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RESEARCH PAPER

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

Alisa Knapman, Marina Santiago and Mark Connor

Australian School of Advanced Medicine, Macquarie University, Sydney, NSW, Australia

Correspondence
Mark Connor, 2 Technology Place, Macquarie University, NSW 2109, Australia. E-mail: mark.connor@mq.edu.au

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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 (MOP receptor) 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 MOP receptor signalling at several signalling pathways using a range of structurally distinct opioid ligands in cells expressing wild-type MOP receptors (MOPr-WT) and the commonly occurring MOP receptor variant, N40D.

EXPERIMENTAL APPROACH

MOPr-WT and MOPr-N40D were stably expressed in CHO cells and in AtT-20 cells. Assays of AC 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 AC inhibition and ERK1/2 phosphorylation. Buprenorphine potency was reduced threefold at MOPr-N40D for K channel activation. Pentazocine efficacy was reduced by 50% for G-protein-gated inwardly rectifying K channel 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 MOP receptors is impaired, and this may have important consequences in a clinical setting for individuals carrying the N40D allele.

Abbreviations

DAMGO, ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin); FSK, forskolin; GIRK, G protein-gated inwardly rectifying K channel; MOP receptor, μ-opioid receptor; *OPRM1*, μ1 opioid receptor gene; PTX, *Pertussis* toxin

Table of Links

TARGETS	LIGANDS
μ opioid receptor	DAMGO
	buprenorphine
	β-endorphin
	endomorphin-1
	endomorphin-2
	fentanyl
	naloxone
	methadone
	morphine
	oxycodone
	pentazocine

This Table lists protein targets and ligands which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

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 and Geisslinger, 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 *et al.*, 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 (MOP receptor, Cox *et al.*, 2014) is a GPCR that is the main site of action for clinically important opioids. The MOP receptor mediates the analgesic and almost all of the adverse effects of these opioid drugs, and it is likely that the genetic variation of *OPRM1*, the gene coding for MOP receptors, 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 and Geisslinger, 2005). The N40D variant is the most common MOP receptor 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 MOP receptor, and removing a putative glycosylation site (Singh *et al.*, 1997).

Many studies have investigated the association between carriers of MOP receptor-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 and 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 and Lotsch, 2009).

Fewer studies have investigated the consequences of the N40D variant on MOP receptor 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 MOP receptors 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 wild-type (WT)-MOP receptors (Befort *et al.*, 2001; Beyer *et al.*, 2004; Kroslak *et al.*, 2007). Other studies have reported both increased and decreased DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) 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 the MOP receptor interacts with many effector and regulatory proteins, changing assay parameters and cellular background may affect MOP receptor 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 GPCRs, the MOP receptor has many active conformations, and in the absence of ligand constantly oscillates through a range of possible active and inactive states (Pineyro and Archer-Lahlou, 2007; Kenakin and Miller, 2010; Manglik *et al.*, 2012). Ligand-biased signalling or functional selectivity has been well characterized, where structurally distinct ligands stabilize 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 MOP 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. Clinically used opioids are chemically diverse, and are likely to have subtle differences in their interaction with structural features of the MOP receptor, 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 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 effect 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

MOP receptor 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 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 synthesized by Genscript (Piscataway, NJ, USA). Cells expressing MOPr-WT or MOPr-N40D were selected using hygromycin B (500 μg·mL^{-1}). Cells were cultured in DMEM containing 10% FBS, 100 U penicillin/streptomycin and 500 μg·mL^{-1} hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 μg·mL^{-1} beyond passage 5.

AtT-20 FlpIn cells were constructed by transfecting Flp-recombinase target site (FRT)/LacZeo2 (Life Technologies, Mulgrave, VIC, Australia) using Fugene. Successfully transfected cells were selected with 100 μg·mL^{-1} zeocin, and stably transfected with MOPr-WT or MOPr-N40D as described for CHO cells. Cells expressing MOP receptors were selected using 100 μg·mL^{-1} hygromycin B. Cells were cultured in DMEM containing 10% FBS, 100 U penicillin/streptomycin and 100 μg·mL^{-1} hygromycin B.

CHO-MOP receptor cells and AtT-20-MOP receptor 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.

MOP receptor expression

Surface expression of MOP receptors was determined on intact CHO-MOP receptor cells by incubation with 0.125–16 nM [^{3}H]-DAMGO (D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; PerkinElmer, Waltham, MA, USA) at 4°C in 50 mM Tris-Cl (pH 7.4) for 2 h. Briefly, 24 h before the assay, cells were detached from flasks with trypsin/EDTA (Sigma, Castle Hill, NSW, Australia) and resuspended in DMEM containing 10% FBS, 100 U penicillin/streptomycin, plus 2 μg·mL^{-1} tetracycline to induce MOP receptor expression. Cells were seeded at a density of 1 × 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 unlabelled 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 RT with 100 μL of 1N NaOH. Then, 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). 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, USA). 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 AC inhibition

Opioid inhibition of AC was measured using an assay of membrane potential (Knapman *et al.*, 2014). The day before the assay, CHO-MOP receptor 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, 100 U penicillin/streptomycin and 15 mM glucose. MOP receptor expression was induced with 2 μg·mL^{-1} tetracycline 24 h before the assay. The cells were plated in a volume of 90 μL in black-walled, clear-bottomed 96-well microplates (Corning, Tewksbury, MA, USA), and incubated overnight at 37°C in ambient CO_2. Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices (Sunnyvale, CA, USA). The dye was reconstituted with assay buffer (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 per well of the dye solution without removal of the L-15, giving an initial assay volume of 180 µL per well. Plates were then incubated at 37°C in ambient CO_2 for 60 min. 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 s for at least 2 min, 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-MOP receptor cells were detached from flasks and plated using the same procedure as for the CHO-MOP receptor 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 s for at least 2 min, 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 s 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 ERK1/2 was measured by ELISA. The day before the assay, CHO-MOP receptor cells were plated and receptor expression was induced as for the membrane potential assay. Cells were plated in 96-well clear microplates (Falcon, Tewksbury, MA, USA). On the day of the assay, cells were deprived of serum for 1 h in 40 µL serum-free L-15 supplemented with 5% BSA. All cell treatments were in a volume of 40 µL unless stated otherwise. Serum-deprived 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 desensitization, 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 room temperature (RT). Cells were washed three 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 h 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 (Thr^{202}/Tyr^{204}) antibody in PBS-T with 1% BSA. Cells were washed three 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 h. Cells were washed four 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 (BMG Labtech, Mornington, VIC, Australia). Cells were then stained with 0.5 $\mu g \cdot mL^{-1}$ DAPI for 10 min at RT, and washed three 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 normalized to DAPI staining to account for differences in cell density between wells. Readings were then normalized to the response of cells treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 10 min.

