# The Effect of μ-Opioid Receptor Polymorphisms on Receptor Signalling Systems

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# Abbreviations

| | |
|---|---|
| 4-AP | 4-aminopyridine |
| aa | amino acid |
| AC | adenylyl cyclase |
| ACTH | adrenocorticotropic hormone |
| Akt | protein kinase B |
| β-CNA | β-chlornaltrexamine |
| β-FNA | β-funal trexamine |
| BSA | bovine serum albumin |
| CaM | calmodulin |
| cAMP | cyclic adenosine monophosphate |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| CHO | Chinese Hamster Ovary |
| CRC | concentration-response curve |
| CRE | cAMP response element |
| DAMGO | [D-$Ala^2$, N-$MePhe^4$, Gly-ol]-enkephalin |
| DMEM | Dulbecco's Modified Eagle Medium |
| DOPr | δ-opioid receptor |
| DPDPE | [2-Dpenicillamin, 5-Dpenicillamin]-enkephalin |
| ECL | extracellular loop |
| EGFR | epidermal growth factor receptor |
| ELISA | enzyme-linked immunosorbent assay |
| EM1 | endomorphin 1 |
| EM2 | endomorphin 2 |
| EPAC | exchange signal activated by cAMP |

| | |
|---|---|
| ERK | extracellular signal regulated kinase |
| FSK | forskolin |
| GIRK | G protein gated, inwardly rectifying potassium channel |
| GLR3 | glycine receptor α3 |
| GPCR | G protein couple receptors |
| GRK | G protein coupled receptor kinase |
| HBSS | Hanks balanced salt solution |
| hMOPr | human μ-opioid receptor |
| HPA | hypothalamic-pituitary-adrenal |
| $I_{Ca}$ | voltage gated Ca channels |
| ICL | intracellular loop |
| $IP_3$ | inositol (1,4,5) triphosphate |
| JNK | Jun N-terminal kinase |
| KO | knock-out |
| KOPr | κ-opioid receptor |
| M-6-G | morphine-6-glucuronide |
| MAPK | mitogen activated protein kinase |
| MOPr | μ-opioid receptor |
| mRNA | messenger ribonucleic acid |
| N/OFQ | nociceptin/orphanin FQ |
| NMDA | *N*-methyl-d-aspartate |
| NOPr | nociceptin/orphanin FQ receptor |
| NR1 | NMDA receptor 1 |
| *OPRD1* | opioid receptor δ 1 |

| | |
|---|---|
| *OPRK1* | opioid receptor κ 1 |
| *OPRL1* | opioid receptor-like 1 |
| *OPRM1* | opioid receptor mu 1 |
| p38MAPK | p38 mitogen activated kinase |
| PAG | periaqueductal grey |
| pDyn | prodynorphin |
| pENK | proenkephalin |
| pERK | phosphorylated ERK1/2 |
| PI3K | phosphatidylinositol-3 kinase |
| PKA | protein kinase A |
| PKC | protein kinase C |
| POMC | proopiomelanocortin |
| PLC | phospholipase C |
| PTX | pertussis toxin |
| SNP | single nucleotide polymorphism |
| STAT5 | signal transducer and activator of transcription 5 |
| TEA | tetraethylammonium |
| TM | transmembrane |
| TRPV1 | transient receptor potential cation channel, subfamily V, member 1 |

## Summary

There is significant variation in individual response to opioid drugs, one cause of which is likely to be polymorphisms on the opioid receptors themselves. The μ-opioid receptor (MOPr) is the primary site of action for most analgesic opioids. Previous studies have identified a number of naturally occurring single nucleotide polymorphisms (SNPs) in the coding region of MOPr. The A118G SNP (N40D), present at allelic frequencies ranging from 10 – 50%, has been associated with diverse phenotypic effects as well as differences in receptor signalling in vitro, with little consistency between studies. Few studies have examined the consequences of other MOPr polymorphisms on receptor function, or potential ligand and pathway specific effects. In this study, the relative potency and efficacy of a range of clinically important and/or structurally distinct opioid ligands were assessed in Chinese hamster ovary (CHO) cells and mouse pituitary neuroblastoma (AtT-20) cells stably transfected with human wild type MOPr and 5 naturally occurring MOPr variants, N40D, A6V, L85I, R260H and R265H. MOPr surface expression levels were similar for all variants examined. MOPr activation was measured using a membrane-potential assay of adenylyl cyclase (AC) inhibition, a whole-cell ELISA of extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, and a membrane-potential assay of G protein-activated potassium channels (GIRKs). In cells expressing MOPr-N40D, buprenorphine inhibition of AC and stimulation of ERK1/2 was significantly reduced, with GIRK activation unaffected. In cells expressing MOPR-C17T, buprenorphine signalling was abolished, with a loss of potency of morphine and other ligands. AC inhibition via non-morphinan opioids was enhanced at L85I, while a significant loss of potency for many opioids was observed at R260H. There were minor alterations in the signalling profile of R265H. These results suggest that MOPr SNPs have the potential to significantly alter receptor function, and may contribute to the individual variability in response to opioid analgesics observed clinically.

## Declaration of originality

I hereby declare that the work presented in this thesis has not been submitted for a higher degree to any other university or institution. To the best of my knowledge this submission contains no material previously published or written by another person, except where due reference is stated otherwise. Any contribution made to the research by others is explicitly acknowledged at the beginning of each Chapter.

Approval from the Institutional Biosafety Committee was obtained to create the cell lines expressing the human MOR variants. These are simple genetically modified organisms that are classified as exempt dealings by the Gene Technology regulator. Biosafety approval numbers are IBC REF: 5201000742, 5201200023.

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# Chapter 1

## INTRODUCTION

Opioid analgesics are the most widely prescribed drugs in the treatment of moderate to severe pain and include drugs such as morphine and oxycodone. Despite their powerful analgesic effects, opioids are associated with a number of side effects including respiratory depression, constipation, nausea, itching and euphoria (Matthes *et al.*, 1996). Furthermore, prolonged use of opioid analgesics can cause the development of tolerance, as well as dependence in some individuals, posing a major challenge in the management of long-term or chronic pain (Moore & McQuay, 2005; Dahan *et al.*, 2010; Noble *et al.*, 2010). Over time, increasing doses of opioids become necessary to maintain analgesia, or maintain addiction in the case of opioid abusers (Buntin-Mushock *et al.*, 2005). The increased toxic side effects associated with escalating doses of opioids can reach unacceptable levels, leading to inadequate pain relief or overdose (Corbett *et al.*, 2006). Despite these limitations, morphine and other opioids are the most effective medications currently available to treat the majority of severe and chronic pain conditions, and remain the gold standard in pain relief (Hanks *et al.*, 2001). As such, the development of improved opioid analgesics with increased analgesic effects, reduced adverse effects and less ability to promote tolerance is an area of intense research.

The degree to which tolerance and adverse effects are observed in individuals varies significantly, limiting the ability of physicians to determine the appropriate dosage to balance adequate pain relief with minimal side effects and tolerance. It is likely that genetic factors contribute to differences in these parameters between individuals (Lotsch & Geisslinger, 2005; Skorpen *et al.*, 2008). The μ-opioid receptor (MOPr) is the primary site

of action for most clinically important opioid drugs, including morphine, and thus differences in MOPr function arising from genetic polymorphisms between individuals may contribute to variation in opioid response (Lotsch & Geisslinger, 2005; Mague & Blendy, 2010). An understanding of how MOPr polymorphisms affect receptor function may assist in predicting an individual's response to opioid drugs, and potentially lead to more rational and individualized pharmacotherapies, maximizing benefits and minimizing harm.

## 1.1 *Opioid Receptors*

Opium and its derivative alkaloids have been used for centuries to relieve pain and alter mood. Morphine, the prototypic opioid alkaloid, was isolated from the opium poppy in 1806, and has since been effectively used for pain relief (van Ree *et al.*, 1999). Specific opiate binding sites were proposed in 1954 (Beckett *et al.*, 1954), and first demonstrated in mammalian tissue in 1973 using radioligand binding studies (Pert & Snyder, 1973; Simon *et al.*, 1973; Terenius *et al.*, 1973). Three opiate receptor subtypes have since been cloned, μ-opioid receptor (MOPr), κ-opioid receptor (KOPr), and δ-opioid receptor (DOPr), and a fourth opioid-related nociceptin/orphanin FQ receptor (NOPr) (Martin *et al.*, 1976; Lord *et al.*, 1977; Meunier *et al.*, 1995). It has been proposed that these major opioid receptor classes can be further divided into receptor subtypes, however there is little convincing evidence for their existence (Alexander *et al.*, 2013; Connor & Kitchen, 2006; Dietis *et al.*, 2011).

Opioid receptors, together with their endogenous opioid ligands, make up a complex neuromodulatory system that plays a major role in the control of pain, motivation and reward. Activation of opioid receptors mediates a range of physiological responses such as analgesia, euphoria, feeding, immunomodulation, hormone release and stress responses, as

well as some autonomic functions including respiration, gastrointestinal motility, blood pressure and thermoregulation (Boom *et al.*, 2012; Brock *et al.*, 2012; Drolet *et al.* 2001). MOPr, DOPr, KOPr and NOPr are differentially expressed throughout the central nervous system (CNS, Mansour *et al.*, 1995, Peckys & Landwehrmeyer, 1999; Reinscheid *et al.*, 2000), the gastrointestinal tract (Sternini, 2001), and peripheral sensory and autonomic nerves (Coggeshall *et al.*, 1997). Some regions express all receptor types, such as the striatum and dorsal horn of the spinal cord, although not necessarily in the same neurons, while other regions express an abundance of one receptor type, for example KOPr in the claustrum (Mansour *et al.*, 1995). There appear to be significant species differences in opioid receptor distribution (Blackburn *et al.* 1988; Peckys & Landwehrmeyer, 1999; Rothman *et al.*, 1992; Sharif & Hughes, 1989). However, rat and human opioid receptor distribution appears to be homologous for many brain regions (Peckys & Landwehrmeyer, 1999).

The four genes that encode the opioid receptor subtypes are *OPRM1* for MOPr, *OPRD1* for DOPr, *OPRK1* for KOPr and *OPRL1* for NOPr (Pawson *et al.*, 2014). All four genes are share a high degree of sequence similarity, and are approximately 60% identical to each other (Law *et al.*, 2000; Satoh & Minami, 1995). The most highly conserved regions are the intracellular loops (85 – 100% identity) and transmembrane domains (75% identity), with greater diversity found within the extracellular N-terminus, the extracellular loops and the intracellular C-terminus (Law *et al.*, 2000; Reinscheid *et al.*, 2000; Satoh & Minami, 1995; Wei & Loh, 2011). Opioid receptors are highly conserved between species, with human opioid receptors 85-90% identical to their rodent counterparts in terms of protein sequence (Kieffer, 1995). Studies in knockout (KO) mice and rats have helped to elucidate the physiological role each of the classes of opioid receptors, and determine ligand selectivity *in vivo*. Although only one gene for each receptor type has been

identified, the diversity of effects arising from opioid receptor activation led to the hypothesis of multiple receptor subtypes. However studies in mice show abolition or a significant reduction of all opioid receptor effects when the corresponding gene is inactivated (Kieffer, 1999; Dietis *et al.*, 2011).

### 1.1.1 *MOPr*

MOPr is the main site of action for morphine and other clinically important opioid analgesics, as demonstrated by a number of studies in *OPRM1* knockout mice. In mice that do not express MOPr, morphine has no analgesic effect, regardless of the dose or route of administration (Fuchs *et al.*, 1999; Matthes *et al.*, 1996; Matthes *et al.*, 1998; Sora *et al.*, 1997; Sora *et al.*, 1999). Furthermore, morphine administration does not cause any of morphine's associated adverse effects, nor generate a reward response or the development of tolerance in knockout mice (Becker *et al.*, 2000; Contarino *et al.*, 2002; Matthes *et al.*, 1996). MOPr knockout mice also show reduced reward in response to alcohol, and in some cases an aversion (Kieffer & Gaveriaux-Ruff, 2002). In the absence of drug, knockout mice demonstrate an increased sensitivity to certain types of pain, and some behavioural differences such as increased anxiety and social withdrawal have been observed (Kieffer & Gaveriaux-Ruff, 2002; Martin *et al.*, 2003). MOPr is the most highly expressed of all the opioid receptor types, and is widely distributed throughout the CNS, with intense labelling in the locus coeruleus, striatum, thalamus and periaqueductal grey (PAG) regions of rat and human brain (Mansour *et al.*, 1994; Mansour *et al.*, 1995; Peckys & Landwehrmeyer, 1999), and the superficial laminae of the spinal cord (Mansour *et al.*, 1994; Arvidsson *et al.*, 1995). MOPr can be expressed on excitatory and inhibitory neurons, both pre- or post-synaptically, and the net effect of MOPr activation may be either an inhibitory effect on ascending pain pathways, or a disinhibitory effect on descending analgesic pathways (Taylor, 2009).

### 1.1.2 *DOPr*

Although the existence of MOPr and KOPr had already been proposed based on experiments in vivo, DOPr was the first opioid receptor to be isolated, and was extracted from mouse vas deferens (Lord *et al.*, 1977). DOPr has been associated with several physiological functions including analgesia, locomotion, reproduction and mood (Chung & Kieffer, 2013; Gaveriaux-Ruff & Kieffer, 2011; Wylot *et al.*, 2013). DOPr knockout mice show no or subtle changes in sensitivity to acute thermal, chemical or mechanical pain, but show significantly increased neuropathic and inflammatory pain, suggesting endogenous DOPr activation may modulate chronic pain (Gaveriaux-Ruff *et al.*, 2008; Gaveriaux-Ruff *et al.*, 2011; Nadal *et al.*, 2006; Pradhan *et al.*, 2013). Activation of DOPr with selective agonists such as DPDPE ([2-Dpenicillamin, 5-Dpenicillamin]-enkephalin) and deltorphin II result in analgesia, however the activity of DPDPE and deltorphin II are maintained, reduced or abolished in DOPr knockout mice depending on the assay and route of administration (Scherrer et al., 2004; Zhu et al., 1999). DOPr selective agonists also show reduced activity in MOPr knockout mice (Fuchs *et al.*, 1999; Matthes *et al.*, 1998), suggesting a functional interaction of DOPr and MOPr *in vivo* (Gomes *et al.*, 2011; Stockton & Devi, 2012). DOPr knockout mice have been shown to exhibit increased anxiety and depressive-like behaviours (Filliol *et al.*, 2000; Roberts *et al.*, 2001; Lutz & Kieffer, 2013), changes in endocrine function (Wylot *et al.*, 2013), and altered learning and reward behaviours (Le Merrer *et al.*, 2013; Nieto *et al.*, 2005).

### 1.1.3 *KOPr*

Activation of KOPr is associated with many effects including analgesia, immunomodulation, stress response and dysphoria (Liu-Chen, 2004). Studies in mice lacking the *OPRK1* gene showed no change in sensitivity to mechanical or thermal pain, but a significantly increased response to visceral pain (Simonin *et al.*, 1998). The

analgesic, hyperlocomotor and aversive effects of the KOPr selective agonist U50-488H were absent in *OPRK1* knockout mice (Simonin *et al.,* 1998). KOPr knockout mice also display an increase in generation of antibodies in response to immunisation (Gaveriaux-Ruff *et al.,* 2003), and a decrease in self-administered alcohol (Kovacs *et al.*, 2005). Activation of KOPr causes dysphoria, particularly in response to stress, and decreases reward response stimulated by other non-opioid drugs such as cocaine and ethanol (Charbogne *et al.*, 2014, Land *et al.*, 2008, McLaughlin *et al.*, 2003). KOPr agonists are therefore of particular interest as potential therapeutics for the treatment of addiction (Wee & Koob, 2010), and their ability to produce analgesia with a low potential for abuse (Wang *et al.*, 2010).

### 1.1.4 *NOPr*

NOPr has little affinity for ligands of the classical opioid receptors, and as such was characterized as belonging to the opioid receptor family a number of years after MOPr, DOPr and KOPr (Meunier *et al.*, 1995). Nociceptin, a selective endogenous NOPr agonist, stimulates a wide and often conflicting range of responses, including hyperalgesia, analgesia, allodynia, as well as anxiolytic and locomotor effects, modulation of other opioid responses and changes in learning and memory (Meis, 2003). Mice lacking the *OPRL1* gene display both increased, decreased and unchanged sensitivity to pain, depending on the assay conditions (Meunier *et al.*, 1997). Studies in NOPr knockout mice have also demonstrated both a suppressive and facilitative role for the receptor in reward and addiction to opioids and other drugs of abuse (Marquez *et al.*, 2008a; Marquez *et al.*, 2008b; Rutten *et al.*, 2011; Sakoori & Murphy, 2007), changes in locomotion and mood behaviours (Rizzi *et al.*, 2011) and both increased and unchanged anxiolytic effects (Gavioli *et al.*, 2007). The conflicting nature of many of these findings highlights the

importance of specific neuronal circuits in the physiological effects of NOPr activation (Heinricher, 2003).

## 1.2 *Opioid receptors are G-protein coupled receptors*

MOPr and the other opioid receptors were cloned in the 1990s and characterized as Type A or rhodopsin-like G-protein coupled receptors (GPCRs, (Bunzow *et al.*, 1994; Chen *et al.*, 1993; Evans *et al.*, 1992; Kieffer *et al.*, 1992; Li *et al.*, 1993; Meng *et al.*, 1993; Mollereau *et al.*, 1994; Thompson *et al.*, 1993; Yasuda *et al.*, 1993). More recently, crystal structures have been solved for MOPr (Manglik *et al.*, 2012), DOPr (Granier *et al.* 2012), KOPr (Wu *et al.*, 2012) and NOPr (Thompson *et al.*, 2012), confirming the presence of classical GPCR characteristics**.** GPCRs are the most abundant class of membrane proteins and act as cellular switches, mediating a vast array of physiological processes. All GPCRs are characterised by 7 membrane spanning helices connected by 3 intracellular loops alternating with 3 extracellular loops, as well as an extracellular N-terminus containing multiple N-glycosylation sites, and an intracellular carboxyl tail containing phosphorylation sites (Law *et al.*, 2000; Rana *et al.*, 2001). GPCRs signal via heterotrimeric guanine nucleotide binding proteins or G-proteins, which associate with the intracellular face of the receptor. G-proteins are composed of 3 subunits, Gα, Gβ and Gγ. When the receptor is in an inactive state, Gα is bound to GDP and is tightly associated with the obligate heterodimer Gβγ. Ligand binding to the extracellular surface or transmembrane domains of GPCRs induces a conformational change in the Gα subunit, facilitating exchange of GDP for GTP and causing Gα and Gβγ to dissociate. Both Gα and Gβγ are then able to couple to distinct cellular signalling pathways and effector molecules (McCudden *et al.*, 2005).

## 1.3 *MOPr Structure and G-Protein Signalling*

The recent crystallisation of mouse MOPr bound to the selective irreversible antagonist β-funaltrexamine (β-FNA) has provided a number of insights into important structural features of the receptor (Manglik *et al.*, 2012). Compared with other GPCRs, the ligand-binding pocket of MOPr is relatively exposed to the extracellular surface, which likely contributes to the rapid dissociation kinetics observed for many opioid ligands. The binding pocket is made up of elements of transmembrane (TM) domains and extracellular loops (ECLs), including TM3, TM5, TM6 and TM7, and possibly residues from ECL2 (Manglik *et al.*, 2012; Serohijos *et al.*¸ 2011). Coupling of the receptor to associated G-proteins and effector molecules is mediated by intracellular loops (ICLs), including ICL2 and ICL3, as well as by the C-terminal region (Gether, 2000). The intracellular regions, in particular the C-terminal tail, also contain a number of important phosphorylation sites that regulate receptor desensitisation, internalisation and resensitisation (Williams *et al.*, 2013).

MOPr preferentially couples to the pertussis toxin (PTX)-sensitive $G\alpha_{i/o}$ family of G-proteins, as well as the closely related PTX-insensitive $G\alpha_z$ and $G\alpha_{16}$ (Chakrabarti *et al.*, 1995; Connor & Christie, 1999; Garzon *et al.*, 1997; Laugwitz *et al.*, 1993). One of the hallmarks of MOPr signalling is the inhibition of adenylyl cyclase (AC) via $G\alpha_{i/o}$ subunits. Co-stimulation of associated Gβγ subunits activates G protein-coupled inwardly rectifying potassium channels (GIRKs) and inhibits voltage-gated calcium channels ($I_{Ca}$). Other MOPr signalling pathways include phosphorylation of mitogen-activated kinases (MAPKs), activation of phospholipase C (PLC), and the release of calcium from intracellular stores (Connor & Henderson, 1996; Law *et al.*, 2000). MOPr also signals via G-protein independent pathways, examples of which include beta-arrestin (β-arr)-mediated extracellular-regulated protein kinase 1/2 (ERK1/2) phosphorylation (Zheng *et al.*, 2008),

signal transducer and activator of transcription 5 (STAT5) phosphorylation (Mazarakou & Georgoussi, 2005), and Src-mediated Ras/Raf-1 recruitment (Zhang *et al.*, 2013).

## 1.4 *MOPr Effectors*

### 1.4.1 *MOPr inhibition of adenylyl cyclase*

One of the major effectors coupled to MOPr is AC. Activation of MOPr results in the inhibition of AC and subsequent downregulation of cAMP, an important second messenger (Connor & Christie, 1999). MOPr signalling via AC is highly complex and is AC isozyme specific (Avidor-Reiss *et al.*, 1997). Ten isoforms of AC have been identified, which are differentially regulated by various Gα proteins, Gβγ proteins and other regulatory molecules such as $Ca^{2+}$/calmodulin (Sadana & Dessauer, 2009). Acute morphine treatment inhibits $G\alpha_s$-stimulated AC5 and AC6 activity and potentiates $G\alpha_s$-stimulated AC7 acitivty (Avidor-Reiss *et al.*, 1997; Kim *et al.*, 2006; Yoshimura *et al.*, 1996). MOPr can also affect AC activity by alternate pathways. Basal AC2, 4 and 7 activity can be upregulated by PKC phosphorylation, and basal AC1 and 8 activity is stimulated by $Ca^{2+}$/calmodulin. MOPr is capable of modulating both PKC and intracellular $Ca^{2+}$, both of which were involved in the opioid stimulation of cAMP in SK-N-SH cells (Sarne *et al.*, 1998). Opioid regulation of AC isoforms differs between acute and chronic exposure (Nevo *et al.*, 2000; Schallmack *et al.*, 2006). Upregulation of AC activity following chronic opioid exposure, termed "superactivation", contributes to the development of opioid tolerance and withdrawal symptoms (Christie, 2008), and is also isozyme specific (Avidor-Reiss *et al.*, 1997).

AC catalyses the formation of cyclic adenosine monophosphate (cAMP), an important second messenger involved in a multitude of cellular processes including regulation of ion channels, release of neurotransmitters and gene transcription (Skalhegg & Tasken, 1997). One of the major actions of cAMP is the activation of cAMP-dependent protein kinase A

(PKA). PKA phosphorylates several ion channels important in nociception, including transient receptor potential cation channel, subfamily V, member 1 (TRPV1), *N*-methyl-d-aspartate (NMDA) receptor 1 (NR1) and glycine receptor α3 (GLR3, Hucho & Levine, 2007). Pronociceptive GPCRs often cause elevation of cAMP levels (Basbaum *et al.*, 2009; Hucho & Levine, 2007) and elevated cAMP levels increase neuronal excitability in animal models of acute and chronic pain (Cunha *et al.*, 1999).

cAMP can also signal independently of PKA via exchange signals activated by cAMP (EPACs). EPACs are involved in many of the physiological actions of cAMP, including maintenance of circadian rhythms, memory, wound healing and nerve regeneration (Borland *et al.*, 2009). EPACs have been shown to contribute to inflammatory pain by mediating cAMP to PKC signalling, which can in turn regulate ion channels such as TRPV1 (Griffin *et al.*, 2005; Hucho *et al.*, 2005). Although the effects of MOPr-mediated inhibition of EPACs via AC inhibition has not yet been described, EPAC mRNA and protein expression in dorsal root ganglia are increased in mouse models of neuropathic pain (Eijkelkamp *et al.*, 2013), and decreasing EPAC levels in vivo has been shown to prevent the development of chronic hyperalgesia and inflammatory pain in transgenic mice (Wang *et al.*, 2013). Another PKA-independent consequence of acute AC inhibition is the modulation of voltage-dependent ion channels ($I_h$, Ingram & Williams, 1994; Svoboda & Lupica, 1998). $I_h$ is a non-selective cation channel that is activated at hyperpolarized membrane potentials, causing an inward current that depolarizes the membrane back towards threshold. Elevated cAMP causes $I_h$ to be activated at less negative potentials, and thus the decrease in cAMP following MOPr activation reduces activation of $I_h$ channels, reducing neuronal excitability (Ingram & Williams, 1994).

### 1.4.2 *MOPr activation of potassium channels*

MOPr activation of potassium channels causes a net efflux of intracellular $K^+$ ions, resulting in membrane hyperpolarisation and subsequent inhibition of neuronal firing (Law *et al.*, 2000). MOPr-mediated hyperpolarisation of neurons was first demonstrated in guinea pig locus coeruleus neurons in 1980, although at that point the mechanism had not been defined (Pepper & Henderson). It has since been demonstrated that MOPr can activate several types of potassium channels, GIRK ($K_{ir}3$ channels), voltage-gated $K^+$ ($K_v$) channels, and possibly ATP-sensitive $K^+$ ($K_{ATP}$) channels, although direct evidence for this last interaction is lacking. GIRK channels are tetramers composed of GIRK1 – 4 subunits (Hibino *et al.* 2010). Upon stimulation of MOPr, GIRKs are activated via coupling of a Gβγ subunit to each of the GIRK subunits, resulting in efflux of intracellular $K^+$ and membrane hyperpolarisation, ultimately reducing neuronal excitability (Corey & Clapham, 2001). MOPr couples to GIRK1/2 channels expressed post-synaptically in the spinal cord (Marker *et al.*, 2005). Studies in GIRK1/2 knockout mice show a significant reduction in morphine-induced spinal antinociception (Ikeda *et al.*, 2000; Marker *et al*, 2002; Mitrovic *et al.*, 2003; Marker *et al.*, 2004), and met-enkephalin-induced hyperpolarisation of locus ceruleus neurons (Torrecilla *et al.*, 2002). DAMGO has also been shown to activate GIRKs pre- and post-synaptically in GABAergic neurons in rat periaqueductal grey (PAG, Vaughan *et al.*, 2003).

MOPr activation of $K_v$ channels causes inhibition of GABA-mediated neurotransmission, resulting in disinhibition of descending antinociceptive pathways (Vaughan *et al.*, 1997). The activation of $K_v$ channels by MOPr was first demonstrated in non-pyramidal hippocampal neurons in rats (Wimpey & Chavkin, 1991), and has also been demonstrated in rat PAG (Vaughan & Christie, 1997) and basolateral amygdala neurons (Finnegan *et al.*, 2006). The identification of specific $K_v$ channels activated by MOPr has been addressed

using knockout mice, as $K_v$ channel blockers such as 4-aminopyridine (4-AP) and tetraethylammonium (TEA) are non-selective. Mice with the $K_v1.1$ gene inactivated showed lower morphine-induced antinociception compared with wild-type mice (Galleotti *et al.*, 1997; Clark & Tempel, 1998), indicating that activation of $K_v1.1$ channels plays a role in MOPr-mediated analgesia.

Another type of inwardly rectifying $K^+$ channel regulated by GPCRs is the $K_{ATP}$ channel. Both $G_{i/o}$ α and βγ subunits are able to open $K_{ATP}$ channels (Sanchez *et al.*, 1998; Wada *et al.*, 2000). $K_{ATP}$ channel blockers such as glibenclamide and gliquidone antagonise the antinociceptive effects of morphine in rodent spinal cord, brain, and peripheral neurons, whereas $K_{ATP}$ channel openers such as diazoxide potentiate the analgesic effects of morphine (Ocana *et al.*, 2004) and fentanyl (Rodrigues *et al.*, 2005), however there is no direct evidence of MOPr activation of these channels.

### 1.4.3 *MOPr inhibition of calcium channels*

One of the inhibitory actions of opioids is the suppression of neurotransmitter release by inhibiting presynaptic $Ca^{2+}$ channels and subsequent neuronal firing in the peripheral and central nervous systems. MOPr couples primarily to voltage-gated $Ca^{2+}$ channels, including N-, L- and P/Q-type $Ca^{2+}$ channels (Law *et al.*, 2000). GPCR inhibition of $I_{Ca}$ channels is predominantly mediated by the direct coupling of Gβγ subunits to the channel, physically altering the conformation of the channel and inhibiting $Ca^{2+}$ ion influx (Dolphin, 2003). $I_{Ca}$ are composed of a considerable number of possible combinations of channel subunits, which may be differentially regulated by GPCRs (Randall, 1998). Furthermore, there are multiple subtypes of both Gβ and Gγ proteins, resulting in a vast number of possible GPCR-channel complexes (Tedford & Zamponi, 2006). Adding to the complexity of GPCR modulation of $I_{Ca}$ channels is the ability of both Gα and Gβγ to

regulate the channels via second messenger systems such as PLC and cAMP, which are modulated by MOPr (Diverse-Pierluissi *et al.*, 2000), and by G protein independent mechanisms (Iegorova *et al.*, 2010). The functional coupling of MOPr to N-type and P/Q-type $I_{Ca}$ has been observed in rat spinal DRG (Schroeder & McCleskey, 1993; Rusin & Moises, 1995; Schroeder *et al.*, 1991), as well as a number of brain regions including rat LC (Connor *et al.*, 1999a; Ingram *et al.*, 1997), mouse PAG (Connor *et al.*, 1999b; Kim *et al.*, 1997), and in heterologous expression systems (Seward *et al.*, 1991; Borgland *et al.*, 2003; Morikawa *et al.*, 1995).

### 1.4.4 *MOPr release of intracellular calcium*

Alteration of the intracellular concentration of $Ca^{2+}$ ($[Ca^{2+}]_i$) is a key regulator of many cellular processes. In addition to preventing the elevation of $[Ca^{2+}]_i$ by inhibiting $I_{Ca}$, MOPr activation can also elevate $[Ca^{2+}]_i$ by mobilising $Ca^{2+}$ from intracellular stores in the endoplasmic reticulum (ER, Samways & Henderson, 2006). In some expression systems MOPr activation alone can stimulate $Ca^{2+}$ release from ER (Harrison *et al.*, 1999; Hauser *et al.*, 1996; Zimprich *et al.*, 1995), but in many other instances MOPr potentiates the $Ca^{2+}$ mobilisation stimulated by co-activation of $G_q$ coupled receptors (Connor & Henderson, 1996; Okajima *et al.*, 1993). The mechanism for this receptor cross-talk is not fully understood, but is likely to involve MOPr mediated modulation of PLC and inositol (1,4,5) triphosphate ($IP_3$, Werry *et al.*, 2003; Zimprich *et al.*, 1995).

Activation of MOPr usually results in a suppression of neurotransmitter release, due to inhibition of $Ca^{2+}$ cellular influx (Christie *et al.*, 2000). On the other hand, the elevation of $[Ca^{2+}]_i$ resulting from intracellular $Ca^{2+}$ mobilisation may be sufficient to stimulate neurotransmitter release (Xu & Gintzler, 1992). Other studies have shown that elevation of $[Ca^{2+}]_i$ is important in mediating morphine induced analgesia. PLC$\beta_3$ knockout mice

show a reduced response to morphine (Xie *et al.*, 1999), and when various components of the $IP_3$ pathway are blocked or inhibited, mice also show a reduced sensitivity to opioid induced analgesia (Aoki *et al.*, 2003; Narita *et al.*, 2000). MOPr elevation of $[Ca^{2+}]_i$ can also activate MAP kinases, which are involved in a wide range of cellular processes (Law *et al.*, 2000).

### 1.4.5 *MOPr and the MAP kinase cascade*

As well as the regulation of cellular signalling and neurotransmission, in recent years it has become apparent that opioid receptors are intimately involved with many other cellular signalling processes such as cell proliferation, differentiation and apoptosis. In many cases, GPCR control of these processes is via activation of mitogen-activated kinases (MAPKs; Luttrell, 2002). The 3 major classes of mammalian MAPKs are the extracellular-signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs) and the p38 kinases (p38MAPKs; Law *et al.*, 2000). All 3 classes can be stimulated, i.e. phosphorylated following activation of $G_i$ G-proteins via multiple signalling pathways involving both Gα and Gβγ-coupled mechanisms. $G\alpha_i$ can activate ERK1/2 via $G\alpha_i$-mediated inhibition of AC, which decreases the inhibitory effect of PKA on c-Raf. The Ras-c-Raf signalling module is then able to activate ERK1/2 via phosphorylation by MEK1/2 (Goldsmith & Dhanasekaran, 2007). Gβγ has also been shown to stimulate ERK1/2 directly via Ras-dependent pathways, although the mechanism by which Gβγ couples to Ras varies between receptors, cell types and assay conditions (Luttrell, 2002). ERK1/2 can be activated via G-protein independent mechanisms involving β-arrestin (Macey *et al.*, 2006; Shenoy & Lefkowitz, 2005; Zheng *et al.*, 2008), and G-protein receptor kinases (GRKs, Macey *et al.*, 2006). MOPr stimulation of ERK1/2 has been observed in a number of heterologous expression systems via various signalling mechanisms, including activation of phospholipase C (PLC), phosphatidylinositol-3 kinase (PI3K), epidermal growth factor

receptor (EGFR) transactivation, and intracellular $Ca^{2+}$ release (Law *et al.*, 2000). The ability of MOPr to activate ERK1/2 varies with cell type (Mouledous *et al.*, 2004; Trapaidze *et al.*, 2000), and ERK1/2 regulation also differs with acute and chronic MOPr activation (Bilecki *et al.*, 2005; Mouledous *et al.* 2004). ERK1/2 signalling is intimately involved in the long-term cellular adaptations to chronic MOPr stimulation, although whether MOR activates these pathways directly is unclear (Christie, 2008; Polakiewicz *et al.*, 1998; Tan *et al.*, 2003b).

There is some evidence for the involvement of JNKs and p38MAPKs in MOPr signalling, with some studies showing JNKs are activated in response to MOPr stimulation (Kam *et al.*, 2004; Melief *et al.*, 2010). Another study reported increased MOPr expression via p38MAPks when JNKs were inhibited (Wagley *et al.*, 2013). JNKs have also been implicated in the development of MOP tolerance (Melief *et al.*, 2010), but not desensitisation (Levitt & Williams, 2012).

### 1.4.6 *Other MOPr effectors*

The signalling cascades activated by MOPr are complex, and involve many additional effector molecules including protein kinase B (Akt). Akt mediates cellular processes such as growth, survival, and glucose uptake, and in the case of opioid signalling, is thought to play a neuroprotective role (Muller *et al.*, 2004). MOPr activation has been shown to cause an increase of phosphorylated Akt (pAkt) via PI3K mediated pathways in CHO cells and in a brain-region specific manner in rat brain. More recently, several novel signalling pathways have been identified for MOPr, including STAT5 phosphorylation (Mazarouki & Georgoussi, 2005) and Src-mediated Ras/Raf-1 recruitment (Zhang *et al*, 2013) in heterologous expression systems. In both of these studies, activated MOPr was converted into a receptor tyrosine kinase-like entity. Ligand-binding to MOPr caused

phosphorylation of the conserved AA motif at residues 336-339. The phosphorylated motif was shown to be a docking site for the direct binding and phosphorylation of STAT5, which regulates gene transcription (Mazarouki & Georgoussi, 2005). Phosphorylated $Tyr^{336}$ also served as a docking site for the small regulatory molecule son-of-sevenless (Zhang 2013). MOPr-mediated phosphorylation son-of-sevenless caused recruitment and activation of Ras/Raf1, which in turn was shown to upregulate AC activity (Zhang 2013).

### 1.4.7 *MOPr desensitisation and endocytosis*

Acute MOPr stimulation causes rapid desensitisation and internalisation of the receptor, and chronic MOPr activation results in long-term cellular adaptations that ultimately lead to opioid tolerance (Christie, 2008). The development of desensitisation and tolerance following receptor activation is a characteristic of GPCRs. A generally accepted mechanism for the rapid desensitisation of GPCRs is receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and subsequent β-arrestin binding. In this model, GRKs are recruited by Gβγ and phosphorylate agonist occupied GPCRs, causing recruitment and binding of β-arrestins to the phosphorylated receptor, uncoupling it from associated G-proteins and thus preventing further signalling (Kelly *et al.*, 2008). The receptor/arrestin complex is then trafficked to clathrin-coated pits and internalised, whereupon it is either recycled to the plasma membrane or else targeted to lysosomes for degradation (Qiu *et al.*, 2003). Receptor endocytosis is thought to contribute directly to MOPr desensitisation by reducing the number of available receptors at the plasma membrane, yet morphine, which is well known to produce tolerance, does not appear to induce significant levels of MOR endocytosis, suggesting additional pathways for the development of receptor desensitisation and tolerance (Alvarez *et al.*, 2002).

The third intracellular loop and the C-terminal tail of GPCRs are important domains for the regulation of receptor phosphorylation, desensitisation and internalisation (Lefkowitz, 1998). Hierarchical, multi-site phosphorylation of the C-terminal domain is required for receptor endocytosis (El Kouhen *et al.,* 2001). While morphine induces phosphorylation at the S375 residue of the C-terminal domain, DAMGO and other agonists that can efficiently stimulate receptor endocytosis cause subsequent phosphorylation of the T370 residue, which in turn results in endocytosis (Doll *et al.,* 2011, Grecksch *et al.,* 2011). The mechanism for receptor desensitisation also appears to be ligand-dependent. DAMGO has been shown to induce MOPr desensitisation via the GRK/β-arrestin dependent pathway, whereas morphine does not, except in the case of GRK2 overexpression (Zhang *et al.*, 1998). Morphine seems able to induce desensitisation in the absence of β-arrestin, via pathways involving PKC (Bailey *et al.*, 2009; Chu *et al.*, 2008; Chu *et al.*, 2010; Johnson *et al.*, 2006). There is some evidence to suggest that the desensitisation pathways activated are directly related to efficacy of the ligand at the receptor, that is, morphine is unable to activate a sufficient number of Gβγ subunits to recruit GRKs to the receptor (Connor *et al.*, 2004; Hull *et al.,* 2010; McPherson *et al.*, 2010). However, the efficacy of opioid ligands in activating G-proteins has been shown to be distinct from their ability to promote MOPr endocytosis (Borgland *et al.,* 2003). Furthermore, there is additional evidence that morphine binding to MOPr results in a specific ligand/receptor conformation that PKC recognizes and is able to bind to (Ingram & Traynor, 2009). Although the mechanisms for activation of specific MOPr endocytotic and desensitisation pathways are not well understood, it is clearly a more complex system than one based solely on ligand efficacy, and may be dependent on specific receptor conformations induced by individual ligands.

## 1.5 *MOPr Ligands*

### 1.5.1 *Endogenous MOPr ligands*

Shortly after the discovery of opioid receptors, the endogenous opioids met- and leu-enkephalin were characterized (Hughes *et al.*, 1975), followed by β-endorphin (Li & Chung, 1976) and the dynorphins (Goldstein, 1979). Since then, a number of endogenous opioid peptides have been identified. Most of these originate from one of three distinct precursor proteins; proenkephalin (pEnk), prodynorphin (pDyn), and proopiomelanocortin (POMC, Akil *et al.*, 1984). Endogenous opioids derived from these precursors bind to MOPr, DOPr and KOPr with varying affinities, however have no affinity for NOPr (Pawson *et al.*, 2014). pEnk gives rise to met- and leu-enkephalin, which show highest affinity for DOPr, lower affinity for MOPr, and little affinity for KOPr. POMC encodes β-endorphin as well as other non-opioid peptides like adrenocorticotropic hormone (ACTH; Akil *et al.*, 1984). β-endorphin couples to MOPr and DOPr, with low affinity for KOPr. pDyn contains coding for the dynorphins, which preferentially bind to KOPr, but also couple to MOPr and DOPr (Pawson *et al.*, 2014). The highly selective MOPr agonists endomorphin-1 (EM1) and endomorphin-2 (EM2) have been isolated from various tissues (Wang *et al.*, 2002b; Zadina *et al.*, 1997), however the coding gene and precursor protein have yet to be identified. It is thus unclear as to whether EM1 and EM2 are produced endogenously. The principle endogenous ligand for NOPr is nociceptin or orphanin FQ (N/OFQ), which is derived from prepronociceptin. N/OFQ does not act at any of the classical opioid receptors, nor is it antagonized by naloxone (Pawson *et al.*, 2014).

### 1.5.2 *Exogenous MOPr ligands*

The archetypal MOPr alkaloid agonist is morphine. Morphine has approximately 50 times higher affinity for MOPr than DOPr, and has been shown to have both full and partial agonist activity, depending on assay conditions (Pawson *et al.*, 2014). The morphine

metabolite, morphine-6-gluconuride (M6G) is biologically active (Yoshimura *et al.*, 1973). M6G has a similar affinity to morphine for MOPr, but has been shown to have a higher agonist efficacy than morphine (Osborne *et al.*, 2000). A number of structurally related, semisynthetic morphine derivatives including buprenorphine and oxycodone have been developed, as well as fully synthetic morphine analogues such as methadone. Non-morphinan synthetic alkaloids include the highly efficacious anilidopiperidine fentanyl (Pawson *et al.*, 2014). D-Ala2-MePhe4-Glyol5-enkephalin (DAMGO) is a synthetic peptide that was designed to be a biologically stable analogue of the DOPr-preferring enkephalins, but has a high affinity and selectivity for MOPr (Dhawan *et al.*, 1996). MOPr antagonists include naloxone, which has highest affinity for MOPr, but also binds to DOPr and KOPr, and naltrexone, which has little selectivity for MOPr. The MOPr antagonists with highest selectivity are cyclic peptides related to somatostatin such as CTOP and CTAP (Dhawan *et al.*, 1996).

### 1.5.3 *Ligand affinity and efficacy*

The action of ligands at a receptor can be measured by two separate properties: the ability of the ligand to bind to the receptor, termed ligand affinity, and the ability of the ligand to elicit a physiological effect at the receptor, termed ligand efficacy. Ligand affinity is commonly measured using radioligand-binding assays to determine the concentration of ligand that occupies 50% of the receptor at equilibrium. Ligand "efficacy" is generally measured using concentration-response curves (CRCs) for a particular effector pathway, e.g. cAMP accumulation. However, there is some confusion over the use of the term "efficacy". Technically, ligand efficacy refers to the ability of the ligand to activate the receptor and elicit a cellular response (Kelly, 2013). The maximum response that a ligand can elicit is tissue-dependent due to a range of factors including differing levels of g proteins or other effector molecules. Thus, measuring the maximum effect of a ligand at a

specific signalling pathways is actually a measure its "intrinsic activity" rather than its efficacy, as two ligands may have a similar maximum response in a particular system or tissue, with very different values of efficacy. Nevertheless, the terms "efficacy" and "intrinsic activity" are both widely and interchangeably used. Throughout this thesis, the term "efficacy" has been used rather than intrinsic activity to describe the maximum effect of a ligand for each particular system. Full agonists are ligands that elicit the maximum response possible for the system. Partial agonists will cause a sub-maximal response of the system even when all receptors are occupied by ligand. Antagonists are ligands that have affinity for a receptor without causing an observable effect, and inverse agonists bind to receptor and inhibit its constitutive activity (Costa & Herz, 1989) The efficacy of a ligand is tissue-dependent, as receptor expression and the available pool of G-proteins and effector molecules can vary between cell types (Atwood *et al.*, 2011). Furthermore, it is now apparent that ligands vary in their ability to stimulate specific signalling pathways, and thus ligand efficacy is also pathway dependent (Kenakin & Miller, 2010).

## 1.6 *Ligand-biased signalling at MOPr*

The early conception of GPCR signalling was that these receptors acted as simple on/off switches with ligand efficacy essentially linear, i.e. after receptor activation, an agonist would have a similar efficacy across the full spectrum of GPCR effectors (Karlin, 1967; Samama *et al.*, 1993). It was thought that GPCRs oscillated between a single active and inactive state, and that ligands only differed in their ability to stabilise the active state. Differences in the pharmacological profile of various drugs were attributed to differences in tissue characteristics, as well as the specific kinetics and metabolism of the drug. Attempts to discover new GPCR drugs with improved pharmacological profiles were focused on designing highly selective compounds for receptor subtypes with distinct anatomical distribution or pharmacological properties. It is now known that GPCRs

continually oscillate through a range of active and inactive states, each of which may be differentially stabilized by receptor agonists and antagonists (Kenakin & Miller, 2010). Receptor ligands can bind with high or low affinity to particular conformations of the receptor, and the resulting ligand/receptor complexes may vary in their ability to couple to different G-protein subtypes and other effectors such as β-arrestin. This in turn can result in preferential activation of a subset of receptor signalling pathways (Ghanouni *et al.*, 2001). There is a growing body of evidence demonstrating ligand-directed activation of a range of GPCRs (Evans *et al.*, 2011; Rajagopal *et al.*, 2013), including MOPr (DeWire *et al.*, 2013; Pineyro *et al.*, 2007). Plasmon-waveguide resonance (PWR) spectroscopy has demonstrated that DOPr agonists, antagonists and inverse agonists stabilise the receptor in different conformations, furthermore, that that the conformations stabilised are ligand-specific (Alves *et al.*, 2003; Salamon *et al.*, 2002). MOPr agonists have been shown to differ in their ability to stimulate various G-protein subtypes in HEK293 cells. Morphine and buprenorphine activated $G\alpha_{oA}$ to a greater extent than $G\alpha_{i1}$, whereas DAMGO, fentanyl, met-enkephalin, leucine-enkaphalin and methadone activated $G\alpha_{i1}$ to the same or greater extent than $G\alpha_{oA.}$ The affinities of the agonists for the G-protein subtypes also differed, with DAMGO, E2 and morphine showing significantly greater potencies at $G\alpha_{oA,}$ and E1 showing greater affinity for $G\alpha_{i1}$ (Saidak *et al.*, 2006). Agonist selectivity for G-protein activation has been demonstrated in vivo, with some opioid drugs producing different levels of analgesia in $G\alpha_{i1}$ and $G\alpha_{oA}$ knockdown mice (Sanchez-Blanquez *et al.*, 1999). A topic of particular interest at present is the identification of ligands biased towards either G-protein coupled pathways or G-protein independent pathways such as β-arrestin recruitment (Violin & Lefkowitz, 2007). Studies in mice injected with antisense $G\alpha_{i}$ oligodeoxynucleotides showed an attenuation of the antinociceptive effects of morphine (Raffa *et al.*, 1994; Sanchez-Blanquez *et al.*, 1993; Sanchez-Blanquez *et al.*, 1995), whereas β-arrestin KO mice show a reduction in the adverse effects associated with

morphine (Raehal & Bohn, 2011; Raehal *et al.*, 2005). This has led to the hypothesis that the desirable characteristics of opioids such as analgesia are mediated by G protein-coupled pathways, while the adverse effects such as tolerance and dependence are mediated via β-arrestin coupled pathways. As such, current efforts to design improved opioid drugs are often focused on developing ligands with significant bias towards G-protein coupled pathways (DeWire *et al.*, 2013). Although the connection between specific ligand/receptor conformations, differential G-protein and β-arrestin activation, and altered signalling has not yet been demonstrated conclusively for MOPr, it is a reasonable supposition that the different effects elicited by various MOPr ligands are due at least in part to differential activation of MOPr effectors.

## 1.7 *OPRM1 Polymorphisms*

A major limitation of opioid prescription for the treatment of pain is the highly variable response observed between individuals, both in the analgesic and adverse effects (Skorpen *et al.*, 2008). The risk associated with potentially serious side-effects such as respiratory depression can limit opioid dosing resulting in inadequate pain relief for many individuals (Boom *et al*., 2012). Furthermore, some patients will respond to certain opioid drugs and not others, for example many patients who do not respond to methadone treatment for opioid dependence may respond to buprenorphine (Gerra *et al.*, 2014). Several parameters contribute to individual response to opioids, such as drug absorption, distribution and metabolism, as well as the intrinsic efficacy of the drug at the receptor. Genetic factors are likely to play a role in variability of all of these parameters. A number of polymorphisms in the MOPr gene, *OPRM1,* occur naturally within the population. *OPRM1* single nucleotide polymorphisms (SNPs) have been identified in the promoter, intron and coding regions, some of which result in an amino acid change and are associated with functional consequences in vivo or in vitro (Lotsch & Geisslinger, 2005). One SNP of particular

interest clinically is the N40D variant, which occurs at relatively high frequencies ranging from 10 – 50% in various populations (Mura *et al.*, 2013). This SNP, an A > G nucleotide substitution at position 118 in exon 1 of *OPRM1*, causes an asparagine to aspartic acid exchange at residue 40 on the extracellular N-terminal domain of MOPr, removing a putative glycosylation site (Singh *et al.*, 1997). This SNP was first identified by Bergen *et al.*, (1997), and in 1998, Bond *et al.* reported that β-endorphin had a threefold higher affinity for MOPr-D40 expressed in AV-12 cells and a threefold higher potency for GIRK activation in *Xenopus* oocytes, although subsequent studies have failed to replicate these findings (Befort *et al.*, 2001; Beyer *et al.*, 2004; Kroslak *et al.*, 2007). Since then a large number of studies have examined associations between the N40D variant and a plethora of clinical effects. Carriers of the G118 allele showed an increased requirement for morphine or fentanyl to treat cancer pain (Campa *et al.*, 2008; Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007) and post-operative pain (Chou *et al*, 2006a; Chou *et al.*, 2006b; Fukuda *et al.*, 2010; Sia *et al.*, 2008; Tan *et al.*, 2009; Zhang *et al.*, 2010; Zhang *et al.*, 2011), and in some cases the associated adverse effects such as nausea were also reduced (Sia *et al.*, 2008; Tan *et al.*, 2009; Tsai *et al.*, 2010). Analgesia, pupillary constriction and nausea were reduced in response to M6G in G118 carriers (Lotsch *et al.*, 2002; Romberg *et al.*, 2005; Skarke *et al.*, 2003). Overall, these data point to a reduced opioid response in G118 carriers, however a recent meta-analysis concluded that the association is weak, applies to homozygous carriers only, and is of little clinical relevance (Walter & Lotsch, 2009).

Several studies have reported an increased susceptibility to heroin abuse and/or dependence in carriers of the G118 allele (Bart *et al.*, 2004; Drakenburg *et al.*, 2006; Kapur *et al.*, 2007; Szeto *et al.*, 2001), while others have found no association or a protective effect of G118 (Bond *et al.*, 1998; Glatt *et al.*, 2007; Shi *et al.*, 2002; Tan *et al.*, 2003a). Similarly, studies examining association between the G118 allele and the risk of alcohol

dependence have reported a positive association (Bart *et al.*, 2005; Miranda *et al.*, 2010; Nishizawa *et al.*, 2006; Rommelspacher *et al.*, 2001), a negative association (Du & Wan, 2009; Town *et al.*, 1999), and no association (Bergen *et al.*, 1997; Gscheidel *et al.*, 2000; Kim *et al.*, 2004a; Loh *et al.* 2004). However, carriers of the G118 allele appear to consistently show an increased response to alcohol as measured by subjective reports of positive affect (Miranda *et al.*, 2010; Ray & Hutchison, 2004; Ray & Hutchison, 2007; Ray *et al.*, 2010), cue-induced alcohol cravings (Van den Wildenberg *et al.*, 2007; Wiers *et al.*, 2009), and PET scans of striatal dopamine release (Ramchandani *et al.*, 2011). The endogenous opioid system is involved in the rewarding properties of alcohol, and to this end MOPr antagonists have been used as a pharmacological treatment of alcohol dependence (Thorsell, 2013). Carriers of G118 reported significantly decreased alcohol induced euphoria when treated with naltrexone compared with A/A individuals (Setiawan *et al.*, 2011). Meta-analyses of studies examining the effectiveness of naltrexone treatment for alcoholism have suggested carriers of G118 may respond more favourably with lower relapse rates, although these findings are not consistent (Chamorro *et al.*, 2012; Coller *et al.*, 2011; Thorsell, 2013).

The apparent differences in the response of G118 carriers to alcohol may be mediated in part by changes in activation of the hypothalamic-pituitary-adrenal axis (HPA). The endogenous opioid system and the HPA axis are activated in response to alcohol consumption (Gianoulakis, 1998), as well as stress (Drolet *et al.*, 2001). Studies examining the association between N40D and activation of the HPA axis have shown an increased cortisol response to naloxone (Chong *et al.*, 2006; Hernandez-Avila *et al.*, 2007; Wang *et al.*, 2002a), a dampened adrenocorticorticotropin (ACTH) response to stress (Ducat *et al.*, 2013), and increased basal cortisol levels with no changes in ACTH (Hernandez-Avila *et al.*, 2003). Taken together, these results are suggestive of increased cortisol activity in

G118 carriers, possibly contributing to stress-induced alcohol consumption and improved response to naltrexone therapy for alcoholism.

Another less common *OPRM1* SNP has been identified within the N-terminal region of MOPr. The C17T variant, resulting in an alanine to valine amino acid substitution at aa6 on the N-terminal tail, is quite rare in the overall population but can reach allelic frequencies of more than 20% in some Indian and African populations (Crowley *et al.*, 2003; Kapur *et al.*, 2007). This variant has been associated with the risk of alcohol, cocaine, tobacco but not opioid use in African-American women (Crystal *et al.*, 2012). Similarly, other studies have demonstrated a trend towards a higher frequency of T17 carriers in substance abusers, however the small sample sizes of the studies, as well as population-dependent nature of the frequency of the SNP makes it difficult to draw any conclusions from these results (Berrettini *et al.*, 1997; Rommelspacher *et al.*, 2001; Compton *et al.*, 2003; Crowley *et al.*, 2003). There is no clinical information relating to other, less common MOPr polymorphisms, however some functional differences have been observed in receptor signalling (see Chapter 2, Knapman & Connor, 2014).

## 1.8 *MOPr variants and differential signalling*

The idea that specific properties of ligands may stabilise receptor conformations that preferentially activate particular signalling pathways can be expanded to include properties of the receptor as well. Genetic polymorphisms causing amino acid changes may cause subtle differences in the structural flexibility of the receptor, resulting in a set of possible receptor conformations that differ to those exhibited by the wild-type receptor, both in the agonist-occupied and unoccupied states. This idea provides a mechanism by which genetic variation between individuals could translate to variation in response to opioid analgesics.

Differences in receptor function have been observed in MOPr receptor variants, however much of the evidence is contradictory and inconclusive, possibly due in part to differences in cellular expression systems, the signalling pathway and ligands assayed, as well as the failure of many of studies to take into account receptor reserve. Although no conclusive evidence is available as to the effect of MOPr polymorphisms on individual response to opioid analgesics, these findings suggest that genetic variation in the MOR may contribute to the highly variable response and warrant further investigation. Chapter 1 has described MOPr function and signalling, particularly in the context of changing conformational states arising from interaction between the receptor and distinct ligands. In the next chapter, my published review in the *British Journal of Pharmacology* extends this and discusses what has been reported about the functional effects of MOPr SNPs.

