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NOP and mu opioid receptors: novel ligands and therapeutic opportunities

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Abstract

The aim of the present study was to characterize novel ligands and investigate new therapeutic opportunities regarding the N/OFQ – NOP receptor and classical opioid systems. NOP and opioid receptors are 7TM receptors coupled with inhibitory G proteins, which upon activation lead to inhibition of cAMP levels and calcium currents, and opening of potassium channels. The N/OFQ – NOP receptor and classical opioid systems control different biological functions both in the central nervous system and in the periphery, including pain transmission and modulation of emotional states.

Regarding the NOP receptor, the pleiotropic actions of N/OFQ limited the development of NOP ligands as innovative drugs. The pharmacological concept of functional selectivity (aka biased agonism) might be useful for amplifying beneficial actions and/or counteracting side effects. To investigate the potential of biased agonism at the NOP receptor, new molecules displaying a large bias factor toward G protein and/or arrestin are needed. In our labs, we investigated the effector specific (G protein vs arrestin) structure-activity relationship of the NOP receptor full agonist N/OFQ(1-13)-NH_2 and its derivatives in BRET studies. The best results in terms of biased factor were achieved when modifications with positively charged peptide sequences or linear aliphatic chains were applied to the C terminus of N/OFQ(1-13)-NH_2: these chemical changes generated NOP receptor agonists biased toward G protein. Furthermore, experiments were also carried out to evaluate the *in vivo* effect of NOP ligands already described *in vitro* Ro 65-6570, AT-403 and MCOPPB on analgesia and locomotor activity. Noteworthy, Ro 65-6570 demonstrated a slightly higher therapeutic index (antinociceptive vs sedative effects) in βarr2(-/-) mice. However, all NOP agonists displayed very similar therapeutic index in wild type mice despite significant differences in G protein biased agonism. In conclusion the different ability of inducing G protein versus βarr2 recruitment of a NOP agonist cannot be applied to predict its analgesic versus sedative properties. Additionally, *in vivo* experiments regarding stress coping strategies behavior and NOP receptor antagonists were carried out in a separate set of experiments, in which animals treated with NOP

antagonist SB-612111 develop less depression like behavior after exposure to stress, thus indicating a preemptive effect in the acquisition of depression behavior of NOP receptor antagonists, which had not yet been reported in literature.

Regarding the opioid receptor family, novel synthetic opioids were pharmacologically characterized *in vitro*. Among these, MT-45 behaved as a potent selective mu agonist with a slightly higher efficacy than morphine in dynamic mass redistribution assay. Moreover, fentanyl derivatives were characterized *in vitro* using calcium mobilization and BRET assays. The different chemical modifications observed in the fentanyl derivatives did not modify mu receptor selectivity and produced marginal effects on agonist potency. Interestingly enough, 4-Fluoro-Butyrylfentanyl and Furanylfentanyl behaved as partial agonists in the calcium mobilization assay and as G protein biased agonists in BRET experiments. In fact, when tested as antagonists for the β arresting pathway in the presence of fentanyl, both compounds displayed high antagonist potency.

Finally, continuing the development of new pharmacological tools, a last study focused on the validation of a new synthetic methodology designed for obtaining bifunctional heterotetrabranched peptide ligands. Such innovative molecules could be of interest when investigating theconsequences of the simultaneous activation and/or blockage of different peptidergic receptors. Based on data in literature that support that mixed NOP/mu agonist may act as safer analgesics,a small series of heteromultimeric peptide derivatives targeting the NOP and mu opioid receptors were synthesized, Among these,H-PWT1-N/OFQ-[Dmt^1]dermorphin demonstrated a similar and high agonist potency at both the NOP and mu receptors, thus demonstrating the usefulness of this new synthetic approach that is extremely versatile and applicable to different peptide sequences whose pharmacological activity can be combined for generating dual acting compounds.

In conclusion, the research activity performed during the PhD program shed contributed to increase our knowledge on new strategies, e.g. NOP receptor biased agonists and mu/NOP bivalent ligands, which can be useful for the development of innovative and safer analgesic drugs

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1. INTRODUCTION

1.1 G Protein Coupled Receptors

G-protein coupled receptors (GPCRs) represent the biggest and most diversified group of membrane receptors. GPCRs are able to transduct a large number of extracellular stimuli, such as peptides, ions, small molecules, photons, and proteins and pass forward these extracellular signals in the form of downstream pathways. GPCRs are characterized by seven transmembrane helices structure (Figure 1.1.1), with the N-terminal portion in contact with the extracellular environment and the C-terminal portion in contact with intracellular cytosol. The third cytoplasmic loop and the C-terminal portion interact with the G protein. GPCRs are the biggest protein family in the human proteome. These receptors can be divided into larger classes (i.e. secretin, , glutamate, frizzled/taste2, adhesion 002 rhodopsin, and other 7TM receptor families) and finally subdivided into smaller families based on sequence homology (e.g. opioid receptors belong to the somatostatin-opioid-galanin (SOG) cluster of γ-group/rhodopsin family of GPCRs (Fredriksson et al., 2003).

Signal transference is mediated through binding between receptors and various intracellular proteins located alongside the membrane (e.g., kinases, heterotrimeric G-proteins, and arrestins) (Rajagopal et al., 2010), which subsequently activate effectors that trigger cellular and physiological responses. GPCRs signalling is implicated in numerous diseases, in fact, approximately 30% of all drugs nowadays in the clinic target GPCRs for medical therapeutics (Wise et al., 2002). Therefore, comprehension of how these receptors elicit their biological function is of great interest in the pursue to safer, more efficacious and better tolerated drugs.

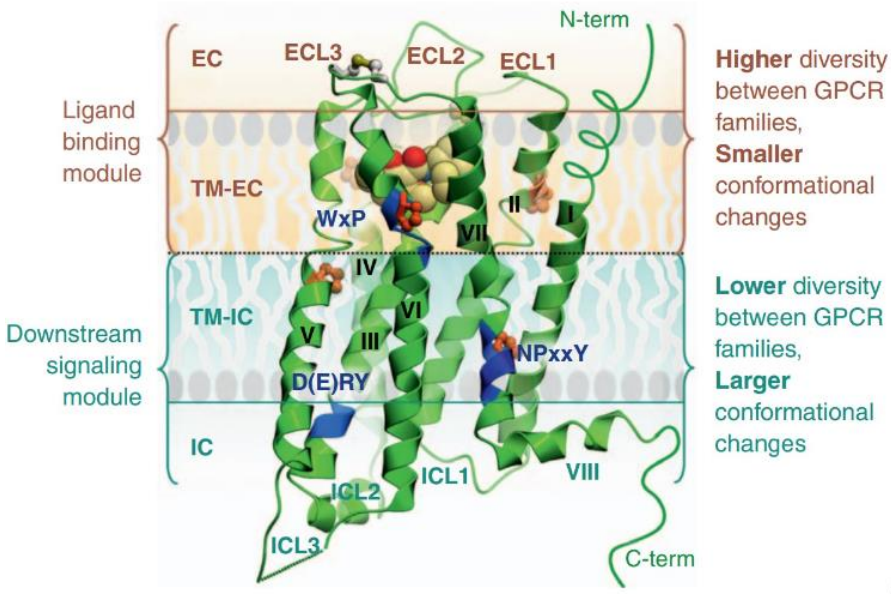


Figure 1.1.1 General architecture and modularity of GPCRs. N-terminal extracellular domains (top side), C-terminal intracellular domains (bottom side). Image taken from (Katritch et al., 2012)

Structural data of active and inactive GPCRs conformation as well as site by site mutations analysis of GPCRs sequence are now widely accessible; however the mechanisms by which molecules that bind GPCRs are able to elicit their signaling have not yet fully comprehended. To better understand and establish the GPCR activation processes, the classical model for GPCR activity is still widely accepted. In such model, upon receptor binding by the agonist, the GPCR causes the receptor to assume a conformation that results in the activation of heterotrimeric G proteins. This activation takes part after the exchange of guanosine diphosphate (GDP) for guanosine-5'-triphosphate (GTP) by the Gα subunit of the G protein, which cause the separation of the heterotrimeric protein complex into Gα and Gβγ subunits. The separation of this complex leads to the production and further signalling by intracellular second messengers, such as cyclic AMP, diacylglycerol and calcium. The signalling evoked by the activated conformation of the GPCR is finished after phosphorylation of the cytoplasmic chains in difference sites as well as in the tail of the GPCR, which occurs mostly by GPCR kinases (GRKs). After phosphorylation of multiple sites, the binding of arrestins (i.e. β-arrestin 1 and 2) take place with sub consequent desensitization of the receptor and final internalization carried by clathrin-coated pits (Lefkowitz and Shenoy, 2005). In such scenario, heterotrimeric G proteins are the ones responsible for signal transduction and β-arrestins regulate receptor desensitization and internalization, with a crucial role in regulating GPCR signalling. It is now known, however, that β-arrestins may act not only as regulators of GPCR desensitization, but also as proteins able to transduct the GPCR signalling through various mediators such as mitogen-activated protein kinases, proto-oncogene tyrosine-protein kinase SRC, nuclear factor-κB and phosphoinositide 3-kinase. Furthermore, biochemical data show that the β arrestins mediated signaling may have different functions and may play a different role in physiological responses when compared to the responses elicited by G proteins *per se* (Lefkowitz and Shenoy, 2005; Rajagopal et al., 2010) (Figure 1.1.2).

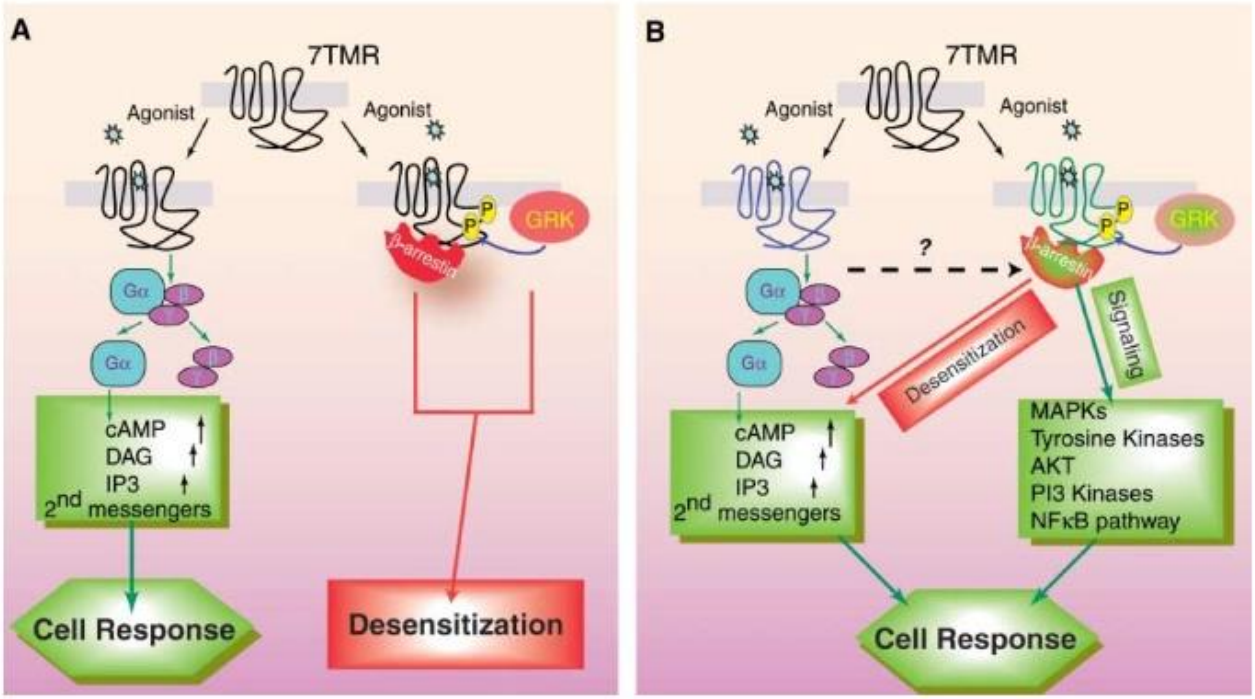


Figure 1.1.2 Signal transduction by seven transmembrane receptors. (A) Classical paradigm. The active form of the receptor (R*)stimulates heterotrimeric G proteins and is rapidly phosphorylated by G protein–coupled receptor kinases (GRKs), which leadsto β-arrestin recruitment. The receptor is thereby desensitized, and the signalling is stalled. (B) New paradigm. β-arrestins notonly mediate desensitization of G protein–signalling but also act as signal transducers themselves.Image taken from (Lefkowitz and Shenoy, 2005)

The biological role of arrestins regarding the classical opioid receptor family was assessed by Bohn and collaborators *in vivo* using knockout mice for the β-arrestin 2 gene, in which these animals, after administration of morphine, displayed an enhanced analgesic potency as well as prolongation of its analgesic effect (Bohn et al., 1999), with remarkable reduced tolerance liability (Bohn et al., 2002) compared to their wild type counterparts. In addition, mice lacking the β-arrestin 2 gene seem to display less of the classical side effects profile related to opioid treatment (respiratory depression and constipation) after morphine administration when compared to wild type littlermates (Raehal et al., 2005). With that in mind, it is postulated that some agonists may act as biased agonists which are ligands able to bind a single receptor and differentially activate some of its pathways over others, for example G-protein over arrestin or vice versa. It is crucial to find new molecules with such unique characteristics (biased towards G protein or towards arrestin) to be able to analyze if these findings in fact can generate more tolerable opioid analgesics.

Biased ligands may have important implications in the development of new drugs. In theory, it is possible that a biased agonist towards a specific pathway, which selectively activates a single pathway of transduction, could be able to maximize therapeutic biological responses and

minimize side effects commonly seen in unbiased agonists. Some examples of this innovative strategy have already been described in the literature. MOP opioid receptor agonists, which are biased towards G protein recruitment, are in development as highly tolerable analgesics, since despite produce a potent antinociception effect, do not induce the effects common side effects to the use of morphine, such as reduced motility gastrointestinal and respiratory activity suppression (DeWire et al., 2013). Similarly, GPR109 receptor agonists biased towards the G protein pathway show reduced serum fatty acid levels without inducing skin flushing (Walters et al., 2009). Furthermore, agonists of AT1 receptor (angiotensin II type 1 receptor) biased towards the recruitment of β-arrestin 2 can act as effective drugs for the treatment of heart failure (Violin et al., 2010). Also in others systems such as serotonergic and dopaminergic have already been found ligands biased to a specific signaling pathway which understand the role of each pathway in generating the biological responses (Allen et al., 2011; Bohn &Schmid, 2010).

Based in the above mentioned findings it has been proposed that opioid receptor biased agonists towards the receptor/G-protein conformation instead of that of the receptor/arrestin interaction may display *in vivo* higher efficacy and/or better tolerability (Violin et al., 2014)

1.2. N/OFQ -NOP system

Nociceptin/Orphanin FQ (N/OFQ) is a heptadecapeptide (FGGFTGARKSARKLANQ) that has been isolated from an animal brain extract by two different groups of researchers, independently, in 1995, which binds the N/OFQ peptide (NOP) receptor with high affinity (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ is derived from a preproN/OFQ peptide precursor (ppN / OFQ), which sequence shares a high degree of homology throughout species, structured by 176 amino acids in humans, 181 in rats, and 187 in mice. Furthermore, the ppN/OFQ gene resembles those of the opioid peptide precursors, specially preproenkephalin and preprodynorphin, which suggests a common ancestor for these peptide precursors (Mollereau et al., 1996). N/OFQ is structurally related to opioid peptides, however the presence of phenylalanine instead of tyrosine in position 1 causes that this peptide does not bind to classical opioid receptors (Reinscheid et al., 1995). Moreover, N/OFQ is the first ligand discovered with the application of reversed pharmacology. Before molecular biology techniques became popular, ligands would be firstly discovered and then their receptors determined by classical pharmacology approaches. Nowadays, most GPCRs are identified using their DNA sequences and which can initially have no match to known natural ligands and get thus classified as orphan GPCRs. Homology cloning was applied for discovering the endogenous agonist of the orphan NOP receptor and subsequent transfected in mammalian cells; these tissue extracts were prepared from the brain, purified, and fractionated in a

series series of rounds. The extracts were then further analyzed regarding the ability to modify cAMP levels in both cells expressing the NOP receptor and in wild type cells. By the use of this approach, N/OFQ was finally isolated and characterized (Meunier et al., 1995; Reinscheid et al., 1996).

The NOP receptor is coupled to the $G_{i/o}$ protein, so its activation leads to the inhibition of both cAMP levels (Wu et al., 1997) and calcium channels (Connor et al., 1996b) and to stimulation of potassium currents (Vaughan & Christie, 1996). Therefore, NOP signaling induces inhibitory effects, causing reduction of neurotransmission (Schlicker & Morari, 2000) and/or inhibition of neuronal firing (Heinricher, 2003), depending on the site (pre- or postsynaptic) NOP receptor expression. Similarly to other GPCRs, it is known that the NOP receptor may interact (and signal downstream pathways) with G-proteins but also with other proteins, such as GRKs and arrestins (Soergel et al., 2014).

The NOP receptor shares high structural similarities with opioid receptors, which suggests that the four receptors (MOP, KOP, DOP and NOP) evolved from a common ancestor. In fact, amongst opioid peptides, N/OFQ shares similar sequence to that of dynorphin A. For this reason, the NOP receptor is represented as opioid-related rather than from the opioid receptor family (Alexander et al., 2019). In fact, the crystal structure of the human NOP receptor was observed in complex with the antagonist compound-24 (C-24) (Thompson et al., 2012a) which showed some crucial discrepancies in the binding pockets of NOP when compared to the classical opioid receptors (Thompson et al., 2012a; Filizola and Devi, 2013) (Figure 1.2.1).

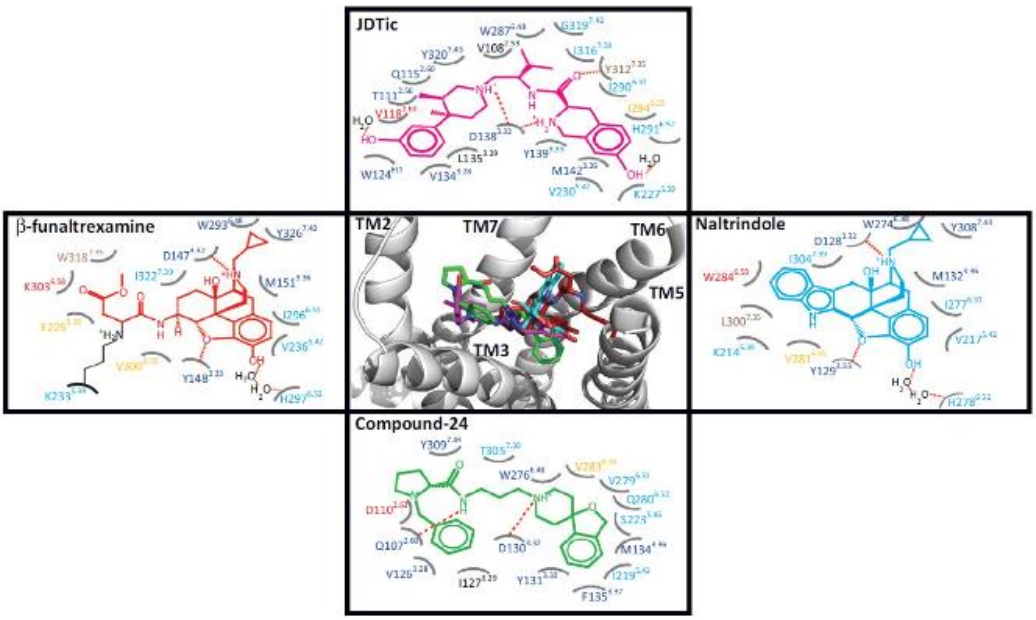


Figure 1.2.1. Overlay of the crystallized opioid ligands in a representative opioid receptor crystal structure along with schema of their interaction modes in each crystal structure. The central panel shows an overlay of β-funaltrexamine (red), naltrindole (cyan), JDTic (magenta), and compound 24 (green) in the MU receptor crystal structure, which is partially shown in a grey cartoon representation. Interaction schema for β-funaltrexamine, naltrindole,JDTic, and compound 24 in the mu opioid (MU), delta opioid (DOP), kappa opioid (KOP), and NOP receptor crystal structures are shown in the left, right, upper, and lower panels, respectively. Identical residues in all four receptors are shown in blue. Identical resides in MU, DOP, and KOP but unique to NOP are shown in cyan. Divergent residues in all four opioid receptors are shown in red. Divergent residues in MU, DOP, and KOP but not NOP are shown in brown. Unique residues to either MU, DOP, or KOP are shown in orange. Image taken from (Filizola and Devi, 2013).

After its discovery, the N/OFQ-NOP system has been widely examined both by academic and industrial groups, which resulted in the discovery of a series of selective NOP receptor ligands (Mustazza and Bastanzio, 2011; Calo and Guerrini, 2013). Following the discovery of such compounds, the role of this system in physiology and pathology states has been, at least in some measure, decoded. N/OFQ and the NOP receptor are involved in the regulation of diverse biological functions regarding pain, learning and memory, food intake, mood and anxiety, locomotion, cough and micturition reflexes, cardiovascular homeostasis, intestinal motility and immune responses (Lambert, 2008) (Figure1.2.2.).

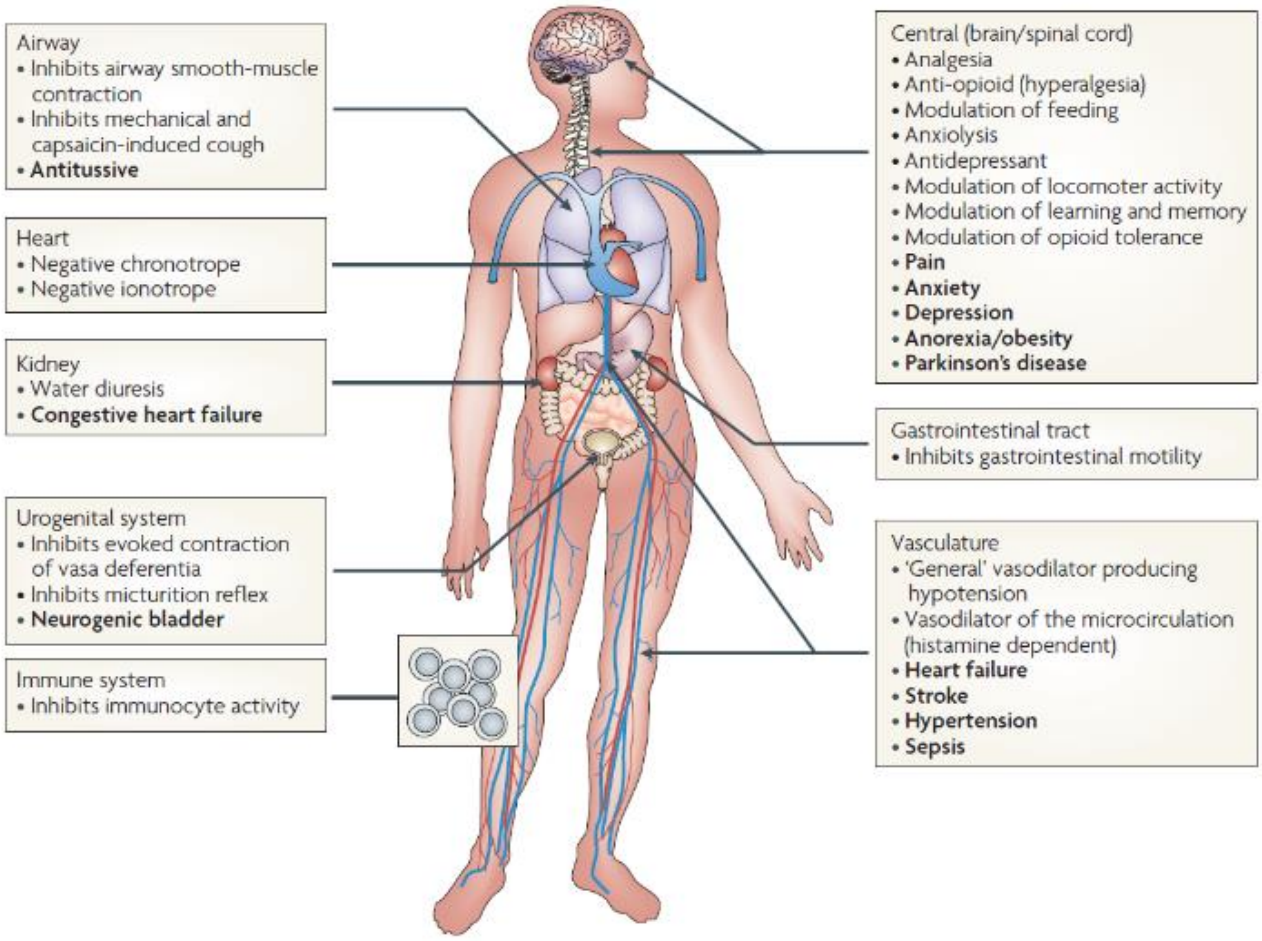


Figure 1.2.2. Pleiotropic effects of nociceptin/orphanin FQ (N/OFQ) on major organ systems. Potential clinical indications are noted in bold.Image taken from (Lambert, 2008).

Since the first studies conducted in rodents, the role of N/OFQ in pain transmission has been observed. After the administration of N/OFQ, hyperalgesia, allodynia and analgesia has been described. Furthermore, when administered supraspinally, N/OFQ is able to enhance pain sensitivity in rodents (Meunier et al., 1995; Reinscheid et al., 1995). These hyperalgesic effects of N/OFQ was only seen after intracerebroventricular (i.c.v.), rather than after intrathecal (i.t.) administration. It has been reported that N/OFQ in supraspinal pain modulation mostly acts counteracting the analgesic effects elicited by opioid administration, thus working as (Grisel and Mogil, 2000). In fact, this anti-opioid role of N/OFQ has been confirmed by results obtained in different conditions, for example it was reported that N/OFQ counteracts the analgesic effects of morphine (Grisel et al., 1996; Zhu et al., 1997; Calò et al., 1998; Bertorelli et al., 1999), and of a series of selective opioid receptor agonists (King et al., 1998). Noteworthy, these "functional opioid antagonism" effects of N/OFQ are susceptible to tolerance liability (Lutfy et al., 1999)

N/OFQ was able to block morphine-induced place preference (Ciccocioppo et al., 2000). Such effect was later observed in other drugs of abuse that act at the dopaminergic

mesocorticolimbic circuits, such as cocaine, alcohol and amphetamine (T. Zaveri, 2011). The reason why N/OFQ mitigates rewarding effects evoked by these drugs may be by directly inhibiting NOP expressing dopaminergic mesocorticolimbic neurons (Murphy et al., 1996).

The peptide N/OFQ is involved in learning and memory, being able to inhibit long-term potentiation in rat hippocampal slices (Yu et al., 1997). These findings were corroborated from similar studies using mice knockout for NOP receptor (NOP(-/-)), which not only displayed enhanced learning ability, but also showed better memory than wild type animals (Manabe et al., 1998).

NOP antagonists are reported to evoke antidepressant like effects in behavioural despair models, such as forced swimming test in mice (Redrobe et al., 2002). Clinical studies suggest that N/OFQ levels are increased in patients living with major depression. Moreover, it has been reported that N/OFQ may inhibit noradrenaline and serotonin release from the cerebral cortex as well as neuronal firing in the dorsal raphe and locus coeruleus. Assuming the inhibitory effects of N/OFQ on the release of neurotransmititters, chronic stress conditions could stimulate the release of N/OFQ, which may reduce monoaminergic signaling. By counteracting these effects of N/OFQ, NOP antagonists may enhance levels of noradrenaline and serotonin, thus restoring monoaminergic transmission. Therefore, NOP receptor antagonists, may obtain their antidepressant effect by the same outcome of that of classical antidepressants, i.e., an increase in concentrations of monoamines (Gavioli and Calo', 2013). Also, it has been reported in literature that N/OFQ affects the brain response to stress and anxiety, indeed N/OFQ play a role in counteracting behaviors related to stress, promoting anxiolytic like effects. The mechanisms by which N/OFQ as well as NOP agonists elicit an anxiolytic phenotype after adminstration are not completely understood, but findings suggest the involvement of GABAA receptor signalling. However CRFergic and serotonergic pathways could also play a crucial role in inducing their anxiolytic effects (Gavioli and Calo', 2013). The inhibitory locomotor effect at higher doses of N/OFQ limit the development of innovative anxiolytic drugs with NOP agonists.

In fact, the endogenous peptide N/OFQ has been reported to inhibit the spontaneous locomotor activity in rodents, such inhibitory effect of N/OFQ is erased with the administration of NOP antagonists and/or in NOP(-/-) mice. The N/OFQ-NOP receptor system, however, does not seem to tonically control spontaneous locomotion, once the administration of NOP antagonists *per se* do not change locomotor parameters and knockout animals do not display a specific locomotor phenotype when compared to their wild type littermates. Noteworthy, intra nigral administration of the selective NOP antagonist UFP-101, dose-dependently enhanced rat performance in the drag and rotarod tests (Marti et al., 2004), in addition NOP(-/-) mice outperformed wild type animals in the

same assays. These results suggest that endogenous N/OFQ may indeed exert, at least partially, an inhibitory influence over motor activity that becomes relevant at higher doses. Other findings with studies in models of Parkinson's disease suggest that NOP receptor antagonists could represent a new strategy for treating hypokinetic disorders and proposed the blockage of the NOP receptor for treating Parkinson (Mercatelli et al., 2020).

N/OFQ-NOP receptor system also plays a role in food intake control, with supraspinal N/OFQ (1–10 nmol) injections enhancing food intake in rats. This effect was also observed with a series of NOP synthetic agonists. Moreover, receptor antagonists and knockout studies confirmed the role of NOP receptor in this biological function (Micioni Di Bonaventura et al., 2019).

The importance of the N/OFQ-NOP receptor system as target for innovative drug development has been demonstrated in several studies, such as that NOP receptor agonists as novel analgesics, as anxiolytic drugs, as well as to treat abuse liability, and for the treatment of cough and urinary incontinence. Under other conditions, NOP receptor antagonists can be useful in the development of innovative drugs for treating, major depression and Parkinson's disease (Lambert, 2008).

1.3 Classical opioid receptor system

The history of classical opioid receptors precedes that of the NOP receptor. Beckett and Casy in 1954 postulated the existence of receptors for opiate drugs (Beckett and Casy, 1954) based on their structure activity relationship studies with synthetic opiates related to antinociceptive activity. These receptors were named opioids, because of the revelation of endogenous peptides that displayed similar effects to those of opiate drugs. Portoghese and colleagues (1965) theorized the presence of more than one opioid receptor type. Endogenous opioid systems are importantly implicated in a wide variety of biological states such as sensory, motivational, emotional, and cognitive functions. Such neuropeptides act as inhibitory transmitters, which can balance neurotransmission across different neuronal circuits, setting thresholds for neuron firing. The first demonstration of opioid receptors binding pockets were achieved by receptor binding studies with radio labeled molecules such as naloxone and etorphine (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). The first findings that these receptors did not unite in a homogeneous group was published in 1976 (Martin et al., 1976). The theorized receptors were named after the compounds used in these studies that were able to activate each distinct receptor, i.e. the mu, for morphine receptor and the kappa for ketocyclazocine receptor. Afterwards, further pharmacological studies of opioid peptide effects in guinea-pig ileum and mouse vas deferens evidenciated the presence of a third opioid receptor named the delta, for deferens receptor (Lord et al., 1977). The three opioid

receptors, mu, delta, and kappa have later been cloned and these recombinant receptors displayed binding and functional features consistent with their native analogues (Evans et al., 1992; Kieffer et al., 1992; Kieffer, 1995; Onogi et al., 1995).

Regarding endogenous opioid peptides, three different groups of opioid peptides have been described: endorphins, enkephalins, and dynorphins; each family derives from a distinct precursor protein, prepro-opiomelanocortin (POMC), prepro-enkephalin, and prepro-dynorphin, respectively. Each precursors is encoded by different genes and undergo complex cleavages and posttranslational changes resulting in the synthesis of numerous active peptides. The mammal opioid peptides share a common amino terminal sequence of Tyr-Gly-Gly-Phe (followed by Leu or Met), which is called the opioid message domain. This sequence is followed by carboxy terminal extensions resulting in peptides ranging from 5 to 31 residues. Worthy of mention, POMC can also result also into non opioid peptides such as adrenocorticotropic hormone, melanocyte stimulating hormone, and β-lipotropin. Proenkephalin displays multiple copies of met-enkephalin, as well as one copy of leu-enkephalin. Prodynorphin displays three peptides of differing lengths which begin with the leu-enkephalin sequence: dynorphin A, Dynorphin B, and neoendorphin. A comparison of opioid peptide sequences is can be seen in Table 1.3.1

Other relevant opioid peptides are endomorphin 1 (EM-1) which was first described in 1997 in the bovine brain (Zadina et al., 1997) and endomorphin-2 (EM-2) which is found aside with EM-1 in the human brain cortex (Hackler et al., 1997). Their sequences are Tyr-Pro-Trp-Phe-$NH_2$and Tyr-Pro-Phe-Phe-NH_2, for EM-1 and EM-2, respectively (Table 1.3.1). Both peptides showed high affinity for the mu opioid receptor with Ki values being 1.1 and 1.3 nM, respectively (Hackler et al., 1997), being more than 4000 fold selective for mu when compared to their selectivity to other opioid receptors. Radioimmunological and immunocytochemical studies showed that endomorphins are widely distributed in the human, bovine, and rodent central nervous system. EM-1 is widely distributed in the brain, being specially abundant in the limbic areas such as nucleus accumbens, the cortex, the amygdala, the thalamus, the hypothalamus, the striatum, and the dorsal root ganglia. By contrast, EM-2 is more common in the spinal cord and lower brainstem, hypothalamus, the nucleus of the solitary tract, with lower concentrations of EM-2 observed in the nucleus accumbens, the stubstantianigra, the nucleus raphe magnus, the ventral tegmental area, pontine nuclei and the amigdala. The effects prompted by the exogenous administration of EM-1 and EM-2 endogenous neuropeptides are comparable to that of distinct mu selective opioid peptides (Fichna et al., 2007).

Opioid receptors are coupled to Gi proteins, which upon receptor binding result in the inhibition of adenylyl cyclase activity and voltage gated calcium channels and stimulation of potassium currents, which also happens upon NOP receptor binding of agonists; These receptors are

coupled to second-messenger pathways, e.g. MAP kinases and phospholipase C mediated cascades. Moreover, other interactions of the activated receptor with GRKs and β-arrestins are also involved in the opioid receptors regulation and downstream cascade. Prolonged exposure to opioids leads to changes at multiple levels within these signalling cascades that can be correlated to physiological effects such as tolerance, sensitization, and withdrawal (Brunton et al., 2011). According to Bohn and collaborators, the recruitment of β-arrestin after the activation of the opioid receptor can lead to some undesirable phisyological responses that are not considered therapeutic, such as respiratory depression and constipation. Such assumption has yet to be confirmed by future studies.

Table 1.3.1 Amino acid sequence of opioid peptides.

