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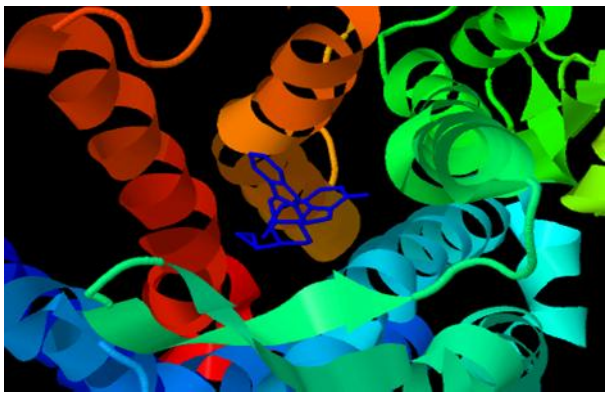
The mechanism of constitutive activity in δ and μ opioid receptors



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1 INTRODUCTION

1.1 THE G PROTEIN-COUPLED RECEPTORS (GPCRS)

Multicellular organisms need a finely controlled system of communication among individual cells. Every cell receives information in the form of multiple extracellular signals, such as neurotransmitters, hormones, growth factors and other molecules, including drugs. Many of these signaling molecules do not need to penetrate into the cells to produce effects, because they can interact with receptors located on the extracellular side of cell membranes, and the signal can be transduced, amplified and converted to the final physiological effect (Cabrera-Vera et al., 2003). Many types of receptors exist, but more than 80% of extracellular signals mediate their effects by binding to GPCRs, which represent the 1-5% of total proteins. About the 1% of the entire human genome encodes GPCRs and these receptors are the target of many types of drugs (Birnbaumer *et al.*, 1990).

Endogenous ligands are known for more than 200 GPCRs, but the studies about the human genome suggest that there are between 800 and 1000 genes codifying for GPCRs (Hill, 2006). Excluding the olfactory types, there are about 100 orphan GPCRs, that may represent future targets in the development of new drugs (Chung *et al.*, 2008).

1.1.1 GPCR families

According to gene sequences, GPCRs are classified into five main large families, named *Glutamate* (Class C), *Rhodopsin* (Class A), *Adhesion* (Class B), *Frizzled* (Class F) and *Secretin* (Class B), according to the GRAFS classification (Fredriksson *et al.*, 2003). Every family includes receptors that share similar characteristics, but there are common characteristics also among different families, indicating that all GPCRs were derived from a common ancestral gene (Krishnan *et al.*, 2012).

The *Glutamate* family consists of 22 members in humans. Most of these receptors have long and bilobate N-termini called “Venus flytraps”, which

enclose the endogenous ligand binding region. Furthermore, the N-terminal region of the majority of human receptors is also characterized by the presence of a cysteine-rich domain (CRD or NCD3G). The family includes glutamate metabotropic receptors (GRM), calcium-sensing like receptors (CASR), taste receptors (TAS1) and some orphan GPCRs (Kunishima *et al.*, 2000).

The *Rhodopsin* family constitutes the largest family of GPCRs in vertebrates, enclosing 683 members in humans (e.g., adrenoceptors, opioid receptors and histamine receptors). Members of this family are further classified into four main groups, termed α-, β-, γ-, and δ-group, and 13 major subfamilies.

These GPCRs are typically characterized by short N-termini and can interact with a broad variety of ligands (e.g., amine, peptide, hormone protein) (Lagerstrom and Schioth, 2008).

The *Adhesion* family is the second largest according to the GRAFS GPCR classification, with 33 members in human (e.g., CD97 antigen receptor). This family is characterized by long N-terminal sequences which contain multiple functional domains and display numerous glycosylation sites (Lagerstrom and Schioth, 2008).

The *Frizzled* family of GPCRs consists of 10 receptors (FZD1–10) in humans. They are the receptors for the Wnt proteins and play a key role in tissue polarity and cell signaling. The family members are characterized by the CRD_FZ domain or FZ domain which has 10 conserved cysteine residues (Dann *et al.*, 2001).

The *Secretin* family seems to have been evolved from the *Adhesion* family and consists of 15 members in human (e.g., the corticotropin-releasing hormone receptor, CRHR). This family members are characterized by an N-terminal domain of 60-80 amino acids, containing conserved cysteine residues. This family includes receptors for ligands such as glucagon, growth hormone-releasing hormone, parathyroid hormone, secretin and other paracrine peptides (Nordstrom *et al.*, 2009).

1.1.2 The molecular anatomy of a GPCR

GPCRs are modular proteins essentially consisting of 3 fundamental regions: an extracellular exposed N-terminus, a membrane-embedded bundle of seven transmembrane helices and an intracellular C-terminus (Hamm, 1998). Major post-translational modifications occur at the N-terminus of many GPCRs that exhibit a variable number of glycosylation sites, and at the C-terminus where highly conserved cysteine residues can be palmitoylated. These post-translational modifications can influence the structure and the function of GPCRs (Qanbar and Bouvier, 2003).

The seven transmembrane helices (TM1 – TM7), consisting of 20-28 hydrophobic amino acids, are connected by three extracellular (ECL1-ECL3) and three intracellular (ICL1-ICL3) loops. GPCRs interact with G proteins through ICL2, ICL3 and the C-terminal. The extracellular loops, the N-terminal regions and the residues exposed to the inner cavity formed by the extracellular half of the transmembrane bundle, are all involved to varying degrees in ligand recognition and binding (Kobilka, 2007).

This molecular organization, originally deduced by a clever cooperation between molecular modeling and experimental site-directed mutagenesis work (Fanelli and De Benedetti, 2011), has been more recently confirmed by the X-ray crystallography analysis of several GPCRs, such as rhodopsin (Palczewski *et al.*, 2000), opsin (Scheerer *et al.*, 2008), β_1 and β_2 adrenoceptors (Warne *et al.*, 2008; Rasmussen *et al.*, 2011a; Rosenbaum *et al.*, 2011), δ, μ and κ opioid receptors (Granier *et al.*, 2012; Manglik *et al.*, 2012; Wu *et al.*, 2012).

1.1.3 Signal transduction

The consequence of ligand binding to a GPCR is the activation of the associated heterotrimeric G protein. G proteins consist of α-, β- and γ-subunits. The human genome encodes 16 Gα, 5 Gβ and 14 Gγ subunits. G proteins are divided into four families, according to the differences in signal transduction of the subunit: G_s, $G_{i/o}$, G_q and G_{12} (Milligan and Kostenis, 2006). The interaction between ligand and receptor induces a conformational change in the receptor that leads to an increased affinity for one or more

specific Gα subunits. Consequently, the Gα-subunit switches from the inactive state, tightly bound to GDP, to the active state, which has low affinity for guanine nucleotides. This favors exchange of GTP for GDP and dissociation of the Gβγ complex (Oldham and Hamm, 2006). The intrinsic GTPase activity of the α-subunit reconverts GTP in GDP. Thus, the enzymatic activity of the heterotrimeric G proteins is a balance between the rate of exchange GDP/GTP and the rate of hydrolysis of GTP (Higashijima *et al.*, 1987).

1.1.3.1 G protein pathways

Many different effector pathways are activated or inhibited through this common mechanism.

For example, $G\alpha_s$ and $G\alpha_{i/o}$ can control, respectively, the stimulation and the inhibition of adenylyl cyclase, which catalyzes the formation of cyclic AMP (cAMP) from ATP (Gilman, 1995). The cAMP acts as an intracellular second messenger, which mediates the activation of PKA, a serine/threonine protein kinase. PKA consists of one regulatory and one catalytic subunit, which form an inactive complex in absence of cAMP. Enhanced cAMP levels bind to the regulatory subunit, thus causing subunits dissociation and liberation of the catalytically active kinase, which can phosphorylate several specific substrates (Taussig and Gilman, 1995). In addition to phosphorylate important regulatory proteins of the cytosol, PKA can translocate into the nucleus, where it controls gene transcription by activation of transcription factors, such as CREB (cAMP responding element binding protein) (Delghandi *et al.*, 2005). Therefore, cAMP controls a great number of cellular processes, including metabolic reactions, ion channels activity and gene transcription. The cyclic nucleotide can also directly bind to cationic membrane channels, inducing a flow of Na^+ and Ca^{2+} inside the cells (Neves *et al.*, 2002).

The cAMP system is also subjected to several mechanisms of inactivation. cAMP itself is rapidly degraded by powerful phosphodiesterases that hydrolyze the nucleotide into 5'-AMP. Moreover, PKA activity can be interrupted by protein phosphatases (Gilman, 1987).

Another important intracellular pathway under control of G proteins is Ca^{2+} signaling. This is the main route of action of the $G\alpha_q/G\alpha_{11}$ group of G proteins. They activate phospholipase C, which catalyzes the hydrolysis of the phospholipid phosphatidylinositol-diphosphate (PIP_2), thus generating two second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG) (Strathmann and Simon, 1990). IP_3 promotes on binding to a specific intracellular channel the transient enhancement of cytosolic levels of calcium; DAG activates specific serine/threonine protein kinases (PKC) (Taylor *et al.*, 1990).

Other Gα-subunits, such as $G\alpha_{12}/G\alpha_{13}$, are implicated in the regulation of Ras homology (Rho) guanine nucleotide exchange factors (RhoGEFs), a monomeric protein which induces the activation of a specific protein kinase (ROCKS). This pathway is involved in many cellular processes, including the progression of cellular cycle, cytoskeletal remodeling and chemotaxis (Riobo and Manning, 2005).

In all activated heterotrimeric G protein, regardless of the type of intracellular signaling in which they are involved, the GTPase activity of the α-subunit is considered a major mechanism of signal quenching, since it favors the stabilization of the GDP-bound inactive form. This GTP hydrolysis can be regulated by additional proteins, such as RGS (Regulators of G-protein Signaling) which interact directly with the Gα-subunits and other components of the signal pathway (Ross and Wilkie, 2000).

1.1.3.2 The role of the Gβγ subunit

Also the Gβγ subunits are involved in the regulation of a broad variety of signal transduction pathways upon their release. For example, they activate some isoforms of PLC and adenylyl cyclase, they control the MAP-kinase cascade, the activation of specific chloride channels and the inhibition of calcium channels (Angermann *et al.*, 2006; Diel *et al.*, 2006; Ohori *et al.*, 2007). Gβγ subunits are also involved in processes of endocytosis and desensitization of GPCRs, because they can bind specific G protein receptors kinases (GRK), thus assisting receptor phosphorylation, which is a fundamental step for the regulation of arrestin-mediated rapid receptor endocytosis (Tesmer *et al.*, 2005) (see below). The signals mediated via Gβγ

subunits seem to play a particularly important role in $G_{i/o}$ coupled receptors, probably because the greater expression levels of $G\alpha_{i/o}$ subunit can cause release of high concentrations of Gβγ (Casey *et al.*, 1991).

1.1.3.3 G protein independent signaling

In addition to G protein-mediated signal transduction, ligand binding of GPCRs can promote phosphorylation of residues located in the ICL2, ICL3 and C-terminal regions by GRKs (G-protein-coupled receptor kinases). GRKs are cytosolic serine/threonine protein kinases that translocate in membrane following ligand binding (Mushegian *et al.*, 2012). An important group of *scaffold* cytosolic proteins, β-arrestins, are recruited to the membrane and bind the phosphorylated receptor, sterically hindering receptor-G protein interaction and causing internalization of the activated receptor (Goodman *et al.*, 1996; Lefkowitz, 1998). This internalization occurs in specific clathrin-rich membrane domains, and requires the action of the GTPase protein dynamin, which promotes the formation of invaginated vesicles and their merging with tubuloreticular endosomes. Proton pumps present in endocytic vesicles maintain low *p*H values, which favor dissociation between ligand and receptor. The ligand-free receptors can either be degraded in lysosomes or recycled back to the plasma membrane through sorting mechanisms that are still poorly understood (Smythe, 2003). β-arrestins, in addition to mediate interruption of receptor signaling through internalization, can also form complexes with several signaling proteins, including components of MAP-kinase cascade, like Raf-1, MEK1 and ERK (DeFea *et al.*, 2000; Luttrell *et al.*, 2001; Ahn *et al.*, 2003). Thus, arrestin are now considered alternative transduction proteins, capable to mediate a secondary wave of signaling, events that include ERK (Extracellular-signal Regulated Kinases) and several others potential effectors, as recently discovered by phosphoproteomic analysis (Kendall *et al.*, 2011; Fereshteh *et al.*, 2012).

1.1.4 Constitutive activity of GPCRs

It is now clear that many GPCRs are constitutively active, i.e., they are able to interact with G proteins also in absence of ligand, and the level of this spontaneous interaction can be decreased by ligands called inverse agonists (Costa and Cotecchia, 2005).

The first evidence of spontaneous activity dates back to about 30 years ago, when Cerione and colleagues, using purified β_2-adrenoceptors (β_2ARs) and G_s proteins reinserted into lipid vesicles, demonstrated that in absence of ligand a significant fraction of receptors was functionally coupled to G protein; this constitutive activation could be inhibited by an antagonist (Cerione *et al.*, 1984). Although the work was criticized on the ground that the artificial reconstitution system used in those experiments could be itself responsible for the observed constitutive activation (Pedersen and Ross, 1985), similar findings were reported later (Costa and Herz, 1989), demonstrating that in native membranes obtained by neuroblastoma cells naturally expressing high levels of δ opioid receptors, the GTPase activity of $G_{i/o}$ proteins, measured in absence of ligand, could be inhibited by some antagonists (inverse agonists) but not by others (neutral antagonists).

Similar observations were also made in other GPCRs, like muscarinic acetylcholine receptors (Hilf and Jakobs, 1992), and at different levels of post-receptor signaling, such as β_2-AR activation of adenylyl cyclase (Gotze and Jakobs, 1994).

1.1.4.1 Constitutively active mutants (CAMs)

Further studies discovered that discrete mutations in the sequence of GPCRs can lead to constitutive activation.

The α_{1B}-adrenoceptor (α_{1B}AR), a G_q-coupled receptor, was the first GPCR in which point mutations were shown to trigger receptor activation. In particular, a conservative substitution of Ala^{293} with Leu in the C- terminus region of the 3d IC loop resulted in marked agonist-independent activity (Cotecchia *et al.*, 1990). Subsequent studies indicated that the constitutive activity caused by such mutations could trigger a proto-oncogenic phenotype of the α_{1B}AR (Allen *et al.*, 1991).

Later, the same residue Ala^{293} of the α_{1B}AR was systematically replaced with all the natural amino acids, demonstrating that every mutation conferred different levels of constitutive activation (Kjelsberg *et al.*, 1992). A similar phenomenon was observed in the β_2AR (Samama *et al.*, 1993), coupled to G_s, and in the α_2-adrenoceptor (α_2AR) (Ren *et al.*, 1993), coupled to G_i, confirming that spontaneous receptor activity was a general phenomenon and did not depend on the particular subtype of G protein with which the receptor interacts.

Also in the α_{1B}AR was for the first time shown that mutagenesis of the Asp^{142} residue, which is part of the highly conserved motif Glu/AspArgTyr (E/DRY) shared by all rhodopsin-like receptors in the cytosolic end of helix 3 and is the major site of receptor-G protein interaction, resulted in high constitutive activity (Scheer *et al.*, 1997). Mutation of the acidic residue of the E/DRY motif produced constitutive activation in several other receptors, including rhodopsin (Cohen *et al.*, 1993), the β_2AR (Rasmussen *et al.*, 1999), the histamine H_2 (Alewijnse *et al.*, 2000) and H_4 (Schneider *et al.*, 2010), the vasopressin V_2 (Morin *et al.*, 1998) and muscarinic M_1 (Lu *et al.*, 1997) receptors.

Studies on constitutively active mutants (CAMs) of GPCRs had two important consequences. First, they provided novel insight into the mechanism of receptor activation, with the consequent modification of the model of receptor activation (see below). Second, they stimulated the search and the discovery of naturally occurring GPCRs mutations associated with several human genetic diseases. For instance, activating mutations in the human thyrotropin-stimulating hormone (TSH) receptor are involved in thyroid adenomas (Parma *et al.*, 1993) and activating mutations of the human luteinizing hormone (LH) receptor are involved in familial male precocious puberty (Shenker *et al.*, 1993). Inverse agonists, which unlike neutral antagonist are capable to inhibit the constitutive activation of GPCRs, might be beneficial in cases where receptor mutations are the pathogenic cause of disease (Bond and Ijzerman, 2006).

1.1.4.2 Wild type constitutively active GPCRs

The physiological relevance of the differences in constitutive activity among native GPCRs remains a mystery. In addition to δ opioid receptor, other wild type GPCRs were shown to exhibit constitutive activity, under some experimental conditions, such as β_1AR (Engelhardt *et al.*, 2001), β_2AR (Chidiac *et al.*, 1994), α_{2A}AR (Wade *et al.*, 2001), μ opioid (Connor and Traynor, 2010), cannabinoid CB_1 and CB_2 (Ross *et al.*, 1999) and angiotensin AT_1 (Qin *et al.*, 2009) receptors.

In general, spontaneous receptor activity can be detected at best in artificially transfected systems and it is difficult to reveal *in vivo*, also due to the impossibility to clearly exclude a contribution from endogenous ligands to basal activity (Milligan, 2003). However, there are some exceptions represented, for example, by the ghrelin GHS-R1a receptor, involved in food intake, whose suppression has been shown to be associated with a short-stature phenotype (Damian *et al.*, 2012).

1.2 PHARMACOLOGICAL RECEPTOR THEORY

The concept of receptor, which is today at the heart of modern pharmacology, was introduced about hundred years ago, when it became clear that extremely small concentration of chemical substances could lead to profound changes in physiological systems, and that discrete structural modifications of those molecules could generate large differences in biological responses. Yet the concept of receptor failed to gain immediate acceptance and for many decades encountered the resistance and the skepticism of a large body of the scientific community. Until fifty years ago, when theoretical models of receptor action, which preceded the experimental discovery of receptors by many years, were shown to accurately predict the relationships between ligand concentrations and biological response (Maehle *et al.*, 2002). Receptors, therefore, were initially discovered as mathematical rather than biological entities.

1.2.1 Classical theory of receptor activation

There are two "pioneers" responsible for setting the stage underlying the evolution of the concept of receptor: the Berlin immunologist Ehrlich (1854–1915) and the Cambridge physiologist Langley (1852–1926).

Ehrlich proposed the existence in cells of "chemoreceptors" for dyes and other inhibitory substances, suggesting that they could differ in human host and in parasites, and that such a difference could be exploited in therapy (Ehrlich and Morgenroth, 1957).

Langley, during his studies on the effects produced by atropine and pilocarpine, introduced the idea that such chemicals interacted with specific receptive substances (i.e., receptors) present in muscle tissues. He suggested that the interaction could result either in activation, with consequent propagation of the signal throughout the tissue, or in the blockade of the receptive substance. (Langley, 1878,1905).

Starting from such initial postulates, the "classical theory" of receptor action took shape as the chronological evolution of several concepts described first by Clark (Clark, 1933) and later modified by many other researchers: Gaddum (Gaddum, 1937), Ariëns (Ariens, 1954), Stephenson (Stephenson, 1956), Schild (Schild, 1957) , Paton (Paton, 1961), Rang (Paton and Rang, 1965), Van Rossum (Van Rossum, 1966), Waud (Waud, 1968), Furchgott (Furchgott, 1972), Colquhoun (Colquhoun, 1973), MacKay (MacKay, 1977) and Ehlert (Ehlert, 1988).

Clark proposed in 1933 the "receptor-occupation theory" (Clark, 1933,1937), according to which the biological response produced by a ligand H is directly proportional to the number of occupied receptors R, and thus to the concentration of the ligand-receptor complex HR.

$$H + R \leftrightarrow HR \Rightarrow\Rightarrow\Rightarrow Biological\ response$$
$$Response = k\,[HR]$$

The maximal response occurs when all the receptors are occupied. The binding of a ligand H to a receptor R was assumed to follow mass-action law and can be described according to the Langmuir adsorption isotherm:

$$\rho = \frac{[HR]}{[R_t]} = \frac{[H]}{[H] + K_H}$$

where [HR] is the amount of complex formed between ligand and receptor and $[R_t]$ is the total number of receptor molecules. The ratio ρ indicates to the fractional binding by a molar concentration of ligand [H] with an equilibrium dissociation constant of K_H.

This theory, however, does not explain why some ligand-receptor complexes can produce a biological response whereas others do not. In other words, it does not tell the reason why ligands can be agonists or antagonists of the receptor.

To solve this problem, Ariëns introduced in 1954 the concept of *intrinsic activity*, α, which was defined as a molecular parameter describing the fraction of maximal response that can be induced by any agonist-receptor complex relative to a standard "full agonist" in the same system (full agonist: α=1; partial agonist: 0<α<1; antagonist: α=0) (Ariens, 1954).

The equation for response became:

$$Response = \frac{[H]\alpha}{[H] + K_H}$$

where K_H is the equilibrium dissociation of the agonist-receptor complex.

This "occupation theory" of receptor action was still a "primitive" description of receptor phenomenology. In particular it could not explain the common finding that the same agonist can produce full maximal responses in some tissues but not in others. It is evident that the intrinsic activity as defined by Ariëns was not a true "molecular" parameter, which should by definition be invariant and independent of the biological tissue in which the receptor is present.

