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CICLO XXI

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NOCICEPTIN/ORPHANIN FQ RECEPTOR LIGANDS: PHARMACOLOGICAL STUDIES

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List of abbreviations

aa	amino acid
Ac	acetyl
Ach	acetylcholine
Aib	amino isobutyric acid
ANOVA	One-Way Analyses of Variance
APN	Aminopeptidase N
Arg	Arginine
AUC	area under curve
bNST	bovine Nocistatin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CPP	conditioned place preference
CHO	chinese hamster ovary
CNS	central nervous system
Compound 24	1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4'-piperidin-1-yl]propyl}
Compound 35	(D)-1-Benzyl-pyrrolidine-2-carboxylic acid {3-[4-(2,6-dichloro-phenyl)-piperidin-1-yl]-propyl}-amide
CRF	corticotropin release factor
DA	dopamine
DAG	diacylglycerol
DOP	delta opioid peptide
DPDPE	[D-Pen^2,D-Pen^5]enkephalin
EDTA	ethylenediamine-tetraacetic acid
EL	extracellular loop
EP 24.11	endopeptidase 24.11
EP 24.15	endopeptidase 24.15
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
GABA	γ-aminobutyric acid
GDP	guanosin diphosphate
GPCR	G-protein coupled receptor
gpI	guinea pig ileum
GTP	guanosin 5'-triphosphate
GTPγS	guanosine-5'-O-(3-thiotriphosphate)
[^{35}S]GTPγS	guanosine-5'-[γ-^{35}S]thiophosphate
5-HT	serotonin
HBSS	Hanks' Balanced Salt Solution
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HTS	high throughput screening
i.c.v.	intracerebroventricular(ly)
i.p.	intraperitoneo(ly)
IP_3	inositol 1,4,5-trisphosphate
i.t.	intrathecal(ly)
i.v.	intravenous(ly)
IUPHAR	International Union of Pharmacology (acronym)
III-BTD	(3S,6S,9R)-2-oxo-3-amino-7-thia-1-aza-bicyclo[4.3.0]nonane-9-carboxylic acid
(±)J-113397	(±)trans-1-[1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one

JTC-801	N-(4-amino-2-methylquinolin-6-yl)-2-(4-ethylphenoxymethyl)benzamide hydrochloride
KOP	kappa opioid peptide
MCOPPB	1-[1-(1-Methylcyclooctyl)-4-piperidinyl]-2-[(3R)-3-piperidinyl]-1H-benzimidazole
MOP	mu opioid peptide
mRNA	messenger ribonucleic acid
mVD	mouse vas deferens
NA	noradrenaline
NalBzOH	naloxone benzoyhlydrazone
NNC 63-0532	(8-Naphthalen-1-ylmethyl-4-oxo-1-phenyl-1,3,8-triaza-spiro[4.5]dec-3-yl)-acetic acid methyl ester
N/OFQ	Nociceptin/orphanin FQ
NOP receptor	Nociceptin/orphanin FQ peptide receptor
NOP(+/+), NOP(-/-)	NOP receptor knockout and wildtype
NSB	non specific binding
NST	nocistatin
oGPCR	orphan G-protein coupled receptor
***Oprl 1* gene**	NOP receptor gene
ORL-1 receptor	Opioid receptor-like 1
PAG	periaqueductal gray
PCPB	2-(3,5-dimethylpiperazin-1-yl)-1-[1-(1-methylcyclooctyl)piperidin-4-yl]-1H-benzimidazole
PCR	polymerase chain reaction
PLC	phospholipase C
ppN/OFQ	prepronociceptin
ppN/OFQ(-/-)	prepronociceptin gene deficient
PVN	paraventricular nucleus
Ro-65-6570	(8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4,5]decan-4-one)
Ro-64-6198	[(1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triaza-spiro[4,5]decan-4-one]
SAR	structure-activity relationship
RT-PCR	reverse transcriptase polymerase chain reaction
rVD	rat vas deferens
SB-612111	(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol
SCH 221510	8-[bis(2-Methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol
s.e.m.	standard error of the mean
TM	transmembrane
Tris	Tris-(hydroxymethyl)-aminomethane
TTX	tetrodotoxin
TRK-820	((-)-17-cyclopropylmethyl-3,14b-dihydroxy-4,5a-epoxy-6b-[N-methyl-trans-3-(3-furyl)acrylamide]morphinan hydrochloride)
UFP-101	[Nphe^{1}Arg^{14}Lys^{15}]N/OFQ-NH_2
UFP-102	[(pF)Phe^{4}Arg^{14}Lys^{15}]N/OFQ-NH_2
UFP-103	[Phe$^{1}\psi$(CH_2-NH)Gly^{2}(pF)Phe^{4}Arg^{14}Lys^{15}]N/OFQ-NH_2
UFP-111	[Nphe^{1}(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2
UFP-112	[(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2
UFP-113	[Phe$^{1}\psi$(CH_2-NH)Gly^{2}(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2
ZP120	Ac-RYYRWKKKKKKK-NH_2

Abstract (University of Leicester Format)

Nociceptin/orphanin FQ receptor ligands: pharmacological studies

The neuropeptide nociceptin/orphanin FQ (N/OFQ) selectively binds and activates the N/OFQ peptide (NOP) receptor. At cellular level N/OFQ inhibits cAMP accumulation and Ca^{2+} conductance and stimulates K^+ currents. N/OFQ regulates several biological functions both at central (pain, locomotion, memory, emotional responses, food intake) and peripheral (airways, cardiovascular, genitourinary and gastrointestinal systems) sites. Potent and selective NOP ligands are now required for investigating the roles played by NOP receptors in pathophysiological studies and for firmly defining the therapeutic indications of NOP receptor ligands.

A novel assay to screen NOP receptor ligands has been validated with a large panel of ligands: the $G\alpha_{qi5}$ chimeric protein has been used to force the NOP receptor to signal through the Ca^{2+} pathway in CHO cells. $[Ca^{2+}]_i$ levels were monitored using the fluorometer FlexStation II. Data are in general agreement with classical G_i driven assay systems.

The NOP peptide partial agonist, ZP120 was extensively characterized *in vitro* using electrically stimulated isolated tissues (mouse and rat vas deferens) and *in vivo* with the tail withdrawal assay. The selective involvement of the NOP receptor in the actions of ZP120 has been demonstrated in NOP(-/-) mice studies.

A detailed pharmacological characterization of the recently identified non-peptide antagonist Compound 24 has been performed. Moreover in the context of a SAR study on Compound 24, a novel NOP ligand named Compound 35 was identified. Compound 24 and Compound 35 bound the human recombinant NOP receptor expressed in CHO_{hNOP} cell membranes with high affinity (pK_i values 9.62 and 9.14, respectively). Our findings derived from functional studies on CHO_{hNOP} and bioassay studies on native receptors demonstrated that Compound 24 and Compound 35 behave as potent, competitive and selective non-peptide NOP antagonists. Finally, the NOP antagonist properties of Compound 24 have been confirmed *in vivo* in the mouse tail withdrawal assay.

Summary

N/OFQ and its receptor share high sequence similarity with opioid peptides, particularly with dynorphin A, and their receptors. However N/OFQ and dynorphin use distinct molecular pathways to bind and activate their cognate receptors. Thus, N/OFQ and NOP receptor represent a novel peptidergic system, which is pharmacologically distinct from the opioid systems. At cellular level N/OFQ inhibits cAMP accumulation and Ca^{2+} conductance and stimulates K^+ currents, and *in vivo* it modulates a variety of biological functions: nociception, food intake, memory processes, anxiety, locomotor activity, gastrointestinal motility, cardiovascular and renal functions, micturition and cough reflexes.

The pharmacological characterization of new ligands for this peptide/receptor system has been the main aim of the studies performed during this Ph.D. program. In close collaboration with Prof Lambert's group (University of Leicester) and with Medicinal Chemistry group of Prof. Salvadori (University of Ferrara), we performed a series of studies on novel ligands for the NOP receptor. The most important areas are summarized as below:

1) Pharmacological profile of NOP receptors coupled with calcium signalling via the chimeric protein $G\alpha_{qi5}$

In this study the $G\alpha_{qi5}$ protein was used to force the human NOP receptor to signal through the Ca^{2+} pathway in CHO cells. $[Ca^{2+}]_i$ levels were monitored using the FlexStation II fluorometer and the Ca^{2+} dye Fluo 4 AM. Concentration response curves were generated with a panel of full and partial agonists while NOP antagonists were assessed in inhibition response curves.

The following rank order of potency of antagonists was measured: SB-612111 > J-113397 = Trap-101 $\geq$ UFP-101 > [Nphe^1]N/OFQ(1-13)-NH_2 >> naloxone, which is in good agreement with the literature. The rank order of potency of full and partial agonists is also similar to that obtained in previous studies with the exception of a panel of ligands (UFP-112, Ro 64-6198, ZP120, UFP-113) whose potency was relatively low in the $G\alpha_{qi5}$ - NOP receptor calcium assay. Interestingly, these NOP ligands are characterized by slow kinetics of interaction with the NOP receptor, as demonstrated by bioassay experiments. This study demonstrated that the FlexStation II - $G\alpha_{qi5}$ - NOP receptor calcium assay represents an adequate and useful screening tool for NOP receptor ligands, particularly for antagonists.

2) Further studies on the pharmacological features of the nociceptin/orphanin FQ receptor ligand ZP120

In previous studies, the effects of ZP120 were found to be sensitive to J-113397 in mouse tissues while resistant to UFP-101 in rat tissues. The aim of this study was to further investigate the pharmacological profile of ZP120 using mouse and rat preparations, J-113397 and UFP-101, as well as NOP(-/-) mice. Electrically stimulated mouse and rat vas deferens were used to characterize the pharmacology of ZP120 *in vitro*. For *in vivo* studies the tail withdrawal assay was performed in wild type (NOP(+/+)) and NOP(-/-) mice. In the mouse and rat vas deferens ZP120 mimicked the effects of N/OFQ showing higher potency but lower maximal effects. In both preparations, J-113397 antagonized N/OFQ and ZP120 effects with similar pK_B values (≈ 7.8). UFP-101 antagonized the actions of N/OFQ (pK_B values ≈ 7.3) but did not modify the effects of ZP120. The inhibitory effects of N/OFQ and ZP120 were no longer evident in vas deferens tissues taken from NOP(-/-) mice. In NOP(+/+) mice subjected to the tail withdrawal assay, ZP120 (1 nmol) mimicked the pronociceptive action of N/OFQ (10 nmol), producing longer lasting effects. The effects of both peptides were absent in NOP(-/-) animals. The NOP receptor ligand ZP120 is a high potency NOP selective partial agonist able to evoke long-lasting effects; its diverse antagonist sensitivity in comparison with N/OFQ may derive from different modality of binding to the NOP receptor.

3) Pharmacological characterization of the nociceptin/orphanin FQ receptor non-peptide antagonist Compound 24

Compound 24, 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4'-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide was recently identified as a NOP ligand. In this study, the *in vitro* and *in vivo* pharmacological profile of Compound 24 was investigated. *In vitro* studies were performed measuring receptor and [^{35}S]GTPγS binding and calcium mobilization in cells expressing the recombinant NOP receptor as well as using N/OFQ sensitive tissues. *In vivo* studies were conducted using the tail withdrawal assay in mice. Compound 24 produced a concentration-dependent displacement of [^{3}H]N/OFQ binding to CHO_{hNOP} cell membranes showing high affinity (pK_i 9.62) and selectivity (1000 fold) over classical opioid receptors. Compound 24 antagonized with high potency the following *in vitro* effects of N/OFQ: stimulation of [^{35}S]GTPγS binding in CHO_{hNOP} cell membranes (pA_2 9.98), calcium mobilization in CHO_{hNOP} cells expressing the $G\alpha_{qi5}$ chimeric protein (pK_B 8.73), inhibition of electrically evoked twitches in the mouse (pA_2 8.44) and rat (pK_B 8.28) vas deferens, and in the guinea pig ileum (pK_B 9.12). In electrically stimulated tissues, Compound 24 up to 1 μM did not modify the effects of classical opioid receptor agonists. Finally *in*

vivo, in the mouse tail withdrawal assay, Compound 24 at 10 mg/kg antagonized the pronociceptive and antinociceptive effects of 1 nmole N/OFQ given supraspinally and spinally, respectively. The present study demonstrated that Compound 24 is a pure, competitive, and highly potent non-peptide NOP receptor selective antagonist.

4) Blending of chemical moieties of NOP receptor ligands: identification of a novel antagonist

In the present investigation, we performed a structure-activity analysis of Compound 24, focussing on its N-benzyl D-Pro, amide bond, and benzoisofurane moieties. This latter structure was substituted with moieties taken from known non-peptide NOP receptor ligands. Twelve Compound 24 derivatives were synthesised and tested in binding experiments performed on CHO_{hNOP} cell membranes. Compound 24 displayed a pK_i value of 9.62 while the analogues modified on the N-benzyl D-Pro and amide bond moieties showed very low affinities. In contrast, Compound **35** in which the benzoisofurane was substituted with the 2,6-dichlorophenyl moiety of the NOP antagonist SB-612111, showed high affinity (pK_i 9.14). This novel compound was pharmacologically characterized in various assays where it consistently behaved as a pure, potent (pA_2 in the range 8.0 – 9.9), competitive and NOP selective antagonist. Collectively the present results indicate that the N-benzyl D-Pro and amide bond of Compound 24 are crucial for biological activity. Moreover, a novel interesting NOP receptor antagonist was identified by blending chemical moieties taken from different NOP receptor ligands.

1. INTRODUCTION

1.1 Orphan G-protein coupled receptors and the reverse pharmacology approach

G-protein coupled receptors (GPCRs) are one of the largest family of proteins that are the main modulators of intercellular interactions and regulate activities in the human body and in particularly in the central nervous system (CNS). There are numerous GPCRs in living organisms, but the function of many is still unknown. The human genome encompasses ~ 800 GPCRs, of which more than half are olfactory and/or taste GPCRs. They are targets of most of the primary messengers including the neurotransmitters, all the neuropeptides, the glycoprotein hormones, lipid mediators and other small molecules; thus have considerable pharmaceutical interest. Drugs that are acting on GPCRs are used to treat numerous disorders. More than 30 % of the approximately 500 clinically used drugs, are modulators of GPCRs function, representing around 9 % of global pharmaceutical sales, making GPCRs the most successful of any target class in terms of drug discovery (Drews, 2000).

367 transmitter GPCRs have been identified within the human genome, the majority of these GPCRs have been identified on the basis of their sequence similarities, either by homology cloning or by bioinformatics analyses. Many of these receptors are currently ‘orphans’.

The first step in the characterization of new orphan GPCRs is the search of the activating ligand. As the genomes of most studied model organism have now been sequenced, the process of discovery of GPCRs-ligand pairs has been reversed. Until recently, neuropeptides have been traditionally identified either on the basis of their chemical characteristics (Tatemoto *et al.*, 1980) or of their effects in particular assay systems (Erspamer *et al.*, 1978). Although highly successful, these approaches had reached a stand still by the mid 80’s.

Through DNA recombination techniques, it is now possible to transfect the sequence of an orphan receptor of which the function is not yet known, into an appropriate cellular expression system. This leads to the use of orphan receptors as baits to isolate their natural ligands from mixtures of synthetic ligands, including known GPCR ligands, naturally occurring bioactive molecules of unknown function and randomized compounds in high-throughput screening (HTS). This approach has been named “reverse pharmacology”. Thus, drug identification precedes the mechanistic understanding of mode of action of the drug candidate. The expression system provides the necessary trafficking and G-protein-signalling machinery to enable the successful identification of the activating ligand. By exposing the transfected cell to a tissue extract containing the natural ligand of the orphan receptor, a change in intracellular second messengers will be induced and will serve as a parameter to monitor orphan receptor ligand purification. Despite the logic of the theory,

the process is not simple, since the physical nature of the ligand and the type of the second messenger response that it will generate, are unknown. However, structural features in an orphan GPCR will determine its relationship to known receptors and will help in evaluating the nature of the receptor's ligand and its activity. Indeed, an orphan receptor which is related, even to a low degree, to a particular receptor family has a higher probability of sharing a ligand of the same physical nature and a coupling to similar G proteins. Notably this strategy has already led to several significant discoveries. The orphan receptor strategy was first proven to be successful with the discovery of the neuropeptide N/OFQ, the subject of this thesis, as the endogenous ligand of the oGPCR ORL-1 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

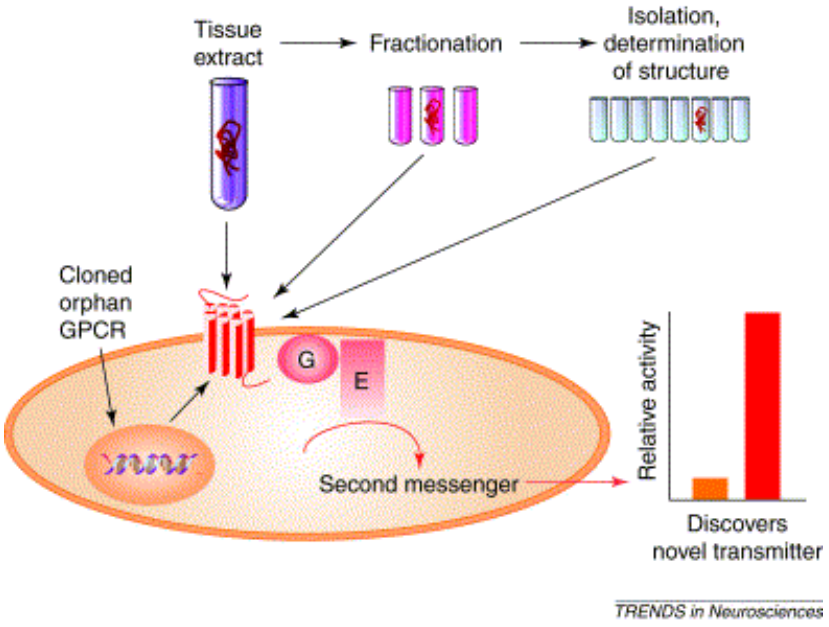


Figure 1. The orphan receptor strategy (Civelli *et al.*,TRENDS in Neurosciences Vol.24 No.4 April 2001). The orphan receptor strategy was developed to identify the natural ligands of orphan G-protein-coupled receptors (GPCRs) with the aim of discovering novel transmitters (defined in the main text). This strategy involves: (1) expression of the cloned orphan GPCR in an heterologous cell line; (2) exposure of this transfected cell line to a tissue extract that is expected to contain the natural ligand; (3) recording of the change in second messenger response elicited by activation of the orphan GPCR; (4) fractionation of the tissue extract and isolation of a surrogate, the active component; (5) determination of the chemical structure of the active component and (6) chemical synthesis of the active component and demonstration that it exhibits identical activity to that of the purified ligand.

This first successful example of orphan receptor strategy was followed by the identification of other novel bioactive peptides such as: hypocretins and orexins, prolactin-releasing peptide,

apelin, ghrelin, melanin-concentrating hormone, urotensin II, neuromedin U, metastin, neuropeptide B, neuropeptide W and neuropeptide S. Each of these discoveries was a landmark in its field (Civelli, 2005). The success in GPCRs deorphanization led to the approach being used by the pharmaceutical industry (Wise *et al.*, 2004), which had mastered the HTS of thousands of ligands. This led to thousands of potential transmitters and unexpected ligands (also of non-peptide nature) being tested on dozens of oGPCRs and a revival of the reverse pharmacology approach. Table 1 summarizes the transmitters of peptidic and non-peptidic nature identified as ligands of oGPCRs after 1995 (Civelli, 2005).

Table 1. Transmitters identified as ligands of oGPCRs after N/OFQ; taken from Civelli (2005).

Date	Transmitters found[b]	
	By synthetic ligand binding	In tissue extracts
1995	–	N/OFQ
1996	C3a	–
1997	LTB_4	–
	Latrotoxin	–
1998	S1P	Hypocretins and orexins
	LPA	PrRP
	–	Apelin
1999	LTD_4	Ghrelin
	MCH	MCH
	UII	UII
	Motilin	–
2000	NMU	NMU
	UDP-glucose	–
	SPC	–
	LTB_4	–
	Histamine	–
	Prostaglandin D_2	–
	LTC_4 and LTD_4	–
	NPFF and NPAF	–
	hRFRP-1 and hRFRP-3	–
2001	LPC	Metastin
	SPC	–
	ADP	–
	Psychosine	–
	Trace amines	–
2002	5-Oxo-ETE	NPB and NPW
	Bile acids	Adenine
	PK1 and PK2	PK1 and PK2
	BAM22	–
	Relaxin	–
2003	Bradykinin	Relaxin 3
	QRFP	–
	Cortistatin	–
	Medium and long fatty acids	–
	Nicotinic acid	–
	Proton	–
2004	β-Alanine	Succinate
	α-Ketoglutarate	NPS
	AMP and adenosine	–

1.2 The NOP receptor

Pharmacological studies have defined at least three subtypes of opioid receptors, termed μ, δ and κ receptors, that are involved in the mediation of the numerous effects, like analgesia, respiratory depression, miosis, constipation, sensation of well being, tolerance and dependence.

The nomenclature for the opioid receptors remains controversial. A 1996 review and proposal for a novel nomenclature (Dhawan *et al.*, 1996) based on guidelines from NC-IUPHAR has not been widely accepted by the research community. The 1996 proposal recommended replacement of the terms μ, δ, and κ with the terms OP_3, OP_1, and OP_2, respectively. However, in the three years or more since the publication of this recommendation, almost all papers referring to opioid receptors have continued to use the well-established Greek symbol nomenclature. Since Greek nomenclature gave many problems in manuscript preparation and particularly WEB searches, this was substituited with terminology more consistent with the overall guidelines of NC-IUPHAR that named the opioid receptors as: DOP, MOP, and KOP (Cox *et al.*, 2000).

Molecular cloning of the DOP receptor (Evans *et al.*, 1992; Kieffer *et al.*, 1992) was soon followed by the cloning of the KOP and MOP receptors (Chen *et al.*, 1993; Yasuda *et al.*, 1993). Further attempts to clone additional opioid receptor types and/or subtypes, by hybridization screening at low stringency with opioid receptor cDNA probes, or using probes generated by selective amplification of genomic DNA with degenerate primers, led several laboratories to isolate a cDNA encoding a homologous protein with a high degree of sequence similarity to the opioid receptors (Bunzow *et al.*, 1994; Chen *et al.*, 1994; Fukuda *et al.*, 1994; Lachowicz *et al.*, 1995; Mollereau *et al.*, 1994; Wang *et al.*, 1994).

The novel clone Opioid Receptor Like-1 (ORL-1) receptor displayed approximately 50 % identity with the traditional opioid receptors overall, with the transmembrane regions showing even higher homologies of up to 80 %. Despite the close homology to the other opioid receptors, opioid ligands displayed very low affinities towards ORL-1 receptor, thus it was considered an orphan receptor. ORL-1 receptor is a typical GPCR with seven predicted transmembrane domains (Figure 2).

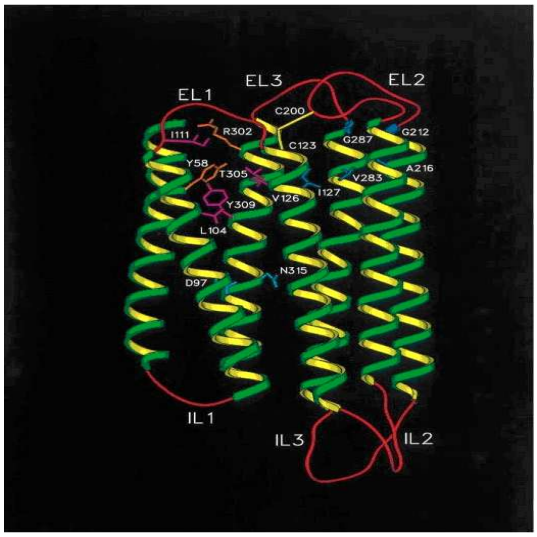


Figure 2. Schematic representation of ORL-1 receptor from Topham *et al.* (1998). TM helices are numbered 1 to 7. E/IL: Extracellular/Intracellular Loop. Visible at the C-terminal of TM 6 is the Gln 286 (human receptor numbering) side chain.

ORL-1 was subsequently named NOP (Nociceptin/Orphanin FQ Peptide) receptor according to the IUPHAR nomenclature (Cox *et al.*, 2000). The ORL-1 receptor was identified in different species and showed substantial sequence identities (>90%) between species variants, namely the human (hNOP, (Mollereau *et al.*, 1994)), rat (XOR1 (Wang *et al.*, 1994); ROR-C (Fukuda *et al.*, 1994); LC132 (Bunzow *et al.*, 1994), C3 (Lachowicz *et al.*, 1995)), mouse (MOR-C (Nishi *et al.*, 1994)) and pig (NOP receptor, (Osinski *et al.*, 1999b)).

The human NOP receptor protein consists of 370 amino acids (Mollereau *et al.*, 1994) and contains seven transmembrane (TM) domains. The N-terminal 44 amino acids contain 3 consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr). There are also sites for potencial phosphorylation by protein kinase A (in the third intracellular loop) and protein kinase C (in the second intracellular loop and the C-terminal).

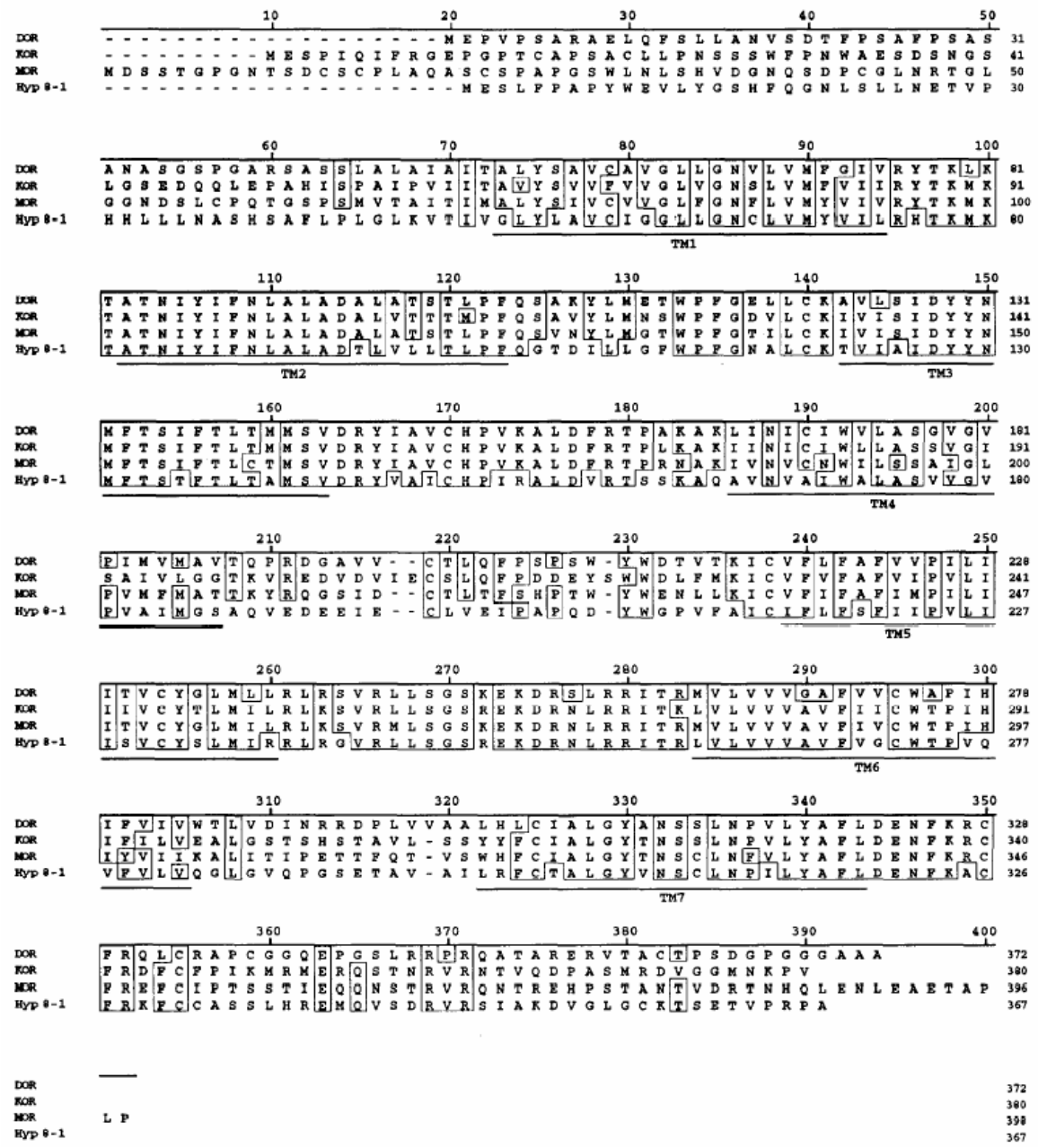


Figure 3. Alignment of the amino acid sequence of the rat NOP receptor (Hyp 8-1) with the amino acid sequences of the rat brain DOP, MOP and KOP. Sequences identical in at least 3 of 4 aligned sequences are boxed. Gaps in the alignment are indicated by a dash (-). Putative transmenbrane regions are underlined. Taken from Wick *et al.* (1994).

The NOP sequence has 57-58% aa (amino acid) identity to each of the rat MOP (Chen *et al.*, 1994), DOP (Fukuda *et al.*, 1993) and KOP (Minami *et al.*, 1993). This percent identity is slightly lower than those obtained when the sequences of opioid receptors are compared to each other (62-67%).

The conservation among the four receptors is highest (>70%) in the II, III, and VII transmembrane domains, and approximately 50% in the I, V and VI, but significantly lower (24%) in the IV. This high level of sequence conservation within the transmembrane domains lends weight to the view that the NOP receptor contains a TM binding pocket that is the structural equivalent of alkaloid binding pocket of the opioid receptors. Indeed, the NOP receptor has retained the ability, with low affinity, to bind and/or respond to opioid receptor ligands, agonist and/or antagonist such as etorphine and diprenorphine (Mollereau *et al.*, 1994), buprenorphine (Wnendt *et al.*, 1999), lofentanil (Butour *et al.*, 1997), and naloxone benzoylhydrazone (Noda *et al.*, 1998).

The NOP receptor gene, *Oprl1*, is located at the q13.2-13.3 region of the human chromosome 20 (Peluso *et al.*, 1998) and has been mapped to the distal region of the mouse chromosome 2 (Nishi *et al.*, 1994). In terms of intron-exon organization, the NOP receptor gene is nearly identical to that of the MOP, DOP, and KOP receptors, suggesting that the four genes have evolved from a common ancestor and hence belong to the same family (Meunier, 1997). Indeed, the NOP receptor appears to be evolutionary as old as the opioid receptors, since NOP receptor-like genomic sequences have been reported in teleost (Darlison *et al.*, 1997), in cartilaginous fish (Li *et al.*, 1996), in sturgeon (Danielson *et al.*, 2001) and in zebra fish (Gonzalez-Nunez *et al.*, 2003).

Although pharmacological studies have not firmly established the existence of NOP receptor subtypes (Calo *et al.*, 2000b), NOP receptor heterogeneity is still an open question. NOP receptor heterogeneity may result from differential expression of NOP splice variants. So far, five splice variants of NOP mRNA have been isolated. One, identified in rat (Wang *et al.*, 1994), encodes a NOP variant with an insertion (intron 5) in the second extracellular loop. The second splice variant, exhibiting an in frame deletion of 15 nucleotides at the 3' end of the TMD 1 coding region (Halford *et al.*, 1995; Wick *et al.*, 1994), does encode a functional receptor and has already been isolated from human tissue (Peluso *et al.*, 1998). Further, insertions of exons 3 and 4 (Curro *et al.*, 2001) after the first coding exon (exon 2) in rats result in three additional splice variants (Pan *et al.*, 1998), which again encode truncated and not functional receptors.

1.3 Identification of nociceptin/orphanin FQ

In 1995 Meunier *et al.* and Reinsheid *et al.* simultaneously described N/OFQ as the endogenous ligand for ORL-1, now known as NOP. CHO cells expressing the orphan ORL-1 were used to identify the endogenous ligand. Based on structural similarities with the known opioid receptors, both the chemical nature of the endogenous ligand (peptide) and the consequences of receptor activation (inhibition of cyclic AMP) were assumed to be similar to those of classical opioids. Consequently, cells were stimulated with forskolin to activate adenylyl cyclase and

increase intracellular cAMP. As a Gi/o-coupled orphan receptor, endogenous agonists at this receptor will inhibit the formation of cAMP. Extracts from rat (Meunier *et al.*, 1995) or pig (Reinscheid *et al.*, 1995) brain were screened. Fractions that were able to inhibit the adenylyl cyclase activity were further fractionated through reverse-phase high-performance liquid chromatography. The purification and mass spectrometry analyses identified a heptadecapeptide (Figure 4), the sequence of which was determined. The synthetic peptide was shown to have high affinity (in the nanomolar range) and to strongly inhibit forskolin-induced accumulation of cAMP in CHO cells expressing the NOP receptor (EC_{50} about 1 nM), while showing no activity in non transfected cells (Meunier *et al.*, 1995). Moreover, when tested *in vivo* by intracerebroventricular (i.c.v.) injection in mice, the peptide induced hyperalgesia in the hot plate (Meunier *et al.*, 1995) and tail flick (Reinscheid *et al.*, 1995) tests. Decrease in the locomotor activity but no analgesia was also observed in the hot plate test (Reinscheid *et al.*, 1995).

The group of Meunier termed the novel peptide nociceptin, based on apparent pro-nociceptive properties, while that of Reinscheid named it orphanin FQ, as ligand of an orphan receptor, whose first and last amino acids are Phe (F) and Gln (Q), respectively.

N/OFQ shares sequence homologies with the opioid peptide ligand dynorphin A (Figure 4); despite the structural similarities these peptides are functionally quite distinct. N/OFQ has no significant affinity for any of the opioid receptors (Reinscheid *et al.*, 1998).

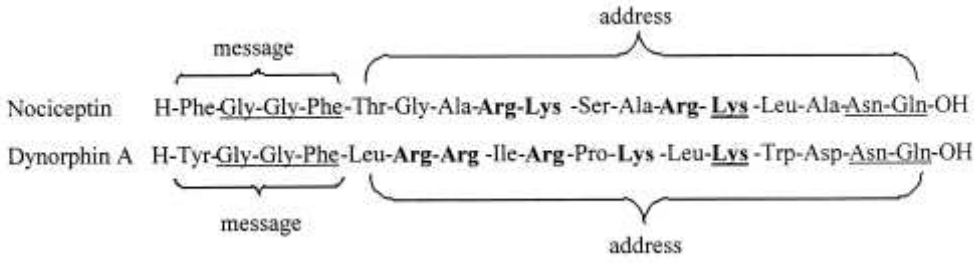


Figure 4. Structural similarities between dynorphin A and N/OFQ amino acid sequences (Guerrini *et al.*, 2000b).

The N-terminal tetrapeptide sequences (message domains) of the two peptides are very similar, with the only difference of the first amino acid residue (Phe in N/OFQ and Tyr in dynorphin A); the C-terminal parts (address domains) of the two molecules are both enriched in positively charged residues, such as arginine and lysine, even if distributed in different positions.

N/OFQ is a heptadecapeptide cleaved from the polypeptide precursor preproN/OFQ (ppN/OFQ). ppN/OFQ 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) (Figure 5). The ppN/OFQ gene, that has been isolated from human, mouse and rat, is highly conserved in the three species.

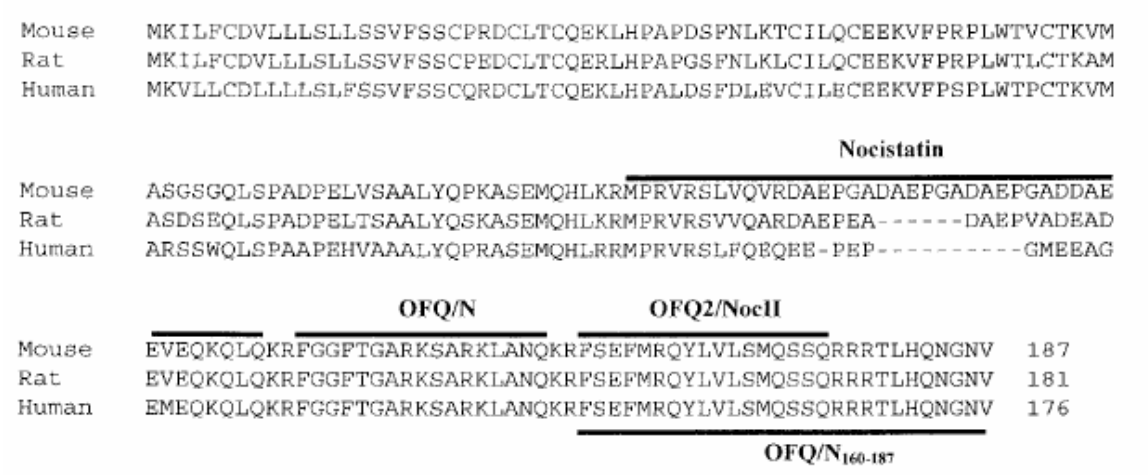


Figure 5. Amino acid sequence for N/OFQ precursor, ppN/OFQ (Calo *et al.*, 2000b).

