BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00944.x
www.brjpharmacol.org

RESEARCH PAPER

Agonists at the δ-opioid receptor modify the binding of μ-receptor agonists to the μ–δ receptor hetero-oligomer

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Keywords
μ-opioid receptor; δ-opioid receptor; heteromer; binding pocket; internalization; clathrin; chronic morphine exposure

Received
29 September 2009
Revised
19 April 2010
Accepted
10 June 2010

BACKGROUND AND PURPOSE
μ- and δ-opioid receptors form heteromeric complexes with unique ligand binding and G protein-coupling profiles linked to G protein α z-subunit ($G\alpha_z$) activation. However, the mechanism of action of agonists and their regulation of the μ–δ receptor heteromer are not well understood.

EXPERIMENTAL APPROACH
Competition radioligand binding, cell surface receptor internalization in intact cells, confocal microscopy and receptor immunofluorescence techniques were employed to study the regulation of the μ–δ receptor heteromer in heterologous cells with and without agonist exposure.

KEY RESULTS
$G\alpha_z$ enhanced affinity of some agonists at μ–δ receptor heteromers, independent of agonist chemical structure. δ-Opioid agonists displaced μ-agonist binding with high affinity from μ–δ heteromers, but not μ receptor homomers, suggestive of δ-agonists occupying a novel μ-receptor ligand binding pocket within the heteromers. Also, δ-agonists induced internalization of μ-opioid receptors in cells co-expressing μ- and δ-receptors, but not those expressing μ-receptors alone, indicative of μ–δ heteromer internalization. This dose-dependent, *Pertussis* toxin-resistant and clathrin- and dynamin-dependent effect required agonist occupancy of both μ- and δ-opioid receptors. In contrast to μ-receptor homomers, agonist-induced internalization of μ–δ heteromers persisted following chronic morphine exposure.

CONCLUSIONS AND IMPLICATIONS
The μ–δ receptor heteromer may contain a novel δ-agonist-detected, high-affinity, μ-receptor ligand binding pocket and is regulated differently from the μ-receptor homomer following chronic morphine exposure. Occupancy of both μ- and δ-receptor binding pockets is required for δ-agonist-induced endocytosis of μ–δ receptor heteromers. δ-Opioid agonists target μ–δ receptor heteromers, and thus have a broader pharmacological specificity than previously identified.

Abbreviations
con A, concanavalin A; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH2; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; deltorphin II, [D-Ala^2]deltorphin II; DPDPE, [D-Pen^2, D-Pen^5]enkephalin, DYN K44A, dynamin-dominant negative mutant; endomorphin-1, Tyr-Pro-Trp-Phe-NH_2; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; Leu-enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH; Met-enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH; mRFP, monovalent red fluorescent protein; PTX, *Pertussis* toxin; SDS, sodium dodecyl sulphate; SNC80 (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; UFP-512, H-Dmt-Tic-NH-CH(CH_2-COOH)-Bid

Introduction

Opioid receptors activated by their ligands are crucial modulators of many biological functions including pain perception, motivation, locomotion, hormone secretion and reward (for review, see Waldhoer *et al.*, 2004). Opioid receptors are members of the rhodopsin family of G protein-coupled receptors (GPCRs) and transduce their signals by activating inhibitory G proteins leading to inhibition of adenylyl cyclase, opening of inward rectifying potassium channels and inhibition of voltage-gated calcium channels. Three types of opioid receptors have been identified by molecular cloning studies: μ-, δ- and κ-opioid receptors (Kieffer *et al.*, 1992; Chen *et al.*, 1993; Li *et al.*, 1993; nomenclature follows Alexander *et al.*, 2009).

However, pharmacological studies in brain and other tissues predict a greater number of opioid receptor subtypes (Traynor and Elliott, 1993), which may result from direct receptor–receptor interactions (Rothman and Westfall, 1982; Levac *et al.*, 2002; Rutherford *et al.*, 2008). Indeed, we and others have shown that co-expressed μ- and δ-opioid receptors formed a hetero-oligomeric complex that functioned as a novel signalling unit, displayed a unique ligand binding profile and coupled to a different signal transducer G protein (George *et al.*, 2000; Gomes *et al.*, 2000; 2004; Fan *et al.*, 2005; Law *et al.*, 2005; Hasbi *et al.*, 2007). The μ–δ receptor heteromer displayed an altered affinity rank order for opioid agonists and had lower but equivalent affinities for the μ-receptor agonist DAMGO and the δ-receptor agonist DPDPE. Furthermore, some endogenous opioid receptor agonists such as Leu-enkephalin displayed greater affinity for the μ–δ receptor heteromer than the individual μ- or δ-opioid receptor. Whereas *Pertussis* toxin (PTX) is known to abolish high-affinity binding to μ- and δ-opioid receptor homomers as well as adenylyl cyclase inhibition and signalling by these receptors when expressed individually, μ–δ receptor heteromer ligand binding parameters and function were PTX resistant (Pak *et al.*, 1999; George *et al.*, 2000; Fan *et al.*, 2005). Recently, we demonstrated that the μ–δ receptor heteromer preferentially coupled to G protein α z-subunit ($G\alpha_z$), a PTX-insensitive GTP-binding protein α subunit, whereas the individual μ- and δ-opioid receptor homomers activated the PTX-sensitive $G\alpha_i$ protein (Fong *et al.*, 1988; Fan *et al.*, 2005; Hasbi *et al.*, 2007). In addition, $G\alpha_z$ was found to affect ligand binding affinities for the μ–δ receptor heteromer, not the μ- and δ-receptor homomers; however, it was not clear whether this was a universal or agonist-specific effect (Fan *et al.*, 2005). The switch in G protein specificity that we identified indicated that the μ–δ receptor heteromer had a distinct cellular mechanism of action compared to μ- and δ-receptor homomers. The μ–δ receptor complex has been demonstrated in native tissue where it may have a role in analgesia (Gomes *et al.*, 2004).

The nature of the ligand binding pocket of the μ–δ receptor heteromer, the effect of $G\alpha_z$ coupling on ligand binding and the regulation of this receptor complex under basal conditions and following agonist exposure have not been fully explored. This is a necessary step for delineating further the physiological relevance of the μ–δ receptor heteromer and determining its suitability as a potential novel therapeutic target. The results of this study provide evidence for δ-receptor agonists competing with high affinity for μ-receptor agonist binding to the μ–δ receptor heteromer. This did not occur for the μ-receptor homomer, which suggested that the μ–δ receptor heteromer may contain a novel μ-receptor ligand binding pocket detected by δ-receptor agonists. In addition, δ-agonist-induced internalization of the μ–δ receptor heteromer required the occupancy of both constituent μ- and δ-opioid receptors, involved the clathrin and dynamin endocytic machinery and was still present in a cellular model of morphine tolerance.

Methods

cDNA Constructs

The transformer site-directed mutagenesis kit (Clontech, Mountain View, CA, USA) was used to insert epitopes into μ- and δ-opioid receptor as previously described (George *et al.*, 2000). To generate $_{cMyc}$-μOR and $_{FLAG}$-δOR, the c-Myc (EQKLISEEDL) or FLAG (DYKDDDDK) epitopes were inserted after the NH_2-terminal start methionine of rat μ- or δ-opioid receptor cDNAs respectively. cDNAs were then inserted together or separately into the mammalian expression vector pBudCE4.1 (Invitrogen, Burlington, ON, Canada). To generate green fluorescent protein-tagged μ-opioid receptors (μOR_{GFP}) and monovalent red fluorescent protein (mRFP)-tagged δ-opioid receptors (δOR_{mRFP}) in pEGF-N1 (Invitrogen), the GFP and mRFP sequences were inserted at the carboxy termini of the μ-receptor or δ-receptor, respectively, as described previously (Hasbi *et al.*, 2007). Sequencing ensured the correct orientation of the polymerase chain reaction products in the expression vector and absence of sequence errors. cDNA-encoding rat $G\alpha_z$ was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen), and sequenced to ensure accuracy and correct orientation. The dominant-negative dynamin

mutant DYN K44A construct in pCB1 vector was a kind gift from Dr Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA, USA), and was generated as described (Zhang *et al.*, 1996). Cells were transfected with 2 μg of $G\alpha_z$ or DYN K44A cDNA using LipofectAMINE (Invitrogen) according to the manufacturer's protocols. Cells were used 48 h post-transfection.

