# Clinical opioids differentially induce co-internalization of μ- and δ-opioid receptors

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## Abstract

Opioid receptors play an important role in mediating the spinal analgesia. The μ-opioid receptor is the major target of opioid drugs widely used in clinics. However, the regulatory mechanisms of analgesic effect and tolerance for clinical μ-opioid receptor-targeting opioids remain to be fully investigated. Previous studies showed the interaction of δ-opioid receptor with μ-opioid receptor to form the μ-opioid receptor/δ-opioid receptor heteromers that could be processed in the degradation pathway after δ-opioid receptor agonist treatment. Here, we showed that clinical μ-opioid receptor-targeting opioids, morphine, fentanyl, and methadone, but not tramadol, caused μ-opioid receptor co-internalization with δ-opioid receptors in both transfected human embryonic kidney 293 cells and primary sensory neurons. Prolonged treatment of morphine led to μ-opioid receptor co-degradation with δ-opioid receptors. Furthermore, fentanyl and methadone, but not tramadol, induced the drug tolerance similar to morphine. Thus, the clinical μ-opioid receptor-targeting opioids including morphine, fentanyl, and methadone induce μ-opioid receptor co-internalization with δ-opioid receptors, which may be involved in the analgesic tolerance of these opioids.

## Introduction

Opioid receptors are recognized as important targets for the spinal analgesia. The μ-opioid receptor (MOR) undertakes the major part of analgesic effect by binding both endogenous opioid peptides and exogenous opioid analgesics.[1] Morphine and other MOR-targeting opioid drugs are strong analgesics used in clinics, but long-term administration may result in many side effects including tolerance, dependence, and addiction.[1,2] The δ-opioid receptor (DOR), another type of opioid receptor, is distributed in small- and large-diameter neurons of the dorsal root ganglion (DRG) and could be co-expressed with MORs.[3–5] Blockage of DORs enhances morphine-mediated analgesia and prevents the development of morphine tolerance,[6,7] and disruption of DOR phosphorylation also attenuates morphine tolerance.[8] Moreover, deletion of DOR leads to the reduction of morphine tolerance without change of morphine-mediated analgesia.[9–11] Therefore, DOR is involved in the development of MOR-mediated morphine tolerance.

As G protein-coupled receptors (GPCRs), MORs and DORs form heteromers which show different properties

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in trafficking and pharmacology from MORs alone.[12–15] After agonist-induced activation, MORs or DORs are phosphorylated and internalized under the help of clathrin. The internalized receptors are recognized by different sorting proteins which may decide their destination: recycling endosome or lysosome.[16,17] GPCR-associated sorting protein binds the cytoplasmic tail of DOR and targets DORs to lysosomes, while imidazoline receptor antisera-selected protein modulates MOR and sorts MORs to the recycling pathway.[18–20] However, our previous study reveals that the post-endocytic MOR/DOR heteromers are processed in proteasomes or lysosomes for degradation following the DOR agonist stimulation, resulting in the reduction of MORs on the cell surface.[21] The MOR/DOR heteromers, but not MORs alone, are able to recruit β-arrestin2.[22,23] In β-arrestin2 knock-out mice, morphine-induced spinal analgesia is enhanced and morphine tolerance is delayed, while morphine-mediated supraspinal analgesic tolerance disappears.[24–26] Notably, MOR/DOR heteromers form distinctive conformation that provides a possibility of different destiny for MORs after receptor internalization, contributing to opioid-induced tolerance.

The trafficking pathway of MORs induced by morphine and other MOR-targeting opioid drugs including fentanyl, methadone, and tramadol has not been systematically investigated. Although the experiments were performed in both animal and human embryonic kidney (HEK)293 cells, the detection of MOR internalization after morphine treatment remains uncertain.[27–29] However, after methadone treatment, the MOR/DOR heteromers are targeted for degradation, but MORs alone are still in relatively more stable state.[30] Since MORs are often co-expressed with DORs in nervous system, detection of the trafficking pathway of MOR/DOR heteromers after treatment with MOR-targeting opioid drugs is more significant. On the other hand, prolonged morphine treatment causes the insertion of DORs into the plasma membrane of neurons[31–33] and increases the abundance of the MOR/DOR heteromers on the cell surface,[34] which provides opportunity for MOR co-internalization with DORs. Furthermore, receptor internalization is often considered as a mechanism of drug tolerance.[1,17] Correlation of MOR internalization with drug tolerance induced by clinical MOR-targeting opioids is certainly worth to be studied.

In the present study, we examined the MOR co-internalization with DORs induced by the clinical MOR-targeting opioids. We showed that morphine, fentanyl, and methadone, but not tramadol, caused MOR co-internalization with DORs in both transfected HEK293 cells and primary sensory neurons expressing these two receptors. Prolonged morphine treatment led to MOR co-degradation with DORs. Correlatively, morphine, fentanyl, and methadone, but not tramadol,

induced dramatic drug tolerance. This study may provide an underlying mechanism for clinical opioid-induced tolerance.

## Methods

### Cell culture and transfection

HEK293 cells were cultured with minimal essential medium (Invitrogen) that contained 10% fetal bovine serum (Invitrogen), and transfected with plasmids including hemagglutinin (HA)-tagged MOR (HA-MOR), Myc-tagged DOR (Myc-DOR) or vector using lipofectamine transfection.[21] The cells were processed for various assays after 24 h.

The DRGs from mice were digested with 1 mg/ml collagenase, 0.4 mg/ml trypsin, and 0.1 mg/ml DNase I (Sigma) at 37°C for 30 min and further triturated. Then, dissociated DRG neurons were processed to electroporation with plasmids including HA-MOR and Myc-DOR by Amaxa electroporation nuclear I, and cultured with Dulbecco's Modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum for two to three days.

### Drug treatment

D-Ala$^2$, N-MePhe$^4$, Gly-ol-enkephalin (DAMGO; Tocris) was dissolved in distilled water and deltorphin I (Delt I; Bachem) was dissolved in DMSO. Opioid drugs including morphine (Shenyang No. 1 Pharmaceutical Co., Northeast Pharmaceutical Group, China), fentanyl (Yichang Humanwell Pharmaceutical Co., China), tramadol (Grünenthal GmbH), and methadone (The Central Pharmaceutical Co., China) were diluted with extracellular solution in cultured cells or saline for mouse subcutaneous injection. These opioid drugs were added to HEK293 cells or neurons for indicated time before fixation or lysis.