Analysis of the nucleotide sequence of the preproN/OFQ gene revealed structural and organisational characteristics very similar to those of the opioid peptide precursors, in particular preproenkephalin and preprodynorphin, suggesting that these peptides derive from a common ancestor (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996).

The ppN/OFQ gene is located on human chromosome 8 (8p21) (Mollereau *et al.*, 1996). In the ppN/OFQ sequence there are several pairs of basic amino acids that present possible sites of cleavage for precursor maturation or for transcriptional regulation (Zaveri *et al.*, 2000). Therefore, several biologically relevant peptides may derive from the N/OFQ precursor (Figure 5). Apparently two additional peptides are excised from the same precursor: orphanin FQ2 and nocistatin (Okuda-Ashitaka *et al.*, 1998). None of them bind to NOP receptor (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996), and until now specific receptors for them have not been identified. The peptide following the N/OFQ sequence, is a heptadecapeptide terminating with the couple FQ (orphanin FQ2): it 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 (NST), has been reported to act as a functional antagonist of N/OFQ (Okuda-Ashitaka *et al.*, 1998). In most studies, NST was found to be inactive *per se*, but was able to reverse several effects of N/OFQ, 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.*, 1998), impairment of learning and memory in mice (Hiramatsu *et al.*, 1999a), stimulation of food intake in rats (Olszewski *et al.*, 2000). Moreover, NST can, *per se*, cause antinociception after i.c.v. administration in the rat carrageenan test (Nakagawa *et al.*, 1999) or after intratechal (i.t.) administration in the rat formalin test (Yamamoto *et al.*, 2001). Interestingly, nocistatin or its C-terminal hexapeptide exerts anxiogenic-like effects in mice; in fact it has been reported that the C-terminal hexapeptide (the most conserved region among species), administered i.c.v., exerts clear anxiogenic-like effects in mice, in contrast to N/OFQ, that in the same experimental model, acts as an anxiolytic (Gavioli *et al.*, 2002). Very recent findings demonstrated that the opposite effects of N/OFQ and NST on supraspinal pain modulation result from their opposing effects on the excitability of central amygdala nucleus-periaqueductal gray projection (CeA-PAG) neurons. Electrophysiological studies showed that N/OFQ hyperpolarized CeA-PAG projection neurons by enhancing an inwardly rectifying potassium conductance. In contrast, NST depolarized CeA-PAG neurons by causing the opening of TRPC cation channels via a G(αq/11)-PLC-PKC pathway (Chen *et al.*, 2009).

In vitro studies demonstrated that bovine nocistatin (bNST) inhibited the K^+-induced [^{3}H]5-HT release from mouse cortical synaptosomes, displaying similar efficacy but lower potency than N/OFQ; this inhibitory effect was not prevented either by the NOP receptor antagonist UFP-101, or by the non-selective opioid receptor antagonist, naloxone. In contrast to N/OFQ, bNST reduced [^{3}H]5-HT release from synaptosomes obtained from NOP receptor knockout mice (Fantin *et al.*, 2007).

1.4 Metabolism of N/OFQ

Degradation of the N/OFQ peptide is principally via aminopeptidase N (APN), which releases the inactive N/OFQ(2-17) and endopeptidase (EP), which cleaves a variety of bonds to release inactive fragments, Figure 6 (Calo *et al.*, 2000b). Endopeptidase 24.15 (EP 24.15) (Montiel *et al.*, 1997) acts on the peptide bonds Ala^7-Arg^8, Ala^{11}-Arg^{12}, Arg^{12}-Lys^{13} and releases inactive compounds; endopeptidase 24.11 (EP 24.11) acts on the cleavage site Lys^{13}-Leu^{14} and plays a major role in the initial stage of N/OFQ metabolism in mouse spinal cord (Sakurada *et al.*, 2002). C-terminal degradation also leads to a reduction in binding affinity of N/OFQ for NOP, loss of the 4 amino acids from the C-terminal tail as in N/OFQ(1-13) results in a 30-fold reduction in potency (Butour *et al.*, 1997). However, amidation of C-terminus of N/OFQ(1-13) restores ligand affinity and potency, consequently N/OFQ(1-13)-NH_2 is the shortest sequence retaining the full biological activity of the endogenous ligand (Guerrini *et al.*, 1997).

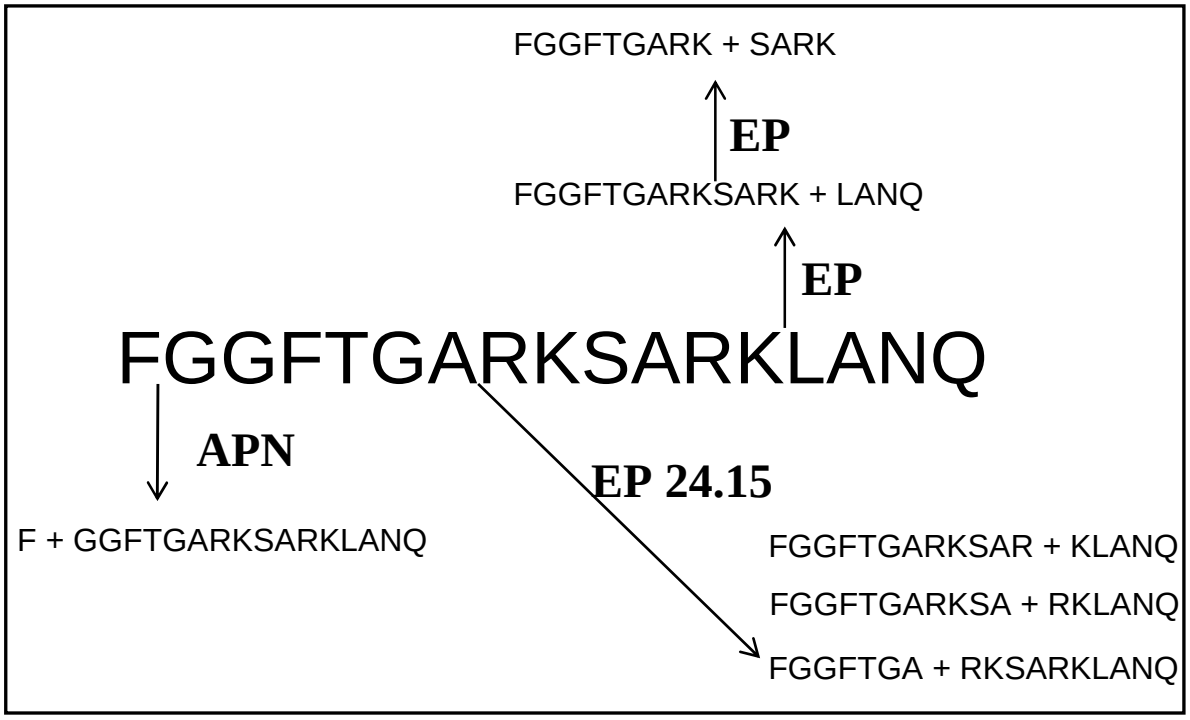


Figure 6. N/OFQ metabolism by aminopeptidase N (APN) and endopeptidases (EP), from Calo *et al.* (2000b).

1.5 NOP receptor and N/OFQ localization

The regional distribution of N/OFQ and the NOP receptor have been well described (Bunzow *et al.*, 1994; Fukuda *et al.*, 1994; Houtani *et al.*, 2000; Lachowicz *et al.*, 1995; Letchworth *et al.*, 2000; Mollereau *et al.*, 1994; Neal *et al.*, 1999a; Neal *et al.*, 1999b; Nothacker *et al.*, 1996; O'Donnell *et al.*, 2001; Riedl *et al.*, 1996; Wick *et al.*, 1995). These series of publications provide detailed descriptions of the distribution of the NOP receptor, mRNA and binding in the brain which are beyond the scope of this work. They report a good correlation between receptor binding distributions and those seen with in situ hybridization. Regions with NOP receptor binding typically express NOP mRNA as well, although the levels of mRNA and NOP binding do not always closely match (Neal *et al.*, 1999a).

The NOP receptor is widely expressed in the CNS, in particular in the forebrain (cortical areas, olfactory regions, limbic structures: hippocampus and amygdala, thalamus), throughout the brainstem (central periaqueductal gray, substantia nigra, several sensory and motor nuclei), and in both dorsal and ventral horns of the spinal cord (Mollereau *et al.*, 2000; Neal *et al.*, 1999a). The distribution patterns have suggested the involvement of the NOP receptor system in motor and

balance control, reinforcement and reward, nociception, stress response, sexual behaviour, aggression and autonomic control of physiological processes (Neal *et al.*, 1999a).

It is worthy of mention that NOP receptors co-express with MOP receptors in the dorsal horn of the spinal cord, the hippocampal formation and the caudate putamen (Judd *et al.*, 1996; Letchworth *et al.*, 2000) in the midbrain periaqueductal gray and the nucleus raphe magnus (Houtani *et al.*, 2000). Distribution of NOP does not always overlap that of opioid receptors: these anatomical differences may provide a possible explanation for the different *in vivo* actions of N/OFQ and opioids (Ikeda *et al.*, 1998; Monteillet-Agius *et al.*, 1998; Sim *et al.*, 1997).

Due to the diffuse distribution of N/OFQ peptide and NOP receptor, this novel system is associated with a large number of physiological responses and probably contributes to homeostasis by modulating neuronal circuitry (Mollereau *et al.*, 2000). This might explain why the NOP(-/-) mice do not display an obvious phenotype (other than the unrestrained nociceptive response and dysregulation of hearing ability) (Nishi *et al.*, 1997) and also why pharmacological effects of N/OFQ are sometimes contradictory (e.g. in pain and locomotor tests) depending on the locus of injection and dose (Mollereau *et al.*, 2000).

The NOP receptor mRNA has also been identified in the peripheral nervous system and several other organs. It is expressed in peripheral ganglia and in the immune system. It has been detected in rat intestine, vas deferens, skeletal muscles and spleen (Wang *et al.*, 1994) in porcine gastrointestinal tract and kidney (Osinski *et al.*, 1999b), in several guinea pig ganglia (Fischer *et al.*, 1998), also in rat retina and heart (Mollereau *et al.*, 2000).

Peluso and colleagues (Peluso *et al.*, 1998) were the first to describe the distribution of NOP receptor transcripts in man, in different brain regions by RT-PCR technique: the highest amplification was observed in cortical areas (the frontal and temporal cortex), in the hypothalamus, mamillary bodies, the substantia nigra, and thalamic nuclei. Transcripts have also been detected in limbic structures (the hippocampus and amygdala), brainstem (the ventral tegmental area, the locus coeruleus) and the pituitary gland. This distribution, which is similar to that of rodents, suggests the participation of the NOP receptor in numerous human physiological functions, such as emotive and cognitive processes, neuroendocrine and sensory regulation.

Berthele and colleagues (Berthele *et al.*, 2003) studied the differential expression of NOP receptors in the human brain (cortex, basal ganglia, hippocampal area and cerebellum) by utilizing on-section ligand binding corroborated with mRNA detection on parallel sections of the same brain tissues. In general, [^{3}H]-N/OFQ ligand binding and NOP receptor mRNA expression were widespread and indicative of a considerable high NOP receptor expression in these anatomical regions. [^{3}H]-N/OFQ ligand binding and NOP mRNA expression studies showed that the highest

amounts of NOP receptor were observed in the cerebellum, in the cortex (cingulate and prefrontal cortex), in the striatum (caudate nucleus and the putamen) and in the lamina II, followed by laminae III, V and VI, in the principal neurons of the dentate gyrus and in the hippocampal area (Berthele *et al.*, 2003).

The localization of N/OFQ-immunoreactive fibers and terminals and/or the localization of the ppN/OFQ mRNA correspond reasonably well with the NOP receptor. Limbic areas highly express N/OFQ, in particular the bed nucleus of the stria terminals, and the amygdala nuclei (Boom *et al.*, 1999; Neal *et al.*, 1999b). A matching pattern of N/OFQ and NOP receptor expression in the human and rodent central nervous system has been observed (Berthele *et al.*, 2003; Peluso *et al.*, 1998; Witta *et al.*, 2004). As with the receptor, N/OFQ immunoreactivity and mRNA levels detected using in situ hybridization are closely correlated. N/OFQ is found in lateral septum, hypothalamus, ventral forebrain, claustrum, mammillary bodies, amygdala, hippocampus, thalamus, medial habenula, ventral tegmentum, substantia nigra, central gray, interpeduncular nucleus, locus coeruleus, raphe complex, solitary nucleus, nucleus ambiguous, caudal spinal trigeminal nucleus, and reticular formation, as well the ventral and dorsal horns of the spinal cord (Neal *et al.*, 1999b). Recently N/OFQ was immunolocalized in rat lateral and medial olivocochlear efferents (Kho *et al.*, 2006). Although N/OFQ and opioid peptides show a similar distribution, they are not colocalized in nociceptive centres such as the dorsal horn, the sensory trigeminal complex or the periaqueductal gray (Schulz *et al.*, 1996).

In the periphery, mRNA of N/OFQ was detected in rat ovary, in human spleen, lymphocytes, and fetal, but not adult kidney (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996). Furthermore, it has been shown that, under physiological conditions, N/OFQ is present in the human plasma (~ 10 pg/ml) (Brooks *et al.*, 1998). In several pathological conditions such as postpartum depression (Gu *et al.*, 2003), Wilson's disease (Hantos *et al.*, 2002), hepatocellular carcinoma (Horvath *et al.*, 2004) and in acute and chronic pain states (Ko *et al.*, 2002b), plasma levels of N/OFQ resulted increased. In contrast, lower N/OFQ plasma levels have been observed in patients suffering from fibromyalgia syndrome (Anderberg *et al.*, 1998), cluster headache (Ertsey *et al.*, 2004) and migraine without aura (Ertsey *et al.*, 2005). After all, a very recent findings indicate that N/OFQ plasma levels are increased in sepsis condition (Williams *et al.*, 2008).

1.6 Cellular effects of N/OFQ

The cellular actions of the classical opioid receptors (MOP/KOP/DOP) and the NOP receptor have been shown to be pertussis toxin sensitive and therefore couple to inhibitory G-

proteins i.e. G-proteins with $G_{i/o}$ alpha subunits (Reinscheid *et al.*, 1996). G-proteins are membrane bound/associated heterotrimeric proteins composed of α, β, γ subunits. There are four major classes of G proteins including G_i/G_o, G_s, G_q.

Activation of NOP, similar to MOP, KOP, and DOP opioid receptors activation, leads to: (i) closing of voltage sensitive calcium channels, (ii) stimulation of potassium efflux leading to hyperpolarisation and (iii) reduced cyclic adenosine monophosphate (cAMP) production via inhibition of adenylyl cyclase. Overall this results in reduced neuronal cell excitability leading to a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release (Figure 7) (Hawes *et al.*, 2000).

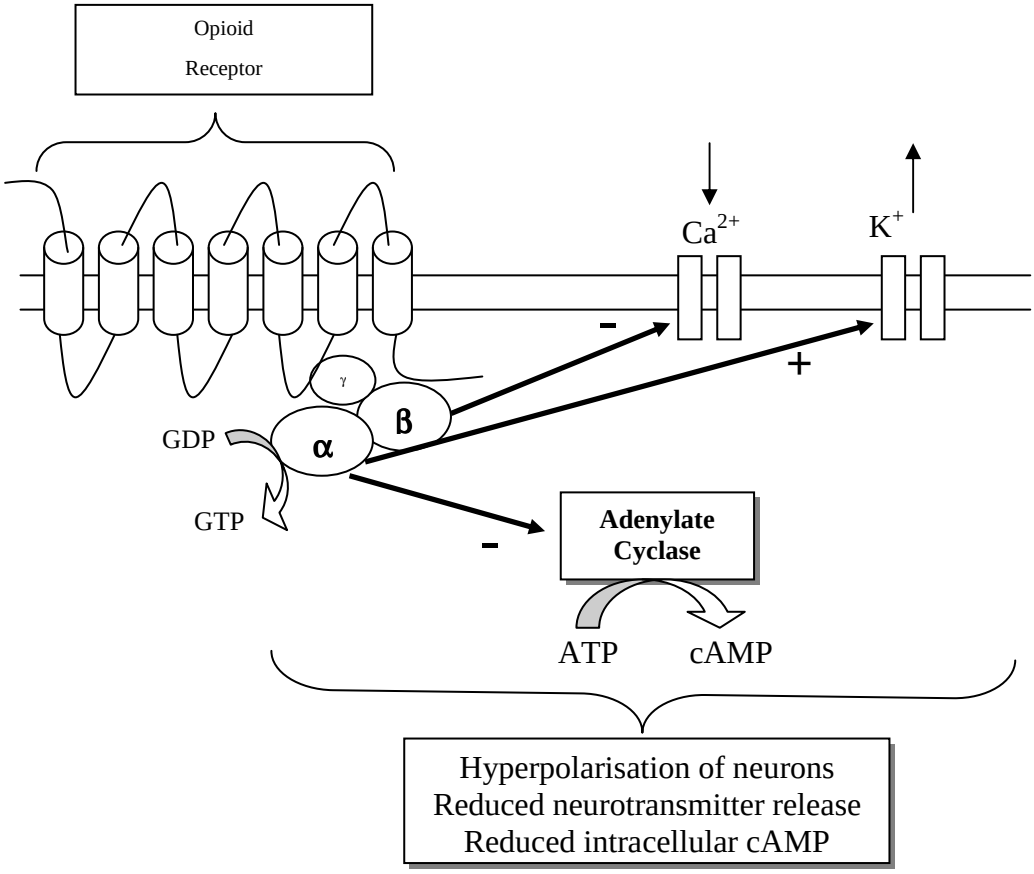


Figure 7. Schematic representation of intracellular responses to NOP receptor activation.

N/OFQ inhibits forskolin-stimulated cellular cAMP production. Forskolin is used to directly activate adenylyl cyclase and consequently increase cAMP production. Elevating cAMP via other receptor driven systems (e.g. via the D1 dopamine receptor) is also inhibited by N/OFQ (Chan *et*

al., 1998). Both the endogenous and recombinant NOP receptors are capable of inhibiting the formation of cAMP with remarkably consistent EC_{50} values in a range of cell systems.

The peptide also inhibits several types of voltage-gated Ca^{2+} channels: for example in human SH-SY5Y neuroblastoma cells it produces a partial inhibition of N-type Ca^{2+} conductance with an IC_{50} value of about 40 nM (Connor *et al.*, 1996a), and in dissociated rat hippocampal neurones the peptide partially inhibits the three major types of Ca^{2+} channels, L, N and P/Q (Knoflach *et al.*, 1996). The inhibition is no longer seen after β pertussis toxin treatment and cannot be prevented by high doses of naloxone. N/OFQ has been shown to mediate a pronounced inhibition of N-type calcium channels, whereas other calcium channel subtypes were not affected.

N/OFQ has also been reported to increase the inwardly rectifying K^+ conductance in rat brain slices containing the dorsal raphe nucleus (Vaughan *et al.*, 1996), the locus coeruleus (Connor *et al.*, 1996b), and the periaqueductal grey (Vaughan *et al.*, 1997), in hippocampal slices (Madamba *et al.*, 1999) and cultured hippocampal neurones (Amano *et al.*, 2000).

Collectively, these data are consistent with the hypothesis that N/OFQ acts primarily to reduce synaptic transmission and neuronal excitability in the nervous system (Meunier, 1997). In the CNS, studies using synaptosomes and brain slices revealed that N/OFQ inhibits the release of noradrenaline (NA), serotonine (5-HT), dopamine (DA), acetylcholine (Ach), γ-aminobutyric acid (GABA), and glutamate (Schlicker *et al.*, 2000).

In the peripheral nervous system, studies showed the general modulatory effects (mostly inhibitory) of N/OFQ on neurotransmitter release from sympathetic, parasympathetic and nonadrenergic-noncholinergic sensory endings. In the respiratory, cardiovascular, genitourinary and gastrointestinal systems N/OFQ exerts inhibitory effects (Giuliani *et al.*, 2000). Several isolated tissues from different species have been shown to be sensitive to N/OFQ. In particular, the electrically stimulated mouse and rat vas deferes and the guinea pig ileum have been described and used extensively in opioid receptor pharmacology: the guinea pig ileum, whose myenteric neuronal network contains mainly MOP receptors (Paton, 1957) has been shown to respond to N/OFQ (Calo *et al.*, 1997; Calo *et al.*, 1996; Zhang *et al.*, 1997); the mouse vas deferens whose nerve terminals contain mainly DOP receptors (Hughes *et al.*, 1975) and the rat vas deferens, whose nerves contain an uncharacterized opioid receptor (Lemaire *et al.*, 1978), have also been reported to be N/OFQ sensitive preparations (Berzetei-Gurske *et al.*, 1996; Calo *et al.*, 1997; Calo *et al.*, 1996; Nicholson *et al.*, 1998; Zhang *et al.*, 1997). The twitch response in the three preparations is due to nerve activation and subsequent release of neurotransmitter since they are blocked by tetradotoxin (TTX). The release of NA from the sympathetic nerves is the major cause of the contractions of mouse and rat vas deferens, since they are blocked by the α-1 adrenoceptor antagonist prazosin. NOP receptors

appear to be localized in sympathetic terminals since N/OFQ inhibits twitch evoked by electrical field stimulation, but does not modify contractions to exogenous NA (Calo *et al.*, 1996). Similar results were obtained in the guinea pig ileum since atropine and TTX sensitive contractions derive from the release of Ach from cholinergic terminals of the myenteric plexus without affecting responses to exogenous Ach, thus demonstrating the prejunctional localization of the NOP receptor. In the three tissues the inhibitory effect of N/OFQ is not influenced by naloxone suggesting that classical opioid receptors are not targeted by the peptide.

A similar picture has been found regarding the inhibitory effects of N/OFQ on sensory fibers on the guinea pig bronchus (Fischer *et al.*, 1998; Rizzi *et al.*, 1999b), cholinergic contractions of human bronchus (Basso *et al.*, 2005), renal pelvis and heart (Giuliani *et al.*, 1996; Giuliani *et al.*, 1997a). The rat anococcygeus has also been described as a preparation, in which N/OFQ produces a concentration-dependent inhibition of the adrenergic motor response to electrical field stimulation, but does not affect the response to exogenous NA. In addition, selective opioid ligands do not exert any effect on this preparation, suggesting that in this preparation the NOP receptor occurs without the co-presence of the classical opioid receptors (Ho *et al.*, 2000). In all the preparations analysed above, the N/OFQ-NOP receptor system displays a prejunctional inhibitory function, as do classical opioid receptors.

1.7 Biological effects of N/OFQ

Due to the widespread distribution of N/OFQ and NOP receptor, this peptidergic system is involved in a wide range of physiological responses with effects noted in the nervous system (central and peripheral), the cardiovascular system, the airways, the gastrointestinal tract and immune system. The role of this peptidergic system has been explored intensely with the pharmacological and biological tools available, such as i) antisense oligonucleotides targeting NOP receptor or ppN/OFQ gene, ii) antibodies directed against N/OFQ, iii) transgenic mice in which the receptor or the peptide precursor genes have been genetically eliminated, iv) available stable and highly potent antagonists. Some of the more well-studied and noteworthy biological actions modulated by this system will be described below.

Pain regulation

Since the identification of N/OFQ there has been intense interest in the role of this peptide in pain processing. This is based on various factors, including the similarity of distribution of receptor and peptide to classical opioids within the defined pain pathway and the structural similarity to classical opioids. Application of N/OFQ has been shown to cause hyperalgesia, allodynia and

analgesia. These conflicting findings are confounded by species and or strain differences in test animals, known to be fundamental in the supraspinal effects of nociception (Mogil *et al.*, 1999). However, the route of administration and nociceptive paradigm under investigation are of paramount importance.

Supraspinal level

N/OFQ was shown to increase pain sensitivity in mice and rats in the two initial studies of the functions of this peptide (hence the name nociceptin; (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995)). However, the hyperalgesic effect of N/OFQ was only seen after intracerebroventricular (i.c.v.), but not after intrathecal (i.t.) administration. It has been demonstrated that N/OFQ's most prominent role in supraspinal pain modulation is a "functional opioid antagonism" directed against many different opioid receptor agonists (Mogil *et al.*, 2001). Since behavioural testing in pain models, in particular i.c.v. and i.t. injections, expose animals to acute stress, the apparent pronociceptive action seen in the initial studies may thus be interpreted as the reversal of stress-induced antinociception rather than as a genuine pronociceptive or hyperalgesic effect (Zeilhofer *et al.*, 2003). I.c.v. injection of N/OFQ was stressful, resulting in a release of central endogenous opioid peptides with their effects subsequently reversed by the delivered dose of N/OFQ (Lambert, 2008). The suggestion for an anti-opioid role of N/OFQ has since been corroborated by results obtained in a variety of assays: indeed, it has been shown that N/OFQ counteracts the analgesic effect of the endogenous opioids (Tian *et al.*, 1997a; Tian *et al.*, 1997b) or that of exougenously applied morphine (Bertorelli *et al.*, 1999; Calo *et al.*, 1998b; Grisel *et al.*, 1996; Zhu *et al.*, 1997) or that of selective opioid receptor agonists (King *et al.*, 1998). Worthy of mention is the fact that tolerance develops to the antiopioid effects of N/OFQ (Lutfy *et al.*, 1999).

Since the NOP 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 N/OFQ and opioid peptides and their respective receptors on the neuronal networks involved in pain transmission at the supraspinal level. Many studies demonstrate that the net effects of N/OFQ on nociception at supraspinal sites strongly depend on the activation state (resting versus sensitized) of pain controlling neuronal circuits (Zeilhofer *et al.*, 2003).

It is possible that N/OFQ plays a role in the physiological modulation of pain signals under normal or pathologic conditions. This question can be best answered through the use of N/OFQ receptor antagonists, or transgenic mice lacking the NOP receptor gene and with antisense oligonucletides. Indeed, the involvement of the NOP receptor in N/OFQ effects on nociception is supported by the following evidence: i) the pronociceptive action of N/OFQ is no longer present in

NOP(-/-) mice (Nishi *et al.*, 1997; Noda *et al.*, 1998); ii) antisense oligonucleotides targeting the NOP receptor prevent the effect of N/OFQ (Tian *et al.*, 1997b; Zhu *et al.*, 1997); the pronociceptive effect of N/OFQ is reversed by NOP selective antagonists: [Nphe^1]N/OFQ(1-13)-NH_2 (Calo *et al.*, 2000a; Di Giannuario *et al.*, 2001; Rizzi *et al.*, 2001b), UFP-101 (Calo *et al.*, 2002b), J-113397 (Ozaki *et al.*, 2000; Yamamoto *et al.*, 2001) and SB-612111 (Rizzi *et al.*, 2007a; Zaratin *et al.*, 2004).

Spinal level

Many lines of evidence indicate that the spinal cord is an equally important CNS area for nociceptive processing and its modulation by N/OFQ and classical opioids. Neurons and fiber networks containing N/OFQ mRNA and N/OFQ like immunoreactivity have been located in the dorsal spinal cord (Lee *et al.*, 1997; Mamiya *et al.*, 1998; Meis *et al.*, 1998; Meunier *et al.*, 1995), and endogenously released N/OFQ can be detected following electrical field stimulation of the spinal cord (Lai *et al.*, 2000).

The role of N/OFQ in modulating pain threshold in the spinal cord is controversial. Although some studies reported that i.t. injection of N/OFQ produces hyperalgesia/allodynia (Hara *et al.*, 1997; Inoue *et al.*, 1999) others found no effect (Grisel *et al.*, 1996; Reinscheid *et al.*, 1995). Most of the studies, however demonstrated that i.t. N/OFQ induces an antinociceptive effect similar to that evoked by classical opioid receptor agonists (Candeletti *et al.*, 2000b; Erb *et al.*, 1997; Hao *et al.*, 1998; Kamei *et al.*, 1999; King *et al.*, 1997; Nazzaro *et al.*, 2007; Wang *et al.*, 1999; Xu *et al.*, 1996). While tolerance develops to the antinociceptive effect of i.t. N/OFQ upon repeated administration, there is no cross tolerance with morphine, suggesting that different receptors are involved in the actions of the two agents (Hao *et al.*, 1997). Differences in animal species or even in strains, as well as in N/OFQ doses used, may account for the conflicting results reported with N/OFQ in the spinal cord. Worthy of mention is the work of Inoue *et al.* (1999) showing the dose response curve to N/OFQ is bell-shaped: very low doses of peptide (fmol range) cause hyperalgesia, while at higher doses (nmol range) N/OFQ is antinociceptive and blocks the scratching, biting and licking induced by i.t. substance P.

Extremely interesting is the latest study of Ko and colleagues (Ko *et al.*, 2006) as this is the first to document the inhibitory action of spinal N/OFQ in a primate species. The behavioural study showed that i.t. administration of N/OFQ dose-dependently produced antinociception in monkeys that was blocked by a NOP receptor antagonist, J-113397, but not by naltrexone. These results provide the first functional evidence of spinal N/OFQ-induced antinociception in primates and indicate that activation of spinal NOP receptors may be a potential target for spinal analgesics.

Collectively, the cellular mechanisms of the pronociceptive effects of N/OFQ in the spinal cord are still rather obscure, whereas inhibition of excitatory synaptic transmission presents as a clearly defined cellular mechanism underlying the spinal analgesia. The combination of opioid-like analgesia by N/OFQ at the level of the spinal cord with functional opioid antagonism at supraspinal sites, where most of the unwanted effects of classical opioids arise, has promoted the idea that NOP receptor agonists might be better tolerated spinally acting analgesics. It should however be noted that the only well studied non-peptide NOP receptor agonist Ro 64-6198 was anxiolytic, but not antinociceptive in acute pain models (Jenck *et al.*, 2000); Ro 64-6198 was recently reported to have an antiallodynic effect mediated by NOP receptors in a neuropathic pain model (Obara *et al.*, 2005). Nevertheless, further studies with other NOP receptor agonists and in chronic pain models are desirable.

Nociceptive responses to acute noxious heat in NOP(-/-), ppN/OFQ(-/-) and double knockout mice were indistinguishable from those of NOP(+/+). However, NOP(-/-), ppN/OFQ(-/-) and double knockout mice showed markedly stronger nociceptive response during prolonged nociceptive stimulation (Depner *et al.*, 2003). These results indicate that the N/OFQ system contributes significantly to endogenous pain control during prolonged nociceptive stimulation but does not affect acute pain sensitivity (Depner *et al.*, 2003).

There have been attempts to correlate human plasma N/OFQ levels with pain but the results of these studies have been contradictory. An increase in N/OFQ levels in acute and chronic pain compared with controls was reported (Ko *et al.*, 2002b). However, a decrease in N/OFQ levels was noted in fibromyalgia syndrome (Anderberg *et al.*, 1998) and cluster headache (Ertsey *et al.*, 2004).

Modulation of locomotor activity

One of the seminal investigations of N/OFQ reported a dose-dependent decrease in locomotor activity (i.e., hypolocomotion) when the peptide was given supraspinally (Reinscheid *et al.*, 1995). This effect was significant only after i.c.v. application of 10 nmol N/OFQ. This finding was later confirmed by other authors in mice (Nishi *et al.*, 1997; Noble *et al.*, 1997; Noda *et al.*, 1998).

Repeated daily N/OFQ injections result in rapid development of tolerance to this depressor effect on locomotion behaviour (Devine *et al.*, 1996). The action of N/OFQ is insensitive to naloxone (Noble *et al.*, 1997), while it is reversed by NalBzOH (Noda *et al.*, 1998). The locomotor-inhibiting effects of N/OFQ seen in NOP(+/+) animals were not seen in the NOP(-/-) mice, confirming the involvement of the NOP receptor in the effect (Nishi *et al.*, 1997; Noda *et al.*, 1998).

However the spontaneous locomotor activity of NOP(-/-) mice is not different from that displayed by NOP(+/+) littermates which suggest that the N/OFQ-NOP receptor system does not play a tonic role in the physiological regulation of locomotion. When microinjected directly into the hippocampus or ventromedial hypothalamus, but not the nucleus accumbens, high doses of N/OFQ (10–25 nmol) significantly decrease locomotor activity (Sandin *et al.*, 1997).

N/OFQ 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 N/OFQ has been related to the anxiolitic-like actions of the peptide (Jenck *et al.*, 1997). Thus, N/OFQ shows a biphasic dose response curve for locomotor activity: stimulation at low doses (0.01-0.1 nmol), inhibition at high doses (1-10 nmol).

It has been demonstrated that firing activity of dopaminergic cells of the substantia nigra, which express NOP receptors, is inhibited by microinjection of N/OFQ (Marti *et al.*, 2004b). When microinjected into the substantia nigra, N/OFQ reduces dopamine release in the striatum and locomotor activity (Marti *et al.*, 2004b). Conversely, the NOP receptor antagonists, UFP-101 and J-113397, injected into the substantia nigra, enhanced striatal dopamine release and facilitated motor performance (Marti *et al.*, 2004b). These data confirm that improvement in locomotor activity is due to the enhanced striatal dopamine release caused by blockade of endogenous N/OFQ signalling. The inhibitory role played by endogenous N/OFQ on motor activity was additionally strengthened by the finding that mice lacking the NOP receptor gene outperformed wild-type mice on the exercise stimulated locomotion (rotarod test) (Marti *et al.*, 2004b). Microinjection of UFP-101 into the substantia nigra also reversed akinesia in haloperidol-treated (Marti *et al.*, 2004a) or 6-hydroxydopamine-hemilesioned rats (Marti *et al.*, 2005). Enhancement of N/OFQ expression and release was observed in the latter parkinsonism model (Marti *et al.*, 2005). Haloperidol-induced motor impairment and the dopaminergic neuronal toxicity induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, but not methamphetamine, were partially abolished in ppN/OFQ(-/-) mice (Brown *et al.*, 2006; Marti *et al.*, 2005). Increased locomotor activity was observed in NOP(-/-) mice (Marti *et al.*, 2004b) and in rats treated with antisense-NOP (Blakley *et al.*, 2004) or antisense-ppN/OFQ (Candeletti *et al.*, 2000a). These studies suggest that endogenous N/OFQ might have a negative regulation in striatal dopamine levels and motor activity.

In the last few years, using a battery of behavioural tests, the group of prof. Morari was first to show that NOP receptor antagonists like J-113397 attenuated parkinsonian-like symptoms in 6-hydroxydopamine hemilesioned rats by reducing glutamate release in the SN whereas deletion of the NOP receptor gene conferred mice partial protection from haloperidol-induced motor depression (Marti *et al.*, 2005), They subsequently showed that coadministration of the NOP

receptor antagonist J-113397 and L-DOPA to 6-hydroxydopamine hemilesioned rats produced an additive attenuation of parkinsonism: J-113397 and L-DOPA decreased thalamic GABA release and attenuated akinesia, their combination resulting in a more profound effect (Marti *et al.*, 2007). Very recently, it has been demonstrated that Trap-101, a non-peptide NOP antagonist, changes motor activity in naive rats and mice and alleviates parkinsonism in 6-hydroxydopamine hemilesioned rats: Trap-101 stimulates motor activity at 10 mg/kg and inhibits it at 30 mg/kg (Marti *et al.*, 2008); such dual action was observed in NOP(+/+) but not in NOP(-/-) mice suggesting a specific involvement of NOP receptors (Marti *et al.*, 2008). Overall, the present studies provides novel insights into the mechanisms underlying the antiparkinsonian action of NOP receptor antagonists that may be used alone or as an adjunct to L-DOPA in the therapy of Parkinson's disease. This indication has also been confirmed in non-human primates (Viaro *et al.*, 2008; Visanji *et al.*, 2008).

Anxiolytic-like action

Different laboratories have reported anxiolytic-like effects in response to intracerebroventricular administration of N/OFQ in rodents in several models of anxiety.

The peptide and its receptor are found in a number of central nervous system loci involved in emotion and stress regulation, including the amygdala, septal region, locus coeruleus, and hypothalamus (Gavioli *et al.*, 2006).