Expression in mammalian cells

Human embryonic kidney (HEK) 293T HEK cells (American Type Culture Collection, Manassas, VA, USA) were maintained as a monolayer at 37°C with 5% CO_2 saturation in advanced minimal essential medium supplemented with 6% fetal bovine serum and antibiotics (Invitrogen). Cells were stably transfected with the pBudCE4.1 expression vector containing μ- and/or δ-opioid receptor using LipofectAMINE reagent (Invitrogen). Clones expressing each of the receptors at a density of 150–175 fmol·mg^{-1} were used (150–350 fmol·mg^{-1} total receptor protein).

Cell membrane preparations

Cells were rinsed with phosphate-buffered saline (PBS), suspended, pelleted and lysed by polytron homogenization in a 5 mM Tris–HCl and 2 mM EDTA solution containing a protease inhibitor cocktail (5 μg·mL^{-1} leupeptin, 10 μg·mL^{-1} benzamidine and 5 μg·mL^{-1} soy bean trypsin inhibitor) as previously described (George *et al.*, 2000). Unbroken cells and nuclei were pelleted by centrifugation at 100× *g*. The supernatant was centrifuged at 40 000× *g* for 20 min at 4°C to prepare the crude membrane fraction (P2). Membrane protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) as per the manufacturer's protocol.

Competition radioligand binding

Experiments were performed in duplicate on cell membrane preparations with increasing concentrations of competing ligand (10^{-12} to 10^{-4} M). Both [^{3}H]-diprenorphine (50 Ci·mmol^{-1}) and [^{3}H]-DAMGO (36.8 Ci·mmol^{-1}) were purchased from Perkin Elmer Life Sciences (Waltham, MA, USA). The concentration of radioligand used approximated its K_D (1 nM [^{3}H]-diprenorphine and 2 nM [^{3}H]-DAMGO). Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Gaithersburg, MD, USA) using GF/C filters (Whatman, Florham Park, NJ, USA). Filters were washed with cold 50 mM Tris–HCl buffer (pH 7.4), placed in vials containing scintillation fluid and counted for tritium.

PTX treatment

Cells were pretreated with 100 ng·mL^{-1} PTX (Sigma, St Louis, MO, USA) for 24 h prior to radioligand binding experiments.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Membrane proteins (20 μg protein/lane) were resolved on a 10% Tris–glycine precast gel (Novex, San Diego, CA, USA) under denaturing conditions by SDS–PAGE, and then electroblotted onto a polyvinylidene difluoride membrane as described previously (Fan *et al.*, 2005). Immunoreactivity was revealed by incubating in $G\alpha_z$ or $G\alpha_{i3}$ antibody diluted 1:250 (Sc-388 or Sc-262, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA), HRP-conjugated goat–anti-rabbit secondary antibody diluted 1:1000 (Bio-Rad) and enhanced chemiluminescent reagents (KPL, Gaithersburg, MD, USA), and then exposing to film. Immunoreactive bands are expected at 41 kDa for $G\alpha_z$ (Premont *et al.*, 1989) and $G\alpha_{i3}$ (Holz *et al.*, 1989) as reported in the literature. Blots were stripped using β-mercaptoethanol buffer and reprobed with GAPDH antibody diluted 1:10 000 (Abcam, Cambridge, MA, USA), which served as the loading control.

Chronic morphine treatment

Cells were treated with 10 μM morphine sulphate in Advanced-MEM for 48 h. Cells were washed three times in PBS (pH 7.4) prior to incubation in medium with or without opioid receptor agonists.

Intact cell internalization assay

μ-Opioid receptor binding was performed on intact cells stably expressing these receptors using [^{3}H]-DAMGO at its K_D (2 nM). [^{3}H]-DAMGO would only label cell surface receptors because it is a hydrophilic peptide ligand and so does not penetrate the plasma membrane (Blake *et al.*, 1997; Koch *et al.*, 1998; Szeto *et al.*, 2001). Non-specific binding was assessed using 10 μM naloxone. Cells were pretreated with agonist for 1 h at 37°C, rinsed, treated with [^{3}H]-DAMGO for 4 h at 4°C, then rinsed. Cell surface radioactivity was measured using the liquid scintillation counter. The disappearance of [^{3}H]-DAMGO binding sites on the cell surface served as an indicator of μ-receptor internalization. In separate experiments, δ-receptor internalization was assessed using 1 nM [^{3}H]-diprenorphine following the same protocol as above.

Cell surface μ-receptor immunofluorescence

Cells stably expressing $_{cMyc}$-μOR and $_{FLAG}$-δOR were seeded onto a 96-well plate at a density of 5×10^4

cells per well. Cells were pretreated with 10 μM agonist for 1 h at 37°C, rinsed and then fixed using 4% paraformaldehyde prior to incubation with blocking solution (4% bovine serum albumin) to prevent binding of antibody to non-specific sites. Cells were then incubated with mouse monoclonal anti-cMyc 4A6 antibody (05-724, Upstate, Billerica, MA, USA) for 2 h at room temperature, then rinsed and incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (A11001, Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Cells were rinsed again and signals were collected using a plate reader spectrofluorometer (Victor, Perkin Elmer Instruments). The decrease in cell surface receptor immunofluorescence served as an indicator of μ-receptor internalization.

Receptor visualization using confocal microscopy

Cells were transfected with cDNAs encoding μOR_{GFP} and δOR_{mRFP}. Forty-eight hours post-transfection, living cells were treated with 100 nM agonist or vehicle, and examined on the LSM 510 Zeiss confocal microscope (Carl Zeiss, Toronto, ON, Canada).

Data analysis

Statistical analyses and graph generation were performed using GraphPad Prism software 3.01 (San Diego, CA, USA). The results were presented as means ± SEM. Data from competition radioligand binding experiments were analysed by non-linear least square regression. An *F*-test was used to compare the coefficients of the goodness-of-fit and to determine whether a two- or a one-site binding was a statistically significant better fit. The unpaired Student's *t*-test was used to assess statistical significance. $P < 0.05$ was deemed significant.

Materials

Deltorphin II, DAMGO, DPDPE, naltrindole hydrochloride, SNC80, Leu-enkephalin, Met-enkephalin, endomorphin-1 and naloxone hydrochloride were purchased from Sigma. CTOP was purchased from Tocris (Ellisville, MO, USA). Morphine sulphate was purchased from BDH Chemicals (Toronto, ON, Canada). UFP-512 was synthesized by Balboni *et al.* (2002). Concanavalin A (con A) was purchased from Calbiochem (Los Angeles, CA, USA).

Results

Modulatory role of $G\alpha_z$ on peptidic and non-peptidic agonist affinities for the μ–δ receptor heteromer

The affinities of peptidic (endogenous and synthetic) and non-peptidic μ- and δ-receptor agonists were compared in cells co-expressing μ- and δ-opioid receptors in the absence or presence of transfected $G\alpha_z$ using competition of antagonist [^{3}H]-diprenorphine (non-selective μ/δ antagonist) binding. Whereas $G\alpha_i$ proteins are ubiquitously expressed in HEK cells, $G\alpha_z$ is not abundantly expressed (Supporting Information Figure S1; Fan *et al.*, 2005). Transfection resulted in an approximately threefold increase in $G\alpha_z$ expression over endogenous levels, as determined by densitometry analysis (ImageJ software, NIH, Bethesda, MD, USA). $G\alpha_z$ co-expression significantly increased high-affinity binding (K_H) of the δ-receptor agonist DPDPE by 10-fold, the endogenous agonist Leu-enkephalin by threefold and the μ-receptor agonist DAMGO by 10-fold for the μ–δ receptor hetero-oligomer (Table 1). $G\alpha_z$ co-expression resulted in an overall leftward shift of the competition curves, resulting in higher affinities both at the high- and low-affinity sites. In contrast, high-affinity binding of the synthetic δ-receptor agonists UFP-512 and SNC80, and the endogenous μ-receptor agonist endomorphin-1 was unaltered by $G\alpha_z$ co-expression (Table 1).

