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RESEARCH PAPER

Morphine-induced internalization of the L83I mutant of the rat μ-opioid receptor

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BACKGROUND AND PURPOSE

Naturally occurring single-nucleotide polymorphisms (SNPs) within GPCRs can result in alterations in various pharmacological parameters. Understanding the regulation and function of endocytic trafficking of the μ-opioid receptor (MOP receptor) is of great importance given its implication in the development of opioid tolerance. This study has compared the agonist-dependent trafficking and signalling of L83I, the rat orthologue of a naturally occurring variant of the MOP receptor.

EXPERIMENTAL APPROACH

Cell surface ELISA, confocal microscopy and immunoprecipitation assays were used to characterize the trafficking properties of the MOP-L83I variant in comparison with the wild-type receptor in HEK 293 cells. Functional assays were used to compare the ability of the L83I variant to signal to several downstream pathways.

KEY RESULTS

Morphine-induced internalization of the L83I MOP receptor was markedly increased in comparison with the wild-type receptor. The altered trafficking of this variant was found to be specific to morphine and was both G-protein receptor kinase- and dynamin-dependent. The enhanced internalization of L83I variant in response to morphine was not due to increased phosphorylation of serine 375, arrestin association or an increased ability to signal.

CONCLUSIONS AND IMPLICATIONS

These results suggest that morphine promotes a specific conformation of the L83I variant that makes it more liable to internalize in response to morphine, unlike the wild-type receptor that undergoes significantly less morphine-stimulated internalization, providing an example of a ligand-selective biased receptor. The presence of this SNP within an individual may consequently affect the development of tolerance and analgesic responses.

LINKED ARTICLES

This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-2

Abbreviations

DAMGO, [D-Ala[2], *N*-MePhe[4], Gly-ol]encephalin; DNM, dominant negative mutant; GRK, G-protein receptor kinase; MOP receptor, μ-opioid receptor; SNP, single-nucleotide polymorphism

Introduction

Naturally occurring, non-synonymous single-nucleotide polymorphisms (SNPs) in a GPCR can result in modifications in surface receptor expression, as well as changes in receptor signalling and trafficking and alterations to ligand profiles (Seifert and Wenzel-Seifert, 2002; Conn *et al.*, 2007; Fortin *et al.*, 2010). Because of the central role of the μ-opioid receptor (MOP receptor; receptor nomenclature conforms to Alexander *et al.*, 2013) in analgesia and opioid drug abuse, numerous studies have investigated the potential contribution of alternative splicing and naturally occurring polymorphisms in the MOP receptor gene (*OPRM1*), to substance abuse susceptibility and inter-individual variability in pharmacological responses to opioid drugs (LaForge *et al.*, 2000; Lotsch and Geisslinger, 2005; Zhang *et al.*, 2005; Ravindranathan *et al.*, 2009; Mague and Blendy, 2010). Determining the effects of various SNPs can provide important insights into the signalling and regulation of MOP receptors.

The process of MOP receptor regulation has usually been considered to follow typical GPCR paradigms that include desensitization by means of G-protein receptor kinase (GRK)- and arrestin-dependent pathways. In addition to the regulation of MOP receptors by this canonical pathway, MOP receptors can also be desensitized by a heterologous, PKC-dependent mechanism (Bailey *et al.*, 2009b), and it is now thought that the mechanism of desensitization and internalization is determined in part by the efficacy of the opioid agonist (Bailey *et al.*, 2006; 2009a,b; Johnson *et al.*, 2006; Kelly *et al.*, 2008; Groer *et al.*, 2011). High-efficacy agonists such as [D-Ala^2, *N*-MePhe^4, Gly-ol] encephalin (DAMGO) and etorphine induce GRK- and arrestin-dependent MOP receptor desensitization and internalization, whereas morphine typically produces PKC-dependent desensitization with little or no internalization in cultured cells expressing either endogenous or recombinant MOP receptors as well as in mature neurones (Arden *et al.*, 1995; Keith *et al.*, 1998; Bailey *et al.*, 2003; 2004; von Zastrow *et al.*, 2003; Johnson *et al.*, 2006). However, the regulation of MOP receptors may yet be more complex; for example, it has been demonstrated that internalization of the MOP receptor is not a necessity for receptor resensitization (Arttamangkul *et al.*, 2006; Doll *et al.*, 2011). Further, data from arrestin-3 knockout mice show that agonist-induced acute MOP receptor desensitization is unaltered in these animals (Walwyn *et al.*, 2007; Arttamangkul *et al.*, 2008; Dang *et al.*, 2009; 2011; Quillinan *et al.*, 2011).

A rare naturally occurring non-synonymous SNP in the human MOP receptor, L85I (allelic frequency: 0.002), has previously been identified in the San Diego Sibling Pair study. This study consisting of a cohort of 550 subjects aged 18–29, aimed to identify families with a high prevalence of alcohol susceptibility genes (Wilhelmsen *et al.*, 2003; Schuckit *et al.*, 2005). The L85I variation occurs in transmembrane one of the receptor and has been reported to promote receptor internalization in response to acute morphine stimulation, an effect not seen in the wild-type (WT) receptor (Ravindranathan *et al.*, 2009). Additionally, the L85I variant receptor is reported by the same authors to be resistant to both morphine tolerance and cAMP superactivation following prolonged morphine exposure when stably expressed in HEK 293 cells. In the current study we investigated in detail the effect of this mutation on MOP receptor function by comparing the molecular regulation of trafficking and signalling of the L83I (rat orthologue of L85I) variant with that of the WT-MOP receptor. The results indicate that the mutation induces a highly selective increase in morphine-induced trafficking of the MOP receptor, which is both GRK- and dynamin-dependent.

Methods

Cell culture, DNA constructs and transfection

HEK 293 cells were maintained in DMEM supplemented with 10% FCS and 100 U mL^{-1} penicillin-streptomycin at 37°C in a humidified atmosphere of 95% air, 5% CO_2. The cloning and stable expression of the rat MOP receptor tagged at the N-terminus with haemagglutinin (HA)-tag in HEK 293 cells has previously been described (Rivero *et al.*, 2012). The GeneTailor™ Site-Directed Mutagenesis System Kit (Invitrogen, Paisley, UK) was used to introduce the L83I point mutation into the pcDNA3.1-HA-MOP receptor construct. The nucleotide sequence of the receptor coding region was confirmed by DNA sequencing. The construct was transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen), and stable transfectants selected in the presence of 600 μg·mL^{-1} geneticin. Single clones were isolated after 2–3 weeks of culture and screened for expression by surface receptor ELISA and radioligand binding assays. An appropriate clone with similar receptor expression to the HEK-MOP receptor cell line was selected for further experimentation. For culture of HEK-MOP receptor and HEK-MOP-L83I receptor cells, the media were supplemented with 250 and 400 μg·mL^{-1} geneticin respectively. For transient transfection, HEK 293 cells were grown in 60 or 100 mm dishes to 70–90% confluence and transfected with 3–5 μg of DNA using Lipofectamine 2000 (Invitrogen).

Receptor internalization assessed by ELISA

Changes in cell surface expression of HA-tagged receptors were assessed by ELISA as described previously (Mundell *et al.*, 2006). For investigations into the constitutive internalization, cells were pre-labelled with primary antibody at 4°C for 1 h before incubation with serum-free media for 0–30 min at 37°C. Changes in surface receptor expression were subsequently determined by normalizing data from each treatment group to corresponding control surface receptor levels determined from cells not exposed to opioid agonists.

Immunofluorescence microscopy

Cellular distribution of HA-tagged receptors was assessed by immunofluorescence microscopy in HEK 293 cells as described previously (Mundell *et al.*, 2006). Cells were imaged using a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM16000 inverted epifluorescence microscope with a pLApoBL 63× oil immersion objective.

