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Structure–activity studies on nociceptin/orphanin FQ: from full agonist, to partial agonist, to pure antagonist

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Abstract

A heptadecapeptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) was identified from rat brain and from porcine brain as a ligand for OP_4, a new G-protein coupled receptor that is similar in sequence to opioid receptors. The OP_4 receptor is widely expressed in the nervous system where it mediates a broad range of physiological functions. The new peptide, nociceptin (NC), has a primary sequence recalling that of opioid peptides. Despite the homologies (a) of the OP_4 receptor with known opioid receptors, especially the OP_2 (κ) receptor, and (b) of NC with opioid peptides, particularly dynorphin A, the two biological systems have different anatomical locations and chemical requirements for activation. NC does not bind to opioid receptors, and mammalian opioid peptides do not interact with the OP_4 receptor. The presence of Phe in position 1 and Arg in position 8, appear to be instrumental to exclude NC from interacting with the opioid receptors. Contrary to opioid peptides which strikly require Tyr in position 1, the active core that activates the OP_4 appears to be towards the centre of the peptide molecule and includes Phe^4. Based on the message/address model, several changes have been made in the *N*-terminal tetrapeptide Phe-Gly-Gly-Phe (message) and a few also in the *C*-terminal of the template NC(1–13)–NH_2, a fragment that acts as a full agonist both in vitro and in vivo. Subtle changes of the *N*-terminal sequence, especially at Phe^1, led to the discovery of peptide antagonists ([$Phe^1\Psi(CH_2–NH)Gly^2$]–NC(1–13)–NH_2 and [$Nphe^1$]–NC(1–13)–NH_2). The first compound has been widely used to characterize NC actions in the periphery and in the central nervous system. It has been shown to act mainly as an antagonist outside the brain and as an agonist in the central nervous system. [$Nphe^1$]–NC(1–13)–NH_2 on the contrary, acts as antagonist both in the periphery and in the brain. These first peptide prototypes may soon be followed by non-peptide compounds, some of which, are already described in patent literature.

Keywords: Nociceptin; Orphanin FQ; Nociceptin analogs; OP_4, Bioassay, mouse vas deferens; Receptor binding

1. Introduction

Several groups of investigators have described a cDNA, from different species, encoding a protein with a primary sequence comparable to that of the opioid receptors [2–9]. This new opioid like receptor (ORL_1), recently named OP_4 in line with the proposal by Hamon et al. [1][1], is a G protein coupled receptor that shares a high degree of homology, especially in the transmembrane domains, with the cloned OP_3 (μ), OP_1 (δ) and OP_2 (κ) receptors. However native opioid peptides and synthetic ligands for OP_1, OP_3 or OP_2 receptors, were found to be unable to bind to this 'orphan receptor' [2–4,7–9].

In 1995, two research groups [10,11] identified a novel peptide neurotransmitter whose structure shows

Abbreviations: Cha, 3-cyclohexyl-L-alanine; Dmt, 2′,6′-dimethyl-L-tyrosine; NalBzoH, naloxone benzoylhyrazone; Mc 2266, (−)-(1R,5R,9R)-5,9-diethyl-2-(3-furylmethyl)-2′-hydroxy-6,7-benzomorphan; Mr 2267, enantiomer of Mr 2266; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Vra, 5-aminovareric acid.

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[1] Nociceptin–orphanin FQ (NC/OFQ): in this review the peptide is indicated as NC. Opioid receptor like 1 (ORL_1): the nociceptin receptor is indicated as OP_4, in accord with the proposal by Hamon [1]. The nociceptin/nociceptin receptor system is indicated as NC/OP_4.

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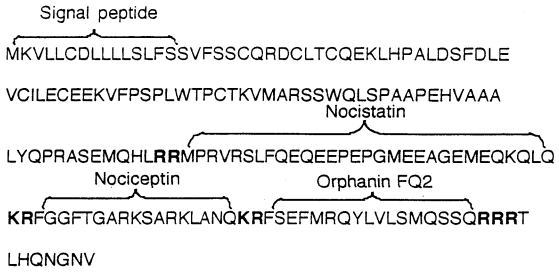


Fig. 1. Sequence of human nociceptin precursor. Putative proteolytic cleavage motifs are shown in bold.

similarities with those of enkephalins, endorphins and dynorphins, three members of the heterogeneous opioid peptide system.

The new ligand was named 'nociceptin' (NC) because of its ability to reduce threshold to painful stimuli [11], or 'orphanin FQ' (OFQ) because it is the natural ligand of the orphan receptor and has Phe (F) at the *N*- and Gln (Q) at the *C*-terminal end [10].

OP_4 receptor localization performed in the rat brain with NC-stimulated [^{35}S]GTPγS binding, indicated the existence of a high density of receptor in the cortex, hippocampus and hypothalamus with a specific anatomical distribution substantially different from those of the opioid receptors [12,13]. The OP_4 receptor protein has also been shown to be present outside the central nervous system, for instance in the rat intestine, the skeletal muscle, the vas deferens, and the spleen [4], as well as in some cells of the immune system [3,14,15], where the mRNA of OP_4 has been demonstrated.

The distribution of OP_4 transcripts in the brain and the spinal cord as well as the results of numerous functional assays suggest that this receptor may play a role in pain and analgesia (see for reviews, [16–18], in locomotion [10], cognitive processes and memory [19,20], feeding behavior [21,22], and neuroendocrine secretions [23,24]. In the periphery, the interaction of NC with OP_4 leads to inhibition of the release of neurotransmitter from the sympathetic [25], parasympathetic [26,27] and sensory nerves [28–31]. NC was also reported to induce diuresis and antinatriuresis [32], bradycardia and hypotension [33] and to inhibit the micturition reflex [34].

2. The NC precursor

Nociceptin is a neuropeptide of 17 amino acids derived from a larger precursor, prepronociceptin, whose gene has been isolated from various species and found to be highly conserved [35,36]. This precursor contains other biologically active peptides, such as nocistatin which has been shown to functionally antagonize some actions of NC [37], and orphanin FQ2 [38] which has been reported to be a relevant neuropeptide with important physiological actions (Fig. 1).

The polypeptide precursor gene is organized in a similar manner as the genes encoding opioid peptide precursors such as prepro-enkephalins, prepro-opiomelanocortin and prepro-dynorphins [35,36]. Nociceptin shows sequence similarity with opioid peptides and in particular with dynorphin A, the physiological ligand of the OP_2 opioid receptor. The message domain is probably coincident with the sequence of the four *N*-terminal residues (Phe-Gly-Gly-Phe) with the marked difference for the *N*-terminal amino acid, which is Phe instead of Tyr, which notoriously is the essential component of ligands for all opioid receptors [39]. The highly basic *C*-terminal address domain of NC differs from that of dynorphin mainly in a detailed distribution of the basic residues. In this regard, it is worthy of mention that the negatively charged second extracellular loop, EL2, of the OP_4 and of the OP_2 receptor has been associated with selectivity for endogeneous NC and dynorphin A [40–42]; these two peptides at physiological pH have, respectively, four or five positively charged residues in the address domain (Fig. 2).

3. Pharmacological characterization of the OP_4 receptor

The OP_4 receptor belongs to the family of G-protein coupled receptors which are characterized by seven transmembrane spanning domains and shares sequence identity of almost 60% with OP_3, OP_2 and OP_1 receptors [17]. In order to probe its functional structure, a molecular model of the receptor has been built, comprehensive of the TM domains and the extra- and intracellular loops; its second extracellular loop (EL2) is rich in acidic residues, and is very similar to that of the OP_2 receptor [8,43].

NC	F-G-G-F-T-G-A-**R**-**K**-S-A-**R**-**K**-L-A-N-Q
Dynorphin A	Y-G-G-F-L-**R**-**R**-I-**R**-P-**K**-L-**K**-W-D-N-Q
β-Endorphin	Y-G-G-F-M-T-S-E-**K**-S-Q-T-P-L-V-T-L-F-**K**-N-A-I-I-**K**-N-A-Y-**K**-**K**-G-E
[Leu^5]-enkephalin	Y-G-G-F-L
[Met^5]-enkephalin	Y-G-G-F-M

Fig. 2. Structural comparison of nociceptin and mammalian opioid peptide ligands. Basic amino acids are indicated in bold.

Table 1
Binding affinities and pharmacological potencies of nociceptin [a]

	Receptor binding, (pK_i)		Functional test (pEC_{50})	
	CHO_{hOP4}	Mouse brain	CHO_{hOP_4}	mVD
NC	9.9	8.7	9.1	7.8

[a] Binding and functional data on CHO_{hOP_4} are from [45], data on mouse brain and vas deferens are from [49] and [70], respectively. pEC_{50} is the negative logarithm to base ten of the molar concentration of agonist that produces 50% of the maximal effect. pK_i is the negative logarithm to base ten of the inhibitory binding constant, K_i.

Synthetic peptides in radiolabelled form, [^{125}I]–[Tyr^{14}]–NC [10] and [^{3}H]–NC [44], have been extensively used to analyze the interaction of different ligands with the OP_4 receptor. Binding assays were performed in membrane preparations derived from either CHO [45,46] or HEK293 [47] cells, expressing the OP_4 protein, or in homogenates from rat [44,46], mouse [48,49] and guinea pig [50] brains.

OP_4 functions were investigated also in peripheral tissues; thus, NC was found to be inactive both as stimulant and as inhibitor of smooth muscle tone in several preparations [27], whereas it inhibited the contractions induced by electrical field stimulation in the mouse vas deferens (mVD) [25,51], the guinea pig ileum (gpI) [27], renal pelvis [29], and bronchus [28,52]. The mVD and gpI contain OP_4 and classical opioid receptors: OP_1 in the mVD [53] and OP_3 in the gpI [54]. NC showed approximately the same potency in the two preparations, being slightly more potent in the guinea pig ileum. The inhibitory effect exerted by NC in the two preparations was not affected by naloxone or by some more selective opioid receptor antagonists [27].

The pharmacological profile of the NC/OP_4 system is presented in Table 1, by showing: (a) the binding affinities of NC to membranes of CHO cells transfected with the human recombinant OP_4 receptor, (b) the affinities of NC for the specific OP_4 sites that are present in the mouse brain homogenate, and (c) the potencies of NC as inhibitor of the forskolin induced cAMP accumulation in CHO_{hOP_4} and of the contractions induced by electrical stimulation in the mVD. The affinity of NC for the sites expressed in the transfected system is extremely high (100–200 pM); that for the native site of the mouse brain is approximately tenfold lower (2 nM) and the potency that have been estimated from the biological activities are even lower (by approximately 100-fold) (10–20 nM). Such differences are frequently observed in peptide pharmacology (e.g. kinins [55], neurokinins [56]) and have been attributed to different receptor accessibility.

