with Tyr or with the aliphatic residues Leu or Cha, which are tolerated with full retention of agonistic activity, even if the replacement with Tyr gives a compound that interacts also with opioid receptors.[26,42] Other substitutions of the aromatic Phe^1, as with (pCH_3)Phe, strongly reduce activity.

Results summarized in Tables 2 and 3 show that compounds **1a, 2a, 5a, 7a,** and **8a** are potent agonists, as active as NC(1−17)NH_2, and they are full agonists in the mVD and in CHO_{NCR} cells. In addition they show high affinities (pK_i from 8.4 to 9.1) in the binding assay. The shorter sequences (compounds **3a** and **4a**) show decreased affinity by approximately 30-fold (compound **3a**) or by >1000-fold (compound **4a**). Compound **6a** is a low-potency agonist in agreement with its low binding affinity. Some of the biological data obtained in the mVD with these compounds have already been communicated.[27]

The compounds of the **b** series (Xaa$^1\Psi$(CH_2-NH)Gly^2) are inactive as agonists in the mVD: only the Tyr^1 and Leu^1 derivatives show weak agonist activities. Affinities in the binding assay are quite high for compounds **1b, 2b, 5b,** and **8b**, weaker for **3b** and **6b**, and very weak, if any, for compound **4b**. Some of them, for instance compounds **1b** and **2b**, act as antagonists of average potencies; compounds **3b**, **4b**, and **6b** are weak antagonists; compounds **5b** and **8b** should be considered as partial agonists. Compound **7b** is inactive not only as agonist but also as antagonist. In CHO_{NCR} cells the compounds of the **b** series all behaved as agonists showing a rank order potency similar to those obtained in the mVD and in the binding assay.

Compounds of the **c** series have no agonistic effects; two of them, compounds **1c** and **2c**, act as antagonists of average potency both in the mVD (Table 2) and in CHO_{NCR} cells (Table 3); all other peptides are inactive also as antagonists. When tested for their ability to compete with [^{3}H]NC-NH_2 for the NC site in mouse brain membranes, compounds **1c** and **2c** show average values of affinity ($pK_i \approx 7$), compounds **3c** and **5c**−**8c** show $pK_i \approx 5.5$, and compound **4c** has a pK_i value below 5.

Ala-scan studies on NC from different laboratories demonstrated that replacement of Phe^1 appears to be critical for receptor occupation[43] and activation.[44] The insertion of a pseudopeptide bond between Phe^1 and Gly^2, as in compound **2b**, maintains affinity but reduces the ability to activate the NC receptor. This reduced efficacy led to antagonism in the mVD but not in CHO_{NCR} cells where the compound behaved as a full agonist. Reports from various laboratories have shown that compound **2b** may behave as an antagonist, partial agonist, or full agonist depending on the preparation/assay. In several isolated tissues[18,29−32] and in the cardiovascular system of the mouse in vivo, compound **2b** behaves as an antagonist, while this pseudopeptide is a potent agonist in several tests on central actions of NC.[11,12,34−37] The full agonist activity of compound **2b** in CHO_{NCR} cells has been confirmed in different laboratories (present data and[33,45,46]). The reasons for the dual behavior of compound **2b** are at present unknown; however, recent findings suggest that the intrinsic activity of the compound may depend on the number of NC receptors expressed in a given preparation[47] (see ref 38 for a detailed discussion of this topic). Collectively, these findings indicate that compound **2b** (as well as the other molecules of the **b** series) should be viewed as a low-efficacy agonist.

Contrary to compound **2b**, compound **2c** acts as a pure NC receptor antagonist both in the mVD and in CHO_{NCR} cells. In addition, [Nphe^1]NC(1−13)NH_2 competitively and selectively antagonized the effects of NC in several

Table 2. Binding Affinities and Functional Activities of NC-Related Peptides in Mouse Tissues

		Xaa^1-Gly^2 (**a** series)				Xaa$^1\Psi$(CH$_2$-NH)Gly^2 (**b** series)				Nxaa^1-Gly^2 (**c** series)			
		mVD bioassay				mVD bioassay				mVD bioassay			
compd[a]	Xaa^1	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B	binding pK_i	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B	binding pK_i	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B	binding pK_i
1, NC(1−17)NH$_2$	Phe	7.7 (0.1)	−79 ± 4	ND	9.1 (0.07)	inactive		7.0 (0.6)	7.7 (0.08)	inactive		6.3 (0.4)	6.9 (0.14)
2, NC(1−13)NH$_2$	Phe	7.8 (0.01)	−84 ± 4	ND	9.1 (0.04)	inactive		6.8 (0.2)	8.0 (0.1)	inactive		6.4 (0.3)	7.0 (0.1)
3, NC(1−12)NH$_2$	Phe	6.1 (0.1)	−79 ± 7	ND	7.6 (0.06)	inactive		5.2 (0.4)	6.4 (0.07)	inactive		inactive	5.5 (0.06)
4, NC(1−9)NH$_2$	Phe	inactive		inactive	<5	inactive		5.1 (0.4)	<5	inactive		inactive	<5
5, [Tyr^1]NC(1−13)NH$_2$	Tyr	7.6 (0.6)	−89 ± 1	ND	8.4 (0.1)	crc incomplete		5.7 (0.5)	7.0 (0.1)	inactive		inactive	5.5 (0.1)
6, [(pMe)Phe^1]NC(1−13)NH$_2$	(pMe)Phe	5.6 (0.4)	−70 ± 2	ND	6.7 (0.07)	inactive		5.7 (0.2)	6.2 (0.04)	inactive		inactive	5.6 (0.05)
7, [Cha^1]NC(1−13)NH$_2$	Cha	7.9 (0.3)	−84 ± 8	ND	9.0 (0.1)	inactive		I	6.5 (0.1)	inactive		inactive	5.6 (0.07)
8, [Leu^1]NC(1−13)NH$_2$	Leu	7.6 (0.3)	−80 ± 9	ND	8.6 (0.04)	crc incomplete		5.4 (0.3)	7.8 (0.1)	inactive		inactive	5.6 (0.08)

[a] The synthesis and bioassay data for compounds **1**−**4** and **8** of the **a** series and for compound **2** of the **b** series have been already published (see refs 27, 28). The antagonistic properties of these compounds were tested using NC(1−13)NH$_2$ as agonist. For pEC_{50}, pK_B, and pK_i values the confidence limits 95% are given in parentheses. ND: not determined because these compounds are full agonists; inactive: inactive up to 10 μM; crc incomplete: only a slight effect (<50% inhibition) was detected at the highest concentration tested (10 μM). None of the effects of these compounds were affected by 1 μM naloxone. These data are mean of at least 5 (bioassay) or 4 (binding) experiments.

