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Themed Section: Opioids: New Pathways to Functional Selectivity

REVIEW

Cellular signalling of non-synonymous single-nucleotide polymorphisms of the human μ-opioid receptor (OPRM1)

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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 gene (*OPRM1*) have been postulated to contribute to this variability. Although many studies have investigated the clinical influences of these μ-opioid receptor 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 μ-opioid receptor SNPs affect their expression, coupling to second messengers, desensitization and regulation is necessary to understand how subtle changes in receptor structure can impact individual responses to opioids. To date, the few functional studies that have investigated the consequences of SNPs on the signalling profile of the μ-opioid receptor *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 data available on the effects of μ-opioid receptor polymorphisms on receptor function, expression and regulation *in vitro*, and discuss the limitations of the studies to date. Whether or not μ-opioid receptor 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 μ-opioid receptor function.

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Abbreviations
aa, amino acid; β-CNA, β-chlornaltrexamine; CaM, calmodulin; CaMK II, Ca^{2+}/calmodulin-dependent PK II; Ca_V, voltage gated Ca channels; CRE, cAMP response element; DAMGO, [D-Ala^2, N-$MePhe^4$, Gly-ol]-enkephalin; GIRK, G protein gated, inwardly rectifying potassium channel; GRK, GPCR kinase; ICL, intracellular loop; M-6-G, morphine-6-glucuronide; *OPRM1*, μ-opioid receptor 1 gene; pERK, phosphorylated ERK1/2; SNP, single-nucleotide polymorphism; 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 and 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 and 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.*, 1998). 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 and Geisslinger, 2005; Skorpen and 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 (Matthes *et al.*, 1996; Alexander *et al.*, 2013a), making this receptor a prime candidate for contributing to the genetic component of inter-individual differences in opioid response. The μ-opioid receptor is a typical class A GPCR (Alexander *et al.*, 2013a). Many single-nucleotide polymorphisms (SNPs) within the *OPRM1* 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.*, 2000; Ikeda *et al.*, 2005; Lotsch and Geisslinger, 2005; Ravindranathan *et al.*, 2009; Fortin *et al.*, 2010). There are good reasons to consider the potential of μ-opioid receptor SNPs to contribute to the clinical variability of opioid responses. GPCR signalling is complex, with the notion of simple, linear and robust rearrangements of protein structure being required for signal transduction no longer accepted. Thus, the possibility that single aa 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 and Steinberg, 2013). Furthermore, 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 μ-opioid receptor 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 the μ-opioid receptor that have been described (Mizoguchi *et al.*, 2012; Pasternak and Pan, 2013). Although the functional role of alternatively spliced *OPRM1* transcripts is not yet well established, a single non-synonymous aa 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 μ-opioid receptor 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 and Geisslinger, 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 μ-opioid receptor function, and in this review, we focus on the effects of naturally occurring, non-synonymous SNPs in the coding region of *OPRM1* on μ-opioid receptor function. The SNPs considered here, the corresponding aa exchanges and their position on the μ-opioid receptor are summarized in Table 1 and Figure 1.

Table 1

Summary of non-synonymous μ-opioid receptor variants in the protein coding region, their corresponding *OPRM1* SNP, exon and μ-opioid receptor protein domain

AA exchange	μ-Opioid receptor 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
V293A	TM6	877 G > A	3

See references in the text for original reports.

The μ-opioid receptor

The μ-opioid receptor is a class A rhodopsin-like GPCR, with a relatively short extracellular N-terminal domain (66 aa),

