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COORDINATORE Prof. Francesco Di Virgilio

Pharmacological profile of nociceptin/orphanin FQ receptor – characterization of novel peptide and non peptide ligands

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Table of contents

Abbreviation List

BRET	Bioluminescence Resonance Energy Transfer
cAMP	cyclic adenosine monophosphate
CHO	Chinese Hamster Ovary cells
CPP	conditioned place preference
$CL_{95\%}$	95% confidence limits
ECL	Extracellular Cellular Loop
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GPCR	G protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanosine-5'-triphosphate
HEK293	Human Embrionic Kidney 293 cells
i.c.v.	intracerebroventricular
i.t.	intratechal
i.v.	intravenous
ICL	intracellular loops
IUPHAR	International Union of Basic and Clinical Pharmacology
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
N/OFQ	Nociceptin/Orphanin FQ
NMDA	N-methyl-D-aspartate receptor
NOP	Nociceptin Opioid Peptide receptor
NOP(+/+) and NOP(-/-)	NOP receptor wild type and knockout
PKC	protein kinase C
ppN/OFQ	N/OFQ precursor protein
PTX	pertussis-toxin
PWT	peptide welding technology
ROCK	Rho-associated coiled-coil-containing protein kinase
S.E.M.	standard error of the mean
TM	transmembrane

1. INTRODUCTION

1.1 Nociceptin/Orphanin FQ – NOP receptor system

A novel receptor was cloned by homology with the classical opioid receptors, immediately after the cloning of delta, mu and kappa opioid receptors. This receptor is a seven transmembrane-spanning G protein-coupled receptor (GPCR), like the opioid receptors, and was called opioid receptor like 1, ORL1, from the first research group who discovered it (Mollereau *et al.,* 1994). After the first paper, other cloning papers called the same receptor with different name, such as LC132, XOR1, kappa 3, ROR-C, C3 (Bunzow *et al.*, 1994; Fukuda *et al.*, 1994; Wang *et al.*, 1994; Lachowicz *et al.*, 1995; Pan *et al.*, 1995). Despite the close homology with opioid receptors, this orphan receptor, did not appear to bind or be activated by standard opioid ligands at low concentrations, when transfected into mammalian cells. However, it was activated by high concentrations of the non-selective opioid agonist etorphine and inhibited by very high concentrations of the universal antagonist naloxone (Mollereau *et al.*, 1994). Furthermore, it was clearly coupled to G_i protein, like classical opioid receptors, because receptor activation inhibited adenylyl cyclase (Mollereau *et al.*, 1994). One year later two groups identified an endogenous neuropeptide of 17 amino acids from rat and porcine brain; the peptide bound with high affinity and activated the ORL1 receptor, as determined by inhibition of adenylyl cyclase activity in cells transfected with ORL1 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). This was the first successful example of "reverse pharmacology" i.e. the identification of endogenous ligands subsequent to the cloning of the receptor (Civelli *et al.*, 2013). This heptadecapeptide was called nociceptin for its ability to decrease hot plate latency when administered intracerebroventricularly (i.c.v.) in mice (Meunier *et al.*, 1995), and orphanin FQ to denote a ligand for an orphan receptor with the first and last amino acids Phe and Gln. The International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature for this receptor and peptide is now officially NOP (Nociceptin Opioid Peptide) receptor and N/OFQ (Cox *et al.*, 2015). The importance of the N/OFQ – NOP receptor system in physiological processes is suggested by its widespread distribution in the brain, spinal cord, and peripheral organs, 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 (Lambert, 2008). Thus, the NOP receptor is emerging as a potential target for the development of innovative drugs. Structure activity 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 (Calo and Guerrini, 2013). 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 *et al.*, 2011; Zaveri, 2016). These pharmacological tools together with genetic models such as NOP receptor knockout mice (Nishi *et al.*, 1997) and rats (Homberg *et al.*, 2009), and N/OFQ precursor protein (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. Results from these studies proposes that the most promising indications for NOP agonists are anxiety, pain, cough and urinary incontinence due to overactive bladder, while for antagonists depression, Parkinson's disease and possibly drug abuse (Toll *et al.*, 2016; Gavioli *et al.*, 2013; Witkin *et al.*, 2014).

1.1.1 Nociceptin/Orphanin FQ

Approximately one year after the discovery of the NOP receptor, two groups identified the neuropeptide N/OFQ with the following primary structure FGGFTGARKSARKLANQ. It is worth mentioning that N/OFQ was the first ligand discovered by reverse pharmacology, the strategy that allows to identify endogenous ligands of previously orphan receptors (Civelli *et al.*, 1998). Before this strategy became commonly adopted, ligands were first discovered and then, their receptors identified by classical pharmacological approaches. Nowadays, GPCRs are identified on the basis of their DNA sequences, and thus are initially unmatched to known natural ligands and classified as orphan GPCRs. Based on the NOP similarities with opioid receptors, it has been assumed that the chemical nature of the endogenous ligand and the consequences of receptor activation (inhibition of cyclic adenosine monophosphate (cAMP)) would be similar to those of classical opioids. The orphan receptor was transfected in Chinese Hamster Ovary (CHO) cells that 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. Then peptide 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 N/OFQ was isolated and its sequence determined (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

Within a short time from 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 bioassays, N/OFQ inhibited electrically induced contractions of the vas deferens, ileum and myenteric plexus preparations. Moreover, when tested in vivo by 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 opioid receptor antagonists emphasising the pharmacological difference between classical opioid and the N/OFQ – NOP system.

N/OFQ has different interesting characteristics, such as the Phe-Gly-Gly-Phe amino terminal that is very similar of the Tyr-Gly-Gly-Phe found in all opioid peptides, and the structure of N/OFQ is relatively similar to dynorphin A in terms of the number of Lys and Arg residues (Figure 1.1). Furthermore, the gene structure of the prepropeptide is also similar to the opioid peptide genes (Mollereau *et al.*, 1996b; Nothacker *et al.*, 1996).

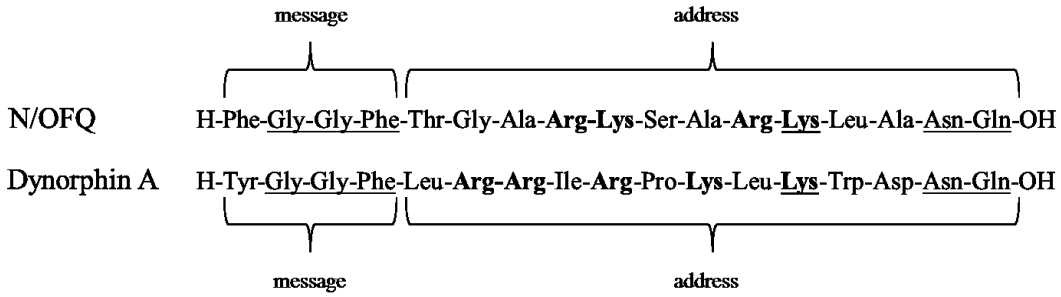


Figure 1.1. Structural similarities between N/OFQ and dynorphin A amino acid sequences.

In the amino acidic sequence of N/OFQ there are two distinct regions with different functional roles: the "message domain" that is responsible for receptor interaction and activation, and is constituted by the amino terminal tetrapeptide, while the remaining 13 amino acids form the "address domain", that contributes to recognize the NOP receptor. Various structure activities relationship studies established the amino acids important for the biological activity of N/OFQ. In particular, Ala-scan (substitution of amino acids with Ala residues) and D-scan studies (substitution of natural L conformation amino acids with D conformation amino acids; Calo and Guerrini, 2013) have demonstrated that: i) positions 1, 4, 8 (Phe, Phe, Arg) have a primary role in NOP receptor occupation since their substitution with other amino acids results in a considerable loss of receptor affinity; ii) Gly residues in positions 2 and 3 define the correct conformation of N/OFQ. Their substitution of other bulky or D conformation amino acids leads to loss of affinity and biological activity; iii) C-terminus is less sensitive to substitution of single amino acids. However, the positively charged amino acids in positions 8, 9 and 12, 13 (Arg^{8}-Lys-Ser-Ala-Arg-Lys^{13}) are very important for receptor binding.

N/OFQ derives through enzymatic cleavage from ppN/OFQ that also liberates other biologically active peptides, discovered in mouse brain and spinal cord (Mallimo *et al.*, 2013). ppN/OFQ is formed from 181 and 176 amino acids in mouse and in human, respectively (Mollereau *et al.*, 1996b). The 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.*, 1996b). 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 *et al.*, 2010). Sequence of ppN/OFQ contains the typical organizational and structural features of classical opioid precursors. ppN/OFQ starts with an amino terminal highly conserved signal peptide necessary for its secretion. 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. ppN/OFQ codes for two additional neuropeptides, nocistatin and N/OFQ II, that are immediately upstream and downstream, respectively, of N/OFQ (Meunier *et al.*, 1995; Okuda-Ashitaka *et al.*, 1998; Reinscheid *et al.*, 2000; Mallimo *et al.*, 2013; Figure 1.2).

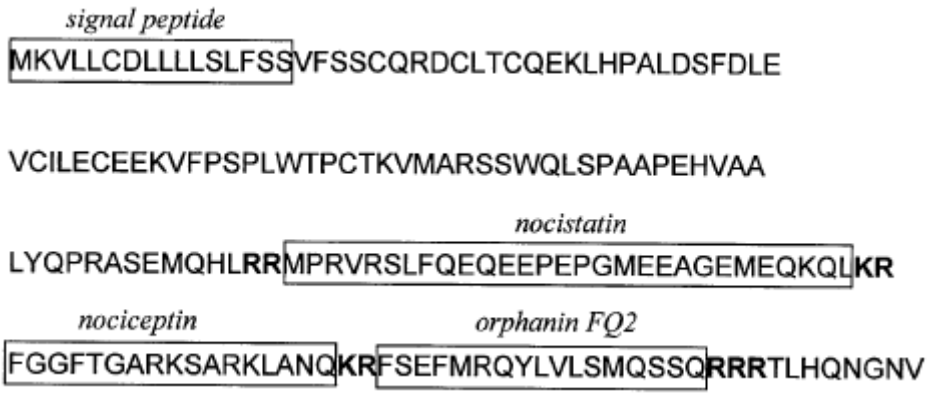


Figure 1.2. Sequence of human nociceptin precursor. Putative proteolytic cleavage motifs are shown in bold. Image taken from (Calo *et al.*, 2000c).

Nocistatin 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 *et al.*, 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 *et al.*, 1997; Rossi *et al.*, 1998). In situ hybridization and immunohistochemistry results demonstrate a diffuse distribution of N/OFQ in the central nervous system of adult male rat. These findings are in general

agreement with early in situ hybridization studies on the distribution of ppN/OFQ in rats and mice. In these studies, ppN/OFQ messenger RNA (mRNA) was demonstrated in several brain areas, including the cortex, striatum, lateral septum, bed nucleus of the stria terminalis, thalamus, amygdala, hypothalamus, substantia nigra, central gray, raphe nuclei, spinal trigeminal nucleus, and spinal cord dorsal horn (Neal *et al.*, 1999b). Little is known regarding the biosynthesis of N/OFQ, apart from the involvement of prohormone convertase 2 as suggested by knockout 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 *et al.*, 1997). Peptidase inhibitors have been demonstrated to increase N/OFQ potency, suggesting that peptidases play a role in regulating N/OFQ signalling (Montiel *et al.*, 1997). Indeed the inhibitory effect of N/OFQ in the human vas deferens can be detected in the presence of a cocktail of peptidase inhibitors, but not in their absence (Bigoni *et al.*, 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 *et al.*, 1997). However, amidation of C-terminus of N/OFQ(1-13) restores ligand affinity and potency, consequently N/OFQ(1-13)-NH_2 is the shortest sequence retaining the full biological activity of the endogenous ligand (Guerrini *et al.*, 1997).

1.1.2 NOP receptor

The NOP receptor is part of GPCR Class A (rhodopsin-like) receptors, similar classical opioid receptors, thank to high homology of several amino acids and motif, in particular in transmembrane helices and intracellular loops. NOP is a protein of 370 amino acids with several degrees of homology in the helices with classical opioid receptors. In particular, NOP receptor has greater than 70% of the amino acids residues in the second, third and seventh helices (TM2, TM3, and TM7); 50% of residues in TM1, TM5, and TM6; 24% of residues conserved between NOP and classical opioid receptors (Meunier *et al.*, 2000). Also in the intracellular loops (ICL) of NOP receptor show high homology with opioid receptors, particularly ICL3 that connects TM5 and TM6 and is involved in receptor activation and interaction with G proteins. Instead, the extracellular loops (ECL) have low sequence conservation. Is worth of mention, that ECL2 of the NOP but not of classical opioid receptors, is involved in receptor activation (Toll *et al.*, 2016). The structures of all four opioid receptors were solved in their inactive, antagonist-bound conformations (Granier *et al.*, 2012; Manglik *et al.*, 2012; Thompson *et al.*, 2012; Wu *et al.*, 2012). The NOP receptor was first crystallized in its inactive form bound to the antagonist C-24 (Thompson *et al.*, 2012), and recently

with the two antagonists SB-612111 and C-35 (Miller *et al.*, 2015). In both the SB-612111- and C-35-bound structures, NOP receptor adopts a very similar conformation as in the previously determined NOP-C-24 structure. All three co-crystallized ligands contain a piperidine group whose protonated nitrogen participates in a salt-bridge interaction with $D130^{3.32}$ (superscripts refer to the Ballesteros-Weinstein numbering of the TM helix residue). Piperidine is a ubiquitous building block in NOP ligand design, and the piperidine- $D130^{3.32}$ salt bridge common to these structures offers a direct rationalization for the high affinities of this ligand class. Although the $D130^{3.32}$ residue is conserved in all four opioid receptors, it plays a crucial role in binding of the highly selective endogenous agonist N/OFQ. Because of this, interactions between $D130^{3.32}$ and ligands likely contribute to affinity rather than efficacy or selectivity (Figure 1.3; Miller *et al.*, 2015). Indeed the N-terminal sequence Phe-Gly-Gly-Phe of N/OFQ binds deep the transmembrane binding pocket, where the N-terminal amino group of N/OFQ makes an essential anchoring charge interaction with the conserved $D130^{3.32}$. It has been seen that several residues in the NOP receptor are critical to its selectivity against the opiate alkaloids, particularly antagonists. Mutating these residues to those of the opioid receptor at the corresponding position, preserves the agonist/antagonist nature of opiate alkaloids as they interact with the mutant receptor (Meng *et al.*, 1998). One of these residues is Q280, and mutagenesis of this amino acid in TM6 in NOP receptor to H. The mutation affects neither the affinity, nor the potency of N/OFQ to inhibit adenylyl cyclase by NOP receptor. In contrast, this mutation increases affinity of opioids such as the agonists lofentanil, etorphine and dynorphin A, and the antagonists diprenorphine and nor-BNI (Mollereau *et al.*, 1996a). Another mutation is the replacement of Q280 in A amino acid. This change of amino acids leads to reduce the potency of NOP activation by N/OFQ and the non-peptide agonists SCH-221510 (Thompson *et al.*, 2012). The NOP and classical opioid receptors although share greater structural similarities, the first uses an activation mechanism markedly distinct and the ECL2 is an absolute requirement for activation of the NOP but not opioid receptors (Daga *et al.*, 2012).

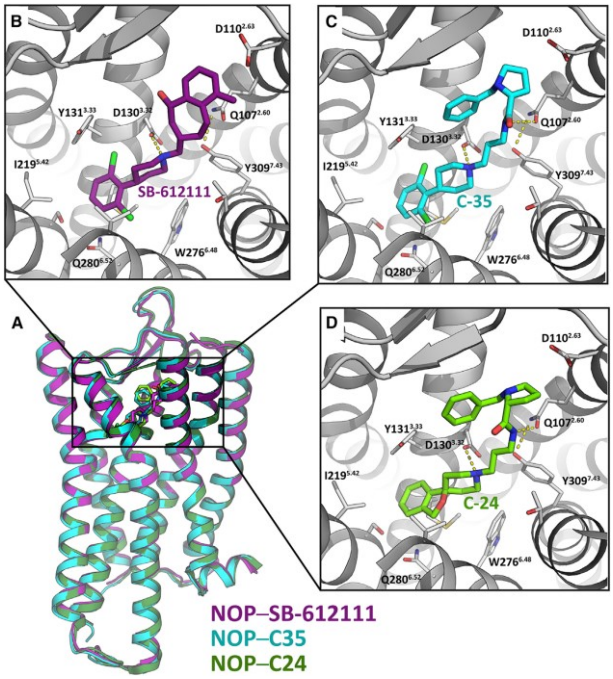


Figure 1.3. Crystal structure of human NOP bound to thermally stabilizing antagonists (panel A). NOP co-crystal structure and ligand binding modes reveal a highly conserved receptor structure when bound to (panel B) SB-612111 (purple), (panel C) Compound-35 (C-35, cyan), and (panel D) Banyu Compound-24 (C-24, green). Image taken from (Miller *et al.*, 2015).

The NOP receptor is expressed in multiple brain regions, and is involved in many central processes including pain, learning and memory, emotional states, neuroendocrine control, food intake, and motor control. In many of these neuronal pathways, there is also considerable overlap between the location of the NOP receptor and its endogenous peptide N/OFQ, as determined by immunohistochemistry and in situ hybridization (Neal *et al.*, 1999b). NOP receptors are found in high numbers in pain-related brain regions including the periaqueductal gray, thalamic nuclei, somatosensory cortex, rostral ventral medulla, lateral parabrachial nucleus, spinal cord, and dorsal root ganglia (Neal *et al.*, 1999a; Florin *et al.*, 2000). NOP receptors are also highly expressed in regions involved in reward and drug abuse, such as ventral tegmental area, nucleus accumbens, prefrontal cortex, and central amygdala (Neal *et al.*, 1999a). In each of these brain regions, NOP receptor activation reduces the release of the neurotransmitters that mediate rewarding effects. Recently studies in knock-in mice for NOP-eGFP, have demonstrated the location of the NOP receptor in similar brain regions as those described above. In addition, NOP-eGFP receptors have been found in the dorsal horn of the spinal cord and in dorsal root ganglia (Ozawa *et al.*, 2015). NOP receptor seems to be involved in different functions depending on where it is: in spinal cord might have a regulatory mechanism in the control of chronic mechanical allodynia (Neumann *et al.*, 2008; Basbaum *et al.*, 2009), while in dorsal root ganglia neurons may to be involved in both heat

and mechanical pain (Basbaum *et al.*, 2009; Cavanaugh *et al.*, 2009). NOP-eGFP receptors also are co-localized with mu opioid receptors in peptidergic C-nociceptors. These results and the similar location of NOP and mu receptors in the spinal cord probably explain the ability of NOP receptor agonists to mediate an anti-nociceptive response when administered intrathecally (i.t.). The tissue localization of the NOP receptor has also been analysed in peripheral nervous system and other organs. NOP receptor mRNA has 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). 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 central nervous system functions. The primary structure of NOP receptor is highly conserved in mammalian, in fact mouse and human have in common more than 95% of sequence. The NOP receptor gene is located on chromosome 20 (region q13.2-13.3) in human and on chromosome 2 in mouse. The coding sequence is similar to other opioid receptors mu, delta and kappa, suggest that these four genes have evolved from common ancestor (Dreborg *et al.*, 2008).

1.1.3 Biological activities of Nociceptin/Orphanin FQ – NOP receptor system

Signal transduction pathways

Like all GPCRs, the activation of the NOP receptor is followed by the dissociation of Gα and Gβγ subunits with consequent activation of various effector pathways (Childers *et al.*, 1978; Childers *et al.*, 1979). In particular, NOP receptor activation promotes guanine nucleotide exchange (a 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. The NOP receptor couples to pertussis-toxin (PTX)-sensitive G-proteins, including Gαi, to cause inhibition of cAMP formation (Zhang *et al.*, 2012), but it can promiscuously couples to other G proteins (Chan *et al.*, 1998). Like opioid receptors, also the NOP receptor couples to Kir3 and Ca^{2+} channels via Gβγ pathways. Opening of Kir channels causes cellular hyperpolarization and inhibits neuronal activity, while Gβγ causes a reduction in Ca^{2+} currents sensitive to P/Q-type, N-type, and L-type channel blockers (Zhang *et al.*, 2012). Recent studies reported that NOP receptor uses Rho-associated coiled-coil-containing protein kinase (ROCK) in the regulation of voltage-dependent Ca^{2+} channels (Mittal *et al.*, 2013). All known classes of GPCRs couple to various intracellular kinase cascades. The NOP receptor is involved in protein kinase C (PKC), phospholipase A2 and C activation (Armstead, 2002; Fukuda *et al.*, 1998; Yung *et al.*, 1999), as well as three mitogen-

activated protein kinase (MAPK) cassettes (Zhang *et al.*, 2012). In particular, N/OFQ causes NOP receptor-mediated increase in ERK 1/2 phosphorylation levels in heterologous expression system, such as CHO and Human Embryonic Kidney 293 (HEK293) cells (Zhang *et al.*, 2012). Furthermore, NOP receptor activates p38 signalling via protein kinase A and PKC pathways (Zhang *et al.*, 1999). Other studies reported that N/OFQ could induce phosphorylation of c-Jun N-terminal kinase (JNK), and that this activation occurred in both a PTX-sensitive and -insensitive manner. JNK signalling is probably mediated through G-protein coupled receptor kinase 3 (GRK3) and arrestin, and this pathway is NOP-mediated and PTX-insensitive (Zhang *et al.*, 2012). NOP receptor signalling is summarized in Figure 1.4.

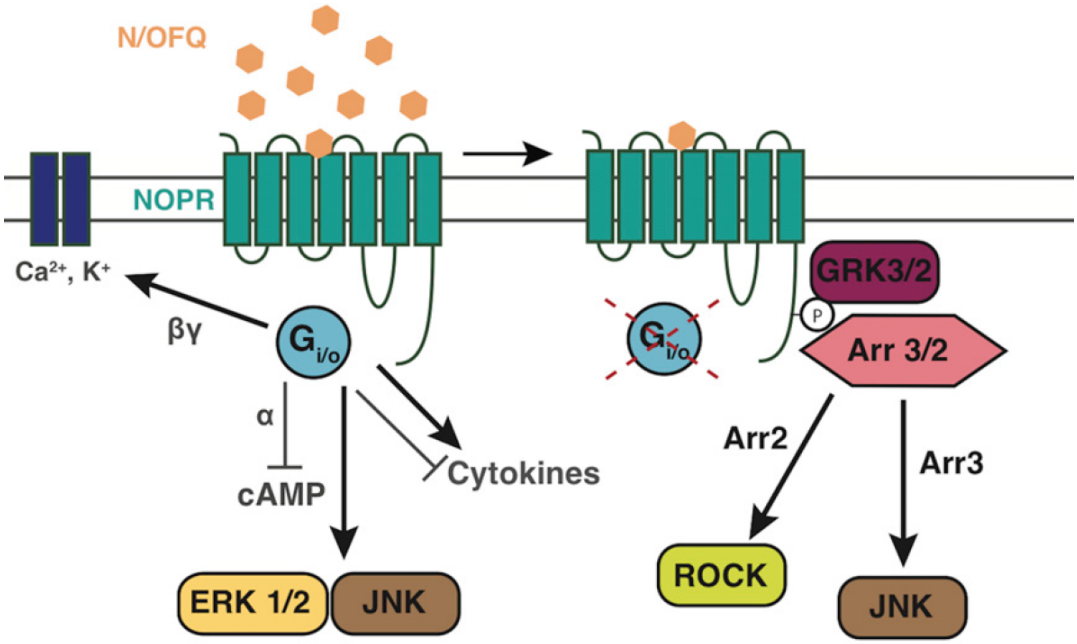


Figure 1.4. Summary of NOP receptor signal transduction and trafficking pathways. Image taken from (Toll *et al.*, 2016).

NOP receptor signal transduction is still under active investigation. The activation of NOP receptor with N/OFQ produces rapid and robust receptor internalization over time. The mechanism of receptor regulation, like for classical opioid receptors, is formed from multi steps including phosphorylation, internalization, and downregulation or recycling. The inactivation and subsequent receptor internalization is due to phosphorylation of carboxyl-terminal serine residues by G protein-coupled receptor kinases (GRK), followed by recruitment of endocytic machinery through the binding of arrestin. For the NOP receptor, serine residues in carboxyl-terminal have a key role in trafficking, homologous desensitization, and arrestin-dependent MAPK signalling. Indeed, mutation of different serine blocks the NOP internalization and homologous desensitization. Furthermore, NOP internalization was absent when β-arrestin 2 but not β-arrestin 1, was knocked down (Zhang *et*

al., 2012). Recent studies have seen that compounds acting as partial agonists in NOP/G protein interaction, behave as antagonists with little to no activity in NOP/arrestin interaction (Chang *et al.*, 2015b; Malfacini *et al.*, 2015).

Some ligands may activate all the pathways down-stream of a GPCR, whereas others may elicit distinct conformational changes within the receptor, promoting the interaction with a specific set of signal transducers and resulting in a selective modulation of intracellular signalling. Therefore, a GPCR activated by a ligand may result in the activation of both G protein and arrestin-mediated signalling cascades, leading to a full response, whereas some ligands may preferentially activate G-protein- but not arrestin mediated signal transduction cascade, or vice versa arrestin but not G-protein mediated actions (Bedini, 2015). This ability of a ligand to activate a subset of signalling cascades at a particular GPCR is named "functional selectivity". Oven the past few years, the phenomenon of functional selectivity has been increasingly explored, providing a novel potential for GPCR-targeted therapies with improved safety and fewer side effects (White *et al.*, 2015). In the opioid field, several biased agonists have been investigated as new selective drugs for a wide range of targets and disease states: mu G protein biased agonists have been proposed as novel analgesics; delta G protein biased agonists are being considered for Parkinson disease, pain, and depression; kappa G protein biased agonists might represent novel analgesics with lower addiction liability and fewer side effects (White *et al.*, 2015). For example, it has been seen that kappa receptor-mediated G protein signalling induces analgesia and aversion, whereas β-arrestin 2 signalling may be associated with motor incoordination (White *et al.*, 2015). For the mu receptor, it has been seen in β-arrestin 2 knockout mice that morphine produced analgesia with reduced respiratory and gastrointestinal side effects (Thompson *et al.*, 2015). Therefore, it was developed the new molecule TRV130. It is a mu G protein biased agonist that elicits robust G protein signalling with far less β-arrestin recruitment and receptor internalization. In mice and rats, TRV130 is potent analgesic while causing less gastrointestinal dysfunction and respiratory suppression than morphine at equianalgesic doses (DeWire *et al.*, 2013). Recently, TRV130 has been investigated in clinical phase II trials for acute pain. The compound produced analgesia in moderate-to-severe acute pain showing no serious adverse events (that limited conventional opioids), with effectiveness similar to morphine (Viscusi *et al.*, 2016). To date there is very limited evidence about the involvement of arrestins in the biological actions of NOP receptor agonists. It has been reported that β-arrestin 1 is involved in the inhibitory action of NOP receptor agonists on locomotor activity (Mittal *et al.*, 2013). Moreover, recent studies (Asth *et al.*, 2016) suggest that the actions of NOP ligands on anxiety and depression are better predicted by their efficacy at β-arrestin 2 rather than G protein. Further studies are needed to asses systematically the effects of NOP agonists toward G protein and arrestin on the biological

actions mediated by the N/OFQ – NOP receptor system, in combination with the analysis of the phenotype and response to NOP agonists of β-arrestin 2 knockout mice. The results of these studies will reveal the therapeutic indications for which bias NOP agonists may act as more effective or better tolerated drugs.

Cellular actions

In almost all neuronal types tested, N/OFQ via NOP receptor activates inwardly rectifying potassium conductance and inhibits Ca^{2+} channels. Although these general inhibitory actions of N/OFQ are essentially indistinguishable from the actions of mu, delta, and kappa receptors at the cellular level, differences in the global actions of N/OFQ compared to classical opioids may derive from differences in their receptor distribution. For example, in "off" cells of the rostral ventromedial medulla, opioids receptors are likely presynaptic, whereas NOP receptors are likely postsynaptic. It is possible that minor differences in the cellular actions of N/OFQ and classical opioids may contribute to differences in their overall physiological roles. For example: i) N/OFQ affects T-type Ca^{2+} current in dorsal root ganglia cells, whereas classical opioids do not; ii) the action of N/OFQ on damaged sensory neurons is increased whereas the action of classical opioids is decreased; iii) there may be differences in the ability of opioids and N/OFQ to modulate glutamate response, where N/OFQ acts to suppress glutamate transmission (Moran *et al.*, 2000).
NOP receptor activation results in a general decrease in monoamine release, such as norepinephrine in cerebral cortical slices (Schlicker *et al.*, 2000), dopamine in striatal slices, nucleus accumbens and ventral tegmental area (Vazquez-DeRose *et al.*, 2013). In particular, activation of the NOP receptor inhibits tyrosine hydroxylase phosphorylation, dopamine synthesis, and dopamine receptor signalling, suggesting that NOP receptors tightly regulate dopamine transmission at multiple levels (Olianas *et al.*, 2008), and so to have an important implications in Parkinson's disease, reward, and addiction-related disease states (Nazzaro *et al.*, 2010). NOP receptor is also involved in glutamate and γ-aminobutyric acid (GABA) release, by virtue of its presynaptic localization, and inhibits neuronal firing. In particular, Lu and colleagues have found that N/OFQ into via ventrolateral periaqueductal gray produced robust decrease in serotonin, norepinephrine and GABA, and increase in glutamate release in the spinal dorsal horn (Lu *et al.*, 2010).

Biological actions

The N/OFQ – NOP receptor system is involved in a wide range of physiological responses with effects noted in the central nervous system, the cardiovascular system, the airways, the gastrointestinal tract, the urogenital tract and the immune system (Figure 1.5).

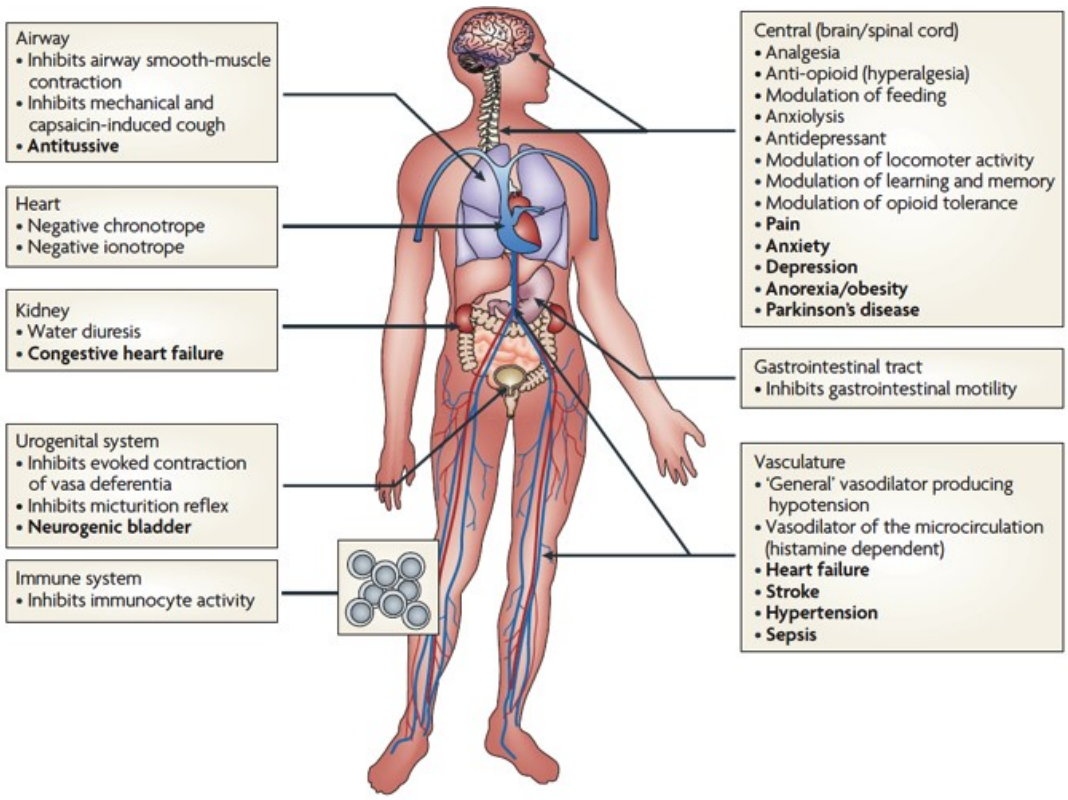


Figure 1.5. Pleiotropic effects of nociceptin/orphanin FQ (N/OFQ) on major organ systems. Potential clinical indications are noted in bold. Image taken from (Lambert, 2008).

Analgesia – The first studies on N/OFQ found that i.c.v. administration in mice decreased the latencies in the hot plate and tail flick tests, suggesting that N/OFQ had nociceptive rather anti-nociceptive activity like opioid ligands (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). Further studies in mice indicated the antiopioid activity of N/OFQ, where it is able to block the antinociceptive activity of mu, delta and kappa analgesics (Mogil *et al.*, 1996). NOP receptor agonists also block stress-induced analgesia. While naloxone attenuates a portion of stress-induced analgesia, N/OFQ blocks it completely, indicating that the endogenous peptide blocks both an opioid as well as non-opioid components of stress-induced analgesia (Rizzi *et al.*, 2001b). The N/OFQ – NOP receptor system have an important role on pain modulation, since NOP receptors are expressed at peripheral, spinal and supraspinal sites of the ascending and descending pain pathways. N/OFQ and NOP receptor agonists can evoke different responses in function of type of pain (acute, neuropathic, and inflammatory), route of administration, and species. In rodent models of acute

pain, at supraspinal level (thalamus, amygdala, periaqueductal gray, and rostral ventral medulla) N/OFQ and NOP receptor agonists inhibit the firing rate of neurons and elicit pronociceptive effects or reduce opioid-induced antinociception. On the contrary, at spinal level evoke antinociceptive effects and potentiate morphine-induced antinociception. Also at peripheral level cause antinociceptive effects. In rodent models of neuropathic pain at supraspinal level, N/OFQ and NOP receptor agonists decrease cold allodynia; at spinal level inhibit thermal hyperalgesia, mechanical and cold allodynia, and flexor reflex. Moreover, there is synergistic interaction with morphine to suppress mechanical hyperalgesia. Also at peripheral level there is an inhibition of mechanical and cold allodynia. In rodent models of inflammatory pain, at supraspinal level N/OFQ and NOP receptor agonists increase pain response and antagonize opioid analgesia. At spinal level, evoke analgesic effect and decrease thermal hyperalgesia flexor reflex. While at peripheral level there is an inhibition of articular mechanosensivity and of colonic hyperalgesia (Schroder *et al.*, 2014). The net effect of systemically administered NOP receptor agonists on nociception is proposed to depend on the relative contribution of peripheral, spinal and supraspinal sites of action, which in turn may depend on experimental conditions (Figure 1.6).

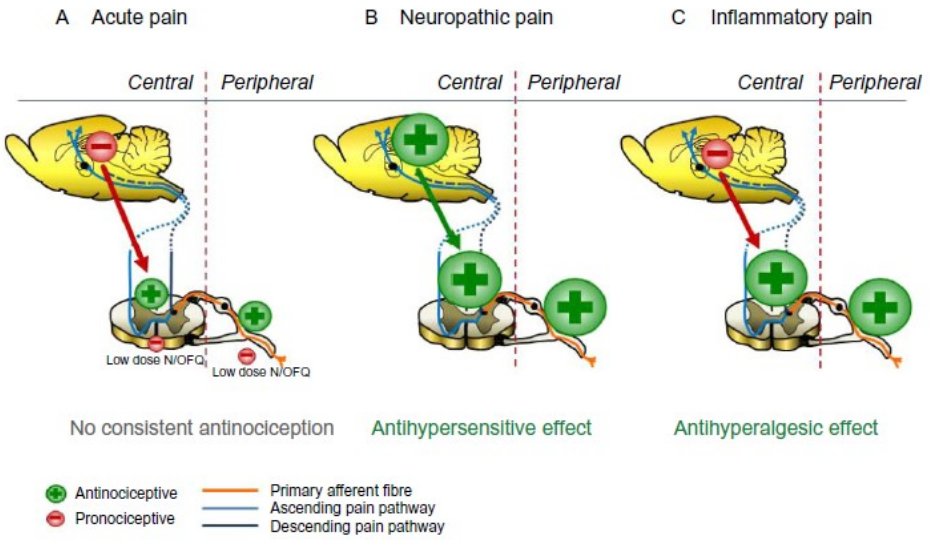


Figure 1.6. Schematic presentation summarizing the effects of NOP receptor activation on nociceptive processing at peripheral, spinal and supraspinal sites, and resulting analgesic effects of systemically administered NOP receptor agonists under conditions of acute, neuropathic and inflammatory pain in rodents. (A) NOP receptor agonists were largely ineffective in acute pain after systemic administration as activation of supraspinal NOP receptors counteracted spinally and peripherally mediated antinociception. Pronociceptive effects were also elicited by low concentrations of N/OFQ at peripheral and spinal sites. (B) In contrast, systemic administration of NOP receptor agonists elicited antihypersensitive effects in neuropathic pain as, here, activation of supraspinal NOP receptors did not counteract, but contributed to analgesic efficacy. In addition, peripheral, spinal and supraspinal NOP receptors were up-regulated and functionally sensitized. (C) Inhibition of nociceptive processing elicited by activation of functionally sensitized peripheral and spinal NOP receptors is hypothesized to overcome pronociceptive effects of supraspinal NOP receptor activation, thus leading to antihyperalgesic efficacy after systemic administration of NOP receptor agonists in inflammatory pain. Larger symbols indicate up-regulation/functional sensitization of NOP receptors. Image taken from (Schroder *et al.*, 2014).

As in rodents, peptide NOP receptor agonists inhibited thermal nociception in non-human primates at both peripheral and spinal levels, and were not evident pronociceptive effects at low doses of N/OFQ (Ko *et al.*, 2009a). In contrast to results obtained in rodents, where systemic administration of NOP agonist Ro 64-6198 did not exert antinociceptive effects (Jenck *et al.*, 2000; Kotlinska *et al.*, 2003b), in non-human primates Ro 64-6198 showed potent efficacious thermal antinociception without motor and sedative sides effects (Ko *et al.*, 2009b). Therefore, a profound species difference exists between rodents and non-human primates in terms of antinociceptive efficacy and tolerability of systemically administered selective NOP receptor agonists. Thus in non-human primates than in rodents, N/OFQ and NOP receptor agonists evoke antinociceptive effects either spinally (Ko *et al.*, 2006) or supraspinally (Ding *et al.*, 2015), and these effects are naloxone-insensitive and without typical opioid-sides effects, such as itch, respiratory depression, and abuse liability (Lin *et al.*, 2013). As with acute pain, N/OFQ exerts antiallodynic and antihyperalgesic effects after i.t. administration in models of neuropathic and inflammatory pain (Hao *et al.*, 1998). Furthermore, both ppN/OFQ(-/-) and NOP(-/-) mice display increased inflammatory hyperalgesia in the formalin assay, but not in an acute pain assay (Depner *et al.*, 2003), similar to NOP(-/-) rats (Rizzi *et al.*, 2011).

Drug abuse – Areas involve in reward mechanisms of different drugs of abuse such as opioids, cocaine, amphetamine, nicotine, and cannabis sativa derivatives are several (nucleus accumbens, amygdala, ventral tegmental area, and medial prefrontal cortex). In animals, abuse liability is measured with several behavioural paradigms including drug self-administration studies and development of a conditioned place preference (CPP). Several studies demonstrates that i.c.v. administered N/OFQ can block CPP induced by morphine, cocaine, alcohol, and methamphetamine (Ciccocioppo *et al.*, 2000; Kotlinska *et al.*, 2002; Kotlinska *et al.*, 2003a; Zhao *et al.*, 2003; Sakoori *et al.*, 2004). N/OFQ also blocks self-administration of alcohol (Ciccocioppo *et al.*, 2004). Furthermore, it has been seen that NOP agonists are able to reduce extracellular dopamine levels in the nucleus accumbens; and block a drug-induced increase (Lutfy *et al.*, 2001). In contrast, N/OFQ was not able to attenuate heroin self-administration in rats (Walker *et al.*, 1998). However, N/OFQ is neither rewarding nor aversive (Devine *et al.*, 1996a). The ability of NOP agonists to attenuate self-administration of any abuse drug is not clear. Studies of self-administration in rats, showed that N/OFQ and Ro 64-6198 block ethanol self-administration, but these experiments were conducted in alcohol preferring rat strains (Ciccocioppo *et al.*, 2004; Economidou *et al.*, 2008). Other studies found a decrease of alcohol drinking by NOP agonists, only in rats with a previous history of alcohol dependence but not in unselected or non-dependent rat lines (Ciccocioppo *et al.*, 2014; de

Guglielmo *et al.*, 2015). An elegant recent study conducted by Cippitelli and colleagues showed a new surprising role for both NOP receptor agonists and antagonists in nicotine and alcohol self-administration. It has been seen that NOP receptor agonists did not modify alcohol self-administration in normal non-dependent Wistar rats. To further investigate the effect of NOP receptor agonism on both nicotine and alcohol self-administration, post-dependent and non-dependent rats were pretreated with the non-peptide NOP agonist AT-202 (previously coded as SR16835) prior to self-administration sessions. Rather than attenuating self-administration of either nicotine or alcohol, AT-202 dose dependently increased responding for nicotine in both groups of animals, while significantly stimulating alcohol self-administration at only a single dose in the nicotine post-dependent group. Conversely, when the rats were pretreated with potent and selective NOP antagonist SB-612111, nicotine and alcohol self-administration was reduced in both post-dependent and non-dependent animals (Cippitelli *et al.*, 2016). The mechanism by which the NOP receptor antagonist reduces nicotine and alcohol self-administration is unclear. There are significant difference in the nature of CPP and self-administration paradigms. First, most CPP experiments have demonstrated that NOP receptor agonists block the acquisition of CPP. In fact, Shoblock and colleagues (Shoblock *et al.*, 2005) demonstrated that Ro 64-6198 blocked the acquisition and reinstatement, but not the expression of morphine CPP. In contrast, in self-administration studies, NOP receptor ligands are tested on the maintenance or expression of drug responding. Therefore, it is possible that NOP agonists block acquisition, but antagonists can block expression of drug taking or drug reward. Second, there are differences in the pattern of drug administration in the two models. CPP generates a rather modest history of drug administration (generally 3-4 days of administration), while self-administration requires many days or weeks of repeated administration prior to drug testing. Finally, CPP requires response-independent forced drug exposure, while self-administration uses volitional and self-regulated drug intake (Cippitelli *et al.*, 2016). This is not the only case reported the effect of an antagonist in drug abuse. Another very recent study demonstrated that the novel NOP antagonist LY2940094 synthetized by Ely Lilly laboratories, dose dependently reduced homecage ethanol self-administration in rats, without affecting food or water intake or general locomotor activity, an effect that was maintained without evidence of tolerance for up to four days of subchronic dosing (Rorick-Kehn *et al.*, 2016). These findings were confirmed with genetic deletion of NOP receptor in rats that confers resilience to drug abuse (cocaine, heroin and alcohol self-administration) and support a role for NOP receptor antagonism as a potential treatment option for drug addiction (Kallupi *et al.*, 2016).

