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RESEARCH PAPER

A6V polymorphism of the human μ-opioid receptor decreases signalling of morphine and endogenous opioids *in vitro*

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BACKGROUND AND PURPOSE

Polymorphisms of the μ opioid receptor (MOPr) may contribute to the variation in responses to opioid drugs in clinical and unregulated situations. The A6V variant of MOPr (MOPr-A6V) is present in up to 20% of individuals in some populations, and may be associated with heightened susceptibility to drug abuse. There are no functional studies examining the acute signalling of MOPr-A6V *in vitro*, so we investigated potential functional differences between MOPr and MOPr-A6V at several signalling pathways using structurally distinct opioid ligands.

EXPERIMENTAL APPROACH

CHO and AtT-20 cells stably expressing MOPr and MOPr-A6V were used. AC inhibition and ERK1/2 phosphorylation were assayed in CHO cells; K channel activation was assayed in AtT-20 cells.

KEY RESULTS

Buprenorphine did not inhibit AC or stimulate ERK1/2 phosphorylation in CHO cells expressing MOPr-A6V, but buprenorphine activation of K channels in AtT-20 cells was preserved. [D-Ala2, N-MePhe4, Gly-ol]-enkephalin, morphine and β-endorphin inhibition of AC was significantly reduced via MOPr-A6V, as was signalling of all opioids to ERK1/2. However, there was little effect of the A6V variant on K channel activation.

CONCLUSIONS AND IMPLICATIONS

Signalling to AC and ERK via the mutant MOPr-A6V was decreased for many opioids, including the clinically significant drugs morphine, buprenorphine and fentanyl, as well endogenous opioids. The MOPr-A6V variant is common and this compromised signalling may affect individual responses to opioid therapy, while the possible disruption of the endogenous opioid system may contribute to susceptibility to substance abuse.

Abbreviations

AC, adenylyl cyclase; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin; GIRK, G-protein–gated inwardly rectifying K^+ channel; MOPr, μ opioid receptor; *OPRM1*, opioid receptor mu 1; PBS-T, PBS plus Tween; PTX, *Pertussis* toxin; SNP, single-nucleotide polymorphism.

Tables of Links

TARGETS
GPCRs[a]
5-HT_{2B} receptor
DOPr, δ opioid receptor
KOPr, κ opioid receptor
Melanocortin MC_4 receptor
MOPr, μ opioid receptor
Ion channels[b]
GIRK, $K_{ir}3.x$ channels
Enzymes[c]
AC, adenylyl cyclase

LIGANDS
Buprenorphine
DAMGO
Endomorphin-1
Endomorphin-2
β-Endorphin
Fentanyl
Forskolin
Morphine
Naloxone
Oxycodone
Pentazocine

These Tables list key protein targets and ligands in this article 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 are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c; see also Cox *et al.*, 2014).

Introduction

Opioid analgesics, including morphine, are the most effective treatments for moderate to severe pain. Opioid consumption is also associated with unwanted effects including respiratory depression, nausea, constipation and sedation. Analgesic tolerance also often develops with continued use (Matthes *et al.*, 1996; Boom *et al.*, 2012). Opioids are characterized as having a relatively narrow therapeutic window, and for most opioids, there is a balance in dosing necessary to achieve a therapeutic effect without producing dangerous side effects such as respiratory depression (Somogyi *et al.*, 2007). The degree to which individuals experience opioid-induced analgesia and adverse effects is highly variable and difficult to predict (Merikangas *et al.*, 1998; Lotsch and Geisslinger, 2005), which can lead to inadequate pain relief for many individuals as dosing of opioids may be restricted in order to avoid serious adverse events (Skorpen and Laugsand, 2008).

Individual differences in opioid requirements and pain perception are likely to, at least in part, have a genetic basis (Crist and Berrettini, 2014). The μ opioid receptor (MOPr) is the principal target for most clinically prescribed opioid analgesics, including morphine, buprenorphine and oxycodone (Matthes *et al.*, 1996; Nozaki and Kamei, 2007; Virk *et al.*, 2009). Naturally occurring, non-synonymous genetic variants are found in the coding region of the gene for MOPr (opioid receptor mu 1, *OPRM1*), and some of these may affect individual opioid responses (Lotsch and Geisslinger, 2005; Somogyi *et al.*, 2007). The most prevalent *OPRM1* variants are N40D (MOPr-N40D, rs1799971) and A6V (MOPr-A6V, rs1799971), single-nucleotide polymorphisms (SNPs) in the N-terminal domain of MOPr. MOPr-N40D, present at allelic frequencies ranging from 10 to 50% in various populations, has been studied extensively and associated with a diverse range of clinical outcomes, including differences in pain perception, opioid requirements and predisposition to substance abuse; however, these associations have not been consistently found (Walter and Lotsch, 2009; Mague and Blendy, 2010; Diatchenko *et al.*, 2011; Klepstad *et al.*, 2011; Mura *et al.*, 2013). Likewise, *in vitro* studies of MOPr show differences between MOPr-N40D and wild-type receptor (MOPr-WT) signalling, but there is little consistency among studies (Knapman and Connor, 2015).

Despite being reported at allelic frequencies of at least 20% in African American and northern Indian populations (see Hoehe *et al.*, 2000; Crowley *et al.*, 2003; Kapur *et al.*, 2007; Crystal *et al.*, 2010), MOPr-A6V has received far less attention than MOPr-N40D. The V6 allele may occur at a higher frequency in substance abusers (Berrettini *et al.*, 1997; Rommelspacher *et al.*, 2001; Comptom *et al.*, 2003; Crowley *et al.*, 2003; Crystal *et al.*, 2010), but the effects of the V6 polymorphism on analgesic responses is unknown. Only two studies have examined MOPr-A6V SNP signalling *in vitro*. Regulation of cAMP-dependent gene transcription in HEK-293 cells transiently expressing MOPr or MOPr-A6V by [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO), endomorphin-1 or Leu-enkephalin did not differ (Fortin *et al.*, 2010). When the V6 variant was expressed on the MOPr-1A splice variant backbone in HEK-293 cells also expressing a chimeric G-protein (Ravindranathan *et al.*, 2009), the maximal effect of DAMGO, but not morphine to mobilize intracellular Ca was increased at the MOPr-1A-V6 variant compared with MOPr-1A-A6. These studies provide little insight into how the MOPr-A6V polymorphism affects acute MOPr signalling, so in this study, we investigated the coupling of hMOPr-WT and hMOPr-A6V to signalling pathways in CHO cells and AtT-20 cells using 11 opioid ligands chosen for their clinical importance and/or distinct structural features. We found that the valine to alanine change at position 6 of MOPr decreased the ability of the receptor to inhibit AC and to stimulate phosphorylation of ERK1/2 in CHO cells. Notably, buprenorphine signalling to both AC inhibition and ERK1/2 phosphorylation was abolished in MOPr-A6V cells. In contrast, the effects of the A6V substitution on activation of G-protein–gated inwardly

rectifying potassium channels (GIRK, composed of $K_{ir}3.x$ subunits) were very modest. These data suggest that the common A6V polymorphism of MOPr results in a receptor that may show pathway-specific changes in function.

Methods

MOPr transfection and cell culture

CHO-FRT-TRex cells were stably transfected with a pcDNA5 construct encoding the haemagglutinin-tagged human MOPr or MOPr-A6V cDNA together with the pOG44 (Flp recombinase) plasmid using Fugene (Promega, Alexandria, NSW, Australia, Knapman *et al.*, 2014a). Receptor constructs were synthesized by Genscript (Piscataway, NJ, USA). Cells expressing MOPr-WT or MOPr-A6V were selected using hygromycin B (500 $\mu g \cdot mL^{-1}$) and cultured in DMEM supplemented with 10% FBS, 100 µg penicillin, 100 µg streptomycin and 500 µg hygromycin B per mL up to passage 5. Hygromycin was reduced to 200 $\mu g \cdot mL^{-1}$ after passage 5. MOPr expression was induced 24 h before assays by adding tetracylcine, 2 $\mu g \cdot mL^{-1}$.