### Immunostaining

The living HEK293 cells and cultured DRG neurons expressing HA-MOR and Myc-DOR were incubated with mouse antibody against HA (1:100; Sigma) and rabbit antibody against Myc (1:100; Sigma) for 30 min at 37°C. Then, cells were treated with different opioid drugs and/or LysoTracker Red (1:500; Molecular Probes) for 30 min or 90 min at 37°C. For examining receptor recycling, cells were given additional 1 h or 2 h at 37°C to recover from stimulation. Finally, cells were fixed with 4% paraformaldehyde and incubated with Alexa-Fluor-conjugated secondary antibodies (1:500; Molecular Probe) for 45 min at 37°C. The cells were mounted and scanned using a Nikon A1 confocal microscope (Nikon) or an Olympus FV10I confocal microscope (Olympus).

The intensities of the intracellular/membrane immunofluorescence versus the total immunofluorescence were quantified using ImageJ software (Wayne Rasband, National Institutes of Health) and indicated as the percentage of MOR distribution in intracellular compartment and membrane. In order to count the number of MOR-positive puncta and calculate the co-localization ratio of MOR with LysoTracker Red, confocal images were performed for deconvolution via Huygens Essential software (Scientific Volume Imaging, the Netherlands). For quantitative analysis of HEK293 cells, 20–35 cells from each experiment and 65–100 cells from three independent experiments were collected for each group; for quantitative analysis of DRG neurons, 10–20 cells from each experiment and 40–45 cells from three independent experiments were collected for each group.

### In situ proximity ligation assay (PLA)

The interaction of HA-MOR and Myc-DOR expressed in cultured DRG neurons was detected by in situ PLA. Cultured DRG neurons were fixed with 4% paraformaldehyde and incubated with mouse antibody against HA (1:1000), rabbit antibody against Myc (1:1000), and chicken antibody against β-tubulin III (TUJ1, 1:2000; Abcam) overnight at 4°C. For detection of the interaction between surface HA-MOR and Myc-DOR, living DRG neurons were incubated with mouse antibody against HA (1:100) and rabbit antibody against Myc (1:100) for 30 min at 37°C and then fixed and incubated with chicken antibody against TUJ1 overnight at 4°C.

After the incubation with primary antibodies, the minus probe against mouse and the plus probe against rabbit conjugated with different oligonucleotides were added to cells for 1 h at 37°C. When the minus and plus probes bound in close proximity, their oligonucleotides joined to form a close circle under the help of ligation solution for 30 min at 37°C. Then, a rolling-circle amplification reaction using the ligated circle as template was carried out in the amplification solution containing fluorescein-labeled oligonucleotides for 100 min at 37°C. The fluorescent signals representing binding probes were regarded as the location of HA-MOR and Myc-DOR interaction.

### Cell-surface biotinylation and immunoblotting

Cell-surface biotinylation was performed to detect the cell-surface proteins. The cells were labeled with 0.25 mg/ml biotin for 45 min at 4°C. Cell lysates were collected with ice-cold cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40), and biotinylated surface proteins were precipitated with NeutrAvidin beads. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred, probed with specific antibodies, and visualized with enhanced chemiluminescence (Bio-Rad). The primary antibodies contained guinea pig antibody against MOR (1:2000; Abcam), rabbit antibody against DOR (1:2000; Alomone), mouse antibody against transferrin receptor (TfR, 1:5000; Invitrogen), rabbit antibody against MOR (1:300; Neuromics), or rabbit antibody against DOR (1:1000; Chemicon). The amount of cell-surface receptor was quantified from the intensities of immunoblotting bands for MOR versus TfR from three to four independent experiments using ImageJ software. For examining the recovery of cell-surface receptors after drug treatment, cells were treated with the drug for 30 min and further 1-h or 2-h recovery without drug, and then labeled with biotin. In this experiment, in order to rule out the interruption of new protein insertion into membrane, cells were pre-treated with 10 μM cycloheximide to inhibit new protein synthesis. For examining the drug-induced degradation of cell-surface receptors, cells were pre-labeled with biotin and then treated with the drugs for 90 min.

### Detection of receptor ubiquitination

To detect the ubiquitination level of MOR and DOR, mouse DRG tissues were collected 60 min after subcutaneous injection of 5 mg/kg morphine in cell lysis buffer containing 10 mM N-ethylmaleimide and 6 M urea was added after tissues lysed. Then, the suspension was diluted to 2 M urea and processed to be immunoprecipitated by 2 μg rabbit antibody against MOR (Dia Sorin) or rabbit antibody against DOR (Chemicon) separately. Samples were subjected to immunoblotting with primary antibodies including rabbit antibody against MOR (1:1000; Dia Sorin), rabbit antibody against DOR (1:1000; Chemicon), or mouse antibody against ubiquitin (1:1000; Santa Cruz).

### Analysis of single-cell RNA-sequencing

According to our RNA-sequencing data from 197 mouse DRG neurons and established 10 types for DRG neurons,[35] a heatmap showing the expression patterns of opioid receptor mu 1 (Oprm1), opioid receptor delta 1 (Oprd1), opioid receptor kappa 1 (Oprk1), opioid receptor-like 1 (Oprl1), tachykinin 1 (Tac1), and neurofilament, heavy polypeptide (Nefh) in different neuron types was generated by R-Studio software.

### Behavior tests

All the experiments complied with the policy about animal use. Opioid-induced analgesia was assessed by tail immersion test. One-third of mouse tail was immersed in 52°C water, and the time of tail flicking

out from water was recorded. The basal response of mice was first examined, and then opioid drugs were administrated subcutaneously (s.c.). The post-drug latency of mice was recorded 30 min after the drug treatment. The cutoff time was set at 10 s to avoid scald. Antinociception was evaluated by the change of tail response after the drug treatment: % maximum possible effect (M.P.E) = $100 \times$ (post latency − basal latency)/ (10 − basal latency).[21]

## Statistics analysis

Statistical analysis was carried out with GraphPad Prism software, and all the data were showed as mean ± SEM. For comparing two groups of data, two-tailed, paired or unpaired Student's $t$ test was used. Comparison of drug-induced changes affected by two factors was performed by two-way analysis of variance. The difference was considered significant when p < 0.05.