isolated tissues (rat vas deferens, guinea pig ileum and renal pelvis, mouse colon) showing pA_2 values ranging from 6.0 to 6.4.[48,49] More importantly, it is also active in vivo where it antagonizes the pronociceptive and antimorphine actions of intracerebroventricularly applied NC, measured in the mouse tail withdrawal assay,[49] and the stimulatory effect of NC on food intake in the rat.[50]

Some conclusions can be drawn from the data presented above. The correlation between the values of apparent affinity (pEC_{50}/pK_B) obtained in the mVD and in the CHO_{NCR} assays and the values of actual affinity (pK_i) obtained in the binding assay for the same compounds is high, as indicated by the value of the correlation coefficients (mVD − CHO_{NCR} $r = 0.80$; mVD − mB $r = 0.88$; CHO_{NCR} − mB $r = 0.90$); this suggests that the same receptor site is expressed in the three preparations. However, the actual affinity values measured in the binding assay and the potencies estimated in the CHO_{NCR} assays were always higher, on average by 10−30-fold, than those of the potencies evaluated by the bioassay in the mVD. This is quite common in peptide pharmacology (see Regoli et al.[51] for the kinin, Regoli et al.[52] for the neurokinin, and Knapp et al.[53] for the opioid field) and has been attributed to the different accessibility of the receptors in the preparations (cell or cell membranes and intact tissues). It is assumed that the whole receptor population can be easily reached in a suspension of cells or plasma membranes, while the receptors that are present in the various layers of smooth muscle cells constituting the mVD are not. Another possible explanation for the different potencies obtained in the different assays is represented by the different buffers utilized especially in terms of concentration of Na^+ which may affect ligand affinity, particularly for agonists.

Some interesting points have emerged from the structure−activity study performed with the compounds of the **a**−**c** series. The use of the pseudopeptide bond (CO−NH → CH_2−NH) between Xaa^1 and Gly^2 reduces the efficacy of all compounds. This reduction in efficacy is evident in the mVD assay (where the compound of the **b** series behaves as NCR antagonists) but not in the CHO_{NCR} assays. This different behavior may depend on the fact that our CHO_{NCR} cells express a very high number of receptors (B_{max} about 1700 fmol/mg protein[45]) which represents a large receptor reserve. Therefore, compounds which are actually partial agonists may elicit maximal effect similar to those evoked by full agonists. The results obtained by Toll[47] in CHO expressing different levels of NCR support this interpretation. Compound **2b** behaves as a receptor agonist in cells with high receptor number, while it behaves as an antagonist in cells with low receptor number.[47] The fact that compounds of the **b** series behave as antagonists in the mVD suggests that (i) the occupation of the receptor by the antagonist requires the modified message (Xaa$^1\Psi$-(CH_2-NH)Gly-Gly-Phe) and a critical C-terminus chain, whose optimum is the nonapeptide NC(5−13), similar to the requirements for agonists (see **a** series); (ii) Phe^1, although structurally affected by the presence of the pseudopeptide bond (**b** series), contributes to the antagonist affinity better than any other substitution, by aromatic residues (Tyr, (pCH_3)Phe) or aliphatic residues

Table 3. Functional Activities of NC-Related Peptides in CHO_{hNCR}

		Xaa^1-Gly^2 (**a** series)			Xaa$^1\Psi$(CH_2-NH)Gly^2 (**b** series)			Nxaa^1-Gly^2 (**c** series)		
compd[a]	Xaa^1	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B	agonist pEC_{50}	agonist E_{max} (%)	antag pK_B
1, NC(1−17)NH_2	Phe	9.77 (0.20)	103 ± 1	ND	9.14 (0.20)	102 ± 1	ND	inactive*		6.17 (0.35)
2, NC(1−13)NH_2	Phe	9.49 (0.21)	104 ± 1	ND	8.65 (0.20)	104 ± 3	ND	inactive*		6.12 (0.28)
3, NC(1−12)NH_2	Phe	8.54 (0.27)	100 ± 3	ND	6.59 (0.24)	109 ± 3	ND	inactive		inactive
4, NC(1−9)NH_2	Phe	6.74 (0.30)	97 ± 7	ND	crc incomplete		ND	inactive		inactive
5, [Tyr^1]NC(1−13)NH_2	Tyr	10.05 (0.29)	101 ± 1	ND	7.97 (0.22)	102 ± 1	ND	crc incomplete		5.28 (0.66)
6, [(pMe)Phe^1]NC(1−13)NH_2	(pMe)Phe	7.98 (0.19)	103 ± 3	ND	6.47 (0.20)	77 ± 2	ND	crc incomplete		ND*
7, [Cha^1]NC(1−13)NH_2	Cha	10.04 (0.34)	104 ± 1	ND	7.20 (0.10)	107 ± 1	ND	6.21 (0.41)	61± 13	ND
8, [Leu^1]NC(1−13)NH_2	Leu	9.79 (0.56)	102 ± 1	ND	7.36 (0.55)	101 ± 7	ND	crc incomplete		ND*

[a] The syntheses for compounds **1**−**4** and **8** of the **a** series and for compound **2** of the **b** series have been already published.[27,28] The data of compounds **1**−**4** of the **a** series and compounds **2b** and **2c** are taken from refs 45, 65. The antagonistic properties of these compounds were tested using NC(1−13)NH_2 as agonist. For pEC_{50} and pK_B values the confidence limits 95% are given in parentheses. ND: not determined because these compounds are full agonists; ND*: not determined as there was >50% inhibition at the highest concentration used (10 μM); inactive: inactive up to 10 μM; inactive*: small inhibition (<15% at 10 μM); crc incomplete: <50% inhibition at the highest concentration tested (10 μM). These data are mean of at least 3 separate experiments.

(Leu or Cha), in contrast with what happens for compounds of the **a** series, which are all agonists of high potency, with the only exception of compound **6a**.

The C → N shift of the Phe^1 side chain leads to complete elimination of efficacy thus giving pure antagonists. In fact the antagonist effects of compounds **1c** and **2c** are similar in the mVD and in CHO_{NCR} cells. This modification leads however to an important reduction of affinity. In addition, this C → N shift is inappropriate for any other residue than Phe, even in binding; all compounds with residues other than Phe in position 1 (compounds **5c**−**8c**) show affinities at least 30-fold lower than compounds **1c** and **2c**. Again, the whole nonapeptide chain (5−13) at the C-terminus is needed for receptor occupation, since shorter sequences, as in compounds **3c** and **4c**, are inactive.

