Title

A novel G protein-biased agonist at the μ opioid receptor induces substantial receptor desensitization through G protein-coupled receptor kinase

Short Running Title

G protein-biased μ opioid agonist induces receptor desensitization

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S.G., A.E.C., R.H., Y.L., L.T., A.K., S.S., G.H., E.K., and C.P.B. participated in research design. S.G. conducted the BRET assays, the ELISA assays and the brain slice electrophysiology. N.K.B. conducted the MOPr phosphorylation assays. A.D., S.M.H., Y.L., and L.T. contributed new reagents. S.G. and N.K.B performed data analysis. S.G., G.H., E.K., and C.P.B. wrote or contributed to the writing of the manuscript. All authors contributed to revision of the manuscript.

Word Count

3911, excluding abstract, methods, figure legends and references.

Abstract

Background and Purpose:

G protein-biased μ opioid receptor (MOPr) agonists have the potential to induce less receptor desensitization and tolerance than balanced opioids. Here we demonstrate that the cyclic endomorphin analogue Tyr-c[D-Lys-Phe-Tyr-Gly] (Compound 1) is a G protein-biased MOPr agonist and characterise its ability to induce rapid receptor desensitization in mammalian neurones.

Experimental Approach:

The signalling and trafficking properties of opioids were characterised using BRET assays, ELISA and phosphosite-specific immunoblotting in HEK 293 cells. Desensitization of opioid-induced currents was studied in rat locus coeruleus neurones using whole-cell patch clamp electrophysiology. The mechanism of Compound 1-induced MOPr desensitization was probed using kinase inhibitors.

Key Results:

Compound 1 is a G protein-biased MOPr agonist with a similar intrinsic activity for G protein signalling as morphine. As predicted for a G protein-biased MOPr agonist, Compound 1 induced minimal agonist-induced internalization and minimal phosphorylation at intracellular MOPr serine/threonine residues known to be involved in GRK-mediated desensitization. However, Compound 1 induced robust rapid MOPr desensitization in locus coeruleus neurons, to a greater degree than morphine. The extent of Compound 1-induced desensitization was unaffected by activation or inhibition of PKC but was significantly reduced by inhibition of G protein-coupled receptor kinase (GRK).

Conclusion and Implications:

Compound 1 is a novel G protein-biased MOPr agonist which induces substantial rapid receptor desensitization in mammalian neurons. Surprisingly, Compound 1-induced desensitization was demonstrated to be GRK-dependent despite its G protein-bias. Our findings refute the assumption that G protein-biased agonists will evade receptor desensitization and tolerance.

Word Count: 249

Bullet Point Summary

'What is already known'

- G protein-biased μ opioid agonists may induce less receptor desensitization and tolerance than typical opioids.

'What this study adds'

- The cyclic endomorphin analogue 'Compound 1' is a novel G protein-biased μ opioid partial agonist.
- Compound 1 induced substantial μ opioid receptor desensitization through G protein-coupled receptor kinase (GRK).

'Clinical Significance'

- There are numerous mechanisms through which μ opioid receptors can desensitise.
- G protein-biased agonists may not evade receptor desensitization or tolerance.

Key Words

opioids, opiates, biased agonism, receptor desensitization, electrophysiology, G protein-coupled receptor kinases, arrestins

Abbreviations

aCSF, artificial cerebrospinal fluid; Compound 1, Tyr-*c*[D-Lys-Phe-Tyr-Gly]; CPD101, Compound 101, 3-[[[4-methyl-5-(4-pyridyl)-4H-1,2,4-triazole-3-yl] methyl] amino]-N-[2-(trifuoromethyl) benzyl]benzamidehydrochloride; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin; GF109203X, 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide; GIRK, G protein-activated inwardly rectifying potassium channel; GRK, G protein-coupled receptor kinase; GSK650394, 2-cyclopentyl-4-(5-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-benzoic acid; LC, locus coeruleus; MOPr, μ opioid receptor; NA, noradrenaline; NLX, naloxone; PMA, phorbol 12-myrtistrate 13-acetate; PRK2, protein kinase C-related protein kinase; PZM21, 1-[(2S)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-[(2S)-1-(thiophen-3-yl)propan-2-yl]urea; RlucII, *Renillia* luciferase II; ROCK2, Rho-associated protein kinase 2; SGK1, serum and glucocorticoid-regulated kinase; Y-27632, *trans*-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride.

Main Text

Introduction

Opioids, such as morphine, remain invaluable therapeutic drugs for the acute treatment of severe pain. This is despite their propensity to elicit significant on-target adverse effects, including respiratory depression and constipation as well as the rapid development of tolerance, all of which limit their clinical utility especially for chronic pain states.

Opioids elicit their physiological effects through activation of the μ-opioid receptor (MOPr) (Matthes et al., 1996), a GPCR that can signal through activation of $G_{i/o}$ proteins as well as arrestin proteins. In recent years, the field has focussed much attention on the concept of biased agonists at GPCRs (Kelly, 2013; Kenakin, 2019) in the hope of developing novel opioid analgesics with reduced adverse effects.

The drive for harnessing biased agonism at MOPr is derived from early observations of the effects of morphine in arrestin-3 (alternatively termed β-arrestin 2) knockout mice. The original report that the respiratory and gastrointestinal adverse effects of morphine were reduced in arrestin-3 knockout mice, while its analgesic properties were retained (Bohn et al., 1999; Raehal et al., 2005), led to the hypothesis that G protein signalling was responsible for opioid-induced analgesia, whilst arrestin-3 signalling was responsible for the adverse effects of MOPr activation. Consequently, many groups have sought to develop novel MOPr ligands that preferentially activate G proteins over arrestin-3 recruitment, G protein-biased agonists, in the hope that they are effective analgesics with reduced respiratory and gastrointestinal adverse effects (DeWire et al., 2013; Manglik et al., 2016; Schmid et al., 2017; Conibear & Kelly, 2019). However recent studies have refuted this hypothesis by clearly demonstrating that respiratory depression and constipation are not arrestin-dependent (Kliewer et al., 2019; Kliewer et al., 2020).

A further possible advantage of G protein-biased MOPr agonists might be that they induce less tolerance than conventional, 'balanced' agonists. A key cellular mechanism that contributes to the development of tolerance to MOPr agonists is agonist-induced desensitization of MOPr (Bailey et al., 2009a). The canonical mechanism underlying desensitization of GPCRs is by GRK-dependent phosphorylation of serine and threonine residues, primarily on the C-terminal tail of the receptor, and subsequent recruitment of arrestin-3 (Williams et al., 2013). The arrestin-bound receptor is unable to promote further G protein activation due to steric hindrance, and so is desensitized.

Given the GRK/arrestin mediated pathways of MOPr desensitization, it could be hypothesised that G protein-biased MOPr agonists, which would inherently have low coupling to arrestin pathways, would cause less MOPr desensitization than a balanced MOPr agonist. This suggests that G protein-biased agonists could be clinically beneficial as they would produce less tolerance. This hypothesis is supported by the attenuation of MOPr desensitization and opioid tolerance in both arrestin-3 knockout mice (Bohn et al., 2000) and in mice expressing mutant, phosphosite-deficient MOPrs (Kliewer et al., 2019).

Our current knowledge of tolerance induced by G protein-biased MOPr agonists is limited, and variable. Oliceridine (TRV130) and PZM21 have both been suggested to be G protein-biased MOPr agonists (DeWire et al., 2013; Manglik et al., 2016; but this is disputed (Hill et al., 2018b; Gillis et al., 2020). In mice, oliceridine has been shown to induce less antinociceptive tolerance than morphine after 3-4 day repeated administration (Altarifi et al., 2017; Liang et al., 2019), whereas PZM21 produces robust antinociceptive tolerance in mice over 4 days (Hill et al., 2018b). SR17018, another MOPr agonist suggested to be G protein-biased (Schmid et al., 2017), although this was not observed by Gillis et al., (2020), was reported to not produce analgesic tolerance after 6 days of repeated oral dosing (Grim et al., 2020).

In the potential absence of GRK & arrestin-dependent receptor desensitization for G protein-biased agonists, other mechanisms could regulate receptor desensitization and tolerance. For example, PKC has been demonstrated to mediate MOPr desensitization induced by morphine in rat locus coeruleus (LC) neurons, but not MOPr desensitization induced by DAMGO (Bailey et al., 2004). These observations translate to *in vivo* tolerance, with tolerance to the antinociception and respiratory depression induced by morphine and oxycodone, but not by DAMGO, in mice being dependent on PKC (Hull et al., 2010; Withey et al., 2017; Hill et al., 2018a). It is reasoned that mechanisms of agonist-selective desensitization are dependent on agonist efficacy: with lower efficacy agonists such as morphine and oxycodone desensitizing predominantly though PKC, whereas higher efficacy agonists such as DAMGO desensitize through GRK/arrestin pathways (Kelly et al., 2008). Given that the purportedly G protein-biased agonists reported to date have similar, or lower, efficacy than morphine (Yudin et al., 2019; Gillis et al., 2020), we hypothesised that these agonists might desensitize MOPr and induce tolerance predominately through PKC, not GRK, in a comparable manner to morphine and oxycodone.

In this study, we examine the ability of G protein-biased MOPr agonists to induce receptor desensitization in rat LC neurons. We report that the cyclic endomorphin analogue Tyr-*c*[D-Lys-Phe-Tyr-Gly] (Li et al., 2016) (Compound 1) is a G protein-biased agonist at MOPr which unexpectedly induces substantial receptor desensitization in LC neurones through a GRK-dependent, PKC-independent mechanism. These findings cast doubt on the assumption that G protein-biased agonists will evade receptor desensitization and tolerance.