Despite its structural similarity to opioid peptide ligands, the authors of the initial reports indicated that, when injected intracerebroventricularly in the mouse, NC exerts a direct hyperalgesic effect [10,17]. Additional work has now confirmed that NC has a potent hyperalgesic activity at supraspinal level, while at spinal level it produces analgesia (see [18], for a review). In addition, NC was found to be able to completely block supraspinal antinociception produced either by morphine [57] or selective opioid receptor agonists [58]. Data of functional assays were validated by the results at cellular level where OP_4 activation, via Gi/Go-proteins, inhibits forskolin-induced accumulation of cAMP in cells expressing the OP_4 receptor, see for reviews [16,17]. Activation of K^+ conductance [59] and inhibition of Ca^{2+} entry through voltage sensitive Ca^{2+} channels [60] have also been reported as cellular mechanisms of NC actions.

4. Metabolism of nociceptin

Degradation of NC has been studied in vitro, in mouse brain cortical slices [61] and in freshly drawn human blood [62]. In the first report, degradation was measured in the presence or in absence of peptidase inhibitors: it was shown that the critical sites of enzymatic cleavages are Phe^1–Gly^2, Ala^7–Arg^8, Ala^{11}–Arg^{12} and Arg^{12}–Lys^{13} bonds. Aminopeptidase N and endopeptidase 24.15 are the two most important enzymes involved in the metabolism of NC. This was confirmed by the potentiation of the behavioral effects mediated by NC observed in the mouse in the presence of an inhibitor of aminopeptidase N (bestatin) and the inhibitor of endopeptidase 24.15, ($Z-_{(L,D)}$PheΨ(PO_2-CH_2)$_{(L,D)}$ Ala-Arg-Phe) [63]. In human blood, it was found that cleavage of the peptide linkage Phe^1–Gly^2 was the predominant biotransformation pathway; cleavage at basic amino acid residues were also observed although not as major sites of breakdown [62]. From these studies it appears that NC is more resistant to biotransformation by human blood, in vitro, than dynorphin A. Recently, in vivo metabolism of NC in rat hippocampus has been reported [64]; it has been shown that the pathway of degradation does not involve aminopeptidase(s), but only endopeptidase(s), at sites that are preceded by paired basic residues (see Fig. 3). These findings were confirmed using several cell lines in cultures [65].

5. Activities and potencies of nociceptin and truncated sequences

The receptor affinities (mouse brain) and pharmacological activities (mVD) of NC and some truncated analogs are reported in Table 2.

Firstly, the NC amide (NC–NH_2) has been shown to have the same pharmacological activity as the naturally

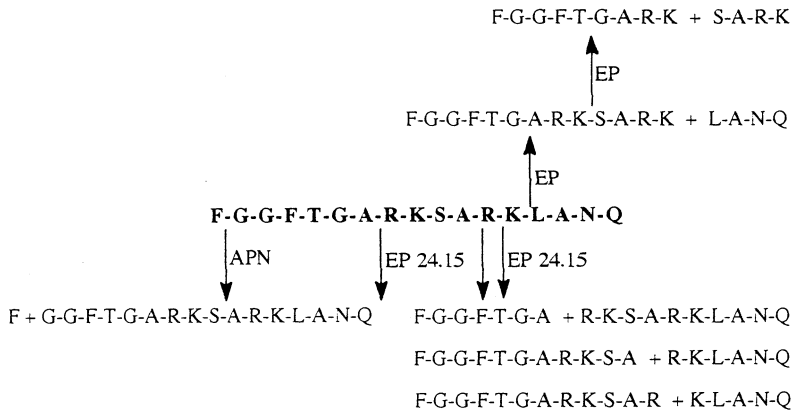


Fig. 3. Principal in vitro and in vivo cleavage site(s) of nociceptin. Fragments resulting from proteolytic hydrolysis are indicated. Proteolytic enzymes: APN, aminopeptidase N from human serum and mouse brain; EP 24.15, endopeptidase 24.15 from mouse brain: EP uncharacterized endopeptidase(s).

occurring peptide and even shows a slightly higher receptor affinity in the binding assay. Dooley and Houghten [44] also reported comparable receptor affinity for NC and NC–NH_2 in rat brain membranes. Secondly, several authors have investigated the minimum sequence required for receptor binding and full biological activity. Deletion of four *C*-terminal residues as in NC–(1–13) gives analogs with different activities and receptor affinities depending on the *C*-terminal chemical function: NC–(1–13)–NH_2 is a full agonist with comparable potency and OP_4 receptor affinity as NC [46,51,52,66–69], while the free acid, NC–(1–13)–OH, is considerably less potent and looses receptor affinity [45,64,70,71]. These findings confirm those reported by Dooley et al. [44] with the progressive *C*-terminal sequence deletion from NC–NH_2 to NC–(1–13)–NH_2. The same author have reported that the four amide fragments NC(1–16)–NH_2, NC(1–15)–NH_2, NC(1–14)–NH_2 and NC(1–13)–NH_2 have similar receptor affinities as the natural peptide in the rat brain membranes. Butour et al. [45] reported that NC–(1–13) shows 1/30 of the affinity of the parent peptide in CHO_{OP4} cell membranes and tenfold less potency when tested as inhibitor of forskolin-induced cAMP accumulation in intact CHO_{OP4} cells. Stepwise shortening NC–(1–13)–NH_2 down to NC–(1–4)–NH_2 resulted in a marked decrease in potency and receptor affinity down to inactivity. Cationic residues (Arg or Lys) in NC(1–13)–NH_2 seem to play a pivotal role in assuring the peptide interaction with the OP_4 receptor. In fact, the removal of Lys^{13} as in NC(1–12)–OH leads to inactivity. This appears however to be partly due to metabolic degradation, since the fragment in which the *C*-terminal amidation protects from degradation by carboxypeptidase(s), maintains some activity. Such protection may account for the residual activity of some of the amide peptides with respect to the free acids.

Further reduction of the *C*-terminal sequence, down to NC(1–9) leads to total loss of activity, even when the *C*-terminal acid group is amidated. NC truncated peptides obtained by deletion from *N*-terminal sequence are devoid of receptor affinity in the mouse brain and of activity on the mouse vas deferens (Table 2). Butour et al. [45] have; however, reported that NC(6–17) and NC(12–17) exhibit fairly high affinity in CHO_{OP4} cells and full agonist activity, as determined by the inhibition of forskolin-induced cAMP accumulation in CHO_{hOP4} cells. The results obtained by Butour et al. raise the question of localization of the message sequence of the NC peptide. The *N*-terminal tetrapeptide, F–G–G–F, has been shown by us [70,72] and other

Table 2
Pharmacological activities and binding affinities of NC and its truncated sequences [a]

Peptide	Bioassay (mVD) pEC_{50}	Receptor binding (mouse brain) pK_i
NC	7.8	8.7
NC–NH_2	7.7	9.1
NC–(1–13)–OH	5.6	6.9
NC–(1–13)–NH_2	7.7	9.1
NC–(1–12)–OH	<5	<5
NC–(1–12)–NH_2	6.1	7.6
NC–(1–11)–NH_2	5.5	5.7
NC–(1–9)–NH_2	<5	<5
NC–(1–5)–NH_2	<5	<5
NC–(1–4)–NH_2	<5	<5
NC–(2–17)–NH_2	<5	n.d.
NC–(13–17)–OH	<5	<5
NC–(13–17)–NH_2	<5	n.d.

[a] pEC_{50} and pK_i as in Table 1. All compounds are full agonist, n.d., not determined. The effects of these compounds were not affected by 1 μM naloxone.

workers [47,73], to be the message domain of NC. The findings of Butour et al., for instance with NC(6–17), point to the fact that the basic core of NC might be a major determinant for the biological interaction of the NC with the OP_4 receptor. This question is also raised by the report of Dooley et al. who recently, found a series of highly basic hexapeptides with affinities and potencies in the nM range for the OP_4 [74]. It must however be considered that the Dooley peptides have three aromatic residues (two Tyr and a Trp) that may be instrumental for receptor activation (see below).

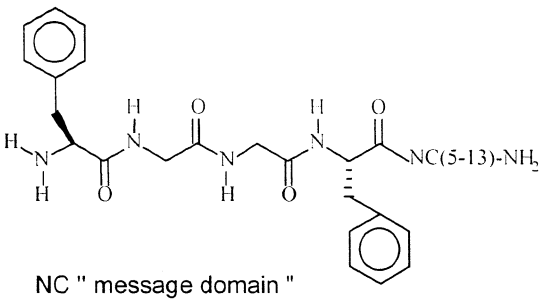
6. The NC(1–13)–NH_2 template

6.1. The *N*-terminal tetrapeptide F–G–G–F

A systematic structure–activity study of NC-related peptides suggests that, as in the case of opioid peptides [39,40,75,76], the message domain of NC coincides with the *N*-terminal tetrapeptide F–G–G–F, then leaving to the highly basic *C*-terminal sequence NC(5–13) the function of address. Taking NC(1–13)–NH_2 as a template, a series of analogs were prepared to explore the role of each residue in the *N*-terminal tetrapeptide, on the assumption that it might contain the active group(s) of NC. Results of biological activities in the mVD of this series of peptides are shown in Table 3.

They indicate firstly: that the *N*-terminal acetylation or the mono or dialkylation leads to significant decrease or total elimination of biological activities. Decrease of *N*-terminal nucleophilicity by acetylation may reduce cationic interaction with the side-chain of the Asp residue which has been shown to be present in the second extracellular loop (EL2) of the NC receptor [43], as well as in those of many receptors of biogenic amines, as noradrenaline (α and β receptors), serotonin, dopamine, and classical opioid receptors [77,78]. Secondly, Phe[1] can be replaced with well-positioned aromatic (Tyr or Dmt) or aliphatic (Cha, Leu) residues without loss of activity, while any spatial displacement of the aromatic group (D-Phe) or spatial encumbrance (Tic) leads to inactivity. Some activity (although reduced by two orders of potency) is found with Phe(NMe) and Phe(pMe) in position one of NC(1–13)–NH_2. A large number of analogs were studied to determine the role of the spacer Gly[2]–Gly[3], which can be drastically modified in the opioid sequences [79,80], (see discussion below). This appears not to be the case for NC, since the elimination of one or both Gly as well as the replacement of Gly[2] with Pro, Phe, D-Phe gives inactive compounds. Replacement of Gly[2] with D-Ala or Sar is associated to a marked decrease (almost two orders of potency) of activity: similar results were obtained with the replacement of Gly[3] made alone (with Phe or Arg) or combined with change or removal of Gly[2]. Some activity is observed in the extended chain (Gly[3]) or by the use of D-Phe in position 3. A few compounds in which Phe[4] was replaced terminate Table 3 and indicate that this position (in contrast to position one) only tolerates the presence of another aromatic

Table 3
Pharmacological activities of NC(1–13)–NH_2 analogs modified in the message domain [a]