Peptide	**amino acidic sequence**
Enkephalins	YGGFM
	YGGFL
Dynorphins	YGGFLRRIRPKLKWDNQ
	YGGFLRRQFVVT
	YGGFLRKYPK
Endorphin	YGGFMTSEKSQTPLVTLKNAIIKNAYKKGE
Endomorphin 1	YPWF
Endomorphin 2	YPFF
N/OFQ	FGGFTGARKSARKLANQ

1.4 Bivalent ligands of opioid receptor in analgesia

For a long while, selective agonists for the opioid receptors constitute the preferable treatment for diverse typologies of pain. Drugs like fentanyl and morphine, both agonists for the mu opioid receptor, represent the golden standard of analgesia. However, their robust action in terminating pain signalling is associated with important side effects such as constipation, respiratory depression and, after prolonged treatment, dependence and tolerance. Such profile of side effect has led to the search of new analgesics that are equally potent but present less side effects.

Evidence from studies in animal models of acute pain and chronic neuropathic pain in rodents (Courteix et al., 2004) demonstrate a synergic effect after spinal administration of morphine

and N/OFQ. Also in non human primates the administration of NOP agonists seems to potentiate the antinociceptive effect of morphine and mu agonists in general (Cremeans et al., 2012). It is reasonable to postulate that bivalent ligands for the mu and NOP receptors can evoke strong analgesic effect. Such synergism between both receptors could even reduce the necessary dose to elicit analgesia, thus eliciting less side effects and making drugs safer and better tolerated.

More recently, bifunctional NOP/mu agonists BU08028 e SR16435 have been reported to generate antinociceptive actions in models of inflammatory and neuropathic pain, with enhanced potency when compared to selective NOP and mu ligands (Sukhtankar et al., 2013; Ding et al., 2016). In 2014, cebranopadol has been developed by the pharma company Grünenthal, which is a bivalent NOP/mu agonist. The compound has shown an effect about 100 times more potent than morphine and also, longer lasting. Regarding the classical side effects associatedto mu receptor activation, cebranopadol presents a therapeutic index, between its analgesic effect and respiratory depression effect, that is wider when compared to that of fentanyl (Calo et al., 2018). In addition, the results of phase III clinical trials in cancer patients also reported a better efficacy and therapeutic index compared to the standard opioid ligands (Eerdekens et al., 2019).

1.5 Peptide Welding Technology (PWT)

Peptides can potentially constitute innovative drugs, based on their high selectivity and low toxicity. The pharmacokinetics of such compounds represent however an important limit for what regards their use as pharmaceuticals: that is because peptides generally get metabolized rapidly by surrounding peptidases which reduces drastically the half life and duration of action of these compounds, thus representing a challenge in the development of peptide drugs .

More recently, the development of drugs with peptide structure has increased, thanks to new chemical strategies aiming in making peptides more resistant to peptidases. An example is the peptide N-metilation, which consists in the simple replacement of an H with a methylgroup: this generates a conformational change of the peptidestructure, preventing the access of the peptide into the catalic pocket of proteases and therefore, and thus prolonging the peptide half time (Chatterjee et al., 2013). Another example is peptide cyclization, which is achieved by reaction of different chemical groups that can generate disulfide or lattamebridges. Such molecules are in fact more resistant to peptidases and are also cell permeable, with retained ability to recognize specific intracellular targets (Diderich and Heinis, 2013; Lian et al., 2014). Thanks to these strategies, cyclosporine is one of the very few peptide compounds that can be orally administrated(Aguirre et al., 2016). An approach that has been reported efficacious in the effort to enhance peptide resistance to peptidases and therefore enhance their life time in the body is the synthesis of multimeric

molecules. Such molecules are constituted of a central structure called “core”, from which can be connected various numbers of peptides. The first example of peptide multimeric molecule is the octameric immunogenic synthesized by Chang et al, with the aim to generate antibodies against the alfa subunit of G protein (Chang et al., 1988). The same structure has been successively used to synthesize tetrameric molecules derived from neuropeptides such as neurotensin, enkephalin and N/OFQ (Bracci et al., 2003).These molecules have shown an improvement in stability in human plasma and rat brain membrane extracts, while maintaining a high affinity for the target receptor. The major limitation of this multimerization strategy is the complexity of their synthesis: the larger the molecule, the more difficult the synthesis and purification become. Precisely for this reason, a strategy was devised in the laboratories of the University of Ferrara, called Peptide Welding Technology (PWT). The PWTstrategy involves a two-step procedure: the peptide sequences and the core are separately synthesized and purified, and are subsequently joined through an highly stereospecific chemical reaction.

In particular, in the case of homomeric PWTs, the "thiol-Michael reaction" is used, an extremely chemoselective reaction between a thiol (eg the side chain of a Cys residue) and an α, β-unsaturated carbonyl group which results in the formation of a thioether. The reactants are added in a 1: 1 proportion since they react completely with each other and form only the product, without the formation of secondary products. To investigate the relationship between PWT architecture and pharmacological activity, three types of PWT cores were tested:

- PWT1 provides a three-residue Lys architecture, originally tested by Chang et al;
- PWT2 provides a cyclam structure;
- PWT3 consists of symmetrically branched ethylenediamine.

Four maleimides are then connected to each of the cores, while a residue of Cys is added to each of the four peptide sequences. The Cys residueprovides the thiol group (-SH) which promotes the addition of the peptide to the α, β-unsaturated carbonyl group of maleimides. This means that there must be no Cys residues inside the biologically active portion of the peptide that must be connected to the core. The Cys residue to be reacted is added to the opposite terminuswith respect to the one containing the pharmacophore. The reaction lasts about ten minutes and has a virtual yield of 100%, thus making PWT a very advantageous method for synthesizing tetrameric peptides (Guerrini et al., 2014).

The data obtained on homomeric PWTs in vitro demonstrate the same efficacy as peptides in their linear form, with variable increase in potency. The in vivo tests, on the other hand, were

very positive, with a robustincrease in potency (from 3 to 30 fold) and especially in the duration of action (up to 50 times, for supraspinal administration). It has been hypothesized that this is due to the tetrameric structure of PWT, which makes the peptides less susceptible to the action of peptidases (Calo et al., 2018)

2. AIM OF THESIS

The present study was aimed to increase our knowledge and investigate the potential of novel pharmacological strategies to generate innovative drugs acting at NOP and MU opioid receptors. In particular studies were performed to assess the potential value of the biased agonism and bifunctional ligand strategies in the field of opioid receptors including NOP . Moreover novel biological actions of opioid ligands as well as innovative and interesting MU selective and mixed mu/NOP agonists were discovered in the frame of the present studies.

3. MATERIALS AND METHODS

3.1 Drugs and reagents

3.1.1 *In Vitro*

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).

NOP ligands

N/OFQ(1-13)-NH_2, N/OFQ-NH_2, N/OFQ and all N/OFQ peptide derivatives were synthesized in the Department of Chemical and Pharmaceutical Sciences of the University of Ferrara, Italy, and stock solution were made in bidistilled water (1 mM) and stored at -20 °C. The homotetrameric PWT1 derivative NOP PWT1-N/OFQ was synthesized in house, stock solutions were made in DMSO at the concentration of 10 mM. The NOP antagonist SB-612111 was purchased from Tocris Bioscience, Bristol, UK.

Opioid ligands

Stock solutions of DPDPE, morphine, dynorphin A, dermorphin were solubilized in bidistilled water at a final concentration of 1 mM. MT-45 and morphine were purchased from LGC Standards (LGC Standards S.r.L., Sesto San Giovanni, Milan, Italy). MT-45 was solubilized in DMSO at a final concentration of 10 mM, and kept at – 20°C until use. Fentanyl was from Bio-Techne (UK) (authorization SP/060 25/05/2018 to GC) and all the fentanyl derivatives acetylfentanyl, acryloilfentanyl, butyrylfentanyl, 4-fluoro-butyrylfentanyl, furanylfentanyl and ocfentanyl were provided by Prof. Matteo Marti and were solubilised in DMSO at a final concentration of 10 mM. The linear homomers Dermorphin(O2Oc)2NH_2 [Dmt^1]dermorphin as well as the homotetrameric PWT1 derivatives PWT1- dermorphin; PWT1-dermorphin(O2Oc)2; PWT1-[Dmt^1]dermorphin were synthesized in house and solubilised in DMSO at the concentration of 10 mM. All stock solutions were stored at -20 °C. Naloxone was purchased from Tocris (Bristol, UK).

Bivalent ligands

As for the bivalent NOP/mu, compounds were synthetized as previously described by Pacifico et al. (2019) , which generated the following heteromeric compounds: H-PWT1-N/OFQ-

dermorphin(7a); H-PWT1-N/OFQ-dermorphin(O2Oc)2; H-PWT1-N/OFQ-[Dmt1]dermorphin. Stock solutions of 1mM in DMSO were kept at -20°C.

3.1.2 *In Vivo*

NOP ligands

The NOP agonists Ro 65-6570 (synthesized and purified in house at the Department of Chemistry and Pharmaceutical Sciences, University of Ferrara, Italy), MCOPPB (Sigma Aldrich, St. Louis, MO, USA), and AT-403 (synthesized at Astraea Therapeutics, Mountain view, CA), and the NOP antagonist SB-612111 (Tocris Bioscience, Bristol, UK), and the tricyclic antidepressant nortriptyline (Sigma Aldrich, St. Louis, MO, USA) were used. The stock solutions of NOP ligands were prepared in 100% dimethylsulfoxide (DMSO), stored at -20 °C and dissolved in saline solution (NaCl 0.9%) just before the experiments. The final concentration of DMSO did not exceed 1%. Nortriptyline was solubilized in saline. NOP ligands were injected intraperitoneally (i.p.), in a volume of 10 ml/kg, 30 min prior to acute stress exposure (inescapable electric footshock or forced swim stress), while nortriptyline was injected 60 min before stressful situation. Control groups were treated with the same vehicle by the same via and volume of administration as treated group. Animals were randomly assigned to the treatment

3.2 *In vitro* studies

3.2.1 Calcium mobilization assay

CHO cells stably coexpressing the human recombinant mu or kappa receptors with the C-terminally modified $G\alpha_{qi5}$ and CHO cells coexpressing the delta receptor and the $G\alpha_{qG66Di5}$ chimeric protein were generated as previously described (Camarda and Calo). 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 (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml), geneticin (G418; 200 µg/ml) and hygromycin B (100 µg/ml). Cell cultures were kept at 37 °C in 5% CO_2 / humidified air. When confluence was reached (3-4 days), cells were sub-cultured as required using trypsin / EDTA and used for experimentation. Cells were seeded at a density of 50,000 cells / well into 96-well black, clear-bottom plates. After 24 hours incubation the cells were loaded with Hank's Balanced Salt Solution (HBSS) supplemented with 2.5 mM probenecid, 3 µM of the calcium sensitive fluorescent dye Fluo-4 AM, 0.01% pluronic acid and 20 mM HEPES (pH 7.4) for 30 min at 37 °C. Afterwards the loading solution was aspirated and a washing step with 100 µl / well of HBSS, HEPES (20 mM, pH 7.4), 2.5 mM probenecid and 500 µM Brilliant Black was

carried out. Subsequently 100 µl / well of the same buffer was added. After placing cell culture and compound plates into the FlexStation II (Molecular Devices, Sunnyvale, CA, USA), the changes in fluorescence of the cell-loaded calcium sensitive dye Fluor-4 AM were measured, after the addition of 50 µl/well of compound.

3.2.2 Bioluminescence energy transfer assay (BRET)

Human embryonic kidney (HEK293) cells permanently co-expressing the different pairs of fusion proteins NOP-RLuc/Gβ1-RGFP and NOP-RLuc/β-arrestin 2-RGFP and SH-SY5Y cells stably co-expressing the different pairs of fusion proteins mu-RLuc/Gβ1-RGFP and mu-RLuc/β-arrestin 2-RGFP or delta-RLuc/Gβ1-RGFP and delta-RLuc/β-arrestin 2-RGFP were used. Cells were prepared and cultured as described previously by Molinari (2010) and Malfacini (2015). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) / HAMS F12 (1:1) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), streptomycin (100 µg ml^{-1}), L-glutamine (2 mM), fungizone (1 µg ml^{-1}), geneticin (G418; 200 µg ml^{-1}) and hygromycin B (100 µg ml^{-1}) in a humidified atmosphere of 5% CO_2 at 37 °C. For G-protein experiments enriched plasma membrane aliquots from transfected cells were prepared by differential centrifugation; cells were detached with PBS/EDTA solution (1 mM, pH 7.4 NaOH) then, after 5 min 500 g centrifugation, Dounce-homogenized (30 strokes) in cold homogenization buffer (TRIS 5 mM, EGTA 1 mM, DTT 1 mM, pH 7.4 HCl) in the presence of sucrose (0.32 M). Three following centrifugations were performed at 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 (Molinari et al., 2010; Malfacini et al., 2015). The protein concentration in membrane preparations was determined using the QPRO-BCA kit (CyanagenSrl, Bologna, IT) and the Ensight (Perkin Elmer, Waltham, US) in spectrophotometer mode.

Luminescence in membranes was recorded in 96-well untreated white opaque microplates, while in whole cells was recorded in 96-well sterile poly-D-lysine-coated white opaque microplates (PerkinElmer, Waltham, MA, USA) using the luminometer Victor 2030 (PerkinElmer, Waltham, MA, USA). For the determination of receptor/G-protein interaction, membranes (3 µg of protein) prepared from cells co-expressing NOP (or mu or delta)/RLuc and Gβ1/RGFP were added to wells in DPBS. For the determination of receptor/β-arrestin2 interaction, cells co-expressing NOP (or mu or delta)/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 5µM 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. In antagonism experiments, antagonists were incubated 15 min before adding N/OFQ(1-13)-NH_2. BRET ratio was measure 15 min after agonist injection. All the experiments were performed at room temperature.

3.2.3 Dynamic mass redistribution assay

Chinese hamster ovary (CHO) cells stably expressing the human mu or kappa (provided by LToll (Torrey Pines Institute for Molecular Studies, Port St. Lucie, USA) ordelta (provided by E Varga (The University of Arizona, USA), opioid receptors were maintained in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM): F12(1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and geneticin (G418, 400 µg/ml) to maintain expression of target receptor. The cells were cultured at 37 °C in 5% CO2 humidified air. DMR experiments were conducted as previously described (Malfacini et al., 2018). When confluence was reached, cells were sub-cultured using trypsin/EDTA and used for experiments. CHO cells were used as control. Cells were seeded into Enspire^{TM}-LC 384-well fibronectin-coated plates and cultured for 20 h to form a confluent monolayer in the cell culture medium. Cells were seeded at a density of 20,000 cells/well/30 µl. On the day of the experiment, cells were manually washed twice and maintained with the assay buffer for 90 min before the DMR experiment. DMR was monitored in real time with a temporal resolution of 44 s throughout the assay. The present study was performed at 37 °C using the EnSight Multimode Plate Reader (PerkinElmer). A 5-min baseline was established. Next, compounds were added manually in a volume of 10 µl and compound-triggered DMR signals were recorded for 60 min. Maximum picometer (pm) modification (peak) was used to determine the agonist response after baseline normalisation.

3.3 *Ex vivo* studies

3.3.1 Genotyping NOP(+/+) and NOP(-/-) mice

All experimental procedures adopted in this study were as humane as possible, and complied with the European Communities Council directives (2010/63/E) and Italian regulations (D.Lgs, 26/2014). Protocols were approved by the Animal Welfare Body of the University of Ferrara and by the Italian Ministry of Health (License N° 302/2017). In vivo studies have been reported according to the ARRIVE guidelines (Kilkenny et al., 2010). Male CD-1 mice (ENVIGO, Udine, Italy, 8-12 weeks old) were used in this study together with male mice lacking the NOP receptor (NOP(-/-))

and wild-type (NOP(+/+)) mice (8-12 weeks old). Details about the generation of mutant mice have been published previously (Bertorelli, 2002; Nishi, 1997). In our laboratories, NOP(-/-) and NOP(+/+) mice have been backcrossed on CD-1 strain. NOP(+/+) and NOP(-/-) littermates were obtained by mating NOP(+/-) mice. All mice were genotyped using the polymerase chain reaction (PCR). DNAs were prepared from ear biopsies. DNA extraction was performed using the KAPA Mouse Genotyping Kit (KAPA Biosystems, Wilmington, Massachusetts, USA). The primers used were: primer 1, NOP-Forward (5'-TGCCTCTGTGTGTGTCATCA-3), primer 2, NOP-Reverse (5'-GTTCCCCCTTCACAGATTGA-3') and primer 3, LacZ-Reverse (5'-GACAGTATCGGCCTCAGGAA-3'). The sequences of interest were amplified by 2 distinct amplification reactions:

Primer Pair 1 (NOP-Forward + NOP-Reverse): 464bp long,it can only be amplified from the wild type allele.

Primer Pair 2 (NOP-Forward + LacZ-Reverse): 730bp long,it can only be amplified from the mutant allele.

These primers promoted the amplification of two different sequences and allowed to discriminate NOP(-/-), NOP(+/-), and NOP(+/+)mice. The wild type allele for homozygous mice (NOP(+/+)) was amplified by the primers pair 1; homozygous mice for the mutant allele (NOP(-/-)) were only amplified by one amplification, derived by the primers pair 2; heterozygous mice (NOP(+/-)) will have all two amplifications reactions. The presence of amplified DNA in both reactions was confirmed performing electrophoresis for 30 minutes in a 1% w/v agarose gel in TAE 1x buffer. Mice were housed in a specific pathogen free animal facility, in 425 × 266 × 155 mm polycarbonate cages (Tecniplast, VA, Italy), 3-4 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 (Tecniplast, VA, Italy) and nesting materials were present in each cage.

Strategy of amplification

Figure 3.3.1.1. 1 shows the wild type allele of the NOP receptor gene along with the mutant allele obtained by homologous recombination lacking the P1 exon in the protein-coding sequence (Nishi et al. 1997). Black boxes parallel to the sequences represent the positions of the primers used in the project:

NOP-Forward: common for both sequences;

NOP-Reverse: specific for the wild type allele;

LacZ-Reverse: specific for mutant allele.

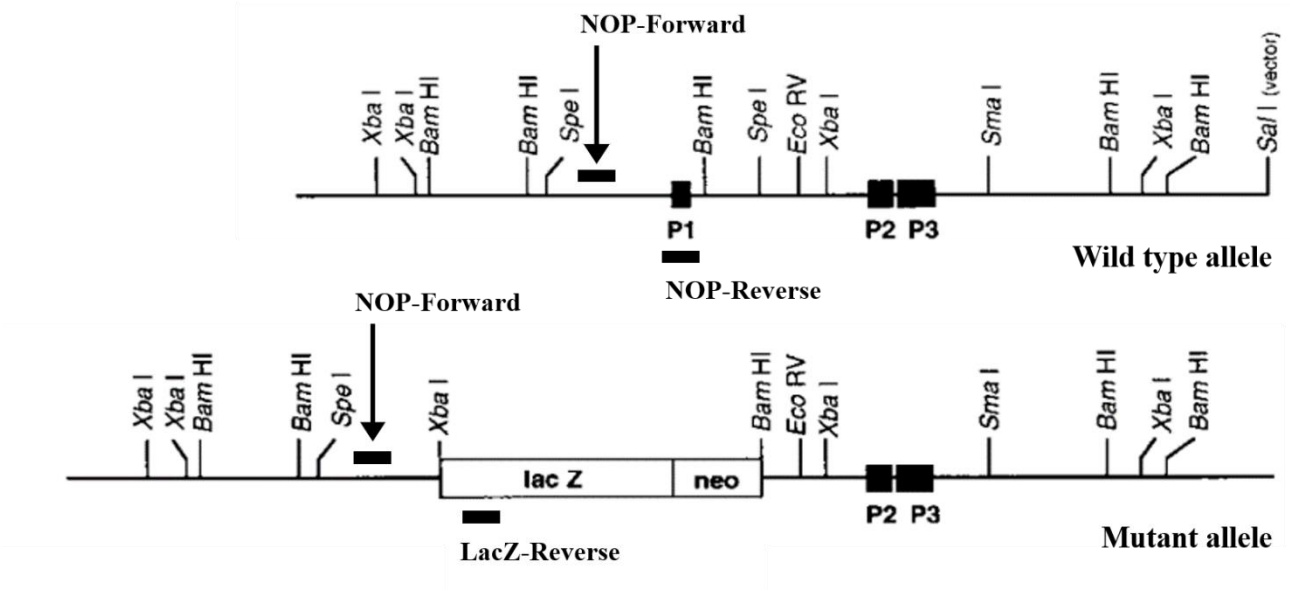


Figure 3.3.1.1. Structures of the wild type allele of nociceptin receptor gene and the mutant allele, obtained by homologous recombination, lacking the P1 exon protein-coding sequence.

These primers allow the amplification of two different sequences and allow to discriminate NOP(-/-), NOP(+/-), and NOP(+/+)mice, (Figure 3.3.1.2.). Primer Pair 1 (NOP-Forward + NOP-Reverse): 464bp long,it can only be amplified from the wild type allele.Primer Pair 2(NOP-Forward + LacZ-Reverse): 730bp long,it can only be amplified from the mutant allele.

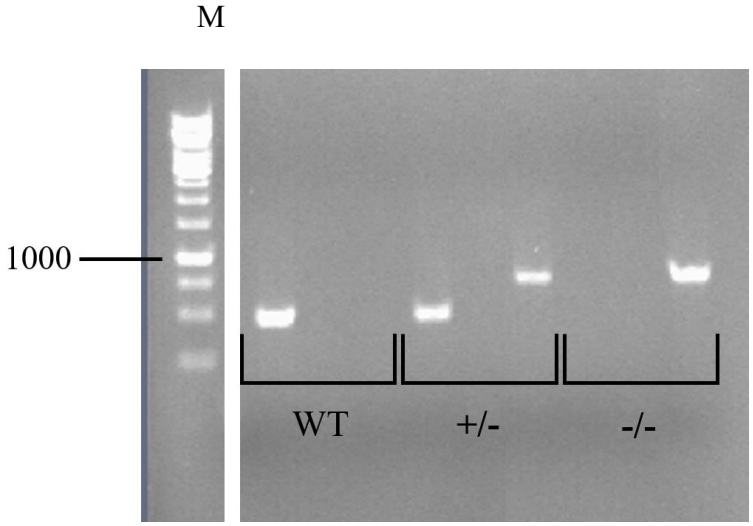


Figure 3.3.1.2. A sample amplification run on agarose gel (1% W/V) from three mice with known genotypes. For each mouse two amplifications have been performed.

Homozygous mice for the wild type allele (NOP(+/+)) will be only amplified by one amplification, derived by the primers pair 1; homozygous mice for the mutant allele (NOP(-/-)) will be only amplified by one amplification, derived by the primers pair 2; heterozygous mice (NOP(+/-)) will have all two amplifications.

DNA extraction

DNA was extracted from a small piece of the ear of each animal using the KAPA Mouse Genotyping Kit (Sigma-Aldrich). The KAPA Mouse Genotyping kit consistsof an Extract Enzyme and an Extract Buffer used together with sterile distilled water, in a total volume of 100 µl (see Table 1).

Table 3.3.1.1. Specific volumes required for the DNA extraction using the KAPA Mouse Genotyping Kit, with a final volume of 100 µl for each tissue sample.

Component	Per 100 µL reaction[1]	Final conc.
PCR-grade water	88 µL	N/A
10X KAPA Express Extract Buffer	10 µL	1X
1 U/µL KAPA Express Extract Enzyme	2 µL	2 U/rxn
Mouse tissue	As required	2 mm section

The KAPA Express Extract system contains a thermostable protease, designed for rapid sample lysis in athermocycler. Once all the required components were added to the tissue, samples were placed in the thermocyclerfor a two steps incubation for the lysis of the tissue and subsequent enzyme inactivation (see Table 2)The extracted DNA is stable at -20°C for at least 6 months.

Table 3.3.1.2. ThermocyclerDNA extraction protocol performed for each tissue sample.

Step	Temperature	Duration
Lysis	75°C	10 min
Enzyme inactivation	95°C	5 min

Polymerase Chain Reaction (PCR) - Primer design

All specific primers (Table 3) were designed on the sequences to be amplifiedusing BLAST alignments.The properties of the primers, including melting temperature (Tm) and secondary structures, were checked using the online service Primer3plus(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were synthetized by Invitrogenand shipped desalted and dried.

Table 3.3.1.3. List of primers used with sequence, melting temperature and length in bp

Name	Sequence	Tm	length
NOP-Forward	5'-TGCCTCTGTGTGTGTCATCA-3	59,9°C	20 bp
NOP-Reverse	5'-GTTCCCCCTTCACAGATTGA-3'	59,9°C	20 bp
LacZ-Reverse	5'-GACAGTATCGGCCTCAGGAA-3'	59,7°C	20 bp

PCR reactions

All PCR reactions was performed using 20 ng of template and were set up in thin walled 0.2ml PCR tubes with 20µl reaction volumes.

A typical reaction mix included:10µl 2X KAPA2G Fast (Hot Start) Genotyping Mix with dye2, 1µl forward primer(10pmol), 1µl reverse primer (10pmol), 20 ng template DNA, nuclease free water to 20µl. Typical PCR cycling conditions were: 95°C for 3 minutes, 5 cycles of 95°C for 20seconds, 66-56°C for 20 seconds (minus 2°C/cycle) and 72°C for 1 minute, 30 cycles of 95°C for 20 seconds, 56°C for 20 seconds and 72°C for 1 minute with a final extension step of 72°C for 1 minute.

Analytical agarose gel electrophoresis

All PCR reactions were analyzed for the presence of the desired product by analyticalTAE agarose gel electrophoresis.A 1Kb GeneRuler™ DNA ladder (Thermo-Fermentas) was typically loaded on each gel, in a total sample volume of 4µl. Typical voltage and current settings were 50V/200mA with an average run times between 30 to 45 minutes.

For each DNA extracted, two amplifications were performed using specific primer pairs. For each amplification reaction a common primer was also used together with a second primer to evaluate the presence of the sequences expressed by wildtype (+/+) genotype (reaction 1: NOP Forward + NOP Reverse primer) and knockout (-/-) genotype (reaction 2: NOP Forward + LacZ Reverse primer) for the NOPreceptor. In each gel, 13 different DNA were analyzed for the presence of the amplified reaction by primers pair 1 and 2. Upon electrophoresis, individuals were classified as:

Amplified by primers pair 1 only: presence of NOP-Forward and NOP-Reverse sequence, which indicates a wildtype genotype (+/+ for the NOP receptor).Amplified by primers pair 2 only: presence of a NOP-Forward and LacZ-Reverse sequence, which indicates a homozygous mutant genotype (-/- for the NOP receptor). Both Reactions amplified: presence of both sequences, which indicates a heterozygous genotype (+/- for the NOP receptor).

In Figure 2.3.1.:3 all types of genotype are displayed, based on the amplification of the 2 DNA reactions previous described. Samples 1, 2 and 5 only displayed the amplification derived by primers pair 2, which consists of a knockout NOP (-/-) genotype, whereas sample number 3 displayed only the amplification derived by primers pair 1 which is representative of a wildtype genotype.

A sample free of DNA, containing only sterile water was also used during all steps (extraction + amplification + gel running) to evaluate if there was any contamination during any step of PCR. There was no DNA amplification in the last sample due to the lack of contamination for all the steps performed, which validates all other PCR reactions.

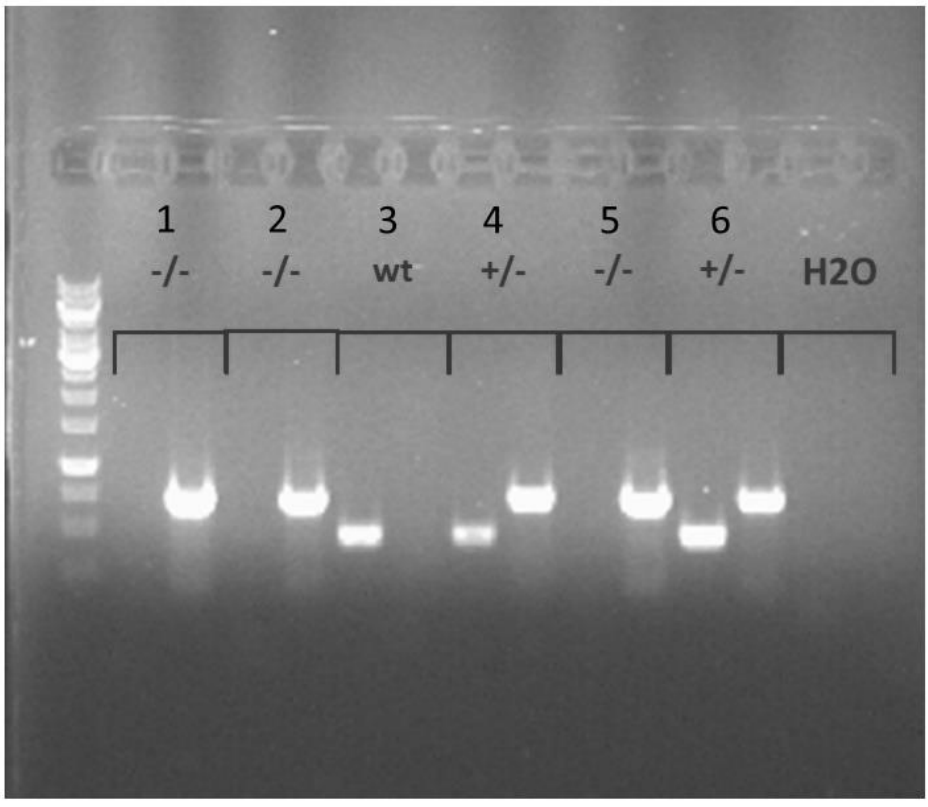


Figure 3.3.1.3. Representative bands of 2 PCR reactions from 6 different mice + Blank to evaluate DNA contamination.

3.3.2 Genotyping βarr2 (+/+) and βarr (-/-) mice

βarr2(-/-) mice were purchased from The Jackson Laboratory (JAX stock #011130; Bohn et al., 1999) and C57BL/6J mice were used as controls. All animals were housed and bred in the Animal Facility of the University of Ferrara LARP, in specific pathogen free conditions, and genotyped by according to The Jackson Laboratory protocol 23872, version 1.2. for βarr2(-/-) mice. Mice were housed in 425 × 266 × 155 mm cages (Tecniplast, MN, Italy), under standard conditions (22°C, 55% humidity, 12h light–dark cycle, lights on 7.00 am) with food (4RF, Mucedola, Italy) and water ad libitum. A mouse red house (Tecniplast, VA, Italy) and nesting materials were present in each cage. Mice 8 – 12 weeks old were used. Each animal was used only once and killed with CO2 overdose at the end of the experiment.

Based on Jackson's Lab protocol, the following primers were used to confirm the genoptype of the colonies:

Table 3.3.2.1 List of primers used with sequence according to Jackson Lab protocol 23872.

PRIMER	SEQUENCE 5' → 3'	PRIMER TYPE	REACTION	LENGTH
10126	GAT CAA AGC CCT CGA TGA TC	Wild type	B	606bp
10127	ACA GGG TCC ACT TTG TCC A	Common	A, B	~300bp and 606bp
10128	GCT AAA GCG CAT GCT CCA GA	Mutant	A	~300bp

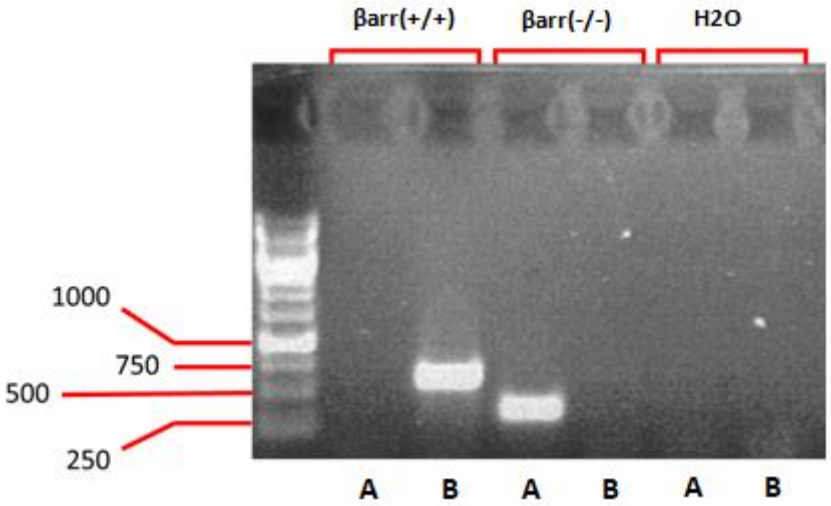


Figure 3.3.2.1. Representative bands of 2 PCR reactions A and B, from 2 different genotype + Blank to evaluate DNA contamination.

3.4 *In vivo* studies

3.4.1 Locomotor activity (evaluate spontaneous locomotor activity for 30 or 60 minutes)

For LA experiments the ANY-maze video tracking system was used (Ugo Basile, application version 4.52c Beta). Mice were positioned in square plastic cages (40 cm × 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), total time immobile (the amount of seconds the animal stays immobile during the test; the animal is considered immobile when 90% of his image remains in the same place for at least 2.5 s), and the number of animal rearings (the number of beam breaks due to vertical movements). Locomotor activity can be thus evaluated at different time points, such as 30 or 60 minutes.

3.4.2 Open field (evaluate spontaneous locomotor activity for 10 minutes and anxiety like behavior)

The OF test was performed using the ANY-maze video tracking system (Ugo Basile, application version 4.52c Beta). Briefly the mouse was placed in a square plastic cage (40×40 cm) and ambulatory behaviour was monitored for 10 min. The central zone of the open field was defined as the central 20×20 cm square. Horizontal activity was monitored by a camera. Four mice were monitored in parallel in each experiment. The parameters measured were the same consideredin the LA assay. The number of entries in the central zone and the time spent by the animal in thecentral area of the field were also measured. An entry in the central zone occurred when the entirearea of the animal was in the central square and the time in the central zone is defined as the amountof time in seconds that the animal spent in the central square.

3.4.3 Rotarod test (evaluate locomotion impairment and sedative effect)

To investigate potential effects on motor coordination we performed a rotarod test using a constant speed device (Ugo Basile, Varese, Italy). Mice were trained at 15 rpm for 120 sec 1 day before the experiment. Motor performance was calculated as time (sec) spent on the rod. A cut-off time of 120 sec was chosen.

3.4.4 Tail withdrawal (evaluate thermal pain)

The test was performed by dipping the distal 1 cm of tail in the water at 52°C. Tail-retraction times were determined visually and recorded with a hand-operated digital stopwatch. The maximal submersion time (cut-off) was limited to 10 sec.

3.4.5 Elevated plus maze (elavuate anxiety like behavior)

The EPM assay was carried out essentially as previously described by Asth et al., (2016). EPM apparatus (Hamilton–Kinder, Poway, CA, USA) consists of two open arms (30x5x0.6 cm), which are facing two opposite wall-enclosed arms (30x5x20 cm) connected by a central platform (5x5 cm) elevated 50 cm from the floor. A red light was focused on the central platform (100 lux). Animals were placed at the centre of the maze, with the head facing an open arm. The number of entries and the time spent in both closed and open arms and some ethological variables (rearing, head dipping and stretch attend postures) were recorded during a 5min period by an experienced observer. An entry was scored as such only when the animal placed all four limbs into any given arm. The ratio of 'time spent in the open arms divided by time spent in all (open and closed) arms'and 'number of entries into open arms divided by total entries into all arms' was calculated and

multiplied by 100, to yield the percentage of time spent in and the frequency of entries into open arms, respectively.

