Studies on the biased signalling of some novel delta opioid receptor agonists

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Abstract

The delta opioid receptor (DOR) is a G protein-coupled receptor (GPCR) which is important in the regulation of neuronal function, predominantly via coupling to heterotrimeric $G_{i/0}$ proteins. The receptor has been shown to be a potential target for the treatment of chronic pain and affective disorders. Although a large number of opioid agonists exist, their properties vary widely, at least partly due to their differential coupling to post-receptor signalling systems, a phenomenon referred to as ligand-biased signalling or functional selectivity.

The aim of the current project was to examine the signalling properties of a set of established and novel DOR agonists in an attempt to identify compounds that have biased signalling profiles. It was hypothesized that DOR agonists with partial efficacy regarding *β*-arrestin recruitment would be less liable to induce receptor internalization and desensitization of G protein-mediated signalling than full agonists.

Chinese hamster ovary (CHO) cells, stably transfected with GFP-tagged human (h)DOR and CHO-K1 and U2OS cells over-expressing hDOR were exposed to a number of novel selective DOR agonists compared with the commercially available agonists, SNC80, ADL5859 and DADLE. The compounds' potencies and efficacies were measured in four different assay systems; 1. Inhibition of forskolin-stimulated cyclic AMP accumulation, 2. Extracellular signal-regulated kinase ($ERK_{1/2}$) phosphorylation using an immunocytochemical In-cell Western (I-CW) assay, 3. *β*-arrestin 2 recruitment and 4. hDOR internalization. The latter two assays employed DiscoverX Enzyme Fragment Complementation technology.

An attempt to develop a secreted placental alkaline phosphatase (SPAP) reporter gene assay to measure DOR-mediated cyclic AMP inhibition was not successful.

All of the ligands were nearly full agonists in relation to cyclic AMP inhibition although some were less efficacious than the standard SNC80 regarding ERK1/2 activation. Their absolute potencies and rank orders of potency in inhibiting cyclic AMP and activating ERK1/2 were quite different, although both signalling systems were apparently Gi/o- protein mediated.

In contrast, the agonists exhibited a full range of efficacies and potencies in both *β*-arrestin 2 recruitment and hDOR internalization assays and there was a significant correlation between the maximum efficacies of the compounds in the two assays.

A potential relationship between *β*-arrestin 2 recruitment/ hDOR internalization and desensitization of agonist-induced cyclic AMP accumulation was explored. Responses to the highly arrestin-recruiting agonists SNC80 and DADLE desensitized fully after extended exposure, whereas the novel partial agonists PN6047 and OPD00003 resisted desensitization. Bias factors were calculated for the agonist set and both PN6047 and OPD00003 were found to be significantly biased towards G protein-mediated cyclic AMP inhibition.

In conclusion, this study reports, for the first time, a detailed characterization of signalling bias for a set of selective DOR agonists in cells over-expressing human DORs. The findings suggest that it is potentially possible to predict wanted and unwanted properties of agonists by determining post-receptor signalling profiles *in vitro* which will facilitate the discovery and development of novel therapeutics based on the DOR.

Acknowledgments

First and foremost, I thank almighty Allah for his enormous blessings. I would like to convey my heartfelt gratitude to my academic advisor, Professor Dave Kendall, for accepting me into his research group and providing me with such a rewarding graduate school experience by giving me intellectual freedom, engaging me in new ideas, and demanding a high quality of work in all my endeavours, it is all highly appreciated! Although your decision of early retirement from the University of Nottingham caused a great deal of distress for me at the time, but your support never ended even if you were away. It has been an honour to be your last PhD student at the University of Nottingham. I would also like to sincerely thank my supervisor, Dr. Stephen Alexander for the unending support and encouragement in real tense situations and for steering me through my lab work.

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Declaration

I declare that all the work in this thesis was done by myself, except where mentioned in the acknowledgements and relevant sections.

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To my son Rayyan Junaid

Chapter 1. General introduction

1.1. The history of opioids

Opioids are some of the earliest drugs used for their medicinal and euphorigenic properties and are amongst the most addictive substances known to man (Rosenblum *et al.,* 2008). Various preparations of opium poppy (*Papaver somniferum*) have been used to provide symptomatic relief of pain while its major active principal, morphine, continues to be one of the most widely used analgesic compounds and remains the gold standard drug in terms of relieving moderate to severe pain (Pasternak, 2011). Conventionally, the term opiate refers to alkaloids and close structural analogues derived from the opium poppy, whereas, an opioid is a compound (endogenous, natural, synthetic or semi-synthetic), acting on an opioid receptor. However, the two terms are often and incorrectly used interchangeably. Opioids can be classified into three groups; i) naturally occurring compounds, such as morphine, codeine and thebaine ii) chemically modified natural compounds (semi-synthetic) such as hydromorphone, buprenorphine and hydrocodone iii) completely artificial compounds (synthetic) such as fentanyl, tramadol and meperidine (pethidine).

Well known tincture forms of opium, laudanum (16th century) and paregoric (early 18th century) were used for their antidiarrheal, antitussive and analgesic actions, eventually making their way into pharmacopoeias (Strathern, 2005). Opiates were used extensively and without any governmental control during the nineteenth century as pain relievers, sedatives and for recreational purposes, yet they were not considered a menace and little concern was displayed about their negative effects. Furthermore, there was no indication that opiate addiction was a significant problem in the nineteenth-century. Opiate use spread throughout the world to every major civilization in Asia and Europe and the drugs were used to treat pain

and many other ailments (Schiff, 2002; Askitopoulou *et al*., 2002; Booth, 1996; Dikotter *et al.,* 2004).

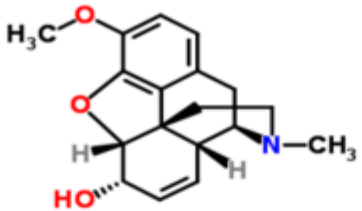
Morphine was first isolated in 1805 by the German Friedrich Sertürner. He originally named the substance morphium after the Greek god of dreams, Morpheus, for its tendency to induce sleep. Dr Charles Wood, a Scottish physician, invented the hypodermic syringe in 1853-1855, which was widely employed as a means to inject morphine to relieve pain from neuralgia (Katz *et al.,* 2007, Meldrum, 2003; Hosztafi, 2001); however, the use of morphine injection later increased, with addicts using it as a means of administering the drug rapidly to enhance euphoria (the “rush”).

Codeine, a less potent opiate than morphine was isolated from opium in 1830 to replace raw opium for medical purposes and is still used as a cough remedy; it is the most widely used opiate worldwide. Heroin (diacetylmorphine, “diamorphine”) was synthesized in 1874 by boiling acetic anhydride with anhydrous morphine which resulted in one of the most potent opioids. It was briefly promoted as being more effective and less addictive than morphine and soon became popular worldwide. In 1898, it was originally marketed as a cough medicine which could also be used as an analgesic. It could be considered as the first synthetic pro-drug marketed as a non-addictive analgesic; however, contrary evidence steadily emerged, eventually leading to the discontinuation of its widespread pharmaceutical manufacturing. Heroin-assisted treatment (HAT), however, is still licensed and is available in the United Kingdom and some other European countries, in which injectable heroin is prescribed to some opiate addicts who do not benefit from, or cannot tolerate, treatment with one of the more established drugs; methadone or buprenorphine (Booth, 1996).

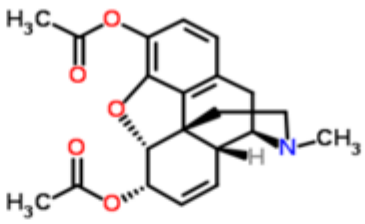
A number of other semi-synthetic drugs such as pethidine and methadone were developed by manipulating the structure of morphine in an attempt to develop derivatives that would retain analgesic activity with reduced side effects (1939). Like heroin, methadone and pethidine were also claimed to be non-addictive at their time of introduction. Further structure-activity relationship (SAR) studies on pethidine led to the synthesis of the potent short-acting opioids, fentanyl and sufentanyl (1962). Later, a series of other potent morphine-like drugs were developed from thebaine, including etorphine (used as veterinary analgesic and anaesthetic) and an etorphine analogue, buprenorphine (used as veterinary analgesic/anaesthetic and HAT). A chemically similar drug to morphine called N-allylnormorphine (nalorphine) emerged as the first opiate antagonist, which could reverse the respiratory depression produced by morphine. It also displayed an anti-nociceptive action almost comparable to morphine and, therefore, was regarded as a mixed agonist/antagonist. However, due to severe psychomimetic effects such as dysphoria, anxiety and hallucinations, it was later discarded (Glatt, 2012). The introduction of the semi-synthetic opioid antagonist, naloxone (approved by the FDA in 1971) resolved the issue, having a complete lack of analgesic, respiratory depressant and addictive properties, but able to antagonize completely the analgesic and respiratory depressant actions of morphine (Pasternak, 2011). Naltrexone, another opioid antagonist has, in general, been better studied for treating alcoholism than opioid addiction, despite originally being approved by the FDA in 1984 for opioid addiction (Galantar, 2008). The chemical structures of some commonly used opioid agonists and antagonists are shown in Figure1.1.



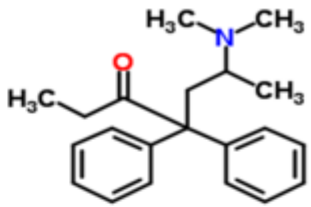
Morphine



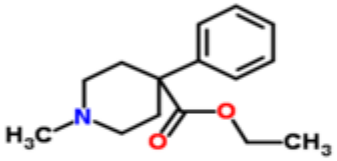
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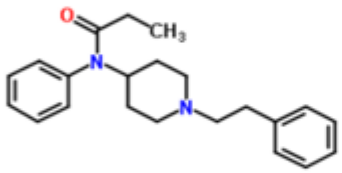
Heroin



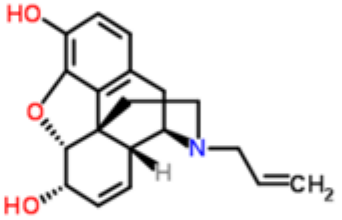
Methadone



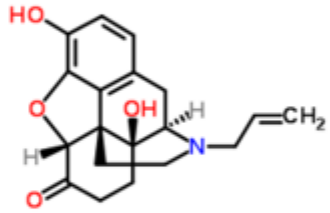
Pethidine



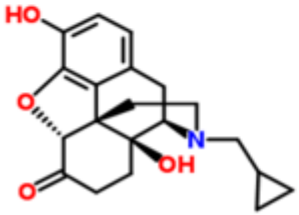
Fentanyl



Nalorphine



Naloxone



Naltrexone

Figure 1.1. Chemical structures of some commonly used opioid agonists and antagonists

1.2. Opioid receptors and the discovery of endogenous opioids

It is now known that the endogenous opioid system includes cell surface G protein-coupled receptors (GPCRs) which are integral membrane proteins. Occupation of these receptors by opioids predominantly leads to activation of inhibitory G proteins (heterotrimeric $G_{i/o}$ proteins) and regulation of many other intracellular processes (Zöllner and Stein, 2007; Jordan *et al.,* 2000; Sobczak *et al.,* 2014). The identification of multiple opioid receptors required the discovery of selective agonists and antagonists, the development of sophisticated assay techniques and, ultimately, cloning of the receptor proteins. The early studies by Pert and Snyder played a key role in paving the way for many of the current sophisticated advances in the field of opioid pharmacology. They were able to characterize the pharmacology of opioid receptors by making use of ligand binding techniques on brain extracts. These technical approaches also led to the identification and characterization of several neurotransmitters in the 1970s. The use of radiolabelled drugs, coupled with rapid but exhaustive washing in order to get rid of interfering substances, such as salts and endogenous nucleotides, in the tissue extracts made the system work effectively and, eventually, allowed multiple samples to be compared easily and to screen thousands of compounds at the microgram level (Pasternak, 2011).

The findings of Snyder and Pert sparked an interest in finding the endogenous ligands of these opioid receptors. In 1975, the two pentapeptides, Met-enkephalin (Tyr-Gly-Gly-Phe-**Met**) and Leu-enkephalin (Tyr-Gly-Gly-Phe-**Leu)** with opiate-like activity were discovered in extracts from porcine brain. Soon after this important discovery, Li and Chung (1976), reported the sequence of a neuropeptide with opioid activity, called *β*-endorphin ("*β* end bound to opioid receptors"). Other less

abundant forms named *α* and γ were also detected but they were found to have only weak biological effects. It is now known that all endogenous opioid peptides are derived from four distinct precursor proteins i.e., pro-enkephalin A (PENK-A), pro-dynorphin (PDYN) or pro-enkephalin B (PENK-B), pro-opiomelanocortin (POMC), and pro-nociceptin/orphanin FQ, which are products of separate genes (Pasternak, 2011).

All endogenous opioid peptides share the same enkephalin sequence at the N-terminus as stated above, with different extensions at the C-terminus. The pro-opioid proteins are synthesized in the cell nucleus and subsequently transported to the nerve cell terminals from where they are released. The active peptides are hydrolyzed from the large precursor proteins by their corresponding proteases. In addition to spinal and centrally mediated analgesia, endogenous opioids exert peripheral actions via receptors expressed on peripheral neurons contributing to antinociception associated with the inflammation process (Filip *et al.,* 2005; Pawlak *et al.,* 2001; Pasternak, 2011).

These endogenous opioids possess tyrosine as an N-terminal amino acid residue, which is essential for opioid activity and resistance to enzymatic degradation (Ramamoorthy and Balasubramanian, 1992). The majority of opioid peptides undergo rapid enzymatic degradation by proteases (Egleton *et al.,* 1998). For instance, the extensive metabolic degradation of Met-enkephalin (*Tyr*-Gly-Gly-Phe-**Met**) leads to the formation of Gly-Gly-Phe-**Met** which is devoid of opioid-like activity. Most of the extracellular peptidases are membrane-bound exo- and endopeptidases, of which aminopeptidase N (APN) is a crucial enzyme. APN is a transmembrane protease present in a wide range of human tissues and cell types (endothelial, epithelial, fibroblast, leukocyte), which is able to modulate bioactive

peptide responses (pain management, vasopressin release) and to influence immune functions and other major biological events (cell proliferation, secretion, invasion, angiogenesis). Natural and synthetic inhibitors of APN activity have been characterized and are known to modulate bioactive peptide responses. APN is responsible for the degradation of several biologically active peptide substrates including enkephalins, neurokinin A and *β*-endorphin (Miller *et al.,* 1994; Bauvois and Dauzonne, 2006; Luan and Xu, 2007). Modifying the peptides at the N-terminal with appropriate residues can appreciably enhance their biological activity. Degradative enzymes generally prefer the L-configuration rather than the unnatural D-configuration; thus, substituting a D-amino acid in place of an L-amino acid might limit or possibly prevent extensive proteolytic activity. The amino acid sequences of some selected endogenously expressed opioids are shown in Table 1.1.

Endogenous opioids	**Amino acid sequence**
[Leu^5]enkephalin	*Tyr*-Gly-Gly-Phe-Leu
[Met^5]enkephalin	*Tyr*-Gly-Gly-Phe-Met
Dynorphin A	*Tyr*-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg–Pro-Lys–Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin B	*Tyr*-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
a-Neoendorphin	*Tyr*-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
β-Neoendorphin	*Tyr*-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro
β_h-Endorphin	*Tyr*-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Endomorphin-1	*Tyr*-Pro-Trp-Phe-NH_2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
Orphanin FQ/nociceptin	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln

Table 1.1 Amino acid sequences of selected endogenous opioids

1.3. Classic opioid receptor family

It was becoming clear that the actions of opioid agonists, antagonists, and mixed agonist-antagonists could best be differentiated and explained on the basis of multiple opioid receptors. Opioid pharmacology took a major step forward with the cloning of genes that encode different opioid receptors. At present, four distinct subtypes of opioid receptors have been studied, including mu (μ) opioid receptor, or MOR (named after **M**orphine); kappa (k) opioid receptor, or KOR (named after the first ligand, **K**etocyclazine); delta (δ) opioid receptor, or DOR (named after the tissue it was first identified in; vas **D**eferens of the mouse); and opioid receptor-like orphan receptor (ORL), also called nociception, or orphanin FQ receptor (NOP), which was added to the list in 1994. The latter can interact with opioid-like ligands, orphanin-FQ and nociception, but not with classical opioid agonists or antagonists. All four subtypes were cloned in the beginning of the 1990s (Chen *et al.,* 1993; Darland *et al.,* 1998; Evans *et al.,* 1992; Kieffer *et al.,* 1992).

Animal models with genetic deletion of each of these receptors (knockout mice) as well as mice lacking two or three of the receptors have been generated, which provide a means of determining which receptor subtypes mediate which effects (wanted and unwanted) of the opioids.

The affinities of endogenous opioids and their precursors for different opioid receptors are summarized in Table 1.2.

Precursor protein (pro-peptide)	Endogenous opioid peptide	Preferential receptor			
		MOR	KOR	DOR	NOP
Pro-opiomelanocortin (POMC)	*β*-endorphin (*β*-END)	✓✓✓	✓✓✓	✓✓✓	×
Pro-enkephalin-A (PENK-A)	Met-enkephalin	✓✓	×	✓✓✓	×
	Leu- enkephalin	✓	×	✓✓✓	×
Pro-enkephalin-B (PENK-B)	Dynorphin and *α*-neoendorphin	✓✓	✓✓✓	✓	✓

Table 1.2 Endogenous opioid peptide agonists' selectivity for the different opioid receptor subtypes; ✓✓✓= high affinity; ✓✓= intermediate affinity; ✓= low affinity; ×= no affinity (Modified from Rang, Dale and Ritter, 2011).

Opioid receptors exist not only in the nervous system (Xia and Haddad, 2001; Abbadie *et al.,* 2004; Beadles-Bohling and Wiren, 2005), but are also widely distributed in peripheral tissues including heart, lungs, liver, gastrointestinal and reproductive tracts (Wittert *et al.,* 1996; Villemagne *et al.,* 2002). However, the expression of these receptors varies significantly among different organs as well as among different animal species (Barry and Zuo, 2005). At the cellular level, MOR and KOR are mainly localized at the plasma membrane in soma, dendrites and nerve terminals. MORs can exist pre-synaptically or post-synaptically depending upon the cell type. MORs are expressed primarily in the cortex, limbic system and brain stem of the central nervous system (CNS) and gastrointestinal

tract (GIT) (Le Merrer *et al.,* 2009). KORs are expressed widely throughout the brain (hypothalamus, periaqueductal gray and claustrum) and spinal cord (substantia gelatinosa) (Fine and Portenoy, 2004; Mansour *et al.,* 1994). It has been established that receptors in different parts of the cells show distinct functional actions. For example, activation of MORs localized in the somato-dendritic compartment reduces the generation of action potentials, whereas, MORs located at terminals have inhibitory effects on transmitter release (Williams *et al.,* 2013). In addition, different signalling pathways can be associated with cellular receptor distribution. In the gastrointestinal tract, MORs are widely distributed in the submucosa (Bagnol *et al.,* 1997), as well as in the ileal mucosa where they influence ion transport changes. Stomach and proximal colon have the most dense MOR and KOR distribution (Fickel *et al.,* 1997). A detailed account on the distribution of DORs is given in section 1.6.

1.4. Opioid receptor; crystal structure and oligomerization

The recent advances in membrane protein crystallization (Chun *et al.,* 2012), which enabled the determination of high-resolution crystal structures of various GPCRs, including those of all four opioid receptor subtypes (Fenalti *et al.,* 2014; Granier *et al.,* 2012; Manglik *et al.,* 2012; Thompson *et al.,* 2012; Wu *et al.,* 2012) marked the beginning of a new era in opioid research by revealing important details of ligand-receptor interactions at the orthosteric binding site (i.e., the site at which endogenous opioid ligands bind), or allosteric sites (e.g., the much anticipated sodium binding site (Fenalti *et al.,* 2014)). The knowledge of crystal structures of opioid receptors provides a new dimension for the structure-guided drug discovery (Filizola and Devi, 2013). According to latest reports, it is speculated that μ and δ

ligands interact with residues at the two transmembrane (TM) helices: TM6 and/or TM7, whereas ligands for the *k* receptor interact with residues on TM2 and TM3. However, further studies are ongoing employing X-ray crystallography and computational mapping server (Fourier-Transform mapping 'FTMap') to predict the binding pockets (*hot spots*) for various opioids ligands within the crystal structure of major opioid receptor subtypes, (Shang and Filizola, 2015). The FTMap algorithm based crystal structures for the three opioid receptors are shown in figure 1.2.

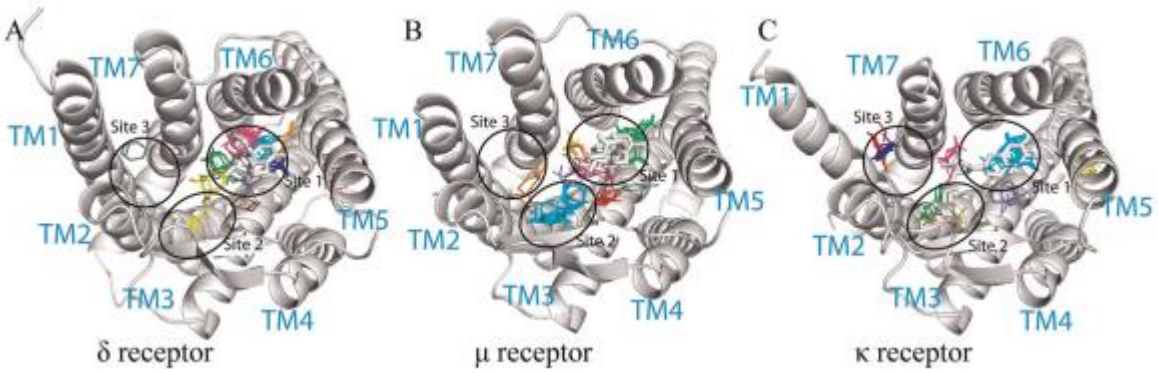


Figure 1.2 FTMap results showing δ (A), μ (B), and κ (C) receptor crystal structures, with the potential binding sites on each receptor (Shang and Filizola, 2015).

On the other hand, oligomerization or receptor-receptor interactions of GPCRs remains controversial; it continues to generate interest and is an increasingly evident phenomenon as opioid receptor dimers may be novel targets for the development of new opioid drugs. Oligomers can be of two types: homodimers (same receptor) and heterodimers (different receptor type) (Figure 1.4). The existence of these GPCR homomers and heteromers has been shown in transfected cell line systems, cell lines, and primary cultures and in some cases *in vivo* (Rios *et al.,* 2001; Prinster *et al.,* 2005). The first evidence of opioid receptor

oligomers came from Devi's group in 1997 when they visualized homodimerization of the DOR (Cvejic and Devi, 1997) and found that DORs can exist as homodimers and agonist stimulation causes their dissociation. Western blotting of CHO cells stably expressing FLAG-tagged DOR exhibited a band of about 120 kDa, which is twice the size of a single DOR. This was the first suggestion that the receptors homodimerized, at least when over-expressed.

In another piece of work, Devi and the colleagues found that KOR and DOR form heterodimeric complexes, which appear to alter the trafficking properties of these receptors. They showed how agonist-induced internalization of DOR is substantially reduced in cells expressing delta and kappa receptors (Jordan and Devi, 1999). Devi's group not only identified the existence of MOR/DOR heterodimers but also revealed that MOR/DOR heterodimers have distinct ligand binding and signal transduction properties (Gomes *et al.,* 2000), suggesting that heterodimerization may affect the regulation of biological activity: modulation of ligand binding, alteration in G protein activation, and novel signalling pathways (Devi, 2001; Terrillon & Bouvier, 2004; Waldhoer *et al.,* 2004; Morse *et al*., 2013). DOR agonists increase antinociceptive effects of MOR agonists in many preclinical assays of acute nociception, indicating that DOR signalling interacts synergistically with the MOR system, resulting in pain modulation (Negus *et al.,* 2012). Some studies have also shown that opioid receptors can heterodimerize with other classes of GPCR. For example, MOR can interact and potentially associate with cannabinoid receptor 1 (CB1R) (Rios *et al*., 2006; Hojo *et al.,* 2008).

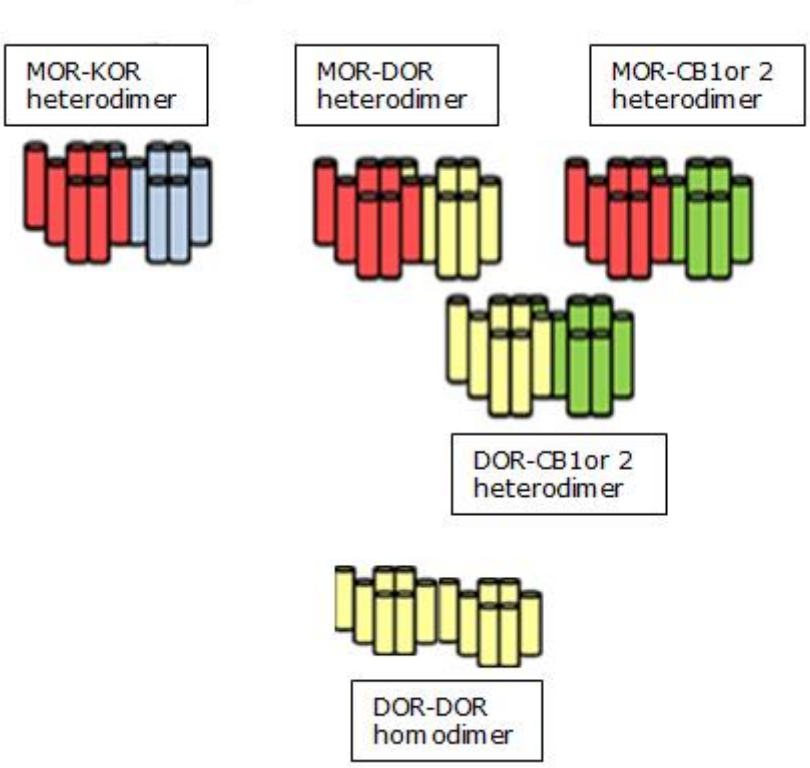


Figure 1.3 Interaction between similar or different GPCR types (opioids and cannabinoids) and their co-expression to form high molecular weight complexes. These oligomeric forms may influence each other's properties. GPCRs shown in red, blue, yellow and green represent MOR, DOR, KOR and CB1R or 2 respectively, either homodimerized or heterodimerized together.

1.5. GPCR signal transduction mechanisms

In mammals, the G protein-coupled receptors (GPCRs) or seven transmembrane spanning receptors (7-TMRs) form the most abundant superfamily of membrane-bound proteins which are expressed in virtually every tissue in the body and influence nearly all physiological responses (Vassilatis *et al.,* 2003). Members of the GPCR family share the same basic architecture, an extracellular amino-terminal segment, an intracellular carboxy-terminal tail and a middle segment

containing seven transmembrane domains (7TM *α*-helices). The physical location and disposition of GPCRs spanning the cell's plasma membrane connect extra- and intracellular environments and provide a direct mechanism for the transduction of extracellular messages into intracellular responses.

1.5.1. Opioid receptor signalling

1.5.1.1. Activation of heterotrimeric G proteins by opioids

Heterotrimeric G proteins (G*α*, G*β*/*Gγ* subunits) serve as molecular switches in response to diverse extracellular signals. The GPCRs identify extracellular signals and connect them to the G proteins, which further transduce them to appropriate downstream effectors and, thereby, play an important role in many signalling pathways. There are four main classes of GPCRs (Gi /Go, Gs, Gq and G12/13) (Schroder *et al.,* 2010; Baltoumas *et al*., 2013). GPCRs are linked to the heterotrimeric G proteins at the cytoplasmic domain and consist of three different subunits**:** G*α* (**~**33-55 kDa), G*β* (~35 kDa) and GϒΥ (**~**15 kDa).

It is generally accepted that the opioid receptors transduce inhibitory signals via G*α*i/o. Studies with G*α*i-specfic antibodies suggested that Gi2 mediates the pertussis toxin (PTx) sensitive DOR inhibition of adenylyl cyclase (AC) activity in NG108-15 cells, while Go mediates MOR inhibition of the AC activity in SH-SY5Y cells and brain membranes. However, the overexpression of the Gz α-subunit with the DOR resulted in PTx-insensitive agonist inhibition of the AC. Hence, the inhibition of AC by the DOR is mediated by the G protein α-subunits, either the G*α*i/o or the Gz (Pasternak, 2011). Under basal conditions, the *βγ* complex and

the guanosine diphosphate (GDP)-bound *α* subunit are associated in the form of a heterotrimer. Upon activation by an opioid agonist, a conformational change of the cytoplasmic domain of the receptor occurs which promotes the exchange of GDP for GTP on the G protein *αi* subunit. The GTP-bound *α* subunit disassembles from the activated receptor and from the *βγ* complex. Both the disengaged subunits are now free to interact with specific intracellular second messenger systems and ion channels (Kieffer and Evans, 2009). G*α*i inhibits adenylyl cyclase (AC) and thus the production of cAMP, whereas G*βγ* directly interacts with different ion channels in the membrane, causing activation of potassium conductance by stimulating inwardly rectifying K^+ channels (GIRKs) and inhibiting calcium channels, thus leading to inhibition of neurotransmitter release in excitable cells (Lüscher and Slesinger, 2010). These phenomena collectively participate in the reduction of nociception mediated by opioid agonists. The kinetics of the G protein signalling are regulated by a family of proteins called Regulators of G protein Signalling, which act as GTPase accelerating proteins. Regulators of G protein Signalling can dramatically accelerate the deactivation rate of G protein signalling by eventually hydrolysing its bound GTP back to GDP, allowing for re-association with G*βγ* and a resetting of the system for another round takes place.

The most convincing evidence that opioid receptors predominantly belong to the Gαi-coupled GPCRs comes from their sensitivity PTx which inactivates (by ADP-ribosylating) the G*αi* subunit of the signalling complex, thus removing the inhibitory component of the pathway causing blunting of agonist signals (Jiang and Bajpayee, 2009). PTx locks the *α* subunits at the cysteine residues into an inactive state (GDP-bound form), rendering them unable to inhibit adenylyl cyclase (AC), which leads to the enhanced accumulation of cAMP (Mangmool and Kurose, 2011).

Other research on opioid receptors describes them as pleiotropic, capable of binding to and activating more than one G protein subtype (Neve, 2009; Kenakin, 2002). Coupling of the opioids to the PTx-insensitive Gs or Gq proteins has been reported after prolonged opioid exposure (Wong *et al*., 1992). It has been observed that opioid activation remained unaltered by PTx, but was sensitive to pre-treatment with cholera toxin, a G*α*s protein activator. In a separate study, morphine administration at very low doses induced a paradoxical effect, in which a decrease in pain threshold was observed (hyperalgesia) (Angst *et al.,* 2003; Guignard *et al.,* 2000; Compton *et al.,* 2003). Some studies have demonstrated that opioids can exert stimulatory effects either at doses well below those producing neuronal inhibition or after chronic exposure. The ultra-low doses of an opioid agonist activate a Gs-coupled excitatory mode of the opioid receptor to activate AC and increase neuronal excitability. An *in vivo* study on morphine demonstrated the presence of a nociceptive inositol signalling pathway stimulated by low concentrations of the agonist, hinting at a dual action of opioids at different doses (Galeotti *et al.,* 2006).

AC is generally accepted to be regulated by G*α*-subunits in opioid receptor signalling. On the one hand, acute activation of inhibitory $G_{i/o}$-coupled receptors leads to inhibition of AC, whereas on the other, chronic activation of such receptors can lead to an increase in cAMP accumulation. This phenomenon, observed in many cell types, has been referred to as cAMP overshoot or AC superactivation. It has been reported to be linked with G*βγ* subunits and may play a role in opioid tolerance and the manifestation of opioid withdrawal (Rubenzik *et al.,* 2001). In addition to modulation of potassium and calcium conductances, the G*βγ* heterodimer can also activate other downstream effectors, including mitogen-activated protein kinases (MAPK), phospholipase C (PLC), non-receptor tyrosine

kinases (e.g. Src family), and N-type calcium channels (Clapham and Neer, 1993; Milligan and Kostenis, 2006; Dupre *et al.,* 2009).

1.5.1.2. Modulation of ion channel function and transmitter release

As discussed earlier, the overall effect of opioid drugs is to inhibit neurotransmitter release by inhibiting voltage-gated Ca^{2+} channels and activating the G protein-coupled inwardly rectifying voltage-gated K^+ channels (GIRK, Kir) (Law *et al.,* 2000). All three opioid receptors can modulate pre- and postsynaptic Ca^{2+} channels, suppress Ca^{2+} influx and, thereby, attenuate excitability in many neuronal systems and/or reduce the release of pronociceptive neurotransmitters such as substance-P (a pro-nociceptive and pro-inflammatory neuropeptide) which are released from central and peripheral terminals of sensory neurons (Law *et al.,* 2000; Kondo *et al.,* 2005; Wang *et al.,* 2010). Opening of K^+ channels allows K^+ efflux, leading to neuronal membrane hyperpolarization and reduced excitability (Law *et al.,* 2000; Zollner and Stein, 2007; Luscher and Slesinger, 2010; Nockemann *et al*, 2013). Opioids also inhibit Na^+ channels, I_h channels (hyperpolarization-activated nonselective cation channels, transient receptor potential vanilloid-1 (TRPV1) channels, and acid-sensing ion channels (ASICs) in dorsal root ganglion (DRG) neurons, as well as excitatory postsynaptic currents evoked by glutamate receptors in the spinal cord (Endres-Becker *et al.,* 2007; Spahn *et al.,* 2013; Cai *et al.,* 2014; Ingram and Williams, 1994; Gold and Levine, 1996). This results in decreased transmission of nociceptive stimuli at all levels of the CNS and contributes to profoundly reduced perception of pain.

Opioid inhibition of GABA release in local circuits, first observed in the hippocampus, has become a common observation that accounts for indirect excitatory (disinhibitory) effects of opioids. In the ventral tegmental area (VTA), GABA interneurons containing opioid receptors innervate the dopaminergic projection neurons. Agonist binding to MOR negatively modulates GABA transmission onto dopaminergic neurons, resulting in their disinhibition (Johnson and North, 1992). This GABA-mediated control of dopamine transmission onto target brain regions likely underlies, at least in part, the rewarding properties of opioids (Tzschentke, 1998; Fenu *et al.,* 2006).

Initially, opioids were considered to mediate inhibition of Ca^{2+} via G*α* subunits (Hescheler *et al.,* 1987; Moises *et al.,* 1994). However, it is now accepted that G*βγ*, rather than G*α* subunits mediate opioid agonist inhibition of Ca^{2+} channels (Ikeda, 1996). It is also known that, although G*βγ* subunits are responsible for mediating the inhibition of Ca^{2+} channels, the G*α* subunits are indispensable in coupling opioid receptors to the channels, as demonstrated with G*α* knockout mice studies in which the ability of the opioid agonists to inhibit Ca^{2+} channels in dorsal root ganglion (DRG) neurons of these G*α* null mice was significantly impaired (Jiang *et al.,* 1998). DORs have been shown to inhibit the L, N, P and Q high voltage-activated Ca^{2+} channels in neostriatal (Stefani *et al.,* 1994), postganglionic sympathetic (Motin *et al.,* 1995), and sensory (Acosta and Lopez, 1999) neurons, as well as clonal cell models such as neuronal F11 (Nah *et al.,* 1997) and NG 108-15 cells (Morikawa *et al.,* 1999; Toselli *et al.,* 1999).

The G*βγ* subunits are known to regulate GIRKs (Kir) that are involved in preventing neuronal excitation. Of at least 12 distinct channel subunits, the $K_{ir}3.1$ is the major channel type regulated by G*βγ* subunits (Pasternak, 2011). Electrophysiological

studies have shown that DORs can activate the Kir currents via PTx-sensitive G proteins in the rat locus coeruleus (North *et al.,* 1987) and substantia gelatinosa neuron preparations (Grudt and Williams, 1993). The overall actions of opioids on different effector systems are shown in Figure 1.4.

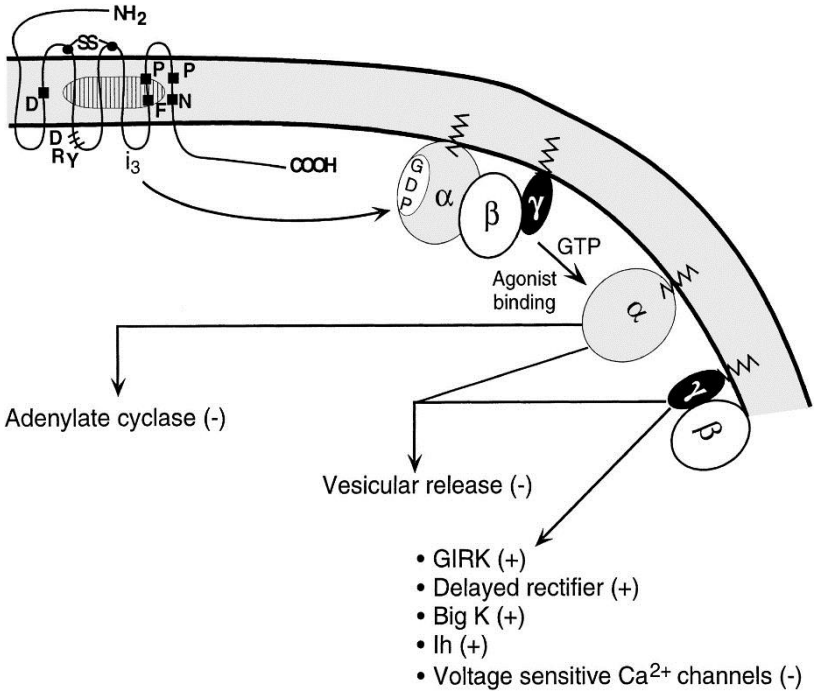


Figure 1.4 An illustration of different effector-activation pathways by opioids, such as adenylyl cyclase, inhibition of vesicular release and interaction with different ion channels by GTP-bound *α*-subunits as well as free *βγ* subunits of PTx sensitive G proteins (Williams *et al.,* 2001).

1.5.1.3. Activation of mitogen-activated protein (MAP) kinase cascades

The mammalian MAP kinases may be divided into three major families: the extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNK 1-3) and p38, each of which represents a separate signalling pathway. MAPK cascades are considered to be central building blocks in the intracellular signalling network. The ERK pathway mediates nuclear signalling and involves multiple protein-protein interactions, translocations, and phosphorylation events. The activation of this pathway by opioids, like other effectors, is sensitive to PTx (Williams *et al.,* 2001).

Multiple pathways can lead to activation of the ERK cascade. Generally, acute application of opioid agonists leads to the release of G*βγ*- subunits which can i) stimulate phospholipase C and cause the release of Ca^{2+} from intracellular storage sites and the production of diacylglycerol, which in turn activates protein kinase C, ii) recruit to the membrane proteins such as Ras-GRF, and iii) activates the phosphatidylinositol- 3 kinase (Pi-3 kinase) (Williams *et al.,* 2001). Transmission of signals via these cascades is initiated by activation of two upstream protein kinases, called Ras (a small GTP-binding protein), which is followed by the activation of the Raf protein-serine/threonine kinase, thus leading to a sequential activation of several sets of cytoplasmic protein kinases. Each of the cascades is generally composed of three core tiers: MAPKKK (Raf), MAPKK ($MEK_{1/2}$), and MAPK ($ERK_{1/2}$). $MEK_{1/2}$ is a dual-specificity protein kinase that can phosphorylate both threonine and tyrosine residues and thus acts as both a tyrosine kinase and serine/threonine kinase. One or more kinase components in each of these tiers phosphorylate and activate components in the next tier until a downstream component phosphorylates target regulatory molecules that initiate the required physiological process. MEKs are upstream kinases which

phosphorylate and activate their only known substrates, ERK 1 and 2, that in turn phosphorylate a large number of substrates throughout the cell, including various transcription factors and immediate early genes products. All of these cascades cooperate in transmitting signals from many extracellular ligands and, thereby, determine the cell's fate upon distinct extracellular stimulations. The best-characterized forms of MAP kinase in mammalian cells belong to the ERK family, which participates in the regulation of proliferation, differentiation, survival, learning, apoptosis, morphology determination and oncogenic transformation. The DOR agonist SNC80 has been shown to promote neural differentiation from multipotent neural stem cells obtained from embryonic C3H mouse forebrain. Thus, DOR activation is proposed to cause neuronal differentiation (Georgranta *et al.,* 2013). ERKS are the protein products of two genes, ERK_1 (a 44 kDa product) and ERK_2 (42 kDa), due to which $ERK_{1/2}$ is often described as P44/42 MAPK (Raman and Cobb, 2003; Kuida and Boucher, 2004; Bogoyevitch, 2004; Rubinfeld, and Seger, 2005). The commercially available anti-phospho-$ERK_{1/2}$ (mouse monoclonal) and anti-total $ERK_{1/2}$ (mouse polyclonal) antibodies are generally used to determine the phosphorylated and total ERK content respectively in different cell lines, upon mitogenic stimulation. Activated ERKs have been demonstrated to phosphorylate a large number of substrates. Some of these substrates are localized in the cell’s cytoplasm, while others are phosphorylated by ERKs further downstream, after translocation into the nucleus (Yoon and Seger, 2006), and eventually activate a series of transcription factors as shown in Figure 1.5.