AtT20-FlpIn cells (Knapman *et al.*, 2014b) were stably transfected with MOPr-WT or MOPr-A6V as described earlier. Cells expressing MOPr were selected using 100 $\mu g \cdot mL^{-1}$ hygromycin B and cultured in DMEM supplemented with 10% FBS, 100 µg penicillin, 100 µg streptomycin and 100 µg hygromycin B per mL.

Transfection of the FlpIn cells should lead to insertion of the MOPr constructs into the same site in each cell, meaning that the receptors are under the same transcriptional control. However, because the cells are not then clonally selected, the range of cellular phenotypes present in the CHO-FRT-TRex and AtT-20-FlpIn populations will be preserved.

Cells were passaged at 80% confluency and used for up to 30 passages. Cells for assays were grown in 75-cm^2 flasks and used when at greater than 90% confluence.

Expression of MOPr

Expression of MOPr on the cell surface of CHO cells was determined as described previously (Knapman *et al.*, 2014b). Briefly, intact cells were incubated with 0.125–16.0 nM [^{3}H]-DAMGO; PerkinElmer, Waltham, MA, USA) for 2 h at 4°C in 50 mM Tris-Cl (pH 7.4). Non-specific binding was determined in the presence of unlabeled DAMGO (10 µM) and represented 15 ± 1% of total binding for CHO-MOPr-WT and 12 ± 1% for CHO-MOPr-A6V. Bound [^{3}H]-DAMGO was 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 per well was determined with a BCA Protein Assay Kit (Pierce, Rockford, MD, USA) according to the manufacturer's instructions. All experiments were performed three times each in triplicate.

Membrane potential assay of adenylyl cyclase (AC) inhibition

Inhibition of AC activity was measured using a membrane potential-sensitive dye as described in Knapman *et al.*, (2014a). Briefly, 24 h prior to the assay, CHO-MOPr cells were resuspended in Leibovitz's L-15 media supplemented with 1% FBS, 100 µg penicillin and 100 µg streptomycin per mL, 15 mM glucose 2 $\mu g \cdot mL^{-1}$ tetracycline. Cells were plated in a volume of 90 µL per well in black-walled, clear-bottomed 96-well microplates (Corning, Castle Hill, Australia), and incubated overnight at 37°C in ambient CO_2. Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue, Molecular Devices, Sunnyvale, CA, USA). 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. Cells were loaded with 90 µL per well of the dye solution without removal of the L-15 and incubated at 37°C for 60 minutes in ambient CO_2. Fluorescence was measured using a FlexStation 3 microplate reader (Molecular Devices, excitation 530 nm/565 nm). Baseline readings were taken every 2 s for at least 2 m, at which time, forskolin plus opioid or forskolin plus vehicle was added in a volume of 20 µL. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. The final concentration of solvents (DMSO or ethanol, as noted) was not more than 0.1%.

Membrane potential assay of GIRK channel activation

Opioid activation of endogenous GIRK channels in AtT-20 cells was also measured using the blue membrane potential-sensitive dye, as previously described (Knapman *et al.*, 2013). AtT-20-MOPr cells were prepared as outlined earlier, without the addition of tetracylcine. Assays were conducted as outlined earlier.

ELISA of ERK1/2 phosphorylation

Opioid-mediated ERK1/2 phosphorylation was measured by ELISA, as described in Knapman *et al.*, 2014b. Briefly, 24 h before the assay, CHO-MOPr cells were plated in 96-well clear microplates (Falcon, Macquarie Park, NSW, Australia) and receptor expression was induced with tetracycline. Cells were serum-starved for 1 h in 40 µL of L-15 supplemented with 5% BSA before the assay. Cells were treated by adding drug or vehicle diluted in serum- and BSA-free L-15 (40 µL added). Preliminary time-course experiments indicated that a 5 min drug treatment produced robust ERK1/2 phosphorylation without desensitization. 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. Cells were washed three times with 300 µL PBS and permeabilized with 0.1% Triton-X in PBS for 30 min at room temperature. Triton-X was removed, and cells incubated for 2 h at room temperature with blocking solution consisting of 5% BSA in PBS with 0.01% Tween-20 (PBS-T). Blocking solution was removed, 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, Castle Hill, NSW, Australia) at room temperature 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 μg·mL^{-1} DAPI for 10 min at room temperature, and washed three times with 300 μL PBS-T. Fluorescence was read in the Pherastar microplate reader (excitation 358 nm, emission 461 nm). Absorbance readings were normalized to DAPI staining to account for any differences in cell density among wells. Readings were then normalized to the response of cells treated with 100 nM phorbol myristate (PMA) for 10 min.

Data analysis

Data are expressed as mean ± SEM of at least five determinations, made in duplicate or triplicate, unless otherwise noted. Concentration–response curves (CRCs) were fit with a four-parameter logistic equation using Graphpad Prism. CRCs from separate experiments were pooled and compared with two-way ANOVA to determine if there was a difference in drug effect among genotypes. E_{max} and pEC_{50} values derived from individual experiments were compared using unpaired Student's *t*-test if the ANOVA indicated that the drug effect was different among genotypes. Comparisons between maximum agonist responses amoung drugs were made by comparing E_{max} values derived from individual experiments using one-way ANOVA followed by Dunnett's *post hoc* test, corrected for multiple comparisons. All other comparisons were made using an unpaired Student's *t*-test. $P < 0.05$ was considered significant.

Materials

Tissue culture reagents and buffer salts were from Invitrogen (Mulgrave, Vic., Australia) or Sigma unless otherwise noted. DAMGO, endomorphin-1, endomorphin-2 and Met-enkephalin were purchased from Auspep (Tullamarine, Australia). Morphine, fentanyl and pentazocine were a kind gift from the Department of Pharmacology, University of Sydney. Buprenorphine and oxycodone were from the National Measurement Institute (Lindfield, Australia). β-endorphin was from Genscript. Forskolin and naloxone were from Ascent Pharmaceuticals (Bristol, UK). *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).

Results

MOPr expression in CHO cells

MOPr-WT and MOPr-A6V expression in CHO cells was controlled by a tetracycline-sensitive repressor. After 24 h of treatment with tetracycline, cell surface receptor number was similar in MOPr-WT or MOPr-A6V CHO cells, as measured by [^{3}H]DAMGO binding (see Figure 1). The B_{max} for CHO-MOPr-WT cells was 280 ± 20 fmol·mg^{-1} total protein and 301 ± 25 fmol·mg^{-1} for CHO-MOPr-A6V ($P > 0.05$). The affinity for [^{3}H]DAMGO was also similar between MOPr-WT- and MOPr-A6V-expressing cells, with K_d of 0.75 ± 0.10 nM and 0.55 ± 0.05 nM, respectively ($P > 0.05$).

Inhibition of AC

The AC activator forskolin (300 nM) produced a rapid decrease in the fluorescence of a membrane potential-sensitive dye in both MOPr-WT or MOPr-A6V CHO cells (Figure 2A; Knapman *et al.*, 2014a) corresponding to a membrane hyperolarization. Changes in fluorescence were quantified 5 min after forskolin application. Forskolin (300 nM) produced a similar hyperpolarization in CHO-MOPr-WT cells (41 ± 1% decrease in fluorescence) and CHO-MOPr-A6V cells (39 ± 2% decrease, $P > 0.05$). Addition of the MOPr-selective peptide agonist DAMGO together with forskolin produced a concentration-dependent inhibition of the forskolin-stimulated hyperpolarization in both cell lines (Figure 2). We have shown previously that the effects of DAMGO in this assay were sensitive to naloxone and inhibited by overnight pretreatment with PTX (Knapman *et al.*, 2014a). Opioids applied by themselves did not affect the membrane potential of CHO-MOPr cells, with the exception of high concentrations of pentazocine (30 μM) and methadone (10 μM), both of which caused small, transient, naloxone-insensitive increases in fluorescence (<10%).

