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DOTTORATO DI RICERCA IN FARMACOLOGIA E ONCOLOGIA MOLECOLARE

CICLO XXIV

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Nociceptin/orphanin FQ – NOP receptor system: novel genetic and pharmacological tools

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ABSTRACT

The neuropeptide nociceptin/orphanin FQ (N/OFQ) selectively binds and activates the N/OFQ peptide (NOP) receptor. In cells expressing the NOP receptor N/OFQ inhibits cAMP accumulation and Ca^{2+} conductance and stimulates K^+ currents. Via these cellular mechanisms N/OFQ regulates several biological functions in the central nervous system (pain, locomotion, memory, emotional responses, food intake), as well as in the periphery (airways, cardiovascular, genitourinary and gastrointestinal systems). Several research tools including knockout mice and NOP selective agonists and antagonists have been developed in the past and used to investigate the role played by this peptidergic system in pathophysiology and to identify possible therapeutic indications of NOP receptor ligands. The aim of the present study was to make available to the scientific community novel genetic and pharmacological tools to speed up the process of target validation of the NOP receptor.

Knockout rats for the NOP receptor gene (NOP(-/-)) have been recently generated. These animals were used in the present study to investigate their emotional (open field, elevated plus maze, and forced swimming test), locomotor (drag and rotarod test), and nociceptive (plantar and formalin test) phenotype in comparison to NOP(+/+) littermates. The results were in line with previous findings obtained with selective NOP receptor antagonists in mice and rats and with mouse knockout studies and indicated that the blockage of N/OFQergic signalling elicits antidepressant and motor stimulant effects.

A detailed pharmacological characterization of novel NOP receptor non peptide ligands has been performed. The compound GF-4 displayed high affinity and potency at recombinant human NOP receptor associated with pure antagonist properties. This profile was confirmed in N/OFQ sensitive animal tissues. In vivo GF-4 elicited, similar to other NOP antagonists, beneficial effects in animal models of Parkinson disease. The NOP non-peptide agonists Ro 65-6570, SCH 221510 and compound 6d were characterized in vitro using a calcium mobilization assay and electrically stimulated mouse and rat vas deferens tissues. The results of these studies demonstrated that Ro 65-6570 and SCH-221510 behaved as full agonists showing however some level of NOP selectivity in rat, but not mouse, tissues. Compound 6d did not display NOP selectivity.

Finally, mixed NOP/MOP receptor agonists were generated. [Dmt^1]N/OFQ(1-13)NH_2 was selected as the most potent compound. The mixed NOP/MOP full agonist activity and high affinity of [Dmt^1]N/OFQ(1-13)NH_2 was confirmed at human recombinant receptors in receptor and [^{35}S]GTPγS binding studies, at rat spinal cord receptors in [^{35}S]GTPγS binding experiments, and at guinea pig receptors inhibiting neurogenic contractions in the ileum. In vivo in the mouse tail withdrawal assay in mice [Dmt^1]N/OFQ(1-13)NH_2 was also able to elicit a robust antinociceptive effect being more potent than N/OFQ (by 30 fold) and morphine (by 3 fold). The antinociceptive properties of spinal [Dmt^1]N/OFQ(1-13)NH_2 were confirmed in non human primate studies. Collectively these results demonstrate that [Dmt^1]N/OFQ(1-13)NH_2 behaves as mixed NOP/MOP agonist and susbtantiate the suggestion that such mixed ligands are worthy of development as innovative spinal analgesics.

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 a large variety of functions in the human body and in particularly in the central nervous system. 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. In fact, more than 30% of the approximately 500 clinically used drugs, are modulators of GPCRs function, representing around 9% of global pharmaceutical sales, and 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 still 'orphans', i.e. its endogenous ligand is unknown.

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. In the past, 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. 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 Nociceptin/Orphanin FQ (N/OFQ), the subject of this thesis, as the endogenous ligand of the orphan GPCR Opioid Receptor-Like 1 (ORL-1) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

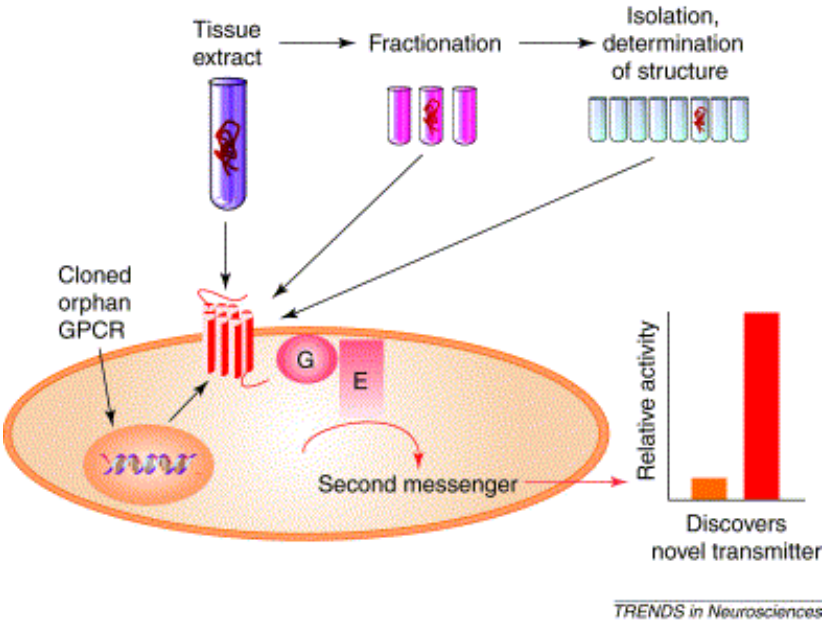


Figure 1.1. The orphan receptor strategy (Civelli *et al.*, 2001). The orphan receptor strategy was developed to identify the natural ligands of orphan G-protein-coupled receptors (GPCRs) with the aim of discovering novel transmitters (defined in the main text). This strategy involves: (1) expression 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 high throughput screening (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 orphan GPCRs and a revival of the reverse pharmacology approach. Table 1.1 summarizes the transmitters of peptide and non-peptide nature identified as ligands of orphan GPCRs after 1995 (Civelli, 2005).

Table 1.1. Transmitters identified as ligands of previously orphan GPCRs 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 of opioid drugs, such as 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 gaves many problems in manuscript preparation and particularly WEB searches, this was substituted 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, named 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 with 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 1.2).

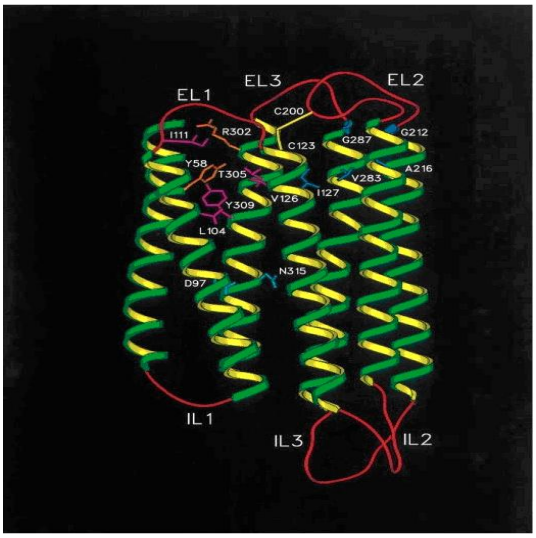


Figure 1.2. Schematic representation of ORL-1 receptor from (Topham *et al.*, 1998)). TM helices are numbered 1 to 7. E/IL: Extracellular/Intracellular Loop. Visible at the C-terminal of TM 6 is the Gln 286 (human receptor numbering) side chain.

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 (Mollereau *et al.*, 1994), rat (Wang *et al.*, 1994; Fukuda *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995), mouse (Nishi *et al.*, 1994) and pig (NOP (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 potential phosphorylation by protein kinase A (in the third intracellular loop) and protein kinase C (in the second intracellular loop and the C-terminal).

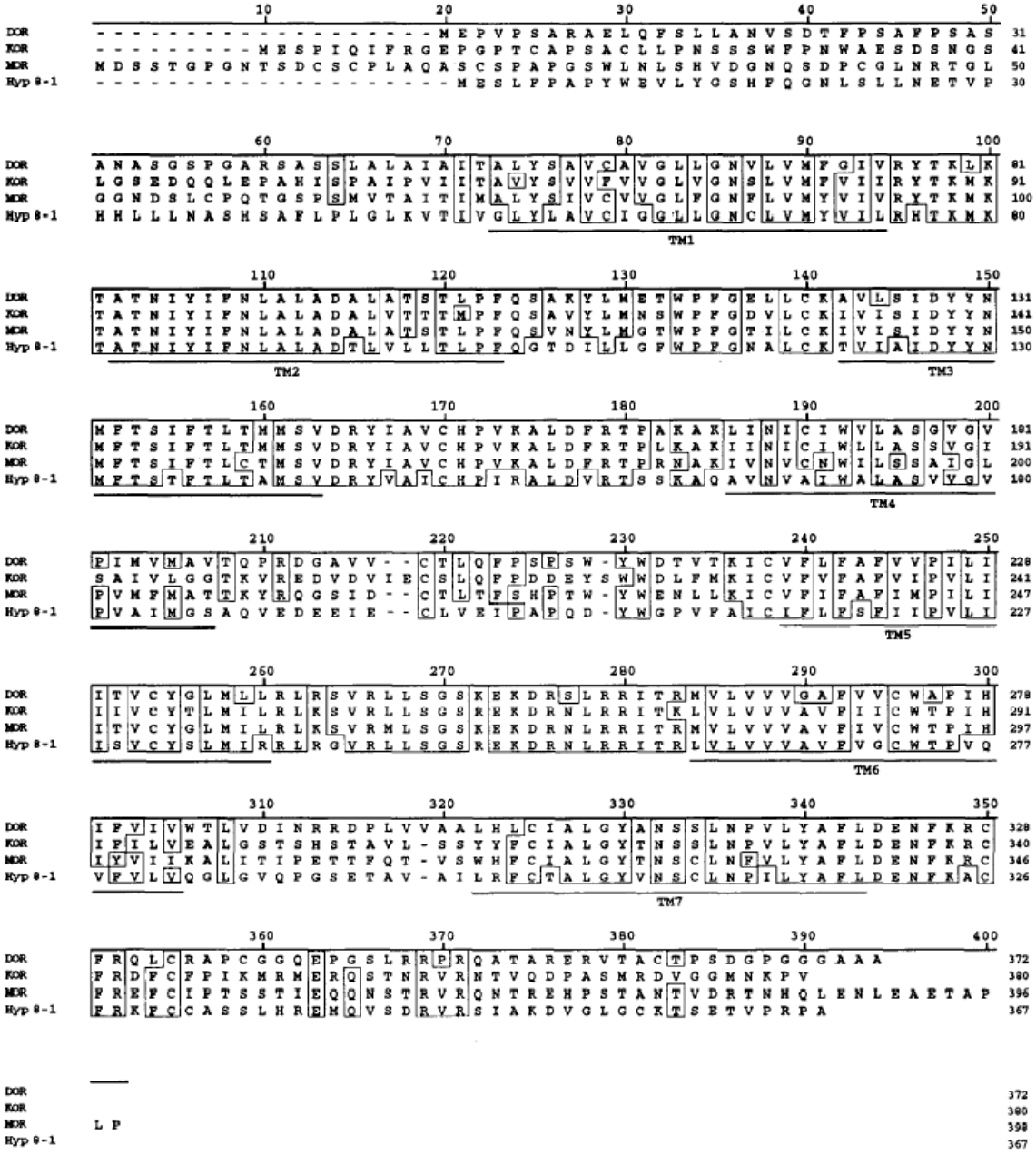


Figure 1.3. Alignment of the amino acid sequence of the rat NOP receptor (Hyp 8-1) with the amino acid sequences of the rat brain DOP, MOP and KOP receptors. Sequences identical in at least 3 of 4 aligned sequences are boxed. Gaps in the alignment are indicated by a dash (-). Putative transmenbrane regions are underlined. Taken from Wick *et al.* (1994).

The NOP sequence has 57-58% amino acid (aa) 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.

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 (Anton *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).

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 (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 (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).

1.3 Nociceptin/orphanin FQ

In 1995 Meunier and Reinsheid simultaneously described N/OFQ as the endogenous ligand for ORL-1, now known as NOP. CHO cells expressing the orphan receptor were used to identify its 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 1.4), 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 tests (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 1.4); 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).

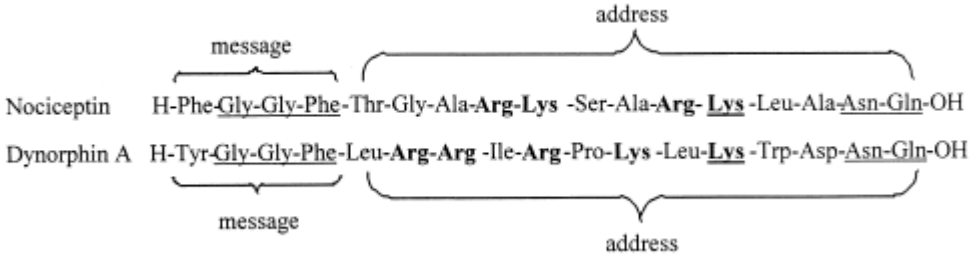


Figure 1.4. Structural similarities between dynorphin A and N/OFQ amino acid sequences (Guerrini *et al.*, 2000b).

The N-terminal tetrapeptide sequences (message domain) 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 domain) 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 peptide 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 1.5). The ppN/OFQ gene, that has been isolated from human, mouse and rat, is highly conserved in the three species.

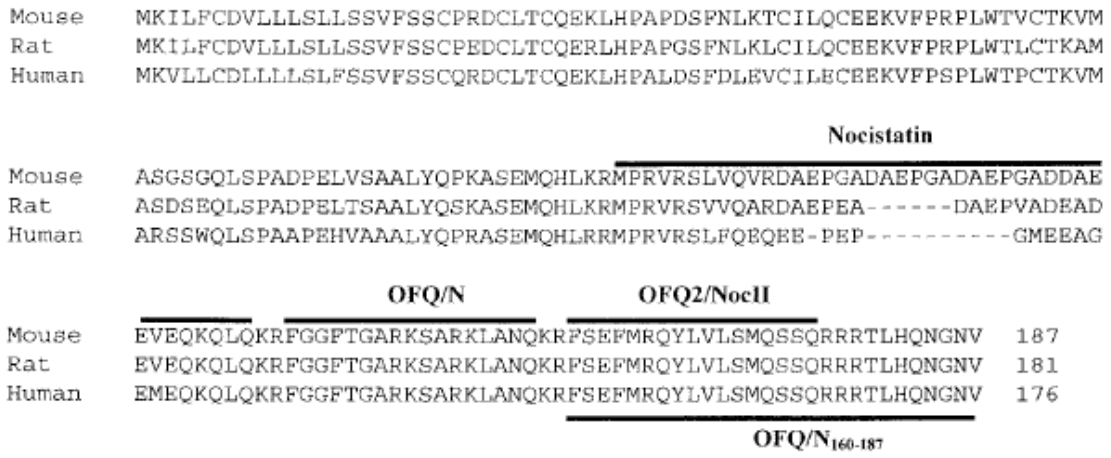


Figure 1.5. Amino acid sequence for N/OFQ precursor, ppN/OFQ (Calo *et al.*, 2000b).

Analysis of the nucleotide sequence of the ppN/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 1.5). Apparently two additional peptides are excised from the same precursor: N/OFQ2 and nocistatin (Okuda-Ashitaka *et al.*, 1998). None of them bind to the 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 (N/OFQ2): 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.*, 1999), 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 $G\alpha_{q/11}$-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).

The localization of N/OFQ-immunoreactive fibres 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). This N/OFQ distribution in the central nervous system suggests that the peptide is potentially involved in the regulation of a variety of brain functions, including emotional processing, learning and memory, locomotion, reward, pain transmission, and autonomic regulation of peripheral organs and systems.

In the periphery, mRNA of pp\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).

Little is known about the biosynthesis of N/OFQ, apart from the involvement of prohormone convertase 2 as demonstrated by studies performed in mice knockout for this enzyme (Allen *et al.*, 2001). As far as N/OFQ metabolism is concerned, different studies demonstrated the involvement of aminopeptidase N (APN) that generates [desPhe^{1}]N/OFQ a peptide lacking affinity for the NOP receptor. However different endopeptidases are also involved in N/OFQ metabolism. endopeptidase (EP) cleaves a variety of bonds to release inactive fragments, Figure 1.6 (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, where 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).

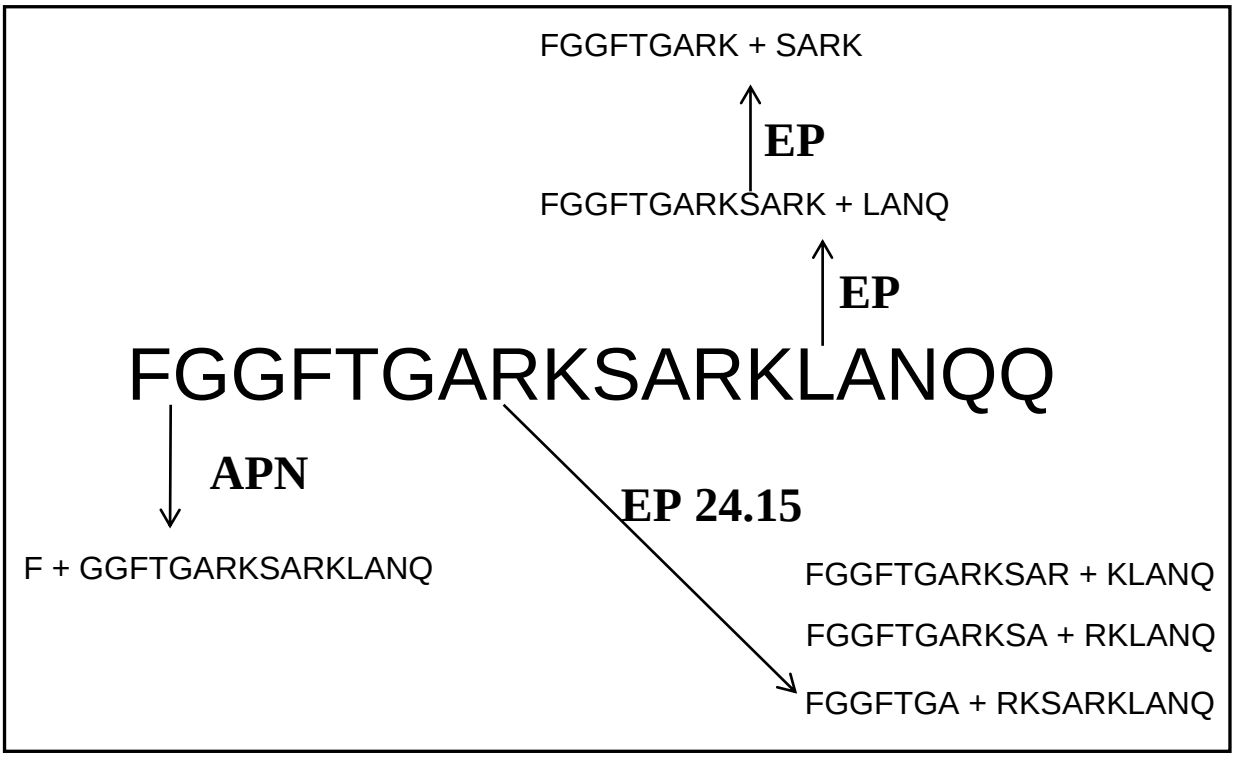


Figure 1.6. N/OFQ metabolism by aminopeptidase N (APN) and endopeptidases (EP), from (Calo *et al.*, 2000b).

1.4 N/OFQ and NOP receptor system: Cellular and Biological actions

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 1.7) (Hawes *et al.*, 2000).

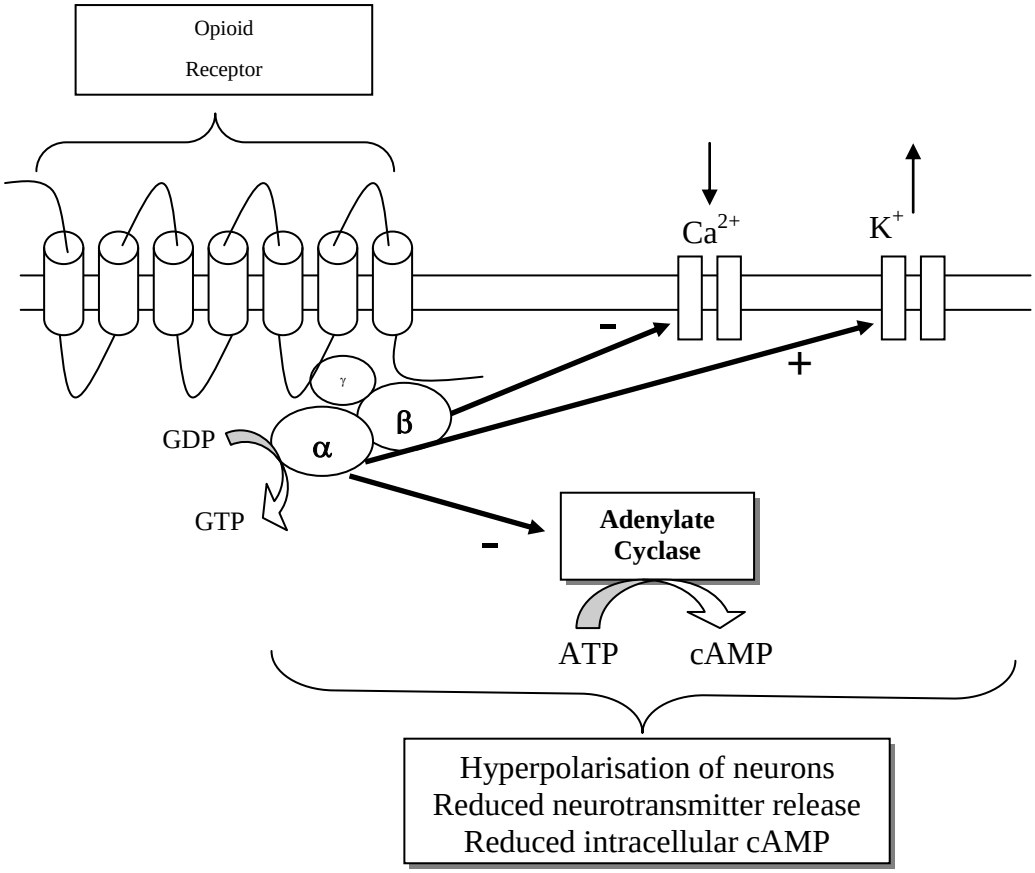


Figure 1.7. Schematic representation of intracellular responses to NOP receptor activation.

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.*, 1996b), 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.*, 1996a), 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 deferens 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.*, 1997b); 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.*, 1997b). The twitch response in the three preparations is due to nerve activation and subsequent release of neurotransmitter since they are blocked by tetradotoxin. 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 N/OFQ inhibited atropine and tetradotoxin sensitive contractions derived 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 fibres on the guinea pig bronchus (Fischer *et al.*, 1998; Rizzi *et al.*, 1999b), renal pelvis and heart (Giuliani *et al.*, 1996; Giuliani *et al.*, 1997b), and cholinergic contractions of human bronchus (Basso *et al.*, 2005). 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.

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, the

genitourinary 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) selective and potent antagonists. Some of the more well-studied and noteworthy biological actions modulated by this system will be described below.

1.4.1 Pain regulation

Rodent studies

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.

When administred supraspinally, 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 most prominent role of N/OFQ 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. 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 (Mogil *et al.*, 2001). 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 exogenously 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. A cellular model explaining the antiopioidergic action of supraspinal N/OFQ focalizes on brain stem, in particular the nucleus raphe magnus of the RVM, the major site of supraspinal N/OFQ effects on pain processing. In this brain region, different types of neurons, so called ON and OFF cells, can be distinguished. ON cells fire immediately before a nociceptive reaction, while OFF cells are inhibited by the GABAergic ON cells and therefore silent at the same time. Activation of OFF cells induces spinal antinociception via descending antinociceptive tracts. MOP opioids inhibit ON cells and thereby cause a subsequent disinhibition of the antinociceptive OFF cells. By contrast, N/OFQ inhibits nearly all cell types in the RVM. Via a direct inhibition of OFF cells, N/OFQ counteracts the disinhibitory effects of MOP agonists on these cells and thereby reverses opioid-induced supraspinal analgesia. The same mechanism may also account for the apparent hyperalgesic effect of N/OFQ, providing a cellular basis for the reversal of stress-induced analgesia by N/OFQ. These 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 (figure 1.8) (Zeilhofer and Calo, 2003).

The involvement of the NOP protein in the actions of N/OFQ on pain transmission has been investigated through the use of receptor antagonists, 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.*, 2002), J-113397 (Ozaki *et al.*, 2000; Yamamoto *et al.*, 2001) and SB-612111 (Rizzi *et al.*, 2007a; Zaratin *et al.*, 2004).

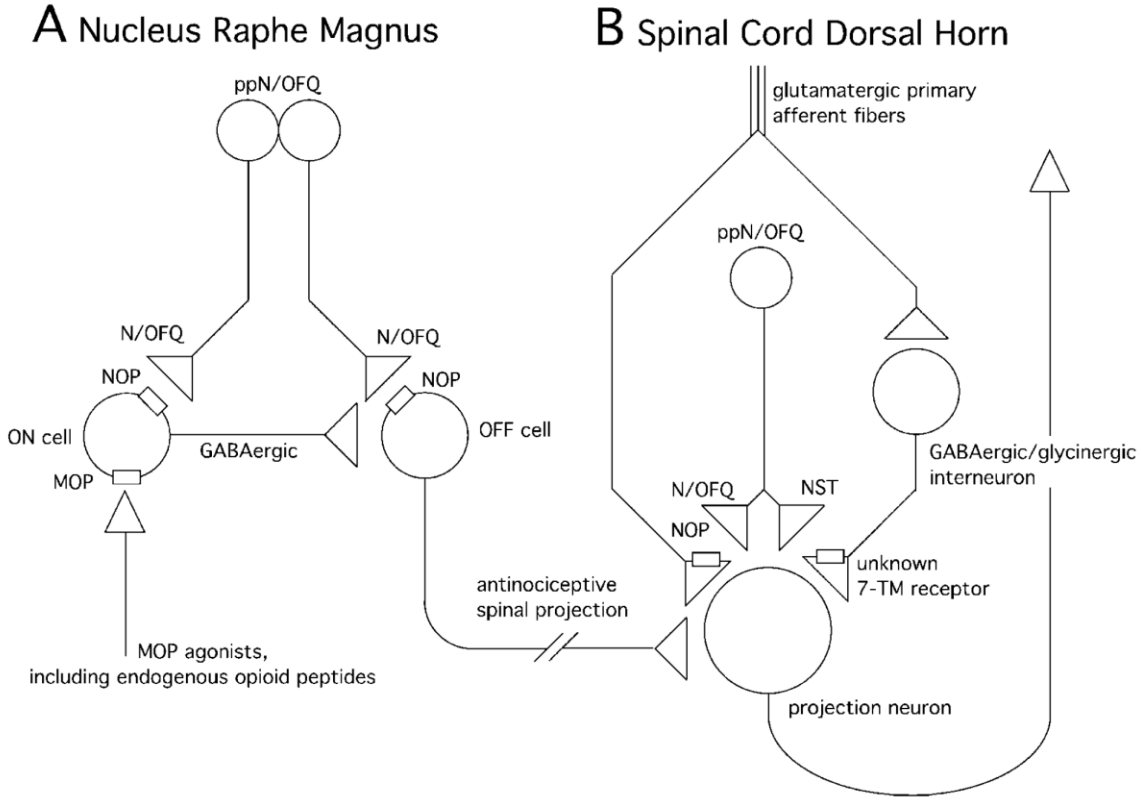


Figure 1.8. Schematic drawing showing how N/OFQ modulates synaptic transmission in the nucleus raphe magnus of the brain stem (A) and in the spinal cord dorsal horn (B). A, in the brain stem, MOP agonists including endogenous opioid peptides inhibit so-called secondary or ON cells. These cells are GABAergic and, in turn, inhibit descending antinociceptive OFF or primary cells. By inhibiting the ON cells, μ-opioids cause a disinhibition of OFF cells and elicit thereby antinociception. By contrast, N/OFQ inhibits both cell types at the same time. Under resting conditions, N/OFQ probably exerts no net effect on nociception. However, when MOP receptors on ON cells are activated N/OFQ can reverse this analgesia by inhibiting in addition OFF cells. B, in the spinal cord, N/OFQ selectively inhibits the release of glutamate and leaves the release of the inhibitory neurotransmitters glycine and GABA unaffected. Inhibition of glutamate release and the subsequent inhibition of nociceptive transmission through the spinal cord probably underlie the analgesic effect of nanomolar doses of spinally applied N/OFQ. NST by contrast reduces selectively the release of GABA and glycine via so far unknown 7-transmembrane receptor, but spares the release of glutamate.

