British Journal of Pharmacology (2000) 129, 1261–1283

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REVIEW

Pharmacology of nociceptin and its receptor: a novel therapeutic target

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Nociceptin (NC), alias Orphanin FQ, has been recently identified as the endogenous ligand of the opioid receptor-like 1 receptor (OP_4). This new NC/OP_4 receptor system belongs to the opioid family and has been characterized pharmacologically with functional and binding assays on native (mouse, rat, guinea-pig) and recombinant (human) receptors, by using specific and selective agonists (NC, NC(1–13)NH_2) and a pure and competitive antagonist, [Nphe^1]NC(1–13)NH_2. The similar order of potency of agonists and affinity values of the antagonist indicate that the same receptor is present in the four species. OP_4 is expressed in neurons, where it reduces activation of adenylyl cyclase and Ca^{2+} channels while activating K^+ channels in a manner similar to opioids. In this way, OP_4 mediates inhibitory effects in the autonomic nervous system, but its activities in the central nervous system can be either similar or opposite to those of opioids. *In vivo* experiments have demonstrated that NC modulates a variety of biological functions ranging from nociception to food intake, from memory processes to cardiovascular and renal functions, from spontaneous locomotor activity to gastrointestinal motility, from anxiety to the control of neurotransmitter release at peripheral and central sites. These actions have been demonstrated using NC and various pharmacological tools, as antisense oligonucleotides targeting OP_4 or the peptide precursor genes, antibodies against NC, an OP_4 receptor selective antagonist and with data obtained from animals in which the receptor or the peptide precursor genes were knocked out. These new advances have contributed to better understanding of the pathophysiological role of the NC/OP_4 system, and ultimately will help to identify the therapeutic potential of new OP_4 receptor ligands.
British Journal of Pharmacology (2000) **129,** 1261–1283

Keywords: Nociceptin/orphanin FQ; nociceptin receptor; recombinant and native receptors; agonists; antagonists; receptor characterization and classification

Abbreviations: CHO, Chinese hamster ovary; EFS, electrical field stimulation; [F/G]NC(1–13)NH_2, [Phe$^1\psi$(CH_2NH)Gly^2]nociceptin(1–13)NH_2; NC, nociceptin/orphanin FQ; [Nphe^1]NC(1–13)NH_2, [Nphe^1]nociceptin(1–13)NH_2; OP_4, nociceptin receptor

Introduction

The history of the NC/OP_4 receptor system began in 1992, with the cloning of the OP_1 (δ) receptor (Evans *et al*., 1992; Kieffer *et al*., 1992), followed shortly by that of the OP_2 (κ) and OP_3 (μ) receptors (Chen *et al*., 1993; Yasuda *et al*., 1993). These three opioid receptors show about 60% homology. Further screening of cDNA libraries with low stringency oligonucleotide probes led, in 1994, to the discovery of an 'opioid receptor like' (ORL_1) sequence by several investigators (Bunzow *et al*., 1994; Fukuda *et al*., 1994; Mollereau *et al*., 1994; Nishi *et al*., 1994; Wang *et al*., 1994). This new receptor shows overall 60% homology with the opioid receptors (80% in the 2nd, 3rd and 7th TM domains), much less in the N-terminal and some extracellular loops and again high homology in some intracellular loops. ORL_1 shows substantial sequence identities (>90%) between species variants, namely the human (Mollereau *et al*., 1994), rat (Bunzow *et al*., 1994; Chen *et al*., 1994; Lachowicz *et al*., 1995; Wang *et al*., 1994), mouse (Nishi *et al*., 1994) and pig (Osinski *et al*., 1999a). Therefore, on structural grounds, the ORL_1 and opioid receptors belong to the same family. Initial pharmacology (before NC was actually discovered) showed that the ORL_1 receptor, stably transfected into Chinese hamster ovary (CHO) cells, could be activated by the nonselective opioid receptor agonist ethorphine and blocked by the opioid receptor antagonist diprenorphine, each at micromolar concentrations (Mollereau *et al*., 1994). Naloxone however, a nonselective opioid receptor antagonist showed very little affinity, if any, for ORL_1 (Mollereau *et al*., 1994). ORL_1 is considered by some experts as an opioid receptor and has been given (according to the new nomenclature for opioid receptors: Dhawan *et al*., (1996)) the name OP_4 (Hamon, 1998), while in other classifications ORL_1 has been left out from the opioid family of receptors (Dhawan *et al*., 1998), or included as ORL_1 (Receptors and ion channel nomenclature supplement, *Trends Pharmacol. Sci.*, 1999). In peripheral tissues, the distribution of ORL_1 (intestine, vas deferens, spleen, etc.), the cell type(s) that express ORL_1 (predominantly neurons) and the cellular mechanisms of action (activation of K^+ and inhibition of Ca^{2+} channels, inhibition of cyclic AMP accumulation) are probably the same as for opioid receptors and may derive from the activation of the same type of G proteins (see Meunier, 1997 for a review). The actions of ORL_1 receptors in peripheral tissues are indeed very similar to those evoked by opioid receptors. The distribution of ORL_1 in the brain (rat, mouse) (Anton *et al*., 1996; Ikeda *et al*., 1998; Monteillet-Agius *et al*., 1998; Neal *et al*., 1999), as evaluated by immunohystochemistry and *in situ*

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hybridization, is widespread but expression is particularly high in cortico-limbic areas, hypothalamus, various brain stem nuclei, and spinal cord. The distribution of ORL_1 appear to be similar in some and different in other neuronal circuits as the opioids receptors. These anatomical similarities and differences may help understanding why ORL_1 and opioid receptors mediate similar, different and even opposite effects in the central nervous system (see section on 'Biological activities').

ORL_1 did not remain an orphan receptor for long, since only a year after its identification, towards the end of 1995, the endogenous ligand was identified, simultaneously, by two groups of investigators. Whereas the French group named it nociceptin (Meunier *et al*., 1995), the Swiss group called it orphanin FQ (Reinscheid *et al*., 1995). It is a heptadecapeptide containing several cationic residues (see primary structure in Table 1), which has rapidly become the target of numerous investigators. The current review attempts to focus on the pharmacological issues revealed by the already more than 350 papers published so far on the NC/OP_4 system.

Peptide and receptor nomenclature

In 1987 the International Union of Pharmacology (IUPHAR) established a Nomenclature Committee for mammalian receptors, recommending that a given receptor should be named after its endogenous ligand(s), attributed a progressive number (1, 2, etc) to identify the different receptor types, and that the use of Greek symbols as well as of the letter R (to designate receptor) should be avoided (see Girlestone, 1998). In 1996, the Opioid Receptor Sub Committee proposed a new nomenclature consistent with the IUPHAR guidelines (Dhawan *et al*., 1996). Thus the members of the opioid receptor family should be indicated as OP (for Opioid Peptides) with numeric subscripts indicating the chronological cloning of the receptor types, i.e. OP_1 OP_2 and OP_3 corresponding to the δ-, κ-, and μ-opioid receptor types, respectively. This proposal has been ignored, however, by most of the authors publishing in this field. The situation has been further complicated after the identification of NC and its receptor. In fact, on structural and transductional grounds, the receptor for NC should be viewed as a member of the opioid receptor family which, however, is insensitive to naloxone while interancting with other opioid receptor ligands, such as naloxone benzoylhydrazone (NalBzOH) and buprenorphine. To date, the following names and abbreviations have been used for the peptide: nociceptin (NC, Noc, Noci), orphanin FQ (OFQ or oFQ), nociceptin/orphanin FQ (N/OFQ). A discussion of the advantage and disadvantages of the name nociceptin vs orphanin FQ may be found in a recent review by Civelli *et al*. (1998). As for its receptor, it has been differently named opioid like receptor 1 (ORL_1), nociceptin receptor (NCR or NocR), orphanin FQ receptor (OFQR), nociceptin-orphanin FQ receptor (NOR, in analogy with the MOR, DOR and KOR terminology) and finally OP_4 (in line with the recent IUPHAR recommendations, Hamon (1998).

These issues have recently been debated at the International Narcotics Research Conference meeting in Saratoga Springs (July 10–15, 1999) during a short symposium on opioid receptor nomenclature chaired by B. Cox and C. Chavkin, in order to collect suggestions for a unified nomenclature in line with the new edition of the IUPHAR Compendium of receptor characterisation and classification, which will be published in the near future. It is hoped that the experts in the field will

Table 1 Chemical names and structures of opoid receptor ligands

Name	*Chemical structure*	*MW*	*Reference*
Morphine	(5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol	285.3	Gulland & Robinson, 1923
Naloxone	(5α)-4,5-epoxy-3,14-dihydroxy-17-(2-propenyl)-morphinon-6-one	327.4	Olofson *et al*., 1977
[Leu^5]enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH	555.6	Hughes *et al*., 1975b
[Met^5]enkephalin	H-Tyr-Gly-Gly-Phe-Met-OH	573.6	Hughes *et al*., 1975b
DPDPE	H-Tyr-c(D-Pen-Gly-Phe-D-Pen)-OH	645.8	Mosberg *et al.,* 1983
Naltrindole	17, cyclopropilmethyl-6,7-deihydro-4,5-epoxy-3,14-dihydroxy-6,7,2′,3′-indolmorphinan	451.0	Portoghese *et al*., 1988
Dynorphin A	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH	2147.5	Chavkin *et al*., 1982
Dynorphin A(1-8)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-OH	981.2	Corbett *et al*., 1982
U69593	(5α,7α,8β)-(-)-N-methyl-2-phenyl-N-[7-(pirrolidin-1-yl)-1-oxaspiro-4,5-dec-8-yl]-phenyl-acetamide	356.3	Lahti *et al*., 1985
nor-Binaltorphimine	17,17′-bis(cyclopropylmethyl)-6-6′,7,7′-tetra-dehydro-4,5α4′,5′α-diepoxy-6,6′-imino-7,7′-bimorphinan-3,3′,14,14′-tetrol	734.7	Portoghese *et al*., 1987
β-endorphin	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gln-OH	3438.0	Li & Chung, 1976
Endomorphin 1	H-Tyr-Pro-Trp-Phe-NH_2	610.7	Zadina *et al*., 1997
Endomorphin 2	H-Tyr-Pro-Phe-Phe-NH_2	571.7	Zadina *et al*., 1997
DAMGO	H-Tyr-D-Ala-Gly-MePhe-Gly-ol	513.6	Handa *et al*., 1981
CTOP	H-D-Phe-c(Cys-Thr-D-Trp-Orn-Thr-Pen)-Thr-NH_2	1062.4	Pelton *et al*., 1985
NC	H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH	1809.1	Meunier *et al*., 1995; Reinscheid *et al*., 1995
NC(1-13)NH_2	H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	1382.0	Calo' *et al*., 1996; Dooley & Houghten, 1996
Roche compound	9-(8-chloro-1,2,3,4-tetrahydro-napthyl-2)-1-phenyl-1,3,8-traza-spiro[4,5]decan-4 one	396.0	Adam *et al*., 1998
Ac-RYYRWK-NH_2	Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2	1012.1	Dooley *et al*., 1997
[F/G]NC(1-13)NH_2	H-PheΨ(CH_2-NH)Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	1368	Guerrini *et al*., 1998
[Nphe^1]NC(1-13)NH_2	H-Nphe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-NH_2	1382	Calo' *et al*. 2000
NalBzOH	6-desoxy-6-benzoylhydrazido-N-allyl-14-hydroxydihydronomorphinone	355.4	Luke *et al*., 1988
Banyu compound	1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one	399.6	Ozaki *et al*., 1998

accept the NC/OP_4 nomenclature that is utilized throughout this article.

Basic pharmacological tools and features of OP_1, OP_2, OP_3, and OP_4 receptors

The three opioid receptors are indicated as OP_1, OP_2, and OP_3 (Dhawan *et al*., 1996) by the current nomenclature recommended by IUPHAR, and by the former nomenclature in Greek letter (δ, κ, and μ); OP_4 is also indicated by the initial name ORL_1. The cloned human receptors are identified by their genetic code; they are 7TM receptors coupled with $G_{i/o}$ proteins. Endogenous ligands that show some selectivity for one or the other receptor are listed, together with the shortest sequences of natural peptides that maintain consistent biological activities (Table 2). While enkephalins (OP_1) and endomorphins (OP_3) do not contain cationic residues, the shortest active sequences that activate OP_2 and OP_4, namely Dyn(1–8) and NC(1–13)NH_2, have two (Arg^6–Arg^7) and four (Arg^8, Lys^9, Arg^{12}, Lys^{13}) positively charged groups respectively that are pivotal for peptide-receptor interaction. While all natural ligands for OP_1, OP_2 and OP_3 receptors have Tyr^1, which is instrumental for the opioid receptor activation, ligands of the OP_4 receptor have a Phe at the N-terminal end (Table 1). For all receptors, at least one synthetic selective agonist (a few non-peptide or pseudopeptides) is available (see chemical names and structures in Table 1). For each receptor, at least one antagonist is listed: these compounds are non peptides (natrindole and nor-BNI) or pseudopeptides (CTOP and [$Nphe^1$]NC(1–13)NH_2). Non peptide ligands (both agonist and antagonists) for OP_4 receptors have already been identified (see chemical names and structures in Table 1).

