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TESI DI LAUREA

Pharmacological evaluation of Gα specific coupling at the NOP receptor

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Abstract (ENG)

This study is focused on a peculiarity of G protein-coupled receptors (GPCRs), the so called ‘biased agonism’. This property is an intense ambit of investigation in drug discovery and development because of the potential in dissecting a given GPCR transduction routes with a single molecule. Biased agonists are defined as those molecules capable of triggering a subset of all possible GPCR conformations and signaling outcomes physiologically activated by the endogenous agonist. In turn, a biased agonist should be capable of modulating a part of the biological effects of the GPCR.

We employed the nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor because of the vastity of action modulated including pain, sedation, sepsis, stress and anxiety disorders, addiction and substance abuse, neurological conditions, cough, and neurogenic bladder. Therefore, a molecule capable of tackling a subset of these actions would be highly beneficial. Previous research indicated NOP agonists favor G protein recruitment over βArrestins and that G proteins are the main players of NOP signaling.

Here, we focused on NOP-Gα selectivity, analyzing the interaction with various G proteins, particularly the inhibitory G protein Gz. The endogenous peptide N/OFQ exhibits a preference for inhibitory G proteins, emphasizing specificity in modulating inhibitory signaling pathways. Twenty, among synthetic peptides and non-peptide agonists, showed various pharmacological effects at different transducers, that, however, are not largely dissimilar across transducers.

Our results suggest limited conformational changes at the NOP receptor and, with this study we speculate on the NOP receptor's adeptness at unbiased activation of Gαi1, Gαi2, Gαi3, GαoA, GαoB, and Gαz. The findings advocate for innovative pharmacophores to deepen the understanding of NOP signaling, overcome existing limitations, and advance therapeutic possibilities in various medical domains.

Abstract (ITA)

Questo studio farmacologico si concentra su una particolarità dei recettori accoppiati alle proteine G (GPCR), chiamata 'agonismo biased'. Questa proprietà è un ambito di intensa indagine nella scoperta e nello sviluppo di farmaci a causa della possibilità di sezionare alcune vie di trasduzione di un dato GPCR con una singola molecola. Gli agonisti biased sono definiti come molecole capaci di attivare un sottoinsieme di tutte le conformazioni recettoriali e di vie del segnale attivate a livello fisiologico da un agonista endogeno. Un agonista biased dovrebbe essere in grado di modulare una parte degli effetti biologici del GPCR.

Ci siamo focalizzati sul recettore del peptide nocicettina/orfanina FQ (N/OFQ) (NOP) a causa della vastità delle azioni da esso modulate, tra cui dolore, sedazione, sepsi, disturbi dello stress e dell'ansia, dipendenza e abuso di sostanze, condizioni neurologiche, tosse e vescica neurogena. Pertanto, una molecola che abbia come bersaglio un sottoinsieme di queste azioni sarebbe altamente benefica. Ricerche precedenti indicavano che gli agonisti NOP favoriscono il reclutamento di proteine G rispetto alle β-arrestine e che le proteine G sono i principali attori dei segnali mediati dall'attivazione del recettore NOP.

Quindi ci siamo concentrati sulla selettività NOP-Gα, analizzando l'interazione con varie proteine G, in particolare con la proteina G inibitoria Gz. Il peptide endogeno N/OFQ mostra una preferenza per le proteine G inibitorie, sottolineando la specificità nella modulazione delle vie di segnalazione inibitorie. Venti tra peptidi sintetici e agonisti non peptidici hanno mostrato vari effetti farmacologici su diversi trasduttori, che tuttavia non sono molto dissimili tra i trasduttori.

I nostri risultati suggeriscono limitate variazioni conformazionali nel recettore NOP e, con questo studio, speculiamo sull'abilità del recettore NOP di attivare in modo biased i trasduttori Gαi1, Gαi2, Gαi3, GαoA, GαoB e Gz. I risultati sottolineano la necessità di farmacofori innovativi per approfondire la comprensione delle vie di segnale del recettore NOP e per superare le limitazioni esistenti e avanzare nelle possibilità terapeutiche in vari settori medici.

1. Introduction

1.1 G protein-coupled receptors

G Protein-Coupled Receptors (GPCRs) represent one of the largest and most diverse families of cell surface receptors, playing a pivotal role in mediating cellular responses to a wide array of physiological stimuli. Also known as seven-transmembrane receptors, GPCRs transduce extracellular signals into intracellular responses by activating intracellular signaling pathways.

All GPCRs share a common structural motif characterized by seven transmembrane alpha-helical segments connected by three extracellular and three intracellular loops. The extracellular domains facilitate ligand binding, while the intracellular loops and carboxyl-terminal tail are involved in G protein coupling and signal transduction. GPCRs ligands are incredibly diverse, ranging from small molecules (such as neurotransmitters and hormones) to larger peptides, light, odorants, mechanical stressors, and ions. GPCRs participate in an extensive range of physiological processes, including sensory perception, neurotransmission, immune response, and hormonal regulation. The functional diversity of GPCRs is attributed to the numerous ligands they can bind and the ability to activate multiple downstream signalling pathways (Lefkowitz, 2007).

GPCR classification - As far as GPCRs classification is concerned, the GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin) nomenclature is among the most used and categorizes all GPCR into five main families based on the type of endogenous ligands they bind. This nomenclature provides a useful framework for understanding the diversity and functional roles of GPCRs. However, in the last decade the “A, B, C, F” nomenclature is gaining consensus because is based on sequence and structure similarities, and, in the last decade a revolution in terms of structural biology characterized this field of research. Specifically, Class A (Rhodopsin-like), such as Adrenergic receptors, dopamine receptors, serotonin receptors, and opioid receptors. Class B (Secretin Receptor Family), such as Glucagon receptor and corticotropin-releasing hormone receptor. Class C (Metabotropic Glutamate Receptor Family), such as Metabotropic glutamate receptors and GABA-B receptors. Class F (Frizzled/Taste2 Family), such as Frizzled receptors and SMO (involved in Wnt signalling) and Taste2 receptors (involved in taste sensation). In addition, the comprehension of the role of adhesion GPCRs (aGPCR) is currently object of intense research.

GPCR coupling - Upon ligand binding, GPCRs undergo conformational changes that facilitate the activation of heterotrimeric G proteins located on the intracellular side of the cell membrane. G proteins consist of Gα, Gβ, and Gγ subunits, and the activation of GPCR by the agonist promotes the exchange of GDP for GTP on the Gα subunit, triggering its dissociation from the βγ dimer. Both Gα and Gβγ subunits then modulate the activity of effector proteins, such as adenylyl cyclase, phospholipase C, or ion channels, leading to the generation of second messengers and downstream cellular responses. The G protein signal is then terminated when the Gα subunit hydrolyze GTP into

GDP and the heterotrimer reassociate in its inactive form. In addition, as part of the desensitization process, activated GPCRs are phosphorylated by GPCRs kinases (GRKs). GRKs are recruited to the cell membrane upon GPCR activation and phosphorylate serine and threonine residues on the intracellular loops and C-terminal tail of the receptor. These events facilitate the binding of arrestins to the receptor. Arrestins are proteins that act as scaffolds and play a critical role in regulating GPCR function. Upon binding, arrestins sterically hinder further G protein coupling, effectively desensitizing the receptor to further activation by the agonist. The arrestin binding not only prevents further G protein activation but also promotes the internalization of the GPCR-arrestin complex. This complex is often internalized via clathrin-coated pits, leading to endocytosis of the receptor. Once internalized, the GPCR-arrestin complex can follow different fates. It may be dephosphorylated and recycled back to the cell membrane for further signaling, or it may be directed to lysosomes for degradation (Rosenbaum et al., 2009; Hauser et al., 2017).

1.2 Heterotrimeric G proteins

As previously stated GPCRs orchestrate a diverse array of physiological and pathological processes by employing G proteins as molecular switches in their intricate signaling pathways. The initial identification of key G proteins, including Gs, Gt, Gi, and Go, marked a milestone in understanding the underlying mechanisms (Neves et al., 2002). Taking advantage of cDNA cloning techniques, scientists unveiled an extensive repertoire of G proteins and their subunits, adding intricacy to the GPCR signaling landscape.

Comprising α, β, and γ subunits, G proteins constitute a complex signaling machinery. The human genome encodes 35 genes dedicated to G proteins, with 16 governing α-subunits, 5 orchestrating β-subunits, and 14 overseeing γ-subunits (Milligan and Kostenis, 2006). Within the Gα subunits, a categorical division into four major families - Gαi, Gα12, Gαs, and Gαq - occurs, each intricately linked to distinct signaling pathways.

Gq proteins activate phospholipase C, leading to the production of inositol trisphosphate (IP3) and diacylglycerol (DAG). This activates downstream effectors, including protein kinase C, influencing diverse cellular functions (Wettschureck and Offermanns, 2005) and together with **G11** it can regulate p63-RhoGEF therefore being implicated in oncogene activation. **G15/16** proteins belong to the Gq family and are highly expressed in cells of the blood line (Kozasa et al., 1993; Lippert et al., 1997; Swift et al., 2000). Of note, such G proteins are very promiscuous, therefore they have been employed to force the coupling of otherwise not calcium stimulating GPCRs in calcium mobilization assays (Connor and Christie, 1999).

Gs proteins stimulate adenylyl cyclase, increasing intracellular cAMP levels. They are involved in activating protein kinase A (PKA) and influencing cellular responses such as metabolism and neurotransmission (Birnbaumer, 2007). **GsS** and **GsL** are ubiquitous while **Golf** is expressed mainly in olfactory neurons and certain CNS ganglia.

G12/13 proteins regulate Rho family GTPases, impacting the cytoskeleton and cell motility. They are implicated in processes such as cell migration and oncogenic transformation (Wettschureck and Offermanns, 2005).

Gi proteins inhibit adenylyl cyclase, reducing intracellular cAMP levels, and modulate ion channels. They play crucial roles in neurotransmission and hormonal regulation (Milligan and Kostenis, 2006). **Go** proteins also inhibit adenylyl cyclase and are involved in neuronal function and modulation of neurotransmitter release (Wettschureck and Offermanns, 2005). **GGust** (a.k.a. Gt3, therefore belonging to the Gi/o/t/z family of Gα proteins) is primarily a taste-specific G protein alpha-subunit (Spielman, 1998) and it is coupled with the taste receptors, a small subfamily of the class C GPCR. When expressed in taste buds in the mouth it can recognize the bitterness, the sweetness and the umami, but since the taste receptors are present in various organs and not only in the mouth, the name could

be misleading. Non gustatory taste receptors are indeed expressed also in the gastrointestinal epithelium, respiratory epithelium, urethra, pancreatic β-cells, heart, thyroid gland, testis and others (Töle et al., 2019). **Gz**, a member of the Gi/o/t/z family, plays a unique role in signal transduction. The Gz protein is primarily coupled to receptors that inhibit adenylyl cyclase, leading to a decrease in intracellular cAMP levels. Structurally, Gz shares similarities with other Gαi/o family members, featuring an N-terminal helical domain, a switch region, and a C-terminal alpha helix (Oldham and Hamm, 2008). Upon activation by GPCRs, Gz undergoes a conformational change, facilitating the exchange of GDP for GTP, thereby activating the protein. Gz activation results in the modulation of various effectors, contributing to the regulation of cellular functions. One prominent downstream target is the inhibition of adenylyl cyclase, leading to decreased cAMP levels. Additionally, Gz can modulate ion channels and activate mitogen-activated protein kinase (MAPK) pathways, influencing cell proliferation and differentiation (Wettschureck and Offermanns, 2005). The diverse signaling pathways activated by Gz confer it with multifaceted roles in physiology. Gz is implicated in neuronal function, where it regulates neurotransmitter release and neuronal excitability. Pivotal investigations by Yang and co-workers (Yang et al., 2000) on Gz null mice underlined i) impaired platelet aggregation (mice were more resistant to thromboembolism), ii) more pronounced responses to cocaine, iii) diminished analgesic effects of morphine, iv) ablation of catecholamine reuptake inhibitors antidepressant effects. Unfortunately, such very important findings did not have follow-ups. Here, by focusing on Gz activation, we aim to open to a renewed aspect of GPCR signaling research. However, further research into the specific roles and regulatory mechanisms of Gz signaling pathways will enhance our understanding of its importance in health and disease. Only recently, the role for the axis GPR176-Gz in circadian pacemaker neurons in the suprachiasmatic nucleus (SCN) in the brain has been shown (Yang et al., 2000), possibly highlighting a broader role of Gz in sleep modulation.

1.3 Biased Agonism

Our understanding about GPCR function posits that ligands induce a conformational change in the receptor, leading to the activation of G proteins and subsequent cellular responses. Biased agonism introduced the idea that ligands can selectively engage specific signaling pathways, resulting in diverse physiological outcomes. The phenomenon involves G protein bias, where ligands favor activation of a particular G protein (e.g., Gαs, Gαi, Gαq), and arrestin bias, where ligands favor arrestin-mediated signaling, influencing receptor desensitization and internalization (Smith and Rajagopal, 2016).