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 fibres networks containing ppN/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.*, 1998; 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.

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 rodent 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 (e.g. formalin, zymosan A, writhing tests, SBL response to i.t. substance P), but does not affect acute (tail flick/immersion test) pain sensitivity (Depner *et al.*, 2003).

Similar to what seen at the spinal level in the periphery both pro and antinociceptive effects were reported for N/OFQ. For instance intradermal administration of very low doses of N/OFQ stimulates the flexor reflex in mice. This effect involves stimulation of the release of substance P from peripheral nerve endings. However at higher doses N/OFQ prevented the facilitatory effect of substance P (Inoue *et al.*, 1999). In addition several groups reported the ability of N/OFQ to inhibit neuropeptide release from peripheral sensory neuron terminals in different organs including the airways, heart, and renal pelvis (Giuliani *et al.*, 2000; Giuliani *et al.*, 1996; Lee *et al.*, 2006).

Primate studies

As far as the role of N/OFQ in modulating pain in primate species is concerned, extremely interesting are the studies of Ko and colleagues, that demonstrated that in non human primates spinal administration of N/OFQ or synthetic NOP ligands i) does not elicit any effect at low doses, ii) in the nanomolar range of doses induces a robust antinociceptive action that is sensitive to NOP antagonists but not naltrexone, iii) in contrast to morphine, does not induce pruritus and iv) elicits a synergistic antinociceptive effect when given in association with morphine (Ko *et al.*, 2006). Thus evidence coming from non human primates strongly suggests NOP receptor agonists as spinal analgesics. Interestingly, this same research group demonstrated that the NOP selective non peptide agonist Ro 64-6198 given systemically is able to induce dose dependent antinociceptive effects in non human primates while being inactive in rodents. In monkeys antinociceptive doses of alfentanil

(and in generally of opioids) are associated with respiratory depression, itch/scratching responses and reinforcing effects under self-administration procedures while in parallel experiments antinociceptive doses of Ro 64-6198 did not produced these side effects (Ko *et al.*, 2009). Further studies are however needed to draw firm conclusions on the therapeutic potential of systemic NOP agonists as analgesics.

1.4.2 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; Rizzi *et al.*, 2001b).

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 NOP(-/-) mice, confirming the involvement of the NOP receptor in this 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 spontaneous 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.*, 1997). 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 showed that NOP receptor antagonists such as 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, these studies provide 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 primate studies (Viaro *et al.*, 2008; Visanji *et al.*, 2008).

1.4.3 Anxiety

Different laboratories have reported anxiolytic-like effects in response to i.c.v 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 (Neal *et al.*, 1999a; Neal *et al.*, 1999b).

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 the elevated plus-maze test (Gavioli *et al.*, 2002; Vitale *et al.*, 2006), in the holeboard test (Kamei *et al.*, 2004), and in the defence test battery (Griebel *et al.*, 1999). 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 has been corroborated by the findings that ppN/OFQ deficient mice display an increased susceptibility to acute and repeated stress, as compared to their wild-type littermates (Koster *et al.*, 1999). In a 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 compounds are orally active and showed anxiolytic-like effects 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 more 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 (Gavioli *et al.*, 2008; Uchiyama *et al.*, 2008). 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.

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).

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 understanding of N/OFQ role in this field.

1.4.4 Mood

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.*, 2007; Gavioli *et al.*, 2004; Redrobe *et al.*, 2002).

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). 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).

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.*, 2007b).

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.

1.4.5 Food intake

Soon after the isolation of N/OFQ, Pomonis and colleagues (1996) showed that supraspinal N/OFQ (1–10 nmol) increased food intake in satiated rats. 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.*, 2000b).

N/OFQ hyperphagia can be blocked by antisense treatment to NOP mRNA (Leventhal *et al.*, 1998)), competitive NOP antagonism (Polidori *et al.*, 2000b) 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.*, 2007b).

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 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.*, 2000b). 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 in rats (Polidori *et al.*, 2000b), the N/OFQ-NOP receptor system does not play a major role in controlling food intake induced by food deprivation. N/OFQ may also increase food intake by decreasing the release of the anorectic peptide cocaine and amphetamine regulated transcript and/or increasing the release of the orexigenic peptide Agouti related protein (Bewick *et al.*, 2005). In addition there is evidence that corticosterone and central glucocorticoid receptors are involved in the orexigenic action of N/OFQ. All these studies indicate that the hyperphagic and the anti-anorectic effect of N/OFQ are mediated by separate brain structures and those synthetic N/OFQ agonists might have therapeutic potential as orexigenic drugs (Ciccocioppo *et al.*, 2004; Economidou *et al.*, 2006b).

1.4.6 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 NOP receptor activation.

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.*, 2008a).

Other studies conduced on NOP(-/-) mice reported that mice are more sensitive to the rewarding effect of cocaine (Marquez *et al.*, 2008a), nicotine (Sakoori *et al.*, 2009), methamphetamine and alcohol (Sakoori *et al.*, 2008). It was also previously reported that systemic administration of J-113397 potentiates the acquisition of cocaine-induced CPP in mice (Marquez *et al.*, 2008b). In line with these findings are the studies conduced by Rutten and colleagues (2011) on NOP knockout rats and rats treated with J-113397, which demonstrates that both pharmacological blockade and genetical ablation of the NOP receptor facilitates morphine-induced reward as assessed in a CPP paradigm in rats.

Recent human studies indirectly support the involvement of the NOP system in alcohol and drug abuse/dependence as well. For example, Kuzmin et al. (2009) reported that alcoholic patients

showed reductions in the expression of the ppN/OFQ gene and the NOP receptor gene in the hippocampus and amygdala, respectively. Furthermore, evidence from human genetic studies implies an association between alcohol and drug abuse and several single nucleotide polymorphisms (SNPs) of the genes. As such, it was demonstrated that genetic variants of the NOP gene were associated with vulnerability to develop opiate addiction in a Caucasian population (Briant *et al.*, 2003) and to play a role in Type I and Type II alcohol dependence in a Scandinavian population (Huang *et al.*, 2008). Although neither gene appeared to be associated with alcohol or drug dependence, two SNPs in ppN/OFQ showed a marginal association with alcoholism and one with drug dependence, and two SNPs in NOP were marginally associated with opioid dependence (Xuei *et al.*, 2008). Therefore, further research is required before definitive conclusions can be drawn on the possible involvement of the NOP system in human drug abuse and addiction.

Also 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 properties of drugs.

1.4.7 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). Later, 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. Knockout mice lacking the NOP receptor displayed facilitated learning and memory in the water maze task and enhanced LTP induction in the

hippocampal CA1 region (Manabe *et al.*, 1998). The impairment of learning induced by N/OFQ can be reversed by nocistatin (Hiramatsu *et al.*, 1999) 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). A recent study showed that intracerebroventricular or intrahippocampal infusions of N/OFQ impair long-term memory formation in the mouse object recognition task (Kuzmin *et al.*, 2009). 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.

1.4.8 Effects in the gastro intestinal system

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.*, 1997; Zhang *et al.*, 1997a), 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, similar to opioids, central administration of N/OFQ also inhibits colon transit in the mouse (Osinski *et al.*, 1999a). On the other hand, Taniguchi and colleagues (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-evoked 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 acting both at central and peripheral levels (Morini *et al.*, 2005). This effect is mediated by the NOP receptor since the selective NOP 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 as well as pathological conditions (Agostini *et al.*, 2009; Broccardo *et al.*, 2008).

1.4.9 Effects in the airways

N/OFQ was found to inhibit neurogenic contractions of the guinea pig isolated bronchus (Fischer *et al.*, 1998; Rizzi *et al.*, 1999b), of the rat trachea and bronchus (Wu *et al.*, 2000), 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 (Anton *et al.*, 1996), which provides polysynaptic inputs to second-order neurons that modulate respiratory neuron activities (Reynolds *et al.*, 2004).

The antitussive effect of N/OFQ 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 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).

1.4.10 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.*, 1997a). In addition, the bradycardic effects of N/OFQ were reduced by vagotomy, indicating that N/OFQ increased parasympathetic activity (Giuliani *et al.*, 1997a).

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 vasodilatation 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 possibly due to mast-cell release of histamine.

1.4.11 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 of low 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 the NOP partial agonists 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.*, 1999a). 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 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, 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. In fact, ZP120 was selected for clinical development as treatment of acute decompensated heart failure

1.4.12 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 investigations. 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 (Kapusta *et al.*, 1999a), is inactive in these patients (Lazzeri *et al.*, 2003). Moreover, a more 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. Moreover, Malaguti and colleagues in 2007 performed the neurophysiological assessment of the nociceptive flexion reflex in four healthy subjects and in five patients with lower urinary tract symptoms to investigate the N/OFQ neuronal site and functional mechanism of action. N/OFQ seems to selectively inhibit vesical sensory innervation in patients with lower urinary tract symptoms as it exerts a tonic inhibitory modulation of the nociceptive reflex, which is mediated by descending pathways (Malagutti *et a., 2007*). In healthy subjects N/OFQ modulation of the nociceptive reflex is not functionally active. These findings seem to provide evidence that N/OFQ is involved in the pathophysiology of lower urinary tract symptoms and make them attractive targets for new therapies.

1.4.13 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).

Carvalho and colleagues (2008) performed a caecal ligation/perforation model of sepsis and found that administration of N/OFQ in rats exacerbated the inflammatory process and increased mortality. Animals treated with N/OFQ had 100% mortality, compared with 70% in the control untreated group and 50% in those treated with NOP antagonist UFP-101 (Carvalho *et al.*, 2008). N/OFQ treatment also increased plasma concentrations of TNFα and IL-1β. In addition, using anaesthetized (but non-septic) rats, Brookes and colleagues (2007) showed that N/OFQ produced an inflammatory response. In mesenteric vessels, there was vasodilatation, macromolecular leak, and leucocyte adhesion (Brookes *et al.*, 2007).

Conversely, intracerebroventricular administration of N/OFQ led to reduced cytokine production by peritoneal macrophages in rats undergoing exploratory laparotomy (Zhao *et al.*, 2002). It is possible that there is a difference in the immune response to N/OFQ between peripheral and central administration, and this is an avenue for further investigation.

Further supporting evidence for the role of N/OFQ in the inflammatory response comes from gene NOP knockout mice where the gene for NOP is absent. A study of mice with induced colitis compared NOP(+/+) with NOP(−/−) mice (Kato *et al.*, 2005). Administration of oral dextran sulphate sodium caused bloody diarrhoea in the NOP(+/+) group but normal stools in the NOP(−/−) group. On histological examination, the colon of NOP(+/+) mice had crypt distortion and increased number of lymphocytes, macrophages, and neutrophils (evidence of colitis), compared with normal crypts and reduced number of inflammatory cells in the NOP(−/−) group. This demonstrated that the absence of NOP significantly reduced the inflammatory response to a known pro-inflammatory stimulus. There is a need for further observational and mechanistic studies in

patients with established inflammatory processes or sepsis. These studies may facilitate the design of appropriate clinical studies to evaluate NOP ligands as modifiers of the inflammatory response. In a small study of 21 critically ill patients admitted to ICU with a diagnosis of sepsis, plasma N/OFQ concentrations over four consecutive days were measured. Plasma concentrations of N/OFQ at ICU admission were increased in patients who subsequently died (n=4) compared with those who survived (n=17) (Williams *et al.*, 2008). More data are required to confirm these findings.

1.5 Knockout animals

Transgenic animals in particular receptor knockout represent essential research tools in modern pharmacology. For instance receptor knockout animals allow researchers to perform simple and meaningful experiments to investigate the in vivo selectivity of action of standard and novel receptor ligands and the involvement of the receptor in the control of a given biological function. In most cases the phenotype of receptor knockout animals is similar to what observed in normal animals after the administration of a selective receptor antagonist. An example of the massive increase in knowledge deriving from the use of knockout mice is summarized in the review by Kieffer (1999): "mice lacking opioid receptors or opioid peptides have been produced by gene targeting, providing molecular tools to study opioid function in vivo. Observations on mutant mice have shed new light on the mode of action of opioids, opioid receptor heterogeneity and interactions, and the involvement of each component of the opioid system in mouse physiology".

As far as the N/OFQ-NOP receptor system is concerned, transgenic knockout mice lacking functional expression of the NOP receptor gene (Oprl1; chromosome 2, 110cM) or the ppN/OFQ gene (Npnc1; genomic location unknown) are available to the scientific community. NOP(-/-) mice were first generated in 1997 (Nishi et al., 1997). Autoradiography studies demonstrated complete loss of N/OFQ binding in the brain of these animals. Subsequent in vitro functional studies demonstrated that in tissues taken from NOP(-/-) mice N/OFQ no longer elicits any effect. This includes data from bioassay studies (contractile action in the colon (Di Giannuario *et al.*, 2001),

inhibitory effect in the electrically stimulated vas deferens(Carra *et al.*, 2005a), inhibition of capsaicin induced bronchoconstriction (D'Agostino *et al.*, 2010)), to neurochemical investigations (inhibition of serotonin release from cerebral cortex synaptosomes (Mela *et al.*, 2004)), as well as to electrophysiological studies (inhibition of excitatory transmission in the spinal cord (Ahmadi *et al.*, 2001)). In addition, in vivo studies on NOP(-/-) mice demonstrated that the N/OFQ actions examined to date are solely mediated by NOP receptor activation. Receptor knockout studies are available in the literature regarding the following biological actions of N/OFQ: supraspinal pronociceptive (Nishi *et al.*, 1997; Noda *et al.*, 1998) and spinal antinociceptive effects (Nazzaro *et al.*, 2007), induction of bradycardia, hypotension and diuresis (Burmeister *et al.*, 2008), stimulation of food intake (Rizzi *et al.*, 2007b) and inhibition of locomotor activity (Marti *et al.*, 2004b; Nishi *et al.*, 1997; Noda *et al.*, 1998).

NOP(-/-) mice were also used for investigating their phenotype. Nociceptive responses to acute noxious heat in NOP(-/-) were indistinguishable from those of NOP(+/+) mice (Depner *et al.*, 2003; Di Giannuario *et al.*, 2001; Nishi *et al.*, 1997). However, NOP(-/-) showed markedly stronger nociceptive responses during prolonged nociceptive stimulation (i.e. the formalin test). 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; Rizzi *et al.*, 2006). NOP(-/-) mice presented an antidepressant phenotype in the forced swimming and tail suspension test (Gavioli *et al.*, 2003). Several lines of evidence also suggest that endogenous N/OFQ has an important role in anxiety and stress regulation. No enhanced anxiety was shown 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). Increased locomotor performance in rotarod assay was observed in NOP(-/-) mice (Marti *et al.*, 2004b). This study suggest that endogenous N/OFQ signalling might have a negative regulation on motor activity. NOP(-/-) mice show greater learning ability and have better memory retention than wild-type control mice. Knockout mice displayed facilitated learning and memory in the water maze task and enhanced LTP induction in the hippocampal CA1 region (Manabe *et al.*, 1998).

Mice knockout for the ppN/OFQ gene have been also generated (Koster *et al.*, 1999). However these ppN/OFQ(-/-) animals were only used in a small number of studies. The limited information available suggests that these animals behave in a similar manner as NOP(-/-) mice in terms of pain transmission (Depner *et al.*, 2003) while they display differences from receptor knockout animals in terms of response to stress (Kest *et al.*, 2001; Ouagazzal *et al.*, 2003; Reinscheid *et al.*, 2002). However parallel experiments with NOP(-/-) and ppN/OFQ(-/-) mice should be performed before drawing firm conclusions on behavioural differences between the two mutant genotypes. Moreover the following consideration is worthy of mention. As indicated in section 1.3 the ppN/OFQ gene codes for, in addition to N/OFQ, other biologically active peptides. Since ppN/OFQ(-/-) mice do not express N/OFQ as well as the other peptides encoded by the same gene caution should be exerted in interpreting behavioral differences between ppN/OFQ(-/-) and ppN/OFQ(+/+) mice as solely due to the lack of the N/OFQ peptide.

1.6 Pharmacology of NOP receptors

From the numerous modulatory actions of N/OFQ on several biological functions, it is clear that NOP receptor 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 low metabolic stability and limited distribution. In contrast, non-peptide molecules 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). C terminal truncation studies 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 crucial 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 preparation under study. Thus, while [F/G]N/OFQ(1–13)-NH_2 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.*, 2003). 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 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.*, 2003).

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.*, 2000). This peptide was the first NOP pure antagonist of peptide nature; 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 (Calo *et al.*, 2000a) and reversed the effects of N/OFQ on memory (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.*, 2000a), 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 higher 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).

Furthermore in a series of structure-activity studies (Guerrini *et al.*, 2005) the chemical modifications which reduce ([$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]) 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, [$Nphe^1Arg^{14}Lys^{15}$]N/OFQ-NH_2 (UFP-101) (Calo *et al.*, 2002), and [(pF)$Phe^4Arg^{14}Lys^{15}$]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.*, 2006a).

A very potent partial agonist [Phe^{1}ψ(CH_2-NH)-Gly^2(pF)Phe^{4}Arg^{14}Lys^{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. UFP-101 (like other selective NOP antagonists) also 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).Vitale an colleagues (2009) demonstrated that chronic treatment with UFP-101 produces antidepressant-like effects in rats subjected to CMS. Moreover several works by Morari and colleagues demonstred the anti-akinetic action of UFP-101 in parkinsonian rats and mice, when administred in the substantia nigra reticulate (Marti *et al.*, 2004a; Marti *et al.*, 2004b; Marti *et al.*, 2010).

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 the 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 different tests, UFP-112 mimicked the N/OFQ effects, with higher potency and longer lasting effects: antinociception (in rodents and monkeys), increase of food intake in mice, inhibition of locomotor activity, of ethanol consumption, of gastric motility, decrease in heart rate, blood pressure and urinary sodium excretion with increase in urine flow (Calo *et al.*, 2011; Rizzi *et al.*, 2007b).

Small peptide ligands

The small peptides in this group were 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.*, 1999b).

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 by our group (Carra *et al.*, 2005a). 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). This study was confirmed by Bojnik and colleagues (Bojnik *et al.*, 2010) using different biochemical and pharmacological techniques, showing that Ac-RYYRIK-ol has agonist and antagonist effects toward the NOP receptors; this is likely due to its partial agonist pharmacological activity .

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 (Fischetti *et al.*, 2009; 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).

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 assessed 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 HTS in pharmaceutical industry laboratories. Since the NOP receptor displays high homology with opioid receptors, the search for non-peptide NOP ligands was initiated by examining small-molecule opioid ligands, such as: i) DOP-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).

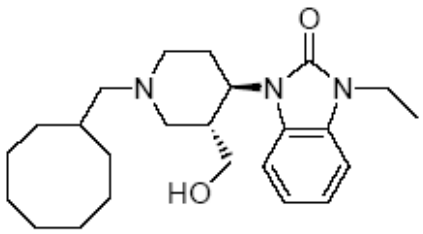
The non-peptide NOP receptor ligands can be broadly divided into six structural classes. It is noteworthy that many of these ligands were first reported in the patent literature (patents from Pfizer, Banyu Pharmaceutical Co., Hoffmann La Roche, EuroCeltique S.A., NovoNordisk, Schering-Plough, Smith Kline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

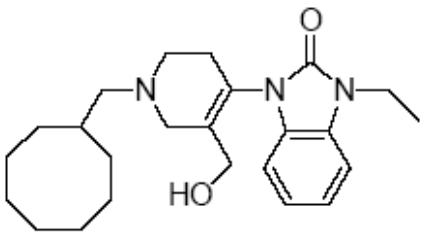
ii) *Benzimidazopiperidines*: The first non-peptide pure NOP antagonist to be reported was a benzimidazolinone, J-113397 (Figure 1.9), reported by Banju researchers (Kawamoto *et al.*, 1999; Ozaki *et al.*, 2000). J-113397 was shown to bind with nanomolar affinity to NOP receptors and to display 100-300 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 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 non-peptide NOP receptor antagonist most 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 1.9), 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 facilitated motor performance in naive rats and mice and alleviated parkinsonism in 6-hydroxydopamine hemilesioned rats (Marti *et al.*, 2008).



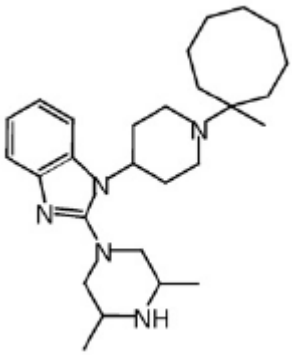
J-113397



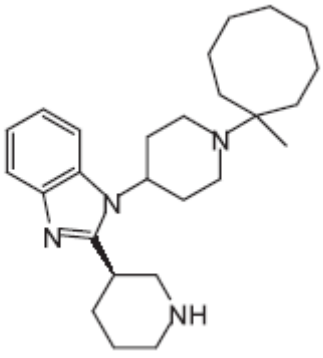
Trap-101

Figure 1.9. 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 1.10). 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. *In vivo* MCOPPB (10 mg/kg, p.o.) elicited anxiolytic-like effects in mice without affecting locomotor activity or memory (Hirao *et al.*, 2008b).



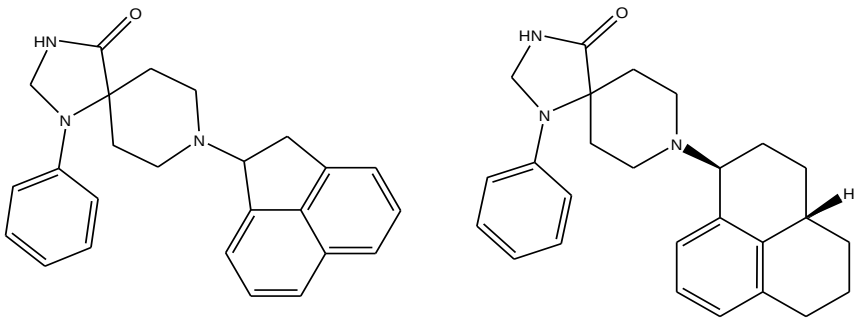
PCPB



MCOPPB

Figure 1.10. 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, 1998) (Figure 1.11). 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; Rutten *et al.*, 2011). 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 due to residual agonist activity of this compound at MOP receptors (Economidou *et al.*, 2006a). For review see Shoblock (2007).



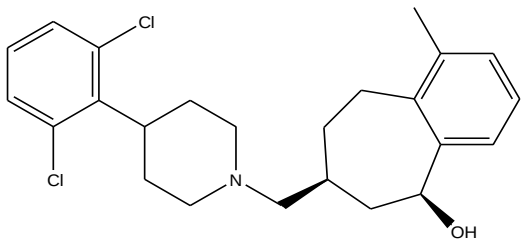
Ro 65-6570 **Ro 64-6198**

Figure 1.11. 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 at human NOP but only a 12-fold selectivity 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. SB-612111 (Zaratin *et al.*, 2004) was patented by GlaxoSmithKline (Figure 1.12). 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 a recent study conduced on rat midbrain periaqueductal gray slices, a crucial site for morphine-induced supraspinal analgesia as well as the site of action that N/OFQ reverses morphine-induced analgesia. SB-612111 was able to block the activation of inwardly rectifying K+ (GIRK) channels induced by N/OFQ (Chee *et al.*, 2011; Liao *et al.*, 2011). *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.

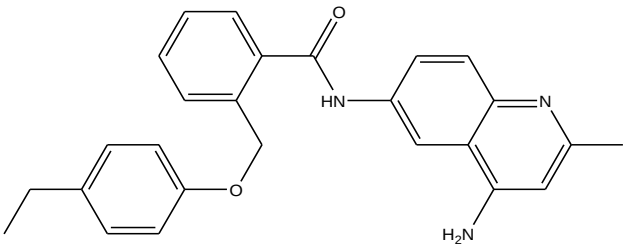


SB-612111

Figure 1.12. Structure of SB-612111.

v) *4-Aminoquinolines:* These are an entirely different chemical class of NOP ligands disclosed by Japan Tobacco Inc. in a patent (Shinkai *et al.*, 2000). The optimized ligand, JTC-801 (Figure 1.13), was obtained through an extensive structure-activity study. Detailed pharmacological

studies with JTC-801 were 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. However, JTC-801 in the same assay didn't show an appreciable selectivity over classical opioid receptors, and this was confirmed by receptor binding assays performed by Lambert and colleagues (personal communication). 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. Moreover, a Schild-plot analysis showed that this compound behaves as non surmountable antagonist, unlike piperidine and spiropiperidine ligands (Sestili *et al.*, 2004). Other JTC-801 analogues with NOP antagonistic properties have been described in a patent by Japan Tobacco Inc (Shinkai *et al*., 2006).

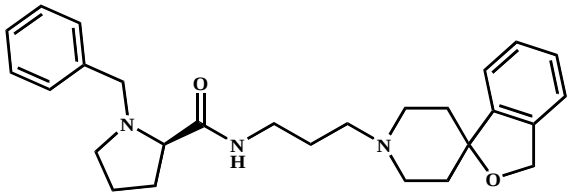


JTC-801

Figure 1.13. Structure of JTC-801, a 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*: 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 1.14) 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). These pharmacological features of Compound 24 were confirmed by Fischetti and colleagues (Fischetti *et al.*, 2009) in various assays and preparations expressing the human recombinant as well as the animal native receptors. In addition, the NOP selective antagonist properties of Compound 24 was confirmed in vivo in mice subjected to the tail withdrawal assay. Systemically administered Compound 24 was also able to improve motor activity in hemiparkinsonian rats acting via the blockade of nigral NOP receptors (Volta *et al.*, 2011)



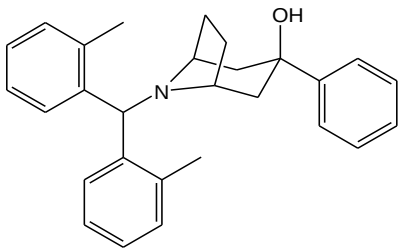
Compound 24

Figure 1.14. 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. Trapella and colleagues (Trapella *et al.*, 2009) performed SAR studies onCompound 24 structure; 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. in vitro in various assays, Compound 35 consistently behaved as a pure, highly competitive and NOP selective antagonist. However compound 35 was found inactive when challenged against N/OFQ in vivo in the mouse tail withdrawal assay. Thus, the usefulness of the novel NOP ligand compound 35 is limited to in vitro investigations.

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 1.15). 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 1.15. Structure of SCH 221510.