The potencies of all compounds included in Table 2 have been established with biological assays and are indicated in brackets. Naloxone acts as an antagonist of OP_1, OP_2, and OP_3 receptors with different potencies (pA_2), but is inactive on OP_4 receptor.

The endogenous ligand for the OP_4 receptor

The same heptadecapeptide isolated by Meunier *et al*. (1995) and Reinscheid *et al*. (1995) is encoded in the gene sequences of several species, even though there are differences in the NC precursor identified in the mouse (Houtani *et al*., 1996; Mollereau *et al*., 1996; Pan *et al*., 1996), rat (Mollereau *et al*., 1996; Nothacker *et al*., 1996) and man (Mollereau *et al*., 1996; Nothacker *et al*., 1996). The NC precursor consists of a 181 amino acids in the rat, 176 amino acids in humans, and 187 amino acids in the mouse (Mollereau *et al*., 1996; Nothacker *et al*., 1996). Analysis of the nucleotide sequence of the preproNC gene revealed structural and organisational characteristics very similar to those of the opioid peptide precursors, in particular preproenkephalin and preprodynorphin, suggesting that these peptide precursors may derive from a common ancestor (Mollereau *et al*., 1996; Nothacker *et al*., 1996). In the preproNC sequence there are several pairs of basic amino acids that represent possible sites of cleavage for precursor maturation. Therefore, several biologically relevant peptides may derive from the NC precursor. The structure of the NC precursor and its components is given in Figure 1A. In particular, two peptides which are potentially derived from the NC precursor have been synthesized and evaluated for biological activity. Neither of them bind to the OP_4 receptor (Mollereau *et al*., 1996; Nothacker *et al*., 1996). The first is, like NC, an heptadecapeptide terminating with the couple FG (orphanin FQ2), which has been found to be biologically active, stimulating locomotor activity in mice (Florin *et al*., 1997), inducing antinociception both spinally and supraspinally (Rossi *et al*., 1998), and inhibiting gastrointestinal transit (Rossi *et al*., 1998). The second peptide, named nocistatin, has been reported to act as a functional antagonist of NC (Okuda-Ashitaka *et al*., 1998). In most studies, nocistatin was found to be inactive per se, but was able to reverse several effects of NC, such as induction of allodynia after spinal administration in mice (Minami *et al*., 1998; Okuda-Ashitaka *et al*., 1998), inhibition of glutamate release from rat brain slices (Nicol *et al*., 1998a), impairment of learning and memory in mice (Hiramatsu & Inoue, 1999). Moreover, nocistatin can, *per se*, cause antinociception after i.c.v. administration in the rat carrageenan test (Nakagawa *et al*., 1999) or after i.t. administration in the rat formalin test (Yamamoto & Sakashita, 1999).

Biologically active peptides are brought to maturation by enzymes of the furin group, which cut the amino acid sequence at pairs of cationic residues. Once released from the neurons, NC is further metabolized to inactive fragments by different types of proteolytic enzymes. Montiel *et al*. (1997) studied NC metabolism in mouse brain cortical slices and reported that NC inactivation is due to the action of aminopeptidase N (APN) which releases NC(2–17), a C-terminal fragment which does not bind to OP_4 receptor sites (Dooley & Houghten, 1996), and of endopeptidase 24.15 (EP 24.15) which by acting at the peptide bonds Ala^7–Arg^8, Ala^{11}–Arg^{12}, and Arg^{12}–Lys^{13}, also releases inactive compounds. On the other hand,

Table 2 Opoid receptors and their ligands

Receptor name	*OP_1*	*OP_2*	*OP_3*	*OP_4*
Previous name	δ	κ	μ	ORL_1
Coupling	$G_{i/o}$	$G_{i/o}$	$G_{i/o}$	$G_{i/o}$
Structure/7TM	h372 P41143	h380 P41145	h400 $P35372^{AS}$	g370 $P41146^{AS}$
Natural ligands	Leu-enkephalin (7.9)	dynorphin (8.5)	β-endorphins (7.5)	nociceptin (7.9)
	Met-enkephalin (7.9)	dynorphin(1-8) (7.9)	endomorphins (8.4)	
Synthetic agonists	DPDPE	U69593	DAMGO	NC(1-13)NH_2
	(8.4)	(7.2)	(8.4)	(7.9)
Selective antagonists	Natrindole	Nor-BNI	CTOP	[$Nphe^1$]NC(1-13)NH_2
	(9.2)	(10)	(8.0)	(6.0)
Non-selective	Naloxone	Naloxone	Naloxone	Naloxone
antagonists	(7.5)	(8.0)	(8.8)	Inactive

Naloxone acts as receptor antagonist at OP_1, OP_2, OP_3 but not OP_4 sites. Non peptide agonists and antagonists for OP_4 receptors have been recently described and patented by Hoffman La Roche (Adam *et al*., 1998) and Banyu (Ozaki *et al*., 1998), respectively pEC_{50} and pA_2 values are indicated in brackets for agonists and antagonists, respectively: these values have been obtained in the electrically stimulated mouse vas deferens (OP_1 and OP_4), rabbit vas deferens (OP_2) and guinea pig ileum (OP_3) in our and other laboratories (Calo' *et al*., 1997; 2000; Corbett *et al*., 1982; Guerrini *et al*., 1998; Schiller *et al*., 1992; Zadina *et al*., 1997).

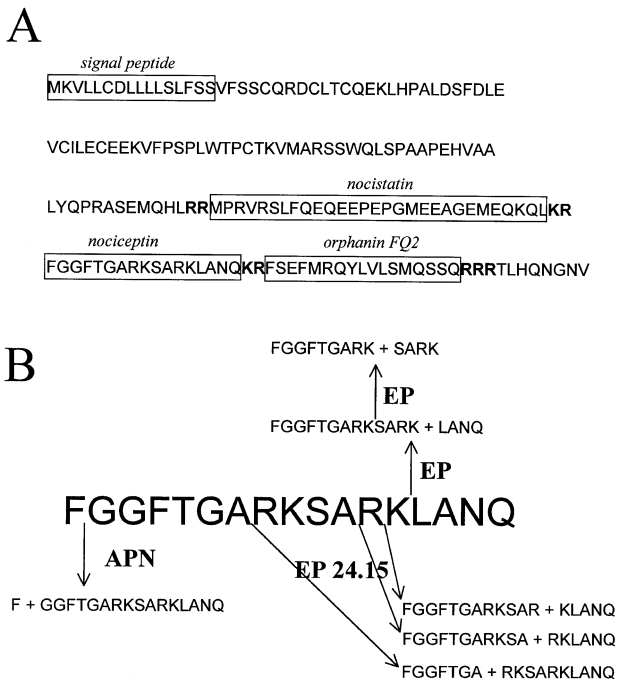

Figure 1 (A) the NC precursor. (B) NC metabolism. APN, aminopeptidase N; EP endopeptidase.

Table 3 Affinities and potencies of OP_4 receptor agonists and antagonists on native and recombinant receptors

	Receptor binding (pKi)		*Functional assay* (pEC_{50}/pA_2)	
Preparation	CHO_{hOP4}	*mBM*	CHO_{hOP4}	*mVD*
NC	9.7	8.7	9.8	7.8
$NC(1\text{-}13)NH_2$	10.4	9.1	9.5	7.9
$NC(1\text{-}9)NH_2$	8.1	<5	6.7	<5
$[Nphe^1]NC(1\text{-}13)NH_2$	8.4	6.9	6.0	6.0

CHO_{hOP4}: Chinese hamster ovary cells expressing the human recombinant OP_4 receptor; mBM: mouse brain membranes; mVD: mouse vas deferens. Receptor binding data have been obtained in CHO_{hOP4} and mouse brain membranes using as a radioligand $[^{125}I]Tyr^{14}NC$ and $[^3H]NCNH_2$, respectively. Functional assays were inhibition of forskolin-stimulated cyclic AMP accumulation and inhibition of electrically-induced twitches, respectively, in CHO_{hOP4} cells and isolated mouse vas deferens. The data are from the following papers: Calo' *et al*., 2000; Okawa *et al*., 1999; Varani *et al*., 1998. pK_i: the negative logarithm to base 10 of the dissociation equilibrium constant measured in displacement studies. pEC_{50}: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. pA_2: the negative logarithm to base 10 of the molar concentration of the antagonist that makes it necessary to double the concentration of agonist needed to elicit the original submaximal response.

endopeptidase 24.11 (enkephalinase) seems to not be involved in the degradation of NC. These findings were later confirmed by showing that a mixture of inhibitors of endopeptidase 24.15 and APN potentiated the behavioural effects of NC in mice (Noble & Roques, 1997). The critical role of aminopeptidase in NC metabolism has also been suggested by Yu *et al*. (1996), who studied NC biotransformation in human blood. NC metabolism has also been evaluated *in vivo* in the rat hippocampus (Sandin *et al*., 1999), where the peptide is first converted to NC(1–13) and then, probably by the same enzyme, to NC(1–9). Similar results were also obtained *in vitro* using enzymes from different cell cultures, namely U1690 human lung carcinoma cells, SHSY5Y human neuroblastoma cells, and primary culture from rat brain cortex cells (Vlaskovska *et al*., 1999). Metabolic pathways of NC are indicated in Figure 1B.

Pharmacological characterization of the NC/OP_4 receptor system

Basic pharmacological parameters for receptor characterisation include pK_i and pEC_{50}/pA_2 (for agonists and antagonists, respectively) values determined in binding and functional assays on recombinant and native receptors. An example is presented in Table 3, where NC, $NC(1\text{–}13)NH_2$ (the template we have adopted for structure-activity studies, see below), $NC(1\text{–}9)NH_2$ and a recently identified OP_4 antagonist, $[Nphe^1]NC(1\text{–}13)NH_2$ (Calo' *et al*., 2000; Guerrini *et al*., 1999) have been studied in CHO cells expressing the human recombinant OP_4 receptor (CHO_{hOP4}) and in native mouse OP_4 receptors. Using a few agonists and one antagonist, we were able to characterize OP_4 receptor with the two classical pharmacological criteria: the order of potency of agonists and the affinity of a competitive antagonist (Schild, 1973). The two receptors analysed in Table 3 appear to be of the same type since both criteria are fulfilled and, in particular, the absolute value of the antagonist affinity is the same in the two functional assays. Major differences (>1.0 log units) are observed for agonist affinities and activities: they may be attributed to differences in transduction coupling between receptors expressed in transfected cells and in the isolated organ. In the former preparation, agonists are more potent by at least 1.5 log units. Methodological details of these assays are to be found in the publications quoted in the footnotes of Table 3, which also provide definitions of the three pharmacological parameters, according to the IUPHAR recommendations (Jenkinson *et al*., 1995).

A detailed characterization of native OP_4 receptors from various species (mouse, guinea-pig, and rat) is presented in Table 4. With minor discrepancies, the three couples of binding and functional assays depicted show the same order of potency of agonists, namely

$$NCNH_2 \geq NC(1-13)NH_2 \geq NC > NC(1-12)\ NH_2 >> NC(1-9)NH_2$$

and very similar pA_2 values for $[Nphe^1]NC(1\text{–}13)NH_2$ (6.0–6.4) and for $[F/G]NC(1\text{–}13)NH_2$ (6.8–7.0). In contrast to $[Nphe^1]NC(1\text{–}13)NH_2$, which did not show any residual agonist activity in these preparations, $[F/G]NC(1\text{–}13)NH_2$ displayed a small but consistent residual agonistic activity, especially in the rat vas deferens (Bigoni *et al*., 1999a; Okawa *et al*., 1999). It is therefore proposed that the OP_4 receptor is the same in the mouse, the guinea-pig, and the rat.

Results obtained on the human OP_4 receptor transfected into CHO cells are presented in Table 5. With minor discrepancies, the order of potency of agonists and the value of antagonistic potency of $[Nphe^1]NC(1\text{–}13)NH_2$ (pA_2 6.0) evaluated in the functional assay are the same as those determined in other animal tissues. Interestingly, $[F/G]NC(1\text{–}13)NH_2$ acts as a full agonist in CHO_{hOP4} cells (Butour *et al*., 1998; Okawa *et al*., 1999; Wnendt *et al*., 1999). A detailed discussion of the dual behaviour of this compound is presented in the section 'The OP_4 receptor ligand $[F/G]NC(1\text{–}13)NH_2$'. Taken together these data are consistent with the proposal that the OP_4 receptors present in four different species are the same pharmacological entity.

