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## RESEARCH PAPER

# Prolonged morphine treatment alters $\delta$ opioid receptor post-internalization trafficking

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## BACKGROUND AND PURPOSE

The  $\delta$  opioid receptor (DOP receptor) undergoes internalization both constitutively and in response to agonists. Previous work has shown that DOP receptors traffic from intracellular compartments to neuronal cell membranes following prolonged morphine treatment. Here, we examined the effects of prolonged morphine treatment on the post-internalization trafficking of DOP receptors.

## EXPERIMENTAL APPROACH

Using primary cultures of dorsal root ganglia neurons, we measured the co-localization of endogenous DOP receptors with post-endocytic compartments following both prolonged and acute agonist treatments.

## KEY RESULTS

A departure from the constitutive trafficking pathway was observed following acute DOP receptor agonist-induced internalization by deltorphin II. That is, the DOP receptor underwent distinct agonist-induced post-endocytic sorting. Following prolonged morphine treatment, constitutive DOP receptor trafficking was augmented. SNC80 following prolonged morphine treatment also caused non-constitutive DOP receptor agonist-induced post-endocytic sorting. The  $\mu$  opioid receptor (MOP receptor) agonist DAMGO induced DOP receptor internalization and trafficking following prolonged morphine treatment. Finally, all of the alterations to DOP receptor trafficking induced by both DOP and MOP receptor agonists were inhibited or absent when those agonists were co-administered with a DOP receptor antagonist, SDM-25N.

## CONCLUSIONS AND IMPLICATIONS

The results support the hypothesis that prolonged morphine treatment induces the formation of MOP–DOP receptor interactions and subsequent augmentation of the available cell surface DOP receptors, at least some of which are in the form of a MOP/DOP receptor species. The pharmacology and trafficking of this species appear to be unique compared to those of its individual constituents.

## LINKED ARTICLES

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

## Abbreviations

DAMGO, [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin; DELT, [D-Ala<sup>2</sup>]-deltorphin II; DOP receptor,  $\delta$  opioid receptor; DRG, dorsal root ganglia; KOP receptor,  $\kappa$  opioid receptor; LAMP1, lysosomal-associated membrane protein 1; MOP receptor,  $\mu$  opioid receptor; SDM-25N, (4bS,8R,8aS,14bR)-5,6,7,8,14,14b-hexahydro-7-(2-methyl-2-propenyl)-4,8-methanobenzofuro[2,3-a]pyrido[4,3-b]carbazole-1,8a(9H)-diol hydrochloride; SNC80, (+)-4-[( $\alpha$ R)- $\alpha$ -((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide; TBS, tris-buffered saline

## Introduction

Opioids mediate their pharmacological effects via activation of three types of GPCRs:  $\mu$  opioid receptor (MOP receptor),  $\delta$  opioid receptor (DOP receptor) and  $\kappa$  opioid receptor (KOP receptor) (Alexander *et al.*, 2013). The availability of opioid receptors at the cell surface is under complex control and is regulated by a number of processes that allow the cell to respond to external stimuli. Opioid receptors traffic regularly to and from the cell surface (Décaillot *et al.*, 2008; for review, see Bernard *et al.*, 2006; Hanyaloglu and von Zastrow, 2008; von Zastrow, 2010; Williams *et al.*, 2013) and such trafficking is fundamental to their function. Desensitization, internalization and recycling of opioid receptors to the plasma membrane have significant functional consequences, affecting both analgesia and tolerance (Groer *et al.*, 2007; Kim *et al.*, 2008; He *et al.*, 2009; Pradhan *et al.*, 2010; for review, see Law and Loh, 1999; Zuo, 2005; Cahill *et al.*, 2007; Martini and Whistler, 2007).

Receptor internalization upon agonist exposure is a well-documented phenomenon for many GPCRs including opioid receptors. Following endocytosis, receptors may undergo recycling back to the plasma membrane, which is thought to underlie re-sensitization, or may undergo targeting to the degradation pathways, a key process in receptor down-regulation. Many GPCRs have been categorized as either 'class A' (recycled) or 'class B' (degraded) depending upon the relative stability of the GPCR–arrestin interaction (Drake *et al.*, 2006). Such differential trafficking involves a number of different intrinsic and extrinsic regulatory mechanisms, including ubiquitination, specific sequence direction and Src (for review, see Hanyaloglu and von Zastrow, 2008; Hislop and von Zastrow, 2011; Nagi and Piñeyro, 2011). Both MOP and DOP receptors are internalized constitutively and in response to activation by agonists. They are, however, trafficked differently after internalization: the MOP receptor is recycled to the cell surface (class A), whereas the DOP receptor is trafficked to lysosomes (class B) and degraded (Law and Loh, 1999; Tanowitz and von Zastrow, 2002; Whistler *et al.*, 2002) (Figure 6A).

A growing body of literature exists revealing that expression of analgesic tolerance following prolonged morphine exposure requires cell surface expression of DOP receptors (Zhu *et al.*, 1999; George *et al.*, 2000; Gomes *et al.*, 2004; Abul-Husn *et al.*, 2007; Rozenfeld *et al.*, 2007; He *et al.*, 2011). We, and others, have demonstrated that prolonged morphine treatment causes the trafficking of DOP receptors from intracellular compartments to neuronal cell membranes (Cahill *et al.*, 2001; Morinville *et al.*, 2003; 2004a; Lucido *et al.*, 2005; Gendron *et al.*, 2006). This effect does not occur with acute treatment and appears to be distinct from the trafficking of DOP receptors elicited by acute nociceptive input (Morinville

*et al.*, 2003; Gendron *et al.*, 2006). Prolonged morphine-induced DOP receptor trafficking is also dependent on MOP receptors (Morinville *et al.*, 2003). What remains unclear and has not been investigated is whether the fate of opioid receptors following prolonged stimulation with morphine differs from the internalization pathways that ensue following short-term agonist exposure.

The dependence of the morphine-induced DOP receptor trafficking on MOP receptors may simply reflect a role for MOP receptor signalling. However, notably similar MOP receptor-dependent DOP receptor trafficking occurs following chronic, but not acute, pain (Morinville *et al.*, 2004b; Gendron *et al.*, 2006; Cahill *et al.*, 2007; Holdridge and Cahill, 2007; Holdridge *et al.*, 2007). In this case, obligate MOP receptor signalling seems less likely. An alternative hypothesis is that tolerance is due to the functional interaction of MOP with DOP receptors in a hetero-oligomeric complex. A requirement for MOP receptors in order to form MOP/DOP receptor heteromers would explain the MOP dependence of both morphine- and pain-induced DOP receptor trafficking on MOP receptors. That is, the DOP receptors trafficked to the cell surface in both cases are in the form of MOP/DOP receptor heteromers. The existence of opioid receptor heteromers has been demonstrated by immunoblotting and co-immunoprecipitation (Gomes *et al.*, 2000), immunocytochemistry (Décaillot *et al.*, 2008; Gupta *et al.*, 2010), and bioluminescence and Foerster resonance energy transfer (Hasbi *et al.*, 2007). Opioid receptor heteromers are functional receptors (Law *et al.*, 2005). There is ample evidence that MOP/DOP receptor heterodimers exist *in vivo*. MOP and DOP receptors are co-expressed in dorsal root ganglia (DRG) neurons (Fields *et al.*, 1980; Wang *et al.*, 2008; 2010; Chieng and Christie, 2009; Beaudry *et al.*, 2011; Erbs *et al.*, 2014) (but see Scherrer *et al.*, 2009) and on axon terminals (Arvidsson *et al.*, 1995) within the superficial dorsal horn, which supports the possibility of a MOP/DOP receptor species. More convincing are the demonstrations that there is a direct physical interaction between the two receptors in the spinal cord (Gomes *et al.*, 2004; Erbs *et al.*, 2014) and the immunohistochemical labelling of MOP/DOP receptor heteromers in the brain and on DRG neurons (Gupta *et al.*, 2010). We have also demonstrated that prolonged morphine treatment increases the abundance of MOP/DOP receptor heteromers in DRG neurons and several brain regions (Gupta *et al.*, 2010). Opioid heteromers identified in heterologous systems have pharmacological profiles unique from that of any constituent alone (Fan *et al.*, 2005) and similar to that of previously described opioid receptor subtypes (Jordan and Devi, 1999; Portoghese and Lunzer, 2003; Waldhoer *et al.*, 2005). *In vivo*, MOP-DOP receptor heteromerization appears to be required for full functional competence of MOP receptors (Walwyn *et al.*, 2009).