Methods

Cell Culture

Human embryonic kidney 293 (HEK 293) cells (HEK293T; CCLV Cat# CCLV-RIE 1018, RRID:CVCL_0063) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 $U.ml^{-1}$ penicillin and 100$\mu g.ml^{-1}$ streptomycin at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cells were grown to approximately 80% confluency in 10-cm dishes before transient transfection using Lipofectamine 2000 (Invitrogen, CA, USA).

BRET Assays

In order to determine the ability of MOPr ligands to activate $G\alpha_i$ G proteins we utilised a $BRET^2$-based assay to study the separation of labelled $G\alpha_{i1}$ and $G\gamma_2$. In the assay, the separation of BRET donor and acceptor (the labelled G protein subunits) is used as a measure of G protein activation. HEK 293 cells were transiently transfected with rat HA-MOPr, $G\alpha_{i1}$-*Renillia* luciferase II (RlucII) and GFP_{10}- $G\gamma_2$, with 3 μg of each cDNA construct transfected per 10-cm dish. Similarly, to study agonist-induced arrestin recruitment, HEK 293 cells were transiently transfected with human MOPr-RlucII and either arrestin-3-GFP_{10} or arrestin-2-GFP_{10}, with 5 μg of each cDNA construct transfected per 10-cm dish. In arrestin-3 recruitment assays with GRK2 overexpression, cells were additionally transfected with 5 μg wild type GRK2.

Prior to assaying, cells were resuspended in phenol red free DMEM and transferred to a 96-well plate at 90 μl per well. Agonists were preincubated with cells at 37°C for 2 min for the G protein activation assay, or 10 min for assays of arrestin recruitment, prior to BRET readings under the same conditions. Coelenterazine 400a (5 μM final concentration) was added 5 seconds prior to plate reading. Measurements of BRET were taken using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) using the following filters: acceptor, 515 $\pm$ 30 nm; and donor, 410 $\pm$ 80 nm. BRET signals were quantified by calculating the ratio of the light emitted by the acceptor (GFP_{10}) over that emitted by the donor (RlucII).

ELISA

Agonist-induced loss of cell-surface MOPr was assessed by an enzyme-linked immunosorbent assay (ELISA) as previously described (Rivero et al., 2012). HEK 293 cells were transiently transfected with rat HA-MOPr pcDNA, 5 μg per 10-cm dish, as described above. Transfected cells were seeded onto 24-well cell culture plates lined with 0.1 $mg.ml^{-1}$ poly-L-lysine 24 h prior to assaying. Cells were then incubated with opioid agonists diluted in serum-free phenol red free DMEM for 15 to 60 min at 37°C, before the medium was aspirated and the reaction was fixed with 3.7% (v/v) formaldehyde. Cells were then incubated with an anti-HA primary antibody (Biolegend Cat# 901516, RRID:AB_2820200, 1:1000) for 1 h at room temperature to label surface receptors. The secondary goat-anti-mouse antibody conjugated to alkaline phosphatase (Sigma Aldrich Cat# A5153, RRID:AB_258225, 1:1000) was then incubated for 1 h, before a colourometric alkaline phosphatase substrate (Thermo Fisher Scientific, Loughborough,

UK) was added and incubated at 37°C. Absorbance (405 nm) of the samples was then assayed in a microplate reader. Changes in surface receptor expression were calculated by normalising absorbance values to those from untreated HEK 293 cells expressing MOPr (100%) and non-transfected, untreated HEK 293 cells (0%).

Phosphosite-specific immunoblotting

Agonist-induced MOPr phosphorylation was assessed using Western blotting as previously described (Gillis et al., 2020). HEK 293 cells stably expressing mouse HA-MOPr were seeded on 6-cm cell culture dishes, maintained in DMEM High Glucose supplemented with 5% (v/v) fetal bovine serum, 1% glutamine and streptomycin/penicillin. At 90% confluency, cells were incubated with opioid ligands for 30 min before lysis in a radioimmunoprecipitation buffer (50 mM tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease and phosphatase inhibitors (complete Mini and PhosSTOP, Roche Diagnostics, Mannheim, Germany). HA-tagged MOPr was enriched using Pierce HA epitope tag antibody agarose beads (Thermo Fisher Scientific, Schwerte, Germany). Samples were then incubated in SDS sample buffer at 43°C for 25 min to elute the proteins from the beads. Samples were then separated and resolved on 8% SDS-polyacrylamide gels and after electroblotting, PVDF membranes were incubated with either anti-pT370, anti-pS375, anti-pT376, or anti-pT379 antibodies, followed by detection using a chemiluminescence detection system. Blots were then stripped and incubated again with a phosphorylation-independent anti-HA to confirm equal well loading. Agonist-induced phosphorylation was quantified using the image processing software Fiji (Fiji, RRID:SCR_002285).

Brain Slice Preparation

Male Wistar Rats (4 weeks old) (RRID:RGD_737929; originally purchased from Charles River then bred at the University of Bath for > 10 years) were anaesthetized via intraperitoneal injection of 160 $mg.kg^{-1}$ ketamine and 20 $mg.kg^{-1}$ xylazine and then decapitated. The brains were rapidly removed and submerged in ice-cold cutting solution composed of (in mM): 20 NaCl, 2.5 KCl, 1.6 NaH_2PO_4, 7 $MgCl_2$, 85 sucrose, 25 D-glucose, 60 $NaHCO_3$, and 0.5 $CaCl_2$ and saturated with 95% O_2/5% CO_2. Horizontal brain slices (230 μm thick) containing the LC were prepared using a vibratome (DTK-1000, DSK, Kyoto, Japan). Immediately after cutting, slices were then incubated in warm (32°C) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 11.1 D-glucose, 21.4 NaHCO3, 2.4 $CaCl_2$, and 0.1 ascorbic acid, saturated with 95% O_2/5% CO_2, and were left to equilibrate for at least 1 h before recording.

All animal care and experimental procedures were in accordance with the UK Animals (Scientific Procedures) Act 1986, the European Communities Council Directive (2010/63/EU), the ARRIVE guidelines (Kilkenny et al., 2010) and the University of Bath ethical review document.

Whole-Cell Patch-Clamp Recordings

Brain slices were submerged in a slice chamber and superfused with a continuous flow (2-3 $ml.min^{-1}$) of aCSF at 32°C. Slices were visualised using oblique optics on a BX51W1 upright microscope (Olympus;

Southend-on-Sea, UK) and the surfaces of individual cell somata were cleared of debris by the gentle applied flow of aCSF from a pipette. Whole-cell voltage-clamp recordings were made using recording electrodes (3-5MΩ) filled with an intracellular solution containing (in mM) 115 potassium gluconate, 10 HEPES, 11 EGTA, 2 $MgCl_2$ 10 NaCl, 2 MgATP, and 0.25 Na_2GTP, and pH 7.3 and with an osmolarity of 270 $mOsm.L^{-1}$. Recordings of whole-cell currents were filtered at 2 kHz using an Axopatch 200B amplifier (Axon Instruments Inc, CA, USA) and digitised with a sampling rate of 10 kHz (Digidata 1440A, Axon Instruments Inc, CA, USA). Recordings were analysed off-line using WinEDR (University of Strathclyde, Glasgow, UK).

LC neurones were voltage-clamped at -60mV with a correction made for a -12mV liquid junction potential. Activation of MOPr evoked GIRK currents, providing a real-time measurement of MOPr activation which could be monitored continuously with the use of whole-cell patch-clamp recordings. All drugs were applied in the superfusing solution at known concentrations. MOPrs and α_2-adrenoceptors couple to the same population of GIRK channels in LC neurones (North & Williams, 1985). To control for variation when making comparisons of peak evoked-current magnitude between individual cells, currents were normalised to the magnitude of the maximal α_2-adrenoceptor-mediated current in the same cell, evoked by 100 μM noradrenaline (NA). All experiments studying α_2-adrenoceptor-mediated currents were conducted in the presence of 1 μM prazosin and 3 μM cocaine. Desensitization of opioid-evoked currents was quantified by expressing the magnitude of the decline in current as a percentage of the initial peak evoked current. Naloxone (1 μM) was applied after each opioid application to antagonise the response completely. A higher naloxone concentration (10 μM) was used in later experiments using Compound 1 (Figure 3-4) in order to overcome the relatively slow rate of its reversal in LC neurones (Figure 2D). The magnitude of NA (100 μM) evoked currents was assessed before and after the recording of opioid-evoked currents in each cell, to provide a reporter of potential current rundown and/or heterologous desensitization.

Data Analysis

All values are expressed as mean ± S.E.M., where $n \geq 5$. BRET and ELISA experiments were run in duplicate and triplicate respectively and averaged to form an individual repeat (n). For LC electrophysiology experiments, each separate repeat of an experimental condition (n) represents experiments in brain slices derived from separate animals. Differences in group size in LC electrophysiology experiments occur in some cases (Figures 2E-G) due to running the same protocol again for other investigations. In other cases (Figures 4E-J) controls or key findings were repeated when examining new experimental conditions, in order to assess and control for potential variation between experiments performed at different times. Both data collection and analysis were conducted unblinded for practical reasons, as the experimenter had to apply the pharmacological agents themselves. This manuscript complies with BJP's recommendations and requirements on experimental design and analysis (Curtis et al., 2018).