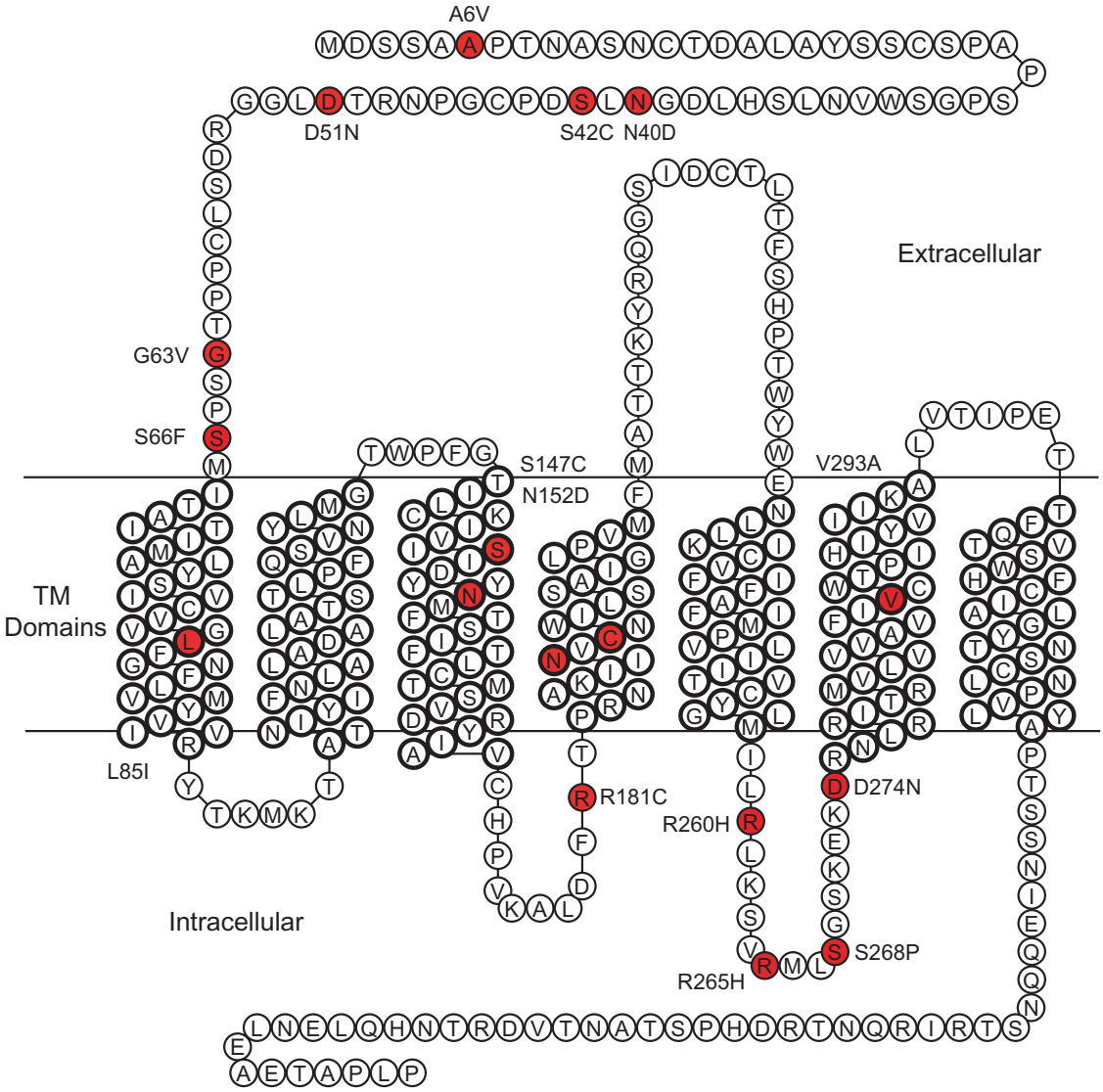

Figure 1

Naturally occurring, non-synonymous *OPRM1* variants reported, and their position on the μ-opioid receptor protein. Residues where an aa exchange occurs are indicated in red.

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 2 (Serohijos *et al.*, 2011; Manglik *et al.*, 2012). G protein interactions are mediated through intracellular domains, including intracellular loops (ICLs) 2 and 3, and the C-terminal region. The intracellular regions of the μ-opioid receptor, particularly the C-terminal domain, also contain important phosphorylation sites regulating receptor desensitization, internalization and resensitization (Williams *et al.*, 2013).

The μ-opioid receptor modulates a diverse range of physiological systems, including nociception and analgesia, reward and euphoria, immune function, stress responsivity, respiration and gut motility (Jordan and Devi, 1998; Kreek *et al.*, 2005). The most well-characterized signalling pathways of the μ-opioid receptor proceed via activation of heterotrimeric G proteins or β-arrestin (Law *et al.*, 2000). The μ-opioid receptor can couple to a number of different G proteins, including *Pertussis* toxin-sensitive $G\alpha_{i/o}$ subunits, the closely related $G\alpha_z$, and $G\alpha_{16}$ (Connor and Christie, 1999). Canonical coupling of the μ-opioid receptor includes $G\alpha_{i/o}$ inhibition of AC, Gβγ subunit activation of G protein-coupled, inwardly rectifying potassium channels (GIRKs; Alexander *et al.*, 2013b) and inhibition of voltage gated Ca^{2+} channels (Ca_V), as well as activation of MAPK. Examples of G protein-independent signalling of μ-opioid receptors include

β-arrestin-mediated ERK1/2 activation (Zheng *et al.*, 2010), signal transducer and activator of transcription 5 phosphorylation (Mazarakou and Georgoussi, 2005) and Src-mediated Ras/Raf-1 recruitment (Zhang *et al.*, 2013).