Drugs and chemicals

Unless otherwise stated, tissue culture reagents and buffer salts were from Life Technologies 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. Fentanyl (Andrews Laboratories) was a gift from the Department of Pharmacology, Sydney University. FSK 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 (Catalogue #9101) and anti-rabbit IgG HRP-lined antibody (Catalogue #7074) were from Cell Signaling Technologies, Danvers, MA, USA.

Data

Unless otherwise noted, data are expressed as mean ± SEM of at least five determinations made in duplicate or triplicate. Concentration-response curves were fit with a four parameter logistic equation using Graphpad Prism (Graphpad). E_{max} and pEC_{50} values were derived from individual experiments and compared using Student's unpaired *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 are 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

MOP receptor 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 MOP receptor expression with tetracycline, specific binding of [^{3}H]-DAMGO was similar for both variants, indicating similar

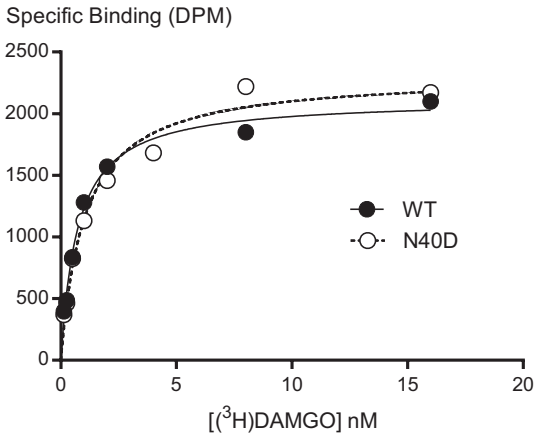

Figure 1

Saturation binding curve of [^{3}H]-DAMGO in intact CHO-MOPr-WT and CHO-MOPr-N40D cells, 24 h 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 ± SEM of triplicate determinations from a single experiment. The assay was repeated three times.

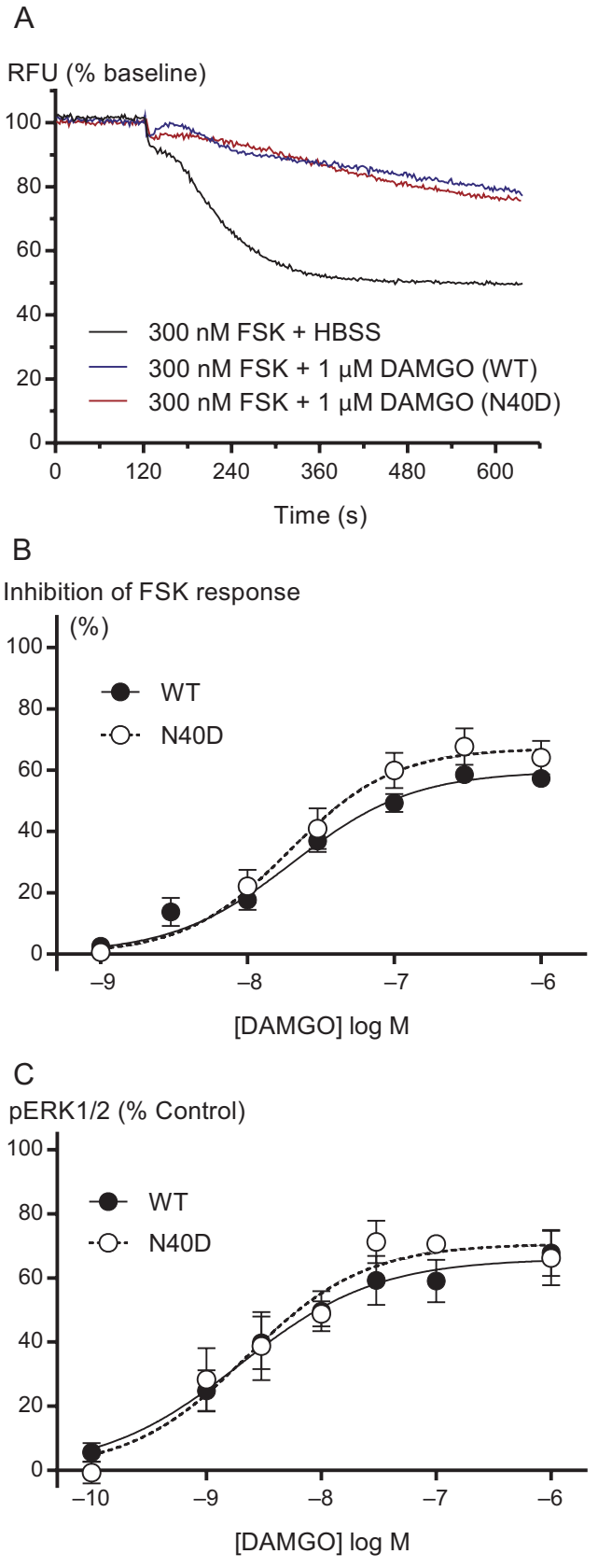

Figure 2

DAMGO inhibits AC and activates ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D. AC 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 FSK + vehicle (HBSS) to MOPr-WT, and 300 nM FSK + 1 μM DAMGO to MOPr-WT and MOPr-N40D cells. Drugs were added at 120 s. Changes in raw fluorescent units (RFU) are normalized to predrug values. (B) DAMGO inhibited FSK-stimulated AC hyperpolarization 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 ± SEM of pooled data from five to six independent determinations performed in duplicate.

levels of surface receptor expression (see Figure 1). The B_{max} for CHO-MOPr-WT cells was 280 ± 20 fmol·mg^{-1} total protein and 356 ± 18 fmol·mg^{-1} 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).