3.4.6 Forced swimming test (evaluate depressive like behavior)

The FST was performed as described by Porsolt *et al.* (1977) and Asth et al. (2016). Mice were placed individually in polyethylene cylinders (18.5 cm high, 12.5 cm diameter), containing water (25 ± 1 °C, 13.5 cm deep), for two swimming sessions: an initial 15 min training session on day 1, which was followed, 24 h later (day 2), by a 5 min test session. Results were relative to this 5 min test session. The immobility time (i.e. the time spent floating in the water without struggling) was recorded. Scoring was performed by an observer blind to the animal genotype. At the end of the swimming sessions, the animal was removed from the cylinder, dried with paper towels, placed in an individual cage to rest and recover, and then it was returned to its collective home cage.

3.4.7 Inescapable electric footshock task (evaluate depressive like behavior)

This is a classical animal model of acute stress with a well accepted cut-off to select resilient and susceptible (termed 'helpless') phenotypes (Pfau and Russo, 2015). Additionally, helpless mice develop a range of behavioral and neuroendocrinal changes that mimic the symptoms of depression

(Vollmayr and Gass, 2013). To develop these experiments, animals were individually placed in a Plexiglas box with a stainless steel grid floor (0.3 x 1 cm) attached to an electric shock generator (Ugo Basile, Gemonio, VA, Italy). The apparatus is divided in two compartments (47 x 18 x 25 cm) by a guillotine door (12 x 25 cm). A protocol with 2 consecutive induction sections was chosen, based on a previous pilot study, with the aim to induce the helpless phenotype in the 50% of mice. Under these experimental conditions, mice were subjected to unpredictable (0.5 mA, 1-10 s automatically randomized shock duration, 1-20 s automatically randomized interval) and inescapable (guillotine door closed) electric footshocks during 2 consecutive days (induction sessions). Twenty-four hours after the last induction session, mice were exposed to the screening session. During the screening session the guillotine door that divides the two compartments is open and the mouse can terminate the electric shock moving through the door to the other side of the apparatus (escapable electric footshocks). During the screening session mice were exposed to 30 escapable electric footshocks (0.5 mA) with a cut off duration of 20 s. A mouse is defined 'helpless' when it fails escaping for 20 or more times. The administration of drugs was performed before each induction sessions, and mouse behavior was assessed, 24 h later, in the screening session, without any drug treatment

3.4.8 Escapable electric footshock task (evaluate memory)

This series of experiments were performed in order to evaluate drug effects on mouse behavior under an aversive operant conditioning task (i.e., escapable electric footshock). In this protocol, during the induction sessions, mice learn that they can terminate the footshock by changing the side of the box. Thus, this protocol can be used to assess the effect of drugs and genotype on the cognitive performance. The active doses of NOP agonists Ro 65-6570 and MCOPPB and NOP(-/-) and NOP(+/+) mice were tested under this experimental condition. Electric footshocks were delivered to mice placed in the chamber with the guillotine door open during all the 3 days of experiment (i.e., 2 induction sessions and 1 test session). The number of cycles, footshocks intensities and duration of each experimental session was the same as in the inescapable electric footshock. On days 1 and 2, prior to the induction sessions mice were treated with the NOP agonists or vehicle and, on day 3, the number of escapes from the electrified chamber was recorded. NOP(-/-) and NOP(+/+) mice did not receive any treatment.

3.4.9 Formalin test (evaluate inflammatory pain)

The procedure fromHunskaar and Hole(1987)was established in our laboratories (Rizzi et al., 2006, 2016). Approximately 30 min before testing, mice were individually placed in transparent observations chambers (32 cm high, 24 cm diameter) for adaptation. Then the animal was taken out

of the chamber, and 30 µl of a 1.5% formalin solution were injected into the dorsal surface of the right hind paw. Immediately after formalin injection, each mouse was returned to the observation chamber, and time (s) spent by the animal displaying pain-related behaviors was measured with a handheld stopwatch for each 5 min block for 45 min after formalin injection. The nociceptive behaviors consisted of licking, biting and lifting of the injected paw. Time spent by the animal showing all these pain-related behaviors was cumulatively measured and expressed as seconds of nociceptive behavior/min. The cumulative response times during 0–10 min and during 15–45 min were regarded as first and second phase, respectively.

3.5 Data analysis and terminology

The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Neubig et al., 2003) (All data were analyzed using Graph Pad Prism 6.0 (La Jolla, CA, USA). In vitro studies: concentration-response curves were fitted using the four parameters nonlinear regression model:

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

Data are expressed as mean ± sem of n experiments performed in duplicate. Agonist potency was expressed as pEC_{50}, which is the negative logarithm to base 10 of the agonist molar concentration that produces 50% of the maximal possible effect of that agonist. In vivo studies: data are expressed as mean ± sem of n animals. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post hoc test, as specified in figure legends. Differences were considered statistically significant when $p < 0.05$.

Antagonist potencies were derived from GaddumSchild 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 concentration response curves of each compound were fitted to the Black-Leff operational model described by Black and Leff (1983).

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

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):

$$\text{Bias factor} = \Delta\log(\tau/K_A)_{\text{Gprotein}} - \Delta\log(\tau/K_A)_{\beta\text{-arrestin2}}$$

Bias factors were calculated using N/OFQ(1-13)-NH_2 for NOP dermorphin for mu, and deltorphin A for delta as standard unbiased ligand. Bias factors are expressed as the mean of at least 5 independent experiments and $CL_{95\%}$are indicated. Biased factor is considered statistically different from 0 when 0 is not included in $CL_{95\%}$. α values have been statistically analyzed with one way ANOVA followed by the Dunnett's post hoc test for multiple comparisons; P values less than 0.05 were considered statistically significant.

For therapeutic index calculation in vivo data from formalin (second phase) and locomotor activity (total distance travelled) tests have been expressed as % of control, where control corresponds to vehicle treated animals. Dose-response curves to agonists were fitted to the classical four-parameter logistic nonlinear regression model. Bottom and top were constrained to 0 and 100% respectively. ED_{50} is the dose of the agonist that produces 50% of the maximal effect of that agonist. For each agonist therapeutic index was calculated as the ratio ED_{50} in the locomotor activity test / ED_{50} in the formalin test.

4. RESULTS AND DISCUSSION

4.1 NOP Receptor

4.1.1 NOP biased agonism

4.1.1.1. *In vitro* pharmacological characterization of palmitoyled peptides as biased ligands for the NOP receptor

The concept of biased agonism at the mu opioid receptor has been widely discussed in literature(Gillis et al. 2020; Kliewer et al. 2020; Bohn, 1999; Bohn et al., 2003; Mann et al., 2019), in which compounds that display a biased activation profile towards G protein show a wider therapeutic index when compared to unbiased ligands (DeWire et al., 2013; Manglik et al., 2017; Grim et al., 2019). Regarding the NOP receptor, few studies evaluated the ability of ligands to discriminate between G protein and arrestins(Chang et al., 2015; Malfacini et al., 2015; Asth et al., 2016). In fact, in the NOP receptor field, the lack of molecules with distinct biased profile (toward G protein and/or toward arrestin) make it difficult to evaluate the potential benefits of a NOP receptor biased agonist in vivo. Therefore, the aim of the present session was to investigate the effector specific (G protein vsarrestin) structure-activity relationship of the NOP peptide ligand N/OFQ(1-13)-NH_2 (the shorter N/OFQ sequence acting as a potent NOP full agonist (Calò et al., 1996; Preti et al., 2019) in order to identify NOP receptor biased agonists.

The ability of already described (compounds **1-38**)and novel (compounds **39-56**)N/OFQ(1-13)-NH_2derivatives to promote NOP-G protein and NOP-arrestin interaction was investigated using the bioluminescence resonance energy transfer (BRET) assay, originally set up for the study of biased ligands for the classical opioid receptors(Molinari et al., 2010).This assay has been further extended to the NOP receptor and validated using a large panel of standard NOP ligands(Malfacini et al., 2015).Moreover, novel NOP ligands have been also characterized using this NOP BRET assay including the mixed NOP/opioid receptor agonists cebranopadol(Rizzi et al., 2016)DeNo,

(Bird et al., 2016)and PWT2-[Dmt^1]N/OFQ(1-13)(Cerlesi et al., 2017), and the NOP selective agonistsAT-403 (Ferrari et al., 2017) and AT-090(Asth et al., 2016).

Regarding the known previously described peptides **1-38,** there are Ala-Scan (Table 4.1.1.1.1) and D-scan derivatives (Table 4.1.1.1.2) of thestandard N/OFQ(1-13)-NH_2, as well asanalogues modified either in the amino acid side chain or in the peptide bond (Table 4.1.1.1.3 and 4.1.1.1.4) along with N/OFQ analogues modified at the 7- or 11- positions of the address domain (Table 4.1.1.1.5). In addition, newly synthesized derivatives, compounds 39-56 were tested in the BRET assay to evaluate their ability to promote NOP-G protein and NOP-arrestin interaction and results are reported in Tables4.1.1.1.6 and 4.1.1.1.7. These novel compounds are the result of C terminal conjugation of N/OFQ(1-13)-NH_2 with moieties of variable length, lipophilicity/hydrophilic, net charge and aromaticity. Lastly, the modifications able to render compounds more biased were then applied to other representative opioid ligands to investigate if the pharmacological effect due to structure modification was specific for the N/OFQ-NOP receptor system. Regarding the opioid receptor family, the most interesting modifications were tested at the BRET assay for the mu and delta receptor as seen in compounds 57-59 in Tables 4.1.1.1.9 and 4.1.1.1.10.

In the first series of studies we assessed in parallel experiments the ability to promote NOP/G protein and NOP/βarrestin2 interaction of N/OFQ(1-13)-NH_2, of its Ala- (**1-11**; Table 4.1.1.1.1) and D-scan (**12-21**; Table 4.1.1.1.2) derivatives as well as of analogues modified either in the amino acid side chain or in the peptide bond (**22-33**; Table 4.1.1.1.3). N/OFQ(1-13)-NH_2 promoted NOP/G protein interaction in a concentration-dependent manner with high potency (pEC_{50} 8.80). All Ala-scan derivatives showed similar maximal effects as N/OFQ(1-13)-NH_2 with the exception for [Ala^2]N/OFQ(1-13)-NH_2 that behaved as a low potency partial agonist. Moreover, compounds replaced in position 1, 4 and 8 displayed very low potency being able to promote the NOP/G protein interaction only at micromolar concentrations. N/OFQ(1-13)-NH_2 stimulated the interaction of the NOP receptor with β-arrestin 2 in a concentration-dependent manner with high

potency (pEC_{50} 8.26). Ala-scan derivatives of N/OFQ(1-13)-NH_2 compounds **1-11** stimulated the interaction of the NOP receptor with β-arrestin 2 displaying the same rank of potency displayed in NOP/G protein experiments (Table 4.1.1.1.1).

Regarding NOP/G protein interaction, D-scan derivatives **12-21** displayed variable results depending on the position of the amino acid investigated, with an overall reduction in potency values. The inversion of the configuration of the chiral amino acids of the message domain produced drastic (> 100 fold) reduction of potency. When the same modification was applied in position 7 to 9 a moderate (>10 fold) reduction in potency was observed while modifications of the C terminal of the peptide produced minor effects on peptide potency (< 10 fold). Regarding NOP/β-arrestin 2 interaction D-scan derivatives of N/OFQ(1-13)-NH_2 showed the same rank of potency displayed in NOP/G protein experiments (Table 4.1.1.1.2).

Table 4.1.1.1.1. Effects of N/OFQ(1-13)-NH_2 and its Ala scan derivatives in NOP/G protein and NOP/β-arrestin 2 experiments.

		NOP/G protein			NOP/β-arrestin 2			
		pEC_{50} ($CL_{95\%}$)	CR	α ± S.E.M.	pEC_{50} ($CL_{95\%}$)	CR	α ± S.E.M.	Biasfactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	8.80 (8.34-9.26)	1	1.00	8.26 (8.11-8.41)	1	1.00	0.00
1	[Ala^1]N/OFQ(1-13)-NH_2	crc incomplete	~700	/		inactive		/
2	[Ala^2]N/OFQ(1-13)-NH_2	6.36 (5.89-6.83)	275	0.54 ± 0.07*		inactive		/
3	[Ala^3]N/OFQ(1-13)-NH_2	7.88 (6.79-8.96)	8	0.87± 0.02	7.54 (7.22-7.86)	5	0.65± 0.02*	-0.21 (-0.96-0.55)
4	[Ala^4]N/OFQ(1-13)-NH_2	crc incomplete	~1000	/		inactive		/
5	[Ala^5]N/OFQ(1-13)-NH_2	7.12 (6.61-7.64)	48	0.86 ± 0.02	6.13 (5.55-6.72)	135	0.72 ± 0.10*	0.40 (-0.24-1.05)
6	[Ala^6]N/OFQ(1-13)-NH_2	7.66 (6.59-8.72)	14	0.93 ± 0.04	7.10 (6.64-7.56)	14	0.97 ± 0.04	0.04 (-0.60-0.67)
7	[Ala^8]N/OFQ(1-13)-NH_2	crc incomplete	~1000	/		inactive		/
8	[Ala^9]N/OFQ(1-13)-NH_2	8.14 (6.88-9.41)	5	0.97 ± 0.04	7.77 (7.43-8.11)	3	1.03 ± 0.07	-0.15 (-0.79-0.49)
9	[Ala^{10}]N/OFQ(1-13)-NH_2	9.25 (7.82-10.68)	0.35	0.94 ± 0.04	8.43 (7.91-8.95)	0.68	1.00 ± 0.04	-0.08 (-0.67-0.50)
10	[Ala^{12}]N/OFQ(1-13)-NH_2	7.31 (6.41-8.22)	31	0.91 ± 0.04	6.85 (6.57-7.14)	26	0.99 ± 0.05	-0.15 (-0.78-0.49)
11	[Ala^{13}]N/OFQ(1-13)-NH_2	7.91 (7.24-8.59)	8	0.96 ± 0.06	7.88 (7.75-8.01)	2	1.03 ± 0.05	-0.48 (-1.14-0.18)

*$p<0.05$ vs N/OFQ(1-13)NH_2 one-way ANOVA followed the Dunnett's post hoc test. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate; CR, concentration ratio;crc, concentration-response curve; $CL_{95\%}$, 95% confidence limits; SEM, standard error of the mean.

Table 4.1.1.1.2. Effects of N/OFQ(1-13)-NH_2 and its D scan derivatives in NOP/G protein and NOP/β-arrestin 2 experiments.

		NOP/G protein			NOP/β-arrestin 2			
		pEC_{50} ($CL_{95\%}$)	CR	α ± S.E.M.	pEC_{50} ($CL_{95\%}$)	CR	α ± S.E.M.	Biasfactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	8.37 (8.30-8.45)	1	1.00	8.02 (7.79-8.24)	1	1.00	0.00
12	[DPhe^{1}]N/OFQ(1-13)-NH_2	6.02 (5.36-6.69)	224	0.70 ± 0.03*	crc incomplete			/
13	[DPhe^{4}]N/OFQ(1-13)-NH_2	5.99 (5.39-6.58)	240	0.79 ± 0.03*	crc incomplete			/
14	[DThr^{5}]N/OFQ(1-13)-NH_2	crc incomplete			inactive			/
15	[DAla^{7}]N/OFQ(1-13)-NH_2	7.26 (7.01-7.52)	13	0.95 ± 0.03	6.76 (6.56-6.96)	18	0.84 ± 0.03*	0.21 (-0.28-0.70)
16	[DArg^{8}]N/OFQ(1-13)-NH_2	7.03 (6.64-7.42)	22	0.95 ± 0.02	6.55 (6.31-6.79)	30	0.79 ± 0.02*	0.26 (-0.25-0.77)
17	[DLys^{9}]N/OFQ(1-13)-NH_2	7.01 (6.85-7.17)	23	0.94 ± 0.02	6.31 (6.00-6.62)	51	0.85 ± 0.06*	0.24 (-0.25-0.73)
18	[DSer^{10}]N/OFQ(1-13)-NH_2	7.80 (7.51-8.09)	4	1.08 ± 0.03	7.65 (7.49-7.80)	2	1.01 ± 0.03	-0.07 (-0.52-0.39)
19	[DAla^{11}]N/OFQ(1-13)-NH_2	7.40 (7.03-7.78)	9	1.07 ± 0.02	7.14 (7.01-7.27)	8	0.97 ± 0.04	0.14 (-0.31-0.60)
20	[DArg^{12}]N/OFQ(1-13)-NH_2	7.63 (7.38-7.88)	5	1.06 ± 0.01	7.39 (7.21-7.57)	4	0.94 ± 0.04	0.26 (-0.19-0.71)
21	[DLys^{13}]N/OFQ(1-13)-NH_2	8.34 (8.12-8.55)	1	1.07 ± 0.02	8.23 (8.12-8.34)	0.62	1.01 ± 0.04	0.03 (-0.42-0.48)

*$p<0.05$ vs N/OFQ(1-13)NH_2 one-way ANOVA followed by Dunnett's post hoc test. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

As far as N/OFQ(1-13)-NH_2 analogues modified in the amino acid side chain of the message domain are concerned, all compounds behaved as full agonistsregarding NOP/G protein interactions with the exception of [Nphe^1]N/OFQ(1-13)-NH_2 (**27**) that displayed no efficacy. Moreover, the potency of these compounds was similar to that of N/OFQ(1-13)-$NH_2$with the exception of [(pF)Phe^4]N/OFQ(1-13)-NH_2 (**29**) that was 5 fold more potent and [Trp^4]N/OFQ(1-13)-NH_2(**24**) and [D-Ala^2]N/OFQ(1-13)-NH_2 (**26**) that were approximately 10 fold less potent. Finally the replacement of the first peptide bond with Ψ(CH_2-NH) (**30**) or Ψ(CH_2-S) (**31**) caused reduction or elimination of peptide efficacy, respectively. Regarding NOP/β-arrestin 2 interaction N/OFQ(1-13)-NH_2 analogues showed the same rank of potency displayed in NOP/G protein experiments (Table 4.1.1.1.3). The behavior of [(pF)Phe^4]N/OFQ(1-13)-NH_2 (**29**) was however slightly different: in fact, as mentioned above, it displayed, compared to N/OFQ(1-13)-NH_2,increased potency in NOP/G protein while similar potency in NOP/β-arrestin 2experiments. This increase of NOP/G protein potency caused a small (3 fold) but statistically significant bias toward G protein for this NOP ligand.

Compounds inactive as agonists were tested as antagonists against N/OFQ (Table 4.1.1.1.4) 10 μM [Nphe^1]N/OFQ(1-13)-NH_2(**27**) antagonized N/OFQ stimulatory effects showing similar pA_2 values in NOP/G protein and NOP/β-arrestin 2 experiments. The addition of a methyl group on Nphe as in the [(S)MeNphe^1]N/OFQ(1-13)-NH_2 (**28**) produced a slight increase in potency with no changes in antagonist activity. [Phe^{1}Ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2 (**30**) behaved as partial agonist in NOP/G protein and as pure antagonist in NOP/β-arrestin 2 studies; its agonist potency for promoting NOP/G protein interaction and its antagonist potency for blocking NOP/β-arrestin 2interaction were in the range 7.83-8.23. Finally the substitution of the CH_2-NH bond between Phe^1 and Gly^2 with CH_2-S(compound **31**) caused a complete elimination of efficacy in NOP/G protein experiments that was however associated with an approximately 10 fold reduction in antagonist potency.

Table 4.1.1.1.3. Effects of N/OFQ(1-13)-NH_2 and its derivatives (compounds **22**– **33)** in NOP/G protein and NOP/β-arrestin 2 experiments.

		NOP/G protein			NOP/β-arrestin 2			
		pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	BiasFactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	8.70 (8.34-9.06)	1	1.00	8.28 (8.03-8.53)	1	1.00	0.00
22	[Cha^1]N/OFQ(1-13)-NH_2	8.93 (8.57-9.29)	0.6	1.08±0.04	8.28 (7.88-8.67)	1	1.03±0.05	0.20 (-0.36-0.76)
23	[Leu^1]N/OFQ(1-13)-NH_2	8.19 (7.88-8.51)	3	1.06±0.02	7.87 (7.69-8.04)	3	1.04±0.06	-0.08 (-0.53-0.37)
24	[Trp^4]N/OFQ(1-13)-NH_2	7.63 (7.15-8.11)	12	1.01±0.01	7.17 (7.00-7.33)	13	0.77±0.03*	0.11 (-0.43-0.65)
25	[D-Phe^3]N/OFQ(1-13)-NH_2	inactive			inactive			/
26	[D-Ala^2]N/OFQ(1-13)-NH_2	7.57 (7.28-7.86)	13	1.03±0.03	7.16 (6.94-7.34)	13	0.95±0.05	0.20 (-0.26-0.66)
27	[$Nphe^1$]N/OFQ(1-13)-NH_2	inactive			inactive			/
28	[(S)βMe$Nphe^1$]N/OFQ(1-13)-NH_2	inactive			inactive			/
29	[(pF)Phe^4]N/OFQ(1-13)-NH_2	9.34 (9.14-9.50)	0.2	1.08±0.04	8.40 (8.11-8.69)	0.8	0.93±0.04	0.59# (0.13-1.05)
30	[Phe^1ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2	8.05 (7.66-8.44)	4	0.59±0.03 *	inactive			/
31	[Phe^1ψ(CH_2-S)Gly^2]N/OFQ(1-13)-NH_2	inactive			inactive			/
32	[Asn^5]N/OFQ(1-13)-NH_2	8.03 (7.45-8.60)	5	1.01±0.03	7.56 (7.11-8.00)	5	1.03±0.07	-0.12 (-0.59-0.35)
33	[Val^5]N/OFQ(1-13)-NH_2	7.94 (7.47-8.42)	6	0.94±0.05	7.38 (7.03-7.74)	8	0.93±0.06	0.09 (-0.38-0.56)

*: $p<0.05$ vs N/OFQ(1-13)NH_2 one-way ANOVA followed by the Dunnett's post hoc test.#: statistically different from 0. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

Table 4.1.1.1.4. Antagonist potency of N/OFQ(1-13)-NH_2derivatives in NOP/G protein and NOP/β-arrestin 2 experiments.

		NOP/G protein	NOP/β-arrestin 2
		pA_2 ($CL_{95\%}$)	
27	[Nphe^1]N/OFQ(1-13)-NH_2	7.51 (6.83-8.19)	7.13 (6.85-7.41)
28	[(S)βMeNPhe^1]N/OFQ(1-13)-NH_2	7.86 (7.28-8.44)	7.67 (6.91-8.43)
30	[Phe^{1}ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2	8.23 (7.24-9.22)	7.83 (7.64-8.02)
31	[Phe^{1}ψ(CH_2-S)Gly^2]N/OFQ(1-13)-NH_2	6.88 (6.00-7.76)	6.99 (6.86-7.12)

Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

Concerning N/OFQ analogues modified in the address domain(Table 4.1.1.1.5), all derivatives displayed full agonists activity at NOP/G protein interactions with minor modifications of potency: the most potent agonist was [Aib^7]N/OFQ-NH_2(**34**) and the least potent was [AC_3C^7]N/OFQ-NH_2 (**36**). As far asNOP/β-arrestin 2 interactionsare concerned, N/OFQ analogues showed the same rank of potency displayed in NOP/G protein experiments.

Table 4.1.1.1.5. Effects of N/OFQ-NH_2 and its derivatives (compounds **34**-**38**) in NOP/G protein and NOP/β-arrestin 2 experiments.

		NOP/G protein			NOP/β-arrestin 2			
		pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	BiasFactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	8.08 (7.70-8.45)	1	1.00	8.01 (7.80-8.22)	1	1.00	0.00
34	[Aib^7]N/OFQ-NH_2	8.92 (8.68-9.17)	0.2	1.08±0.04	8.40 (8.25-8.55)	0.4	0.98±0.05	0.46 (-0.08-1.01)
35	[Aib^{11}]N/OFQ-NH_2	8.35 (7.84-8.86)	0.5	1.07±0.03	8.21 (8.13-8.29)	0.6	0.95±0.03	0.29 (-0.20-0.79)
36	[AC_3C^7]N/OFQ-NH_2	7.67 (7.11-8.23)	3	1.13±0.04	7.56 (7.40-7.73)	3	1.01±0.04	0.40 (-0.11-0.90)
37	[AC_5C^7]N/OFQ-NH_2	8.10 (7.59-8.61)	1	1.11±0.02	8.10 (7.86-8.34)	0.8	1.02±0.03	0.08 (-0.41-0.58)
38	[AC_5C^{11}]N/OFQ-NH_2	8.61 (8.20-9.03)	0.3	1.09±0.04	8.22 (8.08-8.35)	0.6	1.05±0.03	0.31 (-0.19-0.81)

Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

The above mentioned series of experiments were aimed at investigating the possible biased agonism activity of known N/OFQ analogues including Ala- and D-scan derivatives(Dooley and Houghten, 1996; Reinscheid et al., 1996) as well as peptides replaced in different amino acid positions or containing peptide bond modifications(Dooley and Houghten, 1996; Reinscheid et al., 1996; Guerrini et al., 1997, 2001; Calo' et al., 1998; Zhang et al., 2002; Guerrini et al, 2000; Guerrini et al, 2001 Guerrini et al., 2003; Arduin et al., 2007).

The results obtained with these peptides in the NOP/G protein BRET assay perfectly confirmed previous findings regarding the crucial role of the side chain of residues in position 1, 2, 4, 8 and of the chirality of residues in position 1, 4 and 5.(Dooley and Houghten, 1996; Reinscheid et al., 1996). Moreover, as previously demonstrated, the first peptide bond and the benzyl moiety at position 1 affect ligand efficacy(Calo' et al., 1998; Guerrini et al., 2001)while alpha helix inducing amino acids at the 7 and 11 positions (Zhang et al., 2002; Arduin et al., 2007) and the introduction of a fluorine atom in para position of Phe^4 promote an increase of agonist potency(Guerrini et al., 2001).

In addition, these experiments allowed appreciating features of some NOP peptide ligands not detected in previous studies. For instance, [(S)βMeNphe^1]N/OFQ(1-13)-NH_2 (**28**) was slightly more potent than [Nphe^1]N/OFQ(1-13)-NH_2 (**27**) both in NOP/G protein and NOP/β-arrestin 2 experiments while it was reported as equipotent in previous studies(Guerrini et al., 2001)[Phe^{1}Ψ(CH_2-S)Gly^2]N/OFQ(1-13)-NH_2 (**31**) contrary to [Phe^{1}Ψ(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2 (**30**) behaved as pure antagonist in NOP/G protein experiments; this has been not appreciated in previous bioassay experiments performed in the electrically stimulated mouse vas deferens possibly because of off target agonist effects (Guerrini et al., 2003).

Results obtained with these peptides in the NOP/β-arrestin 2 assay were virtually super imposable to those of the NOP/G protein assay with the exceptions of compounds **29** and **30**. In fact, compound **29** displayed a small (3 fold) but statistically significant bias toward G protein. On the other hand, compound **30** ([Phe^1(CH_2-NH)Gly^2]N/OFQ(1-13)-NH_2) in line with previous

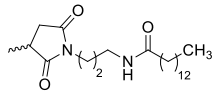
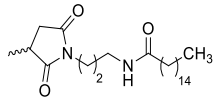
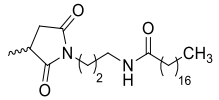
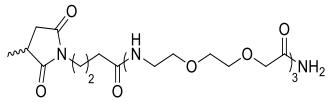
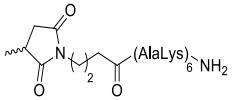
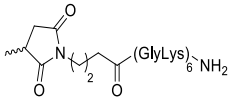
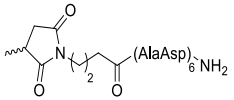
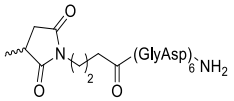
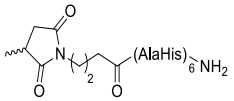
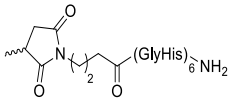
findings(Malfacini et al., 2015) behaved as a partial agonist inNOP/G protein and as pure antagonist inNOP/β-arrestin 2 studies. This is an interesting feature and compound **30**has been used as pharmacological tool for performing initial studies on NOP functional selectivity that demonstrated that the action of a NOP ligand onemotional states is better predicted based on its β-arrestin 2 rather than G protein efficacy(Asth et al., 2016). Apart from these exceptions, all modified peptides behaved as unbiased NOP receptor agonists. This result is somewhat unexpected since similar subtle chemical modifications were sufficient for generating biased agonists when applied to other peptide sequences including angiotensin, apelin, glucagone-like peptide, and parathormone (Gesty-palmer et al., 2010; Godin et al., 2012; Brame et al., 2015; Tan et al., 2018). It could be speculated that for the NOP receptor the chemical requirements of peptide agonists for promoting the interaction of the receptor with G protein are very similar to those required to promote receptor/arrestin interaction. However, we cannot exclude that future studies may eventually identify small chemical modifications of the primary sequence of N/OFQ able to produce strongly biased agonists for the NOP receptor.

Collectively, the results obtained with the known N/OFQ derivatives demonstrated that their pharmacological activity was virtually identical in NOP/G protein and NOP/β-arrestin 2 experiments. In other words all these compounds behaved, similarly to the natural peptide, as unbiased NOP agonists. Interestingly, a certain degree of biased agonism toward G protein has been detected in previous studies by investigating the pharmacological activity of tetrameric N/OFQ derivatives including PWT2-N/OFQ (Malfacini et al., 2015) and PWT2-[Dmt^1]N/OFQ(1-13)(Cerlesi et al., 2017). These multimeric ligands can be considered as N/OFQ related peptides modified with a rather hindered chemical group at the C terminal.

Anchored on the previous findings, we designed novel N/OFQ derivatives modified at the C terminus with various chemical moieties with different functionality, including lipophilic (compounds **39-41**), hydrophilic (compound **42**), positively charged (compounds **43, 44**), negatively charged (compounds **45, 46**), and aromatic (compounds **47, 48**) moieties.The results

obtained with these novel compounds are summarized in Table 4.1.1.1.6. Compounds **39-41**displayed, compared to N/OFQ(1-13)-NH_2,higher potency at NOP/G protein and lower potency at NOP/β-arrestin2 thus behaving as agonistsbiased toward G protein. Similar results were obtained with positively charged peptides (compounds **43, 44**) whose bias factor was however lower than that of compounds functionalized with fatty acid chains. A statistically significant bias toward G protein was also displayed by negatively charged peptides (compounds **45, 46**) but their bias factor was low and associated with reduced agonist potency as well. Finally, C-terminal modification of N/OFQ(1-13)-$NH_2$with hydrophilic neutral (compound **42**) or aromatic (compound **47, 48**) moieties did not produce significant changes in the pharmacological activity of the peptide.

Table 4.1.1.1.6. Effects of N/OFQ(1-13)NH_2 and its derivatives from **39** to **48** in NOP/G protein and NOP/β-arrestin 2.

			NOP/G protein			NOP/β-arrestin 2			
		X	pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	pEC_{50} ($CL_{95\%}$)	CR	α ± SEM	Biasfactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	-	8.08 (7.73-8.42)	1	1.00	8.00 (7.79-8.22)	1	1.00	0.00
39	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		9.54 (8.61-10.48)	0.03	1.01 ± 0.06	7.61 (7.29-7.93)	2	1.05 ± 0.04	1.55* (1.09-2.01)
40	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		9.27 (8.95-9.58)	0.06	1.03 ± 0.06	7.26 (6.91-7.61)	5	1.08 ± 0.04	2.01* (1.55-2.47)
41	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.80 (7.99-9.61)	0.2	0.92 ± 0.22	7.22 (6.79-7.65)	6	0.93 ± 0.07	1.82* (1.37-2.27)
42	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		7.82 (7.58-8.05)	2	0.98 ± 0.07	7.90 (7.67-8.13)	1	0.97 ± 0.03	-0.20 (-0.65-0.25)
43	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		9.10 (8.89-9.31)	0.1	0.93 ± 0.03	7.76 (7.47-8.06)	2	0.93 ± 0.02	1.18* (0.73-1.64)
44	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.96 (8.63-9.28)	0.1	0.99 ± 0.03	7.84 (7.70-7.99)	1	0.98 ± 0.06	1.11* (0.66-1.57)
45	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		7.62 (7.31-7.93)	3	0.97 ± 0.05	7.03 (6.68-7.38)	9	0.94 ± 0.03	0.51* (0.06-0.96)
46	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		7.75 (7.59-7.92)	2	0.85 ± 0.07	7.31 (7.02-7.61)	5	0.99 ± 0.04	0.81* (0.05-1.57)
47	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.10 (7.83-8.36)	1	0.92 ± 0.07	8.03 (7.79-8.27)	1	1.01 ± 0.04	-0.05 (-0.50-0.40)
48	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.37 (7.86-8.89)	0.5	0.93 ± 0.04	7.90 (7.56-8.23)	1	0.98 ± 0.02	0.17 (-0.29-0.62)

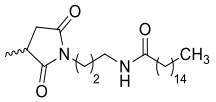
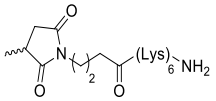
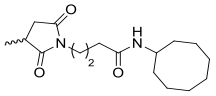
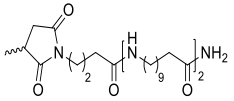
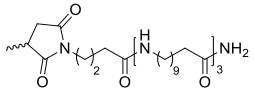
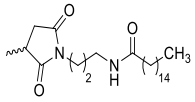
*: statistically different from 0. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

In order to further investigate the contribution to G protein bias of positive charges and lipophilicity of C-terminal modified N/OFQ(1-13)-NH_2 analogues, a second series of peptides was synthesized and tested and the relative results were summarized in Table 4.1.1.1.7. Compound **49** characterized by a $(Lys)_6$ moiety displayed a larger increase in potency at NOP/G protein than NOP/β-arrestin 2 interaction showing a statistically significant bias factor of approximately 10 fold. The use of a cyclic aliphatic moiety (compound **50**) or of chains generated using two (compound **51**) or three (compound **52**) amino undecanoic acidscaused a similar reduction of NOP/G protein andNOP/β-arrestin 2 potency, thus producing unbiased NOP agonists. The shift of position of the palmitoyl moiety produced different results depending on the substituted amino acid. In particular, similar results were obtained when the palmitoyl moiety has been located in position 14 (compound **40**) and 11 (compound **54**), while, when the same moiety was introduced in position 10 (compound **53**), 12 (compound **55**) and 13 (compound **56**) it produced a variable decrease in potency followed by a consistent decrease in the bias factor.

The addition of neutral hydrophilic (**42**) or aromatic (**47**, **48**) moieties did not change the unbiased profile of the reference peptide. On the contrary, the addition of charged moieties particularly in the case of positively charged peptide sequences elicited a shift toward G protein biased agonism (**43**, **44** and **49**). This effect was mainly due to an increase in agonist potency for receptor/G protein interaction associated with no changes for receptor/arrestin interaction. The higher bias was detected with compound **43** characterized by the dipeptide sequence Ala-Lys repeated 6 times. However, similar results were obtained with compound **44** and **49** in which the charges were organized in different ways. Thus for promoting G protein bias the presence of C terminal positive charges seems more important than their spatial distribution and orientation. Interestingly enough, the potent and selective NOP agonist UFP-112(Rizzi et al., 2007) that has an extra couple of positively charged residues (Arg^{14}-Lys^{15}) displayed a small but statistically significant G protein bias (0.71)(Malfacini et al., 2015). Charged residues may promote receptor interaction via ionic bonds with acidic residues of which the second extracellular loop of the NOP

receptor is rich (Meunier et al., 2000; Tancredi et al., 2005; Daga and Zaveri, 2012; della Longa and Arcovito, 2016). This proposal has been corroborated by results obtained in molecular modeling studies based on the crystal structure of the NOP receptor (see for details figure 4e in Thompson et al., 2012b). As a speculative hypothesis, we might suggest that this mechanism is more effective for NOP conformations interacting with G proteins than those interacting with arrestins.