All data fitting and statistical analyses were made using GraphPad Prism (RRID:SCR_002798, v8.0). Concentration-response data were analysed using nonlinear curve fitting to obtain EC_{50} and E_{max} values

for $G\alpha_i$ activation and arrestin-3 recruitment. The Hill slope and the bottom asymptote of the curve were constrained to 1 and 0 respectively.

The statistical analyses used for each individual experiment are described in the respective figure legend. Statistical significance was reported if $p < 0.05$. Briefly, one-way ANOVA was used to compare agonist efficacies, peak responses, induced desensitization and Ser375 phosphorylation. Post-hoc Tukey or Dunnett tests were conducted only if F was significant and variance was homogenous. Total loss of surface receptor over time in the MOPr ELISA was assessed by determining the total area under the curve (AUC) for drug over the time-course. Statistical differences in AUC were then assessed using a one sample t test against 0.

Materials

All reagents were purchased from Sigma Aldrich (Gillingham, UK) except for DAMGO [[D-Ala^2, N-MePhe^4, Gly-ol^5]-enkephalin] (Bachem, Bubendorf, Switzerland), morphine sulphate (MacFarlane Smith, Edinburgh, UK), naloxone hydrochloride, Takeda compound 101 (CPD101) [3-[[[4-methyl-5-(4-pyridyl)-4H-1,2,4-triazole-3-yl] methyl] amino]-N-[2-(trifuoromethyl) benzyl]benzamidehydrochloride] (HelloBio, Bristol, UK), prazosin hydrochloride, GF109203X [2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide], PMA [phorbol 12-myrtistrate 13-acetate], GSK650394 [2-cyclopentyl-4-(5-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl-benzoic acid] and Y-27632 [*trans*-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride] (Tocris, Abingdon, UK). PZM21 [1-[(2S)-2-(dimethylamino)-3-(4-hydroxyphenyl)propyl]-3-[(2S)-1-(thiophen-3-yl)propan-2-yl]urea] hydrochloride was kindly provided by Dr Alexander Disney (University of Bath, UK). Compound 1 [Tyr-*c*[D-Lys-Phe-Tyr-Gly]] was kindly provided by Dr Yangmei Li (University of South Carolina, SC, USA) and also synthesised by Biomatik Corporation (Ontario, Canada). Data using both sources of Compound 1 are presented in this paper.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to Pharmacology 2019/2020 (Alexander et al., 2019).

Results

Signalling Profile of MOPr Ligands

In order to characterise the *in vitro* signalling profile of Compound 1, we examined its ability to activate $G\alpha_i$ and recruit arrestin-3 in HEK 293 cells expressing recombinant MOPr using BRET technology.

Compound 1 (3 nM – 30 μM) produced concentration-dependent $G\alpha_i$ activation in HEK 293 cells expressing MOPr (Figure 1A). The responses to Compound 1 were compared to those elicited by two well-characterised MOPr ligands, DAMGO (a full agonist) and morphine (a partial agonist), and to PZM21, a putative G protein-biased agonist at MOPr (Manglik et al., 2016) (Figure 1A). BRET responses were fitted to concentration-response curves in order to determine the potency (EC_{50}) and the maximum responses (E_{max}) for each ligand for each signalling pathway (Table 1). In systems of low receptor reserve, the maximum responses produced by partial agonists (their intrinsic activity) can be utilised as a robust indicator of their intrinsic efficacy, where the utilisation of the operational model is limited by the large fitting error associated with derived parameters due to variable curve fit quality in the case of weak partial agonists (Kelly, 2013; Dekan et al., 2019). The maximum response to DAMGO for $G\alpha_i$ activation was significantly higher than those elicited by morphine, PZM21 and Compound 1, showing that they are all partial agonists in this system. PZM21 produced a significantly lower maximum response than morphine for $G\alpha_i$ activation suggesting it has lower efficacy, in agreement with findings from Hill et al. (2018b), Yudin et al. (2019) and Gillis et al. (2020). There was no statistical difference between the maximum responses elicited by Compound 1 and morphine, indicating that they have similar efficacies for $G\alpha_i$ activation.

Compound 1 (3 nM – 30 μM) produced a small concentration-dependent recruitment of arrestin-3 in HEK 293 cells expressing MOPr (Figure 1B). The efficacy of Compound 1 for arrestin-3 recruitment was significantly lower than that of partial agonist morphine (Table 1). There was no significant difference between the efficacy of PZM21 and morphine for arrestin-3 recruitment.

The fact that Compound 1 produced $G\alpha_i$ activation with the same efficacy as morphine but induced low arrestin-3 recruitment suggests that Compound 1 is a G protein-biased agonist at MOPr relative to morphine. Despite observed differences in maximal responses between the agonists, the rank order of agonist potency was conserved between $G\alpha_i$ activation and arrestin-3 recruitment (Table 1). This suggests that differences in the signalling profiles of these ligands are driven by efficacy, not affinity.

The $\Delta\Delta\log(\tau/KA)$ approach is the standard method of quantifying biased agonism. However, the utilisation of the operational model limits its power when assessing the responses of weak partial agonists due to the large fitting error associated with derived parameters resulting from variable curve fit quality (Kelly, 2013; Dekan et al., 2019). This approach is therefore inappropriate for these data. Instead we have assessed biased agonism using the 'Normalised ΔE_{max}' approach (Dekan et al., 2019), which allows for the quantification of biased signalling for partial agonists using efficacy alone. This is possible in this case as all test agonists (morphine, PZM21, and Compound 1) are partial agonists relative to DAMGO in both

assays. Compound 1 has a significantly higher normalised ΔE_{max} compared to morphine (Figure 1C) and therefore relative to morphine it is a G protein-biased agonist.

Opioid-Induced MOPr Desensitization in LC Neurons

LC neurones provide a convenient model for the study of MOPr desensitization. This is in part due to their homogeneous postsynaptic expression of MOPrs. Additionally, the expression of δ-opioid receptors (DOPr) and κ-opioid receptors (KOPr) in LC neurones is localised to presynaptic sites only (McFadzean et al., 1987; van Bockstaele et al., 1997). Thus, GIRK responses observed to opioid agonists in LC neurones are mediated solely through activation of MOPr.

In rat LC neurons, a receptor saturating concentration of DAMGO (10 μM, Figure 2A), morphine (30 μM, Figure 2B), PZM21 (30 μM, Figure 2C) and Compound 1 (30 μM, Figure 2D) each evoked outward potassium currents through GIRK channels. At these supramaximal concentrations, the peak amplitude of GIRK currents elicited by partial agonists can be used as an approximation of their E_{max} values. This estimate is only appropriate if agonists themselves do not block GIRK channels (Rodriguez-Martin et al., 2008), which was later confirmed in the case of Compound 1 (Figure 3C & D). The peak responses of morphine, PZM21 and Compound 1 were all significantly lower than that of DAMGO (Figure 2E), demonstrating that they are partial agonists in this system. The peak responses elicited by morphine and Compound 1 were not statistically different from each other, whereas the peak response elicited by PZM21 was significantly lower than that elicited by morphine. These findings are in agreement with those from the heterologous expression system used for $G\alpha_i$ activation BRET (Figure 1A).

DAMGO (10 μM) induced currents underwent marked desensitization over the 10 min of exposure (Figure 2A, F, G), indicative of rapid agonist-induced MOPr desensitization. Morphine (30 μM) induced significantly less MOPr desensitization than DAMGO (Figure 2B, F, G). PZM21 (30 μM) also induced low amounts of MOPr desensitization, as would be expected for a partial agonist with lower efficacy than morphine (Figure 2C, F, G). Unexpectedly, Compound 1 (30 μM) induced significantly more MOPr desensitization than morphine (Figure 2D, F, G).

Noradrenaline (NA; 100 μM) was applied before and after each opioid application as an indicator of both heterologous desensitization and current run-down over time (Harris & Williams, 1991; Llorente et al., 2012). The magnitude of NA-evoked currents after opioid administration compared with those before was similar with all opioid agonists tested (Figure S1). This suggests that the high degree of desensitization induced by Compound 1 was not due to heterologous desensitization.

To test that the decline in Compound 1-induced current was not due to degradation of the compound over time, Compound 1 was applied for 10 min, then a fresh solution of Compound 1 (30 μM) was applied (Figure 3A & B). The application of fresh Compound 1 did not increase the GIRK current, demonstrating that the decline in the response to Compound 1 over time was not due to drug degradation.

Another potential explanation for the rapid decline in Compound 1-evoked GIRK currents is that Compound 1 not only activates GIRK channels through MOPr, but also acts as a GIRK channel blocker

with slower kinetics of action, similar to the effect of methadone (Rodriguez-Martin et al., 2008). To investigate this, we assessed the ability of Compound 1 (30 μM) to inhibit GIRK currents evoked by a submaximal concentration of NA (3 μM), in the presence of naloxone (10 μM) to block activation of MOPr by Compound 1. Application of Compound 1 had no effect on NA-evoked currents (Figure 3C & D), indicating that the decline in Compound 1-evoked currents was not due to GIRK channel inhibition.