The μ-opioid receptor, like all GPCRs, has many active conformations (Pineyro and Archer-Lahlou, 2007; Kenakin and 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 stabilization 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 μ-opioid receptor 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, aa changes resulting from SNPs have the potential to affect μ-opioid receptor 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 μ-opioid receptor SNPs

Most functional studies of human (h) μ-opioid receptors 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 μ-opioid receptor 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 seems to be functional differences between μ-opioid receptor 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 μ-opioid receptor sequence is limited by the experimental conditions used to study them.

N-terminal domain SNPs

N40D

The N40D variant is the most commonly occurring *OPRM1* SNP, with an allelic frequency ranging from 10 to 50% within various populations (Mura *et al.*, 2013). The N40D SNP is in the N-terminal extracellular domain of the μ-opioid receptor, and removes one of five 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 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 the treatment (Walter and Lotsch, 2009), although the number of studies available for review was small. The D40 allele has also been associated with an increased, decreased or unchanged susceptibility to drug use and dependence (reviewed in Mague and Blendy, 2010).

Regulation of N40D expression

Regardless of any impact on the function of the μ-opioid receptor, the possibility that the nucleotide or aa substitutions may affect μ-opioid receptor expression levels needs to be considered. There is some evidence for reduced μ-opioid receptor expression associated with the G118 allele (or its murine orthologue). It was reported that in the cortex and the pons from the brains of A118G heterozygotes, there was significantly less G118 mRNA (1.5–2.5-fold) than A118 mRNA (Zhang *et al.*, 2005). 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 up-regulation of μ-opioid receptor 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.*, 2005; Oertel *et al.*, 2009). In mice, the molecular mass of μ-opioid receptors in 112G/G animals (55 kDa) is lower than 112A/A mice (62 kDa), whereas the molecular mass of deglycosylated μ-opioid receptors 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 μ-opioid receptor expression compared with A/A mice

(Magué *et al.*, 2009; Wang *et al.*, 2012). Findings of lower expression extend to cells lines (Zhang *et al.*, 2005; Huang *et al.*, 2012), with a shorter half-life of D40 (12 h) compared with N40 (28 h) in CHO cells. Enzymatic deglycosylation of the μ-opioid receptor 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 opioid requirement in D40 carriers (Mura *et al.*, 2013).

Second messenger coupling

The consequences of the N40D substitution on the signalling profile of the μ-opioid receptor 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 μ-opioid receptor SNP was a threefold increase in the affinity of β-endorphin for the D40 variant and a threefold increase in the potency of β-endorphin to activate GIRK channels co-expressed with D40 in *Xenopus laevis* oocytes (Bond *et al.*, 1998). No differences in binding or signalling were reported for other opioids including ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO), endomorphins 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 *et al.* (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 sevenfold 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 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 measure μ-opioid receptor activity after prolonged incubation with agonist, and the response measured reflects the integrated outcome of acute inhibition of AC as well as agonist-dependent uncoupling, internalization and possible recycling or degradation of the receptor, any of which could be altered by the D40 polymorphism (Connor *et al.*, 2004; Dang and Christie, 2012). Likewise, possible differences in the acute regulation of D40 and N40 variants of the μ-opioid receptor 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 Ca^{2+} channels in acutely transfected sympathetic neurons (Margas *et al.*, 2007), HEK293 cells (Lopez Soto and Raingo, 2012) as well as mice 'humanized' with A118 and G118 knock-in (Mahmoud *et al.*, 2011; Ramchandani *et al.*, 2011). Opioid receptor inhibition of Ca_V is via direct Gβγ-subunit inhibition of channel gating. In both HEK293 cells and sympathetic neurons, DAMGO inhibited Ca_V more potently in cells expressing the D40 variant, with morphine also being more potent at D40 in sympathetic neurons (Margas *et al.*, 2007; Lopez Soto and Raingo, 2012; 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 Ca_V in sympathetic neurons suggest that there may be genuine differences in N40 and D40 signalling via this pathway. By contrast, trigeminal neuron Ca_V from 'humanized' N40 and D40 mice was inhibited in an essentially identical manner by DAMGO (Ramchandani *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 the observed differences. Finally, it should be noted that the humanized N40/D40 μ-opioid receptors 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 (Ramchandani *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 PKA activity and phosphorylated ERK1/2 (pERK) levels in response to a single concentration of morphine (1 μM) applied for 5 min 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 the 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 μ-opioid receptor (Table 1). A6V is quite common in some populations, but not others, having been reported at allelic