Table 4.1.1.1.7. Effects of N/OFQ(1-13)NH_2 and compounds **40**, **49-56** in NOP/G protein and NOP/β-arrestin 2.

			NOP/G protein			**NOP/β-arrestin 2**			
		X	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	Biasfactor ($CL_{95\%}$)
	N/OFQ(1-13)-NH_2	-	9.18 (9.03-9.34)	1	1.00	8.08 (7.82-8.34)	1	1.00	0.00
40	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		9.76 (9.36-10.16)	0.26	1.18±0.04	7.78 (7.33-8.23)	2	0.86±0.11	1.30# (0.77-1.83)
49	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		10.06 (9.79-10.34)	0.13	1.04±0.02	8.22 (8.03-8.40)	0.73	1.02±0.08	0.83# (0.44-1.22)
50	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.40 (8.25-8.56)	6	0.98±0.01	7.56 (7.24-7.87)	3	0.99±0.04	-0.27 (-0.66-0.12)
51	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.29 (8.00-8.58)	8	1.02±0.03	7.33 (7.13-7.54)	6	0.94±0.03	0.07 (-0.32-0.46)
52	[Cys(X)^{14}]N/OFQ(1-14)-NH_2		8.19 (7.88-8.50)	10	0.96±0.01	7.23 (6.98-7.49)	7	0.79±0.04*	0.09 (-0.32-0.50)
53	[Cys(X)^{10}]N/OFQ(1-13)-NH_2		9.54 (9.28-9.82)	0.44	1.03±0.03	7.72 (7.46-7.98)	2	0.96±0.10	0.91# (0.51-1.30)
54	[Cys(X)^{11}]N/OFQ(1-13)-NH_2		9.36 (8.97-9.74)	0.67	1.04±0.03	7.46 (7.13-7.78)	4	0.88±0.08	1.11# (0.72-1.50)
55	[Cys(X)^{12}]N/OFQ(1-13)-NH_2		9.28 (9.17-9.39)	0.80	1.02±0.02	7.65 (7.46-7.84)	3	0.89±0.05	0.87# (0.48-1.26)
56	[Cys(X)^{13}]N/OFQ(1-13)-NH_2		8.90 (8.63-9.16)	2	0.96±0.02	7.26 (7.11-7.40)	7	0.87±0.08	0.70# (0.32-1.09)

*: $p<0.05$ vs N/OFQ one-way ANOVA followed by the Dunnett's post hoc test; # statistically different from 0. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

The most interesting results have been obtained by introducing lipophilic linear aliphatic moieties at the C-terminus of N/OFQ(1-13)-NH_2. Small differences were obtained with chains of different lengths i.e. 14, 16, 18 carbon atoms (compounds **39**-**41**). On the contrary, the linear structure of the chain seems to be important since compound **50**, with a cyclooctane ring, displayed reduced potency and behaved as an unbiased agonist. Moreover, the linear moieties must be fully aliphatic since their substitution with amphipathic sequences (compounds **51** and **52**) reduced their potencies and totally eliminated the biased profile of the agonists. The importance of the palmitoylation site has been investigated with compounds **53-56**. The shift of the palmitoyl group from position 14 to position 11, 10, 12, and 13 caused a progressive reduction of the G protein bias.

For the latter two compounds it should be noted that the Cys residue needed for palmitoylation substituted positively charged aminoacids (Arg^{12} or Lys^{13}) that are important for NOP binding in agreement with previous results (Dooley and Houghten, 1996; Reinscheid et al., 1996; Guerrini et al., 1997). Altogether these findings suggest that a linear aliphatic chain, particularly the palmitoyl group at the C terminal of N/OFQ(1-13)-NH_2, promoted a large (10-100 fold) G protein biased agonism due to increased NOP/G protein potency associated with slight reduction of NOP/β-arrestin 2 potency. The mechanism by which C-terminal modifications of N/OFQ(1-13)-NH_2 promote biased agonism toward G protein is at present unknown. To the best of our knowledge, there is a single example of naturally occurring palmitoylated ligands for GPCR. Wnt are a family of proteins that must be palmitoylated to exert their biological effects through the activation of Frizzled receptors. Recent crystallographic studies demonstrated that Wnts use the fatty acid as a hotspot residue to engage its receptor (Janda et al., 2015). However, it is unlikely that such mechanism might be relevant for the interaction of compound **40** with the NOP receptor since NOP and Frizzled receptors are phylogenetically very far from each other (Fredriksson et al., 2003).

Moreover, it is worthy of mention that compound **40** is structurally similar to pepducins, lipidated peptides of 10-20 amino acid residues with sequences derived from the receptor

intracellular loops or C-terminus (Carr and Benovic, 2016). Pepducins, most probably acting intracellular, (Tsuji et al., 2013)are able to modulate GPCR signalling sometimes acting as biased agonists either toward G protein (Quoyer et al., 2013; Carr et al., 2014; Gabl et al., 2017) or arrestin (Carr et al., 2016). However, it is again unlikely that compound **40** acts as a pepducin since this peptide is palmitoylated at the C-terminus while pepducins at the N-terminus and, more importantly, there is no homology between N/OFQ sequence and that of the intracellular loops and C-terminus of the NOP receptor (Mollereau et al., 1994). The lipophilicity of the palmitoyl moiety may favor the insertion of the peptide into the plasma membrane or it may directly interact with the NOP transmembrane domains. In both cases, this mechanism may favor G protein- rather than arrestin-preferring NOP active conformations.

To get insights regarding the mechanism of action of compounds **40** and **43,** their effects on NOP/G protein interaction were challenged with the NOP selective antagonist SB-612111 (Zaratin et al., 2004; Spagnolo et al., 2007) and compared to those obtained with the standard agonist N/OFQ(-1-13)-NH_2. The antagonist did not produced any effect per se but elicited a rightward parallel shift of the concentration response curve to N/OFQ(-1-13)-NH_2 without modifying the agonist maximal effect; a pA_2 value of 8.05 ($CL_{95\%}$ 7.74 – 8.37) was derived from these experiments. Similar results were obtained using compound **40** and **43** as NOP agonists; the pA_2values of SB-612111 calculated from these experiments were 7.95 ($CL_{95\%}$ 7.43 – 8.47) and 7.77 ($CL_{95\%}$ 7.32 – 8.21), respectively (see Figure 4.1.1.1.1). Moreover, SB-612111 competitively antagonized the effects of N/OFQ (Malfacini et al., 2015; Ferrari et al., 2020) in a similar matter of N/OFQ(-1-13)-NH_2, compounds **40** and **43**, showing similar pA_2 values as shown in Figure 4.1.1.1.1. This demonstrated that, similar to N/OFQ and N/OFQ(1-13)-NH_2, compounds **40** and **43** activate the NOP receptor by interacting with the orthosteric binding pocket that has been described at atomic level in previous NOP/C-24(Thompson et al., 2012a) and NOP/SB-612111 (Miller et al., 2015) crystal structure studies.

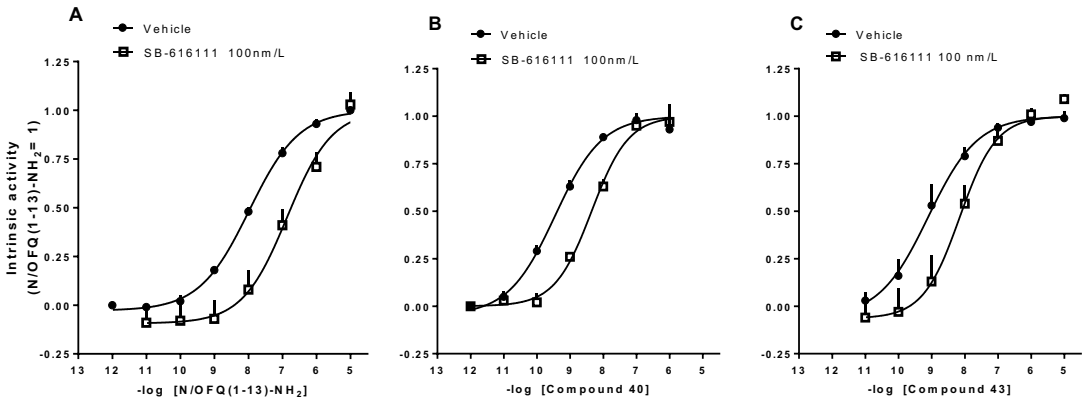
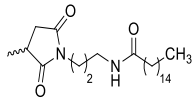
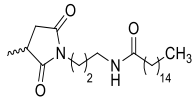
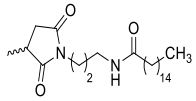


Figure 4.1.1.1.1. BRET assay**.** NOP/G-protein interaction experiments. Concentration-response curves toN/OFQ(1-13)-NH_2 (panel A), compound 40(panel B) and compound 43(panel C), in absence (vehicle) and presence of the NOP antagonist SB-61211 at 100nm/L. Data are the mean ± S.E.M. of 4 separate experiments made in duplicate**.**

Finally, in order to investigate if the effects of palmitoylation of the peptide C-terminus are specific for N/OFQ and the NOP receptor or might influence the pharmacology of other opioid systems, this chemical modification has been applied to dermorphin, deltorphin A, and Leu-enkephalin, (Hughes et al., 1975; Negri et al., 2000)and the peptides evaluated at mu (Table 4.1.1.1.9) and delta (Table 4.1.1.1.10) opioid receptors. Dermorphin promoted mu receptor interaction with G protein and arrestin with similar potency while producing incomplete concentration response curve at the delta receptor. Opposite results were obtained with deltorphin A that promoted delta receptor interaction with G protein and arrestin with similar potency while eliciting stimulatory effects at the mu receptor only at micromolar concentrations. Leu-enkephalin produced similar stimulatory effects at mu and delta receptor being slightly more potent on the latter. Palmitoylation of the C terminus of dermorphin (compound **57**) increased peptide potency both in mu/G protein and mu/β-arrestin 2 experiments. In addition, **57** was able to stimulate delta receptor interaction with both G protein and β-arrestin 2 although with lower potency compared to the mu receptor. Palmitoylation of the C terminus of deltorphin A (**58**) increased peptide potency in delta/G protein but not delta/β-arrestin 2 studies thus displaying a bias factor of 1.06. In addition, Compound **58**was able to stimulate mu receptor interaction with both G protein and β-arrestin 2

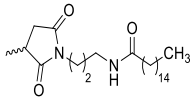
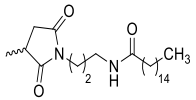
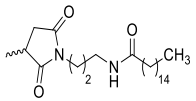
although with lower potency compared to the delta/G protein experiments. Finally, when C-terminal palmitoylation was applied to Leu-enkephalin (compound **59**) it caused a large increase in receptor/G protein potency both at the mu and delta receptor. However, this was associated with a slight increase in mu/β-arrestin 2 potency and with a slight decrease in delta/β-arrestin 2 potency. Thus, compound **59** behaved as a G protein biased delta agonist with a bias factor of 1.70. Collectively the results obtained by the insertion of a palmitoyl moiety at the C terminal of N/OFQ or opioid peptides consistently produced a rather large (10-100 fold) increase of agonist potency in G protein experiments. On the contrary, the effect of this chemical modification in arrestin experiments was variable depending on the receptor under evaluation: increase in potency for the mu opioid receptor no changes or little decrease in potency for the delta and NOP receptors. These combined actions make palmitoylated peptides G protein biased agonists for NOP and delta receptor and unbiased agonists for the mu receptors.

Table 4.1.1.1.9. Effects of Dermorphin, Deltorphin A, Leu-Enkephalin and their C-terminal palmitoylate analogues **57**-**59** in mu/G protein and mu/β-arrestin 2 experiments

			mu/G protein			**mu-RLuc/β-arrestin 2**			
		X	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	BiasFactor ($CL_{95\%}$)
	Dermorphin	-	6.89 (6.24-7.54)	1	1.00	7.09 (6.80-7.38)	1	1.00	0.00
57	[Cys(X)^{8}]Dermorphin(1-8)-NH_2		8.84 (8.65-9.03)	0.01	0.93±0.10	7.96 (7.65-8.28)	0.13	1.18±0.07	0.64 (-0.13-1.42)
	Deltorphin A	-	crc incomplete			inactive			
58	[Cys(X)^{8}]Deltorphin A(1-8)-NH_2		7.29 (6.59-7.99)	~0.005	1.02±0.06	7.42 (7.19-7.66)	/	0.87±0.07	/
	Leu-Enkephalin	-	6.75 (6.57-6.92)	1	0.82±0.05	6.10 (5.85-6.36)	1	0.68±0.10	0.00
59	[Cys(X)^{6}]Leu-Enkephalin-(1-6)-NH_2		8.39 (7.99-8.79)	0.02	0.83±0.08	6.64 (6.28-7.00)	0.29	0.47±0.10*	0.75 (-0.73-2.24)

*: $p<0.05$ vsDermorphin, one-way ANOVA followed by the Dunnett's post hoc test. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.RLuc, renilla luciferase.

Table 4.1.1.1.10. Effects of Dermorphin, Deltorphin A, Leu-Enkephalin and their C-terminal palmitoylate analogues **57**-**59** in delta/G protein and delta/β-arrestin 2 experiments.

			delta/G protein			**delta/β-arrestin 2**			
		X	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	pEC_{50} ($CL_{95\%}$)	CR	α±SEM	BiasFactor ($CL_{95\%}$)
	Dermorphin	-	crc incomplete			crc incomplete			
57	[Cys(X)^8]Dermorphin(1-8)-NH_2		7.23 (6.52-7.95)	~0.006	0.74±0.11	6.21 (5.75-6.67)	~0.02	0.44±0.03*	/
	Deltorphin A	-	7.58 (6.93-8.22)	1	1.00	7.83 (7.70-7.96)	1	1.00	0.00
58	[Cys(X)^8]Deltorphin A(1-8)-NH_2		8.69 (8.33-9.06)	0.08	0.96±0.07	7.65 (7.26-8.03)	2	0.51±0.05*	1.06* (0.27-1.84)
	Leu-Enkephalin	-	6.92 (6.50-7.33)	1	0.87±0.04	7.37 (6.83-7.91)	1	0.93±0.06	0.00
59	[Cys(X)^6]Leu-Enkephalin(1-6)-NH_2		8.09 (7.44-8.74)	0.07	0.91±0.08	6.88 (6.62-7.14)	3	0.50±0.02*	1.70* (0.90-2.50)

*: $p<0.05$ vs Deltorphin A, one-way ANOVA followed by the Dunnett's post hoc test. *: statistically different from 0. Data are expressed as the mean ± sem of 5 independent experiments made in duplicate.

CONCLUSIONS

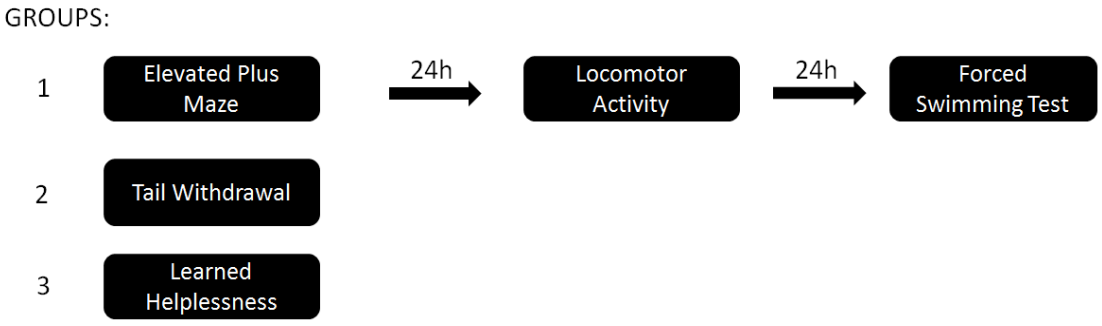
Finally, the present section was aimed at the identification of NOP receptor peptide biased agonists. Subtle chemical modifications in the N/OFQ(1-13)-NH_2 sequence even if able to produce large changes in ligand potency and/or efficacy did not provide useful information for the design of NOP biased agonists. Biased agonism toward G protein can be obtained by N/OFQ(1-13)-NH_2C-terminal modifications with positively charged peptide sequences or linear aliphatic chains. The best results in terms of bias factor were obtained with the palmitoyl moiety. This chemical modification was also applied to mu and delta receptor peptide ligands; palmitoyled peptides consistently behaved as highly potent agonists for receptor/G protein interaction acting as G protein biased agonists for NOP and delta receptors and as unbiased agonistsfor the mu receptor. Further studies are needed to understand the mechanism by which C-terminal palmitoylation modulates the pharmacological profile of peptide agonists. Nevertheless, palmitoylation of biologically active peptides can be proposed as a chemical probe for generating highly potent agonists and in some cases G protein biased agonists.

4.1.1.2. Biased agonism *in vivo*

4.1.1.2.1 Phenotype of beta arrestin2 knock-out mice

A lot of effort has been made to understand how β-arrestin2 can affect physiological responses to drugs. However, little is known about how the lack of βarrestin2 affects basal behavior. For that reason, the understanding of changes of phenotype of C57BL/6 βarr2(-/-) mice in a series of behavioral assays related to locomotor activity, anxiety, depression and, more importantly, pain is of particular interest to further investigate drug effects in these mice as well as theirC57BL/6 wildtype littermates (βarr2(+/+)). C57BL/6 βarr2(-/-) mice were generated by inactivation of the gene *Arrb2*^{tm1Rjl} by homologous recombination as previously described by Bohn (1999) and then purchased from Jackson Laboratory.

Firstly, to improve animal welfare throughout experimentation, the 3Rs (Replacement, Reduction and Refinement) principle developed by Russell & Burch (1959) was adopted. Following the reduction principle, animals were divided into 3 groups (Figure 4.1.1.2.1) related to how stressful each behavioral test is, from leastto most severe. By our experimental design, after the more severe assay animals were immediately sacrificed to minimize suffering.



re Figure **4.1.1.2.1.** Experimental design to evaluate behavioral phenotype of βarr2(-/-) and βarr2(+/+) mice with the most stressful assays been performed at the last day of experiments for each group.

To investigate the effect of genotype on anxiety the Elevated Plus Maze Test was performed whereas spontaneous locomotor activity was recorded performing Open Field and Locomotor Activity assays in parallel. Regarding depression-like behavior, the Forced Swimming Test as well as the Learned Helplessness test were performed. Moreover, it is reported in literature that β-arrestin2 may play a key role in nociception (Bohn, 1999), thus we studied the phenotype to thermal and inflammatory pain with the tail withdrawal assay and Formalin Test.

Genotype comparison on locomotor activity

Open field assay was performed to evaluate basal spontaneous locomotion, vertical exploration (number of rearings) and anxiety related parameters (entries in the central zone and time spent in the central zone) for 10 minutes. There was no difference between genotype in all parameters measured (Figure 4.1.1.2.2).

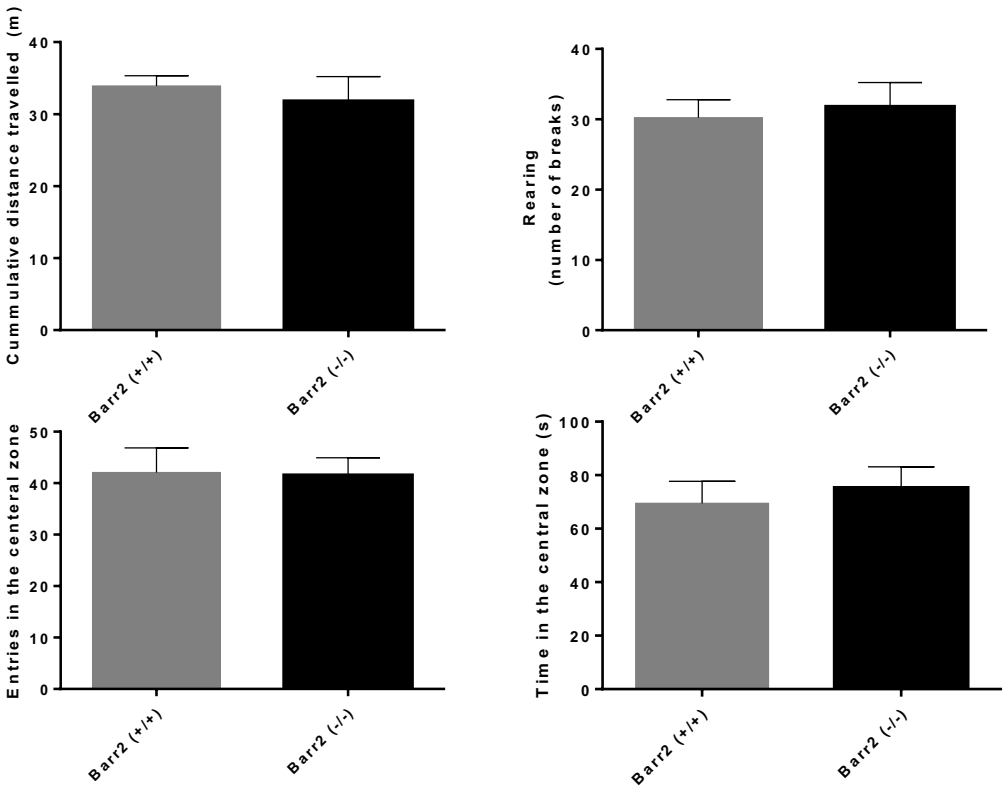


Figure 4.1.1.2.2.Open field assay in C57BL6 βarr2(+/+) and βarr2(-/-) mice. Effects of genetic background on the cummulative distance travelled (top left panel), number of rearings (top right panel), number of entries in the central zone (bottom left panel) and the time spent in the central zone (bottom right panel). Data are mean ± s.e.m. of at least 7-10 mice per group.

After the 10 minutes measurement of the Open Field assay, a locomotor activity assay was carried out to further analyze the spontaneous locomotion up to 60 minutes. The βarr2(-/-) mice did not show any difference in total distance travelled, in total time immobile and in the number of rearings compared to βarr2(+/+) (Figure 4.1.1.2.3).

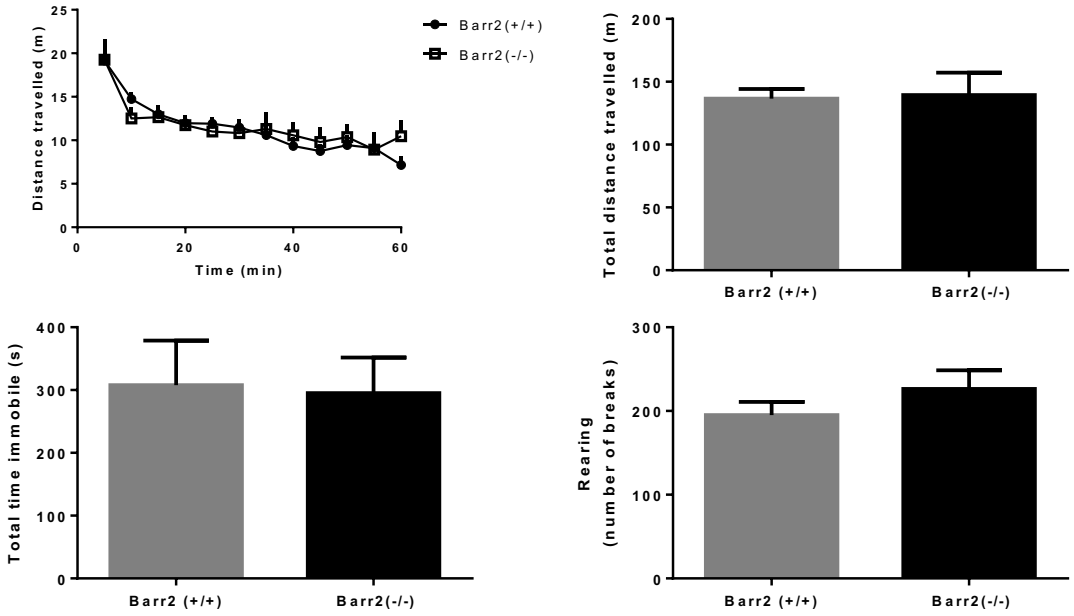


Figure 4.1.1.2.3. Locomotor activity assay performed in C57BL6 βarr2(+/+) and βarr2(-/-) mice. Effect of genotype on the distance travelled across time (top left panel), on the cumulative total distance travelled (top right panel), immobility time (bottom left panel) and number of rearings (bottom right panel). Data are mean ± s.e.m. of 7-10 mice per group.

Genotype comparison on anxiety-like behavior

The phenotype of βarr2(-/-) in comparison with βarr2(+/+) mice subjected to the elevated plus maze was investigated. There was no difference of phenotype between strains. In this test, βarr2(+/+) displayed the percentage of time spent and entries in open arms of 36± 1.6% and 42±1.7%, respectively whereas βarr2(-/-) mice displayed 44±6.5% and 40±4.7% (Figure 4.1.1.2.4). All the behavioral parameters measured in the EPM in βarr2(+/+) and βarr2(-/-) animals are summarized in table 4.1.1.2.1. No differences in the frequency of stretch attend postures, rearings and head dipping were observed (Table 4.1.1.2.1).

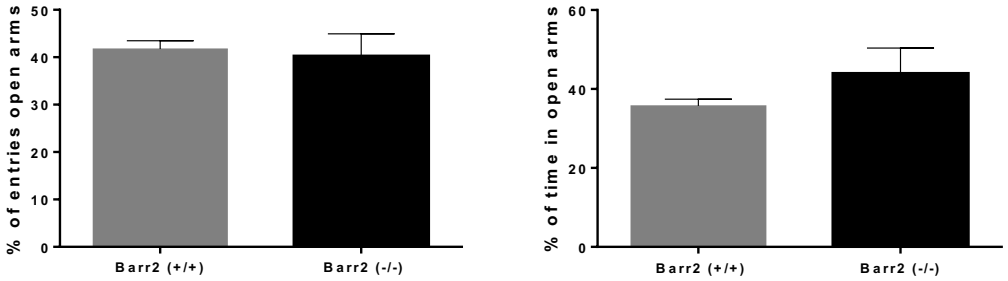


Figure 4.1.1.2.4 Elevated plus maze test performed with C57BL6 βarr2(+/+) and βarr2(-/-) mice. Effects of genotype on the percentage of entries in(left panel) and percentage of total time spent in open arms (right panel). Data are mean ± s.e.m. of 7-10 mice per group.

Table 4.1.1.2.1 Effects of genotype on various behavioral parameters (raw data) displayed by βarr2(+/+) and βarr2(-/-) mice subjected to the EPM.

Genotype	*Time in open arms (s)*	*Entries in open arms*	*Time in closed arms (s)*	*Entries in closed arms*	*Stretch attend posture*	*Head dipping*	*Rearing*
βarr2(+/+)	72±4.3	9.8±0.7	128.8±4.1	13.8±1.2	11±0.6	22.5±2.3	20.3±1.8
βarr2(-/-)	90±12.4	8.7±1.2	115.8±13.6	12.4±0.8	9.4±0.8	22.2±2.76	21.8±4.3

Genotype comparison on depressive-like behavior

No differences were observed in the immobility time of βarr2(+/+) and βarr2(-/-) animals in the Forced Swim Test performed as previously described by Gavioli (2003).

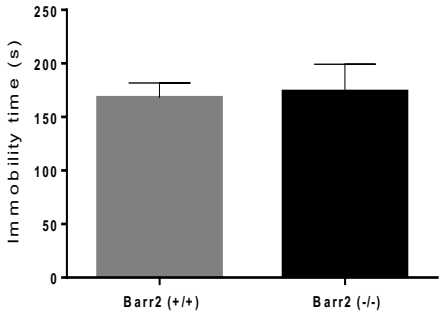


Figure 4.1.1.2.5Forced swimming test in C57BL6 βarr2 (+/+) and βarr2 (-/-) mice. Effects of genotype on immobility time. Data are mean ± s.e.m. of 7-10 mice per group.

To further analyze mice behavior regarding depression-like phenotype, the learned helplessness model was performed as described by Holanda et al., 2019. Interestingly enough, in the learned helplessness paradigm βarr2(-/-) mice exposed to inescapable foot shocks did not develop the helpless behavior (Figure 4.1.1.2.6, left panel), being able to escape at least 10 times (out of 30) in the test session (Figure 4.1.1.2.6, right panel), with the number of behaviors described as failures is significantly higher than the escapes in the screening session, thus displaying an antidepressant-like phenotype when compared to their wildtype littermates.

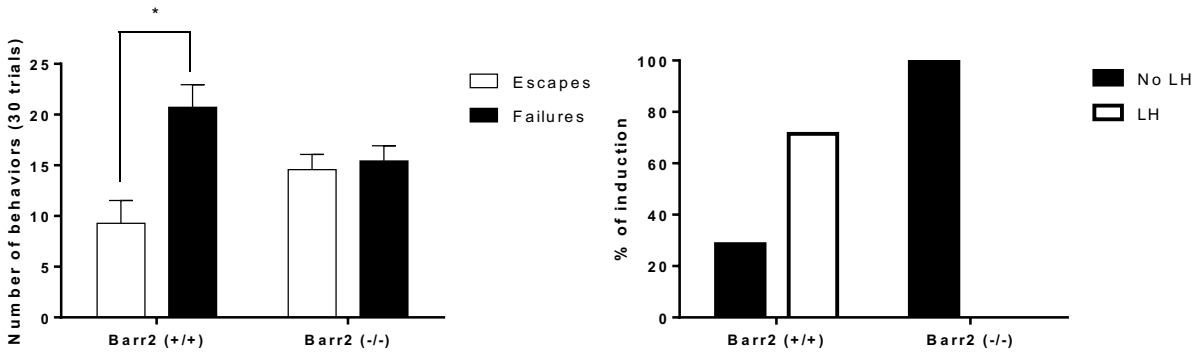


Figure 4.1.1.2.6 Learned Helplessness Model of Depression. Phenotype of βarr2(+/+) and βarr2(-/-) mice that underwent the learned helplessness paradigm. Effects of genotype on the number of behaviors during test session (left panel). Data are mean ± s.e.m. of escape/failures during the test session of 7 mice per group.*<0.05 vs escapes. Percentage of mice that develop the learned helpless/no helpless phenotype (right panel). Data are presented as the percentage of helpless/no helpless of 7 mice/group.

Genotype comparison on nociceptive behavior

To access basal thermal nociception, the tail withdrawal assay was performed as described by Hunter et al., 1997. No difference was observed between genotype.

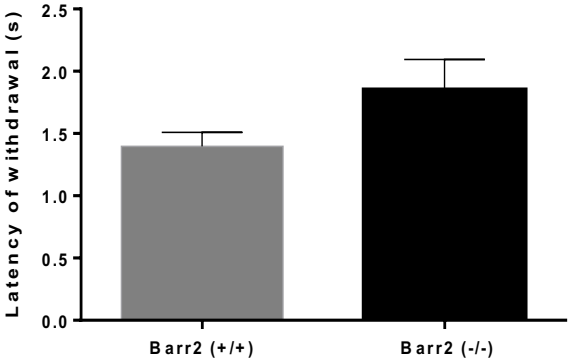


Figure 4.1.1.2.7 Mouse tail withdrawal assayofC57BL6 βarr2(+/+) and βarr2(-/-) mice displaying the latency of tail withdrawal in seconds with water at 52°C.Data are mean ± s.e.m. of 9-10 mice per group.

To further investigate the effect of genotype in inflammatory pain, 30 µl of 1.5% formalin solution was injected into the dorsal surface of theright hind paw of mice to elicit the biphasic nociceptive response typical to the formalin test (Rizzi et al., 2016). After formalin injection, the first phase lasted 10 minutes, followed by a second phase of nociception behavior that lasts approximately 30 minutes. Regarding the first phase, no differences between genotype were observed. In the second phase, however, βarr2(-/-) mice displayed significantly lower nociceptive behavior when compared to the C57BL6 βarr2(+/+) mice. To achieve more robust data, some mice were added to the formalintest, thus displaying a bigger number of mice when compared to the tail withdrawal test.

It is reported in literature that mice lacking β-arrestin2 display enhanced morphine analgesia, thus being more sensible to the analgesic effect elicited by opioid drugs when compared to wildtype mice(Bohn, 1999). Little is known, however, about the role of endogenous opioids and how stress can affect nociceptive behavior in these mice. To investigate the role of theendogenous opioid system in modulating nociceptive behavior under stressful conditions, the stress-induced analgesia (SIA) assay was performed as previously described by Rizzi *et al*., 2001. According to Rizzi, after exposure to low severity swims session with water at 32°Cmice displayed SIA that was sensitive to naloxone, thus suggesting the involvement of the endogenous opioid system. It is worth mentioning that a different strain of mice (Swiss) was used by Rizzi et al. After subjected to the swim session, βarr2(+/+)did not display any differences in tail withdrawal latency when comparedto naïve mice. Noteworthy, βarr2(-/-) mice displayed a significantly higher latency in tail withdrawal 5 minutes after undergoing the swim session (Figure 4.1.1.2.8)

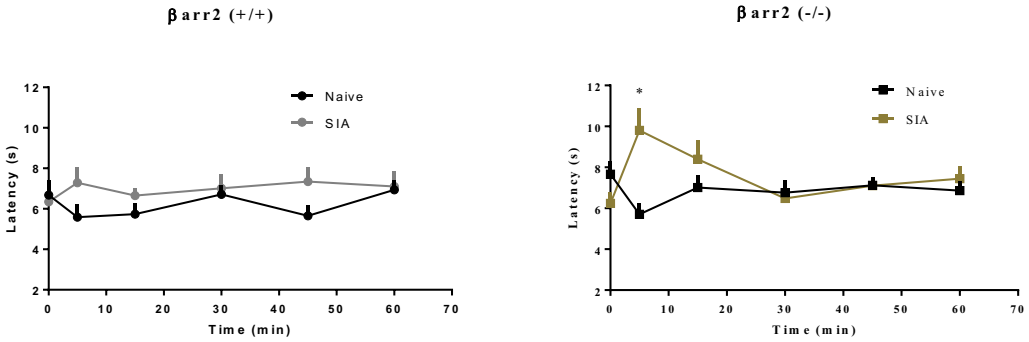


Figure 4.1.1.2.8 Effects of Stress Induced Analgesia across time in the Tail Withdrawal assay compared with naïve mice. Each point represents the mean (12 animals per group) with SEM.*P<0.05 versus naive according to analysis of variance(ANOVA) followed by the Dunnett's test.

To investigate if the SIA observed was due to endogenous opioid signalling, naloxone (3mg/kg) was injected in mice subjected to swim sessions and their latency of tail withdrawal was recorded. Pre-treatment with naloxone reduced the time of tail withdrawal when compared to βarr2(-/-) mice injected with saline (Figure 4.1.1.2.9).