## Results

### MOR-targeting opioid-induced MOR internalization with DORs

To determine the effects of MOR-targeting opioid drugs on the internalization of opioid receptors, we first examined morphine-induced trafficking of surface MORs in HEK293 cells transfected with plasmids expressing HA-MOR alone or co-transfected with plasmids expressing HA-MOR and Myc-DOR (Figure 1(a) and (b)). HEK293 cells lack endogenous MOR/DOR. Because HA and Myc tags were placed in the N-terminals of opioid receptors, we used mouse against HA antibody and rabbit against Myc antibody to pre-label the opioid receptors in the plasma membrane of living HEK293 cells and then examine the drug-induced translocation of pre-labeled surface MORs and DORs. Immunostaining showed that a 30-min treatment with morphine induced an increase of the intracellular punctum structure of pre-labeled surface MORs in a dose-dependent manner (Figure 1(a) and (c)), suggesting the internalization of surface MORs. The levels of morphine-induced MOR internalization in HEK293 cells co-expressing MORs and DORs were more pronounced than that in cells expressing MORs alone (Figure 1(a)–(c)). Obviously, morphine also caused co-internalization of MORs with DORs in HEK293 cells co-expressing MOR/DOR (Figure 1(b)). Thus, the MOR in MOR/DOR heteromer is prone to be internalized compared to MOR homomer after morphine administration.

The morphine-induced co-internalization of MORs and DORs was further confirmed by examining the amount of receptors remaining on the cell surface. A 30-min treatment with 1 µM or 10 µM morphine was applied to HEK293 cells co-expressing MORs and DORs, and the cell-surface proteins were precipitated by the experiment with cell-surface biotinylation. Immunoblotting of surface proteins with the antibodies against opioid receptors showed that a 30-min treatment with morphine reduced the surface MORs and DORs similar to a potent selective MOR agonist, DAMGO, but relatively weaker (Figure 1(d) and (e)). Furthermore, either 10 µM morphine or 10 µM DAMGO displayed a stronger effect than 1 µM drug treatment (Figure 1(d) and (e)). The total protein levels of MORs and DORs were unaffected by 30-min morphine or DAMGO treatment (Figure (d) and (f)).

The other clinically and widely used opioids including fentanyl, tramadol, and methadone mostly target MORs. We detected the effects of these opioid drugs on the internalization of receptors. A 30-min treatment with fentanyl and methadone caused MOR co-internalization with DORs similar to DAMGO and morphine in HEK293 cells co-expressing MORs and DORs in a dose-dependent manner (Figure 2(a), (c), (d), and (f)). Methadone induced the highest level of MOR internalization besides DAMGO in HEK293 cells co-expressing MORs and DORs; however, tramadol did not have this effect (Figure 2(a)–(f)). Taken together, morphine, fentanyl, and methadone cause an obvious internalization of MORs in HEK293 cells co-expressing MORs and DORs.