2. AIMS

The general objective of the present work has been to increase the knowledge related to the neurobiology and pharmacology of the N/OFQ – NOP receptor system and to provide the scientific community with new tools and ideas useful for validating the NOP receptor as target for innovative drugs. In particular the following knockout and pharmacological studies have been performed:

- Detailed investigation of the phenotype of rats lacking of the NOP receptor (NOP(-/-)). The anxiety- and mood-related behaviours and locomotor and nociceptive phenotype of NOP(-/-) rats have been investigated and compared to those of their wild type littermates. The absence of functional NOP receptors was proved in vitro by comparing the effect of N/OFQ on electrically stimulated vas deferens taken from NOP(+/+) and NOP(-/-) animals.
- Pharmacological characterization of novel non peptide ligands selective for the NOP receptor. In the frame of these studies we characterized the pharmacological profile of i) a novel NOP receptor antagonist coded as GF-4, and ii) a short panel of NOP receptor agonists.
- Design, synthesis and characterization of novel peptides as mixed NOP/MOP agonists. SAR studies on Phe^1 of N/OFQ and related peptides allowed the identification of [Dmt^1]N/OFQ(1-13)-NH_2 as the most interesting compound that has been further pharmacologically characterized in vitro and in vivo. [Dmt^1]N/OFQ(1-13)-NH_2 has been demonstrated in rodent and non human primate studies to be a promising molecule and represent a prototype of innovative spinal analgesics.

To pharmacologically characterize the above mentioned compounds, *in vitro* studies on N/OFQ-sensitive isolated tissues from different species and on cells expressing the recombinant NOP and classical opioid receptors, were performed. Moreover, *in vitro* and *in vivo* experiments were performed using normal mice as well as NOP(+/+) and NOP(-/-) mice.

3. MATERIALS & METHODS

3.1 Drugs and reagents

The peptides used in this study were synthesized in the laboratory of Prof Salvadori (Department of Pharmaceutical Sciences, 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).

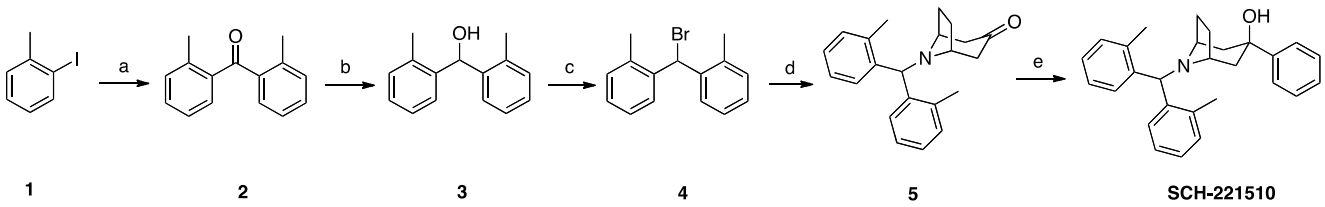
Ro 65-6570 was synthetized as reported by (Rover *et al.*, 2000). SCH 221510 was sinthetized in house as reported in figure 3.1, compound 6d was sinthesized by Prof. Mustazza group (Dipartimento del Farmaco dell' Istituto Superiore della Sanità di Roma) (Mustazza *et al.*, 2008).

GF-4 was synthesized in house following the procedures described in detail in (Trapella *et al.*, 2006)(see page 694, scheme 1, the only modification is the last step of the synthesis (j) where in stead of lithyumaluminiumhydride was used the methyl magnesium bromide to obtain compound GF-4).

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 (Trapella *et al.*, 2006). The compound SB-612111 was purchased from Tocris ltd. (U.K.).

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), [*leucyl*-^{3}H]UFP-101 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, peptides were dissolved in sterile saline solution just before injections.



Conditions: a) $Co_2(CO)_8$, MW, 10s, 130°C; b) $NaBH_4$, THF, quant.; c) THF, $SOBr_2$, quant.; d) Tropanone, alpha Chloroethyl chloroformate, DCE, K_2CO_3 68%; e) C_6H_5Li, THF, 58%.

Figure 3.2. Procedure adopted for the synthesis of SCH-221510.

3.2 In vitro studies

Receptor or GTPγ[^{35}S]binding assay

3.2.1CHO expressing the recombinant NOP and classical opioid receptors

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 classical opioid (DOP, MOP and 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).

3.2.2 Cell harvesting and membranes preparation

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 (HEPES (10 mM), EDTA (1.1 mM), NaCl (154 mM), pH 7.4 with NaOH) and gentle agitation. Cells were suspended in either wash buffer (displacement assay)(Tris-HCl (50 mM), $MgSO_4$ (5 mM), pH 7.4 with KOH for experiments of N/OFQ or UFP-101 displacement, where for displacement diprenorphine a Mg^{++} free solution was used) or homogenising buffer (GTPγ[^{35}S] assay)(Tris-HCl (50 mM), pH 7.4 with KOH), 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 (composed by Tris-HCl (50 mM), $MgSO_4$ (5 mM), pH 7.4 with KOH, 0.5% BSA for N/OFQ or UFP-101 displacement binding experiments, a Mg free solution for the diprenorphine displacement binding)(Tris-HCl (50 mM), EGTA (0.2 mM), NaCl (100 mM), $MgCl_2$ (1 mM), pH 7.4 with NaOH for GTPγ[^{35}S] experiments) and the total protein content determined as set out below.

3.2.3 Rat spinal cord and cerebral cortex membrane preparation

The spinal cord and cerebral cortex were taken from male albino Sprague Dooley rats (200-250 g) The tissues were suspended in homogenising buffer (GTPγ[^{35}S] assay), homogenised using an Ultra Turrax, for 10 seconds followed by 2 consecutive 5-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.

3.2.4 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 3.2) from which sample protein concentrations were determined.

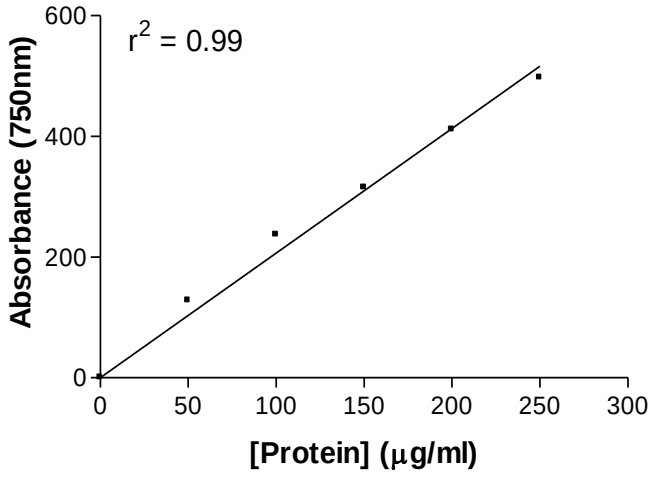


Figure 3.2. Example of protein assay standard curve used to determine the protein mass of unknown samples.

3.2.5 Displacement binding assay

100 μg protein of CHO_{NOP} homogenate were assayed in a total volume of 0.5 ml comprising competition homogenising buffer, with the addition of 10 μM peptidase inhibitors (amastatin, bestatin, captopril and phosphoramidon), 0.9 nM [*Leucyl*-^{3}H]UFP-101 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_{MOP} and CHO_{DOP}) or 150 μg (CHO_{KOP}) 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.

3.2.6 [^{35}S]GTPγS stimulation binding assay

Experimentation was performed essentially as described by (Berger *et al.*, 2000b). Freshly prepared CHO_{NOP} membranes (50 μg), CHO_{MOP} (50 μg), or rat spinal cord (100 μg) or cerebral cortex membranes (50 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 1 pM – 10 μM. NSB was determined in the presence of 100 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.

Calcium mobilization assay

3.2.7 Experimental protocols

CHO cell lines stably co-expressing NOP or classical opioid receptors and the C-terminally modified $G\alpha_{qi5}$ were generated as previously described by (Camarda *et al.*, 2009) Camarda and Calo (in press). CHO_{MOP}, CHO_{DOP}, CHO_{KOP} and CHO_{NOP} 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 x). 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.

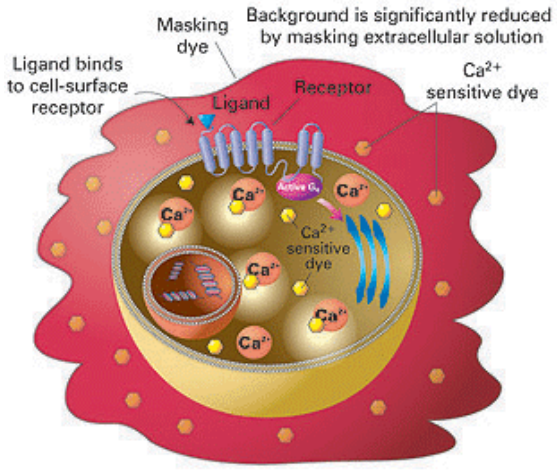


Figure 3.3. Diagram depicting the experimental protocol of the calcium mobilization assay. Cells are incubated with Fluo-4 AM, de-esterification of the ester group (AM) traps the dye in the cells and further leakage of the dye is prevented by blockage of organic anion-transport inhibitors using probenecid. Background fluorescence is reduced by addition of Brilliant Black dye which blocks extracellular signalling from any leaked Fluo-4.

3.2.8 Cell counting

Accurate numbers in a cell suspension can be calculated by counting the cells in a cell counting chamber (Burker's chamber, Figure 3.4). 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.

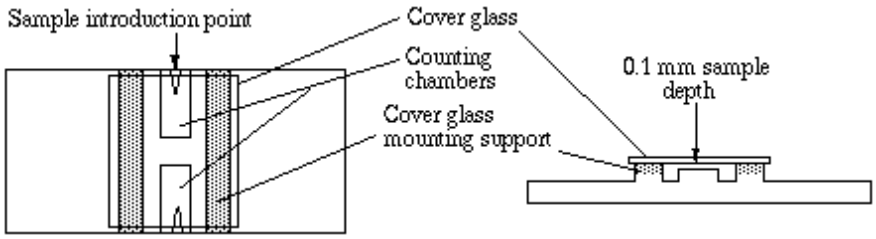


Figure 3.4. Burker's chamber.

Under a microscope the number of cells in diagonally opposite counting areas were counted, Figure 3.5.

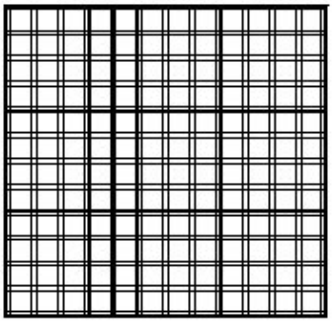


Figure 3.5. Schematic representation of the counting grid of the Burker's chamber.

The Burker's chamber is formed of 3 x 3 major squares, each of these major squares is subdivided into a grid of 4 x 4 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.

3.2.9 Instruments

$[Ca^{2+}]_i$ levels were monitored using a FlexStation II fluorimeter (Figure 3.6). 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.

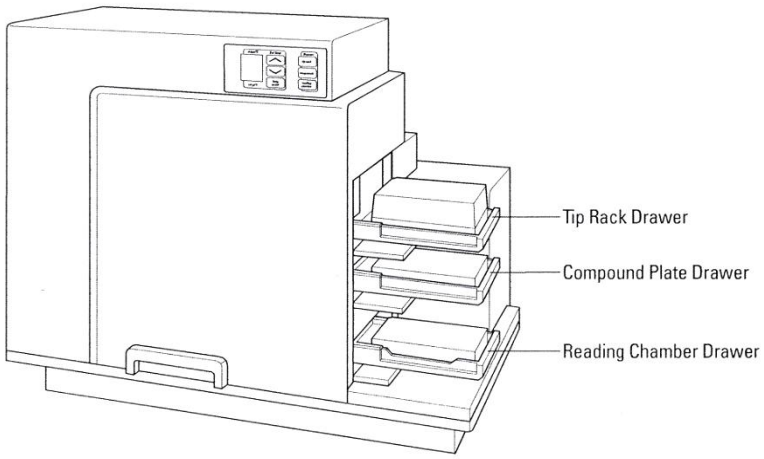


Figure 3.6. Diagram of FlexStation II used for calcium mobilization assay.

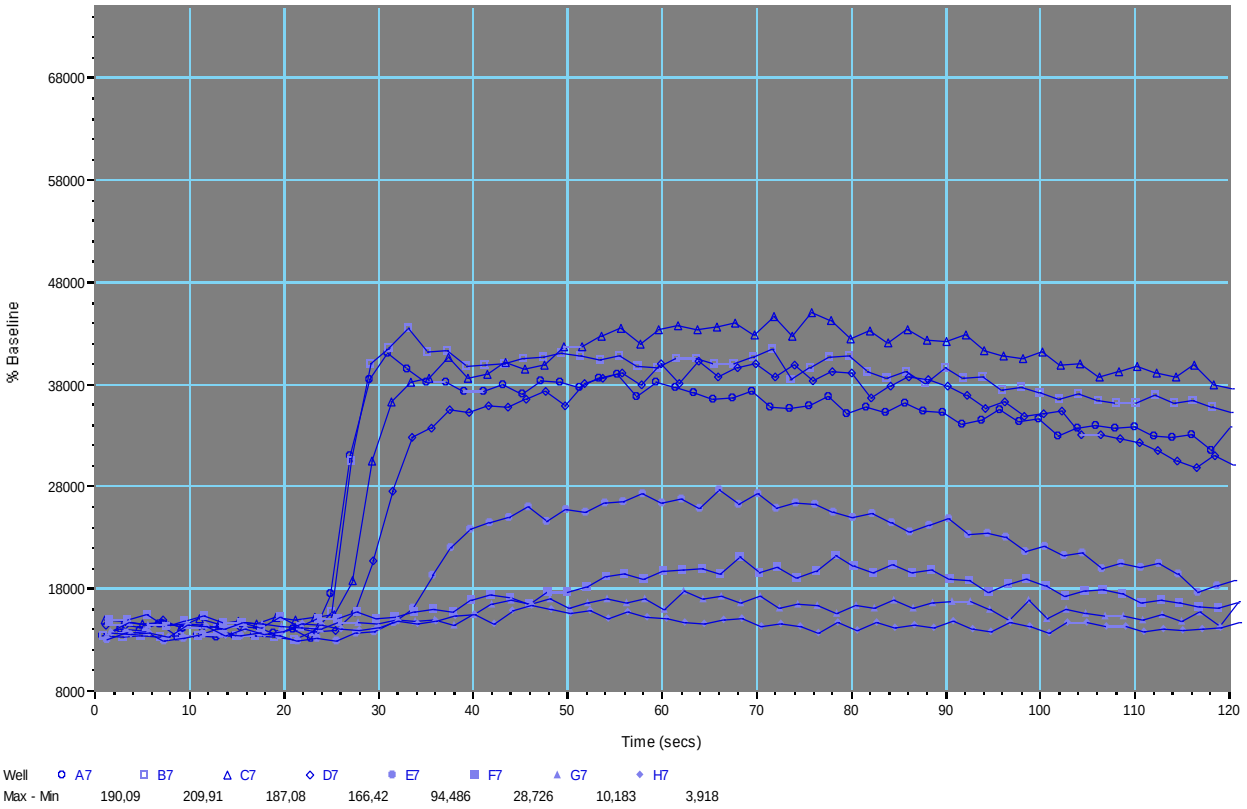


Figure 3.7. Raw data of calcium mobilization assay: concentration response curve (from 1 pM to 1 µM) to N/OFQ obtained in CHO cells expressing the recombinant human NOP receptor and the chimeric $G\alpha_{qi5}$ protein .

Isolated tissues

3.2.10 Methods

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*.

3.2.11 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 (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 bromide 2.2 mM and benadril 1.37 mM. The solution was 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. A resting tension of 0.3 g was applied to the mVD, 1 g to the gpI and rVD.

3.2.12 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, receptor antagonists, at adequate concentrations, were added to the medium 15 min before performing the concentration response curve to agonists.

3.2.13 Instruments

For the *in vitro* bioassays two chamber-glass bathes for isolated organs were utilized (Figure 3.8). 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).

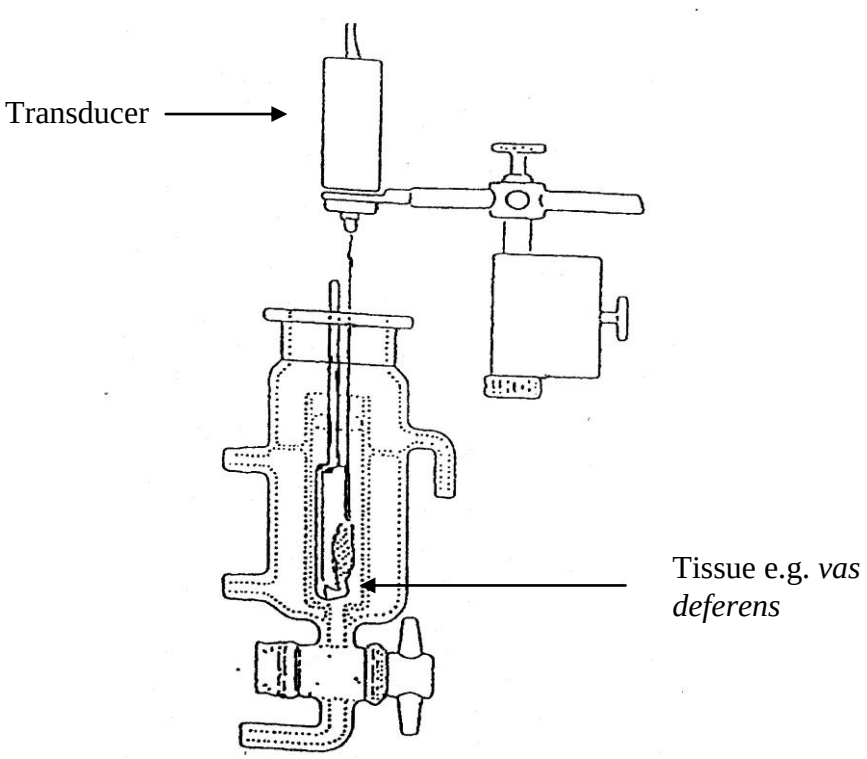


Figure 3.8. Diagram of the tissue chamber used for isolated tissues assays.

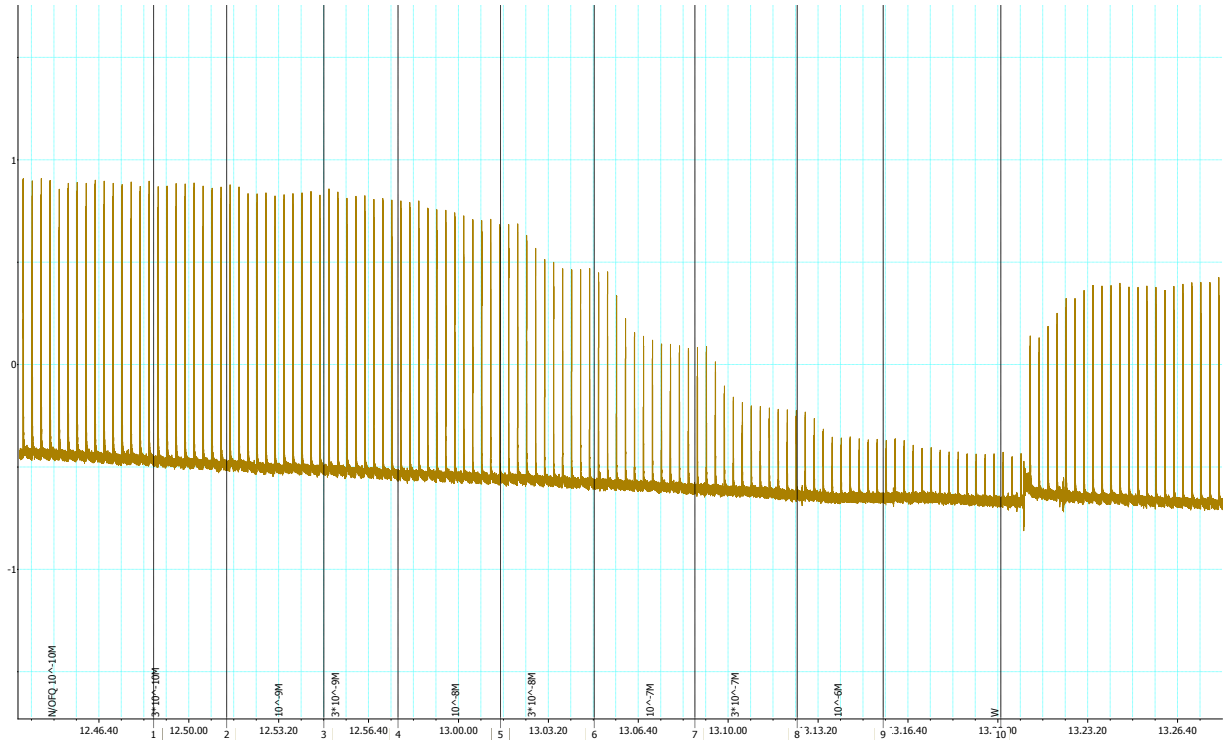


Figure 3.9. Raw data of isolated tissues assay: concentration response curve to N/OFQ obtained in the mouse vas deferens.

3.3 In vivo studies

Experimental protocol

3.3.1 Animals

Mice

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.t. injections (5 µl/mouse) were adapted according to the method of (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. 2 hours before the i.t. injection a cutaneous incision was performed on the mice back under isofluoran anaesthesia.

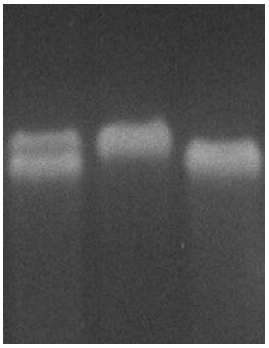


Figure 3.10. PCR analysis of tail biopsies taken from NOP(+/-),NOP(+/+) or NOP(-/-) CD1. The 372 bp PCR product was from NOP(+/+) mice, while the 384 bp was from the knokout litermates. The NOP(+/-) mice showed both the products.

NOP(-/-) rats

Experiments were conducted using 11 NOP(+/+) and 11 NOP(-/-) male littermate rats supplied by GenOway (Lyon, France). These rats were generated in a Brown Norway background and subsequently backcrossed on a Wistar background for four generations as previously described in detail (Homberg *et al.*, 2009). The animals were 3 month old on arrival in our animal facility and were housed under standard conditions (12 h light/dark cycle, lights on at 7.00, temperature 21 ± 1°C, 60% relative humidity, food and water available ad libitum) in groups of 3-4 rats per cage. All experiments were performed between 9.00 and 13.00.

The different series of experiments were performed according to the following schedule: week I elevated plus maze, week II open field, week III forced swimming test, week V plantar test and formalin test. At the end of this schedule, during week VI rats were killed and their vasa deferentia collected and used for in vitro studies. The average body weight of NOP(+/+) and NOP(-/-) rats was 300 ± 9 and 288 ± 11 g on arrival in our animal facility, and 384 ± 9 and 374 ± 9 g after experiments at week VI, respectively. An expert observer who was blind to the animal genotype made all behavioural measurements.

3.3.2 Methods

Mouse Tail withdrawal assay

Male Swiss albino mice weighing 25–30 g were used. Animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and Italian national regulations (D.L. 116/92). They were housed in 425x266x155 mm cages (Techniplast, Milan, Italy), fifteen animals cage^{-1}, under standard conditions (22 °C, 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 for at least 5 days before experiments began. Each mouse was used only once. I.t. (5 µl mouse^{-1}) injections were given according to the procedure described by Hylden and Wilcox (1981). All experiments were started at 10:00 am. The mice were placed in a holder and the distal

half of the tail was immersed in water at 52 °C. Withdrawal latency time was measured by an experienced observer blind to drug treatment. A cut-off time of 10 s was chosen to avoid tissue damage. For each experiment sixteen mice were used by randomly assigning four animals to each treatment group. The experiment was repeated four times; therefore, each experimental point is the mean of the results obtained in 16 mice. Tail-withdrawal latency was determined immediately before and 15, 30, 60, 120, and 180 min after i.t. injection of vehicle (saline), morphine (0.1 – 10 nmol), N/OFQ (1 - 100 nmol), N/OFQ(1-13)-NH_2 (1 - 100 nmol) or [Dmt^1]N/OFQ(1-13)-NH_2 (0.01 – 10 nmol).

Rat elevated Plus Maze

The procedure was carried out essentially as previously described by Pellow *et al.* (Pellow *et al.*, 1985). The elevated plus maze apparatus (Campden Instruments, Loughborough, UK) consists of two open arms (50 x 10 cm) which face two opposite closed arms (50 x 10 cm, 40 cm high) connected by a central platform (10 x 10 cm) elevated 86 cm from the floor. A red dim light (~ 100 lux) was focused on the central platform. Each rat was placed on the platform, facing an open arm, and allowed to explore the apparatus for 5 min. The time spent in and the number of entries into the open and closed arms were recorded during the test. An individual entry was recorded when the animal entered the arm with all the four paws. The incidence of ethological parameters such as stretch-attend postures, head-dipping over the edges, grooming and rearing behaviour, and number faecal pellets were also recorded. Between each trial, the apparatus was cleaned and dried.

Rat open field

This assay was performed by placing rats, individually, into a white wooden arena (50 x 50 cm, closed by walls (45 cm high) divided into 25 squares. This test was performed under dim red light (~ 100 lux). The animal was placed in the center of the arena and then allowed to explore the novel environment for 5 min. The number of crossings into peripheral and central zones, the time spent in

the central area and the number of rearings were recorded. Between each trial, the apparatus was cleaned and dried.

Rat forced Swimming

Rats were placed, individually, in Plexiglas cylinders (46 cm high, 20 cm in diameter) containing water (24-26°C, 30 cm deep), for two swimming sessions: an initial 15 min training session, which was followed, 24 h later, by a 5 min test session. At the end of each swimming session, the animal was removed from the cylinder, dried with paper towels, placed in an individual cage to rest and recover for 15 min and then returned to its collective home cage. The following behavioural parameters, previously shown to be reliable and validated for the detection of antidepressant drug effects (Detke *et al.*, 1995), were scored cumulatively in the second (test) swimming session: i) immobility time (i.e. the time spent floating in the water without struggling, making only those movements necessary to keep the head above the water), ii) swimming time (i.e. the time spent making active swimming motions to move around the cylinder), iii) climbing time (i.e. the time spent making active movements with its forepaws in and out of the water, directed specifically to the cylinder wall). Between each trial, the cylinder was cleaned and the water changed.

Rat plantar test

Changes in thermoceptive responses were evaluated according to (Hargreaves *et al.*, 1988) using a Plantar test apparatus (Ugo Basile, Varese, Italy). On the day of the experiment, rats were individually placed in transparent observation chambers (32 cm high, 24 cm diameter) for adaptation. After 1 h of habituation, the plantar surface of the left hind paw was exposed to a beam of radiant heat though the glass floor. The radiant heat source consisted of an infrared bulb (IR 90). A photoelectric cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. The cutoff time was set at 10 s in order to prevent tissue

damage. Three responses were obtained for each animal at 10 min intervals and averaged. Between each trial, the apparatus was cleaned and dried.

Rat formalin test

Rats were placed individually in a transparent chamber (32 cm high, 24 cm diameter) with a mirror placed on the back to aid observation for 45 min before the beginning of the test. Under light isoflurane (4%) anesthesia (loss of spontaneous movement with preservation of spontaneous respiration, blink and pinnae reflexes) 50 µl of a 2% formalin solution was injected subcutaneously into the dorsal surface of the right hind paw with a 27ga needle (BD, Drogheda, Ireland). Immediately after injection, the rat was returned to the observation chamber. Within 1 min of formalin injection, the rat displayed the behaviour typical of this assay, i.e. it held the injected paw just off the floor. During this period spontaneous flinching of the injected paw was observed. Flinching is characterized by a rapid and brief withdrawal or flexion of the injected paw. This pain-related behaviour was quantified by counting the number of flinches with a hand-held stopwatch in 5-min blocks for 60 min following formalin injection and expressed as the number of nociceptive behaviours per min. Cumulative response times during the 0-10 min and 15-60 min periods were regarded as I° and II° phase, respectively. Between each trial, the apparatus was cleaned and dried.