Anxiety and depression – It has been seen that the N/OFQ – NOP receptor system is involved in emotional disorders including anxiety and depression (Gavioli *et al.*, 2006; Jenck *et al.*, 1997; Redrobe *et al.*, 2002; Gavioli *et al.*, 2003; Gavioli *et al.*, 2004). NOP and N/OFQ are located in areas that are crucial to mood control such as amygdala, hippocampus, thalamus and cortical processing areas (Gavioli *et al.*, 2006). Low doses of N/OFQ (0.1-3 nmol) administered by i.c.v. produced anxiolytic effects and reduces the susceptibility to stress in rodents with similar results obtained with benzodiazepine (Jenck *et al.*, 1997). It has been demonstrated that ppN/OFQ(-/-) mice have an anxiogenic phenotype and increased susceptibility to stress (Koster *et al.*, 1999). Different studies reported the anxiolytic action of NOP receptor agonists including Ro 65-6570, Ro 64-6198 and SCH-221510 (Wichmann *et al.*, 1999; Jenck *et al.*, 2000; Varty *et al.*, 2005; Varty *et al.*, 2008). The level of anxiety does not change with the use of NOP antagonists, denoting a lack of N/OFQ-mediated tone in the control of this behaviour. On the contrary, NOP antagonists display an antidepressant profile and indicate central N/OFQ-mediated tone in mood control (Redrobe *et al.*, 2002; Gavioli *et al.*, 2003; Gavioli *et al.*, 2004). In fact, administration of both peptide (UFP-101) and non-peptide (SB-612111 and J-113397) NOP antagonists induces antidepressant effects in the forced swimming test in mice and rats. Furthermore, NOP(-/-) mice and rats compared to NOP(+/+) animals, showed a reduction of immobility time in the same assay (Gavioli *et al.*, 2003; Rizzi *et al.*, 2011). Recently, it has been seen that the novel non-peptide NOP receptor antagonist LY2940094 induced antidepressant effects in rats and in the first clinical trial in patients with major depressive disorder. The results of these studies indicate that occupancy of NOP receptors results in antidepressant-like but not anxiolytic behavioural effects in rodents and suggest the possibility of an antidepressant effect in depressed patients (Post *et al.*, 2016).

Learning and memory – N/OFQ inhibits memory and learning processes and cognitive functions. When injected in the hippocampus, N/OFQ impairs spatial memory (Sandin *et al.*, 1997). In accordance to these observations, NOP(-/-) mice showed better learning ability and longer lasting memory than NOP(+/+) mice (Manabe *et al.*, 1998). Initial studies suggested a dose-dependent biphasic effect of an intrahippocampal infusion of N/OFQ, in which low doses (0.33 to 1 nmol) enhanced memory in the water maze test and high doses (>5 nmol) resulted in an impairment. Interestingly, this effect is rescued by NOP receptor antagonists. The impairment of learning and memory by single dose of N/OFQ or NOP receptor agonists has been consistently reported in different tasks that are highly dependent on hippocampal and amygdala functioning, when N/OFQ or NOP receptor agonists are administered through different routes (Andero, 2015). The regulation by N/OFQ of ion channel activity may cause the inhibition of neuronal excitability, long term

potentiation in the hippocampus and release of neurotransmitters, including substance P, dopamine, acetylcholine, noradrenaline, GABA and glutamate, which promote synaptic plasticity and memory formation. The functional antagonism revealed between NOP and NMDA receptors at behavioural and cellular levels suggests that under pathological conditions normal endogenous N/OFQ release may exacerbate cognitive impairments linked to deficiency of glutamatergic signalling. Dysfunction of glutamatergic signalling is a central event in the pathogenic cascades of Alzheimer's disease and possibly other forms of dementias. It is therefore plausible that enhanced secretion of N/OFQ may be one component of the pathogenic processes underlying the cognitive deterioration seen in Alzheimer's disease and other dementias. Until recently, the new possibilities that targeting of NOP receptor may offer in the treatment of cognitive decline has stayed largely unexplored because of the lack of selective and brain-penetrant NOP receptor antagonists, but the development of synthetic selective NOP receptor antagonists (J-113397, Trap-101, SB-612111, C-24, LY2940094), opens up the way not only for enhancing understanding of NOP receptor function but also for exploring its utility as a target for cognitive enhancement (Abdel-Mouttalib, 2015).

Food intake – Several studies performed in rats have shown that the i.c.v. administration of N/OFQ or NOP receptor agonists evokes robust orexigenic effects (Olszewski *et al.*, 2004). It has been seen that N/OFQ induced a robust hyperphagic effect also in sated mice. The endogenous peptide induced its maximal effect at 1 nmol, while at 10 nmol there was a reduction of hyperphagic effect, that can be probably due to the inhibitory action on motor activity elicited by high doses of the peptide. Both pharmacological and knockout findings converge indicating that the orexigenic effects induced by N/OFQ are exclusively due to NOP receptor activation. In fact, the orexigenic action of N/OFQ was prevented by SB-612111 and no longer evident in NOP(-/-) animals (Rizzi *et al.*, 2007a). Furthermore, Polidori *et al*. (2000) showed that i.c.v. injection of selective NOP receptor peptide antagonist [Nphe^1]N/OFQ(1-13)-NH_2 prevented the orexigenic action of N/OFQ and also significantly reduced food intake induced by food deprivation in rats. In contrast, when tested in mice that were food deprived, SB-612111 did not modify the food intake of the animals; suggest that in mice, unlike in rats, the N/OFQ – NOP receptor system does not play a major role in controlling food intake induced by food deprivation. This conclusion is supported by the results obtained evaluating the food intake in NOP(+/+) and NOP(-/-) mice, where there were no differences in the two groups (Rizzi *et al.*, 2007a). It is however worthy of mention that recent evidences demonstrated that a systemic administration of the NOP receptor antagonist LY2940094 can reduce feeding behaviour and body weight in rodents. Moreover, the hypophagic effect of LY2940094 is NOP dependent and not due to off-target or aversive effects. Thus, NOP receptor

antagonist LY2940094 may be useful in treating disorders of appetitive behaviour such as binge eating disorders, food choice, and overeating, which lead to obesity and its associated medical complications and morbidity (Statnick *et al.*, 2016).

Locomotor activity – First studies on N/OFQ found that its administration modified the motor function in mice and rats. In particular, low doses of N/OFQ given i.c.v. increased locomotor activity (Kuzmin *et al.*, 2004), whereas high dose reduced locomotor activity (Reinscheid *et al.,* 1995; Devine *et al.*, 1996b). Studies with intracerebral injections of N/OFQ revealed that the peptide is involve on central dopamine systems regulating motor activity. In fact, the N/OFQ effect caused by low doses did not block by naloxone, but by dopamine D_1 and D_2 receptor antagonists, suggesting that the stimulation was mediated by enhanced dopaminergic transmission (Florin *et al.*, 1996). Furthermore, administration of N/OFQ reduced the ability of rats to run on a rotarod, while administration of NOP antagonist UFP-101 improved motor performance (Marti *et al.*, 2009). These findings confirmed the role of N/OFQ – NOP receptor system as a modulator of dopaminergic regulation of motor function, and were supported by knockout studies where NOP(-/-) mice were able to stay on the rotarod for a longer period of time than NOP(+/+) animals (Marti *et al.*, 2004a). These results suggest that the block of NOP receptor might alleviate the symptoms of Parkinson's disease. Indeed in the 6-hydroxydopamine-treated hemi-parkinsonian rat, UFP-101 and J-113397 relieved hypokinesia, and mice knockout for the NOP receptor were more resistant to haloperidol-induced akinesia compared with NOP(+/+) mice (Marti *et al.*, 2005). Also studies conducted in non-human primates showed that the block of NOP receptor with J-113397 attenuated the Parkinsonian-like symptoms (Viaro *et al.*, 2008). It has been seen that J-113397 and L-DOPA decreased thalamic GABA release and attenuated akinesia, with a profound effect. These actions were prevented using tetrodotoxin or the GABA(A) receptor antagonist bicuculine in the substantia nigra reticulata, demonstrating that J-113397 and L-DOPA exert their antiparkinsonian action through overinhibition of nigrothalamic transmission and suggest that NOP receptor antagonists may be useful together with L-DOPA for Parkinson's disease (Marti *et al.*, 2007). In contrast to these findings, it has been seen that NOP receptor agonists improved L-DOPA induced dyskinesia in models of Parkinson at doses that do not impair motor function in normal rats. Thus, NOP receptor agonists might be useful tools in the treatments to alleviate the dyskinesias that torment Parkinson's disease patients in the later stages of their disease. This relief by NOP receptor agonists is probably mediated primarily in the striatum (Marti *et al.*, 2012), whereas the beneficial effects of NOP receptor antagonists appears to be primarily mediated by antagonism of endogenous N/OFQ in the substantia nigra reticulata (Marti *et al.*, 2004a). This is an interesting example of how the

N/OFQ – NOP receptor system is involved with its activation or inhibition, to alleviate various motor symptoms in one disease that is Parkinson.

Cardiovascular, renal and gastrointestinal tract systems – In anaesthetized and conscious laboratory animals N/OFQ produces hypotension and bradycardia (Malinowska *et al.*, 2002; Kapusta, 2000). This occurs following intravenous (i.v.; Bigoni *et al.*, 1999) and i.c.v. (Kapusta *et al.*, 1999) administration and is absent in NOP(-/-) mice (Burmeister *et al.*, 2008). The effects occur at both central and peripheral sites. Moreover, i.v. infusion of N/OFQ produces diuresis and antinatriuresis, and decreases renal sympathetic nerve activity (Kapusta, 2000; Bigoni *et al.*, 1999; Kapusta *et al.*, 2005). Intravenous N/OFQ produces vasodilation without involves nitric oxide (Champion *et al.*, 2002). N/OFQ inhibits contractility of the gastrointestinal tract in a wide range of species and at most sites along the gastrointestinal tract (Osinski *et al.*, 2000). In addition, N/OFQ prevents gastric damage that is induced by intragastric ethanol (Morini *et al.*, 2005) and cold-restraint stress (Grandi *et al.*, 2007).

Inflammation and sepsis – The first indication that the N/OFQ – NOP receptor system may be involved with regulation of immune responses was in 1995, when NOP mRNA was found on mouse and human lymphocytes and lymphocytic cell lines. Then, NOP mRNA was identified in the thymus, lymph nodes, spleen and splenocytes of pigs and additional types of human leucocytes. N/OFQ can neither be seen as having anti-inflammatory nor pro-inflammatory effects on the immune system and is often referred to the literature as having modulatory effects. An increase of N/OFQ can lead to an increase of neutrophil rolling and adhesion, vascular permeability, histamine release, recruitment of inflammatory cells, vasodilation and upregulation of adhesion molecules. These effects lead to a decrease survival in septic rats and in human patients with sepsis. On the other hand, a decrease of N/OFQ can lead to a decrease recruitment of inflammatory cells, vasodilation, histamine release and reduce leucocyte rolling. These effects lead to an increase survival in septic rats and in human patients with sepsis. Taken together, data from these studies suggest that the N/OFQ - NOP system upregulation may be harmful to health, whereas a decrease could be advantageous (Thomas *et al.*, 2014). For these reasons, it is interesting to use NOP receptor antagonists in inflammatory conditions and sepsis. SB-612111 significantly ameliorated the clinical disease course of mice with dextran sodium sulphate-induced colitis (Alt *et al.*, 2012). N/OFQ administration increased mortality in the cecal ligation and puncture model of sepsis in rats, while the treatment with the NOP selective antagonist UFP-101 decreased mortality (Carvalho *et al.*, 2008).

Airways – N/OFQ inhibits cough in guinea pigs (Lee *et al.*, 2006) and in cats (Bolser *et al.*, 2001), and inhibits ex vivo airway contractility in various species including human (Faisy *et al.*, 2004). The antitussive effects can be mimicked by the non-peptide NOP agonist Ro 64-6198 in a J-113397-sensitive manner (McLeod *et al.*, 2004). Another non-peptide NOP agonist SCH-486757 was more potent than common antitussive such as codeine and dextromethorphan in a guinea pig capsaicin cough model, without producing tolerance after 5 days of treatment, neither displaying abuse liability. SCH-486757 antitussive effect was blocked by J-113397 but not by naltrexone (McLeod *et al.*, 2010). 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 *et al.*, 2010). Recent studies showed that N/OFQ reduced the inflammatory immune T helper 2-like microenvironment in a conventional murine model of asthma (airway hyperresponsiveness), confirming that N/OFQ – NOP receptor system may represent a novel pharmacological tool to modulate the immune microenvironment during asthmatic conditions (Spaziano *et al.*, 2016).

Urogenital system – The Menarini researchers were the first to demonstrate that i.v. 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 receptor is present at several sites of integration of the micturition reflex and that its activation produce inhibitory effects (Lecci *et al.*, 2000). Based on these findings, Lazzeri and co-workers performed the first clinical investigation with N/OFQ by testing the urodynamic effects of intravescical application of the peptide in normal subjects and in patients with overactive bladder and demonstrating a robust inhibitory effect of the peptide in patients (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). Thus, NOP agonist could be promising drug in patients suffering from urinary incontinence due to overactive bladder, where the intravescical instillation of N/OFQ produced robust beneficial effects both in acute and chronic (10 days) studies (Lazzeri *et al.*, 2001; Lazzeri *et al.*, 2006).

1.2 NOP receptor ligands

The widespread distribution of the NOP receptor and N/OFQ throughout the central nervous system and in many peripheral organs is now well documented from studies in rodents, non-human primates and humans. To investigate the biological roles of this peptidergic system, a large number of NOP ligands are now available. Structure activity relationship studies on N/OFQ, screening of peptide combinatorial libraries, and other techniques performed in industrial laboratories, have generated NOP-selective peptide and non-peptide ligands encompassing full and partial agonist as well as pure antagonist activities.

1.2.1 Peptide ligands

N/OFQ related peptides

N/OFQ(1-13)-NH_2 – From a great number of structure activities relationship studies have been performed on the endogenous peptide sequence of the NOP receptor, N/OFQ(1-13)-NH_2 is the shortest truncated version of N/OFQ that maintains maximal affinity and full agonist activity at NOP receptors, both in vitro and in vivo (Calo *et al.*, 2000a).

N/OFQ-NH_2 – Amidation of the carboxyl terminus of N/OFQ make it less susceptible to carboxypeptidases, and this likely slightly increased peptide potency both in vitro and in vivo (Calo *et al.*, 2000c).

[$Arg^{14}Lys^{15}$]N/OFQ – It has been seen the importance for NOP receptor binding of Arg-Lys amino acids in N/OFQ sequence. On this bases [$Arg^{14}Lys^{15}$]N/OFQ was developed (Okada *et al.*, 2000) and it displayed full agonist activity and approximately 10 fold higher potency than N/OFQ in various bioassay and cellular studies (Rizzi *et al.*, 2002b; Basso *et al.*, 2005; Trombella *et al.*, 2005). Subsequent to i.c.v. injection in mice, this peptide produced pronociceptive effects in the tail-withdrawal assay and inhibited locomotor activity, being approximately 30 fold more potent than N/OFQ and producing longer lasting effects (Rizzi *et al.*, 2002b).

UFP-112 – [(pF)$Phe^4Aib^7Arg^{14}Lys^{15}$]N/OFQ-$NH_2$ (UFP-112) is an NOP receptor ligand designed using a combination of several chemical modifications in the same peptide sequence that increase NOP receptor affinity/potency and/or reduce susceptibility to enzymatic degradation. (Rizzi *et al.*,

2007b). UFP-112 is a NOP receptor full agonist, showing a potency up to 100 fold higher than N/OFQ in isolated tissues, indicating that the combined chemical modifications of this compound, elicited synergistic rather than additive effects on peptide potency (Calo *et al.*, 2011). UFP-112 mimicked N/OFQ showing its inactivity in NOP(-/-) tissues, and it is significantly more stable than N/OFQ, exhibiting a plasma $t_{1/2}$ threefold longer than that of N/OFQ, a difference that was even more pronounced in brain homogenates (Rizzi *et al.*, 2007b). In vivo UFP-112 mimicked the effects of N/OFQ in several assays being more potent and eliciting longer lasting effects (reviewed in (Calo *et al.*, 2011)). Of note, UFP-112 acts as a potent and long lasting analgesic after spinal administration in non-human primates (Hu *et al.*, 2010).

PWT-N/OFQ – Recently a novel and facile chemical strategy for the synthesis of tetrabranched peptides named peptide welding technology (PWT; Guerrini *et al.*, 2014) was used to synthetize three N/OFQ tetrabranched derivatives containing different cores (PWT1, PWT2, and PWT3). PWT derivatives of N/OFQ behaved as high affinity, potent full agonists in various assays including receptor binding, GTPγ[^{35}S] binding, calcium mobilization, and in the electrically stimulated mouse vas deferens bioassay. In the locomotor activity test in mice, PWT-N/OFQ derivatives mimicked the inhibitory effects of N/OFQ, showing 40 fold higher potency and primarily longer lasting action. Indeed, the action of endogenous peptide disappears 1 hour after i.c.v. injection, whereas PWT derivatives showed their action up to 24 hours. Furthermore, like N/OFQ, PWT2-N/OFQ did not show its inhibitory effects in NOP(-/-) mice in the locomotor activity test (Rizzi *et al.*, 2014). PWT2-N/OFQ by spinal administration in mice, was able to produce analgesic effects both in nociceptive (tail withdrawal) and neuropathic (chronic constriction injury) pain models. These findings were confirmed in non-human primates where PWT2-N/OFQ mimicked the antinociceptive effects of N/OFQ, being approximately 100 fold more potent, with longer lasting effects up to 24 hours (while N/OFQ action lasted for 2.5 hours) after spinal injection (Rizzi *et al.*, 2015).

[*F/G]N/OFQ(1-13)-NH$_2$* – Modifications of the conformational freedom or of the spatial disposition of Phe^1 relative to Phe^4 reduces peptide efficacy (Guerrini *et al.*, 1998). Moreover, Gly^2-Gly^3 dipeptide acts a conformation-inducing spacer between the pharmacophores Phe^1 and Phe^4 (Daga *et al.*, 2012). Reduction of the Phe^1-Gly^2 peptide bond in N/OFQ increase the conformational freedom, producing a loss of efficacy and thus generating the first N/OFQ-related peptide showing partial agonist efficacy [F/G]N/OFQ(1-13)-NH$_2$ (Calo *et al.*, 1998; Guerrini *et al.*, 1998). According to the type of preparation or assay used, this peptide behaved as partial or full agonist or

even as a pure antagonist. For example, [F/G]N/OFQ(1-13)-NH_2 administered by i.c.v. in mice, behaved as full agonist mimicking the pronociceptive effect of N/OFQ in the tail withdrawal assay, while in the locomotor activity test [F/G]N/OFQ(1-13)-NH_2 showed a partial agonist activity. On the contrary, in cardiovascular studies [F/G]N/OFQ(1-13)-NH_2 behaved as NOP antagonist blocking N/OFQ-induced bradycardia and hypotension (Calo *et al.*, 1998; Rizzi *et al.*, 2001a; Madeddu *et al.*, 1999). This variable pharmacological activity is most likely due to the low efficacy agonist proprieties of this ligand whose final effect strongly depends upon the receptor reserve and the resulting stimulus-response coupling of the preparation/function under study. This interpretation has been confirmed testing the peptide in the same cells by modifying NOP receptor density as the only variable (McDonald *et al.*, 2003).

UFP-113 – The chemical modifications [(pF)Phe^4], [Aib^7], and [Arg^{14}Lys^{15}] that increase peptide affinity/potency have been combined with [F/G] to generate UFP-113 (Arduin *et al.*, 2007), a NOP ligand with 100 fold increase in potency and longer duration of action. In various assay in vitro UFP-113 behaved similar to [F/G]N/OFQ(1-13)-NH_2 as a NOP partial agonist (Arduin *et al.*, 2007; Camarda *et al.*, 2009; Malfacini *et al.*, 2015). UFP-113 by i.t. administration elicited a dose dependent antinociception in the rat paw pressure test, mimicking N/OFQ action, and this effect was no longer evident in NOP(-/-) rats (Micheli *et al.*, 2015). In contrast, UFP-113 by i.c.v. administration in mice behaved as NOP antagonist showing antidepressant effect in the forced swimming test (Asth *et al.*, 2016).

[Nphe^1]N/OFQ(1-13)NH_2 – [Nphe^1]N/OFQ(1-13)NH_2 is the first NOP receptor peptide antagonist reported in literature. This peptide bound selectively to recombinant NOP receptors, competitively antagonized the inhibitory effects of N/OFQ on cAMP accumulation in CHO cells (Calo *et al.*, 2000b), and blocked in a competitive manner the contractile effect of N/OFQ but not of endomorphin-1 in the mouse colon (Rizzi *et al.*, 1999). It also antagonized the N/OFQ action in electrically stimulated isolated tissues of the mouse, rat and guinea pig. In vivo, [Nphe^1]N/OFQ(1-13)NH_2 prevented the pronociceptive and antimorphine actions of i.c.v. N/OFQ (Calo *et al.*, 2000b). These initial findings were later confirmed and extended in a large body of studies reviewed in Calo and Guerrini (2013).

UFP-101 – By combining the [Nphe^1] chemical modification that eliminates efficacy with the [Arg^{14}Lys^{15}] modification that increases ligand potency, the potent NOP selective antagonist UFP-101 was generated (Calo *et al.*, 2002). UFP-101 has been demonstrated to reverse many of the

biologic actions of N/OFQ including locomotor activity, pain transmission, neurochemical actions, food intake, cardiovascular, kidney and gastric functions, memory, drug reward, hypothalamic-pituitary-adrenal axis responses, anxiety, and depression (Calo *et al.*, 2005; Calo and Guerrini, 2013). A few years ago, it was found a tritiated version of UFP-101 that was useful for receptor binding studies using recombinant NOP receptors as well as animal tissues (Ibba *et al.*, 2008).

N/OFQ unrelated peptides

Ac-RYYRWK-NH_2 – This peptide was identified from a large peptide combinatorial library by Dooley *et al.* (1997). Ac-RYYRWK-NH_2 behaved as potent and selective NOP receptor partial agonist. Similar to [F/G]N/OFQ(1-13)-NH_2, in different assays Ac-RYYRWK-NH_2 behaved as full or partial agonist or even as a pure NOP antagonist (reviewed in Toll *et al.* (2016)).

ZP120 – The SIP technology was used to generate this compound from Ac-RYYRWK-NH_2 (Rizzi *et al.*, 2002a). In vitro in electrically stimulated mouse and rat vas deferens, ZP120 showed similar efficacy as Ac-RYYRWK-NH_2, but approximately 10 fold higher potency. Interestingly, in calcium mobilization assay in cells co-expressing the NOP receptor and chimeric G proteins, the potency of ZP120 was relatively low (Rizzi *et al.*, 2002a; Fischetti *et al.*, 2009b; Camarda *et al.*, 2009). In vivo in locomotor activity and tail withdrawal studies in mice, ZP120 displayed very high potency and long duration of action (Rizzi *et al.*, 2002a), and these effects were no evident in NOP(-/-) mice (Fischetti *et al.*, 2009b). Furthermore, this compound was tested in humans as acquaretic and reached phase II clinical trials for acute decompensated heart failure, but was stopped due to an unexpected drop in systolic and diastolic blood pressure in patients. ZP120 is now under clinical development for treatment-resistant systolic hypertension (Villar *et al.*, 2016).

1.2.2 Non-peptide ligands

The data produced using NOP peptide agonists and antagonists has greatly increased our knowledge on the effects of the selective activation and blockage of NOP receptors on a large number of peripheral and central systems. However, peptides have some drawbacks that do not allow to be used as drugs. Indeed, the high susceptibility to enzymatic degradation with the poor pharmacokinetic proprieties, in particular the inefficient transport across the blood-brain barrier, limit their usefulness to target central nervous system-disorders. For these reasons, the development

of orally active, brain-penetrant, non-peptide molecules are necessary to perform clinical investigation aimed at identifying their efficacy in patients and eventually their place in therapy.

Agonists

Ro 64-6198 – This compound was between the first small-molecule NOP agonists reported by Hoffman La-Roche (Rover *et al.*, 2000; Wichmann *et al.*, 2000). Ro 64-6198 has a subnanomolar binding affinity for NOP, 100 fold binding selectivity over mu, and negligible affinity for kappa and delta receptors (Wichmann *et al.*, 2000; Dautzenberg *et al.*, 2001). Ro 64-6198 contributed to the identification of anxiety, neurophatic pain, drug abuse, cough, and possibly anorexia as possible therapeutic indications for NOP receptor agonists. This molecule was also extremely useful in the identification of NOP receptor agonist side effects including motor disturbance, impairment of memory, and hypothermia (Shoblock, 2007). Ro 64-6198 showed significant anxiolytic activity in many different assay paradigms (Jenck *et al.*, 2000; Wichmann *et al.*, 2000). In vitro studies reported that Ro 64-6198 activated G-protein-coupled inwardly rectifying K^+ channels in rat periaqueductal gray slices mimicking N/OFQ, but with lower maximal effects and affecting only a subset of N/OFQ sensitive neurons (Chiou *et al.*, 2004). In these experiments, Ro 64-6198 displayed a very low kinetic of action that was also reported, together with slowly reversible effects, in N/OFQ-sensitive electrically stimulated tissues (Rizzi *et al.*, 2001c). Antagonist studies demonstrated that the selectivity of action of this compound seems to be variable depending on species and tissues. Indeed in the rat vas deferens, Ro 64-6198 behaved as a NOP selective agonist, in the guinea pig ileum as a NOP/opioid mixed agonist, whereas in the mouse vas deferens the action of Ro 64-6198 was not affected in the presence of selective NOP receptor antagonists or naloxone or the combination of naloxone and NOP antagonists (Rizzi *et al.*, 2001c). These differences across rodent species have been seen in vivo studies about the anxiolytic potential of Ro 64-6198 after systemic administration. For example, in mice Ro 64-6198 showed significant anxiolytic activity only at doses at which it adversely affected general neurological functions such as rotarod performance, locomotor activity, and body temperature (Varty *et al.*, 2005). Therefore, in mice there is no dose separation between anxiolytic doses and doses that disrupt general behaviour. On the contrary, in rats Ro 64-6198 showed distinct dose separation of at least >3 fold between the doses at which anxiolytic effects are observed and those at which decreased activity is observed. Furthermore, Ro 64-6198 showed significant anxiolytic activity comparable to benzodiazepines with a better side effect profile at efficacious doses (Jenck *et al.*, 2000; Varty *et al.*, 2005; Goeldner *et al.*, 2012). In addition, Ro 64-6198 was reported to counteract the rewarding and reinforcing

properties of morphine and ethanol, with the lack of conditioned place preference in rodents (Jenck *et al.*, 2000; Le Pen *et al.*, 2002) and lack of self-administration in monkeys (Ko *et al.*, 2009b). The systemic injection of Ro 64-6198 does not modify nociceptive pain transmission in rodents, as demonstrated in the tail flick, tail immersion, tactile or cold water stimulation, and foot shock test (Jenck *et al.*, 2000; Obara *et al.*, 2005; Varty *et al.*, 2005; Reiss *et al.*, 2008). However, an exception of this rule is the mouse hot plate test where systemic Ro 64-6198 produced modest antinociceptive effects (Reiss *et al.*, 2008; Chang *et al.*, 2015a). Another dimension to species differences in the pharmacological profile of Ro 64-6198 was revealed by Ko *et al.* (2009b), in non human primates, which showed that Ro 64-6198 given systemically, did not cause sedation or other adverse effects (respiratory depression and itch/scratching responses) at doses at which it exhibited significant antinociceptive efficacy (Podlesnik *et al.*, 2011). In fact, Ro 64-6198 produced robust antinociception against an acute noxious stimulus (50°C water) and capsaicin-induced allodynia in monkeys. These effects are counteracted in presence of the NOP antagonist J-113397, but not naltrexone. In the rat sciatic nerve injury model, Ro 64-6198 given intrathecally or intraplantary produced antyallodyinic that were sensitive to NOP antagonists (Obara *et al.*, 2005). Similar results were obtained in response to systemic injection of Ro 64-6198 in monkeys (Sukhtankar *et al.*, 2014). Another effect due to systemic injection of Ro 64-6198 is the inhibition of the cough reflex in guinea pigs (McLeod *et al.*, 2004). Although Ro 64-6198 has been extensively characterized in vivo and shown to have good brain penetration (Jenck *et al.*, 2000; Varty *et al.*, 2005), it was not further developed for any of the above indications likely because its oral bioavailability in multiple preclinical species was poor (Heinig *et al.*, 2010).

Ro 65-6570 – This compound is a congener from the same family of Ro 64-6198, described by Hoffman la Roche (Wichmann *et al.*, 1999). Ro 65-6570 binds with subnanomolar affinity to the human NOP receptor, displaying 10 fold selectivity over opioid receptors and inhibited cAMP formation with maximal effects similar to N/OFQ and a value of potency 10 fold lower. These effects are counteracted by NOP antagonists (Hashiba *et al.*, 2001). Ro 65-6570 showed dose-dependent anxiolytic effects in the elevated plus maze test in rats in doses at which it did not affected motor performance (Wichmann *et al.*, 1999). The anxiolytic like effects of Ro 65-6570 in the mouse elevated plus maze were antagonized by SB-612111 and no longer evident in NOP(-/-) animals (Asth *et al.*, 2016). Furthermore, Ro 65-6570 produced hypnotic effects in mice (Byford *et al.*, 2007), and reduced reward mechanisms of the abuse of drugs, particularly opioids (Rutten *et al.*, 2010). Similar to Ro 64-6198, Ro 65-6570 showed robust peripheral antihyperalgesic activity when

administered intraplantarly to rats with diabetic polyneuropathy (Schiene *et al.*, 2015), and antinociceptive effects in mice subjected to the formalin assay (Byford *et al.*, 2007).

MT-7716 – This molecule was synthetized by Mitsubishi Pharma Corporation. MT-7716 (previously referred to as W-212393) is a NOP agonist able to cross the blood-brain barrier with high affinity for both rat and human NOP receptors. MT-7716 induced a phase advance at circadian time, and this effect was prevented by the NOP antagonist J-113397. Therefore, MT-7716 may represent a tool for the treatment of circadian rhythm disorders such as jet lag or delayed sleep phase syndrome (Teshima *et al*., 2005). Other studies showed that MT-7716 dose-dependently decreases GABAergic transmission and blocks the ethanol-induced increase in GABA release (Kallupi *et al.*, 2014), and that chronic activation of NOP receptors by MT-7716 leads to a pronounced inhibition of alcohol drinking lasting for several days after treatment discontinuation (Ciccocioppo *et al.*, 2014). Recent studies displayed that MT-7716 reduces alcohol self-administration and stress-induced reinstatement of alcohol seeking. However, these effects were observed only in rats with a history of ethanol dependence. These findings, in particular the anti-stress effects of MT-7716 suggest that blood brain barrier penetrating NOP agonists may have clinical potential as complements to presently available anti-craving pharmacotherapies, that are ineffective in preventing stress-induced alcohol seeking (de Guglielmo *et al.,* 2015).

SCH-221510 – This molecule was characterized by Schering-Plough Corporation (Ho *et al.*, 2009). SCH-221510 in preclinical test in rats and guinea pigs, showed anxiolytic effects similar to benzodiazepines and evoked anxiolytic effects without damage locomotor activity in the elevated plus maze test in rats. Even though this compound was shown to have oral efficacy in anxiety assays, it seems likely that its further development was negatively impacted by its poor distribution into the brain (Varty *et al.*, 2008).

SCH-486757 – SCH-486757, developed by Schering-Plough Corporation, is the first NOP agonist used in human clinical tests (Ib/II) as antitussive. SCH-486757 acted as high affinity NOP full agonist and was extensively characterized in experimental models of cough (guinea pigs capsaicin-induced cough and cat mechanical-induced cough) (McLeod *et al.*, 2010). However, the clinical study investigating the antitussive activity of this compound was terminated due to lack of significant efficacy and therapeutic window. The high does at which cough suppression was observed also resulted in significant somnolence in patients (Woodcock *et al.*, 2010). Furthermore, SCH-486757 reduced fertility and embryonic development in rats (Enright *et al.*, 2012).

AT-202 – AT-202 (also named SR16835) was synthetized from Astraea Therapeutics. AT-202 is a NOP full agonist that was tested in acute and chronic pain in vivo assay: in tail flick test it did not evoke any analgesic effect, while in the spinal nerve ligation model showed antinociceptive and antialloidynic effects, which are reversed by NOP but not opioid antagonists (Khroyan *et al.*, 2011). Recent studies have demonstrated that AT-202 shows opposite effects in conditioned place preference and self-administration in the assumption of nicotine and alcohol in rats. In particular, pretreatment with the NOP agonist increased rather than decreased nicotine-maintained lever pressing and did so in both nicotine dependent and non-dependent groups (Cippitelli *et al.*, 2016).

MCOPPB – This molecule was synthetized at Pfizer (Hayashi *et al.*, 2009) as NOP agonist with high affinity for the human NOP receptor and moderate selectivity over classical opioid receptors. In an ex vivo binding study, MCOPPB inhibited neuronal signalling trough the NOP receptor in the mouse brain, suggesting that it penetrated into the brain after oral administration. Furthermore, MCOPPB evoked anxiolytic effects without damage locomotor activity and memory in the Vogel test in mice, and was still effective as an anxiolytic agent even after repeated administration for 5 days (Hirao *et al.*, 2008). Recently studies have been demonstrated that MCOPPB was 10 and 100 fold more potent than N/OFQ and SCH-221510 in cAMP functional assay, and this molecule showed a bias toward G protein (Chang *et al.*, 2015b).

Antagonists

J-113397 – J-113397 was the first small molecule NOP antagonist to be reported in literature (Kawamoto *et al.*, 1999). Like Ro 64-6198 for agonists, J-113397 allowed to discovery the first therapeutic indications for the NOP antagonists. J-113397 bound with low nanomolar affinity the NOP receptor and displayed high selectivity for NOP over classical opioid receptors, although other laboratories reported a significantly less NOP receptor selectivity, particularly with respect to the mu receptor (Zaratin *et al.*, 2004). In vivo, J-113397 inhibited hyperalgesia elicited by N/OFQ in mouse tail-flick test (Ozaki *et al.*, 2000), and reduced the immobility time in mice in the forced swimming test without modify locomotor activity (Redrobe *et al.*, 2002). Interestingly, J-113397 attenuates Parkinson-like symptoms in rodent and in non-human primate models of Parkinson's disease (Viaro *et al.*, 2008; Marti *et al.*, 2005; Marti *et al.*, 2004b). These results were confirmed with other NOP antagonists suggesting that NOP antagonists may represent a novel approach to treat symptoms in Parkinson's disease.

SB-612111 – This molecule was synthetized by Smithkline Beecham as a novel NOP selective and competitive antagonist. SB-612111 showed subnanomolar affinity for the recombinant human NOP and high (>150 fold) selectivity over classical opioid receptors (Zaratin *et al.*, 2004). Compared with J-113397, SB-612111 showed higher affinity and selectivity (Spagnolo *et al.*, 2007). SB-612111 competitively antagonized the effects of N/OFQ in several in vitro assays, such as [^{35}S]GTPγS binding and cAMP accumulation, as well as in isolated peripheral tissues of mice, rats and guinea pigs and in mouse cerebral cortex synaptosomes (Spagnolo *et al.*, 2007). In vivo, SB-612111 dose dependently blocked the hyperalgesia elicited by an i.c.v. injection of N/OFQ (Zaratin *et al.*, 2004) as well as antinociception produced by an i.t. injection of N/OFQ (Rizzi *et al.*, 2007a). Furthermore, SB-612111 was able to fully prevent the orexigenic effect of N/OFQ in food intake studies performed in sated mice, and reduced the immobility time in the mouse forced swim and tail suspension tests. The antidepressant-like effect elicited by SB-612111 in the forced swim test was reversed by the i.c.v. injection of N/OFQ and no longer evident in NOP(-/-) mice (Rizzi *et al.*, 2007a). SB-612111 was also shown to ameliorate parkinsonian symptoms in the 6-hydroxydopamine hemilesioned rat model of Parkinson's disease and synergize with L-DOPA to provide motor benefit in parkinsonian rats (Marti *et al.*, 2013). Recent studies demonstrated a new role of NOP antagonist in drug abuse. Indeed, it was seen that SB-612111 reduced nicotine self-administration in both dependent and non-dependent rats at 5 and 10 mg/kg and alcohol self-administration was reduced when both groups of rats were treated with 10 mg/kg, a dose also found to weakly reduce locomotor behaviour (Cippitelli *et al.*, 2016). Another recent study reported the effect of SB-612111 on operant alcohol self-administration in rats. Results revealed that the NOP antagonist attenuated ethanol drinking in wild type, but not in the NOP(-/-) line (Kallupi *et al.*, 2016). Furthermore, it has been seen that antagonist potency is correlated with a ligand's ability to induce receptor stability (T_m) and crystallogenesis. Using this screening strategy, the NOP crystal in complex with SB-612111 has been recently solved (Miller *et al.*, 2015).

C-24 – This compound was identified from a library in Banyu laboratories as NOP antagonist. It demonstrated subnanomolar affinity for the NOP receptor associated with extraordinary selectivity (>9000 fold). C-24 inhibited N/OFQ stimulatory effects with subnanomolar potency in [^{35}S]GTPγS binding studies (Goto *et al.*, 2006) and these results were confirmed and extended in subsequent studies in transfected cells and smooth muscle preparations (Fischetti *et al.*, 2009a). In vivo, C-24 displayed good brain penetration and was able to fully prevent the locomotor depressant action of a NOP agonist in mice (Goto *et al.*, 2006), and antagonized both the pronociceptive action of N/OFQ given supraspinally and the antinociceptive effect of spinal N/OFQ (Fischetti *et al.*, 2009a). This

antagonist was the first molecule used to solve the crystal structure of human NOP receptor allowing to show substantial conformational differences in the pocket region between NOP and the classical opioid receptors (Thompson *et al.*, 2012).

LY2940094 – Very recently it was discovered a novel orally-bioavailable selective NOP antagonist that showed antidepressant efficacy in patients with major depressive disorder (Post *et al.*, 2016). Furthermore, this compound attenuated ethanol self-administration and ethanol-motivated behaviours, stress-induced ethanol seeking, and ethanol-induced stimulation of brain reward pathways in lines of rats that exhibit excessive ethanol consumption, suggesting that LY2940094 may have potential therapeutic utility in treating alcohol addiction (Rorick-Kehn *et al.*, 2016). Moreover, LY2940094 decreased feeding behaviour and body weight in rodents. Thus, it may be useful in treating disorders of appetitive behaviour such as binge eating disorders, food choice, and overeating, which lead to obesity and its associated medical complications and morbidity (Statnick *et al.*, 2016).

The above mentioned studies show that the NOP-targeted therapies may have key advantages over existing approaches in several therapeutic areas, notably chronic pain and anxiety for NOP agonists and psychiatric disorders for NOP antagonists. Other therapeutic areas under active investigation include treatment of gastrointestinal, inflammatory disorders, and possibly Parkinson's disease (Zaveri, 2016).

2. AIM OF THE STUDY

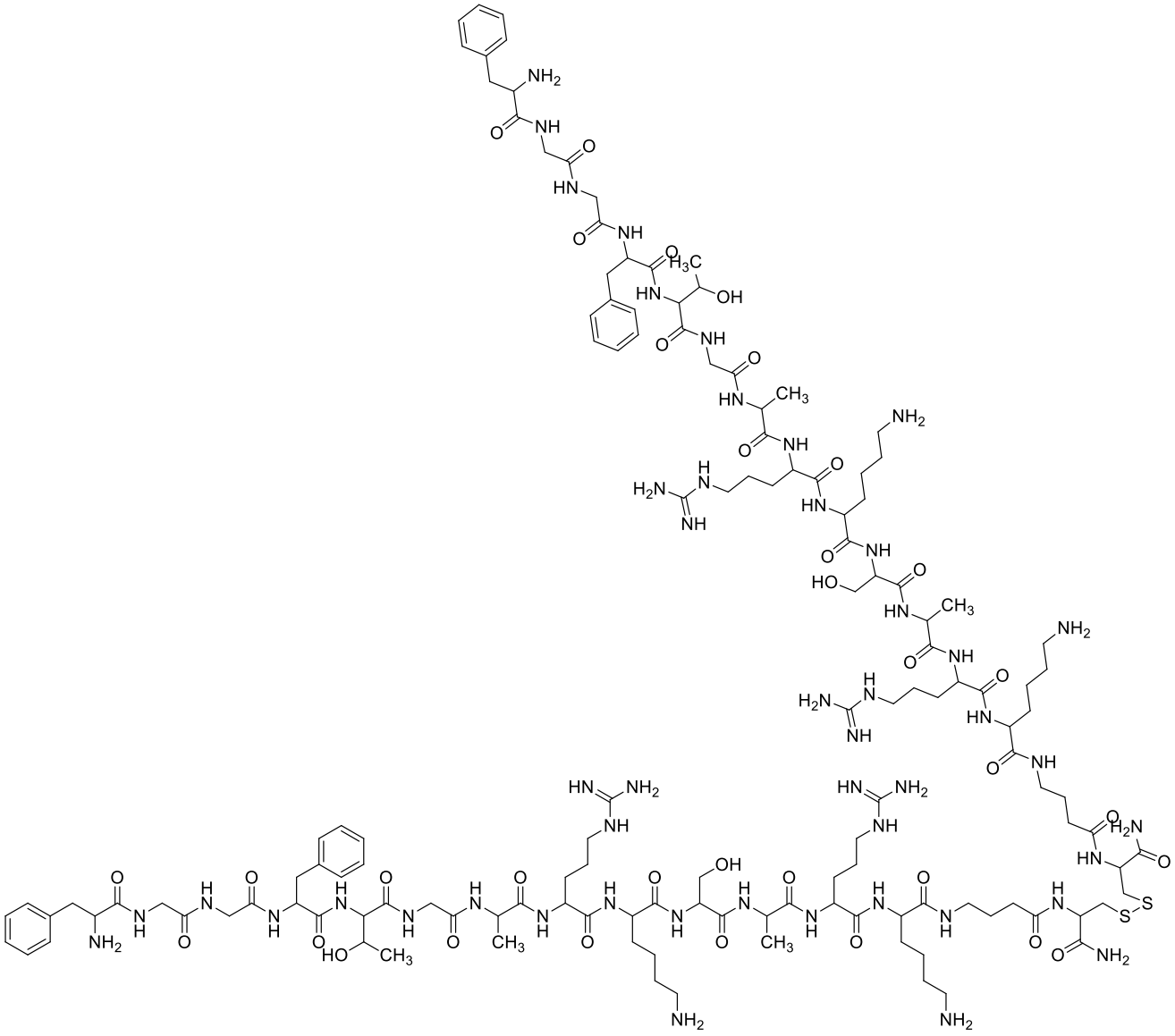
Given the widespread distribution of the N/OFQ – NOP receptor system in areas of the brain and periphery, it is clear that this system plays an important role in several physiological processes. Therefore, it is important the study of both NOP receptor agonists and antagonists, because both NOP receptor activation and blockade can be useful therapeutic strategies for different diseases. The comparison of the action of several chemically different compounds is needed to firmly classify a given beneficial action as a drug class effect. Thus, the present study was focused on the in vitro pharmacological characterization of novel NOP ligands. The present thesis is constituted of two main chapters related to NOP ligands of peptide and non-peptide nature: the first chapter reports on N/OFQ dimeric compounds and the new tetrabranched derivative of the NOP antagonist UFP-101; the second chapter reports on the parallel analysis and comparison of seven non-peptide NOP agonists that were previously described in literature by different laboratories, the pharmacological characterization of novel AT compounds acting as NOP receptor partial agonists, and the in vitro characterization of the NOP and opioid receptor antagonist AT-076. All these ligands have been evaluated in different assays including receptor binding, stimulation of [^{35}S]GTPγS binding, calcium mobilization in cells co-expressing recombinant human receptors and chimeric G proteins, a BRET assay that measures receptor/G protein and receptor/β-arrestin 2 interaction, electrically stimulated mouse vas deferens and guinea pig ileum bioassays, and mouse colon bioassay. PWT2-UFP-101 has been also evaluated in vivo in mice in the forced swimming test and spontaneous locomotor activity assays.