Important examples of G protein vs. Arrestin bias are i) TRV130/Oliceridine a mu agonist when coupled with mu stimulates preferentially G protein without activating βarrestin. TRV130 therapy resulted in significant antinociception, gastrointestinal function inhibition, and a low abuse-related consequence with reduced tolerance to the medication (Altarifi et al., 2017). ii) TRV027 is an AT1 receptor βArrestin biased agonist. Many of the critical functions of the AT1R, which keep blood pressure within a healthy range, are thought to be mediated by canonical signaling via G proteins. On the other hand, activation of the β-arrestin pathway causes a decrease in AT1R functions through desensitization and internalization and so aids in accurately regulating the time course of AT1R signals in response to a stimulus (Altarifi et al., 2017). TRV027 (Sar-Arg-Val-Tyr-Ile-His-Pro-d-Ala-OH) is a β-arrestin biased ligand that promotes the recruitment of β-arrestin and activates numerous kinase pathways. TRV027 improves cardiomyocyte contractility via the β-arrestin pathway, whereas sartans decrease arterial mean pressure by blocking the G protein-dependent pathway.

The evidence that different ligands might be able to activate different signaling pathways by acting at the same receptor emerged in preliminary studies where different G proteins were differentially activated by chemically similar agonists (Kolb et al., 2022).

This was however largely overlooked in the last decades and only limited attention given to biased agonism within the different G proteins. Nowadays, thanks to innovative approaches it is possible to finely follow the activation paradigms of different G proteins. Both in vitro (Grundmann et al., 2018) and in vivo (Wettschureck et al., 2004) genetic tools allowed to disentangle several important features of diverse G protein signal -function relationship. In terms of pharmacological tools, the use of molecules like i) pertussis toxin (PTX), as Gi/o inhibitor; ii) cholera toxin (CTX), as Gs stimulator; and iii) FR/YM, as Gq/11 inhibitors (Schrage et al., 2015; Malfacini et al., 2019) facilitated the understanding of G protein function, at least at the “subfamily” level of resolution. More recently, BRET and complementation approaches allowed the researchers to discriminate between different G proteins (even of the same subfamily) in recombinant systems (Mende et al., 2018; Hauser et al., 2022; Burghi et al., 2023). One important example is the “trupath” approach, by Olsen (2020), as

BRET-based method (Olsen et al., 2020), and the “nanobit” system (Inoue et al., 2019), as complementation tool.

In this thesis, we based our research on the work of Olsen and co-workers, in the original manuscript it has been attempted to engineer all Gα subunits, improved their propensity to evoke BRET signal in several pairing combinations (of Gβ and Gγ), tested few GPCRs for their capacity to trigger the response at each transducer. For instance, the kappa receptor was evaluated with 8 ligands on 7 (cAMP inhibiting) transducers. Crucially and differently from other researchers, Olsen and colleagues made their assay available to the scientific community through Addgene. Thanks to this, studies adopting trupath to evaluate fine mechanisms of G protein activation were reported (Knight et al., 2021), together with kappa receptor signaling evaluation (Lieb et al., 2021), H1 (Singh et al., 2022), A2B receptor (Voss et al., 2022), and M2 receptor pharmacology (Jiang et al., 2022), and on the role of RAMP in signaling alteration (Glenn et al., 2023). In addition, the potential of such biosensors in studying GPCR-G protein preferences has been reviewed in (Olsen and English, 2023). Finally, biased agonism within different G proteins is a poorly studied phenomenon that could be very useful to dissect the different effects of pleiotropically acting GPCRs which stimulation would lead to a wide spectrum of effects both therapeutic and adverse. In this study, we will investigate a class A GPCR, the human nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor, and try to understand more about G protein bias, with particular focus on the Gz coupling.

1.4 N/OFQ- NOP receptor system

The **N/OFQ - NOP receptor system** is a very intriguing pharmacological target because of the pleiotropicity of action exerted and its peculiar pharmacology. Chronologically, several discoveries were crucial for the understanding of this system. In 1994, Mollereau and co-workers cloned the gene encoding the NOP receptor, initially named ORL1 (opioid receptor-like 1). In this study, authors provided prediction into sequence and structure features of the NOP receptor (Mollereau et al., 1994). Afterwards, Meunier in parallel with Reinscheid isolated a novel central nervous system-expressed peptide, which they named nociceptin and orphanin FQ, respectively. The experimental paradigm followed by Meunier and by Reinscheid is known as “reversed pharmacology”. In this case, authors basically separated the tissue mixture in different high pressure liquid chromatography (HPLC) eluates, after measuring a positive response in a specific assay where the NOP receptor was present at high level of expression, they proceeded in further rounds of purification up to N/OFQ was identified. Therefore, both groups demonstrated that N/OFQ selectively activates an orphan GPCR, the NOP receptor (Meunier et al., 1995; Reinscheid et al., 1995).

Like classical opioid receptors (namely, mu, delta, and kappa receptors), the NOP receptor is a class A GPCR. NOP shares about 50% of its amino acid sequence with classical opioid receptors. NOP couples to pertussis toxin-sensitive G proteins it is activated, similarly to the other members of the opioid receptor family. Upon NOP-G protein activation, adenylyl cyclase is inhibited, with consequent cAMP levels decrease, potassium conductance is stimulated, and voltage-sensitive calcium channels are blocked (Ubaldi et al., 2021).

However, N/OFQ - NOP receptor system pharmacology is rather peculiar. NOP receptor is neither bound by other endogenous peptides, nor by opioid alkaloids and naloxone (Toll et al., 2016). N/OFQ is a 17-aa neuropeptide (YGGFLRRIRPKLKDHPHYNKTF) that, in turn, although very similar to Dynorphin A - an endogenous opioid peptide - does not bind the classical opioid receptors.

In terms of **structural biology**, key studies have focused on elucidating the arrangement of NOP receptor’s transmembrane helices, especially those critical for ligand binding and receptor activation. Crystallographic studies of related receptors, such as the mu-opioid receptor, have contributed to predicting ligand-binding pockets within the NOP receptor and that a sodium ion bound to a conserved site in the transmembrane domain stabilizes the inactive conformation of the receptor (Thompson et al., 2012; Miller et al., 2015). The extracellular and intracellular loops of the NOP receptor are very important for ligand recognition, receptor activation, and interaction with downstream signaling partners. These domains contribute to the receptor's selectivity and efficacy. While experimental structures were limited up to the recent discovery of the active structure of the NOP receptor by cryo-em (Wang et al., 2023), computational models provided insights into the conformational dynamics of these crucial regions (Thompson et al., 2012).

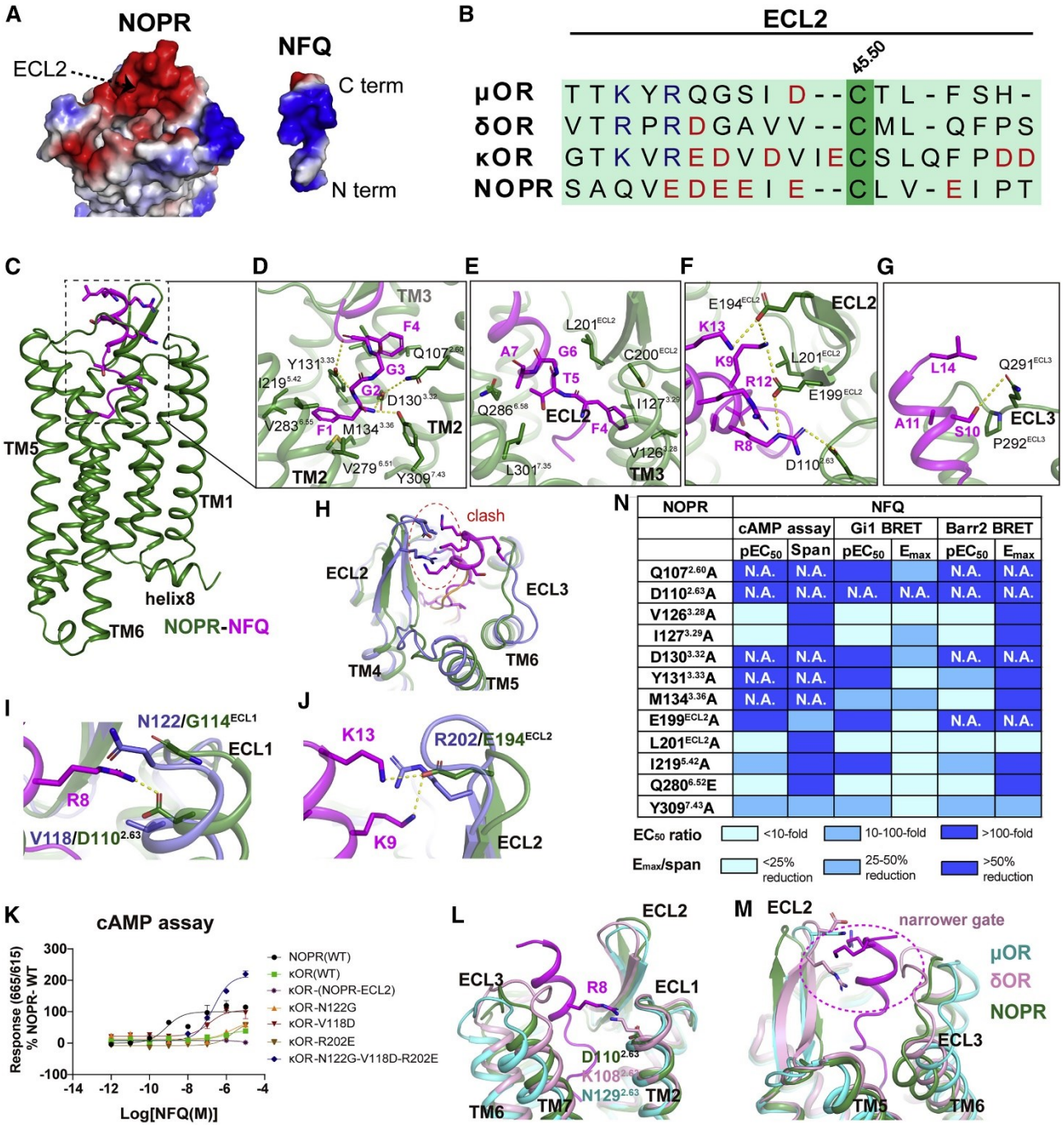


Figure 1.5. Selectivity of N/OFQ toward the NOP receptor (Wang et al., 2023).

This paradigm of know-how (inactive and active structures of the NOP receptor) not only empowers the modeling of cutting-edge antagonists but also extends to the creation of entirely novel agonists, thereby unlocking the potential for the generation of pioneering biased agonists.

What is making the pharmacology of the N/OFQ - NOP receptor system is not only the very high selectivity of peptide and receptor, but also the pleiotropic roles exerted.