FRET assays

HEK 293 cells were transfected with 0.6 μg MOP receptor-YFP or MOP-L83I receptor-YFP, respectively, 0.6 μg GRK2 and 0.8 μg Arr3-CFP using Effectene (Qiagen, Hilden, Germany). Cells were seeded on round polylysine-coated coverslips

(25 mm diameter). Forty-eight hours after transfection, coverslips were observed using an imaging setup as described previously (Milde *et al.*, 2013), except that the light source was a CoolLed pE-2 (CoolLed Ltd., Andover, UK). Cells were illuminated with 425 nm light for 40–60 ms with a frequency of 2 Hz. Fluorescence was measured at 488 ± 20 nm (F_{488}) and 534 ± 10 nm (F_{534}). After acquisition, images were corrected for background fluorescence, and the F_{534} signal was additionally corrected for bleed-through from the F_{488} fluorescence, yielding F_{CFP} and F_{YFP} respectively. F_{CFP}/F_{YFP} is the 'FRET ratio', and for averaging, this is arbitrarily set to 0 at a defined time point (1 s before starting with the morphine perfusion), giving $\Delta F_{CFP}/F_{YFP}$. On- and off-rates were fitted to monoexponential functions using GraphPAD Prism (GraphPAD Software, CA, USA). If a fit showed a $R^2 < 0.5$ or the error of the determined time constant was >10%, it was discarded.

Immunoprecipitation

Detection of serine 375 phosphorylation by immunoprecipitation and Western blotting was carried out as previously described (Rivero *et al.*, 2012). The extent of Ser^{375} phosphorylation and receptor immunoprecipitation was determined by densitometric analysis of resulting autoradiographs.

Co-immunoprecipitation and cross-linking

Co-immunoprecipitation of the MOP receptor and arrestins was performed using a protocol previously described (Groer *et al.*, 2007) with some modifications. Cells were washed three times with PBS, pH 8 and then treated with agonist for 5 min at 37°C. The cross-linking reagent, disuccinimidyl suberate (DSS; 1 mM; Thermo Fisher Scientific, Hemel Hempstead, UK) was added to the cells and incubated at room temperature for 30 min with gentle agitation. The reaction was quenched by the addition of 1M Tris, pH 7.4 (50 mM final concentration). Cells were collected and washed four times with TBS and resuspended in co-immunoprecipitation lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM PMSF, 1 mM sodium orthanovandate, 1 mM NaF, and complete protease inhibitors (Roche Diagnostics, Mannheim, Germany)] and solubilised overnight at 4°C. Cell lysates were clarified by centrifugation at 9660× *g* and then pre-cleared by incubation with protein G/A-agarose at 4°C for 1 h. Receptors were immunoprecipitated with anti-HA conjugated agarose beads (Sigma, Gillingham, UK) for 2 h at 4°C. Beads were washed three times with co-immunoprecipitation lysis buffer and proteins were eluted by the addition of 200 mg·mL^{-1} HA peptide (Sigma, Poole, UK) before the addition of NuPAGE LDS sample buffer (Invitrogen) supplemented with 50 mM DTT and boiling. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with a polyclonal pan-arrestin antibody (1:500; Abcam, Cambridge, UK), and then stripped and reprobed with a monoclonal anti-HA (1:1000; Covance; Cambridge Bioscience, Cambridge, UK). Proteins were detected by enhanced chemiluminescence.

Phospho-ERK1/2 immunoblot assay

Cells were seeded into 12-well plates and deprived of serum for 18 h before agonist treatment. Following agonist exposure, cells were washed twice with ice-cold PBS and lysed directly into 1X NuPAGE LDS sample buffer (Invitrogen) supplemented with 50 mM DTT. The cells were scraped on ice, and samples were repeatedly drawn through a 21-G needle and syringe before being boiled for 3 min. Proteins were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted for phosphorylated ERK (phosphor-p44/42 MAPK; 1:1000: Cell Signalling; New England Biolabs, Hitchin, UK). Blots were stripped and reprobed for total ERK (p44/42 MAPK; 1:1000; Cell Signalling) to normalize the extent of phosphorylated ERK among the samples by densitometry.

cAMP competitive enzyme immunoassay

cAMP measurements were performed using the Direct cAMP enzyme immunoassay kit (Sigma), according to the protocol supplied by the manufacturer. For inhibition of AC, cells were seeded into 12-well tissue culture dishes coated with 0.1 mg·mL^{-1} poly-L-lysine for 24 h before experimentation. Cells were pre-incubated with 100 μM IBMX for 3 min before incubation with increasing concentrations of either DAMGO or morphine (1 nM–10 μM) in the presence of 10 μM forskolin in 500 μL serum-free DMEM for 5 min at 37°C. The reaction was stopped by replacing the media with 0.1 M HCl. After a 20 min incubation, the lysates were centrifuged at 600× *g* for 2 min, and the supernatant was used directly in the assay. Each sample was assayed in duplicate. Inhibition of cAMP formation was calculated as % inhibition of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

Ligand binding assay

Cells were grown in 75 cm^2 flasks until approximately 90% confluent. The cells were washed twice with ice-cold PBS before being dislodged with cell dissociation buffer (Life Technologies, Paisley, UK), pelleted by centrifugation at 377× *g* for 3 min at 4°C. Membranes were prepared as previously described (McPherson *et al.*, 2010).

Membrane pellets were resuspended in assay buffer (100 mM Tris, 200 mM NaCl, 8 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, O.2% (w v^{-1}) BSA, pH 7.4), and 10 μg of membrane protein was incubated with increasing concentrations of [^{3}H]-naloxone (0.06–30 nM). Non-specific binding was determined by carrying out parallel determinations in the presence of excess unlabelled naltrexone (10 μM). All binding reactions were prepared in 500 μL volumes and performed in duplicate. The binding reaction was allowed to proceed for 2 h at room temperature with gentle agitation. Reactions were terminated by the addition of 3 mL of ice-cold wash buffer (50 mM Tris, pH 7.4) and rapid filtration under vacuum through glass fibre (GF/B) filters followed using a Brandel cell harvester. The amount of bound [^{3}H]-naloxone to membranes on individual filters was quantified by liquid scintillation counting.

[^{35}S]-GTPγS binding

For the [^{35}S]-GTPγS binding assay membranes were prepared as for the ligand binding assay. Experiments were carried out as previously described (Johnson *et al.*, 2006). To calculate the amount of agonist-induced binding, non-specific binding was subtracted from all dpm values obtained. The data were then normalized to basal values.

Data analysis

All data are presented as mean ± SEM. Data were analysed using GraphPAD Prism. The EC_{50} and maximum response

values (E_{max}) for concentration–response curves were obtained by fitting data from individual experiments to sigmoidal curves with a variable slope, with mean and SEM derived from individual values obtained from each experiment. Statistical significance was assessed using one of the following; Student's one-sample *t*-test, Student's unpaired two-tailed *t*-test, a one-way or two-way ANOVA followed by Bonferroni's post-test as indicated.

Materials

The GRK2-K220R dominant negative mutant (DNM) has been described previously (Johnson *et al.*, 2006). Tissue culture media and reagents were purchased from Invitrogen. The GRK 2 (C-15) rabbit polyclonal antibody was from Santa Cruz Biotechnology (Heidleberg, Germany). DAMGO was purchased from Bachem AG (Bubendorf, Switzerland), morphine HCl from (Mcfarlane Smith, Edinburgh, UK), etorphine hydrochloride from (RTI NIDA, Research Triangle Park, NC, USA), while forskolin and IBMX were from Tocris Bioscience (Bristol, UK). [^{35}S]-GTPγS (462.5 MBq·mL^{-1}) and [^{3}H]-naloxone (37 MBq·mL^{-1}) were purchased from Perkin Elmer (Beaconsfield, UK). All other biochemical reagents were obtained from Sigma.