Investigating Kinase Involvement in Compound 1-Induced MOPr Desensitization

Previous studies have shown that the agonist-specific mechanisms of MOPr desensitization are via GRK/arrestin or PKC with GRK/arrestin predominating with higher efficacy agonists (such as DAMGO) and PKC predominating with lower efficacy agonists (such as morphine) (Kelly et al., 2008; Bailey et al., 2009b). In LC neurones activation of PKC has been shown to enhance MOPr desensitization induced by morphine and, to a lesser extent, Met-Enkephalin (Bailey et al., 2004). In order to investigate the mechanism(s) underlying Compound 1 desensitization, brain slices were preincubated for 20 min prior to Compound 1 application with either the PKC inhibitor GF109203X (1 μM), PMA (1 μM), a direct activator of PKC, or the GRK inhibitor Compound 101 (CPD101; 30 μM). Compound 1-induced MOPr desensitization was assessed after 20 min application of DMSO (0.1% v/v) to serve as a baseline control for these experiments (Figure 4A). Neither inhibition nor activation of PKC had any effect on Compound 1-induced desensitization (Figure 4C, D, F, H, I). The ability of PMA (1 μM) to increase agonist-induced MOPr desensitization in rat LC neurones was validated in the case of morphine (Figure S2B, E, F). Similarly, GF109203X (1 μM) was demonstrated to effectively reverse PMA-evoked increases in morphine-induced MOPr desensitization (Figure S2C, E, F). However, surprisingly given Compound 1 only weakly recruited arrestins to MOPr (Figure 1B), inhibition of GRK with CPD101 significantly inhibited Compound 1-induced desensitization (Figure 4B, F, G).

Although CPD101 is an effective GRK inhibitor, it can also to some extent inhibit protein kinase N2 (PRK2), serum/glucocorticoid-regulated kinase (SGK1) and Rho-associated coiled-coil protein kinase 2 (ROCK2) (Lowe et al., 2015). To test whether the effects of CPD101 were through these kinases rather than GRK, slices were preincubated with both GSK650394 (10 μM) and Y-27632 (50 μM), collectively inhibiting PRK2, SGK1 and ROCK2. These kinase inhibitors did not affect Compound 1-induced MOPr desensitization (Figure 4F, J). Preincubation with any of the kinase inhibitors tested did not affect the amplitude of Compound 1-induced currents (Figure 4E). These data suggest that, despite being G protein-biased, Compound 1 caused rapid MOPr desensitization by a GRK-dependent mechanism.

Mechanisms Underlying Compound 1-Induced Desensitization In Vitro

While the low level of arrestin-3 recruitment by Compound 1 (Figure 1B) suggests it would induce minimal MOPr internalisation, Compound 1 induced robust receptor desensitization via GRK in LC neurones (Figure 4B, F). To further investigate the GRK/arrestin coupling of Compound 1, we measured agonist-induced MOPr internalisation by ELISA in HEK 293 cells expressing HA-MOPr. While application of DAMGO (10 μM) and morphine (30 μM) produced significant reductions of surface MOPr expression over 60 mins of incubation, Compound 1 (30 μM) and PZM21 (30 μM) did not induce loss of surface receptor

expression (Figure 5A, B). This indicates that Compound 1 did not induce MOPr internalisation in this recombinant system, in agreement with its weak arrestin-3 coupling (Figure 1B).

A potential explanation for the marked desensitization in LC neurones not translating to arrestin-3 recruitment and receptor internalization in our recombinant systems might be different levels of GRK expression between the two systems. To investigate this, we examined the ability of Compound 1 to induce arrestin-3 recruitment in MOPr expressing HEK 293 cells overexpressing GRK2. Under these conditions, as expected (Zhang et al., 1998), the signal level for agonist-induced arrestin-3 recruitment was markedly increased (Figure 5C), with the maximum response of DAMGO increasing around 4-fold compared to recruitment in cells with endogenous GRK expression (Figure 1B). Despite this, the rank order of intrinsic activity of these ligands remained the same, with Compound 1 producing much lower levels of arrestin-3 recruitment than morphine (Figure 5C).

Another possibility is that Compound 1 is not desensitizing through arrestin-3 in LC neurons, but instead through recruitment of arrestin-2 (alternatively termed β-arrestin 1). To investigate this, we examined arrestin-2 recruitment using BRET in HEK 293 cells. The relative signal level for arrestin-2 recruitment was considerably lower than that for arrestin-3 recruitment (Figure 5D) (Cheng et al., 1998). While the full agonist DAMGO produced small but detectable concentration-dependent arrestin-2 recruitment, no measurable response was observed for the partial agonists morphine, PZM21 and Compound 1 (Figure 5E). These findings suggest that arrestin-2 was not responsible for Compound 1-induced MOPr desensitization in LC neurons.

Given that Compound 1-induced MOPr desensitization in LC neurones was inhibited by the GRK inhibitor CPD101, it is possible that Compound 1 could be inducing MOPr desensitization through a GRK-dependent, but arrestin-independent, mechanism. To examine this, we studied the ability of Compound 1 to induce multisite C-terminal tail phosphorylation of MOPr compared to our reference agonists, using phosphosite-specific antibodies. As has previously been demonstrated (Just et al., 2013), the full agonist DAMGO (10 μM) induced phosphorylation at multiple C-terminal serine (S) and threonine (T) sites on MOPr (Figure 6A). The partial agonist morphine (30 μM) induced phosphorylation largely restricted to S375. Compound 1 (30 μM) induced minimal phosphorylation, limited to S375, at significantly lower levels compared to morphine (Figure 6B). These data are consistent with Compound 1 being G protein-biased, and demonstrate that it induces minimal phosphorylation at residues known to be involved in GRK/arrestin-mediated MOPr desensitization and trafficking.

Discussion

Here we report that the cyclic endomorphin analogue Compound 1 is a novel G protein-biased agonist at the MOPr, with equivalent efficacy to morphine for G protein activation but inducing significantly less arrestin-3 recruitment, receptor internalisation and S375 phosphorylation. Despite this, Compound 1 induced substantial MOPr desensitization in LC neurons, to a greater degree than morphine. Surprisingly, Compound 1-induced MOPr desensitization was not mediated by PKC but was inhibited by the GRK inhibitor CPD101. This finding demonstrates that the receptor desensitization induced by Compound 1 is GRK-dependent in spite of its low arrestin recruitment, indicating the presence of a novel GRK-dependent, arrestin-independent mechanism of agonist-induced desensitization at MOPr.

Compound 1-Induced Desensitization does not involve PKC

PKC has been shown to play a substantial role in MOPr desensitization. Previous studies (Bailey et al., 2004; Johnson et al., 2006) have suggested that the relative magnitudes of PKC- and GRK-dependent MOPr desensitization correlate with agonist efficacy. Morphine, Met-Enkephalin and DAMGO have rank order of intrinsic efficacies: DAMGO>Met-Enkephalin>morphine. DAMGO-induced MOPr desensitization is largely GRK-dependent, morphine has the largest PKC-dependent, and smallest GRK-dependent, component of MOPr desensitization, Met-Enkephalin is intermediate.

Given that Compound 1 has equivalent intrinsic efficacy to morphine, it might have been expected that Compound 1-induced MOPr desensitization would be dependent upon PKC activation, as previously seen for morphine in LC neurons. However, we observed no PKC-mediated component of Compound 1-induced desensitization. While Compound 1 has equivalent intrinsic efficacy for G protein activation to morphine, it is G protein-biased and therefore may stabilise a different active conformational state of MOPr to morphine (Schneider et al., 2016; Dekan et al., 2019). This active state may evade PKC-mediated desensitization such that even the low level of GRK activation by Compound 1 may be sufficient to induce MOPr desensitization.

Potential Non-Canonical Roles of GRK2/3 in Compound 1-Induced Desensitization

Precisely how Compound 1 would induce MOPr desensitization via GRK, but not arrestins, is unknown at present. The potential that off-target effectors inhibited by CPD101 were responsible for Compound 1-induced MOPr desensitization was discounted, as inhibitors of known non-GRK targets of CPD101 did not affect Compound 1-induced desensitization.

One possibility is that Compound 1 phosphorylates residues outside of the archetypal residues examined in this study, which were restricted to the C-terminal tail of the receptor (Just et al., 2013; Miess et al., 2018). The phosphorylation of a number of alternative residues outside the C-terminal tail of MOPr has been implicated in its desensitization (Williams et al., 2013). For example, GRK3-dependent phosphorylation of T180 in the second intracellular loop has been demonstrated to be important in DAMGO-induced MOPr desensitization, but not MOPr internalization, in recombinant oocytes and AtT20 cells (Celver et al., 2004; Celver et al., 2001). Phosphorylation of intracellular loop serine/threonine residues has also been demonstrated as a mechanism of desensitization and internalization at other

class A GPCRs (Pals-Rylaarsdam & Hosey, 1997; Clayton et al., 2014). Additionally, a phosphorylation-independent role for GRK2 in sterically inhibiting G-protein signalling has been described at mGluR1 and mGluR5 (Dhami et al., 2005; Ribeiro et al., 2009). GRK2 could potentially be mediating Compound-1 induced desensitization through a similar mechanism.

$G\beta\gamma$ sequestration has been demonstrated as a potential GRK-dependent mechanism of heterologous desensitization for GIRK currents linked to GPCRs, including MOPr, in a recombinant system (Raveh et al., 2010). However, MOPr desensitization in mature rat LC neurones is largely homologous, occurring at the receptor level, as GIRK currents evoked by α_2-adrenoceptors are not substantially reduced after exposure to DAMGO (Harris & Williams, 1991; Llorente et al., 2012). Similarly, the finding here that Compound 1 did not induce marked heterologous desensitization of NA-evoked currents relative to other opioids (Figure S1), suggests that Compound 1-induced desensitization is occurring at the level of the receptor.