Monkey Tail withdrawal assay

Ten adult intact male and female rhesus monkeys (*Macaca mulatta*) with body weights ranging between 6.8 and 12.5 kg were used. The monkeys were housed individually with free access to water and were fed approximately 25 biscuits (Purina Monkey Chow; Ralston Purina, St. Louis, MO) and fresh fruit daily. No monkey had exposure to any opioid drug one month before the present study. The monkeys were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The studies were conducted in accordance with the University Committee on the Use and Care of Animals in the University of Michigan (Ann Arbor,

MI) and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, MD).

The warm water tail-withdrawal assay was used to evaluate thermal antinociceptive effects of the test compound (Ko *et al.*, 2006). Briefly, monkeys were seated in primate restraint chairs, and the lower part of their shaved tails (approximately 15 cm) were immersed in a thermal flask containing water maintained at either 42, 46, or 50^{o}C. Tail-withdrawal latencies were measured using a computerized timer by an experimenter who did not know dosing conditions. In each test session, monkeys were evaluated once with three temperatures given in a random order. If the monkeys did not remove their tails within 20 s (cutoff), the flask was removed and a maximum time of 20 s was recorded. Test sessions began with determining a control value at each temperature. Subsequent tail-withdrawal latencies were determined every 30 min after intrathecal administration. The same group of subjects (n=4) was tested in a 3-hr time course by using a single dosing procedure.

Scratching behavior, inferred as a response to itch sensation (Ko *et al.*, 2004), was recorded on videotape while the monkeys were in their home cages. A scratch was defined as one short-duration (< 1 s) episode of scraping contact of the forepaw or hind paw on the skin surface of other body parts. Scratching responses were scored by individuals who were blinded to experimental conditions. Each recording session was conducted for 15 min per test session that occurred every 30 min after intrathecal administration. The same group of subjects (n=6) was tested in a 3-hr time course by using a single dosing procedure.

For i.t. administration in monkeys, [Dmt^{1}]N/OFQ(1-13)NH_2 was administered at a total volume of 1 ml. The detailed description for intrathecal drug delivery can be referred to previous studies (Ko *et al.*, 2006). All experiments using intrathecal administration in monkeys were conducted with a 10-day inter-injection interval.

3.4 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 x and figure x 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).

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.

[^{35}S]GTPγS data are expressed as stimulation factor i.e. the ratio between specific agonist stimulated [^{35}S]GTPγS binding and basal specific binding. Calcium mobilization data are expressed as fluorescence intensity units (FIU) in percent over the baseline. Isolated tissues data are expressed as percent of the control 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. Concentration response curve to agonists were fitted with the following equation:

$$Effect = baseline + (Emax-baseline)/(1+10^{((LogEC_{50} - X)*HillSlope)})$$

where X is the agonist concentration. Curve fitting was performed using PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.)

Surmountable 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, 1973). 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. 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). the pK_B for insurmountable antagonists evaluated at different concentrations against the concentration response curve to N/OFQ were calculated using the following equation:

$$K_B = [\text{antagonist}]/(\text{slope} - 1)$$

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 rat studies statistical analyses were performed (Prism software; GraphPad Software, San Diego, California) on raw data using Student *t*-test with the only exception of the drag and rotarod data, which have been analyzed by repeated measures two-way ANOVA (genotype and day) followed by Bonferroni's test for multiple comparison. P values < 0.05 were considered statistically significant. For in vivo mouse studies tail withdrawal latency data are shown in time course experiments as mean ± SEM. Each data point was obtained from 16 animals. These data were used for calculating the area under the curve (AUC, latencies x time) to better display the dose response curves to the different agonists. AUC data were statistically analysed using one way ANOVA followed by the Dunnett test for multiple comparison. The criterion for significant differences was set at $p<0.05$. For monkey studies mean values (mean ± SEM) were calculated from individual values for all behavioral endpoints. Comparisons were made for the same monkeys across all test sessions. Data were analyzed by a two-way repeated analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. The criterion for significant differences was set at $p<0.05$.

4. RESULTS AND DISCUSSION

4.1 Knockout studies - Phenotype of NOP(-/-) rats

Recently, knockout rats for the NOP receptor gene have also been generated using target-selected N-ethyl-N-nitrosourea (ENU)-driven mutagenesis. Autoradiographic studies demonstrated that [^{3}H]N/OFQ binding was completely absent in brain slices of NOP(-/-) rats with no compensatory changes in classical opioid receptor expression (Homberg *et al.*, 2009). In the present study these NOP(-/-) rats were used to investigate anxiety- and mood-related behaviour (open field, elevated plus maze, and forced swimming test), locomotor (drag and rotarod test), and nociceptive (plantar and formalin test) phenotypes in comparison to their NOP(+/+) littermates. In addition, N/OFQ sensitivity has been assessed in electrically stimulated vas deferens tissues taken from NOP(+/+) and NOP(-/-) rats.

As shown in figure 4.1, in the elevated plus maze assay rats lacking the NOP receptor gene exhibited a statistically significant reduction in the time spent in the open arms as well as of the number of open arm entries compared to their wild type littermates. Moreover, as described in table 4.1, NOP(-/-) rats spent more time in the closed arms and exhibited less head-dipping and rearing behaviors than NOP(+/+) animals.

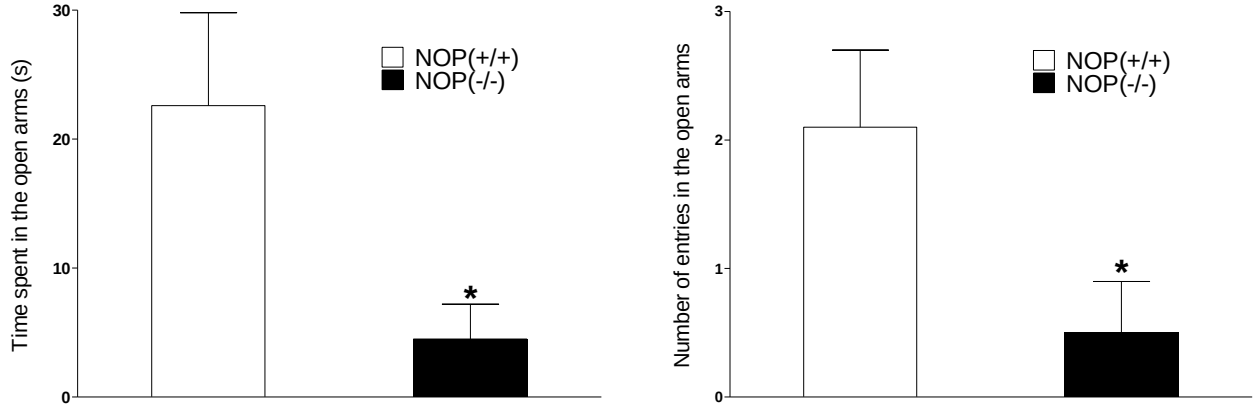


Figure 4.1. Elevated Plus Maze. Time spent (left panel) and number of entries (right panel) into the open arms by NOP(+/+) and NOP(-/-) rats. Sample size was 11 NOP(+/+) and 11 NOP(-/-) animals. All data represent mean ± SEM. *p < 0.05 compared to NOP(+/+) rats according to Student's *t*-test for unpaired data. $t_{DF} = 2.53_{20}$ and 2.13_{20} for left and right panel, respectively.

Table 4.1. Behavioural parameters displayed by NOP(+/+) and NOP(-/-) rats in the elevated plus maze. Sample size was 11 NOP(+/+) and 11 NOP(-/-) animals. All data represent mean ± SEM. *p < 0.05 compared to NOP(+/+) rats according to Student's *t*-test for unpaired data. NS, non statistically significant.

	NOP(+/+)	**NOP(-/-)**
Time in open arms (s)	22.6 ± 7.2	4.5 ± 2.7*
Time in closed arms (s)	171.5 ± 7.0	224.3 ± 10.7*
Number of open arm entries	2.1 ± 0.6	0.5 ± 0.4*
Number of arm entries	6.9 ± 0.7	4.3 ± 0.8*
Number of stretch attend postures	2.6 ± 0.5	1.5 ± 0.3
Number of head-dipping	4.8 ± 0.9	1.4 ± 0.5*
Grooming (s)	6.8 ± 1.6	3.9 ± 0.9
Number of rearings	25.1 ± 1.2	15.8 ± 1.9*
Number of faecal pellets	0.6 ± 0.4	2.5 ± 0.8*

In the open field, the behaviour of NOP(-/-) rats showed no statistically significant differences compared to that of NOP(+/+) littermates, although the latter animals showed a trend towards a reduction in all of the measured parameters (figure 4.2) .

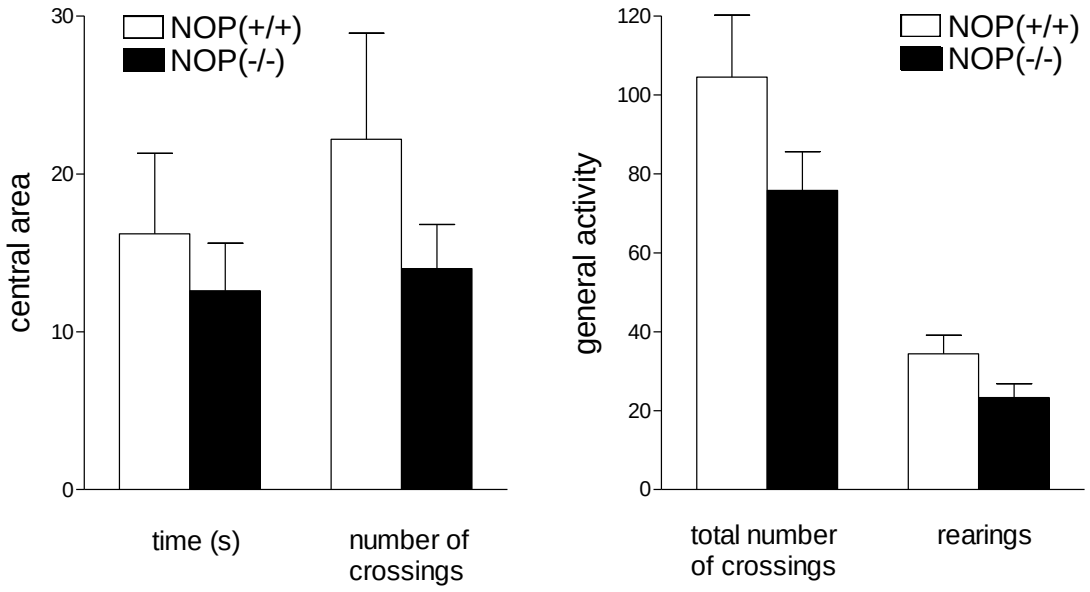


Figure 4.2. Open field test. Time spent and number of crossings into the central area (left panel) and total number of crossings and rearings (right panel) displayed by NOP(+/+) and NOP(-/-) rats subjected to the test. Sample size was 11 NOP(+/+) and 11 NOP(-/-) animals. All data represent mean ± SEM.

As shown in figure 4.3, in the forced swimming assay rats lacking the NOP receptor gene exhibited a statistically significant reduction in immobility time associated with an increase in climbing and swimming time.

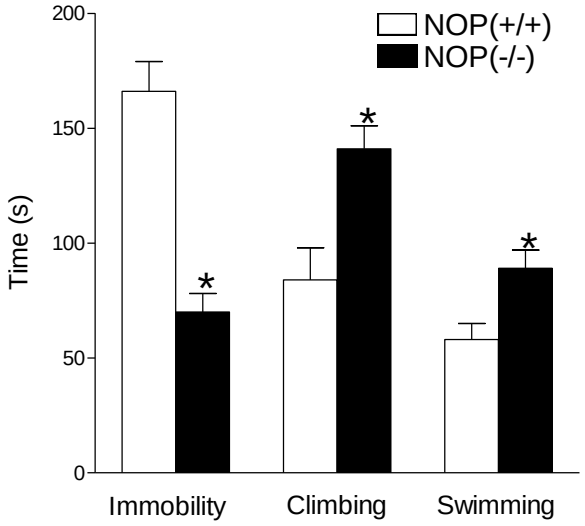


Figure 4.3. Forced Swimming test. Immobility, climbing and swimming time displayed by NOP(+/+) and NOP(-/-) rats subjected to the test. Sample size was 11 NOP(+/+) and 11 NOP(-/-) animals. All data represent mean ± SEM. *$p < 0.05$ compared to NOP(+/+) rats according to Student's t-test for unpaired data.

In the plantar test there were no differences in paw withdrawal latency between NOP(+/+) (5.2 ± 0.3 s) and NOP(-/-) rats (5.7 ± 0.2 s).

Rats receiving 50 µl of saline into the dorsal surface of the right hind paw did not show any pain related behaviour (data not shown, n=4 for both genotypes). In contrast, intraplantar injection of 50 µl of a 2% formalin solution into the dorsal surface of the right hind paw produced clear nociceptive responses in NOP(+/+) rats which lasted for the time course of the experiment. Of note, these animals did not display the typical biphasic response to formalin, showing approximately 7 responses per min over the time course of the experiment (figure 4.4, top panel). Genetic ablation of the NOP receptor gene produced a statistically significant increase in nociceptive behaviour of the mutant rats and in these animals the response to formalin injection was clearly biphasic (figure 4.4, top panel). As shown in figure 4.4 bottom panel NOP(-/-) rats displayed a statistically significantly higher nociceptive response to formalin then their NOP(+/+) littermates both in the I° and II° phase of the assay.

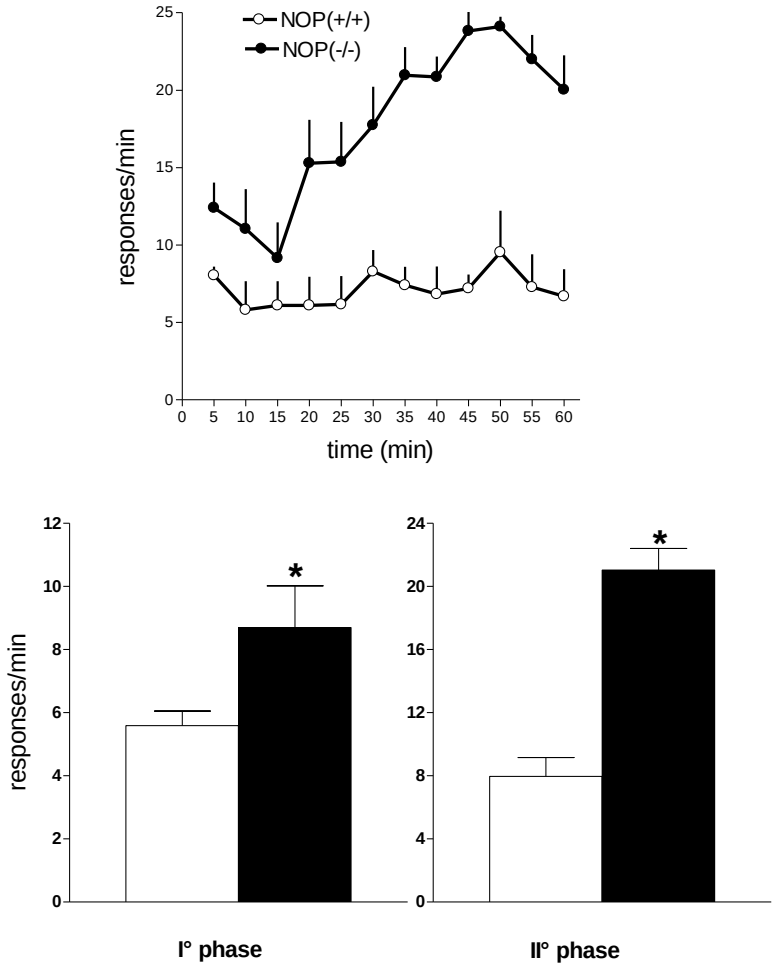


Figure 4.4. Time course of formalin-induced pain behaviour in NOP(+/+) and NOP(-/-) (top panel). The total nociceptive behaviour (responses/min) is plotted versus time (min). Formalin-induced pain behaviour during I° and II° phases (bottom panels). Sample size was 7 NOP(+/+) and 7 NOP(-/-) animals. * $p < 0.05$ vs NOP(+/+) according to the Student's t-test for unpaired data. $t_{DF} = -2.22_{12}$ and -7.18_{12} for left and right bottom panel, respectively.

Finally the electrically-stimulated rVD was used as an in vitro pharmacological preparation to assess the effects of N/OFQ in tissues taken from NOP(+/+) and NOP(-/-) animals. In NOP(+/+) tissues, N/OFQ inhibited in a concentration-dependent manner the electrically-induced twitches with a pEC_{50} of 7.13 and an E_{max} of 90 ± 1%. In contrast, the peptide was inactive in tissues taken from NOP(-/-) animals up to 3 μM (figure 4.5, left panel). In parallel experiments, the opioid receptor agonist etorphine displayed similar potency and efficacy in vas deferens tissues taken from NOP(+/+) (pEC_{50} 8.36 and 95 ± 2%, respectively) and NOP(-/-) (8.23 and 97 ± 1%, respectively) rats (figure 4.5, right panel).

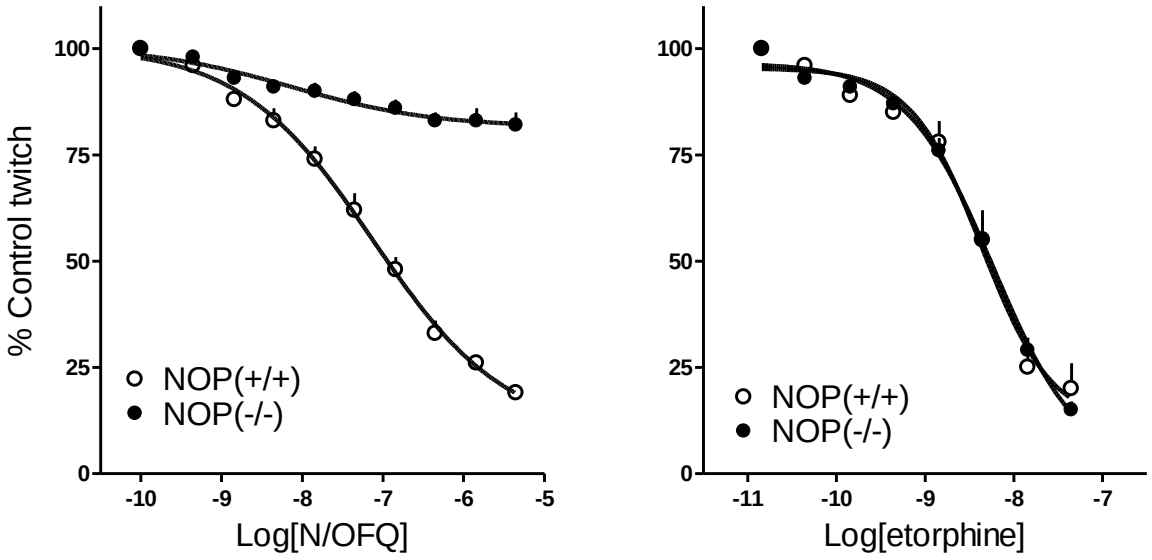


Figure 4.5. Electrically stimulated rat vas deferens. Concentration response curve to N/OFQ (left panel) and etorphine (right panel) in tissues taken from NOP(+/+) and NOP(-/-) rats. Points indicate the means and vertical lines the S.E.M. of three separate experiments.

The present study investigated the anxiety- and mood-related behaviour and locomotor and nociceptive phenotype of NOP receptor knockout rats. The results clearly indicated that the N/OFQ-NOP receptor system plays an important role in controlling anxiety- and mood-related behaviours, exercise driven locomotion and nociception. These results are in line with previous findings obtained with selective NOP receptor antagonists in mice and rats as well as with NOP(-/-) mice. Clearly N/OFQergic control over these biological functions appears to be maintained across animal species, experimental conditions and different behavioural assays. These observations may be relevant in the identification of the therapeutic indications (and contraindications) of NOP receptor antagonists. Moreover the present findings indicate that the NOP(-/-) animals generated by (Homberg *et al.*, 2009) are a useful model for investigating the range of biological functions controlled by the N/OFQ – NOP receptor system in rats. This is of particular importance for neuropharmacological studies since most of our knowledge in the field is based on rat models of pathology.

In the electrically-stimulated rVD taken from NOP(+/+) rats N/OFQ produced a concentration-dependent inhibition of the twitch response with maximal effects and potency values similar to those described in the literature (Bigoni *et al.*, 1999). This action was no longer evident in tissues taken from NOP(-/-) animals. In contrast, the inhibitory effects elicited by the classical opioid receptor agonist etorphine were superimposable in NOP(+/+) and NOP(-/-) rat tissues. Collectively these findings demonstrated that the gene knockout technology used by (Homberg *et al.*, 2009)was indeed successful and the lack of [^{3}H]N/OFQ binding to brain slices of NOP(-/-) rats is associated with a loss of NOP mediated biological actions.

As far as anxiety related behaviours are concerned, NOP(-/-) rats displayed an anxiety-like phenotype in the elevated plus maze but not in the open field, as previously shown for NOP(-/-) mice (Gavioli *et al.*, 2007). Importantly no differences were recorded for spontaneous locomotor activity between the two genotypes in the open field test. Therefore elevated plus maze results are not confounded by this parameter. These findings are in line with a large body of evidence demonstrating that N/OFQ given i.c.v. (Gavioli *et al.*, 2008; Gavioli *et al.*, 2002; Griebel *et al.*, 1999; Jenck *et al.*, 1997; Kamei *et al.*, 2004; Uchiyama *et al.*, 2008; Vitale *et al.*, 2006) as well as non peptide NOP agonists given systemically (Hayashi *et al.*, 2009; Hirao *et al.*, 2008a; Jenck *et al.*, 2000; Varty *et al.*, 2005; Varty *et al.*, 2008) promote anxiolytic-like actions in rodents. The mechanism underlying these anxiolytic-like effects are not fully understood (for details on this issue see (Gavioli *et al.*, 2006)), but recent findings indicate that $GABA_A$ receptor signalling might be involved (Gavioli *et al.*, 2008; Uchiyama *et al.*, 2008). NOP receptor selective antagonists such as J-113397 (Hirao *et al.*, 2008a; Varty *et al.*, 2005) and UFP-101 (Gavioli *et al.*, 2006) did not elicit anxiogenic actions *per se* at doses able to prevent the anxiolytic-like effect of NOP agonists. This contrasts with the anxiety-like phenotype displayed by NOP(-/-) rats (present results) and mice (Gavioli *et al.*, 2007). However, it is worthy to note that in the above studies the experimental conditions were possibly optimized for detecting anxiolytic-like effects of NOP agonists rather than anxiogenic effects of NOP antagonists. Further studies are needed to investigate under which

conditions endogenous N/OFQ-NOP receptor signalling is activated to control stress and anxiety levels. This information is particularly relevant since anxiety states might represent a contraindication to the clinical use of NOP antagonists.

As far as emotional responses are concerned, NOP(-/-) rats displayed a robust antidepressant-like phenotype in the forced swimming test indicating that blockade of N/OFQ-NOP receptor signalling in the brain produces antidepressant-like effects. This indication is corroborated by pharmacological and genetic evidence. Indeed, NOP(-/-) mice showed an antidepressant-like phenotype in the tail suspension and forced swimming tests (Gavioli *et al.*, 2003; Gavioli *et al.*, 2004), while selective NOP receptor antagonists ([Nphe^{1}]N/OFQ(1-13)NH_2, UFP-101, J-113397, and SB-612111) elicited dose-dependent antidepressant-like effects in these tests (Gavioli *et al.*, 2006; Gavioli *et al.*, 2003; Gavioli *et al.*, 2004; Redrobe *et al.*, 2002; Rizzi *et al.*, 2007a). Differences in immobility time between NOP(+/+) and NOP(-/-) rats might reflect differences in their locomotor performance (see below). However, we found no correlation (r^2 always < 0.06) between locomotor activity (drag and rotarod tests; data not shown) and immobility time in the forced swimming test; this holds true for NOP(+/+) and NOP(-/-) rats. Thus, it can be proposed that changes in locomotor activity do not bias the results of the forced swimming test. Recent findings support this proposal. In fact, UFP-101 produced antidepressant-like effects in mice after bilateral injection into the dorsal hippocampus without modifying locomotor performance (Goeldner *et al.*, 2010). More importantly, UFP-101 was recently shown to elicit antidepressant-like effects in the chronic mild stress assay in rats measuring a behavioural parameter (i.e. sucrose solution intake) that is predictive for antidepressant-like effects and not affected by changes in locomotor activity (Vitale *et al.*, 2009). To further confirm the involvement of N/OFQ in mood regulation, plasma N/OFQ levels were found to be significantly increased in depressed patients compared to normal subjects (Wang *et al.*, 2009). Similar results were found in patients with postpartum depression compared to healthy women; in this latter case a significant negative correlation between N/OFQ and 5-HT levels was also reported (Gu *et al.*, 2003). Overall, these

studies support the proposal that the NOP receptor represents a candidate target for the development of innovative antidepressant drugs.

Spontaneous locomotion in the open field was similar for NOP(+/+) and NOP(-/-) rats. However when animals were forced to perform a sustained motor task such as in the drag and rotarod assay, NOP(-/-) rats outperformed NOP(+/+) animals. This result suggests that the inhibitory influence of endogenous N/OFQ over motor activity becomes relevant during exercise rather than at rest. In fact, systemic administration of J-113397 or central administration of UFP-101 facilitated in a dose-dependent manner rat performance in the drag and rotarod tests (Marti *et al.*, 2004b). NOP(-/-) mice also outperformed NOP(+/+) mice in the same assays (Marti *et al.*, 2004b). Consistently, systemic administration of J-113397 and its analogues Trap-101 and GF-4 increased motor activity in NOP(+/+) but not in NOP(-/-) mice (Marti *et al.*, 2008; Viaro *et al.*, 2008; Volta *et al.*, 2010). In addition, preliminary evidence that J-113397 facilitates motor performance in non human primates has been presented (Viaro *et al.*, 2008). Collectively, these findings corroborate the view that NOP receptor blockade may represent an innovative strategy for the control of hypokinetic disorders. Indeed, NOP receptor antagonists attenuated motor deficits in rodent (Mabrouk *et al.*, 2010; Marti *et al.*, 2005; Marti *et al.*, 2008; Viaro *et al.*, 2010; Viaro *et al.*, 2008; Volta *et al.*, 2010) and non human primate (Viaro *et al.*, 2008; Visanji *et al.*, 2008) models of parkinsonism. The finding that N/OFQ levels are 3.5-fold elevated in the cerebrospinal fluid of parkinsonian patients compared to controls (Marti *et al.*, 2010) may provide a rational for developing NOP receptor antagonists as drugs to treat Parkinson's disease.