The percentage of receptors in the agonist-detected high-affinity state (% HA) was not significantly different following $G\alpha_z$ co-expression, except in the case of Leu-enkephalin and UFP-512 where it was reduced. This change in % HA did not seem to relate to the effect of $G\alpha_z$ coupling on agonist affinities, because the affinity of Leu-enkephalin was enhanced upon $G\alpha_z$ co-expression, whereas that of UFP-512 was not altered. Overall, $G\alpha_z$ enhanced the affinity of some agonists at the μ–δ receptor complex, an effect independent of agonist chemical structure.

In order to assess true agonist affinity at the $G\alpha_z$-coupled μ–δ receptor complex, PTX (100 ng·mL^{-1} overnight) was used to inactivate $G\alpha_{i/o}$ proteins and to minimize the contribution of $G\alpha_{i/o}$-coupled receptors to the high-affinity binding detected. PTX treatment resulted in the emergence of a high-affinity binding site for the δ-agonist SNC80 with a 160-fold increase in affinity, whereas Leu-enkephalin-detected high-affinity states were not significantly enhanced (Table 1). PTX was necessary to reveal the affinity of SNC80 at the μ–δ receptor complex.

Comparison of the δ-agonist-detected ligand binding pocket in the μ–δ receptor heteromer and the μ-receptor homomer

To examine δ-receptor agonist displacement of μ-receptor agonist binding from the μ–δ receptor heteromer and the μ-receptor homomer, competition radioligand binding was performed using

Table 1

Effect of $G\alpha_z$ on peptidic and non-peptidic agonist affinities for the μ–δ opioid receptor heteromer (μ–δOR)

	μ–δOR			μ–δOR + $G\alpha_z$			μ–δOR + $G\alpha_z$ + PTX		
	K_H (nM)	K_L (nM)	% HA	K_H (nM)	K_L (nM)	% HA	K_H (nM)	K_L (nM)	% HA
DPDPE	32 ± 11	16 161 ± 1662	48 ± 4.8	3.0 ± 1.0*	123 ± 49	33 ± 4.6	nd	nd	nd
Leu-Enkephalin	5.9 ± 1.2	201 ± 42	62 ± 5.2	1.9 ± 0.5*	38 ± 6.8	34 ± 6.0*	0.9 ± 0.1	157 ± 46	36 ± 5.2
DAMGO	28 ± 6.0	3774 ± 312	32 ± 3.8	2.9 ± 0.7*	2161 ± 519	31 ± 5.0	nd	nd	nd
Endomorphin-1	148 ± 37	7305 ± 702	21 ± 2.4	154 ± 34	13 193 ± 2725	28 ± 2.0	nd	nd	nd
UFP-512	1.7 ± 0.1	1165 ± 101	58 ± 3.4	1.5 ± 0.1	887 ± 29	47 ± 1.0*	nd	nd	nd
SNC80	n/a	298 ± 10	n/a	n/a	309 ± 22	n/a	1.9 ± 0.5***	263 ± 85	33 ± 2.8

Values shown represent mean ± SEM of $n = 3$–6 experiments performed in duplicate. Competition radioligand binding was performed using the non-selective μ/δ antagonist [^{3}H]-diprenorphine. An *F*-test demonstrated that a two-site binding curve provided a statistically significant better fit for SNC80 binding in PTX-treated cells expressing μ-receptors, δ-receptors and $G\alpha_z$ ($P < 0.05$). The unpaired Student's *t*-test was used to compare K_H and % HA values.

K_H, agonist-detected high-affinity site binding constant; K_L, agonist-detected low-affinity site binding constant; % HA, percentage of receptors in the agonist-detected high-affinity state; nd, not determined; n/a, not applicable.

*$P < 0.05$, ***$P < 0.001$ relative to cells expressing μ- and δ-receptors.

[^{3}H]-DAMGO in cells co-expressing μ- and δ-opioid receptors or only expressing μ-receptors. DAMGO is a highly selective μ-receptor agonist having approximately 1600-fold higher affinity for μ- than δ-receptors (George *et al.*, 2000). Furthermore, [^{3}H]-DAMGO binding was inhibited by the selective μ-receptor antagonist naltrexone with similar affinity both in cells co-expressing μ- and δ-opioid receptors ($K_i = 0.51 \pm 0.05$ nM; $n = 3$) or only expressing μ-receptors ($K_i = 0.49 \pm 0.13$ nM; $n = 3$). At the concentration used, DAMGO did not detect δ-receptors as confirmed by the lack of binding in cells expressing δ-receptors alone (Figure 1C). All δ-receptor agonists tested detected a single low-affinity binding site in cells expressing μ-receptors, as indicated by monophasic competition of [^{3}H]-DAMGO binding (Figure 1; Table 2). In contrast, a high-affinity binding site emerged when δ-receptor agonists competed for [^{3}H]-DAMGO binding in cells co-expressing μ- and δ-receptors (Figure 1; Table 2). All of SNC80, UFP-512, Deltorphin II and DPDPE displaced [^{3}H]-DAMGO binding with statistically significantly higher affinities (380-, 165-, 1800- and 200-fold, respectively) in cells co-expressing μ- and δ-receptors compared to cells expressing μ-receptors alone (Table 2), and this was shown by a leftward shift in the agonist competition curve (Figure 1A,B). The percentage of receptors in the δ-agonist-detected high-affinity state was approximately 20–30%. The finding that δ-receptor agonists had higher affinities for the [^{3}H]-DAMGO site in cells co-expressing μ- and δ-opioid receptors, compared to those expressing only μ-receptors, suggested that the μ-receptor ligand binding pocket within the μ–δ receptor heteromer may be different from that in the μ-receptor homomer. To confirm that the binding pocket detected by [^{3}H]-DAMGO was composed of μ-receptors, we pretreated cells with 50 nM of the irreversible μ-receptor antagonist β-funaltrexamine. This concentration was selected because it resulted in approximately 80% inhibition of [^{3}H]-DAMGO binding in cells expressing μ-receptors and only 30% inhibition of [^{3}H]-diprenorphine binding in cells expressing δ-receptors (Supporting Information Figure S2). In membranes from β-funaltrexamine-treated cells co-expressing μ- and δ-opioid receptors or expressing only μ-receptors, specific [^{3}H]-DAMGO binding was greatly attenuated and SNC80 could not displace [^{3}H]-DAMGO (data not shown), indicating that δ-receptor agonists did indeed compete for binding within a ligand binding pocket comprising μ-receptors. These findings may suggest that δ-receptor agonists detect and occupy a novel μ-receptor ligand binding site with high affinity only when the μ-receptor is complexed with the δ-receptor.

In separate experiments, SNC80 competition for [^{3}H]-DAMGO binding was assessed in cells transfected with additional $G\alpha_z$ in the absence and presence of PTX pretreatment. SNC80 displaced [^{3}H]-DAMGO binding with high nanomolar affinity under all these conditions, suggesting that the binding pocket detected is unique to the μ–δ receptor heteromer as it was PTX resistant.

Opioid agonist-induced internalization of the μ–δ receptor heteromer

To determine how opioid ligands modulated the density of cell surface μ–δ receptor heteromers, an