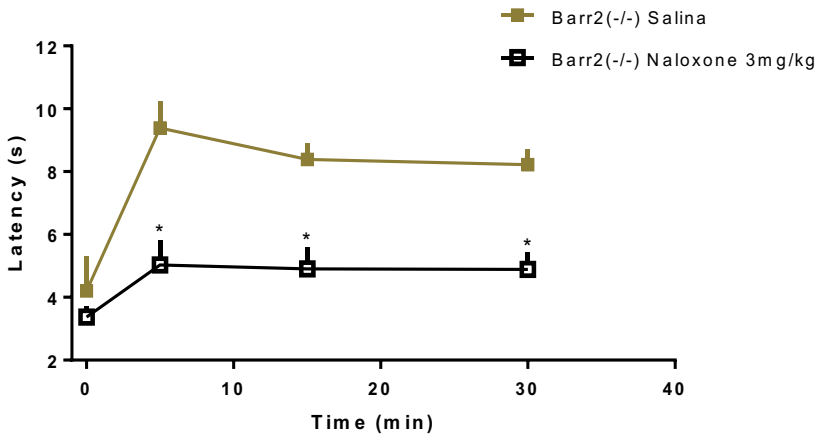


Figure 4.1.1.2.9 Nociceptive responses in βarr2 (-/-) mice forced to swim for 3 min at 32°C treated with saline or naloxone 3 mg/kg,s.c..Each point represents the mean (7 animals per group) S.E.M. .*P<0.05 versus saline (Student's t-test for unpaired data).

The current study reports the behavioral phenotype of mice lacking β-arresting 2 in different behavioral parameters related to locomotion, anxiety, depression and nociception. Importantly, to the intent of investigating the analgesic effect of compounds in these mice, their phenotype related to pain was of particular interest.

No differences were observed between βarr2(+/+) and βarr2(-/-) mice in terms of locomotor activity. This data is important because modifications of locomotor activity can bias all other behavioral tests performed and suggest that β-arrestin 2 might not have any key role in controlling locomotor activity. These results are in line with other studies in whichβ-arrestin 2 deficient mice (mixed background 129/Sv × C57BL/6J)did not display differences in ambulatory distance in different protocols of open field assay when compared to the wildtype mice(Beaulieu et al., 2005; David et al., 2010). Although the present results are in line with literature, it is worth mentioning that one research group recorded significantly lower locomotor activity in βarr2(-/-) mice when compared to their wildtype litters mates in the open field assay (Beaulieu et al., 2008).

Similarly, to evaluate the anxiety-like behavior of mice, the open field and elevated plus maze assays were performed. Regarding the open field assay, no differences between genotype were observed in the time spent in the center zone as well as number of entries in the center. The findings of the open field corroboratethe results obtained in the elevated plus maze, in which no differences between genotype were observed. This may indicate that the lack of β-arrestin 2 does not alter anxiety levels under our experimental conditions. In line with these findings Beaulieu *and* collaborators reported that βarr2 (-/-) mice do not display any basal difference in the Light-Dark Test. Differently, David et al., 2010reported an anxious-like phenotype of βarr2 (-/-) mice treated with vehicle (mixed background 129/Sv × C57BL/6J) when compared to their wildtype littermates in the open field test and novelty suppressed feeding test. These discrepant results might be related to the different experimental procedures. In fact, the present experiments were performed in naïve mice, with slight animal handling, which minimizes stress to the animal. Whereas in the study quoted above animals were treated with vehicle injection, which *per se* is a stressful stimulus that can enhance anxiety parameters (Du Preez et al., 2020).

To assess the depressant-like behavior of mice, the forced swimming test was performed as well as the learned helplessness model, in which some rodents display the helpless condition after being subjected to a protocol of uncontrollable and unpredictable electric footshocks (O' Neil and Moore, 2003). Regarding the FST, βarr2(+/+) and βarr2(-/-) mice spent the same amount of time immobile. Such phenotype was also reported in the tail suspension test performed by Beaulieu *et al*., with no differences in immobility time between genotype. Differently, a study conducted by Chiang *et al*. demonstrated that βarr2(-/-) mice showedreduced immobility time when compared to βarr2(+/+), thus displaying an antidepressant-like behavior. It is worth mentioning that our forced swim test protocol consists of a 2 days test (training session + test session) which enhances the immobility time of mice and is ideal for screening antidepressant compounds, whereas Chiang *et al*

performed a single day forced swim of six minutes with no training session.Similar to Chiang findings, in the learned helplessness model, βarr2(-/-) mice did not develop the helplessness phenotype, being able to escape the shocks at least 10 (out of 30) times in the screening session, thus displaying an antidepressant phenotype.βarr2(+/+) mice displayed significantly higher number of failure compared to escape behaviors, with 70% of the βarr2(+/+) mice developing the helpless phenotype in the screening session. By contrast, none of the βarr2 (-/-)mice tested under these experimentalconditions developed the helpless phenotype.

4.1.1.2.2 Effects of Ro 65-6570 in βarr2(+/+) and βarr2(-/-) mice

The NOP selective agonist Ro 65-6570 has been reported in literature to have analgesic effects (Rizzi et al., 2016) as well as a prominent sedative effect that makes the range of therapeutic dose very narrow. Therefore, we assessed the analgesic and sedative effect of Ro65-6570 in βarr2(+/+) and βarr2(-/-) mice.

In the formalin test, the intraplantar injection of 20 μL of 1.5% formalin solution into the dorsal surface of the right hind paw produced a biphasic nociceptive response. The I° phase started immediately after formalin injection and lasted for 10 min, while the II° phase was prolonged, starting approximately 15–20 min after the injection and lasting for about 40 min. No differences were detected in βarr2(+/+) and βarr2(-/-) mice treated with vehicle. Ro65-6570, 1 mg/kg, i.p., did not modify the first phase of the test in both genotypes. In the second phase of the assay, Ro 65-6570 reduced nociceptive behaviour of βarr2(-/-) but not βarr2(+/+) mice (Figure 4.1.1.2.10).

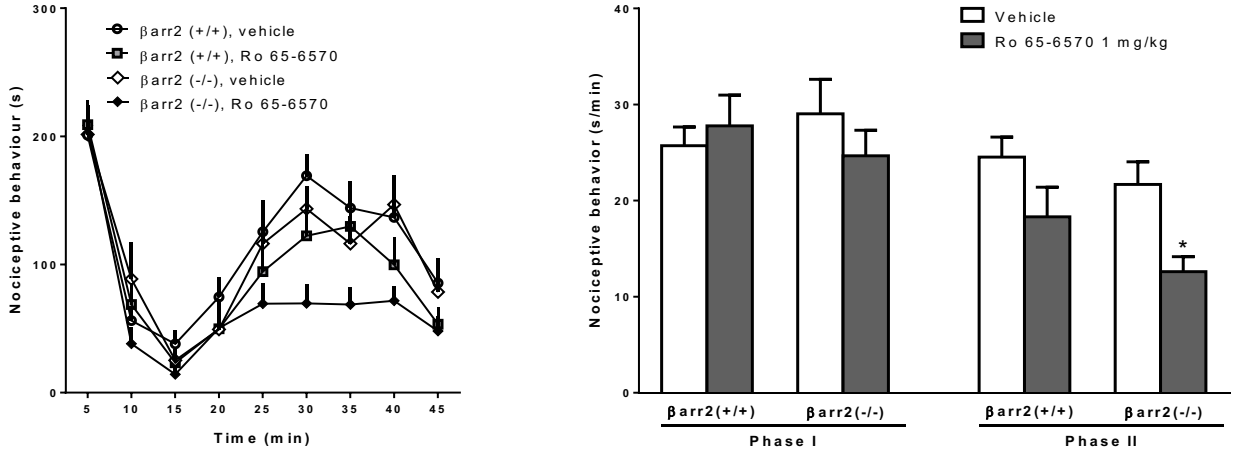


Figure 4.1.1.2.10. Formalin test in βarr2(+/+) and βarr2(-/-) mice, effect of Ro 65-6570 1 mg/kg, i.p. Left panels: time course of formalin-induced pain behaviour. Right panels: cumulative formalin-induced pain behaviour during the I° and II° phases. Each point represents the mean ± sem of 10 mice / group. Two-way ANOVA (treatment x genotype) revealed an effect of Ro-656570 in the second phase $F(1, 35) = 10.72$. *$p < 0.05$vs vehicle, Bonferroni's test.

Regarding sedation, coordination impairment elicited by the Ro 65-6570 was investigated using the rotarod assay. The NOP agonist was tested in dose response curves. As shown in figure 4.1.1.2.11, no differences were detected between βarr2(+/+) and βarr2(-/-) mice treated with vehicle on the rotarod. Ro65-6570 evoked a significant impairment of motor performance of βarr2(+/+) at the doses of 3 and 10 mg/kg. Differently, Ro 65-6570 significantly reduced the time spent on the rod by βarr2(-/-) mice only at the 10 mg/kg dose.

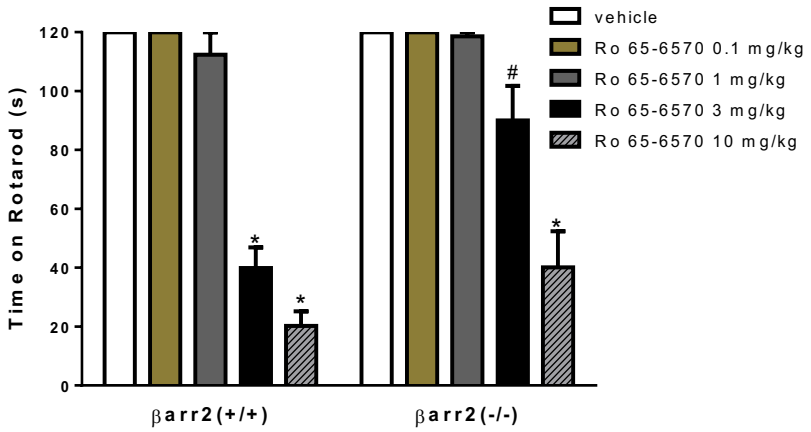


Figure 4.1.1.2.11.Rotarod test in βarr2(+/+) and βarr2(-/-) mice, effect of Ro65-6570 (0.1 – 10 mg/kg). Each point represents the mean ± sem of 8 mice / group.Two-way ANOVA treatment x genotype revealed an effect of treatment, genotype, and their interaction ($F(4, 70) = 78.77$, $F(1, 70) = 13.68$, $F(4, 70) = 5.30$). *$p < 0.05$ vs vehicle, #$p<0.005$ vsβarr2 (+/+), Bonferroni's test.

Using βarr2(-/-) mice, we demonstrated increased sensitivity to NOP agonist induced analgesia and less liability to induce sedation and motor impairment in the absence of βarr2 mediated signalling.

No phenotype differences were detected between βarr2(+/+) and βarr2(-/-) mice regarding the sensitivity to formalin and their motor performance on the rotarod. Anyway, they have shown different sensitivity to the analgesic and sedative effects of Ro 65-6570. Specifically, Ro 65-6570 1 mg/kg failed to produce analgesic effects in βarr2(+/+) but was effective in βarr2(-/-) mice. The absence of effect of Ro 65-6570 1 mg/kg in wild-type mice can be ascribed to the fact that C57BL/6J mice are less sensitive to the analgesic effects of NOP agonists than CD-1 mice used in our previous studies (Rizzi et al., 2016). Unfortunately, dose higher than this could not be used because of the motor impairment effect that represents a confounding factor. Mice lacking the βarr2(-/-) protein resulted more sensitive to the analgesic effects of Ro 65-6570, that at 1 mg/kg significantly reduced pain related behaviours due to the formalin administration.This parallels findings that morphine analgesia is potentiated in βarr2(-/-) mice (Bohn et al., 1999; Neto et al.,

2020) and in phosphorylation-deficient mu knock-in mice (Kliewer et al., 2019) and can be interpreted assuming that βarr2 dependent signal is not involved in inducing NOP mediated analgesia but βarr2 acts (as demonstrated for several GPCRs) as a desensitizing element of NOP agonists analgesia. Of note, events of phosphorylation and internalization have already been described for the NOP receptor (Corbani et al., 2004; Zhang et al., 2012; Mann et al., 2019b).

As far as sedative effects are concerned, βarr2(-/-) mice resulted less sensitive than βarr2(+/+) mice to the effects of Ro 65-6570 in the rotarod, with the 3 mg/kg dose being active in wild-type animals but not in mice lacking the βarr2 protein gene. Thus, the βarr2 protein seems to contribute, at least in part, in mediating the effect of NOP agonists on locomotion, since the absence of this protein renders mice less sensitive to the locomotor impairment related to Ro 65-6570.

In fact, mice lacking βarr2 displayed no effect to locomotor impairment induced by lithium in a novelty-induced locomotor activity when compared to vehicle treated mice. A behaviour not shared by their wild type littermates, in which a single dose of lithium was able to reduce significantly ambulatory exploration in a novel environment (Beaulieu. 2008) This led us to speculate that NOP agonists biased away from the βarr2 can be effective analgesic with less sedative side effects.

4.1.1.2.3 In vivo actions of NOP receptor ligands showing different degree of biased agonismin CD-1 wild type mice

This above mentioned experiment with βarr2(-/-) mice prompted us to hypothesize that NOP agonist biased toward G protein can be more effective analgesics with higher therapeutic window vs the sedative / motor impairment side effect. To test this attractive hypothesis we used the three NOP agonists Ro 65-6570, AT-403, and MCOPPB performing Formalin Test and locomotor activity assay in CD-1 wildtype mice

All the compounds have been deeply characterized in vitro in our laboratories in a panel of assays(Ferrari et al., 2017).From this study all the compounds resulted full NOP agonists, with the following rank order of potency MCOPPB > AT-403 > Ro 65-6570. Approximately, MCOPPB showed 3 fold and 30 fold higher potency than AT-403 and Ro 65-6570, respectively. The high potency of MCOPPB is reported also in other studies (Hirao et al., 2008a; Hayashi et al., 2009; Mann et al., 2019b). Interestingly, the three compounds displayed different G protein vs βarr2 bias factor values, in particular AT-403 is the only balanced NOP ligand of the series, MCOPPB shown a bias factor of 0.97 and Ro 65-6570 shown the higher bias factor of 1.64 (Ferrari et al., 2017). A similar bias factor value for MCOPPB has been estimated in a separate study(Chang et al., 2015).

Firstly, to evaluate the range of dose in which an analgesic effect could be tested without biased results due to sedation or motor impairment, the locomotor activity test was performed.

Effect of NOP ligands AT-403, MCOPPB and Ro65-6570 in locomotor activity

In the locomotor activity test, CD-1 mice treated with vehicle travelled ~ 120 m, spent ~ 600 min immobile, and performed ~ 500 rearings over the time course of the experiment. AT-403 and Ro65-6570 fully inhibited mouse locomotor activity at the dose of 1 and 10 mg/kg, respectively, being inactiveat lower doses. On the contrary, MCOPPB did not significantly change animal locomotion in the range of doses examined (Figure 4.1.1.2.12).To investigate the receptor mechanism involved in the sedative action of AT-403 and Ro 65-6570, the drugs were tested in NOP(-/-) mice. As shown in Figure 4.1.1.2.13, 1 mg/kg AT-403 and 10 mg/kg Ro-656570 fully inhibited locomotion in NOP(+/+) mice while they were completely inactive in NOP(-/-).

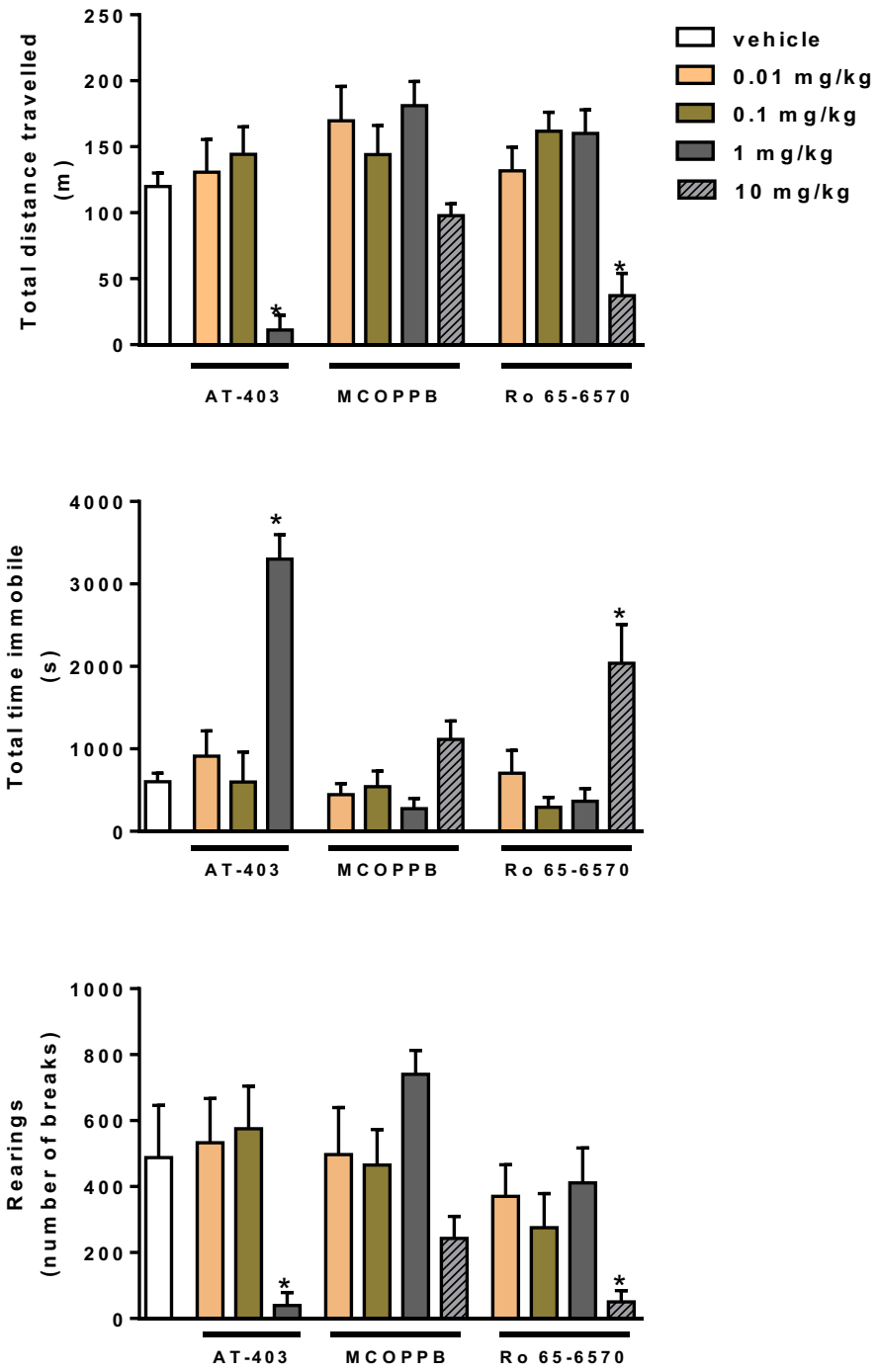


Figure 4.1.1.2.12 Locomotor activity test in CD-1 mice, dose response curves to AT-403, MCOPPB and Ro-656570. Top panel: cumulative distanced travelled in 60 min, central panel: total time spent immobile in 60 min, bottom panel: cumulative number of rearings performed in 60 min. Each point represents the mean ± sem of 6 mice / group. One-way ANOVA revealed an effect of NOP agonists in the total distance travelled F (11, 63) = 8.00, immobility time F (11, 63) = 13.53, and number of rearings F (11, 63) = 3.76. *p < 0.05vs vehicle, Dunnett's test.

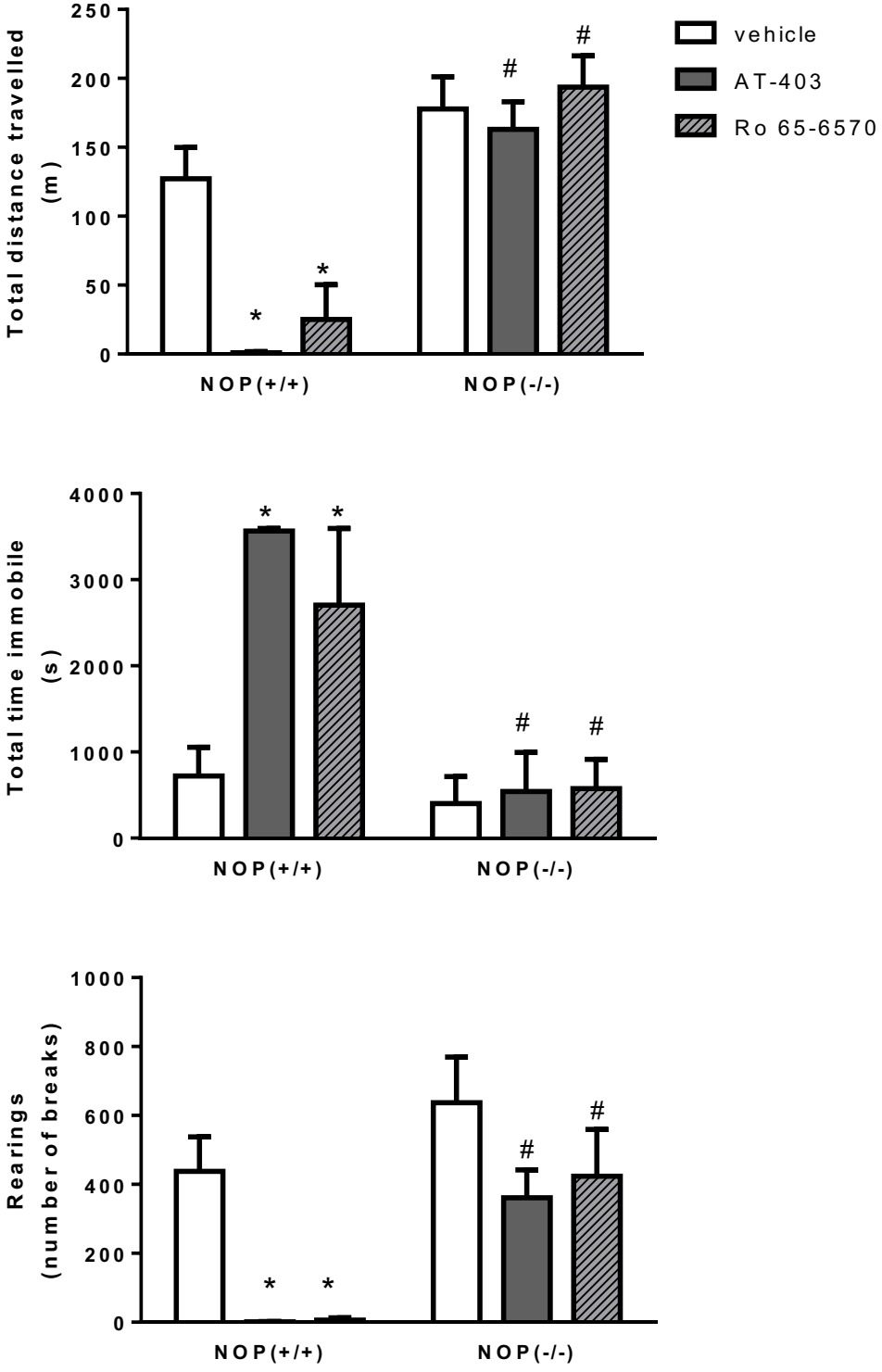


Figure 4.1.1.2.13. Locomotor activity test in CD-1 mice, dose response curves to AT-403, MCOPPB and Ro-656570. Top panel: cumulative distanced travelled in 60 min, central panel: total time spent immobile in 60 min, bottom panel: cumulative number of rearings performed in 60 min. Each point represents the mean ± sem of 6 mice / group. One-way ANOVA revealed an effect of NOP agonists in the total distance travelled F (11, 63) = 8.00, immobility time F (11, 63) = 13.53, and number of rearings F (11, 63) = 3.76. *p < 0.05vs vehicle, Dunnett's test.

Effect of NOP ligands AT-403, MCOPPB and Ro65-6570 in formalin test

After excluding the doses that elicited motor impairment, Formalin Test was performed as mentioned before according to Rizzi et al., 2016**.** AT-403 inhibited both the I° and the II° phase of the assay at 0.1 mg/kg. A complete dose response curveto this compound could not be obtained due to its effects on locomotor performance at high doses. MCOPPBproduced dose-dependent antinociceptive effects, being active at 1 and 10 mg/kg, for the I° and the II° phase, respectively (Figure 4.1.1.2.14). The dose response curve to Ro 65-6570 in CD-1 mice has been previously performed in our laboratory (Rizzi et al., 2016).

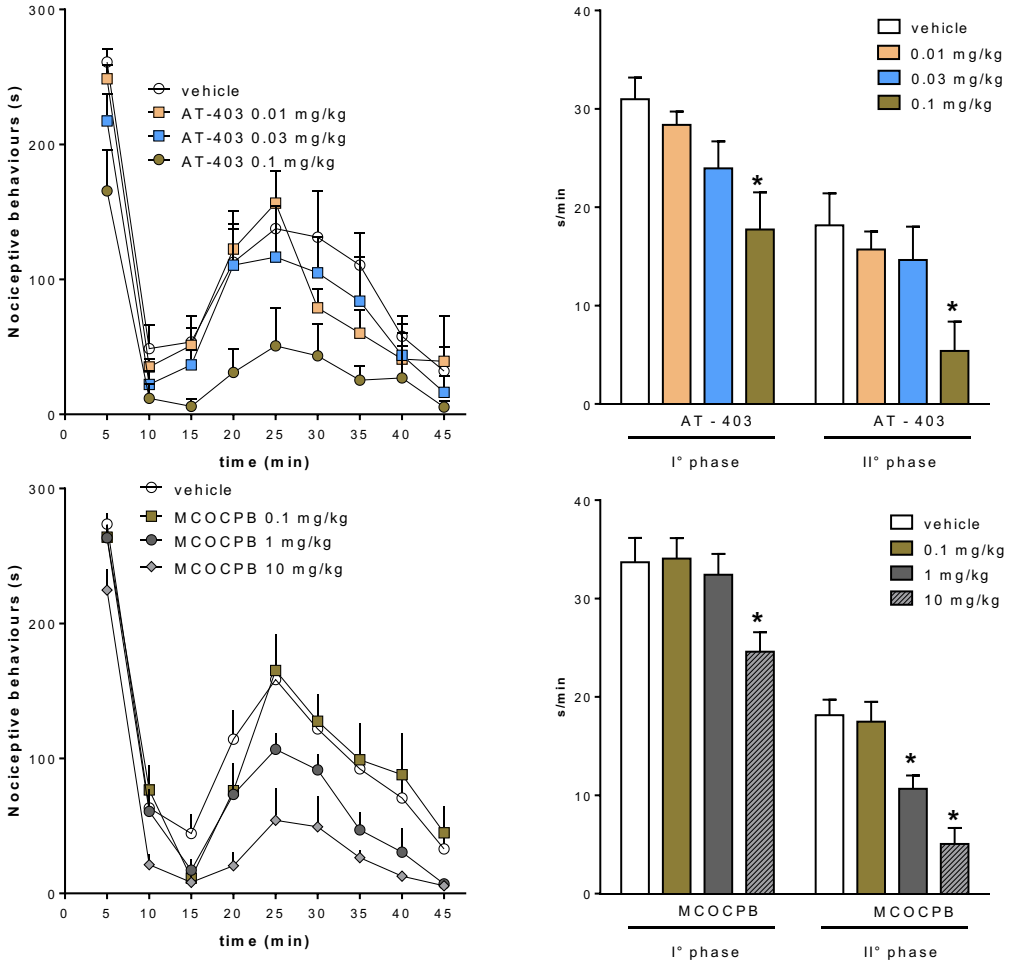


Figure 4.1.1.2.14. Formalin test in CD-1 mice, dose response curves to AT-403and MCOPPB. Left panels: time course of formalin-induced pain behaviour. Right panels: cumulative formalin induced pain behaviour during the I° and II° phases. Each point represents the mean ± sem of 8 mice / group. One-way ANOVA revealed an effect of AT-403 in the first F (3, 28) = 4.71 and second phase F (3, 28) = 3.65 and an effect of MCOPPB in the first F (3, 28) = 4.05 and second F (3, 28) = 13.98 phase,. *p< 0.05vsvehcle,Dunnett's test.

To analyse the therapeutic index of these compounds, a dose response curve for each compound was made in which the effect of a certain dose can be correlated with its biological effect regarding analgesic effect (formalin) and motor impairment (LA). In such graphics, the x axis displays the range of doses used whereas the y axis displays the biological effect normalized as a % of the control response (Figure 4.1.1.2.15).

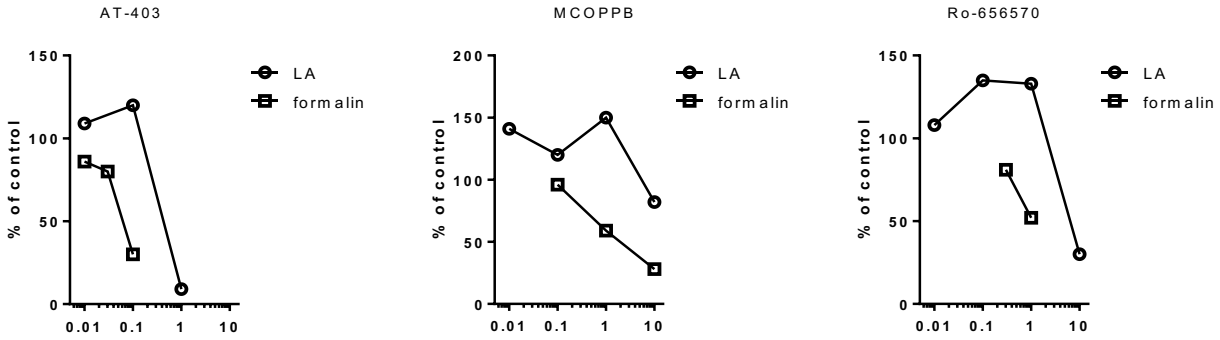


Figure 4.1.1.2.15 Dose response curves showingvalues as per cent of control for the analgesic effect in the formalin test (formalin) and sedative effect in locomotor activity (LA) of all compounds tested in CD-1 wild type mice.

To calculate the therapeutic index (analgesic vs sedative effects) of the three NOP agonists, the ED_{50s} were extrapolated from the dose response curves is shown in Figure 4.1.1.2.15. Data for Ro65-6570 in the formalin assay have been published previously (Rizzi et al., 2016). For the ED_{50s} calculation the bottom and the top of the curves have been constrained to 0 and 100%, respectively. Therapeutic indexes of 13, 10, and 9 have been calculated for AT-403, MCOPPB, and Ro-656570, respectively. No correlation between these values and the bias factors previously reported(Ferrari et al., 2017) has been detected, data are summarized in Table 4.1.1.2.2.

Table 4.1.1.2.2: ED_{50}, therapeutic index and bias factor for AT-403, MCOPPB, and Ro-656570

	ED_{50} - Formalin test	ED_{50} - LA test	therapeutic index	bias factor[a]
AT-403	0.07	0.88	13	0.16
MCOPPB	1.10	10.88	10	0.97
Ro-656570	1.04[b]	9.79	9	1.64

[a]data from (Ferrari et al., 2017); [b]data from (Rizzi et al., 2016)

In mice both MCOPPB and AT-403 produced analgesic effects in the formalin assay(Figure 4.1.1.2.14), being active from 1 and 0.1 mg/kg, respectively. Of note, the second phase of the assay seems more sensitive to the analgesic effect of NOP agonist, suggesting an involvement of the NOP receptor mainly in regulating the inflammatory phase of the test. A similar analgesic effects was recorded for Ro 65-6570 in the formalin assay at the dose of 1 mg/kg (Rizzi et al., 2016). Thus, all the NOP agonist tested produced analgesic effects, with the following rank order of potency AT-403 > MCOPPB = Ro 65-6570. What is unexpected from these data is the low in vivo potency of MCOPPB. Anyway, our finding are in line with previously in vivo studies that reported MCOPPB active as anxiolytic only at the 10 mg/kg dose after oral administration (Hirao et al., 2008a). This in vivo low potency of MCOPPB can be ascribe to pharmacokinetic issues. Anyway, the compound has been reported to be able to cross the blood brain barrier after oral administration and to produce in the mouse brain a NOP occupancy higher than 50% in the whole mouse brain(Hayashi et al., 2009)

As far as the reduction of locomotor activity is concerned, both AT-403 and Ro 65-6570significantly reduced mouse locomotion at 1 and 10 mg/kg doses, respectively. This effect is selectively due to the activation of the NOP receptor, since it completely disappeared in NOP(-/-) mice. Thus, the balanced agonists AT-403 and our best biased ligand Ro 65-6570 produced the same effect on locomotor activity, suggesting no correlation between the activation of the βarr2 dependent pathway and side effects on locomotion. Differently, MCOPPB failed to produce significant sedative effects until 10 mg/kg. Literature data reported the inactivity of this compound also at 30 and 50 mg/kg (Hirao et al., 2008a). The reason for this in vivo pharmacological profile is not known, we can speculate that, despite the NOP occupancy higher than 50% measured in the whole mouse brain (Hayashi et al., 2009), at 10 mg/kg i.p. this compound is not able to reach in a sufficient amount those specific brain area important for the effects of NOP agonists on locomotion (i.e. substantia nigra, Marti et al., 2009).

What is clear from these experiments is that, despite their different in vitro profile, all the NOP agonists tested displayed the same therapeutic index. In particular, no differences were detected between AT-403 (balanced agonist) and Ro 65-6570 (biased agonist with higher bias factor), indicating that the different ability of a NOP agonist of inducing G protein or βarr2 recruitment in vitro do not affect its ability of producing analgesia or sedation in vivo. Our pharmacological results counteract the hypothesis that the functional selectivity phenomenon at the NOP receptor can be exploited to obtain analgesics devoid of sedative effects;however some caution should be adopted in the interpretation of these results. In particular, the biased ligands used in this study shown bias

factor of 0.94 (MCOPPB) and 1.64 (Ro 65-6570). These values mean that the difference in the recruitment of G protein vsβarr2 is limited to 10 fold for MCOPPB and 40 fold for Ro 65-6570. It is possible that these bias factors are too low to have in vivo biological implications. For instance, it has been reported that both MCOPPB and the analogous of Ro 65-6570, Ro 64-6198, are able to induce NOP receptor phosphorylation and internalization similarly to the natural ligand N/OFQ, both in cells and in mice (Mann et al., 2019), demonstrating that βarr2 dependent biological events can occur after the treatment with these ligands.It is worthy of mentionat this regard that the NOP agonist cebranopadol displays a very large bias towards G protein in vitro (Rizzi et al., 2016) and acts in vivo as a potent analgesic devoid of sedative effects (Schunk et al., 2014; Rizzi et al., 2016). However, the in vivo pharmacological actions of cebranopadol cannot be simply attributed to its NOP agonist activity since this molecule is able to simultaneously activate NOP and classical opioid receptors (Calo and Lambert, 2018; Tzschentke et al., 2019). Unfortunately, nothing is known about the functional selectivity of these compounds for the different types of inhibitory G proteins that can be activated by the NOP receptor, this notion could help to explain a possible significance of biased agonism for the NOP receptor.

Finally we would like to underline some limitations of the present study. First, the estimate of the NOP agonist therapeutic indexes is far from being optimal since some ED_{50} values could be only roughly estimated. Second, some comparisons between set of data have to be looked at with caution because different strains(C57BL/6J vs. CD-1) of mice and drug route of administration(i.p. vs. i.v.) have been used. Third, no information is available on the pharmacokinetic properties of the drugs used in this study; such information could significantly contribute to correctly interpret in vivo findings. However, at least in our opinion, the above mentioned limitations do not substantially compromise the major finding of the study that is lack of correlation between in vitro biased agonism and in vivo therapeutic index of NOP agonists.