In conclusion, this study demonstrated that a different orientation of the aromatic residue Phe^1 of NC(1−13)NH_2, as obtained with a pseudopeptide bond between Phe^1 and Gly^2, leads to reduced efficacy. The displacement of the benzyl side chain by one atom, as in Nphe^1 (**c** series), completely eliminates agonistic activity and provides a pure antagonist at the NC receptor. While [Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1−13)NH_2 maintains some residual agonistic activity and is considered to be a partial agonist with different efficacy depending on the preparations, [Nphe^1]NC(1−13)NH_2 is completely inactive as an agonist and behaves as a pure NC receptor antagonist in vitro on native and recombinant NC receptors (present data) as well as in vivo.[49,50] Although weak (pK_i = 7.0; pK_B = 6.3) this new compound provides a new lead for future development of NCR antagonists.

Experimental Section

Materials. Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland). *N*-Benzylglycine was from Aldrich (Milwaukee, WI). Boc-(pMe)Phe-OH was from RSP (Worcester, MA). The resin [5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid]poly(ethylene glycol)/polystyrene suport (Fmoc-PAL-PEG-PS) was from Millipore (Waltham, MA). Naloxone was from Tocris Cookson (Bristol, U.K.). Stock solutions (1 mmol) of peptides were made in distilled water and kept at −20 °C until use. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) had the following composition (in mM): NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. All other reagents were from Sigma Chemical Co. (Poole, U.K.) or E. Merck (Darmstadt, Germany) and were of the highest purity grade available.

Peptide Purification and Analytical Determinations. Crude peptides were purified by preparative reversed-phase HPLC using a Water 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 50 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% solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min was adopted for the elution of peptides.

Analytical HPLC analyses were performed on a Bruker liquid chromatography LC 21-C instrument fitted with a Alltech C_{18} column (4.6 × 150 mm, 5-μm particle size) and equipped with a Bruker LC 313 UV variable-wavelength detector. Recording and quantification were accomplished with a chromatographic data processor coupled to an Epson computer system (BX-10). Analytical determination and capacity factors (K') of the peptides were determinated using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 mL/min using the following linear gradients: (I) from 0% to 50% B in 25 min and (II) from 0% to 20% B in 25 min. All analogues showed less than 1% impurities 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 G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix. The values are expressed as MH^+.

TLC was performed on precoated plates of silica gel F254 (Merck, Darmstadt, Germany) using the following solvent systems: (III) AcOEt/*n*-hexane (1:1, v/v), (IV) CH_2Cl_2/methanol (9.5:0.5, v/v), (V) CH_2Cl_2/methanol (9:1, v/v), (VI) CH_2Cl_2/methanol/toluene (17:2:1, v/v/v). Ninhydrin (1%) or chlorine iodine spray reagents were employed to detect the peptides. Elemental analyses were performed by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara.

Optical rotations were determined using a Perkin-Elmer 241 polarimeter with a 10-cm cell using methanol as the solvent and at a peptide concentration of 1%. ^{1}H NMR spectroscopy

was obtained with a 200-MHz Bruker instrument and are recorded in δ units.

General Procedures for the Solid-Phase Synthesis of a and c Series Peptides. As an illustrative example the synthesis of [Nphe^1]NC(1−17)NH$_2$ (**1c**) is described. Fmoc-PAL-PEG-PS resin (0.21 mmol/g, 0.2 g) was treated with 20% piperine/DMF and linked with ***N***$^\alpha$-Fmoc-N^γ-tritylglutamine, via its *N*-hydroxybenzotriazole (HOBt) active ester formed in situ with DIPCDI. The following N^α-Fmoc amino acids were sequentially coupled to the growing peptide chain: N^α-Fmoc-N^γ-(Trt)-Asn, N^α-Fmoc-Ala, N^α-Fmoc-Leu, N^α-Fmoc-N^ϵ-(Boc)-Lys, N^α-Fmoc-N^ω-(Pmc)-Arg, N^α-Fmoc-Ala, N^α-Fmoc-*O*-(tBu)-Ser, N^α-Fmoc-N^ϵ-(Boc)-Lys, N^α-Fmoc-N^ω-(Pmc)-Arg, N^α-Fmoc-Ala, N^α-Fmoc-Gly, N^α-Fmoc-*O*(tBu)-Thr, N^α-Fmoc-Phe, N^α-Fmoc-Gly, N^α-Fmoc-Gly, and Boc-Nphe[54] in the final acylation step of the peptide synthesis. All the N^α-Fmoc amino acids or Boc-Nphe (4 equiv) were coupled to the growing peptide chain by using 1,3-diisopropylcarbodiimide (4 equiv) and 1-hydroxybenzotriazole (4 equiv) in DMF, and the coupling reaction time was 1 h. Piperidine (20%)/DMF 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 [Nphe^1]-NC(1−17)-NH$_2$-resin. The other peptides **1**−**8** in **a** and **c** series were synthesized in a similar manner. The protected peptide-resin was treated with reagent K[55] (TFA/H$_2$O/phenol/ethanedithiol/thioanisole, 82.5:5:5:2.5:5; v/v; 10 mL/0.2 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 ether. The crude peptide was purified by preparative reverse phase HPLC to yield a white powder after lyophilization.

General Procedures for the Synthesis of b Series Peptides. As an illustrative example the synthesis of [Phe$^1\Psi$(CH$_2$NH)Gly^2]NC(1−17)NH$_2$ (**1b**) is described. Fragment NC(2−17)-PAL-PEG-PS-resin (0.2 g, 0.21 mmol/g, 0.042 mmol) prepared as reported above, was swelled in methanol containing 1% (v/v) acetic acid (2 mL). After 20 min, a solution of Boc-Phe-CHO (0.016 g, 0.063 mmol) and NaBH$_3$CN (0.008 g, 0.13 mmol) dissolved in methanol (0.4 mL) was added and the reaction mixture stirred for 1 h. After this time, the resin was washed with methanol and treated with reagent K as for the synthesis of compound **1c**. The other peptides of b series were prepared in a similar manner.