Table 4 Receptor binding and biological activities of nociceptin and nociceptin-related peptides at native OP_4 receptors of the mouse, the guinea pig and the rat

Species	*Mouse*		*Guinea-pig*		*Rat*	
Technique	*Binding*	*Bioassay*	*Binding*	*Bioassay*	*Binding*	*Bioassay*
Preparation	*Forebrain membranes*	*Vas deferens*	*Brain membranes*	*Ileum*	*Brain membranes*	*Vas deferens*
	pK_i	pEC_{50}/pA_2	pK_i	pEC_{50}/pA_2	pK_i	pEC_{50}/pA_2
Agonists						
NC	8.7	7.8	8.6	8.1	10.3	7.2
desPhe^{1}NCNH$_2$	ND	< 5	ND	< 5	ND	< 5
NCNH$_2$	9.1	8.0	8.9	8.1	9.6	7.8
NC(1-13)NH$_2$	9.1	7.9	9.0	8.1	9.7	6.9
NC(1-12)NH$_2$	7.6	6.1	ND	7.1	9.4	6.2
NC(1-11)NH$_2$	5.7	5.5	ND	5.3	8.5	5.0
NC(1-9)NH$_2$	< 5	< 5	< 5	< 5	7.8	< 5
NC(1-5)NH$_2$	< 5	< 5	< 5	< 5	ND	< 5
Antagonists						
[F/G]NC(1-13)NH$_2$	8.0	6.8	7.9	7.0	9.3	6.8
[Nphe^1]NC(1-13)NH$_2$	6.9	6.0	ND	6.4	8.1	6.2
naloxone	< 5	< 6	< 5	< 6	< 6	< 6

In binding experiments the radioligands were [^{3}H]NCNH$_2$ (mouse and guinea pig) and [^{125}]Tyr^{14}NC (rat). pK_d and B_{max} (fmol mg prot^{-1}) were 9.3 and 94 mouse; 9.0 and 320 guinea-pig; 10.3 and 180 rat. ND: not determined. pEC_{50}, pA_2, pK_i as in Table 3. The data are from Bigoni *et al*., 1999a; Calo' *et al*., 1997; 2000; Okawa *et al*., 1999; Varani *et al*., 1998; 1999.

The comparison of the absolute values obtained in binding and functional assays reveals major differences between the affinity constants (pK_i values) measured in binding assays and the pEC_{50}/pA_2 values determined in functional assays. The pK_i values are consistently higher by 0.5 – 1.5 log units in the mouse and guinea-pig preparations, where a tritiated ligand has been used, whereas they were 1.8 – 3.5 log units higher in the rat and human preparations, where binding was measured with an iodinated ligand. The pK_i values of near ten obtained in the rat and human receptor with NC are at least 1.5 log units higher than those estimated in the mouse and guinea-pig preparations. Further studies and the use of the tritiated ligands in the human and rat receptors are needed before considering the possibility of any species-related OP_4 receptor subtypes.

Table 5 Receptor binding and biological activities of nociceptin and nociceptin-related peptides at recombinant human OP_4 receptors expressed in CHO cells

Preparation	*CHO_{hOP4}*	
Technique	*Binding*	*Cyclic AMP assay*
	pK_i	pEC_{50}/pA_2
Agonists		
NC	9.7	9.8
NCNH$_2$	9.6	9.8
NC(1-13)NH$_2$	10.4	9.5
NC(1-12)NH$_2$	8.7	8.5
NC(1-11)NH$_2$	8.2	7.5
NC(1-9)NH$_2$	8.1	6.7
[F/G]NC(1-13)NH$_2$	10.2	8.6
Antagonists		
[Nphe^1]NC(1-13)NH$_2$	8.4	6.0
Naloxone	< 6	< 6

CHO_{hOP4}: Chinese hamster ovary cells expressing the human recombinant OP_4 receptor. The radioligand used was [^{125}I]Tyr^{14}NC (pK_d9.7; B_{max} 1700 fmol mg prot^{-1}). pEC_{50}, pA_2, pK_i as in Table 3. The data are from the following papers: Calo' *et al*., 2000; Okawa *et al*., 1999.

In vitro *functional assays in isolated organs*

In experiments designed to (a) characterize the myotropic responses of isolated organs to NC and related peptides, (b) determine the type of action (direct, indirect), (c) identify the endogenous mediator if any of the recorded effect, and (d) obtain indications of the anatomical localizations and functional characteristics of OP_4 receptor, NC and NC(1 – 13)NH$_2$ were used as reference agonists. The three isolated tissues analysed in Table 4 are induced to contract by electrical stimulation, using stimulus intensity and characteristics that are suitable to field stimulate neurons and nerve terminals but not the muscle cells. They have been described and used before in opioid receptor pharmacology: thus, the guinea-pig ileum, whose myenteric neuronal network contains mainly OP_3 receptors (Paton, 1957), has been shown to respond to NC and related peptides (Calo' *et al*., 1996; 1997; Zhang *et al*., 1997), the mouse vas deferens, whose nerve terminals contain mainly OP_1 receptors (Hughes *et al*., 1975a), and the rat vas deferens, whose nerves contain an uncharacterized opioid receptor, have also been reported to be NC sensitive preparations (Berzetei-Gurske *et al*., 1996; Bigoni *et al*., 1999a; Calo' *et al*., 1996; 1997; Nicholson *et al*., 1996; Zhang *et al*., 1997). The twitch responses in the three preparations are due to nerve activation and subsequent release of neurotransmitter since they are blocked by tetrodotoxin. The contractions of the mouse and rat vas deferens derive largely from the release of noradrenaline from the sympathetic nerves, since they are blocked by the α_1 adrenoceptor antagonist prazosin. OP_4 receptors appear to be localized in the sympathetic terminals since NC inhibits twitches evoked by electrical field stimulation (EFS), but does not modify contractions to exogenous noradrenaline (Calo' *et al*., 1996). Similar results were also obtained in the guinea-pig ileum. In this preparation, NC inhibits atropine and tetrodotoxin sensitive neurogenic contractions without affecting responses to exogenous acetylcholine, thus demonstrating the prejunctional localization of the OP_4 receptor.

In the three tissues, the inhibitory effect of NC is not influenced by naloxone, indicating that the other opioid receptor types present are not targeted by the peptide. A similar picture has been found regarding the inhibitory effects of NC on sensory fibers of the guinea-pig bronchus (Fischer *et al*., 1998; Rizzi *et al*., 1999a), renal pelvis (Giuliani & Maggi, 1996), and heart (Giuliani & Maggi, 1997). For instance, EFS of the guinea-pig isolated renal pelvis induces tetrodotoxin-sensitive contractile responses which are mediated by

tachykinin release from sensory nerves, since they are blocked by a selective NK-2 receptor antagonist (MEN 10,376, Maggi *et al*., 1992). The contractile effect of EFS, but not that of exogenous tachykinins, was markedly reduced by NC whose effect was insensitive to naloxone (Bigoni *et al*., 1999a; Giuliani & Maggi, 1996).

From the above, it is evident that OP_4 receptor is found in sympathetic, parasympathetic and sensory nerves, similar to the classical opioid receptors. This conclusion is also supported by the findings by Giuliani & Maggi (1997) who showed that NC modulates the complex-nerve mediated response of the guinea-pig left atrium: using adequate pharmacological tools, this response can be dissected into various components that include sympathetic, parasympathetic and CGRP-releasing sensory nerves (Giuliani & Maggi, 1997).

In all the preparations analysed above, the NC/OP_4 receptor system displays a prejunctional inhibitory function, as do the classical opioid peptides/receptors, possibly through the same basic cellular mechanisms, namely: (a) activation of K^+ conductance, as demonstrated in central neurons of the rat (Connor *et al*., 1996; Vaughan & Christie, 1996; Vaughan *et al*., 1997); (b) inhibition of Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, as demonstrated in isolated central neurons (Knoflach *et al*., 1996); (c) inhibition of cyclic AMP accumulation (Meunier *et al*., 1995; Reinscheid *et al*., 1995). However, in other preparations, such as rat or mouse colon (Corbett *et al*., 1998; Osinski *et al*., 1999b; Rizzi *et al*., 1999b; Taniguchi *et al*., 1998; Yazdani *et al*., 1999), NC has been shown to induce naloxone-resistant tetrodotoxin-sensitive contractions which could derive from the inhibition of the release of endogenous relaxing agents, whose nature is at present unknown (Yazdani *et al*., 1999).

Structure-activity study of NC-related peptides: from agonist to antagonist

From a large series of analogues of the template NC(1–13)NH_2, which were recently analysed and discussed (for a review see Salvadori *et al*., 1999), a number of compounds have been listed in Table 6 to enable structureactivity relationship analysis. Major purposes have been to: (a) see if the working hypothesis of dividing the peptide into 'address' (binding) and 'message' (activation) domains, which has been applied successfully to opioid peptides (Portoghese, 1989; Schwyzer, 1986), could also be used for NC; and (b) present and discuss the conceptual frame that led to the recent identification of a partial agonist (Calo' *et al*., 1998a; Guerrini *et al*., 1998) and an antagonist of OP_4 receptors (Calo' *et al*., 2000; Guerrini *et al*., 1999). The abbreviated structures and biological activities of 19 compounds are summarized in Table 6, together with their actual affinities, as measured with binding assays on plasma membranes from mouse brain (Varani *et al*., 1998). The compounds were all tested for their potential agonistic activities, which are expressed in terms of pEC_{50}, and, when found inactive or very weak, they were also tested as antagonists against NC(1–13)NH_2, the reference agonist. This compound was compared to the natural peptide NC in all sets of assays. Furthermore, a binding assay using a new OP_4 receptor ligand [^{3}H]$NCNH_2$ recently described by Varani *et al*. (1998), provided a fairly accurate measure of affinities for agonists, partial agonists and antagonists, allowing the evaluation of the influence of each chemical modification on OP_4 receptor affinity.

As shown in Figure 2, a good correlation is generally found between pEC_{50}/pA_2 and K_i values measured for the native mouse receptor using bioassay and binding data.

Table 6 Structure activity-studies

	Mouse vas deferens functional assay		*Mouse forebrain membranes*
	Agonist	*Antagonist*	*binding*
	pEC_{50}	pA_2	pK_i
NC	7.8	–	8.7
$NCNH_2$	8.0	–	9.1
NC(1-13)	5.6	–	6.9
$NC(1\text{-}13)NH_2$	7.9	–	9.1
$NC(1\text{-}9)NH_2$	< 5	–	< 5
$[Leu^1]NC(1\text{-}13)NH_2$	7.6	–	8.6
$[Leu^4]NC(1\text{-}13)NH_2$	< 5	< 5	5.0
$[Tyr^1]NC(1\text{-}13)NH_2$	7.6	–	8.3
$[Tyr^4]NC(1\text{-}13)NH_2$	5.0	–	ND
$[F/G]NC(1\text{-}13)NH_2$	–	6.8	8.0
$[L/G]NC(1\text{-}13)NH_2$	crc incom.	5.4	7.8
$[Y/G^1]NC(1\text{-}13)NH_2$	crc incom.	5.7	7.0
$[Nphe^1]NC(1\text{-}13)NH_2$	–	6.0	7.0
$[Nleu^1]NC(1\text{-}13)NH_2$	< 5	< 5	5.6
$[Ntyr^1]NC(1\text{-}13)NH_2$	< 5	< 5	5.5
$[Lys^8]NC(1\text{-}13)NH_2$	5.0	–	6.6
$[Arg^9]NC(1\text{-}13)NH_2$	7.6	–	ND
$[Lys^{12}]NC(1\text{-}13)NH_2$	7.1	–	ND
$[Arg^{13}]NC(1\text{-}13)NH_2$	7.4	–	ND

ND: not determined. Crc incom: concentration-response curve incomplete. pEC_{50}, pA_2, pK_i as in Table 3. The data are from the following papers: Calo' *et al*., 1998a; Guerrini *et al*., 1997; Varani *et al*., 1998.

These two sets of data have therefore been used to determine what alterations each chemical modifications introduced in the tridecapeptide NC(1–13)NH_2, exerted on ligand affinity (address?) and activity (message?). Reducing the C-terminal end by four residues from NC(1–17) to NC(1–13) leads to a marked 2 log unit loss of potency. Further shortening to NC(1–9) leads to complete loss of activity. The lower potency of NC(1–13) appears to derive largely from metabolic degradation, perhaps by carboxypeptidases (Sandin *et al*., 1999), since protection of the C-terminal end with amidation is sufficient to maintain full potency and activity (see biological activities and binding affinities in Table 4 and in Bigoni *et al*., 1999a; Calo' *et al*., 1996; 1998b; Dooley & Houghten, 1996; Kapusta *et al*., 1999; Rizzi *et al*., 1999a,b; Varani *et al*., 1999; Okawa *et al*., 1999). Amidation of the carboxyl group in NC(1–17), to yield NC(1–17)NH_2 does not significantly enhance (or very little) potency and activity in *in vitro* studies, but can enhance potency to values greater than for NC in some *in vivo* assays (Bertorelli *et al*., 1999a; Calo' *et al*., 1999). A few analogues of NC(1–13)NH_2 were selected to analyse the N-terminal tetrapeptide FGGF, which has been assumed to contain the message domain of NC (Guerrini *et al*., 1997), in analogy with opioids. The replacement of Phe^1 by Leu has no effect, while that of Phe^4 eliminates activity and drastically reduces affinity by more than 3 log units. The same substitutions have the opposite effects when applied to opioid peptides; those in position 1 leading to inactivity and those in position 4 preserving binding and activity. From these data it was concluded that, since the active site of opioids is Tyr^1, the corresponding site of NC should be Phe^4. Indeed, the replacement of Phe^1 by Tyr gives a compound that acts on OP_4 as well as on OP_2 and OP_3 receptors (Calo' *et al*., 1997; Shimohigashi *et al*., 1996; Varani *et al*., 1999). The Phe^4 appears to be instrumental for activation of OP_4 receptor, as indeed Reinscheid *et al*. (1996) showed that $[Tyr^4]$NC displays

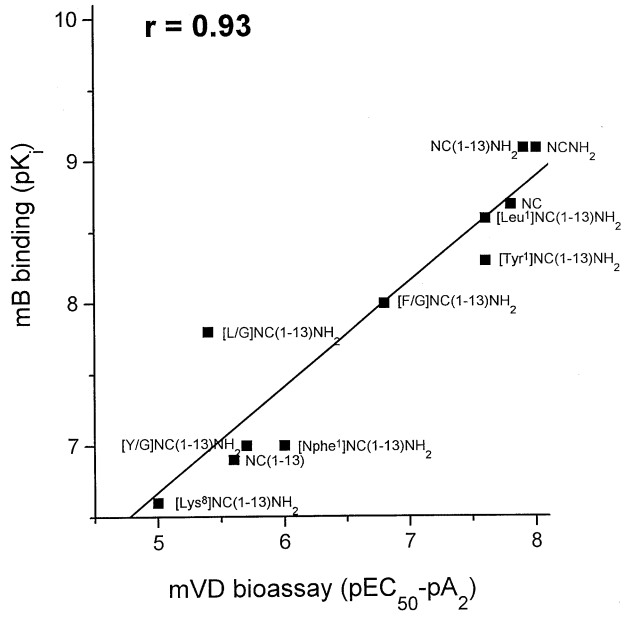

Figure 2 Correlation between binding and bioassay data on mouse OP_4 receptors. mB, mouse brain membranes; mVD, mouse vas deferens.

only a small fraction of the activity of NC, indicating that minor changes of Phe[4] may be associated to drastic loss of potency. This has been confirmed in the sequence NC(1–13)NH_2 (Table 6). In other compounds, changes were made at the N-terminal to simultaneously modify the spatial orientation of Phe[1] and prevent peptide degradation by aminopeptidases. Reduction of the bond Phe[1]-Gly[2] from CONH to CH_2NH yielded a new compound, [F/G]NC(1–13)NH_2, which was found to exert antagonistic activities in several pharmacological preparations, while acting as a partial or even full agonist in others (Table 9 and 10). A detailed discussion of the data obtained with [F/G]NC(1–13)NH_2 by several laboratories may be found in the section 'The OP_4 receptor ligand [F/G]NC(1–13)NH_2'.