Peptide	Bioassay (mVD) pEC_{50}
NC(1–13)–NH_2	7.8
Modification at Phe[1]	
Ac–NC(1–13)–NH_2	5.8
[Phe(*N*,*N*-diallyl)[1]]–NC(1–13)–NH_2	i
[Phe(NMe)[1]]–NC(1–13)–NH_2	6.0
[D-Phe[1]]–NC(1–13)–NH_2	i
[Phe(pMe)[1]]–NC(1–13)–NH_2	5.6
#[Tyr[1]]–NC(1–13)–NH_2	7.6
#[Dmt[1]]–NC(1–13)–NH_2	7.9
[Tic[1]]–NC(1–13)–NH_2	i
[Cha[1]]–NC(1–13)–NH_2	7.9
[Leu[1]]–NC(1–13)–NH_2	7.5
Modification at the spacer Gly[2]–Gly[3]	
[des-Gly[2]]–NC(1–13)–NH_2	i
[des-Gly[2,3]]–NC(1–13)–NH_2	i
[D-Ala[2]]–NC(1–13)–NH_2	6.0
[Sar[2]]–NC(1–13)–NH_2	5.7
[Phe[2]]–NC(1–13)–NH_2	i
[D-Phe[2]]–NC(1–13)–NH_2	i
[Pro[2], des-Gly[3]]–NC(1–13)–NH_2	i
[D-Ala[2], des-Gly[3]]–NC(1–13)–NH_2	i
[Ala[2,3]]–NC(1–13)–NH_2	i
[Phe[3]]–NC(1–13)–NH_2	i
[D-Phe[3]]–NC(1–13)–NH_2	5.7
#[Arg[3]]–NC(1–13)–NH_2	i
[β-Ala[2], des-Gly[3]]–NC(1–13)–NH_2	i
[Gaba[2], des-Gly[3]]–NC(1–13)–NH_2	i
[Vra[2], des-Gly[3]]–NC(1–13)–NH_2	i
[Phe–$(Gly)_3$–Phe]–NC(5–13)–NH_2	5.5
Modification at Phe[4]	
[D-Phe[4]]–NC(1–13)–NH_2	i
[Trp[4]]–NC(1–13)–NH_2	6.4
[Tic[4]]–NC(1–13)–NH_2	i
[Leu[4]]–NC(1–13)–NH_2	i

[a] pEC_{50} as in Table 1. i, inactive at 10 μM: all compounds are full agonists. The effects of these compounds were not affected by 1 μM naloxone, except where indicated by #.

(Trp), however with marked loss of potency in biological and binding assays. All other substitutions, especially with Leu lead to inactive compounds, suggesting the need of aromaticity in position 4 for OP_4 receptor activation.

Results summarized in Table 3 point to important differences between opioids and NC/OP_4 system with respect to the function of the *N*-terminal residue. Tyr^1 of opioids is essential for receptor (OP_1, OP_2, OP_3) activation: any replacement of Tyr^1 with Phe, Leu, Ala is incompatible with activity [81]. On the contrary, OP_4 receptor accepts aromatic (Phe, Tyr, Dmt) or aliphatic (Cha, Leu) residues, however with a definite side-chain size, since [Ala^1]–NC has been shown to be inactive [44,47]. We have therefore suggested that Phe^1 of NC is instrumental for binding to the OP_4 receptor (desPhe^1–NC is inactive) as well as for positioning the other aromatic group (Phe^4) on the OP_4 receptor. The *N*-terminal amino group is required for interaction (presumably with the Asp residue of the third receptor domain), since its acetylation is not tolerated (Table 3): *N*-terminal diallylation reduces activity and does not lead to antagonism, contrary to opioid peptides [82,83]. The spacer Gly^2–Gly^3 appears to be extremely critical for the NC/OP_4 interaction, much more than for the three opioid systems, as any change of spacing, spatial conformation or the reduction of rotational freedom is followed by extreme loss of potency. Again, this is different from opioids, which have been shown to accept quite different spacer profiles (e.g. Tyr-Gly-Gly-Phe; Tyr-D-Xaa-Phe; Tyr-Pro-Phe; Tyr-Tic-Phe-Phe; Tyr-Tic) [79,84–86].

Despite the limited number of analogs available to date, it has been suggested that the active functional site of NC is Phe^4; the residue in this position has to be aromatic and its position for the optimum interaction with the OP_4 receptor appears to be very critical, as suggested by the first partial agonists and antagonists that have been recently discovered [87,88].

The active groups of NC appear therefore to be located towards the middle of the molecule definitely including Phe^4 and some unidentified residues, perhaps in line with the recent findings by Butour et al. [45].

6.2. The C-terminal nonapeptide NC(5–13)

A series of analogs of NC(1–13)–NH_2, modified in the address domain (NC(5–13)) are analyzed in Table 4.

It has been suggested that charged residues (Arg and Lys) in the *C*-terminal nonapeptide NC(5–13) are important for the interaction with OP_4 [44,47]. Such interactions probably occur with the second extracellular loop (EL2) of the OP_4 that is rich of acidic residues (Asp and Glu). Replacement of the first couple of the charged residues, Arg^8–Lys^9, or that of Arg^{12} with Ala, gives an inactive compound which does not even bind to OP_4 (Varani et al., personal communication); conversely deletion of Arg^{12}, gives an analog that shows binding affinity two log units less than NC(1–13)–NH_2, but does not activate the OP_4 receptor.

When tested as antagonist against NC(1–13)–NH_2 in the mVD, [Ala^{12}]–NC(1–13)–NH_2 does not show any antagonist activity. These data confirm the report of Reinscheid et al. [47] in the alanine-subtituted NC peptides; in this study, position 8 of NC appears to be most critical for receptor interaction, as measured by its ability to inhibit forskolin-stimulated cAMP accumulation. Arg^8 appears to be critical not only for its positive charge(s), but also because the strongly basic guanidino function (pK_a 12.5) in the side-chain can permit strong interaction with acidic residues of OP_4. In fact, the replacement of Arg^8 with Lys, a residue that at physiological pH also brings a positive charge due to its amino function in the side-chain, brings a significant loss of activity and potency, indicating the instrumental role of the guanidino group and its right distance from the peptide backbone for the interaction with OP_4 (see Table 4). The same behavior is shown by modified sequence (Arg–Lys → Lys–Arg), confirming the strict requirement of Arg in position 8 for receptor activation. Charged residues in position 9, 12 and 13 are not so critical, the only requirement is the presence of a positive charged residue (Arg or Lys).

From these data and from the data reported in the literature we therefore conclude that: (a) the presence of Arg residue in position 8 of the NC and NC(1–13)–NH_2 is an absolute requirement for receptor inter-

Table 4
Pharmacological activities of NC(1–13)–NH_2 analogs modified in the address domain [a]

H-Phe-Gly-Gly-Phe-Thr^5-Gly^6-Ala^7-Arg^8-Lys^9-Ser^{10}-Ala^{11}-Arg^{12}-Lys^{13}-NH_2
NC 'address domain'

Peptide	Bioassay, mVD pEC_{50}
NC(1–13)–NH_2	7.75
[Pro^6]–NC(1–13)–NH_2	5.9
[D-Ala^7]–NC(1–13)–NH_2	5.85
[Lys^8]–NC(1–13)–NH_2	5.0
[Arg^9]–NC(1–13)–NH_2	7.59
[Lys^8,Arg^9]–NC(1–13)–NH_2	5.41
[$Ala^{8,9}$]–NC(1–13)–NH_2	i
[Pro^{11}]–NC(1–13)–NH_2	5.67
[Ala^{12}]–NC(1–13)–NH_2	i
[Lys^{12}]–NC(1–13)–NH_2	7.11
[Arg^{13}]–NC(1–13)–NH_2	7.43
[Lys^{12},Arg^{13}]–NC(1–13)–NH_2	7.2

[a] pEC_{50} as in Table 1. i, inactive at 10 μM: all compounds are full agonists. The effects of these compounds were not affected by 1 μM naloxone.

action; (b) this positive residue might interact with the side-chain of Glu or Asp residue in the receptor and in this way it could anchor the peptide to the receptor, permitting the access of the *N*-terminal sequence (message) of NC and congeners to the receptor pocket (a cavity formed by helices 3, 5, 6 and 7) where the message may be envisaged for OP_4 activation; (c) the second couple of basic residues does not have stringent requirements in terms of side-chains; however, the presence of a second cationic region in position 12–13, is needed to determine an optimal interaction with OP_4. It is interesting to point out that the absence of an arginine residue in position 8 of all mammalian opioid peptides, including dynorphins, should contribute to exclude these peptides from interacting with OP_4 [41,89].

In another study, we induced changes in the secondary structure of NC(1–13)–NH_2 by replacing Ala^7 with its enantiomer or by replacing Gly^6 or Ala^{11} with Pro. [Pro^6]– and [D-Ala^7]–NC(1–13)–NH_2 show interesting pharmacological behaviors, since they bind fairly well to OP_4, but do not (or very little) activate the receptor. In the D-amino acid-scanning study by Reinscheid et al., [D-Ala^7]–NC displayed receptor binding affinity similar to NC but no activity and a weak antagonism, expressed by its ability to reverse the effect of NC (10 nM) on forskolin-stimulated cAMP in transfected cells. It thus appears that conformational constrains induced by Pro or D-Ala (as in [D-Ala^7]–NC(1–13)–NH_2) between the message and the charged residues of the address or between the cationic regions (as in [Pro^{11}]–NC(1–13)–NH_2) reduce the ability of the template to activate the receptor. Data shown in Tables 3 and 4 led us to work in the message domain to find antagonists for OP_4.

7. Discovery of OP_4 receptor antagonists

The development of potent antagonists acting on different members of the G-protein seven transmembrane domain superfamily of receptors, is of great interest for studies aimed at elucidating the functional role of many endogenous biological systems. Without antagonists, classification of receptors remains inadequate and in the case of the NC/OP_4 system, it will be impossible to know if NC biological actions are mediated by the same receptor type or by multiple receptors. There are not definite principles to develop antagonists of peptide hormones or neurotransmitters; in fact there are many examples of antagonists discovered by serendipity [90,91] or by examining large series of natural or synthetic compounds (e.g. chemical libraries, [92]; some workers have used extensive SAR studies and prepared numerous analogs of the naturally occurring peptide ligands [93].

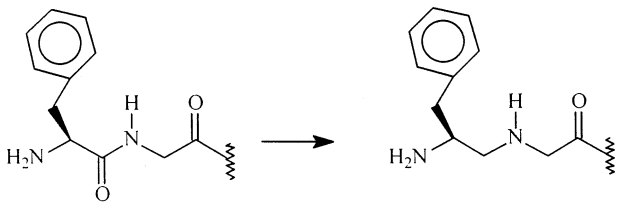
Such an approach has been adopted by our group, using as a template NC(1–13)–NH_2, a potent agonist of the OP_4 receptor. Our study began with an attempt to protect NC(1–13)–NH_2 from degradation by aminopeptidase(s): different strategies were adopted: (a) Gly^2 was replaced with D-Ala; (b) the peptide bond between Phe^1–Gly^2 was modified by *N*-methylation or by reduction to amino function; (c) the side-chain of the first residue was displaced from chiral carbon to nitrogen. Analogs were tested as usual and, when found inactive as agonists, the peptides were assayed as antagonists against the reference agonist NC(1–13)–NH_2 in the mVD and as competitors of the binding of [^{3}H]NC–NH_2 in mouse brain membranes. Data are presented in Tables 5 and 6.