Table 2

Summary of the key findings about μ-opioid receptor SNP signalling

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^{-1}	6.1 pmol·mg^{-1}	Befort *et al.*, 2001
	Similar cAMP inhibition. Reduced D40 expression.	9.1 – Mor 8.8 – M-6-G 7.9 – β-end	9.0 – Mor 8.8 – M-6-G 7.8 – β-end	4.8 pmol·mg^{-1}	0.63 pmol mg^{-1}	Beyer *et al.*, 2004
	Three times increased β-endorphin affinity for D40 than WT, and three times 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^{-1}	830 fmol mg^{-1}	Deb *et al.* 2010
	Similar inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.8 – DAM 8.9 – End-1 8.5 – L-Enk	Not provided	Similar to WT	Fortin *et al.*, 2010
	Decreased agonist potency to inhibit AC in D40-HEK293 and D40-AV-12 cells	8.6 – DAM 8.4 – Mor 8.3 – Meth 8.4 – β-End	8.1 – DAM* 7.8 – Mor* 7.8 – Meth* 8.1 – β-End	Not provided	66% of WT	Kroslak *et al.*, 2007
	Decreased morphine potency for Ca_V inhibition in mouse trigeminal ganglion cells expressing 'humanized' D40.	7.3 – Mor 7.2 – Fent	6.6 – Mor* 7.0 – Fent*	Not provided	Similar to WT	Mahmoud *et al.*, 2011
	Increased DAMGO and morphine potency for Ca_V inhibition at D40 expressing rat SCG cells.	7.5 – DAM 7.1 – Mor 7.1 – M-6-G 7.1 – End-1	7.8 – DAM* 7.4 – Mor* 7.1 – M-6-G 7.1 – End-1	Not provided	Not provided	Margas *et al.*, 2007
	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^{-1}	114 fmol mg^{-1}	Oertel *et al.*, 2009
	No difference in DAMGO potency at D40 for Ca_V inhibition in 'humanized' mouse trigeminal ganglion cells.	7.2 – DAM 6.3 – β-End	7.1 – DAM 6.2 – β-End	Not provided	Similar to WT	Ramchandani *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	Lopez 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 8.8 – End-1 8.4 – L-Enk	8.6 – DAM 8.7 – End-1 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 MOR1A backbone.	7.5 – DAM 7.4 – Mor	7.9 – DAM 7.3 – Mor	5.6 pmol·mg^{-1}	5.8 pmol·mg^{-1}	Ravindranathan *et al.*, 2009
S42C	Decreased agonist potency for intracellular Ca release at C42 on MOR1A backbone.	7.5 – DAM 7.4 – Mor	>6.8 – DAM* >6.8 – Mor*	2.7 pmol·mg^{-1}	5.8 pmol·mg^{-1}	Ravindranathan *et al.*, 2009
D51N	Similar inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.6 – DAM 8.8 – End-1 8.4 – L-Enk	Not provided	Similar to WT	Fortin *et al.*, 2010