The presence of a phenyl ring in the first position is not required for agonists, while it appears to be required for antagonism. Indeed, Phe[1] cannot be replaced by Leu in [F/G]NC(1–13)NH_2, because the new compound still binds to the receptor but does not block it. A strong reduction of potency is also observed when F/G is replaced by Y/G. Further modifications in position 1, particularly the displacement of the phenyl ring to the exterior by one atom, as in [Nphe[1]]NC(1–13)NH_2, led to a compound that shows low potency, but appears to act as a pure antagonist (Rizzi *et al.*,

Table 7 Effects of [Nphe[1]]NC(1-13)NH_2

In vitro studies

Preparation	*pEC_{50}*	*Nociceptin*	*[Nphe^1]NC(1-13)NH_2 pA_2*	*Type of antagonism*	*Reference*
CHO_{hOP4}	9.8	Inhibition of forskolin stimulated cAMP accumulation	6.0	competitive	Calo' *et al.*, 2000
Mouse vas deferens	7.8	Inhibition of electrically induced twitches	6.0	competitive	Calo' *et al.*, 2000
Mouse colon	8.7	Contraction	6.0	competitive	Rizzi *et al.*, 1999b
Rat vas deferens	7.2	Inhibition of electrically induced twitches	6.2	competitive	Calo' *et al.*, 2000
Guinea pig renal pelvis	7.4	Inhibition of electrically induced contractions	6.6	competitive	Calo' *et al.*, 2000
Guinea pig ileum	8.1	Inhibition of selectrically induced twitches	6.4	competitive	Calo' *et al.*, 2000
Rabbit ileum	8.2	Inhibition of spontaneous contractions	6.8	competitive	Pheng *et al.*, 1999
Rat CC synaptosomes	7.9	Inhibition of [^{3}H]5-HT release	6.7	competitive	Sbrenna *et al.*, 2000
Rat brain membranes	8.0	Stimulation of GTPγS binding	7.0	competitive	Berger *et al.*, unpub

In vivo studies

Test route of administration	*Effective dose*	*Nociceptin*	*Effective dose*	*[Nphe^1]NC (1-13)NH_2*	*Reference*
Mouse TW (icv)	1 nmol	Reduction of TW latencies	30 nmol	antagonist*	Calo' *et al.*, 2000
Mouse TW (icv)	1 nmol	Inhibition of morphine induced analgesia	30 nmol	antagonist*	Calo' *et al.*, 2000
Rat TF (it)	5 nmol	Increase in TF latency	200 nmol	antagonist	Candeletti *et al.*, unpub
Mouse LA (icv)	1 nmol	Reduction of spontaneous locomotor activity	10 nmol	antagonist	Calo' *et al.*, 2000
Mouse cardiovascular (iv)	10 nmol Kg^{-1}	Inhibition of HR and BP	100 nmol Kg^{-1}	antagonist	Madeddu *et al.*, unpub
Mouse WM (iv)	10 ng	Neurotoxicity	100 ng	antagonist*	Gressens *et al.*, unpub
Rat food intake (3V)	1.68 nmol	Stimulation of food intake	16.8 nmol	antagonist*	Polidori *et al.*, 2000
Rat GI transit (iv)	100 nmol Kg^{-1}	Inhibition of GI transit	1000 nmol Kg^{-1}	antagonist	Casati *et al.*, unpub

*In these assays [Nphe[1]]NC(1-13)NH_2 *per se* induces changes opposite to that evoked by NC: this suggests a tonic control of these biological functions by nociceptinergic pathways. CC: cerebral cortex; TW: tail withdrawal assay; TF: tail flick assay; LA locomotor activity assay; GI: gastrointestinal; WM: white matter. pEC_{50}, pA_2, as in Table 3.

1999b; Calo' *et al*., 2000). In fact, this new compound is devoid of residual agonistic activities in a variety of bioassays, antagonizes NC effects in both peripheral and central nervous system as well as in a variety of *in vivo* assays (see Table 7).

In addition, [Nphe^1]NC(1–13)NH$_2$ antagonizes the agonistic effects of [F/G]NC(1–13)NH$_2$ in some preparations, such as CHO$_{hOP4}$ (Hashimoto *et al*., 2000), the isolated mouse colon (Rizzi *et al*., 1999b) and rabbit ileum (Pheng *et al*., manuscript in preparation) or *in vivo* in the mouse tail withdrawal assay (Calo' G, Rizzi A and Regoli D., unpublished observation).

The last four compounds of Table 6 were prepared to explore the role of cationic residues in the middle and at the C-terminal end of NC(1–13)NH$_2$. Essential for receptor occupation is Arg^8, which cannot be replaced, even with Lys, while in the other positions (9, 12 and 13) Arg and Lys are equally accepted (interchangeable).

In the next two sections, we will discuss in more detail the properties of the most important new OP$_4$ receptor ligands we have identified: [F/G]NC(1–13)NH$_2$ and [Nphe^1]NC(1–13)NH$_2$. However, new ligands for the OP$_4$ receptors have also been identified by other investigators. Kobayashi *et al*. (1997) found that some sigma receptor ligands (carbetapentane and rimcazole) act as antagonists of the OP$_4$ receptor, albeit non-selectively and with low potency. From a combinatorial library containing more than 52 million different hexapeptides, Dooley *et al*. (1997) have identified 15 compounds that show high affinities for the OP$_4$ receptor but also display partial agonistic activities which limits their usefulness as pharmacological tools. Naloxone benzoylhydrazone (NalBzOH), a non-selective opioid receptor ligand (Paul *et al*., 1990), was also reported to competitively block some effects of NC, with low potency (pA$_2$ values 6.0–6.5) (Bigoni *et al*., 1999b; Bucher, 1998; Mamiya *et al*., 1999; Nicholson *et al*., 1998a; Noda *et al*., 1998; Sbrenna *et al*., 1999; Schlicker *et al*., 1998; Seki *et al*., 1999), but this compound also binds with high affinity to OP$_2$ and OP$_3$ receptors (Paul *et al*., 1990). Very recently, new ligands with antagonistic properties on the OP$_4$ receptor have been identified from a conformationally constrained peptide combinatorial library (Becker *et al*., 1999). Although poorly selective, these novel ligands provide good templates for the development of non-peptidic ligands. Other non-selective ligands for the OP$_4$ receptor include Mr 2266 (Bauer *et al*., 1999), TRK820 (Seki *et al*., 1999), and buprenorphine (Wnendt *et al*., 1999). Detailed pharmacological analyses of these compounds are outside the purpose of the present review and will be presented in a special issue of Peptides dedicated to the NC/OP$_4$ system (Calo' *et al*., manuscript in preparation).

The OP$_4$ antagonist [Nphe^1]NC(1–13)NH$_2$: characterization and uses

[Nphe^1]NC(1–13)NH$_2$ has been found to act as antagonist in a variety of *in vitro* and *in vivo* assays. The pA$_2$ values obtained in functional *in vitro* assays and the effective doses estimated in *in vivo* assays for this ligand are presented in Table 7.

[Nphe^1]NC(1–13)NH$_2$ displays low potency (pA$_2$ 6.0–6.6). However, as it is the first pure OP$_4$ receptor antagonist to be identified thus far, this compound has allowed us to characterize several biological effects of NC clearly mediated through OP$_4$ receptors. The type of data that were utilised for quantification of the antagonistic effects of [Nphe^1]NC(1–13)NH$_2$ and determining the type of antagonism exerted by this compound is exemplified in Figure 3.

NC concentration-dependently reduces twitch responses of the mVD elicited by electrical field stimulation. Increasing concentrations of [Nphe^1]NC(1–13)NH$_2$, induce a gradual displacement of the curve to NC, yielding a linear Schild plot, with a slope close to 1.0, demonstrating that the compound acts as a competitive antagonist. Similar results have been obtained using human recombinant receptors expressed in CHO cells for NC-induced inhibition of cyclic AMP accumulation (Calo' *et al*., 2000) or on native receptors inhibiting the release of 5-HT from rat cerebral cortex slices

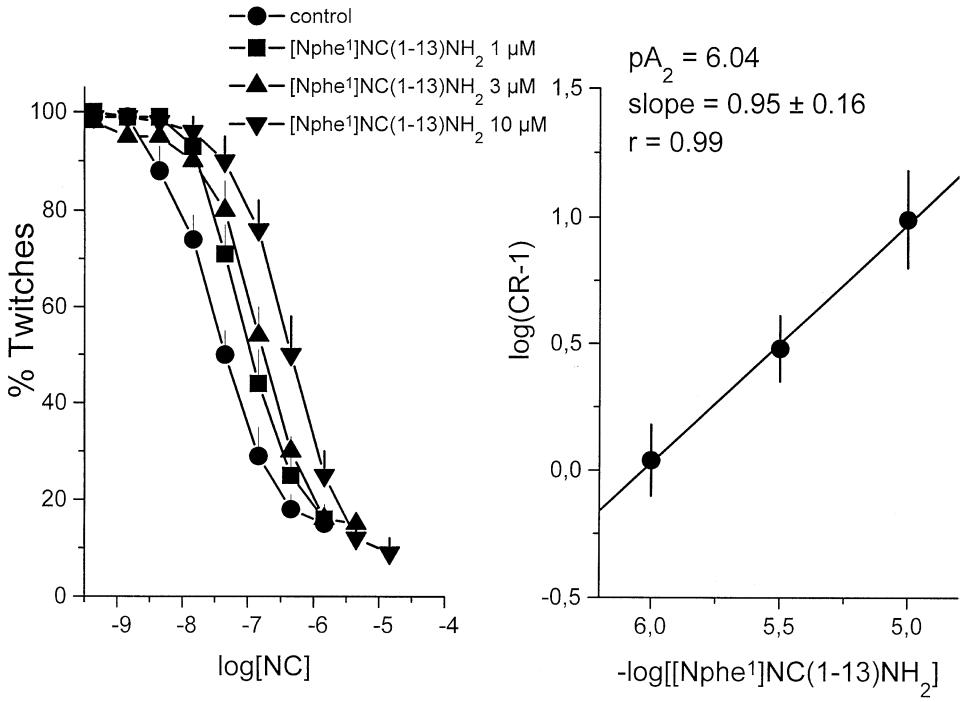

Figure 3 Schild plot of the antagonistic effect of [Nphe^1]NC(1–13)NH$_2$ on NC-induced depression of neurogenic contractions in the electrically stimulated mouse vas deferens.

Table 8 pA_2 values of [Nphe^1]NC(1-13)NH_2 obtained against several NC receptor ligands in the isolated mouse colon and in CHO cells expressing the human recombinant OP_4 receptor

Preparation	*Mouse colon*	*CHO_{hOP4}*
NC action	*Contractile effect*	*Inhibition of forskolin-induced cyclic AMP accumulation*
NC	6.0	6.2
NC(1-13)NH_2	6.0	6.1
[F/G]NC(1-13)NH_2	5.9	6.5
Ac-RYYRWK-NH_2	6.1	6.5

[Nphe^1]NC(1-13)NH_2 applied at 10 μM did not show any contractile effect in the mouse colon, while it causes a maximal inhibition of cyclic AMP accumulation $<15\%$ in CHO_{hOP4} cells. These daya are from Rizzi *et al*., 1999b, and Hashimoto *et al*., 2000.