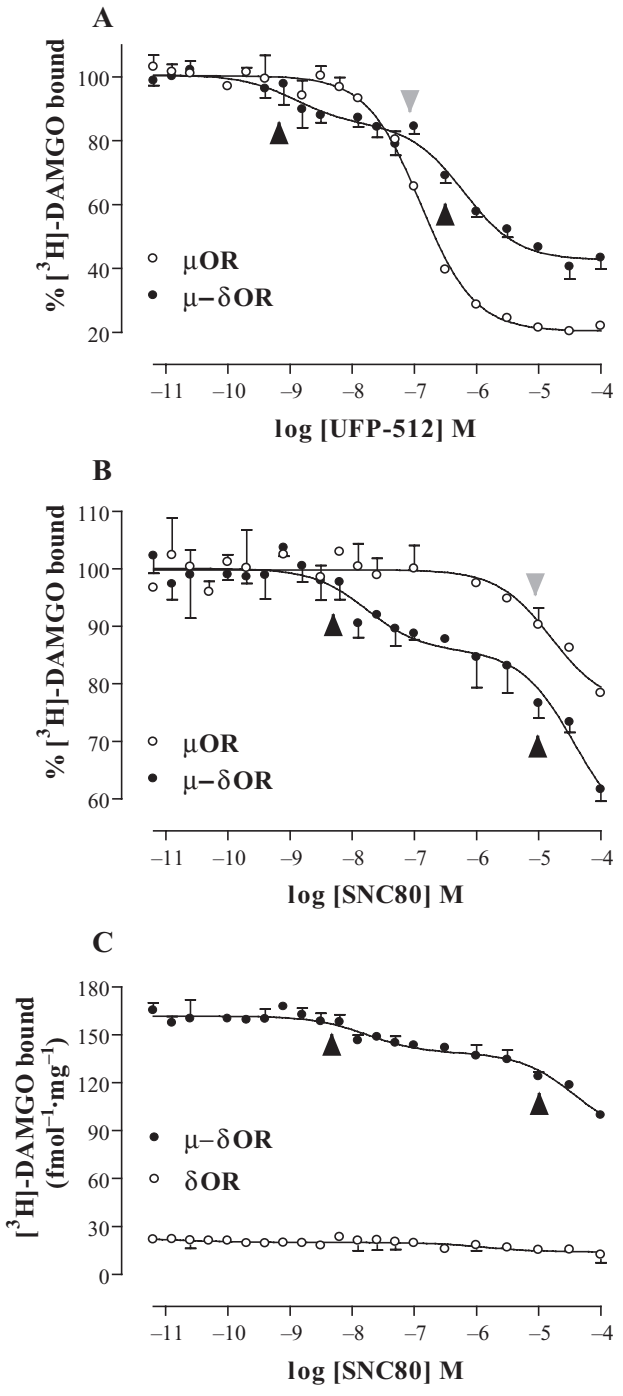

Figure 1

δ-Opioid agonist detection of the μ-ligand binding pocket in cells co-expressing μ- and δ-opioid receptors (μOR; δOR) or expressing only μ- or δ-receptors. Competition of [^{3}H]-DAMGO binding to membranes from HEK 293T cells expressing only μ-receptors or μ- and δ-receptors by (A) UFP-512 or SNC80 (B), and cells expressing μ- and δ-receptors or only δ-receptors by SNC80 (C). Grey and black arrowheads indicate high and low ligand binding affinities in cells expressing μ-receptors only or the μ–δ receptor heteromer respectively. Cells expressed 150–350 fmol·mg^{-1} of total receptor protein, whereby each receptor was expressed at a density of 150–175 fmol·mg^{-1}. Results shown are mean ± SEM, and curves are representative of n = 3–6 experiments performed in duplicate.

internalization assay in intact cells was performed to track the disappearance of cell surface [^{3}H]-DAMGO labelling as an index of μ-receptor internalization in cells co-expressing μ- and δ-opioid receptor. δ-Agonist-induced internalization of μ-receptor in this expression background would serve as an index of μ–δ receptor heteromer internalization. All endogenous and synthetic μ- and δ-opioid agonists tested induced internalization of μ-receptors following agonist (10 μM for 1 h) exposure (Figure 2). Agonist-induced internalization was dose dependent (Supporting Information Figure S3). As expected, μ-receptor agonists induced significantly greater internalization of μ-receptors than δ-receptor agonists, as they may be expected to induce internalization of both μ–δ receptor heteromers and μ-receptor homomers present in the cells. The endogenous opioid agonists endomorphin-1, and Met- and Leu-enkephalin resulted in 50–60% internalization, whereas the μ-receptor agonist DAMGO induced greater attenuation of cell surface μ-receptors (Figure 2). δ-Receptor agonists resulted in loss of cell surface μ-receptors, with UFP-512, SNC8O, deltorphin II and DPDPE inducing internalization of between 48 and 22% of the cell surface receptors, respectively (Figure 2, bars 4–9). Vehicle-treated cells, where no receptor internalization occurred, served as controls.

Internalization of μ-receptors induced by δ-receptor agonists was also analysed using amino terminal epitope-tagged $_{cMyc}$μOR and $_{FLAG}$δOR in a cell surface receptor immunofluorescence assay. The decrease in cell surface cMyc immunofluorescence served as an indicator of μ-receptor internalization. SNC80 treatment (10 μM for 1 h) induced a 20% decrease in cell surface μ-receptors, which was comparable to the internalization by SNC80 measured using radioligand binding. δ-Agonist-induced disappearance of cell-surface μ-receptor labelling in cells co-expressing μ- and δ-receptors suggested that the receptor population internalized were likely to be μ-receptors oligomerized with δ-receptors. These findings confirm that the μ–δ receptor heteromer was expressed on the cell surface and that its function was regulated by both endogenous and synthetic opioid receptor agonists.

Visualization of δ-agonist-induced co-internalization of μ- and δ-opioid receptors in living cells

Internalization of μ-receptors induced by δ-receptor agonists was visualized by real-time confocal microscopy. Cells co-expressing δOR_{mRFP} and μOR_{GFP} were treated with 100 nM UFP-512, and the endocytosis of cell surface receptors was observed. Under basal conditions, μ- and δ-receptor fluorescence was

Table 2

δ-Opioid receptor agonist affinities for the μ-ligand binding pocket in cells co-expressing μ- and δ-opioid receptors (μ–δOR) compared to cells expressing μ-opioid receptors alone (μOR)

	μOR K_i (nM)	K_H (nM)	μ–δOR K_L (nM)	% HA
SNC80	9220 ± 1358	24 ± 7.0**	2606 ± 723	27 ± 2.3
UFP-512	83 ± 9.0	0.5 ± 0.1***	259 ± 60	25 ± 3.5
DPDPE	3120 ± 490	15 ± 4.0**	1774 ± 254	16 ± 2.0
Deltorphin II	6230 ± 1098	3.5 ± 1.2**	5150 ± 1177	21 ± 3.4

Competition radioligand binding was performed using [^{3}H]-DAMGO. Values shown represent mean ± SEM of $n = 3$–6 experiments performed in duplicate. Cells expressed 150–350 fmol·mg^{-1} of total receptor protein. An *F*-test was used to determine whether a two-site or a one-site binding curve provided a statistically significant better fit for δ-agonist binding to the μ-receptor ligand binding pocket in cells co-expressing μ- and δ-receptors (SNC80: $P < 0.0001$; UFP-512, $P < 0.0001$; Deltorphin II, $P < 0.001$; DPDPE, $P < 0.05$).
K_i, agonist-detected affinity site binding constant; K_H, agonist-detected high-affinity site binding constant; K_L, agonist-detected low-affinity site binding constant; % HA, percentage of receptors in the agonist-detected high-affinity state.
**$P < 0.01$, ***$P < 0.001$ relative to cells expressing μ-receptors alone.

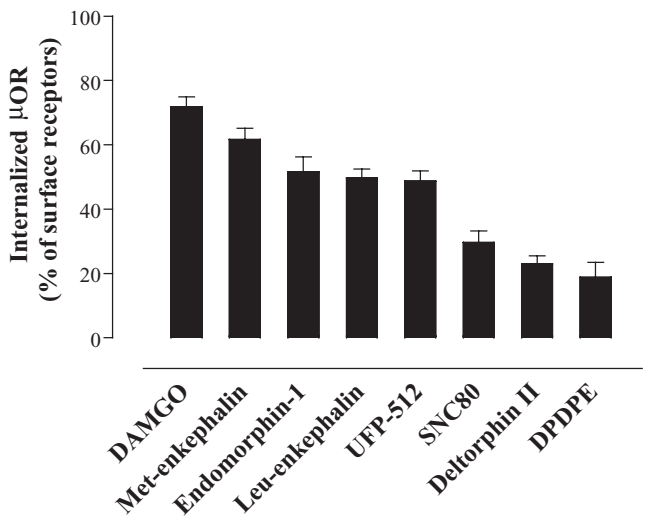

Figure 2

Agonist-induced internalization of cell surface μ-opioid receptors (μOR) detected by [^{3}H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-receptors (δOR). Cells were treated with 10 μM agonist for 1 h at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± SEM for $n = 3$–5 experiments performed in triplicate.

present mostly on the cell surface (Figure 3A–C). Agonist treatment resulted in receptor clustering and inward movement of cell surface μ- and δ-opioid receptors seen as intracellular yellow puncta (Figure 3D–F, arrows). The cell membrane outline became less defined, consistent with drug-induced receptor movement. These observations support our findings from the intact cell internalization assay, indicating that this δ-receptor agonist induced internalization of both μ- and δ-opioid receptors. In separate experiments, UFP-512 induced a dose-dependent internalization of δ-receptors expressed alone, but had no effect (at concentrations between 0.1 and 10 μM) on μ-receptors expressed alone (Supporting Information Figure S4).