AC inhibition

In CHO-MOP receptor cells loaded with membrane potential dye, addition of the AC activator FSK (300 nM) produced a rapid decrease in fluorescence, consistent with hyperpolarization of the cells (Figure 2A; Knapman *et al.*, 2014). The hyperpolarization stabilized 5 min after the addition of FSK, at which point measurements were taken. FSK (300 nM) produced a similar hyperpolarization in CHO-MOPr-WT (42 ± 1% decrease in fluorescence) and CHO-MOPr-N40D (44 ± 2%

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} (%)		pEC_{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***	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 methadone are set in bold (one-way ANOVA, followed by Student's *t*-test, corrected for multiple comparisons, $P < 0.05$). *E_{max} or pEC_{50} significantly different between MOPr-WT and MOPr-N40D ($P < 0.05$).

decrease) cells. ($P > 0.05$). Simultaneous addition of the prototypical MOP receptor selective peptide agonist DAMGO with FSK resulted in a concentration-dependent inhibition of the FSK-stimulated hyperpolarization 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 PTX (Knapman *et al.*, 2014). Application of opioids alone did not affect the membrane potential of CHO-MOP receptor 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 (Nguyen *et al.*, 1998; Matsui and Williams, 2010).

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-MOP receptor or the N40D variant to determine whether the N40D amino acid substitution could affect MOP receptor signalling in a ligand-selective manner. The endogenous opioid β-endorphin has previously been shown to have a threefold 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 was 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 MOP receptors could have accentuated a general difference in transduction efficiency between MOPr-WT and the MOPr-N40D. However, the low efficacy MOP receptor 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).

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 MOP receptors, stimulation of ERK1/2 phosphorylation by MOP receptors. 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 normalized against 100 nM PMA applied for 10 min. 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

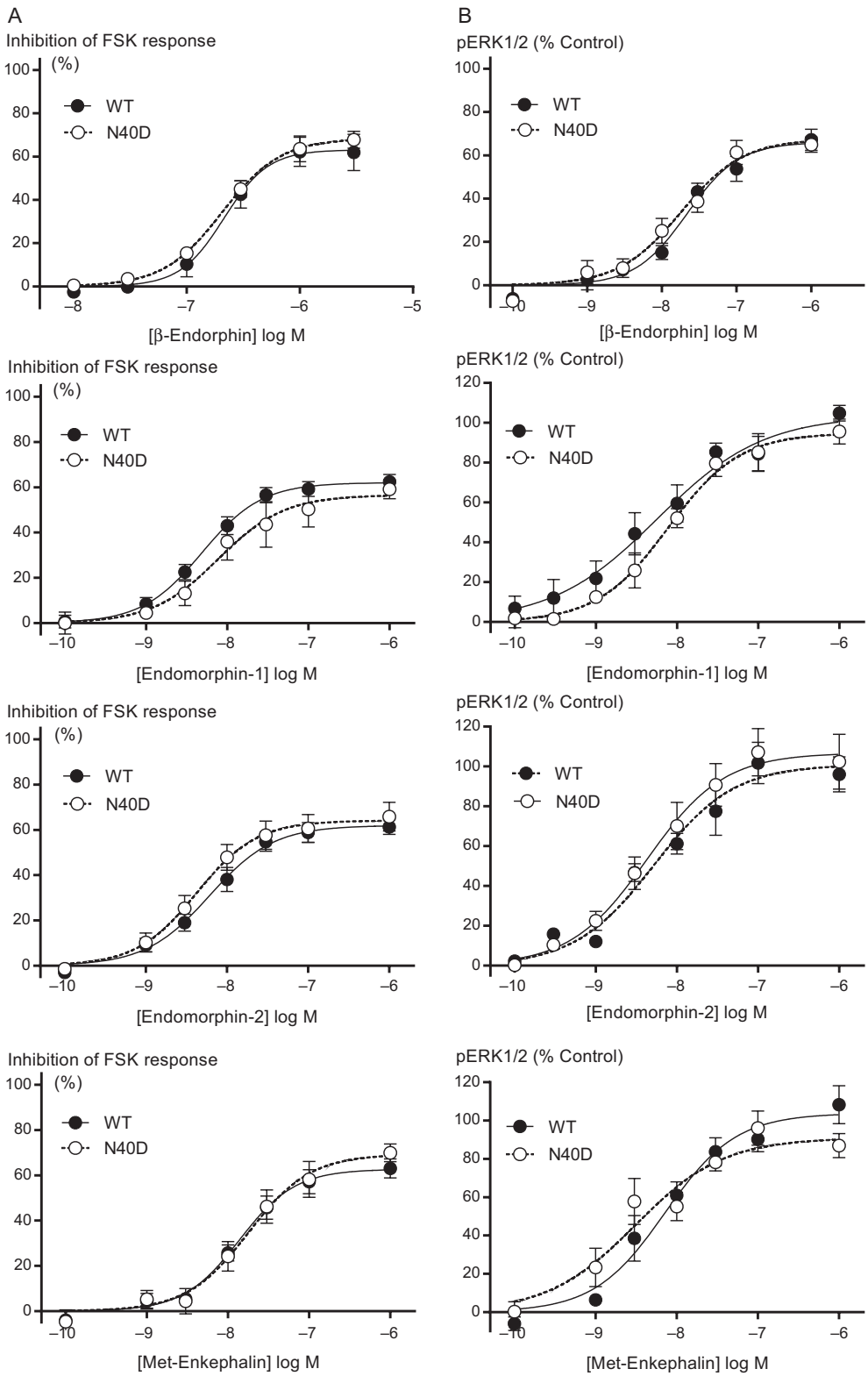

Figure 3

Endogenous opioids inhibit AC and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D. AC inhibition and levels of ERK phosphorylation were determined as described in the Methods. (A) β-endorphin, endomorphins-1 and -2, and met-enkephalin inhibited FSK-stimulated AC hyperpolarization of MOPr-WT or MOPr-N40D to a similar degree and with a similar potency ($P > 0.05$). (B) β-endorphin, endomorphi-1 and -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 ± SEM of pooled data from five to six independent determinations performed in duplicate.