Further refinements due to the work of Stephenson and Furchgott (Furchgott, 1972), brought to the final model of receptor action that is still useful today. The biological response to a ligand H is described as the "unknown" function of the *Stimulus*:

$$Response = f\left[\frac{[H] \cdot \varepsilon[R_t]}{[H] + K_H}\right]$$

where the stimulus is defined by the expression given in parenthesis, and resembles Ariëns formulation. Now it is clear that there are two system-independent molecular parameters that determine the magnitude of the stimulus: the quantity ε named intrinsic efficacy indicates the competence of a drug-receptor complex to trigger a biological signal, and K_H is the equilibrium dissociation constant of the drug-receptor complex. In addition, there are two system-dependent parameters: the receptor density $[R_t]$ and the function *f*, which relates the initial strength of the biological stimulus to the tissue response. Thus, intrinsic efficacy is now defined as a true molecular property of the ligand-receptor complex and must be invariant wherever that receptor is expressed. But the biological impact of intrinsic efficacy can vary widely across different cells, because it depends on the number of expressed receptor and on the differences in sensitivity of the signaling pathways to the activated complex (the stimulus-response function). (Kenakin, 2004).

1.2.1.1 Affinity and intrinsic efficacy

The *affinity* of a drug for a receptor defines the strength of interaction between the two species, i.e., it is a measure of the capability of a drug to bind the receptor.

The property that confers to a molecule the ability to change the receptor in such a way as to make it capable to generate biologically readable signals is termed *intrinsic efficacy*.

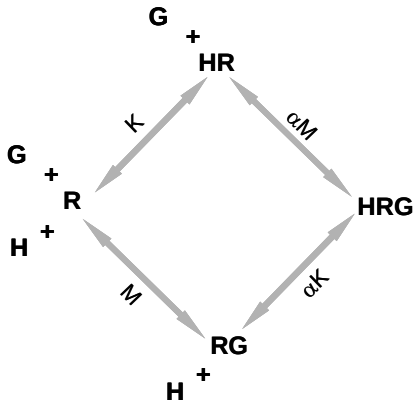
Both the parameters predict the potentiality of a drug to have a pharmacological effect. According to the "classical theory" of receptor activation, intrinsic efficacy and affinity depend only on the chemical identity of the ligand and the receptor. In this definition is included the concept of invariability of the effect, i.e., it is not important where the interaction take place, even if the biological effect may change depending by different network signaling. On this theory is based the classical classification of ligands as agonists or antagonists: an *agonist* is a ligand that has intrinsic

efficacy (on binding to the receptor can generate a biological "stimulus"), while an *antagonist* is a ligand that lacks intrinsic efficacy (Kenakin, 2009).

1.2.2 The ternary complex model

The idea that intrinsic efficacy can be defined as a molecular property that depends only on ligand and receptor is obviously in contrast with what we know today about the functional chemistry of GPCRs. These macromolecules do not execute any intrinsic biochemical reaction, except ligand binding. In other words, they cannot produce any biologically relevant "stimulus" by themself. What triggers a biological signal, instead, is the G protein to which the receptor will associate in response to ligand binding. Therefore, a realistic molecular definition of intrinsic efficacy cannot be made without taking into consideration that the minimal functional unit required for receptor action involves the interaction among three molecular entities: the ligand, the receptor and the G protein heterotrimer. Moreover, the existence of constitutive receptor activation cannot be explained by classical receptor theory, because it implies that the "biological stimulus" can also occur in the absence of ligand.

To account for the effects of guanine nucleotides on GPCR agonist affinity and to explain numerous studies suggesting that GPCRs, when activated by an agonist, could couple to a hypothetical (yet unknown at the time) GTP-binding protein, DeLean and colleagues proposed the "ternary complex model" in 1980 (De Lean *et al.*, 1980). According to the model, agonist binding at the extracellular site of the receptor enhances the affinity of the receptor for the G protein interacting at the intracellular site. Thus the process of receptor activation can be understood according to the thermodynamic principles that describe the interaction of a macromolecule (the receptor R) with two different ligands (the ligand H and the G protein G) interacting at distinct sites, as shown in the following reaction scheme:



The formation of the final ternary complex state HRG, starting from the initial free receptor state R, involves two "unconditional" binding constants K and M (which respectively describe the formation of the binary association complexes HR and RG) and the free-energy coupling constant α (Weber, 1975) which accounts for the cooperative effect that each of the two ligands on binding propagates to the binding site of the other. The closed thermodynamic box that describes the reactions indicates that the free-energy change underlying α is conserved between the two binding processes. Therefore, the effect that G protein binding exerts on the binding of agonist to R must be energetically identical to effect that agonist binding to R exerts on the binding of G protein.

It is clear that α provides an exact chemical definition of ligands efficacy at GPCRs and, at the same time, the TCM provides a simple explanation for the existence of constitutive receptor activation. If $\alpha > 1$ (i.e., the free energy change associated with α has negative sign) the ligand H enhances the binding of receptor to G and thus acts as a receptor agonist. If $\alpha = 1$ (i.e., the free energy change is zero), it means that the ligand H has no influence on the interaction of the receptor with the G protein. However, since a ligand with $\alpha=1$ can still occupy the receptor binding site it will compete with agonist binding, thus behaving as a "neutral" antagonist. Finally, if $\alpha < 1$, the binding of H can reduce the spontaneous interaction between R and G. This results in negative efficacy and inverse agonism.

To explain the effect of mutations that enhance constitutive activation of GPCRs according to the TCM, we can assume that such mutations can cause

a large increase of the affinity constant M, which controls the association between R and G. Such a change will results in enhanced spontaneous coupling between R and G in the absence of ligand, but also in an increased apparent affinity of the ligands which is larger, the greater is the ligand efficacy α. In fact, according to the TCM the apparent dissociation constant of the ligand is given by the following equation:

$$K_{app} = K_D \cdot \frac{\left(1/M + [G]\right)}{\left(1/M + \alpha[G]\right)}$$

where K_D is the true dissociation constant of the ligand (i.e., the reciprocal of the ligand affinity K) and K_{app} is the dissociation constant measured in ligand-binding experiments. It's evident from the expression above that as M increases, the value of the apparent dissociation constant is reduced by a factor that depends on the value of α, reaching the limit value K_D/α as $M \to \infty$.

Ligand binding studies in the constitutively activated β_2AR confirmed such a prediction, since the mutation decreased the dissociation constant of full agonists such as adrenaline or isoproterenol to a much greater extent than that of partial agonists such as dobutamine or dichloroisoproterenol (Samama *et al.*, 1993). Surprisingly, however, this efficacy-related increase of ligand apparent affinity induced by the CAM was also observed under experimental conditions in which the interaction between receptor and G protein was abolished or made negligible (i.e., solubilised and purified receptors or large overexpression of receptor in transfected cells).

Thus, to provide a better explanation of the constitutively active receptor phenotype, the "ternary complex" model was modified in the so called "extended ternary complex" (ETC) model, proposed by Samama and colleagues in 1993 (Samama *et al.*, 1993).

In the ETC model it is assumed that receptors exist in equilibrium between two states, R and R*. Only the "active" state, R*, can interact with G protein. Now the efficacy of ligands depends on two coupling constants, α and β. The first is defined exactly as in the original TCM. The second, β, represents the

energetic effect of ligands on the equilibrium that controls the transition between R and R*. Agonists (β >1) shifts the equilibrium towards R*, inverse agonists (β <1) do the opposite, while neutral antagonists (β = 1) do not perturb this equilibrium. Constitutive activation occurs if a mutation increases the equilibrium between R and R*, thus making the spontaneous interaction between R* and G more probable.

1.2.2.1 Implications of the ternary complex model

The "ternary complex" model and its extensions introduce novel insight in the concept of ligand efficacy.

First, unlike the "classical theory" where *intrinsic efficacy* is specified as a dimensionless and relative quantity with no precise chemical meaning, the TCM defines efficacy as a physical quantity. In fact, the concept of free-energy coupling, originally proposed by Weber (which stands behind the TCM α), is a mass-action-law based interpretation of an exact thermodynamic potential: the so called total binding potential (Л) previously developed by J. Wyman in his linkage theory. Both represent the way to express in terms of macroscopic thermodynamics and chemical energetics the size of the intramolecular changes underlying the long-range cooperativity that links binding events occurring at distant regions of the same protein macromolecule. This cooperativity is also a natural measure of the strength of allosteric effects in proteins. Allostery and cooperativity are a fundamental property in the functional chemistry of proteins: the key to understanding regulation of biological activity. Therefore, the pharmacological concept of efficacy represents the allosteric properties of receptor and transduction proteins, and raises the same questions that are generally posed in relating structure and function in non-receptor proteins.

Second, as defined in the TCM, ligand efficacy (i.e., the free-energy coupling constant α) depends on – and can only be quantified with respect to – three molecules: the ligand, the receptor and the transduction protein. Since it is now clear that the same type of GPCR can interact with several kinds of G proteins and also with non G protein transducers such as arrestins, it makes no sense to think that efficacy can be defined as a property of only ligand and

receptor. Implicit in this concept is that a ligand that shows negative coupling towards G-protein interaction might display positive coupling towards a different transducer that interacts with the receptor. For example, an antagonist at the receptor-Gs interaction can potentially be agonist, even a full agonist, at the receptor-arrestin interaction. This phenomenon – called "biased agonism" – has been recently experimentally demonstrated in the angiotensin AT1 receptor (Kenakin, 2007; Rajagopal *et al.*, 2010).

In conclusion, the recognition that ligand efficacy corresponds to the allosteric property behind receptor-transducer interactions extends the scale of agonism. In constitutively active GPCRs, *positive agonists* are ligands which enhance the interaction receptor-transduction protein; *inverse agonists* are ligands with negative intrinsic efficacy, thus decreasing the spontaneous interaction receptor-transduction protein; *neutral agonists* or *antagonists* are ligands without efficacy, which can only inhibit the effect of both positive and inverse agonists by interfering with the occupation of the receptor binding site. The findings that distinct arrestins and receptor kinase subtypes have differential and competing capabilities in silencing or directing receptors towards alternative signaling (Kim *et al.*, 2005; Ren *et al.*, 2005), add a new level of complexity to the assessment of the pharmacological potential of inverse ligands, but also novel opportunities in the process of drug discovery.

Yet, borne in the classical receptor theory there is a fundamental concept that remains intact and is still valid today: the Stephenson's idea that biological response is an *unknown function* of the stimulus originated at the molecular level. Only today, as systems biology unveils the complex behaviour of biosignaling networks, we can fully appreciate why this was the most ingenious part of the model itself. Translated in modern language, this means that regardless of the molecular details through which a ligand can "manipulate" the receptor-transducer interaction, what determines the biological impact of such interactions are the signal-processing mechanisms of the cell; this processing can vary across different cell types and may change under diverse physiological or pathological conditions. Therefore, biological and molecular efficacy, although obviously related, are neither the same thing, nor it is possible to learn about the first by studying only the

second, or vice versa. We need precise molecular information from X-ray crystallography and novel spectroscopic methods to understand how any ligand structure can have "molecular efficacy" at the receptor-transducer complex, and thus learn to design new improved molecules. But we still need competent and improved cellular and animal models of human disease to measure "biological efficacy" and the true therapeutic potential of the molecular efficacy of any novel drug.

1.3 THE OPIOID SYSTEM

Opioid receptors are widely distributed in the central nervous system where they mediate the principal effects of endogenous and exogenous opioid ligands. Such effects include analgesia, feeding, euphoria, anxiety and the release of several hormones. They are also located in autonomic nerves, where they mediate depressive influences on the respiratory, cardiovascular and gastrointestinal systems (Bodnar, 2011).

1.3.1 Opioid receptors

Four receptors are included in this class: δ or DOP, κ or KOP, μ or MOP and NOP (Cox *et al.*, 2009).

The mouse DOP receptor was the first opioid receptor to be cloned in 1992 by two different groups (Evans *et al.*, 1992; Kieffer *et al.*, 1992), followed by the cloning of all the opioid receptor subtypes in different species, including human (Knapp *et al.*, 1994; Mansson *et al.*, 1994; Mollereau *et al.*, 1994; Wang *et al.*, 1994b). Several different splice variants for every receptor gene have also been identified.

Because early ligand-binding studies on the cloned NOP found very low levels of binding by known opioid ligands, this receptor was considered to be an orphan receptor and termed "orphanin FQ", "nociceptin" receptor or "ORL-1", for opioid receptor-like 1. This receptor has the 67% of sequence identity with the other opioid receptors in transmembrane domains (TMs) (Wang *et al.*, 1994a). However, soon after cloning the orphan receptor, the

endogenous peptide ligand for NOP was identified in rat and porcine brain tissue and termed nociceptin (Meunier *et al.*, 1995) or orphanin FQ (OFQ) (Reinscheid *et al.*, 1995).

All four opioid receptors are seven-transmembrane domains GPCRs, coupled to heterotrimeric $G_{i/o}$ proteins. Thus, on ligand binding, they inhibit adenylyl cyclase, with the consequent decrease of cAMP levels and PKA activity. These events contribute to the hyperpolarization of neuronal cell, which, however, primarily results from the direct ability of $G_{i/o}$ proteins to mediate via Gβγ release opening of K^+ channels and the closure of Ca^{2+} channels. This increases the threshold of neuronal excitability and reduces presynaptic neurotransmitter release (Svoboda and Lupica, 1998).

Opioid receptors can also interact with pertussis toxin-insensitive G proteins, such as G_z and G_{16} proteins (Connor and Christie, 1999) but the signaling resulting from such interactions is not clear.

1.3.1.1 Architecture of opioid receptors

There are highly conserved residues in the sequence of the four opioid receptors, in particular within the TMs, while the extracellular loops (ECL) are the regions with the greatest differences among subtypes (Chen *et al.*, 1993). The sequence identity of the transmembrane domains is 76% between MOP and DOP, 73% between MOP and KOP, and 74% between DOP and KOP (Mollereau *et al.*, 1994) (Kieffer, 1995).

On the basis of mutational analysis, chimeric receptor studies, and computational modeling, it has been suggested that all opioid receptors may share a common ligand-binding pocket cavity between TM3, TM5, TM6 and TM7 (Quock *et al.*, 1999).

This cavity is partially covered by the ECLs. Differences in ECLs and in the residues of the extracellular ends of the TM segments play a role in ligand selectivity and explain different binding affinity for distinct opioid receptor types (Takemori and Portoghese, 1992; Metzger and Ferguson, 1995). Ligand binding and selectivity are conferred through the recognition of two distinct structural elements of the ligand molecule. On binding, the N-terminal tyrosine of the peptides, or the analogue amine function present in alkaloids and other non-peptide ligands, lies at the bottom of the binding cavity and

interacts primarily with residues that are common to all opioid receptors. This initial step contributes to orientate chemically different fragments of the ligand toward the extracellular surface of the TMs, thus favoring the interaction with several residues that differ among the respective opioid receptors and form the borders of the binding cavity and the ECLs. (Pogozheva *et al.*, 1998). In addition to distinct transmembrane residues, agonist ligand selectivity for MOP, DOP and KOP receptors has been attributed to the ECL1 and ECL3 for the MOP (Ulens *et al.*, 2000), the ECL3 for the DOP (Befort *et al.*, 1996) and the ECL2 for the KOP (Zhang *et al.*, 2002). The NOP lacks some of the conserved amino acids near the top of each TMs and displays very low affinity for opioid ligands which show promiscuous high affinity for all the other subtypes (Meunier *et al.*, 2000).
All such data have been largely confirmed by the recent results of the crystallization of DOP (Granier *et al.*, 2012), MOP (Manglik *et al.*, 2012), KOP (Wu *et al.*, 2012) and NOP (Thompson *et al.*, 2012) receptors.

1.3.1.2 Constitutive activity of δ and μ opioid receptors

The constitutive activity of DOP has been extensively demonstrated by studies based on [^{35}S]GTPγS binding either in cells expressing the native receptor, such as neuroblastoma cells (Costa and Herz, 1989; Costa *et al.*, 1990; Szekeres and Traynor, 1997), or in transfected cell lines (Chiu *et al.*, 1996; Mullaney *et al.*, 1996; Merkouris *et al.*, 1997; Hosohata *et al.*, 1999; Neilan *et al.*, 1999; Labarre *et al.*, 2000; Zaki *et al.*, 2001).
The physiological and therapeutic relevance of inverse agonists for delta opioid receptors is still not completely understood. The therapeutic utility of the inverse agonists depends on the extent of constitutive delta opioid receptor activity in native tissues and on the role of constitutive delta opioid receptor activity in the pathophysiology of diseases. For example, it was suggested that selective delta opioid antagonists could be therapeutically useful as immunosuppressant agents in organ transplants (House *et al.*, 1995) and in chronic inflammatory diseases (Spetea *et al.*, 2001). Moreover, data suggest that DOP receptors may regulate mood, and a potential role of constitutive activity in these functions was suggested on a speculative basis (Filliol *et al.*, 2000).

MOP has also been shown to exhibit basal signaling activity in SH-SY5Y cells and in transfected HEK293 cells (Wang *et al.*, 1994c; Burford *et al.*, 2000; Liu and Prather, 2001; Liu *et al.*, 2001; Wang *et al.*, 2001; Guarna *et al.*, 2003; Statnick *et al.*, 2003; Wang *et al.*, 2004; Lam *et al.*, 2011).
Constitutive activity was also documented for KOP (Becker *et al.*, 1999).
While the existence of basal activity for DOP is readily detectable, it is more difficult to demonstrate constitutive activity at MOP and KOP. It was shown that DOP, MOP and KOP receptors display apparent constitutive activity following chronic exposure to morphine or receptor-selective agonists (Wang *et al.*, 2007) and, in fact, the basal activity of MOP is clearly detectable only under such conditions (Wang *et al.*, 1994c). I must be considered that in this type of experiments it is impossible to clarify if the observed basal effect is due to true constitutive activation or to a residual ligand-induced effect, due to the difficulty to effectively remove the incubated ligands from the preparation. Thus the conclusions about constitutive activation drawn from such experiments are doubtful, to say the least. It was suggested, on the ground of pure speculation, that ligand-induced constitutive activity may contribute to development of tolerance and dependence. It was also proposed that the elevated basal signaling in MOP following prolonged agonist treatment might involve calmodulin (CaM), which was shown to potentially act as a constraint at the third intracellular loop of the MOP. In non-agonist exposed state of the receptor, CaM binds to the MOP and competes for G protein coupling, thereby inhibiting constitutive activity. After prolonged morphine treatment, CaM is released from the receptor, thus resulting in an elevated ligand-independent receptor activity (Sadee *et al.*, 2005).

1.3.2 Opioid ligands

Opioid receptors can interact with a great variety of substances. Every ligand can exhibit a divergent profile of selectivity in binding to different opioid receptors subtypes.
With the exception of nociceptin/OFQ, the endogenous ligand of NOP, which has a phenylalanine residue at the N-terminal (Varani *et al.*, 1999), the essential prerequisite shared by all peptides that bind opioid receptors is the

presence of a free tyrosine residue at the N-terminal. Indeed, all opioid ligands, including alkaloids, despite their chemical diversity, have in their structure a phenolic hydroxyl separated by six carbons from a positively charged nitrogen, which mimics the N-terminal tyrosine of all endogenous opioid peptides (Loew *et al.*, 1978).

1.3.2.1 The Dmt-Tic pharmacophore

The condensation of the two unnatural amino acids, 2',6'-dimethyltyrosine (Dmt) and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) generates an active pharmacophore for DOP and MOP receptors.

The discovery of the potent peptide agonists delthorphin (DOP-selective) and dermorphin (MOP-selective) in frog skin, led to a study in which the sequence of the two compounds was systematically altered, in order to identify important functional groups and the minimal sequence required for opioid receptor recognition, while maintaining or improving DOP or MOP receptor affinity and selectivity (Melchiorri and Negri, 1996; Lazarus *et al.*, 1999). The fortuitous replacement of the D-amino acid at the second position with the heteroaromatic residue Tic, led to the discovery that the sequence H-Tyr-Tic-Phe-(Phe)-OH [TIP(P)] exhibited highly selective DOP antagonism (Schiller *et al.*, 1992; Schmidt *et al.*, 1997). Subsequently, the truncation of the C-terminus of TIP(P) to form H-Tyr-Tic-NH_2 and H-Tyr-Tic-OH produced the first opioid dipeptide antagonist (Temussi *et al.*, 1994). Despite the low potency of this compound, the finding was a fundamental step for the identification of the minimal components required by a peptide for interacting with DOP.

Later, through the dimethylation of the 2' and 6' position of the tyramine, the tyrosine analogue Dmt was obtained (Salvadori *et al.*, 1995). Whereas the substitution of Dmt into compounds like [2-D-penicillamine, 5-D-penicillamine]-enkephalin (DPDPE) did not produced great gain of affinity (Chandrakumar *et al.*, 1992a; Chandrakumar *et al.*, 1992b), the introduction of the same residue into the dipeptide H-Tyr-Tic-OH increased DOP affinity more than 8000-fold (Salvadori *et al.*, 1995). Further investigation on such dipeptide analogue demonstrated that N-alkylation can increase the biological activity of the compound by 10- to 20-fold (Salvadori *et al.*, 1997). Some of

these compounds, first identified as antagonists, have been later recognized as inverse agonists on DOP (Labarre *et al.*, 2000; Martin *et al.*, 2002; Tryoen-Toth *et al.*, 2005).
All these data not only demonstrated that the replacement of a single amino acid residue can have dramatic effects on the bioactivity of opioid peptides, but have also made the Dmt-Tic pharmacophore a starting point for the development of novel opioid ligands (Balboni *et al.*, 2002a; Balboni *et al.*, 2002b; Balboni *et al.*, 2003,2004; Balboni *et al.*, 2005; Balboni *et al.*, 2008).