Role of δ- and μ-opioid receptors in δ-agonist-induced internalization of the μ–δ receptor heteromer

To probe the involvement of δ- and μ-receptors in δ-agonist-induced internalization of μ-receptors in cells co-expressing μ- and δ-receptors, cells were pretreated with the δ-receptor antagonist naltrindole or the μ-receptor antagonist CTOP (Figure 4A). Naltrindole abolished SNC80- and deltorphin II-induced internalization. Blocking the μOR with CTOP abolished UFP-512, SNC80- and deltorphin II-induced internalization (Figure 4A), providing evidence that internalization of μ-receptors induced by δ-receptor agonists required the occupancy of both μ- and δ-receptor binding pockets within the heteromer. DAMGO-induced internalization was significantly attenuated by naltrindole and abolished by CTOP, suggesting that both δ- and μ-opioid receptors may be required for DAMGO-induced internalization of the μ-δOR receptor complex. On the other hand, endomorphin-1-induced internalization was unaffected by δOR blockade with naltrindole, but was abolished by blocking the μOR with CTOP, suggesting that this endogenous μ-receptor agonist may not occupy the δ-receptor binding pocket within the μ–δ receptor heteromer, or that it may induce internalization of the heteromer by a different mechanism. Given that internalization of μ-receptors induced by

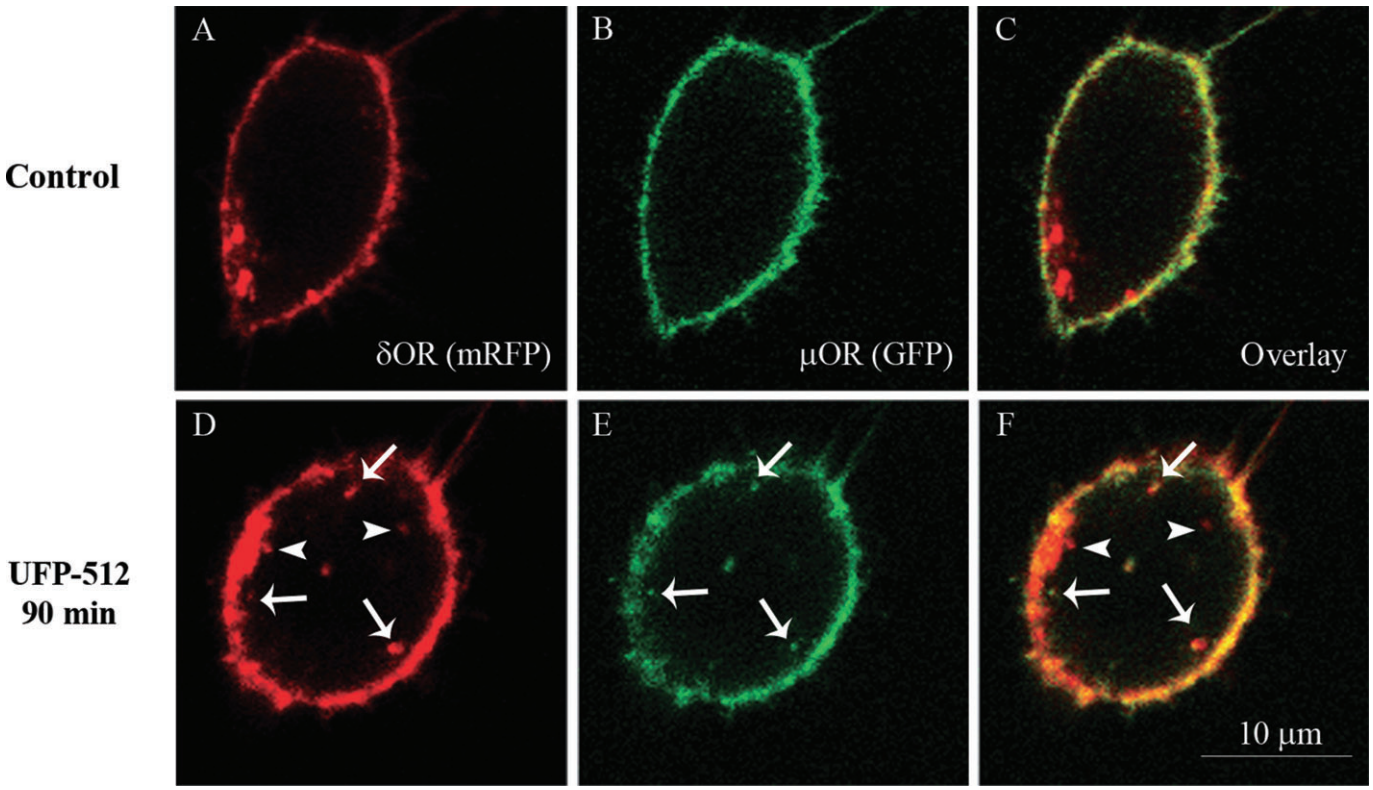

Figure 3

Agonist-induced internalization of mRFP-tagged δ-opioid receptors (δOR) and GFP-tagged μ-receptors (μOR) in a living HEK 293T cell followed in real time. The cell was treated with the δ-receptor agonist UFP-512 (100 nM) and observed under the Zeiss LSM 510 confocal microscope. (A–C and D–F) The cell prior to drug treatment and following UFP-512 treatment for 90 min at room temperature respectively. Agonist treatment resulted in receptor clustering and inward movement of cell surface μ- and δ-receptors. Arrows point to μ- and δ-opioid receptor co-internalization, and arrowheads point to δ-opioid receptor internalization. Red and green represent δ-receptor and μ-receptor fluorescence respectively. This is a representative example of a total sample of 16 cells. Overlay is shown in yellow. Scale bar = 10 μm.

δ-receptor agonists required occupancy of both δ- and μ-receptors, and that these agonists displaced μ-receptor agonist binding with high affinity from the μ–δ receptor heteromer, the receptors internalized were likely to be μ-receptors that were complexed with δ-receptors.

Opioid agonist-induced internalization profile of the μ-receptor homomer

To examine δ-agonist effects at the μ-receptor homomer, internalization was investigated in intact cells singly expressing this receptor by tracking the disappearance of cell surface [^{3}H]-DAMGO binding (Figure 4B). The δ-receptor agonists UFP-512, SNC80, deltorphin II and DPDPE did not induce μ-receptor internalization (Figure 4B), whereas Met-enkephalin, endomorphin-1 and DAMGO induced about 50% internalization of cell surface μ-receptors.

Effect of PTX treatment or Gα$_z$ transfection on agonist-induced internalization of the μ–δ receptor heteromer

To determine whether internalization of the μ–δ receptor heteromer was PTX sensitive, and to investigate the effect of Gα$_{i/o}$ inactivation on internalization, cells were pretreated with PTX for 24 h prior to the internalization assay (Figure 5A). PTX treatment significantly attenuated Met-enkephalin- and DAMGO-induced internalization, but had no effect on that induced by endomorphin-1 (Figure 5A). SNC80-, deltorphin II- and DPDPE-induced internalization of the μ–δ receptor heteromer was not affected by PTX treatment. To determine the effect of Gα$_z$ on internalization of the μ–δ receptor heteromer, agonist-induced endocytosis was assessed in the presence and absence of transfected Gα$_z$ (Figure 5B). In contrast to its modulatory role on agonist affinities, Gα$_z$ had no additional effect on μ- or δ-receptor agonist-induced internalization of the μ–δ receptor heteromer. Thus, inactivation of Gα$_{i/o}$