## Post-endocytic trafficking of internalized MORs

Previous studies report that internalized MORs are largely recycled to the cell surface after DAMGO treatment, and internalized DORs are mainly processed to degradation in the lysosomal compartments after treatment with DOR agonist.[36–39] The post-endocytic process of internalized MORs induced by morphine was examined in HEK293 cells co-expressing MORs and DORs. We detected the distribution of cell-surface MORs after a 30-min treatment of opioid drugs and additional 1-h or 2-h recovery without drugs. During recovery after drug treatment, the internalized MORs may be recycled back to cell surface or retained in intracellular compartments. Consistent with previous reports, DAMGO caused internalized MORs partially recycling back to cell membrane, and a selective DOR agonist, Delt I, led internalized MORs retained intracellular in HEK293 cells co-expressing MORs and DORs (Figure 3(a) and (b)). Importantly, in morphine-treated HEK293 cells co-expressing MORs and DORs, the internalized MORs were mainly distributed in intracellular components with DORs after a 2-h recovery (Figure 3(a) and (b)). This phenomenon was confirmed with the
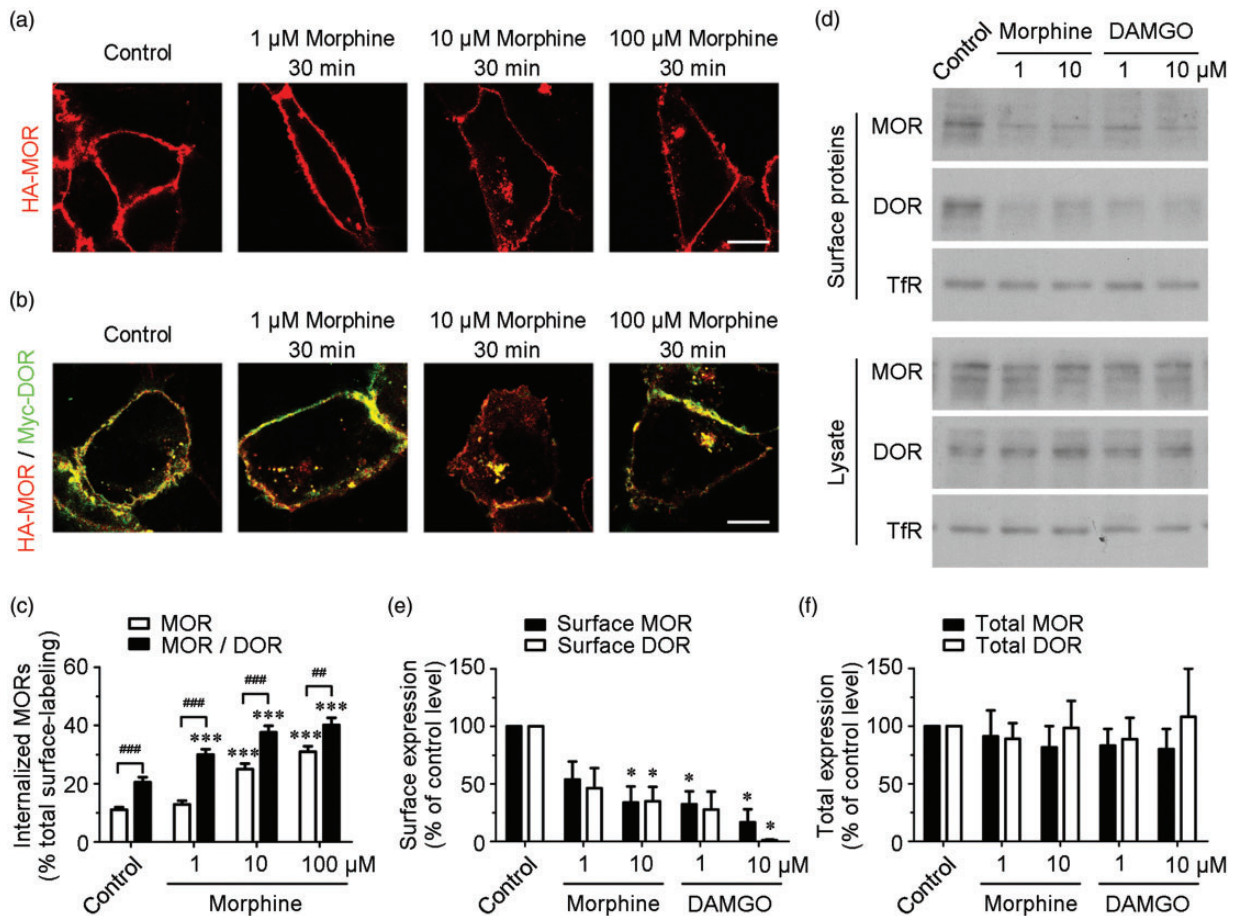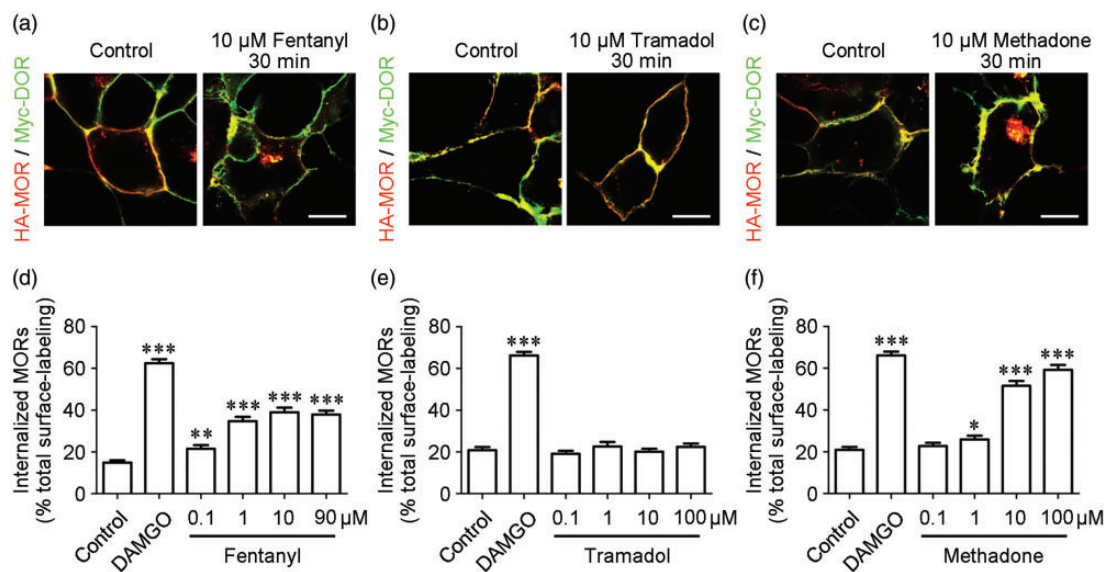
**Figure 1.** Morphine induces co-internalization of MORs with DORs. ((a)–(c)) HA-MOR or/and Myc-DOR expressed on the cell surface were pre-labeled with antibodies against HA (red) or/and Myc (green) in living HEK293 cells, and then treated with morphine for 30 min ((a) and (b)). Quantitative data of internalized MORs were calculated from the intensities of intracellular immunofluorescence versus the total immunofluorescence (c). In control cells, the pre-labeled MORs or/and DORs were mainly localized on the cell surface. Morphine induced MOR internalization in a dose-dependent manner. In HEK293 cells expressing MOR alone, a 30-min treatment with 10 μM and 100 μM, but not 1 μM, morphine induced an increase of intracellular puncta representative of MOR internalization. In HEK293 cells co-expressing MOR/DOR, a 30-min treatment with 1 μM, 10 μM, and 100 μM morphine caused even remarkable MOR internalization in the intracellular puncta partially co-localized with DORs. The results are presented as the mean ± SEM (n = 80–100 cells). Scale bar, 10 μm. ***$p < 0.001$ versus control group and ##$p < 0.01$, ###$p < 0.001$ versus indicated group. ((d)–(f)) Representative immunoblotting (d) and quantitative data ((e) and (f)) showed that in HEK293 cells co-expressing HA-MOR and Myc-DOR, the levels of MORs and DORs on the cell surface were reduced after 30-min treatment with morphine or DAMGO. Transferrin receptor (TfR) served as a control for protein loading. The results are presented as the mean ± SEM (n = 4). *$p < 0.05$ versus control group.
MOR: μ-opioid receptor; DOR: δ-opioid receptor; DAMGO: D-Ala², N-MePhe⁴, Gly-ol-enkephalin.

biochemical detection of cell-surface MORs and DORs. After 2-h recovery, MORs and DORs were detected a little on cell surface in morphine-treated HEK293 cells, and total MORs were decreased which might be morphine-induced degradation (Figure 3(c)–(e)). Therefore, morphine-induced internalized MORs tend to retain in intracellular compartments.