A number of standard behavioural assays reveal the ability of supraspinal N/OFQ to block fear and anxiety in both rats and mice (Jenck *et al.*, 1997). Interestingly, these effects of N/OFQ were evident at relatively low doses (<1 nmol), which do not modify animal gross behaviour and are inactive with regard to other functions (i.e., nociception, food intake, etc.). Later, other laboratories confirmed the anxiolytic-like effects of the natural peptide N/OFQ in mice in the elevated plus-maze test (Gavioli *et al.*, 2002), in the holeboard test (Kamei *et al.*, 2004), and in the defence test battery (Griebel *et al.*, 1999; Vitale *et al.*, 2006). Furthermore, at relatively low doses, several non-peptide agonists from Roche (Ro 65-6570 and Ro 64-6198) are generally reported as anxiolytic (Varty *et al.*, 2005; Wichmann *et al.*, 1999). After peripheral administration in the range of doses 0.1–3 mg/kg, Ro 64-6198 promoted anxiolytic-like effects in rats in the elevated plus-maze, fear-potentiated startle, and operant conflict tests (Jenck *et al.*, 2000). This hypothesis has been corroborated by the findings that genetically N/OFQ precursor deficient mice display an increased susceptibility to acute and repeated stress, as compared to their wild-type littermates (Koster *et al.*, 1999). In a recent study from Schering-Plough the non-peptide agonist SCH 221510 was shown to be anxiolytic but with a reduced side-effect profile when compared with

benzodiazepines (Varty *et al.*, 2008). Pfizer has recently reported two new non-peptide NOP receptor agonists: PCPB (Hirao *et al.*, 2008a) and MCOPPB (Hayashi *et al.*, 2009; Hirao *et al.*, 2008b); both orally active compounds showed anxiolytic-like effect in mice.

Importantly, very little information is present in the literature regarding the effects of selective NOP receptor antagonists on anxiety. It has been reported that the non-peptide molecule J-113397 at 10 mg/kg i.p. antagonized the anxiolytic-like effect of Ro 64-6198 in the conditioned lick suppression test in rats without having any effect in this assay *per se* (Varty *et al.*, 2005). Although very few studies have been performed to date on this topic, the available evidence obtained with two chemically unrelated NOP antagonists, i.e., J-113397 and UFP-101 (Gavioli *et al.*, 2006; Vitale *et al.*, 2006), suggest that the acute blockade of NOP receptor does not modify the level of anxiety in rodents. In other words, these results suggest that N/OFQergic signalling does not tonically control anxiety-related behaviour.

Surprisingly, Fernandez *et al.* (2004) observed anxiogenic-like effects of N/OFQ given by i.c.v. injection in several anxiety-related procedures (i.e., open field, elevated plus maze, and dark-light preference) in rats. Explanations given by the authors are a difference in baseline stress between studies, and/or strain differences.

Even though a control experiment suggested that the anxiogenic-like effects were, at least in part, independent of effects on locomotion (Fernandez *et al.*, 2004), a recent study by Vitale *et al.* (2006) may suggest the opposite. They replicated the anxiogenic-like effects of N/OFQ in the rat elevated plus-maze test, but observed anxiolytic-like effects after 2 subsequent administrations of N/OFQ (Vitale *et al.*, 2006). This change from anxiogenic- to anxiolytic-like effect of N/OFQ was accompanied by tolerance to the hypolocomotor effects of N/OFQ, suggesting that the anxiolytic-like effects of acute N/OFQ were masked by hypolocomotor effects.

So far the anxiolytic mechanisms of N/OFQ are not well understood. It is likely that N/OFQ effects on anxiety may depend on the ability of this peptidergic system to modulate endogenous 5-HTergic pathways since 5-HT is considered to play a pivotal role in the control of anxiety and fear (Millan, 2003). Moreover, recent findings suggested that the anxiolytic-like effects of N/OFQ might be mediated via activation of the GABA/benzodiazepine system and the effects of N/OFQ may relate to the regulation of anxiety states in the amygdala (Gavioli *et al.*, 2008; Uchiyama *et al.*, 2008).

Several lines of evidence also suggest that endogenous N/OFQ has an important role in anxiety and stress regulation. Enhanced anxiety was shown in ppN/OFQ(-/-) mice (Kest *et al.*, 2001; Ouagazzal *et al.*, 2003; Reinscheid *et al.*, 2002) and antisense-NOP treated rats (Blakley *et al.*, 2004), but not in NOP(-/-) mice (Mamiya *et al.*, 1998). Gavioli *et al.* (2007) demonstrated that

there are no clear differences between NOP(-/-) and NOP(+/+) mice in some classical models of anxiety (open-field, hole-board and marble-burying tests). In contrast, when subjected to other models of anxiety such as novelty-suppressed feeding behaviour and the elevated T-maze test, NOP(-/-) mice display lower anxiety-related behaviours compared to NOP(+/+) mice (Gavioli *et al.*, 2007). In the elevated plus-maze and light-dark box, NOP(-/-) mice displayed increased anxiety-related behaviour (Gavioli *et al.*, 2007).

Interestingly, the impact of ppN/OFQ deletion on the anxiety-like behaviours is more significant in group-housed, as compared with individual-housed mice, and male mice were more susceptible than females (Ouagazzal *et al.*, 2003). In mice, differences in anxiety states are associated with differences in G protein coupling efficiency in the nucleus accumbens (but not in 12 other brain regions) (Le Maitre *et al.*, 2006). A likely explanation of this finding is that the observed increase in coupling in non-anxious mice leads to increased N/OFQ-mediated transmission and thus protects from anxiety (Le Maitre *et al.*, 2006).

It has also been suggested that N/OFQ can act as a functional corticotrophin releasing factor (CRF) antagonist, since it is able to revert the hypophagia induced by either stress or the central administration of CRF (Ciccocioppo *et al.*, 2001). Since CRF is a major mediator of stress and a potent anxiogenic agent, the functional relationships between the N/OFQ and CRF systems are worthy of further investigation aimed at clarifying the mechanisms by which N/OFQ exerts its anxiolytic-like effects.

However, the information that has been available to date has been too limited to propose a mechanistic interpretation of the anxiolytic-like effects of N/OFQ. Other studies aimed to the identification of the brain areas involved in this action are needed for a better understand N/OFQ role in this field.

Antidepressant-like action

Studies performed in rodents subjected to behavioural despair tests support a role of the N/OFQ-NOP receptor system in the modulation of mood behaviours.

NOP receptor antagonists, including [Nphe^{1}]N/OFQ(1-13)-NH_2, J-113397, UFP-101 and SB-612111 reduced immobility time in both the forced swim and tail suspension tests. N/OFQ (i.c.v.) alone did not affect immobility time (Gavioli *et al.*, 2004; Redrobe *et al.*, 2000; Rizzi *et al.*, 2007a).

In our laboratories, using a combined pharmacological and genetic approach, we demonstrated that blockade of N/OFQ-NOP receptor signalling in the brain produces antidepressant-like effects in the mouse and rat forced swimming test and in the mouse tail

suspension test. I.c.v. injection of N/OFQ did not induce any behavioural modification in mice, but the co-administration of 1 nmol of N/OFQ reversed the antidepressant-like effect induced by the NOP receptor antagonists UFP-101 (Gavioli *et al.*, 2003; Gavioli *et al.*, 2004). In addition, N/OFQ (1 nmol) also reverted the effects induced by the non-peptide NOP receptor antagonist J-113397 in the mouse forced swimming test (Gavioli *et al.*, 2006). Whereas the immobility time in NOP(-/-) mice is less than that in NOP(+/+), the antidepressant-like effects of NOP receptor antagonists were not observed in NOP(-/-) mice (Gavioli *et al.*, 2003), suggesting that endogenous N/OFQ plays a role in those depression-like behaviours. Treatment with UFP-101 (10 nmol) reduced immobility time in NOP(+/+) mice, while it was inactive in mice lacking the NOP receptor (Gavioli *et al.*, 2003). Systemic administration of J-113397 (20 mg/kg) promoted a statistically significant reduction in immobility time in the forced swimming test in NOP(+/+), but not in NOP(-/-) animals. Additionally, SB-612111 (10 mg/kg, i.p.) reduced the immobility time in NOP(+/+) mice while being inactive in NOP(-/-) animals (Rizzi *et al.*, 2007a).

Moreover, it has been demonstrated that antidepressant-like effects elicited by the selective NOP receptor antagonist UFP-101 are probably due to the block of the inhibitory effects of endogenous N/OFQ on brain monoaminergic (in particular serotonergic) neurotransmission (Gavioli *et al.*, 2004).

Vitale and colleagues investigated the effect of UFP-101 in the chronic mild stress paradigm in rats; UFP-101 (10 nmol/rat, i.c.v. continuously infused by means of minipumps for 24 days) did not influence sucrose intake in non stressed animals, but reinstated the basal sucrose consumption in stressed animals, beginning from the second week of treatment as did fluoxetine (10 mg/kg, i.p.), used as reference drug (Vitale, 2008).

There is just one human study (Gu *et al.*, 2003) in which plasma N/OFQ level was elevated in post-partum depressive women. This limited small study agrees with the notion that post-partum depression results from reduced 5-HT levels and that this is accompanied by elevated N/OFQ with the increase in N/OFQ possibly causing the fall in 5-HT (Lambert, 2008). Thus, NOP receptor antagonists may have the potential to be novel antidepressants.

Overall, these pharmacological and genetic findings suggest that the blockade of N/OFQ signaling induces antidepressant-like effects in rodents. Importantly, all these observations were performed after acute drug administration, and no data are currently available subsequent to chronic drug exposure. Thus, further studies aimed at the evaluation of the antidepressant-like effects of NOP receptor antagonists in animal models based on chronic stress may generate important information about the role of this peptidergic system in mood disorders.

Food intake

Soon after the isolation of N/OFQ, Pomonis and colleagues (Pomonis *et al.*, 1996) showed that supraspinal N/OFQ (1–10 nmol) increased food intake in the satiated rat. N/OFQ effects are short-lasting, specific to food intake with neither water intake nor 1% sucrose intake affected, and accompanied by transient hypolocomotion (Polidori *et al.*, 2000a).

N/OFQ hyperphagia can be blocked by antisense treatment to NOP mRNA (Leventhal *et al.*, 1998), competitive NOP antagonism (Polidori *et al.*, 2000a) and functional antagonism by nocistatin (Olszewski *et al.*, 2000). Surprisingly, naloxone/naltrexone pretreatment also blocks N/OFQ effects on food intake (Leventhal *et al.*, 1998; Pomonis *et al.*, 1996), although this is probably due to classical opioid receptors being involved in feeding control at a distal site or affecting motivational processes related to food intake. In addition, it was shown that the orexigenic action of 1 nmol of N/OFQ was prevented by SB-612111 (1 mg/kg) and no longer evident in NOP(-/-) animals, indicating that the orexigenic effects induced by N/OFQ are exclusively due to NOP receptor activation (Rizzi *et al.*, 2007a).

The orexigenic action of N/OFQ is suggested to be attributed to both the inhibition of anorexigenic systems and the activation of orexigenic systems (Olszewski *et al.*, 2004). N/OFQ has been found to inhibit pathways that promote termination of food intake in the hypothalamic satiety centers, such as oxytocinergic neurons in the paraventricular nucleus and neurons in the arcuate nucleus (Olszewski *et al.*, 2004).

Moreover, Ciccocioppo *et al.* (2004) found that N/OFQ, at doses without hyperphagic effects, inhibited stress-induced anorexia and that this anti-anorexic effect is due to the fact that N/OFQ acts as a functional antagonist of corticotrophin-releasing factor (CRF) at the bed nucleus of the stria terminalis (Ciccocioppo *et al.*, 2004). [Nphe^1]N/OFQ(1-13)-NH_2 did not affect food consumption *per se* in satiated rats, but reduced that in food-deprived rats (Polidori *et al.*, 2000a). UFP-101 also did not affect free feeding in the rat (Economidou *et al.*, 2006b). This suggested that endogenous N/OFQ plays a role in orexigenic tone in response to food deprivation but not in normal feeding. On the contrary, Rizzi *et al.* (2007a) showed that the antagonist SB-612111 (1 and 10 mg/kg, i.p.), tested in food deprived mice, did not modify food intake. Thus, the data obtained with SB-616211 suggest that in mice, unlike rats (Polidori *et al.*, 2000a), the N/OFQ-NOP receptor system does not play a major role in controlling food intake induced by food deprivation.

All these studies indicate that the hyperphagic and the anti-anorectic effect of N/OFQ are mediated by separate brain structures and that synthetic N/OFQ agonists might have therapeutic potential as orexigenic drugs (Ciccocioppo *et al.*, 2004; Economidou *et al.*, 2006b).

Reward and addiction

In animal models aimed at elucidating the rewarding properties of drugs of abuse the conditioned place preference (CPP) test is commonly used. In this assay N/OFQ has been shown to reduce CPP to alcohol (Ciccocioppo *et al.*, 1999; Kuzmin *et al.*, 2003), amphetamines (Kotlinska *et al.*, 2003), cocaine (Kotlinska *et al.*, 2003; Sakoori *et al.*, 2004), and morphine (Sakoori *et al.*, 2004) indicating that this peptide was reducing reward to these stimuli. N/OFQ alone was inactive. All these experiments measured the acquisition or reinstatement of drug preferences, either as a conditioned response (place preference) or self-administration of the drug itself. However, it should be mentioned that N/OFQ failed to block heroin self-administration (Walker *et al.*, 1998). Another study also showed that N/OFQ was effective in preventing stress-induced alcohol-seeking behaviour but not cocaine-seeking behaviour (Martin-Fardon *et al.*, 2000). Finally, one study demonstrated that N/OFQ was able to block sensitization to cocaine, independent of context (Lutfy *et al.*, 2002).

Since the mesolimbic dopaminergic system plays a pivotal role in opioid rewarding properties (Wise, 1989), it has been suggested that N/OFQ attenuates conditioned place preference to any type of drug of abuse by inhibiting its stimulatory effect on mesolimbic dopamine release from the nucleus accumbens (Murphy *et al.*, 1999). In fact, i.c.v. N/OFQ effectively inhibits dopamine release (as evaluated by *in vivo* microdialysis) in the nucleus accumbens of the rat stimulated by systemically injected morphine (Di Giannuario *et al.*, 1999). Alternatively, the inhibitory effects of N/OFQ could be explained by the finding that N/OFQ inhibits GABAergic transmission and blocks ethanol-induced increase of GABA release in the central amygdala (Roberto *et al.*, 2006). Interestingly, Ciccocioppo *et al.* (2007) found that buprenorphine, a partial agonist at MOP and NOP receptors, increased alcohol intake at lower doses through MOP receptors while decreased it at higher doses through NOP receptors. It is suggested that the therapeutic potential of buprenorphine in drug addiction might be attributed to its NOP receptor activation, but not to its activation of classical opioid receptors.

Recent findings demonstrated that the psychostimulant and rewarding actions of buprenorphine were enhanced in NOP(-/-) mice as compared to their NOP(+/+) littermates. However, these actions of morphine were not altered in mutant mice. Buprenorphine displaced specific binding of [^{3}H]-N/OFQ in brain homogenates of NOP(+/+) mice; together these results suggest that the ability of buprenorphine to interact with NOP receptor compromises its acute motor stimulatory and rewarding actions (Marquez *et al.*, 2008).

Even though more work is obviously necessary to fully understand the effects of N/OFQ on drug reward and the mesolimbic dopamine system, it appears that N/OFQ agonists might provide useful compounds to control the rewarding aspects of drugs.

Learning and memory

N/OFQ may play a role in memory and learning processes since there is a high density of NOP receptors in the anterior cingulate, frontal cortex, basolateral complex of the amygdala and hippocampus. In fact N/OFQ injected into the hippocampus impairs spatial learning (Sandin *et al.*, 1997) and *in vitro* it inhibits synaptic transmission and long-term potentiation in rat hippocampal slices (Yu *et al.*, 1997). Recently it was also seen that endogenously released N/OFQ interacts with noradrenergic activity within the basolateral complex of the amygdala in modulating memory consolidation (Roozendaal *et al.*, 2007).

In line with these findings, NOP(-/-) mice show greater learning ability and have better memory retention than wild-type control mice (Manabe *et al.*, 1998). The impairment of learning induced by N/OFQ can be reversed by nocistatin (Hiramatsu *et al.*, 1999b) or by the non-selective NOP receptor antagonist NalBzOH (Mamiya *et al.*, 1999). Moreover, a peptidic NOP receptor antagonist Ret-Noc-OMe, has been reported to strengthen memory retention in a passive avoidance test in mice (Jinsmaa *et al.*, 2000).

It is worthy of note that pre-treatment with [Nphe^1]N/OFQ(1-13)-NH_2, a NOP receptor antagonist, prevented NOP-induced deficits. Using a pure pharmacological approach, i.e. NOP receptor blockade, a role for the N/OFQ and its receptor in learning and memory has been demonstrated (Redrobe *et al.*, 2000).

Very recent study showed that intracerebroventricular or intrahippocampal infusions of N/OFQ impair long-term memory formation in the mouse object recognition task. The synthetic NOP receptor agonist Ro 64-6198, administered systemically, also produced amnesic effects that were blocked by coinfusion of the NOP receptor antagonist UFP-101, into the dorsal hippocampus. In contrast, Ro 64-6198 had not effect on short-term memory or recall performances (Goeldner *et al.*, 2008). Immunoblotting analysis revealed a strong suppressive action of Ro 64-6198 on learning-induced upregulation of hippocampal extracellular signal-regulated kinase (ERK) phosphorylation, which is crucial for long-term information storage (Goeldner *et al.*, 2008). Thus, N/OFQ-NOP receptor system negatively regulates long-term recognition memory formation through a hippocampal ERK signalling mechanism (Goeldner *et al.*, 2008).

Collectively, these findings suggest that the N/OFQ-NOP receptor system may play negative roles in learning and memory, and that NOP receptor antagonists might be worthy of testing as drugs for memory disorders.

Effects in the gastrointestinal tract

Like morphine or other opioid receptor agonists, N/OFQ 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.*, 1999b), rats (Yazdani *et al.*, 1999) and rabbits (Pheng *et al.*, 2000). This depressor effect is resistant to blockade by naloxone. In contrast, N/OFQ causes concentration-dependent contractions in proximal rat colon, without changes in stomach, jejunum or ileum (Taniguchi *et al.*, 1998; Yazdani *et al.*, 1999). N/OFQ also contracts proximal and distal segments of mouse colon (Menzies *et al.*, 1999; Osinski *et al.*, 1999a; Rizzi *et al.*, 1999a).

In vivo, similarly to opioids, central administration of N/OFQ also inhibits colon transit in the mouse (Osinski *et al.*, 1999a). On the other hand, Taniguchi and collegues (Taniguchi *et al.*, 1998) reported that N/OFQ administered subcutaneously in rats actually accelerated transit rate in the large intestine, an action opposite to that induced by morphine or selective opioid receptor agonists. Broccardo and colleagues demonstrated that the NOP receptor antagonist [Nphe^1]N/OFQ(1-13)-NH_2 blocked the N/OFQ-evocked gastrointestinal anti-transit effect (Broccardo *et al.*, 2004). It is worthy of note that [Nphe^1]N/OFQ(1-13)-NH_2 *per se* stimulated gastric acid secretion.

Distal colonic contractions induced by N/OFQ were also dose-dependently antagonized by the NOP non-peptide antagonist J-113397 that behaves as selective NOP antagonist in the rat colon (Tada *et al.*, 2002).

All these findings led suggest that the N/OFQ-NOP receptor system is pharmacologically distinct from opioids but functionally very similar, and could represent a new target for the development of drugs (NOP receptor agonists) to reduce intestinal motility.

In addition to the well-characterized inhibition of gastric motility, recent studies demonstrated that N/OFQ increases gastric mucosal resistance to ethanol induced lesions by operating both in the central nervous system and in the periphery (Morini *et al.*, 2005). This effect is mediated by the NOP receptor since the selective N/OFQ antagonist UFP-101 completely prevents the protective effects of N/OFQ (Morini *et al.*, 2005). There is evidence for central and peripheral components to the regulation of gastrointestinal function: vagal cholinergic and sympathetic

pathways mediate the central activity of N/OFQ, whereas vagal non-muscarinic pathways mediate the peripheral activity of the peptide (Broccardo *et al.*, 2004; Ishihara *et al.*, 2002).

An elegant study recently showed that N/OFQ and UFP-112, a novel highly potent NOP agonist, when administered intracerebroventrically and intraperitoneally decreased bead expulsion time and reduce the percentage of rats with castor oil-induced diarrhoea; UFP-112 showed greater efficacy, higher potency and longer-lasting effects than N/OFQ (Broccardo *et al.*, 2008). These findings indicate that, in the rat, the central and peripheral N/OFQ system has an inhibitory role in modulating distal colonic propulsive motility under physiological and pathological conditions (Broccardo *et al.*, 2008).

Further studies are necessary to investigate the mechanism(s) accounting for this interesting effect of N/OFQ.

Effects in the airways

N/OFQ was found to inhibit contractions of the guinea pig isolated bronchus (Fischer *et al.*, 1998; Rizzi *et al.*, 1999b) of the rat isolated trachea and bronchus (Wu *et al.*, 2000) induced by electrical field stimulation and of the cholinergic contractions of human bronchus (Basso *et al.*, 2005).

N/OFQ inhibited the cough responses provoked by capsaicin in guinea pigs or by mechanical stimulation of intrathoracic airways in cats (Bolser *et al.*, 2001; McLeod *et al.*, 2001). These antitussive actions might be mediated at both central and peripheral sites.

N/OFQ decreased capsaicin-induced Ca^{2+} influx in nodose ganglia (McLeod *et al.*, 2004), the sensory ganglia involved in the cough reflex (Reynolds *et al.*, 2004), and the airway contraction in a manner blocked by tertiapin, an inwardly rectifying K^+ channel blocker (Jia *et al.*, 2002). In the brain, there are dense NOP receptors in the medullar nucleus tractus solitarius (Judd *et al.*, 1996), which provides polysynaptic inputs to second-order neurons that modulate respiratory neuron activities (Reynolds *et al.*, 2004).

This antitussive effect can be mimicked by the non-peptide agonist Ro 64-6198 in a J-113397-sensitive manner (McLeod *et al.*, 2004). Codeine is the current gold-standard antitussive agent but has a poor side-effect profile that is typical of MOP opioid receptor agonists (such as nausea, constipation, tolerance and dependence). So, orally active NOP agonists might represent a viable alternative for the treatment of cough (Lambert, 2008).

Cardiovascular system

When given intravenously (i.v.) in anaesthetised rats, N/OFQ induces transient hypotension and bradycardia (Champion *et al.*, 1997; Giuliani *et al.*, 1997b). 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 N/OFQ and that these effects are not restricted to the rat. Interestingly, N/OFQ 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.*, 1999). The effects occur at both central and peripheral sites. The most compelling evidence for peripheral effects is in the hypotension and bradycardia produced by intravenous administration of N/OFQ, a peptide that does not cross the blood-brain barrier (Lambert, 2008). It has been suggested that as the sympatholytic guanethidine reduced the hypotensive effects of N/OFQ, then this peptide acts to inhibit sympathetic control of the cardiovascular system (Giuliani *et al.*, 1997b). In addition, the bradycardic effects of N/OFQ were reduced by vagotomy, indicating that N/OFQ increased parasympathetic activity (Giuliani *et al.*, 1997b).

On the other hand, N/OFQ has 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 N/OFQ in different species.

N/OFQ also produces vasodilation in several isolated arteries of the cat (Gumusel *et al.*, 1997) and in pial arteries of the pig (Armstead, 1999) and in the mesenteric resistance arteries of the rat (Champion *et al.*, 1998). These studies also demonstrated that the vasodilator responses to N/OFQ were not prevented by naloxone, nitric oxide synthase inhibitors, atropine, phentolamine or by a CGRP receptor antagonist. Recently a study showed that in a rat mesenteric microcirculation model, intravenous administration of N/OFQ dilated arterioles and venules (Brookes *et al.*, 2007); dilation of these non-innervated vessels was blocked by histamine antagonists and mast-cell stabilizers, suggesting that the N/OFQ-mediated dilation of the microcirculation is secondary to mast-cell release of histamine.

Renal function

The i.v. infusion of N/OFQ produces a marked increase in urine flow rate and a decrease in urinary sodium excretion (Kapusta *et al.*, 1997). Concurrent with diuresis, N/OFQ infusion produced hypotension with no change in heart rate; this is in contrast to the concurrent bradycardia and hypotension elicited by N/OFQ when this peptide is administered as an i.v. bolus (Bigoni *et al.*, 1999; Champion *et al.*, 1997; Giuliani *et al.*, 1997b; Madeddu *et al.*, 1999), intrathecally (Lai *et al.*, 2000), or when microinjected into the lateral cerebroventricle (Kapusta *et al.*, 1999a; Kapusta *et al.*,

1999b; Kapusta *et al.*, 1997; Shirasaka *et al.*, 1999). Low doses of N/OFQ (i.v. infusion) also tended to decrease urinary sodium excretion without changes in heart rate or mean arterial pressure. These findings also suggest that the i.v. infusion lower doses of N/OFQ can be used to separate the cardiovascular and renal responses produced by this compound, with the peptide having a more pronounced effect on the renal handling of water (Kapusta, 2000). Following i.c.v. microinjection, N/OFQ produced a marked diuresis, antinatriuresis and renal sympathoinhibition in conscious rats (Kapusta *et al.*, 1999a; Kapusta *et al.*, 1999b; Kapusta *et al.*, 1997; Shirasaka *et al.*, 1999)

It is worthy of note that a class of NOP ligands, the partial agonists (i.e. Ac-RYYRWK-NH_2, Ac-RYYRIK-NH_2 (Dooley *et al.*, 1997), [F/G]N/OFQ(1-13)-NH_2 (Guerrini *et al.*, 1997), ZP120 (Larsen *et al.*, 2001) have been shown to behave as full agonists on cardiovascular and renal functions, mimicking the effects of N/OFQ, when given i.c.v. (Kapusta *et al.*, 1999b). In contrast, the i.v. bolus injection of the same NOP receptor partial agonists produced responses unlike N/OFQ; N/OFQ evoking profound bradycardia and hypotension with no change in urine output, and i.v. bolus NOP receptor partial agonists eliciting water diuresis without altering cardiovascular function (Kapusta *et al.*, 2005a; Kapusta *et al.*, 2005b). Indeed, Kapusta et al. showed that ZP120 (i.v. bolus or i.v.infusion) produced, in rats, a sodium-potassium-sparing aquaresis and a mild vasodilatory response without reflex tachycardia (Kapusta *et al.*, 2005b).

Activation of NOP receptors in the paraventricular nucleus (PVN) of the hypothalamus by N/OFQ produces bradycardia, renal sympathoinhibition, and water diuresis. Recently, the group of Kapusta (Krowicki *et al.*, 2006) showed that endogenous N/OFQ produces a tonic inhibition on PVN activity since UFP-101, when injected into the PVN, increased heart rate and renal sympathetic nerve activity and decreased urine flow rate.

In summary, it was seen that in conscious rats NOP receptor partial agonists produced functionally selective effects on cardiovascular and renal function ranging from full agonist (i.c.v., cardiovascular depressor; i.c.v. and i.v., water diuresis), partial agonist (i.v., submaximal hypotension without altering heart rate) to antagonist (i.v., blockade of N/OFQ-evoked bradycardia and hypotension) behaviour. Based on their ability to produce a selective water diuresis after i.v. bolus injection without apparent adverse cardiovascular or CNS effects, it can be proposed that metabolically stable NOP receptor partial agonists (e.g., ZP120; (Kapusta *et al.*, 2005b)) may be useful therapeutically as novel peripherally acting aquaretics for the acute management of severe water retention and/or hyponatremia; ZP120 was, hence, filed for phase II clinical trials for the treatment of acute decompensated heart failure.

Micturition reflex

In anaesthetised rats, i.v. N/OFQ produced a dose-dependent suppression of the micturition reflex induced by bladder distension or by topical application of capsaicin (Giuliani *et al.*, 1998). Similar results were obtained by administering the peptide i.c.v. or i.t. indicating that N/OFQ inhibits the micturition reflex by acting at peripheral, spinal and supraspinal sites (Lecci *et al.*, 2000).

All these effects are not affected by naloxone, thus excluding the involvement of opioid receptors. These animal studies were later confirmed in clinical studies. Indeed, the urodynamic and clinical effects of N/OFQ were evaluated in normal subjects and in patients with neurogenic bladder. A preliminary report (Lazzeri *et al.*, 2001) and a subsequent randomized, placebo controlled, double-blind study (Lazzeri *et al.*, 2003) demonstrated that intravescical instillation of 1 μM N/OFQ solution produce an inhibitory effect on micturition reflex in selected groups of patients suffering from neurogenic incontinence but not in normal subjects. These effects of N/OFQ are due to its ability to selectively activate the NOP receptor as suggested by the fact that [desPhe^1]N/OFQ, a N/OFQ metabolite which does not bind NOP receptor, is inactive in these patients (Lazzeri *et al.*, 2003). Moreover, a recent study (Lazzeri *et al.*, 2006) demonstrated that a daily treatment with 1 mg N/OFQ intravescically for 10 days, but not the placebo, inhibited the micturition reflex in patients suffering from neurogenic incontinence, thus demonstrating the clinical efficacy of a prolonged NOP receptor agonist treatment. Based on these findings N/OFQ selective and potent peptide agonists with long lasting effects *in vivo* may be proposed as innovative drugs for treating patients suffering from neurogenic incontinence.

Immune system

NOP receptors and N/OFQ are widely distributed throughout the immune system. NOP receptor mRNA and protein have been found in a variety of immune cells including mouse lymphocytes (Halford *et al.*, 1995), human peripheral blood mononuclear cells (Wick *et al.*, 1995) and human circulating granulocytes, lymphocytes and monocytes (Fiset *et al.*, 2003; Peluso *et al.*, 1998). Neutrophils are thought to be a source of N/OFQ in inflammatory tissues (Fiset *et al.*, 2003). N/OFQ can function as an immunosuppressant by suppressing antibody production in mouse lymphocytes, by decreasing proliferation of phytohemagglutinin-stimulated PBMCs, and by inhibiting mast cell function (Civelli, 2008). In addition, it was shown that N/OFQ stimulates human monocyte chemotaxis via NOP receptor activation (Trombella *et al.*, 2005). The importance of the N/OFQ system in the immune response, however, remains to be clearly defined.

1.8 Pharmacology of NOP receptors

From the numerous modulatory actions of N/OFQ in several pathways, it is clear that NOP may represent an important molecular target for the development of novel therapeutics for several pathological conditions. The identification of new molecules possibly of non-peptide nature that selectively activate (agonists) or block (antagonists) the NOP receptor will represent a major achievement in this research field, providing pharmacological tools for clarification of the physiological and pathophysiological roles of the this new system and ultimately for the identification of possible therapeutic agents acting at the NOP receptor.

Peptide compounds usually show high selectivity and specificity but metabolic instability and limited distribution. In contrast, non-peptides demonstrate better pharmacokinetic features while their specificity is often low. There is an evident interest of academia and pharmaceutical companies in developing both agonist and antagonist ligands for the NOP receptor as potential drugs for various human disorders (see series of patents quoted by Zaveri (2003) and (Bignan *et al.* (2005)).

Peptide ligands

N/OFQ shows a significant homology with dynorphin A. The first four amino acids differ from the canonical opioid sequence only by the presence of Phe^1 instead of Tyr^1. This difference may be sufficient to prevent N/OFQ binding to opioid receptors. In fact, replacement of Phe^1 by Tyr^1 results in a peptide that also binds the opioid receptors (Calo *et al.*, 1997; Varani *et al.*, 1999). Amidation of the C-terminus (N/OFQ-NH_2) maintains full potency and activity (Guerrini *et al.*, 1997). Early studies to determine the minimum active sequence showed that up to four C-terminal amino acids can be deleted without loss of activity. Although the free acid N/OFQ(1–13)-OH loses receptor affinity, amidation of the C-terminus to give N/OFQ(1–13)-NH_2 restores potency and agonist activity comparable to the parent peptide (Calo *et al.*, 1996; Dooley *et al.*, 1996; Reinscheid *et al.*, 1996). C-terminal amidation protects from degradation by carboxypeptidases and is now a standard feature of most N/OFQ-based peptide ligands. In fact, the truncated peptide N/OFQ(1–13)-NH_2 has been used as a chemical template for SAR studies aimed to investigation of novel ligands for NOP receptor.

Initial structure-activity studies on N/OFQ(1–13)-NH_2 by Guerrini *et al.* (1997) determined that the N-terminal peptide FGGF is essential for activity and that Phe^4 and Phe^1 appear to be important for receptor activation. Further studies on N-terminal modification resulted in the discovery of a purported NOP antagonist in which the Phe^1-Gly^2 amide bond was replaced with a pseudopeptide (CH_2-NH) bond (Calo *et al.*, 1998a; Guerrini *et al.*, 1998). This peptide [$Phe^1\Psi$(CH_2-

NH)Gly^2]N/OFQ(1–13)-NH_2 abbreviated as [F/G]N/OFQ(1–13)-NH_2, was shown to behave as a selective and competitive antagonist in the electrically stimulated guinea pig ileum and mouse vas deferens (Guerrini *et al.*, 1998). This report initiated a surge of *in vitro* and *in vivo* studies (Calo *et al.*, 2000a) which showed that this peptide behaved as an antagonist, partial agonist, or even full agonist, depending on the tissue preparation. Thus, while it showed different levels of partial agonist activity in [^{35}S]GTPγS assays in CHO cells transfected with human or mouse NOP (Berger *et al.*, 2000b; Burnside *et al.*, 2000), it showed full agonist activity in several *in vivo* CNS assays (Calo *et al.*, 1998b; Carpenter *et al.*, 1998; Grisel *et al.*, 1998; Xu *et al.*, 1998). McDonald and colleagues demonstrated that agonism is primarily dependent upon receptor density and coupling efficiency (McDonald *et al.*, 2003a). As these parameters are tissue/model dependent, intrinsic activity in different tissues can vary. Using the ecdysone-inducible expression system containing the human NOP receptor (hNOP) expressed in Chinese hamster ovary cells they performed [^{35}S]GTPγS binding and inhibition of adenylyl cyclase studies to examine the activity of a range of partial agonists. They found that profile of [F/G]N/OFQ(1–13)-NH_2 can be manipulated to encompass full and partial, agonism along, with antagonism (McDonald *et al.*, 2003a).

Further modifications of the N/OFQ N-terminus led to the design of [Nphe^1]N/OFQ(1–13)-NH_2 by transposition of the Phe^1 side chain from the α-carbon of Phe^1 to the N-terminal nitrogen (Guerrini *et al.*, 2000a). This peptide was the first pure NOP peptide antagonist; it had low potency (pA_2 values 6.0–6.4) (Calo *et al.*, 2000a) but was devoid of any residual agonist activity. This modified N/OFQ peptide selectively antagonized the effects of N/OFQ *in vitro* in various isolated tissues and in CHO cells expressing the human recombinant NOP receptor (Berger *et al.*, 2000b; Calo *et al.*, 2000a; Hashimoto *et al.*, 2000). *In vivo*, i.c.v. administration of this peptide inhibited the pronociceptive and antiopioid actions of N/OFQ (Rizzi *et al.*, 2000) and reversed the effects of N/OFQ on memory impairment (Redrobe *et al.*, 2000), food intake (Polidori *et al.*, 2000a), and locomotor activity (Rizzi *et al.*, 2001a). This compound, *per se*, is able to induce changes opposite to that evoked by N/OFQ such as antinociception (Calo *et al.*, 2000a), prevention of ibotenate induced neurotoxicity (Laudenbach *et al.*, 2001) and inhibition of food intake (Polidori *et al.*, 2000b), facilitation of the flexor reflex with no depression (Xu *et al.*, 2002). Importantly, a study by Di Giannuario and colleagues has shown that no tolerance develops to the antinociceptive action of this antagonist, unlike with opioid analgesics, suggesting that NOP antagonists can be developed as a novel class of supraspinal analgesics (Di Giannuario *et al.*, 2001).

Modification of N/OFQ and N/OFQ(1–13)-NH_2 has also produced peptide agonists more potent than N/OFQ. Okada *et al.* (2000) reported the synthesis of [Arg^{14}Lys^{15}]N/OFQ, which had 3-fold greater binding affinity than N/OFQ at human NOP and was 17 times more potent in the

[^{35}S]GTPγS functional assay. [Arg^{14}Lys^{15}]N/OFQ was the first NOP receptor agonist more potent than the natural ligand, in addition its effects are long lasting *in vivo* (Rizzi *et al.*, 2002c).

Guerrini *et al.* (2001) focused their attention on the Phe^4 residue and found that para-substituted electron-withdrawing groups such as pF and pNO_2 increased binding affinity to NOP receptor 5- and 3-fold, respectively. These agonist peptides were more potent than N/OFQ at recombinant hNOP and at native NOP receptor sites expressed in isolated tissues (Bigoni *et al.*, 2002; McDonald *et al.*, 2002). These agonists also display longer duration of action *in vivo* in several assays, compared to N/OFQ (Rizzi *et al.*, 2002b).