Nociceptive threshold in the plantar test was similar in NOP(+/+) and NOP(-/-) rats. This result suggests that endogenous N/OFQ-NOP receptor signalling does not control the responses to acute noxious stimulation. This statement is supported by evidence obtained with NOP(-/-), ppN/OFQ(-/-), and NOP(-/-)/ppN/OFQ(-/-) mice (Carra *et al.*, 2005b; Depner *et al.*, 2003; Nishi *et al.*, 1997; Ueda *et al.*, 1997) whose nociceptive response to acute stimulation is superimposable to that of their wild type littermates. Consistently, selective NOP receptor antagonists (e.g. J-113397, SB-

612111, Comp 24) do not modify pain threshold to acute noxious stimulation (Fischetti *et al.*, 2009; Ozaki *et al.*, 2000; Rizzi *et al.*, 2007a; Zaratin *et al.*, 2004). By contrast, when subjected to the formalin test NOP(-/-) rats displayed a robust pronociceptive-like phenotype, as observed with different knockout mice genotypes (Depner *et al.*, 2003; Rizzi *et al.*, 2006). In addition, systemic administration of J-113397 as well as intrathecal injection of UFP-101 elicited pronociceptive effects in normal mice subjected to the formalin assay (Rizzi *et al.*, 2006). Similar results were obtained after intrathecal injection of J-113397 in rats (Yamamoto *et al.*, 2001). Thus, converging pharmacological and genetic findings suggests that endogenous N/OFQergic signalling is activated during prolonged noxious stimulation and elicits antinociception. Since the effect of the peptide on pain transmission at supraspinal and spinal levels are opposite (i.e. supraspinal pronociceptive effect vs spinal antinociceptive action (Zeilhofer and Calo, 2003)) it could be proposed that, at least under the present experimental conditions, spinal antinociceptive action prevails over supraspinal pronociceptive effects (as discussed in details in (Rizzi *et al.*, 2006)). Interestingly, spinal levels of N/OFQ increased in response to formalin injection (Candeletti S., personal communication). The antinociceptive properties of spinal N/OFQ are well documented in rodents (Zeilhofer and Calo, 2003) and were recently confirmed in non human primates (Ko *et al.*, 2009; Ko *et al.*, 2006). The mechanism by which N/OFQ promotes spinal antinociception is probably related to its ability to inhibit excitatory neurons and the synaptic release of glutamate (Liebel *et al.*, 1997; Luo *et al.*, 2002). Collectively it appears that blocking the endogenous N/OFQ-NOP receptor signalling produces pronociceptive effects during tonic nociceptive stimulation. Chronic pain may therefore represent a contraindication for NOP selective antagonists.

4.2 Pharmacological studies

NOP selective ligands

The non-peptidic antagonist GF-4

To date, J-113397 represents the most used 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. The compound coded as Trap-101, 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 but as selective (>100 fold) as J-113397 (racemic mixture) for NOP over classical opioid receptors.. *In vivo*, Trap-101 changed motor activity in naive rats and mice and alleviated parkinsonism in 6-hydroxydopamine hemilesioned rats (Marti *et al.*, 2008). The aim of the present study was the *in vitro* pharmacological characterization of a Trap-101 analogue, named GF-4, obtained introducing in the Trap-101 structure two methyl groups in the hydroxymethyl function at the carbon 3 of the piperidine ring (Fig. 4.6).

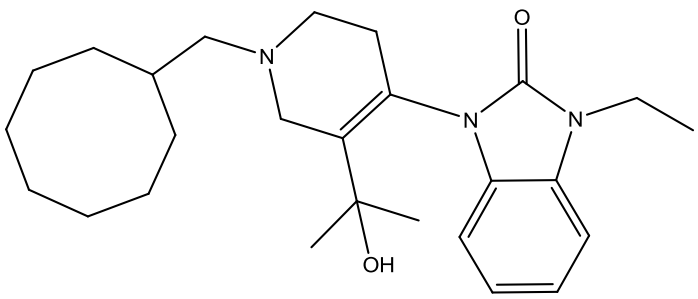


Figure 4.6. Structure of GF-4.

In receptor binding experiments performed on CHO_{NOP} cell membranes GF-4 displaced [^{3}H]N/OFQ in a concentration dependent manner showing nanomolar affinity (pK_i 7.46, Table 4.2). In parallel experiments J-113397 and Trap-101 displayed pKi values of 8.58 and 8.36. Under the same experimental conditions, GF-4 did not bind to the DOP receptor and showed lower affinities for MOP and KOP receptors (pK_i 6.78 and 6.84, respectively) (Table 4.2).

Table 4.2. Receptor binding profile of GF-4 to human recombinant NOP and classical opioid receptors expressed in CHO cells. Data are mean ($CL_{95\%}$) of 3 separate experiments.

	pK_i values ($CL_{95\%}$)			
	CHO_{hNOP}	CHO_{hMOP}	CHO_{hDOP}	CHO_{hKOP}
	N/OFQ	Endomorphin-1	Naltrindole	Norbinaltorphimine
Standard ligand	**9.27** (9.07-9.47)	**8.41** (8.15-8.67)	**9.46** (9.12-9.80)	**9.93** (9.8-10.06)
GF-4	**7.46** (7.09-7.83)	**6.78** (6.57-6.99)	**<6**	**6.84** (6.52-7.16)

In calcium mobilization experiments performed on CHO_{NOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein N/OFQ evoked a concentration dependent stimulation of calcium release displaying high potency (pEC_{50} 9.24 ($CL_{95\%}$ 9.10 – 9.38)) and maximal effects (≈ 200% over the basal values).

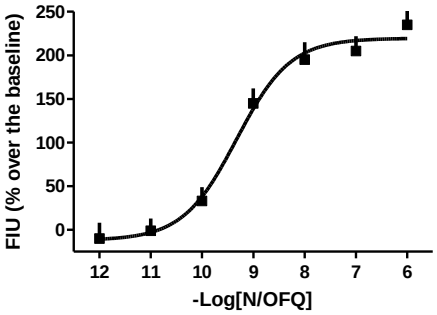


Figure 4.7. Concentration response curve to N/OFQ in calcium mobilization experiments performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. N/OFQ effects was expressed as % over the baseline. Data are the mean of 4 separate experiments performed in duplicate.

Up to 10 µM concentrations, J-113397, Trap-101 and GF-4 did not stimulate calcium release. Inhibition response experiments were performed by testing increasing concentrations of J-113397, Trap-101 and GF-4 (10 pM – 10 µM) against a fixed concentration of N/OFQ (10 nM), approximately corresponding to the EC_{80}. As shown in Figure 4.8, J-113397, Trap-101 and GF-4 were able to inhibit in a concentration-dependent manner the stimulatory effect of N/OFQ, showing similar pIC_{50} values. pK_B values of 7.88, 7.93 and 7.27 were calculated from these experiments for J-113397, Trap-101 and GF-4, respectively (Table 4.3).

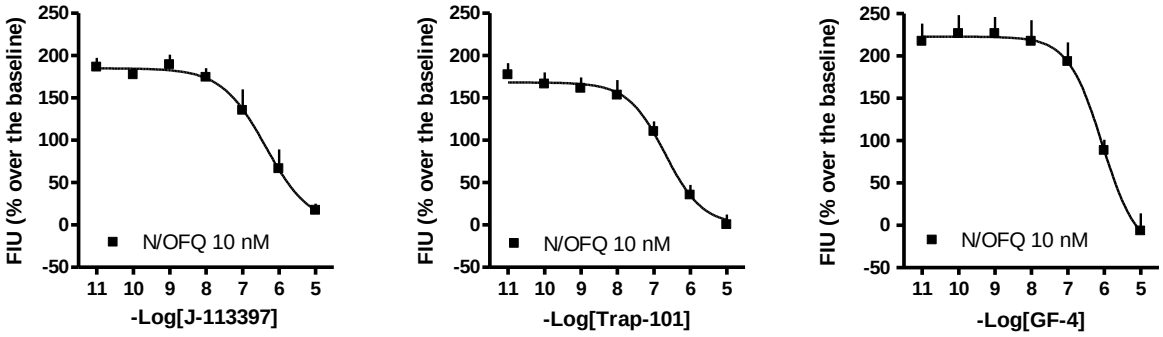
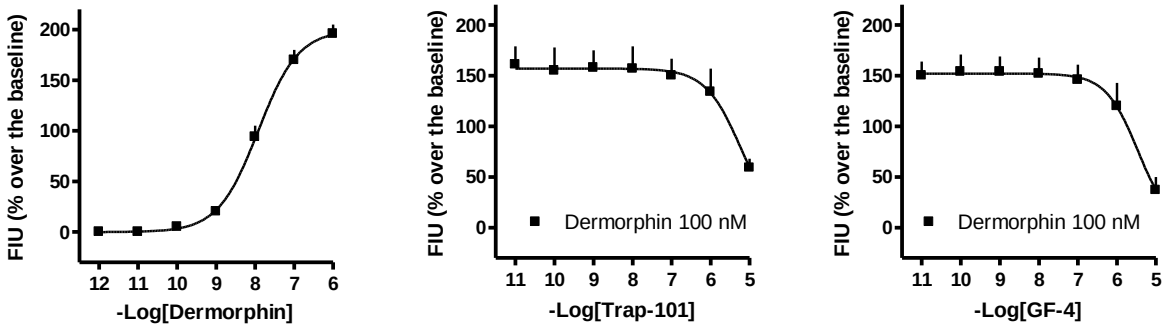


Figure 4.8. Inhibition experiments obtained by challenging 10 nM N/OFQ with increasing concentrations of NOP receptor antagonists in the calcium mobilization assay performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Data are the mean of 4 separate experiments performed in duplicate.

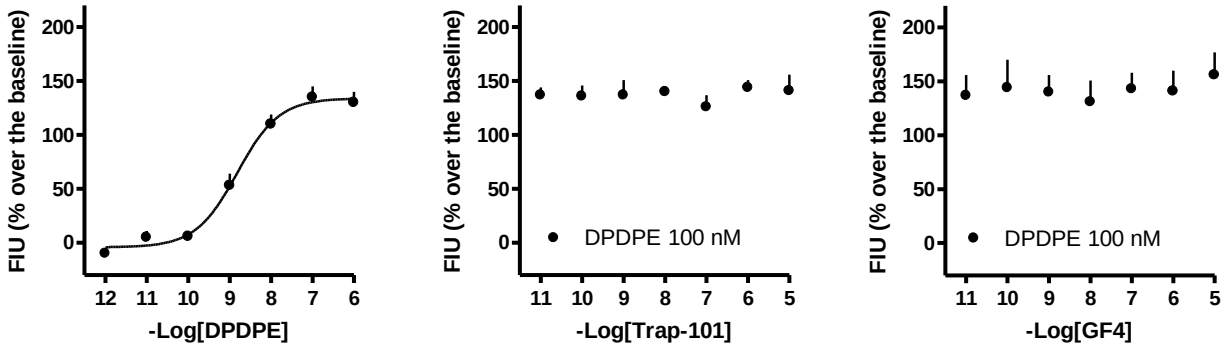
To assess the selectively of action of GF-4 similar experiments were performed in CHO cells stably expressing $G\alpha_{qi5}$ and classical opioid receptors. Dermorphin, DPDPE and Dynorphin A were used in these experiments as agonists for MOP, DOP and KOP receptors, respectively. They

produced a concentration dependent stimulation of calcium 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%. GF-4, inactive up to 1 µM against DPDPE and Dynorphin A, inhibited the effect of the MOP receptor agonists Dermorphin showing pK_B value of 6.48. In parallel experiments Trap-101 inhibited the stimulator effect of Dermorphin with a potency value close to that of GF-4 (pK_B 6.24) (Figure 4.9, Table 4.3).

CHO_{hMOP}



CHO_{hDOP}



CHO_{hKOP}

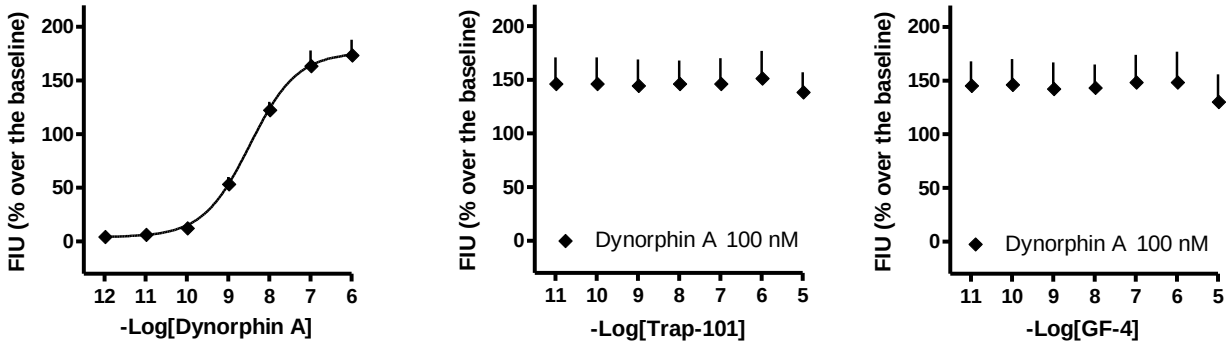


Figure 4.9. Inhibition experiments obtained by challenging 100 nM Dermorphin (top panel), 100 nM DPDPE (middle panel) and 100 nM Dynorphine A (bottom panel) with increasing concentrations of Trap-101 and GF-4 in the calcium mobilization assay performed in CHO co-expressing the classical opioid receptors and the $G\alpha_{qi5}$ protein. Data are the mean of 4 separate experiments performed in duplicate.

In parallel experiments Naloxone was inactive up to 1 µM against N/OFQ while inhibited the effects of the classical opioid receptors agonists showing higher potency at MOP (pK_B 9.09) than KOP (pK_B 7.14) and DOP (pK_B 7.32) (Table 4.3).

Table 4.3. Antagonist potencies of GF-4 and naloxone evaluated in calcium mobilization experiments performed in CHO cells expressing NOP or classical opioid receptors and the $G\alpha_{qi5}$ protein. Data are mean ($CL_{95\%}$) of 4 separate experiments

	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)
Trap-101	**7.93** (7.25-8.61)	**6.24** (5.90-6.58)	**< 6**	**< 6**
GF-4	**7.27** (6.69-7.85)	**6.48** (6.07-6.89)	**< 6**	**< 6**

J-113397, Trap-101 and GF-4 were assessed against N/OFQ in the electrically stimulated mouse and rVD. In the mVD N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner (pEC_{50} of value 7.55, E_{max} = 76 ± 2% inhibition of control twitch). The antagonists were tested at the single concentration of 100 nM against the effects of N/OFQ. J-113397, Trap-101 and GF-4 up to 1 µM did not modify the electrically induced twitch response. However, the antagonists produced a rightward shift of the concentration response curve to N/OFQ without significantly affecting the maximal agonist response. The pK_B values extrapolated from these experiments were 8.13, 7.46 and 7.82 for J-113397, Trap-101 and GF-4, respectively (Figure 4.10).

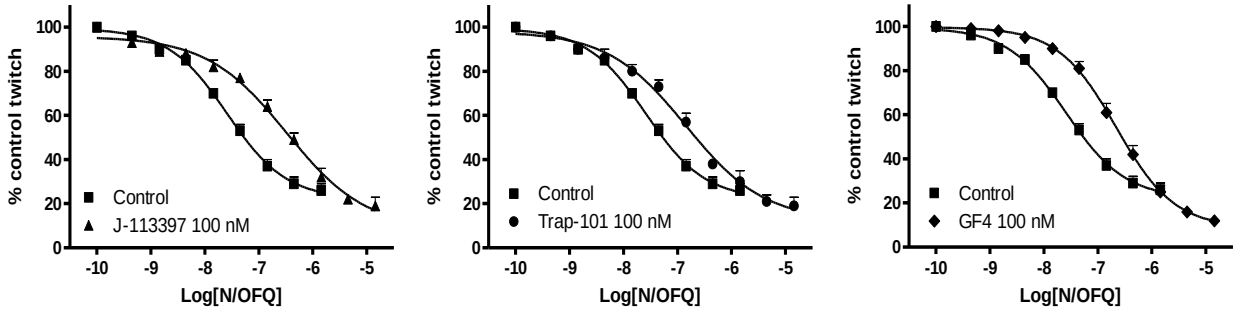


Figure 4.10. Concentration-response curve to N/OFQ obtained in the absence and presence of J-113397, Trap-101 and GF-4 (100 nM) in the electrically stimulated mouse vas deferens. The values are means ± SEM of 4 separate experiments.

In the rVD N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner (pEC_{50} of value 7.51, E_{max} = 78 ± 2% inhibition of control twitch). J-113397, Trap-101 and GF-4 were inactive up to 1 μM, while tested at the single concentration of 100 nM produced a rightward shift of the concentration response curve to N/OFQ without significantly affecting the maximal agonist response. The estimated pK_B values were 7.37, 7.53 and 7.30 for J-113397, Trap-101 and GF-4, respectively (Figure 4.11).

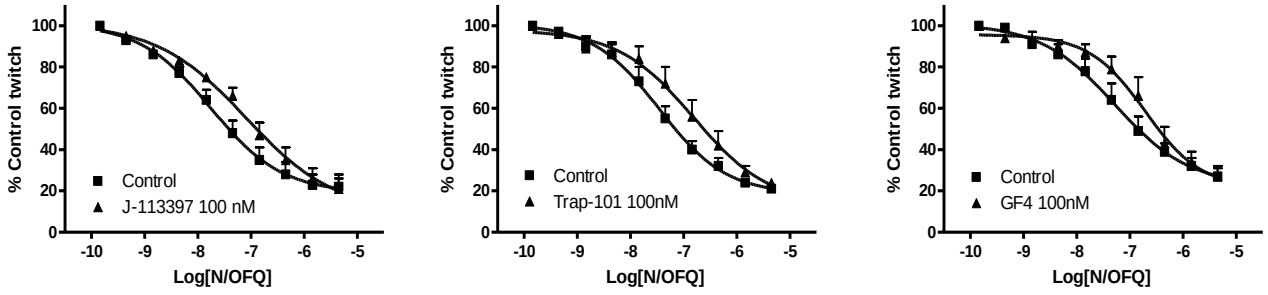


Figure 4.11. Concentration-response curve to N/OFQ obtained in the absence and presence of J-113397, Trap-101 and GF-4 (100 nM) in the electrically stimulated rat vas deferens. The values are means ± SEM of 4 separate experiments. Antagonist pK_B values obtained from these experiments are summarized in Table 3.

Table 4.4. pK_B values of J-113397, Trap-101 and GF-4 vs. N/OFQ in the electrically stimulated mouse and rat vas deferens.

	Electrically Stimulated Tissues	
	Mouse Vas Deferens	*Rat Vas Deferens*
	pK_B	pK_B
J-113397	**8.13** (7.69-8.57)	**7.37** (6.88-7.86)
Trap-101	**7.46** (6.97-7.92)	**7.53** (6.73-8.33)
GF-4	**7.82** (7.61-8.03)	**7.30** (6.58-8.02)

GF-4 is a novel NOP receptor antagonist generated from the Trap-101 structure. In vitro, GF-4 antagonized N/OFQ actions at human recombinant and rodent native NOP receptors in a concentration-dependent and competitive manner (pK_B 7.27-7.88) without exerting primary effects. The affinity at recombinant NOP receptors (~35 nM, pK_i 7.45) is ~100-fold lower than those of the most potent nonpeptide NOP receptor antagonists thus described, namely Compound 24 (0.27 nM; (Goto *et al.*, 2006))and SB-612111 (0.33 nM; (Zaratin *et al.*, 2004)), ~10-fold lower than that of J-113397 and Trap-101 (see also (Ozaki *et al.*, 2000; Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004)) and closer to that of JTC-801 (8.2 nM (Shinkai *et al.*, 2000); 30.8 nM(Zaratin *et al.*, 2004)). In vitro potency values in recombinant and native preparations are in line with this rank order, although the differences are less pronounced. For instance, in calcium mobilization assay, GF-4 was ~57-fold less potent than Compound 24 (Fischetti *et al.*, 2009) and ~5-fold less potent than Trap-101. In the mVD, GF-4 was only ~5-fold less potent than SB-612111 (Spagnolo *et al.*, 2007) and Compound 24 (Fischetti *et al.*, 2009), and ~2-fold less potent than J-113397. In this preparation, GF-4 was found ~2-fold more potent than Trap-101 (see also (Trapella *et al.*, 2006)) while in the rVD the reverse was true, Trap-101 being more potent than GF-4. Since no species differences with respect to responsiveness to NOP receptor ligands have been observed in these preparations, it is likely that these slight discrepancies in potency are due to experimental variability. Therefore, Trap-101 and GF-4 can be considered equipotent in isolated tissues.

Original binding and functional experiments in recombinant systems (Ozaki *et al.*, 2000) revealed that J-113397 is at least 350-fold selective over MOP and >1000-fold selective over DOP and KOP receptors. More recent studies (Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004), confirmed the high selectivity over DOP receptors but found the selectivity over KOP (30-fold) and, particularly, MOP (10-15-fold) receptors to be much lower than previously reported. Introduction of a double bond in the J-113397 molecule (i.e. elimination of chirality) did not change selectivity as shown by a comparative study between J-113397 and Trap-101 (Trapella *et al.*, 2006). Conversely, the introduction of a tertiary alcohol function had dramatic effects, since selectivity over MOP receptors dropped from ~49-fold (Trap-101) to ~6-fold (GF-4). This finding questions about the usefulness of GF-4 in in vivo experiments. The group of prof. Morari conduced a pharmacological characterization of GF-4 in vivo; in vivo selectivity of GF-4 was studied in NOP receptor knock-out mice (NOP(-/-)). The antiparkinsonian potential of GF-4 was investigated in 6-OHDA hemilesioned rats through a battery of previously validated behavioral tests: the bar, drag and rotarod tests (Marti *et al.*, 2005; Marti *et al.*, 2008; Marti *et al.*, 2007; Viaro *et al.*, 2008). Finally, to unravel the circuitry involved in motor actions of GF-4, GABA and GLU release was monitored in the lesioned SNr and ipsilateral VMTh in animals subjected to microdialysis and simultaneously performing the bar test. In these experiments, despite its poor in vitro selectivity, GF-4 was selective for NOP receptors in mice up to 30 mg/Kg. Interestingly, as previously shown for J-113397 (Viaro *et al.*, 2008) and Trap-101 (Marti *et al.*, 2008), GF-4 was able to exert dual control over stepping activity, namely facilitation at low doses and inhibition at high ones. These effects were mediated by NOP receptors since GF-4 was ineffective in NOP(-/-) mice. Antiparkinsonian properties of GF-4 were investigated in 6-hydroxydopamine hemilesioned rats. GF-4 ameliorated akinesia, bradykinesia and overall gait ability in the 0.1-10 mg/Kg dose range, but inhibited motor activity at 30 mg/Kg. To investigate the circuitry underlying motor facilitating and inhibitory effects of GF-4, microdialysis coupled to behavioral testing (akinesia test) was performed. An anti-akinetic dose of GF-4 (1 mg/Kg) reduced glutamate (GLU) and enhanced GABA release in SNr,

while the pro-akinetic dose of GF-4 (30 mg/Kg) evoked opposite effects. Moreover, the anti-akinetic dose of GF-4 reduced GABA and increased GLU release in ventro-medial thalamus, the pro-akinetic dose decreasing GABA without affecting GLU release in this area.

Altogether, these results indicate that the chemical modification of Trap-101, obtained by introducing two methyl groups in the hydroxymethyl function at the position 3 of the piperidine nucleus, did not alter the pharmacological activity of the compound (a pure and competitive NOP receptor antagonist) but slightly reduced its potency at recombinant NOP receptors and, more dramatically, in vitro selectivity over classical opioid receptors. Nevertheless, GF-4 was NOP receptor selective in vivo, replicating the antiparkinsonian effect and neurochemical changes typical of its parent compounds, and being as potent as J-113397 (Marti *et al.*, 2007). Further studies are needed to elucidate whether the in vivo gain in potency is due to greater metabolic stability, possibly to greater resistance to cytochrome oxidation as predicted on the basis of the structural changes made (Trapella *et al.*, 2006). The present data offer new insights into the structural requirements for optimal antagonist activity at NOP receptors and selectivity over classical opioid receptors. Moreover, they confirm that NOP receptor antagonists are able to attenuate parkinsonian-like symptoms via re-setting of GLU and GABA inputs upon nigro-thalamic GABA projection neurons (Marti *et al.*, 2005; Marti *et al.*, 2008; Marti *et al.*, 2007).

Pharmacological studies

The non-peptidic agonists Ro 65-6570, SCH-221510 and Compound 6d

Ro 64-6198 is a high affinity (pK_i 9.41) and highly selective (120-, 229- and 3548-fold over MOP, KOP and DOP, respectively) ligand for the NOP receptor. Ro 64-6198 is a full agonist for stimulation of [^{35}S]GTPγS binding and inhibition of forskolin-stimulated cAMP formation. In addition, the authors completed a number of in vivo behavioural tests and showed an impressive anxiolytic profile for this compound comparable to benzodiazepines (Jenck *et al.*, 2000).

The pharmacological profile and in vivo activity of Ro- 646198 have been confirmed by several groups (Shoblock, 2007).Another Ro 64-6198 chemically similar compound, named Ro 65-6570, was identified as a non-peptide NOP agonist with high affinity and moderate selectivity over the opioid (MOP,DOP,KOP) receptors (Wichmann *et al.*, 1999). In this study, we have evaluated the *in vitro* pharmacological profile of Ro 65-6570 and compared this with N/OFQ on NOP recombinant receptor (in Chinese hamster ovary (CHO) cells expressing the human NOP (CHO_{hNOP})) and on native receceptor (in the electrically stimulated mouse and rVD). In addition, we compared these responses with those obtained by two novel non-peptide NOP agonists: SCH-221510 (Varty *et al.*, 2008) and Compound 6d (Mustazza *et al.*, 2008).

Calcium mobilization experiments were first performed. In CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein, N/OFQ evoked a concentration dependent stimulation of calcium release displaying high potency (pEC_{50} 9.30 ($CL_{95\%}$ 9.20 – 9.39)) and maximal effect (246% over the basal values). The non-peptide ligand, Ro 65-6570, stimulated calcium levels in a concentration-dependent manner with a maximal effect (E_{max} 267%) similar to that of N/OFQ while exhibiting a 30 fold lower potency (pEC_{50} 7.95 ($CL_{95\%}$ 7.84 – 8.10)). Under the same experimental conditions, SCH 221510 showed maximal effect (≈ 170% over the basal values) similar to that of N/OFQ (165%) and a potency that was 100 fold lower (pEC_{50} 6.76 ($CL_{95\%}$ 6.60 – 6.92)) than that of

N/OFQ (pEC_{50} 8.47 ($CL_{95\%}$ 8.37– 8.56)). In another series of experiments compound 6d was tested: in this series of experiments N/OFQ mobilized intracellular calcium with potency of 8.92 ($CL_{95\%}$ 8.79 – 9.05) and maximal effect of 180% over the baseline. Compound 6d mimicked the N/OFQ effects with lower potency (pEC_{50} 7.36($CL_{95\%}$ 7.32 – 7.40)) and similar maximal effect (E_{max} 146%).