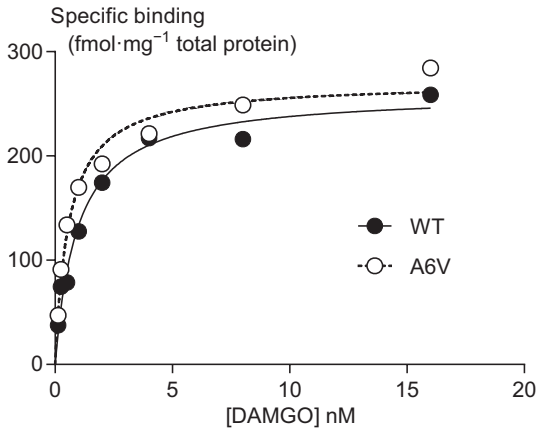

Figure 1

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

In cells expressing MOPr-A6V, DAMGO inhibition of the forskolin-induced hyperpolarization was significantly reduced compared with cells expressing MOPr-WT (Table 1). A two-way ANOVA indicated the DAMGO CRCs differed between CHO-MOPr-WT and CHO-MOPr-A6V cells ($P < 0.001$, Figure 2).

We determined the potency and maximum effect of a range of opioid ligands to investigate whether the A6V substitution affected MOPr inhibition of AC in a ligand-selective manner. In CHO-MOPr-A6V cells, morphine inhibition of forskolin effects was less than in cells expressing MOPr-WT ($P < 0.05$, Table 1). A two-way ANOVA indicated a significant difference between the morphine CRC for MOPr-WT and MOPr-A6V ($P < 0.0001$, Figure 3). We had previously shown that the partial agonist buprenorphine had a reduced efficacy to inhibit AC in cells expressing the MOPr-N40D variant, which is also an amino acid substitution in the N-terminal region of

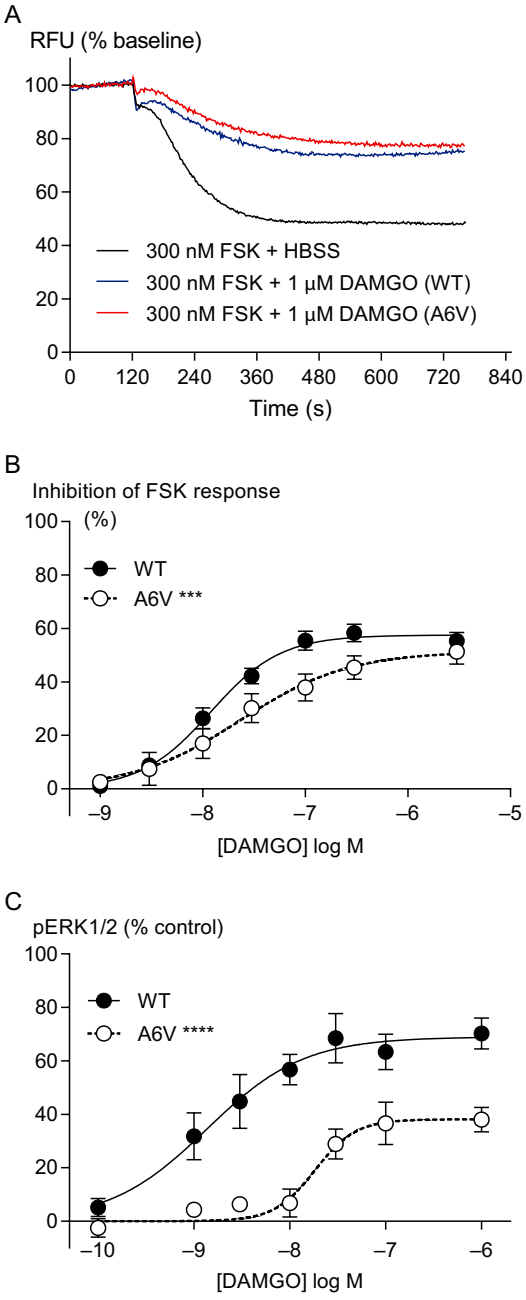

Figure 2

DAMGO inhibits AC and activates ERK1/2 in CHO cells expressing MOPr-WT or MOPr-A6V. AC inhibition and levels of ERK1/2 phosphorylation were determined. (A) Raw trace showing decrease in fluorescent signal following application of 300 nM forskolin (FSK) + HBSS, and 300 nM forskolin + 1 μM DAMGO at 120 s. (B) DAMGO inhibition of forskolin-stimulated AC-mediated hyperpolarization in CHO cells expressing MOPr-WT or MOPr-A6V was significantly affected by the A6V variant. ***$P < 0.001$; significantly different from WT; two-way ANOVA. (C) DAMGO-stimulated ERK1/2 phosphorylation in CHO-MOPr cells was significantly affected by the A6V variant. ****$P < 0.0001$; significantly different from WT; two-way ANOVA. Maximum ERK1/2 phosphorylation by 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± SEM of pooled data from at least five to six independent determinations performed in duplicate.

MOPr (Knapman *et al.*, 2014b). In the present series of experiments, buprenorphine inhibited the forskolin response with high potency but a low E_{max} in cells with MOPr-WT (Table 1) but in CHO-MOPr-A6V cells, buprenorphine signalling was abolished (Figure 3). The maximum inhibition of the forskolin response by pentazocine (10 μM) was not different between CHO-MOPr-WT and CHO-MOPr-A6V cells (Table 1); however, two-way ANOVA showed a significant difference between the pentazocine CRCs in the two cell lines. ($P < 0.05$, Figure 3). At concentrations higher than 10 μM, pentazocine has been reported to block K^+ channels (Nguyen *et al.*, 1998), so we did not test concentrations above 10 μM although the pentazocine response had not reached an obvious maximum. For this reason, we did not calculate a pEC_{50} for pentazocine inhibition of AC.

We also examined the effect of MOPr-A6V on AC inhibition by the endogenous opioids, β-endorphin and Met-enkephalin and the putative endogenous MOPr agonists endomorphin-1 and endomorphin-2. β-endorphin signalling by the A6V variant was decreased (two-way ANOVA, $P < 0.0001$, Figure 4, Table 1). CRCs for Met-enkephalin, endomorphin-1 and endomorphin-2 differed significantly between CHO-MOPr-WT and CHO-MOPr-A6V cells (two-way ANOVA, $P < 0.01$, Figure 4); however, the E_{max} and pEC_{50} for endomorphin-1 calculated from individual experiments in CHO-MOPr-WT and CHO-MOPr-A6V were similar (Table 1). For endomorphin-2, E_{max} values for MOPr-WT and MOPr-A6V did not differ significantly; however, its potency was reduced at MOPr-A6V ($P < 0.05$, Table 1). Likewise, maximum Met-enkephalin inhibition of the forskolin effect was similar between variants, but the pEC_{50} values at MOPr-WT and MOPr-A6V were significantly different ($P < 0.05$, Table 1).

The effects of some other clinically important opioids – fentanyl, methadone and oxycodone – were also examined. For each drug, there was a significant effect of the A6V variant on the CRCs (two-way ANOVA, $P < 0.01$, Figure 5). When calculated from individual experiments, the E_{max} and EC_{50} of fentanyl and oxycodone to inhibit the forskolin response was similar in CHO-MOPr-WT and CHO-MOPr-A6V. The maximum inhibition of the forskolin hyperpolarization by methadone did not differ between MOPr-WT and MOPr-A6V although the pEC_{50} values were different ($P < 0.05$, Table 1).

Opioid-mediated phosphorylation of ERK1/2

We next determined if the A6V SNP affected opioid signalling in CHO cells through another important pathway activated by MOPr, stimulation of ERK1/2 phosphorylation. Agonist stimulation of ERK1/2 phosphorylation was normalized to that produced by the PKC activator, PMA (100 nM). The response to PMA was similar between CHO-MOPr-WT and CHO-MOPr-A6V, with absorbance readings of 0.60 ± 0.06 and 0.53 ± 0.09, respectively ($P > 0.05$). Preincubation of cells with naloxone (1 μM, 5 min) blocked the increase in pERK1/2 produced by EC_{80} concentrations of all opioids. Treatment of cells with 200 ng·mL^{-1} PTX overnight blocked activation of ERK1/2 by DAMGO, and DAMGO did not elicit a response in cells where receptor expression had not been induced. DAMGO-stimulated ERK1/2 phosphorylation was significantly reduced in CHO-MOPr-A6V when compared with CHO-MOPr-WT cells (two-way ANOVA, $P < 0.0001$, Figure 2; Table 2).