In this study, we investigated the post-internalization trafficking of DOP receptors following prolonged morphine treatment, a condition which we suspect induces MOP–DOP receptor interactions. We used cultured sensory neurons and measured the co-localization of DOPr and several post-endocytotic compartments following acute treatment with MOP or DOP receptor agonists. The use of this neuronal model enabled us to assess endogenous DOP receptor trafficking in a non-transfected, non-immortalized model. We showed that prolonged morphine treatment alters DOP receptor agonist-induced trafficking in response to both DOP and MOP receptor agonists.

## Methods

### *DRG cultures*

The lumbar DRGs of two adult, male Sprague Dawley rats (~200 g; Charles River, Quebec, Canada) were used to produce each culture. All experimental protocols were approved by the Queen's University Animal Care Committee and complied with the policies and directives of the Canadian Council on Animal Care; appropriate measures were taken to reduce the pain or discomfort of experimental animals. Each rat was deeply anaesthetized using halothane and killed by decapitation. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The body was sprayed with 70% ethanol and wiped with clean, 70% ethanol-soaked gauze. An incision was made through the superficial layers dorsal to the spinal column. The column was transected in the thoracic and sacral regions and the spinal cord was removed by spinal ejection with sterile, ice-cold HBSS. The length of spinal column was removed and placed in sterile, ice-cold HBSS. In a sterile laminar flow hood, the remaining muscle on the column was debulked and the column bisected lengthwise along the coronal plane. Twelve lumbar DRGs were isolated from each cord and placed in cold Neurobasal-A medium containing 100 000 U L<sup>-1</sup> penicillin/100 mg L<sup>-1</sup> streptomycin. The DRGs were cut using scissors to open their connective tissue envelopes and incubated in a shaking water bath at 37°C with 0.25% collagenase-D (Roche, Laval, Quebec, Canada) for 2 h. After collagenase digestion, the DRGs were spun down at 900× *g* for 2 min and transferred to medium containing 0.25% trypsin for 30 min. After enzymatic dissociation, the DRGs were titrated using fire-polished pipettes. The dissociated cells were again spun down and transferred to the final culture medium, Neurobasal-A augmented with 10% FBS, 0.5 mM L-glutamine, 0.1 µg·mL<sup>-1</sup> nerve growth factor 7 s and containing 100 000 U L<sup>-1</sup> penicillin/100 mg L<sup>-1</sup> streptomycin. The medium and cells were passed through a 70 µm filter and pre-plated on an untreated 12 cm plastic Petri dish and placed in a 37°C, 5% CO<sub>2</sub> incubator for 2 h to reduce glial cell population in the culture. After pre-plating, the cells still in solution were collected and plated on 12-round glass coverslips in a 24-well plate. The coverslips were pre-coated with poly-D-lysine and laminin to facilitate cell adherence. Cultures were incubated at 37°C with 5% CO<sub>2</sub> for a total of 4 days before experimentation.

At the time of experimentation, the cultured cells were firmly adhered to the glass coverslips. The overall cell density was approximately 50–70% confluence. The majority of cells were glia, but neurons were plentiful (>100 per 12 mm round coverslip) and readily identifiable by morphology; neurons extended higher from the glass coverslip, with notably rounder cell bodies than glial cells (Figure 1A). These morphologically identifiable neurons were the same as those identified by microtubule-associated protein 2 immunofluorescent labelling (Figure 1B), which also revealed the growth of numerous fine, distinct processes. The cultured neurons varied in size, with cell bodies between 10 and 40 µm in diameter (Figure 1C). Both the range and the frequency of the observed neuronal cell body sizes were in line with previous findings for similar DRG neuronal cultures (von Banchet *et al.*, 2007).

### *DOP receptor knockout DRG cultures*

Cultures were also prepared using DOP receptor knockout and corresponding wild-type littermate mice (Filliol *et al.*, 2000) for use in antibody validation. This was conducted as described earlier with the following exceptions: DRGs were collected from one mouse to produce each culture; for each culture, cells were plated on four wells of a 24-well plate.

### *Prolonged morphine treatment*

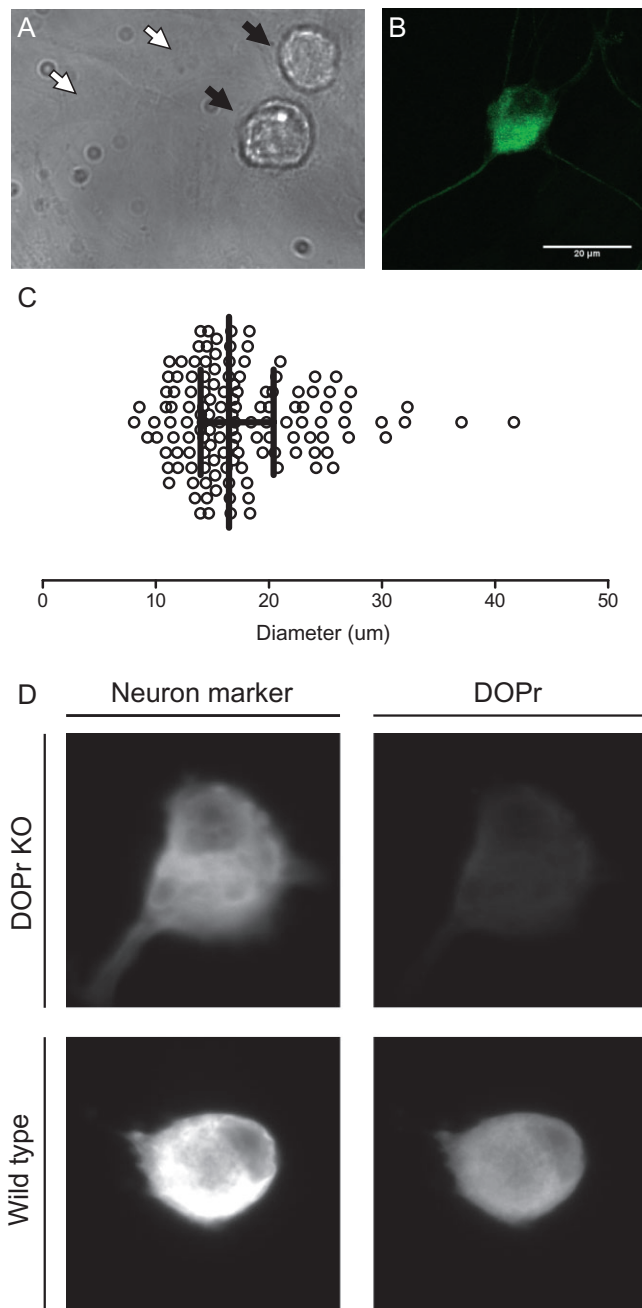
For morphine-treated cells, morphine sulfate (Sandoz Canada, Quebec, Canada) was added to the culture medium for the final 48 h of incubation at a final concentration of 10 µM. Vehicle was added to the culture medium of control cells.