3. RESULTS AND DISCUSSION

3.1 PEPTIDE LIGANDS

3.1.1 N/OFQ dimeric compounds

N/OFQ(1-13)NH_2, a useful pharmacophore for the design of NOP dimeric ligands



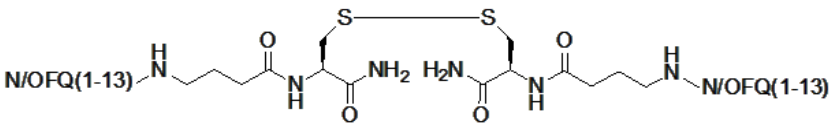
Despite very high primary sequence homology (about 60%) between classical opioid and NOP receptors, N/OFQ activates with high affinity and selectivity the NOP receptor and opioid ligands do not interact with NOP (Lambert, 2008). The reasons for such distinct pharmacology of NOP compared to classical opioid receptors have been recently unravelled at atomic level since the 3D structure of NOP and opioid receptors were solved (Filizola *et al.*, 2012). In particular, crucial structural rearrangements were evident by comparing NOP with kappa where replacements of only a few key residues in helices V and VI promotes an extensive reshaping of the binding pocket associated with an alternative coordination of water molecules. Since the beginning of modern pharmacology, G protein-coupled receptors have been considered to exist and exert their biological actions as monomers. However, in the past years a growing number of studies suggested that GPCRs are able to cross-react, forming homo- and heterodimers and/or oligomers; this process might be important in modulating different receptor functions (Casado *et al.*, 2009; Hiller *et al.*, 2013; Ferre *et al.*, 2014). In the opioid receptor field, evidence for delta homodimers (Cvejic *et al.*, 1997) as well as heterodimers (e.g. delta-kappa (Reinscheid *et al.*, 1995), delta-mu (Gomes *et al.*, 2004), kappa-mu (Wang *et al.*, 2005)) has been reported. These studies suggested that oligomerization of opioid receptors plays a role in receptor activation and internalization and generates novel properties of ligand binding. In parallel, Portoghese and co-workers identified dimeric ligands for opioid receptor heterodimers delta-kappa (Bhushan *et al.*, 2004) (KDN series) and delta-mu (Daniels *et al.*, 2005) (MDAN series) that were of great value for studying the biological effects associated with opioid receptors oligomerization. The KDN series was obtained combining the delta antagonist pharmacophore naltrindole and the kappa antagonist guanidinonaltrindole while the MDAN series was obtained by combining together the mu agonist oxymorphone with the delta antagonist naltrindole. Flexible spacers with length spanning from 15 to 23 atoms have been employed to link the different pharmacophores. Surprisingly, in both series of compounds the best results were obtained with compounds (KDN-21 and MDAN-21) with a spacer of 21 atoms. As far as opioid peptide ligands are concerned, delta receptor homodimeric ligands generated using the enkephalin tetrapeptide Tyr-Gly-Gly-Phe and the opioid related sequence Tyr-D-Ala-Gly showed increased delta receptor potency and selectivity compared with the corresponding monomers (Shimohigashi *et al.*, 1982; Shimohigashi *et al.*, 1989). Finally, using NOP and mu co-transfected cells (Pan *et al.*, 2002; Evans *et al.*, 2010; Majumdar *et al.*, 2011) and rat dorsal root ganglia lysate (Evans *et al.*, 2010) the existence of mu-NOP heteromers have been postulated. NOP-mu heterodimers might be implicated in NOP and mu receptor trafficking (Evans *et al.*, 2010) and can be considered as a novel pharmacological target for the development of analgesics without the classical side effects of opioid drugs (Majumdar *et al.*, 2011). In this study

the design, synthesis and in vitro pharmacological characterization of a series of 12 peptide NOP ligands obtained by dimerization of N/OFQ related sequences are described. The novel ligands were assayed in calcium mobilization studies performed in cells co-expressing the recombinant human NOP and the chimeric G protein $G\alpha_{qi5}$ (Camarda *et al.*, 2009) and, a subset of compounds, in bioassay studies performed with the electrically stimulated mouse vas deferens (Calo *et al.*, 1996).

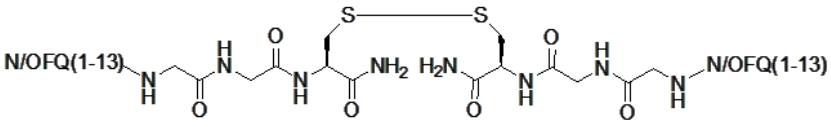
Materials and methods

Drugs – All cell culture media and supplements were from Invitrogen (Thermo Fisher Scientific Inc. MA, USA). All other reagents were from Sigma Chemical Co. (Poole, U.K.) and were of the highest purity available. All N/OFQ dimeric compounds described here (see chemical structures in Figure 3.1.1) were synthesized in the Prof. Guerrini laboratory (Department of Pharmaceutical Sciences and LTTA, University of Ferrara, Ferrara, Italy). N/OFQ dimeric compounds were solubilized in bidistilled water at a final concentration of 1 mM. Stock solutions of ligands were stored at -20 °C. The buffers for successive dilutions are described in the detail in each assay section.

Compound 1



Compound 2



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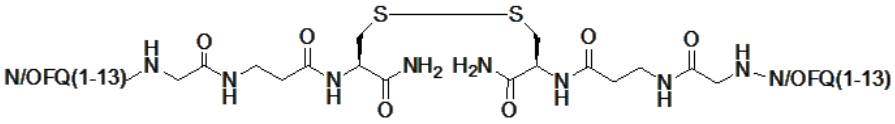

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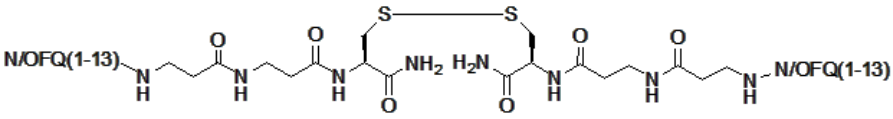

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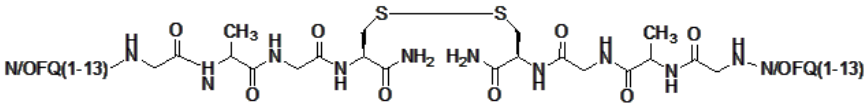
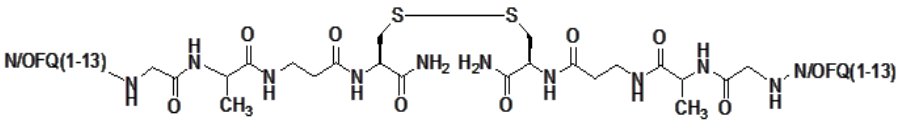
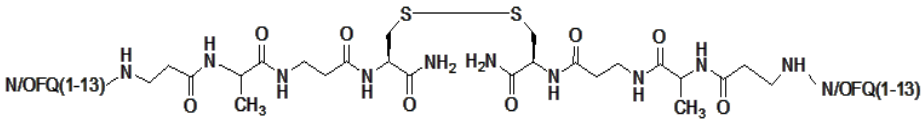


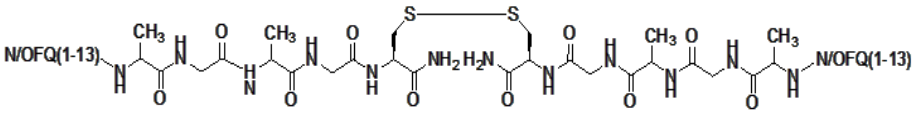
Compound 6



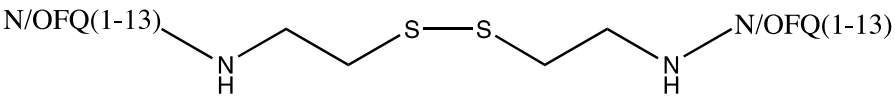
Compound 7



Compound 8

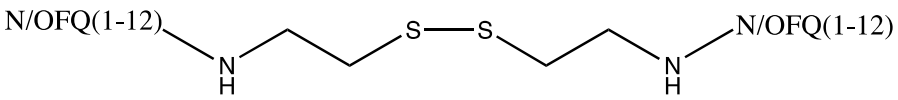


Compound 9



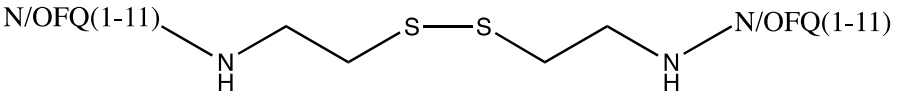
Compound 10 N/OFQ(1-12)-NH_2

Compound 11



Compound 12 N/OFQ(1-11)-NH_2

Compound 13



Compound 14 N/OFQ(2-12)-NH_2

Compound 15

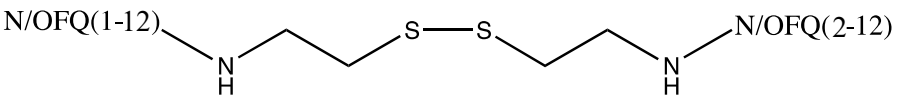


Figure 3.1.1. Chemical structures of N/OFQ dimeric compounds.

Calcium mobilization assay – Chinese Hamster Ovary (CHO) cells stably co-expressing the human NOP and the C-terminally modified $G\alpha_{qi5}$ were generated as previously described (Camarda *et al.*, 2009). Cells were maintained in culture medium consisting of Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/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 testing. 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 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 a washing step with 100 µl / well of Hank's Balanced Salt Solution (HBSS), HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 µM Brilliant Black (Aldrich) was carried out. Subsequently 100 µl/well of the same buffer was added. Serial dilutions of stock solutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% of bovine serum albumin, BSA, Sigma Chemical Co., Poole, UK). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA 94587, US), 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. The buffer for dilutions of the compounds is HBSS / HEPES (20 mM) or phosphate buffered solution (both containing 0.03% BSA fraction V).

Electrically stimulated mouse vas deferens – 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.*, 2010). 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 9927, 19/04/2013). The experiments were performed on the mouse vas deferens. The tissues were taken from male CD-1 mice (16 – 18 g, Harlan, Ud, Italy). Mice were housed in 425 x 266 x 155 mm cages (Techniplast, Mi, Italy), 8 per cage, all under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 7:00 am), with food for mice (4RF, Mucedola, Mi, Italy) and water *ad libitum*. A mouse red house (Techniplast, Va, Italy) and nesting materials were present in each cage for mice.
The day of the experiment the animals were sacrificed with CO_2 overdose. Bioassay experiments were performed as previously described by Calo *et al.* (1996). The tissues were suspended in 5 ml organ bath containing Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10). The Krebs solution was oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C and at resting tension 0.3 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. The 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, Colorado Springs, 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 to agonists were performed (0.5 log unit steps). Compounds were

diluted using the Krebs solution. A total number of 16 mice were used for the present in vitro studies.

Data analysis and terminology – The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig *et al.*, 2003). All data are expressed as the mean ± standard error of the mean (S.E.M.) of at least 4 experiments. For potency values 95% confidence limits ($CL_{95\%}$) were indicated. Efficacy was expressed as E_{max}, the agonist maximal effect. In calcium mobilization experiments, maximum change in fluorescence, expressed as % over the baseline fluorescence (fluorescence intensity unit, FIU), was used to determine agonist response. Electrically stimulated tissues data are expressed as % of the control twitch induced by electrical field stimulation. Agonist potencies are given as pEC_{50} i.e. the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1 + 10^{(LogEC_{50} - Log[compound]) * Hillslope})}$$

EC_{50} is the concentration of agonist producing a 50% maximal response. Curves fitting were performed using PRISM 6.0 (GraphPad Software In., San Diego, USA).

Results

Ligand design – As recently and nicely reviewed (Shonberg *et al.*, 2011), the choice of the pharmacophore, the attachment point selected for linking the two pharmacophores, and the length of the spacer are crucial parameters for the design of bivalent ligands. In a first series of compounds (compounds **1**-**8**, Table 3.1.1), the smallest fragment maintaining the same potency and efficacy of the natural peptide (Calo *et al.*, 1996; Guerrini *et al.*, 1997) i.e. N/OFQ(1-13) has been employed as peptide pharmacophore. As far as attachment point is concerned, extensive structure activity studies demonstrated that the C-terminal part of N/OFQ is an appropriate attachment point for peptide modifications. (Calo and Guerrini, 2013; Guerrini *et al.*, 2014). Regarding the spacer length, C-terminal elongation of the N/OFQ(1-13) sequence with Gly, Ala, β-Ala, Gaba and Cys in various combinations was used to produce dimeric N/OFQ(1-13) derivatives with spacers from 18 to 32 atoms. These spacer lengths were selected in order to cover the range that has been demonstrated to be optimal for targeting putative dimeric opioid receptors (Bhushan *et al.*, 2004; Daniels *et al.*, 2005; Yekkirala *et al.*, 2013). Dimeric peptide NOP ligands of this series have been generated by

the formation of a disulphide bridge between two monomers containing a thiol moiety at the C-terminus. To explore the possible differences in the pharmacological activity of monomeric versus dimeric peptide pharmacophores, in a second series of dimeric compounds the pharmacophore N/OFQ(1-13) has been shortened by C-terminal deletion of Lys^{13} and Arg^{12} (compounds **11** and **13**, Table 3.1.2). These chemical modifications have been previously reported to induce a progressive loss of peptide affinity and potency (Guerrini *et al.*, 1997; Dooley *et al.*, 1996; Varani *et al.*, 1998). In addition, to interpret the results obtained with the above mentioned dimeric ligands, the heterodimeric derivative N/OFQ(1-12)-N/OFQ(2-12) has been synthesized (compound **15**, Table 3.1.2). In this series, the dimers contained the same 2,2'- disulfanediyldiethanamine spacer.

Calcium mobilization assay and electrically stimulated mouse vas deferens – Table 3.1.1 summarized the results obtained in CHO_{NOP} cells co-expressing the chimeric $G\alpha_{qi5}$ protein with a series of dimeric derivatives of N/OFQ(1-13)-NH_2 characterized by a spacer length of 18 – 32 atoms. N/OFQ(1-13)-NH_2 evoked a concentration dependent stimulation of calcium release displaying high potency and maximal effects (Table 3.1.1). The concentration response curve to N/OFQ(1-13)-NH_2 was virtually superimposable to that of natural peptide N/OFQ (data not shown). Dimeric N/OFQ(1-13)-NH_2 derivatives (compounds **1-8**) displayed maximal effects similar to N/OFQ(1-13)-NH_2 thus behaving as NOP full agonists (data not shown). The potency of these derivatives was slightly lower (3-5 fold) than that of the standard (Table 3.1.1). Thus the biological activity of this series of compounds is comparable to that of the standard pharmacophore and not affected by ligand dimerization and spacer length.

Table 3.1.1. Effects of N/OFQ(1-13)-NH_2 and its dimeric derivatives in calcium mobilization experiments performed in CHO_{NOP} cells stably expressing the $G\alpha_{qi5}$ protein.

	pEC_{50} ($CL_{95\%}$)	**E_{max} ± S.E.M.**
N/OFQ(1-13)-NH_2	9.60 (9.40-9.79)	163 ± 32%
Compound 1	9.01 (8.83-9.19)	171 ± 63%
Compound 2	8.90 (8.66-9.15)	153 ± 44%
Compound 3	9.10 (8.81-9.38)	179 ± 63%
Compound 4	9.10 (8.98-9.22)	171 ± 30%
Compound 5	9.15 (8.97-9.32)	156 ± 23%
Compound 6	9.15 (8.96-9.34)	162 ± 62%
Compound 7	9.04 (8.80-9.27)	161 ± 48%
Compound 8	9.14 (8.96-9.33)	172 ± 46%

Table 3.1.2 summarized the results obtained in the calcium assay as well as in the electrically stimulated mouse vas deferens with homodimeric and heterodimeric derivatives of N/OFQ related peptides (compounds **9-15**). As already said, the N/OFQ(1-13)-NH_2 pharmacophore displayed similar maximal effect and potency as the natural peptide both in the calcium mobilization and in the mouse vas deferens assay. The deletion of the C terminal Lys^{13} did not modified the action of the peptide in the calcium mobilization assay while it caused a 27 fold loss of potency in the mouse vas deferens assay (peptide **10**). Further deletion of Arg^{12} caused a reduction of peptide potency in both assays (peptide **12**). In line with results reported in Table 3.1.1, dimerization of N/OFQ(1-13)-NH_2 did not modify the biological activity of peptide **9**, irrespective of the different spacer. This also applies to the action of compound **11** in the calcium mobilization assay. However the dimerization of the N/OFQ(1-12)-NH_2 sequence restored full biological activity in the mouse vas

deferens assay. Similar results were obtained comparing the action of compounds **13** and **12** in the calcium assay although in this case the recovery of biological activity was not complete. To investigate the mechanism by which dimerization elicits the recovery of biological activity of N/OFQ(1-12)-NH_2, compound **15** was synthesized and tested. This compound is generated by linking N/OFQ(1-12)-NH_2 with the biologically inactive sequence N/OFQ(2-12)-NH_2 (**14**). As shown in Table 3.1.2 compound **15** elicited similar effects as compound **11** in both the assays.

Table 3.1.2. Effects of N/OFQ(1-13)-NH_2 and its dimeric derivatives in calcium mobilization experiments performed in CHO_{NOP} cells stably expressing the $G\alpha_{qi5}$ protein and in the electrically stimulated mouse vas deferens.

	Ca^{2+}mobilization		**mVD**	
	pEC_{50} ($CL_{95\%}$)	**E_{max} ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**E_{max} ± S.E.M.**
N/OFQ(1-13)-NH_2	9.83 (9.51-10.16)	199 ± 20%	7.02 (6.94-7.10)	89 ± 3%
Compound 9	9.40 (9.15-9.66)	213 ± 24%	7.31 (7.14-7.49)	88 ± 3%
Compound 10	9.70 (9.41-9.98)	204 ± 16%	5.59 (5.22-5.95)	60 ± 4%
Compound 11	9.38 (9.06-9.70)	205 ± 18%	7.27 (7.09-7.45)	84 ± 5%
Compound 12	8.21 (8.11-8.32)	182 ± 34%	5.28 (5.05-5.51)	65 ± 5%
Compound 13	8.87 (8.58-9.16)	193 ± 22%	5.93 (5.81-6.05)	85 ± 4%
Compound 14	inactive		inactive	
Compound 15	9.49 (9.07-9.91)	205 ± 27%	7.31 (7.18-7.44)	92 ± 3%

Discussion

This study reports on the design, synthesis and pharmacological activity of dimeric ligands of the NOP receptor. The dimeric ligands were generated using as pharmacophore the peptide sequence of

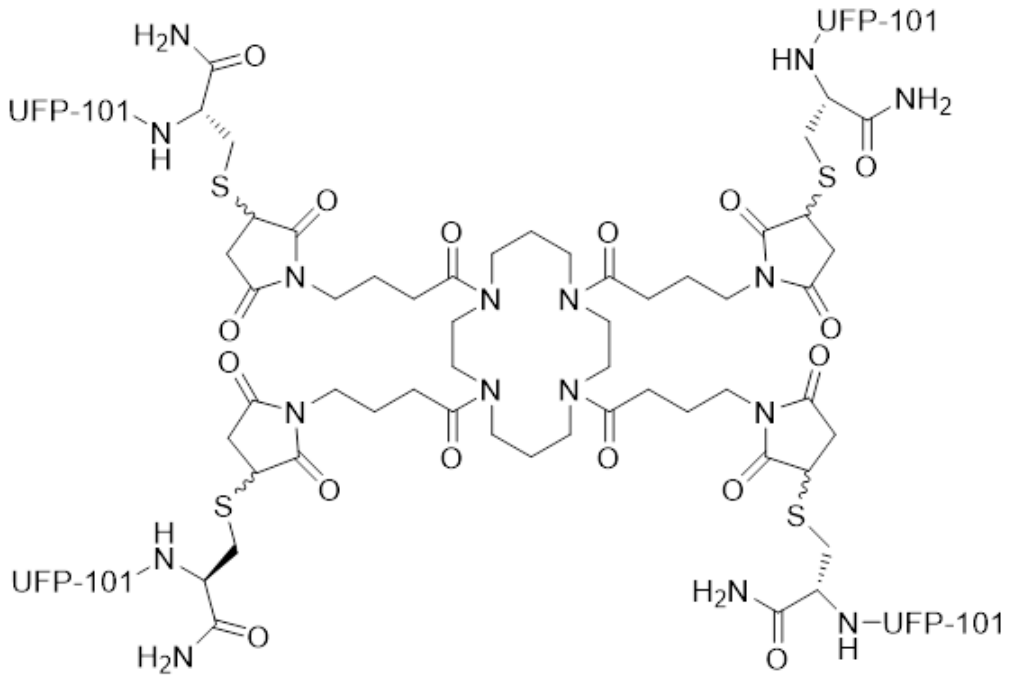
N/OFQ. This pharmacophore was linked with spacers of different length covering the range that has been previously and successfully employed for the generation of dimeric ligands for opioid receptors. Dimerization did not modify the pharmacological activity of the pharmacophore. However, when dimeric compounds were obtained with a low potency peptide pharmacophore, dimerization recovered ligand potency; this effects depends on the doubling of the C-terminal address sequence rather than the presence of an additional message sequence. In the calcium assay the peptide (N/OFQ(1-13)-NH_2) NOP pharmacophore displayed full agonist activity and values of potencies in line with previous studies (Camarda *et al.*, 2009). The pharmacological activity of N/OFQ(1-13)-NH_2 has been compared with that of a series of homodimeric derivatives (compounds **1-8**) with a spacer spanning from 18 to 32 atoms. No significant differences were measured both in terms of efficacy and potency between the dimeric derivatives and the pharmacophore. These results demonstrated that the attachment point selected for generating dimeric derivatives does not affect the pharmacological activity of the pharmacophore. Of note, an increase in agonist potency was reported for opioid receptor homo (Shimohigashi *et al.*, 1989; Shimohigashi *et al.*, 1982) and heterodimers (Bhushan *et al.*, 2004; Daniels *et al.*, 2005) linked with spacers of a similar length as those used in the present investigation. On the contrary the dimeric ligands we generated displayed similar potency as the single pharmacophore. Interestingly, similar findings were previously obtained with a dimeric derivative of [$Arg^{14}Lys^{15}$]N/OFQ (Ligeti *et al.*, 2005). Collectively this evidence suggests that despite their dimeric nature, these compounds bind the NOP receptor as the single pharmacophore. In other terms we were not able to provide pharmacological evidence for the presence of NOP receptor dimers in the preparations under study. To investigate the possibility to generate novel NOP ligands by the design of dimeric derivatives of shorter peptide pharmacophores, compounds **9-15** were synthesized and assayed. In line with previous findings (Guerrini *et al.*, 1997; Dooley *et al.*, 1996) shortening of N/OFQ sequence to 12 and 11 amino acids produced a progressing loss of agonist potency underlining the crucial role of Arg^{12}-Lys^{13} for NOP binding. Of note, in the mouse vas deferens assay the drop in ligand potency was evident already with N/OFQ(1-12) (compound **10**) while in the calcium assay it was clear with N/OFQ(1-11) (compound **12**). This difference is most probably due to the high versus low stimulus / response coupling that characterizes the calcium versus vas deferens assay, respectively (Camarda *et al.*, 2009). Interestingly enough, the dimerization of compounds **10** and **12** produced a recovery in ligand potency by 30 fold (compound **11**) in the vas deferens and 4 fold in the calcium assay (compound **13**). This recovery in ligand potency may in theory derives from the presence in the dimeric compound of the extra $F^1G^2G^3F^4$ message sequence or of the extra $T^5G^6A^7R^8K^9S^{10}A^{11}R^{12}$ address sequence. This issue has been addressed by synthesizing and testing the heterodimeric

compound **15** that lacks the Phe[1] in the extra message domain; this chemical modification is known to fully eliminate NOP binding (Dooley *et al.*, 1996; Connor *et al.*, 1998; Calo *et al.*, 2000b). Compound **15** displayed similar efficacy and potency as compound **11** thus demonstrating the importance of the extra address rather than message sequence in the recovery of ligand potency induced by dimerization. This finding also underlines the importance of the use of negative controls (i.e. the use of an inactive pharmacophore) to deeply investigate the pharmacological activity of dimeric molecules. This allows to correctly interpret the changes in activity as due to the bivalent nature versus the overall chemical structure and/or conformation of the new compound.

In conclusion, the present study demonstrated that peptide homodimeric NOP ligands displayed a pharmacological activity similar to the single pharmacophore. Hence, at least under the present experimental conditions, there is no evidence for NOP receptor homo-dimerization. Recovery of activity shown by some dimers points to the importance of the presence of positively charged residues within the address region of the ligands. The information generated by the present structure-activity studies may be useful for the design of novel peptide NOP ligands.

3.1.2 PWT2-UFP-101

PWT2-UFP-101



UFP-101 = Nphe[1]-Gly-Gly-Phe-Thr-Gly-Ala-Arg[8]-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Asn-Gln[17]

A novel chemical strategy for the facile synthesis of tetrabranched peptides named peptide welding technology (PWT) has been developed and validated in our laboratories (Guerrini *et al.*, 2014). PWT derivatives of different peptide sequences nociceptin/orphanin FQ (N/OFQ) (Rizzi *et al.*, 2014; Rizzi *et al.*, 2015), tachykinins (Ruzza *et al.*, 2014), neuropeptide S (Ruzza *et al.*, 2015), and more recently [Dmt^1]N/OFQ(1-13)-NH_2 (Cerlesi *et al.*, 2016) have been synthesized and pharmacologically characterized in vitro and in vivo. In general PWT derivatives displayed in vitro similar potency, selectivity of action, and pharmacological activity as the linear cognate peptide while in vivo they demonstrated higher potency associated to longer lasting effects. All the above mentioned studies were performed with peptides acting as receptor agonists. In the present study the PWT approach has been applied for the first time to a peptide sequence characterized by receptor antagonist activity namely UFP-101. UFP-101 ([$Nphe^1Arg^{14}Lys^{15}$]N/OFQ-NH_2) is a N/OFQ related peptide acting as N/OFQ receptor (NOP) antagonist. This peptide was designed by combining in the same molecule the $Nphe^1$ modification that confers antagonist properties (Calo *et al.*, 2000b) and the $Arg^{14}Lys^{15}$ modification that increases ligand affinity and potency (Okada *et al.*, 2000; Rizzi *et al.*, 2002b). After its identification (Calo *et al.*, 2002), UFP-101 has been widely used as pharmacological tool for demonstrating the involvement of the NOP receptor in the actions of N/OFQ and of synthetic NOP agonists, for investigating the biological actions controlled by the N/OFQ-NOP receptor system, and for foreseeing the therapeutic indications of selective NOP receptor antagonists (reviewed in Calo and Guerrini, 2013; Toll *et al.*, 2016).

Thus, the aim of the present study was the pharmacological characterization of the tetrabranched derivative PWT2-UFP-101. In vitro PWT2-UFP-101 has been evaluated in a BRET based assay measuring NOP/G protein interaction and in the electrically stimulated mouse vas deferens. In vivo PWT2-UFP-101 has been tested in mice in the forced swimming test and spontaneous locomotor activity assay. In all the assays the action of PWT2-UFP-101 was compared to that elicited by the linear peptide UFP-101.

Material and Methods

Drugs and reagents – All cell culture media and supplements were from Invitrogen (Thermo Fisher Scientific Inc. MA, USA). All other reagents were from Sigma Chemical Co. (Poole, U.K.) and were of the highest purity available. Native coelenterazine (CLZN, 5 mM, EtOH) was from Synchem UG & Co. KG (Altenburg, Germany). N/OFQ, UFP-101, and PWT2-UFP-101 were synthesized in the Prof. Guerrini laboratory (Department of Pharmaceutical Sciences and LTTA,

University of Ferrara, Ferrara, Italy). DPDPE was from Tocris Bioscience (Bristol, UK). For in vitro experiments all compounds were solubilized in bidistilled water at a final concentration of 1 mM. For in vivo experiments UFP-101 and PWT2-UFP-101 were solubilized in saline at final concentrations of 5 mM. Stock solutions of ligands were stored at -20 °C. The buffers for successive dilutions are describe in the detail in each assay section.

BRET assay – Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 °C. Cell line permanently co-expressing the different pairs of fusion proteins (NOP-RLuc/Gβ1-RGFP) was prepared using the pantropic retroviral expression system by Clontech as described previously (Malfacini *et al.*, 2015). 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 (4 °C) 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 10 min 1000 g (4 °C) and the supernatants kept. Two 20 min 24,000 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 *et al.*, 1987). The protein concentration in membrane preparations was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and the spectrophotometer Beckman DU 520 (Brea, CA, USA).
Luminescence in membranes was recorded in 96-well untreated white opaque microplates, (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030 (PerkinElmer, Waltham, MA, USA). For the determination of NOP/G-protein interaction, membranes (3 µg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP were added to wells in DPBS. 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 min before reading luminescence. All the experiments were performed at room temperature.

Electrically stimulated tissues – The experiments were performed on the mouse vas deferens. The tissues were taken from male CD-1 mice 16 – 18 g (Harlan, Ud, Italy). Mice were housed in 425 x 266 x 155 mm cages (Techniplast, Mi, Italy), 8 per cage, under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 7:00 am), with food (4RF, Mucedola, Mi, Italy) and

water *ad libitum*. A mouse red house (Techniplast, Va, Italy) and nesting materials were present in each cage for mice.

The day of the experiment the animals were sacrificed with CO_2 overdose. The experimental protocol was approved by the Ethical Committee for the Use of Laboratory Animals of the University of Ferrara and by the Italian Ministry of Health (authorization number 9927, 19/04/2013). The animals were treated in accordance with 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.*, 2010). Bioassay experiments were performed as previously described by Calo *et al.* (1996). The tissues were suspended in 5 ml organ bath containing Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10). The Krebs solution was oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C and at resting tension 0.3 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. The 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, Colorado Springs, 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 to agonists were performed (0.5 log unit steps). Antagonists were injected into the baths 15 minutes before performing concentration response curves to agonists. Compounds were diluted using the Krebs solution. A total number of 24 mice were used for the present in vitro studies.

All experimental procedures adopted for in vivo studies were as humane as possible complied with the ARRIVE guidelines (Kilkenny *et al.*, 2010), the European Communities Council directives (2010/63/EU) and national regulations (D.L. 26/2014). Protocols were approved by the University of Ferrara and by the Italian Ministry of Health (Protocol No. 316/2013-B). Male CD-1 (8 – 12 weeks old) were used in this study. Mice were housed in 425 × 266 × 155 mm cages (Techniplast, VA, Italy), 5 mice/cage, under standard conditions (22°C, 55% humidity, 12h light/dark cycle, lights on 7.00 am) with food (4RF, Mucedola, MI, Italy) and water *ad libitum*. A mouse red house (Techniplast, VA, Italy) and nesting materials were present in each cage. Each animal was used only once and killed with CO_2 at the end of the experiment. A total number of 189 mice was used for the present study.

PWT2-UFP-101 and UFP-101 were given intracerebroventriculary (i.c.v., 2 µl mouse). Free hand i.c.v. injections were given, under isofluorane anaesthesia (just sufficient to produce a loss of the righting reflex), in the left ventricle according to literature procedures (Laursen *et al.*, 1986).

Forced swimming test – The test was carried out according to (Porsolt *et al.*, 1977). Briefly, the test consists in placing mice, individually, in a Plexiglas cylinders (18.5 cm high, 12.5 cm diameter, water 13.5 cm deep) partially filled with water (25 ± 0.5 °C), for two swimming sessions: an initial 15-min training session, which was followed, 24 h later, by a 5 min test session. The time each animal remained immobile (immobility time) during the 5 min test was recorded. Animals were judged to be immobile when they ceased struggling/swimming and remained floating motionless in the water, making only those movements necessary to keep their heads above the water line. At the end of each swimming session, the animals was removed from the cylinder, dried with paper towels, placed in an individual cage for rest and recover over 15 min, and then returned to its collective home cage. Between each trial, the cylinder was cleaned and the water changed. UFP-101 and PWT2-UFP-101 were injected i.c.v. at different times (5 min, 1, 2, 3 hours) before the test.

Spontaneous locomotor activity – For these experiments the ANY-maze video tracking system was used (Ugo Basile, application version 4.52c Beta). Mice were positioned in a square plastic cage (40 × 40 cm), one mouse per cage. Four mice were monitored in parallel. Mouse horizontal activity was monitored by a camera while vertical activity was measured by an infrared beam array. The parameters measured were cumulative distance travelled (total distance in m that the animal travelled during the test), immobility time (the animal is considered immobile when 90% of it remains in the same place for a minimum of 2.5 s), and the number of rearings (the number of beam breaks due to vertical movements; this input is triggered when the beam is interrupted for a minimum of 200 ms). UFP-101 and PWT2-UFP-101 were injected i.c.v. 1 hour before the test.

Data analysis and terminology – The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Alexander *et al.*, 2013). All data are expressed as the mean ± standard error of the mean (S.E.M.) of *n* experiments. For potency values 95% confidence limits ($CL_{95\%}$) were indicated.

BRET data are calculate 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. Maximal effects elicited by the ligands are expressed as intrinsic activity α using N/OFQ as

standard full agonist. Electrically stimulated tissues data are expressed as % of the control twitch induced by electrical field stimulation.

Agonist potencies are given as pEC_{50} i.e. the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{((LogEC_{50} - Log[compound])*Hillslope)})}$$

EC_{50} is the concentration of agonist producing a 50% maximal response. Curves fitting were performed using PRISM 6.0 (GraphPad Software In., San Diego, USA).

Antagonist potencies were 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. The type of antagonism exerted by PWT2-UFP-101 was assayed by using the Schild analysis. The Schild plot was analyzed by linear regression to derive the pA_2 value of the antagonist (Kenakin, 2004).

In vivo data are expressed as mean ± S.E.M. of *n* animals. Data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test, as specified in figure. Differences were considered statistically significant when $p < 0.05$.

Results

BRET assay – Membrane extracts taken from HEK293 cells stably expressing the human NOP receptor (NOP-RLuc) and the $G\beta_1$ subunit (Gβ1-RGFP) were used in concentration response experiments to evaluate the NOP/G-protein interaction. N/OFQ promoted NOP/G-protein interaction in a concentration-dependent manner with high potency (pEC_{50} 9.40 (7.90-10.89)) and maximal effects corresponding to 0.94 ± 0.02 stimulated BRET ratio; all the intrinsic activities were computed as fraction of N/OFQ maximal stimulated BRET ratio (N/OFQ = 1.00). UFP-101 and PWT2-UFP-101 promoted NOP/G protein interaction with maximal effects of 0.14 ± 0.03 and 0.31 ± 0.08, respectively. The low maximal effects produced by UFP-101 and PWT2-UFP-101 did not allow to estimate precisely their potenty (Figure 3.1.2).

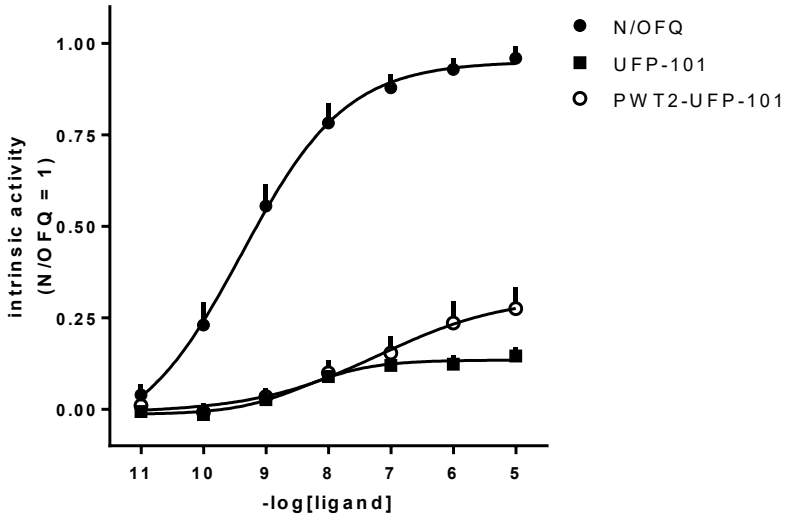


Figure 3.1.2. BRET assay. Concentration response curves to N/OFQ, UFP-101 and PWT2-UFP-101 in promoting NOP/G-protein interaction. Data are the mean ± S.E.M. of 3 experiments performed in duplicate.

UFP-101 and PWT2-UFP-101 were tested as antagonists in the BRET NOP/G-protein interaction assay against the concentration response curve to N/OFQ. UFP-101 at a single concentration of 100 nM shifted to the right the N/OFQ concentration response curve without affecting the agonist maximal effect; a pA_2 of 8.32 (7.79-8.85) was derived from these experiments (Figure 3.1.3A). PWT2-UFP-101 was challenged at different concentrations (3 nM - 100 nM) against the concentration response curve to N/OFQ. Schild plot analysis of these data yielded a pA_2 value of 8.58 (8.09-9.08) (Figure 3.1.3B).

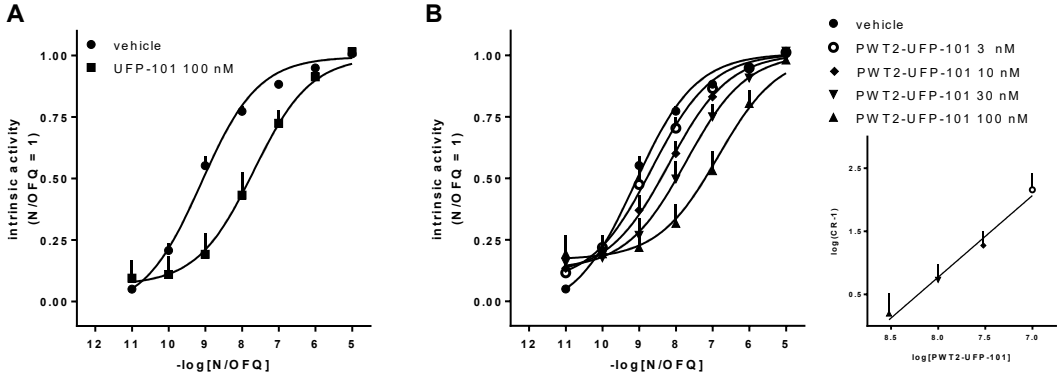


Figure 3.1.3. NOP/G-protein interaction. Concentration response curve to N/OFQ obtained in the absence (vehicle) and presence of UFP-101 100 nM (panel A) and of increasing concentrations of PWT2-UFP-101 (3, 10, 30 and 100 nM) (panel B), the corresponding Schild plot is shown in panel B inset. Data are the mean ± S.E.M. of 5 experiments.

Mouse vas deferens – In the electrically stimulated mouse vas deferens, N/OFQ inhibited the twitch response in a concentration dependent manner with pEC_{50} 7.16 (7.07 – 7.25), E_{max}= 88 ± 2%. The

delta receptor agonist DPDPE mimicked the effect of N/OFQ being more potent and producing higher maximal effect (pEC_{50} 8.26 (8.16 – 8.36), E_{max}= 100 %) (Figure 3.1.4).

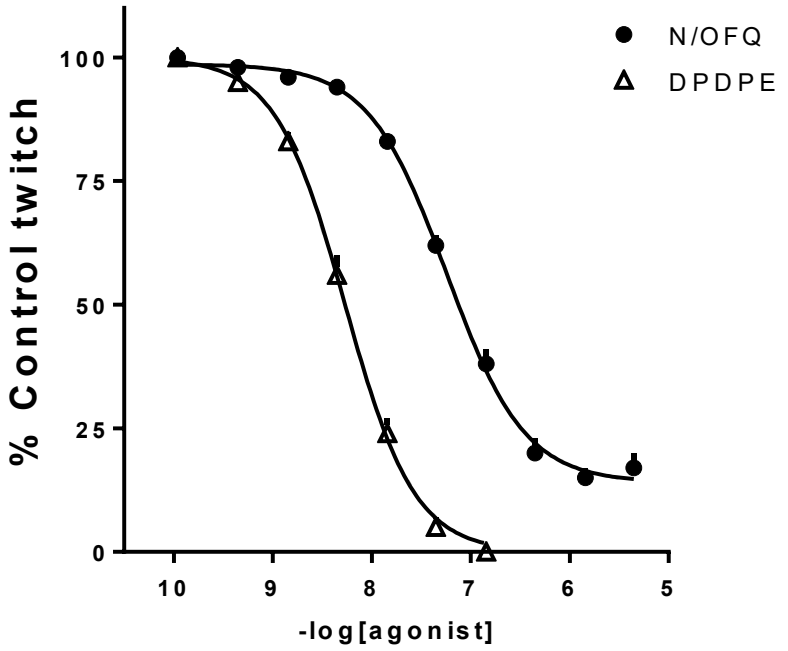


Figure 3.1.4. Electrically stimulated mouse vas deferens. Concentration response curves to N/OFQ and DPDPE. Data are the mean ± S.E.M. of 5 separate experiments.

The selective NOP receptor antagonist UFP-101 1 μM was challenged against N/OFQ. This antagonist produced a rightward shift of the concentration response curve to N/OFQ without affecting the agonist maximal effects: a pA_2 value of 7.02 (6.63-7.42) was derived from these experiments (Figure 3.1.5A). The new tetrabranched compound PWT2-UFP-101 tested as agonist up to 1 μM did not modify per se the electrically induced twitch. However, increasing concentration of PWT2-UFP-101 (10 nM – 1000 nM) produced a concentration dependent rightward shift of the concentration response curve to N/OFQ without significantly affecting the maximal agonist effect (Figure 3.1.5B). Schild analysis of these data (Figure 3.1.5B) yielded a pA_2 value of 7.59 (7.36-7.82).

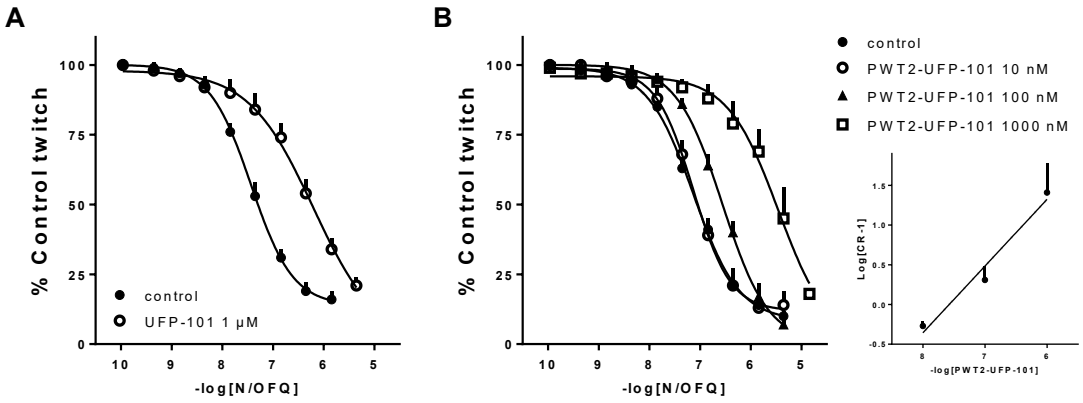


Figure 3.1.5. Electrically stimulated mouse vas deferens. Concentration response curves to N/OFQ obtained in the absence (control) and presence of UFP-101 1µM (panel A), and of increasing concentrations of PWT2-UFP-101 (10, 100 and 1000 nM) (panel B). The corresponding Schild plot is shown in panel B inset. Data are the mean ± S.E.M. of at least 5 separate experiments.

Then we tested PWT2-UFP-101 against the delta selective agonist DPDPE. PWT2-UFP-101 1 µM slightly displaced the concentration response curve of DPDPE with a pA_2 value of 5.95 (5.59 – 6.32) (Figure 3.1.6).

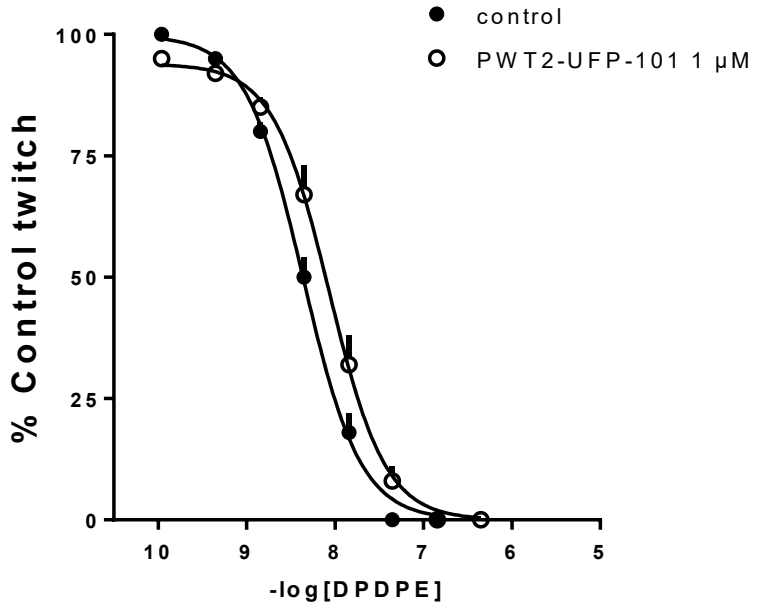


Figure 3.1.6. Electrically stimulated mouse vas deferens. Concentration response curves to DPDPE obtained in the absence (control) and presence of PWT2-UFP-101 1µM. Data are the mean ± S.E.M. of 3 separate experiments.

