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Pharmacological characterization of novel ligands acting as NOP/opioid receptor agonists

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Dottoranda
Dr.ssa Cerlesi Maria Camilla

Tutore
Prof. Calo' Girolamo

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…il genio può essere confinato dentro un guscio di noce e ciò nonostante abbracciare tutta la pienezza della vita.

THOMAS MANN

Abstract

The opioid receptor family comprises the classical opioid receptors mu (MOP), delta (DOP), and kappa (KOP) and a fourth member the nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor that, based on its distinct pharmacology, has been referred to as a non opioid branch of the opioid receptor family. Opioid drugs (e.g., morphine or fentanyl), mainly targeting MOP receptors, remain the most powerful analgesics available for pain relief. However, the use of opioid drugs is associated with several side effects including respiratory depression, constipation, tolerance and abuse liability. Moreover while the effects of opioids against acute nociceptive pain are brilliant their effectiveness in chronic pain patients particularly in the case of neuropathic pain is often disappointing. Thus there is a large medical need regarding novel drugs for the treatment of chronic (particularly neuropathic) pain. The N/OFQ-NOP receptor system modulates various biological functions including pain transmission. Recent evidence obtained in rodent and non-human primate studies suggests that the simultaneous activation of NOP and MOP receptors elicits super-additive analgesic effects in animal models of pain. Thus mixed NOP/MOP agonists are worthy of development as innovative analgesics.

The aim of the present study was the pharmacological characterization of novel ligands designed to act as mixed NOP/opioid receptor agonists. These compounds have been designed, synthesized and purified in the Department of chemical and pharmaceutical sciences of our University by the research groups of Claudio Trapella and Remo Guerrini. Mixed NOP/MOP agonists were generated by complimentary strategies using a single non-selective pharmacophore or two distinct pharmacophores each selective for the NOP or MOP receptor linked together with an appropriate chemical spacer to generate chimeric compounds. These chemical approaches were applied at both peptide and non-peptide molecules, obtaining the following mixed NOP/MOP ligands: the non-selective compounds PWT2-[Dmt^{1}] and cebranopadol and the chimeric compounds DeNo and RR4-Ro. The pharmacological profiles of these molecules were assayed *in vitro* in several assays including receptor binding, stimulated [^{35}S]GTPγS binding, cyclic AMP formation, Western Blotting-MAPKinase detection, calcium mobilization studies performed in cells co-expressing the human recombinant receptors and chimeric G-proteins, bioluminescence resonance energy transfer (BRET) experiments investigating receptor interaction with G-protein and β-arrestin 2, and bioassay studies in isolated tissues. Moreover the effects of DeNo were also assessed *in vivo* in the rat paw pressure test, while those of cebranopadol in the mouse tail withdrawal and formalin tests.

We applied to the known universal opioid receptor agonist [Dmt^1]N/OFQ(1-13)NH_2 the recently discovered PWT technology that allows the facile synthesis its tetrabranched derivative. PWT-[Dmt^1] showed *in vitro* the same pharmacological activity, potency and selectivity of action of the parent peptide. Studies are under way for investigating the effects of PWT-[Dmt^1] *in vivo* after spinal administration in non human primates; it will be particularly interesting to measure the duration of action of PWT-[Dmt^1] in fact previous studies with various peptide sequences demonstrated that this pharmacological parameter is greatly prolonged by the PWT chemical modification.

The peptide molecule DeNo has been synthesized by linking the MOP selective agonist dermorphin with the NOP selective agonist N/OFQ. DeNo mimicked dermorphin effects in preparations expressing MOP receptor and N/OFQ actions in those expressing NOP receptors. In the guinea pig ileum, a pharmacological preparation expressing both MOP and NOP receptors, the biological activity of this compound was clearly antagonized only using a cocktail of MOP and NOP selective antagonists. Despite its MOP/NOP agonist *in vitro* pharmacological profile, DeNo shows only weak antinociceptive properties *in vivo* after spinal administration in rats.

Cebranopadol behaved as universal opioid receptor agonist in calcium mobilization studies and similar results were obtained in the BRET assay where cebranopadol behaved as a potent full agonist at NOP and MOP receptors. Interestingly cebranopadol displayed low potency in promoting MOP/β-arrestin 2 interaction and no efficacy at NOP/β-arrestin 2; thus cebranopadol acts as a G-protein biased agonist particularly at the NOP receptor. The evaluation of the pharmacological effects of cebranopadol in tissues was made difficult by its very low kinetic of action. *In vivo* cebranopadol was able to produce potent and long lasting analgesic effects that were due to the simultaneous activation of NOP and MOP receptors. Importantly, contrary to classical opioid drugs, the analgesic potency of cebranopadol was higher in animal models of inflammatory than nociceptive pain.

Finally non-peptide chimeric compounds were generated tethering the NOP agonist Ro 65-6570 with fentanyl derivatives of the RR series. A large series of experiments i.e. receptor binding, calcium mobilization, and bioassay were performed in order to characterize the pharmacological effects of the standard molecules (Ro 65-6570 and fentanyl), and of compounds of the RR series. From these studies RR4 and RR9 were selected as the best molecules. The chemistry needed for linking those molecules to Ro 65-6570 was very demanding and only a small amount of the chimeric compound RR4-Ro was obtained. In [^{35}S]-GTPγS binding assay RR4-Ro displayed similar potency and efficacy as Ro 65-6570

in NOP expressing cells and similar potency but reduced efficacy compared to fentanyl in MOP cells, thus acting as mixed NOP full agonist/MOP partial agonist.

In conclusion the present study investigated in great detail the pharmacological profile of several different molecules designed to act as mixed NOP/opioid receptor agonists thus providing to the scientific community novel tools useful for investigating the therapeutic potential of this class of compounds as innovative analgesics.

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1. Introduction

1.1. Brief history of opioid systems

Opium and its derivatives have been used for centuries, both in a medicinal and 'recreational' manner. While morphine and its derivatives are used since the 18th century as analgesics for the treatment of acute and chronic pain, the history of classical opioid receptor did not come up until the midway of the 20th century. In fact, only in 1954 Beckett and Casy proposed the existence of receptors for opiate drugs following their structure-activity relationship studies of synthetic opioid analgesic activity (Beckett & Casy, 1954). Now it is understood that morphine and other opioid drugs act on an endogenous opioidergic system, which is not only involved in setting pain (nociceptive) threshold and controlling nociceptive processing but also participates in modulation of many biological activities such as gastrointestinal, endocrine, cardiac, respiratory, emotional, immune and autonomic functions as well as a possible role in cognition, obesity, neurodegenerative disorders, drug abuse and addiction. Initially, the names of these receptors derived from the prototypic drugs used to identify them, i.e. morphine for μ (mu) and ketocyclazocine for κ (kappa) (Martin, Eades, Thompson, Huppler & Gilbert, 1976) or from their anatomical distribution as vas deference for δ (delta) (Lord, Waterfield, Hughes & Kosterlitz, 1977). Then, they are called opioid since the discovery of endogenous peptides with effects similar to those of opiate drugs. The existence of more than one opioid receptor type and that multiple modes of interaction of ligands with opioid receptors were possible, arose from structure-activity relationship studies of Portoghese and colleagues in 1965 (Portoghese, 1965). In 1973, three separate group succeeded almost simultaneously in showing through receptor binding studies in brain homogenates the presence of opiate binding site in the central nervous system (Kuhar, Pert & Snyder, 1973) (Hiller, Pearson & Simon, 1973) (Terenius, 1973) and soon afterwards these receptors were found to have a non uniform distribution there (Hiller, Pearson & Simon, 1973), (Kuhar, Pert & Snyder, 1973). Later followed the discovery of the Met-enkephalin and Leu-enkephalin effects (Kosterlitz & Waterfield, 1975); like the enkephalins, also β-endorphin proved to have a high affinity for brain opioid receptors (Birdsall & Hulme, 1976) and in 1981 were identified another group of peptides structurally related to the enkephalins (Goldstein,

Fischli, Lowney, Hunkapiller & Hood, 1981). The first of these was named dynorphin. Finally, also a new family of peptides were discovered, now collectively known as deltorphins (Erspamer et al., 1989). However the first definitive evidence that these receptors did not form a homogeneous population was provided in 1976 (Martin, Eades, Thompson, Huppler & Gilbert, 1976), where was suggested the presence of three types of receptors, concluding in the early 1990s when genes encoding for the opioid receptor were finally cloned (Evans, Keith, Morrison, Magendzo & Edwards, 1992) (Kieffer, Befort, Gaveriaux-Ruff & Hirth, 1992) (Chen, Mestek, Liu, Hurley & Yu, 1993) (Yasuda et al., 1993) (Minami et al., 1993). Homology cloning led to the identification of a fourth opioid like receptor that shared significant sequence homology with the opioid receptors. Recently it has been included in the opioid receptor family and is termed the Nociceptin/Orphanin FQ peptide receptor (NOP). Receptor nomenclature has changed numerous times in the past years but currently, the governing body on receptor nomenclature: the International Union of Basic and Clinical Pharmacology (IUPHAR) has amended the present phrasing to refer the four opioid receptor: MOP (μ), DOP (δ), and KOP (κ) (Alexander et al., 2013). Due to their affinity for the opioid antagonist naloxone, the MOP, DOP and KOP are termed as the "classical opioid receptors", unlike the NOP that displays no affinity for this antagonist compound. Only compound dynorphin A (endogenous ligand for the KOP receptor) displays weak affinity for NOP, otherwise the NOP receptor displays no affinity for endogenous opioids other than Nociceptin/Orphanin FQ (N/OFQ). Therefore, NOP is often referred to as a "non-classical" opioid receptor. All four receptors are a sub-part of a larger family of receptors, known as G-protein coupled receptors (GPCR) sharing the similar seven-transmenbrane topology (Figure 1).

GPCRs represented the most common class of receptors, for this reason, they have been classified into major classes and further separated into sub-families based on common characteristics or sequence similarities. Opioid receptors are members of the largest sub-family; Class A, rhodopsin-like receptor family (Fredriksson, Lagerstrom, Lundin & Schioth, 2003). Pharmacologists have studied receptors for more than a century but a molecular understanding of their properties has emerged only during the past 30-35 years. Developments and discoveries primarily during the 1970s and 1980s led to current concepts about by far the largest, most versatile and most ubiquitous group of seven-transmenbrane (7TM) receptors, also known as G-protein-coupled receptors (GPCRs) (Pierce, Premont & Lefkowitz, 2002). There are almost 1000 genes encoding such

receptors in the human genome and these receptors regulate virtually all known physiological process in mammals. Their central importance and relevance to the current clinical practice of medicine is reflected in the plethora of drugs that target these receptors including neurotransmitters, neuropeptides, glycoproteins, hormones, lipid mediators and small molecules. In fact, a substantial proportion of all worldwide prescription drug sales today are generally attributed to drugs that target the 7TM receptors either directly or indirectly as agonists or antagonists (Gudermann, Nurnberg & Schultz, 1995). Signal transmission occurs through the interaction between receptors and different intracellular proteins (e.g. heterotrimeric G-proteins, kinases, and arrestins (Rajagopal, Rajagopal & Lefkowitz, 2010)), which then activate downstream effectors and trigger cascades of cellular and physiological responses. The GPCR receptor is characterized by a seven-transmenbrane spanning structure exposing the N-terminus to the extracellular domain and the C-terminus to the intracellular domain (Figure 1). The structure of the GPCR binding pocket varies from receptor to receptor however is always expressed on the extracellular domain.

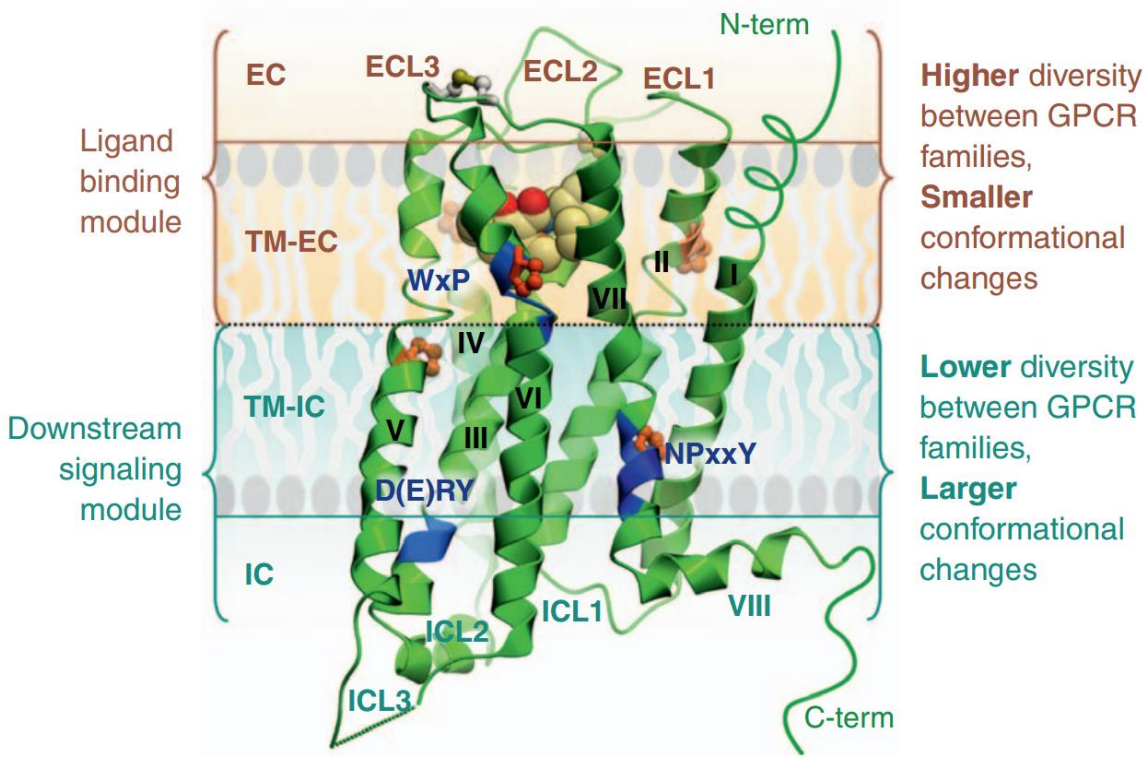


Figure 1. General architecture and modularity of GPCRs. N-terminal extracellular domains (top side), C-terminal intracellular domains (bottom side). Image taken from (Katritch, Cherezov & Stevens, 2012).

Such as for opioid receptors, GPCRs have no direct link with effector proteins; instead the message is relayed via a heterotrimeric complex, named as G-protein. It consists of three sub-units termed α, β and γ, among which the first mentioned, has a guanonucleotide-binding pocket, where a guanosine 5'-diphosphate (GDP) molecule is housed when the receptor is in an inactivate state. Following GPCR receptor activation, the α subunit exchanges GDP with a guanosine trisphosphate (GTP) and separates from the other subunits βγ, both from the receptor and each other. At this point, the separated subunits can influence effector proteins, such as adenyl cyclase (AC) or ion channels within the cell. In literature are known several subtypes of G-protein α subunits able to interact with various effector molecules. To name few examples, these include: $G\alpha_s$ (increases adenyl cyclase activity-increasing production of cyclic AMP), $G\alpha_{i/o}$ (inhibits adenyl cyclase-decreasing cyclic AMP) and $G\alpha_q$ (activates phospholipase C, leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacyl glycerol and inositol triphosphate) proteins (Nature Reviews Drug Discovery, 2004). It is clear that is essential to increase the knowledge on the pharmacology of these receptors, to uncover the processes regulated by these receptors, their mechanism at molecular level, in order to discover more efficacious and better tolerated drugs targeting this receptors.

In the characterization of new orphan GPCRs, the first step 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 on the basis of their chemical characteristics (Tatemoto & Mutt, 1980) or of their effects in particular assay systems (Erspamer, Melchiorri, Erspamer & Negri, 1978). Now, through DNA recombination techniques, it is possible to transfect the sequence of an orphan receptor of which the function is not yet known, into an appropriate cellular expression system. This process includes the use of orphan receptors as baits to isolate their natural ligands from mixtures of synthetic ligands such us known GPCRs ligands, naturally bioactive molecules of unknown function, or randomized compounds in high-throughput screening. This approach has been termed "reverse pharmacology" (Chung, Pohl, Zeng, Civelli & Reinscheid, 2006). 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 (Figure 2). 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 advent of the orphan receptor strategy was confirmed to be successful with the discovery of the neuropeptide Nociceptin/Orphanin FQ (N/OFQ) as the endogenous ligand of the orphan GPCR Opioid Receptor-Like 1 (ORL-1) (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997) (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997).

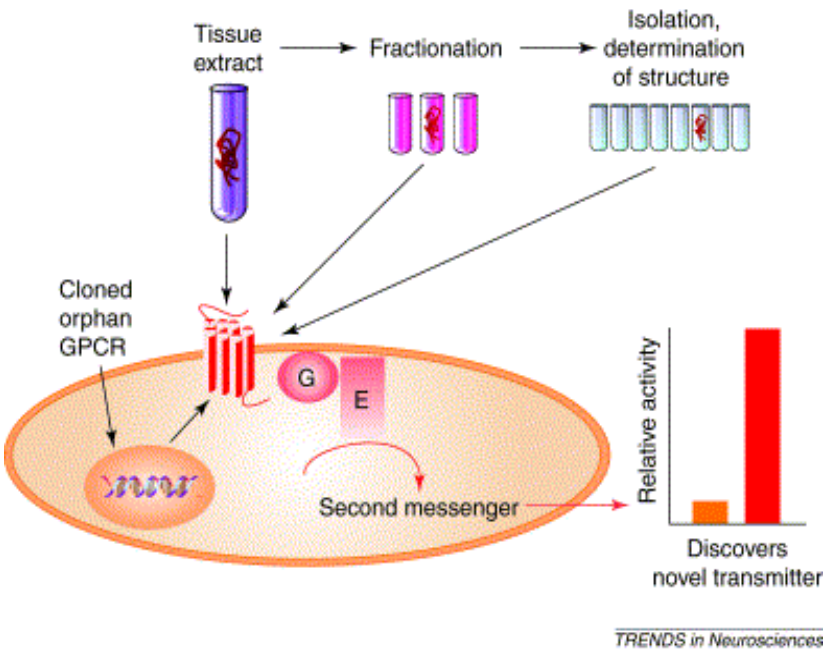


Figure 2. The orphan receptor strategy (Civelli, 2005). The orphan receptor strategy was developed to identify the natural ligands of orphan G-protein-coupled receptors (GPCRs) with the aim of discovering novel transmitters (defined in the main text). This strategy involves: (1) expression of the cloned 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.

1.2. Opioids

1.2.1. OP peptides

In terms of pharmacological activity the opioid receptors are activated by both endogenous and synthetic ligands, whose discovery played an outstanding role for identifying the biological roles played by OP receptors. Not unexpectedly, each of the opioid peptide is made as part of a larger precursor protein. The endogenous opioid peptides are derived from three opioid prohormone precursors by selective cleavages predominantly at basic and pair basic residues: pre-proenkephalin (ppENK), pre-proopiomelanocortin (POMC) and pre-prodynorphin (PDYN) (Table 1). These precursors are encoded by distinct gene and are subjected to many post-translational modification and cleavages resulting in the synthesis of multiple active peptides that act on the classical opioids receptors. The peptides associated with the classical opioid receptors share a common N-terminal sequence of Tyr-Gly-Gly-Phe (followed by Leu or Met) that represents the opioid message domain. This motif is accompanied by various carboxyl terminal extensions and can yield a peptide that ranges in length from 5 to 31 residues (Evans, 2004) (Kostenis et al., 2005). The endogenous peptides for DOP from proenkephalin that gives rice to four Met-enkephalin and a single copy of Leu-enkephalin. Additional larger fragments of proenkephalin have been isolated from tissue. These may be incompletely processed or, possibly, opioid ligands in their own right. More recently have also been isolated selective DOP compounds named as deltorphins, which possess a D-amino acid in the sequence. Prodynorphin also gives rise to several biologically active KOP peptides all of which contain the Leu-enkephalin sequence. These include dynorphin A, dynorphin B, α-neoendorphin and β-neondorphin. Dynorphin A, obtained from porcine pituitary is the putative endogenous ligand for KOP receptor (Chavkin, James & Goldstein, 1982). Proopiomelanocortin is the precursor for the opioid peptide β-endorphin along with other non-opioid related peptides such as corticotropin, melanotropin and β-lipotropin. Beta-endorphin has agonist activity at all three classical opioid receptors, Leu-enkephalin shows high affinity for the DOP receptor, Met- enkephalin has a high affinity for the DOP receptor and some affinity for the MOP receptor, while the dynorphins primarily show affinity for the KOP receptor (Kostenis et al., 2005). Other important opioid peptides are endomorphin 1 (EM-1) that was identified in 1997 in the bovine brain (Hackler, Zadina, Ge & Kastin, 1997) and endomorphin-2 (EM-2) found together with EM-1 in the human

brain cortex (Hackler et al., 1997). The sequences of these peptides are Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2, for EM-1 and EM-2, respectively. They showed high affinity and selectively for the MOP receptor (Hackler, Zadina, Ge & Kastin, 1997). Presently, the precursor protein for the two endogenous MOP peptides is unknown (Dhawan et al., 1996). Endogenous opioid ligands are shown in Table 1 and 2 with accompanying receptor selectivity. In total the three precursor described above give rise to more than 20 candidate opioid ligands and that there were many potential ligands, gave credence to the suggestion, during the opioid pharmacology research mention previously, that there might be more than one opioid receptor.

Table 1. Various mammalian endogenous opioids peptides (Janecka, Fichna & Janecki, 2004).

Precursor	Endogenous peptide	Amino acid sequence	Affinity for opioid receptors
Pro-enkephalin	[Met]enkephalin [Leu]enkephalin Metorphamide	Tyr-Gly-Gly-Phe-Met Tyr-Gly-Gly-Phe-Leu Tyr-Gly-Gly-Phe-Met-Arg-Phe Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-NH_2	δ, μ (δ>>μ)
Pro-opiomelanocortin	β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu	μ, δ (μ=δ)
Pro-dynorphin	Dynorphin A Dynorphin A(1-8) Dynorphin B α-Neoendorphin β-Neoendorphin [Leu]enkephalin	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro Tyr-Gly-Gly-Phe-Leu	κ, μ, δ (κ>>μ and δ)
Pro-nociceptin	Nociceptin	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln	ORL
Unknown	Endomorphin-1 Endomorphin-2	Tyr-Pro-Trp-Phe-NH_2 Tyr-Pro-Phe-Phe-NH_2	μ

Table 2. The table above displays various endogenous and synthetic opioids compounds with their selectivity (RANG H., 2007).

	MOP	DOP	KOP	NOP
Opioid Peptide				
β-Endorphin	***	***	***	-
Leu-enkephalin	*	***	-	-
Met-enkephalin	**	***	-	-
Dynorphin A&B	**	*	***	*
Dermorphin	***	*	-	-
N/OFQ	-	-	-	***
Opioid Clinical Drugs				
Morphine	***	*	*	-
Pethidine	***	*	*	-
Diamorphine	***	*	*	-
Fentanyl	***	*	-	-
Meperidine	**	*	*	-
Partial Agonists				
Buprenorphine	(***)	-	**	*
Pentazocine	*	*	**	-
Antagonists				
Naloxone	***	*	**	-
Naltrexone	***	*	***	-

***: Agonist activity; partial agonists in parenthesis; ***: antagonists activity; -: weak or no activity.

1.2.2. OP receptors

Opioid receptors belong to the super-family of G-protein coupled receptor (GPSRs), which are by far the most abundant class of cell-surface receptors, and also the targets of about on third of approve and marketed drugs. They resided in different part of the body, they are most abundant in the central nervous system (CNS), but have also been localized in many peripheral tissues (e.g. brain, spinal cord, digestive tract, heart, lungs, liver, reproductive tract, etc.). However, the expression and distribution of these receptors vary significantly among different organs and animal species (Barry & Zuo, 2005). They are widely studied due to their crucial role in pain management and analgesia, drug abuse/addiction, mood disorders and all many other biological effects. As mentioned before, it is now clearly established that there are three defined types of classical opioid receptors reported as MOP, DOP and KOP, activated by endogenous peptides, such as endomorphin, enkephalins and dynorphins, but also by naturally occurring alkaloids and other semisynthetic or synthetic ligands. The three opioid receptors show a high degree of sequence homology, approximately 65 percent, with highest similarity in the transmenbrane spanning regions and intracellular loops. The MOP receptor is 66 percent identical to the DOP receptor and 68 percent to the KOP, while the two latter receptors share a 58 percent identity in their respective amino acid sequences. A common opioid receptor-binding pocket within the helical transmenbrane core has been postulated based upon modelling and structure activity studies (Metzger & Ferguson, 1995) and all three receptors have the aspartate-arginine-tyrosine (DRY) sequence and aspartates in the second and third transmenbrane spanning regions that are conserved among GTP binding G-protein linked receptors. The greatest divergence in sequence between the receptors occurs at extracellular (N-terminus) domains and these regions are important for ligand selectivity (Kane, Svensson & Ferguson, 2006). Likewise, have been identified helical domain-mediated mechanisms for opioid receptors activation within the membrane core receptor domain, which is highly similar across the three receptors (Decaillot, Befort, Filliol, Yue, Walker & Kieffer, 2003). Using bioinformatics method and comparing available sequences database was noted that the protein sequence of the MOP receptor is most identical to that of the DOP receptor (and vice versa), while the sequence of the KOP to that of the NOP receptor (Stevens, 2009). It is greater interest to note that the target of most clinically used opioids is the MOP receptor and its relative selective agonist are the most efficacious type of opioid analgesics in the clinic and in animal models. MOP was the last of the classical opioid

receptors to be cloned and it is expressed from the gene OPMRM1. The receptor is located throughout the central nervous system, in areas involved in sensory and motor function including regions concerned with the integration and perception of these senses (e.g. cerebral cortex, hippocampus and amygdala). High densities of MOP receptors are found in the caudate putamen, presynaptically on primary afferent neurons within the dorsal horn of the spinal cord and in the periaqueductal grey (PAG). In the dorsal horn, their activation causes the inhibition of glutamate release and thus the transmission of nociceptive signals from both Aδ and C fibers. The PAG is involved in the central control of nociceptive pathway, thus activation of MOP receptors in this area is believed to provoke analgesia through inhibition of the neurotransmitter γ-amino butyric acid (GABA), one of the main inhibitory neurotransmitters in the brain that acts to reduce or prevent antinociceptive efferent outflow from the PAG (Bee and Dickenson, 2009). In the region of the nucleus raphe magnus, two different types of neurons can be distinguished: ON and OFF cells. The MOP receptor can be found also on ON cells, and when an agonist, such as morphine or fentanyl, activates the MOP receptor it results in direct inhibition of these cells leading to an increase in the nociceptive signal to the dorsal horn from the descending inhibitory tract (Bee and Dickenson, 2009). Instead causing analgesia, prolonged activation of the MOP receptor can also causes moderate to severe side effects, as tolerance to the drug, constipation and, in severe cases, respiratory depression. Further, numerous are the physiological functions to be controlled by MOP receptors. These include the cardiovascular system, thermoregulation, hormone secretion, locomotors activity and immune function. The crystal structure of MOP recently has been solved and as for other GPCRS, the structure consists of seven TM alpha helices that are connected by three extracellular loops and three intracellular loops (Figure 3). Interestingly, unlike previously crystallized GPCRs, the MOP receptor's binding pocket is exposed to the extracellular surface (Granier et al., 2012). The crystallized structure presented provides the first high-resolution insight into a peptide receptor activated by small molecules, some of which are the oldest used drugs in human history as morphine. This structure will enable the application of new approaches to develop more selective drugs and may provide novel insight into the role of oligomerization in GPCR function.

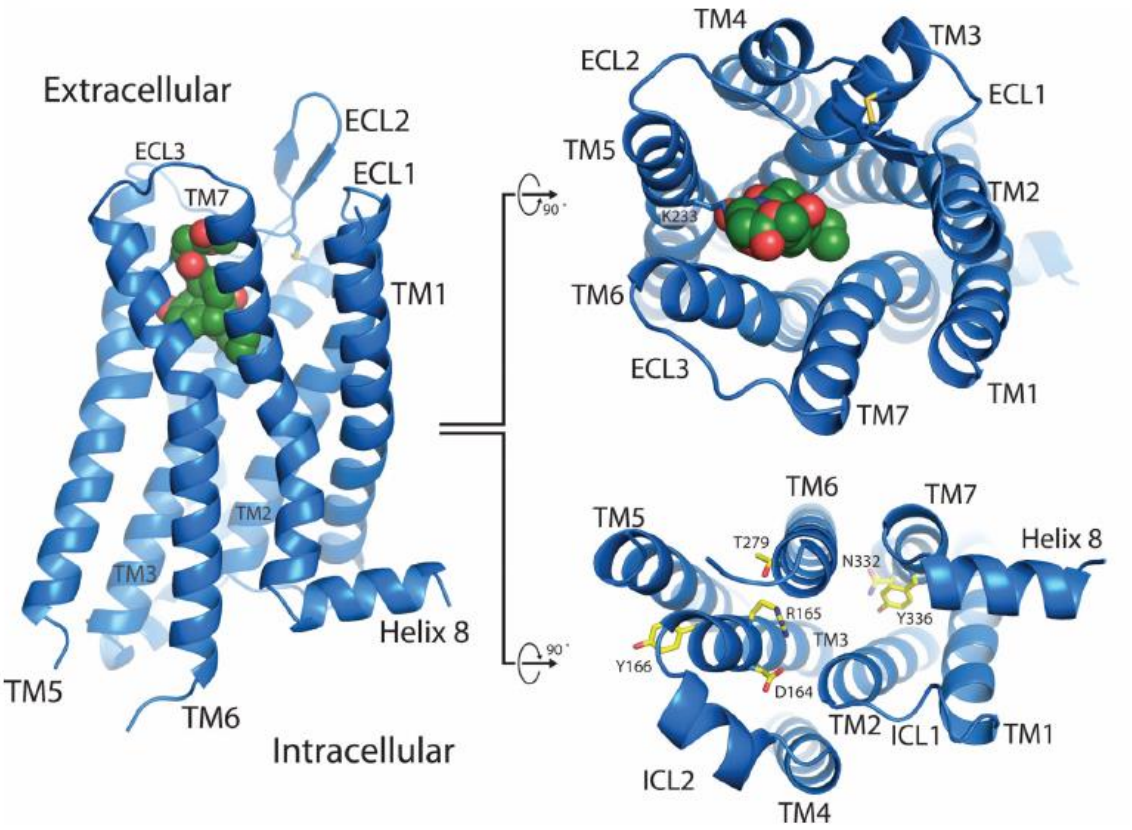


Figure 3. Overall view of the MOP receptor structures (Granier et al., 2012).

The KOP receptor was the second of the opioid receptor family to be cloned. It is located throughout the CNS, mostly being located in the diencephalic and limbic areas, brain stem and spinal cord. KOP receptors have been implicated in the regulation of several physiological responses, including nociception, stress, mood, diuresis, feeding, gut motility, neuroendocrine secretions and control of immune functions. On the other hand, the clinical relevance of KOP agonists is limited by their side effects, in particular dysphoria, and psychotomimesis, but also depression and anxiety, sedation and dependence. Nevertheless, therapeutically the advantage of the KOP receptor agonists as alternatives to MOP analgesics is that they have low abuse potential and produce minimal effects on gastrointestinal transit and do not cause respiratory depression over time (Kivell & Prisinzano, 2010) (Vanderah, 2010). The crystal structure of the KOP receptor has demonstrated a large binding pocket with a number of potential anchoring points for ligands (Figure 4). These unique features explain diversity of drugs able to interact with KOP. Thus, the recent breakthroughs in elucidation the high resolution structure of KOP receptor in complex with small molecule and peptide ligands are providing a molecular

framework for understanding opioid drug action and thereby affords the discovery of new drugs with ideal pharmacological properties (Thompson et al., 2012).

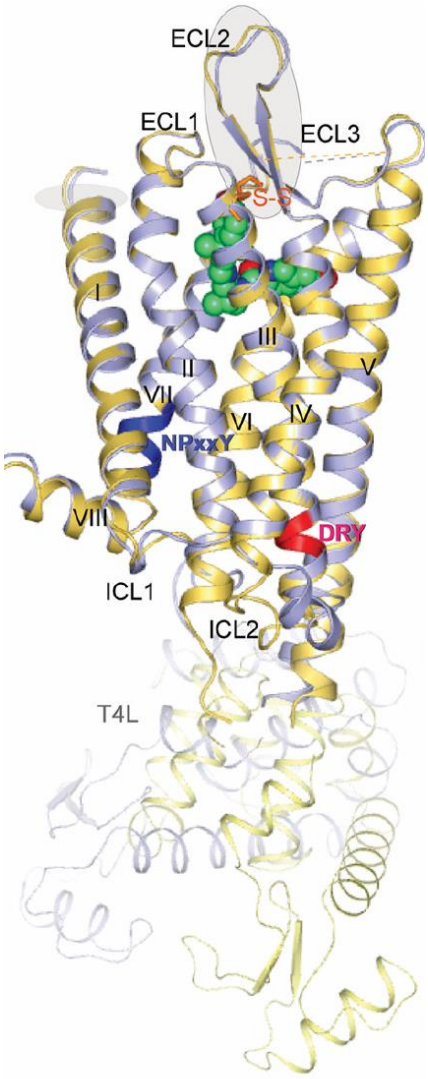


Figure 4. Overall view of the KOP receptor structures in complex with JDTic (Thompson et al., 2012).

The DOP receptor was the first receptor to be cloned. It is the less widely distributed compared to the other receptors. High density is found and in areas such as the nucleus accumbens, caudate putamen, olfactory bulb and cerebral cortex (Kostenis et al., 2005). DOP receptors are located on primary afferents and act to inhibit the release of neurotransmitters from presynaptic c-terminals. Activation of the DOP receptor can lead to analgesic actions due to their location within spinal and supraspinal sites (Kostenis et al., 2005) DOP receptors have been relatively understudied; however have been revealed its importance in a number of physiological processes without evoking many of the adverse effects associated with MOP agonists, including additive liability and constipation, even if

evidences reported respiratory depression. Currently there are no clinically available DOP-specific drugs available. Although DOP agonists are poor analgesics in acute pain, they are highly effective in animal models of chronic inflammatory and neuropathic pain (Nadal, Banos, Kieffer & Maldonado, 2006). The DOP receptor has been implicated in the treatment of emotional disorders, neurological disorders and negatively influencing reward and addiction (Pradhan, Befort, Nozaki, Gaveriaux-Ruff & Kieffer, 2011). A work in DOP receptor KO mice has demonstrated an increased anxiety, indicating the potential for DOP agonists in affective disorders (Gaveriaux-Ruff & Kieffer, 2002) and studies on the same mice has revealed that they display hyperlocomotor activity, may dampen locomotor behaviour. With regards to potential unwanted effects, DOP agonists have been shown to be proconvulsive (Jutkiewicz, Baladi, Folk, Rice & Woods, 2006). Structurally, DOP shares a number of highly conserved features with the other opioid receptors. It has a typical seven transmembrane domain, with high similarity in structure to that of the other opioid receptors. Its binding pocket, like the other member of the opioid receptor family, can be split into two sections. The lower section is highly conserved amongst all of the opioid receptors, while the upper section confers ligand selectivity (Figure 5) (Granier et al., 2012).

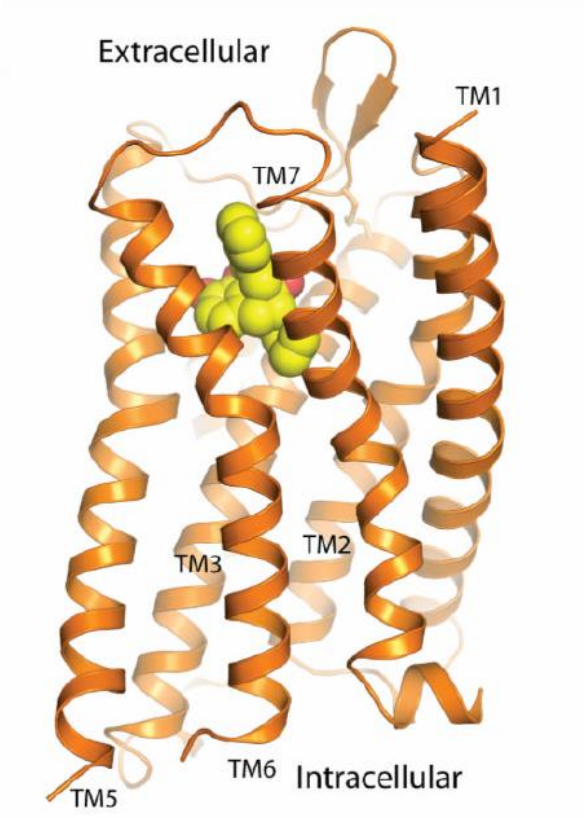


Figure 5. Overall view of the DOP receptor structures (Granier et al., 2012).

1.2.3. Cellular and biologic actions

Cellular actions

As mentioned previously, the opioid receptors are GPCRs, precisely, coupled to a $G\alpha_{i/o}$ sub-type of G-proteins. The classical model of opioid receptor activation processes could be generalized for all the three classical opioid receptors (MOP/KOP/DOP). The binding of a ligand to the receptor causes a conformational modification of the receptor that results in the activation of the associated heterotrimeric $G\alpha_{i/o}$ proteins, involving the exchange of bound guanosine diphosphate (GDP) for guanosine-5'-triphosphate (GTP) by the Gα subunit of the G-protein and the dissociation of the heterotrimeric protein complex into Gα and Gβγ subunits. This dissociation then promotes various consequent signalling events controlled by second messenger systems, such as those involving cyclic AMP, diacylglycerol and calcium. Thus, the activation of the classical opioid receptors results in the closing of voltage sensitive calcium channels (VSCC); stimulation of potassium efflux causing membrane hyperpolarization; and reduced cyclic adenosine monophosphate (cAMP) production via inhibition of adenylyl cyclase. Overall, this results in reduced neuronal cell excitability promoting a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release (Figure 6). Signalling by the activated conformation of the GPCR opioid receptors is terminated by phosphorylation of the cytoplasmic loops and tail of the GPCR, which is catalysed predominantly by GPCR kinases (GRKs). This results in the binding of arrestins (i.e. β-arrestin 1 and 2) and consequent desensitization followed by internalization into clathrin-coated pits (Lefkowitz & Shenoy, 2005). Therefore, in the classical model, heterotrimeric G-proteins mediate signal transduction and β-arrestins mediate receptor desensitization and internalization.

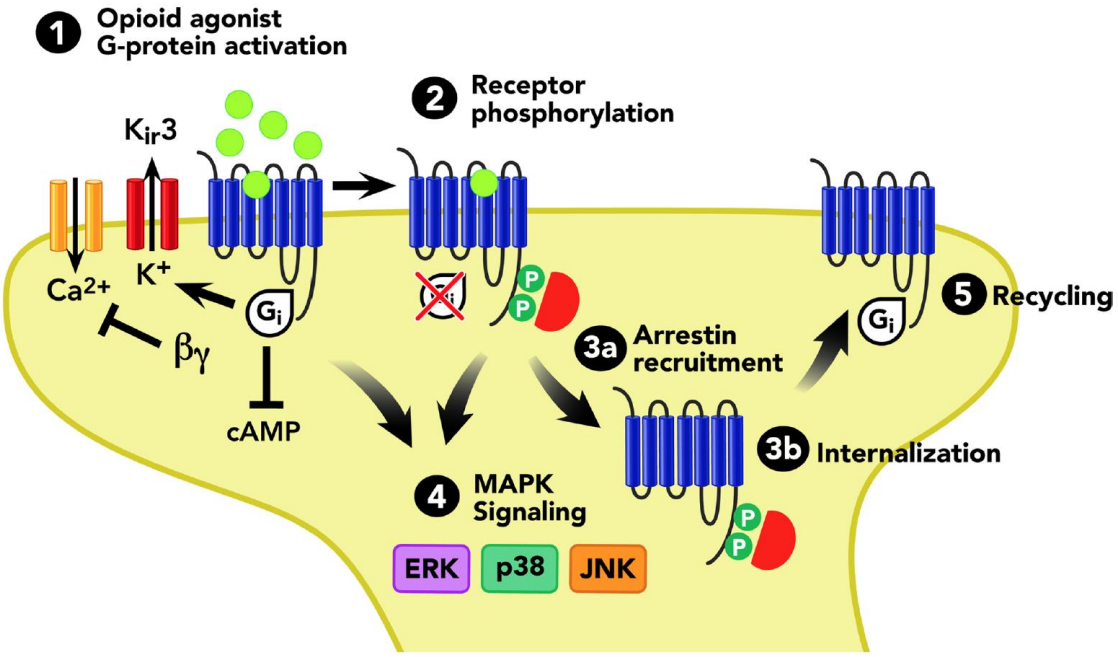


Figure 6. Summary of opioid receptor signalling (Al-Hasani & Bruchas, 2011).

As far as opioid receptor actions are concerned, not only interaction via ion channels and neurotransmitter release are known, but also further downstream signalling events. In fact, it has been proposed that opioid receptors interact with a family of serine/threonine kinases, the mitogen-activated protein kinases (MAPK), which are divided into three classes: extracellular signal-regulated kinases (ERK1/2), the p38 MAPKs and the c-Jun N-terminal kinase/Stress-activated protein kinases (JNK/SAPK) (Fukuda, Kato, Morikawa, Shoda & Mori, 1996). This pathway regulates DNA synthesis, cell growth, apoptosis and regulation of nuclear transcription factors, in particular, their signalling pathway are influential in opioid receptor desensitization and neuronal survival or apoptosis (Polakiewicz, Schieferl, Gingras, Sonenberg & Comb, 1998). The activation of the MAPK pathways mediated by opioid receptors occurs through multiple routes. The βγ subunit of the G-protein effects ERK1/2 activity through interactions with a member of the small GTPase family, Ras (Belcheva et al., 1998). Furthermore, opioid inhibition of both protein kinase A (PKA) and stimulation protein kinase C (PKC) affects ERK 1/2 and p38 signalling (Zhang, Xin, Wu, Zhang, Ma & Pei, 1999). Recently, it has been demonstrated that β-arrestin 1 and 2 proteins are involved in opioid activation of MAPK signalling (Strungs & Luttrell, 2014). It seems that the modulation of members of the MAPK family by opioids depends on the methods of opioid receptors activation and thus, by changing the duration or intensity of stimulation on the MAPK pathway, the kinase activity can be

targeted to perform specific functions (Strungs & Luttrell, 2014). After receptor activation, a number of cellular mechanisms occur to regulate GPCR signalling. Thus, the receptors stop registering an on-going stimulus, in a phase that is called "desensitisation", which occurs through receptor phosphorylation, either through heterologous protein kinases such as PKA or PKC, or, mainly, through a family of kinases called the G-protein receptor kinases (GRKs), as an homologous phosphorylation (Pitcher, Freedman & Lefkowitz, 1998). The latter kind of phosphorylation leads to recruitment of β-arrestins (β-arrestin 1 or β-arrestin 2), sorting and either recycling to the surface or degradation of the receptors (Shenoy & Lefkowitz, 2011). The ability of β-arrestin proteins to mediate numerous signalling pathways, as well as affecting receptor recycling or degradation, has led to the hypothesis of differential phosphorylation of receptors. In this case, varying phosphorylation sites would allow various patterns, and/or numbers, of β-arrestin recruitment allowing for multiple signalling pathways.

Biological actions

Opioids are the most widely used and effective analgesics for the treatment of pain and related disorders and in the last century have been made huge strides in the development of novel molecules within the fields of receptor pharmacology and medicinal chemistry. In addition to pain, opioids are frequently used in the treatment of numerous other disorders and diseases including diarrhoea, cough, post-operative pain and cancer (Table 3), considering then, that they are critical in the modulation of pain behaviour and nociception. The action of opioid compounds is mediated through activation of specific opioid receptors, witch are expressed throughout the nociceptive neural circuitry, in critical region of the central and peripheral nervous systems (including in reward and emotional-related brain structures), in neuroendocrine and immune systems, in mucosal cells and also in many peripheral organs systems (e.g. hear, lungs, liver, gastrointestinal and reproducing tracts) (Wittert, Hope & Pyle, 1996). It highlight how further studies are imperative to fully understand the wide field of opioid pharmacology and the multifunctional role of opioids compounds in the pathophysiology of diverse diseases and biological phenomenon of therapeutic interest. Opioid peptide receptors in CNS represent the most extensive and diverse peptidergic transmission system and are widely involved in various pleiotropic functions. They are essential for various physiological functions (pain modulation,

locomotion, mood, diuresis, thermoregulation and stress, along with regulatory function in respiratory, gastrointestinal and cardiovascular systems) on the other hand, abuse of opioid compounds leads to addiction and tolerance, witch greatly affects brain function and body homeostasis. Recent studies show that opioid receptors are involved in the regulation in ischemic or stress conditions of ionic homeostasis that is vital for normal functioning of neurons (Kang et al., 2009). Opioid receptors affects cell proliferation (Malendowicz, Rebuffat, Tortorella, Nussdorfer, Ziolkowska & Hochol, 2005) and other findings have mooted the concept that DOP receptors play a crucial role in neurogenesis and neuroprotection (Narita et al., 2006). It has been suggested that endogenous opioids especially DOP, play a significant role in the hibernation state of mammalian (Chen, Mestek, Liu, Hurley & Yu, 1993).

Pain – A majority of studies on opioids are associated with analgesia. Many acute insults results in the release of endogenous opioids and their increased levels in blood, witch counteracts the noxious stimulus in pain as stressed-induced analgesia by opioids. For instance, significantly high levels of circulating β-endorphin were detected following muscle injury, fixed-pressure haemorrhagic shock and lipopolysaccharide (LPS) administration in animal models, which indicated the endogenous opioid system activation (Molina, 2002). Similarly, stress-induced elevated production and release of endogenous opioids have been demonstrated in clinical studies, such us in patients after oral, gynaecological and abdominal surgeries (Troullos, Hargreaves & Dionne, 1997) (Kho, Kloppenborg & van Egmond, 1993). Importantly, stress-induced analgesia can be partially reversed by the broad-spectrum opioid antagonist naloxone (Yamada & Nabeshima, 1995), underlining the involvement of endogenous opioids in this process. It is generally accepted that opioids tonically regulate nociceptive information producing analgesia. Today, opiate drugs are widely used in the treatment of pain and many of the currently available opioid analgesics exert their effects primarily through the MOP receptors, indicating a vital role of this receptor in pain modulation. Strong evidence showed that MOP receptor plays a central role in analgesia. For examples, MOP(-/-) mice have increased sensitivity to heat, suggesting the existence of a MOP-mediated tone in thermal nociception (Kieffer & Gaveriaux-Ruff, 2002) and a reduction in stress-induced analgesia, while DOP- and KOP-deficient mice did not exhibit any alteration in heat perception (LaBuda, Sora, Uhl & Fuchs, 2000). Furthermore, in mice lacking of the MOP receptor, the analgesic effects of DOP agonists are either unchanged or diminished (Gaveriaux-Ruff & Kieffer, 2002), while

MOP agonists failed to exhibit analgesia (Matthes et al., 1996). However DOP agonists can enhance the analgesic potency and efficacy of MOP agonists and DOP antagonists can prevent or reduce the development of tolerance and physical dependence to MOP agonists (Ananthan, 2006). The recorded analgesia could be a result of the cross-reactivity of DOP agonists at MOP receptors *in vivo* (Gaveriaux-Ruff & Kieffer, 2002). Also the KOP receptor seems to play a role in pain modulation. Even if KOP(-/-) mice did not exhibit any alteration in the perception of thermal or mechanical pain, they showed an enhanced pain response to the peritoneal acetic acid injection (Gaveriaux-Ruff & Kieffer, 2002). Anyway compared to the other two opioid receptors, KOP receptor chiefly mediates analgesia to visceral pain (Gebhart, 2000).

Drug abuse and addiction – Unfortunately excessive use of opioid leads to addiction and tolerance in nervous system, becoming considered as a neurological pathology due to the effects on brain function. Unfortunately, the mechanisms underlying neurological disorder caused by opioid addiction are not still clear yet, however it is likely that the neuronal basis of positive reinforcement relies on activation of dopaminergic neurons resulting in an increased dopamine release in the mesolimbic brain structures and several aspects of opioid dependence and withdrawal syndrome are also related to noradrenergic peptidergic systems. An important role in neurochemical mechanisms of opioid reward, dependence and vulnerability to addiction has been ascribed to the activation of MOP and KOP receptors. Opioid abuse causes to opioid tolerance in the nervous system. Many mechanisms of receptor regulation such as desensitization and internalization are described to be involved in receptor tolerance and adaptation (Koch & Hollt, 2008). Different important processes including up-regulation of cAMP/PKA, cAMP response and MAPK cascades have been identified to influence tolerance and withdrawal in opioid sensitive neurons and also synaptic plasticity during the cycles of intoxication and withdrawal (Christie, 2008). Intracellular molecules of signal transmission, i.e. G proteins, cyclic AMP, MAP kinases, and some transcription factors are also involved in opioid tolerance and dependence. In general, drug abuse induces adaptive changes in opioid receptors that occur following acute (desensitization and internalization) and chronic (adaptive tolerance and down-regulation) administration (Harrison, Kastin & Zadina, 1998).

Emotional response – Although it is poorly documented in pharmacology compared to other opioid biological functions, the opioid system has a role in regulating other emotional

responses. In particular, the DOP receptor acts as natural inhibitor of stress and anxiety (Saitoh, Yoshikawa, Onodera & Kamei, 2005) and its activation produces antidepressant and anxiolytic effects in rodent models. DOP agonists have been shown to increase expression of brain-derived neurotrophic factor (BDNF) mRNA, an effect in common with some antidepressants, which may be important for the clinical efficacy of antidepressant drugs (Torregrossa, Jutkiewicz, Mosberg, Balboni, Watson & Woods, 2006). It has also been reported that similarly to the like most clinically used antidepressants, the selective KOP ligand nor-BNI after centrally administrations up-regulated BDNF mRNA expression in the rat hippocampus (Zhang, Shi, Woods, Watson & Ko, 2007). These evidence further imply that the central activation of the KOP receptor may mediate antidepressant-like effects by BDNF gene up-regulation. In addition, stress contributes to opioid-induced neuronal activation and mice exposed to stress and morphine showed region-specific increased level of the KOP receptor (Vien, Gleason, Hays, McPherson, Chavkin & Juul, 2009).

Epileptic seizures – The role of the opioid receptor system in epileptogenesis and epileptic seizure has been proposed in literature, but it still remain controversial, so increased familiarity with the effects of opioid action on epilepsy is necessary. However, the MOP and KOP receptors are described to be involved in hypoxic preconditioning against seizures in the brain by the fact that an episode of normobar hypoxia reduced the susceptibility to convulsions induced by pentylenetetrazol, which could be mimicked by both MOP or KOP agonists (Rubaj, Gustaw, Zgodzinski, Kleinrok & Sieklucka-Dziuba, 2000). Certain experimental models of epilepsy have showed that endogenous opioid (e.g. enkephalin and dynorphin) levels are greatly increased in the brain during epileptic seizures (Tortella & Long, 1985) (Schwarzer, 2009) and opioid receptors are up regulated following spontaneous epileptic seizure (Hammers et al., 2007). Nevertheless it is not clear if this represents a compensatory mechanism against epilepsy because multiple factors determine the anticonvulsant or proconvulsant role of opioids in the brain. For example, morphine depresses electrographic seizure activity under a low concentration, while enhances seizure activity under high concentrations with an apparent dose-dependent manner (Honar et al., 2004). The non-peptide DOP agonist, SCN 80 produced convulsions in the rat both in low or high dose, but had little effects in rhesus monkeys (Negus, Gatch, Mello, Zhang & Rice, 1998). Thus, further studies are strongly required to clarify the role of the opioid system in this field.

Immune function – Opioid system is involved in immune regulation. It mediates immunosuppression, although its actions are complicated and not completely understood. Acute and chronic opioid administration is known to have inhibitory effects on humoral and cellular immune responses as antibody production, natural killer cell activity, cytokine expression and phagocytic activity (Vallejo, de Leon-Casasola & Benyamin, 2004) (Jamali, Bamdad, Soleimanjahi, Pakdel & Arefian, 2007). Experiments performed both *in vivo* and *in vitro* showed that stimulation of opioid receptor exerts suppression of multiple components of the immune defence response such as natural killer (NK) cell activity (Carr, Gebhardt & Paul, 1993), neutrophil complement and immunoglobulin receptor expression (Welters et al., 2000), chemokine-induced chemotaxis (Grimm et al., 1998) and phagocytosis (Menzebach, Hirsch, Nost, Mogk, Hempelmann & Welters, 2004). It was demostreted that following i.c.v. injection of β-endorphin in rats, the mitogen-induced spleen lymphocyte DNA synthesis, hemolysin formation and IgG production were reduced significantly, indicating a role of the central opioid system in immune regulation (Bai, Du & Zheng, 1999). There are evidence for all three classical opioid receptors. Pretreatment with the KOP selective ligand U50 488 significantly reduced lipopolysaccharide (LPS)-stimulated interleukin-6 (IL-6) production (Parkhill & Bidlack, 2006). Treatment of normal human astrocytes with morphine leads to significant down-regulation of gene expression for β-chemokines, MCP-1, and MIP-1β (Mahajan, Schwartz, Aalinkeel, Chawda, Sykes & Nair, 2005). Acute administration of morphine significantly decreased NK cell cytotoxicity and interferon-γ mRNA levels, and increased the mortality rate of mice infected with herpes simplex virus 1 (Jamali, Bamdad, Soleimanjahi, Pakdel & Arefian, 2007). As far as the DOP receptor is concerned, its activation through the DOP agonist DPDPE, triggers monocyte adhesion (Pello et al., 2006), while the non-peptide SNC 80 significantly stimulated rat thymic and human leukocyte chemotaxis (Ordaz-Sanchez et al., 2003). It has been proposed that activation of the MOP receptor favours a proinflammatory effect, while activation of KOP induces an anti-inflammatory response via the down-regulation of cytokine, chemokine and chemokine receptor expression (Finley, Happel, Kaminsky & Rogers, 2008). In summary, opioid modulation of the immune response in animals is mediated through the direct interaction with opioid receptors expressed by immune cells.

Feeding and obesity – Opioid receptors also participate in the regulation of feeding by agonists and antagonists ligands, of which correspond stimulatory and inhibitory effects respectively. Same data suggest a role for opioids in the control of appetite for specific

macronutrients and in the stimulation of intake based on already existing diet or taste preferences and in controlling intake motivated by hedonics rather than by energy needs (Gosnell & Levine, 2009). Agonist stimulation of opioid receptors increases feeding in rodents, while opioid antagonists inhibit food intake and weight gain in ob/ob mice (Marczak et al., 2009). For instance, the opioid antagonist, LY255582, produces a sustained reduction in food intake and body weight in rodent models of obesity likely through a combination of the MOP, KOP and DOP-receptor activity (Gackenheimer et al., 2005). In addiction are reported studies regarding the involvement of opioid receptors as obesity-related factor in affecting body weight and obesity and the MOP receptor stimulation preferentially increases the intake of a high fat diet.