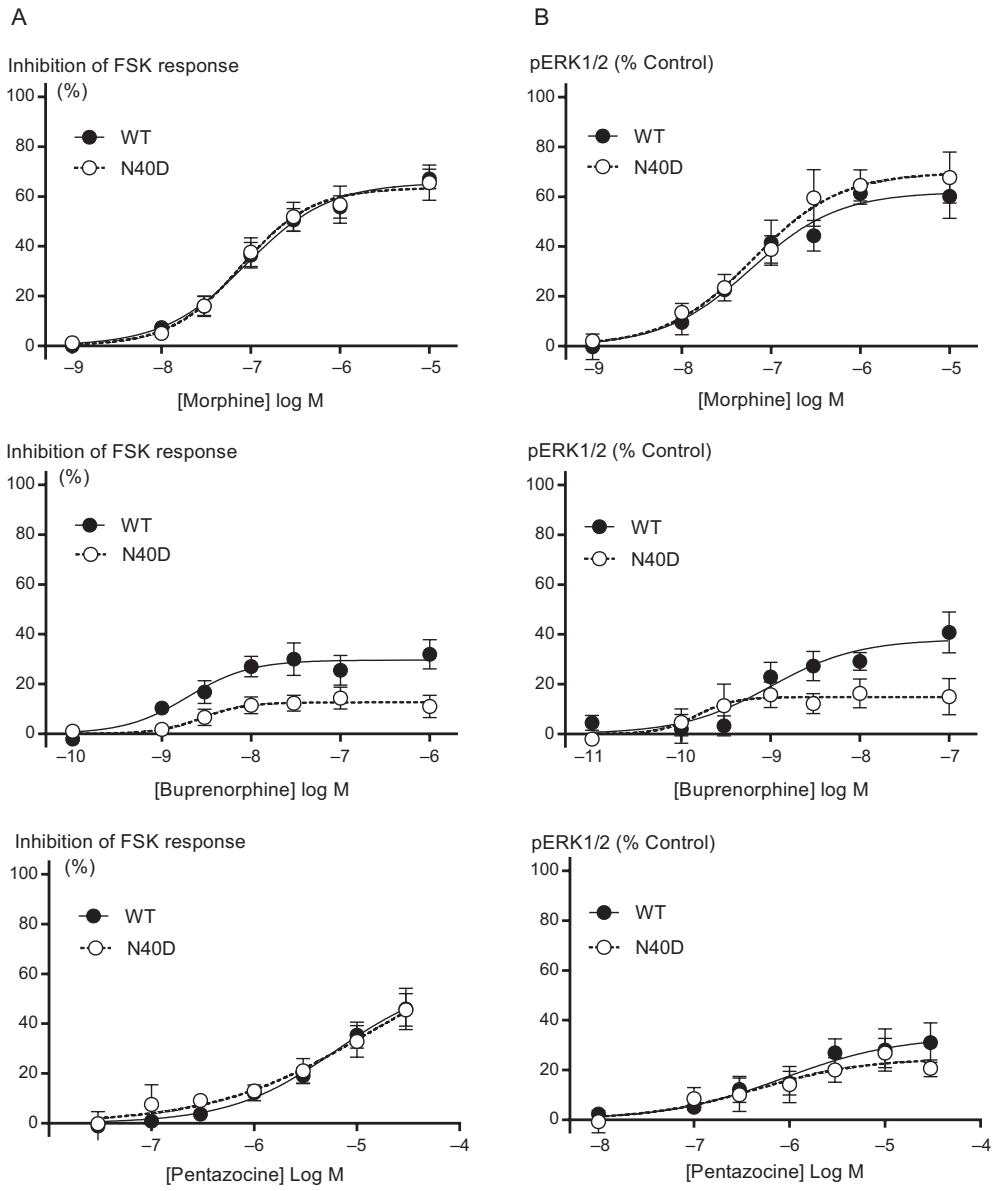

Figure 4

Buprenorphine inhibits AC and activates ERK1/2 less effectively in CHO cells expressing MOPr-N40D. AC inhibition and levels of ERK phosphorylation were determined as described in the Methods. (A) Buprenorphine E_{max} for inhibition of FSK-stimulated AC hyperpolarization was decreased from 35 ± 6 % in CHO-MOPr-WT to 16 ± 4% in CHO-MOPr-N40D ($P < 0.05$). Morphine and pentazocine inhibited AC hyperpolarization 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 ± SEM of pooled data from five to six independent determinations performed in duplicate.

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 E_{max} of 72 ± 5% in CHO-MOPr-N40D (Figure 2, Table 2). Pre-incubation of cells with 1 μM naloxone for 5 min blocked the response for all opioids tested. Application of DAMGO to cells treated overnight with 200 ng·mL^{-1} 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 MOP receptor 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