Forced swimming test – In a first series of experiments we compare the effect of equi-active doses of UFP-101 and PWT2-UFP-101 injected 5 min before the test. As shown in Figure 3.1.7, UFP-101

reduced immobility time of mice subjected to the forced swimming test in a statistically significant manner, while under the same experimental conditions, PWT2-UFP-101 was completely inactive.

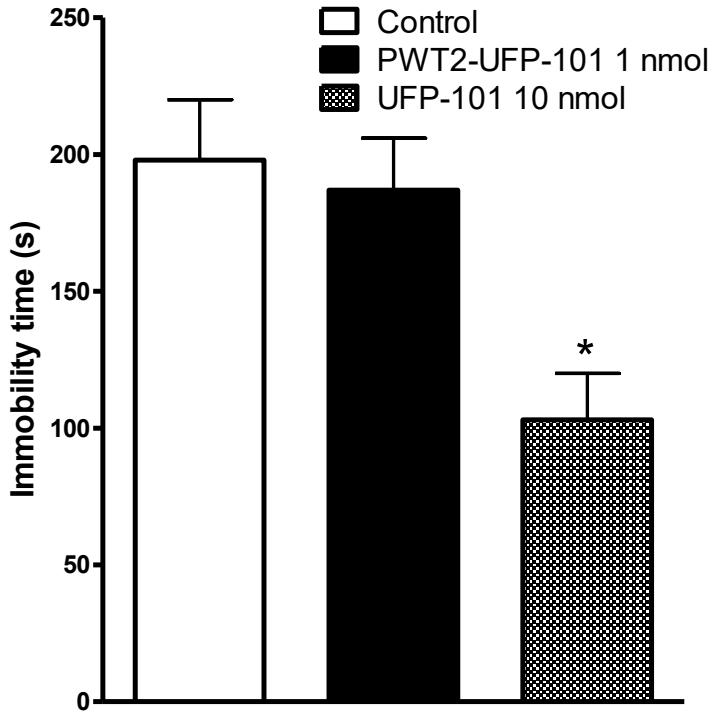


Figure 3.1.7. Effect of UFP-101 (10 nmol) and PWT2-UFP-101 (1 nmol) on the immobility time of mice subjected to the forced swimming test. Data are mean ± S.E.M. of 12 mice per group. One-way ANOVA followed by the Dunnett's post hoc test, *$p<0.05$ vs saline.

Therefore, we increase the pre-treating time to 60 min and we injected the mice with increasing doses of PWT2-UFP-101. As shown in figure 3.1.8, PWT2-UFP-101 (0.01 – 1 nmol) reduced immobility time of mice subjected to the forced swimming test in a dose dependent manner, evoking statistically significant effects at the dose of 0.1 and 1 nmol.

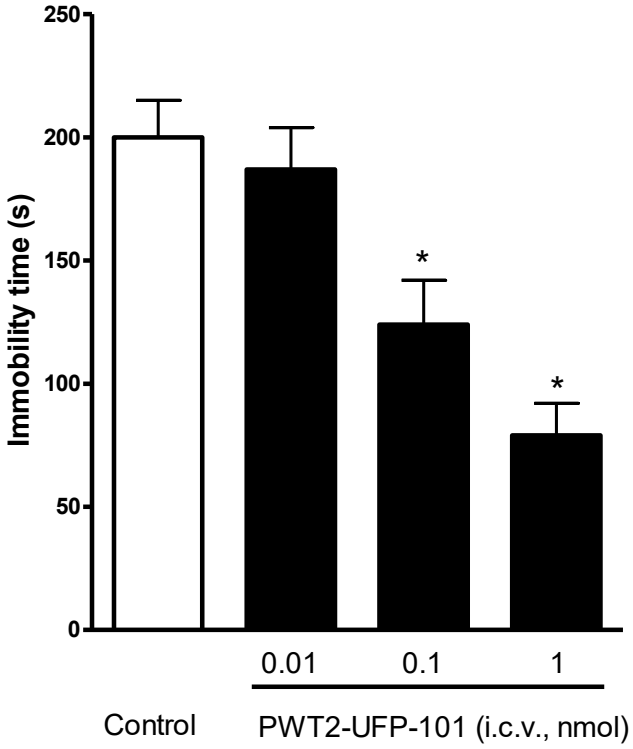


Figure 3.1.8. Effects of PWT2-UFP-101 (0.01, 0.1 and 1 nmol injected) 1 hour before starting the forced swimming test. Data are mean ± S.E.M. of 12 mice per group. One-way ANOVA followed by the Dunnett's post hoc test, *p<0.05 vs saline.

Finally, in order to investigate kinetic of action of the two ligands we pre-treated the mice with equi-active doses of UFP-101 and PWT2-UFP-101 1, 2 or 3 hours before performing the test. As shown in figure 3.1.9, 10 nmol UFP-101 and 1 nmol PWT2-UFP-101 reduced immobility time of mice subjected to the forced swimming test evoking statistically significant effects after 1 and 2 h pre-treatment, while they were completely inactive after 3 hours.

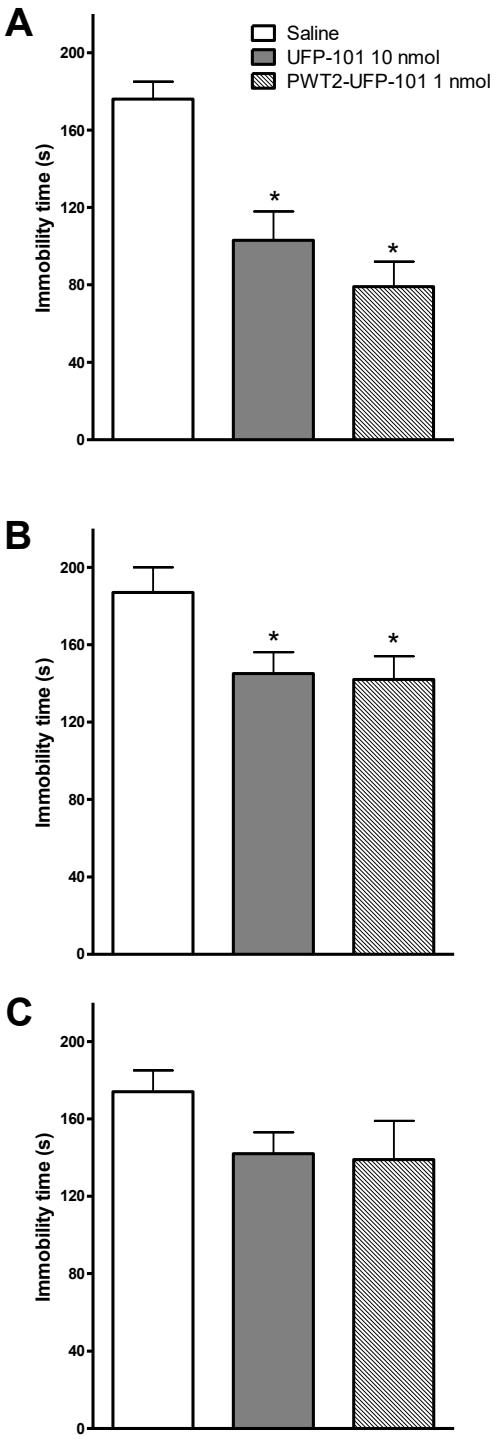


Figure 3.1.9. Effects of UFP-101 (10 nmol) and PWT2-UFP-101 (1 nmol) 1 hour (panel A), 2 hour (panel B) and 3 hour (panel C) before starting the forced swimming test. Data are mean ± S.E.M. of 12 mice per group. One-way ANOVA followed by the Dunnett's post hoc test, *$p<0.05$ vs saline.

Locomotor activity – As shown in Figure 3.1.10, mice pre-treated i.c.v., 60 min before the test, with 10 nmol UFP-101 showed similar spontaneous locomotor activity as control animals, while those treated with the PWT derivative displayed a statistically significant decrease in total distance travelled and in number of rearing.

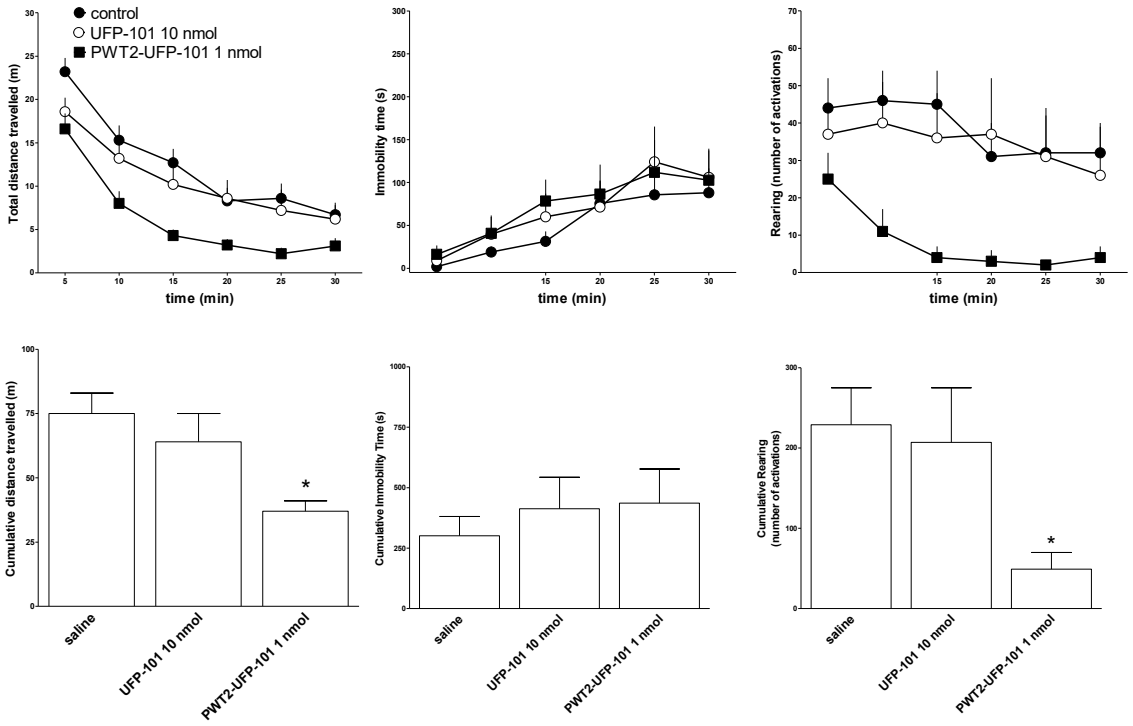


Figure 3.1.10. Effects of UFP-101 (10 nmol) and its PWT derivative (1 nmol) on mouse locomotor activity after 1 hour from injection. Results are shown as time course in the top panels and as cumulative effects in the bottom panels. Data are mean ± S.E.M. of 12 animals for each point. *p < 0.05 according to one way ANOVA followed by the Dunnett's post hoc test for multiple comparisons.

Discussion

The present study investigated the pharmacological profile of PWT2-UFP-101, a tetrabranched derivative of the NOP receptor antagonist UFP-101. In vitro, PWT2-UFP-101 antagonized N/OFQ effects at human recombinant and mouse native receptor acting as a competitive antagonist, and showing similar potency as UFP-101. In vivo in the mouse forced swimming assay, PWT2-UFP-101 elicited antidepressant-like effects being approximately 10 fold more potent that UFP-101. However PWT2-UFP-101 but not UFP-101 produced an inhibitory effect on mouse locomotor activity; this action may limit the usefulness of PWT2-UFP-101 as pharmacological tools for in vivo studies. Taken together these results demonstrated that the PWT strategy can be applied to peptide antagonists increasing their in vivo potency.

The BRET based NOP/G protein assay has been previously set up and validated using a large panel of NOP ligands (Malfacini *et al.*, 2015), obtaining results in line with literature findings (Toll *et al.*, 2016). The usefulness of this assay was later confirmed in a series of studies investigating novel NOP ligands (Rizzi *et al.*, 2016; Bird *et al.*, 2016; Asth *et al.*, 2016). Of note this assay was also successfully used for selecting NOP ligands for receptor crystallization studies (Miller *et al.*, 2015). In this assay PWT2-UFP-101 competitively antagonized N/OFQ stimulatory effects showing a value of potency similar to that of UFP-101. Thus, the PWT chemical modification did not modify the NOP antagonist activity and the potency of UFP-101. This finding obtained for the first time with a peptide antagonist is in line with previous results obtained by applying the PWT to various peptide sequences with agonist activity including N/OFQ (Rizzi *et al.*, 2014), substance P, and neurokinin A and B (Ruzza *et al.*, 2014), neuropeptide S (Ruzza *et al.*, 2015) and, more recently, [Dmt^1]N/OFQ(1-13)-NH_2 (Cerlesi *et al*., 2016). In addition the PWT modification did not affect the competitive nature of UFP-101 antagonism. This suggests that the N-terminal tetrapeptide (Nphe^1-Gly^2-Gly^3-Phe^4) of both UFP-101 and of its PWT derivative interacts with the binding pocket of the NOP receptor in a very similar manner. The atomic details of this interaction have been elucidated by docking UFP-101 to the crystal structure of the inactive state of the NOP receptor (Thompson *et al.*, 2012; and Figure 2 of Calo and Guerrini, 2013). It is worthy of mention that both UFP-101 and PWT2-UFP-101 displayed some residual agonist activity in this assay. This result is not completely unexpected. In fact, despite a very large body of evidence that demonstrated that UFP-101 behaves in vitro and in vivo as a NOP antagonist (reviewed in Calo *et al.* (2005) and Calo and Guerrini, 2013), there are also some few results suggesting that the reduction of ligand efficacy produced by [Nphe^1] could not be complete. In GTPγS binding studies performed with the NOP-G_o fusoprotein GTP concentrations modulated the pharmacological behavior of both [Nphe^1]N/OFQ(1-13)-NH_2 and UFP-101 from pure antagonism to robust partial agonism (T. Costa, personal communication). Moreover in electrophysiological studies in neurons microinjected with a plasmid coding for the NOP receptor the non peptide NOP antagonists C-24 and Trap-101 behaved as pure antagonists in control neurons and as inverse agonists in transfected neurons. On the contrary, UFP-101 acted as a pure antagonist in control cells while displayed a partial agonist behavior in transfected neurons (Mahmoud *et al.*, 2010).

The electrically stimulate mouse vas deferens has been identified as N/OFQ sensitive pharmacological preparation soon after the discovery of the peptide (Berzetei-Gurske *et al.*, 1996; Calo *et al.*, 1996). Since then this preparation has been proven extremely useful as bioassay for performing structure activity studies on N/OFQ (reviewed in Calo and Guerrini, (2013)), and more

in general, for evaluating the pharmacological profile of novel NOP ligands at native NOP receptors (see Tables 2 and 3 in Toll *et al.* (2016)). In this preparation UFP-101 antagonized N/OFQ inhibitory effects with a value of potency (7.02) in line with previous findings (7.29, (Calo *et al.*, 2002)). The Schild analysis of the action of PWT2-UFP-101 confirmed the NOP antagonist activity and competitive behavior of the interaction with N/OFQ and yielded a value of potency of 7.59 slightly higher than that of the linear peptide. Importantly, when challenged against the delta receptor selective agonist DPDPE, PWT2-UFP-101 displayed a value of potency more than 30 fold lower. Of note, previous studies with UFP-101 demonstrated very high NOP selectivity in bioassay studies (Calo et al., 2002). Thus the present results suggests that the PWT modification does promote a certain reduction of the UFP-101 selectivity of action. This is somewhat unexpected since no changes of ligand selectivity were observed in previous studies where the selectivity a several peptide agonists was maintained by their PWT derivatives (Rizzi *et al.*, 2014; Ruzza *et al.*, 2014; Ruzza *et al.*, 2015; Cerlesi *et al*., 2016).

The promising in vitro pharmacological profile of PWT2-UFP-101 prompted us to test the compound in vivo in the mouse forced swimming test. In this assay the i.c.v. injection of peptide NOP antagonists as well as the systemic injection of brain penetrant non peptide NOP antagonists elicits antidepressant like effects (reviewed in Gavioli *et al.* (2013)). These findings were corroborated by knockout studies that demonstrated that both NOP(-/-) mice (Gavioli *et al.*, 2003) and rats (Rizzi *et al.*, 2011) displayed an antidepressant phenotype in the forced swimming assay. In addition, the recently identified NOP selective antagonist LY2940094 promoted antidepressant effects in rodents and, more importantly, displayed antidepressant efficacy in patients with major depressive disorder (Post *et al.*, 2016). PWT2-UFP-101 displayed slow developing effects in the forced swimming test since its effects were evident after 60 but not 5 min from injection. This finding is in line with previous studies demonstrating that PWT derivatives of N/OFQ and substance P displayed slow developing effects (Rizzi *et al.*, 2014; Ruzza *et al.*, 2014). PWT2-UFP-101 promoted dose dependent antidepressant like action eliciting statistically significant effects starting from the dose 0.1 nmol. Thus compared with UFP-101 (Gavioli *et al.*, 2003) its tetrabranced derivative was approximately 10 fold more potent. This is also in line with previous studies in which PWT compounds of various peptide agonists showed in vivo increased potency by 10 to 40 fold (Rizzi *et al.*, 2014; Ruzza *et al.*, 2014; Ruzza *et al.*, 2015). A separate series of experiments were performed for investigating the duration of action of PWT2-UFP-101. In this studies the effect of equiactive doses of UFP-101 and PWT2-UFP-101, i.e. 10 and 1 nmol, respectively, were compared at different pretreatment times. The compounds elicited statistically

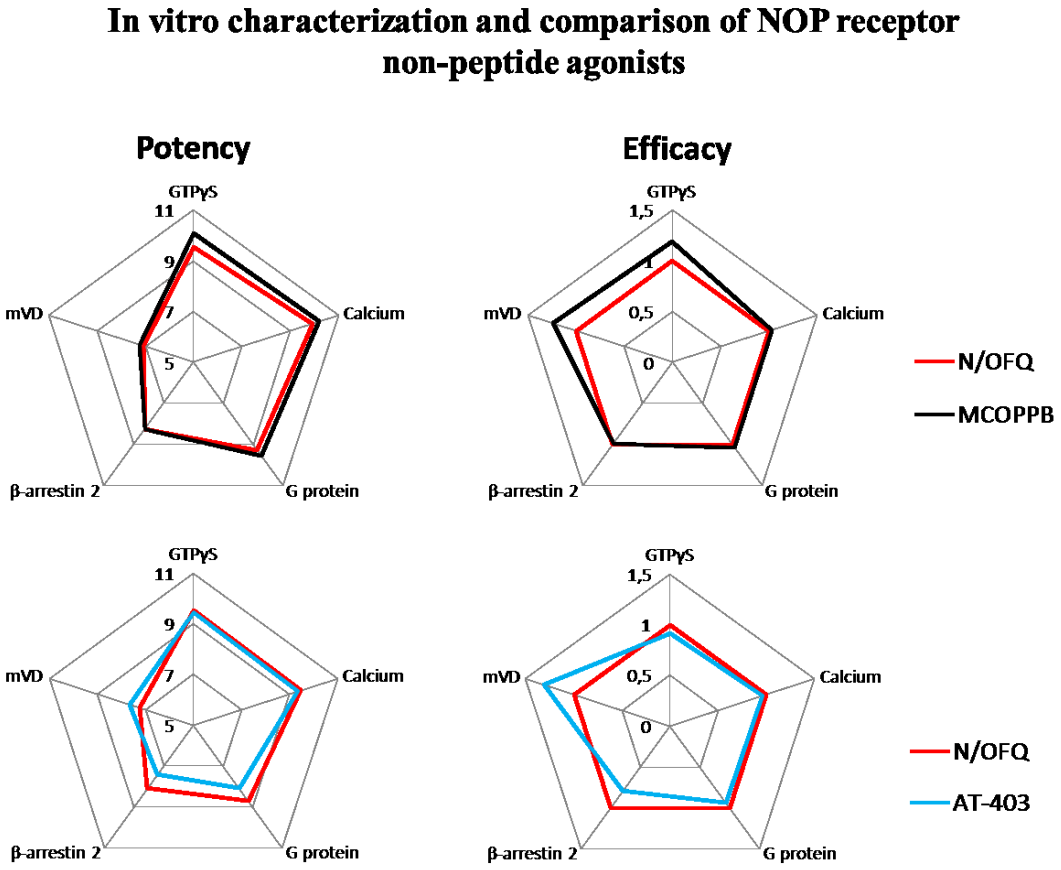
significant effects when injected 1 and 2 h before the assay, but not when the injection was performed 3 h before the assay. These results demonstrated that there are no major difference in terms of duration of action between UFP-101 and its tetrabranced derivative. This result contrasts with previous findings demonstrating long lasting actions of PWT derivatives (Rizzi *et al.*, 2014; Ruzza *et al.*, 2014; Ruzza *et al.*, 2015; Cerlesi *et al*., 2016) which has been interpreted as due to lower susceptibility to peptidases (Bracci *et al.*, 2003). Eventually the presence of an unnatural amino acid residue at the N terminus of UFP-101 may already confer to this peptide some resistance to peptidases that is not further increased by the PWT chemical modification. However is should be also noted that [Dmt^1]N/OFQ(1-13)-NH_2 contains an unnatural residue in position 1 but nevertheless its PWT derivative displayed longer lasting antinociceptive action when injected spinally in non human primates (Molinari *et al.*, 2013; Cerlesi *et al*., 2016).

False positive results may be obtained in behavioral assays with drugs that affect locomotion (Bourin *et al.*, 2001). Thus the possible effects of UFP-101 and PWT2-UFP-101 on mouse spontaneous locomotor activity were evaluated in the open field test. In line with previous findings (Calo *et al.*, 2002; Gavioli *et al.*, 2003), UFP-101 did not modify the animal motor behavior. On the contrary, PWT2-UFP-101 produced a robust inhibition of horizontal and vertical motor activity without affecting immobility time in the open field test. It is unlikely that this effect may bias the interpretation of the results obtained with PWT2-UFP-101 in the forced swimming assay since the compound promoted a reduction of immobility time i.e. promoted swimming behavior; eventually this action of PWT2-UFP-101 may cause un underestimation of its antidepressant like effect in the forced swimming assay. However, the inhibitory effect elicited by PWT2-UFP-101 on locomotion may clearly limits the usefulness of this compound as pharmacological tool for in vivo investigations.

In conclusions, the present study evaluated the in vitro and in vivo actions of PWT2-UFP-101, a tetrabranced derivative of the peptide NOP antagonist UFP-101. In vitro PWT2-UFP-101 maintains the antagonist activity, competitive behavior, and potency of the linear peptide. In vivo in the mouse forced swimming test PWT2-UFP-101 mimicked the antidepressant like effects of UFP-101 being however 10 fold more potent. Taken together these results suggests that the PWT strategy can be applied to peptide antagonists to increase their in vivo potency. This proposal needs to be experimentally validated by designing, synthesizing and pharmacologically evaluating different PWT derivatives of peptides acting as receptor antagonists.

3.2 NON-PEPTIDE LIGANDS

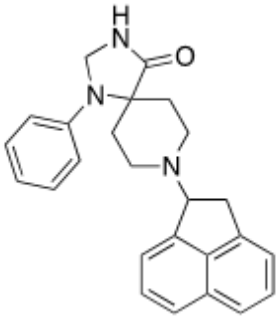
3.2.1 Non-peptide NOP full agonists



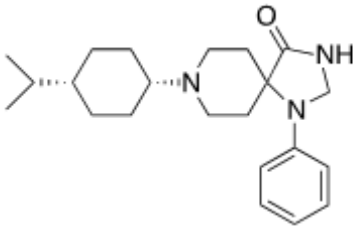
Several NOP receptor ligands have been identified and characterized by academic and industrial laboratories (Mustazza *et al.*, 2011; Zaveri, 2016). Selective NOP antagonists have been investigated as antidepressants (Gavioli *et al.*, 2013; Post *et al.*, 2016) and for Parkinson's disease treatment (Marti *et al.*, 2004b; Marti *et al.*, 2010) whereas selective non-peptide NOP agonists have shown significant efficacy for the treatment of anxiety (Gavioli *et al.*, 2006) and pain (Schroder *et al.*, 2014; Toll *et al.*, 2016).

The aim of the present study was to perform a detailed characterization and comparison of the pharmacological profiles of available non-peptide NOP agonists. The compounds investigated were Ro 65-6570, Ro 2q, SCH-221510, MCOPPB, AT-403, AT-202 and SCH-486757 (see chemical structures in Figure 3.2.1). These compounds were described in the literature as NOP selective agonists and evaluated in vivo for their anxiolytic like effects (Ro 65-6570 (Wichmann *et al.*, 1999), SCH-221510 (Varty *et al.*, 2008), MCOPPB (Hirao *et al.*, 2008)), antitussive (SCH-486757 (Woodcock *et al.*, 2010) or antinociceptive (AT-202 (also named SR16835) (Khroyan *et al.*, 2011)) actions. To the best of our knowledge Ro 2q has not been tested in vivo. AT-403 is recently identified novel compound synthetized by Prof. Nurulain Zaveri at Astraea Therapeutics laboratories.

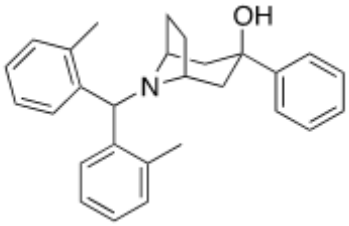
These NOP ligands were investigated and compared in vitro in a panel of biochemical assays of downstream signal transduction as well as in the ex vivo mouse vas deferens assay. The following assays were used: GTPγ[^{35}S] binding assay performed in membranes of cells expressing the human NOP receptor, calcium mobilization assay performed in cells expressing the human NOP or classical opioid receptors and chimeric G proteins, bioluminescence resonance energy transfer (BRET) based assay for studying NOP receptor interaction with G protein and arrestin and the electrically stimulated vas deferens bioassay performed with tissues taken from wild type (NOP(+/+)) and NOP knockout (NOP(-/-)) mice. N/OFQ and the NOP selective antagonist SB-612111 (Zaratin *et al.*, 2004) were used as standard NOP ligands.



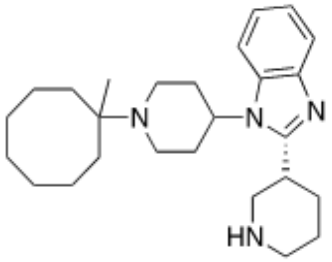
Ro 65-6570



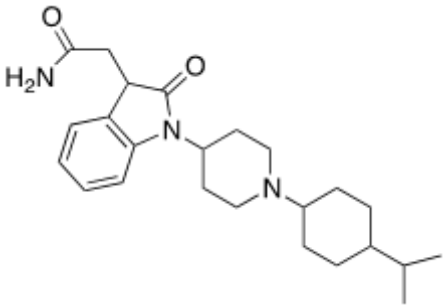
Ro 2q



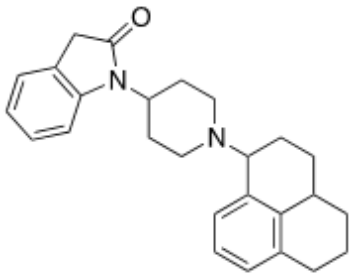
SCH-221510



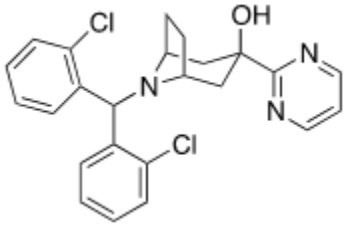
MCOPPB



AT-403



AT-202



SCH-486757

Figure 3.2.1. Chemical structures of the non-peptide NOP agonists evaluated in the present study.

Materials and methods

Drugs and reagents – N/OFQ, Ro 65-6570 and Ro 2q, were synthesized in the Department of Chemical and Pharmaceutical Sciences of the University of Ferrara, Italy. MCOPPB and U-69593 were purchased from Sigma-Aldrich (Schnelldorf, Germany). SCH-221510, SNC-80 and SB-612111 were purchased from Tocris (Bristol, UK). Fentanyl was from SALARS (Como, Italy). AT-403 and AT-202 were synthesized at Astraea Therapeutics (Mountain view, CA, USA). SCH-486757 was synthesized at Chiesi (Parma, Italy). Stock solution of N/OFQ, and fentanyl were made in bidistilled water (1 mM), those of MCOPPB, Ro 65-6570, Ro 2q, SCH-221510, SNC-80, U-69593, SCH-486757 and SB-612111 in DMSO (10 mM), those of AT-403 and AT-202 in 30% HP-cyclodextrin in water (10 mM) and stored at -20 °C.
All cell culture media and supplements were from Invitrogen (Paisley, UK) or EuroClone (Milano, Italy). All other reagents were from Sigma Chemical Co. (Poole, UK) and were of the highest purity available. Native coelenterazine (CLZN, 5 mM, EtOH) was from Synchem UG & Co. KG (Altenburg, Germany).

GTPγ[^{35}S] binding assay – Chinese Hamster Ovary (CHO) cells stably expressing the human NOP receptor were grown DMEM/Hams F12 1:1. The media contained streptomycin (100 μg/ml), fungizone (2.5 μg/ml), penicillin (100 IU/ml) and 10% fetal bovine serum. Stock media containing G418 (200 μg/ml) and hygromycin B (200 μg/ml) was used to maintain CHO_{NOP} expression. Cells were were kept at 37°C in 5% CO_2/humidified air and used for experiments once confluent. Homogenization/wash buffer consisting 50 mM Tris and 0.2 mM EGTA pH 7.4 with NaOH for CHO_{NOP} was used. Membranes were centrifuged at 20374 g 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 buffer and the protein concentration was determined by Lowry assay (Lowry *et al.*, 1951). 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 ligand (N/OFQ) or non-peptide NOP ligands (1 pM – 10μM) were 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. Radioactivity was determined following an 8 h extraction of filters in ScintiSafe Gel using liquid scintillation spectroscopy (Bird *et al.*, 2016).

Calcium mobilization assay – Chinese Hamster Ovary (CHO) cells stably co-expressing the human NOP, or kappa, or mu opioid receptors and the C-terminally modified $G\alpha_{qi5}$ and CHO cells co-expressing the delta opioid receptor and the $G\alpha_{qG66Di5}$ protein were generated as previously described (Camarda *et al.*, 2009; Camarda *et al.*, 2013). Cells were maintained in culture medium consisting of Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/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 testing. 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 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 a washing step with 100 µl / well of Hank's Balanced Salt Solution (HBSS), HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 µM Brilliant Black (Aldrich) was carried out. Subsequently 100 µl/well of the same buffer was added. Serial dilutions of stock solutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% of bovine serum albumin, BSA, Sigma Chemical Co., Poole, UK). After placing both plates (cell culture and compound plate) into the FlexStation II (Molecular Device, Union City, CA 94587, US), fluorescence changes were measured after 10 min of stabilization. On-line additions were carried out in a volume of 50 µl/well. In antagonism type experiments the antagonists were injected in the well 24 min before adding the agonist and, to facilitate drug diffusion into the wells, the experiments were performed at 37 °C and three cycles of mixing (25 µl from each well moved up and down 3 times) were performed immediately after antagonist injection.

BRET assay – Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml) 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 and NOP-RLuc/β-arrestin 2-RGFP) were prepared using the pantropic retroviral expression system by Clontech as described previously (Malfacini *et al.*, 2015). 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 (4 °C) 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 10 min 1000 g (4 °C) and the supernatants kept. Two 20 min 24,000 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 *et al.*, 1987). The protein concentration in membrane preparations was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and the spectrophotometer Beckman DU 520 (Brea, CA, USA).

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 NOP/G-protein interaction, membranes (3 μg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of NOP/β-arrestin 2 interaction, cells co-expressing NOP/RLuc and β-arrestin 2/RGFP were plated 24 h before the experiment in poly-D-Lysine treated plates (100,000 cells/well). 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 min before reading luminescence. All the experiments were performed at room temperature.

Electrically stimulated mouse vas deferens – 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.*, 2010). 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 9927, 19/04/2013). The experiments were performed on the mouse vas deferens. The tissues were taken from male CD-1 mice (16 – 18 g, Harlan, Ud, Italy) and from NOP(+/+) and NOP(-/-) mice (16 – 18 g). Details about the generation of mutant mice have been published previously (Nishi *et al.*, 1997; Bertorelli *et al.*, 2002). NOP(+/+) and NOP(-/-) mice have been backcrossed on CD-1 strain in our laboratories. Mice were housed in 425 x 266 x 155 mm cages (Techniplast, Mi, Italy), 8 per cage, all under standard conditions (22°C, 55% humidity, 12 h light/dark cycle, light on at 7:00 am), with food for mice

(4RF, Mucedola, Mi, Italy) and water *ad libitum*. A mouse red house (Tecniplast, Va, Italy) and nesting materials were present in each cage for mice.

The day of the experiment the animals were sacrificed with CO_2 overdose. Bioassay experiments were performed as previously described by Calo *et al.* (1996). The tissues were suspended in 5 ml organ bath containing Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10). The Krebs solution was oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C and at resting tension 0.3 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. The 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, Colorado Springs, 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 to agonists were performed (0.5 log unit steps). Compounds were diluted using Krebs or saline solution. A total number of 62 mice were used for the present in vitro studies.

Data analysis and terminology – The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig *et al.*, 2003). All data are expressed as the mean ± standard error of the mean (S.E.M.) of at least 4 experiments. For potency values 95% confidence limits ($CL_{95\%}$) were indicated. Efficacy was expressed as E_{max}, the agonist maximal effect. GTPγ[^{35}S] data are expressed as stimulation factor that is the ratio between specific agonist stimulated GTPγ[^{35}S] binding and basal specific binding. In calcium mobilization experiments, maximum change in fluorescence, expressed as % over the baseline fluorescence (fluorescence intensity unit, FIU), was used to determine agonist response. BRET data are expressed as ratio between CPS measured for the RGFP and RLuc light emitted using 460(25) and 510(10) filters (PerkinElmer, Waltham, MA, USA), respectively. Maximal agonist effects were expressed as fraction of the N/OFQ maximal effects which was determined in every assay plate. Bioassay data are expressed as % of the control twitch induced by electrical field stimulation. Agonist potencies are given as pEC_{50} i.e. the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1 + 10^{((LogEC_{50} - Log[compound]) * Hillslope)})}$$

EC_{50} is the concentration of agonist producing a 50% maximal response. Curves fitting were performed using PRISM 6.0 (GraphPad Software In., San Diego, USA).

Antagonist potencies were derived from Gaddum Schild equation:

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

assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist (Kenakin, 2004).

Bias factors were calculated by choosing N/OFQ as standard unbiased ligand.

The concentration response curves of each compound were fitted to the Black-Leff operational model described by Nagi and Pineyro (2016):

$$response = \frac{[A^n]\tau^n E_m}{[A]^n\tau^n + ([A] + K_A)^n}$$

where [A] is the agonist concentration, the maximal response of the system is given by E_m, *n* is a fitting parameter for the slope, the affinity of the agonist is represented by the equilibrium dissociation constant of the agonist-receptor complex (K_A), and the efficacy of the agonist is defined by τ. τ and K_A are descriptive parameters of intrinsic efficacy and binding affinity and may be directly obtained by fitting experimental data to the operational equation and can be expressed as "transduction coefficients" $\log(\tau/K_A)$. The relative efficiency of agonists producing activation of any pathways can thus be quantified with a "normalized" transduction coefficient, namely $\Delta\log(\tau/K_A)$. Finally, the bias factors were calculated as difference between $\Delta\log(\tau/K_A)$ values for a given agonist between the pathways (G protein and β-arrestin 2):

$$bias\ factor = \Delta log(\tau/K_A)_{\,G\ protein} - \Delta log(\tau/K_A)_{\beta-arrestin\ 2}$$

bias factors are expressed as the mean ± S.E.M. of at least 5 independent experiments.

Data have been statistically analyzed with Student's t test; P values less than 0.05 were considered statistically significant.

Results

GTPγ[^{35}S] binding assay – In CHO_{NOP} membranes N/OFQ stimulated the binding GTPγ[^{35}S] in a concentration dependent manner with high potency (pEC_{50} 9.54 (9.36-9.72); Figure 3.2.2). The non-peptide NOP agonists mimicked the stimulatory action showing similar maximal effects (α in the range 0.88 – 1.19). The two Roche compounds and SCH-221510 were approximately 10 fold less potent than N/OFQ. On the contrary MCOPPB was 3 fold more potent than N/OFQ (Figure 3.2.2A). AT-403 displayed similar potency as N/OFQ, while AT-202 and SCH-486757 were approximately 50 and 150 fold less potent (Figure 3.2.2B).

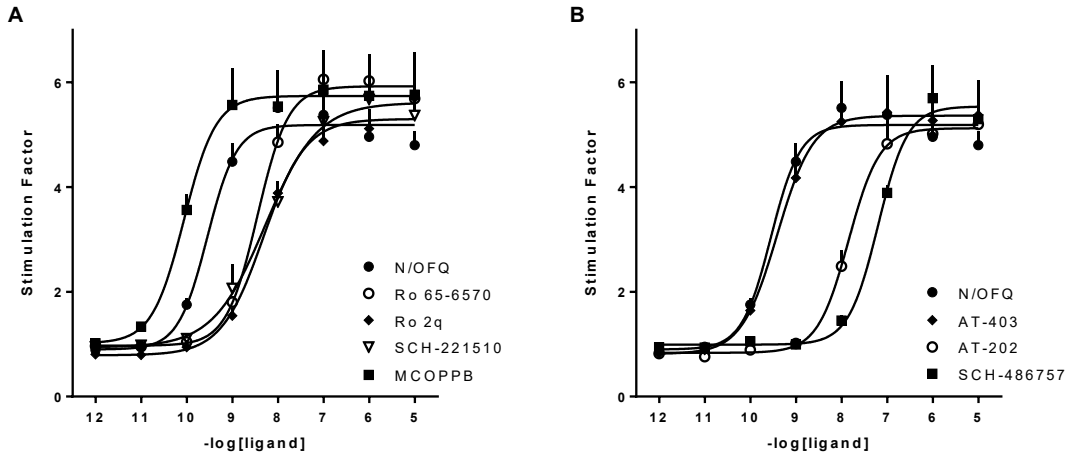


Figure 3.2.2. GTPγ[^{35}S] binding experiments. Concentration response curves to N/OFQ, Ro 65-6570, Ro 2q, SCH-221510 and MCOPPB (panel A), and to N/OFQ, AT-403, AT-202 and SCH-486757 (panel B) in membranes of CHO_{NOP} cells. Data are the mean ± S.E.M. of 6 separate experiments.

Calcium mobilization assay – In CHO_{NOP} cells expressing chimeric G proteins, the natural NOP ligand N/OFQ increased in a concentration dependent manner the intracellular calcium levels with high potency (pEC_{50} 9.92 (9.51-10.32)). MCOPPB was slightly more potent than N/OFQ while Ro 65-6570, Ro 2q, and SCH-221510 were 10 to 30 fold less potent (Figure 3.2.3A). In a separate series of experiments, AT-403 showed similar potency as N/OFQ while AT-202 and SCH-486757 were more than 100 fold less potent (Figure 3.2.3B).

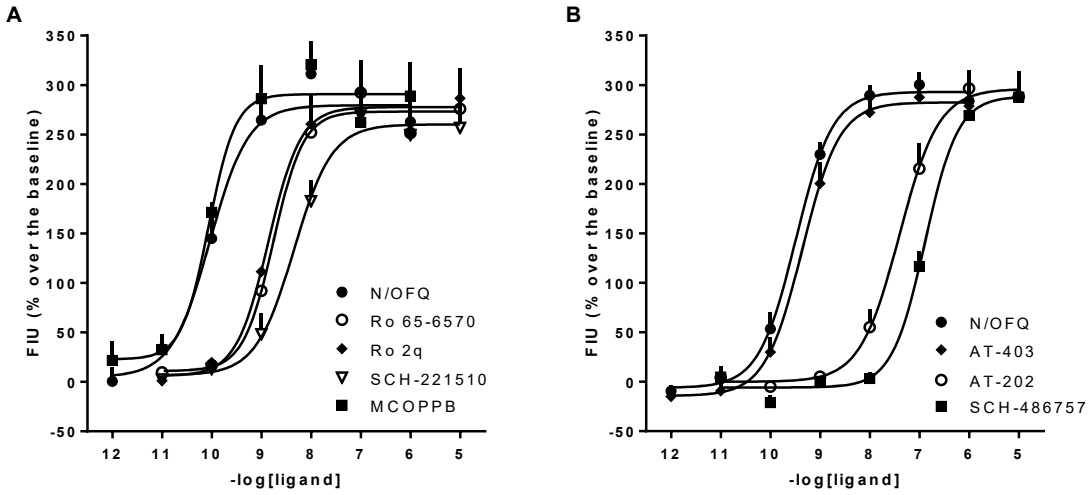


Figure 3.2.3. Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curves to N/OFQ, Ro 65-6570, Ro 2q, SCH-221510 and MCOPPB (panel A) and to N/OFQ, AT-403, AT-202 and SCH-486757 (panel B). Data are expressed as mean ± S.E.M. of at least 4 separate experiments made in duplicate.

The effects of N/OFQ and of the seven NOP agonists were challenged with the selective NOP antagonist SB-612111 in order to investigate the involvement of the NOP receptor in their action. As shown in Figure 3.2.4A, 100 nM SB-612111 was able to rightward shift the concentration

response curve to N/OFQ without modifying the agonist maximal effects. A pA_2 of 8.91 (8.58-9.25) was obtained from these experiments. Similar results were obtained when the antagonist was tested against Roche compounds, SCH-221510 and MCOPPB (pA_2 in the range 8.99 - 9.25; Figure 3.2.4).

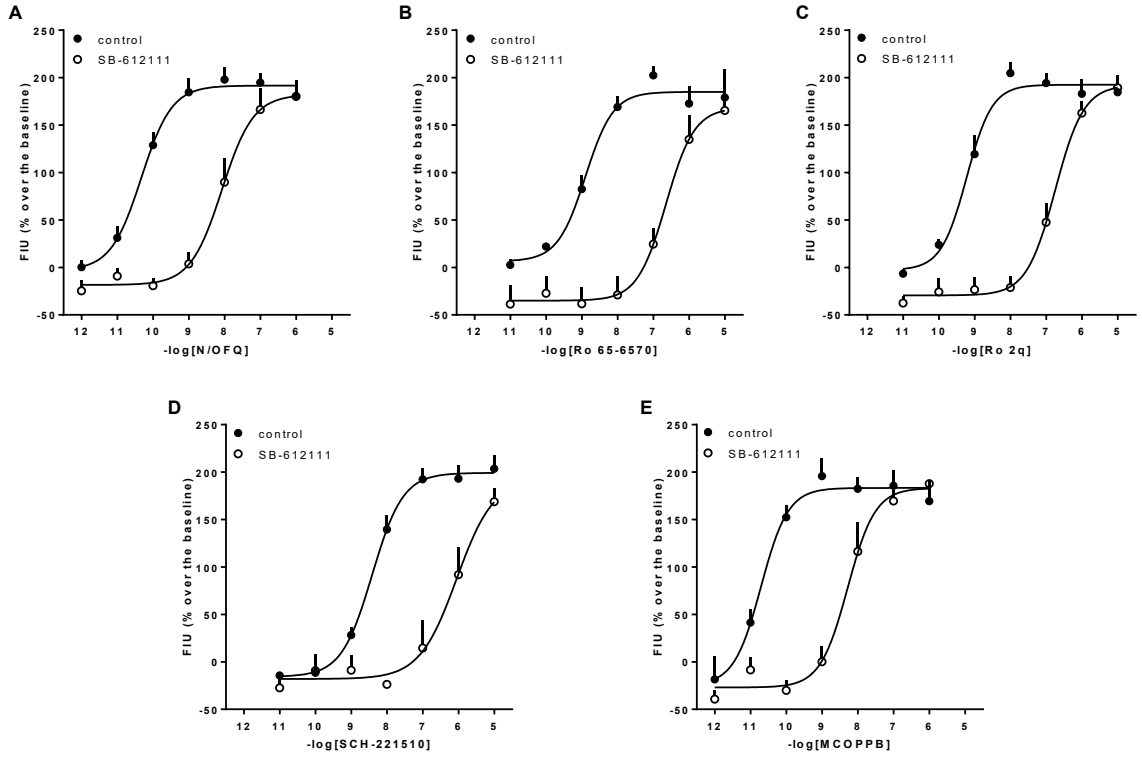


Figure 3.2.4. Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curves to N/OFQ (panel A), Ro 65-6570 (panel B), Ro 2q (panel C), SCH-221510 (panel D) and MCOPPB (panel E) in absence (control) and in presence of SB-612111 100 nM. Data are expressed as mean ± S.E.M. of at least 4 separate experiments made in duplicate.