### *Acute agonist/antagonist treatment*

Cells were treated with one of three opioid agonists: DAMGO, DELT or SNC80. The original growth medium was removed and replaced with medium containing DAMGO, DELT or SNC80 at 1 µM. The medium was formulated as follows: Neurobasal-A augmented with 10% FBS, 0.5 mM L-glutamine and 100 000 U L<sup>-1</sup> penicillin/100 mg L<sup>-1</sup> streptomycin. The cells were incubated with the acute agonists for 1 h at 37°C. Control cells were vehicle-treated. For acute antagonist co-treatment experiments, cells treated with DAMGO, DELT, SNC80 and vehicle were co-treated with SDM-25N at 50 nM. SDM-25N is a small-molecule DOP receptor antagonist that exhibits greater selectivity for DOP over MOP receptors (200- to >2000-fold selectivity) than naltrindole (McLamore *et al.*, 2001). SDM-25N has been characterized in binding, GTPγS and smooth muscle assays, and has been used *in vivo*, in brain slices and in cultured cells (Chen *et al.*, 2007; Blomeley and Bracci, 2011; Southern *et al.*, 2013). The dose used was chosen based on those reports to maximize DOP receptor selectivity.

### *Immunofluorescence cytochemistry*

Cultured cells, on coverslips, were washed with cold Tris-buffered saline (TBS) to remove the culture medium and any drugs. Cells were then fixed by immersion in 4% paraformaldehyde for 10 min at 37°C and washed with cold TBS. Fixed



### Figure 1

Cultured DRG neurons were morphologically identifiable. (A) Neurons (black arrows) are easily distinguished from co-cultured glial cells (white arrows). (B) The same cells morphologically identified as neurons showed positive immunolabelling for microtubule-associated protein 2, a common neuronal marker. Scale bar shows 20  $\mu\text{m}$ . (C) Cultured DRG neurons vary in size. A subset of DRG neurons was analysed for size; the cell body diameter at the plane corresponding to the centre of the nucleus was measured. Bars show quartiles. (D) Anti-DOP receptor antibody specificity was validated by labelling DRG neuronal cultures from DOP receptor knockout (KO) and wild-type animals.

cells were blocked by incubating in 300  $\mu\text{L}$  of TBS supplemented with 5% BSA, 5% normal goat serum (NGS) and 0.1% Triton X-100 at room temperature for 2 h. After being blocked, the cells were incubated for 48 h at 4°C in 300  $\mu\text{L}$  TBS supplemented with 1% NGS and 0.1% Triton X-100 and containing the primary antisera at appropriate concentrations. The cells were double-labelled using rabbit anti-DOP receptor diluted 1:1500 (MyBioSource, San Diego, CA, USA; MBS316175, lot 1B03801, directed against DOP receptor residues 360–372) and one of mouse anti-Rab5 diluted 1:750 (Sigma-Aldrich, St. Louis, MO, USA; R7904, lot 129K4799, directed against Rab5 residues 2–14), mouse anti-Rab11 diluted 1:500 (Millipore, Billerica, MA, USA; 05-853, lot 2011576) or goat anti-LAMP1 (lysosomal-associated membrane protein 1) diluted 1:750 (Santa Cruz, Dallas, TX, USA; SC8098, lot D0113, directed against LAMP1 c-terminus). Cells were then washed with TBS and incubated in 300  $\mu\text{L}$  of identically supplemented TBS containing the appropriate secondary antibodies conjugated to Alexa fluorophores diluted 1:200 for 2 h at room temperature. Alexa-488 conjugated donkey anti-rabbit was used to fluorescently label DOP receptors and Alexa-594 conjugated to either goat anti-mouse or donkey anti-goat was used to fluorescently label one of the internalization compartments of interest. Finally, the cells were washed with TBS and mounted on Superfrost Plus slides (Fisher Scientific, Ottawa, Ontario, Canada) using aquamount (Polyscience, Niles, IL, USA). The slides were kept in the dark at 4°C until imaging. Anti-DOP receptor antibody specificity was validated by labelling DRG neuronal cultures from DOP receptor knockout and wild-type animals (Figure 1D).

### Confocal microscopy

Immunoreactive neurons were imaged using a Leica SP2 scanning laser confocal microscope (Leica Microsystems, Wetzlar, Germany). Neurons were identified morphologically and imaged at 100 $\times$  magnification with 488 and 543 nm wavelength excitation. Emissions corresponding to each Alexa-488 and Alexa-568 were detected by independent photomultiplier tubes to yield an image pair, with one image showing the fluorescently labelled DOP receptors and the other image showing the fluorescently labelled internalization compartment of interest. Approximately 20 randomly selected fields of view containing 1–2 neurons were imaged from each unique drug pair–antibody pair condition per experiment. One image pair was captured per field of view at a focal plane transecting the nucleus of the neuron(s). Images were digitally captured as 8 bit uncompressed TIFF files using Leica Confocal Software (Leica Microsystems) on a connected computer and saved for offline analysis.

### Co-localization analysis

Confocal image pairs were analysed using ImageJ (v1.45s; NCBI). Eight-bit image pairs were merged as red and green channels to a single RGB image. Neurons of interest were selected free-hand. Co-localization analysis was performed using the PSC Colocalization plugin (v0.9.01; Andrew French, University of Nottingham, Nottingham, UK) to measure co-localization as previously described (French *et al.*, 2008). A threshold value of 40 was used and the Pearson's



co-localization coefficient was recorded for each neuron analysed. Approximately 15–25 neurons were analysed for each unique drug pair–antibody pair condition per experiment. Across all conditions and all experiments, a total of 3018 neurons were analysed. For each experiment, the Pearson's coefficients recorded for each antibody pair were normalized such that the mean of the values for the prolonged vehicle-treated, acute vehicle-treated condition was zero. Subsequent analyses used these normalized co-localization scores. The results from three (DOP receptor-Rab11 agonist only and all antagonist experiments) or four (DOP receptor-Rab5 and DOP receptor-LAMP1 agonist only) experiments were pooled. Pooled Pearson's coefficients were analysed by two-way ANOVA followed by Tukey's HSD (honest significant difference) test using R (v2.15.0 x86\_64; R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean  $\pm$  95% confidence interval using Prism 5 for Windows (v5.0.1; GraphPad Software, San Diego, CA, USA).

## Reagents

Neurobasal-A, HBSS, FBS, nerve growth factor 7 s, laminin and Alexa-conjugated secondary antibodies were purchased from Life Technologies (Burlington, Ontario, Canada). Penicillin/streptomycin, trypsin, L-glutamine and poly-D-lysine were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin (DAMGO), [D-Ala<sup>2</sup>]-deltorphin II (DELTA), (+)-4-[( $\alpha$ R)- $\alpha$ -((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide (SNC80) and (4bS,8R,8aS,14bR)-5,6,7,8,14,14b-hexahydro-7-(2-methyl-2-propenyl)-4,8-methanobenzofuro[2,3-a]pyrido[4,3-b]carbazole-1,8a(9H)-diol hydrochloride (SDM-25N) were purchased from Tocris (Bristol, UK). Milli-Mark pan neuronal marker (MAB2300, Lot 2383597) was purchased from Millipore.

## Results

### Assessment of DOP receptor co-localization with post-endocytic compartments

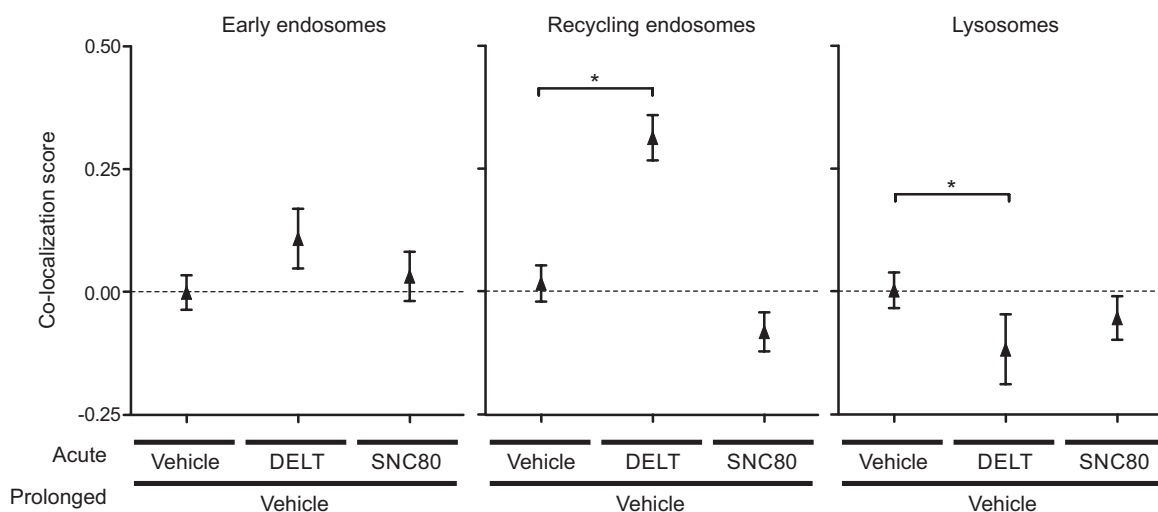
We measured DOP receptor co-localization with markers for three post-endocytic compartments: Rab 5, marks early endosomes; Rab 11, marks recycling endosomes; and LAMP 1, marks lysosomes. DOP receptor co-localization with these compartments was assessed following prolonged and acute opioid receptor agonist treatment, with and without acute DOP receptor antagonist.