Respiratory control – In a heterogeneous way, opioid receptors affect ventilation, leading to respiratory depression due to a direct action on respiratory-generating structures in the brain (Pattinson, 2008). At this regard, it has been demonstrated that there are high densities of opioid receptors in the brain areas related to respiration and local application of opioid agonists to these areas depressed the activity of respiratory related neurons (Mutolo, Bongianni, Einum, Dubuc & Pantaleo, 2007). Interestingly, MOP activation shows a marked decrease in respiratory frequency and complete apnoea, less pronounced by DOP activation while absent upon KOP activation (Mutolo, Bongianni, Einum, Dubuc & Pantaleo, 2007). In fact, it is well established that opioid drugs depress the rate and depth of breathing, blunt respiratory responsiveness to CO_2 and hypoxia, increase upper airway resistance and reduce pulmonary compliance. These disturbances are mainly due to the MOP and DOP receptors activation, involving specific types of respiratory-related neurons in the ventrolateral medulla and the dorsolateral pons (Lalley, 2008). Under certain conditions, this adverse effect may be lethal. In general, severe stress (haemorrhagic shock, trauma, bacterial infection) resulting in a massive release of endogenous opioids, or an overdose of opioid analogues (drug abuse, addiction, and uncontrolled use for pain relief) can cause a severe respiratory depression, hypotension, and even death. All this firmly explains why the undesirable side effects of opioids accompanying their extensive clinical use for pain relief are an important concern.

Gastrointestinal function – MOP, DOP and KOP receptors have been localized in the gastrointestinal tract of rodents and humans, however their relative distribution varies with gastrointestinal region and species (Holzer, 2009). MOP receptor agonists as morphine and

loperamide slow gastrointestinal transit by their effects on the circular and longitudinal muscle of the intestine (Corazziari, 1999). Endomorphin-1 and 2 induces a gastro-protective action and shows that endogenous opioids may have a central role in maintaining gastric mucosal integrity (Gyires & Zadori, 2008). Other studies reported improvement using opioid compounds in treating irritable bowel syndrome and gastrointestinal disorders including diarrhoea. Opioid peptides (β-endorphin, enkephalins and dynorphins) are present in the heart and myocardial cells are sites of opioid peptide synthesis, storage and release (Barron, Jones & Caffrey, 1995).

Cardiovascular regulation – Animal experiments have demonstrated that the opioid system can modulate hemodynamic and cardiovascular activity. Opioid peptides as β-endorphin (Forman, Estilow & Hock, 1989), enkephalins (Lang et al., 1983) and dynorphins (Weihe, McKnight, Corbett & Kosterlitz, 1985), were found in the heart. Myocardial cells are sites of opioid peptide synthesis; storage and release (Barron, Jones & Caffrey, 1995) and thus their levels are elevated during episodes of stress, such as ischemia (Eliasson et al., 1998). In conscious squirrel monkeys there are evidence that same KOP agonist elicited an increase heart rate with a little effect on blood pressure via a combined action on central and peripheral receptors. This effect was not mimicked by morphine and was reversed by the opioid antagonist naltrexone thus demonstrating the specific involvement of the KOP receptor in eliciting this action (Schindler et al., 2007). In addition, both DOP (Schultz, Hsu & Gross, 1997) and KOP (Wu, Li & Wong, 1999) receptors have been shown to mediate cardioprotection by preconditioning with myocardial ischemia and metabolic inhibition, one of the consequences of ischemia. MOP receptor has been shown to be involved in the regulation of cardiovascular function, otherwise, the contrary results obtained, indicate that the cardiovascular effects are likely to be site-action dependant.

Neurodegenerative and other diseases – Substantial data demonstrated the role of opioids receptors in many diseases, e.g. multiple sclerosis, rheumatoid arthritis, Parkinson, L-DOPA-induced dyskinesia symptoms, even in the treatment itching and sleep disturbances. Although the side effects of opioid receptors activation under certain conditions represents the other side of the coin, the opioid system is considered as one of the most complex and interwoven neurotransmitter system in the body, more intricate and in-depth studies are needed to understand the functions in which it is involved because the majority of the

research are focused on pain modulation and drug addiction via MOP activation in the CNS. Other potential roles of the opioid system throughout the body still remain to be clarified. With the advent of new approaches and pharmacological techniques, opioid pharmacology is poised for same major breakthroughs in the next future. It is hopeful that new molecular and cellular discoveries will lead better opioid analgesics in the clinic with a discrete risk of addiction and tolerance. Furthermore, it is likely that forefront studies will continue to reveal novel uses for opioids in the treatment of a variety of diseases.

Table 3. Organ system effects exerted by various clinically available endogenous and synthetic opioids agonists (Al-Hasani & Bruchas, 2011).

Organ Systems	Effects	Additional information
Central Nervous system	↑ Analgesia	
	↑ Euphoria	leading to risk of addiction and abuse
	↑Sedation	
	↓ Rate of respiration	
	↓ Cough reflex	Codeine used for treatment of pathological cough
	↑ Miosis-Constriction of the pupils	
	↑ Truncal rigidity	Most apparent when using fentanyl, sufentanil, alfentanil
	↑ Nausea and vomiting	
Peripheral	**Gastrointestinal system**	
	↑ Constipation	
	↓ Gastric motility	
	↓ Digestion in the small intestine	
	↓ Peristaltic waves in the colon	
	↑ Constriction of biliary smooth muscle	
	↑ Esophageal reflux	
	Other smooth muscle	
	↑ Depression of renal function	
	↓ Uterine tone	
	↑ Urinary retention	
	Skin	
	↑ Itching and sweating	
	↑ Flushing of the face, neck and thorax	
	Cardiovascular system	
	↓ Blood pressure and heart rate if cardiovascular system is stressed	
	Immune System	
	↓ Formation of rosettes by human lymphocytes	
	↓ Cytotoxic activity of natural killer cells	
	Other	
	Behavioral restlessness	

1.2.4. OP receptor ligands

A wide range of exogenous compounds is also available for OP receptors, which comprise natural compounds, synthesized compounds to peptides found in animals. The prototypical MOP agonists are the natural alkaloids, represented by the morphine extracted and purified from opium, along with codeine. Morphine and other opiates are widely used in clinical practice for severe pain syndromes or for anaesthetic purposes. Morphine is primarily a selective agonist ligand for the MOP receptor, due to its sufficiently low affinities for the DOP and KOP receptors (Magnan, Paterson, Tavani & Kosterlitz, 1982). Semi-synthetic alkaloids derivatives can be obtained through chemical modification such as hydromorphone and hydrocodone. Within synthetic agonists, the piperidine opioid derivatives, whose structures bear no resemblance to morphine, fentanyl and remifentanil are the most potent compounds. In general, most clinically available opioids act solely at the MOP receptor. Nevertheless, compound as buprenorphine has partial agonist activity at MOP and NOP receptors, while acts as antagonist at KOP. Some other opioid drugs have mixed actions at different opioid receptors. For examples, pentazocine behaves as an antagonist at MOP receptor but a partial agonist at DOP and KOP receptors. Currently, there are no clinically selective drugs available that work via DOP, KOP or NOP receptors. Noteworthy are as selective synthetic compounds: DAMGO for MOP (probably the most selective one), DPDPE and SNC-80 for DOP and U-69,593 for KOP. Listed among compounds with antagonist activity there are naloxone described as universal opioid antagonist, while CTOP, naltrendole and nor-binaltorphine (nor-BNI) as selective antagonists for the MOP, DOP and KOP receptors, respectively. The most frequently used opioid antagonists are synthetic alkaloids such as naloxone and naltrexone. Naloxone, the first pharmacologically pure antagonist identified, is considered a universal, non-selective opioid antagonist. The action of an agonist is characterized as opioid only if its effects are "naloxone-reversible". Although naloxone and its analogue naltrexone bind to all three opioid receptors, they have the highest affinity for the MOP. The analgesic properties of opioid compounds are undoubted, as far as therapeutics effects are concerned, morphine, buprenorphine, methadone, fentanyl, tapetandol, etc. are common used for the treatment of the pain. Otherwise, the clinical utility of opioids continues to be limited by a compromise between efficacy and side effects. As a matter of fact, after prolonged administration, they are marred by important common adverse effects as constipation, respiratory depression, nausea, drug abuse, tolerance liability and limited efficacy in certain disease characterized

by neuropathic pain states. Moreover long-term use of opioids can lead to dependence and addiction. At this purpose much efforts have been spent for developing more effective and well tolerate drugs by research groups both industrial and academic and these researches are still on going to obtain novel and innovative drug candidates.

1.3. N/OFQ – NOP receptor system

After the cloning of the three opioid receptors, different research groups simultaneously identified an orphan G protein-coupled receptor characterized by a overall structural homology whit the classical opioid receptors MOP, DOP and KOP as high as the opioid receptors have with each other (Bunzow et al., 1994). However, surprisingly, when transfected into mammalian cells the orphan receptor did not bind neither appear to be activated by any opioid ligand at low concentrations. Nevertheless, it was activated by high concentrations of the opiate agonist etorphine and inhibited by very high concentrations of naloxone. Based on these findings the receptor was referred to as opioid receptor-like 1 (ORL-1) receptor (Mollereau et al., 1994). As the first successful example of reverse pharmacology (Chung, Pohl, Zeng, Civelli & Reinscheid, 2006), ORL-1 cDNA was transfected into cells that were exposed to either rat brain or porcine pituitary extracts resulting in a revolutionary discovery. In fact, two different laboratories almost simultaneously and independently achieved the purification of the natural ligand that bound to ORL-1 with high affinity and activated the receptor (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997) (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997). The new ligand was a 17 amino acid neuropeptide, which showed structural homology with endogenous opioid peptides, particularly dynorphin A, however the presence of Phe in position 1 instead of Tyr makes the peptide highly selective for its receptor over classical opioid receptors. The peptide was called nociceptin for its ability to elicit hyperalgesia after supraspinal administration in mice (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997) or orphanin FQ for its ability to recognize an orphan receptor and for its first and last amino acid residues (F and Q) (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997). The peptide is now referred to as nociceptin/orphanin FQ and abbreviated as N/OFQ and the receptor as N/OFQ peptide receptor and abbreviated as NOP. Despite the structural similarities, N/OFQ does not activate MOP, DOP and KOP receptors. In addition, classical opioid ligands have negligible binding affinity for NOP. These differences in ligand selectivity likely arise due to small number of residues different between NOP and other opioid receptors and subsequent changes in the structure of the binding pocket of NOP (Thompson et al., 2012). For this reason, the NOP receptor is currently classified as a non-classical opioid member of the opioid receptor family according to IUPHAR recommendations (Alexander et al., 2013). The N/OFQ-NOP receptor system is widely distributed both in the nervous and peripheral systems where it

modulates several different biological functions including pain, mood and anxiety, food intake, learning and memory, locomotion, intestinal motility, cardiovascular homeostasis and immune responses (Ibba et al., 2008). Thus, the NOP receptor is emerging as a potential target for the development of innovative drugs. Structure relationship studies performed on the N/OFQ sequence allowed generating NOP selective peptide ligands encompassing full and partial agonist, as well as pure antagonist pharmacological activity. In addition, medicinal chemistry efforts mainly performed in industrial laboratories made available to the scientific community small molecules acting as selective NOP receptor agonists and antagonists (Mustazza & Bastanzio, 2011). These pharmacological tools together with genetic models such as NOP receptor knockout mice (Nishi et al., 1997) and rats (Homberg, Mul, de Wit & Cuppen, 2009) and ppN/OFQ knockout mice (Koster et al., 1999) were instrumental to investigate the consequences of the activation or blockage of the NOP receptor, thus suggesting possible therapeutic indications of drugs interacting with this receptor. The evidence coming from these studies suggests that the most promising indications for NOP agonists are anxiety, drug abuse, cough and pain, while for antagonists are depression and Parkinson's disease.

1.3.1. N/OFQ

N/OFQ was identified (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997), (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997) as a 17-residue long peptide with the following primary structure FGGFTGARKSARKLANQ (Figure 7). It is worth mentioning that N/OFQ was the first ligand discovered by reverse pharmacology, the strategy that allows identifying the endogenous ligands of previously orphan receptors (Chung, Pohl, Zeng, Civelli & Reinscheid, 2006). Before this molecular technique became commonly adopted, firstly ligands had been discovered and then, their receptors found by classical pharmacological approaches. Nowadays, most GPCRs are identified on the basis of their DNA sequences and thus are initially unmatched to known natural ligands and classified as orphan GPCRs. The discovery of the endogenous ligand of the NOP receptor, it has been hypothesized thinking that the chemical nature of the ligand and the consequences of receptor activation (inhibition of cyclic AMP) were similar to those of classical opioids, based on structural similarities with the known opioid receptors. The orphan receptor was cloned and transfected in CHO cells which were stimulated with forskolin to activate adenylyl cyclase and increase intracellular cAMP assuming that the activation of a $G_{i/o}$-coupled orphan receptor will inhibit the formation of cAMP. After, tissue extracts were prepared from the brains and screened. Fractions that were able to inhibit the adenylyl cyclase activity in cells transfected with the NOP receptor gene but not in wild type cells were fractionated several times and finally purified until N/OFQ was isolated and its sequence determined (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997) (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997).

Within a short time of its discovery, N/OFQ was shown to induce a variety of intracellular effects. The peptide displayed high affinity for its receptor and strongly inhibited forskolin-induced accumulation of cAMP in CHO cells expressing the NOP receptor, while showing no activity in non-transfected cells. N/OFQ was able to modulate the biochemical properties of cells, alter the electrophysiological properties of neurons and to affect their transmitter release. In bioassay, N/OFQ inhibited electrically induced contractions of the vas deferens, ileum and myenteric plexus preparations. Moreover, when tested *in vivo* by intracerebroventricular (i.c.v.) injection in mice, the peptide induced hyperalgesia in the hot plate and tail flick tests. Importantly, none of these effects were blocked by opiate antagonists emphasising the pharmacological difference between the opioid and the N/OFQ-NOP systems.

The primary structure of the neuropeptide appeared immediately interesting considering that there are striking sequence similarities between N/OFQ and opioid peptides. The Phe-Gly-Gly-Phe N-terminal is reminiscent of the canonical Try-Gly-Gly-Phe found in all mammalian opioid peptides. The preproN/OFQ precursor protein (ppN/OFQ) exhibits a similar structure as opioid peptide precursors: the biologically active sequences of ligands are located on the C-terminal domain and seven Cys residues are found conserved at the N-terminus of ppN/OFQ, ppdynorphin and ppenkephalin. Further, the gene structure of ppN/OFQ is similar to the opioid peptide genes and mapped, in humans, to the chromosomal location 8p21 (Nothacker et al., 1996) (Mollereau et al., 1996). All together, these data support the view that neuropeptide precursors of the opioid and N/OFQ systems have been originated from common ancestral genes (Sundstrom, Dreborg & Larhammar, 2010). N/OFQ shares sequence homologies with the opioid peptide dynorphin A (Figure 7). Excepted for the amino acid in position 1 (Phe in N/OFQ and Tyr in dynorphin A), the message domain (N-terminus) of the two peptides is very similar. In addiction, as dynorphin A, N/OFQ is a highly basic peptide. The address domains (C-terminus) of the two molecules are both enriched in positively charged residues (Lys and Arg), even if distributed in different positions.

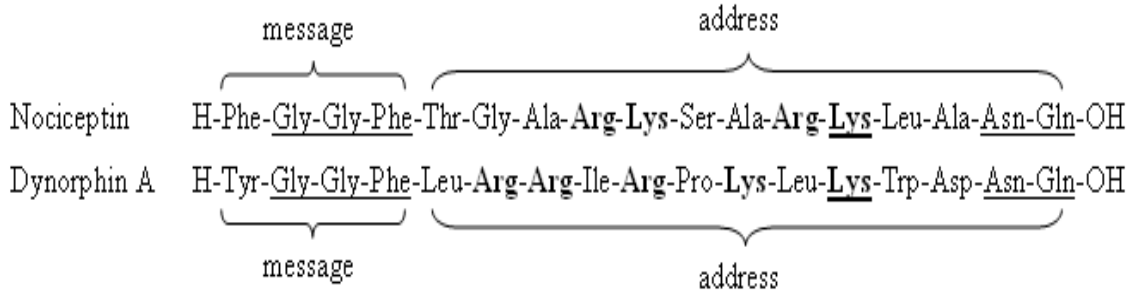


Figure 7. Structural similarities between dynorphin A and N/OFQ amino acid sequences (Calo et al., 2000).

Despite the structural similarities, these peptides are functionally quite distinct. N/OFQ has no significant affinity for any of the opioid receptors nor the opioid peptides elicit biological activity at the NOP receptor (Jenck et al., 1997). N/OFQ has evolved features that specifically exclude opioid binding, thus, although the N/OFQ and opioid systems are evolutionary related, they have evolved to be pharmacologically distinct. As all bioactive peptides, N/OFQ is synthesizes as part of the larger polypeptide precursor ppN/OFQ. Its sequence contains the typical organizational and structural features of classical opioid precursors. It starts with an amino terminal highly conserved signal peptide necessary for

its secretion. Especially, at the C-terminus, the sequence of the mature peptide is flanked by canonical Lys-Arg excision motifs, indicating that its maturation requires trypsin-like cleavages. However, these are not the only putative sites for precursor processing. In fact, in addiction to framing N/OFQ, ppN/OFQ sequence contains other cleavage sites, which generate further biologically active peptides such as a 28-residue long peptide, a 17-residue long peptide named N/OFQ II and a 19 amino acid peptide termed nocistatin (Okuda-Ashitaka et al., 1998) (Figure 8).

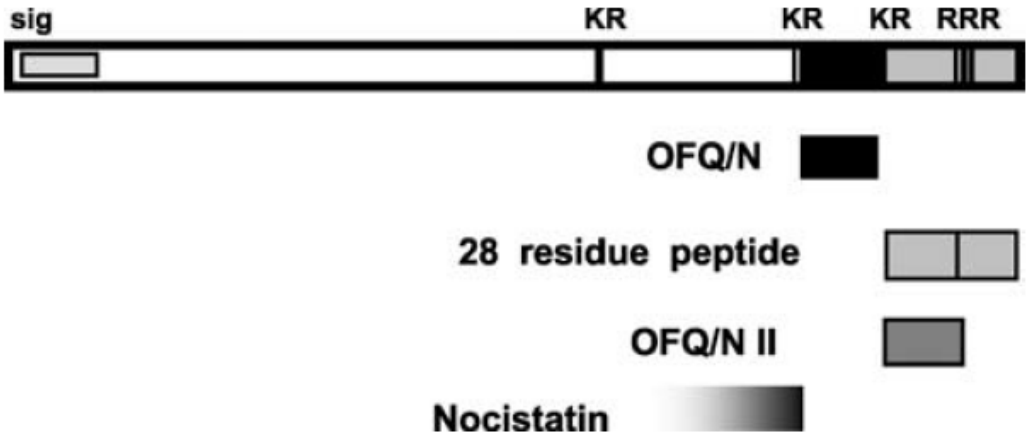


Figure 8. Synthesis of N/OFQ from the structure of the N/OFQ precursor (Civelli, 2008).

The latter peptide in most cases does not produce any effect per se but is able to counteract the action of N/OFQ (e.g. N/OFQ-induced allodynia and hyperalgesia), without binding to the NOP receptor (Okuda-Ashitaka & Ito, 2000). Similarly, N/OFQ II is unable to bind the NOP receptor, but stimulates locomotor activity in mice and exhibits effects on pain transmission (Florin, Suaudeau, Meunier & Costentin, 1997) (Rossi, Mathis & Pasternak, 1998). Regarding the biosynthesis of N/OFQ little is known, apart from the involvement of prohormone convertase 2 as suggested by knockout mice studies (Allen et al., 2001). As far as N/OFQ metabolism is concerned, metallopeptidases play a major role as endopeptidase 24.15 and aminopeptidase N, that generates [desPhe^{1}]N/OFQ a peptide lacking affinity for the NOP receptor (Montiel, Cornille, Roques & Noble, 1997). Peptidase inhibitors have been demonstrated to increase N/OFQ potency, suggesting that peptidases play a role in regulating N/OFQ signalling (Montiel, Cornille, Roques & Noble, 1997). Indeed the inhibitory effect of N/OFQ in the human vas deference can be detected in the presence of a cocktail of peptidase inhibitors, but not in their absence (Bigoni, Calo, Guerrini, Strupish, Rowbotham & Lambert, 2001). Degradation at the C-terminal domain

leads to a reduction in NOP binding affinity of N/OFQ, 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, Moisand, Mazarguil, Mollereau & Meunier, 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).

1.3.2. NOP receptor

Unlike the classical opioid receptor, DOP, KOP and MOP, which were delineated by pharmacological criteria in the 1970s and 1980s, relatively recently, several laboratories through molecular cloning and characterization of an orphan GPSRs, isolated a cDNA encoding a homologous protein with a high sequence similarity to the classical opioid receptors. It was the discovery of the actually termed NOP receptor; appertained to the family of GPCR, Class A, rhodopsin-like receptors due to the presence of several conserved amino acids and motifs in the transmembrane helices and intracellular loops, like the OP receptors. Amino acids residues in the second, third and seventh helices (TM2, TM3 and TM7) are about 70% conserved between NOP and opioid receptors, approximately 50% in the TM1, TM5 and TM6, but only 24% in the TM4. Although NOP shares sequence similarities with opioid receptors, it has a markedly distinct pharmacology, featuring activation by the endogenous peptide N/OFQ, unique selectivity for synthetic ligands with no affinity for classical opioid drugs and ligands. The NOP receptor was identified showing substantial sequence identities in several mammalian species, particularly in human and mouse (95%). The human NOP receptor protein consists of 370 amino acids and its gene (OPRl1) is located in the chromosome 20 (Peluso, LaForge, Matthes, Kreek, Kieffer & Gaveriaux-Ruff, 1998), while it has been mapped in the distal region of the mouse chromosome 2 (Nishi, Takeshima, Mori, Nakagawara & Takeuchi, 1994). In terms of intron-exon organization, the NOP receptor gene is nearly identical to that of the opioid receptors, suggesting that they have evolved from a common ancestor (Stevens, 2009) and thus, belong to the same family (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997). As far as the receptor heterogeneity is concerned, it still remains an open question for the NOP receptor. Similar to opioid receptors, the NOP receptor gene undergoes alternative splicing processes (Inoue, Kobayashi, Kozaki, Zimmer & Ueda, 1998; Peluso, LaForge, Matthes, Kreek, Kieffer & Gaveriaux-Ruff, 1998). However, the biological significance of these splice variants is unknown since pharmacological studies did not firmly established the existence of functionally distinct NOP receptor subtypes. The NOP receptor is widely expressed in the CNS. Quantitatively, prominent receptor expression was observed in the neocortex, cingulate and piriform cortex, hippocampus, anterior olfactory nucleus, cortical amygdala, claustrum, and endopiriform nucleus. Moderate contents of NOP receptor were found in the central and medial amygdala, dentate gyrus, subiculum, entorhinal cortex, dorsal and ventral pallidum,

triangular and medial septum, medial preoptic area, mammillary bodies, and parafascicular and posterior thalamic nuclei, but also at a lesser level in the olfactory system, lateral septum, basal forebrain, thalamus, and hypothalamus (Neal, Mansour, Reinscheid, Nothacker, Civelli & Watson, 1999) (Mollereau et al., 1994). The tissue localisation of the N/OFQ system has also been analysed in peripheral nervous system and other organs. NOP receptor mRNA has also been detected in peripheral ganglia and in the immune system; the intestine, the vas deferens, the skeletal muscles, the liver and spleen (Wang et al., 1994). Such discrepancy between ligand and receptor distribution have been described, may underline the difference among N/OFQ sites of synthesis and site of actions. Collectively, the broad distribution of NOP mRNA and N/OFQ binding sites, which mark the site of action of the endogenous ligand, supports an extensive role for the N/OFQ system in a multitude of CNS functions. It is worthy of mention that NOP receptors can form heterodimers with classical opioid receptors, particularly with MOP (Civelli, Saito, Wang, Nothacker & Reinscheid, 2006). It is believed that this may play a role in receptor regulation and function by altering receptor binding, signalling and trafficking. Although MOP/NOP receptor heterodymerization has yet to be demonstrated in the brain, both receptors are co-expressed in neurones of the PAG, RVM, hypothalamus, dorsal root ganglion, trigeminal ganglion, locus coeruleus and nucleus tractus solitaries (Donica, Awwad, Thakker & Standifer, 2013). The potential for MOP/NOP heterodimerization in these regions may provide an additional level of MOP and NOP receptor modulation of nociceptive processing. However, development of additional tools will be necessary to move this discussion past the speculative stage. For instance, heterodimers were shown to associate N-type calcium channels, with activation of MOP receptors triggering N-type channel internalisation, but only in the presence of NOP. Furthermore, the heterodimers attenuated the NOP inhibition of N-type channels (Evans et al., 2010). If this mechanism is operative in primary sensory neurones as well as in brain areas relevant for nociceptive transmission, it may have potentially profound effects on nociceptive processing. Following the rapid explosion of GPCR crystal structures determinations, not too long ago, also the structure of the NOP receptor was solved in its inactive in complex with the peptide mimetic antagonist compound C-24 (Figure 9), revealing atomic details of ligand-receptor recognition and selectivity. The high level of sequence conservation within the transmembrane domains among the four receptors, 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. This concept was grounded on the bases that the NOP

receptor has retained the ability, with low affinity, to bind and/or respond to opioid receptor ligands, agonist and/or antagonist (Mollereau et al., 1994) (Manabe et al., 1998). Evidence occurred from the crystal structure of the NOP receptor reveals, as expected, that the ligand-binding pocket is contained within the transmembrane helices, with residues from TM3, TM5, TM6 and TM7 interacting with the ligand in the binding pocket. Similarly, molecular modelling of the complex of the peptide agonist N/OFQ with homology models of the NOP receptor (Akuzawa, Takeda & Ishiguro, 2007; Daga & Zaveri, 2012; Topham, Mouledous, Poda, Maigret & Meunier, 1998) show that the N-terminal sequence F-G-G-F of N/OFQ binds deep in the transmembrane binding pocket, where the N-terminal amino group of N/OFQ makes an essential anchoring charge interaction with the conserved Asp130$^{3.32}$ (superscripts refer to the Ballesteros-Weinstein numbering of the TM helix residue), present in all the opioid receptors as well as in biogenic amine GPCRs. An extensive array of site-directed mutagenesis studies carried out with NOP receptors show that there are only 4-5 amino acid residues in NOP that afford the exquisite selectivity of N/OFQ for NOP receptors and prevent binding of small-molecule morphinan opioid ligands to the NOP receptor. Thus, mutation of certain NOP receptor residues to their corresponding specific conserved opioid receptor residues confers a functional opioid alkaloid binding site in NOP receptors, which binds opioid antagonists with high affinity, without adversely affecting N/OFQ binding significantly (Meng et al., 1998). This study was supported by the previous observation that mutagenesis of Q280 in TM6 in NOP to His, a TM6 residue conserved in all three opioid receptors results in an increased affinity of opioid agonists lofentanil, etorphine and dynorphin A, and antagonists diprenorphine and nor-BNI, without affecting N/OFQ binding or potency significantly (Mollereau et al., 1996). Besides, a Glu280A mutation was shown to reduce the potency of receptor activation by N/OFQ and the NOP agonist SCH 221510 by several orders of magnitude (Thompson et al., 2012). Farther, mutation of Glu286$^{6.58}$ near the extracellular end of TM6 completely nullifies activation by N/OFQ, but not affects the binding affinity for the mutated NOP receptor, suggesting a very specific role for this residue during activation after N/OFQ binding, even though it does not contribute to N/OFQ binding affinity (Mouledous, Topham, Moisand, Mollereau & Meunier, 2000). In conclusion, the new released crystal structures of NOP and classical opioid receptors suggest that, albeit the chemical moieties responsible for the opioid ligand efficacy interact similarly within the seven TM helical bundles, two different regions of the binding pocket (the upper region defined by TM5, TM6 and TM7 and the region defined by TM2 and TM7) are involved in

interactions with the chemical moieties responsible for the opioid selectivity. These findings are extremely valuable for structure-based design, optimization and screening of future agonist or antagonist selective compounds. The NOP crystal structure represents an unprecedented tool to understand the structural requirements for NOP ligand selectivity and their modes of binding as essential details providing a new structural template for the design of novel NOP ligands acting on the NOP receptor.

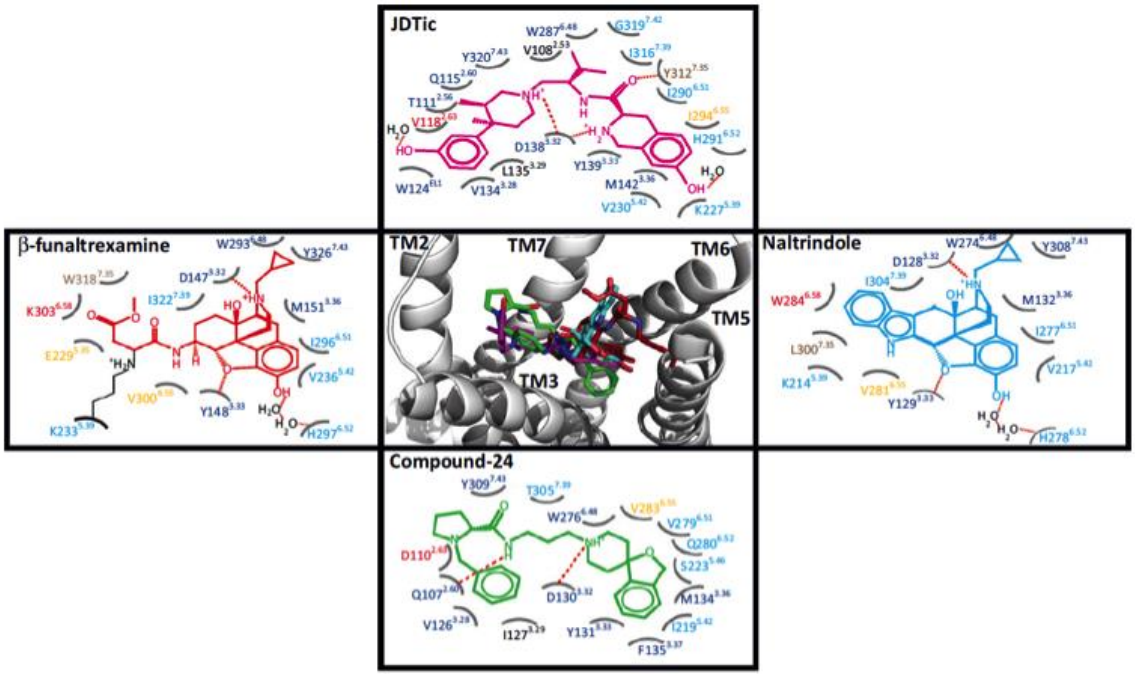


Figure 9. Overlay of the crystallized opioid ligands in a representative opioid receptor crystal structure along with schema of their interaction modes in each crystal structure. The central panel shows an overlay of β-funaltrexamine (red), naltrindole (cyan), JDTic (magenta), and compound 24 (Sullivan, Von Korff, Banta-Green, Merrill & Saunders) in the MOP receptor crystal structure, which is partially shown in a grey cartoon representation. Interaction schema for β-funaltrexamine, naltrindole, JDTic, and compound 24 in the mu opioid (MOP), delta opioid (DOP), kappa opioid (KOP), and NOP receptor crystal structures are shown in the left, right, upper, and lower panels, respectively. Identical residues in all four receptors are shown in blue. Identical resides in MOP, DOP, and KOP but unique to NOP are shown in cyan. Divergent residues in all four opioid receptors are shown in red. Divergent residues in MOP, DOP, and KOP but not NOP are shown in brown. Unique residues to MOP, DOP, or KOP are shown in orange. Image taken from (Filizola & Devi, 2013).

1.3.3. Cellular and biological actions

Cellular actions

The NOP receptor, like all GPCRs including classical opioid receptors, couples to pertussis toxin-sensitive and -insensitive inhibitory G-proteins ($G_{i/o}$) (Abdulla & Smith, 1997). The NOP receptor activation, promotes the Gα and Gβγ subunits dissociation, clearly stimulates guanine nucleotide exchange and triggers intracellular signalling events, involved in various effector pathways (Childers & Snyder, 1978). For instance, agonist stimulation of the NOP receptor inhibits cyclic adenosine monophosphate (cAMP) production via inhibition of adenylyl cyclase. NOP receptor, likewise opioid receptors, couples to Kir3 and Ca^{2+} channels via Gβγ pathways (Connor & Christie, 1998) and after its activation, it promotes the opening of Kir channels and the inhibition of N-type calcium conductance that reflected in the reduction in Ca^{2+} currents sensitive to P/Q-type, N-type, and L-type channel blockers (Connor, Vaughan, Chieng & Christie, 1996). Overall this results in reduced neuronal cell excitability causing a reduction in transmission of nerve impulses along with inhibition of neurotransmitter release (Figure 10) (Hawes, Graziano & Lambert, 2000). Although it has not been well characterized in physiologically relevant systems, it seems that the NOP receptors can promiscuously couple to other G-proteins as demonstrated in heterologous expression studies and SH-SY5Y cells (Belcheva et al., 1998). The NOP receptor couples to various intracellular kinase cascades, in particular to protein kinase A (PKA), protein kinase C (PKC) pathways, and the more recently appreciated signalling through mitogen-activated protein kinase (MAPK) cassettes. NOP receptor activity provokes activation of PKC, phospholipase A_2 and C as well as all three MAPK cassettes. NOP receptor induced extracellular-signal regulated kinase (ERK) phosphorylation, in particular increases in ERK 1/2-phosphorylation levels (Zhang et al., 2012). The phosphorylation of p38 MAPK has been demonstrated NOP-mediated via PKA and PKC pathways (Zhang, Xin, Wu, Zhang, Ma & Pei, 1999). N/OFQ promotes phosphorylation of c-Jun N-terminal Kinase (JNK) in a time and concentration-dependent manner (Alexander et al., 2013) and it happens in a PTX-sensitive and insensitive manner, the latter, mediated through G-protein coupled receptor kinase 3 (GRK3) and arrestin 3 (Zhang et al., 2012). The ability of N/OFQ to promote kinase activation and channel modulation directly influences the neurotransmitter release, immune function, and transcriptional activation.

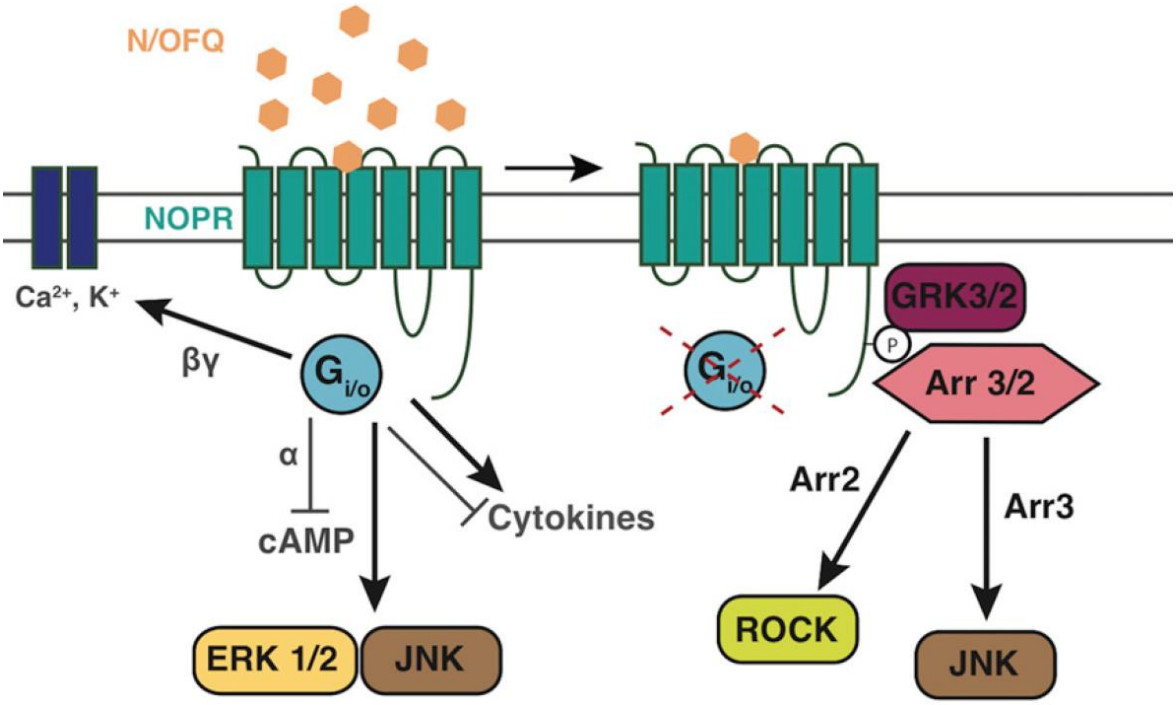


Figure 10. Summary of NOP receptor signal transduction and trafficking pathways (Lawrence Toll, 2016).

Biological actions

The role of the N/OFQ-NOP receptor system in physiological and behavioural responses immediately started after the discovery of N/OFQ as the endogenous ligand of the NOP receptor. During the period 1995-99, the majority of the studies relied principally on administration of the natural peptide N/OFQ. Soon after, the arising interest from industrial and academic researchers was crucial for the identification of selective NOP agonists and antagonists, and the generation of transgenic animal models, particularly knockouts. Together these tools have permitted us to elucidate the biological functions controlled by the N/OFQ-NOP receptor system in the organism (Figure 11).

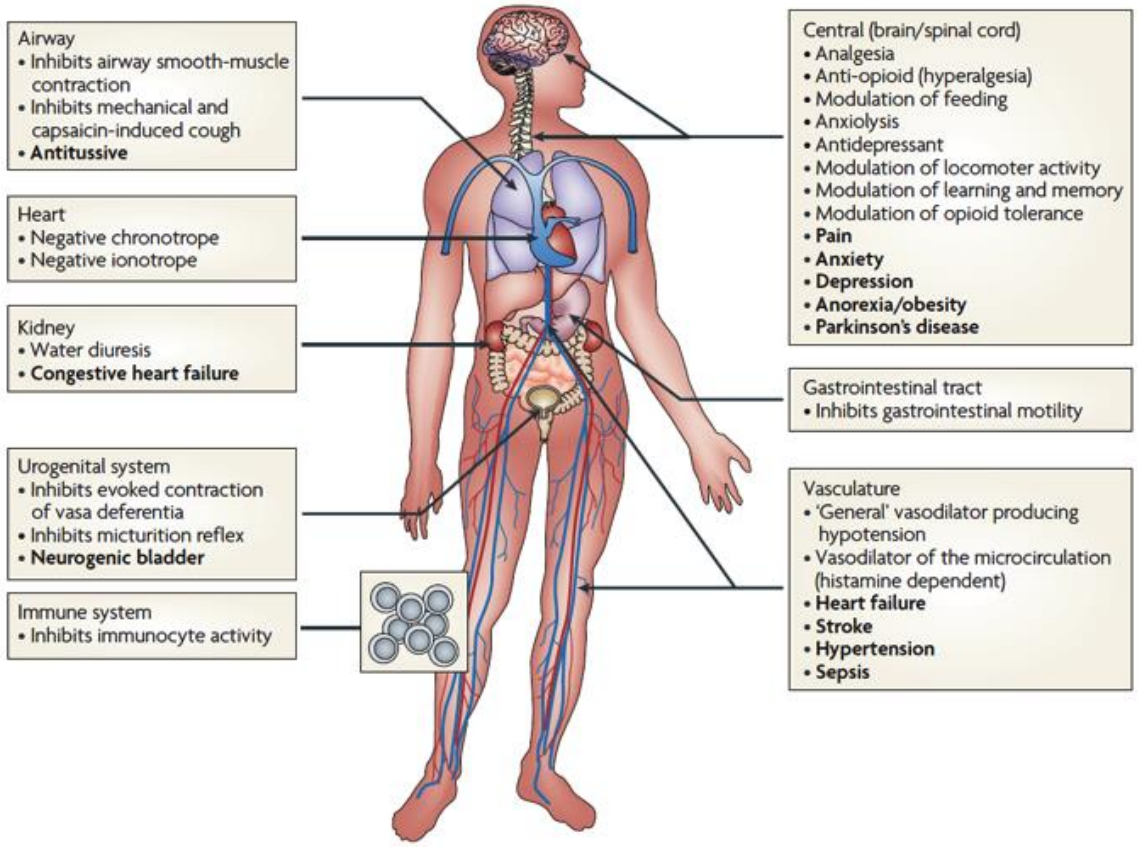


Figure 11. Pleiotropic effects of nociceptin/orphanin FQ (N/OFQ) on major organ systems. Potential clinical indications are noted in bold. Image taken from (Ibba et al., 2008).

Pain – Based on various factors, including the similarity of chemical structure, distribution and post-receptor transduction of the N/OFQ-NOP receptor with the classical opioid systems within the defined pain pathway, several studies have been pointed to understand the role of N/OFQ in the pain processing. N/OFQ has been reported to cause hyperalgesia, allodynia, analgesia, and even nociceptive behaviours (Mogil & Pasternak, 2001) (Depner, Reinscheid, Takeshima, Brune & Zeilhofer, 2003). However, although results obtained are controversial one point in common is that N/OFQ effects on nociception are strongly dependent by the range of doses and the route of administration. Since the original description by Meunier, i.c.v. administration in mice of the endogenous peptide produced an unexpected hyperalgesia (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997), decreasing latencies in hot plate and tail flick. Soon after, this effect was shown to derive from a reversal of stress-induced analgesia caused by i.c.v injection (Mogil, Grisel, Reinscheid, Civelli, Belknap & Grandy, 1996). Subsequent studies demonstrated that N/OFQ attenuate the antinociceptive action elicited by MOP, DOP and KOP receptor agonists (Mogil & Pasternak, 2001). One explanation for the attenuated opioid-induced

analgesia exerted by N/OFQ could be found in a direct inhibition of the descending antinociceptive pathway which is itself indirectly activated (or disinhibited) by chronic opioids administration (Alexander et al., 2013). At this regard, receptor knockout (Ueda, Yamaguchi, Tokuyama, Inoue, Nishi & Takeshima, 1997) as well as antagonist studies (Chung, Pohl, Zeng, Civelli & Reinscheid, 2006) supported this mechanism demonstrating that the blockage of the N/OFQ-NOP receptor signalling counteracts morphine tolerance. Also NOP receptor agonists block the stress-induced analgesia. It was highlight from the fact that, while NOP receptor agonists halts it completely, naloxone only attenuates a portion of stress-induced analgesia, demonstrating that N/OFQ blocks both an endogenous opioid as well as non-opioid components of stress-induced analgesia (Bigoni et al., 2000). On the contrary, administration of N/OFQ into the spinal cord generated opposite results. At very low doses when administered i.t. in rodents, N/OFQ elicits pronociceptive effects, while at higher doses promotes a direct antinociception and potentiates morphine action (Homberg, Mul, de Wit & Cuppen, 2009) (Watanabe, Yano, Horie & Yamamoto, 1997). These site-specific opposite effects of N/OFQ on pain transmission could be explain by the distinct localisation of OP and NOP receptors in pain relevant neuronal networks. Some evidence from literature supports this interpretation. In the nucleus raphe magnus of the RVM, that is the major neuroanatomical site involved in the supraspinal anti-opioid effect of N/OFQ on pain processing, there are expressed two types of cells: the ON and OFF cells (Figure 12). In this brain region, MOP receptors are found on primary ON cells. Conversely, NOP receptors are found on both ON and OFF cells. GABAergic ON cells fire immediately before a nociceptive reaction and inhibit the action of OFF cells and in turn, ON cells are inhibited by opioids acting at MOP receptors. OFF cells project back to the spinal dorsal horn, (also called as the descending inhibitory control circuitry), to decrease the ascending nociceptive information. When morphine inhibits the ON cell this disinhibits the OFF cell, leading to an antinociceptive effect through the block of the descending pain signal. N/OFQ inhibits both the ON and OFF cells. Thus, via a direct inhibition of the OFF cell, N/OFQ produces an increase in nociceptive traffic, counteracting the disinhibitory effect of MOP agonists. Clearly, this inhibition of the OFF cell would reverse any actions of opioids at the ON cell; generating an anti-opioid action and providing a cellular basis for the reversal of stress-induced analgesia mediated by N/OFQ (Figure 12) (Depner, Reinscheid, Takeshima, Brune & Zeilhofer, 2003). This mechanism is in line with the fact that the NOP receptor activation blocks MOP opiate-mediated antinociceptive activity in naïve animals but induces apparent analgesic activity in morphine tolerant animals

(Alexander et al., 2013). These experiments demonstrated how the ultimate result of NOP receptor activation strongly depends on the activation state (resting versus sensitized) of pain controlling, while activation of MOP receptors has invariant antinociceptive activity.

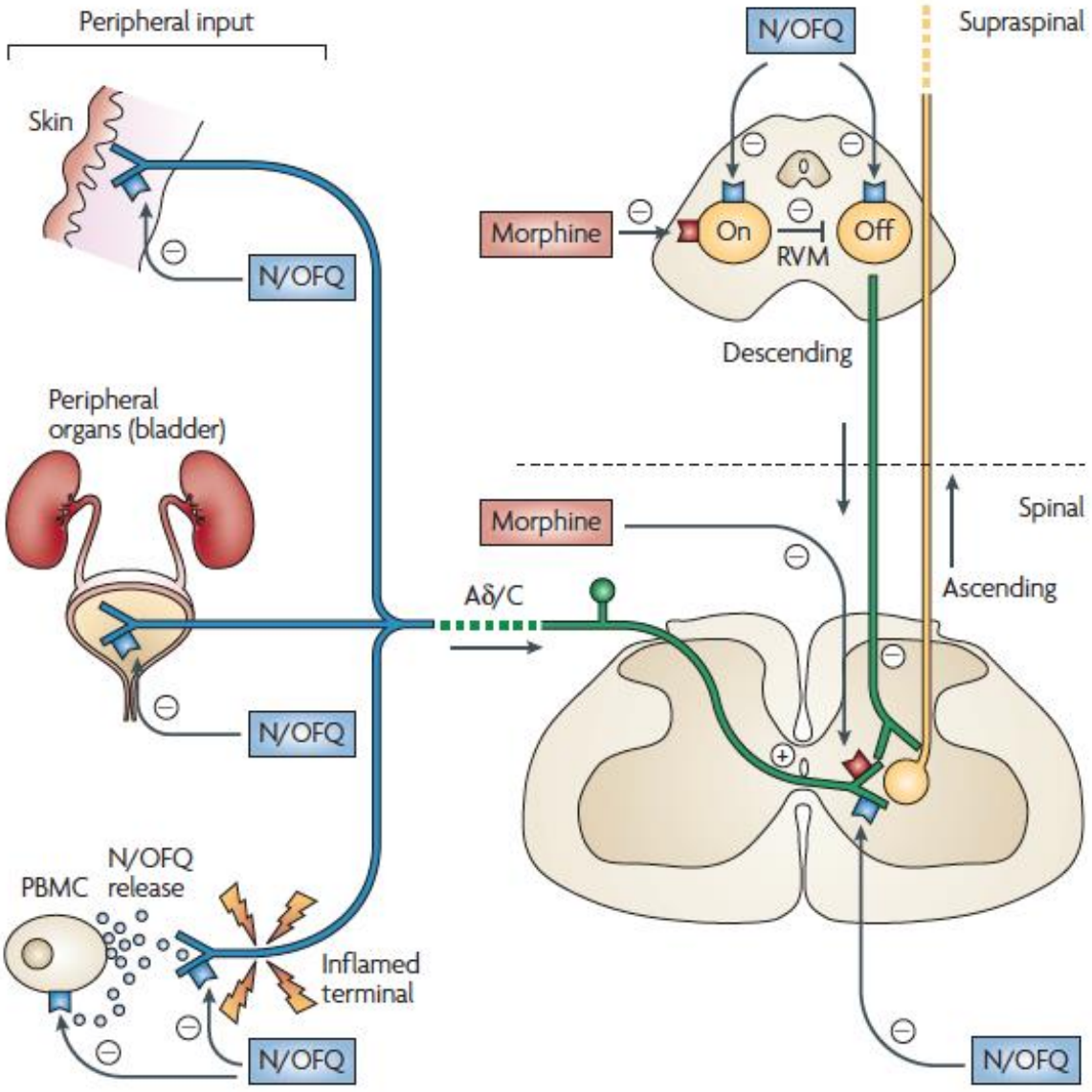


Figure 12. Schematic description of the interrelationship between the anatomical site(s) underlying the actions of N/OFQ on pain (Ibba et al., 2008).

The antinociceptive effect mediated by intratelcal administration of N/OFQ at nanomolar doses, was corroborated in other studies assessed in a wide variety of animal models, including phasic (mechanical or thermal) as well as of tonic (inflammatory or neuropathic) pain. Further, the spinal antinociceptive action of N/OFQ is consistent with the well-documented ability of the peptide to block excitatory (glutamate) transmission in the dorsal horn of the spinal cord (Depner, Reinscheid, Takeshima, Brune & Zeilhofer, 2003). The

spinal antinociceptive action of N/OFQ was demonstrated to be a consequence of the N/OFQ-NOP receptor signalling activation in the spinal cord by a prolonged, but not acute nociceptive stimuli. As well as the chronic neuropathic or inflammatory pain is concerned, N/OFQ has anti-allodynic and anti-hyperalgesic activity after i.t. administration in model of acute pain (Hao, Xu, Wiesenfeld-Hallin & Xu, 1998). This finding holds true in experiments conducted with NOP(-/-) and ppN/OFQ(-/-) mice (Depner, Reinscheid, Takeshima, Brune & Zeilhofer, 2003), receptor antagonists (Inoue et al., 2003) and rat NOP(-/-) (Calo et al., 2011), which display increased inflammatory hyperalgesia in the formalin assay, but not in an acute pain assay. With regard to NOP agonists, UFP-112 was antinociceptive in the mouse tail flick assay following intrathecal administration (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007), as well as intrathecal administrations of Ro 64-6198 in rats, that produced anti-allodynic effects against thermal and mechanical stimuli (Obara, Przewlocki & Przewlocka, 2005). Together, peptidic and non-peptidic NOP agonists were able to block pain behaviours in rodents in response to acute noxious stimulus or chronic pain conditions. These studies suggest that the NOP system may be recruited differently in different pain modalities, also considering that the levels of NOP receptors and N/OFQ change in chronic or inflammatory pain states maybe due to a sensitization of the NOP system (Sun, Wang, Zhao, Chang & Han, 2001) (Altier et al., 2006). Several groups have reviewed the i.c.v. application of NOP antagonists based on the concept that if N/OFQ induced pain, antagonist might show antinociceptive behaviour. Results of such studies are complicated to interpret and there is a reasonably consistent view that peptide but not small non-peptide antagonists have antinociceptive effects when administered i.c.v. (Di Giannuario et al., 2001) (Bigoni et al., 2002) (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007). Nevertheless, this observation is not fully consistent in the literature. At this regard, the NOP receptor antagonist, JTC 801, appears to have naloxone-irreversible antinociceptive activity in both acute and chronic pain models when administered systemically (Suyama, Kawamoto, Gaus & Yuge, 2003) (Tamai, Sawamura, Takeda, Orii & Hanaoka, 2005), while the majority of selective antagonists do not have any effect on latencies in tail withdrawal assays in naïve animals. Regarding non-human primates studies, spinal administration of N/OFQ or synthetic NOP ligands did not evoke any effect at low doses, on the contrary at high doses induced a significant antinociceptive effect from noxious thermal stimulus with a magnitude of effects similar to that of clinically available MOP agonists such as morphine and fentanyl. The antinociception induced by i.t. N/OFQ was sensible only to the action of the selective

antagonist J-113397 and not to the action of the classic opioid antagonist naltrexone, indicated a NOP receptor-mediated antinociception (Hu, Calo, Guerrini & Ko, 2010). Worthy to mention is that NOP mediated analgesia was not characterized by scratching at antinociceptive doses unlike the MOP agonists, and when given with morphine, N/OFQ produced a synergistic antinociceptive effect suggesting that the NOP receptor could be a viable target for spinal analgesia devoid of itch common side effects. (Altier et al., 2006) (Camarda et al., 2009). Furthermore, i.t injection of UFP-112 in monkeys provoked antinociceptive effects in a dose dependent manner in the acute thermal nociception and capsaicin-induced allodynia assays (Brighton, Rana, Challiss, Konje & Willets, 2011). These effects were fully reversed by J-113397 and not by the classical opioid antagonist naltrexone demonstrating the involvement of the NOP receptor in this response. In terms of the magnitude of effectiveness and duration of action under the same experimental conditions, UFP-112 was more potent than intratecal morphine, while unlike morphine; UFP-112 did not produce scratching responses in monkeys. In addiction, i.t. inactive doses of UFP-112 and morphine produced significant antinocicption effects where given in combination without increasing scratching responses. Together these preclinical studies indicate the NOP agonists as potential spinal analgesics devoid of itch and promising candidate for future clinical studies. On the contrary, not many studies have investigated the antinociceptive effects of systemically administration of NOP agonists in rodents and non-human primates and results obtained are not as consistent as their spinal actions. Further investigations are needed. However, similarly to the spinal level, in the periphery N/OFQ provoked both pro and antinociceptive effects. For example, intradermal administration of N/OFQ stimulates the flexor reflex in mice at very low doses, involving the stimulation of the release of substance P from peripheral nerve endings. In contrast, at higher doses N/OFQ prevented the facilitator effect of substance P (Inoue, Kobayashi, Kozaki, Zimmer & Ueda, 1998) In addition, several groups have divulged that N/OFQ is able to stop the neuropeptide release from peripheral sensory neuron terminals in different organs including the airways (Shah, Page & Spina, 1998), heart (Giuliani & Maggi, 1997), and renal pelvis (Bigoni et al., 1999). In non-human primates the co-administration of N/OFQ with capsaicin into the tail dose dependently inhibited thermal nociception suggesting that activation of peripheral NOP receptors produces antinociceptive effects (Bregola et al., 2002). In addition, also other NOP agonists systemically administrated were investigated in non-human primates in three different pain models. In particular, compound Ro 64-6198 produced significant antinociception mediated by NOP receptors

against acute thermal nociception, capsaicin-induced thermal allodynia, and carrageenan-induced thermal hyperalgesia (Camarda et al., 2009). In the last two assays, effects and potency of the compounds were demonstrated being comparable to that of the MOP agonist alfentanil at antinociceptive doses. Intramuscolar injection of Ro 64-6198 did not cause scratching, respiratory depression and reinforcing effects, unlike alfentanil, in a wide dose range. Taken together, these studies provide functional evidence that NOP agonists may have a therapeutic value as systemic analgesics without causing scratching, respiratory depression and abuse liability. Antinociceptive effects of NOP agonists seem to vary between rodents and monkeys. Anatomical studies reveal that there are differences between rodents and primates in terms of the distribution of N/OFQ and its receptors (Bridge, Wainwright, Reilly & Oliver, 2003). For instance, degree of physiological outcome from activating supraspinal, spinal and peripheral NOP receptors together following systemic administration of NOP agonists may vary across species. Collectively these results have propelled scientists thinking which effects could be involved from the action of a multi-targeted ligand, based on a rational approach to modulate complementary pharmacology of two targets.