1.3.2.2 Classification of opioid ligands

Opioid ligands can be divided in four broad classes (Brownstein, 1993):

1) Endogenous opioid peptides, which are naturally produced in the body (enkephalins, endorphins, dynorphins and endomorphins).
2) Opium alkaloids, contained in the resin of opium poppy plant (*Papaver somniferum*), such as morphine and codeine.
3) Semi-synthetic opiates, such as heroin, oxycodone and buprenorphine.
4) Fully synthetic opioid, such as methadone and fentanyl, which have structures unrelated to the opium alkaloids.

Although often used as synonymous, the terms "opiate" and "opioid" have slightly different meanings: the first indicates a drug derived from opium; the second refers to a fully synthetic compound, but is more often used to describe the entire class of substances interacting with opioid receptors (Wilson, 1960).

The compounds interacting with opioid receptors, including agonists, inverse agonists and neutral antagonists, display remarkably different chemical structures. Therefore they can also be classified on the basis of their chemical structures (Zaveri *et al.*, 2001; Zaveri, 2003; Porreca and Woods, 2004; Berger and Whistler, 2010):

a) Peptide ligands, such as endogenous peptides and their derivatives (e.g., DADLE), dermorphin and deltorphin.
b) Pseudopeptides, i.e., molecules formed by a peptide portion and a non-proteinogenic spacer, such as several Dmt-Tic (2',6'-

dimethyltyrosine-1,2,3,4-tetrahydroquinoline-3-carboxylate) derivatives.

c) Morphine derivatives (esters and ethers), such as heroin and naloxone.
d) Thebaine derivatives, such as oxymorphone, oxycodone and buprenorphine.
e) Benzomorphan derivatives, such as pentazocine, bremazocine and ethylketocyclazocine.
f) Piperidines derivatives, such as meperidine, fentanyl and lofentanyl.
g) Benzhydrylpiperazine derivatives, such as SNC 80 (4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide) and SNC 121 (4-[(R)-[(2S,5R)-2,5-dimethyl-4-propylpiperazin-1-yl]-(3-methoxyphenyl)methyl]-N,N-diethylbenzamide).
h) Diphenylpropylamine derivatives, such as methadone, propoxyphene and dextropropoxyphene.

1.4 THEORY OF BRET (BIOLUMINESCENCE RESONANCE ENERGY TRANSFER)

Resonance energy transfer (RET) between a donor-acceptor pairs of chromophores linked to different proteins or protein domains has been extensively used to investigate nanoscale molecular interactions in protein biochemistry (Stryer and Haugland, 1967). In RET, a fluorescent donor can transfer the energy of the excited state to a proximal chromophore acceptor. This results in a decrement of the half-life of the excited state, which is revealed by a reduction of fluorescence intensity emitted by the donor, and the simultaneous enhancement of fluorescence intensity emitted by the acceptor (Selvin, 2000). As theorized by Förster, the efficiency of this process depends on the spectral characteristics of the two chromophores, their relative orientation in space and the sixth inverse potency of their distance (Förster, 1948). Thus, under controlled experimental conditions, two chromophores with compatible spectral characteristics, (i.e., spectra that are

overlapped as much as possible), can be used to measure intermolecular distances spanning between 20-80 Ångstrom.

1.4.1 The BRET assays

Around the ‘90s, RET was principally employed in structural biochemistry, to study the intramolecular distances between protein or purified nucleic acid domains (Miki *et al.*, 1986; dos Remedios *et al.*, 1987; Clegg *et al.*, 1992). Later, the identification of green fluorescent protein (GFP) variants, like cyan (CFP) and yellow (YFP) fluorescent protein, and the possibility to engineer chimeric fusion proteins, has made RET-based techniques attractive also for cell biology experiments (Overton and Blumer, 2002; Nobles *et al.*, 2005; Marullo and Bouvier, 2007). In fact, genetically encoded chimaeras consisting of the proteins under study fused to donor and acceptor fluorescent proteins, can be transfected in cells and their interaction can be monitored by recording the variations in RET signals.

One more recent variation of this technique consist in the use of a luminescent-bound donor with a compatible fluorescent protein acceptor. This kind of RET induced by bioluminescence is called BRET, to distinguish it from FRET that uses the fluorescence resonance energy transfer (Prinz *et al.*, 2006). BRET has two advantages over conventional FRET. Firstly, it does not require incident light, which can be damaging to living cells under several conditions. Secondly, it may lead to assays with better signal-to-noise ratio, because endogenous luminescence is far lower than autofluorescence in mammalian cells (Boute *et al.*, 2002; Milligan, 2004).

There are some limitations in the use of RET due to the lack of precise information about the spatial orientation of the donor-acceptor pair and the relative positions of chromophores in the target molecules. This is mostly dependent on the synthetic strategies employed and on the type of studied macromolecules. For these reasons, RET is more applicable and useful in the study of variations of relative distances (i.e., conformational changes), rather than in the measure of absolute distances (i.e., changes of intermolecular distance due to association or dissociation) (Audet *et al.*, 2008). Furthermore, when using GFP chimaeras, there are additional uncertainties in data

interpretation, which are due to limitations posed by steric hindrance. In fact, the intrinsic chromophore of GFP is located into a rigid *β-barrel* structure that limits the range of reachable intermolecular distances; this is often responsible for the poor sensitivity and spatial resolution of genetically-encoded probes (Tsien, 1998). Therefore, it must be borne in mind that results based on the use of GFP and the variation of RET that are measured in such type of experiments are intrinsically ambiguous, because they can reflect with the same probability either conformational/rotational changes between the two partners, or changes in intermolecular distances due to the true association between the two molecules (Gales *et al.*, 2006).

1.4.2 The natural BRET in coelenterates

Resonance energy transfer phenomena exist in nature and are often responsible for the characteristic bioluminescence that is observed in many eukaryotic and prokaryotic organisms (Cormier *et al.*, 1974; Shimomura and Johnson, 1975; O'Kane and Prasher, 1992; Tu and Mager, 1995).

One example is given by the marine coelenterate *Renilla reniformis*, (commonly known as *sea pansy*) which lives in the ocean of northeast Florida.

Renilla cells have particular intracellular compartments, called lumisomes, which contain two proteins: the luciferase (Rluc) and the Green Fluorescent Protein (RGFP) (Anderson and Cormier, 1973). When studied as isolated molecule, the oxidation of the natural substrate coelenterazine by Rluc results in emission of photons displaying a broad-band spectrum of blue light (λ_{max}, 480 nm). When Rluc is in close proximity of RGFP, highly efficient energy transfer occurs and the emission is converted in a narrow-band spectrum of green light (λ_{max}, 505 nm) (Wampler *et al.*, 1971).

The RET efficiency of this natural process is close to 100%, in fact there is almost total disappearance of the original emission peak of the donor when the reaction occurs with a large excess of RGFP (Ward and Cormier, 1976; Matthews *et al.*, 1977a; Hart *et al.*, 1979). This high efficiency depends on two principal factors. The first is the high degree of overlapping existing between the emission spectrum of coelenterazine and the absorption

spectrum of RGFP. The second is the existence of a spontaneous binding interaction between Rluc and RGFP, with an apparent K_d located in the sub-millimolar range (Matthews *et al.*, 1977b; Hart *et al.*, 1979). The direct interaction between the two partners can presumably optimize spatial orientation and the critical donor-acceptor distance, so that the energy transfer can occur with maximal efficiency.

1.4.2.1 Quantum yield enhancement of luminescence

An interesting property in the natural BRET in *Renilla* proteins is the of 3-4-fold increase of the apparent quantum yield of luminescence in the luciferase reaction, measured by emission of photons per mole of coelenterazine, when the interaction Rluc-RGFP occurs, with respect to the same reaction in the absence of the acceptor. This effect is substrate-dependent. For example, the didehydroxylated analogue of coelenterazine (bisdeoxycoelenterazine, or coelenterazine 400a) on reacting with Rluc produces an emission spectrum which is shifted by almost 100 nm towards the ultraviolet region (λmax, 390 nm) and displays extremely low quantum yield. However, in presence of RGFP, the quantum yield of the emission is enhanced about 50-100-fold. Under these conditions, the interaction between the two partners produces a net appearance of green light that is virtually undetectable when Rluc does not interact with RGFP (Hart *et al.*, 1979; Molinari *et al.*, 2008).

Therefore, the use of the didehydroxylated analogue of coelenterazine is particularly advantageous when the BRET signal is very weak.

The mechanism of the enhancement of quantum yield is not completely understood. It has been hypothesized that the responsible mechanism could be the protection from solvent quenching that the binding of RGFP confers to the coelenterazine binding pocket of Rluc. Clearly there must be an allosteric effect that RGFP exerts on Rluc, which enhances the maximal intrinsic catalytic rate of the enzyme and contributes to the observed increase of quantum yield (Hart *et al.*, 1979; Loening *et al.*, 2006; Molinari *et al.*, 2008).

1.4.2.2 Measuring of protein-protein interactions by Renilla chromophores: high efficiency BRET

BRET was employed for many years in the study of a wide variety of proteins interaction, including nucleic acids, GPCRs and nuclear cofactors (Angers *et al.*, 2000; Germain-Desprez *et al.*, 2003; Subramanian *et al.*, 2004; Charest *et al.*, 2005). In many cases, the Rluc donor of luminescence is paired with GFP acceptors (or similar spectral variants) derived from *Aequorea Victoria*. These proteins do not interact with Rluc like the natural RGFP partner, thus neither the optimum of efficiency nor the apparent enhancement of quantum yield can occur. The consequence is that these assays have low sensibility and an unfavorable signal-to-noise ratio. In practice, the benefits of the lower background of BRET compared to FRET is offset by the smaller efficiency of the RET process, as often observed in many assays, even when donors and acceptors are fused in tandem within a single polypeptide chain (Pfleger and Eidne, 2006).

The reason for using fluorescent acceptors from different species is that the existence of spontaneous interactions between donor-acceptor probes that are used as RET reporters is considered a limitation. It is thought that a spontaneous interaction between reporters might perturb the natural interaction occurring between the protein partners under study (Gales *et al.*, 2006). This might be true if the affinity that drive the interaction between the partners has similar dimensions of that existing between the reporters.

However, it has been exhaustively demonstrated that no detectable spontaneous interaction can occur between Rluc and RGFP reporters when they are co-expressed as individual proteins in mammalian cells. This lack of spontaneous interaction may seem a paradox, considering that in living *Renilla* cells the two proteins interact with high efficiency. In reality the intrinsic binding affinity between Rluc and RGFP is very low and it was probably over estimated in the original study, where the binding was measured under very low ionic strength conditions that tend to stabilize even the weakest protein-protein interactions. On the other hand, it is likely that the high concentrations of proteins that results from compartmentalization into luminosomes and from additional membrane docking mechanisms inside

those organelles are the key factors that make possible the interaction between Rluc and RGFP which takes place in *Renilla* cells.
Thus, Rluc and RGFP can be used as a donor-acceptor pair reporter to develop BRET assays characterized by optimal signal-to-noise ratio, accuracy and sensitivity (Molinari *et al.*, 2008).

2 AIM OF THE WORK

Although many GPCRs were identified as constitutively active, i.e., able to couple to G proteins also in absence of ligand, the mechanisms that are responsible of this phenomenon are not yet clear.

There are two principal factors that limit the study of constitutive activation. One is the difficulty to quantify precisely the amount of receptor activity in the absence of ligand. Another is the lack of congeneric series ligands exhibiting both positive and negative efficacy, where the change of efficacy can be related to discrete chemical changes of a shared structural scaffold.

In this thesis I developed an assay based on BRET, in which receptor is tagged at the C-terminal with a luminescent donor and the Gβ subunit of G protein is tagged at the N-terminal with a fluorescent acceptor. The extent of receptor-G protein coupling can be measured as change of the RET signal in membranes prepared from cells expressing the two tagged proteins. In this assay, the addition of GDP to the membranes can inhibit ligand-induced activity and abolish spontaneous receptor activity. Thus, the BRET signal in the presence of GDP marks the level of "zero coupling" between receptor and G protein, and the net difference between absence and presence of the guanine nucleotide is a precise measure of the constitutive activation. Ligands that enhance the BRET signal above the basal level are positive agonists, whereas those inhibiting the basal signal are inverse agonists.

Using this assay, I have been able to compare both the spontaneous and the ligand-induced interaction with G proteins of δ and μ opioid receptors (DOP and MOP, respectively). I decided to investigate a series of 35 different analogues. All these ligands have a common peptidomimetic structure: the Dmt-Tic (2',6'-dimethyltyrosine-1,2,3,4-tetrahydro-quinoline-3-carboxylate) scaffold (see Introduction). As shown in the results, these ligands exhibit a wide range of efficacy at both receptors, spanning both the negative and the positive spectrum of possible effects.

Although DOP-R and MOP-R are coded by distinct receptor genes, they are closely related in terms of sequence identity and homology, functional

interaction with G protein subtypes, and structural configuration of the ligand binding site, as recently shown in X-rays studies. Despite such similarities, the two receptors exhibit a very different degree of constitutive activation. This makes them an ideal experimental system to investigate the difference of constitutive receptor activity that occurs between native receptors. In fact, due to the low levels of spontaneous activity in MOP a side-by-side comparison between the two opioid receptors has never been made.

3 MATERIALS AND METHODS

3.1 REAGENTS AND DRUGS

Cell culture media, reagents, and fetal calf serum were from Invitrogen; restriction enzymes were from New England Biolabs. Coelenterazine and bisdeoxycoelenterazine (or coelenterazine 400a, sold as *Deep Blue*^{TM}) were from Biotium Inc. DADLE was from Bachem, ICI 174,864 was from Tocris, GDP Tris Salt was from Sigma-Aldrich and all other ligands were synthetized in the University of Ferrara.

3.2 PLASMID CONSTRUCTS

SH-SY5Y human neuroblastoma cells were engineered with genes expressing for fusion proteins. All constructs were synthesized by PCR.Human MOPR and DOPR Rluc-tagged fusion proteins were made by replacing stop codons with a sequence encoding a 10-mer linker peptide (GPGIPPARAT) and cloned into the expression vector pRluc-N1 (PerkinElmer, Life science). MOPR-Rluc and DOPR-Rluc inserts were then transferred into the retroviral expression vector pQIXN (Clontech). Bovine $G\beta_1$ N-terminally tagged with RGFP (Prolume) were built by linking the RGFP sequence without its stop codon to Ser^2 through a 21-mer linker peptide (EEQKLISEEDLGILDGGSGSG), and cloned into the retroviral expression vector pQIXH (Clontech). The N-termini of human βArr2 after removal of the start codon were tethered to the C-terminus of RGFP through a 13-mer linker peptide (EEQKLISEEDLRT) and subcloned in pQIXH.

3.3 CELL CULTURE AND TRANSFECTION

Recombinant retroviruses expressing receptor-Rluc or $G\beta_1$-RGFP or βArr2-RGFP fusion proteins were prepared by transfection of packaging cell with different retroviral vectors using FuGENE^{TM}. Fugene is a non-liposomial

agent that binds DNA. The complexes Fugene-DNA are stable and can be added directly into cell cultures, releasing DNA into the cytosol by endocytosis without cytotoxic effects. Cells were allowed to increase the viral titre for 48–72 h before collecting the virus-containing supernatants. Cells were infected with the $G\beta_1$-RGFP or βArr2-RGFP retrovirus in the presence of 8 μg/ml polybrene for 48 h, and selected under hygromycin (100 μg/ml). The polyclonal cells expressing $G\beta_1$-RGFP or βArr2-RGFP were later super-infected with the different receptor-Rluc retroviruses and selected under G418 (600 μg/ml). SH-5YSY cells permanently expressing for DOPR or MOPR tagged at the C-terminal with Rluc in association with Gβ1or βArr2 tagged at the N-terminal with RGFP were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12, supplemented with 10% (v/v) fetal calf serum, 100 μg/ml hygromycin B and 400 μg/ml G418 in a humidified atmosphere of 5% CO_2 at 37°C. HEK293 cells were grown in identical conditions in DMEM.

3.4 EXPRESSION LEVELS OF LUMINESCENT AND FLUORESCENT CHIMERIC PROTEINS

The levels of fusion proteins expressed in transfected cells were determined by measuring the intrinsic luminescence (DOPR-Rluc or MOPR-Rluc) or fluorescence (RGFP-$G\beta_1$) in membrane preparations. For bioluminescence, 6 duplicate serial dilutions of each cell extract (1–20 μg of protein) or membrane preparation (0.2–8 μg) in PBS were counted in the luminometer without filters using an automated protocol; to each sample 500 nM (final) of coelenterazine was automatically injected, and after a delay of 2 s total light emission was counted at 0.5-s intervals for 5 s. Integrated photon counts were plotted as a function of protein concentration, and the luminescence (counts/μg of protein) of the membrane/extract was computed by linear regression of the data. To record fluorescence, corresponding dilutions of the samples were measured in a Packard FluoroCount plate fluorometer using 450(20)-nm and 510(20)-nm filter sets for excitation and emission, respectively. Intrinsic fluorescence (RFU/μg) was computed by linear regression of the data after subtraction of background.

3.5 PREPARATION OF PURIFIED MEMBRANES

Cells were previously thawed (10x 10^6/ml), grown in flaks to reach confluence of 80-90% and pelleted using Dulbecco's phosphate buffer saline (PBS) containing 1mM ethylenediaminetetraacetic acid (EDTA) at 1,000 x g for 10 min at 4°C. The obtained pellets were frozen at –80°C. Later, membranes were obtained by differential centrifugation in gradient of sucrose. All procedures were carried out at 0-4°C. Pellets were homogenized in 5 mM Tris-HC1, pH 7.4, containing 1 mM EGTA, 1 mM dithiothreitol, and 0.32 mM sucrose, with a Dounce homogenizer (pestle A, 30 strokes). The homogenate was centrifuged at 1,000 x g for 10 min. The supernatant was kept, and the pellet was centrifuged in the presence of the original volume of buffer at 1,000 x g for 10 min. This step was repeated.

The three supernatants were pooled and centrifuged at 24,000 x g for 20 min. The pellet was washed twice, resuspending in the original volume of the buffer without sucrose and centrifuged at 24,000 x g for 20 min. The pellet was finally resuspended in bidistillate water and frozen in fractions at -80°C.

3.6 QUANTIFICATION OF MEMBRANE PROTEINS

The amount of total proteins in membranes was determined by Lowry's method (Lowry *et al.*, 1951). The method is based on the reduction of Folin® (Folin-Ciocalteau reagent, which consists of sodium tungstate molybdate and phosphate) by aromatic amino acid residues in presence of copper ions and in alkaline conditions. The result is the formation of a blue color complex with maximum absorption at 750 nm wavelength. The values of absorbance of samples can be compared with a standard curve of Bovine Serum Albumin (BSA) at known concentrations to obtain the concentration of total proteins.

Two solutions are needed:

1) Reagent A, containing one part of CTC ($CuSO_4$ + 50 H_2O + 2 Na and/or K Tartrate + 10% Na_2CO_3), two parts of SDS 5%, and one part of NaOH 0.8N.

2) Reagent B, containing one part of Folin® 2N (Fischer Scientific) and five parts of bidistillate water.

Analytic samples and increasing concentration of BSA (400 µl) were prepared in plastic cuvettes. Then, 200 µl of the reagent A were added. After

additional 10 min at RT, 400 µl of the reagent B were also added. After 30 min, samples were read at the spectrophotometer (λ=750 nm), comparing the values of absorbance of samples with the values of absorbance of BSA standard curve.

3.7 LUMINESCENCE RECORDING OF RECEPTOR-TRANSDUCER INTERACTIONS

Luminescence was recorded in sterile 96-well white plastic plates (Packard Opti-plate) using a plate luminometer (Victorlight, PerkinElmer).

For the determination of receptor/Gβ1 interactions, membranes (5µg of proteins) prepared from cells co-expressing DOPR/Rluc or MOPR/Rluc with RGFP/Gβ1 were added to wells in 90 µl of Dulbecco's phosphate buffer saline (PBS) containing coelenterazine (500 nM) for 10 min. Next, different concentrations of ligands in 10 µl of BSA 0.1% were added to the wells using a multichannel pipette and incubated for an additional 3 min before reading luminescence.

For the kinetics assays, the RET ratio was first recorded in the luminometer for 1-2 minutes in membranes (5 µg) preincubated with coelenterazine (final concentration, 500 nM) to assess the basal level. Next, ligands were automatically injected and the variation of RET was continuously recorded.

For the determination of receptor/βArr2 interactions on attached monolayers, cells were plated 24 h before the experiment ($1x10^5$ cells/well). The assay was started by replacing medium with 90 µl of PBS containing the luciferase substrate analogue bisdeoxycoelenterazine (bDOC, 5 µM). After 2 min, 10 µl of PBS containing different concentrations of ligands was added to the wells and incubated for 3 min before counting.

3.8 EVALUATION OF DIRECT EFFECTS OF LIGANDS ON LUCIFERASE ACTIVITY

Dmt-Tic derivatives contain aromatic rings in their structure that can affect the enzymatic activity of Rluc directly, binding to enzymatic site, or indirectly, by optical effects (Auld *et al.*, 2008). All ligands were tested at

100 μM on cytosol extract of COS-7 cells transfected with Rluc cDNA, on cytosol extract of COS-7 cells transfected with thrombin cleavable fusion protein RGFP-Thr-Rluc (Molinari *et al.*, 2008), on membranes obtained by 2B2 cells expressing for receptor-Rluc and mt-RGFP (Molinari *et al.*, 2010) and on membranes obtained by SH-SY5Y expressing for receptor-Rluc.
For membranes, the assays were performed in 96-well white plastic plates (Packard Opti-plate) as previously described for receptor/Gβ1 interaction, using a single concentration of ligand.
For cytosol extracts, bioluminescence was recorded as described above (see "**Expression levels of luminescent and fluorescent chimeric proteins**").