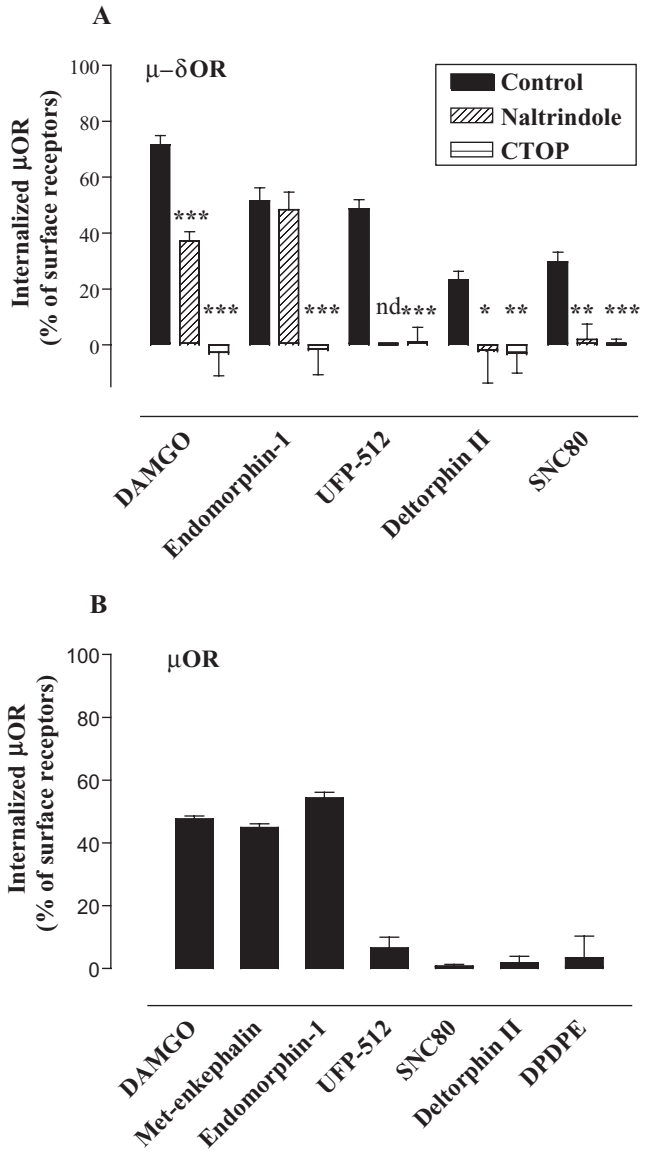

Figure 4

Agonist-induced internalization of cell surface μ-opioid receptors (μOR) detected by [^{3}H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-receptors (δOR), and treated with 100 nM naltrindole or 1 μM CTOP (A), or only expressing μ-receptors (B). Cells co-expressing μ- and δ-receptors were pretreated with 100 nM naltrindole or 1 μM CTOP for 30 min at 37°C, rinsed, then treated with 10 μM agonist for 1 h at 37°C (A). Cells expressing only μ-receptors were treated with 10 μM agonist for 1 h at 37°C (B). Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells, and are expressed as mean ± SEM for $n = 3$–5 experiments performed in triplicate. Nd = not determined. Statistical significance in (A) was determined using an unpaired Student's *t*-test [$^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to cells not pretreated with naltrindole or CTOP].

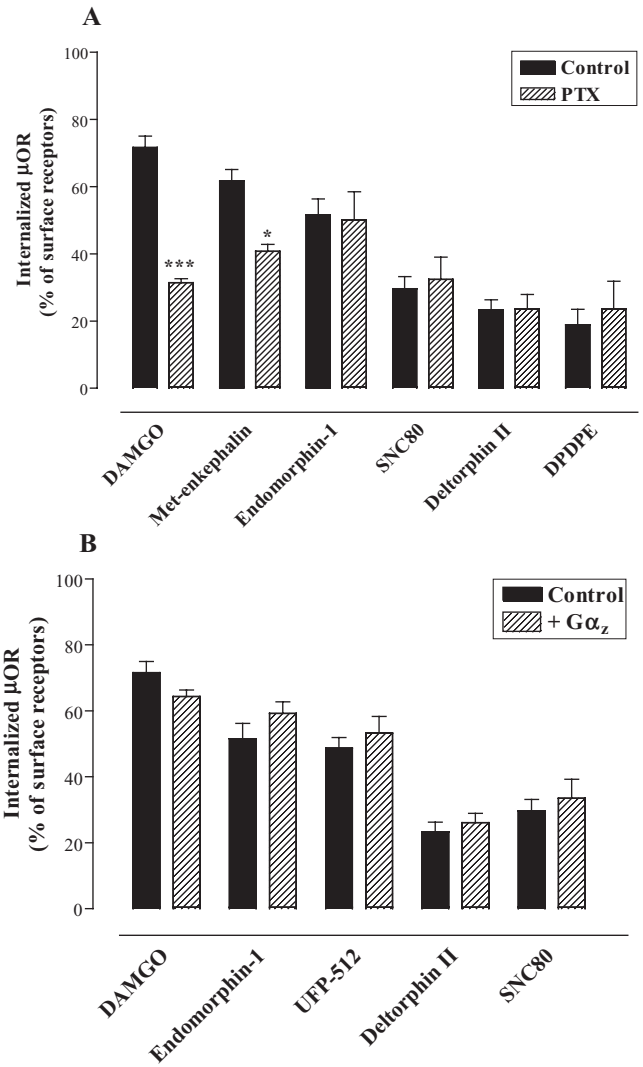

Figure 5

Effect of PTX treatment or $G\alpha_z$ transfection on agonist-induced internalization of cell surface μ-receptors (μOR) detected by [^{3}H]-DAMGO in intact HEK 293 cells co-expressing μ- and δ-receptors (δOR). Cells were pretreated with 100 ng·mL^{-1} PTX for 24 h, and rinsed (A) or transfected with $G\alpha_z$ 48 h (B) prior to 10 μM agonist treatment for 1 h at 37°C. Data shown represent a percentage of the vehicle-treated control cells and are expressed as mean ± SE for $n = 3$ experiments performed in triplicate. Statistical significance was determined using an unpaired Student's *t*-test [$^*P < 0.05$, $^{***}P < 0.001$ relative to cells not pretreated with PTX or not transfected with additional $G\alpha_z$].

or transfection of additional $G\alpha_z$ did not affect δ-agonist-induced internalization of the μ–δ receptor heteromer.

Effect of disruption of the clathrin–dynamin endocytic machinery on δ-agonist-induced internalization of the μ–δ receptor heteromer

To investigate the pathway by which δ-agonists induced internalization of the μ–δ receptor heteromer, cells were either co-treated with the clathrin

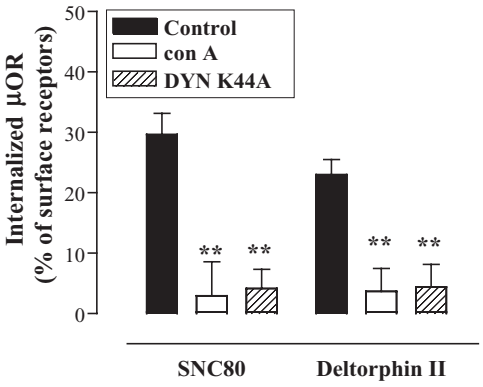

Figure 6

Effect of con A treatment or co-expression of the dynamin-dominant-negative mutant DYN K44A on agonist-induced internalization of cell surface μ-opioid receptors (μOR) detected by [^{3}H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-receptors (δOR). Cells were co-treated with 275 μg·mL^{-1} con A and 10 μM agonist for 1 h at 37°C, or were transfected with the DYN K44A construct 48 h prior to agonist treatment. Data are expressed as a percentage of the receptor density in vehicle-treated control cells and as mean ± SEM for $n = 3$ experiments performed in triplicate. Statistical significance was determined using an unpaired Student's *t*-test [**$P < 0.01$ relative to cells not co-treated with con A or not expressing DYN K44A].

endocytosis disrupter, con A, or transfected with the dynamin-dominant-negative construct DYN K44A. Both con A and DYN K44A abolished SNC80- and deltorphin II-induced internalization (Figure 6), suggesting that the μ–δ receptor heteromer utilized the clathrin and dynamin endocytic machinery for internalization. In separate control experiments, transfection of empty vector had no effect on internalization induced by δ-receptor agonists.