Anxiety and depression – The current market has a wide range of drugs for the treatment of anxiety and depression, but often they are characterized by poor efficacy, tendency to tolerability and compliance problems. Thus, one of the most highly investigated actions of N/OFQ is its ability to counteract stress related behaviours and promote anxiolytic like effects. Pivotal studies demonstrated that N/OFQ acts as anxiolytic in several benzodiazepine-sensitive behavioural tests (Jenck et al., 1997) and the systemic administration of the selective NOP agonist Ro 64-6198 generates similar results (Jenck et al., 2000), importantly without showing tolerance liability (Dautzenberg et al., 2001). Furthermore anxiolytic-like effects were reported for several non-peptide NOP agonists (Ro 65-6570 (Wichmann, Adam, Rover, Cesura, Dautzenberg & Jenck, 1999) and SCH 221510 (Varty et al., 2008)). These results were later confirmed in different assays, species and laboratories, including knockout studies. Indeed, ppN/OFQ(-/-) mice show a greater tendency towards anxiety-like behaviour when exposed to a novel and threatening environment (Koster et al., 1999), ppN/OFQ(-/-) mice display a milder anxiogenic phenotype in the same related assay, when compared with wild type animals (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007). The mechanisms by which N/OFQ exerts its anxiolytics effects are not fully understood. It has been suggested the involvement of

$GABA_A$ receptor signalling, however CRFergic and serotonergic pathways might be implicated (Gavioli & Calo, 2006). The availability of NOP selective antagonists accompanied with NOP(-/-) animals models made possible to investigate the consequences of blocking endogenous N/OFQergic signalling and to foresee the possible therapeutic indications of drugs acting as NOP blockers. As far as the use of NOP antagonists is concerned, the lack of their effect on model of anxiety was contrasted by antidepressant action of J-113397 and Nphe[1] in the forced swimming test in mice (Redrobe, Calo, Regoli & Quirion, 2002). Subsequent studies involving the use of different NOP antagonists (e.g. UFP-101 and SB-612111) corroborate the initial evidence. In addiction, NOP(-/-) mice and rats display an antidepressant like phenotype (Gavioli et al., 2003) (Gavioli et al., 2004) (Calo et al., 2011). Furthermore, very recently was published an interesting study aimed to valuate the novel, potent, and selective antagonist, LY2940094, based on the hypothesis that blockade of NOP receptors would induce antidepressant effects. At this purpose, targeting the NOP receptors with LY2940094, antidepressant-like effects have been observed in rodent models. More importantly, LY2940094 showed a robust antidepressant efficacy in the treatment of patients with major depressive disorder (MDD), providing the first human evidence that the blockade of NOP receptor signalling represents a promising strategy for the treatment of MDD (Kieffer & Gaveriaux-Ruff, 2002). Taken together these data indicate that NOP agonists are anxiolytic and that antagonists are antidepressant. Anyway, larger studies are required to confirm this finding and the clinical trial of antagonist are eagerly awaiting. The precise neuroanatomical sites of the anxiolytic/antidepressant behaviours are largely unknown, however it has been proposed that NOP antagonists may achieve a similar endpoint to that of classical antidepressants, by increasing cortical synaptic monoamines concentrations by blocking both pre- and post-synaptic inhibitory actions of endogenous N/OFQ on monoaminergic neurons (Gavioli & Calo, 2006).

Drug abuse – Drug abuse has an incredible socioeconomic impact and is relatively difficult to treat. The conditioning place preference (CPP) test, that pairs administration of the drug with a particular set of environmental clues, is commonly adopted to elucidate the rewarding properties of drugs of abuse. In this assay, N/OFQ reduced CPP to alcohol, amphetamine, cocaine and morphine indicating that it was depressing reword to these stimuli (Khroyan et al., 2011). Conversely, N/OFQ alone was inactive demonstrating that it lacks intrinsic motivational properties (Devine, Reinscheid, Monsma, Civelli & Akil,

1996). Of particular relevance to the control of rewarding functions exerted by the endogenous N/OFQ-NOP system was emphasized since the pharmacological blockage or the genetic knockout of the NOP receptor potentiates the rewarding properties of morphine in rats Sakoori, 2008 #123} and of methamphetamine and ethanol in NOP(-/-) mice (Rutten, De Vry, Bruckmann & Tzschentke, 2011). A substantial amount of current information on addiction and reward comes from studies using alcohol. In this respect the Marchigian Sardinian alcohol-preferring (msP) rat has provided some interesting insights. For example, i.c.v. administration of the peptides UFP-102 and UFP-112 reduced alcohol consumption in this assay. Of particular relevance, it has been observed that N/OFQ blocks reinstatement of alcohol-seeking behaviour in alcohol-preferring rats (Ciccocioppo, Cippitelli, Economidou, Fedeli & Massi, 2004), and Ro 64-6198 blocks morphine place preference in mice (Shoblock, Wichmann & Maidment, 2005). One mechanism whereby NOP agonists attenuate reward elicited by drugs of abuse is by directly inhibiting dopaminergic mesocorticolimbic neurons, which express the NOP receptor (Murphy, Ly & Maidment, 1996).

Learning and memory – The first observation that the N/OFQ system may be involved in memory was reported by showing that the performance of NOP(-/-) mice in assay such as the water maze, passive avoidance and fear conditioning, was better than that of wild-type mice. These findings have been corroborated by studies that demonstrated that supraspinal administrations of N/OFQ impair learning and memory performance in mice and rats in water maze (Sandin, Ogren & Terenius, 2004), fear conditioning (Mamiya et al., 2003), Y-maze and passive avoidance tests (Mamiya, Noda, Nishi, Takeshima & Nabeshima, 1999). However systematic studies on the possible cognitive enhancing properties of selective NOP antagonists have not yet been performed.

Food intake – N/OFQ system has also been shown to be involved in the modulation of food intake. I.c.v. injections of N/OFQ increase food consumption in satiated and food-deprived rats (Olszewski & Levine, 2004). Then, N/OFQ inhibits stress-induced anorexia without eliciting hyperphagia, maybe acting in this case as a functional antagonist of the corticotrophin-releasing factor system in the bed nucleus of the stria terminalis (Ciccocioppo, Cippitelli, Economidou, Fedeli & Massi, 2004). However, when administered i.c.v., the peptidergic antagonist [Nphe^{1}]N/OFQ(1-13)-NH_2 did not affect food consumption per se in satiated rats, on the contrary, it reduced that in food-deprived

rats (Polidori, Calo, Ciccocioppo, Guerrini, Regoli & Massi, 2000). Also the peptide antagonist UFP-101 did not affect free feeding in the rat (Ciccocioppo, Economidou, Rimondini, Sommer, Massi & Heilig, 2007). A possible interpretation of these evidence could be that the endogenous N/OFQ plays a role in orexigenic tone in response to food deprivation but not in normal feeding. On the contrary, it has been shown that the antagonist SB-612111 did not modify food intake when tested in food deprived mice (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007), leaving to think that, unlike rats, in mice the N/OFQ-NOP receptor system does not play a major role in controlling food intake induced by food deprivation. All together, it indicates that the hyperphagic and the anti-anorectic effect of N/OFQ are mediated by separate brain structures and synthetic N/OFQ agonists might have therapeutic potential as orexigenic drugs (Ciccocioppo, Economidou, Rimondini, Sommer, Massi & Heilig, 2007) (Ciccocioppo, Cippitelli, Economidou, Fedeli & Massi, 2004). Worthy to be mentioned is a recent work, examining the effects of the NOP antagonist LY2940094 on feeding in a variety of behavioural models. LY2940094 inhibited fasting-induced feeding in wild, but not in NOP(-/-) mice, which instead exhibited a baseline phenotype of reduced fasting-induced feeding, relative to wild-type littermate controls. LY2940094 inhibited the overconsumption of a palatable high-energy diet in lean rats, reducing caloric intake to control chow levels. Furthermore, in dietary-induced obese rats, LY2940094 inhibited feeding and body weight regain induced by a 30% daily caloric restriction. Among the several aspects examined, LY2940094 in dietary-induced obese mice was also able to decreased 24-hour intake of a high-energy diet made freely available. In addiction, the hypophagic effect of LY2940094 is NOP receptor dependent and not due to off-target or aversive effects. Collectively, this research demonstrated that the systemically administration of the NOP receptor antagonist LY2940094 can reduce feeding behaviour and body weight in rodents, hypothesizing a therapeutic potential of NOP antagonists in the treatment of appetitive behaviour disorders (Statnick et al., 2016).

Locomotor activity – Since its first identification, N/OFQ reported to significantly induce a dose-dependent decrease in spontaneous locomotor activity when administered supraspinally (Butour, Moisand, Mazarguil, Mollereau & Meunier, 1997). Afterwards, different laboratories confirmed this effect in mice and rats and demonstrated the exclusive involvement of the NOP receptor with receptor antagonist and knockout studies (Nishi et al., 1997). Interestingly, either in the drag and rota rod tests the rat performance was

facilitated in a dose-dependent manner by systemic administration of J-113397 or by intranigral injection of UFP-101. In the same assay NOP(-/-) outperformed NOP(+/+) mice (Kostenis et al., 2005). This evidence was later corroborated by superimposable findings obtained in NOP receptor knockout rats (Calo et al., 2011), confirming that endogenous N/OFQ may indeed exert an inhibitory influence over motor activity that becomes relevant during exercise rather than at rest. According with these observations, was also reported that systemic administration of J-113397 and its analogues Trap-101 and GF-4 increased motor performance in normal rats and in NOP(+/+) mice but were ineffective in NOP(-/-) animals (Marti, Trapella & Morari, 2008) (Volta, Mabrouk, Bido, Marti & Morari, 2010). Given that, it is likely that the NOP receptor blockade may represent a new strategy for the control of hypokinetic disorders. A series of studies demonstrated that NOP receptor antagonists attenuated motor deficits in rodent and non-human primate models of Parkinson disease including 6-hydroxydopamine (Kostenis et al., 2005), haloperidol (Kostenis et al., 2005) and reserpine (Volta, Mabrouk, Bido, Marti & Morari, 2010). In addiction, the finding that N/OFQ levels were found higher in the cerebrospinal fluid of parkinsonian patients than the controls (Calo et al., 2002) strengthens the proposal that NOP receptor antagonists are worthy of development as innovative drugs for Parkinson's disease.

Cardiovascular, renal and gastrointestinal tract systems – By acting on both central and peripheral nervous systems, N/OFQ modulates the functioning of several organs and systems. In anaesthetized and conscious laboratory animals N/OFQ produces hypotension and bradicardia (Champion & Kadowitz, 1997) at both central and peripheral sites. This occurs following intravenous and i.c.v. administration and is absent in NOP(-/-) mice (Burmeister, Ansonoff, Pintar & Kapusta, 2008). Moreover, intravenous infusion of N/OFQ stimulates diuresis and in particular acquaresis (Kapusta, Sezen, Chang, Lippton & Kenigs, 1997), while decreases renal sympathetic nerve activity (Calo et al., 2005). Intravenous N/OFQ produces vasodilation, without involving nitric oxide in the dilator response (Champion, Bivalacqua, Zadina, Kastin, Hyman & Kadowitz, 2002). N/OFQ controls several gastrointestinal functions under physiological as well as pathological conditions. It inhibits contractility of the gastrointestinal tract in a wide range of species and at most sites along gastrointestinal tract (Osinski & Brown, 2000). In addition, N/OFQ prevents gastric damage induced by gastric ethanol (Morini, De Caro, Guerrini, Massi & Polidori, 2005) and cold-restrain stress (Grandi, Solenghi, Guerrini, Polidori, Massi &

Morini, 2007). Furthermore, NOP seems to have a role in the inflammatory bowel disease (Kato et al., 2005).

Inflammation and sepsis – The NOP system is present in immune cells and N/OFQ modifies immunocyte functions. N/OFQ affects tissue perfusion, increases capillary leakage and inflammatory markers, and leads to immune cell chemotaxis (Gavioli & Romao, 2011). Considering that NOP activation produces bradycardia and hypotension, it was hypothesized that the block of the NOP receptor may elicit beneficial effects in some inflammatory diseases. In fact, it was published that NOP(-/-) mice are less vulnerable than wild type animals in the dextran sodium sulphate murine model of colitis. In addition, the expression level of mucosal addressin cell adhesion molecule-1 and infiltrating cells were significantly decreased in NOP(-/-) compared to NOP(+/+) mice (Kato et al., 2005). Recently, by receptor antagonist studies was confirmed the proposal that N/OFQ-NOP receptor signalling deteriorates colonic inflammation. For instance, SB-612111 significantly ameliorated the clinical disease course of mice with dextran sodium sulphate-induced colitis (Alt et al., 2012). Another condition in which the block of the NOP receptor may provide beneficial effects is sepsis. In fact, systemic N/OFQ administration increased mortality in the cecal ligation and puncture model of sepsis in rats and more importantly treatment with the NOP selective antagonist UFP-101 decreased mortality in the same model (Carvalho et al., 2008). There is also clinical evidence for increased plasma N/OFQ concentrations in septic patients; in fact, plasma N/OFQ levels were reported higher in patients with sepsis who died compared with those who survived (Varty et al., 2008). Although more preclinical and clinical studies are needed, the available evidence suggests that NOP antagonists may elicit beneficial effects in inflammatory bowel disease and sepsis.

Airway – NOP agonists are under clinical development as antitussive agents (McLeod, Tulshian & Sadeh, 2011). N/OFQ inhibits cough in guinea pigs, in cats and inhibits *ex vivo* airway contractility in various species including humans (Faisy et al., 2004). The non-peptide agonist Ro 64-6198 can mimic the antitussive effect in a J-113397 manner (McLeod et al., 2004). Similar results were obtained by testing in various preclinical models of cough a large series of non-peptide NOP agonists, among which compound SCH 486757 showed the best favourable antitussive profile (McLeod et al., 2010). In a guinea pig capsaicin cough model, SCH 486757 displayed similar antitussive efficacy as codeine,

hydrocodone, dextromethorphan and baclofen, without producing tolerance after 5 days of treatment, neither displaying abuse liability. SCH 486757 effects were reversed by the NOP receptor antagonist J-113397 but not by naltrexone. However, despite its favourable antitussive profile in preclinical animal models, in patients the effectiveness of SCH 486757 was limited by its tendency to produce somnolence (Woodcock, McLeod, Sadeh & Smith, 2010). Further studies are therefore needed before drawing firm conclusions on the therapeutic value of NOP agonists as innovative antitussive drugs.

The urogenital system – The Menarini researchers were the first to demonstrate that intravenous administration of N/OFQ in rats inhibits the micturition reflex, but not the local bladder contraction, induced by topical capsaicin. This effect was no longer evident in capsaicin-pretreated rats indicating the involvement of capsaicin-sensitive nerve fibres innervating the urinary bladder. A series of elegant rodent studies indicated that NOP receptors are present at several sites of integration of the micturition reflex and that their activation produces inhibitory effects (Lecci, Giuliani, Meini & Maggi, 2000). Based on these findings, Lazzeri and co-workers performed the first clinical investigation with N/OFQ by testing the urodynamic effects of intravesical application of the peptide in normal subjects and in patients with overactive bladder and demonstrating a robust inhibitory effect of the peptide (Lazzeri et al., 2001). A follow up study demonstrated the clinical efficacy of N/OFQ during 10 days of intravescical treatment supporting the use of NOP receptor agonists as innovative drugs for the control of incontinence due to detrusor over activity (Lazzeri et al., 2006).

It is clear that N/OFQ exhibit a broad pharmacological spectrum of actions and as such selective NOP receptor ligands have therapeutic potential for clinical development. Indeed, many efforts have been spent in the last year to generate highly potent and selective ligands encompassing pure antagonists and partial or full agonist activities, which allowed the scientific community to collect a large body of evidence regarding the biological functions controlled by the N/OFQ-NOP receptor system. As a matter of fact, until now NOP agonists were useful to improve our knowledge on the effects mediated by the selective activation of NOP receptor in the periphery, including in the respiratory, gastrointestinal, genitourinary, cardiovascular, and renal systems as well as in the central nervous system for the control of the response to stress and of anxiety levels, pain transmission, regulation of food intake, control of locomotor and memory functions, and drug addiction. In particular, NOP agonist could be promising drug in patients suffering

from urinary incontinence due to overactive bladder, where the intravesical instillation of N/OFQ produced robust beneficial effects both in acute and chronic (10 days) studies (Lazzeri et al., 2001; Lazzeri et al., 2006). Whatever, long term clinical studies are yet necessary to validate NOP agonists as drugs for patients affected from this conditions. Another indication for which peptide NOP agonists can be assessed as drugs is spinal analgesia, based on the fact that today there are only two drugs formally approved for this indication: morphine and the N type calcium channel blocker ziconotide (Williams, Day & Heavner, 2008). As far as renal functions are concerned, full and partial NOP agonist are able to elicit similar diuretic, in particular aquaretic, effects (Calo et al., 2005). However i.v. administration of NOP partial agonists produce per se negligible effects on cardiovascular functions, acting as antagonists, while full agonists consistently evoke bradycardia and hypotension (Calo et al., 2005). Therefore, the use of NOP receptor partial agonists (but not full agonists) may be useful for conditions such as congestive heart failure for which aquaresis is beneficial while cardiovascular depressor effects are unwanted.

Similar considerations can be made for NOP selective antagonists, which contributed to increase our knowledge on the role of the endogenous N/OFQ-NOP receptor system in controlling several biological functions, and to demonstrate the involvement of the NOP receptor in several *in vitro* and *in vivo* actions of N/OFQ. In addition, it has been reported that NOP antagonists are able not only to block N/OFQ actions but also to produce per se opposite effects on some biological functions such as locomotor performance on the rota rod (Kostenis et al., 2005), stress induced analgesia (Bigoni et al., 2000), and mortality in an animal model of sepsis (Carvalho et al., 2008). Furthermore, chemically different NOP antagonists produced antidepressant like effects in the forced swimming, tail suspension and chronic mild stress assays (Gavioli & Calo, 2013). This has been recently confirmed in the clinic demonstrating antidepressive effectiveness of the NOP antagonist LY2940094 in patients (Post et al., 2015). Finally, a large series of preclinical studies reviewed in (Tekes et al., 2013) corroborate the proposal that NOP antagonists are worthy of development as innovative drugs for the treatment of Parkinson disease. Limited information also suggests that NOP antagonists may exert beneficial effects in some inflammatory conditions such as ulcerative colitis (Kato et al., 2005) (Alt et al., 2012) and sepsis (Carvalho et al., 2008).

1.3.4. NOP receptor ligands

Peptide ligands – The paucity of selective ligands and especially of antagonists, have hampered the investigation of the N/OFQ-NOP system from an experimental point of view. Fortunately, the increasingly interest of the N/OFQ-NOP receptor system as target of innovative drugs prompted several groups of researchers to develop structure-activity relationship (SAR) programmes focused at the identification of NOP ligands. The first generation SAR studies on N/OFQ allowed to identify several peptide NOP ligands useful for pharmacological as well as pathophysiological investigations, including the truncated and amidated N/OFQ(1-13)-NH_2, the smallest fragment able to mimic the N/OFQ actions maintaining the same potency and efficacy of the natural peptide (Calo et al., 1996); the partial agonist [$Phe^1\psi(CH_2\text{-}NH)Gly^2$]N/OFQ(1-13)-$NH_2$ ([F/G]N/OFQ(1-13)-NH_2) (Guerrini et al., 1998); the potent full agonist [(pF)Phe^4]N/OFQ(1-13)-NH_2 (Bigoni et al., 2002) (Bigoni et al., 2002) and the low potency pure antagonist [$Nphe^1$]N/OFQ(1-13)-NH_2 (Alexander et al., 2013). In the early 00's other chemical modifications were described leading to the generation of interesting peptide NOP ligands. For example, the replacement of Leu-Ala with an extra couple of Arg-Lys residues in position 14-15 was convenient for generating [Arg^{14},Lys^{15}]N/OFQ, a highly potent and selective NOP ligand with long lasting effects *in vivo* (Okada et al., 2000). The substitution of Aib in position 7 and/or 11 of N/OFQ generated potent NOP agonists (Zhang, Miller, Valenzano & Kyle, 2002). Subsequent studies investigated the pharmacological profile of second-generation peptide NOP ligands obtained by combining in the same molecule the above-mentioned chemical modifications. With this strategy the following highly potent and NOP selective peptide ligands were obtained: the full agonist [(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (UFP-112) (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007), the partial agonist [$Phe^1\psi(CH_2$-NH)Gly^2(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (UFP-113) (Arduin et al., 2007) and the pure antagonists [$Nphe^1Arg^{14}Lys^{15}$]N/OFQ-NH_2 (UFP-101) (Bigoni et al., 2002). These NOP ligands have been used worldwide to perform pharmacological as well as pathophysiological studies. The *in vitro* and *in vivo* pharmacological profile of UFP-112 and UFP-101 has been reviewed in (Calo et al., 2011) and (Calo et al., 2005), respectively. UFP-112 is a full agonist at NOP receptor and more potent than N/OFQ in isolated tissues. In tissues taken from NOP(-/-) mice UFP-112 is inactive. Further, exhibiting plasma half life ($t_{1/2}$) longer than the natural peptide, UFP-112 is considerably more stable (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007). More in general detailed information

about NOP receptor peptide ligands can be found in (Calo G, 2013). Not so far, the laboratory of Guerrini validated a novel and facile chemical strategy, named peptide-welding technology (PWT), for the synthesis of tetra branched derivatives of N/OFQ characterized by high yield and purity of the desired final product (Bigoni, Calo, Guerrini, Strupish, Rowbotham & Lambert, 2001). PWT derivatives were pharmacologically characterized as NOP ligands *in vitro*, behaving in several assays as high affinity, potent full agonists at human NOP recombinant receptor on cells as well as in animal tissues. Moreover, in *in vivo* experiments, N/OFQ PWT derivatives mimicked the inhibitory effects exerted by the natural peptide on locomotor activity, with higher potency and longer lasting (24 h) action. The effects of PWT-N/OFQ were no longer evident in NOP(-/-) mice (Guerrini et al., 2014). When assessed both in nociceptive and neuropathic pain models, after i.t. administrations in mice and non human primated PWT-N/OFQ produced robust and long lasting antinociceptive effects (Rizzi et al., 2015). Altogether these findings demonstrated that the PWT chemical strategy can be successfully applied to the N/OFQ peptide sequence to generate tetra branched compounds distinguished by a pharmacological profile superimposable to that of the endogenous peptide in terms of NOP selective full agonism associated to high potency and prolonged *in vivo* action.

N/OFQ unrelated peptide ligands – In 1997, Dooley et al. identified from a large peptide combinatorial library five hexapeptides showing high affinity and selectivity for the NOP receptors. In particular Ac-RYYRWK-NH_2 and Ac-RYYRIK-NH_2 were described as potent and NOP selective partial agonists (Dooley et al., 1997). Ac-RYYRWK-NH_2 was later used as template to generate other interesting NOP ligands such as the peptide ZP120 a selective NOP partial agonist with prolonged actions *in vivo* (Bigoni et al., 2002) and developed for its aquaretic activity (Calo et al., 2005). Another example coming from the combinatorial peptide chemical approach is III-BTD a NOP antagonist with mixed agonist activity at classical opioid receptors (Becker et al., 1999; Bigoni et al., 2000).

Non-peptide ligands – As mentioned before, the different biological functions controlled by N/OFQ-NOP system stimulated the industrial interest in this field of research and several companies activated medicinal chemistry programs aimed at the identification and characterization of drug-like non-peptide NOP ligands. Thus, at the beginning of the 00' were identified the first interesting and useful molecules, such as J-113397, the first non-peptide NOP antagonist (Ozaki et al., 2000). Roche researchers disclosed a series of novel

spiropiperidines, between these Ro 64-6198 a high potent and selective NOP agonist (Jenck et al., 2000) and Ro 65-6570 (Wichmann, Adam, Rover, Cesura, Dautzenberg & Jenck, 1999). The latter was identified as the most interesting molecule of the series after observing that it bound with high affinity to the NOP receptor displaying 10 fold selectivity over opioid receptors in a NOP selective antagonist reversible manner (Hashiba et al., 2001). Later, other important molecules were published including the highly potent antagonists SB-612111 (Zaratin et al., 2004) and C-24 (Goto et al., 2006). Compound SB-612111 was patented by GlaxoSmithKline and described as a high affinity and selective NOP receptor antagonist both *in vitro* and *in vivo*. These results were confirmed when tested in our laboratories (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007), as a matter of fact SB-612111 displayed subnanomolar affinity for the human recombinant NOP receptor and high selectivity (>150 fold) over classical opioid receptors compared to the NOP antagonist J-113397. Furthermore, SB-612111 antagonised N/OFQ effects with a competitive mode of interaction (Zaratin et al., 2004). *In vivo* studies was demonstrated that given i.p up to 3 mg/kg in the mouse tail withdrawal assay, SB-612111 prevented the pronociceptive and antinociceptive action of N/OFQ given i.c.v and i.t., respectively (Gavioli, Rizzi, Marzola, Zucchini, Regoli & Calo, 2007). All these molecules were and are widely used as standards ligands for the NOP receptor. Finally, SCH 221510 discovered by Schering-Plough binds with high affinity to the NOP receptor and was shown to be anxiolytic with a reduced side-effect profile when compared with benzodiazepines (Varty et al., 2008).

1.4. MOP and NOP receptors and analgesia

For centuries, opioids receptor agonists were and still are the most effective and widely used analgesic drugs for the management of moderate to severe forms of pain. In particular, the most clinically used compounds are MOP agonists such as fentanyl or morphine. However, the analgesic value of these drugs is compromised due to unwanted side effects, such as nausea, vomiting, constipation and respiratory depression. In addition drug abuse and tolerance liability are important long-term side effects associated with the use of opioids. To treat obstetric, post-operative and cancer-related-pain, morphine can be administered spinally becoming one of the most significant breakthroughs in pain management, although this is also associated with unwanted side effects e.g. pruritus. Spinal analgesia is also an option when a patient develops tolerance to the analgesia induced by systemic opioids. However some patients do not only develop tolerance to long-term intrathecal opioid treatment but also show opioid-induced hyperalgesia, that occurs when a patient receiving opioid treatment become more sensitive to certain painful stimuli (Mercadante, Ferrera, Villari & Arcuri, 2003). In addition, there are contradictory reports on the effectiveness of opioids in the treating neuropathic pain (Arner & Meyerson, 1988). On the other hand, activation of the NOP receptor by NOP ligands produces either antinociceptive activity or inhibition of opiate-related antinociception, depending upon the route of administration, dose, and time course of action. Though systemic administration, NOP receptor agonists does not induce antinociception in most acute pain models in rodents, but also is reported that activation of NOP receptors blocks opiate-mediated reward and reduces opiate tolerance development. Hence, appear very clear that there is a crucial need to identify novel analgesic targets that can provide effective opioid-like analgesia associated with fewer side effects and reduced abuse liability. With this in mind, scientists have addressed their attention to the synthesis of novel bivalent NOP/MOP compounds to test the hypothesis that they could have antinociceptive activity with reduced tolerance development, utility as non-addicting analgesics and drug abuse treatment with lower propensity of withdrawal-related effects. In order to understand events and mechanism underlying modulation of pain sensitivity by opioids, many studies have been carried out using either acute or chronic intrathecal administration of MOP agonists. Spinal delivery of morphine was shown to be active in response to acute noxious thermal stimulus (Gupta, Verma, Ahuja, Srivastava, Wadhwa & Ray, 2007), to attenuate mechanical allodynia in rats with peripheral nerve injury (Zhao, Tall, Meyer & Raja,

2004), and reduce sensitivity to thermal, mechanical and cold stimuli following acute paw inflammation in rodents (Alt et al., 2012). At the same time, these effects are accompanied by the onset of tolerance and development of opioid-induced hyperalgesia in rodents (Zhang et al., 2012). Similarly, also in non-human primates studies, the spinal analgesia induced by morphine in a dose dependent manner was associated with phenomena of itch and scratching (Alexander et al., 2013), which represented the most common side effect of spinal administration of MOP agonists. Hence, research identifying analgesics agents that do not induce itch responses are necessary. On the other hand, spinal administration of NOP agonists has antinociceptive effects in rodents under both acute and chronic pain conditions without producing sedation or motor dysfunction (Tian et al., 1997). Several evidence coming from rodents as well as non human primates studies suggest that the simultaneous activation of MOP and NOP receptor may represent a promising strategy for the development of innovative analgesics. In fact, the spinal activation of NOP receptors can potentiates MOP-mediated antinociception. Indeed, intratecally injected N/OFQ potentiated morphine induced antinociception without affecting the motor function in the rat tail flick test assay (Tian et al., 1997). Similar effects have been obtained with Ro 64-6198 systemically co-administered with sub-threshold doses of morphine, where the drug combination promoted the attenuation of heat sensitivity in the hot plate test in mice (Goeldner, Reiss, Wichmann, Meziane, Kieffer & Ouagazzal, 2008). Systemic injection of morphine with intrathecal injection of N/OFQ resulted in strong potentiation of analgesia in rats with diabetic neuropathy (Courteix, Coudore-Civiale, Privat, Pelissier, Eschalier & Fialip, 2004). In the same study, isobolographic analysis assessed in neuropathic rats with chronic constriction of the sciatic nerve, showed that intrathecal co-administration of morphine and N/OFQ suppressed mechanical hyperalgesia in a superadditive manner, thus demonstrating that the co-activation of NOP/MOP receptors is an effective analgesic strategy in both acute and chronic pain rodent models. Switching to studies performed in non-human primates, firstly was assumed that the two independent components NOP and MOP can equally contribute to analgesia, afterwards was tested if their co-activation could be able to elicit synergistic analgesic effects. Thus, N/OFQ combined with a single intrathecal dose of morphine, has potentiated in dose-dependent manner morphine analgesia against noxious thermal stimuli. N/OFQ did not attenuate intrathecal morphine-induced scratching responses and did not produce motor-related side effects in monkeys (Camarda et al., 2009). Interesting results were observed also using inactive doses of UFP-112 and morphine that when given as cocktail blocked capsaicin-induced thermal

allodynia. This synergistic effect was not attenuated when the selective antagonists J-113397 and naltrexone were administered alone, but only after their co-administration. It is worthy of note that the antiallodynic effects achieved by simultaneous activation of MOP and NOP receptors did not elicit scratching responses in monkeys (Brighton, Rana, Challiss, Konje & Willets, 2011). The synergistic antinociceptive effect produced by co-activation of NOP and MOP receptors also occurs after systemic administration of drugs in primates. In fact, when the NOP agonists Ro 64-6198 or SCH 221510 were systemically co-administered with buprenorphine, synergistic antinociceptive effects were obtained. Moreover the drug cocktail produced full antinociception without detectable respiratory depression or scratching responses (Alexander et al., 2013). It is worth noting that the NOP antagonist J-113397 only blocked NOP agonist-induced antinociception, but could neither enhanced nor attenuate buprenorphine-induced antinociception in monkeys (Alexander et al., 2013). Reassuming, the activation of NOP receptors did not attenuate but instead potentiated buprenorphine's antinociception mediated by MOP receptors in primates. Together, these preclinical studies provided pharmacological evidence that simultaneous activation of NOP and MOP receptors produce synergistic analgesic effects with reduced side effects at the systemic level in non-human primates and may prove to be a promising therapeutic strategy to achieve optimum analgesic action. As far as tolerance liability is concerned, the co-activation of NOP and MOP receptors is particularly important also in this context. Indeed, compounds that can activate both NOP and MOP receptors allow that less receptor pools are recruited to achieve final analgesia and so, more receptors will be available for the subsequent treatments, thus slowing the development of tolerance. In other words, bifunctional NOP/MOP ligands may display reduced tolerance liability as compared to selective agonists. In conclusion, given that: i) the co-activation of NOP and MOP receptors can provide a wider therapeutic window through their synergic antinociception effect associated with reduced side effect; ii) the activation of the NOP receptor is considered to have anti-addiction property, whereby, bifunctional ligands may display reduced risk of being abused and iii) their analgesic function might be also associated with slower development of tolerance. Thus the present knowledge suggests that novel mixed NOP/MOP agonists may be valuable as future analgesics drugs.

In the last years some groups of research investigated the pharmacological properties of known compounds in terms of NOP and MOP activity or identified and evaluated novel compounds that bind to NOP and MOP receptors with different degrees of affinity and efficacy. The well-known opiate buprenorphine, although with moderate affinity, was

found to activate NOP receptors (Wnendt, Kruger, Janocha, Hildebrandt & Englberger, 1999), which apparently leads to some of its biological properties. Buprenorphine is also a MOP partial agonist with a complex pharmacology such as a very shallow dose response curve for antinociceptive activity in the tail withdrawal assay, in fact, at higher doses its antinociception dose response curve results in an inverted U shape. It has been demonstrated that the efficacy of buprenorphine continued to increase at higher doses in NOP(-/-)mice (Lutfy & Cowan, 2004), suggesting that at higher doses, the NOP agonist activity of buprenorphine can interfere with its MOP analgesic component. With respect to alcohol consumption, buprenorphine shows a biphasic effect, increasing the substance consumption at low doses and eliciting opposite effects at high doses. However, in Marchigian Sardinian alcohol-preferring (msP) rats, the i.c.v. administration of the NOP antagonist UFP-101 reverses the high dose buprenorphine-induced inhibition and results in the continued increase in alcohol consumption. In addition, in the same experiments the buprenorphine-induced increased alcohol consumption at low doses was blocked by naloxone (Ciccocioppo, Economidou, Rimondini, Sommer, Massi & Heilig, 2007). Therefore, low doses of buprenorphine have MOP-mediated effects (increases drinking), while at high doses it has NOP-mediated activity (attenuated drinking). These results giving weigh to think that buprenorphine can activate both MOP and NOP receptors in situ, and also they validate the hypothesis that in mixed NOP/MOP compounds, the NOP agonist counterpart can maintain analgesic activity with reduced abuse liability. Unlike buprenorphine, characterized by a significantly higher affinity at the opioid receptors rather than at NOP, novel compounds were designed using a NOP scaffold with the aim to obtain various affinities and activities at both receptors. The first compound tested was SR16435. It displayed high affinity and potent partial agonist activity at both MOP and NOP receptors (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009), potent antinociceptive activity in the radian heat tail flick assay and when given daily at its antinociceptive EC_{50} dose, unlike morphine, it was able to reduce tolerance development. Nevertheless, this compound induces a CPP equal to that of morphine. In experiments aimed to study the ability to moderate the reward induced by the MOP component, SR16507 behaves as a full agonist at the NOP receptor and partial agonist at MOP, albeit showing similar high affinity at both receptors. SR16507 has a very potent antinociceptive activity, attenuates morphine CPP but still induces a modest CPP per se (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009). Compound SR14150 is a selective NOP and weak MOP agonist showing naloxone reversible antinociceptive activity (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009) but

without inducing CPP (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009). These findings suggest that the possibility to create a profile with both NOP and MOP agonist activity maintaining antinociceptive response at the expense of the reward effect diminished by the presence of the NOP component. In addition, this series of compounds underlines that the presence of NOP agonist activity also attenuates the antinociceptive activity of the MOP component, as demonstrated by potentiation of the antinociception by co-administration of the NOP receptor antagonist SB-612111 (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009). Compound SR16835 is a selective NOP full agonist and weak MOP agonist, it does not have acute antinociceptive activity in the tail flick test nor does induce a CPP, while it attenuates morphine CPP (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009). Taken together, these studies suggest that a NOP/MOP profile can be found to own antinociceptive activity associated with reduced reward and tolerance development, confirming the observation that a compound with sufficient NOP agonist activity might have potential as a drug abuse medication. Under conditions of chronic pain, the analgesic properties of these compounds appear to be quite different. Interestingly, in mechanical allodynia pain model tested in spinal nerve ligated mice, the non-selective MOP/NOP partial agonist SR14150 is anti-allodynic, but its activity is blocked by SB-612111 instead naloxone. Furthermore, SR16835, was able to attenuate SNL-induced mechanical allodynia in a SB-612111 sensitive manner, while was inactive in blocking tail flick acute pain (Khroyan et al., 2011). These results can be explained considering that chronic pain as well as chronic inflammation induce changes in the levels of NOP receptor mRNA, N/OFQ peptide levels, and ppN/OFQ mRNA levels in rodents (Itoh, Takasaki, Andoh, Nojima, Tominaga & Kuraishi, 2001) (Witta, Buzas & Cox, 2003) and humans (Raffaeli et al., 2006), and once again suggest that NOP agonists might have better success in the treatment of chronic or inflammatory rather than nociceptive acute pain. Collectively, the results coming from rodents' studies claim that the MOP component is required to obtain antinociceptive activity after systemic administration while NOP receptor activation attenuates both analgesia and reward. Overall, it seems possible to elicit optimum analgesia without the risk of abuse liability by balancing the selectivity of a bifunctional ligand between NOP and MOP receptors while still maintaining partial agonism at both receptors (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009) (Khroyan et al., 2007). However, results obtained in non-human primates experiments seem to be different as discussed previously. In fact, the antinociceptive activity of buprenorphine, due to mu receptor activation, was completely blocked by naltrexone and potentiated rather than inhibited by the NOP agonists SCH

221510 and Ro 64-6198 in rhesus monkeys. Based on the relevant evidence of a synergistic antinociceptive effect due to the simultaneous activation of spinal NOP and opioid receptors (Camarda et al., 2009), is worthy to mention the non-selective NOP/MOP agonist peptide [Dmt^1]N/OFQ(1-13)-NH_2. Taking a step back, the design of the non-selective [Dmt^1]N/OFQ(1-13)-NH_2 compound was based on the following evidence: N/OFQ(1-13)-NH_2 maintains the same potency and efficacy as the natural peptide (Calo et al., 1996); a reduction in NOP selectivity over the classical opioid receptor was obtained substituting the Phe^1 with Tyr in N/OFQ and N/OFQ(1-13)-NH_2 sequences (Varani et al., 1999); the increase in ligand potency was observed through substitution of Try^1 with Dmt in opioid peptide sequences (Salvadori et al., 1995). The pharmacological profile assessed both *in vitro* and *in vivo* confirmed that this design strategy was successful. Indeed, the novel peptide behaves as NOP/opioid receptor universal full agonist in various *in vitro* assays performed on recombinant human receptors and, in the bioassay of the guinea pig ileum, where the inhibitory action of the peptide was particularly sensitive to the co-application of J-113397 and naloxone, known as NOP and MOP selective antagonists, respectively (Molinari et al., 2013). Interestingly, *in vivo* experiments in the tail withdrawal assay performed in monkeys, [Dmt^1]N/OFQ(1-13)-NH_2 produced a significant dose-dependent antinociceptive effect against acute thermal nociception in monkeys after i.t. administration, not only more potent than that evocated by N/OFQ, but also longer-lasting and not associated with scratching (Altier et al., 2006). Collectively these findings corroborated the idea that non-selective NOP/opioid agonists may behave as innovative spinal analgesics and prompted us to use [Dmt^1]N/OFQ(1-13)-NH_2 as a drug-template for the synthesis of a PWT compound. Finally, considerable attention should be given to the most preclically tested mixed NOP/opioid receptor agonist, synthesized by Grunenthal researchers, known as cebranopadol (previously called GRT-6005) (Schunk et al., 2014). In binding experiment it has been described to have nanomolar affinity at NOP, MOP and KOP receptors, with approximately 20 nM affinity at DOP receptors (Linz et al., 2014). In [^{35}S]GTPγS binding experiments it displays full efficacy at NOP, MOP and DOP receptors and lower efficacy at KOP. More interestingly, when administered i.v and p.o. it have been demonstrated to evoke very potent antinociceptive effects in acute pain models in rats, and similarly in chronic pain models, being in both cases more potent than morphine. Furthermore it showed significantly lower side effects when compared with standards opioids, in fact also using high doses of the compound motor coordination and respiratory process were little affected (Linz et al., 2014). Its antinociceptive activity was described

longer-lasting to that of morphine with reduced tolerance development in the chronic pain assays. Tolerance to the drug took 26 days of dosing to appear, whereas 11 days are enough for morphine (Lambert, Bird & Rowbotham, 2015). Currently, cebranopadol remains the first in class NOP/MOP full agonist that is in Phase II clinical trials for both acute and chronic pain and its apparent clinical success, at least to this point, demonstrates the potential clinical usefulness of its particular receptor profile, pointing up the relevance of mixed NOP/MOP bivalent compound as potent analgesic with reduced side effects profile.

1.5. Aim of the study

The MOP receptor has been and still is the most important target for the development of analgesics drugs, particularly for the treatment of severe pain. Recent evidence reviewed in the section 1.4. of the introduction obtained both in rodents and in non-human primates, strongly suggest that mixed NOP/MOP receptors agonists are worthy of development as innovative analgesics. To validate this concept experiments should be performed by testing the analgesic properties of chemically different molecules able to simultaneously activate the NOP and MOP receptors in the same range of doses. Thus the present study was focused on the design, synthesis and *in vitro* pharmacological characterization of novel NOP/MOP bivalent compounds. To obtain molecules able to act as NOP/MOP bivalent ligands two distinct chemical strategies were adopted: i) use of a non selective pharmacophore able to recognise with similar potency the two receptor sites, ii) use of two distinct pharmacophores each selective for one receptor tethered together with a proper spacer thus generating a chimeric compound. The above mentioned chemical strategies were applied both at peptide and non peptide molecules thus generating the following mixed NOP/MOP ligands: the non selective compounds PWT2-[Dmt^1] (peptide) and cebranopadol (non peptide) and the chimeric compounds DeNo (peptide) and RR-Ro (non peptide). The present thesis is constituted of four chapters in which the detailed pharmacological characterization of these four compounds is presented as briefly summarised below.

Non selective peptide – PWT2-[Dmt^1]

The *in vitro* pharmacological profile of the tetrabranched derivative of [Dmt^1]N/OFQ(1-13)-NH_2 has been evaluated in different assays including receptor binding, stimulation of [^{35}S]GTPγS binding, calcium mobilization in cells co-expressing recombinant receptors and chimeric G-proteins, and a BRET assay that measures receptor/G-protein and receptor/β-arrestin 2 interaction.

Chimeric peptide – DeNo

DeNo has designed as MOP-NOP ligand by linking the selective peptide agonists dermorphin and N/OFQ. DeNo was assayed in comparison with N/OFQ and dermorphin in the following tests for its ability to: i) increase Ca^{2+} in cells co-expressing recombinant receptors and the chimeric protein $G\alpha_{qi5}$, ii) stimulate the binding of GTPγ[^{35}S], iii) inhibit

cAMP formation, iv) activate MAPKinase, v) stimulate receptor/G-protein and arrestin interaction, vi) inhibit the electrically stimulated guinea pig ileum and vii) to produce analgesia via the intrathecal route in rats.

Non-selective non-peptide – cebranopadol

Using an original chemical strategy cebranopadol has been synthesized in house and further characterized *in vitro* in the following assays: i) calcium mobilization studies performed in cells expressing the human recombinant receptors and chimeric G-proteins, ii) bioluminescence resonance energy transfer BRET studies investigating its ability to promote NOP and mu receptor interaction with G-protein and β-arrestin 2, and iii) bioassay studies in isolated tissues. Moreover, cebranopadol has been evaluated *in vivo* in mice subjected to the tail withdrawal assay and the formalin test.

Chimeric non-peptide – RR-Ro

In this chapter firstly is reported the *in vitro* characterization of a series of fentanyl derivatives (RR compounds) furnished by Prof. Vardanyan in order to select the most convenient MOP pharmacophore to be used for generating chimeric compounds. Then, is presented the *in vitro* pharmacological evaluation of the chimeric compound RR4-Ro, synthesized joining together the fentanyl derivative RR4 with the selective NOP agonist Ro 65-6570. For this study the following assays were used: receptor binding in membranes taken from CHO cells expressing the human recombinant NOP or OP opioid receptors, and on the same preparation the stimulated GTPγ[^{35}S] binding studies, calcium mobilisation studies performed in cells co-expressing the NOP and MOP receptors and the chimeric protein $G\alpha_{qi5}$, and finally the electrically stimulated guinea pig ileum.

2. Results and discussion

2.1. Peptides ligands

2.1.1. PWT2-[Dmt^1]

Recently we developed an innovative chemical strategy, named PWT, for the facile synthesis of peptide tetrabranched derivatives (Bigoni, Calo, Guerrini, Strupish, Rowbotham & Lambert, 2001). The PWT strategy has been validated with several peptide sequences including N/OFQ (Guerrini et al., 2014), neuropeptide S (Rizzi et al., 2015), and tachykinins (substance P, neurokinin A and B) (Rizzi et al., 2014). Previous studies demonstrated that [Dmt^1]N/OFQ(1-13)-NH_2 behaves as an universal agonist for NOP and classical opioid receptors (Molinari et al., 2013). The purpose of the present study was to investigate the *in vitro* pharmacological profile of PWT2-[Dmt^1], a novel tetrabranched derivative of [Dmt^1]N/OFQ(1-13)-NH_2 (Figure 13). The effects of PWT2-[Dmt^1] were assessed in different *in vitro* assays including receptor binding, stimulation of [^{35}S]GTPγS binding, calcium mobilization in cells co-expressing recombinant receptors and chimeric G-proteins, and a BRET assay that measures receptor/G-protein and receptor/β-arrestin 2 interaction.

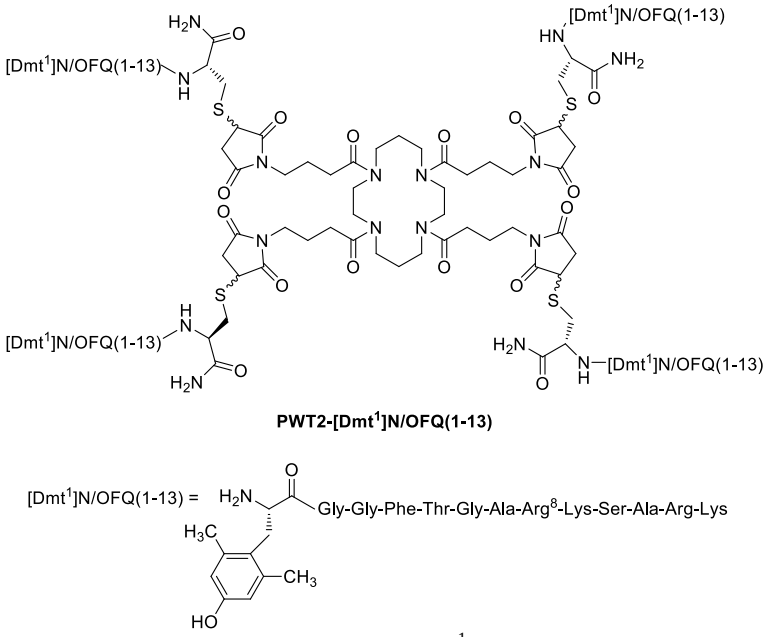


Figure 13. Chemical structure of the compound PWT2-[Dmt^1].

Materials and Methods

Membrane preparation – Cells were harvested, homogenized, and membrane fragments were resuspended in a wash buffer either consisting of 50 mM Tris-HCl pH to 7.4 with KOH, for CHO_{MOP}, CHO_{DOP} and CHO_{KOP}; or supplemented with 5 mM $MgSO_4$ for CHO_{NOP} cells in displacement binding assays, or in an homogenization buffer (50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH) in [^{35}S]GTPγS functional assays. The membrane suspensions were centrifuged and homogenized at 13,500 rpm at 4°C for 10 min repeating this process a total of 3 times. The resulting pellet was resuspended in an appropriate volume of the desired buffer and protein concentration measured using the Lowry assay (Lowry, Rosebrough, Farr & Randall, 1951).

Displacement binding assay – Membrane protein (20~40 µg) was incubated in 0.5 ml of 50 mM Tris, 0.5% BSA and ~0.8 nM [^{3}H]-DPN for classical opioid or ~0.8 nM [^{3}H]-UFP-101 for NOP receptors, as well as varying concentrations (1 pM - 10 µM) of the control ligands and test compounds. Non-specific binding was determined in the presence of 10 µM naloxone or 1 µM of N/OFQ for classical opioid and NOP receptors, respectively. Samples were incubated for 1 h at room temperature, following which reactions were terminated by vacuum filtration, onto PEI-soaked Whatman GF/B filters, using a Brandel harvester. The buffer for dilutions of the compounds is that describe above.

Stimulated [^{35}S]GTPγS binding assay – Membrane protein (20~40 µg) was incubated in 0.5 ml volume of 50 mM Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 100 mM NaCl, 0.1% BSA, 0.15 mM bacitracin; pH 7.4, GDP (33 µM), and ~150 pM [^{35}S]GTPγS. A range of concentrations of N/OFQ, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-Dmt (1 pM - 10µM) was added prior to incubation. Non-specific binding was determined in the presence of unlabelled GTPγS (10 µM) (McDonald, Calo, Guerrini & Lambert, 2003). Samples were incubated for 1 h at 30°C with gentle agitation. Reactions were terminated by vacuum filtration through dry Whatman GF/B filters, using a Brandel harvester. The buffer for dilutions of the compounds is that describe above.

Calcium mobilization assay – CHO cells stably co-expressing human recombinant NOP, MOP or KOP receptor and the C-terminally modified $G\alpha_{qi5}$ (Conklin, Farfel, Lustig, Julius & Bourne, 1993) chimeric protein and cells co-expressing the DOP receptor and the

$G\alpha_{qG66Di5}$ (Kostenis et al., 2005) chimeric protein were generated as previously described (Camarda et al., 2009), (Camarda & Calo, 2013). Cells were cultured in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM)/HAMS F12 (1:1) supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml). Cell cultures were kept at 37°C in 5% CO_2/humidified air. When confluence was reached (3-4 days), cells were sub-cultured as required using trypsin/EDTA and used for experimentation. Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. After 24 hours incubation the cells were loaded with Hank's Balanced Salt Solution (HBSS) supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM, 0.01% pluronic acid and 20 mM HEPES (pH 7.4) for 30 min at 37°C. Afterwards the loading solution was aspirated and a washing step with 100 µl/well of HBSS, HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 µM Brilliant Black was carried out. Subsequently 100 µl/well of the same buffer was added. After placing cell culture and compound plates into the FlexStation II (Molecular Devices, Sunnyvale, CA, USA), fluorescence changes were measured after 10 min of stabilization at 37°C. On-line additions were carried out in a volume of 50 µl/well.

BRET assay – Human Embryonic Kidney (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 100 units ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin sulphate, human SH-SY5Y neuroblastoma cells were grown in Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% (v/v) foetal calf serum, 100 units ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin sulphate either in a humidified atmosphere of 5% CO_2 at 37°C. Cells lines permanently co-expressing the different pairs of fusion proteins, NOP-RLuc/Gβ1-RGFP (HEK-293), NOP-RLuc/β-arrestin 2-RGFP (HEK-293), MOP-RLuc/Gβ1-RGFP (SH-SY5Y) and MOP-RLuc/β-arrestin 2-RGFP (SH-SY5Y) were prepared using the pantropic retroviral expression system by Clontech as described previously (Molinari, Casella & Costa, 2008). For G-protein experiments enriched plasma membrane aliquots from transfected cells were prepared and quantified as previously described in details (Corrado et al., 2015). Luminescence in membranes was recorded in 96-well untreated white opaque microplates, while in whole cells was recorded in 96-well sterile poly-D-lysine-coated white opaque microplates for HEK-293 cells and in untreated white opaque microplates for SH-SY5Y cells (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030

(PerkinElmer, Waltham, MA, USA). For the determination of receptor/G-protein interaction, membranes (3 μg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP, MOP/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of receptor/β-arrestin 2 interaction, cells co-expressing NOP/RLuc and β-arrestin 2/RGFP, MOP/RLuc and β-arrestin 2/RGFP were plated 24 h before the experiment (100,000 cells $well^{-1}$) in poly-D-Lysine treated plates for HEK-293 and in untreated plates for SH-SY5Y cells. The cells were prepared for the experiment substituting the medium with PBS with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM). Coelenterazine at a final concentration of 5 μM was injected 15 minutes prior reading the cell plate. Different concentrations of ligands in 20 μl of PBS - BSA 0.01 % were added and incubated 5 or 60 min before reading luminescence. All the experiments were performed at room temperature.

Materials – All cell culture media and supplements were from Invitrogen (Paisley, U.K.). All other reagents used were from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany) and were of the highest purity available. The reference molecules, dermorphin and N/OFQ were synthesized in house (Department of Chemical and Pharmaceutical Sciences, University of Ferrara) as previously described (Calo et al., 1997), while DPDPE, and dynorphin A were bought from Tocris Bioscience (Bristol, UK). Tritiated UFP-101 ([^{3}H]-UFP-101) was synthesized as described previously (Ibba et al., 2008). Tritiated diprenorphine ([^{3}H]-DPN) was purchased from Perkin Elmer. Native coelenterazine (CLZN, 5 mM, EtOH) was from Synchem UG & Co. KG (Altenburg, Germany). [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] were synthesized and purified in the laboratories of Prof. Remo Guerrini (Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Italy). Stock solutions (1 mM) of peptides and the new compound PWT2-[Dmt^1] were made in ultrapure water and all stored at - 20°C until use. The successive dilutions were made in HBSS/HEPES (20 mM) buffer (containing 0.005 % Bovine Serum Albumin (BSA) fraction V to avoid licking) in the calcium assay and PBS/BSA (0.01 %) buffer in the BRET assay.

Synthesis of PWT2-[Dmt^1] – PWT2 derivative of [Dmt^1]N/OFQ(1-13)-NH_2 ([Dmt^1]) was prepared by using a convergent synthetic approach and methodology previously applied for the synthesis of PWT derivatives of nociceptin/orphanin FQ peptide (Guerrini et al., 2014), neropeptide S (Ruzza et al., 2015) and tachykinin (Ruzza et al., 2014). Firstly,

[Cys14][Dmt^1] was synthesised by solid phase method with an automatic solid phase peptide synthesizer Syro II (Biotage, Uppsala Sweden) using Fmoc/tBu chemistry (Benoiton, 2005). The resin 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA (Rink amide MBHA resin) was used as a solid support. The resin was treated with 40% piperine/ N,N-dimethylformamide (DMF) and linked with Fmoc-Cys(Trt)-OH by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) as the coupling reagent. The following Fmoc amino acids were sequentially coupled to the growing peptide chain: Fmoc-Lys(Alexander et al.)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ala-OH, Fmoc-Ser(tBu)-OH, Fmoc-Lys(Alexander et al.)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, and Fmoc-Dmt-OH. All the Fmoc amino acids (4 equiv) were coupled to the growing peptide chain by using HATU (4 equiv) in DMF in the presence of an equimolar concentration of 4-methylmorpholine (NMM), and the coupling reaction time was 1h. To improve the analytical profile of the crude peptide, capping with acetic anhydride (0.5M/DMF) in the presence of NMM (0.25M/DMF) (3:1 v/v; 2mL / 0.2 g of resin) was performed at any step. 40% Piperidine/DMF was used to remove the Fmoc. The protected peptide-resin was treated with reagent B (Sole', 1992) (trifluoroacetic acid (TFA) / H_20 / phenol / triisopropylsilane 88 : 5 : 5: 2; v/v; 10 mL / 0.2 g of resin) for 1.5 h at room temperature. After filtration of the resin, the solvent was concentrated in vacuum and the residue triturated with ether. Crude [Cys^{14}][Dmt^1] was purified by preparative reversed-phase HPLC using a Water Delta Prep 3000 system with a Jupiter column C_{18} (250 x 30 mm, 300 A, 15 □m spherical particle size). The column was perfused at a flow rate of 20 mL/min with a mobile phase containing solvent A (5%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 5 to 70% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) over 25 min for the elution of the peptide. Purified [Cys^{14}][Dmt^1] was reacted in solution with PWT2 core in a classical thio-Michael reaction using experimental conditions previously optimized for the synthesis of nociceptin/orphanin FQ tetra branched derivatives (Guerrini et al., 2014) and the PWT2-[Dmt^1] purified using the same HPLC conditions employed for the purification of the linear [Cys^{14}][Dmt^1]. Analytical HPLC analyses were performed on a Beckman 116 liquid chromatograph equipped with a Beckman 166 diode array detector. Analytical purity of [Cys^{14}][Dmt^1] and PWT2-[Dmt^1] were determined using a Luna C_{18} column (4.6 x 100 mm, 3 □m particle size) with the above solvent system (solvents A and B) programmed at a flow rate of 0.5 mL / min using a linear gradient from 0% to 80% B

over 25 min. Final product showed ≥ 95% purity when monitored at 220 nm. Molecular weight of PWT2-[Dmt[1]] was in accord with the expected molecular formula.