The insertion of a pseudo-peptide bond (CO–NH → CH_2–NH) between Phe^1 and Gly^2 maintains good affinity but eliminates the ability of the peptide to activate the OP_4 receptor and gives a receptor antagonist. Antagonism is obtained with both the full length peptide NC–NH_2 and the truncated template NC(1–13)–NH_2 with comparable potencies and binding affinities (Table 5): however, [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2 requires L-chirality of Phe^1, since the D-Phe^1 diastereomer is inactive. The antagonist is selective for the NC receptor; in fact, as shown in Fig. 1, the inhibitory effect of NC in the mVD is not modified by naloxone, but it is reduced by [$Phe^1\Psi$-(CH_2–NH)Gly^2]–NC(1–13)–NH_2: conversely, the effect of the OP_1 receptor selective agonist, [D-Ala^2]–deltorphin I, is antagonized by naloxone but is not affected by [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2 (see Fig. 4). Similarly, the interactions of selective OP_3 or OP_2 agonists on the respective functional sites are not modified by the OP_4 receptor antagonist [72]. Similar results were also obtained by studying the binding of [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2 to OP_4, OP_1, OP_2, and OP_3 sites in guinea pig brain membranes [94].

The displacements to the right of the NC concentration–response curves by [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2 (Fig. 4, left panel) as well as that of [D-Ala^2]–deltorphin I by naloxone (Fig. 4, right panel) are parallel to the control curves, suggesting that the antagonists are competitive. [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2 represents the first example of a selective OP_4 receptor antagonist. In fact, only antagonists which act non-selectively and with low affinity [95–97] or compounds that act as partial agonists [74] were reported before (see below).

From a chemical point of view, the replacement of CO by CH_2 eliminates the possibility of acting as a H-bond acceptor: other manipulations of the peptide bond between Phe^1–Gly^2 by methylation, preserving the carbonyl function, gives an agonist with decreased activity (see Table 1). Replacement of the amide with an

Table 5
Pharmacological activities and binding affinities of NC(1–13)–NH_2 analogs [a]



Peptide	Bioassay, mVD		Receptor binding (mouse brain)
	Agonist pEC_{50}	Antagonist pA_2	pK_i
NC	7.8		8.7
NC(1–13)–NH_2	7.8		9.1
Modification at the Phe[1]–Gly[2] peptide bond, [Xaa[1]Ψ(CH_2–NH)Gly[2]]			
[Phe[1]ΨGly[2]]–NC–NH_2	i	7.0	7.7
[Phe[1]ΨGly[2]]–NC(1–13)–NH_2	i	6.8	8.0
[D-Phe[1]ΨGly[2]]–NC(1–13)–NH_2	i	i	6.8
[Phe[1]ΨGly[2]]–NC(1–12)–NH_2	i	5.2	6.4
[Phe[1]ΨGly[2]]–NC(1–9)–NH_2	i	5.1	<5
[Cha[1]ΨGly[2]]–NC(1–13)–NH_2	i	i	6.5
[Phe(pMe)[1]ΨGly[2]]–NC(1–13)–NH_2	i	5.7	6.2
[Tyr[1]ΨGly[2]]–NC(1–13)–NH_2	i	5.7	7.0
[Leu[1]ΨGly[2]]–NC(1–13)–NH_2	i	5.4	7.8
[Phe[1]ΨGly[2],Leu[4]]–NC(1–13)–NH_2	i	i	n.d.

[a] pEC_{50} and pK_i as in Table 1. pA_2 is the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response; the antagonistic properties of these compounds were tested using NC(1–13)–NH_2 as agonist. i, inactive at 10 μM; n.d., not determined.

amino function increases the flexibility of the *N*-terminal portion of the molecule, which also becomes more basic, and this may prevent receptor activation.

As reported above, position one of the ligand NC(1–13)–NH_2 can be modulated in different ways, e.g. by replacing Phe with Tyr, Dmt or with the aliphatic residues Cha or Leu, which are both tolerated with full retention of agonist activity; however the replacement with Tyr or Dmt gives compounds that interact also with opioid receptors. These same residues have been coupled through a pseudopeptide bond to Gly[2] (see compounds in Table 5) but all have shown antagonistic activities well below (by 1.0 to 1.7 log units) that of [Phe[1]Ψ(CH_2–NH)Gly[2]]–NC(1–13)–NH_2.

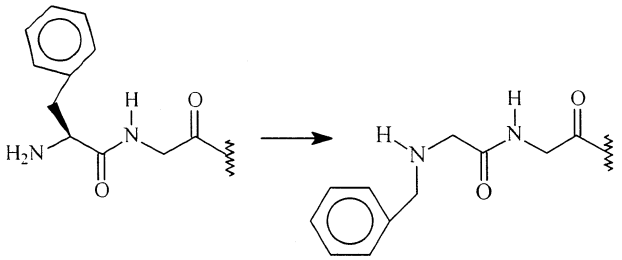
[Leu[1]Ψ(CH_2–NH)Gly[2]]–NC(1–13)–NH_2 binds but does not antagonize and [Phe[1],Ψ(CH_2–NH)Gly[2], Leu[4]]–NC(1–13)–NH_2 is inactive, as expected by the replacement of Phe[4] with Leu in the agonist [70]. Other substitutions of the aromatic Phe[1], as with p(Me)Phe, dramatically reduce the activity. Finally, sequence deletion of charged residues from NC(1–13)–NH_2 down to NC(1–9)–NH_2 gives reduction of activity or inactivity. The fact that other compounds act as antagonists suggests that: (a) the occupation of the receptor by the antagonist requires the modified message (Xaa[1]Ψ(CH_2–NH)–Gly–Gly–Phe) and a critical *C*-terminal chain, whose optimum is the nonapeptide NC(5–13), similar to the requirements for agonists (see Table 5); (b) Phe[1] contributes to the antagonist activity better than any other substitute, including aromatic (Tyr or p(Me)Phe), aliphatic residues of small (Leu) or rather large (Cha) size; this is in contrast with the behavior of agonist compounds (Table 3) which are all high affinity ligands with the exception of [p(Me)Phe[1]]–NC(1–13)–NH_2 (see Table 3); (c) the aromatic residue in position four is required for both agonist and antagonist activities.

[Phe[1]Ψ(CH_2–NH)Gly[2]]–NC(1–13)–NH_2 acts as an antagonist of the OP_4 receptor in the mouse vas deferens [87], as well as in a variety of in vitro NC sensitive preparations [46,52,66,98,99] and in some in vivo assays [66,69,100]. However, this compound maintains partial [97,101,102] or full agonist activities [46,67,68,103–106], mimicking the actions of NC in most central nervous system assays and in CHO_{hOP_4} cells [46,107,108]. This dual behavior limits the usefulness of this compound for OP_4 receptor characterization and for elucidating the role of the NC/OP_4 system in the central nervous system.

To identify a pure receptor antagonist, devoid of residual agonist activity, we prepared other compounds, modified in the Phe[1] residue; these are reported in Table 6. In the design of these compounds we consid-

ered an alternative way to bring Phe in a variety of geometries and chemical environments in order to maximize the chances of favorable interactions with the target receptor. Moving the first side-chain attachment from chiral carbon to nitrogen, we obtained *N*-alkyl glycines that are not found in nature and have been used to prepare peptoids (oligomers of *N*-substituted glycines, [109]). Peptoid versions of naturally occurring peptides have been found to maintain full affinities while showing either agonist or antagonist activities

Table 6
Pharmacological activities and binding affinities of NC(1–13)–NH_2 analogs [a]



Peptide	Bioassay, mVD		Receptor binding (mouse brain)
	Agonist	Antagonist	
	pEC_{50}	pA_2	pK_i
NC	7.8		8.7
NC(1–13)–NH_2	7.8		9.1
Modification at the Phe[1] residue, [Nxaa[1]]			
[Nphe[1]]–NC–NH_2	i	6.3	6.9
[Nphe[1]]–NC(1–13)–NH_2	i	6.4	7
[Nphe[1]]–NC(1–12)–NH_2	i	i	5.5
[Nphe[1]]–NC(1–9)–NH_2	i	i	<5
[Ncha[1]]–NC(1–13)–NH_2	i	i	5.6
[Nphe(pMe)[1]]–NC(1–13)–NH_2	i	i	5.6
[Ntyr[1]]–NC(1–13)–NH_2	i	i	5.5
[Nleu[1]]–NC(1–13)–NH_2	i	i	5.6
[Nphe[1],Leu[4]]–NC(1–13)–NH_2	i	i	n.d.

[a] pEC_{50} and pK_i as in Table 1. pA_2 as in Table 5. i, inactive at 10 μM; n.d., not determined.

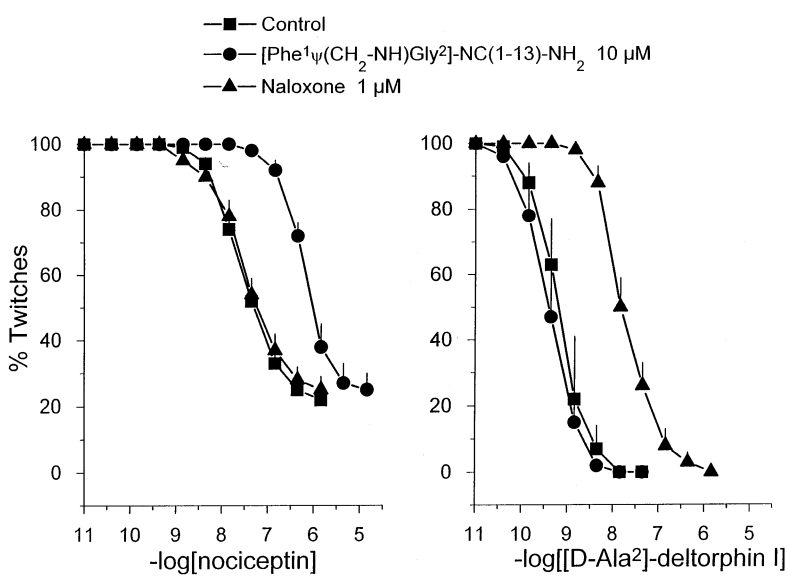


Fig. 4. Effects of naloxone (1 μM) and [$Phe^1\Psi(CH_2$–NH)–Gly^2]–NC(1–13)–NH_2 (10 μM) on concentration–response curves to nociceptin (left panel) and to [D-Ala^2]–deltorphin I (right panel) in the electrically stimulated mouse vas deferens. Data are mean ± SEM of at least five experiments.