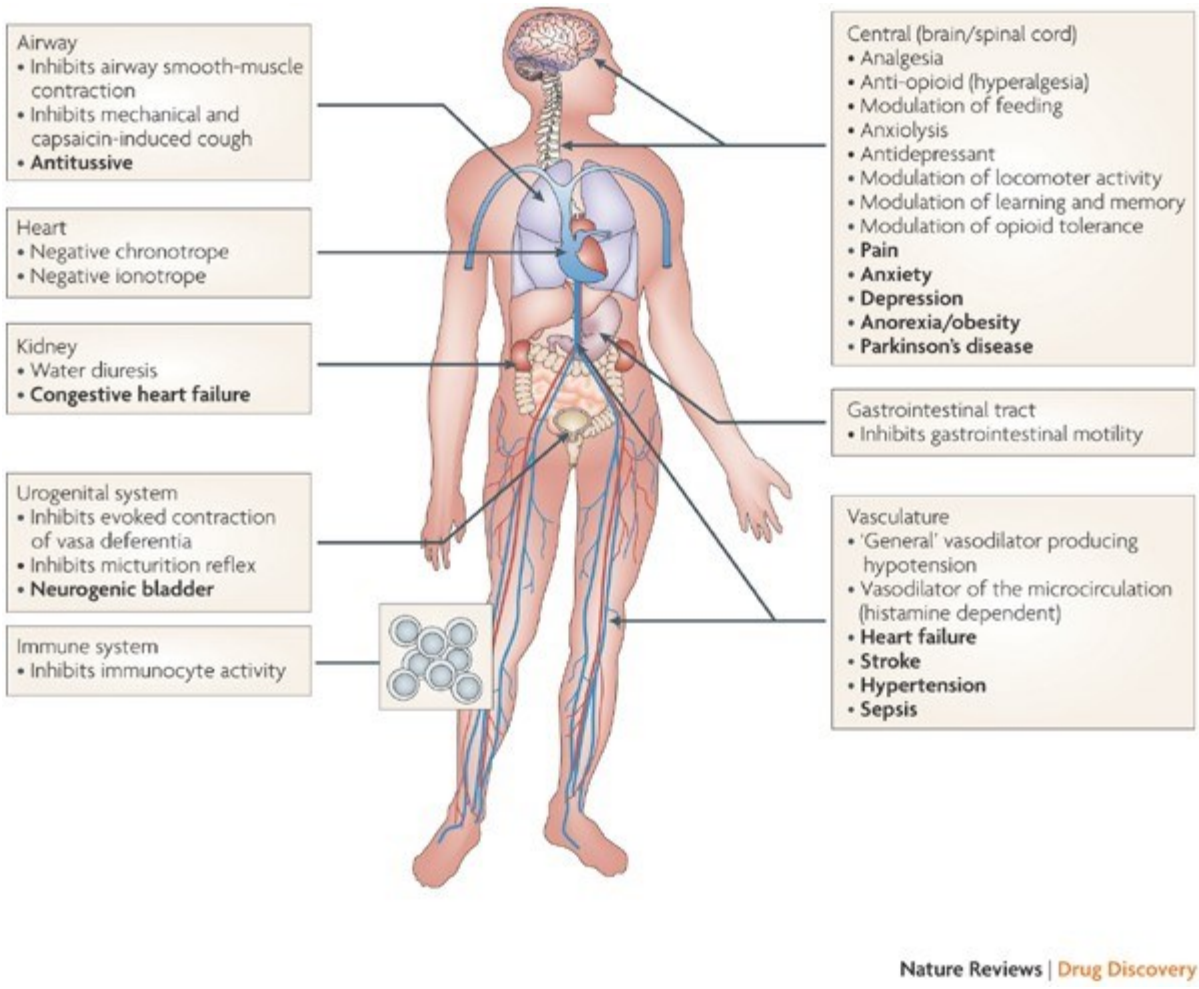


Figure 1.6. Pleyotropic effects of the NOP receptor (Lambert, 2008).

The therapeutic potential of modulating the NOP receptor is still object of intense investigation. In a nutshell: pain, sepsis, stress and anxiety disorders, addiction and substance abuse, neurological conditions, cough, and neurogenic bladder incontinence are among the most investigated (Lambert, 2008). Only this year (i.e. 2023), a careful evaluation of the role of the N/OFQ-NOP receptor system in altering the sleep behavior has been reported (Morairty et al., 2023). Almost parallelly, the hypnotic effects of a selective NOP receptor partial agonist (Sunobinop) have been reported (Whiteside et al., 2023). In terms of pain management, preclinical studies suggest that activation of the NOP receptor produces analgesic effects, indicating its potential as a target for pain medications. Additionally, in both rodents and non-human primates the concomitant activation of NOP and mu receptors leads to synergistic antinociceptive effects with no increase of the most common opioid side effects (Hu et al., 2010; Molinari et al., 2013; Rizzi et al., 2015; Cerlesi et al., 2017). This evidence (NOP/mu simultaneous activation) has been instrumental in the development of Cebranopadol, a potent analgesic under clinical evaluation (Linz et al., 2014). As far as stress and anxiety is concerned, modulating the NOP receptor may offer therapeutic benefits in conditions characterized by heightened stress and anxiety, providing a novel avenue for treatment (Preti et al., 2019). Importantly,

preclinical studies suggest that targeting the NOP receptor may offer a novel approach to managing substance abuse and addiction disorders (Ciccocioppo et al., 2019).

This plethora of biological effects is associated with high potential for new drug development and patients' life amelioration. However, a systemic activation of the NOP receptor will simultaneously modulate all of such "situations" afore mentioned, and this may bring with it many challenges. The possibility with "biased agonists" to trigger some but not all the biological outputs of a given receptor can be advantageous also in targeting the NOP receptor.

2. Aim of the study

This thesis aims to elucidate the in vitro pharmacology of the human NOP receptor by employing an innovative assay capable of measuring NOP-mediated activation across diverse G proteins, including Gz, Gust, Gq, G15, GsS, and GsL. This assay is called *trupath*. While comprehensively assessing the receptor's pharmacological profile with a panel of 21 NOP receptor agonists, we specifically focus on Gz due to its predominant interaction (with the NOP receptor) and significance in neuronal contexts. The primary objective is to unravel the distinct activation patterns induced by NOP agonists, providing a detailed understanding of the pharmacology of action leading in turn to different intracellular pathways involvement. We will also compare our results with a previous study within our research group and integrate findings from the literature, ultimately contributing to the broader comprehension of NOP receptor pharmacology and facilitating the dissection of various biological roles exerted by NOP agonists.

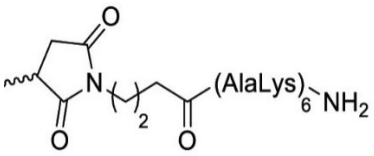
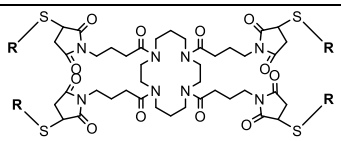
3. Materials and Methods

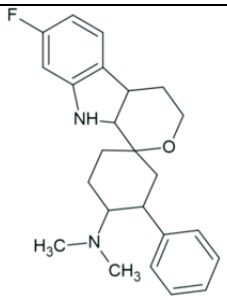
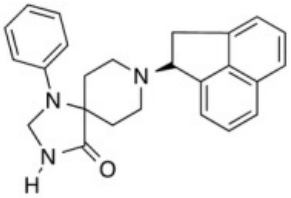
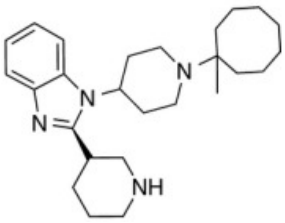
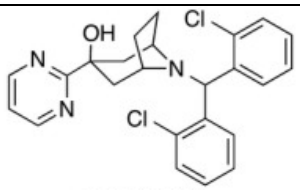
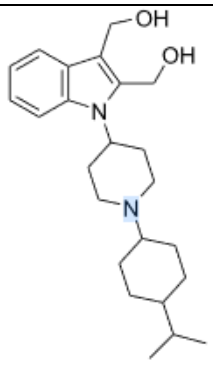
3.1 Drugs and reagents – The nociceptin/orphanin FQ (N/OFQ) peptide was synthesized in-house. Cebranopadol was a gift from Prof. Trapella (University of Ferrara, Italy). The peptides UFP 113, UFP 112, Ac-RYYRIK-NH_2, Ac-RYYRWK-NH_2, PWT2-N/OFQ, N/OFQ-palmitic/Comp 40, comp 3, comp 26, comp 22, comp 29, comp 43 were a gift from Prof. Guerrini (University of Ferrara, Italy). The non-peptide molecules MCOPPB, Ro65-6570, SCH-486757 were a gift from Chiesi (Italy). AT-390, AT-312, AT-090, AT-202 and AT-403 were a gift from Dr. Nurulain T. Zaveri (Astraea Therapeutics, Mountain View, CA, USA). Concentrated solutions of ligands were made in ultrapure water or dimethyl sulfoxide and kept at – 20°C until use. Reagents used were from Merck / Millipore Sigma (Burlington, MA, USA) and were of the highest purity available. Purple coelenterazine (CLZN, 5 mM, EtOH) was from NanoLight Technology (White Mountain, AZ; USA).

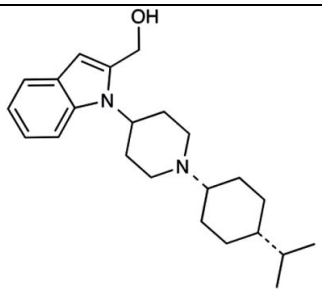
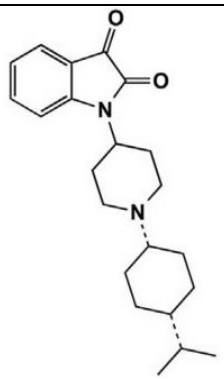
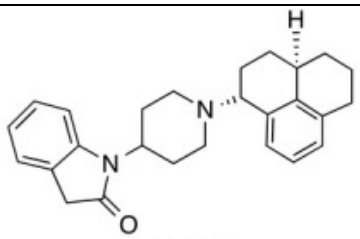
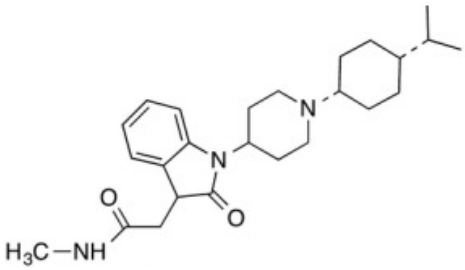
Here as follows is an overview of the molecules object of this study (**Table 1**).

Table 3.1. Common name, structure, and general information of given molecules.

NAME	STRUCTURE	INFO	REF
N/OFQ	FGGFTGARKSARKLANQ	endogenous neuropeptide, selective for the NOP receptor	(Meunier et al., 1995; Reinscheid et al., 1995)
Comp 3	[Ala^3]N/OFQ(1–13)-NH2	N/OFQ(1-13)-NH2 derivative, slightly less potent than N/OFQ	(Pacifico et al., 2020)
Comp 22	[Cha^1]N/OFQ(1–13)-NH_2	N/OFQ(1-13)-NH2 derivative, slightly more potent than N/OFQ	(Pacifico et al., 2020)
Comp 26	[D-Ala^2]N/OFQ(1–13)-NH_2	N/OFQ(1-13)-NH2 derivative, slightly less	(Pacifico et al., 2020)

		potent than N/OFQ	
Comp 29	[(pF)Phe^4]N/OFQ(1–13)-NH_2	N/OFQ(1-13)-NH2 derivative, more potent than N/OFQ	(Pacifico et al., 2020)
Comp 40	[Cys^{14}Palmitoyl]N/OFQ(1–14)-NH_2	N/OFQ(1-13)-NH2 derivative, more potent than N/OFQ	(Pacifico et al., 2020)
Comp 43	[Cys(X)^{14}]N/OFQ(1–14)-NH_2 X, 	N/OFQ(1-13)-NH2 derivative, more potent than N/OFQ	(Pacifico et al., 2020)
Ac-RYYRIK-NH2	Ac-RYYRIK-NH2	NOP partial agonist	(Dooley et al., 1997)
Ac-RYYRWK-NH2	Ac-RYYRWK-NH2	NOP agonist	(Dooley et al., 1997)
PWT2-N/OFQ	 R, [Cys^{18}]N/OFQ	Long-lasting potent NOP agonist	(Guerrini et al., 2014; Rizzi et al., 2015)
UFP 112	[(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2	Long-lasting potent NOP agonist Formerly in clinical evaluation for neurogenic bladder	(Arduin et al., 2007)
UFP 113	Phe^{1}Psi(CH_2-NH)Gly^2(pF)Phe^{4}Aib^{7}Arg^{14}Lys^{15}]N/OFQ-NH_2	highly potent NOP partial agonist	(Arduin et al., 2007)

Cebranopadol		NOP/opioid agonist. Under clinical evaluation for different types of pain	(Linz et al., 2014)
Ro 65-6570		Non peptide; NOP agonist Anxiolytic-like effects	(Wichmann et al., 1999)
MCOPPB		Non peptide; NOP very potent agonist. Anxiolytic-like effects	(Hirao et al., 2008)
SCH 486757		Non peptide; low potency NOP agonist; It was under clinical evaluation as anti-cough treatment	(McLeod et al., 2010)
AT-390		Non peptide; NOP receptor agonist	(Arcuri et al., 2018)

AT-312		Non peptide; NOP receptor agonist	(Zaveri et al., 2018)
AT-090		Non peptide; Potent NOP receptor partial agonist	(Ferrari et al., 2016)
AT-202		Non peptide; potent NOP receptor agonist	(Cippitelli et al., 2016)
AT-403		Non peptide; very potent NOP receptor agonist	(Ferrari et al., 2017)

3.2 Constructs - Plasmids are short, circular, double helix extrachromosomal DNA found primarily in bacteria and are used as vectors in various types of experiments in molecular biology. Their main features are i) the origin of replication (ORI), ii) the sites of restriction, iii) and the antibiotic(s) resistance. Here as follows, a scheme of the plasmid coding for Gαz-RLuc