3.9 DATA ANALYSIS

RET ratios were determined as the ratios of high energy (donor) and low energy (acceptor) emissions, recording by reading each well sequentially through two different filters.
Two different protocols were used depending on the luciferase substrate employed in the experiment. For native coelenterazine, the donor/acceptor emission maxima are only 30 nm apart.
Because the Rluc/coelenterazine spectrum shows almost identical light emission at 450 and 510 nm (λ ratio 510/450 ≈ 1.06), we used this property to correct the ratio of donor/acceptor emission for spectral overlap. Samples were counted using two band pass filters (blue, 450/20 nm, and green, 510/20 nm, 3rd Millenium, Omega Optical, Brattleboro, VT). The emission of RGFP at 450 nm is negligible, so the fraction of light due to donor emission can be eliminated by the subtraction of blue filter counts from the green counts.
Thus, the overlap corrected RET ratio can be calculated as:

$$RET\ ratio = \left(\frac{cps_G \cdot T_B}{cps_B \cdot T_G}\right) - 1$$

where, cps_G and cps_B indicate, respectively, photon counts per second recorded through the green and blue filter, and T_G and T_B are the relative

transmittance of the filters, as reported by the manufacturer (0.86 and 0.77, respectively). This ratio was ≈ 0 ± 0.05 in samples containing only luciferase. For the bis-deoxycoelenterazine analogue (λ_{max} 398 nm), spectral overlap is negligible (acceptor-donor λ_{max} difference ≈ 110 nm) and light was recorded through blue short-pass (450 nm cut-off) and green long-pass (490 nm cut-off) filters (3rd Millennium, Omega Optical). RET was quantified as a simple ratio of cps between the two filters:

$$RET\ ratio_{(bDOC)} = \frac{cps_G}{cps_B}$$

RET Ratio and RET $Ratio_{(bDOC)}$ have different scales and they can be directly compared.

Concentration-response curves were obtained using increasing concentrations of each ligand in 96-well plastic plates.

For the determination of receptor/Gβ1 interaction, twelve ligands in singlicate were tested in each plate, and every experiment included the reading of four plates in sequence, to have results about all ligands obtained in the same day. The enkephalin analogue DADLE, the guanonucleotide GDP and the inverse agonist ICI-174,864 were always included in all experiments as references and as test for inter-plate variability.

For the determination of receptor/βArr2 interaction, four ligands in duplicate wells were tested in each plate. DADLE was included in each plate. In the both types of assay, every experiment was performed three times.

Concentration-response curves were analyzed by nonlinear curve fitting to the general logistic function

$$R_{BRET} = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

where, R_{BRET} is BRET ratio; *x* is ligand concentration; *a* and *d*, upper and lower asymptotes, *c* is the ligand concentration yielding half-maximal RET change (EC_{50}), and *b* is the slope factor (in interaction receptors-Gβ1, positive or negative for agonism or inverse agonism, respectively) at *c*. The significance of the difference of fitted parameters among ligands were

assesses according to the extra sum of squares principle (DeLean *et al.*, 1978).

Next, all data points were converted to fractional extent of absolute receptor coupling (FRC) by subtracting the maximal inhibition of BRET produced by GDP and dividing for the maximal stimulation induced by DADLE:

$$FRC = \frac{R_{BRET} - d_{GDP}}{a_{DADLE} - d_{GDP}}$$

where d_{GDP} and a_{DADLE} are the best fitting computed parameters shared for the set of fitted curves. Transformed data were re-fitted with the equation above, to compute ligands E_{max} and EC_{50} values. The *FRC* in the absence of ligand represents the level of constitutive receptor activation of each receptor.

Intrinsic activity (E_{max}) and EC_{50} values presented in the paper were measured and averaged from at least three independent experiments performed on membranes obtained from different batches of cells.

For interaction receptor/βARR2, the relative Emax of all ligands (intrinsic activities) were computed as fraction of the Emax for DADLE, after subtraction of the RET signal recorded in the absence of ligand.

Kinetics data were normalized by setting the 0 point at the time of the first injection (i.e. the time point that triggered the syringe injector was subtracted from all time points).

4 RESULTS

4.1 EXPRESSION STOICHIOMETRY OF CHIMERIC PROTEINS

A meaningful comparison of the extent of G protein coupling between MOP and DOP receptor requires that the membrane expression levels of the two receptors and the relative stoichiometry between luminescent receptor and fluorescent Gβ-subunit in the membranes are similar. The expression levels of the chimeric proteins can be measured taking advantage of the intrinsic luminescence (receptors) and fluorescence (Gβγ) of the fused reporter proteins.

Thus, we measured the level of intrinsic luminescence and fluorescence present in the membranes of all the cell lines engineered during this study, including HEK293 (human embryonic kidney) and SH-SY5Y (human neuroblastoma) cells. Cell lines expressing similar levels of DOP, MOP and $G\beta_1$ with virtually identical donor/acceptor ratios were selected for this study. The results of this analysis are summarized in Table 1.

Table1. Expression levels of chimeric receptors and $G\beta_1$ subunits in HEK293 and SH-SY5Y cell lines. Intrinsic luminescence and fluorescence were measured in membrane preparations as described in *Materials and Methods.* Both cell lines expressed similar levels of receptors and Gβ1 subunit.

Cell line (*recorded in membrane preps.*)	Fluorescence ($RFU/10^4$)	Luminescence ($cps/10^5$)	Ratio (RFU/cps)
SH-SY5Y Rluc-DOPR/RGFP-$G\beta_1$	0.31 ± 0.03	2.94 ± 0.13	0.011
SH-SY5Y Rluc-MOPR/RGFP-$G\beta_1$	0.21 ± 0.03	2.02 ± 0.04	0.010
HEK293 Rluc-DOPR/RGFP-$G\beta_1$	0.84 ± 0.05	6.72 ± 0.7	0.013
HEK293 Rluc-MOPR/RGFP-$G\beta_1$	0.81 ± 0.03	4.71 ± 1.5	0.017

4.2 EVALUATION OF DIRECT EFFECTS OF LIGANDS ON LUCIFERASE ACTIVITY

Another important criterion that affects the validity of an assay based on BRET is that the chemicals used in the experiments should not interfere aspecifically with the enzymatic activity of the light-emitter Rluc. It is known that chemical structures containing aromatic rings can affect the enzymatic activity of Rluc directly, probably by binding to the substrate site of the molecule. Furthermore, there is also the possibility that at high concentration a substance endowed with light absorption in the wavelengths that are used for the assay may interfere through aspecific optical effects.

To evaluate such possibilities, all ligands used in this study, including GDP, were tested at 100 μM on several preparations. We used cytosolic extracts of both COS-7 cells transfected with Rluc cDNA and with the thrombin cleavable fusion protein RGFP-Thr-RLuc (Molinari *et al.*, 2008), to verify the direct activity of ligands on the enzyme and on the interaction between Rluc and RGFP that generates the RET signal. We also used 2B2 cells expressing a luminescent receptor and a membrane–bound form of the fluorescent protein mt-RGFP (Molinari *et al.*, 2010), to test for unspecific effects that might occur only in the presence of the plasma membranes and would thus escape detection in experiments made using cytosolic extracts.

The results of this analysis (see Appendix, Table A1) indicated that most Dmt-Tic analogues at high concentrations (100 μM) can produce small but detectable inhibitory effects (10 - 30%) on the luciferase activity of Rluc, whereas neither the enkephalin analogue DADLE nor GDP are active at this concentration. However, at least 11 analogues produced greater level of inhibition (30-60 %), and three ligands, (i.e. the Dmt analogues Tic-Ph and Bid-Bzl, and the reference alkaloid BNTX), inhibited enzymatic activity up to 70-80%, under such conditions. The consequence of this luciferase inhibitory activity on the BRET ratio was investigated using a fusion protein between Rluc and RGFP. We found that the ratio of donor-acceptor emissions could compensate for the reduction of luciferase activity in almost all cases, as most of the ligands, regardless of their Rluc inhibitory activity, produced negligible or undetectable effects on the BRET ratio of the fused reporter construct. However, the ligands with the strongest stimulatory effect

could cause a slight (< 2%) aspecific increase of the BRET ratio, although this effect was not consistently observed in all experiments.
In summary, we concluded from such experiments that concentrations of DMT-Tic analogues greater than 10 μM should be avoided in this type of assay. As a consequence, some analogues with low potency for the MOP receptor (e.g. analogues carrying an amino acid residue at the C-terminus) could not be studied using a full concentration-response curve and the exact maximal stimulatory effect of these ligands could not be experimentally determined. For this reason this group of ligand is often not included in the comparisons made throughout the study.

4.3 COMPARISON OF LIGANDS EFFECTS IN HEK293 AND SH-SY5Y CELLS

In a previous study the activity of several opioid ligands in promoting MOP and DOP coupling to G protein and arrrestin was investigated in engineered HEK293 cells. Because δ and μ opioid receptors *in vivo* are principally expressed in neuronal cells, we decided to use a neuronal-like cell for this study. We choose to use the cell line SH-SY5Y, originally isolated from a human neuroblastoma (Biedler *et al.*, 1973; Biedler *et al.*, 1978). This line was shown to express several markers typical of neuronal cells and it has been extensively used in many neurochemical studies (Kazmi and Mishra, 1987; Prather *et al.*, 1994; Bayerer *et al.*, 2007).
As a first step in this investigation we compared the characteristics of the opioid receptor-Gβγ interaction between the two types of cell lines: the previously studied HEK293 cells and the new engineered neuroblastoma line
Although the receptors and $G\beta_1$-subunit are in the same ratio in both cell lines, (see 4.1 and Table 1) we found that both the net agonist-stimulated BRET signal (measured using the enkephalin analog DADLE) and the net GDP-inhibited BRET signal (which is a measure of constitutive receptor coupling in the absence of ligands) were slightly greater in SH-SY5Y than in HEK293 cells (data not shown). This slight enhancement of receptor coupling between the two cell lines presumably reflects the larger amount of $G\alpha_o$ subunits that is present in the neuronal cells. In fact, the interaction between the receptor and the $G\beta_1$-subunit is strictly Gα-subunit mediated, and

it is therefore influenced by the levels of α subunits expressed in the membrane.

To compare the pharmacology of opioid-mediated coupling in the two cell lines we measured the maximal coupling induced by opioids agonists exhibiting different extent of intrinsic activity. As shown in Fig. 1*ab*, the plots of relative ligand intrinsic activities obtained in HEK293 and SH-SY5Ymembranes for both DOP and MOP receptors display a perfect linearity, with slopes not significantly differing from one. Thus, despite the greater efficiency of receptor-Gβγ coupling exhibited by neuroblastoma cells, it is clear that the relative ability of agonists to promote coupling is identical in the two cells.

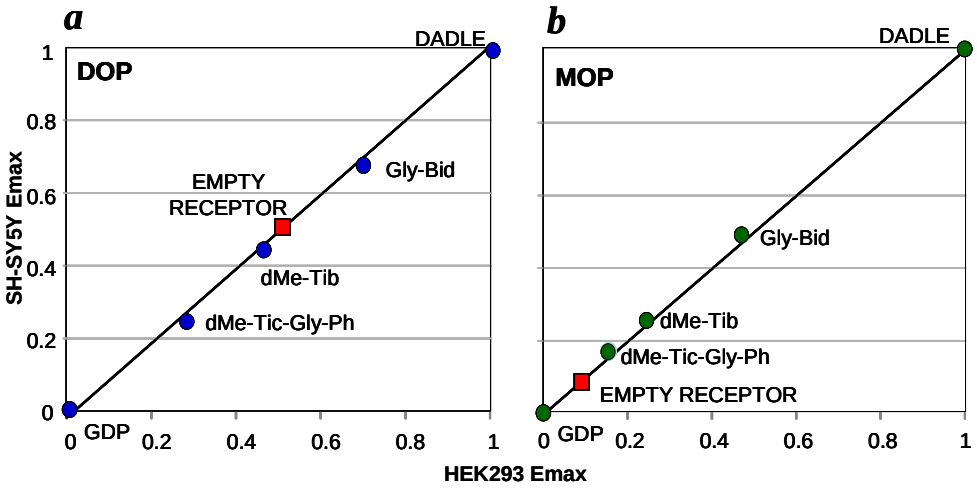


Figure1. The comparison of the effect of different ligands obtained in both HEK293 and SH-SY5Y cells.
The maximal G protein-coupling (Emax) recorded for different ligands at SH-SY5Y cells is shown in function of the maximal coupling of the same ligands at HEK293 cells.
Data are linearly related with unitary slope at (*a*) DOP and (*b*) MOP receptors. The red squares represent the coupling of the empty receptor.

4.4 KINETICS OF RECEPTOR- Gβγ INTERACTION MEASURED BY BRET

To investigate the potency and the maximal coupling effect of the agonists, concentration-response curves of the ligands must be obtained under conditions in which the change of BRET ratio reaches steady-state levels. We have therefore undertaken a number of experiments to evaluate the kinetics of the BRET signal in response to the addition of agonist, inverse agonist and GDP. The data in Fig.2 document experiments made on DOP receptors, since the lower level of constitutive activation exhibited by MOP receptor does not allow a reliable measurement of the kinetics of the inverse agonist. However the kinetic effect of the agonist DADLE on MOP receptor was very similar to that shown for DOP receptor in Fig.2.

The injection of the full agonist DADLE produced enhancement of basal RET signal, which was very rapid in the first 30 s and was followed by a slower approach to a steady-state level within the next 4-5 min (Fig. 2*a*). The addition of the inverse agonist ICI 174,864 diminished the basal RET in a mirror-like fashion, indicating that ligands can enhance or decrease receptor coupling through a process that exhibits essentially similar kinetic properties (Fig. 2*b*). In contrast, the addition of GDP (100 μM), either during agonist-induced enhancement (Fig. 2*c*) or inverse agonist-induced inhibition of RET signal (Fig. 2*d*), produced a very fast reversal of coupling activity.

On the basis of such data, concentration-response curve of the ligands were obtained using incubation times of 5 min. In addition, the total counting of the luminescence in each multiwell plate (about 2.5 min for a 96 wells plate) was repeated sequentially at least 3 times. The final BRET ratios data used in each experiment were averaged from all the repeated countings to insure steady-state stability of the determination.

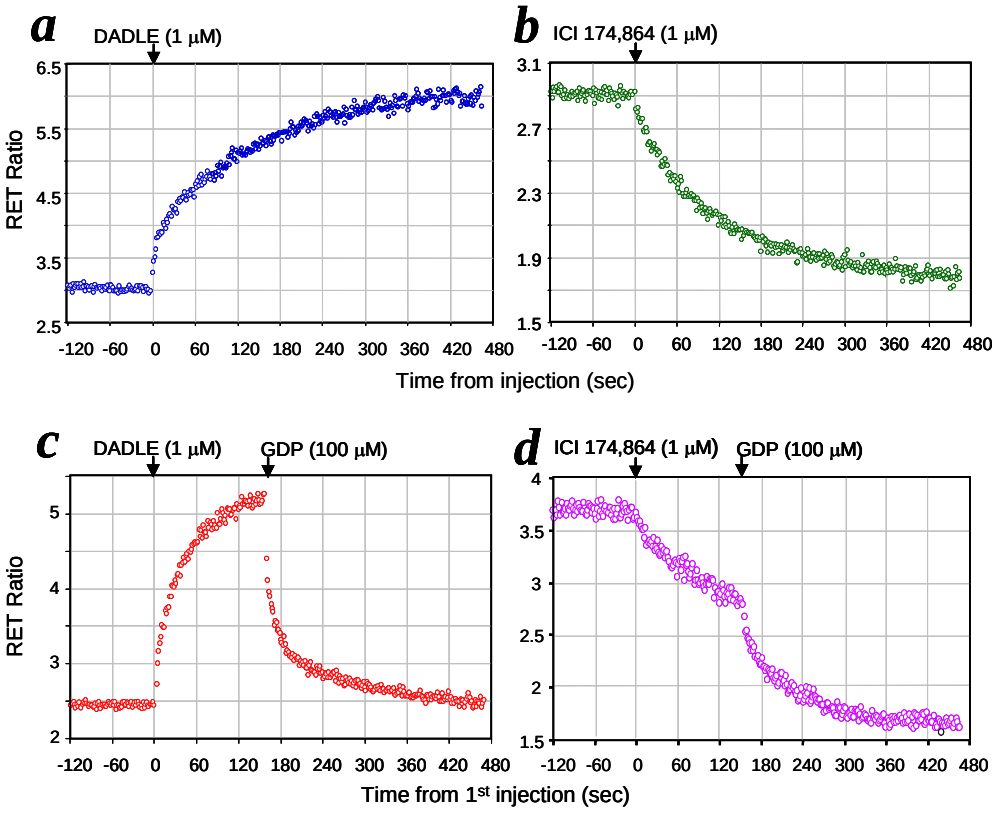


Figure2. Kinetics of RET ratio variation with positive agonist, inverse agonist and GDP in membranes prepared by HEK293 cells expressing for DOP-Rluc and Gβ1-RGFP.

a, membranes injected with the agonist DADLE 1 µM at time 0. The enhancement of basal RET is very fast in the first 30 s, followed by a slower approach to the steady state in the next 4-5 min.

b, membranes injected with the inverse agonist ICI 174,864 1 µM at time 0. The decrement of basal RET follows a kinetic similar to the enhancement induced by agonist.

c, membranes injected with DADLE 1 µM at time 0, and later with GDP 100 µM. GDP produces a very fast reversal of DADLE effect.

d, membranes injected with ICI 174,864 1 µM at time 0 and later with GDP 100 µM. GDP rapidly abolishes the residual G protein coupling.

4.5 CHEMICAL CHARACTERISTICS OF DMT-TIC LIGANDS

The pentapeptide ligand ICI-174,864 represents the prototypic inverse agonist at DOP receptor, and for many years it was the only available antagonist known to have negative efficacy.

Subsequently, it was reported that a ligand derived from the Dmt-Tic pharmacophore (Fig. 3), obtained by the condensation of the two unnatural amino acids, 2',6'-dimethyltyrosine (Dmt) and 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid (Tic), and characterized by a more constrained structure than ICI-174,864, also exhibited potent inverse agonism at DOP receptor.

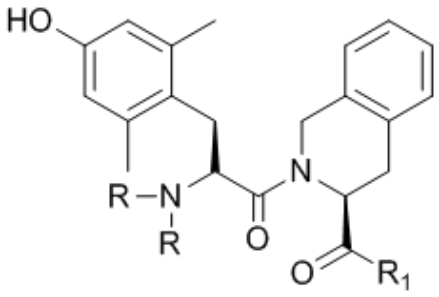


Figure3. The Dmt-Tic pharmacophore. Ligands tested in this thesis are derived from the common structural scaffold of the Dmt-Tic, obtained by the condensation of the two unnatural amino acids, 2',6'-dimethyltyrosine (Dmt) and 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and characterized by a more constrained structure than ICI-174,864. The Dmt-Tic pharmacophore can be variously substituted in R_1.

It has been extensively demonstrated that modifications of the Dmt-Tic structure can lead to analogues exhibiting a wide range of efficacy at DOP and MOP receptors (Balboni *et al.*, 2002b; Balboni *et al.*, 2005). We thus suspected that many other ligands with negative efficacy could exist within the numerous analogues of this class of substance that have been synthesized during the past 15 years. In collaboration with the chemists in the University of Ferrara, we selected a total of 35 compounds derived from the Dmt-Tic pharmacophore. Although the selected compounds are only a minority, compared to the total of available structures that were produced, they were chosen in such a way as to constitute a representative sample of the conformational space of the Dmt-Tic pharmacophore which has been explored through chemical synthesis to date.

Within the selected 35 Dmt-Tic ligands, it is possible to recognize four principal chain extensions that allow dividing four distinct chemical sub-classes of compounds:

1) Tripeptides, in which a third amino acid residue extend the C-terminal of the Dmt-Tic structure;
2) Compounds with a third aromatic group at the C-terminal, that is a "Bid" (1*H*-benzimidazol-2-yl) derivative;
3) Compounds with a "Tib" (3-(1*H*-benzimidazol-2-yl)-1,2,3,4-tetrahydroisoquinolin-2-yl) extension, in which the C-terminal amidic group of the Dmt-Tic is cyclized;
4) Compounds extended at the C-terminal with a third phenyl nucleus linked through a chain of variable length.

Since the IUPAC nomenclature of Dmt-Tic analogues is rather cumbersome, we have established short alternative names that are based on the principal chemical differences characterizing every structure. The structures, names and the short abbreviations of the ligands used in this study are given in detail in the Appendix. (see Appendix, Table A2).

4.6 DOP AND MOP HAVE DIFFERENT LEVELS OF CONSTITUTIVE ACTIVITY

Concentration-response curves describing the change of RET signal in response to the increase in concentration of the ligand were constructed for all 35 Dmt-Tic derivatives. Enriched membrane preparations obtained from the two SH-SY5Y cell lines co-expressing either the DOP or the MOP receptor tagged with Rluc in combination with RGFP-fused $G\beta_1$ were used for such experiments. The use of isolated membranes allows studying receptor-G protein coupling in the absence of arrestins, which are instead likely to interfere with the interaction when studies are performed using intact cells. Since the RET signal can be abolished upon binding of GDP to the endogenous Gα subunits, the difference in basal RET signal between absence or presence of GDP provides a quantitative assessment of the extent of receptor constitutive activation in the system.