Amino Aldehydes. The Boc-protected amino aldehydes were prepared as reported,[39] using WSC instead of 1,1′-carbonyldiimidazole. The Boc-amino acids (Boc-Leu-OH, Boc-Phe-OH, Boc-Cha-OH, Boc-Tyr(tBu)-OH, and Boc-(pMe)Phe-OH) (1 mmol) dissolved in DMF (5 mL) were reacted with *N*,*O*-dimethylhydroxylamine hydrochloride (1.5 mmol) in the presence of WSC (1.1 mmol), HOBt (1.1 mmol) and TEA (1.5 mmol). The reaction was stirred at room-temperature overnight, diluted with 0.5 N HCl (50 mL) and extracted with AcOEt (3 × 30 mL). The organic phase was washed with saturated aqueous NaHCO$_3$ (3 × 15 mL), brine (3 × 15 mL), dried over Na$_2$SO$_4$ and then filtered and concentrated in vacuo to yield the Boc-protected amino acid *N*,*O*-dimethylhydroxamate. The hydroxamate was then reduced to the corresponding aldehyde with lithium aluminum hydride according to the published procedure. The crude Boc-amino aldehyde was used immediately, without further purification, for the synthesis of compounds **1b**−**8b**. The analytical data of: Boc-Leu-CHO and Boc-Phe-CHO,[56] Boc-Cha-CHO,[39] and Boc-Tyr(tBu)-CHO[57] are in accordance with the literature.

Boc-(pMe)Phe-N(CH$_3$)-OCH$_3$: yield 93%; mp 94−96 °C; TLC R_f 0.43 (III); $[\alpha]^{20}_D$ 4.38; ^{1}H NMR (CDCl$_3$) δ 1.36 (s, 9H), 2.28 (s, 3H), 2.91 (dd, 2H, J = 14.5, 7.6 Hz), 3.12 (s, 3H), 3.67 (s, 3H), 4.72 (m, 1H), 5.12 (bs, 1H), 7.11 (s, 4H). Anal. (C$_{17}$H$_{26}$N$_2$O$_4$) C, H, N.

Boc-(pMe)Phe-CHO: yield 89%; mp 86−88 °C; TLC R_f 0.84 (III); $[\alpha]^{20}_D$ −28.4; ^{1}H NMR (CDCl$_3$) δ 1.43 (s, 9H), 2.32 (s, 3H), 3.09 (m, 2H), 4.42 (m, 1H), 5.03 (bs, 1H), 7.14 (s, 4H), 9.62 (s, 1H). Anal. (C$_{15}$H$_{21}$NO$_3$) C, H, N.

Boc-*N*-alkylglycines. The N-substituted glycine ethyl esters were prepared following the procedure of Skiles et al.[58] by treatment of known primary amines: isobutylamine and cyclohexylmethylamine with ethyl bromoacetate or alternatively by reductive alkylation of 4-hydroxybenzaldehyde or *p*-tolualdehyde with glycine ethyl esters in the presence of NaCNBH$_3$. The successive protection of the amino function with Boc[59] and the hydrolysis of ethyl ester gave the Boc-protected *N*-alkylglycine employed for the synthesis of compounds **1c**−**8c**.

Nleu-OEt. Ethyl bromoacetate (1.1 mL, 10 mmol) in THF (10 mL) was added dropwise to a chilled (0 °C) solution of isobutylamine (1 mL, 10 mmol) and Et$_3$N (1.4 mL, 10 mmol) in THF (30 mL). After the addition was complete the mixture was warmed to room temperature and then stirred overnight. The precipitate Et$_3$N·HBr was filtered and washed with a small amount of THF. The filtrate was concentrated under reduced pressure and the residue was purified by silica gel column chromatography with solvent system (IV). The product was an oil: yield 0.68 g (43%); TLC R_f (IV) 0.56; ^{1}H NMR (CDCl$_3$) δ 0.82 (d, 6H, J = 6.4 Hz), 1.27 (t, 3H, J = 7.1 Hz), 1.75 (m, 1H), 3.25 (m, 3H), 3.56 (m, 2H), 3.96 (q, 2H, J = 7.1 Hz).

Ncha-OEt. This compound was synthesized as described for Nleu-OEt. The product was an oil: yield 0.93 g (47%); TLC R_f (IV) 0.51; ^{1}H NMR (CDCl$_3$) δ 1.17 (m, 5H), 1.28 (m, 4H), 1.73 (m, 5H), 2.7 (bs, 1H), 2.96 (m, 2H), 3.7 (m, 2H), 4.15 (q, 2H, J = 6.8 Hz).

Ntyr-OEt. Glycine ethyl ester hydrochloride (1.8 g, 13 mmol) and 4-hydroxybenzaldehyde (1.2 g, 10 mmol) were dissolved in absolute EtOH (50 mL), and then NaCNBH$_3$ (1.38 g, 22 mmol) was added portionwise. The reaction was stirred at room temperature for 16 h. The EtOH was removed under reduced pressure and the residue dissolved in EtOAc. The organic layer was washed with NaHCO$_3$ (5%) and brine, dried, and evaporated to dryness. The residue was purified by silica gel column chromatography with solvent system (V). The product was an oil: yield 0.77 g (37%); TLC R_f (V) 0.53; ^{1}H NMR (CDCl$_3$) δ 1.17 (t, 3H, J = 6.9 Hz), 3.82 (m, 2H), 3.89 (m, 2H), 4.16 (q, 2H, J = 6.9 Hz), 5.27 (bs, 2H), 6.83 (d, 2H, J = 6.5 Hz), 7.15 (d, 2H, J = 6.5 Hz).

N(pMe)phe-OEt. This compound was synthesized as described for Ntyr-OEt. The product was an oil: yield 0.93 g (45%); TLC R_f (V) 0.72; ^{1}H NMR (CDCl$_3$) δ 1.15 (t, 3H, J = 6.9 Hz), 2.25 (s, 3H), 2.8 (bs, 1H), 3.66 (m, 2H), 3.88 (m, 2H), 4.14 (q, 2H J = 6.9 Hz), 7.14 (s, 4H).

Boc-Nleu-OEt: yield 87%; oil; TLC R_f 0.89 (VI); ^{1}H NMR (CDCl$_3$) δ 0.86 (d, 6H, J = 6.5 Hz), 1.27 (t, 3H, J = 6.9 Hz), 1.39 and 1.43 (s, 9H, Boc of two conformers), 1.8 (m, 1H), 3.1 (m, 2H), 3.86 and 3.95 (s, 2H, CH$_2$ of two conformers), 3.98 (q, 2H, J = 6.9 Hz).

Boc-Ncha-OEt: yield 84%; oil; TLC R_f 0.93 (VI); ^{1}H NMR (CDCl$_3$) δ 1.15 (m, 5H), 1.32 (m, 4H), 1.45 and 1.48 (s, 9H, Boc of two conformers), 1.79 (m, 5H), 3.15 (m, 2H), 3.92 and 3.96 (s, 2H, CH$_2$ of two conformers), 4.12 (q, 2H, J = 6.8 Hz).