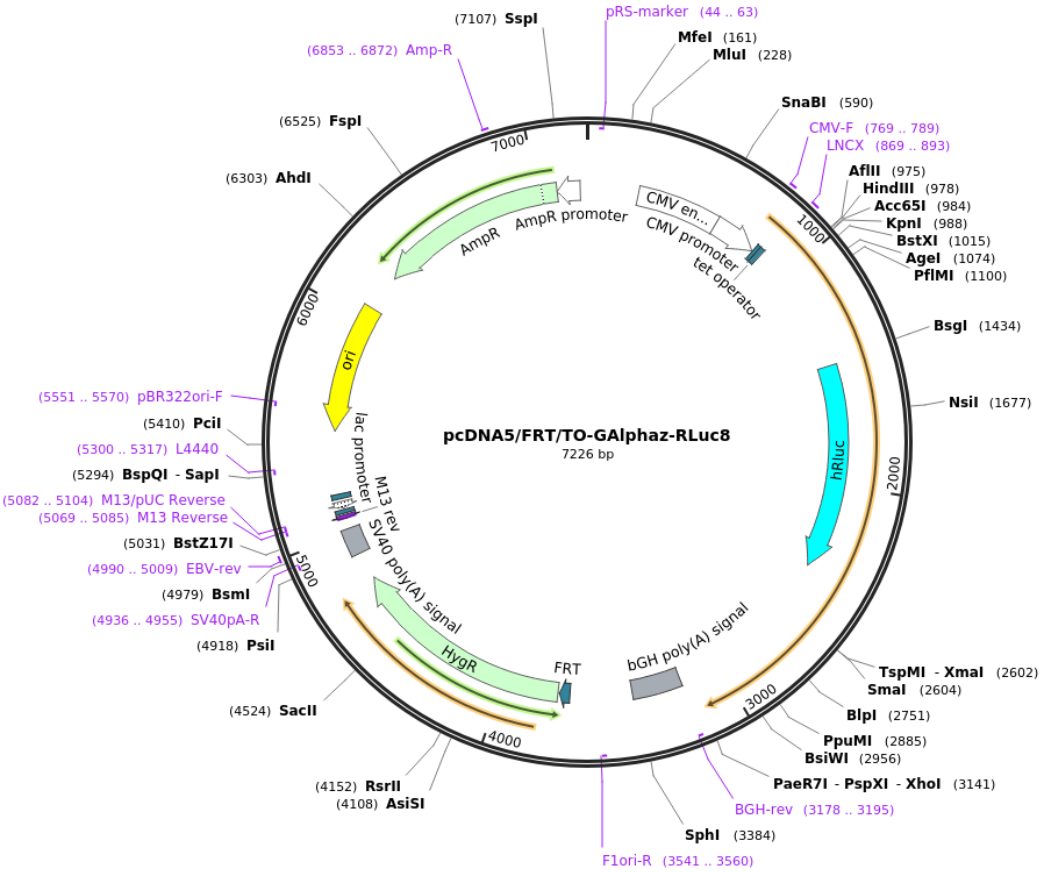


Fig. 3.1. Construct map for Gαz-RLuc. The image was taken from Addgene (https://www.addgene.org/140978/) and depict the plasmid "pcDNA5/FRT/TO-GAlphaz-RLuc8".

Plasmids are generally used to overexpress a given RNA or protein of interest. The proteins of interest in the present study were the G protein subunits alfa, beta and gamma (**Table 2**). The plasmids used in the experiment were a gift from Bryan Roth ((Addgene Kit #1000000163). Bacteria cells harboring the needed plasmids were conserved in a - 80° C freezer until use.

Table 3.2. Combinations of all heterotrimeric G proteins contained in the trupath (original) kit. The main focus of this study will be on Gz (grey)

Gα-RLuc	Gγ-GFP2	Gβ
i1	γ9	β3
i2	γ8	β3
i3	γ9	β3
oA	γ8	β3
oB	γ8	β3
Z	γ1	β3
Gustducin	γ1	β3

sS	γ9	β3
sL	γ1	β1
Q	γ9	β3
11	γ13	β3
15	γ13	β3
12	γ9	β3
13	γ9	β3

3.3 Plasmid isolation - *E. coli* grows in a liquid LB medium (a pre-mixed powder containing Tryptone, NaCl and Yeast Extract) with antibiotic (ampicillin) dissolved in purified water. The Petri dishes were prepared using the liquid broth with agar agar powder.

The frozen bacteria were picked with a tip, that then was placed in an Eppendorf vial with 1 ml of LB medium without antibiotic. The inoculated culture was leaved to reconstitute at room temperature for one hour, then 200 µl were spread using a streak plate method and put in an incubator at 37° C for 24 h.

Three colonies were picked from each plate and inoculated in vials with 4 ml of LB medium, put in a shaking incubator at 37° C for two days and let them grow for two days.

About 150 µL bacteria solution aliquot was kept aside for each of the three preparations. The remaining bacteria solution were lysated and then, using a classical filter column protocol, the plasmids were extracted, concentrated, and purified, then they were quantified by the nanodrop. Once selected the sample with the more concentrated plasmids, the aliquot was expanded in 200 mL of fresh LB medium (in the presence of ampicillin) and after approximatively 24 and 36 hours was added chloramphenicol to increase the plasmids copy number. Chloramphenicol is a well-known inhibitor of bacterial ribosomal activity, the addition of such antibiotic would impair most of bacteria metabolisms, therefore advantaging plasmid synthesis. The extraction/purification protocol was carried out with the Machery-Nagel kit and plasmids were collected at -20° C until use.

3.4 Cell culture - In our experiment cell line HEK293 was used. It is a relatively easy to grow and to transfect by various techniques (https://www.atcc.org/products/crl-1573). This cell line was stably transfected with the human NOP receptor, using the antibiotic Hygromicin B for the selection (gift from Prof. Dave G Lambert). The cells were kept in liquid nitrogen until use. $HEK293_{NOP}$ grow in adhesion on sterile pre-coated flasks or Petri dishes, in an incubator at 37 ° C with 5 % of CO_2 and regular condition of humidity, in a high glucose DMEM complemented with 10 % (v/v) FBS, 1% (v/v) of L-glutammine, 1% (v/v) of penicillin/streptomycin, and 100 µg/mL Hygromycin B.

3.5 Cell transfection - Cells were rinsed with PBS without Ca2+ and Mg2+, detached using ~3 ml of tripsin (inactivated 3 minutes later by adding an equal volume of complete medium) and counted with the CounterCoulter (Agilent). The cells were centrifuged at 250 rpm for 5 minutes to eliminate the tripsin, the pellet was resuspended with new medium. Then about 5 million cells were taken and seeded in a 10 cm Petri dish. Cells were let adhere in the incubator for at least 4-6 hours.

6 µg DNA of each Gα, Gβ and Gγ subunit were added to 500 µl of PBS with Ca2+ and Mg2+, while 54 µg of polyethylenimine (PEI) were added another 500 µl of PBS with Ca2+ and Mg2+. Both solutions were vortexed separately and subsequently the PEI solution was added to that with one, the mixed solution was then vortexed and let it sit at room temperature in order to create complexes. After 25 minutes the solution was added gently drop by drop to the adhered cells on the Petri dish.

After about 24 hours the cells were detached and counted again and plated in a poly-D-lysine coated white, clear bottom 96-well plates (Greiner Bio-One, Monroe, NC), at about 50.000-60.000 cells per well.

3.6 BRET – The transfer of energy from the donor to the acceptor into a Bioluminescence Resonance Energy Transfer (BRET) couple, is possible only if the two molecules are near to each other (<10 nm) and in a proper orientation, and so the high energy molecule (the donor) can excite the highly sensitive molecule (the acceptor).

There are different techniques for biological analysis that involve photoactive molecules, for example FRET (Fluorescence Resonance Energy Transfer) and BRET (Bioluminescence Resonance Energy Transfer). FRET requires external excitation for the donor and because of that it has a higher background noise. BRET is more sensitive than FRET and returns more stable and reliable signals. In BRET two proteins of interest are separately attached to a luciferase and a fluorescent protein (**Fig. 2**). There is various combinations of pairs, and so various wavelength windows and different types of BRET.

To analyze and decipher the activities of complex biological systems is fundamental to have an enzymatic and cellular signaling biosensor. Historically GPCR activity was assessed by enzymatic transduction cascades and to amplify and detect the signal second messengers and gene activation was measured. These methods allow the evaluation of the intermediate or final stages of the cascade, but this can lead to interferences and unwanted interactions, so a more direct measurement is needed.

In our experiments we used TRUPATH (TRansdUcer PATHways), an open-source BRET-based platform that allows the analysis of the GPCR transducerome (i.e. all G protein that transduce signal upon GPCR activation). This is a standardized and near-complete toolkit that minimize the experimental differences allowing a direct comparison for all Gα (Gα14 and Gαolf not present).

The BRET pair is composed by a Renilla luciferase (RLuc8) and GFP2 covalently attached to the alpha and gamma subunits, respectively. The BRET signal is an indication of proximity, and thus of interaction between Gα and Gβγ (i.e. high BRET low activation). The ligand-induced reduction in the BRET signal is an indication of the activation of the GPCR.

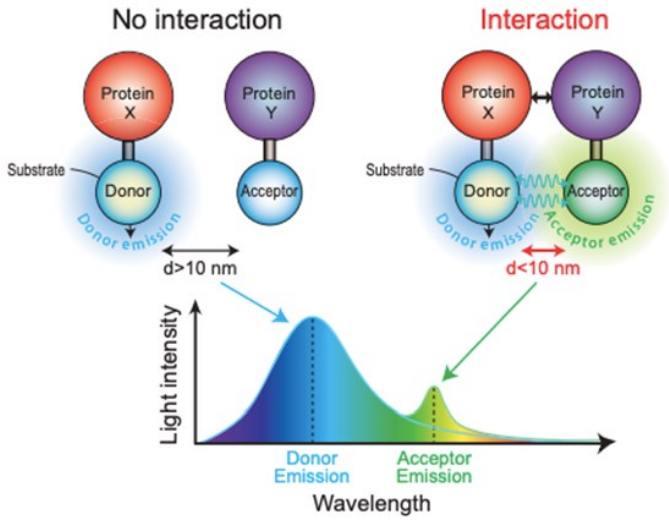


Fig. 3.2. **Prototypical example of BRET**

The day of the experiment, the medium was discarded, and cells were rinsed once with PBS with Ca2+ and Mg2+. 80 µl of a buffer solution made by HBSS/HEPES and 2 µM purple coelenterazine (the luciferase oxidizes the coelenterazine, that emits light). Coelenterazine is less bright than other luciferins but has an excellent signal-to-noise ratio.

Serial dilutions of the compounds were prepared in PBS with Ca2+ and Mg2+. A 96-well clear plate was prepared, and PBS added with 0.01% BSA. 20 µl of the drug solution were added to the cells in duplicate, then the plate was placed immediately in the luminometer Victor Nivo (Perkin Elmer) and at least three readings were performed. Every well was read at two different wavelengths, at 405 nm and 530 nm. The mean of the first three ratios between the measurement at 530 nm (GFP2) and 405 nm (RLuc8) was used in the data analysis.

3.7 Data analysis - In this study, we employed terminology and computations consistent with the recommendations of the International Union of Basic and Clinical Pharmacology (IUPHAR)(Kolb et al., 2022; Neubig et al., 2003). We performed basic statistical evaluation of our datasets with Excel for Microsoft 365 (Microsoft, Redmond, Washington, US) and Prism 8.4 (GraphPad Software Inc., San Diego, CA, US). We expressed experimental points as mean ± sem of n experiments. We applied the one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple

comparisons. We fitted all concentration-response curves to agonists to the four-parameter logistic nonlinear regression model as follows:

$$\text{Effect} = \frac{\text{basal} + (\text{Emax} - \text{basal})}{(1 + 10[(\text{LogEC50} - \text{Log[ligand]}) \text{ x Slope}])}$$

We expressed agonists' potency (pEC_{50}) values as the mean with computed confidence limits ($CL_{95\%}$), while maximal effects (Emax) in percentage ± sem of inhibition of the basal BRET value. We finally considered p values < 0.05 as statistically significant.

4. Results

We built on a previous set of experimental data dealing with the propensity of the human NOP receptor to mediate the activation of all pertussis toxin-sensitive G proteins (i.e Gi1, Gi2, Gi3, GoA, GoB). Here, we assessed the selectivity of the endogenous peptide of the NOP receptor N/OFQ to the most important other families of G proteins (i.e. Gq and Gs). We then focused on the neuronally relevant inhibitory G protein Gz, fully characterizing a comprehensive panel of NOP receptor ligands encompassing full and partial agonists of both peptide and non-peptide nature.

4.1 Expression of Gα transducers in HEK293$_{NOP}$ cells – Implying that RLuc functions only if present in the cells at high levels, we compared the RLuc activity of all Gα transducers in a set of control experiments. In these experiments, we obtained high levels of relatively comparable expression (no significant difference by one-way ANOVA) of all transducers independently of the construct.