### Deltorphin II, but not SNC80, induces DOP receptor recycling

We first examined DOP receptor internalization trafficking following acute treatment with a DOP receptor agonist in prolonged vehicle-treated neurons (Figure 2). In vehicle-treated neurons, acute DELT increased DOP receptor co-localization with recycling endosomes ( $P < 0.0001$ ) and decreased DOP receptor co-localization with lysosomes ( $P = 0.0033$ ). There was no effect on DOP receptor co-localization with early endosomes ( $P = 0.0713$ ). Acute SNC80 had no effect on DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes  $P = 0.9885$ ; DOP receptor-recycling endosomes  $P = 0.2638$ ; DOP receptor-lysosomes  $P = 0.6132$ ).

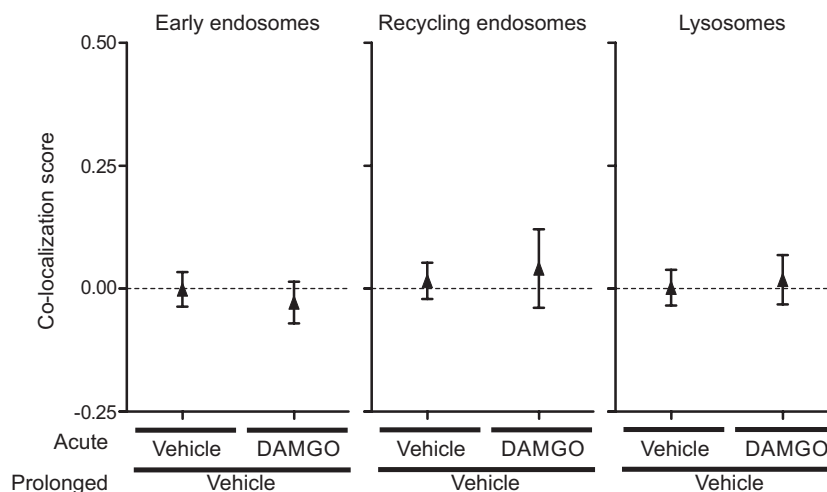
### DAMGO does not affect DOP receptor trafficking

We also examined DOP receptor internalization trafficking following acute MOP receptor agonist treatment in prolonged vehicle-treated neurons (Figure 3). In vehicle-treated neurons, the MOP receptor agonist DAMGO had no effect on



**Figure 2**

Deltorphin II, but not SNC80, induces DOP receptor recycling. Cultured DRG neurons underwent prolonged treatment with vehicle and acute treatment with vehicle, DELT or SNC80. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. In vehicle-treated neurons, acute DELT, but not SNC80, increased DOP receptor co-localization with recycling endosomes and decreased DOP receptor co-localization with lysosomes. Data are shown as mean  $\pm$  95% confidence interval. \* $P < 0.05$ .



**Figure 3**

DAMGO does not affect DOP receptor trafficking. Cultured DRG neurons underwent prolonged treatment with vehicle and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. In vehicle-treated neurons, DAMGO had no effect on DOPr co-localization with any of these compartments. Data are shown as mean  $\pm$  95% confidence interval.

DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes  $P = 0.9174$ ; DOP receptor-recycling endosomes  $P = 0.9989$ ; DOP receptor-lysosomes  $P = 0.9457$ ).

### *Constitutive DOP receptor trafficking is augmented following morphine treatment*

We assessed the effect of prolonged morphine treatment on DOP receptor internalization trafficking (Figure 4A). Prolonged morphine treatment increased DOP receptor co-localization with early endosomes ( $P = 0.0002$ ) and lysosomes ( $P = 0.0013$ ). There was no effect on DOP receptor co-localization with recycling endosomes ( $P = 0.7834$ ).

### *SDM-25N inhibits DOP receptor trafficking augmentation following morphine treatment*

We treated neurons acutely with the DOP receptor antagonist SDM-25N (Figure 4B) and observed that prolonged morphine treatment increased DOP receptor co-localization with early endosomes ( $P = 0.0415$ ) but decreased DOP receptor co-localization with lysosomes ( $P < 0.0001$ ). There was no effect on DOP receptor co-localization with recycling endosomes ( $P = 0.0579$ ).

### *Deltorphin II and SNC80 induce DOP receptor recycling following morphine treatment*

We next examined DOP receptor internalization trafficking following acute DOP receptor agonist treatment in prolonged morphine-treated neurons (Figure 5B). Following morphine treatment, acute DELT had no effect on DOP receptor co-localization with early endosomes ( $P = 0.9430$ ), increased DOP receptor co-localization with recycling endosomes ( $P < 0.0001$ ) and decreased DOP receptor co-localization with lysosomes ( $P = 0.0118$ ) compared with prolonged morphine, acute vehicle-treated neurons. Similarly, following prolonged

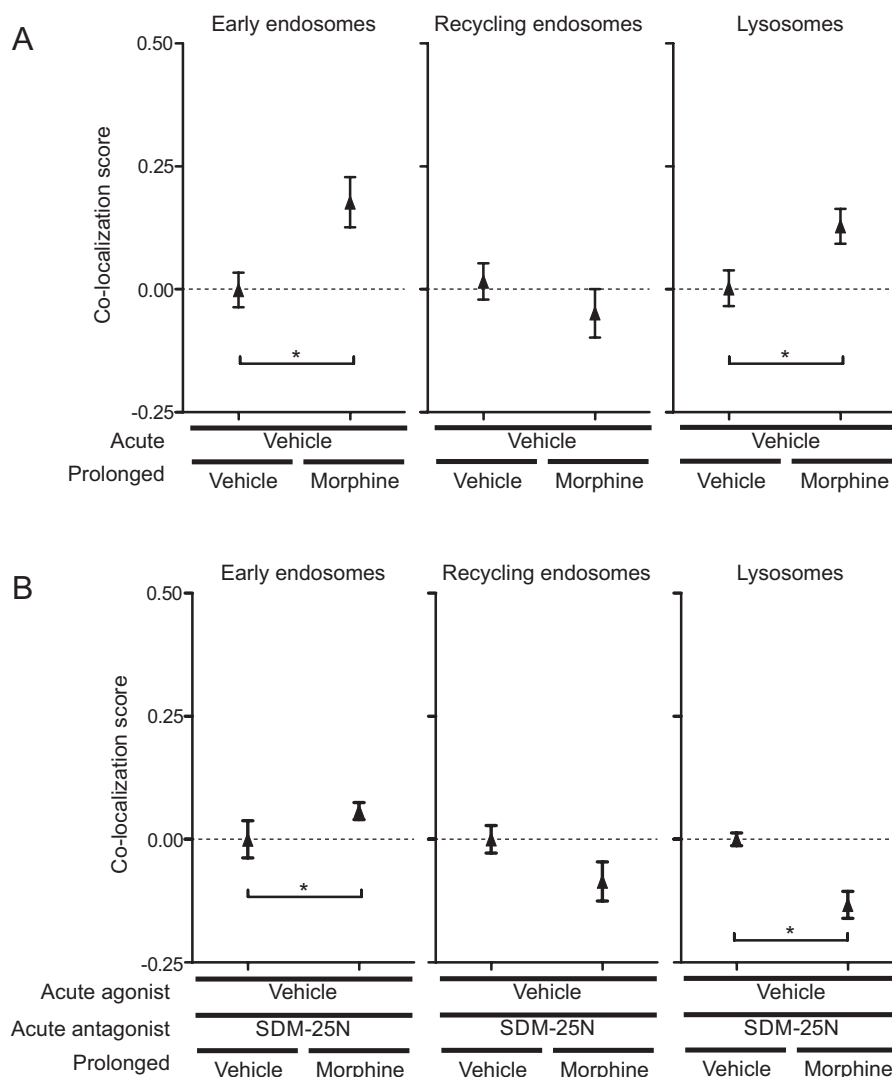
morphine treatment, acute SNC80 had no effect on DOP receptor co-localization with early endosomes ( $P = 0.9099$ ), increased DOP receptor co-localization with recycling endosomes ( $P = 0.0102$ ) and decreased DOP receptor co-localization with lysosomes ( $P = 0.0129$ ) compared with prolonged morphine, acute vehicle-treated neurons.