We further explore whether morphine-induced internalized MORs subsequently enter the degradation pathway. Immunostaining of cell-surface pre-labeled MORs and DORs showed that consistent with our previous study,[21] a 90-min Delt I treatment caused robust MOR

entry to LysoTracker-labeled lysosomes with DORs, and DAMGO induced much weaker effect in HEK293 cells co-expressing MORs and DORs (Figure 4(a) and (b)). Importantly, a 90-min morphine treatment also led to a certain extent of internalized MOR entry to lysosomes with DORs in HEK293 cells co-expressing MORs and DORs (Figure 4(a) and (b)). Immunoblotting further showed that the level of cell-surface pre-labeled MORs was significantly reduced with DORs after a 90-min morphine treatment in HEK293 cells co-expressing MORs and DORs, which was similar to Delt I (Figure 4(c) and (d)). The total protein levels of MORs

**Figure 2.** Clinical MOR-targeting opioids induce co-internalization of MORs with DORs. HA-MOR and Myc-DOR expressed on the cell surface were pre-labeled with antibodies against HA (red) and Myc (green) in living HEK293 cells and then treated with opioid drugs for 30 min ((a)–(c)). Quantitative data of internalized MORs were calculated from the intensities of intracellular immunofluorescence versus the total immunofluorescence ((d)–(f)). In HEK293 cells co-expressing MOR/DOR, a 30-min treatment with fentanyl and methadone, but not tramadol, induced MOR co-internalization with DORs in a dose-dependent manner similar to morphine. DAMGO (1 μM) was chosen as a positive control for inducing MOR internalization. The results are presented as the mean ± SEM (n = 80–100 cells). Scale bar, 10 μm. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the corresponding control group.
MOR: μ-opioid receptor; DAMGO: D-Ala[2], N-MePhe[4], Gly-ol-enkephalin.

and DORs were also decreased after a 90-min morphine treatment, which was weaker than Delt I (Figure 4(e)). Taken together, these data suggest that morphine causes internalized MORs partial entry to lysosomes for degradation with DORs.

## MOR-targeting opioid-induced MOR internalization with DORs in primary sensory neurons

Given MOR-targeting opioids could induce co-internalization and co-degradation of MORs with DORs in HEK293 cells, we assessed the situation in primary sensory neurons. Previous studies have proved the co-existence of MORs and DORs in the DRG neurons.[3,40] Recently, our single-cell RNA-sequencing work has classified somatosensory neurons into 10 types: C1 represented by galanin (*Gal*), C2 by natriuretic peptide B (*Nppb*), C3 by tyrosine hydroxylase (*Th*), C4 by MAS-related G-protein-coupled receptor A3 (*Mrgpra3*), C5 by MAS-related G-protein-coupled receptor D (*Mrgprd*), C6 by *Mrgprd* and the S100 protein, beta polypeptide (*S100b*), and large neurons (C7–C10) by *S100b*.[35] The sequencing data also confirmed *Oprm1* (gene encoding MOR), *Oprd1* (gene encoding DOR), *Oprk1*, *Oprl1*, *Tac1,* and *Nefh* existed in multiple types of DRG neurons. *Oprm1* and *Oprd1*

could be co-expressed in small neurons (C1, C2, and C4–C6 types) and large DRG neurons (C8 type) (Figure 5 (a)). Among all *Oprd1*-expressing DRG neurons, 77% of them showed *Oprm1*-positive; 86% of small *Oprd1*-positive neurons, and 69% of large *Oprd1*-positive neurons also expressed *Oprm1* (Figure 5(b)). Our data indicated the co-existence of *Oprm1* and *Oprd1* non-selectively occurred in different types of DRG neurons.

The interaction between MORs and DORs was further examined in single DRG neuron cultured from mice. Due to the lack of suitable antibodies against MOR and DOR for immunostaining from different species, we transfected the plasmids expressing HA-MOR and Myc-DOR and used antibodies against HA and Myc tags to label MORs and DORs in DRG neurons. In situ PLA showed immunofluorescent signals in the cell membrane pre-labeled surface HA and Myc in DRG neurons co-expressing HA-MOR and Myc-DOR (Figure 5(c)), suggesting that HA-MOR and Myc-DOR are localized in close proximity and interacted on the cell surface. In situ PLA with antibodies against HA and Myc also exhibited immunofluorescent signals in the cell body and neurites of DRG neurons (Figure 5(c)). Thus, MORs and DORs are not only co-localized but also interact in single primary sensory neuron.