Another cyclic peptide having an intramolecular disulphide bridge: cyclo [Cys^{10}, Cys^{14}]N/OFQ(1-14)-NH_2, was reported to be the first conformationally restricted derivative among the N/OFQ analogues. This peptide has agonist activity and may serve as a good template for studying the bioactive conformation of N/OFQ (Ambo *et al.*, 2001). Combination of the cyclic analog with Nphe^1 which previously led to pure antagonism, provided a partial agonist c[Nphe^{1}Cys^{10},^{14}]N/OFQ(1-14)-NH_2 (Kitayama *et al.*, 2003).

Furthermore in a series of structure-activity studies (Guerrini *et al.*, 2005) the chemical modifications which reduce ([Phe^{1}Ψ(CH_2-NH)Gly^2]) or eliminate ([Nphe^1]) agonist efficacy, in the N/OFQ-NH_2 structure, were combined with those which increase agonist potency i.e. [(pF)Phe^4] and [Arg^{14}Lys^{15}]. This study led to the identification of a very potent antagonist, [Nphe^{1}Arg^{14}Lys^{15}]N/OFQ-NH_2 (UFP-101) (Calo *et al.*, 2002b), and [(pF)Phe^{4}Arg^{14}Lys^{15}]N/OFQ-NH_2 (UFP-102) (Carra *et al.*, 2005b), a highly potent and selective full agonist at NOP receptors. The gain in potency was accompanied by slow onset and relatively long duration of action that was observed in *in vitro* and especially in *in vivo* assays (Carra *et al.*, 2005b; Economidou *et al.*, 2006b). A very potent partial agonist [Phe^{1}ψ(CH_2-NH)-Gly^2(pF)Phe^{4}Arg^{14}Arg^{15}]N/OFQ-NH_2 (Guerrini *et al.*, 2005) was also identified.

Regarding the potent and selective antagonist UFP-101 a detailed summary of its pharmacological characterization was reported by our group (Calo *et al.*, 2005). Collectively data obtained *in vitro* in a variety of preparations with different approaches demonstrated that UFP-101 behaves as a potent, competitive and selective antagonist at NOP receptors. *In vivo*, UFP-101 has been tested against N/OFQ in a series of experiments aimed at the investigation of the role of the N/OFQ-NOP receptor system in regulating various biological functions including pain transmission, locomotor activity, mood-related behaviours, drug abuse, food intake and cardiovascular, renal and gastrointestinal function. It has been demonstrated that UFP-101 antagonizes the following actions of N/OFQ: hyperalgesia, reversal of stress-induced analgesia, inhibition of locomotor activity, stimulation of diuresis in mice, bradycardia, hypotension and

reduction of plasma NE levels in guinea pig ileum, stimulation of food intake and spinal analgesia in rats. Moreover UFP-101 (like other selective NOP antagonists) produced antidepressant-like effects in normal mice in the forced swimming or the tail suspension test. In mice lacking the NOP receptor gene these actions are absent (Calo *et al.*, 2005).

Recently (Economidou *et al.* (2006b) verified the hyperphagic effect of 3 new peptide agonists, termed OS-500, OS-462 and OS-461 which display an affinity for NOP receptors in the subnanomolar range: no selectivity data were provided.

Previous structure–activity and NMR studies on N/OFQ demonstrated that Aib substitution of Ala^{7} and/or Ala^{11} increases peptide potency through an alpha helix structure induction mechanism (Zhang *et al.*, 2002). Based on these findings Arduin *et al.* (2007) synthesised a series of N/OFQ-NH_2 analogues substituted in position 7 and 11 with Ca,a-disubstituted cyclic, linear and branched amino acids. None of the 20 novel N/OFQ analogues produced better results than [Aib^{7}]N/OFQ-NH_2. Thus, this substitution was combined with other chemical modifications known to modulate peptide potency and/or efficacy generating [$Nphe^{1}Aib^{7}Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (coded as UFP-111), [(pF)$Phe^{4}Aib^{7}Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (UFP-112) and compound [$Phe^{1}\Psi(CH_2–NH)Gly^{2}(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (UFP-113). These novel peptides behaved as highly potent NOP receptor ligands showing full (UFP-112) and partial (UFP-113) agonist and pure antagonist (UFP-111) activities in a series of *in vitro* functional assays performed on pharmacological preparations expressing native as well as recombinant NOP receptors (Arduin *et al.*, 2007). *In vitro* data obtained in the electrically stimulated mouse vas deferens demonstrated that UFP-112 behaved as a high potency (pEC_{50} 9.43) full agonist at the NOP receptor. UFP-112 effects were sensitive to the NOP antagonist UFP-101 but not naloxone and no longer evident in tissues taken from NOP(-/-) mice. *In vivo*, in the mouse tail withdrawal assay, UFP-112 (1–100 pmol, i.c.v.) mimicked the actions of N/OFQ producing pronociceptive effects after i.c.v. administration and antinociceptive effects when given i.t. In both cases, UFP-112 was approximately 100 fold more potent than the natural peptide and produced longer lasting effects. UFP-112 also mimicked the hyperphagic effect of N/OFQ producing a bell shaped dose response curve, this effect was absent in NOP(-/-) mice. Equi-effective high doses of UFP-112 (0.1 nmol) and N/OFQ (10 nmol) were injected i.c.v. in mice and spontaneous locomotor activity recorded for 16 h. N/OFQ produced a clear inhibitory effect which lasted for 60 min while UFP-112 elicited longer lasting effects (> 6h). UFP-112 (0.1 and 10 nmol/kg, i.v.) produced a marked and sustained decrease in heart rate, blood pressure, and urinary sodium excretion and a profound increase in urine flow (Rizzi *et al.*, 2007b).

Small peptide ligands

The small peptides in this group are identified by screening of synthetic peptide combinatorial libraries. Peptide III-BTD was identified from a combinatorial library of β-turn constrained peptides (Becker *et al.*, 1999). This conformationally restricted peptide is a mixed NOP antagonist / opioid agonist (Bigoni *et al.*, 2000b). Five hexapeptides (Ac-RYYRIK-NH_2, Ac-RYYRWK-NH_2, Ac-RYYRWR-NH_2, Ac-RYYLWR-NH_2, Ac-RYYKWK-NH_2) with high affinity and selectivity for the NOP receptor were identified from a peptide library containing about 52 million compounds made considering all the natural amino acids except cysteine (Dooley *et al.*, 1997). Similar to [F/G]N/OFQ(1-13)-NH_2, these peptides were partial agonists whose final effects vary from full agonist to antagonist depending on the tissue and system used in the study (Berger *et al.*, 1999; Berger *et al.*, 2000a; Dooley *et al.*, 1997; Ho *et al.*, 2000; Mason *et al.*, 2001; Rizzi *et al.*, 1999a). These hexapeptides, the shortest peptide sequences interacting with the NOP receptor, have been used as chemical templates for SAR studies. The head to tail cyclization of Ac-RYYRWK-NH_2 produced a drastic decrease in binding affinity (Thomsen *et al.*, 2000b) while the N-terminal acylation with a pentanoyl group (Judd *et al.*, 2003) or the replacement of the $Tyr^{2,3}$ residues with (pF)Phe (Judd *et al.*, 2004) led to the discovery of high affinity low efficacy NOP receptor ligands. The N-terminal alkylation of the central core YYRW with groups bearing a guanidine function generated a NOP receptor agonist (Ishiama *et al.*, 2001). Modifications on the Trp (W) were also preformed (Carra *et al.*, 2005a) by our group. Finally, substitution of the C-terminal amide with an alcoholic function produced Ac-RYYRIK-ol, a NOP receptor ligand that displays high affinity (pK_i 7.91) for NOP receptor expressed in rat brain membranes. Ac-RYYRIK-ol antagonized N/OFQ effects in the vas deferens while it mimicked N/OFQ action in the colon. *In vivo*, the peptide consistently behaved as a NOP receptor agonist mimicking the supraspinal pronociceptive, orexigenic, and motor inhibiting actions and the spinal antinociceptive effects of N/OFQ (Gunduz *et al.*, 2006).

SinVax Inc. proposed the compound pentanoyl-RYYRWR-NH_2 as NOP receptor antagonist. In [^{35}S]GTPγS assays performed in CHO cells transfected with human NOP it displayed a very high affinity with a very low agonist activity (pA_2 value of 8.99). When tested *in vivo*, this compound had a modest analgesic effect, somewhat less than has been reported with other NOP antagonists. Moreover this compound inhibited morphine-induced analgesia suggesting some agonist activity *in vivo* (Judd *et al.*, 2003).

In order to improve the stability and therapeutic utility of these small peptide ligands, a novel technology called structure inducing probes (SIP) (Larsen, 1999) was applied to the hexapeptide Ac-RYYRWK-NH_2 (Dooley *et al.*, 1997), resulting in the design of the peptide Ac-

RYYRWKKKKKKK-NH_2 (ZP120) (Larsen *et al.*, 2001). ZP120 behaved as potent and selective NOP receptor partial agonist whose *in vivo* effects are long lasting (Rizzi *et al.*, 2002a) and, after i.v. administration, confined to periphery. This pharmacological profile makes ZP120 an interesting drug candidate especially for those indications (i.e. aquaresis (Kapusta, 2000)) for which NOP partial agonists that produce renal but not cardiovascular effects are more selective than full agonists which are known to elicit both renal and cardiovascular actions (Kapusta *et al.*, 2002).

This hypotesis was recently confirmed by Kapusta and collegues that candidate ZP120 as aquaretic drug for its selectivity to produce renal but not cardiovascular effects (Kapusta *et al.*, 2005b). The further pharmacological characterization of ZP120 is one of the goals of this dissertation project.

Although the design and pharmacological characterization of peptide ligands for NOP has facilitated great advances in elucidating the functional role of the N/OFQ-NOP receptor system, the therapeutic utility of NOP ligands, particularly for neurological disorders, can only be realized with potent non-peptide ligands. This is because as compared to peptide ligands, non-peptide would be expected to be more resistant to enzymatic breakdown following oral or parenteral administration and allow greater penetration into the CNS where they may have therapeutic utility. Many pharmaceutical companies and different groups have discovered potent non-peptide agonists and antagonists. These are summarized below.

Non-peptide ligands

Non-peptide ligands are generally discovered via high-throughput screening (HTS) in pharmaceutical industry laboratories. Since the NOP receptor represents the non-opioid branch of the opioid family, the search for non-peptide ligands was initiated by examining small-molecule opiate ligands. Among these compounds: i) σ-receptor ligands carbetapentane and rimcazole (Kobayashi *et al.*, 1997); ii) MOP receptor ligands lofentanil (an anilidopiperidine) and etorphine (an oripavine derivative) (Butour *et al.*, 1997); iii) anilidopiperidines, morphinans and benzomorphan classes of opiate ligands (Hawkinson *et al.*, 2000); iv) MOP receptor ligand buprenorphine (Wnendt *et al.*, 1999); v) naloxonebenzoylhydrazone (NalBzOH) (Noda *et al.*, 1998); vi) the 5-HT partial agonist spiroxatrine (Zaveri *et al.*, 2001).

The non-peptide NOP receptor ligands can be broadly divided into six structural classes. It is noteworthy that many of these ligands were first reported in the patent literature (patents from Pfizer, Banyu Pharmaceutical Co., Hoffmann La Roche, EuroCeltique S.A., NovoNordisk, Schering-Plough, Smith Kline Beecham, Japan Tobacco Inc, Toray Industries, etc). However, the

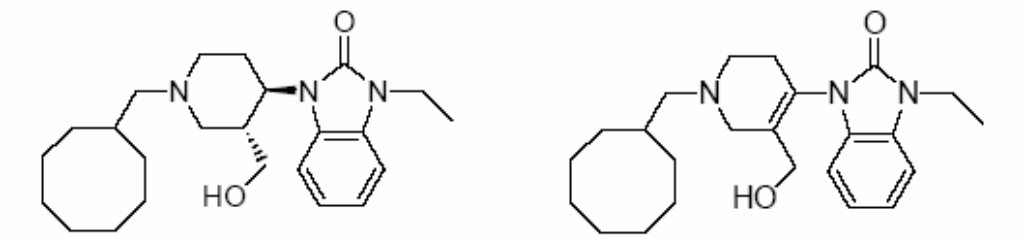
biological data of some of them are still not available, thus making it difficult to define clearly the structural requirements for NOP receptor affinity and selectivity.

i) *Morphinan-based ligands*: Among this group TRK-820 was reported to antagonize the effects of N/OFQ on cAMP accumulation in CHO_{hNOP} cells (Seki *et al.*, 1999). Thus, the morphinan skeleton may provide a good lead for a unique profile of NOP antagonism coupled with opioid agonist activity for a novel class of analgesics.

ii) *Benzimidazopiperidines*: The first non-peptide pure NOP antagonist to be reported was a benzimidazolinone, J-113397 (Figure 8), reported by Kawamoto *et al.* (1999) and patented by Banyu Pharmaceutical Co. (Ozaki *et al.*, 1998). J-113397 was shown to bind with nanomolar affinity to NOP receptors and to display 100-300 fold selectivity over classical opioid receptors (Hashiba *et al.*, 2001; Ozaki *et al.*, 2000). J-113397 antagonized N/OFQ effects at human NOP receptor in a competitive manner with pA_2 values in the range of 7.5 – 8.9 in cAMP and [^{35}S]GTPγS assays (Bigoni *et al.*, 2000a; Hashiba *et al.*, 2002a; Hashiba *et al.*, 2002b; Ozaki *et al.*, 2000). The selective antagonist properties of J-113397 were confirmed at native NOP receptors expressed in isolated tissues (Bigoni *et al.*, 2000a; Tada *et al.*, 2002) and in brain preparations evaluated with biochemical (Olianas *et al.*, 2002), neurochemical (Marti *et al.*, 2003; Rominger *et al.*, 2002) and electrophysiological (Chiou *et al.*, 2002; Luo *et al.*, 2002) techniques. J-113397 was also investigated *in vivo* where, in the range of 1-30 mg/kg, it prevented the actions of N/OFQ on pain transmission (Ko *et al.*, 2002a; Ozaki *et al.*, 2000; Ueda *et al.*, 2000), on airways (Corboz *et al.*, 2001) and the chough reflex (Bolser *et al.*, 2001; McLeod *et al.*, 2002), and on gastrointestinal functions (Ishihara *et al.*, 2002; Tada *et al.*, 2002). Moreover J-113397 produced *per se* pronociceptive effects in the rat (Yamamoto *et al.*, 2001) and mouse (Rizzi *et al.*, 2006) formalin test, antidepressant like effects (similar to NOP receptor peptide antagonists, (Gavioli *et al.*, 2003; Redrobe *et al.*, 2002) in the forced swimming test (Redrobe *et al.*, 2002), reduction of kainate induced seizures (Bregola *et al.*, 2002), potentiation of buprenorphine analgesia in wild type but not in NOP knockout mice (Lutfy *et al.*, 2003), and facilitation of striatal dopamine release and locomotor performance on the rotarod in rats (Marti *et al.*, 2004b). This latter effect was later confirmed in 6-hydroxydopamine lesioned animals (Marti *et al.*, 2005).

To date, J-113397 represents the most potent and selective non-peptide NOP receptor antagonist widely used in pharmacological studies. However, the synthesis, purification, and enantiomer separation of this molecule, which contains two chiral centers, is rather difficult and low-yielding. A series of simplified J-113397 analogues was synthesized and tested to investigate

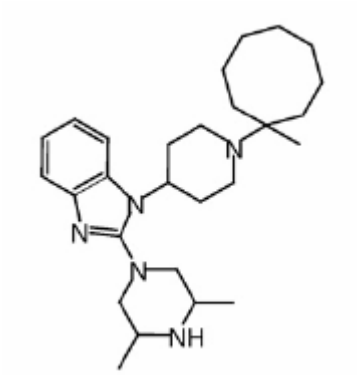
the importance of the stereochemistry and the influence of the substituents at position 3 of the piperidine nucleus and on the nitrogen atom of the benzimidazolidinone nucleus. The compound coded as Trap-101 (Figure 8), an achiral analogue of J-113397, combines a pharmacological profile similar to that of the parent compound with a practical, high-yielding preparation (Trapella *et al.*, 2006). In *in vitro* N/OFQ sensitive preparations Trap-101 was a NOP selective antagonist with a potency 2-3 fold lower than the reference compound J-113397. *In vivo*, Trap-101 changed motor activity in naive rats and mice and alleviated parkinsonism in 6-hydroxydopamine hemilesioned rats: Trap-101 stimulated motor activity at 10 mg/kg and inhibited it at 30 mg/kg (Marti *et al.*, 2008). Trap-101 could be use as a novel template for a structure-activity study aimed at establishing the importance of both the C3 hydroxymethyl function and of the benzimidazolidinone nitrogen substituent.

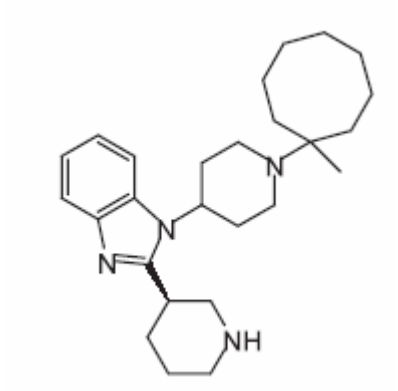


J-113397 **Trap-101**

Figure 8. Structures of non-peptide NOP antagonist J-113397 and Trap-101.

Pfizer has also reported a new series of benzimidazoles as NOP agonists, among them PCPB and MCOPPB (Figure 9). PCPB bound to the NOP receptor in mouse brain membranes (K_i = 0.12 nM) and to recombinant human NOP receptor (K_i = 2.1 nM). Orally administered PCPB (30 mg/kg) exhibited anxiolytic activity in mice subjected to the Vogel conflict test that was comparable to the maximal response induced by diazepam (Hirao *et al.*, 2008a). MCOPPB showed a high affinity for the human NOP receptor (pK_i = 10.07) and selectivity for the NOP receptor over other members of the opioid receptor family: 12, 270 and >1000 fold more selective for the NOP receptor than for the MOP, KOP, and DOP receptors, respectively; *in vivo* MCOPPB (10 mg/kg, p.o.) elicited anxiolytic-like effects in mice without affecting locomotor activity or memory. On the other hand, the benzodiazepine-type anxiolytic agent diazepam caused memory deficits (Hirao *et al.*, 2008b).

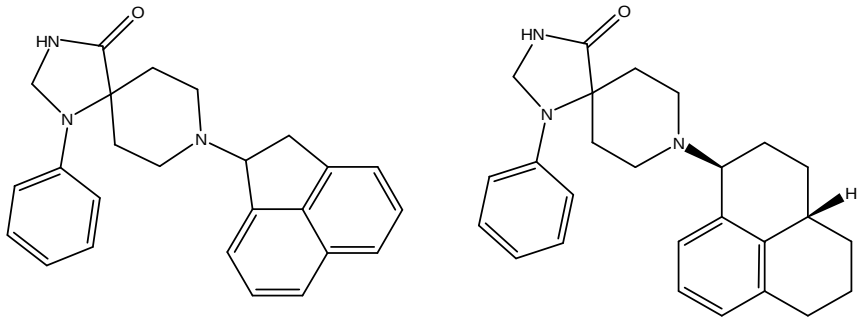




PCPB **MCOPPB**

Figure 9. Structures of non-peptide NOP agonist PCPB and MCOPPB.

iii) *Spiropiperidines*: Hoffmann La Roche disclosed a series of 1,3,8-triazaspiro[4,5]decan-4-ones, discovered through high throughput screening. Among them Ro 65-6570 and Ro 64-6198 were two of those ligands widely used as pharmacological tools (Adam *et al.*, 1998) (Figure 10). Although Ro 65-6570 was found to show anxiolytic effects (Wichmann *et al.*, 1999), it was only 5 to 10-fold selective over opioid receptors (Hashiba *et al.*, 2001). Ro 64-6198, on the other hand, is far more selective and has shown an impressive anxiolytic profile comparable to benzodiazepines, in several *in vivo* anxiety paradigms (Jenck *et al.*, 2000; Le Pen *et al.*, 2002). As an agonist only slightly less potent than N/OFQ itself (Hashiba *et al.*, 2002b), Ro 64-6198 can potentially be used as a therapeutic agent in disorders where a NOP agonist may prove beneficial, such as anorexia (Ciccocioppo *et al.*, 2002), anxiety (Jenck *et al.*, 2000), and inhibition of drug reward pathways (Dautzenberg *et al.*, 2001). However, Ro 64-6198 was found not to affect cocaine-induced conditioned place preference (Kotlinska *et al.*, 2003). Moreover, at higher doses, Ro 64-6198 was found to have affinity for dopamine and sigma receptors (Jenck *et al.*, 2000) and increased alcohol drinking in genetically selected alcohol-preferring Marchigian Sardinian rats while other NOP agonists such as UFP-102 and UFP-112 reduce alcohol drinking; an effect probably induced by residual agonist activity of this compound at MOP receptors (Economidou *et al.*, 2006a). For review see Shoblock (2007).



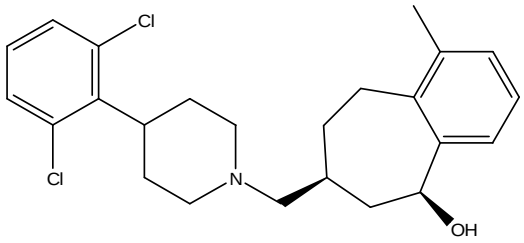
Ro 65-6570 **Ro 64-6198**

Figure 10. Structures of Hofmann-La Roche lead compounds Ro 65-6570 and Ro 64-6198.

Interestingly, Novo Nordisk has also reported on the synthesis and characterization of 1,3,8-triazaspirodecanones, similar to the Roche compounds, starting with spiroxatrine as their lead (Thomsen *et al.*, 2000a). Their best ligand, NNC 63-0532 had binding affinity of 6.3 nM against human NOP but only a 12-fold selectivity for NOP over classical opioid receptors. This low selectivity was also seen in electrically stimulated mouse vas deferens where NNC 63-0532 produced a concentration-dependent inhibition of the electrically induced twitches showing, in comparison with N/OFQ, lower potency and higher maximal effects. In addition, contrary to N/OFQ, the effects of NNC 63-0532 were insensitive to the NOP selective antagonist UFP-101 but were prevented by naloxone (Guerrini *et al.*, 2004). Recently it was seen that NNC 63-0532 (0.01 nM-10 µM) like N/OFQ induces a concentration-dependent endocytosis and recycling of the N/OFQ receptor. This mechanism contributes to maintain receptor signaling as it counteracts desensitization development and enhances a compensatory upregulation of adenylyl cyclase activity (Spampinato *et al.*, 2006).

iv) *Aryl piperidines*: Designing compounds in this group led several pharmaceutical companies (Schering Plough, Roche etc) to obtain patents. Recently SB-612111 was patented by GlaxoSmithKline (Zaratin *et al.*, 2004) (Figure 11). The results describe SB-612111 as a high affinity and broadly selective NOP receptor antagonist *in vitro* and *in vivo*. Furthermore SB-612111 can resensitize mice to morphine in animals which had been chronically treated with opiate, suggesting utility of this class of NOP receptor antagonist in prolonging the analgesic action of morphine (Zaratin *et al.*, 2004). SB-612111 was synthesized by our laboratories and investigated *in vitro* and *in vivo*. *In vitro* SB-612111 displayed subnanomolar affinity for the NOP receptor and high selectivity over classical opioid receptors (Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004).

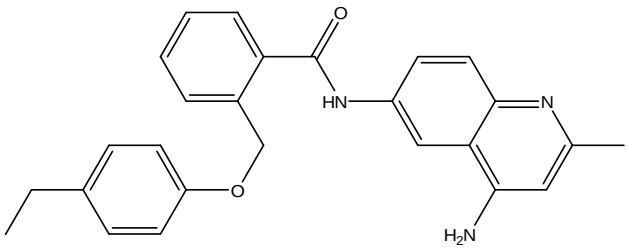
Functional studies ([^{35}S]GTPγS binding and cAMP accumulation) in CHO cells expressing the human NOP receptor demonstrated pure, competitive and high potency antagonism exerted by this molecule against N/OFQ (pK_B value of 9.70 and 8.63 in the [^{35}S]GTPγS binding and cAMP accumulation experiments, respectively (Spagnolo *et al.*, 2007). In isolated peripheral tissues of mice, rats, and guinea pigs and in mouse cerebral cortex synaptosomes preloaded with [^{3}H]5-HT, SB-612111 competitively antagonized the inhibitory effects of N/OFQ, with pA_2 values in the range of 8.20 to 8.50 (Spagnolo *et al.*, 2007). *In vivo*, in the mouse tail withdrawal assay, SB-612111 given i.p. up to 3 mg/kg prevented the pronociceptive and the antinociceptive action of 1 nmol of N/OFQ given i.c.v. and i.t., respectively (Rizzi *et al.*, 2007a). In food intake studies performed in sated mice, SB-612111 (1 mg/kg i.p.) had no effect on food consumption but fully prevented the orexigenic effect of 1 nmol of N/OFQ i.c.v. (Rizzi *et al.*, 2007a). In the mouse forced swimming and tail suspension tests, SB-612111 (1-10 mg/kg) reduced immobility time. The antidepressant-like effect elicited by SB-612111 in the forced swimming test was reversed by the i.c.v. injection of 1 nmol of N/OFQ and was no longer evident in mice knockout for the NOP receptor gene (Rizzi *et al.*, 2007a). In conclusion, SB-612111 is among the most potent and NOP-selective non-peptide antagonists identified to date.



SB-612111

Figure 11. Structure of SB-612111.

v) *4-Aminoquinolines:* These are an entirely novel chemical class of NOP ligands disclosed by Japan Tobacco Inc. in a patent (Shinkai *et al.*, 2000). The optimized ligand, JTC-801 (Figure 12), was obtained through an extensive structure-activity study of lead compound 25. Detailed pharmacological studies with JTC-801 were recently reported (Yamada *et al.*, 2002). Its binding affinity for hNOP was 44.5 nM. It completely antagonized the inhibition of cAMP accumulation by N/OFQ. Furthermore, when administered *in vivo* orally or i.v., at doses of 0.1-1 mg/kg, it antagonized N/OFQ induced allodynia in mice and increased latency in the mouse hot plate test. These effects were not inhibited by naloxone.

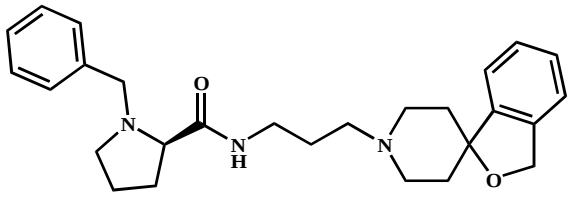


JTC-801

Figure 12. Structure of JTC-801, a novel Japan Tobacco Inc compound.

JTC-801 was chosen as a candidate for clinical trials for analgesia because of its oral bioavailability profile, which was more favourable than that of some other more potent analogs in this series. It was seen that JTC-801 alleviates heat-evoked hyperalgesia in chronic constriction injury rats (Suyama *et al.*, 2003).

vi) *N-benzyl-D-proline*: Recently Banyu Pharmaceuticals discovered a novel class of NOP antagonists using a focused library approach starting from a moderately active hit compound found in their chemical collection. The N-benzyl-D-proline analogue (Compound 24) (Figure 13) showed significantly improved antagonistic activity when compared with other reported NOP antagonists and showed good brain penetrability and *in vivo* antagonistic activity (Goto *et al.*, 2006).



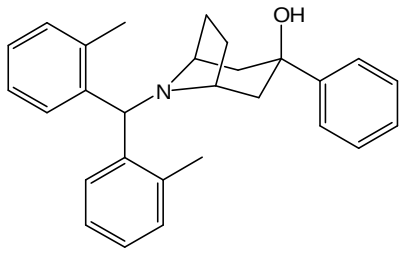
Compound 24

Figure 13. Structure of Compound 24, a novel Banyu Pharmaceuticals compound.

Based on this novel antagonist structure we performed a structure activity analysis of Compound 24, focusing on its N-benzyl-D-proline, amide bond and benzoisofurane moieties; this latter structure was substituted with moieties taken from known non-peptide NOP ligands such as Ro 64-6198, SB-612111 and J-113397. Twelve new derivates were synthesized and evaluated for

their ability to bind the human recombinant NOP receptor. The molecule showing the highest affinity (Compound 35) has been further characterized. The pharmacological characterization of both Compound 24 and Compound 35 is one of the major goals of this thesis.

Finally, there is a recent description of a 4-aryl-tropane NOP agonist, SCH 221510 (Varty *et al.*, 2008) a new molecule discovered by Schering-Plough (Figure 14). SCH 221510 binds with high affinity (K_i 0.3 nM) to the NOP receptor and was shown to be anxiolytic with a reduced side-effect profile when compared with benzodiazepines (Varty *et al.*, 2008).



SCH 221510

Figure 14. Structure of SCH 221510.

Table 2. NOP receptor ligands available to date.

	N/OFQ-RELATED PEPTIDE ANALOGUES	NON-PEPTIDES
AGONISTS	♦ N/OFQ ♦ N/OFQ-NH_2 ♦ [Tyr^1]N/OFQ ♦ N/OFQ(1-13)-NH_2 ♦ [$Arg^{14}Lys^{15}$]N/OFQ ♦ [(pF)Phe^4]N/OFQ(1-13)-NH_2 ♦ [(pF)$Phe^4Arg^{14}Lys^{15}$]N/OFQ-NH_2 *(UFP-102)* ♦ [(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ *(UFP-112)* ♦ OS-461, OS-462, OS-500	♦ Ro 65-6570 ♦ NNC-63-0532 ♦ Ro 64-6198 ♦ SCH 221510 ♦ PCPB ♦ MCOPPB
PARTIAL AGONISTS	♦ [$Phe^1\psi(CH_2\text{-}NH)\text{-}Gly^2$]N/OFQ(1-13)-$NH_2$ ♦ [$Phe^1\psi(CH_2NH)Gly^2$(pF)$Phe^4Arg^{14}Lys^{15}$]N/OFQ-NH_2 *(UFP-103)* ♦ [$Phe^1\psi(CH_2NH)Gly^2$(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ *(UFP-113)*	
ANTAGONISTS	♦ [$Nphe^1$]N/OFQ(1-13)-NH_2 ♦ [$Nphe^1Arg^{14}Lys^{15}$]N/OFQ-NH_2 *(UFP-101)* ♦ [$Nphe^1$(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ *(UFP-111)*	♦ NalBzoH ♦ J-113397 ♦ JTC-801 ♦ SB-612111 ♦ Trap-101 ♦ Compound 24

1.9 Aims

The main objectives of this work have been to:

- Produce a detailed characterization of the pharmacological profile of NOP receptors coupled with calcium signalling via the chimeric protein $G\alpha_{qi5}$ using a large panel of full and partial agonists as well as pure antagonists. $[Ca^{2+}]_i$ levels were monitored using the fluorometer FlexStation II. This has the potential to provide a rapid screening tool for novel NOP ligands.
- Further investigate the pharmacological profile of ZP120 using mouse and rat preparations, J-113397 and UFP-101, as well as NOP(-/-) mice.
- Fully characterize the pharmacological profile of the novel non-peptide NOP antagonist Compound 24.
- Screen a new series of non-peptide ligands deriving from SAR studies on the potent antagonist Compound 24. Compound 35, the best compound of this series, has been further investigated.

To pharmacologically characterize these molecules, *in vitro* studies on N/OFQ-sensitive isolated tissues from different species and on transfected cells expressing the recombinant NOP and classical opioid receptors, were performed. In addition, *in vitro* experiments were also assessed in CHO cells co-expressing the recombinant NOP and classical opioid receptors and the chimeric protein $G\alpha_{qi5.}$ Moreover, some compounds were studied using an *in vivo* assay in mice, the tail withdrawal assay. Some *in vitro* and *in vivo* experiments were performed using NOP(+/+) and NOP(-/-) mice.

2. MATERIALS & METHODS

2.1 Drugs and reagents

The peptides used in this study were synthesized in the laboratory of Prof Salvadori (Department of Pharmaceutical Sciences and Biotechnology Centre, University of Ferrara) using standard solid-phase synthesis techniques and purified using High Pressure Liquid Chromatography, according to previously published methods (Guerrini *et al.*, 1997). Amino acids, protected amino acids, and chemicals were purchased from Bachem, Novabiochem, Fluka (Switzerland) or Chem-Impex International (U.S.A).

The compound [F/G]N/OFQ(1-13)-NH_2 was prepared as described by Guerrini *et al.* (2003), [Nphe^1]N/OFQ(1-13)-NH_2 was obtained by the shift of the side chain of Phe^1 from the C to the N atom in the template N/OFQ(1-13)-NH_2. NNC 63-0532-COOH was obtained following literature methods (Watson *et al.*, 1999) and UFP-111, UFP-112 and UFP-113 were prepared as previously described (Arduin *et al.*, 2007). Ac-RYYRIK-NH_2, Ac-RYYRIK-ol and all the other modified hexapeptides were synthesized accordingly to Kocsis *et al.* (2004) in the laboratory of Dr Anna Magyar (Research Group of Peptide Chemistry, Eötvös Loránd University, Budapest, Hungary) using solid-phase peptide synthesis techniques.

ZP120 was provided by Zealand Pharma (Copenhagen, Denmark), [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and dynorphin A were purchased from NeoMPS (Strasbourg, France).

Ro 64-6198 was provided by Dr. Wichmann (Hoffmann-La Roche, Basel, Switzerland). The non-peptide compound Compound 24 was synthesized following the procedures described in detail by Goto *et al.* (2006).

J-113397 was prepared as a racemic mixture, according to De Risi *et al.* (2001), Trap-101 is obtained through treatment with $LiAlH_4$ on a common intermediate from the J-113397 synthesis. The compound SB-612111 was synthesized following the experimental conditions and protocols described in detail in the patent (WO 03/040099 A1) by Palombi *et al.* (2003).

Captopril, amastatin, bestatin, phosphoramidon, naloxone, bovine serum albumin (BSA), guanosine 5'-O-(3-thiotri-phosphate) (GTPγS), GDP, unlabelled GTPγS, bacitracin and probenecid were from Sigma Chemical Co. (Poole, U.K.) or E. Merck (Darmstadt, Germany). All tissue culture media and supplements were from Invitrogen (Paisley, U.K.). [^{35}S]GTPγS (1250 Ci mmol^{-1}), [^{3}H]Diprenorphine ([^{3}H]DPN, 75-133 Ci/mmol) were from Perkin Elmer Life Sciences (Boston, Mass., USA) and [*leucyl*-^{3}H]N/OFQ ([^{3}H]-N/OFQ, 150 Ci/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other consumables and reagents were of the highest purity available.

For *in vitro* experiments, the peptides were solubilized in H_2O and stock solutions (1 mM or 2 mM) were stored at -20 °C until use; the non-peptide compounds were solubilized in dimethyl sulfoxide at a final concentration of 10 mM, and the successive dilutions were made in saline or water, stock solutions were kept at -20 °C until use. For *in vivo* studies, Compound 24 was dissolved in 2% DMSO and 10% encapsin (hydroxypropyl-beta-cyclodextrin) just before performing the experiment.

2.2 Buffer composition

- *Tissue culture buffers:*

  Harvest: HEPES (10 mM), EDTA (1.1 mM), NaCl (154 mM), pH 7.4 with NaOH.

- *[^{35}S]GTPγS buffers:*

  [^{35}S]GTPγS Homogenising: Tris-HCl (50 mM), EGTA (0.2 mM), pH 7.4 with NaOH.

  [^{35}S]GTPγS Assay: Tris-HCl (50 mM), EGTA (0.2 mM), NaCl (100 mM), $MgCl_2$ (1 mM), pH 7.4 with NaOH.

- *[^{3}H]-N/OFQ competition buffers:*

  Wash/Homogenising: Tris-HCl (50 mM), $MgSO_4$ (5 mM), pH 7.4 with KOH.

  Assay: Tris-HCl (50 mM), $MgSO_4$ (5 mM), pH 7.4 with KOH, 0.5% BSA.

- *[^{3}H]-Diprenorphine competition buffers:*

  Wash/Homogenising: Tris-HCl (50 mM), pH 7.4 with KOH.

  Assay: Tris-HCl (50 mM), pH 7.4 with KOH, 0.5% BSA.

- *Calcium mobilization assay buffers:*

  HBSS (Hanks' Balanced Salt Solution): KCl (5.4 mM), KH_2PO_4 (0.44 mM), NaCl (137 mM), $NaHCO_3$ (4.2 mM), Na_2HPO_4 x 7 H_2O (0.25 mM), $CaCl_2$ (1.3 mM), $MgSO_4$ x 7 H_2O (1 mM), glucose (5 mM).

  Loading solution: cell culture medium, HEPES (20 mM), probenecid (2.5 mM), calcium-sensitive fluorescent dye Fluo 4AM (3 µM), pluronic acid (0.01%).

  Dye loading solution: HBSS, HEPES (20 mM), probenecid (2.5 mM), Brilliant Black (500 µM).