Results

Internalization of MOP-L83I receptors in response to morphine

Initial experiments investigated the ability of peptide and non-peptide opioid agonists to induce internalization of the MOP-L83I variant. HEK 293 cells were transiently transfected with either HA-tagged WT-MOP receptors or the MOP-L83I variant to investigate if there were any differences in agonist-stimulated receptor internalization (Figure 1A). The rate of internalization of the MOP receptor has been reported with time constants ($t_{1/2}$) generally in the order of less than 5 min and reaching steady state by 30 min (Law *et al.*, 2000; Borgland *et al.*, 2003; Johnson *et al.*, 2006). Receptor saturating concentrations of each agonist were therefore applied for 5 min in these experiments to allow sufficient time for receptor internalization without measurements being confounded by receptor recycling (Alvarez *et al.*, 2002; Borgland *et al.*, 2003). In agreement with previous studies (Borgland *et al.*, 2003; Johnson *et al.*, 2006; Ravindranathan *et al.*, 2009), the WT-MOP receptor remained primarily on the cell surface after activation with morphine (30 μM; 1.6 ± 7.8% internalization) whereas activation with either DAMGO (10 μM) or etorphine (10 μM) induced substantial internalization (33.9 ± 3.0% and 28.1 ± 5.5% internalization, respectively). In contrast, the MOP-L83I variant, internalized significantly in response to morphine (27.2 ± 4.1% internalization; $P < 0.05$), in addition to internalizing to both DAMGO and etorphine to an extent comparable with that of the WT-MOP receptor (36.8 ± 4.3% and 33.8 ± 5.0% internalization, respectively). No significant differences were observed in the internalization of the MOP-L83I receptor compared to the WT-MOP receptor induced by leu-enkephalin (10 μM), or by pentazocine (10 μM) or buprenorphine (10 μM), the latter two of which produced relatively little internalization of either receptor construct (Figure 1A). Further, the extent of internalization induced by a submaximal concentration of DAMGO (300 nM) was found to be similar between the WT-MOP receptors and MOP-L83I variant (data not shown).

To assess whether the observed increase in morphine-induced internalization of the MOP-L83I variant receptor could be explained in part by an increase in constitutive internalization, we assessed the time course of constitutive internalization for both HA-tagged WT-MOP and MOP-L83I receptors by ELISA in HEK 293 cells stably expressing either receptor construct. Both the WT-MOP and MOP-L83I receptor underwent a small amount of constitutive internalization (Figure 1B); however, no significant difference was observed in the constitutive internalization of the MOP-L83I variant in comparison with WT-MOP receptor.

To investigate these internalization profiles further, we studied the cellular distribution of the HA-tagged WT-MOP receptors and MOP-L83I receptors by confocal immunofluorescence microscopy. As depicted in Figure 1C, the HA-tagged receptors (as visualized with a fluorescein-conjugated secondary antibody) were localized at the plasma membrane before agonist stimulation (Figure 1C, upper panels). The WT-MOP receptor displayed little internalization in response to morphine stimulation (30 μM; 5 and 30 min). However, the variant displayed substantial internalization in response to both DAMGO and morphine as demonstrated by the formation of punctate spots within the cytosol of the cell, supporting the ELISA data.

Mechanisms of agonist-induced internalization of MOP-L83I receptors

To investigate the molecular mechanisms that may mediate the differences observed in morphine-induced internalization of the MOP-L83I receptor in comparison with that of the WT-MOP receptor, we employed both pharmacological and molecular approaches. We began initially using dynasore, a cell-permeable small molecule that inhibits dynamin (Macia *et al.*, 2006), to evaluate the dynamin-dependency of WT-MOP and MOP-L83I receptor internalization. HEK 293 cells transiently transfected with either HA-tagged WT-MOP or MOP-L83I receptors were pre-incubated with either DMSO (0.05%) or dynasore (40 μM) 15 min before stimulation with either DAMGO (10 μM; 5 min) or morphine (30 μM; 5 min). Changes in cell surface receptor expression were assessed by ELISA. As shown in Figure 2A, pretreatment with dynasore effectively attenuated the DAMGO-induced internalization of both WT-MOP and MOP-L83I receptors, in addition to inhibiting morphine-induced internalization of MOP-L83I receptors. Pre-incubation with dynasore had no apparent effect on the small amount of morphine-induced internalization of the WT-MOP receptor.

To investigate the GRK-dependency of internalization of MOP-L83I receptors we overexpressed a GRK2-DNM (K220R), which is devoid of kinase activity and has been shown to selectively antagonise GRK2 (Ghadessy *et al.*, 2003; Johnson *et al.*, 2006). HEK 293 cells were transiently transfected with either HA-tagged WT-MOP or MOP-L83I receptors, with or without the GRK2-DNM and challenged with either DAMGO (10 μM; 5 min) or morphine (30 μM; 5 min). Expression of the GRK2-DNM inhibited the internalization of the WT-MOP receptor in response to DAMGO (Figure 2B), but