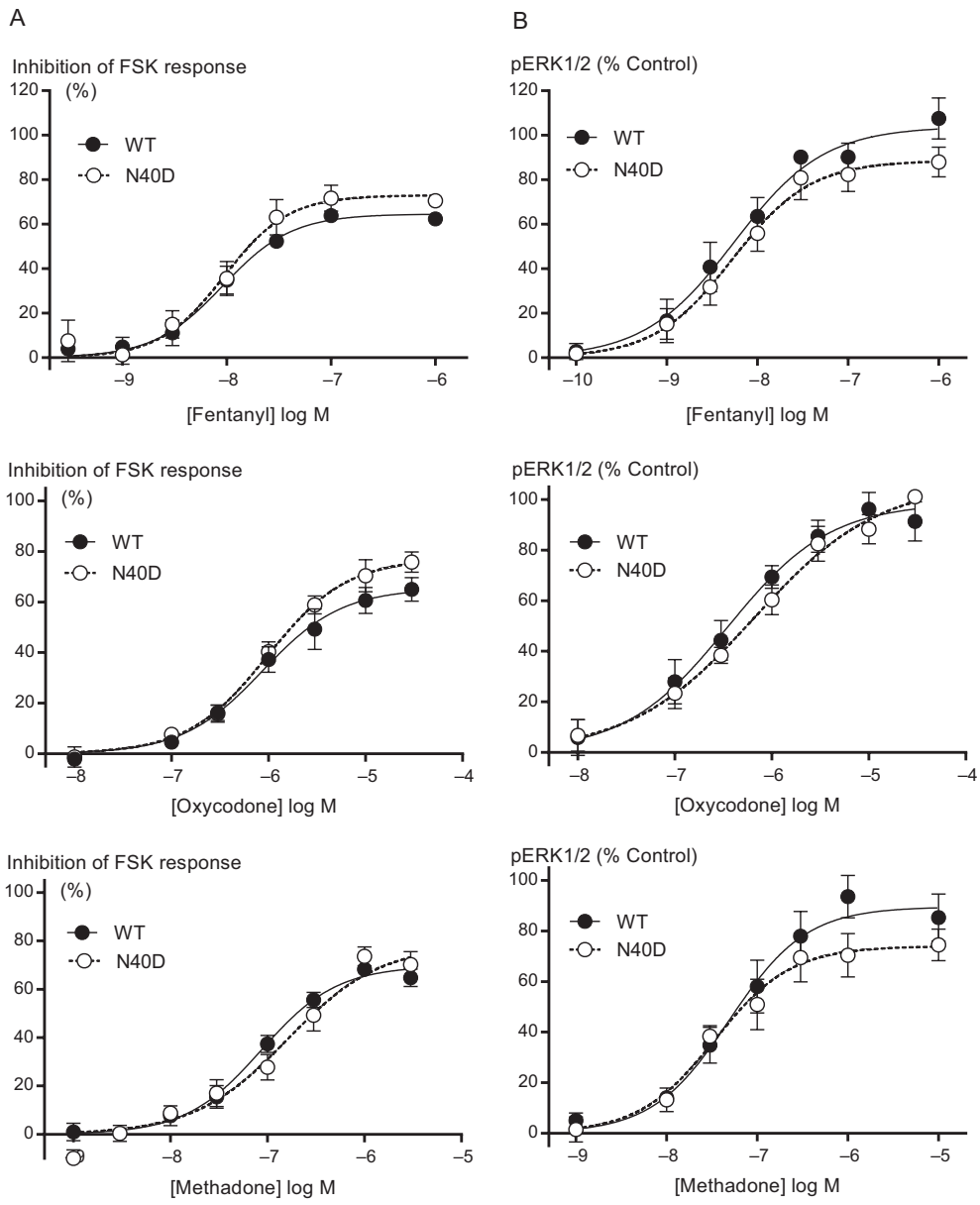

Figure 5

Fentanyl, oxycodone and methadone inhibit AC and activate ERK1/2 in CHO cells expressing MOPr-WT or MOPr-N40D. AC inhibition and levels of ERK phosphorylation were determined as described in the Methods. (A) Fentanyl, oxycodone and methadone inhibited FSK-stimulated AC hyperpolarization 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 ± SEM of pooled data from five to six independent determinations performed in duplicate.

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 β-endorphin for both MOPr-WT and MOPr-N40D. DAMGO had a significantly lower E_{max} than β-endorphin to inhibit AC in MOPr-WT but not in 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

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} (%)		pEC_{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***	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 set in bold (one-way ANOVA, followed by Student's *t*-test, corrected for multiple comparisons, $P < 0.05$). *E_{max} or pEC_{50} significantly different between MOPr-WT and MOPr-N40D ($P < 0.05$).

this assay, endomorphin-2. Morphine had a significantly lower E_{max} than endomorphin 2 to stimulate ERK1/2 phosphorylation in MOPr-WT but not in 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 MOP receptors 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 hyperpolarization from GIRK activation (Figure 6). Sub-maximal membrane hyperpolarization produced by low-efficacy agonists such as buprenorphine or low concentrations of high-efficacy agonists such as DAMGO rapidly returned towards baseline within 2 min after addition; however, maximum membrane hyperpolarization 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 pEC_{50} 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 ($P < 0.05$).

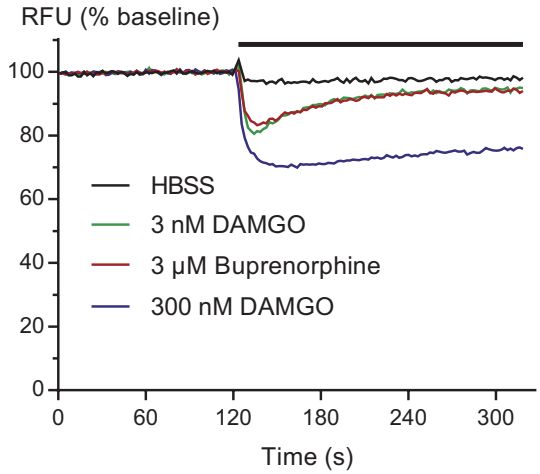

Figure 6

DAMGO causes membrane hyperpolarization in AtT-20 cells expressing MOPr-WT. Raw trace showing decrease in fluorescent signal following application of vehicle (HBSS), 3 nM DAMGO, 300 nM DAMGO or 3 μM buprenorphine to AtT20 cells expressing MOPr-WT, corresponding to membrane hyperpolarization 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 six individual experiments, each performed in duplicate.