- *Bioassay buffers composition:*

  The tissues were suspended in the following Krebs solution (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. For the experiments on the mouse vas deferens and rat vas deferens, a Mg^{++}-free and 1.8 mM $CaCl_2$ Krebs solution were used, respectively. For the experiments on guinea pig ileum the normal medium was added with hexamethonium (0.0008%) and benadryl (0.00001%).

2.3 In vitro studies

CHO expressing the recombinant NOP/DOP/MOP/KOP receptors

2.3.1 Cell harvesting and membranes preparation

Chinese Hamster Ovary cells (CHO) stably expressing the human NOP receptor (CHO_{hNOP}) cells were cultured consisting of Dulbecco's MEM/HAMS F12 (50/50) supplemented with 5% foetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml), geneticin (G418; 200 μg/ml) and hygromycin B (200 μg/ml) at 37˚C in 5% CO_2/humidified air. CHO cell stocks expressing the human DOP/MOP/KOP receptors ($CHO_{MOP/DOP/KOP}$) were maintained in Ham F12 containing 10% FCS, 100 IU/ml P, 100 μg/ml S and 400 μg/ml G418, for CHO non-transfected cells G-418 and hygromycin B were omitted. Cell cultures were kept at 37˚C in 5% CO_2/humidified air. In all cases experimental cultures were free from selection agents (hygromycin B, G418). When confluence was reached (3-4 days), cells were sub-cultured as required using trypsin//EDTA and used for experimentation. Cells were harvested from sterile tissue culture flasks using harvest buffer and gentle agitation. Cells were suspended in either wash buffer (displacement assay) or homogenising buffer (GTPγ[^{35}S] assay), homogenised using an Ultra Turrax, for 10 seconds followed by 6 consecutive 1-second bursts. The homogenate was then centrifuged at 13,500 rpm for 10 min at 4°C, this was carried out a total of three times. The membrane fraction was resuspended in an appropriate volume of assay buffer and the total protein content determined as set out below.

2.3.2 Displacement binding assay

5 μg protein of CHO_{hNOP} homogenate were assayed in a total volume of 0.5 ml comprising competition homogenising buffer supplemented with 0.5% (w:v) BSA, 10 μM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon), 0.2 nM [*Leucyl*-^{3}H]N/OFQ and 100 nM – 0.1

pM of competing ligands. Non-specific binding (NSB) was determined in the presence of 1 μM N/OFQ. 50 μg (CHO_{hMOP}), 25 μg (CHO_{hDOP}) and 40 μg (CHO_{hKOP}) membrane protein were incubated in 0.5 ml homogenising buffer supplemented with 0.5% BSA, approximately 0.7 nM [^{3}H]Diprenorphine. NSB binding was defined in the presence of 10 μM naloxone. Reactions were incubated for 1 hour at room temperature and harvested under vacuum filtration using a Brandel cell harvester. Whatman GF/B filters were soaked in 0.5% polyethylenimine, to reduce NSB, and loaded onto the harvester wet. Radioactivity was determined following filter extraction (8 hours, Optiphase Safe) using liquid scintillation spectroscopy.

2.3.3 [^{35}S]GTPγS stimulation binding assay

Experimentation was performed essentially as described by Berger *et al.* (2000b). Freshly prepared CHO_{hNOP} membranes (20 μg) were incubated in 0.5 ml volumes of buffer consisting Tris (50 mM), EGTA (0.2 mM), GDP (100 μM), bacitracin (0.15 mM), BSA (1 mg/ml), peptidase inhibitors (amastatin, bestatin, captopril, phosphoramidon; 10 μM), [^{35}S]GTPγS (~150 pM) and ligands in the concentration range of 10^{-12} – 10^{-5} M. NSB was determined in the presence of 10 μM unlabelled GTPγS. Assays were incubated for 1 h at 30^{o}C with gentle shaking and bound and free radiolabel were separated by vacuum filtration onto Whatman GF/B filters. Polyethylenimine was not used. In all cases radioactivity was determined following filter extraction (8 hours) using liquid scintillation spectroscopy.

2.3.4 Protein assay

The protein concentration was determined for membrane fractions using the method of Lowry (Lowry *et al.*, 1951): BSA protein standards at set concentrations of 0, 50, 100, 150, 200, 250 μg protein/ml were made up in 0.1 M NaOH. Samples of unknown protein concentration were diluted in 0.1 M NaOH. 0.5ml volumes of standards and samples were incubated for 10 min in 2.5 ml of solution consisting of, A ($NaHCO_3$ in 0.1 M NaOH) B (1% $CuSO_4$) and C (2% Na^+ K^+ tartrate) mixed to the ratio 100:1:1. Folin's reagent (diluted 1:4 in dH_2O) was then added and incubated at room temperature for a further 30min. The absorbance at 750 nm for standards and samples was then determined using a spectrophotometer. Linear regression of the known BSA protein concentrations was used to produce a standard curve (Figure 15) from which sample protein concentrations were determined.

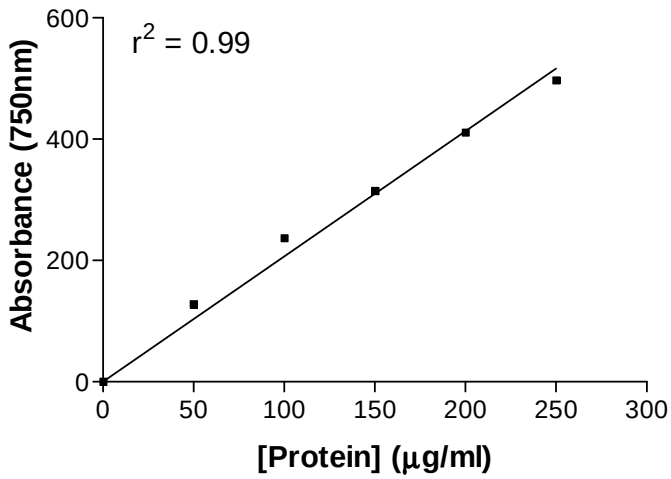


Figure 15. Example of protein assay standard curve used to determine the protein mass of unknown samples.

Calcium mobilization assay

2.3.5 Experimental protocols

CHO cell lines stably co-expressing MOP/DOP/KOP/ NOP receptors and the C-terminally modified $G\alpha_{qi5}$ were generated as described by Camarda *et al.* (2009). CHO_{hMOP}, CHO_{hDOP}, CHO_{hKOP} and CHO_{hNOP} stably expressing the $G\alpha_{qi5}$ protein were seeded at a density of 40,000 cells/well into 96-well black, clear-bottom plates. After 24 hours incubation the cells were loaded with medium supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM and 0.01% pluronic acid, for 30 min at 37 °C (Figure 16). Afterwards the loading solution was aspirated and 100 µl/well of assay buffer: HBSS buffer supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 µM Brilliant Black was added. Stock solutions (1 mM) of ligands were made in distilled water and stored at -20 °C. Serial dilutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured at room temperature. On-line additions were carried out in a volume of 50 µl/well.

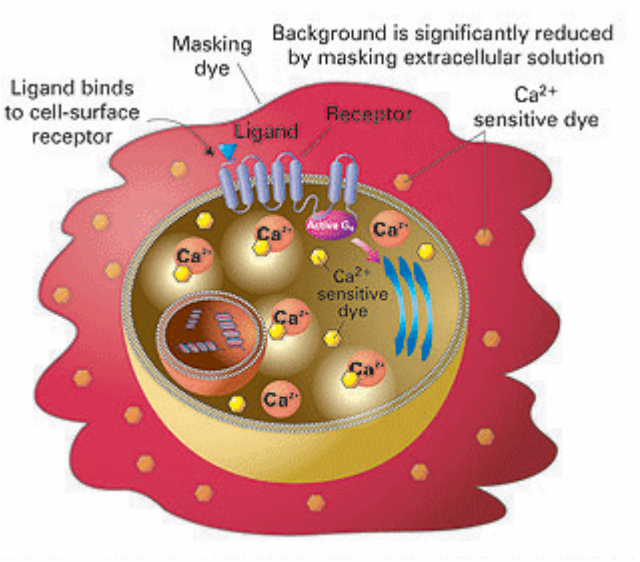


Figure 16. Diagram depicting the experimental protocol of the calcium mobilization assay. Cells are incubated with Fluo-4 AM, de-esterification of the ester group (AM) traps the dye in the cells and further leakage of the dye is prevented by blockage of organic anion-transport inhibitors using probenecid. Background fluorescence is reduced by addition of Brilliant Black dye which blocks extracellular signalling from any leaked Fluo-4.

2.3.6 Cell counting

Accurate numbers in a cell suspension can be calculated by counting the cells in a cell counting chamber (Burker's chamber, Figure 17). A small volume of the cell suspension (10 µl) was pipetted onto the chamber, the capillary action under the cover slip will draw the suspension into the counting chamber. The space between the cover slip and the counting chamber ensures a specific volume of cell suspension is present.

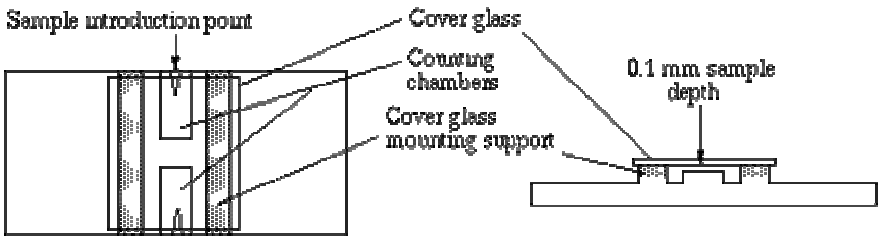


Figure 17. Burker's chamber.

Under a microscope the number of cells in diagonally opposite counting areas were counted, Figure 18.

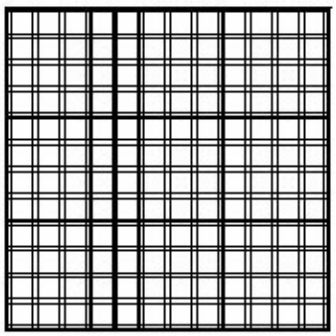


Figure 18. Schematic representation of the counting grid of the Burker's chamber.

The Burker's chamber is formed of 3x3 major squares, each of these major squares is subdivided into a grid of 4x4 squares. The number of cells present in three major cells are counted, cells in contact with two of the squares sides are included and the average taken. The volume of a major square is 0.1 mm^3 which is equal to 0.0001 ml. To determine the number of cells per ml the average number of cells determined is increased by a factor of 10^4.

2.3.7 Instruments

$[Ca^{2+}]_i$ levels were monitored using a FlexStation II fluorimeter (Figure 19). The FlexStation II system includes:

- Xenon-lamp light source
- Automatic eight-channel pipettor
- Tip rack drawer
- Compound plate drawer
- Reading chamber drawer

The Xenon-lamp light source and dual monochromators permit the use of essentially all dual-wavelength dyes for functional cellular assays.

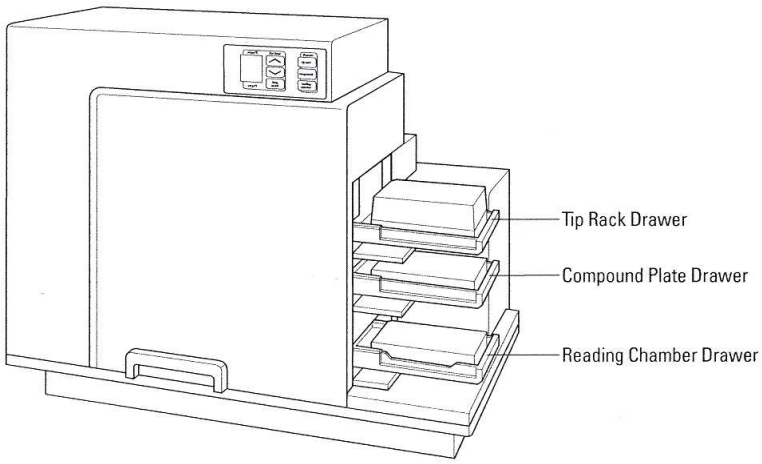


Figure 19. Diagram of FlexStation II used for calcium mobilization assay.

Isolated tissues

The *in vitro* experiments were performed on mouse vas deferens (mVD), rat vas deferens (rVD) and guinea pig ileum (gpI). The animals (Morini, Reggioemilia, Italy) were handled according to guidelines published in the European Communities Council directives (86/609/EEC), National regulation (D.L 116/92). They were housed in 425 x 266 x 155 mm cages (Techniplast, Milan, Italy), fifteen animals/cage, under standard conditions (22ºC, 55 % humidity, 12-h light/dark cycle, light on at 7:00 am) with food (MIL, standard diet; Morini, Reggio Emilia, Italy) and water *ad libitum*.

2.3.8 Tissue preparation

Tissues were taken from male Swiss mice (25-30 g), guinea pigs (300-350 g) and Sprague Dowley rats (300-350 g). On the day of the experiments the animals were killed by a lethal injection of urethane. From the mouse and rat the prostatic portion of the vas deferens was isolated, and prepared according to Hughes *et al.* (1975) and Schulz *et al.* (1979), respectively; from the guinea pig segments of ileum (1.5-2 cm in length) were taken as described by Paton (1957). The tissues were suspended in 5 ml organ baths containing heated Krebs solution oxygenated with 95% O_2 and 5% CO_2 (pH 7.4). The temperature was set at 33 °C for the mVD and at 37 °C for the other tissues (Hughes *et al.*, 1975; Paton, 1957; Schulz *et al.*, 1979). A resting tension of 0.3 g was applied to the mVD, 1 g to the gpI and rVD.

2.3.9 Experimental protocols

The mVD, gpI and rVD were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 msec duration and 0.05 Hz frequency. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006; UgoBasile s.r.l., Varese, Italy). After an equilibration period of about 60 min the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration-response curves to N/OFQ, N/OFQ related peptides, or to opioid ligands were performed (0.5 log unit steps). The concentration-response curve consists of progressive administration of increasing concentrations of peptide without changing the Krebs solution in which the tissue is bathed; the injection of a certain concentration of ligand must be done only when the previous concentration produced a stable effect (plateau). About 1 hour with 3 changes of Krebs solution (wash out) is needed to the tissue to recover the original twitch.

When required, in all the preparations described above, the NOP receptor antagonists, at adequate concentrations, were added to the medium 15 min before performing the challenge with agonists.

2.3.10 Instruments

For the *in vitro* bioassays two chamber-glass bathes for isolated organs were utilized (Figure 20). The outer chamber contains water heated at 33 or 37 °C, while the inner chamber contains 5 ml of oxygenated Krebs solution. One end of the tissue is fixed to the bottom side of the inner chamber and the other end is linked to a force transducer by a surgery thread. The role of the transducer is to convert the mechanical signal in electrical signal, then amplified and recorded with a PC-based acquisition system Power Lab 4/25 (model ML845, ADInstrument, USA).

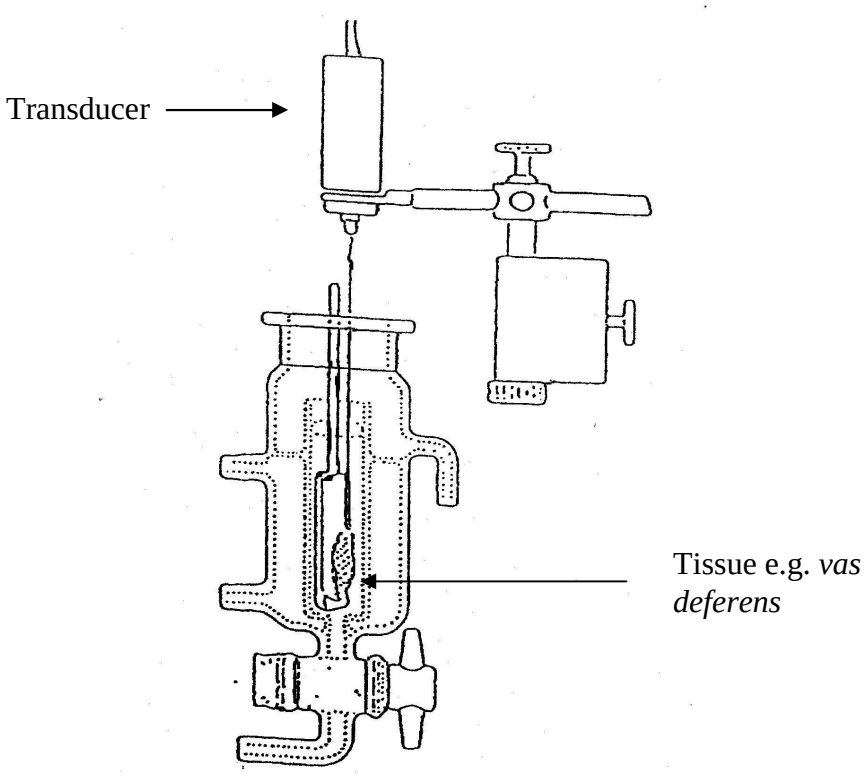


Figure 20. Diagram of the tissue chamber used for isolated tissues assays.

2.4 In vivo studies

Experimental protocol

2.4.1 Animals

Male Swiss and male CD1/C57-BL6J/129 NOP(+/+) and NOP(-/-) mice weighing 20-25 g were used. All transgenic animals were genotyped by PCR. Details of the generation and breeding of mutant mice have been published previously (Gavioli *et al.*, 2003). The animals were handled as described above in the isolated tissues section.

I.c.v. injections (2 µl/mouse) were given, under light isofluoran anesthesia (just sufficient to produce a loss of the righting reflex), in the left ventricule according to the procedure described by Laursen and Belknap (Laursen *et al.*, 1986). Briefly, the syringe was held at an approximate 45° angle to the skull. Bregma was found by lightly rubbing the point of the needle over the skull until the suture was felt. Once found, care was taken to maintain the approximate 45° angle and the needle was inserted about 2 mm lateral to the midline. The skull is relatively thin at this point, so only mild pressure was required to insert and remove the needle. The solutions of drugs were then injected slowly (2 µl in about 20 s).

I.t. injections (5 µl/mouse) were adapted according to the method of Hylden and Wilcox (Hylden *et al.*, 1980). A 28-gauge stainless steel needle attached to a 50 µl 65 Hamilton microsyringe was inserted, with an angle of about 20° in the spinal subarachnoid space between the L5 and L6 segments in mice. 1-3 hours before the i.t. injection a caudal cutaneous incision was performed under isofluoran anesthesia.

I.p. injections 100 µl/mouse of non-peptide compounds were given 30 min before N/OFQ administration.

2.4.2 Tail withdrawal assay

All experiments were started at 10.00 a.m. and performed according to the procedure previously described in detail (Calo *et al.*, 1998b). Briefly, the mice were placed in a holder and the distal half of the tail was immersed in water at 48 °C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut off time of 20 s was chosen to avoid tissue damage. For each experiment four mice were randomly assigned to each experimental group, and the experiment was repeated at least four times: therefore each experimental point is the mean of the results obtained in $\geq$16 mice.

Tail-withdrawal latency was determined immediately before and 5, 15, 30, and 60 min after i.c.v. or i.t. injection of saline (control) or N/OFQ (1 nmole). Compound 24 (10 mg/kg) was given

i.p. 30 min before N/OFQ administration. Increased and decreased tail withdrawal latencies compared with baseline indicated antinociceptive and pronociceptive effects, respectively.

2.5 Data analysis and terminology

All data are expressed as means ± standard error of the mean (s.e.m.) of *n* experiments. For potency values 95% confidence limits were indicated. Data have been statistically analyzed with the Student's *t* test for unpaired data or one way ANOVA followed by the Dunnett's test, as specified in table and figure legends; p values less than 0.05 were considered to be significant. The pharmacological terminology adopted in this manuscript is consistent with the IUPHAR recommendations (Neubig *et al.*, 2003).

[^{35}S]GTPγS data are expressed as a stimulation factor i.e. the ratio between agonist-stimulated [^{35}S]GTPγS specific (minus NSB) binding and basal specific binding. Receptor binding data are expressed as pK_i derived from the Cheng and Prusoff (Cheng *et al.*, 1973) equation:

$$K_i = IC_{50} / (1+([R]/K_D))$$

where IC_{50} is the concentration of the competitor producing 50 % displacement, [R] is the concentration of the radiolabel and K_D is the radiolabel affinity for the receptor under investigation. The [^{3}H]N/OFQ K_D was 83 pM while those of [^{3}H]Diprenorphine were 125, 323, and 134 pM (in-house laboratory values) at MOP, DOP, and KOP, respectively. pK_i is the antilogarithm of the K_i values obtained after the calculations.

Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline. Isolated tissue data are expressed as percent of the twitch induced by electrical field stimulation.

Agonist potencies are given as pEC_{50} = the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. Concentration response curve to agonists were fitted with the following equation:

$$\text{Effect} = \text{baseline} + (E_{max}\text{-baseline})/(1+10\text{^}((\text{Log}EC_{50} - X)*\text{HillSlope}))$$

where X is the agonist concentration.

The E_{max} is the maximal effect that an agonist can elicit in a given tissue. In the electrically stimulated tissues, the E_{max} of agonists is expressed as % of inhibition of the control twitch.

Antagonist potencies are expressed in terms of pA_2. pA_2 is the negative logarithm to base 10 of the antagonist molar concentration that makes it necessary to double the agonist concentration to elicit the original response (Schild, 1997). The pA_2 values are calculated using Schild's linear regression, that correlates the log of concentrations of antagonists (x axis) to the log of (CR-1) (y axis), where CR is the ratio between the EC_{50} (nM) values of agonist, in the presence and in absence of antagonist. The value of x for y=0 represents the pA_2 value, and the slope not significantly different from the unity means that the antagonist is competitive. When one single concentration of antagonist is utilized, the pK_B value is calculated with the Gaddum Schild equation:

$$K_B = ((CR - 1)/[antagonist])$$

assuming a slope equal to unity. Curve fitting was performed using PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.)

For calcium mobilization experiments pK_B values were derived from inhibition response curves using the following equation:

$$K_B = IC_{50}/([2 + ([A]/EC_{50})^n]^{1/n} - 1)$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the Hill coefficient of the concentration response curve to the agonist (Kenakin, 2004). In addition the pK_B for Compound 24 evaluated at different concentrations against the concentration response curve to N/OFQ was calculated using the following equation:

$$K_B = [antagonist]/(slope - 1)$$

where slope is calculated from a double-reciprocal plot of equieffective concentrations of agonist in the absence and presence of antagonist (Kenakin, 2004).

For *in vivo* studies, raw data from tail withdrawal experiments were converted to the area under the time versus tail withdrawal latency curve (AUC min/s), as described by Grisel *et al.* (1996). For ZP120 experiments AUC data for the time interval 0-180 min was calculated and used for statistical analysis. For Compound 24 experiments AUC data for the time interval (0-15 and 0-30 min for i.c.v. and i.t. studies, respectively) was used for statistical analysis.

3. RESULTS & DISCUSSION

3.1 Pharmacological profile of NOP receptors coupled to calcium signalling via the chimeric protein $G\alpha_{qi5}$

Innovative drugs interacting selectively with the NOP receptor are under development in several companies. However target validation studies in this field are limited by the relative paucity of pharmacological tools which are the non-peptide agonist Ro 64-6198 (Jenck *et al.*, 2000) and the antagonists J-113397 (Ozaki *et al.*, 2000) and SB-612111 (Zaratin *et al.*, 2004), and a few peptide ligands including the antagonist UFP-101 (Calo *et al.*, 2002b), the partial agonists [F/G]N/OFQ(1-13)-NH_2 (Guerrini *et al.*, 1998), Ac-RYYRWK-NH_2 (Dooley *et al.*, 1997) and ZP120 (Rizzi *et al.*, 2002a), and the agonists UFP-102 (Carra *et al.*, 2005b) and UFP-112 (Rizzi *et al.*, 2007b) (for reviews see (Calo *et al.*, 2005; Chiou *et al.*, 2007; Lambert, 2008; Shoblock, 2007; Zaveri, 2003).

The identification of novel GPCR ligands are nowadays mainly based on the use of automated fluorometers and calcium dyes. To extend this approach to G_i coupled receptors several strategies have been developed including the use of chimeric G proteins (Conklin *et al.*, 1993; Kostenis *et al.*, 2005). This strategy has been validated with a large panel of G_i coupled receptors (Kostenis *et al.*, 2005) including classical opioid and the NOP receptor (Coward *et al.*, 1999). However, in the study by Coward *et al.* (1999) only N/OFQ has been used as NOP agonist and no receptor antagonists were tested. Thus, in the present study we performed a detailed characterization of the pharmacological profile of NOP receptors coupled to calcium signalling via the chimeric protein $G\alpha_{qi5}$ using a large panel of full and partial agonists as well as pure antagonists.

Results

Figure 21 (left panel) displays the response in terms of calcium mobilization of CHO_{hNOP} cells expressing the $G\alpha_{qi5}$ protein to increasing concentrations of N/OFQ: the peptide evoked immediate calcium transients in a concentration-dependent manner displaying high potency (pEC_{50} ≈ 9.5) and maximal effects (≈ 200% over the basal values). N/OFQ was found completely inactive up to 1 µM concentrations in wild type CHO stably expressing the $G\alpha_{qi5}$ protein. In CHO_{hNOP} cells (not expressing the $G\alpha_{qi5}$ protein) N/OFQ produced a modest stimulatory effect (≈ 50% over the basal values) only at high concentrations i.e. 0.1 and 1 µM. On the contrary ATP produced superimposable concentration response curves (pEC_{50} ≈ 6, E_{max} ≈ 250%) in the three cell lines (i.e. CHO_{hNOP}, CHO-$G\alpha_{qi5}$, and CHO_{hNOP}-$G\alpha_{qi5}$).

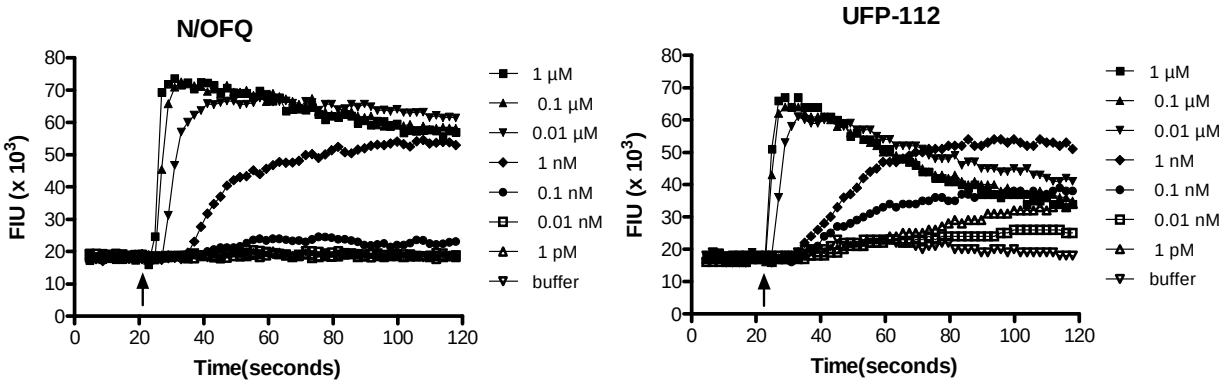


Figure 21. Raw data from a single representative experiment of the concentration response curve to N/OFQ (left panel) and to UFP-112 (right panel) in calcium mobilization experiments performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein.

In order to perform a detailed characterization of NOP receptors coupled to calcium signalling via the chimeric protein $G\alpha_{qi5}$ a large panel of NOP receptor agonists, partial agonists and antagonists were tested. The pharmacological activities of all these ligands are summarized in Table 3 in terms of pEC_{50} and E_{max} values for full and partial agonists and pK_B values for antagonists.

Table 3. Effects of N/OFQ and NOP receptor ligands in CHO cells coexpressing the $G\alpha_{qi5}$ chimeric protein and the human NOP receptor.

	Agonist		**Antagonist**
	pEC_{50} ($CL_{95\%}$)	**E_{max} ± s.e.m.**	**pK_B ($CL_{95\%}$)**
N/OFQ	9.54 (9.27-9.81)	167 ± 12%	-
N/OFQ(1-13)-NH_2	9.30 (8.84-9.76)	161 ± 12%	-
UFP-112	9.05 (8.39-9.71)	173 ± 29%	-
Ro 64-6198	7.98 (7.45-8.51)	178 ± 24%	-
NNC 63-0532	6.80 (6.60-7.00)	111 ± 23%	-
[F/G]N/OFQ(1-13)-NH_2	8.03 (7.45-8.61)	91 ± 16%	-
Ac-RYYRWK-NH_2	8.68 (8.07-9.29)	97 ± 34%	-
Ac-RYYRIK-NH_2	8.16 (7.80-8.52)	99 ± 22%	-
Ac-RYYRIK-ol	8.05 (7.45-8.65)	170± 23%	-
UFP-113	7.97 (7.38-8.56)	104 ± 29%	-
ZP120	7.15 (6.60-7.70)	97 ± 26%	-
UFP-101	Inactive		7.66 (7.06-8.26)
Trap-101	Inactive		7.93 (7.39-8.47)
J-113397	Inactive		7.88 (7.43-8.33)
[Nphe^1]N/OFQ(1-13)-NH_2	Inactive		6.29 (5.89-6.75)
SB-612111	Inactive		8.16 (7.55-8.77)
Naloxone	Inactive		Inactive

Data are means ± s.e.m. of 4 separate experiments made in duplicate.
Inactive: inactive up to 10 µM.

As shown in Figure 22, concentration response curves to NOP receptor agonists were performed and compared to that to N/OFQ. All the agonists tested evoked immediate calcium transients similar to those stimulated by N/OFQ (see as an example the raw data relative to an UFP-112 experiment in Figure 21 right panel). The peptides N/OFQ(1-13)-NH_2 and UFP-112 mimicked the effects of N/OFQ showing similar potency and maximal effects as the natural peptide. The non-peptide ligands Ro 64-6198 and NNC 63-0532 stimulated calcium levels in a concentration-

dependent manner with maximal effects similar to those of N/OFQ but approximately 30 and 500 fold lower potency.

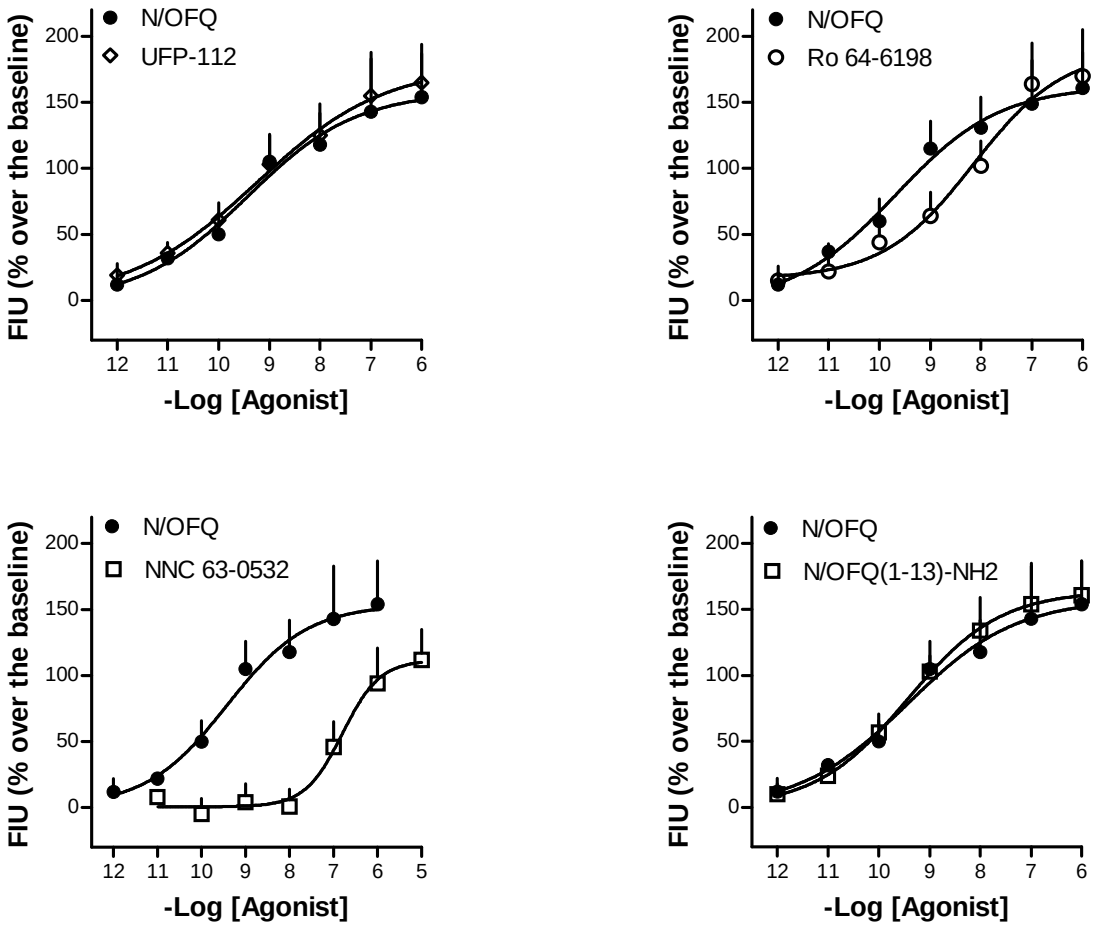


Figure 22. Concentration response curve to NOP receptor agonists in calcium mobilization experiments performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Ligand effects were expressed as % over the baseline. Data are the mean of 4 separate experiments performed in duplicate.

Under the same experimental conditions, the pharmacological profile of a series of NOP receptor partial agonists was investigated (Figure 23). Although the maximal effects elicited by these peptides were lower that those of N/OFQ these differences did not reach statistical significance (see the comparison of N/OFQ and NOP ligands maximal effects obtained in parallel experiments showed in Figure 22). Ac-RYYRWK-NH_2, Ac-RYYRIK-NH_2, Ac-RYYRIK-ol, [F/G]N/OFQ(1-13)-NH_2, and UFP-113 displayed approximately 30 fold lower potency than N/OFQ. ZP120 was found to be the weaker compound of the series; in fact, ZP120 was able to stimulate calcium levels only at very high concentrations i.e. 100 and 1000 nM.

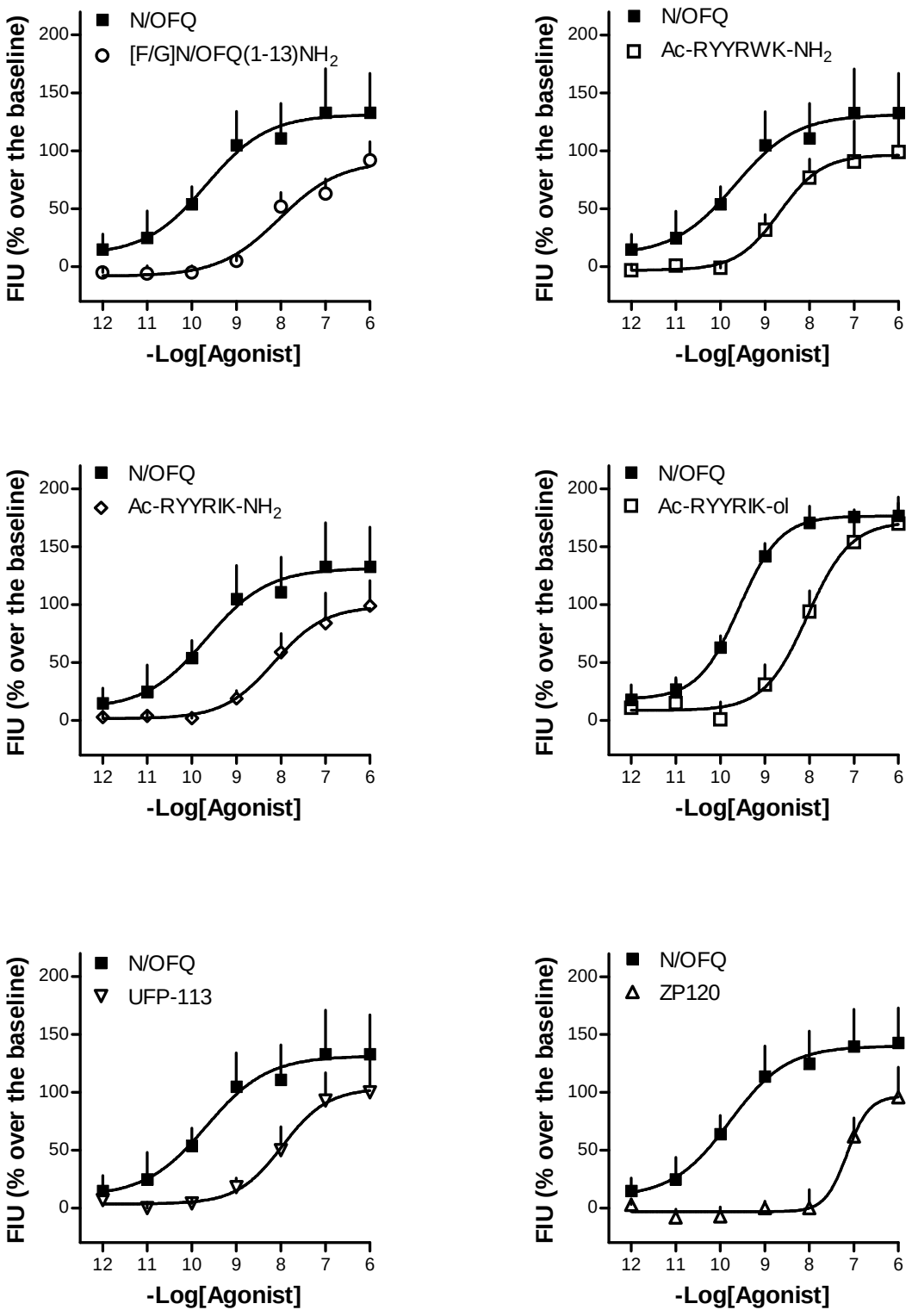


Figure 23. Concentration response curve to NOP receptor partial agonists in calcium mobilization experiments performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Ligand effects were expressed as % over the baseline. Data are the mean of 4 separate experiments performed in duplicate.