Table 7
Pharmacological activities of miscellaneous compounds interacting with the OP_4 receptor [a]

Comp.	Receptor binding	Functional test		
		Agonist		Antagonist
	pK_i	pED_{50}	α^E	pA_2
NC	9.37 (mouse)	8.83 (rat)	1.00	
Ac-Arg-Tyr-Tyr-Arg-Trp-Arg-NH_2	9.22	9.28	0.89	
Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2	9.15	9.28	0.69	
Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2	8.82	8.93	0.74	
Ac-Arg-Tyr-Tyr-Lys-Trp-Arg-NH_2	8.83	8.71	0.81	
Ac-Arg-Tyr-Tyr-Lys-Trp-Lys-NH_2	9.14	9.01	0.78	
NC	9.88 (human)	9.10 (human)	1.00	
Dynorphin A	6.96	<5		
Etorphine	6.28	6.34	1.00	
Lofentanil	7.62	8.14	1.00	
NC	10.09 (human)	9.84 (human)	1.00	
NalBzoH	7.26	5.80	0.44	6.3
NC	8.87 (mouse)	7.76 (mouse)	1.00	
Mr 2266	4.8			5.77
Mr 2267	5.14			5.64

[a] pEC_{50} and pK_i as in Table 1. pA_2 as in Table 5. The data summarized in this table are from Refs. [74] (first series), [45] (second series), [112] (third series), and [113] (last series).

[110,111]. The functional groups in the peptoid residue can be readily altered, with changes in the rigidity, complexity, and diversity of the designed analogs. In the NC(1–13)–NH_2, the C→N shift of the side-chain leads to antagonism, weaker (by about fivefold) than the corresponding [$Phe^1\Psi(CH_2$–NH)Gly^2]–NC(1–13)–NH_2. However, this modification leads to complete elimination of residual agonist activity: in fact, [$Nphe^1$]–NC(1–13)–NH_2 acts as a pure OP_4 antagonist. This C→N shift is inappropriate for any of the functional groups considered out of the benzyl pharmacophore as indicated by the total loss of agonist and antagonist activities of the compounds reported in Table 6. Again, the whole nonapeptide chain (5–13) at the *C*-terminal and the aromatic residue in the fourth position are needed for receptor interaction. In summary, transforming the peptide bond between Phe^1 and Gly^2 from amide to amine function leads to a receptor antagonist of the functional sites mediating the peripheral actions of NC. The displacement of the benzyl side-chain of Phe^1 by one atom, as in $Nphe^1$ series, completely eliminates agonist activities and provides a pure antagonist for the OP_4 receptors that is present in the mVD and in several other preparations such as the guinea pig ileum and renal pelvis, and the rat vas deferens [88]. In addition, [$Nphe^1$]–NC(1–13)–NH_2 binds selectively to recombinant OP_4 receptors expressed in CHO cells, and competitively antagonizes the inhibitory effects of NC on cAMP accumulation in the same preparation [88]. Although weak (pK_i 7.0; pA_2 6.4) this new compound provides a new lead for future development of OP_4 receptor antagonists. The results obtained with analogs of the template NC(1–13)–NH_2 confirm our previous suggestions that OP_4 receptor agonism and antagonism are modulated in different way compared to classical opioid receptors [70,72].

8. Miscellaneous compounds interacting with OP_4 receptors

In spite of the structural and trasductional homology between the classical opioid receptors and the recently discovered OP_4, the pharmacological profile of the new receptor appears to be well distinct. Dynorphin A, the physiological ligand of the OP_2 receptor, is the only opioid peptide that shows some affinity for the OP_4. In cells transfected with the human recombinant OP_4, Dyn A binds the receptor at concentration 850-fold higher than those of NC (see Table 7) in line with the findings by Reinscheid et al. [47] who reported that Dyn A interacts with rat OP4 transfected at even higher concentration, (2300-fold compared to NC).

Despite this modest OP_4 receptor affinity, Dyn A is inactive as inhibitor of the forskolin induced accumulation of cAMP in CHO_{OP4} cells (see Table 7). It could be concluded that even if Dyn A has a similar number of positive charged residues in the *C*-terminal sequence as NC (see Fig. 1), a different distribution of these charges (in particular in position 8) hinders the interaction of Dyn A with OP_4. On the other hand, NC shows very modest if any potency on opioid receptors, probably

because the amino terminal residue, Phe[1], is incompatible with opioid receptor activation [81]. This conclusion is supported by the finding that replacement of Phe[1] with Tyr, confers to NC conspicuous affinities and activities for the opioid receptors, while maintaining good potency on OP_4 [27].

Dooley et al. [74] reported a series of hexapeptides completely unrelated to the natural ligand NC, identified from a combinatorial library containing more than 52 million peptides. Five hexapeptides have affinity for the OP_4 receptor in the nM range quite similar to NC. In different pharmacological tests, as the stimulation of [^{35}S]GTPγS binding, the inhibition of forskolin stimulated cAMP in CHO_{OP_4} (see Table 7, and the electrically stimulated contractions in the mVD, the Dooley's compounds have shown partial agonist activities [74]. The hexapeptide amides are acetylated at the *N*-terminal amino function and are endowed with basic residues flanked by aromatic residues. It is reported that the interaction of positively charged hexapeptides with OP_4 receptor could be compared with shorter *C*-terminal fragments of NC, as NC(6–17), suggesting that the basic core of the NC sequence could represent a message of the NC peptide [45]. However, it is important to remember that the presence of three aromatic residues in these hexapeptide sequences appear to be essential for the activity and that two aromatics (Phe) are present in the so-called message domain of NC.

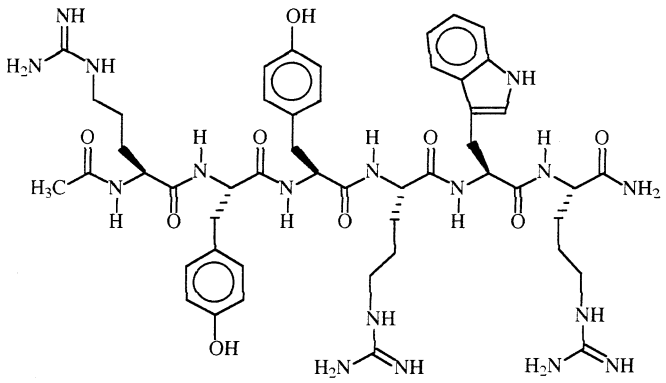
Several non peptide compounds have been reported as stimulants or inhibitors of OP_4. Thus ethorphine, a potent non selective opioid receptor agonist, used initially to monitor the activation of OP_4 [7], was found to bind to OP_4 with affinity 4000-fold less than NC; however, etorphine is only 500-fold less potent as activator of OP_4 (see Table 7). Further, Butour et al. [45] reported that lofentanil, a piperidino derivative with high opioid receptor affinity and pharmacological activity, shows quite good OP_4 affinity (only 200-fold less than NC), and activates the receptor when applied at nM concentrations (see Table 7). Fentanil, a close structural analog of lofentanil, has only marginal affinity and does not activate OP_4.

Naloxone benzoylhydrazone (NalBzoH), a mixed agonist/antagonist of the classical opioid receptors, with some selectivity for the OP_2 receptor, has been reported to compete with [^{3}H]NC binding in CHO_{rOP_4} [96] and CHO_{hOP_4} [112] cell membranes. In the latter cells, NalBzoH has partial agonist activity in the cAMP accumulation assay [112]. NalBzoH competitively antagonizes NC effects on NE release in cerebral cortex slices in vitro [97] and blocks NC-induced hyperalgesia and hypolocomotion in vivo [96].

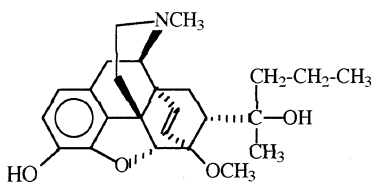
Schlicker's group [113] has recently reported that Mr 2266 is an antagonist at OP_2 and OP_4 receptors. In mouse brain cortex membranes, the binding of OP_4 receptor agonist [^{3}H]–NC was equipotently inhibited by Mr 2266 and its enantiomer Mr 2267 (see Table 7), whereas the binding of the OP_2 receptor agonist [^{3}H]–U69,593 was inhibited by Mr 2266 more potently than by its enantiomer Mr 2267. The stereoselective antagonism of Mr 2266 at OP_2 receptors does not extend to OP_4 sites.

The activities of opiates such as lofentanil, etorphine, NalBzoH, Mr 2266 and Mr 2267 on OP_4 indicate that the requirements of peptide ligands as NC(1–13)–NH_2, are not as strict as they appeared to be initially and can possibly be overcome. Basic residues which are present in the *C*-terminal sequence of NC, may be required to establish multiple interactions between the natural peptides and the acidic EL2 loop of the OP_4 receptor; however, small molecules without charges can still penetrate into the active site of OP_4, as they do in the classical opioid and numerous other receptors [114]. In analogy, also dynorphin peptides strictly require a *C*-terminal basic core to interact with EL2 loop of the OP_2 receptor with high affinity and selectivity: however, recently discovered non-peptide ligands, as U50-488 and related analogs, interact selectively with OP_2, despite the absence of a basic core [115]. The structures of the above discussed opiate ligands which interact with OP_4 may resemble the bioactive conformation of the NC-message domain, Phe-Gly-Gly-Phe (especially that of lofentanil which is more active on OP_4 than the other compounds). In fact, *N*-phenyl substitution of the propion amide function of lofentanil (as a counterpart of the 3-hydroxyl substitution in the morphinans or 2′-hydroxyl substitution in the 6,7-benzomorphan A-ring) by avoiding phenol function, may facilitate the interaction with the OP_4 receptor. Because the *N*-terminal residue of NC lacks the hydroxyl group, it should be assumed that a 6,7-benzomorphan or a morphinan analog (specifically a fentanil analog that lacks the hydroxyl function) could interact in a more efficiently way with the OP_4 receptor. The further substitution of the piperidine ring as in lofentanil compared to fentanil, by improving the bioactive conformation of the *N*-phenyl ring could create additional OP_4 interactions with specific ester function. These non-peptide templates could serve to develop new analogs that could be useful to better understanding the pharmacology of the OP_4 receptor.

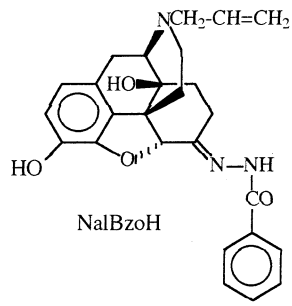
Recently, new 2-oxoimidazole and 1,3,8-triazaspiro-[4,5]-decan-4-on derivatives were reported and patented by Banyu Pharmaceutical [116] and by Hofmann La Roche [117]. The compound of Banyu is expected to act as a potent antagonist, while those reported by Roche investigators are agonists of the OP_4 receptor. The general structures of these compounds are reported in Fig. 5.



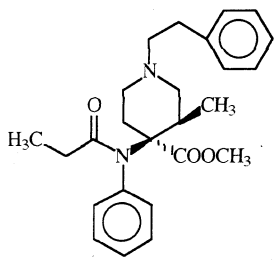
Ac-Arg-Tyr-Tyr-Arg-Trp-Arg-NH_2



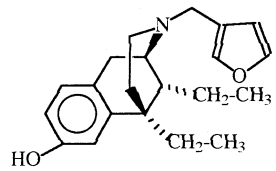
Etorphine



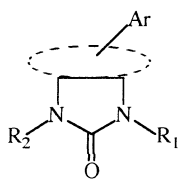
NalBzoH



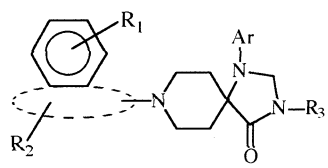
Lofentanil



Mr 2266



2-oxoimidazole derivatives



1,3,8-triazaspiro-[4,5]-decan-4-on derivatives

Fig. 5. Structure of miscellaneous compounds interacting with the OP_4 receptor.