(Sbrenna *et al*., 2000). In addition, [Nphe^1]NC(1 – 13)NH_2 acts as a pure NC receptor antagonist also in mouse brain slices, where it prevents NC-induced GTPgammaS binding without showing any residual agonistic activity (Berger *et al*., manuscript in preparation). In the mouse colon (Rizzi *et al*., 1999b) and in CHO_{hOP4} cells (Hashimoto *et al*., 2000), [Nphe^1]NC(1 – 13)NH_2 was also tested against several ligands (NC(1 – 13)NH_2, [F/G]NC(1 – 13)NH_2, AcRYYRWK-NH_2) that are expected to act on the OP_4 receptor. Results presented in Table 8 indicate that the different compounds act indeed on OP_4 receptor since their effects are inhibited by [Nphe^1]NC(1 – 13)NH_2, but not by naloxone. In contrast, the effect elicited by endomorphin 1 in the mouse colon was not affected by [Nphe^1]NC(1 – 13)NH_2, while it was fully prevented by naloxone (Rizzi *et al*., 1999b). As expected, [Nphe^1]NC(1 – 13)NH_2 showed very similar pA_2 values when tested against different OP_4 receptor agonists.

[Nphe^1]NC(1 – 13)NH_2 has been also demonstrated to be active *in vivo*. Indeed, this compound antagonizes: (i) NC (i.c.v.) induced reduction of latencies and inhibition of morphine evoked analgesia in the tail withdrawal test in the mouse (Calo' *et al*., 2000); (ii) NC (i.t.) evoked analgesia in the rat (Candeletti S., personal communication); NC (i.v.) induced bradycardia and hypotension in the mouse (Madeddu P., personal communication); and NC (i.c.v.) stimulated food intake in the rat (Polidori *et al*., 2000). In some cases, as for instance in the studies on pain threshold in the mouse (Calo' *et al*., 2000) or on food intake in the rat (Polidori *et al*., 2000), [Nphe^1]NC(1 – 13)NH_2 was found to induce *per se* changes opposite to those evoked by NC. This may well suggest a tonic control of these biological functions by nociceptinergic pathways. A detailed discussion of these findings, as well as their relevance for the understanding of the physiological role of the NC/OP_4 system, can be found in the section dedicated to the biological actions of NC.

The OP_4 receptor ligand [F/G]NC(1 – 13)NH_2

[F/G]NC(1 – 13)NH_2 has been identified in the frame of a SAR study (Calo' *et al*., 1998a) and is reported to act as a selective NC receptor antagonist *in vitro* in the electrically stimulated mouse vas deferens and guinea pig ileum (Guerrini *et al*., 1998). Since, various studies have been published on the actions of this pseudopeptide in a variety of *in vitro* and *in vivo* assays. The results of these studies have been summarized in Table 9 (*in vitro* studies) and 10 (*in vivo* studies).

From these data it is evident that [F/G]NC(1 – 13)NH_2 may act as an antagonist, as a partial agonist or even as a full agonist, depending on the preparation. In *in vitro* studies performed in isolated tissues, [F/G]NC(1 – 13)NH_2 mainly acts as an antagonist (pA_2 around 7) but there are exceptions, such as the rat and mouse colon (Corbett *et al*., 1998; Rizzi *et al*., 1999b) or the rabbit ileum (Pheng *et al*., 1999), where the pseudopeptide acts as a full agonist and is subject to antagonism by [Nphe^1]NC(1 – 13)NH_2 (Pheng *et al*., 1999; Rizzi *et al*., 1999b). In recombinant systems showing high level of expression of the OP_4 receptor, [F/G]NC(1 – 13)NH_2 consistently behaves as a full agonist (Butour *et al*., 1998; Okawa *et al*., 1999; Wnendt *et al*., 1999). Again, its effects are antagonized by [Nphe^1]NC(1 – 13)NH_2 (Hashimoto *et al*., 2000). However, in N1E-115 cells expressing low levels of native OP_4 receptors, [F/G]NC(1 – 13)NH_2 acts as a partial agonist, either when stimulating [^{35}S]-GTPgammaS binding or when inhibiting forskolin-stimulated [^{3}H]-cyclic AMP formation (Olianas *et al*., 1999). Partial agonistic activity of [F/G]NC(1 – 13)NH_2 has been also demonstrated in studies in which neurotransmitter release was measured (noradrenaline, Schlicker *et al*., 1998; or 5HT, Sbrenna *et al*., 2000); Siniscalchi *et al*., 1999) and in electrophysiological studies in which K^+ currents were monitored (Allen *et al*., 1999; Chiou, 1999). In *in vivo* assays, [F/G]NC(1 – 13)NH_2 mainly acts as a full agonist, mimicking several effects elicited by NC, when injected i.t. or i.c.v. (see Table 10). However, in few cases, the pseudopeptide behaves as a partial agonist (Bigoni *et al*., 1999a; Calo' *et al*., 1999) or as a pure antagonist (Armstead, 1999; Chu *et al*., 1999a,b; Gressens *et al*., 1999; Madeddu *et al*., 1999).

Interpretation of these data has been attempted by assuming the existence of multiple OP_4 receptor types (Butour *et al*., 1998; Calo' *et al*., 1998b; Xu *et al*., 1998). However, this interpretation is ruled out by the demonstration that binding of NC completely disappears in mice in which the OP_4 gene has been knocked out (Clarke *et al*., 1999). All NC binding sites appear to be the product of a single gene, that coding for the ORL_1/OP_4 receptor. Splice variants of the ORL_1 gene have been described in the literature for the rat and mouse receptor (Pan *et al*., 1998; Peluso *et al*. 1998; Wang *et al*., 1994; Wick *et al*., 1994; 1995). These variants may account for the different pharmacological actions of [F/G]NC(1 – 13)NH_2 in the various tests. However, no differences in the pharmacological profiles of these variants have been reported up to now. Alternatively, [F/G]NC(1 – 13)NH_2 may actually be a 'low efficacy agonist' whose final effect (antagonist, partial or full agonist) depends on the stimulus-response efficiency of the preparation under study. The findings by Toll *et al*. (1998) corroborate this hypothesis; [F/G]NC(1 – 13)NH_2 acts as an antagonist in transfected cells expressing low levels of OP_4 receptor, while it acts as a partial or full agonist in cells expressing high receptors levels. This elegant demonstration also illustrates the limits and the great possibilities of the molecular biology approach applied to pharmacological investigations.

Independently from the pharmacological behaviour (full or partial agonist, antagonist) of [F/G]NC(1 – 13)NH_2 in the different assays, the pseudopeptide is on average 10 fold less potent than NC *in vitro* (Table 9). This [F/G]NC(1 – 13)NH_2/NC ratio of potency perfectly matches with the ratio of actual affinities obtained in receptor binding experiments (Okawa *et al*., 1999; Varani *et al*., 1998; 1999). However, the ratio of potency [F/G]NC(1 – 13)NH_2/NC obtained in *in vivo* studies (Table 9) is near 1 or even less. This difference can be explained assuming a higher metabolic stability of the pseudopeptide as compared to the naturally occurring peptide. Interestingly, already in the first description of [F/G]NC(1 –

Table 9 Effects of [F/G]NC(1-13)NH_2: *in vitro* studies

Preparation	*pEC_{50}*	*Nociceptin*	*pEC_{50} or pA_2*	*[F/G]NC(1-13)NH_2*	*Reference*
CHO_{ORL1}	9.00	Inhibition of forskolin stimulated cyclic AMP accumulation	8.12	Full agonist	Butour *et al.*, 1998
CHO_{ORL1}	9.78	Inhibition of forskolin stimulated cyclic AMP accumulation	8.65	Full agonist	Okawa *et al.*, 1999
CHO_{ORL1}	9.09	Inhibition of forskolin stimulated cyclic AMP accumulation	7.07	Full agonist	Wnendt *et al.* 1999
Mouse CC slices	7.5	Inhibition of electrically induced noradrenaline release	7.0-7.2	Partial agonist	Schlicker *et al.*, 1998
Rat CC slices	6.54	Inhibition of electrically induced 5-HT release	6.5	Partial agonist	Siniscalchi *et al.*, 1999
Rat CC slices	7.29	Inhibition of KCl induced glutamate release	7.39	Full agonist	Okawa *et al.*, 1999
Rat CC synaptosomes	7.88	Inhibition of KCl induced 5-HT release	7.22	Partial agonist	Sbrenna *et al.*, 1999
Rat RVM neurons	8.5	Inhibition of electrical activity	ND	Antagonist	Chu *et al.*, 1999b
Rat MVN slices	≈ 7	Inhibition of electrical activity	ND	Antagonist	Sulaiman *et al.*, 1999
Mouse vas deferens	7.6	Inhibition of electrically induced twitches	6.7	Antagonist	Guerrini *et al.*, 1998
Guinea-pig ileum	7.9	Inhibition of electrically induced twitches	7.0	Antagonist	Guerrini *et al.*, 1998
Guinea-pig bronchus	7.1	Inhibition of electrically induced contraction	ND	Antagonist	Rizzi *et al.*, 1999a
Guinea-pig bronchus	≈ 7	Inhibition of electrically induced contraction	ND	Antagonist	Shah *et al.*, 1998
Guinea-pig renal pelvis	7.4	Inhibition of electrically induced contraction	ND	Antagonist	Bigoni *et al.*, 1999a
Rat vas deferens	6.6	Inhibition of electrically induced twitches	6.7	Antagonist	Okawa *et al.*, 1999
GTPγS binding rat brain	8.2	Stimulation of [^{35}]-GTPγS binding	8.6	Antagonist	Nicholson *et al.*, 1998b
Rat colon	9.5	Contraction	8.4	Full agonist	Corbett *et al.*, 1998
Mouse colon	8.9	Contraction	8.2	Full agonist	Corbett *et al.*, 1998
Rabbit ileum	8.2	Inhibition of spontaneous contractions	7.3	Ful agonist	Pheng *et al.*, 1999
Rat amygdala neurons	7.5	Increase in K^+ conductance	ND	Antagonist	Meis & Pape, 1998
Rat PAG slices	7.3	Increase in K^+ conductance	7.1	Partial agonist	Chiou, 1999
Rat SCN neurons	7.6	Increase in K^+ conductance	ND	Partial agonist	Allen *et al.*, 1999
Rat AN and VMH slices	8.1	Increase in K^+ conductance	ND	Partial agonist	Emmerson & Miller, 1999
Mouse N1E-115 cells	8.1	Stimulation of [^{35}S]-GTPγS binding	6.9	Partial agonist	Olianas *et al.*, 1999
Mouse N1E-115 cells	9.6	Inhibition of forskolin stimulated cyclic AMP accumulation	8.5	Partial agonist	Olianas *et al.*, 1999

CC: cerebral cortex; RVM: rostral ventrolateral medulla; MVN: medial vestibular nucleus; SCN: suprachiasmatic nucleus; AN: arcuate nucleus; VMH: ventromedial hypothalmus; PAG: periaqueductal grey. ND: not determined. pEC_{50} and pA_2 as in Table 3.

Table 10 Effects of [F/G]NC(1-13)NH_2: *in vivo* studies

Test/route of administration	*Effective dose*	*Nociceptin*	*Effective dose*	*[F/G]NC (1-13)NH_2*	*Reference*
Rat cardiovasc. (iv)	30 nmol Kg^{-1}	Inhibition of heart rate and blood pressure	300 nmol Kg^{-1}	Partial agonist	Bigoni *et al.*, 1999a
Mouse cardiovasc. (iv)	10 nmol Kg^{-1}	Inhibition of heart rate and blood pressure	10 nmol Kg^{-1}	Antagonist	Madeddu *et al.*, 1999
Rat cardiovasc and kidney (icv)	10 μg	Inhibition of heart rate and blood pressure-diuresis	10 μg	Full agonist	Kapusta *et al.*, 1999
Mouse TW (icv)	1 nmol	Reduction of tail withdrawal latencies	1 nmol	Full agonist	Calo' *et al.*, 1998b
Mouse TW (icv)	1 nmol	Inhibition of morphine induced analgesia	1 nmol	Full agonist	Calo' *et al.*, 1998b
Mouse TF (icv)	5 nmol	Inhibition of morphine induced analgesia	3 nmol	Full agonist	Grisel *et al.*, 1998
Mouse spontaneous LA	1 nnol	Inhibition spontaneous locomotor activity	1 nmol	Partial agonist	Calo' *et al.*, 1999
Mouse hot plate (it)	1 nnol	Increase of jumping latency	1 nnol	Full agonist	Bertorelli *et al.*, 1999b
Rat TF (icv)	1 nmol	Reduction of tail withdrawal latencies	1 nmol	Full agonist	Candeletti *et al.*, 1998
Rat TF (icv)	1 μg	Reduction of tail withdrawal latencies	1 μg	Full agonist	Wang *et al.*, 1999
Rat TF (it)	2 nmol	Induction of analgesia	2 nmol	Full agonist	Candeletti *et al.*, 1998
Rat TF (it)	1 μg	Induction of analgesia	1 μg	Full agonist	Wang *et al.*, 1999
Rat flexor reflex (it)	0.55 nmol	Inhibition of the flexor reflex	0.55 nmol	Full agonist	Xu *et al.*, 1998
Rat dorsal horn (it)	50 μg	Inhibition of noxious evoked electrical activity	25 μg	Full agonist	Carpenter & Dickenson, 1998
Rat food intake (3V)	210 pmol	Stimulation of food intake	26 pmol	Full agonist	Polidori *et al.*, 1999
Rat arthritis (icv)	1 nmol	Inhibition of morphine induced analgesia	1 nmol	Full agonist	Bertorelli *et al.*, 1999a
Rat RVM (intracerebral)	10 nmol	Inhibition of heart rate and blood pressure	10 nmol	Antagonist	Chu *et al.*, 1999a
Mouse seizures (icv)	10 nmol	Anticonvulsant action in pentylenetetrazole treated mice	10 nmol	Full agonist	Zgodzinski *et al.*, 1999
Mouse WM (iv)	10 μg	Neurotoxicity	10 ng	Antagonist	Gressens *et al.*, 1999
Piglet vasodilation	1 μM	Induction of pial artery dialation	1 μM	Antagonist	Armstead, 1999

TW: tail withdrawal assay; TF: tail flick assay; LA: locomotor activity; RVM: rostral ventrolateral medulla; WM: white matter.