We next assessed whether MOR-targeting opioids induced the co-internalization of MORs with DORs in cultured DRG neurons co-expressing HA-MOR and
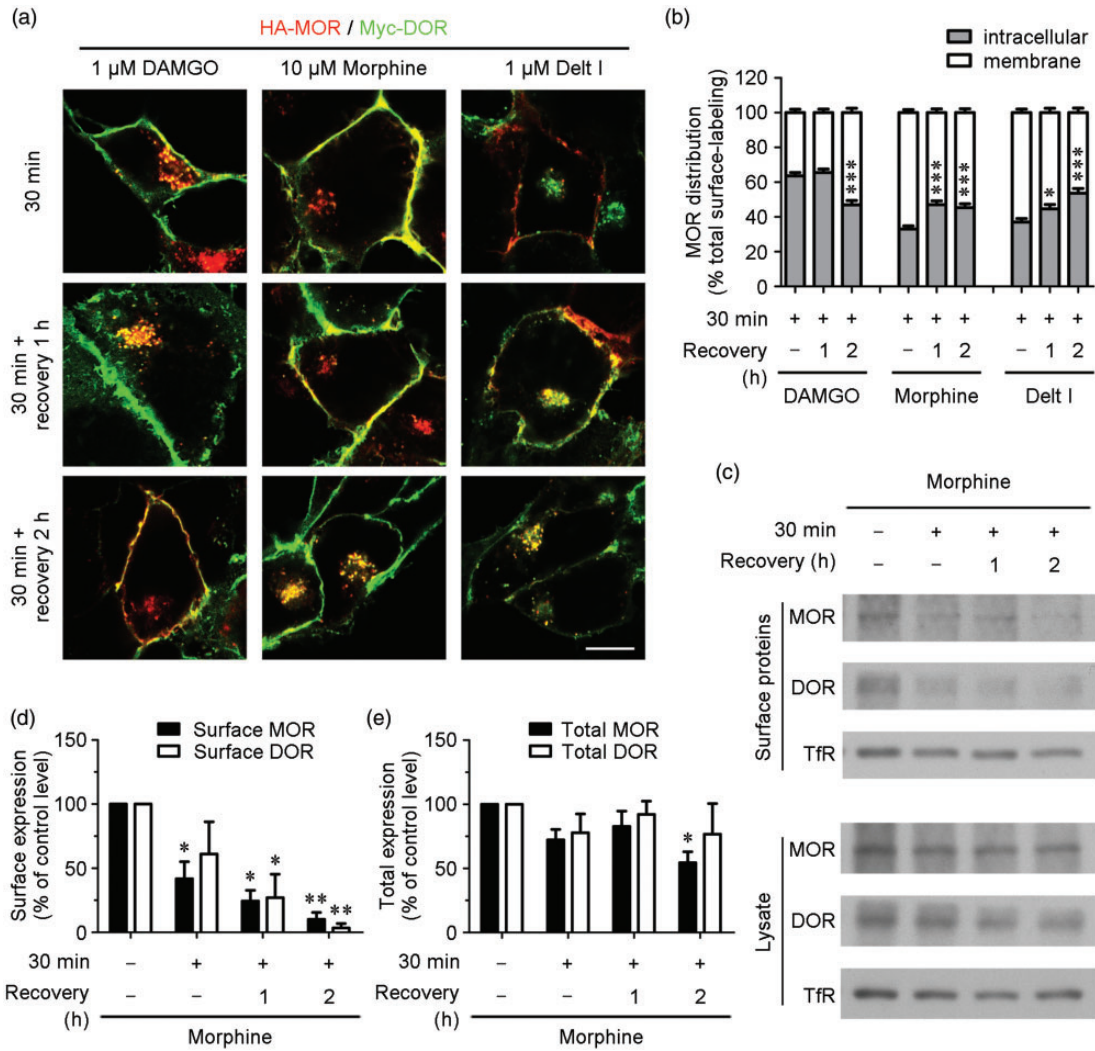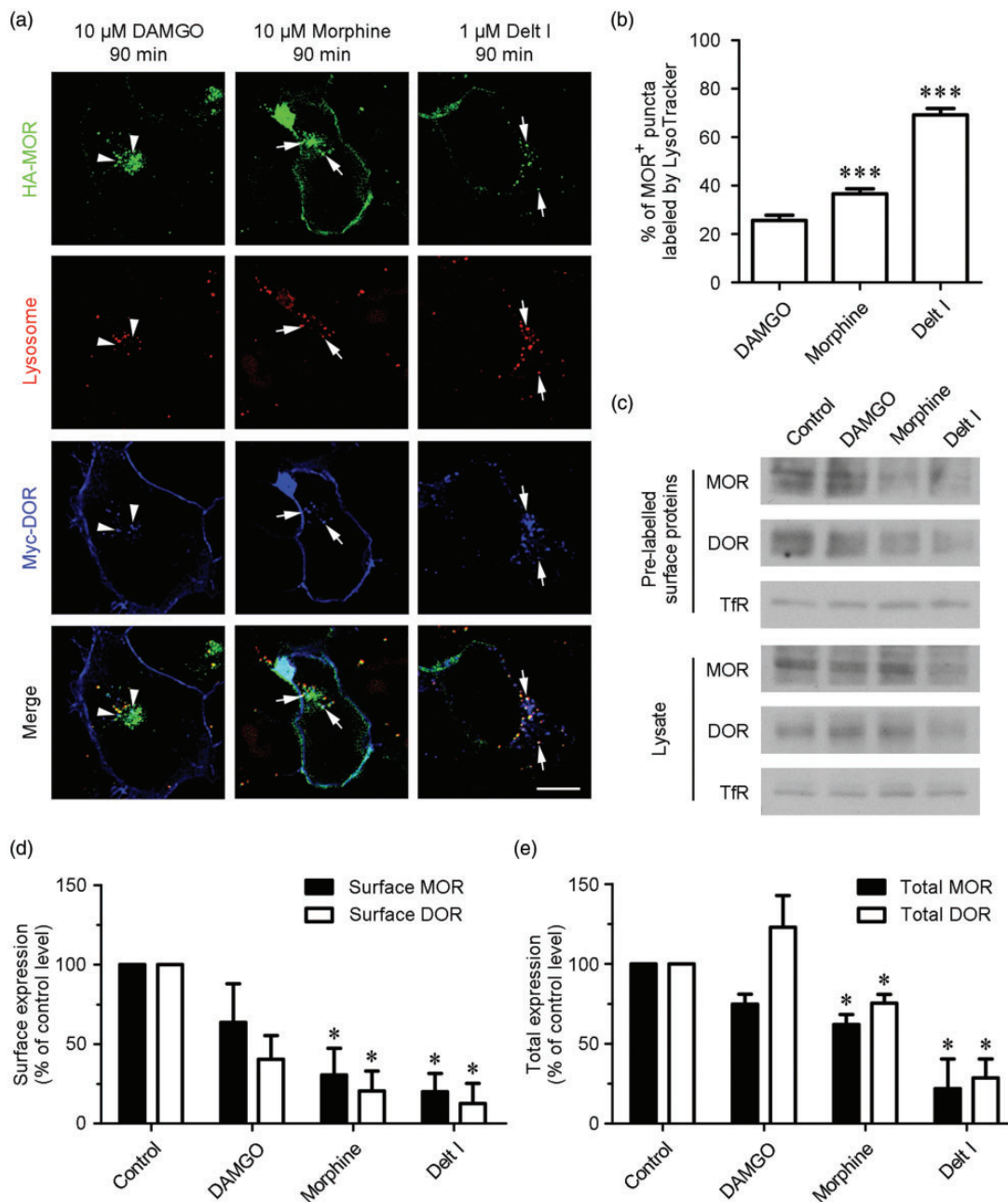
**Figure 3.** MORs internalized by morphine are retained intracellularly with DORs. ((a) and (b)) HA-MOR and Myc-DOR expressed on the cell surface were pre-labeled with antibodies against HA (red) and Myc (green) in living HEK293 cells and then treated with drugs for 30 min and given additional 1 h or 2 h to recover from stimulation (a). Quantitative data of pre-labeled cell-surface MOR distribution were calculated from the intensities of intracellular/membrane immunofluorescence versus total immunofluorescence (b). Compared with DAMGO, morphine-caused internalized MORs were distributed more likely in the intracellular compartment with DORs after recovery similar to Delt I. The results are presented as the mean ± SEM (n = 65–85 cells). Scale bar, 8 μm. *p < 0.05 and ***p < 0.001 versus corresponding group without recovery. ((c)–(e)) Representative immunoblotting (c) and quantitative data ((d) and (e)) showed that in HEK293 cells co-expressing HA-MOR and Myc-DOR, the levels of MORs and DORs on the cell surface were reduced after 30-min treatment with 10 μM morphine and further decreased after additional recovery. TfR served as a control for protein loading. The results are presented as the mean ± SEM (n = 4). *p < 0.05 and ** p < 0.01 versus the corresponding control group.
MOR: μ-opioid receptor; DOR: δ-opioid receptor; DAMGO: D-Ala$^2$, N-MePhe$^4$, Gly-ol-enkephalin; Delt I: deltorphin I; TfR: transferrin receptor.