The concentration-response curves were analyzed using a non-linear fitting procedure with a 4-parameters logistic model (see Data analysis) to compute the level of receptor-G protein coupling at maximal ligand concentration (intrinsic activity, I.A.) and the concentration of ligand at which half of the maximal effect was produced (EC_{50}). Both such parameters contain information about the efficacy of each ligand to promote the formation of the receptor-G protein complex. However, neither parameter can provide a simple measure of such a quantity, because both are affected in nonlinear fashion by the differences in affinity between receptors and G protein. Yet, while the EC_{50} (also called "potency" through this paper) additionally reflects the differences in receptor binding affinities of the ligands, the ligands intrinsic activity do not depend on binding affinity, and are thus proportional, although non linearly, to ligand efficacy.

As described in *Materials and Methods*, to facilitate the comparison of G protein coupling between the two receptors, the measured BRET ratios were converted into fractional extent of absolute receptor coupling (FRC). To do so, the maximal inhibition computed from the concentration-response of GDP (i.e. the "zero" coupling baseline) was subtracted from all experimental points, to eliminate the part of BRET signal that cannot be attributed to functional changes of receptor-G protein coupling. In addition these net changes of BRET response were divided by the maximal stimulation induced by the full agonist DADLE in either receptor system. Thus, in both DOP and MOP receptors the level of FRC spans between 0 and 1, where 0 represents the point of no interaction and 1 represents the maximal level of receptor-G protein interaction. This allows comparing the fraction of coupling induced by ligands or spontaneously formed in their absence (i.e., constitutive coupling) on the same scale in both receptors. Thus both constitutive activation and ligands intrinsic activity values are normalized to maximal effect of the endogenous enkephalin DADLE.

To illustrate how different ligands can change the FRC in the two receptors we plot typical concentration-response curves for a number of Dmt-Tic ligands along with the DADLE and GDP curves in Fig. 4. It is evident from such data that there is major difference in spontaneous coupling between DOP and MOP receptors (Fig. 4 *ab*, *shaded areas*). Constitutive coupling

was consistently measured to be 4-5 folds greater at DOP than at MOP receptor in all experiments. It is also evident from the graph that one Dmt-Tic ligand that produces strong inhibition of basal coupling in the DOP receptor (i.e. acts as an inverse agonist) stimulates FRC above the basal level in the MOP receptor (i.e. acts as a partial agonist). The reference inverse agonist ICI-174,864 can inhibit coupling in DOP receptor to a level close to the GDP baseline. Consistent with the low binding affinity of this peptide for the MOP receptor, ICI-174,864 produced little effect on FRC in MOP, and, only at very high concentrations, a small stimulatory effect was observed.

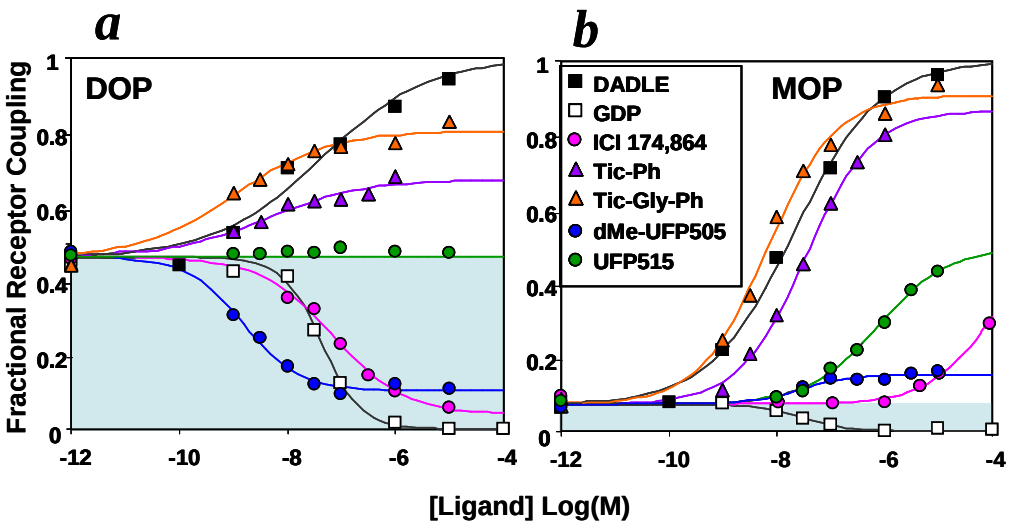


Figure4. Concentration-response curves of ligands at DOP and MOP for interaction receptors/Gβ1 recorded in purified membranes.
The graphs show the curves of some Dmt-Tic analogues obtained in (*a*) DOP and (*b*) MOP receptors. The Fractional Receptor Coupling of each ligand was computed after subtracting the maximal inhibitory effect of GDP from all the data and dividing for the maximal effect of DADLE. Best fitting theoretical curves (solid lines) were computed as described in *Materials and Methods*. The color shaded areas of graphs represent the extent of constitutive activity of both receptors, which is far greater at DOP than at MOP.

4.7 INTRINSIC ACTIVITIES AND POTENCIES OF THE DMT-TIC LIGANDS AT MOP AND DOP RECEPTORS

Concentration-response curves like those shown in Fig.4 were obtained for all 35 ligands. A curve for DADLE and for GDP was always included in each microwell plate, to allow measurement of minimal and maximal coupling in every experiment, and to correct for inter-assay variability. Values of intrinsic activity (I.A.) and potency, expressed as pEC_{50} values (i.e., the negative log of the EC_{50}) were computed by computer fitting of curves obtained in at least three independent experiments.

All data averaged from all experiments along with the computed standard errors are reported in Table 2.

In examining Table 2, note that the level of constitutive coupling (Empty receptor) measured in all the experiments at DOP and MOP receptors is shown in the first row of each I.A. column. All ligands with intrinsic activity values lower than this value are inverse agonists whereas those exhibiting larger values are agonists. A few ligands with intrinsic activity similar to the level of constitutive coupling can be considered neutral antagonists. It is clear from the inspection of Table 2 that at least 16 ligands (i.e. more than 40 % of the compounds) displayed varying degree of negative efficacy at DOP receptors, whereas no Dmt-Tic ligand had a level of intrinsic activity exhibiting a statistically significant lower value than the constitutive coupling of the MOP receptor.

Two known inverse agonists (ICI-174,864 and the alkaloid 7-Benzylidenenaltrexone (BNTX)) included in this analysis for comparative purpose, were inverse agonist at DOP in this assays, confirming that the BRET system used in this study provides data in line with previous results obtained through different methods. However both BNTX and probably also ICI-174,864 display partial agonism for MOP receptors. Likewise, all the 16 Dmt-Tic ligands that act as inverse agonists at the DOP receptors display some extent of positive efficacy at the MOP receptor.

Inspection of the pEC_{50} values reported in Table 2 also shows that most Dmt-Tic derivatives tend to exhibit more potency at DOP than at MOP receptors.

These data are in line with the findings that most Dmt-Tic derivatives tend to

Table2. Intrinsic activities (FRC) and potencies (pEC_{50}) of ligands for receptor-G protein coupling.
The values of intrinsic activity (I.A.) of each ligand were computed as Fractional Receptor Coupling (FRC) (See Data Analysis). Data are the means (±S.E.) of the indicated number of experiments (n). For ligands with intrinsic activities non measurable (N.M.) at MOP, the means of experimental values obtained at the maximum concentration of ligand (10 μM) is reported in the column Exp.E_{max}.

Ligand	DOPR/Gβ1			MOPR/Gβ1			
	I.A.(FRC) (±S.E.)	pEC_{50} (±S.E.)	n	I.A.(FRC) (±S.E.)	Exp. E_{max} (±S.E.)	pEC_{50} (±S.E.)	n
Empty receptor	0.48 (0.007)		**12**	0.09 (0.007)			**12**
DADLE	1.00 (0.018)	7.60 (0.071)	**12**	1.00 (0.039)		7.84 (0.035)	**12**
ICI 174,864	0.07 (0.013)	7.20 (0.032)	**12**	N.M.	0.43 (0.052)	<=4	**9**
Tic-Ala	0.34 (0.027)	8.31 (0.051)	**3**	N.M.	0.32 (0.036)	<=5	**3**
Tic-Asp	0.49 (0.017)	-	**3**	N.M.	0.54 (0.029)	<=5	**3**
Tic-Asn	0.25 (0.028)	7.92 (0.094)	**3**	N.M.	0.68 (0.021)	<=6	**3**
Tic-DAsn	0.27 (0.027)	8.04 (0.088)	**3**	N.M.	0.48 (0.087)	<=5	**3**
Tic-Glu	0.89 (0.009)	7.92 (0.081)	**3**	N.M.	0.30 (0.053)	<=5	**3**
Tic-DGlu	0.79 (0.010)	7.09 (0.127)	**3**	N.M.	0.26 (0.039)	<=4	**3**
Tic-Gln	0.31 (0.026)	8.35 (0.069)	**3**	N.M.	0.24 (0.039)	<=4	**3**
Tic-DGln	0.36 (0.026)	7.98 (0.139)	**3**	N.M.	0.37 (0.045)	<=5	**3**
Tic-Arg	0.81 (0.035)	6.63 (0.266)	**3**	0.76 (0.042)		6.36 (0.097)	**3**
Tic-Lys(Ac)	0.17 (0.025)	8.16 (0.146)	**3**	N.M.	0.26 (0.036)	<=5	**3**
Tic-DLys(Ac)	0.25 (0.026)	8.36 (0.063)	**3**	N.M.	0.65 (0.030)	<=6	**3**
Tic-Lys	0.48 (0.004)	-	**3**	0.74 (0.040)		6.07 (0.023)	**3**
Tic-Gly	0.63 (0.012)	7.18 (0.080)	**3**	N.M.	0.68 (0.029)	<=6	**3**
Tic-Ser	0.60 (0.026)	7.33 (0.385)	**3**	0.73 (0.030)		6.13 (0.056)	**3**
Bid-Bzl	0.71 (0.013)	7.19 (0.059)	**3**	0.84 (0.053)		7.03 (0.069)	**3**
Bid-Propen	0.59 (0.026)	7.52 (0.294)	**3**	0.85 (0.015)		7.56 (0.124)	**3**
Bid-cPropyl	0.61 (0.036)	6.81 (0.174)	**3**	0.86 (0.009)		7.36 (0.089)	**3**
UFP512	0.91 (0.032)	8.95 (0.108)	**3**	0.83 (0.019)		7.63 (0.051)	**3**
UFP502	0.90 (0.025)	8.08 (0.324)	**3**	0.82 (0.018)		7.93 (0.110)	**3**
dMeUFP502	0.74 (0.024)	8.01 (0.377)	**3**	N.M.	0.20 (0.034)	<=6	**3**
C1-Bid	0.75 (0.024)	7.63 (0.301)	**3**	0.75 (0.030)		7.29 (0.076)	**3**
dMe-C1-Bid	0.36 (0.028)	8.90 (0.176)	**3**	0.19 (0.038)		6.55 (0.069)	**3**
Gly-Bid	0.64 (0.039)	7.86 (0.428)	**3**	0.71 (0.033)		6.90 (0.069)	**3**
dMe-Gly-Bid	0.15 (0.027)	8.49 (0.223)	**3**	0.09 (0.008)		7.40 (0.133)	**3**
Tib	0.81 (0.038)	8.14 (0.366)	**3**	0.89 (0.018)		7.40 (0.160)	**3**
dMe-Tib	0.40 (0.035)	8.62 (0.211)	**3**	0.38 (0.054)		6.67 (0.063)	**3**
Tic-Ph	0.70 (0.018)	8.11 (0.200)	**3**	0.95 (0.040)		7.52 (0.078)	**3**
dMe-Tic-Ph	0.28 (0.037)	8.66 (0.057)	**3**	0.52 (0.122)		6.25 (0.377)	**3**
Tic-Gly-Ph	0.84 (0.017)	8.58 (0.247)	**3**	0.93 (0.013)		8.21 (0.038)	**3**
dMe-Tic-Gly-Ph	0.23 (0.029)	8.61 (0.151)	**3**	0.24 (0.038)		7.31 (0.030)	**3**
UFP505	0.60 (0.031)	7.71(0.523)	**3**	0.92 (0.012)		7.71 (0.061)	**3**
dMe-UFP505	0.12 (0.020)	8.46(0.199)	**3**	0.23 (0.039)		7.39 (0.204)	**3**
UFP515	0.48 (0.011)	-	**3**	0.56 (0.039)		6.43 (0.165)	**3**
UFP501	0.13 (0.031)	8.41 (0.071)	**3**	N.M.	0.06 (0.021)	<=6	**3**
TIC	0.13 (0.020)	8.37 (0.024)	**3**	0.09 (0.020)		6.31 (0.345)	**3**
BNTX	0.36 (0.039)	8.34 (0.135)	**3**	0.24 (0.040)		8.24 (0.157)	**3**

have higher affinities for DOP receptors as reported in previous studies (Balboni *et al.*, 2003,2004; Balboni *et al.*, 2005).

As mentioned before, the low potency of many compounds for the MOP receptor prevented an exact determination of EC_{50} and intrinsic activity. For such ligands under "*Experimental* E_{max} (*Exp.* E_{max})" in Table 2 we report the FRC value measured the maximal concentration of ligand tested in the experiments (10 μM).

4.8 EFFECTS OF LIGANDS ON RECEPTOR-ARRESTIN COUPLING

We were intrigued by the large number of inverse agonists found in the G protein coupling assay for DOP receptor. It has been suggested that inverse agonists of the DOP receptor are actually "biased agonists" since they were reported to stimulate ERK1/2 phosphorylation presumably via interaction of the receptor with arrestin. We thus decided to measure the activity of the Dmt-Tic analogues in a receptor-arrestin binding assay.

SH-SY5Y cells engineered with RGFP-arrestin2 (βARR2) were additionally transfected with the two luminescent receptor chimeras and used to evaluate the activity of the Dmt-Tic analogues. The receptor-arrestin coupling was measured in intact cells because βARR2 is a cytosolic protein. As previously described (Molinari *et al.*, 2010), the interaction receptor-arrestin is only marginally affected by treatment of cells with pertussis toxin, suggesting that the assessment of ligand efficacy for this interaction is not altered by the concurrent interaction of the receptor with G proteins and the consequent signaling.

All the Dmt-Tic derivatives were first tested at a single saturating concentration (10 μM) on both DOP and MOP receptors (data not shown) to rapidly screen the entire set of ligands. Concentration-response curves for enhancement of RET were constructed only for ligands showing detectable effects (i.e., enhancement of RET ratio > 0.1). (Fig. 5*ab*)

Table 3 lists E_{max} and pEC_{50} data of the ligands that exhibited a measurable effect on receptor-arrestin coupling in DOP and MOP receptors. Note that roughly only one third of the ligands were found to produce agonistic effects on this interaction (Table 3). Moreover, for the MOP receptor only three of

the ligands had EC_{50} values low enough to perform a full concentration-response curve.

Unlike G protein-receptor interaction, we found no detectable constitutive activity in DOP and MOP receptors for arrestin recruiting. Although the basal BRET signal was slightly greater in DOP than in MOP receptor expressing cells, no ligand-mediated inhibition was observed, indicating that such a difference cannot be attributed to divergences of constitutive arrestin recruiting by unoccupied receptors.

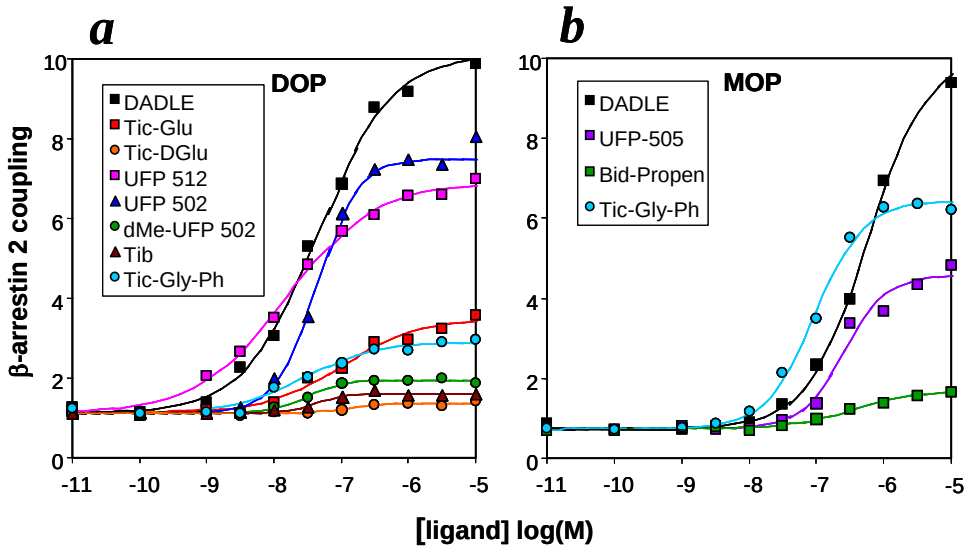


Figure5. Concentration-response curves of DOP and MOP for interaction receptors/βARR2 recorded in intact cells.
Data are expressed as β-arrestin 2 coupling, and represent the enhancement of RET Ratio as a function of increasing concentrations of the indicated ligands. Best fitting theoretical curves (solid lines) were computed as described in *Materials and Methods.*
The picture shows, in addition to DADLE, (*a*) 7 of 10 Dmt-Tic ligands with detectable effects at DOP and (*b*) all the 3 ligands detectable at MOP.

Table3. Intrinsic activities (Emax) and potencies (pEC50) of ligands for receptor-β arrestin 2 interactions.
a, Data of E_{max} and *p*EC50 measured at DOP; *b*, Data of E_{max} and pEC_{50} measured at MOP. Concentration-response curves were analyzed as described in *Materials and Methods.* E_{max} values were obtained after subtracting the basal BRET from all the experimental points and then dividing for the maximal effect of DADLE, which was present in every experiment. Data are the means (±S.E.) of the indicated number of experiments (n). NM, not measurable.

a

Ligand	DOPR/βARR2		
	Emax (±S.E.)	pEC_{50} (±S.E.)	n
DADLE	1.00 (0.015)	7.41 (0.786)	**12**
Tic-Glu	0.25 (0.007)	6.70 (0.158)	**12**
Tic-DGlu	0.02 (0.002)	6.62 (0.204)	**3**
Bid-Bzl	0.04 (0.007)	6.54 (0.092)	**3**
UFP 512	0.63 (0.037)	7.68 (0.139)	**3**
UFP 502	0.70 (0.026)	7.43 (0.059)	**4**
dMe-UFP 502	0.10 (0.006)	7.43 (0.045)	**3**
C1-Bid	0.03 (0.005)	7.03 (0.145)	**3**
Tib	0.07 (0.007)	7.26 (0.161)	**3**
Tic-Ph	0.04 (0.004)	7.23 (0.251)	**3**
Tic-Gly-Ph	0.22 (0.013)	7.74 (0.105)	**4**

b

Ligand	MOPR/βARR2			
	Emax (±S.E.)	Exp. Emax (±S.E.)	pEC_{50} (±S.E.)	n
DADLE	1.00 (0.027)		6.18 (0.148)	**8**
Bid-Bzl	N.M.	0.03 (0.002)	<=6	**2**
Bid-Propen	0.06 (0.021)		6.44 (0.071)	**3**
Bid-cPropyl	N.M.	0.06 (0.003)	<=6	**2**
UFP 502	N.M.	0.07 (0.007)	<=6	**2**
Gly-Bid	N.M.	0.04 (0.006)	<=6	**2**
Tib	N.M.	0.30 (0.063)	<=6	**2**
Tic-Ph	N.M.	0.50 (0.028)	<=6	**2**
Tic-Gly-Ph	0.57 (0.042)		6.96 (0.042)	**3**
UFP 505	0.45 (0.039)		6.54 (0.169)	**3**

4.8.1 Comparison of ligand intrinsic activities for arrestin and G protein coupling

The comparison of intrinsic activity between arrestin and G protein coupling at DOP receptors display a pattern similar to that observed in a previous investigation on opioid receptors, where a wider range of active opioid structures was examined (Molinari *et al.*, 2010). For this comparison, the Relative E_{max} of ligands for receptor/Gβ1 and receptors/βARR2 interactions

were computed after subtraction of the RET signal recorded in the absence of ligand (Fig. 6). All the Dmt-Tic agonists that were examined displayed lower intrinsic activity at arrestin than at G protein, and the extent of discrepancy was inversely related to the level of intrinsic activity.
It is also important to emphasize that none of ligands exhibiting inverse agonists in the G protein assay displayed detectable agonism (i.e. biased agonism) for arrestin.