Table 2

Continued

MOPr variant	Key observations	pEC_{50} WT	pEC_{50} SNP	B_{max} WT	B_{max} Var	Reference
G63V	Similar inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	9.0 – DAM 8.9 – End-1 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 8.8 – End-1 8.4 – L-Enk	8.2 – DAM* 8.3 – End-1* 7.7 – L-Enk*	Not provided	Similar to WT	Fortin *et al.*, 2010
L85I (L83I)	Increased morphine stimulated endocytosis in I83-HEK293 cells. Decreased agonist efficacy in inhibition of AC and ERK phosphorylation.	6.7 – DAM 6.7 – Mor	6.5 – DAM 6.9 – Mor	1.8 pmol mg^{-1}	2.7 pmol mg^{-1}	Cooke *et al.*, 2014
L85I	Increased morphine stimulated endocytosis in I85-HEK293 cells. Increased AC super activation in I85 HEK-293 cells. No change in agonist potency.	7.5 – DAM 7.4 – Mor	7.9 – DAM 7.7 – Mor	5.6 pmol mg^{-1}	5.2 pmol mg^{-1}	Ravindranathan *et al.*, 2009
S147C	Decreased agonist potency for inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.3 – DAM* 8.4 – End-1* 7.9 – L-Enk*	Not provided	Similar to WT	Fortin *et al.*, 2010
	Increased agonist potency for intracellular Ca release at C147 on MOR1A backbone.	7.5 – DAM 7.4 – Mor	7.9 – DAM* 8.3 – Mor*	5.6 pmol·mg^{-1}	5.0 pmol·mg^{-1}	Ravindranathan *et al.*, 2009
N152D	Decrease in morphine affinity for D152 in COS cells.	N/A	N/A	5.5 pmol·mg^{-1}	1.9 pmol·mg^{-1}	Befort *et al.*, 2001
R181C	HEK-293 cells expressing C181 failed to signal via DAMGO or morphine.	7.5 – DAM 7.4 – Mor	N/A	5.6 pmol·mg^{-1}	3.5 pmol·mg^{-1}	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 MOR1A backbone.	7.5 – DAM 7.4 – Mor	>6.8 – DAM* >6.8 – Mor*	5.6 pmol·mg^{-1}	4.4 pmol·mg^{-1}	Ravindranathan *et al.*, 2009
R260H	Decreased basal GTPγS activity at H260 in HEK293 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^{-1}	3.9 pmol·mg^{-1}	Wang *et al.*, 2001
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^{-1}	4.6 pmol·mg^{-1}	Befort *et al.*, 2001
	Decreased agonist potency for inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.0 – DAM* 8.1 – End-1* 7.6 – L-Enk*	Not provided	Similar to WT	Fortin *et al.*, 2010
	Decreased basal GTPγS activity at H265 in HEK293 cells. Slight decrease in maximal morphine stimulated GTPγS at H265. Decreased affinity of H265 for CaM binding, and decreased desensitization following morphine pretreatment.	8.4 – Mor	8.5 – Mor	3.5 pmol·mg^{-1}	4.2 pmol·mg^{-1}	Wang *et al.*, 2001

Table 2

Continued

MOPr variant	Key observations	pEC_{50} WT	pEC_{50} SNP	B_{max} WT	B_{max} Var	Reference
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 6.5 – β-End 6.2 – Mor	6.4 – DAM* 5.9 – β-End* 5.8 – Mor*	5.5 pmol·mg^{-1}	3.6 pmol·mg^{-1}	Befort *et al.*, 2001
	Decreased potency of DAMGO and endomorphin-1 for inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.2 – DAM* 8.4 – End-1* 7.9 – L-Enk	Not provided	Similar to WT	Fortin *et al.*, 2010
	Decreased GTPγS binding, slower desensitization and decreased AC inhibition in response to DAMGO.	N/A	N/A	643 fmol·mg^{-1}	340 fmol·mg^{-1}	Koch *et al.*, 2000
	Decreased morphine potency at P268 for inhibition of cAMP accumulation.	7.0 – Mor	6.3 – Mor*	3.5 pmol·mg^{-1}	4.5 pmol·mg^{-1}	Wang *et al.*, 2001
D274N	Increased agonist potency for inhibition of cAMP-stimulated CRE transcription.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	9.1 – DAM* 8.3 – End-1* 8.6 – L-Enk*	Not provided	Similar to WT	Fortin *et al.*, 2010
V293I	Unchanged in agonist potency for inhibition of cAMP accumulation.	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	8.8 – DAM 8.8 – End-1 8.4 – L-Enk	Not provided	Similar to WT	Fortin *et al.*, 2010

*$P < 0.05$, from original publications. Abbreviations: β-End, β-endorphin; DAM, DAMGO; End-1, endomorphin 1; Fent, fentanyl; L-ENK, [Leu]^{5}enkephalin; Meth, methadone; Mor, morphine; MOR1A, μ-opioid receptor 1A splice variant; SCG, superior cervical ganglion.