The availability of non peptide selective ligands (either agonists and antagonists) of the OP_4 receptor will definitely be crucial for elucidating the physiological and pathophysiological roles of the NC/OP_4 system.

9. Conclusions

Similar to mammalian opioid peptides, (whose *N*-terminal tetrapeptide is Tyr-Gly-Gly-Phe), the *N*-terminal sequence of NC, (Phe-Gly-Gly-Phe) is considered to be the message portion of the peptide. For receptor activation opioid peptides require Tyr in position 1 while Phe in position 4 appears to be essential for activation of OP_4. In favor of this interpretation, [Tyr^1]–NC can activate both some classical opioid (OP_3 and OP_2) and the OP_4 receptors.

OP_4 and OP_2 receptors require charged residues (Arg and Lys) in the *C*-terminal sequence of the natural ligands, NC and dynorphin A. However, these basic

residues are not equally distributed in the two ligands. While Arg^8 is the essential residue for the interaction of NC with OP_4, dynorphin peptides require Arg in positions 6 and 7. Despite their homologies, the two endogenous systems show definite and specific agonist chemical requirements.

Peptide antagonists for the two systems have been described. [$Phe^1\Psi(CH_2–NH)Gly^2$]–NC(1–13)–NH_2 (which actually has to be considered as a low-efficacy agonist) and [$Nphe^1$]–NC(1–13)–NH_2 for OP_4 and [*N*,*N*-diallylTyr^1, D-Pro^{10}]–Dyn A(1–11) for the OP_2. Both groups of agents derive from modification of the *N*-terminal tetrapeptide; however, OP_4 antagonists require the maintenance of a critical spacing between the two aromatic rings, while OP_2 receptor antagonists require firstly the *N*-terminal dialkylation. Several patterns of messages are tolerated by the opioid receptors, such patterns are however not accepted by OP_4, probably because they imply drastic displacement of Phe^4. Subtle displacements such as those obtained with [$Phe^1\Psi(CH_2–NH)Gly^2$]–NC(1–13)–NH_2 or with moving by one atom the side-chain of Phe^1, (as in [$Nphe^1$]–NC(1–13)–NH_2) allow Phe^4 to keep most of its binding capability while losing (in part or completely) its ability to activate OP_4. Emerging non-peptide molecules indicate that, as for many other GPCRs, specific address sequences are required for binding of naturally occurring peptide ligands; they may; however, not be essential for occupation (especially by antagonists) or even for activation of the active receptor site which are embedded in transmembrane receptor domains. This working hypothesis should facilitate the design of non-peptide agonists and antagonists which will be essential for elucidating the physiopathological roles of the NC/OP_4 system.

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References

[1] M. Hamon, The new approach to opioid receptors, N. S. Arch. Pharmacol. 358 (1998) SA 5.3.
[2] J.R. Bunzow, C. Saez, M. Mortrud, C. Bouvier, J.T. Williams, M. Low, D.K. Grandy, Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type, FEBS Lett. 347 (1994) 284–288.
[3] M.J. Wick, S.R. Minnerath, X. Lin, R. Elde, P.Y. Law, H.H. Loh, Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors, Brain Res. Mol. Brain Res. 27 (1994) 37–44.
[4] J.B. Wang, P.S. Johnson, Y. Imai, A.M. Persico, B.A. Ozenberger, C.M. Eppler, G.R. Uhl, cDNA cloning of an orphan opiate receptor gene family member and its splice variant, FEBS Lett. 348 (1994) 75–79.
[5] Y.X. Pan, J. Cheng, J. Xu, G. Rossi, E. Jacobson, J. Ryan-Moro, A.I. Brooks, G.E. Dean, K.M. Standifer, G.W. Pasternak, Cloning and functional characterization through antisense mapping of a kappa 3-related opioid receptor, Mol. Pharmacol. 47 (1995) 1180–1188.
[6] M.A. Osinski, M.S. Pampusch, M.P. Murtaugh, D.R. Brown, Cloning, expression and functional role of a nociceptin/orphanin FQ receptor in the porcine gastrointestinal tract (in process citation), Eur. J. Pharmacol. 365 (1999) 281–289.
[7] C. Mollereau, M. Parmentier, P. Mailleux, J.L. Butour, C. Moisand, P. Chalon, D. Caput, G. Vassart, J.C. Meunier, ORL_1, a novel member of the opioid receptor family-cloning, functional expression and localization, FEBS Lett. 341 (1994) 33–38.
[8] J.E. Lachowicz, Y. Shen, F.J. Monsma, D.R. Sibley, Molecular cloning of a novel G protein coupled receptor related to the opiate receptor family, J. Neurochem. 64 (1995) 34–40.
[9] Y. Chen, Y. Fan, J. Liu, A. Mestek, M. Tian, C.A. Kozak, L. Yu, Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family, FEBS Lett. 347 (1994) 279–283.
[10] R.K. Reinscheid, H.P. Nothacker, A. Bourson, A. Ardati, R.A. Henningsen, J.R. Bunzow, D.K. Grandy, H. Langen, F.J. Monsma Jr., O. Civelli, Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor, Science 270 (1995) 792–794.
[11] J.C. Meunier, C. Mollereau, L. Toll, C. Suaudeau, C. Moisand, P. Alvinerie, J.L. Butour, J.C. Guillemot, P. Ferrara, B. Monserrat, H. Mazarguil, G. Vassart, M. Parmentier, J. Costentin, Isolation and structure of the endogenous agonist of opioid receptor-like ORL_1 receptor, Nature 377 (1995) 532–535.
[12] L.J. Sim, R.Y. Xiao, S.R. Childers, Identification of opioid receptor-like (ORL_1) peptide-stimulated [S-35]GTP gamma S binding in rat brain, Neuroreport 7 (1996) 729–733.
[13] L.J. Sim, S.R. Childers, Anatomical distribution of mu, delta, and kappa opioid- and nociceptin/orphanin FQ-stimulated [35*S*]guanylyl-5′-*O*-(gamma-thio)-triphosphate binding in guinea pig brain, J. Comp. Neurol. 386 (1997) 562–572.
[14] J. Peluso, K.S. LaForge, H.W. Matthes, M.J. Kreek, B.L. Kieffer, C. Gaveriaux-Ruff, Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells, J. Neuroimmunol. 81 (1998) 184–192.
[15] W.P. Halford, B.M. Gebhardt, D.J. Carr, Functional role and sequence analysis of a lymphocyte orphan opioid receptor, J. Neuroimmunol. 59 (1995) 91–101.
[16] O. Civelli, H.P. Nothacker, R. Reinscheid, Reverse physiology: discovery of the novel neuropeptide, orphanin FQ/nociceptin, Crit. Rev. Neurobiol. 12 (1998) 163–176.
[17] J.C. Meunier, Nociceptin/orphanin FQ and the opioid receptor-like ORL_1 receptor, Eur. J. Pharmacol. 340 (1997) 1–15.
[18] T. Yamamoto, N. Nozaki-Taguchi, Y. Sakashita, S. Kimura, Nociceptin/orphanin FQ: role in nociceptive information processing, Prog. Neurobiol. 57 (1999) 527–535.
[19] J. Sandin, J. Georgieva, P.A. Schott, S.O. Ogren, L. Terenius, Nociceptin/orphanin FQ microinjected into hippocampus impairs spatial learnig in rats, Eur. J. Neurosci. 9 (1997) 194–197.
[20] T. Manabe, Y. Noda, T. Mamiya, H. Katagiri, T. Houtani, M. Nishi, T. Noda, T. Takahashi, T. Sugimoto, T. Nabeshima, H. Takeshima, Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors, Nature 394 (1998) 577–581.