### *SDM-25N blocks deltorphin II- and SNC80-induced DOP receptor recycling following morphine treatment*

Using prolonged morphine-treated neurons, we co-treated acutely with SMD-25N and each DOP receptor agonist (Figure 5C). When co-administered with the DOP receptor antagonist, acute DELT decreased DOP receptor co-localization with early endosomes ( $P = 0.0048$ ) and had no effect on DOP receptor co-localization with either recycling endosomes ( $P = 0.9310$ ) or lysosomes ( $P = 0.9999$ ) compared with prolonged morphine, acute vehicle, acute SDM-25N-treated neurons. SNC80, when acutely co-administered with the DOP receptor antagonist, had no effect on DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes  $P = 0.6857$ ; DOP receptor-recycling endosomes  $P = 0.9674$ ; DOP receptor-lysosomes  $P = 0.0529$ ) compared with prolonged morphine, acute vehicle acute SDM-25N-treated neurons.

### *DAMGO induces DOP receptor internalization and recycling following morphine treatment*

Finally, we examined DOP receptor internalization trafficking following acute MOP receptor agonist treatment in prolonged morphine-treated neurons (Figure 6B). After morphine treatment, acute DAMGO increased DOP receptor co-localization with early endosomes ( $P < 0.0001$ ) and recycling endosomes ( $P < 0.0001$ ) compared with prolonged morphine, acute vehicle-treated neurons. There was no effect on



**Figure 4**

Constitutive DOP receptor trafficking is augmented following morphine treatment. (A) Cultured DRG neurons underwent prolonged treatment with vehicle or morphine and acute treatment with vehicle. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. Prolonged morphine treatment increased DOP receptor co-localization with early endosomes and lysosomes. (B) Neurons underwent prolonged treatment with vehicle or morphine and acute treatment with SDM-25N or vehicle. Prolonged morphine and acute DOP receptor antagonist increased DOP receptor co-localization with early endosomes and decreased DOP receptor co-localization with lysosomes. Data are shown as mean  $\pm$  95% confidence interval. \* $P < 0.05$ .

DOP receptor co-localization with lysosomes ( $P = 0.9738$ ). We also conducted preliminary time-course experiments, which revealed DAMGO-induced DOP receptor trafficking was early and sustained (Table 1).

### *SDM-25N blocks DAMGO-induced DOP receptor trafficking following morphine treatment*

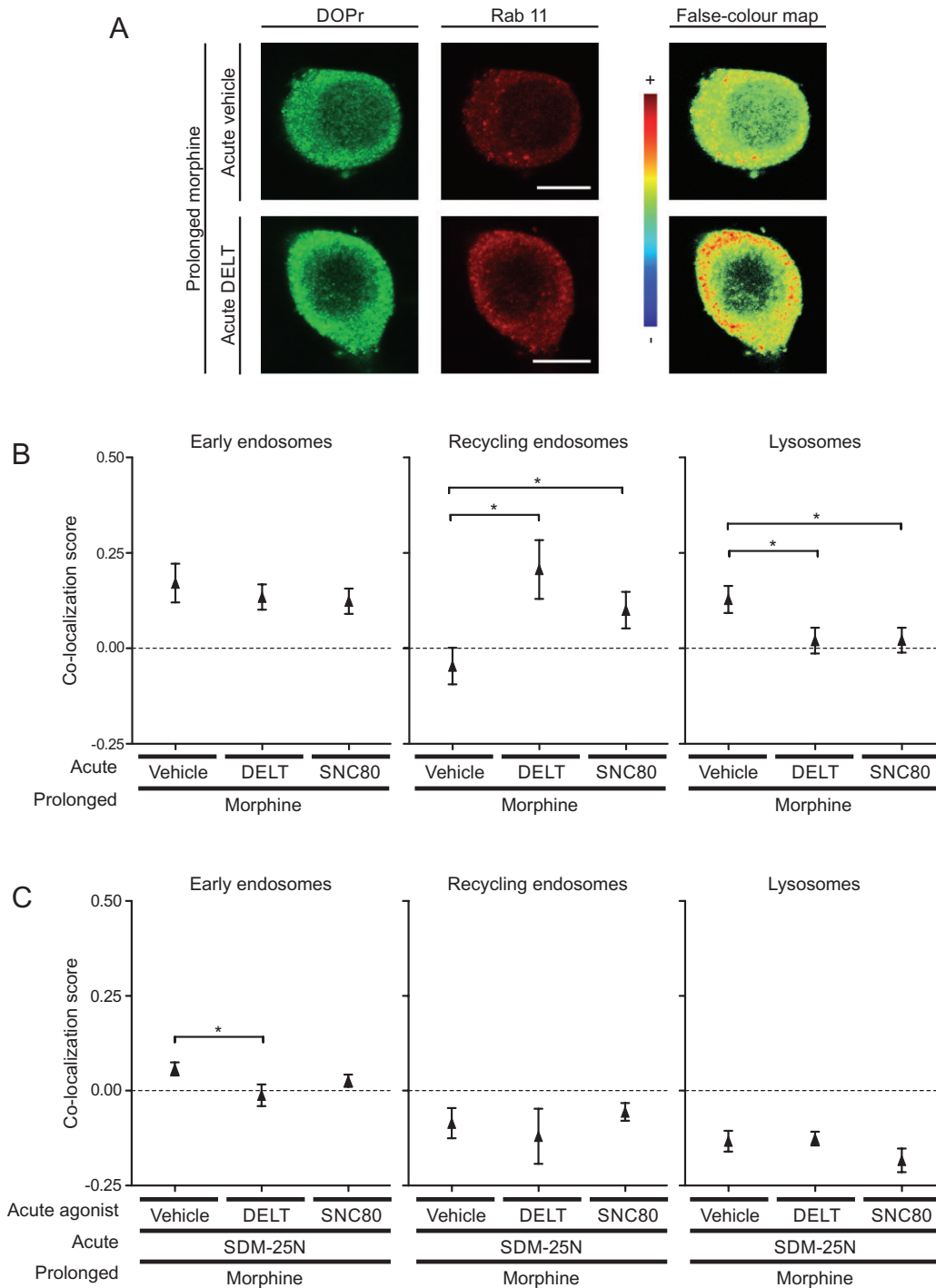
We acutely co-treated prolonged morphine neurons with SDM-25N and DAMGO (Figure 6C). When co-administered with the DOP receptor antagonist, DAMGO decreased DOP receptor co-localization with early endosomes ( $P = 0.0196$ ) and had no effect on DOP receptor co-localization with recy-

cling endosomes ( $P = 1.0000$ ) or lysosomes ( $P = 0.6691$ ) compared with prolonged morphine, acute vehicle, acute SDM-25N-treated neurons.