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; Comptom *et al.*, 2003; Crowley *et al.*, 2003).

There are no studies comparing acute A6 and V6 signalling on the predominant isoform of the μ-opioid receptor. In an assay of cAMP-dependent gene transcription, no difference in potency was found for DAMGO, endomorphin 1 or leu-enkephalin in HEK293 cells expressing A6 and V6 (Table 2, Fortin *et al.*, 2010). The A6V variant was studied on the MOR1A splice variant sequence expressed in HEK293 cells, where DAMGO but not morphine showed a higher maximum effect at V6- than A6-MOR1A in an assay of intracellular Ca release catalysed by a transiently transfected chimeric G protein. No differences in internalization 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 the μ-opioid receptor are unclear, but suggest that further work is warranted.

S42C, D51N, G63V and S66F

Other rare polymorphisms within the N-terminal domain of the μ-opioid receptor 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.* 2008) and examined in a cAMP-dependent gene transcription assay (Fortin *et al.*, 2010). Neither D51N nor G63V showed any differences to wild-type (WT) μ-opioid receptors 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).

TM domain SNPs

L85I (TM1)

The TM helices of μ-opioid receptors 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 orthologue) activity; however, morphine displays an enhanced capacity to promote internalization 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 internalization of both variants, suggesting that they may form functional dimers (Ravindranathan *et al.*, 2009).

Previous studies have shown that the WT-μ-opioid receptor 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 desensitization 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-μ-opioid receptor has been shown to be a poor substrate for GPCR kinase (GRK) subtypes 2/3 phosphorylation, which is required for endocytosis (Doll *et al.*, 2012), internalization of the I83 μ-opioid receptor 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 μ-opioid receptor endocytosis (El Kouhen *et al.*, 2001), and while morphine induces phosphorylation of the S375 residue on the C-terminus of the μ-opioid receptor, DAMGO also efficiently stimulates phosphorylation of T370 after S375 phosphorylation, resulting in receptor internalization (Doll *et al.*, 2011; Grecksch *et al.*, 2011). Morphine-stimulated internalization of I83 was not due to enhanced phosphorylation of S375 compared with the WT-μ-opioid receptor, but T370 phosphorylation was not investigated (Cooke *et al.*, 2014). The observations that the I83/85 μ-opioid receptor show apparently decreased signalling efficacy compared with enhanced receptor trafficking in response to morphine increase 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 μ-opioid receptor 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 μ-opioid receptor activation, one of the most well described of these is the up-regulation 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 internalization (Wang *et al.*, 2003). Ravindranathan *et al.* tested the I85 variant for changes in AC superactivation. Cells expressing the L/I85 μ-opioid receptor variant were chronically treated with morphine for 14 h. Upon morphine withdrawal, cells expressing the I85 μ-opioid receptor showed a significantly lower level of cAMP compared with WT cells (2.5- and 1.5-fold cAMP levels of morphine naive cells respectively). Upon a 4 h 'acute' rechallenge with 10 nM morphine, cAMP levels were again significantly lower in the I85 expressing cells, indicating a reduction in AC superactivation and morphine tolerance.

S147C and N152D (TM3)

Computational modelling and X-ray crystallography studies have shown TM domains 3, 5 and 6 to be of particular importance in the formation of the ligand-binding pocket of the μ-opioid receptor (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.*, 2001; 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 WT-μ-opioid receptor backbone, C147 was modestly less effective at supporting agonist-mediated inhibition of cAMP-dependent gene transcription (Fortin *et al.*, 2010). Whether this discrepancy is because of the different receptor backgrounds or whether it hints at a reciprocal change in the capacity of μ-opioid receptors 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).

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 aa exchange in μ-opioid receptors. I293 was reported to signal in an identical manner to V293 (Fortin *et al.*, 2010) and there is no clinical information about this phenotype.

ICL SNPs

The ICL domains of the μ-opioid receptor 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 internalization (Lefkowitz, 1998). GPCRs with the ICL2 and ICL3 domains deleted cannot couple to G proteins but can retain 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 μ-opioid receptor agonist efficacy, and also to increase the constitutive activity of μ-opioid receptors (Li *et al.*, 2001; Clayton *et al.*, 2010).