Effect of chronic morphine exposure on agonist-induced internalization of the μ–δ receptor heteromer

To investigate the internalization profile of the μ–δ receptor heteromer in a morphine tolerance model, cells were pretreated with 10 μM morphine sulphate for 48 h prior to the internalization assay (Figure 7A). SNC80-, deltorphin II- and DPDPE-induced internalization was not altered by chronic morphine exposure. DAMGO-induced internalization was significantly attenuated, whereas the effects of endomorphin-1 remained intact following morphine exposure. In contrast, this morphine regimen almost abolished DAMGO- and endomorphin-1-induced internalization in cells expressing only μ-receptors (Figure 7B).

Discussion and conclusions

This report provides evidence for δ-opioid receptor agonists displacing μ-receptor agonist binding with

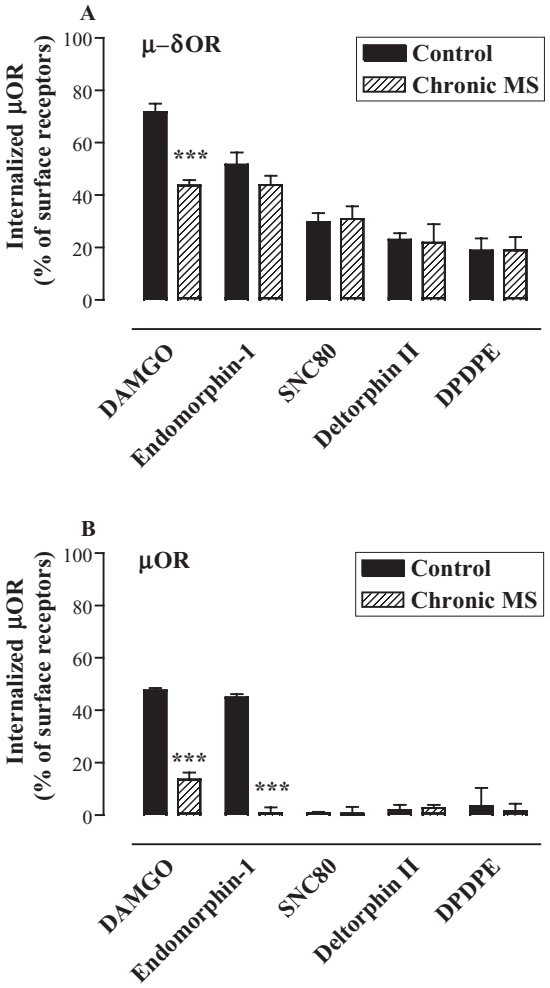

Figure 7

Effect of chronic morphine exposure on agonist-induced internalization of cell surface μ-opioid receptors (μOR) detected by [^{3}H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-receptors (δOR; A) or expressing only μ-receptors (B). Cells were pretreated with 10 μM morphine sulphate (MS) for 48 h, washed, then incubated with medium with or without 10 μM agonist for 1 h at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells, and are expressed as mean ± SEM for $n = 3$–4 experiments performed in triplicate. Statistical significance was determined using an unpaired Student's *t*-test [***$P < 0.001$ relative to cells not pretreated with morphine].

high affinity from the μ–δ receptor heteromer, but not the μ-receptor homomer, which suggested that the μ–δ receptor heteromer may contain a novel δ-agonist-detected μ-receptor ligand binding pocket that differed from that within the μ-receptor homomer. The novel conformation of μ- and δ-opioid receptors induced by their hetero-oligomerization resulted in association with $G\alpha_z$ and the enhanced affinities of some agonists at the μ–δ receptor heteromer. This receptor complex was expressed on the cell surface and internalized in

response to endogenous and synthetic agonists via the clathrin and dynamin endocytic machinery. Internalization of μ-receptors induced by δ-receptor agonists within the μ–δ receptor heteromer required occupancy of both μ- and δ-opioid receptor constituents. In contrast to the μ-receptor homomer, μ–δ receptor heteromer internalization occurred after chronic morphine exposure without evidence of attenuation. The finding that δ-receptor agonists displaced μ-receptor agonist binding from the μ–δ receptor heteromer and induced its internalization suggests that these ligands may have unique pharmacological specificity at the heteromer.

G protein coupling plays an important role in modulating the conformation of opioid receptors (Yan *et al.*, 2008). Because we showed that the μ–δ opioid receptor heteromer preferentially coupled to $G\alpha_z$ (Fan *et al.*, 2005; Hasbi *et al.*, 2007), agonist affinities at the $G\alpha_z$-coupled receptor complex were investigated. We transfected $G\alpha_z$ because the endogenous expression levels of this Gα subunit are low compared to other $G\alpha_{i/o}$ proteins, which are highly expressed in our cellular background. Enhanced affinities for select agonists were demonstrated at the μ–δ receptor-$G\alpha_z$ complex, an effect that was agonist specific and did not depend on ligand chemical structure. The shift in high- and low-affinity states we observed cannot be explained by the ternary complex model of ligand binding to GPCRs, which postulates that these receptors exist in G protein-coupled (high affinity) or -uncoupled (low affinity) states (DeLean and Lefkowitz, 1980). The latter is a simplistic interpretation of the results and does not account entirely for the multiple affinity states detected by radioligand binding studies which can result from multiple factors such as G protein coupling, ligand co-operativity and different oligomeric states (Chidiac *et al.*, 1997; Green *et al.*, 1997; Kara *et al.*, 2010). We had previously demonstrated that $G\alpha_z$ had no effect on binding affinities of selective μ- or δ-receptor agonists, at their individual receptors, which is consistent with the finding that these homomers preferentially couple to, and interact with, the PTX-sensitive $G\alpha_i$ proteins (Fan *et al.*, 2005). Whereas PTX treatment is known to abolish high-affinity agonist binding to μ- and δ-receptor homomers, it resulted in the emergence of a high-affinity SNC80 binding site in the μ–δ receptor heteromer. In the absence of PTX, multiple binding sites could not be resolved on a multiphasic competition curve likely accounting for the monophasic SNC80-[^{3}H]-diprenorphine competition curve. PTX, by inactivating $G\alpha_i$ proteins and removing the contribution of any $G\alpha_i$-coupled receptors to the binding detected by [^{3}H]-diprenorphine, unmasked the true affinity of SNC80 at the μ–δ receptor heteromer. Our finding that the associated G protein enhanced the affinity of select ligands for opioid receptors has also been documented for the κ-receptor and opioid receptor–G protein fusion constructs (Snook *et al.*, 2008; Yan *et al.*, 2008). In the case of κ-receptors, use of the substituted cysteine accessibility method showed that two- to threefold changes in agonist affinity at the differentially coupled receptor were due to conformational changes induced by G protein binding to the receptor (Yan *et al.*, 2008). As changes in agonist affinity reflect receptor conformational changes, the $G\alpha_z$-coupled μ–δ receptor heteromer probably had an altered conformation.

Agonists of δ-opioid receptors displaced the selective μ-receptor agonist [^{3}H]-DAMGO from its binding site with high affinity in cells co-expressing μ- and δ-receptors, but not in those expressing μ-receptors individually, which suggested that the μ–δ receptor heteromer may contain a novel μ-receptor ligand binding pocket that differed from the one within the μ-receptor homomer. Co-expression of δ-receptor with the μ-receptor is likely to alter the latter receptor's conformation, an effect that would only occur if the two receptors were physically interacting, as in a receptor hetero-oligomer. The binding site labelled by [^{3}H]-DAMGO was comprised of μ-receptors because its binding was abolished following pretreatment with an irreversible μ-receptor antagonist at a concentration that selectively inhibited μ-receptors. Secondly, this selective μ-agonist radioligand did not detect δ-receptors at the concentration used, as confirmed by the lack of binding in cells that expressed δ-receptors alone. Thus, δ-receptor agonists may not simply be binding δ-receptors alone, but also occupying the μ-receptor binding pocket within the μ–δ receptor heteromer with high affinity. Therefore, the behavioural effects of δ-receptor agonists *in vivo* may not result solely from the activation of δ-receptors, but also from their ability to occupy the μ-receptor binding pocket with the μ–δ receptor heteromer. Overall, these findings suggest that δ-receptor agonists have previously unrecognized high affinity for the μ–δ receptor heteromer and provide a possible explanation for the attenuation of δ-receptor agonist-mediated effects in μ-receptor gene-deleted animals.