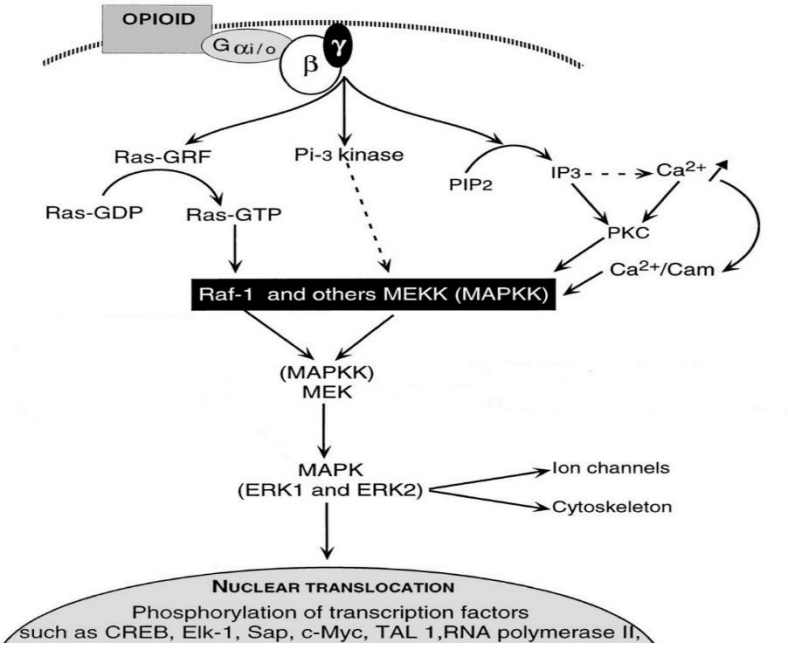


Figure 1.5 Acute application of opioid agonists leads to the release of G*βγ*-subunits which can **i)** stimulate phosopholipase C and cause the release of Ca^{+2} from intracellular storage sites and the production of diacylglycerol, which in turn activate the protein kinase C, **ii)** recruit to the membrane proteins such as Ras-GRF, **and iii)** activate the phosphatidylinositol 3-kinase (Pi-3 kinase) and ultimately result in a three-tier regulatory cascade (MAPK, MAPKK and MAPKKK). ERK1/2 then leads to the phosphorylation of cytoplasmic and nuclear substrates (Adapted and modified from Williams *et al.,* 2001).

Activation of MOR, KOR or DOR in various cell models results in the activation of ERK1/2 (Fukuda *et al.,* 1996; Li and Chang, 1996, Tso *et al.,* 2000; Wilson *et al.,* 1997; Hawes *et al.,* 1998; Burt *et al.,* 1996; Bohn *et al.,* 2000), JNKs (Bae *et al*., 2003a) and p38 (DeWire *et al*., 2007). Furthermore, opioid receptors have been reported to regulate brain ERK1/2 activity as demonstrated by the *in vitro* opioid stimulation of ERK1/2 in the ventral tegmental area (Lesschera *et al.,* 2003) and by the *in vivo* activation of ERK1/2 in distinct brain regions.It is now evident that In

addition to their classic roles in desensitization and internalization, β-arrestins can also act as signalling scaffolds for many pathways and, in particular, those of the MAPKs. ERK activation that is mediated by *β*-arrestins seems to have different consequences at the biochemical level from those activated by G proteins. *β*-arrestin mediated phosphorylated ERK is retained in endocytic vesicles and needs further investigation (DeWire *et al*., 2007; DeFea *et al*., 2000).

Like other GPCRs, opioid receptors have been shown to cause the activation of MAPK pathways, including the Ras-Raf-ERK cascade, in a variety of cells *in vitro* (Belcheva *et al.,* 2002; Derkinderen *et al.,* 2003; Galve-Roperh *et al.,* 2002) and *in vivo* (Derkinderen *et al.,* 2003; Eitan *et al.,* 2003; Rubino *et al.,* 2004). Opioids have been known to elevate ERK phosphorylation by transactivation of epidermal growth factor receptors (EGFRs) in human embryonic kidney cells (HEK-293) and in astrocytes (Belcheva *et al.,* 2001; Eisinger and Schulz, 2004), and by transactivation of fibroblast growth factor receptors (FGFRs) in rat C6 glioma cells (Belcheva *et al.,* 2002).

Because of the DOR's critical role in pain and mood regulation, DOR-dependent MAPK signalling requires further investigation, which may reveal novel mechanisms for DOR regulation of neural activity (Al-Hasani and Bruchas, 2011).

1.6. Regulation of DOR expression

The synthesis of the DOR protein, its insertion into the plasma membrane and removal from the membrane are all tightly regulated. The native protein is synthesized in the endoplasmic reticulum and delivered to the Golgi apparatus

where glycosylation takes place and the protein is then packaged into exocytic vesicles for insertion into the plasma membrane (Cahil *et al.,* 2007). Unlike MORs and KORs, DORs were thought to exist predominantly (> 90%) at intracellular sites (Cahill *et al.,* 2001; Wang and Pickel, 2001; Gendron *et al.*, 2006) until, experiments with mice expressing fluorescently tagged DORs revealed that there was strong membrane localization of DORs *in vivo* (Scherrer *et al.,* 2009). The possible reasons for these differences between the numerous studies and reviews showing intracellular DOR labelling and membrane labelling remain largely unknown and continue to be matters of controversy. However, at least some of the previous studies using DOR antibodies were probably flawed after concerns were raised about poor antibody specificity. Although green fluorescent protein (GFP) tagging of DORs is a powerful *in vivo* technique, it requires careful interpretation given that GFP is a bulky protein that may interfere with the DOR trafficking machinery. Further investigation is required in both cases, and it is plausible that both concepts are partly correct; the levels of DOR expressed on the plasma membrane may well be higher than originally hypothesized, but a large intracellular pool of DOR protein remains. Nevertheless, DOR seems to be a dynamic opioid receptor that can readily traffic in response to agonists. Prolonged exposure with morphine treatment is reported to promote targeting of DORs to the cell surface in the dorsal horn of the rat spinal cord (Cahill *et al.,* 2001b; Morinville *et al.,* 2003). However, this up-regulation of DOR was shown to be dependent on MOR activity because blocking or deleting MOR genetically (MOR knockout) prevented the effect. On the other hand, inflammatory and pro-nociceptive agents such as capsaicin, bradykinin and ATP also promote trafficking of the DOR from the intracellular storage sites to the cell surface where they can be activated by agonists (Patwardhan *et al.,* 2005; Zhang *et al.,* 2006). Like MOR and KOR, desensitization of DOR is controlled via phosphorylation (Hasbi *et al.,* 2000; Law

et al., 2000). G protein receptor kinases (GRKs) are known to cause C-terminal phosphorylation, leading to receptor desensitization to further agonist stimulation. Several residues within the C-terminal region of the DOR were shown to be critical for opioid receptor regulation. In DOR, Ser363 is described as the key phosphorylation site. This residue is phosphorylated by GRK-2 (Marie *et al.,* 2008; Aguila, 2007; Bradbury *et al.,* 2009; Guo *et al.,* 2000; Pei *et al.,* 1995). Other studies have demonstrated that other amino acid residues are involved in DOR regulation. For example, Thr353 was found to be crucial for [D-Ala2, D-Leu5]-enkephalin (DADLE)-mediated down-regulation of DOR. Furthermore, ligand-specific variability in agonist-dependent DOR phosphorylation has been observed with differences between SCN80 and [D-Pen2,5]enkephalin (DPDPE)-bound conformations recruiting kinases with various efficacies and potencies (Varga *et al.,* 2004).

1.7. Molecular mechanisms of opioid tolerance

Possible contributions to opioid tolerance at the receptor level include receptor desensitization, internalization and post-endocytic processing of the receptor *(*recycling or degradation*)*. The cellular dynamics of delta opioid tolerance are complex with several potential contributors, including the possible involvement of both cell-dependent mechanisms such as GRKs (Guo *et al*., 2000; Marie *et al*., 2008), PKCs (Ueda *et al*., 1995) and *β*-arrestins (Qiu *et al.,* 2007), and/or, ligand-dependent functional selectivity towards a given receptor (Pradhan *et al*., 2010, 2016). Thus, overcoming opioid tolerance has proven to be a continuing challenge as it depends on multiple mechanisms.

1.7.1. Opioid receptor desensitization, internalization and recycling

One important feature of biological systems is that they tend to eliminate their sensitivity to continuous outside stimulus to prevent over-stimulation, thereby maintaining biological equilibrium. Desensitization is a component of such phenomenon and is observed in diverse organisms and biological regulation processes (Lohse, 1993; Lachowicz *et al.,* 1995). Receptor desensitization is the progressive loss of receptor function subsequent to prolonged or repeated exposure to an agonist (Dang and Christie, 2012). Desensitization is typically divided into three main steps: receptor phosphorylation by GRKs, endocytosis and sorting (recycling or downregulation) (Aguila *et al.,* 2007), i.e. with prolonged agonist exposure, there is a decrease or downregulation of the functional receptor content.

Few reports distinguish opioid receptor desensitization from cellular tolerance. In chronically morphine-treated animals, examination of MOR activity on potassium channel conductance shows an inhibition as compared to naive animals which is not reversible even after 6 hours in morphine-free medium; this is cellular tolerance (Levitt and Williams, 2012). In contrast, desensitization may be defined as a reduction of signal transduction from opioid receptors after acute activation by agonists that recover when cells or tissues are placed in the agonist-free medium. The first work investigating the molecular mechanisms underlying opioid receptor desensitization were reported more than 30 years ago (Gahwiler, 1981; Law *et al.,* 1982). It was reported that after internalization, DOR prefers to traffic into lysosomes and degrade finally (Tsao and von Zastrow, 2000). In contrast, MOR

has been shown to prefer to recycle and resensitize (Koch *et al.,* 1998; Wolf *et al.,* 1999).

Initially, studying desensitization was made possible by using experimental models endogenously expressing opioid receptors such as animal brain membranes or cell lines (NG 108-15, SH-SY5Y, SK-N-SH, SK-N-AS). Since the cloning of the first opioid receptors, those models have been largely superseded by systems (HEK, CHO, COS-7, *Xenopus laevis* oocyte) heterologously over-expressing human opioid receptors (ORs) but their cellular characteristics are different from neurons in which ORs are endogenously expressed. Desensitization of ORs has been studied with respect to different signalling pathways including AC inhibition, activation of MAP kinases, *β*-arrestin recruitment, inhibition of voltage-gated calcium channels and activation of GIRK channels (Allouche *et al.,* 2014; Lowe *et al.,* 2002; Qui *et al.,* 2007; Connor *et al.,* 2004).

1.7.2. Role of *β*-arrestins

Phosphorylation of all the opioid receptor subtypes by GPCR kinases (GRK-2 or -3) leads to arrestin 2 and/or arrestin 3 recruitment. GRKs are a family of Ser/Thr protein kinases that specifically recognise the agonist-activated G protein-coupled receptor as substrates. Arrestin molecules are key proteins that bind to phosphorylated GPCRs and lead to uncoupling of the receptor from their respective G proteins, even in the continued presence of agonist resulting in diminished G protein-mediated signalling (functional desensitization), sequestration, sorting and ultimately they assist in determining receptor fate. Mammalian cells express four arrestin protein subtypes (arrestins 1-4), of which

arrestin 2 and 3 were named *β*-arrestins 1 and 2 (*β*-arr1 and 2) respectively; they showed a preference for binding to the β_2-adrenergic receptor. Arrestins typically exist in a phosphorylated state in the cytosol and are dephosphorylated upon binding to activated GPCR at the plasma membrane (DeWire *et al.,* 2007).

Originally identified as negative regulators of GPCR activity, *β*-arrestins are now well known as multifunctional adaptor proteins that transduce signals to diverse intracellular signalling pathways, and thus play a critical role in the regulation of a range of post-receptor intracellular processes such as regulation of desensitization, internalization, endocytosis, ion transporters and ion channels (Shenoy and Lefkowitz, 2011; Pierre-Yvesal *et al.,* 2016; Gainetdinov *et al.,* 2004; Schmid and Bohn, 2009). Arrestin binding is known to sterically block G protein activation and prevent some forms of signalling (membrane-delimited ion channel regulation), arrestin has a scaffolding function that enables opioid receptor activation of the ERK1/2 cascade.

The ligand bound GPCR becomes a substrate for GRKs, followed by arrestin recruitment which bind directly to GRK-phosphorylated receptor. A polar core located in the hinge region between the two globular domains of the arrestin interacts with GRK-phosphorylated residues on the receptor tail, displacing the arrestin C terminus and exposing the concave surface of the globular domains to interact with the receptor. It has been suggested that the receptor can either be dephosphorylated and reactivated (re-sensitized) or β-arrestin-2 can be recruited to the phosphorylated receptors, prolonging desensitization by signalling for receptor internalization into the endosome. The receptor can either be degraded or dephosphorylated in the endosome causing β-arrestin-2 to fall off the receptor, which signals for receptor recycling back to the plasma membrane for another

round of opioid agonist receptor activation (Gurevich and Gurevich, 2006; Williams *et al.,* 2013).

Mounting evidence suggests that *β*-arrestins induce agonist-dependent endocytosis into the clathrin-coated vesicles (Figure 1.6) (Whalen *et al.,* 2011). *β*-arrestins mediated signals have distinct biochemical and functional consequences from those mediated by G proteins and, currently, pharmacologists are keen to identify biased ligands that could preferentially signal through, either Gi protein or *β*-arrestin-mediated cascades. Interestingly, morphine was shown to exert enhanced analgesia and reduced respiratory depression and constipation in a β-arrestin 2 knockout mouse (Raehal et al., 2005). Evidence of the involvement of β-arrestin 2 in the mediation of the characterisocs adverse effects of opioids has prompted the search for opioids that do not induce β-arrestin 2 recruitment.

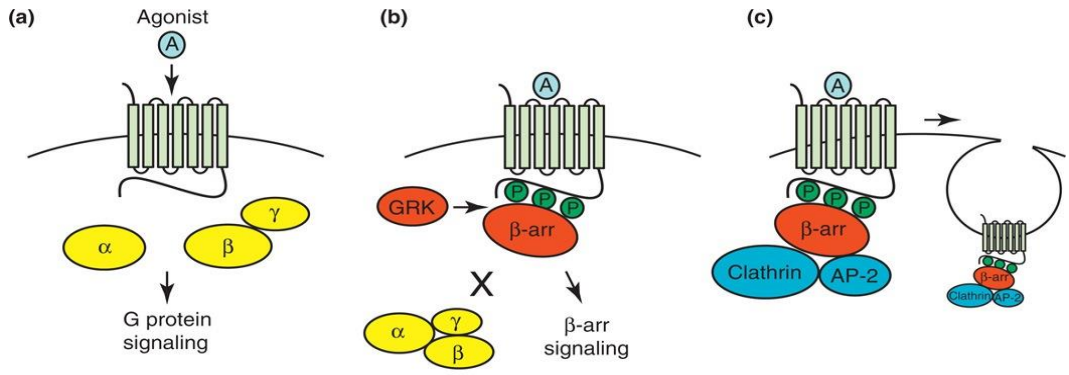


Figure 1.6 GPCR signalling and regulation by the GRKs and *β*-arrestins. (**a**) Agonist-mediated GPCR activation. (**b**) Subsequent desensitization of G protein signalling and activation of *β*-arrestin signalling **(c)** and *β*-arrestin mediated GPCR internalization involving adapter protein-2 (AP-2) and clathrin (Whalen *et al.,* 2011).

For continued GPCR signalling, desensitized receptors need to re-establish their responsiveness to extracellular signals through a receptor re-sensitization (recycling) process (Figure 1.7) (Kenakin, 2006). The sorting of DORs to these alternative destinations has also been shown to be linked to *β*-arrestin 2 (Zhang *et al.,* 2008).

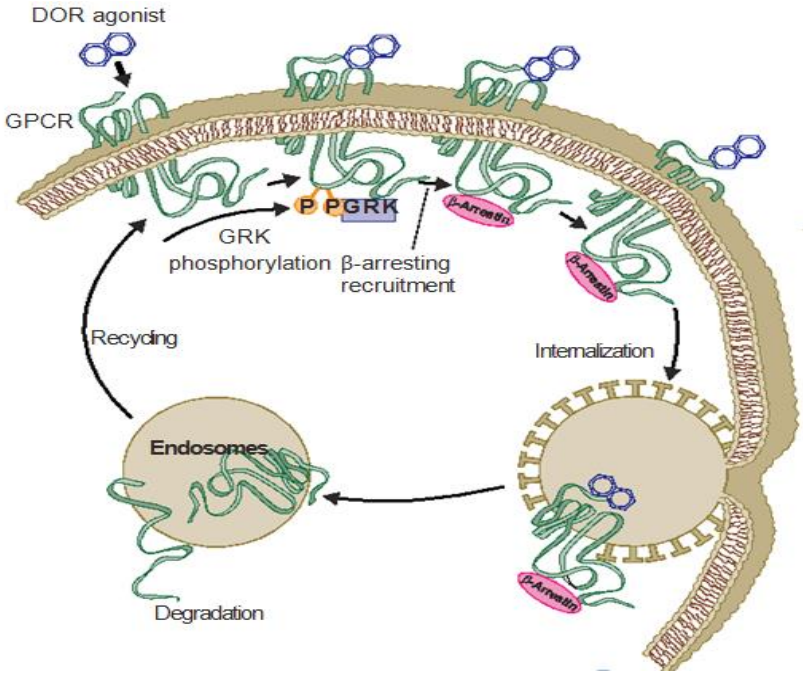


Figure 1.7 Internalization of GPCRs. Conformational change in the GPCR after activation by a DOR ligand and subsequent phosphorylation, leading to β-arrestin binding, which precedes internalization of the receptor. Receptors then are either degraded in endosomes or recycled to the cell surface (Adapted and modified from Kenakin, 2006).

Studies using MOR agonists indicate that different opioids induce different degrees of tolerance. For example, morphine produces similar analgesic tolerance as oxycodone (Madia *et al.,* 2009), but more so than methadone (Kim *et al.,* 2008) or

etorphine (Madia *et al.,* 2009). Since internalization efficacy of these agonists is also distinct (Arttamangkul *et al.,* 2008), tolerance has been linked to differences in agonist ability to recruit *β*-arrstins and induce MOR sequestration (Martini and Whistler, 2007). DOR agonists with distinct internalization profiles also differ in the generation of tolerance. In particular, administration of the internalizing agonist SNC80 resulted in acute analgesic tolerance that was not observed following the administration of non-internalizing AR-M1000390 (Pradhan *et al.,* 2009). On the other hand, alternative reports indicate that DOR internalization and tolerance may not be strictly related. For example, deltorphin II and SB235863 have similar internalization capacities as SNC80, but, unlike the latter, they maintain their analgesic efficacy after acute administrations (Beaudry *et al.,* 2009). Furthermore, despite differences in triggering internalization, long-term administration of SNC80 and of AR-M1000390 both eventually result in the generation of tolerance (Pradhan *et al.,* 2010). From a therapeutic perspective, these findings imply that the hunt for low *β*-arrestin recruiters and non-internalizing DOR agonists is a potentially fruitful strategy.

1.8. Opioids in clinical use; wanted and unwanted effects

The clinical efficacy of opioids is undisputed in acute (e.g., post-operative) and cancer pain; however, their long-term use has met with increasing scrutiny in the contexts of chronic pain, mainly because of their negative effects (side effects and tolerance) (Stein, 2016). The relative potencies of different opioids, as well as the side-effect profiles, can vary from patient to patient (Pasternak, 2011). The major opioids in current clinical practice are listed in Table 1.3.

Category	Agonist	Therapeutic use
	Morphine	Analgesic
	Hydromorphone	Analgesic
	Levorphanol	Analgesic
	Oxymorphone	Analgesic
	Oxycodone	Analgesic
	Meperidine	Analgesic
	Methadone	Analgesic, treatment of opiate addiction
	Fentanyl	Analgesic
	Alfentanyl	Anesthetic
	Sufentanil	Anesthetic
	Etorphine	Anesthetic, immobilizing agent for large animals
	Diphenoxylate	Anti-diarrheal (no penetration across blood brain barrier)
	Loperamide	Anti-diarrheal (no penetration across blood brain barrier)
Weak agonists	Codeine	Analgesic, anti-tussive
	Hydrocodone	Analgesic
Weak agonist/ monoamine reuptake inhibitor	Tramadol	Analgesic
Partial agonist	Buprenophine	Analgesic, treatment of opiate addiction
Mixed agonists/ antagonists	Nalbuphine	Analgesic, as a supplement to balanced anaesthesia
	Pentazocin	Analgesic
Antagonists	Naloxone	Treatment of opioid overdose
	Naltrexone	Treatment of alcohol and opioid overdose/ addiction

Table 1.3 Opioid drugs in clinical use (Modified from Pasternak, 2011).

The most common side-effects associated with the opioid drugs are constipation (which has a very high incidence) and nausea. Opioid receptors are present throughout the GIT and stimulation of these receptors by opioids can cause uncoordinated peristaltic contractions leading to constipation. Other common side effects include itching, bradycardia/ hypotension, dizziness, euphoria, sedation and respiratory depression. The side effects may be severe enough to require opioid discontinuation and contribute to under-dosing and inadequate analgesia (Neve, 2009; Kieffer and Evans, 2002; Palos *et al.,* 2004; Stein *et al.,* 2003; Corbett *et al.,* 2006). Although traditionally, the degranulating effect (histamine release) of MOR agonists on mast cells was assumed to be the essential mechanism implicated for itching, it is now clear that they can also induce itching as a side effect by their spinal actions at the spinal cord level. Neurons of the dorsal horn in the pain circuit have an inhibitory effect on spinal itch neurons. Opioids are known to weaken these inhibitory actions rendering the itch neurons active, without stimulation of the primary afferent peripheral nerves (Ganesh and Maxwell, 2007). Opioids generally reduce the tone of sympathetic nervous system and can cause bradycardia: the exception is pethidine, which causes tachycardia possibly related to the structural similarity between pethidine and atropine. The nucleus accumbens and the ventral tegmental area are known to be a part of the brain's 'reward pathway'. Heroin and morphine inhibit the release of *γ*-aminobutyric acid (GABA), thus reducing the inhibitory effect of GABA on dopaminergic neurones. The increased activation of dopaminergic neurones and the release of dopamine results in sustained activation of the post-synaptic membrane. Opioids stimulate the chemoreceptor trigger zone (CTZ) located in the area postrema in the medulla oblongata causing nausea and vomiting. They exert an antitussive (cough suppressant) effect by producing depression of neurons in the medulla; codeine has been effectively used owing to its potent inhibitory effect on the cough reflex

while meperidine has a weak effect. Opioids stimulate the Edinger-Whesphal nucleus of the oculomotor nerve which activates the parasympathetic stimulation of the iris sphincter muscle and the ciliary muscle to produce miosis (pupillary constriction) (Lalley, 2008), even in the dark. Because little or no tolerance (see below) develops to miosis, this is often used clinically as a diagnostic sign of opioid abuse. Respiratory depression is induced via activation of MOR at specific sites in the CNS including the pre-Bötzinger complex, a respiratory rhythm generating area in the pons and medulla (Boom *et al.,* 2012). Although MOR agonists are still the mainstay of analgesic therapy to relieve severe pain, their therapeutic value is seriously hampered by their tendency to cause tolerance and addiction following repeated or prolonged administration.

KOR agonists produce mild antinociception in animal models of pain; however, they are also dysphoric, sedating, and may cause psychotomimetic effects (Schiller, 2010). Ligands displaying partial agonism at the KOR have been produced as potential addiction therapies. Some drugs with mixed MOR and KOR profiles (e.g. nalbuphine and pentazocine) have been used clinically as analgesics for their mu agonist properties (Trescot *et al.,* 2008). Because KOR agonists can offer analgesia and suppress the drug reward response, they were initially proposed as non-addictive analgesics. However, clinical trials on selective KOR agonists that freely enter the CNS (*e.*g. ICI199441, enadoline, and spiradoline) have been reported to produce unpleasant central side effects, such as dysphoria, sedation and diuresis.

Tolerance is a neuroadaptive process, in which the magnitude of a drug effect decreases with the repeated administration of the same dose, or that increasing doses are needed to produce the same level of therapeutic response. In

pharmacological terms, tolerance may be illustrated quantitatively by a rightward shift in the dose-response curve. Depending on its time-frame, tolerance may be classified as acute (minutes to hours; also called tachyphylaxis) occurring after only one or two drug administrations or chronic (days to weeks) (Christie, 2008).

Tolerance poses significant limitations in opioid use as increasing doses of the drug may cause toxic effects and physical dependence (Connor *et al.,* 2004). In addition to analgesic tolerance, some tolerance also develops to euphoria, sedation, while miosis and respiratory depression effects are generally maintained. Significant tolerance to nausea and constipating effects of opioids is not common. Tolerance had been viewed as a consequence of decreased number of functional receptors at the cell membrane. However, more recent work on opioid signalling has demonstrated that down-regulation of opioid receptors does not occur with all opioid agonists and the mechanism is far more complex than previously thought (McQuay, 1999; Pasternak, 2011). With all this in mind, the current direction of opioid research is focused on separating the beneficial functions of opioids from their undesirable effects.

1.9. Delta opioid receptor (DOR), as an evolving therapeutic target

DORs are expressed ubiquitously throughout the body and CNS and are, thus, positioned to play a role in a multitude of diseases. DOR expression is often dynamic, with many reports of increased expression during exposure to some chronic states such as stress, inflammation, or neuropathy or ethanol consumption, and thus DOR agonists become more potent and efficacious in these conditions in mediating specific DOR responses. A large structural variety in DOR ligands that

have moderate to high affinity for the DOR implies potentially different mechanisms of activating the receptor. These combined features of DOR pharmacology illustrate the potential benefit of designing tailored or biased DOR ligands (Cahill *et al.,* 2007; Cahill *et al.,* 2003; Cahill *et al.,* 2001b; Commons, 2003; Kabli and Cahill, 2007; van Rijn *et al.,* 2012b; Zhang *et al.,* 2006b).

At the cellular level, DOR activation, similar to MOR activation, results in decreased presynaptic Ca^{2+} influx, which inhibits release of neurotransmitters, and an increase in post-synaptic K^{+} efflux, which hyperpolarizes the neuron (decreases responsiveness to excitability input). DORs are located throughout the pain transmission pathway (peripheral sensory nerves, dorsal root ganglia, spinal cord, ascending neuronal tracts, rostral ventromedial medulla, periaqueductal gray and other brain regions) (Al-Hasani and Bruchas, 2011). It has been demonstrated recently that DOR activation at the central terminals of myelinated mechanoreceptors (A fibres) depresses synaptic input to the spinal dorsal horn, via the inhibition of voltage-gated calcium channels (Bardoni *et al.,* 2014). DOR-selective agonists display anti-allodynic properties in murine models of touch-evoked neuropathic and inflammatory pain, and DOR knockout mice exhibit increased mechanical hypersensitivity after peripheral injury (Gaveriaux-Ruff and Kieffer, 2011). These findings suggested that DORs may be expressed by cutaneous mechanosensory A fibres and that DOR-mediated regulation of these afferents could counteract nerve injury-associated mechanical hypersensitivity. A number of studies have reported the anti-nociceptive activity of DOR agonists against persistent pain and neuropathic pain in mouse, rat and monkey models (sciatic nerve or spinal nerve ligation) (Gaveriaux-Ruff and Kieffer, 2011; Kabli and Cahill, 2007). It was reported that DOR knockout mice displayed increased pain responses in models of neuropathic (Nadal *et al.,* 2006) and inflammatory pain

(Gaveriaux-Ruff and Kieffer, 2011), indicating that DOR density and activity is upregulated in these two states.

DOR agonists inhibit the hyperalgesia that develops during inflammation (Fraser *et al.,* 2000). The anti-inflammatory effects are apparently independent of cyclooxygenase (COX) inhibition, as DOR agonists do not produce COX-associated adverse effects and, in fact, militate against the gastric mucosal lesions induced by a number of standard NSAIDs (Gyires and Ronai, 2001; Scoto and Parenti, 1993). In a mouse model of bone cancer pain (C3H/HeJ mice with injected tibial osteosarcoma), peri-tumoral injection of the DOR-selective peptide agonist DPDPE (D-Pen2,5-enkephalin) attenuated paw thermal withdrawal latencies, an effect that was sensitive to a selective DOR antagonist (naltrindole), but not a selective KOR antagonist (nor-binaltorphimine; nor-BNI) (Menéndez *et al.,* 2003). In the same model, the highly DOR-selective (1700-fold over MOR) peptide agonist (D-Val2, Ala5-enkephalin) produced dose-related and naltrindole-sensitive inhibition of tactile allodynia that developed over 10-18 days (Brainin-Mattos *et al.,* 2006).

Activation of DORs appears to mediate antinociception and anti-hyperalgesia in many conditions including stress, chronic pain, opioid-induced hyperalgesia and tolerance (Pasternak, 2011). According to some studies, DOR activation does not result in many of the adverse effects associated with MOR agonists, including addictive liability (Stevenson *et al.,* 2005; Codd *et al.,* 2009), respiratory depression (Takita *et al.,* 1997; Codd *et al.,* 2009) and constipation (Petrillo *et al.,* 2003; Codd *et al.,* 2009) and so could offer a promising alternative for management of certain painful states (Pradhan *et al.,* 2010). Although agonists at DOR are not highly effective in alleviating acute pain, selective activation of the

receptor has been demonstrated to relieve chronic inflammatory and neuropathic pains (Kabli and Cahil, 2007), suggesting a functional up-regulation of the DOR, possibly due to increased translocation of DORs to the plasma membrane of the cell. Electron microscopy has revealed that DORs are normally located sub-plasmalemmally and tissue insult or inflammation relocates them to the plasma membrane (Cahill *et al.,* 2003; Gendron *et al.,* 2006). Studies conducted on genetically modified mice show that DORs, fluorescently tagged with Green Fluorescent Protein (GFP) are, however, normally found at the cell surface in the central and peripheral nervous systems. These differences in receptor localization have led to some uncertainty as to how DORs are up-regulated in chronic pain states, although the differences might be artefacts of the GFP labelling (see section 1.7.1 above).

The functional role of peripheral opioid receptors (PORs) in attenuating the excitability of neurons in pain and hyperalgesia has been demonstrated for decades (Ferreira *et al.,* 1979a,b; Sachs *et al.,* 2004; Stein *et al.,* 2003), and animal data provide crucial information supporting the role of DORs under various pain conditions (Garlicki *et al.,* 2006; Guan *et al.,* 2008; Núñez *et al.,* 2007; Obara *et al.,* 2009). Consistent with the animal data, the peripheral anti-nociceptive effects of opioids have been reported in patients with chronic rheumatoid and osteoarthritis, ischemic pain, dental pain, pancreatitis, and postoperative visceral pain upon local application of opioids to these tissues (Dionne *et al.,* 2001; Duckett *et al.,* 1997; Eisenach *et al.,* 2003; Keskinbora *et al.,* 2009; Likar *et al.,* 2001; Modi *et al.,* 2009; Rorarius *et al.,* 1999).

There is no dispute that peripheral, spinal and supraspinal opioid receptors act together in mediating opioid-induced analgesia, however, the relative contribution

of each site to a given analgesic effect after the systemic (intravenous or oral) administration of an opioid agonist has not been examined thoroughly. Some studies suggest that systemically (Lewanowitsch and Irvine, 2002; Shannon and Lutz, 2002) and even centrally (intracerebroventricularly) injected opioids may act largely through DORs (King *et al.,* 2001). Tissue damage has been seen to stimulate the expression of DORs, which likely leads to a concomitant increase of their function.

The anatomical distribution of DORs includes brain regions that, in addition to sensation and perception of pain, are associated with sensory information, emotional processing, reward and impulsivity, and other processes. Therefore, they are believed to have roles in modulating diverse behaviours and cellular functions. As opioids are known for their euphoric actions, it is not surprising that mice lacking the MORs and DORs show alterations in mood states. But unlike the MOR, deletion of DOR increases anxiety and depressive-like symptoms in mice (Filliol *et al.,* 2000). The DOR is expressed in regions commonly associated with depression and mood disorders including hippocampus, nucleus accumbens, olfactory bulb and frontal cortex which are associated with motor and motivational control (Tejedor-Real *et al.,* 1998; Saitoh *et al.,* 2012). DORs are also expressed in brain regions involved in stress responses: the amygdala, locus coeruleus (Alvira-Botero and Garzon, 2006; Scherrer *et al.,* 2006) and regions of the hypothalamus where they are co-localized with stress hormones and can regulate corticotropin-releasing hormone release (Williams and Milner, 2011).

The anxiolytic actions of DOR ligands and their reversal by appropriate delta antagonists has been extensively studied now which has led to proposals that these compounds may have potential as novel anti-depressants with much better

pharmacological profiles than the commonly available tricyclic antidepressants (Jutkiewicz, 2006).

The antitussive effects of opioids, including DOR selective opioids, are attributed to be due to their actions in the CNS but also in part, from the localization of opioid receptors on sensory nerve fibres in the vagus nerve in the lung (Belvisi and Hele, 2009).

DOR agonists are promising potential therapies for migraine. In an acute and chronic model of migraine pain, the two DOR agonists, SNC80 and ARM390 effectively reversed hyperalgesia evoked by the known migraine trigger, nitroglycerine (NGT) (Pradhan *et al.,* 2014). NGT-evoked sensory hypersensitivity in rodents has been developed as a model for the cutaneous allodynia (Bates *et al*., 2010; Pradhan *et al*., 2013), photophobia and vasodilation of meningeal blood vessels (Greco *et al.,* 2011; Markovics *et al.,* 2012) associated with migraine. Thus, DOR agonists may be well positioned as novel migraine therapies; however, further research is required to verify these effects.

Parkinson's disease is a neurodegenerative disorder of the CNS, caused by a loss of dopaminergic neurons in the substantia nigra, leading to muscle rigidity and lack of movement control due to the inability of the circuit to properly relay signals (Davie, 2008). DOR-selective ligands may be of therapeutic value in Parkinson's Disease considering that DORs are expressed in parts of the basal ganglia (Mansour *et al.,* 1987; Scherrer *et al.,* 2006) and several DOR agonists are reported to induce hyperlocomotion when administered systemically (Perrine *et al.,*

2006), intracerebroventricularly, or into the nucleus accumbens (Havemann *et al.,* 1983; Churchill *et al.,* 1995; Ingman *et al.,* 2003; Katsuura and Taha, 2010).

Expression of DORs on cardiac myocytes (Ventura *et al.,* 1989) has made these receptors interesting targets to study in ischemia and stroke. DORs are highly expressed in the sino-atrial node of the heart where they can modulate vagal transmission (Deo *et al.,* 2008). In particular, DOR agonists may protect against ischemic injury, indicating their role in pre-conditioning protection against ischemic heart disease (Johnson and Freiberg, 2010). Some studies have suggested a role for DORs in human immunodeficiency virus (HIV) infection. It has been shown that DORs are expressed in cells of immune system such as T-cells, neutrophils, monocytes, and macrophages (Pasternak, 2011), which play a key role in combating infections, and that the DOR agonists SNC80 and deltorphin inhibit the infection rate of HIV into T-cell derived Jurkat cells (Sharp *et al.,* 1998). The observation that DOR expression is upregulated in human peripheral blood lymphocytes after stimulation with phytohaemagglutinin (Sharp *et al.,* 2001), which differentiates these cells to activated T-like cells (O'Donovan *et al.,* 1995), increases its functional relevance as a potential (co-) therapy of HIV infection.

1.10. DOR-selective agents in research

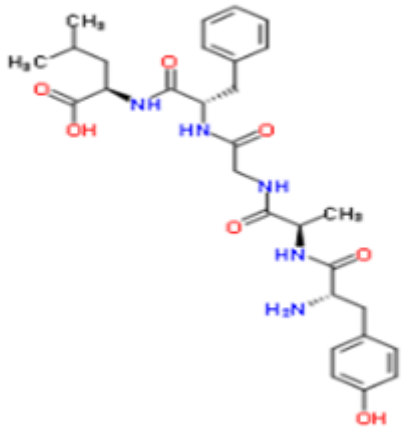
A number of DOR-selective agonists have been synthesized so far. The first generation of non-peptide compounds produced non-lethal convulsions in rodents and non-human primates, but subsequent generations of drugs were designed away from this problem. To date, only three DOR-selective compounds have reached clinical trials for the treatment of pain or depression, but no DOR selective

drugs have been approved by the Federal Drug Administration (FDA) and some candidates have failed in phase 2 clinical trials, highlighting current difficulties in developing effective and safe delta opioid-based therapies (van Rijn *et al.,* 2013). Endogenous opioids with DOR affinity have been isolated from humans (e.g. Met- and Leu-enkephalin) as well as from other species such as frogs (e.g. deltorphin I and II) (Erspamer *et al.,* 1989). Some of these peptides have served as templates for other selective, potent and/or stable peptide agonists that are less prone to enzymatic degradation, such as DADLE [D-Ala^2, D-Leu^5]-Enkephalin, DSLET [D-Ser2-Leu-Enkephalin-Thr6], DPDPE [d-Pen2,d-Pen5]-Enkephalin and the tetrapeptide JOM-13 (David *et al.,* 1982; Kosterlitz *et al.,* 1980; Mosberg *et al.,* 1983; Mosberg and Kroona, 1992). A major concern with these compounds from a therapeutic point of view is their limited ability to penetrate the blood-brain barrier (BBB), although some reports have suggested the existence of pumps that can transport DPDPE and deltorphin II across the BBB (Fiori *et al*., 1997; Williams *et al.,* 1996). To remove this obstacle, numerous small non-peptide, DOR-selective alkaloids have been developed to enhance penetration. Some of these compounds are morphinan derivatives (BNTX, NTI, SoRI-9409), whereas, others use different alkaloid scaffolds such as diarylmethylpiperazines (SNC80, ARM1000390, AZD2327) (Calderon, 2011), isoquinoline (TAN-67, KNT-127), spiro (ADL5747) and diarylmethylenepiperidine (JNJ-39204880). A summary of the characteristics of selected DOR agonists is presented below. The bivalent ligands have been designed in part to test the hypothesis that DORs heterodimerize with MORs to form an opioid receptor target with unique functionality (Abdelhamid *et al.,* 1991; Gendron *et al.,* 2007b; Gomes *et al.,* 2004; van Rijn *et al.,* 2012b; van Rijn and Whistler, 2009).The bi-functional ligands would fit the hypothesis that ligands acting on DOR and MOR can produce a unique functional response that is different from selective DOR or MOR ligands functionality.

1.10.1. Peptide agonists

DADLE or ([D-Ala^2, D-Leu^5]- enkephalin) is a peptide DOR agonist from the early days of DOR research. The compound was designed to protect the enkephalins from peptidases by D-amino acid substitution at positions 2 and 5. Initially it was considered as a selective ligand; however, it showed only ten-fold selectivity for the DOR over MOR (Pasternak, 2011).

Intracerebroventricular (icv) administration of another selective DOR peptide ligand DPDPE [D-Pen2,5]enkephalin produces antinociception (Ossipov *et al.,* 1995). However, due to low bioavailability, the clinical use of peptide agonists is limited.



DADLE

Figure 1.8 Chemical structure of the 'peptide' standard/ selective DOR agonist, DADLE used in the present study.

1.10.2. Non-peptide agonists

SNC80 was the first non-peptide selective DOR agonist (Calderon *et al.,* 1994) and it is about 500-fold DOR/MOR and 250-fold DOR/KOR selective, respectively (Bilsky *et al.,* 1995). It displays antinociceptive (Gallantine and Meert, 2005), antidepressant (Jutkiewicz, 2005) and anxiolytic (Perrine *et al.,* 2006) activity in animal models. However, it produces convulsions at high doses and, therefore, it cannot be used clinically (Broom *et al.,* 2002a, b). The pro-convulsive effect of SNC80 was initially suggested to be mediated through DORs; however, its mechanistic link to the receptor was not established. SNC80 and related compounds are commonly classified as having a diarylpiperidine chemical structure (Figure 1.9). Pro-convulsant activity produced by SNC80 and its analogues may be dependent upon the features of the structure and not of DOR function *per se* (Saitoh and Yamada, 2012).