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,

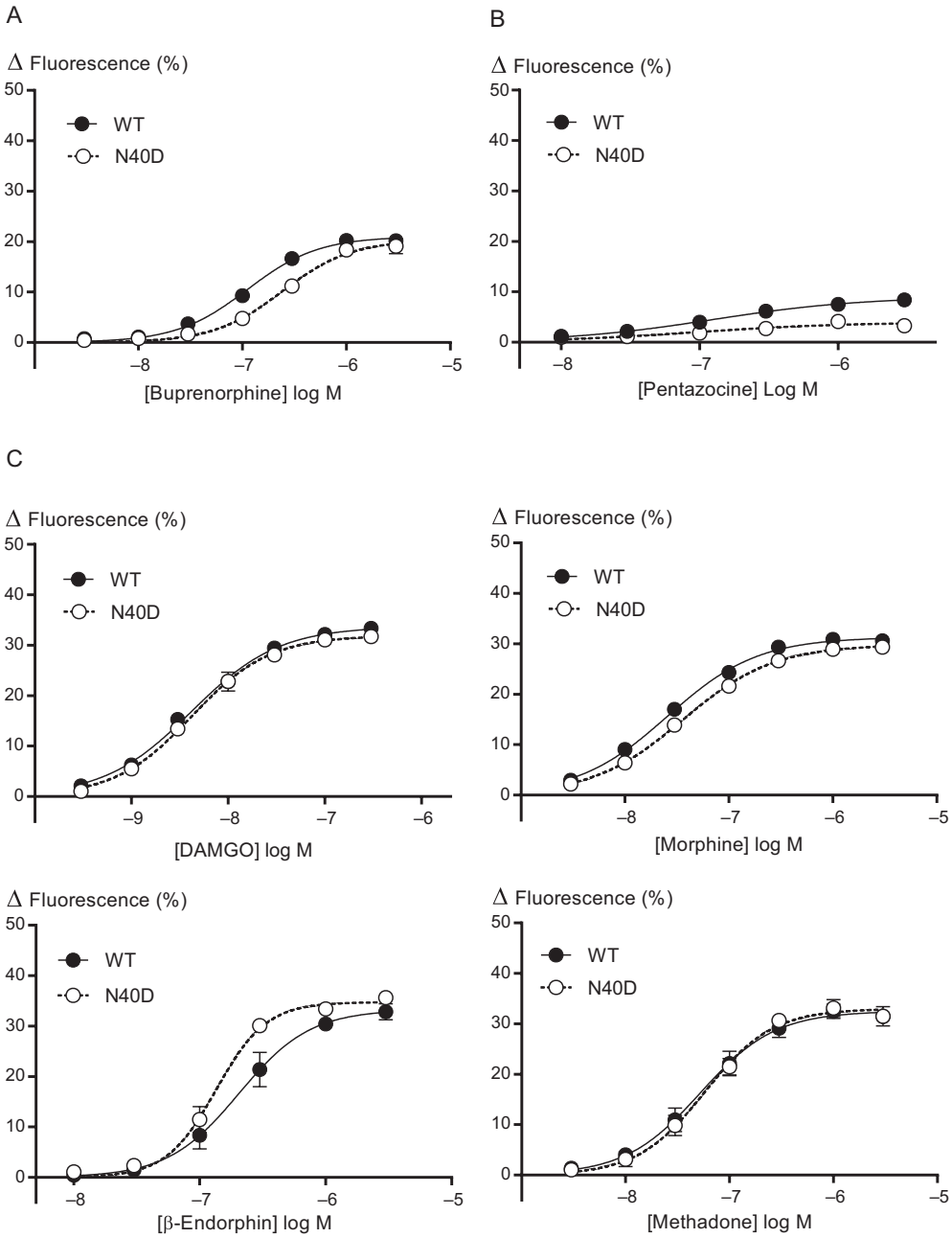

Figure 7

Buprenorphine is less potent at activating GIRK in AtT20 cells expressing MOPr-N40D than in those expressing MOPr-WT. 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 a 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 ± SEM of pooled data from five to six independent determinations performed in duplicate.

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.

Discussion and conclusions

We have shown that the commonly occurring MOP receptor 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 AC inhibition and ERK1/2 phosphorylation in CHO-MOPr

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*
Pentazocine	**8 ± 1**	**4 ± 1***	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 set in bold (one-way ANOVA, followed by Student's *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).

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 three-fold. 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 (Zhang *et al.*, 2005; Kroslak *et al.*, 2007; Mague *et al.*, 2009; Oertel *et al.*, 2012). MOP receptor expression was similar for both variants in the cell lines used in our assays. The N40D variant causes the removal of a putative N-linked glycosylation site, which has been shown to result in decreased MOP receptor glycosylation and stability in CHO cells (Huang *et al.*, 2012). In our study, MOP receptor 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 the MOP receptor may affect the ability of some opioid ligands to effectively transduce signals to the intracellular effectors of MOP receptor. GPCRs constantly oscillate through a range of possible conformations, and different ligands can more effectively stabilize a subset of receptor conformations (Kenakin and 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 and Archer-Lahlou, 2007). The involvement of the N-terminal domain in MOP receptor 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 MOP receptor and other GPCRs. Antibodies generated against the region proximal to N40D on the N-terminal domain of MOP receptor show differential recognition of activated receptors, suggesting this region undergoes conformational changes upon ligand-binding (Gupta *et al.*, 2007; 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 the MOP receptor, implying that conformational changes associated with receptor coupling to associated G-proteins may influence all domains of the MOP receptor. A similar effect was seen in other GPCRs examined in this study, including the δ-opioid receptor and the cannabinoid CB_1 receptor (Gupta *et al.*, 2007). N-terminal polymorphisms affect signalling in other related GPCRs. N-terminal single nucleotide polymorphisms (SNPs) in the $5\text{-}HT_{2B}$ 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 MC_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 MOP receptors, or affect the efficacy of ligand-directed MOP receptor 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. MOP receptor 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, 2005). Whereas AC inhibition is mediated by a single Gα subunit, GIRK channels require the binding of four 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 (Margas *et al.*, 2007; Lopez Soto and Raingo, 2012) via N40D, no change in DAMGO signalling (Ramchandani *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 CHO cells expressing MOPr-WT and MOPr-N40D 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.

It is interesting to note that DAMGO and morphine had similar efficacies to promote ERK phosphorylation in MOPr-WT cells, but that this efficacy was significantly less than for endomorphin 2 and appeared less than several other ligands such as met-enkephalin and oxycodone. There is little, if any, data examining the efficacy for different MOP receptor ligands activating ERK or comparing efficacy of ERK activation with other effectors. The apparently high efficacy for ERK phosphorylation of endomorphin 2 and oxycodone bears further examination in light of the increasing interest in signalling bias at MOP receptors, particularly as endomorphin-2 has been identified as a significantly biased agonist for recruitment of arrestin3 over G-protein activation (Rivero *et al.*, 2012), and either pathway can potentially contribute to ERK activation. Many previous studies and our preliminary time course data indicated that μ-opioid activation of ERK peaks at around 5 min, the time point we chose for our assay. However, it is possible that subtle differences in the time course of ERK activation mean that DAMGO efficacy is slightly underestimated at 5 min compared with some other ligands.

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 MOP receptor 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 homozygous and heterozygous carriers (e.g. Bond *et al.*, 1998; Bortsov *et al.*, 2012; Song *et al.*, 2013; Solak *et al.*, 2014), and for the few studies that consider both genotypes the data are 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 MOP receptors and other Class A GPCR in native tissue (Herrick-Davis *et al.*, 2013; Malik *et al.*, 2013), and cells expressing both N40 and D40 receptors could have two 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 MOP receptor-L85I variant that undergoes endocytosis in response to morphine were co-expressed in HEK293 cells. Both MOPr-WT and MOPr-L85I internalized 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 internalized 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 MOP receptor 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 MOP receptor variants and buprenorphine, and our data suggest 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 MOP receptor function across cell types and signalling pathways. Further functional studies examining other MOP receptor 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 to 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.