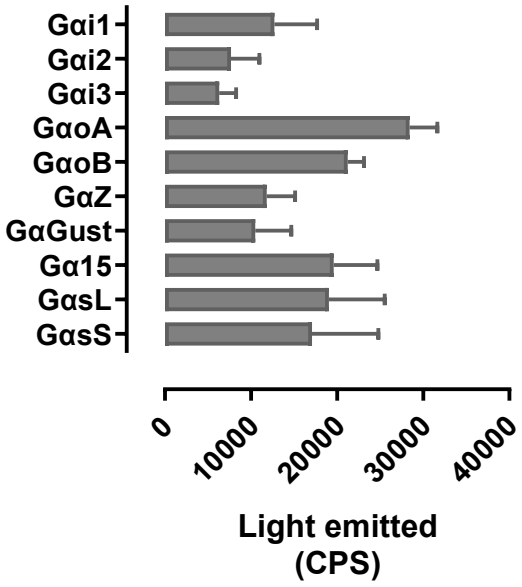


Fig. 4.1. Expression of different Gα-RLuc. Data are mean ± sem of n ≥ 3 made in octuplicate.

4.2 Effects of N/OFQ on all Gα transducers - N/OFQ could foster the dissociation of the Gα-Gβγ heterotrimer in Gαi1, Gαi2, Gαi3, GαoA, and GαoB (previous data). In addition, N/OFQ triggered the activation of Gαz, GαGust, and Gα15. Experiments carried out with Gαq, GαsS, and GαsL displayed (close to) no activation of these transducers (**Fig. 2**). Although all transducers herein adopted were thoroughly validated by Olsen and co-workers with recombinantly expressing GPCRs, It is worth mentioning that this approach was totally insensitive to any stimuli at endogenously expressed GPCRs (data not shown).

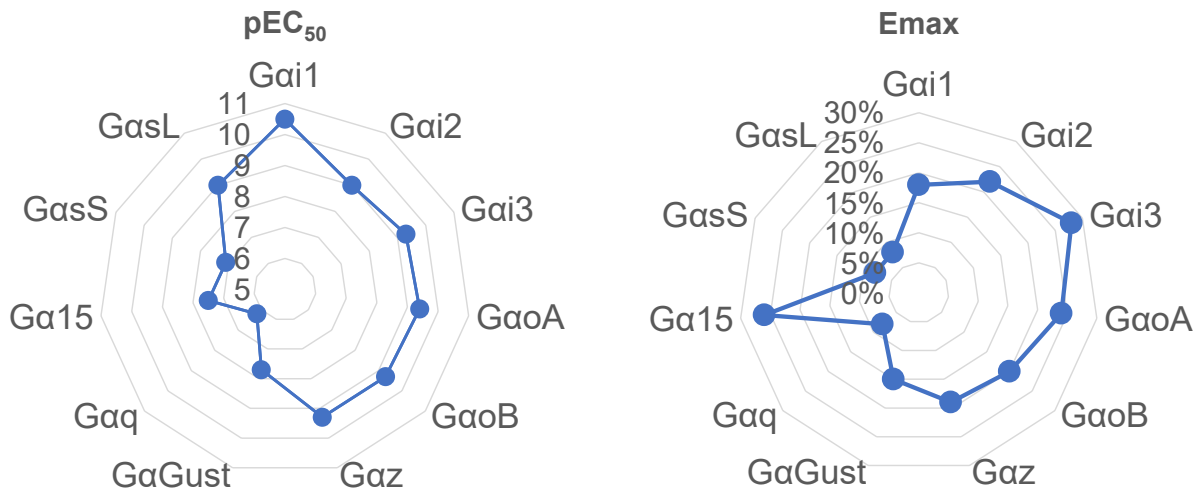


Fig. 4.2. N/OFQ potency and maximal effects on tested transducers. Spider plots generated for potency (pEC_{50}) and maximal effects (absolute % of basal BRET inhibition) at all different transducers tested.

4.3 Activities of NOP receptor endogenous agonist on NOP-Gz activation – We tested increasing concentrations of N/OFQ on NOP-Gz activation. The endogenous peptide could foster about 20% inhibition of the basal (Emax 81%) and displayed a potency value of 9.3 (**Fig. 3**).

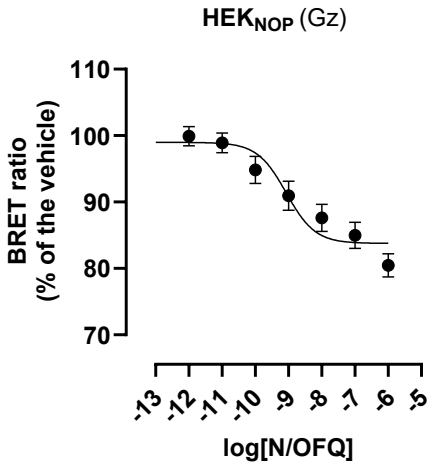


Fig. 4.3. Concentration-response curve to N/OFQ on NOP-Gz activation. Data are expressed as mean ± sem of 17 experiments performed in duplicate.

4.4 Effects of peptides UFP-113, UFP-112, and PWT2-N/OFQ on NOP-Gz – All synthetic peptides mimicked the stimulatory effects of N/OFQ with similar maximal effects. Regarding potency, UFP-112 was the most potent, followed by UFP-113 and PWT2-N/OFQ (Fig. 4).

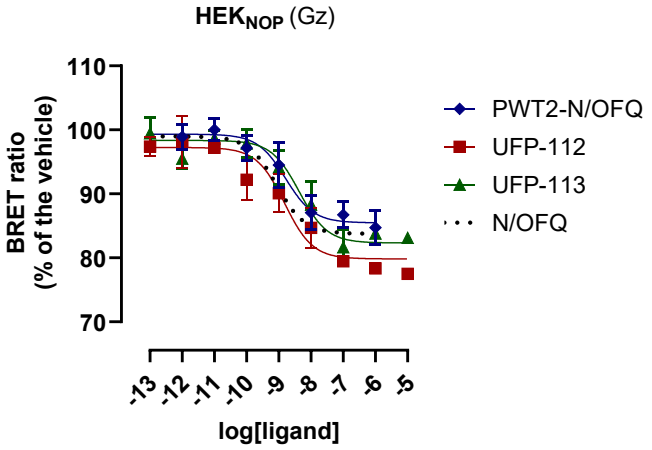


Fig. 4.4. Concentration-response of N/OFQ derivatives UFP-113, UFP-112, and PWT2-N/OFQ on NOP-Gz activation. Data are expressed as mean ± sem of at least 3 experiments performed in duplicate.

4.5 Effects on NOP-Gz activation by non-peptide standards Ro 65-6570, SCH-486757, and MCOPPB – Non-peptide standard agonists displayed maximal effects statistically similar to that of the endogenous peptide N/OFQ. In terms of potency, MCOPPB was the most potent by far (25-fold more than N/OFQ), while Ro 65-6570 was slightly less potent than the endogenous peptide, and SCH-486757 was more than 300-fold less potent than N/OFQ (**Fig. 5**).

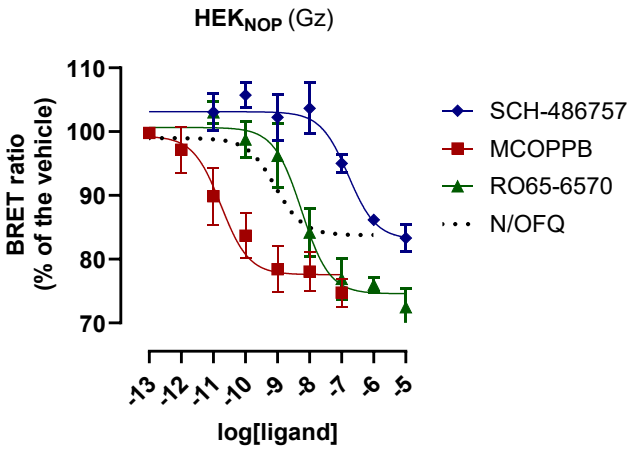


Fig. 4.5. Concentration-response of standard non-peptide NOP agonists on NOP-Gz activation. Data are expressed as mean ± sem of at least 3 experiments performed in duplicate.

4.6 Hexapeptides Ac-RYYRIK-NH2 and Ac-RYYRWK-NH2 effects on NOP-Gz. Ac-RYYRIK-NH2 and Ac-RYYRWK-NH2 maximal effects were not significantly different than that of N/OFQ, with potency values slightly higher for Ac-RYYRIK-NH2 than Ac-RYYRWK-NH2 (**Fig. 6**).

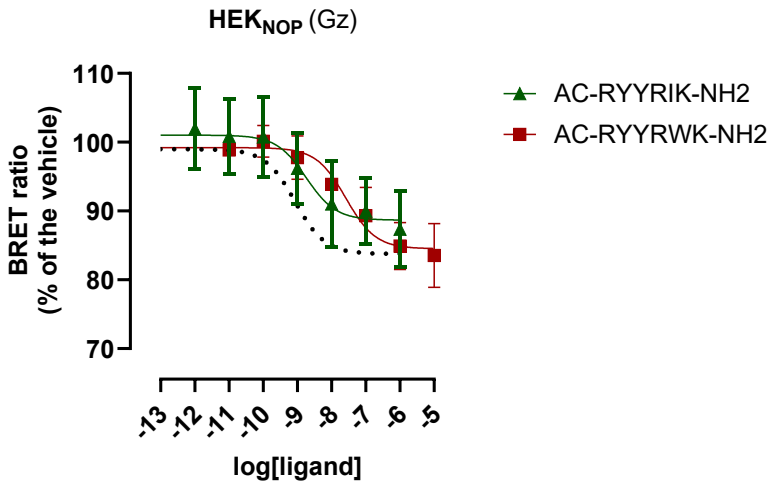


Fig. 4.6. Concentration-response of hexapeptides on NOP-Gz activation. Data are expressed as mean ± sem of at least 5 experiments performed in duplicate.

4.7 Derivatives of N/OFQ(1-13)-NH2 and effects on NOP-Gz - Comp 3, Comp 26, Comp 22, Comp 29, Comp 40, and Comp 43 all behaved as moderate potent NOP receptor full agonists with values of potency ranging from 8.7 to 8.1 (**Fig. 7A** and **B**).

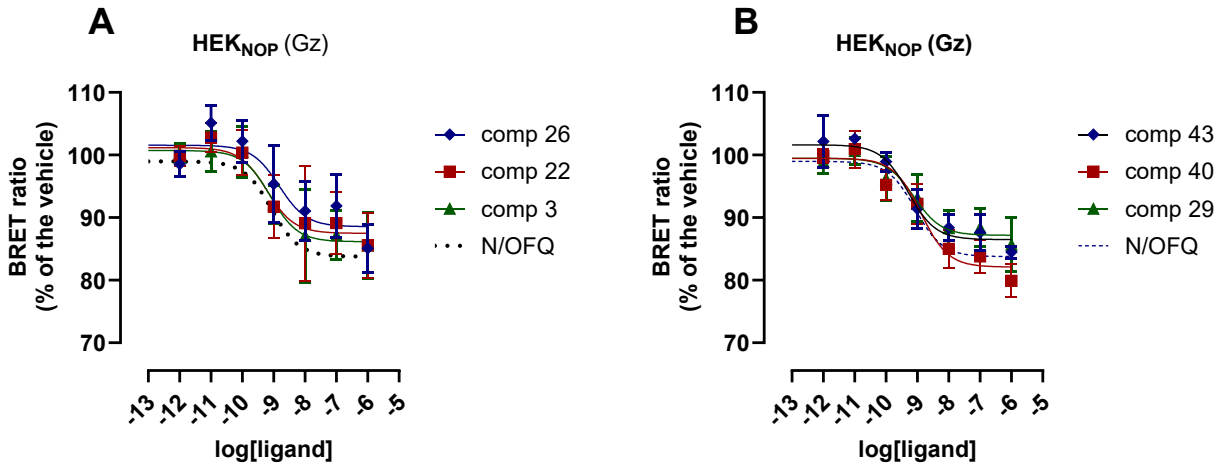


Fig. 4.7. Concentration-response of N/OFQ(1-13)-NH$_2$ derivatives on NOP-Gz activation. Data are expressed as mean ± sem of at least 3 experiments performed in duplicate.

4.8 Cebranopadol – This molecule could evoke similar maximal effects than N/OFQ, however, with about 100-fold lower potency (**Fig. 8**).

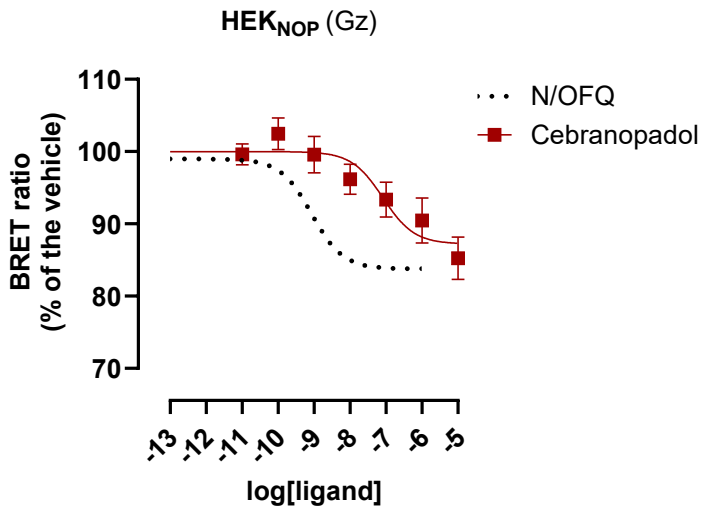


Fig. 4.8. Concentration-response of Cebranopadol on NOP-Gz activation. Data are expressed as mean ± sem of at least 4 experiments performed in duplicate.