DPI-221 exhibits a high affinity and selectivity for DOR with fewer propensities to cause seizures than previous DOR agonists (Holt *et al.,* 2005). It exerts limited effect on respiratory function or respiratory depression (Gengo *et al.,* 2003a).

The Adolor (ADL) compounds are two of the few DOR agonists to have entered clinical trials: ADL5747 for the treatment of postherpetic neuralgia (NCT01058642) and ADL5859 (Figure 1.9) for treatment of acute dental pain after third-molar extraction (NCT00993863) and osteoarthritis of the knee (NCT00979953). ADL5747 and ADL5859 are structurally distinct DOR full agonists with high affinity and selectivity. They possess good oral bioavailability and are devoid of seizure induction (Le Bourdonnec *et al.,* 2008, 2009). There were small, albeit non-

significant reductions, in pain scores due to both agonists in the clinic but the trials were flawed as the positive comparator used in the trials (oxycodone) was without effect.

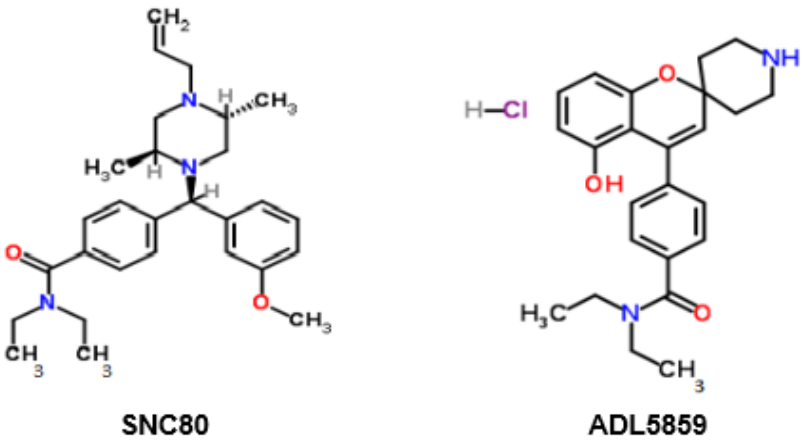
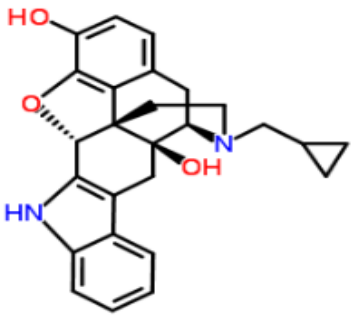


Figure 1.9 Chemical structures of the two stanadard DOR agonists; an example of the 'non-peptide' ligands used in the current study is presented.

AZD2327 is a full agonist at human DORs. It possesses antidepressant and anxiolytic activity in animal models (Hudzik *et al.,* 2011). It is non-seizurogenic in rats but causes convulsions in mice, dogs and primates (Hudzik *et al.,* 2014). AZD2327 was evaluated in Phase 2 clinical trial for the treatment of anxious major depressive disorder (NCT00759395). No statistical analyzes are reported, but there does not appear to be a separation from placebo regarding the primary outcome measures (Richards *et al*., 2016).

1.10.3. Non-peptide antagonist

Naltrindole (Figure 1.10) is a highly potent, highly selective delta opioid receptor antagonist (Granier *et al.,* 2012), which unlike peptide antagonist can cross the blood brain barrier.



Naltrindole

Figure 1.10 Chemical structure of the standard/ selective DOR antagonist, naltrindole used in the present study.

1.11. Basic G protein coupling concepts

The action of an agonist at GPCR is characterized by three fundamental properties, i) agonist affinity, which is its ability to bind to the receptor, ii) its binding sustainability (dissociation rate, receptor coverage) iii) and its ability to change the receptor behaviour and produce a response by modulating the downstream signalling systems. RP Stephenson (1956) in his observations on guinea pig ileum reported that agonists with equal potency could produce a different maximal response. He named this property e*fficacy*. It was further postulated that the relationship between the receptor occupancy and the response is a non-linear

function (Furchgott, 1966; Stephenson, 1956). This concept gave rise to the development of 'receptor reserve theory' which is defined as the ability of the agonist to produce a maximal response (E_{max}) through only fractional occupancy of the available receptor population in a system. Efficacy depends on different factors, such as the intrinsic efficacy of the agonist and/or, signal amplification of the system, where, steps in a signalling cascade allow sequential increases in the magnitude of a response (Kenakin, 2013; Randall *et al.,* 2012). Agonists and antagonists at GPCR can display a range of efficacies as shown in Figure 1.11.

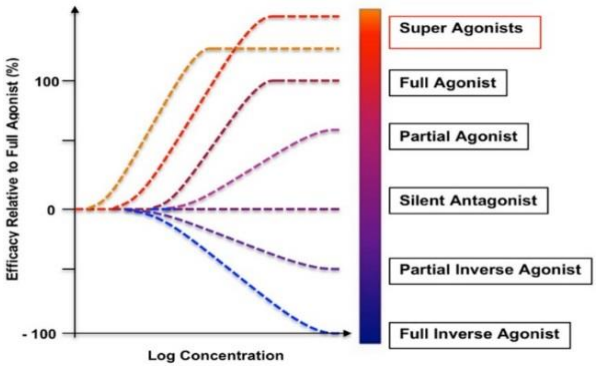


Figure 1.11 The range of different efficacies displayed by given agonists and antagonists at GPCRs (Szkudlinski, 2015).

The potency of an agonist is determined by the affinity of drug at the receptor and the number of receptors available and is represented by its EC_{50} value. The efficacy (E_{max}) of an agonist refers to the strength of response by an agonist and is related to the maximum effect produced by a drug in a system (Figure 1.12) and is generally independent of potency and affinity. In summary, potency is a relative measure on the concentration scale, whereas, efficacy is a relative measure on the response scale.

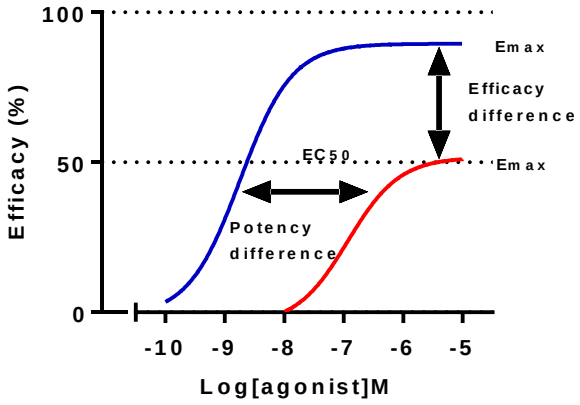


Figure 1.12 A typical pharmacological concentration-response curve using a linear Y-axis; showing difference in potencies and efficacies of two ligands (blue and red curves), and a Log_{10} X-axis; the curves that are generated are sigmoidal curves. The midpoint of the curves (50% response) represents the effective concentration 50 (EC_{50}). Efficacy (E_{max}) is shown as the measurement of the maximal response attained by the two agonists. Both measures are used to rank agonists.

Commonly employed functional assays to monitor the pharmacological characteristics of G_i- and G_s- coupled GPCRs involve substantial signal amplification between ligand binding and the final measured response. Partial agonists are known to occupy all the available receptors but are unable to elicit the same maximal response seen with full agonists, thus falling short of producing a maximal response as seen with a full agonist. Full agonists on the other hand could only occupy a fraction of receptors to generate a maximal response. The remaining unoccupied receptors are referred to as 'spare receptors' and full agonists are described as having a receptor reserve. Some tissues or cells express more receptors than needed to obtain a full response, thus for agonists with high intrinsic efficacy, the maximum response can be elicited without occupying all the receptors (Randall *et al.,* 2012).

As efficacy refers to the functional effects of a drug, it must be defined using functional tests and the effects of a set of drugs are therefore tested in a concentration-response experiment. The E_{max} is predominantly used to assess the efficacy of a ligand, which is calculated by the upper asymptote of the concentration-response curve. The efficacy concept has been defined and re-defined many times since its introduction in different eras, with the current view advocating that a given ligand may exhibit different efficacies in different signalling environments, hinting at the phenomenon of 'functional selectivity' or *'biased agonism'* (Kenakin, 2013).

1.12. Biased agonism as a novel concept in drug development

The old dogma of GPCR signalling or the traditional receptor theory which accounts for only a single form of efficacy, in which agonists can differ in the magnitude of their effects but not in the set of downstream pathways stimulated, behaving as full or partial agonists has been thoroughly disproven on many fronts for more than a decade now.

According to historical 'two-state' receptor model (active and inactive states), a simple on-off switching model was suggested for the GPCRs, in which binding of the agonist to the receptor shifts the receptor equilibrium from the predominantly inactive conformation/ state (R) to the active conformation/ sate (R*) (Samama *et al.,* 1993). Therefore, inverse agonists would tend to shift the equilibrium of the constitutively active receptor back to the inactive conformation. In this two-state model, partial agonists generate a submaximal response even by occupying all the available receptors. The idea that a receptor can adopt more than one activated

conformation (R_S*) was derived from the concept of agonist-directed trafficking or biased agonism (Kenakin, 1995). For “biased” agonism to be possible, there must be at least two distinct active receptor conformations that can be distinguished functionally (i.e., coupling differentially to the downstream effectors). In this case, ligands could conceivably display selectivity for the different active states, just as they can between the inactive and active states, thus allowing a ligand to “bias” the signalling. Conceptually, GPCRs can be visualized as oscillating amongst a series of conformational intermediates influenced by both agonists and effector proteins which can generate diverse functional responses in different pathways that cannot be explained by the traditional receptor theory. Different ligands regulate the GPCR activity by shifting the conformational equilibrium and the shape of this landscape (Wisler *et al.,* 2014). This appears to be also the case for opioid receptors. A biased opioid, therefore, should shift the receptor’s conformational equilibrium to a specific state and/ or states that preferentially activates a specific downstream signalling network (e.g., activating G protein-dependent or independent, β-arrestin signalling), thereby optimizing therapeutic action. A detailed characterization and comparison of these distinct conformations by a variety of biophysical and X-ray crystallographic techniques is required which will provide new insights into the structural basis of biased GPCR signalling.

It is realized that there are limitations in bias estimation on the basis of classic pharmacological parameters in different signalling systems. For example, in assays with significant receptor reserve, both full and partial agonists will reach close to a maximal response. Whereas in assays with little to no receptor reserve, full agonists reach higher maximal responses than partial agonists (Figure 1.13). Potencies are similarly affected by receptor reserve, because in assays with high levels of receptor reserve, a full agonist would have a greater leftward shift

compared with a partial agonist from its dissociation constant. However, in assays with lower levels of receptor reserve, these shifts do not correlate in a linear fashion (Rajagopal *et al.,* 2011).

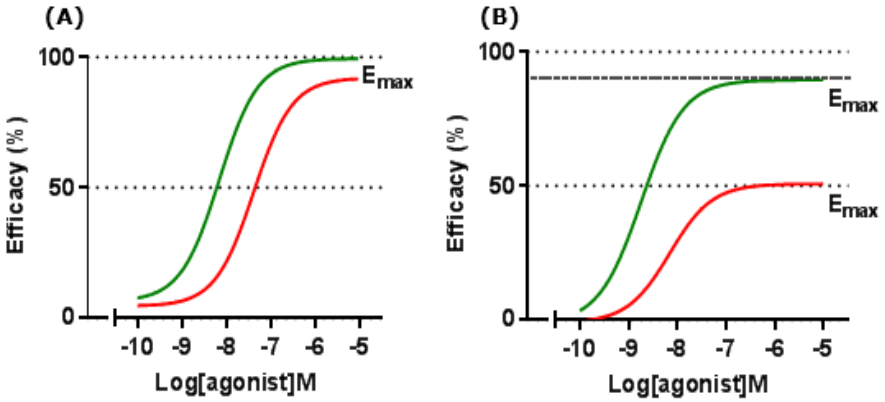


Figure 1.13 Limitations in quantifying ligand bias. (A) shows a system with high receptor reserve; both full (green) and partial (red) agonists reach close to a maximum response. (B) shows an assay with less receptor reserve in which a full agonist (green) reach a higher maximal response than the partial agonist (red).

The concept of efficacy thus becomes complex because ligands can have multiple efficacies in different signalling environments that translate into multiple observed activities as shown in Figure 1.14.

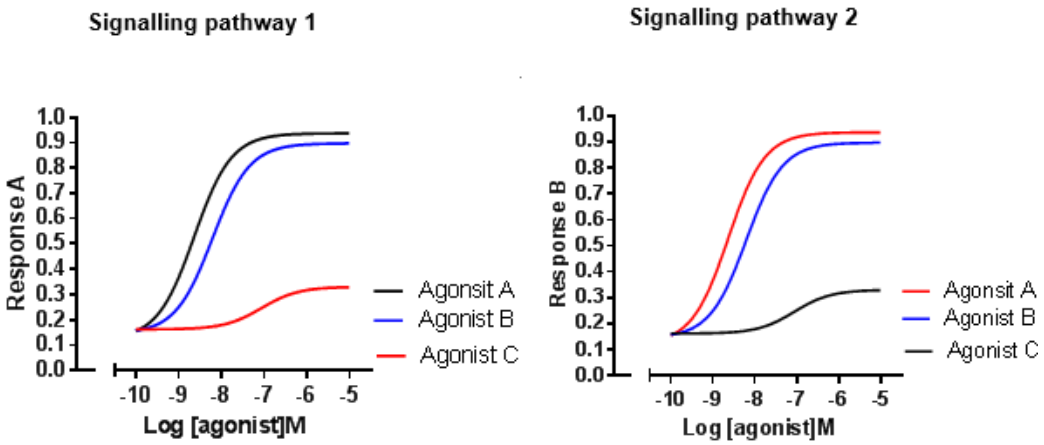


Figure 1.14 Concentration-response curves of three agonists (A, B and C), engaging two distinct signalling systems (signalling pathway 1) and (signalling pathway 2). It can be seen that agonist B produces comparable responses in signalling pathway 1 (as a function of responses in signalling pathway 2). The Agonist C produces a considerably lower response in signalling pathway 2 as a function of its response in pathway 1, indicating a bias towards pathway 1. Agonist A shows more bias towards signalling pathway 1 than pathway 2.

While MOR continues to be the main focus for biased agonism in opioid pharmacology thus far, there has also been a notable amount of research done on DOR and KOR. It has been seen that agonists at DOR may vary in their efficacy, desensitization, internalization and ultimately tolerance profiles *in vivo*. The idea of biased agonism has been suggested to be crucial for drug development, as this phenomenon raises the possibility of selecting or designing novel ligands that differentially activate only a subset of functions of a single receptor action.

The bias is usually not absolute and it is probably rare for a biased ligand at a GPCR to fully activate one pathway and be completely inactive at another For instance, opioids are indicated for pain but they produce unwanted effects such as respiratory depression, tolerance (Raehal and Bohn, 2014) and even alcohol

preference (Li *et al*., 2013), which have been suggested to be linked to the recruitment of *β*-arrestins. Thus an opioid agonist that stimulates the desired Gi/o mediated intracellular signalling pathway without much *β*-arrestin interaction would be predicted to be a superior analgesic choice (Figure 1.15). For example, TRV130 (also called oliceridine) is a pathway-selective MOR agonist that is under evaluation in human clinical trials for the treatment of acute/ severe pain. It mediates robust G protein signalling, with a potency and efficacy similar to morphine, but with far less *β*-arrestin 2 recruitment and receptor internalization and displays a higher therapeutic index than morphine in rodent models (Chen *et al.,* 2013).

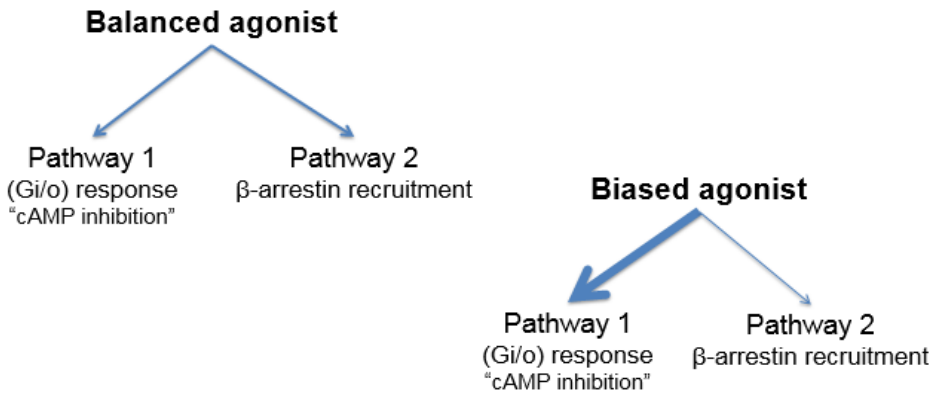


Figure 1.15 Balanced agonists have the potential to equally interact with two or more distinct pathways (e.g., pathways 1 and 2), whereas, a biased agonist preferentially engages one pathway (Pathway 1) i.e., displaying better efficacy in activating pathway 1 over pathway 2 i.e., showing partial efficacy in pathway 2.

The ability of an agonist to favour some particular cellular signalling pathways over others has been named in many different ways including biased agonism, functional selectivity, ligand-directed signalling, ligand-biased efficacy, collateral efficacy, pluridimensional efficacy, agonist-dependent signalling, stimulus

trafficking, protean agonism, differential engagement and selective receptor modulation (Smith and O'Malley; 2004; Urban *et al.,* 2007; Kenakin, 2007; Galandrin *et al.,* 2007; Rajagopal *et al.,* 2010; Vaidehi and Kenakin, 2010).

The two domains of opioid drug discovery and development that are affected by biased agonism are ligand detection (screening) and lead optimization. For screening, the assays need to be sensitive and simple to effectively decode the pharmacology of series of novel ligands. Relying on conventional radioligand binding assays and second-messenger screens (e.g. cAMP analysis) may fall short of detecting all possible efficacies present in a molecule. In contrast, assays detecting more comprehensive and amplified downstream signals e.g. ERK1/2 phosphorylation, *β*-arrestin recruitment and receptor internalization assays may serve as useful tools for detailed pre-clinical evaluation of both standard and novel DOR ligands. Other GPCR screening approaches include the establishment of stable cell lines for second messenger and reporter gene systems for detection of transcriptional regulation of promoter elements activated by GPCRs in drug screening programs (Hill *et al*., 2010).

Thus, the use of more focused high-throughput screening assays with a wider reach of both proximal and distal signalling mechanisms is important to capture fuller sets of receptor functions which could assist in covering all the relevant signal transduction pathways. However, the goal of developing perfectly biased ligands that could solely activate or inhibit one particular pathway without hitting other targets is a daunting challenge.

1.12.1. Quantification of agonist bias

Several analytical methods have been proposed to quantify ligand bias by using concentration-response curve data, of which most valuable and applicable approach is the operational model (OM) developed by Black and Leff (1983). This model has some interesting characteristics because it helps differentiate between properties of the drug (i.e., receptor affinity, intrinsic efficacy) and properties of the system (i.e., receptor density and E_{max}) and how the two come together to produce a pharmacological effect. The model used in the present study is derived from the Black-Leff approach and is based on the concentration-response curve data fitted to the following equation (Thompson *et al.,* 2015):

$$Y = bottom + \frac{top - bottom}{1 + 10^{(logEC50 - log\,[A])}}$$

$$Y = basal + \frac{(E_m - basal)(\frac{\tau}{K_A})^n[A](\frac{\tau}{K_A})[A]^n}{[A]^n(\frac{\tau}{K_A})^n + (1 + \frac{[A]^n}{K_A})}$$

Where, E_m is the maximum response produced in the system, basal is the basal level of the response, K_A denotes the functional equilibrium dissociation constant of the agonist (A) for the receptor. [A] is the concentration of agonist, and τ is the index of functional or intrinsic efficacy (i.e.,$\tau = \frac{R_T}{K_E}$), where R_T is the total number of receptors and K_E is the coupling efficiency of the agonist-occupied receptor). Therefore, τ comprises both agonist-specific factors (intrinsic efficacy) and tissue-dependent elements (receptor density and the ability of the system to convert a receptor stimulus into response), n is the slope of the transducer function that links

occupancy to response. Whereas, K_A is specific for the ligand-receptor coupling and τ is influenced by both ligand and the pathway. Therefore, an optimal scale to estimate biased agonism should include both τ and K_A values.

The ratio τ/K_A is determined as a logarithm i.e., $\log(\tau/K_A)$ (or, logTR here) and is referred to as '*transduction coefficient',* defined as the efficiency of the agonist to activate a signalling pathway, which can be measured by fitting the concentration-response curves to the operational model. The relative power of the agonists to activate each pathway is quantified by a normalized transduction coefficient i.e., ΔlogTR, to a reference ligand, in order to cancel the impact of the system bias and observational bias. ΔlogTR is thus a theoretically complete term to describe the power of a ligand to active a cellular pathway; which incorporates both elements of efficacy and affinity. Considering that the most common difference between systems is receptor density, ratios of ΔlogTR account for these and should be system-independent measures of the relative power of ligands to activate pathways. In fact, it has been shown experimentally that ΔlogTR values for muscarinic agonists do not change with a change in receptor density (Kenakin *et al.,* 2012).

It is noteworthy that the activity of agonists must be compared with the same reference ligand in each assay; hence an ideal reference ligand needs to activate most of the signalling pathways while possessing a relatively high efficacy for the desired cascade(s) (Thompson *et al.,* 2014). Actual agonist bias for different pathways can be calculated by comparing the ΔlogTR values of a given agonist across different pathways to calculate the relative transduction ratios ΔΔlogTR, which is important to eliminate the effects of system and observation bias (Kenakin *et al.*, 2012, Thompson *et al.,* 2015).

Actual ligand bias for different pathways can be calculated using the following equation:

$$\Delta\log TR = \log TR_{(\text{TEST LIGAND})} - \log TR_{(\text{REFERENCE LIGAND})}$$

The ligands' effectiveness toward each pathway, relative to the reference compound is then calculated as the inverse logarithm of the ΔlogTR:

$$\text{Relative efficacy} = 10^{\Delta \log TR}$$

Ligand bias or bias factor (ΔΔlog TR) is calculated as the difference between the ΔlogTR values:

$$\Delta\Delta\log TR = \Delta\log TR_{(L1:P1)} - \Delta\log TR_{(L2:P2)}$$

Where, L1 is ligand 1, P1 is pathway 1, L2 is ligand 2, and P2 is pathway 2

1.13. Project Aim

The aim of the current project was to examine the signalling properties of a number of established and novel DOR agonists in an attempt to identify compounds that have biased signalling profiles. It was hypothesized that DOR agonists with partial efficacy regarding *β*-arrestin recruitment would be less liable to induce receptor internalization and desensitization of G protein-mediated signalling and eventually (although outside the current remit) reduced analgesic tolerance *in vivo*.

Chapter 2. Development of a secreted placental alkaline phosphatase (SPAP) reporter gene assay for the study of delta opioid receptor-mediated signalling.

2.1. Introduction

The process of novel drug development is complex and can be approached in a variety of ways when undertaking a ligand characterization exercise. An early part of the experimental process often involves screening of compound libraries, using high-throughput screening (HTS) technology or, at least, investigation of relatively large numbers of compounds over a range of concentrations. The effect of a drug on an organism involves interactions at multiple cellular levels and, therefore, a compound should ideally be tested in more than one signalling pathway, the choice of which is of paramount importance. Cell-based screening assays are considered to be more biologically relevant surrogates than broken cell-based biochemical assays in order to predict the response of the organism as they involve integrated signalling systems.

Since the description of 3′-5′-cyclic adenosine monophosphate (cAMP) in the 1950s as the first accepted intracellular “second messenger”. It has remained the object of intense scientific interest and has been found to have essential roles in many intracellular signalling pathways in both prokaryotic and eukaryotic cells (Beavo and Brunton, 2002; Perera and Nikolaev, 2013; Scott *et al.,* 2012). The cAMP signalling pathway comprises multiple components and modulates a range of downstream cascades (described in section 1.5.1.1.) The pathway is responsible for delivering signals from GPCRs to membrane components, such as ion channels, and intracellular targets, including genes, through a cAMP-dependant protein kinase known as protein kinase A (PKA). The intracellular events take place when cAMP molecules bind to each of the two regulatory subunits of PKA, which then undergoes a conformational change that releases its catalytic subunits. The free subunits can then catalyse the phosphorylation of other

enzymes, ion channels or transcription factors such as cAMP response element-binding protein (CREB) in the nucleus. CREB is phosphorylated at the Ser133, enabling it to bind to the corresponding cAMP response element (CRE) sequence in the promotor region of target genes, which induces transcription and protein expression. PKA activation is dependent on the intracellular levels of cAMP and downregulation of PKA signalling occurs by a feedback mechanism involving phosphodiesterase (PDE) which quickly converts cAMP to AMP, thus depleting the activated PKA levels. cAMP signalling is widely held to be synonymous with the activity of its binding partner, protein kinase A (PKA).

G protein-coupled receptors associated with the stimulatory G protein, Gs, mediate increases in cAMP, while receptors, including the opioid receptor family, coupled to G*αi/*o proteins inhibit adenylyl cyclase. Pharmacological as well as genetic inhibition of PKA, results in a reduction of inflammatory mediator-induced hyperalgesic behaviour (Aley and Levine, 1999; Malmberg *et al.,* 1997). cAMP has been widely implicated in pain and nociceptor sensitization. Intradermal injection of the adenylyl cyclase activator forskolin (Taiwo and Levine, 1991) produces robust sensitization to physical stimuli (hyperalges1ia) and sensitization of nociceptive fibres (Kress *et al.,* 1996).Thus, it is important to understand how novel drugs aimed at controlling chronic pain regulate cAMP levels.

Several *in vitro* approaches to determining cAMP levels have been developed, some of which are anti-cAMP- antibody-based techniques, such as radio-immuno-assay (RIA) which is a method for indirect detection of cAMP. Another common method is the radiolabelled cAMP accumulation assay in which the ATP content of cells is labelled with tritium-containing adenine; however, both assays are time-consuming and require the purchase of expensive, legally regulated radioactive

materials and their subsequent disposal. Furthermore, RIA is not applicable for use in living cells and tissues. Over the last 5-10 years, high-throughput screening technologies for direct cAMP measurements have been established in pharmaceutical companies and academic research groups. These assays, such as the DiscoverX HitHunter™ cAMP kits based on enzyme complementation techniques, although very expensive, are extremely sensitive have few safety issues and are now largely taking over from the conventional tools and techniques.

cAMP can also be determined by indirect methods, including the use of reporter gene assays such as the use of secreted alkaline phosphatase (SPAP). A reporter gene is a sequence of DNA whose product is synthesised in response to activation of the signalling cascade under investigation (Hill *et al.,* 2001). According to Cullen and Malim (1992), reporter gene assays are sensitive, convenient and highly quantitative. With regard to sensitivity, the assays are more than sensitive enough to detect activation of GPCRs expressed even at relatively low levels. In terms of ease of use, the SPAP assay does not require any extraction or solubilization steps and it allows for the use of standard 24, 48 or 96-well assay plate formats and plate readers. All of these factors simplify automation and reduce costs. Reporter gene technology (RGT) offers an alternative to biochemical assays of intracellular messengers in living cells. RGT measures the activity of a desired gene and is often used as a means of indirect determination of ligand potency and efficacy (Hill *et al.,* 2001). As a general rule, the gene of interest is introduced into the model cell of choice (typically Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells) which subsequently enters the nucleus and is integrated into the chromosomal DNA. Integration into host chromosomes is a rare event and, thus, the process usually results in short-term gene expression in dividing cells. However, stable transfection is often desirable in which the foreign gene is

integrated into the cells' genome, which is transferred to daughter cells, under appropriate conditions. In order to maintain a homogenous population of cells containing the desired phenotype, the transfected cells are subjected to an antibiotic selection and cloning process. The desired gene is co-transfected with another gene for antibiotic resistance (such as the neomycin resistance gene, neo) and then the transiently transfected cells are exposed to an appropriate antibiotic for selection (such as G418 for neo-transfected cells). Only the stably transfected cells with resistance to the antibiotic will survive in long-term cultures.

The reporter gene product could be either extracellular or intracellular; the commonly used extracellular reporter genes include secreted placental alkaline phosphatase (SPAP, also abbreviated as SEAP) and *β*-lactamases, the products of which are secreted into the extracellular space which can be recovered for quantification. Luciferases, *β*-galacatosides and green fluorescent protein (GFP) are the intracellular reporters for studying gene delivery and regulation.

SPAP is a mutated form of human placental alkaline phosphatase lacking the last 24 amino acids at the C-terminal end of the protein. Removal of these amino acids prevents the enzyme from anchoring to the plasma membrane, and it is consequently secreted from the cell and can be detected by sampling the culture medium. Its activity in the extracellular medium is directly proportional to changes in intracellular concentrations of SPAP mRNA and protein (Kain, 1997). SPAP has the unusual properties of extreme heat stability, unlike endogenous alkaline phosphatases; therefore, endogenous alkaline phosphatase activity in transfected cells can be minimised by pre-treatment of samples at 65°C (Yang *et al.,* 1997). An additional advantage of the SPAP assay is that harvested supernatants

(containing SPAP) can be frozen down for further analysis because SPAP activity remains un-altered by freezing and thawing of the media.

As our goal was to establish a stably transfected DOR cell line that constitutively expresses a marker gene to trace the receptor location in addition to its activity, it was decided to fuse our receptor to the GFP which is a fluorescent genetic reporter, also referred to as “imaging marker gene” (IMG). GFP has evolved from a nearly unknown protein to a commonly used tool/ marker in wide domains of molecular biology and drug discovery. GFP possesses the property of emitting light in the green wavelength upon excitation with an appropriate UV light source without the need for any additional substrate or cofactor. GFP is composed of 238 amino acids with a molecular mass of 26.9 kD. After incorporation or transfection into appropriate cells, it can act as a fluorescent tag that can be visualised non-invasively, exhibiting bright green fluorescence when exposed to light in the blue to ultraviolet range. The IMGs can be incorporated with other genes of interest (the delta opioid receptor (DOR) in the present study) so that co-expression of both genes takes place and this can be visualised, serving as a tracer or optical signature to monitor successful gene transfer. GFP is particularly useful due to its stability, enabling its use in living systems for locating or tracking cellular events and quantifying molecules. Furthermore, it appears that fusion of GFP to a protein often does not alter the function or location of the protein (Tsien*, 1998;* Zimmer, 2002; Phillips, 2001).

While most small fluorescent molecules such as FITC (fluorescein isothiocyanate) can induce strong phototoxicity caused by the generation of free radicals, GFP is usually much less harmful when illuminated in cultured cells (Komorowski *et al.,* 2010).

2.2. Aims and objectives

The aim of this study was to develop a simple and efficient screening assay that could be used to identify and quantify the interaction of novel DOR agonists with Gi-protein mediated signalling.

In order to address this aim, the following objectives were established:

- To transfect CHO cells stably expressing the SPAP gene (containing multiple cAMP-response elements in its promotor region (CHOCRE-SPAP)) with a GFP-labelled human delta opioid receptor construct.
- To validate a DOR-mediated inhibition of cAMP assay in the cells.
- To monitor the cellular localization of GFP-labelled DORs after transfection.

2.3. Materials

Cell culture reagents were purchased from Sigma Chemical (Poole, Dorset, UK) and fetal bovine serum (FBS) was obtained from PAA Laboratories (Teddington, Middlesex, UK). X-tremeGENE 9 reagent was from Roche Diagnostics GmbH, Germany and GFP-tagged plasmid (pCMV FLAG-DOR-GFP) was a kind gift from Dr. Nick Holliday (Associate Professor of Pharmacology, School of Life Sciences, University of Nottingham, UK). The CHOCRE-SPAP stable cell line, under hygromycin selection, was donated by Prof. Jillian Baker, the Institute of Cell Signalling, University of Nottingham, UK. Rolipram and forskolin were purchased from Sigma-Aldrich. SNC80 and DADLE were purchased from Tocris Bioscience and Abcam respectively. Hygromycin and G418 were from Roche and

Calbiochem, Germany respectively. All other reagents were supplied by Sigma Chemicals.

2.4. Methods

2.4.1. G418 kill curve

The optimal concentration of G418 required for selection (the lowest concentration required to kill non-transfected cells) was determined. The CHOCRE-SPAP cells conferring resistance to hygromycin were allowed to grow in complete growth medium, DMEM/F12, containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine in a 37°C humidified 5% CO_2/ 95% air atmosphere. The cells were plated in a 12-well plate at 70% confluence in 1 ml medium. Selection was begun 24 hours after plating cells and maintained with a range of limiting dilutions of G418 at concentrations of 1 mg/ml, 800 µg/ml, 600 µg/ml, 400 µg/ml and 200 µg/ml, each in duplicate including a no-antibiotic (control) as shown in Figure 2.1. The cells were washed with PBS to remove dead cells and the selection media was changed every 3 days and wells examined daily under an inverted light microscope to assess cell viability.

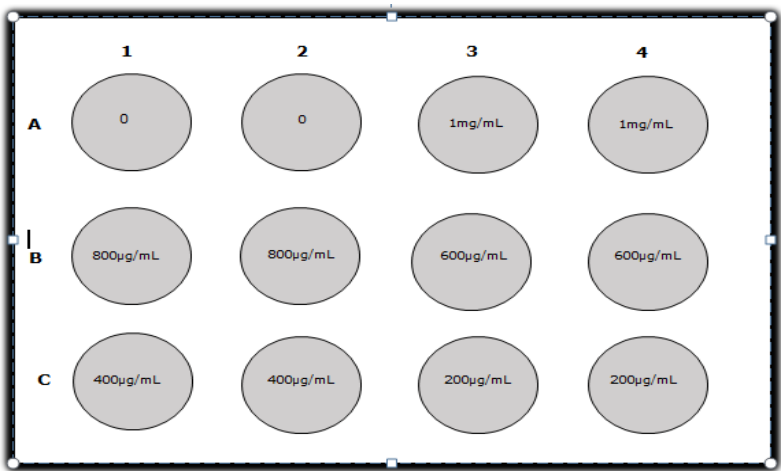


Figure 2.1 12-well plate layout for G418 kill curve

After 7 days, the percentage of surviving cells was assessed via visual examination using light microscopy. The lowest concentration of antibiotic that killed the cells was chosen for further experiments.

2.4.2. Cell culture and transfection

The SPAP secreting Chinese hamster ovary cells (CHOCRE-SPAP) under the transcriptional control of a six CRE promoter (McDonnell *et al.,* 1998), conferring resistance to hygromycin, were co-transfected with a GFP-tagged DOR construct. For this purpose stably transfected CHOCRE-SPAP cells were plated in a 6-well plate in duplicate at a seeding density of $1x10^5$ cells per well to achieve a confluence of 40-60%. The transfection mixture consisting of a C-terminal plasmid: green fluorescent protein (GFP)-tagged human delta opioid receptor (hDOR) (1 µg/1µL PCMV flag delta opioid GFP- p3.1 neo) and X-treme gene HP transfection reagent (used as per manufacturer's instructions), under neomycin (G418) selectivity, was added dropwise into one of the duplicate wells, while keeping the other well as control (cells with transfection reagent only and no GFP-tagged plasmid). Cells were grown at 37°C in Dulbecco's modified Eagle's medium/ nutrient mix F12 (DMEM/F12) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine in a humidified 5% CO2 : 95% air atmosphere. Once cells reached 70-80% confluency (about 3 days), the media containing selection antibiotics hygromycin (200 µg/ml) and G418 (1 mg/ml) was refreshed. After 3-4days, visible cell populations were observed in transfected culture due to the presence of the resistant gene, whereas, cells in non-transfected culture died. The transfected populations were allowed to grow and later passaged into larger T25 and then T75 culture flasks. The cells were observed daily so they would not become confluent and the selection media was changed every 2-3 days to ensure

there was enough hygromycin and G418 for the selection. Over time this will select for cells that have stably incorporated the GFP-tagged DOR into their genomic DNA.

2.4.3. Establishment of CHOCRE-SPAP-hDOR stable cell line

In order to ensure long-term transfection, the individual cells containing transfected DNA in their genome were isolated and propagated. For this purpose, the SPAP-secreting GFP labelled CHO-DOR cells (CHOCRE-SPAP-hDOR) were dilution cloned to achieve higher expression of DOR. Cultured cells were harvested by trypsinization, combined and seeded in a 96-well culture plate.

2.4.3.1. Selection and isolation of clones

To generate a stable cell line expressing both the CRE reporter promoter and the GFP-tagged DOR, the pooled transfected cell line was used for limited dilution cloning. For this, cells were serial diluted to achieve 1 cell per well in a 96-well culture plate using DMEM/F12 with 10% FBS and 2 mM L-glutamine, containing G418 (1 mg/ml).

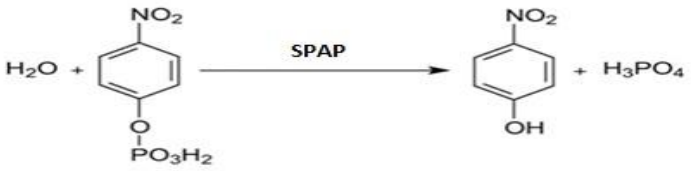
A 10 ml cell suspension containing 100 cells per ml was prepared. The cells were seeded in a 96-well culture plate, adding 100 µl per well to achieve a cell density of 1 cell per well. The cells were allowed to grow in a humidified 5% CO2: 95% air atmosphere for 5 hours. The wells were inspected for the presence of single cell clones under an inverted light microscope. The wells containing 1 cell per well were identified and marked after 18-24 hours of seeding. The wells containing only one

cell were allowed to reach confluence for another 7 days, routinely changing the culture media every 3 days to allow clones to expand further, using G418 (1 mg/ml for the GFP-tagged DOR) and hygromycin (200 µg/ml for the CRE-SPAP reporter), 11 different clones were isolated from the mixed population using fluorescent microscope and labelled as B3, B6, C5, C8, D7, F8, G6, H10, H11 and H12 according to the well label in the 96-well plate. The confluent cultures from the labelled wells were transferred to a 12-well culture plate for further growth and expansion once cultures became confluent in the 96-well plate. Cells were allowed to grow further for two weeks, changing the media every 2-3 days. Upon confluency, all clones were transferred to separate T25 culture flasks and labelled accordingly. SPAP assay (section 2.4.3.2) was performed on all the GFP-positive clones to compare the potential inhibitory effects of SNC80 on forskolin-mediated SPAP activity in our clones. Once expression was verified, the 2 best clones (G6 and F8) were then picked and cultured separately and expanded into T75 flasks. The cells were split every third day for two weeks to ensure that the selected clones become stable. Early passage cells were banked in liquid nitrogen for future experiments.

2.4.3.2. **SPAP assays**

CRE-mediated reporter gene activation (secreted placental alkaline phosphatase, SPAP activity) was measured after the cells were serum-starved for 24 hours. Briefly, cells were incubated with DOR agonists for 20-30 min prior to incubation with forskolin for a further 4 hours at 37°C. The drug media was replaced with serum-free media (SFM) and incubated for a further 1 hour at the same 37°C, 5% CO_2 atmosphere. The supernatant was harvested for SPAP activity and collected in Eppendorf tubes and assayed on the same day or frozen at -20°C. The frozen

samples were thawed out and heated at 65°C for 30 min to inactivate all endogenous alkaline phosphatases (SPAP is stable at 65°C). The samples were cooled to room temperature and added to 96-well culture plates to quantify the secreted CRE- dependent SPAP activity by following the colour change caused by the hydrolysis of the exogenously introduced chromogenic substrate, p-nitrophenol phosphate (pNPP), 5mM solution in diethanolamine (DEA) buffer. The plates were then incubated at 37°C for 25-30 min in the dark. The absorbance at 405 nm was measured on a Molecular Devices 307 plate reader.



pNPP p-nitrophenol (yellow)

Figure 2.2 *p*-nitrophenyl phosphate (pNPP) is a non-proteinaceous substrate, SPAP catalyses the hydrolysis of pNPP to *p*-nitrophenol, a chromogenic (yellow) product, with absorbance read at 405 nm.

The experiments were carried out in 24, 48 and 96-well plate formats on different occasions and optimized for various parameters in order to achieve the best possible response.