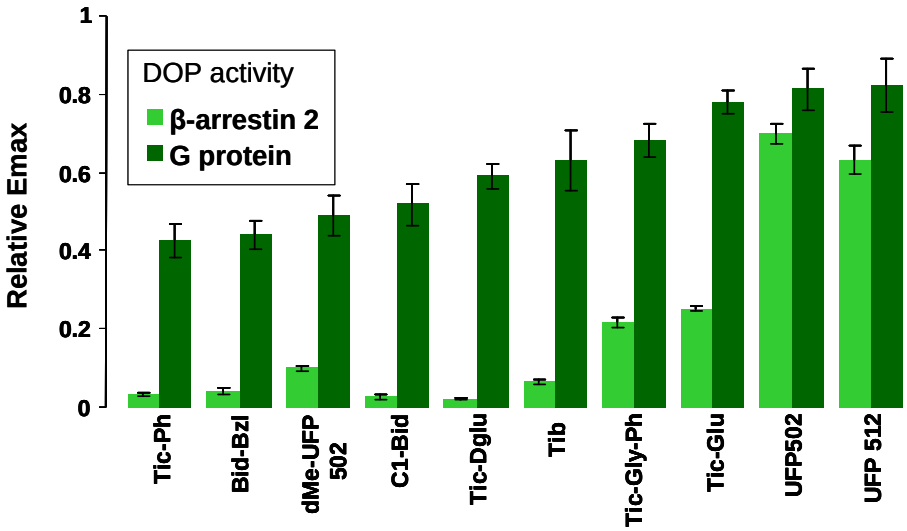


Figure6. Comparison between Relative E_{max} of ligands in interaction DOP/Gβ1 and DOP/βARR2.
The Relative E_{max} of ligands for both receptor/Gβ1and receptor/βARR2 interactions were computed after subtracting the RET signal recorded in the absence of ligand and dividing by the maximal effect of DADLE.
Only ligands with detectable E_{max} in both interactions are shown.
All ligands loose efficacy in the interaction with β-arrestin 2.

4.9 THE EFFECT OF MAGNESIUM ON THE CONSTITUTIVE ACTIVITY OF DOP

It is well known that magnesium promotes the interaction between receptor and G protein, while GDP and GTP have an opposite effect. Magnesium is

known to bind tightly to the guanine nucleotide binding site of the Gα subunits, where it forms coordination bonds involving also the anionic phosphates of the nucleotide. In addition, there is experimental evidence suggesting that Mg^{2+} might also establish lower affinity interactions with the receptor itself or at the interface between receptor and G protein.

We wondered if the higher constitutive activity of DOP receptors could be related to an altered sensitivity of the system to the effects of Mg^{2+} or rather reflect an intrinsic difference of the two GPCRs which does not depend on external agents.

To compare the effect magnesium on the G protein coupling activity of DOP and MOP receptors we measured how increasing concentrations of the divalent cation affected the BRET signal either in the absence of ligand or in the presence of saturating concentrations of a full agonist or an inverse agonist. (Fig. 7).

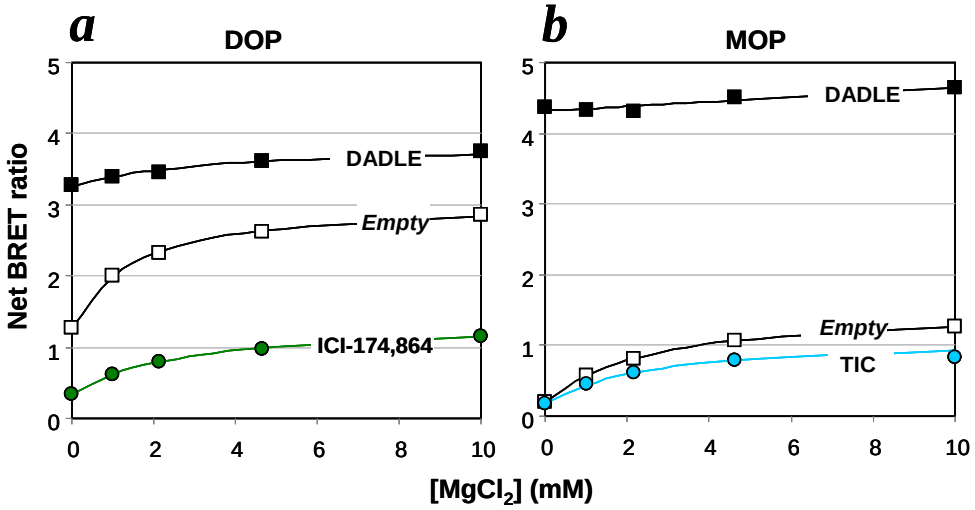


Figure7. The effect of magnesium at DOP and MOP receptors.
Data of Net BRET Ratio for empty and ligand-bound receptors in presence of increasing concentration of $MgCl_2$ were obtained subtracting by all experimental points the BRET ratio recorded for GDP at each concentration of $MgCl_2$. The effect of GDP is not shown in the graph.
Magnesium enhances the basal activity of both empty (*a*) DOP and (*b*) MOP receptors (white squares) of about 2 and 1 unit of BRET, respectively. The effect of magnesium is greater at the empty receptor than in the presence of ligands.

In both receptors Mg^{2+} increased the coupling of empty receptors (constitutive coupling) more efficiently than that of receptor occupied by ligands. In fact, the enhancement of constitutive coupling occurred at lower cation concentrations and resulted in a greater net increase of coupling (about 2 and 1 BRET units over the GDP baseline in DOP and MOP receptors), when compared to the same effects observed in the presence of ligands (Fig 7ab).

However, the large difference in constitutive activity between DOP and MOP was not altered by magnesium and remained roughly constant at all cation concentrations. Note that the level of MOP spontaneous coupling induced by the highest concentrations of Mg^{2+} is still slightly lower than the level of DOP spontaneous coupling observed in the absence of the ion (Fig 7ab).

Using DOP receptors, we also studied the effect of magnesium on the levels of G protein coupling induced by saturating concentrations of ligands exhibiting a wide range of intrinsic activities (Fig. 8).

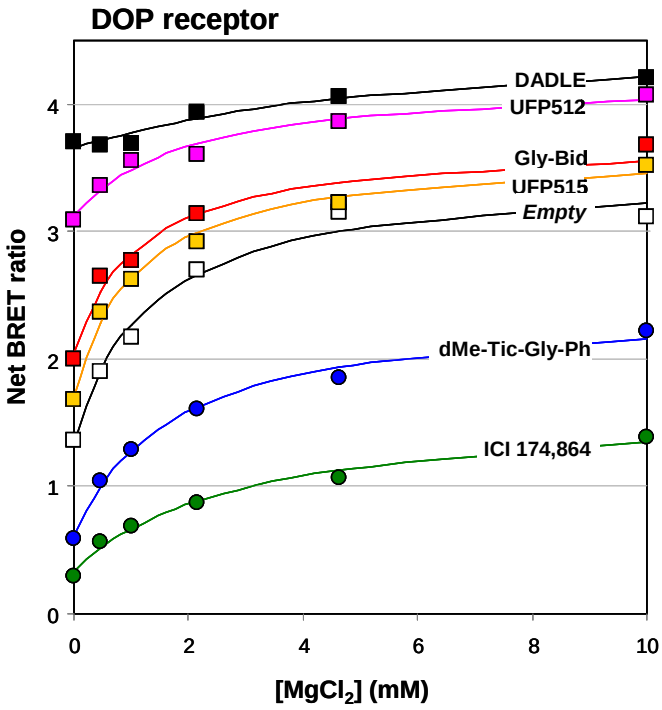


Figure8. The effect of magnesium at empty and ligand-bound DOP receptor.
Data are shown as in Figure7. The effect of magnesium was tested at DOP in the empty state and in presence of saturating concentrations of the indicate ligands, which cover a wide range of efficacy. The effect of magnesium is larger at empty receptor and gets progressively smaller as the receptor is occupied by ligands with increasing levels of either positive or negative intrinsic activity.

It is clear from this experiment that the effect of the ion becomes progressively smaller as the receptor is occupied by ligands with increasing levels of efficacy. This suggests a cooperative effect between magnesium and ligand-induced coupling, but also indicates that the two processes are not additive. As a consequence of this interaction, the net fraction of constitutive receptor activation is much larger in the presence than in the absence of magnesium.

4.10 COMPETITIVE INHIBITION OF POSITIVE AND INVERSE AGONISTS

The intrinsic activity of the Dmt-Tic derivate UFP 515 at DOP receptors was very similar to the level of receptor coupling in the empty form (see Table 2). Thus the characteristics of this ligand are close to those theoretically expected for a “pure” neutral antagonist. According to receptor theory, this ligand should competitively inhibit the effects of both agonists and inverse agonists.

To verify this prediction, concentration-response curves of an agonist (UFP 512) and of an inverse agonist (Tic-Lys(Ac)) were obtained both in the absence and in the presence of UFP 515.

As shown in Fig. 9, the neutral antagonist induced a rightward shift of the concentration-response curves of both ligands, without significantly altering their maximal effects on G protein coupling. This pattern of inhibition is diagnostic of competitive behavior and indicates that all three ligands occupy in a mutually exclusive fashion the same binding pocket of the receptor. A similar pattern of antagonism was observed on the concentration-response curves of ICI-174,864 and DADLE (data not shown).

Overall, these data indicate that Dmt-Tic analogues and their positive and negative effects on receptor-G protein coupling are mediated via occupation of the same orthologous GPCR binding site where endogenous pentapeptides enkephalins act.

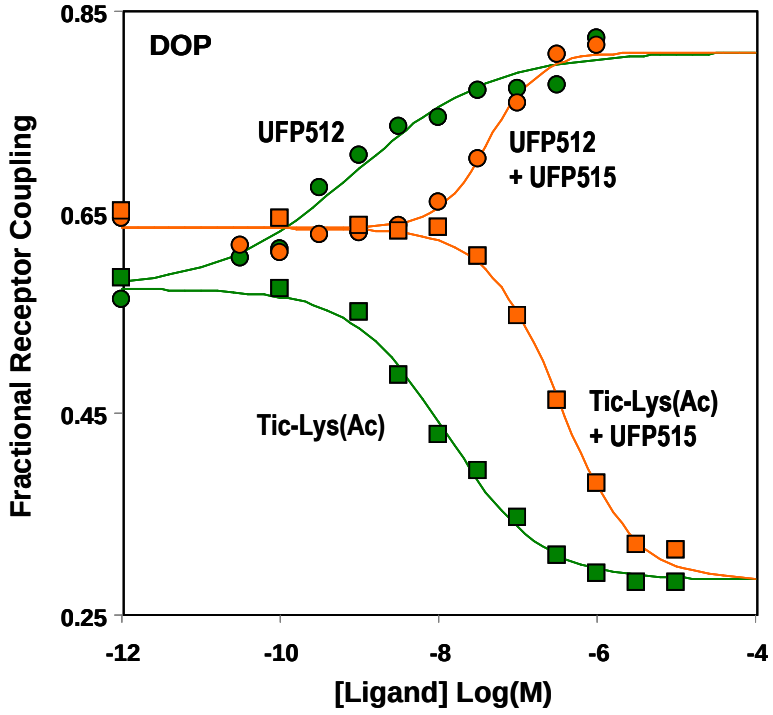


Figure9. Competitive antagonist on both positive and inverse agonist in interaction DOP/Gβ1.
Data are shown as in Figure 4.
The addition of UFP 515 (50 nM) produced about 50-fold shift in the EC_{50} of UFP 512 and about 30-fold shift in EC_{50} of Tic-Lys(Ac), leaving unchanged the intrinsic activities of both compounds.
In fact, when the curves were refitted by constraining the intrinsic activities of both ligands in absence and in presence of UFP 515, the difference of extra sum of squares was not significant.

4.11 CONSERVED INTRINSIC ACTIVITIES AT DOP AND MOP RECEPTORS

A close inspection of the ligand intrinsic activities for G protein coupling listed in Table 2 shows a puzzling paradox. When compared with the level of spontaneous coupling measured in each receptor, the data indicate that a large number of the ligands are inverse agonists with variable levels of negative efficacy for the DOP receptor, since they inhibit, to various degrees, the

constitutive activation of the receptor. In contrast, the same compounds display varying levels of positive efficacy in the MOP receptor, because they clearly increase G protein coupling above the level of constitutive activation in this receptor subtype. Therefore, when examined with respect to the level of spontaneous coupling, most Dmt-Tic peptides appear to undergo a dramatic "inversion" of efficacy on passing from MOP to DOP receptors (Table 2).

However, if we compare the intrinsic activities of the ligands directly across the two receptors, regardless of the receptor differences in spontaneous coupling, it is evident that there is a remarkable correspondence. In fact, the majority of ligands show very similar level of intrinsic activity in the two receptors. Since the intrinsic activity for G protein coupling is related to efficacy, this direct comparison suggests that most ligands have similar or identical efficacy at the two receptors.

To further analyze such conflicting deductions, all the ligand intrinsic activities for MOP and DOP receptors were plotted in Fig. 10 (Note, however, that ligands with non-measurable intrinsic activity values in the MOP receptor cannot be included in this analysis).

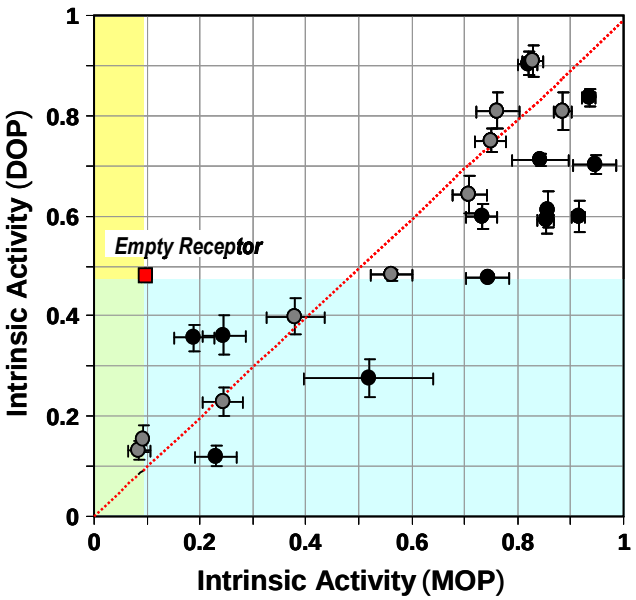


Figure10. The comparison of intrinsic activities at DOP and MOP receptors in G protein coupling. The intrinsic activity values for each ligand measurable both at DOP and MOP from Table2 are plotted against each other. Ten ligands (grey dots) displayed not statistically different intrinsic activities at both receptors; fourteen ligands (black dots) displayed differences, but the divergence in intrinsic activity was small. Colored areas indicate the extent of constitutive activity in each receptor.

For at least ten of the 24 analogues under comparison, intrinsic activity values were not statistically different in the two receptors (grey dots in Fig. 10). But also in the remaining ligands that display significant differences, the divergences in intrinsic activity values between the receptors are very small. The overall trend implied by the data is that Dmt-Tic ligands tend to promote similar levels of receptor-G protein coupling at DOP and MOP receptors. Indeed, regression analysis of the entire set of data indicates a highly significant correlation between DOP and MOP intrinsic activities.

To gain more insight, we analyzed a subset of the ligands with similar intrinsic activity in both systems. Concentration-response curves were obtained in parallel assays using DOP and MOP membranes.Simultaneous global fitting of the entire set of curves for both receptors shows that the intrinsic activity values of the ligands are perfectly conserved in the two receptors, regardless of whether the ligand changes the coupling below the DOP or above the MOP basal activity level. (Fig. 11*ab*).

Thus, the reason why some ligands show as inverse agonists at DOP and positive agonists at MOP seems to depend on the much greater level of spontaneous coupling existing in DOP compared to MOP receptors.

4.12 LIGAND STRUCTURAL FEATURES RELATED TO INVERSE AGONISM

On comparing the structures of Dmt-Tic ligands (Appendix, Table A2) with the intrinsic activity data summarized in Table 2, we identified two types of modifications of the ligand molecule that are apparently involved in the emergence of inverse agonism. Interestingly, such modifications involve the opposite ends of the peptide scaffold of Dmt-Tic.

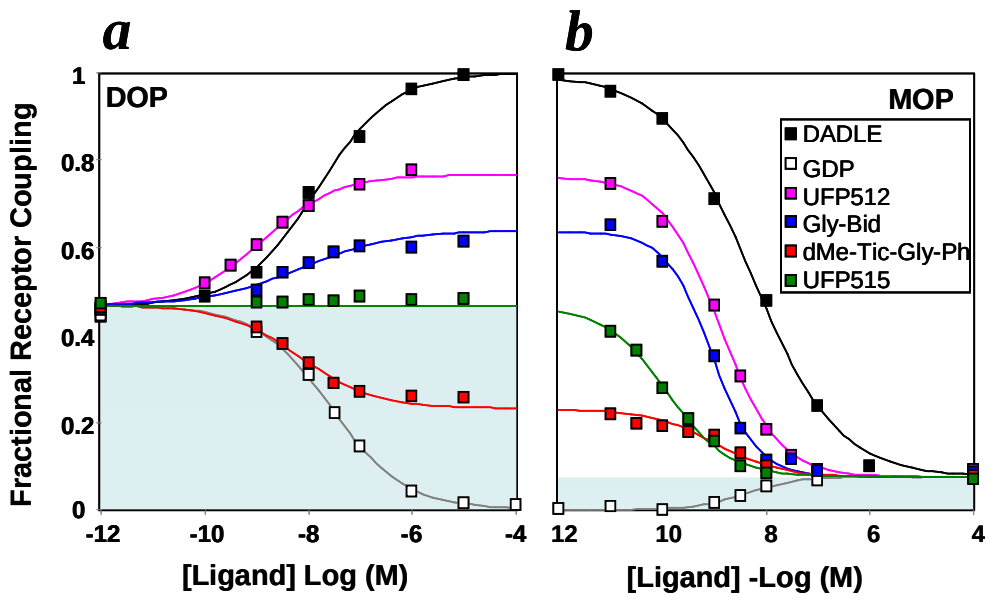


Figure11. Concentration-response curves for receptors-G protein coupling of ligands with identical intrinsic activity at DOP and MOP.
Curves for DOP are represented on left (*a*), where Fractional Receptor Coupling (see Fig.4) is plotted as function of logarithm of molar concentration. Curves for MOP are represented on right (*b*), where Fractional Receptor Coupling is plotted as function of negative logarithm of molar concentration. This specular representation of data facilitates the comparison of the intrinsic activity of each ligand in both receptors, which is identical. Dashed lines represent the best fit of curves, obtained as described in *Materials and Methods*. These curves were re-fitted by constraining the intrinsic activity of each ligand in both receptors. The difference of extra sum of squares was not significant after constraining.

4.12.1 Position of an anionic carboxyl group at the ligand C-terminal

The first modification regards the series of Dmt-Tic analogues characterized by the extension of the C-terminal with a third amino acid residue. In this group of ligands, the transfer of an anionic carboxylate group from the side-chain to the C-terminal of the molecule always reduces ligand intrinsic activity below the level of constitutive receptor coupling, thus generating inverse agonists at DOP receptor (Fig. 12*a*). For example, the compound Tic-Glu-NH_2 (COOH on the side-chain) is a partial agonist, but the closely

related analogue Tic-Gln-OH, in which the carboxylic group is relocated to the terminal end, displays strong inverse agonism. The optical enantiomeric forms of these ligands (Tic-DGln and Tic-DGlu) show exactly the same effect (Fig. 12*b*). Likewise, replacement of Asp-NH2 with Asn-OH also converted a close-to-neutral antagonist into inverse agonist (Fig. 12*b*). Because such compounds have very low potency at MOP receptor, no intrinsic activity data could be obtained, and the comparative effect of such a modification cannot be evaluated.

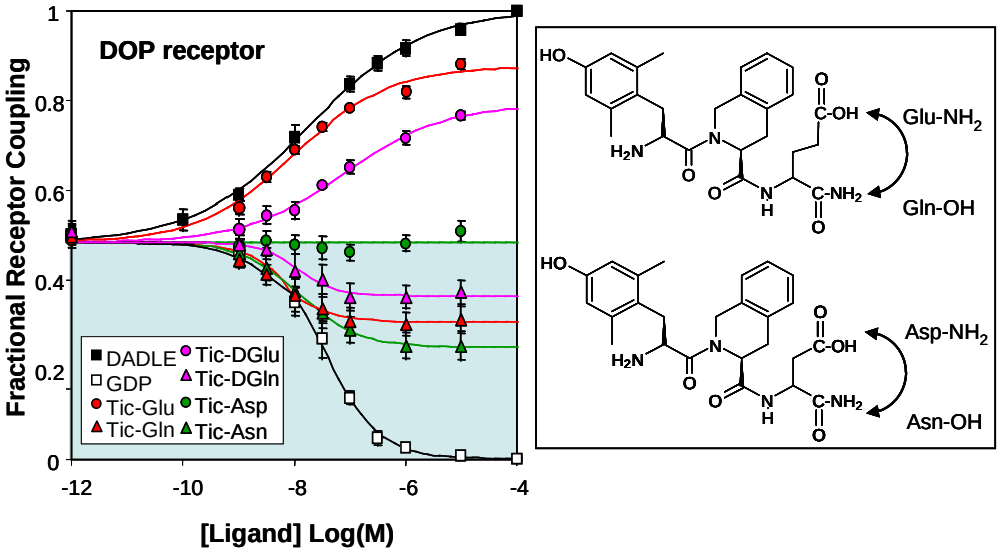


Figure12. Shift of a free carboxylic group at the C-terminus of the Dmt-Tic.
a, Concentration-response curves for Dmt-Tic ligands extended with amino acid residues obtained at DOP are shown in the graph. Ligands that are positive or neutral agonists (colored circles) have a carboxylic group on the side chain of the molecule. Ligands that are inverse agonists (colored triangles) have a free carboxylic group at the C-terminal. The shift of a free carboxylic group from the side chain to the C-terminal of the ligands, as schematically illustrated in (*b*), generates inverse agonists at DOP receptor.
The efficacies of these ligands are not measurable at MOP receptors (see Table 2).

4.12.2 Dimethylation of the ligand N-terminal

The second important modification is the dimethylation of the N-terminal of the Dmt-Tic. In this case, a comparison between both opioid receptors is possible because the intrinsic activities of most of the ligands carrying such modifications are measurable in both systems.