Myc-DOR. Immunostaining showed that a 30-min treatment with 10 μM morphine, fentanyl, and methadone, but not tramadol, induced an increase of the intracellular punctum structures representative of the internalized MORs with DORs in DRG neurons pre-labeled surface MORs and DORs (Figure 6(a) and (b)), consistent with the results in transfected HEK293 cells (Figures 1(b) and 2(a)–(c)). The cell-surface biotinylation and immunoblotting of surface proteins with the antibodies against

opioid receptors showed that a 30-min treatment with 10 μM morphine reduced the surface MORs and DORs (Figure 6(c) and (d)). The total protein levels of MORs and DORs were unaffected by 10 μM morphine (Figure 6(e)). Furthermore, immunoprecipitation of opioid receptors combined with immunoblotting using antibody against ubiquitin showed that the ubiquitination levels of opioid receptors in DRG tissues were significantly increased 60 min after subcutaneous injection

**Figure 4.** Prolonged morphine treatment causes MOR co-degradation with DORs. ((a) and (b)) HA-MOR and Myc-DOR expressed on the cell surface were pre-labeled with antibodies against HA (green) and Myc (blue) in living HEK293 cells and then treated with drugs and LysoTracker (red) for 90 min. Quantitative data showed the percentage of MOR-positive puncta labeled by LysoTracker (b). In HEK293 cells co-expressing MOR/DOR, a certain extent of internalized MORs were sorted to lysosomes with DORs (arrows) after morphine treatment in contrast to a robust entry induced by Delt I and a weak effect caused by DAMGO. Most of DAMGO-induced co-internalized MORs and DORs did not enter the lysosomes (arrowheads). In order to count the number of MOR-positive puncta, confocal images were performed for deconvolution. The results are presented as the mean $\pm$ SEM (n $=$ 65–85 cells). Scale bar, 8 $\mu$m. ***p $<$ 0.001 versus corresponding group treated with DAMGO. ((c)–(e)) Representative immunoblotting (c) and quantitative data ((d) and (e)) showed that in HEK293 cells co-expressing HA-MOR and Myc-DOR, 10 $\mu$M morphine resulted in a significant decrease of pre-labeled cell-surface MOR/DOR and total MOR/DOR similar to 1 $\mu$M Delt I. TfR served as a control for protein loading. The results are presented as the mean $\pm$ SEM (n $=$ 3). *p $<$ 0.05 and ***p $<$ 0.001 versus the corresponding control group.

MOR: $\mu$-opioid receptor; DOR: $\delta$-opioid receptor; DAMGO: D-Ala[2], N-MePhe[4], Gly-ol-enkephalin; Delt I: deltorphin I; TfR: transferrin receptor.
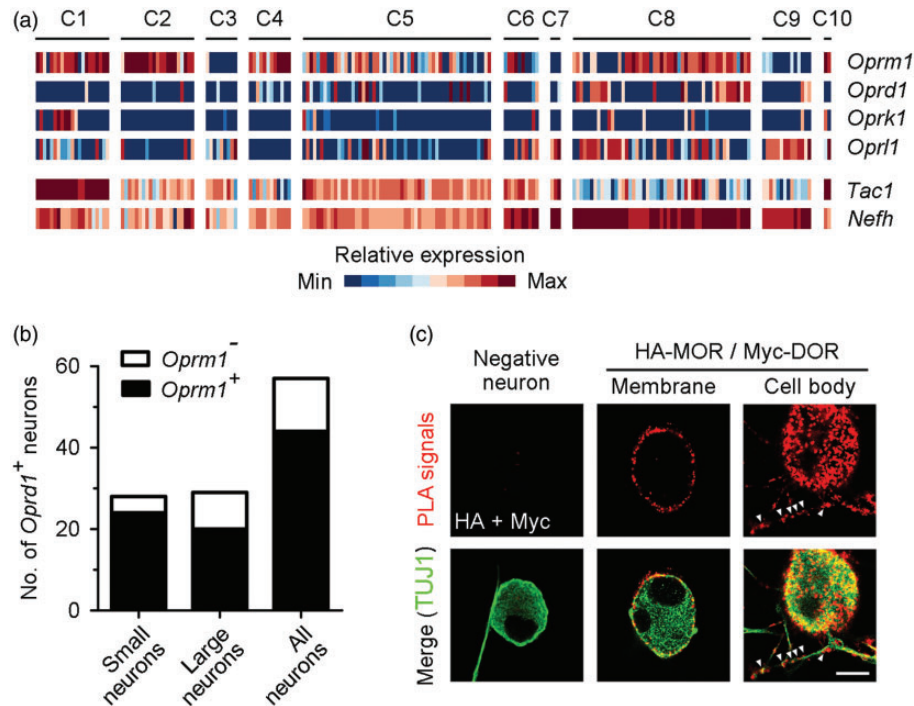
**Figure 5.** *Oprm1* and *Oprd1* are co-expressed in primary sensory neuron. (a) A heatmap from single-cell RNA-sequencing data showed the expression patterns of *Oprm1, Oprd1, Oprk1, Oprl1, Tac1*, and *Nefh* in different mouse DRG neuron types. Relative expression level of individual gene was indicated from minimum (Min) to maximum (Max). Co-expression of *Oprm1* and *Oprd1* dispersedly existed in multiple types of DRG neurons. (b) Quantitative data showing the number of *Oprm1*-positive and negative neurons in the *Oprd1*-expressing DRG neurons. The *Oprd1*-expressing DRG neurons were divided into small neurons (cross-sectional area $< 800$ $\mu m^2$) and large neurons. (c) Interaction of MORs and DORs on the cell membrane and in the cell body and neurites of primary sensory neuron. Cultured DRG neurons were transfected with HA-MOR and Myc-DOR, while negative neurons did not express both. HA-MOR and Myc-DOR on the cell surface were labeled with antibodies against HA and Myc in living DRG neurons and then performed for PLA. HA-MOR and Myc-DOR in the cell body and neurites (arrowheads) were labeled with antibodies against HA and Myc after fixation. PLA signals (red) were generated by probe-linked secondary antibodies to indicate HA-MOR interacted with Myc-DOR. TUJ1 (green) served as a neuronal marker. Scale bar, 10 $\mu$m.