Mutations in ICL2 have also been shown to affect receptor uncoupling and desensitization (Celver *et al.*, 2001; 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, 1999). Mutations within the ICL3 of the μ-opioid receptor 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 and 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 mobilization 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-chlornaltrexamine (β-CNA; 10 μM, 18 h) increased cell surface receptor expression, with naltrexone treatment producing levels similar to WT-μ-opioid receptor. 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 (Petäjä-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 aberrant 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 μ-opioid receptors 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-μ-opioid receptors, 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 Ca^{2+}/calmodulin (CaM)-dependent PK II (CaMK II) phosphorylation site (Koch *et al.*, 2000) and insertion of an aa, proline, that is likely to significantly disrupt the structure of ICL3. Most studies have found that P268 or the rat orthologue 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 the μ-opioid receptor affect the signalling of the receptor, but changes in the signalling profile of the μ-opioid receptor resulting from ICL3 SNPs are likely to be expressed in situations other than acute μ-opioid receptor signalling because ICL domains of GPCRs interact with effectors involved in receptor regulation and adaptive processes such as receptor down-regulation (Lefkowitz, 1998). The ICL3 domain of the μ-opioid receptor has multiple consensus phosphorylation sites, as well as a putative CaM-binding domain (Wang *et al.*, 1999; Koch *et al.*, 2000). Sustained exposure to high concentrations of agonist produces down-regulation of receptor protein in cell lines, and the ICL3 variants R260H, R265H and S268P were down-regulated (~80%) to a similar degree to WT receptors by 10 μM DAMGO (Befort *et al.*, 2001). Functionally, P268 μ-opioid receptor-mediated inhibition of AC desensitized with a similar time course to P268, while desensitization of P268-mediated activation of GIRK was slower and incomplete when compared with P268 when the proteins were expressed in *Xenopus* oocytes (Koch *et al.*, 2000).

In addition to phosphorylation sites, μ-opioid receptor ICL3 contains a putative CaM-binding site. It has been sug-

gested that CaM competes with G protein coupling at ICL3, and regulates basal μ-opioid receptor signalling (Wang *et al.*, 1999). Wang *et al.* (2001) investigated the interaction of CaM with μ-opioid receptor ICL3 domains by incubating CaM with short peptides derived from ICL3 sequences as well as full-length μ-opioid receptors purified from transfected HEK293 cells. The ICL3 H260 peptide showed a 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 μ-opioid receptor variants bound to CaM. The broader significance of these findings has 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-μ-opioid receptors in HEK293 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 μ-opioid receptor activation in many studies do not reflect acute, real-time, naturalistic signalling of the μ-opioid receptor. The μ-opioid receptor undergoes rapid desensitization and internalization following agonist exposures of 5–10 min (Connor *et al.*, 2004). Thus, the reporter gene assays used for facile quantification of μ-opioid receptor function measure the summed effects of μ-opioid receptor activation, desensitization, internalization and resensitization, 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.

The μ-opioid receptor is expressed in a wide variety of human cell types, and subtle changes in μ-opioid receptor 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 μ-opioid receptor 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 μ-opioid receptor 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 μ-opioid receptor 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 μ-opioid receptors 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-CAN, 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 characterization 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 and 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 μ-opioid receptor signalling pathways has barely been touched on, as have possible ligand-specific changes in this coupling. Several

studies have examined the trafficking of μ-opioid receptor variants in response to a limited range of agonists (Ravindranathan *et al.*, 2009; Cooke *et al.*, 2014), but the effect of μ-opioid receptor SNPs on the rapid desensitization of signalling that precedes receptor internalization remains unknown. The way in which μ-opioid receptor SNPs may affect the occurrence or function of putative μ-opioid receptor dimers has received limited attention (Ravindranathan *et al.*, 2009), even though most carriers of variant μ-opioid receptor alleles will be heterozygous for the WT receptor.

Understanding how μ-opioid receptor SNPs affect cellular signalling is important for predicting the potential clinical or phenotypic consequences of these variants in humans. However, understanding other aspects of μ-opioid receptor function such as the regulation of gene expression in response to environmental or epigenetic factors, and the function of μ-opioid receptors in the wide range of human cells that normally express it, are equally important and more difficult to achieve. Nevertheless, understanding the consequences of expressing a particular μ-opioid receptor 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 the μ-opioid receptor, but a key element would be knowing what version of the μ-opioid receptor a patient had, and knowing which of the many opioid analgesics available had the best pharmacodynamic profile at that variant.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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