CPD101 did not fully inhibit the desensitization induced by Compound 1. Our findings are similar to those examining the effect of CPD101 on DAMGO, Met-Enkephalin and morphine-induced desensitization in rat LC neurones (Lowe et al., 2015). Residual desensitization could be indicative of the presence of an additional mechanism or, potentially, non-phosphorylation dependent $G\beta\gamma$ sequestration by GRK (Raveh et al., 2010). Alternatively, the lack of complete inhibition of MOPr desensitization could be due to incomplete inhibition of GRK by CPD101.

Efficacy and Other Confounding Factors in Biased Signalling

A further finding from this study regards the putatively G protein-biased agonist PZM21. While no functional selectivity was detected for PZM21 compared with morphine, in this paper, PZM21 had significantly lower E_{max} values in the G protein BRET assay and lower magnitude of peak response in LC neurones at a supramaximal concentration when compared to morphine. These data indicate that it is a low efficacy MOPr agonist, which could explain its lower efficacy for arrestin recruitment, rather than biased signalling. Indeed, in the initial study describing PZM21 (Manglik et al., 2016), PZM21 has lower G protein E_{max} values data to morphine (in assays with lower amplification factors), a finding repeated by similar studies from other groups (Hill et al., 2018b; Yudin et al., 2019; Gillis et al., 2020).

Comparisons between an agonist's intrinsic activities for different signalling pathways of different receptor reserve can provide misleading results (Kelly, 2013; Conibear & Kelly, 2019) and can result in compounds being incorrectly identified as biased agonists. In the case of Compound 1, we have determined that its intrinsic efficacy for G protein signalling in both recombinant expression systems and in rat LC neurons, a system of physiological receptor expression, is equivalent to that of morphine (Table 1, Figure 2E), indicating that efficacy is not a confounding factor when comparing the signalling of these agonists.

Low intrinsic efficacy might also drive the low levels of tolerance observed with the putatively G protein-biased agonists TRV130 (Altarifi et al., 2017; Liang et al., 2019) and SR-17018 (Grim et al., 2020). Similarly, recent studies from Gillis et al. (2020) showed that both TRV130 and SR-17018 have significantly lower intrinsic efficacy for G-protein activation than morphine in numerous recombinant

systems, which could explain their purportedly improved side effect profiles. Given that Compound 1 has a similar intrinsic efficacy to morphine, it is proposed that differences in induced receptor desensitization are not efficacy-dependent.

Conclusions

In conclusion, we have determined that Compound 1 is a G protein-biased MOPr agonist which induces substantial receptor desensitization in LC neurons. Compound 1-induced MOPr desensitization appears to occur through a novel GRK-dependent, arrestin-independent mechanism. Our findings therefore cast doubt on the assumption that by evading canonical pathways of receptor desensitization, G protein-biased agonists will evade receptor desensitization and subsequent tolerance.

Acknowledgements

S.G. was funded by an A.J. Clark studentship from the British Pharmacological Society (BPS). This work was supported by the following grants: National Institute of Health (NIH, USA) Grant RO1DA036975 to G.H.; National Institute on Drug Abuse (NIDA, USA) Grant RO1DA07315 to S.M.H.;

The funders of the study had no role in the study design, data collection, data analysis, data interpretation or writing of the report.

Conflict of Interest

The authors declare no conflicts of interest.

Declaration of Transparency and Scientific Rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Figure Legends

Figure 1

Opioid-induced $G\alpha_i$ activation and arrestin-3 recruitment in HEK 293 cells transiently expressing recombinant MOPrs. (A) DAMGO, morphine, PZM21 and Compound 1 produced concentration-dependent activation of $G\alpha_i$ as measured as a decrease in BRET signal occurring between $G\alpha_{i1}$-RlucII & GFP_{10}-$G\gamma_2$ proteins. (B) DAMGO, morphine, PZM21 and Compound 1 produced concentration-dependent recruitment of arrestin-3 translocation as measured by an increase in BRET signal from association of MOPr-RlucII and arrestin-3-GFP_{10}. (C) Compound 1 displayed bias for G protein activation over arrestin-3 recruitment relative to morphine when comparing efficacies through the Normalised ΔE_{max} approach (Dekan et al., 2019). Fitted values for agonist pEC_{50} and E_{max} are presented in Table 1. Data are representative of mean ± SEM, n = 5. * P < 0.05, one-way ANOVA with post-hoc Dunnett's test vs morphine.

Figure 2

Rapid desensitization of opioid-evoked currents in rat LC neurons. (A-D) Representative traces showing outward potassium currents recorded from rat LC neurones in response to receptor-saturating concentrations of DAMGO (10 μM), morphine (30 μM), PZM21 (30 μM) or Compound 1 (30 μM) over at least 10 minutes of application. Scale bars are representative of 50pA and 5 minutes. NLX, naloxone (1 μM). (E) The average peak GIRK currents elicited by opioids in rat LC neurones applied at receptor-saturating concentrations, normalised to the maximal α_2-adrenoceptor-mediated current evoked by NA (100 μM) in the same cell. (F) Time-courses for desensitization of GIRK currents evoked by receptor-saturating concentrations of opioids in rat LC neurones post-peak response. (G) Averaged data for percentage of desensitization induced by all agonists 8 minutes post-peak response. For E-G, data is presented as mean ± SEM, where n = 5-10. For E, † P < 0.05, significantly different from all respective values for other agonists. * P < 0.05, significantly different from respective morphine value. *ns* P > 0.05, no difference to respective morphine value. One-way ANOVA with post-hoc Tukey test. For G, † P < 0.05, significantly different from the respective DAMGO value. * P < 0.05, significantly different from the respective morphine value. One-way ANOVA with post-hoc Tukey test.

Figure 3

Apparent Compound 1-induced desensitization is not a product of peptide degradation or indirect GIRK channel blockade. (A) A representative trace showing decline of a Compound 1 (30 μM)-evoked potassium current in a rat LC neuron. Decline of the evoked current was unaffected when the superfused Compound 1 which had been applied for 10 minutes had freshly prepared ('fresh') Compound 1 (30 μM) added. Compound 1-evoked currents were fully reversed with naloxone (NLX; 10 μM). (B) Pooled data from experiments of the type presented in A. The Compound-1 evoked GIRK current, expressed as a %

of the peak GIRK current, was unaffected by the application of 'fresh' Compound 1 as previously described. (C) A representative trace of potassium current evoked by a submaximal concentration (3 μM) of noradrenaline (NA) in a rat LC neuron. The NA-evoked current was assessed in the presence of prazosin (1 μM), cocaine (3 μM) and NLX (10 μM). With MOPr blocked by NLX, the application of Compound 1 (30 μM) had no indirect effect on the NA-evoked current. NA-evoked currents were reversed with phentolamine (10 μM; PA). (D) Pooled experiments of the type presented in C. There was no change in the amplitude of NA-evoked currents, expressed as a % of the plateau NA-induced current, upon the application of Compound 1. (A & C) Scale bars are representative of 20pA and 5 minutes. (B & D) Data are presented as mean ± SEM, where n = 5.

Figure 4

Compound 1-induced MOPr desensitization in rat LC neurones is inhibited by CPD101. (A-D) Representative traces of Compound 1 (30 μM)-evoked potassium currents in rat LC neurones exposed to DMSO (A; 0.1 %), CPD101 (B; 30 μM), GF109203X (C; 1 μM) or PMA (D; 1 μM) for 20 minutes before and during Compound 1 application. All Compound 1-evoked currents were reversed with naloxone (NLX; 10 μM). Scale bars represent 20pA and 5 minutes. (E) Pooled data from experiments of the type presented in A-D assessing the peak GIRK current evoked by Compound 1 after 20 minutes preincubation with the described inhibitors. Responses were normalised to the maximal α_2-adrenoceptor-mediated current evoked by NA (100 μM) in the same cell. Pooled data for Compound 1-evoked currents in the presence of GSK650394 (10 μM) and Y27632 (50 μM) is also presented. (F) Pooled data for percentage of desensitization induced by Compound 1 in the described conditions 10 minutes after peak Compound 1 response. (G-J) Pooled time-courses for the desensitization of Compound 1-evoked GIRK currents in rat LC neurones post peak response after preincubation with CPD101 (G), GF109203X (H), PMA (I) or GSK650394 & Y-27632 (J). Control: 0.1% DMSO. (E-J) All data are presented as mean ± SEM, where n = 5-11. (E & F) * $P < 0.05$, one-way ANOVA with post-hoc Tukey test.

Figure 5

Compound 1 evokes minimal MOPr internalisation and arrestin recruitment. (A) Surface loss of rat HA-MOPrs transiently expressed in HEK 293 cells assessed by ELISA using an anti-HA antibody to label surface receptors upon incubation with DAMGO (10 μM), morphine, PZM21 or Compound 1 (30 μM). (B) The area under the curve (AUC) for agonist-induced loss of surface MOPr over the time course presented in A. * $P < 0.05$, denotes values significantly different to 0 in a one-sample t test. (C) Opioid-induced arrestin-3 recruitment in HEK 293 cells transiently expressing recombinant MOPrs and GRK2. DAMGO, morphine, PZM21 and Compound 1 produced concentration-dependent recruitment of arrestin-3-GFP_{10} translocation to MOPr-RlucII. GRK2 overexpression caused an increase in the signal level, but the relative intrinsic activities of these agonists were retained (see Figure 1B). (D & E) Opioid-induced arrestin-2 recruitment in HEK 293 cells transiently expressing recombinant MOPr. DAMGO alone

promoted concentration-dependent recruitment of arrestin-2-GFP_{10} to MOPr-RlucII. (E), but raw signal levels were negligible when compared to DAMGO-induced arrestin-3 recruitment in a similar BRET assay (D; Figure 1B). All data are presented as mean ± SEM, where *n* = 5.