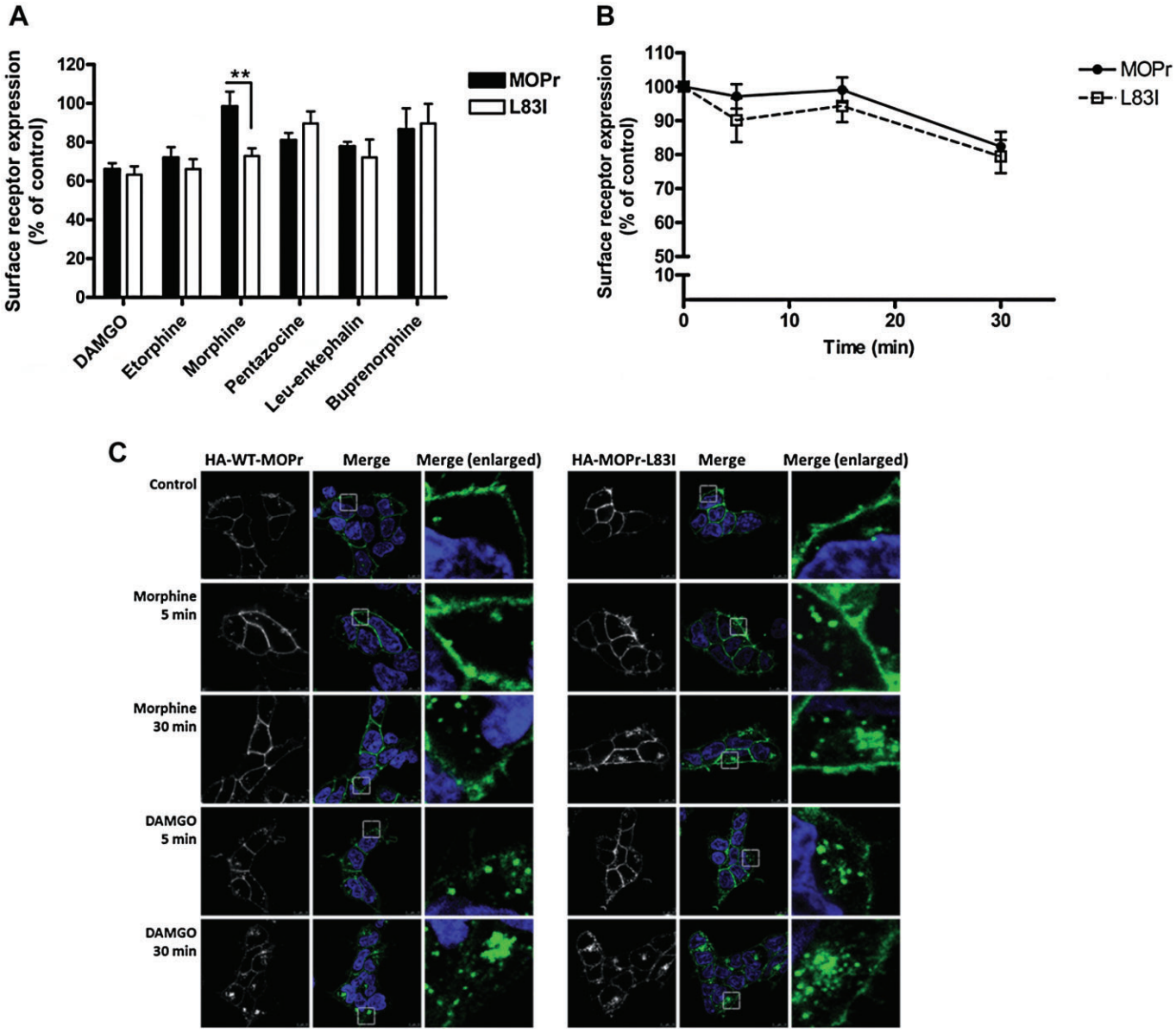

Figure 1

Morphine induces internalization of the MOP-L83I receptor (L83I) variant in comparison with the WT-MOP receptor (MOPr). Internalization (expressed as a percentage of surface expression in untreated cells) was assessed by ELISA in HEK 293 cells transiently transfected with either HA-tagged WT-MOP or MOP-L83I receptor. (A) Cells were challenged with DAMGO (10 μM), morphine (30 μM), etorphine (10 μM), pentazocine (10 μM), leu-enkephalin (10 μM) or buprenorphine (10 μM) for 5 min to induce internalization. Data represent mean ± SEM (n = 3–6). (B) Time course of constitutive internalization. HEK 293 cells stably expressing HA-tagged WT-MOP or MOP-L83I receptors were incubated with anti-HA antibody to label surface receptors at 4°C. Cells were washed before incubation with serum-free media for 0–30 min at 37°C. Data represent mean ± SEM (n = 4). For (A) and (B), statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (*P < 0.05). (C) Receptor distribution assessed by immunofluorescence confocal microscopy. Cell surface receptors were labelled at 4°C with anti-HA antibody before stimulation with morphine (30 μM) or DAMGO (10 μm) for 5 or 30 min at 37°C. Fluorescein-conjugated secondary antibody was used to visualize HA-tagged receptors (green) and DAPI to stain nuclei (blue), higher magnification inserts are included from regions within the cells, identified by the white boxes. Data are representative confocal images from three independent experiments.

had little effect on the small amount of morphine-induced internalization of the WT-MOP receptor. Additionally, the GRK2-DNM inhibited internalization of the MOP-L83I variant in response to DAMGO, for the morphine-induced internalization of the variant a similar inhibitory trend was observed, although this did not reach statistical significance. However, the extent of morphine-induced internalization of the variant was found to be significantly different from controls in the absence but not in the presence of the GRK2-DNM (one-sample t-test; P < 0.05). Overexpression of the GRK2-DNM was confirmed by Western blot analysis in each experiment (Figure 2C).

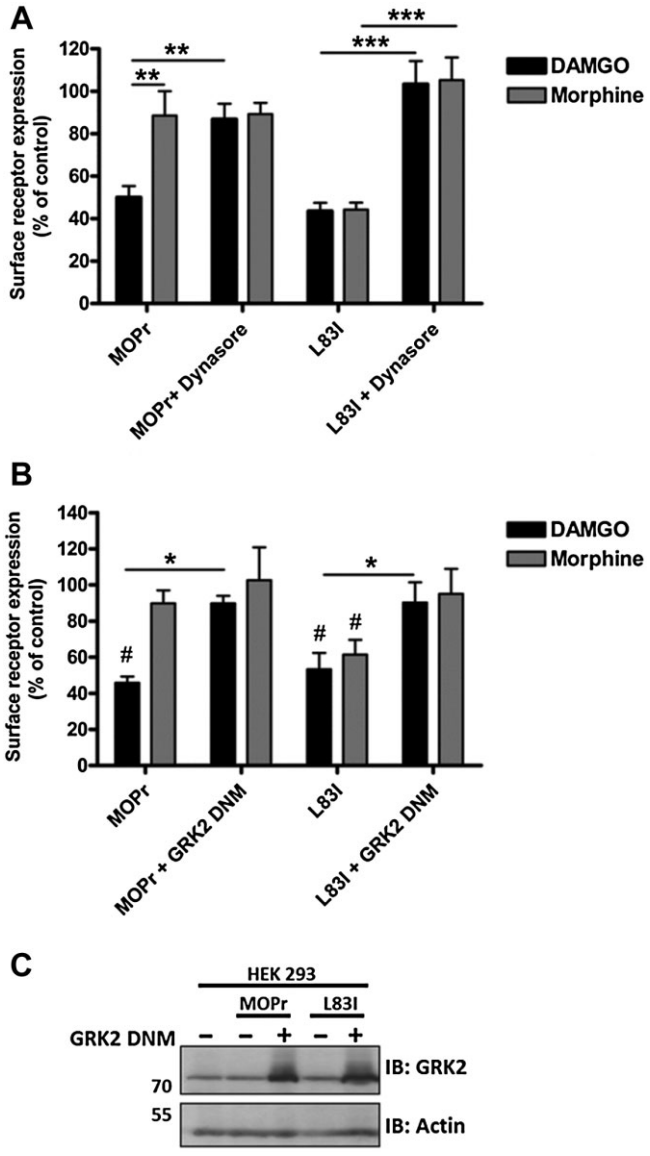

Figure 2

Dynamin- and GRK2-dependence of morphine-induced internalization of MOP-L83I receptors (L83I). (A) HEK 293 cells transiently transfected with HA-WT-MOP or HA-MOP-L83I receptors were incubated with media containing 0.05% (v v^{-1}) DMSO or Dynasore (40 μM) for 15 min before agonist stimulation. (B) HEK 293 cells transiently transfected with HA-MOP or HA-MOP-L83I receptors with or without a GRK2 DNM (K220R). Cells were challenged with either DAMGO (10 μM) or morphine (30 μM) for 5 min to induce internalization. Changes in cell surface receptor expression were determined by ELISA. (C) Representative immunoblot of GRK2-DNM overexpression. Data represent mean ± SEM (n = 3). Statistical analysis was performed either by two-way ANOVA followed by Bonferroni *post hoc* test (*P < 0.05; **P < 0.01; ***P < 0.001), or by one-sample t-test (#P < 0.05) to compare the statistical difference from control values.

Of the 20 potential phosphorylation sites present in the intracellular loops and C-terminus of the MOP receptor (Chavkin *et al.*, 2001), serine 375 in the C-terminal tail has been the most extensively studied and identified as a probable target for GRK-mediated phosphorylation of the receptor (Schulz *et al.*, 2004; Zhang *et al.*, 2009; Lau *et al.*, 2011). Using a commercially available anti-phosphoserine 375 antibody we assessed the ability of either DAMGO (10 μM; 5 min) or morphine (30 μM; 5 min) to induce phosphorylation of this residue in HEK 293 cells stably expressing either HA-tagged WT-MOP or MOP-L83I receptors. As shown in Figure 3A and quantified in Figure 3B, DAMGO induced substantial phosphorylation of this residue in both the WT-MOP receptor and the MOP-L83I variant whereas, morphine induced far less phosphorylation of this residue in both the WT-MOP receptor and the MOP-L83I variant.