## Discussion and conclusions

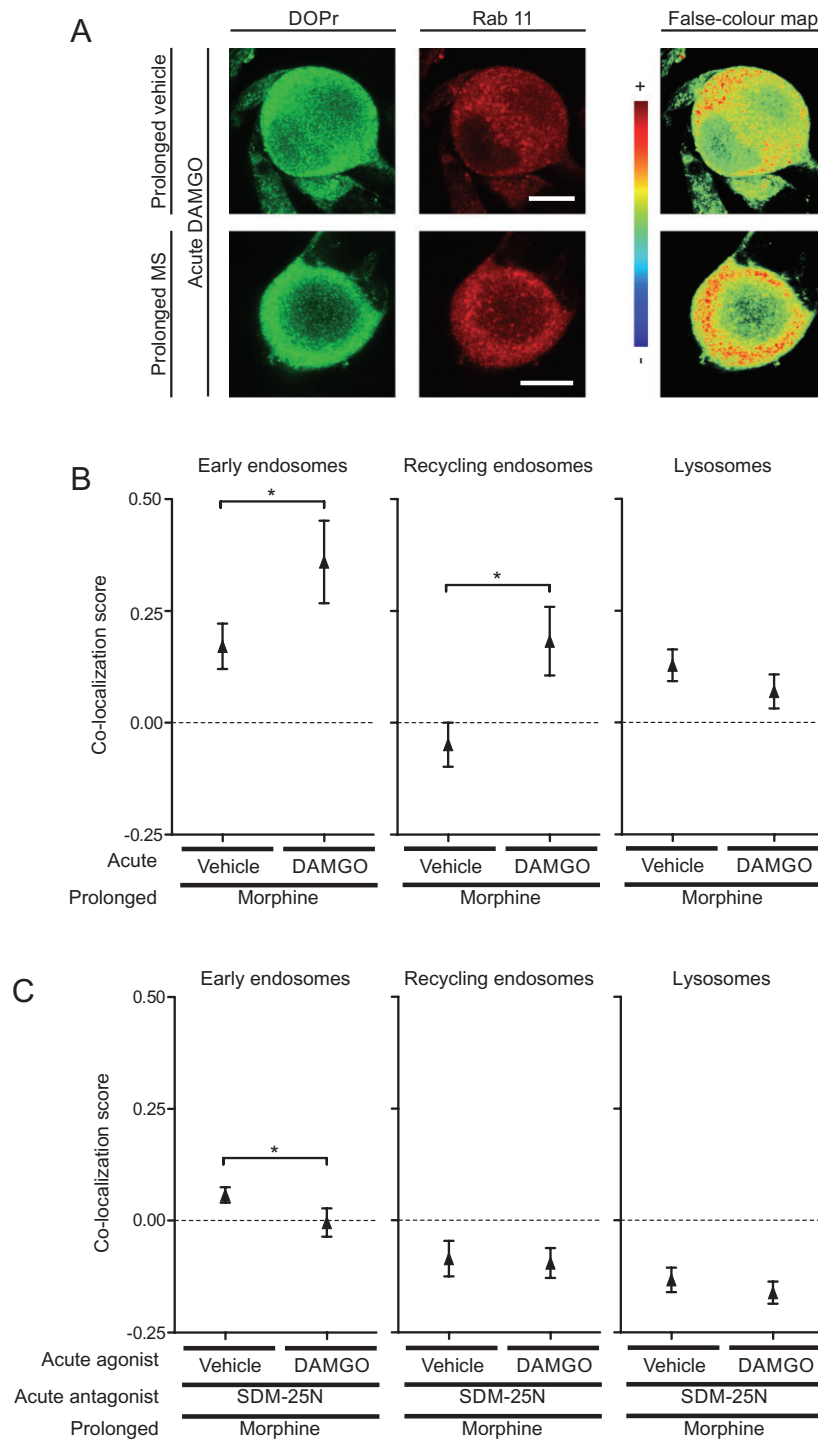
In this study, we have used co-localizational analysis of immunolabelling to quantify endogenous DOP receptor association with three post-endocytic compartments following various ligand treatments, both prolonged and acute. Co-localization with the compartment markers is interpreted, directly, as co-localization with those compartments and,





**Figure 5**

Deltorphin II and SNC80 induce DOP receptor recycling following morphine treatment. (A) Example confocal photomicrographs of two neurons. The upper neuron was treated with prolonged morphine and acute vehicle. The lower neuron was treated with prolonged morphine and acute DELT. Both neurons were immunolabelled for DOP receptor (green, left) and Rab 11 (recycling endosomes, red, centre). False-colour maps of co-localization were generated for each neuron, illustrating per-pixel DOP receptor co-localization with recycling endosomes (heat map, right). Scale bars show 10  $\mu$ m. (B) Cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle, DELT or SNC80. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. Following morphine treatment, both acute DELT and SNC80 increased DOP receptor co-localization with recycling endosomes and decreased DOP receptor co-localization with lysosomes. (C) Neurons underwent prolonged treatment with morphine and acute treatment with SDM-25N and vehicle, DELT or SNC80. Following morphine treatment and DOP receptor antagonist co-treatment, acute DELT decreased DOP receptor co-localization with early endosomes. Neither DELT nor SNC80 had any other effects on DOP receptor co-localization with the compartments. Data are shown as mean  $\pm$  95% confidence interval. \* $P < 0.05$ .



## Figure 6

DAMGO induces DOP receptor internalization and recycling following morphine treatment. (A) Example confocal photomicrographs of two neurons. The upper neuron was treated with prolonged vehicle and acute DAMGO. The lower neuron was treated with prolonged morphine and acute DAMGO. Both neurons were immunolabelled for DOP receptors (green, left) and Rab 11 (recycling endosomes, red, centre). False-colour maps of co-localization were generated for each neuron, illustrating per-pixel DOP receptor co-localization with recycling endosomes (heat map, right). Scale bars show 10  $\mu\text{m}$ . (B) Cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. Following morphine treatment, acute DAMGO increased DOP receptor co-localization with early endosomes and recycling endosomes. (C) Neurons underwent prolonged treatment with morphine and acute treatment with SDM-25N and vehicle or DAMGO. Following morphine treatment and DOP receptor antagonist co-treatment, acute DAMGO only decreased DOP receptor co-localization with early endosomes. Data are shown as mean  $\pm$  95% confidence interval. \* $P < 0.05$ .

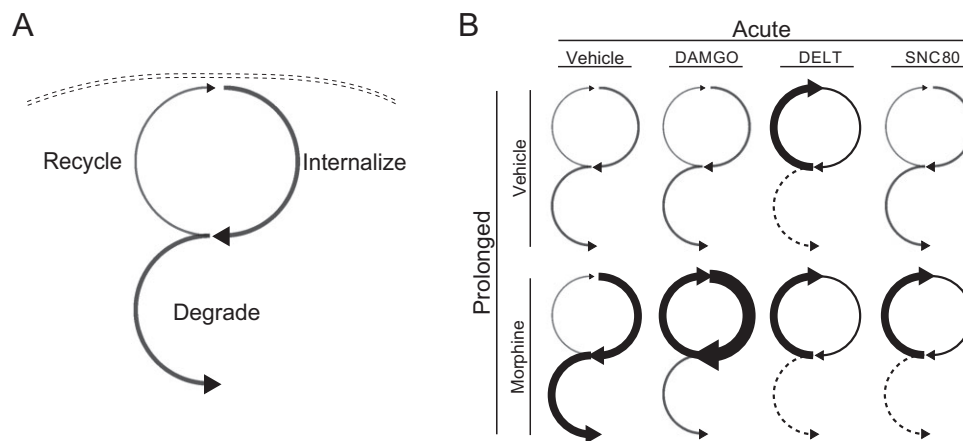
**Table 1**

DAMGO induction of DOP receptor recycling is early and sustained following morphine treatment

Prolonged	Acute	Time (min)	DOPr-Rab5	DOPr-Rab11	DOPr-Lamp1
Morphine	Vehicle	15	0.17 ± 0.03	-0.05 ± 0.02	0.13 ± 0.02
Morphine	DAMGO	30	0.15 ± 0.02	0.23 ± 0.03*	0.01 ± 0.03*
		60	0.20 ± 0.04	0.17 ± 0.04*	0.08 ± 0.03
			0.36 ± 0.05*	0.18 ± 0.04*	0.07 ± 0.02

In a preliminary experiment, cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson's co-localization coefficient following 15, 30 and 60 min acute treatments. Data are reported as mean ± 95% confidence interval. *n* = 1–4 independent cultures; 15–25 neurons imaged per culture.