Table 1

Summary of opioid efficacy and potency in assays of AC inhibition in CHO cells expressing MOPr-WT and MOPr-A6V

AC inhibition	E_{max} (%)		pEC_{50}	
Opioid	**WT**	**A6V**	**WT**	**A6V**
β-Endorphin****	73 ± 4	50 ± 4# $P = 0.005$	6.8 ± 0.1	6.4 ± 0.1# $P = 0.04$
Fentanyl**	70 ± 6	68 ± 6	8.0 ± 0.2	7.6 ± 0.1
Morphine****	66 ± 4	39 ± 4# $P = 0.001$	7.0 ± 0.1	6.1 ± 0.3# $P = 0.01$
Oxycodone***	65 ± 5	69 ± 8	5.8 ± 0.3	5.4 ± 0.4
Methadone****	65 ± 3	52 ± 9	7.0 ± 0.1	6.4 ± 0.1# $P = 0.005$
Met-enkephalin**	64 ± 4	67 ± 6	7.9 ± 0.1	7.2 ± 0.1# $P = 0.005$
Endomorphin-1***	64 ± 5	61 ± 5	8.2 ± 0.1	7.9 ± 0.2
Endomorphin-2****	65 ± 7	56 ± 6	8.2 ± 0.1	7.7 ± 0.1# $P = 0.02$
DAMGO***	62 ± 3	52 ± 3# $P = 0.038$	7.9 ± 0.1	7.6 ± 0.2
Buprenorphine	**38 ± 7**	**No Response**	8.4 ± 0.3	No Response
Pentazocine*	**36 ± 5**	**24 ± 7**	N/A	N/A

Assays were performed as described. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with E_{max} significantly lower than β-endorphin are highlighted bold (one-way ANOVA, followed by Dunnett's *post hoc* test corrected for multiple comparisons, $P < 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, opioids with CRCs significantly different between variants; two-way ANOVA. #MOPr-A6V E_{max} and pEC_{50} values significantly different from MOPr-WT; unpaired Student's *t*-test, *P* values listed in the table).

ERK1/2 phosphorylation elicited by morphine was also significantly reduced at MOPr-A6V, compared with MOPr-WT (two-way ANOVA, $P < 0.0001$, Figure 3). The pEC_{50} for morphine did not differ significantly between variants (Table 2). Buprenorphine failed to promote ERK1/2 phosphorylation in cells expressing MOPr-A6V (Figure 3, Table 2). ERK1/2 phosphorylation stimulated by pentazocine was also altered at MOPr-A6V when compared with MOPr-WT (two-way ANOVA, $P < 0.0001$, Figure 3). The small and inconsistent responses to pentazocine in MOPr-A6V cells meant that it was not possible to accurately determine EC_{50}s for each experiment; however, the response to pentazocine at the highest concentration tested (30 μM) was significantly reduced ($P < 0.05$, Table 2).

The maximum ERK1/2 phosphorylation elicited by β-endorphin was similar between CHO-MOPr-WT and CHO-MOPr-A6V; however, two-way ANOVA showed a significant effect of MOPr-A6V on the β-endorphin CRC ($P < 0.0001$, Figure 4). CRCs for Met-enkephalin, endomorphin-1 and endomorphin-2 differed at MOPr-A6V (two-way ANOVA, $P < 0.0001$, Figure 4) with the E_{max} significantly decreased for each peptide, and the endomorphin-1 pEC_{50} altered ($P < 0.05$, Table 2).

The A6V variant showed less stimulation by MOPr activation, of ERK1/2 phosphorylation in response to fentanyl, methadone and oxycodone, with CRCs significantly different to WT receptors (two-way ANOVA, $P < 0.0001$, Figure 5). Maximal ERK1/2 phosphorylation by fentanyl, methadone and oxycodone was significantly decreased at MOPr-A6V ($P < 0.05$, Table 2).

In summary, AC inhibition and ERK1/2 phosphorylation was reduced for most opioids in CHO-MOPr-A6V cells, although the effect was more pronounced for ERK1/2 phosphorylation. The maximum effect and potency of DAMGO, morphine, and β-endorphin to inhibit AC were significantly affected by the A6V variant, while the potencies of endomorphin-2, Met-enkephalin and methadone were also affected (Table 1). By contrast, in the assay of ERK1/2 phosphorylation, the maximum response was reduced by 35–50% for every opioid tested other than β-endorphin, where the E_{max} was not affected, but pEC_{50} was, and buprenorphine, where signalling was completely abolished (Table 2).

GIRK channel activation in AtT-20 cells

Stimulation of MOPr produces Gβγ subunit-mediated opening of GIRK, resulting in membrane hyperpolarization. AtT-20 cells express endogenous GIRK channels and represent a robust system for studying this important signalling pathway (Knapman *et al.*, 2013). Application of opioids to AtT-20 cells expressing either MOPr-WT or MOPr-A6V resulted in a concentration-dependent decrease in the fluorescence of a membrane potential-sensitive dye, corresponding to membrane hyperpolarization (Figure 6). There was no significant difference in DAMGO-stimulated GIRK activation between AtT-20 cells expressing MOPr-WT or MOPr-A6V (two-way ANOVA, Figure 7, Table 3).

Morphine activated GIRK in AtT20-MOPr-WT cells to the same E_{max} as DAMGO but with a slightly lower potency (Table 3). In AtT20-MOPr-A6V cells, morphine E_{max} was