4.9 Effects of AT-390, AT-312, AT-090, AT-202, and AT-403 on Gz dissociation by NOP activation – All compounds displayed slightly higher effects than that of N/OFQ although not significantly. AT-403 was the most potent of the series, followed by AT-090, AT-390, AT-202, and AT-312.

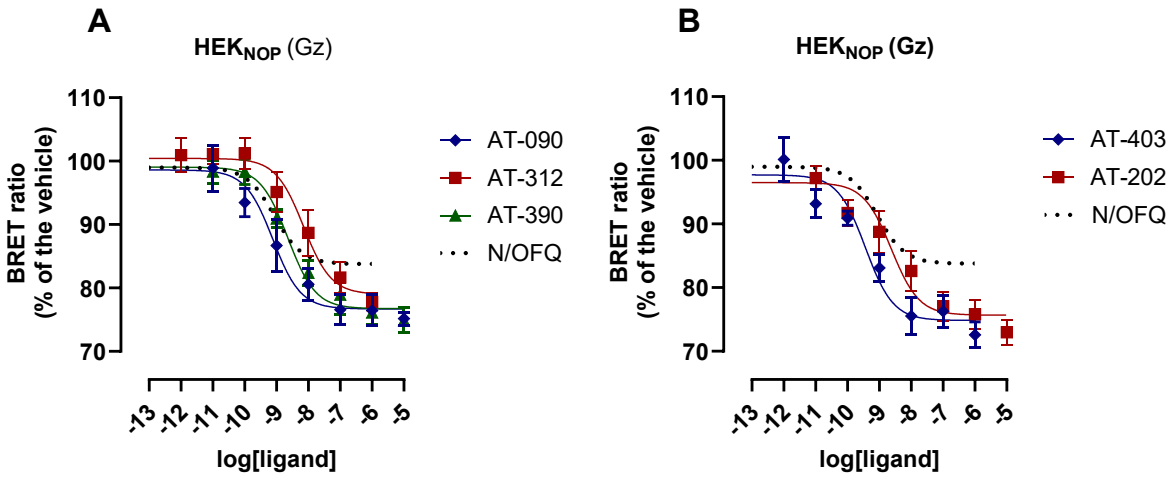


Fig. 4.9. Concentration-response of AT-compounds on NOP-Gz activation. Data are expressed as mean ± sem of at least 5 experiments performed in duplicate.

All data are summed up in **Table 1**. Comparative analysis of all 21 agonists over Gi1, Gi2, Gi3, GoA, GoB, and Gz are presented in **Fig. 10**.

Table 4.1. Pharmacological parameters at NOP receptor Gz activation

	pEC_{50} ($CL_{95\%}$)	**Emax ± sem**	**n**
N/OFQ	9.3 (8.6-10.0)	80.9 ± 1.7	17
UFP-113	8.4 (7.8-9.0)	82.9 ± 7.1	3
UFP-112	8.8 (5.6-12.1)	77.7 ± 1.8	3
PWT2-N/OFQ	8.8 (8.0-9.5)	85.2 ± 2.1	5
Ro 65-6570	8.3 (7.0-9.3)	74.4 ± 1.7	3
SCH-486757	6.8 (6.5-7.1)	83.0 ± 1.2	3
MCOPPB	10.7 (9.1-12.3)	77.0 ± 2.8	3
Ac-RYYRIK-NH_2	8.7 (6.9-10.5)	85.9 ± 3.9	5
Ac-RYYRWK-NH_2	8.1 (6.9-9.2)	86.6 ± 4.0	5
Comp 3	8.5 (5.6-11.5)	83.8 ± 4.8	3
Comp 22	8.5 (4.8-12.2)	84.2 ± 6.2	3
Comp 26	8.1 (4.3-11.8)	84.1 ± 5.2	3
Comp 29	8.5 (4.0-13.1)	84.1 ± 3.6	3
Comp 40	8.7 (7.5-9.9)	80.9 ± 2.1	6
Comp 43	8.5 (4.2-12.7)	84.1 ± 2.1	3
Cebranopadol	7.3 (5.5-9.2)	84.7 ± 3.9	4
AT-090	9.2 (8.4-10.1)	78.3 ± 1.2	5
AT-312	8.3 (7.2-9.4)	78.3 ± 1.2	5
AT-390	8.6 (7.8-9.3)	76.3 ± 1.6	5
AT-202	8.6 (7.4-9.8)	74.9 ± 2.2	5
AT-403	9.4 (7.9-10.8)	74.5 ± 1.9	5

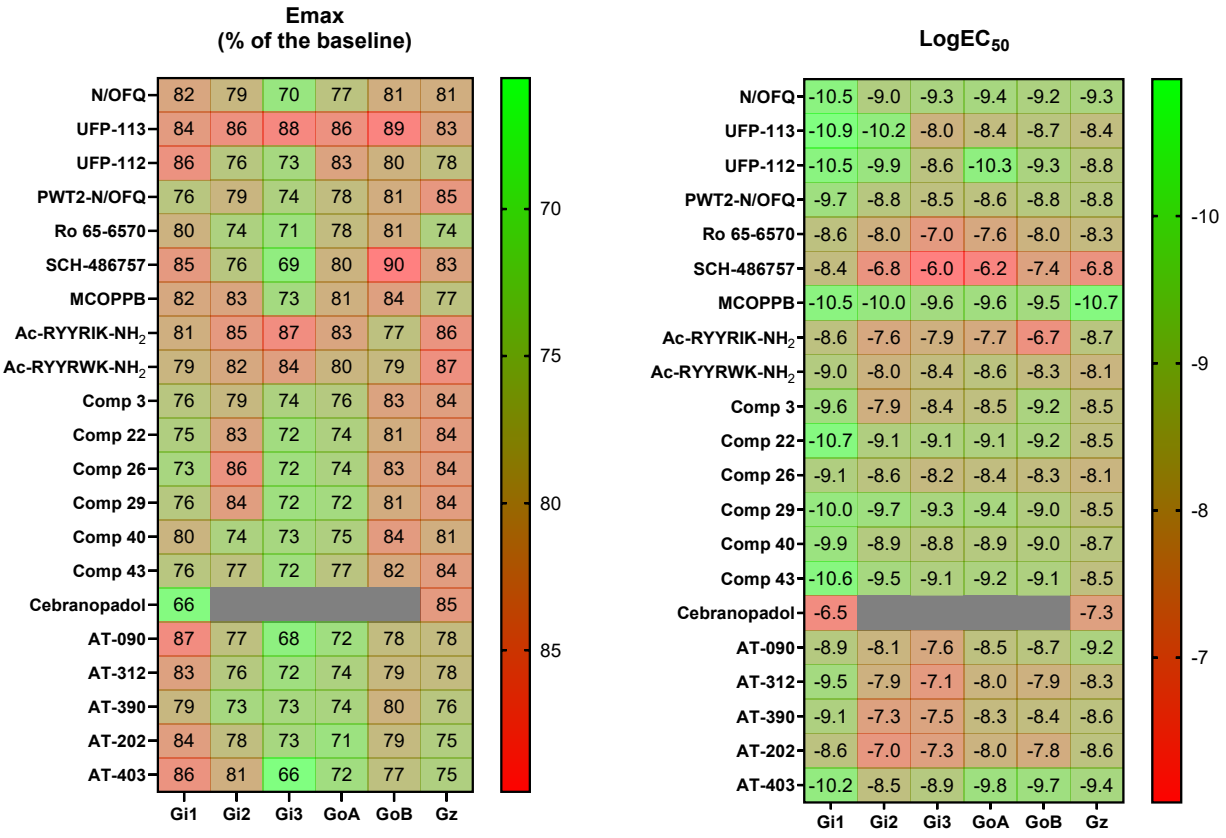


Figure 4.10. Heatmaps of pharmacological effects of NOP agonists at the indicated transducers (red to green, low to high efficacy/potency).

5. Discussion and Conclusions

Previous studies investigated the biased agonism between the interactions NOP-G protein and NOP-βArrestins (Chang et al., 2015; Malfacini et al., 2015; Ferrari et al., 2016; Mann et al., 2019; Azevedo Neto et al., 2021). Overall, such studies always highlighted that NOP agonists were either unbiased or favored G protein recruitment over βArrestins. Without entering the details, this seems to be a hallmark among NOP and the classical opioid receptors. Moreover, given the recognized significance of G proteins in GPCR signaling (Grundmann et al., 2018), we directed our attention toward exploring the potential of biased agonism across G proteins. Our hypothesis rested on the idea that the impact of NOP receptor activation on different G protein subtypes and downstream pathways could vary depending on the chemical diversity of the ligands, mirroring such observations made in the context of G protein versus βArrestins interactions. Investigating the functional selectivity of NOP receptor ligands becomes pivotal in unraveling how this receptor can be finely tuned for therapeutic applications.

The extensive dataset presented in this thesis advances our understanding of NOP receptor pharmacology, specifically focusing on its interaction with various G proteins, with a spotlight on the neuronally relevant inhibitory G protein, Gz. The outcomes of this study contribute (prospectively) to the broader comprehension of the nuanced signaling pathways orchestrated by the NOP receptor and its ligands.

In this thesis, we focused on the NOP-Gα selectivity analysis of N/OFQ and twenty diverse agonists. The endogenous peptide N/OFQ revealed a distinct preference for pertussis toxin-sensitive G proteins (Gi1, Gi2, Gi3, GoA, GoB, previous datasets), including the inhibitory Gz and Gust, and the promiscuous G15, over Gq and Gs, emphasizing the specificity of N/OFQ for modulating inhibitory signaling pathways. These findings align with previous knowledge but extend our understanding to encompass a more comprehensive set of G proteins, laying the foundation for a more detailed map of NOP receptor-mediated signaling cascades. N/OFQ effects on NOP-Gz activation were concentration-dependent, with relatively high maximal effects, and high potency. Overall, synthetic peptides and non-peptide agonists mirrored the endogenous peptide's effects with different potencies not discrepant from literature data.

5.1 Comparison of Gz and Gi1, Gi2, Gi3, GoA, GoB and literature data

Here, as follows, we will enter the details of each compound, comparing our results on NOP-Gz with literature data and the previous evaluation of Gi1, Gi2, Gi3, GoA, and GoB.

Potency values derived for N/OFQ were high (9.0-10.5) for all transducers. Generally, this assay gives high values of potency, higher than that found in the NOP-G protein interaction (8.44), stimulation of GTPγS[35S] binding (8.75), and electrically stimulated mouse Vas Deferens (mVD) (7.47). Of note, in the inhibition of forskolin (FSK)-induced cAMP, values of potency were more similar

to what obtained for the Gα-Gβγ dissociation assay (9.54) (Toll et al., 2016). We might speculate that, despite the relatively poor level of signal amplification of the trupath approach - we measure the activation of G proteins, the very beginning of the GPCR signaling cascade - this assay leads to high potency values for agonists because the extensive protein engineering effort made to achieve high BRET values, could have led to a facilitated Gα-Gβγ dissociation compared to the native conditions, hence, shifting to the left our concentration-response curves.

UFP-113 is a N/OFQ derivative that showed very high potency at Gi1 and Gi2, while lower at GoB, GoA, and Gi3. These compounds on NOP-Gz displayed similar activities as at GoB, GoA, and Gi3 in terms of potency. In terms of efficacy, UFP-113 Emax was always lower than that of N/OFQ, same but much less important trend was observed at Gz. In terms of literature data, UFP-113 is reported as a highly potent and selective NOP receptor partial agonist (Arduin et al., 2007; Malfacini et al., 2015; Toll et al., 2016). UFP-112 was synthesized through a series of chemical modifications to the N/OFQ core, resulting in enhanced affinity/potency for the NOP receptor and increased resistance to enzymatic degradation (Arduin et al., 2007; Rizzi et al., 2007). This compound behaved as a full agonist. Potency values were slightly higher/equal than that of N/OFQ at all transducers apart Gi3 and Gz. Literature data showed high values of potency in the stimulation of GTPγS[35S] binding (10.55) and in the inhibition of FSK-stimulated cAMP levels (10.34) (Calo' et al., 2011), being in general more potent than N/OFQ. PWT2-N/OFQ is a tetrabranched derivative of N/OFQ (Guerrini et al., 2014; Rizzi et al., 2014), described as NOP receptor selective agonist with potent and prolonged antinociceptive effects (Rizzi et al., 2014). Here, we confirmed PWT2-N/OFQ as full agonist, but we also observed a slightly decreased potency in all transducers compared to N/OFQ.