13)NH_2 (Guerrini *et al*., 1998) we suggested that, due to the presence of the amide at the C-terminus and of the pseudopeptide bond at the N-terminus, [F/G]NC(1–13)NH_2 should be more stable than NC: this was confirmed by the following line of evidences: (i) the potency ratio [F/G]NC(1–13)NH_2/NC is 10 fold higher *in vitro* (where peptidase activity is probably less relevant) than *in vivo*; (ii) in some *in vivo* studies [F/G]NC(1–13)NH_2 shows a longer duration of action compared to NC (Bertorelli *et al*., 1999a; Kapusta *et al*., 1999; Wang *et al*., 1999a; Xu *et al*., 1998), (iii) *in vitro* in the rat vas deferens peptidase inhibitors increase the potency of NC but not that of [F/G]NC(1–13)NH_2 (Okawa *et al*., 1999).

In the first reports [F/G]NC(1–13)NH_2 was been proposed as a selective OP_4 receptor ligand. This conclusion was based on the fact that the pseudopeptide does not modify the actions of opioid receptor agonists in several bioassays (Calo' *et al*., 1998a; Guerrini *et al*., 1998). These functional data have been recently confirmed in binding experiments (Varani *et al*., 1999) by showing that the affinity of [F/G]NC(1–13)NH_2 is higher for NC sites (K_i 12 nM) than for opioid sites (OP_3: K_i 800 nM; OP_2: K_i 2630 nM; OP_1: K_i >10000 nM) expressed in guinea-pig brain membranes. However, in recent experiments performed in rats (Carpenter & Dickenson, 1998; Sbrenna *et al*., 1999), it has been reported that part of the effects of [F/G]NC(1–13)NH_2 can be reversed by naloxone. Therefore, we can not completely exclude (at least in the rat) some interactions of [F/G]NC(1–13)NH_2 with classical opioid receptors.

Biological actions of nociceptin

The biological actions exerted by NC have been extensively covered in other review articles (see, for instance, Civelli *et al*., 1998; Darland *et al*., 1998; Henderson & McKnight, 1997; Meunier, 1997; Taylor & Dickenson, 1998). Here we will briefly review the major effects of this new peptide, focusing on the involvement of the endogenous NC/OP_4 receptor system in these various actions. The role of the endogenous NC/OP_4 receptor system in the different central nervous functions have been explored to date (a) with antisense oligonucleotides targeting OP_4 receptor or preproNC genes, (b) with antibodies directed against NC, or (c) using mice in which the receptor or the peptide precursor genes have been genetically eliminated. While describing and discussing the findings obtained with these approaches, we will compare the actions of these various pharmacological and biological tools with those obtained with the first OP_4 receptor selective antagonist, [Nphe^1]NC(1–13)NH_2.

The distribution of NC and its receptor in the central nervous system of rodents has been extensively and elegantly investigated with *in situ* hybridization and immunohystochemical techniques (Anton *et al*., 1996; Ikeda *et al*., 1998; Monteillet-Agius *et al*., 1998; Neal *et al*., 1999). In addition, Darland *et al*. (1998) have compiled maps that detail the expression of OP_4 receptors and preproNC mRNAs in the rat brain. The reader is referred to the above mentioned papers for detailed coverage of this topic, which goes beyond the aims of the present article. Some of the major points are briefly summarized here. Both the receptor (Anton *et al*. 1996; Ikeda *et al*., 1998) and the peptide (Ikeda *et al*., 1998; Neal *et al*., 1999) are widely expressed in the central nervous system suggesting that the NC/OP_4 receptor system may be involved in the modulation of a variety of central nervous system functions. The OP_4 receptor is mainly expressed on nerve fibres, although in few brain areas, including the hippocampus, it is also expressed on neuronal cell bodies (Anton *et al*., 1996), suggesting that most of the actions of NC are likely to be presynaptic. The peptide is mostly expressed in middle-sized neurons, probably interneurons, although it is also found rarely in large projection-type neurons (Ikeda *et al*., 1998). Moreover, the distribution of NC generally matches that of the OP_4 receptor (Darland *et al*., 1998). This anatomical organization implies that the NC/OP_4 receptor system may modulate local circuitry. As underscored by Darland *et al*. (1998), in most studies aimed at investigating the effects of NC on various central nervous functions, the peptide has been administered i.c.v. or i.t. Only a few studies have investigated the actions of NC within discrete brain areas. This latter experimental paradigm, together with the development of selective OP_4 receptor antagonists will lead, in the near future, to major advances in our understanding of the physiological roles of this system within the neuronal networks relevant to the various functions.

Nociceptin and pain threshold: supraspinal and spinal effects

Supraspinal level Already in the first papers in which the identification of NC was reported (Meunier *et al*., 1995; Reinscheid *et al*., 1995), it was shown that, contrary to opioids (the classical analgesic agents), the i.c.v. injection of NC has a pronociceptive effect, i.e. it reduces the tail flick (Reinscheid *et al*., 1995) or hot plate response latency (Meunier *et al*., 1995). Similar results were subsequently obtained in the land snail (Kavaliers & Perrot-Sinal, 1996), mice (Calo' *et al*., 1998b; Nishi *et al*., 1997) and rats (Candeletti *et al*., 1998; Wang *et al*., 1999a; Zhu *et al*., 1996). A direct pronociceptive role of NC was questioned by Mogil *et al*. (1996a), who reported that i.c.v. injection of NC does not produce hyperalgesia, but rather reverses opioid-mediated stress-induced antinociception in different algesiometric assays and reverses morphine induced-analgesia. This suggestion for an anti-opioid role of NC has since been corroborated by results obtained in a variety of assays; indeed, NC has been shown to counteract the analgesic effect of the endogenous opioid system (Tian *et al*., 1997a; 1998; Zhu *et al*., 1996; 1997), or that of exogenously applied morphine (Bertorelli *et al*., 1999a; Calo' *et al*., 1998b; Grisel *et al*., 1996; Tian *et al*., 1997b; Zhu *et al*., 1997), or that of selective opioid receptor agonists (King *et al*., 1998; Mogil *et al*., 1996b), including endomorphins (Wang *et al*., 1999b). Worthy of mention is the fact that tolerance develops to the antiopioid effects of NC (Lutfy *et al*., 1999).

The involvement of the OP_4 receptor in these effects is suggested by the following lines of evidence: (i) the pronociceptive action of NC is no longer present in OP_4 receptor knockout mice (Nishi *et al*., 1997; Noda *et al*., 1998); (ii) antisense oligonucleotides targeting the OP_4 receptor prevent the effect of NC (Tian *et al*., 1997b; Zhu *et al*., 1997); (iii) the pronociceptive effect of NC is reversed by NalBzOH, a non-selective opioid receptor ligand (Noda *et al*., 1998). These observations are corroborated by the recent findings that the selective OP_4 receptor antagonist [Nphe^1]NC(1–13)NH_2 prevents the pronociceptive and anti-morphine actions of NC in the mouse tail withdrawal assay (Calo' *et al*., 2000). Moreover, this compound causes *per se* a robust, naloxone-resistant, antinociceptive effect and is able to potentiate by 3 fold at relatively low doses the analgesic effect of morphine, suggesting that nociceptinergic pathways controlling pain threshold are tonically activated at the supraspinal level. Interestingly, another low affinity NC receptor antagonist, retronociceptin methyl-ester (Ret-Noc-OMe), has been found to act as an analgesic following i.c.v. administration in mice

(Yoshikawa *et al*., 1999), and similar results were also obtained using NalBzOH in wild type but not in OP_4 receptor knockout mice (Noda *et al*., 1998).

The block of NC/OP_4 signalling not only raises pain threshold and potentiates the effect of endogenous and exogenous opioids, but probably prevents at least in part the development of tolerance to the analgesic actions of opioids. This consideration is based on the following findings: (i) in mice knocked out for the OP_4 receptor, a partial loss of tolerance to morphine analgesia has been demonstrated (Ueda *et al*., 1997); (ii) morphine tolerance can be partially reversed by i.c.v. injection of anti-NC antibodies (Tian *et al*., 1998); and (iii) there is an accelerated production and release of NC in the brain of chronic morphine tolerant rats (Yuan *et al*., 1999). These data would seem to suggest that the NC/OP_4 receptor system may well subserve an important modulatory influence on development of morphine tolerance. Nevertheless, this proposal still depends on further studies, using OP_4 receptor antagonists, to be validated.

Little is known about the mechanism(s) by which NC counteracts the analgesic action of opioids. Since the OP_4 receptor and classical opioid receptors largely share the same transductional mechanisms, it is reasonable to speculate that their opposite effects on pain threshold are due to distinct localisations of NC and opioid peptides and their respective receptors on the neuronal networks involved in pain transmission. Several lines of evidence support this hypothesis. Although NC and opioid peptides show a similar distribution, they are not co-localized in nociceptive centres such as the dorsal horn, the sensory trigeminal complex or the periaqueductal grey (Schulz *et al*., 1996). The OP_4 receptor and μ-opioid receptors are not co-localized in areas involved in pain processing (Monteillet-Agius *et al*., 1998). In addition, the anatomical distribution of the OP_4 receptors is quite distinct to that seen for opioid-stimulated [^{35}S]-GTPγS binding (Sim & Childers, 1997).

The anti-opioid effect of NC has been evaluated in a few discrete brain areas. In the rostral ventromedial medulla of the rat, NC exerts a marked inhibitory action on all neurones, and can block the activation of some cells by opioids (Heinricher *et al*., 1997). Moreover, coadministration of NC attenuates the antinociceptive effect of DAMGO in the tail flick test, when applied within the rostral ventromedial medulla. A similar picture has also been found when studying the effects of NC within the periaqueductal grey where NC inhibits virtually all neurons present (Vaughan *et al*., 1997), and is also able to attenuate the analgesic effect of locally injected morphine (Morgan *et al*., 1997). It is therefore suggested that NC reverses opioid analgesia by inhibiting neuronal output from the periaqueductal grey (Morgan *et al*., 1997). Taken together, these findings corroborate the suggestion that the different actions of NC and opioids on nociception may be ascribed to a differential localization of their receptors in critical neuronal networks.

The analysis presented above contains an indication that OP_4 receptor antagonists could provide a new class of supraspinally acting analgesics which could enable a reduction in morphine dosage, thus minimising the risk of tolerance and dependence.

Spinal level The role of NC in modulating pain threshold in the spinal cord is controversial. Although some studies reported that i.t. injection of NC produces hyperalgesia/allodynia (Hara *et al*., 1997; Inoue *et al*., 1999; Minami *et al*., 1997; Okuda-Ashitaka *et al*., 1996; Sakurada *et al*., 1999), others found no effect (Grisel *et al*., 1996; Reinscheid *et al*., 1995). However most of the studies demonstrated that i.t. NC induces an antinociceptive effect similar to that evoked by classical opioid receptor agonists (Bertorelli *et al*., 1999b; Candeletti *et al*., 1998; Erb *et al*., 1997; Hao *et al*., 1998; Kamei *et al*., 1999; King *et al*., 1997; Tian *et al*., 1997b; Wang *et al*., 1999a; Xu *et al*., 1996; Yamamoto *et al*., 1997a,b). While tolerance develops to the antinociceptive effect of i.t. NC upon repeated administrations, there is no crosstolerance with morphine, suggesting that different receptors are involved in the actions of the two agents (Hao *et al*., 1997). The view that NC is a spinal analgesic is further substantiated by electrophysiological findings showing a major inhibitory action of NC on neuronal activity in the spinal dorsal horn (Faber *et al*., 1996; Liebel *et al*., 1997; Stanfa *et al*., 1996). Differences in animal species, or even in strains, which have been reported to be very important in determining the supraspinal effects of NC (Mogil *et al*., 1999), as well as in NC doses used, may account for the conflicting results reported with NC in the spinal cord. Worthy of mention is the work of Inoue *et al*. (1998; 1999), and Sakurada *et al*. (1999) showing that dose-response curve to NC is bell-shaped: very low doses of peptide (fmol range) cause hyperalgesia with a maximum at 1–10 fmol. At higher doses (nmol range), NC is antinociceptive and blocks the scratching, biting and licking induced by i.t. substance P (Inoue *et al*., 1999). Both actions of NC are surely mediated by the OP_4 receptor, since they are absent in $OP_4^{-/-}$ mice (Inoue *et al*., 1999). They also involve the activation of substance P signalling since (a) they are blocked by the NK-1 receptor antagonist CP 96345, or capsaicin pretreatment, (b) they disappear in mice lacking the tachykinin 1 gene. As pointed out by Inoue *et al*. (1999) OP_4 receptors are present on both peripheral and central nerve endings (or terminals) of sensory neurons, where they can trigger release of neuropeptides, particularly substance P. Functional OP_4 receptors are also found postsynaptically on spinal neurons, where they exert an antinociceptive effect by negatively modulating substance P signalling. The differential sensitivity of the pre- and post-synaptic sites to NC is of such magnitude (fmol vs nmol, respectively) to suspect the existence of two different functional sites. However, the fact that both pre- and post-synaptic actions of NC were eliminated in $OP_4^{-/-}$ mice argues against such a proposition, even though this does not rule out the alternative possibility of splice variants of the receptor (Inoue *et al*., 1999).