In a separate series of experiments 100 nM SB-612111 was challenged versus N/OFQ, AT compounds and SCH-486757. Similar values of pA_2 were obtained for SB-612111 against the natural peptide (pA_2 8.45), AT-403 (pA_2 8.73) and AT-202 (pA_2 8.54; Figure 3.2.5). In presence of SB-612111, SCH-486757 was able to elicit a weakly stimulatory response only at the higher concentration tested (Figure 3.2.5D). Therefore, it was not possible to estimate the pA_2 value of SB-612111 against this agonist.

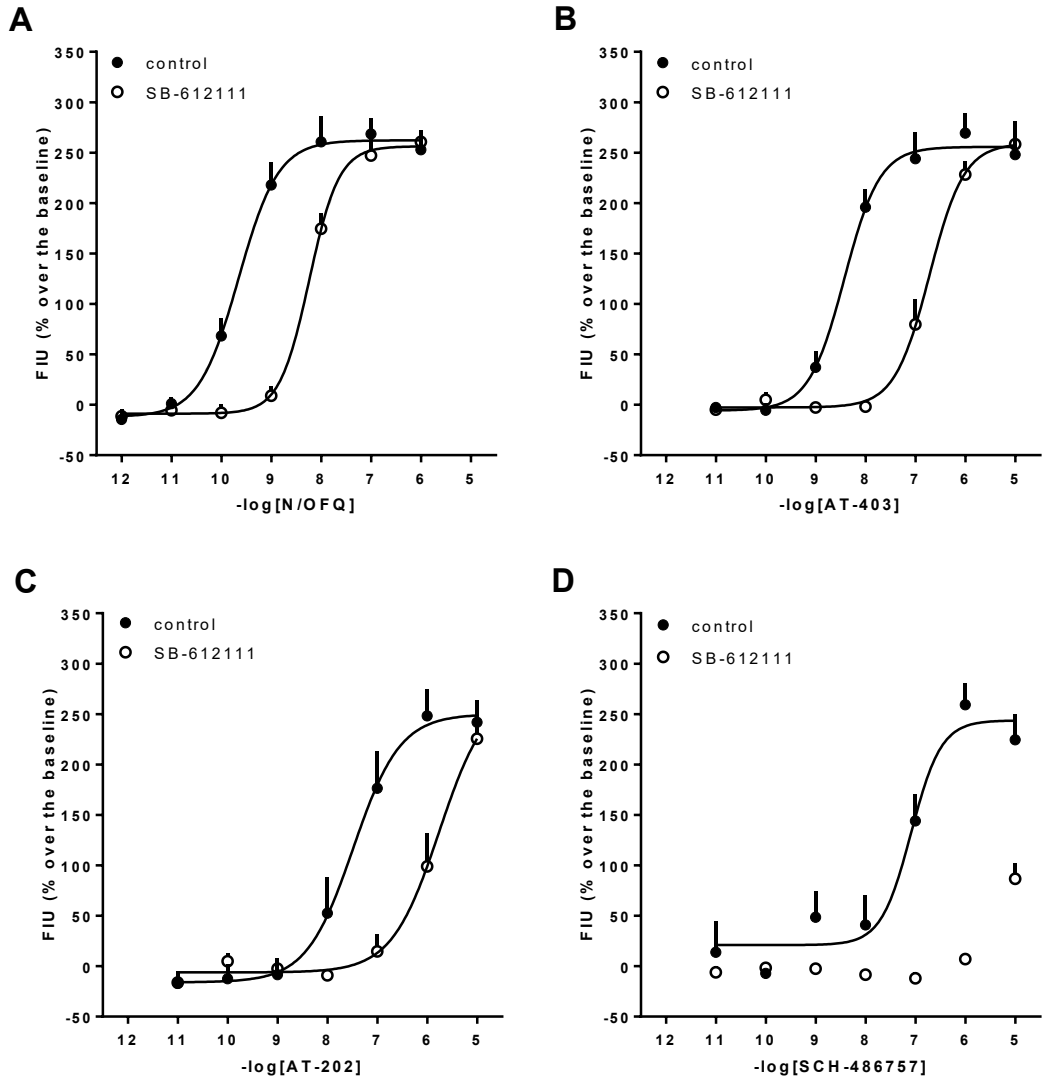


Figure 3.2.5. Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curves to N/OFQ (panel A), AT-403 (panel B), AT-202 (panel C) and SCH-486757 (panel D) in absence (control) and in presence of SB-612111 100 nM. Data are expressed as mean ± S.E.M. of at least 4 separate experiments made in duplicate.

The NOP selectivity of the compounds over classical opioid receptors was assessed using CHO cells coexpressing classical opioid receptors and chimeric G proteins and, as standard opioid receptor agonists, fentanyl, SNC-80, and U-69593 (Figure 3.2.6). In CHO_{mu} cells, fentanyl stimulated in a concentration dependent manner the release of intracellular calcium with high potency (pEC_{50} 8.84 (8.76-8.93)); Ro 65-6570, Ro 2q, SCH-221510, and MCOPPB mimicked the action of fentanyl being 100 – 300 fold less potent (Figure 3.2.6A). Similar results were obtained with AT-403 while AT-202 and SCH-486757 were able to elicit a weakly stimulatory response only at micromolar concentrations (Figure 3.2.6B). N/OFQ, SNC-80 and U-69593 were completely inactive (data not shown).

In CHO_{kappa} cells, the standard agonist U-69593 increased the intracellular calcium concentrations in a concentration dependent manner with high potency (8.51 (8.24-8.78)). SCH-221510 and MCOPPB stimulated calcium mobilization but with low potency and efficacy while the two Roche compounds elicited a weakly stimulatory response only at micromolar concentrations (Figure 3.2.6C). As shown in Figure 3.2.6D AT compounds and SCH-486757 were completely inactive at the kappa receptor, and similar results were obtained with N/OFQ, fentanyl and SNC-80 (data not shown).

On the delta receptor, the standard agonist SNC-80, stimulated the release of intracellular calcium in a concentration dependent manner with moderate potency (7.44 (6.75-8.13)). At this receptor Ro 65-6570, Ro 2q, SCH-221510 and MCOPPB were able to elicit a weakly stimulatory response only at the higher concentration tested (Figure 3.2.6E). Similar results were obtained with AT compounds and SCH-486757 (Figure 3.2.6F). N/OFQ, fentanyl and U-69593 were found inactive on delta receptor (data not shown).

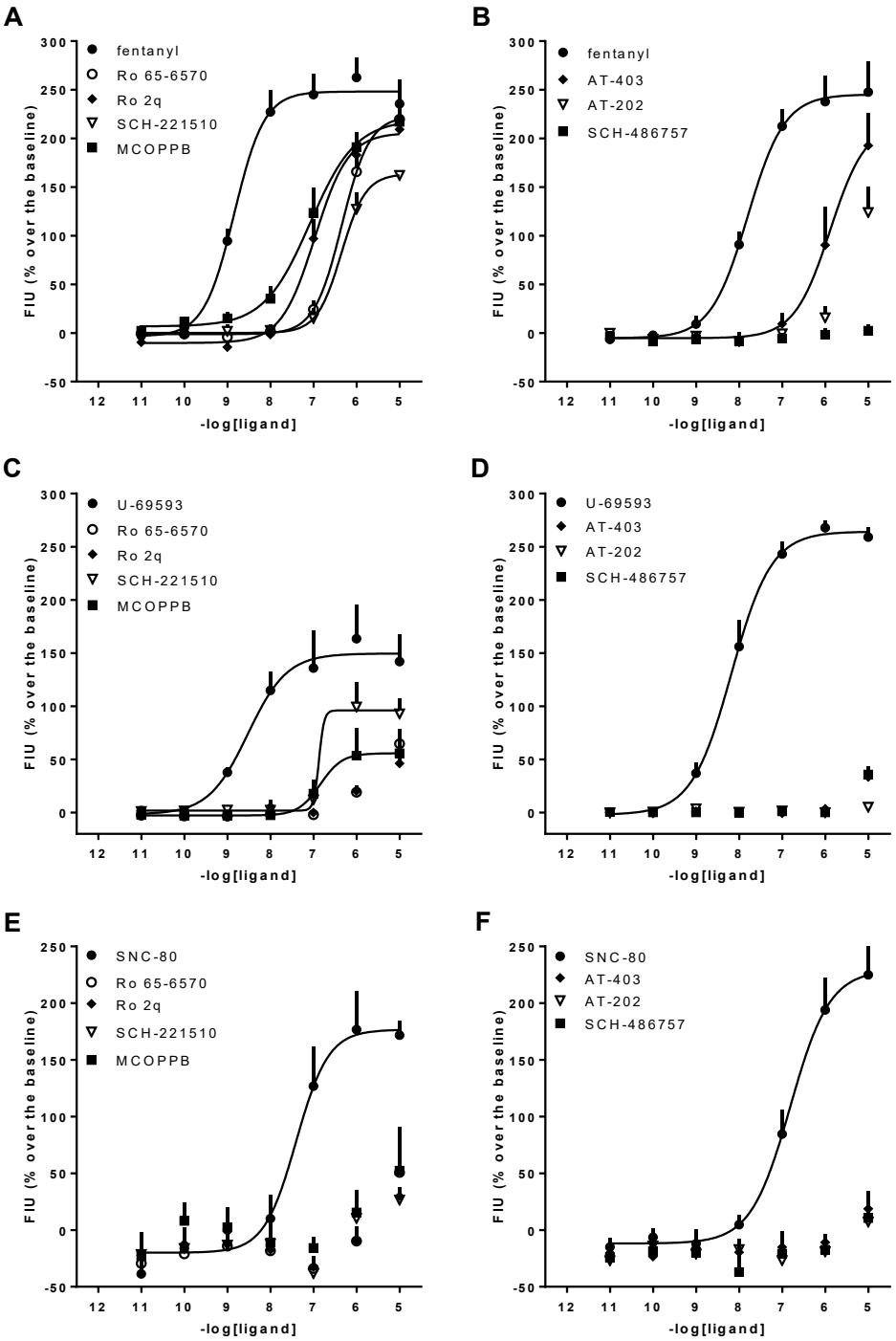


Figure 3.2.6. Calcium mobilization assay performed in CHO cells expressing opioid receptors and chimeric G proteins. Concentration response curves to opioid receptor standard agonists and to NOP receptor agonists in cells expressing the mu (panels A and B), the kappa (panels C and D), and delta (panels E and F) opioid receptors. Data are expressed as mean ± S.E.M. of at least 4 separate experiments made in duplicate.

BRET assay – Membrane extracts taken from HEK293 cells stably coexpressing the NOP/RLuc and the Gβ1/RGFP fusoproteins were used to evaluate NOP/G-protein interaction. In the first series of the experiments, N/OFQ promoted NOP/G-protein interaction in a concentration dependent manner with high potency (pEC_{50} 9.27 (9.12–9.41)) and maximal effect of 0.29 ± 0.01 stimulated BRET

ratio. All compounds showed similar maximal effects as N/OFQ. Ro 65-6570, Ro 2q, and SCH-221510 mimicked N/OFQ action showing 16, 28, and 36 fold lower potency, respectively. On the contrary MCOPPB stimulated NOP/G protein interaction in a concentration dependent manner with a potency 2 fold higher than N/OFQ (Figure 3.2.7A). In the second series of the experiments, N/OFQ promoted NOP/G-protein interaction in a concentration dependent manner with high potency (pEC_{50} 8.71 (8.52–8.90)) and maximal effect of 0.27 ± 0.03 stimulated BRET ratio. AT-403, AT-202, and SCH-486757 mimicked N/OFQ action showing similar maximal effects but 4, 90 and 135 fold lower potency (Figure 3.2.7B).

Whole HEK293 cells stably expressing the NOP/RLuc and the β-arrestin 2/RGFP fusoproteins were used to evaluate NOP/β-arrestin 2 interaction. N/OFQ stimulated the interaction of the NOP receptor with β-arrestin 2 in a concentration dependent manner with high potency (pEC_{50} 8.24 (7.88–8.59)) and maximal effects corresponding to 0.10 ± 0.01 stimulated BRET ratio. MCOPPB promoted NOP/β-arrestin 2 interaction with similar potency and efficacy as N/OFQ. Roche compounds and SCH-221510 displayed reduced potency and efficacy (Figure 3.2.7C). In a separate set of the experiments N/OFQ produced a stimulatory response showing high potency (pEC_{50} 8.10 (7.94–8.25)) and maximal effects corresponding to 0.09 ± 0.01 stimulated BRET ratio. AT-403 and AT-202 mimicked N/OFQ action showing similar maximal effects but lower potency by 5 and 100 fold respectively. SCH-486757 slightly stimulated NOP/β-arrestin 2 interaction only at micromolar concentrations (Figure 3.2.7D).

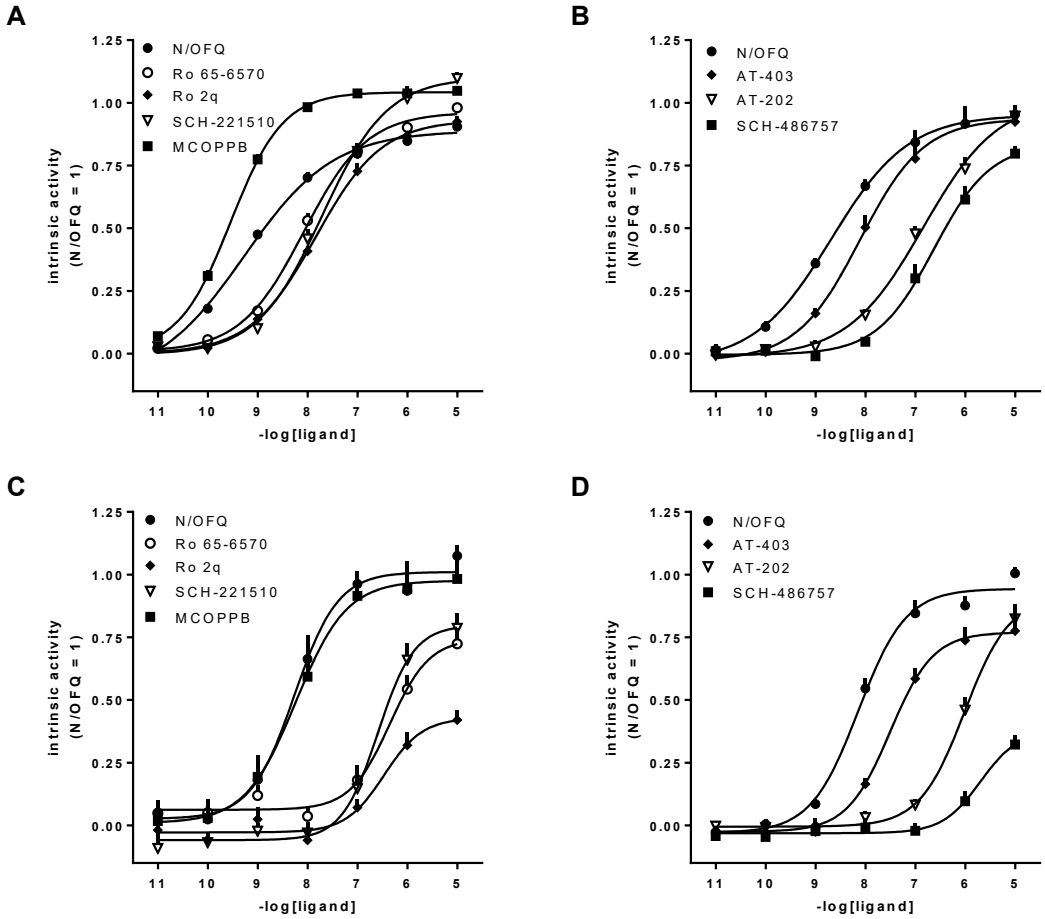


Figure 3.2.7. BRET assay. Concentration response curve to N/OFQ and NOP agonists in promoting NOP/G protein interaction (panels A and B) and NOP/β-arrestin 2 interaction (panels C and D). Data are the mean ± S.E.M. of 5 separate experiments made in duplicate.

Figures 3.2.8 and 3.2.9 display the comparison of the effects of each compound on NOP/G protein and NOP/arrestin interaction. Compared to the natural NOP agonist N/OFQ, all compounds displayed higher potency and/or efficacy at G protein than arrestin with the only exception of AT-403. These results were used for calculating the bias factor of the ligands: all compounds displayed statistically significant bias toward G protein with Ro 65-6570 showing the larger bias (1.64). The only compound showing a bias factor (0.16) not statistically different from 0 was AT-403 (Table 3.2.1).

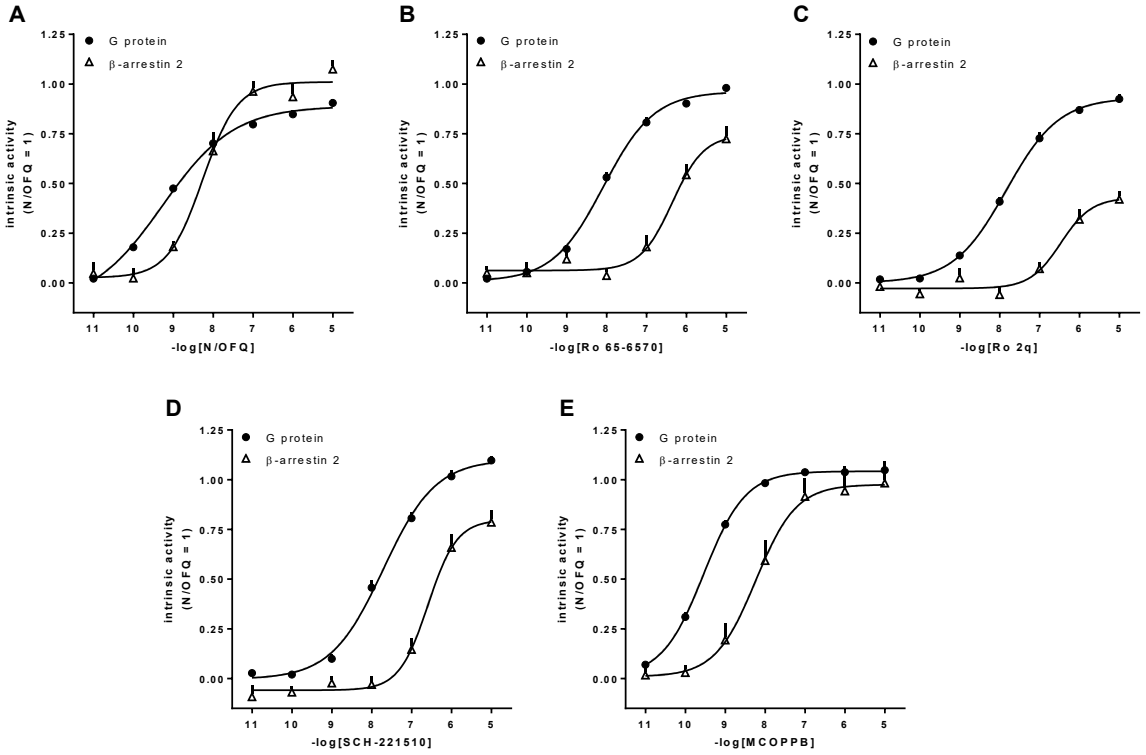


Figure 3.2.8. BRET assay. Comparison of the effect of N/OFQ (panel A), Ro 65-6570 (panel B), Ro 2q (panel C), SCH-221510 (panel D) and MCOPPB (panel E) at G protein and β-arrestin 2. Data are the mean ± S.E.M. of 5 separate experiments made in duplicate.

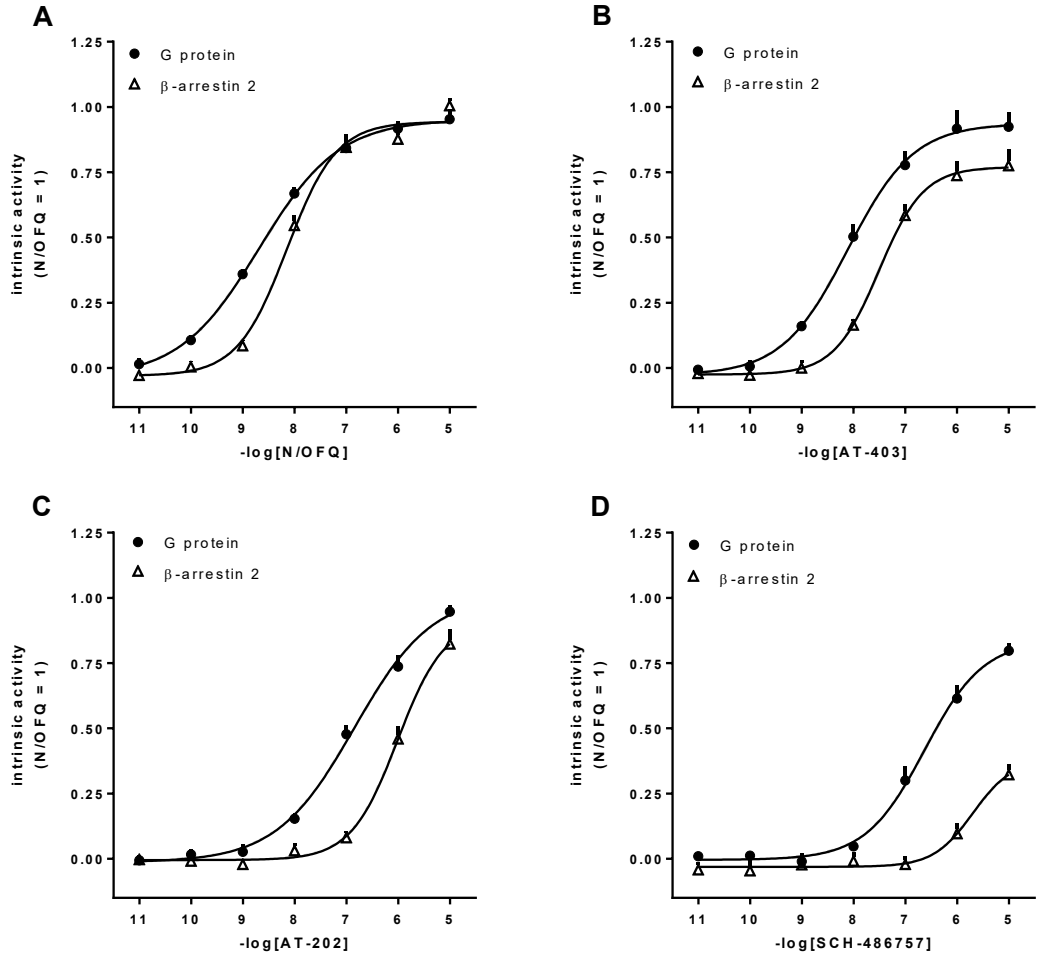


Figure 3.2.9. BRET assay. Comparison of the effect of N/OFQ (panel A), AT-403 (panel B), AT-202 (panel C) and SCH-486757 (panel D) at G protein and β-arrestin 2. Data are the mean ± S.E.M. of 5 separate experiments made in duplicate.

Electrically stimulated mouse vas deferens – N/OFQ inhibited the electrically induced contractions of the tissues in a concentration dependent manner with a potency value of 7.07 (6.98–7.15) and maximal effect of 76 ± 2%. MCOPPB, Roche compounds and SCH-221510 mimicked the inhibitory effect of N/OFQ showing however higher maximal effects. As far as potency is concerned MCOPPB displayed similar potency as N/OFQ while Ro 2q, Ro 65-6570, and SCH-221510 were 2, 4, and 11 fold less potent, respectively (Figure 3.2.10A). In a separated series of the experiments, N/OFQ inhibited the twitch response with a potency value of 7.23 (7.14–7.32) and maximal effect of 78 ± 2%. AT compounds mimicked the N/OFQ action with higher maximal effects; AT-403 was slightly more potent than the peptide while AT-202 was 6 fold less potent (Figure 3.2.10B). In the electrically stimulated mouse vas deferens, SCH-486757 was inactive up to 1 μM displaying inhibitory effects only at higher concentrations. However similar inhibitory effects were elicited by the injection of vehicle (5% DMSO for 10 μM SCH-486757). Thus, its low

solubility associated with low potency did not allow to investigate the action of SCH-486757 in this preparation.

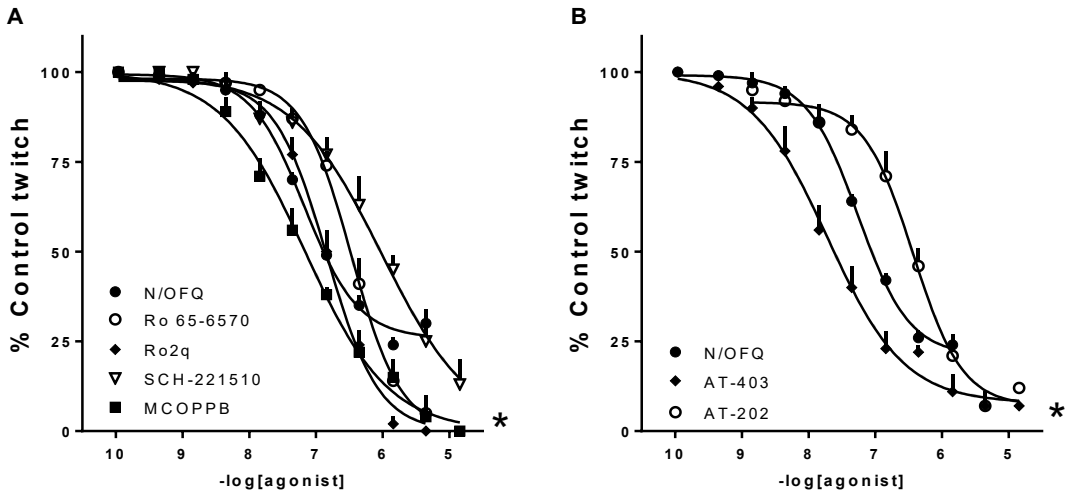


Figure 3.2.10. Mouse vas deferens bioassay. Concentration response curves to N/OFQ and to NOP agonists. Data are the mean ± S.E.M. of at least 5 experiments. *P<0.05 vs N/OFQ (Student t test).

Of note, the kinetic of action of non-peptide NOP agonist in the mouse vas deferens was very different to that of N/OFQ. In fact the peptide elicited a rapid inhibition of the electrically induced twitch and its effect was rapidly and fully reversible after washing. On the contrary, the inhibitory effects of non-peptide NOP agonists were slow to develop and somewhat resistant to washing. As examples, representative tracings of the concentration response curves to N/OFQ and Ro 65-6570 are shown in figure 3.2.11.

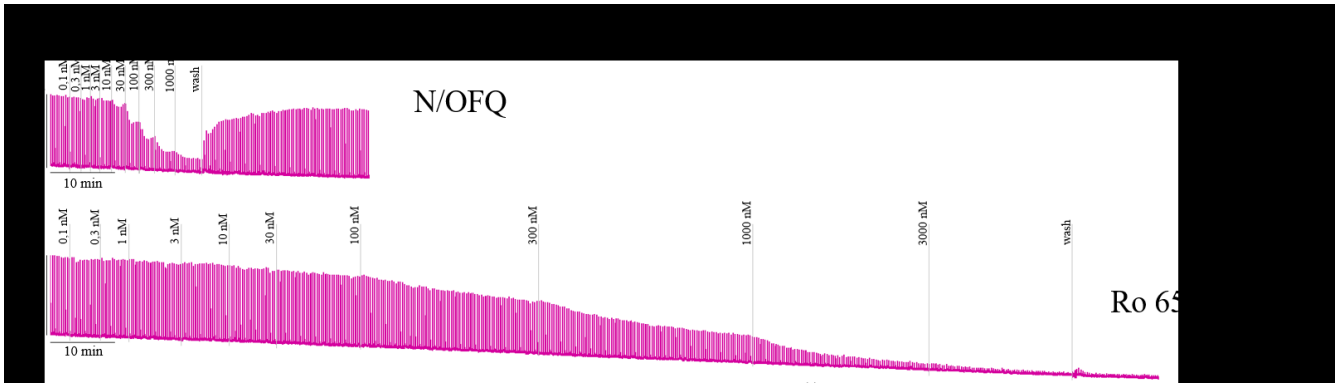


Figure 3.2.11. Representative tracings of the concentration response curve to N/OFQ and Ro 65-6570 in the electrically mouse vas deferens.

To investigate the receptor(s) involved in the action of non-peptide NOP agonists in the electrically stimulated mouse vas deferens knockout studies were performed. The concentration response curves to the delta selective agonist DPDPE were superimposable in tissues taken from NOP(+/+) and NOP(-/-) mice (Figure 3.2.12A). On the contrary, N/OFQ inhibited the electrically induced contractions of tissues taken from NOP(+/+) but not NOP(-/-) mice (Figure 3.2.12B). All non-

peptide agonists were able to elicit their inhibitory effects in both types of tissues being however more potent in NOP(+/+) tissues. This difference in potency ranged from 2 fold for SCH-221510 (Figure 3.2.12E) to 30 fold for AT-403 (Figure 3.2.12H).

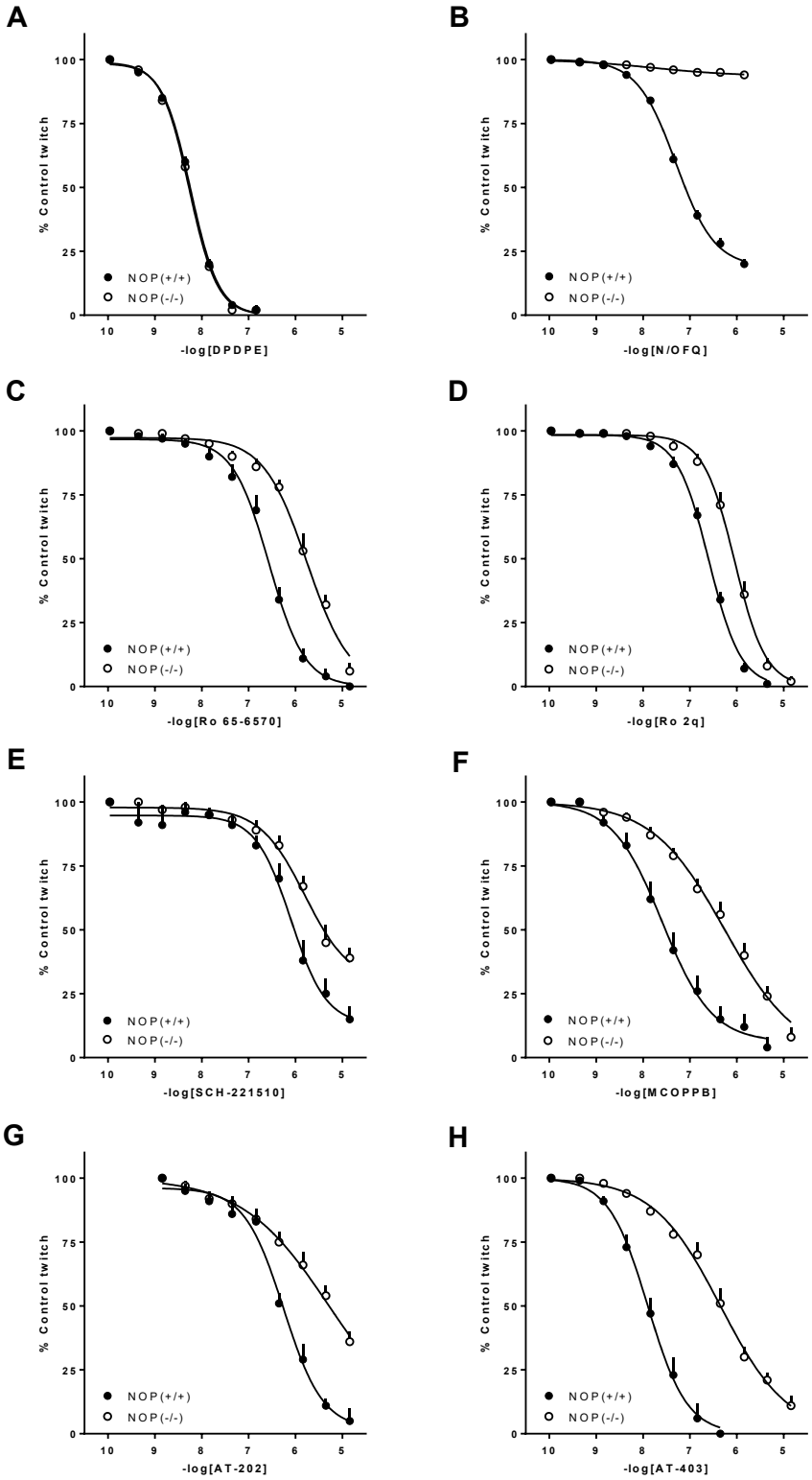


Figure 3.2.12. Mouse vas deferens bioassay. Concentration response curve to DPDPE (panel A), N/OFQ (panel B), Ro 65-6570 (panel C), Ro 2q (panel D), SCH-221510 (panel E), MCOPPB (panel F), AT-202 (panel G) and AT-403 (panel H) in tissues taken from NOP(+/+) and NOP(-/-) mice. Data are the mean ± S.E.M. of at least 5 experiments.

All the results obtained in the present study are schematically summarized in table 3.2.1 where non-peptide molecules are compare to N/OFQ in terms of potency, efficacy, selectivity of action and biased agonism behaviour.

Table 3.2.1. Summary of the pharmacological profile of NOP agonists in comparison with N/OFQ.

	GTPγ[^{35}S]		Calcium mobilization			BRET					mouse Vas Deferens		
						G protein		β-arrestin 2					
	CR	α	CR	α	selectivity (NOP vs OP)	CR	α	CR	α	bias factor	CR	α	selectivity (+/+ vs -/-)
N/OFQ	1	1.00	1	1.00	>10000	1	1.00	1	1.00	0.00	1	1.00	>100
Ro 65-6570	12	1.11	14	0.98	270	16	0.96	65	0.74	1.64	4	> 1	7
Ro 2q	14	0.88	10	0.99	100	28	0.93	63	0.43	0.93	1.70	> 1	3
SCH-221510	13	1.04	36	0.94	60	36	1.10	55	0.84	1.10	11	> 1	2
MCOPPB	0.30	1.19	0.52	1.03	1500	0.53	1.04	1	0.99	0.97	0.71	> 1	14
AT-403	1.20	0.91	1	0.96	1500	4	0.94	5	0.79	0.16	0.40	> 1	30
AT-202	48	0.95	120	1.01	250	90	0.96	105	0.89	0.46	6	> 1	9
SCH-486757	155	1.12	380	0.99	80	135	0.82	178	0.36	0.81		-	

Discussion

In the present study a detailed in vitro pharmacological characterization and comparison of a panel of non-peptide NOP agonists has been presented. The compounds have being previously identified by pharmaceutical companies including Roche (Ro 65-6570 (Wichmann *et al.*, 1999), Ro 2q (Rover *et al.*, 2000)), Schering-Plough (SCH-221510 (Varty *et al.*, 2008), SCH-486757 (McLeod *et al.*, 2010)), Astraea Therapeutics (AT-202 (Toll *et al.*, 2009), AT-403) and Pfizer (MCOPPB (Hirao *et al.*, 2008)). These molecules were assayed and compared at human recombinant receptors in the GTPγ[^{35}S] binding assay, in the calcium mobilization assay performed in cells expressing chimeric G proteins, and in a BRET based assay useful for studying NOP interaction with G proteins and arrestins. Moreover, the non-peptide NOP agonists were tested in the electrically stimulated mouse vas deferens to assess their pharmacological actions at native NOP receptors. Results obtained in this study demonstrated that all compounds behaved as NOP full agonists displaying the same rank order of potency in the different assays. MCOPPB and AT-403 were the most potent and NOP selective compounds; they are valuable pharmacological tools to be used for future in vitro and particularly in vivo studies aimed to investigate the therapeutic potential of NOP agonists.

The first series of experiments investigated the pharmacological profile of the compounds in GTPγ[^{35}S] binding assay performed in membranes of CHO cells expressing the human NOP receptor. All molecules produced maximal effects similar to N/OFQ thus behaving as NOP full agonists confirming data obtained in the original papers (Rover *et al.*, 2000; Varty *et al.*, 2008; Hayashi *et al.*, 2009; Toll *et al.*, 2009; McLeod *et al.*, 2010). This also applies to AT-403 for which Astraea Therapeutics internal results demonstrated NOP full agonist activity in the GTPγ[^{35}S] assay (N. Zaveri personal communication). The following rank order of potency was obtained:

MCOPPB > AT-403 > Ro 65-6570 = Ro 2q = SCH-221510 > AT-202 > SCH-486757.

This is also generally in line with affinity and/or potency values reported in the original papers quoted above although direct comparison of data generated in different laboratories should be avoided. It is however worthy of mention that both in the present results and in the original reports (Hirao *et al.*, 2008; Hayashi *et al.*, 2009; N. Zaveri personal communication), the only compounds showing affinity/potency similar to N/OFQ values were MCOPPB and AT-403.

The same pharmacological behaviour (i.e. full agonism) and a similar rank order of potency was obtained in calcium mobilization studies performed in cells co-expressing the NOP receptor and chimeric G proteins. This further confirms that the pharmacological profile of the NOP receptor is not affected by the aberrant signalling generated by chimeric G protein as demonstrated in previous studies using a large panel of standard NOP ligands (Camarda *et al.*, 2009). Moreover, the stimulatory effects elicited by N/OFQ and all compounds were similarly sensitive to the NOP competitive antagonist SB-612111 (Zaratin *et al.*, 2004; Spagnolo *et al.*, 2007; Marti *et al.*, 2013). In fact, the agonist maximal effects were maintained in the presence of antagonist and, more importantly, similar pA_2 values were calculated for SB-612111 against N/OFQ and all non-peptide agonists. Thus, these results suggest that these non-peptide ligands bind the NOP receptor through the same binding pocket recognized by N/OFQ and SB-612111. Of note, this binding pocked has recently been described at the atomic level by solving the structure of the NOP crystal in complex with SB-612111 (Miller *et al.*, 2015). Further efforts are now needed to solve the structure of the NOP receptor in its active form in complex with an agonist (as recently reported for the mu opioid receptor (Huang *et al.*, 2015)) in order to obtain useful information for structure based rational design of novel NOP agonists.

Together with potency and efficacy, selectivity of action is another crucial pharmacological feature of a given ligand. This aspect has been investigated in calcium assays performed on cells expressing chimeric G proteins and classical opioid receptors. This assay has been previously validated (Camarda *et al.*, 2013) using a panel of opioid receptor ligands. Based on the results of opioid selectivity studies, NOP agonists can be classified into three groups displaying i) moderate (< 100 fold, Schering-Plough compounds and Ro 2q), ii) good (approx. 300 fold, Ro 65-6570 and AT-202), and very good (> 1000 fold, MCOPPB and AT-403) selectivity. Thus, among the compounds evaluated, MCOPPB and AT-403 were both the most potent and the most selective non-peptide NOP agonists. Of note, the NOP selectivity of the best non-peptide molecule is still far from that displayed by the natural peptide N/OFQ.

An aspect of GPCR pharmacology that has been recognized as extremely important in the recent years is functional selectivity or biased agonism (Kenakin, 2015). These terms indicate that some ligands acting on the same receptor are able to stimulate one pathway (i.e. G protein) over another (i.e. arrestin); these compounds are referred to as biased agonists. Thus to investigate functional selectivity at the NOP receptor we recently developed a BRET based assay that allows to measure NOP interaction with G protein and β-arrestin 2. This assay was first set up and validated with a

large series of NOP standard ligands (Malfacini *et al.*, 2015) and then used for investigating the actions of novel compounds (Asth *et al.*, 2016; Bird *et al.*, 2016; Rizzi *et al*., 2016). In the NOP/G protein interaction assay, all compounds promoted NOP/G protein interaction with maximal effects similar to N/OFQ thus behaving as full agonists and displayed a rank order of potency superimposable to that obtained in GTPγ[^{35}S] binding and calcium mobilization studies. These results further corroborate the robustness of this pharmacological assay previously suggested by the high correlation coefficient obtained by comparing the BRET G protein and GTPγ[^{35}S] binding results of a large panel of standard NOP ligands (Malfacini *et al.*, 2015). When evaluated in the NOP/β-arrestin 2 BRET assay, all compounds displayed reduced potency and/or efficacy. Thus all compounds behaved as NOP agonists biased towards G protein. The amount of bias was different among the different ligands with Ro 65-6570 showing the higher value (bias factor 1.64) and AT-202 the lower value (bias factor 0.46). This result is not unexpected in fact a G protein biased agonism behaviour has been previously reported for some non-peptide NOP agonists (Chang *et al.*, 2015b; Malfacini *et al.*, 2015; Rizzi *et al*., 2016). In addition similar findings were reported for mu and delta opioid receptor non peptide agonists (Molinari *et al.*, 2010). The reason(s) of this phenomenon are unknown. It has been suggested that the receptors of the opioid family might be characterized by more restrictive chemical requirements for arrestin than G protein efficacy (Molinari *et al.*, 2010). Alternatively, we proposed that the bias toward G protein of synthetic agonists might derive from the G protein signalling based primary screening assays (e.g. cAMP, GTPγ[^{35}S], calcium mobilization) that have been used to identify and optimize these compounds (Malfacini *et al.*, 2015). Clearly further investigations and particularly effector specific structure activity studies may allow this open question to be answered.

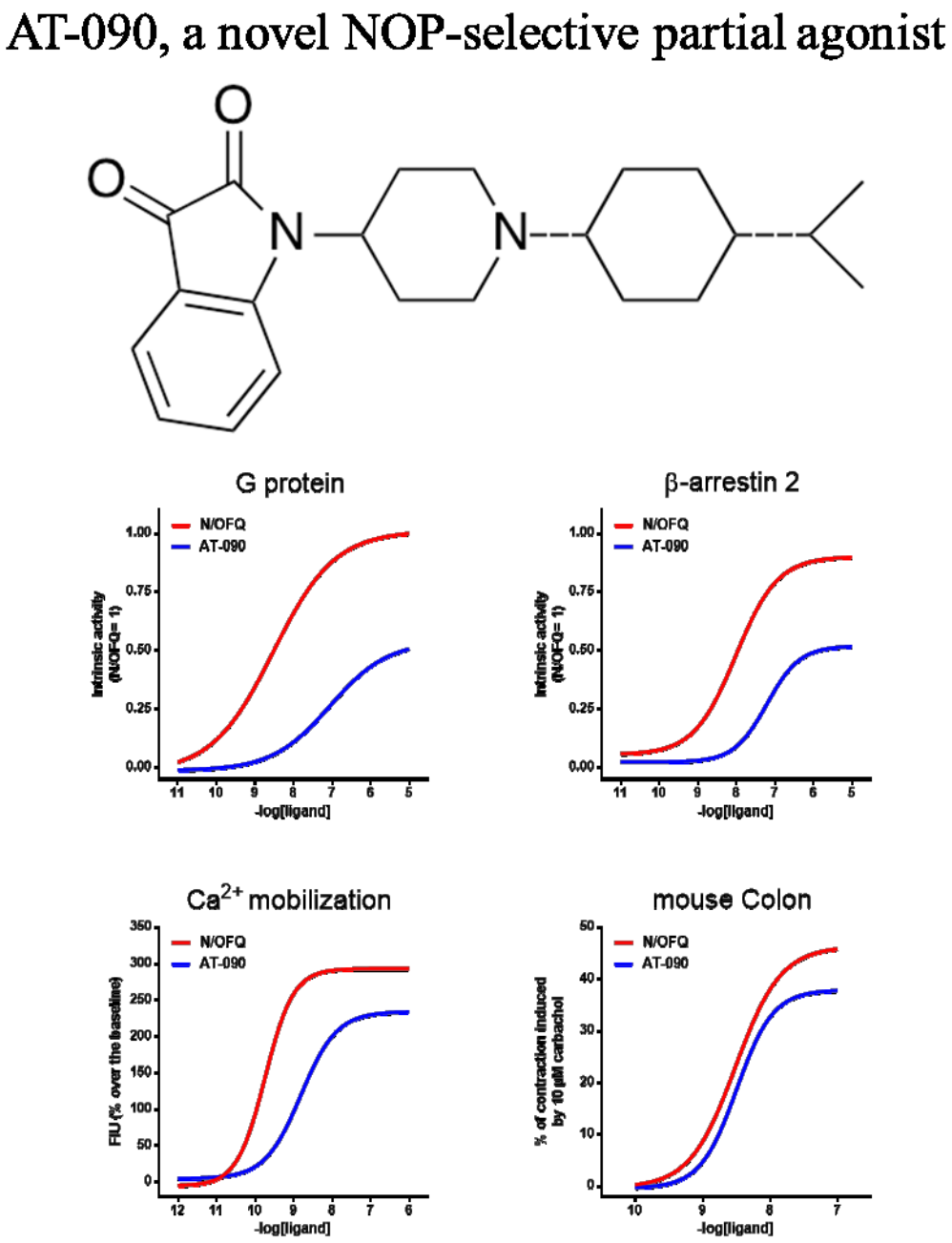
Interestingly, AT-403 seems to represent the exception to this rule. In fact, AT-403 generated similar concentration response curves in promoting NOP/G protein and NOP/β-arrestin 2 interaction in a similar manner as the unbiased endogenous agonist N/OFQ. This is quantified by the AT-403 bias factor (0.16) that is not statistically different from 0. Thus, to the best of our knowledge, AT-403 is the only example of a NOP receptor non-peptide unbiased full agonist. This compound could serve as a hit for future structure activity studies aimed at generating NOP receptor agonists biased toward β-arrestin 2. Such compounds would be of exceptional value for investigating functional selectivity in vivo and assessing the therapeutic potential of biased agonists in the NOP receptor field.