Internalization plays a key role in regulating receptor number at the cell surface and is an important mechanism by which receptor function is tightly regulated (Ferguson *et al.*, 1998). Reduction of cell surface binding of the μ-agonist [^{3}H]-DAMGO was used as a quantitative measure of μ-receptor endocytosis. [^{3}H]-DAMGO would only label cell surface receptors because it is a hydrophilic peptide

ligand and so does not penetrate the plasma membrane (Blake *et al.*, 1997; Koch *et al.*, 1998; Szeto *et al.*, 2001). In addition, incubation with [^{3}H]-DAMGO was conducted at 4°C to prevent any possible internalization of [^{3}H]-DAMGO bound to the μ-receptor and to prevent recycling of receptors to the cell surface (Blake *et al.*, 1997; Segredo *et al.*, 1997; Koch *et al.*, 1998; Toews, 2000). This protocol has been validated as a sensitive and reliable approach by several other groups (Segredo *et al.*, 1997; Koch *et al.*, 1998). Cell surface immunofluorescence experiments tracking the μ-receptor confirmed that decreased [^{3}H]-DAMGO binding represented μ-receptor endocytosis and was not merely a result of loss of affinity for the radioligand or persistent receptor occupancy by δ-receptor agonists. Internalization of μ-receptors induced by δ-receptor agonists was indicative of μ–δ receptor heteromer internalization because this effect occurred only in cells co-expressing μ- and δ-receptors, and not in those expressing μ-receptors alone. Occupancy of both μ- and δ-opioid receptor binding pockets was necessary for δ-agonist-induced internalization of the μ–δ receptor heteromer, as endocytosis was abolished by the highly selective μ- and δ-receptor antagonists CTOP (Gulya *et al.*, 1988) and naltrindole (Portoghese *et al.*, 1988) respectively. DAMGO-induced internalization was attenuated by naltrindole and abolished by CTOP, suggesting that δ-receptors may also be required for DAMGO-induced internalization of this receptor complex. In contrast, endomorphin-1-induced internalization was insensitive to δ-receptor blockade, suggesting that this agonist may induce internalization of the heteromer via a different mechanism or that it may occupy a different ligand binding pocket within the receptor complex.

Consistent with the findings from ligand binding studies, confocal imaging experiments showed co-internalization of both μ- and δ-opioid receptors following δ-agonist treatment of living cells. Further, δ-agonist-induced internalization of the μ–δ receptor heteromer occurred via a clathrin- and dynamin-dependent mechanism. The finding that the μ–δ receptor heteromer readily internalized in response to a wide array of agonists may be explained by evidence that the μ–δ receptor heteromer is constitutively associated with β-arrestin 2, a key component of the endocytic pathway (Rozenfeld and Devi, 2007). Thus, δ-agonists not only displaced μ-agonist binding with high affinity; they also induced internalization of the μ–δ receptor complex.

Internalization of individually expressed μ-opioid receptors by their selective agonists is PTX sensitive, whereas internalization of δ-receptors is PTX resistant (Remmers *et al.*, 1998; Zaki *et al.*, 2000; Bradbury *et al.*, 2009). Internalization of the μ–δ receptor heteromer, as assessed by δ-agonist-induced internalization of μ-receptors, was PTX resistant and was not enhanced by transfection of additional $G\alpha_z$, suggesting that endogenous levels of this G protein are sufficient or that this process may not involve G proteins. PTX attenuated DAMGO-induced internalization, consistent with the notion that a proportion of μ-opioid receptors internalized in cells co-expressing μ- and δ-opioid receptors are homomeric. The lack of PTX effect on endomorphin-1-induced internalization suggests that a large proportion of the μ-opioid receptors internalized by endomorphin-1, in cells co-expressing μ- and δ-opioid receptors, may be within a heteromeric complex.

The adaptations in opioid receptor trafficking that occur following prolonged morphine treatment are of clinical significance (Connor *et al.*, 2004; Christie, 2008). However, the regulation of the μ–δ receptor heteromer under these conditions is not known. In this study, the chronic morphine exposure paradigm used produces a cellular model of morphine tolerance (Sharma *et al.*, 1975; Avidor-Reiss *et al.*, 1995; Blake *et al.*, 1997; Finn and Whistler, 2001). Whereas chronic morphine exposure has been shown to impair internalization of the individual μ- and δ-opioid receptors by their selective agonists (Eisinger *et al.*, 2002), we found that δ-agonist-induced internalization of the μ–δ receptor heteromer still occurred without impairment. Chronic morphine exposure resulted in a reduction in, but not a loss of, DAMGO-induced internalization. Consistent with the possibility that receptors internalized by endomorphin-1 in cells co-expressing μ- and δ-opioid receptors are mostly heteromeric, endocytosis induced by this agonist was not impaired by chronic morphine exposure. Because internalization is a measure of function, our findings suggest that μ–δ receptor heteromer function was not impaired in a paradigm of morphine tolerance, and imply that targeting the μ–δ receptor heteromer may be a better therapeutic strategy in treating conditions that require prolonged exposure to morphine.

In conclusion, this study provides novel insights into the nature of the ligand binding pocket of the μ–δ opioid receptor heteromer and its regulation following exposure to a range of opioid agonists. δ-Receptor agonists displaced μ-receptor agonist binding from the μ–δ receptor heteromer with high affinity, and regulated the intracellular trafficking of the μ–δ receptor heteromer through the clathrin and dynamin endocytic machinery. Internalization of μ–δ receptor heteromers by δ-agonists required

occupancy of both μ- and δ-opioid receptor binding pockets, and remained intact in a model of morphine tolerance. Our finding that δ-receptor agonists target the μ–δ receptor heteromer in addition to the δ-opioid receptor suggests that these agonists may have unique pharmacological specificity for the heteromer. This receptor complex represents a novel opioid signalling complex, and understanding its pharmacology and regulation will provide further insight into the functions of the opioid system and the possible identification of selective compounds targeting the μ–δ receptor heteromer exclusively.

Acknowledgements

This work was funded by a grant from the Canadian Institutes of Health Research and an Ontario Mental Health Foundation Research Studentship (N.K.). S.R.G. holds a Tier I Canada Research Chair in Molecular Neuroscience.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Expression of $G\alpha_z$ and $G\alpha_i$ in HEK 293T cells. Membrane preparations (20 μg protein/lane) from cells were denatured and analysed by electrophoresis on 10% Tris–glycine gels. Proteins were electroblotted onto a polyvinylidene difluoride membrane, and immunoblotted using $G\alpha_z$ or $G\alpha_{i3}$ antiserum diluted 1:250. Cells co-expressing μ- and δ-opioid receptors were (lane 2) or were not (lanes 1 and 3) transfected with additional $G\alpha_z$. Immunoblotting with $G\alpha_z$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as

reported previously (Premont *et al.*, 1989). Similarly, immunoblotting with $G\alpha_{i3}$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as reported previously (Holz *et al.*, 1989). Blots were stripped and reprobed with GAPDH antibody diluted 1:10 000 to serve as the loading control.

Figure S2 Determination of β-funaltrexamine concentration required to block binding to μ-opioid receptors with the least effect on δ-opioid receptors. Competition of [^{3}H]-DAMGO (A) or [^{3}H]-diprenorphine (B) binding to membranes from HEK 293T cells expressing μ-opioid receptors (shaded circles) or δ-opioid receptors (open circles) by β-funaltrexamine.

Figure S3 Dose dependence of agonist-induced internalization. Cell surface μ-opioid receptors were detected by [^{3}H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-opioid receptors. Cells were treated with 1 or 10 μM agonist for 1 h at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± SEM for $n = 3$–5 experiments performed in triplicate.

Figure S4 UFP-512-induced internalization of cell surface μ-opioid receptors or δ-opioid receptors detected by [^{3}H]-DAMGO or [^{3}H]-diprenorphine, respectively, in intact HEK 293T cells expressing μ- or δ-opioid receptor individually. Cells were treated with 10, 1 or 0.1 μM agonist for 1 h at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± SEM for $n = 3$ experiments performed in triplicate.

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