Figure 6

Agonist-induced phosphorylation of MOPr. (A) HEK 293 cells stably expressing mouse HA-MOPr were incubated with increasing concentrations of Compound 1 (1 nM – 30 μM) or supramaximal concentrations of DAMGO (10 μM), morphine (30 μM) or PZM21 (30 μM) for 30 minutes. Cells were lyzed and immunoblotted for MOPr phosphorylated at Thr^{370} (pT370), Ser^{375} (pS375), Thr^{376} (pT376) or Thr^{379} (pT379). Blots were then stripped and immunoblotted with anti-HA antibody to confirm equal loading of HA-MOPr. Blot is representative of five individual experiments. The position of molecular mass markers are indicated on the left (in KDa). (B) Pooled data from experiments as depicted in A, quantifying MOPr Ser375 phosphorylation induced by supramaximal concentrations of opioids as a factor of total receptor loaded. Data are presented as mean ± SEM, where *n* = 5. * *P* < 0.05, significantly different from unstimulated control values, one-way ANOVA with post-hoc Tukey test.

Supplementary Figure 1

Examined opioids have no distinct heterologous desensitization effect on noradrenaline-evoked GIRK currents in rat LC neurones. The magnitude of noradrenaline (100 μM) evoked GIRK currents was assessed before and after the application of described opioids at receptor saturating concentrations (10 μM DAMGO, 30 μM morphine, PZM21 and Compound 1). Noradrenaline-evoked currents were determined in the presence of prazosin (1 μM) and cocaine (3 μM). Data are presented as mean ± SEM, where *n* = 4 – 8. One-way ANOVA indicated there was no significant difference between described groups (*P* = 0.33).

Supplementary Figure 2

Morphine-induced MOPr desensitization in rat LC neurones is enhanced by activation of PKC. (A-C) Representative traces of morphine (30 μM)-evoked potassium currents in rat LC neurones exposed to DMSO (A; 0.1 %), PMA (B; 1 μM) or PMA & GF109203X (C; both at 1 μM) for 20 minutes before and during morphine application. All morphine-evoked currents were reversed with naloxone (NLX; 10 μM). Scale bars represent 20pA and 5 minutes. (D) Pooled data from experiments of the type presented in A-C assessing the peak GIRK current evoked by morphine after 20 minutes preincubation in described conditions. Responses were normalised to the maximal α_2-adrenoceptor-mediated current evoked by NA (100 μM) in the same cell. (E) Pooled time-courses for the desensitization of morphine-evoked GIRK currents in rat LC neurones post peak response after preincubation with DMSO (A), PMA (B) or PMA & GF109203X (C). (F) Pooled data for percentage of MOPr desensitization induced by morphine in the described conditions 10 minutes after peak morphine response. (D-F) Control: 0.1% DMSO. All data are

presented as mean ± SEM, where *n* = 5. (D) One-way ANOVA indicated there was no significant difference between described groups (*P* = 0.22). (F) * *P* < 0.05, *ns P* > 0.05, one-way ANOVA with post-hoc Tukey test.

Figure 1

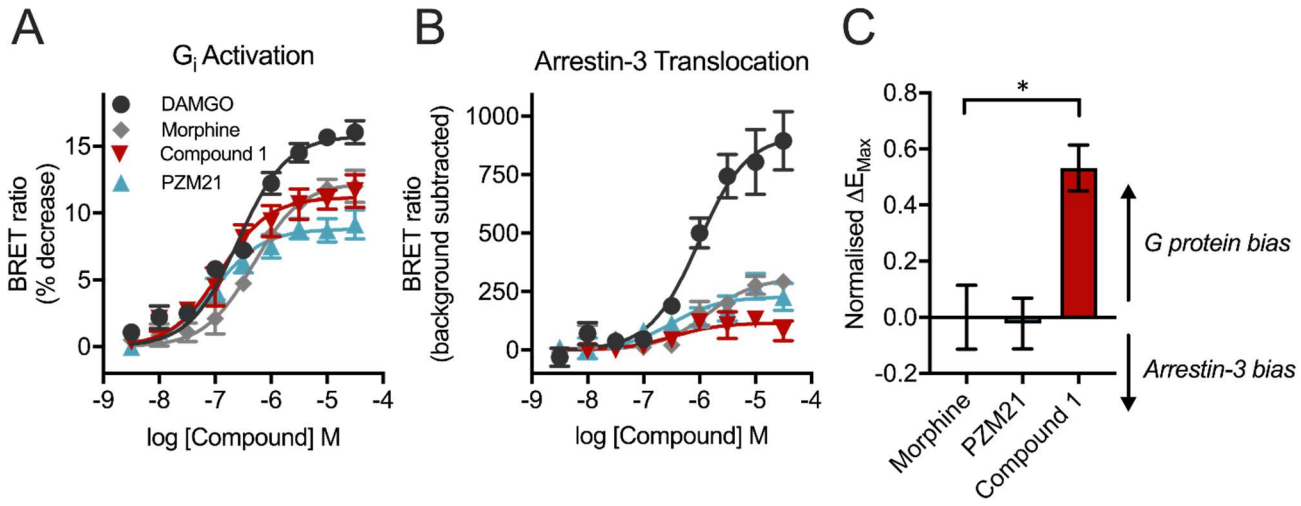

A
G_i Activation
DAMGO
Morphine
Compound 1
PZM21
BRET ratio (% decrease)
log [Compound] M
B
Arrestin-3 Translocation
BRET ratio (background subtracted)
log [Compound] M
C
Normalised ΔE_{Max}
*
G protein bias
Arrestin-3 bias
Morphine
PZM21
Compound 1

Figure 2

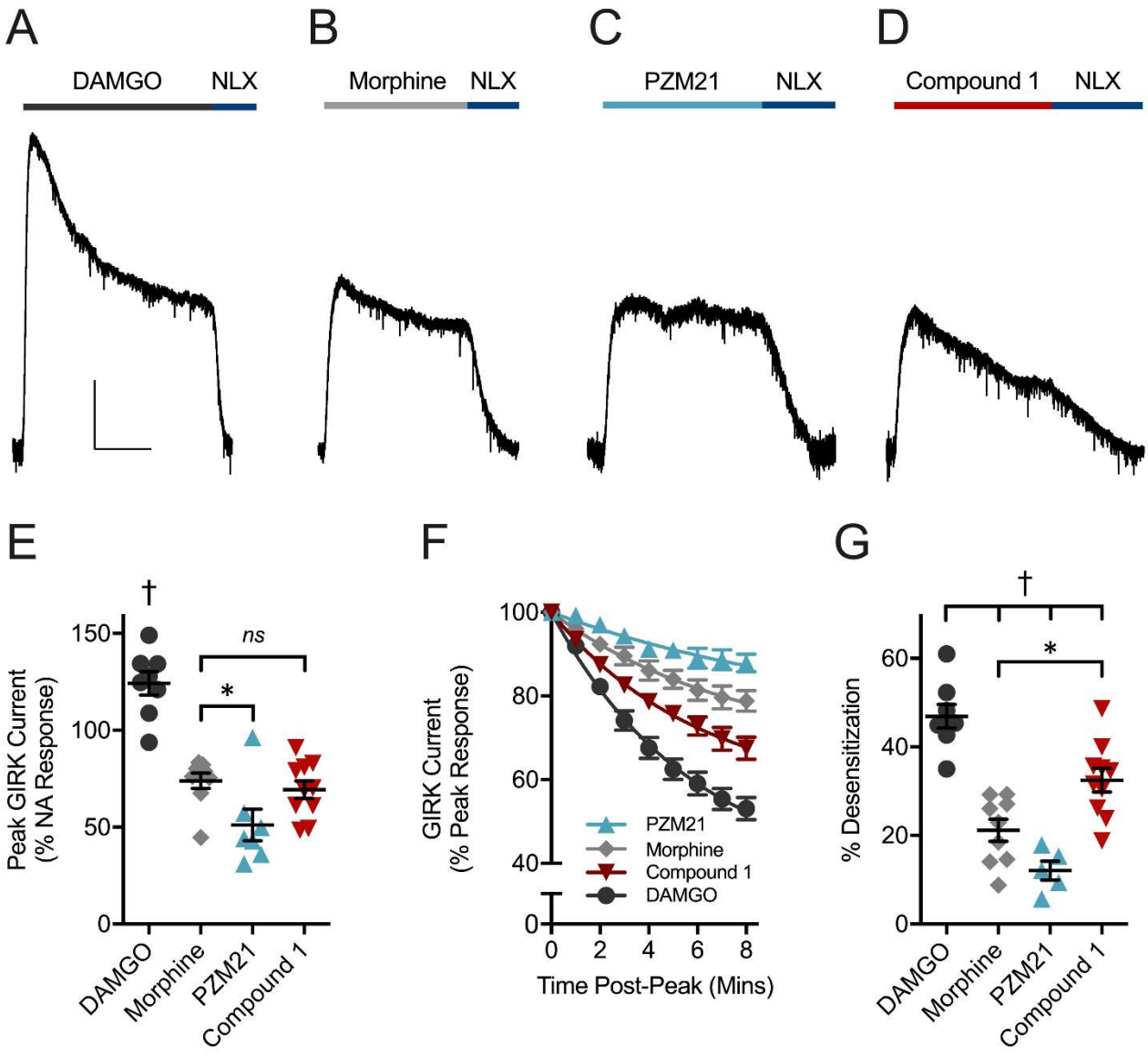

A
DAMGO
NLX
B
Morphine
NLX
C
PZM21
NLX
D
Compound 1
NLX
E
Peak GIRK Current
(% NA Response)
150
100
50
0
†
ns
*
DAMGO
Morphine
PZM21
Compound 1
F
GIRK Current
(% Peak Response)
100
80
60
40
0
PZM21
Morphine
Compound 1
DAMGO
0
2
4
6
8
Time Post-Peak (Mins)
G
% Desensitization
60
40
20
0
†
*
DAMGO
Morphine
PZM21
Compound 1