# Chapter 2

## Cellular signalling of non-synonymous single nucleotide polymorphisms of the human μ-opioid receptor

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## Summary

There is significant variability in individual responses to opioid drugs, which is likely to have a significant genetic component. A number of non-synonymous single nucleotide polymorphisms (SNPs) in the coding regions of the μ-opioid receptor (MOPr) gene (OPRM1) have been postulated to contribute to this variability. Although many studies have investigated the clinical influences of these MOPr variants, the outcomes are reported in the context of thousands of other genes and environmental factors, and we are no closer to being able to predict individual response to opioids based on genotype. Investigation of how MOPr SNPs affect receptor expression, coupling to second messengers, desensitisation and regulation is necessary to understand how subtle changes in receptor structure can impact individual response to opioids. To date, the few functional studies which have investigated the consequences of SNPs on the signalling profile of the MOPr *in vitro* have shown that the common N40D variant has altered functional responses to some opioids, while other, rarer, variants display altered signalling or agonist-dependent regulation. Here, we review the available data on the effects of MOPr polymorphisms on receptor function, expression and regulation *in vitro*, and discuss the limitations of the studies to date. Whether or not MOPr SNPs contribute to individual variability in opioid responses remains an open question, in large part because we have relatively little good data about how the amino acid changes affect MOPr function.

Keywords: A118G; pharmacogenomics, analgesia, addiction, tolerance, dependence

Abbreviations

| | |
|---|---|
| aa | amino acid |
| AC | adenylyl cyclase |
| β-CNA | β-chlornaltrexamine |
| CaM | calmodulin |
| cAMP | cyclic adenosine monophosphate |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| CRE | cAMP response element |
| DAMGO | ([D-$Ala^2$, N-$MePhe^4$, Gly-ol]-enkephalin) |
| ECL | extracellular loop |
| ELISA | enzyme-linked immunosorbent assay |
| ERK | extracellular signal regulated kinase |
| GIRK | G protein gated, inwardly rectifying potassium channel |
| GRK | G protein coupled receptor kinase |
| hMOPr | human μ-opioid receptor |
| HPA | hypothalamic-pituitary-adrenal |
| $I_{Ca}$ | voltage gated Ca channels |
| ICL | intracellular loop |
| M-6-G | morphine-6-glucuronide |
| MAP kinase | mitogen activated protein kinase |
| MOPr | μ-opioid receptor |
| mRNA | messenger ribonucleic acid |
| *OPRM1* | opioid receptor mu 1 |
| pERK | phosphorylated ERK1/2 |

| | |
|---|---|
| PKA | protein kinase A |
| PTX | pertussis toxin |
| SNP | single nucleotide polymorphism |
| STAT5 | signal transducer and activator of transcription 5 |
| TM | transmembrane |

***Introduction***

Opioid analgesics are the most important classes of drug used for the treatment of moderate to severe pain. Opioids elicit powerful analgesic effects, yet they are also associated with a number of adverse effects such as respiratory depression, constipation, nausea and sedation (Moore & McQuay, 2005; Dahan *et al.,* 2010; Noble *et al.,* 2010). The development of tolerance to opioid analgesia, coupled with the associated adverse effects, limits the usefulness of opioid therapy in the treatment of long-term and chronic pain. Opioid misuse is also a major social problem in many countries (Dhalla *et al.*, 2011)**.**

There is significant variation between individuals in both the analgesic effect of opioid drugs and the degree of adverse effects experienced. The risk of serious adverse events such as respiratory depression can limit dosing with the result that many individuals receive inadequate pain relief (Skorpen & Laugsand, 2008). Furthermore, as tolerance develops over time, the escalating opioid doses that are required to maintain adequate analgesia can cause intolerable side effects (Corbett *et al.,* 2006). There is also an apparently heritable predisposition towards opioid abuse and addiction (Merikangas *et al.*, 2008). A number of elements may affect final individual response to opioids including drug absorption, distribution and metabolism, as well as the intrinsic efficacy of the drug at the receptor and variation in receptor signalling function, agonist regulation and downstream effector pathways. Genetic factors such as differences in protein sequence, regulatory element function and potentially complex epigenetic regulation of protein expression contribute to variability in all these parameters (Lotsch *et al.,* 2005; Skorpen & Laugsand, 2008). Understanding these components could result in the ability to better predict clinical outcomes when prescribing opioid analgesics, reducing the number of patients receiving an inappropriate dose of opioid by potentially limiting the development

of tolerance and dependence. Rational dosing would also likely increase the number of patients who benefit from opioid therapy.

Clinically important opioid analgesics act by binding to the μ-opioid receptor (MOPr) (Matthes *et al.*, 1996; Alexander *et al.*; 2011), making MOPr a prime candidate for contributing to the genetic component of inter-individual differences in opioid response. The MOPr is a typical Class A G protein coupled receptor (GPCR, Alexander *et al.*; 2011). Many single nucleotide polymorphisms (SNPs) within the *ORPM1* gene have been identified in humans, and a number of these are non-synonymous changes in the coding regions, meaning that there is an amino acid (aa) substitution resulting in an alternative receptor isoform (LaForge *et al.*, 2001; Ikeda et al., 2005; Lotsch *et al.*, 2005; Ravindranathan *et al.*, 2009; Fortin *et al.*, 2010). There are good reasons to consider the potential of MOPr SNPs to contribute to the clinical variability of opioid responses. GPCR signalling is complex, with the notion of simple, linear and robust re-arrangements of protein structure being required for signal transduction no longer accepted. Thus, the possibility that single amino acid substitutions can lead to subtle or profound changes in the way receptors signal is very real, and has been demonstrated for several GPCRs (Thompson *et al.*, 2008; Zhang *et al.*, 2013a). Further, commonly prescribed opioids such as morphine and buprenorphine have relatively low efficacy, and even modest differences in receptor expression or efficiency of signal transduction could have a significant impact on individual response to these drugs. Finally, clinically used opioids are chemically diverse, and are likely to have subtly different structural features of the MOPr determining their signalling – potentially leading to distinct effects of non-synonymous SNPs on different drugs.

An additional level of complexity when considering the functional consequences of SNPs arises from the large number of putative splice variants of MOPr which have been described (Mizoguchi *et al.*, 2012; Pasternak & Pan, 2013). Although the functional role of alternatively spliced *OPRM1* transcripts is not yet well established, a single non-synonymous amino acid change could conceivably have distinct effects on different splice variants of the receptor. For the most part, this remains unexplored.

Many studies have examined potential associations between MOPr SNPs and various clinical outcomes, such as the degree of pain relief in response provided by opioids or the prevalence of substance abuse. These clinical reports are often contradictory and there is no clear consensus as to the effect of any polymorphism on disease susceptibility or the outcomes of drug administration. This is presumably in part due to relatively small sample sizes in most studies, as well as a range of confounding influences such as overall genotype and environment (reviewed in Lotsch *et al.*, 2005). Far fewer studies have investigated the molecular consequences of *OPRM1* SNPs on receptor function and signalling *in vitro*, and results from these studies are also conflicting. Nevertheless, *in vitro* experiments have led to intriguing insights into MOPr function, and in this review we focus on the effects of naturally occurring, non-synonymous SNPs in the coding region of *OPRM1* on MOPr function. The SNPs considered here, the corresponding amino acid exchanges and their position on the MOPr are summarized in Table 1 and Figure 1.

***The μ-opioid receptor***

The MOPr is a Class A rhodopsin-like GPCR, with a relatively short extracellular N-terminal domain (66 aa), 7-membrane spanning domains and an intracellular carboxy-terminal "tail" (70 aa) that includes a putative "helix 8" domain tethered to the plasma membrane by a palmitoyl residue (Manglik *et al.*, 2012). Opioid ligands are thought to

approach the receptor from the extracellular space, engaging with the receptor by interacting with a binding pocket formed by elements of transmembrane (TM) domains TM3, TM5, TM6 and TM7, and possibly residues in extracellular loop (ECL) 2 (Serohijos *et al.*, 2011; Manglik *et al.*, 2012). G-protein interactions are mediated through intracellular domains, including intracellular loops (ICL) 2 and 3, and the C-terminal region. The intracellular regions of MOPr, particularly the C-terminal domain, also contain important phosphorylation sites regulating receptor desensitisation, internalisation and resensitisation (Williams *et al.*, 2013).

MOPR mutation diagram

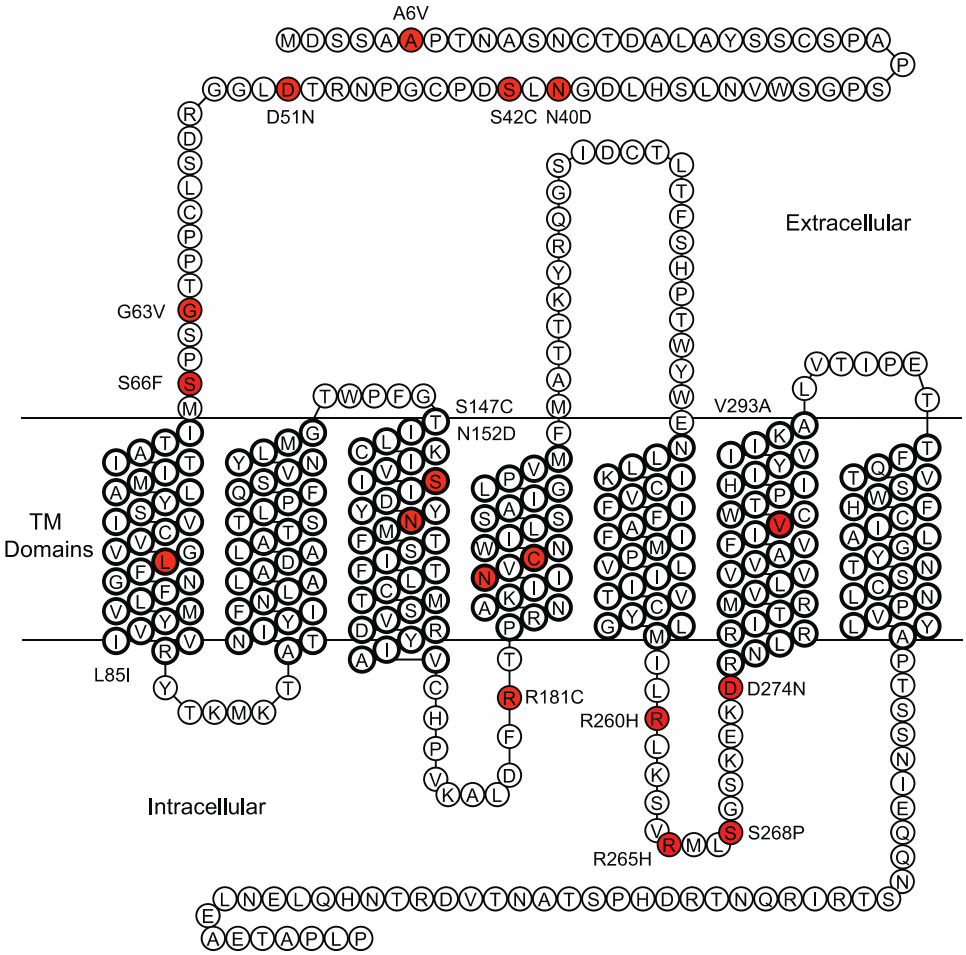


**Figure 1:** Naturally occurring, non-synonymous *OPRM1* variants reported, and their position on the MOPr protein. Residues where an amino acid exchange occurs are indicated in red.

The MOPr modulates a diverse range of physiological systems, including nociception and analgesia, reward and euphoria, immune function, stress responsivity, respiration and gut motility (Jordan & Devi, 1998; Kreek *et al.,* 2005). The most well characterized signalling pathways of MOPr proceed via activation of heterotrimeric G proteins or β-arrestin (Law *et al.* 2000). MOPr can couple to a number of different G proteins, including pertussis-toxin (PTX) sensitive $G\alpha_{i/o}$ subunits, the closely related $G\alpha_{z,}$ and $G\alpha_{16}$ (Connor & Christie, 1999). Canonical coupling of MOPr includes $G\alpha_{i/o}$ inhibition of adenylyl cyclase (AC), Gβγ subunit activation of G protein-coupled inwardly rectifying potassium channels (GIRKs) and inhibition of voltage gated $Ca^{2+}$ channels ($I_{Ca}$), as well as activation of MAP kinases. Examples of G protein-independent signalling of MOPr include β-arrestin-mediated extracellular signal regulated kinase 1/2 (ERK1/2) activation (Zheng *et al,* 2008), signal transducer and activator of transcription 5 (STAT5) phosphorylation (Mazarakou & Georgioussi, 2005) and Src-mediated Ras/Raf-1 recruitment (Zhang *et al.,* 2013b).

The MOPr, like all GPCRs, has many active conformations (Pinyero *et al.*, 2007; Kenakin & Miller, 2010; Manglik *et al.*, 2012). In their unbound state, GPCRs constantly oscillate through a range of possible conformational states. Ligands bind to GPCRs and stabilize subsets of conformational states, some of which couple to and activate downstream effectors (agonists), while other are not coupled to effectors, and when ligands bind they prevent downstream signalling (antagonists). The stabilisation of subsets of conformations by a ligand may lead to preferential activation of a restricted set of signalling pathways, leading to ligand-specific patterns of signalling and receptor regulation – also known as ligand-biased signalling or functional selectivity. The MOPr binds an array of structurally diverse ligands and interacts with many effector and regulatory proteins providing a fertile system for ligand biased signalling. (Kenakin, 2002; Massotte *et al.*, 2002; Saidak *et al.*, 2006).

The corollary of structurally distinct agonists and effector molecules preferentially coupling via subsets of receptor conformations is that changes in the molecular structure of the receptor itself are likely to affect receptor conformation (Abrol *et al.,* 2013; Cox, 2013). Thus, amino acid changes resulting from SNPs have the potential to affect MOPr signalling globally or in a ligand-dependent manner by affecting the ability of a ligand to bind to the receptor, altering the conformation of the ligand-receptor complex and/or affecting the ability of this complex to couple to G-proteins and associated signalling or regulatory pathways.

***Functional studies of MOPr SNPs***

Most functional studies of human MOPr (hMOPr) use heterologously expressed receptors in an immortalized cell line such as CHO-K1, HEK-293 or AtT-20. The *physiological* relevance of subtle differences in signalling exhibited by MOPr variants in these highly engineered expression systems is difficult to predict, and making direct comparisons between receptor signalling profiles in different expression systems may be problematic as different cell lines vary in the available pool of G-proteins, effector molecules and regulatory proteins (e.g. Atwood *et al.,* 2012). Nevertheless, μ-opioid receptors are naturally expressed in a wide variety of cell types, and there is unlikely to be "one true path" for receptor activation and regulation. Thus, studies in diverse systems are probably necessary to capture the possible consequences of variations in receptor structure. However, in order to make comparisons between polymorphic variants meaningful, careful attention needs to be paid to receptor expression levels and the nature of the signalling assays (Connor *et al.,* 2004). While there seem to be functional differences between MOPr SNPs and the most common form of the receptor, many variants have been superficially described and making firm conclusions about the consequences of variations in MOPr sequence is limited by the experimental conditions used to study them.

**Table 1:** Summary of non-synonymous MOPr variants in the protein coding region, their corresponding *OPRM1* SNP, exon and MOPr protein domain. See references in the text for original reports.

| AA Exchange | MOPr Domain | SNP | Exon |
|---|---|---|---|
| N40D | N-terminus | 118 A > G | 1 |
| A6V | N-terminus | 17 C > T | 1 |
| S42C | N-terminus | 124 T > A | 1 |
| D51N | N-terminus | 151 G > A | 1 |
| G63V | N-terminus | 188 G > T | 1 |
| S66F | N-terminus | 197 C > T | 1 |
| L85I | TM1 | 253 C > A | 1 |
| S147C | TM3 | 440 C > G | 2 |
| N152D | TM3 | 454 C > G | 2 |
| R181C | ICL2 | 541 C > T | 2 |
| N190K | ICL2 | 570 A > T | 2 |
| C192F | TM4 | 575 G > T | 2 |
| R260H | ICL3 | 779 G > A | 3 |
| R265H | ICL3 | 794 G > A | 3 |
| S268P | ICL3 | 802 T > C | 3 |
| D274N | ICL3 | 820 G > A | 3 |
| V293I | TM6 | 877 G > A | 3 |

### *N-terminal domain SNPs*

#### N40D

The N40D variant is the most commonly occurring *OPRM1* SNP, with an allelic frequency ranging from 10 - 50% within various populations (Mura *et al.*, 2013). The N40D SNP is in the N-terminal extracellular domain of MOPr, and removes one of 5 putative asparagine-linked glycosylation sites in this region (Table 1, Singh *et al.*, 1997). First reported by Bergen *et al.* (1997), many studies have examined associations between the D40 allele and physiological and clinical parameters including nociception, altered response to opioid analgesics, opioid and alcohol dependence and hypothalamic-pituitary-adrenal (HPA) axis responses (Kreek *et al*, 2005; Walter and Lotsch, 2009). Most of the association studies report that carriers of the D40 allele have a reduced response to opioids, although some studies have reported the opposite, and others no effect at all (reviewed in Diatchenko *et al.* 2011). A recent meta-analysis of the clinical effects of the N40D variant in pain management concluded that knowing a patient's allele(s) at position 118 in *OPRM1* would have little impact on treatment (Walter & Losch, 2009), although the number of studies available for review was small. The D40 allele also has been associated with an increased, decreased or unchanged susceptibility to drug use and dependence, (reviewed in Mague *et al.*, 2010).

#### Regulation of N40D expression

Regardless of any impact on the function of the μ-opioid receptor, the possibility that the nucleotide or amino acid substitutions may affect MOPr expression levels needs to be considered. There is some evidence for reduced MOPr expression associated with the G118 allele (or its murine ortholog). It was reported that in cortex and pons from the brains of A118G heterozygotes, there was significantly less G118 mRNA (1.5 - 2.5-fold) than A118 mRNA (Zhang *et al.,* 2005a). A similar reduction in mRNA was found in a

knock-in mouse model with an orthologous A112 to G112 mutation (Mague *et al.,* 2009). A potential explanation for the reduced levels of G118 mRNA was provided by Oertel *et al.,* (2012), who deduced that the G118 allele has an extra methylation site introduced by the guanine nucleotide, which was suggested to inhibit compensatory upregulation of MOPr mRNA in chronic opioid users. It is possible that this epigenetic regulation also results in lower levels of G118 mRNA in basal conditions.

The loss of the potential glycosylation site, N40, may also contribute to lower cell surface receptor levels for the D40 allele, although this has not been consistently reported (Zhang *et al.,* 2005a; Oertel *et al.,* 2009). In mice, the molecular mass of MOPr in 112G/G animals (55 kDa) is lower than 112A/A mice (62 kDa), whereas the molecular mass of deglycosylated MOPr was identical (42 kDa) for both variants, indicating less glycosylation in 112G/G mice (Huang *et al.,* 2012). The G/G mice also have lower MOPr expression compared with A/A mice (Mague *et al.,* 2009; Wang *et al.,* 2012). Findings of lower expression extend to cells lines (Zhang *et al.,* 2005a; Huang *et al.,* 2012), with a shorter half life of D40 (12 hr) compared with N40 (28 hr) in CHO cells. Enzymatic deglycosylation of MOPr also decreased receptor expression in HEK-293 cells by 90% (Kroslak *et al.,* 2007). Decreased mRNA stability, potential epigenetic repression and incomplete glycosylation could all contribute to reduced D40 receptor expression, potentially providing a mechanism for greater opioids requirement in D40 carriers (Mura *et al.,* 2013).

**Second Messenger Coupling**

The consequences of the N40D substitution on the signalling profile of MOPr are not well understood and despite the intense research into the clinical effects of the D40 variant, only a handful of functional studies in cells have been performed on this variant (Table 2). The

first reported functional consequences of a MOPr SNP was a three-fold increase in the affinity of β-endorphin for the D40 variant and a three-fold increase in the potency of β-endorphin to activate GIRK channels co-expressed D40 in *Xenopus laevis* oocytes (Bond *et al.*, 1998). No differences in binding or signalling were reported for other opioids including DAMGO, endomorphin 1 and 2, and enkephalins. Unfortunately, these provocative results were based on very limited quantification of the cellular responses to activation of the N40 and D40 alleles, and no statistical analysis was included. Subsequent studies looking at different signalling pathways in other expression systems have failed to find differences in β-endorphin potency at N40 and D40 (Befort *et al.*, 2001; Beyer *et al.*; 2004; Kroslak *et al.*, 2007).

N40 inhibition of AC has been examined in several studies in HEK 293 cells (Beyer *et al.* 2004; Kroslak *et al.*, 2007; Fortin *et al.*, 2010). Unfortunately, these studies did not use N40 and D40 cell lines with equivalent receptor expression, and receptor reserve for AC inhibition was not assessed. Beyer and colleagues (2004) found no differences in the effects of morphine, morphine-6-glucuronide (M-6-G) or β-endorphin to inhibit acute cyclic adenosine monophosphate (cAMP) accumulation despite 7 fold lower expression of D40 than N40 in their cells. Fortin *et al.* (2010) also found no difference in how DAMGO, endomorphin-1 or leu-enkephalin modified cAMP-dependent gene transcription in cells acutely transfected with D40 and N40 constructs. This strategy produced apparently equivalent levels of receptor expression (measured by enzyme-linked immunosorbent assay [ELISA]) but the absolute levels were not reported. By contrast, Kroslak *et al.* (2007) reported a *decreased* potency of morphine, methadone and DAMGO but not β-endorphin to inhibit cAMP accumulation in cells expressing D40, however, this was associated with a 66% lower expression of D40 compared with N40. It is difficult to explain the differences between these studies, particularly in the absence of information

about relative efficacy. Studies using cAMP-dependent gene expression assays to measure MOPr activity have prolonged incubation with agonist and the response measured reflects the integrated outcome of acute inhibition of AC as well as agonist-dependent uncoupling, internalisation and possible recycling or degradation of the receptor, any of which could be altered by the D40 polymorphism (Connor *et al.*, 2004; Dang & Christie 2012). Likewise, possible differences in the acute regulation of D40 and N40 variants of the MOPr over the time course of acute cAMP accumulation assays could also confound their outcomes (Connor *et al.*, 2004).

The activity of N40 and D40 have also been compared by measuring inhibition of $I_{Ca}$ in acutely transfected sympathetic neurons (Margas *et al.*, 2007), HEK293 cells (Lopez Soto & Raingo, 2012) as well as mice "humanized" with A118 and G118 knock-in (Mahmoud et al., 2011; Ramchandari *et al.*, 2011). Opioid receptor inhibition of $I_{Ca}$ is via direct Gβγ-subunit inhibition of channel gating. In both HEK293 cells and sympathetic neurons, DAMGO inhibited $I_{Ca}$ more potently in cells expressing the D40 variant, with morphine also being more potent at D40 in sympathetic neurons (Lopez Soto & Raingo, 2012; Margas *et al.*, 2007, Table 2). Interestingly, the potency of endomorphin 1 and M-6-G was not different between N40 and D40 in sympathetic neurons. Although the relative expression levels of each receptor were not determined in these studies, the selective enhancement of DAMGO and morphine coupling to $I_{Ca}$ in sympathetic neurons suggest that there may be genuine differences in N40 and D40 signalling via this pathway. By contrast, trigeminal neuron $I_{Ca}$ from "humanized" N40 and D40 mice was inhibited in an essentially identical manner by DAMGO (Ramchandari et al., 2011) and fentanyl (Mahmoud et al., 2011), but morphine was less potent and had a lower efficacy in the neurons from the D40 mice (Mahmoud *et al.*, 2011). These results are essentially opposite to those found in the acutely transfected cell lines. There is no ready explanation for these

differences, although differences in receptor expression cannot be ruled out. It is likely that HEK293 cells, rat sympathetic neurons and mouse trigeminal neurons express different complements of Gα and βγ subunits, which may also contribute to observed differences. Finally, it should be noted that the humanized N40/D40 MOPr are hybrids, with only the first exon of the human receptor inserted into mouse, meaning that the receptors are human/mouse chimeras. The receptors had a similar affinity for DAMGO (Ramchandari *et al.,* 2011), but their signalling properties have not been well characterized.

Deb *et al*. (2010) expressed N40 and D40 variants of the μ-opioid receptor in the mouse neuroblastoma cell line Neuro2A, with radioligand binding experiments indicating similar levels of receptor expression. Using measurements of protein kinase A (PKA) activity and phosphorylated ERK1/2 (pERK) levels in response to a single concentration of morphine (1 μM) applied for 5 minutes or 6 days, the investigators found differences in PKA and pERK levels between N40 and D40 expressing cells after 6 days only. Unfortunately, the basal PKA activity and acute agonist-stimulated ERK phosphorylation differed significantly between the cell lines, making sensible interpretations difficult. The differences in the signalling responses of the cells could be due to expression of the different opioid receptor variants, or could have arisen due to variations in the phenotype of different Neuro2A cells at the time of clonal selection.

**A6V**

The A6V variant is located at the N-terminus of MOPr (Table 1). A6V is quite common in some populations but not others, having been reported at allelic frequencies ranging from less than 1% in Caucasian and east Asian populations (Rommelspacher *et al.*, 2001; Tan *et al.,* 2003) to upwards of 20% in African-American and northern Indian populations

(Crowley *et al.*, 2003; Kapur *et al.*, 2007). Few studies have investigated the clinical effects of this polymorphism. Crystal *et al.* (2012) reported an association between the T/T genotype in African-American women and the risk of alcohol, cocaine, tobacco but not opioid use. Other studies have demonstrated a trend towards a higher frequency of V6 in individuals with substance abuse, however these studies have lacked sufficient statistical power due to small sample sizes, and the confounding factor of overall genotype (Berrettini *et al.*, 1997; Rommelspacher *et al.*, 2001; Compton *et al.*, 2003; Crowley *et al.*, 2003).

There are no studies comparing acute A6 and V6 signalling on the predominant isoform of MOPr. In an assay of cAMP-dependent gene transcription, no difference in potency was found for DAMGO, endomorphin-1 or leu-enkephalin in HEK-293 cells expressing A6 and V6 (Table 2, Fortin et al., 2010). The A6V variant was studied on the MOR1A splice variant sequence expressed in HEK 293 cells, where DAMGO but not morphine showed a higher maximum effect at V6- than A6-MOR1A in an assay of intracellular Ca release catalyzed by a transiently transfected chimeric G protein. No differences in internalisation of the V6-MOR1A receptor in response to DAMGO and morphine were observed (Ravindranathan et al., 2009). The significance of these findings for more naturalistic coupling of MOPr are unclear, but suggest that further work is warranted.

**S42C, D51N, G63V and S66F**

Other rare polymorphisms within the N-terminal domain of MOPr have been identified within the population but no clinical or phenotypic information is available (Table 1). The S42C variant resulted in reduced receptor expression and coupling to intracellular calcium release when assayed on the MOR1A splice variant background (Ravindranathan *et al*, 2009, Table 2)..

Several extracellular domain polymorphisms for which there is no phenotypic data were identified on the GPCR Natural Variant database (Kazius *et al.* 2007) and examined in a cAMP-dependent gene transcription assay (Fortin *et al.*, 2010). Neither D51N nor G63V showed any differences to wild type MOPr in this assay. However, the S66F variant showed a reduction in the potency of DAMGO and endomorphin-1, but not leu-enkephalin to inhibit AC (Table 2; Fortin *et al.*, 2010).

***Transmembrane Domain SNPs***

**L85I (TM1)**

The transmembrane helices of MOPr are important elements of the ligand-binding pocket, and they are essential for transmitting information from the extracellular surface to the intracellular signalling domains and also participate in the formation of oligomers (Serohijos *et al.*, 2011; Manglik *et al.* 2012). The L85I variant, in TM1 (Table 1), was first reported by Ravindranathan *et al* (2009). Although there is no information about the phenotype of people carrying the I85 allele, it has an interesting functional profile *in vitro*. Both DAMGO and morphine have a moderately lower efficacy in signalling assays measuring I85 (or I83 – the rat ortholog) activity, however morphine displays an enhanced capacity to promote internalisation of the I85/I83 variant (Ravindranathan *et al.*, 2009; Cooke *et al.*, 2014). Co-expression of the I85 and L85 receptors results in morphine promoting internalisation of both variants, suggesting that they may form functional dimers (Ravindranathan *et al.*, 2009).

Previous studies have shown that WT-MOPr internalizes relatively poorly in response to morphine, and there is also evidence that high efficacy agonists such as DAMGO and etorphine appear to induce receptor desensitisation by different mechanisms to partial agonists such as morphine (Ueda *et al.*, 2001; Borgland *et al.*, 2003; Johnson *et al.*, 2006;

Kelly *et al.*, 2008; Bailey *et al.* 2009). Intriguingly, while morphine activated MOPr has been shown to be a poor substrate for G protein-coupled receptor kinase (GRK) subtypes 2/3 phosphorylation, which is required for endocytosis (Doll *et al.*, 2012), internalisation of I83 MOPr was significantly attenuated with overexpression of a GRK2 dominant negative mutant, suggesting this variant is better able to recruit GRK2 (Cooke *et al.*, 2014). Hierarchical, multi-site phosphorylation is required for efficient MOPr endocytosis (El Kouhen *et al.*, 2001) and while morphine induces phosphorylation of the S375 residue on the C-terminus of MOPr, DAMGO also efficiently stimulates phosphorylation of T370 after S375 phosphorylation, resulting in receptor internalisation (Doll *et al.*, 2011, Grecksch *et al.*, 2011). Morphine stimulated internalisation of I83 was not due to enhanced phosphorylation of S375 compared with WT MOPr, but T370 phosphorylation was not investigated (Cooke *et al*., 2014). The observations that I83/85 MOPr show apparently decreased signalling efficacy compared with enhanced receptor trafficking in response to morphine underscore the likelihood that distinct receptor conformations underlie each of these processes. It will be interesting to see whether it is possible to further define the structural elements in the region of L85 that are involved in MOPr signalling and phosphorylation, and whether it will be possible to independently manipulate these properties of the agonist/receptor complex.

Compensatory changes in cell signalling processes are associated with chronic MOPr activation, one of the most well described of these is upregulation of AC activity that results in "superactivation" of AC upon opioid withdrawal (Sharma *et al.*, 1975; Avidor-Reiss *et al.*, 1996; Whistler *et al.*, 1999). It has also been suggested that these compensatory changes are limited by agonist-induced receptor internalisation (Wang *et al.*, 2003). Ravindranathan *et al.* tested the I85 variant for changes AC superactivation. Cells expressing L/I85 MOPr variant were chronically treated with morphine for 14h. Upon

morphine withdrawal, cells expressing I85 MOPr showed a significantly lower level of cAMP compared with WT cells (2.5 fold and 1.5 fold cAMP levels of morphine naive cells respectively). Upon a 4hr "acute" rechallenge with 10 nM morphine, cAMP levels were again significantly lower in the I85 expressing cells, indicating a reduction AC superactivation and morphine tolerance.

**S147C and N152D (TM3)**

Computational modeling and x-ray crystallography studies have shown transmembrane domains (TM) 3, 5 and 6 to be of particular importance in the formation of the ligand-binding pocket of MOPr (Serohijos *et al.*, 2011; Manglik *et al.*, 2012). Two polymorphisms in TM3 have been detected within the population, S147C and N152D (Table 1), both occurring at frequencies of < 1% (Bergen *et al.*, 1997; Uhl et *al.*, 1999; Befort *et al.*, 2000; Ravindranathan *et al.*, 2009). No information on the clinical phenotype of C147 or D152 carriers is available, and limited functional studies have been published. When expressed on the MOR1A splice variant backbone C147 appeared to support an increased efficacy and potency for DAMGO and morphine to stimulate intracellular calcium release when compared with S147 (Ravindranathan *et al.* 2009), however, when expressed on the wild type MOPr backbone, C147 was modestly less effective at supporting agonist-mediated inhibition of cAMP-dependent gene transcription (Fortin *et al.* 2010). Whether this divergence is because of the different receptor backgrounds or whether it hints at a reciprocal change in the capacity of MOPr to activate different signalling pathways remains unknown. The N152D SNP appears to have reduced affinity for morphine but not opioid peptides, unfortunately it was not possible to measure receptor activity, apparently due to low overall expression (Befort *et al.*, 2001).

**Table 2:** Summary of the key findings about MOPr SNP signalling. * P < 0.05, from original publications. Abbreviations: β-End, β-endorphin; CaM, calmodulin; DAM, DAMGO, [D-Ala$^2$, NMe-Phe$^4$, Gly-ol$^5$]-enkephalin; End-1, endomorphin 1; Fent, fentanyl; L-ENK, [Leu]$^5$enkephalin; Meth, methadone; Mor, morphine; MOR-1A, μ-opioid receptor 1A splice variant; M-6-G, morphine-6-glucuronide; PKA, protein kinase A; SCG, superior cervical ganglion

| MOPr Variant | Key Observations | $pEC_{50}$ WT | $pEC_{50}$ SNP | $B_{max}$ WT | $B_{max}$ Var | Reference |
|---|---|---|---|---|---|---|
| N40D | Unchanged agonist affinity. Similar DAMGO stimulated GTPγS activation. | 7.0 - DAM | 6.7 - DAM | 5.5 pmol/mg | 6.1 pmol/mg | Befort *et al.,* 2001 |
| | Similar cAMP inhibition. Reduced D40 expression. | 9.1 - MOR<br>8.8 - M-6-G<br>7.9 - β-end | 9.0 - MOR<br>8.8 - M-6-G<br>7.8 - β-end | 4.8 pmol/mg | 0.63 pmol/mg | Beyer *et al.,* 2004 |
| | 3 x increased β-endorphin affinity for D40 than WT, and 3x increased potency for GIRK activation in D40 expressing *Xenopus* oocytes . | Not provided | Not provided | Not provided | Not provided | Bond *et al.*, 1998 |
| | Different N/D40 stimulated PKA activity and ERK1/2 phosphylation after chronic morphine treatment. | N/A (1 μM morphine only) | N/A (1 μM morphine only) | 835 fmol/mg | 830 fmol/mg | Deb *et al.* 2010 |
| | Similar inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.8 - DAM<br>8.9 - End-1<br>8.5 - L-Enk | Values not provided | Similar to WT | Fortin *et al.*, 2010 |
| | Decreased agonist potency to inhibit AC in D40-HEK-293 and D40-AV-12 cells | 8.6 - DAM<br>8.4 - Mor<br>8.3 - Meth<br>8.4 - β-End | 8.1 - DAM*<br>7.8 - Mor*<br>7.8 - Meth*<br>8.1 - β-End | Not provided | 66% of WT | Kroslak *et al.*, 2007 |
| | Decreased morphine potency for $I_{Ca}$ inhibition in mouse trigeminal ganglion cells expressing “humanized” D40. | 7.3 - Mor<br>7.2 - Fent | 6.6 - Mor*<br>7.0 - Fent* | Not provided | Similar to WT | Mahmoud *et al.*, 2011 |
| | Increased DAMGO and morphine potency for $I_{Ca}$ inhibition at D40 expressing rat SCG cells. | 7.5 - DAM<br>7.1 - Mor<br>7.1 - M-6-G<br>7.1 - End-1 | 7.8 - DAM*<br>7.4 - Mor*<br>7.1 - M-6-G<br>7.1 - End-1 | Not provided | Not provided | Margas *et al.*, 2007 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **N40D (cont.)** | Decreased D40 expression in $S_{II}$ region of cortex in post-mortem brain.. $S_{II}$ region-specific decrease in DAMGO efficacy in D40 carriers. | 5.9 - DAM | 6.0 - DAM | 97 fmol/mg | 114 fmol/mg | Oertel *et al.*, 2009 |
| | No difference in DAMGO potency at D40 for $I_{Ca}$ inhibition in "humanized" mouse trigeminal ganglion cells. | N/A | N/A | Not provided | Similar to WT | Ramchandari *et al.*, 2011 |
| | Increased DAMGO potency for $Ca_V2.2$ inhibition in D40 HEK-293 cells. | 8.6 - DAM | 9.5 - DAM* | Not provided | Not provided | Soto & Raingo, 2012 |
| | Lower mRNA levels of G118 allele for heterozygous A118G carriers in post-mortem brain. Decreased G118 mRNA and 10-fold decreased D40 expression in CHO-K1 cells. | N/A | N/A | Not provided | Not provided | Zhang *et al.*, 2005 |
| **A6V** | Similar inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.6 - DAM<br>8.7 - End-1<br>8.2 - L-Enk | Not provided | Similar to WT | Fortin *et al.*, 2010 |
| | Unchanged agonist efficacy and potency for intracellular Ca release at A/V6 on MOR-1A backbone. | 7.5 - DAM<br>7.4 - Mor | 7.9 - DAM<br>7.3 - Mor | 5.6 pmol/mg | 5.8 pmol/mg | Ravindranathan *et al.*, 2009 |
| **S42C** | Decreased agonist potency for intracellular Ca release at C42 on MOR-1A backbone. | 7.5 - DAM<br>7.4 - Mor | > 6.8 - DAM*<br>> 6.8 - Mor* | 2.7 pmol/mg | 5.8 pmol/mg | Ravindranathan *et al.*, 2009 |
| **D51N** | Similar inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.6 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | Not provided | Similar to WT | Fortin *et al.*, 2010 |
| **G63V** | Similar inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 9.0 - DAM<br>8.9 - End-1<br>8.5 - L-Enk | Not provided | Similar to WT | Fortin *et al.*, 2010 |
| **S66F** | Decreased potency of DAMGO and endormorphin-1 at F66 for inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.2 - DAM*<br>8.3 - End-1*<br>7.7 - L-Enk* | Not provided | Similar to WT | Fortin *et al.*, 2010 |

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| **L85I (L83I)** | Increased morphine stimulated endocytosis in I83-HEK293 cells. Decreased agonist efficacy in inhibition of AC and ERK phosphorylation. | 6.7 - DAM<br>6.7 - Mor | 6.5 - DAM<br>6.9 - Mor | 1.8 pmol/mg | 2.7 pmol/mg | Cooke *et al.*, 2014 |
| **L85I** | Increased morphine stimulated endocytosis in I85-HEK293 cells. Increased AC superactivation in I85 HEK-293 cells. No change in agonist potency. | 7.5 - DAM<br>7.4 - Mor | 7.9 - DAM<br>7.7 - Mor | 5.6 pmol/mg | 5.2 pmol/mg | Ravindranathan *et al.*, 2009 |
| **S147C** | Decreased agonist potency for inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.3 - DAM*<br>8.4 - End-1*<br>7.9 - L-Enk* | Values not provided | Similar to WT | Fortin *et al.*, 2010 |
| | Increased agonist potency for intracellular Ca release at C147 on MOR-1A backbone. | 7.5 - DAM<br>7.4 - Mor | 7.9 - DAM*<br>8.3 - Mor* | 5.6 pmol/mg | 5.0 pmol/mg | Ravindranathan *et al.*, 2009 |
| **N152D** | Decrease in morphine affinity for D152 in COS cells. | N/A | N/A | 5.5 pmol/mg | 1.9 pmol/mg | Befort et al., 2001 |
| **R181C** | HEK-293 cells expressing C181 failed to signal via DAMGO or morphine. | 7.5 - DAM<br>7.4 - Mor | N/A | 5.6 pmol/mg | 3.5 pmol/mg | Ravindranathan *et al.*, 2009 |
| **N190K** | Decreased K190 expression in HEK-293 cells. Treatment with naloxone and naltrexone both increased K190 expression and inhibition of cAMP-stimulated CRE transcription. | Not provided | Not provided | Not provided | N/A | Fortin *et al.*, 2010 |
| **N192F** | Decreased agonist potency at F192 for intracellular calcium release in HEK-293 cells expressing F192 on MOR-1A backbone. | 7.5 - DAM<br>7.4 - Mor | > 6.8 - DAM*<br>> 6.8 - Mor* | 5.6 pmol/mg | 4.4 pmol/mg | Ravindranathan *et al.*, 2009 |
| **R260H** | Decreased basal GTPγS activity at H260 in HEK-293 cells. Slight decrease in morphine stimulated GTPγS at H260, and slight decrease in affinity of H260 for CaM. | 8.4 - Mor | 8.6 - Mor | 3.5 pmol/mg | 3.9 pmol/mg | Wang *et al.*, 2001 |

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|---|---|---|---|---|---|---|
| **R265H** | Decreased basal GTPγS activity at H260 in COS cells. Slight decrease in maximal DAMGO stimulated GTPγS at H260. | 7.0 - DAM | 6.9 - DAM | 5.5 pmol/mg | 4.6 pmol/mg | Befort et al., 2001 |
| | Decreased agonist potency for inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.0 - DAM*<br>8.1 - End-1*<br>7.6 - L-Enk* | Values not provided | Similar to WT | Fortin *et al.*, 2010 |
| | Decreased basal GTPγS activity at H265 in HEK-293 cells. Slight decrease in maximal morphine stimulated GTPγS at H265. Decreased affinity of H265 for CaM binding, and decreased desensitisation following morphine pretreatment. | 8.4 - Mor | 8.5 - Mor | 3.5 pmol/mg | 4.2 pmol/mg | Wang *et al.*, 2001 |
| **S268P** | No of agonist-stimulated GTPγS binding in COS cells. Decreased agonist potency and efficacy at P268 for inhibition of cAMP accumulation. | 7.2 - DAM<br>6.5 - β-End<br>6.2 - Mor | 6.4 - DAM*<br>5.9 - β-End*<br>5.8 - Mor* | 5.5 pmol/mg | 3.6 pmol/mg | Befort et al., 2001 |
| | Decreased potency of DAMGO and endomorphin-1 for inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.2 - DAM*<br>8.4 - End-1*<br>7.9 - L-Enk | Values not provided | Similar to WT | Fortin *et al.*, 2010 |
| | Decreased GTPγS binding, slower desensitisation and decreased AC inhibition in response to DAMGO. | N/A | N/A | 643 fmol/mg | 340 fmol/mg | Koch *et al.*, 2000 |
| | Decreased morphine potency at P268 for inhibition of cAMP accumulation. | 7.0 - Mor | 6.3 - Mor* | 3.5 pmol/mg | 4.5 pmol/mg | Wang *et al*., 2001 |
| **D274N** | Increased agonist potency for inhibition of cAMP-stimulated CRE transcription. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 9.1 - DAM*<br>8.3 - End-1*<br>8.6 - L-Enk* | Values not provided | Similar to WT | Fortin *et al.*, 2010 |
| **V293I** | Unchanged in agonist potency for inhibition of cAMP accumulation. | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | 8.8 - DAM<br>8.8 - End-1<br>8.4 - L-Enk | Values not provided | Similar to WT | Fortin *et al.*, 2010 |

**C192F (TM4)**

One SNP in TM4 of OPRM1 has been identified; C192F (Ravindranathan *et al.* 2009). When expressed on the MOR1A splice variant backbone, C192F showed significant decreases in DAMGO and morphine potency to mobilize calcium in HEK293 cells transfected with an engineered G protein. No phenotypic information is available.

**V293I (TM6)**

Shi *et al.* (2002) detected a V293I amino acid exchange in MOPr. I293 was reported to signal in an identical manner to V293 (Fortin *et al.*, 2010) and there is no clinical information about this phenotype.

***Intracellular Loop SNPs***

The intracellular loop (ICL) domains of MOPr form major elements of the cytoplasmic interface between the receptor and intracellular effector proteins. ICL2 and 3 have been shown to be of key importance in G-protein coupling of GPCRs, as well as being involved in regulatory processes such as receptor phosphorylation, uncoupling and internalisation (Lefkowitz, 1998). GPCRs with the ICL2 and ICL3 domains deleted cannot couple to G-proteins but can retaining their ligand-binding properties, and there are a number of examples of ICL SNPs affecting selectivity of G-protein coupling (Capeyrou *et al.*, 1997; Visiers *et al.*, 2001; Goldfeld *et al.*, 2011; Zheng *et al.* 2013).

ICL2 contains the highly conserved E/DRY motif, mutations in which have been shown to reduce MOPr agonist efficacy, and also to increase constitutive activity of MOPr (Li *et al.*, 2001; Clayton *et al.*, 2010). Mutations in ICL2 have also been shown to affect receptor uncoupling and desensitisation (Celver *et al.*, 2001; Celver *et al.*, 2004). ICL3 is highly

conserved among all opioid receptor types and has been shown to be involved in basal and agonist-stimulated G-protein coupling, β-arrestin recruitment and contains multiple phosphorylation consensus sequences (Merkouris *et al.*, 1996; Georgoussi *et al.*, 1997; Wang, 1999b). Mutations within the ICL3 of MOPr have been shown to differentially affect agonist potency and efficacy (Chaipatikul *et al.*, 2003). In addition to their role in acute signalling and short-term regulatory processes, the intracellular domains of GPCRs may be of importance in long-term adaptations to chronic opioid exposure, and contribute to the development of opioid tolerance (Chavkin *et al.*, 2001; Koch & Hollt, 2008; Williams *et al.*, 2013).

**R181C (ICL2)**

The R181C variant was reported by Ravindranathan *et al.* (2009). Interestingly, C181 appears to have an unchanged affinity for DAMGO, but it fails to promote calcium mobilisation or be internalized in response to agonist. Whether the receptor is unable to signal to all effectors remains to be established.

**N190K (ICL2)**

The rare N190K variant is located at the base of TM4, and was originally reported as an ICL2 SNP (Table 1; Fortin *et al.* 2010). Total K190 expression in HEK293 cells is lower than N190, but cell surface expression is almost absent. DAMGO fails to signal through K190, although it is not clear if this is because of the inaccessibility of the intracellular receptor or a change in the transduction of peptide agonist signals. Interestingly, treatment of the K190 variant with the non-peptide, cell permeable opioid receptor ligands naltrexone, naloxone, buprenorphine or β-CNA (10 μM, 18h) increased cell surface receptor expression, with naltrexone treatment producing levels similar to WT-MOPr. Small, membrane permeable ligands can "rescue" misfolded or immature GPCR, including

opioid receptors, by stabilizing a more native-type conformation in the endoplasmic reticulum and allowing the protein to enter the appropriate secretory pathway (Petaja-Repo *et al.*, 2002; Ulloa-Aguirre et al., 2004; Chen *et al.,* 2006). Fascinatingly, naloxone and naltrexone were also apparently agonists at K190, producing significant inhibition of cAMP-stimulated reporter gene transcription after prolonged treatment (Fortin *et al.*, 2010). This suggests that K190 is not misfolded/misconfigured to such a degree that it cannot recognize G proteins, but that it nonetheless has an abberant native conformation.

Four rare, naturally occurring SNPs present on ICL3 have been described, R260H (Bond *et al.*, 1998), R265H (Hoehe *et al.*, 2000; Befort *et al.*; 2001; Wang *et al.*, 2001), S268P (Hoehe *et al.*, 2000) and D274N (Wang *et al.*, 2001; Table 1). The importance of ICL3 in the regulation and signalling of MOPr has prompted investigation of the functional consequences of ICL3 polymorphisms, despite their rarity within the population.

**R260H, R265H, S268P**

The R260H and R265H variant receptors exhibited very similar ability to bind opioids and be activated by morphine or DAMGO, with minor differences in agonist-stimulated GTPγS binding potentially accounted for by small differences in receptor expression or the proportion of receptors on the cell surface. An intriguing finding was that basal GTPγS activity was significantly lower in cells expressing H260 or H265, suggesting a lower constitutive activity (Befort *et al,* 2001; Wang *et al.*, 2001).

Assays of cAMP accumulation have provided inconsistent results with respect to H260 or H265 signalling. Wang *et al.* (2001) found no differences in morphine potency or efficacy for inhibition of forskolin-stimulated radiolabelled cAMP accumulation in cells expressing WT-MOPr, H260 or H265 while Befort *et al.* (2001) also found no differences between

H265 and WT in a cAMP response element (CRE) reporter gene assay (see Table 2). By contrast, Fortin *et al.* (2010) used a different CRE reporter assay and found a decrease in potency of DAMGO, endomorphin-1 and leu-enkephalin signalling through both H260 and H265. It is difficult to directly compare these studies as Fortin *et al.* (2010) did not quantify receptor expression, but the relatively high levels of receptor expression in the cells used by Wang *et al.* (2001) and Befort *et al.* (2001) could conceivably reduce the sensitivity of the assay to detect differences in agonist potency at the variant receptors.

A third ICL3 variant, S268P, results in the loss of a putative Ca2+/calmodulin-dependent protein kinase II (CaMK II) phosphorylation site (Koch *et al.*, 2000) and insertion of an amino acid, proline, that is likely to significantly disrupt the structure of ICL3. Most studies have found that P268 or the rat ortholog S266P (Koch *et al.*, 2000) have a significantly reduced signalling capacity, although the extent of this depends somewhat on the assay conditions used (Koch *et al.*, 2000, Befort *et al.* 2001; Wang *et al.*, 2001; Fortin *et al.* 2010; Table 2). The reduction in signalling does not seem to be associated with a change in ligand affinity for the receptor (Koch *et al.*, 2000; Befort *et al.* 2001), but it is unclear what the relative contributions of the loss of the potential phosphorylation site or the introduction of the proline residue are to the observed *in vitro* phenotype.

Mutations in ICL3 of MOPr affect signalling of the receptor, but changes in the signalling profile of MOPr resulting from ICL3 SNPs are likely to be expressed in situations other than acute MOPr signalling because ICL domains of GPCRs interact with effectors involved in receptor regulation and adaptive processes such as receptor downregulation (Lefkowitz, 1998). The ICL3 domain of MOPr has multiple consensus phosphorylation sites, as well as a putative calmodulin binding domain (Wang *et al.*, 1999a; Koch *et al.* 2000). Sustained exposure to high concentrations of agonist produces downregulation of

receptor protein in cell lines, and the ICL3 variants R260H, R265H and S268P were downregulated (~ 80%) to a similar degree to WT receptors by 10 μM DAMGO (Befort *et al.*, 2001). Functionally, P268 MOPr-mediated inhibition of AC desensitized with a similar time course to T268, while desensitisation of P268-mediated activation of GIRK was slower and incomplete when compared to T268 when the proteins were expressed in *Xenopus* oocytes (Koch *et al.*, 2000).

In addition to phosphorylation sites, MOPr ICL3 contains a putative calmodulin (CaM) binding site. It has been suggested that CaM competes with G-protein coupling at ICL3, and regulates basal MOPr signalling (Wang *et al.*, 1999). Wang *et al.* (2001) investigated interaction of CaM with MOPr ICL3 domains by incubating CaM with short peptides derived from ICL3 sequences as well as full length MOPr purified from transfected HEK-293 cells. The ICL3 H260 peptide showed marginal reduction of CaM binding but the ICL3 H265 and P268 peptides bound CaM significantly less well. A similar pattern was observed in western blots of full-length MOPr variants bound to CaM. The broader significance of these findings have not been firmly established.

**D274N**

The D274N variant has received much less attention than ICL3 variants discussed earlier. It was originally reported by Wang *et al.* (2001), but not investigated until the study of Fortin *et al.* (2010). DAMGO and leu-enkephalin showed a slight increase in potency for inhibition of cAMP accumulation at N274, while endomorphin-1 potency was significantly increased when compared with WT-MOPr in HEK-293 cells (see Table 2). No change in DAMGO efficacy was observed. These results are in direct contrast to other ICL3 variants, where receptor signalling tended to be reduced.

***Limitations of extant functional SNP studies***

The interpretation of studies of opioid receptor function in vitro, and the extent to which fruitful comparisons can be made between studies are subject to several important caveats. These extend beyond the everyday differences in the way that laboratories perform studies, and can limit the confidence we have in our understanding of the impact non-synonymous SNPs have on *OPRM1* function. Firstly, many studies do not quantify receptor expression, either in the whole cell or on the cell surface. While it is unrealisitic to expect "physiological" expression levels (whatever they may be) in all expression systems, high levels of receptor can lead to significant receptor reserve or exaggerated coupling to effectors not normally accessed by the receptor. Receptor reserve is an important issue that has apparently rarely been considered, and even modest differences in receptor expression could significantly affect the signalling profile of important partial agonists such as morphine, and spare receptors may mask subtle differences between variant signalling.

Secondly, the techniques used to measure MOPr activation in many studies do not reflect acute, real-time, naturalistic signalling of MOPr. MOPr undergoes rapid desensitisation and internalisation following agonist exposures of 5-10 mins (Connor *et al.,* 2004). Thus, the reporter gene assays used for facile quantification of MOPr function measure the summed effects of MOPr activation, desensitisation, internalisation and resensitisation, and this may obscure differences between variants at any of these points. Clonal selection of transformed cells during establishment of cell lines expressing variants may contribute to signalling differences observed between variants, and this is rarely controlled for with experiments on endogenous GPCR in each cell line used. These shortcomings are common to many studies of cell signalling in heterologous systems, and to an extent come with the

territory, but they are especially important to consider and try and minimize given the potentially subtle nature of changes produced by SNPs.

MOPr is expressed in a wide variety of human cell types, and subtle changes in MOPr signalling arising from SNPs are likely to differ between tissue and cell type. As such, it is difficult to lay out an "ideal" strategy for investigating functional consequences of MOPr SNPs. In reality, studies undertaken in a variety of heterologous expression systems are probably useful for capturing the range of signalling and regulatory differences that may be produced by MOPr variants (e.g. Charfi *et al.,* 2013). However, simple measures that might enable more confident assertions that differences seen might represent more than just an experimental quirk would include using similar expression systems when attempting to make direct comparisons between the effects of changes in MOPr sequence and/or the effects of multiple ligands, controlling for receptor expression and reserve, and examining as many effectors as possible in similar conditions. Practical steps towards this include the use of cell lines with defined recombination sites to allow the construction of multiple clones on an isogenic background (e.g. FlpIn cells, Knapman *et al.,* 2014) and the use of inducible expression systems or transient transfections to minimize the effects of prolonged expression of MOPr on cell phenotype and perhaps gain some ability to titrate the amount of cell surface receptor (e.g. Fortin *et al.,* 2010; Knapman *et al.,* 2014). It is always useful to use assays that capture the kinetics of drug/receptor/second messenger activity, rather than simply endpoint assays (e.g. Johnson *et al.,* 2006; Cawston *et al.,* 2013; Knapman *et al.,* 2013, 2014; Tudashki *et al.,* 2014) and it is also important to have a system where changes in efficacy can be readily determined, whether by use of pharmacological tools or by choosing cell lines where there are a minimum of spare receptors. Defining receptor reserve using irreversible antagonists such as β-funaltrexamine or β-chlornaltrexamine and then fitting data to operational models (e.g.

Borgland *et al.*, 2003; Rivero *et al.*, 2012; Kelly, 2013) can allow for precise determination of rank orders of agonist efficacy and uncover differences in signalling across different effectors in the same cell, enabling a more complete characterisation of the consequences of changes in receptor sequence. All these ideas have been extensively reviewed in the context of defining ligand bias and allostery at GPCRs, and there is no reason they should not be applied when it is the receptor that changes rather than the ligand (Kenakin & Christopoulos, 2013).