As far as potency is concerned, the non-peptide Ro 65-6570 showed the following rank order: Gi1 ≥ Gz ≥ Gi2 = GoB > GoA > Gi3, being always less potent than N/OFQ. What we show here is overall in line with that obtained in the cAMP inhibition assay by (Hashiba et al., 2001) (Ro 65-6570 8.7 and N/OFQ 9.6), but also NOP-G protein (Ro 65-6570 7.8 and N/OFQ 8.4) (Malfacini et al., 2015). SCH-486757 is as well a non-peptide agonist, this compound was clinically evaluated for the treatment of cough (McLeod et al., 2010). Unfortunately (at least for the treatment of cough (Morairty et al., 2023)), this molecule development was discontinued because accompanied by sedation effects. On the NOP-trupath, SCH-486757 potency was low, ranking Gi1 >> GoB > Gi2 = Gz > GoA ≥ Gi3, being approximately always 100-fold less potent than N/OFQ. In the stimulated GTPγ[35S] binding, in the calcium mobilization, in the NOP-G protein, in the NOP-βArrestin, and in the electrically stimulated mVD assays (Ferrari et al., 2017); the compound's potency was always lower than that of N/OFQ. MCOPPB is a highly potent non-peptide selective NOP receptor agonist. Here, the rank order of potency was Gz ≥Gi1 > Gi2 > Gi3 ≥ GoA ≥ GoB. In addition, MCOPPB was consistently more potent than N/OFQ on all paradigms here described. In line, Ferrari and co-workers investigated the effects

of MCOPPB in several assays (including stimulated GTPγS[35S] binding, calcium mobilization with chimeric G proteins, and NOP-G protein interaction) achieving a pharmacological profile close to that obtained by trupath (Ferrari et al., 2017).

Compounds Ac-RYYRIK-NH2 and Ac-RYYRWK-NH2 are hexapeptides coming from a pivotal combinatorial chemistry study (Dooley et al., 1997). Both displayed moderate/high potency values at all transducers with the latter being almost always, more potent than Ac-RYYRIK-NH2 (Gi1, Gi2, Gi3, GoA, GoB). From literature data, Ac-RYYRIK-NH2 showed in the stimulation of GTPγS[35S] binding (8.2) and inhibition of cAMP levels (7.9) moderate values of potency (Mason et al., 2001), in accordance with our results. Ac-RYYRWK-NH2 was described as a more potent agonist at the GTPγS[35S] binding (9.1), and at the inhibition of cAMP levels (8.7) (Mason et al., 2001). This is true for all transducers but Gz, where we observed a potency reversal. Nevertheless, caution must be paid in drawing conclusion from this point, in fact, potency values measured for both peptides at Gz are not significantly differing (overlapping confidence limits).

Comp 3, Comp 22, Comp 26, Comp 29, Comp 40, and Comp 43 were designed by Pacifico and co-workers (Pacifico et al., 2020) on the core of N/OFQ(1-13)-NH_2. Literature data is present solely in the NOP-G protein and NOP-βArrestin 2 interaction assays; here, based on divergence between G protein and βArrestin2 recruitment, we chose to evaluate these six molecules on the trupath approach. Very unexpectedly, in this assay compounds effects were similar to that of N/OFQ with no significant evidence for transducer selectivity.

Cebranopadol is a non-peptide molecule under clinical development for treating pain. Previous reports on animal models of pain highlighted a synergistic anti-nociceptive effect of NOP and mu receptors stimulation without increase side effects (Hu et al., 2010). Cebranopadol can activate NOP and mu receptors with similar potency (Linz et al., 2014), therefore, taking advantage of the NOP-mu synergism of action for eliciting a pronounced anti-nociceptive effect with few side effects. In the NOP-trupath approach, Cebranopadol was active only on Gz > Gi1. This is quite surprising, in several reports Cebranopadol was of similar potency as N/OFQ. However, further evaluation of Cebranopadol kinetics on NOP-trupath might underline a slow onset of action, leading to underestimation of its potency, in line with what described by Rizzi and co-workers (Rizzi et al., 2016). Nevertheless, the quite high potency of Cebranopadol at NOP-Gz might be reminiscent of some sort of G protein bias to be furtherly addressed.

Finally, we evaluated the non-peptide small molecules by Dr. Zaveri: AT-390, AT-312, AT-090, AT-202, and AT-403. All such molecules but AT-403 were of equivalent / lower potency than N/OFQ. In

terms of transducer specificity, we did not observe significant bias within G proteins. AT-403 potency was in very high, in line with literature data (Ferrari et al., 2017).

5.2 Conclusions and perspectives

In this work, even with a heightened expression of the NOP receptor in HEK293 cells, the signal-to-noise ratio remained surprisingly low, potentially attributed to the transient nature of transduced G proteins. Given the challenge of establishing stable cell lines with all G protein components and the receptor due to multiple antibiotic resistance enzymes, a recent breakthrough involving an IRES-based construct incorporating all G protein components in a single entity presents a promising avenue for upcoming transduceromic studies. However, it is noteworthy that results obtained with the trupath assay might be influenced by extensive protein engineering efforts aimed at enhancing BRET coupling efficiency, highlighting the need for alternative approaches capable of studying biased agonism at physiological receptor and transducer levels.

In this extensive comparative study involving 21 NOP agonists, we examined their effects on the most relevant coupling G proteins of the NOP receptor (Masuho et al., 2023). Leveraging the Gα-Gβγ dissociation assay (trupath), our findings demonstrate its efficacy in discriminating potencies among molecules, a trend consistent with previous reports (Knight et al., 2021; Lieb et al., 2021; Jiang et al., 2022; Singh et al., 2022; Voss et al., 2022; Glenn et al., 2023). Intriguingly, despite the chemical diversity and pharmacological variances among tested compounds, none exhibited a profound discrepancy among transducers when compared to N/OFQ. This observation suggests a potential limitation in the diversity of conformational changes elicited at the NOP receptor, prompting future endeavors in innovative structure-based drug design to uncover novel pharmacophores capable of selectively activating a subset of G proteins.

Speculatively, the NOP receptor, distinct from some highly bias-prone GPCRs, seems adept at channeling the perturbations induced by its activation toward Gαi1, Gαi2, Gαi3, GαoA, GαoB, and Gz facilitating a parallel and unbiased activation of these transducers. This leads us to contemplate that allosteric modulation of the NOP receptor could differentially trigger such transducers. In essence, our study not only unveils the intricate pharmacological profile of the human NOP receptor but also hints at potential avenues for innovative drug design targeting specific G proteins, fostering a more comprehensive understanding of biased agonism in NOP receptor signaling.

In summary, our study did not reveal an obvious reversal in the behavior of specific compounds across transducers (*no significative "biased agonism"*). The interpretation of this 'discovery' is complex. The findings presented herein, while acknowledging its complexity, contribute to unraveling the intricate landscape of NOP receptor pharmacology. The dataset strongly advocates for the adoption of entirely innovative pharmacophores. This will be crucial for achieving a more profound understanding of NOP signaling, addressing existing limitations, and paving the way for therapeutic advancements in various medical areas.

6. References

Altarifi, A.A., David, B., Muchhala, K.H., Blough, B.E., Akbarali, H., and Negus, S.S. (2017). Effects of acute and repeated treatment with the biased mu opioid receptor agonist TRV130 (oliceridine) on measures of antinociception, gastrointestinal function, and abuse liability in rodents. J Psychopharmacol 31: 730–739.

Arcuri, L., Novello, S., Frassineti, M., Mercatelli, D., Pisanò, C.A., Morella, I., et al. (2018). Anti-Parkinsonian and anti-dyskinetic profiles of two novel potent and selective nociceptin/orphanin FQ receptor agonists. Br J Pharmacol 175: 782–796.

Arduin, M., Spagnolo, B., Calò, G., Guerrini, R., Carrà, G., Fischetti, C., et al. (2007). Synthesis and biological activity of nociceptin/orphanin FQ analogues substituted in position 7 or 11 with Cα,α-dialkylated amino acids. Bioorg Med Chem 15: 4434–4443.

Azevedo Neto, J., Ruzza, C., Sturaro, C., Malfacini, D., Pacifico, S., Zaveri, N.T., et al. (2021). Functional Selectivity Does Not Predict Antinociceptive/Locomotor Impairing Potencies of NOP Receptor Agonists. Front Neurosci 15: 657153.

Birnbaumer, L. (2007). The discovery of signal transduction by G proteins: a personal account and an overview of the initial findings and contributions that led to our present understanding. Biochim Biophys Acta 1768: 756–771.

Burghi, V., Paradis, J.S., Officer, A., Adame-Garcia, S.R., Wu, X., Matthees, E.S.F., et al. (2023). Gαs is dispensable for β-arrestin coupling but dictates GRK selectivity and is predominant for gene expression regulation by β2-adrenergic receptor. Journal of Biological Chemistry 299:.

Calo', G., Rizzi, A., Cifani, C., Micioni Di Bonaventura, M.V., Regoli, D., Massi, M., et al. (2011). UFP-112 a potent and long-lasting agonist selective for the Nociceptin/Orphanin FQ receptor. CNS Neurosci Ther 17: 178–198.

Cerlesi, M.C., Ding, H., Bird, M.F., Kiguchi, N., Ferrari, F., Malfacini, D., et al. (2017). Pharmacological studies on the NOP and opioid receptor agonist PWT2-[Dmt1]N/OFQ(1-13). Eur J Pharmacol 794: 115–126.

Chang, S.D., Brieaddy, L.E., Harvey, J.D., Lewin, A.H., Mascarella, S.W., Seltzman, H.H., et al. (2015). Novel Synthesis and Pharmacological Characterization of NOP Receptor Agonist 8-[(1S,3aS)-2,3,3a,4,5,6-Hexahydro-1H-phenalen-1-yl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one (Ro 64-6198). ACS Chem Neurosci 6: 1956–1964.

Ciccocioppo, R., Borruto, A.M., Domi, A., Teshima, K., Cannella, N., and Weiss, F. (2019). NOP-Related Mechanisms in Substance Use Disorders. Handb Exp Pharmacol 254: 187–212.

Cippitelli, A., Schoch, J., Debevec, G., Brunori, G., Zaveri, N.T., and Toll, L. (2016). A key role for the N/OFQ-NOP receptor system in modulating nicotine taking in a model of nicotine and alcohol co-administration. Sci Rep 6:.

Connor, M., and Christie, M.J. (1999). OPIOID RECEPTOR SIGNALLING MECHANISMS. Clin Exp Pharmacol Physiol 26: 493–499.

Dooley, C.T., Spaeth, C.G., Berzetei-Gurske, I.P., Craymer, K., Adapa, I.D., Brandt, S.R., et al. (1997). Binding and In Vitro Activities of Peptides with High Affinity for the Nociceptin/Orphanin FQ Receptor, ORL1 1.

Ferrari, F., Cerlesi, M.C., Malfacini, D., Asth, L., Gavioli, E.C., Journigan, B. V., et al. (2016). In vitro functional characterization of novel nociceptin/orphanin FQ receptor agonists in recombinant and native preparations. Eur J Pharmacol 793: 1–13.

Ferrari, F., Malfacini, D., Journigan, B. V., Bird, M.F., Trapella, C., Guerrini, R., et al. (2017). In vitro pharmacological characterization of a novel unbiased NOP receptor-selective nonpeptide agonist AT-403. Pharmacol Res Perspect 5:.

Glenn, N.A.K., Finlay, D.B., Carruthers, E.R., Mountjoy, K.G., Walker, C.S., and Grimsey, N.L. (2023). RAMP and MRAP accessory proteins have selective effects on expression and signalling of the CB1 , CB2 , GPR18 and GPR55 cannabinoid receptors. Br J Pharmacol.

Grundmann, M., Merten, N., Malfacini, D., Inoue, A., Preis, P., Simon, K., et al. (2018). Lack of beta-arrestin signaling in the absence of active G proteins. Nat Commun 9:.

Guerrini, R., Marzola, E., Trapella, C., Pela', M., Molinari, S., Cerlesi, M.C., et al. (2014). A novel and facile synthesis of tetra branched derivatives of nociceptin/orphanin FQ. Bioorg Med Chem 22: 3703–3712.

Hauser, A.S., Attwood, M.M., Rask-Andersen, M., Schiöth, H.B., and Gloriam, D.E. (2017). Trends in GPCR drug discovery: new agents, targets and indications. Nat Rev Drug Discov 16: 829–842.

Hauser, A.S., Avet, C., Normand, C., Mancini, A., Inoue, A., Bouvier, M., et al. (2022). Common coupling map advances GPCR-G protein selectivity. Elife 11:.