Arrestins are known to be recruited to activated and GRK-phosphorylated GPCRs, where they function to uncouple the receptor from its cognate G-protein and also recruit the receptor to clathrin-coated pits for internalization (Gainetdinov *et al.*, 2004; Jean-Alphonse and Hanyaloglu, 2011). The ability of the MOP-L83I receptor to recruit arrestin following agonist stimulation in comparison with the WT-MOP receptor was investigated using FRET assays in intact cells. HEK 293 cells were transiently co-transfected with either WT-MOP receptor-YFP or MOP-L83I receptor-YFP, respectively, arrestin-3-CFP and GRK2; 48 h later, FRET was monitored following morphine (30 μM) and DAMGO (10 μM) application. The GRK2 was included in order to enhance the FRET signal. Analysis of the agonist-induced FRET ratio showed that both DAMGO and morphine induced a rapid increase in the FRET signal although morphine produced a smaller increase in the FRET ratio in comparison with DAMGO. However, there was no difference in the association between arrestin-3-CFP and the WT-MOP receptor or the L83I variant (Figure 3C). Further, no significant difference was observed in the kinetic parameters for the interaction between arrestin-3 and the receptors (Supporting Information Table S1). To further investigate the interaction between arrestins and the receptors in the absence of overexpressed arrestin or GRK2, co-immunoprecipitation assays were performed (Figure 3D). HEK 293 cells stably expressing either HA-tagged WT-MOP or MOP-L83I receptors were stimulated with either DAMGO (10 μM; 5 min) or morphine (30 μM; 5 min), cellular proteins were then cross-linked with DSS (1 mM) to stabilize interactions, the HA-tagged receptors immunoprecipitated, and proteins then resolved by SDS-PAGE. DAMGO induced arrestin association with both the WT-MOP and the MOP-L83I receptor. The extent of arrestin association was repeatedly found to be comparable between the two receptors. Using this protocol, we were unable to detect any morphine-induced arrestin association with either the WT-MOP receptor or MOP-L83I variant.

Functional analysis of the MOP-L83I variant

Saturation binding of [^{3}H]-naloxone to membranes of HEK 293 cells stably expressing either HA-tagged receptor construct was initially performed (Figure 4A). B_{max} values for the WT-MOP and MOP-L83I receptor were calculated to be 0.95 ± 0.12 pmol·mg^{-1} and 1.46 ± 0.23 pmol·mg^{-1} respectively. To assess if the observed increase in morphine-induced internalization of MOP-L83I receptors was accompanied by changes in G-protein signalling, we utilized both a [^{35}S]-GTPγS binding assay and a cAMP assay. We initially investigated the ability

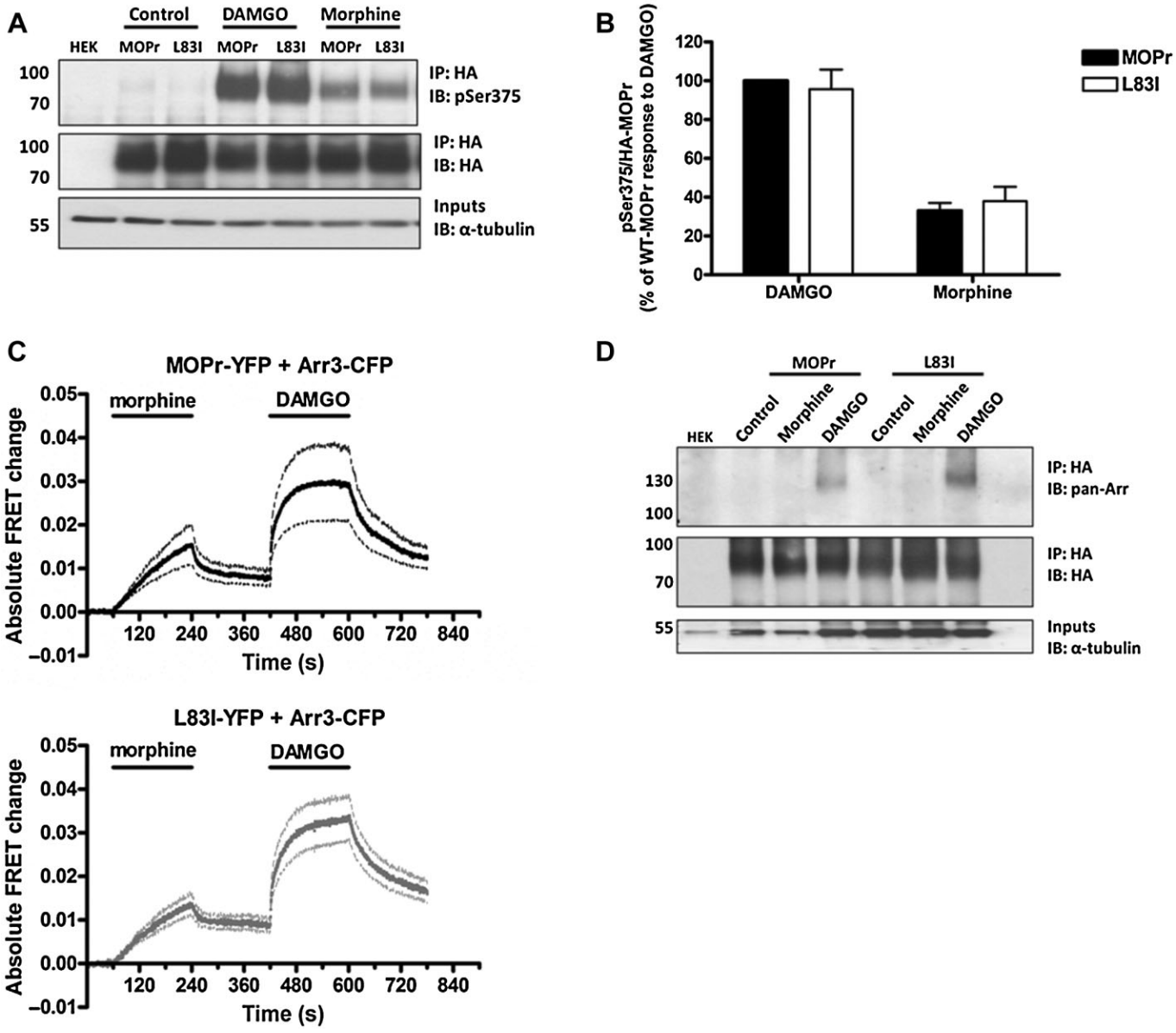

Figure 3

Comparison of agonist-induced phosphorylation of serine 375 and arrestin association with the WT-MOP receptor (MOPr) and MOP-L83I receptor (L83I). HEK 293 cells stably expressing either construct were stimulated with either DAMGO (10 μM) or morphine (30 μM) for 5 min. HA-tagged receptors were immunoprecipitated using anti-HA monoclonal antibody and proteins were resolved by SDS-PAGE. (A) Changes in receptor phosphorylation at serine 375 were evaluated using an anti-phosphoserine 375 specific antibody (pSer375). Blots were then stripped and reprobed to assess the relative expression levels and pull-down efficiency of each of the receptor constructs using an anti-HA antibody. The expression of α-tubulin from the whole-cell lysates was used as a loading control. (B) Densitometric analysis of DAMGO- and morphine-induced phosphorylation of serine 375, data are shown as the percent of the DAMGO-induced phosphorylation of the WT-MOP receptor and represent mean ± SEM ($n = 5$). Statistical analysis was performed by either a one-sample *t*-test to compare DAMGO stimulation between receptor constructs or by unpaired two-tailed Student's *t*-test for comparing the effects of morphine. No statistical significance was observed. (C) HEK 293 cells transiently transfected with WT-MOP-YFP or MOP-L83I-YFP receptors, GRK2 and arrestin-3-CFP were perfused with 30 μM morphine followed by 10 μM DAMGO. FRET between the receptor constructs and arrestin-3 was measured every 0.5 s with an epifluorescence microscope. Shown are averaged traces for WT-MOP-YFP receptors ($n = 15$ cells) or MOP-L83I-YFP receptors ($n = 14$ cells). The kinetics of arrestin-receptor interaction was found to be similar for both the WT-MOP and MOP-L83I receptors (Supporting Information Table S1). (D) HEK 293 cells stably expressing HA-MOP or HA-MOP-L83I receptors were stimulated with either DAMGO (10 μM) or morphine (30 μM) for 5 min. Proteins were cross-linked using a cell-permeable cross-linking reagent DSS (1 mM), before receptors were immunoprecipitated with anti-HA-agarose beads, and the proteins resolved by SDS-PAGE. Co-immunoprecipitation of arrestins was assessed with a pan-arrestin antibody (pan-Arr), blots were then stripped and reprobed with anti-HA antibody to assess total receptor content. Shown is a representative immunoblot ($n = 3$). Densitometry was not performed for these experiments because of the effects of irreversible cross-linking on the receptor immunoblots, which results in the formation of multiple immunoreactive bands complicating densitometric analysis, and the low signal-to-noise ratio of the pan-arrestin immunoblots.