Some evidence has also been obtained in favour of a role of endogenous spinal NC in modulating pain threshold. For instance, it has been demonstrated that i.t. injection of antibodies against NC decrease the antinociceptive effects of electroacupuncture (Tian *et al*., 1998).

Anxiolytic-like action of nociceptin

In 1997 Jenck *et al*. (1997) demonstrated that NC can act as an anxiolytic, attenuating the behavioural inhibition of animals acutely exposed to stressful/anxiogenic conditions. Given i.c.v. NC, at relatively low doses (0.1–3 nmol), induced anxiolytic-like effects in several behavioural paradigms, each generating different types of anxiety (light-dark preference, elevated plus-maze, exploratory behaviour of an unfamiliar environment, pharmacological anxiogenesis, operant conflict), leading to the proposal that the peptide may act as an endogenous regulator of acute anxiety. This view has been recently corroborated by studies in knockout animals (Koster *et al*., 1999; Reinscheid *et al*. 1999) showing that genetically engineered NC precursor-deficient mice display an increased susceptibility to acute and repeated stress, as compared to their wild-type littermates. The

anxiolytic-like properties of NC have also been investigated in the mouse defence test battery (Griebel *et al*., 1999). Unlike the classical anxiolytic drug diazepam, which affects all defensive responses, NC clearly reduced only defensive upright postures and biting reactions which are the typical reactions to highly stressful stimuli, suggesting that the peptide may not be primarily involved in all anxiety-related responses, but may actually play a role in the adaptative responses to unavoidable or extreme stress stimuli. The anxiolytic mechanisms of NC are at present largely unknown, but may be related to the inhibitory action of NC on serotoninergic mechanisms exerted at two different levels: on dorsal raphe nucleus neurons, where NC causes inhibition by increasing K^+ conductance (Vaughan & Christie, 1996), and on cortical serotoninergic nerve terminals, where NC inhibits 5-HT release (Sbrenna *et al*., 2000; Siniscalchi *et al*., 1999; Werthwein *et al*., 1999).

Recently some nonpeptide OP_4 receptor agonists have been identified by investigators at Hoffmann La Roche (Adam *et al*., 1998). These compounds displayed anxiolytic-like properties in the elevated plus-maze test in rats (Wichmann *et al*., 1999). These findings suggest that OP_4 receptor agonists may provide a new class of anxiolytic drugs devoid of any motivational effects, such as abuse liability (Devine *et al*., 1996a). They also provide ground for an intensive research programme to be pursued in both academic and industrial laboratories (Adam *et al*., 1998; Wichmann *et al*., 1999).

Modulation of spontaneous locomotor activity

Reinscheid *et al*. (1995) were the first to show that i.c.v. NC administration (in the range of 1–10 nmol) inhibits spontaneous locomotor activity in mice, a finding later confirmed by several authors (Calo' *et al*., 1999; Devine *et al*., 1996b; Nishi *et al*., 1997; Noble & Roques, 1997; Noda *et al*., 1998). Repeated daily NC injections result in rapid development of tolerance to this depressor effect of the peptide on locomotion and rearing activity (Devine *et al*., 1996b). As is typical for various NC-induced effects, this inhibitory action of NC is insensitive to naloxone (Noble & Roques, 1997), but is reversed by NalBzOH (Noda *et al*., 1998), and is undetectable in $OP_4^{-/-}$ knockout mice (Nishi *et al*., 1997). Nevertheless, the basal locomotor activity of $OP_4^{-/-}$ mice is not different from that displayed by wild-type littermates which suggests that the NC/OP_4 receptor system does not play a tonic role in the physiological regulation of locomotion. This view is further strengthened by the fact that: (i) a mixture of peptidase inhibitors potentiates the inhibitory effect of exogenously applied NC on locomotion by about 10 fold, but does not modify this behaviour when applied alone (Noble & Roques, 1997); and (ii) NalBzOH, at doses sufficient to prevent the inhibitory effect of NC, does not increase *per se* spontaneous locomotor activity.

NC has also been reported to stimulate locomotor activity and exploratory behaviour at very low doses (0.01–0.1 nmol) (Florin *et al*., 1996). This effect of NC has been related to the anxiolytic-like actions of the peptide (Jenck *et al*., 1997). Thus, NC shows a bell-shaped dose response curve for locomotor activity: stimulation at low doses (0.01–0.1 nmol), inhibition at high doses (1–10 nmol). Although bell-shaped curves are typical of conventional anxiolytic drugs such as benzodiazepines, the nature of the two opposite effects of NC has not been clarified.

Stimulation of food intake

The i.c.v. injection of NC has been shown to stimulate food intake in satiated rats (Pomonis *et al*., 1996). This orexigenic action is prevented by naloxone, unlike most NC-mediated effects, suggesting that the peptide stimulates food intake by activating an opioidergic neuronal pathway. Similar orexigenic effects of NC can also be detected following injection into discrete brain areas, such as the nucleus accumbens shell or the ventromedial hypothalamic nucleus (Stratford *et al*., 1997). It appears likely that this action involves neuronal depression within this satiety center as NC has been reported to hyperpolarize, in a concentration-dependent manner, the neurones of the ventromedial hypothalamus (Lee *et al*., 1997).

Polidori *et al*., (1999), have shown that the brain regions surrounding the third ventricle are the areas most sensitive to the orexigenic effects of NC. They also showed that [F/G]NC(1–13)NH_2 is 10 fold more potent than NC for stimulation of food intake (Polidori *et al*., 1999). Furthemore, the orexigenic effects of NC are clearly due to OP_4 receptor activation since they can be blocked either by antisense oligonucleotides targeting the receptor (Leventhal *et al*., 1998) or by the OP_4 receptor antagonist [Nphe^1]NC(1–13)NH_2 (Polidori *et al*., 2000). While the OP_4 receptor antagonist [Nphe^1]NC(1–13)NH_2 alone is ineffective in modifying food consumption in satiated rats it effectively reduces that induced by food deprivation. Thus, nociceptinergic pathways may well be activated by food deprivation to intervene in food intake regulation (Polidori *et al*., 2000). It is, therefore, conceivable that OP_4 receptor antagonists may constitute new anorectic agents alongside the neuropeptide Y receptor antagonists (Rowland *et al*., 1996).

Nociceptin and rewarding effects of drugs

Unlike classical opioids, NC fails to produce conditioned place preference or aversion (Devine *et al*., 1996a). Furthermore another study, carried out in alcohol preferring rats (Ciccocioppo *et al*., 1999) has shown that while acute i.c.v. injection of NC just before access to ethanol increases ethanol intake, similar i.c.v. injections for 7 days result in a progressive decrease in ethanol consumption. Once the subchronic NC treatment was terminated, rats progressively recovered their usual ethanol intake. On the other hand, NC also significantly reduces the increase in time that is spent in the ethanol-paired compartment after conditioning. Thus, NC seems to negatively modulate the rewarding properties of ethanol. Likewise NC also reduces the development of place preference to morphine (Angeletti *et al*., 1999; Murphy *et al*., 1999a) as well as opioid-induced locomotion in the rat (Grandy *et al*., 1999). Since the mesolimbic dopaminergic system plays a pivotal role in opioid rewarding properties (Wise, 1989), it has been suggested that NC attenuates conditioned place preference to morphine by inhibiting the alkaloid stimulatory effect on mesolimbic dopamine release (Murphy *et al*., 1999a). In fact, i.c.v. NC effectively inhibits dopamine release (as evaluated by *in vivo* microdialysis) in the nucleus accumbens of the rat stimulated by systemically injected morphine (Pieretti & Di Giannuario, 1999). Notwithstanding, NC has been reported to be ineffective in altering heroin self administration rate (Walker *et al*., 1998) or the development of cocaine sensitization (Narayanan & Maidment, 1999). Further studies are therefore needed to elucidate the role of the endogenous NC/OP_4 receptor system in regulating the mesolimbic dopaminergic system and the rewarding properties of drugs of abuse, and hence the usefulness of NC receptor agonists for controlling drug self administration.

Inhibitory effect of nociceptin on memory process

The first indications for a role of the NC/OP_4 system in learning and memory came from the observations that NC injection into the hippocampus impairs spatial learning (Sandin *et al*., 1997) and that *in vitro* it inhibits synaptic transmission and longterm potentiation in rat hippocampal slices (Yu *et al*., 1997; Yu & Xie, 1998). In line with these findings, OP_4 receptor knockout mice show greater learning ability and have better memory retention than wild-type control mice (Manabe *et al*., 1998). In additon, hippocampal slices of OP_4 receptor-deficient mice display greater long-term potentiation in CA1 region than those of control mice. The impairment of learning induced by NC can be reversed by nocistatin (functional antagonist of NC; Hiramatsu & Inoue, 1999) or by the nonselective OP_4 receptor antagonist NalBzOH (Mamiya *et al*., 1999). Moreover, a peptidic OP_4 receptor antagonist Ret-Noc-OMe, has been reported to potentiate memory retention in a passive avoidance test in mice (Yoshikawa *et al*., 1999).

Collectively, these findings suggest that the NC/OP_4 receptor system may play negative roles in learning and memory, and that OP_4 receptor antagonists may be interesting drugs for the alleviation of memory disorders. Much remains to be done in this field.

Nociceptin/OP_4 receptor system and epilepsy

NC has been shown to inhibit the release of glutamate from rat cerebral (Nicol *et al*., 1996) and cerebellar (Nicol *et al*., 1998b) cortex slices. Moreover, several studies indicate that NC counteracts glutamate-mediated excitatory transmission in various central nervous system areas (Allen *et al*., 1999; Faber *et al*., 1996; Vaughan *et al*., 1997; Wang *et al*., 1996; Yu & Xie, 1998). Since glutamate plays a key role in epilepsy (Bradford, 1995), it is reasonable to expect that NC may exert an anti-epileptic action. Indeed, NC has been reported to inhibit kindling development in rats (Gutierrez *et al*., 1998) and pentylenetetrazole-induced seizures in mice (Zgodzinski *et al*., 1999). Interestingly, pronociceptin gene expression is dramatically enhanced in the thalamic reticular nucleus during kainate-induced seizures in rats (Bregola *et al*., 1999). These initial studies suggest that the NC/OP_4 receptor system may have a role to play in seizure/epilepsy, and also that further studies into the potential effectiveness of OP_4 receptor agonists as anti-epileptic drugs should be encouraged.

Inhibition of neurotransmitter release from central neurones

NC has also been reported to inhibit neurotransmitter release in the central nervous system. This has been shown *in vitro* for (i) noradrenaline (mouse, rat and guinea-pig cerebral cortex slices (Schlicker *et al*., 1998); mouse hippocampus, hypothalamus and cerebellum (Werthwein *et al*., 1999)); (ii) serotonin (rat (Sbrenna *et al*., 2000; Siniscalchi *et al*., 1999) and mouse (Werthwein *et al*., 1999) cortex); (iii) glutamate (rat cerebral (Nicol *et al*., 1996) and cerebellar (Nicol *et al*., 1998b) cortex); (iv) dopamine (midbrain primary cultures (Murphy *et al*., 1999b)); and (v) acetylcholine (rabbit retina (Neal *et al*., 1997)). These effects of NC were not affected by naloxone, ruling out the involvement of classical opioid receptors.

NC has also been demonstrated to modulate neurotransmitter release *in vivo*. Thus, intracerebroventricular injection of NC in the anaesthetized rat decreased dopamine release in the nucleus accumbens (Murphy *et al*., 1996), an effect that could be reproduced by NC perfusion into the ventral tegmental area (Murphy & Maidment, 1999). It may therefore be related to inhibition of the mesolimbic dopaminergic pathway. In this model, NC perfusion also increased local extracellular levels of glutamate and GABA (Murphy & Maidment, 1999). Worthy of mention is the fact that NC has also been reported to inhibit dopamine release from the nucleus accumbens stimulated by morphine (Pieretti & Di Giannuario, 1999).

Dual probe microdialysis in the awake rat has revealed that NC perfusion in the substantia nigra pars reticulata increases local extracellular glutamate levels *via* naloxone-insensitive mechanisms (Marti *et al*., 1999). This stimulation was associated with a decrease in acetylcholine release in the ipsilateral striatum, an effect possibly related to inhibition of the nigrostriatal dopaminergic pathway (Marti *et al*., 1999).

NC perfusion in the striatum stimulated local dopamine release but this was blocked (although at very high doses) by naloxone, suggesting the involvement of classical opioid receptors (Konya *et al*., 1998).