Hirao, A., Imai, A., Sugie, Y., Yamada, Y., Hayashi, S., and Toide, K. (2008). Pharmacological characterization of the newly synthesized nociceptin/orphanin FQ-receptor agonist 1-[1-(1-methylcyclooctyl)-4-piperidinyl]-2-[(3R)-3-piperidinyl]-1H-benzimidazole as an anxiolytic agent. J Pharmacol Sci 106: 361–368.

Hu, E., Calò, G., Guerrini, R., and Ko, M.C. (2010). Long-lasting antinociceptive spinal effects in primates of the novel nociceptin/orphanin FQ receptor agonist UFP-112. Pain 148: 107–113.

Inoue, A., Raimondi, F., Kadji, F.M.N., Singh, G., Kishi, T., Uwamizu, A., et al. (2019). Illuminating G-Protein-Coupling Selectivity of GPCRs. Cell 177: 1933-1947.e25.

Jiang, Y., Yeasmin, M., Gondin, A.B., Christopoulos, A., Valant, C., Burger, W.A.C., et al. (2022). Importance of receptor expression in the classification of novel ligands at the M2 muscarinic acetylcholine receptor. Br J Pharmacol.

Knight, K.M., Ghosh, S., Campbell, S.L., Lefevre, T.J., Olsen, R.H.J., Smrcka, A. V., et al. (2021). A universal allosteric mechanism for G protein activation. Mol Cell 81: 1384-1396.e6.

Kolb, P., Kenakin, T., Alexander, S.P.H., Bermudez, M., Bohn, L.M., Breinholt, C.S., et al. (2022). Community guidelines for GPCR ligand bias: IUPHAR review 32. Br J Pharmacol 179: 3651–3674.

Kozasa, T., Hepler, J.R., Smrcka, A. V., Simon, M.I., Rhee, S.G., Sternweis, P.C., et al. (1993). Purification and characterization of recombinant G16α from Sf9 cells: Activation of purified phospholipase C isozymes by G-protein α subunits. Proc Natl Acad Sci U S A 90: 9176–9180.

Lambert, D.G. (2008). The nociceptin/orphanin FQ receptor: A target with broad therapeutic potential (Nat Rev Drug Discov).

Lefkowitz, R.J. (2007). Seven transmembrane receptors: something old, something new. Acta Physiol (Oxf) 190: 9–19.

Lieb, A., Thaler, G., Fogli, B., Trovato, O., Posch, M.A., Kaserer, T., et al. (2021). Functional Characterization of Spinocerebellar Ataxia Associated Dynorphin A Mutant Peptides. Biomedicines 9:.

Linz, K., Christoph, T., Tzschentke, T.M., Koch, T., Schiene, K., Gautrois, M., et al. (2014). Cebranopadol: a novel potent analgesic nociceptin/orphanin FQ peptide and opioid receptor agonist. J Pharmacol Exp Ther 349: 535–548.

Lippert, E., Baltensperger, K., Jacques, Y., and Hermouet, S. (1997). Gα16 protein expression is up- and down-regulated following T-cell activation: Disruption of this regulation impairs activation-induced cell responses. FEBS Lett 417: 292–296.

Malfacini, D., Ambrosio, C., Gro', M.C., Sbraccia, M., Trapella, C., Guerrini, R., et al. (2015). Pharmacological profile of nociceptin/orphanin FQ receptors interacting with G-proteins and β-arrestins 2. PLoS One 10:.

Malfacini, D., Patt, J., Annala, S., Harpsøe, K., Eryilmaz, F., Reher, R., et al. (2019). Rational design of a heterotrimeric G protein subunit with artificial inhibitor sensitivity. Journal of Biological Chemistry 294: 5747–5758.

Mann, A., Mouledous, L., Froment, C., O'neill, P.R., Dasgupta, P., Gunther, T., et al. (2019). Agonist-selective NOP receptor phosphorylation correlates in vitro and in vivo and reveals differential post-activation signaling by chemically diverse agonists. Sci Signal 12:.

Mason, S.L., Ho, M., Nicholson, J., and McKnight, A.T. (2001). In vitro characterization of Ac-RYYRWK-NH2, Ac-RYYRIK-NH2 and [Phe1CΨ(CH2-NH)Gly2] nociceptin(1-13)NH2 at rat native and recombinant ORL1 receptors. Neuropeptides 35: 244–256.

Masuho, I., Kise, R., Gainza, P., Moo, E. Von, Li, X., Tany, R., et al. (2023). Rules and mechanisms governing G protein coupling selectivity of GPCRs. Cell Rep 42:.

McLeod, R.L., Tulshian, D.B., Bolser, D.C., Varty, G.B., Baptista, M., Fernandez, X., et al. (2010). Pharmacological profile of the NOP agonist and cough suppressing agent SCH 486757 (8-[Bis(2-Chlorophenyl)Methyl]-3-(2-Pyrimidinyl)-8-Azabicyclo[3.2.1]Octan-3-Ol) in preclinical models. Eur J Pharmacol 630: 112–120.

Mende, F., Hundahl, C., Plouffe, B., Skov, L.J., Sivertsen, B., Madsen, A.N., et al. (2018). Translating biased signaling in the ghrelin receptor system into differential in vivo functions. Proc Natl Acad Sci U S A 115: E10255–E10264.

Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., et al. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. Nature 377: 532–535.

Miller, R.L., Thompson, A.A., Trapella, C., Guerrini, R., Malfacini, D., Patel, N., et al. (2015). The Importance of Ligand-Receptor Conformational Pairs in Stabilization: Spotlight on the N/OFQ G Protein-Coupled Receptor. Structure 23: 2291–2299.

Milligan, G., and Kostenis, E. (2006). Heterotrimeric G-proteins: a short history. Br J Pharmacol 147 Suppl 1:

Molinari, S., Camarda, V., Rizzi, A., Marzola, G., Salvadori, S., Marzola, E., et al. (2013). N/OFQ(1-13)-NH2 a universal opioid agonist. Br J Pharmacol 168: 151–162.

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., et al. (1994). ORL1, a novel member of the opioid receptor family. Cloning, functional expression and localization. FEBS Lett 341: 33–38.

Morairty, S.R., Sun, Y., Toll, L., Bruchas, M.R., and Kilduff, T.S. (2023). Activation of the nociceptin/orphanin-FQ receptor promotes NREM sleep and EEG slow wave activity. Proc Natl Acad Sci U S A 120:.

Neves, S.R., Ram, P.T., and Iyengar, R. (2002). G protein pathways. Science 296: 1636–1639.

Oldham, W.M., and Hamm, H.E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol 9: 60–71.

Olsen, R.H.J., DiBerto, J.F., English, J.G., Glaudin, A.M., Krumm, B.E., Slocum, S.T., et al. (2020). TRUPATH, an open-source biosensor platform for interrogating the GPCR transducerome. Nat Chem Biol 16: 841–849.

Olsen, R.H.J., and English, J.G. (2023). Advancements in G protein-coupled receptor biosensors to study GPCR-G protein coupling. Br J Pharmacol 180: 1433–1443.

Pacifico, S., Ferrari, F., Albanese, V., Marzola, E., Neto, J.A., Ruzza, C., et al. (2020). Biased Agonism at Nociceptin/Orphanin FQ Receptors: A Structure Activity Study on N/OFQ(1-13)-NH2. J Med Chem 63: 10782–10795.

Preti, D., Caló, G., and Guerrini, R. (2019). NOP-targeted peptide ligands. Handb Exp Pharmacol 254: 17–36.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., et al. (1995). Orphanin FQ: A neuropeptide that activates an opioidlike G protein-coupled receptor. Science (1979) 270: 792–794.

Rizzi, A., Cerlesi, M.C., Ruzza, C., Malfacini, D., Ferrari, F., Bianco, S., et al. (2016). Pharmacological characterization of cebranopadol a novel analgesic acting as mixed nociceptin/orphanin FQ and opioid receptor agonist. Pharmacol Res Perspect 4: e00247.

Rizzi, A., Malfacini, D., Cerlesi, M.C., Ruzza, C., Marzola, E., Bird, M.F., et al. (2014). In vitro and in vivo pharmacological characterization of nociceptin/orphanin FQ tetrabranched derivatives. Br J Pharmacol 171: 4138–4153.

Rizzi, A., Spagnolo, B., Wainford, R.D., Fischetti, C., Guerrini, R., Marzola, G., et al. (2007). In vitro and in vivo studies on UFP-112, a novel potent and long lasting agonist selective for the nociceptin/orphanin FQ receptor. Peptides (N.Y.) 28: 1240–1251.

Rizzi, A., Sukhtankar, D.D., Ding, H., Hayashida, K., Ruzza, C., Guerrini, R., et al. (2015). Spinal antinociceptive effects of the novel NOP receptor agonist PWT2-nociceptin/orphanin FQ in mice and monkeys. Br J Pharmacol 172: 3661–3670.

Rosenbaum, D.M., Rasmussen, S.G.F., and Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. Nature 459: 356–363.

Schrage, R., Schmitz, A.L., Gaffal, E., Annala, S., Kehraus, S., Wenzel, D., et al. (2015). The experimental power of FR900359 to study Gq-regulated biological processes. Nat Commun 6:.

Singh, K., Senatorov, I.S., Cheshmehkani, A., Karmokar, P.F., and Moniri, N.H. (2022). The Skeletal Muscle Relaxer Cyclobenzaprine Is a Potent Non-Competitive Antagonist of Histamine H1 Receptors. J Pharmacol Exp Ther 380: 202–209.

Smith, J.S., and Rajagopal, S. (2016). The β-Arrestins: Multifunctional Regulators of G Protein-coupled Receptors. J Biol Chem 291: 8969–8977.

Spielman, A.I. (1998). Gustducin and its role in taste. J Dent Res 77: 539–544.

Swift, S., Sheridan, P.J., Covic, L., and Kuliopulos, A. (2000). PAR1 thrombin receptor-G protein interactions: Separation of binding and coupling determinants in the Gα subunit. Journal of Biological Chemistry 275: 2627–2635.

Thompson, A.A., Liu, W., Chun, E., Katritch, V., Wu, H., Vardy, E., et al. (2012). Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. Nature 485: 395–399.

Töle, J.C., Behrens, M., and Meyerhof, W. (2019). Taste receptor function. Handb Clin Neurol 164: 173–185.

Toll, L., Bruchas, M.R., Calo', G., Cox, B.M., and Zaveri, N.T. (2016). Nociceptin/orphanin FQ receptor structure, signaling, ligands, functions, and interactions with opioid systems. Pharmacol Rev 68: 419–457.

Ubaldi, M., Cannella, N., Borruto, A.M., Petrella, M., Micioni Di Bonaventura, M.V., Soverchia, L., et al. (2021). Role of nociceptin/orphanin FQ-NOP receptor system in the regulation of stress-related disorders. Int J Mol Sci 22:.

Voss, J.H., Mahardhika, A.B., Inoue, A., and Müller, C.E. (2022). Agonist-Dependent Coupling of the Promiscuous Adenosine A2BReceptor to Gα Protein Subunits. ACS Pharmacol Transl Sci 5:.

Wang, Y., Zhuang, Y., DiBerto, J.F., Zhou, X.E., Schmitz, G.P., Yuan, Q., et al. (2023). Structures of the entire human opioid receptor family. Cell 186: 413-427.e17.

Wettschureck, N., Moers, A., and Offermanns, S. (2004). Mouse models to study G-protein-mediated signaling. Pharmacol Ther 101: 75–89.

Wettschureck, N., and Offermanns, S. (2005). Mammalian G proteins and their cell type specific functions. Physiol Rev 85: 1159–1204.

Whiteside, G.T., Kyle, D.J., Kapil, R.P., Cipriano, A., He, E., Zhou, M., et al. (2023). The nociceptin/orphanin-FQ receptor partial agonist sunobinop promotes non-REM sleep in rodents and patients with insomnia. J Clin Invest.

Wichmann, J., Adam, G., Röver, S., Cesura, A.M., Dautzenberg, F.M., and Jenck, F. (1999). 8-acenaphthen-1-yl-1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one derivatives as orphanin FQ receptor agonists. Bioorg Med Chem Lett 9: 2343–2348.

Yang, J., Wu, J., Kowalska, A.M., Dalvi, A., Prevost, N., O'Brien, P.J., et al. (2000). Loss of signaling through the G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci U S A 97: 9984–9989.

Zaveri, N.T., Marquez, P. V., Meyer, M.E., Polgar, W.E., Hamid, A., and Lutfy, K. (2018). A Novel and Selective Nociceptin Receptor (NOP) Agonist (1-(1-((cis)-4-isopropylcyclohexyl)piperidin-4-yl)-1H-indol-2-yl)methanol (AT-312) Decreases Acquisition of Ethanol-Induced Conditioned Place Preference in Mice. Alcohol Clin Exp Res 42: 461–471.