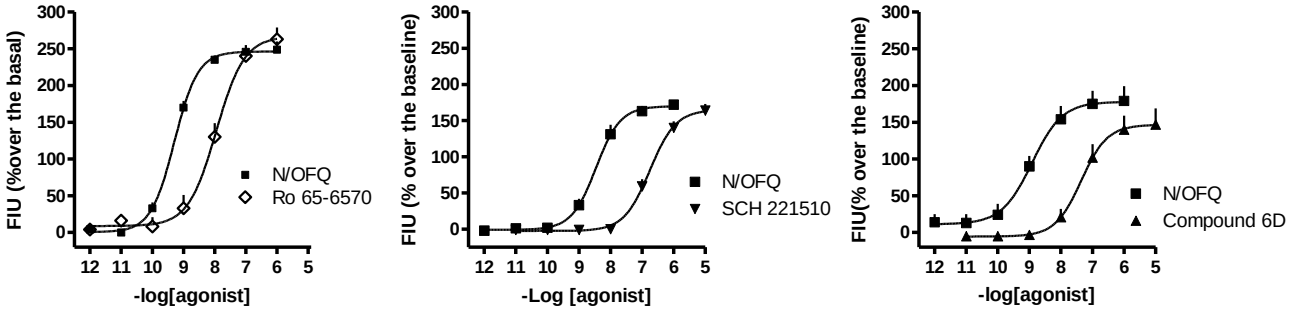


Figure 4.12. Concentration response curve to N/OFQ and Ro 65-6570 (left panel), SCH 221510 (middle panel), and Compound 6d (right panel) in calcium mobilization experiments performed in CHO_{hNOP} cells stably expressing the $G\alpha_{qi5}$ protein. Compounds effects was expressed as % over the baseline. Data are the mean of 4 separate experiments performed in duplicate.

In the isolated mVD N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner (pEC_{50} 7.64 ($CL_{95\%}$ 7.53 –7.75), E_{max} = 69± 2% inhibition of control twitch). Ro 65-6570 mimicked the inhibitory effect of N/OFQ with a pEC_{50} value of 6.79 ($CL_{95\%}$ 6.40 –7.18) appearing about 10 fold less potent than N/OFQ and producing a maximal effect value significantly higher than that of the peptide (90± 3%) (fig. 4.13, left panel). The inhibitory effect induced by N/OFQ takes place immediately after adding the peptide to the bath, whereas Ro 65-6570 induced a very slow inhibitory effect which reaches the plateau only after 15-20 min. SCH 221510 effects in the mVD appeared similar to that observed with Ro 65-6570. Indeed, SCH 221510 showed a pEC_{50} value of 6.56 ($CL_{95\%}$ 6.27 –6.85) that was 10 fold lower than that calculated for N/OFQ in the same experimental set (pEC_{50} of value 7.55 ($CL_{95\%}$ 7.46 – 7.64), E_{max} = 69± 2% inhibition of control twitch) (fig. 4.13, middle panel). SCH 221510, as Ro 65-6570, displayed very slow kinetics, with the plateau reached after 15-20 min from the injection.

Compound 6d was then tested; it behaved as an agonist less potent than Ro 65-6570 and SCH 221510, showing a potency value of 5.56 ($CL_{95\%}$ 5.36-5.86). In the same experiments N/OFQ showed potency values 30 fold higher (pEC_{50}=7.14) (figure 4.13, right panel).

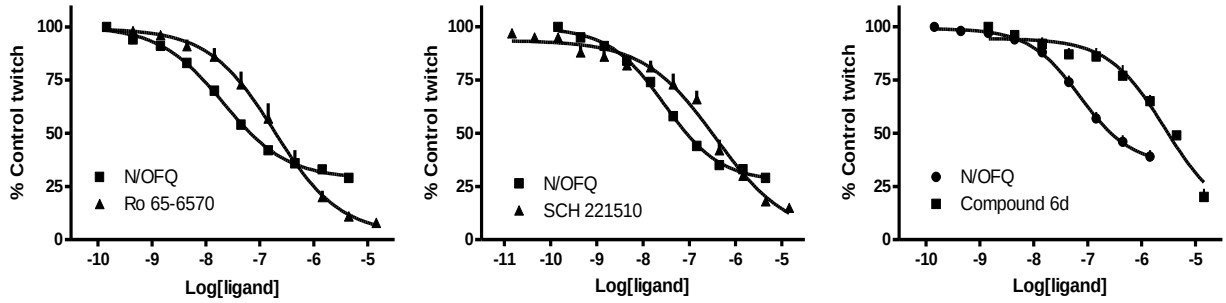


Figure 4.13. Concentration response curve to N/OFQ and Ro 65-6570 (left panel), SCH 221510 (middle panel), and Compound 6d (right panel) in the electrically stimulated mouse vas deferens. The values are means ± SEM of 5 separate experiments.

As shown in figure 4.14, the effects of N/OFQ and the non-peptide agonists were evaluated in the presence of the NOP selective antagonist, J-113397 (1 μM). J-113397 did not modify *per se* the control twitches, and produced a rightward shift of the concentration-response curve to N/OFQ showing a pK_B value of 7.88. At the contrary, 1 μM J-113397 did not significantly modify the concentration-response curve to Ro 65-6570 (figure 4.14, top right panel). Under the same experimental conditions, the effect of SCH 221510 was evaluated in the presence of J-113397 1 μM (figure 4.14, bottom left panel). The concentration response curve to SCH 221510 was not modified by the antagonist. Finally, compound 6d was tested in presence of J-113397 (1 μM). The antagonist was poorly effective against compound 6d (figure 4.14, bottom right panel).

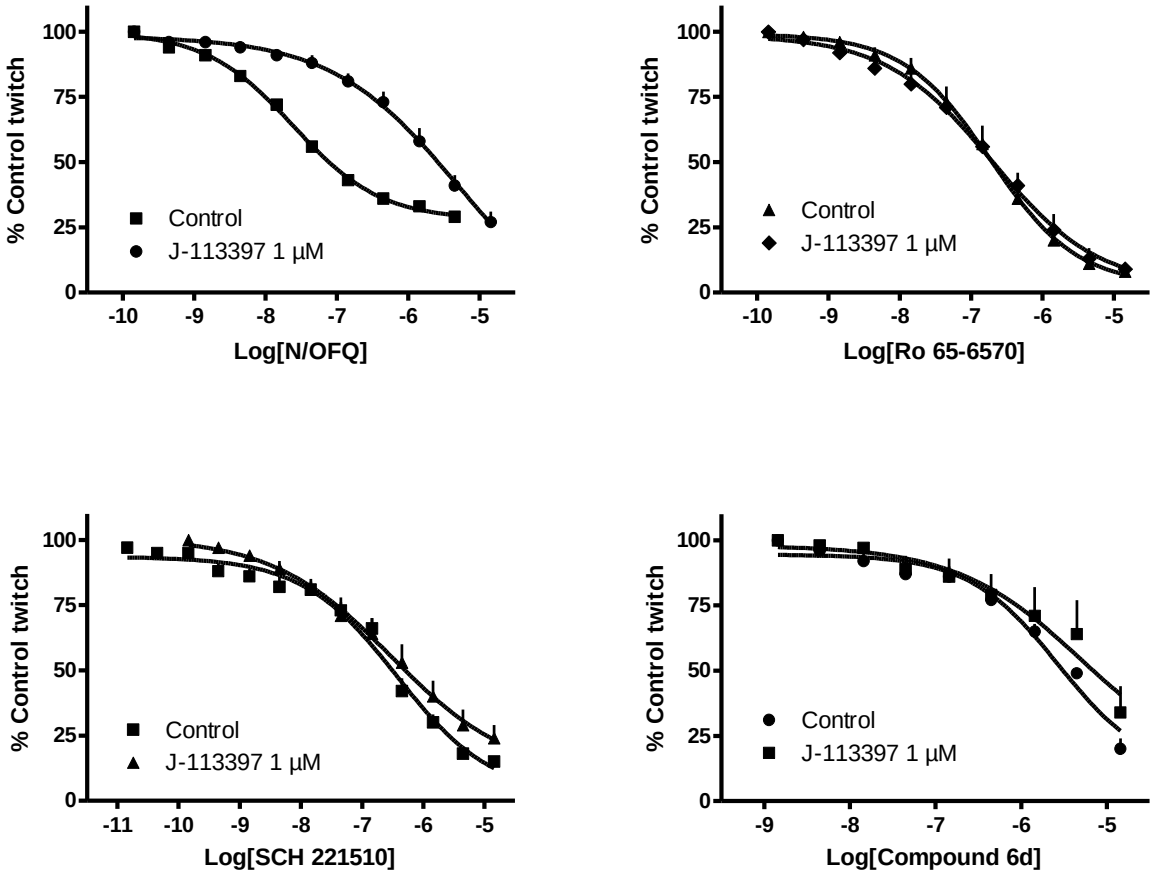


Figure 4.14. Concentration-response curve to N/OFQ (top left panel), Ro 65-6570 (top right panel), SCH 221510 (bottom left panel), Compound 6d (bottom right panel) obtained in the absence and presence of J-113397 (1 µM) in the electrically stimulated mouse vas deferens. The values are means ± SEM of 5 separate experiments.

All the compounds were then tested in tissues taken from NOP(-/-) animals.

In NOP(+/+) tissues, Ro 65-6570 mimicked the inhibitory effect of N/OFQ (pEC_{50} 7.66 (CL $_{95\%}$ 7.50 –7.77), E_{max} = 72± 3%) showing lower potency (pEC_{50} 6.86 (CL $_{95\%}$ 6.08–7.64)) and higher maximal effect (E_{max} = 94± 2%). In tissues taken from NOP(-/-) mice, N/OFQ was found inactive while Ro 65-6570 produced similar inhibitory effects as those measured in NOP(+/+) tissues (pEC_{50} 7.05 (CL $_{95\%}$ 6.54 –7.56), E_{max} = 95± 1%) (fig. 4.15 top panels). SCH 221510, examined in NOP(+/+) tissues.

In NOP(-/-) tissues, SCH 221510 showed a pEC_{50} value of 5.85(CL $_{95\%}$ 5.04 –6.64) and a maximal effect of 73 ± 5% (fig. 4.15 bottom left panel). Compound 6d was then tested: it displayed similar low potency in both NOP(+/+) and NOP(-/-) tissues (figure 4.15 bottom right panel).

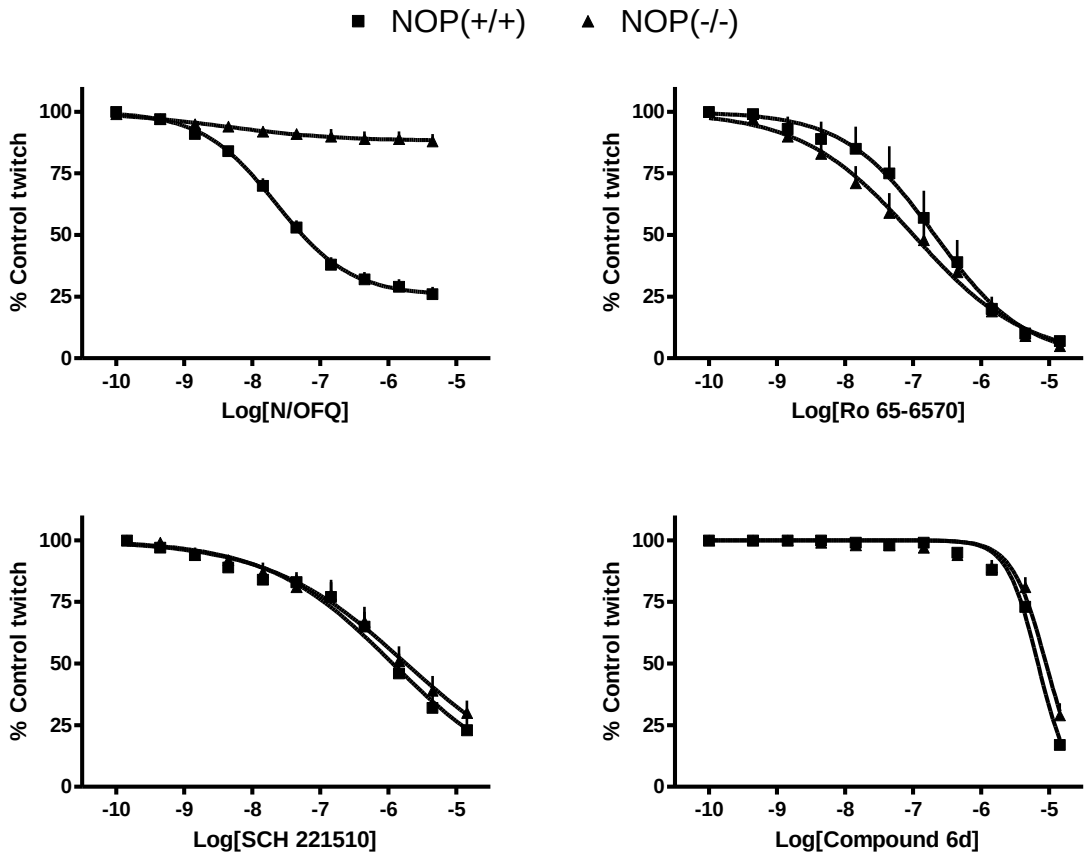


Figure 4.15. Concentration-response curve to N/OFQ, Ro 65-6570, SCH 221510 and Compound 6d obtained in vasa deferentia taken from NOP(+/+) and NOP(-/-) mice. The values are means ± SEM of 3 separate experiments.

In the rVD N/OFQ inhibited the twitch response to electrical field stimulation in a concentration dependent manner (pEC_{50} of value 7.24, Emax = 80 ± 2% inhibition of control twitch).

Ro 65-6570 mimicked the inhibitory effect of N/OFQ with a pEC_{50} value of 7.11 (CL 95% 6.77 – 7.45) and producing a maximal effect value similar to that of N/OFQ (85± 4%, figure 4.16).

In these tissues, SCH 221510 inhibited the electrically induced contraction with potency of 5.73 ($CL_{95\%}$ 4.82-6.63) and with maximal effects of 80 ± 5%, where N/OFQ showed a potency value of 7.26 ($CL_{95\%}$ 6.91-7.61) and E_{max} = 80 ± 4%.

In a parallel set of experiments the compound 6d effects were tested. In these tissues N/OFQ inhibited the twitch in a concentration-dependent manner (pEC_{50} = 6.93 ($CL_{95\%}$ 6.78 – 7.08) E_{max} = 90 ± 3%). At the contrary, Compound 6d showed a concentration dependent stimulatory action, augmenting the tissues contraction with on effect of 380 ± 35% at 10µM.

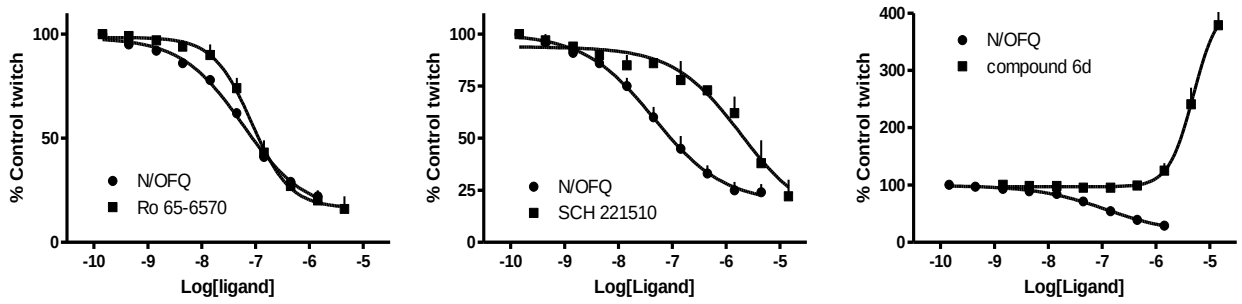


Figure 4.16. Concentration-response curve to N/OFQ and Ro 65-6570 (left panel), SCH 221510 (middle panel), and Compound 6d (right panel) in the electrically stimulated rat vas deferens. The values are means ± SEM of 4 separate experiments.

Also in this case, the effects of the three compounds were evaluated in the presence of the NOP selective antagonist, J-113397 (1 µM). J-113397 produced a rightward shift of the concentration-response curve to N/OFQ showing a pK_B value of 7.73. 1 µM J-113397 modified the concentration-response curve to Ro 65-6570 reducing its maximal effect. Under the same conditions, J-113397 1 µM produced a marked shift of the SCH 221510 concentration-response curve (figure 4.17). Compound 6d was also tested in presence of J-113397. The antagonist was inactive against the stimulatory effect of Compound 6d (figure 4.17).

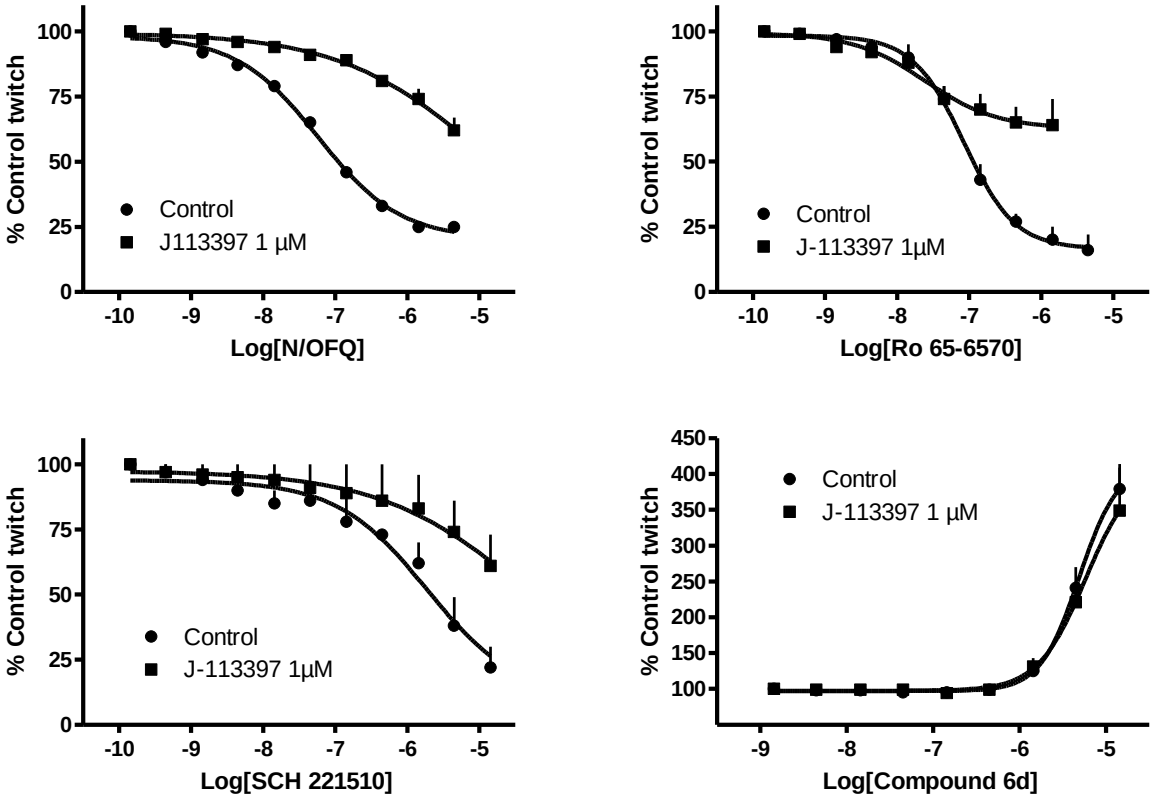


Figure 4.17. Concentration-response curve to N/OFQ (top left panel), Ro 65-6570 (top right panel), SCH 221510 (bottom left panel), Compound 6d (bottom right panel) obtained in the absence and presence of J-113397 (1 µM) in the electrically stimulated rat vas deferens. The values are means ± SEM of 5 separate experiments.

A final note is referred to the compound kinetics: N/OFQ showed a fast kinetic reaching the plateau of action in approximately 1 min and eliciting effects that were easily reverible by washing the tissues, on the contrary Ro 65-6570, SCH 221510 and compound 6d showed a slow kinetic profile (15-20 min needed to reach the plateau), and produced non reversible effects. In fact, while N/OFQ crc could be repeated in the same tissue, the crc to the other compounds could not be repeated in the same tissue.

As mentioned in the introduction after more than ten years from its discovery (Jenck *et al.*, 2000), Ro 64-6198 still represents the most potent and selective and the most used NOP non peptide agonist. This molecule has been and still is surely useful for increasing our knowledge regarding the

N/OFQ - NOP receptor system and the possible therapeutic indications of drugs interacting with the NOP receptor (Shoblock, 2007). However to firmly identify drug class effects the actions of Ro 64-6198 must be compared to those elicited by other, possibly chemically unrelated, non peptide compounds acting as selective NOP agonists. To this aim the molecules Ro 65-6570 (Wichmann *et al.*, 1999), SCH 221510 (Varty *et al.*, 2008) and compound 6d (Mustazza *et al.*, 2008) were selected and characterized in vitro at human recombinant and animal native NOP receptors.

The full agonist properties of the three molecules at human recombinant NOP receptors were confirmed in calcium mobilization studies. In fact all compounds mimicked the stimulatory action of N/OFQ eliciting similar maximal effects. The potency of Ro 65-6570, SCH 221510 and compound 6d were lower than that of the natural peptide. This is in line with previous studies performed in different laboratories in which these compounds were evaluated in receptor binding as well as functional assays (either cAMP levels or stimulated GTPγS binding) (Hashiba *et al.*, 2001; Mustazza *et al.*, 2008; Varty *et al.*, 2008). However it is also worthy of mention that the potency of these molecules in the present calcium assay (N/OFQ concentration ratio from 22 to 51) is lower compared to that reported in literature for instance in the GTPγS assay (concentration ratio from 1 to 10). Similar results were reported for Ro 64-6198 that displayed a N/OFQ concentration ratio of 30 in this calcium assay (Camarda *et al.*, 2009) while its concentration ratio in the GTPγS assay was 1 (Jenck *et al.*, 2000). Thus the present assay seems to underestimate the potency of these ligands. As mentioned in the results, the kinetics of the inhibitory effect elicited by N/OFQ in electrically stimulated tissues is rapid and immediately and completely reversible after washing while that of Ro 65-6570, SCH 221510 and compound 6d (and also of Ro 64-6198, (Rizzi *et al.*, 2001c)) is characterized by slow onset and development, and slow and partial reversibility after washing. The slow kinetic 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 get full activation of NOP receptors with these agonists may be incompatible with the rapid kinetics which characterized the calcium transient response. As a matter of fact, the different kinetics of N/OFQ and the above

mentioned ligands recorded in isolated tissues could not be detected in the present calcium mobilization experiments.

In the electrically stimulated mouse vas deferens N/OFQ inhibited the twitch response in a concentration dependent manner showing values of potency and maximal effects similar to those reported in literature (Calo *et al.*, 1996). The three compounds mimicked N/OFQ effects showing lower potency but higher maximal effects. The lower potency of these compounds is expected based on results obtained at the recombinant NOP receptors while their higher maximal effects may suggest that the NOP receptor is not the exclusive target of action of these molecules. This hypothesis is corroborated by the results obtained in receptor antagonist and knockout studies. In fact in the mouse vas deferens the inhibitory action of N/OFQ is antagonized effectively by the NOP antagonist J-113397 that displayed pK_B values in line with literature findings (Bigoni *et al.*, 2000a). In addition the action of the peptide was no longer evident in tissues taken from NOP(-/-) animals. Thus the results of these experiments clearly demonstrated that the biological effect of N/OFQ in this preparation is entirely due to its ability to activate the NOP protein. On the contrary, the effects of Ro 65-6570, SCH 221510 and compound 6d were resistant to J-113397 and were similar in tissues taken from NOP(+/+) and NOP(-/-) mice. Thus these findings suggest that the three compounds do not behave as selective NOP agonists in this preparation.

Somewhat different results were obtained in the rat vas deferens. In fact in this preparation Ro 65-6570 and SCH 221510 mimicked the inhibitory effects of the natural peptide showing similar maximal effects while compound 6d was found inactive in the nanomolar range of concentration and was able to stimulate the twitch response at micromolar concentrations. The NOP receptor antagonist J-113397 produced a rightward shift of the concentration response curve to N/OFQ and SCH 221510, strongly reduced the maximal effect of Ro 65-6570, and did not modify the stimulatory action of compound 6d. These results demonstrated that in this preparation SCH 221510 behaves as a selective NOP agonist, Ro 65-6570 displayed reduced selectivity for NOP

while compound 6d interact only at high concentration with an undefined site whose activation produces stimulatory effects. The residual effect of Ro 65-6570 in the presence of J-113397 can be possibly be attributed to the activation of classical opioid receptors since this molecule displayed poor selectivity particularly over MOP receptors (~10 fold, (Hashiba *et al.*, 2001)).

Interestingly, similar results were obtained in the past with Ro 64-6198. This compound, similar to SCH 221510 and to a lesser extend to Ro 65-6570, elicited inhibitory effects in the rat vas deferens that were sensitive to NOP antagonists (J-113397 and [Nphe^1]N/OFQ(1-13)-NH_2) while in the mouse vas deferens its action was resistant to the same molecules (Rizzi *et al.*, 2001c).

Collectively the present results confirmed the NOP agonist properties of SCH 221510, Ro 65-6570, and compound 6d but demonstrated important limitations of these molecules related to their selectivity of action. In particular compound 6d displayed NOP antagonist resistant effects in both mouse and rat preparations; SCH 221510 and Ro 65-6570 showed a tissue dependent profile behaving as NOP selective agonists only in the rat tissue. Based on these results compound 6d can not be recommended for in vivo use. Ro 65-6570 and particularly SCH 221510 can be used in vivo together with the standard molecule Ro 64-6198, however caution should be adopted in the interpretation of their effects and antagonist and/or knockout experiments should be always performed to demonstrate the involvement of the NOP receptor in the actions elicited by these molecules.

Pharmacological studies

NOP/MOP mixed agonists: [Dmt^1]N/OFQ(1-13)-NH$_2$

The aim of the present study was the design, synthesis and pharmacological characterization of novel peptides acting as non selective NOP/MOP agonists and their in vivo evaluation as spinal analgesics. Thus, some [X^1] substituted N/OFQ analogues were synthesised and evaluated pharmacologically in calcium mobilization experiments performed in Chinese hamster ovary (CHO) cells expressing the human NOP or MOP receptors as well as the chimeric G protein $G\alpha_{qi5}$ which forces Gi coupled receptors to signal via the PLC-IP$_3$-Ca^{2+} pathway (Camarda *et al.*, 2009; Fischetti *et al.*, 2009). From these experiments [Dmt^1]N/OFQ(1-13)-NH$_2$ was selected as the most potent and least selective NOP/MOP agonist. The pharmacological profile of this peptide was then evaluated in vitro in i) membranes of CHO cells expressing the NOP, MOP, DOP or KOP receptors studied with receptor binding and stimulation of [^{35}S]GTPγS binding experiments for MOP and NOP, ii) membranes of the rat cerebral cortex or spinal cord in the [^{35}S]GTPγS assay, and iii) the electrically stimulated guinea pig ileum, a pharmacological preparation expressing both MOP and NOP receptors. The in vivo activity of [Dmt^1]N/OFQ(1-13)-NH$_2$ was investigated in the mouse tail withdrawal assay by injecting the peptide i.t.. Finally the antinociceptive action of spinal [Dmt^1]N/OFQ(1-13)-NH$_2$ was assessed in non human primates.