***Future Studies***

Areas of great importance for opioid receptor function remain largely unexplored for most SNPs. In particular, the efficiency of coupling of SNPs to the range of possible MOPr signalling pathways has barely been touched on, as have possible ligand-specific changes in this coupling. Several studies have examined the trafficking of MOPr variants in response to a limited range of agonists (Ravindranathan *et al.*, 2009; Cooke *et al.*, 2014), but the effect of MOPr SNPs on the rapid desensitisation of signalling that precedes receptor internalisation remains unknown. The way in which MOPr SNPs may affect the occurrence or function of putative MOPr dimers has received limited attention (Ravindranathan *et al.*, 2009), even though most carriers of variant MOPr alleles will be heterozygous for the wild type receptor.

Understanding how MOPr SNPs affect cellular signalling is important for predicting the potential clinical or phenotypic consequences of these variants in humans. However, understanding other aspects of MOPr function such as the regulation of gene expression in response to environmental or epigenetic factors, and the function of MOPr in the wide range of human cells which normally express it, are equally important and more difficult to achieve. Nevertheless, understanding the consequences of expressing a particular MOPr

variant should one day contribute to a more personalized approach to opioid prescription. The ability to predict the effects of specific opioid drugs in individuals, including side effects and the development of tolerance, would minimize the risk of serious adverse events associated with opioid overdose, while maximizing therapeutic benefits and ensuring individuals receive adequate pain relief. Such prediction would necessarily involve determining the genotype of multiple proteins involved in opioid ligand distribution and metabolism, as well as effectors downstream of MOPr, but a key element would be knowing what version of MOPr a patient had, and knowing which of the many opioid analgesics available had the best pharmacodynamic profile at that variant.

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# Hypotheses and Aims

The overall hypothesis of this thesis is that naturally occurring *OPRM1* SNPs contribute to the variability observed between individuals in response to opioid analgesics by causing changes in MOPr signalling and function. The distinct aims of this study are to:

1. Determine whether MOPr variants will couple to distinct signalling pathways with different efficiencies compared with wild-type MOPr.

2. Determine whether opioid ligands will vary in their ability to stimulate distinct signalling pathways.

3. Determine whether opioid ligands are differentially affected in their ability to stimulate distinct signalling pathways at MOPr variants compared with wild-type MOPr.

# METHODS

The next 2 chapters describe two novel high-throughput screening (HTS) assays that were developed as part of this thesis, “A real-time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese hamster ovary cells” (Chapter 3) and “A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells” (Chapter 4). A detailed protocol for these assays is outlined in the Appendix, “Fluorescence-Based, High-Throughput Assays for μ-Opioid Receptor Activation using Membrane Potential-Sensitive Dye”. These assays were developed in order to address the aims listed above, by enabling simple and rapid measurement of MOPr signalling via 11 opioid ligands at 6 MOPr variants. In subsequent chapters I explore the consequences of MOPr SNPs for receptor signalling via a wide range of opioid agonists utilising these assays, along with a whole-cell ELISA assay for ERK1/2 phosphorylation described in Chapter 5, “Buprenorphine signalling is compromised at the N40D polymorphism of the human μ-opioid receptor in vitro".

# Chapter 3

## A real-time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese hamster ovary cells

**Alisa Knapman, Fe Abogadie, Peter McIntrye, Mark Connor**

This paper was published in 2014 in the *Journal of Biomolecular Screening* **19:** 223-231. Fe Abogadie and Peter McIntyre constructed the T-Rex FlpIn CHO cells that were subsequently used to express MOPr variants. Mark Connor assisted in experimental design and manuscript preparation. All other work is my own.

**ABSTRACT**

Inhibition of adenylyl cyclase (AC) activity is frequently used to measure μ-opioid receptor (MOR) activation. We sought to develop a simple, rapid assay of AC activity in whole cells that could be used to study MOR signalling. Chinese hamster ovary cells expressing human MOR (CHO-MOR cells) were grown in 96-well plates and loaded with membrane potential-sensitive fluorescent dye. CHO-MOR cells were treated with the AC activator forskolin (FSK), with or without simultaneous application of MOR agonists, and the resulting change in fluorescence was measured. CHO-MOR cells hyperpolarised in response to application of FSK ($p$EC$_{50}$ 7.3) or calcitonin ($p$EC$_{50}$ 9.4). A submaximally effective concentration of FSK (300 nM) caused a 52 ± 2% decrease in fluorescence. Simultaneous application of the opioids DAMGO ($p$EC$_{50}$ 7.4, E$_{max}$ 56%), morphine ($p$EC$_{50}$ 7.0, E$_{max}$ 61%) and buprenorphine ($p$EC$_{50}$ 8.6, E$_{max}$ 24%) inhibited the FSK response in a dose-dependent manner, while having no effect by themselves. The effects of DAMGO were blocked by pertussis toxin. This assay represents a simple, robust method for real-time observation of AC inhibition by MOR in CHO cells. It represents an appealing alternative to end-point assays that rely on cAMP accumulation and can avoid potential confounds associated with rapid desensitisation of MOR signalling.

Keywords: opioid, membrane potential, high throughput, cAMP, calcitonin

## INTRODUCTION

Opioid analgesics are the most widely prescribed drugs in the treatment of moderate to severe pain. Despite their powerful analgesic effects, the use of opioids is limited due to the number of associated adverse affects such as respiratory depression, sedation, constipation and nausea, as well as the development of tolerance. Over time, the development of opioid tolerance and physical or psychological dependence may require 10-fold escalations in dose in order to maintain adequate pain relief.[1] Both the analgesic and adverse effects of opioid analgesics are mediated via the μ-opioid receptor (MOR) subtype.[2] MOR mediate their effects via downstream mechanisms including inhibition of adenylyl cyclase (AC) activity via $G\alpha_{i/o}$ subunits, inhibition or activation of ion channels via Gβγ subunits and activation of MAPK signalling via β-arrestin.[3]

One of the hallmarks of MOR receptor activation is the inhibition of AC activity, leading to a decrease in the production of cAMP. Changes in cAMP-dependent signalling are also hallmarks of processes associated with chronic opioid receptor activation.[4,5] cAMP is an important cellular second messenger, mediating a diverse range of physiological processes via activation of cAMP-dependent protein kinase A (PKA), exchange protein directly regulated by cAMP (EPAC) as well as directly via cAMP-gated ion channels.[6,7] The measurement of cAMP accumulation is frequently used as a sensitive endpoint assay in studies of both acute and chronic MOR signalling. A number of techniques have been developed for quantifying cAMP accumulation in recent years, particularly in the rapidly growing field of high-throughput screening (HTS). AC activity can be measured in a number of ways including measurement of the accumulation of [$^3$H]-cAMP, by [$^3$H]-cAMP binding displacement assays, by reporter gene assays utilizing cAMP-dependent transcription factors, by ELISA assays that measure cAMP-like immunoreactivity, and

through measurements of cAMP-dependent protein/protein interactions using FRET.[8] These assays often require cell lysis, are usually single time-point, and require the addition of multiple reagents or transfection of reporter constructs. Importantly, many assays of AC activity require significant incubation times to allow appropriate cAMP accumulation.[9, 10] In studies of MOR signalling, prolonged incubation times can pose a significant and underappreciated problem as MOR signalling undergoes rapid desensitisation followed by receptor internalisation during agonist exposures as short as 5 - 10 minutes.[11] As the incubation periods in most cAMP assays are at least 10 – 20 minutes, during which time opioid exposure is sustained, these assays are likely to be measuring the combined effects of receptor activation, desensitisation, internalisation and even resensitisation.[11,12]

In this study, we sought to develop a simple, rapid assay of AC modulation. Here we report an assay of MOR inhibition of AC in intact CHO cells using a proprietary membrane potential sensitive dye. The assay is rapid, real-time, robust and requires minimal preparation. This assay should also obviate the problem of MOR receptor desensitisation during baseline measurements of AC inhibition.

## MATERIALS AND METHODS

### *MOR transfection and cell culture*

Flp-In T-Rex Chinese hamster ovary (CHO) cells were created as follows. CHO Flp-In cells were grown in minimal essential medium alpha (Invitrogen, Melbourne, Australia) containing 5% fetal bovine serum (FBS) and 100 μg/ml zeocin. They were transfected with pcDNA6TR (tet-repressor plasmid) using Fugene 6 reagent (Promega, Alexandria, Australia) and selected with 10 μg/ml blasticidin and 100 μg/ml zeocin. Six individual clones were isolated and transiently transfected with the plasmid pcDNA5-FRT-TRPV1,

which encodes the Transient Receptor Potential vanilloid 1 receptor (TRPV1) under control of a tetracycline-sensitive repressor. Clones were then tested for successful induction with 1 μg/ml tetracycline using a plated-based calcium assay of TRPV1 receptor activation.[13] One CHO-FRT-TR cell line was chosen and stably transfected with a pcDNA5 construct encoding the haemagglutinin-tagged human μ-opioid receptor cDNA together with the pOG44 (Flp recombinase plasmid) using the transfectant Fugene (Promega). The HA-tagged human μ-opioid receptor was synthesised by Genscript (Piscataway, New Jersey, USA). Cells expressing MOR were selected using hygromycin B (500 μg/mL) and grown to confluency. The selected cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 500 μg/mL hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 μg/mL beyond passage 5. Cells were passaged at 80% confluency as required. Assays were carried out on cells up to 30 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at greater than 80% confluence. The day before the assay cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. hMOR receptor expression was induced with 2 μg/mL tetracyclin 20 hrs prior to the assay. The cells were plated in a volume of 90 μl in black walled, clear bottomed 96-well microplates (Corning) and incubated overnight at 37$^oC$ in ambient $CO_2$.

***Membrane potential assay***

Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer containing (in mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407,

$MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 5). Prior to the assay, cells were loaded with 90 µl/well of the dye solution without removal of the L-15, giving an initial assay volume of 180 µl/well. Plates were then incubated at 37°C at ambient $CO_2$ for 60 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time either drug or vehicle was added in a volume of 20 µl. Further additions were made in volumes of 20 µl, as indicated. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. The final concentration of DMSO was not more than 0.1%, and this concentration did not produce a signal in the assay.

***Drugs and Chemicals***

Unless otherwise noted, tissue culture reagents and buffer salts were from Invitrogen or Sigma. [D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin (DAMGO), was purchased from Auspep (Tullamarine, Australia). Morphine was a kind gift from the Department of Pharmacology, University of Sydney. Buprenorphine was from the National Measurement Institute (Lindfield, Australia). Rp-8-(4-chlorophenylthio)adenosine-3',5'- cyclic mono-phosphorothioate, (Rp-8-CPT-cAMPS), Sp-8-(4-chlorophenylthio)adenosine- 3', 5'- cyclic monophosphorothioate (Sp-8-CPT-cAMPS) and 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, acetoxymethyl ester (8-CPT-2Me-cAMP) were from Biolog (Bremen, Germany). Membrane potential dye (blue) was from Molecular Devices (Sunnyvale, Califormia, USA). Calcitonin was from Bachem (Bubendorf, Switzerland). Nigericin was from Enzo Life Sciences (Farmingdale, NY,

USA). Forskolin, naloxone, H89, staurosporine, glibenclamide and 4-aminopyridine were from Ascent Pharmaceuticals (Bristol, UK). 1,9-dideoxyforskolin and tetraethylammonium (TEA) were from Sigma Aldrich (Castle Hill, Australia). Pertussis toxin (PTX) and VU-591 were from Tocris Bioscience (Bristol, UK). Charybdotoxin (CHX) was from Alexis Biochemicals (San Diego, US). KT-5720 was from Cayman Chemicals (Michigan, US).

***Data***

Unless otherwise noted, data is expressed as mean ± s.e.m. of at least 5 determinations made in duplicate or triplicate. Concentration response curves were fit with a 4 parameter logistic equation using Graphpad Prism (Graphpad). Statistical comparisons were made with an unpaired Student's T-test, unless otherwise noted. $P < 0.05$ was considered significant. All channel and receptor nomenclature is consistent with the British Journal of Pharmacology Guide to Receptors and Channels. [14]

To calculate Z', a measure of the robustness of the assay and indication of its suitability for HTS, the assay was performed on three separate occasions using assay buffer as the minimum response and either 300 nM FSK or 300 nM FSK with 3 μM DAMGO as the maximum response in 96-well plates. The Z' factor was calculated as outlined in Zhang *et al*.[15]

**RESULTS**

***Hyperpolarisation of CHO cells by calcitonin and forskolin***

CHO cells express calcitonin receptors, $G_s$-coupled G protein-coupled receptors (GPCR) which stimulate AC.[16] In CHO-MOR cells loaded with the proprietary membrane potential dye, addition of rat calcitonin produced an immediate decrease in fluorescence, consistent with hyperpolarisation of the cells (Figure 1A). The fluorescent signal continued to slowly

decrease for 10 min after the addition of calcitonin, after which fluorescent signals remained stable for the remainder of the assay. The decrease in fluorescence was concentration dependent, with $E_{max}$ of 46 ± 3% and $pEC_{50}$ of 9.4 ± 0.1 (n = 5, Figure 1B). Addition of forskolin (FSK) to CHO cells loaded with membrane potential dye produced a similar decrease in fluorescence to that observed with calcitonin, with the fluorescent signal stabilising 5 min after the addition of FSK (Figure 1A). The decrease in fluorescence for FSK was concentration dependent, with a maximal effect ($E_{Max}$) of 52 ± 2% and $pEC_{50}$ of 7.3 ± 0.1 (n = 6, Figure 1B). The AC-inactive FSK analogue 1,9-dideoxyforskolin (1 µM) did not produce a change in fluorescence (n = 5, Figure 3C, P > 0.1).

***Opioid inhibition of adenylyl cyclase***

Application of opioids alone did not affect the membrane potential of CHO-MOR cells. However, when the MOR agonist DAMGO was added together with a sub-maximally effective concentration of FSK (300 nM), DAMGO inhibited FSK induced membrane hyperpolarisation in a concentration dependent manner, consistent with inhibition of cAMP generation (Figure 2A). This response was blocked by naloxone (1 µM, Figure 2A). We measured the effects of opioids on the FSK-induced hyperpolarisation 5 min after co-application of the drugs. DAMGO inhibited the FSK-induced hyperpolarisation with a $pEC_{50}$ of 7.4 ± 0.1 and a maximum inhibition of 56 ± 3%. Maximum DAMGO inhibition of FSK-induced hyperpolarisation was reduced to 40 ± 2% ($p < 0.02$) when measured 10 minutes after FSK addition, while the potency was unchanged. Pretreatment of cells overnight with pertussis toxin (200 ng/ml) significantly reduced the inhibition of the FSK response by DAMGO (1 µM), inhibition was 81 ± 12% in control, and 11 ± 0.5 % after PTX treatment ($P < 0.01$, n=3). The change in fluorescence produced by FSK was unaffected by PTX treatment (46 ± 2% in control; 45 ± 5% after PTX, P = 0.73). FSK-

induced hyperpolarisation was observed in CHO-MOR cells where MOR expression had not been induced by tetracycline, however the hyperpolarisation was not inhibited by opioids (data not shown). DAMGO (1 µM) also reduced membrane hyperpolarisation produced by 10 nM calcitonin from 40 ± 1.6% to 23 ± 1.2% ($P < 0.01$).

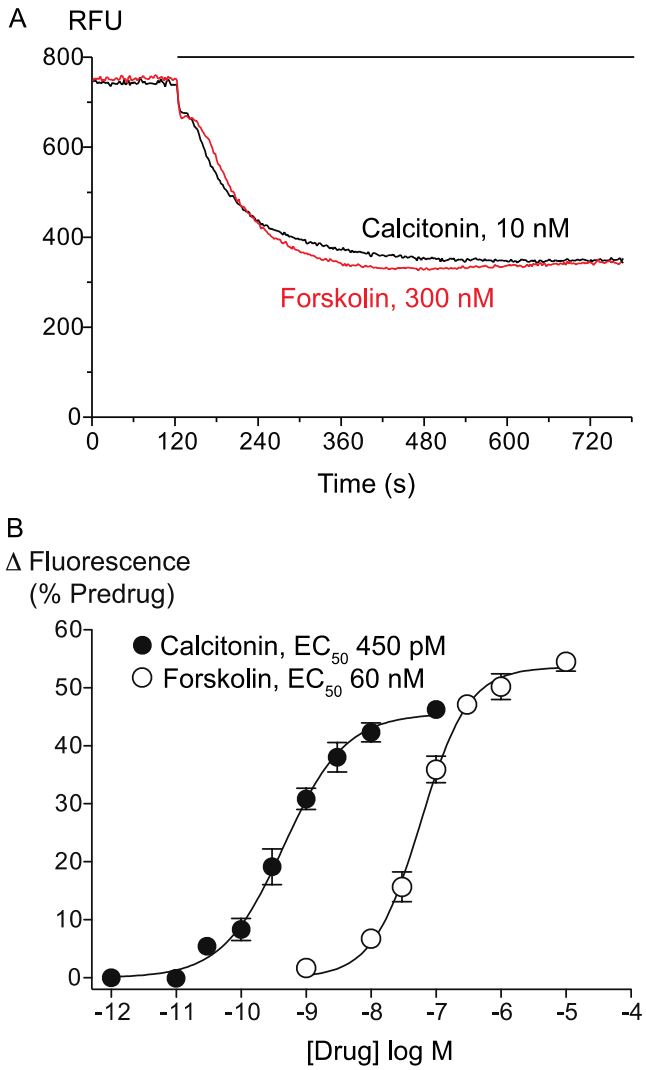
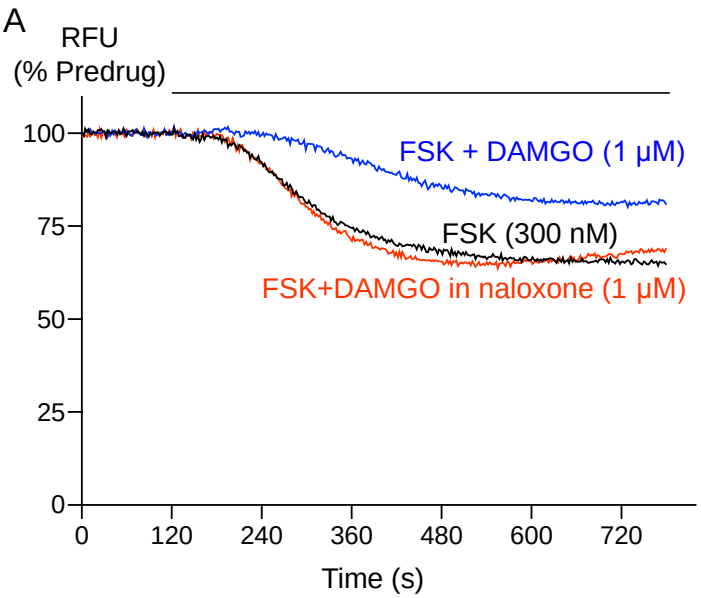



**Figure 1: Stimulating adenylyl cyclase hyperpolarises CHO cells**. The membrane potential of CHO cells was determined as outlined in the Methods. Both calcitonin and forskolin hyperpolarise CHO-MOR cells in a concentration dependent manner. A) Representative traces showing the raw fluorescence (RFU) from individual wells of a 96-well plate. Calcitonin (10 nM) or forskolin (1 µM) were added to CHO-MOR cells for the duration of the bar. B) Concentration response curves illustrating the effects of calcitonin and forskolin on the membrane potential of CHO-MOR cells. The calcitonin $E_{max}$ was 46 ±

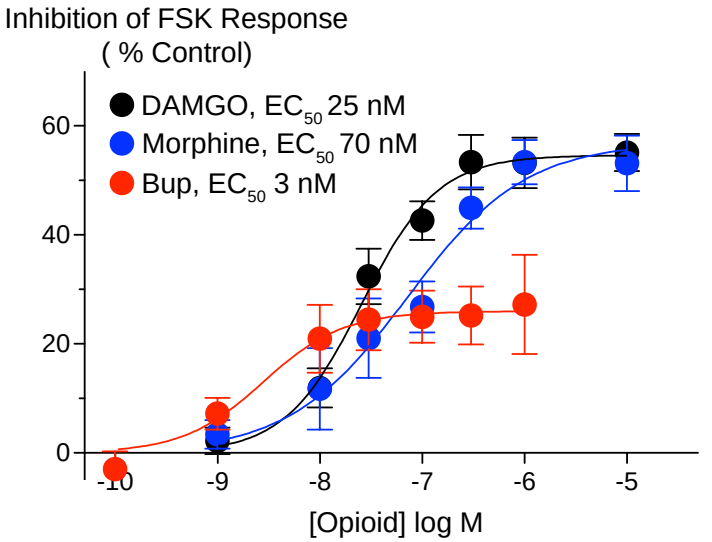
3% and $pEC_{50}$ was 9.4 ± 0.1. Forskolin $E_{max}$ 52 ± 2% and $pEC_{50}$ was 7.3 ± 0.1. Each point represents the mean ± s.e.m. of at least 5 experiments performed in triplicate.

**Figure 2: Opioids inhibit forskolin-stimulated membrane hyperpolarisation in CHO cells.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** Example traces showing the effects of forskolin (FSK, 300 nM), FSK and DAMGO (1 µM) applied simultaneously and FSK and DAMGO added in the presence of naloxone (NAL, 1 µM) which had been added 10 minutes earlier. FSK and DAMGO were added for the duration of the bar. **B)** Concentration-response curves for morphine, buprenorphine and DAMGO inhibition of the hyperpolarisation produced by FSK (300 nM). DAMGO inhibition of FSK stimulated AC activation was concentration-dependent, with an $E_{max}$ of 56 ± 3 %, and $pEC_{50}$ of 7.4 ± 0.1, morphine had an $E_{max}$ of 61 ± 7% and $pEC_{50}$ of 7.0 ± 0.2, Buprenorphine showed partial agonist activity with $E_{max}$ of 24 ± 4% and $pEC_{50}$ of 8.6 ± 0.5. **C)** Preincubation of the cells with increasing concentrations of naloxone (3, 10, 100 nM) produced a parallel shift in the concentration-response curve of morphine, with a $pA_2$ of -8.5 ± 0.1, (2.9 ± 0.5 nM, n=4).

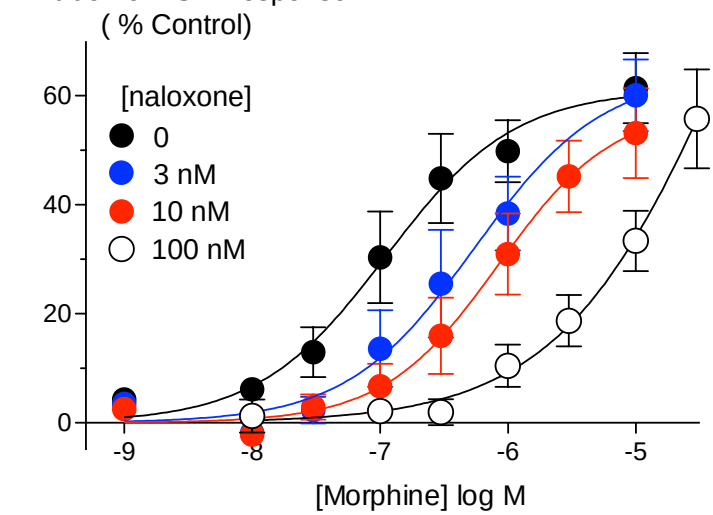
A


RFU
(% Predrug)
100
75
50
25
0
FSK + DAMGO (1 μM)
FSK (300 nM)
FSK+DAMGO in naloxone (1 μM)
0
120
240
360
480
600
720
Time (s)

B


Inhibition of FSK Response
( % Control)
DAMGO, EC50 25 nM
Morphine, EC50 70 nM
Bup, EC50 3 nM
60
40
20
0
-10
-9
-8
-7
-6
-5
[Opioid] log M

C


Inhibition of FSK Response
( % Control)
[naloxone]
0
3 nM
10 nM
100 nM
60
40
20
0
-9
-8
-7
-6
-5
[Morphine] log M

We assessed the capacity of this assay to reliably detect agonists of differing efficacy by examining the effects of morphine and buprenorphine (Figure 2B). Morphine and buprenorphine have previously been shown to have partial agonist activity at MOR.[17,18] Both agonists inhibited FSK-stimulated AC activation. Morphine had a similar efficacy as DAMGO, with $E_{max}$ of 61 ± 7%, and $pEC_{50}$ of 7.0 ± 0.2. Buprenorphine showed lower efficacy with $E_{max}$ of 24 ± 4%, and $pEC_{50}$ of 8.6 ± 0.5. Addition of increasing concentrations of naloxone produced a parallel shift in the concentration response curve for morphine, with a $pA_2$ of -8.5 ± 0.1 (2.9 ± 0.5 nM, n=3), a value consistent with the reported binding affinity of naloxone at human MOR[19] (Figure 2C).

***Mechanism of forskolin induced hyperpolarisation of CHO-MOR cells***

We sought to determine the mechanism by which AC activation caused membrane hyperpolarisation in CHO-MOR cells. The increase in cAMP resulting from AC activation can lead to activation of PKA, EPAC, or cAMP-gated ion channels.[6-7, 20] The membrane permeable cAMP analogue Sp-8-CPT-cAMPs (100 µM), a direct activator of PKA, mimicked the FSK response, producing a 44 ± 4% decrease in fluorescence (n=5, Figure 3A). Rp-8-CPT-cAMPs, a cAMP analogue that inhibits activation of PKA, did not produce a change in membrane fluorescence. The hyperpolarisation produced by Sp-8-CPT-cAMPs was not affected by DAMGO (Figure 3B), consistent with Sp-8-CPT-cAMPs producing its effects downstream of AC, potentially by acting directly on PKA. However, moderate concentrations of PKA inhibitors H89 (100 nM - 1 µM), KT5270 (100 nM – 1 µM) and staurosporine (1 µM) did not affect the hyperpolarisation produced by forskolin, and at higher concentrations (10 µM and above) the protein kinase inhibitors produced substantial hyperpolarisations by themselves. The cAMP analog 8-CPT-2Me-cAMP (100 µM), which selectively activates EPAC and not PKA, did not significantly affect cellular fluorescence (n=4, Figure 3C, $P > 0.5$).

Membrane hyperpolarisation usually occurs by efflux of K ions from cells. The non-specific K channel blockers tetraethyl ammonium chloride (TEA, up to 10 mM) or 4 aminopyridine (4-AP**,** up to 1 mM) did not inhibit the FSK-induced hyperpolarisation (Figure 4B). Additionally, the hyperpolarisation produced by FSK was not affected by the more selective $K_{ATP}$ channel blocker glibenclamide (10 μM), the renal outer medullary potassium channel ($K_{ir}$ 1.1) blocker VU-591 (10 μM) or charybdotoxin (10 μM)**,** a blocker of high-conductance Ca activated K channels ($K_{Ca}$1.1). To determine if FSK-induced membrane hyperpolarisation was due to K efflux, the extracellular K concentration ($[K]_{Ex}$) was increased to 30 mM and 75 mM in order to make the reversal potential ($K_e$) for K less negative. This will reduce K efflux and associated membrane potential changes when K channels are opened. $[K]_{Ex}$ was adjusted by dissolving the membrane potential dye in HBSS where NaCl was substituted by KCl. The effects of a maximally effective concentration of FSK (10 μM) was reduced by about 50% in 30 mM $[K]_{Ex}$, and almost completely when $[K]_{Ex}$ was 75 mM (Figure 4A), suggesting FSK-induced membrane hyperpolarisation is mediated by efflux of K from cells. The hyperpolarisations produced by 100 nM, 1 μM and 10 μM FSK in the 3 concentrations of $[K]_{Ex}$ were analysed by 2 way ANOVA and found a main effect of FSK ($P < 0.003$) and $[K]_{Ex}$ ($P < 0.0001$). Subsequent analysis of the effect of $[K]_{Ex}$ on each concentration of FSK using Tukey's multiple comparison test indicated that the hyperpolarisation produced by each concentration of FSK differed for each concentration of $[K]_{Ex}$ ($P < 0.05$ for each). The changes in fluorescence produced by altering the membrane K permeability were independently assessed by incubating CHO-MOR cells with nigericin, a potassium selective antibiotic ionophore.[21] A maximally effective concentration of nigericin (1 μM) produced a decrease in fluorescence signal of 70 ± 3%, which was not decreased any further upon the addition

of 10 μM FSK (Figure 4C). The fluorescent signal observed after nigericin incubation may reflect the signal when the membrane potential of the cells is driven to $E_K$.[21]

**Figure 3: Protein kinase A activators mimic the effects of forskolin.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** The cAMP analog Sp-8-CPT-cAMPs (100 μM, black trace), but not Rp-8-CPT-cAMPs (blue trace) mimicked the effect of FSK (100 nM, red trace). **B)** The hyperpolarisation produced by 30 μM Sp-8-CPT-cAMPs (black trace) was not inhibited by the simultaneous addition of 1 μM DAMGO (blue trace). **C)** The AC-inactive FSK analogue 1,9-ddFSK (1 μM) and the EPAC selective cAMP analogue 8-CPT-2Me-cAMP (100 μM) had little effect on CHO-MOR membrane potential when compared with 1 μM FSK. Bars represent the mean ± s.e.m of 4-5 determinations in triplicate.

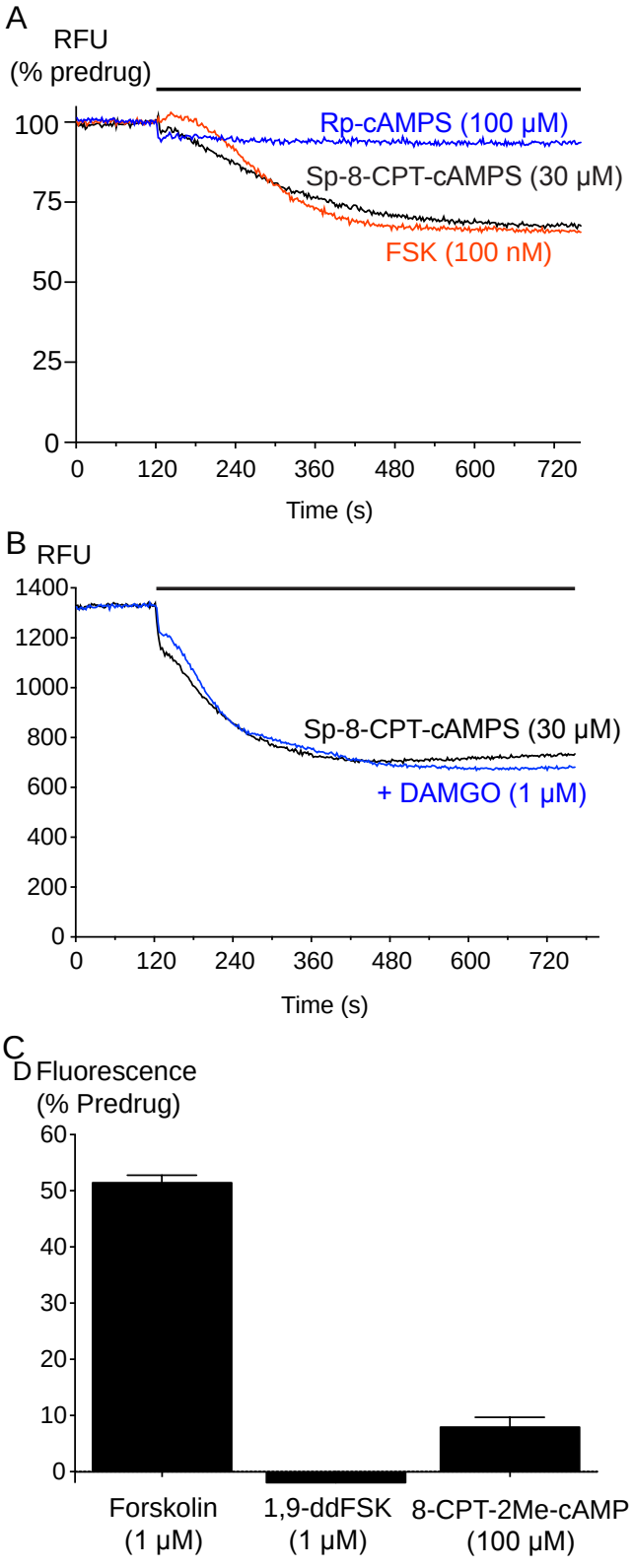
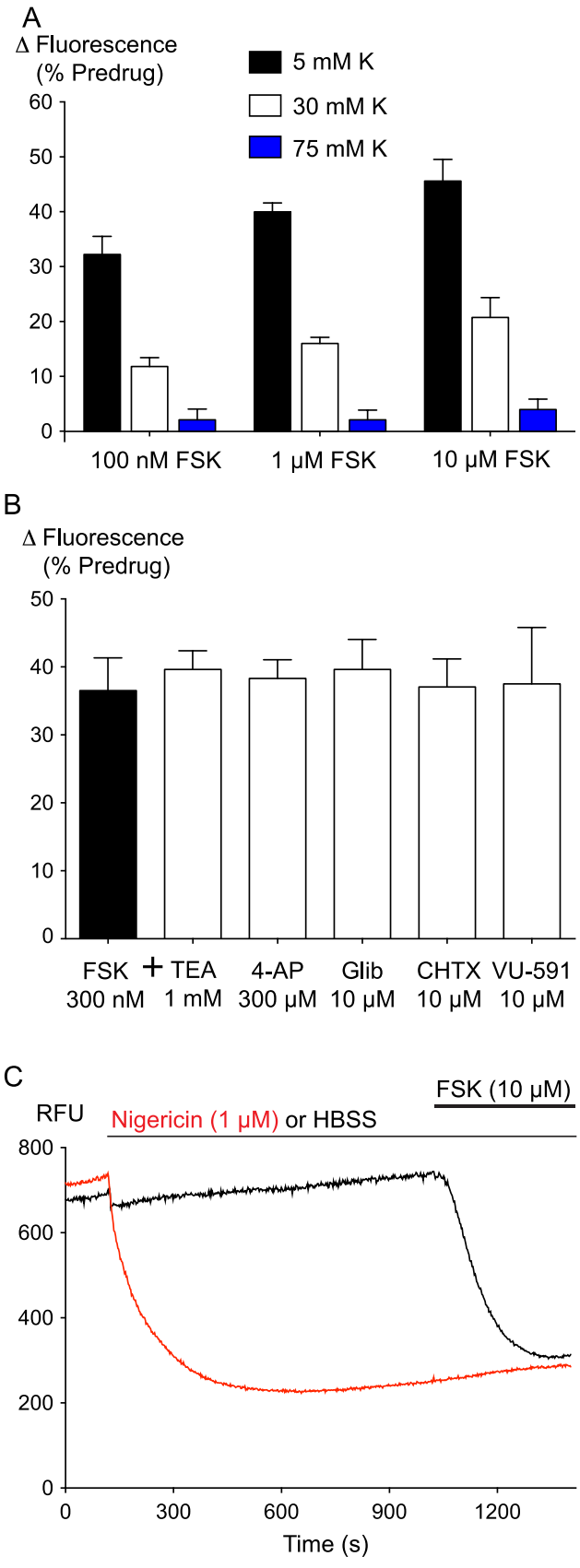

A
RFU
(% predrug)
100
75
50
25
0
Rp-cAMPS (100 µM)
Sp-8-CPT-cAMPS (30 µM)
FSK (100 nM)
0
120
240
360
480
600
720
Time (s)
B
RFU
1400
1200
1000
800
600
400
200
0
Sp-8-CPT-cAMPS (30 µM)
+ DAMGO (1 µM)
0
120
240
360
480
600
720
Time (s)
C
D
Fluorescence
(% Predrug)
60
50
40
30
20
10
0
Forskolin
(1 µM)
1,9-ddFSK
(1 µM)
8-CPT-2Me-cAMP
(100 µM)

**Figure 4: The forskolin-induced hyperpolarisation is dependent on extracellular K concentration.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** The effects of forskolin (FSK) were reduced as the extracellular K concentration was changed from 5 mM to 30 mM or 75 mM. K was replaced with equimolar Na. The hyperpolarisations produced by 100 nM, 1 µM and 10 µM FSK in the 3 concentration of $[K]_{Ex}$ were analysed by 2 Way ANOVA and found a main effect of FSK ($P < 0.003$) and $[K]_{Ex}$ ($P < 0.0001$). **B)** Membrane hyperpolarisation produced by FSK (300 nM) was not affected the non-specific $K^+$ channel blockers tetraethylammonium chloride (TEA) or 4-aminopyridine (4-AP), the selective $K_{ATP}$ channel blocker glibenclamide (Glib), charybdotoxin (CHTX), a blocker of high-conductance Ca activated K channels and the renal outer medullary K channel channel blocker VU-591. **C)** The K selective ionophore nigericin (1 µM) produced a decrease in fluorescence that occluded the effects of a high concentration of FSK (10 µM).


A
Δ Fluorescence
(% Predrug)
5 mM K
30 mM K
75 mM K
60
50
40
30
20
10
0
100 nM FSK
1 μM FSK
10 μM FSK
B
Δ Fluorescence
(% Predrug)
50
40
30
20
10
0
FSK + TEA
300 nM 1 mM
4-AP
300 μM
Glib
10 μM
CHTX
10 μM
VU-591
10 μM
C
FSK (10 μM)
RFU
Nigericin (1 μM) or HBSS
800
600
400
200
0
0
300
600
900
1200
Time (s)

We assessed the suitability of this assay for HTS by calculating the Z' factor, a measure of the assay robustness. An assay with a Z' factor of between 0.5 and 1 is an appropriate assay in terms of signal-to-noise ratio and data reproducibility.[15] The Z' factor for this assay was calculated for both the 300 nM FSK response and for inhibition of the FSK response with 3 μM DAMGO in multiple experiments. The Z' values for FSK alone were 0.7, 0.7, and 0.8, and for FSK + DAMGO were 0.7, 0.7 and 0.7, indicating that the assay is suitable for HTS.

**DISCUSSION**

In this study, we have developed a real time, no wash, fluorescence-based assay for MOR-mediated inhibition of AC in intact CHO-K1 cells. We used a proprietary membrane potential sensitive dye from Molecular Devices to measure membrane hyperpolarisation following FSK stimulated AC activation.[22] Activation of endogenous calcitonin receptors in CHO-K1 cells[16] produced a reduction in fluorescence similar to that seen following FSK application, and in both cases this reduction was less than that produced by application of the K-selective ionophore nigericin[21]. The fluorescent signal rapidly and reliably decreased after FSK application, and this decrease in signal was inhibited by the simultaneous application of opioid agonists.

CHO cells are frequently used in assays of opioid inhibition of AC activity. The elevation of cAMP in CHO cells following FSK application, as well as the ability of opioids to inhibit this elevation of cAMP is well documented.[16, 23-24] In this assay, opioid inhibition of FSK-stimulated membrane hyperpolarisation is consistent with opioid inhibition of AC, one of the hallmarks of MOR activation.[3] CHO cells have been shown to express AC subtypes VI and VII.[25] Opioid modulation of AC activity is isozyme specific, with acute opioid treatment shown to inhibit $G_s$-stimulated AC-VI activity and potentiating $G_s$-

stimulated AC-VII.[26] Because AC-VII has been reported to be insensitive to FSK,[32] we chose to use FSK rather than calcitonin as the AC-stimulating agent, as this should avoid the confounding effects of opposite MOR modulation of the $G_s$-mediated stimulation of AC-VI and AC-VII.

Morphine and buprenorphine are lower efficacy agonists at MOR[17,18], however the measured efficacy of a compound depends on the assay being used. In the present assay, morphine acted as a full agonist, with an $E_{max}$ similar to that of DAMGO. This likely reflects the relatively low receptor occupancy required for AC inhibition, particularly as we were stimulating AC with a submaximally effective concentration of forskolin. Many previous studies have reported morphine to be a full agonist in assays of AC inhibition.[4,28]

The measurement of MOR inhibition of AC is often performed using end-point assay techniques with lengthy inhibition times. As shown in our assay, some sensitivity would be lost using this approach. Inhibition of the FSK-stimulated membrane hyperpolarisation peaked at approximately 5 minutes after the addition of FSK and opioid. After this time, the fluorescent signal gradually decreased further. A single time-point measurement of cAMP accumulation after 10 minutes in our assay shows a reduction in the efficacy of DAMGO in AC inhibition, with $E_{max}$ decreased from 56% to 40%, possibly reflecting receptor desensitisation. While we chose to measure at a time point when the FSK-induced hyperpolarisation signal had plateaued, it would be a simple matter to measure at any time point after addition of the drugs, enabling the virtually instantaneous measurement of cell responses after the addition of FSK and/or opioid. Depending on the agonist employed, MOR rapidly desensitises and/or internalises, with up to 50% of MOR internalized within 5 minutes of agonist exposure in some cells.[11-12,20] AC assays using single time point measurement of cAMP accumulation after incubation times of up to 20

minutes are measuring the summed output of signalling from activated, desensitized and internalized MOR, and thus give little insight into the real-time dynamics of the effects of MOR activation on cAMP mediated signalling.

The measurement of real-time AC inhibition has previously only been achievable by the use of complex techniques such as transfection of reporter genes or proteins with cAMP binding domains, or bioluminescence resonance energy transfer (BRET) assays.[8, 29-32] These assays can be very useful for studying cAMP signalling, although each has its potential weaknesses as well as strengths.[8] Bioluminescence assays such as the Glosensor (Promega) can provide a continuous reading of cAMP levels, allowing kinetic studies and repeated drug applications[30]. However, this assay requires transfection of a luciferase sensor construct with cAMP binding domains and use of specialized reagents, in addition to any stable transfections of the receptors of interest. For the kind of studies described in this paper – inhibition of cAMP accumulation by a $G_i/G_o$-coupled GPCR, the developers of the Glosensor assay recommend a 5-10 minute pre-incubation with agonist before the addition of FSK, which is likely to be unsuitable for studies of rapidly desensitizing GPCR such as MOR.[30] The assay described here is simpler than these assays, requiring transfection of the receptor of interest only and addition of a single assay reagent. The drug responses can be observed immediately following agonist addition. The Z' calculated for this assay were similar to those reported for the Glosensor assay.[31,32] However, as the plate reader format means that we are unable to study recovery of AC activity following wash of agonists, and the kinetic and stoichiometric relationship between the FSK-induced rise in AC levels and hyperpolarisation of the CHO cells is unknown.

Activation of the endogenous $G_s$-coupled CTR receptors in CHO cells caused membrane hyperpolarisation similar to that produced by the AC activator FSK. Membrane

hyperpolarisation in CHO cells by AC activation has not been shown previously, so we sought to determine the mechanism by which membrane hyperpolarisation occurs. Endogenous K channels are not well defined in CHO cells, and CHO cells are frequently used as heterologous expression systems for recombinant K channels due to the apparent low levels of native K channel activity.[33] The non-specific K channel inhibitors TEA and 4-AP did not inhibit FSK induced membrane hyperpolarisation, nor did the more specific K channel inhibitors glibenclamide, VU-591 or charybdotoxin. However, when the reversal potential for K was made less negative by increasing $[K]_{Ex}$, membrane hyperpolarisation was reduced, and hyperpolarisation was essentially abolished when $[K]_{Ex}$ was increased to 75 mM. Furthermore, the FSK induced membrane hyperpolarisation was mimicked and occluded by the addition of the K-selective ionophore nigericin. This suggests that FSK induced membrane hyperpolarisation is due to the movement of K ions through native K channels in CHO cells.

Membrane hyperpolarisation resulting from application of FSK and calcitonin is due to the activation of AC.[16,22] The resulting elevation of cAMP levels in the cell leads to the activation of PKA, as well as EPACs. The cAMP analogue Sp-8-CPT-cAMPs, a direct activator of PKA, mimicked the effects of FSK, while Rp-8-CPT-cAMPs, an inhibitor of cAMP activation of PKA, was inactive. 8-CPT-2Me-cAMP, a selective activator of EPAC, also produced no effect. Together these data are consistent with the idea that membrane hyperpolarisation is occurring via PKA activation. However, we were unable to inhibit the effects of FSK with modest concentrations of protein kinase inhibitors, and higher concentrations of these drugs themselves hyperpolarized the CHO cells. Novel cAMP-dependent signal transduction pathways are still being discovered,[34] and it may be that the observed hyperpolarisation of CHO cells is mediated by such a mechanism.

Assays of AC inhibition represent one of the most straightforward ways of studying Gα (as opposed to Gβγ) signalling in a high throughput environment. The assay described here offers a novel approach for measuring MOR-mediated AC inhibition in intact CHO cells, and has the advantages of being both real-time and reflecting the naturalistic coupling of MOR to the signalling pathway. The lack of a defined mechanism for the hyperpolarisation does not detract from the utility of the assay for acute studies, however, the assay may not be suitable for studies of more complex signalling cascades such as those potentially underlying agonist-induced receptor regulation. Nevertheless, our results show the membrane potential assay to be a rapid, reliable and inexpensive method for assessing opioid activation of MOR in CHO cells, and may be scaled up to enable high-throughput screening. Coupled with our recent description of a HTS-appropriate membrane potential assay of Gβγ signalling in AtT-20 cells[35], it is clear that multiple aspects of GPCR signalling can be studied in a relatively simple, non-invasive and efficient manner using simple reagents which report changes in basic cellular properties such as membrane potential.

**Acknowledgements**

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# Chapter 4

## A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells

**Alisa Knapman, Marina Santiago, Yan Ping Du, Philip R. Bennallack, Macdonald J. Christie and Mark Connor**

This paper was published in 2013 in the *Journal of Biomolecular Screening* **18:** 269-276. Yan Ping Du and Macdonald Christie constructed AtT-20 cells stably expressing mouse MOPr, and quantified receptor expression. Marina Santiago and Phillip Bennallack designed and performed desensitisation experiments. Mark Connor assisted in experimental design and manuscript preparation. All other work is my own.

**ABSTRACT**

Opioids are widely prescribed analgesics, however their use is limited due to development of tolerance and addiction, as well as high variability in individual response. The development of improved opioid analgesics requires high-throughput functional assays to assess large numbers of potential opioid ligands. In this study, we assessed the ability of a proprietary "no wash" fluorescent membrane potential dye to act as a reporter of μ-opioid receptor (MOR) activation and desensitisation via activation of G protein-coupled inwardly rectifying potassium channels. AtT-20 cells stably expressing mouse MOR were assayed in 96-well plates using the Molecular Devices FLIPR membrane potential dye. Dye emission intensity decreases upon membrane hyperpolarisation. Fluorescence decreased in a concentration-dependent manner upon application of range of opioid ligands to the cells, with high efficacy agonists producing a decrease of 35% to 40% in total fluorescence. The maximum effect of morphine faded in the continued presence of agonist, reflecting receptor desensitisation. The effects of opioids were prevented by prior treatment with pertussis toxin and blocked by naloxone. We have demonstrated this assay to be an effective method for assessing ligand signalling at MOR which may potentially be scaled up as an additional HTS technique for characterizing novel opioid ligands.

## INTRODUCTION

Opioid analgesics are the most widely prescribed drugs in the treatment of moderate to severe pain. Despite their powerful analgesic effects, the use of opioids in the treatment of chronic pain can be problematic due to the development of tolerance and physical or psychological dependence[1]. Over time, increasing doses of opioids become necessary to maintain analgesia. The increased toxic side effects such as respiratory depression, constipation and nausea associated with escalating doses of opioids can reach unacceptable levels, leading to inadequate pain relief or overdose. Opioids, however, continue to be the mainstay of chronic pain treatment due to a lack of suitable alternative drugs. As such, there is a substantial need for the development of new opioid analgesics, with reduced adverse effects and a decreased ability to produce tolerance. The μ opioid receptor (MOR) is the primary site of action for most clinically important opioid drugs, and as such is the major target for the development of improved opioid analgesic drugs[2]. It is generally accepted that there are not functionally important subtypes of MOR[3], and so aside from novel formulations or delivery strategies, the development of new pharmacotherapies targeting MOR is likely to focus on subtleties of receptor signalling and regulation.

MOR is a member of the G protein-coupled receptor (GPCR) superfamily, ubiquitous cell-surface receptors that act as cellular switches to regulate most cellular signalling processes[4]. GPCR agonists stabilise active conformations of their receptors, leading to signalling via both the α and βγ subunits of the associated heterotrimeric G protein complex and sometimes also via non-G protein-dependent pathways[5]. GPCR signalling is complex, with different ligands preferentially activating (or inhibiting) distinct signalling pathways at the same receptor[5]. There is also increasing evidence that distinct ligands also differentially engage pathways which regulate receptor activity during prolonged agonist exposure, a topic of intense investigation with regard to opioid receptors[6,7,8]. These ideas

have lead to renewed interest in the possibility of developing ligands targeting MOR which engage only subsets of signalling systems or regulatory pathways, potentially leading to drugs with more favourable clinical profiles.

Drug development typically involves the screening of large libraries of lead compounds to identify those capable of binding to and signalling via a receptor. The vast array of lead compounds available requires high-throughput screening methods for efficient detection of potential therapeutic compounds. Radioligand binding studies are often used to identify candidates, but determination of ligand efficacy requires some sort of signalling response. For opioid receptors, this can be achieved using cell lines expressing engineered G proteins which couple to processes such as intracellular calcium ($[Ca]_i$) mobilisation[9], cAMP-dependent gene transcription or more traditional assays of adenylyl cyclase (AC) activity which require harvesting or lysing of cells[10]. In this study we sought to develop a minimally invasive assay that reflected a naturalistic coupling of MOR and which could potentially be used to readily examine agonist regulation of receptor signalling. In mouse pituitary AtT-20 cells, heterologously expressed MOR inhibit native calcium channels ($I_{Ca}$)[11] and activate endogenous G protein gated inwardly rectifying potassium channels[12] (GIRKs), and in both cases this signalling is subject to rapid, agonist-induced regulation. We assessed the suitability of a proprietary fluorescent membrane potential assay to act as a robust reporter of MOR activation and desensitisation in AtT-20 cells, with the view to potentially providing an assay for high throughput screening of both these important aspects of MOR function.

## MATERIALS AND METHODS

### *FLAG-MOR Transfection and Cell Culture*

Mouse AtT-20 neuroblastoma cells were stably transfected with the cDNA encoding the FLAG epitope-tagged mouse μ-opioid receptor using the transfectant Lipofectamine (Gibco BRL) as previously described[11]. The pcDNA3 FLAG-MOR construct was a kind gift from Dr. Lakshmi Devi (Mt Sinai School of Medicine, New York, USA). Geneticin (500 μg/ml) was added to select for clones expressing FLAG-MOR protein. During *in situ* identification of positive clones using Alexa-488 coupled FLAG-MOR antibody (Sigma, F7425), 48 potentially suitable single cells were transferred to single wells using a micropipette and were grown to confluence for subsequent determination of cell surface MOR binding density. MOR binding density was determined on intact cells by incubation with increasing triplicate concentrations of [$^3$H] DAMGO (0.125 - 32 nM; Perkin Elmer, USA) at 4$^o$C in 50mM Tris-Cl, pH7.4 for 2h. Briefly, approximately 1 x $10^5$ cells were plated in 24-well plate coated with poly-L-lysine overnight. Cells were then rinsed gently twice with 50mM Tris-Cl, pH7.4, placed on ice and incubations in the radioligand were commenced. Non-specific binding (less than 2% of total binding at [$^3$H] DAMGO 5 nM) was determined in the presence of unlabelled DAMGO (10 μM). At the end of the incubation plated cells were rinsed three times with 1 ml 50mM Tris-Cl, pH 7.4 at 4$^o$C. Cells in each well were then digested for 1 h at room temperature with 100 μl of 1N NaOH. 100 μl 1N HCl was then added to each well and collected into scintillation vials and bound ligand determined using a liquid scintillation counter (Packard Tricarb, USA). Specific binding was plotted, and Kd and Bmax for each clone determined using GraphPad Prism. One clone expressing a moderate density of surface FLAG-MOR was selected for subsequent experiments. The Kd for [$^3$H]-DAMGO binding was 1.2 nM and receptor density was 10.2 pmol/mg protein. After counting cell numbers used for protein determination it was estimated that 2.5 x $10^7$ cells yielded one mg protein. Therefore 10.2

pmol/mg protein represents approximately 2.5 x $10^5$ receptors per cell. The selected clone of AtT-20 cells stably expressing mouse FLAG-MOR was then cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100U penicillin/streptomycin and 300µg/mL G418. Cells were passaged at 80% confluency as required. Assays were carried out on cells up to 25 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at 90% confluence. The day before the assay cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. The cells were plated in volume of 90 µl in black walled, clear bottomed 96-well microplates (Corning) and incubated overnight in ambient $CO_2$.

***Membrane Potential Assay***

Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer supplied with the kit or with a low-K modification. The standard assay buffer contained (mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, KCl 5.33, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, Glucose 5.56 (pH 7.4, osmolarity 315 ± 5). The modified buffer was formulated without the addition of 5.33 mM KCl. Taking into account the K concentration of L-15, the final in-well concentrations of K were 5.55 mM (standard) and 2.88 mM (low K) respectively. Prior to the assay, cells were loaded with 90 µl/well of the dye solution without removal of the L-15, giving an initial assay volume of 180 µl/well. Plates were then incubated at 37°C at ambient $CO_2$ for 45 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time either drug or vehicle was added in a volume of 20 µl. Further additions were made in volumes of 20 µl, as indicated. The

background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition, which was less than 2 % for drugs dissolved in assay buffer or DMSO. The final concentration of DMSO was not more than 0.1%.

***Data Analysis and Calculation of Z' values***

Concentration response data was analysed using PRISM (GraphPad Software Inc., San Diego, CA), using four-parameter non-linear regression to fit concentration-response curves. The time course of morphine-induced receptor desensitisation was fit with a single phase exponential to obtain an estimated t/2 for the process. To calculate Z', a measure of the robustness of the assay and its suitability for HTS, the membrane potential assay was performed on 3 separate occasions using assay buffer as the negative control and 300 nM DAMGO as the positive control in 96-well plates. The Z' factor was calculated as outlined in Zhang *et al.*, (1999)[13].

**RESULTS**

In AtT-20 cells loaded with the proprietary membrane potential dye, addition of the peptidergic MOR agonist DAMGO or the prototypic alkaloid opioid morphine produced a rapid decrease in fluorescence, consistent with hyperpolarisation of the cells (Figure 1). It took about 30s for high concentrations of morphine to maximally hyperpolarise the cells (Table 1). The decrease in fluorescence produced by morphine was concentration dependent and reversed by addition of the opioid receptor antagonist naloxone (Figure 2A). Addition of increasing concentrations of naloxone produced a parallel shift in the concentration response curve for morphine, with a $pA_2$ of -8.5 ± 0.1, (2.9 ± 0.5 nM, n=3), a value consistent with the reported binding affinity of naloxone at mouse MOR (2 nM)[14]

(Figure 2B). Pretreatment of cells overnight with pertussis toxin (200 ng/ml) prevented the decrease in fluorescence by DAMGO and morphine (Figure 3). Addition of morphine or DAMGO to AtT-20 cells not transfected with MOR produced no change in fluorescence.