The effects of a panel of peptide and non-peptide antagonists have been also evaluated. Inhibition response experiments were performed by testing increasing concentrations of antagonists (10 pM – 10 µM) against a fixed concentration of N/OFQ (10 nM), approximately corresponding to the EC_{80}.

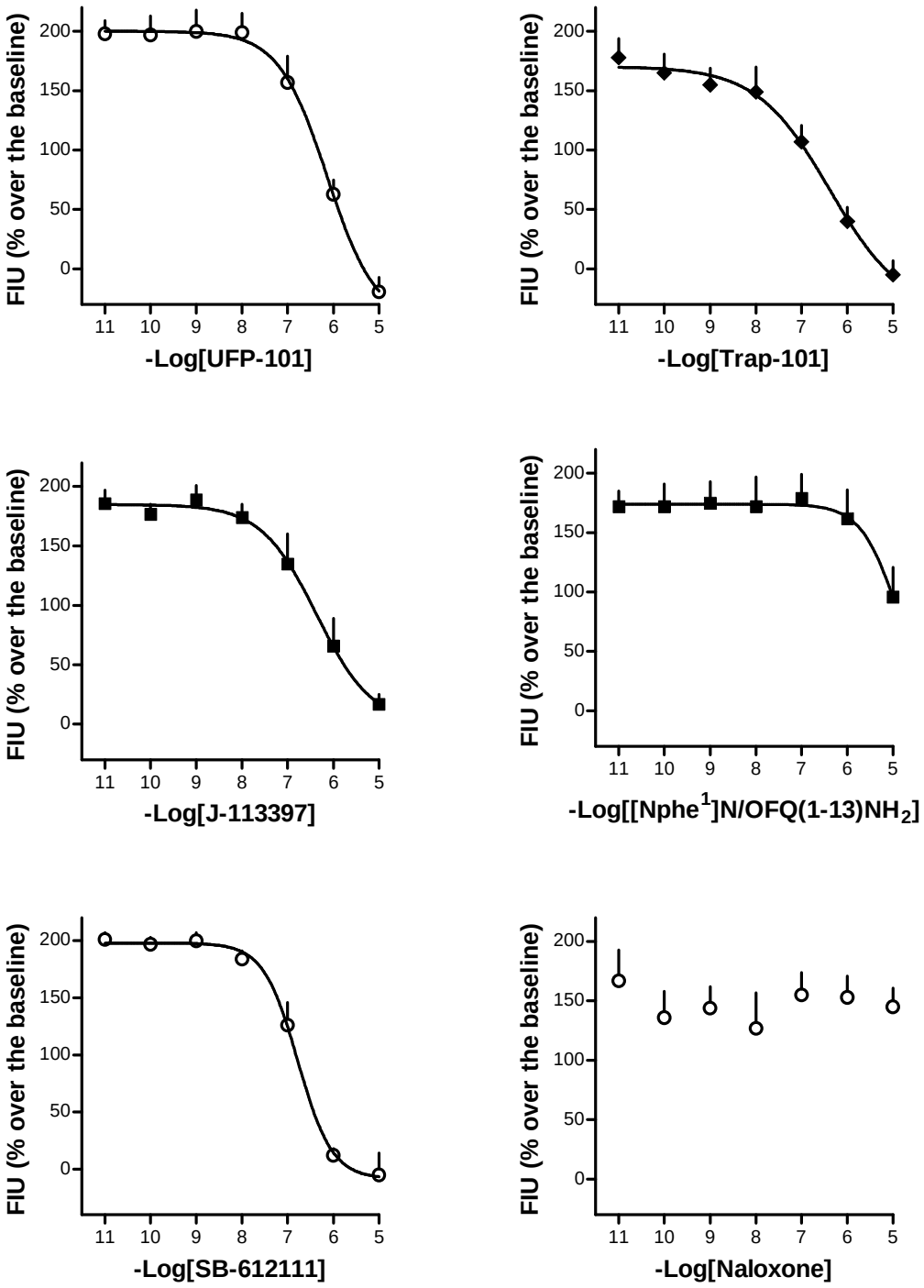


Figure 24. Inhibition experiments obtained by challenging 10 nM N/OFQ with increasing concentrations of NOP receptor antagonists in the calcium mobilization assay performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. N/OFQ effects were expressed as % over the baseline. Data are the mean of 4 separate experiments performed in duplicate.

As shown in Figure 24, UFP-101, J-113397 and Trap-101 were able to inhibit in a concentration manner the stimulatory effect of 10 nM N/OFQ, showing similar pIC_{50} values. pK_B values of 7.66, 7.88 and 7.93 were calculated from these experiments for UFP-101, J-113397 and Trap-101, respectively. [Nphe^1]N/OFQ(1-13)-NH_2 behaved as a low potency antagonist since it was able to counteract the effect of N/OFQ only at the higher concentration tested, i.e. 10 µM. Assuming a complete inhibition for higher concentrations, a pIC_{50} of 4.92 was calculated for [Nphe^1]N/OFQ(1-13)-NH_2 which corresponds to a pK_B value of 6.29. SB-612111 was the most potent antagonist, with a pIC_{50} of 6.69 and a pK_B of 8.16. Naloxone, up to 10 µM, did not modify the actions of 10 nM N/OFQ.

Finally, a Schild analysis has been performed for the compound J-113397 which has been tested at the concentrations of 0.1, 1 and 10 µM against the effects of increasing concentrations of N/OFQ. As showed in Figure 25, J-113397 produced a rightward shift of the concentration response curve to N/OFQ in a concentration-dependent manner without modifying the maximal effect elicited by the agonist. The corresponding Schild plot was linear with a slope value not significantly different from the unity. The calculated pA_2 value, 7.32, is close to the potency value (7.88) obtained in inhibition response experiments.

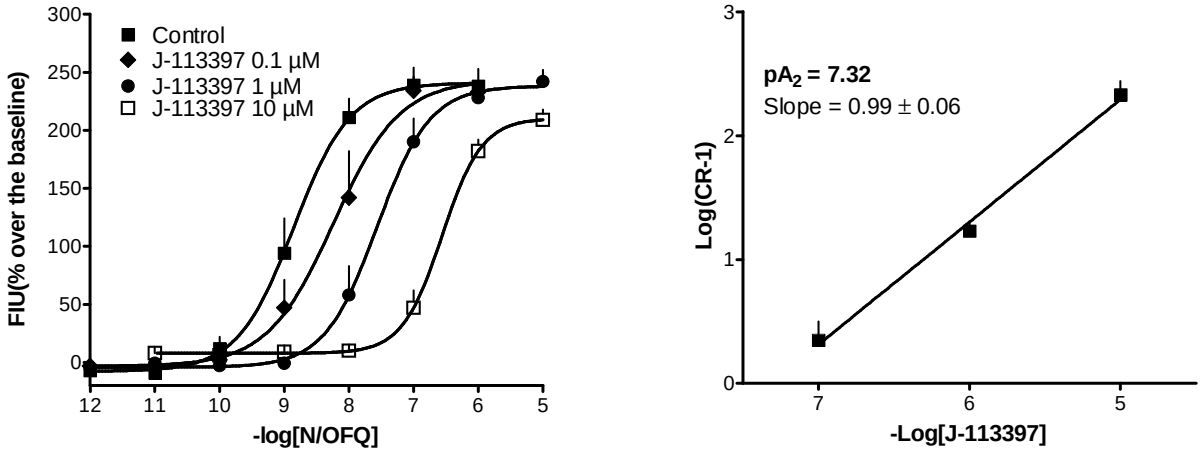


Figure 25. Concentration-response curve to N/OFQ obtained in the absence (control) and in presence of increasing concentrations of J-113397 (0.1-10 µM) (left panel); the corresponding Schild plot is shown in the right panel. Data are the mean of 4 separate experiments performed in duplicate.

Discussion

The results of the present study confirmed previous findings (Coward *et al.*, 1999) demonstrating that it is possible to use the $G\alpha_{qi5}$ chimeric protein to force NOP receptors to signal via the calcium pathway. Moreover, the detailed pharmacological characterization of NOP receptors artificially coupled to calcium signalling demonstrated that this strategy is not associated with major distortions of the pharmacological profile of this receptor. However some important pharmacological differences were demonstrated comparing the present data with those from literature for a subset of partial or full agonists characterized by slow kinetics of interaction with the NOP receptor.

In CHO_{hNOP}, CHO-$G\alpha_{qi5}$, and CHO_{hNOP}-$G\alpha_{qi5}$ cell lines ATP stimulated calcium mobilization generating superimposable concentration response curves. This demonstrates that the expression of the chimeric protein does not interfere with normal calcium signalling. On the contrary, N/OFQ consistently stimulated calcium mobilization only in the CHO_{hNOP}-$G\alpha_{qi5}$ cells, indicating that this response derives from the ability of the chimeric protein to force the NOP receptor to signal via calcium. The efficiency of this artificial coupling, measured in terms of N/OFQ E_{max}, indicated a signal to noise ratio in the range 2.5 – 3. Although details regarding this point are not presented in the (Coward *et al.*, 1999) paper, the analysis of the results reported in Figure 2 of that article suggests a similar efficiency. Thus the calcium mobilization response to N/OFQ in CHO_{hNOP}-$G\alpha_{qi5}$ cells is large and robust enough for performing both agonist and antagonist protocols.

To comprehensively characterize NOP receptors coupled to calcium signalling full and partial agonists as well as pure antagonists were used. As far as antagonists are concerned, all the compounds tested were found to be inactive *per se* up to 10 µM while they inhibited in a concentration-dependent manner the effect evoked by 10 nM N/OFQ. The following order of potency of antagonist has been recorded:

SB-612111 > J-113397 = Trap-101 $\geq$ UFP-101 > [Nphe^1]N/OFQ(1-13)-NH_2 (naloxone inactive). These results are superimposable to those obtained by testing the antagonists in [^{35}S]GTPγS binding experiments performed on CHO_{hNOP} cell membranes (McDonald *et al.*, 2003b; Spagnolo *et al.*, 2007; Trapella *et al.*, 2006) as well as in mouse vas deferens bioassay studies (Bigoni *et al.*, 2000a; Calo *et al.*, 2000a; Calo *et al.*, 2002b; Spagnolo *et al.*, 2007; Trapella *et al.*, 2006). The correlation analysis of the present data with those previously obtained in the mouse vas deferens assay (data taken from the quoted articles) yielded a determination coefficient r^2 of 0.90. Therefore it can be proposed that at least for ligand devoid of efficacy (i.e. pure antagonists), the $G\alpha_{qi5}$ NOP receptor calcium assay generate results superimposable to those obtained with classical

techniques useful for studying G_i coupled receptors. This applies not only to antagonist potency but also to the type of antagonism as demonstrated, at least for J-113397, by the results of the Schild analysis. In fact a competitive type of NOP antagonism was demonstrated for J-113397 both by the present study and by previous investigations performed in the mouse vas deferens preparation (Bigoni *et al.*, 2000a).

All the NOP receptor full agonists evaluated in the present study were able to evoke maximal effects similar to those obtained with N/OFQ with the following order of potency: N/OFQ = N/OFQ(1-13)-NH_2 > UFP-112 > Ro 64-6198 > NNC 63-0532

These results are only partially in agreement with findings from literature. In fact, data regarding N/OFQ, N/OFQ(1-13)-NH_2 and NNC 63-0532 perfectly match those obtained with [^{35}S]GTPγS binding (McDonald *et al.*, 2003b; Thomsen *et al.*, 2000b) and mouse vas deferens and guinea pig ileum assays (Calo *et al.*, 1996; Guerrini *et al.*, 2004). On the contrary, in the same assays Ro 64-6198 displayed potency values similar to those of N/OFQ (Hashiba *et al.*, 2002b; Jenck *et al.*, 2000; Rizzi *et al.*, 2001c) while UFP-112 was found to be 30 fold more potent than the naturally occurring peptide (Arduin *et al.*, 2007; Rizzi *et al.*, 2007b). Thus, the potency of both Ro 64-6198 and UFP-112 is significantly lower in the $G\alpha_{qi5}$ NOP receptor calcium assay than in other assays. The interpretation of these discrepant results is far from obvious. The chemical nature of the small molecule Ro 64-6198 and that of the N/OFQ analogue UFP-112 is very different suggesting that chemical features are not relevant. It is worthy of note that isolated tissue experiments demonstrated an important characteristic which is common to UFP-112 and Ro 64-6198. Indeed, as described in detail in previous publications (Arduin *et al.*, 2007; Rizzi *et al.*, 2007b), the kinetics of the inhibitory effect elicited by N/OFQ on the electrically induced twitch response is rapid and immediately and completely reversible after washing while that of both UFP-112 and Ro 64-6198 is characterized by slow onset, and slow and partial reversibility after washing. The slow kinetics of action of these ligands may be relevant for the estimation of their potency in the $G\alpha_{qi5}$ NOP receptor calcium assay. In fact, the long time required to obtain full activation of NOP receptors with these agonists may be incompatible with the rapid kinetics which characterized the calcium transient response. The different kinetics of N/OFQ and UFP-112 recorded in isolated tissues could not be detected in the present calcium mobilization experiments (see Figure 21). However, we cannot exclude that the relative low potency displayed by UFP-112 and Ro 64-6198 in the calcium assay merely depends on subtle changes of the receptor pharmacological profile induced by the chimeric protein.

Results obtained with the panel of NOP partial agonists are different from those described in the literature in terms of ligand efficacy and, for a subset of compounds, ligand potency. All the ligands evaluated in the present study induced maximal effects lower than that of the natural peptide N/OFQ although these differences did not reach statistical significance. Thus ligand efficacy estimated in the $G\alpha_{qi5}$ NOP receptor calcium assay is higher than in other assays. However this result is not completely unexpected. NOP receptor partial agonists displayed consistent efficacy often behaving as full agonists in the cAMP assay (Butour *et al.*, 1998; Dooley *et al.*, 1997; Kapusta *et al.*, 2005b; Mason *et al.*, 2001) while the same compounds showed limited and sometimes negligible efficacy in [^{35}S]GTPγS binding studies (Berger *et al.*, 2000b; Dooley *et al.*, 1997; Kocsis *et al.*, 2004; Mason *et al.*, 2001). Thus, the estimated efficacy of this kind of ligand strongly depends on the efficiency of the stimulus-response coupling which is different in the different assays. Those tests in which signal amplification phenomena make the efficiency of the stimulus-response coupling high (i.e. cAMP and calcium assays) tend to overestimate efficacy while those tests in which there is no amplification and the efficiency of the stimulus-response coupling is low (i.e. [^{35}S]GTPγS binding) tend to underestimate efficacy. This concept has been experimentally validated in the study by McDonald *et al.* (2003a) where stimulus-response coupling of both the cAMP and [^{35}S]GTPγS binding assays was modulated by changing the number of membrane receptors using a NOP inducible expression system. In this study partial agonist efficacy could be manipulated to encompass full and partial agonism along with pure antagonism (McDonald *et al.*, 2003a).

In the present study the partial agonist order of potency was as follows: Ac-RYYRWK-NH_2 > Ac-RYYRIK-NH_2 = Ac-RYYRIK-ol = [F/G]N/OFQ(1-13)-NH_2 = UFP-113 > ZP120. Similarly to that found with full agonists these results are only partially in line with literature findings. Indeed, the following order of potency, which is in good agreement with the present results, has been described using classical tests for Gi coupled receptors: Ac-RYYRWK-NH_2 > Ac-RYYRIK-NH_2 = Ac-RYYRIK-ol ≥ [F/G]N/OFQ(1-13)-NH_2 (Dooley *et al.*, 1997; Gunduz *et al.*, 2006; Kocsis *et al.*, 2004; Mason *et al.*, 2001). In contrast, UFP-113 (Arduin *et al.*, 2007) and ZP120 (Kapusta *et al.*, 2005b; Rizzi *et al.*, 2002a) displayed potency values 10-30 fold higher than their templates ([F/G]N/OFQ(1-13)-NH_2 and Ac-RYYRWK-NH_2 respectively). Interestingly, in the electrically stimulated mouse vas deferens ZP120 showed kinetics of action comparable to those described for Ro 64-6198 and UFP-112 (inhibitory effects slow to develop and slowly and only partially reversible after washing (Rizzi *et al.*, 2002a). This kind of evidence is not available for UFP-113 because this peptide produced variable agonist effects mainly behaving as a NOP antagonist in the mouse vas deferens (Arduin *et al.*, 2007). However, a similar type of kinetics of action can be

hypothesized for UFP-113 based on its close structural similarities with UFP-112 (Arduin *et al.*, 2007). Therefore, as proposed for the NOP full agonists Ro 64-6198 and UFP-112, the relatively low potency of UFP-113 and ZP120 in the $G\alpha_{qi5}$ NOP receptor calcium assay may be the consequence of the long time required to activate the NOP receptor with these ligands which is not compatible with the rapid kinetics of the calcium transient response.

In conclusion, the present study confirmed and extended previous findings demonstrating that the chimeric protein $G\alpha_{qi5}$ is indeed capable of coupling to NOP receptors to the Ca^{2+} signalling pathway. Moreover, results obtained from the detailed pharmacological characterization of the NOP receptor with the $G\alpha_{qi5}$ calcium assay demonstrated that this approach represents a very useful strategy for the screening of NOP receptor antagonists. Results obtained with this class of ligands in the $G\alpha_{qi5}$ NOP receptor calcium assay are superimposable to those collected with G_i based biochemical assays performed at recombinant or native NOP receptors or with bioassays performed on isolated tissues expressing native NOP receptors. In contrast, the usefulness of this assay for the screening of partial and full agonists appears to be limited by its tendency to estimate high efficacy for partial agonists and low potency for partial and full agonists characterized by slow kinetics of action.

3.2 Further studies on the pharmacological features of the nociceptin/orphanin FQ receptor ligand ZP120

ZP120 is NOP receptor ligand (Larsen *et al.*, 2001) which has been generated by applying the structure inducing probes technology (Larsen, 1999) to the NOP selective partial agonist Ac-RYYRWK-NH_2 (Dooley *et al.*, 1997) with the aim of improving its metabolic stability and potency without modifying its pharmacodynamic properties. This strategy appeared to be successful since ZP120 has been demonstrated in *in vitro* experiments to bind to the NOP receptor with high affinity, to inhibit forskolin induced cAMP accumulation in $HEK293_{hNOP}$ cells and electrically induced contractions of the mouse vas deferens showing compared to N/OFQ higher potency and lower maximal effects (Kapusta *et al.*, 2005b; Rizzi *et al.*, 2002a). Furthermore in *in vivo* experiments ZP120 mimicked N/OFQ actions including the pronociceptive and locomotor inhibitory effects after supraspinal administration (Rizzi *et al.*, 2002a) and the diuretic effects after intravenous administration (Kapusta *et al.*, 2005b), showing a longer duration of action. Interestingly, unlike N/OFQ, ZP120 did not modify *per se* cardiovascular parameters but rather

antagonized the bradycardic and hypotensive action of N/OFQ (Kapusta *et al.*, 2005b). This confirmed the findings obtained with other NOP receptor partial agonists i.e. [F/G]N/OFQ(1-13)-NH_2, Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2 and corroborate the proposal that NOP receptor partial agonists produce functionally selective effects on cardiovascular and renal functions ranging from full agonist (water diuresis) to antagonist (bradycardia and hypotension) action (Kapusta *et al.*, 2005a).

As far as the ZP120 receptor mechanism is concerned, we have previously reported that the effects of this peptide in the mouse vas deferens assay are resistant to naloxone while sensitive to the NOP selective antagonist J-113397 (Ozaki *et al.*, 2000) which displayed similar pA_2 values against ZP120 (7.80) and N/OFQ (7.81) (Rizzi *et al.*, 2002a). Recently, it has been reported that N/OFQ and ZP120 are both able to inhibit in a concentration-dependent manner the contractions evoked by electrical field stimulation in rat mesenteric arteries (Simonsen *et al.*, 2008). However, in this preparation, the NOP selective peptide antagonist UFP-101 (Calo *et al.*, 2005; Calo *et al.*, 2002b) antagonized the action of N/OFQ but not that of ZP120 (Simonsen *et al.*, 2008). Several important differences in terms of molecules (J-113397 vs UFP-101), preparations (vas deferens vs mesenteric arteries), and species (mouse vs rat) used in the two above mentioned studies do not allow an easy interpretation of these contrasting findings. Therefore the aim of the present study was to extensively investigate the pharmacological features of ZP120 by comparing its effects with N/OFQ in the mouse and rat vas deferens and by challenging the actions of these NOP agonists with both J-113397 and UFP-101. Moreover the *in vitro* (inhibition of electrically stimulated vas deferens) and *in vivo* (supraspinal pronociceptive effect) actions of ZP120 were reassessed in tissues taken from and in mice knockout for the NOP receptor gene, respectively.

Results

Electrically stimulated isolated tissues - The NOP receptor ligands N/OFQ and ZP120 inhibited the electrically induced contraction of the mouse and rat vas deferens in a concentration-dependent manner (Figure 26). N/OFQ displayed similar values of potency ($pEC_{50} \approx 7.3$) and maximal effects (≈ 80% inhibition of control twitch) in mouse and rat tissues. ZP120 ($pEC_{50} \approx 8.8$) was about 30 fold more potent than N/OFQ but produced maximal effects (≈ 60% inhibition of control twitch) significantly lower than those of the natural peptide in both preparations.

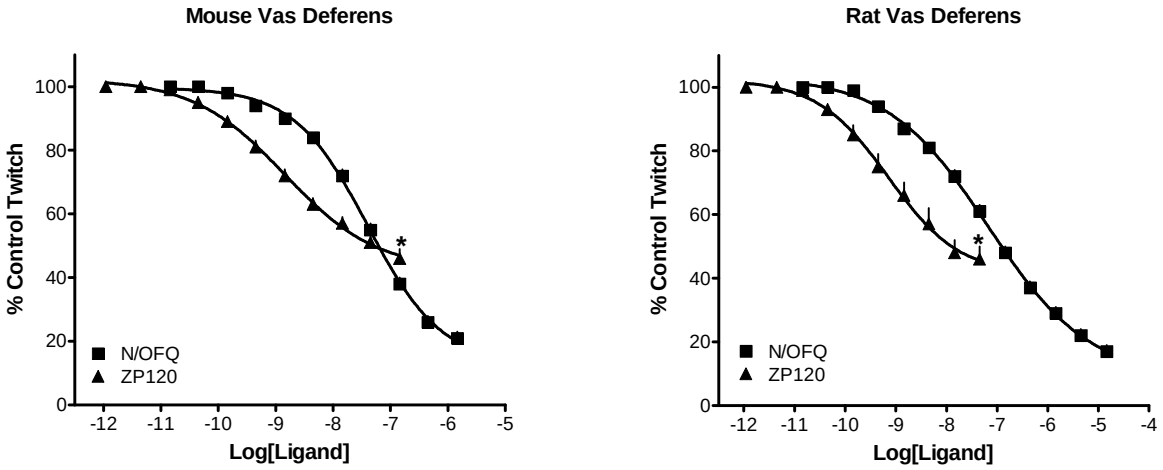


Figure 26. Concentration response curves to N/OFQ and ZP120 in the electrically stimulated mouse (left panel) and rat (right panel) vas deferens. Data are the mean ± s.e.m. of 5 separate experiments. *****$p<0.05$ vs N/OFQ, according to the Student *t*-test.

In addition and confirming previous findings obtained in the mouse vas deferens (Rizzi *et al.*, 2002a), the kinetics of the inhibitory effects elicited by ZP120 was different from that of N/OFQ in both preparations. The action of the natural peptide took place immediately after adding the peptide to the bath, was rapidly reversible after washing, and could be repeated in the same tissue, on the contrary, the effects of ZP120 were slow to develop, slowly reversible after washing, and could not be repeated in the same tissue. Typical tracings showing the concentration response-curve to N/OFQ and ZP120 in the rat vas deferens are displayed in Figure 27.

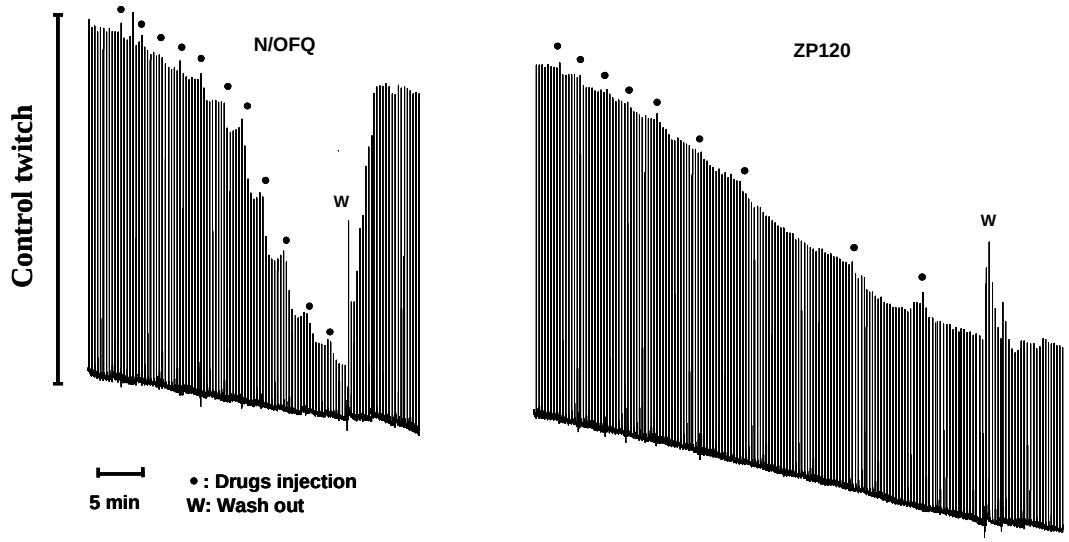


Figure 27. Typical tracings showing the concentration response curve to N/OFQ (0.1 nM-10 µM, left panel) and ZP120 (10 pM-100 nM; right panel) in the electrically stimulated rat vas deferens.

As shown in the Figure 28, the effects of N/OFQ and ZP120 were evaluated in the presence of the NOP selective antagonists UFP-101 (1 µM) and J-113397 (100 nM) in both preparations.

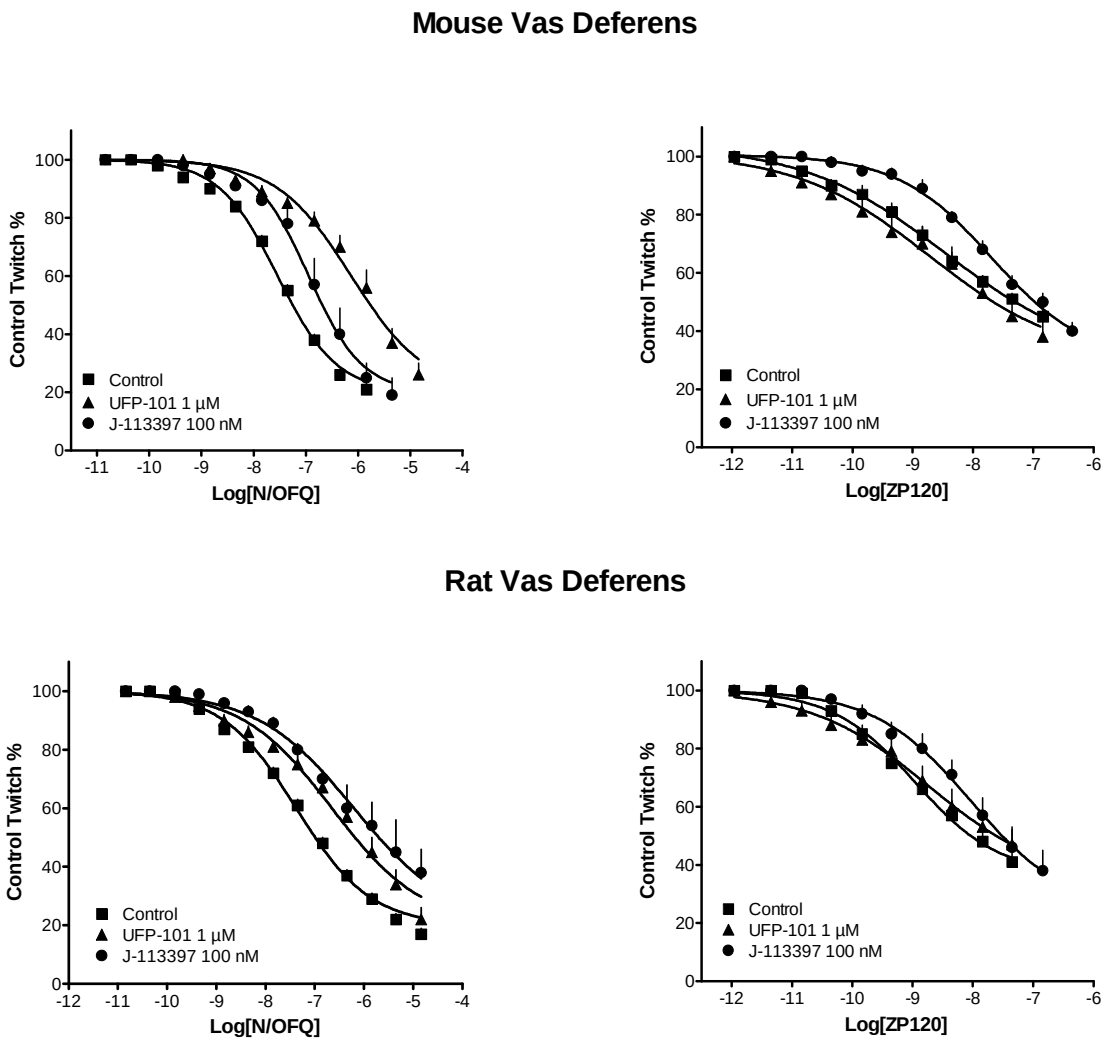


Figure 28. Concentration-response curves to N/OFQ (left panels) and ZP120 (right panels) measured in the absence and in presence of UFP-101 (1 µM) and J-113397 (100 nM) in the electrically stimulated mouse (top panels) and rat (bottom panels) vas deferens. Data are the mean ± s.e.m. of 4 separate experiments.

Both the antagonists did not modify *per se* the control twitches. In the mouse and rat vas deferens J-113397 produced a rightward shift of the concentration-response curve to N/OFQ without significantly affecting the maximal agonist response. J-113397 pK_B values derived from these experiments were 7.69 and 8.04, respectively. Superimposable results were obtained by challenging J-113397 vs ZP120. J-113397 competitively antagonized ZP120 effects in the mouse

and rat tissues with the following pK_B values: 7.76 and 7.89. In the mouse and rat vas deferens the NOP selective antagonist UFP-101 displaced to the right the concentration-response curve to N/OFQ yielding pK_B values of 7.37 and 6.79, respectively. UFP-101 did not modify the inhibitory effect of ZP120 in both preparations. Antagonist pK_B values obtained from these experiments are summarized in Table 4.

Table 4. pK_B values of UFP-101 and J-113397 vs N/OFQ and ZP120 in the electrically stimulated mouse and rat vas deferens. Data are mean of 4 separate experiments. CL_{95}%: confidence limit.

	Mouse Vas Deferens		**Rat Vas Deferens**	
	pK_B (CL_{95}%)		*pK_B (CL_{95}%)*	
	UFP-101	***J-113397***	***UFP-101***	***J-113397***
N/OFQ	7.37 (7.13-7.61)	7.69 (7.17-8.22)	6.79 (6.45-7.13)	8.04 (7.54-8.54)
ZP120	<6	7.76 (7.67-7.85)	<6	7.89 (7.02-8.76)

The effects of N/OFQ and ZP120 and those elicited by the selective DOP agonist DPDPE, were investigated in the electrically stimulated mouse vas deferens taken from NOP(+/+) and NOP(-/-) mice (Figure 29).

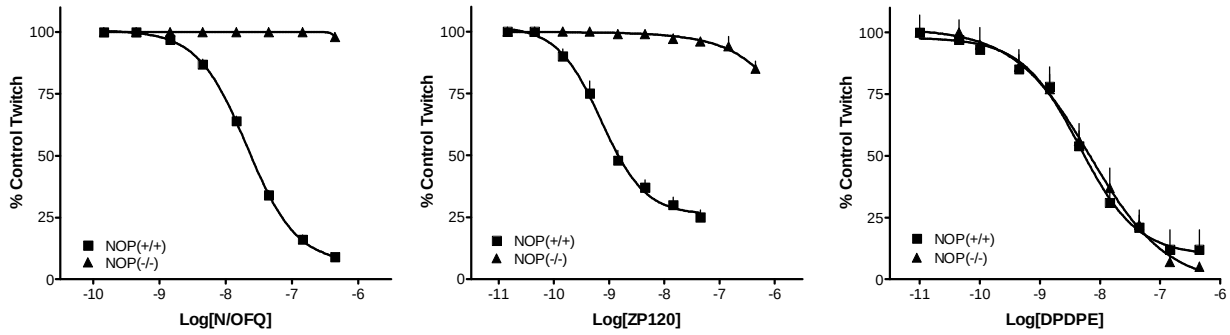


Figure 29. Electrically stimulated mouse vas deferens. Concentration response curve to N/OFQ, ZP120 and DPDPE in tissues taken from NOP(+/+) and NOP(-/-) mice. Data are the mean ± s.e.m. of 4 separate experiments.

In NOP(+/+) tissues, ZP120 mimicked the inhibitory effect of N/OFQ (pEC_{50} 7.62; E_{max} 91 ± 1%) showing higher potency (pEC_{50} 9.10) but lower maximal effects (E_{max} 73 ± 3%). In tissues taken from NOP(-/-) mice, N/OFQ was found inactive and ZP120 slightly inhibited the electrically induced contraction only at the highest concentration tested (0.3 μM). In parallel experiments, the DOP receptor selective agonist DPDPE displayed similar potency (pEC_{50} 8.40 and 8.20) and maximal effects (E_{max} 93 ± 3% and 91 ± 5%) in tissues taken from NOP(+/+) and NOP(-/-) mice, respectively. The values of potency and maximal effects derived from concentration response curves to N/OFQ and ZP120 in the electrically stimulated vas deferens taken from rats and mice (Swiss, NOP(+/+) and NOP(-/-)) are summarized in Table 5.

Table 5. Effects of N/OFQ and ZP120 in the electrically stimulated mouse and rat vas deferens. Data are mean of 5 separate experiments. $CL_{95\%}$: confidence limit. ND: could not be determined. *p<0.05 vs N/OFQ, according to the Student *t*-test.

	Mouse Vas Deferens						**Rat Vas Deferens**	
	Swiss mice		NOP(+/+) **mice**		NOP(-/-) **mice**			
	pEC_{50} ($CL_{95\%}$)	*E_{max} ± s.e.m.*	*pEC_{50} ($CL_{95\%}$)*	*E_{max} ± s.e.m.*	*pEC_{50} ($CL_{95\%}$)*	*E_{max} ± s.e.m.*	*pEC_{50} ($CL_{95\%}$)*	*E_{max} ± s.e.m.*
N/OFQ	7.37 (7.27-7.47)	80 ± 2%	7.62 (7.56-7.68)	91 ± 1%	< 6	ND	7.25 (7.08-7.42)	83 ± 2%
ZP120	8.78 (8.61-8.95)	56 ± 3%*****	9.10 (8.88-9.32)	73 ± 3%*****	< 6	ND	8.80 (8.41-9.19)	64 ± 4%*****

Mouse tail withdrawal assay - NOP(+/+) mice injected i.c.v. with saline did not show any modification of gross behaviour. Those injected with high doses of N/OFQ (10 nmol) or ZP120 (1 nmol) displayed a clear decrease in locomotor activity and muscular tone, ataxia, and partial loss of the righting reflex. These actions on mice gross behaviour were previously reported in Swiss mice both for N/OFQ (Calo *et al.*, 1998b) and ZP120 (Rizzi *et al.*, 2002a). The i.c.v. injection of these peptides in NOP(-/-) mice did not produce any obvious modifications of their spontaneous behaviour.