To compare the change of activity due to methylation, we plot the intrinsic activity values of the dimethylated and non-methylated analogues measured in DOP receptors as a function of the same values measured in MOP receptors (Fig. 13*a*). In this graph, arrow vectors trace the distance between the activity of unsubstituted and substituted analogues, and thus indicate the joint variation of intrinsic activity caused by the modification of the ligand in the two systems. Such vectors have different lengths, because dimethylation has a different effect depending on the peptide analogue to which it is applied.

Yet, all vectors show similar slopes and are all roughly parallel to the line of perfect correlation. This means that both direction and magnitude of the loss of intrinsic activity caused by N-methylation is perfectly conserved in DOP and MOP receptors. In other words, this modification produces an identical reduction of efficacy, and presumably acts through and identical mechanism, in the two receptors.

However this identical loss of ligand efficacy leads us to a quite different interpretation if we relate the change of ligand intrinsic activity to the respective levels of spontaneous coupling in the two receptors. In fact, dimethylation lowers the intrinsic activity of most ligands below the empty receptor level in the DOP receptor, thus it appears to "reverse" from positive to negative the efficacy of ligands. In contrast, the same reduction of intrinsic activity at the MOP receptor results in levels that are still above the constitutive receptor coupling, therefore methylation seems to merely diminish the extent of partial agonism of the ligands in this case.

These data further strengthen the notion that Dmt-Tic analogues have similar efficacy in the two receptors, despite the divergent directions that the changes of intrinsic activity take with respect to each empty receptor baseline.

We also analysed the effect of methylation on the pEC_{50} of the ligands (Fig. 13*b*). In this case the effects were clearly divergent in the two receptors. At

the DOP receptor the double alkylation increased the potency of most ligands, whereas at the MOP receptor there were minor and less consistent effects. These data are in line with previous investigations showing that methylation produces significant and often selective increases of the Dmt-Tic ligand binding affinity for the DOP receptor.

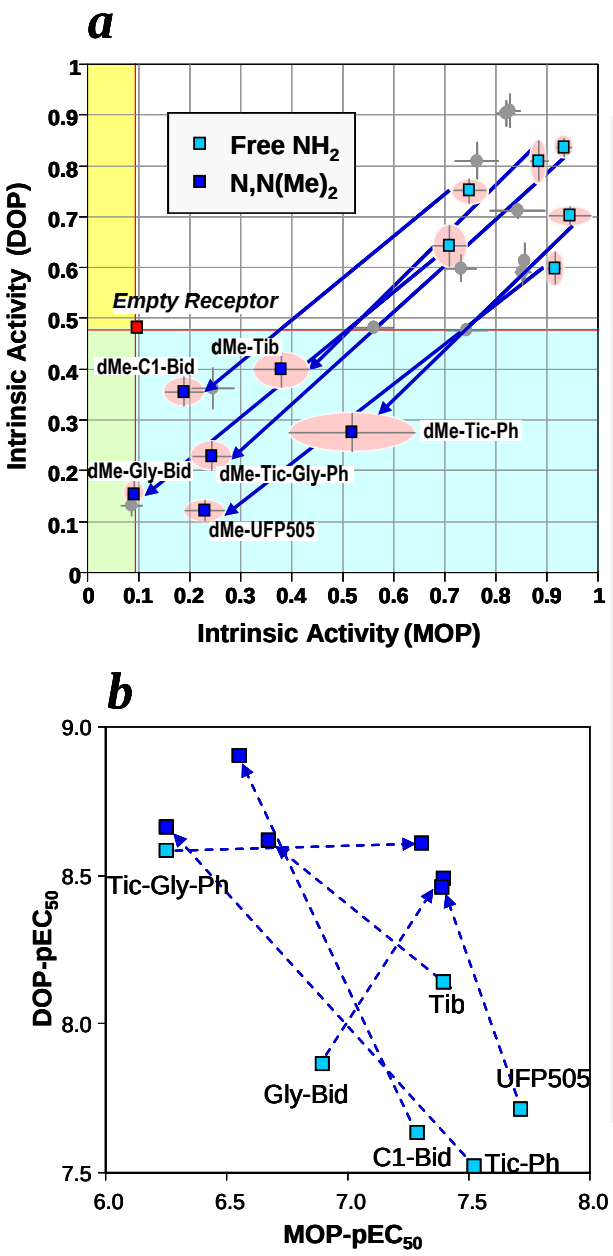


Figure13. The effect of dimethylation at the N-terminus of the Dmt-Tic.
The cyan squares represent the non-methylated ligands, while the blue squares represent the dimethylated ligands.
The arrows connect the unsubstituted and substituted form of the same ligand.
a, Data are plotted as in Fig.10. The double methylation of the N-terminal of ligands produces loss of efficacy at both receptors. The effect is identical for DOP and MOP receptors (arrow directions and slopes). The similar loss of efficacy produces inverse agonism only in the DOP receptor because the greater level of constitutive activity.
b, The pEC_{50} of the same ligands of (*a*) at both receptors are plotted against each other. The double alkylation increased potency in 5 out of 6 ligands at DOP receptor, but produced reductions or enhancements of comparable size in the ligands pEC_{50} at MOP receptors (arrow direction).

4.13 THE SHIFT OF GDP APPARENT AFFINITY AND LIGANDS EFFICACY

As shown in previous sections of this thesis, GDP can inhibit in a concentration-dependent fashion receptor-G protein coupling both in the absence or the presence of ligand. These concentration-response curves, and particularly the IC_{50} of the nucleotide that can be computed from them, have an important meaning. In fact, at any GPCR system GDP and receptor agonist act as two allosteric effectors that are linked by a negative cooperative effect through their distinct binding sites in the receptor-G protein assembly. GDP binding reduces the agonist apparent affinity and, vice versa, the apparent affinity of GDP is reduced by agonist binding. The size of this cooperative effect provides a measure of agonist efficacy.

Taking advantage of the BRET assay, we thus measured the efficacy of Dmt-Tic analogues at MOP and DOP receptors using this alternative strategy. Concentration-response curves of GDP were obtained on empty receptors and in presence of saturating concentrations of several different ligands and the data measured in DOP and MOP receptors can be compared.

Fig. 14*ab* shows an example of the GDP inhibitory curves obtained in DOP and MOP receptors. The apparent Ki (i.e. the $IC_{50)}$ of the nucleotide is progressively shifted to the right (i.e. its values becomes larger) in the presence of ligands displaying greater levels of intrinsic activity, and this pattern is virtually identical in MOP and DOP receptors. Note that the agonists with the highest level of intrinsic activity also diminish the maximal inhibitory effect of GDP, in line with the allosteric nature of this interaction.

Moreover, these experiments also allow quantifying the effect that the empty receptor has on the apparent Ki of GDP, i.e.: the “efficacy” of constitutive receptor activation. Although the difference in the inhibition of constitutive coupling by GDP between MOP and DOP is small, we averaged the data from many experiments to compare the Ki values measured in the two receptors. As shown in Fig. 15, the apparent Ki of GDP for inhibiting spontaneous coupling is larger at DOP than at MOP receptors, consistent with the much greater level of constitutive activation in the former.

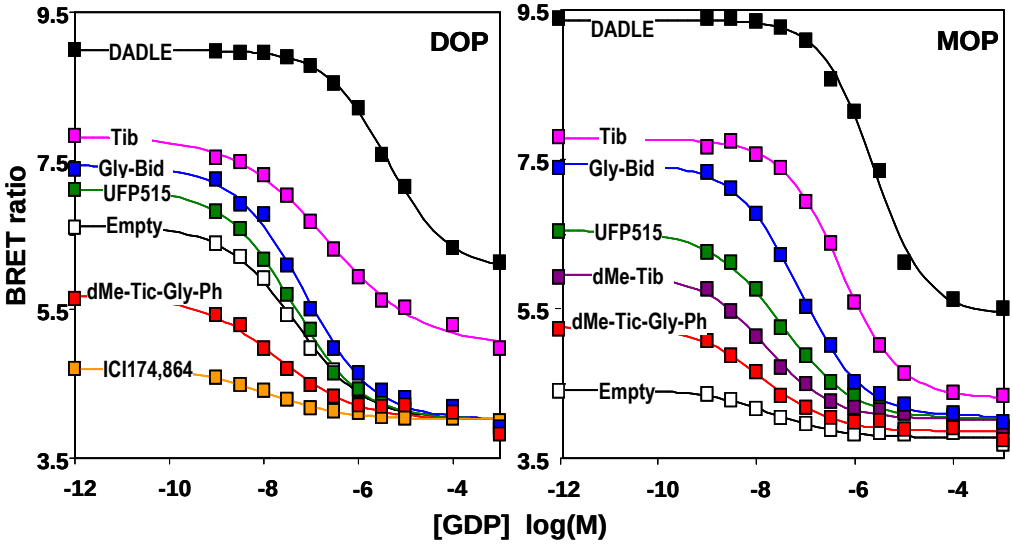


Figure14. The shift of GDP curves on empty and ligand-bound DOP and MOP receptors.
GDP curves were obtained at (*a*) DOP and (*b*) MOP receptors by recording the change of BRET ratio in presence of saturating concentrations of the indicate ligands and at the empty receptors (white squares). The potency of GDP decreases with the enhancement of intrinsic activity of ligands. For ligands with great values of intrinsic activity (e.g. DADLE, black squares), GDP is unable to bring the receptor-G protein complex to basal level.

4.13.1 Ligand intrinsic activity and the shift of GDP apparent affinity

A subset of ligands covering the full range of efficacy was chosen to examine their effects on the Ki for GDP, which was computed and averaged from several experiments similar to that illustrated in Fig. 14. In Fig. 16 we compare the ability of ligands (i.e. their intrinsic activity) in promoting fractional receptor coupling with the corresponding effect on the Ki of GDP measured in MOP and DOP receptors. In this graph, GDP apparent affinity (*y-axis*) is given as *pKi* (i.e. the negative log of the IC_{50}) while the ligands intrinsic activities (*x-axis*) as FRC were taken from Table 2. These relationships are very similar in the two receptors and show a characteristic curvilinear trend. In the lower range of intrinsic activity, large differences of

FRC correspond to minimal changes of GDP Ki, whereas the opposite occurs at larger values of intrinsic activity.

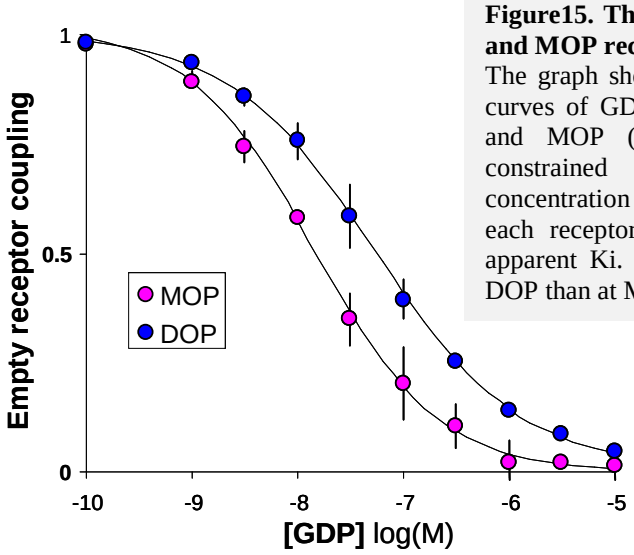


Figure15. The Ki of GDP at empty DOP and MOP receptors.
The graph shows the averages of different curves of GDP at empty DOP (blue dots) and MOP (pink dots) receptors, both constrained between 0 (maximal concentration of GDP) and 1 (basal level of each receptor) to appreciate the shift of apparent Ki. The apparent Ki is larger at DOP than at MOP.

This is due to the fact that the free-energy associated with the cooperative effect existing between ligand and GDP represents a smaller fraction of the overall free-energy change measured through the Ki of GDP. Thus, when this cooperativity is small (low ligand efficacy) its effect on the overall Ki is negligible and comparable to the experimental error of the measurement, whereas at high levels of ligand efficacy the effect of cooperativity on Ki is well detectable.

There was a subtle difference between MOP and DOP receptors in the 0.3-0.6 range of intrinsic activity, but it's hard to assess if this difference can be accounted by experimental error or represent an intrinsic difference between the two receptors, potentially related to their divergence in constitutive activity. Note that both in MOP and DOP receptor the intrinsic activity of the empty receptor state was well aligned with the overall relation described by the intrinsic activity of the ligands.

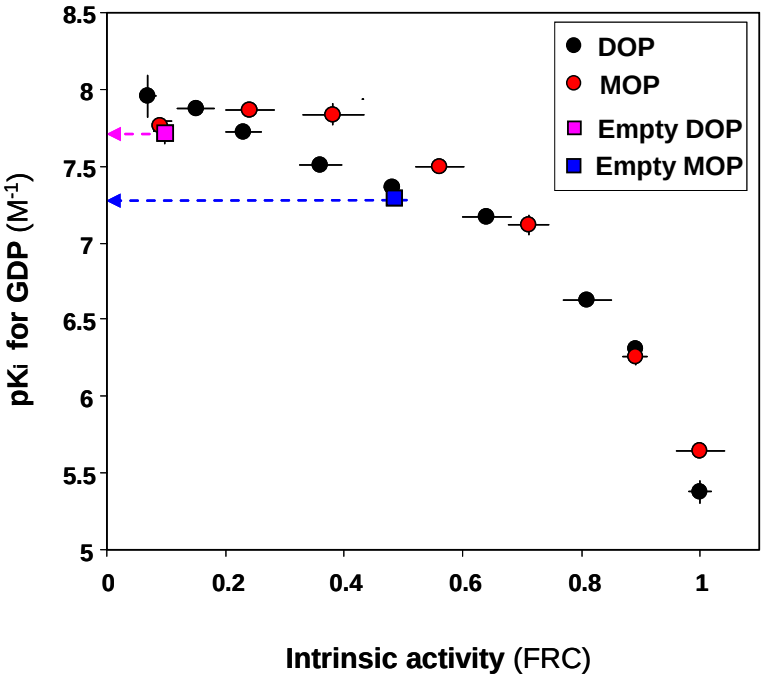


Figure16. The comparison between the apparent affinity of GDP and the intrinsic activities of ligands.
The values of *p*Ki computed by GDP curves obtained in presence of saturating concentrations of ligands are plotted in function of the intrinsic activities of ligands (Table2). The trend is similar for both DOP (black dots) and MOP (red dots) receptors. The major difference is for empty DOP (blue square) and MOP (pink square) and reflects the difference in apparent Ki of GDP at both receptors (see Fig.15).

To obtain a more direct measurement of the cooperative effect between ligand and GDP, we computed for each ligand the ratios of GDP Ki measured in the absence and in the presence of saturating concentration of the ligand. In fact, when two ligand binding processes are linked through cooperativity, the "shift" in affinity of one ligand caused by raising the concentration of the second ligand to "infinite" values directly measures the overall free energy-coupling existing between the two ligands binding sites.
As shown in Fig. 17, these free-energy coupling values measured for the same ligands in MOP and DOP receptors display a linear relation with unitary slope. However, the line is shifted from the zero point.

The unitary slope of this relationship indicates that any given change of ligand structure results in identical changes of negative allosteric coupling (i.e. of ligand efficacy) in the two proteins. However, the nonzero intercept indicates that all values of free-energy coupling of the ligands at the DOP receptor are uniformly smaller and shifted away from those at the MOP receptor by a constant factor. This factor (~ 1 *RT* unit of free-energy) has the same size of the shift in GDP Ki induced by the constitutive activity of the DOP receptor (Fig.15).

Thus, this analysis suggests that there is an energetic link between constitutive activation of the receptor and the efficacy of all ligands.

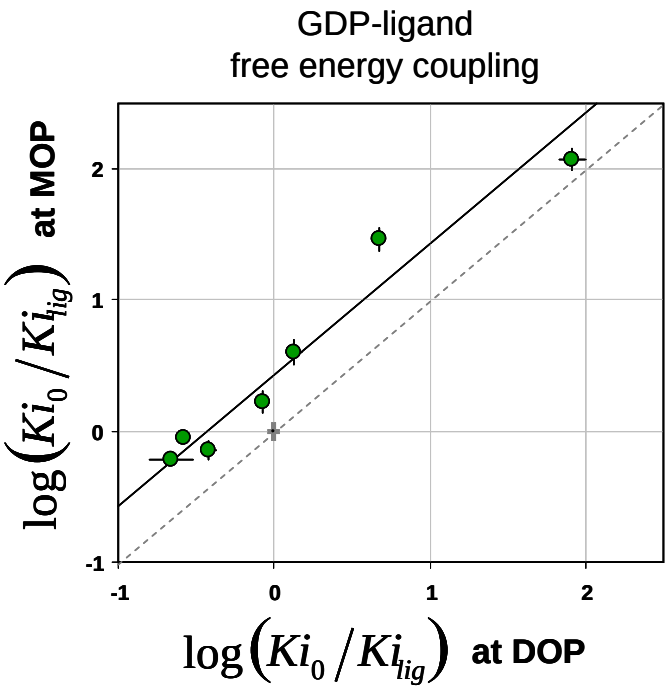


Figure17. The allosteric effect of ligands on GDP.
The free-energy coupling values for DOP and MOP are plotted against each other. Data are expressed as$\log(Ki_0 / Ki_{lig})$, where Ki_0 is the apparent affinity of GDP measured in absence of ligand, and Ki_{lig} is the apparent affinity of GDP measured in presence of saturating concentrations of each ligand.
The allosteric effect of some ligands, calculated by the shift of GDP curves, is perfectly conserved in both receptors. Data are linearly related with unitary slope (solid line), but shifted from the zero point (dashed line) of a constant factor (~ 1 RT unit), which reflects the difference of the two receptors in the empty form (see Fig.15).

5 DISCUSSION

In this thesis I studied the G protein coupling activity of DOP and MOP receptors and the effect of 35 congeneric opioid analogues derived from the Dmt-Tic pharmacophore (Balboni *et al.*, 2002a; Balboni *et al.*, 2003; Balboni *et al.*, 2005). Unlike previous investigations, the interaction between opioid receptors and G proteins was studied here directly, using a BRET technique that can monitor the change of association between the two proteins in isolated membranes.

The data obtained by BRET reflect the direct interaction between receptor and transduction protein, therefore they are not affected by mechanisms of amplification or attenuation that are caused by the cascade of biochemical reactions existing in signal transduction pathways, and which confound the interpretation of the results in studies based on the indirect measurement of biological signals. The BRET assay used in this study is based on the change of RET emission due to altered proximity between the luminescent donor located on the receptor C-terminus and the fluorescent acceptor fused to the N-terminus of Gβ1. As we have demonstrated in earlier work (Molinari *et al.*, 2010), this interaction is mediated by the endogenous Gα subunits in the plasma membrane of transfected cells. Since receptor agonists enhance RET signals, this indicates that the increased microassociation of the receptor-G protein complex mediated by Gα brings the receptor C-terminus and the N-terminus of Gβγ at closer distance, thus allowing a more efficient energy transfer between the fused protein reporters Rluc and RGFP.

In principle the BRET assay used in this study is similar to assays based on the binding of radiolabeled GTPγS in membranes, since both determinations can give direct information about receptor-G protein interaction. However, the determination of intrinsic activity of ligands measured in a typical GTPγS binding assay is strongly biased by the high concentration of GDP that is used in this reaction, and which is required to achieve a favorable signal-to-noise ratio. In contrast the BRET assay in the membranes can be studied under very simple reaction conditions.

In addition there are two important differences between the two types of determinations.

First, GTPγS binding gives information about the affinity of the nucleotide binding site of Gα, while BRET reports the formation or the rearrangement of receptor-Gα-Gβγ complexes.

Second, since the BRET signal originating from the spontaneous interaction between receptor and G protein is completely abolished by GDP, we have a simple way to quantify the level of receptor coupling in the absence of ligand (constitutive activation). Therefore, while in GTPγS binding we are limited to define the activity of receptor as the difference between the absence and presence of ligand, in the BRET assay we can measure and quantify both the extent of constitutive G protein coupling and ligand-induced coupling on the same scale, by taking the difference between the recorded signal and that measured at saturating concentration of GDP. As long as the amount of luminescent and fluorescent proteins is similar across different cell membranes, these data allow comparing different receptor for both constitutive and ligand-induced activities.

The receptor-G protein coupling behaviour described in this BRET membrane assay closely resembles the behaviour of ternary complex formation recently described in studies on purified β_2-adrenoceptor and heterotrimeric Gs (Chung *et al.*, 2011; Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b). In both cases, agonists cause association of the complex through positive cooperativity, while guanine nucleotides (either GDP or GTPγS) promote destabilization.

In this study, we also examined a set of constrained ligand structures based on the peptidomimetic scaffold of the Dmt-Tic, to test their ability to alter G protein coupling in DOP and MOP receptors. In GPCRs studies, the availability of ligand congeners exhibiting gradual variations from positive to negative values of efficacy is a rare event. Even when several inverse agonists are known for a given GPCR subtype, they often belong to a different chemical class than the agonist or the neutral antagonist. We suspected on setting up this study that the vast variety of Dmt-Tic analogues that have been synthesized thus far could hide a “treasure chest” of still

unknown inverse agonists for the opioid receptor. The results of this thesis prove this suspicion to be true.
There are several novel results reported in this PhD thesis.

First, we found that the major difference between wild-type DOP and MOP receptors is the extent of constitutive activation. The spontaneous coupling of the DOP receptor to G proteins was consistently 4-5 times greater than that observed for the MOP receptor. This difference is so large that we might indeed consider wild type DOP receptor as a constitutively-active mutant of the MOP receptor. We also found that this difference is not the results of an increased sensitivity of the DOP receptor to allosteric ligands. In fact our studies on the effect of Mg^{2+} ions demonstrate that the gap in constitutive activation between DOP and MOP receptors remains constant, despite the strong effects that this allosteric regulator has on the G protein coupling of both receptors. Therefore, constitutive activation reflects an intrinsic difference in structural properties between DOP and MOP receptors.