Studies with recombinant receptors expressed in heterologous systems allow a very precise estimate of pharmacological parameters and the evaluation of the drug effect on different biochemical pathways. However, important insights into drug action may come from the analysis of drug effects in "real life" with the use of isolated tissues. With this aim we investigated the action of non-peptide NOP agonist in the electrically stimulated mouse vas deferens, a N/OFQ sensitive pharmacological preparation (Berzetei-Gurske *et al.*, 1996; Calo *et al.*, 1996). In this preparation N/OFQ concentration-dependently inhibited the neurogenic contraction elicited by electrical field stimulation with potency values and maximal effects in line with previous studies (Calo *et al.*, 1996; Spagnolo *et al.*, 2007). The non-peptide agonists mimicked the inhibitory effects of the peptide showing a very different kinetic of action characterized by slow onset and development of their effects and resistance to wash. This kind of kinetic action has been previously reported for other non-peptide NOP agonists including Ro 64-6198 (Rizzi *et al.*, 2001c) and a different series of AT compounds (Ferrari *et al.*, 2016). As far as potency is concerned the rank order of agonist potency obtained in the mouse vas deferens assay was superimposable to that obtained at the human recombinant receptor further corroborating our proposal that the human and murine NOP receptor display a very similar if not identical pharmacological profile (Calo and Guerrini, 2013; Toll *et al.*, 2016). Importantly, the maximal effects elicited by non-peptide agonists were significantly higher than those of N/OFQ thus suggesting off target effects. This was confirmed in knockout studies. Indeed, the action of N/OFQ was completely lost in tissues taken from NOP(-/-) mice, whereas the non-peptide agonists displayed inhibitory effects in tissue taken from both NOP(+/+) and NOP(-/-) animals, although with lower potency in NOP(-/-) tissues. This difference in potency was 14 and 30 fold for MCOPPB and AT-403, respectively and less than 10 fold for the other compounds. The implications of these results are twofold. On one hand, these results suggest that the selectivity for NOP over classical opioid receptors evaluated in recombinant systems is largely overestimated compared to the results obtained in the knockout tissues which should be considered the real acid test for ligand selectivity. On the other hand, these results confirm and extend those obtained at recombinant receptors demonstrating that MCOPPB and particularly AT-403 are considerably more NOP selective than the other non-peptide agonists even if, in absolute terms, their selectivity of action is still far away from that of N/OFQ. Although we consider these findings on NOP selectivity important, they should not be overemphasized. In fact a large body of evidence (recently reviewed in Toll *et al.* (2016) and Zaveri (2016)) demonstrated that the in vivo actions of NOP agonists are sensitive to selective NOP antagonists and/or no longer evident in NOP(-/-) mice. As an example we recently demonstrated that Ro 65-6570, displaying only 4 fold selectivity in the mouse vas deferens produces in vivo in the elevated plus maze test robust anxiolytic like effects. This action of

Ro 65-6570 could be prevented by the injection of SB-612111 and could be seen in NOP(+/+) but not NOP(-/-) mice (Asth *et al.*, 2016).

Collectively the present study demonstrated that the ligands evaluated behaved as NOP full agonists with the following rank order of potency: MCOPPB > AT-403 > Ro 65-6570 = Ro 2q > SCH-221510 > AT-202 > SCH-486757 both at recombinant and native NOP receptors. MCOPPB and AT-403 were not only the most potent agonists but also the most NOP selective as suggested by both recombinant opioid receptors and NOP knockout tissue studies. Moreover, the pharmacological activity of MCOPPB and AT-403 is not superimposable since BRET studies demonstrate that the former compound behaves, similar to the other non-peptide agonists, as a biased agonist toward G protein while the latter, similar to N/OFQ, as an unbiased agonist. The present detailed analysis and comparison of non-peptide NOP agonists is of value in selecting the best pharmacological tools for investigating in vivo biological functions controlled by the N/OFQ – NOP receptor system and ultimately for assessing the therapeutic potential of NOP agonists.

3.2.2 AT partial agonists



Several NOP receptor ligands have been identified and characterized by academic and industrial laboratories (Mustazza *et al.*, 2011; Zaveri, 2016). Selective NOP antagonists have been investigated as antidepressants (Gavioli *et al.*, 2013; Post *et al.*, 2016) and for Parkinson's disease treatment (Marti *et al.*, 2013; Marti *et al.*, 2004b; Marti *et al.*, 2010) whereas selective non-peptide NOP agonists have shown significant efficacy for anxiety (Gavioli *et al.*, 2006; Shoblock, 2007; Witkin *et al.*, 2014) and pain (Schroder *et al.*, 2014; Toll *et al.*, 2016). Recently, NOP receptor partial agonists have also been developed and investigated in models of anxiety and depression (Asth *et al.*, 2016; Ross *et al.*, 2015).

With our active work in the design and synthesis of novel NOP ligands, we have discovered several peptide (Calo and Guerrini, 2013) and non-peptide (Zaveri et al., 2013) NOP ligands as useful pharmacological tools. The novel non-peptide NOP ligands AT-001, AT-004, AT-035, AT-090, and AT-127 were recently identified and their initial characterization in the GTPγS binding assay using the human NOP receptor transfected Chinese hamster ovary (CHO) cells showed that these compounds had partial agonist activity with efficacies ranging from 0.21 to 0.61 (N/OFQ 1.00). Since the maximal effects elicited by partial agonists strongly depend on the efficiency of the stimulus response coupling of the different preparations, the aim of the present study was to characterize the in vitro functional efficacy of these compounds in detail in several other in vitro biochemical assays of downstream signal transduction as well as in ex vivo assays with N/OFQ sensitive rodent tissues. The following in vitro assays were used: receptor binding and [^{35}S]GTPγS binding assay, a calcium mobilization assay performed in cells expressing the human NOP or classical opioid receptors and chimeric G proteins, a bioluminescence resonance energy transfer (BRET) based assay for studying NOP receptor interaction with G protein and arrestin, the electrically stimulated mouse vas deferens and the mouse colon bioassays. The effect of AT compounds were compared to those of standard NOP ligands such as the agonists N/OFQ and Ro 65-6570 and the antagonist SB-612111.

Materials and methods

Drugs and reagents – N/OFQ and Ro 65-6570 were synthesized and purified in the Prof. Guerrini's laboratories (Department of Chemical and Pharmaceutical Sciences, University of Ferrara). SB-612111 was purchased from Tocris (Bristol, UK). AT-001 [(1-(1-((1s,4s)-4-isopropylcyclohexyl)piperidin-4-yl)indoline], AT-004 [1-(1-((1s,4s)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indole], AT-035 [(1-(1-((1s,4s)-4-

isopropylcyclohexyl)piperidin-4-yl)-1H-indol-3-yl)methanamine], AT-090 [1-(1-((1s,4s)-4-isopropylcyclohexyl)piperidin-4-yl)indoline-2,3-dione], and AT-127 [(Z)-3-(hydroxyimino)-1-(1-((1s,4s)-4-isopropylcyclohexyl)piperidin-4-yl)indolin-2-one] were synthesized at Astraea Therapeutics, Mountain view, CA, USA. Chemical structures of AT compounds are showed in Figure 3.2.13. Stock solution of N/OFQ was made in bidistilled water (1 mM), that of Ro 65-6570 and SB-612111 in DMSO (10 mM) whereas the AT compounds were solubilized in 10% Tween 20 in DMSO (10 mM) and stored at -20 °C.

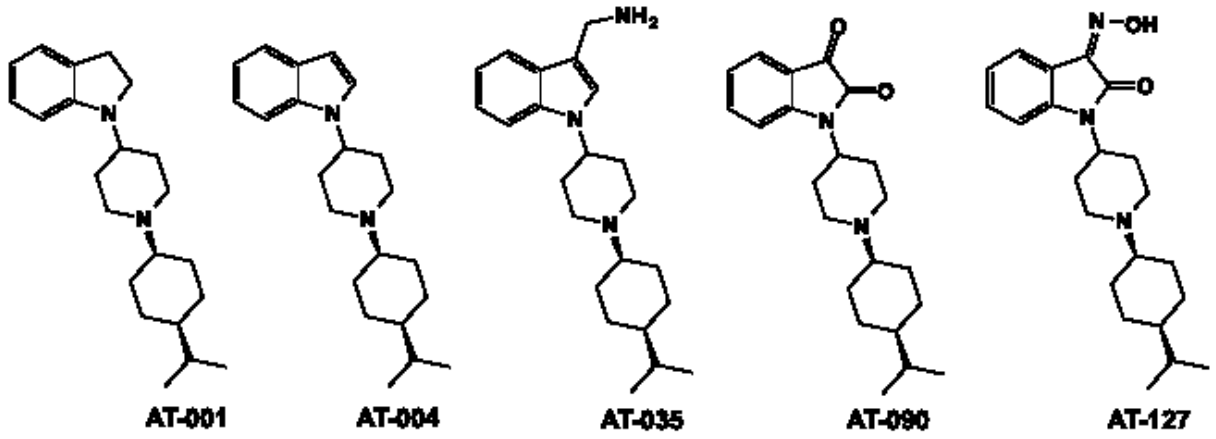


Figure 3.2.13. Chemical structures of AT-001, AT-004, AT-035, AT-090 and AT-127.

Receptor Binding – The human NOP, mu, delta and kappa opioid receptors were stably expressed in CHO cells. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of G418 (0.4 mg/ml) and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. Binding to cell membranes was conducted in a 96-well format, as described previously (Adapa *et al.*, 1997). Cells were removed from the plates by scraping with a rubber policeman, homogenized in Tris buffer using a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27,000 g for 15 min. The pellet was resuspended in 50 mM Tris, pH 7.5, and the suspension incubated with [^{3}H]N/OFQ, [^{3}H]DAMGO, [^{3}H]DPDPE, or [^{3}H]U69593, for binding to NOP, mu, delta, or kappa opioid receptors, respectively. The total volume of incubation was 1.0 ml and samples were incubated for 60-120 min at 25˚C. The amount of protein in the binding reaction varied from approximately 15 to 30 µg. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT) with glass fiber filters. Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute. IC_{50} values were determined using at least six concentrations of each peptide analog, and calculated using Graphpad/Prism (ISI, San Diego, CA). K_i values were determined by the method of Cheng *et al.* (1973).

[^{35}S]GTPγS binding assay – CHO cells stably expressing human NOP or mu opioid receptors were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of G418 (0.4 mg/ml) and 0.1% penicillin/streptomycin, in 100-mm plastic culture dishes. [^{35}S]GTPγS binding was conducted basically as described by Traynor *et al.* (1995). Cells were scraped from tissue culture dishes into 20 mM HEPES, 1 mM EDTA, then centrifuged at 500 g for 10 min. Cells were resuspended in this buffer and homogenized using a Polytron homogenizer. The homogenate was centrifuged at 27,000 g for 15 min and the pellet resuspended in Buffer A, containing 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension was recentrifuged at 27,000 g and suspended once more in Buffer A. The pellet was sometimes frozen at -70˚C prior to use. For the binding assay, membranes (8-15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM), and the appropriate compound, in a total volume of 1.0 ml for 60 min at 25˚C. Samples were filtered over glass fiber filters using a Tomtec 96 harvester (Orange, CT). Bound radioactivity was counted on a Pharmacia Biotech beta-plate liquid scintillation counter (Piscataway, NJ) and expressed in counts per minute.

Calcium mobilization assay – CHO cells stably co-expressing the human NOP, kappa, or mu opioid receptors and the C-terminally modified $G\alpha_{qi5}$ and CHO cells expressing the delta opioid receptor and the $G\alpha_{qG66Di5}$ protein were generated as previously described (Camarda *et al.*, 2013; Camarda *et al.*, 2009). Cells were maintained in culture medium consisting of Dulbecco's MEM/HAM'S F-12 (50/50) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutammine (2 mM), fungizone (1 μg/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 testing. 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 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 (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid and 500 μM Brilliant Black (Aldrich) was added. Serial dilutions of stock solutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% of bovine serum albumin, BSA, Sigma Chemical Co., Poole, UK). After placing both plates (cell culture and compound plate) 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. In antagonism experiments the antagonists were injected in the well 24 min before adding the agonist. To facilitate drug

diffusion into the wells in antagonist type experiments, studies were performed at 37 °C and three cycles of mixing (25 µl from each well moved up and down 3 times) were performed immediately after antagonist injection.

BRET assay – Human Embryonic Kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml) 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 and NOP-RLuc/β-arrestin 2-RGFP) were prepared using the pantropic retroviral expression system by Clontech as described previously (Malfacini *et al.*, 2015). 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) and then, after 5 min, subjected to 500 g (4 °C) centrifugation and 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 subsequent centrifugations were performed at 1000 g (4 °C) and the supernatants retained. 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 *et al.*, 1987). The protein concentration in membrane preparations was determined using the QPRO-BCA kit (Cyanagen Srl, Bologna, IT) and the spectrophotometer Beckman DU 520 (Brea, CA, USA).

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 NOP/G-protein interaction, membranes (3 µg of protein) prepared from cells co-expressing NOP/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of NOP/β-arrestin 2 interaction, cells co-expressing NOP/RLuc and β-arrestin 2/RGFP were plated 24 h before the experiment in poly-D-Lysine treated plates (100,000 cells / well). 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 min before reading luminescence. All the experiments were performed at room temperature.

Bioassays – All experiments involving animals were performed according to the European Communities Council directives (2010/63/EU) and Italian regulations (D.L.26/2014). Protocols were approved by Ethic Committees for Animal Use of the University of Ferrara and by the Italian Ministry of Health (Protocol No. 10086). These studies have been reported according to ARRIVE guidelines (Kilkenny *et al.*, 2010). Male CD-1 mice (12 - 16 weeks old, 30 – 35 g, Harlan, Udine, Italy) were used together with NOP(+/+) and NOP(-/-) mice (8 – 12 weeks old). Details about the generation of mutant mice have been published previously (Bertorelli *et al.*, 2002). NOP(+/+) and NOP(-/-) mice have been recently backcrossed on CD-1 strain in our laboratories. Mice were housed in 425 × 266 × 155 mm cages (Techniplast, VA, Italy), 5 mice/cage, under standard conditions (22°C, 55% humidity, 12 h light–dark cycle, lights on 7.00 am) with food (4RF, Mucedola, MI, Italy) and water *ad libitum*. A mouse red house (Techniplast, VA, Italy) and nesting materials were present in each cage. Each animal was killed with CO_2 before tissues extraction. A total number of 94 mice was used for the present study.

The mouse vas deferens tissues were prepared as previously described (Calo *et al.*, 1996). Tissues were suspended in 5 ml organ baths containing heated Krebs solution (composition in mM: NaCl 118.5, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10 and $CaCl_2$ 2.5) oxygenated with 95% O_2 and 5% CO_2. The bath temperature was set at 33 °C. Tissues were continuously stimulated through two platinum ring electrodes with supramaximal rectangular pulses of 1 ms duration and 0.05 Hz frequency. A resting tension of 0.3 g was applied to the tissues. The electrically evoked contractions (twitches) were measured isotonically with a strain gauge transducer (Basile 7006, UgoBasile s.r.l., Varese, Italy) and recorded with the PC based acquisition system Power Lab (ADInstrument, USA). Following an equilibration period of 60 min, the contractions induced by electrical field stimulation were stable. At this time, cumulative concentration-response curves to N/OFQ, Ro 65-6570, and AT compounds were performed (0.5 log unit steps). All the compounds inactive as agonist were tested as antagonists. In antagonism experiments the concentration response curve to N/OFQ has been performed in absence and in presence of a fixed concentration of antagonist, incubated for 15 min.
Segments of mouse colon (approximately 1 cm length) were prepared as previously described (Rizzi *et al.*, 1999) to record isometric smooth muscle contractions. Briefly, the preparations were mounted longitudinally under 1 g tension in an organ bath (5 ml) containing Krebs at 37ºC and continuously gassed with 5% CO_2 and 95% O_2. Tissues were equilibrated for 60 min with washing every 10 min. For recording the maximal contractile response of the tissues 10 μM carbachol was used. The concentration-response curves to N/OFQ and AT-compounds were determined non-cumulatively by adding different concentrations of compound to the bath every 20 min followed by

washing. Since its effects were resistant to washing, single concentrations of Ro 65-6570 were tested in each tissue.

Data analysis and terminology – The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig *et al.*, 2003). All data are expressed as means ± S.E.M of *n* experiments. For potency values 95% confidence limits ($CL_{95\%}$) are reported. In calcium mobilization experiments, maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response. For BRET experiments receptor-transducer interactions were 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 were expressed as stimulated BRET ratio obtained by subtracting the vehicle value to that measured in the presence of ligand. Agonist intrinsic activity was expressed as fraction of the N/OFQ E_{max}; the maximal effect of N/OFQ has been assayed in all the plates. For the electrically stimulated mouse vas deferens data the effect of agonists was expressed as % of inhibition of the control twitch, while for mouse colon data as percent of the contraction elicited by 10 μM carbachol. Non-linear regression analysis using GraphPad Prism software (6.0) allowed logistic iterative fitting of the resultant responses and the calculation of agonist potencies and maximal effects. Agonists potencies were given as pEC_{50} (the negative logarithm to base 10 of the molar concentration of an agonist that produces 50 % of the maximal possible effect). Antagonist potencies were expressed as pK_B, calculated using the following equation: $pK_B = \log(CR-1) - \log[A]$, where CR is the ratio between the EC_{50} (nM) values of the agonist in the presence and absence of antagonist and [A] is the concentration (M) of antagonist used. Data have been statistically analyzed with the Student's t test; P values less than 0.05 were considered statistically significant. Bias factors were calculated by choosing the standard NOP agonist N/OFQ, as standard unbiased ligand. The concentration response curves of each compound were fitted to the Black-Leff operational model described in Nagi and Pineyro (2016):

$$response = \frac{[A^n]\tau^n E_m}{[A]^n\tau^n + ([A] + K_A)^n}$$

where [A] is the agonist concentration, the maximal response of the system is given by E_m, *n* is a fitting parameter for the slope, the affinity of the agonist is represented by the equilibrium dissociation constant of the agonist-receptor complex (K_A), and the efficacy of the agonist is defined by τ. τ and K_A are descriptive parameters of intrinsic efficacy and binding affinity and may be directly obtained by fitting experimental data to the operational equation and can be expressed as "transduction coefficients" $\log(\tau/K_A)$. The relative efficiency of agonists producing activation of any pathways can thus be quantified with a "normalized" transduction coefficient, namely

$\Delta\log(\tau/K_A)$. Finally, the bias factors were calculated as difference between $\Delta\log(\tau/K_A)$ values for a given agonist between the pathways (G protein and β-arrestin 2):

$$bias\ factor = \Delta\log(\tau/K_A)_{G\ protein} - \Delta\log(\tau/K_A)_{\beta-arrestin\ 2}$$

bias factors are expressed as the mean ± S.E.M. of at least three independent experiments.

Results

Receptor binding assay – The new AT series of ligands showed high binding affinity for the NOP receptor, with K_is ranging from 3–10 nM at the recombinant human NOP receptor transfected into CHO cells (Table 3.2.2). These NOP ligands showed significantly lower binding affinities for the mu and kappa opioid receptors, showing NOP selectivity ranging from 17 (AT-035) to 61 fold (AT-127). All AT compounds showed poor if any affinity for the delta opioid receptor (data not shown).

[^{35}S]GTPγS binding assay – Agonist potency and efficacy were measured in the GTPγS binding assay using membranes from CHO cells expressing NOP or mu receptors. Maximal effects were normalized to that elicited by the full agonists N/OFQ and DAMGO for NOP and mu receptors, respectively. All the new AT compounds had intrinsic activities ranging from 0.21 to 0.61 of the response to N/OFQ, thus showing partial agonist activity at NOP. AT-001, AT-004, AT-035 and AT-090 showed partial agonist efficacy at the lower end of the spectrum (0.21 – 0.36), whereas AT-127 showed a higher level of efficacy, i.e. 61% of N/OFQ response (Table 1). As far as potency is concerned, the EC_{50} values of AT compounds were 5-40 fold higher than their K_i. The rank order of the potency was AT-127 > AT-090 = AT-001 > AT-035 = AT-004. At the mu receptor all AT compounds displayed lower potency and efficacy with the only exception of AT-001 that produced higher maximal effects at mu than at NOP receptor (Table 3.2.2).

Table 3.2.2. Receptor binding and stimulated [^{35}S]GTPγS binding of AT compounds in membranes of CHO cells stably expressing the human recombinant receptors.

	Receptor Binding K_i (nM)			[^{35}S]GTPγS NOP		[^{35}S]GTPγS mu	
	NOP	mu	kappa	EC_{50} (nM)	α	EC_{50} (nM)	α
AT-001	10.3 ± 0.03	604.35 ± 5.02	562 ± 150	54.5 ± 17.55	0.33 ± 0.03	1781 ± 337	0.48 ± 0.10
AT-004	9.80 ± 0.86	375.5 ± 36.5	1593.6 ± 57	188.2 ± 32.8	0.25 ± 0.09	231.7 ± 10	0.16 ± 0.08
AT-035	3.27 ± 0.3	65.29 ± 2.42	1736.8 ± 172	121.2 ± 51.7	0.36 ± 0.06	410 ± 105	0.11 ± 0.03
AT-090	5.61 ± 1.72	95.35 ± 3.46	232.9 ± 18.1	50.1 ± 6.4	0.21 ± 0.06	FLAT	0.06 ± 0.03
AT-127	1.18 ± 0.2	71.65 ± 27.8	149.2 ± 18.3	15.5 ± 3.1	0.61 ± 0.02	59.2 ± 3.0	0.37 ± 0.02

K_i values for standard NOP (N/OFQ), mu (DAMGO), and kappa (U69593) ligands were 0.12, 2.96, and 1.05, respectively.
EC_{50} values of standard agonists N/OFQ at NOP and DAMGO at mu receptor were 3.6 and 32.6, respectively.

Calcium mobilization assay – In CHO cells coexpressing the NOP receptor and the $G\alpha_{qi5}$ chimeric protein, N/OFQ increased intracellular calcium levels in a concentration dependent manner with high potency and maximal effects. Ro 65-6570 mimicked N/OFQ action showing similar maximal effects but 10-fold lower potency (Figure 3.2.14A). AT compounds stimulated calcium mobilization showing maximal effects slightly lower than that of N/OFQ (α range 0.73 – 0.82) and the following rank order of potency AT-127 = AT-090 > AT-035 > AT-001 > AT-004 (Figure 3.2.14B).

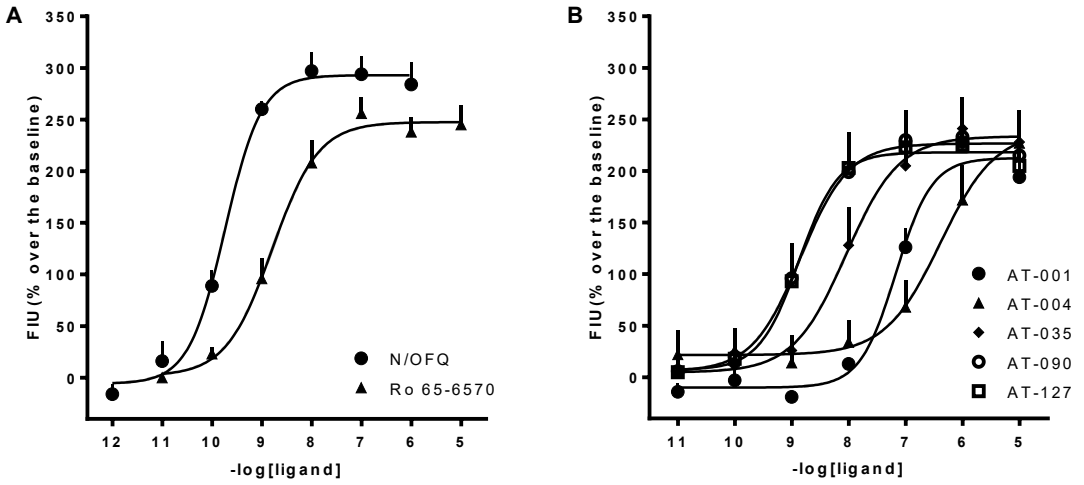


Figure 3.2.14. Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

The effects of standard agonists and the most potent AT compounds was challenged with the NOP antagonist SB-612111 in order to investigate the involvement of the NOP receptor in their action. As shown in Figure 3.2.15A, 100 nM SB-612111 was able to shift the concentration response curve of N/OFQ to the right, without modifying the agonist maximal effect. A similar effect was obtained for agonist response curves for Ro 65-6570, AT-090, and AT-127 in presence of the antagonist (Figure 3.2.15B, 3.2.15C and 3.2.15D). SB-612111 pK_B values calculated from these experiments were in the range 8.49 – 8.72.

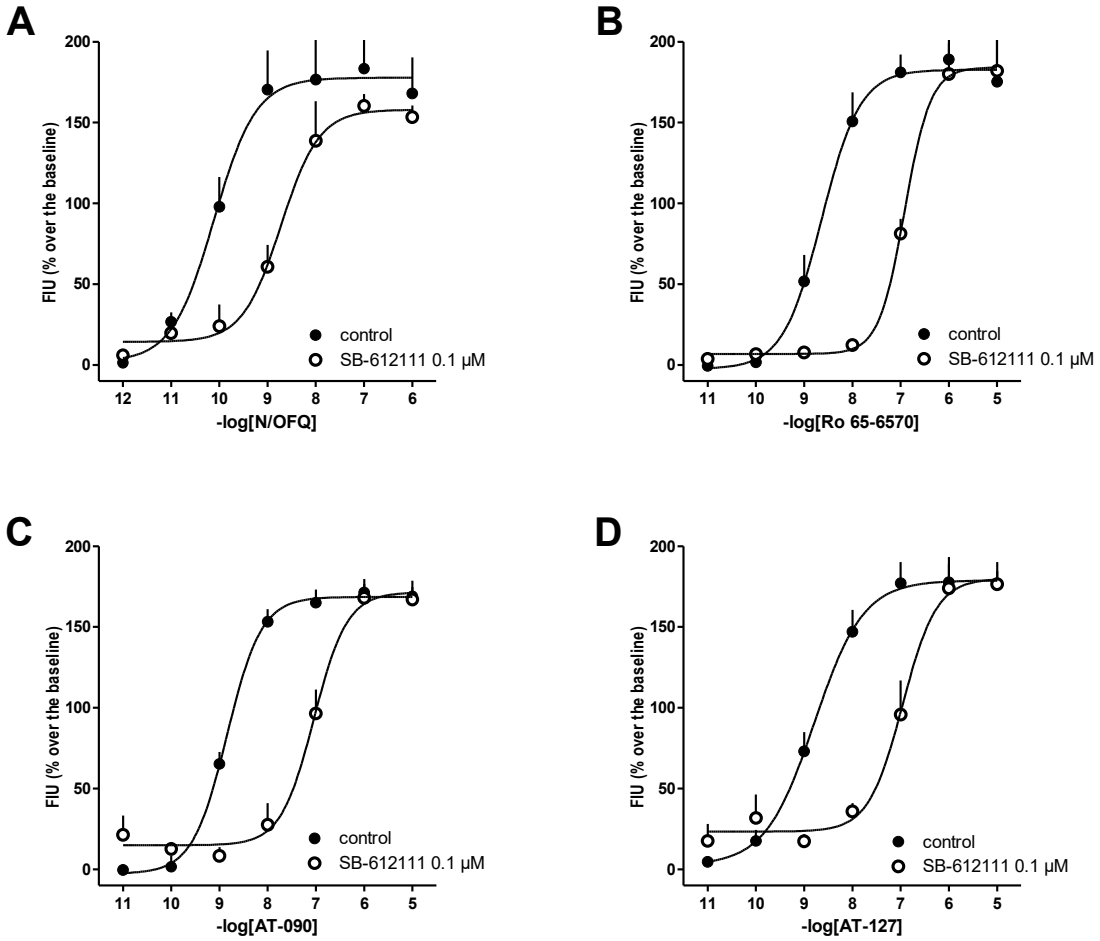


Figure 3.2.15. Calcium mobilization assay performed in CHO cells expressing the NOP receptor and the chimeric $G\alpha_{qi5}$ protein. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in absence and in presence of SB-612111 0.1 μM. The following values of pK_B were calculated for SB-612111: 8.49 (7.80 – 9.18) vs N/OFQ, 8.72 (8.20 – 9.24) vs Ro 65-6570, 8.65 (8.22 – 9.08) vs AT-090, 8.59 (7.53 – 9.65) vs AT-127. Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

The selectivity of the AT compounds over classical opioid receptors was assessed using as standard opioid receptor agonists dermorphin, DPDPE, and dynorphin A. The results of these experiments are summarized in Table 3.2.3. N/OFQ, dermorphin, DPDPE, and dynorphin A behaved as selective agonists for the NOP, mu, delta, and kappa receptor, respectively. Ro 65-6570 displayed relatively high (at least 100 fold) selectivity for the NOP receptor. AT compounds showed moderate (AT-004 and AT-035) to high (AT-090 and AT-127) NOP selectivity with the only exception of AT-001 that displayed similar potency at NOP and kappa receptors.

Table 3.2.3. Calcium mobilization assay performed in CHO cells expressing NOP or classical opioid receptor and chimeric G proteins. Potencies and efficacy of standard agonists and AT compounds.

	NOP		**mu**		**kappa**		**delta**	
	pEC_{50} ($CL_{95\%}$)	α ± S.E.M.	pEC_{50} ($CL_{95\%}$)	α ± S.E.M.	pEC_{50} ($CL_{95\%}$)	α ± S.E.M.	pEC_{50} ($CL_{95\%}$)	α ± S.E.M.
N/OFQ	9.77 (9.52–10.02)	1.00	inactive		inactive		inactive	
Ro 65-6570	8.75 (8.15–9.40)	0.95±0.05	< 6		crc incomplete		inactive	
dermorphin	inactive		8.31 (7.90–8.72)	1.00	inactive		5.98 (5.76–6.10)	0.8 ± 0.07
DPDPE	inactive		inactive		inactive		8.23 (7.72–8.74)	1.00
dynorphin A	inactive		6.49 (5.96–7.02)	0.73±0.11	9.26 (8.69–9.83)	1.00	6.40 (5.87–6.93)	0.75±0.13
AT-001	7.13 (6.79–7.47)	0.73±0.03	inactive		7.12 (6.42–7.84)	0.95 ± 0.01	inactive	
AT-004	6.41 (5.87–6.95)	0.82±0.07	inactive		inactive		inactive	
AT-035	7.96 (7.36–8.56)	0.82±0.08	crc incomplete		inactive		inactive	
AT-090	8.84 (7.99–9.69)	0.80±0.11	crc incomplete		crc incomplete		inactive	
AT-127	8.85 (8.23–9.47)	0.79±0.12	crc incomplete		inactive		crc incomplete	

Inactive: inactive up to 1 μM; crc incomplete: weak stimulatory effect at 1-10 μM. Data are the mean of three separate experiments performed in duplicate.

BRET assay – Membrane extracts taken from HEK293 cells stably coexpressing the NOP/RLuc and the Gβ1/RGFP fusoproteins were used to evaluate NOP/G-protein interaction. N/OFQ promoted NOP/G-protein interaction in a concentration-dependent manner with high potency (pEC_{50} 8.52 (8.46 – 8.58)) and maximal effect of 0.34 ± 0.02 stimulated BRET ratio. The intrinsic activities of the compounds under study were computed as fraction of N/OFQ maximal-stimulated BRET ratio (N/OFQ = 1.00). Ro 65-6570 mimicked N/OFQ action showing similar maximal effects but 4-fold lower potency (pEC_{50} 7.90 (7.42 – 8.39); Figure 3.2.16A). For assessing whether compounds affect luciferase activity, AT compounds were assayed at 1 and 10 μM employing cell membranes obtained from HEK293 expressing NOP/RLuc and Gβ1/RGFP. AT-001, AT-004 and AT-035, at 10 μM but not at 1 μM, inhibited the light emitted by RLuc. On this basis the concentration response curves to these compounds were carried using 1 μM as maximal concentration. AT-090 and AT-127 stimulated NOP/G-protein interaction in a concentration-dependent manner showing lower potency and efficacy compared with N/OFQ. Potency values of 7.19 (7.12 – 7.26) and 6.81 (6.53 – 7.09) with maximal effects of 0.49 ± 0.08 and 0.69 ± 0.09 were calculated for AT-090 and AT-127, respectively. AT-001, AT-004 and AT-035 were only poorly active in promoting NOP/G-protein interaction, generating incomplete concentration response curves (Figure 3.2.16B).

Whole HEK293 cells stably expressing the NOP/RLuc and the β-arrestin 2/RGFP fusoproteins were used to evaluate the NOP/arrestin interactions. N/OFQ stimulated the interaction of the NOP receptor with β-arrestin 2 in a concentration dependent manner with high potency (pEC_{50} 8.00 (7.91 – 8.09)) and maximal effects corresponding to 0.11 ± 0.01 stimulated BRET ratio. The intrinsic activities of the compounds under study were computed as fraction of N/OFQ maximal-stimulated BRET ratio (N/OFQ = 1.00). Ro 65-6570 mimicked N/OFQ action displaying similar maximal effect but 50-fold lower potency (Figure 3.2.16C). AT-090 and AT-127 mimicked the stimulatory effect of N/OFQ showing lower potency and efficacy (pEC_{50} 6.96 (6.11 – 7.81) and maximal effects of 0.57 ± 0.08 for AT-090 and pEC_{50} 6.38 (5.81 – 6.95) and maximal effects of 0.60 ± 0.13 for AT-127). The other compounds were nearly inactive in stimulating NOP/β-arrestin 2 interaction (Figure 3.2.16D).

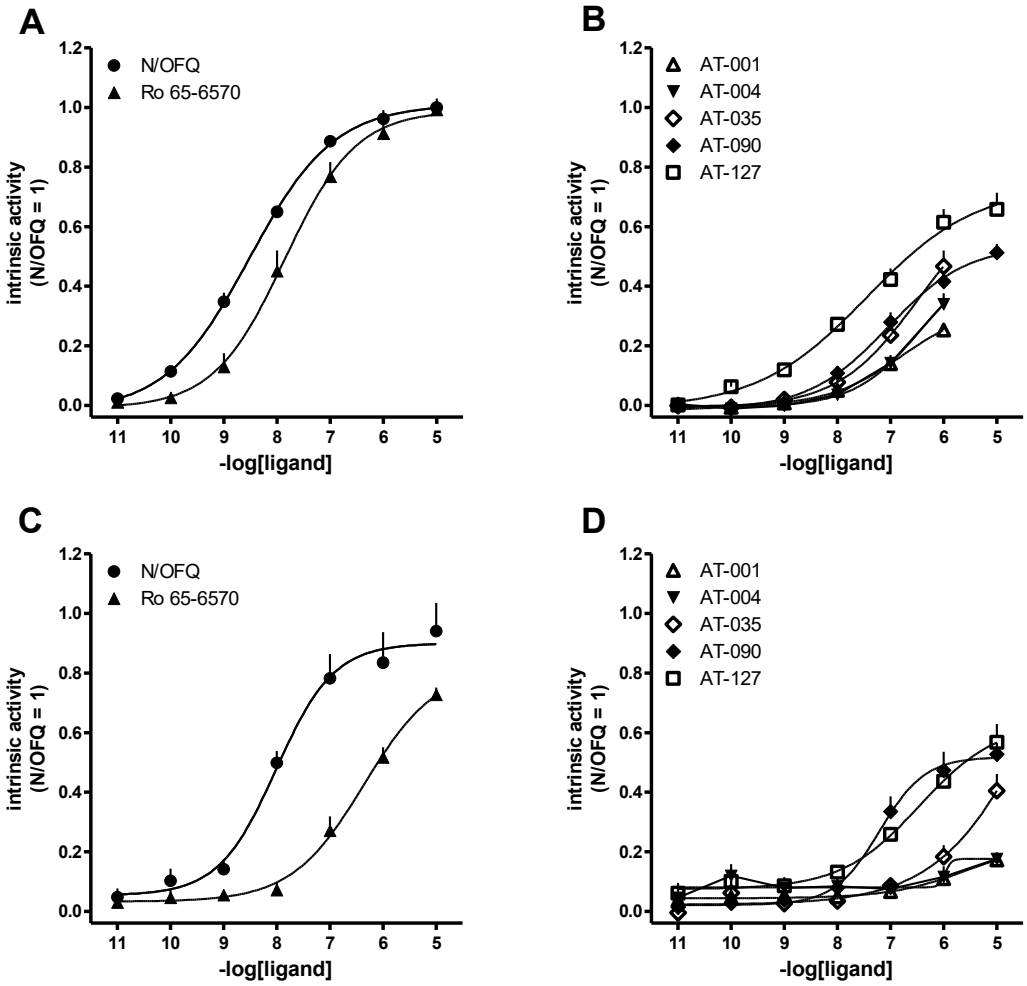


Figure 3.2.16. BRET assay. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B) in NOP/G protein interaction. Concentration response curve to N/OFQ and Ro 65-6570 (panel C) and to AT compounds (panel D) in NOP/β-arrestin 2 interaction. Data are the mean ± S.E.M. of at least 3 experiments.

Figure 3.2.17 illustrates the comparison of the concentration response curves of standard agonists and the two best AT compounds on NOP/G protein and NOP/arrestin interaction. N/OFQ displayed slight lower potency at arrestin compared to G protein. Similar results were obtained with AT-127. Ro 65-6570 was approximately 40 fold more potent in the G protein assay. Of note AT-090 displayed the same potency in the two assays. To obtain a quantitative measure of biased agonism, the bias factor values were calculated for Ro 65-6570 and the two active AT compounds using N/OFQ as reference agonist. Ro 65-6570 displayed a 10 fold biased behavior towards G protein (1.00 ± 0.20). AT-090 and AT-127 showed bias factor values of -0.78 ± 0.27 and 0.27 ± 0.25, respectively.

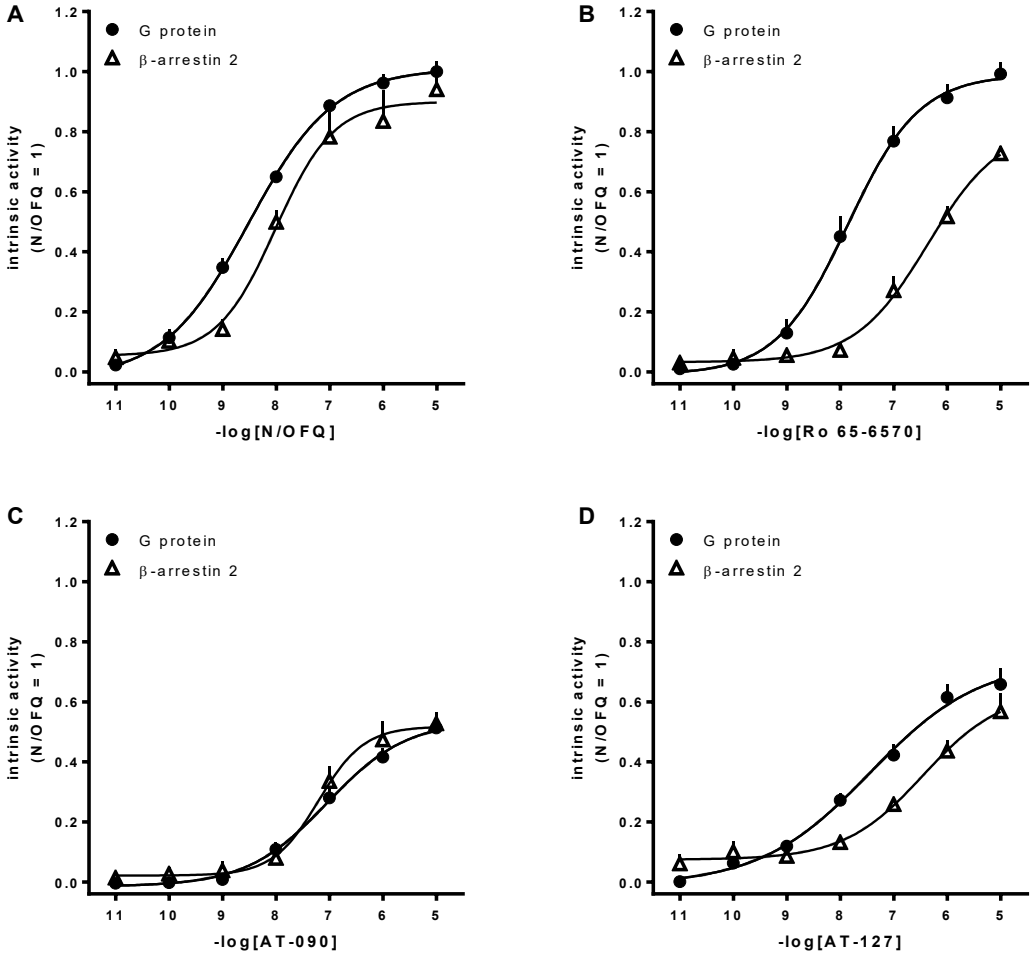


Figure 3.2.17. NOP/G protein and NOP/β-arrestin 2 interactions. Concentration response curves to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C), and AT-127 (panel D). Data are the mean ± S.E.M. of at least 3 experiments.

Mouse vas deferens – N/OFQ inhibited the electrically induced contractions of mouse vas deferens tissue in a concentration-dependent manner with a potency value of 7.27 (7.21 – 7.33) and maximal effect of 81 ± 1 % of the control twitch. Ro 65-6570 mimicked the inhibitory effect of N/OFQ but showing lower potency (pEC_{50} 6.53 (6.34 – 6.72)) and higher maximal effects (Figure 3.2.18A). In this preparation AT-004 and AT-035 were inactive. AT-001 at micromolar concentrations slightly increased the twitch response to electrical stimulation. These compounds were then tested as antagonists at 1 μM against N/OFQ. The peptide elicited superimposable concentration response curve in the presence and in the absence of these molecules (data not shown). On the contrary, AT-090 and AT-127 mimicked the inhibitory effect of N/OFQ but with lower potency (pEC_{50} 6.77 (6.55 – 6.99) and 6.50 (6.26 – 6.74), respectively) and higher maximal effects ($\alpha > 1.0$) (Figure 3.2.18B).