\**P* < 0.05 compared with acute vehicle-treated neurons.



**Figure 7**

Prolonged morphine alters DOP receptor post-internalization trafficking. (A) Schematic summary of constitutive DOP receptor trafficking: internalization via early endosomes and ultimate degradation at lysosomes. Recycling is not considered to play a major role. (B) Schematic summary of experimental findings. In prolonged vehicle-treated neurons, DELT induced DOP receptor recycling and reduced degradation; no other acute condition affected DOP receptor trafficking. Prolonged morphine treatment (acute vehicle) augmented DOP receptor constitutive trafficking. Following prolonged morphine treatment, both DELT and SNC80 induced DOP receptor recycling and reduced degradation. Notably, DAMGO induced DOP receptor internalization and recycling in prolonged morphine-treated, but not control, neurons.

more abstractly, with the processes in which those compartments participate, namely internalization, degradation and recycling. This abstraction is helpful in understanding the results, but it is important to recognize that the results remain proxies for these overarching processes. These processes, in their totalities, are broader and more complex than the tell-tales we measure (for review, see Hanyaloglu and von Zastrow, 2008; von Zastrow, 2010; Williams *et al.*, 2013). However, these results do present a broad survey of post-internalization DOP receptor trafficking in which interesting comparisons can be made.

As class B GPCRs, constitutively internalized DOP receptors are trafficked via early endosomes ultimately to lysosomes where they are degraded (Figure 7A). Our results, however, indicate a departure from the constitutive trafficking pathway in many cases following acute agonist-induced internalization (Figure 7B). Consistent with previously published results obtained in immortalized neurons and expression systems, DOP receptors were found to be associated with

lysosomes following acute agonist treatment. However, acute administration of the DOP receptor peptide agonist DELT to control, prolonged vehicle-treated sensory neurons induced DOP receptor recycling. This increase in DOP receptor co-localization with subcellular compartments associated with receptor re-sensitization pathways was accompanied by a reduction in DOP receptor trafficking to lysosomes. This rules out the possibility that the observed increase in DOP receptor recycling was due to a global increase in DOP receptor internalization trafficking, as that explanation would require a concomitant, substantial increase in DOP receptor trafficking to lysosomes. Rather, these data show that DELT induced a shift in DOP receptor trafficking from the constitutive, lysosome-terminated pathway to recycling of the receptor back to the cell surface. DOP receptor post-internalization trafficking appears not to be a pre-determined track, but rather open to agonist influence. That is, the determinant of post-endocytic sorting is not DOP receptor *per se* but instead the event which triggered internalization.

It should be noted that while we observed increased DOP receptor recycling and decreased degradation, we did not detect any change in DOP receptor internalization (DOP receptor-early endosome co-localization). There are two possible explanations. Firstly, that there was, in fact, no net change in DOP receptor internalization. Agonist-induced DOP receptor internalization may have replaced constitutive DOP receptor internalization. On normal DOP receptor-expressing neurons, the vast majority of DOP receptors are at intracellular sites. There is a paucity of DOP receptors on the cell surface (Petaja-Repo *et al.*, 2000; Cahill *et al.*, 2001). The DELT-induced internalization of DOP receptors may pre-empt its normal, constitutive internalization either by design (i.e. constitutive internalization is only triggered in the absence of induced internalization) or by competition (i.e. there is only a sufficient pool of surface receptors for one internalization process). This would lead to no net change in DOP receptor internalization and co-localization with early endosomes, while preserving down-stream changes in trafficking. Secondly, we may have failed to detect a change in DOP receptor internalization. The methodology we used to assess DOP receptor trafficking provides a measure of DOP receptors associated with post-endocytic compartments at a specific time point. In this case, all measurements were made on neurons fixed after 60 min of acute agonist exposure. It is possible that there was a DELT-induced change in DOP receptor internalization, which happened and ended within that time. That is, the DOP receptor internalization had already occurred and DOP receptor-early endosome associations had returned to baseline levels by the time we washed and fixed the cells. This would have resulted in our temporally failing to detect the change.

Acute administration of SNC80 to control neurons had no effect on DOP receptor trafficking. It seems unlikely that SNC80-induced internalization simply replaced constitutive internalization exactly, yet this result does not, at first, seem to agree with the well-established ability of SNC80 to induce DOP receptor internalization (Pradhan *et al.*, 2010). The most likely explanation is that while SNC80 can induce DOP receptor internalization, there are simply too few DOP receptors on the surface of normal neurons for the non-peptide agonist's effects to be detectable. In this case, DELT appears to be better able to induce DOP receptor trafficking. Such variability in the ability to induce receptor internalization and trafficking is well established for both DOP (Pradhan *et al.*, 2010) and MOP receptor (Martini and Whistler, 2007) agonists. More broadly, these findings also fit with the growing body of work describing ligand-specific sorting in opioid receptors (Marie *et al.*, 2003; Audet *et al.*, 2005; 2008; 2012; Hong *et al.*, 2009; for review, see Piñeyro and Archer-Lahlou, 2007; Nagi and Piñeyro, 2011; Williams *et al.*, 2013). These results may also reflect DELT and SNC80 acting on different DOP receptor populations. As a peptidic ligand, DELT would largely be restricted to acting on surface DOP receptors, whereas SNC80, a small molecule, would not have that limitation. As a result, SNC80's additional actions on intracellular DOP receptors may contribute to the differences observed.

Since prolonged morphine treatment causes MOP receptor-dependent trafficking of DOP receptors to neuronal cell membranes (Cahill *et al.*, 2001; Morinville *et al.*, 2003; 2004a; Lucido *et al.*, 2005; Gendron *et al.*, 2006), we exam-

ined whether endogenous DOP receptor post-internalization trafficking in neurons was altered by prolonged morphine treatment. Constitutive DOP receptor trafficking was augmented following prolonged morphine treatment; we observed increased DOP receptor internalization and trafficking to lysosomes. This is consistent with an increase in the cell surface DOP receptor population. With more surface DOP receptors, there are more DOP receptors constitutively internalized, whereupon they follow the typical pathway to degradation in lysosomes. With acute administration of the DOP receptor antagonist SDM-25N, we continued to observe increased DOP receptor internalization but decreased trafficking to lysosomes. As SDM-25N was administered acutely, without acute agonist treatment, it is not surprising that effects of prolonged morphine were still apparent, in the form of increased DOP receptor internalization. It is interesting, though, that lysosomal trafficking of DOP receptors decreased. These apparently opposite changes may represent a combination of stabilization of DOP receptors by SDM-25N against the background of an increased DOP receptor surface population.

Acute administration of DELT to morphine-treated neurons induced DOP receptor recycling and decreased DOP receptor degradation (Figure 7B). This is a preservation of the same effect as observed in control neurons. Once again, DELT induced DOP receptor trafficking different from the constitutive pathway. Interestingly, we were again unable to detect a change in DOP receptor internalization following acute DELT compared to control neurons, which received acute vehicle and the same prolonged treatment. Once again both possible explanations are valid. There may be no net change in DOP receptor internalization; constitutive internalization was augmented following prolonged morphine, and DELT-induced internalization may have replaced some of the constitutive traffic. Alternatively, we may still be temporally failing to detect the DELT-induced changes in internalization.