Results summarized in Figure 30 (left panel) show that baseline tail withdrawal latency of NOP(+/+) mice was 5.04 ± 0.73 s. In animals treated with saline this parameter was stable around 5-6 s over the time course of the experiment. The i.c.v. administration of 10 nmol N/OFQ produced an immediate (peak effect at 5 min) pronociceptive action which lasted for approx 1 h. The injection of 1 nmol ZP120 mimicked the pronociceptive effect of N/OFQ, however it was characterized by a slow onset of action (peak effect at 120-180 min) and was longer lasting with the

reduction of tail withdrawal latency still evident after 3h from peptide injection. This pattern of effects of N/OFQ and ZP120 is superimposable to that previously reported in Swiss mice (Calo *et al.*, 1998b; Rizzi *et al.*, 2002a).

In line with previous findings (Nishi *et al.*, 1997), baseline latency of NOP(-/-) mice (5.21 ± 0.90 s) was similar to that of NOP(+/+) mice. The injection of saline in NOP(-/-) animals produced a slight increase in tail-withdrawal latency which was then stable over the time course of the experiment (Figure 30, right panel). In NOP(-/-) mice, the i.c.v. injection of both N/OFQ and ZP120 did not produce any effect with tail withdrawal latencies superimposable to that of saline treated animals (Figure 30, right panel).

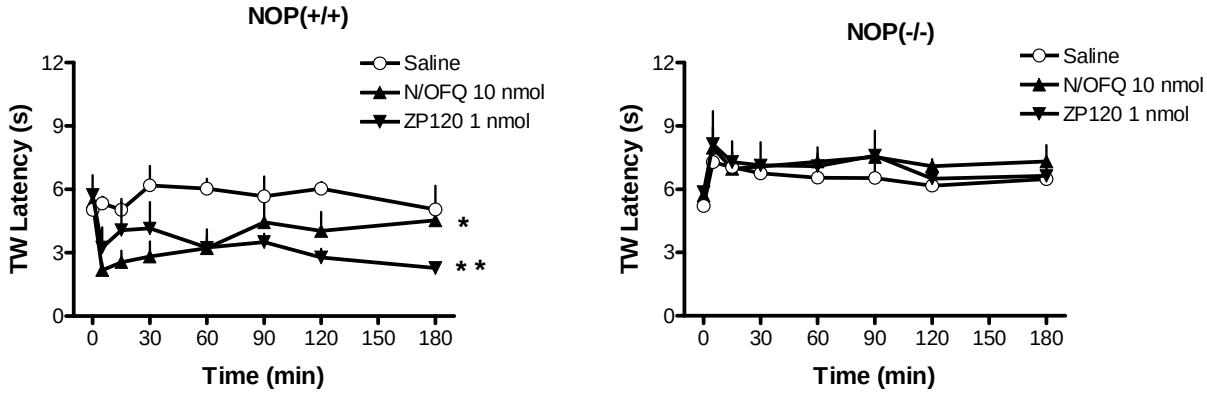


Figure 30. Effects of 10 nmol N/OFQ and 1 nmol ZP120 given i.c.v. in the tail withdrawal assay performed in NOP(+/+) (left panel) and NOP(-/-) (right panel) mice. Data are the mean ± s.e.m. of 4 separate experiments. *p<0.05 and **p<0.01 versus saline according to ANOVA followed by the Dunnett's test.

Discussion

This study provides novel evidence that confirms and extends our knowledge of the pharmacological profile of ZP120 in rodents. This peptide behaves as a selective NOP receptor partial agonist and displays kinetics of action characterized by slow onset and long lasting effects. The antagonist sensitivity of ZP120 effects (J-113397 sensitive, naloxone and UFP-101 resistant) is not superimposable to that of the endogenous NOP ligand N/OFQ (J-113397 and UFP-101 sensitive, naloxone resistant). However the exclusive involvement of the NOP receptor protein in the *in vitro* as well as *in vivo* effects of ZP120 (and N/OFQ) was clearly demonstrated by knockout studies. Therefore the different sensitivity of N/OFQ and ZP120 to UFP-101 can be attributed to their diverse way of binding to and activating the NOP receptor.

The present data obtained in parallel experiments performed on mouse and rat tissues confirmed previous finding (Kapusta *et al.*, 2005b; Rizzi *et al.*, 2002a; Simonsen *et al.*, 2008) demonstrating that ZP120 behaves as a NOP receptor partial agonist. The following evidence and considerations make this pharmacological feature of ZP120 extremely important. Partial agonists may discriminate between the different pharmacological effects evoked by full agonists in different systems/organs depending on the efficiency of the stimulus-response coupling that characterize each of the systems/organs (Kenakin, 2004). This has been demonstrated for the cardiovascular and renal effects of NOP ligands. I.v. administration of the full agonist N/OFQ produce in rodents cardiovascular inhibitory effects (i.e. hypotension and bradycardia) associated with diuretic, in particular aquaretic, effects (Kapusta, 2000). In contrast, i.v. administration of NOP receptor partial agonists ([F/G]N/OFQ(1-13)-NH_2, Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2) only mimicked the renal effects of N/OFQ without modifying cardiovascular parameters (Kapusta, 2000). This same pattern of effects was demonstrated for ZP120 (Kapusta *et al.*, 2005b). Therefore in water-retaining states with hyponatremia such as heart failure, NOP receptor partial agonists like ZP120 represent a better therapeutic option than full agonists. In addition to partial agonist behaviour, ZP120 displays slow onset, long lasting effects with high potency. These features have been confirmed in the present study both *in vitro* and *in vivo* and are in line with that reported in the literature. ZP120 has been shown to mimic with higher potency and longer lasting effects the following N/OFQ actions: inhibition of locomotor activity and pronociceptive effects after supraspinal administration in mice (Rizzi *et al.*, 2002a) and sodium-sparing aquaretic activity after i.v. administration in rats (Kapusta *et al.*, 2005b). Collectively these pharmacological features make ZP120 a promising and innovative drug candidate for treating heart failure (Lambert, 2008).

The receptor mechanisms involved in ZP120 actions was investigated by using two chemically unrelated and selective NOP receptor antagonists the peptide UFP-101 (Calo *et al.*, 2005; Calo *et al.*, 2002b) and the non-peptide J-113397 (Ozaki *et al.*, 2000) as well as mice knock out for the NOP receptor gene (Nishi *et al.*, 1997). UFP-101 and J-113397 represent standard antagonists for NOP receptor investigations with more than 50 papers reported in literature for each of these molecules describing their antagonist properties vs N/OFQ (Chiou *et al.*, 2007; Lambert, 2008). In line with this picture, in the present study both molecules competitively antagonized N/OFQ effects in mouse and rat tissues showing the expected order of potency of antagonists i.e. J-113397 ($pK_B \approx 7.8$) > UFP-101 ($pK_B \approx 7.3$). J-113397 was also found to be active against ZP120 whose effects were antagonized in both preparations with pK_B values superimposable to those obtained against N/OFQ. In contrast, UFP-101 was found inactive against ZP120. Similar findings were obtained in separate studies where the effects of N/OFQ and ZP120 in the mouse vas deferens

were found to be sensitive to J-113397 (Rizzi *et al.*, 2002a) while those of N/OFQ but not ZP120 to be sensitive to UFP-101 in rat mesenteric arteries (Simonsen *et al.*, 2008). Since we investigated in parallel studies both rat and mouse preparations obtaining consistent pharmacological results in tissues from the two species, we can rule out that species specific NOP receptor isoforms play a role in the pattern of antagonist sensitivity of ZP120 effects. As mentioned above ZP120 differs from N/OFQ in terms of kinetics of action and this might be relevant for antagonist sensitivity. However, other NOP selective peptide agonists such as UFP-102 (Carra *et al.*, 2005b) and UFP-112 (Arduin *et al.*, 2007) characterized by kinetics of action superimposable to that of ZP120 were found to be sensitive to the antagonist effect of UFP-101 (Carra *et al.*, 2005b; Rizzi *et al.*, 2007b), thus suggesting that the agonist kinetics of action are not important for the pattern of antagonist sensitivity. A different receptor mechanism can be suggested to interpret the diverse antagonist sensitivity of ZP120 and N/OFQ. However, the *in vitro* and *in vivo* knockout studies performed in the frame of the present study clearly demonstrated that the effects of both agonists, which are no longer evident in NOP(-/-) tissues and animals, are due to the selective activation of the NOP receptor protein. Therefore, the most reasonable suggestion that can be proposed to collectively interpret these findings is that by Simonsen *et al.* (2008) i.e. N/OFQ and the hexapeptide Ac-RYYRWK-NH_2 (from which ZP120 derives) have distinct modes of interaction with the NOP receptor and UFP-101 is able to discriminate between them by blocking N/OFQ binding but not that of ZP120. This suggestion is corroborated by the following evidence: in elegant photoaffinity labelling studies the group of Meunier investigated the NOP receptor regions that bind N/OFQ and hexapeptides; in particular N/OFQ binds to extracellular loop III and transmembrane helix VII (Mouledous *et al.*, 2000) while the hexapeptides bind to transmembrane helix II (Bes *et al.*, 2003). However, some overlapping of the NOP receptor regions that recognized N/OFQ and hexapeptides should be hypothesized because the binding of N/OFQ and hexapeptides to the NOP receptor is mutually exclusive as demonstrated by receptor binding studies (Dooley *et al.*, 1997; Thomsen *et al.*, 2000b) and by functional antagonist studies performed in those preparations/assays in which the hexapeptides (or their analogues) displayed small or no efficacy and where a competitive type of interaction has been reported for these peptides. These antagonist studies were performed investigating [^{35}S]GTPγS binding to G proteins in membranes and sections of rat brain (Berger *et al.*, 1999), chronotropic effect on neonatal rat cardiomyocytes (Berger *et al.*, 1999), the electrically stimulated mouse vas deferens (Gunduz *et al.*, 2006; Li *et al.*, 2008) or the cardiovascular parameters of the rat (Kapusta *et al.*, 2005a). On the other hand, the NOP receptor site recognized by UFP-101, which might be similar but not identical to that of N/OFQ, is not overlapping with that of the hexapeptides and this may explain the lack of effect of UFP-101 against ZP120 reported by

Simonsen *et al.* (2008) in rat mesenteric arteries and confirmed in the present study in vas deferens tissues from rats and mice.

In conclusion, the present investigation demonstrated via *in vitro* and *in vivo* receptor knockout studies that ZP120 is a highly selective NOP receptor ligand. Moreover a diverse antagonist sensitivity of N/OFQ (J-113397 and UFP-101 sensitive) and ZP120 (J-113397 sensitive and UFP-101 resistant) which can not be explained in terms of species specific NOP receptor isoforms, diverse agonist kinetics of action, or different receptor mechanism, was reported. This diverse antagonist sensitivity may derive from different mechanisms of binding to the NOP receptor for N/OFQ, ZP120, and UFP-101. Collectively this study provides further evidence that ZP120 behaves as a selective NOP receptor partial agonist characterized by high potency and able to evoke slow onset long lasting effects. Collectively these pharmacological features make ZP120 not only a useful pharmacological tool to be used in preclinical investigations but also a drug candidate for exploiting the therapeutic benefits derived by a prolonged partial activation of peripheral NOP receptors.

3.3 Pharmacological characterization of the nociceptin/orphanin FQ receptor non-peptide antagonist Compound 24

A novel NOP receptor non-peptide antagonist, 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),4'-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide, has been recently identified by Banyu investigators and named Compound 24 (Goto *et al.*, 2006). The synthesis of this novel ligand is relatively easy and the overall yield relatively high (25% in our laboratory, 31% in Goto *et al.* (2006)). This is particularly true when compared to the synthesis of other non-peptide NOP antagonists such as J-113397 and SB-612111 whose synthesis is very difficult and of low overall yield (≈ 1% for both molecules in our laboratories, C. Trapella personal communication).

Thus, the aim of the present study was the synthesis and detailed investigation of the pharmacological profile of Compound 24. The novel ligand was investigated *in vitro* in receptor binding and [^{35}S]GTPγS experiments performed in CHO_{hNOP} cell membranes, in calcium mobilization experiments performed in CHO_{hNOP} cells expressing the $G\alpha_{qi5}$ protein, and in N/OFQ sensitive isolated tissues. Finally, the *in vivo* actions of Compound 24 were assessed in mice using the tail withdrawal assay.

Results

Receptor binding assay - In receptor binding experiments performed on CHO_{hNOP} cell membranes Compound 24 displaced [^{3}H]N/OFQ in a concentration-dependent manner with subnanomolar affinity (pK_i 9.62, Table 6). Under the same experimental conditions, Compound 24 did not bind the hDOP receptor and showed low affinities for MOP and KOP sites (pK_i 6.72 and 6.47, respectively). In contrast, the universal opioid receptor ligand naloxone did not bind the NOP receptor but displayed the expected rank order of affinity at classical opioid receptors i.e. MOP (pK_i 9.25) > KOP (pK_i 8.35) > DOP (pK_i 7.67) (Table 6).

Table 6. Affinities of Compound 24 and naloxone at NOP and classical opioid receptors expressed in CHO cell membranes.

receptor	**NOP**	**MOP**	**DOP**	**KOP**
radioligand	[^{3}H]N/OFQ	[^{3}H]DPN	[^{3}H]DPN	[^{3}H]DPN
naloxone[a]	< 6	9.25 (9.04 – 9.46)	7.67 (7.59 – 7.75)	8.35 (8.20 – 8.50)
Compound 24	9.62 (9.47 – 9.77)	6.72 (6.43 – 6.97)	< 6	6.47 (6.20 – 6.74)

a: naloxone affinities at classical opioid receptors are from Vergura *et al.* (2008). Data are mean ($CL_{95\%}$) of 3 separate experiments.

[^{35}S]GTPγS binding assay - In CHO_{hNOP} cell membranes N/OFQ stimulated [^{35}S]GTPγS binding in a concentration-dependent manner with a pEC_{50} value of 8.95 ± 0.05 and E_{max} of 11.71 ± 0.37 (Figure 31, left panel). The antagonistic properties of Compound 24 were evaluated over the 1-100 nM concentration range, in order to obtain data for a Schild analysis. Compound 24 up to 10 µM did not elicit any stimulation of [^{35}S]GTPγS binding in CHO_{hNOP} cell membranes, however it produced a concentration-dependent and parallel shift of the concentration response curve to N/OFQ without modifying the maximal effects induced by the agonist (Figure 31, left panel). Schild analysis of the data (Figure 31, right panel) demonstrated a linear (r^2 = 0.95) plot with a slope not significantly different from unity. The extrapolated pA_2 value was 9.98.

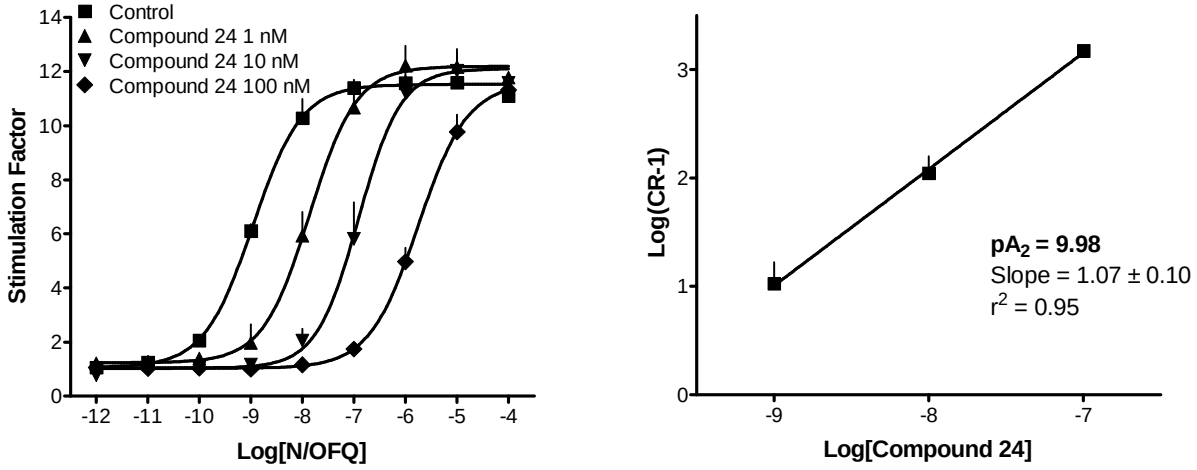


Figure 31. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (1 – 100 nM) in the [^{35}S]GTPγS binding assay performed on CHO_{hNOP} cell membranes. The relative Schild Plot is shown in the right panel. Data are mean ± s.e.m. of 4 separate experiments.

Calcium mobilization assay - In CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein N/OFQ evoked a concentration-dependent stimulation of calcium release (pEC_{50} 9.24 ($CL_{95\%}$ 9.10 – 9.38)). Compound 24 was inactive *per se* but in the range 0.01 nM–10 µM concentration-dependently inhibited calcium mobilization induced by 10 nM N/OFQ with a pK_B value of 9.03 ± 0.20 (Table 7).

Table 7. Antagonist potencies of Compound 24 and naloxone evaluated in calcium mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and the $G\alpha_{qi5}$ protein.

receptor	**NOP**	**MOP**	**DOP**	**KOP**
agonist	N/OFQ 10 nM	Dermorphin 100 nM	DPDPE 100 nM	Dynorphin A 100 nM
naloxone	< 6	9.09 (8.73-9.45)	7.32 (6.11-8.53)	7.14 (6.60-7.68)
Compound 24	9.03 (8.83-9.23)	< 6	< 6	< 6

Data are mean ($CL_{95\%}$) of 4 separate experiments.

Naloxone was inactive up to 1 µM. To assess the selectively of action of Compound 24 similar experiments were performed in CHO cells stably expressing $G\alpha_{qi5}$ and classical opioid

receptors. Dermorphin, DPDPE and dynorphin A were used in these experiments as agonists for MOP, DOP and KOP receptors, respectively. They produced a concentration-dependent stimulation of calcium mobilization with the following values of pEC_{50} and E_{max}: dermorphin 7.93 ($CL_{95\%}$ 7.67 – 8.19), 196 ± 9%; DPDPE 8.82 ($CL_{95\%}$ 8.43 – 9.21), 130 ± 10%; dynorphin A 8.47 ($CL_{95\%}$ 8.16 – 8.78) 174 ± 14%. Naloxone inhibited the effects of these agonists showing higher potency at MOP (pK_B 9.09) than KOP (pK_B 7.14) and DOP (pK_B 7.32) (Table 7). In contrast Compound 24 was inactive up to 1 µM against DPDPE, dynorphin A, and dermorphin (Table 7).

To investigate the type of antagonism produced by Compound 24 in calcium mobilization experiments a Schild analysis has been performed by testing this molecule at various concentrations (0.01, 0.1 and 1 µM) against the concentration-response curve to N/OFQ. As shown in Figure 32, Compound 24 produced a rightward shift of the concentration-response curve to N/OFQ in a concentration-dependent manner. However, the maximal effects elicited by N/OFQ appear to be slightly but significantly reduced by Compound 24. A pK_B value of 8.73 was derived from these experiments. It is worthy of note that this value is close to that obtained in inhibition response experiments (9.03).

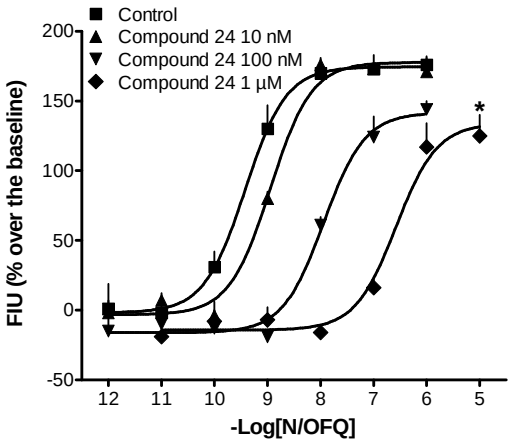


Figure 32. Concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (10 nM - 1 µM) in the calcium mobilization assay performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Data are mean ± s.e.m. of 4 separate experiments performed in duplicate. *p<0.05 versus control according to ANOVA followed by Dunnett's test.

Electrically stimulated isolated tissues - Compound 24 was assessed against N/OFQ in the electrically stimulated mouse and rat vas deferens and guinea pig ileum. In the mouse vas deferens N/OFQ inhibited the twitch response to electrical field stimulation in a concentration-dependent

manner (pEC_{50} value 7.46, Figure 33 left panel). Compound 24, tested over the concentration range 10 nM -1 µM, did not modify *per se* the electrically-induced twitches, but displaced to the right the concentration response curve to N/OFQ in a concentration-dependent manner. Curves obtained in the presence of Compound 24 were parallel to the control (Figure 33, left panel) with no modification of the agonist maximal effect. The corresponding Schild plot was linear (r^2 = 1.00) with a slope not significantly different from unity, yielding a pA_2 value of 8.44 (Figure 33, right panel).

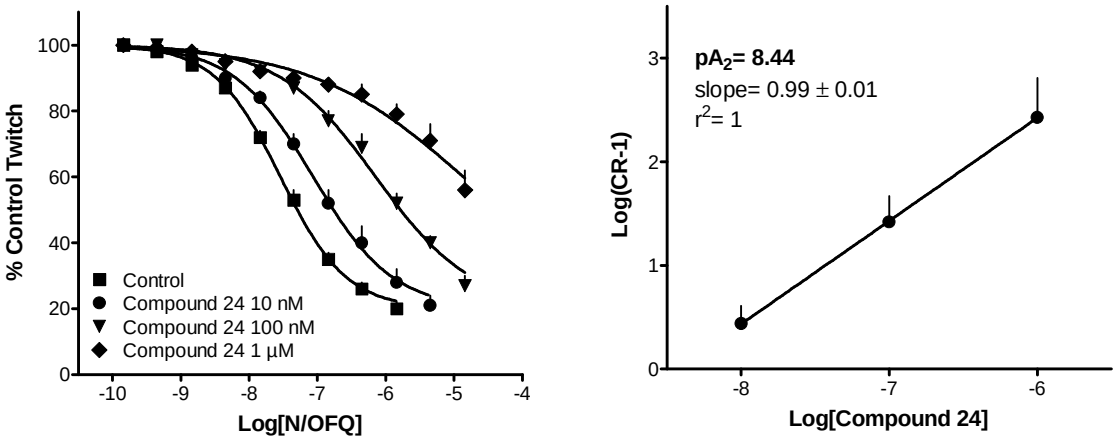


Figure 33. Left panel: concentration response curves to N/OFQ obtained in the absence (control) and presence of increasing concentrations of Compound 24 (10 nM – 1 µM) in the electrically stimulated mouse vas deferens. The relative Schild Plot is shown in the right panel. Data are mean ± s.e.m. of 4 separate experiments.

In the rat vas deferens and in guinea pig ileum Compound 24 was tested at the single concentration of 100 nM against the effects of N/OFQ. In both preparations the concentration response curves to N/OFQ obtained in the absence and presence of Compound 24 were parallel and reached similar maximal effects. The estimated pK_B values were 8.28 ± 0.12 and 9.12 ± 0.19 in the rat vas deferens (Figure 34, left panel) and guinea pig ileum (Figure 34, right panel), respectively. In these preparations, Compound 24 up to 1 µM was *per se* inactive.

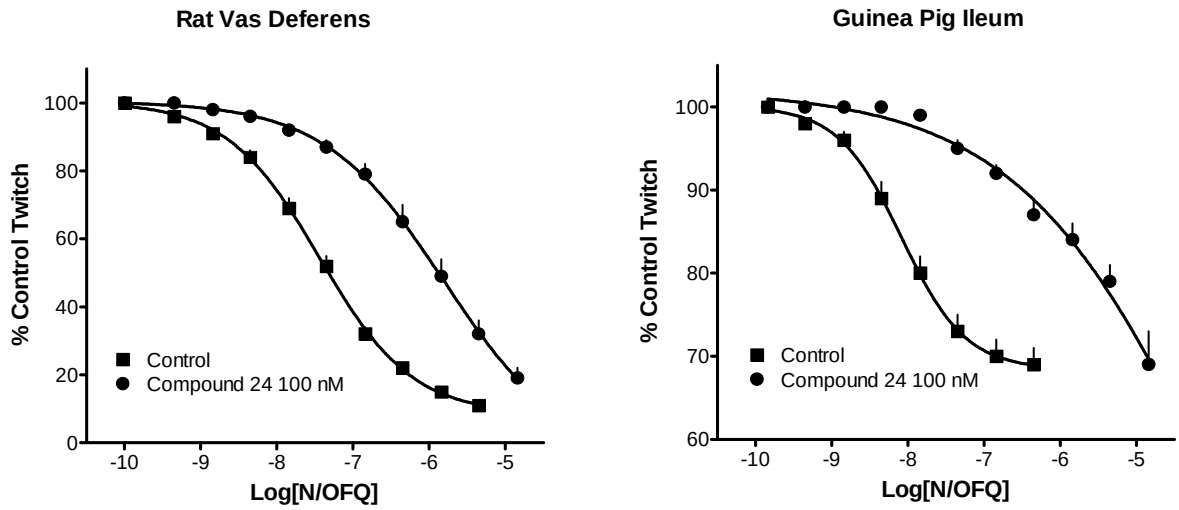


Figure 34. Concentration response curves to N/OFQ obtained in the absence (control) and presence of 100 nM Compound 24 in the electrically stimulated rat vas deferens (left panel) and guinea pig ileum (right panel). Data are mean ± s.e.m. of 4 separate experiments.

Finally, Compound 24 at 1 µM did not modify the inhibitory effects of DPDPE in the mouse vas deferens (control: pEC_{50} (95% confidence limit) 8.38 (8.20 - 8.56), E_{max}, 98 ± 1%; 1 µM Compound 24: pEC_{50} 8.30 (7.80 - 8.80), E_{max}, 99 ± 1%) or those evoked by dermorphin in the guinea pig ileum (control: pEC_{50} 8.52 (8.35 - 8.69), E_{max}, 98 ± 3%; 1 µM Compound 24: pEC_{50}, 8.51 (8.35 - 8.67), E_{max}, 95 ± 3%).

Tail withdrawal assay - In tail withdrawal experiments, mice injected with vehicle (either i.c.v. or i.t.) displayed tail withdrawal latencies of approximately 5 s that were stable over the time course of the experiment (Figure 35). In line with previous studies, N/OFQ (1 nmole) applied i.c.v. significantly reduced tail withdrawal latency with a maximal effect (about 50% reduction in tail withdrawal latency) obtained at 5 min ($AUC_{[0\text{-}15\ min]}$ vehicle 77 ± 5; 1 nmole of N/OFQ 51 ± 3, $p < 0.05$). The i.p. administration of Compound 24 up to 10 mg/kg did not modify, *per se*, tail withdrawal latencies ($AUC_{[0\text{-}15\ min]}$, 80 ± 7) but prevented the pronociceptive effects of the natural peptide ($AUC_{[0\text{-}15\ min]}$, 66 ± 5) (Figure 35, left panel). When the same dose of N/OFQ was administered i.t., a statistically significant antinociceptive effect was recorded ($AUC_{[0\text{-}30\ min]}$ vehicle, 165 ± 14; 1 nmole of N/OFQ 346 ± 25, $p < 0.05$) (Figure 35, right panel). This antinociceptive effect was reduced by Compound 24 ($AUC_{[0\text{-}30\ min]}$, 226 ± 25) (Figure 35, right panel).

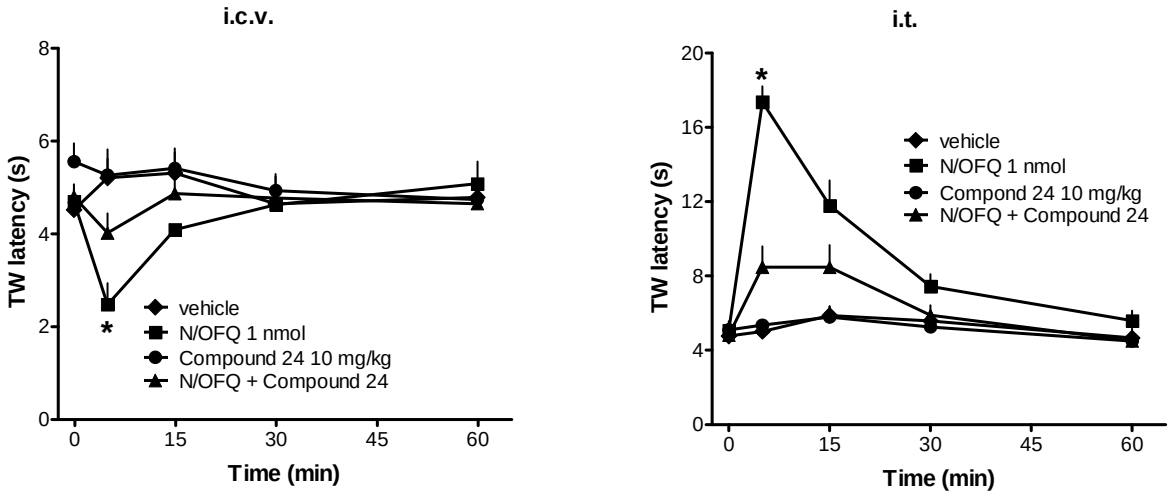


Figure 35. Mouse tail withdrawal assay. Effects of Compound 24 (10 mg/kg i.p., 30 min pre-treatment) on the pronociceptive or antinociceptive effects induced by 1 nmole N/OFQ injected i.c.v. (left panel) or i.t. (right panel). Data are mean ± s.e.m. of 4 separate experiments. *p<0.05 versus vehicle according to ANOVA followed by the Dunnett's test.

Discussion

The present study extend previous findings (Goto *et al.*, 2006) demonstrating that Compound 24 binds with high affinity to the NOP receptor and behaves as a pure and potent NOP receptor antagonist with high selectivity over classical opioid receptor. These pharmacological features of Compound 24 were consistently observed in various assays and preparations expressing the human recombinant as well as the animal native receptors. In addition, the NOP antagonistic properties of Compound 24 have been confirmed *in vivo* in mice in the tail withdrawal assay. Therefore, Compound 24 represents a valuable research tool that should be included in the class of selective NOP receptor antagonists and used in future target validations studies.

In receptor binding studies Compound 24 displayed very high affinity for the NOP receptor. The pK_i value calculated from the present experiments (9.62) is virtually superimposable to that previously reported by Goto *et al.* (2006) (pIC_{50} 9.57). These values of affinity are similar to that reported for SB-612111 and higher than that of J-113397 (Ozaki *et al.*, 2000; Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004). In functional studies performed on the human recombinant receptor ([^{35}S]GTPγS binding and calcium mobilization assays) and on animal native receptors from various species (mouse, rat, guinea pig) Compound 24 consistently behaved as a pure NOP receptor antagonist showing, in line with receptor binding studies, high values of potency (range 8.28 – 9.12). The only result which was out of this range is that obtained in [^{35}S]GTPγS binding studies where Compound 24 displayed a pA_2 of 9.98. However, this result, which is again superimposable

to that obtained by Goto *et al.* (2006) (pIC_{50} 9.82), is expected on the basis of what we have observed with several NOP receptor antagonists, including the peptides [Nphe^1]N/OFQ(1–13)-NH_2 and UFP-101 (Calo *et al.*, 2005; Calo *et al.*, 2002a), and the non-peptides J-113397, Trap-101, and SB-612111 (Spagnolo *et al.*, 2007; Trapella *et al.*, 2006), that consistently showed, in this particular assay, values of potency approximately 10 fold higher than in the other tests. As previously suggested (Spagnolo *et al.*, 2007), this might be due to the higher receptor accessibility in membranes (where the [^{35}S]GTPγS binding assay is performed) than in whole cells or tissue preparations (where the other assays are performed). Despite this minor difference, very similar antagonist potency values were obtained in the different assays for Compound 24 demonstrating the recombinant and native as well as species-specific NOP receptors are similarly sensitive to this antagonist. This also applies to the NOP receptors expressed in rat periaqueductal gray slices (Yan-Yu *et al.*, 2008) and sympathetic neurons (Ruiz-Velasco *et al.*, 2008) although no quantitative data were reported in these latter studies. The consistency of Compound 24 potency values among preparations and species corroborates previous findings obtained with peptide (Calo *et al.*, 2000a; Calo *et al.*, 2005; Calo *et al.*, 2002a; Calo *et al.*, 2002b) and non-peptide (Bigoni *et al.*, 2000a; Ozaki *et al.*, 2000; Spagnolo *et al.*, 2007; Trapella *et al.*, 2006; Zaratin *et al.*, 2004) NOP antagonists and enable the following rank order of antagonist potency to be proposed: Compound 24 (≈8.5) = SB-612111 (≈8.5) > J-113397 (≈8.0) > UFP-101 (≈7.5) = Trap-101 (≈7.5) > [Nphe^1]N/OFQ(1–13)-NH_2 (≈6.5) as NOP receptor fingerprint.

With respect to the type of antagonism exerted by Compound 24, results obtained in the [^{35}S]GTPγS binding and mouse vas deferens assay by performing concentration response curve to N/OFQ in the presence of increasing concentrations of antagonist are clearly compatible with a competitive type of interaction between Compound 24 and N/OFQ. Similar results were obtained by testing Compound 24 in the calcium mobilization assay where a rightward displacement of the concentration response curve to N/OFQ was recorded in response to increasing concentration of antagonist. However in these experiments Compound 24 at the highest concentrations tested produced a slight but statistically significant reduction of N/OFQ maximal effect. This apparent insurmountable antagonism behaviour can be attributed to i) the transient nature of the calcium response that may not allow equilibrium between agonist–antagonist competition to be reached thus generating depression of the agonist response in the presence of high concentrations of antagonist (Kenakin, 2004), ii) lack of stirring in the 96 well plate which is another source of hemiequilibrium conditions (Kenakin, 2004), iii) a combination of the two factors. The observation that a truly competitive antagonist produces a reduction of agonist maximal effects in calcium mobilization

assay is not uncommon. For instance, the urotensin-II receptor antagonists urantide and UFP-803 competitively antagonized the contractile effects of urotensin-II in the rat aorta bioassay while depressed maximal responses to the agonist in calcium experiments performed on cells expressing the rat recombinant urotensin-II receptor (Camarda *et al.*, 2006). On this basis, we propose to classify Compound 24 as a competitive NOP receptor antagonist.

Selectivity of action along with high affinity/potency and pure antagonist activity is another important feature of a valuable research tool. This property of Compound 24 has been evaluated in three sets of experiments over classical opioid receptors. In receptor binding studies Compound 24 displayed approximately 1000 fold selectivity over classical opioid receptors. A superimposable value of selectivity was found in functional studies performed measuring calcium mobilization in cells expressing the $G\alpha_{qi5}$ chimeric protein. Finally at least 300 fold selectivity of Compound 24 for NOP over MOP and DOP was obtained in bioassay experiments. These data confirm the impressive selectivity profile of Compound 24 reported by Goto *et al.* (2006). Thus, in terms of selectivity over classical opioid receptor Compound 24 seems to be similar to SB-612111 (Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004) and UFP-101 (Calo *et al.*, 2002b) and certainly more selective then J-113397 (Ozaki *et al.*, 2000; Spagnolo *et al.*, 2007) for which NOP independent effects were reported *in vivo* (Koizumi *et al.*, 2004). However more comprehensive selectivity studies are needed against a large panel of receptors and ion channels to firmly classify Compound 24 as a highly selective NOP receptor antagonist.