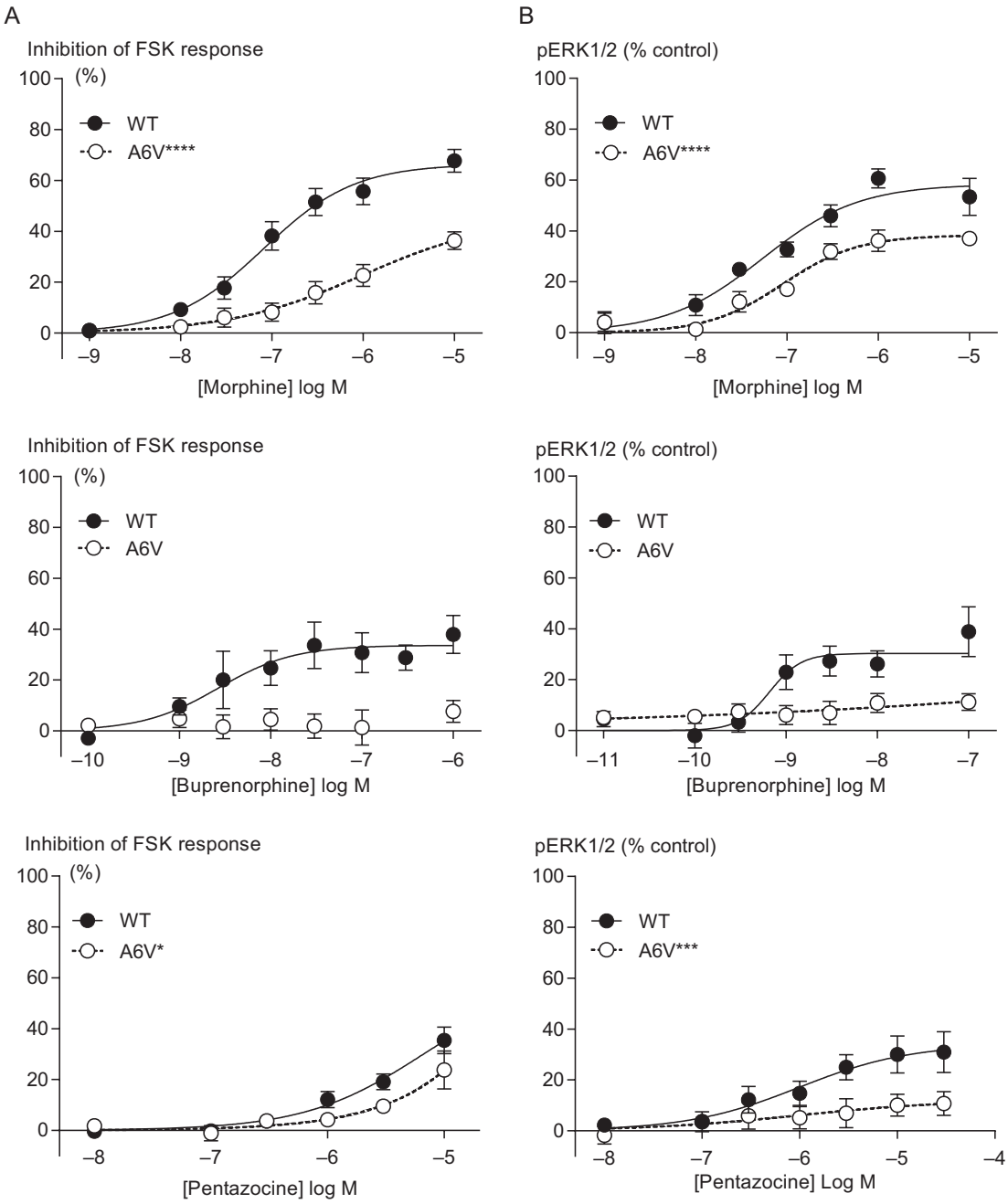

Figure 3

Morphine, buprenorphine and pentazocine inhibition of AC and activation of ERK1/2 is compromised in CHO cells expressing MOPr-A6V. AC inhibition and levels of ERK phosphorylation were determined. (A) Morphine and pentazocine inhibition of forskolin-stimulated AC activity was decreased at MOPr-A6V compared with MOPr-WT. *P < 0.05, ****P < 0.0001; significantly different from WT; two-way ANOVA. Buprenorphine signalling was abolished at MOPr-A6V. (B) Morphine and pentazocine (two-way ANOVA,) stimulated ERK1/2 phosphorylation was significantly decreased at MOPr-A6V compared with MOPr-WT. ***P < 0.001, ****P < 0.0001; significantly different from WT; two-way ANOVA. Buprenorphine signalling was abolished at MOPr-A6V. Maximum ERK1/2 phosphorylation via 100 nM PMA was used as a control for pERK1/2 experiments. Data represent the mean ± SEM of pooled data from five to six independent determinations performed in duplicate.

slightly, but significantly reduced (P < 0.05, Table 3) and a two-way ANOVA indicated a significant difference between morphine CRCs at MOPr-WT and MOPr-A6V in AtT20 cells (P < 0.0001, Figure 7). The maximal buprenorphine-stimulated GIRK activation and pEC_{50} did not differ between cells expressing MOPr-WT and MOPr-A6V; however two-way ANOVA indicated a significant effect of the A6V variant on overall buprenorphine signalling (P < 0.05, Figure 7). Interestingly, buprenorphine appears to be slightly more efficacious in AtT20-MOPr-A6V cells compared with AtT-MOPr-WT

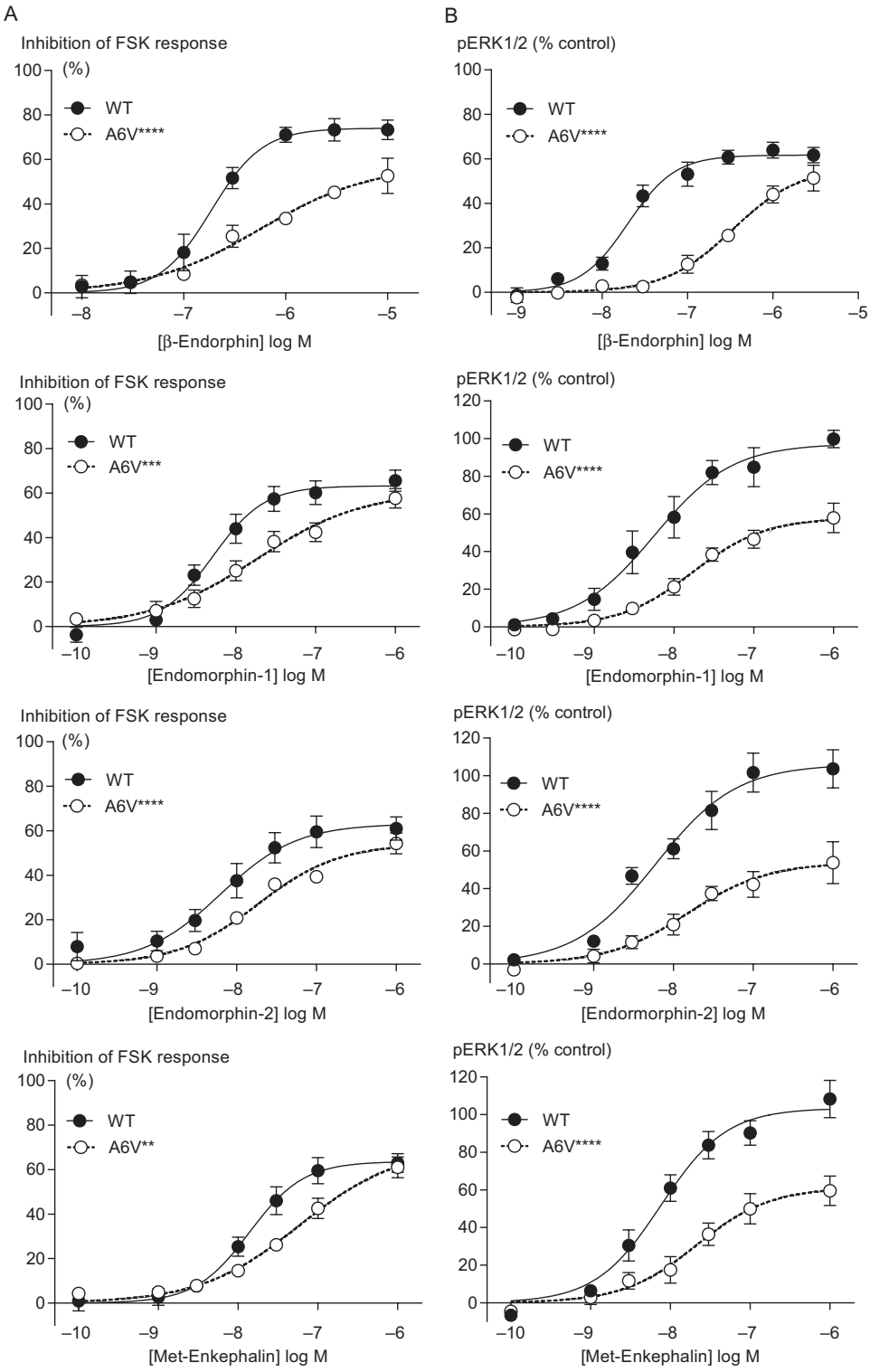

Figure 4

Endogenous opioid inhibition of AC and activation of ERK1/2 is significantly affected by the A6V variant in CHO cells expressing MOPr. AC inhibition and levels of ERK phosphorylation were determined. (A) β-endorphin, endomorphins-1 and 2, and Met-enkephalin inhibition of the forskolin-stimulated AC response was significantly different between MOPr-WT and MOPr-A6V, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; significantly different from WT; two-way ANOVA. (B) β-endorphin, endomorphins-1 and 2, and Met-enkephalin activation of ERK1/2 was significantly different between MOPr-WT and MOPr-A6V. $^{****}P < 0.0001$; significantly different from WT; two-way ANOVA. 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.