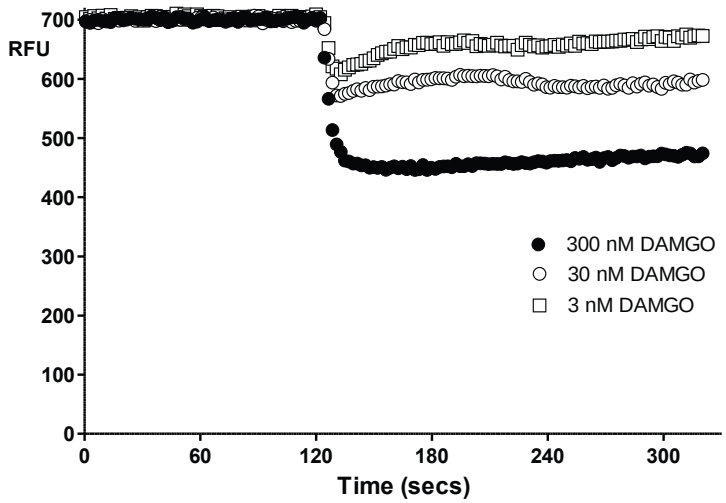
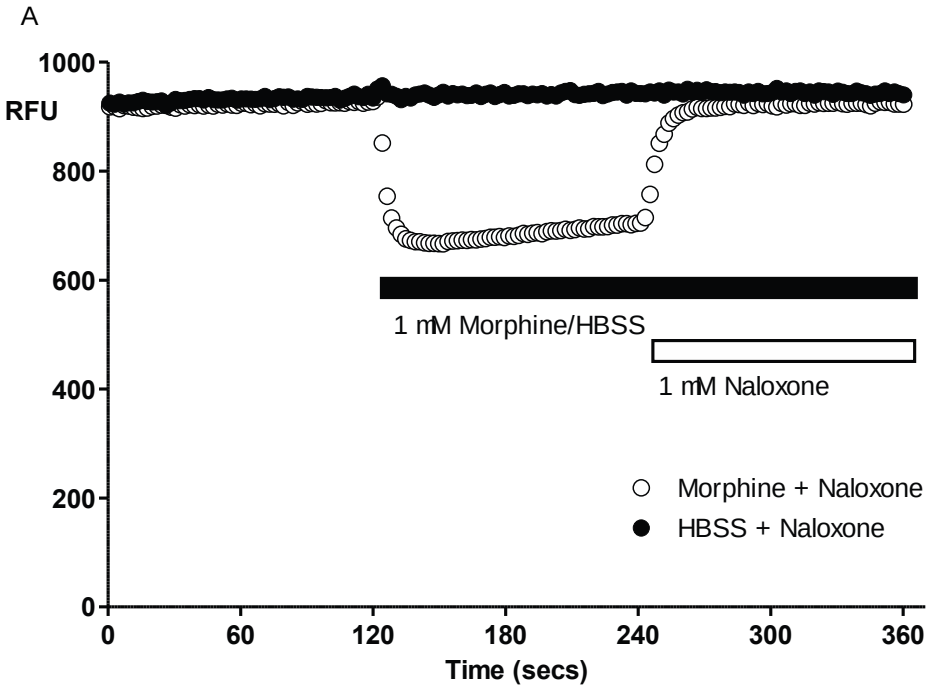


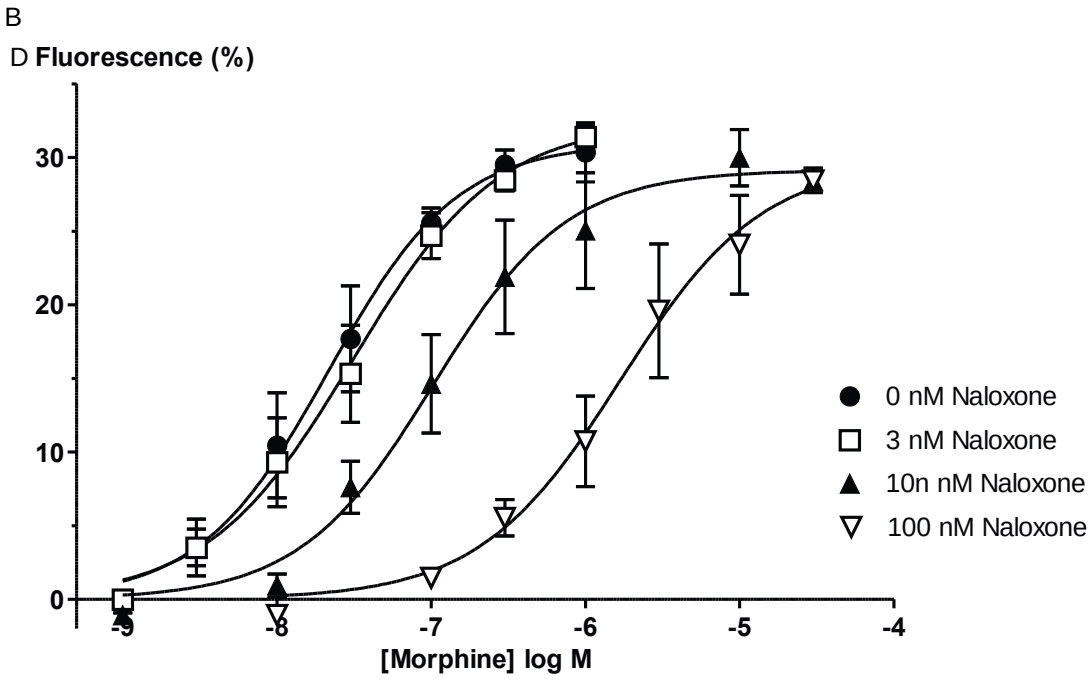

**Figure 1:** Example traces of fluorescent signal in the membrane potential assay over 300 sec. Baseline readings were taken every 2 sec for 120 sec, at which point increasing concentrations of DAMGO (3 nM, 30 nM, 300 nM) were added to AtT-20MOR cells, resulting in concentration-dependent decreases in fluorescent signal as shown.

Using the assay buffer supplied with the kit, the maximal decrease in fluorescence produced by DAMGO was 31 ± 1% (n=6). We sought to optimise the assay by decreasing extracellular $[K]_{Ex}$ from 5.6 mM to 2.9 mM in order to make the reversal potential ($K_e$) for K more negative. The reduction in $[K]_{Ex}$ was achieved by dissolving the dye in saline containing no added KCl. The reduction of $[K]_{Ex}$ resulted in maximally effective concentrations of DAMGO producing a 7 % greater decrease in total fluorescence ($P < 0.01$, Students T-test). The $E_{max}$ and $pEC_{50}$ for DAMGO in 5.6 mM $[K]_{Ex}$ were 31 ± 1% and 8.3 ± 0.1 respectively, while in 2.9 mM $[K]_{Ex}$, $E_{max}$ and $pEC_{50}$ for DAMGO were 38 ± 2% and 8.3 ± 0.1 respectively (Figure 4).

The maximum response elicited by DAMGO was similar to that elicited by somatostatin ($E_{max}$ = 39 ± 3.2%, $pEC_{50}$ 8.7), which acts at endogenous $sst_2$ and $sst_5$ receptors to activate GIRK channels in AtT-20 cells[15]. We independently assessed the changes in fluorescence produced by altering the membrane $K^+$ permeability by incubating AtT-20 cells with nigericin, a potassium selective antibiotic ionophore[16]. A maximally effective concentration of nigericin (1 μM) produced a decrease in fluorescence signal of 50 ± 3%, which was not decreased any further upon the addition of 300 nM DAMGO. The fluorescent signal observed after nigericin incubation may reflect the signal when the membrane potential of the cells is driven to $E_K$[16] (Figure 5).

**Figure 2:** (A) Trace of fluorescent signal illustrating the reversal of DAMGO stimulated decrease in signal by MOR antagonist naloxone. 1 µM DAMGO/HBSS was added to AtT-20MOR cells at 120 secs, followed by addition of 1 µM naloxone at 240 secs. The DAMGO stimulated decrease in fluorescent signal was completely reversed by naloxone. Naloxone had no effect when applied without previous addition of DAMGO. (B) Concentration response curve for morphine, both alone and with the addition of 3 nM, 10 nM and 100 nM naloxone. The addition of naloxone produced a parallel shift in the morphine concentration response curve with a $pA_2$ of -8.5 ± 0.1, (2.9 ± 0.5 nM, n=3).


A
1000
RFU
800
600
400
200
0
0
60
120
180
240
300
360
Time (secs)
1 mM Morphine/HBSS
1 mM Naloxone
Morphine + Naloxone
HBSS + Naloxone


B
D Fluorescence (%)
30
20
10
0
-9
-8
-7
-6
-5
-4
[Morphine] log M
0 nM Naloxone
3 nM Naloxone
10n nM Naloxone
100 nM Naloxone

We assessed the capacity of the fluorescence assay to reliably detect opioid ligands of differing efficacy by treating AtT-20MOR cells with a range of structurally distinct opioid ligands. All agonists tested produced a concentration-dependent decrease in cellular fluorescence. High efficacy agonists such as fentanyl, DAMGO and β-endorphin produced a maximum decrease in fluorescence of 35-40%, while morphine, buprenorphine and pentazocine were shown to be partial agonists (Figure 6). A rank order of ligand efficacy was established (Table 1).

We assessed the suitability of this assay for HTS by calculating the Z' factor, a measure of the assay robustness. An assay with a Z' factor of between 0.5 - 1 is an excellent assay in terms of signal/noise ratio and data reproducibility[13]. The Z' factor for this assay was calculated in multiple experiments, with Z' values of 0.6, 0.7 and 0.7, indicating that the assay is suitable for HTS.

A hallmark of opioid signalling is agonist-dependent desensitisation, where continuous application of agonist results in a relatively rapid decline in receptor activation. The decrease in fluorescence produced by application of high concentrations of morphine faded in the continued presence of agonist, reaching a plateau after about 30 minutes (Figure 7A). In order to assess whether the decline in fluorescence reflected a change in MOR signalling, we incubated cells with a high concentration of morphine (1 μM) and then challenged them with subsequent addition of 10 μM morphine. The response to 10 μM morphine declined significantly over time. When fit with a one phase exponential association function, the peak response to 10 μM morphine declined with a τ of 490 s (95 % C.I. 413–603 s) to a maximum inhibition of 72 % (95 % C.I. 67–76 %, , Figure 7B). In order to assess whether the decline in morphine effectiveness reflected heterologous desensitisation, we repeated the experiments using 1 μM somatostatin as the challenge

drug. The response to somatostatin also declined during continuous morphine exposure (τ of 460 s, 95 % C.I. 331-748 s), the maximum inhibition of the somatostatin response was 30 % (95 % C.I. 27-34 %). Preincubation with naloxone (1 μM) did not affect the hyperpolarisation produced by somatostatin (10 nM; control 36 ± 1 %, in naloxone 35 ± 1 %).

**Table 1:** Potencies and efficacies of the range of structurally distinct opioid ligands tested using the membrane potential assay in AtT-20 cells. Ligands are ranked in order of efficacy. Ligands with $E_{max}$ significantly different to that of DAMGO are marked with * ($P < 0.05$, extra sum of squares F test). Latency is the time taken for the peak signal to be reached after a maximal concentration of drug was added.

| OPIOID AGONIST | *p*$EC_{50}$ | $E_{max}$ (%) | Hill Slope | Latency (s) |
|---|---|---|---|---|
| DAMGO | 8.3 ± 0.1 | 38 ± 2 | 0.8 ± 0.1 | 29 ± 1 |
| Endomorphin 2 | 8.4 ± 0.1 | 37 ± 2 | 1.0 ± 0.1 | 32 ± 2 |
| Endomor phin 1 | 8.7 ± 0.1 | 36 ± 1 | 0.9 ± 0.1 | 27 ± 1 |
| B-endorphin | 7.0 ± 0.1 | 36 ± 3 | 1.2 ± 0.2 | 33 ± 4 |
| Fentanyl | 9.3 ± 0.1 | 35 ± 1 | 1.0 ± 0.1 | 27 ± 2 |
| Met-Enkephalin * | 8.5 ± 0.1 | 34 ± 1 | 0.8 ± 0.1 | 30 ± 3 |
| Methadone * | 7.6 ± 0.1 | 32 ± 1 | 1.2 ± 0.2 | 29 ± 3 |
| Morphine * | 7.7 ± 0.1 | 31 ± 1 | 1.0 ± 0.2 | 29 ± 2 |
| Oxycodone * | 6.7 ± 0.1 | 28 ± 1 | 1.2 ± 0.1 | 24 ± 3 |
| Buprenorphine * | 8.0 ± 0.1 | 21 ± 1 | 0.8 ± 0.3 | 12 ± 4 |
| Pentazocine * | 6.2 ± 0.2 | 5 ± 1 | 1.6 ± 0.3 | 18 ± 2 |

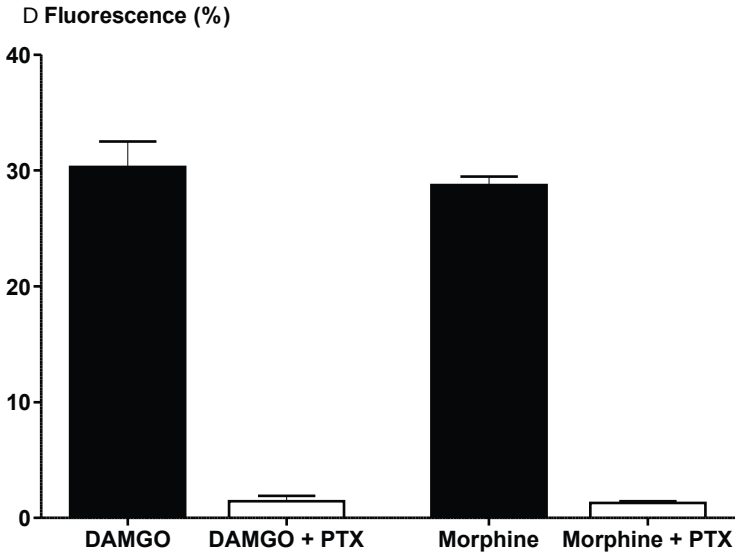



**Figure 3:** The decrease in fluorescent signal observed with 30 nM DAMGO or 100 nM morphine was abolished following overnight incubation of AtT-20-MOR cells with 200ng/mL PTX.

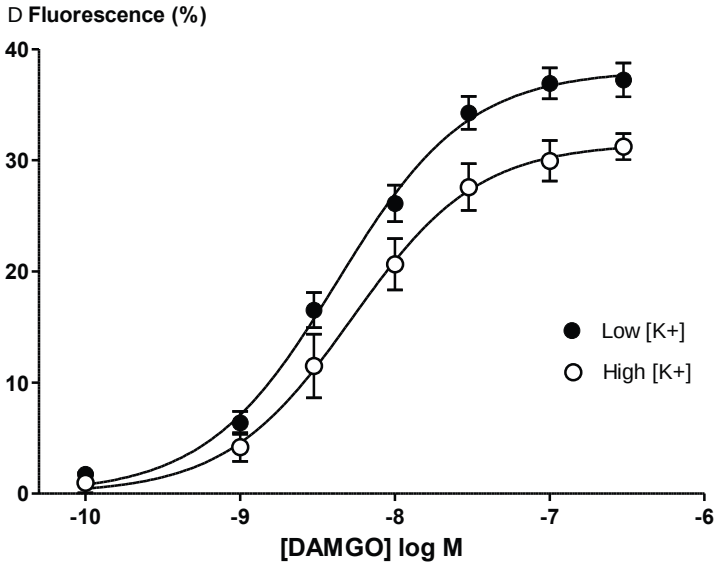



**Figure 4:** Decreasing extracellular potassium concentration ($[K]_{Ex}$) increases the maximum change in fluorescent signal observed with DAMGO. Concentration-response curves were generated for DAMGO activation of GIRK channels in both low $[K]_{Ex}$ (2.9 mM) and high $[K]_{Ex}$ (5.5 mM) conditions. $E_{max}$ in low $[K]_{Ex}$ was 38 ± 2%, 21% higher than in high $[K]_{Ex}$ ($E_{max}$ 31± 1%; $P < 0.01$).

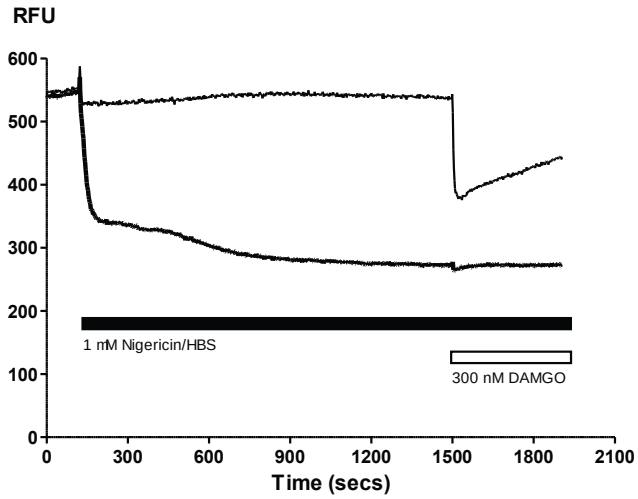



**Figure 5:** Treatment of AtT-20-MOR cells with nigericin, a K selective antibiotic ionophore, caused a greater decrease in fluorescent signal than the maximally effective concentration of DAMGO ($P < 0.05$). 1 µM nigericin (heavy trace), or vehicle (light trace) was added to cells at 120 secs. Nigericin caused a decrease of 50 ± 3% in fluorescent signal. Membrane potential was allowed to reach equilibrium before the addition of 300 nM DAMGO at 1500 sec, which did not cause any further decrease in signal.

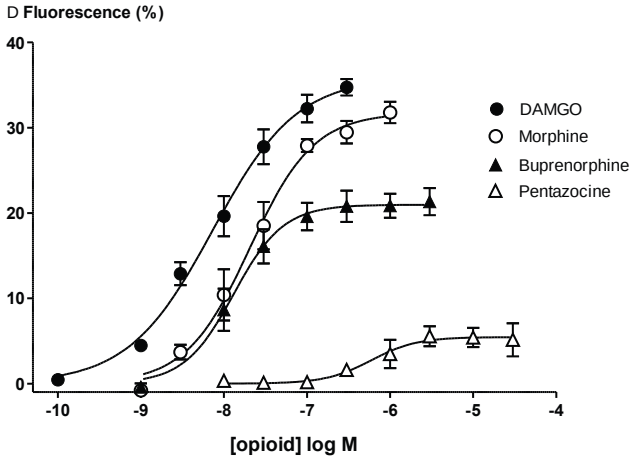
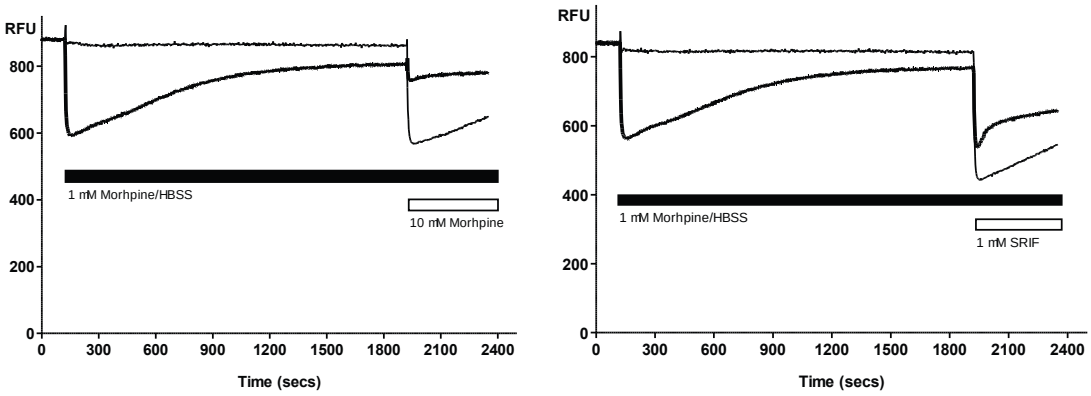


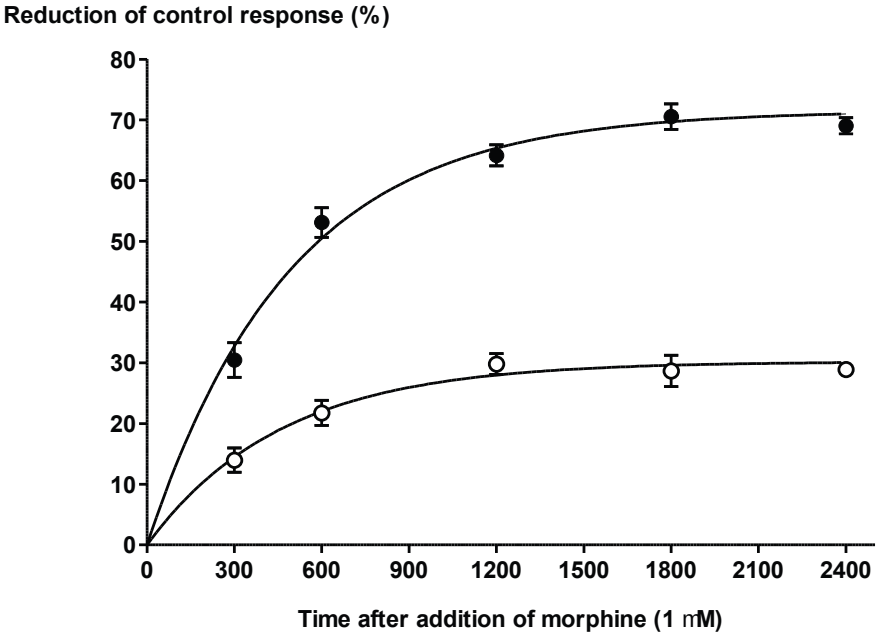

**Figure 6:** Concentration response curves for DAMGO, morphine, buprenorphine and pentazocine activation of GIRK channels, illustrating the differences in agonist potency and efficacy. Morphine, buprenorphine and pentazocine all showed partial agonist activity with Emax values of 31 ± 1%, 21 ± 1% and 5 ± 1% respectively.

**Figure 7:** Desensitisation of MOR signalling in AtT-20 cells. Continuous application of morphine (1 µM) reduces the response to a subsequent addition of a high concentration of morphine or SRIF. Panel A shows example traces from an experiment where a high concentration of morphine (10 µM) or SRIF (1 µM) was added 30 minutes after the desensitizing concentration of 1 µM morphine (heavy trace) or 30 minutes after the addition of vehicle (light trace). Panel B shows the time course of the decline in response to 10 µM morphine or 1 µM SRIF in the continued presence of 1 µM morphine. Data are expressed as a percentage of the control response to drug added at the same time after the run commenced, and represent the mean ± s.e.m. of 3 - 8 determinations, each in duplicate or triplicate. The data was fit with a single exponential function to derive a t/2 for desensitisation (490s for morphine/morphine, 460s for morphine/somatostatin).

A


RFU
800
600
400
200
0
1 mM Morhpine/HBSS
10 mM Morhpine
0 300 600 900 1200 1500 1800 2100 2400
Time (secs)
RFU
800
600
400
200
0
1 mM Morhpine/HBSS
1 mM SRIF
0 300 600 900 1200 1500 1800 2100 2400
Time (secs)

B


Reduction of control response (%)
80
70
60
50
40
30
20
10
0
0 300 600 900 1200 1500 1800 2100 2400
Time after addition of morphine (1 mM)

## DISCUSSION

In this study, we have developed a real time, no wash, fluorescence based assay for MOR-mediated hyperpolarisation of intact AtT-20 cells. We used a proprietary membrane potential sensitive dye from Molecular Devices to measure membrane hyperpolarisation following MOR mediated GIRK channel activation[12]. The fluorescent signal rapidly and reliably decreased after opioid application, and this decrease in signal could be completely reversed by the opioid naloxone applied for up to at least 2 hours after agonist, indicating the stability of the dye signal and capacity of the systems to report continued activation of the μ-receptors for prolonged periods, albeit in the face of receptor desensitisation. The ability to continuously measure the consequences of opioid receptor activation in cells for such prolonged periods of time has only previously been possible using high resistance electrode recordings from single neurons in brain slices[7,17]. Activation of endogenous sst receptors in AtT-20 cells[15] produced a reduction in fluorescence similar that seen following MOR activation, and in both cases this reduction was less than that produced by application of the K-selective ionophore nigericin[16].

The potency and efficacy of the opioid agonists correlate well with previous studies in AtT-20 cells, and with studies in native neurons where activation of GIRK channels or inhibition of $I_{Ca}$ was used to determine agonist intrinsic activity[11,18,19,20]. The data we obtained with the hyperpolarisation assay is similar to that we obtained when we measured MOR inhibition of calcium currents in these cells – the rank order of potency in both studies is DAMGO > methadone = morphine > pentazocine[11], and in both studies morphine and pentazocine have reduced efficacy when compared with DAMGO. The only notable inconsistencies are the apparently greater efficacy of endomorphin 1 and 2 compared with methadone in the present study. Many studies have reported the endomorphins as being partial agonists[18,21], while methadone has been reported to be an

agonist with a similar efficacy to DAMGO[11,22,23]. The most likely explanation for the discrepancy in our study is that methadone appeared to have a reduced maximal effect because of its propensity to block K channels, including the GIRK channels likely to contribute to the hyperpolarisation measured in the present study[22,24]. Conversely, the observation that the maximum effect of the endomorphins was similar to that of well recognized high efficacy agonists suggests the presence of some spare receptors in our system, as noted previously with similar cell lines[11]. It is also possible that the efficacy discrepancies reflect subtle bias in ligand signalling to one pathway over another in different tissues, and it is worth noting that endomorphins have recently been reported to show such bias in assays of β-arrestin recruitment[25,26].

Continuous application of opioid agonists usually results in desensitisation of receptor signalling, a complex process that may involve receptor phosphorylation and sequestration. The apparent stability of the membrane potential assay led us to explore whether it could be used to investigate μ-opioid receptor desensitisation. The hyperpolarisation produced by high concentrations of morphine or DAMGO appeared to wane over time, and when the cells were challenged with a concentration of agonist that should saturate the cell surface receptors there was a marked decrease in this response after only a few minutes exposure to agonist, consistent with analogous studies performed in locus coeruleus neurons[27] or cell lines transfected with μ-opioid receptors and GIRK channels[28]. We were able to make use of the endogenous sst receptors in the AtT-20 cells to determine whether exposure to desensitizing concentrations of opioid agonist affected signalling through other receptors. The time constant for the desensitisation of signalling produced by morphine was slower than that reported in our previous study of opioid signalling which utilized inhibition of $I_{Ca}$ in AtT-20 cells, this may reflect a distinct recruitment of desensitisation processes by morphine in intact cells compared with cells dialyzed by patch-clamp recording[11]. A

major limitation of using the Flexstation for desensitisation assays is the inability to wash off drugs, which makes it impossible to do experiments where receptor function is repeatedly probed with concentrations of drugs that activate only a portion of receptors[11,27]. Nevertheless, the potential of this assay to quickly test multiple agonists or putative modulators of receptor desensitisation in an intact system makes it an attractive option.

GIRK channel activation has most commonly been assessed using electrophysiological techniques, but assays potentially suitable for high throughput screening have been reported utilizing thallium flux[29] or commercially available membrane potential dyes[30,31]. Thallium is toxic and apparently unsuitable for all cell lines[31] while other membrane potential sensitive dyes (Di-Bac, HLB 021-152) give results qualitatively similar to those reported here[30,31]. The $EC_{50}$ value for the somatostatin-induced hyperpolarisation of our AtT-20 cells was 2 nM, very similar to that previously reported using another dye (4 nM)[31]. However, it should be noted that under our standard conditions the proprietary dye gives a change in fluorescence of approximately 40% following GPCR activation, which compares with changes of approximately 10% using $DiBAC_4$ in HL-1 or HLB 021-152 in AtT-20 cells[30,31]. Removal of media and/or washing or the cells is also required during dye loading of commercial dyes used previously, this increases assay time and may promote cell detachment from the microplate wells[31,32].

This assay has a number of advantages as a rapid screen for assessing ligand potency and efficacy at μ-opioid receptors. It is rapid, real-time, only requires the addition of a single reagent and is it performed in intact cells. When compared with assays of Gα subunit activation – either [$^{35}$S]GTPγS binding assays or assays which directly measure agonist-stimulated GTPase activity[33], the GIRK assay is far simpler, requires no handling of radioactivity and provides a real time measure of receptor activation rather than a single

point determination. However, the Gα subunit activation assays will provide a more sensitive discriminator of efficacy in cells with a low to moderate expression of receptors because the assay is constrained only by the amount of accessible Gα subunit in the cells, and this is usually not limiting[33]. By contrast, assays of AC regulation via measurement of cAMP accumulation have been adapted for use in whole cells, however these assays usually require incubation steps and either cell lysis followed by addition of several reagents or the use of cells transfected with enzymes which catalyse the production of a fluorescent substrate or with fluorescently labelled reporter proteins[10]. Assays of AC activity can be very sensitive and also detect cAMP levels over a large range of concentrations but in general when assaying the activity of $G_i/G_o$-coupled receptors such as the μ-opioid receptor it is usually necessary to artificially elevate cAMP levels with forskolin in order to obtain an appropriate signal window for determining AC inhibition by the $G_i/G_o$-derived Gα subunits. Nevertheless, assays of AC inhibition represent the most flexible and straightforward way of studying Gα (as opposed to Gβγ) signalling in a high throughput environment.

While both GTPγS binding and cAMP accumulation assays have been widely used for studying desensitisation of opioid receptor signalling, the usual requirement for control cells to be incubated with agonist for at least 10-15 minutes makes interpretation of the data problematic, as receptor desensitisation will be occurring during this time (e.g. Figure 7)[7].

Other relatively straightforward strategies for real-time measurement of MOR-mediated signalling involve measuring $[Ca]_i$ concentration using $Ca^{2+}$ sensitive fluorescent dyes. This approach provides relatively rapid real time response, and assays of $[Ca]_i$ concentration can be amenable to studying receptor desensitisation[34,35]. Interestingly, we

were unable to activate intracellular Ca release in AtT-20 cells via MOR or any of the $G\alpha_q$ receptors reportedly expressed in this cell line[36] (data not shown).

This assay offers an alternative approach for measuring MOR activation and desensitisation by targeting a naturalistic Gβγ-mediated signalling pathway. Our results show the membrane potential assay to be a rapid, reliable and inexpensive method for identifying ligands that modulate GIRK activity, and may be scaled up to enable high-throughput screening for novel opioid drugs.

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# RESULTS

The following chapters describe the consequences of MOPr SNPs on receptor signalling via a range of clinically important and/or structurally distinct opioid ligands. Chapter 5, “Buprenorphine signalling is compromised at the N40D polymorphism of the human μ-opioid receptor in vitro", describes how the common N40D variant significantly reduces maximum buprenorphine inhibition of AC and phosphorylation of ERK1/2, and reduces buprenorphine potency for GIRK activation. Chapter 6, “The A6V polymorphism of the human μ-opioid receptor negatively impacts signalling of morphine and endogenous opioids in vitro” reports the aberrant signalling of a range of opioid ligands at the relatively common A6V MOPr variant. Chapter 7, “Mu-opioid receptor polymorphisms differentially affect receptor signalling via adenylyl cyclase inhibition and ERK phosphorylation”, describes the effects of the L85I, R260H and R265H variants on the ability of MOPr to couple to pathways of AC inhibition and ERK1/2 phosphorylation.

# Chapter 5

## Buprenorphine signalling is compromised at the N40D polymorphism of the human μ-opioid receptor in vitro

**Alisa Knapman, Marina Santiago and Mark Connor**

This paper was submitted for peer review to the *British Journal of Pharmacology* on 23/4/14. Marina Santiago constructed AtT20-MOPr cells and performed GIRK activation assays. Mark Connor assisted with experimental design, data analysis and manuscript preparation. All other work is my own.

**SUMMARY**

***Background and Purpose***

There is significant variation in individual response to opioid drugs, which may result in inappropriate opioid therapy. Polymorphisms of the μ-opioid receptor (MOPr) may contribute to individual variation in opioid response by affecting receptor function, and the effect may be ligand-specific. We sought to determine functional differences in MOPr signalling at several signalling pathways using a range of structurally distinct opioid ligands in cells expressing wild-type MOPr and the commonly occurring MOPr variant, N40D.

***Experimental Approach***

MOPr-WT and MOPr-N40D were stably expressed in CHO cells and in AtT-20 cells. Assays of adenylyl cyclase inhibition and ERK1/2 phosphorylation were performed on CHO cells, and assays of K activation were performed on AtT-20 cells. Signalling profiles for each ligand were compared between variants.

***Key Results***

Buprenorphine efficacy was reduced by over 50% at MOPr-N40D for adenylyl cyclase inhibition and ERK1/2 phosphorylation. Buprenorphine potency was reduced threefold at MOPr-N40D for K channel activation. Pentazocine efficacy was reduced by 50% for GIRK activation at MOPr-N40D. No other differences were observed for any other ligands tested.

***Conclusions and Implications***

The N40D variant is present in 10 - 50% of the population. Buprenorphine is a commonly prescribed opioid analgesic, and many individuals do not respond to buprenorphine therapy. This study demonstrates that buprenorphine signalling to several effectors via the N40D variant of MOPr is impaired, and this may have important consequences in a clinical setting for individuals carrying the N40D allele.

***Abbreviations***

| | |
|---|---|
| AC | adenylyl cyclase |
| BSA | bovine serum albumin |
| CHO | Chinese Hamster Ovary |
| DAMGO | ([D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin) |
| DMEM | Dulbecco's Modified Eagle Medium |
| ERK | extracellular signal regulated kinase |
| FSK | forskolin |
| GIRK | G protein-gated inwardly rectifying K channel |
| GPCR | G protein couple receptors |
| HBSS | Hanks balanced salt solution |
| MOPr | μ-opioid receptor |
| OPRM1 | opioid receptor mu 1 |
| PTX | pertussis toxin |

## INTRODUCTION

Opioids are powerful analgesics used for the clinical management of moderate to severe pain. Opioid use is associated with adverse effects such as respiratory depression, nausea, constipation and sedation, and tolerance and dependence can develop with continued opioid use. There is significant variation between individuals in response to opioid drugs, both in the analgesic and adverse effects (Lotsch *et al.,* 2005). This variation can lead to restricted dosing of opioid analgesics due to the unpredictability of serious adverse events such as respiratory depression, resulting in inadequate pain relief for many individuals (Skorpen & Laugsand, 2008). The inter-individual variability observed in response to opioids is likely to be caused, at least in part, by genetic differences in proteins responsible for drug absorption, distribution and metabolism, as well as differences in drug/receptor interactions and receptor signalling (Somogyi *et al.,* 2007). A clearer understanding of the genetic factors contributing to individual response to opioids could result in the ability to better predict the outcomes of opioid administration in individuals, leading to more rational choice of drug and dose, thus potentially limiting adverse effects and the development of tolerance and dependence.

The μ-opioid receptor (MOPr, Cox *et al.,* 2014) is a G-protein coupled receptor (GPCR) that is the main site of action for clinically important opioids. MOPr mediates the analgesic and almost all of the adverse effects of these opioid drugs, and it is likely that genetic variation of *OPRM1,* the gene coding for MOPr, could contribute to individual variation in the response to opioid analgesics. A number of non-synonymous allelic variants of *OPRM1* have been identified within the population, each resulting in an alternative receptor isoform (Lotsch *et al.*, 2005). The N40D variant is the most common MOPr variant, occurring at an allelic frequency of 10-50% in various populations (Mura *et al.*, 2013). This variant arises from an A > G substitution at nucleotide 118, resulting in an

asparagine to aspartic acid amino acid exchange in the N-terminal domain of MOPr, and removing a putative glycosylation site (Singh *et al.*, 1997).

Many studies have investigated the association between carriers of MOPr-N40D and various clinical outcomes, such as the degree of pain relief from opioid analgesics, and the susceptibility to substance abuse. A number of these studies suggest that carriers of the D40 allele require higher doses of post-operative opioid analgesics, however other studies have shown an increased sensitivity to opioids and a reduced perception of pain (Diatchenko *et al.*, 2011; Mura *et al.*, 2013). There are also reports of an increased susceptibility to alcohol and heroin abuse in D40 carriers, but an improved response to naltrexone treatment of alcoholism (Mague & Blendy, 2010). Despite the volume of research into the effect of the N40D variant on disease outcomes, many of the reports are contradictory and there is no clear consensus as to the effect of the N40D variant on the outcomes of opioid administration or disease susceptibility (Walter & Lotsch, 2009).

Fewer studies have investigated the consequences of the N40D variant on MOPr signalling and function in vitro, and the results of these studies are also conflicting. One study reported a threefold increase in β-endorphin affinity for MOPr heterologously expressed in AV-12 cells, and a similar increase in β-endorphin potency for G protein-gated inwardly rectifying K channel (GIRK) activation in *Xenopus* oocytes (Bond *et al.*, 1998). Subsequent studies have found no differences in β-endorphin potency between N40D- and WT-MOPr (Befort *et al.*, 2001; Beyer *et al.*, 2004; Kroslak *et al.*, 2007). Other studies have reported both increased and decreased DAMGO and morphine potency, differences in regulatory processes with chronic opioid treatment, and brain region-dependent differences in expression and signalling, however there is little consistency in these reports

(reviewed in Knapman & Connor, 2014). As MOPr interacts with many effector and regulatory proteins, changing assay parameters and cellular background may affect MOPr signalling in different ways. Furthermore, most studies have used only a single or a small subset of opioid ligands, and may have failed to capture ligand-specific differences in N40D signalling.

Like all G protein couple receptors (GPCR), MOPr has many active conformations, and in the absence of ligand constantly oscillates through a range of possible active and inactive states (Pinyero *et al.*, 2007; Kenakin & Miller, 2010; Manglik *et al.*, 2012). Ligand-biased signalling or functional selectivity has been well characterised, where structurally distinct ligands stabilise the receptor in a restricted subset of conformations, preferentially activating certain effectors. The corollary of this phenomenon is that changes in amino acid residues arising from genetic polymorphisms may also affect conformation of the receptor (Abrol *et al.*, 2013; Cox, 2013). Thus, the N40D variant may affect MOPr signalling globally or in a ligand-dependent manner by affecting the ability of a ligand to bind to the receptor, altering the conformation of the ligand-receptor complex and/or affecting the ability of this complex to couple to G-proteins and associated signalling or regulatory pathways. Clinically used opioids are chemically diverse, and are likely to have subtle differences in their interaction with structural features of the MOPr, potentially leading to distinct effects of the N40D variant on different drugs. In this study, the ability of human MOPr-WT and MOPr-N40D to couple to several distinct signalling pathways was investigated in Chinese hamster ovary (CHO) cells and mouse AtT-20 cells using 11 clinically important and/or structurally distinct opioid ligands. We found that the N40D variant had a negative impact on the signalling of the commonly prescribed opioid buprenorphine in all of our assays. In addition, the efficacy of pentazocine was

significantly reduced for GIRK activation in AtT-20 cells expressing N40D. No differences in signalling via other opioids, including β-endorphin, were observed.

## METHODS

### *MOR transfection and cell culture*

CHO-FRT-TRex cells were stably transfected with a pcDNA5 construct encoding the haemagglutinin-tagged human μ-opioid receptor cDNA together with the pOG44 (Flp recombinase plasmid) using the transfectant Fugene (Promega), as described in previously (Knapman *et al.*, 2014). The HA-tagged human wild-type μ-opioid receptor (MOPr-WT) and the μ-opioid receptor containing the D40 variant (MOPr-N40D) were synthesised by Genscript (Piscataway, New Jersey, USA). Cells expressing MOPr-WT or MOPr-N40D were selected using hygromycin B (500 μg/mL). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 500 μg/ml hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 μg/ml beyond passage 5.

AtT-20 FlpIn cells were constructed by transfecting Flp-recombinase target site (FRT)/LacZeo2 (Life Technologies) using Fugene. Successfully transfected cells were selected with 100 μg/ml zeocin, and stably transfected with MOPr-WT or MOPr-N40D as described for CHO cells. Cells expressing MOPr were selected using 100 μg/ml hygromycin B. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 100 μg/ml hygromycin B.

CHO-MOPr cells and AtT-20-MOPr cells were passaged at 80% confluency as required. Assays were carried out on cells up to 30 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at greater than 90% confluence.

***MOPr receptor expression***

Surface expression of MOPr was determined on intact CHO-MOPr cells by incubation with 0.125 – 16 nM [$^3$H]-DAMGO (D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin; PerkinElmer, Waltham, MA) at 4 °C in 50 mM Tris-Cl (pH 7.4) for 2 h. Briefly, 24 hours before the assay, cells were detached from flasks with trypsin/EDTA (Sigma) and resuspended in DMEM containing 10% FBS, 100U penicillin/streptomycin, plus 2 µg/ml tetracycline to induce MOPr expression. Cells were seeded at a density of $1 \times 10^5$ cells per well in 24-well plates pre-coated with polylysine and grown overnight at 37° C. On the day of the assay, cells were washed twice gently with 50 mM Tris-Cl (pH 7.4) and incubated for 2 h on ice with 0.125 – 16 nM [$^3$H]-DAMGO. Non-specific binding was determined in the presence of unlabeled DAMGO (10 µM). Non-specific binding was 15 ± 1% of total binding for CHO-MOPr-WT, and 11 ± 1% in CHO-MOPr-N40D. At the end of the incubation, plated cells were washed three times with 50 mM Tris-Cl (pH 7.4) at 4 °C. Cells in each well were then digested for 1 h at room temperature with 100 µl of 1N NaOH. 100 µl 1N HCl was added to each well and collected into scintillation vials and bound ligand determined using a liquid scintillation counter (Packard Tricarb, Perkin Elmer,Waltham MA, USA). Receptor density ($B_{max}$) and affinity ($K_d$) were calculated using a one-site binding curve fitted using GraphPad Prism (GraphPad Software, La Jolla, CA). Protein concentration was determined with a BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. All experiments were performed three times in triplicate.

***Membrane Potential Assay of Adenylyl Cyclase Inhibition***

Opioid inhibition of adenylyl cyclase (AC) was measured using an assay of membrane potential (Knapman *et al.,* 2014a). The day before the assay, CHO-MOPr cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. MOPr receptor expression was induced with 2 μg/ml tetracycline 24 hours prior to the assay. The cells were plated in a volume of 90 μl in black walled, clear-bottomed 96-well microplates (Corning), and incubated overnight at 37$^{o}$C in ambient $CO_2$. Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer (Hanks balanced salt solution, HBSS) containing (in mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 5). Prior to the assay, cells were loaded with 90 μl/well of the dye solution without removal of the L-15, giving an initial assay volume of 180 μl/well. Plates were then incubated at 37°C in ambient $CO_2$ for 60 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time forskolin (FSK, an activator of AC) and either opioid or vehicle was added in a volume of 20 μl. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. The final concentration of DMSO was not more than 0.1%, and this concentration did not produce a signal in the assay.

***Membrane Potential Assay of GIRK Channel Activation***

Opioid activation of endogenous GIRK channels in AtT-20 cells was measured using a membrane potential sensitive dye, as previously described (Knapman *et al.*, 2013). AtT-20-MOPr cells were detached from flasks and plated using the same procedure as for the CHO-MOPr cells in the AC inhibition assay, with no addition of tetracycline. Blue membrane potential dye was reconstituted and loaded onto cells, and the assay was performed in the same way as the AC inhibition assay. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time opioid or vehicle was added in a volume of 20 µl. Measurements were taken at the peak decrease in signal from baseline, approximately 10-20 sec after drug addition. The background fluorescence of cells without dye or dye without cells was negligible. The final concentration of DMSO was not more than 0.1%, and this concentration did not produce a signal in the assay.

***ELISA of ERK1/2 phosphorylation***

Opioid-induced phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2) was measured by ELISA. The day before the assay, CHO-MOPr cells were plated and receptor expression was induced as for the membrane potential assay. Cells were plated in 96-well clear microplates (Falcon). On the day of the assay, cells were serum-starved for 1 hr in 40 µl serum-free L-15 supplemented with 5% bovine serum albumin (BSA). All cell treatments were in a volume of 40 µl unless stated otherwise. Serum-starved cells were treated with drug or vehicle diluted in serum-free L-15. Preliminary experiments indicated that drug treatment for 5 min was optimal to produce robust ERK1/2 phosphorylation without inducing desensitisation, thus all drug treatments were for 5 min unless otherwise indicated. After drug application, the reaction was stopped by inverting the plates to remove the drug solution, placing the plates on ice, and immediately fixing the cells with 4% paraformaldehyde for 15 min at RT. Cells were washed 3 times with 300 µl PBS, then

permeabilized with 0.1% Triton-X in PBS for 30 min at RT. Triton-X was removed, and cells were incubated for 2 hr at RT with blocking solution consisting of 5% BSA in PBS with 0.01% Tween-20 (PBS-T). Blocking solution was removed, then cells were incubated overnight at 4°C with a 1:500 dilution of rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody in PBS-T with 1% BSA. Cells were washed 3 times with 300 µl PBS-T, and incubated with 1:5000 anti-rabbit IgG HRP-linked antibody in PBS-T with 1% BSA for 2 hr. Cells were washed 4 times with 300 µl PBS-T, and incubated with 3,3',5,5'-tetramethylbenzidine (Sigma) at RT in the dark for 45 min. The reaction was stopped with 1M HCl. Absorbance was read at 450 nm using a BMG Pherastar FS microplate reader. Cells were then stained with 0.5 µg/mL DAPI for 10 min at RT, and washed 3 times with 300 µl PBS-T. Fluorescence was read in the Pherastar microplate reader with cells excited at a wavelength of 358 nm and emission measured at 461 nm. Absorbance readings were normalised to DAPI staining to account for differences in cell density between wells. Readings were then normalised to the response of cells treated with 100 nM PMA for 10 min.

***Drugs and Chemicals***

Unless otherwise noted, tissue culture reagents and buffer salts were from Invitrogen or Sigma. DAMGO, endomorphin-1, endomorphin-2 and met-enkephalin were purchased from Auspep (Tullamarine, Australia). Morphine and pentazocine were a kind gift from the Department of Pharmacology, University of Sydney. Buprenorphine and oxycodone were from the National Measurement Institute (Lindfield, Australia). β-endorphin was from Genscript (Piscataway, New Jersey, USA). Fentanyl (Andrews Laboratories) was a gift from the Department of Pharmacology, Sydney University. Forskolin and naloxone were from Ascent Pharmaceuticals (Bristol, UK). 1,9-dideoxyforskolin and tetraethylammonium (TEA) were from Sigma Aldrich (Castle Hill, Australia). Pertussis

toxin (PTX) was from Tocris Bioscience (Bristol, UK). Phospho-ERK1/2 antibody (Catalog #9101) and anti-rabbit IgG HRP-lined antibody (Catalog #7074) were from Cell Signalling Technologies, Danvers, Massachusetts, USA.

### *Data*

Unless otherwise noted, data is expressed as mean ± s.e.m. of at least 5 determinations made in duplicate or triplicate. Concentration response curves were fit with a 4 parameter logistic equation using Graphpad Prism (Graphpad). $E_{max}$ and $pEC_{50}$ values were derived from individual experiments and compared using unpaired Student's T-test. $P < 0.05$ was considered significant. Full agonist activity was determined by comparing $E_{max}$ values derived from individual experiments using one-way ANOVA, corrected for multiple comparisons. Pooled data from replicate experiments is shown in the Figures for ease of reference. All channel and receptor nomenclature is consistent with the British Journal of Pharmacology/IUPHAR Concise Guide to Pharmacology (Alexander et al., 2013).

## RESULTS

### *MOPr Expression in CHO-K1 cells*

CHO-K1 cells were stably transfected with either MOPr-WT or the MOPr-N40D variant, with receptor expression controlled by a tetracycline-sensitive repressor. After induction of MOPr expression with tetracycline, specific binding of [$^3$H]DAMGO was similar for both variants, indicating similar levels of surface receptor expression (see Figure 1). The $B_{max}$ for CHO-MOPr-WT cells was 280 ± 20 fmol/mg total protein and 356 ± 18 fmol/mg for CHO-MOPr-N40D ($P > 0.05$). The affinity for [$^3$H]DAMGO was also similar between for WT-MOPr and N40D-MOPr expressing cells, with $K_D$ of 0.75 ± 0.10 and 0.65 ± 0.2, respectively ($P > 0.05$).

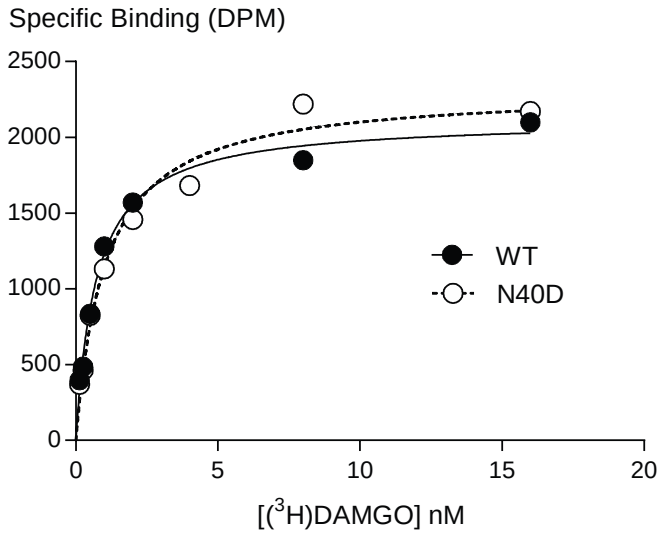



**Figure 1: Saturation binding curve of [$^3$H]DAMGO in intact CHO-MOPr-WT and CHO-MOPr-N40D cells, 24 hr after induction of receptor expression with tetracycline.** Radioligand binding was carried out as described in the Methods. No significant difference in $B_{max}$ or $K_d$ was observed between cells expressing MOPr-WT or MOPr-N40D ($P > 0.05$). Each point represents the mean ± s.e.m. of triplicate determinations from a single experiment. The assay was repeated 3 times.

***Adenylyl Cyclase Inhibition***

In CHO-MOPr cells loaded with membrane potential dye, addition of the adenylyl cyclase activator forskolin (FSK, 300 nM) produced a rapid decrease in fluorescence, consistent with hyperpolarisation of the cells (Figure 2; Knapman *et al.*, 2014). The hyperpolarisation stabilised 5 min after the addition of FSK, at which point measurements were taken. FSK (300 nM) produced a similar hyperpolarisation in CHO-MOPr-WT (42 ± 1 % decrease in fluorescence) and CHO-MOPr-N40D (44 ± 2 % decrease) cells. ($P > 0.05$). Simultaneous addition of the prototypical MOPr selective peptide agonist DAMGO with FSK resulted in a concentration-dependent inhibition of the FSK-stimulated hyperpolarisation in both cell lines (Figure 2). DAMGO maximally inhibited the FSK response by 60 ± 2 %, with $pEC_{50}$ of 7.7 ± 0.1 in CHO-MOPr-WT cells (Table 1). DAMGO inhibited FSK similarly in cells expressing N40D, $E_{max}$ was 67 ± 6 % and $pEC_{50}$ 7.7 ± 0.1 ($P > 0.05$). We have reported previously that the effects of DAMGO in this assay were sensitive to naloxone and strongly inhibited by overnight pretreatment with pertussis toxin (Knapman *et al.*, 2014). Application of opioids alone did not affect the membrane potential of CHO-MOPr cells with the exception of 10 μM methadone and 30 μM pentazocine, which both caused a small, transient increases in fluorescence (< 10%). These effects were not naloxone sensitive and were not observed at lower concentrations of drug. They are consistent with previously reported inhibition of K channels by these drugs (Matsui and Williams, 2010; Nguyen *et al.*, 1998).

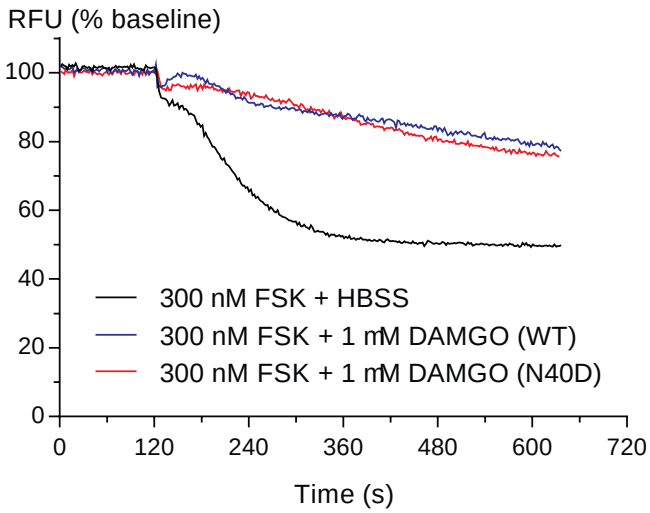
Because of the uncertainty surrounding the effects of N40D polymorphism on opioid actions, we measured the potency and efficacy of a range of clinically important and/or structurally distinct opioid ligands in CHO cells expressing WT-MOPr or the N40D variant to determine whether the N40D amino acid substitution could affect MOPr-signalling in a ligand-selective manner. The endogenous opioid β-endorphin has

previously been shown to have a three-fold increase in potency at GIRK channel activation in *Xenopus* oocytes expressing N40D-MOPr (Bond *et al.*, 1998). In the present assay, there was no difference in β-endorphin potency or efficacy between CHO-MOPr-WT cells and CHO-MOPr-N40D cells (Figure 3, Table 1). AC inhibition by the putative endogenous opioid ligands endomorphin-1 and endomorphin-2, as well as met-enkephalin were also similar in cells expressing MOPr-WT or MOPr-N40D receptor (Figure 3, Table 1).

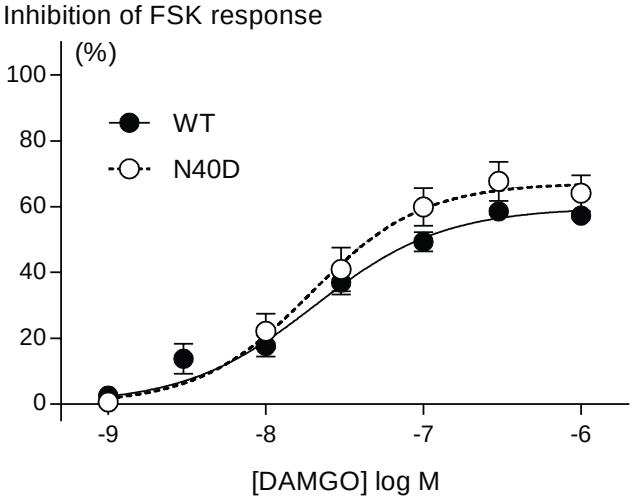
The efficacy and potency of the prototypical opioid alkaloid morphine was not different in cells expressing the N40D variant (see Figure 4, Table 1). Interestingly, the semisynthetic morphine derivative buprenorphine had a significantly lower efficacy for AC inhibition in CHO cells expressing MOPr-N40D when compared with MOPr-WT. The buprenorphine $E_{max}$ was 35 ± 6% in CHO-MOPr-WT cells, and 16 ± 4% in CHO-MOPr-N40D cells, a reduction of over 50% ($P < 0.05$). Buprenorphine potency was similar at the N40D variant (see Figure 4, Table 1). The low efficacy of buprenorphine at MOPr could have accentuated a general difference in transduction efficiency between MOPr-WT and the MOPr-N40D. However, the low efficacy MOPr agonist pentazocine inhibited AC to a similar degree in cells expressing MOPr-WT or MOPr-N40D (Figure 4, Table 1), indicating that the low efficacy of buprenorphine *per se* was not responsible for reduced signalling at the N40D variant. No difference in AC signalling was observed between cells expressing MOPr-WT or the MOPr-N40D variant when challenged with fentanyl, methadone or oxycodone (Figure 5, Table 1).