Boc-Ntyr-OEt: yield 85%; oil; TLC R_f 0.81 (VI); ^{1}H NMR (CDCl$_3$) δ 1.22 (t, 3H, J = 7.2 Hz), 1.44 and 1.47 (s, 9H, Boc of two conformers), 3.86 and 3.89 (s, 2H, CH$_2$ of two conformers), 4.23 (m, 4H), 6.81 (m, 2H), 7.11 (m, 2H).

Boc-N(pMe)phe-OEt: yield 89%; oil; TLC R_f 0.91 (VI); ^{1}H NMR (CDCl$_3$) δ 1.23 (t, 3H J = 7.1 Hz), 1.46 (s, 9H), 2.28 (s, 3H), 3.83 and 3.92 (s, 2H, CH$_2$ of two conformers), 4.15 (q, 2H, J = 7.1 Hz), 4.44 and 4.51 (s, 2H, CH$_2$ of two conformers), 7.16 (s, 4H).

Boc-Nleu-OH: yield 93%; mp 71−73 °C; TLC R_f 0.33 (VI); ^{1}H NMR (CDCl$_3$) δ 0.89 (d, 6H, J = 6.5 Hz), 1.43 and 1.47 (s, 9H, Boc of two conformers), 1.8 (m, 1H), 3.08 (m, 2H), 3.90 and 3.97 (s, 2H CH$_2$ of two conformers). Anal. (C$_{11}$H$_{21}$NO$_4$) C, H, N.

Boc-Ncha-OH: yield 89%; mp 64−68; TLC R_f 0.37 (VI); ^{1}H NMR (CDCl$_3$) δ 1.15 (m, 2H), 1.37 (m, 4H), 1.42 and 1.46 (s, 9H, Boc of two conformers), 1.75 (m, 5H), 3.12 (m, 2H), 3.89 and 3.97 (s, 2H CH$_2$ of two conformers). Anal. (C$_{14}$H$_{25}$NO$_4$) C, H, N.

Boc-Ntyr-OH: yield 91%; mp 92−95 °C; TLC R_f 0.31 (VI); ^{1}H NMR (CDCl$_3$) δ 1.46 and 1.48 (s, 9H, Boc of two conform-

ers), 3.88 and 3.91 (s, 2H CH_2 of two conformers), 4.43 (m, 2H), 6.79 (m, 2H), 7.13 (m, 2H). Anal. ($C_{14}H_{19}NO_5$) C, H, N.

Boc-N(pMe)phe-OH: yield 86%; mp 84−86 °C; TLC R_f 0.4 (VI); ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9H), 2.33 (s, 3H), 3.80 and 3.94 (s, 2H CH_2 of two conformers), 4.47 and 4.50 (s, 2H CH_2 of two conformers), 7.13 (s, 4H). Anal. ($C_{15}H_{21}NO_4$) C, H, N.

mVD studies. Swiss male mice weighing 25−30 g were used. The bioassay experiments were performed as previously described.[27] Briefly, the vas deferens (mVD) was prepared according to Hughes et al.[60] and suspended in 10-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 0.1-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 on a Linseis multichannel chart recorder (model 2005). After an equilibration period of about 2 h the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration−response curves for NC and NC-related peptides were performed (0.5 log unit steps). When required, antagonists (10 μM) were added to the Krebs solution 15 min before performing concentration−response curves for agonists. pK_B values were calculated by mean of the Gaddum−Schild equation: pK_B = −log((concentration ratio − 1)/[antagonist]), assuming a slope equal to 1.

Binding Studies on Mouse Brain Membranes. Swiss male mice weighing 25−30 g were used. The binding experiments were performed as previously described.[41] Briefly, the mice were decapitated and the forebrain was dissected on ice. The tissue was disrupted in a Polytron homogenizer (setting 5) in 20 volumes of 50 mM Tris HCl, 2 mM EDTA, 100 μM phenylmethanesulfonyl fluoride (PMSF) at pH 7.4. The homogenate was centrifuged at 40000*g* for 10 min and the pellet was resuspended in the same buffer. After 30 min of incubation at 37 °C, the membranes were centrifuged and the resulting pellets were stored at −80 °C. Prior to freezing, an aliquot of the homogenate was removed for protein assay according to a Bio Rad method,[61] using bovine albumin as reference standard. The final pellet was resuspended in the same incubation buffer at a concentration of 200 μg protein/100 μL and this homogenate was used for the binding assay. Inhibition experiments were carried out in duplicate in a final volume of 250 μL in test tubes containing 0.5 nM [^{3}H]$NCNH_2$, 50 mM Tris HCl buffer, 2 mM EDTA, 100 μM PMSF at pH 7.4, mouse forebrain membranes (200 μg protein/assay), and different concentrations of the ligand under study. Details about the synthesis of the radioligand [^{3}H]$NCNH_2$ have been already reported.[41] Moreover, the inhibitory binding constant, K_i, was calculated from the IC_{50} value according to the Cheng−Prusoff equation.[62] Nonspecific binding was defined as the binding observed in the presence of 10 μM NC(1−17)NH_2 (compound **1a**) and was about 30% of the total binding. Incubation time was 120 min at 25 °C, based on the results of previous time-course experiments.[41] Bound and free radioactivities were separated by filtering the assay mixture through Whatman GF/C glass-fiber filters using a Brandel cell harvester. The incubation mixture was diluted with 3 mL of ice-cold incubation buffer and then vacuum-filtered rapidly and the filters were washed three times with 3 mL of incubation buffer. The filter-bound radioactivity was counted in a Beckman LS-1800 spectrometer (efficiency 55%). Under the experimental conditions here described we demonstrated that the mouse forebrain membranes express a single class of binding sites for [^{3}H]$NCNH_2$ with a K_d value of 0.55 nM and a B_{max} value of about 100 fmol/mg protein. Moreover the pharmacological profile of such a site was superimposable to that of the NC receptor expressed in the mVD.[41]

Inhibition of cAMP Accumulation in CHO Cells. cAMP accumulation was measured in whole CHO_{NCR} cells incubated in 0.3-mL volumes of Krebs-HEPES buffer containing BSA as described in detail by Okawa et al.[45] In addition, 1-isobutyl-4-methylxanthine (1 mM) and forskolin (1 μM) were also included. Concentration−response curves to NC-related peptides were performed, the maximum concentration tested was 10 μM. All incubations were for 15 min at 37 °C. In antagonist studies the effects were tested against compound **2a** (the reference agonist). cAMP was extracted and assayed as previously described.[45] pK_B values were calculated by mean of the Gaddum−Schild equation: pK_B = −log((concentration ratio − 1)/[antagonist]), assuming a slope equal to 1.