Data analysis and terminology – All data are expressed as means ± standard error of the mean (SEM) of at least 3 experiments performed in duplicate. For potency values 95% confidence limits were indicated. The pharmacological terminology adopted in this report is consistent with the IUPHAR recommendations (Alexander et al., 2013). Receptor binding data are expressed as % displacement. [^{35}S]GTPγS data are expressed as stimulation factor that is 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. BRET data are calculated as BRET ratio between CPS measured for the RGFP and RLuc light emitted using 460(25) and 510(10) filters (PerkinElmer, Waltham, MA, USA), respectively. Data are expressed as stimulated BRET ratio obtained by subtracting the vehicle value to that measured in the presence of ligand. Affinity values are showed as pK_i calculated using the Cheng-Prusoff equation:

$$pK_i = \log\left[\frac{IC_{50}}{1+\frac{[L]}{K_D}}\right]$$

Where IC_{50} is the concentration of ligand that produces 50% inhibition of the specific binding, [L] is the concentration of free radioligand and K_D is the dissociation constant of the radioligand for the receptor. Agonist potencies were given as pEC_{50} that is 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 four parameter logistic nonlinear regression model:

$$Effect = baseline + \frac{E_{max} - baseline}{1+10^{(\log EC_{50} - X)\cdot n}}$$

Where EC_{50} is the concentration of agonist producing a 50% maximal response, X is the agonist concentration and n is the Hill coefficient of the concentration response curve to the agonist. In representative tables maximal effects elicited by the ligands are expressed as intrinsic activity (α) calculated as the ratio between the E_{max} of the ligand under investigation and that elicited by a standard full agonist. Curve fittings were performed

using Graph Pad PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.). Data obtained with calcium mobilization and BRET assays have been statistically analysed with one way ANOVA followed by the Dunnett's test for multiple comparisons, while data obtained with receptor binding assays (receptor binding and stimulated [^{35}S]GTPγS) with one way ANOVA followed by the Bonferroni's test for multiple comparisons. In both cases p values less than 0.05 were considered to be significant.

Results

Displacement Binding assay – In CHO_{NOP} cell membranes, N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2 displaced the binding of [^{3}H]-UFP-101 in a concentration dependent and saturable manner with high and similar values of affinity, i.e. 10.12 and 10.39 respectively. PWT2-[Dmt^1] displayed a significant decrease in affinity (pK_i 9.13) when compared to the patent compound [Dmt^1]N/OFQ(1-13)-NH_2 (Figure 14A). Moreover, at high concentrations it showed an increased ability than N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2 to displace [^{3}H]-UFP-101 possibly reflecting artefacts due to the low specific activity of the radioligand and its high non specific binding. In order to understand this action we performed the same experiment comparing the concentration response curves to PWT2-[Dmt^1] in the range 1 pM - 10 μM, with that (in the range 1 nM - 10 □M) in the presence of 1 μM of N/OFQ as further specific competitor for the NOP receptor binding sites, and with that obtained in the range 1 pM - 100 nM. Thus, as shown in Figure 15, we demonstrated that the ability to produce a displacement of the radioligand higher than 100 % at high PWT2-[Dmt^1] concentrations compared to the parent peptide and the standard N/OFQ is most probably due to non specific displacement of the radioligand. At MOP sites dermorphin, [Dmt^1]N/OFQ(1-13)-NH_2 and its tetrameric derivative displaced the binding of [^{3}H]-DPN in a concentration dependent and saturable manner. [Dmt^1]N/OFQ(1-13)-NH_2 (pK_i 10.4) demonstrated higher affinity than dermorphin (9.14), while the affinity of the tetrameric compound was superimposable to that of dermorphin (9.25) (Figure 14B). At CHO_{KOP} membranes, dynorphin A and [Dmt^1]N/OFQ(1-13)-NH_2 displaced the binding of [^{3}H]-DPN in a concentration dependent and saturable manner with high and similar (10.30 and 10.38) pK_i values. PWT2-[Dmt^1] showed a ~18 fold loss in affinity compared to its patent compound (Figure 14C). Furthermore, all tested compounds displaced the binding of [^{3}H]-DPN at the DOP receptor in a concentration dependent and saturable manner showing the following rank order of affinity: [Dmt^1]N/OFQ(1-13)-NH_2 > DPDPE > PWT2-[Dmt^1] (Figure 14D). The values of affinity of [Dmt^1]N/OFQ(1-13)-NH_2 and its tetrabranced derivative are summarized in Table 4.

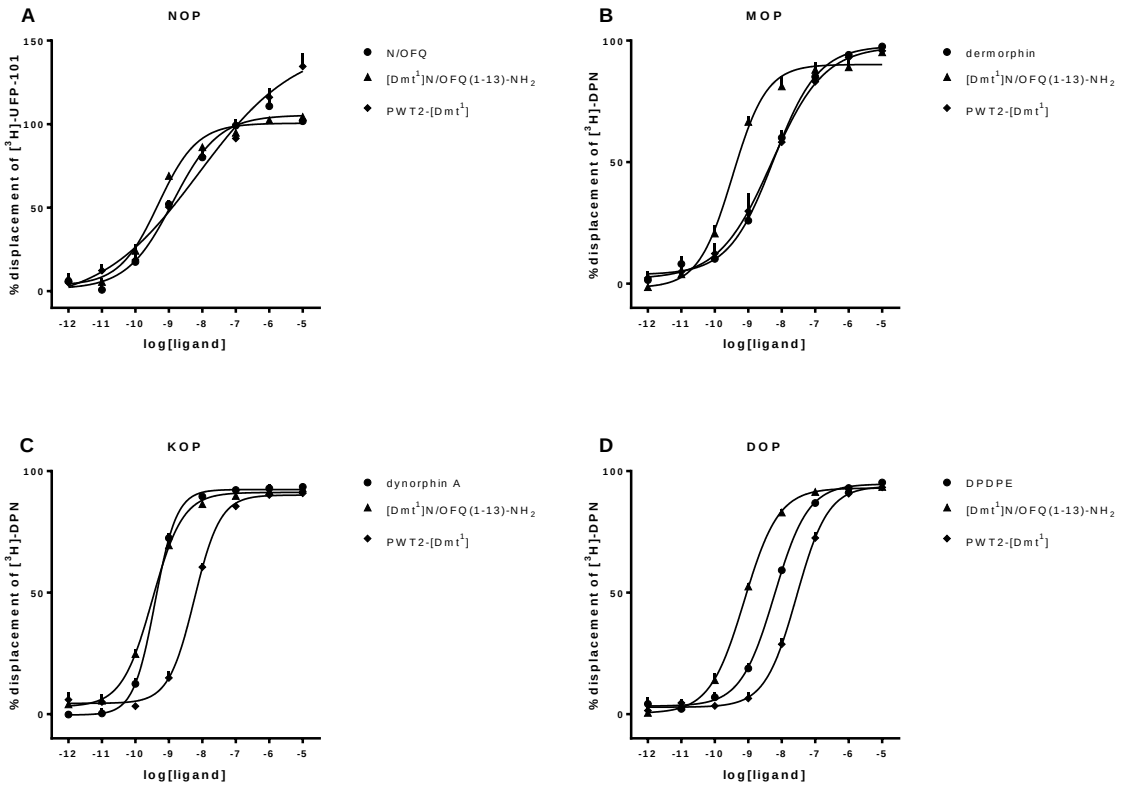


Figure 14. Displacement binding experiments. Displacement of [^{3}H]UFP-101 at CHO_{NOP} cell membranes and of [^{3}H]DPN at $CHO_{MOP/KOP/DOP}$ by respective control ligand, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1]. Data are the mean ± SEM of at least 5 separate experiments performed in duplicate.

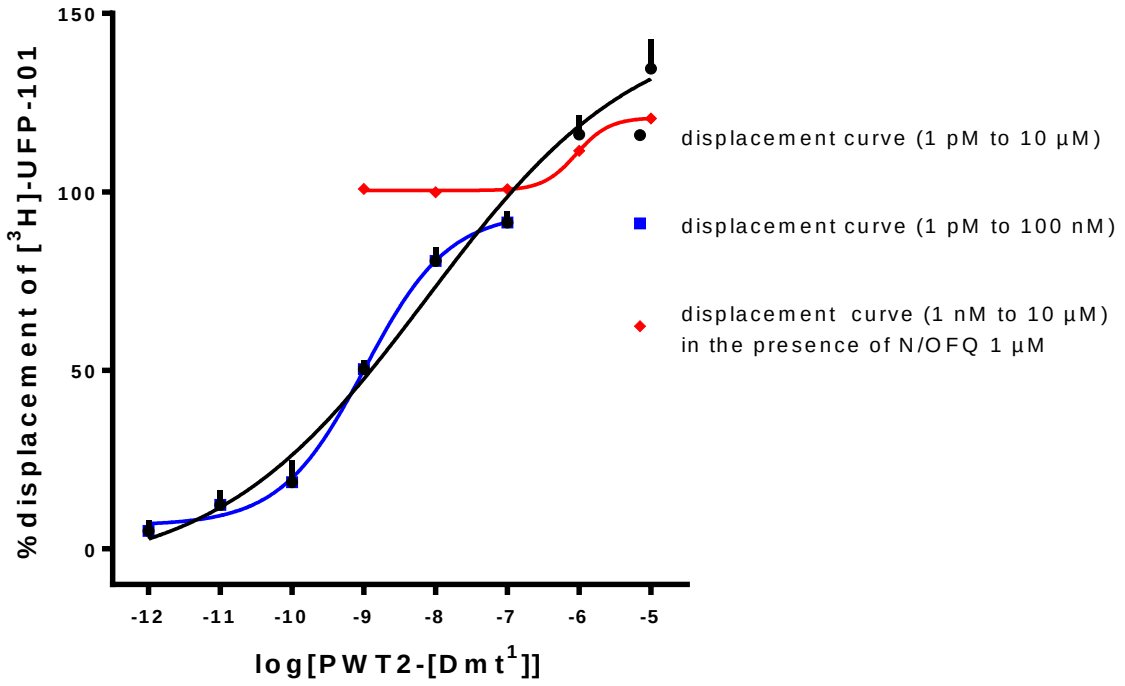


Figure 15. Comparison of three different ranges of concentration in displacement experiments of [^{3}H]UFP-101 at CHO_{NOP} cell membranes by PWT2-[Dmt^1].

Table 4. Displacement binding experiments. pK_i values of [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] in CHO cells expressing the NOP and classical opioid human recombinant receptors.

	NOP	MOP	KOP	DOP
		$pK_i(CL_{95\%})$		
[Dmt^1]N/OFQ (1-13)-NH_2	10.39 (9.31-10.48)	10.04 (9.43-10.65)	10.38 (10.31-10.44)	9.99 (9.82-10.16)
PWT2-[Dmt^1]	9.13* (7.80-10.45)	9.25 (8.73-9.77)	9.13 (9.02-9.25)	8.42* (8.22-8.62)

*Significantly different from the patent compound [Dmt^1]N/OFQ(1-13)-NH_2, $p < 0.05$ with one way ANOVA followed by the Bonferroni's test for multiple comparisons. Control ligands N/OFQ, dermorphin, dynorphin A and DPDPE used for the NOP, MOP, KOP and DOP receptors respectively.

[^{35}S]GTPγS stimulation binding – N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2 stimulated the [^{35}S]GTPγS binding in a concentration dependent and saturable manner in membranes prepared from CHO cells expressing the NOP receptor. [Dmt^1]N/OFQ(1-13)-NH_2 showed a slightly higher value of potency (9.46) and similar maximal effects (3.22 ± 0.22%) compared to those of the standard N/OFQ (pEC_{50} 9.33, E_{max} 2.95 ± 0.10%)). PWT2-[Dmt^1] mimicked the stimulating effect of the patent compound with similar potency and maximal effects (Figure 16A). In CHO_{MOP} cell membranes, dermorphin and [Dmt^1]N/OFQ(1-13)-NH_2 stimulated the binding of [^{35}S]GTPγS in a concentration dependent and saturable manner. [Dmt^1]N/OFQ(1-13)-NH_2 showed a 3 fold increased potency (8.58) and similar efficacy (3.06 ± 0.04%) compared to dermorphin (pEC_{50} 8.02, E_{max} 2.88 ± 0.05%). PWT2-[Dmt^1] behaved as the patent compound showing no significant differences both in terms of potency (8.51) and maximal effects. (2.84 ± 0.11%) (Figure 16B). At CHO_{KOP}, dynorphin A, [Dmt^1]N/OFQ(1-13)-NH_2 and the PWT compound stimulated the [^{35}S]GTPγS binding in a concentration dependent and saturable manner showing similar values of potency (9.19, 9.14, 9.20 respectively). [Dmt^1]N/OFQ(1-13)-NH_2 and its PWT derivative produced a significant higher maximal responses (2.51 ± 0.17%, 2.43 ± 0.08%) compared to that elicited by the endogenous compound dynorphin A (2.03 ± 0.04%) (Figure 16C). In parallel experiments performed in membranes expressing the DOP receptor, the standard compound DPDPE, [Dmt^1]N/OFQ(1-13)-NH_2 and its PWT derivative stimulated the binding of [^{35}S]GTPγS in a concentration dependent and saturable manner. The three compounds showed similar values of potency and efficacy

(Figure 16D). The values of potency and efficacy of [Dmt[1]]N/OFQ(1-13)-NH_2 and its PWT derivative are summarized in Table 5.

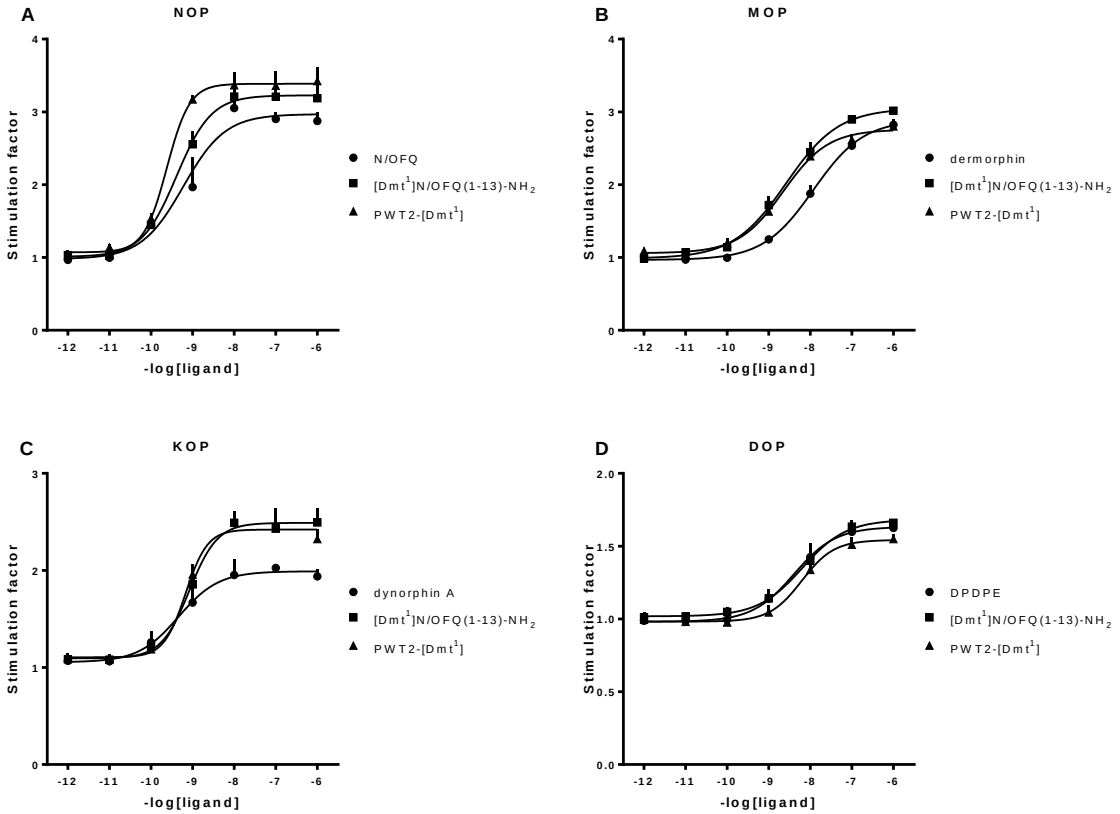


Figure 16. [^{35}S]GTPγS binding experiments. Concentration response curves to respective control ligand, [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]] in membranes of CHO cells stably expressing the NOP and classical opioid human recombinant receptors. Data are the mean ± SEM for n ≥ 5 separate experiments.

Table 5. [^{35}S]GTPγS assay performed in CHO cells expressing the NOP and classical opioid human recombinant receptors.

	NOP		MOP		KOP		DOP	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
[Dmt^{1}]N/OFQ (1-13)-NH_2	9.46 (9.20-9.72)	1.18 ± 0.16	8.58 (8.17-9.00)	1.10 ± 0.04	9.14 (9.43-8.86)	1.47 ± 0.17	8.17 (7.46-8.88)	1.09 ± 0.08
PWT2-[Dmt^{1}]	9.61 (9.36-9.86)	1.23 ± 0.13	8.51 (7.70-9.32)	0.98 ± 0.07	9.20 (8.94-9.45)	1.40 ± 0.11	8.16 (7.49-8.83)	0.87 ± 0.07*

*Significantly different from the patent compound [Dmt^{1}]N/OFQ(1-13)-NH_2, $p < 0.05$ Bonferroni test for multiple comparisons. Intrinsic activity (α) relative to full agonist control ligand. Control ligands N/OFQ, dermorphin, dynorphin A and DPDPE used for the NOP, MOP, KOP and DOP receptors respectively.

Calcium mobilization assay – In $CHO_{NOP+}G\alpha_{qi5}$ cells, N/OFQ increased in a concentration dependent manner the intracellular calcium levels, with high potency (9.59) and maximal effects of 316 ± 51% over the basal value. In parallel experiments [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] mimicked the effect of the N/OFQ with similar maximal effects but showing reduced potency by 3 and 58 fold, respectively (Figure 17A). In CHO cells stably expressing the chimeric protein $G\alpha_{qi5}$ and the human MOP receptor, dermorphin produced a concentration dependent stimulation of calcium mobilization displaying high potency (8.19) and maximal effects (345 ± 14% over basal). [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] elicited the same stimulatory effect of the standard peptide, however with slightly lower maximal effects (306 ± 10% and 283 ± 3%) and potencies by 3 and 23 fold for [Dmt^1]N/OFQ(1-13)-NH_2 and tetrameric derivative, respectively (Figure 17B). In CHO_{KOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein the reference agonist dynorphin A evoked a concentration dependent stimulation of calcium release displaying high potency (8.54) and maximal effects of 206 ± 31 % over the basal values. In parallel experiments [Dmt^1]N/OFQ(1-13)-NH_2 stimulated calcium release with 8 fold lower potency (7.66) and maximal effects (191 ± 26 %) not far from those of the dynorphin A. PWT2-[Dmt^1] mimicked the stimulatory effect of the parent compound showing similar maximal effects associated however with a large loss of potency (Figure 17C). Finally in CHO_{DOP} cells stably expressing the $G\alpha_{qG66Di5}$ chimeric protein the reference agonist DPDPE evoked a concentration dependent stimulation of calcium release displaying a potency of 8.15 and maximal effects of 238 ± 27% over the basal values. Compounds [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] elicited slightly lower maximal effects and displayed values of potency 8 and 23 fold lower than DPDPE (Figure 17D). The values of potency and efficacy of [Dmt^1]N/OFQ(1-13)-NH_2 and its PWT derivative are summarized in Table 6.

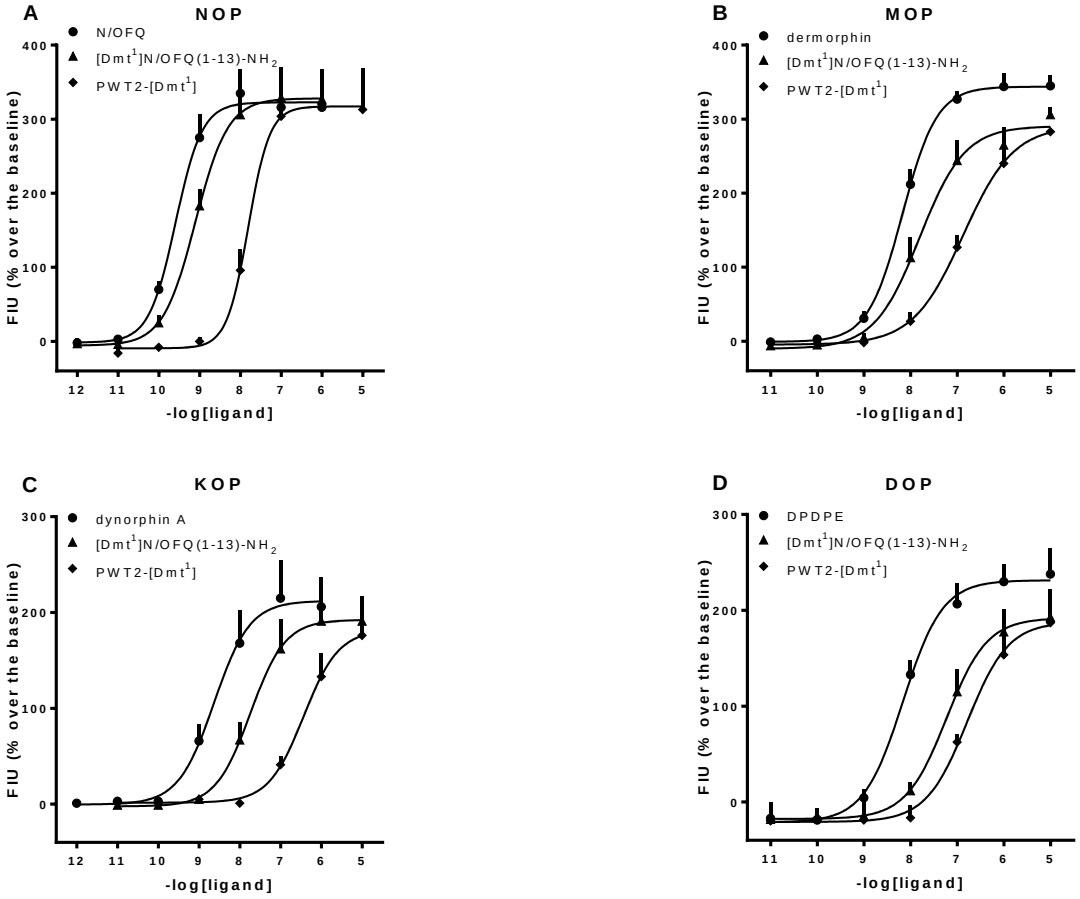


Figure 17. Concentration response curves to standard agonists, [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]] in CHO cells stably expressing the NOP and classical opioid receptors and chimeric G-proteins. Data are the mean ± SEM for at least 4 separate experiments performed in duplicate.

Table 6. Effects of [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]] at NOP and classical opioid receptors coupled with calcium signalling via chimeric G-proteins.

	NOP		MOP		KOP		DOP	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
[Dmt[1]]N/OFQ (1-13)-NH_2	9.18 (8.71-9.64)	0.99 ± 0.04	7.78 (7.14-8.42)	0.85 ± 0.04	7.66 (7.37-7.95)	0.92 ± 0.03	7.24 (6.92-7.57)	0.83 ± 0.07
PWT2-[Dmt[1]]	7.83 (7.62-8.03)	0.98 ± 0.08	6.82 (6.20-7.43)	0.87 ± 0.04	6.40 (6.20-6.60)	0.86 ± 0.03	6.79 (6.42-7.16)	0.80 ± 0.08

inactive means that the compound was inactive up to 1 μM. N/OFQ, dermorphin, dynorphin A and DPDPE were used as reference agonists for calculating intrinsic activity at NOP, MOP, KOP, and DOP receptor respectively.

BRET assay – The ability to promote receptor/G-protein and receptor/β-arrestin 2 interaction of standard ligands, (i.e. N/OFQ for the NOP receptor and dermorphin for the MOP receptor), [Dmt^1]N/OFQ(1-13)-NH_2 and its PWT derivative, has been assessed with a BRET based assay. As shown in Figure 18A, in membrane prepared from cells HEK-293 expressing the NOP receptor N/OFQ, [Dmt^1]N/OFQ(1-13)-NH_2 and its PWT derivative promoted receptor/G-protein interaction in a concentration dependent manner showing similar values of potency and maximal effects. Under the same experimental conditions, membrane extracted from SH-SY5Y cells were used to evaluate the activity of the compounds at the MOP receptor. Dermorphin promoted MOP/G-protein interaction in a concentration dependent manner displaying high potency and maximal effect. [Dmt^1]N/OFQ(1-13)-NH_2 mimicked the stimulating effect of the standard with a similar maximal response associated with 6 fold higher potency. PWT2-[Dmt^1] promoted MOP/G-protein interaction with a potency close to that of the patent compound. Interestingly PWT2-[Dmt^1] at micromolar concentrations elicited stimulatory effect higher than those of the standard (Figure 18B). All data are summarized in Table 7.

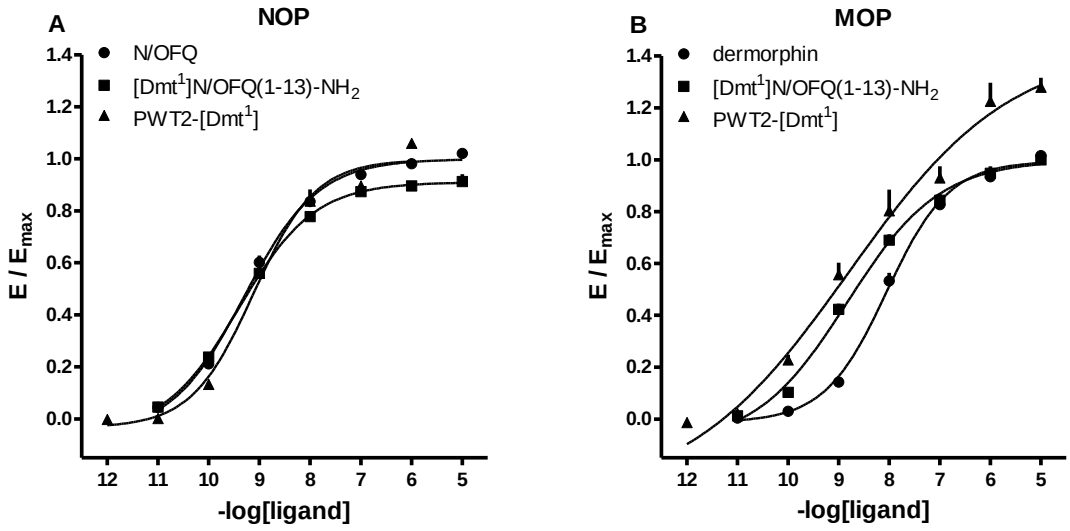


Figure 18. Concentration response curves to N/OFQ, dermorphin, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] in CHO cells stably expressing the NOP/RLuc (panel A) or MOP/RLuc (panel B) receptors and the Gβ1/RGFP protein. Data are the mean ± SEM for at least 3 separate experiments performed in duplicate.

Table 7. Potencies (pEC_{50}) and efficacy (α) of standard agonists, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] in promoting NOP/Gβ1 and MOP/Gβ1 interaction.

	Gβ1-protein			
	NOP		MOP	
	pEC_{50} ($CL_{95\%}$)	α ± SEM	pEC_{50} ($CL_{95\%}$)	α ± SEM
N/OFQ	9.30 (9.15-9.45)	1.00	crc incomplete[a]	
Dermorphin	crc incomplete[a]		8.03 (7.82-8.24)	1.00
[Dmt^1]N/OFQ(1-13)-NH_2	9.42 (9.15-9.69)	0.91 ± 0.02	8.80 (8.53-9.07)	9.42 (9.15-9.69)
PWT2-[Dmt^1]	9.16 (8.86-9.46)	1.04 ± 0.04	9.16 (8.93-9.39)	9.16 (8.86-9.46)

N/OFQ and dermorphin were used as reference agonists for calculating intrinsic activity at NOP and MOP receptors respectively. *Significantly different from the patent compound [Dmt^1]N/OFQ(1-13)-NH2. [a]crc incomplete means that maximal effects could not be determined due to the low potency of the compound. These data are taken from previous experiments.

In HEK-293 cells expressing the NOP receptor N/OFQ promoted receptor/β-arrestin 2 interaction in a concentration dependent manner displaying high potency and maximal effects. [Dmt^1]N/OFQ(1-13)-NH_2 mimicked the stimulatory effect of the standard with similar potency but with a lower maximal response. PWT2-[Dmt^1] showed a lower potency and efficacy compared to those of the standard and the patent compound behaving as partial agonist (Figure 19A). In SH-SY5Y cells expressing the MOP receptor dermorphin promoted receptor/β-arrestin 2 interaction in a concentration dependent manner displaying high potency and maximal effects. [Dmt^1]N/OFQ(1-13)-NH_2 demonstrated a similar potency associated with a reduction of maximal effects. PWT2-[Dmt^1] acted as its patent compound however with a slightly lower potency and higher efficacy (Figure 19B). All data are summarized in Table 8.

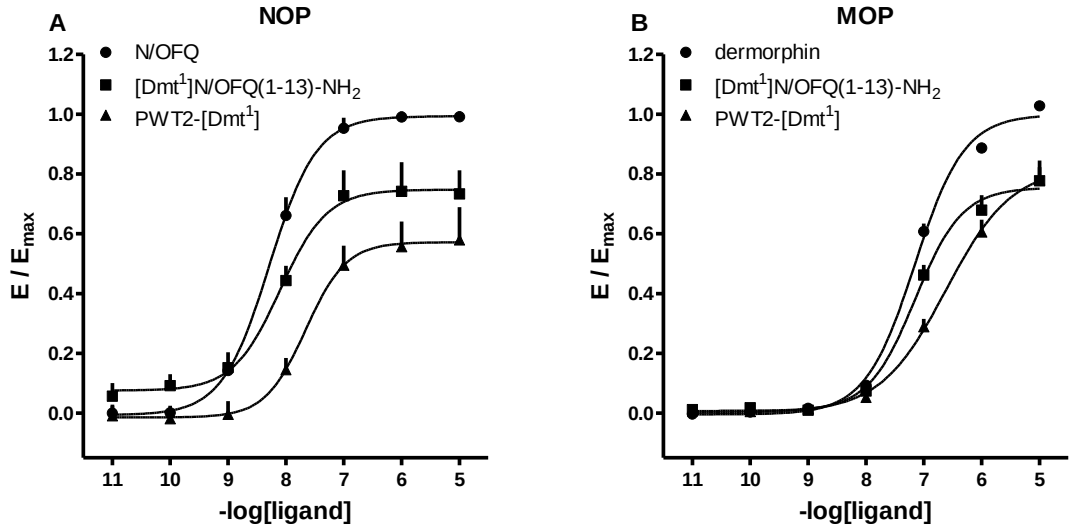


Figure 19. Concentration response curves to N/OFQ, dermorphin, [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]] in CHO cells stably expressing the NOP/RLuc (panel A) or MOP/RLuc (panel B) human recombinant receptors and the β-arrestin 2/RGFP protein. Data are expressed as the mean ± SEM for at least 4 separate experiments performed in duplicate.

Table 8. Potencies (pEC_{50}) and efficacy (α) of standard agonists, [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]] in promoting NOP/β-arrestin 2 and MOP/ β-arrestin 2 protein interactions.

	β-arrestin 2			
	NOP		**MOP**	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
N/OFQ	8.25 (7.99-8.52)	1.00	inactive[a]	
Dermorphin	inactive[a]		7.15 (7.05-7.25)	1.00
[Dmt[1]]N/OFQ(1-13)-NH_2	8.15 (7.79-8.51)	0.75 ± 0.09	7.13 (7.07-7.19)	0.76 ± 0.05
PWT2-[Dmt[1]]	7.67 (7.41-7.93)	0.58 ± 0.10	6.64 (6.50-6.78)	0.82 ± 0.07

N/OFQ and dermorphin were used as reference agonists for calculating intrinsic activity at NOP and MOP receptors respectively. [a]inactive means that the compound was inactive up to 1 μM. These data are taken from previous experiments.

In Figure 20, concentration response curves to standard agonists, [Dmt^1]N/OFQ(1-13)-NH_2, and PWT2-[Dmt^1] at G-protein and arrestin are plotted in the same graph. It can be seen that the PWT modification favoured G-protein vs arresting potency and efficacy; this applies both to the NOP and MOP receptor. This is made clearer in Figure 21 where the bias plot obtained by plotting the amount of signal produced in the G-protein pathway as a function of equal amounts of signal produced in the arresting pathway in response to equimolar concentrations of agonist (Kenakin & Christopoulos, 2013).

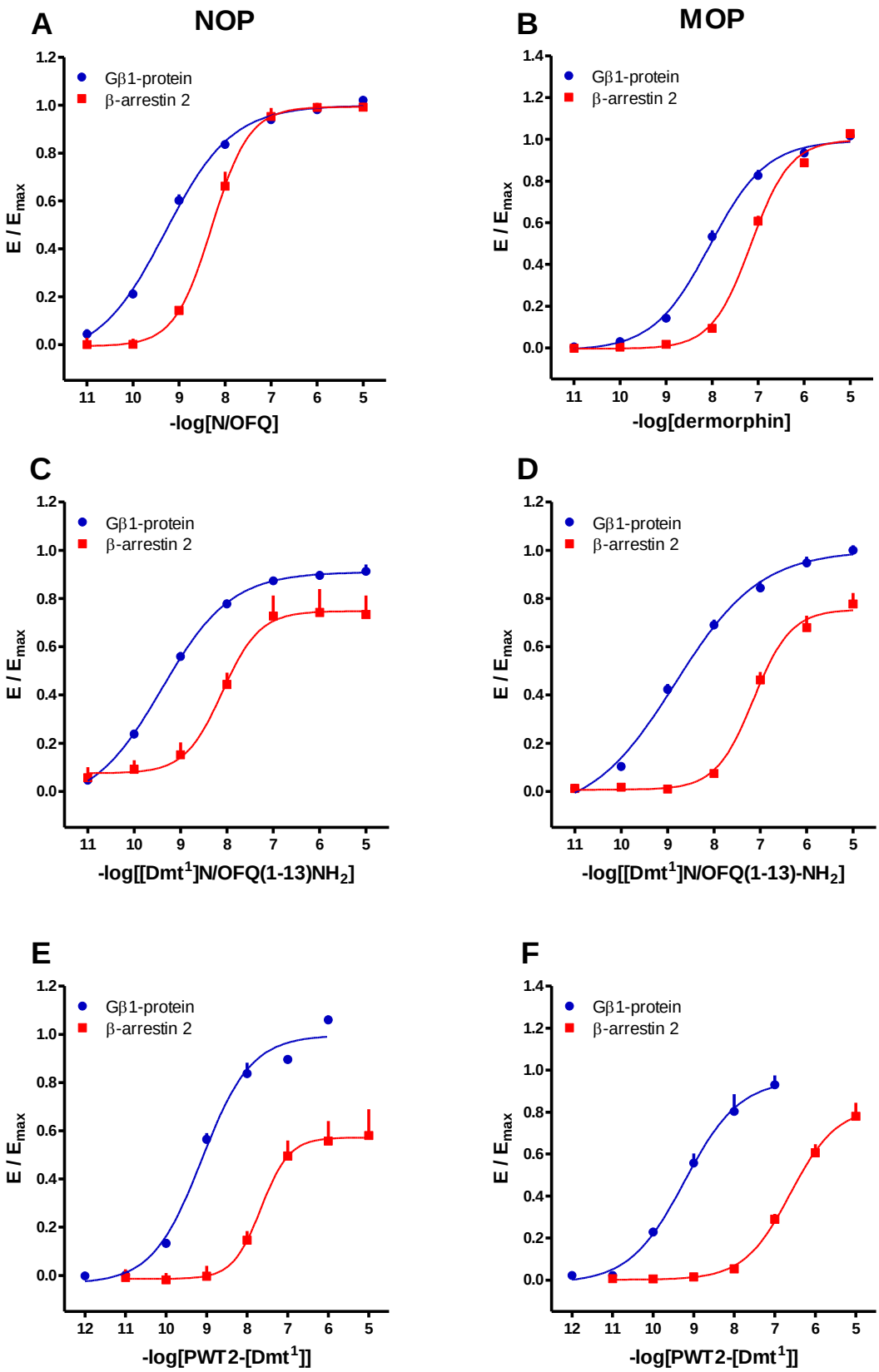


Figure 20. Concentration response curves to N/OFQ, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] (panel A, C and E) in cells stably expressing the NOP/RLuc and the Gβ1/RGFP or β-arrestin 2/RGFP proteins. Concentration response curves to dermorphin, [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] (panel B, D and F) in cells stably expressing the MOP/RLuc and the Gβ1/RGFP or β-arrestin 2/RGFP proteins. Data are expressed as the mean ± SEM for at least 4 separate experiments performed in duplicate.

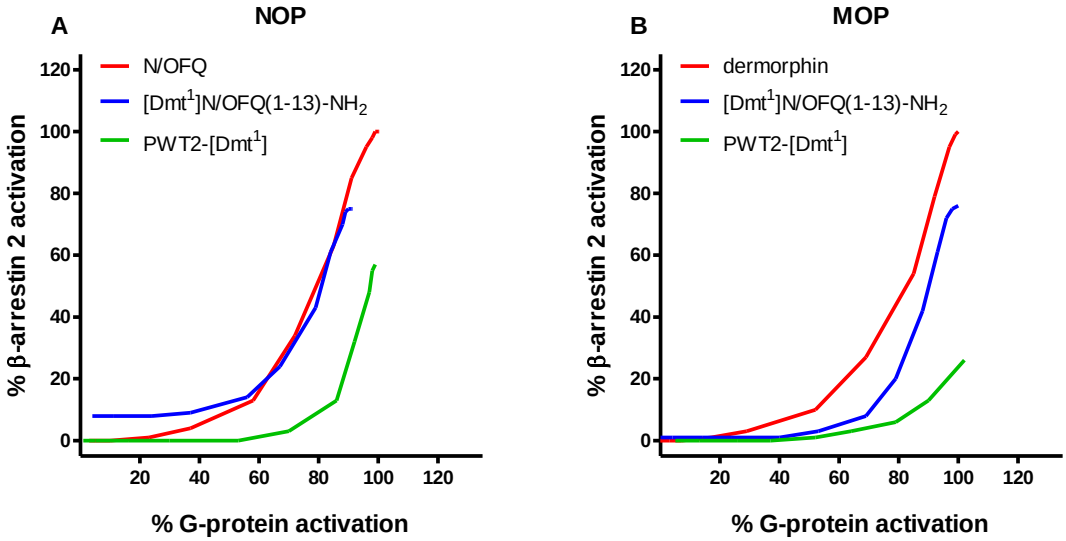


Figure 21. Bias Plot showing the profile of [Dmt^1]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^1] in comparison with the standard agonists N/OFQ for NOP (panel A) and dermorphin for MOP (panel B).

Discussion

Previous studies demonstrated that [Dmt[1]]N/OFQ(1-13)-NH_2 behaves as a potent agonist for NOP and OP receptors and elicits robust antinociceptive effects after spinal administration in non human primates (Molinari et al., 2013). The recently discovered PWT technology (Bigoni, Calo, Guerrini, Strupish, Rowbotham & Lambert, 2001) has been applied to [Dmt[1]]N/OFQ(1-13)-NH_2 generating the tetrabranched derivative PWT2-[Dmt[1]]. In receptor binding studies PWT2-[Dmt[1]] displayed approximately 10 fold lower affinity than the parent peptide but maintained the same profile of selectivity. In functional studies performed with different assays PWT2-[Dmt[1]] always behaved, similar to [Dmt[1]]N/OFQ(1-13)-NH_2, as a full agonist. Moreover PWT2-[Dmt[1]] potency was in general similar to that of the parent peptide. Interestingly enough BRET studies investigating receptor/G-protein and receptor/β-arrestin 2 interaction demonstrated that the PWT chemical modifications promoted G-protein biased agonism at both NOP and MOP receptors. Thus this study corroborated previous findings (Guerrini et al., 2014), (Micheli et al., 2015), (Rizzi et al., 2014), (Rizzi et al., 2015) demonstrating that the PWT approach can be applied to the peptide sequence of different GPCR peptide agonists without modifying their selectivity of action and pharmacological activity. Moreover this study provided the scientific community a novel tool for investigating the consequences of the simultaneous activation of NOP and OP receptors under physiological as well as pathological conditions.

In receptor binding studies performed on membranes from cells expressing the recombinant human receptors standard ligands for NOP and OP receptors i.e. N/OFQ, dermorphin, dynorphin A and DPDPE displayed high affinity for their respective receptors with pK_i values in line with previous findings (Molinari et al., 2013). [Dmt[1]]N/OFQ(1-13)-NH_2 was able to displace radioligand binding at the four receptors showing the following rank order of affinity NOP = MOP = KOP > DOP. These results perfectly confirmed previous studies both in terms of absolute values and rank order of affinity (Molinari et al., 2013) corroborating the proposal of [Dmt[1]]N/OFQ(1-13)-NH_2 as an universal opioid receptor ligand. PWT2-[Dmt[1]] was also able to bind to NOP and classical opioid receptors. Interestingly in NOP membranes PWT2-[Dmt[1]] displaced an amount of radioactivity higher than N/OFQ. We interpret these perplexing results as due to the presence of displaceable non-specific binding, a phenomenon relatively common when N/OFQ or N/OFQ related peptides are used as radioligands (Dooley & Houghten,

2000). Compared to [Dmt[1]]N/OFQ(1-13)-NH_2, PWT2-[Dmt[1]] displayed approximately 10 fold lower affinity at all the receptors; as a consequence the profile of selectivity of PWT2-[Dmt[1]] is identical to that of parent peptide. These results are similar to those previously obtained with PTW2-N/OFQ that maintained the high NOP selectivity for NOP over OP receptors typical of the natural peptide (Guerrini et al., 2014). However it must be noted that PTW2-N/OFQ displayed slightly higher affinity than N/OFQ while the opposite is true for [Dmt[1]]N/OFQ(1-13)-NH_2 and PWT2-[Dmt[1]].

In stimulated [^{35}S]GTPγS experiments, standard agonists displayed high potency and efficacy for their receptors. Data obtained at NOP and MOP receptor are superimposable to our previous findings (DOP and KOP were not assessed in (Molinari et al., 2013)). [Dmt[1]]N/OFQ(1-13)-NH_2 mimicked the stimulatory effects of standard agonists producing a concentration dependent stimulation of [^{35}S]GTPγS binding and eliciting maximal effects not significantly different from the standards. As far as potency is concerned the following rank order of potency has been obtained NOP $\geq$ KOP $\geq$ MOP $\geq$ DOP which is similar to that obtained in receptor binding experiments and, limited to NOP and MOP, in previous [^{35}S]GTPγS experiments (Molinari et al., 2013). In parallel experiments PWT2-[Dmt[1]] mimicked the effects of [Dmt[1]]N/OFQ(1-13)-NH_2 displaying similar potency and maximal effects at all the four receptor. Thus these results demonstrated that the application of the PWT technology to the [Dmt[1]]N/OFQ(1-13)-NH_2 sequence does not modify its pharmacological activity i.e. full agonism. This finding is in line with a rather large series of previous studies in which the pharmacological activity of different peptide sequences including the full agonists N/OFQ (Guerrini et al., 2014), substance P, neurokinin A and B (Rizzi et al., 2014), neuropeptide S (Rizzi et al., 2015), dermorphin (F. Ferrari, unpublished), and the pure antagonist UFP-101 (A. Rizzi, unpublished) was perfectly maintained by PWT derivatives.

The calcium mobilization assay used to characterize the pharmacological profile of PWT2-[Dmt[1]] has been validated in previous studies. In fact, 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 to investigate the pharmacological profile of human classical opioid receptors although, in this case, the panel of ligands investigated was relatively small (Camarda & Calo, 2013). The results obtained in the above mentioned studies are virtually identical to those described in the literature using classical assays for G_i-coupled receptors such as

inhibition of cAMP levels or stimulation [^{35}S]GTPγS in cells expressing recombinant receptors, and inhibition of electrically induced contractions in isolated tissues. Moreover this assay has been successfully used to pharmacologically characterize several novel ligands for both classical opioid (Piekielna et al., 2015) (Piekielna et al., 2015) (Ben Haddou et al., 2014) (Perlikowska et al., 2014) (Ben Haddou et al., 2014) as well as NOP (Corrado et al., 2015) (Bird et al., 2015) (Guerrini et al., 2014) receptors.

In the calcium mobilization studies the standard ligands displayed values of potency and selectivity profile in line with previous studies (Camarda & Calo, 2013). [Dmt^{1}]N/OFQ(1-13)-NH_2 mimicked the stimulatory effects of standards at the four receptors displaying similar maximal effects and the following rank order of potency NOP > MOP = KOP > DOP. Superimposable results were previously obtained both in term of absolute values of potency and profile of selectivity (Molinari et al., 2013). PWT2-[Dmt^{1}] was also able to stimulate in a concentration dependent manner calcium mobilization in the four cell lines. Compared to [Dmt^{1}]N/OFQ(1-13)-NH_2 the tetrabranched derivative showed similar maximal effects and profile of selectivity but reduced potency by 3 to 30 fold. This result is in line with receptor binding findings but contrasts with those obtained in the stimulated [^{35}S]GTPγS binding assay. It is worthy of mention at this regard that similar findings i.e. reduced potency of PWT derivatives in the calcium assay compared to other functional assay have been previously obtained with N/OFQ (Guerrini et al., 2014), substance P, neurokinin A and B (Rizzi et al., 2014), and dermorphin (F. Ferrari, unpublished) but not with neuropeptide S (Rizzi et al., 2015). The calcium mobilization assay is based on the aberrant signalling generated by the chimeric protein that forces G_i coupled receptors to signal via the calcium pathway. Therefore, it is possible that the aberrant signalling may induce modifications of the pharmacological profile of the receptor. In previous studies (see references quoted above), a large panel of NOP and OP receptor ligands was evaluated in the calcium assay, and the results obtained were the same as those from classical G_i-based assays and tissue studies. Clearly this large evidence argues against the proposal of changes in the receptor pharmacological profile due to aberrant signalling. Thus, other reasons may account for the discrepant results regarding the potency of PWT derivatives estimated in the calcium mobilization compared to others assays. A feature which seems typical of PWT derivatives (but which has not been evaluated for PWT2-[Dmt^{1}]) is a rather slow kinetic of action as revealed in isolated tissue experiments. (Camarda et al., 2009) showed that the calcium mobilization assay tends to underestimate the potency of agonists characterized by a slow interaction with the NOP receptor such as

the peptides UFP-112, UFP-113, and ZP120, and the non-peptide Ro 64-6198. This phenomenon may derive from the non-equilibrium conditions that characterize the calcium assay. In fact, the relatively long time needed to obtain full receptor activation by slowly equilibrating agonists is not compatible with the rapid and transient nature of the calcium response. It is likely that this may account for the underestimation of the PWT derivative potency in the calcium assay. Interestingly, Charlton and Vauquelin (2010) (Charlton & Vauquelin, 2010) investigated this hypothesis in two different experimental systems modelled to mimic the [^{35}S]GTPγS binding and the calcium assay with two different agonists: a high affinity slowly associating ligand (L1) and a lower affinity fast onset ligand (L2). The simulation displayed an opposite rank order of potency of agonists in the two assays with L1 > L2 in the system mimicking the [^{35}S]GTPγS assay and L2 > L1 in the system mimicking the calcium assay (for details see Figure 4 in (Charlton & Vauquelin, 2010)). Thus, the results of this simulation match our experimental data obtained with PWT derivatives of various peptide sequences, thus suggesting that the underestimation of PWT compounds in the calcium assay is likely to derive from kinetic artefacts. It should be however added that no evidence about the possible slow kinetic of action of PWT2-[Dmt^{1}] is available; this aspect can be possibly evaluated in future studies performed with isolated tissue preparations.

The BRET assay used to investigate the ability of [Dmt^{1}]N/OFQ(1-13)-NH_2 and PWT2-[Dmt^{1}] to promote receptor interaction with G-protein and β-arrestin 2 has been set up in T. Costa laboratories (ISS, Rome). The first study with this assay investigated and compared the G-protein and β-arrestin 2 efficacy of a large series of MOP and DOP ligands (Molinari et al., 2010). Moreover the same assay was also used to study the molecular mechanisms of constitutive activity and inverse agonism at OP receptors (Vezzi et al., 2013). More recently this BRET assay has been extended to the NOP receptor by testing a rather large panel of NOP ligands encompassing full and partial agonism as well as pure antagonism activity (Corrado et al., 2015). The BRET NOP/G-protein assay has been also successfully used to select the best NOP ligands useful for inducing receptor stability and crystallogenesis. Using this strategy, two structures of the NOP receptor in complex with top candidate ligands SB-612111 and C-35 have been obtained (Miller et al., 2015). In addition this assay has been used to characterize in detail the signalling properties of the mixed NOP/MOP ligands DeNo (see section 2.1.2.) and cebranopadol (see section 2.2.1).

In line with previous findings (Corrado et al., 2015), (Molinari et al., 2010), in BRET experiments the standard peptides N/OFQ and dermorphin behaved as potent and selective agonists for NOP and MOP respectively in promoting receptor interaction both with G-protein and β-arrestin 2. [Dmt^1]N/OFQ(1-13)-NH_2 was able to similarly promote receptor interaction with G-protein and β-arrestin at NOP and MOP receptors. The effects of the peptide were similar to those of the standard both in term of potency and maximal effects. The results obtained with the standards and [Dmt^1]N/OFQ(1-13)-NH_2 with the BRET receptor/G-protein assay and with the [^{35}S]GTPγS binding assay are virtually superimposable. This confirms our previous findings obtained with a large panel of NOP (Corrado et al., 2015) and OP (Molinari et al., 2010) receptor ligands. On one hand, this result is not surprising, as both assays are performed in isolated membranes and both measure the same early event of the signalling cascade, i.e., receptor-mediated G-protein activation. On the other hand, the good correlation that has been found in this and previous studies is important, because it suggests that there is no dissociation between ligand-induced coupling to the G-protein and ligand-promoted changes in the nucleotide-exchange properties of the G-protein. Moreover, this robust agreement between receptor/G-protein coupling and receptor stimulated GTPγS binding further demonstrate that the BRET assay used in these studies provides a robust and precise assessment of the ligand ability to activate NOP or OP receptors.

As far as PWT2-[Dmt^1] is concerned, this compound displayed in BRET/G-protein experiments similar potency and efficacy as [Dmt^1]N/OFQ(1-13)-NH_2 at both NOP and MOP receptors while in BRET/□β-arrestin studies it displayed reduced potency (particularly at MOP) associated with reduced efficacy (particularly at NOP). Thus, as clearly showed by the bias plot, PWT2-[Dmt^1] behaved as a G-protein biased agonist both at NOP and MOP receptors. Similar results have been previously obtained by applying the PWT technology to N/OFQ. In fact an inversion in the rank order of potency between N/OFQ and PWT2-N/OFQ was measured in NOP/G-protein and NOP/β-arrestin studies. PWT2-N/OFQ was more potent than the natural agonist in promoting G-protein interaction, but less potent than N/OFQ in inducing arrestin interaction. Thus similar to PWT2-[Dmt^1] also PWT2-N/OFQ behaved as G-protein biased agonist. The interpretation of these findings is far from being obvious. In fact the N-terminal pharmacophoric peptide sequences i.e. Phe-Gly-Gly-Phe and Dmt-Gly-Gly-Phe of N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2, respectively, are identical in the PWT derivatives of the two peptides and recent receptor structure (Thompson et al., 2012), (Miller et al., 2015) and receptor modelling

(Daga & Zaveri, 2012), (Kothandan, Gadhe, Balupuri, Ganapathy & Cho, 2014) studies demonstrated that receptor activation is triggered by the occupation of the NOP receptor binding pocked by these sequences. Eventually when linked together into the PWT structure the N-terminal pharmacophoric sequences lose the ability to adopt some conformational states that are more important for promoting the interaction of the receptor with β-arrestin than with G-protein. However these are mere speculations that should be experimentally validated by solving the structure of the NOP receptor in complex with peptides and their PWT derivatives and G-protein and β-arrestin. Moreover the ability to promote G-protein biased agonism by applying the PWT chemical modification is not a general phenomenon. In fact this has been demonstrated for the PWT derivatives of N/OFQ and [Dmt^1]N/OFQ(1-13)-NH_2 but not for PWT2-dermorphin that in BRET experiments maintained the unbiased behaviour of the naturally occurring peptide dermorphin (F. Ferrari, unpublished).

In conclusion this study demonstrated that the application of the PWT technology to the peptide sequence of [Dmt^1]N/OFQ(1-13)-NH_2 generated a tetrabranched derivative that maintains the universal opioid agonist features of the parent peptide associated with a certain degree of G-protein biased agonism for MOP and NOP receptor. Thus this study provided the scientific community a novel tool for investigating the consequences of the simultaneous activation of NOP and OP receptors under physiological as well as pathological conditions. Considering that the most interesting characteristic of PWT derivatives of bioactive peptides is the ability to display *in vivo* high potency associated to long lasting effects (Micheli et al., 2015), (Rizzi et al., 2015), (Guerrini et al., 2014), (Rizzi et al., 2014), future studies aimed to evaluate the spinal antinociceptive properties of PWT2-[Dmt^1] are crucial and required.

2.1.2. DeNo

While the majority of clinical opioids mainly target the MOP receptor, work in cell and animal models would suggest targeting two or more opioid receptors simultaneously might produce drugs with reduced harmful effects. The NOP receptor is located throughout the pain pathways and has been shown to co-localise in the pain pathways with MOP (Schroder, Lambert, Ko & Koch, 2014). Activation of the NOP receptor has demonstrated several advantages over the classical opioid receptors. For instance, NOP agonists are able to efficiently treat neuropathic pain, a condition which classical opioid do not adequately treat (Lambert, 2008) (Schroder, Lambert, Ko & Koch, 2014). Furthermore, intrathecal co-administration of N/OFQ and morphine in non-human primates led to a potentiation of morphine-induced antinociception, without the associated morphine-induced side effects (itch) (Ko & Naughton, 2009). From a cellular aspect, MOP and NOP have been demonstrated to co-express in close proximity and display differential signalling activity *in vitro*, suggesting the formation of a heterodimer (Evans et al., 2010) (Wang et al., 2005). To further understand the interactions between MOP and NOP, an examination of the interactions of a full agonist dual-targeted drug need to be further examined. In order to further explore the interactions between MOP and NOP we have synthesized a mixed MOP/NOP agonist named DeNo (Figure 22). The MOP agonist component is provided by dermorphin, a peptide isolated from the skin of *Phyllomedusa* frogs (Montecucchi, de Castiglione, Piani, Gozzini & Erspamer, 1981). The NOP component is the endogenous agonist N/OFQ. We have assessed receptor binding, upstream and downstream signalling in cells and tissues and assessed *in vivo* spinal anti-nociceptive effects in rats.