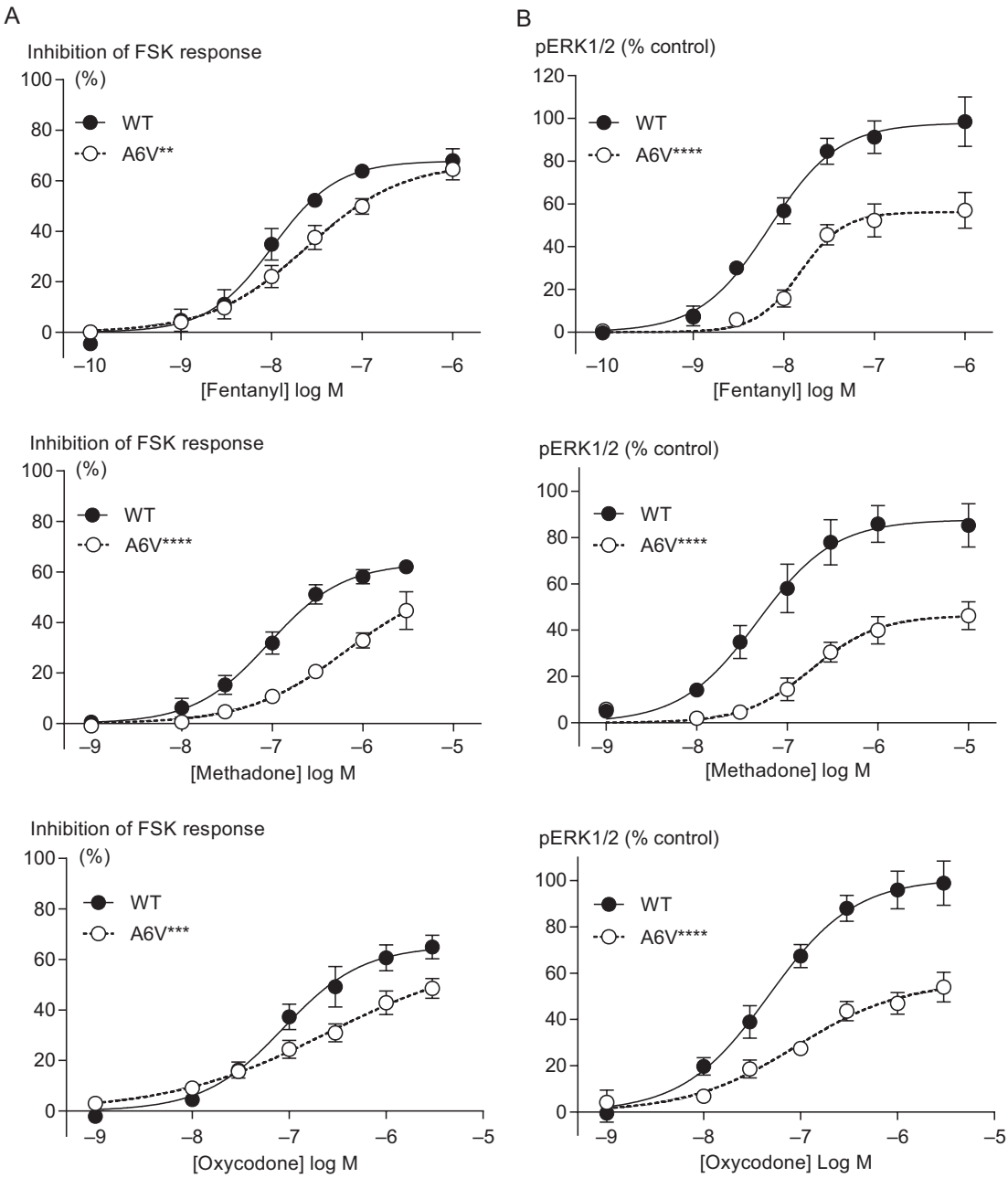

Figure 5

Fentanyl, methadone and oxycodone inhibition of AC and activation of ERK1/2 is significantly affected by the A6V variant in CHO cells expressing MOPr. AC inhibition and levels of ERK phosphorylation were determined. (A) Fentanyl, methadone and oxycodone inhibition of the forskolin-stimulated AC response was s different between MOPr-WT and MOPr-A6V. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, significantly different from WT; two-way ANOVA. (B) Fentanyl, methadone and oxycodone activation of ERK1/2 was significantly different between MOPr-WT and MOPr-A6V. $^{****}P < 0.0001$; significantly different from WT; two-way ANOVA. 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.

cells (Figure 7, Table 3). This is in direct contrast with the effect of the A6V variant on AC and ERK1/2 signalling in CHO cells, where buprenorphine failed to elicit a response. In AtT20-MOPr-WT and AtT20-MOPr-A6V cells, there was no difference in GIRK activation for pentazocine, β-endorphin or methadone (Figure 7, Table 3).

Discussion and conclusions

We have shown that the relatively common MOPr variant A6V has a significant, detrimental effect on the ability of MOPr to couple to effector pathways in CHO cells. In assays of AC inhibition, buprenorphine signalling through MOPr-A6V was

Table 2

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

pERK1/2	E_{max} (%)		pEC_{50}	
Opioid	**WT**	**A6V**	**WT**	**A6V**
Endomorphin-2****	106 ± 7	54 ± 7# $P = 0.003$	8.2 ± 0.1	7.8 ± 0.2
Met-enkephalin****	103 ± 6	61 ± 7# $P = 0.003$	8.1 ± 0.1	7.7 ± 0.2
Oxycodone****	101 ± 6	57 ± 6# $P = 0.01$	6.3 ± 0.1	6.1 ± 0.2
Fentanyl****	98 ± 5	56 ± 4# $P = 0.02$	8.2 ± 0.1	7.8 ± 0.1
Endomorphin-1****	97 ± 7	58 ± 5# $P = 0.03$	8.2 ± 0.1	7.8 ± 0.1# $P = 0.009$
Methadone****	88 ± 6	46 ± 4# $P = 0.0003$	7.3 ± 0.1	6.7 ± 0.1# $P = 0.05$
DAMGO**	**69 ± 5**	**38 ± 4**	**8.8 ± 0.2**	**7.7 ± 0.1# $P = 0.01$**
β-Endorphin**	**62 ± 2**	**56 ± 6**	**7.7 ± 0.1**	**6.4 ± 0.1# $P = 0.009$**
Morphine**	**58 ± 4**	**38 ± 3# $P = 0.04$**	**7.2 ± 0.1**	**7.0 ± 0.1**
Pentazocine***	**35 ± 9**	**11 ± 5**	**6.0 ± 0.4**	**N/A**
Buprenorphine	**30 ± 4**	**No response**	**9.1 ± 0.2**	**No response**

Assays were performed as described. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with E_{max} significantly lower than endomorphin-2 are highlighted in **bold** (one-way ANOVA, followed by Dunnett's *post hoc* test, corrected for multiple comparisons, $P < 0.05$). ***$P < 0.001$, ****$P < 0.0001$, opioids with CRCs significantly different between variants; two-way ANOVA. #MOPr-A6V Emax and pEC_{50} values significantly different from MOPr-WT; unpaired Student's *t*-test, *P* values listed in the table. It was not possible to accurately determine EC_{50} values for pentazocine at the MOPr-A6V variant, E_{max} was determined at 30 μM.

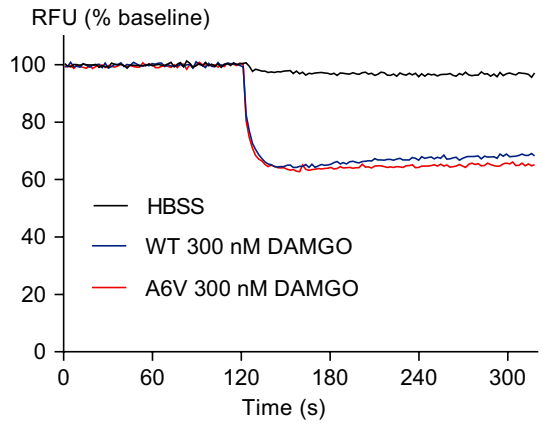

Figure 6

DAMGO causes membrane hyperpolarization in AtT-20 cells expressing both MOPr-WT and MOPr-A6V. Normalized traces showing decrease in fluorescent signal in MOPr-WT (blue) and MOPr-A6V (red) expressing AtT-20 cells following application of 300 nM DAMGO, corresponding to membrane hyperpolarization from GIRK activation. Drug was added at 120 s, the black trace shows changes produced by vehicle (HBSS) addition to MOPr-WT cells.

abolished, and most opioids had reduced potency and/or efficacy in CHO-MOPr-A6V cells. In assays of ERK1/2 phosphorylation, buprenorphine also failed to elicit a response in cells expressing MOPr-A6V and maximum ERK1/2 phosphorylation was significantly decreased for all opioids tested, with the exception of β-endorphin. In marked contrast, MOPr activation of GIRK was not strongly altered by the A6V variant, with the exception of slight inhibition of morphine efficacy. Most strikingly, buprenorphine-stimulated GIRK activation was not compromised in AtT-20-MOPr-A6V cells. These results suggest that the effects of opioid receptor polymorphisms can be pathway-dependent, which is consistent with the idea that different receptor conformations may couple preferentially to different signalling cascades.