2.4.4. Cell imaging using fluorescence microscopy

2.4.4.1. Specimen preparation

12 mm circular glass coverslips #1.5 (170 um thick) were autoclaved prior to mounting cells and transferring to 12-well plates, using fine sterile forceps. The CHOCRE-SPAP-hDOR cells were grown to a confluence of 60-80% on the coverslips in 2 ml of DMEM/F12, containing 10% FBS and 2 mM L-glutamine at 37°C in a humidified 5% CO2, 95% air atmosphere. Two wells were kept as controls (seeded with native CHO-K1 cells). On the next day, the media was aspirated off and the cells were washed once with PBS. The cells were then covered with 2 ml of 4% paraformaldehyde (PFA), diluted in warm PBS to maintain the cellular structures. The cells were left to fix for 15-20 minutes at room temperature, the fixative aspirated off and 2 ml per well of DAPI solution (1:1000; diluted in PBS) was added, making sure that the cells were completely covered. The plates were then incubated for up to 5 minutes at room temperature then rinsed three times with PBS, followed by three washes with distilled water to remove all free DAPI. A small drop of mounting medium (50-90% glycerol in PBS, mixed with an anti-quenching agent, i.e. 0.5% N-propyl gallate which helps prevent photobleaching) was put onto the glass slide. A coverslip was carefully lowered onto the drop of mounting medium in such a way to prevent the formation of bubbles. Finally, edges of the coverslips were sealed with clear nail varnish and left to dry. The prepared slides were then stored in a storage box at 4°C for next day imaging.

2.4.4.2. Imaging

For fluorescence analysis, the fixed GFP-conjugated CHOCRE-SPAP cells were visualised under a Leica inverted DM IRB fluorescence microscope with a 40x oil immersion objective and a highly sensitive charge-coupled device (CCD) colour camera using the Q-imaging software. To visualise nuclei, a nuclear and chromosome counterstain, DAPI (4′,6-diamidino-2-phenylindole) was used, which emits blue fluorescence upon binding to AT regions of DNA. DAPI traverses the intact cell membrane in both live and fixed cells; however, it stains the fixed cells more efficiently than live cells and binds/ labels the nuclei specifically, with little or no cytoplasmic staining.

2.5. Data analysis

Data analysis was performed by Graphpad Prism 7.01 (GraphPad software, Inc, San Diego, CA, USA). Non-linear regression was used to plot and analyze concentration-response curves and to measure EC_{50} values. The standard error of the mean is represented by an error bar on each graph. The statistical differences between mean values were determined using a two-tailed t-test or one-way ANOVA, followed by Dunnett's multiple comparisons tests, where appropriate. A $p<0.05$ was considered significant. The amount of chromogenic product, p-nitrophenol, is determined by reading the absorbance at 405 nm.

2.6. Results

2.6.1. G418 kill curve

After 7 days, no viable cells were present at a concentration of 1 mg/ml G418. This was then chosen as the effective concentration to select for transfected cells.

2.6.2. The model; Optimization of forskolin responses in the reporter assay

The CHOCRE-SPAP cell line transfected with a GFP-labelled DOR was selected as an *in vitro* model for assaying the effects of drugs on the cAMP signalling pathway. Initially, the model had to be validated in order to determine its suitability. In order to detect negative regulation of adenylyl cyclase by DOR ligands, the system was first activated by the diterpene forskolin (FSK), a direct activator of adenylyl cyclase, which increases intracellular levels of cAMP.

An initial experiment was carried out on the transfected cell line to investigate the stimulatory effects of FSK on cAMP accumulation in the absence of any DOR agonist. The cells were grown in a 24-well plate to a confluence of 80-90% and then serum-starved for 24 hours. The cells were exposed to a wide range of FSK concentrations (0.4 µM, 0.8 µM, 1.65 µM, 3.75 µM, 7.50 µM, 15 µM and 30 µM) and incubated for 4 hours at 37°C. The culture media was removed and assayed for SPAP enzyme activity (as described in section 2.4.3.2)

All concentration of FSK provoked a response with a maximum effect observed at the highest concentration (30 µM) employed (Figure 2.3).

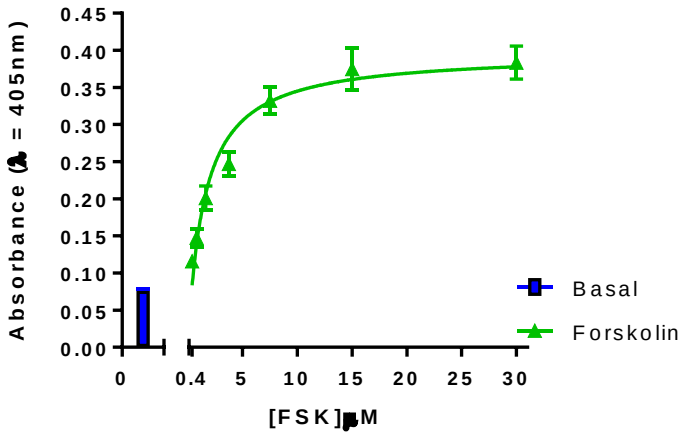


Figure 2.3 FSK concentration-response ranging from 0.4 µM to 30 µM FSK. The supernatant was harvested post-stimulation and quantified for the SPAP enzyme. The curve shows a strong stimulation of the SPAP activity and, by extension, of cAMP levels. Data points are means ± SEM of triplicates in a 24-well plate (n=1).

In another experiment, the effects of FSK were evaluated at lower concentrations in order to identify an appropriate concentration of FSK that would allow the detection of agonist inhibitions of SPAP in further experiments. The FSK-stimulated SPAP response was determined after the cells were incubated for 4 hours at 37°C with a range of FSK concentrations as shown in Figure 2.4. Forskolin (1 µM) produced a significant and marked increase in cAMP accumulation and the logEC_{50} value of FSK was calculated to be -6.6 ± 0.07.

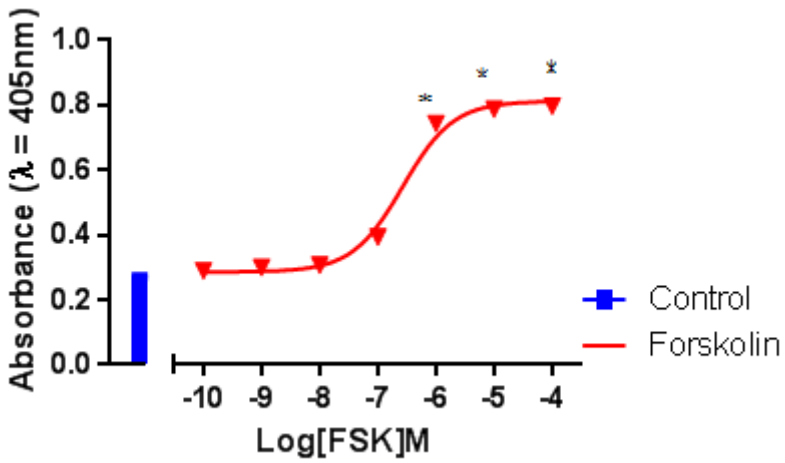


Figure 2.4 Concentration-response curve of forskolin (FSK)-stimulated activation of SPAP in CHOCRE-SPAP-hDOR cells. Data points are means ±SEM of triplicate samples (n=3) (*p < 0.05; One-way ANOVA).

In a separate experiment, the CHOCRE-SPAP cells were treated with the optimal concentration (1 µM) of FSK and incubated at 37°C for different times (15min, 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs and 5 hrs) to identify any differences in the FSK temporal response profile and to determine which time point would provide the maximum cAMP response for use in agonist concentration/response experiments.

The maximum response to FSK occurred at 5hrs (Figure 2.5). Additionally, the incubation time with pNPP, the chromogenic substrate for SPAP, was also tested at different time points (30 min, 1.5 hrs, 2 hrs and 2.5 hrs) and it was also found to increase as a function of the time, showing the largest response at 2.5 hrs and this time point *was* selected *as* the optimal condition for future experiments.

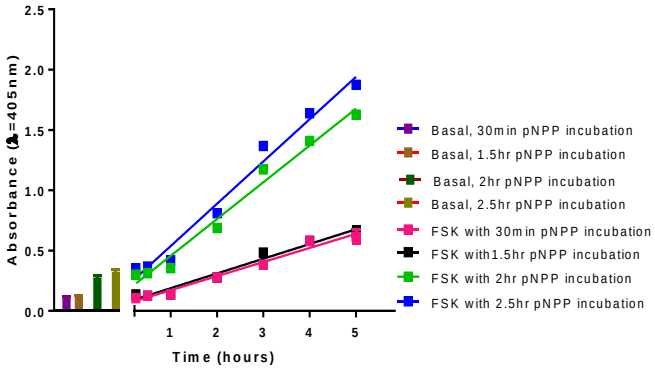


Figure 2.5 Time-course of forskolin (FSK)-stimulated production of SPAP in CHOCRE-SPAP-hDOR cells. Cells were incubated with FSK for 15 min, 30 min, 1hrs, 2 hrs, 3 hrs, 4 hrs and 5 hrs at 37°C. The supernatant was harvested for SPAP after 30 min of incubation at 65°C. The secreted SPAP activity was quantified by adding pNPP solution and the plates were incubated again at 37°C and the absorbance was measured at λ = 405 nm, testing pNPP incubation at different time points (30 min, 1.5 hrs, 2 hrs and 2.5 hrs).

2.6.3. Acute effects of DOR agonists on CREB/CRE-mediated gene transcription

Initially, SNC80 was used as a representative non-peptide DOR agonist to investigate the effects on cAMP signalling linked to CREB/CRE-mediated gene transcription in the pooled transfected cell line. The peptide DADLE was included in some experiments to investigate any possible differences in SPAP activity increases due to the two chemically distinct DOR agonists.

The acute effects of SNC80 were determined in CHOCRE-SPAP-hDOR cells, either alone or in the presence of FSK. Each drug treatment was carried out in triplicate in a 48-well plate. The cells were pre-incubated with 10 µM SNC80 for 15min and 30 min. In other triplicates, the cells were pre-treated with 10 µM SNC80 for 15 to 30 min and then challenged with 10 µM FSK respectively for 4hrs.

FSK was consistently able to produce a marked stimulatory cAMP response in the cells but there were no significant inhibitory effects of SNC80 (One-way ANOVA) at any time-point (Figure 2.6). The experiments were repeated several times and optimized for different conditions (agonist concertation, time points etc.); however, SNC80 was unable to show any significant effect under any of the conditions employed.

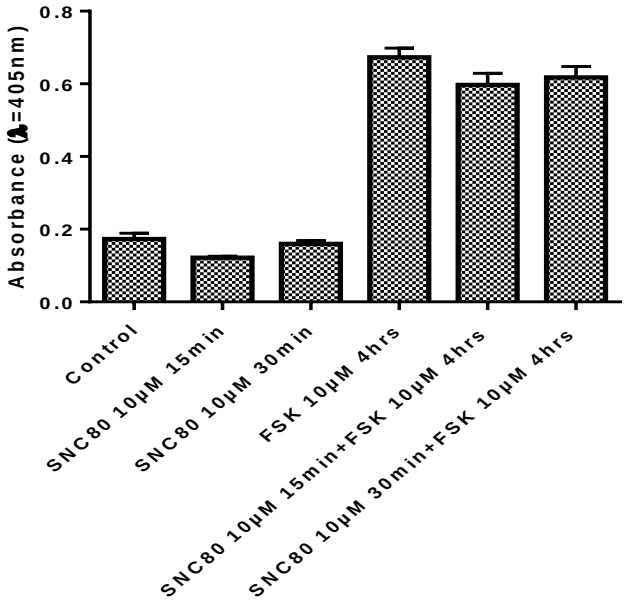


Figure 2.6 Acute effects of SNC80 on CREB/CRE-mediated gene transcription. The CHOCRE-SPAP-hDOR cells were incubated with 10 μM SNC80 for 15 min and 30 min alone to investigate any effects on the basal cAMP formation. The values represent means± SEM from 5 experiments in triplicate. The cells were then stimulated with 10 μM FSK for 4hrs in the presence of SNC80 and the SPAP activity was measured at λ = 405 nm.

In another experiment, cells were pre-exposed to a wide range of SN80 concentrations in the presence of 1 μM FSK (Figure 2.7). Again, SNC80 did not produce any significant inhibition at any concentration.

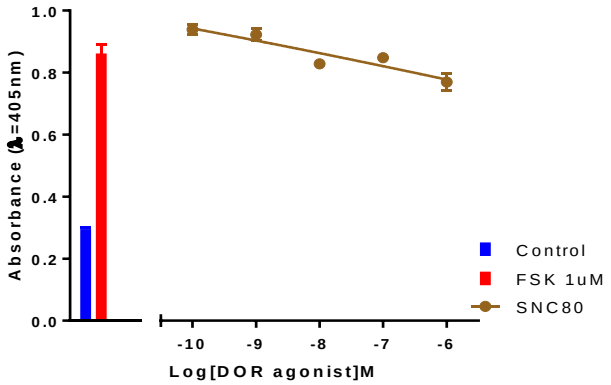


Figure 2.7 SNC80 concentration-response of CHOCRE-SPAP-hDOR cells, incubated with different concentrations of the agonist for 20 min and then challenged with 1 µM FSK. SPAP activity was measured after 4 hours of further incubation. The values represent means±SEM from 5 experiments in triplicate. SNC80 failed to significantly inhibit forskolin-stimulated SPAP activity at all concentrations (unpaired, two-tailed t-test).

2.6.4. Long-term effects of DOR agonists on CREB/CRE-mediated gene transcription

Although there were no significant acute effects of SNC80 on FSK-stimulated SPAP activity, it was decided to test the model cell line for potential long-term DOR agonist effects. The experimental protocol was kept the same as described above but, in this case, the pre-treatment of cells with the agonist was carried out for 2hrs and 3hrs respectively (Figure 2.8). Single concentrations (10 µM) of FSK and SNC80 were used for the assay. SNC80 alone had no statistically significant effect on basal levels whereas, FSK showed consistent stimulation. SNC80 again failed to produce any statistically significant inhibitory effect (one-way ANOVA). At this point, it was decided to carry out dilution cloning, potentially to select cells with higher DOR expression which could enhance the Gi-mediated effects.

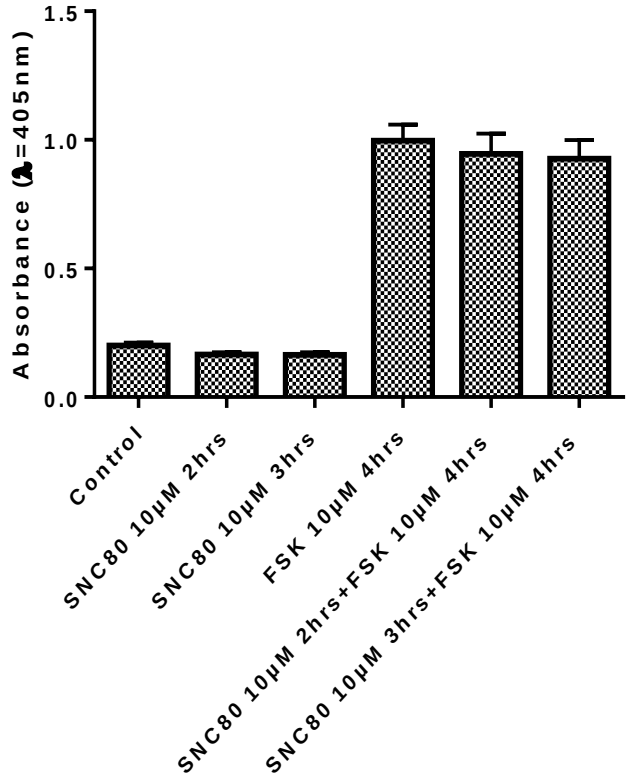


Figure 2.8 Long-term effects of SNC80 on CREB/CRE-mediated gene transcription. The CHOCRE-SPAP-hDOR cells were incubated with SNC80 alone for 2hrs and 3hrs respectively to investigate any long-term endogenous effects. The cells were then stimulated with 10 μM FSK for 4hrs in the presence or absence of 10 μM SNC80 and the cAMP/ SPAP activity was measured at 405nm. The values represent mean±SEM from 3 experiments in triplicate.

2.6.5. Dilution cloning

The pooled GFP-tagged CHOCRE-SPAP-hDOR cells were generated as described in section 2.4.3.1, by taking portions of the 10 clones, each in triplicate of a 96-well plate and treated according to the assay plate layout as shown in Figure 2.9. Only two clones (G6 and F8) were found to show any indication of

agonist-mediated inhibition of the FSK response suggesting higher expression of the GFP-tagged DOR (Figure 2.10).

Basal B3	FSK B3	FSK +SNC80 B3	Basal G6	FSK G6	***FSK +SNC80 G6***
Basal B6	FSK B6	FSK +SNC80 B6	Basal H10	FSK H10	FSK +SNC80 H10
Basal C5	FSK C5	FSK +SNC80 C5	Basal H11	FSK H11	FSK +SNC80 H11
Basal C8	FSK C8	FSK +SNC80 C8	Basal H12	FSK H12	FSK +SNC80 H12
Basal D7	FSK D7	FSK+SNC80 D7			
Basal F8	FSK F8	***FSK +SNC80 F8***			

Figure 2.9 48-well assay plate layout for testing CHOCRE-SPAP–hDOR clones.

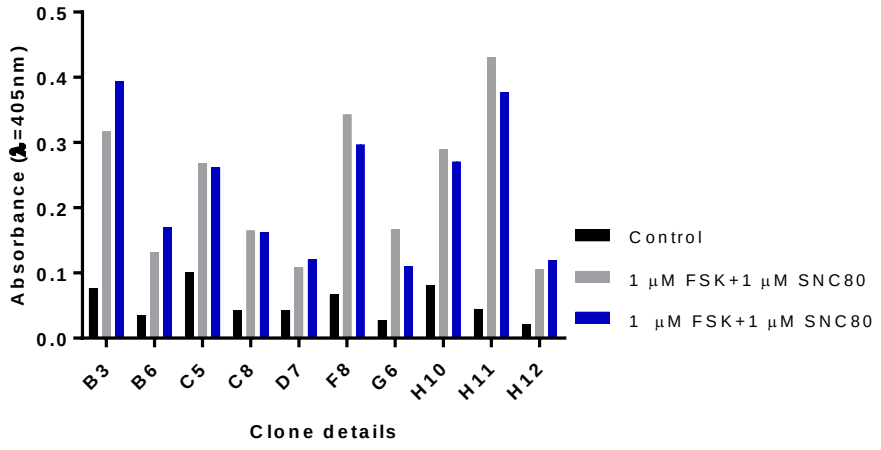


Figure 2.10 SPAP assay performed on 10 CHOCRE-SPAP-hDOR clones (each assayed in triplicate). Result represents a single experiment. All the clones were incubated with 10 µM SNC80 for 20 min and SPAP activity was measured after 4 hrs of incubation with 10 µM FSK in a 48-well plate. The G6 clone apparently showed some inhibition, however, it was not statistically significant (unpaired, two-tailed t test).

The G6 clone was selected for further experimentation due to it exhibiting the greatest apparent inhibition of the FSK response, by SNC80.

2.6.6. Fluorescence Microscopy

In order to estimate the transfection efficiency visually, images of the G6 clone were captured using a Leica DM IRB inverted fluorescence microscope and cells were examined for the presence of GFP, presumably tagged to DORs. The images indicate that a reasonable proportion of the transfected cells showed GFP-conjugated DOR expression (Figure 2.11).

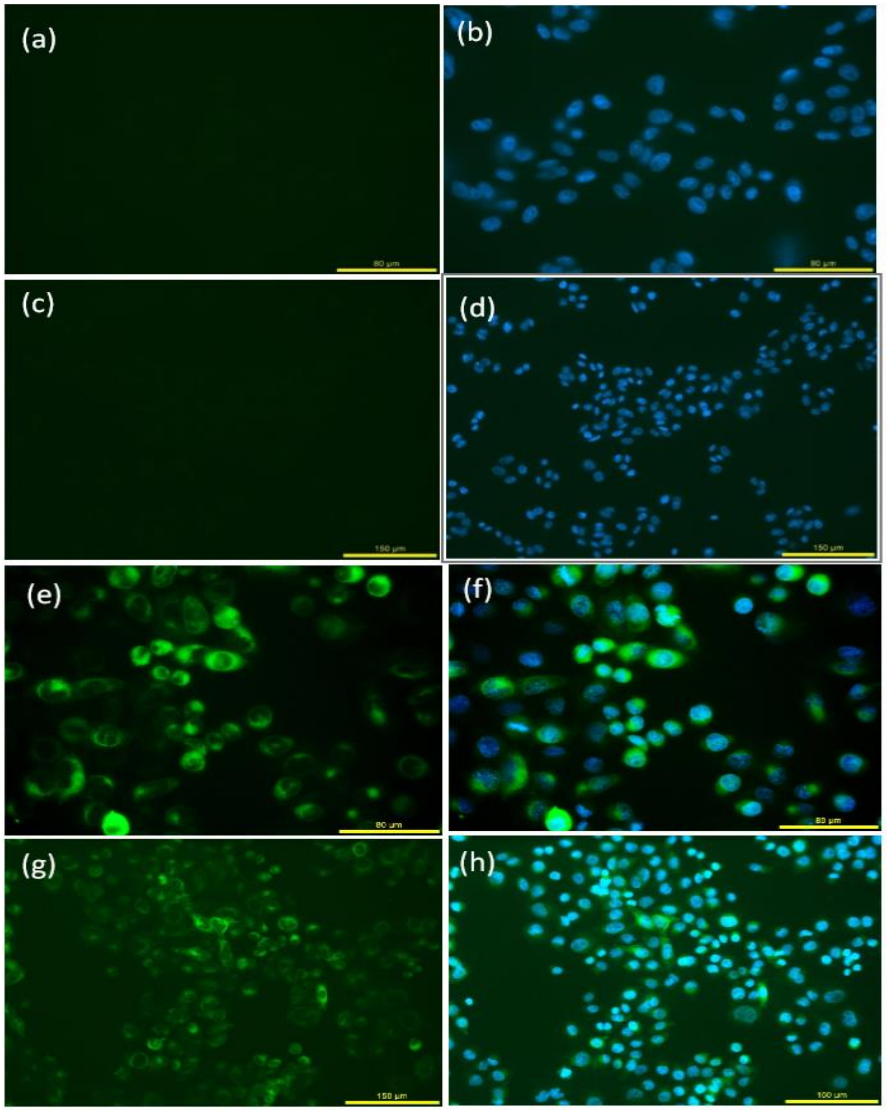


Figure 2.11 The CHO-K1 (native) cells (a, b, c and d) and G6 clone (e, f, g and h); were allowed to grow on coverslips in 12-well plates and fixed using 4% PFA. The cells were then treated with DAPI and rinsed with PBS and mounted on glass slides. GFP images (green) were taken of the fixed cells, using a Leica inverted DM IRB fluorescence microscope with a 40x oil immersion objective and a Q-Imaging CCD colour camera with Q-imaging software. The scale bar represents 80 µM (a b, e and f) and 150 µM (c, d, g and h). Nuclei were visualized by DAPI staining (blue) in the right panels (b, d, f and h).

2.6.7. Confirmation of cloning by cAMP chemiluminescence assay

As a further confirmation of GFP-tagged DOR expression in our clonal cell line, a cAMP chemiluminescence assay (HitHunter™ assay kit, DiscoverX) (method described later in Chapter 3, section 3.4.2.) was performed on the G6 clone, as a direct method to investigate the inhibition of FSK-stimulated cAMP accumulation.

As shown in Figure 2.12, the DOR agonist SNC80 significantly inhibited the cAMP accumulation in the G6 cell line (unpaired, two-tailed t-test; $p < 0.0001$). The inhibition of cAMP accumulation was blocked by the selective DOR antagonist naltrindole (1 μM).

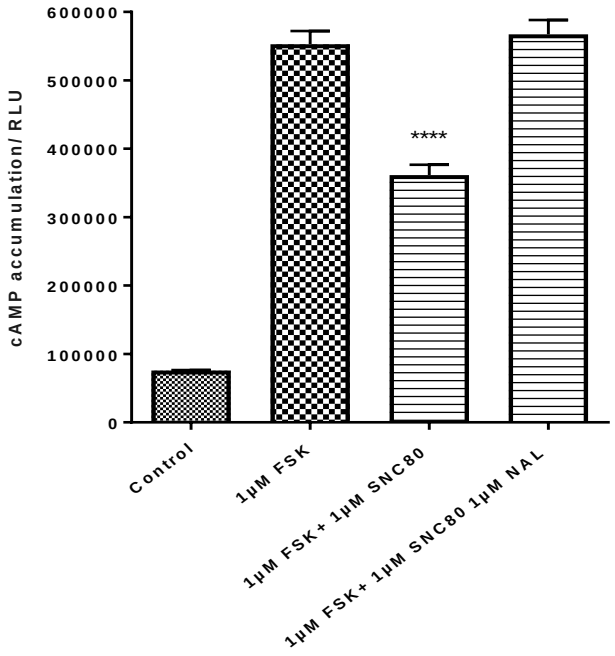


Figure 2.12 HitHunter™ cAMP chemiluminescence assay (DiscoverX assay) performed on G6 clone. FSK (1 μM) stimulated cAMP accumulation was measured using 1 μM SNC80 in the experiment. The values represent mean ± SEM from a single experiment in triplicate. SN80 produced a statistically significant inhibition of FSK mediated cAMP accumulation (unpaired, two-tailed t-test, ****$p < 0.0001$). 1 μM naltrindole (NAL) was able to antagonise the apparent SNC80-mediated inhibition of the FSK response.

2.6.8. Post-cloning determination of short-term effects of DOR agonists on CREB/CRE-DOR mediated gene transcription

Full concentration/response experiments were carried out using the non-peptide DOR agonist SNC80 and the peptide DOR agonist, DADLE to investigate possible structure-depenedent differences in agonist responses. The assay was performed and repeated in 96-well plates to generate more data points. In the case of SNC80, there was a significant inhibition of the FSK-mediated SPAP response only at the highest concentration (1 μM) employed. DADLE also produced a significant

inhibition but only at 10 µM, as shown in Figure 2.13 (one-way ANOVA). The experiments were repeated several times with different experimental conditions, including the use of 0.1% bovine serum albumin (BSA) (diluted in SFM), to prevent potential sticking of the agonists to the plate wells, which could reduce the concentration of the free drug. However, the magnitude of the inhibition could not be improved by any adjustment (data not shown).

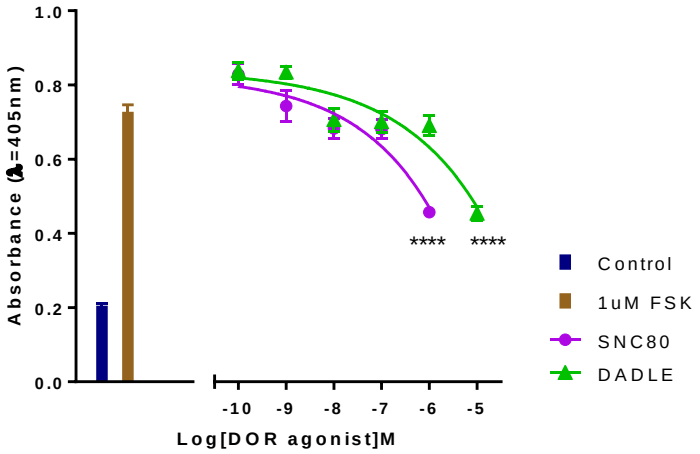


Figure 2.13 Concentration responses of SNC80 and DADLE. Cells (G6 clone) were pre-treated with a range of agonist concentrations for 20 minutes and then stimulated with FSK for 4 hours prior to measurement of supernatant SPAP activity. Data points are means ± SEM of triplicates in a 96-well plate in three independent experiments. Curves were fitted by non-linear regression using GraphPad Prism 7.01. ****$p < 0.0001$ (One-way ANOVA, followed by Dunnett's multiple comparisons test).

2.7. Discussion

The aim of the studies described in the present chapter was to determine whether the SPAP reporter gene assay could be developed for use in identifying and quantifying the interactions of DOR agonists with Gi protein mediated signalling which could aid the investigation of novel drugs' signalling properties.

The DORs couple predominantly to the inhibitory protein (Gi/o), the alpha subunit of which mediates the inhibition of adenylyl cyclase (AC) and subsequent reduction in cellular cAMP levels, although the receptors also engage kinase signalling cascades and the beta/gamma subunit dimers derived from Gai/o proteins can modulate membrane-delimited enzymes (e.g., ACs, PLCb, and channels such as $Ca_v2.1$ and Kir3) (Gendron *et al.,* 2016). One of the key functions of cAMP is to act as a link between cell surface receptors and the nucleus, modulating the function of genes that contain cyclic AMP response elements (CREs) in their promotor regions. This provides an opportunity for developing reporter gene assays as indirect, but relatively facile, reflections of cAMP formation in intact cells.

In the present study, the SPAP assay was chosen instead of using an established radiolabelled cAMP accumulation assay which, despite being a direct second messenger measurement, is an expensive and time-consuming assay, with chances of frequent error due to the multiple assay steps, accompanied by the risks and disposal problems associated with radioactivity.

CRE-SPAP has become one of the most widely used reporter gene assays. Activation of adenylyl cyclase causes conversion of ATP to cAMP, followed by activation of PKA which phosphorylates cAMP response element binding protein (CREB) that ultimately serves as a transcription factor and is the major regulator

of CREs in gene promotor regions (Dwivedi, and Pandey, 2008). The SPAP construct used in the present study contains 6 CREs upstream of the coding region allowing amplification of the phospho-CREB transcriptional response.

The fact that SPAP is extracellularly secreted allows cells transfected with the reporter gene to be used for repeated estimations of cAMP; for example, in time course experiments, as samples of media can be removed and assayed for SPAP without disrupting the cells. The choice of the host cell for reporter gene assays is important and the Chinese hamster ovary (CHO) cells are the most commonly used for studying receptor interactions (Wurm, 2004). As model cells for research, they offer several advantages as they can be easily cultured, express relatively few signalling molecules compared with most primary cultured cells and can be readily transfected with genes of interest.

In the present study, the CHOCRE-SPAP cells were transfected with a construct coding for GFP-tagged DORs to enable the expression level and cellular localization of the receptors to be readily estimated. According to Kallal and Benovic (2000), the advantages of using GFP are numerous; since no co-factors or substrates are required for fluorescence, the cumbersome protocols, potential artefacts, and time and expense in obtaining primary and secondary antibodies can be avoided. The GFP-conjugated cells can be fixed for convenience if desired, because GFP is relatively chemically resistant. GFP is covalently attached to the protein of interest, therefore, potential problems with non-specific fluorescence are avoided. Additionally, GFP allows time-course studies to be performed on living samples in real time so that the dynamics of protein trafficking can be observed (Böhme and Beck-Sickinger, 2009).To date, published accounts of GPCR–GFP fusions are extensive and include over 20 different GPCRs (not including opioid

receptors, however), and when tested in radioligand binding studies with their respective agonists, there appears to be remarkable retention of the normal receptor characteristics within the chimeras (Drmota *et al.,*1998; Barak *et al.,* 1997; Kallal *et al*., 1998). However, the potential disadvantages of GFP must also be considered. First, the expression of GPCR-GFP chimeras in cells produces labelling of protein biosynthetic compartments, such as the endoplasmic reticulum and Golgi, which can partially obscure or complicate the interpretation of results. In addition, one also needs to consider the possibility that production of a GPCR–GFP chimera might result in altered properties of the receptor. Thus, careful experimental comparisons to untagged proteins should be made whenever possible (Kallal and Benovic, 2000) and future studies could be conducted with CHO-CRE-SPAP cells transfected with the native human DOR.

Both Gαs- and Gαq-coupled receptors signal through CREs (Durocher *et al.,* 2000), providing the opportunity to monitor the activity of a wide variety of GPCRs via SPAP assay. For the β_2-adrenoceptor (Gs-coupled), efficacious agonists appeared more potent in the cAMP assay, whereas the less efficacious agonists were more potent in the SPAP assay (Baker *et al.,* 2003b). This was attributed to GRK phosphorylation and internalization of the β_2-adrenoceptor, such that exposure to the efficacious agonists caused a desensitization of the response in the long-term downstream assay (January *et al.,* 1997, 1998; Baker *et al.,* 2003b). Changes in agonist potency between assays and between the different GPCRs are, therefore, likely to be related to different patterns of phosphorylation, internalization and desensitization that occur at different GPCRs (January *et al.,* 1997; Clark *et al.,* 1999).

Fewer reports of studies involving G*α*i compared with G*α*s are present in the literature, probably indicating the relatively fewer successes with Gi-mediated signalling assays and the SPAP system has never been tested for DOR ligands.

In the present study, adherent CHOCRE-SPAP cells, growing in DMEM/F12, supplemented with 10% FBS and 2 mM L-glutamine conferring resistance to hygromycin selection were adapted to grow in T75 flasks and then transfected with a GFP-tagged DOR plasmid when the cells were at P-12. Prior to that, a kill curve was performed on CHOCRE-SPAP cells with limiting concentrations of G418 and consequently 1 mg/ml (which falls within the recommended range), was chosen to be the minimum concentration that killed nearly all cells.

Once transfected with the CRE-SPAP and DOR/GFP constructs, the growth behaviour of the transfected cells gradually improved with time as cells at P-3 had a shorter growth lag phase, achieved higher maximum cell densities, and maintained high viability (≥95%) throughout the adaptation process, ultimately reaching the exponential growth phase (results not shown).

To detect negative regulation of adenylyl cyclase by Gi-coupled DOR receptor, the system must first be activated. To achieve this, FSK, a direct chemical activator of adenylyl cyclase, was used. Preliminary experiments were performed in 24-well plates to investigate the stimulatory effects of FSK on SPAP activation before testing the DOR agonists. Before that, the serum starvation time was optimized for our cell line; 24hrs time gave the lowest basal values (results not shown). The cells were then stimulated with a range of FSK concentrations of (0.4 µM to 30 µM) and the cells were assayed for SPAP activity. Simulations indicated that at 4 and 5 hrs

post-treatment, a large increase in cAMP-induced SPAP generation was observed, with a maximum effect at the highest dose (30 µM), as shown in Figure 2.3.

The potency for FSK may vary depending on cell and assay type (Durocher *et al.,* 2000). 1 µM FSK was selected as an optimal concentration for stimulating the cells significantly (Figure 2.4) whilst potentially allowing the detection of DOR-mediated inhibitions although supramaximal concentrations were also employed in some experiments (Figure 2.3). The $logEC_{50}$ value for FSK was calculated to be -6.6 ± 0.07.

Another experiment demonstrating the time-courses of FSK followed by different pNPP incubation times was performed. A consistent FSK stimulation was observed as a function of time and the 4hr time point was chosen for further experiments (Figure 2.5). When cells were incubated with pNPP over a long period of time, a sustained increase in SPAP response was noticed with the greatest effect seen at 2.5 hrs, which can be attributed to the ability of SPAP to increasingly catalyse the hydrolysis of pNPP to *p*-nitrophenol when provided with a longer incubation time.

Once the FSK concentration-response curve and the resultant $logEC_{50}$ were established, the next step was to introduce the non-peptide DOR agonist, SNC80 into the system, which was investigated for both acute and long-term effects. The transfected cell line was exposed to 10 µM SNC80 alone or in the presence of 10 µM FSK in 48-well plates. FSK showed a robust SPAP response consistent with the previous experiments but SNC80 had no significant effect when pre-incubated with the cells for 30 mins (Figures 2.6 and 2.7).

The same assay was repeated over a longer time course (2 and 3hrs pre-treatment with SNC80) but again with no significant effect (Figure 2.8). At this point, it was

decided to carry out dilution cloning to potentially enhance any DOR-mediated responses.

CHOCRE-SPAP-hDOR cells were isolated from mixed populations of cells and 10 clones were isolated after the cells were dilution cloned and kept under G418 and hygromycin selection, as described in section 2.6.5. The SPAP assay was carried out on all of the clones, of which only 2, G6 and F8 demonstrated any apparent inhibition of the FSK response. The two clones retained stable responses to FSK over periods up to 3 weeks that included cell-freezing and thawing cycles.

The very minor/lack of DOR agonist-mediated inhibition of the SPAP response could have been due to a number of possible reasons:

1. Insufficient total expression of DOR
2. Deficient coupling of DOR to Gi/o
3. Failure to express DORs appropriately in the cell membrane
4. Agonist bias with SNC80 having unusually low efficacy for the DORs in the particular configuration maintained in the CHO-CRE-SPAP cells.
5. Prolonged incubation time (4-6 hrs) due to the distal mechanism of the system, thus causing desensitization of the DOR.

The idea behind generating a fluorescent tagged DOR cell line was to allow visualisation of the receptor in fixed cells. Such fusion proteins are widely used to study protein trafficking (Lippincott-Schwartz and Smith 1997), and their usefulness is amply demonstrated for GPCRs (Kallal and Benovic 2000; McGrath and Daly, 2003). The G6 clone which showed some, albeit still modest, inhibition of the SPAP response displayed a reasonable expression of the GFP-tagged

DORs when viewed under the fluorescent microscope. However, there are some assumptions that need to be considered, the first being that all of the GFP remained coupled to the DORs and that no post-translational modification separating the proteins had occurred. Also, it is assumed that GFP tagging did not alter the ability of the DOR protein to adopt the appropriate conformation to interact with agonist and G protein (see above). It would be interesting to test the ability of DOR agonists to stimulate G protein coupling in a ^{35}S-GTPγS binding assay on membranes from G6 and less active DOR-CRE-SPAP clones.

Visual inspection of the GFP expression appeared to indicate that fluorescence was generated throughout the cells and not selectively at the membrane, suggesting that insertion of the DORs into the plasma membrane, thus allowing access to agonist, was insubstantial. Inflammatory conditions are known to greatly enhance membrane DOR expression and function (at least in neurons) (Cahil *et al.,* 2003) and the cytosolic DORs apparent in the G6 cells might not be able to efficiently interact with agonist.

The lack of substantial agonist effect was not specific to SNC80 as the high potency peptide agonist DADLE was similarly unable to significantly inhibit the SPAP response, except at very high concentration, however, and without investigating the effect of a DOR receptor antagonist, it cannot be assumed that either effects of SNC80 or DADLE were DOR-mediated. However, in later experiments when the usefulness of the cell line was investigated in the HitHunter™ cAMP assay (DiscoverX), a significant inhibition of the FSK-stimulated cAMP was achieved with SNC80 which was completely blocked by the selective DOR antagonist, naltrindole; clearly implying mediation via hDORs (Figure 2.12) at least in this assay format. This implies that the choice of signalling assay can

have a profound effect on the interpretation of the experimental outcomes and indicates that no single approach can be totally reliable.

An additional possible reason for the minor agonist responses could be the fact that the drugs were adsorbed onto the plastic of the multi-wells, thus reducing their effective free concentration. However, the inclusion of 0.1% BSA in drug dilutions did not enhance the responses (data not shown).

A further possibility for the, at best, minor inhibitions of cAMP-dependent SPAP responses by the DOR agonists is that the receptors in these studies also couple positively to AC. It has been demonstrated that some opioids positively regulate adenylyl cyclase activity, in chick heart cells, rat heart and DRG neurons. Some studies have found that the stimulation of adenylyl cyclase was G_s-linked (Ammer and Schulz, 1997; Shen and Crain, 1994), whereas in other studies it was found to occur in a PTX-sensitive ($G_{\alpha i}$) manner involving $\beta\gamma$ subunit stimulation of type II adenylyl cyclase (Kaneko *et al.,* 1994; Tsu *et al.,* 1993; Olianas and Onali, 1993). In SK-N-SH neuroblastoma cells, activation of the MOR stimulates the formation of cAMP in a process that is calcium/calmodulin (Ca^{2+}/CaM) dependent and is secondary to the opening of voltage-sensitive calcium channels (VOCCs). The stimulatory effect is seen only at nM concentrations of MOR agonist, whereas at higher concentrations (0.1 µM) an inhibitory effect is seen. This bimodal effect of stimulation at low concentrations of agonist and inhibition at higher doses was also noted in myenteric plexus (Wang and Gintzler, 1995). In rat olfactory bulb, MOR and DOR agonists were shown to stimulate cAMP formation in a PT_X-sensitive manner at nM and µM concentrations (Olianas and Onali, 1993).

It would be interesting to extend the present studies by investigating the effect of pertussis toxin to block out Gi-mediated responses to the DOR agonists to reveal any positive AC coupling that might counter coincident inhibitory effects.

2.8. Conclusions

The aim of the studies described in this chapter was to develop a simple and efficient assay Gi/o mediated signalling via the DOR receptor. Although the SPAP assay functioned well in that FSK produced robust increases in enzyme activity, reflecting enhanced cAMP levels, the transfected DORs, perhaps for some of the possible reasons discussed above, did not produce a signal of sufficient magnitude to enable it to be employed as a reliable whole cell assay of DOR function.