4.1.2. NOP receptor modulation in acute stress in mice

Besides the previous mentioned effects of NOP agonists to elicit analgesia and sedation *in vivo*, there is in literature a large amount of information supporting antidepressant actions due to the blockage of the NOP receptor signalling (see introduction for detailed information). It is unclear, however, how the endogenous N/OFQ – NOP receptor system is involved in mediating stress coping strategies. Of note, conflicting results have been reported about the expression of the N/OFQ and its receptor NOP in the rodent brain under stressful situations (Devine et al., 2003; Green and Devine, 2009; Delaney et al., 2012; Nativio et al., 2012; Ciccocioppo et al., 2014; Granholm et al., 2015). For example, acute restraint stress enhanced N/OFQ expression in the hippocampus (Nativio et al., 2012), while a reduction of the peptide levels was detected at the basal forebrain under similar conditions (Devine et al., 2003). Concerning the NOP receptor, an increase in the NOP mRNA was detected in the amygdala and hypothalamus of rats exposed to acute stressful stimuli (Ciccocioppo et al., 2014; Green et al., 2009a). By contrast, a reduction in the receptor transcripts was observed at the same brain areas under analogous conditions (Delaney et al., 2012). These contradictory findings may be related to individual susceptibilities to stress that were neglected when interpreting these results. Of note, Der-Avakian et al. (2017) studied the relationship between N/OFQ system expression and mouse behavior after exposure to the social defeat stress. A negative correlation was observed between behavioral performance in a rewarding task (generally compromised during depressive episodes) and ppN/OFQ and NOP mRNA expression in brain areas related to depression (Der-Avakian et al., 2017). This pivotal study, for the first time, linked the overexpression of the endogenous N/OFQ system with a depressive-related phenotype.

Based on this scarce literature, the aim of the present study was investigating how the activation or blockade of the NOP receptor signalling affect stress coping responses. To achieve this aim, two non peptide NOP agonists, Ro 65-6570 (Wichmann et al., 1999) and MCOPPB (Hirao et al., 2008) and one NOP antagonist, SB-612111 (Zaratin et al., 2004), were used . Additionally, the phenotype under stressful condition NOP(/-/-) was assessed. To model stressful situations the inescapable electric foot shock task and the forced swim were used (see materials and methods).

Animal models of acute stress

After genotyping all mice, our experimental protocol was organized following 3 sets of animal models of acute stress (Figure 4.1.2.2) in order to evaluate the effect of NOP modulation (activation or blockage) before exposure to stressful stimuli.

Inescapable electric foot shock task – This is a classical animal model of acute stress with a well-accepted cut-off to select resilient and susceptible (termed 'helpless') phenotypes (Pfau and Russo, 2015). Additionally, helpless mice develop a range of behavioral and neuroendocrinal changes that mimic the symptoms of depression (Vollmayr and Gass, 2013). The administration of drugs was performed before each induction sessions, and mouse behavior was assessed, 24 h later, in the screening session, without any drug treatment

Escapable electric foot shock task – This series of experiments were performed in order to evaluate drug effects on mouse behavior under an aversive operant conditioning task (i.e., escapable electric foot shock). Equal to the inescapable electric foot shock task, the administration of drugs was performed before each induction sessions, and mouse behavior was assessed, 24 h later, in the screening session, without any drug treatment

Forced swim stress - It was performed as described previously by Gavioli et al. (2003). The immobility time (i.e. the time spent floating in the water without struggling) was recorded by an experienced observer. The drug treatments were carried out before the training session.

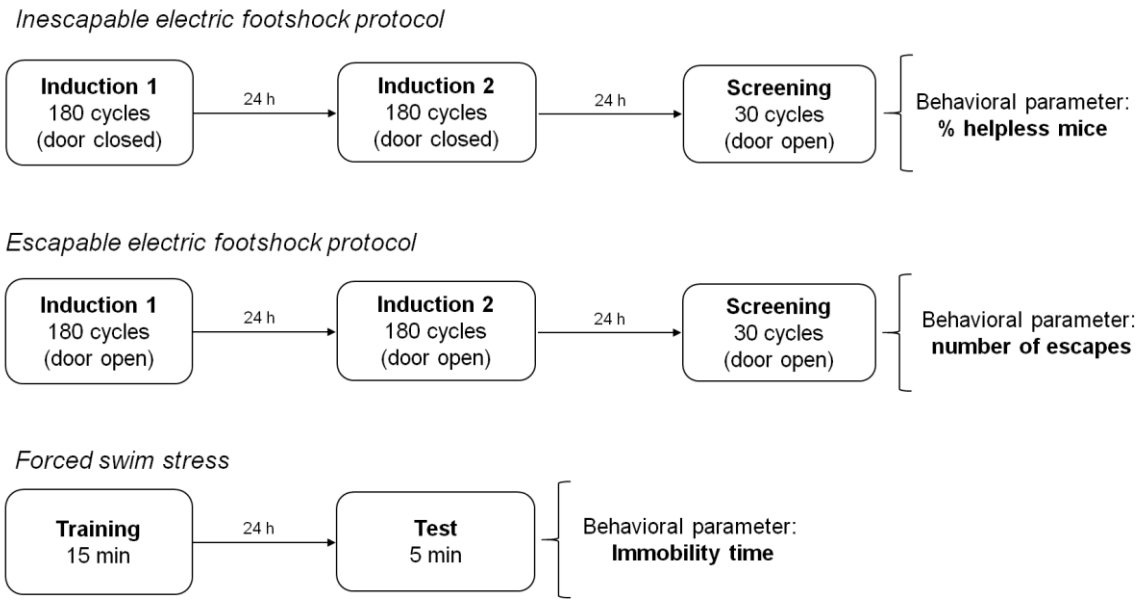


Figure 4.1.2.2. Schematic representation of the procedures and behavioral parameters employed in the electric foot shock tasks and forced swim stress. A mouse is defined helpless when it fails to escape 20 or more times (within 30 trials). Each foot shock cycle includes one electric shock (0.5 mA), with an automatically randomized duration of 1-10 s, plus 1-20 s of interval. When administered, drugs have been injected before exposition to induction sessions of electric foot shocks protocol or training session of the forced swim stress.

Effects of NOP agonists before exposure to acute stress

The systemic administration of two distinct NOP agonists, Ro 65-6570 (0.01 - 1 mg/kg) and MCOPPB (0.1 - 10 mg/kg), before exposure to inescapable electric foot shocks, significantly increased the percentage of helpless mice compared to vehicle-treated animals (Figure 4.1.2.3 left panel, $\chi 2= 118.7$, $P<0.05$, chi-square test; Figure 4.1.2.3 right panel, $\chi 2= 45.3$, $P<0.05$, chi-square test).

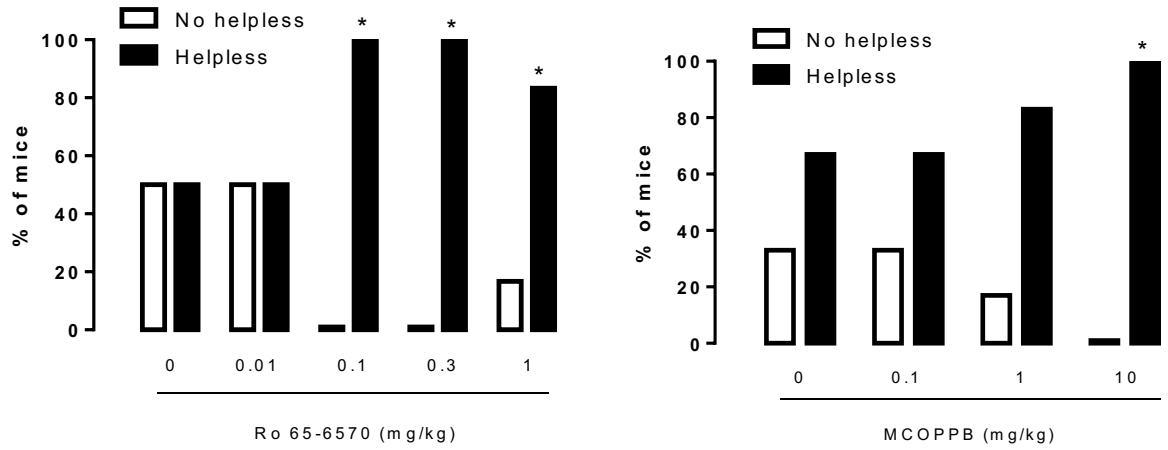


Figure 4.1.2.3. Effects of the administration before exposure to the inescapable electric foot shock of the NOP agonists (left panel) Ro 65-6570 (0.01 - 1 mg/kg, ip) and (right panel) MCOPPB (0.1-10 mg/kg, ip) on the percentage of helpless mice. Data are presented as the percentage of helpless/no helpless mice. A total number of 6 mice/group was used. *p<0.05 vs. vehicle, according to chi-square test followed by adjusted residuals.

A different set of groups were exposed to forced swim stress to confirm findings from inescapable electric foot shock task. Mice pretreated with the NOP agonist Ro 65-6570 (0.01 - 1 mg/kg), administered before the swimming training session, displayed a significant increase in the time spent immobile during the test session compared to controls (Figure 4.1.2.4, two-way RM ANOVA, interaction factor: $(F3,28)=16.32$, $P<0.05$).

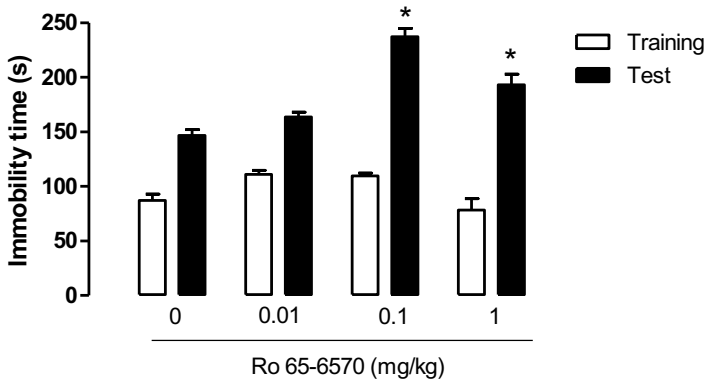


Figure 4.1.2.4. Effects of the administration of the NOP agonist Ro 65-6570 (0.01 - 1 mg/kg, ip) before exposure to forced swim stress on the time spent immobile during the training and test sessions. Data are presented as mean ± SEM of 8 mice/group. *p<0.05 vs. vehicle of its respective session, according to two-way RM ANOVA, Sidak´s post-hoc test.

Pharmacological and genetic blockade of NOP receptor before exposure to acute stress

The effects of the blockade of NOP receptor signalling by using pharmacological (SB-612111) and genetic (NOP(-/-) mice) tools wereinvestigated in the acquisition of the helpless phenotype. As showed in Figure 4.1.2.5 right panel, the systemic administration of SB-612111 at 3 and 10 mg/kg, but not at the lower dose, significantly reduced the percentage of helpless mice compared to vehicle (Figure 4.1.2.5 left panel, χ^2=64.7, P<0.05, chi-square test). By contrast, the administration of the tricyclic antidepressant nortriptyline, at the dose tested (20 mg/kg), did not change the percentage of helpless mice (Figure 4.1.2.5 right panel).

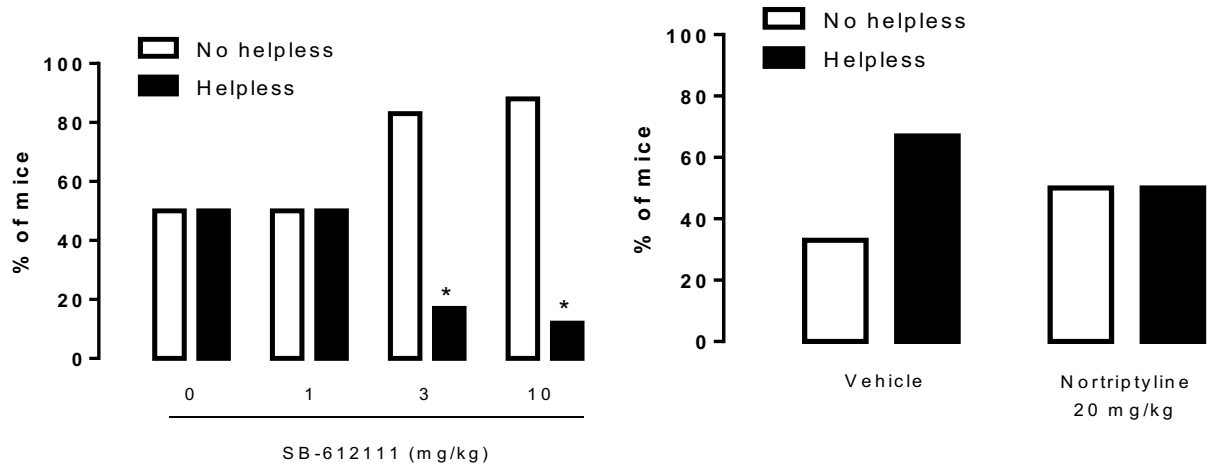


Figure 4.1.2.5. Effects of the administration before exposure to the inescapable electric foot shock of (left panel) the NOP antagonist SB-612111 (1, 3 and 10 mg/kg, ip, 30 min), and (right panel) the tricyclic antidepressant nortriptyline (20 mg/kg, ip, 60 min) on the percentage of helpless mice. A total number of 6-8 mice/group was used. *p<0.05 vs. vehicle, according to chi-square test followed by adjusted residuals.

Similarly to CD-1 mice, approximately 50% of NOP(+/+) mice developed the helpless phenotype after exposure totwo inescapable electric foot shock sessions. By contrast, NOP(-/-) mice displayed a significant resistance to develop the helpless behavior. In fact, none of the NOP(-/-) mice tested under these experimental condition developed the helpless phenotype (Figure 4.1.2.6, χ^2=100.7, P<0.05, chi-square test).

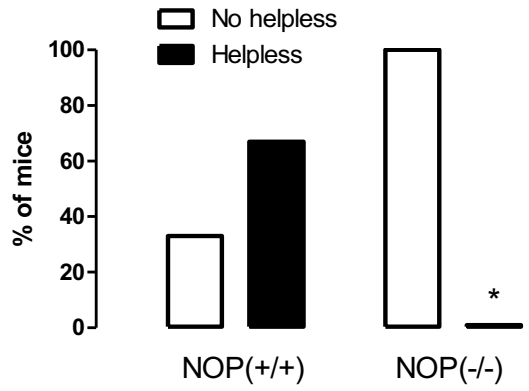


Figure 4.1.2.6. Behavioral phenotype of NOP(+/+) and NOP(-/-) mice exposed to inescapable electric foot shock. Data are presented as the percentage of helpless/no helpless 9-10 mice/group. *p<0.05 vs. NOP(+/+), according to chi-square test followed by adjusted residuals.

The pre-treatment with SB-612111 at 3 and 10 mg/kg, before exposure to forced swim stress, significantly reduced the time spent immobile in the test session compared to controls (Figure 4.1.2.7 left panel; two-way RM ANOVA, interaction factor: $F_{(3,28)}$=23.00, P<0.05). Interesting enough, the administration of nortriptyline (20 mg/kg) did not change the time spent immobile in the test session of the forced swim (Figure 4.1.2.7 right panel).

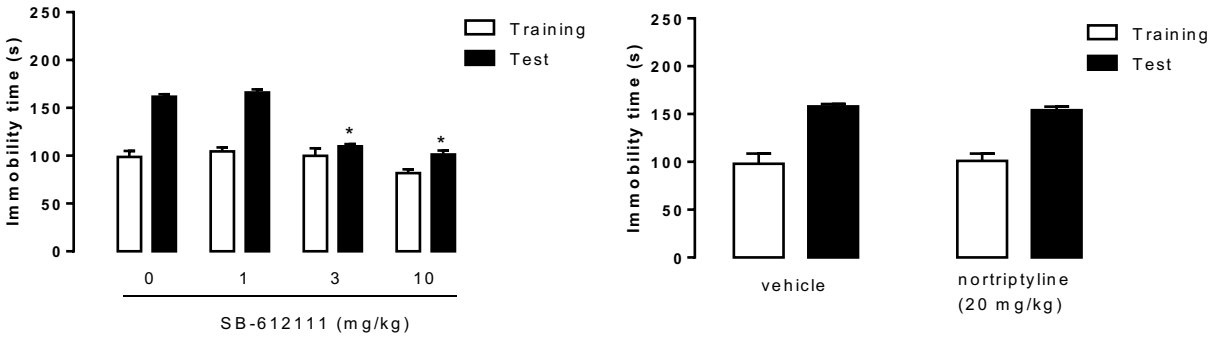


Figure 4.1.2.7. Effects of the administration of (left panel) the NOP antagonist SB-612111 (1-10 mg/kg, ip) and (right panel) the tricyclic antidepressant nortriptyline (20 mg/kg, ip) before exposure to forced swim stress on the time spent immobile during the training and test sessions. Data are presented as mean ± SEM of 8 mice/group. *p<0.05 vs. vehicle of its respective session, according to two-way RM ANOVA, Sidak's post-hoc test.

Effects of NOP ligands and mouse genotype on escapable stressful situation and spontaneous locomotion

Aimed to investigate any potential effects of NOP agonists on the escapable footshock task, the higher active doses of the NOP agonists, Ro 65-6570 (1 mg/kg) and MCOPPB (10 mg/kg), were tested. As showed in Figure 4.1.2.8, the control group escaped 20 times in 30 trials from the electrified chamber during the test session. The administration of MCOPPB pre-induction sessions did not significantly change the number of escapes. Differently, Ro 65-6570 significantly reduced the number of escapes from the electrified chamber (Figure 4.1.2.8 left panel, $F_{(2,15)}$=10.02, P<0.05, one-way ANOVA, Tukey's test).

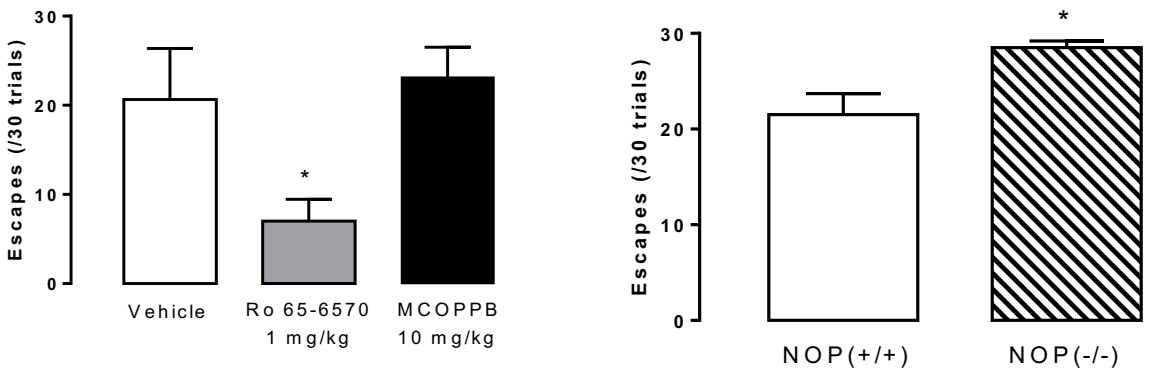


Figure 4.1.2.8. Effects of (left panel) NOP agonists Ro 65-6570 (1 mg/kg, ip) and MCOPPB (10 mg/kg, ip) and (right panel) behavioral phenotype of NOP(+/+) and NOP(-/-) mice in the escapable electric foot shock task. Data are presented as the mean ± SEM of escapes performed during test session in 6 mice/group. *p<0.05 vs. vehicle, according to ANOVA followed by Tukey's test (left) and Student's T test (right).

When tested in the escapable electric footshock task, NOP(+/+) mice were able to avoid electric foot shocks during test session, as showed by the number of escapes which are approximately 20 escapes within 30 trials. NOP(-/-) mice over performed wild-type animals in this task as showed by the significant higher number of escapes displayed during test session (Figure 4.1.2.8 right panel; t(10)=3.02, P<0.05, Student's T test).

Of note, at the doses tested in the inescapable electric footshock task and forced swim stress, neither Ro 65-6570 nor MCOPPB modified mouse locomotor activity compared to control. Only Ro 65-6570 10 mg/kg significantly reduced the distance moved (Figure 4.1.2.9, $F_{(4,28)}$=9.42, P<0.05, ANOVA, Tukey's test). Anyway, this dose was not used for testing the effects of Ro 65-6570 on mouse behavior prior to stress exposure.

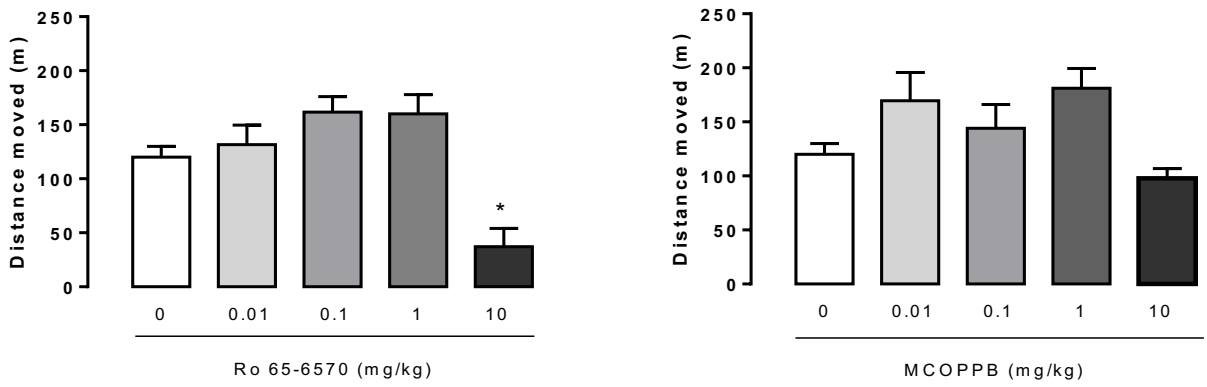


Figure 4.1.2.9. Effects of the systemic administration of (left panel) Ro 65-6570 (0.01 – 10 mg/kg) and (right panel) MCOPPB (0.01 – 10 mg/kg) on the total distance moved in the open field test. Data are the mean ± SEM of 6-7 mice/group. *p<0.05 vs. vehicle, according to one-way ANOVA followed by Tukey´s test.

In the present study, for the first time, using pharmacological and genetic approaches, we showed the involvement of the NOP receptor signalling in mediating stress coping responses. The administration of NOP agonists prior to acute stress increased the percentage of helpless mice in the inescapable electric foot shock task and facilitated the acquisition of the immobile posture in the forced swim stress. By contrast, the administration of the NOP antagonist SB-612111, prior to stress, significantly reduced the percentage of helpless mice, and NOP(-/-) mice when exposed to inescapable electric foot shock did not develop the helpless phenotype. It is relevant to mention that a similar active strategy (i.e., reduction of immobile posture) was observed when mice pretreated with SB-612111 were exposed to the forced swim stress. Thus, the activation of the NOP receptor during stressful events increased the probability of mice to develop depressive-related behaviors. On the contrary, NOPblockage prevented this phenomenon, then contributing to increase resilience to stress.

Considering the scientific claim of testing the effects of drugs on both sexes in preclinical models (Miller et al., 2017), female mice were used for investigating the influence of NOP ligands on stress coping strategies in a different acute stressor, i.e., the forced swim. It is worth of mention that a similar profile of action for NOP ligands was observed in females compared to male mice when exposed to acute stress (i.e., forced swim x inescapable electric foot shock). Additionally, a pilot study using male Swiss mice treated with SB-612111 10 mg/kg was performed in order to exclude sex influences on NOP antagonist-induced increase of stress resilience in the forced swim (Figure 4.1.2.10). Therefore, we can conclude that, despite to be reported a sex influence on the resilience to stress (Wellman et al., 2018) the ability of NOP ligands to manipulate stress coping strategies are not sex-dependent.

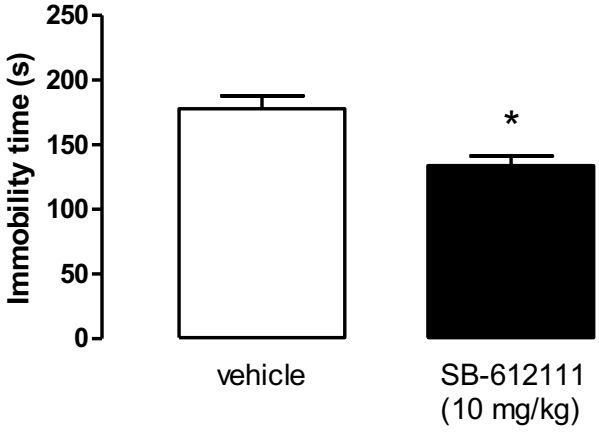


Figure 4.1.2.10. Separate set of data showing the effects of the administration of the NOP antagonist SB-612111 (10 mg/kg, ip) before exposure to forced swim stress on the time spent immobile during the 5-min test session in male Swiss mice. Data are the mean ± SEM of 5 mice/group. *p<0.05 vs. vehicle, according to Student´s T test ($t(8)=3.75$, $P<0.05$).

Our findings also showed that the standard antidepressant nortriptyline when injected prior to inescapable electric footshock and forced swim stress did not change the efficacy of the protocol in inducing helpless phenotype or affecting immobility time compared to controls. Similar results have already been reported for the tricyclic antidepressant imipramine (10 and 30 mg/kg, p.o.) when administered before inescapable footshock task (Takamori et al., 2001). Noteworthy, the ability of NOP antagonists and tricyclic antidepressants of reverting depressive-like behaviors is similar (Rizzi et al., 2007; Medeiros et al., 2015; Holanda et al., 2016; Vitale et al., 2017) but only NOP antagonists when administered before stress are able to affect stress coping responses. Taken together, our findings suggest that the endogenous N/OFQ-NOP receptor system is tonically active and directly involved in the acquisition of depressive-related behaviors during acute stress. On the other hand, acute tricyclic antidepressants-induced increase in monoamines is not sufficient for impairing the development of passive coping strategies. These observations suggest that NOP ligands exert neurochemical actions that go beyond the modulation of monoamine levels and are crucial for acquisition of a depressive-like phenotype.

The distinct profile of NOP antagonists compared to classical antidepressants can come from the relationship between the N/OFQ-NOP receptor system and stress. A growing body of evidence suggests that the activation of NOP receptor increases corticosterone and adrenocorticotrophic hormone (ACTH) circulating levels and activates the hypothalamus-pituitary-

adrenal (HPA) axis (Devine et al., 2001; Leggett et al., 2006). Additionally, changes in the N/OFQ levels after acute stress suggest that this peptide is released under stressful events (Devine et al., 2003), consequently increasing NOP receptor signalling. In this context, the administration of NOP agonists amplifies the activation of the HPA axis and NOP brain signalling, thus potentiating the consequences of stress. In contrast, the blockade of the NOP receptor signalling counteracts stress-induced increase in the N/OFQergic transmission, thus attenuating the behavioral consequences of stress. It is interesting to mention that neuroplastic adaptations on the N/OFQ-NOP receptor signalling can be at least in part explained by the control that glucocorticoids exert on N/OFQ expression. Of note, glucocorticoid responsive elements in the gene encoding N/OFQ in humans have been described (Xie et al., 1999). In line with this hypothesis, it has been shown that glucocorticoids are implicated in the acquisition of helpless behavior during exposure to inescapable foot shocks and facilitation of immobile postures in the forced swim stress ((De Kloet et al., 1988; Báez and Volosin, 1994). Further studies aimed to investigate the involvement of glucocorticoids in NOP agonists-induced depressive-related behaviors are in progress (Holanda et al., 2020).

The effects of NOP ligands and the NOP genotype on memory and on spontaneous locomotion may be confounding factors for the interpretation of the helpless phenotype. Thus some experiments have been performed to investigating this aspect. To assess the cognitive effects of NOP agonists and of the lacking of the NOP protein, mice were subjected to the escapable foot shock task. When administered before the induction sessions in the escapable footshock task, Ro 65-6570, but not MCOPPB, significantly reduced the number of escapes from the electrified chamber; consequently, animals treated with Ro 65-6570 did not acquire and exhibit the operant response that controls aversive events. Memory impairments were previously reported for peptide and non peptide NOP agonists in rodents (Abdel-Mouttalib, 2015; Andero, 2015). However, regarding the results reported in the present study, it is unlikely that NOP agonists-induced memory impairments can bias the interpretation of their action on helpless behavior acquisition. The memory deficits can eventually prevent the acquisition of the helpless phenotype, but this is not the case for mice treated with Ro 65-6570, that, on the contrary, showed higher probability to develop helpless behavior. Of note, MCOPPB does not affect mouse cognition, as showed here (i.e., it did not affect escapable footshock task) and reported (Hirao et al., 2008b) but facilitated the acquisition of helpless phenotype similarly to Ro 65-6570. Additionally, when Ro 65-6570 was administered before exposition to the forced swim stress, it facilitated the acquisition of an immobile posture. Literature data support the fact that immobility behavior during the forced swim does not correlate to cognitive impairments (Borsoi et al., 2014), thus reinforcing the view that NOP agonists-induced

cognitive impairments and passive stress coping responses are distinct actions unrelated to each other. As far as SB-612111 effects on escapable footshock task are concerned, Holanda and colleagues (2016) previously reported that this NOP antagonist do not affect the mouse cognitive performance. Additionally, we observed that NOP(-/-) mice escaped significantly more than wild-type animals from the electrified chamber when assessed in the escapable footshock task. An enhancement of learning and memory abilities in mice with genetic depletion of the N/OFQ precursor (Kuzmin et al., 2009) or of the NOP receptor gene (Nagai et al., 2007) has already been reported. However, learning and memory enhancement displayed by NOP(-/-) mice cannot explain their reduced acquisition of helpless behavior, since during the induction sessions (day 1 and 2) of the inescapable stressful task, animals do not have any opportunity to learn how to avoid the electric foot shocks. Therefore, we believe that during the screening session (day 3), when mice can escape from aversive stimuli, NOP(-/-) mice do not develop a passive strategy and distinct from wild-type mice they assume an active form of stress coping behavior.

Spontaneous locomotion and nociception are two other confounding factors for the interpretation of the NOP ligands-induced increased/decreased acquisition of the helpless behavior. As far as locomotor activity is concerned, we demonstrated that Ro 65-6570 and MCOPPB did not modify spontaneous locomotion at the doses able to modify animal behavior under acute stress. Additionally, data from literature demonstrated that the NOP antagonist SB-612111 do not affect mouse spontaneous locomotion (Rizzi et al., 2007; Silva et al., 2018). Moreover, in line with previous findings (Marti et al., 2004; Nishi et al., 1997), in the present study we detected no differences in the distance moved between NOP(+/+) and NOP(-/-) mice. Concerning the effects of NOP ligands on nociception, a large body of evidence, recently reviewed in Toll et al. (2016), demonstrated that selective NOP agonists and antagonists do not modify acute nociceptive pain transmission. This has been confirmed in the mouse tail withdrawal assay in our laboratories both for Ro 65-6570 (Rizzi et al., 2016) and SB-612111 (Rizzi et al., 2007). To the best of our knowledge no data are reported in the literature regarding MCOPPB in analgesiometric assays.

Finally, NOP(-/-) mice display similar acute nociceptive threshold as wild type mice (Di Giannuario et al., 2001; Nissi et al., 1997). Thus, we propose that the actions of NOP ligands on acquisition of the helpless behavior are genuine and not biased by confounding factors such as locomotion, and nociception and the same can be said for the phenotype displayed by NOP(-/-) mice.

In conclusion, the administration of NOP agonists Ro 65-6570 and MCOPPB prior to stress increased expression of the helpless behavior and facilitate immobile posture. By contrast, the pretreatment with the NOP antagonist reduced the percentage of helpless mice and decreased

immobility time, and similar findings were observed in NOP(-/-) mice, as showed here and elsewhere (Gavioli et al., 2003). Thus, we hypothesized that a potentiation of the N/OFQergic transmission takes place during acute stressful events, such as inescapable electric foot shocks and forced swim stress. In this scenario, the exogenous administration of a NOP agonist facilitates stress-induced susceptible behaviors while the administration of NOP receptor antagonists efficiently counteracts this phenomenon. Finally, these data reinforce the view that NOP receptor signalling is tonically active during acute stress exposure and plays a crucial role in the development of stress-induced depressive-like states.

Taken together the present study demonstrates that the N/OFQ - NOP receptor system is a relevant player in controlling resilience to stress and development of depression. In particular, over activation of this system may increase vulnerability to stress-induced depression, while its blockade exerts protective action by enhancing stress resilience. Importantly, these first findings suggest an innovative therapeutic potential for NOP antagonists as drugs able to prevent the onset of the depressive episodes in vulnerable patients subjected to stressful life events. Preclinical studies aiming to corroborate this hypothesis should be conducted using different animal species and models. As far as clinical trials are concerned, the NOP antagonist BTRX-246040 was well tolerated in normal human subjects and effective in the treatment of depression (Post et al., 2015). This molecule can be now a good candidate to perform a proof-of-concept clinical trial to assess its protective potential in populations with severe risk factors for depression.

4.2 Mu opioid receptor

4.2.1 *In Vitro* characterization of novel mu receptor ligands

The emergence of designed drugs has been increasing worldwide and has become a major public issue. These substances are known as novel psychoactive substances (NPS) which are synthetic alternatives of traditional drugs of abuse (e.g. cannabis, cocaine, morphine and heroin) that are designed to evade international drug controls and laws. Until today, more than 730 new psychoactive substances were identified by the European Monitoring Centre for Drug and Drug Addiction (EMCDDA), among these, 55 were detected for the first time in Europe only in 2018 (EMCDDA 2019). The NPS market comprise a wide range of drugs, such as synthetic cannabinoids, stimulants, novel synthetic opioids (NSO), phenetilamines, dissociative anaesthetics and benzodiazepines (EMCDDA, 2019). The groups of NSO is involved in most lethal overdoses not only in Europebut also in North America that is facing an opioid epidemic nowadays. Overall, 49 NSO have been detected on Europe´s drug market since 2009, including fentanyl, its analogue used in medical therapy (e.g. sufentanyl, alfentanyl and remifentanyl (Lemmens, 1995), novel non-pharmaceutical fentanyl derivatives (e.g. ocfentanyl, furanylfentanyl, acetylfentanyl, carfentanyl, acryloyfentanyl etc..) and other NSO with different chemical structures such as, U-47, U-51, AH-7921 and MT-45 (Armenian et al., 2018; Solimini et al., 2018; Zawilska and Wojcieszak, 2019). Recreational abuse of non-fentanyl compounds is becoming a serious problem, despite the popularity of fentanyl and its analogues, for fatalities and abuse. In fact, the recent reports of abuse highlight the emerging trend of these NSO, and in particular, MT-45 (Domanski et al., 2017; Schneir et al., 2017). In fact, due to the novelty profile of these compounds, their pharmacological characterization in vitro seems of particular interest since there is a largegap of information regarding these newly synthetized illegal mu opioid receptor agonists.

4.2.1.1 MT-45

MT-45 (1-cyclohexyl-4-(1,2-diphenylethyl)piperazine)is a NSO, chemically distinct to other opioid agonists, with a N,N'-disubstitutedpiperazinechemical structure (Figure 4.2.1.1). MT-45 is one of a series of 1-(1,2-diphenylethyl) piperazine analgesics identifiedin the early 1970s by the Dainippon Pharmaceutical Company in Japan, as an alternative to morphine (Natsuka et al., 1975, 1978), commonly known by the abbreviation "MT-45", and also reported in some publications with the abbreviation "I-C6" (Natsuka et al., 1975; Helander et al., 2014).