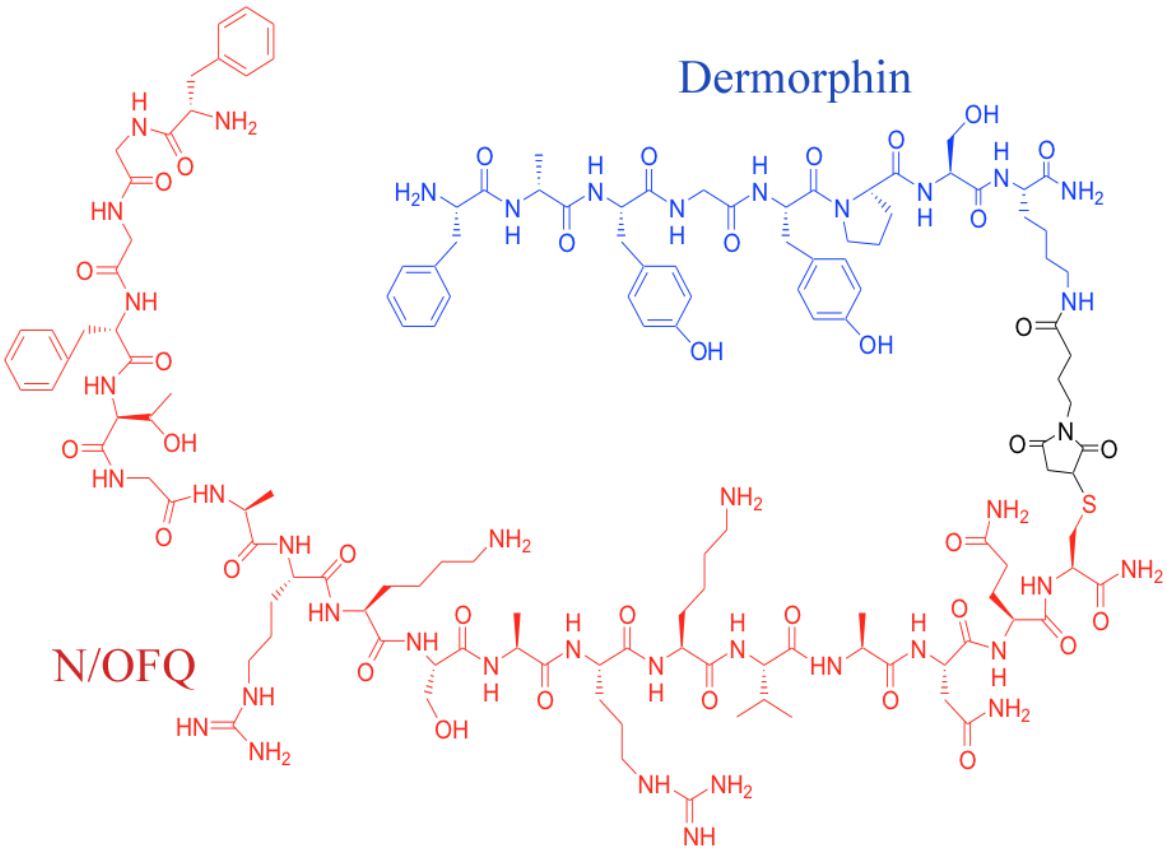


Figure 22. Chemical structure of the compound DeNo.

Materials and Methods

Cells Culture – Cells were grown in either Hams F12 (for Chinese Hamster Ovary; CHO_{MOP}, CHO_{DOP} and CHO_{KOP} cells), DMEM/Hams F12 1:1 (for CHO_{NOP} cells and SH-SY5Y cells) or DMEM (for HEK-293 cells). The media contained 100 µg ml^{-1} streptomycin, 2.5 µg ml^{-1} fungizone, 100 IU ml^{-1} penicillin and 10% foetal bovine serum. G418 (200 µg ml^{-1}) was used to maintain CHO cells expressing classical opioid receptors. Stock media containing G418 (200 µg ml^{-1}) and hygromycin B (200 µg ml^{-1}) was used to maintain CHO_{NOP} cells. HEK-293 cells permanently co-expressing the fusion proteins NOP-Rluc and Gβ1-RGFP or NOP-Rluc and β-arrestin 2-RGFP and SH-SY5Y cells co-expressing the fusion proteins MOP-Rluc and Gβ1-RGFP or MOP-Rluc and β-arrestin 2-RGFP were prepared using the pantropic retroviral expression system by Clontech as described previously (Molinari, Casella & Costa, 2008). Cell cultures were maintained at 37°C in 5% CO_2/humidified air. Cells were used for experiments once confluent. CHO cells stably co-expressing the human recombinant NOP or MOP receptors as well as the C-terminally modified $G\alpha_{qi5}$ protein were generated and used in calcium mobilisation studies

as previously described (Camarda et al., 2009) (Camarda & Calo, 2013). Cells were cultured in culture medium consisting of Dulbecco's MEM/Hams F12 (50/50) supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml). Cell cultures were kept at 37° C in 5% CO_2/humidified air.

Membrane preparation – In the radioligand displacement binding assays, homogenization/wash buffer consisting of 50 mM Tris-HCl pH to 7.4 with KOH, for CHO_{MOP}, CHO_{DOP} and CHO_{KOP} or additional 5 mM $MgSO_4$ for CHO_{NOP} was used. Homogenisation buffer (50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH) was used in GTPγ[^{35}S] assays. Membranes were centrifuged at 13,500 rpm for 10 min at 4°C. This process was repeated at least three times. The resulting pellet was resuspended in an appropriate amount of the necessary buffer and the proteirn concentration was determined by Lowry assay (Lowry, Rosebrough, Farr & Randall, 1951).

Displacement binding assay – Membrane protein (40 µg) was incubated in 0.5ml of 50 mM Tris, 0.5% BSA and ~0.8nM [^{3}H]-DPN (for CHO_{MOP}, CHO_{DOP} and CHO_{KOP}) or ~0.8nM [^{3}H]-UFP-101 (for CHO_{NOP} cells), as well as varying concentrations (10 µM-1pM) of the reference ligand DeNo. Non-specific binding was determined in the presence of 10 µM naloxone for CHO_{MOP}, CHO_{DOP} and CHO_{KOP} or 1µM of N/OFQ for CHO_{NOP} cells. Samples were incubated for 1 hr at room temperature and reactions were terminated by vacuum filtration, onto PEI-soaked Whatman GF/B filters, using a Brandel harvester. The concentration of displacing ligand producing 50% displacement was corrected for the competing mass of radioligand to yield pK_i, a measure of its affinity (Bird et al., 2015).

Calcium mobilisation assay – When confluence was reached (3-4 days), cells were seeded at a density of 50,000 cells/well into 96-well black, clear bottom plates. After 24 hs 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. Afterwards, the loading solution was aspirated and 100 µl/well of assay buffer (Hank's balanced salt solution supplemented with 20 mM HEPES, 2.5 mM probenecid, and 500 µM Brilliant Black (Aldrich)) was added. Serial dilutions of ligands were made in Hank's balanced salt solution/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 37°C. Online additions were carried out in a volume of 50µl/well. Maximum change in fluorescence, expressed in percent of baseline fluorescence, was used to determine agonist response (Camarda & Calo, 2013).

GTPγ[^{35}S] binding assay – Membrane protein (40 µg) was incubated in 0.5 ml volume of 50 mM Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 100 mM NaCl, 0.1% BSA, 0.15 mM bacitracin; pH 7.4, GDP (33 µM), and ~150 pM GTPγ[^{35}S]. Varying concentrations of reference ligands (dermorphin, N/OFQ, dynorphin A and Leu-enkephalin) DeNo (1 pM - 10µM) was added prior to incubation. Non-specific binding was determined in the presence of unlabeled GTPγS (10µM). Samples were incubated at 30°C for 1 h with gentle agitation. Reactions were terminated by vacuum filtration through dry Whatman GF/B filters, using a Brandel harvester (Bird et al., 2015).

cAMP assay – CHO_{MOP} and CHO_{NOP} whole cells were suspended in Krebs/HEPES buffer, containing isobutylmethylxanthine (1mM) and forskolin (1µM). For MOP, dermorphin and DeNo were included at 1µM concentrations. At NOP, N/OFQ and DeNo were included at 1µM concentrations. cAMP was extracted and assayed using a protein binding assay as described previously (Kitayama et al., 2007).

Western blotting-MAPK detection – ERK1/2 and p38 MAPK activity in CHO_{MOP} and CHO_{NOP} cells was detected by Western blotting techniques. CHO_{MOP} and CHO_{NOP} cells were serum starved for 24 hours prior to treatment. Drugs were added for 15 minutes in Krebs Buffer (composition: 115mM NaCl, 4.7mM KCl, 2mM $CaCl_2$, 1.2mM $MgCl_2$, 25mM $NaHCO_3$, 8mM glucose). For MOP: dermorphin and DeNo were added at 1µM concentrations. At NOP: N/OFQ, and DeNo were added at 1µM concentrations. Signalling was terminated via Lysis Buffer [Tris-HCl (pH 7.4), 20 mm; 1% (vol/vol); Triton X-100, 10% (vol/vol); glycerol, NaCl, 137 mm; EDTA, 2 mm; β-glycerophosphate, 25 mm; sodium orthovanidate; 1 mm; phenylmethanesulfonylfluoride, 500 µm; leupeptin, 0.1 mg/ml; benzamidine, 0.2 mg/ml; pepstatin, 0.1 mg/m followed by centrifugation (13,000 rpm, 10 minutes, 4^{o}C), with the supernatant removed and added to an equal volume of 2XSDS buffer (composition: 100mM Tris-HCl (pH 6.8), 2% SDS, 10% Glycerol, 0.1% Bromophenol Blue). Samples were denatured (heated 100^{o}C for 5 minutes) and separated by 10% SDS-PAGE; transferred onto nitrocellulose paper in a semi-dry buffer

(composition: 48mM Tris Base, 39mM Glycine, 0.037% w/v SDS, 20% Methanol) and blocked using conventional western blotting techniques. To detect phosphorylated ERK1/2 and phosphorylated p38 activity, pERK1/2 antibodies (1:6000 dilution) and p38 antibodies (1:3000 dilution), diluted in TBS-T solution (50 mm Tris-base, 150 mm NaCl, 0.1% Tween-20 (vol/vol), pH 7.5) with 0.01% (wt/vol) BSA, were used to probe the membrane and left overnight at 4°C. Horseradish peroxidase-conjugated secondary anti-rabbit antibodies (1hr room temperature, 1:1000 dilution in TBS-T with 5% milk) were used to visualise immune-reactive bands, followed by chemiluminescence detection using the ChemiDoc™ MP Imaging System (Bio-Rad). In order to ensure equal gel loading, membranes were stripped and reprobed for total ERK1/2 and total p38 MAPK. Membranes were incubated in Restore Plus™ (Fisher) for 15 minutes then thoroughly washed in TBS-T, following which they were blocked as previously described. The membrane was then probed using the specific antibody for ERK 1/2 (NEBL, 1:3000 dilution), or p38 (NEBL, 1:3000 dilution), overnight (4°C) followed by addition of the secondary antibody and chemiluminescence detection. Normalisation of total protein levels for each sample was then achieved by representing levels of pERK1/2 and p-p38 as a proportion of total ERK1/2 or total p38 protein. Data were analysed using the "Origin 9" software and images analysed using Image Lab software (Bio-Rad, UK) (Brighton, Rana, Challiss, Konje & Willets, 2011).

BRET Assay – Membrane extracts taken from HEK-293 and SH-SY5Y cells stably expressing respectively NOP-RLuc and MOP-RLuc together with $G\beta_1$-RGFP were used to assess the effects of drugs on receptor/G-protein interaction in concentration response curve experiments. For G-protein experiments enriched plasma membrane aliquots from transfected cells were prepared by differential centrifugation; cells were detached with PBS/EDTA solution (1 mM, pH 7.4 NaOH) then, after 5 min 500 g centrifugation, Dounce-homogenized (30 strokes) in cold homogenization buffer (TRIS 5 mM, EGTA 1 mM, DTT 1 mM, pH 7.4 HCl) in the presence of sucrose 0.32 M. Three following centrifugations were performed at 1000 g (4°C) and the supernatants kept. Two 25,000 g (4°C) subsequent centrifugations (the second in the absence of sucrose) were performed for separating enriched membranes that, after discarding the supernatant were kept in ultrapure water at -80°C (Vachon, Costa & Herz, 1987). The protein concentration in membrane preparations was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and a Beckman DU 520 spectrophotometer (Brea, CA, USA). Luminescence

in membranes was recorded in 96-well untreated white opaque microplates (PerkinElmer, Waltham, MA, USA) using the Victor 2030 luminometer (PerkinElmer, Waltham, MA, USA). For the determination of receptor/G-protein interaction, membranes (3 μg of protein) prepared from cells co-expressing NOP or MOP-RLuc and Gβ1-RGFP were added to wells in Dulbecco's PBS. For the determination of receptor/β-arrestin 2 interaction, cells co-expressing NOP-RLuc and β-arrestin 2-RGFP were plated 24 hs before the experiment in poly-D-Lysine treated plates (100,000 cells/well), while for MOP expressing cells untreated plates were used. The cells were prepared for the experiment substituting the medium with DPBS supplemented with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM). Coelenterazine at a final concentration of 5 μM was injected 15 minutes prior to reading the cell plate. Receptor/G-protein interaction was measured in cell membranes to exclude the participation of other cellular processes (i.e. arrestin recruitment, internalization). Different concentrations of ligands in 20 μL of PBS - BSA 0.03% were added and incubated for an additional 5 min before reading luminescence. All the experiments were performed at room temperature.

Guinea pig ileum bioassay – With approval of Animal Subjects Review Board of the University of Ferrara and from the Italian Ministry of Health (PROT-186) ileum tissues were taken from male albino guinea pigs of 350 – 400 g (Pampaloni, Pisa, Italy). The animals were treated in accordance with European guidelines (86/609/ECC) and national regulations (DL 116/92). They were housed in 560 x 320 x 180 mm cages (Techinplast), three per cage, under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 7:00 h) with food (complete feed for guinea pig, Mucedola, Milano, Italy) and water ad libitum. The day of the experiment the animals were killed with an isofluorane overdose. Bioassay experiments were performed as previously described (Bigoni et al., 1999). The tissues were suspended in 5 ml organ bath containing Krebs solution (composition in 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), hexamethonium bromide 22 μM, benadril 0.34 μM oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 37°C. At resting tension 1 g was applied to the tissues. Tissues were stimulated through two platinum electrodes with supramaximal rectangular pulse of 1 ms duration, 0.05 Hz frequency, 80 V of amplitude. Electrically evoked contractions were measured isotonically by means of Basile strain gauge transducers (Basile 7006; srl Ugo Basile, Varese, Italy) and recorder with a computer-based acquisition system (Power Lab 8, ADInstruments, USA). 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 agonists were constructed (0.5 log unit steps). Antagonists were injected into the baths 15 minutes before constructing agonist concentration response curves.

Animals – For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy), weighing approximately 280-300g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. One rat was housed per cage (size 26x41 cm); animals were fed with standard laboratory diet and tap water ad libitum, and kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence and from the Italian Ministry of Health (N°54/2014-B). Experiments involving animals have been reported according to ARRIVE guidelines (Kilkenny et al., 2011). All efforts were made to minimize animal suffering and to reduce the number of animals used. All animals were monitored daily using a scoring system (based on: Appearance, Food and Water Intake, Clinical Signs, Natural Behaviour and Provoked Behaviour (Wolfensohn S, 2008). Maximum score is 20 and animals reaching 10 are euthanized. In the experiments reported here no animals reached this score and none died before the end of the experiment) and at the end of the experiment were euthanized by CO_2 overdose.

Intrathecal catheterization – Rats were anesthetized with 2% isoflurane and an intrathecal catheter was surgically implanted according to the method of Yaksh & Rudy (1976) (Yaksh & Rudy, 1976). Rats were shaved on the back of the neck and placed in the stereotaxic frame with the head securely held between ear bars. The skin over the nap of the neck was cleaned with ethyl alcohol and incised for 1 cm. The muscle on either side of the external occipital crest was detached and retracted to expose about 3-4 mm^2 of the atlanto-occipital membrane. The membrane was incised by a needle, which led to the

escape of cerebrospinal fluid. The caudal edge of the cut was lifted and about 7.0 cm of 28G polyurethane catheter (0.36 mm outer diameter; 0.18 mm inner diameter; Alzet, USA) was gently inserted into the intrathecal space in the midline, dorsal to the spinal cord until the lumbar enlargement. The exit end of the catheter was connected to 4.0 cm polyurethane (0.84 mm outer diameter; 0.36 mm inner diameter) and was taken out through the skin, flushed with saline solution, sealed and securely fixed on the back of the head with a silk wire. Animals were placed in individual cages until recovery. All animals used during behavioural tests did not show surgery induced motor impairment as evaluated by rota rod test. Animals who presented any kind of motor disability were excluded from the behavioural measurements. Behavioural measurements were performed on 5 rats for each treatment.

Intrathecal drug treatments – Dermorphin and DeNo were dissolved in sterile saline solution. Acute measures were performed after the intrathecal (i.t.) infusion of 0.1 - 1 nmol dermorphin and DeNo. All compounds were administered in a final volume of 10 µl. Behavioural tests were carried out after 15, 30, 45, 60, 90, 120 and 180 min.

Paw Pressure test – Nociceptive threshold was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. (1988) (Leighton, Rodriguez, Hill & Hughes, 1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40g or over 75g during the test before drug administration were rejected. For analgesia measures, mechanical pressure application was stopped at 150g. A researcher blind to drug treatment performed all experiments.

Rota rod test – Rota rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6cm and a non-slippery surface. The rod was placed at a height of 25cm from the base. The rod, 36cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the

basis of the number of falls from the rod in 60s measured 15, 30, 45, 60, 90, 120 and 180 min after treatments.

Materials – The reference molecules, dermorphin, Leu-enkephalin, dynorphin A and N/OFQ were synthesised in house (Department of Chemical and Pharmaceutical Sciences, University of Ferrara). Tritiated UFP-101 ([^{3}H]-UFP-101) was synthesized as described previously (Ibba et al., 2008). Tritiated diprenorphine ([^{3}H]-DPN) and [^{3}H]-cyclic Adenosine monophosphate (cAMP) were purchased from Perkin Elmer. Naloxone was purchased from Sigma-Aldrich Co. (Dorset, U.K.). Naltrindole (DOP antagonist) and [D-Pen^2, D-Pen^5]-enkephalin (DOP agonist, DPDPE) were purchased from Tocris (Abingdon, UK). Antibodies and protein ladders were purchased from Cell Signalling (Boston, MA, USA). All tissue culture media and supplements were obtained from Invitrogen (Paisley, U.K.).

Synthesis of DeNo – DeNo was assembled using a classical thiol-Michael reaction; reacting in solution a thiol function inserted in the N/OFQ sequence with a maleimide function inserted into the dermorphin sequence. In detail, [Cys^{18}]N/OFQ-NH_2 was synthesized and purified in house while the synthesis of dermorphin elongated in the C-terminal with Lys(maleimide) (Figure 22) was performed using classical solid phase peptide synthesis techniques (Calo et al., 2005). Selective deprotection of the Lys side chain of the intermediate [Lys(Dde)^8]dermorphin-resin was achieved following the procedure of Bycroft et al (Barrie W. Bycroft, 1993). To a suspension of protected [Lys(Dde)^8] Der-resin (300 mg) in anhydrous tetrahydrofuran (3 ml), 2% hydrazine in methanol (5 ml) was added. The reaction mixture was stirred under argon for 30 min, then filtered and the resin washed 3 times with dimethylformamide (DMF, 5 ml) and CH_2Cl_2 (5 ml). To a stirred solution of 4-(2,5-dioxo-2,5-dihydro-1Hpyrrol- 1-yl)butanoic acid (0.2 mmol) in (DMF) (5 mL) at 0° C, [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) (0.2 mmol) and 4-methylmorpholine (0.2 mmol) were added. After 10 min, [Lys(free side chain)^8]dermorpin-resin (200 mg, 0.67 meq/gr; 0.13 meq) was added and the reaction mixture stirred at room temperature for 4 hs. The solution was then filtered and the resin washed 5 times with DMF (5 ml) and 3 times with CH_2Cl_2 (5 ml). The protected [Lys(Mal)^8]dermorphin-resin was treated with reagent B (trifluoroacetic acid (TFA) / H_20 / phenol / triisopropylsilane 88 : 5 : 5: 2; v/v; 10 mL / 0.2 g of resin) for 1.5 h at room temperature (Sole', 1992). After filtration of the resin, the

solvent was concentrated under vacuum and the residue triturated with ethyl ether. Crude peptide was purified by preparative reverse phase HPLC and lyophilized. Finally, [Cys^{18}]N/OFQ-NH_2 was reacted with [$Lys(Mal)^8$]dermorphin following a procedure reported in literature and then purified by preparative HPLC to give the desired final product after lyophilisation (Guerrini et al., 2014).

Data analysis – Data are expressed as Mean ± SEM or with confidence intervals as appropriate. For more than 2 groups data are analyzed using ANOVA with post-hoc testing using Dunnett's test as appropriate. Where there are only 2 groups paired or un paired t-tests were used. *P* values of less than 0.05 were considered significant. All curve fitting was accomplished using Graphpad-Prizm (V6). The concentration of drug producing 50% of the maximum response (pEC_{50}) and the maximum response (E_{max}) are quoted. In gpI experiments the antagonist potency (pK_b) is calculated from the rightward shift of the agonist concentration response curve by a fixed antagonist concentration.

Results

Displacement binding assay – In displacement binding studies at CHO_{NOP}, DeNo displaced [^{3}H]-UFP-101 in a concentration dependent and saturable manner. DeNo (10.22) displayed a similar pK_i value, for NOP, to its parent compounds N/OFQ (10.69) (Figure 23A). At CHO_{MOP}, dermorphin and DeNo displaced the binding of [^{3}H]-DPN in a concentration dependent and saturable manner DeNo (pK_i 9.55) demonstrated a significant increase in affinity at MOP, when compared to the parent compound dermorphin (8.69) (Figure 23B). Furthermore, DeNo (7.34) showed affinity for the KOP receptor, whereas the parent compounds (dermorphin and N/OFQ) failed to displace [^{3}H]-DPN at this receptor (Figure 23C). At CHO_{DOP}, DeNo (8.12) demonstrated an increase in affinity compared to its parent compounds. Dermorphin displayed an affinity of 7.17, while N/OFQ failed to displace [^{3}H]-DPN at the DOP receptor (Figure 23D). All data are summarized in Table 9.

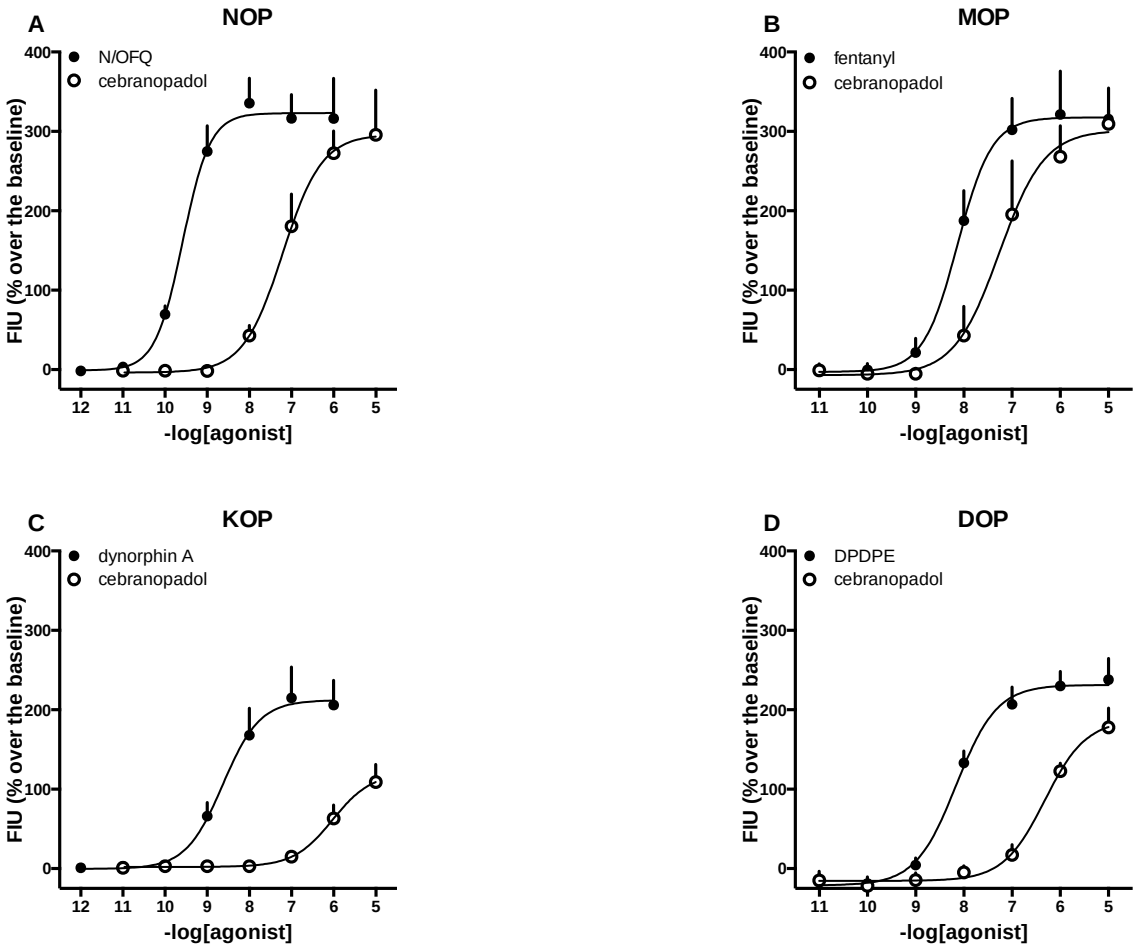


Figure 23. Displacement binding experiments. Displacement of [^{3}H]UFP-101 at CHO_{NOP} cell membranes and of [^{3}H]DPN at $CHO_{MOP/KOP/DOP}$ by respective control ligand and DeNo. Data are means ± SEM of n ≥ 5 experiments for all cell lines.

Table 9. Displacement binding experiments. pK_i values for N/OFQ, dermorphin and DeNo in CHO cells expressing the NOP and classical opioid human recombinant receptors.

	NOP	MOP	KOP	DOP
		pK_i(± SEM)		
N/OFQ	10.69 (±0.10)	inactive	inactive	inactive
dermorphin	inactive	8.69 (±0.10)	7.17 (±0.11)	inactive
DeNo	10.22 (±0.09)	9.55* (±0.10)	7.34* (±0.13)	8.12* (±0.11)

inactive means that the compound was inactive up to 1 µM.*Significantly different from the reference ligands, $p < 0.05$ using one-way ANOVA with Dunnett's correction. Control ligands N/OFQ, dermorphin, dynorphin A and naltrendole used for the NOP, MOP, KOP and DOP receptors respectively.

Calcium mobilisation assay – In CHO cells stably expressing the $G\alpha_{qi5}$ chimeric protein and the human NOP receptor, N/OFQ evoked a concentration dependent stimulation of calcium release with high potency (pEC_{50} 9.85) and maximal effect (279% over basal). DeNo showed similar potency (pEC_{50} 9.69) and maximal effect (244% over basal) to N/OFQ (Figure 24A and Table 10). In CHO_{MOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein, dermorphin produced a concentration-dependent stimulation of calcium mobilisation with high potency (pEC_{50} 8.07) and maximal effects (234% over basal). DeNo showed similar maximal effect (209% over basal) but approximately 10-fold lower potency (pEC_{50} 7.17) (Figure 24B and Table 10).

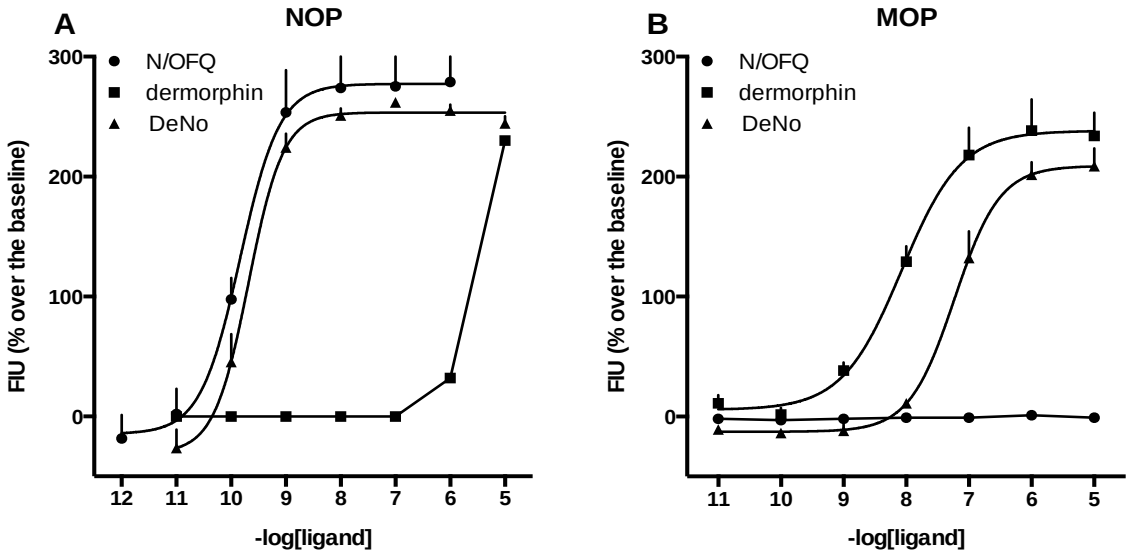


Figure 24. Concentration response curves to N/OFQ, dermorphin and DeNo in calcium mobilisation assay using CHO_{NOP} cells (A) and CHO_{MOP} cells (B). Data are the mean ± SEM of at least three separate experiments.

Table 10. Effects of N/OFQ, dermorphin and DeNo in calcium mobilisation experiments performed in CHO cells stably expressing the human NOP or MOP receptor and the chimeric $G\alpha_{qi5}$ protein.

	NOP		**MOP**	
	pEC_{50} ($CL_{95\%}$)	**E_{max} ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± SEM**
N/OFQ	9.85 (9.37-10.33)	279 ± 44 %	inactive	
dermorphin	crc incomplete		8.07 (7.85-8.29)	234 ± 14 %
DeNo	9.69 (9.35-10.03)	244 ± 6 %	7.17 (6.91-7.43)	209 ± 15 %

inactive means that the compound was inactive up to 1 µM.
crc incomplete means that maximal effects could not be determined due to the low potency of the compound.

GTPγ[^{35}S] assay – At CHO_{NOP}, N/OFQ and DeNo stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner. DeNo produced a maximal response (E_{max} 2.49) similar to that of its parent compound, N/OFQ (2.57). The pEC_{50} value of 9.50 achieved by DeNo was similar to that of N/OFQ (9.05) (Figure 25A). Dermorphin and DeNo stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner at the MOP receptor. DeNo (E_{max} 2.68) demonstrated a similar maximal response to that of dermorphin (2.63). The pEC_{50} values for DeNo (7.77) showed no significant difference to that of the parent compound, dermorphin (7.83) (Figure 25B). At CHO_{KOP}, dynorphin A and DeNo stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner. DeNo (E_{max} 2.36) produced a maximal response similar to that of dynorphin A (2.33). The pEC_{50} value of DeNo (5.91) was significantly lower than that of dynorphin A (9.36) (Figure 25C). Leu-enkephalin and DeNo stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner in membranes expressing DOP receptors. DeNo (E_{max} 1.84) produced a maximal response similar to that of the endogenous DOP receptor peptide, Leu-enkephalin (1.90). However, the pEC_{50} value for DeNo (6.78) was significantly lower than that of Leu-enkephalin (8.50) (Figure 25D). All data are summarized in Table 11.

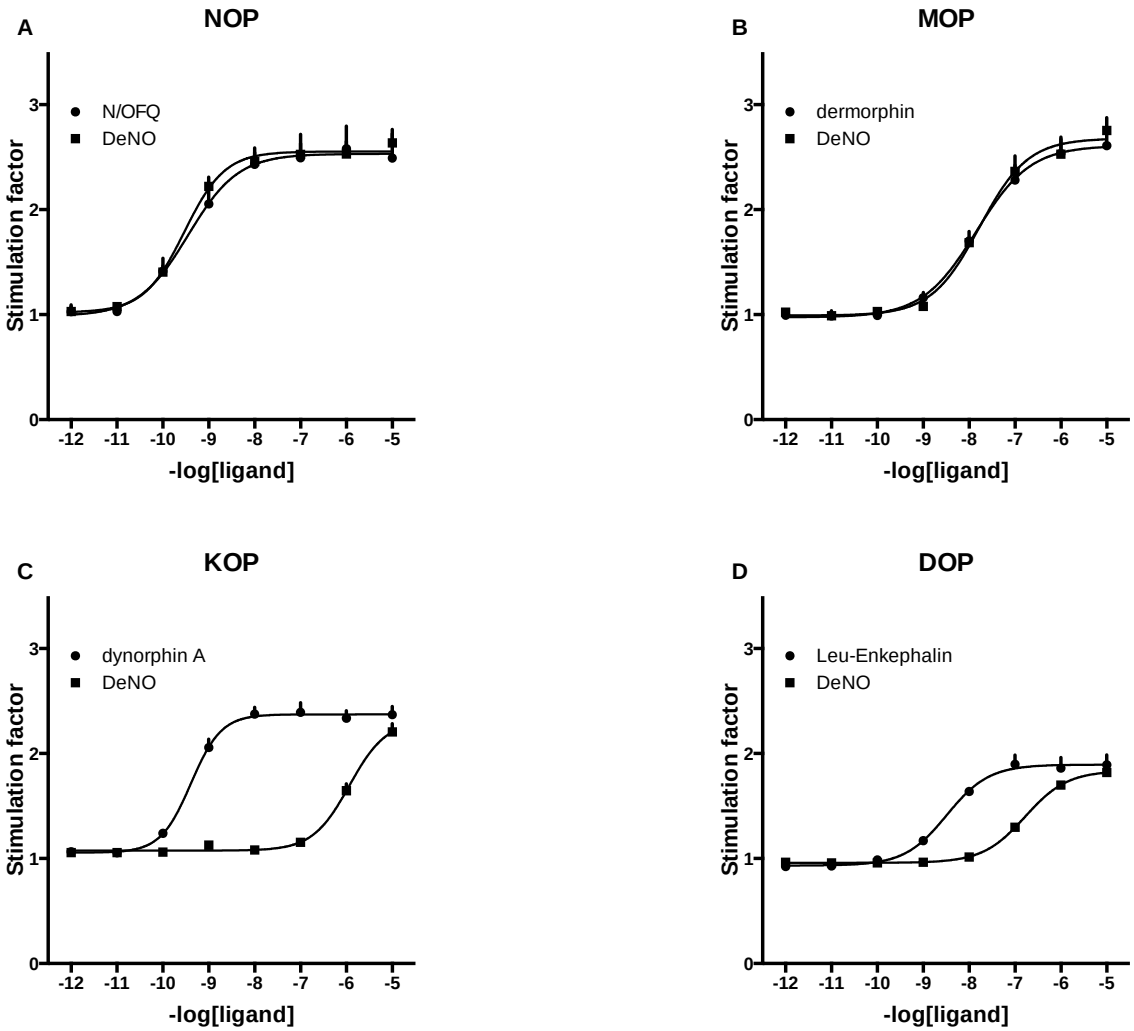


Figure 25. [^{35}S]GTPγS binding experiments. Concentration response curves to respective control ligand and DeNo in membranes of CHO cells stably expressing the NOP and classical opioid human recombinant receptors. Data are mean ± SEM for n=5 experiments.

Table 11. Agonist activity of DeNo in [^{35}S]GTPγS assay performed in CHO cells expressing the NOP and classical opioid human recombinant receptors.

	NOP			MOP			KOP			DOP		
	pEC_{50} ($CL_{95\%}$)	**E_{max} ± SEM**	**α**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± SEM**	**α**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± SEM**	**α**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± SEM**	**α**
Control	9.05 (8.77-9.34)	2.57 ± 0.17	1.00	7.83 (7.45-8.21)	2.61 ± 0.14	1.00	9.34 (9.22-9.49)	2.30 ± 0.09	1.00	8.50 (8.19-8.81)	1.90 ± 0.10	1.00
DeNo	9.50 (9.33-9.68)	2.49 ± 0.25	0.96	7.77 (7.54-7.80)	2.68 ± 0.12	1.03	5.92* (5.78-6.02)	2.23 ± 0.14	0.97	6.78* (6.59-6.97)	1.84 ± 0.03	0.97

*Significantly different from the control ligand, $p < 0.05$ using one-way ANOVA with Dunnett's correction. Intrinsic activity (α) relative to full agonist control ligand. Control ligands: N/OFQ, dermorphin, dynorphin A and naltrendole used for the NOP, MOP, KOP and DOP receptors respectively.

Cyclic AMP assay – To further assess functional activity of DeNo, inhibition of cyclic AMP (cAMP) formation was assessed. Since DeNo demonstrated a higher affinity and potency at NOP and MOP, assays were performed using CHO_{NOP} and CHO_{MOP} cells only. In CHO_{NOP} cells, forskolin stimulation leads to a 21.23 (±3.86) fold increase in cAMP production, when compared to basal activity. The addition of 1µM N/OFQ reverses forskolin stimulated cAMP production. The addition of 1µM DeNo has a similar effect, returning cAMP levels to basal. Addition of forskolin in CHO_{MOP} cells lead to a 24.3 (±1.79) fold increase in cAMP production, when compared to basal activity. Co-incubation of 1µM dermorphin, or 1µM DeNo reverses the effects of forskolin, returning cAMP levels to basal.

Detection of MAPK activity through Western blot Densitometry – In CHO_{MOP} cells, basal activity of phosphorylated p38 (p-p38) was 9.67 ± 1.39% of total. Following stimulation by 1µM dermorphin, this activity rose to 25.02 ± 1.18%, this was statistically significant. Administration of DeNo (28.41 ± 1.27%) led to a similar increase in p-p38 activity. In phosphorylated ERK1/2 (p-ERK1/2) assays, basal activity was measured at 2.75 ± 0.83% of total. The addition of 1 µM dermorphin (34.23 ± 4.97%) or DeNo (34.30 ± 4.5%) produced a statistically significant increase over basal activity (Figure 26A). In CHO_{NOP} cells, phosphorylation of p38 was not detected. In studies assessing the activation of p-ERK1/2, basal activity was measured at 4.24 ±1.26% of total. The addition of 1µM N/OFQ (63.08 ±7.97%) or DeNo (70.46 ±10.63%) produced a statistically significant increase over basal activity (Figure 26B).

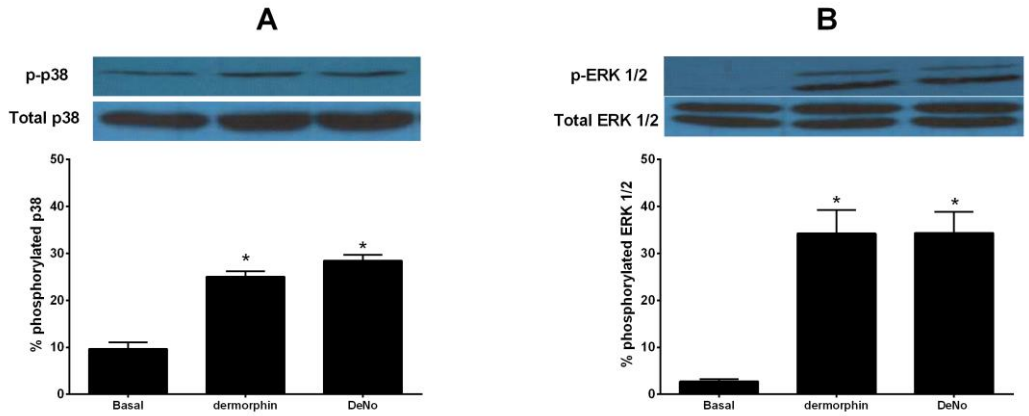


Figure 26. A) The activity of p38 phosphorylation compared to total p38 at CHO_{MOP} caused by dermorphin (1µM) and DeNo (1µM). B) The activity of ERK1/2 phosphorylation compared to total ERK1/2 at CHO_{MOP} caused by dermorphin (1µM) and DeNo (1µM). Data are mean ± SEM for n=5. $p<0.05$, according to ANOVA followed by Dunnett's test for multiple comparison.

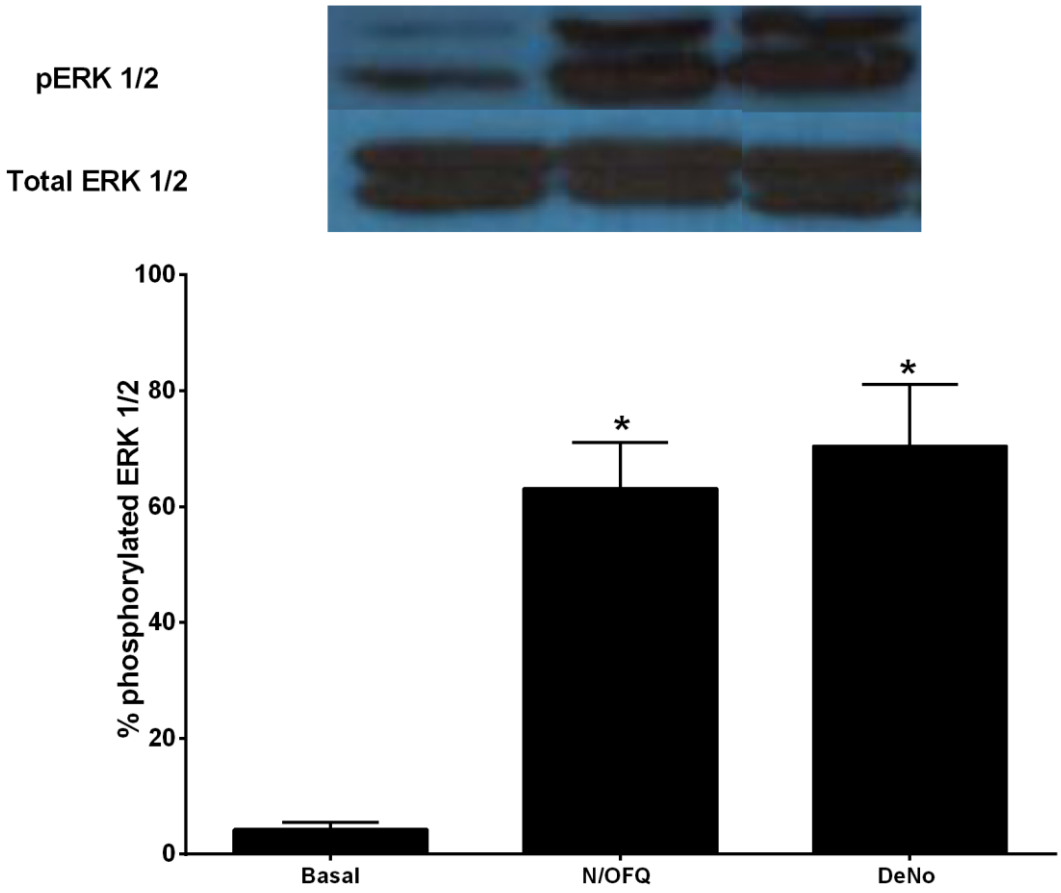


Figure 27. The activity of ERK1/2 phosphorylation at CHO_{NOP} caused by N/OFQ (1μM) and DeNo (1μM). Data are mean ± SEM) for n=5. *p<0.05; according to ANOVA followed by Dunnett's test for multiple comparison.

BRET assay – In HEK-293 membranes, N/OFQ promoted NOP/G-protein interaction in a concentration-dependent manner with high potency (pEC_{50} 9.22) and a maximal effect corresponding to 0.42 ± 0.04 stimulated BRET ratio. Dermorphin was very weak only increasing BRET ratio at micromolar concentrations (Figure 28A). In SH-SY5Y membranes, dermorphin produced a concentration dependent stimulation of the G-protein interaction, also with high potency (pEC_{50} 8.13). In this cell type the maximal effect was larger at 1.39 ± 0.14 stimulated BRET ratio. N/OFQ was very weak increasing BRET ratio only at micromolar concentrations (Figure 28B). Under the same experimental conditions, DeNo was tested in both cells lines. In HEK-293 membranes, DeNo mimicked the stimulatory effect of N/OFQ with similar potency (9.02) and maximal effect (α 1.01) (Figure 28A). In SH-SY5Y membranes, DeNo concentration dependently increased BRET ratio with similar potency and maximal effect (pEC_{50} 8.03 and α 0.98) to dermorphin (Figure 28B). All data are summarized in Table 12.

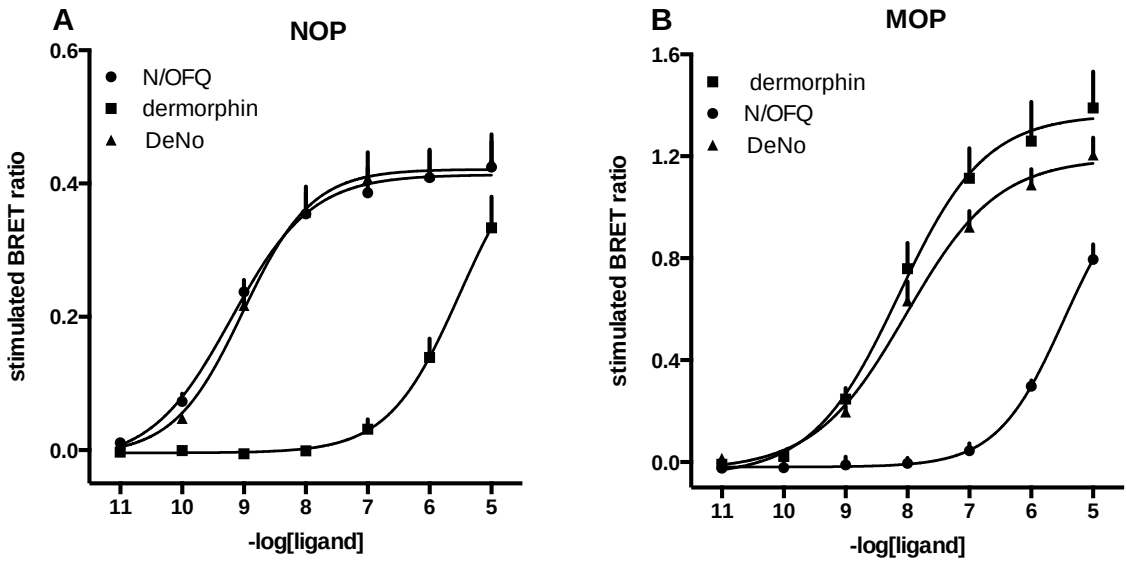


Figure 28. Concentration response curves to N/OFQ, dermorphin and DeNo for receptor/G protein interaction in membranes of cells expressing the NOP (A) and MOP (B) receptors. Data are the mean ± SEM of at least 3 experiments performed in duplicate.

Table 12. Potencies (pEC_{50}) and efficacy (α) of standard agonists and DeNo in promoting NOP/Gβ1-protein and MOP/Gβ1-protein interactions.

	Gβ1-protein			
	NOP		**MOP**	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
N/OFQ	9.22 (9.14-9.30)	1.00	crc incomplete	
dermorphin	crc incomplete		8.13 (7.87-8.39)	1.00
DeNo	9.02 (8.87-9.17)	1.01 ± 0.01	8.01 (7.39-8.63)	0.98 ± 0.03

N/OFQ and dermorphin were used as reference agonists for calculating intrinsic activity at NOP and MOP receptors respectively. crc incomplete means that maximal effects could not be determined due to the low potency of the compound.

HEK-293 and SH-SY5Y cells stably expressing the human NOP or MOP (NOP/MOP-RLuc) receptors and β-arrestin 2-RGFP were used to evaluate NOP and MOP interaction with β-arrestin 2. In HEK-293 cells, N/OFQ promoted NOP/β-arrestin 2 interaction with high potency (pEC_{50} 8.21) and maximal effect corresponding to 0.092 ± 0.003 stimulated BRET ratio. Dermorphin was completely inactive (Figure 29A). In SH-SY5Y cells

dermorphin stimulated MOP β-arrestin 2 interaction with a potency (pEC_{50}) of 7.00. Maximal effect was 0.43 stimulated BRET ratio (again in this cell line simulated ratio was larger). N/OFQ was completely inactive (Figure 29B). Under the same experimental conditions in HEK-293 cells DeNo mimicked the stimulatory effect of N/OFQ (pEC_{50} 8.19 and α 1.03) (Figure 29A). In SH-SY5Y cells DeNo promoted MOP/β-arrestin 2 interaction mimicking the stimulatory effect of dermorphin (α 0.98) but with 4 fold lower potency (pEC_{50} 6.36) (Figure 29B). All data are summarized in Table 13.

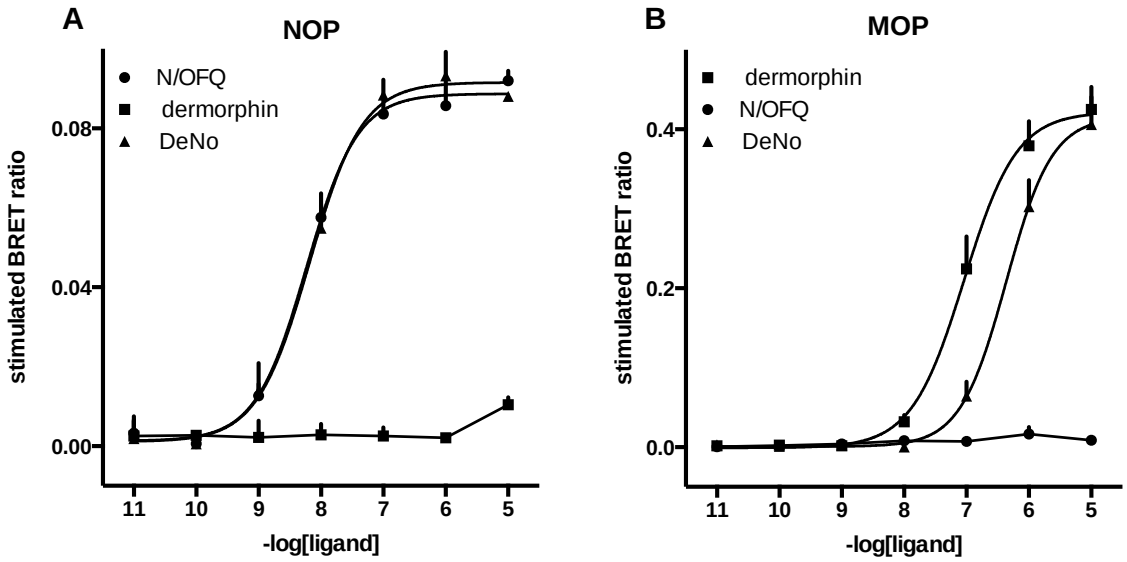


Figure 29. Concentration response curves to N/OFQ, dermorphin and DeNo for receptor/β-arrestin 2 interaction in cells expressing the NOP (panel A) and MOP (panel B) receptors. Data are the mean ± SEM of at least 4 experiments performed in duplicate.

Table 13. Potencies (pEC_{50}) and efficacy (α) of standard agonists and DeNo in promoting NOP/β-arrestin 2 protein and MOP/β-arrestin 2 protein interactions.

	β-arrestin 2			
	NOP		**MOP**	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
N/OFQ	8.21 (7.97-8.45)	1.00	inactive	8.21 (7.97-8.45)
dermorphin	inactive	7.00 (6.59-7.42)	1.00	inactive
DeNo	8.19 (7.93-8.46)	1.03 ± 0.02	6.36 (6.08-6.63)	8.19 (7.93-8.46)

N/OFQ and dermorphin were used as reference agonists for calculating intrinsic activity at NOP and MOP receptors respectively. inactive means that the compound was inactive up to 1 μM.

Guinea pig ileum bioassay – DeNo was assessed in the electrically stimulated guinea pig ileum. In this preparation, concentration response curves to DeNo were assessed in comparison with N/OFQ and dermorphin (Figure 30A). N/OFQ inhibits contractions induced by electrical field stimulation in a concentration dependent manner, with a pEC_{50} of 8.03 (7.84-8.23) and maximal effect of 71 ± 4%. Dermorphin mimicked the effects of N/OFQ with higher potency and maximal effects (pEC_{50} 9.55 (9.33-9.77) E_{max} 86 ± 2%). The new compound, DeNo, inhibited electrically induced twitch, with a potency of 8.63 and maximal effects similar to those of dermorphin (E_{max} 90 ± 1%). In order to determine the site of action of DeNo in the guinea pig ileum, antagonist assays were undertaken. The standard non-selective opioid antagonist naloxone (100 nM) does not affect the inhibitory action of N/OFQ, while 100 nM SB-612111 was able to shift to the right the concentration response curve to N/OFQ with a pK_b of 8.36 (Figure 30B). In contrast, the effects of dermorphin were sensitive to naloxone (pK_b 8.57) but not to SB-612111 (Figure 30C). Finally, the effects of DeNo were challenged with naloxone, SB-612111, and the cocktail of the two antagonists. Naloxone antagonized the inhibitory effect of DeNo producing a rightward shift of the concentration response curve and no modification of maximal effects. A pK_b value of 7.54 was derived from these experiments. SB-612111 was also able to counteract DeNo effects by producing a displacement to the right of the concentration response curve; a pK_b value of 7.07 was derived from these experiments. When the two antagonists were assayed together they displayed a clear additive effect (Figure 30D).

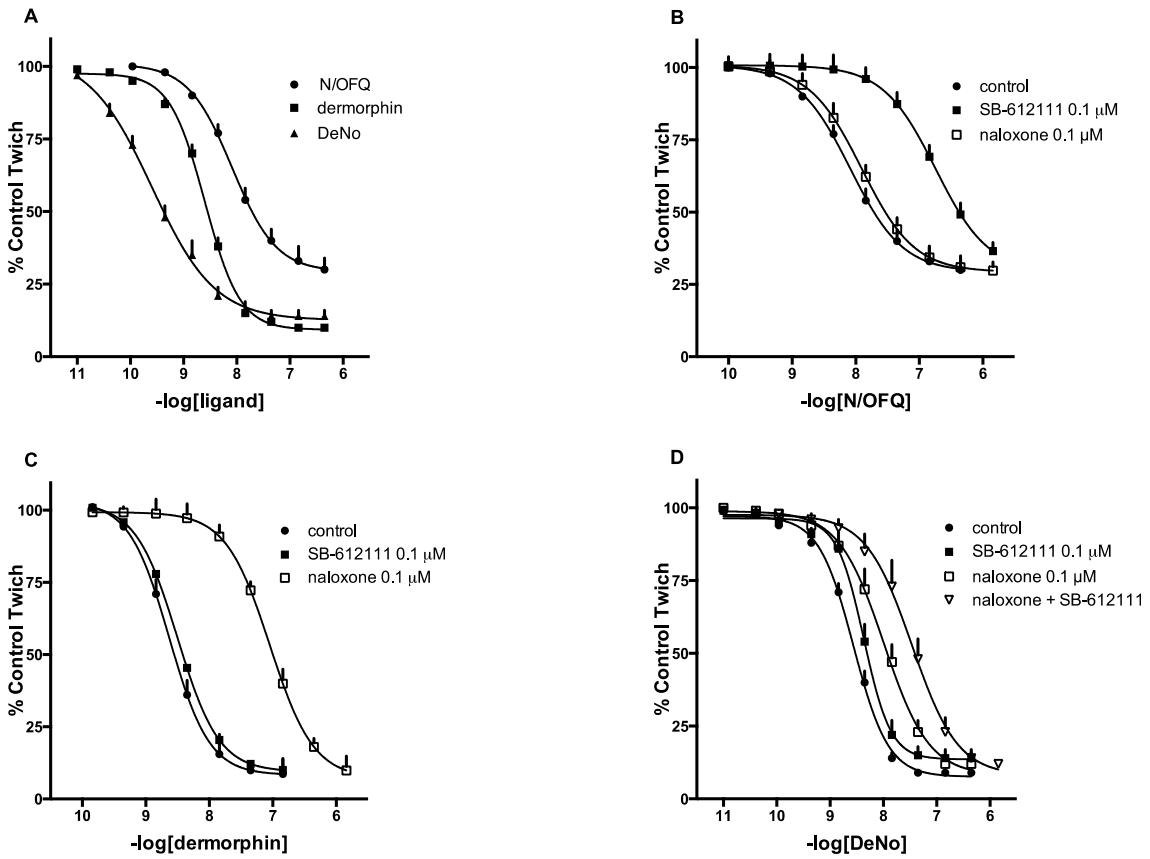


Figure 30. Concentration response curves to N/OFQ, dermorphin and DeNo in the electrically stimulated guinea pig ileum. Effects of naloxone, SB-612111 and a cocktail of both on concentration-response curves to N/OFQ (B), dermorphin (C) and DeNo (D) in electrically stimulated guinea pig ileum. Data are the mean ± SEM for at least n=3.