In CHO$_{NOP}$ cells stably expressing the $G\alpha_{qi5}$ chimeric protein, N/OFQ evoked a concentration dependent stimulation of calcium release displaying high potency (pEC$_{50}$ 9.30 (CL$_{95\%}$ 9.05 – 9.55)) and maximal effect (240 ± 14 % over the basal values) while dermorphin was inactive up to 10 μM (figure 4.18, left panel). Opposite results were obtained in CHO$_{MOP}$ cells stably expressing the $G\alpha_{qi5}$ chimeric protein, where dermorphin concentration dependently stimulated calcium mobilization (pEC$_{50}$ 8.17 (CL$_{95\%}$ 7.93 – 8.41)); E$_{max}$ 130 ± 12 % over the basal values) while N/OFQ was inactive up to 1 μM (figure 4.18, right panel). Under the same experimental

conditions, N/OFQ analogues were assayed in both cell lines. Table 4.5 summarizes the results obtained in this series of experiments. The amide form of N/OFQ displayed similar potency, maximal effects and selectivity of action as the natural peptide. [Tyr^1]N/OFQ-NH_2 displayed a slight reduction in NOP potency (pEC_{50} 9.14 ($CL_{95\%}$ 8.93 – 9.35)) while being able to activate the MOP receptor although only in the micromolar range of concentrations (pEC_{50} 6.07 ($CL_{95\%}$ 5.90 – 6.24)). The substitution of Phe^1 with Dmt produced a reduction of NOP potency by 10 fold (pEC_{50} 8.57 ($CL_{95\%}$ 8.28 – 8.86)) associated with an important increase in MOP potency (pEC_{50} 7.05 ($CL_{95\%}$ 6.55 – 7.55)). Similar results were obtained when the [Tyr^1] and [Dmt^1] modifications were applied to the N/OFQ(1-13)-NH_2 template. N/OFQ(1-13)-NH_2 behaves as a highly potent (pEC_{50} 9.49 ($CL_{95\%}$ 9.42 – 9.56)) and selective NOP agonist. [Tyr^1]N/OFQ(1-13)-NH_2 displayed a slight reduction in NOP potency (pEC_{50} 9.16 ($CL_{95\%}$ 8.91 – 9.41)) and selectivity. [Dmt^1]N/OFQ(1-13)-NH_2 similar to [Dmt^1]N/OFQ-NH_2 behaved as a mixed NOP/MOP agonist showing only 26 fold selectivity for NOP over MOP receptors. From this series of experiments the compound [Dmt^1]N/OFQ(1-13)-NH_2 was selected as the most potent and least selective NOP/MOP agonist.

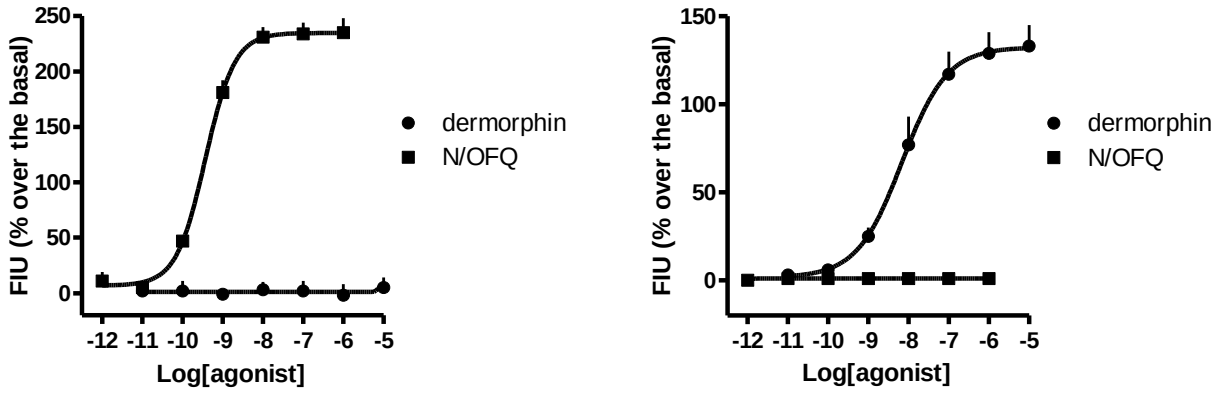


Figure 4.18. Calcium mobilization experiments. Concentration-response curves to N/OFQ and dermorphin in CHO cells stably expressing the $G\alpha_{qi5}$ chimeric protein and the NOP (left panel) and MOP (right panel) human recombinant receptor. Data are the mean ± SEM of 4 separate experiments performed in duplicate.

Table 4.5. Effects of standard and novel agonists in calcium mobilization experiments performed in CHO cells stably expressing the human NOP or MOP receptor and the $G\alpha_{qi5}$ protein.

	NOP		**MOP**		**MOP/NOP**
	$pEC_{50}(CL_{95\%})$	**E_{max}± SEM**	**$pEC_{50}(CL_{95\%})$**	**E_{max}± SEM**	
Dermorphin	**< 5**	-	**8.17** (7.93-8.41)	133 ± 12%	< 0.0007
N/OFQ	**9.30** (9.05-9.55)	235 ± 14%	**< 5**	-	> 20.000
N/OFQ-NH_2	**9.49** (9.42-9.56)	255 ± 13%	**< 5**	-	> 30.000
[Tyr^1]N/OFQ-NH_2	**9.14** (8.93-9.35)	289 ± 14%	**6.07** (5.90-6.24)	121 ± 16%	1174
[Dmt^1]N/OFQ-NH_2	**8.57** (8.28- 8.86)	259 ± 7%	**7.05** (6.55-7.55)	97 ± 10%	33
N/OFQ(1-13)-NH_2	**9.49** (9.42-9.56)	222 ± 10%	**< 5**	-	> 30.000
[Tyr^1]N/OFQ(1-13)-NH_2	**9.16** (8.91-9.41)	235 ± 17%	**6.01** (5.67-6.49)	105 ± 6%	1412
[Dmt^1]N/OFQ(1-13)-NH_2	**8.94** (8.39-9.49)	242 ± 12%	**7.52** (7.18-7.86)	126 ± 18%	26

The values are the means of 3-4 separate experiments performed in duplicate.

[Dmt^1]N/OFQ(1-13)-NH_2 affinity for NOP and classical opioid receptors was assessed in displacement binding experiments performed in membranes of CHO cells transfected with human recombinant receptors and compared with affinities of standard ligands. In CHO_{NOP} cell membranes N/OFQ displaced the radioligand with a pK_i value of 10.18. N/OFQ(1-13)-NH_2 bound the receptor with a pK_i of 10.60. The NOP selective antagonist J-113397 displayed an affinity of 9.44. [Dmt^1]N/OFQ(1-13)-NH_2 displaced the radioligand with a pK_i value of 10.59 (figure 4.19, top left panel). In CHO_{MOP} cell membranes [Dmt^1]N/OFQ(1-13)-NH_2 displaced [^{3}H]DPN with a pK_i of 10.48. The standard ligands dermorphin and naloxone showed pK_i values of 8.90 and 8.95, respectively (figure 4.19, top right panel). In CHO_{DOP} cell membranes [Dmt^1]N/OFQ(1-13)-NH_2 showed a pK_i value of 9.43, where the standard DOP agonist DPDPE, the selective DOP antagonist naltrindole and the non selective opioid receptor antagonist naloxone displaced

[^{3}H]DPN with pK_i values of 7.29, 9.74 and 7.46, respectively (figure 4.19, bottom left panel). Finally [Dmt^1]N/OFQ(1-13)-NH_2 showed a pK_i value of 9.83 in CHO_{KOP} cell membranes. The KOP agonist dynorphin A displayed a pK_i value of 10.71, where the KOP selective antagonist nor-binaltorphimine and the opioid universal ligand naloxone displaced [^{3}H]DPN with pK_i values of 10.14 and 8.44, respectively (figure 4.19, right bottom panel).

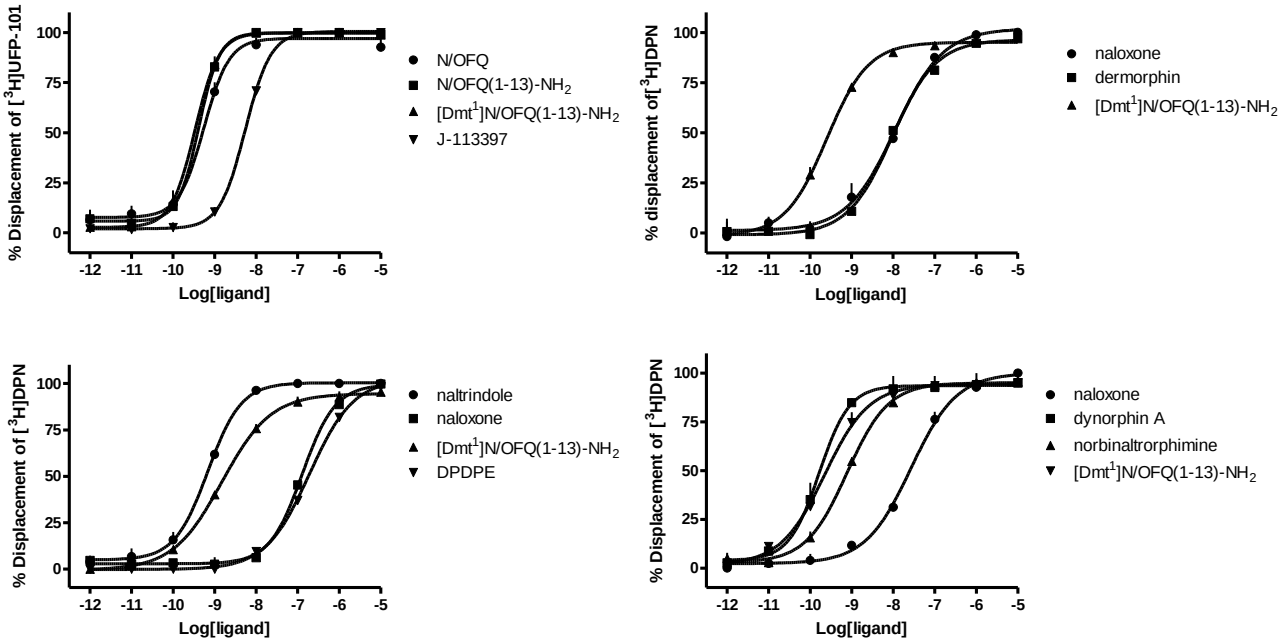


Figure 4.19. Receptor binding experiments. Competition binding curves to [Dmt^1]N/OFQ(1-13)-NH_2 and standard ligands in membranes of CHO cells expressing the NOP (top left panel), MOP (top right panel), DOP (bottom left panel), or KOP (bottom right panel) receptors. [^{3}H]UFP-101 was used as radioligand for the NOP and [^{3}H]DPN for classical opioid receptors. Data are the mean ± SEM of 3 separate experiments performed in duplicate.

In CHO_{NOP} cell membranes N/OFQ stimulated [^{35}S]GTPγS binding in a concentration dependent manner with a pEC_{50} value of 8.52 ($CL_{95\%}$ 7.92 – 9.12) and E_{max} of 4.80 ± 0.37. N/OFQ(1-13)-NH_2 and [Dmt^1]N/OFQ(1-13)-NH_2 mimicked the stimulatory effect of the natural peptide showing similar potency and maximal effects. The MOP agonist dermorphin produced a weak stimulation only at the highest concentration tested i.e. 10 μM. (figure 4.20, left panel). On the contrary, in CHO_{MOP} cell membranes dermorphin stimulated [^{35}S]GTPγS binding in a concentration dependent manner with high potency and maximal effects (pEC_{50} 7.74 ($CL_{95\%}$ 7.56 – 7.91); E_{max} of 5.47 ±

0.13). [Dmt^1]N/OFQ(1-13)-NH_2 mimicked the stimulatory effect of the opioid peptide showing similar maximal effects and even higher potency (pEC_{50} 8.19 ($CL_{95\%}$ 8.00 – 8.39)). In these cell membranes N/OFQ was found inactive up to micromolar concentrations (figure 4.20, right panel).

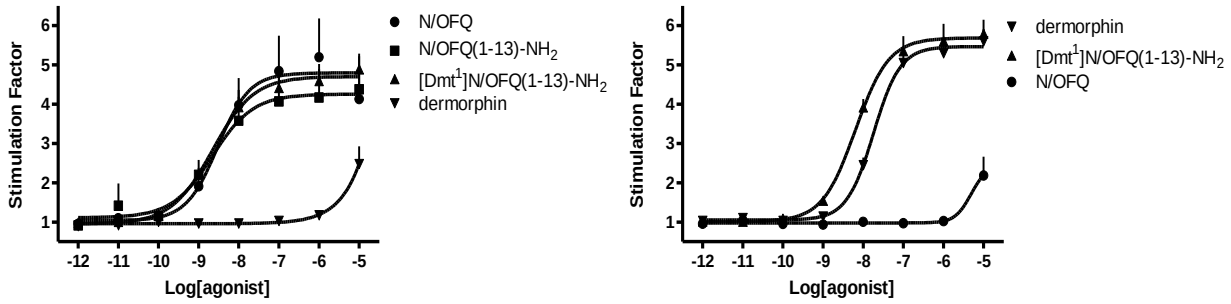


Figure 4.20. [^{35}S]GTPγS binding experiments. Concentration-response curves to N/OFQ, N/OFQ(1-13)-NH_2, dermorphin, and [Dmt^1]N/OFQ(1-13)-NH_2 in membranes of CHO cells stably expressing the NOP (left panel) or MOP (right panel) human recombinant receptors. Data are the mean ± SEM of 5 separate experiments performed in duplicate.

In rat cerebral cortex membranes (figure 4.21) N/OFQ stimulated [^{35}S]GTPγS binding in a concentration dependent manner with a pEC_{50} value of 7.82 ($CL_{95\%}$ 7.49 – 8.15) and E_{max} of 1.40 ± 0.03. N/OFQ(1-13)-NH_2 mimicked the stimulatory effect of N/OFQ with similar maximal effects but higher potency (pEC_{50} 8.48 ($CL_{95\%}$ 8.09 – 8.87)). The MOP agonist dermorphin displayed a relatively low potency in this preparation and this prevented a precise determination of its maximal effects. [Dmt^1]N/OFQ(1-13)-NH_2 produced a stimulation of [^{35}S]GTPγS binding with a pEC_{50} value of 8.01 ($CL_{95\%}$ 7.969 – 8.32) and E_{max} of 1.68 ± 0.04; of note, the maximal effect elicited by [Dmt^1]N/OFQ(1-13)-NH_2 was significantly higher than those produced by the other ligands (figure 4.21).

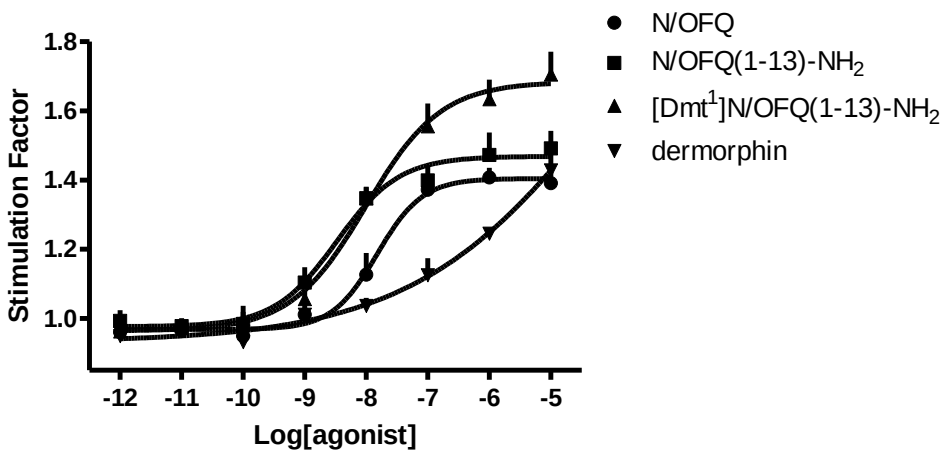


Figure 4.21. [^{35}S]GTPγS binding experiments. Concentration-response curves to N/OFQ, N/OFQ(1-13)-NH_2, dermorphin, and [Dmt^1]N/OFQ(1-13)-NH_2 in membranes of the rat cerebral cortex. Data are the mean ± SEM of 4 separate experiments performed in duplicate.

In rat spinal cord membranes N/OFQ and N/OFQ(1-13)-NH_2 produced superimposable results ($pEC_{50} \approx 7.6$; $E_{max} \approx 1.25$). Dermorphin displayed a lower potency (pEC_{50} of 6.41 ($CL_{95\%}$ 5.66 – 7.17)) but higher maximal effect (1.42 ± 0.10). [Dmt^1]N/OFQ(1-13)-NH_2 produced a stimulation of [^{35}S]GTPγS binding with similar potency to N/OFQ (pEC_{50} 7.81 ($CL_{95\%}$ 7.47 – 8.16)) and maximal effects higher than those elicited by dermorphin (figure 4.22, top left panel). In this preparation the stimulatory effects of N/OFQ, dermorphin and [Dmt^1]N/OFQ(1-13)-NH_2 were challenged with the NOP selective antagonist J-113397 and the universal opioid receptor antagonist naloxone. As shown in figure 4.22 top right panel the effects of N/OFQ were resistant to naloxone while sensitive to J-113397 (pK_B 7.95). On the contrary the action of dermorphin was antagonized by naloxone (pK_B 8.07) but not by J-113397 (bottom left panel). As shown in figure 4.22 bottom right panel, the stimulatory effect elicited by [Dmt^1]N/OFQ(1-13)-NH_2 was sensitive to both naloxone and J-113397. The coapplication of the two antagonists did not produce a further shift of the concentration response curve to [Dmt^1]N/OFQ(1-13)-NH_2.

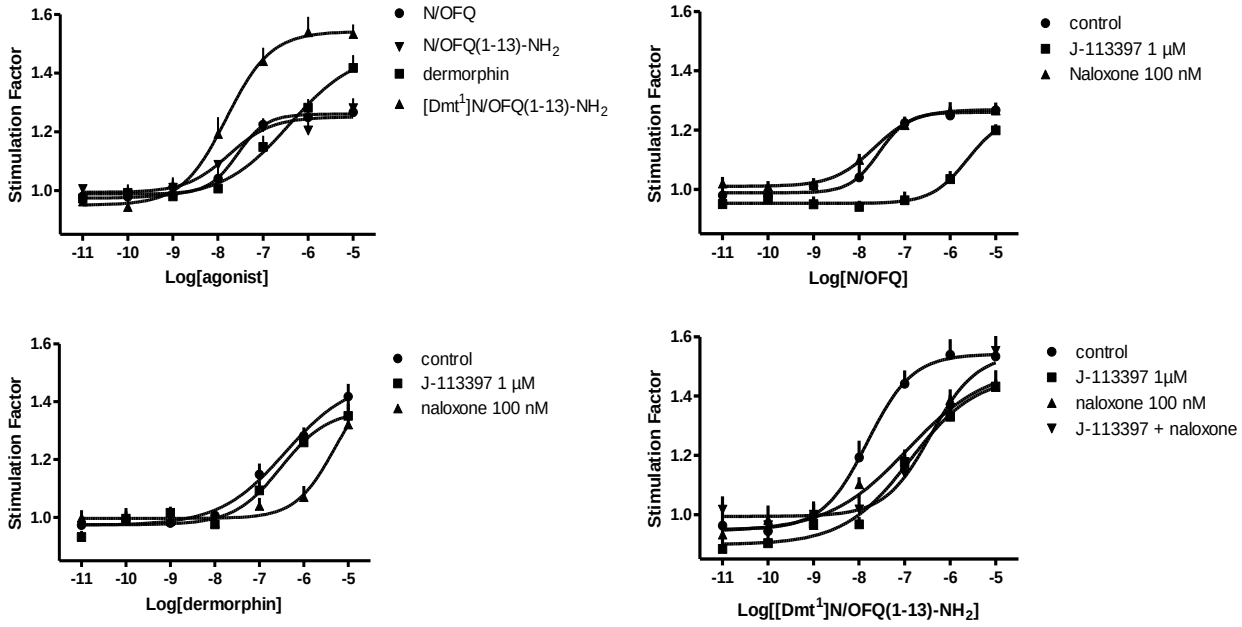


Figure 4.22. [^{35}S]GTPγS binding experiments. Concentration-response curves to N/OFQ, N/OFQ(1-13)-NH_2, dermorphin, and [Dmt^1]N/OFQ(1-13)-NH_2 in membranes of the rat spinal cord (top left panel). Effects of naloxone and J-113397 vs N/OFQ (top right panel), dermorphin (bottom left panel), and [Dmt^1]N/OFQ(1-13)-NH_2 (bottom right panel). Data are the mean ± SEM of 5 separate experiments performed in duplicate.

In the electrically stimulated guinea pig ileum N/OFQ inhibited the twitch response in a concentration dependent manner (pEC_{50} 8.26 ($CL_{95\%}$ 8.16 – 8.36), E_{max} = 40 ± 2% inhibition of control twitch). The MOP receptor agonist dermorphin mimicked the effect of N/OFQ being however more potent (pEC_{50} 8.61 ($CL_{95\%}$ 8.50 –8.72) and efficacious (E_{max} = 80 ± 2% inhibition of control twitch). [Dmt^1]N/OFQ(1-13)-NH_2 inhibited the electrically induced twitch showing similar potency and maximal effects as dermorphin (figure 4.23, top left panel). The inhibitory action of N/OFQ was not affected by naloxone but was antagonized by J-113397 (pK_B 7.87) (figure 4.23, top right panel). In contrast, the effects of dermorphin were sensitive to naloxone (pK_B 8.55) but not J-113397 (figure 4.23, bottom left panel). Finally, the effects of [Dmt^1]N/OFQ(1-13)-NH_2 were challenged with J-113397, naloxone and the cocktail of the two antagonists. As shown in figure 4.23 bottom right panel, naloxone antagonized the inhibitory effect of [Dmt^1]N/OFQ(1-13)-NH_2 producing a rightward shift of the concentration response curve and no modifications of maximal effects; a pK_B value of 7.97 was derived from these experiments. J-

113397 1 µM was also able to counteract [Dmt^1]N/OFQ(1-13)-NH_2 effects by producing a slight displacement to the right of the concentration response curve associated with a reduction in maximal effect; a pK_B value of 5.96 was derived from these experiments. When the two antagonists were assayed together they displayed an additive effect.

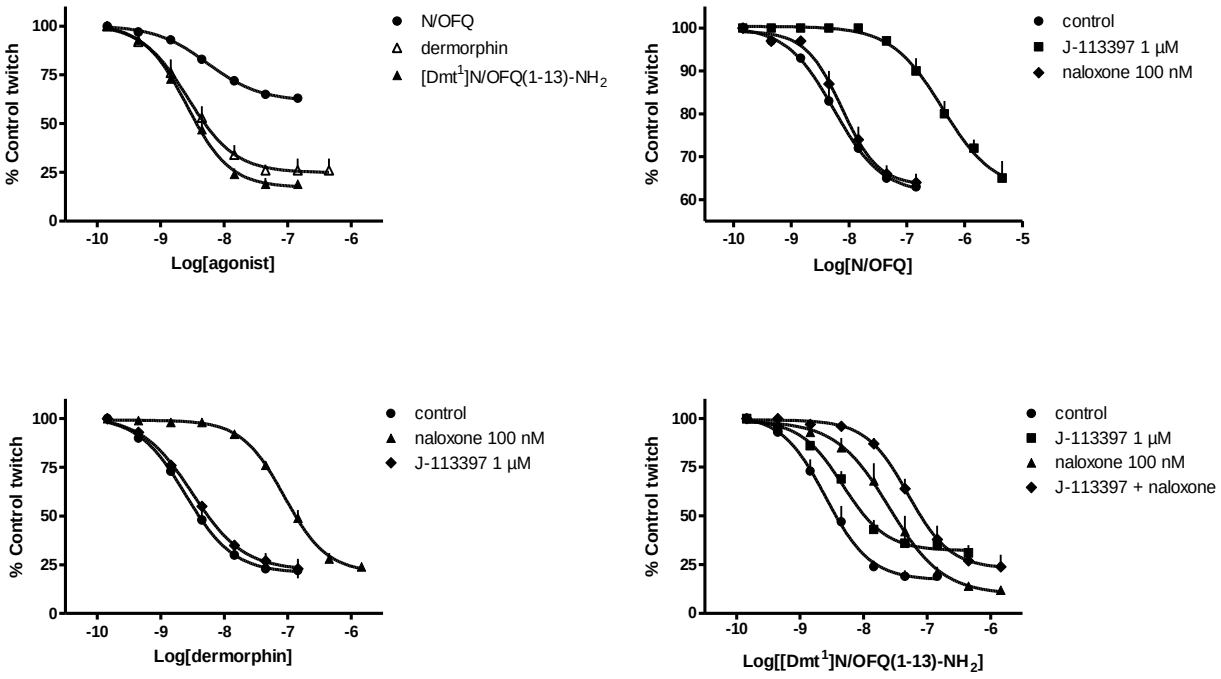


Figure 4.23. Electrically stimulated guinea-pig ileum. Concentration-response curves to N/OFQ, dermorphin, and [Dmt^1]N/OFQ(1-13)-NH_2 (top left panel). Effects of naloxone and J-113397 vs N/OFQ (top right panel), dermorphin (bottom left panel), and [Dmt^1]N/OFQ(1-13)-NH_2 (bottom right panel). Data are the mean ± SEM of 4 separate experiments performed in duplicate.

In the mouse tail withdrawal assay, mice injected i.t. with saline displayed tail withdrawal latencies around 1 s and this value remained stable over the time course of the experiment i.e. 3 h. The i.t. injection of morphine in the dose range 0.1 – 10 nmol produced a dose dependent antinociceptive effect that peaked at 15 min and then slowly and progressively declined (figure 4.24, top panels). Similarly, the i.t. injection of N/OFQ (1 – 100 nmol) elicited antinociceptive effects. The peptide was about 10 fold less potent than the alkaloid. However while the antinociceptive effects of the alkaloid were behaviourally selective (morphine did not modify the animal gross behaviour), those

elicited by N/OFQ were associated with flaccidity of the hind limbs and a consequent reduction of locomotion. These signs were present in approximately 50% of the animals treated with 10 nmol and in 85% of those treated with 100 nmol. The i.t. injection of N/OFQ(1-13)-NH_2 produced antinociceptive effects superimposable to those of the natural peptide in terms of kinetics of action, potency and associated side effects. The i.t. administration of [Dmt^1]N/OFQ(1-13)-NH_2 (figure 4.24, bottom panels) produced dose dependent antinociceptive effects. This peptide displayed very high potency being approximately 30 fold more potent than NOP agonists and 3 fold more potent than morphine. In terms of side effects, at antinociceptive doses [Dmt^1]N/OFQ(1-13)-NH_2 induced flaccidity of the hind limbs in a percent of animals similar to that of N/OFQ and N/OFQ(1-13)-NH_2. However this effect was less pronounced with [Dmt^1]N/OFQ(1-13)-NH_2 than with the other agonists.