Conflict of interest

The authors have no conflicts of interest.

References

Abrol R, Kim SK, Bray JK, Trzaskowski B, Goddard WA 3rd (2013). Conformational ensemble view of G protein-coupled receptors and the effect of mutations and ligand binding. Methods Enzymol 520: 31–48.

Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M *et al.* (2013). The concise guide to PHARMACOLOGY 2013/14: G protein-coupled receptors. Br J Pharmacol 170: 1459–1581.

Allouche S, Polastron J, Hasbi A, Homburger V, Jauzac P (1999). Differential G-protein activation by alkaloid and peptide opioid agonists in the human neuroblastoma cell line SK-N-BE. Biochem J 342: 71–78.

Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011). Expression G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12: 14.

Befort K, Filliol D, Decaillot FM, Gaveriaux-Ruff C, Hoehe MR, Kieffer BL (2001). A single nucleotide polymorphic mutation in the human mu-opioid receptor severely impairs receptor signaling. J Biol Chem 276: 3130–3137.

Belmer A, Doly S, Setola V, Banas SM, Moutkine I, Boutourlinsky K *et al.* (2014). Role of the N-terminal region in G protein-coupled receptor functions: negative modulation revealed by 5-HT_{2B} receptor polymorphisms. Mol Pharmacol 85: 127–138.

Beyer A, Koch T, Schroder H, Schulz S, Hollt V (2004). Effect of the A118G polymorphism on binding affinity, potency and agonist-mediated endocytosis, desensitization and resensitization of the human mu-opioid receptor. J Neurochem 89: 553–560.

Bond C, LaForge KS, Tian M, Melia D, Zhang S, Borg L *et al.* (1998). Single-nucleotide polymorphism in the human mu opioid receptor gene alters β-endorphin binding and activity: possible implications for opiate addiction. Proc Natl Acad Sci USA 95: 9608–9613.

Bortsov AV, Millikan RC, Belfer I, Boortz-Marx RL, Arora H, McLean SA (2012). μ-Opioid receptor gene A118G polymorphism predicts survival in patients with breast cancer. Anesthesiology 116: 896–902.

Corey S, Clapham DE (2001). The stoichiometry of Gβγ binding to G-protein-regulated inwardly rectifying K^+ channels (GIRKs). J Biol Chem 276: 11409–11413.

Cox B (2013). Recent developments in the study of opioid receptors. Mol Pharmacol 83: 723–728.

Cox BM, Christie MJ, Devi L, Toll L, Traynor JR (2014). Challenges for opioid receptor nomenclature. Brit J Pharmacol In Press.

Diatchenko L, Robinson JE, Maixner W (2011). Elucidation of mu-opioid gene structure: how genetics can help predict therapeutic response to opioids. Eur J Pain Suppl 5: 433–448.

Galandrin S, Oligny-Longpre G, Bonin H, Ogawa K, Gales C, Bouvier M (2008). Conformational rearrangements and signalling cascades involved in ligand-biased mitogen-activated protein kinase signalling through the β1-adrenergic receptor. Mol Pharmacol 74: 162–172.

Gerra G, Somaini L, Leonardi C, Cortese E, Maremmani I, Manfredini M *et al*. (2014). Association between gene variants and response to buprenorphine maintenance treatment. Psychiatry Res 215: 202–207.

Gupta A, Decaillot FM, Gomes I, Tkalych O, Heimann AS, Ferro ES *et al*. (2007). Conformation state-sensitive antibodies to G-protein-coupled receptors. J Biol Chem 282: 5116–5124.

Gupta A, Rozenfeld R, Gomes I, Raehal KM, Decaillot FM, Bohn LM *et al*. (2008). Post-activation-mediated changes in opioid receptors detected by N-terminal antibodies. J Biol Chem 283: 10735–10744.

Herrick-Davis K, Grinde E, Cowan A, Mazurkiewicz JE (2013). Fluorescence correlation spectroscopy analysis of serotonin, adrenergic, muscarinic, and dopamine receptor dimerization: the oligomer number puzzle. Mol Pharmacol 84: 630–642.

Huang P, Chen C, Mague SD, Blendy JA, Liu-Chen LY (2012). A common single nucleotide polymorphism A118G of the μ opioid receptor alters its N-glycosylation and protein stability. Biochem J 441: 379–386.

Kenakin T, Miller LJ (2010). Seven transmembrane receptors as shapeshifting proteins: the impact of allosteric modulation and functional selectivity on new drug discovery. Pharmacol Rev 62: 265–304.

Klepstad P, Rakvag TT, Kaasa S, Holthe M, Dale O, Borchgrevink PC *et al*. (2004). The 118 A > G polymorphism in the human μ-opioid receptor gene may increase morphine requirements in patients with pain caused by malignant disease. Acta Anaesthesiol Scand 48: 1232–1239.

Klepstad P, Fladvad T, Skorpen F, Bjordal K, Caraceni A, Dale O *et al*. (2011). Influence from genetic variability on opioid use for cancer pain: a European genetic association study of 2294 cancer pain patients. Pain 152: 1139–1145.

Knapman A, Connor M (2014). Cellular signalling of non-synonymous single nucleotide polymorphisms of the human μ-Opioid Receptor (OPRM1). Br J Pharmacol doi: 10.1111/bph.12644; In Press.

Knapman A, Santiago M, Du YP, Bennallack PR, Christie MJ, Connor M (2013). A continuous, fluorescence-based assay of μ-opioid receptor activation in AtT-20 cells. J Biomol Screen 18: 269–276.

Knapman A, Abogadie F, McIntyre P, Connor M (2014). A real-time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese hamster ovary cells. J Biomol Screen 19: 223–231.

Kroslak T, LaForge KS, Gianotti RJ, Ho A, Nielsen DA, Kreek MJ (2007). The single nucleotide polymorphism A118G alters functional properties of the human mu opioid receptor. J Neurochem 103: 77–87.

Law PY, Yung HW, Loh HH (2000). Molecular mechanisms of opioid receptor signalling. Annu Rev Pharmacol Toxicol 40: 389–430.

Lopez Soto EJ, Raingo J (2012). A118G mu opioid receptor polymorphism increases inhibitory effects on $Ca_V2.2$ channels. Neurosci Lett 523: 190–194.