Chapter 3. Use of direct cAMP accumulation assays to study DOR-mediated effects

3.1. Introduction

Assays measuring cellular levels of cAMP are dependent on the activity of adenylyl cyclase, a key effector enzyme involved in cAMP production which is regulated by GPCRs coupled to G proteins. Both $G\alpha_s$ and $G\alpha_{i/o}$ proteins modulate the activity of adenylyl cyclase; $G\alpha_s$ positively stimulates the activity of adenylyl cyclase, resulting in increased cellular cAMP. In contrast, activation of $G\alpha_i$ leads to a negative regulation of the cyclase and a decrease in cAMP levels. Screening $G\alpha_s$-coupled responses is generally straightforward, whereas, $G\alpha_{i/o}$-coupled effects are more difficult to measure with high precision using cAMP detection methods. This difficulty arises because of the requirement to pre-stimulate adenylyl cyclase with the diterpene, forskolin, or another activator of the cyclase, prior to inhibitory agonist exposure. In addition, to counteract the natural degradation of cAMP to AMP by phosphodiesterase (PDE) enzymes, an inhibitor of PDE *e.g*. rolipram (RLP) is required in the system (Figure 3.1).

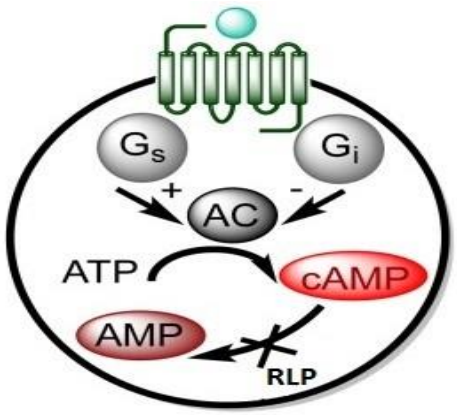


Figure 3.1 Upon ligand binding to the GPCR, activation of G_s or G_i proteins can occur. The G proteins either stimulate (G_s) or inhibit (G_i) the enzyme adenylyl cyclase (AC). AC converts ATP into cAMP. Degradation of cAMP is inhibited by rolipram (RLP) (Norskov-Lauritsen *et al*., 2014).

Chinese hamster ovary or CHO cells are an immortalised cell line developed by Theodore T. Puck in the early 1960s and since then, it has been a cell line of choice because of the cells' rapid growth in suspension culture and their ability to express high amounts of proteins. They are the most commonly used cell line for expression of recombinant protein owing to their relatively low chromosomal number (n=22), and their relative paucity of endogenously expressed proteins. Typically, the cells are cultured as an adherent monolayer in media containing the amino acid proline. CHO-K1 cells over-expressing human DORs, as used in the present study, represent a reusable resource that can be stored under cryogenic conditions for long periods of time (potentially indefinitely), retrieved, and cultured to provide a consistent and reliable level of protein expression (Durocher and Butler, 2009).

The previous chapter described relatively unsuccessful attempts to adapt an indirect reporter gene assay to monitor the effects of novel DOR agonists on inhibition of cAMP; therefore, the studies described in the present chapter were of a more direct nature.

3.1.1. Determination of cAMP accumulation by [^{3}H]-adenine pre-labelling

The conversion of adenosine triphosphate (ATP) to 3',5'-cyclic AMP (cAMP) and pyrophosphate is a central reaction in many receptor-mediated signal transduction systems and the production of cAMP can be used as a readout of receptor activation. [^{3}H]adenine can be used to pre-label pools of intracellular adenine nucleotides, including ATP ([^{3}H]ATP) which is then converted to [^{3}H]cAMP by adenylyl cyclase. In response to the activation of receptors coupled to the Gi/o class of G proteins, such as DOR, the Gi subunit inhibits adenylyl cyclase and

subsequent [^{3}H]cAMP production. Sequential Dowex/alumina column chromatography is used to recover [^{3}H]cAMP from [^{3}H]ATP and other tritium-labelled species (Huang *et al*., 1971; Donaldson *et al*., 1988b; McCrea and Hill, 1996; Baker *et al*., 2003b).

Although laborious, [^{3}H]-adenine-labelling strategies are considered to be a fairly sensitive means of cAMP determination and provide a direct read-out of [^{3}H]cAMP accumulation. It is one of the conventional techniques which can be used to generate potency (EC_{50}) values that are calculated from the agonist concentration-response relationships obtained. The method has proved to be useful in the study of the kinetics of cAMP accumulation. For example, such studies have provided important information on the duration of action of agonists at different GPCRs (e.g. salmeterol on β_1- and β_2-adrenoceptors) (McCrea and Hill, 1996). The assay, however, is cumbersome involving a complex methodology including ionising radiation and multiple wash steps with the requirement for expensive radioactive waste handling.

3.1.2. Enzyme fragmentation complementation (EFC) assay

Along with the conventional [^{3}H]cAMP accumulation assay, in the present chapter, we also report the use of a commercial high-throughput, cell-based, screen for novel DOR agonists, obtained from DiscoverX. We interrogated a small library of novel compounds with DOR agonist potential. We employed CHO-K1-hDOR cells which utilise their natural coupling status to monitor activation of Gαi-coupled receptors. Following ligand stimulation, the DORs functional status is monitored by measuring the cellular cAMP levels using a homogeneous (no wash) immunoassay based on enzyme fragment complementation (EFC) technology,

which uses two fragments of E. coli E-galactosidase (E-gal): a large protein fragment (enzyme acceptor, EA) and a small peptide fragment (enzyme donor, ED). Separately, these fragments are inactive, but in solution, they rapidly complement (recombine) to form an active *β*-gal enzyme, which can hydrolyzes substrate to produce a luminescent signal. In this assay, cAMP from cell lysates and ED-labelled cAMP (ED-cAMP) compete for antibody binding sites. Unbound ED-cAMP is free to complement EA to form active enzyme, which subsequently produces a luminescent signal. The amount of signal produced is proportional to the amount of cAMP in the cell lysate (Figure 3.2). The assay is highly sensitive and can differentiate different compound types such as full and partial agonists or inverse agonists and antagonists. As an additional advantage, it offers a large assay window and works with a low number of cells.

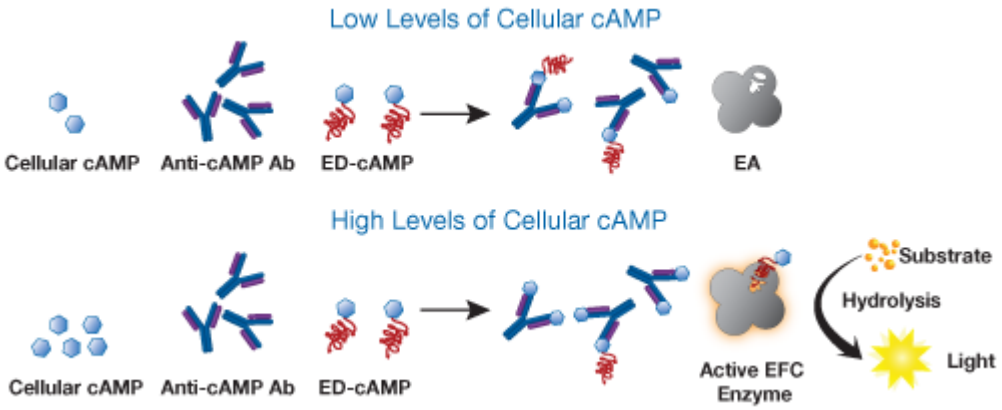


Figure 3.2 Enzyme fragmentation complementation (EFC) assay principle (DiscoverX technologies).

3.2. Aims and objectives

Having been unable to develop a practical SPAP assay for the determination of cAMP inhibition by the novel DOR agonists (Chapter 2), the purpose of this investigation was to estimate the relative potencies and agonist efficacies of several novel selective DOR ligands using more direct *in vitro* cAMP assays. For this purpose, a high expression G*α*i-CHO-K1-hDOR cell line was used to detect forskolin-mediated cAMP accumulation and its inhibition by the DOR agonists as determined in two different cAMP assay systems. The cell line provides an attractive model for investigating agonist potencies because of its relatively high receptor population which should provide clear agonist responses, thus bringing confidence to the data generated.

3.3. Materials

All cell culture plastic-ware was obtained from Corning. The over-expressing hDOR cell line (CHO-K1-hDOR) was purchased from DiscoverX. The standard DOR compounds, SNC80 and DADLE were from Tocris Bioscience and ADL5859 was purchased from Adooq Bioscience. All the novel DOR ligands were supplied by PharmNovo, Sweden. Rolipram and forskolin were purchased from Sigma-Aldrich and pertussis toxin (PTx) from Calbiochem.

ADL5859 and DADLE were stored as single-use aliquots at -20°C, diluted in aqueous stock (10mM) and SNC80 was stored in dimethylsulfoxide DMSO (10 mM stock) at room temperature. The rolipram and FSK stocks (10 mM) were made in DMSO. The novel compounds were also dissolved in DMSO as a

concentrated (10 mM) stock and stored at room temperature in the dark, as per suppliers' instructions.

The radioisotopes [^{3}H]adenine and [^{14}C]cAMP were purchased from Amersham Biosciences, UK. The scintillation fluid cocktail was from Packard Bioscience (Perkin Elmer, UK). All other reagents were obtained from Sigma-Aldrich.

3.4. Methods

3.4.1. [^{3}H]cAMP accumulation assay

CHO-K1-hDOR were grown to confluence in 24-well plates in DMEM/F12, containing 2 mM L-glutamine and 10% FBS at 37°C and 5% CO_2 in a humidified incubator. The cellular ATP pool was metabolically labelled by incubating cells for 24hrs in the presence of [^{3}H]adenine (1 µCi per well in serum-free media (SFM), i.e. 30 µL of [^{3}H]adenine added to 30 ml SFM). Following this, the cells were washed once by the addition and removal of 500 µL per well of PBS to remove most of the extracellular [^{3}H]adenine. The cells were then incubated at 37°C in serum-free media containing different concentrations of the DOR agonists and rolipram, followed by FSK, both at a final concentration of 10 µM. The reaction was terminated by the addition of 50 µL of 1 M HCl to all the wells (to prevent enzymatic activity and permeabilize the cell membrane), followed by the addition of 100 µL of [^{14}C]cAMP solution (100 µL [^{14}C]cAMP in 1 ml ddH_2O per plate) to all wells. 100 µL of [^{14}C]cAMP was also directly added to three scintillation vials representing 100 % (maximum possible) [^{14}C]cAMP detection recovery controls (which can be compared to actual detection of [^{14}C]cAMP recovered through columns). The 24-

well plate was incubated for at least 30 min at 4°C. As cAMP is stable in HCl, this incubation period can be extended if required e.g. overnight.

Dowex alumina columns were used to isolate [^{3}H]cAMP from [^{3}H]ATP. The assay plates were thawed at room temperature and the whole well contents were transferred to the corresponding Dowex /alumina columns. Elution profiles of labelled cyclic AMP together with [^{14}C]cyclic AMP tracer were determined by Dowex-alumina chromatography by the sequential application of 6 ml of 5 mM HCl and 2 ml of 0.1 M ammonium acetate. HCl will remove the majority of adenosine, adenine, xanthine and hypoxanthine contaminants from the samples. Once all HCl and ammonium acetate had run through the columns, 2 ml of 0.1 M ammonium acetate was added to each column. The negatively charged nucleotides (cAMP, AMP, ADP and ATP molecules) are adsorbed into the resin of the columns. The effluents were allowed to drain into a plastic tray and were discarded as radioactive waste. Finally, [^{3}H]cAMP was eluted with the addition of 6 ml of 0.1 M ammonium acetate, collected into scintillation counting vials, and mixed with 10 ml of scintillation fluid. The vials were capped and then counted on a liquid scintillation-counter capable of counting [^{3}H]cAMP and [14]cAMP simultaneously for 4min per vial. Accumulation of [^{3}H]cAMP was expressed as per cent conversion of the [^{3}H] adenine to [^{3}H]cAMP, following correction for the column recovery. The [^{14}C] and [^{3}H] radioisotopes decay by emitting β-particles, which transfer energy to scintillators, which in turn re-emit the absorbed energy in the form of light. These emitted photons were then counted using a scintillation counter whose programming allows calculation of counting efficiency to estimate the amount of [^{3}H]cAMP reflected by ^{3}H disintegrations per minute (dpm).

3.4.2. Enzyme fragmentation complementation (EFC) assay

Levels of cAMP accumulation were determined using the DiscoverX HitHunter® cAMP XS+ assay kit. Cells were seeded into white 96-well assay plates at 20,000 cells per well (unless otherwise stated) in the culture medium, incubated overnight at 37°C. On the day of the assay, the culture medium was aspirated and replaced with assay medium, comprising SFM containing rolipram (1 µM), and FSK (1 µM). Cells were treated in their respective wells with serial dilutions of DOR ligands for 30min, in the presence or absence of the DOR antagonist naltrindole (1 µM), in assay medium in a final assay volume of 30 µL. Each subsequent step involved incubations with the appropriate assay reagents at room temperature.

Once ligand treatment was complete, the assay was terminated by cell lysis via the addition of ED/Lysis CL working solution, followed by the addition of EA reagent. The chemiluminescent signal, corresponding to the cAMP concentration, was detected using a PerkinElmer TopCount NXT™. The data were expressed as cAMP accumulating in the presence of DOR agonist as a percentage of the FSK response (1 µM).

3.5. Data analysis

Curve-fitting and statistical analysis were performed using GraphPad Prism 7.01. Non-linear regression was used to plot and analyze concentration-response curves and to measure EC_{50} and E_{max} values. The data are expressed as percentages of the responses to 1 µM forskolin. Statistical differences between mean values were determined using unpaired, two-tailed t-tests. A p value of <0.05 was considered significant. EC_{50} is the agonist concentration which produced half-maximal

inhibitory responses and E_{max} is the maximum response produced by each agonist (maximum possible =100%, i.e. zero cAMP accumulation).

3.6. Results

3.6.1. cAMP accumulation studies

3.6.2. Radiolabelled cAMP accumulation assay

Preliminary experiments were performed using the [^{3}H]cAMP accumulation assay in order to determine the ability of selected DOR ligands (SNC80, PN6037 and OPD00003) to inhibit forskolin-stimulated cAMP accumulation in the cell line selected (Figure 3.3).

CHO-K1-hDOR cells were subcultured in 96-well plates and treated with increasing concentrations of the DOR agonists on the following day, followed by the phosphodiesterase PDE4 inhibitor rolipram (10 µM) and then challenged with FSK (10 µM). The standard agonist SNC80 and the two novel ligands (OPD00003 and PN6037) produced a concentration-dependent inhibition of forskolin-stimulated cAMP accumulation. The data indicate that all of the agonists tested, PN6037, OPD00003 and SNC80 were full agonists with logEC_{50} values of -9.3 ± 0.15, -9.1 ± 0.08 and -8.4 ± 0.10 respectively. The E_{max} values of the three agonists were calculated on the basis of inhibition of the FSK response and were found to be more than 90%.

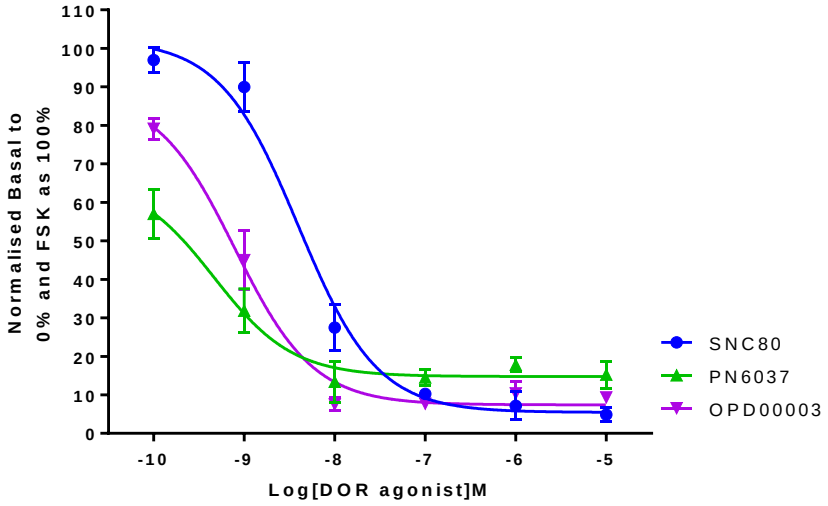


Figure 3.3 Concentration-response curves of three DOR agonists (SNC80, PN6037, and OPD00003) on FSK-stimulated cAMP content in CHO-K1-hDOR cells. cAMP accumulation was measured over 30 min in intact cells in the presence of 10 µM rolipram. The data shown represent mean ± SEM values from 3 independent experiments performed for each ligand in triplicate (n=3) and expressed as a percentage of the 10 µM forskolin response. No significant differences between the maximum values for each of the agonists were seen (One-way ANOVA).The curves were fitted using GraphPad Prism 7.01.

Thus, the [^{3}H]adenine-accumulation assay was seen to be able to measure DOR agonist inhibitions of cAMP accumulation but it was decided to test an alternative, higher throughput assay.

3.6.3. Chemiluminescent cAMP accumulation assay

Assay Optimization

Before testing the whole range of available DOR ligands, some preliminary experiments were performed in this cell-based (Gi-cAMP) assay system to determine the optimum concentration of FSK for use in subsequent experiments. This is important because, if the concentration of FSK is too low, it will not be possible to detect significant agonist activity because the cyclase will not be sufficiently activated to allow the inhibition of Gi/o. FSK concentration-response curve was constructed in the absence of DOR ligands. Cells were incubated with 1 µM rolipram and various concentrations of FSK as shown in Figure 3.4. A concentration-dependent increase in cAMP levels ($logEC_{50}$ for FSK = -6.3 ± 0.12; Figure 3.4), at a cell density of 25,000 cells per well was observed. Another experiment was performed to determine the optimal cell seeding density of CHO-K1-hDOR cells to be used in the assay to provide optimal inhibition of FSK-mediated cAMP. The CHO-K1-hDOR cells at different seeding densities (15, 20, 25,000 cells per well) were treated with the optimized concentration (1 µM) of FSK. An apparent modest effect of the cell number on the basal cAMP levels was observed; however, there was a significant rise in the cAMP levels at all cell densities. 20,000 cells per well was used as optimized cell density in further experiments, producing a consistent response in all the experiments (One-way ANOVA; $p < 0.05$) as shown in Figure 3.5.

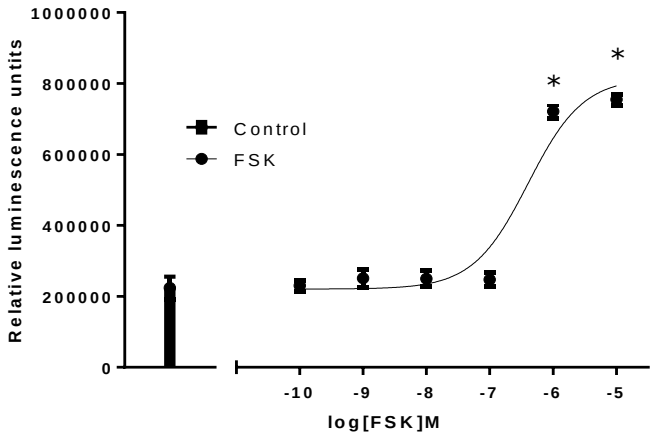


Figure 3.4 Optimization of cAMP assay in CHO-K1-hDOR cells. Cells (25,000 cells per wells) were grown in 96-well plates, pre-treated with rolipram and FSK (both 1 μM) for 5 min before incubation with increasing concentrations of forskolin (10 nM - 10 μM) for 30 min at room temperature. At the end of the incubation, the assay was terminated by the addition of lysis buffer, and the relative levels of cAMP determined using the HitHunter™ assay kit. The curves were fitted using GraphPad Prism 7.01.The data shown represent a single experiment performed in duplicate and is given as the mean ± SEM value. *$p < 0.05$ was considred significant (One-way ANOVA).

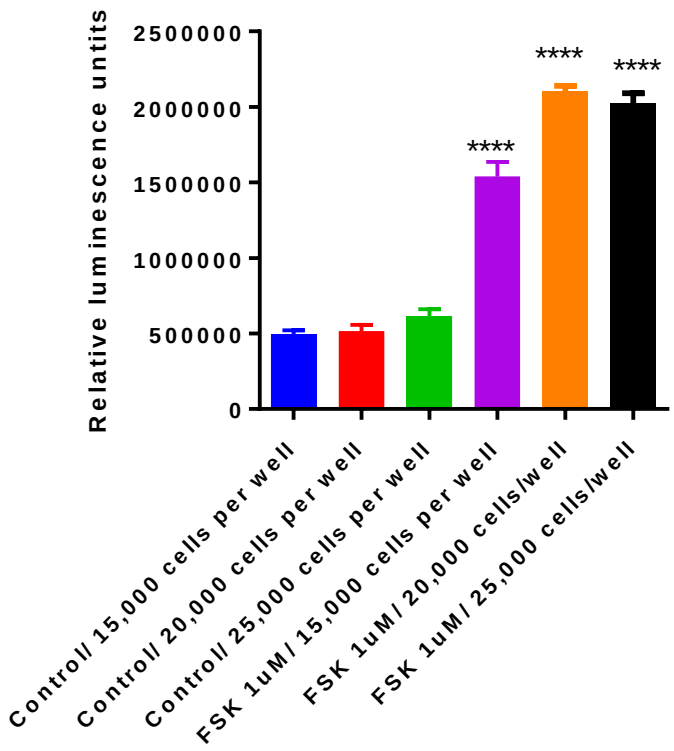


Figure 3.5 Optimization of cAMP assay in CHO-K1-hDOR cells. Cells (15, 20 and 25,000 cells per wells) were grown in 96-well plates, pre-treated with rolipram and FSK (both 1 µM) for 5 min and the remaining steps were carried out as described in the Methods section (3.4.2). Forskolin-stimulated cAMP levels were typically 3-4 fold higher than basal values at 20,000 and 25,000 cells per well seeding densities. The data were analyzed using GraphPad prism 7.01 (****$p \leq 0.0001$; One-way ANOVA).

On the basis of these results, 1 µM FSK and 20,000 cells per well were respectively chosen as optimized concentration and cell seeding number for all subsequent experiments.

3.6.4. DOR-mediated inhibition of FSK-stimulated cAMP accumulation

CHO-K1-hDOR cells treated with forskolin and the phosphodiesterase PDE4 inhibitor rolipram (both 1 µM), were stimulated with increasing concentrations of the standard DOR agonists SNC80, ADL5859 and DADLE, followed by the novel ligands to determine the ability of these compounds to inhibit forskolin-stimulated cAMP accumulation (Figure 3.6). All ligands studied showed a robust inhibitory effect on the forskolin-stimulated cAMP production The rank order of potency (logEC_{50} values) of the agonists acting on CHO-K1-hDOR cells from highest to lowest was PN6037 = PN6047 > DADLE > OPD00005 > OPD00003 > SNC80 = ADL5859 > PN6054 > PN6055. The potencies for the different ligands ranged from 0.04 -140 nM. The most potent novel ligands studied, as measured by their EC_{50} values were PN6037, PN6047, PN6053, OPD00003 and OPD00005 with EC_{50s} of less than 10 nM (Table 3.1).

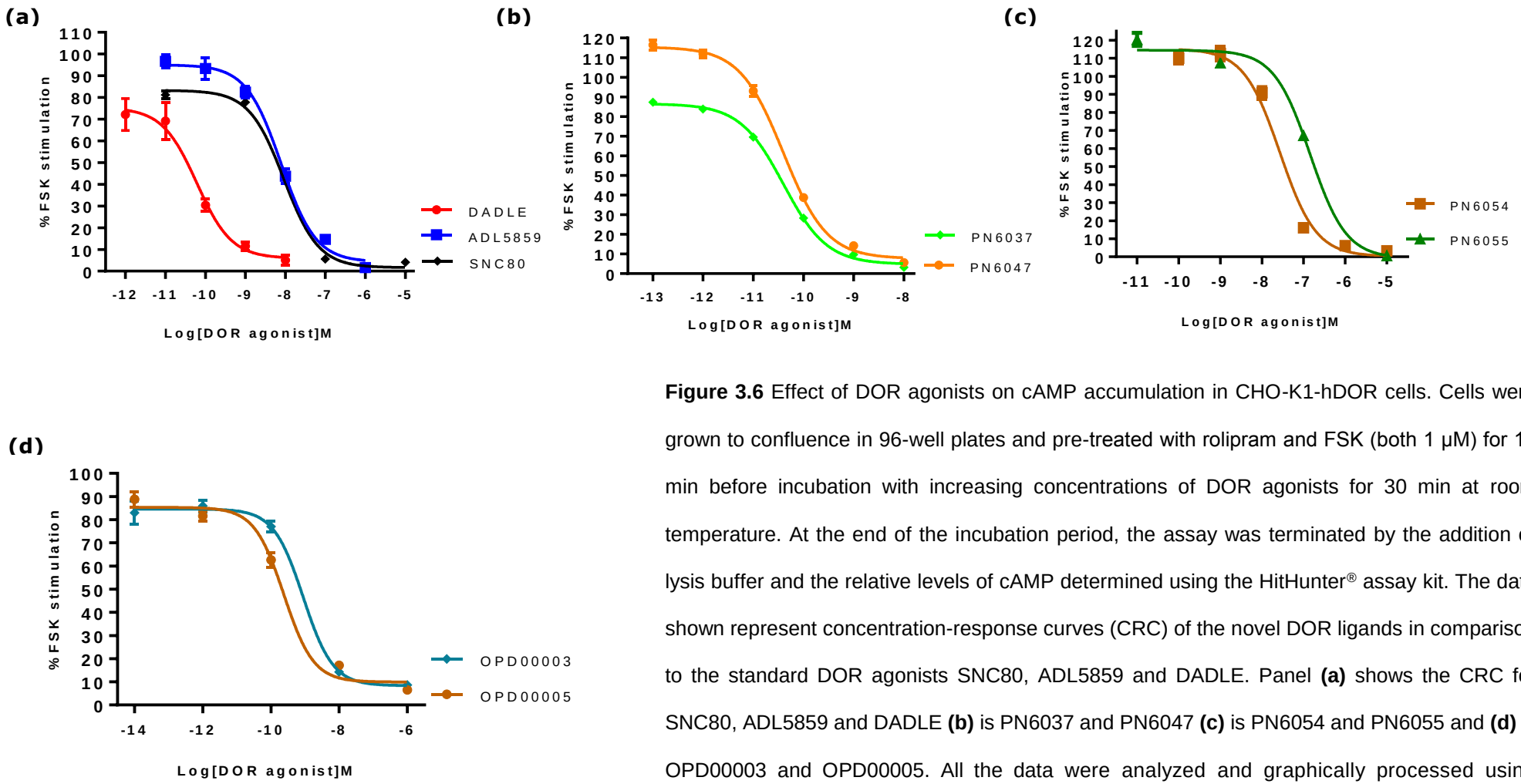


Figure 3.6 Effect of DOR agonists on cAMP accumulation in CHO-K1-hDOR cells. Cells were grown to confluence in 96-well plates and pre-treated with rolipram and FSK (both 1 μM) for 10 min before incubation with increasing concentrations of DOR agonists for 30 min at room temperature. At the end of the incubation period, the assay was terminated by the addition of lysis buffer and the relative levels of cAMP determined using the HitHunter® assay kit. The data shown represent concentration-response curves (CRC) of the novel DOR ligands in comparison to the standard DOR agonists SNC80, ADL5859 and DADLE. Panel **(a)** shows the CRC for SNC80, ADL5859 and DADLE **(b)** is PN6037 and PN6047 **(c)** is PN6054 and PN6055 and **(d)** is OPD00003 and OPD00005. All the data were analyzed and graphically processed using GraphPad Prism 7.01. All values are means ± SEM (n = 6-8) expressed as a percentage of the 1 μM forskolin response. Non-visible SEMs are within the symbols.

All the ligands tested were close to being full agonists. Some of the novel DOR agonists were found to have higher potencies (EC_{50}) values than the three standard DOR agonists when tested for the ability to inhibit FSK-stimulated cAMP accumulation (Table 1.3). PN6037 and PN6047 and both the OPD compounds were found to be significantly more potent than SNC80 in inhibiting forskolin-stimulated cAMP accumulation (One-way ANOVA, $p < 0.05$, $n = 6\text{-}8$), whereas PN6054 (27 nM) and PN6055 (147 nM) were the least potent.

Comparisons were also made for the E_{max} values of the novel agonists compared to the standard SNC80 (One-way ANOVA) (i.e. efficacy= % of the FSK remaining). All the novel DOR agonists except OPD00005 (88%) produced more than 90% inhibition of the FSK response (Table 3.1).

DOR Agonist	EC_{50} (nM)	$LogEC_{50}$ ± SEM	E_{max} ± SEM *(% Inhibition of FSK response)*
SNC80	***9.0***	***-8.1 ± 0.18***	***99 ± 3***
ADL5859	8.0	-8.1 ± 0.06	92 ± 4
DADLE	0.06*	-10.2 ± 0.17	96 ± 2
OPD00003	0.9*	-9.0 ± 0.18	90 ± *2*
OPD00005	0.2*	-9.6 ± 0.09	88 ± *2*
PN6037	0.04*	-10.4 ± 0.04	94 ± *1*
PN6047	0.04*	-10.4 ± 0.04	94 ± *1*
PN6054	27	-7.6 ± 0.05	100 ± *2*
PN6055	147	-6.8 ± 0.05	100 ± *2*

Table 3.1 Determination of potency (EC_{50} and $logEC_{50}$), calculated as concentrations of agonist inhibiting 50% of FSK-stimulated cAMP accumulation in CHO-K1-hDOR cells. Efficacy (E_{max}) of the agonists are expressed as percentage inhibition of the FSK response. The values are the results of the pooled normalized data from 6-8 experiments carried out in triplicate ($p < 0.05$ shows significantly higher potency than SNC80; One-way ANOVA, followed by Dunnett's multiple comparisons test).

3.6.5. Characterization of DOR coupling to G protein in the CHO cell line

The experiments were then repeated in the presence or absence of pertussis toxin (PTx) (100 ng/ml) which ADP-ribosylates and inhibits $G_{i/o}$ proteins (Mangmool and Kurose, 2011). The cells were pre-treated with PTx to determine the role of $G_{i/o}$ protein in the DOR-mediated cAMP response and whether any alternative $G_{i/o}$ protein-independent signalling could be identified. Cells were treated with 1 µM rolipram and FSK and then challenged with different concentrations of SNC80. The

SNC80-mediated inhibition of forskolin-stimulated cAMP was abolished by pertussis toxin pre-treatment, suggesting that the agonist responses were due to coupling of the DOR to either the G_i or G_o class of G proteins (Figure 3.7).

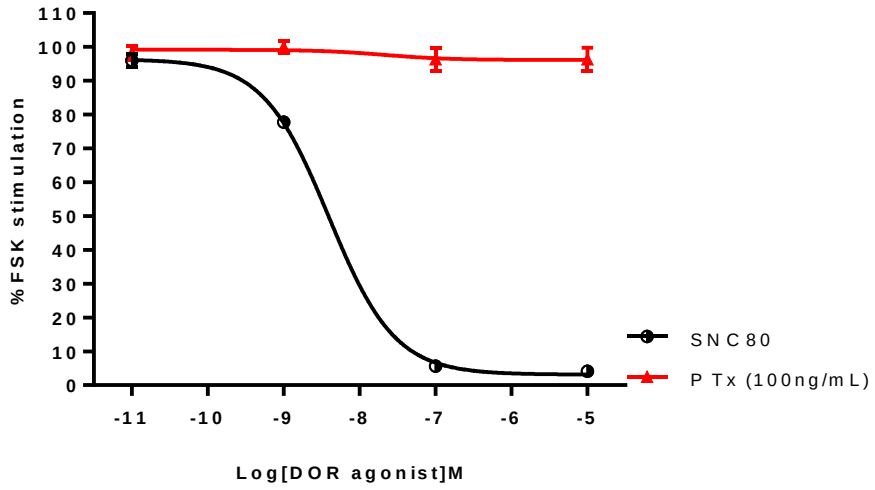


Figure 3.7 Effect of SNC80 on cAMP accumulation in CHO-K1-hDOR cells in the presence or absence of PTx. Cells were pre-treated with PTx (100 ng/ml) for 24 hrs and then treated with rolipram and FSK (both 1μM) for 5 min before incubation with the increasing concentrations of SNC80 for 30 min at room temperature. An SNC80 concentration-response curve was also generated in the absence of PTx. At the end of the incubation period, the assay was terminated by the addition of lysis buffer and the relative levels of cAMP determined using the HitHunter assay kit. The data shown represents mean ± SEM values from 3 individual experiments performed in triplicate. The curves were fitted using GraphPad Prism 7.01

To confirm the role of the DOR in the agonist responses, a 1 μM concentration of each agonist was challenged with the DOR-selective competitive antagonist,

naltrindole (NAL) (1 µM). NAL completely abolished the inhibitory effects of the DOR agonists on cAMP accumulation (Figure 3.8).

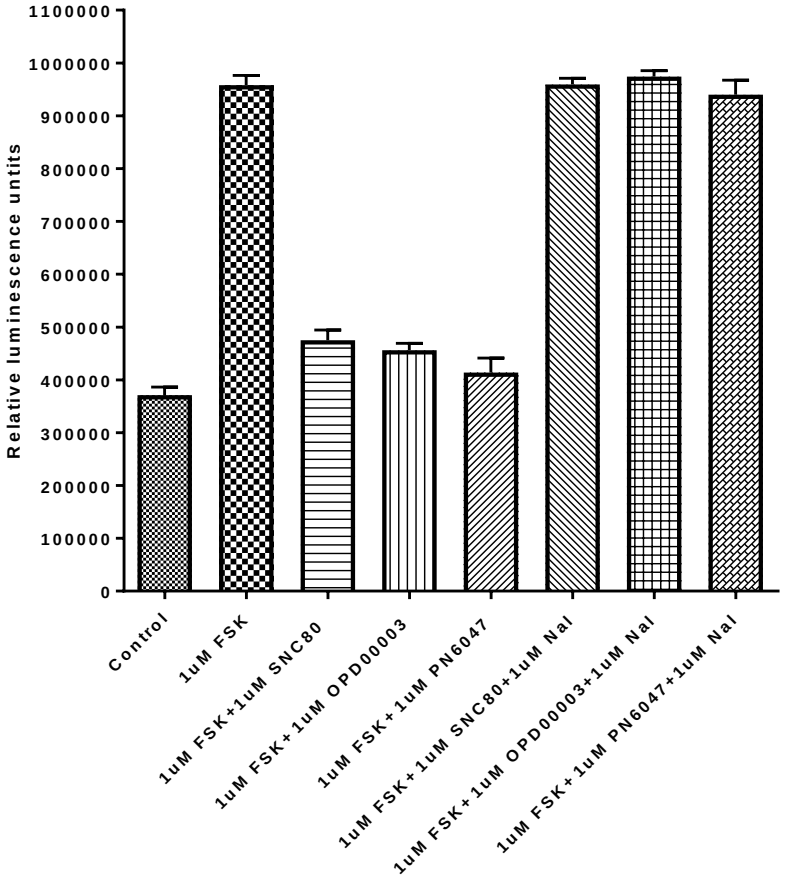


Figure 3.8 Effect of the selective DOR antagonist naltrindole (NAL) on DOR-mediated inhibition of FSK-stimulated cAMP accumulation in CHO-K1-hDOR. 1 µM concentration of rolipram, FSK, DOR agonists and the NAL were used in these experiments. Data represent the mean ± SEM of three independent experiments performed in triplicate. The data shows that NAL completely blocked the agonist response and there were no significant differences between FSK mediated cAMP accumulation and the NAL mediated blockade of the DOR (unpaired two-tailed t-test). The data were analyzed using GraphPad prism 7.01.

3.7. Discussion

The overall aim of the thesis was to look for signalling bias amongst a range of DOR agonists and the work in this chapter tested the ability of the agonists to couple to a key signalling system, i.e. Gi mediated inhibition of cAMP accumulation, in this case in the over-expressing CHO-K1-hDOR cell line. The data presented in this chapter agree with available published data on SNC80, showing it to be an effective agonist in relation to cAMP inhibition, thus establishing that the methods used were valid and could be successfully used to provide useful data for previously uncharacterized non-peptide selective DOR ligands. A well-established method for assessing G-protein activation by opioid receptors is measuring the extent of inhibition of forskolin-stimulated adenylyl cyclase (AC) activity by the opioid ligands (Arden *et al*., 1995; Keith *et al*., 1996). Thus, characterization of our DOR ligands to inhibit cAMP production, described in this chapter, could eventually be used as an index for assessing their activity as a correlate of their analgesic effects.

[^{3}H]-adenine pre-labelling cAMP assays have been widely used for the determination of GPCR ligand efficacy and potency. In the present study, we used a radiolabelled cAMP assay as an initial method to determine the efficacy of our novel DOR ligands. Once confirmed, we then switched over to a higher throughput luminescence-based assay to avoid the use of radioactivity. The non-radioactive approach for cAMP detection selected is based on Enzyme Fragment Complementation (HitHunter™) technology introduced by DiscoverX. In this assay, cellular cAMP competes with cAMP labelled with a small peptide fragment of *β*-galactosidase for binding to an anti-cAMP antibody. The resulting free labelled-cAMP complements with the enzyme fragment, producing active *β*-galactosidase,

which is detected with luminescent substrates (Eglen and Singh, 2003). Although extremely sensitive for the detection of Gα_i-coupled receptor activation, the system requires prior stimulation by compounds such as forskolin.

The set of novel DOR ligands exhibited a range of potencies but very similar efficacies. Comparing potencies of the novel agonists to those of the standard agonists, SNC80 and ADL5859 showed that, PN6037 and PN6047 had a significantly higher potency than the two standard compounds. Comparing the potencies of the novel ligands to the three standard DOR compounds indicate that all of them except perhaps for PN6054 and PN6055 were full agonists, with EC_{50} values of less than 1 nM, and thus they might be considered as useful candidates for further investigation. The EC_{50} value for SNC80 (9.0 nM) in the present investigation was not very different than those reported in the previous studies (15 and 6.3 nM) (Malatynska *et al*., 1996; Clark *et al*., 1997). Some ligands that exhibited lower potencies in this study, such as PN6054 and PN6055, were, however, capable of maximally activating DOR at the higher concentrations (1 and 10 µM). The maximum inhibitory effect of SNC80 in transfected DOR has been reported as 71 ± 5% (Clark *et al*., 1997) and 94 ± 2% (Malatynska *et al*., 1996). Different efficacies and potencies of SNC80 reported in the literature indicate that different E_{max} values of the agonist could be obtained depending upon the signalling systems examined, the assay conditions or the cell types employed. This has an extremely important effect on the drug discovery process for new agonists since it cannot be assumed that a given screening or lead optimization assay would be able to correctly predict the therapeutic behaviour of the tested ligands. Furthermore, it is possible that apparent partial agonists in an over-expressing system might be totally ineffective in a naturally expressing cell line. This amplifies the need to look for methods sensitive enough to detect drug effects

on signalling in “real” cells. Ligand bias must be expressed relative to a reference agonist in any given system. It could be argued that the natural agonist would be a good choice, since differences seen for a synthetic agonist would then predict possible differences from endogenous signalling (Kenakin, 2013).

Biased ligands have the potential to be better therapeutic entities than unbiased ligands (Maudsley *et al*., 2005; DeWire and Violin, 2011). For example, two major GPCR pathways are G-protein activation (e.g. cAMP inhibition/ MAP kinase activation) and *β*-arrestin recruitment. *β*-arrestin 2 recruitment is known to be a switch to turn off G-protein signalling; thus ligands biased against *β*-arrestin *2* recruitment might be expected to yield more persistent G-protein-based signalling and less desensitization (Varga *et al*., 2004; Raehal *et al*., 2005; Groer *et al*., 2007).

The DOR is well known to couple to Gi/o proteins in transfected CHO-K1 cells which, among other functions, inhibits the activity of adenylyl cyclase (Felder *et al.*, 1995; Bonhaus *et al.*, 1998). Stimulation of cells with FSK, a receptor-independent activator of AC, produced a concentration-dependent increase in cAMP levels. All agonists tested produced clear concentration-dependent inhibitions of FSK-stimulated cAMP accumulation. The agonist EC_{50} values generated from the radiolabelled and chemiluminescent assay were not statistically different giving some confidence in the results generated.

The effect of pertussis toxin on DOR modulation of cAMP accumulation was investigated (Figure 3.7). Previous reports have demonstrated that inhibition of $G_{i/o}$-mediated signalling can facilitate receptor coupling to other signalling

mediators, such as Gs proteins (Glass and Felder, 1997; Maneuf and Brotchie, 1997; Bonhaus *et al.*, 1998). On the other hand, application of PTx to the cells exerted a loss of inhibitory effect of SNC80, indicating that the coupling of transfected DOR to endogenous Gs proteins is weak and/or negligible. Finally, the DOR-mediated responses were confirmed by using the selective DOR agonist to block the receptor which successfully abolished the FSK-mediated cAMP inhibition in our cell line (Figure 3.8).