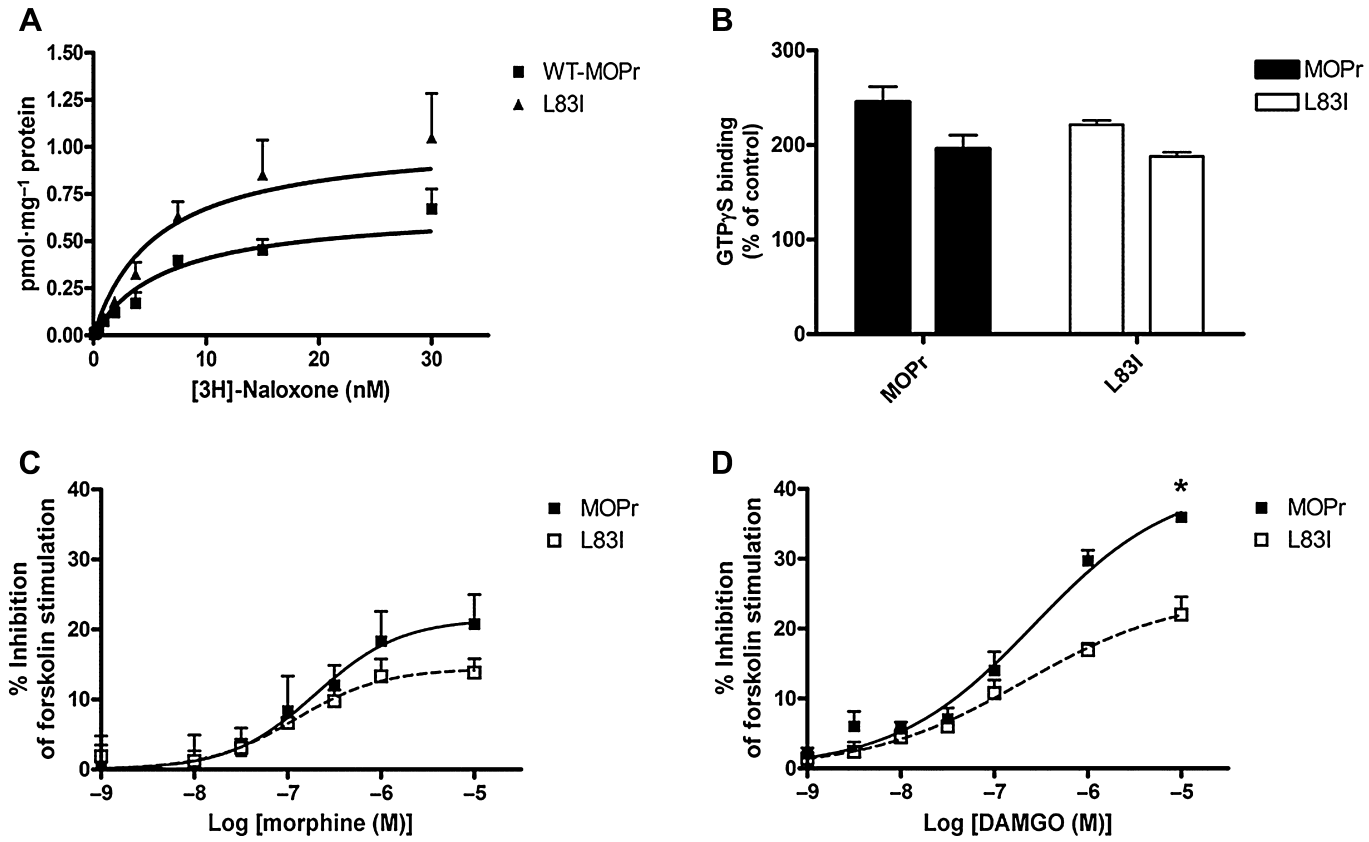

Figure 4

Comparison of G–protein-mediated signalling for WT-MOP receptors (MOPr) and MOP-L83I receptors (L83I). All experiments were performed in HEK 293 cells stably expressing either HA-WT-MOP or HA-MOP-L83I receptors. (A) Saturation analysis of [^{3}H]-naloxone binding. Membrane preparations were incubated for 2 h at 25°C with various concentrations of [^{3}H]-naloxone (0.06–30 nM). Naltrexone (10 μM) was used to determine non-specific binding. Experiments were performed in duplicate and data represent mean ± SEM (n = 4). For graphical representation only, the B_{max} values have been constrained to the mean maximum response for each of the receptors obtained in the four experiments. (B) Binding of [^{35}S]-GTPγS stimulated by 30 min application of either DAMGO (10 μM) or morphine (30 μM). Control was basal [^{35}S]-GTPγS binding. Experiments were performed in triplicate, data represent mean ± SEM (n = 3). Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test. (C and D) Inhibition of forskolin stimulated cAMP accumulation by increasing concentrations of morphine or DAMGO. Cells were pre-incubated with IBMX (100 μM) for 3 min before stimulation with forskolin (10 μM) and either morphine or DAMGO (0.001–10 μM) for 5 min at 37°C. The accumulation of cAMP was measured by competitive enzyme immunoassay. Data were fit to a sigmoidal curve with variable slope using GraphPad Prism. Experiments were performed in duplicate and represent mean ± SEM (n = 4). The agonist minimum response in each case was constrained to zero. Statistical analysis of difference between the EC_{50} values and maximum inhibition (E_{max}) was assessed using Student's unpaired two-tailed t-test (*P < 0.01). EC_{50} values for morphine (WT-MOP receptors: 194 ± 48 nM; MOP-L83I receptors: 125 ± 25 nM) and DAMGO (WT-MOP receptors: 185 ± 28 nM; MOP-L83I receptors: 290 ± 110 nM) were not significantly different.

of supramaximal concentrations of DAMGO or morphine to activate [^{35}S]-GTPγS binding. As shown in Figure 4B, both DAMGO (10 μM) and morphine (30 μM) stimulated [^{35}S]-GTPγS binding. The extent of DAMGO-induced [^{35}S]-GTPγS binding was similar for both the WT-MOP and MOP-L83I receptor. Morphine-induced binding of [^{35}S]-GTPγS was also similar for the wild type and mutant variant. To further support the [^{35}S]GTPγS binding assay experiments, we assessed the ability of morphine and DAMGO (1 nM–10 μM; 5 min) to inhibit forskolin-stimulated cAMP accumulation. As a control we confirmed that forskolin stimulated cAMP accumulation to a comparable level in both stable cell lines (WT-MOP receptor: 2.11 ± 0.06 pmol·mL^{-1} cAMP; MOP-L83I receptor: 2.09 ± 0.04 pmol·mL^{-1} cAMP). Stimulation with either morphine or DAMGO resulted in concentration-dependent inhibition of forskolin-stimulated cAMP accumulation (Figure 4C and D). The EC_{50} values of both morphine and DAMGO were similar for both the WT-MOP receptor and the L83I variant. However, we did observe a difference in the maximum inhibition. The maximum extent of inhibition produced by DAMGO was significantly greater for the WT-MOP receptor (38.7 ± 2.5%) in comparison with the MOP-L83I receptor (25.2 ± 2.4%; P < 0.01), despite the fact that the MOP-L83I stable cell line actually expressed higher levels of receptor (~1.5-fold).