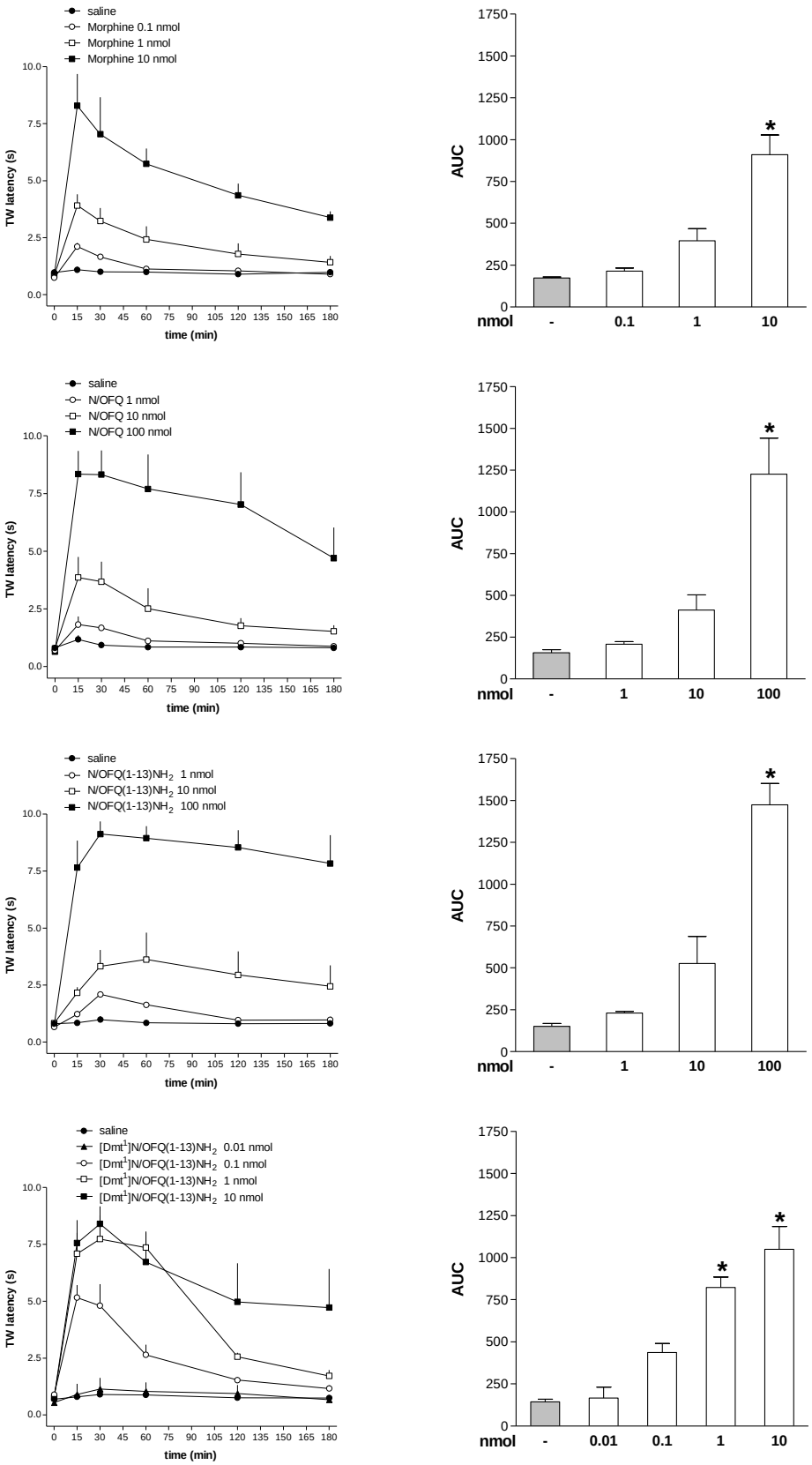


Figure 4.24. Mouse tail withdrawal assay. Dose response curves (left panels) and relative cumulative AUC data (right panels) to i.t. morphine, N/OFQ, N/OFQ(1-13)-NH_2, and [Dmt^1]N/OFQ(1-13)-NH_2. Data are the mean ± SEM of 4 separate experiments (4 mice per treatment per experiment).

In a separate series of experiments subthreshold doses of morphine and N/OFQ(1-13)NH_2 were evaluated alone and in combination (figure 4.25). Morphine 0.1 nmol and N/OFQ(1-13)NH_2 1 nmol produced a slight and short lasting antinociceptive effect that did not reach statistical significance. In contrast, when the two drugs were injected together they produced a robust and statistically significant antinociceptive effect. This effect was larger than double the sum of the single effects (figure 4.25). Interestingly no behavioural side effects (i.e. flaccidity of the hind limbs) were observed in the animals treated with the combination of the two drugs.

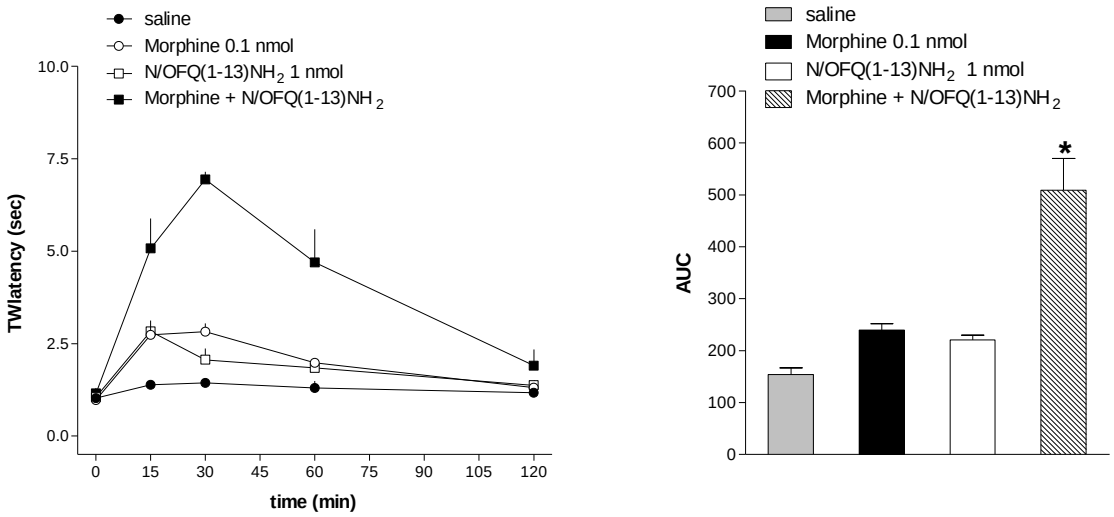


Figure 4.25. Mouse tail withdrawal assay. Effects of morphine 0.1 nmol and N/OFQ(1-13)-NH_2 1 nmol alone and in combination. Data are the mean ± SEM of 4 separate experiments (4 mice per treatment per experiment).

Finally, in collaboration group of prof. Ko, the spinal effects of [Dmt^1]N/OFQ(1-13)NH_2 on pain transmission were evaluated in non human primates (figure 4.26). In the dose range of 1 and 10 nmol the peptide produced dose-dependent antinociceptive effects. Of note at 10 nmol [Dmt^1]N/OFQ(1-13)NH_2 elicited maximal antinociceptive effects (top left panel) without causing itch/scratching responses (top right panel). By contrast, supramaximal doses of peptide i.e. 30 and 100 nmol still produced full antinociceptive effects (bottom left panel) that were however associated with robust scratching responses (bottom right panel).

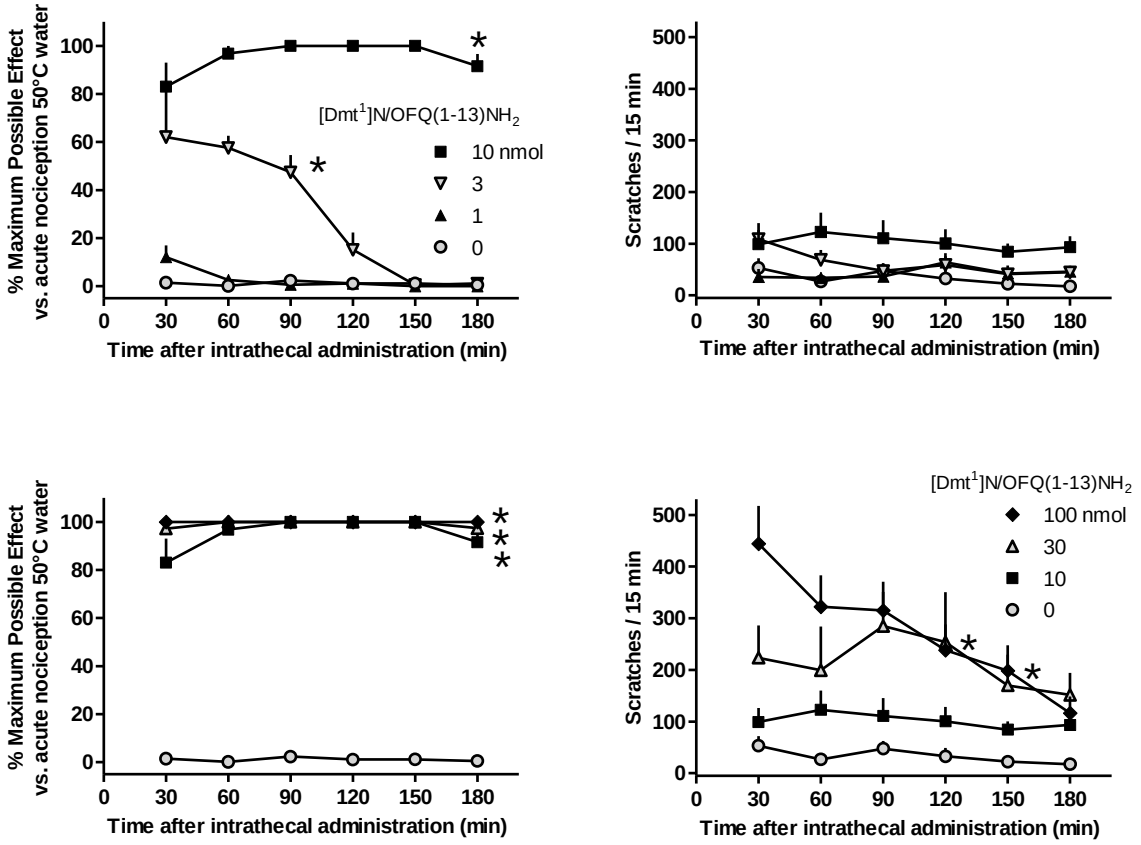


Figure 4.26. Monkey tail withdrawal assay. Effect of low (top panels) and high (bottom panels) doses of [Dmt^1]N/OFQ(1-13)-NH_2 on nociception (left panels) and scratching behaviour (right panels). Data are the mean ± SEM of data (4 separate experiments for left panels and 6 experiments for right panels). The asterisk represents a significant.

Based on the recent evidence of a synergistic antinociceptive effect in response to the simultaneous activation of spinal NOP and MOP receptors (Hu *et al.*, 2010; Ko *et al.*, 2009), the present study was carried out with the aim to identify, pharmacologically characterize, and evaluate as innovative spinal analgesics non selective MOP/NOP agonists. [Dmt^1]N/OFQ(1-13)-NH_2 was identified in the calcium mobilization primary screening assay as the most potent and least selective NOP/MOP agonist. This pharmacological activity of the peptide was then confirmed in various in vitro assays performed on recombinant human receptors (receptor binding, [^{35}S]GTPγS binding) as well as at native animal receptors expressed in the rat cerebral cortex and spinal cord and in the guinea pig ileum. In vivo in the tail withdrawal assay performed in mice and monkeys [Dmt^1]N/OFQ(1-13)-NH_2 produced dose dependent antinociceptive effects. Of note, while [Dmt^1]N/OFQ(1-13)-NH_2

displayed in vitro similar potency to N/OFQ, in vivo in both animal species the peptide was approximately 30 fold more potent. These results corroborate our hypothesis that non selective MOP/NOP agonists may behave as innovative spinal analgesics and candidate [Dmt^1]N/OFQ(1-13)-NH_2 as the prototype of this class of drugs.

The calcium mobilization assay used for screening the novel NOP/MOP receptor ligands has been validated in previous studies. In particular the pharmacological profile of the human NOP receptor coupled with calcium signalling has been assessed with a rather large panel of ligands encompassing full and partial agonist as well as pure antagonist activity (Camarda *et al.*, 2009). Similar experiments were performed investigating the pharmacological profile of human MOP receptor although, in this case, the panel of ligands investigated was relatively small (Camarda and Calo, in press). For both receptors the results obtained with the calcium assay were virtually superimposable to those described in literature using classical assays for Gi coupled receptors (i.e. cAMP levels or stimulation of [^{35}S]GTPγS binding).

The design of non selective NOP/MOP agonists was based on the following evidence: i) N/OFQ(1-13)-NH_2 maintains the same potency and efficacy as the natural peptide (Calo *et al.*, 1997; Varani *et al.*, 1999); the substitution of Phe^1 with Tyr in N/OFQ as well as N/OFQ(1-13)-NH_2 sequences causes a reduction in selectivity for NOP over classical opioid receptors (Calo *et al.*, 1997; Varani *et al.*, 1999); the substitution of Tyr^1 with Dmt in opioid peptide sequences increases ligand potency (Salvadori *et al.*, 1995; Schiller, 2010). The results obtained in the calcium assay demonstrated that this design strategy was indeed successful. In fact, the substitution of Phe with Tyr in position 1 generated less selective peptides. However both [Tyr^1]N/OFQ-NH_2 and [Tyr^1]N/OFQ(1-13)-NH_2 were more than 1000 fold more potent at NOP than at MOP. These results are in line with previous findings. In fact [Tyr^1]N/OFQ-NH_2 and [Tyr^1]N/OFQ(1-13)-NH_2 were able to bind to both NOP and MOP sites in guinea pig brain membranes but with higher affinity at the former receptor (Varani *et al.*, 1999). Moreover in the electrically stimulated guinea pig ileum [Tyr^1]N/OFQ(1-13)-

NH_2 at low concentrations (< 30 nM) produced naloxone resistant inhibitory effects; however, at higher concentrations, the opioid antagonist partially counteracted the action of the peptide(Varani *et al.*, 1999). Finally, when tested in vivo [Tyr^1]N/OFQ mimicked the effect of the natural peptide decreasing systemic arterial pressure in the rat (Champion *et al.*, 1997) and eliciting erectile activity in the cat (Champion *et al.*, 1998). The non-natural amino acid Dmt has been widely and successfully used in the past for generating highly potent ligands for opioid receptors (Briant *et al.*, 2003; Schiller, 2010). The ability of this residue to increase opioid receptor affinity compared to Tyr has been confirmed in the present study. In fact [Dmt^1]N/OFQ-NH_2 and [Dmt^1]N/OFQ(1-13)-NH_2 displayed a slight decrease in NOP potency associated with a substantial increase in potency at MOP receptors. As a consequence, the selectivity of these peptides for NOP over MOP receptors dropped to only ≈ 30 fold. Since [Dmt^1]N/OFQ(1-13)-NH_2 displayed slightly higher potency and lower selectivity compared to [Dmt^1]N/OFQ-NH_2, it was selected as candidate for further studies.

The high NOP/MOP affinity and full agonist activity of [Dmt^1]N/OFQ(1-13)-NH_2 were then confirmed in receptor binding and [^{35}S]GTPγS stimulation binding experiments performed using membranes prepared from CHO cells expressing NOP or classical opioid receptors. In these experiments the peptide displayed extremely high affinity both for NOP and MOP sites. Of note, [Dmt^1]N/OFQ(1-13)-NH_2 also showed high affinity for DOP and KOP. In [^{35}S]GTPγS assay the peptide behaved as potent full agonist both at NOP and at MOP receptors. In these experiments [Dmt^1]N/OFQ(1-13)-NH_2 displayed, in line with calcium mobilization data, higher potency at NOP than at MOP but its ratio of selectivity (2) was substantially lower than that derived from calcium mobilization studies (26). Collectively these results clearly demonstrated that [Dmt^1]N/OFQ(1-13)-NH_2 behaved as a non selective NOP/MOP full agonist at recombinant human receptors.

The pharmacological activity of the peptide was then reassessed at native animal receptors by performing [^{35}S]GTPγS binding experiments with membranes from the rat cerebral cortex and

spinal cord and bioassay experiments in the guinea pig ileum. In line with previous findings N/OFQ and dermorphin stimulated [^{35}S]GTPγS binding in the rat cerebral cortex and spinal cord membranes (Albrecht *et al.*, 1998; Narita *et al.*, 1999; Sim *et al.*, 1997). In both preparations [Dmt^{1}]N/OFQ(1-13)-NH_2 behaved as a potent agonist producing maximal effects higher than those elicited by the selective agonists. The receptor mechanism involved in the stimulant effects of [Dmt^{1}]N/OFQ(1-13)-NH_2 in rat spinal cord membranes has been investigated in receptor antagonist experiments. While the stimulatory effect of N/OFQ and dermorphin were sensitive to J-113397 and naloxone, respectively, that elicited by [Dmt^{1}]N/OFQ(1-13)-NH_2 was counteracted by both molecules. This result suggests that stimulation of [^{35}S]GTPγS binding by N/OFQ and dermorphin derives from the selective activation of NOP and MOP receptors, respectively, while that elicited by [Dmt^{1}]N/OFQ(1-13)-NH_2 is due to the simultaneous activation of both proteins. This view is corroborated by findings obtained in the electrically stimulated guinea pig ileum. In this preparation J-13397 and naloxone selectively antagonized the inhibitory effects of N/OFQ and dermorphin, respectively, with pK_B values similar to those obtained in the rat spinal cord membranes and the literature (Bigoni *et al.*, 2000a; Calo *et al.*, 1997). In contrast, the inhibitory action of [Dmt^{1}]N/OFQ(1-13)-NH_2 was sensitive to both antagonists and a profound shift to the right of the concentration response curve to the agonist was obtained when J-113397 and naloxone were coapplied. Collectively these findings clearly demonstrated that [Dmt^{1}]N/OFQ(1-13)-NH_2 acts as a potent and non selective NOP/MOP receptor full agonist at native animal receptors expressed in the periphery and in the central nervous system.

In the spinal cord NOP and MOP receptor stimulation elicits antinociceptive effects via similar cellular mechanism i.e. presynaptic inhibition of neurotransmitter release from primary sensory neurons (Zeilhofer and Calo, 2003). Moreover recent studies performed in non human primates suggest that the simultaneous activation of both receptors produces synergistic antinociceptive effects (Hu *et al.*, 2010; Ko *et al.*, 2009). This evidence prompted us to assess the spinal

antinociceptive properties of the non selective NOP/MOP agonist [Dmt^1]N/OFQ(1-13)-NH_2. In the mouse tail withdrawal assay morphine given i.t. elicited a dose dependent antinociceptive effect. Similar antinociceptive effects were recorded in response to spinal administration of N/OFQ or N/OFQ(1-13)-NH_2. The two peptides were approximately 10 fold less potent than the alkaloid. However it should be emphasized that while the antinociceptive effects of morphine were behaviourally selective those elicited by N/OFQ (or N/OFQ(1-13)-NH_2) were associated with flaccidity of the hind limbs. Previous receptor antagonist and knockout studies demonstrated the exclusive involvement of NOP receptor activation in the spinal antinociceptive effects of N/OFQ (Fischetti *et al.*, 2009; Nazzaro *et al.*, 2007; Rizzi *et al.*, 2007b) and MOP receptors in that of morphine (Kieffer, 1999). [Dmt^1]N/OFQ(1-13)-NH_2 injected i.t. elicited a robust antinociceptive effect in the mouse tail withdrawal assay. This antinociceptive effect was associated with flaccidity of the hind limbs that was however less pronounced than that in response to NOP selective agonists. The potency of [Dmt^1]N/OFQ(1-13)-NH_2 was approximately 30 fold higher than that of N/OFQ or N/OFQ(1-13)-NH_2. These data contrast to the similar NOP potency displayed by [Dmt^1]N/OFQ(1-13)-NH_2 and N/OFQ or N/OFQ(1-13)-NH_2 in vitro. It has been demonstrated that [desPhe^1]N/OFQ is a major metabolite of N/OFQ when the peptide is given i.t. (Ko *et al.*, 2006). Therefore the presence of the non-natural amino acid Dmt in position 1 may reduce susceptibility to enzymatic degradation. This may cause an increase in peptide potency in vivo where metabolism is likely more relevant than in vitro. However it is unlikely that the huge increase in [Dmt^1]N/OFQ(1-13)-NH_2 potency is solely due to increased metabolic stability. Rather it is suggested that the high potency of the synthetic peptide mainly derives from its ability to simultaneously activate NOP and MOP receptors. This simultaneous receptor activation produced synergistic antinociceptive effects as demonstrated in previous monkey studies performed with subthreshold doses of morphine and N/OFQ (Ko *et al.*, 2009) or the potent and selective NOP agonist UFP-112 (Hu *et al.*, 2010). This same synergistic antinociceptive effect of NOP and MOP receptor activation seems to be operative also in the mouse spinal cord as demonstrated in the present study by the robust antinociceptive

effect obtained in response to coapplication of subthreshold doses of morphine and N/OFQ(1-13)-NH_2.

Finally the spinal antinociceptive properties of [Dmt^1]N/OFQ(1-13)-NH_2 have been evaluated in non human primates. It should be emphasized that in this species antinociceptive effects in response to spinal administration of NOP agonists is behaviourally selective while those elicited by MOP agonists are always associated with scratching (Ko *et al.*, 2004; Ko *et al.*, 2006). In monkeys [Dmt^1]N/OFQ(1-13)-NH_2 induced significant antinociceptive effects at the dose 3 nmol and full antinociception at 10 nmol. Thus, compared to N/OFQ (Ko *et al.*, 2009; Ko *et al.*, 2006), [Dmt^1]N/OFQ(1-13)-NH_2 was found about ~10-30 fold more potent and elicited longer lasting effects. Interestingly, over this range of doses the antinociceptive effect of [Dmt^1]N/OFQ(1-13)-NH_2 was not associated with scratching. At higher supramaximal doses (i.e. 30 and 100 nmol) [Dmt^1]N/OFQ(1-13)-NH_2 was able to induce scratching similar to morphine (Ko *et al.*, 2009; Ko *et al.*, 2006). These results, in line with mouse studies, strongly suggest that the high antinociceptive potency of [Dmt^1]N/OFQ(1-13)-NH_2 may derive from its non selective NOP/MOP agonist activity.

In conclusion the present study describes the design, synthesis and in vitro pharmacological characterization of [Dmt^1]N/OFQ(1-13)-NH_2, a potent non selective NOP/MOP agonist. The spinal administration of this peptide in mice and non human primates elicits potent and antinociceptive effects similar to those produced by a combination of NOP and MOP selective agonists. These results suggest that [Dmt^1]N/OFQ(1-13)-NH_2 could be considered the prototype of a novel class of spinal analgesics worthy of consideration for clinical development.

5. GENERAL CONCLUSIONS

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. This large research effort is documented by the number scientific articles published in this field (nowadays a pubmed search for nociceptin or orphanin/FQ generates more than 1500 results). Our laboratory, in close collaboration with the group of medicinal chemists lead by Prof Salvadori, contributed to this field since its very beginning with the design and synthesis of novel NOP ligands, their in vitro and in vivo pharmacological characterization, and their use together with transgenic animals for target validation studies. The present PhD thesis summarizes the work we performed in the field of N/OFQ and its receptor during the last three years. In particular the following lines of research were developed: investigation of the phenotype of NOP(-/-) rats, in vitro pharmacological characterization of non peptide NOP ligands, and in vitro and in vivo studies on novel peptides acting as mixed NOP/MOP agonists.

The behavioural phenotype of rats knockout for the NOP receptor gene has been investigated. Loss of NOP mediated functions in NOP(-/-) rat tissues were demonstrated in bioassay experiments. In behavioural studies NOP(-/-) rats displayed a robust antidepressant-like phenotype associated with a better exercise-driven motor performance. These results are in line with previous pharmacological studies and suggest that selective NOP receptor antagonists may act as innovative drugs for the treatment of depression and hypokinetic disorders. In addition, NOP(-/-) rats displayed a mild anxiety-like phenotype and clear pronociceptive like phenotype in the formalin assay. These data combined with evidence arising from genetic and pharmacological investigations in rodents call for caution in the use of NOP receptor antagonists in anxious patients and chronic pain states.

Actually, these conditions may possibly represent contraindications for the use of NOP antagonists. These considerations are based on preclinical studies and in some cases on indirect clinical evidence. They might be useful for the design of controlled clinical trials performed with selective and potent NOP antagonists. Such studies will demonstrate the correct place in therapy for these innovative drugs.

As far as non peptide NOP ligands are concerned, studies were performed both on antagonists and agonists. GF-4 is a derivative of the NOP selective antagonist Trap-101; it was obtained by introducing two methyl groups in the hydroxymethyl function at the position 3 of the piperidine nucleus. Such chemical modification did not alter the pharmacological activity of the compound (a pure and competitive NOP receptor antagonist) but slightly reduced its potency at recombinant NOP receptors and, more dramatically, in vitro selectivity over classical opioid receptors. Nevertheless, GF-4 was NOP receptor selective in vivo, replicating the antiparkinsonian effect and neurochemical changes typical of its parent compound, and being as potent as J-113397. Further studies are needed to elucidate whether the in vivo gain in potency of GF-4 is due to greater metabolic stability possibly due to resistance to cytochrome oxidation. The results obtained with GF-4 from one hand offer new insights into the structural requirements for optimal antagonist activity at NOP receptors and selectivity over classical opioid receptors and, from the other hand, confirmed and extended previous findings demonstrating that NOP receptor blockage attenuate parkinsonian-like symptoms in rodents.

In vitro pharmacological studies were also performed with non peptide NOP agonists. These studies confirmed the NOP agonist properties of all the investigated molecules but also demonstrated their limitation in terms of selectivity of action. At present, only little information is available in literature about toxicology, pharmacokinetics, and therapeutic levels of non peptide NOP agonists. Moreover the following issues makes the development of these compounds difficult: most of the studies have been performed in a limited number of species (rats, mice, and guinea pigs) and particularly with a very limited number of molecules (quite often only with Ro 64-6198);

several side effects, including impairments of motor activity, coordination, learning, and memory, as well as hyperphagic and hypothermic effects have been described in rodents; the doses at which these side effects are produced depend on the species (it is therefore difficult predict whether a therapeutic dose range with limited side effects will exist in humans); as previously demonstrated for Ro 64-6198 and confirmed in this study with other molecules there is some tissue-dependent variability of agonist efficacy and particularly selectivity of action. Despite these considerations, the fact that anxiolytic like effects in response to NOP selective agonists have been reported in different laboratories and with chemically unrelated molecules represents a robust experimental evidence that candidates this kind of putative drugs as innovative anxiolytics. Moreover recent findings obtained in non human primates demonstrated interesting antinociceptive properties in response to systemic administration of Ro 64-6198. Thus, the available evidence is still too limited to draw firm conclusions on the potential of non peptide NOP agonists as novel drug to treat anxiety and pain.

I.t infusions of analgesics have been increasingly utilized during the last two decades for the treatment of persistent cancer pain. With recent technological advances in the field, this therapeutic option has been extended to moderate or severe pain related to cancer and non cancer origins. However only morphine and ziconotide have been approved for i.t. administration; thus there is a strong medical need for novel drugs to be used as spinal analgesics. At this regard non selective NOP/MOP agonists such as [Dmt^1]N/OFQ(1-13)-NH_2 may represent an interesting option. Indeed the synergistic antinociceptive effect generated by the simultaneous activation of NOP and MOP receptors may offer important advantages: i) during acute administration a complete analgesic effect can be achieved with reduction or even elimination of the side effects associated to the full activation of a single receptor, ii) during chronic treatment the desired level of analgesia can maintained for longer (i.e. reduction of tolerance liability) since the analgesic action does not derive from the full and prolonged activation of a single receptor. These are however intriguing speculations that need rigorous experimental validation. We have contributed to this line of research by describing the design, synthesis and in vitro pharmacological characterization of

[Dmt^{1}]N/OFQ(1-13)-NH_2, a potent non selective NOP/MOP agonist. The spinal administration of this peptide in mice and non human primates elicits potent and antinociceptive effects similar to those produced by a combination of NOP and MOP selective agonists. These results suggest that [Dmt^{1}]N/OFQ(1-13)-NH_2 could be considered the prototype of a novel class of spinal analgesics worthy of consideration for clinical development.

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Vediamo… soprattutto di tanto tempo passato con persone incredibili, ma partiamo con ordine (chi conosce questo lab, solitamente di ordine ne ha visto molto poco):

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- La noci è ripetibile…. Non sempre…
- L'UFP-112 ha cinetica lenta…. Non in mano a me…
- la tetrodotossina non si lava… più bianco del bianco con Dash ecodosi…
- i tessuti vengono sospesi nella soluzione di Krebs… anche nella birra se necessario

E tanti altri….
Dopo questi fatti ho abbandonato i libri di farmacologia tipo Kenakin per adottare l'unico vero libro di Siensa Farmacologica, il mitico " The Pharmacology Of The Year 2012: love, luck and GPCR" di Fox P. and Branko (2012) (lo trovate su pubmed).

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Un abbraccio
n.n.

Ste

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