In the absence of spare receptors, even small differences in receptor expression could significantly affect the signalling of MOPr variants, potentially providing an explanation for the results in the CHO cells. However, radioligand-binding assays showed that CHO-MOPr-A6V cells had similar surface receptor expression as CHO-MOPr-WT cells. For AtT20-FlpIn cells, we did not measure receptor expression directly; however, the efficacy of the partial agonists buprenorphine and pentazocine were similar in AtT20 cells expressing MOPr-WT and MOPr-A6V, suggesting similar receptor

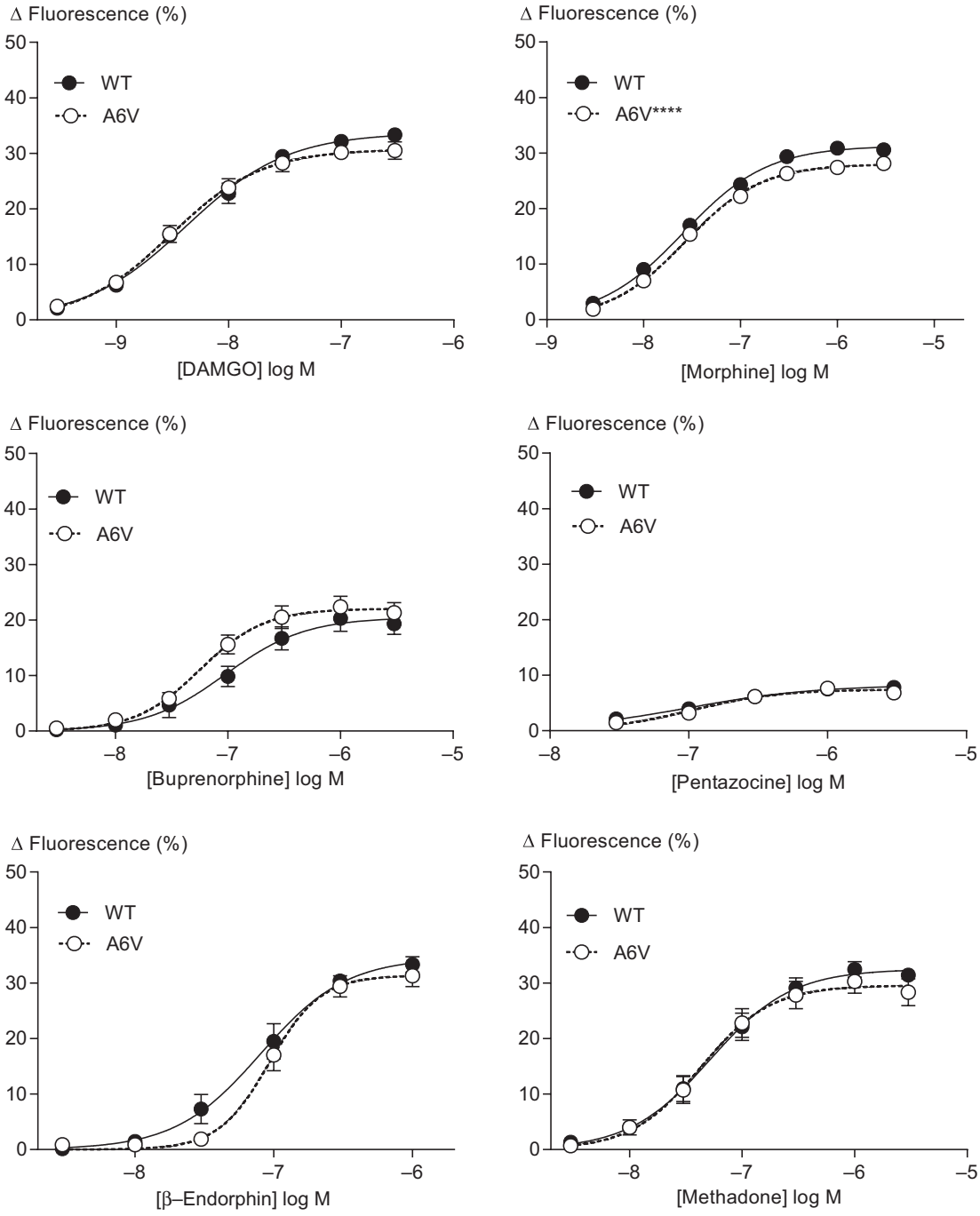

Figure 7

Opioid activation of GIRK is preserved in AtT20 cells expressing MOPr-A6V. GIRK activation was determined. Morphine and buprenorphine activation of GIRK channels was significantly different between MOPr-WT and MOPr-A6V. ****$P < 0.0001$; significantly different from WT; two-way ANOVA. Morphine E_{max} was decreased from 31 ± 1% in AtT20-MOPr-WT to 28 ± 1% in AtT20-MOPr-A6V (Student's *t*-test, $P < 0.05$). Buprenorphine E_{max} and pEC_{50} did not differ significantly between MOPr-WT and MOPr-A6V. Data represent the mean ± SEM of pooled data from five to six independent determinations performed in duplicate.

expression levels. The use of the FlpIn system for receptor expression means that the receptor construct should be integrated at the same location in the genome in each cell line, ensuring each variant is subjected to a similar transcriptional environment (Sauer, 1994). The selection of a population of cells rather than a single clone after MOPr transfection also means that the cell lines are isogenic. The use of an inducible receptor expression system also means that the consequences of any differences between MOPr-WT and MOPr-A6V mRNA or protein stability may be minimized. There is no other

Table 3

Summary of opioid efficacy and potency in assays of GIRK activation in AtT-20 cells expressing MOPr-WT and MOPr-A6V

GIRK activation	E_{max} (%)		pEC_{50}	
Opioid	**WT**	**A6V**	**WT**	**A6V**
DAMGO	34 ± 1	31 ± 1	8.4 ± 0.1	8.5 ± 0.1
β-Endorphin	35 ± 2	32 ± 1	7.0 ± 0.1	7.0 ± 0.1
Methadone	33 ± 1	30 ± 1	7.3 ± 0.1	7.4 ± 0.1
Morphine****	31 ± 1	28 ± 1# $P = 0.02$	7.6 ± 0.1	7.6 ± 0.1
Buprenorphine*	**21 ± 2**	**22 ± 1**	**7.0 ± 0.1**	**7.3 ± 0.1**
Pentazocine	**8 ± 1**	**7 ± 1**	**7.0 ± 0.1**	**7.4 ± 0.1**

Assays were performed as described. Opioids are listed in rank order of maximal effect at MOPr-WT. Opioids with E_{max} significantly lower than DAMGO are highlighted in bold (one-way ANOVA, Dunnett's *post hoc* test, corrected for multiple comparisons, $P < 0.05$). *$P < 0.05$, β-endorphin ****$P < 0.0001$, opioids with CRCs significantly different between variants; two-way ANOVA. #MOPr-A6V E_{max} and pEC50 values significantly different from MOPr-WT; unpaired Student's *t*-test.

information on MOPr-A6V receptor expression in humans or cellular models.

Genetic variation in GPCRs may affect receptor function by altering GPCR conformation, with conformational changes potentially affecting ligand binding to the receptor, and/or affecting the efficacy of the resulting ligand/receptor complex to couple to associated effector molecules (Pineyro and Archer-Lahlou, 2007; Kenakin and Miller, 2010). Our data indicate that the alanine to valine amino acid change in the initial segment of the N-terminal domain of MOPr affects the ability of the receptor to effectively transduce signals from some ligands to an intracellular effect. Most studies examining ligand interaction with GPCRs have focused on regions that form the ligand-binding pocket, namely the transmembrane helices and extracellular loops, and the recently published crystal structures of MOPr and the other two opioid receptors, DOPr and KOPr do not include the N-terminus (Granier *et al.*, 2012; Manglik *et al.*, 2012; Wu *et al.*, 2012). Thus, it may be somewhat surprising that the A6V variant has such a marked effect on MOPr function. However, several studies investigating the functional consequences of the common N-terminal region MOPr variant N40D, have reported altered N40D signalling (see Knapman & Connor, 2015). We have recently shown that buprenorphine efficacy for inhibition of AC and stimulation of ERK1/2 phosphorylation is selectively decreased at the MOPr-N40D variant expressed in CHO cells, and buprenorphine potency for GIRK activation is also decreased in AtT20-MOPr-N40 cells (Knapman *et al.*, 2014b). Buprenorphine was also the most markedly affected opioid at MOPr-A6V, but there are two striking differences between the effects of the N40D polymorphism, and those of the A6V mutant. Firstly, signalling of many opioids to AC and ERK was compromised at A6V receptors and, secondly, coupling of A6V to GIRK was essentially the same as to MOPr-WT, even for the partial agonists, buprenorphine and pentazocine. The N40D variant of MOPr removes a putative glycosylation site in the protein and introduces an extra potential methylation site in the gene, and both these factors are said to contribute to decreased levels of mRNA and receptor expression of MOPr-N40D in animal models and *post mortem* human brain (Zhang *et al.*, 2005; Oertel *et al.*, 2009). The A6V variant does not affect a MOPr glycosylation site, and receptor expression was equivalent between cell lines, suggesting that genetic changes in the N-terminal region of MOPr may be capable of affecting receptor function, independently of receptor glycosylation.