Paw pressure Test – Time-courses were produced for both dermorphin and DeNo given intrathecally (i.t.) in rats subjected to the plantar test. Dermorphin produced significant antinociceptive effects (Figure 31A). Similar effects were measured in response to i.t. DeNo which was less potent producing statistically significant effects only at the top dose of 1 nmol (Figure 31B). However it should be underlined that in the same range of doses both compounds impaired animal performance in the rota rod test (data not shown).

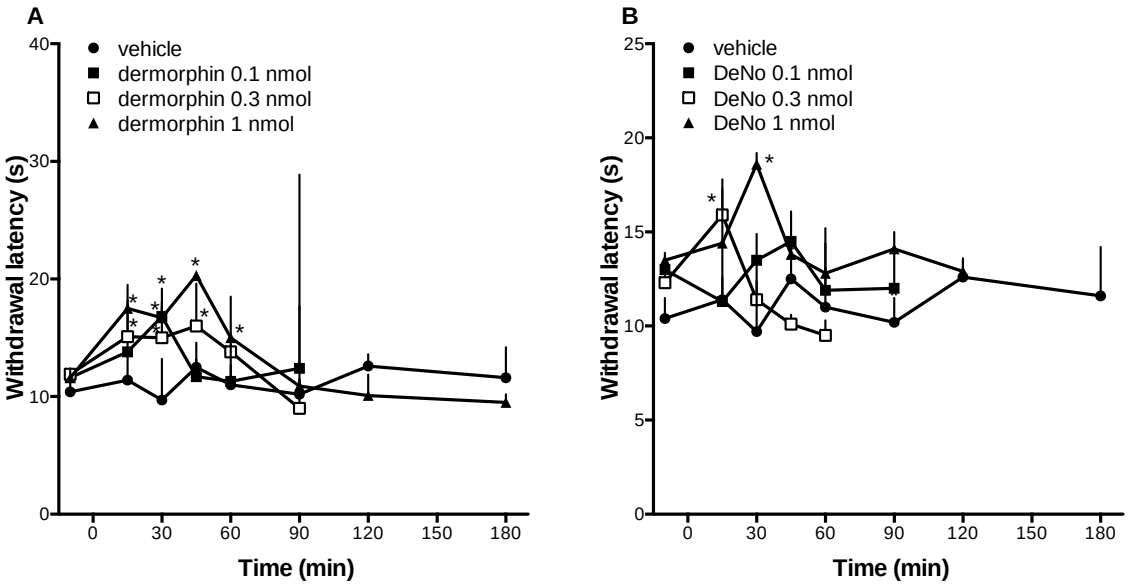


Figure 31. Dose response curve to dermorphin (panel A) and DeNo (panel B) in rats subjected to the paw pressure test. Data are the mean ± SEM of 5 rats in each group. *p<0.05, according to one way ANOVA followed by the Dunnett's test for multiple comparisons.

Discussion

In this study we have characterised a novel peptide bivalent MOP/NOP ligand, DeNo, created by combining dermorphin and N/OFQ. DeNo bound to MOP and NOP receptors and was ~5 fold NOP selective. There was between 1 and 2 logs selectivity over DOP and KOP receptors. At conventional upstream (GTPγ[^{35}S]) and downstream (cAMP) assays and in Ca^{2+} mobilisation experiments using chimeric G-protein constructs DeNo was a full agonist at both MOP and NOP. In BRET assays to assess receptor G-protein interaction and arrestin recruitment DeNo also behaved as a full agonist. Moreover, DeNo activated ERK1/2 as a full agonist at MOP and NOP; there was no activation of p38 at NOP. In a more intact preparation DeNo inhibited contraction of the electrically stimulated gpI via simultaneous activation of NOP and OP receptors and was antinociceptive via the i.t. route in rats.

At the receptor the most striking difference was a log increase in DeNo binding affinity at MOP compared to dermorphin and this may result from the structure of the linker between the two 'pharmacophores'. Comparing CHO data and taking into account different assays/buffer systems there was a marked difference in pK_i/pEC_{50} for MOP falling 60 fold at GTPγ[^{35}S] (upstream) and 331 fold at the chimeric G-protein (downstream). In contrast at NOP there was a 7 and 3 fold difference in affinity compared to potency. As the point of interrogation (assay) moved downstream the difference in potency at MOP and NOP increased; in GTPγ[^{35}S] DeNo was 38 fold more potent at NOP and 331 fold more potent in the chimeric G-protein assay. In all assays DeNo was a full agonist. If there was amplification or a coupling reserve then the potency values should shift leftwards rather than rightwards. This rightward shift was more marked at MOP than NOP and could be explained by buffer composition and end point. It is well documented that the chimeric G-protein assay suffers from hemi-equilibrium problems and we have discussed this in detail previously (Rizzi et al., 2014).

Using BRET based assay, we have been able to assess receptor/G-protein interaction and arrestin recruitment. These end points were examined in HEK cells for NOP and SH-SY5Y cells for MOP; this is a minor drawback in comparison with the other assays reported but because we have used dermorphin and N/OFQ then we can cross compare. In all assays, the parent compounds and DeNo behaved in essentially the same manner (potency) and as full agonists. A possible confounder is that SH-SY5Y cells have been shown to express both the DOP receptor and, more relevantly, the NOP receptor; at very low expression levels (Wu et al., 1997). Previous work with MOP/NOP heterodimers has

demonstrated that activation of NOP often leads to a reduction in the potency of MOP agonists throughout the cell-signalling cascade (Wang et al., 2005). It is possible in this cell line that DeNo is engaging both MOP and NOP. It has been known for some time that opioid receptors couple to MAPK with some variation in coupling (Bobeck, Ingram, Hermes, Aicher & Morgan, 2016) (Goeldner, Reiss, Wichmann, Meziane, Kieffer & Ouagazzal, 2008). In CHO cells expressing recombinant MOP and NOP there were marked differences. ERK1/2 was ubiquitously activated but p38 was only activated by MOP. ERK1/2 is typically involved in proliferative and differentiation responses but there is good evidence for a role in more chronic opioid receptor activation and possibly withdrawal (Christie, 2008) (Mazei-Robison & Nestler, 2012). This kinase along with p38 also plays a role in apoptosis (Wada & Penninger, 2004); the distinction of roles is not clear-cut. In terms of activation, two pathways have been suggested-the first involving a typical G-protein mediated event and the second via an arrestin-MAPK protein scaffold. That arrestin is activated is confirmed by the BRET assay but without full concentration response curves and pertussis toxin sensitivity experiments it is not possible to delineate the relative importance of these pathways. The fact that dermorphin and N/OFQ behaved as full agonists with no striking differences in potency suggests no ligand bias as expected for naturally occurring agonists. We have used the gpI as a more intact bioassay that (1) endogenously expresses both MOP and NOP (Bigoni et al., 1999) and (2) links the *in vitro* recombinant data set to the *in vivo* behavioural experiments. In this preparation N/OFQ was sensitive to the non-peptide antagonist SB-612111 but not naloxone; dermorphin was sensitive to naloxone but not SB-612111. These antagonists displayed lower potency when tested against DeNo whose concentration response curve was additively shifted when a cocktail of antagonists was used. Collectively these results demonstrated that the biological action of DeNo in this preparation is due to the simultaneous activation of NOP and MOP receptors despite the greater loss of potency at MOP than at NOP found in recombinant systems.

In vivo, after spinal administration in rats dermorphin (present results) and N/OFQ (Micheli et al., 2015) elicited similar antinociceptive actions. Under the same experimental conditions DeNo mimicked the effects of dermorphin and N/OFQ being neither more potent nor more effective. This result contrasts with the extensive literature evidence suggesting that the simultaneous activation of MOP and NOP receptor elicits synergistic antinociceptive effects both in rodents (Tian et al., 1997) (Reiss, Wichmann, Tekeshima, Kieffer & Ouagazzal, 2008) and in non-human primates (Ko & Naughton, 2009) (Molinari

et al., 2013) (Cremeans, Gruley, Kyle & Ko, 2012) (Hu, Calo, Guerrini & Ko, 2010). *In vitro* studies clearly demonstrated that DeNo is able to bind and activate both MOP and NOP receptor in the same range of concentrations; this may not be the case *in vivo*. A similar situation has been previously well documented in the studies of the MDAN series of compounds (Daniels, Lenard, Etienne, Law, Roerig & Portoghese, 2005). Under the present experimental conditions there were almost no differences between doses inducing antinociceptive effects and those disrupting animal performance on the rota rod. Further studies are needed to assess in detail any potential spinal antinociceptive actions of DeNo.

In conclusion we have created a novel bivalent MOP-NOP peptide agonist by combining dermorphin (MOP) and N/OFQ (NOP); this molecule behaves essentially as the parent compounds. Despite this promising profile analgesic actions of DeNo are poor in the model employed here. As a mixed molecule this ligand represents a useful addition to the non-peptides BU08028 (Khroyan et al., 2011) (Sukhtankar, Zaveri, Husbands & Ko, 2013), the SRI-International compounds exemplified by SR16435 (Sukhtankar, Zaveri, Husbands & Ko, 2013) (Khroyan et al., 2007) cebranopadol and the peptide [Dmt^1]N/OFQ(1–13)-NH_2. DeNo may be a useful tool, particularly *in vitro* for investigating the consequences of the simultaneous activation of NOP and MOP receptors.

2.2. Non peptides ligands

2.2.1. cebranopadol

Opioids drugs remain the gold standards analgesics for the treatment of the pain relief in spite of their unwanted side effects (Sullivan, Von Korff, Banta-Green, Merrill & Saunders, 2010). Moreover while the action of opioid drugs against acute nociceptive pain is brilliant their effectiveness against chronic, in particular neuropathic, pain is certainly less satisfactory (Cohen & Mao, 2014). Thus, it appear clear that there is a medical need for potent and well-tolerated analgesics able to control chronic and in particular neuropathic pain. The fourth member of the opioid receptor family, the NOP receptor, is also implicated in the modulation of pain responses and its activation affects nociceptive transmission in a complex site- and pain modality-specific manner. NOP selective agonists when given systemically promoted robust antinociceptive action in models of inflammatory and neuropathic pain while they are almost inactive when acute nociceptive pain is evaluated (Linz et al., 2014). Moreover, in non-human primates, NOP agonists produce antinociception by systemic as well as spinal administration without MOP receptor side effects (Linz et al., 2014) (Ko & Naughton, 2009). On the light of recently evidence coming from different laboratories, it was endorse the hypothesis that mixed NOP/MOP receptor agonists may have potential as innovative analgesics. Therefore, to explore the potential benefit of NOP and opioid receptor co-activation, medicinal chemists have generated novel molecules acting as mixed NOP/opioid receptor agonists (Sukhtankar, Zaveri, Husbands & Ko, 2013) (Linz et al., 2014) (Molinari et al., 2013) (Sobczak, Cami-Kobeci, Salaga, Husbands & Fichna, 2014), including the Grunenthal's compound trans-6'-fluoro-4',9'-dihydro-N,N-dimethyl-4-phenyl-spiro[cyclohexane-1,1'(3'H)-pyrano[3,4-b]indol]-4-amine, named cebranopadol (Figure 32), which demonstrated high affinity and efficacy at NOP and opioid, particularly MOP receptors and displayed robust antinociceptive properties in different rat models of pain (Linz et al., 2014). The same compound, becoming the object of this study, was synthesised by Prof. Trappella in the laboratories of the University of Ferrara with a novel chemical strategy allowing a whole high yield of the final product (Sara Bianco & Camilla Cerlesi, 2015). Thus, the aim of the present study was to characterize the pharmacological profile of the novel molecule. *In vitro* cebranopadol was assayed in: i) calcium mobilization studies

performed in cells expressing the human recombinant receptors and chimeric G-proteins, ii) bioluminescence resonance energy transfer BRET studies investigating its ability to promote NOP and MOP receptor interaction with G-protein and β-arrestin 2, and iii) bioassay studies in isolated tissues. Moreover cebranopadol has been evaluated *in vivo* in mice subjected to the tail withdrawal assay and the formalin test.

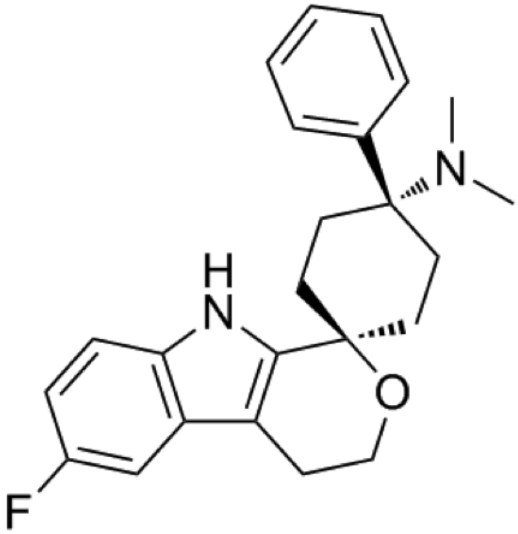


Figure 32. Chemical structure of the compound cebranopadol.

Materials and Methods

Calcium mobilization assay – CHO cells stably co-expressing the human NOP, or KOP, or MOP receptor and the C-terminally modified $G\alpha_{qi5}$ and CHO cells expressing the DOP receptor and the $G\alpha_{qG66Di5}$ protein were generated and cultured as previously described (Camarda et al., 2009) (Camarda & Calo, 2013). Cells were seeded at a density of 50,000 cells $well^{-1}$ into 96-well black, clear-bottom plates. After 24 hours incubation 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. Afterwards the loading solution was aspirated and 100 µl $well^{-1}$ of assay buffer: Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 µM Brilliant Black was added. Serial dilutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.01% BSA fraction V). After placing cell culture and compound plates into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured. On-line additions were carried out in a volume of 50 µl $well^{-1}$. To facilitate drug diffusion into the wells in antagonist type

experiments, the present studies were performed at 37°C and three cycles of mixing (25 µl from each well) were performed immediately after antagonist injection.

BRET assay – Human Embryonic Kidney (HEK-293) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum, 100 units ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin sulphate, human SH-SY5Y neuroblastoma cells were grown in Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% (v/v) foetal calf serum, 100 units ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin sulphate in a humidified atmosphere of 5% CO_2 at 37°C. Cell lines permanently co-expressing the different pairs of fusion proteins, NOP-RLuc/Gβ1-RGFP (HEK-293), NOP-RLuc/β-arrestin 2-RGFP (HEK-293), MOP-RLuc/Gβ1-RGFP (SH-SY5Y), and MOP-RLuc/β-arrestin 2-RGFP (SH-SY5Y) were prepared using the pantropic retroviral expression system by Clontech as described previously (Molinari, Casella & Costa, 2008). For G-protein experiments enriched plasma membrane aliquots from transfected cells were prepared and quantified as previously described in details (Malfacini et al., 2015). Luminescence in membranes was recorded in 96-well untreated white opaque microplates, while in whole cells was recorded in 96-well sterile poly-D-lysine-coated white opaque microplates (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030 (PerkinElmer, Waltham, MA, USA). For the determination of receptor/G-protein interaction, membranes (3 µg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP or MOP/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of receptor/β-arrestin 2 interaction, cells co-expressing NOP/RLuc and β-arrestin 2/RGFP or MOP/RLuc and β-arrestin 2/RGFP were plated 24 hs before the experiment in poly-D-Lysine treated plates (100,000 cells $well^{-1}$). The cells were prepared for the experiment substituting the medium with PBS with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM). Coelenterazine at a final concentration of 5 µM was injected 15 minutes prior reading the cell plate. Different concentrations of ligands in 20 µl of PBS - BSA 0.01 % were added and incubated 5 or 60 min before reading luminescence. All the experiments were performed at room temperature.

Animal welfare and ethical statement – All animal care and experimental procedures conformed to the standards of the European Communities Council directives (2010/63/EU) and national regulations (D.L. 26/2014). Studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2011). The present study was

approved by the Ethical Committee for the Use of Laboratory Animals (CEASA) of the University of Ferrara and by the Italian Ministry of Health (authorization number n° 10086 for isolated tissues and 316/2013-B for *in vivo* studies). Male CD-1 mice (25-35 g, total number 300), and guinea pigs (300-350 g, total number 6) were from Harlan, Natisone Udine, Italy. They were housed in plexiglas cages (425x266x155 mm for mice, 560x320x180 for guinea-pigs, Techniplast, Buguggiate, Italy), under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 07:00 h) with food (standard diet, Mucedola, Settimo Milanese, Italy) and water *ad libitum*. Each cage was provided with a mouse red house (Tecniplast, Buguggiate, Italy) and nesting materials.

Bioassays – Mouse vas deferens and guinea pig ileum tissues were prepared and mounted into organ baths according to procedures previously described in details (Bigoni et al., 1999). Tissues were stimulated through two platinum electrodes with supramaximal rectangular pulse of 1 ms duration, 0.05 Hz frequency, 80 V of amplitude. The electrically evoked contractions were measured isotonically by means of strain gauge transducers (Basile 7006; Ugo Basile srl, Varese, Italy) and recorder with a computer-based acquisition system (Power Lab 8, AD Instruments, USA). After an equilibration period of about 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration response curve (CRC) to N/OFQ, dermorphin, and DPDPE were performed (0.5 log unit steps). In a preliminary series of experiments, performed in the guinea pig ileum, we compare cebranopadol potency by performing cumulative versus consecutive CRC; based on the results of these experiments we decided to perform cebranopadol CRC non-cumulatively. Antagonists experiments using naloxone and SB-612111 were performed using a curative protocol, i.e. naloxone (1 μM) and SB-612111 (1 μM) were injected on the plateau of the effect induced by cebranopadol. Mouse colon tissues were prepared and investigated as previously described in details (Rizzi, Bigoni, Calo, Guerrini, Salvadori & Regoli, 1999). In each tissue single concentration of carbachol (100 μM), N/OFQ (0.1 μM), endomorphin-1 (EM-1) (1 μM) and cebranopadol (1 μM) were added every 30 min and the tissues were washed after testing the effect of compound.

In vivo studies – Each animal was used only once in all *in vivo* models. Animals were assigned randomly to treatment groups. Different treatment doses and vehicle (saline or 8% DMSO) were tested in a randomized fashion and the operator performing the behavioural tests was blinded with respect to the treatments.

Tail withdrawal test – All experiments started at 10.00 a.m. and were performed according to the procedure previously described in detail (Molinari et al., 2013). Fentanyl and cebranopadol were tested in the dose range 0.01 - 1 mg kg^{-1}, while Ro 65-6570 only at 1 mg kg^{-1} because this is the higher dose not affecting animal performance on the rota rod. Results were expressed as maximum possible antinociceptive effect (%MPE). In a first series of experiments we evaluated the time course of the dose response curves (DRC) to each drug. In a second series we performed a cumulative DRC on the same mouse by was measuring tail withdrawal latency at 5 or 30 min (peak effect) after each dose of fentanyl or cebranopadol, respectively. Finally, in the third series of experiments naloxone (1 mg kg^{-1}) or SB-612111 (1 mg kg^{-1}) were injected i.v. (100 µl $mouse^{-1}$) 5 or 30 min respectively before performing DRC to fentanyl or cebranopadol.

Rota rod test – To investigate potential effects on motor coordination a rota rod test was performed using a constant speed device (rota rod for mice Ugo Basile, Varese, Italy). One day prior to the experiment, mice were trained at 15 rpm for 120 sec. Motor performance has been calculated as time (sec) spent on rod measured at the antinociceptive peak effect for each drug (i.e. 5 or 30 min from i.v. injection for fentanyl or cebranopadol and Ro 65-6570, respectively). A cut-off time of 120 sec was chosen.

Formalin test – The procedure and the protocol used for this assay have been previously described in details (Rizzi et al., 2006). All the compounds were injected intravenous (i.v.) (100 µl $mouse^{-1}$) in the anterior caudal vein. Since the duration of action of fentanyl is very short (less than 20 min), this drug was applied (0.01 - 0.1 mg kg^{-1}) 5 min before formalin injection to investigate its effects on the first phase and 15 min after formalin for its effects on the second phase. Ro 65-6570 (0.3 - 1 mg kg^{-1}) and cebranopadol (0.001 - 1 mg kg^{-1}) were applied 30 min before formalin injection.

Materials – All cell culture media and supplements were from Invitrogen (Paisley, UK). All other reagents were from Sigma Chemical Co. (Poole, U.K.) and were of the highest purity available. DPDPE, endomorphin-1, naloxone and SB-612111 were bought from Tocris Bioscience (Bristol, UK). Fentanyl was bought from SALARAS (SP/270, Como, Italy). Native coelenterazine (CLZN, 5 mM, EtOH) was from Synchem UG & Co. KG (Altenburg, Germany). N/OFQ, dermorphin, dynorphin A, Ro 65-6570, and cebranopadol were synthesized in house. Stock solutions (1 mM) of peptides and fentanyl were made in

distilled water. SB-612111, Ro 65-6570 and cebranopadol (10 mM) were solubilized in DMSO. Stock solutions of ligands were stored at -20°C.

Data analysis – The pharmacological terminology adopted in this report is consistent with the IUPHAR recommendations. *In vitro* data were expressed as mean ± SEM of at least four separate experiments. Agonist responses were reported in calcium mobilization studies as maximum change in fluorescence, expressed as percent over the baseline, in BRET experiments as stimulated BRET ratio obtained by subtracting the vehicle value to that measured in the presence of ligand, in bioassay studies on electrically stimulated tissues as percent of the control twitch, and in mouse colon experiments as g of contraction. Non-linear regression analysis using Graph Pad PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Antagonist potencies derived from inhibition response curves have been expressed as pK_B calculated according to the equation reported by Kenakin, (2004) (Kenakin, 2004) while antagonist potencies obtained by testing a fixed concentration of antagonist against the CRC to the agonist were derived from the Gaddum Schild equation:

$$pA_2 = \log\left[\frac{CR - 1}{antagonist}\right]$$

assuming a slope value equal to unity. Agonist potencies were given as pEC_{50}, that is, the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect of that agonist. E_{max} is the maximal effect that an agonist can elicit in a given preparation. Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test or the Student t test. Differences were considered statistically significant when $p < 0.05$.

Results

Calcium mobilization assay – N/OFQ, fentanyl, dynorphin A, and DPDPE were used as standard ligands for NOP, MOP, KOP, and DOP receptors in calcium mobilization studies performed on CHO cells stably expressing chimeric G-proteins and human recombinant receptors. N/OFQ evoked a concentration-dependent stimulation of calcium release displaying high potency and maximal effects in NOP cells while it was inactive in cells expressing classical opioid receptors. Similar findings were obtained with fentanyl and DPDPE that behaved as potent full agonists displaying high selectivity for MOP and DOP receptors, respectively. Dynorphin A was inactive in cells expressing the NOP receptor while behaved as full agonist at classical opioid receptors; as expected dynorphin A displayed the highest potency at the KOP receptor (Figure 33 and Table 14). In parallel experiments, cebranopadol elicited concentration-dependent stimulation of calcium release in the four cell lines. Cebranopadol evoked maximal effects similar to those of standard ligands with the only exception of KOP cells where it behaved as a partial agonist. As far as potency is concerned, cebranopadol displayed similar and relatively high potency at NOP and MOP receptors being approximately 10 fold less potent at DOP and KOP (Figure 33 and Table 14).

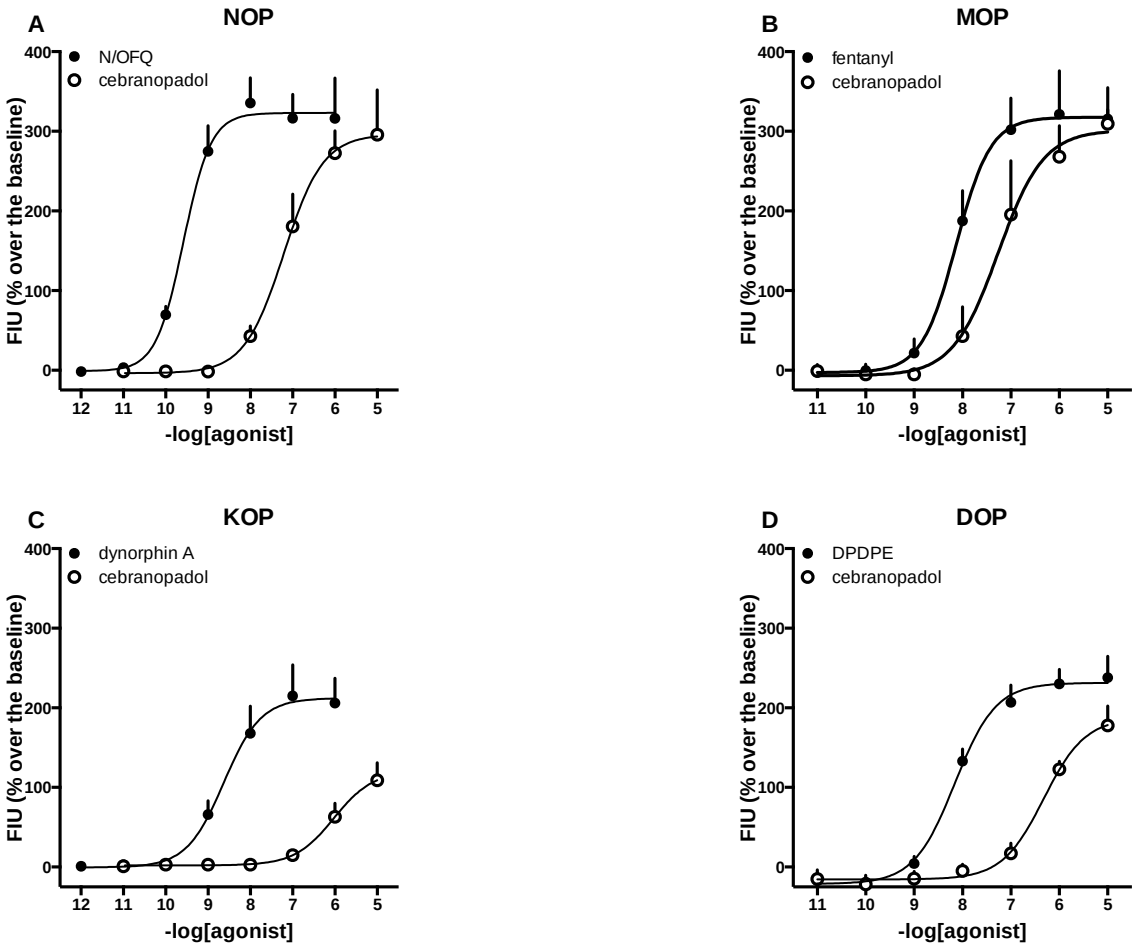


Figure 33. CRC to cebranopadol and standard opioid agonists in calcium mobilization experiments performed on CHO cells co expressing the NOP (A), MOP (B), KOP (C) and DOP (D) receptors and chimeric G-proteins. Data are mean ± SEM of 4 experiments performed in duplicate.

Table 14. Calcium mobilization studies. Potencies and efficacy of N/OFQ, cebranopadol and standard opioid agonists in CHO cells expressing the human NOP or classical opioid receptors and chimeric proteins.

	NOP		MOP		KOP		DOP	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
N/OFQ	9.59 (9.49-9.68)	1.00	inactive		inactive		inactive	
fentanyl	inactive		8.13 (7.90-8.37)	1.00	inactive		inactive	
dinorphin A	inactive		6.67 (6.17-7.17)	0.82 ± 0.10	8.54 (8.18-8.90)	1.00	inactive	6.67 (6.17-7.17)
DPDPE	inactive		inactive		inactive		8.15 (7.88-8.43)	1.00
cebranopadol	7.28 (6.74-7.81)	0.89 ± 0.06	7.20 (6.51-7.88)	0.99 ± 0.05	5.98 (5.69-6.27)	0.55 ± 0.03*	6.31 (5.71-6.90)	0.81 ± 0.06

inactive means that the compound was inactive up to 1 µM.
*$p < 0.05$ according to ANOVA followed by the Dunnett's test for multiple comparisons.
N/OFQ, fentanyl, DPDPE and dynorphin A were used as reference agonist for calculating intrinsic activity at NOP, MOP, DOP and KOP receptors, respectively.

In a separate series of experiments, the effect of cebranopadol and standard agonists at NOP and MOP receptors has been challenged with receptor antagonists such as naloxone and SB-612111. In CHO_{NOP} cells, the CRC to N/OFQ was not affected by 1 μM naloxone while it was rightward shifted in the presence of 100 nM SB-612111 (Figure 33A). Similar results were obtained with cebranopadol whose effects were not sensitive to naloxone while inhibited by SB-612111 (Figure 33C). Of note, the action of the NOP receptor antagonist was larger vs cebranopadol (pA_2 > 10) than vs N/OFQ (pA_2 8.85). In MOP expressing cells the effects of fentanyl were slightly inhibited by 1 μM SB-612111 while 100 nM naloxone produced a large dextral displacement of the CRC (Figure 33B). Very similar results were obtained using as MOP agonist cebranopadol (Figure 33D). Antagonist potencies vs standard ligands and vs cebranopadol in the two cell lines are displayed in Table 15.

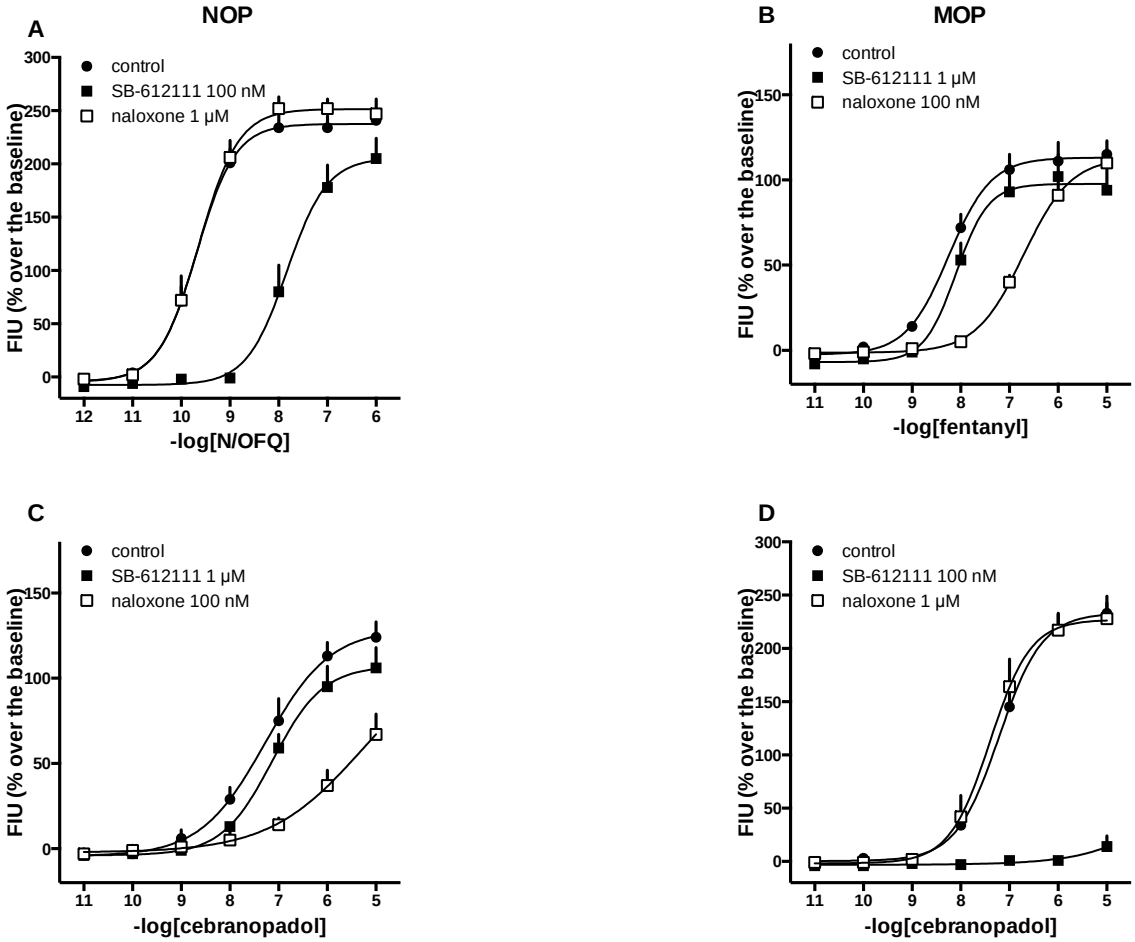


Figure 34. CRC to N/OFQ (A) and cebranopadol (C), in absence and in presence of naloxone or SB-612111 in calcium mobilization experiments performed on CHO cells co-expressing the NOP receptor and the $G\alpha_{qi5}$ chimeric protein. CRC to fentanyl (B) and cebranopadol (D), in absence and in presence of naloxone or SB-612111 in calcium mobilization experiments performed on CHO cells co-expressing the MOP receptor and the $G\alpha_{qi5}$ chimeric protein. Data are mean ± SEM of 4 experiments performed in duplicate.

Table 15. Calcium mobilization assay. Potencies (PK_B values) of SB-61211 and naloxone against N/OFQ or cebranopadol in CHO cells expressing the human NOP or MOP opioid receptors and chimeric G-proteins.

	NOP		MOP	
	vs N/OFQ	**vs cebranopadol**	**vs fentanyl**	**vs cebranopadol**
SB-612111	8.85 (8.30-9.40)	>10	5.86 (5.60-6.11)	6.15 (5.41-6.89)
naloxone	inactive	inactive	8.18 (7.31-9.05)	8.61 (8.07-9.15)

inactive means that the compound was inactive up to 1 μM.

BRET assay – The ability of standard ligands (N/OFQ and the non peptide NOP agonist Ro 65-6570 for the NOP receptor, and dermorphin and fentanyl for the MOP receptor) and cebranopadol to promote receptor/G-protein and receptor/β-arrestin 2 interaction has been assessed with a BRET based assay. As shown in Figure 35 in membrane prepared from NOP expressing cells N/OFQ promoted receptor/G-protein interaction in a concentration dependent manner. Ro 65-6570 and cebranopadol mimicked the effect of the natural peptide eliciting similar maximal effects but showing approximately 10 fold lower potency (Figure 35A). A separate series of experiments was performed prolonging the incubation time with the agonist from 5 to 60 min. Under these experimental conditions the three agonists displayed similar maximal effects and similar potency. This was due to a 3 fold reduction of N/OFQ potency associated with a 3 fold increase in Ro 65-6570 and cebranopadol potency (Figure 35B and Table 16). Membrane extracts taken from SH-SY5Y cells stably expressing the human MOP receptor were used to perform CRC to MOP agonists. After 5 min exposure time, dermorphin and fentanyl promoted MOP/G-protein interaction in a concentration-dependent manner displaying similar maximal effects and potencies (Figure 35C). Under the same experimental conditions, cebranopadol displayed similar maximal effects but 10 fold higher potency than standard agonists (Figure 35C). By prolonging incubation time the potency of the MOP agonists increased with the larger change for cebranopadol (Figure 35D).

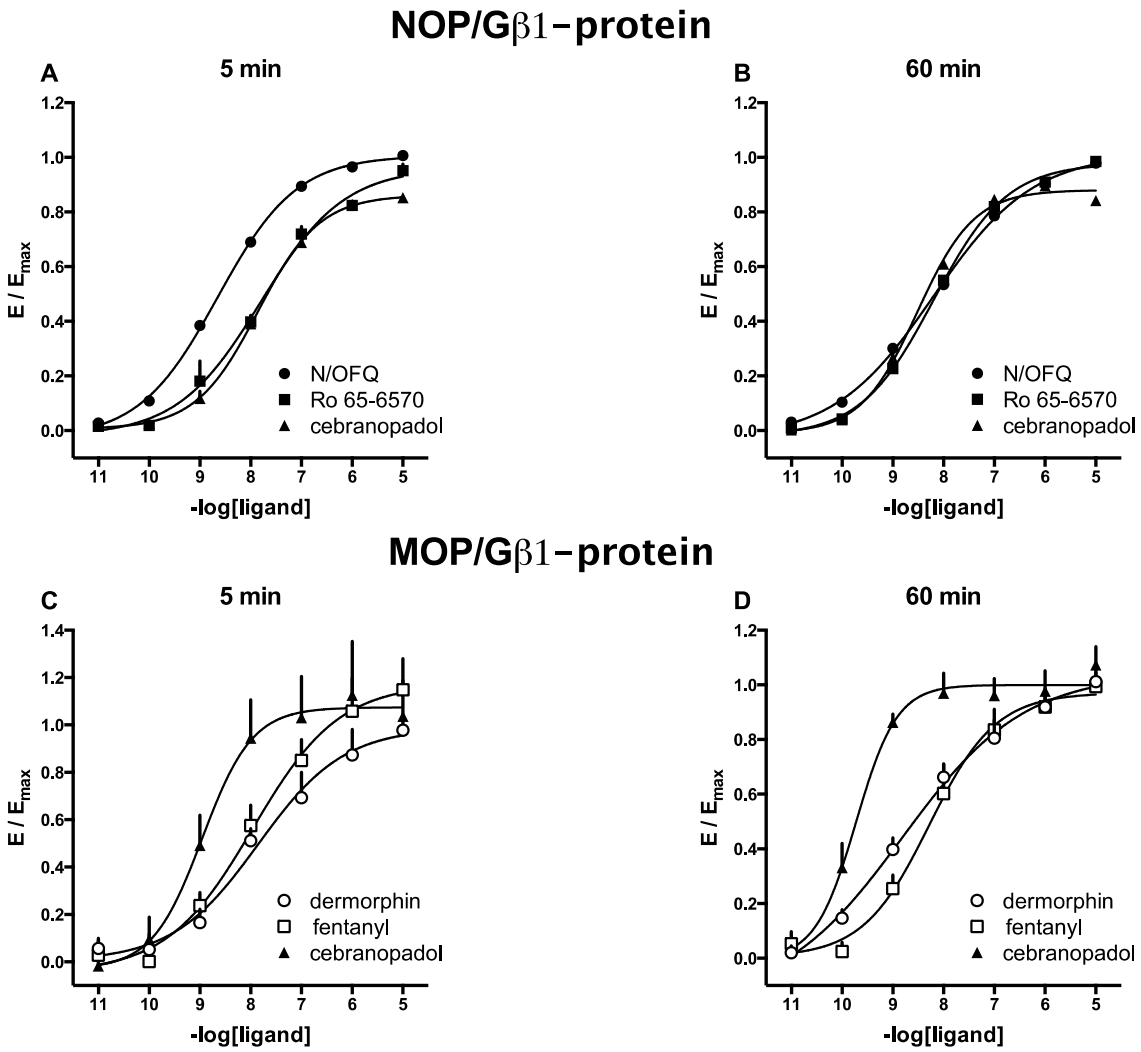


Figure 35. NOP receptor/G-protein interaction experiments: CRC to N/OFQ, Ro 65-6570 and cebranopadol after 5 (A) or 60 min incubation (B); MOP receptor/G-protein interaction experiments: CRC to dermophin, fentanyl and cebranopadol after 5 (C) or 60 min incubation (D). Data are expressed as mean ± SEM of at least 4 separate experiments performed in duplicate.

In whole cells expressing the NOP receptor N/OFQ promoted receptor/β-arrestin 2 interaction in a concentration dependent manner displaying high potency and maximal effects. Ro 65-6570 mimicked the stimulatory effect of N/OFQ showing however lower maximal effects and potency. Cebranopadol, up to 10 μM, did not modify the BRET signal (Figure 36A). Very similar results were obtained by prolonging the agonist incubation time from 5 to 60 min (Figure 36B). In MOP receptor expressing cells, dermorphin, fentanyl and cebranopadol promoted receptor/arrestin interaction in a concentration dependent manner showing similar values of potency. Dermorphin and cebranopadol displayed high maximal effects while fentanyl behaved as a partial agonist (Figure 36C). Prolonging incubation time from 5 to 60 min caused an increase in dermorphin and cebranopadol

potency (Figure 36D). All the data obtained in this series of experiments have been summarized in Table 16.

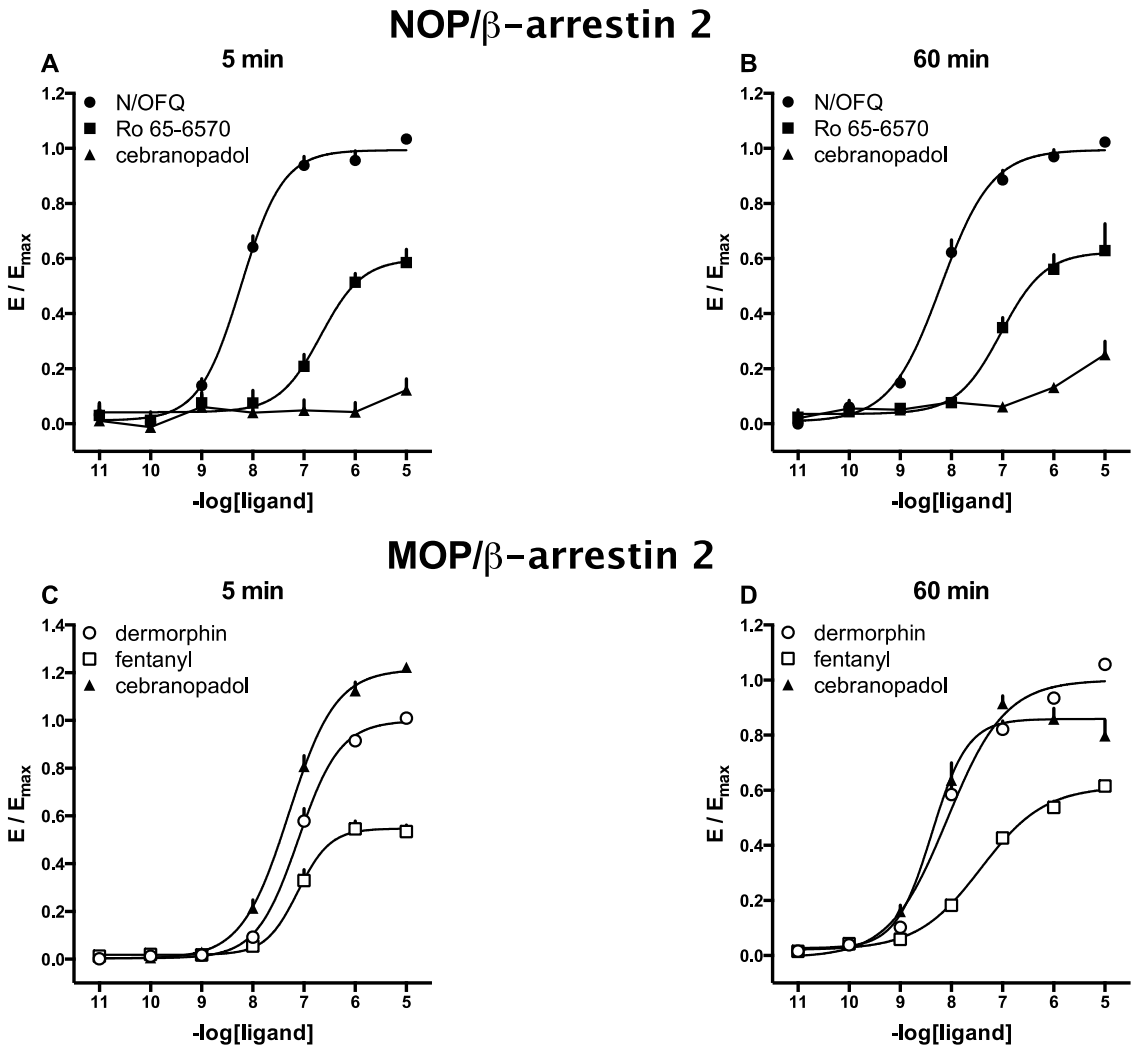


Figure 36. NOP receptor/β-arrestin 2 interaction experiments: CRC to N/OFQ, Ro 65-6570 and cebranopadol after 5 (A) or 60 min incubation (B); MOP receptor/β-arrestin 2 interaction experiments: CRC curves to dermophin, fentanyl and cebranopadol after 5 (C) or 60 min incubation (D). Data are expressed as mean ± SEM of at least 4 separate experiments performed in duplicate.

Table 16. Potencies (pEC_{50}) and efficacies (α) of NOP and MOP receptor agonists in promoting receptor interaction with G-protein and β-arrestin 2 at two different incubation time (5 or 60 minutes).

NOP

	Gβ1-protein				β-arrestin 2			
	5’		60’		5’		60’	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
N/OFQ	8.60 (8.58-8.63)	1.00	8.16 (7.81-8.51)	1.00	8.21 (7.97-8.45)	1.00	8.18 (7.93-8.44)	1.00
Ro 65-6570	7.80 (7.45-8.15)	0.87 ± 0.02	8.17 (7.99-8.34)	0.92 ± 0.01	6.62 (6.02-7.23)	0.65 ± 0.07*	6.99 (6.47-7.50)	0.64 ± 0.09*
cebranopadol	7.87 (7.52-8.21)	0.83 ± 0.01	8.49 (8.43-8.55)	0.86 ± 0.01	inactive	inactive		

MOP

	Gβ1-protein				β-arrestin 2			
	5’		60’		5’		60’	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
dermorphin	7.99 (7.35-8.62)	1.00	8.52 (8.00-9.04)	1.00	7.11 (6.87-7.35)	1.00	8.06 (7.86-8.27)	1.00
fentanyl	7.76 (7.09-8.43)	1.15 ± 0.12	8.28 (7.58-8.98)	0.93 ± 0.04	7.10 (6.83-7.37)	0.55 ± 0.02*	7.41 (7.25-7.57)	0.62 ± 0.03*
cebranopadol	8.85 (8.51-9.18)	1.14 ± 0.13	9.74 (9.19-10.3)	1.00 ± 0.04	7.30 (7.08-7.52)	1.21 ± 0.02	8.36 (8.07-8.65)	0.86 ± 0.04

inactive means that the compound was inactive up to 1 μM. *$p < 0.05$ according to ANOVA followed by the Dunnett’s test for multiple comparisons. N/OFQ and dermorphin were used as reference agonist for calculating intrinsic activity at NOP and MOP receptors, respectively.

Since cebranopadol did now show any efficacy in promoting NOP/β-arrestin 2 interaction, its antagonistic properties were assessed against N/OFQ. As a positive control, the effect of SB-612111 was evaluated in parallel experiments. As shown in Figure 37A in the presence of 100 nM SB-612111 the CRC to N/OFQ was shifted to the right; from these data a pK_B value of 8.44 was calculated. On the contrary cebranopadol did not modify the CRC to N/OFQ; only a slight depression of the maximal effect induced by the agonist was detected in the presence of cebranopadol 1 μM (Figure 37B).

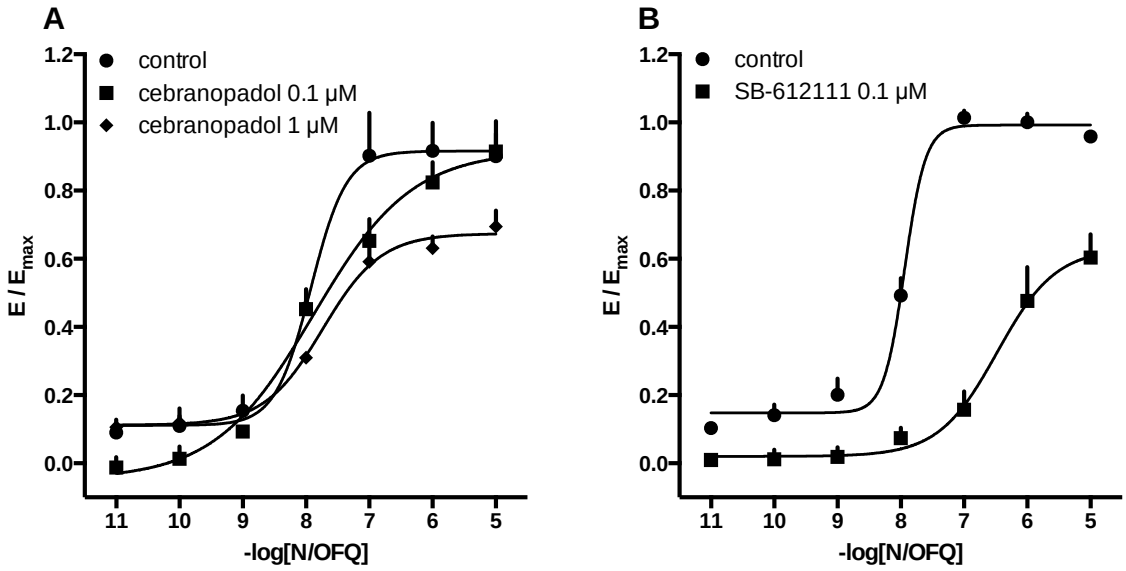


Figure 37. NOP receptor/β-arrestin 2 interaction experiments: CRC to N/OFQ in absence and in presence of SB-612111 (A) or cebranopadol (B). Data are expressed as mean ± SEM of 4 separate experiments performed in duplicate.

Isolated tissues – The effects of cebranopadol were investigated in the electrically stimulated mouse vas deferens and guinea pig ileum in comparison with standard agonists for NOP (N/OFQ) and opioid receptors (DPDPE and dermorphin for the vas deferens and the ileum, respectively). In these tissues standard agonists inhibited the electrically induced twitch response showing a fast kinetic of action and an effect immediately and fully reversible after washing. On the contrary, cebranopadol induced slow developing inhibitory effects that could not be reversed by washing the tissues (see typical tracing in Figure 38).

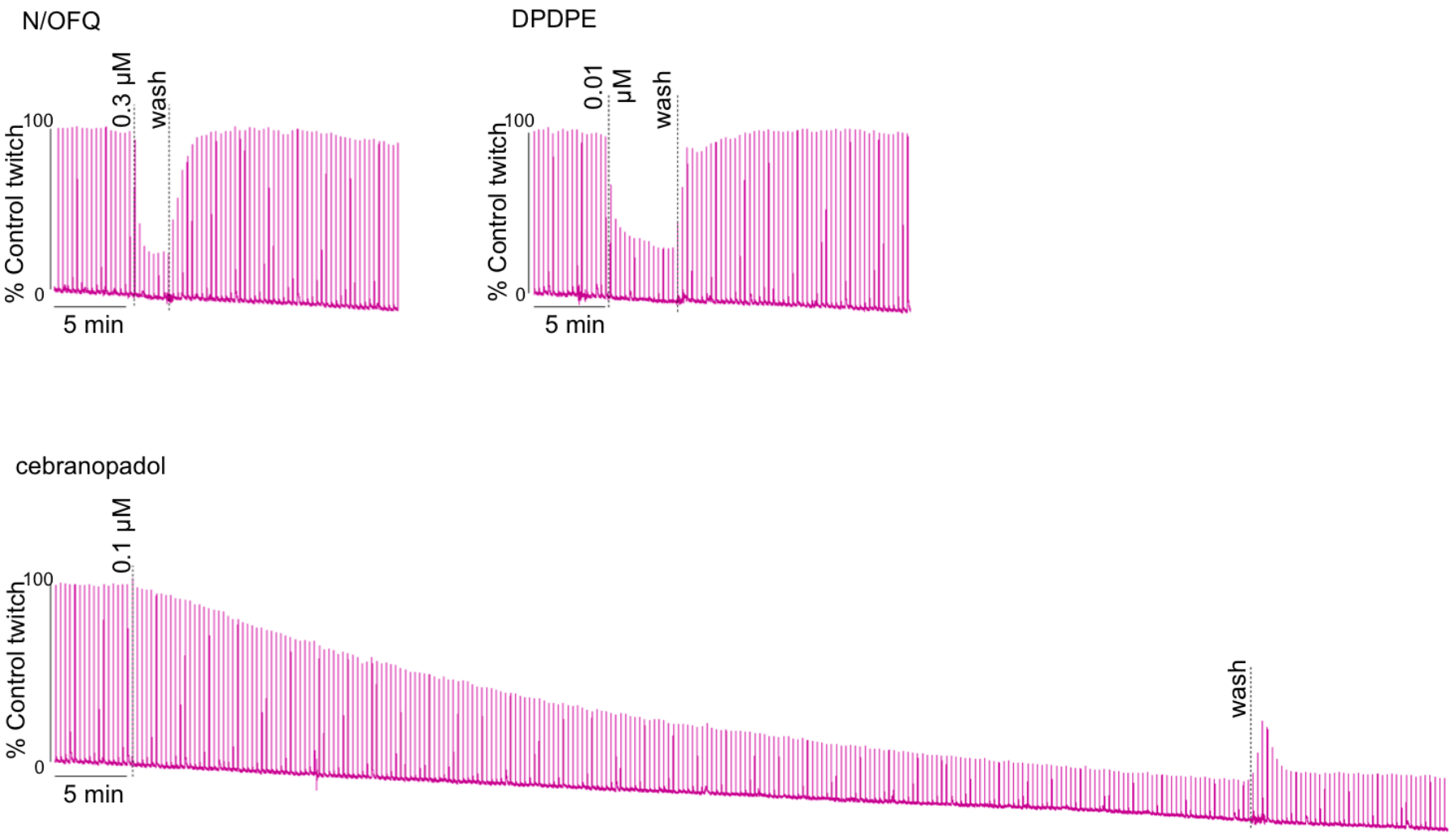


Figure 38. Typical tracings showing the time course of the effect of equieffective concentrations of N/OFQ, DPDPE and cebranopadol in the electrically stimulated mouse vas deferens. Abscissa: time in min. Ordinate: % control twitch.

The extremely slow kinetic displayed by cebranopadol made difficult if not impossible to perform CRC thus the action of different concentrations of drug were assessed in different tissues. In the mouse vas deferens, N/OFQ and DPDPE inhibited the electrically induced contractions in a concentration dependent manner with potency values of 7.06 and 8.25, respectively. Cebranopadol mimicked the inhibitory effect of the peptides showing similar maximal effects and a value of potency of 7.65 (Figure 39A).

In the guinea pig ileum, N/OFQ and dermorphin inhibited the electrically induced contractions in a concentration dependent manner with potency values of 8.15 and 9.07 and maximal effect of 57 ± 2% and 86 ± 2%, respectively. Cebranopadol displayed maximal effect similar to those of dermorphin and a pEC_{50} of 8.24 (Figure 39B). In order to investigate the receptor(s) involved in the action of cebranopadol the inhibitory action elicited by a single concentration of compound was challenged with SB-612111 and naloxone using a curative protocol.