3.8. Conclusions

In the present study, all of the agonists examined were capable of fully inhibiting cAMP signalling, at least when activated directly by FSK, albeit with a range of potencies. In the next chapter, the effects of the same agonists on a different signalling system (MAP kinase activation) coupled to DORs will be examined.

Chapter 4. Investigations of DOR-mediated MAP kinase ($ERK_{1/2}$) signalling

4.1. Introduction

Opioid receptor agonists can be distinguished by their ability to modulate an array of different post-receptor signalling pathways, which is referred to as functional selectivity or ligand-selective signalling (Clarke, 2005; Urban *et al*., 2007). In this chapter, we focused on DOR agonist interactions with extracellular signal-regulated kinase 1 and 2 ($ERK_{1/2}$), members of the mitogen-activated protein kinase (MAPK) family.

ERKs have been shown to play an important role in neuronal plasticity (Grewal *et al*., 1999; Mazzucchelli and Brambilla, 2000; Ji and Woolf, 2001), and could have a role in the development of tolerance and dependence to opioids. In a mu-opioid receptor (MOR)-transfected CHO cell model, ERK activation has indeed been implicated in receptor desensitization by phosphorylation (Polakiewicz *et al*., 1998), a phenomenon that may be related in part to the development of opioid tolerance (Taylor and Fleming, 2001). Moreover, in human embryonic kidney (HEK)-293 cells transfected with the MOR, ERK1 and ERK2 have been shown to play roles in chronic opioid-induced adenylyl cyclase superactivation, a phenomenon potentially underlying symptoms of morphine withdrawal (Tso and Wong, 2001).

The kinetics of $ERK_{1/2}$ phosphorylation by MOR and KOR systems vary, yet both receptors can activate the kinase within 5-10 minutes. MOR-mediated $ERK_{1/2}$ phosphorylation requires phosphokinase C (PKC) activity and MOR-dependent $ERK_{1/2}$ signalling requires G protein receptor kinase 3 (GRK3) and arrestin activation in primary neurons, glial cells and heterologous expression

systems. DORs have also been shown to activate ERK1/2 through G*βγ* and Ras signalling cascades and do not necessarily require receptor internalization or receptor phosphorylation for signalling (Belcheva *et al.,* 1998; Macey *et al*., 2006; Groer *et al*., 2007; Miyatake *et al*., 2009; Kramer and Simon, 2000). Proteomic studies have shown that *β*-arrestins interact with over a hundred binding partners (Xiao *et al.,* 2007). *β*-arrestin dependent MAP kinase activation has been studied extensively as a model for *β*-arrestin dependent signalling. For example, *β*-arrestin dependent ERK activation for the angiotensin 1A receptor is fundamentally distinct from G protein mediated ERK activation, with different kinetics, spatial distribution, and function (DeWire *et al.,* 2008). DOR-dependent ERK1/2 signalling requires further investigation because, coupled with DOR's critical role in the chronic pain and mood regulation, ERK signalling through DOR may reveal a novel mechanism for DOR regulation of neural activity.

The In-Cell Western™ (I-CW) technique employed in this chapter uses quantitative immunocytochemistry and it can be performed in 96- or 384-well microplate formats. The possibility to perform assays in more complex microenvironments such as when screening large compound libraries is an advantage compared with traditional techniques. I-CW combines the specificity of Western blotting with the reproducibility and throughput of immunoassays. Assay steps such as lysate preparation, gel loading, electrophoresis, and membrane transfer are eliminated and I-CW is, therefore, considered ideal for screening cell treatments or drug candidates for effects on target proteins. In I-CW, cells are cultured in microplates and treated with the drugs under investigation. The treated cells are then fixed and permeabilized and stained with primary antibodies (1 or 2 protein targets per well) followed by staining with infrared dye (IRDye®) secondary antibody conjugates. The microplates are imaged and fluorescent signals are quantified from cell

populations in each well; thus relative protein levels can be quantified and the data normalized to correct for well-to-well variation. The technique allows the use of more than one coloured secondary antibody and, thus, it is possible to probe the system simultaneously for two different proteins; in the present case total and phosphorylated ERKs. The total fluorescent signal from a well of a 96-well plate is determined and normalized to a loading control (IRDye® secondary antibodies). Also, this type of readout lends itself to automation and high-throughput analyzes; thus, many quantitative protein analysis assays can be performed at the same time. Western blot analyzes of the phosphorylation of signalling molecules are laborious and difficult to quantify, requiring subjective judgments about which part of the bands to scan and what to designate as a background signal. These considerations predispose the data to variations that can only be overcome by numerous repetitions. Plate-based I-CW assays with a machine-read signal reduce variability and allow simultaneous analysis of a large number of samples.

4.2. Aims and objectives

Having observed that all of the DOR agonists tested were apparently full agonists in relation to inhibitory coupling to adenylyl cyclase signalling (Chapter 3), the aim of this chapter was to determine the ability of the same agonists to couple to MAP kinases. The main objective was to measure the effect of the agonists on $ERK_{1/2}$ phosphorylation using I-CW assays. The $ERK_{1/2}$ activation was investigated in the same cell line (CHO-K1-hDOR), used in cAMP assays which makes the data from the two assays more comparable.

4.3. Materials

All cell culture reagents were purchased from Sigma Chemical (Poole, Dorset, UK), while FBS was obtained from PAA Laboratories (Teddington, Middlesex, UK). Two primary antibodies phospho-p44/42 MAPK (Thr202/Tyr204) (mouse) # 9106; and total p44/42 MAPK (rabbit) # 9102; were supplied by Cell Signalling Technology (New England Biolabs, UK). Secondary antibodies: Odyssey secondary; IRDye®700 Goat anti-Mouse and IRDye®800 Goat anti-Rabbit were both supplied by DAKO, UK.

4.4. Methods

4.4.1. In-Cell Western assay

Levels of MAP kinase phosphorylation were determined using the In-Cell Western™ assay. Cells were seeded into 96-well clear bottom plates, at seeding density of 25,000 cells per well (unless otherwise stated) in culture medium for 24-36 hrs at 37°C in humidified 5% CO_2/95% air atmosphere. The following day, the culture medium was replaced with SFM and the cells re-incubated for 24 hrs at 37°C. The following day, serial dilution of DOR ligands was performed in SFM and added to respective wells to a final volume of 200 µl per well. Assay plates were incubated for the stated period at 37°C during experimentation. After incubation, the assay medium was rapidly aspirated and the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature (100 µl per well). Each reagent was aspirated before the addition of the next reagent. Cells were then permeabilized with 0.1% (v/v) TritonTM X-100 in PBS for 20 min at

room temperature (100µl per well). Non-specific antibody binding sites were blocked with 5% (w/v) milk powder in PBS for 20 min at room temperature with gentle rocking.

The two primary antibodies for anti-total and anti-phosphorylated ERK1/2 (see section 4.3), were diluted together in blocking buffer (1:500) to form primary antibody solution. Cells were incubated with primary antibody solution (100 µl per well) overnight at 4°C with gentle rocking. The next day, the primary antibody solution was aspirated and the cells were washed three times with PBS for 5 min at room temperature with gentle rocking. The appropriate LI-COR IRDye® secondary antibodies were diluted (1:500) in blocking buffer, added to the cells (100 µl per well), and incubated in darkness for 1 hr at 37°C with gentle shaking. For Phospho ERK1/2 secondary antibody Goat anti-mouse IRDye®800 (green) was used and with total ERK1/2 Goat anti-rabbit IRDye® 700 (red) which allowed simultaneous detection of both proteins.

The secondary antibody solution was aspirated and the cells washed three times with wash buffer as before. The LI-COR® Odyssey glass scanner surface was wiped with Millipore-Q water and the plate was scanned, using both 700 nm and 800 nm channels with a resolution of 169 µm, medium quality, and intensity of 3.0 and focus offset of 4 mm. The data were analyzed by LI-COR Odyssey® application software (Image Studio™).

4.5. Data analysis

Blank wells consisting of cells and secondary antibody alone (without primary antibody solution) were used in this assay so as to eliminate any background, resulting from auto-fluorescence and/or any fluorescence from non-specific binding of the secondary antibody. Therefore, the responses generated from blank wells were subtracted from the secondary antibody responses to give a measure of any non-specific binding of the secondary antibody.

The $ERK_{1/2}$ signal was normalized against the total $ERK_{1/2}$ using Microsoft Excel and the signal expressed as a normalized ratio (700/800 nm) of phosphorylated to total $ERK_{1/2}$. Curve-fitting (nonlinear regression) and statistical analysis were performed using GraphPad Prism 7.01. EC_{50} is the molar concentration of agonist required to generate a response 50% of E_{max}, where, E_{max} is the maximum response produced by each agonist relative to the maximum response to the designated standard agonist SNC80 (= 100%).

4.6. Results

4.6.1. Determination of ERK phosphorylation

4.6.1.1. Assay Optimization

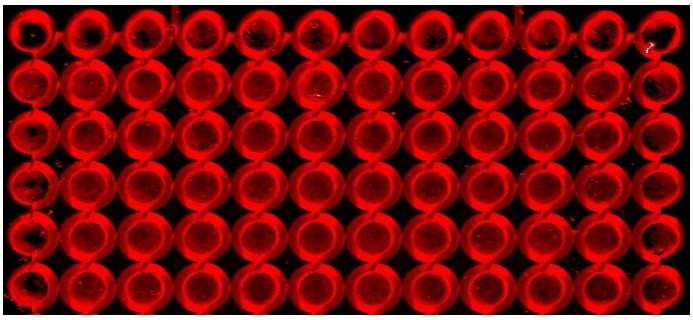
To validate the I-CW technique for testing the DOR ligands used in this study, the assay was optimized to achieve a sizable and reproducible $ERK_{1/2}$ response in

order to illustrate the importance of this technique as a HTS tool for analyzing compound libraries.

4.6.1.1.1. Reduction of edge effect

Preliminary I-CW experiments clearly demonstrated an uneven distribution pattern of cells in the perimeter wells of the 96-well microplates when scanned. It is known that differential evaporation rates and thermal changes in the plate lead to edge effects, thus causing heterogeneity or variability in results. Upon microscopic inspection, cells in the outer wells of the plate appeared clumped towards the outer edges of the wells (Figure 4.1a). Pre-incubation of the plates at room temperature (RT) for 30 minutes immediately after seeding and prior to moving plates to the incubator markedly decreased the visible edge effect (Figure 4.2b). This method was reported by Tanner (2000) to control edge effects on microplates. A nearly similar effect was achieved when blank DMEM media was added to the peripheral wells helping to control the rate of evaporation from the vulnerable wells (not shown). Another method to reduce the edge effect was tested by using a specialized ‘edge 96-well plate’ (from Thermo Scientific Nunc), with a surrounding moat which, when filled with media, acts as an evaporation buffer during longer incubations. This modification gave very similar results (data not shown) to those of the 30-minute pre-incubation at room temperature approach. Since 30 minutes of pre-incubation at RT in our experiments provided a fairly uniform distribution of the cells across the peripheral wells in our cell line, all the experiments in this study were performed using this method.

(a)



(b)

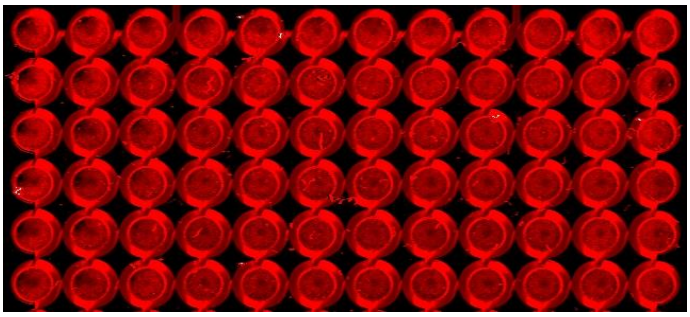


Figure 4.1 Pre-incubation at room temperature for 30min decreases the edge-effect. The cells were assayed as described in Methods, section 4.4.1, using SNC80 (1 µM). Images of 96-well plates seeded with 25,000 cells per well were placed either (**a**) immediately into 37°C incubator, or (**b**) pre-incubated for 30 min at room temperature (22±1°C), prior to incubation at 37°C. Images show cells cultured for 24 hours, fixed, permeabilized, blocked and labelled with primary (ERK1/2) and secondary (IRDye®) antibodies. The images were scanned using LI-COR Odyssey® system (Image Studio™).

4.6.1.1.2. Cell density quantification

To further determine whether cell density affects ERK phosphorylation of CHO-K1-hDOR cells, cell seeding densities were optimized to evaluate assay robustness and performance, The cells were plated at different densities (20, 25 and 30,000 cells per well) into 96-well microtiter plates for 24 hours. Cells were serum-starved for another 24 hours and then stimulated with varying concentrations of SNC80,

which produced an increase in ERK phosphorylation at all cell seeding densities tested (data not shown) with no significant differences between conditions. The wells were not fully confluent when seeded with 20,000 cells per well. The cells produced a much cleaner image, as they were uniformly distributed across all the wells when plated at 25,000 cells per well (plates not shown). The basal levels of ERK phosphorylation for 25,000 cells per well were markedly lower than for the other seeding densities (data not shown). This cell number was chosen for further experimentation.

4.6.1.1.3. Extracellular ATP as a contributor to increased basal ERK signalling

To see whether basal phosphorylation levels could be reduced by apyrase or if it might contribute to an increase in the size of the agonist signal, experiments were performed in the presence or absence of apyrase. Based upon previous studies in other cell lines, it has been suggested that the $ERK_{1/2}$ basal levels could be significantly reduced by the inclusion of apyrase (an ATPase), suggesting a possible involvement of extracellular nucleotide signalling i.e. endogenous ATP release from the cells (Burnstock *et al*., 2010). Therefore, the cells were pre-incubated with apyrase (2 units per ml) for 1 hour at 37°C*,* and assayed as described in Methods section 4.4.1*)*. Apyrase, however, did not significantly reduce the basal levels of ERK phosphorylation, implying that basal phosphorylation levels were not regulated by ambient concentrations of extracellular ATP in this cell line (data not shown). Similarly, apyrase did not have a significant effect on SNC80-stimulated ERK phosphorylation (data not shown).

4.6.1.1.4. Differential effects of DOR agonists in the presence or absence of serum

To determine whether the presence of serum alters the effects of DOR agonists on MAPK activation, we compared the activation of ERK following overnight incubation in serum-replete (10% FBS) versus serum-depleted (no FBS) (Figure 4.4) conditions. There were no significant differences in either basal or SNC80-stimulated ERK phosphorylation between serum-containing and serum-depleted conditions in CHO-K1-hDOR cells (One-way ANOVA).

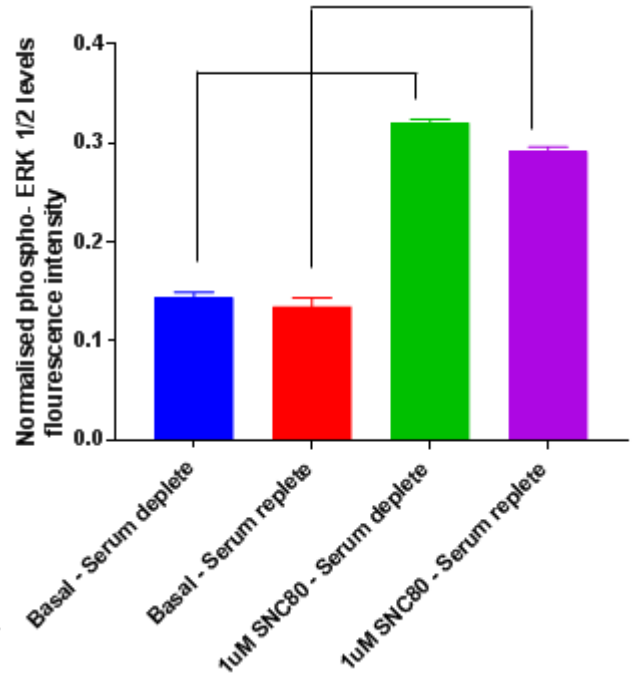


Figure 4.4 Differential effects of SNC80 (1µM) on ERK activation in serum-replete (10% FBS) versus serum-depleted (no FBS) conditions in CHO-K1-hDOR cells. The signal is expressed as a normalized ratio (700/800 nm) of phosphorylated to total ERK1/2. Data shown are mean ± SEM values from three independent experiments performed in triplicate (One-way ANOVA).

4.6.2. Effect of DOR agonists on ERK activation- time course

CHO-K1-hDOR cells were treated with 1 μM concentrations of SNC80 and OPD00003 at varying time points (1-35 min), and the phospho-ERK responses measured. This temporal characterisation was important as subsequent experiments were carried out on the basis of these results. Optimal agonist stimulation time points also provided a means to identify any multiphasic responses. ERK phosphorylation was detectable as early as 1 min after agonist addition with a distinct peak at 3-5 min (Figure 4.5). SNC80 and OPD00003 responses declined over the next 10 mins but were still significantly elevated compared with basal for up to 35 mins (One-way ANOVA).

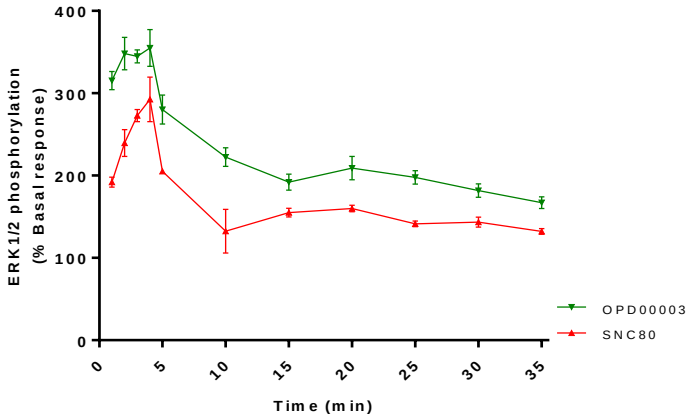


Figure 4.5 Time-course of the effect of DOR agonists (SNC80 and OPD00003) on ERK phosphorylation in CHO-K1-hDOR. Cells were cultured in DMEM-F12 medium for 24 hr, followed by serum-starvation for 24 hr. Cells were stimulated with DOR agonists (1 µM) for the indicated times, followed by rapid fixation with PFA (4% w/v) solution. Detection of total and phospho-ERK levels was as stated in Methods (see 4.4.1). The data were expressed as a percentage of the basal response. The data shown are mean ± SEM values from 3 independent experiments performed in triplicate.

4.6.3. ERK activation, concentration- response to DOR agonists

A stimulation period of 5 min was chosen for DOR concentration-response experiments, representing the peak of the first phase response. All agonists produced a concentration-dependent increase in phospho-ERK levels (Figures 4.6). The rank order of potency (logEC_{50} values) of the agonists acting on CHO-K1-hDOR cells from highest to lowest was SNC80 > PN6047 > OPD00003 > OPD00005 > PN6037 > ADL5859 > PN6054 > DADLE > PN6055. The most potent novel ligands studied, as measured by their EC_{50} values were PN6037, PN6047,

OPD00003 and OPD00005 with EC_{50s} of less than 10 nM (Table 4.1). In comparison with the effects of the agonists on cAMP inhibition (Chapter 3) which reflected nearly full efficacy for all agents, there were clear differences in the maximal effects of the DOR agonists on ERK phosphorylation. With regard to the three standard agonists, SNC80, ADL5859 and DADLE, the latter (a peptide) was clearly much less effective than the non-peptides with an E_{max} less than 40% that of the other two. With the novel agonists, there was a spectrum of efficacies ranging from PN6054 with an efficacy close to that of DADLE to PN6047 with an E_{max} not significantly different from that of SNC80 (One-way ANOVA) (Table 4.1).

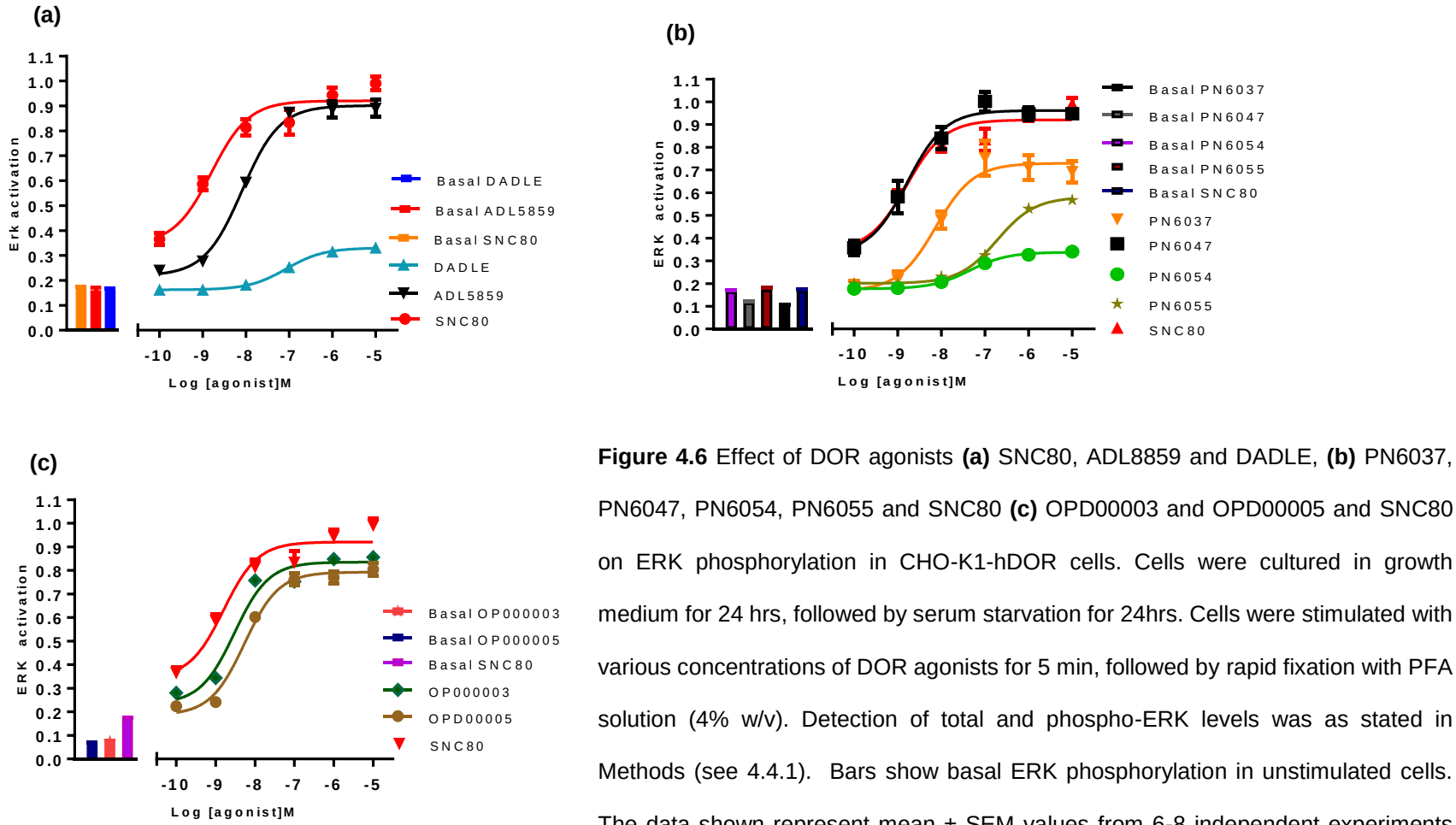


Figure 4.6 Effect of DOR agonists **(a)** SNC80, ADL8859 and DADLE, **(b)** PN6037, PN6047, PN6054, PN6055 and SNC80 **(c)** OPD00003 and OPD00005 and SNC80 on ERK phosphorylation in CHO-K1-hDOR cells. Cells were cultured in growth medium for 24 hrs, followed by serum starvation for 24hrs. Cells were stimulated with various concentrations of DOR agonists for 5 min, followed by rapid fixation with PFA solution (4% w/v). Detection of total and phospho-ERK levels was as stated in Methods (see 4.4.1). Bars show basal ERK phosphorylation in unstimulated cells. The data shown represent mean ± SEM values from 6-8 independent experiments performed in triplicate. The curves were fitted using GraphPad Prism 7.01.

DOR Agonist	EC_{50} (nM)	$LogEC_{50}$ ± SEM	E_{max} ± SEM (% *SNC80*)
SNC80	***1.6***	***-9.0 ± 0.17***	***100 ± 3***
ADL5859	8.0*	-8.0 ± 0.01	100 ± 1
DADLE	86*	-7.0 ± 0.01	36 ± 1*
OPD00003	3.0*	-8.5 ± 0.09	91 ± 3
OPD00005	5.0*	-8.2 ± 0.08	86 ± 3*
PN6037	7.0*	-8.1 ± 0.08	78 ± 3*
PN6047	1.6*	-8.7 ± 0.02	104 ± 1
PN6054	45*	-7.3 ± 0.14	37 ± 4*
PN6055	162*	-6.7 ± 0.06	63 ± 1*

Table 4.1 Potency (EC_{50}, $logEC_{50}$) and efficacy (E_{max}) values for DOR agonist activation of $ERK_{1/2}$ in CHO-K1-hDOR cells. Shown are the mean values ± SEM from 6-8 separate experiments. Experiments were performed as detailed under methods (see 4.4.1), with the maximum response induced by SNC80 defined as 100% ($p < 0.05$ shows significantly less potency/ or efficacy than SNC80; One-way ANOVA, followed by Dunnett's multiple comparisons test).

4.6.4. Mechanism of hDOR- induced ERK activation

To investigate the role of Gi/o proteins in the DOR-mediated phospho-ERK responses, and whether any alternative Gi/o protein-independent signalling could be identified, the experiments were repeated in CHO-K1-hDOR cells with overnight incubation with 100 ng/ml pertussis toxin (PTx) followed by exposure to three different agonists. PTx was able to abolish all DOR-mediated ERK phosphorylation

responses at 5 min (Figure 4.7). The loss of ERK activation suggested that the enhanced MAPK activation was mediated by a PTx-sensitive Gi/o protein.

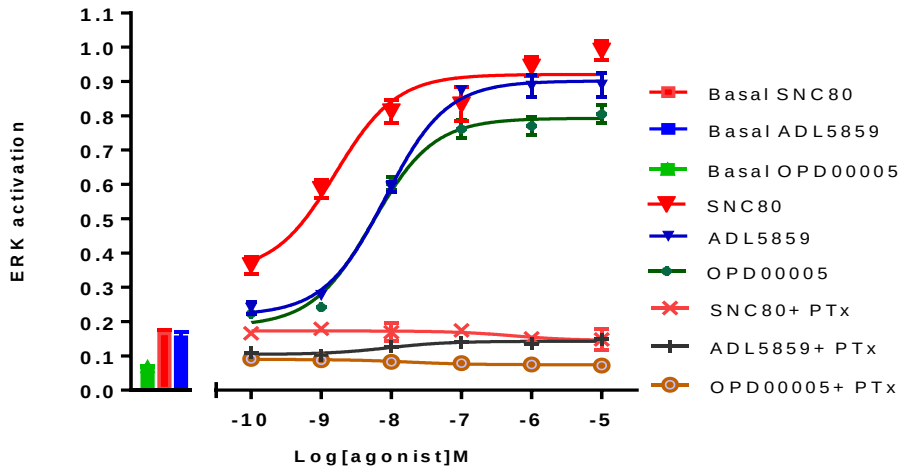


Figure 4.7 Effects of DOR agonists (SNC80, ADL5859 and OPD00005) on ERK phosphorylation in PTx-treated CHO-K1-hDOR cells. 25,000 cells per well were cultured for 24 hrs, followed by serum starvation for 24 hrs in medium containing 200 nM PTx. Cells were stimulated with different concentrations of three DOR agonists, SNC80, ADL5859 and OPD00005 for 5min, followed by rapid fixation with PFA (4% w/v) solution. Detection of total and phospho-ERK levels was carried out as stated in Methods (see 4.4.1). Individual ERK phosphorylation values were normalized to the corresponding total-ERK values. Bars show basal ERK response from unstimulated cells. The data shown are means ± SEM values from 6 independent experiments performed in triplicate.

To confirm the role of the DOR in the agonist responses, the experiments were carried out in the absence and presence of the DOR-selective competitive antagonist naltrindole (1 µM). Naltrindole blocked the stimulatory effects of all the DOR agonists (SNC80, DADLE, PN6047 and OPD00003) as shown in Figure 4.8.

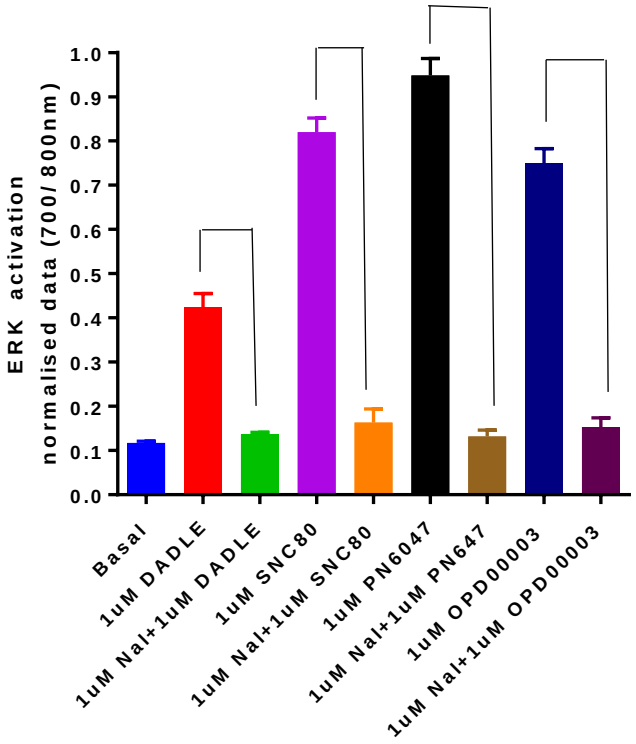


Figure 4.8 Effect of the selective DOR antagonist, naltrindole in CHO-K1-hDOR cells. 1 µM concentrations of each of the DOR ligands (DADLE, SNC80, PN6047 and OPD00003) were co-incubated with an equal concentration of naltrindole in CHO-K-1-hDOR cells. Naltrindole was able to antagonise the DOR-mediated ERK activation by all the ligands.

4.7. Discussion

All of the agonists examined in the cAMP inhibition assays were relatively potent and all appeared to be nearly fully efficacious (Chapter 3). To determine whether the agonists might show biased signalling, these compounds were subjected to further evaluation in an alternative signalling pathway i.e. $ERK_{1/2}$ phosphorylation which has important roles in a large number of cell functions (Roskoski, 2012).

Because GPCR agonists can activate multiple signalling pathways and display biased signalling towards certain pathways, it is crucial to test the ligand efficacies and potencies in different assays since, if a functional assay capturing only one signalling pathway is selected for screening, potentially valuable compounds could be missed if that compound does display biased activity. Therefore, development of signalling-dependent cell-based functional assays can provide more accurate and comprehensive *in vitro* characterization of agonist compounds targeting GPCRs (Zhang and Xie, 2012).

An ideal assay for GPCR ligand screening should be simple, nonradioactive, robust and easily adapted to a microtiter plate format. I-CW is an immunofluorescence-based technique in which the target protein is probed with epitope-specific antibodies in fixed cultured cells in 96-well plates (Daigle *et al.*, 2008). As compared to conventional Western blotting, I-CW is a pharmacologically more suitable assay for screening libraries of compounds. No previous studies have explored the use of I-CW assays in determining ERKs responses to DOR agonist libraries; therefore, it was necessary to determine its feasibility for measuring signalling in our cell line.

Preliminary experiments revealed a clearly visible edge effect in the peripheral wells of the assay plates. The evaporation can occur in all the wells in long-term cultures but is more frequent in corner wells as they are more exposed to the surroundings, and this effect can frequently be seen in 1-3 days of culture. This may also cause culture problems in some sensitive cell types since the medium components, especially the salts, may reach concentrations which are harmful to the cells. It is possible to reduce the evaporation by controlling humidity in the CO_2 incubators and limiting the number of outside inspections as opening the incubator door too frequently is a contributing factor to high rates of evaporation. Moreover, we clearly found lesser volumes of medium in the peripheral wells of the plate, so one intervention could be to add larger volumes of medium in perimeter wells to compensate for the lost water. In the present study, however, we compared the edge effect using pre-incubation of the plates at room temperature (in the laminar hood) for 30 minutes immediately after seeding and prior to moving to the incubator and found that this markedly decreased the visible edge effect (Figure 4.1b). This method was reported by Lundholt *et al* (2003) to control edge effect on microplates. A similar effect was achieved when blank DMEM media was added to the peripheral wells as a measure to allow water loss from the perimeter wells thus controlling the rate of evaporation from the inner wells. However, this results in the loss of a number of data points on each plate so we did not proceed with this approach. We also tested the possible advantages of using a specialised “edge 96-well plate from Thermo Scientific Nunc”, with a surrounding moat which when filled with media acts as an evaporation buffer during longer incubations. This intervention gave comparable results to that of 30-minute pre-incubation at room temperature. However, the results obtained from a 30 minutes pre- incubation at RT indicated a fairly uniform distribution of the cells across the plate at a lower cost. Therefore, all the experiments in this study were performed using this

intervention. However, there is still room for optimising the pre-incubation time for other cell lines.

Initial testing showed that SNC80 was able to produce a phospho-ERK response in CHO-K1-hDOR cells after 5 min stimulation. MOR and KOR stimulation have been demonstrated to initiate ERK1/2 phosphorylation in astrocyte cultures and transfected cell lines. The kinetics of ERK1/2 phosphorylation by MOR and KOR systems vary, yet both receptors can activate ERK1/2 within 5-10 minutes (Belcheva *et al*., 1998; Al-Hasani and Bruchas, 2011). The DOR selective agonist, DPDPE has also been shown to activate ERK1/2 through G*βγ* and Ras signalling cascades (Belcheva *et al*., 1998). Our results in CHO-K1, over-expressing hDOR cells show that the ERK1/2 activation was at a peak after 3-5 minutes of exposure to agonists. Optimization of the cell seeding densities showed that 25,000 cells per well gave the lowest basal phospho-ERK level and a much cleaner agonist ERK1/2 signal. Interestingly, with this cell seeding number the cells reached 100% confluency in 24 hrs while the other cell numbers were either under- or over-confluent. It is of note that the E_{max} of the agonist (SNC80) was apparently different depending on cell seeding density (data not shown), emphasising the need to keep experimental conditions as near to identical as possible when comparing the properties of different agonists in signalling assays.

All cell lines, including CHOs, endogenously express an array of receptors that also signal via MAP kinases, which is why cells were tested in both serum-free and serum-replete conditions, but the basal and agonist-stimulated phospho-ERK levels in both the conditions were not statistically significant (Figure 4.4). However, it was decided to serum starve the cells prior to stimulation with DOR agonists in further experiments to exclude “endogenous” ERK responses to ligands released

from the cells that could cause any interference. Interestingly, the addition of vehicle to test wells without agonist (as a negative control) was able to elicit a small phospho-ERK response in the CHO cells although this was controlled for by expressing data as additions over basal. The possible cause of this vehicle-mediated response was not, however, investigated (data not shown). Many cells release ATP after even very mild stimulation (such as vehicle addition to wells) and so the addition of the ADP/ATP hydrolase enzyme apyrase was examined since this has been previously shown to reduce basal ERK phosphorylation in some cell lines. However, apyrase had no significant effect on either basal or DOR agonist-stimulated ERK activation.

Multiphasic ERK signalling via GPCRs is a commonly observed phenomenon, which can be mediated by G proteins, *β*-arrestins and other scaffolding proteins (Kolch, 2005), as well as via transactivation of receptor tyrosine kinases (Wetzker and Bohmer, 2003). The time course experiments with SNC80 and one of the test ligands, OPD00003 showed that both the compounds were able to promote ERK phosphorylation and the temporal profiles for the two ligands were generally similar to each other, i.e. phospho-ERK levels increased rapidly upon stimulation with agonist, reaching a peak between 3-5 min, after which they steadily declined, presumably due to phosphatase activation, although phosphorylation levels above basal were still evident after 35 mins (Figure 4.5). Since sustained ERK activation could be important in determining phasic agonist responses (e.g. receptor desensitization), it would be interesting to determine complete time courses for some of the other DOR agonists. The sustained ERK activation (minutes to hours) by the 'novel' low *β*-arrestin recruiters (Chapter 5) would be of interest because late phase ERK activation is reported to be associated with an arrestin-dependent activation mechanism (Al-Hasani *et al.,* 2011).

All agonist-mediated phospho-ERK responses at 5 min were clearly shown to be concentration-dependent. Unlike the situation with DOR agonist inhibition of adenylyl cyclase, clear patterns of ERK phosphorylation partial agonism were observed notably when comparing the E_{max} value of DADLE, which was significantly less than that of SNC80. E_{max} values depend not only on ligand properties but also on system determinants such as total receptor number and the maximal response allowed by the system. Based on their E_{max} values, the standard compound ADL5859 and the test ligands, PN6037, PN6047, PN6053, OPD00003 and OPD00005 were full agonists, whereas, PN6054 and PN6055 were partial agonists when compared with SNC80 (Table 4.1). It is also notable that the rank order of agonist potency for cAMP inhibition (PN6037 = PN6047 > DADLE > OPD00005 > OPD00003 > SNC80 = ADL5859 > PN6054 > PN6055) was very different to that for ERK activation SNC80 > PN6047 > OPD00003 > OPD00005 > PN6037 > ADL5859 > PN6054 > DADLE > PN6055).The absolute agonist potencies in the two signalling systems were also very different. Taking the standard peptide agonist DADLE as an example; its EC_{50} regarding cAMP inhibition was 0.06 nM compared with an EC_{50} of 86 nM in the ERK phosphorylation assay. Given that the same high expression DOR cell line was used in both the assays, the low intrinsic efficacy of DADLE in ERK assays cannot be attributed to the low receptor reserve of the system. Moreover, both responses are apparently $G_{i/o}$-mediated and equally sensitive to the DOR antagonist naltrindole, this is a good indication of agonist biased signalling, but care must be exercised over such an interpretation as the assay conditions and end points are very different and these could have a major influence on the calculated potency and efficacy values.

Overall, however, and to the best of my knowledge, this is the first relatively comprehensive characterisation of agonist-dependent ERK signalling in DOR-expressing CHO cells. Whether this apparent agonist bias in relation to cAMP and ERK signalling is reflected in another signalling system (arrestin recruitment) and whether it has any functional significance will be reflected in the next chapters.

5.8. Conclusions

The In-Cell Western assay proved to be an effective tool for measuring DOR agonist activation of $ERK_{1/2}$ signalling. All of the agonists in the set examined enhanced ERK phosphorylation to some extent, although, unlike their effects on cAMP inhibition, they displayed a range of efficacies as well as widely different potencies. This is despite the finding that $ERK_{1/2}$ signalling, like cAMP inhibition, was pertussis toxin-sensitive and presumably Gi/o-mediated.

Chapter 5. A study of the effects of DOR agonists on *β*-arrestin 2 recruitment and receptor internalization and their involvement in agonist desensitization

5.1. Introduction

The purpose of this chapter was to study possible links between *β*-arrestin 2 recruitment by different DOR agonists, their propensity to cause DOR internalization in cells over-expressing hDOR and the relationship of these effects to agonist desensitization/ tolerance.

Clinical tolerance to opioids is a major therapeutic problem and a key challenge of opioid research is to develop agonists that have high analgesic efficacy and a low profile for unwanted side effects, tolerance and dependence (Morgan and Christie, 2011). Tolerance is a phenomenon characterized by decreased responsiveness to the same dose of opioid after repeated use or, in pharmacological terms, a shift to the right in the agonist concentration-response curve. DOR function is known to be modulated by arrestins (Lowe *et al*., 2002; Qiu *et al*., 2007; Raehal and Bohn, 2011) and in various cell models, both *β*-arrestin 1 (arrestin 2) and *β*-arrestin 2 (arrestin 3) can mediate internalization and desensitization after receptor phosphorylation (Qiu *et al*., 2007; Hong *et al*., 2009). Arrestins are key adapter proteins that bind phosphorylated GPCRs to regulate their sequestration and intracellular sorting and, ultimately, they assist in determining receptor fate; either recycling or downregulation via processing in lysosomes (Chapter 1, section 1.7.2).