**Figure 2: DAMGO inhibits adenylyl cyclase and activates ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D.** Adenylyl cyclase inhibition and levels of ERK1/2 phosphorylation were determined as described in the Methods. A) Traces showing changes in fluorescent signal following application of 300 nM forskolin + vehicle (Hank's Balanced Salt Solution, HBSS) to MOPr-WT, and 300 nM forskolin + 1 μM DAMGO to MOPr-WT and MOPr-N40D cells. Drugs were added at 120 sec. Changes in RFU are normalized to predrug values. B) DAMGO inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). C) DAMGO stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

A


RFU (% baseline)
100
80
60
40
20
0
0
120
240
360
480
600
720
Time (s)
300 nM FSK + HBSS
300 nM FSK + 1 mM DAMGO (WT)
300 nM FSK + 1 mM DAMGO (N40D)

B


Inhibition of FSK response
(%)
100
80
60
40
20
0
-9
-8
-7
-6
[DAMGO] log M
WT
N40D

C

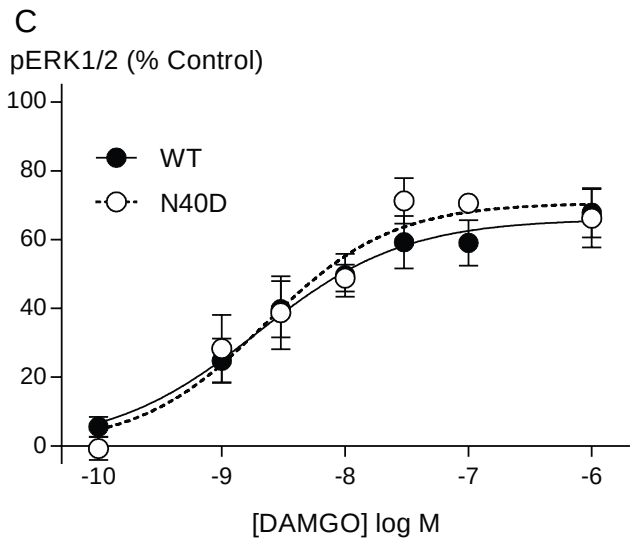

pERK1/2 (% Control)
100
80
60
40
20
0
-10
-9
-8
-7
-6
[DAMGO] log M
WT
N40D

**Figure 3: Endogenous opioids inhibit adenylyl cyclase and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) β-endorphin, endomorphins 1 & 2, and met-enkephalin inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). B) β-endorphin, endomorphins 1 & 2, and met-enkephalin stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

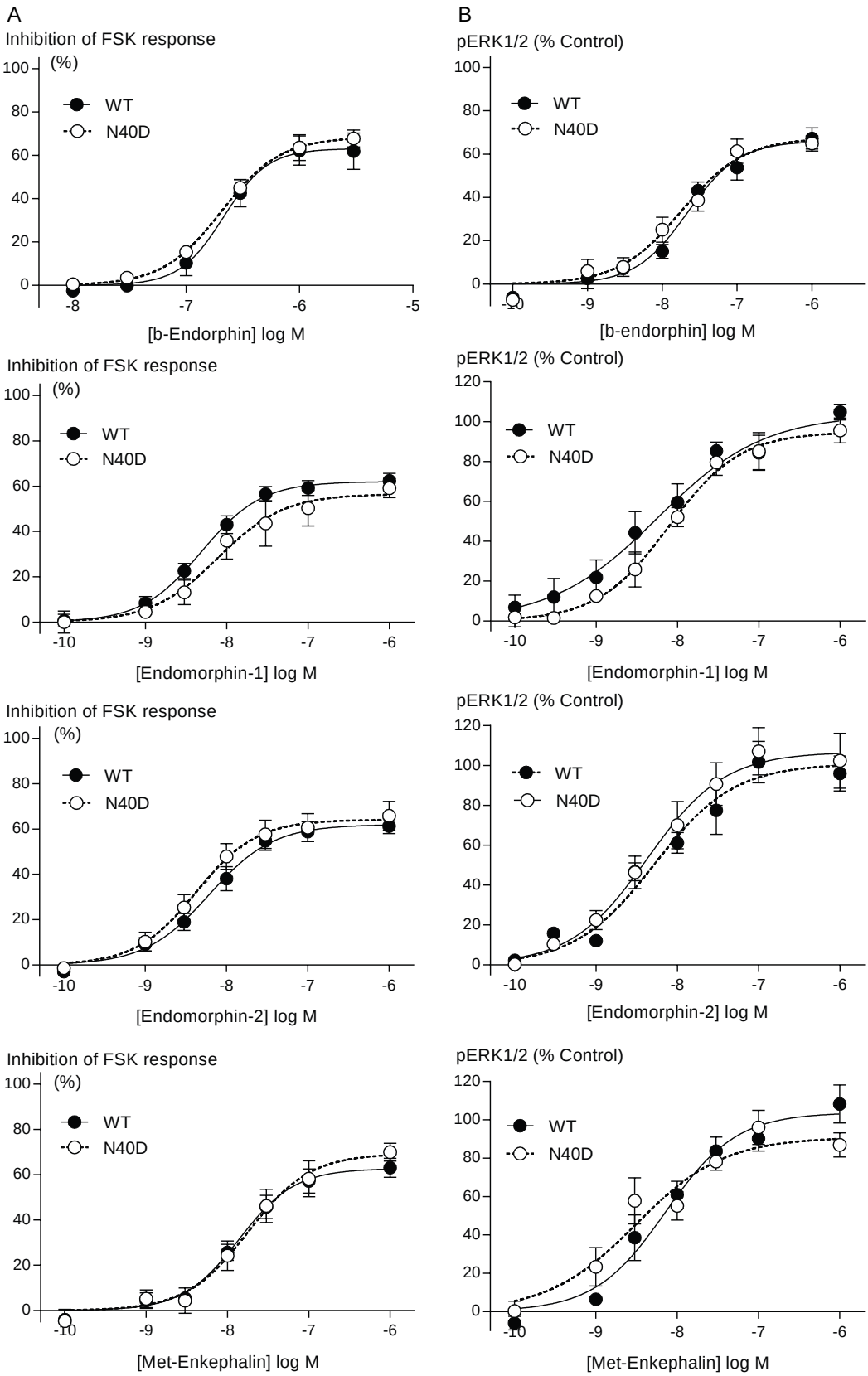

A
Inhibition of FSK response
(%)
WT
N40D
[b-Endorphin] log M
B
pERK1/2 (% Control)
WT
N40D
[b-endorphin] log M
Inhibition of FSK response
(%)
WT
N40D
[Endomorphin-1] log M
pERK1/2 (% Control)
WT
N40D
[Endomorphin-1] log M
Inhibition of FSK response
(%)
WT
N40D
[Endomorphin-2] log M
pERK1/2 (% Control)
WT
N40D
[Endomorphin-2] log M
Inhibition of FSK response
(%)
WT
N40D
[Met-Enkephalin] log M
pERK1/2 (% Control)
WT
N40D
[Met-Enkephalin] log M

**Figure 4: Buprenorphine inhibits adenylyl cyclase and activates ERK1/2 less effectively in CHO cells expressing MOPr-N40D.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) Buprenorphine $E_{max}$ for inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation was decreased from 35 ± 6 % in CHO-MOPr-WT to 16 ± 4% in CHO-MOPr-N40D ($P < 0.05$). Morphine and pentazocine inhibited adenylyl cyclase hyperpolarisation to a similar degree and with similar potency in CHO cells expressing MOPr-WT and MOPr-N40D ($P > 0.05$). B) Buprenorphine $E_{max}$ for stimulation of ERK1/2 phosphorylation was decreased from 35 ± 7 % in CHO-MOPr-WT to 14 ± 6% in CHO-MOPr-N40D ($P < 0.05$). Morphine and pentazocine stimulated ERK1/2 phosphorylation to a similar degree and with similar potency in CHO cells expressing MOPr-WT and MOPr-N40D ($P > 0.05$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

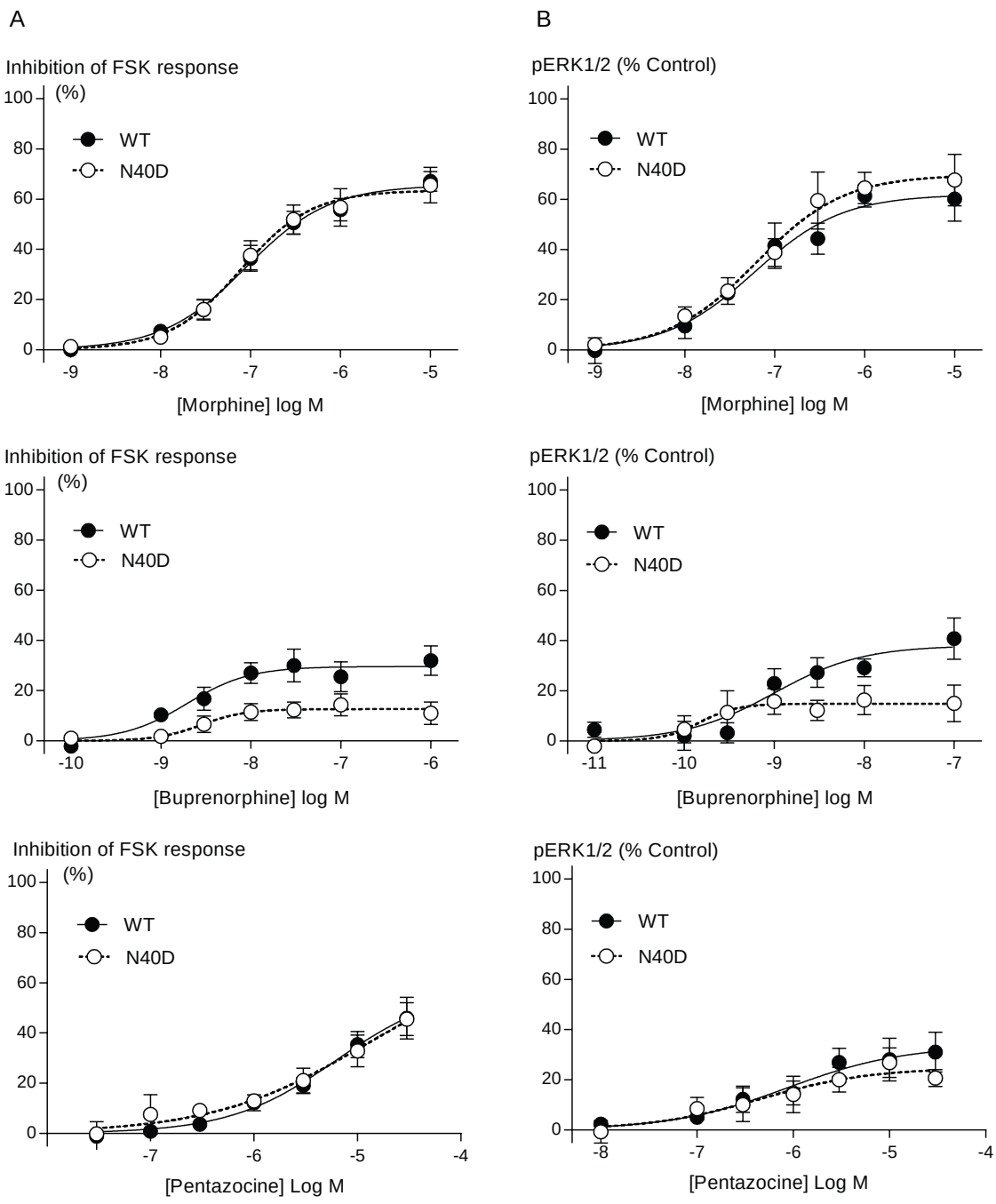

A
B
Inhibition of FSK response
(%)
WT
N40D
[Morphine] log M
pERK1/2 (% Control)
WT
N40D
[Morphine] log M
Inhibition of FSK response
(%)
WT
N40D
[Buprenorphine] log M
pERK1/2 (% Control)
WT
N40D
[Buprenorphine] log M
Inhibition of FSK response
(%)
WT
N40D
[Pentazocine] Log M
pERK1/2 (% Control)
WT
N40D
[Pentazocine] Log M

**Figure 5: Fentanyl, oxycodone and methadone inhibit adenylyl cyclase and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) Fentanyl, oxycodone and methadone inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). B) Fentanyl, oxycodone and methadone stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

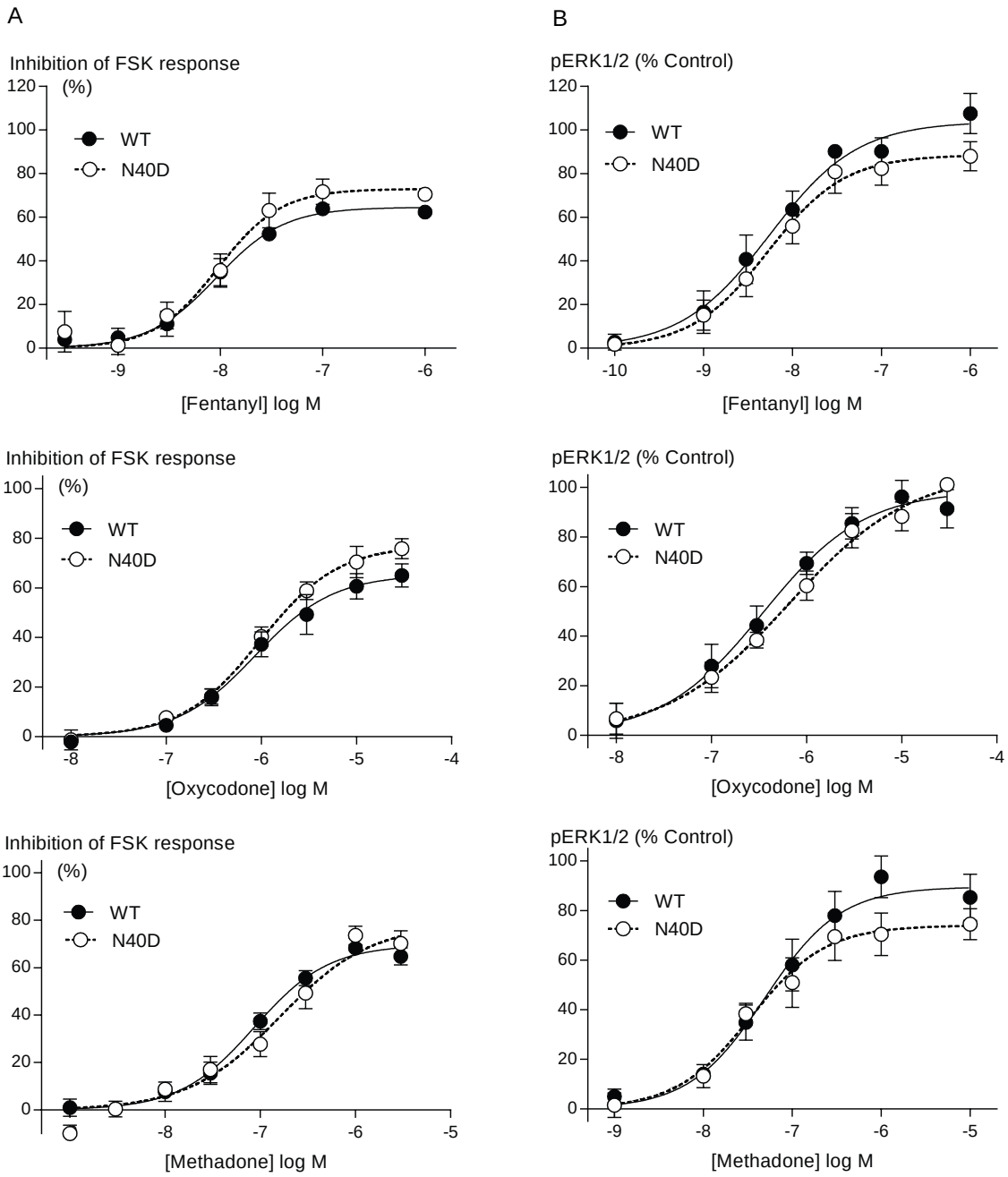

A
B
Inhibition of FSK response
(%)
WT
N40D
[Fentanyl] log M
pERK1/2 (% Control)
[Oxycodone] log M
[Methadone] log M

**Table 1: Summary of opioid efficacy and potency in assays of AC inhibition in CHO cells expressing MOPr-WT and MOPr-N40D.**

| AC inhibition | $E_{max}$ (%) | | *p*$EC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **N40D** | **WT** | **N40D** |
| β-Endorphin | 70 ± 4 | 69 ± 6 | 6.7 ± 0.1 | 6.7 ± 0.1 |
| Methadone | 66 ± 2 | 73 ± 5 | 7.0 ± 0.1 | 7.2 ± 0.6 |
| Met-enkephalin | 66 ± 4 | 73 ± 4 | 7.8 ± 0.1 | 7.7 ± 0.3 |
| Morphine | 66 ± 3 | 70 ± 4 | 7.0 ± 0.1 | 7.0 ± 0.1 |
| Fentanyl | 65 ± 2 | 74 ± 4 | 8.1 ± 0.2 | 8.2 ± 0.1 |
| Oxycodone | 65 ± 5 | 77 ± 6 | 5.8 ± 0.3 | 6.0 ± 0.1 |
| Endomorphin-1 | 62 ± 3 | 61 ± 4 | 8.3 ± 0.1 | 8.3 ± 0.1 |
| Endomorphin-2 | 60 ± 7 | 64 ± 6 | 8.1 ± 0.1 | 8.3 ± 0.1 |
| DAMGO | 60 ± 2 | 67 ± 6 | 7.7 ± 0.1 | 7.7 ± 0.2 |
| Pentazocine | 50 ± 6 | 47 ± 6 | 5.2 ± 0.1 | 4.8 ± 0.6 |
| Buprenorphine | 35 ± 6 | 16 ± 4* (P* = 0.02) | 8.5 ± 0.2 | 8.0 ± 0.2 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than β-endorphin are highlighted in red (One way ANOVA, followed by Students T-test, corrected for multiple comparisons, $P < 0.05$). * $E_{max}$ or *p*$EC_{50}$ significantly different between MOPr-WT and MOPr-N40D ($P < 0.05$).

**Table 2: Summary of opioid efficacy and potency in assays of ERK1/2 phosphorylation in CHO cells expressing MOPr-WT and MOPr-N40D.**

| pERK1/2 | $E_{max}$ (%) | | *p*$EC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **N40D** | **WT** | **N40D** |
| Endomorphin-2 | 109 ± 14 | 107 ± 14 | 8.2 ± 0.2 | 8.3 ± 0.1 |
| Met-Enkephalin | 103 ± 9 | 87 ± 6 | 8.1 ± 0.1 | 8.5 ± 0.3 |
| Oxycodone | 101 ± 8 | 109 ± 6 | 6.5 ± 0.2 | 6.1 ± 0.1 |
| Fentanyl | 99 ± 11 | 89 ± 8 | 8.1 ± 0.1 | 8.4 ± 0.2 |
| Endomorphin-1 | 93 ± 6 | 92 ± 5 | 8.4 ± 0.3 | 8.2 ± 0.1 |
| Methadone | 88 ± 9 | 72 ± 8 | 7.3 ± 0.1 | 7.5 ± 0.1 |
| β-Endorphin | 72 ± 9 | 79 ± 9 | 7.5 ± 0.1 | 7.6 ± 0.1 |
| DAMGO | 67 ± 6 | 72 ± 5 | 8.5 ± 0.1 | 8.5 ± 0.7 |
| Morphine | 65 ± 7 | 76 ± 7 | 7.0 ± 0.1 | 7.2 ± 0.1 |
| Pentazocine | 39 ± 12 | 39 ± 15 | 6.1 ± 0.2 | 6.0 ± 0.6 |
| Buprenorphine | 35 ± 7 | 14 ± 6* (P* = 0.04) | 8.7 ± 0.4 | 9.3 ± 0.1 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than endomorphin-2 are highlighted in red (One way ANOVA, followed by Students T-test, corrected for multiple comparisons, $P < 0.05$). * $E_{max}$ or *p*$EC_{50}$ significantly different between MOPr-WT and MOPr-N40D ($P < 0.05$).

***Opioid-mediated phosphorylation of ERK1/2***

We next examined whether the N40D amino acid substitution leads to alterations in signalling through another important pathway activated by MOPr, stimulation of ERK1/2 phosphorylation by MOPr. We analysed the ability of opioids to stimulate ERK1/2 phosphorylation via MOPr-WT and MOPr-N40D in CHO cells, using a whole-cell ELISA assay.

Opioid responses were normalised against 100 nM PMA applied for 10 minutes. The average PMA response was similar in cells expressing MOPr-WT or MOPr-N40D, with corrected absorbance readings of 0.56 ± 0.07 and 0.60 ± 0.07, respectively ($P > 0.5$). DAMGO-stimulated ERK1/2 phosphorylation was similar between WT and N40D expressing cells, with a $pEC_{50}$ of 8.5 ± 0.1 and $E_{max}$ of 67 ± 6% of the PMA response in CHO-MOPr-WT cells and a $pEC_{50}$ of 8.5 ± 0.7 and Emax of 72 ± 5% in CHO-MOPr-N40D (Figure 2, Table 2). Preincubation of cells with 1 μM naloxone for 5 mins blocked the response for all opioids tested. Application of DAMGO to cells treated overnight with 200ng/mL PTX, or in cells where receptor expression had not been induced did not elicit a response.

The ERK1/2 phosphorylation elicited by β-endorphin stimulation of MOPr-N40D was similar to that of MOPr-WT. β-endorphin $E_{max}$ and $pEC_{50}$ were 72 ± 2% and 7.5 ± 0.1 respectively in CHO-MOPr-WT cells, and 79 ± 9% and 7.6 ± 0.1 respectively in CHO-MOPr-N40D cells ($P > 0.05$). Similarly, no differences in potency or efficacy were observed between MOPr variants for endomorphin-1, endomorphin-2 and met-enkephalin (Figure 3, Table 2).

Buprenorphine efficacy was significantly decreased at the N40D variant. Maximum buprenorphine stimulated ERK1/2 phosphorylation in CHO-MOPr-WT was 35 ± 7%, while in CHO-MOPr-N40D efficacy was 14 ± 6%, a reduction of approximately 60% ($P < 0.05$). Buprenorphine potency did not differ between cells expressing MOPr-WT and MOPr-N40D, with $pEC_{50}$s of 8.7 ± 0.4 and 9.3 ± 0.1 respectively (Figure 4, Table 2). The partial agonists morphine and pentazocine stimulated ERK1/2 phosphorylation similarly at MOPr-WT and MOPr-N40D (Figure 4, Table 2). Fentanyl-, methadone- and oxycodone-stimulated ERK1/2 phosphorylation was also similar in efficacy and potency between CHO-MOPr-WT and CHO-MOPr-N40D cells (Figure 5, Table 2).

There were minor differences in relative agonist efficacy between MOPr-WT and MOPr-N40D. In assays of AC inhibition, endomorphin-1, endormorphin-2, pentazocine and buprenorphine had a significantly lower $E_{max}$ than methadone for both MOPr-WT and MOPr-N40D. DAMGO had a significantly lower $E_{max}$ than methadone to inhibit AC in MOPr-WT but not MOPr-N40D. In assays of ERK1/2 phosphorylation in both MOPr-WT and MOPr-N40D, DAMGO, β-endorphin, pentazocine and buprenorphine had significantly lower $E_{max}$ than the most efficacious agonist in this assay, endomorphin-2. Morphine had a significantly lower $E_{max}$ than endomorphin 2 to stimulate ERK1/2 phosphorylation in MOPr-WT but not MOPr-N40D. As there was no statistically significant difference between variants for $E_{max}$ of methadone, endomorphin-2, DAMGO or morphine in any of our assays, presumably the variance inherent in the AC inhibition and ERK1/2 phosphorylation assays precluded the statistical differentiation of small differences in efficacy between ligands.

***GIRK channel activation in AtT-20 cells***

Activation of MOPr results in activation of GIRK channels via Gβγ subunits. As CHO-K1 cells do not express endogenous GIRK channels, we assessed opioid mediated GIRK activation in AtT-20 cells stably transfected with hMOPr-WT and hMOPr-N40D as previously described (Knapman *et al.*, 2013). In AtT-20 cells loaded with membrane potential sensitive dye, application of opioids resulted in a concentration-dependent decrease in fluorescence from baseline, corresponding to membrane hyperpolarisation from GIRK activation (Figure 6).

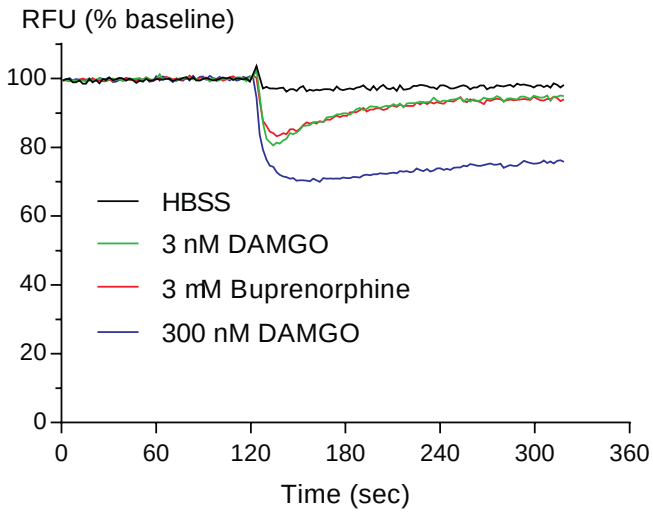


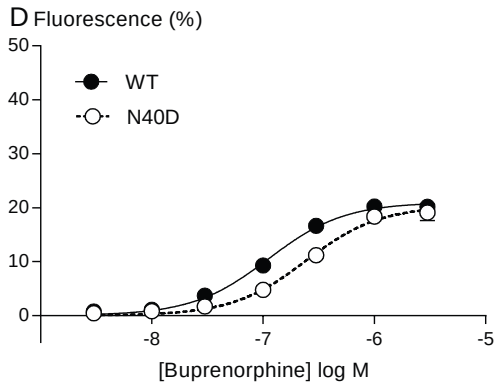

**Figure 6: DAMGO causes membrane hyperpolarisation in AtT-20 cells expressing MOPr-WT.** Raw trace showing decrease in fluorescent signal following application of vehicle (Hank's Balanced Salt Solution, HBSS), 3 nM DAMGO, 300 nM DAMGO or 3 µM buprenorphine to AtT20 cells expressing MOPr-WT, corresponding to membrane hyperpolarisation from GIRK activation. The Y-axis is raw fluorescent units (RFU). Drugs were added for the duration of the bar. The traces are representative of at least 6 individual experiments, each performed in duplicate.

Sub-maximal membrane hyperpolarisation produced by low efficacy agonists such as buprenorphine or low concentrations of high efficacy agonists such as DAMGO rapidly returned towards baseline within 2 mins after addition, however maximum membrane hyperpolarisation produced by high concentrations of high efficacy agonists remained steady over 5 min. (Figure 6). There was no difference in maximum DAMGO-stimulated GIRK activation between AtT20-MOPr-WT cells and AtT20-MOPr-N40D cells, with $E_{max}$ of 34 ± 0.1% and 32 ± 0.1% decrease from baseline, respectively ($P > 0.05$), and *p*EC50 for both variants was 8.4 ± 0.01 (Figure **7**, Table 3).

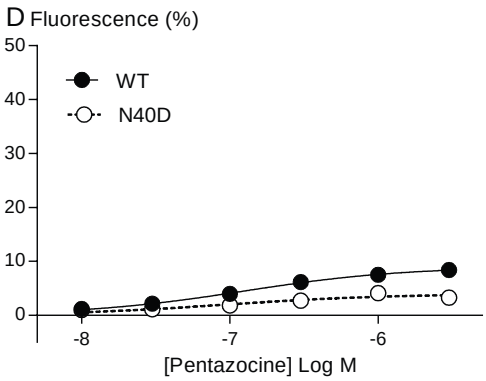
Buprenorphine was less potent for GIRK activation in AtT20-MOPr-N40D cells, with $pEC_{50}$ of 6.7 ± 0.08 compared with $pEC_{50}$ of 7.0 ± 0.07 in AtT20-MOPr-WT cells ($P < 0.05$). Buprenorphine efficacy was unaffected by the N40D variant, MOPr-WT $E_{max}$ was 22 ± 2%, and MOPr-N40D $E_{max}$ was 20 ± 2% ($P > 0.05$). We also observed a significant decrease in the efficacy of the partial agonist pentazocine at MOPr-N40D, the pentazocine $E_{max}$ was 8 ± 1% at MOPr-WT, and 4 ± 1% at N40D although the $pEC_{50}$ was similar between variants (Figure 7, Table 3). Morphine, methadone and β-endorphin signalling was unaffected at the N40D variant (Figure 7, Table 3). As buprenorphine efficacy was similar between variants, and significantly less than higher efficacy agonists, it is unlikely that the difference in pentazocine efficacy is due to lower receptor expression in AtT20-MOPr-N40D cells. Similarly, the partial agonist activity of buprenorphine precludes the possibility of a change in receptor reserve contributing to decreased potency for GIRK activation.

**Figure 7: Buprenorphine is less potent for GIRK activation in AtT20 cells expressing MOPr-N40D.** GIRK activation was determined as described in the Methods. A) Buprenorphine activated GIRK channels to a similar degree in AtT20 cells expressing MOPr-WT and MOPr-N40D, but with threefold lower $pEC_{50}$, from 7.0 ± 0.1 in AtT20-MOPr-WT to 6.7 ± 0.1 in AtT20-MOPr-N40D ($P < 0.05$). B) Pentazocine activated GIRK channels with 50% lower efficacy at MOPr-N40D, $E_{max}$ was decreased from 8 ± 1% in AtT20-MOPr-WT to 4 ± 1% in AtT20-MOPr-N40D ($P < 0.05$). C) DAMGO, morphine, β-endorphin and methadone activated GIRK channels to a similar degree and with similar potency in AtT-20 cells expressing MOPr-WT and MOPr-N40D ($P > 0.05$). Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

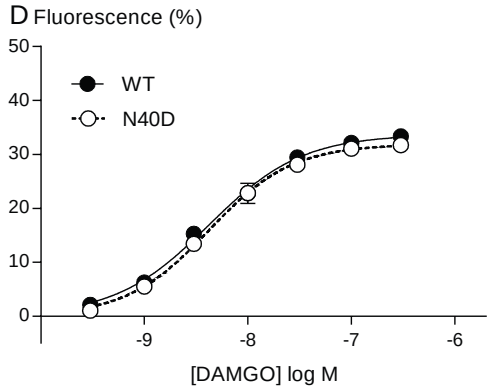
A

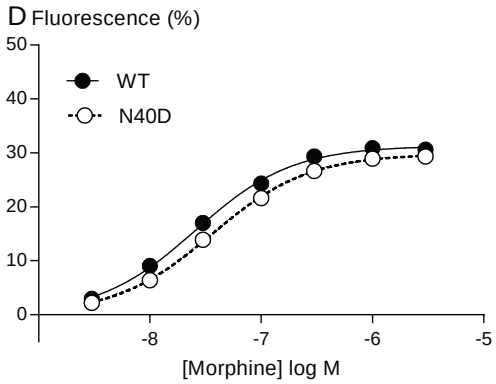

D Fluorescence (%)
WT
N40D
[Buprenorphine] log M

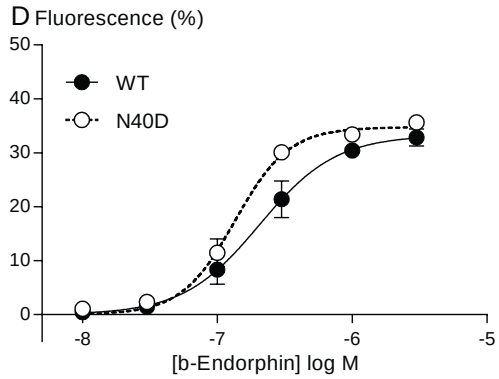
B


D Fluorescence (%)
WT
N40D
[Pentazocine] Log M

C


D Fluorescence (%)
WT
N40D
[DAMGO] log M


D Fluorescence (%)
WT
N40D
[Morphine] log M


D Fluorescence (%)
WT
N40D
[b-Endorphin] log M

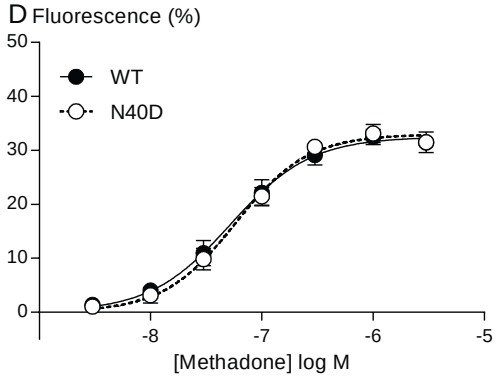

D Fluorescence (%)
WT
N40D
[Methadone] log M

**Table 3: Summary of opioid efficacy and potency in assays of GIRK activation in AtT-20 cells expressing MOPr-WT and MOPr-N40D.**

| GIRK activation | $E_{max}$ (%) | | $pEC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **N40D** | **WT** | **N40D** |
| DAMGO | 34 ± 1 | 33 ± 1 | 8.4 ± 0.1 | 8.4 ± 0.1 |
| β-Endorphin | 33 ± 2 | 34 ± 1 | 6.7 ± 0.1 | 6.9 ± 0.1 |
| Methadone | 33 ± 2 | 33 ± 2 | 7.3 ± 0.1 | 7.3 ± 0.1 |
| Morphine | 31 ± 1 | 30 ± 1 | 7.6 ± 0.1 | 7.4 ± 0.1 |
| Buprenorphine | 22 ± 1 | 20 ± 2 | 7.0 ± 0.1 | 6.7 ± 0.1* (P = 0.04) |
| Pentazocine | 8 ± 1 | 4 ± 1* (P *= 0.002) | 6.3 ± 0.6 | 7.0 ± 0.2 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than DAMGO are highlighted in red (One way ANOVA, followed by Students T-test, corrected for multiple comparisons, $P < 0.05$). $E_{max}$ or $pEC_{50}$ significantly different between MOPr-WT and MOPr-N40D are marked with * ($P < 0.05$, uncorrected for multiple comparisons).

**DISCUSSION and CONCLUSIONS**

We have shown that the commonly occurring MOPr variant N40D is less effectively activated by buprenorphine in assays of AC inhibition, ERK phosphorylation and GIRK activation. Buprenorphine efficacy was reduced by over 50% for adenylyl cyclase inhibition and ERK1/2 phosphorylation in CHO-MOPr cells, with no effect on potency. Buprenorphine efficacy for GIRK activation was not affected by the N40D variant when expressed in AtT-20 cells, but potency was decreased threefold. In assays of GIRK activation, pentazocine efficacy was also significantly decreased, with no change in potency. No other opioids were affected by the N40D variant in any of our assays.

Buprenorphine is a partial agonist, and as such, even modest differences in levels of receptor expression between variants and/or the presence of spare receptors could have a significant impact on its signalling profile. Studies in animal models, post-mortem human brain and heterologous expression systems have suggested the N40D variant causes decreased receptor expression, although reports are inconsistent (Kroslak *et al.*, 2007; Mague *et al.*, 2009; Oertel *et al.*, 2012; Zhang *et al.*, 2005). MOPr expression was similar for both variants in the cell lines used in our assays. Radioligand binding assays showed that CHO-MOPr-N40D cells had a slightly higher cell surface receptor expression level than CHO-MOPr-WT cells, and thus receptor expression levels are unlikely to have contributed to a decrease in buprenorphine efficacy at N40D. The N40D variant causes the removal of a putative N-linked glycosylation site, which has been shown to result in decreased MOPr glycosylation and stability in CHO cells (Huang *et al.*, 2012). In our study, MOPr expression in CHO cells was acutely induced prior to experiments, minimizing possible effects on receptor turnover. For AtT-20 cells, receptor expression was not directly measured, however the use of the FlpIn system for receptor transfection ensures that the receptor construct is inserted only once into the same location in the

genome, thus all cells are subjected to a similar transcriptional environment (Sauer, 1994). Additionally, buprenorphine efficacy was similar in cells expressing MOPr-WT and MOPr-N40D, suggesting similar expression levels.

Our findings suggest that the amino acid change on the N-terminus of MOPr may affect the ability of some opioid ligands to effectively transduce signals to the intracellular effectors of MOPr. GPCRs constantly oscillate through a range of possible conformations, and different ligands can more effectively stabilize a subset of receptor conformations (Kenakin & Miller, 2010). GPCRs undergo further conformational changes upon ligand binding, and the resulting conformation of the ligand-receptor complex may couple differentially to associated G-proteins (Pineyro *et al.*, 2007). The involvement of the N-terminal domain in MOPr conformational changes is not yet well understood, and this region of the receptor is not included in published crystal structures (Manglik *et al.*, 2012)**.** However, activation-dependent changes in this region have been observed in MOPr and other GPCRs. Antibodies generated against the region proximal to N40D on the N-terminal domain of MOPr show differential recognition of activated receptors, suggesting this region undergoes conformational changes upon ligand-binding (Gupta *et al.*, 2007, Gupta *et al.*, 2008). This effect was not seen in cells expressing MOPr-N40D or in cells treated with deglycosylating agents, indicating that agonist binding induces movement of N-glycan chains (Gupta *et al.*, 2008). Furthermore, N-terminal antibody binding was affected by changes in the C-terminal region of MOPr, implying that conformational changes associated with receptor coupling to associated G-proteins may influence all domains of MOPr. A similar effect was seen in other GPCRs examined in this study including the δ-opioid receptor and the cannabinoid CB1 receptor (Gupta *et al.*, 2007). N-terminal polymorphisms affect signalling in other related GPCRs. N-terminal SNPs in the 5-$HT_{2B}$ receptor increased constitutive and agonist-stimulated activity in COS-7 cells

(Belmer *et al.* 2014). Likewise, polymorphisms of the N-terminal region in the melanocortin-4 receptor increased constitutive activity (Srinivasan *et al.*, 2004). Taken together, these results indicate the N-terminal region can undergo substantial structural changes upon receptor activation, and that structural changes arising from genetic variation have the ability to significantly affect receptor function. Thus, the N40D substitution may affect the ability of a ligand to bind to MOPr, or affect the efficacy of ligand-directed MOPr coupling to effector pathways due to altered receptor conformation.

It is not immediately apparent why buprenorphine *efficacy* is affected in CHO cells while buprenorphine *potency* changes in AtT-20 cells, but the differences may be due to different effectors. MOPr inhibition of AC is mediated by $G\alpha_{i/o}$ subunits of the G-protein heterotrimer, whereas GIRK activation occurs via Gβγ subunits (Law *et al.*, 2000). Phosphorylation of ERK1/2 can occur via multiple pathways, and may involve Gα and Gβγ-coupled processes as well as G-protein independent pathways such as β-arrestin (Luttrell *et al.*, 2005). Whereas AC inhibition is mediated by a single Gα subunit, GIRK channels require the binding of 4 Gβγ subunits for activation (Corey and Clapham, 2001). Differences in the ability of the N40D variant to couple to various G-proteins may differentially affect opioid ligand signalling at Gα and Gβγ-mediated pathways (Allouche *et al.*, 1999; Galandrin *et al.*, 2008). Furthermore, cell lines vary in the available pool of G-proteins, effector molecules and regulatory proteins, and opioids may activate a different suite of G-proteins in CHO cells and AtT-20 cells (Atwood *et al.*, 2011). Alternatively, buprenorphine may show functional selectivity towards GIRK activation over AC inhibition or ERK1/2 phosphorylation, although studies of AC inhibition and ERK1/2 phosphorylation in AtT-20 cells are necessary to determine the presence of any functional selectivity.

The N40D variant did not affect signalling of any of the other 10 opioid ligands tested, including β-endorphin. The first in vitro studies of N40D signalling reported a threefold increase in β-endorphin affinity for MOPr-N40D in AV-12 cells, as well as a threefold increase in β-endorphin potency for GIRK activation in *Xenopus* oocytes (Bond *et al.*, 1998). We found no difference for MOPr-N40D in GIRK activation in AtT20 cells, or in AC inhibition or ERK1/2 phosphorylation in CHO cells. Other studies have also failed to find differences in β-endorphin coupling to other signalling pathways in various expression systems (Befort *et al.*, 2001; Beyer *et al.*; 2004; Kroslak *et al.*, 2007). Several studies have investigated the effect of N40D on $I_{Ca}$ channel inhibition, with reports of enhanced DAMGO and morphine signalling (Lopez Soto & Raingo, 2012; Margas *et al.*, 2007) via N40D, no change in DAMGO signalling (Ramchandari *et al.*, 2011), and decreased morphine potency at N40D (Mahmoud *et al.*, 2011). We found no significant differences in DAMGO or morphine signalling between WT-MOPr and N40D-MOPr. Most of the functional studies performed to date have failed to take into account receptor expression and/or receptor reserve, which may contribute to the inconsistency between results. Furthermore, most of the assays were performed under different experimental conditions, making direct comparison between studies difficult (reviewed in Knapman & Connor, 2014). In our study, the assays of AC and pERK1/2 were performed on MOPr-WT and MOPr-N40D expressing CHO cells with an isogenic cellular background, similar levels of receptor expression and in very similar experimental conditions, enabling direct comparisons of opioid potency and efficacy to be made between the variants and signalling pathways.

There are no functional studies investigating the consequences of the N40D variant on buprenorphine signalling published. However, the consistent loss of buprenorphine

effectiveness in several cellular expression systems and distinct signalling pathways suggest that the negative effect of the N40D variant on buprenorphine signalling may be significant in other cell types such as neurons, where MOPr is endogenously expressed. In populations where the N40D variant occurs at a high frequency (Mura *et al.*, 2013), a significant number of people will be homozygous for the 118G allele, however the majority of carriers will be heterozygous for the 118A allele. Few clinical studies distinguish between homo- and heterozygous carriers (e.g. Bond *et al.*, 1998; Borstav *et al.*¸2012; Solak *et al.*, 2014; Song *et al.*, 2013), and for the few studies that consider both genotypes the data is often contradictory and sample sizes are small (Klepstad *et al.*, 2004; Reyes-Gibby *et al.*, 2007). There is still considerable uncertainty about the formation of obligate dimers by MOPr and other Class A GPCR in native tissue (Malik *et al.*, 2013; Herrick-Davis 2013), and cells expressing both N40 and D40 receptors could have 2 populations of receptors independently signalling as monomers or homodimers, or variant receptors could interact and form heterodimers. Functional studies in cells co-expressing both WT and variant receptors have not been performed, largely due to the technical difficulty of accurately quantifying and expressing equivalent amounts of each variant. In one study, HA-tagged MOPr-WT and a FLAG-tagged MOPr-L85I variant that undergoes endocytosis in response to morphine were co-expressed in HEK293 cells. Both MOPr-WT and MOPr-L85I internalised in response to morphine, suggesting the formation of functional heterodimers with L85I showing a dominant phenotype (Ravindranathan *et al.*, 2009), although alternative explanations are also possible. Conversely, when MOPr-WT and a non-functional MOPr-R181C variant were co-expressed, MOPr-WT internalised independently in response to DAMGO, indicating that this variant either fails to form dimers or forms unstable dimers with MOPr-WT (Ravindranathan *et al.*, 2009). These results suggest that the potential for interaction between MOPr variants may be dependent

on the SNP, and the receptor dynamics resulting from co-expression of MOPr-WT and MOPr-N40D warrant further investigation.

Buprenorphine is commonly prescribed as both an analgesic and as an alternative to methadone for the treatment of opioid dependence, and differences in signalling arising from the N40D variant may be of clinical significance (Virk *et al.*, 2009). Most studies investigating differences in opioid requirements for N40D carriers in a clinical setting have not included buprenorphine. For example, a recent large study of European cancer patients investigated the association between the N40D variant and the required dose of opioid analgesics (Klepstad *et al.*, 2011). This study found no association between the N40D variant and dose of morphine, fentanyl or oxycodone used, entirely consistent with our results showing no difference in the activity of these ligands at the N40D variant (Klepstad *et al.*, 2011). Importantly, this study did not examine the interaction between OPRM1 variants and buprenorphine, and our data suggests that this would be worthwhile. One study investigating buprenorphine in N40D carriers reported a shorter opioid withdrawal period for newborns with the N40D variant who were exposed to maternal buprenorphine in utero, supporting the possibility of a reduced response of N40D carriers to buprenorphine (Wachman *et al.*, 2013). Different individual responses to opiate maintenance treatment including buprenorphine are frequently observed among heroin addicts, however attempts to predict responders and non-responders have been unsuccessful, and the effect of the N40D variant has not been investigated (Gerra *et al.*, 2014).

Our results demonstrate that the N40D variant has the potential to affect MOPr function across cell types and signalling pathways. Further functional studies examining other MOPr signalling pathways are required to extend our findings, and investigation into

buprenorphine response in N40D carriers in a clinical setting would be of great interest. The N40D variant is highly prevalent within the population, occurring at allelic frequencies ranging from 10 – 50% (Mura *et al.*, 2013). A decrease in buprenorphine efficacy arising from the N40D variant could be a contributing factor for the lack of response of some individuals to buprenorphine maintenance therapy, and may result in a significant proportion of the population receiving inadequate or inappropriate analgesic therapy. Although individual opioid response is influenced by a number of genetic and epigenetic factors, understanding how the N40D variant affects cellular signalling is a key element in predicting the potential clinical or phenotypic consequences of a particular opioid drug. Prior knowledge of individual genotype could provide valuable insight into the most effective form of opioid therapy, minimizing the risk of serious adverse events associated with opioid overdose, while maximizing therapeutic benefits and ensuring individuals receive adequate pain relief.

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**Author Contributions**

AK, MS and MC designed and analysed experiments, AK and MS conducted the experiments. AK and MC conceived the study and wrote the paper, all authors have seen the final manuscript.

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# Chapter 6

## The A6V polymorphism of the human μ-opioid receptor negatively impacts signalling of morphine and endogenous opioids *in vitro*

**Alisa Knapman, Marina Santiago and Mark Connor**

This paper has been prepared for future submission to the *British Journal of Pharmacology*. Marina Santiago constructed AtT20-MOPr cells and performed GIRK activation assays. Mark Connor assisted with experimental design, data analysis and manuscript preparation. All other work is my own.

**SUMMARY**

***Background and Purpose***

Evidence suggests that polymorphisms of the μ-opioid receptor (MOPr) may contribute to individual variation in opioid response. The A6V variant is present in up to 20% of individuals in some populations, and has been associated with increased susceptibility to substance abuse. To date, no functional studies have examined the effect of A6V on MOPr signalling in vitro. We sought to determine functional differences in MOPr signalling at several signalling pathways using a range of structurally distinct opioid ligands in cells expressing wild-type MOPr and the MOPr variant, A6V.

***Experimental Approach***

MOPr-WT and MOPr-A6V were stably expressed in CHO cells and in AtT-20 cells. Assays of adenylyl cyclase inhibition and ERK1/2 phosphorylation were performed on CHO cells, and assays of GIRK activation were performed on AtT-20 cells. Signalling profiles for each ligand were compared between variants.

***Key Results***

Buprenorphine efficacy was abolished at MOPr-A6V for adenylyl cyclase inhibition and ERK1/2 phosphorylation, but not GIRK activation. DAMGO, morphine and β-endorphin signalling was significantly compromised for AC inhibition, and signalling of all opioids tested was reduced for ERK1/2 phosphorylation. For GIRK activation, morphine signalling was selectively affected by the A6V variant.

***Conclusions and Implications***

The A6V variant is present in 1 - 20% of the population. This variant affects the signalling of many clinically important opioids including morphine, buprenorphine and fentanyl, as well as the signalling of endogenous opioids. This may affect individual response to opioid therapy, and possible disruption of the endogenous opioid system may contribute to susceptibility to substance abuse.

***Abbreviations***

| | |
|---|---|
| AC | adenylyl cyclase |
| BSA | bovine serum albumin |
| CHO | Chinese Hamster Ovary |
| DAMGO | ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) |
| DMEM | Dulbecco's Modified Eagle Medium |
| ERK | extracellular signal regulated kinase |
| FSK | forskolin |
| GIRK | G protein-gated inwardly rectifying K channel |
| GPCR | G protein couple receptors |
| MOPr | μ-opioid receptor |
| OPRM1 | opioid receptor mu 1 |
| PTX | pertussis toxin |

## INTRODUCTION

Morphine and other opioid analgesics are the most effective treatments currently available for moderate to severe pain. In addition to their powerful analgesic effects, opioids are associated with a number of negative side effects including respiratory depression, nausea, constipation and sedation, as well as the development of analgesic tolerance with continued use (Boom *et al.*, 2012; Matthes *et al.*, 1996). Opioids are characterised as having a narrow therapeutic window, and for most opioids there is a fine balance in dosing to achieve a therapeutic dose without producing dangerous side effects such as respiratory depression (Somogyi *et al.*, 2007). Furthermore, the degree to which individuals experience opioid-induced analgesia and adverse effects is highly variable and difficult to predict (Lotsch & Geisslinger, 2005; Merikangas *et al.*, 2008). This can lead to inadequate pain relief for many individuals, as dosing of opioid analgesics may be restricted in order to avoid serious adverse events (Skorpen & Laugsand, 2008).

Individual differences in opioid requirements and pain perception are likely to have a genetic basis (Crist & Berrettini, 2013). The μ-opioid receptor (MOPr) is the primary target for most clinically prescribed opioid analgesics including morphine, buprenorphine and oxycodone (Matthes *et al.*, 1996; Nozaki & Komei, 2007; Virk *et al.*, 2009). A number of naturally occurring, non-synonymous genetic variants have been identified in the coding region of the MOPr gene *OPRM1*, and there is evidence to suggest that some of these may affect individual opioid response (Lotsch & Geisslinger, 2005; Somogyi *et al.*, 2007). The two most common *OPRM1* variants are the N40D variant and the A6V variant, both single nucleotide polymorphisms (SNPs) in the N-terminal region of MOPr. The N40D variant, present at allelic frequencies ranging from 10-50% in various populations, has been studied extensively and associated with a diverse range of clinical outcomes, including differences in pain perception, opioid requirements, and predisposition to substance abuse, however

these associations have not been consistently found (Diatchenko *et al.*, 2011; Klepstad et al., 2011, Mague & Blendy, 2010; Mura *et al.*, 2013; Walter & Lotsch, 2009). Likewise, functional studies of MOPr show differences between N40D and wild-type (WT) receptor signalling, but with little consistency in results (Knapman & Connor, 2014).

Despite being reported at allelic frequencies upwards of 20% in African-American and northern Indian populations (e.g. Hoehe et al., 2000; Crowley *et al*., 2003; Kapur *et al*., 2007, Crystal 2010), the A6V variant has received far less attention than the N40D variant. Data suggest that there may be a higher frequency of the V6 allele in substance-abusing populations (Berrettini *et al.*, 1997; Compton *et al.*, 2003; Crowley *et al.*, 2003; Crystal *et al.*, 2010; Rommelspacher *et al.*, 2001), but the effects of the polymorphism on analgesic responses is unknown. Only two studies have examined the functional consequences of the A6V variant on MOPr signalling in vitro. In an assay of cAMP-dependent gene transcription in HEK-293 cells transiently expressing MOPr, there was no difference in the potency of DAMGO, endomorphin-1 or leu-enkephalin between the A6 and V6 variants (Fortin *et al.*, 2010). Another study expressed the V6 variant on the MOPr-1A splice variant backbone in HEK-293 cells co-transfected transiently with a chimeric G protein (Ravinadrathan *et al.*, 2009). In this study, the maximal effect of DAMGO but not morphine in mediating intracellular Ca release was increased at the MOPr-1A-V6 variant compared with MOPr-1A-A6. These studies provide little insight into how the MOPr-A6V polymorphism affects acute MOPr function, so in this study, we investigated the ability of human MOPr-WT and MOPr-A6V receptors to couple to several different signalling pathways in CHO cells and AtT-20 cells using 11 clinically important and/or structurally distinct opioid ligands. We found that the substitution of valine for alanine at amino acid 6 of MOPr had a significant, negative impact on the ability of the receptor to inhibit adenylyl cyclase (AC) and to stimulate extracellular signal-regulated kinase 1/2 (ERK1/2)

phosphorylation in CHO cells. Notably, buprenorphine signalling was abolished in CHO-MOPr-A6V cells for both AC inhibition and ERK1/2 phosphorylation. By contrast, the effects of the A6V substitution on activation of G protein-gated, inwardly rectifying potassium channels (GIRK, composed of $K_{IR}$ 3.x subunits) were very modest, with only morphine showing any reduction in efficacy. These data suggest that the common A6V polymorphism of MOPr results in a receptor that may show pathway specific changes in function.

## METHODS

### *MOPr transfection and cell culture*

CHO-FRT-TRex cells were stably transfected with a pcDNA5 construct encoding the haemagglutinin-tagged human μ-opioid receptor cDNA together with the pOG44 (Flp recombinase plasmid) using the transfectant Fugene (Promega), as described previously (Knapman *et al.,* 2014). The HA-tagged human wild-type μ-opioid receptor (MOPr-WT) and the μ-opioid receptor containing the V6 variant (MOPr-A6V) were synthesised by Genscript (Piscataway, New Jersey, USA). Cells expressing MOPr-WT or MOPr-A6V were selected using hygromycin B (500 μg/mL). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 500 μg/mL hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 μg/mL beyond passage 5.

AtT-20 FlpIn cells were constructed by transfecting Flp-recombinase target site (FRT)/LacZeo2 (Life Technologies) using Fugene. Successfully transfected cells were selected with 100 μg/mL zeocin, and stably transfected with MOPr-WT or MOPr-A6V as described for CHO cells. Cells expressing MOPr were selected using 100 μg/mL

hygromycin B. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 100 μg/mL hygromycin B.

CHO-MOPr cells and AtT-20-MOPr cells were passaged at 80% confluency as required. Assays were carried out on cells up to 30 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at greater than 90% confluence.