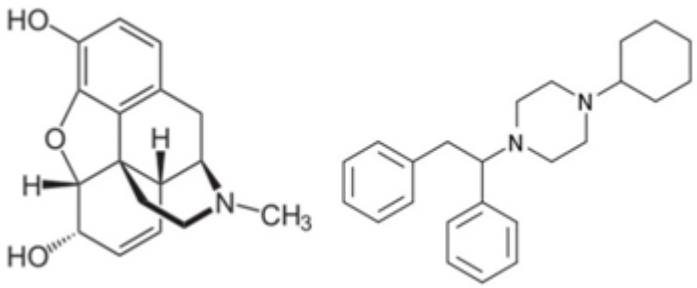


Figure 4.2.1.1. Chemical structures of morphine (left) and MT-45 (1-Cyclohexyl-4-(1,2-diphenylethyl) piperazine; (right).

MT-45 contains an asymmetry centre; thus it is a chiral molecule. Scientific studies revealed that modes of action of MT-45 and are partly different from those of morphine (Natsuka et al., 1975; Fujimura et al., 1978). The stereoisomeric composition of the MT-45 that is on the drug market within the European Union is currently unknown, but evidence from Japan suggests that the products sold in Europe are most likely racemic (EMCDDA, 2015).For the first time, in 2013, in Japan, a study of Kikura-Hanajiri and colleagues classified MT-45 as a new type of psychoactive substances (Coppola and Mondola, 2015). It has been detected in seized material in Japan, the United States and in Europe (Sweden, Belgium and Germany) and was sold online as a "research chemical" (Expert Committee on Drug Dependence Thirty-seventh Meeting Geneva, 16-20 November 2015 WHO).

Little remains clear, however, about how this compound acts regarding its opioid receptor selectivity of action. Therefore, the present study is aimed at investigating the pharmacodynamic profile of the synthetic opioid MT-45 in comparison with morphine; to this aim the in vitro dynamic mass redistribution (DMR) assay was used. DMRis a label-free approach based on an optical biosensor technology (Schröder et al., 2011; Grundmann and Kostenis, 2015) that has been already used for investigating the pharmacological profile of several GPCRs (Schroder et al ., 2011) including opioid receptors (Codd et al., 2011; Morse et al., 2011, 2013). DMR assay offers the possibility to have, in a non-invasive manner, a holistic view of cellular responses after receptor activation.

To evaluate the selectivity of MT-45 towards opioid receptor family, a series of in vitro experiments were performed in chinese hamster ovary (CHO) cells stably expressing the human mu or delta or kappa opioid receptors and their response to MT-45 generated a DMR signal that enabled us to produce a concentration response curve.

DMR effects on mu opioid receptor

In CHO cells stably transfected with the human mu opioid receptor, the standard agonist dermorphin evoked a robust concentration-dependent DMR response, with pEC_{50} of 9.07 and maximal effect of 165 ± 16 pm. Similar effects were obtained with morphine that showed a $pEC_{50}$of 7.03 and maximal effect of 128 ± 11 pm. MT-45 mimicked the action of morphine with similar potency but higher maximal effects (Figure 4.2.1.2 ; panel A). The shape of the DMR response to dermorphin, morphine, and MT-45 were similar (Figure 4.2.1.3).

DMR effects on kappa opioid receptor-

In CHO_{kappa} cells, the standard agonist dynorphin A evoked a DMR response with $pEC_{50}$of 9.08 and maximal effect of 180±55pm. Morphine as well as MT-45 were active only at micromolarconcentrations (Figure 4.2.1.2; panel B).

DMR effects on delta opioid receptor –

In CHO_{delta} cells, the standard agonist DPDPE evoked a robust concentration-dependent DMR response, with pEC_{50} of 10.01 and maximal effect of 220 ± 35 pm(Figure 4.2.1.3; panel C). Morphine displayed a pEC_{50} of 6.5 while MT-45 was less potent showing an incomplete concentration response curve.

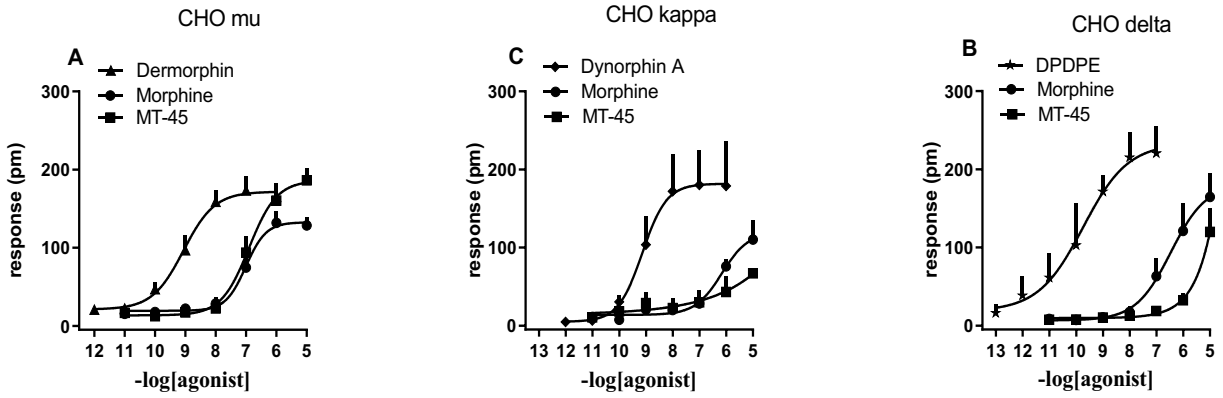


Figure 4.2.1.2. Concentration response curves to dermorphin, and morphine and MT45 tested in CHO_{mu} cells (Panel A). Data are the mean ± s.e.m. of 7 separate experiments made in duplicate. Concentration response curve to the compounds tested in CHOkappa cells (Panel B). Data is represented as mean ± s.e.m. of 4 separate experiments made in

duplicate. Concentration response curve to the compounds tested in CHOdelta cells (Panel C). Data is represented as mean ± s.e.m. of 3 separate experiments made in duplicate.

Importantly, to exclude that the responses observed in DMR were due to off target effects, compounds were also tested in wild type CHO cells at high concentration. As shown in table4.2.1.1, the compounds did not elicited any significant DMR response in wild type CHO cells (Table 4.2.1.1).

Table 4.2.1.1 Effects of high concentrations (1 µM for dermorphin, 10 µM for all other compounds) of mu receptor agonists in CHO_{mu} and CHO_{WT} cells in the DMR assay.

	CHO_{mu} pm ± sem	**CHO_{WT} pm ± sem**
Dermorphin	165 ± 16*	6 ± 4
Morphine	128 ± 11*	10 ± 6
MT-45	186 ± 16*	15 ± 10
buffer	17±5	49±20

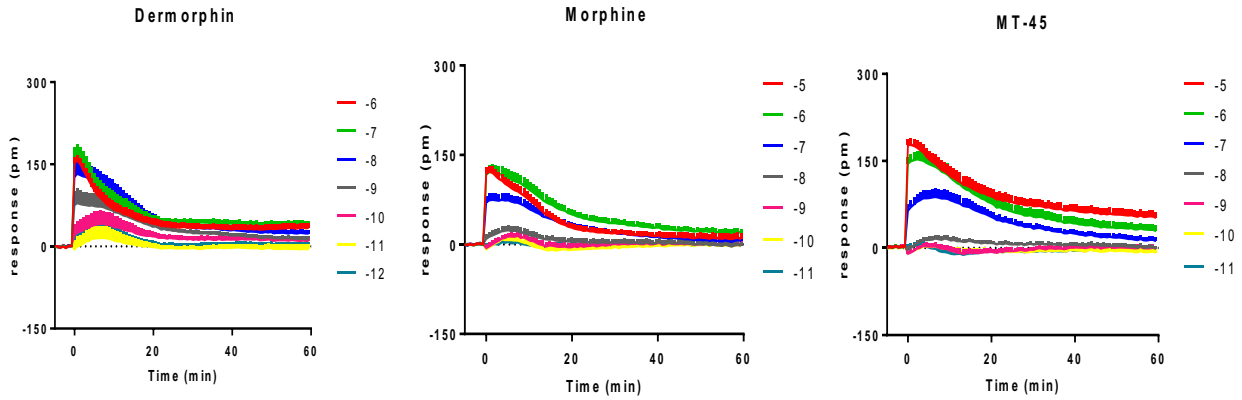


Figure 4.2.1.3. Representative DMR tracings of mu agonists under investigation in CHO cells expressing mu opioid receptor.

Results of DMR experiments have been summarized in Table 4.2.1.2.

Table 4.2.1.2 Potencies (pEC_{50}) and maximal effects of standard agonists (dermorphin, dynorphin A, and DPDPE for mu, kappa, and delta receptors, respectively), morphine and MT-45 on CHO expressing opioid receptors in the DMR assay. Data are mean of at least 3 experiments performed in duplicate.

	CHO_{mu}		**CHO_{kappa}**		**CHO_{delta}**	
Compounds	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem
Standard	9.07 (8.83-9.31)	165±16	9.08 (8.59-9.57)	180±55	10.01 (7.68-12.34)	220±35
Morphine	7.03 (6.72-7.34)	128±11*	6.08 (5.93-6.23)	134±13	6.50 (5.38-7.62)	164±29
MT-45	6.74 (6.37-7.11)	186±16	Crc incomplete		Crc incomplete	

The present in vitro study reveals that standard opioid receptor agonists (dermorphin, dynorphin A, and DPDPE) promoted DMR responses in CHO cells expressing the human recombinant opioid receptors with values of potency and efficacy in line with literature findings. Morphine behaved as expected as a mu receptor preferring agonists and MT-45 mimicked the actions of the alkaloid displaying similar potency but slightly higher efficacy and mu selectivity. The DMR assay is now widely used for pharmacological studies on GPCR including opioid receptors (see references quoted in the introduction).

This assay has been recently set up and validated in our laboratories for investigating the pharmacological profile of the NOP (Malfacini et al., 2018). Under the present experimental conditions, the standard agonists dermorphin, dynorphin A, and DPDPE elicited DMR responses with values of potency similar to what reported in previous DMR studies (Malfacini et al., 2018) as well as in calcium mobilization studies performed in cells co-expressing opioid receptors and chimeric G proteins (Ferrari et al., 2016).

Morphine displayed moderate potency and selectivity for the mu receptor which is again in line with previous DMR (Codd et al., 2011; Morse et al., 2011) as well as calcium mobilization studies (Camarda&Calo' 2013; Rizzi et al., 2016). MT-45 mimicked the stimulatory effects of morphine showing similar potency but higher efficacy and selectivity for the mu receptor. In receptor binding experiments MT-45 displayed higher affinity for mu compared to kappa and delta receptors and behaved as a mu agonist in GTP□S binding studies (Baumann et al., 2018). However, in binding experiments MT-45 displayed approximately 10-fold lower affinity than morphine for the mu receptor while a similar potency was measured in the present experiments.

Regarding its in vivo effects, Bilel and collaborators (2020) assessed the effect of MT-45 compared with morphine in a series of behavioral tests widely used in pharmacology safety studies for the preclinical characterization of new psychoactive substances in rodents (Ossato et al., 2015; Canazza et al., 2016; Fantinati et al., 2017; Marti et al., 2019). In nociception studies, the administration of MT-45 and morphine increased the threshold to acute mechanical and thermal pain stimulus in mice in a dosedependent manner. Differently, MT-45 displayed lower analgesic potency for chemical pain, when compared to morphine. Noteworthy, the pre-treatment with naloxone totally inhibited the analgesic effect induced by MT-45 and morphine.

As far as motor activity is concerned, the systemic administration of MT-45 and morphine (0.01–15 mg/kg) significantly increased the performance of the mice in accelerod test only at the highest dose tested and pre-treatment with 6 mg/kg naloxone inhibited such behavior induced by the two compounds in the accelerod test.

Finally, as respiratory depression is the major cause of opioid-related death (Dahan et al., 2010), the effect of compoundsoncardiorespiratory function was investigated. Both MT-45 and morphine significantly altered the cardiorespiratory parameters when administered to mice at high doses. Specifically, the dose of 30 mg/kg of both opioids was the most effective in reducing the basal heart rate, respiratory rate and SpO_2.

In conclusion, MT-45 behaves in vivo in a similar way compared to morphine, eliciting all classical biological effects linked to mu opioid activation such as analgesia, locomotor impairment and respiratory depression which is in line with the in vitro profile displayed by MT-45 in DMR studies.

4.2.1.2 Fentanyl Derivatives

Novel synthetic opioids are a class of psychoactive substances that are growing in popularity and presenting a significant public health risk; most of the molecules of this class are derivatives of the highly potent analgesic, fentanyl. The aim of this study was to investigate *in vitro* six fentanyl derivatives (see chemical structures in Figure 4.2.1.2.1) in comparison with fentanyl measuring their opioid receptor efficacy, potency, and selectivity as well as their capability to promote the interaction of the mu receptor with G protein and β-arrestin 2.

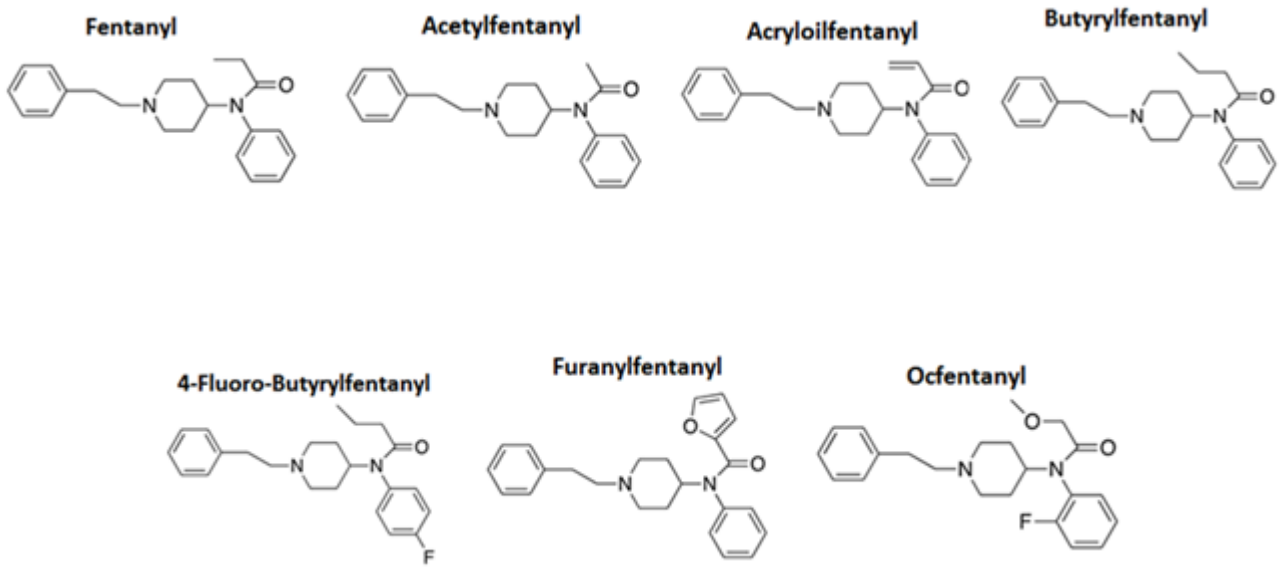


Figure 4.2.1.2.1. Chemical structures of the novel fentanyl derivatives

Calcium mobilization studies

In CHOcells transfected with the human mu opioid receptor and chimeric G protein, the standard agonist dermorphin evoked a robust concentration-dependent stimulation of calcium release displaying high potency (pEC_{50} of 8.19) and maximal effects (319 ± 13% over the basal values). Fentanyl mimicked the stimulatory effect elicited by dermorphin showing similar potency and maximal effects. All fentanyl derivatives were able to activate the mu opioid receptor in a concentration dependent manner with the following rank of potency: Fentanyl = Acryloifentanyl≥Furanylfentanyl = Ocfentanyl≥ 4-Fluoro-Butyrylfentanyl = Butyrylfentanyl>Acetylfentanyl. Regarding ligand efficacy, all compounds were able to elicit

maximal effects similar to that of dermorphin, with the exception of 4-Fluoro-Butyrylfentanyl and Furanylfentanyl that displayed statistically significant lower maximal effects thus behaving as partial agonists (Table 4.2.1.2.1).

In CHOcells stably expressing delta receptors and chimeric G-proteins, the standard agonist DPDPE evoked a robust concentration-dependent stimulation of calcium release displaying high potency (pEC_{50} of 7.47) and maximal effects (230 ± 18% over the basal values). All other compounds were either inactive or displayed incomplete concentration response curves, stimulating calcium mobilization only at micromolar concentrations.

In CHOcells stably expressing kappa receptors and chimeric G-proteins, the standard agonist dynorphin A evoked a robust concentration-dependent stimulation of calcium release displaying very high potency (pEC_{50} of 8.81) and maximal effects (257 ± 34% over the basal values). All other compounds were either inactive or displayed incomplete concentration response curves, stimulating calcium mobilization only at micromolar concentrations.

Table 4.2.1.2.1. Effects of dermorphin, fentanyl and its derivatives in calcium mobilization experiments performed in CHO cells coexpressing opioid receptors and chimeric G-proteins.

COMPOUNDS	mu		delta		kappa	
	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem %	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem %	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem %
Dermorphin	8.19 (8.02 – 8.36)	319 ± 13	Inactive		Inactive	
DPDPE	Inactive		7.47 (7.09-7.85)	230 ± 18	Inactive	
Dynorphin A	Inactive		Inactive		8.81 (8.22-9.40)	257 ± 34
Fentanyl	8.13 (7.73-8.52)	326 ± 13	CRC incomplete		CRC incomplete	
Acetylfentanyl	6.40 (6.17-6.63)	292 ± 13	Inactive		CRC incomplete	
Acryloilfentanyl	8.20 (7.05-9.35)	308 ± 12	CRC incomplete		CRC incomplete	
Butyrylfentanyl	7.32 (6.70-7.93)	283 ± 18	CRC incomplete		CRC incomplete	
4-Fluoro-Butyrylfentanyl	7.50 (6.92-8.09)	258 ± 8*	Inactive		Inactive	
Furanylfentanyl	7.93 (7.57-8.29)	226 ± 7*	Inactive		Inactive	
Ocfentanyl	7.78 (7.50-8.07)	305 ± 16	CRC incomplete		CRC incomplete	

*$p<0.05$ vsdermorphin according to ANOVA followed by the Dunnett test. Data are mean of at least 3 separate experiments made in duplicate.

BRET studies

In the BRET G-protein assay, membrane extracts taken from SH-SY5Y cells stably co-expressing the mu/RLuc and Gβ1/RGFP fusoproteins were used to evaluate receptor/G-protein interaction. Dermorphin promoted mu/G-protein interaction in a concentration dependent manner with pEC_{50} of 7.71 (7.41-8.01) and maximal effect of 0.96 ± 0.11 stimulated BRET ratio. The intrinsic activities of the compounds under study were computed as fraction of the standard ligand dermorphin maximal-stimulated BRET ratio (dermorphin=1.00) (Figure 4.2.1.2.2. panel A). All compounds, including fentanyl, mimicked the maximal effects of dermorphin displaying the following rank of potency: Furanylfentanyl≥Fentanyl=Ocfentanyl=4-Fluoro-Butyrylfentanyl≥Acryloilfentanyl=Butyrylfentanyl>Acetylfentanyl.

Whole SH-SY5Y cells stably expressing the mu/RLuc and the β-arrestin 2/RGFP fusoproteins were used to evaluate mu/β-arrestin 2 interaction. Dermorphin stimulated the interaction of the mu receptor with β-arrestin 2 in a concentration-dependent manner with pEC_{50} 6.96 (6.56-7.37) and maximal effects corresponding to 0.24 ± 0.09 stimulated BRET ratio. As for G protein studies, the intrinsic activities of the compounds were computed as fraction of the standard agonist dermorphin (dermorphin=1.00) (Figure 4.2.1.2.2 panel A). All compounds displayed similar potency in recruiting the β-arrestin 2 pathway with Acetylfentanyl displaying lower potency among compounds. Regarding their efficacy, all compounds behaved as partial agonists for the β-arrestin 2 pathway in comparison with dermorphin whereas 4-Fluoro-Butyrylfentanyl and furanylfentanyl were not able to promote β-arrestin 2 recruitment (Figure 4.2.1.2.2 panel F and panel G).

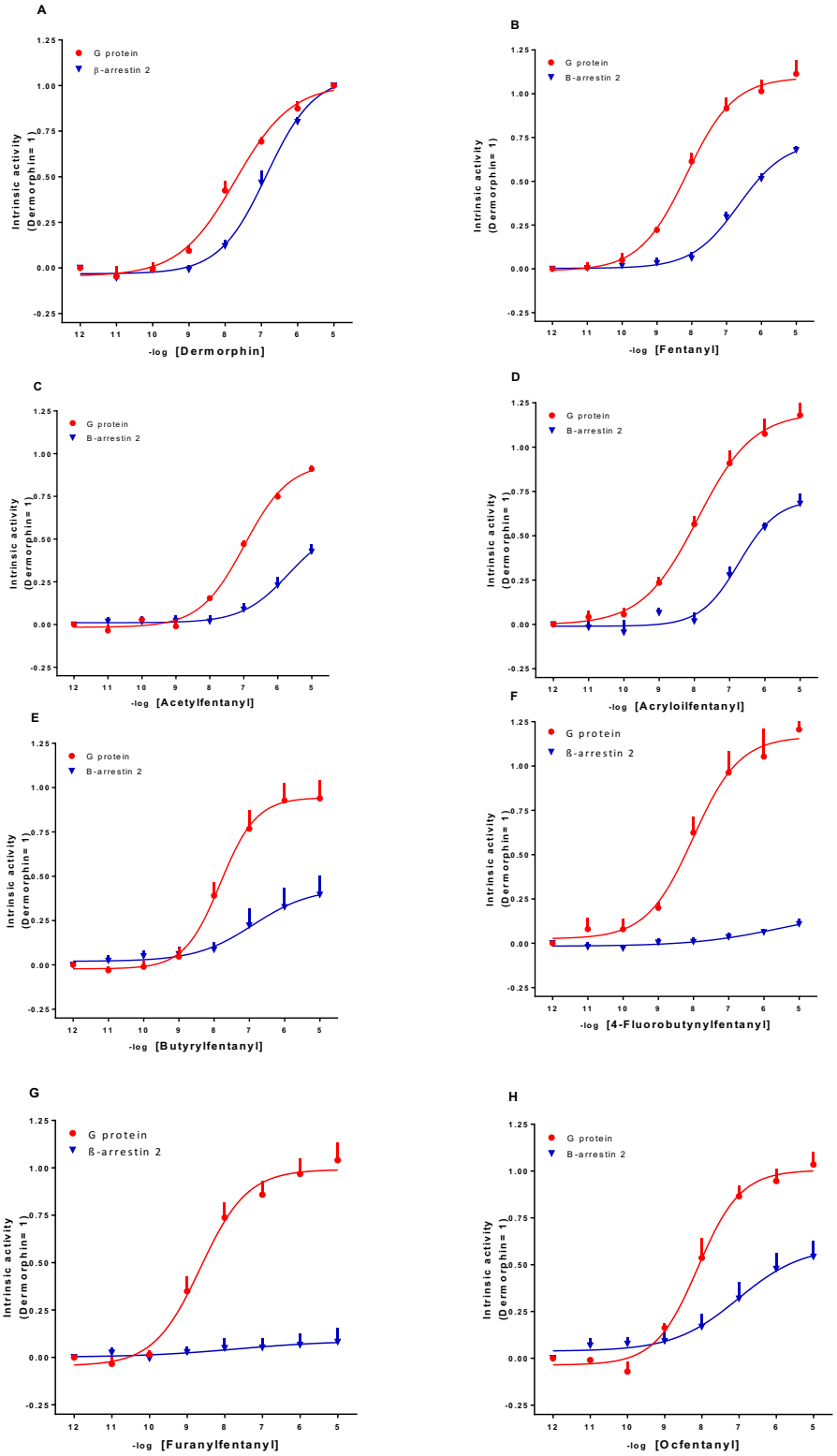


Figure 4.2.1.2.2. BRET assay. Concentration response curve to dermorphin (panel A), fentanyl (panel B) and the fentanyl derivatives under investigation (panel C-H) in promoting mu/G protein and mu/β-arrestin 2 interaction. Data are the mean ± s.e.m. of 5 separate experiments made in duplicate.

Table 4.2.1.2.2. Effects of dermorphin, fentanyl and its derivatives in BRET experiments investigating mu/G protein and mu/β-arrestin 2 interaction.

	mu/G protein		mu/β-arrestin2	
	pEC_{50} ($CL_{95\%}$)	α±SEM	pEC_{50} ($CL_{95\%}$)	α±SEM
Dermorphin	7.71 (7.41-8.01)	1.00	6.96 (6.56-7.37)	1.00
Fentanyl	8.28 (8.06-8.49)	1.11±0.07	6.86 (6.54-7.18)	0.67±0.02*
Acetylfentanyl	6.99 (6.59-7.38)	0.91±0.02	6.05 (5.43-6.68)	0.42±0.4*
Acryloilfentanyl	7.87 (7.49-8.25)	1.18±0.10	6.76 (6.48-7.03)	0.68±0.05*
Butyrylfentanyl	7.87 (7.34-8.40)	0.94±0.10	6.54 (5.83-7.25)	0.39±0.10*
4-Fluoro-Butyrylfentanyl	8.02 (7.65-8.40)	1.20±0.19	Inactive	
Furanylfentanyl	8.66 (8.15-9.16)	1.04±0.09	Inactive	
Ocfentanyl	8.09 (7.63-8.55)	1.03±0.07	7.28 (6.65-7.92)	0.54±0.08*

The fentanyl derivatives 4-Fluoro-Butyrylfentanyl and furanylfentanyl were further investigated as antagonists of fentanyl induced β-arrestin 2 recruitment. At the concentration of 0.1 μM, both compounds were able to shift the concentration response curve to fentanyl to the right with no modification of the agonist maximal effect (Figure 4.2.1.2.3, panel A and B). Furanylfentanyl displayed an higher pA_2 of 8.53, when compared to 4-Fluoro-Butyrylfentanyl, which yielded a pA_2 value of 7.81. This is similar to the higher agonist potency displayed by furanylfentanyl compared to 4-Fluoro-Butyrylfentanyl in mu/G protein experiments.

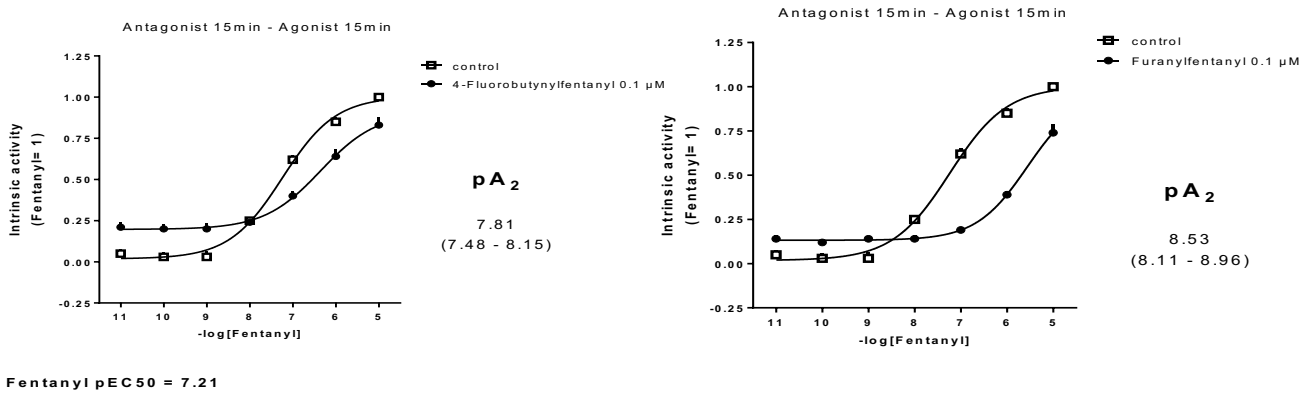


Figure 4.2.1.2.3. BRET assay. Competitive antagonism of 4-Fluoro-Butyrylfentanyl (left panel) and Furanylfentanyl (right panel) against fentanyl in mu/β-arrestin 2 experiments. Data are the mean ± s.e.m. of 5 separate experiments made in duplicate.

These results demonstrated that fentanyl derivatives behaves similar to the parent compound as mu receptor agonists. The different chemical modifications did not modify mu receptor selectivity and produced marginal effects on agonist potency with the exception of Acetylfentanyl that was 10-30 fold less potent than fentanyl. The most interesting differences among the fentanyl derivatives were measured in terms of agonist efficacy; in fact, 4-Fluoro-Butyrylfentanyl and Furanylfentanyl behaved as partial agonists in the calcium mobilization assay and as G protein biased agonists in BRET experiments. Moreover furanylfentanyl displayed values of potency similar to fentanyl. Collectively these pharmacological features make furanylfentanyla very interesting pharmacological tool. In fact, there is in literature an ongoing debate regarding the relationship between in vitro mu receptor partial vs biased agonism and the therapeutic index of opioid analgesics (for reviews of this topic, see Gillis et al., 2020; Neto et al., 2020)

Opioid analgesic effectiveness is limited by a series of side adverse effects including respiratory depression, constipation, immune suppression and with prolonged treatment, tolerance, dependence and abuse liability. Thus there is clearly a huge medical need in terms of novel and safer analgesics. On possible strategy for the development of such drugs has been suggested by genetic studies; in fact in βarr2(-/-) mice morphine analgesia is potentiated (Bohn, 1999) while side effects are reduced (Raehal et al., 2005). These results suggested that mu receptor agonists biased toward G protein might display an enhanced therapeutic index. Several molecules have been later identified as mu receptor G-protein-biased agonists, including oliceridine (TRV130) (DeWire et al., 2013), PZM21 (Manglik et al., 2016) and SR-17018 (Schmid et al., 2017). These compounds consistently displayed in preclinical studies a larger therapeutic index than morphine. Moreover

oliceridine has been recently approved for short term intravenous use in hospitals and other controlled settings(Lambert and Calo, 2020). Collectively these genetic and pharmacological findings corroborate the proposal that G protein biased agonism is a useful strategy for generating safer opioid analgesics.

However, recent findings questioned the above hypothesis. In fact a consortium of three different laboratories reinvestigated the effect of morphine in βarr2(+/+) andβarr2(-/-) mice and found that morphine side effects were similar in the two genotypes (Kliewer et al., 2020). Moreover, elegant studies performed with mice genetically engineered with G-protein-biased mu receptors suggested increased sensitivity of these animals to both analgesic actions and side effects of opioid drugs(Kliewer et al., 2019). In addition, a highly detailed study comparing oliceridine, PZM21 and SR-17018 (among others) in a range of assays showed that these molecules actually behave as mu receptor partial agonists(Gillis et al., 2020a). Moreover, there was a correlation between their therapeutic indices and their efficacies, but not their bias factors.

Overall, the available evidence does not allow to discriminate mu receptor biased vs partial agonism as a strategy for the development of safer opioid analgesics. Thus further studies comparing the in vitro properties of mu ligands with their in vivo therapeutic index are needed and furanylfentanyl should be included in these studies.

4.3 NOP/mu mixed agonists

PWT technology and NOP/mu mixed agonists

Bioactive peptides obtained from natural sources hold great potential for the development of drugs because of their capability to modulate a broad panel of biological functions excelling in potency and selectivity of action with usually low toxicity profile. However, as largely known, peptides suffer from a short duration of action when administered for therapeutic purposes because of their fast degradation by peptidases. Despite this, the number of peptide drugs being placed on the market has been extraordinarily increasing in the last two decades (Fredriksson et al., 2003) In most cases, this has been made possible by the identification of chemical strategies useful for improving peptide resistance to peptidases. In the last few years, with the development of the so-called peptide welding technology (PWT), we provided evidence that the multimerization of bioactive peptides can be one of the possible approaches to be exploited for extending their limited half-life (Rajagopal et al., 2010). The methodology, that is based on a high efficacy chemical synthesis for the generation of tetra-branched peptide ligands, is of particular interest since being an elegant way to improve the drug-likeness and pharmacokinetic properties of bioactive peptides in view of their potential therapeutic employment.

All PWT-derivatives reported to date have been efficiently prepared following a convergent synthetic approach based on the conjugation of a clustering core with four linear monomers of the same target peptide after their independent synthesis (Wise et al., 2002). Three different cores have been exploited, each of which strategically functionalized with four reactive maleimide groups as depicted in figure 4.3.1 (PWT1witha tris-Lysine motif, PWT2 a cyclam-based scaffold and PWT3 a symmetric lysine-ethylendiaminederivative) (Katritch et al., 2012). The classical procedure consisted in a thiol-Michael reaction between the central reactive scaffold and the peptide of interest in which a key cysteine residue was conveniently introduced (Lefkowitz and Shenoy, 2005).

Thanks to thishighly chemo-selective conjugation, different GPCR-targeting peptides have been homotetramerized such as nociceptin/orphanin FQ (N/OFQ) (Lefkowitz and Shenoy, 2005; Rajagopal et al., 2010), N/OFQ related peptide (Lefkowitz and Shenoy, 2005) tachykinins neuropeptide S (Bohn et al., 1999) of note, the resulting multi branched ligands exhibited an in vitro pharmacological profile similar to that of the native peptides. Even more important,a higher potency and a marked prolongation of action in vivo have been generally observed, possibly due to reduced susceptibility to the action of proteolytic enzymes (Bohn et al., 2002). Thus, the first generation of PWT derivatives have been considered as valuable pharmacological tools suitable for investigating

in vivo the effects of the selective and prolonged stimulation/blockage of a single receptor in physiopathological conditions.

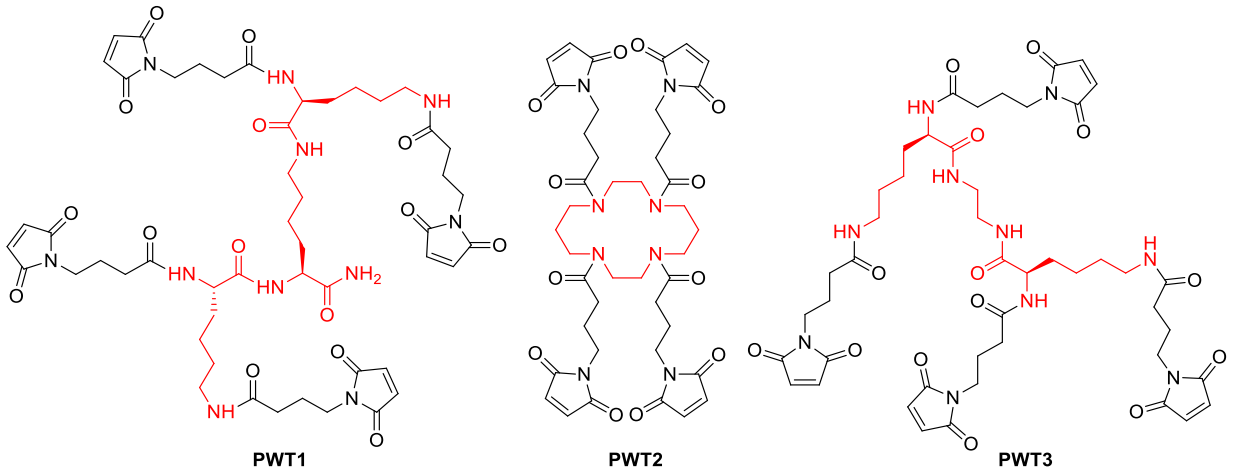


Figure 4.3.1. Chemical structures of the clustering cores used for generating PWT peptides.