Figure 3

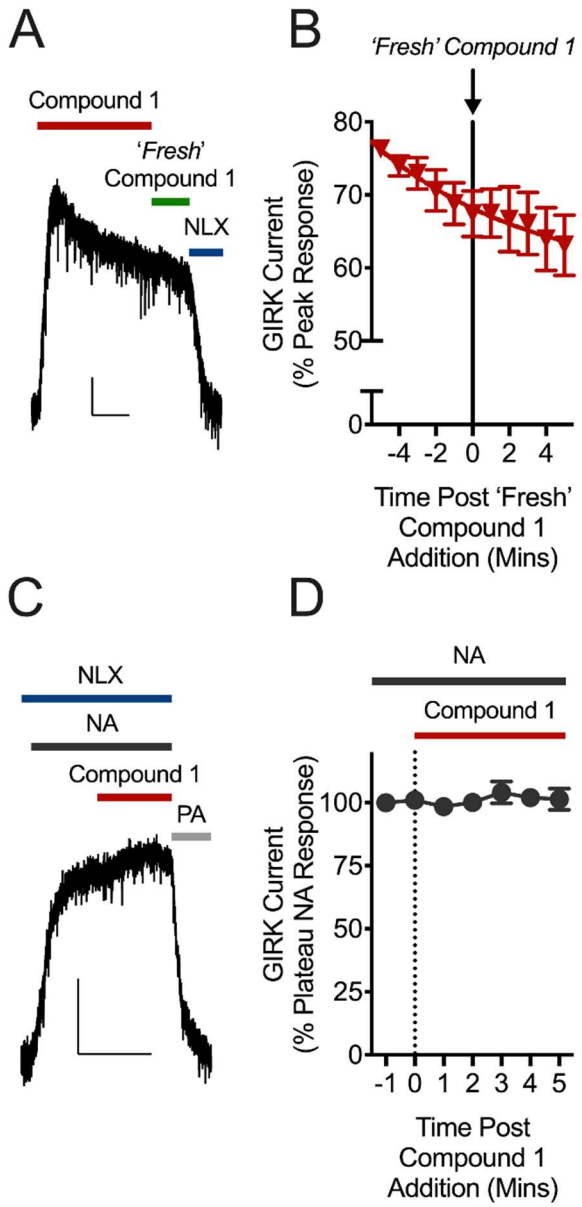

A
Compound 1
'Fresh'
Compound 1
NLX
B
'Fresh' Compound 1
GIRK Current
(% Peak Response)
80
70
60
50
0
-4 -2 0 2 4
Time Post 'Fresh'
Compound 1
Addition (Mins)
C
NLX
NA
Compound 1
PA
D
NA
Compound 1
GIRK Current
(% Plateau NA Response)
100
75
50
25
0
-1 0 1 2 3 4 5
Time Post
Compound 1
Addition (Mins)

Figure 4

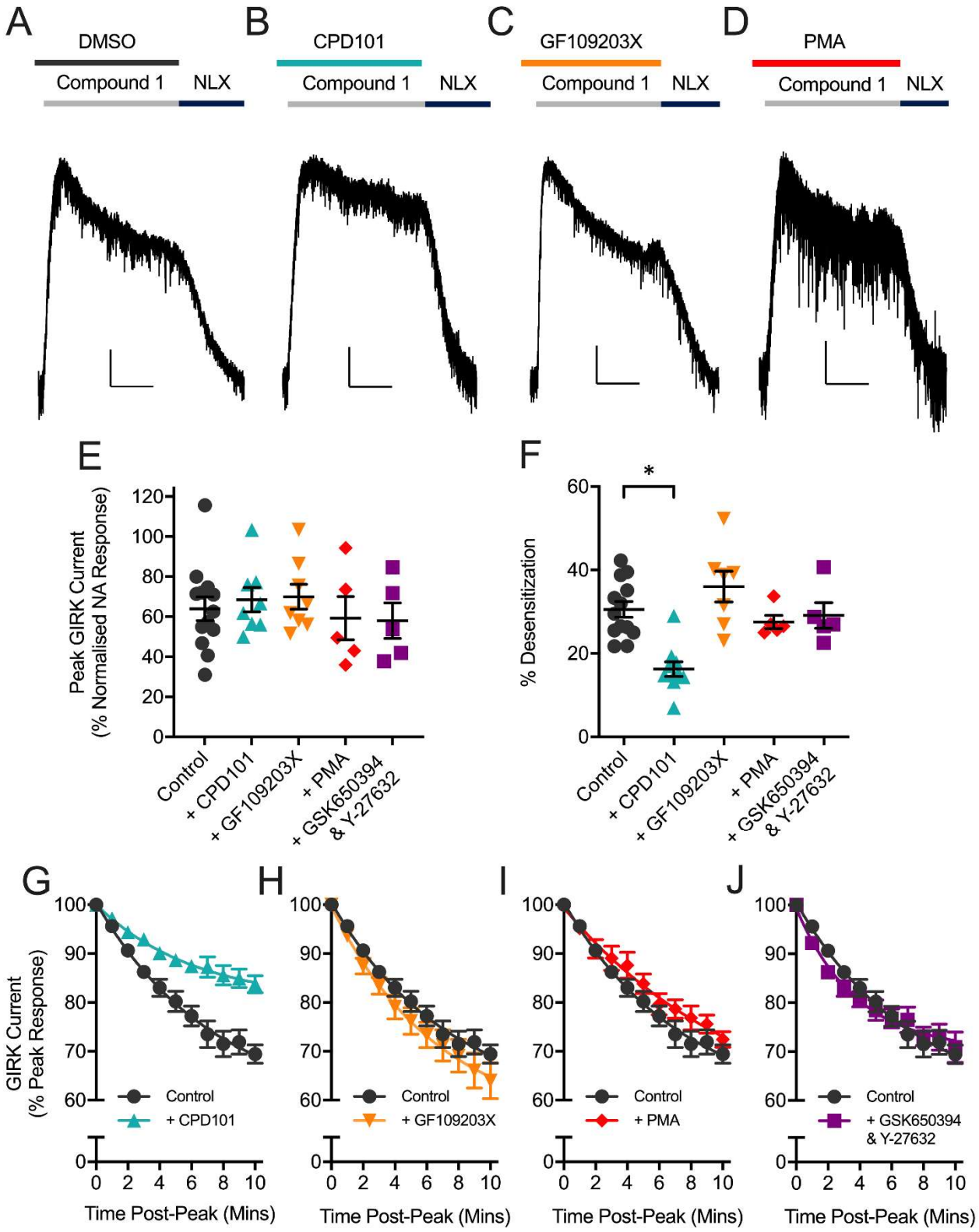

A
DMSO
Compound 1
NLX
B
CPD101
Compound 1
NLX
C
GF109203X
Compound 1
NLX
D
PMA
Compound 1
NLX
E
Peak GIRK Current
(% Normalised NA Response)
120
100
80
60
40
20
0
Control
+ CPD101
+ GF109203X
+ PMA
+ GSK650394
& Y-27632
F
% Desensitization
*
60
40
20
0
Control
+ CPD101
+ GF109203X
+ PMA
+ GSK650394
& Y-27632
G
GIRK Current
(% Peak Response)
100
90
80
70
60
0
Control
+ CPD101
0 2 4 6 8 10
Time Post-Peak (Mins)
H
100
90
80
70
60
0
Control
+ GF109203X
0 2 4 6 8 10
Time Post-Peak (Mins)
I
100
90
80
70
60
0
Control
+ PMA
0 2 4 6 8 10
Time Post-Peak (Mins)
J
100
90
80
70
60
0
Control
+ GSK650394
& Y-27632
0 2 4 6 8 10
Time Post-Peak (Mins)