Stimulation of the opioid receptor has been reported to cause its phosphorylation via GPCR kinase (GRK) and the subsequent recruitment of arrestins, an event that precedes receptor endocytosis (Zuo, 2005; Bohn *et al*., 2000; Hales, 2011; Groer *et al*., 2011; Raehal and Bohn, 2011; Nagi and Piñeyro, 2011; Al-Hasani and Bruchas, 2011; Connor *et al*., 2015). DOR internalization has been observed *in*

vitro for the DOR peptide ligand, [D-Pen^{2,5}]-enkephalin hydrate (DPDPE), deltorphin II and [Met5]enkephalin and the non-peptide agonist BW373U86 (Bradbury *et al.*, 2009). Unlike the MOR, which rapidly recycles to the cell surface, DORs are targeted for lysosomal degradation via the endosomal sorting machinery using ubiquitination-dependent or independent mechanisms (Henry *et al*., 2011) and receptor internalization might be a key step in DOR desensitization. On the other hand, different DOR agonists have been reported to preferentially recruit either of the two *β*-arrestin isoforms, showing bias within the arrestin system, for example, SNC80 is known to be a more efficient recruiter of *β*-arrestin 1 than *β*-arrestin 2 (Pradhan *et al*., 2016).

5.1.1. Principle of *β*-arrestin 2 recruitment assay (DiscoverX)

Ligand-induced *β*-arrestin recruitment activates signalling cascades independent of G-protein signalling. The commercial DiscoverX assay utilises enzyme fragment complementation (EFC) of *β*-galactosidase (*β*-gal) and subsequent enzymatic activity to measure receptor/*β*-arrestin interactions (Figure 5.1). The basis of enzyme fragment complementation (EFC) technology centres on two fragments of *β*-galactosidase (*β*-gal) enzyme: the enzyme acceptor (EA), which lacks the residues 11- 41 and is catalytically inactive, and the enzyme donor (ProLink™/ PK or enzyme donor/ ED), whose structure includes those missing residues. Upon GPCR-*β*-arrestin interaction caused by ligand stimulation, the complementation of EA and ED occurs to form an active *β*-gal enzyme that hydrolyses substrate to generate a detectable luminescent signal which could be read on a luminescence microplate plate reader (Zhao *et al*., 2008, McGuinness *et al.,* 2009).

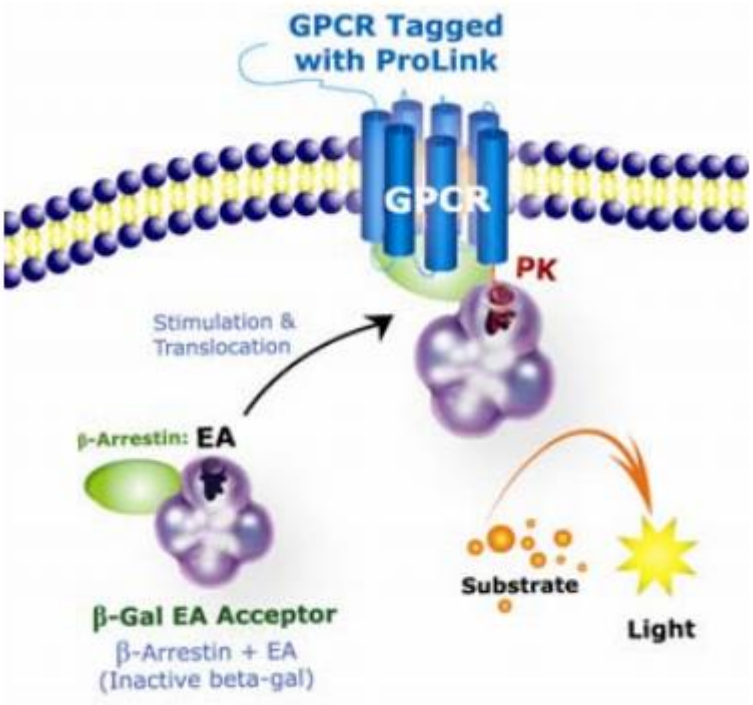


Figure 5.1 PathHunter™ enzyme fragment complementation (EFC) *β*-arrestin recruitment assay principle (DiscoverX, Fremont, CA). The GPCR of interest is fused to the ED peptide, termed ProLink™ (PK) (enzyme donor), which weakly binds a complementary fragment, EA, which is fused to the C-terminus of the arrestin protein. Activation of the PK-tagged GPCR results in recruitment of the *β*arr-EA fusion (Enzyme acceptor). Recruitment of *β*-arrestin by the ligand-activated GPCR results in the complementation of two *β*-galactosidase fragments. The reconstituted holoenzyme catalyses the hydrolysis of a substrate, which generates a chemiluminescent signal.

5.1.2. Principle of internalization assay (DiscoverX)

The small enzyme fragment of *β*-galactosidase (*β*-gal) called ProLink™ (PK) is appended to the C-terminus of the receptor. The larger, complementing enzyme fragment termed Enzyme Acceptor (EA) is localized exclusively on the surface of

early endosomes. Stimulation of the GPCR by a ligand results in receptor activation and induces β-arrestin recruitment followed by internalization of the receptor/arrestin-EA complex in PK-tagged endosomes. These events drive complementation of the two of β-gal enzymes (PK and EA). The resultant increase of β-gal enzyme activity due to the formation of a functional enzyme hydrolyses the substrate to generate a chemiluminescent signal.

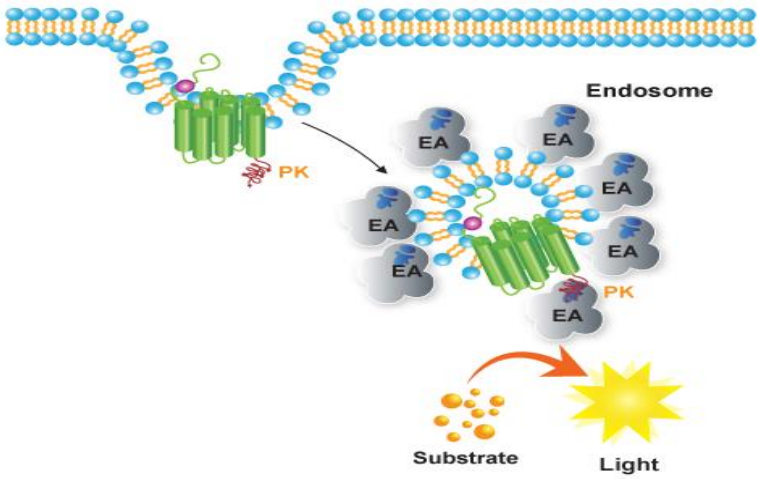


Figure 5.2 PathHunter™ Internalization Assay Principle (DiscoverX, Fremont, CA). The small enzyme fragment of β-gal called ProLink™ (PK) is appended to the C-terminus of the receptor. The larger, complementing enzyme fragment termed Enzyme Acceptor (EA) is localized exclusively on the surface of early endosomes. Stimulation of the receptor results in receptor activation and subsequent internalization and trafficking to cellular endosomes. These events drive complementation of the two of β-gal enzyme fragments. The resultant increase of β-gal enzyme activity can be easily measured using a chemiluminescent substrate.

5.2. Aims and objectives

The main aim of the experiments in this chapter was to study potential agonist-selective signalling via DORs with respect to selective recruitment of *β*-arrestin 2 and to begin to address the hypothesis that arrestin recruitment vs G protein activation bias favours DOR internalization and possibly desensitization and *in vivo* tolerance The objectives of this chapter were, therefore, to measure the effects of a number of DOR agonists on *β*-arrestin 2 recruitment, DOR internalization and desensitization of agonist signalling monitored as agonist-induced inhibition of forskolin-stimulated cAMP accumulation.

5.3. Materials

PathHunter™ GPCR Arrestin 2 and PathHunter™ Internalization cell lines (DiscoverX Corp., Fremont, CA) were used to test the function of DOR agonists. The CHO-K1-hDOR cells (for cAMP determination) and all the ligands used in this study have been discussed in Chapter 3 (Materials section 3.3).

5.4. Methods

For the *β*-arrestin recruitment assay, CHO-K1-OPRD1 cells were seeded at a density of 10,000 cells per well in a 96-well microplate in 100 µL pre-warmed cell plating medium and incubated overnight in a humidified atmosphere at 5% CO_2 and 37 °C. The next day, agonists were serially diluted in serum-free medium (SFM) to generate 9-point dilutions of each ligand (plus vehicle-only as a negative control) and incubated for 1.5 hrs at 37 °C in 5% CO_2. After incubation, appropriate volumes of PathHunter™ detection reagent (DiscoverX) were added according to the manufacturer's instructions. Chemiluminescence indicated as relative luminescence units (RLUs), was measured on a TopCount® NXT™ Plate reader (Perkin Elmer).

In the DOR internalization assay, the U2OS-OPRD1 cells were exposed to agonists for 3 hours at 5% CO_2 and 37 °C. After incubation, appropriate volumes of PathHunter™ detection reagent (DiscoverX) were added according to the manufacturer's instructions. Chemiluminescence indicated as relative luminescence units (RLUs), was measured on a TopCount® NXT™ Plate reader (Perkin Elmer).

In the DOR desensitization experiments, cells were grown to confluence in 96-well microtiter culture plates and then incubated with 1 µM concentrations of SNC80, DADLE,OPD00003 and PN6047 for 48 hours at 37° in 5% CO_2. On the day of assay, the agonist-treated cells were given a 30 min washout period during which the cells were incubated without the agonists in DMEM-F12 growth media. The cells were re-exposed to the agonists and then subsequently challenged with 1 µM forskolin (FSK) in the presence of rolipram and the plates were incubated for 30min at room temperature. At the end of the incubation period, appropriate volumes of PathHunter™ detection reagent (DiscoverX) were added according to the manufacturer's instructions. Chemiluminescence represented as relative luminescence units (RLUs), was measured on TopCount® NXT™ Plate reader (Perkin Elmer). DOR-mediation of the effects was tested by incubation with the selective DOR antagonist, naltrindole (1 µM) .

5.5. Data analysis

Data were analyzed by non-linear regression analysis of sigmoidal concentration-response curves using GraphPad Prism 7.01. CRCs were fitted to obtain EC_{50} values. E_{max} values were compared to the designated standard agonist SNC80 (= 100%).

For statistical analysis, the agonist EC_{50} and E_{max} values were compared with those for standard agonists using one-way or two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test where appropriate. Significance was achieved at $p < 0.05$.

5.6. Results

5.6.1. Measurement of *β*-arrestin recruitment in CHO-K1-OPRD1 cells

The agonists were tested for their ability to promote *β*-arrestin 2 recruitment to the DOR receptor using the PathHunter™ *β*-arrestin assay. All agonists produced a concentration-dependent increase in *β*-arrestin 2 recruitment as measured by an increase in the chemiluminescent signal (Figure 5.3). The rank order of potency ($logEC_{50}$ values) of the agonists, from highest to lowest, was DADLE > OPD00005 = PN6037 > PN6047 = SNC80 = OPD00003 > ADL5859 > PN6054 > PN6055. Whereas, the rank order of E_{max}, from highest to lowest, was SNC80 > DADLE > ADL5859 > PN6037 > PN6047 > PN6054 > PN6055 > OPD00005 > OPD00003 (Table 5.1).

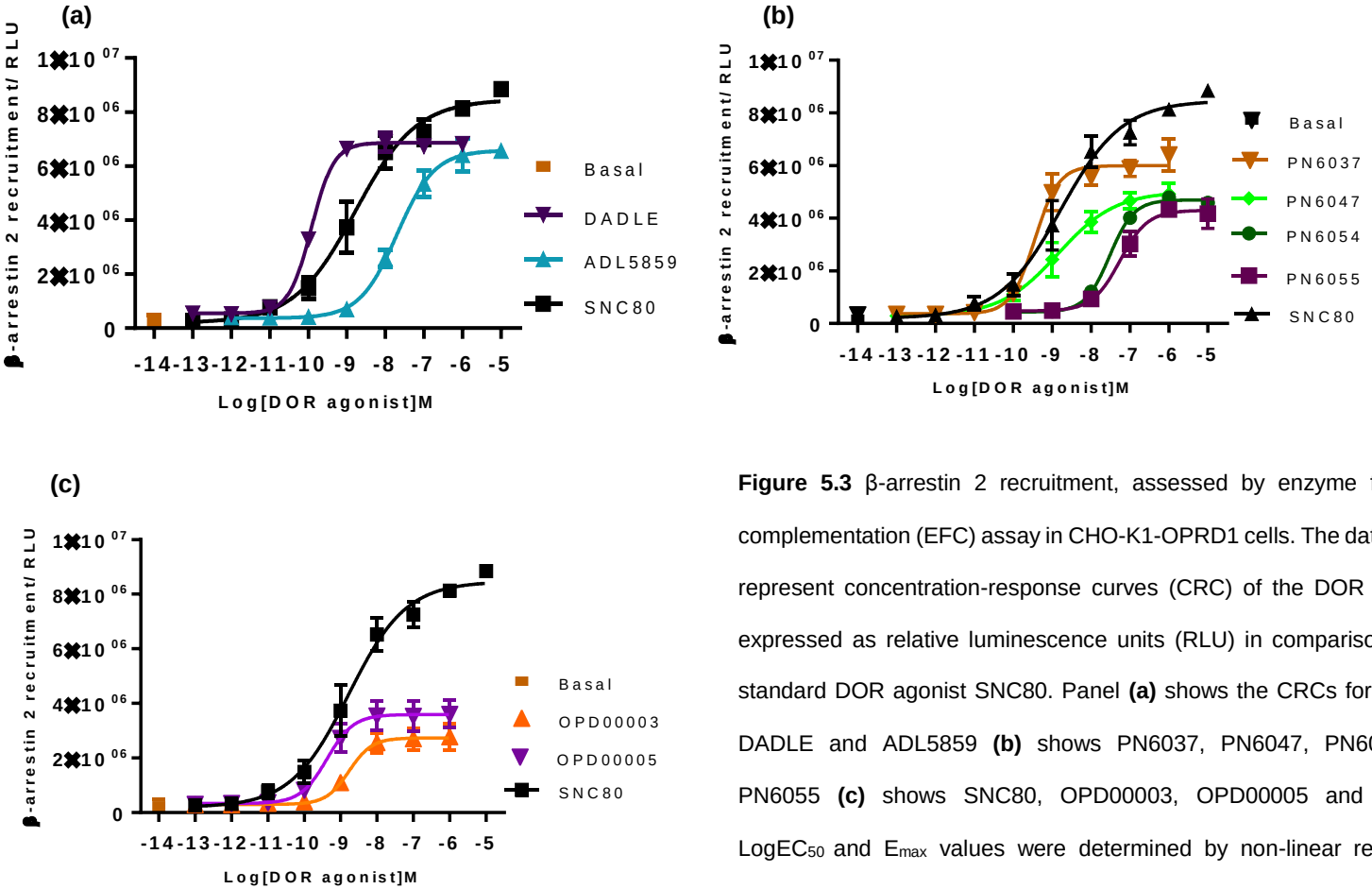


Figure 5.3 β-arrestin 2 recruitment, assessed by enzyme fragment complementation (EFC) assay in CHO-K1-OPRD1 cells. The data shown represent concentration-response curves (CRC) of the DOR agonists expressed as relative luminescence units (RLU) in comparison to the standard DOR agonist SNC80. Panel **(a)** shows the CRCs for SNC80, DADLE and ADL5859 **(b)** shows PN6037, PN6047, PN6054 and PN6055 **(c)** shows SNC80, OPD00003, OPD00005 and SNC80. $LogEC_{50}$ and E_{max} values were determined by non-linear regression analysis of concentration-response curves, using GraphPad Prism 7.01. Statistical analysis was done by One-way ANOVA; $p < 0.05$ was considered significant. All values are means ± SEM (n = 6-8). Error bars represent SEM. Non-visible SEMs are within the symbol.

There were no significant differences in the potencies ($logEC_{50s}$) of all the novel DOR ligands compared to the three standard agonists (SNC80, DADLE and ADL5859) (One-way ANOVA), except for OPD00003, which was similar to SNC80. The EC_{50} values for PN6037, PN6047, OPD00003 and OPD00005 were all less than 1 nM.

However, in contrast to their effects on cAMP and ERK signalling (Chapters 3 and 4), all of the novel DOR ligands showed low efficacies (E_{max} values; Table 5.1) in the *β*-arrestin recruitment assay (One-way ANOVA, $p < 0.05$) compared to SNC80.

DOR Agonist	**EC_{50} (nM)**	**$LogEC_{50}$ ± SEM**	**E_{max} (% SNC80) ± SEM**
SNC80	***2.0***	***-9.0 ± 0.10***	***100 ± 3***
ADL5859	***20***	***-7.7 ± 0.10***	**78 ± *3***
DADLE	***0.01***	***-9.8 ± 0.01***	**82 ± *1***
OPD00003	2.0	-9.0 ± 0.03	32 ± 1*
OPD00005	0.4	-9.5 ± 0.03	42 ± 2*
PN6037	0.4	-9.5 ± 0.02	71 ± 1*
PN6047	0.4	-9.0 ± 0.03	59 ± 2*
PN6054	30	-7.5 ± 0.03	55 ± 2*
PN6055	55	-7.3 ± 0.05	51 ± 2*

Table 5.1 Effects of DOR agonists on *β*-arrestin 2 recruitment in CHO-K1-OPRD1 cells. Potency (EC_{50}, $logEC_{50}$) and efficacy (E_{max}) values are shown from 6-8 separate experiments. Experiments were performed as detailed under Methods (section 5.4), with the maximum response induced by SNC80 defined as 100% for E_{max} calculations. *$p < 0.05$ shows a significant difference in efficacy of novel agonists as compared to SNC80 (One-way ANOVA, followed by Dunnett's multiple comparisons test).

5.6.2. The effects of agonists on DOR internalization in U2OS-OPRD1 cells

In the DOR internalization assay (Figure 5.4, Table 5.2), all of the DOR agonists were able to trigger receptor internalization to a greater or lesser extent. The $logEC_{50}$ values in both the assays were not significantly different (One-way ANOVA), however, the rank order of potencies ($logEC_{50S}$) for the compounds (OPD00003 > PN6037 > DADLE > SNC80 = OPD00005 > PN6047 > ADL5859 > PN6054 > PN6055 were different from those generated in the *β*-arrestin recruitment assay. All of the novel DOR agonists, except PN6037, were partial agonists on the basis of their E_{max} values being significantly different (One-way ANOVA, $p < 0.05$) from the maximum degree of internalization induced by the standard compound SNC80, with some (particularly the OPD compounds) showing very low efficacy. The overall rank order of efficacies, from highest to lowest, was PN6037 > ADL5859 > DADLE > PN6047 > OPD00005 > PN6055 >PN6054 > OPD00003 (Table 5.2).

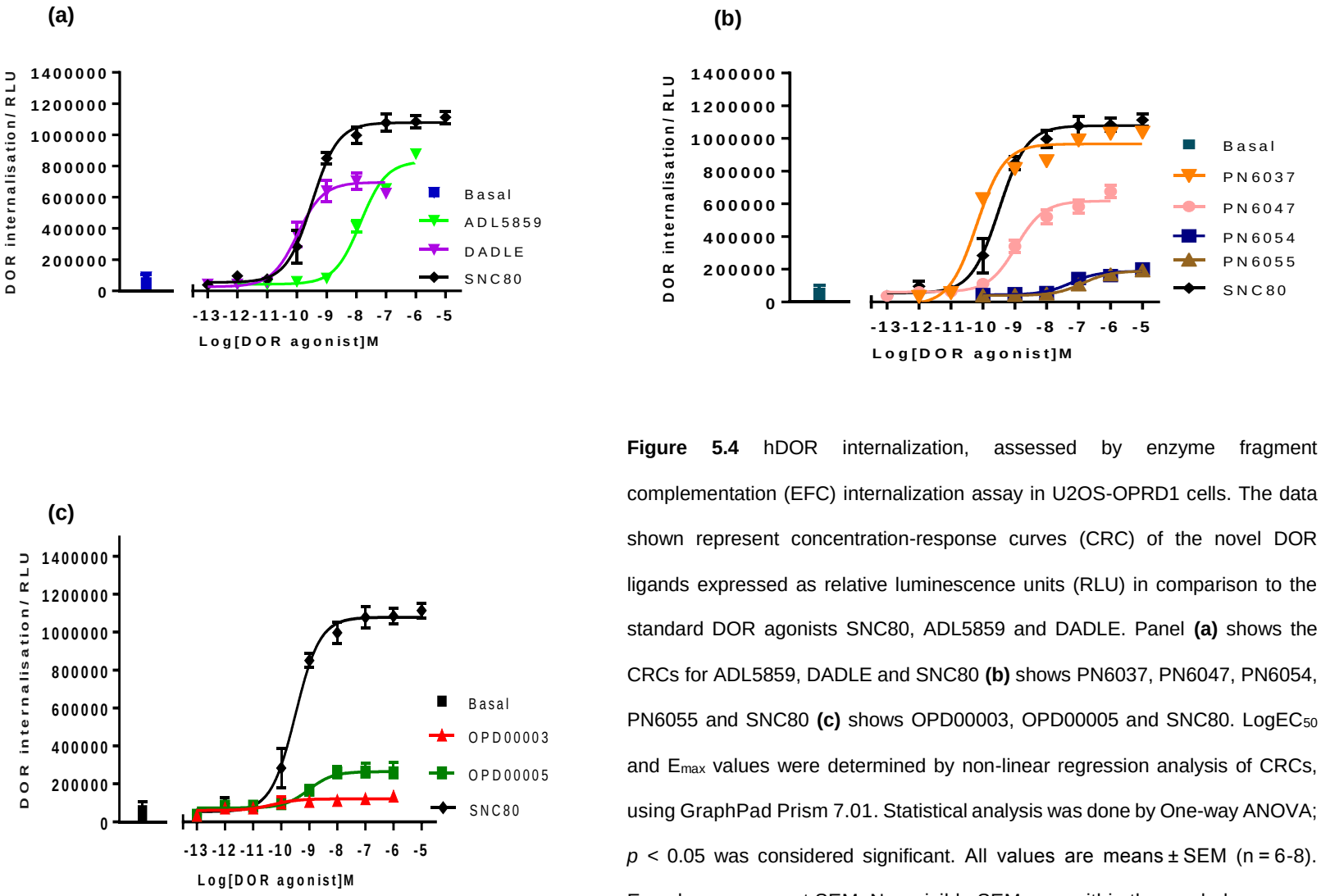


Figure 5.4 hDOR internalization, assessed by enzyme fragment complementation (EFC) internalization assay in U2OS-OPRD1 cells. The data shown represent concentration-response curves (CRC) of the novel DOR ligands expressed as relative luminescence units (RLU) in comparison to the standard DOR agonists SNC80, ADL5859 and DADLE. Panel **(a)** shows the CRCs for ADL5859, DADLE and SNC80 **(b)** shows PN6037, PN6047, PN6054, PN6055 and SNC80 **(c)** shows OPD00003, OPD00005 and SNC80. $LogEC_{50}$ and E_{max} values were determined by non-linear regression analysis of CRCs, using GraphPad Prism 7.01. Statistical analysis was done by One-way ANOVA; $p < 0.05$ was considered significant. All values are means ± SEM ($n = 6$-8). Error bars represent SEM. Non-visible SEMs are within the symbols.

DOR Agonist	EC_{50} (nM)	$LogEC_{50}$ ± SEM	E_{max} (%SNC80) ± SEM
SNC80	***0.32***	***-9.5 ± 0.02***	***100 ± 1***
ADL5859	***13.7***	***-7.8 ± 0.07***	***77 ± 3***
DADLE	***0.10***	***-10.0 ± 0.03***	**64 ± *2***
OPD00003	0.05	-10.3 ± 0.02	11 ± 1*
OPD00005	0.9	-9.5 ± 0.09	25 ± 3*
PN6037	0.06	-10.2 ± 0.10	95 ± 3
PN6047	1.0	-9.0 ± 0.02	57 ± 1*
PN6054	56	-7.2 ± 0.02	17 ± 1*
PN6055	134	-6.8 ± 0.07	18 ± 2*

Table 5.2 Effects of DOR agonists on receptor internalization in U2OS-OPRD1 cells. Potency (EC_{50}, $logEC_{50}$) and efficacy (E_{max}) values are shown of 6-8 separate experiments. Experiments were performed as detailed in Methods (section 5.4), with the maximum response induced by SNC80 defined as 100% for E_{max} calculations. *$p < 0.05$ shows a significantly low efficacy of novel agonists as compared to SNC80 (One-way ANOVA, followed by Dunnett's multiple comparisons test).

5.6.3. Agonist-induced receptor desensitization

Although there were clear differences in agonist-induced *β*-arrestin recruitment through DORs, the functional significance of this selectivity is unclear. Therefore, the effects of some of the agonists which represented a spectrum of arrestin signalling and receptor internalization were tested to see whether such effects

might be reflected in their ability to desensitize, after prolonged exposure, inhibition of cAMP accumulation.

In contrast to the inhibition of FSK-stimulated cAMP accumulation due to short-term exposure to the DOR agonists (Chapter 3), after 48 hrs pre-incubation, neither SNC80 nor DADLE (which were both full agonists in relation to cAMP and arrestin signalling) inhibited the FSK response. However, the OPD compounds and PN6047 (which were partial agonists with regard to arrestin recruitment and DOR internalization) were still able to significantly attenuate FSK-mediated cAMP accumulation after 48 hrs exposure (Figure 5.5). Thus, SNC80 and DADLE had caused a desensitization of the Gi-mediated inhibition of cAMP response, whereas, the OPD compounds and PN6047 either failed to desensitize the response or it had rapidly resensitized during the 30 min washout period. The effects were DOR-mediated as the inhibition of the FSK response was reversed by naltrindole (Figure 5.5).

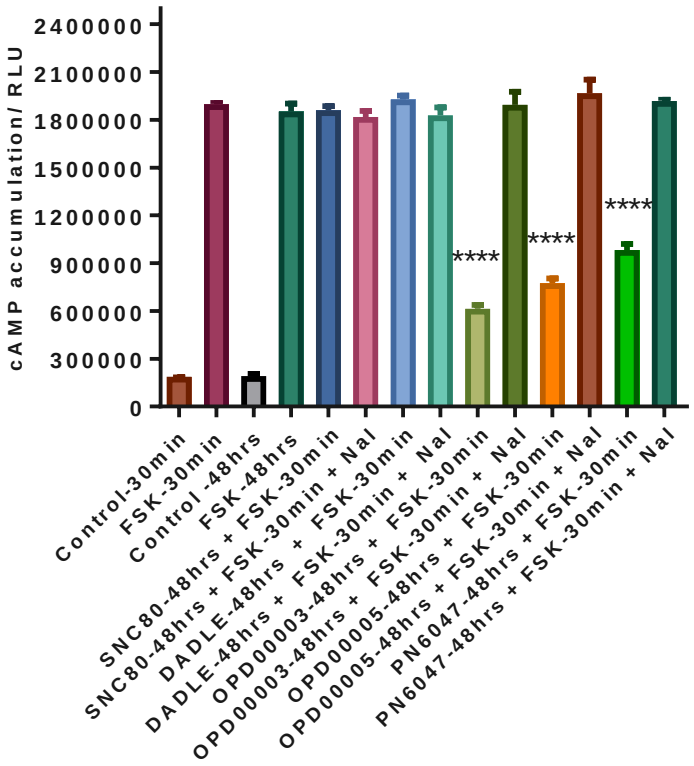


Figure 5.5 Agonist-induced desensitization of cAMP inhibition; CHO-K1-hDOR cells were pre-treated with 1 µM SNC80, DADLE, OPD00003, OPD00005 and PN6047 for 48 hrs at 37°C. After a 30 min washout period, the media containing agonists were replaced and cAMP accumulation was stimulated with FSK (1 µM) for 30 min. Where indicated, the agonist treatments included the selective DOR antagonist, naltrindole (1 µM). Data shown are the means ± SEM of three independent experiments, each assayed in duplicate. ****p < 0.0001, compared with 48 hrs FSK-treated cells (One-way ANOVA, Dunnett's multiple comparisons test).

The effects of shorter-term exposure to the non-desensitizing agonists PN6047 and SNC80 were also compared using the [^{3}H]-adenine pre-labelling cAMP assay (see methods in Chapter 3, section 3.4.1). CHO-K1-hDOR cells were exposed to increasing concentrations of either SNC80 or PN6047 for periods ranging over 10 mins, 2 hrs and 6 hrs and then tested for their inhibitory effects on FSK-stimulated cAMP accumulation.

As expected from previous results (Chapter 3), both compounds, after 10 mins exposure, significantly reduced the effect of FSK, in a concentration-dependent manner. After 2 hrs exposure, the inhibitory effect of SNC80 was attenuated (Figure 5.6 (a)) although the effect of PN6047 was no different to that after 10 mins (Figure 5.6 (b)). After 6 hrs exposure to SNC80, there was no significant effect of any concentration on the FSK response (Figure 5.6 (c)) although there was still, at this time-point, no reduction in the inhibitory effects of PN6047 (Figure 5.6 (d)).

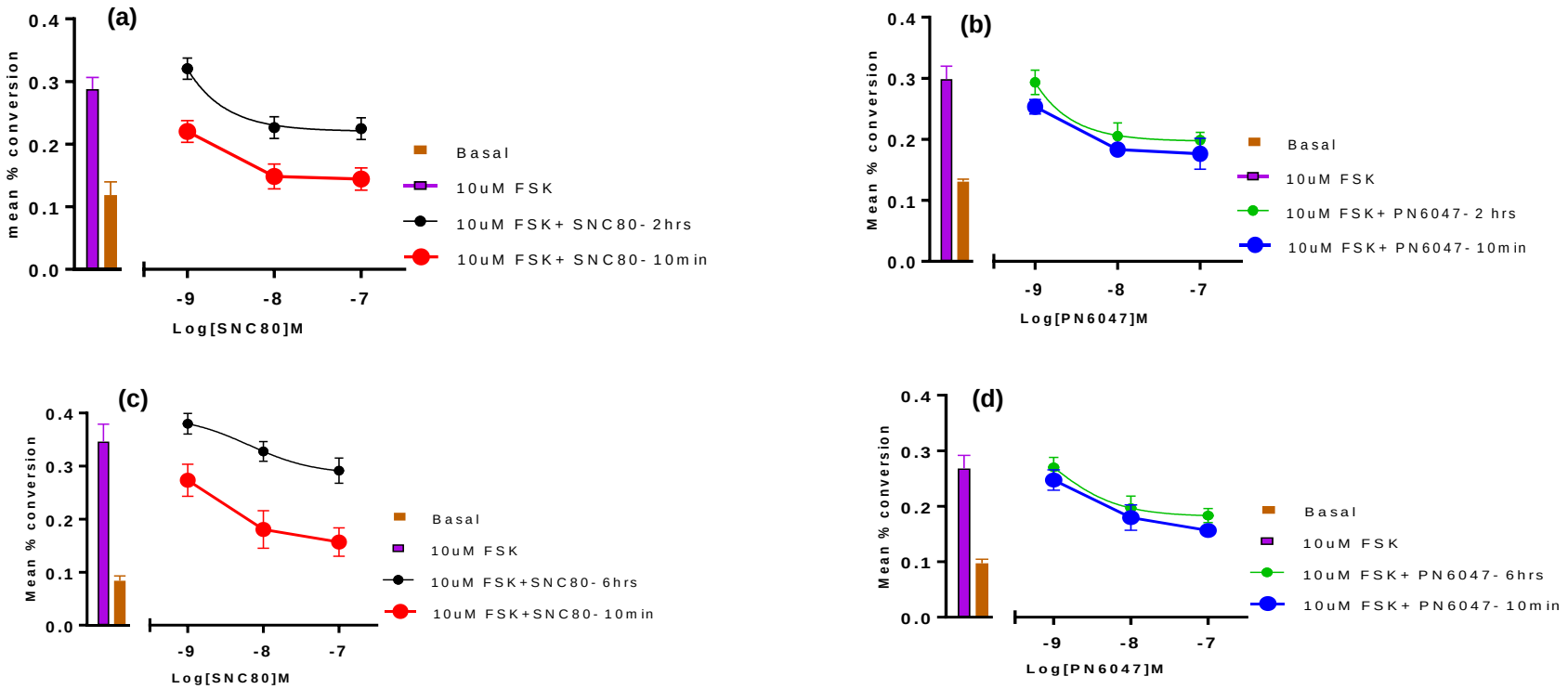


Figure 5.6 Concentration-response curves of SNC80 and PN6047 on FSK-stimulated [^{3}H]cAMP accumulation in CHO-K1-hDOR cells. [^{3}H]adenine-pre-labelled monolayers were treated for 10 min, 2 hr and 6 hrs with different concentrations of the two DOR agonists at 37°C. After treatment, the medium was replaced with fresh growth medium for 30 min and then challenged with 10 µM forskolin in the presence of rolipram, and re-incubated for 20 min at 37°C then assayed for [^{3}H]cAMP levels as described under "Methods", Chapter 3, section 3.4.1). PN6047 significantly inhibited the FSK response at all time points **(b, d)**, whereas, there was significant attenuation of the response to SNC80, seen after 10 mins following 2hr and 6hr incubations **(a, c)** (Two-way ANOVA, Dunnett's multiple comparisons test, $p < 0.05$). The data shown represent means ± SEM values from 3 independent experiments performed for each ligand in triplicate. The curves were fitted using GraphPad Prism 7.01. Non-visible SEMs are within the symbols.

5.7. Discussion

The aim of this chapter was to study arrestin-mediated signalling by a number of delta opioid receptor (DOR) agonists and their propensity to cause desensitization of the human DOR. We addressed the hypothesis that recruitment of *β*-arrestin 2 due to DOR activation is associated with DOR internalization and that this is partially responsible for receptor desensitization, at least of the Gi-mediated inhibition of cAMP accumulation.

G protein-coupled receptor kinases (GRKs) phosphorylate agonist-occupied receptors on serine or threonine residues within the C-terminus or third intracellular loop, creating high-affinity binding sites for arrestins, which translocate from the cytosol to the plasma membrane to physically block receptor-G protein coupling (Stoffel *et al*., 1997; Ferguson, 2001, Luttrell and Gesty-Palme, 2010). There are seven known GRKs and four arrestins. GRK 2 and -3 have C-terminal G*βγ* subunit binding domains that recruit them to the plasma membrane upon G protein activation. Arrestin 1 (visual arrestin) and -4 (cone arrestin) have restricted tissue distribution, localising primarily to visual sensory tissue. The two non-visual arrestins, arrestin 2 (*β*-arrestin1) and 3 (*β*-arrestin 2), are ubiquitously expressed and interact with the vast majority of G protein-coupled receptors (Ferguson, 2001). Arrestins bind directly to GRK-phosphorylated GPCRs, forming a stoichiometric complex that prevents further G protein coupling.

Previous studies demonstrated that DOR internalization was increased by over-expression of both *β*-arrestin 1 and *β*-arrestin 2 (Zhang *et al*., 2005). These authors concluded that endogenous *β*-arrestin 1 functions exclusively in mediating

phosphorylation-dependent receptor internalization, whereas endogenous *β*-arrestin 2, but not *β*-arrestin 1, is required for phosphorylation-independent receptor internalization. That study did not address the possibility that different DOR agonists might be capable of selectively inducing GRK-dependent DOR phosphorylation and thence selective recruitment of arrestins. Qiu *et al* (2007) demonstrated that the DOR agonist D-[Pen2,Pen5]enkephalin could induce receptor internalization and adenylyl cyclase (AC) desensitization following receptor phosphorylation which, they concluded, confers DOR selectivity for *β*-arrestin 2, but again the possibility of agonist functional selectivity was not addressed.

More recently, Pradhan *et al* (2016) demonstrated that DOR agonists can differentially recruit arrestin isoforms. They reported that the high-internalizing agonist SNC80 preferentially recruits *β*-arrestin 1 and that its knock-out increased efficacy. In contrast, the low-internalizing agonists (ARM390 and JNJ20788560) preferentially recruited *β*-arrestin 2 and, surprisingly, its knockout produced acute tolerance and impaired receptor re-sensitization to these agonists. The authors concluded that *β*-arrestin 2 exists in pre-engaged complexes with the DOR at the cell membrane and that low-internalizing agonists promote this interaction. This apparently demonstrates a role for *β*-arrestin1 as a mediator of DOR internalization and desensitization and *β*-arrestin 2 as a mediator of DOR re-sensitization.

In the present study, in contrast to their effects on Gi-mediated cAMP inhibition and ERK phosphorylation in which all agonists were close to being full agonists, the compounds tested showed a spectrum of efficacies with the standard SNC80 showing the greatest effect on *β*-arrestin 2 recruitment while others, for example, the OPD compounds, were clearly partial agonists (Figure 5.3; Table 5.1). When

the effects of the same group of DOR agonists were examined with regard to DOR internalization, in comparison with arrestin recruitment, there were clear differences in both their absolute potencies and their rank orders of potencies indicating that there is not a simple relationship between the agonists' ability to recruit *β*-arrestin 2 and to promote DOR internalization. It must be remembered, however, that the two assays have some quite important differences in their protocols, even in the host cells employed, which could contribute to the different outcomes due to different levels of amplification, for example, differences in host cells, i.e., cell type dependent coupling of DOR to effectors in two cell lines (CHO and hU2OS) would be important to consider while drawing parallels, as there might be differences in releative DOR expression levels and/ or the expression levels of GRKs, and over-expression of recombinant arrestin 2 in both the cell lines.

Nevertheless, it was decided to determine whether the clear differences in the agonists' ability to cause DOR internalization might correspond with their receptor desensitization capacity. Indeed, after prolonged (48hrs) exposure of the DOR-expressing CHO cells to maximally effective concentrations of the highly internalizing agonists SNC80 and DADLE, re-application of the compounds failed to have any effect on forskolin-stimulated cAMP accumulation. In contrast, the OPD compounds and PN6047, which were partial agonists with regard to both *β*-arrestin 2 recruitment and DOR internalization, were still able to attenuate the effects of forskolin on cAMP accumulation after prolonged pre-incubation (Figure 5.5). This difference between effective and poorly DOR internalizing compounds was confirmed when the effects of shorter pre-incubations of lower concentrations of SNC80 and PN6047 were assessed using an alternative [^{3}H]-adenine pre-labelling cAMP assay (Figure 5.6). Whether the low internalizing agonists failed to cause desensitization of the inhibition of cAMP accumulation or, on balance,

promote re-sensitization cannot be inferred from these experiments. It would be interesting to determine whether there is a similar difference in the ability of the agonists to desensitize ERK phosphorylation, another Gi-mediated response.

On the basis of the recent report of Pradhan *et al*. (2016), the results presented in the present chapter are perhaps unexpected. Since these authors concluded that *β*-arrestin 1 (arrestin 2) is a mediator of DOR internalization and *β*-arrestin 2 (arrestin 3) promotes receptor re-sensitization, it might be expected that the agonists that we demonstrated to be highly efficacious *β*-arrestin 2 recruiters (such as SNC80 and DADLE) would be poor DOR internalizers and resistant to causing receptor desensitization, whereas the partial agonists with regard to *β*-arrestin 2 recruitment (e.g. the OPD compounds and PN6047) would be effective DOR internalizers and prone to causing DOR desensitization. This proved not to be the case and there was a positive relationship between *β*-arrestin 2 recruitment and DOR internalization and a negative (albeit based on fewer compounds) relationship between *β*-arrestin 2 recruitment /DOR internalization and receptor desensitization. However, given the apparent controlling role of *β*-arrestin 1 in DOR internalization it will be important to determine the effects of the various agonists on this isoform, although it is hard to imagine that, for example, the effects of SNC80 as a full agonist at *β*-arrestin 2 being offset by an even more effective recruiter of *β*-arrestin 1. Even harder to reconcile would be the partial agonists at *β*-arrestin 2 (e.g. OPD compounds) being even weaker *β*-arrestin 1 recruiters since their efficacy at *β*-arrestin 2 was already very low. Clearly, other elements apart from the arrestins might play crucial roles in determining agonist effects on DOR desensitization.

From studies of peptidic ligands such as ([Leu^5]- and [Met^5]-enkephalins and non-peptidic ligands such as SNC80 and ARM-390, it has been suggested that DOR selective agonists promote profound desensitization compared to non-selective opioid receptor ligands (Marie *et al*.,2003a; Lecoq *et al*., 2004). However, the current study indicates that hDOR desensitization is more complex as the receptor responds differently to a variety of selective, highly potent ligands. We suggest that this behaviour of certain selective DOR compounds could possibly be due to multiple factors, including the chemical nature of the selective ligand used which may drive differential conformation changes of the receptor and thus signalling and/ or sorting to recycling endosomes. Nevertheless, all these data favour the very basic idea that reduction of active DORs from the cell surface by opioids would potentiate their desensitization and thus loss of efficacy.

As from chapter 3, we were able to draw a good correlation between operational efficacy for G protein activation and *β*-arrestin recruitment/ internalization for a series of selective DOR agonists. We observed that a number of full agonists in the cAMP assay were partial agonists in the *β*-arrestin 2 recruitment assay, including OPD00003, OPD00005, and PN6047. To the best of my knowledge, this is the first study comparing agonist potencies and efficacies in three distinct DOR signalling assays (*β*-arrestin, internalization and cAMP assays) for such a wide range of delta opioid compounds.