The results of these studies indicate that NC is a potent modulator of neurotransmitter release in various preparations that are widely used to explore the neurochemical bases of the central actions of the drugs. For NC effects, the following combinations should be considered: (i) inhibition of glutamate release/anti-epileptic action and disruption of spatial memory; (ii) inhibition of serotonin release/anxiolytic action; (iii) inhibition of mesolimbic dopaminergic transmission/anti-rewarding properties; (iv) modulation of striatal dopamine and glutamate/effects on locomotor activity.

Inhibitory effects of nociceptin on the cardiovascular system

When given i.v. to anaesthetized rats, NC induces transient hypotension and bradycardia (Champion & Kadowitz, 1997; Giuliani *et al*., 1997). These effects appear to be mediated by the autonomic nervous system, because hypotension can be prevented by guanethidine pretreatment while bradycardia is reduced by bilateral cervical vagotomy and abolished by a combination of both procedures (Giuliani *et al*., 1997). Similar results have been obtained in conscious rats (Kapusta *et al*., 1997) and mice (Madeddu *et al*., 1999), indicating that anaesthesia does not affect the cardiovascular effects of NC and that these effects are not restricted to the rat. The cardiovascular depressor actions of NC are not modified by naloxone, but are antagonized by [F/G]NC(1 – 13)NH_2 in both mice (Madeddu *et al*., 1999) and rats (Bigoni *et al*., 1999a). Interestingly, NC induces similar cardiovascular effects when injected i.c.v. (Kapusta *et al*., 1997) or into the rostral ventrolateral medulla of the rat (Chu *et al*., 1999a). While [F/G]NC(1 – 13)NH_2 mimics the effects of NC when injected i.c.v. (Kapusta *et al*., 1999), it blocks NC-induced responses if applied on rostral ventrolateral medulla neurons both *in vitro* (Chu *et al*., 1999b) and *in vivo* (Chu *et al*., 1999a), again illustrating the dual agonistic/antagonistic nature of this OP_4 receptor ligand.

These data demonstrate that NC, probably by acting at central sites, exerts a marked inhibitory action on cardiovascular parameters in rodents. On the other hand, NC has recently been shown to increase blood pressure and heart rate in sheep following i.v. administration (Arndt *et al*., 1999). Thus, there may be important differences in the cardiovascular effects of NC in different species.

NC also induces vasodilation in several isolated arteries of the cat (Gumusel *et al*., 1997) and in mesenteric resistance arteries of the rat (Champion *et al*., 1998). This latter study

also demonstrated that vasodilator responses to NC were not prevented by naloxone, nitric oxide synthase inhibitors, atropine, phentolamine or by the CGRP receptor antagonist CGRP(8–37). Moreover, the effect of NC was unchanged in endothelium denuded vessels (Champion *et al*., 1998). Vasodilator effects of NC have also been described also in other vascular beds such as the hindquarters of the rat (Czapla *et al*., 1997), or pial arteries of the pig (Armstead, 1999). This latter effect has been demonstrated to be due to activation of the OP_4 receptors coupled to increased production of cyclic AMP and subsequent activation of K_{ATP} and K_{Ca} channels (Armstead, 1999). In addition, NC has been shown to inhibit electrically-evoked noradrenaline release in the isolated rat tail artery, by acting on prejunctional OP_4 receptors located on postganglionic nerve endings (Bucher, 1998). NalBzOH antagonizes this effect of NC without affecting *per se* noradrenaline release, suggesting that OP_4 receptors are not tonically activated by endogenously-released NC.

Finally, intracavernosal injection of NC induces a pronounced and relatively long-lasting erectile response in the cat (Champion *et al*., 1997).

Nociceptin effects on renal functions

The first study addressing the effects of NC on renal functions was performed by Kapusta *et al*. (1997), who reported that the peptide induces a marked increases in water excretion and decreases in urinary sodium excretion when injected i.v. or i.c.v. These diuretic and antinatriuretic effects of NC were resistant to blockade by the selective kappa-opioid receptor antagonist, nor-binaltorphimine, at a dose which blocked dynorphinA-induced diuresis. Interestingly, i.c.v. injection of [F/G]NC(1–13)NH_2 mimics the action of NC, producing similar, but longer lasting cardiovascular and renal effects (Kapusta *et al*., 1999). These findings indicate that [F/G]NC(1–13)NH_2 acts as an agonist at OP_4 receptors controlling water balance. The longer duration of action of the pseudopeptide probably reflects its higher metabolic stability (see the section on [F/G]NC(1–13)NH_2). As for the mechanism(s) by which NC exerts its diuretic and antinatriuretic actions, it has been reported that NC inhibits both oxytocin and vasopressin neurons, as measured using patch-clamp recording techniques in rat supraoptic nucleus slices (Doi *et al*., 1998). Similarly, NC also depresses guinea-pig supraoptic nucleus neurons acting through two mechanisms, hyperpolarization subsequent to activation of inwardly rectifying K^+ conductance, as well as increase in membrane resistance due to closing of low-resistance gap junctions (Slugg *et al*., 1999). Taken together, these results suggest that the renal actions of NC can be attributed to inhibition of vasopressin secretion from the supraoptic nucleus neurons.

These observations suggest that endogenous NC may be a novel peptide involved in the central control of water and electrolyte balance and ultimately in the regulation of arterial blood pressure. Future analogues of NC may prove to be the first clinically useful water diuretics for patients with water-retaining diseases.

Effects of nociceptin in the gut

The gastrointestinal tract is a major site of action for opioids and opium, which have been used to reduce gut motility since ancient times. Likewise, OP_4 receptor are also expressed in the gut (Wang *et al*., 1994), but little is known about the possible functions they subserve in the tract.

Like to opioids, NC inhibits *in vitro* neurogenic contractions of the stomach and the small intestine in a variety of species, including guinea pigs (Calo' *et al*., 1996; Zhang *et al*., 1997), pigs (Osinski *et al*., 1999a); rats (Yazdani *et al*., 1999); rabbits (Pheng *et al*., 1999). The depressor effect of NC is resistant to blockade by naloxone and appears to be correlated with a reduction of acetylcholine release, at least in the rat (Yazdani *et al*., 1999). On the other hand, NC causes concentration-dependent contractions in colonic smooth muscle strips from rodents (Corbett *et al*., 1998; Osinski *et al*., 1999b; Rizzi *et al*., 1999b; Taniguchi *et al*., 1998; Yazdani *et al*., 1999). This effect is prevented by tetrodotoxin or Ca^{2+}-free medium suggesting the involvement of nerve activation (Yazdani *et al*., 1999). Given the depressor role of NC in neurotransmission at various autonomic neuroeffector functions, it appears plausible to hypothesize that its contractile effect in the colon is due to decreased release of an (unknown) inhibitory neurotransmitter. Very recently, it was shown that the contractions induced by NC and a variety of OP_4 receptor ligands in the mouse colon are selectively antagonized by [Nphe^1]NC(1–13)NH_2, and are thus mediated by such receptors (Rizzi *et al*., 1999b). Like opioids, central administration of NC also inhibits colonic transit in the mouse (Osinski *et al*., 1999b). This effect is also mimicked in the rat by i.v. administration of NC in a [Nphe^1]NC(1–13)NH_2 sensitive manner (Casati *et al*., personal communication). NC also inhibits active anion transport in the intestinal mucosa through a neural mechanism in the pig ileum (Osinski *et al*., 1999b). On the other hand, Taniguchi *et al*. (1998) reported that NC administered subcutaneously actually accelerated transit rate in the large intestine, an action opposite to that induced by morphine or selective opioid receptor agonists. This other unexpected findings raise the question of the role(s) of the NC/OP_4 receptor in the gut and its relation to the opioid system. However, the body of data reported in the literature points to a new regulatory NC/OP_4 receptor system in the gastrointestinal tract, which is pharmacologically distinct from opioids but functionally very similar, that could represent a new target for the development of drugs (OP_4 receptor agonists) to reduce intestinal motility.

Effects in the airways

NC was found to inhibit the contractions of the guinea-pig isolated bronchus induced by electrical field stimulation (Fischer *et al*., 1998). This effect was found to be mediated by a prejunctional mechanism not involving the classical opioid receptors. The same study also disclosed the existence of NC immunoreactive nerve fibers in the airway wall, distinct from the tachykinin-containing fibers. These findings were later validated by the demonstration that the inhibitory effect of NC is effectively and specifically blocked by [F/G]NC(1–13)NH_2 (Rizzi *et al*., 1999a; Shah *et al*., 1998). Direct evidence has been provided for inhibitory actions of NC on neurotransmitter release from cholinergic and peptidergic airways nerves (Patel *et al*., 1997; Shah *et al*., 1998; Helys *et al*., 1997; Nemeth *et al*., 1998). The effect of NC on airways *in vivo* has not yet been investigated.

Inhibition of the micturition reflex

NC has been reported to modulate several autonomic functions, among others the inhibition of the micturition reflex in rats: this effect of NC has been investigated in great detail by the Menarini group (Giuliani *et al*., 1998; 1999; Lecci *et al*., 1999). In anaesthetized rats, i.v. NC produced a dose-

dependent suppression of the micturition reflex induced by distension or topical application of capsaicin (Giuliani *et al*., 1998). Similar results were obtained by administering the peptide i.c.v., but not i.t., indicating that NC inhibits the micturition reflex by acting at peripheral and supraspinal sites (Lecci *et al*., 1999). All these effect are not affected by naloxone, thus excluding the involvement of opioid receptors. Worthy of mention is the fact that NC given i.v. affords long-lasting protection to capsaicin-induced desensitization of afferent nerves, which are known to mediate the chemoceptive micturition reflex in this model. In contrast, NC did not modify the desensitization of the local response to capsaicin (efferent function) (Giuliani *et al*., 1999). These results suggest that NC is able to discriminate between the afferent and efferent functions of capsaicin-sensitive primary sensory neurones, by selectively protecting the afferent function of these nerves (Giuliani *et al*., 1999). The recent identification of OP_4 receptor antagonists of either peptidic (Calo' *et al*., 2000; Guerrini *et al*., 1999) or of non-peptidic nature (Ozaki *et al*., 1998) should be very useful for evaluating the possible pathophysiological involvement of endogenous NC in the micturition reflex in the rat and more generally on the sensory system.

Conclusions

The recent discoveries of ORL1 and its endogenous ligand NC add another member to the already well-defined opioid receptor family. The new system has been named NC/OP_4 receptor, in accord with a proposal presented at the last IUPHAR international congress (Hamon, 1998). The advantages of this nomenclature have been underlined. Structure-activity relationship studies, which have led to identification of potent agonists ($NCNH_2$, $NC(1-13)NH_2$), of a partial agonist ($[F/G]NC(1-13)NH_2$), and a pure antagonist ($[Nphe^1]NC(1-13)NH_2$), have also provided the basic tools for receptor characterization and classification in terms of agonists and antagonists. These tools have been used in studying the basic pharmacology of the system *in vitro*, with classical biological and binding assays performed on native OP_4 receptors from various species, as well as with molecular biology/biochemistry techniques on the human recombinant receptor. Several agonists and a recently discovered competitive antagonist ($[Nphe^1]NC(1-13)NH_2$) have been successfully applied to the characterisation of OP_4 functional receptors and binding sites. Very similar pharmacological profiles have been observed in the various assays using the Schild criteria of agonist order of potency and antagonist affinity. These finding have led us to conclude that the same receptor mediates the actions of the system at different levels (peripheral, spinal, supraspinal) in the same species and in preparations obtained from different species (mouse, rat, guinea-pig, man).

In reviewing the numerous emerging effects of and roles played by NC in the body, we have attempted to select the most consistent effects and identify the potential application of agonists and antagonists of the OP_4 receptor. A broad spectrum of possible therapeutical indications of such compounds include: (a) OP_4 receptor agonists as anxiolytics, stimulants of food intake, intrathecal (spinal) analgesics, suppressants of drug abuse, anti-epileptics and for the management of hyponatremic and water-retaining syndromes; (b) OP_4 receptor antagonists could be tried as anorectics, analgesics (alone or in combination with opioids) or as nootropic agents.

The recent identification of OP_4 receptor agonists (Adam *et al*., 1998; Wichmann *et al*., 1999) and antagonists (Ozaki *et al*., 1998) of non-peptidic nature may provide the tools needed to elucidate the role of the NC/OP_4 receptor system in pathophysiology and to verify the actual value of compounds that activate or inhibit this system, as new potential drugs for treatment of human diseases.

This work was supported by a 60% grant of the University of Ferrara and by a cofin99 grant of the Italian Ministry of the University (MURST). We wish to thank Dr D.G. Lambert (Dept. Anaesthesia, University of Leicester, U.K.) for helpful discussion and Dr G.A. Rae (Dept. Pharmacology, Universidade Federal de Santa Catarina, Florianopolis, Brazil) for improving the style of this paper. Thanks are also due to students and technicians (Raffaella Bigoni, Daniela Rizzi, Giuliano Marzola, Katia Varani) who have contributed substantially to the acquisition of the data quoted in tables and figures, and to collaborators who worked with us on the nociceptin project since its initial phases (D.G. Lambert, M. Massi, M. Morari, C. Bianchi, P.A. Borea, P. Madeddu, S. Candeletti, H. Berger, C.A. Maggi, E. Ongini).

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(Received December 16, 1999
Revised January 5, 2000
Accepted January 10, 2000)