Data Analysis and Terminology. The data are expressed as mean of *n* experiments. For pEC_{50}, pK_B, and pK_i values the confidence limits at 95% ($CL_{95\%}$) are given. A weighted nonlinear least-squares curve-fitting program LIGAND[63] was used for computer analysis of binding inhibition experiments. The pharmacological terminology adopted in this study is in line with the recent IUPHAR recommendations:[64] the agonist apparent affinities 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; apparent affinities of antagonists are given in terms of pK_B which were calculated using the Gaddum−Schild equation: pK_B = log((CR − 1)/[antagonist]), assuming a slope value equal to unity, where CR is the ratio between equieffective concentrations of agonist in the presence and absence of the antagonist. The ligand affinities obtained in binding competition experiments are given as pK_i = the negative logarithm to base 10 of the inhibition equilibrium constant.

Acknowledgment. This work was supported by the Italian Ministry of the University (Cofin 99 Grant) by the University of Ferrara (60% grants), by the Consiglio Nazionale delle Ricerche, and by Prof. G. Smith (Leicester, U.K.) funds. We would like to acknowledge the kind assistance of C. Harrison in performing some of the experiments on CHO_{NCR}.

References

(1) Abbreviations follow the IUPAC−IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides: *J. Biol. Chem.* **1985**, *260*, 1442. Additional abbreviations used herein are as follows: Boc (*tert*-butoxycarbonyl), DIPCDI (1,3-diisopropylcarbodiimide), Fmoc ((9-fluorenylmethyl)oxycarbonyl), Fmoc-PAL-PEG-PS (5-(4′-Fmoc-aminomethyl-3′,5′-dimethoxyphenoxy)-valeric acid on the poly(ethylene glycol)/polystyrene support), EtOAc (ethyl acetate), HOBt (1-hydroxybenzotriazole), MALDI-TOF (matrix assisted laser desorption ionization time-of-flight), Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl), tBu (*tert*-butyl), TEA (triethylamine), TFA (trifluoroacetic acid), THF (terahydrofuran), Trt (trityl), WSC (1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide·HCl).

(2) Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monserrat, B.; Mazarguil, H.; Vassart, G.; Parmentier, M.; Costentin, J. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **1995**, *377*, 532−535.

(3) Reinscheid, R. K.; Nothacker, H. P.; Bourson, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr.; Civelli, O. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* **1995**, *270*, 792−794.

(4) Connor, M.; Vaughan, C. W.; Chieng, B.; Christie, M. J. Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro. *Br. J. Pharmacol.* **1996**, *119*, 1614−1618.

(5) Connor, M.; Yeo, A.; Henderson, G. The effect of nociceptin on Ca2+ channel current and intracellular Ca2+ in the SH−SY5Y human neuroblastoma cell line. *Br. J. Pharmacol.* **1996**, *118*, 205−207.

(6) Meunier, J. C. Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. *Eur. J. Pharmacol.* **1997**, *340*, 1−15.

(7) Civelli, O.; Nothacker, H. P.; Reinscheid, R. Reverse physiology: discovery of the novel neuropeptide, orphanin FQ/nociceptin. *Crit. Rev. Neurobiol.* **1998**, *12*, 163−176.

(8) Murphy, N. P.; Ly, H. T.; Maidment, N. T. Intracerebroventricular orphanin FQ/nociceptin suppresses dopamine release in the nucleus accumbens of anaesthetized rats. *Neuroscience* **1996**, *75*, 1−4.

(9) Nicol, B.; Lambert, D. G.; Rowbotham, D. J.; Smart, D.; McKnight, A. T. Nociceptin induced inhibition of K+ evoked glutamate release from rat cerebrocortical slices. *Br. J. Pharmacol.* **1996**, *119*, 1081−1083.