***MOPr receptor expression***

MOPr surface expression was determined on CHO-MOPr cells by incubating intact cells with 0.125 – 16 nM [$^3$H]-DAMGO (D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin; PerkinElmer, Waltham, MA) for 2 h at 4 °C in 50 mM Tris-Cl (pH 7.4). Briefly, 24 hours before the assay, cells were detached from flasks with trypsin/EDTA (Sigma) and resuspended in DMEM containing 10% FBS, 100U penicillin/streptomycin, plus 2 μg/mL tetracycline to induce MOPr expression. Cells were seeded at a density of $2 \times 10^5$ cells per well in 24-well plates pre-coated with polylysine and grown overnight at 37° C. On the day of the assay, cells were washed twice gently with 50 mM Tris-Cl (pH 7.4) and incubated for 2 h on ice with 0.125 – 16 nM [$^3$H]-DAMGO. Non-specific binding was determined in the presence of unlabeled DAMGO (10 μM). Non-specific binding was 15 ± 1% of total binding for CHO-MOPr-WT, and 12 ± 1% in CHO-MOPr-A6V. At the end of the incubation, cells were washed three times with 50 mM Tris-Cl (pH 7.4) at 4 °C. Cells in each well were then digested for 1 h at room temperature with 100 μL of 1N NaOH. 100 μL 1N HCl was added to each well to stop the reaction and cells were then collected into scintillation vials. Total bound [$^3$H]-DAMGO was determined using a liquid scintillation counter (Packard Tricarb, Perkin Elmer,Waltham MA, USA). Receptor density ($B_{max}$) and affinity ($K_d$) were calculated using a one-site binding curve fitted using GraphPad Prism (GraphPad Software, La Jolla, CA). Protein concentration per well was determined with a

BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions. All experiments were performed three times in triplicate.

***Membrane Potential Assay of Adenylyl Cyclase Inhibition***

Opioid inhibition of adenylyl cyclase was measured using a membrane potential assay as previously described (Knapman *et al.*, 2014). Briefly, 24 hrs prior to the assay, CHO-MOPr cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. MOPr receptor expression was induced with the addition of 2 μg/mL tetracycline. The cells were plated in a volume of 90 μl per well in black walled, clear-bottomed 96-well microplates (Corning), and incubated overnight at 37$^{o}$C in ambient $CO_2$. Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer containing (in mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 5). Prior to the assay, cells were loaded with 90 μl/well of the dye solution without removal of the L-15 and incubated at 37°C for 60 minutes in ambient $CO_2$. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time FSK + opioid or FSK + vehicle was added in a volume of 20 μl. Further additions were made in volumes of 20 μl, as indicated. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. When used, the final

concentration of the solvents DMSO or EtOH was not more than 0.1%, and these concentrations did not produce a signal in the assay.

***Membrane Potential Assay of GIRK Channel Activation***

Opioid activation of endogenous GIRK channels in AtT-20 cells was measured using a membrane potential sensitive dye, as previously described (Knapman *et al.*, 2013). AtT-20-MOPr cells were detached from flasks and plated using the same procedure as for the CHO-MOPr cells in the AC inhibition assay, without the addition of tetracycline. Blue membrane potential dye was reconstituted and loaded onto cells, and the assay was performed in the same way as the AC inhibition assay. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time opioid or vehicle was added in a volume of 20 µl. The background fluorescence of cells without dye or dye without cells was negligible. The final concentration of DMSO was not more than 0.1%, and this concentration did not produce a signal in the assay.

***ELISA of ERK1/2 phosphorylation***

Opioid-mediated ERK1/2 phosphorylation was measured by ELISA, as previously described (Knapman et al., *In Press*). Briefly, 24 hrs before the assay, CHO-MOPr cells were plated in 96-well clear microplates (Falcon) and receptor expression was induced as for the AC inhibition assay. Cells were serum-starved for 1 hr in 40 µL serum-free L-15 supplemented with 5% bovine serum albumin (BSA) before the assay. Cells were treated with drug or vehicle diluted in serum-free L-15 (40 µl added) with no BSA added to minimise background. Preliminary time-course experiments indicated that a 5 min drug treatment was optimal to produce robust ERK1/2 phosphorylation without inducing desensitisation, thus all drug treatments were for 5 min. After drug application, the reaction was stopped by inverting the plates to remove the drug solution, placing the plates on ice,

and immediately fixing the cells with 4% paraformaldehyde for 15 min at RT. Cells were washed 3 times with 300µL PBS, then permeabilized with 0.1% Triton-X in PBS for 30 min at RT. Triton-X was removed, and cells were incubated for 2 hr at RT with blocking solution consisting of 5% BSA in PBS with 0.01% Tween-20 (PBS-T). Blocking solution was removed, then cells were incubated overnight at 4°C with a 1:500 dilution of rabbit α-phospho-p44/42 MAPK (Thr202/Tyr204) antibody in PBS-T with 1% BSA. Cells were washed 3 times with 300µL PBS-T, and incubated with 1:5000 α-rabbit IgG HRP-linked antibody in PBS-T with 1% BSA for 2 hr. Cells were washed 4 times with 300 µL PBS-T, and incubated with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) at RT in the dark for 45 min. The reaction was stopped with 1M HCl. Absorbance was read at 450 nm using a BMG Pherastar FS microplate reader. Cells were then stained with 0.5µg/mL DAPI for 10 min at RT, and washed 3 times with 300 µL PBS-T. Fluorescence was read in the Pherastar microplate reader with cells excited at a wavelength of 358 nm and emission measured at 461 nm. Absorbance readings were normalised to DAPI staining to account for any differences in cell density between wells. Readings were then normalised to the response of cells treated with 100nM PMA for 10 min.

***Drugs and Chemicals***

Tissue culture reagents and buffer salts were from Invitrogen or Sigma unless otherwise noted. Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol acetate (DAMGO), endomorphin-1, endomorphin-2 and met-enkephalin were purchased from Auspep (Tullamarine, Australia). Morphine, fentanyl and pentazocine were a kind gift from the Department of Pharmacology, University of Sydney. Buprenorphine and oxycodone were from the National Measurement Institute (Lindfield, Australia). β-endorphin was from Genscript (Piscataway, New Jersey, USA). Forskolin and naloxone were from Ascent Pharmaceuticals (Bristol, UK). 1,9-dideoxyforskolin and tetraethylammonium (TEA) were

from Sigma Aldrich (Castle Hill, Australia). Pertussis toxin (PTX) was from Tocris Bioscience (Bristol, UK). Phospho-ERK1/2 antibody (Catalog #9101) and anti-rabbit IgG HRP-lined antibody (Catalog #7074) were from Cell Signalling Technologies, Danvers, Massachusetts, USA.

***Data***

Unless otherwise noted, data is expressed as mean ± s.e.m. of at least 5 determinations made in duplicate or triplicate. Concentration response curves (CRCs) were fit with a 4 parameter logistic equation using Graphpad Prism (Graphpad). CRCs from separate experiments were pooled and compared with two-way ANOVA, followed by Bonferroni post-hoc test corrected for multiple comparisons. $E_{max}$ and $pEC_{50}$ values derived from individual experiments were compared using unpaired Student's t-test. Comparisons between maximum agonist responses were made by comparing $E_{max}$ values derived from individual experiments using one-way ANOVA followed by Dunnett-s post-hoc test, corrected for multiple comparisons. All other comparisons were made using an unpaired Student's T-test. $P < 0.05$ was considered significant. Channel and receptor nomenclature is consistent with the British Journal of Pharmacology Concise Guide to Pharmacology 2013/2014 and recent guidelines from IUPHAR (Alexander et al., 2013, Cox et al., 2014).

**RESULTS**

***MOPr Expression in CHO-K1 cells***

CHO-K1 cells were stably transfected with either MOPr-WT or the MOPr-A6V variant, with receptor expression controlled by a tetracycline-sensitive repressor. After 24 h tetracycline induction, surface receptor expression was similar in cells expressing MOPr-WT or MOPr-A6V as indicated by similar levels of specific [$^3$H]DAMGO binding (see Figure 1). The $B_{max}$ for CHO-MOPr-WT cells was 280 ± 20 fmol/mg total protein and 301

± 25 fmol/mg for CHO-MOPr-A6V ($P > 0.05$). The affinity for [$^3$H]DAMGO was also similar between for MOPr-WT and MOPr-A6V expressing cells, with $K_D$ of 0.75 ± 0.10 and 0.55 ± 0.05, respectively ($P > 0.05$).

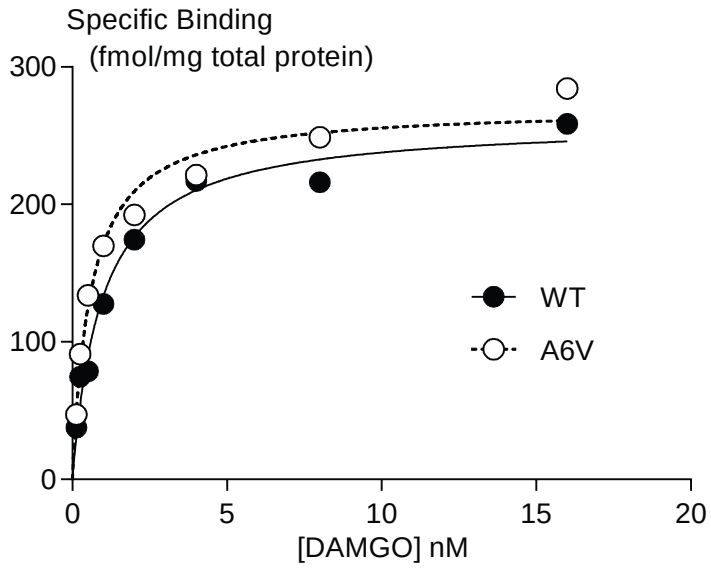



**Figure 1: Saturation binding curve of [$^3$H]DAMGO in intact CHO-MOPr-WT and CHO-MOPr-A6V cells, 24 hr after induction of receptor expression with tetracycline.** Radioligand binding was carried out as described in the Methods. No significant difference in $B_{max}$ or $K_d$ was observed between cells expressing MOPr-WT or MOPr-A6V ($P > 0.05$). Each point represents the mean ± s.e.m. of triplicate determinations from a single experiment. The assay was repeated 3 times.

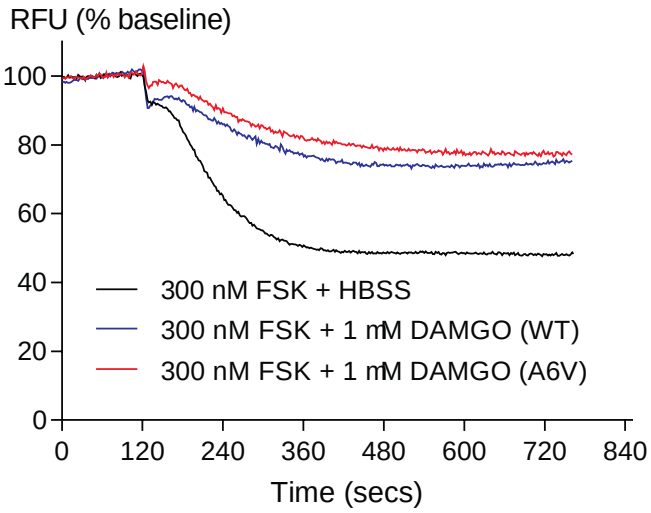
***Adenylyl Cyclase Inhibtion***

In CHO-MOPr cells loaded with membrane potential dye, addition of the AC activator forskolin (FSK, 300 nM) produced a rapid decrease in fluorescence, consistent with hyperpolarisation of the cells (Figure 2; Knapman *et al.*, 2014). The hyperpolarisation stabilised 5 min after the addition of FSK, at which point measurements were taken. FSK (300 nM) produced a similar hyperpolarisation in CHO-MOPr-WT cells (41 ± 1 % decrease in fluorescence) and CHO-MOPr-A6V cells (39 ± 2 % decrease, $P > 0.05$). Simultaneous addition of the MOPr selective peptide agonist DAMGO with FSK resulted in a concentration-dependent inhibition of the FSK-stimulated hyperpolarisation in both cell lines (Figure 2). DAMGO maximally inhibited the FSK response by 62 ± 3 %, with $pEC_{50}$ of 7.9 ± 0.1 in CHO-MOPr-WT cells (Table 1). We have reported previously that the effects of DAMGO in this assay were sensitive to naloxone and strongly inhibited by overnight pretreatment with pertussis toxin (Knapman et al., 2014). Application of opioids alone did not affect the membrane potential of CHO-MOPr cells, with the exception of 30 µM pentazocine and 10 µM methadone, which caused small, transient increases in fluorescence (< 10%). This effect was not naloxone sensitive.

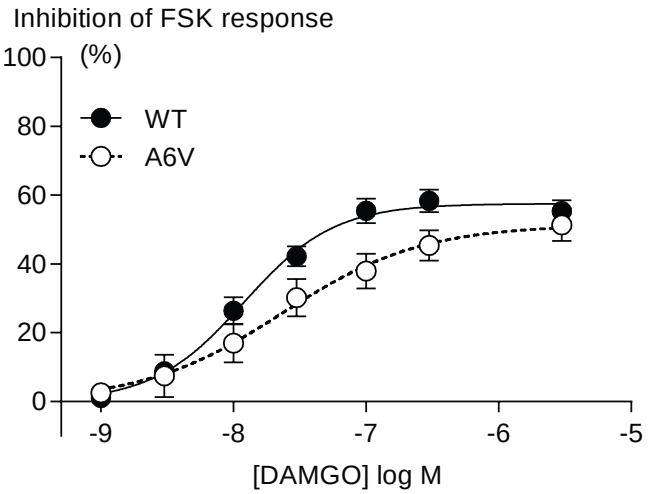
In cells expressing MOPr-A6V, DAMGO inhibition of the FSK-induced hyperpolarisation was significantly reduced compared with cells expressing MOPr-WT, with $E_{max}$ of 52 ± 3%, and $pEC_{50}$ of 7.6 ± 0.2 (Table 1). A 2-way ANOVA indicated the DAMGO CRCs for CHO-MOPr-WT and CHO-MOPr-A6V were significantly different ($P < 0.001$, Figure 2).

**Figure 2: DAMGO inhibits adenylyl cyclase and activates ERK1/2 in CHO cells expressing MOPr-WT or MOPr-A6V.** Adenylyl cyclase inhibition and levels of ERK1/2 phosphorylation were determined as described in the Methods. A) Raw trace showing decrease in fluorescent signal following application of 300 nM forskolin + HBSS, and 300 nM forskolin + 1 μM DAMGO at 120 sec. B) DAMGO inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation in CHO cells expressing MOPr-WT or MOPr-A6V was significantly affected by the A6V variant (2-way ANOVA, $P < 0.001$). C) DAMGO-stimulated ERK1/2 phosphorylation in CHO-MOPr cells was significantly affected by the A6V variant (2-way ANOVA, $P < 0.0001$). Maximum ERK1/2 phosphorylation by 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from at least 5-6 independent determinations performed in duplicate.

A


RFU (% baseline)
100
80
60
40
20
0
0
120
240
360
480
600
720
840
Time (secs)
300 nM FSK + HBSS
300 nM FSK + 1 mM DAMGO (WT)
300 nM FSK + 1 mM DAMGO (A6V)

B


Inhibition of FSK response
(%)
100
80
60
40
20
0
WT
A6V
-9
-8
-7
-6
-5
[DAMGO] log M

C

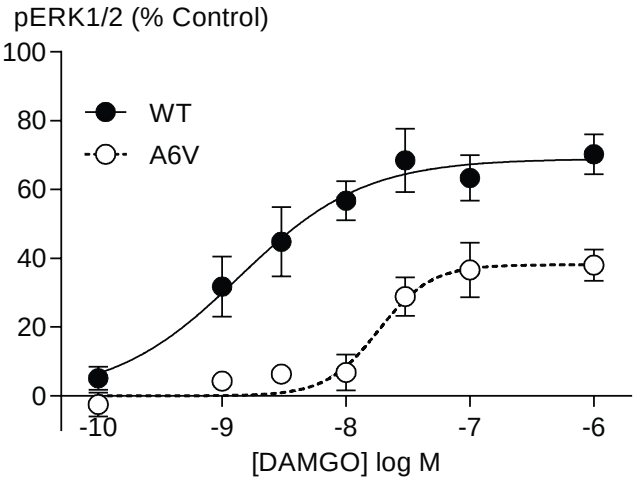

pERK1/2 (% Control)
100
80
60
40
20
0
WT
A6V
-10
-9
-8
-7
-6
[DAMGO] log M

**Table 1: Summary of opioid efficacy and potency in assays of AC inhibition in CHO cells expressing MOPr-WT and MOPr-A6V.**

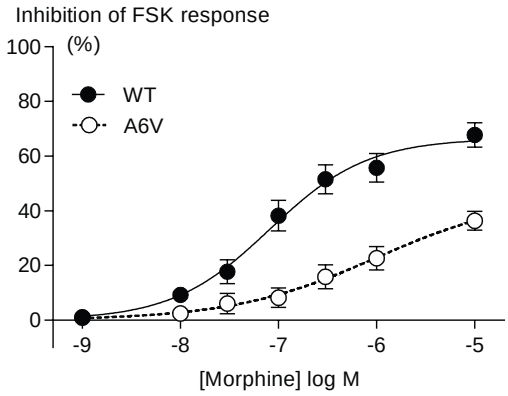
| AC inhibition | $E_{max}$ (%) | | $pEC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **A6V** | **WT** | **A6V** |
| β-Endorphin **** | 73 ± 4 | 50 ± 4* | 6.8 ± 0.1 | 6.4 ± 0.1* |
| Fentanyl ** | 70 ± 6 | 68 ± 6 | 8.0 ± 0.2 | 7.6 ± 0.1 |
| Morphine **** | 66 ± 4 | 39 ± 4* | 7.0 ± 0.1 | 6.1 ± 0.3* |
| Oxycodone *** | 65 ± 5 | 69 ± 8 | 5.8 ± 0.3 | 5.4 ± 0.4 |
| Methadone **** | 65 ± 3 | 52 ± 9 | 7.0 ± 0.1 | 6.4 ± 0.1* |
| Met-Enkephalin ** | 64 ± 4 | 67 ± 6 | 7.9 ± 0.1 | 7.2 ± 0.1* |
| Endomorphin-1 *** | 64 ± 5 | 61 ± 5 | 8.2 ± 0.1 | 7.9 ± 0.2 |
| Endomorphin-2 **** | 65 ± 7 | 56 ± 6 | 8.2 ± 0.1 | 7.7 ± 0.1* |
| DAMGO *** | 62 ± 3 | 52 ± 3* | 7.9 ± 0.1 | 7.6 ± 0.2 |
| Buprenorphine | 38 ± 7 | No Response | 8.4 ± 0.3 | No Response |
| Pentazocine * | 36 ± 5 | 24 ± 7 | N/A | N/A |

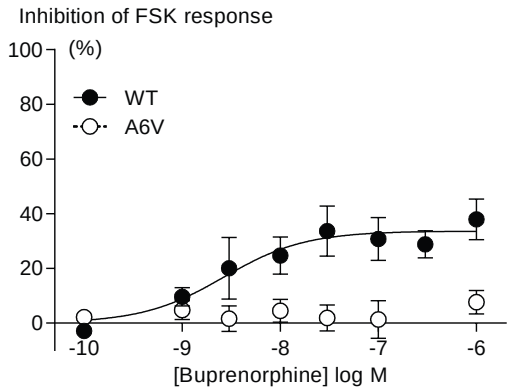
Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than β-endorphin are highlighted in red (1-way ANOVA, followed by Dunnett's post-hoc test corrected for multiple comparisons, $P < 0.05$). Opioids with concentration-response curves significantly different between variants are marked with * (2-way ANOVA, followed by Bonferroni post-hoc test, corrected for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). MOPr-A6V $E_{max}$ and $pEC_{50}$ values significantly different from MOPr-WT are marked with * (unpaired Student's T-test, $P < 0.05$).

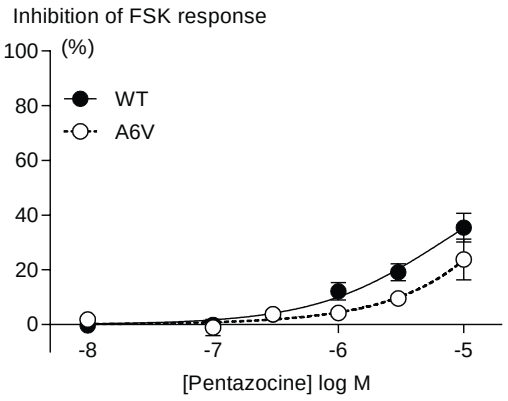
We next determined the potency and efficacy of a range of clinically important and/or structurally distinct opioid ligands in CHO cells expressing WT-MOPr or the MOPr-A6V to determine whether the A6V amino acid substitution affected MOPr inhibition of AC in a ligand-selective manner. In cells expressing MOPr-WT, morphine inhibited the FSK response by 66 ± 4%, with $p\text{EC}_{50}$ of 7.0 ± 0.1. In CHO-MOPr-A6V cells, morphine inhibition of FSK effects was significantly compromised, with a $p\text{EC}_{50}$ of 6.1 ± 0.3 and $E_{max}$ of 39 ± 4% ($P < 0.05$, Table 1). A 2-way ANOVA also indicated a significant difference between the morphine CRC for MOPr-WT and MOPr-A6V ($P < 0.0001$, Figure 3). We have previously shown that the partial agonist buprenorphine has a significantly reduced efficacy for AC inhibition in cells expressing MOPr -N40D, a variant also located on the N-terminal region of MOPr (Knapman *et al., In Press*). Buprenorphine inhibited the FSK response by 38 ± 7% in CHO-MOPr-WT cells, with $p\text{EC}_{50}$ of 8.4 ± 0.3 (Table 1). In CHO-MOPr-A6V cells, buprenorphine signalling was abolished (Figure 3). Maximum inhibition of the FSK response elicited by 10 μM pentazocine did not differ significantly between cells expressing MOPr-WT and MOPr-A6V (Table 1), however 2-way ANOVA showed a significant difference between the CRCs of CHO-MOPr-WT and CHO-MOPr-A6V ($P < 0.05$, Figure 3). At concentrations higher than 10 μM, pentazocine has been reported to block $K^+$ channels (Zhang & Cuevas, 2005), so concentrations above 10 μM were not tested despite pentazocine response not reaching a stable maximum at this concentration. For this reason, it was not possible to calculate $p\text{EC}_{50}$ for pentazocine AC inhibition.

**Figure 3: Morphine, buprenorphine and pentazocine inhibition of adenylyl cyclase and activation of ERK1/2 is compromised in CHO cells expressing MOPr-A6V.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) Morphine and pentazocine inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation was significantly decreased at MOPr-A6V compared with MOPr-WT (2-way ANOVA, $P < 0.05$). Buprenorphine signalling was abolished at MOPr-A6V. B) Morphine and pentazocine inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation was significantly decreased at MOPr-A6V compared with MOPr-WT (2-way ANOVA, $P < 0.001$). Buprenorphine signalling was abolished at MOPr-A6V. Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

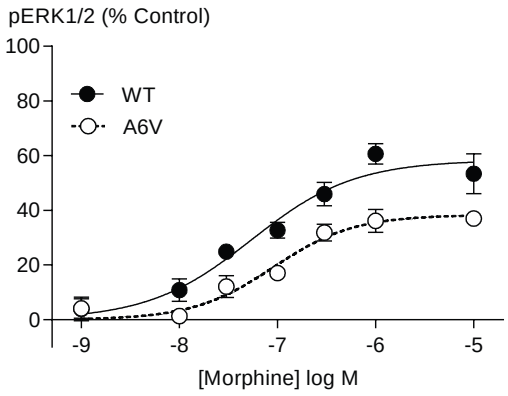
A


Inhibition of FSK response
(%)
WT
A6V
100
80
60
40
20
0
-9
-8
-7
-6
-5
[Morphine] log M


Inhibition of FSK response
(%)
WT
A6V
100
80
60
40
20
0
-10
-9
-8
-7
-6
[Buprenorphine] log M


Inhibition of FSK response
(%)
WT
A6V
100
80
60
40
20
0
-8
-7
-6
-5
[Pentazocine] log M

B


pERK1/2 (% Control)
WT
A6V
100
80
60
40
20
0
-9
-8
-7
-6
-5
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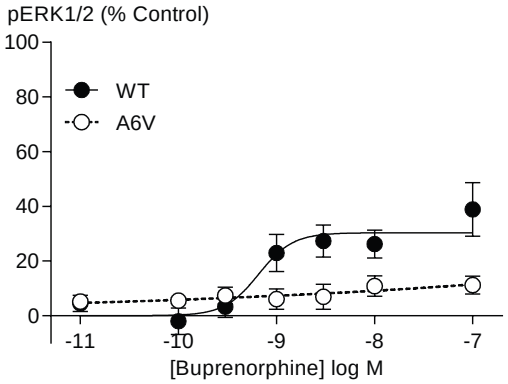
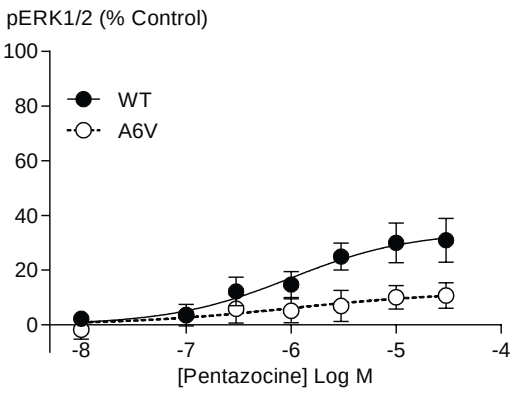

pERK1/2 (% Control)
WT
A6V
100
80
60
40
20
0
-11
-10
-9
-8
-7
[Buprenorphine] log M


pERK1/2 (% Control)
WT
A6V
100
80
60
40
20
0
-8
-7
-6
-5
-4
[Pentazocine] Log M

We next tested the effect of MOPr-A6V on AC inhibition by the endogenous opioids β-endorphin, endomorphins-1 and 2, and met-enkephalin. β-endorphin signalling was negatively affected by the A6V variant (2-way ANOVA, $P < 0.0001$, Figure 4). In CHO-MOPr-WT cells, β-endorphin inhibited the FSK response to a maximum of 73 ± 4%, with $p\mathrm{EC}_{50}$ of 6.8 ± 0.1. Maximum β-endorphin FSK inhibition was significantly decreased in CHO-MOPr-A6V cells with $\mathrm{E}_{max}$ of 50 ± 4% ($P < 0.05$), and $p\mathrm{EC}_{50}$ of 6.4 ± 0.1 was also significantly different to MOPr-WT (Table 1). In cells expressing MOPr-A6V, CRCs for the endogenous opioid ligands endomorphin-1, endomorphin-2 and met-enkephalin differed significantly to CRCs of CHO-MOPr-WT (2-way ANOVA, $P < 0.01$, Figure 4). $\mathrm{E}_{max}$ and $p\mathrm{EC}_{50}$ for endomorphin-1 in cells expressing MOPr-WT and MOPr-A6V were similar (Table 1). For endomorphin-2, $\mathrm{E}_{max}$ values for MOPr-WT and MOPr-A6V did not differ significantly, however $p\mathrm{EC}_{50}$ was affected at 8.2 ± 0.1 and 7.7 ± 0.1 respectively ($P < 0.05$, Table 1). Likewise, maximum met-enkephalin inhibition of the FSK effect was similar between variants, but $p\mathrm{EC}_{50}$ values of 7.9 ± 0.1 at MOPr-WT and 7.2 ± 0.1 at MOPr-A6V were significantly different ($P < 0.05$, Table 1).

CHO-MOPr cells were then challenged with the clinically important opioids fentanyl, methadone and oxycodone. The maximum inhibition of the FSK effect by fentanyl was similar in cells expressing MOPr-WT or MOPr-A6V, however 2-way ANOVA indicated a significant difference in the fentanyl CRCs ($P < 0.01$, Figure 5). There was also a significant effect of the A6V variant on the CRCs of methadone and oxycodone (2-way ANOVA, $P < 0.001$, Figure 5). Maximum FSK inhibition by methadone was not different between MOPr-WT and MOPr-A6V, however $p\mathrm{EC}_{50}$s of 7.0 ± 0.1 and 6.4 ± 0.1 respectively were significantly different ($P < 0.05$, Table 1). Oxycodone $\mathrm{E}_{max}$ and $p\mathrm{EC}_{50}$ were not affected by MOPr-A6V (Table 1).

***Opioid-mediated phosphorylation of ERK1/2***

We next examined whether the A6V amino acid substitution has an impact on opioid signalling through another important pathway activated by MOPr, stimulation of ERK1/2 phosphorylation. We analysed the ability of opioids to stimulate ERK1/2 phosphorylation via MOPr-WT and MOPr-A6V in CHO cells, using a whole-cell ELISA assay. Opioid responses were normalised against 100 nM PMA applied for 10 minutes. The average PMA response was similar in cells expressing MOPr-WT or MOPr-A6V, with corrected absorbance readings of 0.60 ± 0.06 and 0.53 ± 0.09, respectively ($P > 0.05$). Preincubation of cells with 1 µM naloxone for 5 mins blocked the response of $EC_{80}$ concentrations of all opioids used in this assay. Application of DAMGO to cells treated overnight with 200ng/mL PTX, or in cells where receptor expression had not been induced did not elicit a response. DAMGO-stimulated ERK1/2 phosphorylation was significantly affected by the A6V variant in CHO-MOPr cells when compared with WT expressing cells (2-way ANOVA, $P < 0.0001$, Figure 2). DAMGO $E_{max}$ for ERK1/2 phosphorylation was 70 ± 3% of the PMA response in CHO-MOPr-WT cells with a $pEC_{50}$ of 8.6 ± 0.1, and in CHO-MOPr-A6V cells, DAMGO $E_{max}$ was 48 ± 2%, with $pEC_{50}$ of 7.1 ± 0.4 ($P < 0.05$, Table 2). ERK1/2 phosphorylation elicited by morphine was significantly altered at MOPr-A6V when compared with MOPr-WT (2-way ANOVA, $P < 0.0001$, Figure 3). Morphine activated ERK1/2 to a maximum of 58 ± 6% at MOPr-WT, and 39 ± 2% at MOPr-A6V ($P < 0.05$). The $pEC_{50}$ for morphine did not differ significantly between variants (Table 2). Buprenorphine failed to elicit ERK1/2 phosphorylation in cells expressing MOPr-A6V (Figure 3, Table 2). The CRC of ERK1/2 phosphorylation stimulated by pentazocine was altered at MOPr-A6V when compared with MOPr-WT (2-way ANOVA, $P < 0.0001$, Figure 3), however $E_{max}$ and $pEC_{50}$ were not significantly different (Table 2).

**Figure 4: Endogenous opioid inhibition of adenylyl cyclase and activation of ERK1/2 is significantly affected by the A6V variant in CHO cells expressing MOPr.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) β-endorphin, endomorphins 1 & 2, and met-enkephalin inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation was significantly different between MOPr-WT and MOPr-A6V (2-way ANOVA, $P < 0.01$). B) β-endorphin, endomorphins 1 & 2, and met-enkephalin activation of ERK1/2 was significantly different between MOPr-WT and MOPr-A6V (2-way ANOVA, $P < 0.01$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

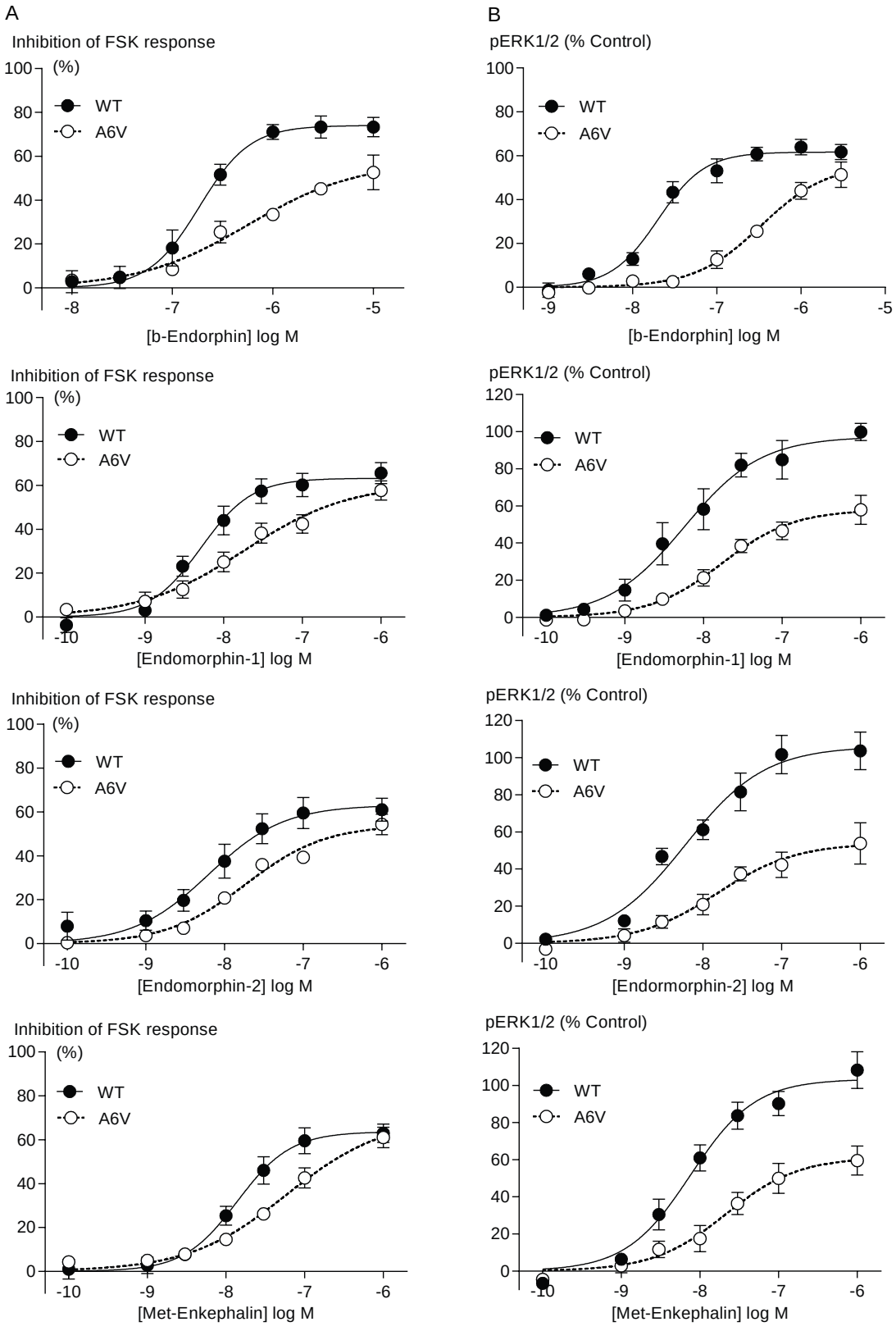

A
Inhibition of FSK response
(%)
WT
A6V
[b-Endorphin] log M
B
pERK1/2 (% Control)
WT
A6V
[b-Endorphin] log M
Inhibition of FSK response
(%)
WT
A6V
[Endomorphin-1] log M
pERK1/2 (% Control)
WT
A6V
[Endomorphin-1] log M
Inhibition of FSK response
(%)
WT
A6V
[Endomorphin-2] log M
pERK1/2 (% Control)
WT
A6V
[Endormorphin-2] log M
Inhibition of FSK response
(%)
WT
A6V
[Met-Enkephalin] log M
pERK1/2 (% Control)
WT
A6V
[Met-Enkephalin] log M

**Table 2: Summary of opioid efficacy and potency in assays of ERK1/2 phosphorylation in CHO cells expressing MOPr-WT and MOPr-A6V.**

| pERK1/2 | $E_{max}$ (%) | | *p*$EC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **A6V** | **WT** | **A6V** |
| Endomorphin-2 **** | 106 ± 7 | 54 ± 7 | 8.2 ± 0.1 | 7.8 ± 0.2 |
| Met-Enkephalin **** | 103 ± 6 | 61 ± 7 | 8.1 ± 0.1 | 7.7 ± 0.2 |
| Oxycodone **** | 101 ± 6 | 57 ± 6 | 6.3 ± 0.1 | 6.1 ± 0.2 |
| Fentanyl **** | 98 ± 5 | 56 ± 4 | 8.2 ± 0.1 | 7.8 ± 0.1 |
| Endomorphin-1 **** | 97 ± 7 | 58 ± 5 | 8.2 ± 0.1 | 7.8 ± 0.1 |
| Methadone **** | 88 ± 6 | 46 ± 4 | 7.3 ± 0.1 | 6.7 ± 0.1 |
| DAMGO **** | 69 ± 5 | 38 ± 4 | 8.8 ± 0.2 | 7.7 ± 0.1 |
| β-Endorphin **** | 62 ± 2 | 56 ± 6 | 7.7 ± 0.1 | 6.4 ± 0.1 |
| Morphine **** | 58 ± 4 | 38 ± 3 | 7.2 ± 0.1 | 7.0 ± 0.1 |
| Pentazocine *** | 34 ± 9 | 12 ± 12 | 6.0 ± 0.4 | 6.0 ± 2.0 |
| Buprenorphine | 30 ± 4 | No Response | 9.1 ± 0.2 | No Response |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than endomorphin-2 are highlighted in red (1-way ANOVA, followed by Dunnett's post-hoc test, corrected for multiple comparisons, $P < 0.05$). Opioids with concentration-response curves significantly different between variants are marked with * (2-way ANOVA, followed by Bonferroni post-hoc test, corrected for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). MOPr-A6V Emax and *p*$EC_{50}$ values significantly different from MOPr-WT are marked with * (unpaired Student's T-test, $P < 0.05$).

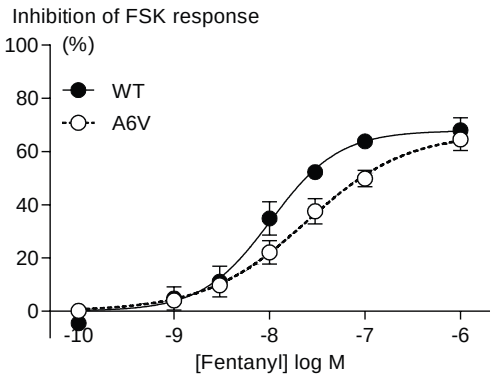
The maximum ERK1/2 phosphorylation elicited by β-endorphin was similar between cells expressing MOPr-WT and MOPr-A6V however 2-way ANOVA showed a significant effect of MOPr-A6V on β-endorphin CRC ($P < 0.0001$, Figure 4). CRCs for endomorphin-1 and endomorphin-2 were also different at MOPr-A6V (2-way ANOVA, $P < 0.0001$, Figure 4), with $E_{max}$ significantly decreased for both opioids, and endomorphin-1 $pEC_{50}$ altered ($P < 0.05$, Table 2). Similarly, $E_{max}$ for met-enkephalin stimulated ERK1/2 phosphorylation was decreased in CHO-MOPr-A6V cells compared with cells expressing MOPr-WT ($P < 0.005$, Table 2), and CRCs were significantly different (2-way ANOVA, $P < 0.0001$, Figure 4).

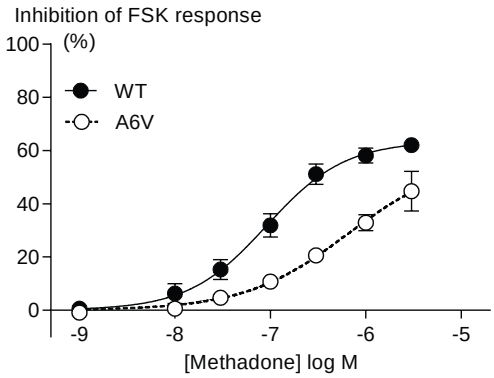
The A6V variant had a negative impact on the ability of MOPr to stimulate ERK1/2 phosphorylation via fentanyl, methadone and oxycodone, with CRCs significantly different to WT (2-way ANOVA, $P < 0.0001$, Figure 5). Maximal ERK1/2 phosphorylation by fentanyl, methadone and oxycodone was significantly decreased at MOPr-A6V ($P < 0.05$, Table 2).

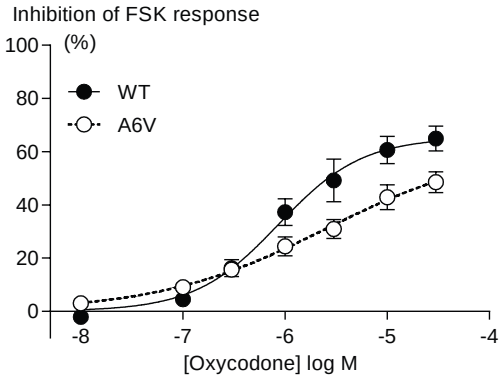
The A6V variant had a negative effect on the signalling of most opioids tested for both AC inhibition and ERK1/2 phosphorylation, however the effect was more pronounced for ERK1/2 phosphorylation. In assays of AC inhibition, the Emax and pEC50 of DAMGO, morphine, and β-endorphin were significantly affected by the A6V variant, and $pEC_{50}$s of endomorphin-2, met-enkephalin and methadone were also affected (Table 1). In assays of ERK1/2 phosphorylation, maximum opioid response was reduced by approximately 35 - 50% for every opioid tested with the exception of β-endorphin, where $E_{max}$ was not affected but $pEC_{50}$ was, and buprenorphine, where signalling was completely abolished (Table 2).

**Figure 5: Fentanyl, methadone and oxycodone inhibition of adenylyl cyclase and activation of ERK1/2 is significantly affected by the A6V variant in CHO cells expressing MOPr.** Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. Adenylyl cyclase inhibition and levels of ERK phosphorylation were determined as described in the Methods. A) Fentanyl, methadone and oxycodone inhibition of forskolin-stimulated adenylyl cyclase hyperpolarisation was significantly different between MOPr-WT and MOPr-A6V (2-way ANOVA, $P < 0.01$). B) Fentanyl, methadone and oxycodone activation of ERK1/2 was significantly different between MOPr-WT and MOPr-A6V (2-way ANOVA, $P < 0.0001$). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

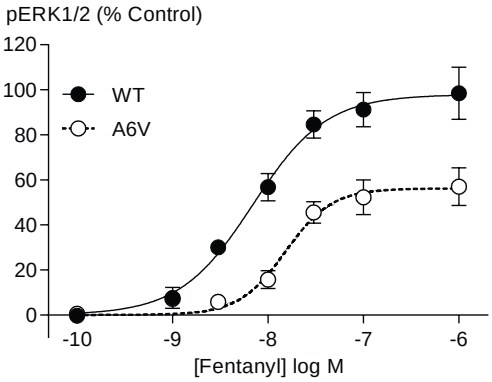
A

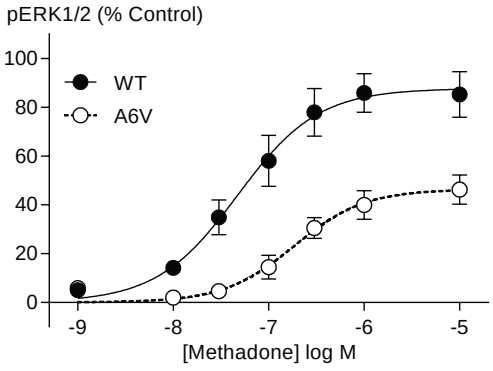

Inhibition of FSK response
(%)
WT
A6V
[Fentanyl] log M

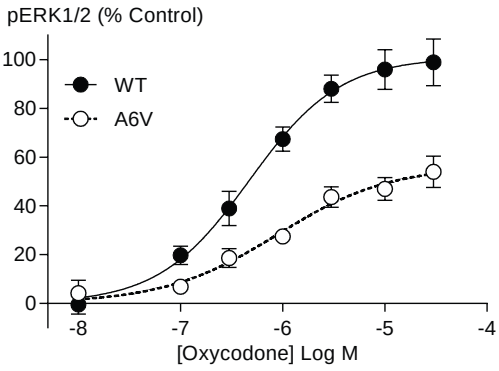

Inhibition of FSK response
(%)
WT
A6V
[Methadone] log M


Inhibition of FSK response
(%)
WT
A6V
[Oxycodone] log M

B


pERK1/2 (% Control)
WT
A6V
[Fentanyl] log M


pERK1/2 (% Control)
WT
A6V
[Methadone] log M


pERK1/2 (% Control)
WT
A6V
[Oxycodone] Log M

***GIRK channel activation in AtT-20 cells***

Activation of MOPr causes GIRK channel activation via Gβγ subunits, resulting in membrane hyperpolarisation. CHO-K1 cells do not express endogenous GIRK channels, so we assessed opioid mediated GIRK activation in AtT-20 cells stably transfected with hMOPr-WT and hMOPr-A6V as previously described (Knapman *et al.*, 2013). In AtT-20 cells loaded with membrane potential sensitive dye, application of opioids resulted in a concentration-dependent decrease in fluorescence from baseline, corresponding to membrane hyperpolarisation from GIRK activation (Figure 6). DAMGO-stimulated GIRK activation in AtT20-MOPr-WT cells and AtT20-MOPr-A6V cells did not differ significantly (2-way ANOVA, Figure **7**). DAMGO $E_{max}$ was 34 ± 1% for cells expressing MOPr-WT, and 31 ± 1% for cells expressing MOPr-A6V. DAMGO potency was also similar between variants, with $pEC_{50}$ values of 8.4 ± 0.1 and 8.5 ± 0 (Table 3).

Morphine activated GIRK in AtT20-MOPr-WT cells to a maximum of 31 ± 1%, with pEC50 of 7.6 ± 0.1. In AtT20-MOPr-A6V cells, morphine $E_{max}$ was significantly reduced, at 28 ± 1% ($P < 0.05$, Table 3). A 2-way ANOVA indicated a significant difference between MOPr-WT and MOPr-A6V morphine CRCs ($P < 0.0001$, Figure 7). Maximum buprenorphine-stimulated GIRK activation and $pEC_{50}$ did not differ between cells expressing MOPr-WT and MOPr-A6V, however 2-way ANOVA showed a significant effect of the A6V variant on buprenorphine signalling overall ($P < 0.05$, Figure 7). Interestingly, buprenorphine appears to be slightly more efficacious in AtT20-MOPr-A6V cells compared with AtT-MOPr-WT cells (Figure 7, Table 3). This is in direct contrast with the effect of the A6V variant on AC inhibition and ERK1/2 phosphorylation in CHO cells, where buprenorphine failed to elicit a response. In AtT20-MOPr-WT and AtT20-MOPr-A6V cells, there was no difference in GIRK activation for pentazocine, β-endorphin or methadone (Figure 7, Table 3).

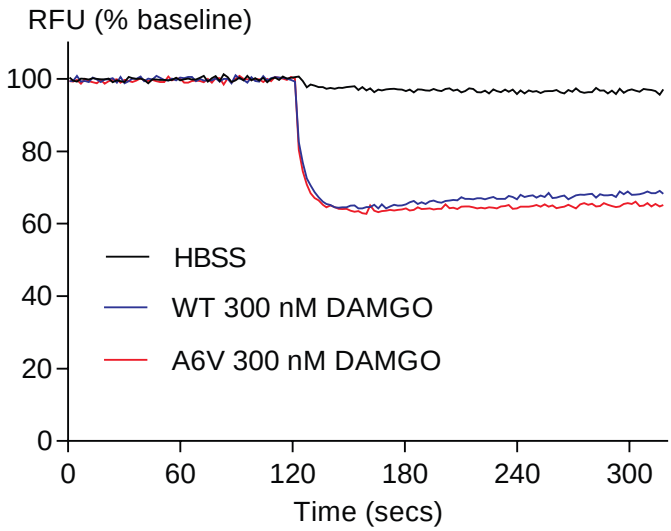
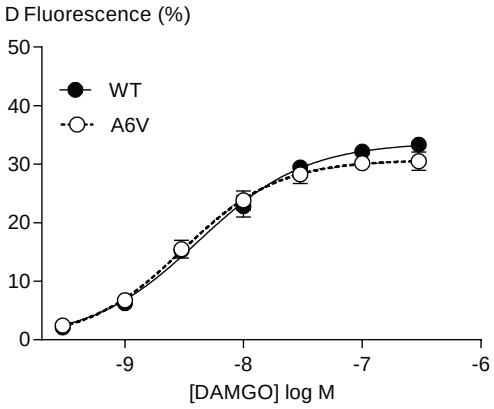
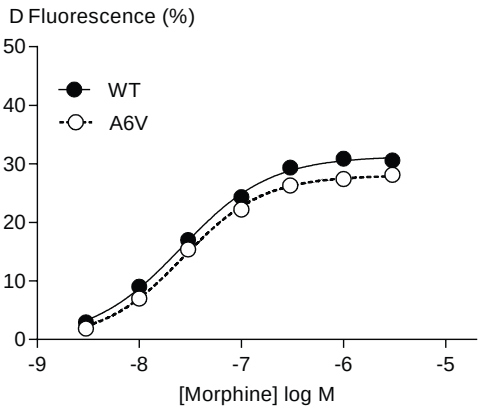
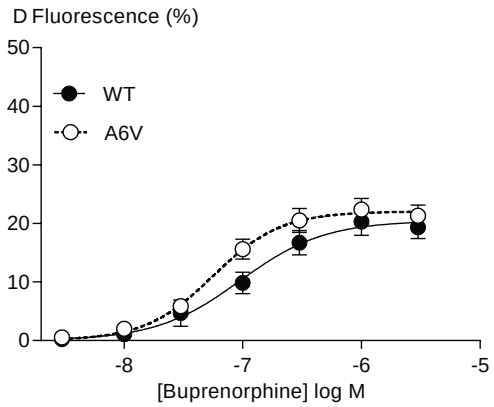


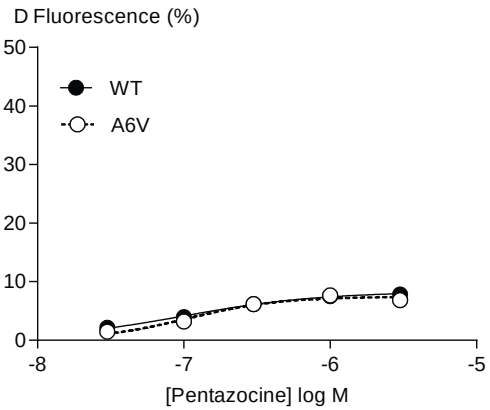

**Figure 6: DAMGO causes membrane hyperpolarisation in AtT-20 cells expressing MOPr-WT and MOPr-A6V.** Raw trace showing decrease in fluorescent signal following application of 300 nM DAMGO or 3 µM buprenorphine at 120 sec, corresponding to membrane hyperpolarisation from GIRK activation.

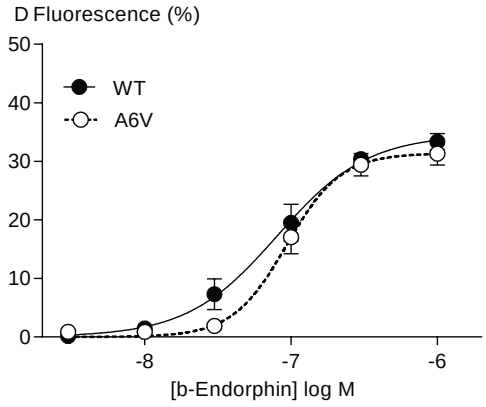
**Figure 7: Morphine activates GIRK less effectively in AtT20 cells expressing MOPr-A6V.** GIRK activation was determined as described in the Methods. Morphine and buprenorphine activation of GIRK channels was significantly different between MOPr-WT and MOPr-A6V (2-way ANOVA, $P < 0.05$). Morphine $E_{max}$ was decreased from 31 ± 1% in AtT20-MOPr-WT to 28 ± 1% in AtT20-MOPr-A6V (Student's T-test, $P < 0.05$). Buprenorphine $E_{max}$ and $pEC_{50}$ did not differ significantly between MOPr-WT and MOPr-A6V. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.


D Fluorescence (%)
WT
A6V
[DAMGO] log M


D Fluorescence (%)
WT
A6V
[Morphine] log M


D Fluorescence (%)
WT
A6V
[Buprenorphine] log M


D Fluorescence (%)
WT
A6V
[Pentazocine] log M


D Fluorescence (%)
WT
A6V
[b-Endorphin] log M

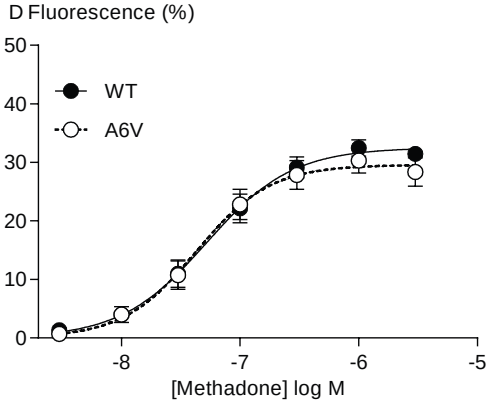

D Fluorescence (%)
WT
A6V
[Methadone] log M

**Table 3: Summary of opioid efficacy and potency in assays of GIRK activation in AtT-20 cells expressing MOPr-WT and MOPr-A6V.**

| GIRK activation | $E_{max}$ (%) | | *p*$EC_{50}$ | |
|---|---|---|---|---|
| **Opioid** | **WT** | **A6V** | **WT** | **A6V** |
| DAMGO | 34 ± 1 | 31 ± 1 | 8.4 ± 0.1 | 8.5 ±0.1 |
| β-Endorphin | 35 ± 2 | 32 ± 1 | 7.0 ± 0.1 | 7.0 ± 0.1 |
| Methadone | 33 ± 1 | 30 ± 1 | 7.3 ± 0.1 | 7.4 ± 0.1 |
| Morphine **** | 31 ± 1 | 28 ± 1 | 7.6 ± 0.1 | 7.6 ± 0.1 |
| Buprenorphine * | 21 ± 2 | 22 ± 1 | 7.0 ± 0.1 | 7.3 ± 0.1 |
| Pentazocine | 8 ± 1 | 7 ± 1 | 7.0 ± 0.1 | 7.4 ± 0.1 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than DAMGO are highlighted in red (1-way ANOVA, Dunnett's post-hoc test, corrected for multiple comparisons, $P < 0.05$). Opioids with concentration-response curves significantly different between variants are marked with * (2-way ANOVA, followed by Bonferroni post-hoc test, corrected for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$). MOPr-A6V Emax and pEC50 values significantly different from MOPr-WT are marked with * (unpaired Student's T-test, $P < 0.05$).