Figure 5

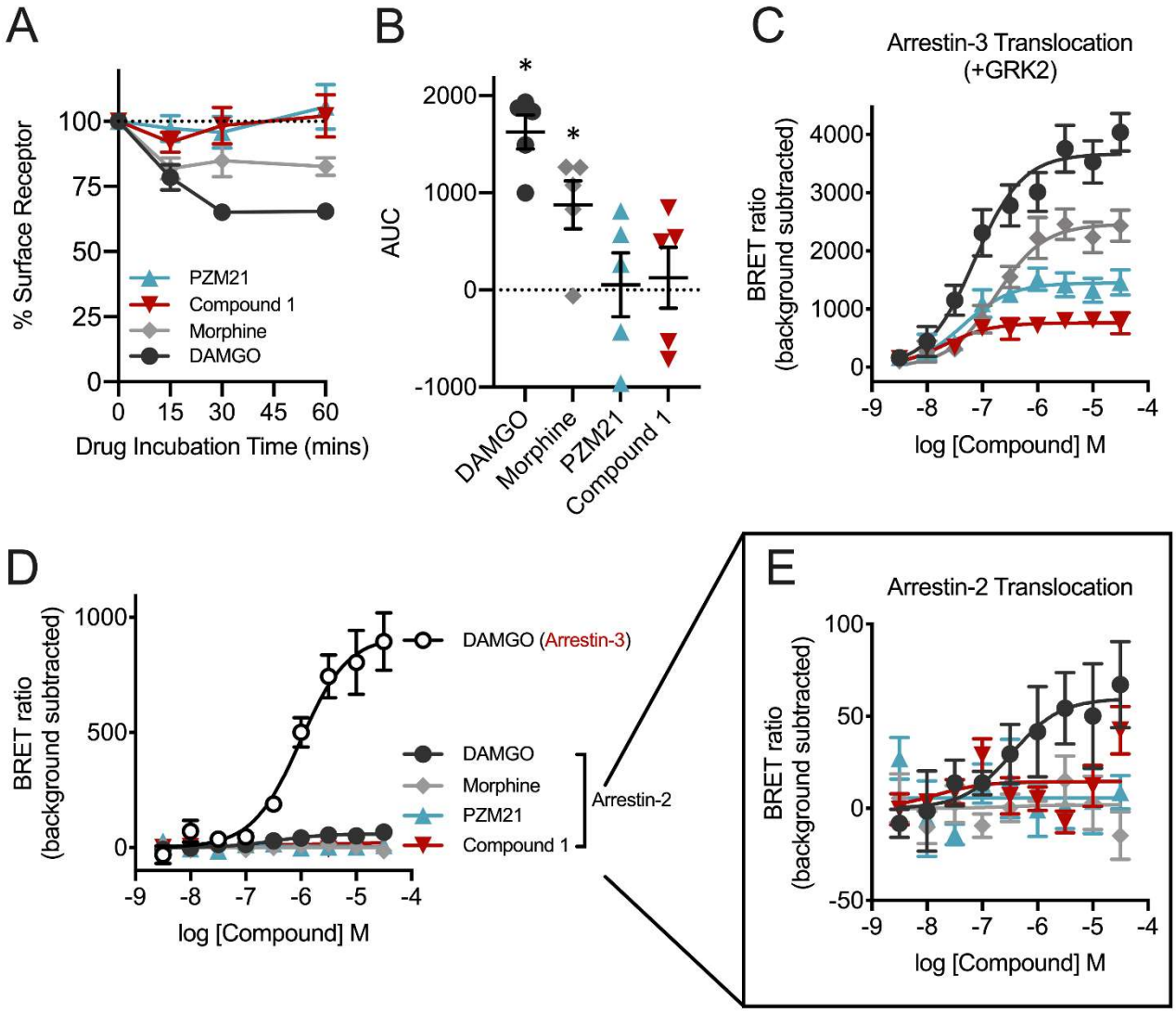

A
% Surface Receptor
100
75
50
25
0
0 15 30 45 60
Drug Incubation Time (mins)
PZM21
Compound 1
Morphine
DAMGO
B
AUC
2000
1000
0
-1000
*
*
DAMGO
Morphine
PZM21
Compound 1
C
Arrestin-3 Translocation
(+GRK2)
BRET ratio
(background subtracted)
4000
3000
2000
1000
0
-9 -8 -7 -6 -5 -4
log [Compound] M
D
BRET ratio
(background subtracted)
1000
500
0
-9 -8 -7 -6 -5 -4
log [Compound] M
DAMGO (Arrestin-3)
DAMGO
Morphine
PZM21
Compound 1
Arrestin-2
E
Arrestin-2 Translocation
BRET ratio
(background subtracted)
100
50
0
-50
-9 -8 -7 -6 -5 -4
log [Compound] M

Figure 6

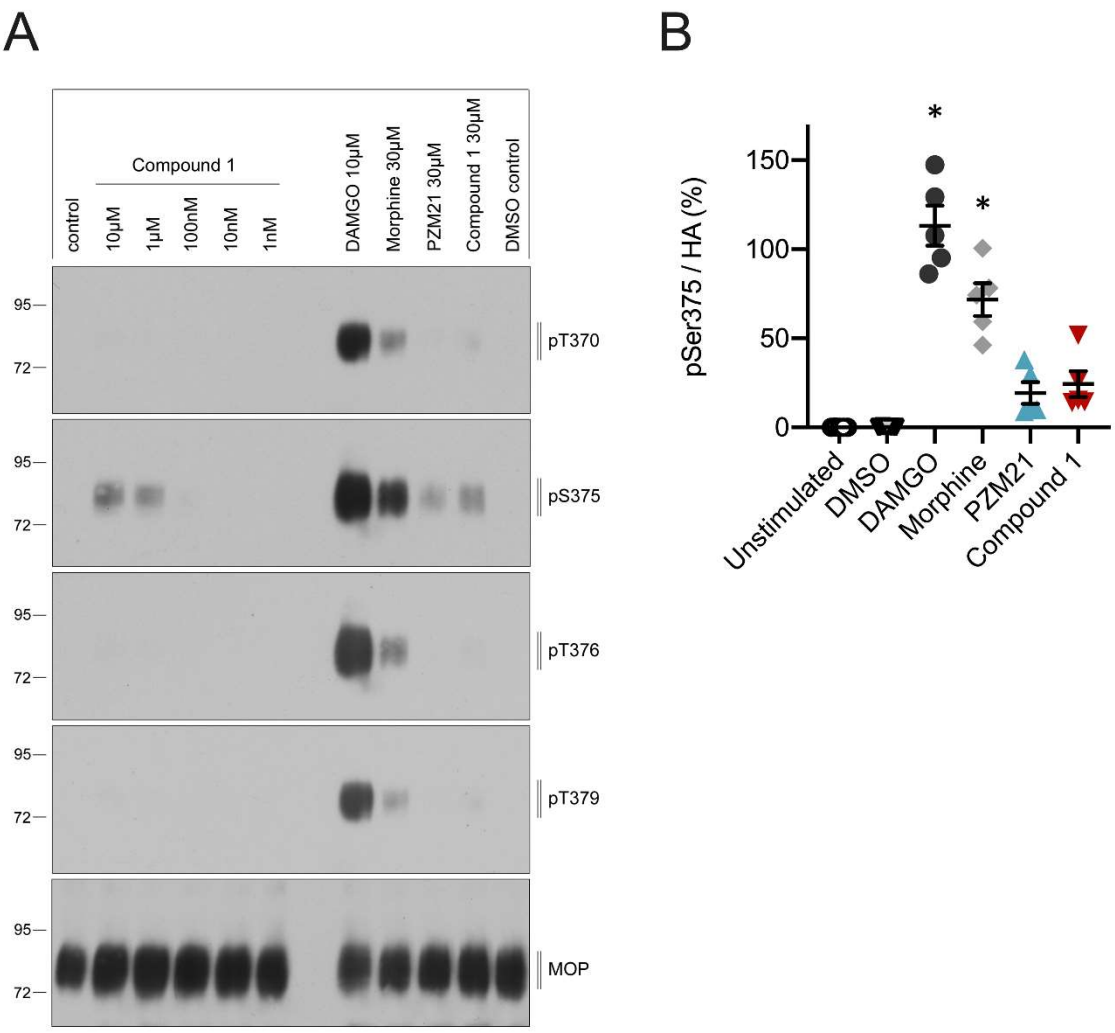

A
Compound 1
control
10μM
1μM
100nM
10nM
1nM
DAMGO 10μM
Morphine 30μM
PZM21 30μM
Compound 1 30μM
DMSO control
95
72
pT370
pS375
pT376
pT379
MOP
B
pSer375 / HA (%)
150
100
50
0
*
*
Unstimulated
DMSO
DAMGO
Morphine
PZM21
Compound 1

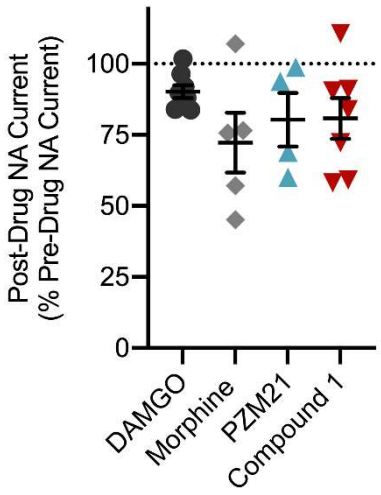
Table 1

pEC_{50} and E_{max} values for DAMGO, morphine, PZM21 and Compound 1 for $G\alpha_i$ activation and arrestin-3 recruitment downstream of MOPr.

	$G\alpha_i$ Activation			Arrestin-3 Recruitment		
	pEC_{50}	E_{max}		pEC_{50}	E_{max}	
Agonist	(M)	BRET Ratio (% decrease)	% DAMGO	(M)	BRET Ratio (baseline subtracted)	% DAMGO
DAMGO	6.6 ± 0.1	15.8 ± 0.5 †	100	6.0 ± 0.1	920 ± 56 †	100
Morphine	6.3 ± 0.1	12.3 ± 0.5	78 ± 3	5.9 ± 0.2	307 ± 33	33 ± 4
PZM21	7.0 ± 0.1	08.8 ± 0.4 *	56 ± 2	6.5 ± 0.2	227 ± 26	25 ± 3
Compound 1	6.9 ± 0.1	11.2 ± 0.5	71 ± 3	6.5 ± 0.4	116 ± 22 *	13 ± 2

† $P < 0.05$, significantly different to respective values of all other agonists. * $P < 0.05$, significantly different to the respective morphine value. One-way ANOVA with post-hoc Tukey test.

Supplementary Figure 1



Supplementary Figure 2