(10) Neal, M. J.; Cunningham, J. R.; Paterson, S. J.; McKnight, A. T. Inhibition by nociceptin of the light-evoked release of ACh from retinal cholinergic neurones. *Br. J. Pharmacol.* **1997**, *120*, 1399−1400.
(11) Schlicker, E.; Werthwein, S.; Kathmann, M.; Bauer, U. Nociceptin inhibits noradrenaline release in the mouse brain cortex via presynaptic ORL1 receptors. *Naunyn-Schmiedeberg's Arch Pharmacol.* **1998**, *358*, 418−422.
(12) Siniscalchi, A.; Rodi, D.; Beani, L.; Bianchi, C. Inhibitory effect of nociceptin on [3H]5-HT release from the rat cerebral cortex slices. *Br. J. Pharmacol.* **1999**, *128*, 119−123.
(13) Sbrenna, S.; Marti, M.; Morari, M.; Calo′, G.; Guerrini, R.; Beani, L.; Bianchi, C. 5-HT efflux from rat cerebrocortical synaptosomes: modulation by opioids and nociceptin. *Br. J. Pharmacol.* **2000**, *130*, 425−433.
(14) Nicholson, J. R.; Paterson, S. J.; Menzies, J. R.; Corbett, A. D.; McKnight, A. T. Pharmacological studies on the "orphan" opioid receptor in central and peripheral sites. *Can. J. Physiol. Pharmacol.* **1998**, *76*, 304−313.
(15) Berzetei-Gurske, I. P.; Schwartz, R. W.; Toll, L. Determination of activity for nociceptin in the mouse vas deferens. *Eur. J. Pharmacol.* **1996**, *302*, R1−R2.
(16) Calo, G.; Rizzi, A.; Bogoni, G.; Neugebauer, V.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. The mouse vas deferens: a pharmacological preparation sensitive to nociceptin. *Eur. J. Pharmacol.* **1996**, *311*, R3−R5.
(17) Patel, H. J.; Giembycz, M. A.; Spicuzza, L.; Barnes, P. J.; Belvisi, M. G. Naloxone-insensitive inhibition of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by the novel opioid, nociceptin. *Br. J. Pharmacol.* **1997**, *120*, 735−736.
(18) Rizzi, A.; Calo, G.; Trevisani, M.; Tognetto, M.; Fabbri, L.; Mapp, C.; Guerrini, R.; Salvadori, S.; Regoli, D.; Geppetti, P. Nociceptin receptor activation inhibits tachykinergic non adrenergic non cholinergic contraction of guinea pig isolated bronchus. *Life Sci.* **1999**, *64*, L157−L163.
(19) Giuliani, S.; Maggi, C. A. Inhibition of tachykinin release from peripheral endings of sensory nerves by nociceptin, a novel opioid peptide. *Br. J. Pharmacol.* **1996**, *118*, 1567−1569.
(20) Giuliani, S.; Maggi, C. A. Prejunctional modulation by nociceptin of nerve-mediated inotropic responses in guinea-pig left atrium. *Eur. J. Pharmacol.* **1997**, *332*, 231−236.
(21) Helyes, Z.; Nemeth, J.; Pinter, E.; Szolcsanyi, J. Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals. *Br. J. Pharmacol.* **1997**, *121*, 613−615.
(22) Madeddu, P.; Salis, M. B.; Milia, A. F.; Emanueli, C.; Guerrini, R.; Regoli, D.; Calo, G. Cardiovascular effects of nociceptin in unanesthetized mice. *Hypertension* **1999**, *33*, 914−919.
(23) Giuliani, S.; Tramontana, M.; Lecci, A.; Maggi, C. A. Effect of nociceptin on heart rate and blood pressure in anaesthetized rats. *Eur. J. Pharmacol.* **1997**, *333*, 177−179.
(24) Champion, H. C.; Czapla, M. A.; Kadowitz, P. J. Nociceptin, an endogenous ligand for the ORL1 receptor, decreases cardiac output and total peripheral resistance in the rat. *Peptides* **1997**, *18*, 729−732.
(25) Kapusta, D. R.; Sezen, S. F.; Chang, J. K.; Lippton, H.; Kenigs, V. A. Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ). *Life Sci.* **1997**, *60*, PL15−PL21.
(26) Calo, G.; Rizzi, A.; Bodin, M.; Neugebauer, W.; Salvadori, S.; Guerrini, R.; Bianchi, C.; Regoli, D. Pharmacological characterization of nociceptin receptor: an in vitro study. *Can. J. Physiol. Pharmacol.* **1997**, *75*, 713−718.
(27) Guerrini, R.; Calo, G.; Rizzi, A.; Bianchi, C.; Lazarus, L. H.; Salvadori, S.; Temussi, P. A.; Regoli, D. Address and message sequences for the nociceptin receptor: a structure-activity study of nociceptin-(1−13)-peptide amide. *J. Med. Chem.* **1997**, *40*, 1789−1793.
(28) Calo, G.; Guerrini, R.; Bigoni, R.; Rizzi, A.; Bianchi, C.; Regoli, D.; Salvadori, S. Structure−Activity Study of the Nociceptin-(1−13)-NH2 N-Terminal Tetrapeptide and Discovery of a Nociceptin Receptor Antagonist. *J. Med. Chem.* **1998**, *41*, 3360−3366.
(29) Guerrini, R.; Calo, G.; Rizzi, A.; Bigoni, R.; Bianchi, C.; Salvadori, S.; Regoli, D. A new selective antagonist of the nociceptin receptor. *Br. J. Pharmacol.* **1998**, *123*, 163−165.
(30) Shah, S.; Page, C. P.; Spina, D. Nociceptin inhibits nonadrenergic noncholinergic contraction in guinea-pig airway. *Br. J. Pharmacol.* **1998**, *125*, 510−516.
(31) Nicholson, J. R.; Mason, S. L.; Lee, K.; McKnight, A. T. The effect of the agonist Ac-RYYRWKNH2 and the antagonist Phepsi(CH2-NH)Gly2]nociceptin(1−13)NH2 at the ORL1 receptor of central and peripheral sites. 29th International Narcotics Research Conference, 1998.
(32) Bigoni, R.; Giuliani, S.; Calo, G.; Rizzi, A.; Guerrini, R.; Salvadori, S.; Regoli, D.; Maggi, C. A. Characterization of nociceptin receptors in the periphery: in vitro and in vivo studies. *Naunyn-Schmiedebergs Arch. Pharmacol.* **1999**, *359*, 160−167.
(33) Butour, J. L.; Moisand, C.; Mollereau, C.; Meunier, J. C. [Phe1psi(CH2-NH)Gly2]nociceptin-(1−13)-NH2 is an agonist of the nociceptin (ORL1) receptor. *Eur. J. Pharmacol.* **1998**, *349*, R5−R6.
(34) Calo, G.; Rizzi, A.; Marzola, G.; Guerrini, R.; Salvadori, S.; Beani, L.; Regoli, D.; Bianchi, C. Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. *Br. J. Pharmacol.* **1998**, *125*, 373−378.
(35) Xu, I. S.; Wiesenfeld-Hallin, Z.; Xu, X. J. [Phe1psi(CH2-NH)-Gly2]-nociceptin-(1−13)NH2, a proposed antagonist of the nociceptin receptor, is a potent and stable agonist in the rat spinal cord. *Neurosci. Lett.* **1998**, *249*, 127−130.
(36) Grisel, J. E.; Farrier, D. E.; Wilson, S. G.; Mogil, J. S. [Phe1psi-(CH2-NH)Gly2]nociceptin-(1−13)-NH2 acts as an agonist of the orphanin FQ/nociceptin receptor in vivo. *Eur. J. Pharmacol.* **1998**, *357*, R1−R3.
(37) Carpenter, K. J.; Dickenson, A. H. Evidence that (Phe1psi-(CH2NH)Gly2)nociceptin(1−13)NH2, a peripheral ORL-1 receptor antagonist, acts as an agonist in the rat spinal cord. *Br. J. Pharmacol.* **1998**, *125*, 949−951.
(38) Calo′, G.; Guerrini, R.; Rizzi, A.; Salvadori, S.; Regoli, D. Pharmacology of nociceptin and its receptor − A novel therapeutic target. *Br. J. Pharmacol.* **2000**, *129*, 1261−1283.
(39) Patel, D. V.; Rielly-Gauvin, K.; Ryono, D. E.; Free, C. A.; Smith, S. A.; Petrillo, E. W. Activated ketone based inhibitors of human renin. *J. Med. Chem.* **1993**, *36*, 2431−2447.
(40) Ho, P. T.; Chang, D.; Zhong, J. W.; Musso, G. F. An improved low racemization solid-phase method for the synthesis of reduced dipeptides (ψCH_2NH) bond isosteres. *Pept. Res.* **1993**, *6*, 10−12.
(41) Varani, K.; Calo, G.; Rizzi, A.; Merighi, S.; Toth, G.; Guerrini, R.; Salvadori, S.; Borea, P. A.; Regoli, D. Nociceptin receptor binding in mouse forebrain membranes: thermodynamic characteristics and structure activity relationships. *Br. J. Pharmacol.* **1998**, *125*, 1485−1490.
(42) Varani, K.; Rizzi, A.; Calo, G.; Bigoni, R.; Toth, G.; Guerrini, R.; Gessi, S.; Salvadori, S.; Borea, P. A.; Regoli, D. Pharmacology of [Tyr1]nociceptin analogues: receptor binding and bioassay studies. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1999**, *360*, 270−277.
(43) Dooley. C. T.; Houghten, R. A. Orphanin FQ: receptor binding and analogue structure activity relationships in rat brain. *Life Sci.* **1996**, *59*, PL23−PL29.
(44) Reinscheid, R. K.; Ardati, A.; Monsma, F. J., Jr.; Civelli, O. Structure−activity relationship studies on the novel neuropeptide orphanin FQ. *J. Biol. Chem.* **1996**, *271*, 14163−14168.
(45) Okawa, H.; Nicol, B.; Bigoni, R.; Hirst, R. A.; Calo, G.; Guerrini, R.; Rowbotham, D. J.; Smart, D.; McKnight, A. T.; Lambert, D. G. Comparison of the effects of [Phe1psi(CH2-NH)Gly2]Nociceptin (1−13)NH2 in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin receptors. *Br. J. Pharmacol.* **1999**, *127*, 123−130.
(46) Wnendt, S.; Kruger, T.; Janocha, E.; Hildebrandt, D.; Englberger, W. Agonistic effect of buprenorphine in a nociceptin/OFQ receptor-triggered reporter gene assay. *Mol. Pharmacol.* **1999**, *56*, 334−338.
(47) Toll, L.; Burnside, J.; Berzetei-Gurske, I. Agonist activity of ORL1 antagonists is dependent upon receptor number. International Narcotics Research Conference, 1998; A89.
(48) Rizzi, A.; Bigoni, R.; Calo′, G.; Guerrini, R.; Salvadori, S.; Regoli D. [Nphe1]nociceptin(1−13)NH2 antagonizes nociceptin effects in the mouse colon. *Eur. J. Pharmacol.* **1999**, *385*, R3−R5.
(49) Calo, G.; Guerrini, R.; Bigoni, R.; Rizzi, A.; Marzola, G.; Okawa, H.; Bianchi, C.; Lambert, D. G.; Salvadori, S.; Regoli, D. Characterization of [Nphe1]NC(1−13)NH2, a new selective nociceptin receptor antagonist. *Br. J. Pharmacol.* **2000**, *129*, 1183−1193.
(50) Polidori, C.; Calo, G.; Ciccocioppo, R.; Guerrini, R.; Regoli, D.; Massi, M. Pharmacological characterization of the nociceptin receptor mediating hyperphagia: identification of a selective antagonist. *Psychopharmacology* **2000**, in press.
(51) Regoli, D.; Gobeil, F.; Nguyen, Q. T.; Jukic, D.; Seoane, P. R.; Salvino, J. M.; Sawutz, D. G. Bradykinins receptor types and B2 subtypes. *Life Sci.* **1994**, *55*, 735−749.
(52) Regoli, D.; Boudon, A.; Fauchere, J. L. Receptors and antagonists for substance P and related peptides. *Pharmacol. Rev.* **1994**, *46*, 551−599.
(53) Knapp, R. J.; Malatynska, E.; Collins, N.; Fang, L.; Wang, J. Y.; Hruby, V. J.; Roeske, W. R.; Yamamura, H. I. Molecular biology and pharmacology of cloned opioid receptors. *FASEB J.* **1995**, *9*, 516−525.
(54) Mouna, A. M.; Nguyen, C.; Rage, I.; Xie, J.; Née, G.; Mazaleyrat, J. P.; Wakselman, M. Preparation of N-Boc N-Alkyl Glycines for peptoid synthesis. *Synth. Commun.* **1994**, *24*, 2429−2435.
(55) King, D. S.; Fields, C. G.; Fields, G. B. A cleavage method which minimizes side reactions following Fmoc solid-phase peptide synthesis. *J. Pept. Protein Res.* **1990**, *36*, 255−266.