**DISCUSSION and CONCLUSIONS**

We have shown that a relatively commonly occurring MOPr variant, A6V has a significant, detrimental impact on the ability of MOPr to couple to effector pathways in CHO cells. In assays of AC inhibition, buprenorphine signalling was abolished at the A6V variant. Most opioids tested had reduced potency in CHO-MOPr-A6V cells compared with CHO-MOPr-WT cells, and the response at maximal concentrations of opioid was significantly reduced for morphine, β-endorphin, methadone and oxycodone. In assays of ERK1/2 phosphorylation, buprenorphine also failed to elicit a response in cells expressing MOPr-A6V. Maximum ERK1/2 phosphorylation was significantly decreased for DAMGO and all other opioids tested in CHO-MOPr-A6V cells, with the exception of β-endorphin. A significant effect of A6V was also apparent at sub-maximal concentrations of β-endorphin, as well as DAMGO, endomorphin-1, endomorphin-2, met-enkephalin, fentanyl and methadone. In contrast to assays of AC inhibition and ERK1/2 phosphorylation, MOPr coupling to GIRK activation did not appear to be significantly affected by the A6V variant. In AtT20-MOPr-A6V cells, GIRK activation was similar for all opioids tested, with the exception of morphine for which a significant effect of the variant was observed at submaximal concentrations, although it seems ulikely this would translate to a significant physiological effect. Most strikingly, buprenorphine-stimulated GIRK activation was not affected by the A6V variant when expressed in AtT-20 cells.

In the absence of spare receptors, even modest differences in levels of receptor expression could have a significant impact on the signalling profile of MOPr variants. There is no information on the effects of the A6V variant on receptor expression in humans or animal models. However, in our assays, MOPr expression was similar for both variants for CHO-K1 cells. Radioligand binding assays showed that CHO-MOPr-A6V cells had similar surface receptor expression as CHO-MOPr-WT cells, and thus receptor expression levels

are unlikely to have contributed to a decrease in opioid signalling at MOPr-A6V. For AtT20-MOPr cells, receptor expression was not directly measured, however the use of the FlpIn system for receptor transfection ensures that the receptor construct is inserted into the same location in the genome. All cells are thus subjected to a similar transcriptional environment (Sauer, 1994). The efficacy of the partial agonists buprenorphine and pentazocine was similar in AtT20 cells expressing MOPr-WT and MOPr-A6V, suggesting similar expression levels.

Our findings suggest that the alanine to valine amino acid change on the N-terminus of MOPr may affect the ability of opioid ligands to bind to MOPr and effectively transduce signal to intracellular effectors. Genetic variation of GPCRs may affect receptor function by altering GPCR conformation, with conformational changes potentially affecting the ability of ligands to bind to the receptor, and/or affecting the efficacy of the resulting ligand/receptor complex in coupling to associated effector molecules (Kenakin & Miller, 2010; Pineyro *et al.*, 2007). Most studies examining ligand interaction with GPCRs have focused on regions that form the ligand-binding pocket, namely the transmembrane helices and extracellular loops, and the recently published crystal structures of MOPr, DOPr and KOPr do not include the N-terminus (Granier *et al.*, 2012; Manglik *et al.*, 2012; Wu *et al.*, 2012). The A6V residue change is located at the distal end of the N-terminal region, and thus it is somewhat surprising that this variant has such a striking impact on MOPr function. However, several studies investigating the functional consequences of the common MOPr variant N40D, which is also present in the N-terminal region of MOPr, have reported altered N40D signalling (reviewed in Knapman & Connor, 2014). We have recently shown that in CHO cells, the MOPr-N40D variant selectively decreases buprenorphine efficacy for AC inhibition and ERK1/2 phosphorylation. Furthermore, buprenorphine potency for GIRK activation was decreased in AtT20-MOPr-N40 cells

(Knapman *et al.*,2014). In the present study, buprenorphine was also the most markedly affected opioid, with its activity completely abolished in CHO-MOPr-A6V cells for both AC inhibition and ERK1/2 phosphorylation. The N40D variant of MOPr removes a putative glycosylation site, which has been associated with decreased levels of mRNA and receptor expression in animal models and post-mortem human brain (Oertel *et al.*, 2009; Zhang *et al.*, 2005). The A6V variant does not affect a MOPr glycosylation site, and receptor expression was equivalent between cell lines, suggesting that genetic changes in the N-terminal region of MOPr may be capable of affecting receptor function independently of receptor glycosylation.

Importantly, the N-terminal region has been shown to undergo activation-dependent changes in MOPr and other GPCRs. Antibodies generated against the N-terminal domain of MOPr show differential recognition of inactive and activated receptors, suggesting this region undergoes conformational changes upon ligand-binding (Gupta *et al.*, 2007, Gupta *et al.*, 2008). Changes in the C-terminal region of MOPr also affected N-terminal antibody binding, implying that all domains of MOPr may be affected by conformational changes associated with receptor coupling to associated G-proteins. A similar effect was seen in other GPCRs examined in this study including the δ-opioid receptor and the cannabinoid CB1 receptor (Gupta *et al.*, 2007). Signalling in some other GPCRs is also affected by N-terminal SNPs. Constitutive and agonist-stimulated activity of the 5-$HT_{2B}$ receptor was increased by an R > G aa substitution at an equivalent position on the N-terminal tail to MOPr-A6V (Belmer *et al.*, 2014). It is perhaps not surprising that the residue change from a large, charged arginine molecule to a small neutral glycine molecule has a significant impact on receptor signalling. In the present study, the A6V substitution on MOPr is a relatively small change with a single methylene addition, and given its position, would generally not be expected to have a significant effect on receptor function. Nonetheless, the

dramatic impact of this alteration on MOPr signalling underscores the potential importance of this region to global GPCR conformation and function (Belmer *et al.* 2014). N-terminal polymorphisms in the melanocortin-4 receptor have also been shown to increase constitutive activity (Srinivasan *et al.*, 2004). The results of these studies suggest the N-terminal region may undergo substantial structural changes upon receptor activation, and that structural changes arising from genetic variation have the ability to significantly affect receptor function.

Intriguingly, although opioid signalling was markedly decreased in CHO cells expressing MOPr-A6V, in AtT20-MOPr cells the A6V variant had little effect on the ability of opioids to activate GIRK channels. This was particularly apparent for buprenorphine, which failed to inhibit AC or stimulate ERK1/2 in CHO cells expressing MOPr-A6V, yet activated GIRK channels to a similar extent as MOPr-WT in AtT20 cells. This data suggests that the A6V polymorphism may result in pathway selective losses of function. MOPr inhibition of AC is mediated by $G\alpha_{i/o}$ subunits of the G-protein heterotrimer, whereas GIRK activation occurs via Gβγ subunits (Law *et al.*, 2000). ERK1/2 phosphorylation can occur via multiple pathways, and may involve Gα and Gβγ-coupled processes as well as G-protein independent pathways such as β-arrestin (Luttrell *et al.*, 2005). At present it is not known which specific subtypes of Gi/o protein couple MOPr to inhibition of AC or activation of ERK in CHO cells, or activation of GIRK in AtT-20 cells. Thus, it is not clear if the selective loss of efficacy in signal transduction mediated by A6V in CHO cells is because the MOPr-A6V couples less well to Gα-mediated process than Gβγ-mediated signalling, or whether it is disruption of coupling to a specific subtype of Gi/o that is responsible for AC inhibition and ERK phosphorylation in CHO cells, but which is not involved in GIRK activation in AtT-20 cells. The apparently greater detrimental effect of the A6V variant on ERK1/2 phosphorylation compared with AC

inhibition in CHO-MOPr cells may also be due to differences in the effector molecules required for activation of these pathways. The apparently greater loss of efficacy for some ligands such as buprenorphine at MOPr-A6V signalling to AC and ERK may also reflect some functional selectivity towards GIRK activation over AC inhibition or ERK1/2 phosphorylation, although studies of AC inhibition and ERK1/2 phosphorylation in AtT-20 cells are necessary to definitively establish the presence of any functional selectivity. Each of the assays utilized here is relatively rapid, with a maximum 5 minute time-course, and a degree of opioid receptor desensitisation can occur over this time. Conceivably, a reduction in AC inhibition or pERK levels at 5 minutes could represent enhanced receptor desensitisation. However, we have not seen any evidence for enhanced desensitisation of MOPr-A6V activation of GIRK at 5 minutes (Santiago and Connor, unpublished observations), and no differences in morphine or DAMGO-induced internalisation were reported for A6V expressed on the MOR1A background (Ravindranathan *et al.,* 2009), suggesting that agonist-induced regulation of MOPr-A6V is not obviously accelerated compared with MOPr-WT.

There are no functional studies investigating the consequences of the A6V variant on acute opioid signalling published. The results from our study demonstrate a significant impact of the A6V variant on the ability of MOPr to activate associated signalling pathways and warrant further investigation with functional studies examining other MOPr signalling pathways using alternative cellular backgrounds.

The A6V variant is present at allelic frequencies of more than 20% in some populations, thus any differences in signalling arising from the A6V variant could be of clinical significance. The A6V variant markedly decreased the effect of a number of clinically prescribed opioids including morphine, one of the most commonly prescribed strong

analgesics worldwide (Hanks *et al.*, 2001; Pergolizzi *et al.*, 2008; Zernikow *et al.*, 2009). Furthermore, the signalling of the endogenous opioids β-endorphin, endomorphins 1 and 2, and met-enkephalin was significantly compromised at MOPr-A6V. Some studies have suggested a higher frequency of the A6V variant in substance abusing populations, and disruption of the normal function of the endogenous opioid system caused by the A6V variant could conceivably contribute to a higher predisposition to substance abuse (Berrettini *et al.*, 1997; Compton *et al.*, 2003; Crowley *et al.*, 2003; Crystal *et al.*, 2010; Rommelspacher *et al.*, 2001).

Our results demonstrate that the A6V variant has the potential to affect MOPr function across cell types and signalling pathways. Most carriers of the 17T allele will be heterozygous with 17C, and at present it is not known how co-expressed MOPr-WT and MOPr-A6V receptors will signal. There is still considerable uncertainty about whether MOPr and other Class A GPCR form obligate dimers in native tissue (Malik *et al.*, 2013; Herrick-Davis 2013), so whether cells expressing A6 and V6 receptors will have a mixture of homo- and heterodimers, or 2 populations of receptor which signal independently is unknown. To date, the effect of MOPr variants expressed together with MOPr-WT has been largely unexplored, largely owing to the technical difficulty of co-expressing accurately quantified and equivalent amounts of each variant. Ravindranathan *et al.* (2009) co-expressed HA-tagged MOPr-WT and FLAG-tagged MOPr variants in HEK293 cells and reported the internalisation of both MOPr-WT and MOPr-L85I in response to morphine, suggesting the formation of functional dimers with L85I showing a dominant phenotype. On the other hand, when MOPr-WT and MOPr-R181C were co-expressed, MOPr-WT internalised independently in response to DAMGO, indicating that this variant either fails to form dimers or forms unstable dimers with MOPr-WT (Ravindranathan *et al.*, 2009). The effect of co-expression on receptor expression and signalling was not

measured, and the physiological relevance is unknown. In any case, potential interactions between WT- and A6V-MOPr require further examination, and the investigation of opioid response in A6V carriers in a clinical setting would also be of great interest.

The A6V variant is highly prevalent in some populations, occurring at allelic frequencies of up to 20% (Crowley *et al.*, 2003; Kapur *et al.*, 2007; Rommelspacher *et al.*, 2001; Tan *et al.*, 2003). A decrease in the efficacy of opioid analgesics in A6V carriers could result in a significant proportion of the population receiving inadequate or inappropriate analgesic therapy. Understanding how the A6V variant affects opioid signalling is an important element in predicting individual response to opioids, and the potential clinical or phenotypic consequences of altered MOPr function. Although individual opioid response is influenced by a number of genetic and epigenetic factors, knowledge of the effect of individual MOPr genotype on opioid response could provide valuable insight into the most effective form of analgesic therapy, particularly in the case of A6V carriers, where opioid efficacy may be significantly diminished. Understanding how potential disruptions in endogenous opioid signalling affect individual phenotype could also be beneficial in elucidating the role of endogenous opioids in physiological processes such as nociception, reward and addiction.

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**Author Contributions**

AK, MS and MC designed and analysed experiments, AK and MS conducted the experiments. AK and MC conceived the study and wrote the paper, all authors have seen the final manuscript.

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# Chapter 7

## Mu-opioid receptor polymorphisms differentially affect receptor signalling via adenylyl cyclase inhibition and ERK phosphorylation

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## INTRODUCTION

Single nucleotide polymorphisms (SNPs) in genes coding for G protein-coupled receptors (GPCRs) can result in aberrant signalling profiles of these receptors (Thompson *et al.*, 2008; Vassart & Costagliola, 2011). An amino acid substitution arising from a non-synonymous SNP may alter the structure and/or conformation of a GPCR in such a way as to affect the ability of a ligand to bind to the receptor and elicit a response. The μ-opioid receptor (MOPr) is a GPCR that is the primary target for most clinically prescribed opioid analgesics including morphine, oxycodone and fentanyl (Matthes *et al.*, 1996; Pawson *et al.*, 2014). Genotyping of the human MOPr gene (*OPRM1*) has revealed the presence of a number of non-synonymous SNPs within the population (Lotsch & Geisslinger, 2005). Individual response to opioids is highly variable, and as such naturally occurring MOPr variants are possible contributors to interindividual differences in opioid response

Differences in individual opioid response have been associated with the two most common *OPRM1* SNPs, the N40D variant and the A6V variant, both of which occur in the N-terminal region of MOPr (Lotsch & Geisslinger, 2005; Somogyi *et al.*, 2007). Other rare *OPRM1* SNPs, present in less than 1% of the population, have received far less attention. These include the L85I variant, present in the first transmembrane domain (TM1) of MOPr, the R181C variant on the second intracellular loop (ICL2), and the R260H, R265H and S268P variants, which affect the third intracellular loop (ICL3) of the receptor ((Lotsch & Geisslinger, 2005; Ravindranathan *et al.*, 2009). The clinical effects of these variants in humans are not known due to their rarity within the population, yet the potential insights that MOPr SNPs can provide into receptor function has prompted several studies investigating the consequences of rare *OPRM1* SNPs in vitro.

The potential for MOPr SNPs to contribute to the clinical variability of opioid responses is very real, and has been demonstrated for several GPCRs (Thompson *et al.*, 2008; Zhang *et*

*al.*, 2013a). Like all GPCRs, the MOPr has many active conformations, which may differentially activate various downstream effector and regulatory molecules (Pinyero *et al.*, 2007; Kenakin & Miller, 2010; Manglik *et al.*, 2012). Single amino acid substitutions can lead to subtle or profound changes in receptor conformation, potentially affecting the signalling pathways activated (Abrol *et al.*, 2013; Cox, 2013). Ligands may also exhibit specific patterns of signalling and receptor regulation by preferentially binding to and stabilising subsets of MOPr conformational states (Kenakin, 2002; Massotte *et al.*, 2002; Saidak *et al.*, 2006). SNPs resulting in single amino acid changes therefore have the potential to affect MOPr signalling globally or in a ligand-dependent manner by affecting the ability of a ligand to bind to the receptor, altering the conformation of the ligand-receptor complex and/or affecting the ability of this complex to couple to G-proteins and associated signalling or regulatory pathways of MOPr.

In this study, we investigated the effect of the L85I, R181C, R260H, R265H and S268P variants on the ability of human MOPr to inhibit adenylyl cyclase and stimulate pERK phosphorylation in CHO cells, using 11 clinically important and/or structurally distinct opioid ligands. We found significant enhancement of adenylyl cyclase inhibition by selected opioids at MOPr-L85I, but this enhancement was not evident in assays of ERK1/2 phosphorylation. MOPr-R260H had a markedly negative impact on opioid signalling in assays of AC inhibition and ERK1/2 phosphorylation, whereas MOPr-R265H signalling was similar to MOPr-WT for most opioids tested.

## METHODS

### *MOR transfection and cell culture*

CHO-FRT-TRex cells were stably transfected with a pcDNA5 construct encoding the haemagglutinin (HA)-tagged human μ-opioid receptor cDNA together with the pOG44 (Flp recombinase plasmid) using the transfectant Fugene (Promega), as described previously (Knapman *et al.,* 2014). The HA-tagged human wild-type μ-opioid receptor (MOPr-WT) and the six variant HA-tagged μ-opioid receptors were synthesised by Genscript (Piscataway, New Jersey, USA). The MOPr variants examined were the A6V variant (MOPr-A6V), the L85I variant (MOPr-L85I), the R181C variant (MOPr-R181C), the R260H variant (MOPr-R260H), the R265H variant (MOPr-265H) and the S268P variant (MOPr-S268P). Cells expressing MOPr were selected using hygromycin B (500 μg/mL). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 500 μg/mL hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 μg/mL beyond passage 5.

CHO-MOPr cells were passaged at 80% confluency as required. Assays were carried out on cells up to 30 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at greater than 90% confluence.

### *MOPr receptor expression*

Surface expression of all MOPr variants was determined on intact CHO-MOPr cells by incubation with [$^3$H]DAMGO (0.125–16 nM; PerkinElmer, Waltham, MA) at 4 °C in 50 mM Tris-Cl (pH 7.4) for 2 h. Briefly, approximately $1 \times 10^5$ cells were plated in a 24-well plate and grown overnight. Cells were then washed gently twice with 50 mM Tris-Cl (pH 7.4) and placed on ice, and incubated for 2h with increasing concentrations of

[$^3$H]DAMGO. Nonspecific binding was determined in the presence of unlabelled DAMGO (10 μM). At the end of the incubation, plated cells were washed three times with 50 mM Tris-Cl (pH 7.4) at 4 °C. Cells in each well were then digested for 1 h at room temperature with 100 μL of 1N NaOH. 100 μL 1N HCl was added to each well and collected into scintillation vials and bound ligand determined using a liquid scintillation counter (Packard Tricarb, Perkin Elmer, Waltham MA, USA). Specific binding was plotted, and $K_d$ and $B_{max}$ for each clone were determined using GraphPad Prism (GraphPad Software, La Jolla, CA). Protein concentration was determined with a BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

***Membrane potential assays***

The day before the assay, cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. MOPr receptor expression was induced in CHO-MOPr cells with 2 μg/mL tetracycline 24 hrs prior to the assay. The cells were plated in a volume of 90 μl in black walled, clear-bottomed 96-well microplates (Corning), and incubated overnight at 37$^o$C in ambient $CO_2$. Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer containing (in mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 5). Prior to the assay, cells were loaded with 90 μl/well of the dye solution without removal of the L-15, giving an initial assay volume of 180 μl/well. Plates were then incubated at 37°C in ambient $CO_2$ for 60 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time either drug or vehicle was

added in a volume of 20 µL. Further additions were made in volumes of 20 µl, as indicated. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. When used, the final concentration of the solvents DMSO or EtOH was not more than 0.1%, and these concentrations did not produce a signal in the assay.

***ELISA of ERK1/2 phosphorylation***

Opioid-mediated ERK1/2 phosphorylation was measured by ELISA, as previously described (Knapman et al., *In Press*). Briefly, 24 hrs before the assay, CHO-MOPr cells were plated in 96-well clear microplates (Falcon) and receptor expression was induced as for the AC inhibition assay. Cells were serum-starved for 1 hr in 40 µL serum-free L-15 supplemented with 5% bovine serum albumin (BSA) before the assay. Cells were treated with drug or vehicle diluted in serum-free L-15 (40 µl added) with no BSA added to minimise background. Preliminary time-course experiments indicated that a 5 min drug treatment was optimal to produce robust ERK1/2 phosphorylation without inducing desensitisation, thus all drug treatments were for 5 min. After drug application, the reaction was stopped by inverting the plates to remove the drug solution, placing the plates on ice, and immediately fixing the cells with 4% paraformaldehyde for 15 min at RT. Cells were washed 3 times with 300µL PBS, then permeabilized with 0.1% Triton-X in PBS for 30 min at RT. Triton-X was removed, and cells were incubated for 2 hr at RT with blocking solution consisting of 5% BSA in PBS with 0.01% Tween-20 (PBS-T). Blocking solution was removed, then cells were incubated overnight at 4°C with a 1:500 dilution of rabbit α-phospho-p44/42 MAPK (Thr202/Tyr204) antibody in PBS-T with 1% BSA. Cells were washed 3 times with 300µL PBS-T, and incubated with 1:5000 α-rabbit IgG HRP-linked antibody in PBS-T with 1% BSA for 2 hr. Cells were washed 4 times with 300 µL PBS-T,

and incubated with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) at RT in the dark for 45 min. The reaction was stopped with 1M HCl. Absorbance was read at 450 nm using a BMG Pherastar FS microplate reader. Cells were then stained with 0.5μg/mL DAPI for 10 min at RT, and washed 3 times with 300 μL PBS-T. Fluorescence was read in the Pherastar microplate reader with cells excited at a wavelength of 358 nm and emission measured at 461 nm. Absorbance readings were normalised to DAPI staining to account for any differences in cell density between wells. Readings were then normalised to the response of cells treated with 100nM PMA for 10 min.

***Drugs and Chemicals***

Tissue culture reagents and buffer salts were from Life Technologies or Sigma unless otherwise noted. Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol acetate (DAMGO), endomorphin-1, endomorphin-2 and met-enkephalin were purchased from Auspep (Tullamarine, Australia). Morphine, fentanyl and pentazocine were a kind gift from the Department of Pharmacology, University of Sydney. Buprenorphine and oxycodone were from the National Measurement Institute (Lindfield, Australia). β-endorphin was from Genscript (Piscataway, New Jersey, USA). Forskolin and naloxone were from Ascent Pharmaceuticals (Bristol, UK). 1,9-dideoxyforskolin and tetraethylammonium (TEA) were from Sigma Aldrich (Castle Hill, Australia). Pertussis toxin (PTX) was from Tocris Bioscience (Bristol, UK). Phospho-ERK1/2 antibody (Catalog #9101) and anti-rabbit IgG HRP-lined antibody (Catalog #7074) were from Cell Signalling Technologies, Danvers, Massachusetts, USA.

***Data***

Unless otherwise noted, data is expressed as mean ± s.e.m. of at least 5 determinations made in duplicate or triplicate. Concentration response curves were fit with a 4 parameter logistic equation using Graphpad Prism (Graphpad). Statistical comparisons were made

with one-way ANOVA followed by Dunnett's post-hoc test corrected for multiple comparisons, or an unpaired Student's T-test not corrected for multiple comparisons. $P < 0.05$ was considered significant. All channel and receptor nomenclature is consistent with the British Journal of Pharmacology Guide to Receptors and Channels (Alexander et al., 2013).

## RESULTS

### *MOPr Expression in CHO-K1 cells*

CHO-K1 cells were stably transfected with MOPr-WT or with MOPr-L85I, MOPr-R181C, MOPr-R260H, MOPr-R265H or MOPr-S268P, with receptor expression controlled by a tetracycline-sensitive repressor. After induction of MOPr expression with tetracycline, specific binding of [$^3$H]DAMGO in cells expressing MOPr-WT was similar to cells expressing MOPr-L85I, MOPr-R181C, MOPr-R260H and MOPr-R265H ($P > 0.05$), however in cells transfected with MOPr-S268P, receptor expression was significantly reduced. $B_{max}$ for CHO-MOPr-WT cells was 280 ± 20 fmol/mg total protein and $B_{max}$ for CHO-MOPr-S268P was 175 ± 18 fmol/mg ($P < 0.05$, Table 1). $K_D$ was similar between cells expressing MOPr-WT and all other variants with the exception of MOPr-R181C, which had a $K_D$ of 2.43 ± 0.53 nM compared with MOPr-WT $K_D$ of 0.75 ± 0.05 ($P < 0.05$, Table 1). As CHO-MOPr-R181C and CHO-MOPr-S268P displayed aberrant DAMGO binding profiles, we have not at this stage conducted further assays using these cell lines.

### *Adenylyl Cyclase Inhibtion*

We have previously reported opioid-mediated inhibition of FSK-stimulated membrane hyperpolarisation in CHO-MOPr cells, and this effect was naloxone and pertussis-toxin sensitive (Knapman *et al.*, 2014). In CHO-MOPr cells loaded with membrane potential

dye, addition of the adenylyl cyclase activator forskolin (FSK, 300 nM) produced a hyperpolarisation in CHO-MOPr-WT of 41 ± 1 % decrease in fluorescence from baseline. FSK stimulated a similar membrane hyperpolarisation in all cell lines ($P > 0.05$, Table 2). The maximal inhibition of the FSK response by DAMGO in CHO-MOPr-WT cells was 64 ± 2 %, with $pEC_{50}$ of 7.9 ± 0.1 (Table 3). DAMGO inhibited FSK similarly in cells expressing MOPr-L85I, MOPr-R260H and MOPr-R265H (Table 3, Figure 1).

We have reported previously that the effects of DAMGO in this assay were sensitive to naloxone and strongly inhibited by overnight pretreatment with pertussis toxin (Knapman et al., 2014). Application of opioids alone did not affect the membrane potential of CHO-MOPr cells, with the exception of 30 µM pentazocine and 10 µM methadone, which caused small, transient increases in fluorescence (< 10%). This effect was not naloxone sensitive.

***Opioid-mediated ERK1/2 Phosphorylation***

We have previously shown that in CHO cells expressing MOPr, application of opioids stimulates ERK1/2 phosphorylation, and this response is blocked by naloxone and pertussis-toxin (Knapman *et al*, *In Press*). Opioid ERK1/2 phosphorylation was normalised against application of 100 nM PMA for 10 min. The average PMA response in cells expressing MOPr-WT was 0.60 ± 0.07 (corrected absorbance reading), and did not differ significantly between cell lines (Table 2). Maximal DAMGO-stimulated ERK1/2 phosphorylation was 64 ± 3% of the PMA response in CHO-MOPr-WT cells, with a $pEC_{50}$ of 7.9 ± 0.1 (Figure 1, Table 4).

**Table 1: Surface receptor expression and DAMGO $K_d$ for MOPr variants in CHO cells**

| CHO-MOPr Variant | $B_{max}$ (fmol/mg) | $K_d$ (nM) |
|---|---|---|
| **Wild Type** | 280 ± 20 | 0.75 ± 0.05 |
| **L85I** | 268 ± 69 | 0.56 ± 0.09 |
| **R181C** | 245 ± 31 | 2.43 ± 0.53* |
| **R260H** | 249 ± 45 | 1.09 ± 0.46 |
| **R265H** | 322 ± 49 | 0.76 ± 0.37 |
| **S268P** | 175 ± 18* | 0.54 ± 0.18 |

Radioligand binding was carried out as described in the Methods. No significant difference in $B_{max}$ or $K_d$ was observed between cells expressing MOPr-WT, MOPr-L85I, R260H or R265H ($P > 0.05$). Cells expressing MOPr-R181C had a significantly different Kd compared to MOPr-WT, and cells expressing S268P expressed significantly less receptor ($P < 0.05$). Further assays were not conducted on these cell lines. The assay was repeated 3 times in triplicate.

**Table 2: Forskolin and PMA responses in MOPr variants**

| CHO-MOPr Variant | **300nM FSK response** (Δ fluorescence from baseline (%)) | **100nM PMA response** (Corrected Abs) |
|---|---|---|
| **Wild Type** | 42 ± 1 | 0.60 ± 0.07 |
| **L85I** | 39 ± 1 | 0.54 ± 0.06 |
| **R260H** | 37 ± 3 | 0.59 ± 0.06 |
| **R265H** | 39 ± 2 | 0.56 ± 0.05 |

MOPr variants did not differ in the response to 300 nM forskolin which was used to stimulate adenylyl cyclase activity ($P > 0.05$). ERK1/2 phosphorylation stimulated by 100 nM PMA was similar in MOPr-WT and MOPr variants ($P > 0.05$). Data is from 6

independent experiments performed in duplicate.

Because of the possibility of ligand-specific and/or pathway specific effects of MOPr polymorphisms, we compared the potency and efficacy of a range of clinically important and/or structurally distinct opioid ligands for both AC inhibition and ERK1/2 phosphorylation in CHO cells expressing MOPr-WT and the MOPr-L85I, MOPr-R260H and MOPr-R265H variants.

***MOPr-L85I***

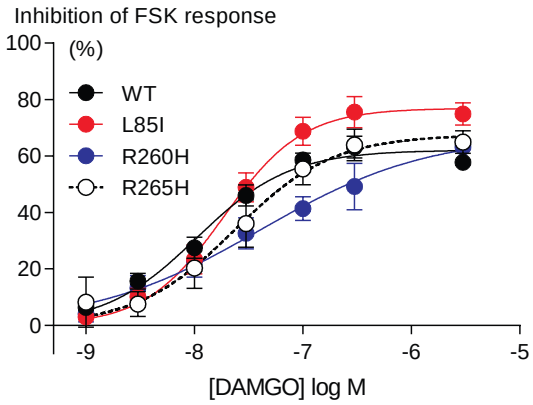
In assays of AC inhibition, DAMGO inhibition of the FSK-stimulated response in CHO-MOPr-L85I cells was significantly higher than in CHO-MOPr-WT, with $E_{max}$ of 77 ± 5% ($P < 0.05$, Figure 1, Table 3). DAMGO potency was not affected by the MOPr-L85I variant, with $pEC_{50}$ of 7.7 ± 0.1 ($P > 0.05$, Table 3). FSK inhibition by the commonly prescribed opioid analgesics morphine, buprenorphine and pentazocine was not significantly different from MOPr-WT at MOPr-L85I (Figure 1, Table 3). At concentrations higher than 10 μM, pentazocine has been reported to block $K^+$ channels (Zhang & Cuevas, 2005), so concentrations above 10 μM were not tested despite pentazocine response not reaching a clear plateau at this concentration. For this reason, it was not possible to calculate $pEC_{50}$ for pentazocine AC inhibition for any variant.

The endogenous opioid β-endorphin inhibited FSK-stimulated hyperpolarisation similarly in cells expressing MOPr-WT or MOPr-L85I. In contrast, signalling of the endogenous opioids met-enkephalin, endomorphin-1 and endomorphin-2 was significantly enhanced in cells expressing MOP-L85I compared with cells expressing MOPr-WT in assays of AC inhibition (Figure 2, Table 3). For met-enkephalin, $E_{max}$ was 63 ± 3% in CHO-MOPr-WT cells, and 99 ± 6% in CHO-MOPr-L85I cells ($P < 0.05$). Met-enkephalin was also more potent at MOPr-L85I, with $pEC_{50}$ of 8.0 ± 0.1, compared with 7.4 ± 0.1 at MOPr-WT ($P <$

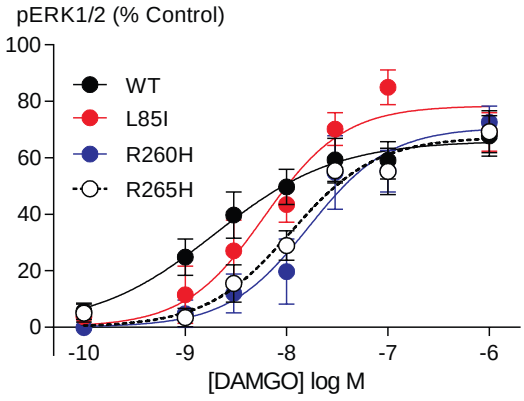
0.05). Endomorphin-1 and endomorphin-2 signalling via AC inhibition was similarly enhanced in CHO-MOPr-L85I cells (Figure 2, Table 3).

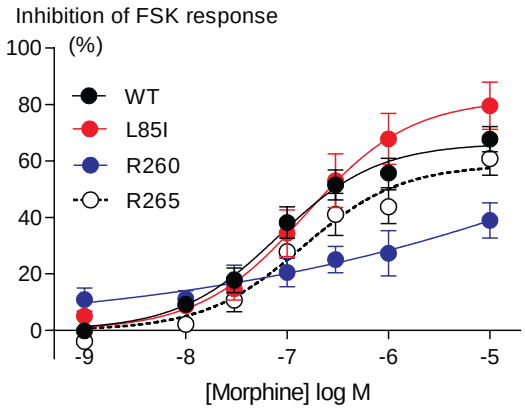
**Figure 1: DAMGO, morphine, buprenorphine and pentazocine inhibit adenylyl cyclase and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr variants.** Adenylyl cyclase inhibition and levels of ERK1/2 phosphorylation were determined as described in the Methods. A) DAMGO, morphine, buprenorphine and pentazocine inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT and MOPr-variants to varying degrees and with different potencies (Table 3). B) DAMGO, morphine, buprenorphine and pentazocine stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-variants to varying degrees and with different potencies (Table 4). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

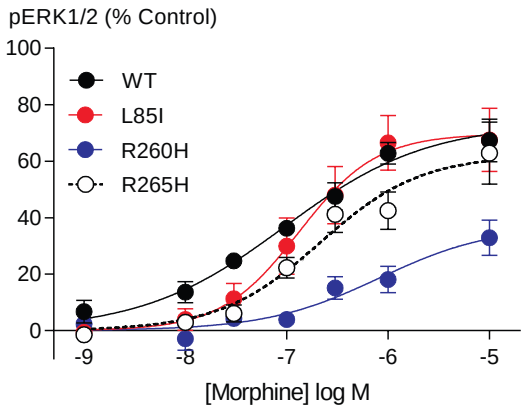
A

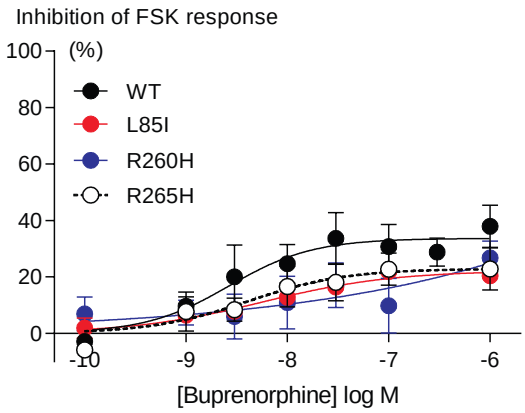

Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[DAMGO] log M

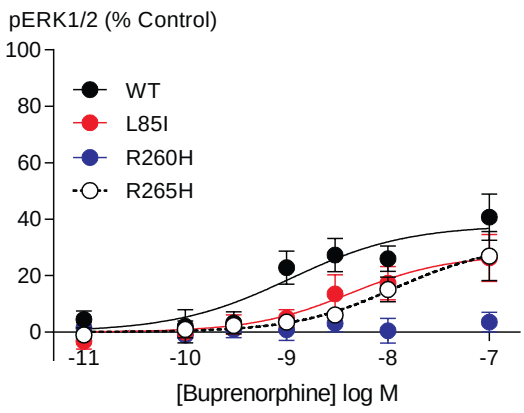
B

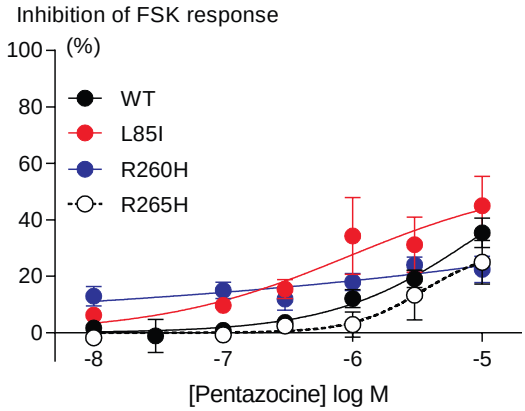

pERK1/2 (% Control)
WT
L85I
R260H
R265H
[DAMGO] log M


Inhibition of FSK response
(%)
WT
L85I
R260
R265
[Morphine] log M


pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Morphine] log M


Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[Buprenorphine] log M


pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Buprenorphine] log M


Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[Pentazocine] log M

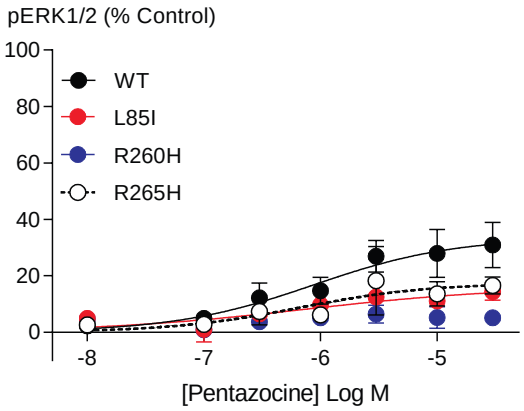
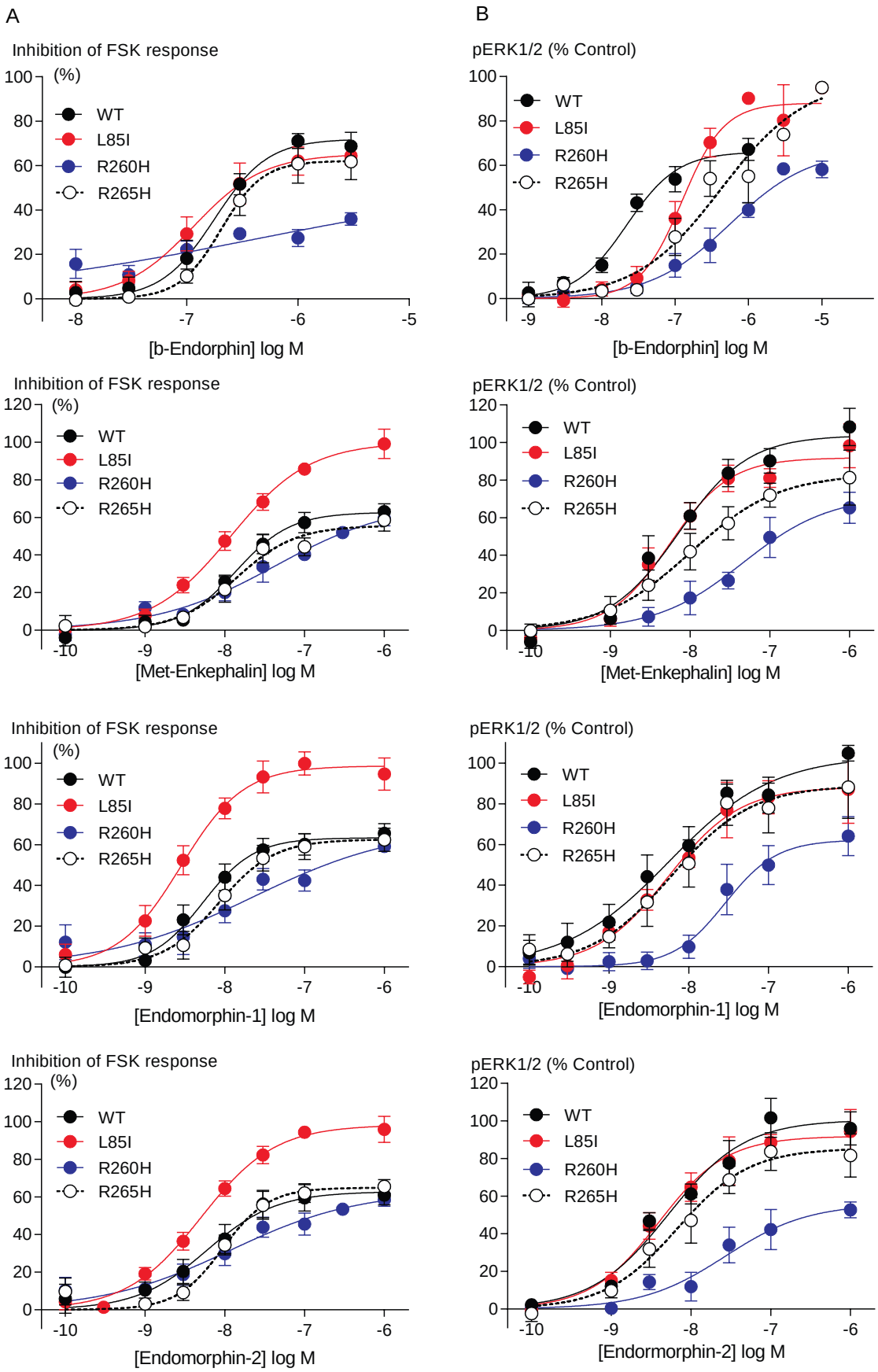

pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Pentazocine] Log M

**Figure 2: Endogenous opioids inhibit adenylyl cyclase and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr variants.** Adenylyl cyclase inhibition and levels of ERK1/2 phosphorylation were determined as described in the Methods. A) β-endorphin, met-enkephalin, endomorphin-1 and endomorphin-2 inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT and MOPr-variants to varying degrees and with different potencies (Table 3). B) β-endorphin, met-enkephalin, endomorphin-1 and endomorphin-2 stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-variants to varying degrees and with different potencies (Table 4). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.


A
Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[b-Endorphin] log M
B
pERK1/2 (% Control)
WT
L85I
R260H
R265H
[b-Endorphin] log M
Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[Met-Enkephalin] log M
pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Met-Enkephalin] log M
Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[Endomorphin-1] log M
pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Endomorphin-1] log M
Inhibition of FSK response
(%)
WT
L85I
R260H
R265H
[Endomorphin-2] log M
pERK1/2 (% Control)
WT
L85I
R260H
R265H
[Endormorphin-2] log M

We then challenged cells with the clinically important opioids methadone, fentanyl and oxycodone. The inhibition of FSK-stimulated membrane hyperpolarisation was significantly increased in cells expressing MOPr-L85I compared with MOPr-WT. Maximal inhibition of the FSK response by methadone was 65 ± 3% in CHO-MOPr-WT cells, with $pEC_{50}$ of 7.0 ± 0.1. In CHO-MOPr-L85I cells, maximal methadone inhibition of the FSK-response was 112 ± 7% ($P < 0.05$), resulting in a slight membrane depolarisation from baseline (Figure 3). Methadone potency was unaffected by MOPr-L85I, with $pEC_{50}$ of 7.1 ± 0.1 ($P > 0.05$, Figure 3, Table 3). The maximally effective concentration of fentanyl also caused a slight membrane depolarisation from baseline in CHO-MOPr-L85I cells (Figure 4). MOPr-L85I $E_{max}$ for fentanyl inhibition of FSK-stimulated membrane hyperpolarisation was 110 ± 9%, compared with MOPr-WT $E_{max}$ of 70 ± 6% ($P < 0.05$, Figure 3, Table 3). Fentanyl potency was similar between MOPr-WT and MOPr-L85I. Oxycodone signalling was not affected by L85I (Table 3).

In assays of ERK1/2 activation, maximal DAMGO stimulation of ERK1/2 phosphorylation was not significantly different between cells expressing MOPr-WT and MOPr-L85I, with $E_{max}$ of 74 ± 6% of the maximum PMA response in CHO-MOPr-L85I cells, and $pEC_{50}$ of 8.6 ± 1 (Figure 1, Table 4). The ERK1/2 phosphorylation elicited by morphine and buprenorphine was not affected by the MOPr-L85I variant, however maximum ERK1/2 phosphorylation stimulated by pentazocine was significantly decreased in cells expressing MOPr-L85I compared with MOPr-WT. $E_{max}$ for pentazocine in CHO-MOPr-WT cells was 44 ± 11%, and 16 ± 3% in CHO-MOPr-L85I cells ($P < 0.05$, Figure 1, Table 4). Pentazocine $pEC_{50}$ was unaffected by MOPr-L85I (Table 4).

Interestingly, the effect of the L85I variant on ERK1/2 signalling via endogenous opioid ligands was a mirror image of the effects observed for AC inhibition. β-endorphin-stimulated ERK1/2 phosphorylation was significantly increased at MOPr-L85I, with $E_{max}$ of 99 ± 4% compared with $E_{max}$ of 72 ± 5% in CHO-MOPr-WT cells ($P < 0.05$, Figure 3, Table 4), although β-endorphin potency was decreased at MOPr-L85I, with $pEC_{50}$ of 6.9 ± 0.1, and 7.7 ± 0.1 at MOPr-WT ($P < 0.05$, Table 4). ERK1/2 phosphorylation stimulated by endomorphin-1, endomorphin-2, and met-enkephalin was not affected by MOPr-L85I (Figure 2, Table 4). Methadone, fentanyl and oxycodone ERK1/2 signalling was similar in CHO-MOPr-WT and CHO-MOPr-L85I cells (Figure 3, Table 4).

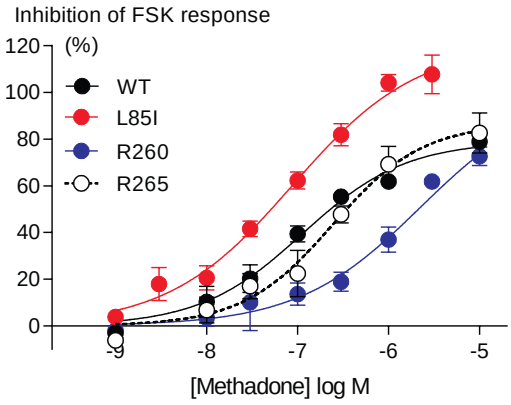
***MOPr-R260H***

In cells expressing MOPr-R260H, DAMGO inhibition of FSK-stimulated membrane hyperpolarisation was not different to that in cells expressing MOPr-WT. DAMGO $E_{max}$ was 64 ± 3%, and pEC50 was 7.6 ± 0.3 (Figure 1, Table 3). Morphine and buprenorphine AC inhibition was not affected by MOPr-R260H (Table 3). In CHO-MOPr-R260H cells, pentazocine signalling was compromised (Figure 1). It was not possible to fit a standard concentration-response curve to the data, as low pentazocine concentrations inhibited AC to a similar degree as maximum pentazocine concentrations.

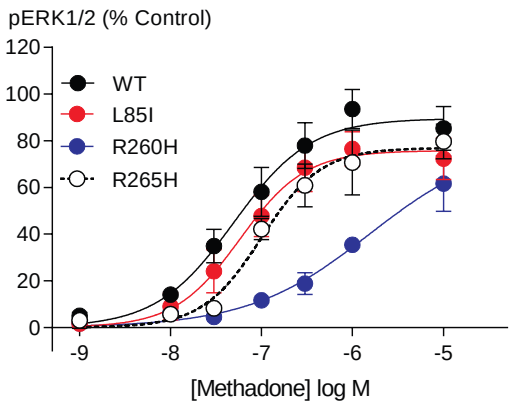
β-endorphin inhibition of the FSK response was significantly decreased in CHO-MOPr-R260H cells compared with CHO-MOPr-WT cells. Maximal β-endorphin-stimulated AC inhibition was 70 ± 4% in CHO-MOPr-WT cells, and 34 ± 3% in CHO-MOPr-R260H cells ($P < 0.05$, Figure 3, Table 3). β-endorphin potency was not different between cells expressing MOPr-WT or MOPr-R260H (Table 3). FSK inhibition by endomorphin-1, endomorphin-2 and met-enkephalin was similar between CHO-MOPr-WT cells and CHO-MOPr-R260H cells (Figure 3, Table 3).

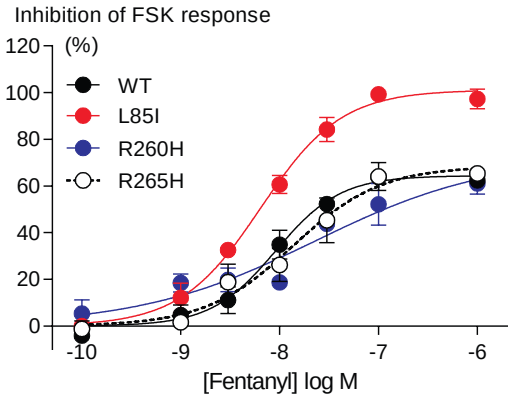
**Figure 3: Methadone, fentanyl and oxycodone inhibit adenylyl cyclase and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr variants.** Adenylyl cyclase inhibition and levels of ERK1/2 phosphorylation were determined as described in the Methods. A) Methadone, fentanyl and oxycodone inhibited forskolin-stimulated adenylyl cyclase hyperpolarisation of MOPr-WT and MOPr-variants to varying degrees and with different potencies (Table 3). B) Methadone, fentanyl and oxycodone stimulated ERK1/2 phosphorylation in cells expressing MOPr-WT or MOPr-variants to varying degrees and with different potencies (Table 4). Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± s.e.m. of pooled data from 5-6 independent determinations performed in duplicate.

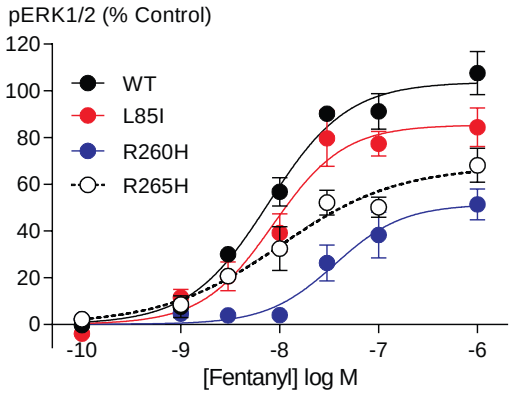
A

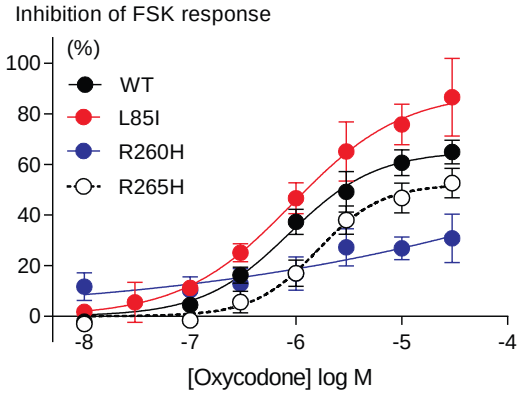

Inhibition of FSK response
(%)
120
100
80
60
40
20
0
WT
L85I
R260
R265
-9
-8
-7
-6
-5
[Methadone] log M

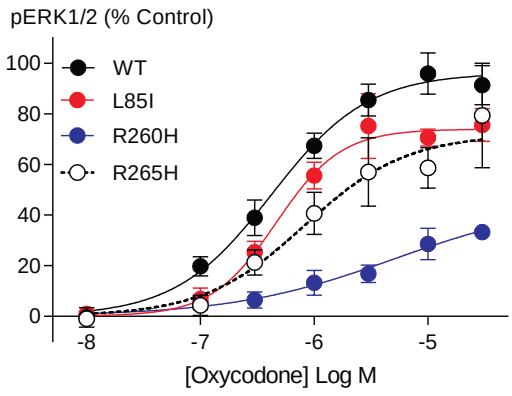
B


pERK1/2 (% Control)
120
100
80
60
40
20
0
WT
L85I
R260H
R265H
-9
-8
-7
-6
-5
[Methadone] log M


Inhibition of FSK response
(%)
120
100
80
60
40
20
0
WT
L85I
R260H
R265H
-10
-9
-8
-7
-6
[Fentanyl] log M


pERK1/2 (% Control)
120
100
80
60
40
20
0
WT
L85I
R260H
R265H
-10
-9
-8
-7
-6
[Fentanyl] log M


Inhibition of FSK response
(%)
100
80
60
40
20
0
WT
L85I
R260H
R265H
-8
-7
-6
-5
-4
[Oxycodone] log M


pERK1/2 (% Control)
100
80
60
40
20
0
WT
L85I
R260H
R265H
-8
-7
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-5
[Oxycodone] Log M

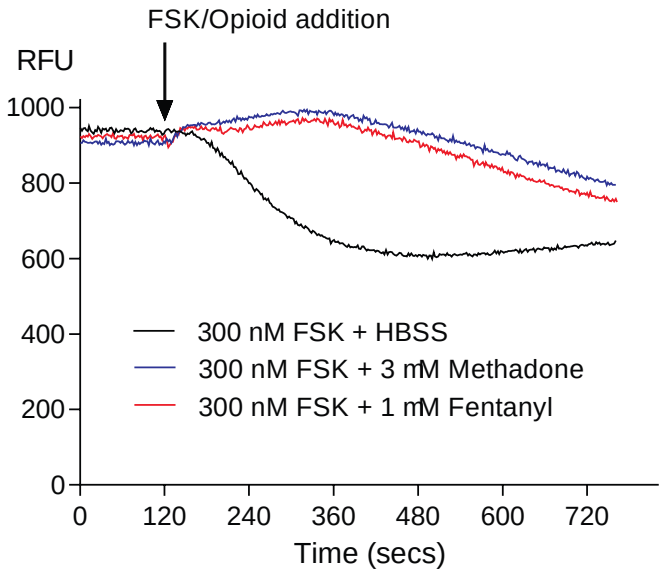


**Figure 4: High concentrations of methadone and fentanyl caused membrane depolarisation in CHO-MOPr-L85I cells.** The membrane potential assay was performed as described in the Methods. Raw trace showing application of 3 μM methadone or 1 μM fentanyl with 300 nM forskolin caused a small (< 10%) increase in fluorescent signal from baseline in CHO-MOPr-L85I cells. Data shown is a representative trace from a single experiment.

In cells expressing MOPr-R260H, maximum methadone inhibition of the FSK response was similar to cells expressing MOPr-WT, however potency was affected, with $pEC_{50}$ of 7.0 ± 0.1 in CHO-MOPr-WT cells, and 5.9 ± 0.2 in CHO-MOPr-R260H cells ($P < 0.05$, Figure 3, Table 3). Maximum oxycodone inhibition of FSK was significantly decreased at MOPr-R260H, with $E_{max}$ of 37 ± 9% compared with $E_{max}$ of 65 ± 4% at MOPr-WT ($P < 0.05$). Oxycodone potency was not affected by MOPr-R260H. Fentanyl signalling was similar between variants. (Figure 3, Table 3).

For assays of ERK1/2 phosphorylation, DAMGO stimulated ERK1/2 similarly in cells expressing MOPr-R260H and MOPr-WT (Figure 1, Table 4). Morphine-stimulated ERK1/2 phosphorylation was significantly compromised at the R260H variant, with $E_{max}$ of 43 ± 7% and $pEC_{50}$ of 6.1 ± 0.3 ($P < 0.05$, Figure 1, Table 4). Buprenorphine and pentazocine failed to elicit significant ERK1/2 phosphorylation in CHO-MOPr-R260H cells (Figure 1, Table 4). ERK1/2 signalling by the endogenous opioids was also compromised. Maximum β-endorphin-stimulation of ERK1/2 was unchanged, but $pEC_{50}$ was significantly affected in cells expressing MOPr-R260H (6.6 ± 0.2, $P < 0.05$, Table 4). For endomorphin-1 and met-enkephalin, $E_{max}$ and $pEC_{50}$ were both affected in CHO-MOPr-R260H cells, whereas for endomorphin-2, only $E_{max}$ was affected (Figure 2, Table 4). The R260H variant also had an impact on the signalling of methadone, fentanyl and oxycodone. Maximum methadone ERK1/2 phosphorylation was similar between variants, but potency was significantly affected, with $pEC_{50}$ of 7.3 ± 0.3 in CHO-MOPr-WT, and 5.5 ± 0.4 in CHO-MOPr-R260H ($P < 0.05$, Figure 3, Table 4). Fentanyl $E_{max}$ and $pEC_{50}$ were both affected by R260H, and maximum oxycodone-stimulated ERK1/2 was significantly decreased, but $pEC_{50}$ was unchanged in CHO-MOPr-R260H (Figure 3, Table 4).