Second, we report in this study 16 new ligand structures that are capable to act as inverse agonists at the DOP receptors and display a remarkable variation in the extent of apparent negative efficacy. We show that these ligands exert their inverse agonistic effect by occupying the same binding site of enkephalins and other opioid ligands, as demonstrated by competition with a pure antagonist. Thus, inverse agonism is a frequent event in ligands based on the Dmt-Tic scaffold, and this suggest that this pharmacophore is an ideal experimental platform for further investigations to gain more insight on the structural-activity relationships of negative efficacy for opioid receptors.

Third, using a BRET-based assay of receptor-arrestin interaction, we found that none of the inverse agonists for the DOP receptor-G protein interaction, nor any of the studied analogues, displayed “biased” agonism for arrrestin. Therefore, the notion that inverse agonists are agonists for arrestin interaction, previously suggested on the basis of ERK signaling studies (Oligny-Longpre *et al.*, 2012), is not generally true. We also found no evidence of constitutive activity of the DOP receptor in interacting with arrestin, suggesting that whatever structural change is responsible for the

increase in constitutive activation of the DOP receptor, such a change affects specifically receptor-G protein interaction.

Fourth, we found two main structural modifications of the Dmt-Tic scaffold that appear to be correlated with the occurrence of inverse agonism in the ligands. Interestingly, these modifications engage opposite sides of the molecule and involve opposite ionic charges. In fact, either the position of an anionic carboxyl group in the C terminal or the double methylation of the cationic amine in the N-terminal was involved in conferring the strongest level of inverse agonism observed for Dmt-Tic ligands in this investigation. This suggests that electrostatic interactions in the receptor binding pocket play a major role in determining negative efficacy. Perhaps the advantage of the Dmt-Tic scaffold compared to conventional peptides in generating ligands with inverse agonism lies in its relatively constrained and rigid structure, which may allow proper positioning of those charges and facilitate their interactions with residues that are probably located on different transmembrane domains of the TM bundle.

However, the most interesting result of this investigation is a surprising new feature of opioid inverse agonism. Ligands that move the interaction into opposite directions from the ligand-free receptor baseline – thus apparently showing opposite efficacy in the two receptors – can induce equal extent of G protein coupling in both receptors, i.e. they show identical intrinsic activity in the two systems. Therefore, our deductions about ligands efficacy depend on whether we evaluate the effects as changes relative to the active level of each empty receptor, or as absolute levels of G protein coupling in the two receptors. According to the former, structures that are negative antagonists for the highly constitutively active DOP receptor become agonists for the low constitutively active MOP receptor. According to the latter, all ligands appear to show the same efficacy in both receptors.
One possible explanation for this conflicting interpretation is that the correspondence of ligands intrinsic activities in MOP and DOP receptor is entirely fortuitous. Despite the similarity, DOP and MOP are two different proteins. Therefore, is perfectly possible that identical ligand structure can produce opposite effects on receptor activation. If so, the ability of ligands to

change receptor activity towards divergent directions in MOP and DOP reflect indeed opposite efficacy in the two systems.

However, the global correlation of ligand intrinsic activity data in MOP and DOP receptors is highly significant, which makes it unlikely that such a correspondence may be a random event. Moreover, there are mechanistic considerations that point to the same conclusion. The identical loss of efficacy caused by N-terminal methylation of the ligands in the two receptors and the overlapping relationships between ligands intrinsic activities and apparent affinity of GDP observed in MOP and DOP receptors, both suggest that the efficacy of Dmt-Tic ligands is perfectly conserved in the two subtypes.

The overall pattern of agonism and inverse agonism documented in this study is in contrast with the prediction of models that are currently used to understand inverse agonism, such as the ternary complex model and/or the extended two-state TCM model. In fact, according to this theory, a change of constitutive activity cannot alter the ability of a given ligand to change G protein coupling above or below the baseline of the empty receptor. For example, ligands that inhibit basal receptor coupling in DOP should still inhibit constitutive activity in MOP, even though in this receptor it would be difficult to distinguish their relative effects because of the narrow range of spontaneous receptor activation.

The data suggest that the mechanisms of receptor activation exerted constitutively or induced by the ligand are different; consequently, the process that leads to receptor coupling in the empty state may not be related to that triggered by occupation of the receptor binding site by a ligand. Yet, in contrast with this indication, we have shown in this study that the effect of empty receptors on GDP affinity is quantitatively identical to that observed for ligands that induce a corresponding level of G protein coupling.

Perhaps the most enlightening result that might provide a clue in solving these questions is shown in the last figure of this thesis (Fig. 17). Quantitative measurements of the free-energy coupling underlying the negative cooperativity between ligands and GDP show that any change of ligand structure results in identical changes of the cooperative effects of the ligands in the two receptors, confirming that the mechanisms of ligand-induced

receptor activation are perfectly conserved in the constitutively active and inactive receptor. However, they also show that in the constitutively active DOP the cooperative effects of all ligands are uniformly reduced by a constant factor which is energetically equivalent to the cooperative effect underlying the constitutive activation of the DOP receptor. This suggests that the constitutive activation of the DOP receptor can reduce a common "energetic barrier" that all ligands must surmount in inducing the G protein coupling state of the receptor.

It is hard to evaluate whether our results represent a general feature of the constitutive activity of all GPCRs, or rather reflect a particular mode of interaction that exists between analogues derived from the Dmt-Tic pharmacophore and opioid receptors. Additional studies in other constitutively active GPCR subtypes will be necessary. Also, X-ray analysis of crystals of opioid receptors bound to ligands derived from the Dmt-Tic scaffold will be mandatory to unravel the structural basis of the mechanism that makes such ligands powerful inverse agonists.

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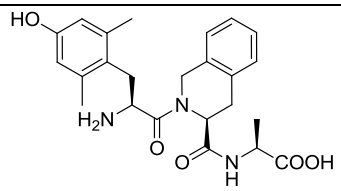
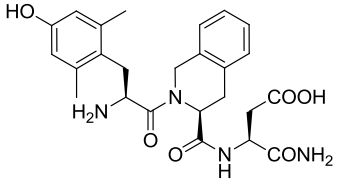
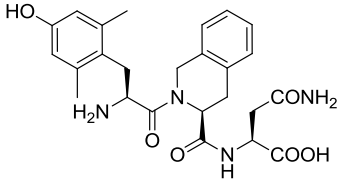
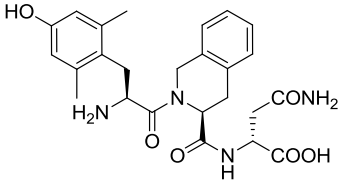
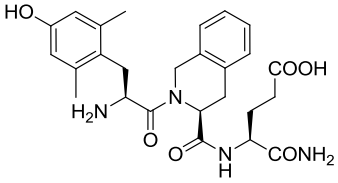
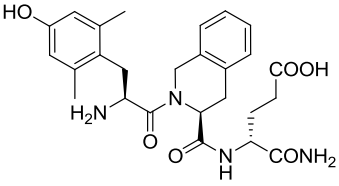
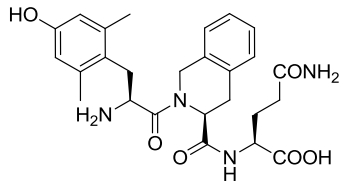
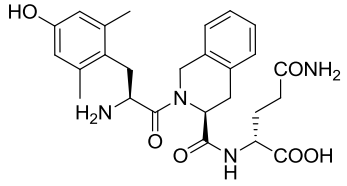
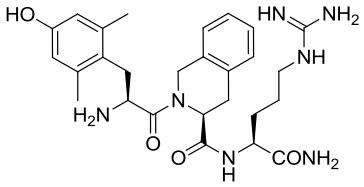
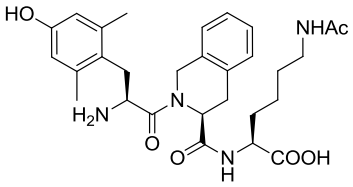
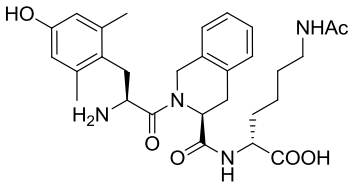
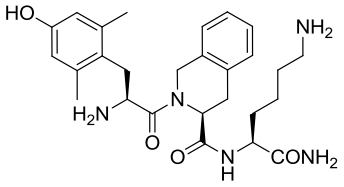
APPENDIX

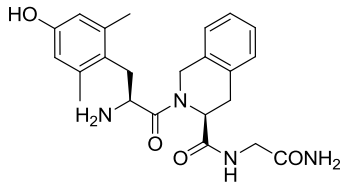
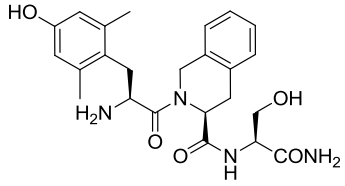
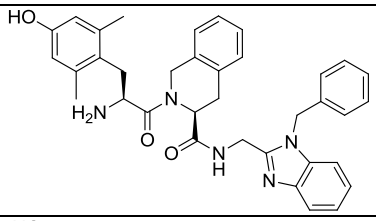
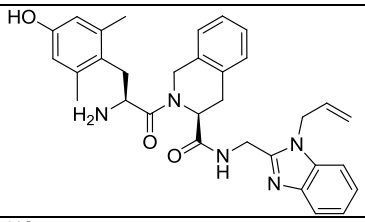
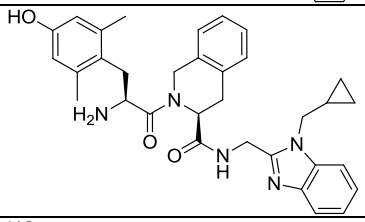
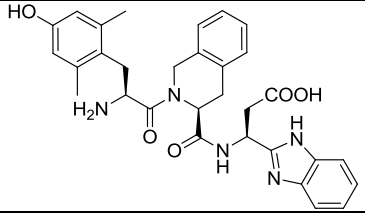
Table A1. Direct effects of ligands at luciferase activity and at BRET Ratio.
The effect at luciferase activity is expressed as the mean (± S.E.) of the percent of inhibition obtained for each ligand. The effect at BRET Ratio is expressed as the mean (± S.E.) of the percent of stimulatory (positive values) or inhibitory (negative values) effects obtained for each ligand.

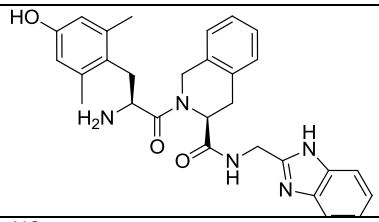
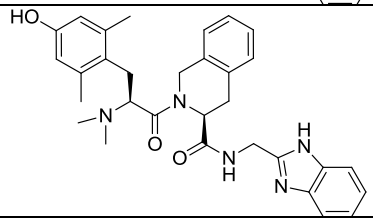
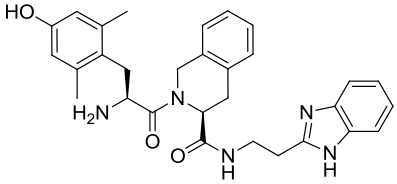
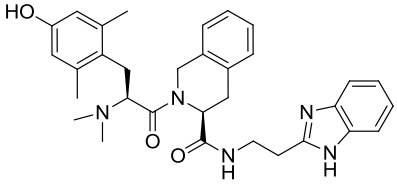
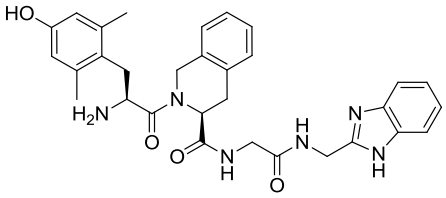
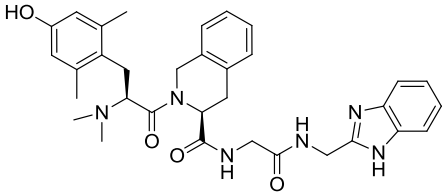
Ligand	% of luciferase inhibition (± S.E.)	% of effect at RATIO (± S.E.)
DADLE	0.15 (0.50)	-0.02 (0.02)
GDP	0.68 (0.76)	0.75 (0.06)
ICI 174,864	31.07 (3.45)	-1.06 (0.81)
Tic-Ala	25.95 (2.80)	-0.72 (1.03)
Tic-Asp	20.93 (3.32)	-2.68 (1.00)
Tic-Asn	23.38 (0.67)	-0.41 (0.34)
Tic-DAsn	26.34 (1.25)	0.84 (0.78)
Tic-Glu	23.96 (2.68)	0.44 (0.06)
Tic-DGlu	21.58 (2.22)	-0.56 (1.68)
Tic-Gln	20.83 (1.26)	1.00 (0.62)
Tic-DGln	24.47 (2.42)	-1.75 (2.43)
Tic-Arg	22.59 (2.84)	2.87 (0.06)
Tic-Lys(Ac)	23.60 (2.45)	1.15 (0.34)
Tic-DLys(Ac)	24.18 (3.10)	-1.25 (0.81)
Tic-Lys	25.03 (2.34)	0.19 (0.25)
Tic-Gly	30.34 (5.82)	2.43 (0.69)
Tic-Ser	24.63 (0.97)	1.37 (1.62)
Bid-Bzl	78.19 (3.53)	12.35 (0.44)
Bid-Propen	53.89 (4.50)	13.54 (0.75)
Bid-cPropyl	58.95 (4.98)	15.03 (0.31)
UFP512	32.58 (4.43)	3.06 (0.94)
UFP502	64.68 (2.85)	20.96 (0.12)
dMeUFP502	48.93 (2.18)	11.54 (0.75)
C1-Bid	42.66 (2.58)	9.54 (2.06)
dMe-C1-Bid	31.94 (4.10)	3.65 (0.97)
Gly-Bid	41.68 (3.56)	8.11 (1.56)
dMe-Gly-Bid	34.51 (3.59)	0.62 (0.19)
Tib	66.01 (2.50)	19.28 (2.56)
dMe-Tib	51.65 (1.69)	13.38 (0.03)
Tic-Ph	84.90 (2.09)	42.33 (3.15)
dMe-Tic-Ph	65.85 (3.31)	22.74 (1.72)
Tic-Gly-Ph	62.19 (3.76)	13.57 (3.03)
dMe-Tic-Gly-Ph	38.06 (1.86)	4.30 (0.75)
UFP505	41.72 (1.42)	6.71 (0.09)
dMe-UFP505	33.84 (2.26)	5.24 (0.37)
UFP515	31.46 (1.62)	6.18 (0.44)
UFP501	23.74 (3.98)	3.81 (0.12)
TIC	32.01 (4.47)	4.74 (0.37)
BNTX	75.06 (1.34)	19.68 (3.40)

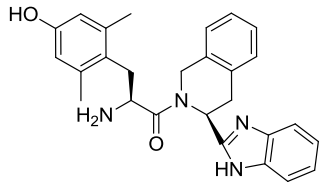
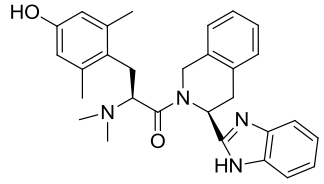
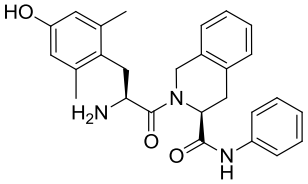
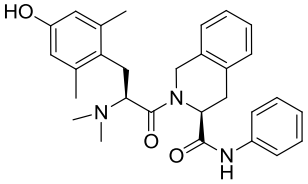
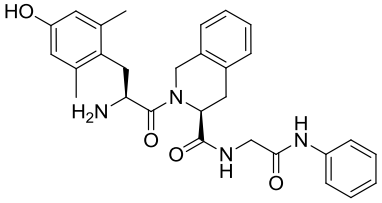
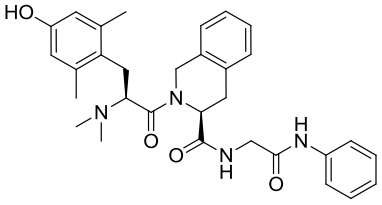
Table A2. The Dmt-Tic compounds.
Structure, names and abbreviations for all the Dmt-Tic ligands tested in this thesis are included in Table.

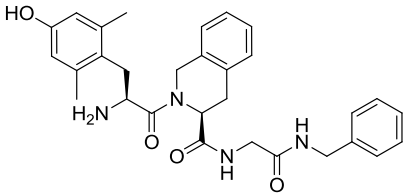
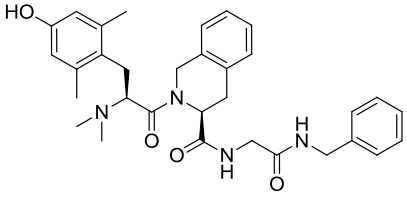
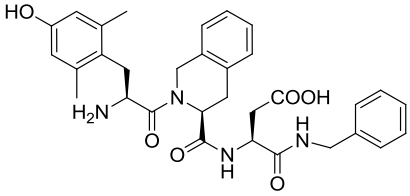
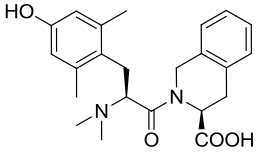
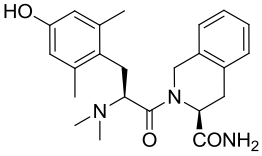
Structure	Full Name	Abbreviation
	**H-Dmt-Tic-Ala-OH**	**Tic-Ala**
	**H-Dmt-Tic-Asp-NH_2**	**Tic-Asp**
	**H-Dmt-Tic-Asn-OH**	**Tic-Asn**
	**H-Dmt-Tic-DAsn-OH**	**Tic-DAsn**
	**H-Dmt-Tic-Glu-NH_2**	**Tic-Glu**
	**H-Dmt-Tic-DGlu-NH_2**	**Tic-DGlu**

Structure	Full Name	Abbreviation
	**H-Dmt-Tic-Gln-OH**	**Tic-Gln**
	**H-Dmt-Tic-DGln-OH**	**Tic-DGln**
	**H-Dmt-Tic-Arg-NH_2**	**Tic-Arg**
	**H-Dmt-Tic-Lys(Ac)-OH**	**Tic-Lys(Ac)**
	**H-Dmt-Tic-DLys(Ac)-OH**	**Tic-DLys(Ac)**
	**H-Dmt-Tic-Lys-NH_2**	**Tic-Lys**

Structure	Full Name	Abbreviation
	**H-Dmt-Tic-Gly-NH_2**	**Tic-Gly**
	**H-Dmt-Tic-Ser-NH_2**	**Tic-Ser**
	**H-Dmt-Tic-NH-CH_2-Bid(CH_2-C_6H_5)**	**Bid-Bzl**
	**H-Dmt-Tic-NH-CH_2-Bid(CH_2-CH=CH_2)**	**Bid-Propen**
	**H-Dmt-Tic-NH-CH_2-Bid(CH_2-c(C_3H_5))**	**Bid-cPropyl**
	**H-Dmt-Tic-NH-CH(CH2-COOH)-Bid**	**UFP512**

Structure	Full Name	Abbreviation
	**H-Dmt-Tic-NH-CH_2-Bid**	**UFP502**
	**N,N$(Me)_2$-Dmt-Tic-NH-CH_2-Bid**	**dMe-UFP502**
	**H-Dmt-Tic-NH-CH_2-CH_2-Bid**	**C1-Bid**
	**N,N$(Me)_2$-Dmt-Tic-NH-CH_2-CH_2-Bid**	**dMe-C1-Bid**
	**H-Dmt-Tic-Gly-NH-CH_2-Bid**	**Gly-Bid**
	**N,N$(Me)_2$-Dmt-Tic-Gly-NH-CH_2-Bid**	**dMe-Gly-Bid**

Structure	Full Name	Abbreviation
	**H-Dmt-Tib**	**Tib**
	**N,N(Me)$_2$-Dmt-Tib**	**dMe-Tib**
	**H-Dmt-Tic-NH-Ph**	**Tic-Ph**
	**N,N(Me)$_2$-Dmt-Tic-NH-Ph**	**dMe-Tic-Ph**
	**H-Dmt-Tic-Gly-NH-Ph**	**Tic-Gly-Ph**
	**N,N(Me)$_2$-Dmt-Tic-Gly-NH-Ph**	**dMe-Tic-Gly-Ph**

Structure	Full Name	Abbreviation
	**H-Dmt-Tic-Gly-NH-CH_2-Ph**	**UFP505**
	**N,N$(Me)_2$-Dmt-Tic-Gly-NH-CH_2-Ph**	**dMe-UFP505**
	**H-Dmt-Tic-Asp-NH-Bzl**	**UFP515**
	**N,N$(Me)_2$-Dmt-Tic-OH**	**UFP501**
	**N,N$(Me)_2$-Dmt-Tic-NH_2**	**TIC**

PUBLICATIONS

1. Molinari P, **Vezzi V**, Sbraccia M, Grò C, Riitano D, Ambrosio C, Casella I, Costa T. (2010). "Morphine-like opiates selectively antagonize receptor-arrestin interactions". *J. Biol. Chem.*, **285(17)**: 12522-35.
2. **Vezzi V**, Guerrini R, Salvadori S, Onaran HO, Costa T. "The mechanism of constitutive activity in δ and μ opioid receptors". *Submitted.*

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