(56) Fehrentz, J. A.; Castro, B. An efficient synthesis of optically active α-(*tert*-butoxycarbonylamino)-aldehydes from α-amino acids. *Synthesis* **1983**, 676−678.
(57) Hann, M. M.; Sammes, P. G.; Kennewell, P. D.; Taylor, J. B. On the double isostere of the peptide bond: preparation of an enkephalin analogue. *J. Chem. Soc. Perkin Trans. I* **1982**, 307−314.
(58) Skiles, J. W.; Fuchs, V.; Miao, C.; Sorcek, R.; Grozinger, K. G.; Mauldin, S. C.; Vitous, J.; Mui, P. W.; Jacober, S.; Chow, G.; Matteo, M.; Skoog, M.; Weldon, S. W.; Possanza, G.; Keirns, J.; Letts, G.; Rosenthal, A. S. Inhibition of human leukocyte elastase (HLE) by N-substitued peptidyl trifluoromethyl ketones. *J. Med. Chem.* **1992**, *35*, 641−662.
(59) Moroder, L.; Hallett, A.; Wunsch, E.; Keller, O.; Wersin, G. Di-*tert*-butyldicarbonat-ein vorteilhaftes Reagenz zur Einfuhurung der-*tert*-butyloxycarbonyl-Schutzgruppe. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651−1653.
(60) Hughes, J.; Kosterlitz, H. W.; Leslie, F. M. Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmacol.* **1975**, *53*, 371−381.
(61) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(62) Cheng, Y. C.; Prusoff, W. H. Relationships between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC50) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.
(63) Munson, P. J.; Rodbard, D. Ligand: a versatile computerized approach for the characterization of ligand binding systems. *Anal. Biochem.* **1980**, *107*, 220−239.
(64) Jenkinson, D. H.; Barnard, E. A.; Hoyer, D.; Humphrey, P. P. A.; Leff, P.; Shankley, N. P. International Union of Pharmacology Commitee on receptor nomenclature and drug classification XI Recommendations on terms and symbols in quantitative pharmacology. *Pharmacol. Rev.* **1995**, *47*, 255−266.
(65) Hashimoto, Y.; Calo′, G.; Guerrini, R.; Smith, G.; Lambert, D. G. Antagonistic effects of [Nphe1]nociceptin(1−13)NH2 on nociceptin receptor mediated inhibition of cAMP formation in Chinese ovary cells stably expressing the recombinant human nociceptin receptor. *Neurosci. Lett.* **2000**, *278*, 109−112.

JM990075H