The innovative purpose of this study is the design, synthesis, and in vitro pharmacological characterization of the first examples of heteromultivalent PWT derivatives of potential interest for the study of multifactorial diseases where the simultaneous modulation of multiple signaling systems can be advantageous compared to the activation/blocking of a single target. To this aim, we focused on the recent evidence that molecules that are able to modulate multiple opioid receptors (currently classified as mu, delta, kappa and NOP, the opioid related N/OFQ peptide receptor) may result into novel opioid analgesics possibly with reduced side effects (Violin et al., 2014). .Most progress has been made in the development of NOP/mu bifunctional agonists that have been demonstrated to relieve some of the side effects of selective mu agonists, including tolerance, dependence, constipation and respiratory depression (Meunier et al., 1995; Reinscheid et al., 1995). Of note, NOP receptor is co-localised with the mu opioid receptor in the pain pathways where they seem to be involved in the formation of heterodimers (Mollereau et al., 1996). In addition the co-administration of N/OFQ and mu receptor agonists (i.e. morphine, buprenorphine) in non-human primates was shown to enhance the antinociceptive effects of classical opioid analgesics reducing their remarkable side effects (Meunier et al., 1995; Reinscheid et al., 1996).

Overall, these findings inspired the synthesis of peptide and non-peptide bifunctionalNOP/mu ligands. Among these, the small molecule agonists BU08028 e SR16435 have emerged for their antihyperalgesic and antiallodynic action in animal models of inflammatory and neuropathic pain with higher potency if compared to selective NOP or mu ligands (Lambert, 2008). AT-121 is another relevant example of bifunctional NOP/muligand that displayed analgesic

properties without opioid side effects in nonhuman primates (Soergel et al., 2014). Furthermore, the mixed opioid/NOP receptors agonist cebranopadol is a novel first-in-class analgesic currently in clinical development by the company Grünenthal for the treatment of different acute and chronic pain states (Alexander et al., 2019). In addition, a few peptide-based bifunctionalNOP/mu receptor ligands have been obtained through the generation of chimeric peptides containing an opioid and a NOPbinding pharmacophore that have been merged in a linear sequence (Thompson et al., 2012a).

A first attempt to combine the PWT technology to the principles of polypharmacology consisted in the homotetramerization of the peptide [Dmt^{1}]N/OFQ(1–13)-NH_2 that behaves as a potent agonist for both NOP and classical opioid receptors and elicits robust anti-nociceptive effects after spinal administration in non-human primates (Thompson et al., 2012a; Filizola and Devi, 2013).PWT2-[Dmt^{1}]N/OFQ(1–13)-NH_2displayed the same potency of the linear parent peptide in vitro and a prolonged action in vivo (Mustazza and Bastanzio, 2011; Calo and Guerrini, 2013). Here we described a new chemical strategy that allowed us to exploit the PWT technology to the obtainment of NOP/mu bivalent heterotetrameric peptides. In particular, we synthetized a novel PWTcore (H-PWT1, depicted in figure 4.3.2) featuring two maleimide and two benzaldehyde groups that have been independently reacted with two chains of N/OFQ, as native ligand of the NOP receptor, and two chains of dermorphine or dermorphine-related peptides, as mu agonists.

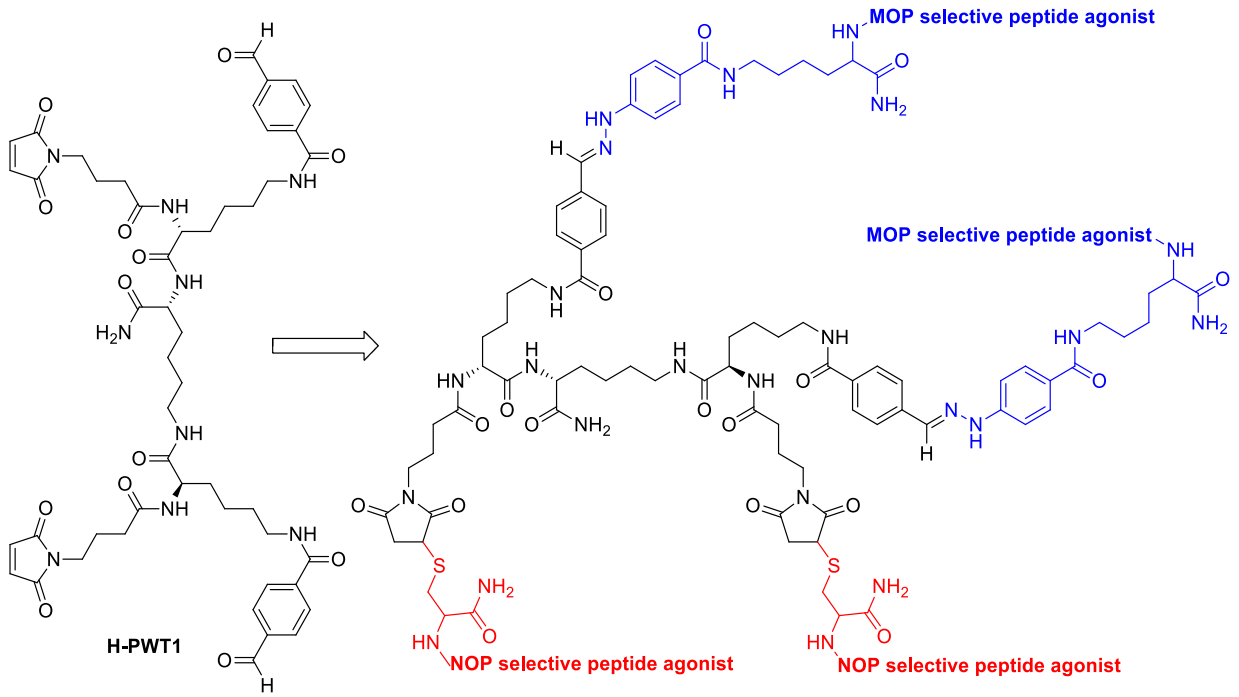


Figure 4.3.2. General design for the generation of heterotetrameric H-PWT1 derivatives as NOP/MU dual acting ligands.

In vitro effects of Heterotetrabranched Peptide Ligands as Bifunctional Agonists of the NOP and mu Opioid Receptors

The described ligands were assayed in calcium mobilization studies performed in CHO cells coexpressing either the human recombinant NOP or mu receptors and the chimeric protein $G\alpha_{qi5}$ (Lambert, 2008). The NOP and mu agonist potencies of the heterotetramers **7a-c** were expressed as pEC_{50}s andcompared to those of the parent peptide monomers and the reference homotetrabranched derivatives investigated under the same experimental conditions (see **Table 4.3.1.).**This calcium mobilization assay has been previously validated with a large panel of NOP and opioid ligands (Meunier et al., 1995; Reinscheid et al., 1995) and the standard agonists used in the present work(N/OFQ and dermorphin) displayed potencies and selectivity profiles consistent with these studies. In particular, N/OFQ evoked a concentration dependent stimulation of calcium release in cells expressing the human NOP receptor with high potency (pEC_{50} = 9.52) and maximal effects (308± 30% over basal values), while dermorphin was inactive up to micromolar concentrations. Conversely, dermorphin behaved as a potent full agonist of the mu receptor (pEC_{50} = 8.09; E_{max}324± 18% over basal values) toward which N/OFQ resulted inactive.

In the first instance, the standard opioid agonists N/OFQ and dermorphin have been tetramerized in compound H-PWT1-N/OFQ-dermorphin (**7a**) that elicited a potent stimulatory action both in cells expressing the NOP receptor and in those transfected with the mu receptor with maximal effects similar to those of the corresponding linear monomers.

These encouraging results demonstrated that the new heteromultimerization strategy did not compromise the pharmacological activity of the starting bioactive peptides, in line with what previously observed in the same assay for most of PWT-based homotetramers (Grisel and Mogil, 2000). Even though H-PWT1-N/OFQ-dermorphin displayed an interesting profile of mixed NOP/mu full agonist, the potency of the compound in activating the NOP receptor (pEC_{50} = 8.09) was almost 30-fold lower if compared to that of unconjugated N/OFQ. Likewise, **7a** resulted 10-fold less potent than dermorphin in stimulating the mu receptor(pEC_{50} = 7.13). This would indicate that theH-PWT1-based assembling of the analyzed bioactive sequences disfavours to some degree their interaction with the respective biological targets. This effect was also evident from the in vitro pharmacological profile of the homotetrabranched derivatives PWT1-N/OFQ (Table 4.3.1.) (Grisel et al., 1996; Zhu et al., 1997; Calò et al., 1998; Bertorelli et al., 1999) and PWT1-dermorphin that behaved respectively, as a NOP-selective and a MU-selective agonist3-10fold less potent than the native peptide precursors.

Table 4.3.1. In vitroeffects of the synthetized compounds in calcium mobilization studies performed on CHO cells coexpressing either the NOP or MU receptor and the $G\alpha_{qi5}$ chimeric protein.

	NOP		MU		NOP/MU
	pEC_{50} ($CL_{95\%}$)	**α ± sem**	**pEC_{50} ($CL_{95\%}$)**	**α ± sem**	
Linear peptide monomers					
N/OFQ	9.52 (8.97-10.06)	1.00	< 6	-	> 1000
Dermorphin	< 6	-	8.09 (7.44-8.74)	1.00	< 0.01
Dermorphin(O2Oc)$_2$NH$_2$	< 6	-	7.74 (7.21-8.27)	0.94 ± 0.06	< 0.1
[Dmt^1]dermorphin	< 6	-	8.76 (8.42-9.10)	0.91 0.07	< 0.01
Homotetrameric PWT1 derivatives					
PWT1-N/OFQ	8.44 (8.13-8.75)	1.08 ± 1.07	< 6	-	> 100
PWT1- dermorphin	< 6	-	7.53 (7.23-7.84)	0.88 ± 0.05	< 0.1
PWT1-dermorphin(O2Oc)$_2$	< 6	-	7.34 (6.81-7.88)	0.80 ± 0.08	< 0.1
PWT1-[Dmt^1]dermorphin	< 6	-	9.64 (9.20-10.08)	1.04 ± 0.06	< 0.001
HeterotetramericH-PWT1derivatives					
H-PWT1-N/OFQ-dermorphin(7a)	8.09 (7.98-8.20)	0.99 ± 0.04	7.13 (6.90-7.37)	0.93± 0.03	0.11
H-PWT1-N/OFQ-dermorphin(O2Oc)$_2$(7b)	8.21 (8.03-8.40)	1.09 ± 0.09	7.42 (7.25-7.59)	0.91 ± 0.03	0.16
H-PWT1-N/OFQ-[Dmt^1]dermorphin(7c)	8.35 (7.71-8.99)	0.88 ± 0.03	8.75 (8.32-9.19)	1.00± 0.04	2.5

Moreover, the bifunctional profile of **7a** was considered suboptimal in view of the unbalanced NOP/mu potency i.e. 10-foldhigher potency for the NOP receptor. This prompted us to investigate suitable strategies for the structural modification of the dermorphin sequence with the aim to increase the mu potency of the resulting heterotetrabranched derivatives. Firstly, we designed compound **7b** (H-PWT1-N/OFQ-dermorphin$(O2Oc)_2$) in which the mu pharmacophore was spaced from the central PWT core through two polyoxyethylene units. The hydrophilic linker was selected because of the low water solubility experienced with the homotetrabranched derivative PWT2-dermorphin previously described (King et al., 1998). In addition, we speculated about the possibility that a flexible linker of the proper length could facilitate the interaction of the message sequence of dermorphin with the binding pocket of the mu receptor. Compared to **7a**, H-PWT1-N/OFQ-dermorphin$(O2Oc)_2$ displayed a substantial maintenance of NOP potency (pEC_{50} = 8.21) but only a marginal increase in mu potency (pEC_{50} = 7.42). However, it has to be considered that the modification of the dermorphin sequence in the derivative dermorphin$(O2Oc)_2$-NH_2 resulted per se slightly detrimental for mu potency. Thus, in the case of **7b** the H-PWT1-derivatization of the dermorphin component did not significantly affect the mu potency of the linear precursor. This was also confirmed in the homotetrameric derivative PWT1-dermorphin$(O2Oc)_2$ displaying similar potency as PWT1-dermorphin.

In the last derivative H-PWT1-N/OFQ-[Dmt^1]dermorphin (**7c**), the dermorphin component was directly linked to the core but modified at the N-terminal portion where the Tyr^1 residue was replaced with a 2,6-dimethyltyrosine.The corresponding linear precursor [Dmt^1]dermorphin was shown to be 5-fold more potent than the native peptide analogue (pEC_{50}= 8.76) confirming the important effect of this chemical modification in promoting the interaction of opioid peptide ligands with the mu receptor (Bryant et al.; Yamamoto et al., 2011; Giri et al., 2015; Marrone et al., 2016). Surprisingly, and unlike most of the previously reported PWT derivatives, the newly described homotetrabranchedPWT1-[Dmt^1]dermorphin exhibited a significantly improved mu-agonist potency compared to the parent peptide monomer (pEC_{50} = 9.64).This would suggest that in the case of compound PWT1-[Dmt^1]dermorphin, the beneficial effect of the introduction of Dmt^1 was amplified by the presence of multiple modified message domains that would cooperate synergistically in mu receptor binding and activation. It can be speculated that the molecular flexibility and the relatively high distance between the four pharmacophores of the investigated multivalent ligand, make it possible the concurrent targeting of functional dimers or oligomers of the mu-opioid receptor(Pascal and Milligan, 2005).

Of relevance for the aim of this work, the introduction of a Dmt^1 residue had also a positive impact on the pharmacological profile of compound **7c**. Indeed, the mu potency of H-PWT1-N/OFQ-[Dmt^1]dermorphin was significantly incremented in comparison with **7a** and **7b** to such an extent that the NOP/mu potency ratio was reversed (NOP pEC_{50} = 8.35, mu pEC_{50} = 8.75). Even in this case, the maximal effects of the compound at both the investigated receptors were comparable to those of the unconjugated peptide precursors with no changes in ligand efficacy. Thus, H-PWT1-N/OFQ-[Dmt^1]dermorphin exhibited a profile of dual acting full agonist with high and balanced potencies for NOP and mu receptors.

In order to confirm this result with a different assay,H-PWT1-N/OFQ-[Dmt^1]dermorphin has been investigated in DMR studies performed in CHO cells expressing the human mu or NOP receptors (Figure 4.3.3). DMR is a label free assay that gives the possibility to measure, in a noninvasive manner, receptor - dependent holistic cellular responses (Schröder et al., 2011). DMR studies have been performed in recent years investigating the pharmacological profile of several GPCR including classical opioid (Morse et al., 2013) and NOP receptors (Malfacini et al., 2018). As shown in Figure 4.3.3, N/OFQ elicited a concentration dependent DMR response in cells expressing the NOP receptor (pEC_{50} 9.48, Emax 236 ± 33 pm) being inactive in mu cells. Opposite results were obtained with dermorphin that evoked DMR responses in cells expressing the mu receptor (pEC_{50}8.98, Emax 152 ± 28 pm) being inactive in NOP cells. Similar results have been previously obtained with both peptides in our laboratories (Malfacini et al., 2018). Importantly N/OFQ and dermorphin up to 1 μM were inactive in wild type CHO cells (data not shown). H-PWT1-N/OFQ-[Dmt^1]dermorphin produced similar DMR responses in cells expressing the NOP or the mu receptors. However, the potency and maximal effects of H-PWT1-N/OFQ-[Dmt^1]dermorphin could not be estimated in these experiments since at 1 μM the tetrameric peptide elicited statistically significant DMR responses in wild type CHO cells (data not shown). This result was not completely unexpected since similar findings has been previously reported in DMR studies investigating the homomeric PWT derivatives of N/OFQ (Malfacini et al., 2018) and neuropeptide S (Ruzza et al., 2018). Corroborating these findings, bioassay studies performed with tissues taken from wild type and NOP receptor knockout mice demonstrated that PWT-N/OFQ displays reduced selectivity than the parent peptide. The implications of these findings are twofold: i) a certain loss of selectivity compared with the parent peptide(s) is probably a common feature of both homo and heteromeric PWT derivatives and ii) the label free DMR assay is superior than single endpoint assays for revealing this aspect. Despite the above mentioned limitations, DMR experiments confirmed that H-PWT1-N/OFQ-[Dmt^1]dermorphin behaves as mixed NOP/mu agonist able to stimulate the two receptors in the same range of concentrations.

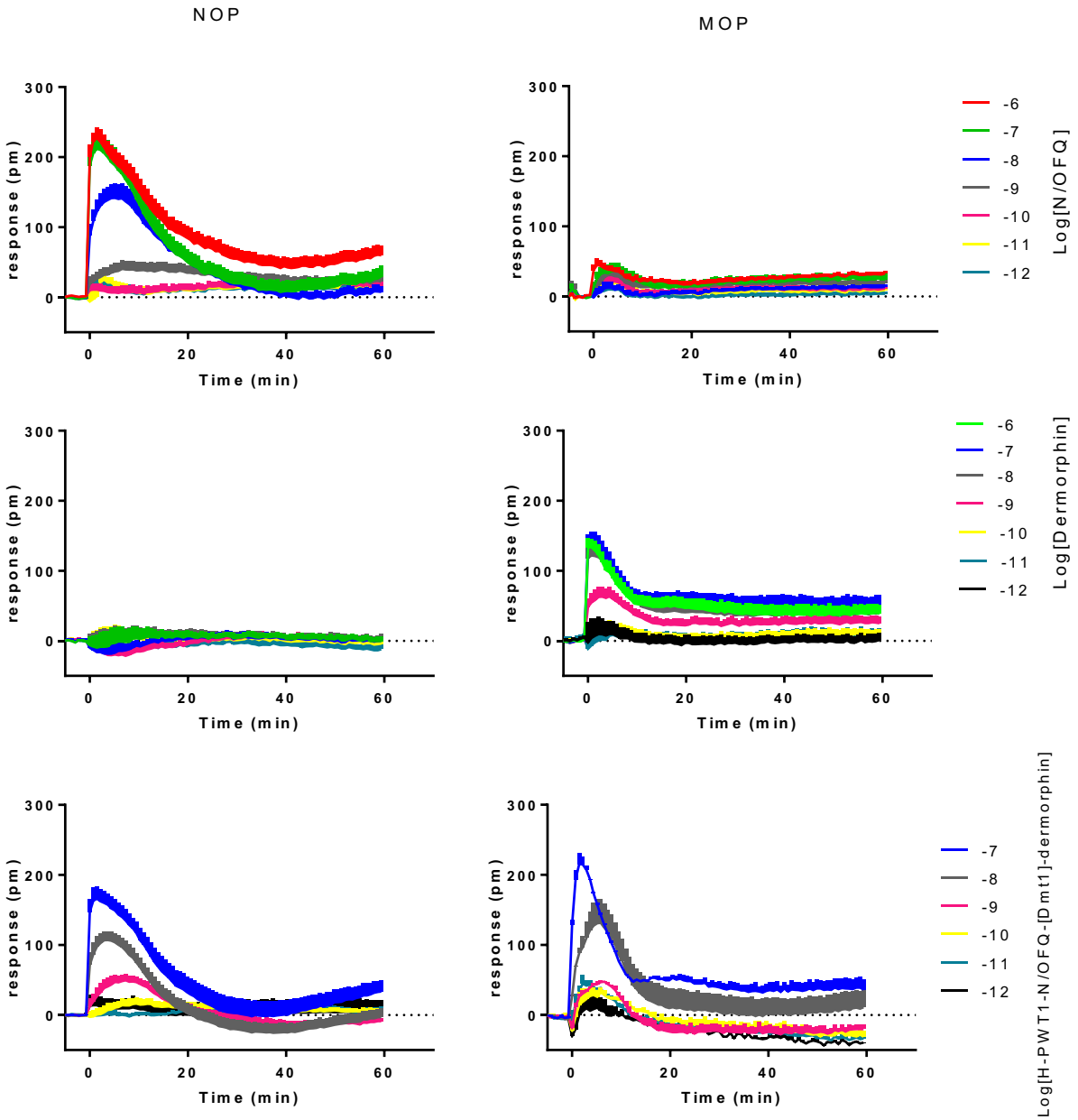


Figure 4.3.3. DMR traces of N/OFQ (top panels), dermorphin (middle panels) and H-PWT1-N/OFQ-[Dmt[1]]dermorphin (bottom panels) in CHO cells expressing the NOP (left panels) and the mu (right panels) receptors. Data are the mean ± sem of 4 experiments performed in duplicate.

In conclusion, with this work we validated an easy accessible synthetic methodology that allows obtaining heterotetramericpeptide ligands with abifunctional pharmacological profile. Overall, the results of this study will let the scientific community to increase the knowledge regarding the design and synthesis of bivalent ligands for peptidergic receptors. As above detailed, the approach has been successfully applied to the identification of the NOP/mu mixed full agonist H-PWT1-N/OFQ-[Dmt^1]dermorphin which exhibited a balanced potency at the investigated targets with EC_{50} values in the low nanomolar range. The available evidence suggests that dual acting NOP/mu agonists may have therapeutic potential in the treatment of pain as non-addictive analgesics and possibly as medications to treat drug abuse (Toll, 2013; Ding et al., 2018; Preti et al., 2019).

The newly disclosed compound H-PWT1-N/OFQ-[Dmt^1]dermorphin can be considered a valuable tool for future in vivo studies aimed at investigating the effects of the simultaneous stimulation of the NOP and mu receptors in disease models. This will allow the understanding and, possibly, the expansion of the therapeutic potential of opioid bivalent ligands for treating any disease that can likely benefit by multi-target drug treatments. In vivo studies will be useful to establish if the newly described methodology can be exploited to enhance the in vivo potency of the investigated peptides and particularly their duration of action, as previously demonstrated with different examples of homotetrameric PWT derivatives (Calo et al., 2018).

Of note, the strategy herein described is extremely versatile and virtually applicable to any peptide sequence, thus, this approach could be translated in the future to the development of bivalent compounds targeting other receptor systems. As a consequence, the achieved results could be extremely helpful to explore new therapeutic perspectives and pave the way to innovative pharmacological treatments for diseases in which the concurrent activation or blockade of two different receptors is more effective / safer than the selective modulation of a single target.

5. GENERAL CONCLUSIONS

The present thesis summarizes the work performed along 3 years focusing our effort on novel pharmacological concepts and strategies useful for the development of innovative drugs targeting the NOP receptor as well as classical opioid receptors. In particular, the role of β-arrestin 2 in NOP receptor signalling and the development of novel NOP ligands with enhanced bias factor towards G protein have been investigated. The hypothesis proposed by Bohn (1999), that β-arrestin2 plays a crucial role in eliciting mu opioid related side effects, led us to investigated whether β-arrestin 2 could be responsible for some of the effects linked to NOP activation, since the NOP receptor shares high homology with classical opioid receptors. To this aim, we performed SAR studies aimed to the identification of peptides able to preferentially activate the G protein pathway. Another strategy for investigating the role of β-arrestin 2 in NOP signalling was that of using genetic tools such as βarr2(−/−) mice. Alongside with investigating the relationship between βarr2 and the NOP receptor, a series of novel mu opioid receptor agonists have been characterized *in vitro* for their ability to activate G protein and to recruit β-arrestin2 pathway in order to extend the panel of pharmacological tools useful for functional selectivity studies in the field of opioid receptors, the ultimate aim of such studies being the development of safer analgesics.

The therapeutic potential of NOP receptor ligands is not limited to analgesia but includes affective disorders. In fact NOP receptor selective antagonists elicit antidepressant effects. The relationship between the N/OFQ-NOP receptor system and individual vulnerability of developing depression in response to stressful stimuli has been investigated in mice with the LH test.

Finally, an increasing body of evidence suggests that mixed mu/NOP agonists have great potential as innovative analgesics. Thus we used the novel chemical strategy named Peptide Welding Technology for generating heteromeric tetrabranched peptides able to simultaneously stimulate NOP and mu opioid receptors..

The *in vitro* studies aimed to identify G protein biased NOP agonists revealed that subtle modifications in the peptide sequence of N/OFQ(1-13)-NH_2,such as Ala- and D-scan as well as chemical modifications of amino acid chains or peptide bonds were able to produce large changes in ligand potency and/or efficacy but no modification of the ligand bias factor. Then a series of modifications were applied to the C terminus of N/OFQ(1-13)-NH_2. The most interesting results were achieved with the use of the palmitoyl moiety; in particular the compoundCys(palmitoyl)^{14}]N/OFQ(1-14)-NH_2displayed a bias factor of 2, which is highest biased factor reported in literature for a NOP agonist. The same chemical modification has been applied to

opioid peptides acting as mu and delta ligands. This chemical modification was also applied to mu and delta receptor peptide ligands; palmitoylated peptides consistently behaved as highly potent agonists for receptor/G protein interactions acting as G protein biased agonists for NOP and delta receptors and as unbiased agonists for the mu receptor. Collectively in the frame of this study [Cys(palmitoyl)^{14}]N/OFQ(1-14)-NH_2 was identified and characterized as the most G protein biased agonist for the NOP receptor. This compound is therefore a very useful pharmacological tools for performing functional selectivity studies in the NOP receptor field.

To investigate the possible impact of NOP functional selectivity in vivo βarr2(−/−) mice were used. The analgesic and sedative effects of the selective NOP agonist Ro65-6570were assessed in βarr2(−/−) mice. Ro 65-6570 elicited an antinociceptive effect in βarr2(−/−)but not βarr2(+/+)mice in the formalin test, thus suggesting that βarr2(−/−) mice are more sensitive to the analgesic effect of NOP agonists. Bohn and collaborators (1999) reported similar findings with morphine. The regulatory role of βarr2 in terminating G protein signalling and subsequent receptor internalization is well known and may explain the results obtained in knockout mice both with Ro 65-6570 and morphine. Moreover, Ro65-6570 was able to dose dependently reduce the time on the Rotarod both in βarr2(−/−) and βarr2(+/+) mice, being slightly more potent in wildtype mice. All together these findings suggest that Ro65-6570 displayed a slightly higher therapeutic index in βarr2(−/−) mice thus suggesting that G protein biased agonists may display a larger therapeutic window. This concept has been investigated by comparing the analgesic vs sedative properties of three different NOP agonists:AT-403 (Ferrari et al., 2017), Ro 65-6570 (Wichmann et al., 1999), and MCOPPB (Hirao et al., 2008b). Importantly these NOP ligands displayed different values of bias factor (i.e. 0.16 for AT-403, 1.64 for Ro 65-6570 and 0.97 for MCOPPB). Dose response studies were performed for each compound and no differences in therapeutic index were observed; in fact all compounds displayed a therapeutic index of approximately 10 despite large difference in their bias factor values. These results suggest that functional selectivity at least in terms of G protein vs βarr2 is not an useful strategy for increasing the tolerability of NOP receptor agonists as innovative analgesics. Further studies possibly comparing the ability of different NOP agonists to activate different G proteins may provide useful information for firmly establish the therapeutic potential of functional selectivity in the NOP receptor field. The recent publication of the TRUPATH platform (Olsen et al., 2020) makes feasible this kind of transducerome studies.

A study has been performed investigating the role of N/OFQergic transmission in the development of depression in response to stressful stimuli. The potential of NOP receptor in modulating mood states has been widely described in literature (Gavioli and Calo', 2013; Witkin et

al., 2014, 2016; Post et al., 2015; Toll et al., 2016; Gavioli et al., 2019), with its activation being linked to anxiolytic-like effects (Hirao et al., 2008b; Asth et al., 2016) and its blockage to antidepressant-like effects in behavioral despair tests (Redrobe et al., 2002; Gavioli et al., 2003, 2004; Post et al., 2015, 2016), inescapable footshocks (Holanda et al., 2016, 2018) and chronic mild stress (Vitale et al., 2009, 2017) in rodents. To shed light into how this peptidergic system modulates the development of depression, NOP agonists (Ro 65-6570 and MCOPPB) and antagonist (SB-612111) were administrated in mice before exposure to uncontrollable, unescapable stress. Interestingly enough, in the group of animals treated with NOP agonists virtually all mice developed helpless behavior after stress exposure. By contrast, the administration of NOP antagonist SB-612111significantly counteracted the development of depressive behavior. Interestingly nortriptyline (20 mg/kg) did not change the acquisition of helpless behavior. Furthermore, no NOP(-/-) knockout mice developed helpless behavior after exposure to stress thus displaying a resilient phenotype. These findings strongly suggest an innovative therapeutic potential for NOP antagonists as drugs able to prevent the onset of depressive episodes in vulnerable patients subjected to stressful life events and contribute to the growing body of evidence that NOP antagonists could represent innovative antidepressant drugs.

There has been as increasing number of illicit synthetic opioids in the so called third wave of the opioid epidemic faced by the United States (Volkow and Blanco, 2020). These new substances aim to evade international drug control and are responsible for an unabated rise of opioid related death by respiratory depression (Compton et al., 2019). Some research activities have been also made in this field regarding novel synthetic opioids, in particular MT-45 and novel non-pharmaceutical fentanyl derivatives that have been detected on Europe´s drug market since 2009 (Solimini et al., 2018). The pharmacological profile of some of these compounds has yet to be studied. To contribute to the investigation of these new substances, compounds were tested in a series of *in vitro* assays aiming to define their pharmacological activity at opioid receptors in terms of potency, efficacy and selectivity of action. Among these molecules there is MT-45, which is chemically distinct from other opioid agonists. On that matter, *in vitro* studies have demonstrated that MT-45 behaved as a full agonist for the mu opioid receptor, differently from morphine which behaved as a partial agonist. Regarding the effects of MT-45at delta and kappa opioid receptors, MT-45mimicked the effects of morphine bothin terms of efficacy and potency. Similar studies were carried out to novel non-pharmaceutical fentanyl derivatives; for these compounds we also evaluated their ability to recruit the β arresting 2 pathway, in order to eventually detect mu receptor biased agonists. All fentanyl derivatives were selective to the mu opioid receptor, displaying different values of potency. Interestingly enough, only two fentanyl derivatives (4-Fluoro-

Butyrylfentanyl and furanylfentanyl) behaved as partial agonists for the mu opioid receptor. When the experiments continued focusing on the compounds ability to recruit theβ arresting 2 pathway, the very same compounds that behaved as partial agonists in calcium studies failed to recruit the β-arresting 2 pathway and acted as antagonists of fentanyl induced β-arresting 2 recruitment.

As mentioned in the thesis, studies in β-arrestin 2 gene knockout (βarr2(-/-)) animals indicate that morphine analgesia is potentiated while side effects are reduced, suggesting that drugs biased away from arrestin may manifest with a reduced-side-effect profile. However, there is controversy in this area with improvement of morphine-induced constipation and reduced respiratory effects in βarr2(-/-) mice. Moreover, studies performed with mice genetically engineered with G-protein-biased mu receptors suggested increased sensitivity of these animals to both analgesic actions and side effects of opioid drugs. Several new molecules have been identified as mu receptor G-protein-biased agonists, including oliceridine (TRV130), PZM21 and SR-17018. These compounds have provided preclinical data with apparent support for bias toward G proteins and the genetic premise of effective and safer analgesics. There are clinical data for oliceridine that have been very recently approved for short term intravenous use in hospitals and other controlled settings. While these data are compelling and provide a potential new pathway-based target for drug discovery, a simpler explanation for the behavior of these biased agonists revolves around differences in intrinsic activity. A highly detailed study comparing oliceridine, PZM21 and SR-17018 (among others) in a range of assays showed that these molecules behave as partial agonists. Moreover, there was a correlation between their therapeutic indices and their efficacies, but not their bias factors. If there is amplification of G-protein, but not arrestin pathways, then agonists with reduced efficacy would show high levels of activity at G-protein and low or absent activity at arrestin; offering analgesia with reduced side effects or 'apparent bias'. Overall, the current data suggests,and we support, caution in ascribing biased agonism to reduced-side-effect profiles for mu-agonist analgesics. The large literature produced in recent years on this topic has been summarized and discussed into a review article (Neto et al., 2020). The same in vitro pharmacological profile of the above mentioned putative biased agonists (oliceridine, PZM21 and SR-17018)was observed in our laboratory with 4-Fluoro-Butyrylfentanyl and furanylfentanyl. Therefore further studies are need to compare in vivo the analgesic properties and side effect profile of 4-Fluoro-Butyrylfentanyl and furanylfentanyl with those of fentanyl. These studies will certainly contribute to understand whether partial agonism or functional selectivity is the key strategy for discovering safer opioid analgesics.

Finally, another strategy to look for safer analgesics is that of targeting simultaneously multiple opioid receptors by developing bivalent compounds (Dietis et al., 2009). In particular accumulating evidence suggest that the simultaneous activation of mu and NOP receptor elicits a synergistic analgesic effects associated to a reduction of side effects (Kiguchi et al., 2020). As a matter of fact the NOP/opioid mixed agonist cebranopadol showed very promising results both in preclinical and clinical studies (Calo and Lambert, 2018; Tzschentke et al., 2019). Peptides due to their high selectivity of action can represent an useful pharmacological tool to guide the development of innovative drugs. However, pharmacokinetic features of peptides are poor particularly in terms of half-life. For that matter, strategies focusing in enhancing peptide resistance to peptidases are of interest, in particular the synthesis and purification of branched peptides, which has been validated as a strategy for generating peptides able to elicit long-lasting actions. Following the idea that NOP and mu concomitant activation might be useful in generating safer analgesics, the peptide welding technology (Calo et al., 2018) was used to generate heterotetrameric peptides targeting the mu and NOP receptors. Among these compounds H-PWT1-N/OFQ-[Dmt^{1}]dermorphin demonstrated a similar and high agonist potency at the NOP and mu receptors. This compound will be certainly a useful tool for further in vivo studies aimed at investigating the therapeutic potential of NOP/mu agonists as innovative analgesics.

In perspective the experimental work summarized in this thesis was aimed at increasing pharmacological knowledge instrumental for the development of innovative opioid receptor ligands with potential for the treatment of pain and depression. After its discovery, the N/OFQ – NOP receptor system has been proposed as an innovative target for drug development. NOP antagonists displayed antidepressant properties in preclinical studies (Gavioli and Calo', 2013) and promising results were obtained in small proof of concept clinical studies (Witkin et al., 2016). Available antidepressant drugs have important limitations the most important being delayed onset of action and high percent of non-responders; hopefully NOP antagonists might not share these limitations with classical antidepressants. In addition, the ability of NOP antagonists, but not classical antidepressants, to counteract the development of depression in response to stressful stimuli is of particular interest and might be exploited therapeutically as preemptive treatment in patients with severe risk factors for depression.

The acute (constipation, respiratory depression) and chronic (tolerance and abuse liability) side effects of classical opioids are still unsolved problems that strongly limit the use of this drugs and our capability to treat pain patients. Thus the identification of safer analgesics is an urgent medical need. A possible strategy for obtaining safer opioid analgesics is that of developing mu

agonists biased toward G protein. This approach has been recently questioned and partial rather than biased agonism seems to be the underlying mechanism for the increased therapeutic index of the novel molecules (Neto et al., 2020). Independently from molecular mechanisms, these studies were useful for patients since oliceridine has been recently approved as a novel opioid analgesic with a therapeutic index higher than morphine (Lambert and Calo, 2020). Another promising strategy for generating safer analgesics is that of targeting multiple opioid receptors. In particular compounds able to simultaneously activate mu and NOP receptors displayed a great potential in preclinical studies in rodents as well as in non human primates (Ding and Ko, 2021). More importantly, the mixed NOP/opioid agonist cebranopadol is now completing the clinical development as innovative analgesic. When cebranopadol will be approved we will be able to collect the needed information to define the place in therapy of NOP/opioid agonists and to appreciate their contribution to increase our ability to treat pain patients.

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