In order to examine MOP receptor signalling distal to G-protein activation, we initially studied the time course of morphine-induced phosphorylation of MAPK (ERK1/ERK2). Morphine (30 μM) induced a transient activation of ERK (Figure 5A), with the time course of activation being similar

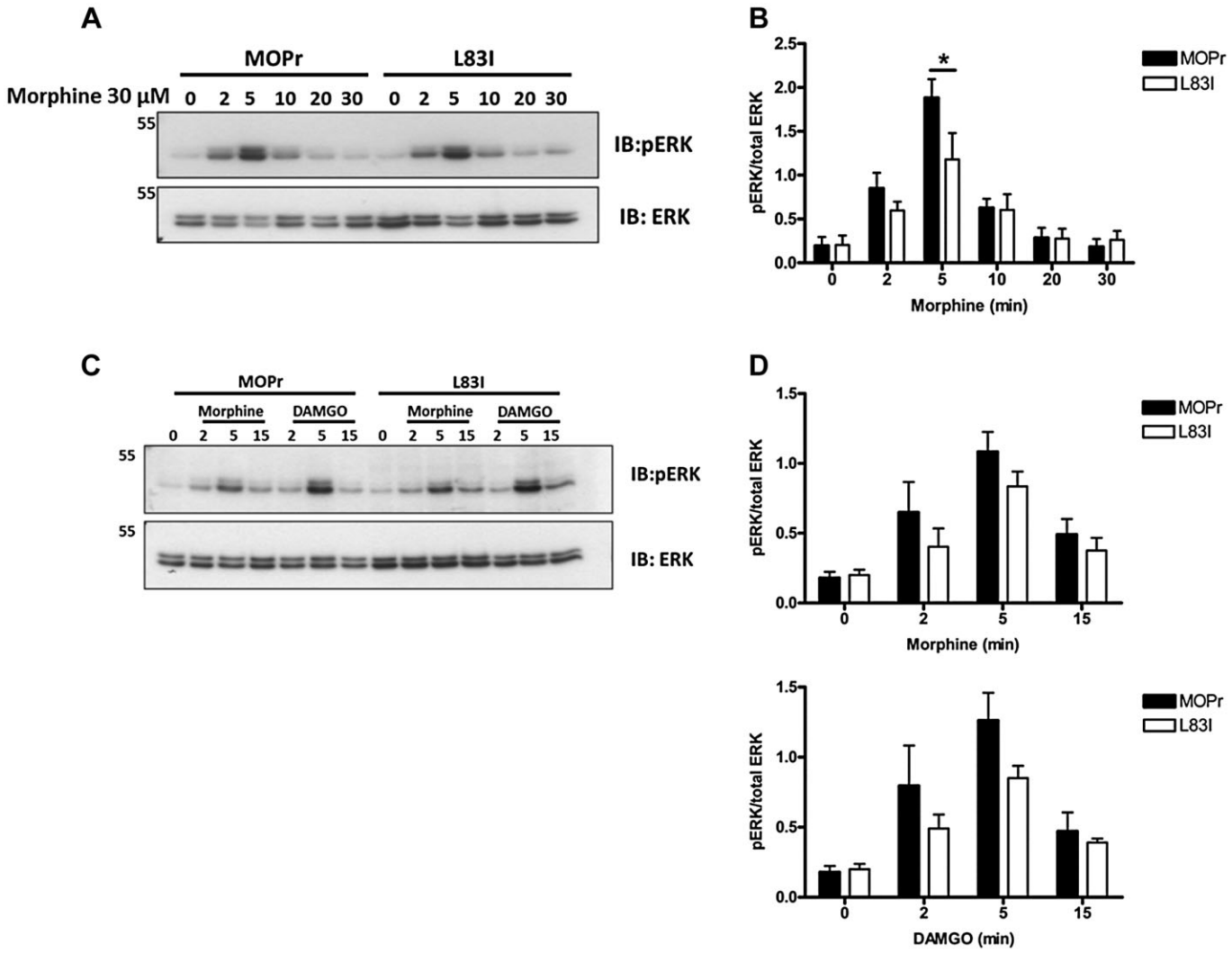

Figure 5

Comparison of WT-MOP receptor (MOPr)- and MOP-L83I receptor (L83I)-induced ERK signalling. All experiments were performed in HEK 293 cells stably expressing either HA-WT-MOP or HA-MOP-L83I receptors. (A) Representative immunoblot of ERK activation induced by a receptor saturating concentration of morphine. Cells were deprived of starved for 18 h and then stimulated with morphine (30 μM) for 0–30 min. (B) Densitometric analysis of morphine-induced ERK activation (n = 3). (C) Comparison of ERK activation induced by a submaximal concentration of morphine or DAMGO. Cells were serum starved for 18 h and then stimulated with either morphine (100 nM) or DAMGO (10 nM) for 0–15 min. (D) Densitometric analysis of ERK activation (n = 3). Proteins from whole-cell lysates were resolved by SDS-PAGE and immunoblotted for phospho-ERK 1/2 (pERK), membranes were stripped and reprobed for ERK. The pERK band intensities were normalized to the corresponding total ERK bands and are presented as the mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Bonferroni *post hoc* test (*P < 0.05).

for both WT-MOP and MOP-L83I receptor, peaking after 5 min of agonist stimulation and returning to basal levels by 20 min. The peak ERK activation induced by morphine at 5 min was observed to be significantly less for the L83I variant compared with WT-MOP receptor (Figure 5B). We then compared the ability of a submaximal concentration of morphine (100 nM) or DAMGO (10 nM) to induce ERK activation, to assess if any differences could be observed at lower agonist concentrations. The time frame and extent of morphine- or DAMGO-induced ERK activation was again found to be similar between the WT-MOP and MOP-L83I receptors (Figure 5C and D).

Discussion

A number of SNPs in the human MOP receptor gene have now been identified, but because the majority of these SNPs have a low allelic frequency, their wider functional significance, if any, has yet to be determined (Cox, 2013), even though some of these SNPs may underlie the individual variability observed in response to opioids (Mague and Blendy, 2010). In the present study, we investigated the functional significance of the L83I variant, by comparing corresponding pharmacological parameters with those of the WT-MOP receptor.

On initial inspection a leucine to isoleucine mutation in the transmembrane domain may not be expected to significantly alter receptor protein structure or function. Indeed, there are numerous occasions when such a substitution has been shown to be conservative in various other proteins (Brosnan and Brosnan, 2006). There are however subtle differences in both the hydrophobicity and side chain shape between leucine and isoleucine, with leucine being more commonly found in α-helices than in β-sheets, whereas the opposite is true for isoleucine (Brosnan and Brosnan, 2006). The two amino acids are also known to play subtly different roles in protein structure, and as such, are not always interchangeable (Brosnan and Brosnan, 2006). For example, sensitivity of the voltage-gated potassium channel subunits of the Shaker family to the antiarrhythmic drug, flecainide, is dependent on the presence of a leucine or isoleucine at a key position within the S6 transmembrane domain of this channel (Herrera *et al.*, 2005). With respect to GPCRs, an isoleucine to leucine mutation in transmembrane domain 5 (I542L) was reported to elevate the signalling capacity of the leutinizing hormone receptor (Kremer *et al.*, 1999). Furthermore, a comprehensive analysis of high-resolution GPCR structures has suggested the existence of 24 conserved inter-transmembrane domain contacts that are thought to be mediated by 36 amino acids that are topologically equivalent (Venkatakrishnan *et al.*, 2013). Interestingly, the L83I resides in close proximity to some of these identified amino acids, notably N86 (1.50 according to Ballesteros–Weinstein numbering) that forms important interactions with transmembrane domains 2 and 7 raising the possibility that the L83I mutation could influence MOP receptor protein structure in significant ways.

In agreement with previous findings (Ravindranathan *et al.*, 2009), we demonstrate that in HEK 293 cells the L83I variant, unlike the WT-MOP receptor, undergoes significant, rapid internalization in response to morphine. DAMGO and etorphine produced comparable extensive internalization of the L83I variant and the WT-MOP receptor; a possible explanation for this observation is that DAMGO and etorphine already promote such robust internalization of the L83I variant such that it is not possible to further increase the rate or extent of receptor internalization. It should be noted, however, that at a submaximal agonist concentration, DAMGO promoted similar levels of WT-MOP receptor and L83I variant internalization. Interestingly, stimulation with other agonists (leu-enkephalin, buprenorphine and pentazocine) that induce varying amounts of WT-MOP receptor internalization (McPherson *et al.*, 2010), failed to produce enhanced internalization of the L83I variant. Therefore the ability of morphine to promote internalization of MOP-L83I receptors is not due to the fact that morphine has lower efficacy for signalling at MOP receptors compared with agonists such as DAMGO and etorphine.