Collectively, these *in vitro* data confirm and extend previous findings (Goto *et al.*, 2006) demonstrating that Compound 24 is a pure, competitive, selective and potent NOP receptor antagonist. This excellent *in vitro* pharmacological profile is associated with good brain penetration after peripheral administration (Goto *et al.*, 2006). Indeed, Compound 24 was reported to be active *in vivo* in mice where at 10 mg/kg it prevented the inhibitory effects on locomotor activity of a non-peptide NOP agonist (Goto *et al.*, 2006). Here we assessed the *in vivo* effects of Compound 24 in the mouse tail withdrawal assay. N/OFQ has been repeatedly demonstrated to exert in rodents pronociceptive and antinociceptive effects following supraspinal and spinal injection, respectively (Zeilhofer *et al.*, 2003). The present results i.e. pronociceptive and antinociceptive response to 1 nmole N/OFQ given i.c.v. and i.t., are therefore in line with the literature. Both of these *in vivo* actions of N/OFQ are due to selective NOP receptor activation as consistently demonstrated by knockout (Nazzaro *et al.*, 2007; Nishi *et al.*, 1997) and receptor antagonist (UFP-101 (Calo *et al.*, 2002b; Nazzaro *et al.*, 2007), J-113397 (Ozaki *et al.*, 2000; Ueda *et al.*, 2000), SB-612111 (Rizzi *et al.*, 2007a; Zaratin *et al.*, 2004)) studies. In line with these findings, Compound 24 at 10 mg/kg

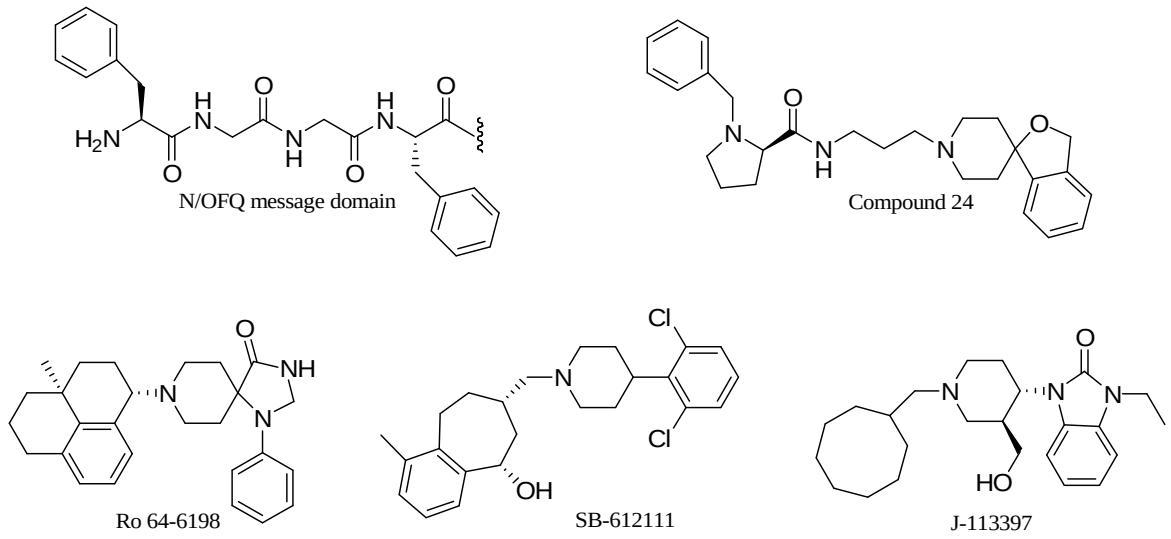
antagonised both the pronociceptive and antinociceptive effects evoked by N/OFQ when given spinally and supraspinally, respectively. This result confirms on the one hand that Compound 24 is an effective antagonist at NOP receptors regulating pain transmission *in vivo* and on the other that the effects of N/OFQ on pain transmission are exclusively due to NOP receptor activation. Interestingly, Compound 24 at 10 mg/kg counteracted rather than fully prevented the effects of 1 nmole N/OFQ. Under the same experimental conditions SB-612111 completely blocked the actions of the same dose of N/OFQ at ten fold lower doses (i.e. 1 mg/kg) (Rizzi *et al.*, 2007a). Therefore SB-612111 appeared to be more potent than Compound 24 *in vivo* in the mouse tail withdrawal assay. Since the *in vitro* potency of the two antagonists is similar (see above), it can be proposed that *in vivo* potency differences may derive from better pharmacokinetic properties of SB-612111 than Compound 24. However, this is merely speculation that required rigorous experimental validation. Finally, the systemic administration of Compound 24 at pharmacologically active doses did not modify *per se* tail withdrawal latencies. Again this is in line with previous findings obtained with both receptor antagonists (Ozaki *et al.*, 2000; Rizzi *et al.*, 2007a; Ueda *et al.*, 2000; Zaratin *et al.*, 2004) and mice knockout for the NOP receptor gene (Nazzaro *et al.*, 2007; Nishi *et al.*, 1997), and indicates that the endogenous N/OFQ-NOP receptor system is not activated by the mild and acute stimulus employed for evoking the nociceptive response in this assay. However, endogenous N/OFQergic signalling can be activated using more intense and prolonged nociceptive stimuli such as formalin. In fact in the mouse formalin assay the spinal antinociceptive action of endogenous N/OFQ seems to prevail over the supraspinal pronociceptive effect as indicated by the pronociceptive phenotype of NOP knockout mice (Depner *et al.*, 2003; Rizzi *et al.*, 2006) (recently confirmed in the acetic acid-induced writhing test by Rizzi *et al.* (2008)) and by the pronociceptive effects elicited by NOP antagonists e.g. systemic J-113397 (Rizzi *et al.*, 2006).

In conclusion the present study demonstrated that Compound 24 is a pure, competitive, selective and potent NOP receptor antagonist. The NOP antagonist properties of Compound 24 were demonstrated in a large panel of *in vitro* assays and *in vivo* in the mouse tail withdrawal assay. Based on its pharmacological profile, Compound 24 should be included (together with J-113397, SB-612111 and UFP-101) in the list of potent and selective NOP receptor antagonists to be tested in future target validation studies to firmly define their therapeutic potential as innovative drugs for treating depression, Parkinson's disease and possibly sepsis.

3.4 Blending of chemical moieties of NOP receptor ligands: identification of a novel antagonist

Compound 24 displays some chemical characteristics typical of N/OFQ related peptides (Figure 36), these include: i) a spacer of 12 atoms between the two phenyl rings which corresponds to the Phe-Gly-Gly-Phe sequence of the N/OFQ message domain; ii) an amide bond which is quite uncommon in non-peptide NOP ligands; iii) a N-benzyl amino acid, a chemical moiety also present in the N-terminal part of the NOP receptor peptide antagonists [Nphe^{1}]N/OFQ(1-13)-NH_2 (Guerrini *et al.*, 2000a) and UFP-101 (Guerrini *et al.*, 2005). Based on these considerations, in the present study, the importance of the N-benzyl D-Pro of Compound 24 was assessed by replacement with L- or D-Phe, and Nphe. In addition, the amide bond of Compound 24 was substituted with other amide bond isosters. Moreover, the Compound 24 spiroisobenzofurane nucleus was replaced with chemical moieties derived from other non-peptide NOP ligands i.e. Ro 64-6198, SB-612111 and J-113397 (Figure 36). The novel molecules were evaluated for their ability to bind the human recombinant NOP receptor expressed in CHO_{hNOP} cell membranes. The molecule showing the highest affinity, i.e. Compound **35** has been further characterized at the recombinant human NOP in the [^{35}S]GTPγS binding and calcium mobilization assays and at native NOP receptors expressed in isolated animal (mouse, rat, guinea-pig) tissues.

Figure 36. Chemical formulae of nociceptin/orphanin FQ receptor ligands.



Results and Discussion

Substitution into Compound 24 structure of the N-benzyl D-Pro with D or L-Phe (compounds **9** and **10**) or Nphe (compound **11**) produced a profound (> 100 fold) loss of NOP

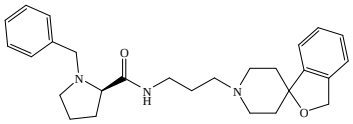
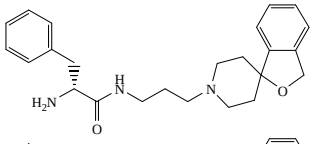
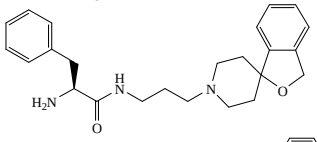
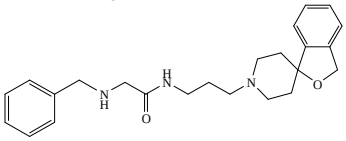
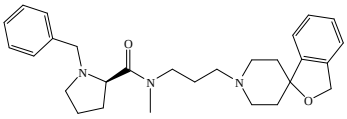
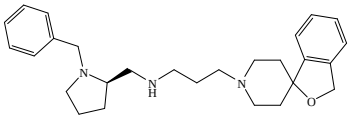
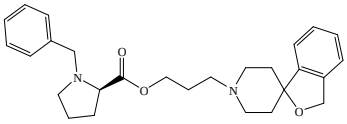
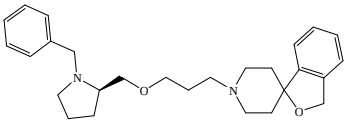
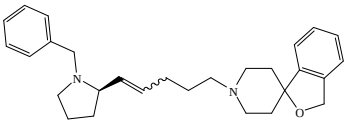
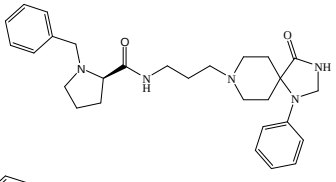
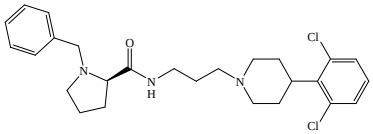
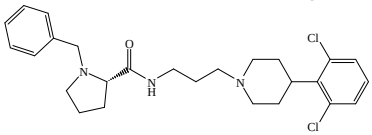
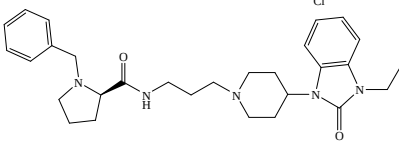
affinity suggesting a pivotal role of the N-benzyl D-Pro moiety for Compound 24 bioactivity (Table 8). This result is not surprising since the simple inversion of Pro chirality was reported to generate an analog 200 fold less potent than Compound 24 (Goto *et al.*, 2006).

Modifications of the amide bond obtained by N-methylation (compound **12**) or by replacement with a methyleneamino (compound **13**), an ester (compound **17**), a methyleneoxy (compound **20**), or an alkene bond (compound **25**), generated a drastic reduction in NOP receptor binding or, in the case of compound **25**, complete loss of affinity. These results indicated that the amide bond represents a chemical feature crucial for the bioactivity of Compound 24. It is worthy of note at this regard, that an amide bond is a relatively uncommon chemical feature in non-peptide NOP ligands. However, this chemical bond is also present in the NOP receptor antagonist JTC-801 and its chemical modification (i.e. N-methylation and retro-inverso bond) was reported to be detrimental to binding affinity (Shinkai *et al.*, 2000). Thus, the amide bond might play a similarly important role in both Compound 24 and JTC-801. In particular, according to the pharmacophoric model for non-peptide NOP ligands proposed by Zaveri *et al.* (2005), the amide bond may be part of the B-moiety that links and contributes to maintain in the correct spatial disposition the A-moiety (important for affinity and selectivity) and the B-moiety (important for efficacy).

Next, we considered replacement of the benzoisofurane in position 4 of the Compound 24 piperidine scaffold with chemical moieties taken from the same position of other high affinity NOP receptor ligands. The substitution in position 4 with 1-phenylimidazolidin-4-one, the chemical group of the NOP receptor agonist Ro 64-6198 (compound **28**), or with 1-ethylbenzoimidazol-2-one, the chemical group of the NOP receptor antagonist J-113397 (compound **41**), generated inactive molecules. Interestingly, the insertion in position 4 of the piperidine scaffold of the 2,6-dichlorophenyl moiety (Compound **35**), a pharmacophore of the NOP receptor antagonist SB-612111, generated a molecule with high affinity for the NOP receptor (pK_i 9.14).

In particular Compound **35** is only 3 fold less potent than Compound 24. The results obtained with this limited series of chimeric compounds suggest that it is possible to combine pharmacophoric moieties taken from different NOP receptor ligands to generate novel biologically active molecules. In particular, the chemical groups benzoisofurane and 2,6-dichlorophenyl seem to contribute to NOP receptor binding in a very similar manner. This is corroborated by the finding that the substitution of D-Pro with L-Pro in the chimeric NOP ligand compound **36** produced a 100-fold reduction in receptor affinity. This result is in agreement with that previously observed with compounds 22 and 23 of the Goto series (Goto *et al.*, 2006). Thus, the D chirality of the Pro residue represents a crucial requirement for both Compound 24 and Compound **35** for high affinity NOP receptor recognition.

Table 8. Binding affinities at CHO_{hNOP} cell membranes of Compound 24 and related compounds.

Compound	*Structure*	pK_i
Compound 24		9.62 ± 0.07
9		7.23 ± 0.19
10		7.23 ± 0.05
11		7.22 ± 0.08
12		5.94 ± 0.28
13		7.31 ± 0.06
17		6.97 ± 0.02
20		6.80 ± 0.80
25		< 5
28		< 5
35		9.14 ± 0.05
36		7.08 ± 0.02
41		< 5

Based on its high affinity for the NOP receptor, Compound **35** was selected for further pharmacological characterization. As shown in Figure 37 (left panel) in CHO_{hNOP} cell membranes N/OFQ concentration-dependently stimulated [^{35}S]GTPγS binding with pEC_{50} and E_{max} values of 8.91 and 10.93 ± 0.18 (stimulation factor), respectively. Over the concentration range of 1 – 100 nM Compound **35** was inactive *per se* but produced a rightward shift of the concentration response curve to N/OFQ in a parallel manner and without modifying the maximal effect. Schild analysis of these data (Figure 37, right panel) is compatible with a competitive type of antagonism with a pA_2 value of 9.91. This potency value is close to that previously reported for Compound 24 by Goto *et al.* (2006) (pIC_{50} 9.82) and by us (see Figure 31) (pA_2 9.98).

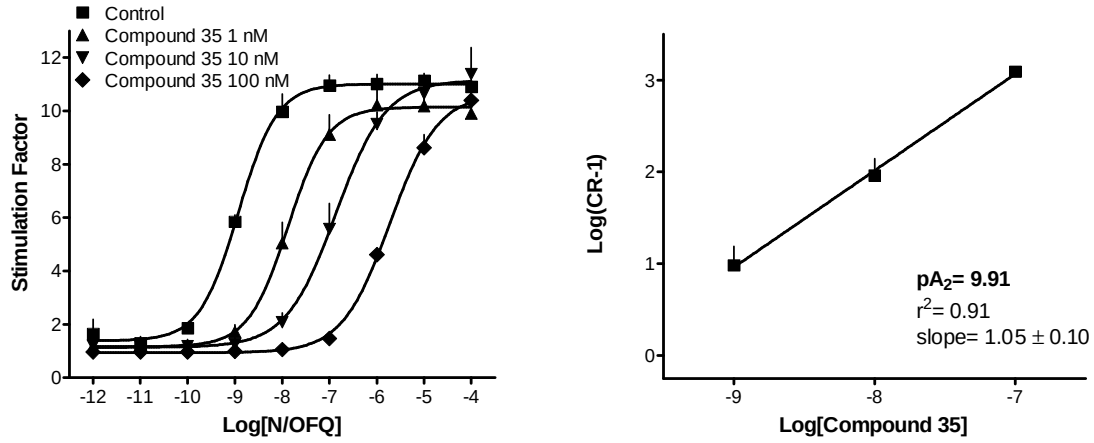


Figure 37. Concentration response curve to N/OFQ in the absence and presence of increasing concentrations of Compound **35** on [^{35}S]GTPγS binding in CHO_{hNOP} cell membranes. The corresponding Schild plot is shown in the right panel. Data are means ± s.e.m. of 4 separate experiments.

These results suggest that the benzoisofurane and 2,6-dichlorophenyl moieties of Compound 24 and Compound **35** play a similar role not only in receptor binding but also in determining pharmacological activity i.e. pure and competitive antagonism. This may derive from the common ability of the spiro junction (Compound 24) and of the 2,6 dichloro substitution (Compound **35**) to favor an orthogonal spatial disposition between their piperidine and phenyl nuclei. Therefore, this conformational feature may likely be crucial for both NOP receptor binding and antagonist activity.

The pharmacological actions of Compound **35** were further assessed at the hNOP receptor coupled to calcium signalling via the chimeric protein $G\alpha_{qi5}$; this assay has been previously

validated with a large panel of NOP receptor full and partial agonists and antagonists (see section 3.1). In CHO cells stably expressing the hNOP receptor and the $G\alpha_{qi5}$ protein, N/OFQ produced a concentration-dependent stimulation of intracellular calcium levels with a pEC_{50} of 9.24 and an E_{max} of 198 ± 12 % over the basal values. Compound **35** was inactive *per se* up to 10 µM while inhibiting in a concentration-dependent manner the stimulatory effect of 10 nM N/OFQ. A pK_B value of 8.47 was derived from these experiments (Table 9). This value of potency is approx 3 fold lower than that obtained with Compound 24 (pK_B 9.03, see Table 7).

Table 9. Compound **35** affinity/antagonist potency in various pharmacological assays.

	CHO_{hNOP} cell membranes		$CHO_{hNOP\ /\ G\alpha qi5}$ cells	Electrically stimulated tissues		
	receptor binding	$[^{35}S]GTP\gamma S$ binding	Ca^{2+} mobilization	mouse vas deferens	rat vas deferens	guinea pig ileum
	pK_i	pA_2	pK_B	pA_2	pK_B	pK_B
Compound **35**	9.14 (9.04 – 9.24)	9.91 (9.23 – 10.59)	8.47 (8.31 – 8.63)	8.00 (7.32 – 8.68)	8.06 (7.58 – 8.54)	8.84 (8.64 – 9.04)

Data are means ($CL_{95\%}$) of at least 4 separate experiments.

The competitive antagonist behaviour of Compound **35** was confirmed at the native NOP receptor expressed in the mouse vas deferens. In this preparation, N/OFQ inhibited electrically evoked twitches in a concentration-dependent manner with pEC_{50} and E_{max} values of 7.49 of -80 ± 2 %, respectively (Figure 38, left panel). Compound **35** was inactive *per se* but caused a concentration-dependent (10 – 1000 nM) and parallel rightward shift of the concentration response curve to N/OFQ without modifying the agonist maximal effects. The relative Schild plot, depicted in Figure 38 (right panel), demonstrated a competitive type of antagonism with a pA_2 value of 8.00. This value of potency is close to that previously reported for Compound 24 i.e. 8.44 (see Figure 33).

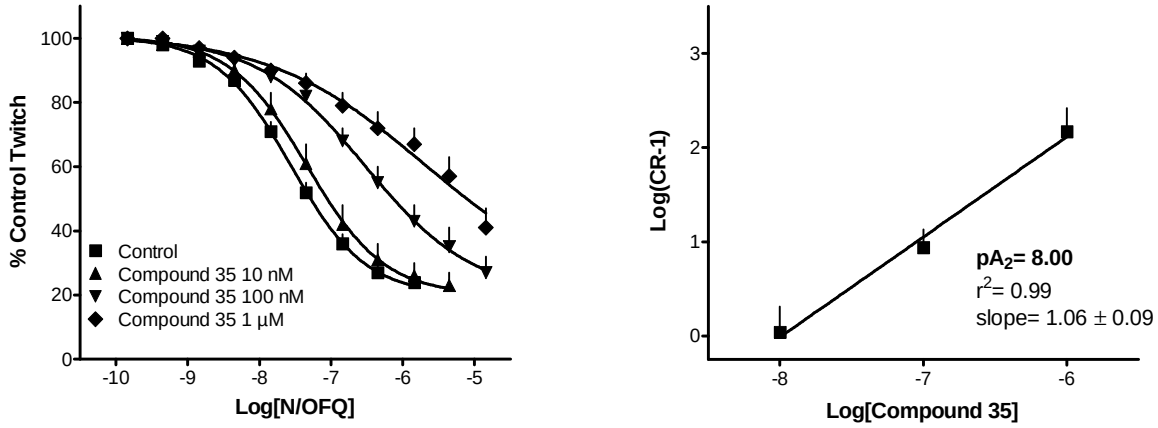


Figure 38 Concentration response curve to N/OFQ in the absence and presence of increasing concentrations of Compound **35** on the electrically stimulated mouse vas deferens. The corresponding Schild plot is shown in the right panel. Data are means ± s.e.m. of 4 separate experiments.

Similar results were obtained in other N/OFQ sensitive preparations such as the rat vas deferens and guinea pig ileum where Compound **35** at 100 nM antagonized N/OFQ inhibitory action with pK_b values of 8.06 and 8.84, respectively (Figure 39, Table 9).

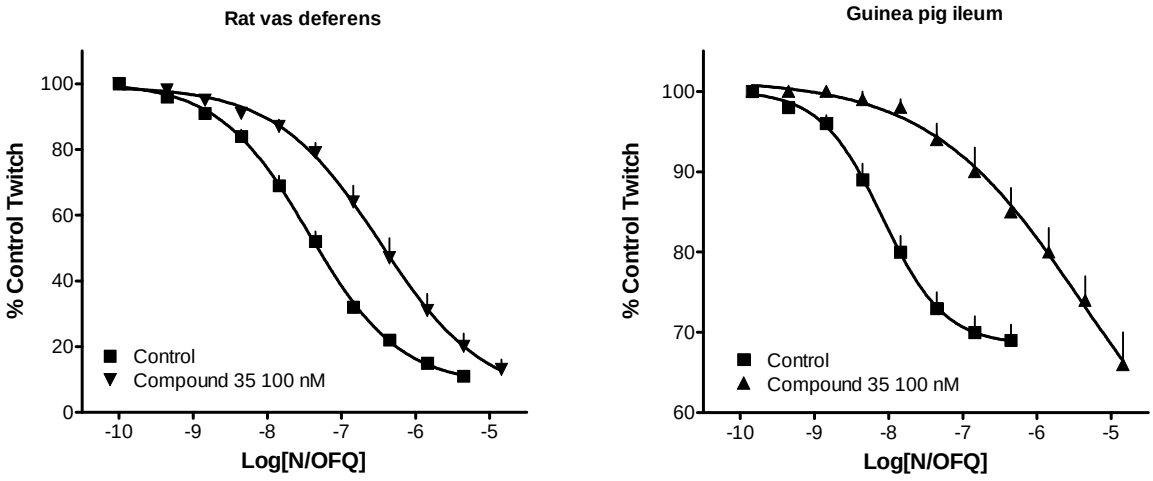


Figure 39. Concentration response-curve to N/OFQ obtained in the absence (control) and presence of Compound **35** (100nM) in the electrically stimulated rat vas deferens (left panel) and in guinea pig ileum (right panel). Data are means ± s.e.m of 3 separate experiments.

Finally the selectively of action of Compound **35** over classical opioid receptors was assessed in animal tissues expressing native receptors and at recombinant human proteins. Compound **35** at 1 μM was inactive *per se* and did not modify the inhibitory effects elicited by the DOP selective agonist DPDPE in the mouse vas deferens (control pEC_{50} 8.38 ($CL_{95\%}$ 8.20 – 8.56), E_{max} -98 ± 1%; 1 μM Compound **35** pEC_{50} 8.44 ($CL_{95\%}$ 8.26 – 8.62), E_{max} -98 ± 1%) or those produced by the MOP agonist dermorphin in the guinea pig ileum (control pEC_{50} 8.52 ($CL_{95\%}$ 8.35 – 8.69), E_{max} -90 ± 3%; 1 μM Compound **35** pEC_{50} 8.44 ($CL_{95\%}$ 8.19 – 8.69), E_{max} -85 ± 5%).

Results obtained with Compound **35** and the universal opioid receptor antagonist naloxone in selectivity studies performed with receptor binding and calcium mobilization assays are summarized in tables 10 and 11, respectively. Compound **35** up to 10 μM did not displace [^{3}H]DPN from DOP sites and showed very low affinity for MOP and KOP receptors (Table 10). In contrast, naloxone did not bind the NOP receptor up to 10 μM, while it displaced [^{3}H]DPN from classical opioid receptors with very high affinity for MOP and lower affinities for KOP and DOP (Table 10).

Table 10. Affinities of Compound **35** and naloxone at NOP and classical opioid receptors expressed in CHO cell membranes.

receptor	**NOP**	**MOP**	**DOP**	**KOP**
radioligand	[^{3}H]N/OFQ	[^{3}H]DPN	[^{3}H]DPN	[^{3}H]DPN
naloxone	< 6	9.25 (9.04 – 9.46)	7.67 (7.59 – 7.75)	8.35 (8.20 – 8.50)
Compound **35**	9.14 (9.04 – 9.24)	6.72 (6.47 – 6.97)	< 6	6.50 (6.37 – 6.63)

Data are mean ($CL_{95\%}$) of 4 separate experiments. Naloxone data on classical opioid receptors are from Vergura *et al.* (2008).

Similar results were found in functional studies performed measuring calcium mobilization in CHO cells stably expressing the hNOP receptor or classical opioid receptors and the $G\alpha_{qi5}$ protein (Table 11). Dermorphin, DPDPE and dynorphin A were used in these experiments as agonists for the MOP, DOP and KOP receptors, respectively. All produced a concentration-dependent increase in calcium levels with the following pEC_{50} values: 7.93 ($CL_{95\%}$ 7.67 – 8.19), 8.82 ($CL_{95\%}$ 8.43 – 9.21), 8.47 ($CL_{95\%}$ 8.16 – 8.78). Naloxone inhibited the effects of these agonists showing higher potency at MOP than KOP and DOP (Table 11), and being inactive against N/OFQ. Compound **35** was at least 300 fold less potent at classical opioid receptors than at the NOP receptor (Table 11).

Table 11. Antagonist potencies of Compound **35** and naloxone evaluated in calcium mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and the $G\alpha_{qi5}$ protein.

receptor	**NOP**	**MOP**	**DOP**	**KOP**
agonist	N/OFQ 10 nM	Dermorphin 100 nM	DPDPE 100 nM	Dynorphin A 100 nM
naloxone	< 6	9.09 (8.73 – 9.45)	7.32 (6.80 – 7.84)	7.14 (6.60 – 7.68)
Compound **35**	8.47 (8.31 – 8.63)	6.11 (5.92 – 6.30)	< 6	< 6

Data are mean ($CL_{95\%}$) of 4 separate experiments made in duplicate.

These pharmacological results confirmed that Compound **35** behaves as a pure, potent and competitive NOP receptor antagonist. Moreover selectivity studies demonstrated that the substitution of the benzoisofurane with the 2,6-dichlorophenyl moiety not only allows maintenance of a high affinity and antagonist potency but also high selectivity of action over classical opioid receptors.

3.5 General conclusions

The present thesis summarizes the work we performed in the last three years in the field of N/OFQ and its receptor. Part of this work was performed during 2007 in Prof. Lambert's laboratories at University of Leicester as part of the Joint Ferrara-Leicester PhD programme.

Following the formal identification of the receptor NOP and of its endogenous ligand N/OFQ, an extensive search has started to assess the biological functions regulated by this peptide-receptor system and to foresee the therapeutic indications of drugs interacting selectively with the NOP receptor. In parallel, academic and industrial laboratories generated new molecules that selectively activate or block the NOP receptor thus providing the pharmacological tools needed for target validation studies.

The major aim of this work was detailed *in vitro* and *in vivo* pharmacological characterization of novel ligands for the NOP receptor designed and synthesized by the medicinal chemistry group of Prof. Salvadori. To this aim, we develop and use different *in vitro* and *in vivo* assays: *in vitro* studies were performed measuring receptor and [^{35}S]GTPγS binding and calcium mobilization in cells expressing the recombinant NOP receptor as well as using N/OFQ sensitive

tissues from different species (mouse, rat, guinea pig). *In vivo* studies were conducted using an analgesiometric assay such as the tail withdrawal tests after both supraspinal and spinal administration. Moreover some *in vitro* and *in vivo* experiments were performed using NOP(+/+) and NOP(-/-) mice for investigating the involvement of the NOP receptor in the actions exerted by the novel ligands.

Our major contributions to this field can be summarized below:

1) We confirmed that it is possible to use the $G\alpha_{qi5}$ chimeric protein to force classical opioid and NOP receptors to signal via the calcium pathway. $[Ca^{2+}]_i$ levels were monitored using the fluorometer FlexStation II. A panel of full and partial agonists as well as antagonists were assessed in calcium mobilization experiments demonstrating that that the FlexStation II – $G\alpha_{qi5}$ NOP receptor calcium assay represents a very useful strategy for the screening of NOP receptor ligands particularly for antagonists. In fact, results obtained with this class of ligands in the $G\alpha_{qi5}$ NOP receptor calcium assay are superimposable to those collected with more classical G_i based biochemical assays performed on recombinant or native NOP receptors or with bioassays performed on isolated tissues expressing native NOP receptors, with the exception of ligands characterized by slow kinetics of interaction with the NOP receptor.

2) Parallel experiments, performed on mouse and rat tissues, confirmed previous finding demonstrating that ZP120 behaves as a high potency NOP selective partial agonist. In fact in both preparations ZP120 mimicked the effects of N/OFQ showing higher potency but lower maximal effects. The effects of N/OFQ and those elicited by ZP120 have been evaluated in the presence of two NOP receptor antagonists: UFP-101 and J-113397. The antagonist sensitivity of ZP120 effects (J-113397 sensitive and UFP-101 resistant) is not superimposable to that of the endogenous NOP ligand N/OFQ (J-113397 and UFP-101 sensitive). However the exclusive involvement of the NOP receptor protein in the *in vitro* as well as *in vivo* effects of ZP120 (and N/OFQ) was clearly demonstrated by knockout studies. Therefore the different sensitivity of N/OFQ and ZP120 to UFP-101 might be attributed to their diverse binding and activation of the NOP receptor. *In vivo*, ZP120 has been shown to mimic N/OFQ actions displaying higher potency and longer lasting effects than the natural ligand. These pharmacological features make ZP120 a promising and innovative drug candidate for treating heart failure and for exploiting the

therapeutic benefits deriving from a prolonged partial activation of peripheral NOP receptors.

3) Compound 24 has been recently identified as a novel non-peptide selective antagonist (Goto *et al.*, 2006). Our major goal was to perform an extensive *in vitro* and *in vivo* pharmacological characterization of the actions of this interesting molecule. Our findings derived from competition binding and functional studies on CHO_{hNOP}, calcium mobilization assay on CHO_{hNOP} co-expressing the $G\alpha_{qi5}$ chimeric protein, bioassay studies on native receptors and *in vivo* in the tail withdrawal assay, demonstrated that Compound 24 behaves as one of the most potent and selective non-peptide NOP antagonists. It is worth noting that synthesis of Compound 24 is relatively easy when compared to that of other non-peptide NOP antagonists such as J-113397 and SB-612111.

4) A novel ligand has been identified from SAR studies of the NOP receptor antagonist Compound 24, this compound is named Compound 35. Based on the novel structure of Compound 24 the group of Prof. Salvadori synthesized twelve new derivates focusing on the N-benzyl-D-proline, amide bond and benzoisofurane moieties. This latter structure was substituted with moieties taken from known non-peptide NOP ligands such as Ro 64-6198, SB-612111 and J-113397. The new derivates were evaluated for their ability to bind the human recombinant NOP receptor and the molecule showing the highest affinity (Compound 35) has been further characterized. *In vitro* studies were performed measuring receptor and [^{35}S]GTPγS binding and calcium mobilization in cells expressing the recombinant NOP receptor as well as using N/OFQ sensitive tissues. The pharmacological results confirmed that Compound 35 behaves as a pure, potent and competitive NOP receptor antagonist. Moreover selectivity studies demonstrated that the substitution of the benzoisofurane with the 2,6-dichlorophenyl moiety not only allows maintenance of a high affinity and antagonist potency but also high selectivity of action over classical opioid receptors. Based on its pharmacological profile Compound 35 might be useful in future *in vivo* studies aimed at investigating the possible therapeutic indications of selective NOP antagonists.

The development of new NOP receptor antagonists has to be considered of primary interest particularly in the field of pain (Fioravanti *et al.*, 2008; Zeilhofer *et al.*, 2003), obesity (Przydzial *et al.*, 2008), memory disorders (Jinsmaa *et al.*, 2000), sepsis (Carvalho *et al.*, 2008; Williams *et al.*, 2008) and mood (Gavioli *et al.*, 2006). In addition, very recent studies performed by the group of Prof. Morari (Marti *et al.*, 2005; Marti *et al.*, 2004a; Marti *et al.*, 2004b; Marti *et al.*, 2008; Marti *et al.*, 2007) provided very robust evidence that NOP antagonists are worthy of development as innovative drugs for Parkinson's disease.

A useful comparison between peptide and non-peptide NOP receptor antagonists is summarized in Table 12.

Table 12. Affinity and potency values of the peptide and non-peptide NOP receptor antagonists in different *in vitro* assays. Data are mean of at least 4 experiments.

	CHO_{hNOP}			**Electrically Stimulated Tissues**		
	Cell membranes		**Whole cells**			
	Receptor Binding	*[^{35}S]GTPγS Binding*	*Calcium Assay*	*mVD*	*rVD*	*gpI*
	pK_i	***pA_2***	***pK_B***	***pK_B***	***pK_B***	***pK_B***
UFP-101	10.24	9.12	7.66	7.29	7.30	7.18
J-113397	8.42	8.71	7.88	7.53	7.97	7.69
Trap-101	8.65	8.55	7.93	7.75	7.68	7.51
SB-612111	9.18	9.70	8.16	8.50	8.20	8.40
Compound 24	9.62	9.98	9.03	8.44	8.28	9.12
Compound 35	9.14	9.91	8.47	8.00	8.06	8.84

As shown in Table 12, all the NOP receptor antagonists were found to bind to the NOP receptor with high affinity (in the nanomolar range). For each compound pA_2 values obtained in [^{35}S]GTPγS binding experiments on CHO_{hNOP} were higher comparing with all the other tests. This might be due to the higher receptor availability in membranes (where the [^{35}S]GTPγS is performed) than in whole cells or tissues preparations (where the other tests are performed). Despite this minor difference, very similar potency values were obtained in different assays for all the antagonists. UFP-101, represents the most potent NOP peptide antagonist. J-113397 has been largely used as a standard non-peptide NOP antagonist. Among non-peptide molecules, SB-612111 is one of the

most potent and selective NOP receptor antagonist identified to date. *In vitro*, in most of the preparations, Compound 24 has shown potency values higher than those obtained by SB-612111. However, the *in vivo* potency of Compound 24 seems to be lower than that of SB-612111. Moreover, synthesis of Compound 24 and Compound 35 is easier compared with that of both J-113397 and SB-612111. Collectively these data suggest the following order of potency of antagonists: Compound 24 = Compound 35 = SB-612111 > J-113397 > UFP-101 = Trap-101. Based on the findings summarized in this thesis Compound 24 and Compound 35 should be included (together with J-113397, SB-612111 and UFP-101) in the list of potent and selective NOP receptor antagonists to be used in future target validation studies for firmly defining the therapeutic potential of selective NOP antagonists as innovative drugs.

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List of Publications

Full papers

- Spagnolo B., Carrà G., Fantin M., **Fischetti C**., Hebbes C., McDonald J., Barnes T.A., Rizzi A., Trapella C., Fanton G., Morari M., Lambert D.G., Regoli D., Calò G. Pharmacological characterization of the nociceptin/orphanin FQ receptor antagonist SB-612111 [(-)-cis-1-methyl-7-[[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl]-6,7,8,9-tetrahydro-5H-benzocyclohepten-5-ol]: in vitro studies. J Pharmacol Exp Ther. 2007;321(3):961-7.
- Rizzi A., Spagnolo B., Wainford R.D., **Fischetti C.**, Guerrini R., Marzola G., Baldisserotto A., Salvadori S., Regoli D., Kapusta D.R., Calo G. In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor. Peptides. 2007;28(6):1240-51.
- Arduin M., Calo' G., Guerrini R., Spagnolo B., **Fischetti C.**, Trappella C., Marzola E., Regoli D., and Salvadori S. Synthesis and biological activity of Nociceptin / Orphanin FQ analogues substituited in position 7 or 11 with Cα,α-dialkylated amino acids. Bioorg Med Chem. 2007;15(13):4434-43.
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- **Fischetti C.**, Rizzi A., Gavioli E.C., Marzola G., Trapella C., Guerrini R., Petersen J.S., Calo G. Further studies on the pharmacological features of the nociceptin/orphanin FQ receptor ligand ZP120. Peptides. 2009; 30:248-55.
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- Trapella C., **Fischetti C.**, Pela'M., Lazzari I., Guerrini R., Calo' G., Lambert D.G., McDonald J., Regoli D., Salvadori S. Blending of chemical moieties of NOP receptor ligands: identification of a novel antagonist. Submitted to J Med Chem.

Abstracts

- **Fischetti C**., Fantin M., Morari M. Nocistatin inhibits 5-hydroxytryptamine release in the mouse neocortex via G protein coupled presynaptic receptors. National Congress of the Italian-Swedish for Neuroscience, Ischia (Italy) 1-4 October 2005 (poster presentation).
- Marti M., Mela F., **Fischetti C**., Morari M. Nociceptin / Orphanin FQ receptor antagonists as a novel therapeutic approach to Parkinson's disease. National Congress of the Italian-Swedish for Neuroscience, Ischia (Italy) 1-4 October 2005 (poster presentation).
- Fantin M., **Fischetti C**., Ballini C., Della Corte L., Marti M., Morari M. Striatal NMDA receptors regulate the striato-pallidal pathway. National Congress of the Italian-Swedish for Neuroscience, Ischia (Italy) 1-4 October 2005 (poster presentation).
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