Lotsch J, Geisslinger G (2005). Are μ-opioid receptor polymorphisms important for clinical opioid therapy? Trends Mol Med 11: 82–89.

Luttrell LM (2005). Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. J Mol Neurosci 26L: 253–264.

Mague SD, Blendy JA (2010). OPRM1 SNP (A118G): involvement in disease development, treatment response, and animal models. Drug Alcohol Depend 108: 172–182.

Mague SD, Isiegas C, Huang P, Liu-Chen LY, Lerman C, Blendy JA (2009). Mouse model of OPRM1 (A118G) polymorphism has sex-specific effects on drug-mediated behavior. Proc Natl Acad Sci USA 106: 10847–10852.

Mahmoud S, Thorsell A, Sommer WH, Heilig M, Holgate JK, Bartlett SE *et al*. (2011). Pharmacological consequence of the A118G μ opioid receptor polymorphism on morphine- and fentanyl-mediated modulation of Ca^{2+} channels in humanized mouse sensory neurons. Anesthesiology 115: 1054–1062.

Malik RU, Ritt M, DeVree BT, Neubig RR, Sunahara RK, Sivaramakrishnan S (2013). Detection of G protein-selective G protein-coupled receptor (GPCR) conformations in live cells. J Biol Chem 288: 17167–17178.

Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, Sunahara RK *et al*. (2012). Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. Nature 485: 321–326.

Margas W, Zubkoff I, Schuler HG, Janicki PK, Ruiz-Velasco V (2007). Modulation of Ca^{2+} channels by heterologously expressed wild-type and mutant human μ opioid receptors (hMORs) containing the A118G single-nucleotide polymorphism. J Neurophysiol 97: 1058–1067.

Matsui A, Williams JT (2010). Activation of μ-opioid receptors and block of $K_{IR}3$ potassium channels and NMDA receptor conductance by *l*- and *d*- methadone in rat locus coeruleus. Br J Pharmacol 161: 1403–1413.

Mura E, Govoni S, Racchi M, Carossa V, Ranzani GN, Allegri M *et al*. (2013). Consequences of the 118A>G polymorphism in the *OPRM1* gene: translation from bench to bedside? J Pain Res 6: 331–353.

Nguyen VH, Ingram SL, Kassiou M, Christie MJ (1998). Sigma-binding site ligands inhibit K^+ currents in rat locus coeruleus neurons *in vitro*. Eur J Pharmacol 361: 157–163.

Oertel BG, Doehring A, Roskam B, Kettner M, Hackmann N, Ferreiros N *et al*. (2012). Genetic-epigenetic interaction modulates μ-opioid receptor regulation. Hum Mol Genet 21: 4751–4760.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP, Davenport AP, McGrath JC, Peters JA, Southan C, Spedding M, Yu W, Harmar AJ; NC-IUPHAR. (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledgebase of drug targets and their ligands. Nucl. Acids Res. 42 (Database Issue): D1098–106.

Pineyro G, Archer-Lahlou E (2007). Ligand-specific receptor states: implications for opiate receptor signaling and regulation. Cell Signal 19: 8–19.

Ramchandani VA, Umhau J, Pavon FJ, Ruiz-Velasco V, Margas W, Sun H *et al*. (2011). A genetic determinant of the striatal dopamine response to alcohol in men. Mol Psychiatry 16: 809–817.

Ravindranathan A, Joslyn G, Robertson M, Schuckit MA, Whistler JL, White RL (2009). Functional characterisation of human variants of the mu-opioid receptor gene. Proc Natl Acad Sci U S A 106: 10811–10816.

Reyes-Gibby CC, Shete S, Rakvag T, Bhat SV, Skorpen F, Bruera E *et al*. (2007). Exploring joint effects of genes and the clinical efficacy of morphine for cancer pain: OPRM1 and COMT gene. Pain 130: 25–30.

Rivero G, Llorente J, McPherson J, Cooke A, Mundell SJ, McArdle CA *et al*. (2012). Endomorphin-2: a biased agonist at the μ-opioid receptor. Mol Pharmacol 82: 178–188.

Sauer B (1994). Site-specific recombination: developments and applications. Curr Opin Biotechnol 5: 521–527.

Singh VK, Bajpai K, Biswas S, Haq W, Khan MY, Mathur KB (1997). Molecular biology of opioid receptors. Neuroimmunomodulation 4: 285–297.

Skorpen F, Laugsand EA, Klepdstad P, Kaasa S (2008). Variable response to opioid treatment: any genetic predictors in sight? Palliat Med 22: 310–327.

Solak O, Erdogan MO, Yildiz H, Ulasli AM, Yaman F, Terzi ES *et al*. (2014). Assessment of opioid receptor μ1 gene A118G polymorphism and its association with pain intensity in patients with fibromyalgia. Rheumatol Int doi: 10.1007/200296-014-2995-1; In Press.

Somogyi AA, Barrat DT, Coller JK (2007). Pharmacogenetics of opioids. Clin Pharmacol Ther 81: 429–444.

Song Z, Du B, Wang K, Shi X (2013). Effects of OPRM1 A118G polymorphism on epidural analgesia with fentanyl during labor: a meta-analysis. Genet Test Mol Biomarkers 17: 743–749.

Srinivasan S, Lubrano-Berthelier C, Govaerts C, Picard F, Santiago P, Conklin BR *et al*. (2004). Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. J Clin Invest 114: 1158–1164.

Virk MS, Arttamangkul S, Birdsong WT, Williams JT (2009). Buprenorphine is a weak partial agonist that inhibits opioid receptor desensitization. J Neurosci 29: 7341–7348.

Wachman EM, Hayes MJ, Brown MS, Paul J, Harvey-Wilkes K, Terrin N *et al*. (2013). Association of OPRM1 and COMT single-nucleotide polymorphisms with hospital length of stay and treatment of neonatal abstinence syndrome. JAMA 309: 1821–1827.

Walter C, Lotsch J (2009). Meta-analysis of the relevance of the OPRM1 118A>G genetic variant for pain treatment. Pain 146: 270–275.

Zhang Y, Wang D, Johnson AD, Papp AC, Sadee W (2005). Allelic expression imbalance of human mu opioid receptor (OPRM1) caused by variant A118G. J Biol Chem 280: 32618–32624.