Consistent with the role of dynamin in the DAMGO-induced internalization of the WT-MOP receptors (Keith *et al.*, 1996; Whistler and von Zastrow, 1998), we demonstrated that dynasore, a non-competitive inhibitor of dynamin (Macia *et al.*, 2006) can also ablate the internalization of MOP-L83I receptors in response to both DAMGO and morphine. However, as dynamin is known to be involved in clathrin-independent internalization (Henley *et al.*, 1998), it remains possible that the MOP-L83I variant undergoes morphine-induced internalization by a clathrin-independent pathway, such as one involving caveolae.

Differential phosphorylation of MOP receptors by opioid agonists has been well documented (Kelly, 2011). DAMGO-induced internalization of the MOP receptor is regulated by GRK-mediated phosphorylation of serine and threonine residues in the C-terminal tail, with GRK2 thought to be the predominant kinase mediating phosphorylation of the receptor (Schulz *et al.*, 2004; Chen *et al.*, 2013). Consistent with the significant increase in internalization of the L83I variant in response to morphine, we found that antagonism of GRK2 with a DNM resulted in an attenuation of the morphine-induced internalization of MOP-L83I receptors. This suggests that GRK2 is required for morphine-induced internalization of MOP-L83I receptors, possibly via direct phosphorylation of the mutant receptor. Of the numerous potential phosphorylation sites present in the WT-MOP receptor C-terminal tail, serine 375 has been the most extensively studied and identified as a probable GRK phosphorylation site (El Kouhen *et al.*, 1999; Schulz *et al.*, 2004; Chen *et al.*, 2013). Mutation of this residue has been reported to strongly inhibit agonist-induced internalization (El Kouhen *et al.*, 1999), and in certain instances, desensitization of the WT-MOP receptor (Ozsoy *et al.*, 2005). Although we observed a decrease in the morphine-induced internalization of MOP-L83I receptors in the presence of GRK2 DNM, levels of morphine-induced phosphorylation of serine 375 were similar across the WT-MOP receptor and L83I variant. These data suggest that enhanced phosphorylation of serine 375 is not the likely mechanism by which morphine induces internalization of the L83I variant. It remains to be determined if the L83I variant is also phosphorylated at a different residue or if GRK2 is modulating receptor internalization in a receptor phosphorylation-independent manner. For example, GRK2 can modulate GPCR trafficking by phosphorylation of non-receptor substrates (Ferreira *et al.*, 2012) or in a phosphorylation-independent manner via direct protein–protein interactions (Evron *et al.*, 2012).

In addition to receptor phosphorylation, there are also agonist-dependent differences in MOP receptor interactions with arrestins-2 and -3. Agonists that promote extensive MOP receptor phosphorylation have been demonstrated to promote robust arrestin-3 recruitment and subsequent internalization (Whistler and von Zastrow, 1998; Johnson *et al.*, 2006; Groer *et al.*, 2007; 2011). In contrast, morphine-induced recruitment of arrestin-3 has only been observed following overexpression of GRK2 or following overexpression of arrestin-3-GFP in the absence of competing endogenous arrestins (Bohn *et al.*, 2004; McPherson *et al.*, 2010; Groer *et al.*, 2011). For arrestin-2, there is far less experimental evidence of agonist-induced recruitment, with only high-efficacy agonists such as DAMGO and etorphine capable of inducing interactions between arrestin-2 and the MOP receptor (Bohn *et al.*, 2004; Johnson *et al.*, 2006). In agreement with these published reports, we observed that both morphine and DAMGO induced an association of arrestin-3 and the WT-MOP or MOP-L83I receptor, in the presence of overexpressed GRK2. However, both the extent of association and the kinetics of association induced by either DAMGO or morphine were found to be similar for the WT-MOP

and MOP-L83I receptors. In experiments investigating the agonist-induced association of endogenous arrestin with each of the receptors, we were only able to detect a DAMGO-induced association with the receptor, which was again found to be similar for the WT-MOP and MOP-L83I receptors. It remains possible that morphine induces a weak association between arrestin and the receptor that is below the detection limits of the immunoprecipitation assay used in this study. However, it is difficult to see how such limited arrestin recruitment could lead to such extensive internalization of MOP-L83I receptors. Importantly, the results with arrestin are consistent with the serine 375 phosphorylation data, and taken together imply that the morphine-induced regulation of MOP-L83I receptors is not analogous to the DAMGO-induced regulation of the WT-MOP receptor, and is likely to be independent of both phosphorylation of serine 375 and arrestin.

The present study further demonstrates that the internalization of MOPrL83I receptors by morphine cannot be attributed to an enhancement in efficacy of morphine at this variant, as enhanced morphine-stimulated signalling through multiple pathways was not observed for MOP-L83I receptors. However, and in contrast to Ravindranathan *et al.* (2009), we did observe that in the signalling assays, the MOP-L83I variant elicited slightly lower maximum responses compared with the WT-MOP receptor, despite the fact the MOP-L83I receptor was expressed at a somewhat higher level (approximately 1.5-fold higher) than the WT-MOP receptor. Possibly the mutation does induce a moderate reduction in overall agonist efficacy that is not morphine-specific.

The results of this study suggest that morphine promotes a specific conformation of the L83I variant that is more liable to internalize than the morphine-bound WT receptor. One possible explanation for this phenomenon would be a change in the binding of morphine to the L83I variant. Although it was observed that there was no change in the affinity of [^{3}H]-DAMGO at this variant (Ravindranathan *et al.*, 2009), morphine is structurally distinct from DAMGO, and the two agonists are known to interact differentially within the ligand binding pocket (Law *et al.*, 1999). The altered trafficking of MOP-L83I receptors may potentially be explained by changes in the phosphorylation status of a residue other than serine 375, or an enhancement in the ability of the L83I variant to internalize via either clathrin-dependent or clathrin-independent mechanisms, perhaps through a novel interaction with an endocytic adaptor protein other than arrestin.

Our results show that the MOP-L83I receptor is a ligand-biased receptor compared with the WT-MOP receptor. Presuming that this mutant receptor behaves in a similar fashion in a neuronal context, this has implications for the presence of this SNP within an individual, and how they might respond to morphine versus other opioid agonists. It would be of interest to assess the clinical phenotype of heterozygous individuals, in particular, with respect to pain perception and opioid responsiveness. It is tempting to speculate that such individuals may be more resilient to the development of tolerance to morphine as it has been hypothesized that agonist-induced MOP receptor internalization and presumed resensitization may counteract the development of tolerance (von Zastrow *et al.*, 2003). Certainly, further functional and clinical evaluation of these variants in individuals may help provide further insights into the function and regulation of the MOP receptor.

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Author contributions

A. E. C., E. K., G. H. and S. J. M. conceived the project and designed the experiments. A. E. C., E. K. and G. H. wrote the paper. S. O. generated the MOP-L83I receptor construct, C. K. carried out the FRET experiments, and E. K. carried out the GTPγS. A. E. C. carried out all other experiments.

Conflict of interest

There is no conflict of interest with regard to this work.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12709

Table S1 Kinetic parameters for the interaction of arrestin-3 with MOP and MOP-L83I receptors respectively. Shown are the agonist-induced rate constants for association and dissociation of arrestin-3 with WT-MOP or MOP-L83I receptors. There were no statistically significant differences between WT-MOP and MOP-L83I receptors (Mann–Whitney test, two-tailed). Results from 15 (WT-MOP) or 14 (MOP-L83I) cells were considered, and cells that gave sensible fits, as described in Methods, were included. In particular, the traces upon washout of morphine could not be fit reliably, and therefore the number of cells considered is small.