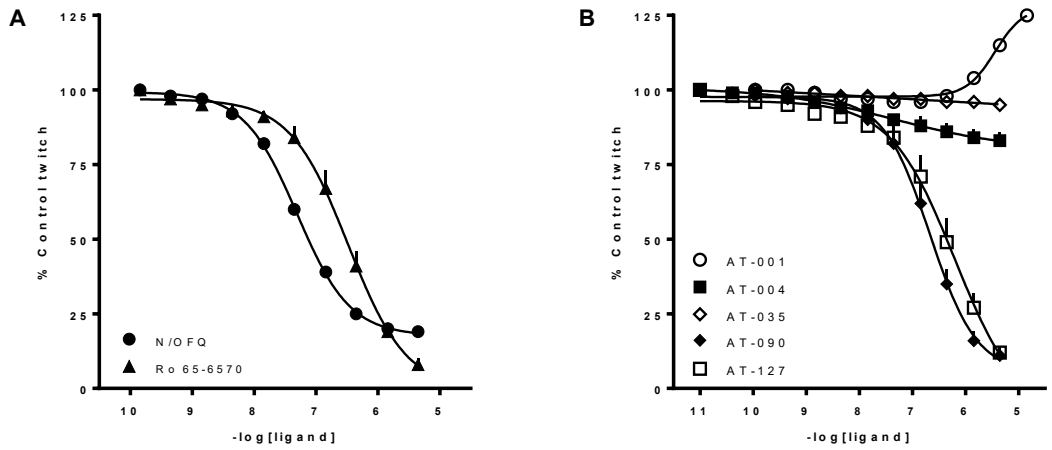


Figure 3.2.18. Mouse vas deferens bioassay. Concentration response curves to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are the mean ± S.E.M. of at least 3 experiments.

Of note, the kinetics of action of NOP agonists were very different (Figure 3.2.19). The action of N/OFQ was very rapid and immediately reversible after washing, on the contrary the effects elicited by Ro 65-6570 were very slow to develop and virtually resistant to wash. AT-090 and AT-127 displayed intermediate kinetics of action.

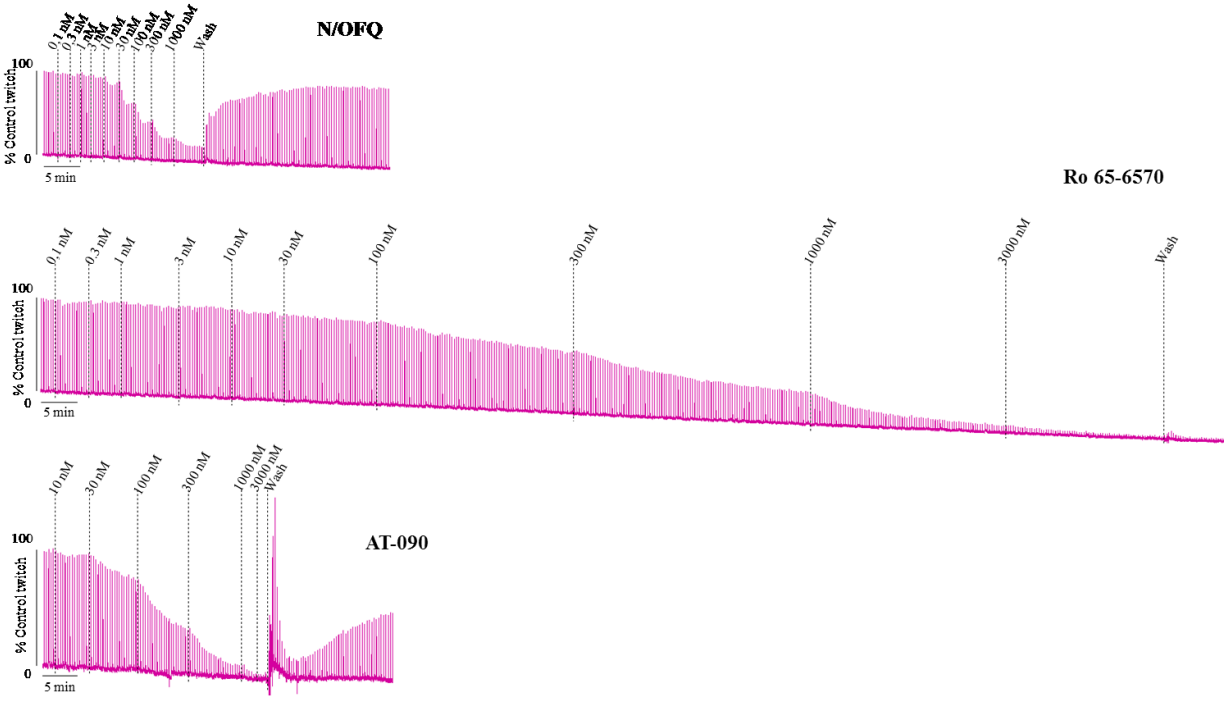


Figure 3.2.19. Representative tracings of tissue contraction in response to N/OFQ, Ro 65-6570 and AT-090 in the electrically stimulated mouse vas deferens.

To investigate the receptor(s) involved in the action of AT-090 and AT-127, both antagonism and knockout studies were performed. SB-612111 0.1 μM shifted to right the concentration response

curve to N/OFQ without changing its maximal effects. A pK_B of 8.69 (7.85 – 9.53) was derived from this experiment (Figure 3.2.20A). At the same concentration, the antagonist was not able to shift to right the concentration response curve to Ro 65-6570 (Figure 3.2.20B). SB-612111 0.1 μM shifted to right the concentration response curve to AT-090 and AT-127 but, compared to N/OFQ, with lower potency (pK_B of 7.41 (6.96 – 7.86) for AT-090 and of 7.95 (7.12 – 8.78) for AT-127, Figure 3.2.20C and 3.2.20D).

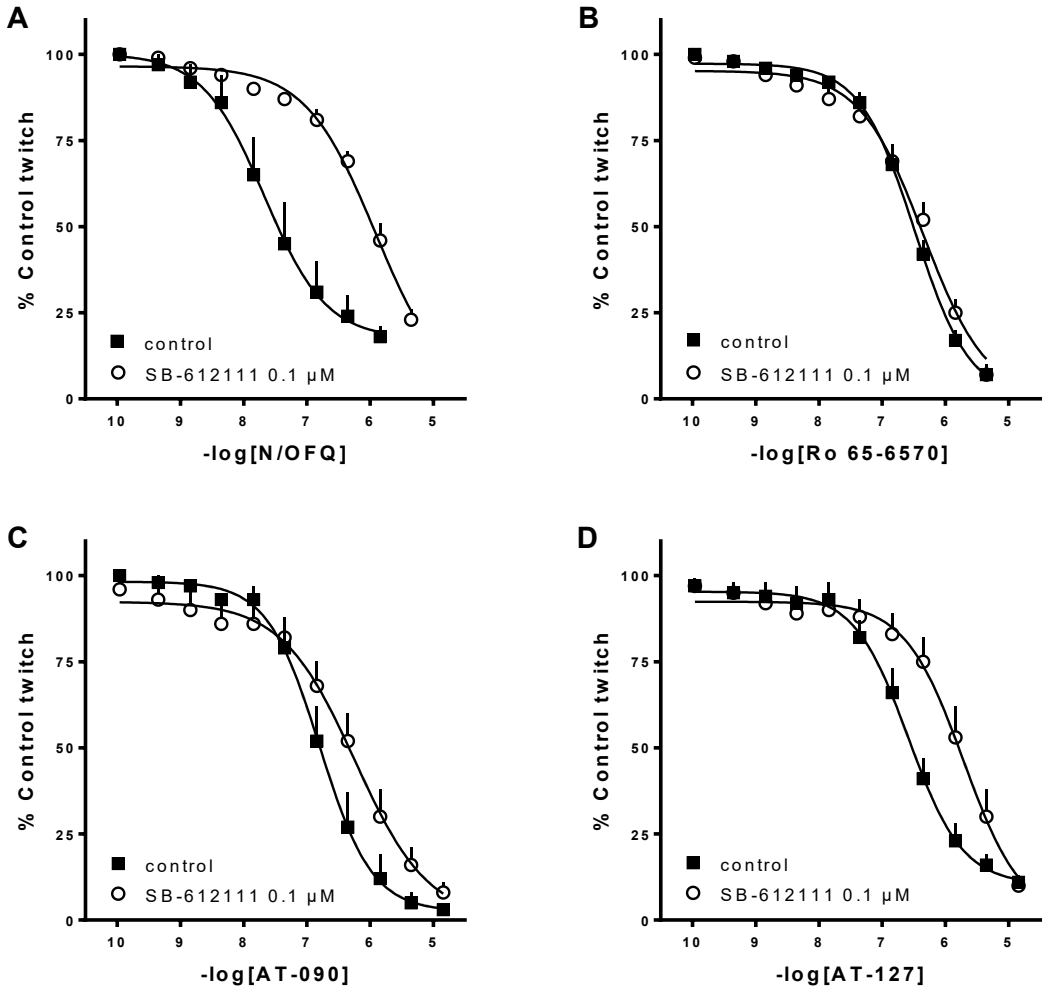


Figure 3.2.20. Mouse vas deferens bioassay. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in absence and in presence of SB-612111 (0.1 μM). Data are the mean ± S.E.M. of at least 3 experiments.

N/OFQ inhibited the electrically induced contractions of tissues taken from NOP(+/+) mice (pEC_{50} 7.48 (7.32 – 7.64), E_{max} = 87 ± 3 %) but not from NOP(-/-) mice (Figure 3.2.21A). The delta opioid receptor agonist DPDPE elicited similar inhibitory effects in tissues taken from NOP(+/+) and NOP(-/-) mice (data not shown). Ro 65-6570 mimicked the inhibitory effects of N/OFQ in both tissues, with a potency value 2-fold lower in NOP(-/-) than NOP(+/+) mice (Figure 3.2.21B). AT-090 and AT-127 inhibited the electrically induced contractions of the mouse vas deferens both in

NOP(+/+) and NOP(-/-) mice, being approximately 10 fold less potent in NOP(-/-) tissues (Figure 3.2.21C and 3.2.21D).

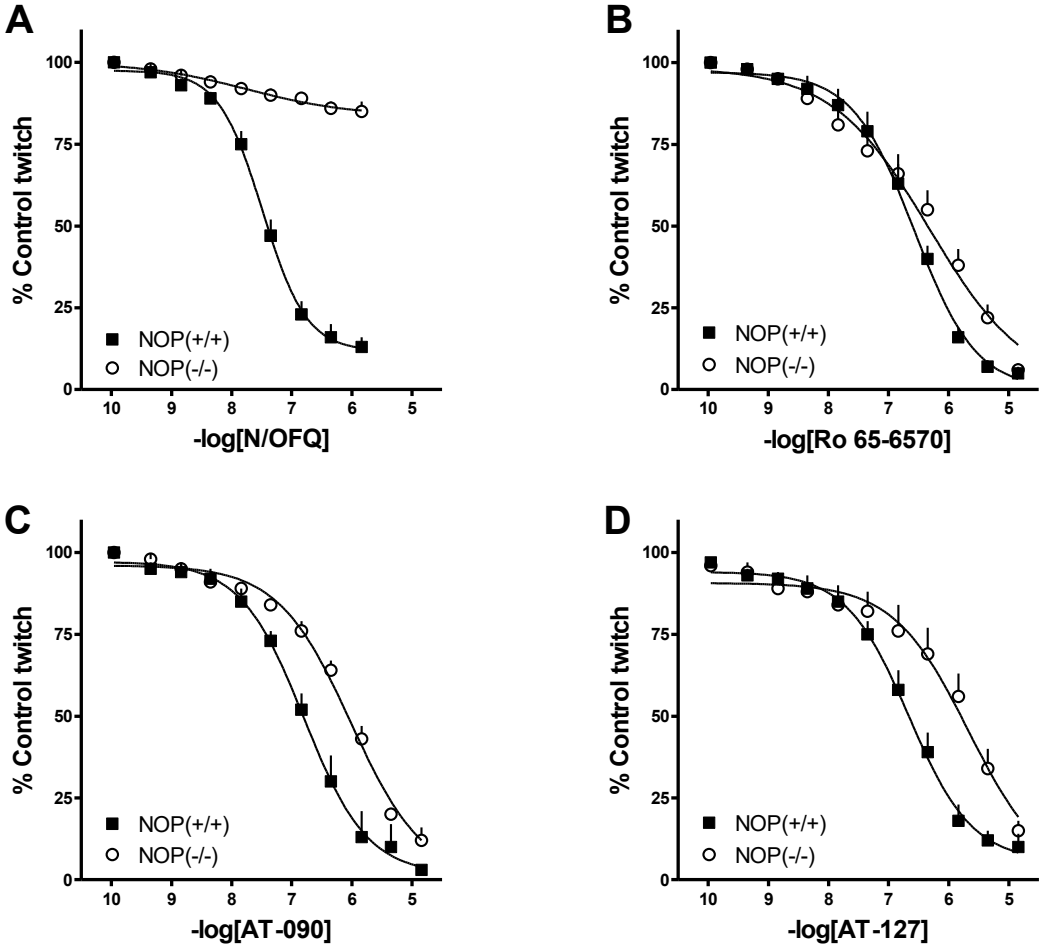


Figure 3.2.21. Mouse vas deferens bioassay. Concentration response curve to N/OFQ (panel A), Ro 65-6570 (panel B), AT-090 (panel C) and AT-127 (panel D) in NOP(+/+) and NOP(-/-) tissues. Data are the mean ± S.E.M. of at least 5 experiments.

Mouse colon – N/OFQ induced a concentration-dependent contraction of the mouse colon with potency of 8.51 (8.43 – 8.58) and an E_{max} = 45 ± 5 % of the contraction induced by 10 μM carbachol (Figure 3.2.22A). The contractile effect elicited by Ro 65-6570 could not be repeated in the same tissue and therefore its concentration response curve was generated using different tissues and only three concentrations i.e. 10, 100, and 1000 nM. As shown in Figure 3.2.22A, Ro 65-6570 elicited maximal effect similar to N/OFQ being however 30-fold less potent. AT-090 and AT-127 induced concentration dependent contractions of the mouse colon tissues with potency and efficacy similar to N/OFQ. A potency value of 8.49 (8.42 – 8.54) and E_{max} = 38 ± 4 % was calculated for AT-090 and a potency value of 8.23 (7.85 – 8.61) and E_{max} = 42 ± 6 % was obtained for AT-127. In the same preparation AT-001 and AT-035 were only poorly active, while AT-004 was inactive up to 10 μM (Figure 3.2.22B). Of note, the kinetic of action of these NOP ligands were different.

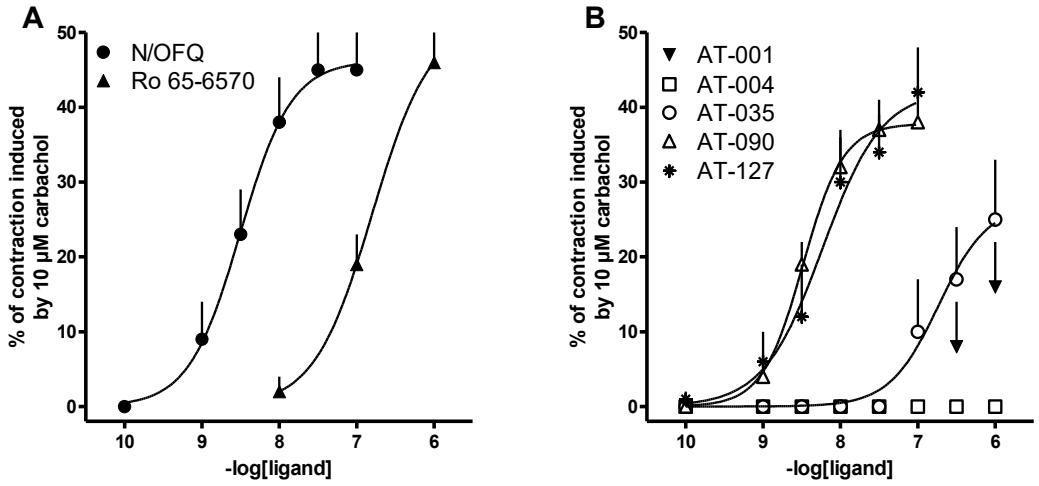


Figure 3.2.22. Mouse colon bioassay. Concentration response curve to N/OFQ and Ro 65-6570 (panel A) and to AT compounds (panel B). Data are the mean ± S.E.M. of at least 3 experiments.

As shown in Figure 3.2.23, all compounds showed a rapid contractile effect, but after washing the tissues N/OFQ-induced contraction rapidly disappeared, while the contraction evoked by Ro 65-6570 and the two AT compounds were less sensitive to washing.

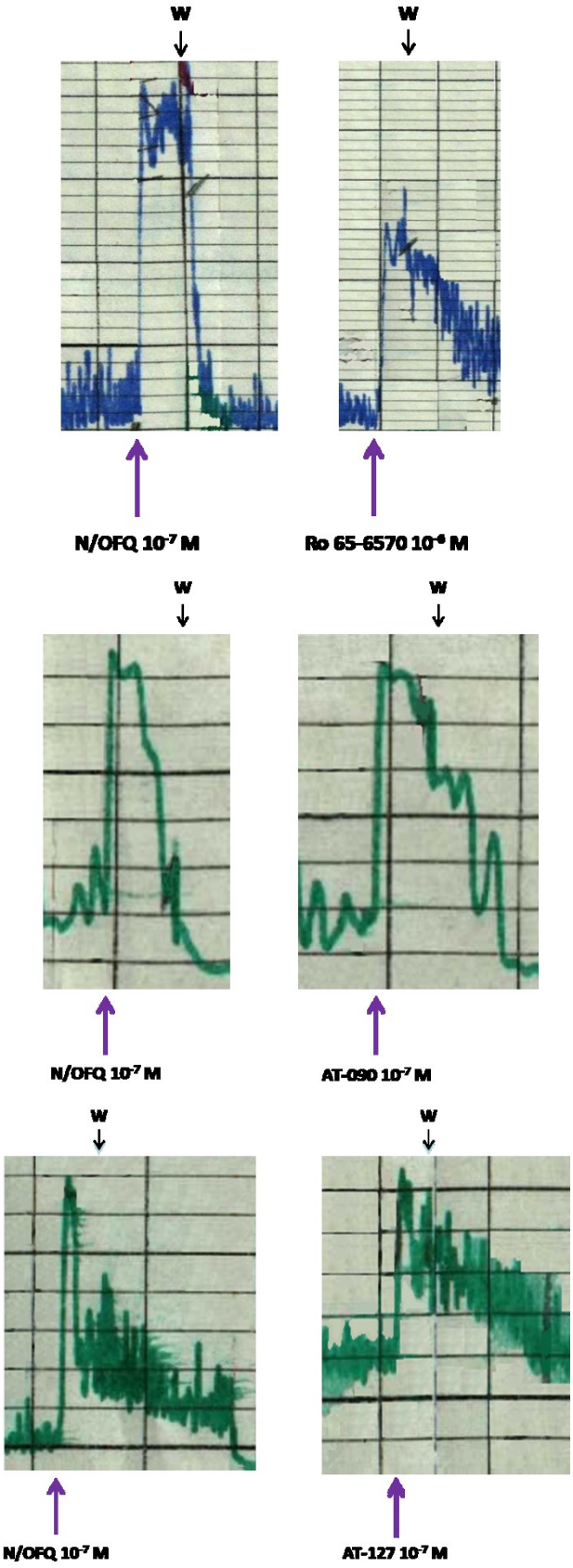


Figure 3.2.23. Typical tracings of N/OFQ, Ro 65-6570, AT-090 and AT-127 in the mouse colon bioassay.

To test the selectivity of action of AT-090 and AT-127 some experiments with tissues extracted from NOP(+/+) and NOP(-/-) mice were performed. N/OFQ 0.1 μM induced a contractile response

in mouse colon from NOP(+/+) mice but not in colon from NOP(-/-) mice. The mu opioid agonist endomorphin-1 1 μM contracted both tissues. The effects elicited by Ro 65-6570 1 μM was similar in tissues from NOP(+/+) and NOP(-/-) mice. Similarly to N/OFQ, both AT-090 0.1 μM and AT-127 0.1 μM were able to induce mouse colon contraction in NOP(+/+) but not in NOP(-/-) mice (Figure 3.2.24).

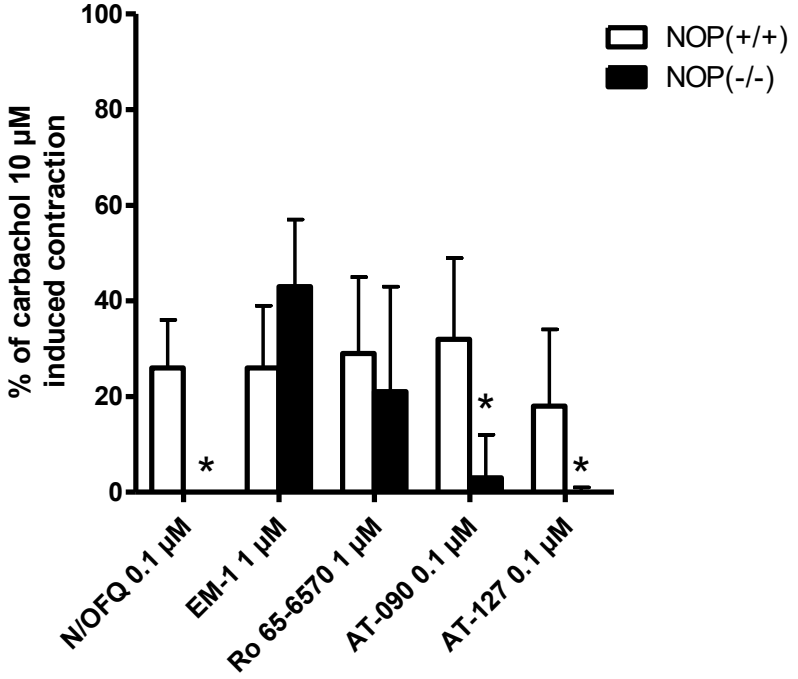


Figure 3.2.24. Mouse colon bioassay. Effects of N/OFQ, EM-1, Ro 65-6570, AT-090 and AT-127 in NOP(+/+) and NOP(-/-) tissues. Data are the mean ± S.E.M. of 6 separate experiments. *p<0.05 vs NOP(+/+) Student t test.

Discussion

In the present study, a detailed in vitro pharmacological characterization of novel NOP partial agonists ligands has been presented. The novel compounds were evaluated at human recombinant receptors in receptor binding, stimulated GTPγS binding, calcium mobilization, and BRET assays. Moreover, the novel compounds were tested in isolated mouse tissues to assess their pharmacological actions at native receptors. Results demonstrated that AT-090 and AT-127 behave as potent NOP receptor partial agonists in all functional assays. Moreover, these compounds displayed a level of NOP selectivity higher than the standard non-peptide NOP agonist Ro 65-6570. Thus, AT-090 and AT-127 might be useful pharmacological tools for investigating the therapeutic potential of NOP partial agonists.

Receptor bindings studies demonstrated that AT compounds behave as high affinity NOP ligands showing a moderate selectivity for NOP over classical opioid receptors. GTPγS binding studies

demonstrated that AT compounds act as NOP partial agonists with AT-127 showing the higher potency and efficacy. These results were substantially confirmed in calcium mobilization studies performed on cells expressing human NOP or classical opioid receptors and chimeric G proteins. This assay has been previously validated for NOP (Camarda *et al.*, 2009) and later for classical opioid receptors (Camarda *et al.*, 2013) and then used for investigating several NOP ligands (Toll *et al.*, 2016). N/OFQ and Ro 65-6570 behaved as full agonists with the non-peptide ligand being, in line with previous findings (Hashiba *et al.*, 2001), 10-fold less potent. The stimulatory effects of these control agonists were mimicked by the AT compounds that showed an order of potency, i.e. AT-127 $\geq$ AT-090 $\geq$ AT-035 > AT-001 $\geq$ AT-004 in line with receptor affinity and GTPγS functional potency. All AT compounds displayed reduced efficacy (α 0.73 – 0.82) although these differences did not reach statistical significance; it is worthy of mention that the calcium mobilization assay tends to overestimate ligand efficacy as previously demonstrated with other NOP partial agonists (Camarda *et al.*, 2009). This is probably due to signal amplification phenomena that make high the efficiency of the stimulus / response coupling in this assay. SB-612111 displayed similar pA_2 values versus standard agonists as well as versus AT compounds (range 8.49 – 8.72) and surmountable antagonist behaviour, in line with its described competitive nature (Spagnolo *et al.*, 2007; Zaratin *et al.*, 2004). These results strongly suggest that AT compounds bind the same NOP site recognized by N/OFQ and SB-612111 and recently described at atomic level (Miller *et al.*, 2015).

As far as selectivity over opioid receptors is concerned, N/OFQ displayed extraordinary high selectivity while Ro 65-6570 showed approximately 100-fold selectivity for NOP. AT-090 and AT-127 displayed similar selectivity as Ro 65-6570 whereas the other AT compounds showed reduced selectivity with AT-001 displaying similar potency at NOP and kappa receptors.

AT compounds were also assessed in a BRET based assay that measures receptor interaction with G protein and β-arrestin 2. This assay was first set up and validated for opioid receptors (Molinari *et al.*, 2010) and recently extended to the investigation of NOP (Malfacini *et al.*, 2015). In the NOP/G protein interaction, Ro 65-6570 behaved as a full agonist showing slightly lower potency compared to the natural peptide. AT-090 and AT-127 mimicked the stimulatory effects of standard agonists but with lower potency and efficacy while the other AT compounds displayed very low potency and their concentration response curves could not be completed. The rank order of potency in this assay is similar to what observed in the other assays. Of note, AT-090 and AT-127 clearly behaved as partial agonists confirming the GTPγS binding results. In fact a high determination coefficient has been previously calculated between the two assays using a large panel of NOP ligands (Malfacini *et al.*, 2015).

In the NOP/β-arrestin 2 assay, Ro 65-6570 behaved as a full agonist showing, however, a large loss of potency compared to the natural peptide. AT-090 and AT-127 mimicked the stimulatory effects of standard agonists showing relatively high potency but lower efficacy. The other AT compounds were virtually inactive. Comparing the ligand efficacy at G protein and β-arrestin 2 suggests that Ro 65-6570 behaved as a G protein-biased agonist. This seems to be the rule for NOP (Malfacini *et al.*, 2015; Chang *et al.*, 2015b) and opioid (Molinari *et al.*, 2010) non-peptide agonists. AT-090 and AT-127 seem to be the exception to this rule. In fact these compounds displayed similar ability to promote NOP/G protein and NOP/arrestin interactions, behaving as the unbiased standard agonist N/OFQ. Further investigations are needed to identify NOP receptor agonists biased toward β-arrestin. The availability of unbiased, G protein and β-arrestin biased agonists would be of paramount value for investigating functional selectivity in vivo and assessing the therapeutic potential of biased agonists in the NOP receptor field.

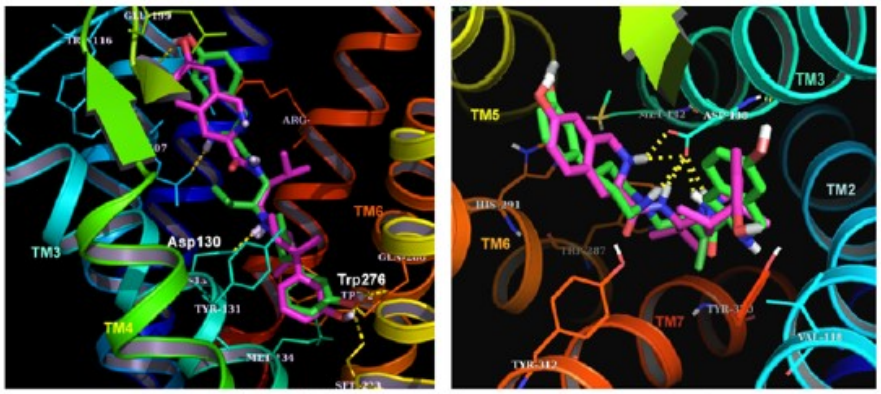
In the mouse vas deferens, N/OFQ showed values of potency and maximal effects in line with previous studies (Calo *et al.*, 1996; Berzetei-Gurske *et al.*, 1996). Ro 65-6570, AT-090 and AT-127 mimicked the action of the peptide, showing lower potency but higher maximal effects thus suggesting possible off-target effects. This was confirmed in receptor antagonist and knockout studies. In fact, SB-612111 antagonized N/OFQ action with a pA_2 value in line with previous results (Spagnolo *et al.*, 2007) while it showed lower pA_2 values against AT compounds and was almost inactive against Ro 65-6570. These results demonstrate that the biological action of N/OFQ in this preparation is solely due to NOP receptor activation while that of Ro 65-6570 mainly derives from the interaction with an unknown inhibitory site. The same can be said for AT-090 and AT-127 however, the amount of effect deriving from NOP receptor activation by these molecules is larger than that of Ro 65-6570. This interpretation is corroborated by findings in tissues from knockout animals. In fact, effect of N/OFQ is completely lost whereas Ro 65-6570 displayed a only slightly reduced potency in NOP(-/-) tissues. AT-090 and AT-127 were almost 10-fold less potent in NOP(-/-) than NOP(+/+) tissues. Collectively, receptor antagonists and knockout studies demonstrate that in the mouse vas deferens the effects of AT compounds and particularly of Ro 65-6570 result from their interaction with the NOP receptor and possibly with an additional inhibitory site. Similar results were obtained previously when investigating the effects of a related NOP agonist Ro 64-6198 in the mouse vas deferens. Notably, the inhibitory effects elicited by Ro 64-6198 were resistant to NOP antagonists ($[Nphe^1]$N/OFQ(1-13)NH_2 and J-113397). Interestingly the concentration response curve for Ro 64-6198 was not affected even in the presence of naloxone or of a cocktail of naloxone and NOP antagonist (Rizzi *et al.*, 2001c), thus suggesting that classical opioid receptors are likely not involved in the action of this ligand. These findings, however, do not

underscore the usefulness of these tools for investigating in vivo the biological actions controlled by the N/OFQ-NOP system. In fact several in vivo actions of Ro 64-6198 and Ro 65-6570 were sensitive to NOP antagonists and no longer evident in NOP(-/-) animals (reviewed in Toll *et al.* (2016)). In addition we recently demonstrated that the anxiolytic-like effect of AT-090 in the mouse elevated plus maze can be detected in NOP(+/+) but not NOP(-/-) mice (Asth *et al.*, 2016).
In line with previous findings in the mouse colon (Rizzi *et al.*, 1999), N/OFQ produced concentration-dependent contractions with high potency. Ro 65-6570 mimicked the N/OFQ effect being 30-fold less potent while AT-090 and AT-127 displayed similar maximal effects and potency as N/OFQ. Interestingly AT compounds behaved as full agonists in this preparation. This is not unexpected since in this preparation, similar to the calcium mobilization assay, the efficiency of the stimulus / response coupling is high as suggested by the full agonist behaviour of known NOP partial agonists (Rizzi *et al.*, 1999). To investigate the receptor involved in the action of AT compounds in this preparation, knockout studies were performed. In line with previous studies (Di Giannuario *et al.*, 2001) the action of N/OFQ was no longer evident in NOP(-/-) tissues while that elicited by the mu-selective agonist endomorphin-1 was unaffected or even increased. The effect of 1 μM Ro 65-6570 was only slightly and non-significantly reduced in NOP(-/-) tissue while that elicited by 0.1 μM of AT compounds was virtually abolished. Thus, these results together with those obtained in the mouse vas deferens suggest that the NOP selectivity of the AT compounds is greater than that of Ro 65-6570.

Collectively the analysis of present results suggests that AT compounds behave as NOP partial agonists with the following rank order of potency: AT-090 $\geq$ AT-127 > AT-035 $\geq$ AT-004 = AT-001. AT-090 and AT-127 displayed high NOP potency similar to that of the standard agonist Ro 65-6570. The rank order of the potency of the functional responses among the various assays appears to be similar. The maximal effects elicited by these partial agonists varies depending on the efficiency of the stimulus / response coupling of the preparation used. AT-090 and AT-127 displayed similar selectivity over classical opioid receptors as Ro 65-6570 in recombinant receptor studies; however at native mouse receptors they displayed higher NOP selectivity than Ro 65-6570. Finally, the NOP partial agonists AT-090 and AT-127 are particularly interesting due to their ability stimulate NOP interaction with both G protein and arrestin with the same potency and efficacy. This is different from other nonpeptide NOP agonists previously examined, which in general tend to show at least some degree of bias toward G protein.

3.2.3 AT-076, a novel opioid and NOP receptor antagonist

From JDTic to AT-076, a novel kappa receptor antagonist



AT-076 (green) and JDTic (magenta) bound to the

NOP receptor KOP receptor

Image taken from Zaveri *et al.*, 2015

The group of research lead by Prof. Nurulain Zaveri at Astraea Therapeutics has provided several important contributions in the field of the design, synthesis and characterization of non-peptide ligands for the NOP receptor (Daga *et al.*, 2014; Zaveri, 2016; Toll *et al.*, 2016). In the frame of a structure activity study on the kappa opioid receptor antagonist JDTic, the Zaveri group of research synthesized the compound AT-076 (Zaveri *et al.*, 2015) (see Figure 3.2.25). In receptor binding studies AT-076 displayed low nanomolar affinity for NOP, mu and kappa opioid receptors and only 10 fold lower affinity for the delta receptor. Moreover AT-076 consistently behaved as a receptor antagonist at NOP and classical opioid receptor showing higher potency at kappa and mu receptors than at delta and NOP receptors. On the bases of these results AT-076 has been proposed as an "opioid pan antagonist" (Zaveri *et al.*, 2015).

Thus the aim of this study was the detailed in vivo pharmacological characterization of AT-076. To this aim the compound has been assessed at recombinant human NOP and classical opioid receptors in calcium mobilization studies performed on cells co-expressing the receptors and chimeric G proteins and at native animal NOP and opioid receptors in bioassay studies performed on the electrically stimulated mouse vas deferens and guinea pig ileum.

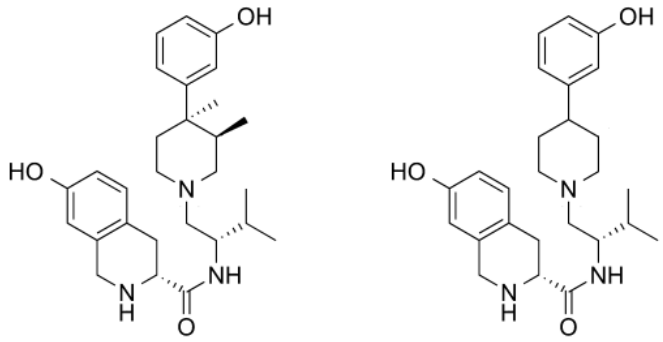


Figure 3.2.25. Chemical structures of JDTic and AT-076.

Materials and methods

Drugs and reagents – All cell culture media and supplements were from Invitrogen (Thermo Fisher Scientific Inc. MA, USA). All other reagents were from Sigma Chemical Co. (Poole, U.K.) and were of the highest purity available.

Dermorphin and N/OFQ were synthesized in the Prof. Guerrini laboratory (Department of Pharmaceutical Sciences and LTTA, University of Ferrara, Ferrara, Italy). DPDPE, dynorphin A, SB-612111 and naloxone were from Tocris Bioscience (Bristol, UK). AT-076 were synthesized in Astraea Therapeutics laboratories (Mountain View, California,USA). Dermorphin, N/OFQ, DPDPE

and dynorphin A were solubilized in bidistilled water at a final concentration of 1 mM, while naloxone at 10 mM. SB-612111 and AT-076 was solubilized in DMSO at a final concentration of 10 mM. Stock solutions of ligands were stored at -20 °C. The buffers for successive dilutions are described in the detail in each assay section.

Calcium mobilization assay – CHO cells stably co-expressing human recombinant NOP, kappa or mu receptor and the C-terminally modified $G\alpha_{qi5}$ and cells co-expressing the delta receptor and the $G\alpha_{qG66Di5}$ chimeric proteins were generated as previously described (Camarda *et al.*, 2013; Camarda *et al.*, 2009). Cells were cultured in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) / HAMS F12 (1:1) supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutammine (2 mM), fungizone (1 µg/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 20 mM HEPES (pH 7.4), 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 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. Serial dilutions of stock solutions of ligands for experimental use were made in HBSS/HEPES (20 mM) buffer (containing 0.03% of bovine serum albumin, BSA, Sigma Chemical Co., Poole, UK). 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. On-line additions were carried out in a volume of 50 µl/well. In antagonism type experiments the antagonists were injected in the well 24 min before adding the agonist and, to facilitate drug diffusion into the wells, the experiments were performed at 37 °C and three cycles of mixing (25 µl from each well moved up and down 3 times) were performed immediately after antagonist injection.

Electrically stimulated tissues – The experiments were performed on the mouse vas deferens and the guinea pig ileum. The tissues were taken from male CD-1 mice 16 – 18 g (Harlan, Ud, Italy) and male albino guinea pigs of 350 – 400 g (Harlan, Ud, Italy). Mice were housed in 425 x 266 x 155 mm cages (Techniplast, Mi, Italy), 8 per cage and guinea pigs were housed in 560 x 320 x 180 mm cages (Techinplast, Mi, Italy), 3 per cage, all under standard conditions (22°C, 55% humidity, 12 h

light/dark cycle, light on at 7:00 am), with food for mice (4RF, Mucedola, Mi, Italy) and complete feed for guinea pigs (Mucedola, Mi, Italy) and water *ad libitum*. A mouse red house (Techniplast, Va, Italy) and nesting materials were present in each cage for mice.

The day of the experiment the animals were sacrificed with CO_2 overdose. The experimental protocol was approved by the Ethical Committee for the Use of Laboratory Animals of the University of Ferrara and by the Italian Ministry of Health (authorization number 9927, 19/04/2013). The animals were treated in accordance with the European Communities Council directives (2010/63/EU) and national regulations (D.L. 26/2014). These studies have been reported according to ARRIVE guidelines (Kilkenny *et al.*, 2010). Bioassay experiments were performed as previously described by Calo *et al.* (1996) for the mouse vas deferens and by Bigoni *et al.* (1999) for the guinea pig ileum. 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). For the experiments on the mouse vas deferens $MgSO_4$-free Krebs solution were used. For the experiments on guinea pig ileum the normal medium was added with hexamethonium bromide 0.022 mM and benadril $3.43x10^{-4}$ mM. The Krebs solution was oxygenated with 95% O_2 and 5% CO_2. The temperature was set at 33°C for the mouse vas deferens and 37°C for the guinea pig ileum. At resting tension 0.3 g was applied to the mouse vas deferens and 1 g to the guinea pig ileum. 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 Basile strain gauge transducers (Basile 7006; srl Ugo Basile, Varese, Italy) and recorder with a computer – based acquisition system (Power Lab 8, ADInstruments, Colorado Springs, 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 to agonists were performed (0.5 log unit steps). Antagonists were injected into the baths 15 minutes before performing concentration response curves to agonists. Compounds were diluted using the Krebs solution. A total number of 20 mice and 6 guinea pigs were used for the present in vitro studies.

Data analysis and terminology – The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig *et al.*, 2003). All data are expressed as the mean ± standard error of the mean (S.E.M.) of *n* experiments. For potency values 95% confidence limits ($CL_{95\%}$) were indicated.

In calcium mobilization experiments, maximum change in fluorescence, expressed as percent over the baseline fluorescence, was used to determine agonist response. Electrically stimulated tissues data are expressed as % of the control twitch induced by electrical field stimulation.

Agonist potencies are given as pEC_{50} i.e. the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect of that agonist. Concentration-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model:

$$\text{Effect} = \text{Baseline} + \frac{(E_{max} - \text{Baseline})}{(1+10^{((LogEC_{50} - Log[compound])*Hillslope)})}$$

Curves fitting were performed using PRISM 6.0 (GraphPad Software In., San Diego, USA).

EC_{50} is the concentration of agonist producing a 50% maximal response.

Three types of experiments were performed to investigated the antagonist potency: i) inhibition-response curves to the antagonist against a fixed concentration of agonist approximately corresponding to its EC_{80}, ii) concentration-response curves to the agonist in absence and in presence of a fixed concentration of antagonist, iii) concentration-response curves to the agonist in absence and in presence of increasing concentrations of AT-076 (Schild analysis).

The antagonist potency for antagonist in inhibition response experiments was expressed as pK_B, which was calculated as the negative logarithm to base 10 of the K_B from the following equation:

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

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the slope coefficient of the concentration-response curve to the agonist (Kenakin, 2014).

When antagonists were assayed at a single concentration against the concentration-response curve to the agonist their pK_B was derived with the Gaddum Schild equation:

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

assuming a slope value equal to unity, where CR indicates the ratio between agonist potency in the presence and absence of antagonist.

When the maximal effects of agonist in the presence and absence of antagonist are statistically different, the slope is calculated from a double-reciprocal plot of equieffective concentrations of agonist in the absence and presence of antagonist (Kenakin, 2014):

$$K_B = \frac{\text{antagonist}}{\text{slope-1}}$$

The type of antagonism exerted by AT-076 at the kappa receptor was assayed by using the Schild analysis.

Results

Calcium mobilization assay – In CHO_{NOP} cells, N/OFQ increased in a concentration dependent manner the intracellular calcium levels, with high potency (pEC_{50} 9.40) and maximal effects of 209 ± 12% over the basal value while opioid receptor agonists were inactive (Table 3.2.4). In CHO_{mu} cells, the mu standard agonist dermorphin stimulated in a concentration dependent manner the release of intracellular calcium with pEC_{50} of 8.67 and maximal effects of 120 ± 6% over the basal value. In these cells N/OFQ and DPDPE were inactive while dynorphin A displayed moderate potency (Table 3.2.4). The kappa standard agonist dynorphin A increased calcium levels in a concentration dependent manner with pEC_{50} of 8.52 and maximal effects of 189 ± 6% over the basal value in CHO_{kappa} cells; all the other agonists were completely inactive (Table 3.2.4). Finally in CHO_{delta} cells the standard agonist DPDPE increased in a concentration dependent manner the intracellular calcium levels with pEC_{50} of 8.39 and maximal effects of 90 ± 15% over the basal value (Table 3.2.4). In these cells N/OFQ was inactive while dynorphin A and dermorphin mimicked the stimulatory effects of DPDPE being however approximately 3 an 100 fold less potent (Table 3.2.4).

Table 3.2.4. Effects of standard agonists in CHO cells expressing the human recombinant receptors and chimeric G proteins in the calcium mobilization assay.

	NOP		**mu**		**kappa**		**delta**	
	pEC_{50} ($CL_{95\%}$)	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**	**pEC_{50} ($CL_{95\%}$)**	**α ± S.E.M.**
N/OFQ	9.40 (8.77-10.03)	1.00	inactive[a]		inactive[a]		inactive[a]	
dermorphin	inactive[b]		8.67 (8.34-9.01)	1.00	inactive[a]		6.43 (5.95-4.91)[a]	1.02[a]
dynorphin A	inactive		6.67 (6.17-7.17)[a]	0.83[a]	8.52 (8.07-8.97)	1.00	7.73 (7.46-8.00)[a]	0.99[a]
DPDPE	inactive		inactive[a]		inactive[a]		8.39 (8.14-8.65)	1.00

[a]: (Camarda *et al*., 2013)
[b]: (Molinari *et al.,* 2013)

The new compound AT-076 tested as agonist up to 10 μM did not modify per se intracellular calcium levels in the four cells lines. Similar results were obtained with SB-612111 and the universal opioid receptor antagonist naloxone.

In inhibition response experiments, in CHO_{NOP} cells, increasing concentrations (10 pM – 10 μM) of SB-612111 and AT-076 were tested against a fixed concentration of N/OFQ 1 nM. SB-612111 inhibited the release of calcium stimulated by N/OFQ in a concentration dependent manner: from these experiments a pK_B value of 7.68 was derived. In parallel experiments, AT-076 was inactive (Figure 3.2.26).