[21] T.R. Stratford, M.R. Holahan, A.E. Kelley, Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake, Neuroreport 8 (1997) 423–426.
[22] J.D. Pomonis, C.J. Billington, A.S. Levine, Orphanin FQ, agonist of orphan opioid receptor ORL_1, stimulates feeding in rats, Neuroreport 8 (1996) 369–371.
[23] W. Bryant, J. Janik, M. Baumann, P. Callahan, Orphanin FQ stimulates prolactin and growth hormone release in male and female rats, Brain Res. 807 (1998) 228–233.
[24] N. Doi, M.B. Dutia, J.A. Russell, Inhibition of rat oxytocin and vasopressin supraoptic nucleus neurons by nociceptin in vitro, Neuroscience 84 (1998) 913–921.
[25] I.P. Berzetei-Gurske, R.W. Schwartz, L. Toll, Determination of activity for nociceptin in the mouse vas deferens, Eur. J. Pharmacol. 302 (1996) R1–R2.
[26] H.J. Patel, M.A. Giembycz, L. Spicuzza, P.J. Barnes, M.G. Belvisi, Naloxone-insensitive inhibition of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by the novel opioid, nociceptin, Br. J. Pharmacol. 120 (1997) 735–736.
[27] G. Calo', A. Rizzi, M. Bodin, W. Neugebauer, S. Salvadori, R. Guerrini, C. Bianchi, D. Regoli, Pharmacological characterization of nociceptin receptor: an in vitro study, Can. J. Physiol. Pharmacol. 75 (1997) 713–718.
[28] Fischer, W.G. Forssmann, B.J. Undem, Nociceptin-induced inhibition of tachykinergic neurotransmission in guinea pig bronchus, J. Pharmacol. Exp. Ther. 285 (1998) 902–907.
[29] S. Giuliani, C.A. Maggi, Inhibition of tachykinin release from peripheral endings of sensory nerves by nociceptin, a novel opioid peptide, Br. J. Pharmacol. 118 (1996) 1567–1569.
[30] S. Giuliani, C.A. Maggi, Prejunctional modulation by nociceptin of nerve-mediated inotropic responses in guinea-pig left atrium, Eur. J. Pharmacol. 332 (1997) 231–236.
[31] Z. Helyes, J. Nemeth, E. Pinter, J. Szolcsanyi, Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals, Br. J. Pharmacol. 121 (1997) 613–615.
[32] D.R. Kapusta, S.F. Sezen, J.K. Chang, H. Lippton, V.A. Kenigs, Diuretic and antinatriuretic responses produced by the endogenous opioid-like peptide, nociceptin (orphanin FQ), Life Sci. 60 (1997) PL15–PL21.
[33] S. Giuliani, M. Tramontana, A. Lecci, C.A. Maggi, Effect of nociceptin on heart rate and blood pressure in anaesthetized rats, Eur. J. Pharmacol. 333 (1997) 177–179.
[34] S. Giuliani, A. Lecci, M. Tramontana, C.A. Maggi, The inhibitory effect of nociceptin on the micturition reflex in anaesthetized rats, Br. J. Pharmacol. 124 (1998) 1566–1572.
[35] C. Mollereau, M.J. Simons, P. Soularue, F. Liners, G. Vassart, J.C. Meunier, M. Parmentier, Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene, Proc. Natl. Acad. Sci. USA 93 (1996) 8666–8670.
[36] H.P. Nothacker, R.K. Reinscheid, A. Mansour, R.A. Henningsen, A. Ardati, F.J. Monsma, S.J. Watson, O. Civelli, Primary structure and tissue distribution of the orphanin FQ precursor, Proc. Natl. Acad. Sci. USA 93 (1996) 8677–8682.
[37] E. Okuda-Ashitaka, T. Minami, S. Tachibana, Y. Yoshihara, Y. Nishiuchi, T. Kimura, S. Ito, Nocistatin, a peptide that blocks nociceptin action in pain transmission, Nature 392 (1998) 286–289.
[38] G.C. Rossi, J.P. Mathis, G.W. Pasternak, Analgesic activity of orphanin FQ2, murine prepro-orphanin FQ141-157 in mice, Neuroreport 9 (1998) 1165–1168.
[39] P.S. Portoghese, Bivalent ligands and the message–address concept in the design of selective opioid receptor antagonists, Trends Pharmacol. Sci. 10 (1989) 230–235.
[40] R. Schwyzer, Estimated conformation, orientation, and accumulation of dynorphin A-(1–13)-tridecapeptide on the surface of neutral lipid membranes, Biochemistry 25 (1986) 4281–4286.
[41] R.K. Reinscheid, J. Higelin, R.A. Henningsen, F.J. Monsma Jr., O. Civelli, Structures that delineate orphanin FQ and dynorphin A pharmacological selectivities, J. Biol. Chem. 273 (1998) 1490–1495.
[42] S. Lapalu, C. Moisand, J.L. Butour, C. Mollereau, J.C. Meunier, Different domains of the ORL_1 and kappa-opioid receptors are involved in recognition of nociceptin and dynorphin A, FEBS Lett. 427 (1998) 296–300.
[43] C.M. Topham, L. Mouledous, G. Poda, B. Maigret, J.C. Meunier, Molecular modelling of the ORL_1 receptor and its complex with nociceptin, Protein. Eng. 11 (1998) 1163–1179.
[44] C.T. Dooley, R.A. Houghten, Orphanin FQ: receptor binding and analog structure–activity relationships in rat brain, Life Sci. 59 (1996) PL23–PL29.
[45] J.L. Butour, C. Moisand, H. Mazarguil, C. Mollereau, J.C. Meunier, Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogs and opioids, Eur. J. Pharmacol. 321 (1997) 97–103.
[46] H. Okawa, B. Nicol, R. Bigoni, R.A. Hirst, G. Calo', R. Guerrini, D.J. Rowbotham, D. Smart, A.T. McKnight, D.G. Lambert, Comparison of the effects of Phe1psi-$(CH_2(NH)Gly^2]$nociceptin(1–13)NH_2 in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin recetors, Br. J. Pharmacol. 127 (1999) 123–130.
[47] R.K. Reinscheid, A. Ardati, F.J. Monsma Jr., O. Civelli, Structure–activity relationship studies on the novel neuropeptide orphanin FQ, J. Biol. Chem. 271 (1996) 14 163–14 168.
[48] J.P. Mathis, J. Ryan-Moro, A. Chang, J.S. Hom, D.A. Scheinberg, G.W. Pasternak, Biochemical evidence for orphanin FQ/nociceptin receptor heterogeneity in mouse brain, Biochem. Biophys. Res. Commun. 230 (1997) 462–465.
[49] K. Varani, G. Calo', A. Rizzi, S. Merighi, G. Toth, R. Guerrini, S. Salvadori, P.A. Borea, D. Regoli, Nociceptin receptor binding in mouse forebrain membranes: thermodynamic characteristics and structure–activity relationships, Br. J. Pharmacol. 125 (1998) 1485–1490.
[50] I.D. Adapa, L. Toll, Relationship between binding affinity and functional activity of nociceptin/orphanin FQ, Neuropeptides 31 (1997) 403–408.
[51] G. Calo', A. Rizzi, G. Bogoni, V. Neugebauer, S. Salvadori, R. Guerrini, C. Bianchi, D. Regoli, The mouse vas deferens: a pharmacological preparation sensitive to nociceptin, Eur. J. Pharmacol. 311 (1996) R3–R5.
[52] A. Rizzi, G. Calo', M. Trevisani, M. Tognetto, L. Fabbri, C. Mapp, R. Guerrini, S. Salvadori, D. Regoli, P. Geppetti, Nociceptin receptor activation inhibits tachykinergic non adrenergic non cholinergic contraction of guinea pig isolated bronchus, Life Sci. 64 (1999) PL157–PL163.
[53] J. Hughes, H.W. Kosterlitz, F.M. Leslie, Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics, Br. J. Pharmacol. 53 (1975) 371–381.
[54] E.A. Gyang, H.W. Kosterliz, Agonist and antagonist actions of morphine-like drugs on the guinea pig isolated ileum, Br. J. Pharmacol. 27 (1966) 514–527.
[55] D. Regoli, F. Gobeil, in: P. Geppetti, W. Muller-Esterl, D. Regoli (Eds.), Peptidergic G Protein Coupled Receptors, IOS Press, Amsterdam, 1999, pp. 64–77.
[56] D. Regoli, A. Boudon, J.L. Fauchere, Receptors and antagonists for substance P and related peptides, Pharmacol. Rev. 46 (1994) 551–599.
[57] J.S. Mogil, J.E. Grisel, R.K. Reinscheid, O. Civelli, J.K. Belknap, D.K. Grandy, Orphanin FQ is a functional anti-opioid peptide, Neuroscience 75 (1996) 333–337.

[58] J.S. Mogil, J.E. Grisel, G. Zhangs, J.K. Belknap, D.K. Grandy, Functional antagonism of mu-, delta- and kappa-opioid antinociception by orphanin FQ, Neurosci. Lett. 214 (1996) 131–134.
[59] M. Connor, C.W. Vaughan, B. Chieng, M.J. Christie, Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus neurones in vitro, Br. J. Pharmacol. 119 (1996) 1614–1618.
[60] M. Connor, A. Yeo, G. Henderson, The effect of nociceptin on Ca^{2+} channel current and intracellular Ca^{2+} in the SH-SY5Y human neuroblastoma cell line, Br. J. Pharmacol. 118 (1996) 205–207.
[61] J.L. Montiel, F. Cornille, B.P. Roques, F. Noble, Nociceptin/orphanin FQ metabolism: role of aminopeptidase and endopeptidase 24.15, J. Neurochem. 68 (1997) 354–361.
[62] J. Yu, B.T. Chait, L. Toll, M.J. Kreek, Nociceptin in vitro biotransformation in human blood, Peptides 17 (1996) 873–876.
[63] F. Noble, B.P. Roques, Association of aminopeptidase N and endopeptidase 24.15 inhibitors potentiate behavioral effects mediated by nociceptin/orphanin FQ in mice, FEBS Lett. 401 (1997) 227–229.
[64] J. Sandin, J. Georgieva, J. Silberring, L. Terenius, In vivo metabolism of nociceptin/orphanin FQ in rat hippocampus, Neuroreport 10 (1999) 71–76.
[65] M. Vlaskovska, L. Kasakov, P. Suder, J. Silberring, L. Terenius, Biotransformation of nociceptin/orphanin FQ by enzyme activity from morphine-naive and morphine-treated cell cultures (in process citation), Brain Res. 818 (1999) 212–220.
[66] R. Bigoni, S. Giuliani, G. Calo', A. Rizzi, R. Guerrini, S. Salvadori, D. Regoli, C.A. Maggi, Characterization of nociceptin receptors in the periphery: in vitro and in vivo studies, Naunyn. Schmiedebergs. Arch. Pharmacol. 359 (1999) 160–167.
[67] D.R. Kapusta, J.K. Chang, V.A. Kenigs, Central administration of [Phe^{1}Psi(CH$_2$(NH)Gly^2]Nociceptin(1–13)–NH$_2$ and orphanin FQ/Nociceptin (OFQ/N) produce similar cardiovascular and renal responses in conscious rats, J. Pharmacol. Exp. Ther. 289 (1999) 173–180.
[68] G. Calo', A. Rizzi, G. Marzola, R. Guerrini, S. Salvadori, L. Beani, D. Regoli, C. Bianchi, Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay, Br. J. Pharmacol. 125 (1998) 373–378.
[69] P. Madeddu, M.B. Salis, A.F. Milia, C. Emanueli, R. Guerrini, D. Regoli, G. Calo', Cardiovascular effects of nociceptin in unanesthetized mice, Hypertension 33 (1999) 914–919.
[70] R. Guerrini, G. Calo', A. Rizzi, C. Bianchi, L.H. Lazarus, S. Salvadori, P.A. Temussi, D. Regoli, Address and message sequences for the nociceptin receptor: a structure–activity study of nociceptin-(1–13)-peptide amide, J. Med. Chem. 40 (1997) 1789–1793.
[71] E. Albrecht, N.N. Samovilova, S. Oswald, I. Baeger, H. Berger, Nociceptin (orphanin FQ): high-affinity and high-capacity binding site coupled to low-potency stimulation of guanylyl-5′-*O*-(gamma-thio)-triphosphate binding in rat brain membranes, J. Pharmacol. Exp. Ther. 286 (1998) 896–902.
[72] G. Calo', R. Guerrini, R. Bigoni, A. Rizzi, C. Bianchi, D. Regoli, S. Salvadori, Structure–activity study of the nociceptin(1–13)–NH$_2$ *N*-terminal tetrapeptide and discovery of a nociceptin receptor antagonist, J. Med. Chem. 41 (1998) 3360–3366.
[73] Y. Shimohigashi, R. Hatano, T. Fujita, R. Nakashima, T. Nose, T. Sujaku, A. Saigo, K. Shinjo, A. Nagahisa, Sensitivity of opioid receptor-like receptor ORL$_1$ for chemical modification on nociceptin, a naturally occurring nociceptive peptide, J. Biol. Chem. 271 (1996) 23 642–23 645.
[74] C.T. Dooley, C.G. Spaeth, I.P. Berzetei-Gurske, K. Craymer, I.D. Adapa, S.R. Brandt, R.A. Houghten, L. Toll, Binding and in vitro activities of peptides with high affinity for the nociceptin/orphanin FQ receptor, ORL$_1$, J. Pharmacol. Exp. Ther. 283 (1997) 735–741.
[75] R. Schwyzer, in: D. Theodoropulos (Ed.), Peptides 1986, W. de Gruyter, New York, 1987, pp. 7–23.
[76] Chavkin, A. Goldstein, Specific receptor for the opioid peptide dynorphin: structure–activity relationships, Proc. Natl. Acad. Sci. USA 78 (1981) 6543–6547.
[77] M.F. Hibert, S. Trumpp-Kallmeyer, A. Bruinvels, J. Hoflack, Three-dimensional models of neurotransmitter G-binding protein-coupled receptors, Mol. Pharmacol. 40 (1991) 8–15.
[78] C.D. Strader, T.M. Fong, M.R. Tota, D. Underwood, R.A. Dixon, Structure and function of G protein-coupled receptors, Annu. Rev. Biochem. 63 (1994) 101–132.
[79] P.W. Schiller, Development of receptor-specific opioid peptide analogs, Prog. Med. Chem. 28 (1991) 301–340.
[80] V.J. Hruby, C.A. Gehrig, Recent developments in the design of receptor specific opioid peptides, Med. Res. Rev. 9 (1989) 343–401.
[81] J.S. Morley, Structure–activity relationships of enkephalin-like peptides, Annu. Rev. Pharmacol. Toxicol. 20 (1980) 81–110.
[82] Choi, T.F. Murray, G.E. DeLander, V. Caldwell, J.V. Aldrich, *N*-terminal alkylated derivatives of [D-Pro^{10}]dynorphin A-(1–11) are highly selective for kappa-opioid receptors, J. Med. Chem. 35 (1992) 4638–4639.
[83] J.S. Shaw, L. Miller, M.J. Turnbull, J.J. Gormley, J.S. Morley, Selective antagonists at the opiate delta-receptor, Life Sci. 31 (1982) 1259–1262.
[84] P.W. Schiller, G. Weltrowska, R. Schmidt, I. Berezowska, T.M. Nguyen, C. Lemieux, N.N. Chung, K.A. Carpenter, B.C. Wilkes, Subtleties of structure–agonist versus antagonist relationships of opioid peptides and peptidomimetics, J. Recept. Signal Transduct. Res. 19 (1999) 573–588.
[85] P.A. Temussi, S. Salvadori, P. Amodeo, C. Bianchi, R. Guerrini, R. Tomatis, L.H. Lazarus, D. Picone, T. Tancredi, Selective opioid dipeptides, Biochem. Biophys. Res. Commun. 198 (1994) 933–939.
[86] S.D. Bryant, S. Salvadori, P.S. Cooper, L.H. Lazarus, New delta-opioid antagonists as pharmacological probes, Trends Pharmacol. Sci. 19 (1998) 42–46.
[87] R. Guerrini, G. Calo', A. Rizzi, R. Bigoni, C. Bianchi, S. Salvadori, D. Regoli, A new selective antagonist of the nociceptin receptor, Br. J. Pharmacol. 123 (1998) 163–165.
[88] G. Calo', R. Guerrini, R. Bigoni, R. Rizzi, G. Marzola, H. Okawa, C. Bianchi, D.G. Lambert, S. Salvadori, D. Regoli, Characterization of [NPhe^1]NC(1–13)NH$_2$, a novel selective nociceptin receptor antagonist (1999), The 30th International Narcotics Research Conference meeting, S1-5, Saratoga Springs, NY, 10–15 July 1999.
[89] A. Mansour, M.T. Hoversten, L.P. Taylor, S.J. Watson, H. Akil, The cloned mu, delta and kappa receptors and their endogenous ligands: evidence for two opioid peptide recognition cores, Brain Res. 700 (1995) 89–98.
[90] P.W. Schiller, T.M. Nguyen, G. Weltrowska, B.C. Wilkes, B.J. Marsden, C. Lemieux, N.N. Chung, Differential stereochemical requirements of mu vs. delta opioid receptors for ligand binding and signal transduction: development of a class of potent and highly delta-selective peptide antagonists, Proc. Natl. Acad. Sci. USA 89 (1992) 11 871–11 875.
[91] J.T. Pelton, K. Gulya, V.J. Hruby, S.P. Duckles, H.I. Yamamura, Conformationally restricted analogs of somatostatin with high mu-opiate receptor specificity, Proc. Natl. Acad. Sci. USA 82 (1985) 236–239.
[92] C.T. Dooley, N.N. Chung, P.W. Schiller, R.A. Houghten, Acetalins: opioid receptor antagonists determined through the use of synthetic peptide combinatorial libraries, Proc. Natl. Acad. Sci. USA 90 (1993) 10 811–10 815.