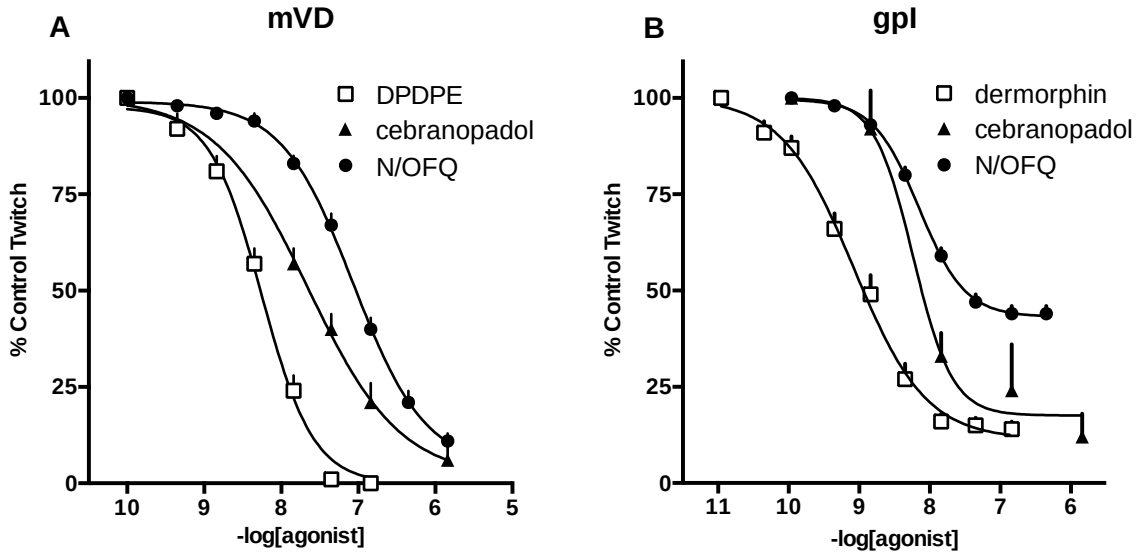


Figure 39. Electrically stimulated mouse vas deferens: CRC to DPDPE, cebranopadol and N/OFQ (A). Electrically stimulated guinea pig ileum: CRC to dermorphin, cebranopadol and N/OFQ (B). Points indicate the means and vertical lines the SEM of at least 4 experiments**.**

As shown in Figure 40 the inhibitory effect elicited by 100 nM cebranopadol can be fully reversed by 1 µM naloxone but not by the same concentration of SB-612111. Similar results were obtained in the mouse vas deferens (data not shown). Finally in order to investigate possible NOP antagonist properties of cebranopadol, CRC to N/OFQ were performed in the absence and presence of the cocktail 1 µM naloxone plus 1 µM

cebranopadol. No statistically significant differences were observed between control (pEC_{50} 7.25, E_{max} 93 ± 7%) and treated tissues (pEC_{50} 7.29, E_{max} 94 ± 6%).

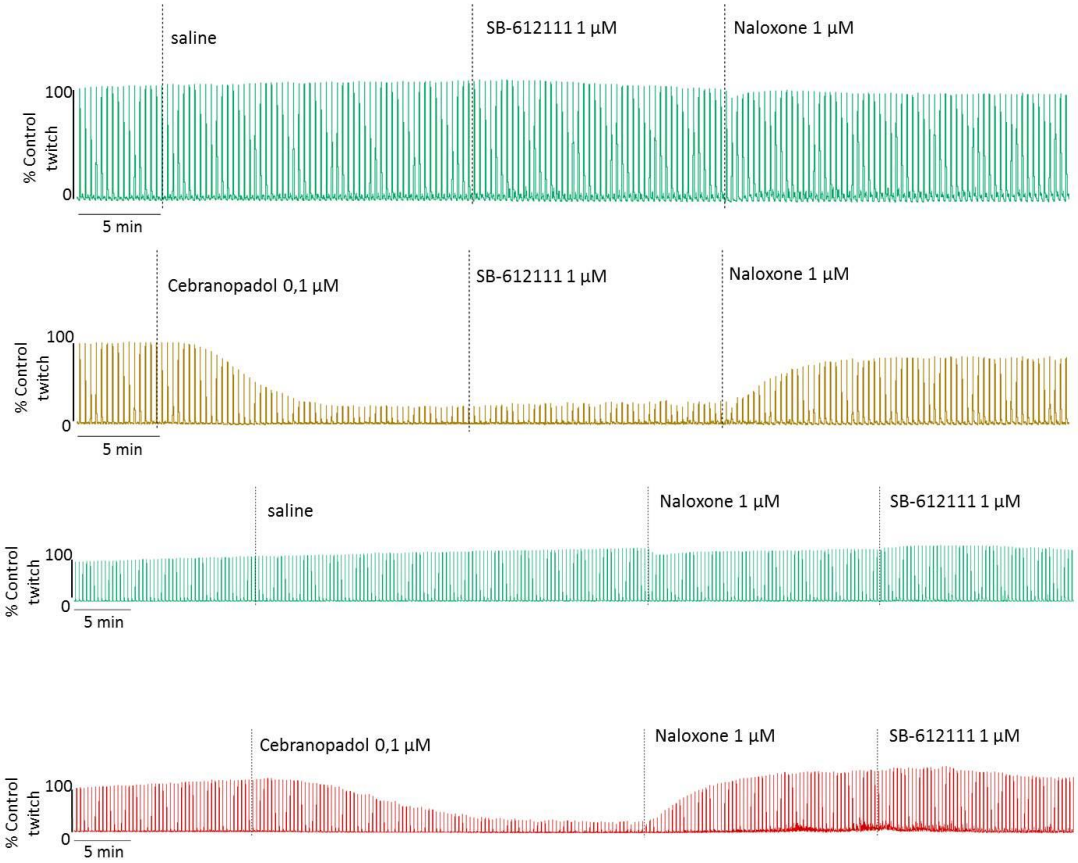


Figure 40. Tracings showing the curative effects of naloxone and SB-612111 against the inhibitory effect of cebranopadol in the electrically stimulated guinea pig ileum. Abscissa: time in min. Ordinate: % control twitch.

In the mouse colon, N/OFQ and EM-1 elicit contractile effects via selective stimulation of the NOP and MOP receptor, respectively (Rizzi, Bigoni, Calo, Guerrini, Salvadori & Regoli, 1999). This preparation was used for investigating the pharmacological action of cebranopadol. Carbachol (10 µM), N/OFQ (0.1 µM), and EM-1 (1 µM) contracted the tissues of 1.98 ± 0.4, 0.94 ± 0.21 and 0.39 ± 0.19 g, respectively. These contractile effects were fully repeatable after 30 min (Figure 41A). Cebranopadol up to 1 µM was completely inactive. However, in tissues previously exposed to cebranopadol the contraction induced by carbachol was unaffected while that evoked by N/OFQ or EM-1 was partially or completely inhibited, respectively (Figure 41B).

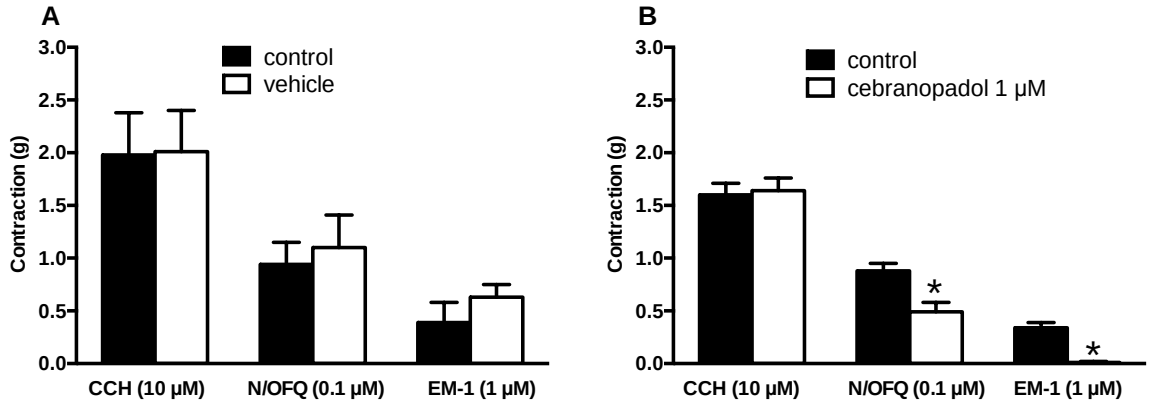


Figure 41. Mouse colon: effects evoked by single concentrations of carbachol, N/OFQ or EM-1 in tissues treated with vehicle (A) or in tissues previously exposed to 1 µM cebranopadol (B). Histograms indicate the means and vertical lines the SEM of 6 experiments. *p < 0.05 vs vehicle according to the Student T test.

Tail withdrawal test – The NOP selective agonist Ro 65-6570 tested up to 1 mg kg^{-1} (i.v.) did not produce statistically significant effects in the mouse tail withdrawal assay (data not shown). Under the same experimental conditions, fentanyl (0.01–1 mg kg^{-1}, i.v.) produced dose-dependent antinociceptive effects. Fentanyl effect peaked 5 min post injection and lasted, with the highest dose, for more that 1 hr (Figure 42A). In the same range of doses cebranopadol mimicked the action of fentanyl but producing longer lasting effects. In particular the increase in tail withdrawal latency induced by cebranopadol peaked at 30 min, remained stable for 2 h, and then slowly returned to basal levels (Figure 42B).

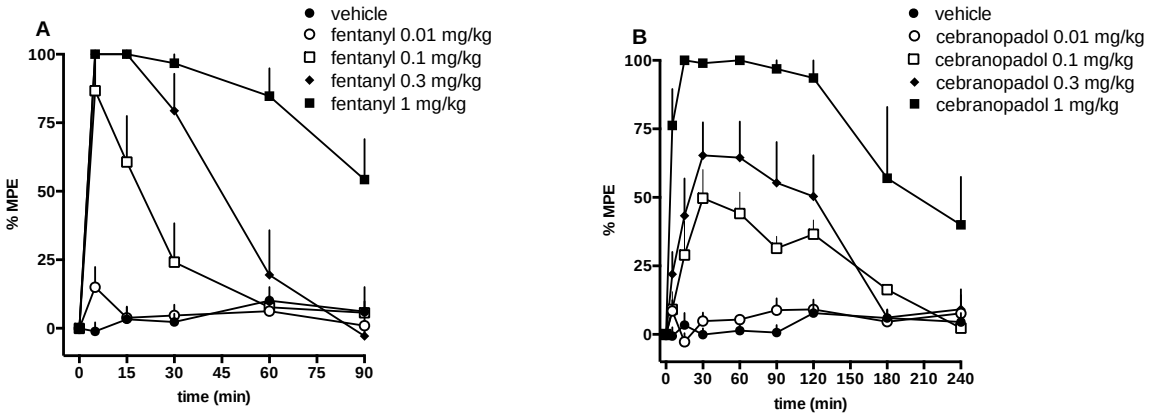


Figure 42. Mouse tail withdrawal assay. DRC to fentanyl (0.01 - 1 mg kg^{-1} i.v.) (A) and cebranopadol (0.01 - 1 mg kg^{-1} i.v.) (B). Each point represents the mean (8 animals per group) and the vertical bars indicate the SEM.

The same animals were evaluated in the rota rod test immediately after the antinociceptive peak effect. Mice treated with 0.3 and 1 mg kg^{-1} of fentanyl and 1 mg kg^{-1} of cebranopadol were hyperactive but they did not fall dawn from the apparatus (data not shown). Moreover mice treated with the highest dose of the two ligands (i.e. 1 mg kg^{-1}) showed the typical Straub's tail. Ro 65-6570 up to mg kg^{-1} did not modify animal performance on the rota rod. However, higher doses (i.e. 3 and 10 mg kg^{-1}) produced a dose dependent and statistically significant disruption of mouse locomotor performance (Figure 43).

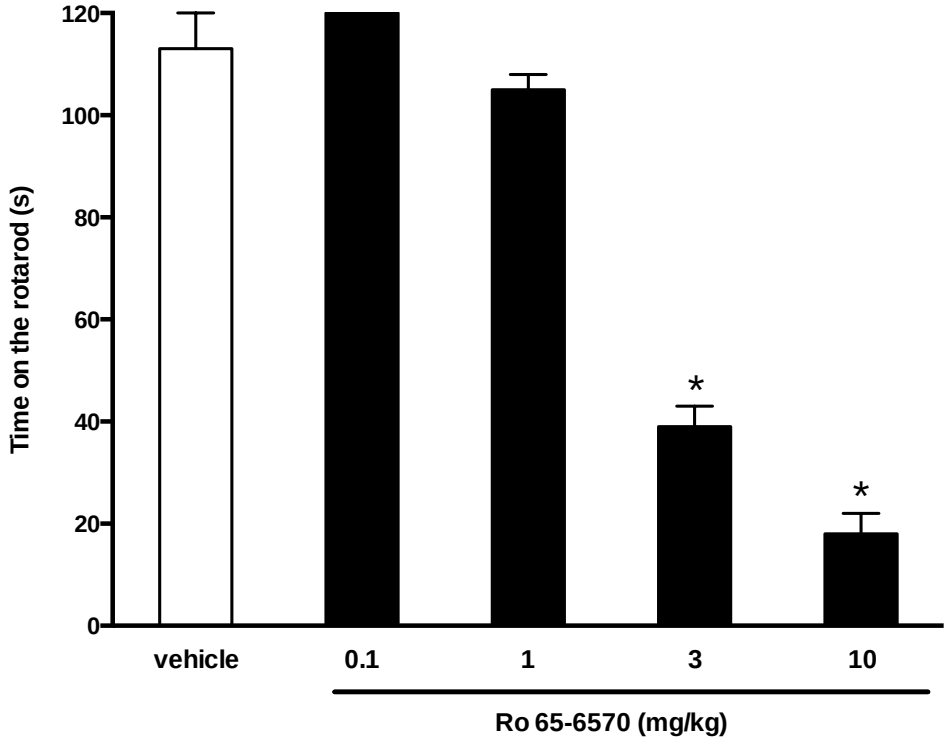


Figure 43. Mouse rota rod test. DRC to Ro 65-6570 (0.1 - 10 mg kg^{-1} i.v.). Histograms indicate the means (8 animals per group) and vertical lines the sem. *p < 0.05 vs vehicle according to ANOVA followed by the Dunnett's test.

To investigate the involvement of opioid and NOP receptors in the antinociceptive action of cebranopadol antagonist studies were performed. In order to reduce the number of employed animals these experiments were made by performing cumulative DRC to the agonist in the same subject (see Material and Methods for details). No signs of distress or overt pain behaviours were observed in these mice after the repeated i.v. injections. The results of this series of experiments are reported in Figure 44. Under these experimental conditions 1 mg kg^{-1} of naloxone or SB-612111 did not evoke any effect per se on tail withdrawal latency (data not shown). In animal treated with vehicle, fentanyl elicited a dose dependent antinociceptive action showing an ED_{50} of 0.03 mg kg^{-1}. Similar results

were obtained in mice treated with 1 mg kg^{-1} SB-612111. On the contrary 1 mg kg^{-1} of naloxone caused a dextral displacement of the DRC to fentanyl to approximately ten fold (Figure 44A). In parallel experiments cebranopadol produced a dose dependent effect with a value of potency of 0.2 mg kg^{-1}. The antinociceptive effects induced by cebranopadol were antagonised in a similar manner by both naloxone and SB-612111 (Figure 44B).

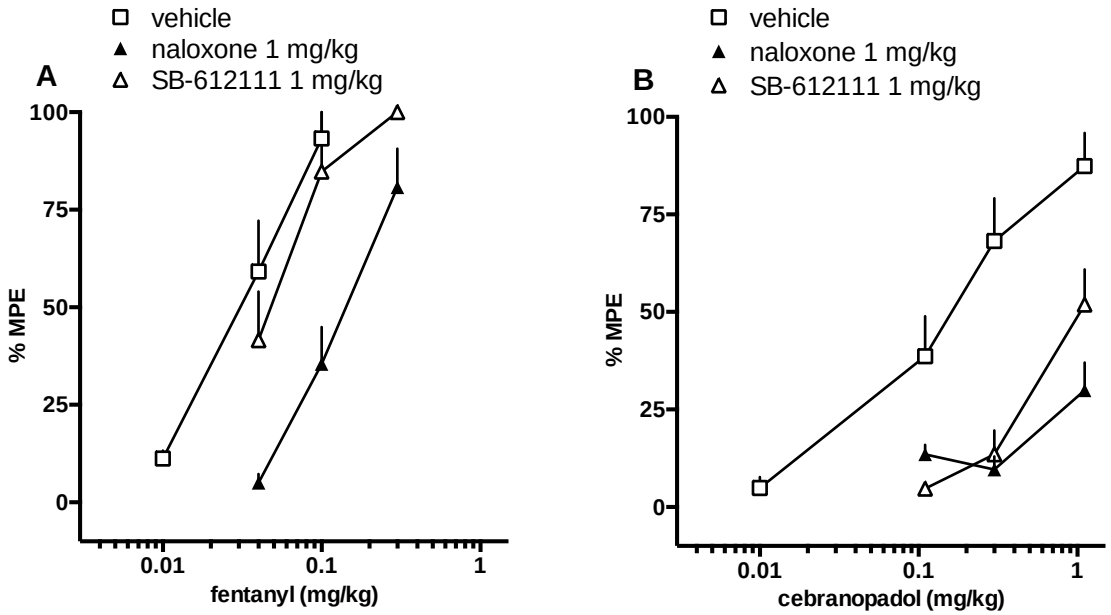


Figure 44. Mouse tail withdrawal assay. Cumulative DRC to fentanyl (Panel A) or to cebranopadol (Panel B) in animal treated with vehicle, naloxone or SB-61211. Data are the means (8 animals per group) and vertical lines the SEM.

Formalin test – The intraplantar injection of 20 µl of 1.5% formalin solution into the dorsal surface of the right hind paw produced a biphasic nociceptive response. The first phase (I° phase) started immediately after formalin injection and lasted for 10 min, while the second phase (II° phase) was prolonged, starting approximately 15-20 min after the injection and lasting for about 40 min. Mice receiving 20 µl of saline or 10% DMSO into the dorsal surface of the right hind paw did not show any pain-related behaviour (data not shown). Fentanyl inhibited in a dose dependent manner both the I° and the II° phase of the assay (Figure 45B) inducing a statistical significant effect at 0.03 and 0.1 mg kg^{-1} (Figure 45A). Form these results ED_{50} of 0.03 and 0.04 mg kg^{-1} were calculated for the I° and the II° phase, respectively. Similar results were induced by Ro 65-6570 that produced a dose dependent antinociceptive effect (Figure 45C). The DRC to this compound is however incomplete (Figure 45C) since doses higher than 1 mg kg^{-1} could not be tested due to their disrupting action on animal locomotor performance. Finally, cebranopadol was also able to inhibit nociceptive behaviour in the formalin assay in a dose dependent manner (Figure 45E) producing statistically significant effect starting from the 0.01 mg kg^{-1} dose. Cebranopadol ED_{50} values for the I° and the II° phase were 0.04 and 0.03 mg kg^{-1}, respectively.

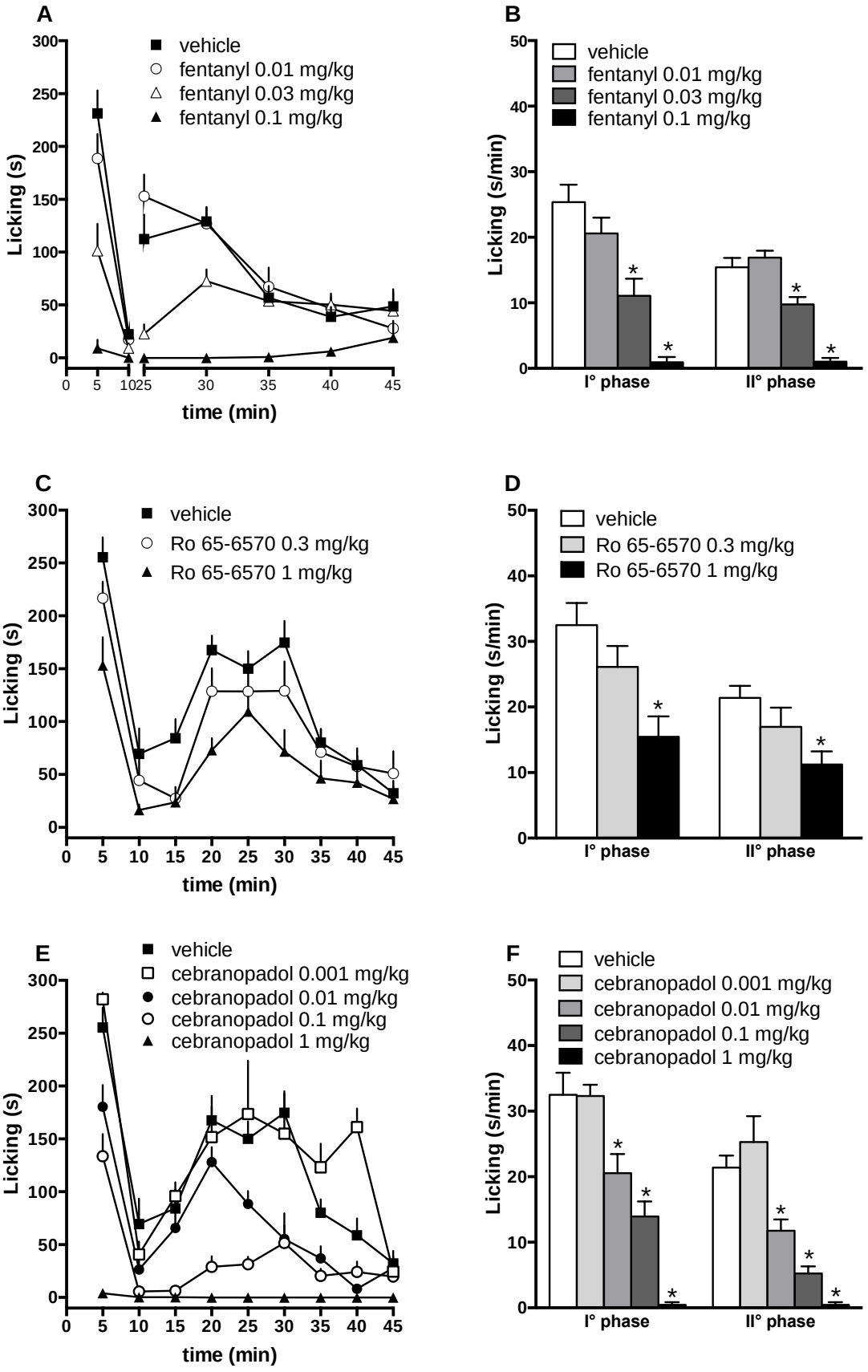


Figure 45. Time course of formalin-induced pain behaviour in mice treated i.v. with fentanyl (0.01 - 0.1 mg kg^{-1}) (A) or Ro 65-6570 (0.3 - 1 mg kg^{-1}) (C) or cebranopadol (0.001 - 1 mg kg^{-1}) (E). B, D and F: formalin-induced pain behaviour during the I° and II° phases. Each points represents the mean (9-11 animals per group) and the vertical bars indicate the sem. *$p < 0.05$ vs vehicle according to ANOVA followed by the Dunnett's test.

Discussion

The present study confirms and extends previous findings (Linz et al., 2014) by demonstrating that cebranopadol acts as a mixed NOP/opioid receptor agonist. In addition results obtained in the BRET assay suggest that cebranopadol behaves as G-protein biased agonist at MOP and particularly at NOP receptors. *In vivo* in mice cebranopadol promoted brilliant antinociceptive effects via simultaneous activation of NOP and opioid receptors. Interestingly, cebranopadol displayed higher analgesic potency against inflammatory than nociceptive pain. Altogether this study support the proposal that cebranopadol represents the prototype of a novel class of analgesics i.e. mixed NOP/opioid agonists characterised by high efficacy particularly for inflammatory/neuropathic pain associated with better tolerability compared to classical opioids (Alexander et al., 2013) (Alexander et al., 2013) (Schroder, Lambert, Ko & Koch, 2014).

In calcium mobilization studies the pharmacological profile of cebranopadol has been investigated and compared to that of standard agonists. N/OFQ, fentanyl, and DPDPE displayed high potency and selectivity for the NOP, MOP and DOP receptor, respectively. Dynorphin A behaved as a non-selective KOP preferring agonist. These results are perfectly in line with previous findings published by us (Camarda et al., 2009) (Camarda & Calo, 2013) and others (Zhang, Wang, Cox & Civelli, 2012). Cebranopadol was able to stimulate calcium mobilization in the four cell clones showing the following order of potency NOP = MOP > DOP $\geq$ KOP. This result is very similar to the profile obtained in receptor binding studies (Linz et al., 2014). As far as efficacy is concerned, cebranopadol behaved as full agonist at NOP, MOP, and DOP receptors and as partial agonist at the KOP. Again this confirms previous findings obtained in the stimulated [^{35}S]GTPγS assay (Linz et al., 2014). Thus calcium mobilization studies perfectly confirmed the proposed pharmacological profile of cebranopadol as a NOP/opioid (MOP preferring) universal agonist. However it is worthy of mention that absolute values of potency in the calcium assay are approximate 30 fold lower that those obtained in the [^{35}S]GTPγS assay (Linz et al., 2014). BRET and bioassay studies demonstrated a slow kinetic of action of cebranopadol (see below) and our previous studies suggest that the calcium assay tends to underestimate the potency of agonists characterized by slow kinetic of action (Camarda et al., 2009) (Rizzi et al., 2014) (Ruzza et al., 2014). Thus, the relatively low potency displayed by cebranopadol in the calcium assay may likely be due to the hemi-equilibrium conditions (Charlton & Vauquelin, 2010) generated by the relatively long time needed to

obtain full receptor activation by this compound on one hand and the rapid and transient nature of the calcium response on the other hand.

The stimulatory effects of cebranopadol at NOP and MOP receptors were challenged with antagonists. SB-612111 antagonized with high potency (8.85) the stimulatory effect of N/OFQ in NOP expressing cells while showing 1000 fold lower potency in MOP cells stimulated with fentanyl. On the contrary naloxone displayed high potency at MOP (8.18) being inactive at NOP receptors. These results perfectly confirmed previous findings both in terms of antagonist potency and selectivity of action (Camarda et al., 2009) (Camarda & Calo, 2013). Similar results were obtained when cebranopadol was used as agonist; in fact its stimulatory effects at MOP receptors were antagonized with high potency (8.61) by naloxone while SB-612111 display low potency (6.15) and opposite results were obtained in NOP cells where cebranopadol effects were resistant to naloxone and sensitive to SB-612111. Of note this antagonist displayed extremely high potency and an apparent insurmountable behaviour when challenged against cebranopadol but not N/OFQ. We interpret this unexpected finding as due to the combination of the slow kinetic of action of cebranopadol with the slow dissociation of this antagonist from the NOP receptor demonstrated in isolated tissue experiments (Arduin et al., 2007). In fact it is known that insurmountable antagonism may became apparent using a competitive antagonist when the time of challenge with the agonist is too short to allow the attainment of a new mass action equilibrium before the response is measured (Vauquelin & Van Liefde, 2006).

The ability of cebranopadol to promote NOP and MOP receptor interaction with G-proteins and arrestins has been evaluated with a BRET assay that has been recently set up and validated with a large series of opioid (Molinari et al., 2010) and NOP (Malfacini et al., 2015) ligands. N/OFQ stimulated NOP/G-protein interaction with high potency and efficacy. The non-peptide NOP agonist Ro 65-6570 mimicked N/OFQ action being, as expected (Rover et al., 2000) (Hashiba et al., 2001), 10 fold less potent. Superimposable results were obtained with cebranopadol. Interestingly prolonging the agonist incubation time from 5 to 60 min caused a slight reduction of N/OFQ potency associated with an increase in that of Ro 65-6570 (2 fold) and cebranopadol (4 fold). Thus non-peptide agonists and in particular cebranopadol display a time dependent increase in potency that probably reflects slow kinetic of receptor interaction. As mentioned before this characteristic may likely explain some findings obtained in the calcium mobilization assay. Dermorphin and fentanyl stimulated MOP/G-protein interaction with similar high potency and efficacy. Cebranopadol mimicked this stimulatory effect with similar efficacy but 10

fold higher potency. Interestingly prolonging the agonist incubation produced a similar increase in agonist potency for dermorphin and fentanyl (3 fold) and a larger increase (8 fold) for cebranopadol. Thus cebranopadol displayed a slow kinetic of action both at NOP and MOP receptor. Moreover it is worthy of note that cebranopadol, despite showing a similar value of binding affinity at both receptors (Linz et al., 2014), was approximately 10 fold more potent at MOP than NOP receptor. Very similar results were obtained by Grunenthal researchers in [^{35}S]GTPγS experiments (Linz et al., 2014). These findings may suggest that the efficacy of cebranopadol in promoting receptor/G-protein interaction and G-protein activation is higher at MOP than at NOP. Interestingly and possibly supporting this proposal, the maximal effects elicited by cebranopadol at MOP receptor were always superimposable to those elicited by standard agonists (DAMGO in [^{35}S]GTPγS and dermorphin in BRET experiments) while at NOP receptor they were always < 90% of the maximal effect elicited by N/OFQ.

N/OFQ stimulated NOP/β-arrestin 2 interaction with high potency and efficacy. In this assay Ro 65-6570 displayed reduced potency and efficacy while cebranopadol was found completely inactive up to micromolar concentrations. Similar results were obtained at 60 min incubation time. In cells expressing the MOP receptor dermorphin displayed high potency and efficacy, fentanyl behaved as partial agonist (in line with previous findings (Molinari et al., 2010)) while cebranopadol behaved as full agonist showing however 30 fold lower potency compare to its ability to promote receptor/G-protein interaction. In general these results indicated that naturally occurring peptides are similarly able to promote interaction of their receptors with both G-protein and arrestin while synthetic agonists are much better in selecting the conformation of the active receptor in complex with G-protein than arrestin. In the opioid receptor field this general rule has been established in a series of studies (Molinari et al., 2010) and recently confirmed for the NOP receptor (Malfacini et al., 2015) (Chang et al., 2015). In particular these results indicate that cebranopadol behaves as G-protein biased agonist; for the MOP receptor this is based on its 30 fold higher potency in promoting receptor/G-protein interaction and for the NOP receptor on its lack of efficacy in promoting the formation of the receptor/arrestin complex. The implications of this *in vitro* pharmacological behaviour in terms of *in vivo* biological actions are not easy to foreseen. As far as the MOP receptor is concerned converging evidence coming from arrestin knockout (Bohn, Gainetdinov, Lin, Lefkowitz & Caron, 2000) (Bohn, Lefkowitz, Gainetdinov, Peppel, Caron & Lin, 1999) (Raehal, Walker & Bohn, 2005) and pharmacological studies (DeWire et al., 2013) indicate that the analgesic

response to receptor activation mainly depends on G-protein while arrestin recruitment and signalling plays a role in tolerance liability and respiratory and gastrointestinal side effects. As far as the NOP receptor is concerned, no direct information is available. However it is possibly worthy of mention the fact that NOP agonists showing a certain degree of bias toward G-protein such as UFP-112 and PWT2-N/OFQ (Malfacini et al., 2015) produced brilliant and long lasting antinociceptive effect after spinal delivery in non human primates (Hu, Calo, Guerrini & Ko, 2010) (Rizzi et al., 2015).

In the electrically stimulated mouse vas deferens N/OFQ and DPDPE elicited fast inhibitory effects that were rapidly and fully reversible after washing. Previous studies demonstrated that the action of N/OFQ is solely due to NOP receptor activation (Calo et al., 2000) and that of DPDPE to the DOP receptor (Vergura et al., 2006). In this preparation cebranopadol produced show developing inhibitory effects that could not be reversed by washing. Similar results were obtained in the guinea pig ileum where cebranopadol action was compared with that of N/OFQ and dermorphin. The kinetic of action of cebranopadol in tissues matches the time dependent increase in potency observed in BRET experiments, strongly suggesting a slow kinetic of receptor interaction for this compound. To investigate the receptors involved in the biological effect of cebranopadol antagonist studies were performed using a curative protocol. The inhibitory effects exerted by cebranopadol both in the mouse vas deferens and in the guinea pig ileum were sensitive to naloxone and resistant to SB-612111. Thus in these preparations cebranopadol behaved as an opioid compound. The reasons for the lack of effect of cebranopadol at NOP receptor in these preparations are unknown. The involvement of PTX sensitive G-proteins in the action of opioids in the guinea pig ileum has been established (Lujan, Lopez, Ramirez, Aguilar, Martinez-Olmedo & Garcia-Sainz, 1984). This information is not available for NOP receptor agonists. The mouse vas deferens and the guinea pig ileum have being used as useful pharmacological preparations for investigating the profile of NOP ligands of peptide nature (Calo G, 2013) and of non-peptide NOP antagonists such as, for example, J-113397 (Bigoni et al., 2000), SB-612111 (Spagnolo et al., 2007), or C-24 (Camarda et al., 2009). The usefulness of these preparations for investigating the pharmacological profile of non-peptide agonists has been not established yet. For instance the effect of the NOP selective non-peptide agonist Ro 64-6198 in the guinea pig ileum could be fully antagonized only using a cocktail of NOP and opioid receptor antagonists and the same cocktail did not affect the inhibitory effect of the compound in the mouse vas deferens (Bigoni et al., 2000). In order to get further information about the effect of cebranopadol in

tissues expressing native NOP and opioid receptors the mouse colon was used. In this preparation NOP and MOP agonists elicit concentration depended contractile effects probably due to inhibition of NO release from NANC-terminals (Rizzi, Bigoni, Calo, Guerrini, Salvadori & Regoli, 1999) (Menzies & Corbett, 2000). In line with previous studies N/OFQ and the MOP selective agonist EM-1 produced robust contractive effects in this preparation. Cebranopadol up to 1 µM was completely inactive. Since the contractions in response to N/OFQ or EM-1 are extremely fast and transient it is possible that the lack of agonist action of cebranopadol is due to its slow kinetic of receptor occupation and activation. However in tissues previously treated with cebranopadol the contractile action of the peptides was reduced (N/OFQ) or completely abolished (EM-1). Thus in this preparation cebranopadol did act as mixed NOP/MOP antagonist.

It has been reported that cebranopadol produced robust antinociceptive effects in a variety of analgesiometric assays in rats and mice (Linz et al., 2014) (Schunk et al., 2014). In the present study we investigated cebranopadol *in vivo* in the mouse tail withdrawal and formalin test and compared its effects with those elicited by agonists selective for the MOP (fentanyl) and NOP (Ro 65-6570) receptors. All compounds were used at doses that did not produce any alteration of animal performance in the rota rod test. Fentanyl elicited robust but short lasting antinociceptive effects in the mouse tail withdrawal assay showing a potency of 0.03 mg/kg, in line with previous findings (Zernig et al., 1995). Ro 65-6570 at 1 mg/kg did not modify tail withdrawal latency. This confirms previous findings demonstrating that selective activation of NOP receptor by the systemic administration of non-peptide agonist is inactive in nociceptive pain models (Jenck et al., 2000) (Dautzenberg et al., 2001). Cebranopadol produced brilliant and long lasting effects very similar in terms of peak effect and duration of action to those reported in rats (Linz et al., 2014) and mice (Schunk et al., 2014). Cebranopadol ED50 in this assay was 0.2 mg/kg.

The antinociceptive action of fentanyl in this assay was sensitive to naloxone but not to SB-612111 demonstrating the exclusive involvement of opioid receptors in its action. On the contrary, both antagonists counteracted the antinociceptive action of cebranopadol. Similar finding were previously obtained measuring mechanical hypersensitivity in the rat spinal nerve ligation model where the inhibitory effects of cebranopadol were prevented both by naloxone and by the NOP selective antagonist J-113397 (Linz et al., 2014). Collectively these findings robustly demonstrate that the antinociceptive action of cebranopadol derives from the simultaneous activation of NOP and opioid receptors. In the formalin assay fentanyl produced a complete and dose dependent antinociceptive action

showing a value of potency 0.03 similar to literature findings (Romero, Miranda & Puig, 2010) and to that obtained in the tail withdrawal assay. On the contrary Ro 65-6570 at doses inactive in the tail withdrawal assay produced in the formalin assay statically significant antinociceptive effects. This finding confirms literature evidence suggesting that in rodents selective NOP receptor agonists, despite being inactive in nociceptive pain models, are able to evoke robust analgesic action in models of inflammatory/neuropathic pain. For a detailed discussion of this topic see (Schroder, Lambert, Ko & Koch, 2014).

In the formalin assay cebranopadol evoked a dose dependent and complete inhibition of the animal nociceptive behaviour with an ED_{50} value of 0.03 mg/lg. The effect of cebranopadol is similar to that of opioids in terms of complete analgesic response and lack of disrupting effect on locomotor activity but is also similar to that of NOP agonists in terms of higher potency against inflammatory vs nociceptive pain. In the present study this difference is of 7 fold; similar results, with even higher potency differences, were obtained in rats using a large panel of analgesiometric assays (Linz et al., 2014). The simultaneous activation of NOP and opioid receptors, which mediated the antinociceptive action of cebranopadol, has been demonstrated to elicit additive (Reiss, Wichmann, Tekeshima, Kieffer & Ouagazzal, 2008) or superadditive (Courteix, Coudore-Civiale, Privat, Pelissier, Eschalier & Fialip, 2004) effects in terms of analgesic action in rodents. In non-human primates, systemic administration of buprenorphine and selective agonists for the NOP receptor (Ro 64-6198 or SCH 221510) determines superadditive antinociceptive effects without the side effects that are observed in response opioids (respiratory depression, pruritus) (Cremeans, Gruley, Kyle & Ko, 2012). Collectively these findings corroborate the proposal that mixed NOP/opioid agonists represent a promising strategy for identify innovative analgesics (Lin & Ko, 2013) (Toll, 2013) (Schroder, Lambert, Ko & Koch, 2014).

Although the present results confirmed the basic features of cebranopadol, i.e. mixed NOP/opioid agonist properties and brilliant antinociceptive action, there are some aspects of the pharmacological profile of this molecule that deserve attention and may at least in part explain some unexpected findings. As briefly mentioned before cebranopadol display the same affinity for NOP and MOP in binding studies while it shows higher functional potency at MOP than at NOP and slightly lower maximal effects compared to standard agonists at NOP but not at MOP. This may suggest partial agonist activity at the NOP receptor. This feature may explain lack of effect in promoting receptor/arrestin interaction since results obtained with large panels of compounds suggest that ligand chemical

requirements for arresting efficacy are more strict than for G-protein efficacy (Malfacini et al., 2015) (Chang et al., 2015). The putative NOP partial agonist activity of cebranopadol may also explain the lack of agonist effect in electrically stimulated tissues. In fact these preparations are characterized by a low efficiency of the stimulus/response coupling as suggested by the fact that NOP partial agonists such as the peptides [F/G]N/OFQ(1-13)-NH_2 or UFP-113 behave as NOP antagonists in the mouse vas deferens and guinea pig ileum (Guerrini et al., 1998) (Arduin et al., 2007). Despite higher efficacy at MOP over NOP receptor the *in vivo* antinociceptive action of cebranopadol seems to be well balanced on the two receptors. This may be possibly explained considering that arrestin counteract G-protein mediated signalling, the analgesic response to opioid is mainly mediated by G-protein signalling, the cebranopadol bias toward G-protein is larger for NOP than for MOP. However some findings of the present study speak, at least in part, against the view of cebranopadol as NOP partial agonist. In fact NOP partial agonists such as [F/G]N/OFQ(1-13)-NH2 or UFP-113 behave as N/OFQ antagonists in the electrically stimulated tissues (Guerrini et al., 1998) (Arduin et al., 2007), and in the BRET arrestin assay (Malfacini et al., 2015). Moreover in BRET studies these molecule showed similar value of potency as receptor/G-protein agonists and as receptor/arrestin antagonists. On the contrary despite its nanomolar agonist potency in the BRET receptor/G-protein assay cebranopadol up to micromolar concentration did not antagonized N/OFQ action in the BRET arrestin assay and in the mouse vas deferens. These findings raise doubts about the ortosteric nature of cebranopadol binding to the NOP receptor that has been proposed based on molecular modelling and docking studies (Schunk et al., 2014) and may suggest a more complex interaction of this ligand to the NOP receptor.

In conclusion the present study further investigated the *in vitro* and *in vivo* profile of cebranopadol. This compound behaves as a mixed NOP/opioid agonist showing biased agonism toward G-protein particularly at the NOP receptor. Cebranopadol promotes *in vivo* brilliant analgesic effects via simultaneous stimulation of NOP and opioid receptors and showing higher potency against inflammatory than nociceptive pain. The present findings together with positive results of phase II clinical studies (Bird et al., 2015) support the proposal that mixed NOP/opioid receptor agonists are worthy of development as innovative analgesics.

2.2.2. RR fentanyl derivatives and RR4-Ro

One of the strategies evaluated for generating NOP/MOP mixed agonists was to design chimeric compounds by linking together non-peptide agonists selective for the NOP and MOP receptors. To this aim the potent NOP agonist Ro 65-6570 which has high affinity and moderate selectivity for the NOP receptor (Wichmann, Adam, Rover, Cesura, Dautzenberg & Jenck, 1999) and the potent and selective MOP agonist fentanyl (Stanley, 1992) have been selected (Figure 46).

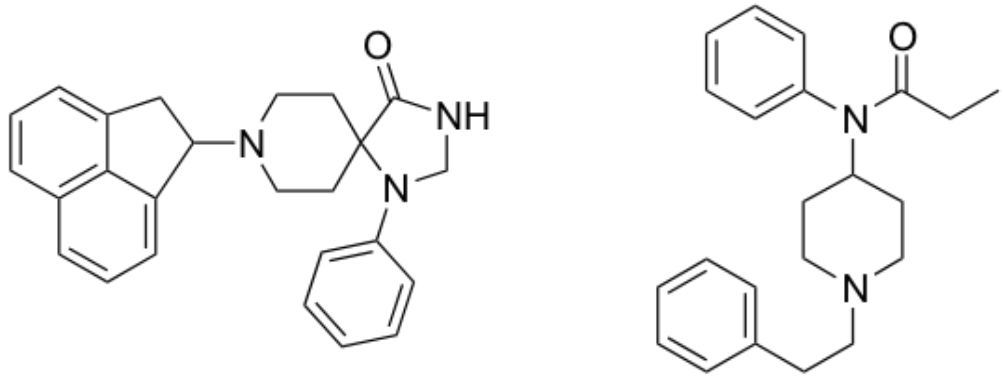
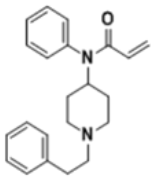
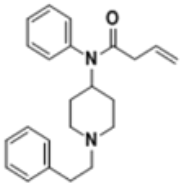
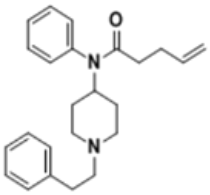
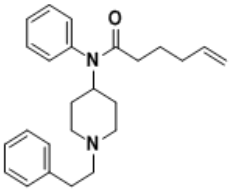
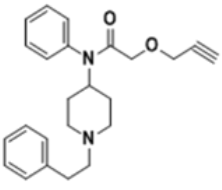
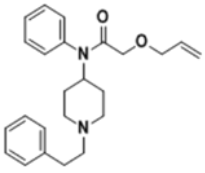


Figure 46. Chemical structures of Ro 65-6570 (left) and fentanyl (right).

This strategy was based on previous studies reporting the discovery of novel DOP/MOP ligands developed using the DOP selective antagonist Dmt-Tic as a pharmacophore. This compound was linked with the MOP/KOP morphinan pharmacophore butorphan to produce a non-selective opioid ligand that substantially maintained the pharmacological features of the parent compounds (Neumeyer et al., 2006). This investigation demonstrated the possibility of grafting different opioid pharmacophores without affecting their original pharmacological activity. In addition the medicinal chemists of our university started collaboration with Prof. Ruben Vardanyan (University of Tucson, Arizona) and Prof. David G. Lambert (University of Leicester, UK) focussed on the design and synthesis of novel DOP/MOP ligands based on the general formula Dmt-Tic-Spacer-fentanyl (Bird et al., 2015). This collaboration was instrumental in the context of the present project for the design and synthesis of NOP/MOP non-peptide bivalent ligands. In fact, our study started with the pharmacological characterisation of functionalized fentanyl derivatives provided by Prof. Vardanyan and named RR compounds (Table 17). These compounds differ in the length of the amide bond in the northern region of fentanyl chemical structure.

Table 17. Chemical structure of the RR compounds. The amide linker length and composition in the northern hemisphere of the fentanyl structure are shown in the right hand column.

Chemical Structure	Name	Amide Bond Extension
	RR4	1 carbon bond
	RR5	2 carbon bonds
	RR6	3 carbon bonds
	RR7	4 carbon bonds
	RR8	Ether spacer and carbon bond
	RR9	Ether Spacer

The pharmacological characterization of these compounds was aimed to select the most convenient MOP pharmacophore to be used for the synthesis of bifunctional NOP/MOP ligands. Thus the aim of this study was twofold: i) *in vitro* pharmacological characterization of the RR series of fentanyl derivatives and selection of the best molecules, and ii) investigation of the pharmacological profile of chimeric compounds. To these aims the following assays were used: receptor binding in membranes taken from CHO cells expressing the human recombinant NOP or OP opioid receptors, this same preparation was used for performing stimulated GTPγ[^{35}S] binding studies, calcium mobilisation studies were performed in cell co-expressing the NOP and MOP receptors and the chimeric protein $G\alpha_{qi5}$ that forces G_i coupled receptor to signal via the PLC-IP_3-Ca^{2+} pathway. Finally ligand effects at native receptors were evaluated in the electrically stimulated the guinea pig ileum, a pharmacological preparation expressing both NOP and MOP receptors.

Materials and methods

Cell Culture – Cell-lines were cultured in 5% carbon dioxide humidified air, at 37°C, and sub-cultured using trypsin EDTA as required. Cells were used experimentally when confluent. $CHO_{MOP/DOP/KOP}$ cells were cultured in Dulbecco's HAMS/F12, which was supplemented with 10% foetal bovine serum (100 IU/ml), streptomycin (100 µg/ml), fungizone (2.5 µg/ml) and geneticin (G418) (400 µg/ml). CHO_{NOP} cells were cultured in Dulbecco's MEM/HAMS F12 (50/50) supplemented with 5% FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml), fungizone (2.5 µg/ml), geneticin (G418) (400µg/ml) and hygromycin B (200µg/ml). CHO cells stably co-expressing human recombinant NOP, MOP or KOP receptor and the C-terminally modified $G\alpha_{qi5}$ (Conklin, Farfel, Lustig, Julius & Bourne, 1993) chimeric protein and cells co-expressing the DOP receptor and the $G\alpha_{qG66Di5}$ (Kostenis et al., 2005) chimeric protein were generated as previously described (Camarda et al., 2009), (Camarda & Calo, 2013). Cells were cultured in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM)/HAMS F12 (1:1) supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml).

Radioligand Binding assay – In order to harvest cells, harvest buffer and gentle agitation of the flasks was required. Cells were collected and centrifuged at 1,500 rpm for 3 minutes before being resuspended in the appropriate buffer. Dependent on the experiment, cells were suspended in either wash buffer for radioligand displacement binding assays (consisting of 50 mM Tris-HCl pH to 7.4 with KOH, for $CHO_{MOP/KOP/DOP}$ and additional 5 mM $MgSO_4$ for CHO_{NOP}) or homogenizing buffer for GTPγ^{35}S assay (consisting of 50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH). An Ultra-Turrax was used, to homogenize the cells, for a minimum of 10 seconds. The homogenate was then centrifuged at 13,500 rpm for 10 minutes, at 4°C. This step is repeated three times. In order to obtain the protein concentrations from the membrane fractions obtained from the previous steps, methods set out by Lowry (Lowry, Rosebrough, Farr & Randall, 1951) were used: BSA protein standards were made in 0.1M NaOH, at set concentrations of 0, 50, 100, 150, 200, 250 µg protein/ml. The membrane fractions were diluted in 0.1M NaOH. 0.5ml volumes of the samples and standards were incubated in 2.5 ml of a solution, which consisted 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, for 10 minutes. Folin's reagent, which is diluted at a 1:4 ratio in H_2O, is then added to the standards and sample and incubated at room temperature for 30 minutes. A spectrophotometer is used to determine the absorbance, at 750 nm, of the standards and samples. A standard curve is produced from the linear regression of the known BSA standards, which then allows the sample protein concentration to be determined from this curve. Displacements curve experiments require the use of a single concentration of radioligand in all experimental tubes. The ligands of interest, however, were used in varying concentrations (1 pM - 10 µM). Dependent on the cell line and its receptor expression between 20-200µg of membrane protein was used in each experiment. $CHO_{MOP/KOP/DOP}$ cells were incubated with ~0.8 nM of [^{3}H]-Diprenorphine, while CHO_{NOP} cells were incubated with ~1 nM of [^{3}H]UFP-101. In order to define non-specific binding, 10 µM of naloxone was used in $CHO_{MOP/KOP/DOP}$ cells, while 1µ M of N/OFQ was used for the CHO_{NOP} cells. The cells were incubated for one hour at room temperature, followed by separation of the free and bound radioligand using vacuum filtration onto polyethylemine (PEI 0.5%)-soaked Whatman glass fibre filters in a Brandel harvester. The collected filters were placed in scintillation fluid for an incubation period of 8 h before being counted in a liquid scintillation spectrometer.

Stimulated [^{35}S]GTPγS binding assay – The cell membranes for this assay are prepared using the methods described above, except with the homogenization buffer already mentioned. Forty micrograms of the CHO membrane protein were resuspended in 0.5 ml volumes of the CHO assay buffer, consisting of 50 mM Tris, 0.2 mM EGTA, 1 mM $MgCl_2$, 100 mM NaCl, 0.1% BSA, bacitracin (0.15 mM), GDP (classical opioids-33 µM; NOP-100 µM) and ~150 pM of GTPγ[^{35}S]. In order to define non-specific binding, 10 µM of non-radiolabelled GTPγS was used in all cell membranes. The assay was incubated for 1 hr in a 30°C degree water bath, before being harvested, in the absence of PEI, using the protocols mentioned previously.

Calcium mobilization assay – When confluence was reached (3-4 days), cells were sub-cultured as required using trypsin/EDTA and used for experimentation. Cells were seeded at a density of 50,000 cells/well into 96-well black, clear-bottom plates. After 24 hours incubation the cells were loaded with Hank's Balanced Salt Solution (HBSS) supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM, 0.01% pluronic acid and 20 mM HEPES (pH 7.4) for 30 min at 37°C. Afterwards the loading solution was aspirated and a washing step with 100 µl/well of HBSS, HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 µM Brilliant Black was carried out. Subsequently 100 µl/well of the same buffer was added. After placing cell culture and compound plates into the FlexStation II (Molecular Devices, Sunnyvale, CA, USA), fluorescence changes were measured after 10 min of stabilization at 37°C. On-line additions were carried out in a volume of 50 µl/well.

Guinea pig ileum bioassay – Tissues were taken from male albino guinea pigs of 350 – 400 g (Pampaloni, Pisa, Italy). The animals were treated in accordance with European guidelines (86/609/ECC) and national regulations (DL 116/92). They were housed in 560 x 320 x 180 mm cages (Techinplast), three per cage, under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 7:00 h) with food (complete feed for guinea pig, Mucedola, Milano, Italy) and water ad libitum. The day of the experiment the animals were killed with an isofluorane overdose. Bioassay experiments were performed as previously described (Bigoni et al., 1999). The tissues were suspended in 5 ml organ bath containing Krebs solution (composition in 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), hexamethonium bromide 22 µM, benadril 0.34 µM oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 37°C. A resting tension

of 1 g was applied to the tissues. Tissues were stimulated through two platinum electrodes with supramaximal rectangular pulse of 1 ms duration, 0.05 Hz frequency, 80 V of amplitude. Electrically evoked contractions were measured isotonically by means of Basile strain gauge transducers (Basile 7006; srl Ugo Basile, Varese, Italy) and recorder with a computer based acquisition system (Power Lab 8, ADInstruments, USA). 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 agonists were constructed (0.5 log unit steps). Antagonists were injected into the baths 15 min before performing concentration response curves to agonists.

Materials – All cell culture media and supplements were from Invitrogen (Paisley, U.K.). All other reagents used were from Sigma Chemical Co. (Poole, UK) or E. Merck (Darmstadt, Germany) and were of the highest purity available. Tritiated UFP-101 ([^{3}H]-UFP-101) was synthesized as described previously ((Ibba et al., 2008)). Tritiated diprenorphine ([^{3}H]-DPN) was purchased from Perkin Elmer. Fentanyl citrate chloridrate used in this study were bought from S.A.L.A.R.S. S.p.A. (Como Italia). The reference molecules, dermorphin, endomorphin-1 and N/OFQ were synthesized in house (Department of Chemical and Pharmaceutical Sciences, University of Ferrara) as previously described (Calo et al., 1997), while Norbinaltorphimine (Nor-BNI), Naltrindole, naloxone and SB-612111 were purchased from Tocris Bioscience (Bristol, UK). Stock solutions (1 mM) of peptides and naloxone, (10 mM) of fentanyl were made in ultrapure water, SB-612111 (10 mM) was solubilized in DMSO. Compounds RR4, RR5, RR6, RR7, RR8, and RR9 were synthesized in the laboratory of Ruben Vardanyan (University of Arizona) and solubilized in DMSO at a final concentration of 10 mM. Compounds Ro 65-6570 and RR4-Ro were synthesized by Prof. Claudio Trapella (University of Ferrara). Ro 65-6570 was synthesized according to published procedures (Wichmann, Adam, Rover, Cesura, Dautzenberg & Jenck, 1999). RR4-Ro was synthesized as described in detail in the next section. Ro 65-6570 and RR4-Ro were solubilized in DMSO at the final concentration of 10 mM. All compound was stored at -20°C until use. The successive dilutions of compounds were made in either wash buffer for radioligand displacement binding assays or homogenizing buffer for GTPγ^{35}S assay (both containing 0.5% Bovine Serum Albumin); HBSS/HEPES (20 mM) buffer (containing 0.005 % BSA fraction V to avoid licking) in the calcium assay and in saline in the bioassay studies.

Synthesis of RR-Ro compounds – This synthetic strategy involved compound Ro 65-6570 as NOP agonist and some fentanyl derivatives as MOP agonists. RR4 and RR9, which have been selected among various fentanyl derivatives based on their high affinity and potency and full agonist activity at the MOP receptor, were conjugated to Ro 65-6570 through thiol-ene reaction that takes advantage of the versatility of the final alkene moiety. This reaction is environment friendly, easy to perform and shows quantitative yields. The synthesis, at the beginning, was expected to functionalize Ro 65-6570 on the amidic nitrogen with bromo-butene to give a compound prone to react with thio-acetic acid in a thiol-ene reaction. Then, the thio-ester obtained was hydrolysed to give a thiole moiety that could react on the alkene of fentanyl derivatives with another thiol-ene reaction but it was observed dimerization of thiole moiety that formed disulphide bridges. The synthetic strategy was re-considered by functionalizing Ro 65-6570 with a bromo-propylamine that reacted with a maleimid derivative that shows an alkene moiety and build the SH moiety on the fentanyl derivatives that were subjected to thiol-ene with thio-acetic acid followed by the hydrolysis step. This last step was conducted with acetyl chloride to avoid dimerization of –SH. The difficulties of this synthesis, apart from dimerization of –SH intermediates, were purification of products and characterization of final compounds due to their large molecular weight. Altogether synthetic, yield, and purification issues did not allow us to obtain RR9-Ro and only few mg of RR4-Ro were available for pharmacological evaluation.