5.8. Conclusions

We observed that the ligands which acted as full agonists in Gi-mediated signalling pathways (Chapters 3 and 4) and displayed a partial agonism with respect to *β*-

arrestin 2 recruitment and internalization, showed sustained activation of the hDOR even after prolonged agonist exposure probably by maintaining an active receptor population at the cell surface. Whether this resistance to DOR desensitization *in vitro* predicts a potentially useful resistance to tolerance in a therapeutic setting remains to be seen.

Chapter 6. General discussion

6.1. General discussion

Conventionally, G protein-coupled receptor (GPCR) activation engages broad networks of signalling pathways; however, it is now well known that GPCRs do not uniformly activate all available cell signalling pathways. This is a phenomenon termed ligand or agonist bias whereby a ligand induces distinct GPCR conformations that allow selective coupling to effectors. Moreover, the concept of drug discovery targeting GPCRs is no longer limited to identifying agonists or antagonists selective for a particular receptor, as the ligands could influence a multitude of downstream effects. Hallmarks of biased agonism include changes in the relative maximal efficacies of a set of compounds across different signalling pathways referred to as “pluridimensional efficacy” (Urban *et al*., 2007; Kenakin, 2011).

The overall aim of the thesis was to investigate the potential for post-receptor signalling bias amongst a range of delta opioid receptor (DOR) agonists in human DOR over-expressing cells. On the basis of results from other GPCR-based studies, it is thought that G protein-biased signalling, in comparison with arrestin-biased signalling, might confer positive properties, such as resistance to agonist tolerance on such compounds. While a few examples of ligand-selective signalling at the DOR were reported prior to the initiation of this project, no comprehensive *in vitro* studies existed which investigated a variety of signal transduction pathways in a range of selective DOR ligands. We initially screened a small library of 16 DOR-selective compounds in different signalling assays of which 6 were selected for detailed evaluation. We have been able to demonstrate that some of the novel ligands employed in this study showed G protein pathway-selectivity, while partially escaping *β*-arrestin 2-dependent desensitization.

The attempt to develop opioid compounds that provide relief from pain without triggering potentially serious side effects including constipation, tolerance and life-threatening respiratory depression has remained a challenging task. Support for the hypothesis that selective post-receptor signalling underlies at least some of the differences in opioid agonist pharmacology came from the altered behavioural responses to morphine in *β*-arrestin 2-knockout mice reported by the Bohn group. They found that arrestin knockout resulted in a marked potentiation and prolongation of the analgesic effect of morphine (in tail-flick and hot-plate analgesia assays), suggesting that mu opioid receptor (MOR) desensitization was impaired and they suggested that inhibition of arrestin recruitment might be a general strategy for enhancing the analgesic effectiveness of opioids (Bohn *et al*., 1999, 2000; Raehal *et al*., 2005).

Similar conclusions were drawn in a very recent report on novel MOR agonists, published in Nature, by Manglik *et al* (2016). They showed that one of their pathway selective compounds, PZM21, led to no detectable *β*-arrestin 2 recruitment and a minimal level of MOR internalization using the PathHunter™ assays (DiscoverX), compared to DAMGO and morphine, which supports the idea that preference for one signalling pathway over another could improve the efficacy of a compound in a real setting. They observed that PZM21 conferred longer-lasting analgesia *in vivo* with decreased respiratory depression and constipation, both key dose-limiting side-effects of classic opioid drugs. They have further shown that analgesia induced by PZM21 was substantially longer than that induced by a maximal dose of morphine, whereas its constipating effect was also significantly less than morphine.

DOR agonists are currently being developed for the treatment of chronic pain and affective disorders (Pradhan *et al*., 2011; van Rijn *et al*., 2013; Gendron *et al*., 2015). Similar to other GPCRs, delta opioid receptors are also modulated by arrestins (Lowe *et al*., 2002; Qiu *et al*., 2007; Raehal and Bohn, 2011).The present study illustrates a similar potential for signalling bias amongst a set of selective DOR agonists and begins to address the possibility that selective signalling, in the DOR system could provide a similar opportunity to develop therapeutics that are analgesics without unwanted side effects.

The study of biased agonism requires targeted assays to detect and quantify the detailed and precise signalling profiles of potentially large numbers of compounds (Urban *et al*., 2007; Kenakin, 2011). The rapid advances in the field of drug discovery and biased nature of the compounds have encouraged the demand for designing high-throughput screens (HTS) to characterize and optimize lead compounds. In the present study, DOR agonist activity was characterized in three different *in vitro* functional assays. The potency and efficacy studies were accomplished via a number of relatively high-throughput methods: a cAMP accumulation assays (HitHunter^{TM} cAMP Assay, DiscoverX) using CHO-K1-hDOR cells, a *β*-arrestin 2 recruitment assay (PathHunter^{TM} *β*-arrestin Assay, DiscoverX), using CHO-K1-hDOR -arrestin 2 cells, and an internalization assay (PathHunter^{TM} internalization assay) using U2OS-hDOR cells. In addition, an In-cell Western (I-CW^{TM}) assay was employed as an immunocytochemical tool for measuring ERK$_{1/2}$ phosphorylation, using CHO-K1-hDOR cells. All of the agonists used in the study produced substantial DOR activation and some displayed significantly different levels of receptor activation in these assays.

In an attempt to employ a robust, and reproducible method to identify and quantify the interactions of DOR agonists with Gi protein-mediated signalling, we started this investigation by attempting to develop a SPAP reporter gene assay. CHOCRE-SPAP cells were transfected with a construct coding for GFP-tagged hDOR s to enable estimation of the expression level and cellular localization of the receptors. Two standard DOR agonist ligands; a non-peptide (SNC80) and peptide (DADLE) failed to show any significant inhibition of the SPAP response, except at a very high concentration. The suitability of the cell line was confirmed by employing it in an alternative, more direct, cAMP accumulation assay (HitHunter^{TM} DiscoberX). Using this approach, SNC80 (at 1 μM, the highest concentration employed), resulted in a substantial inhibition of the FSK-mediated cAMP accumulation, which was, effectively blocked by the selective DOR antagonist, naltrindole (Chapter 2, Figure 2.12). The SPAP assay *per se* functioned well in that forskolin was able to consistently stimulate cAMP accumulation and there appeared to be significant DOR expression as judged by GFP localization. However, the transfected DORs, perhaps for some of the possible reasons detailed in Chapter 2, did not produce an inhibitory signal of sufficient magnitude to enable it to be employed as a reliable whole cell assay of DOR function in further investigations.

The concentration-response curve (CRC) is a way of determining the activity of a compound which conveys information about the potency (EC_{50}) and maximal activity (E_{max}), relative to a standard ligand in a given cell system. These curves can be determined *in vitro* for any defined pharmacological response, such as the production of a receptor-mediated second messenger (e.g., cAMP) or the interaction of the receptor with signalling proteins such as *β*-arrestin or G proteins. Using CRC data, the relative activity of agonists in different signalling pathways can be compared and biased agonism determined. For a possible bias to be

identified at the compound screening stage, it is necessary to separately determine full CRCs for each signalling pathway so that the pathways can be differentiated. If comparing the effects of a set of agonists on different cellular pathways shows differences in the activation of signalling pathways that are not due to observational bias (i.e., the potency of a given ligand can be different in two different assays due to differences in experimental conditions such as temperature, temporal factors and reagents used) or system bias (i.e., bias due to distinct coupling efficiency of the different pathways), they can be caused by *'ligand bias'*. Because different intracellular signalling pathways have various sensitivities to agonists, ligand bias must be detected and quantified by comparing the activity of agonists within one assay to a selected standard agonist (in order to remove observational and system bias). The relative activity of each agonist in one assay can then be compared to its relative activity in other assays (compared to the same reference agonist in each assay) (Kenakin and Christopoulos, 2013). In the present study, we compared the agonists' effects with the reference DOR ligand, SNC80 by employing increasing concentrations and analyzing the $\log EC_{50}$ and E_{max} values of the concentration-response curves produced. All the responses in different assays showed sigmoidal curves, while the majority of responses were maximal at 1 and 10 μM. A number of compounds tested in the cAMP assay (Chapter 3) and I-CW^{TM} assay (Chapter 4) were full agonists on the basis of their E_{max} values (SNC80 used as reference. Most of the ligands exhibited efficacy close to 100% in the cAMP assay; however, they exhibited a range of E_{max} values in ERK phosphorylation, *β*-arrestin 2 and internalization assays (Tables 6.1), indicating the usefulness of the E_{max} parameter for comparing the compound efficacy and thus potential bias in different signalling systems. With regard to agonist potency, the set of compounds displayed a wide range of EC_{50} values and the rank orders of potency ($\log EC_{50}$) differed for each of the signalling systems analyzed, again showing the potential for functional bias

with regard to effective concentrations of different agonists (or doses *in vivo*) producing maximal effects.

	Maximum response (E_{max})			
DOR agonist	**cAMP**	**ERK1/2**	**β-arrestin 2**	**Internalization**
SNC80	**99 ± 3**	100 ± 3	100 ± 3	100 ± 1
ADL5859	**92 ± 4**	100 ± 1	78 ± 3*	77 ± 3*
DADLE	**96 ± 2**	36 ± 1*	82 ± 1	64 ± 2*
OPD00003	**90 ± 2**	91 ± 3	32 ± 1*	11 ± 1*
OPD00005	**88 ± 2**	86 ± 3	42 ± 2*	25 ± 3*
PN6037	**94 ± 1**	78 ± 3	71 ± 1*	95 ± 3*
PN6047	**94 ± 1**	104 ± 1	59 ± 2*	57 ± 1*
PN6054	**100 ± 2**	37 ± 4*	55 ± 2*	17 ± 1*
PN6055	**100 ± 2**	63 ± 1*	51 ± 2*	18 ± 2*

Table 6.1 Summary of maximal responses (E_{max}) induced by the standard and novel DOR agonists as compared in four signalling readouts: cAMP, $ERK_{1/2}$, β-arrestin 2 recruitment and hDOR internalization. Data is taken from tables 3.1, 4.1, 5.1 and 5.2. *$p < 0.05$ shows a significant difference in efficacy of the agonists as compared to its Gi mediated response (cAMP inhibition) (One-way ANOVA, followed by Dunnett's multiple comparisons test).

In the cAMP accumulation assay (Chapter 3), concentration-response curves (CRCs) were generated for the set of ligands used in this study. The novel DOR ligands exhibited a spectrum of different EC_{50} (Table 6.2) values but the $logEC_{50}$ values were not significantly different (Table 6.3). PN6037 and PN6047 had significantly higher potencies than the two standard compounds (SNC80 and ADL5859). All the novel and standard ligands appeared to be full agonists.

Similarly, minimal differences in efficacies were observed for all the ligands.

	Potency (EC_{50})nM			
DOR agonist	**cAMP**	**ERK1/2**	**β-arrestin 2**	**Internalization**
SNC80	***9.0***	***1.6***	**2.0**	***0.32***
ADL5859	8.0	8.0*	**20***	***13.7****
DADLE	0.06*	86*	**0.01***	***0.10***
OPD00003	0.9*	3.0*	2.0	0.05*
OPD00005	0.2*	5.0*	0.4*	0.9
PN6037	0.04*	7.0*	0.4*	0.06*
PN6047	0.04*	1.6	0.4*	1.0*
PN6054	27*	45*	30*	56*
PN6055	147*	162*	55*	134*

Table 6.2 Summary of DOR agonists' potencies (EC_{50s}) in four signalling readouts: cAMP, $ERK_{1/2}$, β-arrestin 2 recruitment and hDOR internalization. Data is taken from tables 3.1, 4.1, 5.1 and 5.2. *$p < 0.05$ shows a significantly less or high potency of the agonists as compared to SNC80 in each signalling output (One-way ANOVA, followed by Dunnett's multiple comparisons test).

	Potency ($LogEC_{50}$)			
DOR agonist	**cAMP**	**ERK1/2**	**β-arrestin 2**	**Internalization**
SNC80	**-8.1 ± 0.18**	-9.0 ± 0.17	-9.0 ± 0.10	-9.5 ± 0.02
ADL5859	**-8.1 ± 0.06**	-8.0 ± 0.01	-7.7 ± 0.10	-7.8 ± 0.07
DADLE	**-10.2 ± 0.17**	-7.0 ± 0.01	-9.8 ± 0.01	-10.0 ± 0.03
OPD00003	**-9.0 ± 0.18**	-8.5 ± 0.09	-9.0 ± 0.03	-10.3 ± 0.02
OPD00005	**-9.6 ± 0.09**	-8.2 ± 0.08	-9.5 ± 0.03	-9.5 ± 0.09
PN6037	**-10.4 ± 0.04**	-8.1 ± 0.08	-9.5 ± 0.02	-10.2 ± 0.10
PN6047	**-10.4 ± 0.04**	-8.7 ± 0.02	-9.0 ± 0.03	-9.0 ± 0.02
PN6054	**-7.6 ± 0.05**	-7.3 ± 0.14	-7.5 ± 0.03	-7.2 ± 0.02
PN6055	**-6.8 ± 0.05**	-6.7 ± 0.06	-7.3 ± 0.05	-6.8 ± 0.07

Table 6.3 Summary of DOR agonists' potencies ($logEC_{50s}$) in four signalling readouts: cAMP, $ERK_{1/2}$, β-arrestin 2 recruitment and hDOR internalization. Data is taken from tables 3.1, 4.1, 5.1 and 5.2. No significant differences were seen for any of the ligands' potencies (One-way ANOVA).

Application of PTx to the cells also blocked the inhibitory effect of SNC80, indicating coupling to the inhibitory *G* protein (G*i)* and a negative regulation of adenylyl cyclase activity (Figure 3.7) The DOR-mediation of responses was confirmed by the use of the DOR antagonist naltrindole which abolished the FSK-mediated cAMP inhibition in the CHO-K1-hDOR cell line (Figure 3.8). In this assay DOR overexpressing cells were used, which are very different, in all probability, from the real target neurons in the CNS and peripheral nervous systems. However, they are, at least, a homogenous source of recombinant human DORs which were tested in more than one assay method, thus giving some confidence in the data generated.

An attempt was made to measure inhibition of FSK-stimulated cAMP in neuroblastoma cells (SH-SY5Y and Neuro-2a) endogenously expressing DORs data not shown). SNC80 and one of the novel ligands, PN6047, were tested and both produced modest cAMP inhibitions at the highest concentration (1 μM) used; however, it was not possible to generate full CRCs for either of these ligands, most likely due to the comparatively low DOR density in the endogenously expressing cell lines.

The assessment of different signalling parameters could be a little difficult to determine when dealing with other variables, including the use of different cell lines, or the use of a modified (in this case GFP-tagged) receptor (Chapter 2), in the case of the reporter gene assay. The present study, however, could be useful as a basic model for the evaluation of the ligand activity and bias in a DOR system and the methods can be trusted as a tool for screening additional compound libraries with potential activity at hDOR.

The literature reports a number of studies evaluating the role of SNC80 at the DOR, with an overwhelming focus on G protein-activated signalling, using cAMP accumulation assays as an end-point (Gharagozlou, 2002; Lecoq *et al.,* 2004; Charfi *et al.,* 2015). Therefore, it was decided to test the compounds' effects on an alternative signalling pathway and, therefore, further evaluation was performed of the ERK1/2 pathway (Chapter 4). The MAP kinases have important roles in a large number of cell functions, including cell cycle progression, cell survival, differentiation, metabolism, proliferation, and transcription (Roskoski, 2012). Prior to conducting a detailed evaluation on the basis of I-CW^{TM} assay, the standard

ligand SNC80 was subjected to Western blot (WB) analysis (results not shown), in which the activation (phosphorylation) of ERK1/2 proteins was confirmed. I-CW™ assay was preferred over conventional WB in further experiments to avoid cumbersome operational steps in WB, keeping in mind that generation of CRCs for a range of agonists was more practicable with I-CW assay. The literature shows that ERK1/2 is activated by DOR selective agonist, DPDPE through G*β*γ and Ras signalling cascades (Belcheva *et al*., 1998). The current study (Chapter 4) expands on previous studies by evaluating ERK1/2 activation following acute exposure to DOR agonists. Previous studies using heterologous cell systems have shown that ERK1/2 activation reaches a peak between 5 and10 minutes of agonist exposure (Belcheva *et al*., 1998). The time course experiments with the present set of agonists generally displayed maximum responses between 3 and 5 minutes (Figure 4.5) and declined over the next 10 minutes, presumably due to activated phosphatase activity. However, levels of phospho-ERK1/2 were still elevated above basal after 35 mins exposure to at least two of the agonists (SNC80 and OPD00003). Thus, signalling via DORs would appear to be a relatively short-term mechanism although time courses of other agonist responses and the consequences of sustained ERK1/2, phosphorylation for example, regarding altered downstream gene transcription would be interesting to study. The inhibition of ERK1/2 activation with the Gi/o protein blocker PTx (Figure 4.7) suggests that the agonists were acting via a Gi/o protein as was the case for cAMP inhibition (Figure 3.7). Although many of the agonists were virtually full agonists in both signalling systems, their absolute potencies and rank orders of potency were quite different, indicating that, although both responses are Gi protein-mediated, they are differentially activated, possibly due to the involvement of other modulating elements, the identities of which are beyond the scope of the present studies to

clarify. It is also important to be mindful of the differences in assay conditions which could contribute to the apparently different outcomes.

As discussed earlier, arrestin recruitment by opioid receptors appears to be involved in the control of receptor internalization/desensitization. *β*-arrestins have been reported to be the negative regulators of and scaffolds for GPCR signalling elements. According to Zhang *et al* (2005), *β*-arrestin 1 and *β*-arrestin 2 preferentially bind to the phosphorylated GPCRs in response to agonist stimulation, resulting in receptor internalization and desensitization.

In Figure 6.1, correlations are shown between agonist potencies ($\log EC_{50s}$) in different assays. Apprarently, there is little correlation, if at all (r^2 =0.90, $p < 0.05$) between arrestin and internalization (Figure 6.1 (d)). Interestingly, when the efficacy (E_{max}) of ech agonist was compared, a smilar correlation (r^2 =0.85, $p < 0.05$) was obtained for arrestin and internalization efficacies (Figure 6.2 (d)), suggesting that the two phenomena are linked. However, again, the agonist potencies and efficacies in other systems were different, indicating that there is not a simple causal relationship.

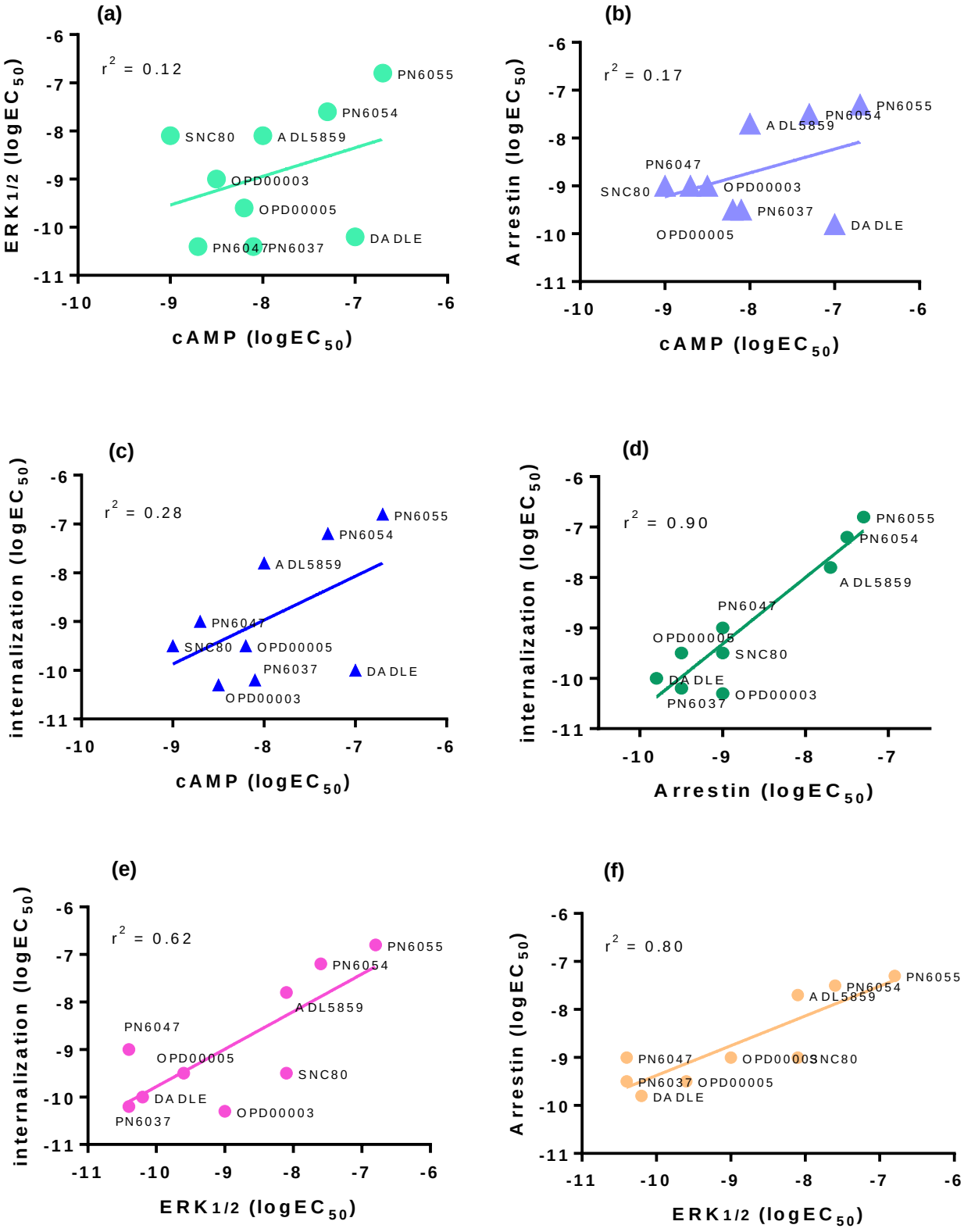


Figure 6.1 Correlation between logEC$_{50}$ values of DOR agonists in different assays. Data analyzed using Prism 7.01 (p value less of than 0.05 was considered significant (One-way ANOVA)).

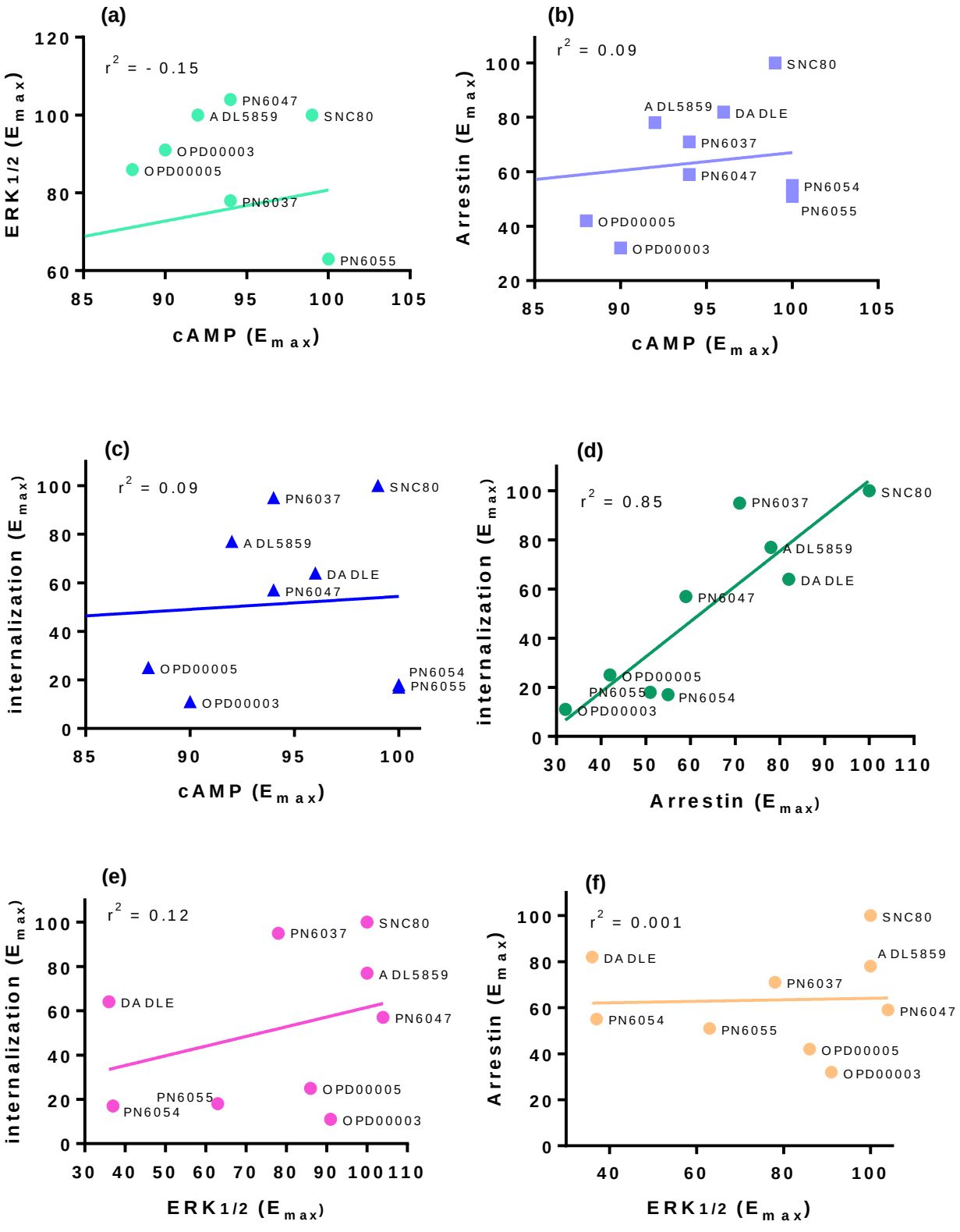


Figure 6.2 Correlation between E_{max} values of DOR agonists in different assays. Data analyzed using Prism 7.01 (p value of less than 0.05 was considered significant (One-way ANOVA)).

As discussed earlier (Chapter 1 and 5), a recently published paper (Pradhan *et al*., 2016), shows that DOR agonists preferentially recruit specific arrestin isoforms in a ligand-selective manner. They showed that the high-internalizing agonist SNC80 preferentially recruited *β*-arrestin 1 and KO of *β*-arrestin 1 resulted in increased potency and duration of action and decreased acute tolerance to this agonist. In contrast, low-internalizing agonists preferentially engaged *β*-arrestin 2. However, in the present study, we observed that SNC80 displayed a robust recruitment of *β*-arrestin 2, which is interesting as it somewhat contradicts the Pradhan group's findings. On the other hand, based on previous studies with arrestin knockout mice and pharmacological studies with biased MOR ligands (Bohn *et al*., 1999, 2000, Raehal, 2005; DeWire, 2013), we anticipated that the partial arrestin recruiters in the hDOR system would also confer persistent cAMP inhibition which was effectively displayed by at least two of the novel ligands, PN6047 and OPD00003. In contrast, the effects of the reference ligand, SNC80, which is a full agonist with regard to both arrestin recruitment and DOR internalization, waned over time indicating a desensitization of DOR-effector coupling (Figure 5.5).

Although maximal efficacy and potency are both indicators for bias measurement, comparing the maximal efficacies or potencies alone is not adequate to determine agonist bias. Comparison of maximum responses cannot differentiate between two full agonists in a system, and using potency values to determine agonist activity is not sufficient in order to separates agonists that produce different maximum effects. Different pharmacodynamic models for bias estimation have been suggested in the literature (i.e., linear model, log-linear model, E_{max} model, sigmoid E_{max} model, logarithmic model), all of which are based on the assumption of a direct proportionality between pharmacological effect and the number of occupied receptors. However, experimental data from different studies have demonstrated

that these assumptions are not valid in many biological systems because the systems are said to have “spare receptors”. Such a requirement has prompted the development of some novel analytical tools, several of which share the operational model of Black and Leff (1983) as a common basis, which is a valuable method to characterize the agonism regardless of the system. To exploit ligand bias in a manner that can assist structure-activity studies or drug discovery, it is necessary to use methods that remove observational biases on functional selectivity. In the present study, we opted for the model which allows ligand affinity for the receptor to be influenced by its signalling partner(s) (Kenakin and Christopoulos, 2013). For this, “functional affinity” or K_A values were calculated as one of the parameters of the concentration-response curve, yielding an estimate of the agonist’s affinity to bind receptor conformation(s), mediating each of the responses. By fitting the CRCs of functional data to the operational model, functional efficacy and affinity can be determined from a parameter defined as “transduction coefficient” ($\log(\tau/K_A)$ or, (logTR), which denotes the effectiveness of a particular ligand-receptor pair to generates a response in a given system (Chapter 1, Section 1.11).

To quantify true ligand bias, i.e., eliminating the influence of system and observational bias, a bias plot was generated in GraphPad Prism 7.01 (Figure 6.3), using the operational model of agonism; the theoretical rationale of which has been described previously (Chapter 1, section 1.11), in which a lack of biased agonism compared to the reference agonist SNC80 will result in values not significantly different from 0 between the two pathways.

ΔΔLogTR (transduction ratio) represents a single fitted parameter sufficient to describe agonism and bias for a given pathway. It is known to be one of the most

robust and widely applicable methods for bias quantification. (Kenakin *et al*., 2012; Thompson *et al*., 2015). The aspect of ligand diversity that is captured by allowing the receptor to adopt distinct ligand affinities for different responses was incorporated into the calculation of bias by comparing transduction coefficients (τ/K_A) instead of operational affinities for each of the functional readouts.

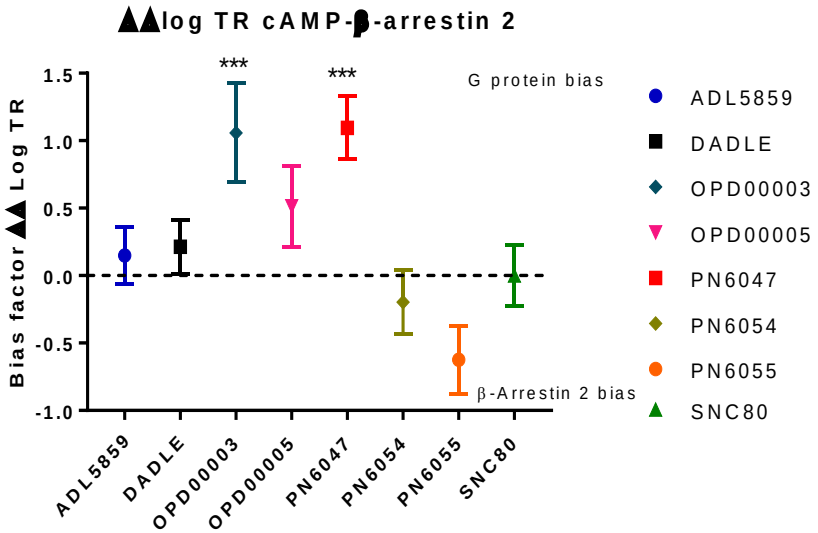


Figure 6.3 Log bias factor (ΔΔLogTR) of all agonists (SNC80, ADL5859, DADLE, OPD00003, OPD00005, PN6047, PN6054 and PN6055) between cAMP and *β*-arrestin 2 recruitment assays**.** ****p* < 0.05 shows a significant difference from SNC80, as determined by One-way ANOVA, followed by Dunnett's multiple comparisons test.

Agonists were assessed for bias between Gi protein activation and β-arrestin 2 recruitment. The responses to SNC80 for both the pathways yielded apparently similar efficacies, revealing SNC80 as functionally distinct from the test compounds. As shown in Figure 6.3, PN6047 and OPD00003 are clearly less effective in stabilization of the β-arrestin signalling states as compared with the G protein signalling, showing substantial bias towards G protein activation, and

hence revealing a rank order of pathway bias (i.e., cAMP > β-arrestin), relative to SNC80 (cAMP = β-arrestin) (One-way ANOVA), whereas PN6055 showed a trend, albeit not significant, towards arrestin bias. OPD00005 showed bias towards Gi signalling, however, the bias did not reach statistical significance. Other compounds were no different to the standard, SNC80.

The use of modern teachniques such as X-ray crystallography (Okada *et al.,* 2001), plasmon waveguide resonance spectroscopy (Hruby *et al.,* 2007), and NMR spectroscopy (Liu *et al.,* 2012) has revealed that each ligand can stabilize a unique subset of receptor conformation that leads to signalling through distinct pathways. The DORs, like other GPCRs, may adopt different active conformations (receptor-effector complexes) with preferential affinities for a subset of effectors (biased agonism) (Salamon *et al.,* 2002; Audet *et al.,* 2008; Audet *et al.,* 2012); an adoption of these distinct conformations in the presence of a given ligand was clearly reflected in our obervations. Kenakin and Christopoulos (2013) report that biased agonism is associated with the molecular structure of the agonist, and that is why this ligand-related behaviour has been termed “agonist-directed trafficking”, “stimulus trafficking” or “functional selectivity” (Clarke, 2005; Urban *et al*., 2007).

It is of note that the differences in receptor reserve and amplification (Rajagopal *et al.,* 2010) and the use of different methodologies and time points (Herenbrink *et al.,* 2016) for different signalling systems can affect the potencies and maximal effects of ligands. According to Eglen *et al* (2007), employing assays with significant amplification, such as second-messenger assays (e.g., cAMP accumulation assays), both full and partial agonists can reach the same maximal response, whereas in assays with little amplification, such as EFC assays that monitor recruitment of β-arrestin to a receptor, partial agonists have lower E_{max}

than full agonists (see chapter 1, section 1.10). Because the signal amplification or the expression levels of the various proteins involved in the activation of the signalling pathways examined may differ across cell lines and thus have an impact on the measures of bias (Thompson *et al.,* 2015), drawing such comparisons is not straightforward, however, a significant reversal in rank order of efficacies or potencies would be evidence for ligand bias (Kenakin, 2007). Importantly, the efficacies for standard ligand SNC80 towards both the pathways (G protein and β-arrestin2 recruitment) were similar, whereas significant reversal of efficacies (E_{max}) was seen for most of the novel ligands (Figure 6.2) in both the assays, however, only two ligands (PN6047 and OPD00003) displayed significant selectivity/ bias in the operational model, clearly reflecting it to be a relevant means of demonstrating agonist bias, thus providing confidence to the data generarted.

One of the limitations in the present study is that the timescale of all the functionally distinct assays employed were different. According to Sheth *et al* (2013) and Herenbrink *et al* (2016), the difference in time points between two cascades under investigation can influence the bias. Therefore, the impact of kinetics on measured drug response should be considred carefully, particularly when comparing transient responses (e.g., ERK/5min), with those measured over longer time scales (e.g., cAMP/30 min, arrestin/90min, internalization/180min, in the present study). This area of bias estimation needs further probing to obtain more specific outcomes.

A more practical question is whether agonist bias has functional consequences; for example, underpinning differences in promoting receptor desensitization and downregulation of responses. The first reports describing a differential desensitization of MOR, DOR, and KOR by various agonists came from Reisine's

group (Blake *et al*., 1997a,b; Bot *et al*., 1997), suggesting that biased agonism could influence desensitization and, as shown in Chapter 5, there were clear differences in the ability of different agonists from the present set of compounds to cause uncoupling of DORs from cAMP inhibition *in vitro*. This might have parallels with the agonists' effects *in vivo* and, indeed, the partial arrestin recruiting agonist PN6047 has recently been shown to induce no tolerance to its ability to reduce mechanical hypersensitivity in a rodent (sciatic nerve ligation) model of neuropathic pain after 28 days of daily administration (von Mentzer *et al*., unpublished).

This indicates that it is possible to achieve an agonist concentration *in vitro,* and perhaps a dose *in vivo that* could nearly maximally activate desirable cAMP signalling whilst not significantly engaging the other unwanted signalling systems. This is illustrated for PN6047 in Figure 6.4.

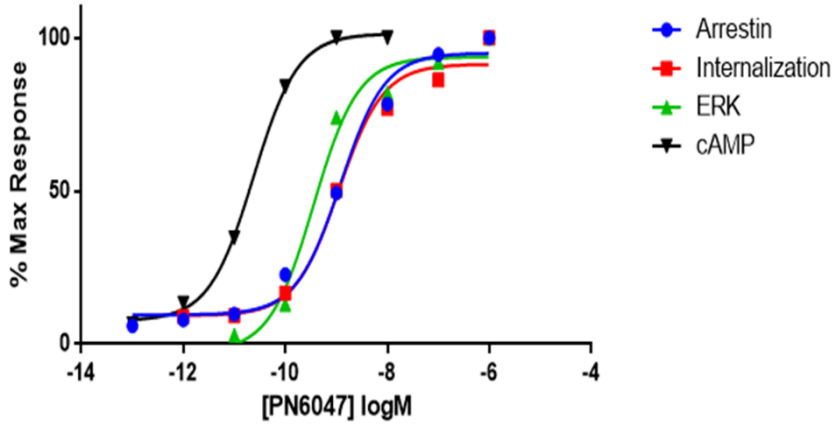


Figure 6.4 A comparison of the potencies of PN6047 in different *in vitro* assays. Data taken from Chapters 2, 3, 4 and 5.

Although the therapeutic potential of DOR agonists is compelling, an important unwanted property that has hampered development is the pro-convulsant effect seen in rodents (Chung *et al*., 2015). However, this appears not to be a property

common to all DOR agonists and whether differences in agonist signalling bias have any relevance to the behavioural properties of DOR agonists' remains to be seen. Interestingly, the cAMP signalling biased PN6047 does not induce convulsions (von Mentzer *et al*., unpublished). It will be crucial in the future to understand the structure-activity relationships (SAR) of agonist bias to facilitate novel therapeutic compound development but this is a topic beyond the scope of this thesis to be tackled by medicinal chemists and other pharmacologists.

In summary, we have characterized agonist biased signalling for a set of standard and novel DOR-selective compounds and have identified a possible mechanistic connection between the drugs' preference for Gi-mediated cAMP inhibition and resistance to receptor desensitization/tolerance. On the other hand, calculating bias factors for the established reference ligands and novel compounds will add a new level of texture in examining drug responses. The present study also provides a framework to systematically compare a large number of DOR ligands for diverse signalling pathways, thereby allowing the classification of compounds into functionally distinct clusters which could have important implications for drug discovery and therapeutic development directed against chronic pain, affective disorders and other neurological conditions.

6.2. Future directions

Some of the findings in this thesis have raised additional questions and highlighted a number of areas of further work which would contribute to our understanding of DOR function in future.

Due to an apparent discrepancy between the results of the present study and the report of Pradhan *et al*., 2016, in which it was concluded that *β*-arrestin 1 recruitment promotes DOR internalization whilst *β*-arrestin 2 mediates DOR re-sensitization, it would be interesting to determine the effects of the DOR agonist set on recruitment of both isoforms. In parallel studies in which the expression of the separate arrestin isoforms is reduced by appropriate siRNAs, determination of the agonists' effects on receptor internalization and desensitization would be instructive.

As mentioned earlier, a major problem with the interpretation of agonist bias in the experiments described in this thesis is that only relatively easy-to-measure downstream signalling has been investigated and the assays were conducted with different cell lines under different experimental conditions. An alternative, although more technically demanding and expensive approach would be to employ phosphoproteomic assays in which cells (transfected or possibly "real" primary cultured cells) are exposed to agonists under identical conditions and the full profiles of downstream phosphorylated proteins determined by mass spectroscopy (Gao *et al*., 2014). This is not, of course, a high-throughput approach and its application to new compound discovery is probably limited.

Given more time and funding, the next step in this project would have been to test these novel ligands in a rat model of chronic or inflammatory pain (e.g. knee osteoarthritis), employing *in vivo* electrophysiology; given the fact that there is an upregulation of delta opioid receptor function following tissue injury associated with chronic pain (Pardhan *et al.,* 2013), it would be interesting to see whether these novel DOR ligands show a profound analgesic efficacy in both behavioural and electrophysiological measures after systemic or local injections. In this context,

the use of both normal animals and disease models will be required to look for the correlations between the full signalling profiles of drug-like DOR agonists and their range of desirable and unwanted effects.

Other potential areas where future work could be warranted include binding studies, ligand-based structure-analysis (similarity searches, pharmacophore screens and quantitative structure-activity relationships) and lead optimization on some of the potential drug candidates explored in the present study.

6.3. Conclusion

The present study helps us recognise that in a given cell system, biased agonists can differentially activate a subset of the receptor's full signalling repertoire. We have demonstrated that there is a practical correlation among the agonists' Gi/o protein activation profiles and their capacity to recruit *β*-arrestin 2 and thus mediate internalization of the hDOR. Importantly, it is reasonable to support that G protein bias over arrestin recruitment could be a practical approach to achieving a sustained effect in a DOR system.

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