**Table 3: Summary of opioid efficacy and potency in assays of AC inhibition in CHO cells expressing MOPr-WT and MOPr-variants.**

| AC inhibition | $E_{max}$ (%) | | | | *p*$EC_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|
| **Opioid** | **WT** | **L85I** | **R260H** | **R265H** | **WT** | **L85I** | **R260H** | **R265H** |
| Fentanyl | 70 ± 6 | 110 ± 9* | 65 ± 2 | 67 ± 3 | 8.0 ± 0.2 | 8.1 ± 0.1 | 6.9 ± 0.6 | 7.4 ± 0.2 |
| β-Endorphin | 70 ± 4 | 66 ± 4 | 34 ± 3* | 65 ± 9 | 6.7 ± 0.1 | 6.9 ± 0.2 | 6.3 ± 1.1 | 6.7 ± 0.1* |
| Morphine | 66 ± 4 | 81 ± 8 | 51 ± 9 | 59 ± 5 | 7.0 ± 0.1 | 6.8 ± 0.1 | 6.7 ± 1.0 | 6.8 ± 0.3 |
| Methadone | 65 ± 3 | 112 ± 7* | 58 ± 3 | 76 ± 7 | 7.0 ± 0.1 | 7.1 ± 0.1 | 5.9 ± 0.2* | 6.9 ± 0.3 |
| Oxycodone | 65 ± 4 | 88 ± 8 | 37 ± 9* | 52 ± 6 | 5.8 ± 0.3 | 6.0 ± 0.2 | 6.0 ± 0.4 | 5.8 ± 0.9 |
| Endomorphin-2 | 65 ± 7 | 97 ± 4* | 66 ± 5 | 66 ± 4 | 8.2 ± 0.1 | 8.3 ± 0.1 | 7.8 ± 0.3 | 8.0 ± 0.1 |
| Endomorphin-1 | 64 ± 5 | 98 ± 6* | 64 ± 4 | 62 ± 6 | 8.2 ± 0.1 | 8.6 ± 0.1* | 7.9 ± 0.3 | 8.1 ± 0.1 |
| DAMGO | 63 ± 3 | 76 ± 5* | 64 ± 3 | 66 ± 4 | 7.9 ± 0.1 | 7.7 ± 0.1 | 7.6 ± 0.2 | 7.6 ± 0.2 |
| Met-Enkephalin | 63 ± 3 | 99 ± 6* | 60 ± 4 | 56 ± 5 | 7.4 ± 0.1 | 8.0 ± 0.1* | 7.5 ± 0.1 | 7.8 ± 0.1* |
| Pentazocine | 35 ± 5 | 38 ± 11 | 20 ± 3* | 18 ± 4* | N/A | N/A | N/A | N/A |
| Buprenorphine | 34 ± 4 | 26 ± 3 | 24 ± 6 | 22 ± 7 | 8.4 ± 0.3 | 7.9 ± 0.3 | 8.4 ± 0.5 | 8.4 ± 0.2 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than fentanyl are highlighted in red (1-way ANOVA, followed by Dunnett's post-hoc test corrected for multiple comparisons, $P < 0.05$). MOPr-variant $E_{max}$ and *p*$EC_{50}$ values significantly different from MOPr-WT are marked with * (unpaired Student's T-test, $P < 0.05$).

**Table 4: Summary of opioid efficacy and potency in assays of ERK1/2 phosphorylation in CHO cells expressing MOPr-WT and MOPr-variants.**

| pERK1/2 | $E_{max}$ (%) | | | | *p*$EC_{50}$ | | | |
|---|---|---|---|---|---|---|---|---|
| **Opioid** | **WT** | **L85I** | **R260H** | **R265H** | **WT** | **L85I** | **R260H** | **R265H** |
| Endomorphin-2 | 104 ± 11 | 97 ± 9 | 55 ± 10* | 88 ± 8 | 8.2 ± 0.2 | 8.3 ± 0.2 | 7.8 ± 0.3 | 8.1 ± 0.2 |
| Oxycodone | 101 ± 10 | 77 ± 6 | 43 ± 4* | 65 ± 5* | 6.4 ± 0.1 | 6.2 ± 0.1 | 5.5 ± 0.4 | 6.2 ± 0.1 |
| Met-Enkephalin | 100 ± 9 | 96 ± 8 | 74 ± 6* | 88 ± 11 | 8.2 ± 0.1 | 8.2 ± 0.1 | 7.2 ± 0.3* | 8.2 ± 0.1 |
| Fentanyl | 99 ± 11 | 87 ± 7 | 51 ± 6* | 77 ± 12 | 8.2 ± 0.1 | 8.0 ± 0.1 | 7.2 ± 0.3* | 7.9 ± 0.3 |
| Endomorphin-1 | 93 ± 5 | 90 ± 9 | 51 ± 11* | 94 ± 11 | 8.5 ± 0.3 | 8.2 ± 0.2 | 7.5 ± 0.2* | 8.1 ± 0.2 |
| Methadone | 88 ± 9 | 77 ± 9 | 63 ± 10 | 83 ± 9 | 7.3 ± 0.1 | 7.2 ± 0.2 | 5.5 ± 0.4* | 6.8 ± 0.1* |
| β-Endorphin | 72 ± 5 | 99 ± 4* | 63 ± 5* | 80 ± 11 | 7.7 ± 0.1 | 6.9 ± 0.1* | 6.6 ± 0.2* | 6.8 ± 0.2* |
| Morphine | 67 ± 8 | 69 ± 11 | 36 ± 6* | 65 ± 11 | 7.2 ± 0.3 | 6.7 ± 0.1 | 6.1 ± 0.3* | 6.6 ± 0.1 |
| DAMGO | 64 ± 6 | 74 ± 6 | 73 ± 4 | 69 ± 10 | 8.6 ± 0.1 | 8.3 ± 0.2 | 7.6 ± 0.3 | 7.9 ± 0.1* |
| Pentazocine | 45 ± 11 | 16 ± 3* | No Response | 20 ± 3 | 5.7 ± 0.4 | 6.5 ± 0.3 | No Response | 6.0 ± 0.5 |
| Buprenorphine | 39 ± 7 | 32 ± 6 | No Response | 29 ± 10 | 8.6 ± 0.4 | 8.4 ± 0.3 | No Response | 8.4 ± 0.4 |

Assays were performed as described in the methods. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with $E_{max}$ significantly lower than endomorphin-2 are highlighted in red (1-way ANOVA, followed by Dunnett's post-hoc test corrected for multiple comparisons, $P < 0.05$). MOPr-variant $E_{max}$ and *p*$EC_{50}$ values significantly different from MOPr-WT are marked with * (unpaired Student's T-test, $P < 0.05$).

***MOPr-R265H***

For cells expressing MOPr-R265H, inhibition of FSK-stimulated membrane hyperpolarisation was similar to cells expressing MOPr-WT for DAMGO, morphine, buprenorphine and pentazocine (Figure 1, Table 3). The signalling of β-endorphin, endormophin-1 and endomorphin-2 was also unaffected by the R265H variant (Figure 2, Table 3). For met-enkephalin, maximal FSK inhibition was similar between CHO-MOPr-WT and CHO-MOPr-R265H, however $pEC_{50}$ was affected, with values of 7.4 ± 0.1 and 7.8 ± 0.1, respectively ($P < 0.05$, Figure 2, Table 3). Fentanyl, methadone and oxycodone inhibition of the FSK-response was not significantly different at the R265H variant (Figure 3, Table 3).

DAMGO elicited a similar degree of ERK1/2 phosphorylation in CHO-MOPr-WT and CHO-MOPr-R265H cells, however DAMGO was less potent at MOPr-R265H compared with MOPr-WT, with $pEC_{50}$ of 7.9 ± 0.1 and 8.6 ± 0.1 respectively ($P < 0.05$, Figure 1, Table 4). Likewise, the maximum morphine response was similar between variants, but morphine was significantly less potent at MOPr-R265H (Table 4). Buprenorphine and pentazocine ERK1/2 phosphorylation was not affected by the MOPr-R265H variant. Maximal β-endorphin stimulation of ERK1/2 by cells expressing MOPr-R265H was similar to cells expressing MOPr-WT, however β-endorphin elicited ERK1/2 phosphorylation with lower potency, with $pEC_{50}$ of 6.8 ± 0.2 ($P < 0.05$, Table 4, Figure 2). Endomorphin-1, endomorphin-2 and met-enkephalin signalling did not differ between variants (Figure 2, Table 4). Fentanyl activation of ERK1/2 was similar at MOPr-WT and MOPr-R265H. Methadone was less potent at R265H, and maximum oxycodone stimulation of ERK1/2 was decreased in CHO-MOPr-R265H cells (Figure 3, Table 4).

## DISCUSSION

A number of MOPr polymorphisms have been identified in the population, yet as most are relatively rare, little is known about the clinical significance for individuals expressing the variants. In this study, we have shown that non-synonymous *OPRM1* SNPs alter the signalling profile of MOPr in vitro, in a ligand-selective and/or pathway-specific manner.

The L85I variant, arising from a 253C > A nucleotide change in *OPRM1*, is a leucine to isoleucine amino acid substitution in the first transmembrane (TM1) domain of MOPr, and was first reported by Ravindranathan *et al.* (2009). The transmembrane helices of GPCRs are essential for transmitting information from the extracellular surface to the intracellular signalling domains, and may also contribute to ligand-selectivity of GPCRs (Laurila *et al.*, 2011; Law *et al.*, 2000; Serohijos et al., 2011; Manglik et al. 2012). In this study, the L85I variant enhanced the signalling of the non-morphinan opioids DAMGO, met-enkephalin, endomorphin-1, endomorphin-2, fentanyl and methadone in assays of AC inhibition, but did not affect the morphinan opioids morphine, buprenorphine or oxycodone signalling. Signalling of the large peptide β-endorphin, and the benzomorphan pentazocine was also unaffected. It seems unlikely that an increase in non-morphinan opioid affinity would directly cause a pathway-specific enhancement of MOPr signalling, with no change in potency. Furthermore, we found no difference in $K_D$ for DAMGO between MOPr-WT and MOPr-L85I in agreement with a previous study (Ravindranathan *et al.*, 2009). MOPr expression levels were similar between CHO cells expressing MOPr-WT and MOPr-L85I, thus the apparent increase in the efficacy of some opioids at MOPr-I85 is not likely to be due to an insufficient amount of receptor to fully inhibit AC in MOPr-WT cells. Another possibility for the ligand- and pathway-selectivity of L85I effects is that the three-dimensional structure of non-morphinan opioids may enable the L85I receptor-ligand complex to adopt a conformation that more effectively couples to effector molecules

involved in AC inhibition. Morphinan opioids and the large β-endorphin molecule may restrict the ability of MOPr-L85I to adopt a similar conformation. The L85I residue change is a relatively small change in terms of hydrophobicity and size, and perhaps would not be expected to result in any major alteration of protein structure or function. However, these amino acids are not always interchangeable (Brosnan & Brosnan, 2006). The two amino acids both play key, but different, roles in protein structure, with leucine found more frequently in α-helices, and isoleucine found more in β-sheets (Brosnan & Brosnan, 2006). This has important implications for stability of the folded protein and the ultimate conformation of the receptor.

As we were unable to measure pentazocine inhibition of AC at maximal concentrations due to non-specific effects, it is unclear whether pentazocine signalling is affected by the L85I variant. It would be of interest to examine other benzomorphans at this variant to determine any enhancement of activity. In assays of ERK1/2 phosphorylation, the enhancement of non-morphinan opioid signalling was absent, indicating that the receptor conformation elicited by non-morphinan opioids did not confer any increase in the ability of MOPr-L85I to stimulate ERK1/2 phosphorylation.

In contrast to our results, previous studies reported DAMGO and morphine signalling was not affected by the L85I variant in assays of intracellular $Ca^{2+}$ release (Ravindranathan *et al.*, 2009), and was moderately decreased in assays of cAMP accumulation at L83I (Cooke *et al.,* 2014). Although our results differ from these observations, the non-morphinan ligands most markedly affected by the L85I variant in our study were not examined. Interestingly, previous studies of the L85I variant or the rat ortholog L83I have demonstrated an enhanced capacity for morphine to promote internalisation of MOPr-L85I/L83I (Cooke *et al.,* 2014; Ravindranathan *et al.,* 2009), possibly due to an increased

capacity for morphine to recruit GRK2 (Cooke *et al.*,2014). The L85I variant was also reported to show reduced tolerance and cAMP superactivation in response to chronic morphine treatement (Ravindranathan *et al.*, 2009). The enhanced L85I/L83I trafficking, reduced adaptations to chronic morphine exposure and apparent decrease in signalling efficacy observed in these studies highlight the probability that distinct receptor conformations underlie each of these processes, and that ligand-specific receptor conformations may selectively enhance some signalling or regulatory processes over others. The apparent contradiction between our results and those of previous studies may also reflect differences in the available pool of effector proteins and regulatory molecules expressed in CHO cells compared with HEK293 cells (Atwood *et al.*, 2011). It would be interesting to see if morphine is capable of promoting L85I internalisation in cell lines such as CHO, and if non-morphinan ligand signalling is enhanced in other cell lines such as HEK-293.

The R260H and R265H variants are both arginine to histidine amino acid substitutions in ICL3, arising from G > A SNPs at nucleotides 779 and 794, respectively. Although the R260H and R265H variants are rare within the population, the importance of ICL3 in MOPr signalling and regulation has prompted investigation into their functional consequences. The ICL domains of MOPr form major elements of the cytoplasmic interface between the receptor and intracellular effector proteins (Lefkowitz, 1998). The highly conserved ICL3 domain has been shown to be involved in basal and agonist-stimulated G-protein coupling, β-arrestin recruitment and contains multiple phosphorylation consensus sequences (Merkouris et al., 1996; Georgoussi et al., 1997; Wang, 1999). In our study, the R260H variant in ICL3 of MOPr had a negative impact on the signalling of β-endorphin, pentazocine and oxycodone in assays of AC inhibition. In assays of ERK1/2 phosphorylation, signalling of all opioids was compromised with the

exception of DAMGO and methadone. Buprenorphine and pentazocine failed to elicit ERK1/2 phosphorylation at MOPr-R260H. The effects of the R265H variant on MOPr signalling were less pronounced, with a small increase in the potency of β-endorphin and met-enkephalin for AC inhibition, and in DAMGO, β-endorphin and methadone potency for ERK1/2 phosphorylation (Table 2). Pentazocine $E_{max}$ for AC inhibition and oxycodone $E_{max}$ for ERK1/2 phosphorylation were decreased at R265H.

Mutations within the ICL3 of MOPr have been shown to differentially affect agonist potency and efficacy (Chaipatikul et al., 2003), however previous studies examining R260H or R265H signalling have provided inconsistent results (Knapman & Connor, 2014). In assays of cAMP accumulation, morphine potency and efficacy for inhibition of FSK-stimulated radiolabelled cAMP accumulation was not affected by the H260 or H265 variants (Wang *et al.*, 2001). Befort et al. (2001) also found no differences between H265 and WT in a cAMP response element (CRE) reporter gene assay. By contrast, Fortin et al. (2010) found a decrease in potency of DAMGO, endomorphin-1 and leu-enkephalin signalling through both H260 and H265, using a different CRE reporter assay. It is difficult to directly compare the results of these studies and ours due to different cell backgrounds, levels of receptor expression and assay conditions used. It is possible that the high level of receptor expression in the cells used by Wang *et al.* (2001) and Befort *et al.* (2001) may have masked the changes in opioid inhibition of AC observed at the R260H and R265H variants in our assay. Furthermore, our results highlight the ligand-selective effects of R260H and R265H on AC inhibition, while previous studies have only examined a limited number of opioids and may have failed to capture ligand-specific differences in variant signalling. The effects of R260H and R265H on MOPr function may also be pathway specific. In our study, the most marked changes in R260H signalling were observed in assays of ERK1/2 phosphorylation, a pathway that has not previously been examined with

regards to MOPr ICL3 variant signalling.

Measurement of the extent of AC inhibition and ERK1/2 phosphorylation was taken 5 min after the application of opioid, during which time significant receptor desensitisation and internalisation can occur (Connor *et al.*, 2004). It is possible that the differences observed in R260H and R265H signalling may reflect differences in receptor regulation rather than a direct effect on receptor signalling, however R260H, R265H and WT-MOPr have previously been shown to be downregulated to a similar degree by high concentrations of DAMGO (Befort *et al.*, 2001). There is also some evidence that R260H and R265H may affect constitutive activity of MOPr, and although this evidence is far from conclusive, must be considered when interpreting results (Befort *et al.*, 2001; Wang *et al.*, 2001).

It is interesting to note that R260H and R265H had quite different effects on MOPr signalling, despite being identical amino acid substitutions in a similar region of MOPr. The ICL3 domain has been shown to be one of the most dynamic regions in MOPr and other GPCRs (Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007; Serohijos *et al.*, 2011), and conformational changes in this domain are related to the rearrangement of TM5 and TM6 upon activation (Rasmussen *et al.*, 2011; Serohijos *et al.*, 2011). It is conceivable that a minor change in the position of an R > H amino acid substitution on the structurally flexibile ICL3 domain could cause different conformations of MOPr, resulting in different patterns of MOPr signalling for R260H and R265H. Furthermore, TM5 and TM6 form important elements of the ligand-binding pocket, and the presence of ligand has been shown to dramatically alter the flexibility of ICL3 in MOPr (Serohijos *et al.*, 2011; Manglik *et al.*, 2012). Aberrant arrangements of TM5 and TM6 resulting from ICL3 polymorphisms could potentially differentially affect the ability of ligand to bind to and activate MOPr-R260H or MOPr-R265H, which is consistent with our observations of ligand-selective changes in ICL3 variant signalling.

In this study, amino acid changes in MOPr arising from naturally occurring SNPs altered the signalling profile of MOPr in a ligand-selective and pathway-specific manner. Although the variants described here are rare within the population, a high number of naturally occurring SNPs have been described (Kazius *et al.*, 2008; Lotsch & Geisslinger, 2005; Ravindranathan *et al.*, 2009), and thus a significant proportion of the population may carry one or more MOPr SNPs. All MOPr variants we examined resulted in alterations to MOPr signalling, suggesting that MOPr function is finely regulated by the composition of amino acids and ultimate conformation of the receptor. It is thus likely that other SNPs found within the population have a high potential to affect receptor function. Presuming the variant receptors behave similarly in a physiological context, this may go some way towards explaining the individual variability in response to opioid analgesics observed within the population. As most individuals would be heterozygous for MOPr SNPs, the physiological significance of changes in MOPr variant signalling in vitro is unclear, and as the rarity of most MOPr SNPs within the population makes clinical studies impractical, further studies in cells expressing both WT and variant MOPr would be of interest. Nevertheless, MOPr SNPs clearly have the potential to alter receptor function, and a clearer understanding of the effects of these variants could provide valuable insights into the function and regulation of MOPr. Ultimately, knowledge of the effect of individual MOPr genotype on the response to a range of opioid analgesics could one day contribute to a more personalised approach to the prescription of opioid analgesic therapy, resulting in maximal therapeutic benefits with reduced risk of adverse effects.

# Chapter 8

## General Discussion

In this thesis, the functional consequences of five non-synonymous, naturally occurring OPRM1 SNPs were examined. The results presented show that each MOPr variant examined caused significant alterations in MOPr signalling, and these changes were ligand-selective and pathway-specific. This supports the notion that naturally occurring polymorphisms of MOPr contribute to the individual variability observed clinically in response to opioid analgesics, and that differences in response may only be manifest for a subset of opioid drugs and/or opioid effects.

The common N40D variant on the extracellular N-terminal domain of MOPr caused a selective impairment of buprenorphine signalling for all effectors examined. The A6V variant, also on the N-terminal domain of MOPr, resulted in a complete abolition of buprenorphine signalling via AC inhibition and ERK1/2 stimulation, and a significant decrease in the efficacy and/or potency of a number of other opioids. It is not immediately apparent how variation in the N-terminal domain of MOPr causes the significant and ligand-selective effects on opioid signalling observed. The N-terminal domain does not appear to form an integral part of the ligand-binding pocket of MOPr (Manglik *et al.*, 2012), however, as this region was not included in the recently solved MOPr crystal structure, its contribution to the conformation of the ligand-binding pocket and the receptor as a whole is not yet understood. The N-terminus contains 5 putative glycosylation sites, one of which is removed by the N40D variant (Singh *et al.*, 1997). This has been associated with reduced receptor expression and mRNA stability (Huang *et al.*, 2012; Mague *et al.*, 2009; Wang *et al.*, 2012; Zhang *et al.*, 2005), yet in our cell lines MOPr-

N40D and MOPr-WT surface receptor expression levels were equivalent. Moreover, the A6V variant does not affect a glycosylation site, suggesting that N-terminal polymorphisms can affect receptor function independently of glycosylation.

An N-terminal polymorphism equivalent to MOPr-A6V in the 5-$HT_{2B}$ receptor, R6G, increased constitutive and agonist-stimulated activity (Belmer *et al.*, 2014), and conformational changes in the N-terminal domain of MOPr have been shown following receptor activation (Gupta *et al.*, 2007; Gupta *et al.*, 2008). Furthermore, truncation, point mutations of phosphorylation sites or β-arrestin binding in the C-terminal domain of MOPr affect the conformation of the N-terminus (Gupta *et al.*, 2008). The reports from these studies and the results presented in this thesis indicate that conformation of the N-terminal and C-terminal domains are mutually dependent, and that changes to either of these regions have the potential to globally alter receptor conformation and function. Changes in the N-terminal region of other GPCRs such as the dopamine $D_2$ and $D_3$ receptors have been shown to affect receptor trafficking and internalisation (Cho *et al.*, 2012; Dong & Wu, 2006; Langelaan *et al.* 2013), and assays investigating the consequences of MOPr N-terminal variants on these aspects of MOPr function would be of interest.

Buprenorphine was more markedly affected by N-terminal polymorphisms compared with other opioids, and appeared to be affected in a pathway-specific manner at N40D and A6V. Buprenorphine efficacy was significantly decreased in assays of AC inhibition and ERK1/2 phosphorylation in CHO-MOPr-N40D cells, yet showed a decrease in potency for GIRK activation in AtT20-MOPr-N40D cells, with no change in efficacy. Buprenorphine did not inhibit AC or stimulate ERK1/2 in CHO-MOPr-A6V, but activated GIRK channels to a similar degree as MOPr-WT in AtT20 cells, suggesting a functional selectivity towards GIRK activation, or some effect of the expression system being used.

Buprenorphine is a partial MOPr agonist and is an antagonist at KOPr (Pawlak *et al.*, 2014). One study has demonstrated pathway-specific effects of buprenorphine at MOPr (Zaki *et al.*, 2000). Buprenorphine showed partial agonist activity for the inhibition of cAMP and stimulation of [$^{35}$S]GTPγS binding in HEK-293 cells, yet caused an increase in surface MOPr expression after treatment, similar to the increased surface receptor expression observed after treatment with the MOPr antagonist naloxone (Zaki *et al.*, 2000). Although significant post-activation trafficking would probably not occur within the time-frame of our assays, the results from the present study and previous reports suggest that buprenorphine may show functional selectivity towards certain signalling pathways. Measuring ERK1/2 phosphorylation in AtT20-MOPr cells could help to determine the presence of functional bias. Examination of the signalling of closely related buprenorphine derivatives (e.g. Cami-Kobeci *et al.*, 2011; Neilan *et al.*, 2004), and biologically active buprenorphine metabolites (Brown *et al.*, 2011) could also provide valuable insight into the involvement of different structural elements of the ligand or receptor in the functional changes observed at MOPr-N40D.

In addition to the abolition of buprenorphine signalling, the A6V variant had a significant impact on the signalling of a range of other opioid analgesics such as morphine and fentanyl, and on endogenous opioids including β-endorphin and endomorphins 1 and 2. There does not appear to be a clear pattern in the opioids affected by A6V, with DAMGO, morphine and β-endorphin affected in AC inhibition assays, all opioids with the exception of β-endorphin and pentazocine affected in ERK1/2 phosphorylation assays, and morphine selectively affected in GIRK activation assays. One of the limitations of assays measuring downstream signalling pathways such as AC inhibition is that there may be significant signal amplification between ligand binding and the final measured response (Hill *et al.*, 2001). Ligands known to have partial agonist activity in other assay formats may manifest

as full agonists in cAMP accumulation assays (Hill *et al.*, 2010). For example, in this study morphine showed full agonist activity for AC inhibition, despite being classed as a partial agonist at MOPr (Pawson *et al.*, 2013). The differences observed between assays of AC inhibition and ERK1/2 activation may be due to a loss of sensitivity in AC inhibition assays due to signal amplification. Assays investigating different effectors in CHO cells and in particular ERK1/2 assays in AtT20 cells could help to further understand whether the differences observed between assays in ligand signalling are due to functional selectivity, assay parameters or the expression system used.

The TM1 L85I variant selectively enhanced the signalling of non-morphinan opioids for AC inhibition, resulting in a maximum FSK inhibition that exceeded the maximum stimulated by full agonists at MOPr-WT. Receptor expression levels of MOPr-WT and MOPr-L85I were equivalent, indicating either an increased efficiency of G protein activation by L85I, or activation of different G protein subtypes. Opioid receptors show ligand-specific patterns of Gα subtype activation (Alves *et al.*, 2004; Massotte *et al.*, 2001; Sanchez-Blazquez *et al.*, 2001; Saidak *et al.*, 2006), which can lead to differential coupling to effector and regulatory molecules (Abrol *et al.*, 2013). CHO cells express AC isozymes VI and VII (Varga *et al.*, 1998). Opioids inhibit AC VI but stimulate AC VII (Avidor-Reiss *et al.*, 1997), thus it seems unlikely that the increased inhibition of AC by MOPr-L85I is caused by differential coupling to AC isozymes. AC VI is inhibited by protein kinase C as well as Gα proteins (PKC; Sadana & Dessauer, 2009). PKC is involved in many MOPr regulatory pathways (Law *et al.*, 2000), and has been suggested to mediate uncoupling and desensitisation of the morphine-activated receptor (Bailey *et al.*, 2009; Kelly *et al.*, 2008). Activation of MOPr by other opioid agonists such as DAMGO results in β-arrestin recruitment and subsequent receptor endocytosis, whereas the PKC-mediated MOPr phosphorylation does not induce internalisation (Williams *et al.*, 2013). Related

GPCRs such as 5-$HT_{2A}$ and dopamine receptors require PKC for endocytosis stimulated by specific ligands, indicating that PKC activation is capable of promoting internalisation (Krillov *et al.*, 2011; Raote *et al.*, 2013). Altered coupling of MOPr-L85I to PKC could lead to changes in AC inhibition, and conceivably contribute to the differences in morphine-stimulated MOPr-L85I internalisation observed in previous studies (Cooke *et al.* 2014; Ravindranathan *et al.*, 2009). The slight depolarisation observed with high concentrations of fentanyl and methadone requires further examination, but may be a reflection of the enhanced activity of L85I.

The R260H variant on ICL3 of MOPr decreased the ability of β-endorphin, oxycodone and pentazocine to inhibit AC, and had a negative impact on ERK1/2 phosphorylation by most opioids tested with the exception of DAMGO and methadone. The effects of the R265H variant on MOPr signalling were more subtle, with minor changes in the signalling of select opioids including β-endorphin, oxycodone and pentazocine. In agreement with these results, previous studies examining R260H and R265H signalling have reported unchanged or minor differences in DAMGO and morphine inhibition of cAMP accumulation (Befort *et al.*, 2001; Wang *et al.*, 2001). Fortin *et al.* (2010) reported decreased DAMGO, endomorphin-1 and leu-enkephalin potency using a different assay of cAMP accumulation. These studies did not examine many of the ligands most markedly affected at R260H/R265H in the present study, e.g. buprenorphine, highlighting the necessity for examining a range of opioids and signalling pathways to capture ligand- and pathway-specific effects of GPCR SNPs (Knapman & Connor, 2014).

The assays used here to examine differences in MOPr variant functional profiles focused on acute signalling pathways, however MOPr also undergoes receptor trafficking and longer-term regulatory and adaptive processes (Williams *et al.* 2013), as well as

constitutive or ligand-independent signalling (Connor & Traynor, 2010). The ICL3 domain is of primary importance in the coupling of MOPr to effector and regulatory molecules, contains several consensus phosphorylation sites, and serves as a docking site for accessory proteins such as β-arrestin (Lefkowitz, 1998). Befort *et al.* (2001) and Wang *et al.* (2001) both reported a decrease in constitutive GTPγS binding for R260H and R265H MOPr variants. The ICL domains have been shown to be involved in stabilisation of the inactive state of Class A GPCRs in the absence of ligand (Chee *et al.*, 2008; Mokrosinksi *et al.*, 2012; Piechowski *et al.*, 2013). MOPr ICL3 SNPs may either stabilize active conformations of the unbound receptor more readily, or enable the ligand-bound receptor to couple more effectively to effector and regulatory molecules (Rana *et al.*, 2001). Constitutively active GPCRs have been reported to undergo receptor desensitisation and other adaptive processes in the absence of agonist (Barak *et al.*, 2001; Wilbanks *et al.*, 2002). Although the marked decrease in ligand-stimulated MOPr-R260H signalling suggests a decreased capability for ligand-stimulated MOPr effector coupling, it is possible that the differences observed for R260H and R265H in assays of "acute" signalling in the present study reflect a certain level of receptor desensitisation arising from differences in constitutive activity. It would certainly be of interest to investigate basal signalling and desensitisation of R260H and R265H to provide further insight into the functional consequences of these SNPs.

It is becoming increasingly apparent that MOPr pharmacology must be considered to be system- and context-dependent. MOPr is expressed in a wide variety of human cell types, and subtle changes in MOPr signalling arising from SNPs may generate distinct signals in different tissues and cell types, reflecting differential binding and stabilisation of distinct conformations of the receptor, varying efficacy of the receptor to couple to different pools of effector and regulatory proteins, and context-specific tissue response to activated

signalling pathways (Kenakin & Miller *et al.*, 2010). For example, [$^{35}$S]GTPγS binding was significantly decreased at MOPr-N40D in the secondary somatosensory area of post-mortem human brain, but was unchanged in the thalamus, despite equivalent receptor expression levels for N40D and WT (Oertel *et al.*, 2009). It is probably necessary to examine MOPr polymorphisms in a variety of heterologous expression systems using a wide range of structurally distinct opioid ligands in order to capture the range of signalling and regulatory differences that may be produced by MOPr variants (Charfi et al., 2013). However, examining as many effectors as possible under similar conditions enables direct comparisons between MOPr variants and/or the effects of multiple ligands. In this project, the ability of MOPr variants to inhibit AC and stimulate ERK1/2 were both examined in CHO cells. In general, changes in signalling arising from MOPr variants were more marked in ERK1/2 assays compared with AC, and a higher proportion of opioids tested showed full agonist activity for AC inhibition than ERK1/2 phosphorylation. This probably reflects the relatively low receptor occupancy required for AC inhibition compared with ERK1/2 phosphorylation, particularly at the sub-maximal concentration of FSK used (Nickolls *et al.*, 2011). The use of a higher concentration of FSK may increase the sensitivity of the AC assay. It would be interesting to observe the effects of a higher FSK concentration on AC inhibition at MOPr-L85I, to help understand the mechanism behind the enhanced AC inhibition compared with MOPr-WT. An unusual finding of this study was the relatively low efficacy of DAMGO in assays of ERK1/2 phosphorylation. For MOPr-WT, maximum DAMGO-stimulated ERK1/2 phosphorylation was approximately 65% of the most efficacious agonist, endomorphin-2. In contrast, DAMGO shows full agonist activity in most published assays. One explanation for our results is that DAMGO may desensitize more rapidly or reach maximum effect more slowly compared with some other agonists tested. All measurements were taken at 5 min, thus further investigation into the ideal measurement time-point for every agonist tested may be of

benefit. Furthermore, the difference between ligand and MOPr-variant effects on AC inhibition and ERK1/2 phosphorylation may also be related to the relative amounts of G protein subtypes in CHO cells. GIRK activation was measured in AtT-20 cells as CHO cells do not express native GIRK channels. Overall, MOPr variants showed less effect on GIRK activation than AC inhibition and ERK1/2 activation, and this was particularly apparent with buprenorphine signalling at MOPr-A6V. The inability of buprenorphine to elicit AC inhibition or pERK1/2 phosphorylation in CHO cells at MOPr-A6V, with an unchanged capability for GIRK activation in AtT-20 cells could reflect some pathway bias, or may be related to the different cellular backgrounds. This would be best investigated by performing the ERK1/2 phosphorylation ELISA in AtT-20 cells to see if opioid signalling is affected by MOPr-variants in a comparable way to ERK1/2 assays carried out in CHO cells. It should be noted that MOPr activation of GIRK activation showed some desensitization over the course of the assay, whereas MOPr inhibition of AC did not. AC inhibition did begin to decrease 5 min after opioid addition, and we have observed further desensitization when assay duration was increased. As both assays were continuous and measurements were taken at the peak of MOPr activity, desensitization of MOPr is not likely to have significantly affected our results.

Another area of interest at present is the identification of ligands biased towards either G-protein coupled pathways or G-protein independent pathways such as β-arrestin recruitment (Violin & Lefkowitz, 2007). There is some evidence that β-arrestin-coupled pathways mediate the adverse effects arising from MOPr stimulation such as tolerance and dependence (Raehal & Bohn, 2011; Raehal *et al.*, 2005), and current efforts to design improved opioid drugs are often focused on developing ligands with significant bias towards G-protein coupled pathways (DeWire *et al.*, 2013). MOPr polymorphisms could also in theory show preferential coupling towards G-protein coupled pathways or G-

protein independent pathways, but to date this has not been explored. The assays used in this project were predominantly focused on G-protein coupled pathways, although ERK1/2 phosphorylation can occur via a number of mechanisms including β-arrestin signalling (Law *et al.*, 2000). Examination of the relative ability of MOPr variants to couple to β-arrestin and G proteins could provide important insights into the structural components involved in these processes as well as the mechanisms behind the pathway-specific effects observed. Defining receptor reserve using irreversible antagonists such as β-funaltrexamine or β-chlornaltrexamine and then fitting data to operational models (e.g. Borgland et al., 2003; Rivero et al., 2012; Kelly, 2013) would also enable the precise determination of rank orders of agonist efficacy and uncover differences in signalling across different effectors in the same cell, enabling a more complete characterisation of the consequences of changes in receptor sequence (Kenakin & Christopoulos, 2013).

One area that has remained largely unexplored is the way in which variant MOPr may interact with WT-MOPr, even though most carriers of variant MOPr alleles will be heterozygous for the wild-type receptor. The MOPr crystallises as parallel dimers tightly associated through TM5 and TM6, and to a lesser extent TM1 and TM2 (Manglik *et al.*, 2012). Ravindranathan *et al.* (2009) co-expressed the L85I variant with MOPr-WT in HEK-293 cells and reported internalisation of both L85I- and WT-MOPr in response to morphine, suggesting the formation of functional MOPr-WT/L85I dimers with L85I showing domainance. Conversely, co-expression of R181C- and WT-MOPr resulted in independent internalisation of MOPr-WT in response to DAMGO, indicating R181C either does not dimerise with WT, or that the interaction is unstable (Ravindranathan *et al.*, 2009). The effects of co-expression of these variants on MOPr signalling were not investigated. Other functional studies of MOPr SNPs have not examined the possible interactions between WT- and variant-MOPr, the formation of homo- or heterodimers, or

the effects of co-expressed MOPr variants on cellular signalling. Although there is growing evidence supporting the ability of MOPr and other GPCRs to form homo- or heterodimers under experimental conditions (Golebiewska *et al.*, 2011; Jordan & Devi, 1999; Juhasz *et al.*, 2008; Milligan, 2009; Zheng *et al.*, 2012), whether this occurs in a physiological setting is still a matter of debate (Chabre & LeMaire, 2005; Herrick-Davis *et al.*, 2013; James *et al.*, 2006; Kuscak *et al.*, 2009; Meyer *et al.*, 2006). In one study, human MOPr was reconstituted in monomeric form following expression in insect cells, and the monomer was shown to be capable of ligand-binding, G protein activation and allosteric modulation (Kuszak *et al.*, 2009), indicating that the formation of oligomers is not necessary for MOPr function. On the other hand, a study using a novel fluorescent correlation spectroscopy technique suggested that freely diffusing class A GPCRs exist primarily as homodimers (Herrick-Davis *et al.*, 2013). In any case, the consequences of co-expression of WT and mutant MOPr alleles is certainly an area requiring further investigation, although limited by the technical difficulty of accurately quantifying and expressing equivalent amounts of variant receptor (Liu *et al.*, 2000). The use of bicistronic vectors, which allow the expression of 2 genes using a single promoter, should in theory result in equal expression of WT-MOPr and variant MOPr, although this is not always the case in practice (Kim *et al.*, 2004b; Pfutzner, 2008). A recent study suggested that insert size may cause the differences in relative transfection efficiency and expression of the two inserts in bicistronic vectors (Payne *et al.*, 2013). As MOPr SNP variants are identical in length, this method may be appropriate for future examination of co-expressed MOPr variants.

The N40D variant is present in up to 50% of individuals in some populations (Mura *et al.*, 2013), while the A6V variant is present in upwards of 20% of individuals from other populations (Crowley *et al.*, 2003; Kapur *et al.*, 2007). Although other identified MOPr

SNPs are rare (< 1%; Lotsch & Geisslinger 2005), a substantial proportion of the population may carry one or more MOPr variants. Each MOPr variant examined in this study had some impact on MOPr signalling, indicating that the ultimate conformation of MOPr is highly sensitive to changes in the amino acid sequence, and that this has the potential to significantly alter receptor signalling and/or regulation. Opioid analgesics remain the mainstay of analgesic therapy, despite the associated adverse effects and the potential for tolerance and dependence to develop. The total number of opioid prescriptions in Australia in 2010 exceeded 11 million (Mabbott *et al.*, 2010). Buprenorphine accounted for almost 10% of total opioid prescriptions, exceeded only by codeine, tramadol and oxycodone (Mabbott *et al.*, 2010). In the present study, buprenorphine signalling was significantly affected by the N40D, A6V and R260H MOPr variants. Other commonly prescribed opioids including oxycodone (24% of total prescriptions) and morphine (6% of total prescriptions, 40% including codeine; Mabbott *et al.*, 2010) were less effective at the A6V variant. Assuming MOPr variants behave similarly in a neuronal context, a significant proportion of the population may be receiving inappropriate analgesic therapy, and could potentially benefit from prescription of an alternative opioid analgesic. Understanding the consequences of expressing a particular MOPr variant should in the future enable the development of personalized prescription plans depending on individual phenotype. Individual opioid response involves multiple proteins involved in opioid ligand distribution and metabolism, as well as effectors downstream of MOPr, which would also be affected by genotype. Neverthelss, knowledge of an indiviudal's MOPr genotype would be a key element in the ability to predict the effects of specific opioid drugs, including side effects and the development of tolerance, minimizing the risk of serious adverse events associated with opioid overdose, while maximizing therapeutic benefits and ensuring individuals receive adequate pain relief.

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# Appendix

## Fluorescence-Based, High-Throughput Assays for μ-Opioid Receptor Activation using Membrane Potential-Sensitive Dye

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**Summary**

The development of new and improved opioid analgesics requires high throughput screening (HTS) methods to identify potential therapeutics from large libraries of lead compounds. Here we describe two simple, real-time fluorescence-based assays of μ-opioid receptor activation that may be scaled up for HTS. In AtT-20 cells expressing the μ-opioid receptor (MOPr), opioids activate endogenous G protein gated inwardly rectifying K channels (GIRK channels), leading to membrane hyperpolarisation. In Chinese hamster ovary cells expressing MOPr, adenylyl cyclase activation via forskolin results in membrane hyperpolarisation, which is inhibited by opioids. Changes in membrane potential can be measured using a proprietary membrane potential-sensitive dye. In contrast to many HTS methods currently available, these assays reflect naturalistic coupling of the receptor to effector molecules.

**Keywords**

GIRK, adenylyl cyclase, μ-opioid receptor, membrane potential, high-throughput screening, AtT-20, CHO, fluorescent assay.

## 1. Introduction

The μ-opioid receptor (MOPr) is a G protein coupled receptor (GPCR), and is the primary target for opioid analgesics ***(1)***. There is increasing evidence that different ligands can stabilise GPCRs including MOPr in various active conformations, leading to the preferential activation of distinct signalling pathways via Gα and Gβγ subunits ***(2 – 3)***. The development of novel opioid pharmacotherapies is now beginning to focus on identifying MOPr ligands that can selectively activate a subset of effector pathways associated with analgesia, without activating pathways leading to adverse effects ***(4-7)***.

Drug development typically involves the screening of large libraries of lead compounds to identify those capable of binding to and signalling via a receptor. The vast array of lead compounds available requires high throughput screening (HTS) methods to enable identification of potential therapeutic compounds. Many of the HTS methods available for opioid receptors involve the expression of highly engineered proteins, require harvesting or lysing cells, and rely on a single endpoint measurement ***(8, 9)***. Here, we describe two simple, real-time assays reflecting a naturalistic coupling of MOPr to Gα and Gβγ proteins, using a proprietary membrane potential sensitive dye.

In mouse pituitary AtT-20 cells, heterologously expressed MOPr activates endogenous G-protein-gated inwardly rectifying potassium channels (GIRKs), via direct coupling of Gβγ subunits to the channels ***(10)***. The resulting membrane hyperpolarisation can be measured using a membrane potential sensitive dye. The extent of hyperpolarisation corresponds to the number of activated GIRK channels, and therefore closely reflects of the degree of MOPr activation ***(11)***.

Inhibition of AC activity leading to a decrease in cAMP production is one of the hallmarks of MOPr activation and measurement of cAMP accumulation is frequently used to examine opioid potency and efficacy. Most cAMP accumulation assays rely on end-point measurements after significant incubation times to allow cAMP accumulation ***(9, 12,13)***, while real-time assays of cAMP accumulation such as the GloSensor assay (Promega), require transfection of sensor constructs with cAMP binding domains and use of specialized reagents. ***(14)***. The assay described here is a real-time, robust assay of AC inhibition in live CHO cells requiring minimal preparation and reagents. Stimulation of cAMP production Chinese hamster ovary (CHO) cells via the AC activator forskolin results in membrane hyperpolarisation, which is inhibited with the simultaneous addition of opioids ***(15)***.

Both assays described here have z-factors of 0.7, indicating sufficient robustness for HTS ***(11, 15, 16)***. These assays offer an alternative approach for measuring MOPr activation by targeting naturalistic signalling pathways. The membrane potential assay is a rapid, reliable, and inexpensive method for identifying ligands that modulate Gα and Gβγ-mediated signalling and may be scaled up to enable HTS for novel opioid drugs.

## 2. Materials

1. FlexStation3 Plate Reader, running at 37$^{o}$C. See Note 1.
2. Incubator with room air, at 37$^{o}$C.
3. FLIPR Membrane Potential Dye (Molecular Devices) – blue rather than red. See Note 2.
4. Assay Buffer – consisting of (in mM): NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, Glucose 5.56, pH 7.4, osmolarity 310-320

5. AtT-20 cells expressing MOPr (GIRK channel activation assay), or CHO-K1 cells expressing MOPr (AC inhibition assay) at 90 % or more confluency in 75 $cm^2$ tissue culture flasks (1 flask per microplate)
6. Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin $ml^{-1}$ and 15 mM glucose. See Note 3.
7. 96-well black-walled, sterile clear-bottomed microplates
8. 96-well clear, v-bottomed microplates
9. 96-well Black FlexStation pipette tips (Molecular Devices)
10. 8-channel multi-pipette
11. Troughs for holding cells and dye while using multichannel pipette.
12. Opioid ligands
13. Forskolin (AC inhibition assay)

**3. Methods**

1. On the day before the assay, harvest cells from single 75 $cm^2$ flask, re-suspend the cells obtained from each flask in 10 mL L-15 media, and plate in a volume of 90 µL/well in a black-walled 96-well plate using 8 channel pipettor. Incubate overnight at 37° C in ambient $CO_2$. See Note 4.

2. Prepare the dye according to the manufacturer's instructions in assay buffer. Prepared dye can be stored at -80° C for several months. See Note 5.

3.1 GIRK Channel Activation Assay (AtT-20 cells)

1. Load the cells with 90 µL/well prepared membrane potential dye. Incubate for a minimum of 45 minutes at 37° C in ambient $CO_2$. Ensure the plate is uncovered for the final 15 mins of incubation. See Notes 6 - 8.
2. Prepare drug solutions: Make up drugs in assay buffer at 10 x final concentration desired. See Note 9. Load 200 µL/well drug solutions or vehicle into wells in v-bottom 96-well plate. See Notes 10 - 11.

   Insert drug plate into appropriate FlexStation drawer and incubate for approx. 10 mins to warm solutions to 37° C. See Note 12.
3. Set up the assay parameters as follows:

   Read Mode: Fluorescence, bottom read

   Excitation wavelength: 530

   Emission wavelength: 565

   Cutoff: Auto

   Readings per well: 6

   PMT: Medium

   Run time: 300 secs

   Interval: 2 secs

   Select appropriate assay plate type and wells to read. See Note 13.

   Compound transfer: Initial volume: 180 µL

   Transfers: 1

   Pipette Height: 190 µL

   Volume: 20 µL

   Rate: 2

Time point: 120 secs – this will allow a stable baseline to be established before drug addition.

Select appropriate compound plate.

Triturate: Select Assay plate, volume 20 µL, 3 cycles, pipette height 150 µL. Pipette Tip Layout: Select columns of tips to be used corresponding to columns of wells to be read. Tips may be re-used up to 3 times for replicates.

Select "Read". See Note 14 - 17.

3.2 Adenylyl Cyclase Inhibition Assay (CHO cells)

1. Load the cells with 90 µL/well prepared membrane potential dye. Incubate for a minimum of 60 minutes at 37° C in ambient $CO_2$. See Note 6 – 8.
2. Prepare drug solutions:

   Forskolin is added simultaneously with drug or vehicle. Make up drugs with forskolin in assay buffer at 10 x final concentration desired, e.g. 3 µM FSK + 10 nM – 10 µM DAMGO for a 1 nM – 1 µM DAMGO concentration response curve. See Note 9. Load 200 µL/well drug solutions or vehicle into wells in v-bottom 96-well plate. See Notes 10 - 11.

   The FlexStation 3 reads column by column, so concentration response curves are best set up within columns rather than across rows. Insert drug plate into appropriate FlexStation drawer and incubate for approx. 10 mins to warm solutions to 37° C. See Note 12.

3. Set up the assay parameters as follows:

   Read Mode: Fluorescence, bottom read

   Excitation wavelength: 530

Emission wavelength: 565

Cutoff: Auto

Readings per well: 6

PMT: Medium

Run time:  720 secs

Interval: 2 secs

Select appropriate assay plate type and wells to read. See Note 13.

Compound transfer:

Initial volume: 180 µL

Transfers: 1

Pipette Height: 190 µL

Volume: 20 µL

Rate: 2

Time point: 120 secs – this will allow a stable baseline to be established before drug addition.

Select appropriate compound plate

Triturate: Select Assay plate, volume 20 µL, 3 cycles, pipette height 150 µL Pipette Tip Layout: Select columns of tips to be used corresponding to columns of wells to be read. Tips may be re-used up to 3 times for replicates.

Select “Read”.  See Notes 13 – 15, 17 - 18.

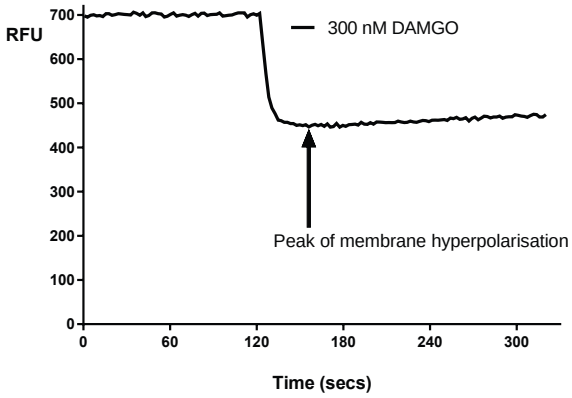


Figure 1

**Figure 1:** Example trace of fluorescent signal in AtT-20-MOPr cells with the addition of 300 nM DAMGO at 120 sec. The rapid drop in fluorescent signal peaks 20 seconds after DAMGO addition, at which point measurements were taken.

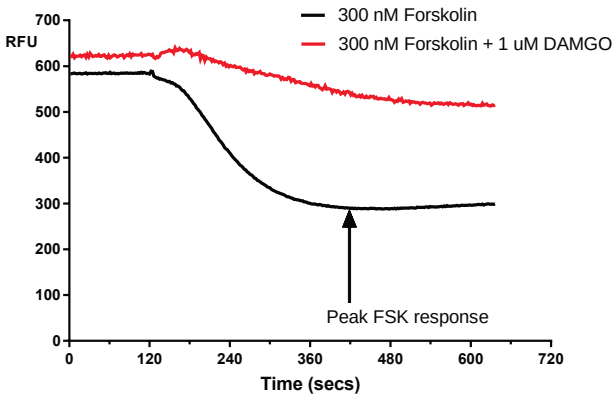


Figure 2

**Figure 2:** Example trace of the fluorescent signal in CHO-MOPr cells with the addition of 300 nM FSK alone or 300 nM FSK + 1 μM DAMGO. The maximum FSK-stimulated decrease in fluorescent signal occurred 5 min after addition, after which time the signal stabilized. Measurements for FSK alone and FSK + opioid were taken at this time-point.

## 4. Notes

1. We used a FlexStation 3 for this assay, however a similar plate reader with a fluidic module could be used for the GIRK assay. The AC assay could potentially be performed as an end-point assay on a static plate reader approximately 5 mins after drug treatment, providing a 300 nM FSK control is included in each column.
2. The membrane potential-sensitive dye is available in a blue or red formulation. We found blue dye to give the largest signal window for our cell lines, however, this may vary. To ensure optimal results both blue and red dyes should be trialled, particularly if adapting the assays for other cell types.
3. Serum-starving the cells synchronises the cell cycle phase and helps to increase reproducibility of the results. Serum starving also prevents cell overgrowing and forming multiple layers in wells.
4. For the cell lines used in these assays, the use of polylysine coated plates was not necessary. We have found that experiments with other AtT-20 lines, such as those expressing the cannabinoid-CB1 receptor, require polylysine coated plates to prevent cells detaching.
5. Membrane potential dye (blue) can be successfully used at half the concentration suggested by the manufacturer for these assays with no appreciable difference in signal, however we observed decreased stability of half-strength dye with longer assays (> 20 min). We have not tested red membrane potential dye at half-concentration.
6. Although the manufacturer's protocol recommends an incubation time of 30 mins with dye, we found that when readings were taken after 30 mins incubation the baseline steadily increased over time, indicating incomplete uptake of dye into cells. An incubation of 45 mins was sufficient to achieve a flat baseline for AtT-20 cells, a

minimum of 60 mins was necessary for CHO cells. This may vary between cell lines and assay conditions. Incubate for sufficient time period to achieve a flat baseline.

7. For the GIRK channel activation assay, an entire plate may be read in approximately one hour, so it is practical to load an entire plate with dye at once with no deterioration of signal. For longer assays such as the AC inhibition assay, dye loading can be staggered so that cells are not loaded with dye for an extended period of time. In this case, reading of half a plate takes approximately 80 mins, so load half a plate with dye, incubate for 80 mins, then immediately prior to reading load the second half of the plate with dye and cover with parafilm, allowing dye to load in the second half of the plate while the first half is being read.
8. Where possible, the assay plate should be incubated in the FlexStation 3 while the dye is loading to minimize temperature changes when transferring between the incubator and Flexstation (in our lab these are located in different rooms). When this is not possible, the assay plate may be incubated in an incubator, and transferred to the FlexStation for the last 15 mins of incubation, uncovered.
9. When making up drug solutions, keep the concentration of any solvents used (e.g. DMSO or ethanol) constant. Also include the same concentration of solvent in the vehicle blank.
10. There should be a minimum of 80 µL of compound in the wells of the compound plate to ensure consistency in compound transfer volume. Compound may be taken from the same column for replicates until volume in well reaches 60 µL.
11. For relatively short assays, no changes in response over time were detected when reading an entire plate, i.e. there appears to be little effect of evaporation of drug or dye from compound and assay plates. For the AC inhibition assay, the compound plate should be only be loaded with the drugs required for the portion of the plate being

read. Load fresh compounds when beginning a new read for the second half of the plate.

12. Some compounds, for example somatostatin, may be unstable for extended time periods at 37°C. In this case, drugs should be kept on ice and loaded into compound plate just prior to reading.
13. For short assays, providing you are not using any unstable drugs, you can select and read the entire plate. Otherwise, select only the columns you will read before loading fresh drug solutions. Note that when using SoftMax Pro with the FlexStation 3, only consecutive columns can be read in a single run.
14. For this assay, on our Flexstation, a baseline of between 600 – 1200 RFU is optimal. These values may vary between machines, but a low baseline does not give a sufficient window for the decreased fluorescent signal associated with cellular hyperpolarisation, and a high baseline can lead to unpredictable results for a number of reasons including too many cells per well, cells which are excessively depolarized or a double addition of dye. More than a single layer of cells can lead to problems with drug access and also loss of cells when drug is added, which leads to a large and immediate drop in signal.
15. The background signals for these assays are low – cells without dye usually read approximately 20-30 RFU (< 5% of the optimal signal), while L-15 plus dye without cells gives a negligible reading. We do not routinely correct for these background signals, but they should checked occasionally by reading a few wells without dye, with and without cells.
16. GIRK Activation Assay Data Analysis: For this assay, we calculated GIRK activation as a percentage decrease in fluorescence from baseline. We calculated the mean of the readings from 30 sec before drug addition, ensuring that the baseline is flat and stable. The peak response occurs rapidly after drug addition, usually within 20 secs (see Fig

1). We took the mean of the lowest reading from the peak response and 2 readings either side, calculating the percentage difference between this value and the mean baseline. We usually include a buffer/solvent blank in each row and subtract the change produced by the 20 µl addition of buffer from all the experimental data. This change is usually less than 5%.

17. If you want to normalize data between assays, the hyperpolarisation produced by activation of endogenous SST receptors in AtT-20 cells can be used, alternatively nigericin (1 µM), a K-selective ionophore, can be used to determine the fluorescence change asscoiated with hyperpolarisation of the cells to $E_K$. Both somatostatin and nigericin produce decreases in fluorescence greater than the maximum produced by opioids.
18. AC Inhibition Data Analysis: For this assay, we calculated AC inhibition as a percentage inhibition of the FSK response. The FSK response was calculated as the maximum % decrease in fluorescence from baseline after addition of FSK alone. We calculated the mean of the readings from 30 sec before drug addition, ensuring that the baseline is flat and stable. The peak response of FSK occurs approximately 5 min after drug addition (see Fig 2). We took the mean of the lowest reading from the peak response and 2 readings either side, calculating the percentage difference between this value and the mean baseline for the maximum FSK response. We took readings at the same timepoint when FSK was added with opioid, and calculated the difference between the FSK response and the FSK + opioid response, and expressed as a percentage inhibition of FSK response. We also included a buffer/solvent blank in each row and subtracted the change produced by the 20 µl addition of buffer containing the solvent for FSK from the experimental data. This change is usually less than 5%.

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