Importantly, the N-terminal region of the receptor has been shown to undergo activation-dependent changes in MOPr and other GPCRs. Evidence that the N-terminal domain undergoes conformational changes when ligands bind, comes from studies showing that antibodies generated against this region of MOPr show differential recognition of inactive and activated receptors (Gupta *et al.*, 2007; 2008). Changes in the intracellular C-terminal region of MOPr also affect N-terminal antibody binding, implying that many domains of MOPr are affected by conformational changes associated with receptor binding to intracellular G-proteins. Signalling in other GPCRs is also affected by N-terminal SNPs. Constitutive and agonist-stimulated activity of the 5-HT_{2B} receptor was increased by an R > G amino acid substitution at position 6 on the 5-HT_{2B} N-terminal domain, underscoring the potential importance of this region to GPCR function (Belmer *et al.*, 2014). Polymorphisms in the N-terminal domain of the melanocortin MC_4 receptor also increase constitutive activity of the receptor (Srinivasan *et al.*, 2004). These data from several different receptors indicate that the N-terminal region of GPCR may undergo substantial conformational changes upon receptor activation, and the structural changes arising from genetically encoded amino acid variation in this domain can significantly affect receptor function.

Intriguingly, although opioid signalling was markedly decreased in CHO cells expressing MOPr-A6V, in AtT20-MOPr cells the A6V variant had little effect on the ability of opioids to activate GIRK channels. These data suggest that the A6V polymorphism may result in pathway-selective losses of function. MOPr inhibition of AC is mediated by $G\alpha_{i/o}$ subunits of

the G-protein heterotrimer, while GIRK are activated by Gβγ subunits (Law *et al.*, 2000). Phosphorylation of ERK1/2 can occur via multiple pathways involving Gα or Gβγ-coupled processes as well as G-protein independent pathways mediated by effectors such as β-arrestin (Luttrell, 2005). It is not known which subtypes of $G_{i/o}$ protein couple MOPr to inhibition of AC or activation of ERK in CHO cells, or activation of GIRK in AtT-20 cells. Thus, it is unclear if the selective loss of efficacy for A6V in CHO cells is because the MOPr-A6V couples less well to Gα-mediated process than Gβγ-mediated signalling, or whether the mutation disrupts receptor interactions with a subtype of G-protein that is responsible for inhibition of AC and/or stimulation of ERK phosphorylation in CHO cells, but which is not involved in activation of GIRK in AtT-20 cells. The apparently greater loss of efficacy for ligands such as buprenorphine at MOPr-A6V when signalling to AC and ERK rather than GIRK may also reflect functional selectivity towards GIRK activation rather than inhibition of AC or stimulation of ERK1/2 phosphorylation. However, studies aimed at establishing such functional selectivity should ideally be carried out in the same cell lines. Each of the assays utilized here is relatively rapid, with a maximum 5 min time-course, but a degree of opioid receptor desensitization can occur over this time (Borgland *et al.*, 2003; Connor *et al.*, 2004). Conceivably, a reduction in AC inhibition or pERK levels at 5 min could represent enhanced receptor desensitization. However, we have not seen any evidence for enhanced desensitization of MOPr-A6V activation of GIRK at 5 min (M. Santiago and M. Connor, unpubl. obs.), and no differences in morphine or DAMGO-induced internalization were reported for A6V expressed on the MOPr1A backbone (Ravindranathan *et al.*, 2009), suggesting that agonist-induced regulation of MOPr-A6V is not obviously accelerated compared with MOPr-WT.

The differences in signalling between wild-type MOPr and the A6V variant could be of clinical significance. The A6V variant markedly decreased the effect of a number of clinically prescribed opioids including morphine, one of the most commonly prescribed potent analgesics worldwide (Hanks *et al.*, 2001; Pergolizzi *et al.*, 2008; Zernikow *et al.*, 2009). Furthermore, the effects of the endogenous opioid peptides, β-endorphin and Met-enkephalin, as well as endomorphins-1 and 2, were significantly compromised at MOPr-A6V. Some studies have suggested a higher frequency of the A6V variant in substance-abusing populations, and disruption of the normal function of the endogenous opioid system caused by the A6V variant could conceivably contribute to a higher predisposition to substance abuse (Berrettini *et al.*, 1997; Rommelspacher *et al.*, 2001; Comptom *et al.*, 2003; Crowley *et al.*, 2003; Crystal *et al.*, 2010).

Our results demonstrate that the A6V variant potentially affects MOPr function in different cell types and across various signalling pathways. Most carriers of the 17T allele will be heterozygous with 17C, and at present it is not known how co-expressed MOPr-WT and MOPr-A6V receptors will signal. There is still considerable uncertainty about whether MOPr and other Class A GPCRs form obligate dimers in native systems (Herrick-Davis *et al.*, 2013; Malik *et al.*, 2013), so whether cells from people who are heterozygotes for the C17T SNP will have a mixture of homo- and heterodimers, or two populations of receptor that signal independently, is unknown. To date, the effect of MOPr variants expressed together with MOPr-WT is largely unexplored, for the most part because of the technical difficulties involved in co-expressing equivalent amounts of each variant. Ravindranathan *et al.* (2009) co-expressed HA-tagged MOPr-WT and FLAG-tagged MOPr variants in HEK293 cells and reported that MOPr-WT and the SNP MOPr-L85I both internalized in response to morphine, in contrast to the lack of internalization of MOPr-WT in response to morphine when MOPr-WT was expressed alone. This suggests the formation of heterodimers with L85I showing a dominant phenotype. On the other hand, co-expression of MOPr-WT and MOPr-R181C resulted in the expected DAMGO-induced internalization of MOPr-WT independent of MOPr-181C, indicating that MOPr-181C either fails to form dimers or forms unstable dimers with MOPr-WT (Ravindranathan *et al.*, 2009). Potential interactions between WT- and A6V-MOPr require further examination, and the assessment of opioid responses in A6V carriers would also be of great interest.

The A6V variant is quite prevalent in some populations, with allelic frequencies of up to 20% (Rommelspacher *et al.*, 2001; Crowley *et al.*, 2003; Tan *et al.*, 2003; Kapur *et al.*, 2007). If the decrease in efficacy of opioid analgesics seen in CHO-MOPr-A6V cells were to be translated functionally in A6V carriers, it could result in a significant number of people receiving inadequate or inappropriate analgesic therapy. Understanding how the A6V SNP affects opioid signalling is an important part in predicting individual response to opioids. Although an individual's response to opioids will be influenced by numerous genetic and epigenetic factors, knowledge of the effect of individual MOPr genotypes on opioid responses could provide valuable insight into what the most effective form of analgesic therapy might be, particularly in the case of A6V carriers, where opioid efficacy may be significantly diminished. Understanding how potential disruptions in endogenous opioid signalling affect individual phenotype could also be beneficial in elucidating the role of endogenous opioids in physiological processes such as nociception, reward and addiction.

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Author contributions

A. K., M. S. and M. C. designed and analysed experiments. A. K. and M. S. conducted the experiments. A. K. and M. C. conceived the study and wrote the paper. All authors have seen the final paper.

Conflict of interest

The authors declare they have no conflicts of interest associated with this work.

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