Trafficking in response to SNC80 changed following prolonged morphine treatment compared with control neurons. Acute administration of SNC80 to morphine-treated neurons induced DOP receptor recycling and decreased DOP receptor degradation (Figure 7B). This unmasking of distinct SNC80-induced trafficking is consistent with the hypothesis that such activity was not observed in control neurons because of the relative paucity of surface DOP receptors. The increase in available DOP receptors following prolonged morphine permits SNC80 to induce DOP receptor trafficking. SNC80's trafficking effects appear to be the same as DELT's; DOP receptor trafficking shifts from the constitutive lysosome-terminated pathway to recycling. In a further similarity to DELT, we observed no change in DOP receptor internalization after acute SNC80, and the same explanations are possible. As would be expected, DOP receptor agonist-induced trafficking was absent when DELT and SNC80 were co-administered with SDM-25N.

It is unlikely that alterations in trafficking in response to DELT and SNC80 are as a result of pharmacological chaperoning of DOP receptors given the similarity of the responses. Previous studies identified that a high percentage of DOP receptors are targeted directly from endoplasmic reticulum to lysosomes with only a small percentage ever maturing and reaching the plasma membrane (Petaja-Repo *et al.*, 2000;

2001). However, non-peptide DOP receptor agonists have been shown to act as chaperones, promoting DOP receptor maturation and thus increasing plasmalemma-associated receptors (Petäjä-Repo *et al.*, 2006). While chaperoning by SNC80 could play a role in the availability of surface DOP receptors for SNC80 to then internalize, DELT would not have such effects given the need for any pharmacological chaperone to be membrane permeable (Petäjä-Repo *et al.*, 2002). Furthermore, in such a case, one would expect the effects of DELT and SNC80 in control neurons to be reversed; that is, the membrane-permeable SNC80, but not DELT, should have chaperoned DOP receptors to the surface to then internalize it. In both cases, the opposite occurred.

Acute administration of DAMGO, a MOP receptor agonist, to control neurons had no effect on DOP receptor internalization. It is possible that such a lack of effect reflects a temporal limitation, but in such a case, DAMGO effects on DOP receptor trafficking would have to normalize within 60 min despite continued agonist application.

Acute administration of DAMGO to morphine-treated neurons, however, induced DOP receptor internalization and recycling. This is a change from control neurons, where DAMGO had no detected effect, consistent with the postulated induction of MOP–DOP receptor interactions by prolonged morphine treatment. Indeed, preliminary findings indicate that the induced DOP receptor recycling is both early and sustained. *Prima facie*, DAMGO would be expected to have no effect on DOP receptor trafficking as it has negligible activity at that receptor. However, the presence of a MOP/DOP receptor species at the cell surface following prolonged morphine could account for the existence of a DOP receptor species upon which DAMGO may act. Interestingly, in this case, we did observe increased DOP receptor internalization but did not observe any change in DOP receptor degradation following 60 min DAMGO exposure. Our preliminary findings, however, indicate that DAMGO may reduce early (15 min) DOP receptor degradation, whereas the increase in internalization occurs following sustained (60 min) exposure. This may reflect DAMGO inducing the recycling of a largely different pool of receptors than those undergoing constitutive trafficking. In this case, DAMGO-responsive DOP receptor would be immediately shifted away from degradation to recycling, resulting in the early reduction in DOP receptor degradation. With continued exposure, the surface population becomes enriched in DAMGO-responsive DOP receptors, ultimately leading to the late increase in DOP receptor internalization and sustained increase in DOP receptor recycling. Such recycling may also involve recycling from superficial endosomes, identified as the Rab5-labelled compartment, consistent with the existence of a distinct pool of MOP/DOP receptors. Further, surface enrichment in DAMGO-responsive DOP receptors may also involve DAMGO-induced export of new or intracellularly reserved DOP receptors. Ultimately, whereas DOP receptor internalization in response to DELT or SNC80 may occur to some extent in place of constitutive internalization, DAMGO internalization may be occurring in addition to it. This is consistent with the presence, post-prolonged morphine, of an additional MOP/DOP receptor species with distinct trafficking characteristics. Notably, the responsiveness of DOP receptor trafficking to DAMGO was abolished by co-treatment with SDM-25N. Together, these

results demonstrate that following prolonged morphine, but not in basal conditions, there is surface availability of a DOP receptor species upon which DAMGO may act to induce internalization trafficking and at which such action may be blocked by a DOP receptor antagonist. These findings support previous reports that prolonged morphine treatment induces an increase in cell surface available DOP receptor and that prolonged morphine treatment induces MOP–DOP receptor interactions, potentially in the form of a MOP/DOP receptor species. These MOP–DOP receptor interactions appear to alter responsiveness to opioid receptor ligands and subsequent post-internalization DOP receptor trafficking.

These findings are consistent with other reports of MOP–DOP receptor interaction effects upon trafficking. Milan-Lobo and Whistler (2011) reported that in a heterologous system, the MOP receptor agonist methadone internalizes both MOP and MOP/DOP receptors. Additionally, He *et al.* (2011) reported that in a heterologous system, DOP receptor agonists induced endocytosis of both DOP and MOP receptors. In this case, DOP receptor agonist-induced endocytosis of MOP receptors was attenuated by disrupting putative MOP–DOP receptor interactions. In both cases, these studies concluded that the existence of a MOP–DOP receptor interaction permitted the apparently paradoxical agonist-induced receptor internalization. These reports agree with both our observation of DAMGO-induced DOP receptor internalization and our interpretation that the internalized DOP receptor were in the form of MOP/DOP receptor heteromers. Furthermore, and in agreement with the finding of altered post-internalization trafficking of MOP/DOP receptors, Milan-Lobo and Whistler reported that an internalized MOP/DOP receptor was handled differently from an internalized MOP receptor, tending towards degradation (Milan-Lobo and Whistler, 2011). Notably, Milan-Lobo and Whistler reported a shift towards degradation for MOP/DOP receptors, while we observed a shift towards recycling. This is, however, not surprising as we each compared against different standards. That MOP/DOP receptors would undergo greater degradation than MOP receptors and also greater recycling than DOP receptors is sensible; MOP/DOP receptor's trafficking behaviour appears to be unique from and intermediate to that of either component individually.

It is important to recognize that we measured DOP receptor sorting to these post-endocytic compartments after 60 min ligand treatment. This provides a 'snapshot' of DOP receptor post-endocytic trafficking across these compartments at that time point but not a summation or time course of trafficking over that time period. Indeed, a comparison of the time courses of post-endocytic trafficking in certain ligand treatment conditions is an interesting future direction.

In conclusion, we have demonstrated that in physiologically relevant neurons expressing endogenous receptors, prolonged morphine treatment augments constitutive DOP receptor trafficking and alters agonist-induced DOP receptor trafficking. Notably, the DOP receptor internalizes and traffics in response to DAMGO, a MOP receptor agonist. These effects are inhibited or absent when agonists are co-administered with a DOP receptor antagonist. These observations are consistent with the hypothesis that prolonged morphine treatment induces the formation of MOP–DOP receptor interactions and subsequent cell surface availability of a



MOP/DOP receptor species. The pharmacology and trafficking of such a species appear to be unique compared to those of its individual constituents and could represent a novel therapeutic target.

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## Author contributions

E. W. O. conceptualized the hypothesis and objectives, conducted the experiments, analysed the data, and wrote and edited the manuscript; L. X. conducted the experiments; M. C. O. supervised and gave feedback in the manuscript; C. M. C. supervised, conceptualized the hypothesis and objectives, and wrote and edited the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Supporting information

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

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- Figure S1** Original image, Figure 5A, upper left.  
**Figure S2** Original image, Figure 5A, upper centre.  
**Figure S3** Original image, Figure 5A, lower left.  
**Figure S4** Original image, Figure 5A, lower centre.  
**Figure S5** Original image, Figure 6A, upper left.  
**Figure S6** Original image, Figure 6A, upper centre.  
**Figure S7** Original image, Figure 6A, lower left.  
**Figure S8** Original image, Figure 6A, lower centre.