Data analysis and terminology – All data are expressed as means ± standard error of the mean (SEM) of at least 3 experiments performed in duplicate. For potency values 95% confidence limits were indicated. The pharmacological terminology adopted in this report is consistent with the IUPHAR recommendations (Alexander et al., 2013). Receptor binding data are expressed as % displacement. [^{35}S]GTPγS data are expressed as stimulation factor that is 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. Affinity values are showed as pK_i calculated using the Cheng-Prusoff equation:

$$pK_i = \log\left[\frac{IC_{50}}{1+\frac{[L]}{K_D}}\right]$$

Where IC_{50} is the concentration of ligand that produces 50% inhibition of specific binding, [L] is the concentration of free radioligand and K_D is the dissociation constant of the radioligand for the receptor. Agonist potencies were given as pEC_{50} that is the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. Maximal effects elicited by the agonists are expressed as intrinsic activity α calculated as ratio between the agonist E_{max} and that elicited by a standard full agonist. Concentration response curve to agonists were fitted with the four parameter logistic nonlinear regression model:

$$\text{Effect} = \text{baseline} + \frac{E_{max} - \text{baseline}}{1 + 10^{(\log EC_{50} - X) \cdot n}}$$

Where EC_{50} is the concentration of agonist producing a 50% maximal response, X is the agonist concentration and n is the Hill coefficient of the concentration response curve to the agonist. In the experiments in which the concentration response curve to the agonist was tested in the absence and presence of a fixed concentration of antagonist, antagonist potency expressed as pK_B was derived from Gaddum Schild equation:

$$pA_2 = \log\left[\frac{CR - 1}{\text{antagonist}}\right]$$

assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist. Curve fittings were performed using Graph Pad PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.). Data obtained with calcium mobilization and BRET assays have been statistically analysed with one way ANOVA followed by the Dunnett's test for multiple comparisons, while data obtained with receptor binding assays (receptor binding and stimulated [^{35}S]GTPγS) with one way ANOVA followed by the Bonferroni's test for multiple comparisons. In both cases p values less than 0.05 were considered to be significant.

Results

Fentanyl derivatives of the RR series

Receptor binding – As shown in Figure 47, the analysis of competitive radioligand binding data in CHO cell membranes expressing the human NOP or the classical OP receptors demonstrate that fentanyl behaves as an high affinity (pK_i 7.98) and selective MOP ligand.

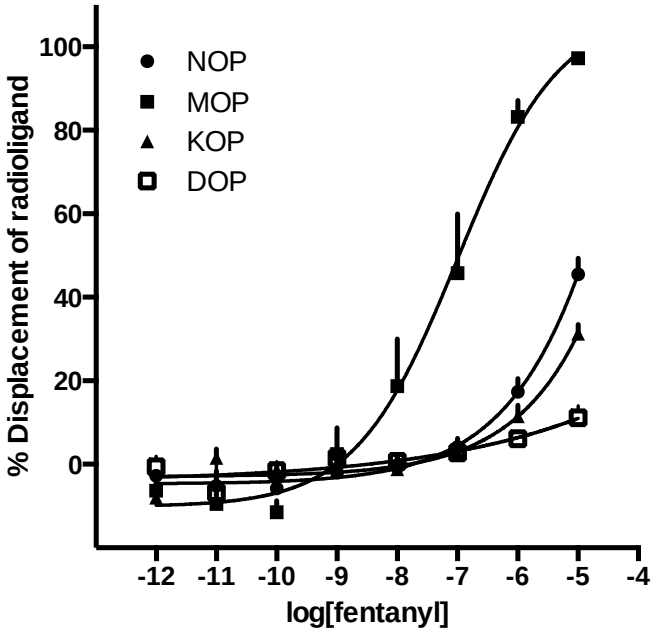


Figure 47. Displacement of tritiated diprenorphine ([^{3}H]-DPN) at $CHO_{MOP/KOP/DOP}$ and of tritiated UFP-101 ([^{3}H]UFP-101) at CHO_{NOP} by fentanyl. Data are shown as mean ± SEM for at least 3 experiments.

Under the same experimental conditions, RR fentanyl derivatives displaced the binding of [^{3}H]-DPN in a concentration dependent and saturable manner in membranes prepared from CHO expressing the MOP receptor. Compounds RR4, RR5 and RR6 showed high and similar values of affinity (pK_i 7.98, 8.16 and 8.01 respectively). RR7 and RR8 displayed a significant lower affinity (6.89 and 6.55). On the contrary RR9 displayed very high affinity (8.69) 5 fold higher than fentanyl (Figure 48).

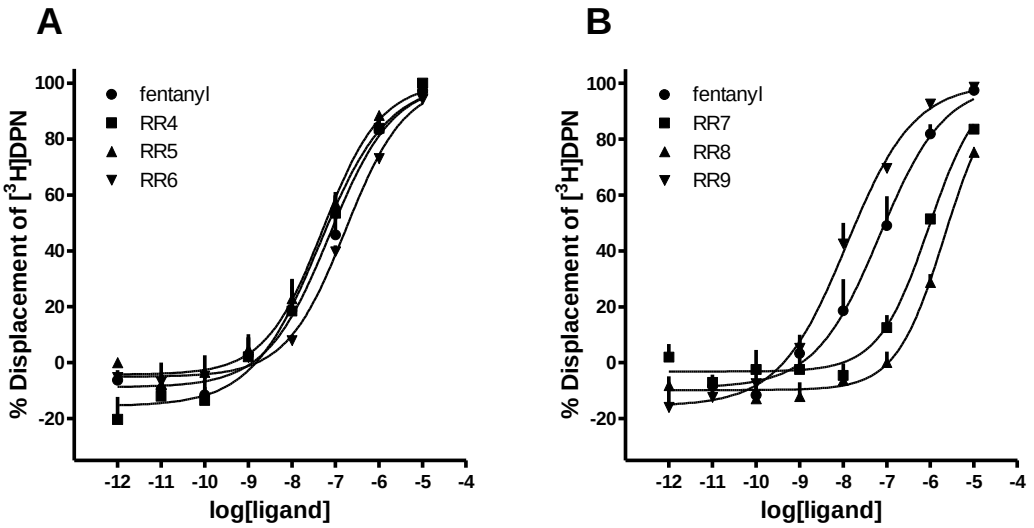


Figure 48. Displacement of tritiated diprenorphine ([^{3}H]-DPN) by fentanyl and the RR compounds in CHO_{MOP} cell membranes. Data are shown as mean ± SEM for 5 experiments.

Binding affinity was also measured in membranes prepared from cells expressing KOP and DOP receptor. At the KOP receptor the standard ligand nor-BNI displaced the binding of [^{3}H]-DPN with a pK_i of 9.95. All RR compounds bound with very low affinity to the KOP receptor (Figure 49).

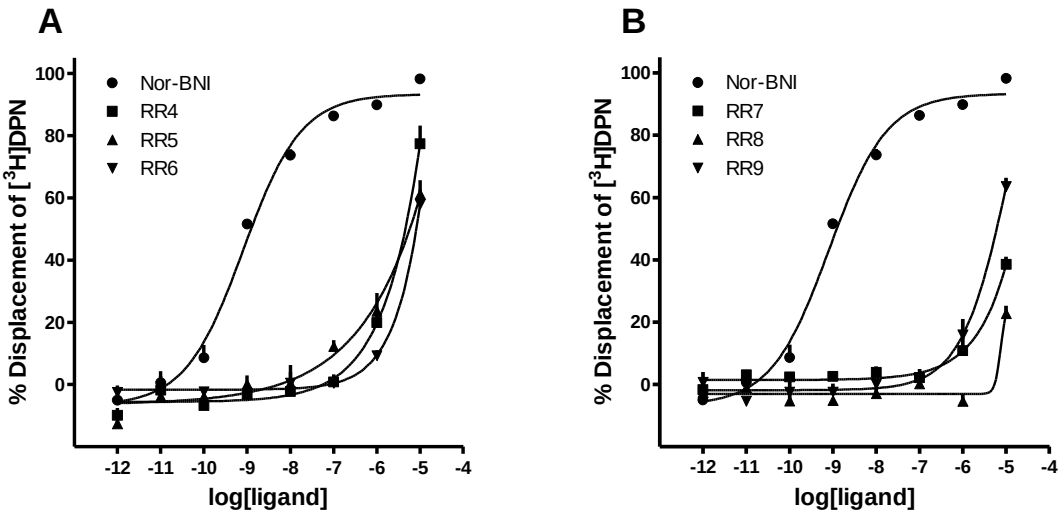


Figure 49. Displacement of tritiated diprenorphine ([^{3}H]-DPN) by nor-BNI and the RR compounds at CHO_{KOP} cell membranes. Data are shown as mean ± SEM for 3 experiments.

At the DOP receptor the control ligand naltrindole displayed a pK_i of 10.02. RR4 and RR9 showed moderate affinity (7.29 and 7.00), all other compounds exhibited lower binding affinity (<7) (Figure 50).

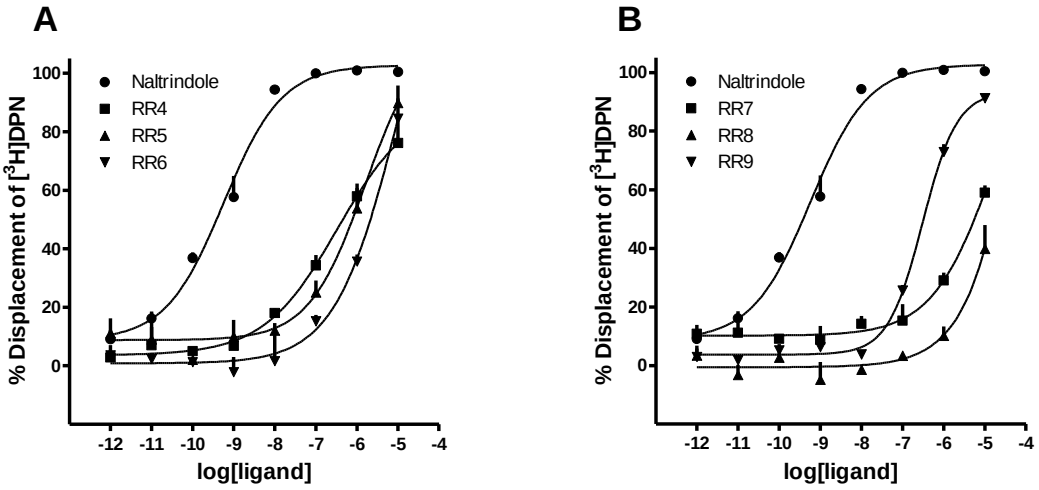


Figure 50. Displacement of tritiated diprenorphine ([^{3}H]-DPN) by naltrindole and the RR compounds at CHO_{DOP}. Data are shown as mean ± SEM for 5 experiments.

In parallel experiments, in membranes of CHO cells expressing the NOP receptor N/OFQ promoted the displacement of the radioligand in a concentration dependent and saturable manner, showing very high affinity (pK_i 9.49). Very low values of affinity were displayed by RR4, RR5, RR6 and RR9. RR7 and RR8 showed negligible affinity (<5) (Figure 51). Results obtained in this series of binding experiments have been summarized in Table 18.

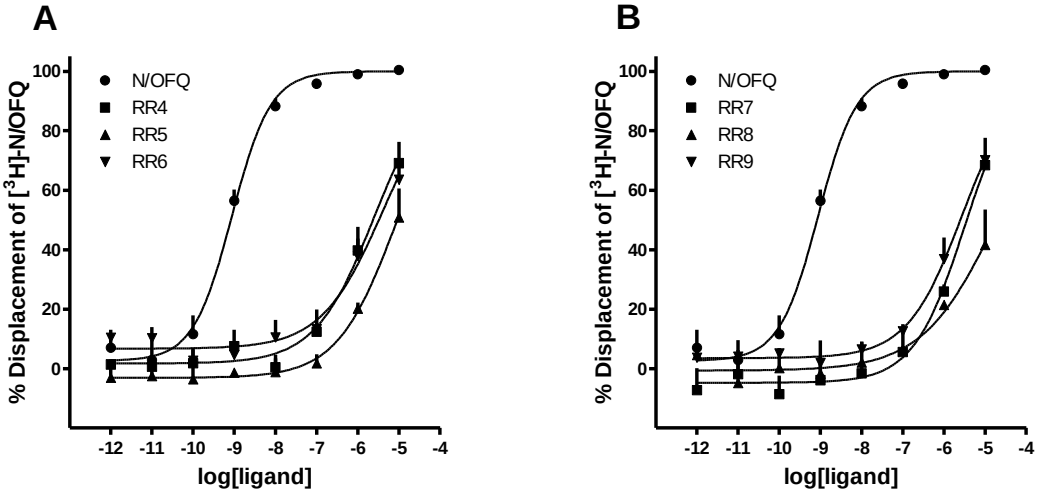


Figure 51. Displacement of tritiated N/OFQ ([^{3}H]-N/OFQ) by N/OFQ and the RR compounds in CHO_{NOP} cell membranes Data are shown as mean ± SEM for 3 experiments.

Table 18. Binding affinity of fentanyl and its RR derivatives at NOP and classical OP receptors.

	MOP	KOP	DOP	NOP
	pK_i ($CL_{95\%}$)			
fentanyl	7.98 (7.34-8.62)	inactive	inactive	inactive
RR4	8.16 (7.39-8.93)	<6	7.29 (6.10-8.47)	6.29 (6.11-6.47)
RR5	8.09 (8.00-8.18)	<6	6.77 (6.47-7.03)	<6
RR6	7.58 (7.50-7.65)	<6	6.28 (4.96-7.60)	<6
RR7	6.89 (6.68-7.09)	<6	<6	<6
RR8	6.55 (6.33-6.77)	6.08 (4.77-7.39)	<6	<6
RR9	8.69 (8.31-9.07)	6.74 (6.66-6.81)	7.00 (5.87-8.13)	<6

Data are mean ± SEM of n=5 (MOP and DOP) or n=3 (NOP and KOP).

Calcium mobilization assay – In CHO_{MOP} cells stably expressing the $G\alpha_{qi5}$ chimeric protein fentanyl evoked a concentration dependent stimulation of calcium release displaying high potency (pEC_{50} 8.19) and maximal effects (209 ± 14 % over the basal values) (Figure 52). The MOP selective peptide agonists dermorphin and endomorphin-1 mimicked the stimulatory effects of fentanyl showing similar potencies and maximal effects (Figure 52).

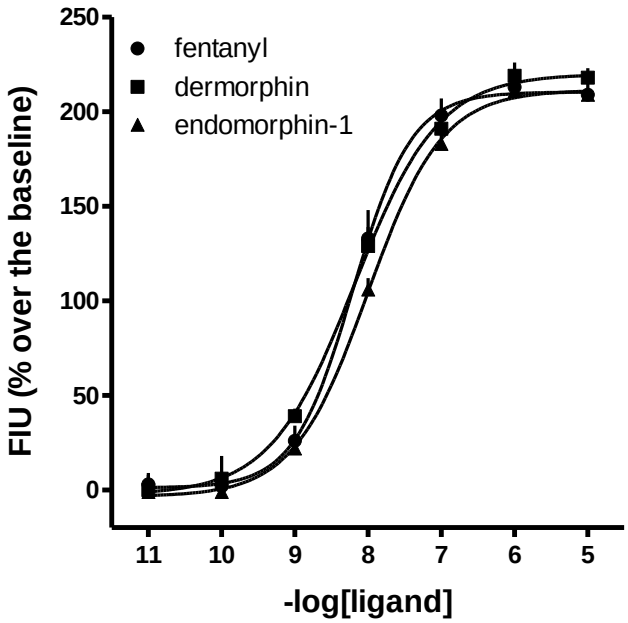


Figure 52. Concentration response curve to fentanyl, dermorphin, and endomorphin-1 in calcium mobilization experiments performed in CHO_{MOP} cells stably expressing the $G\alpha_{qi5}$ protein. Agonist effects are expressed as % over the baseline. Data are the mean ± SEM of ≥ 5 separate experiments performed in duplicate.

RR4 mimicked fentanyl stimulatory action showing similar potency (pEC_{50} 8.23) and maximal effects (209 ± 10 %) (Figure 53A). RR9 displayed maximal effects similar to fentanyl (212 ± 19 %) but slightly lower potency (Figure 53A). The RR5 compound also exhibited relatively high potency (pEC_{50} 7.29), however its maximal effect (157 ± 7 %) was a fraction of that of fentanyl corresponding to a value of α of 0.75 (Figure 53B). RR6 and RR7 compounds displayed low potency and their concentration response curves were incomplete (Figure 53 B and C). Finally, RR8 compound exhibited in this assay only a weak stimulation at the highest concentration tested i.e. 10 μM (34 ± 25%) (Figure 53C). All the results obtained with the RR series of compounds in calcium mobilization studies were summarized in Table 19.

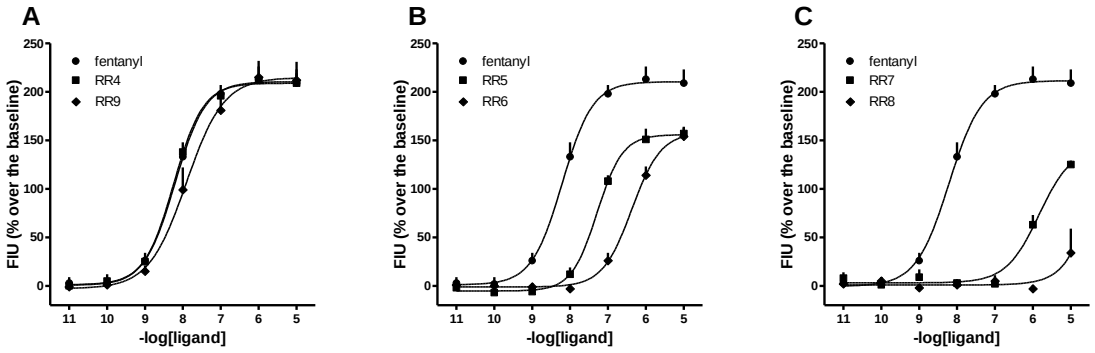


Figure 53. Concentration response curves to RR compounds compared to fentanyl. RR4, RR9 (A), RR5, RR6 (B), RR7 and RR8 (C) in calcium mobilization experiments performed in CHO_{MOP} cells stably expressing the $G\alpha_{qi5}$ protein. Agonist effects are expressed as % over the baseline. Data are the mean ± SEM of 4 separate experiments performed in duplicate.

Table 19. Effects of fentanyl and RR compounds in calcium mobilization experiments performed in CHO_{MOP} cells stably expressing the $G\alpha_{qi5}$ protein.

	pEC_{50} ($CL_{95\%}$)	**α ± SEM**
fentanyl	8.19 (7.95-8.43)	1.00
RR4	8.23 (8.10-8.36)	1.00 ± 0.05
RR5	7.29 (7.22-7.37)	0.75 ± 0.04
RR6	crc incomplete, at 10 µM 154 ± 9 %	
RR7	crc incomplete, at 10 µM 125 ± 4 %	
RR8	crc incomplete, at 10 µM 34 ± 25 %	
RR9	7.88 (7.38-8.39)	1.05 ± 0.04

crc incomplete means that maximal effects could not be determined due to the low potency of the compound.

Guinea pig ileum – The pharmacological activity of peptide MOP agonists, fentanyl and its RR derivatives has been also evaluated in the electrically stimulated guinea pig ileum. In this preparation fentanyl inhibited the electrically induced twitch response in a concentration dependent manner with high potency (pEC_{50} 9.08) and efficacy (E_{max} 88 ± 2%). The peptide dermorphin mimicked the inhibitory effect of the fentanyl with similar values of potency and maximal effects. Endomorphin-1 was also active in this preparation showing slightly lower maximal effects (78 ± 2%) and potency (8.41) (Figure 54).

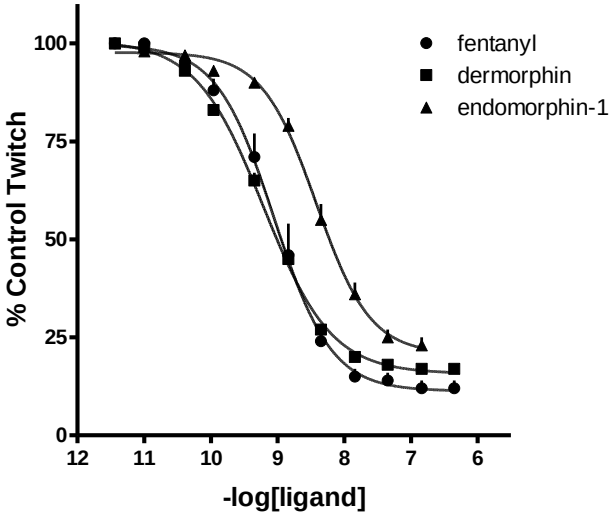


Figure 54. Electrically stimulated guinea pig ileum. Concentration response curves to fentanyl, dermorphin, and endomorphin-1. Data are the mean ± SEM of 6 separate experiments.

In parallel experiments the compounds of the RR series were assessed in comparison with fentanyl. Compounds RR4 and RR9 mimicked the action of fentanyl generating virtually superimposable concentration response curves. (Figure 55A). Compound RR5 also mimicked the inhibitory action elicited by fentanyl showing similar maximal effects but 9 fold lower potency (Figure 55A). The compounds RR6 and RR7 were also able to inhibit the control twitch in a concentration dependent manner but displaying a large loss of potency (Figure 55B). Finally RR8 generated an incomplete concentration response curve (Figure 55B). All results obtained in this preparation have been summarized in Table 20.

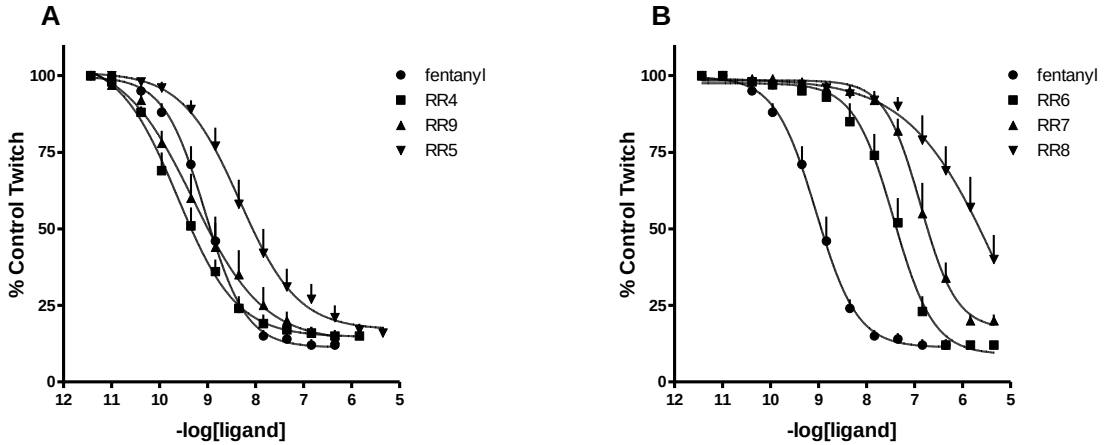


Figure 55. Electrically stimulated guinea pig ileum. Concentration response curve to fentanyl and RR compounds (RR4, RR9, and RR5 panel A, RR6, RR7, RR8 panel B). Data are the mean ± SEM of ≥6 separate experiments.

Table 20. Effects of fentanyl and RR compounds in the guinea pig ileum.

	pEC_{50} ($CL_{95\%}$)	E_{max} ± SEM
fentanyl	9.08 (8.76-9.41)	88 ± 2 %
RR4	9.56 (9.22-9.89)	85 ± 2 %
RR5	8.24 (7.65-8.82)	84 ± 2 %
RR6	7.48 (7.05-7.90)	88 ± 1 %
RR7	6.85 (6.51-7.18)	80 ± 2 %
RR8	crc incomplete	
RR9	9.23 (8.73-9.73)	85 ± 2 %

crc incomplete means that maximal effects could not be determined due to the low potency of the compound.

Based on these results, we selected compounds RR4 and RR9 to be studied in more detail. In order to investigate the receptor involved in the inhibitory action elicited by these compounds, they were challenged with the opioid receptor antagonist naloxone. The antagonist produced a rightward shift of the concentration response curve to fentanyl without significantly affecting the agonist maximal effects. A pA_2 value of 8.67 (7.99-9.35) was derived from these experiments (Figure 56A). At the same concentration, naloxone was able to shift to the right the concentration response curves to RR4 and RR9 with similar values of potency (pA_2 9.08 (8.91-9.25) and 9.16 (8.11-10.21) respectively) (Figure 56B and C).

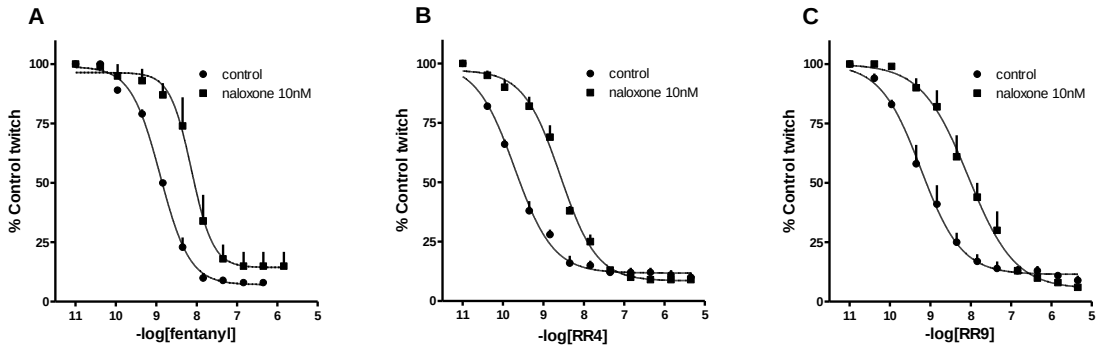


Figure 56. Electrically stimulated guinea pig ileum. Concentration response curve to fentanyl (panel A), RR4 (panel B) and RR9 (panel C) obtained in the absence (control) and presence of naloxone 10 nM. Data are the mean ± SEM of 3 separate experiments.

As shown in Figure 57 there is a good correlation between receptor binding affinity and potency in functional assays of fentanyl and its RR derivatives.

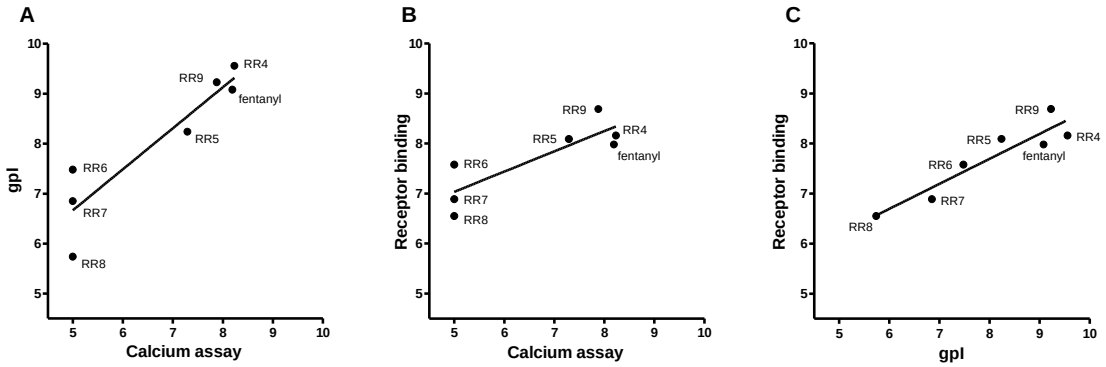
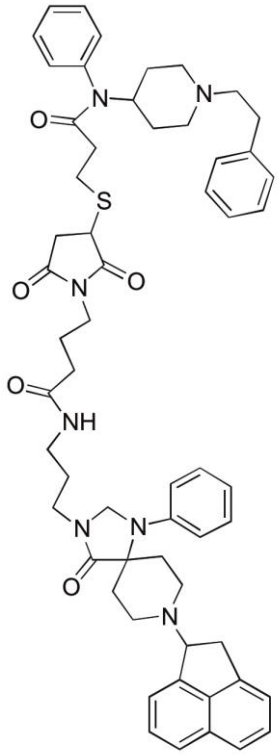


Figure 57. Correlations between receptor binding and functional assay data obtained with fentanyl and its RR derivatives. r^2 0.85 (A), r^2 0.72 (B), r^2 0.87 (C).

RR4-Ro

Based on the above-mentioned results we selected RR4 and RR9 to be used as MOP pharmacophores to generate chimeric ligands via conjugation with Ro 65-6570. However due to the synthetic problems encountered (see section of Materials and Methods) we were able to obtain only few mg of RR4-Ro (Figure 58). The pharmacological activity of this compound has been assessed in the stimulated GTPγ[^{35}S] binding assay.



RR4-Ro

Figure 58. Chemical structure of chimeric compound RR4-Ro.

In membranes from CHO cells expressing the MOP receptor, fentanyl, Ro 65-6570, and RR4-Ro stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner. However the maximal effects elicited by Ro 65-6570 and particularly by RR4-Ro were only a fraction of those elicited by fentanyl. RR4-Ro displayed a value of potency similar to that of fentanyl while Ro 65-6570 was significantly less potent (Figure 59A). In CHO_{NOP} cell membranes, Ro 65-6570 and RR4-Ro stimulated the binding of GTPγ[^{35}S] in a concentration dependent and saturable manner while fentanyl was completely inactive (Figure 59B). The concentration response curves to RR4-Ro and Ro 65-6570 were

virtually superimposable (Figure 59B). The results of these experiments are summarized in Table 21. In summary the compound RR4-Ro behaved as a potent full agonist at the NOP receptor while at the MOP receptor it behaved as a potent low efficacy partial agonist.

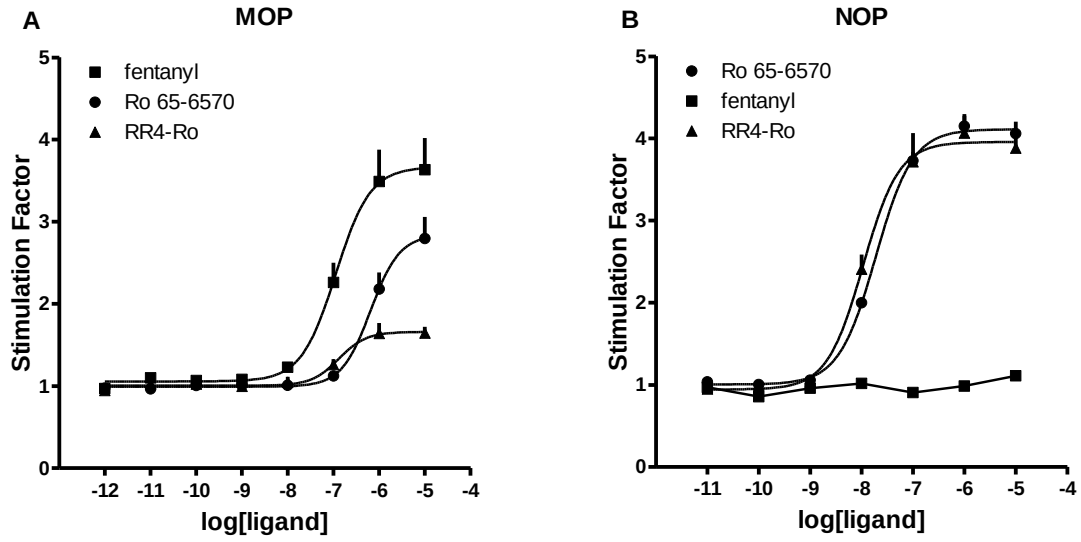


Figure 59. [^{35}S]GTPγS binding experiments. Concentration response curves to fentanyl, Ro 65-6570, and RR4-Ro, in CHO_{MOP} (panel A) and CHO_{NOP} (panel B) cell membranes. Data are the mean ± SEM for n ≥ 5 separate experiments.

Table 21. Potencies and efficacies of fentanyl, Ro 65-6570, and RR4-Ro at MOP and NOP receptors.

	MOP		NOP	
	pEC_{50} ($CL_{95\%}$)	**α ± SEM**	**pEC_{50} ($CL_{95\%}$)**	**α ± SEM**
fentanyl	7.05 (6.93-7.17)	1.00	inactive	
Ro 65-6570	6.17 (6.07-6.27)*	0.69 ± 0.04*	7.73 (7.64-7.82)	1.00
RR4-Ro	6.78 (6.46-7.11)	0.25 ± 0.01*	8.01(7.96-8.06)	0.96 ± 0.02

Intrinsic activity (α) was determined as a ratio between the maximal effect of the tested compound and that of fentanyl in CHO_{MOP} membranes and of Ro 65-6570 in CHO_{NOP} membranes. Fentanyl was inactive at NOP up to 10 μM concentrations. *$p<0.05$ vs the standard agonist, according to ANOVA followed by the Bonferroni's multiple comparison test.

Discussion

One of the chemical strategies investigated in the present thesis for generating NOP/MOP mixed ligands was to link together two small molecule agonists selective for the NOP and MOP receptor. To this aim the NOP agonist Ro 65-6570 and the MOP agonist fentanyl were chosen. We took advantage for this project of an on going collaboration with Prof. Vardanyan who made available to us a series of fentanyl derivatives, named RR compounds. Thus we characterize the RR fentanyl derivatives in a series of pharmacological assays including receptor binding, calcium mobilization, and electrically stimulated guinea pig ileum. These studies consistently demonstrated that RR4 and RR9 behaved as potent full agonists at the MOP receptor; thus these two compounds were selected to be linked to Ro 65-6570 in order to generate novel mixed MOP/NOP agonists. Due to unexpected synthetic problems only few mgs of the compound RR4-Ro have been obtained. GTPγ[^{35}S] binding studies demonstrated that RR4-Ro behaves as a potent mixed NOP full agonist / MOP partial agonist. Thus the linking strategy adopted in this study did not affect the potency and efficacy of the Ro 65-6570 pharmacophore at NOP or the potency of the fentanyl pharmacophore at the MOP receptor but caused an important loss of its efficacy. Collectively this study demonstrated the feasibility of using Ro 65-6570 and fentanyl as pharmacophores for generating chimeric compounds acting as MOP/NOP mixed agonists.

The first series of experiments investigated the receptor binding profile of fentanyl and its RR derivatives at OP and NOP human recombinant receptors. Standard ligands for OP and NOP receptors displayed the expected values of affinity for their respective receptors (Reisine, 1995), (Okawa et al., 1999), thus confirming the robustness of this binding assay. In line with previous studies (Stanley, 1992) (Okawa et al., 1999), fentanyl displayed high affinity associated with very high selectivity for MOP sites. All RR fentanyl derivatives displayed reduced MOP selectivity; this drop in selectivity was approximately of 100 fold compared to fentanyl. As far as affinity is concerned, RR4 and RR5 displayed similar affinity as fentanyl, RR6 showed 3 fold lower affinity then fentanyl while a larger loss in affinity was measured with RR7 and RR8. Of note RR9 showed 3 fold higher affinity than fentanyl. Thus the rank order of MOP affinity of this series of compounds was: RR9 > RR4 = fentanyl = RR5 > RR6 > RR7 = RR8.

The second series of experiments has been performed assessing calcium mobilization in CHO cells co-expressing the human MOP and the G_{qi5} chimeric protein that forces the

receptor to couple with the PLC-IP_3-Ca^{2+} pathway (Camarda et al., 2009). This assay has been validated using a panel of standard OP agonists and antagonists (Camarda & Calo, 2013) and then successfully used for investigating the pharmacological profile of novel OP receptor ligands (Piekielna et al., 2015), (Piekielna et al., 2015), (Ben Haddou et al., 2014), (Ben Haddou et al., 2014). A similar assay has been also set up and used in the laboratory of Prof Civelli (Zhang et al., 2012). In cells co-expressing the MOP receptor and the chimeric protein fentanyl evoked a concentration dependent stimulation of calcium release displaying high efficacy and values of potency (8.19) in line with literature data (e.g. 7.95, (Smart, Hirst, Hirota, Grandy & Lambert, 1997)). The MOP selective standard compounds dermorphin and endomorphin-1 mimicked the action of fentanyl showing similar potency and efficacy as described in previous studies (Camarda & Calo, 2013). In this assay RR4 and RR9 elicited stimulatory effects in a concentration dependent manner showing values of potency and efficacy similar to that of fentanyl. Thus these data demonstrated that the chemical modifications of the RR4 and RR9 structures did not modify either the ligand potency or its efficacy. RR5 displayed approximated 10 fold reduced potency while a larger loss of potency was measured for the other RR compounds. Thus the rank order of potency of the RR fentanyl derivatives in the calcium assay was: RR-4 = fentanyl $\underline{\geq}$ RR-9 > RR-5 > RR-6 = RR-7 > RR-8; this is in good agreement with the rank order of affinity measured in binding studies.

The third series of experiments has been performed in the electrically stimulated guinea pig ileum. This preparation has been used by decades since the report by Paton not only as a useful pharmacological preparation for investigating opioid compounds (particularly MOP ligands) but also for studies about the cellular mechanisms of opioid tolerance and dependence (Paton, 1957). The guinea pig ileum bioassay is particularly useful in the frame of the present investigation; in fact soon after the identification of N/OFQ as the endogenous ligand of the NOP receptor the guinea pig ileum was recognized as a N/OFQ sensitive pharmacological preparation (Calo et al., 1996), (Calo et al., 1997). Then the guinea pig ileum bioassay was later used to investigate pharmacological effects of mixed NOP/OP ligands (Varani et al., 1999), (Molinari et al., 2013), and section 2.1.2. (DeNo) of the present thesis.

In the electrically stimulated guinea pig ileum the peptide standard MOP agonist dermorphin and endomorphin-1 inhibited the twitch response with values of potency and maximal effects in line with previous findings (Bigoni et al., 1999; Tonini et al., 1998). Fentanyl mimicked the inhibitory effects elicited by standard peptide agonists showing a

concentration response curve superimposable to that of dermorphin. The value of fentanyl potency obtained in this study (9.08) is not far from those previously reported e.g. 8.74 (James, Feldman, Schuster, Bilotta, Brackeen & Leighton, 1991), 8.80 (James, Feldman, Schuster, Bilotta, Brackeen & Leighton, 1991). Comparing these results with calcium mobilization data, all agonists displayed full agonist activity and fentanyl and dermorphin showed similar high potency. On the contrary endomorphin-1 displayed lower potency in the guinea pig ileum but not in the calcium mobilization assay. This result is not surprising considering that peptidases are much more expressed in the tissue than in CHO cells and endomorphin-1 is much more susceptible to peptidase degradation than fentanyl (a non peptide molecule) and dermorphin (a peptide containing the D-Ala residue that confers metabolic stability to the peptide sequence).

In the electrically stimulated guinea pig ileum the RR compounds mimicked the inhibitory action exerted by fentanyl. All compounds behaved as full agonists in this preparation showing the following rank order of potency: RR4 $\geq$ RR9 $\geq$ fentanyl > RR5 > RR6 > RR7 > RR8. These results are in good agreement both with receptor binding and with calcium mobilization findings. This is corroborated by the high determination coefficient (r^2) values calculated by comparing the results obtained in the three assays namely 0.85 for gpI/calcium, 0.72 for binding/calcium and 0.87 for binding/gpI. The implications of these findings are the following: i) the MOP bindings sites evaluated in the displacement studies perfectly corresponds to the MOP receptor evaluated in functional studies, ii) the aberrant signalling generated by the chimeric G-protein does not affect the pharmacological profile of the MOP receptor, and iii) no major species specific differences exist between the human and the guinea pig MOP receptor.

Collectively these series of experiments consistently demonstrated that RR4 and RR9 behave as potent full agonists at the MOP receptor. Thus, these two compounds were selected for further studies. In order to investigate the receptor involved in the inhibitory action elicited by RR4 and RR9 in the guinea pig ileum the compounds were challenged against the opioid receptor antagonist naloxone. Naloxone antagonized fentanyl action with a behaviour compatible with a competitive type of antagonism and with a pA_2 value of 8.67. Similar values of naloxone potency were obtained in the present study by challenging the antagonist against RR4 and RR9 and in previous studies against different MOP agonists (James, Feldman, Schuster, Bilotta, Brackeen & Leighton, 1991), (Watanabe, Yano, Horie & Yamamoto, 1997). Collectively these results demonstrated that the biological effects of RR4 and RR9 (as well as of fentanyl) are solely due to MOP

receptor activation. Moreover to assess if the MOP agonist properties of RR4 are maintained *in vivo* this compound has been evaluated in comparison with fentanyl in the mouse tail withdrawal assay (A. Rizzi, unpublished results). After i.p administration of 0.1 mg/kg in mice RR4 elicited a robust antinociceptive action that was similar both in terms of kinetic of action and amount of effect to that elicited by treating animals with the same dose of fentanyl.

Based on the above mentioned findings RR4 and RR9 were selected as MOP pharmacophores to be linked with Ro 65-6570 for generating mixed MOP/NOP agonists. Unfortunately a list of synthetic and purification issues (described in details in the Materials and Methods section) did not allow completing the synthesis of RR9-Ro and only few mgs of RR4-Ro were obtained. Due to the limited amount of compound available, RR4-Ro has been assessed in comparison with RR4 and Ro 65-6570 in the stimulated GTPγ[^{35}S] assay in membranes taken from cells expressing the MOP or NOP receptors. In membranes expressing the MOP receptor fentanyl stimulated GTPγ[^{35}S] assay binding relatively high potency and maximal effects. Ro 65-6570 displayed 10 fold lower potency and statistically significant lower maximal effects thus acting as a MOP partial agonist. The chimeric compound RR4-Ro showed a value of potency similar to fentanyl but associated with an important loss of efficacy (α 0.25) thus acting as a potent low efficacy MOP partial agonist. In membranes expressing the NOP receptor fentanyl was completely inactive. Ro 65-6570 stimulated GTPγ[^{35}S] binding with high potency and efficacy and the chimeric compound mimicked the effect of Ro 65-6570 generating a concentration response curve superimposable to that of the standard. Thus RR4-Ro behaved as a potent NOP full agonist. Collectively these experiments demonstrated that RR4-Ro is as a potent mixed NOP full agonist/MOP partial agonist. Therefore the linking strategies adopted in this study did not affect the NOP potency and efficacy of the Ro 65-6570 pharmacophore and the MOP potency of the fentanyl pharmacophore but promoted an important loss of its efficacy. Alternative linking strategies should be investigated in the future in order to obtain potent mixed MOP/NOP full agonists.

It is worthy of being underlined that RR4-Ro can be used as an valuable pharmacological tools for investigating those conditions in which a full activation of the NOP receptor associated with MOP receptor partial agonism can promote beneficial effects. One of such condition can be drug abuse (Khroyan et al., 2011) as indicated by the following evidence: the compound SR16835 that is a full NOP agonist MOP partial agonist reduced conditional place preference to morphine (Khroyan, Polgar, Jiang, Zaveri & Toll, 2009); recent

evidence obtained in the laboratory of R Ciccocioppo demonstrated that a mixed NOP full agonist/MOP partial agonist is worthy of development as innovative drug to treat cocaine addiction (R. Ciccocioppo, submitted for publication); buprenorphine that is characterized by a complex pharmacological profile including MOP and NOP partial agonism (Wnendt, Kruger, Janocha, Hildebrandt & Englberger, 1999), (Bloms-Funke, Gillen, Schuettler & Wnendt, 2000) (Marquez, Borse, Nguyen, Hamid & Lutfy, 2008) is currently used in the clinical setting to treat opioid addiction (Lobmaier, Gossop, Waal & Bramness, 2010).

In conclusion, in the present study a series of fentanyl derivatives were pharmacologically characterized in detail in order to select the best molecules to be used for generating NOP/MOP mixed agonists. Results obtained in receptor binding, calcium mobilization and bioassay studies consistently demonstrated that RR4 and RR9 behaved as potent full agonists at the MOP receptor. Despite important chemical issue the chimeric compound RR4-Ro has been obtained. GTPγ[^{35}S] binding studies demonstrated that RR4-Ro behaves as a potent mixed NOP full agonist/MOP partial agonist. Collectively this study demonstrated the feasibility of the proposed approach to generate chimeric compounds acting as MOP/NOP mixed agonists. Further studies employing different linker strategies are however needed to obtain MOP/NOP mixed full agonists.

3. General conclusions

For many years opioids have been used as drugs to treat moderate to severe pain. Opioids produce brilliant effects against acute pain however their effects against chronic pain states are reduced by decreased effectiveness and tolerability. Thus novel drugs showing similar analgesic potential as opioids and able to better maintain effectiveness during chronic treatments are highly desirable. One strategy to generate such kind of drugs is to target simultaneously different receptors with multitarget ligands. These compounds can be designed as non-selective ligands (same pharmacophore able to bind different targets) or as bivalent compounds (two selective pharmacophores joined by a linker molecule). Multitarget ligands show advantages compared to the administration of combinations of drugs; in fact, the administration of a single dual target drug shows more predictable pharmacokinetic and pharmacodynamics properties, improved patient compliance and reduced self-dosing errors as well as reduced risk of possible interactions between more drugs.

The NOP receptor, the fourth member of the opioid receptor family, is implicated in the modulation of pain responses. The NOP system in the brain and spinal cord is up regulated under neuropathic and inflammatory pain conditions in rodents, where the activation of spinal NOP receptor is shown to produce antinociception in acute, neuropathic and inflammatory pain (Tian et al., 1997). In non-human primates, NOP agonists produce systemic and spinal antinociception devoid of MOP-associated side effects (Ko & Naughton, 2009) (Hu, Calo, Guerrini & Ko, 2010), making NOP a potential target for spinal analgesia. It has been demonstrated that spinal NOP and MOP receptors drive antinociception in preclinical models. In fact, in rodents spinal administration of NOP agonists potentiated morphine-induced antinociception in the absence of motor dysfunctions (Courteix, Coudore-Civiale, Privat, Pelissier, Eschalier & Fialip, 2004) (Reiss, Wichmann, Tekeshima, Kieffer & Ouagazzal, 2008) and similar results have been obtained in non-human primates, where NOP agonists potentiated MOP-mediated antinociception, (Hu, Calo, Guerrini & Ko, 2010). This evidence suggests that mixed NOP/MOP ligands may have great potential as innovative analgesics. In fact, both industrial and academic researchers focused their effort in the design and synthesis of NOP/OP compounds, including SR series compounds, Grunenthal's GRT6005 known as cebranopadol, BU08070, etc.

In this thesis a rather large research work is summarized regarding the pharmacological characterization of different molecules acting as mixed MOP/NOP receptors agonists. Compounds under study were designed and synthesised with the purpose to make them able to interact simultaneously with both MOP and NOP receptors, and were assessed in several pharmacological assays. In particular, we investigated the pharmacological profile of non-selective compounds and chimeric compounds generated linking together two selective pharmacophores, both of peptide and non-peptide nature. In all assays each compound was tested in comparison with the relative standard ligands for NOP and classical OP receptors.

In the frame of this study, the first compound described is PWT2-[Dmt^1], the tetrabranched derivative of the universal agonist for NOP and classical opioid receptors [Dmt^1]N/OFQ(1-13)-NH_2 (Molinari et al., 2013). The novel compound was generated by applying the innovative chemical strategy PWT developed by Prof. Guerrini and his group (Guerrini et al., 2014) and was assessed in several *in vitro* assays always compared with the patent compound and the standard ligands.

In receptor binding studies PWT2-[Dmt^1] displaced the radioligand binding maintaining the same profile of selectivity of the patent compound. In stimulated [^{35}S]GTPγS experiments, PWT2-[Dmt^1] behaved as a full agonist with similar potency and maximal effects at all the receptors. In the calcium mobilization assay, with a loss in potency the PWT derivative stimulated the calcium release with the profile of selectivity of the patent compound. In BRET experiments, the PWT compound behaved as a G-protein biased agonist both at NOP and MOP receptors, displaying reduced efficacy at NOP and reduced potency at MOP in the stimulation of receptor/β-arrestin 2 interactions.

Collectively, the study proved that PWT2-[Dmt^1] maintains the same selectivity of action of the patent compound and behaves as a full agonist at all the receptors in the functional assays. Furthermore, it also demonstrates that the PWT modification applied to N/OFQ related sequences seems promote G-protein biased agonism (Malfacini et al., 2015). Thus PWT2-[Dmt^1] could be defined as a MOP/NOP bivalent compound, representing a novel tool to investigate the consequences of the simultaneous activation of the NOP and opioid receptors under physiological and pathological conditions. The results obtained with this compound firmly corroborates the proposal that the application of the PWT technology does not affect the pharmacological activity of a given peptide sequence in line with findings obtained investigating PWT derivatives of N/OFQ (Rizzi et al., 2014), substance

P, neurokinin A and B (Ruzza et al., 2014), neuropeptide S (Ruzza et al., 2015), dermorphin (F. Ferrari, unpublished) and UFP-101 (A. Rizzi, unpublished). Moreover, the most peculiar characteristic of PWT compounds is to display *in vivo* high potency associated to long lasting effects compared to the parent peptide sequence (Rizzi et al., 2014) (Ruzza et al., 2014) (Ruzza et al., 2015). In order to assess if this also applies to also to PWT2-[Dmt^1], experiments are under way in Prof. MC. KO laboratories for evaluating the spinal analgesic properties of this novel compound in non human primates and to compare its effect with those previously reported with Dmt^1 (Molinari et al., 2013).

In order to generate a peptide chimeric compound, we synthesized DeNo, bounding together the MOP and NOP selective peptides dermorphin and N/OFQ.

In displacement binding studies, DeNo bound to the NOP and classical OP receptors with a reasonable selectivity for the NOP receptor and significant increased affinity at MOP. It evoked the calcium release with higher potency at NOP receptor, while it stimulated [^{35}S]GTPγS binding as the parent compounds. DeNo inhibited the formation of cAMP both at NOP and MOP receptors. In Western blot experiments, in CHO_{MOP} DeNO produced a significant increase of both phosphorylated p38 and ERK1/2 activities as the standard dermorphin. On the contrary in CHO_{NOP}, there was no activation of p38 activity and only phosphorylation of ERK1/2 was detected. In the BRET assay, DeNo promoted both NOP and MOP/G-protein interaction. On the other hand, it stimulated NOP/β-arrestin 2 interaction similar to N/OFQ, while in the MOP/β-arrestin 2 interaction assay it was 4 fold less potent than dermorphin. In the guinea pig ileum bioassay, DeNo mimicked the inhibitory effects of dermorphin and N/OFQ however its effects were effectively antagonized only using a cocktail of selective antagonists for NOP and MOP receptors. *In vivo* in the rat plantar test, DeNo produced antinociceptive effects similar to those of the standards.

Collectively, results obtained *in vitro* demonstrated that DeNo behaves essentially as a combination of the parent compounds, acting as a full agonist at both NOP and MOP receptors in all assays used in the study. Furthermore, in the guinea pig ileum preparation we demonstrated with receptor antagonists that the biological action of DeNo is due to the simultaneous activation of NOP and MOP receptors. The results obtained with DeNo *in vivo* in the rat plantar test were somewhat disappointing; in fact the compound displayed analgesic effects similar to those of N/OFQ or dermorphin despite the large literature evidence suggesting supradditional interaction between NOP and MOP activation in

analgesiometric assays. Further studies with the used of different analgesiometric assays should be performed before drawing conclusions on the spinal analgesic potential of DeNo. Nevertheless, our *in vitro* results demonstrated that DeNo was able to bind and activate with similar potency both MOP and NOP receptors thus behaving as a novel mixed MOP/NOP bivalent compound.

As far as non-peptide bivalent compounds are concerned, we examined *in vitro* and *in vivo* the pharmacological profile of cebranopadol, the molecule identified and selected by Grunenthal researchers as a mixed NOP/OP agonist (Linz et al., 2014).

In the calcium mobilization assay, cebranopadol acted as a full agonist at NOP, MOP and DOP receptors, while as a partial agonist at KOP. In BRET assay, the G-protein biased agonism profile elicited by cebranopadol was revealed by a lack of efficacy in stimulating NOP/β-arrestin 2 interaction in association with weaker potency in promoting MOP/β-arrestin 2 interaction. In the mouse vase deferens and in the guinea pig ileum bioassays, the inhibitory action of cebranopadol was very slow and not reversed by washing. Surprisingly, in both tissues cebranopadol appeared resistant to the action of the NOP antagonist SB-612111, while acted as mixed NOP/MOP antagonist in the mouse colon bioassay. In the mouse tail withdrawal assay, cebranopadol produced brilliant and long lasting effects counteracted by both naloxone and SB-612111. In the formalin test, cebranopadol exerted a complete and potent analgesic response without altering the locomotor activity of the animals.

Altogether these results demonstrated that cebranopadol is a mixed NOP/opioid agonist, which behaves as a G-protein biased agonist particularly at the NOP receptor, able to evoke robust analgesic effects due to the simultaneous activation of NOP and opioid receptors. Thus our findings corroborate and extended the proposal by Grunenthal researchers that cebranopadol may likely represent the first molecule of a novel class of analgesics: the mixed NOP/OP agonists. This proposal is supported by the successful completion of Phase II clinical studies where cebranopadol was tested in patients suffering from chronic moderate to severe pain related to cancer (Lambert, Bird & Rowbotham, 2015).

Our study also demonstrated that cebranopadol behaves as a G protein biased agonist particularly at the NOP receptor. The implications of this pharmacological activity are not obvious. There is evidence in literature that G-protein biased agonists at the MOP receptor may be better tolerated analgesics and may show reduced tolerance liability (Molinari et al., 2010). On the other hand the investigation of functional selectivity at the NOP receptor

is still in its infancy and studies investigating the relative contribution of G-proteins and arrestins in the biological effects elicited by NOP selective ligands are clearly needed before foreseeing the possible indication of NOP biased ligands.

In the frame of the present study the collaboration with Prof. Vardanyan (Arizona) and Prof. Lambert (UK) were instrumental for the design, synthesis and pharmacological characterization of NOP/MOP non-peptide bivalent ligands obtained linking two selective small molecules: the potent NOP agonist Ro 65-6570 and fentanyl derivatives named RR compounds. Initially, the study focused on the *in vitro* pharmacological characterization of the RR fentanyl derivatives in order to identify the most convenient molecules to be used as MOP pharmacophore. In receptor binding studies, compounds RR4, RR5 and RR9 showed a loss in selectivity compared to fentanyl, but maintained high MOP affinity. In calcium mobilization assay, compounds RR4 and RR9 acted as MOP full agonists. In the electrically guinea pig ileum bioassay, again RR4 and RR9 behaved as potent MOP full agonists and in the tail withdrawal test, compound RR4 elicited a robust antinociceptive action similar to fentanyl. Based on these findings RR4 and RR9 were selected as MOP pharmacophores to be linked with the NOP agonist Ro 65-6570 in order to generate novel mixed MOP/NOP agonists. Due to synthetic and purification issues, we were able to produce only the RR4 derivative bifunctional compound, named RR4-Ro. In stimulated [^{35}S]GTPγS binding experiments RR4-Ro acted as a potent low efficacy MOP partial agonist and potent NOP full agonist. Thus the linker strategy adopted caused a substantial loss of efficacy of the MOP but not NOP pharmacophore; clearly other linker strategies should be conceived and tested in order to obtain a chimeric MOP/NOP full agonist.
However RR4-Ro is able to promote full activation of the NOP receptor associated with a partial activation of the MOP receptor and this capability might be of values for the treatment of drug abuse. Thus it will be interesting exploring the pharmacologic effects of RR4-Ro *in vivo* in models of drug addiction.

In summary the pharmacological characterization of four different mixed NOP/MOP receptor agonists presented in this thesis provides to the scientific community novel compounds to be used as pharmacological research tools for further investigations on the effects trigged by the co-activation of MOP and NOP receptors for the control of pain transmission. Furthermore, we support the idea that this kind of mixed compounds may

likely be the prototypes of a novel class of analgesics characterized by better tolerability and reduced tolerance liability.

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