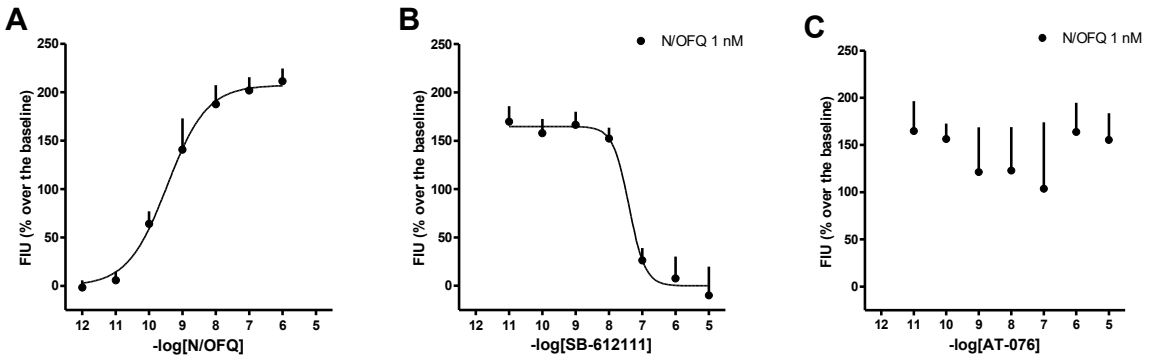


Figure 3.2.26. Calcium mobilization assay performed in CHO_{NOP} cells. Concentration response curve to N/OFQ (panel A), and inhibition response curves of SB-612111 (panel B) and AT-076 (panel C) against N/OFQ 1 nM. Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

In CHO_{mu} cells, dermorphin 10 nM was challenged against increasing concentrations (10 pM – 10 μM) of the universal opioid antagonist naloxone and the new compound AT-076. Both molecules inhibited the effect of dermorphin with pK_B values of 8.40 and 6.88 respectively (Figure 3.2.27).

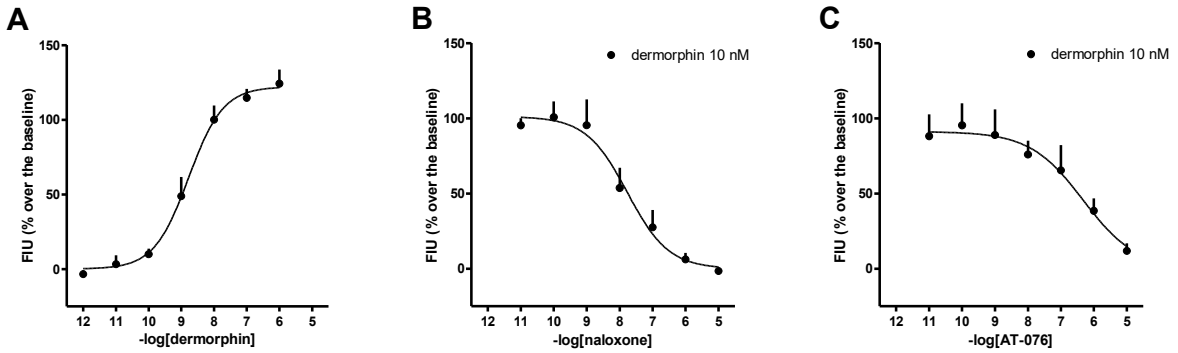


Figure 3.2.27. Calcium mobilization assay performed in CHO_{mu} cells. Concentration response curve to dermorphin (panel A), and inhibition response curves of naloxone (panel B) and AT-076 (panel C) against dermorphin 10 nM. Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

Increasing concentrations (10 pM – 10 μM) of the antagonists naloxone and AT-076 were also tested at the other two opioid receptors. In CHO_{delta} cells, the stimulation induced by DPDPE 10 nM was countered by naloxone (pK_B 7.07), while AT-076 was inactive (Figure 3.2.28).

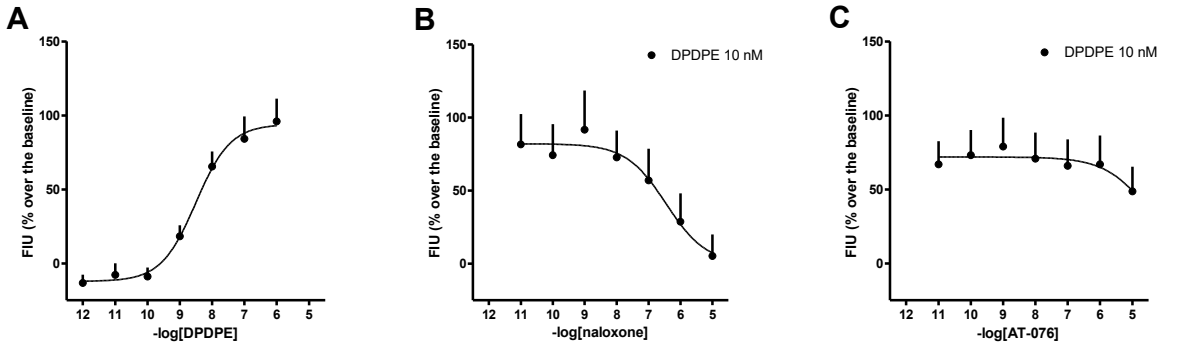


Figure 3.2.28. Calcium mobilization assay performed in CHO_{delta} cells. Concentration response curve to DPDPE (panel A), and inhibition response curves of naloxone (panel B) and AT-076 (panel C) against DPDPE 10 nM. Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

In CHO_{kappa} cells, both naloxone and AT-076 were able to inhibit the stimulatory effect of dynorphin A (10 nM) in a concentration dependent manner with a pK_B values of 6.68 and 10.13, respectively (Figure 3.2.29).

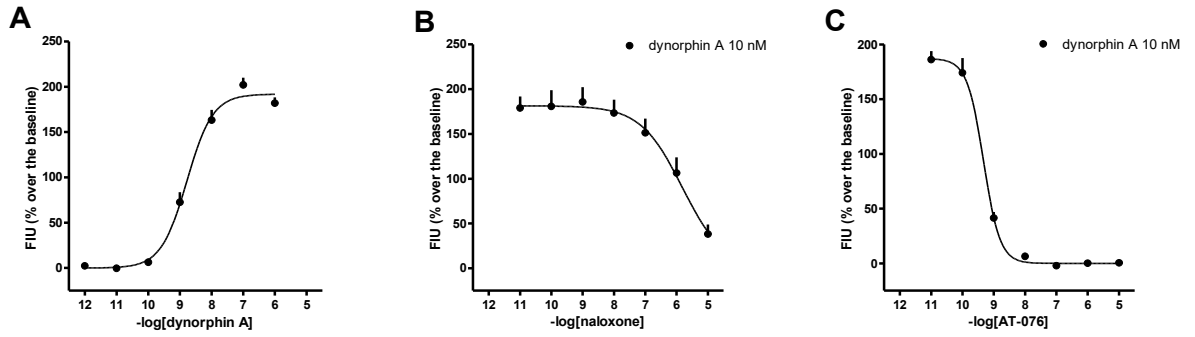


Figure 3.2.29. Calcium mobilization assay performed in CHO_{kappa} cells. Concentration response curve to dynorphin A (panel A), and inhibition response curves of naloxone (panel B) and AT-076 (panel C) against dynorphin A 10 nM. Data are expressed as mean ± S.E.M. of 4 separate experiments made in duplicate.

Then in a separate series of experiments, a fixed concentration of AT-076 was tested against the concentration response curves to standard agonists for NOP and opioid receptors. In parallel experiments the reference antagonists SB-612111 and naloxone were tested.

At the concentration of 0.1 μM, SB-612111 was able to shift to right the concentration response curve to N/OFQ with a pA_2 value of 8.28 (Figure 3.2.30A). On the contrary, AT-076 10 μM did not modify the concentration response curve to N/OFQ (Figure 3.2.30B).

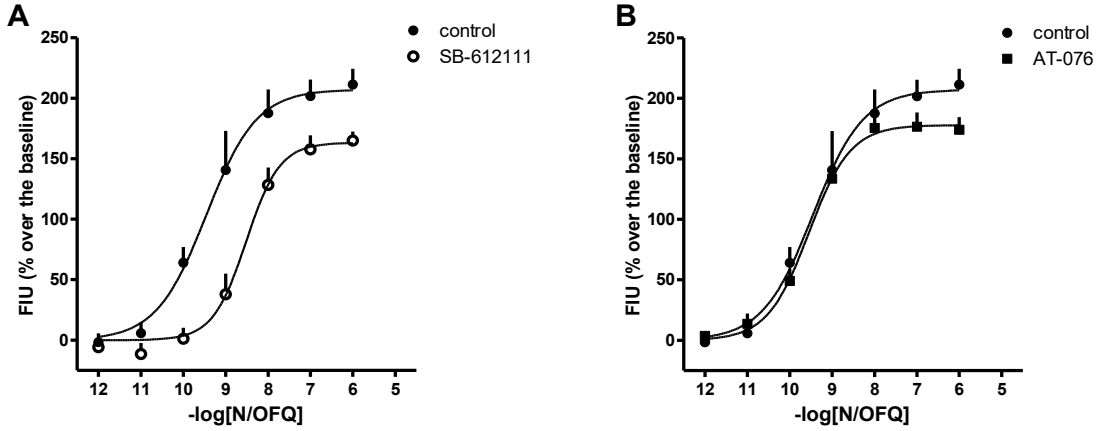


Figure 3.2.30. Calcium mobilization assay performed in CHO_{NOP} cells. Concentration response curve to N/OFQ obtained in the absence (control) and presence of SB-612111 0.1 μM (panel A) and AT-076 10 μM (panel B). Data are the mean ± S.E.M. of 4 separate experiments made in duplicate.

In CHO_{mu} cells, the concentration response curve to dermorphin was assessed in absence and in presence of naloxone 0.1 μM and AT-076 10 μM. Both antagonists were able to displaced to right the curve to dermorphin. A pA_2 values of 8.60 and 6.39 were derived from these experiments, for naloxone and AT-076, respectively (Figure 3.2.31A and 3.2.31B).

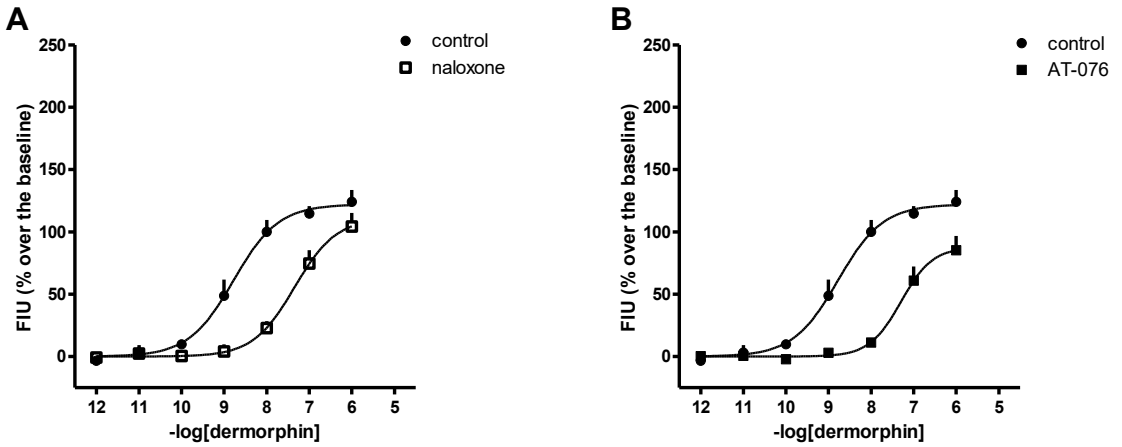


Figure 3.2.31. Calcium mobilization assay performed in CHO_{mu} cells. Concentration response curve to dermorphin obtained in the absence (control) and presence of naloxone 0.1 μM (panel A) and AT-076 10 μM (panel B). Data are the mean ± S.E.M. of 4 separate experiments made in duplicate.

In CHO_{delta} cells, naloxone 0.1 μM was able to shift to right the concentration response curve to DPDPE without changed maximal effects, with a pA_2 value of 7.56. AT-076 10 μM slightly lowered the maximal effects of DPDPE without modify its potency (Figure 3.2.32A and 3.2.32B).

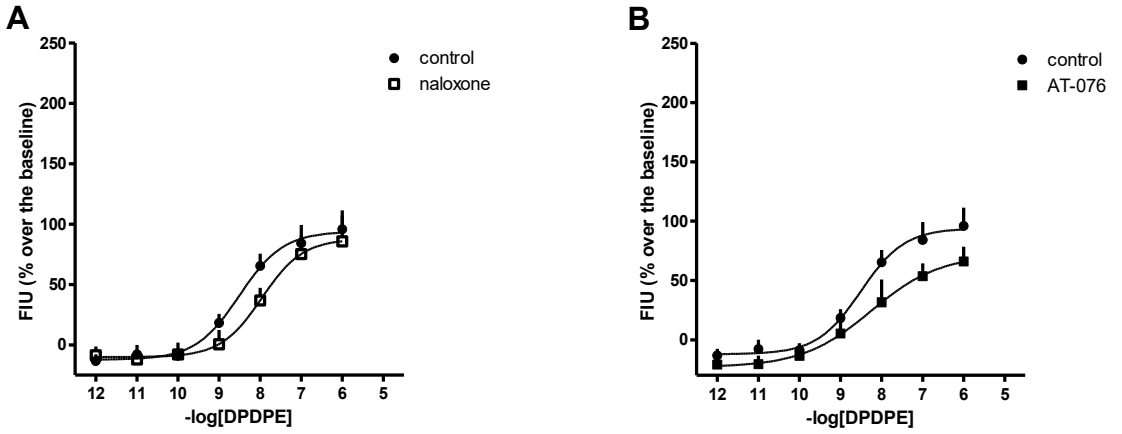


Figure 3.2.32. Calcium mobilization assay performed in CHO_{delta} cells. Concentration response curve to DPDPE obtained in the absence (control) and presence of naloxone 0.1 μM (panel A) and AT-076 10 μM (panel B). Data are the mean ± S.E.M. of 4 separate experiments made in duplicate.

Then, both antagonists were challenged against the concentration response curve to dynorphin A. Naloxone 0.1 μM was able to shift to right the curve to dynorphin A with a pA_2 value of 6.74 (Figure 3.2.33A). AT-076 10 nM displaced the concentration response curve to dynorphin A with a large depression of maximal effects: a pA_2 value of 10.11 was derived from these experiments (Figure 3.2.33B).

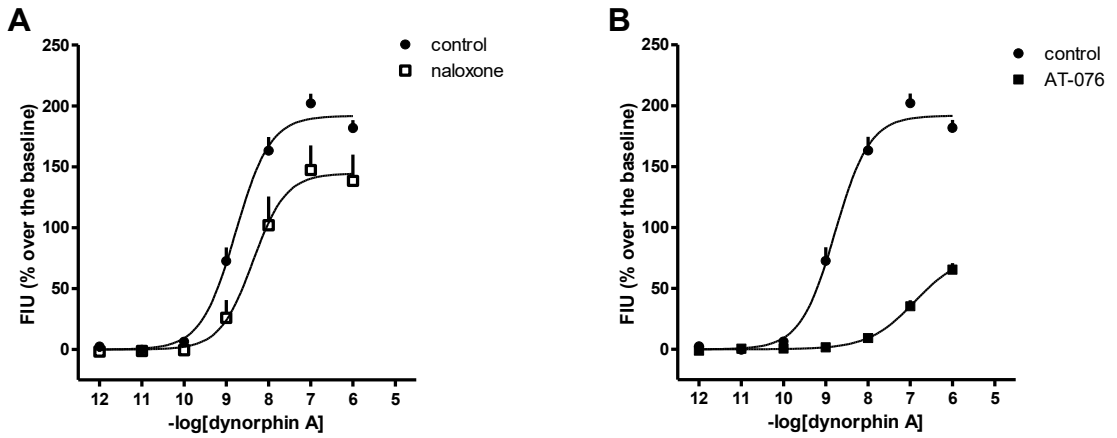


Figure 3.2.33. Calcium mobilization assay performed in CHO_{kappa} cells. Concentration response curve to dynorphin A obtained in the absence (control) and presence of naloxone 0.1 μM (panel A) and AT-076 10 nM (panel B). Data are the mean ± S.E.M. of 4 separate experiments made in duplicate.

The potencies of standard antagonists and of AT-076 obtained in inhibition response curves and in concentration response curve displacement experiments were summarized in table 3.2.5:

Table 3.2.5. Potencies of standard antagonists and AT-076 in CHO cells expressing the human recombinant receptors and chimeric G proteins in the calcium mobilization assay. Potencies obtained with displacement protocol are express as pA_2, while potencies obtained with inhibition protocol are express as pK_B.

	NOP		**mu**		**kappa**		**delta**	
	pA_2 ($CL_{95\%}$)	**pK_B ($CL_{95\%}$)**	**pA_2 ($CL_{95\%}$)**	**pK_B ($CL_{95\%}$)**	**pA_2 ($CL_{95\%}$)**	**pK_B ($CL_{95\%}$)**	**pA_2 ($CL_{95\%}$)**	**pK_B ($CL_{95\%}$)**
SB-612111	8.28 (7.49-9.08)	7.68 (6.94-8.42)	<6		<6		<6	
naloxone	<6[a]		8.60 (8.33-8.86)	8.40 (7.59-9.21)	6.74 (6.29-7.18)	6.68 (6.20-7.15)	7.56 (6.80-8.32)	7.07 (5.06-9.08)
AT-076	<6		6.39 (6.01-6.77)	6.88 (6.23-7.52)	10.11 (9.30-10.92)	10.13 (9.83-10.43)	<6	

[a]: (Camarda *et al.*, 2009)

In order to evaluate the nature of the antagonism exerted by AT-076 at the kappa receptor, the classical Schild analysis was performed. The concentration response curve to dynorphin A was evaluated in absence and presence of increasing concentrations of AT-076 (0.1 nM – 100 nM). As shown in figure 3.2.34, the antagonist was able to rightwardly shift the concentration response

curve to the agonist with a concentration dependent depression of maximal effects. A pK_B value of 10.17 (9.51 – 10.84) was calculated from these experiments. This value is also similar to that obtained in inhibition-response experiments (10.13).

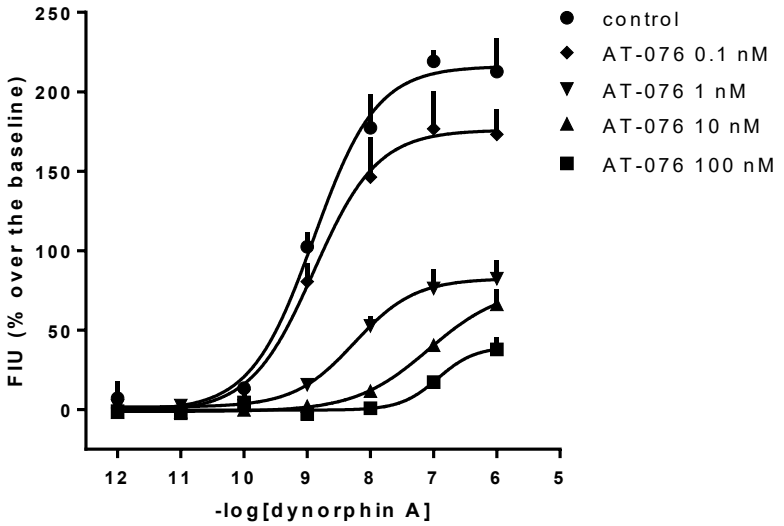


Figure 3.2.34. Calcium mobilization assay performed in CHO_{kappa} cells. Concentration response curve to dynorphin A obtained in the absence (control) and presence of increasing concentrations of AT-076 (0.1; 1; 10; 100 nM). Data are the mean ± S.E.M. of 3 separate experiments made in duplicate.

Mouse vas deferens – In the electrically stimulated mouse vas deferens, N/OFQ inhibited the twitch response in a concentration dependent manner with pEC_{50} 7.21 (7.08 – 7.38), E_{max}= 79 ± 2% and the delta receptor agonist DPDPE mimicked the effect of N/OFQ being more potent and producing higher maximal effect (pEC_{50} 8.15 (8.08 – 8.22), E_{max}= 96 ± 1%) (Figure 3.2.35A and 3.2.35B). The selective NOP receptor antagonist SB-612111 and the opioid receptor antagonist naloxone were tested as agonist up to 1 μM did not modify per se the electrically induced twitch. SB-612111 1 μM and naloxone 1 μM were challenged against N/OFQ and DPDPE. The inhibitory action of N/OFQ was antagonized by SB-612111 but not by naloxone. A pA_2 value of 8.15 was calculated for SB-612111 from this experiment (Figure 3.2.35A). On the contrary, the effects to DPDPE were sensitive to naloxone (pA_2 7.74) but not SB-612111 (Figure 3.2.35B). Then the new antagonist AT-076 was assayed in this preparation. The compound tested as agonist up to 1 μM did not modify per se the electrically induced twitch. At the same concentration AT-076 did not modify the concentration response curves to N/OFQ or DPDPE (Figure 3.2.35C and 3.2.35D).

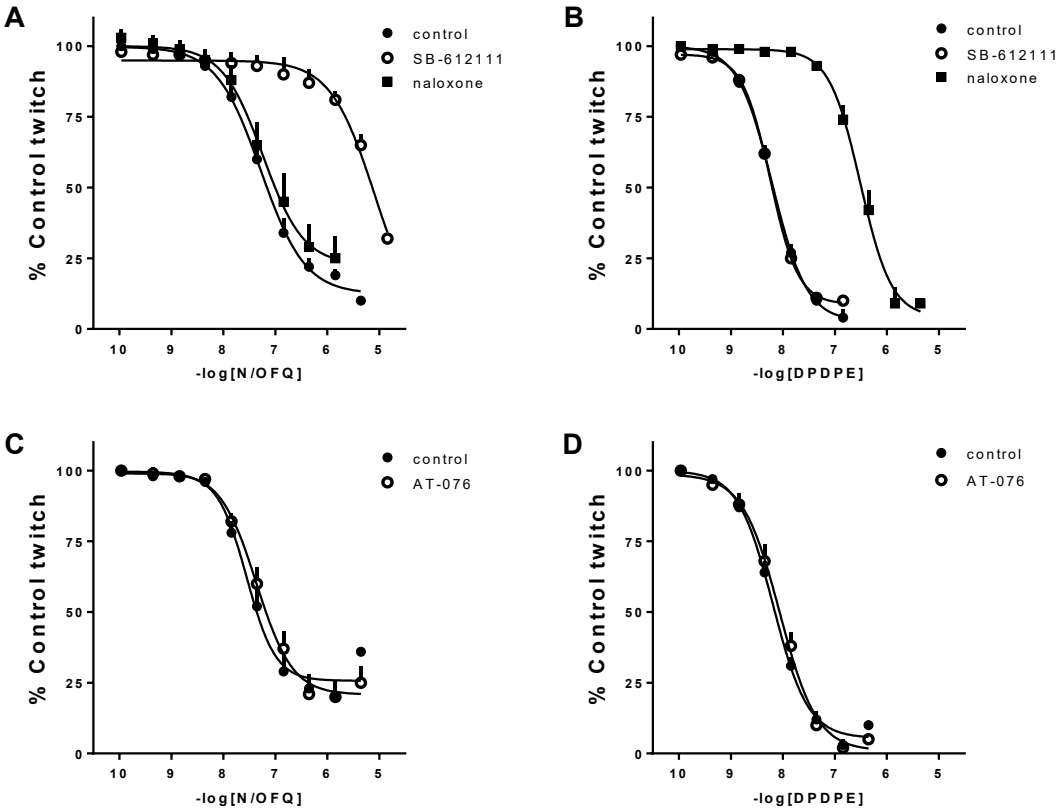


Figure 3.2.35. Electrically stimulated mouse vas deferens. Concentration response curve to N/OFQ (panels A and C) and DPDPE (panels B and D), in absence (control) and presence of SB-612111 1 μM, naloxone 1 μM, and AT-076 1 μM. Data are the mean ± S.E.M. of 4 separate experiments.

Guinea pig ileum – In the electrically stimulated guinea pig ileum, N/OFQ inhibited the electrically induced contraction in a concentration dependent manner with pEC_{50} 7.91 (7.83 – 8.00), E_{max}= 63 ± 2% (Figure 3.2.36A). The mu receptor agonist dermorphin mimicked the effect of N/OFQ being more potent and producing higher maximal effects (pEC_{50} 9.60 (9.45 – 9.74), E_{max}= 92 ± 1%) (Figure 3.2.36B). SB-612111 and naloxone tested as agonist up to 1 μM did not modify the electrically induced twitch. The concentration response curves to N/OFQ and dermorphin were assessed in absence and presence of both antagonists. SB-612111 100 nM was able to shift to right the curve to N/OFQ (pA_2 8.60), while naloxone was inactive (Figure 3.2.36A). In contrast, dermorphin was sensitive to naloxone 100 nM (pA_2 9.01) but not to SB-612111 (Figure 3.2.36B). AT-076 was tested in this preparation as agonist up to 1 μM did not modify per se the electrically induced twitch. At the same concentration the compound slightly displaced the concentration response curve to N/OFQ with a pA_2 value of 6.00 (Figure 3.2.36C). Moreover, AT-076 1 μM was able to shift to right the concentration response curve to dermorphin with a pA_2 value of 7.85 (Figure 3.2.36D).

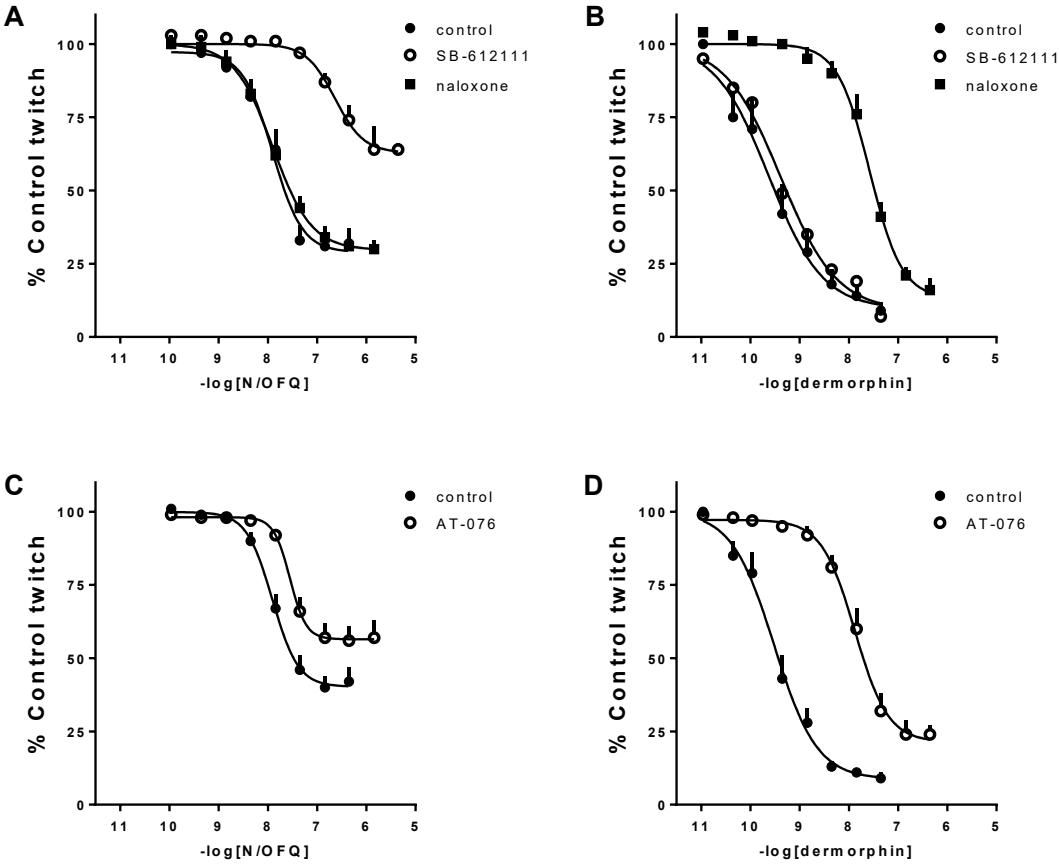


Figure 3.2.36. Electrically stimulated guinea pig ileum. Concentration response curve to N/OFQ (panels A and C) and dermorphin (panels B and D), in absence (control) and presence of SB-612111 100 nM, naloxone 100 nM, and AT-076 1μM. Data are the mean ± S.E.M. of 6 separate experiments.

The potency of standard antagonists and of AT-076 obtained in bioassay studies were summarized in table 3.2.6.

Table 3.2.6. Potencies of standard antagonists and AT-076 in the mouse vas deferens and guinea pig ileum.

	mVD		**gpI**	
	pA_2 ($CL_{95\%}$)		**pA_2 ($CL_{95\%}$)**	
	N/OFQ	DPDPE	N/OFQ	dermorphin
SB-612111	8.15 (6.76-9.55)	inactive	8.60 (8.05-9.15)	inactive
naloxone	inactive	7.74 (7.50-7.99)	inactive	9.01 (8.57-9.45)
AT-076	5.52 (5.04-6.00)	inactive	6.00 (5.81-6.19)	7.85 (7.61-8.09)

Discussion

In the present study the novel non peptide ligand AT-076 previously proposed as universal NOP and classical opioid receptor antagonist (Zaveri *et al.*, 2015) was pharmacologically characterized in vitro at human recombinant receptors and native animal receptors. Confirming previous findings AT-076 consistently behaved as a receptor antagonist. However, the results obtained in calcium mobilization and in bioassays studies demonstrated that AT-076 should be classified as a rather selective kappa receptor antagonist with the following rank of order of potency:

kappa >> mu > delta = NOP.

The first set of data has been obtained in calcium mobilization studies performed in cells expressing NOP or opioid receptors, and chimeric G proteins. This assay has been set up and validated using a series of standard ligands (Camarda *et al.*, 2009; Camarda *et al.*, 2013) and then used for investigating the pharmacological profile of several novel compounds acting at the NOP receptor (Guerrini *et al.*, 2015; Rizzi *et al.*, 2014; Fischetti *et al.*, 2009a), at classical opioid receptors (Adamska *et al.*, 2016; Piekielna *et al.*, 2015; Ben Haddou *et al.*, 2014) or as mixed NOP/opioid ligands (Molinari *et al.*, 2013; Bird *et al.*, 2016; Rizzi *et al*., 2016). Despite the aberrant signaling generated by the chimeric G protein the results obtained in the above mentioned studies were perfectly in line with those obtained with classical assays for Gi/o coupled receptors demonstrating the robustness and usefulness of this assay.

The pharmacological actions of AT-076 were compared with those of standard antagonists i.e. the potent and selective antagonist SB-612111 for the NOP receptor and the non selective opioid receptor antagonist naloxone. In calcium mobilization studies SB-612111 antagonized N/OFQ stimulatory effects with values of potency in line with literature data (Camarda *et al.*, 2009; Marti *et al.*, 2013) being completely inactive at classical opioid receptors, again in line with previous findings (Zaratin *et al.*, 2004; Spagnolo *et al.*, 2007). On the other hand naloxone was inactive at the NOP receptor while it showed the typical order of antagonist potency i.e. mu >> kappa = delta at classical opioid receptors (Marti *et al.*, 2013). In parallel experiments, AT-076 was evaluated as antagonist at NOP and opioid receptors. AT-076 displayed very high potency at kappa receptors, moderate potency at mu receptors and negligible effects at NOP and delta. Thus the following rank order of antagonist potency was measure for AT-076: kappa >> mu >> delta = NOP. It is worthy of mention that the values of potency estimated for standard antagonists and for AT-076 in inhibition response curve and in concentration response curve displacement studies were always very similar further underlining the robustness of the assay and protocol used.

In order to evaluate the nature of the antagonism exerted by AT-076 at the kappa receptor, the classical Schild analysis was performed testing the concentration response curve to dynorphin A in presence of increasing concentrations of AT-076. The antagonist rightwardly shifted the concentration response curve to the agonist but this was associated with a concentration dependent depression of agonist maximal effects, thus suggesting a non competitive interaction with the kappa receptor. However it is known that the calcium mobilization assay is characterized by hemi-equilibrium conditions and these may not allow to reach the equilibrium between the agonist and the antagonist at the receptor binding pocket before the response to the agonist is measured (Charlton *et al.*, 2010). This phenomenon may lead to the classification of non competitive antagonists of molecules actually acting as competitive antagonists. However it is unlikely that this happens for AT-076 since this compound has been previously reported to reduce the maximal effects of non peptide kappa agonist U-69593 in stimulated [^{35}S]GTPγS binding experiments with incubation time of 60 min (Zaveri *et al.*, 2015). Thus previous and present results obtained in different laboratories and with different assays converge in suggesting that AT-076 interact with the kappa opioid receptor as a non competitive antagonist.

Then, the compound was evaluated in the electrically stimulated mouse vas deferens, that allow to investigate the pharmacological activities of new ligands at native NOP (Berzetei-Gurske *et al.*, 1996; Calo *et al.*, 1996) and delta receptors (Hughes *et al.*, 1975). N/OFQ and DPDPE inhibits the electrically induced twitch showing efficacy and potency values in line with literature (Calo *et al.*, 1996; Bigoni *et al.*, 1999; Spagnolo *et al.*, 2007). To investigate the receptor mechanism involved in the actions of N/OFQ and DPDPE, their effects were challenged with SB-612111 and naloxone. SB-612111 antagonized N/OFQ action with high potency (pA_2 8.15) in line with previous data (pA_2 8.50, Spagnolo *et al.*, 2007). As expected (Calo *et al.*, 1996), the effects of N/OFQ were unaffected by naloxone. On the contrary, naloxone antagonized DPDPE action with a pA_2 of 7.74, in line with literature data (pA_2 8.08, Vergura *et al.*, 2008). Is worthy of note that this potency value is similar to that obtained on the delta human receptor (7.56). On the contrary, SB-612111 was found inactive against DPDPE and this confirms previous findings (Spagnolo *et al.*, 2007), Thus these experiments, in line with previous findings, demonstrated that in the mouse vas deferens bioassay the biological actions of DPDPE and N/OFQ are exclusively due to the selective activation of the delta and NOP receptor, respectively. In parallel experiments the effects of N/OFQ and DPDPE were challenged with AT-076. The antagonist antagonized N/OFQ effects with very low potency and was completely inactive against DPDPE. These findings confirmed at native animal receptors

the results obtained on the human receptors in the calcium mobilization assay, and demonstrated that antagonist potency of AT-076 is very low at NOP and delta receptors.

The electrically stimulated guinea pig ileum is a pharmacological preparation used for decades in the field of opioid research and recognized as N/OFQ sensitive preparation soon after the discovery of this peptide (Calo *et al.*, 1997). In line with literature data (Molinari *et al.*, 2013), both N/OFQ and dermorphin inhibited the twitch response with high potency. It is worthy of note that the maximal effects of dermorphin are higher than those of N/OFQ. This can be interpreted assuming a higher expression in this preparation of mu than NOP receptors. The effects of N/OFQ and dermorphin were challenged with the standard antagonists of SB-612111 and naloxone. SB-612111 antagonized N/OFQ action with a pA_2 of 8.60 in line with results obtained in the mouse vas deferens (8.15) and calcium mobilization studies (8.28) as well as previous literature data (8.40, Spagnolo *et al.*, 2007). As expected (Calo *et al.*, 1997) N/OFQ action was not affected by naloxone. On the other hand, SB-612111 was inactive against dermorphin (as described in Spagnolo *et al.*, 2007), while naloxone rightward shifted the concentration response curve to dermorphin with high potency (pA_2 9.01). This value of potency is similar to that obtained in calcium mobilization studies (8.60). Collectively, these findings demonstrate that in this preparation the biological action of N/OFQ and dermorphin is exclusively due to the activation of NOP and mu receptors, respectively.

In parallel experiments, AT-076 antagonized the action of N/OFQ with low potency (pA_2 6.00). This result is in line with the data obtained in the mouse vas deferens and in the calcium mobilization assay, confirming the low potency of AT-076 on the NOP receptor. On the contrary AT-076 antagonized dermorphin action with relatively high potency (pA_2 7.85). This results is in line with the mu antagonist activity of AT-076 proposed by Zaveri *et al.* (2015) and confirmed by the results obtained in the calcium assay. Unexpectedly the value of potency obtained in the guinea pig ileum was approximately 10 fold higher than at human recombinant mu receptor. Species specific differences may account for this discrepancy.

In conclusion, the novel antagonist AT-076 was characterized in vitro to investigate its antagonist proprieties at NOP and opioid receptors. In the calcium mobilization assay on the human recombinant NOP and opioids receptors the compound acts as antagonist with the following rank order of potency: kappa >> mu >> delta = NOP. The moderate potency of AT-076 at mu receptor as well as its very low potency at NOP and delta receptor has been confirmed in bioassay studies. These results are only partially in line with those previously reported in literature (Zaveri *et al.*, 2015). In fact we could confirm in our assay the higher antagonistic potency of AT-076 at mu than delta and NOP reported in [^{35}S]GTPγS studies by Zaveri *et al.* (2015). However our results

strongly differ from those previously reported in terms of kappa receptor antagonists activity; in fact we estimated a subnanomolar potency (pA_2 10) of AT-076 at the kappa receptor while the potency calculated by Zaveri *et al.* (2015) was approximately 30 fold lower (pA_2 8.40). In summary, the present findings indicate that AT-076 should be classified as a quite selective kappa receptor antagonist rather than a pan opioid antagonist.

4. GENERAL CONCLUSIONS

The NOP receptor is the fourth member of the opioid receptor family. Despite the high homology of sequence that is common with classical opioid receptors, the NOP receptor has different pharmacological properties. Given its wide distribution at both central and peripheral levels, the N/OFQ - NOP receptor system plays an important role in several different physiological processes, such as pain, anxiety, reward, depression, memory, cough and micturition reflex, etc. In order to increase our knowledge on the functions controlled by the N/OFQ - NOP receptor system and to foresee the therapeutic indications of innovative drugs acting as NOP ligands, potent and selective agonists and antagonists are needed. In this thesis, the detailed pharmacological characterization of novel peptide and non-peptide NOP ligands encompassing full and partial agonism as well as antagonist activity is presented.

The first part of this thesis reports on the pharmacological characterization of novel NOP receptor ligands of peptide nature. In order to investigate the impact of ligand dimerization on the pharmacological effect of NOP agonists, N/OFQ dimeric compounds were synthesized and characterized in the calcium mobilization assay and in the electrically stimulated mouse vas deferens. The dimeric ligands were generated using as pharmacophore the N/OFQ(1-13)-NH_2 sequence, and this pharmacophore was linked with spacers of different length. The homodimeric derivatives displayed similar values of efficacy and potency compared to N/OFQ in the calcium mobilization assay. Hence, at least under the present experimental conditions, these results do not provide evidence for NOP receptor homo-dimerization. In line with previous findings shortening the N/OFQ sequence to 12 and 11 amino acids produced a progressing loss of potency. Interestingly, the dimerization of these peptides produced a recovery of potency. Studies with heterodimeric compounds demonstrated that the extra address rather message domain is responsible for the recovery of ligand potency induced by dimerization. The information generated by the present structure-activity study may be useful for the design of novel peptide NOP ligands.

The peptide welding technology (PWT) used in the past to generate novel peptide agonists for different GPCR, has been applied for the first time to the NOP antagonist UFP-101, generating the novel compound PWT2-UFP-101. This ligand was assayed in vitro in the BRET based NOP/G protein assay and in the electrically stimulated mouse vas deferens, and in vivo in the mouse forced swimming and in the locomotor activity assays. In vitro, PWT2-UFP-101 maintained the antagonist activity showing similar values of antagonist potency as UFP-101. Furthermore, the PWT modification did not affect the competitive behavior of UFP-101, as demonstrated by Schild

analysis. Then, the PWT compound was tested in vivo where it displayed an antidepressant action in the mouse forced swimming assay being 10 fold more potent than UFP-101. Furthermore, PWT2-UFP-101, but not UFP-101, produced a robust inhibition of horizontal and vertical motor activity in the open field test; this action limits the usefulness of this compound as pharmacological tool for in vivo investigations. Taken together these results suggests that the PWT strategy can be applied to peptide antagonists to increase their in vivo potency. However, this was associated in the case of UFP-101 to a loss of selectivity of action.

In the second part of this thesis, several novel non-peptide ligands for the NOP receptor were investigated. In the first chapter, a detailed characterization and comparison of the pharmacological profiles of the available non-peptide NOP agonists was described. These compounds were assessed in cells based assays including GTPγ[^{35}S] binding, calcium mobilization, NOP/G protein and NOP/arrestin interaction assays. In these assays, all compounds behaved as full agonists with the following rank order of potency: MCOPPB > AT-403 > Ro 65-6570 = Ro 2q = SCH-221510 > AT-202 > SCH-486757. Results obtained with SB-612111, suggest that these non-peptide ligands bind the NOP receptor in the same binding pocket recognized by N/OFQ. Moreover, BRET studies demonstrated that all compounds displayed a certain degree of bias toward G protein. The only exception to this rule was AT-403 that behaved as un unbiased NOP agonist. Finally, bioassay studies performed with tissues taken from wild type and NOP(-/-) mice demonstrated that MCOPPB and AT-403 are the most NOP selective agonists. Collectively the present study demonstrated that MCOPPB and AT-403 are the most potent and selective agonists currently available for the NOP receptor. However the pharmacological profile of the two molecules is not superimposable since the Pfizer compound behaves, similar to the other non-peptide agonists, as a biased agonist toward G protein while the Astraea Therapeutics molecule acts, similar to N/OFQ, as unbiased NOP agonist. Therefore these molecules will be extremely useful in future in vivo studies for investigating functional selectivity in the NOP receptor field and assessing the therapeutic potential of NOP biased agonists.

In the second chapter five non-peptide NOP partial agonists AT-001, AT-004, AT-035, AT-090, and AT-127, were pharmacologically characterized in several in vitro assays. In terms of efficacy, in GTPγ[^{35}S] binding and BRET assays AT compounds behaved as NOP partial agonists, while in calcium mobilization, mouse vas deferens and colon assays, these compounds behaved as full agonists. These differences are most probably due to the efficiency of the stimulus / response coupling of the preparation used. In terms of agonist potency, in all assays the same rank order of potency was obtained i.e. AT-127 $\geq$ AT-090 $\geq$ AT-035 > AT-001 $\geq$ AT-004. Furthermore, AT

compounds showed moderate selectivity for NOP over classical opioid receptors in receptor binding and calcium mobilization assays. In bioassays studies AT-090 and AT-127 showed higher selectivity than the standard NOP agonist Ro 65-6570. Finally, BRET studies demonstrated that AT-090 and AT-127 behave as unbiased NOP partial agonists. Collectively, the analysis of present results suggests that AT-090 and AT-127 are useful pharmacological tools for investigating the therapeutic potential of NOP partial agonists.

In the last chapter of this thesis describes the pharmacological characterization of a novel NOP and opioid receptor antagonist, AT-076, at human recombinant receptors and native receptors expressed in the mouse vas deferens and guinea pig ileum. The pharmacological actions of AT-076 were compared with those of standard antagonists SB-612111 and naloxone, for NOP and classical opioid receptor, respectively. In calcium mobilization studies AT-076 displayed the following rank order of antagonist potency: kappa >> mu >> delta = NOP. These findings were confirmed in isolated tissues. In fact, in the mouse vas deferens AT-076 antagonized N/OFQ effects only at high concentrations being completely inactive against DPDPE. In the guinea pig ileum, AT-076 antagonized dermorphin effects with relatively high potency showing low potency against N/OFQ. In summary, the present findings indicate that AT-076 should be classified as a quite selective kappa receptor antagonist.

In summary, the results summarized in the present thesis provide to the scientific community the detailed pharmacological characterization of novel ligands for the NOP receptor encompassing full and partial agonist, and pure antagonist activities. This information is of great values for the selection of useful pharmacological tools to be used in future in vivo studies aimed at investigating the therapeutic potential of NOP ligands. In particular, compounds such as MCOPPB and AT-403 that are similarly potent and selective for the NOP receptor but display a different ability to promote the interaction of the NOP receptor with arrestin might be extremely useful for investigating NOP functional selectivity. As pointed out in a recent review article (Toll *et al*., 2016) the findings obtained with these compounds will be compared to those coming from genetic studies in which the phenotype and sensitivity to NOP agonists will be assessed in beta arrestin knockout animals. Moreover, medicinal chemistry efforts should be directed to identify selective NOP ligands displaying large bias toward G-protein and arrestin. These molecules associated to the knowledge related to the involvement of G-protein and arrestin signaling in the beneficial action and in the side effects of NOP ligands will allow the rational selection of the best molecules for individual

indications, thus allowing to identify more effective and/or better tolerated innovative drugs acting at the NOP receptor.

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