[93] J.S. Morley, The design of antagonists of regulatory peptides, and comments on their specificity, Prog. Brain Res. 66 (1986) 333–340.
[94] K. Varani, A. Rizzi, G. Calo', R. Bigoni, G. Toth., R. Guerrini, S. Gessi, S. Salvadori, P.A. Borea, D. Regoli, Pharmacology of [Tyr^1]nociceptin analogs: receptor binding and bioassay studies, Naunyn. Schmiedebergs Arch. Pharmacol. (1999), in press.
[95] T. Kobayashi, K. Ikeda, S. Togashi, N. Itoh, T. Kumanishi, Effects of sigma ligands on the nociceptin/orphanin FQ receptor co-expressed with the G-protein-activated K^+ channel in Xenopus oocytes, Br. J. Pharmacol. 120 (1997) 986–987.
[96] Y. Noda, T. Mamiya, T. Nabeshima, M. Nishi, M. Higashioka, H. Takeshima, Loss of antinociception induced by naloxone benzoylhydrazone in nociceptin receptor-knockout mice, J. Biol. Chem. 273 (1998) 18 047–18 051.
[97] E. Schlicker, S. Werthwein, M. Kathmann, U. Bauer, Nociceptin inhibits noradrenaline release in the mouse brain cortex via presynaptic ORL_1 receptors, Naunyn. Schmiedebergs Arch. Pharmacol. 358 (1998) 418–422.
[98] S. Shah, C.P. Page, D. Spina, Nociceptin inhibits non-adrenergic non-cholinergic contraction in guinea-pig airway, Br. J. Pharmacol. 125 (1998) 510–516.
[99] X. Chu, N. Xu, P. Li, J.Q. Wang, The nociceptin receptor-mediated inhibition of the rat rostral ventrolateral medulla neurons in vitro, Eur. J. Pharmacol. 364 (1999) 49–53.
[100] P. Gressens, G. Calo', P. Evrard, Nociceptin potentiates neonatal white matter excitotoxicity, Regul. Pept. 80 (1999) 123.
[101] M.C. Olianas, C. Maullu, A. Ingianni, P. Onali, [Phe$^1\Psi$(CH_2–NH)Gly^2]nociceptin-(1–13)–NH_2 acts as a partial agonist at ORL_1 receptor endogenously expressed in mouse N1E-115 neuroblastoma cells, Neuroreport 10 (1999) 1127–1131.
[102] Siniscalchi, S. Sbrenna, D. Rodi, L. Beani, C. Bianchi, Inhibitory effect of nociceptin on [^{3}H]5-HT release from the rat cerebral cortex in vitro, Br. J. Pharmacol. 126 (1999) 266P.
[103] I.S. Xu, Z. Wiesenfeld-Hallin, X.J. Xu, [Phe$^1\Psi$(CH_2–NH)Gly^2]-nociceptin-(1–13)–NH_2, a proposed antagonist of the nociceptin receptor, is a potent and stable agonist in the rat spinal cord, Neurosci. Lett. 249 (1998) 127–130.
[104] J.E. Grisel, D.E. Farrier, S.G. Wilson, J.S. Mogil, [Phe$^1\Psi$(CH_2–NH)Gly^2](nociceptin-(1–13)–NH_2 acts as an agonist of the orphanin FQ/nociceptin receptor in vivo, Eur. J. Pharmacol. 357 (1998) R1–3.
[105] S. Candeletti, R. Guerrini, G. Calo', S. Ferri, Effects of the nociceptin receptor antagonist [Phe$^1\Psi$(CH_2NH)Gly^{2}NC(1–13)NH_2 on nociception in rats (1998), in 29th International Narcotics Research Conference meeting A170, Garmisch-Partenkirchen, Germany, 20–25 July, 1998.
[106] K.J. Carpenter, A.H. Dickenson, Evidence that [Phe$^1\Psi$(CH_2–NH)Gly^2]-nociceptin(1–13)NH_2, a peripheral ORL_1 receptor antagonist, acts as an agonist in the rat spinal cord, Br. J. Pharmacol. 125 (1998) 949–951.
[107] J.L. Butour, C. Moisand, C. Mollereau, J.C. Meunier, [Phe$^1\Psi$(CH_2–NH)Gly^2]-nociceptin-(1–13)NH_2 is an agonist of the nociceptin (ORL_1) receptor, Eur. J. Pharmacol. 349 (1998) R5–R6.
[108] S. Wnendt, T. Kruger, E. Janocha, D. Hildebrandt, W. Englberger, Agonistic effect of buprenorphine in a nociceptin/OFQ receptor-triggered reporter gene assay, Mol. Pharmacol. 56 (1999) 334–338.
[109] R.J. Simon, R.S. Kania, R.N. Zuckermann, V.D. Huebner, D.A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C.K. Marlowe, et al., Peptoids: a modular approach to drug discovery, Proc. Natl. Acad. Sci. USA 89 (1992) 9367–9371.
[110] D.C. Horwell, The 'peptoid' approach to the design of non-peptide, small molecule agonists and antagonists of neuropeptides, Trends Biotechnol. 13 (1995) 132–134.
[111] R.N. Zuckermann, E.J. Martin, D.C. Spellmeyer, G.B. Stauber, K.R. Shoemaker, J.M. Kerr, G.M. Figliozzi, D.A. Goff, M.A. Siani, R.J. Simon, et al., Discovery of nanomolar ligands for 7-transmembrane G-protein-coupled receptors from a diverse *N*-(substituted)glycine peptoid library, J. Med. Chem. 37 (1994) 2678–2685.
[112] H. Okawa, R.A. Hirst, D. Smart, A.T. McKnight, D.G. Lambert, Studies on the coupling of recombinant ORL_1 receptors to adenylyl cyclase, Br. J. Pharmacol. 123 (1998) 218P.
[113] U. Bauer, M. Nakazi, M. Kathmann, M. Gothert, E. Schlicker, The stereoselective kappa-opioid receptor antagonist Mr 2266 does not exhibit stereoselectivity as an antagonist at the orphan opioid (ORL_1) receptor, Naunyn. Schmiedebergs Arch. Pharmacol. 359 (1999) 17–20.
[114] G.J. Moore, Designing peptide mimetics, Trends Pharmacol. Sci. 15 (1994) 124–129.
[115] D.C. Rees, Chemical structures and biological activities of non-peptide selective kappa opioid ligands, Prog. Med. Chem. 29 (1992) 109–139.
[116] S. Ozaki, H. Kawamoto, Y. Ito, K. Hayashi, Y. Iwasawa, K. Hirano, Preparation of 2-oxaimidazole derivatives as pharmaceuticals, WO9854168-A1 (1998), Japan; C.A. 130:52418q (1999).
[117] F. Jenck, F. Monsma, G. Galley, G. Adam, A. Cesura, S. Rover, J. Wichmann, Preparation of 8-benzocycloalkyl-1,3,8-triazaspiro-[4,5]decan-4-ones as orphanin FQ receptor ligands, EP856514-A1 (1998); C.A. 131:58825n (1999).