*Oprm1*: opioid receptor mu 1; *Oprd1*: opioid receptor delta 1; *Oprk1*: opioid receptor kappa 1; *Oprl1*: opioid receptor-like 1; *Tac1*: tachykinin 1; *Nefh*: neurofilament, heavy polypeptide; MOR: μ-opioid receptor; DOR: δ-opioid receptor; PLA: proximity ligation assay; TUJ1: β-tubulin III.

of 5 mg/kg morphine (Figure 6(f)), suggesting MORs and DORs in the ubiquitination pathway ready for degradation. Thus, several MOR-targeting opioids including morphine, fentanyl, and methadone also induce MOR co-internalization with DORs in primary sensory neuron.

## MOR-targeting opioid-induced drug tolerance

Finally, we evaluated the correlation of MOR-targeting opioid-induced MOR/DOR co-internalization with analgesic tolerance. Given that the decrease of surface opioid receptors after morphine treatment contributed to drug tolerance, morphine tolerance might be related to either the drug dosage or the time of morphine exposure. We performed the tail immersion test to examine the

morphine-induced antinociception and tolerance during 10 days of drug treatment. The antinociceptive effect of morphine was reduced in mice two to four days after daily subcutaneous injection of 2, 5, and 10 mg/kg morphine, respectively (Figure 7(a)). Therefore, analgesic tolerance could be developed by all these dosages of morphine. However, a strong antinociception of 10 mg/kg morphine might partially compensate for morphine-induced MOR internalization and led to delayed tolerance development. To evaluate the effect of MOR internalization on morphine tolerance, we injected 5 mg/kg morphine twice a day to increase the time of morphine exposure. The tail immersion test showed that the analgesic tolerance induced by daily injection twice with 5 mg/kg morphine appeared earlier than that caused by daily single injection with 5 mg/kg morphine (Figure 7(b)). Thus, the development of morphine tolerance could be accelerated by daily increase in the time of morphine exposure.
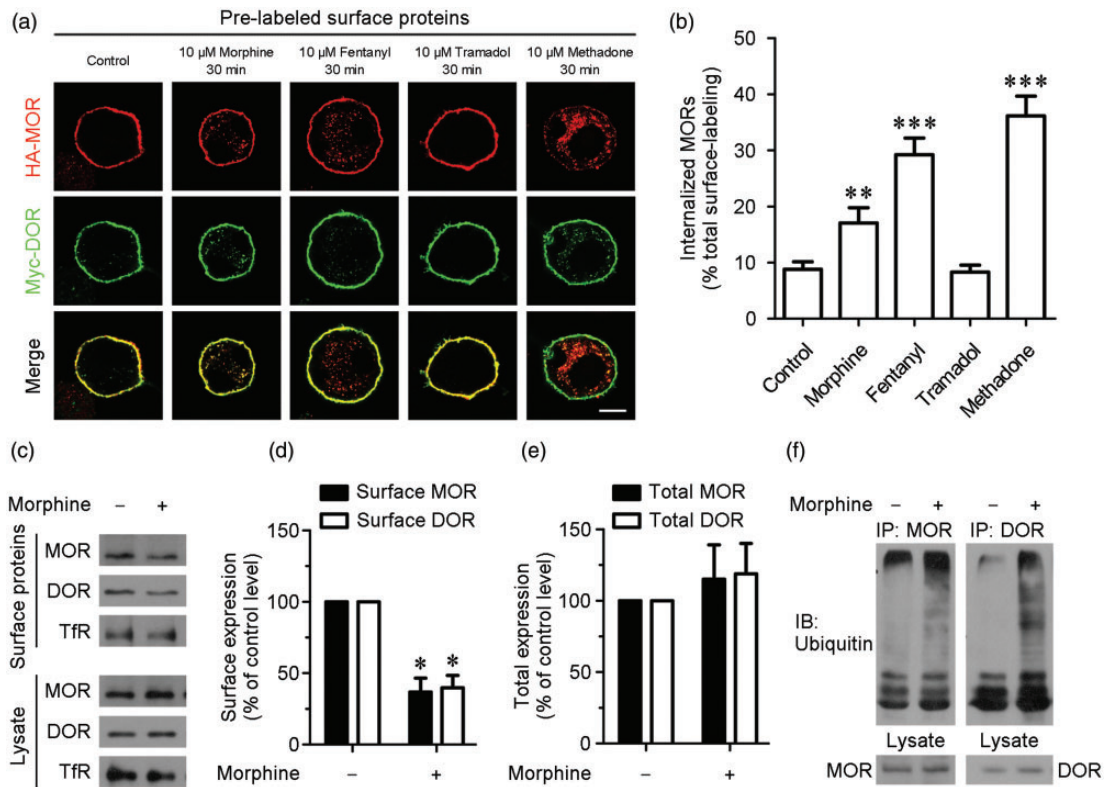
**Figure 6.** Opioids induce MOR co-internalization with DORs in primary sensory neuron. ((a) and (b)) HA-MOR and Myc-DOR expressed on the cell surface were pre-labeled with antibodies against HA (red) and Myc (green) in living DRG neurons and then treated with opioids for 30 min (a). Quantitative data of internalized MORs were calculated from the intensities of intracellular immunofluorescence versus total immunofluorescence (b). In control cells, the pre-labeled MORs and DORs were mainly localized on the cell surface. A 30-min treatment with 10 μM morphine, fentanyl, and methadone, but not tramadol, caused significant MOR co-internalization with DORs. The results are presented as the mean ± SEM (n = 40–45 cells). Scale bar, 10 μm. **p < 0.01 and ***p < 0.001 versus control group. ((c)–(e)) Representative immunoblotting (c) and quantitative data ((d) and (e)) showed that in cultured DRG neurons the levels of MORs and DORs on the cell surface were reduced after 30-min treatment with 10 μM morphine. TfR served as a control for protein loading. The results are presented as the mean ± SEM (n = 3). *p < 0.05 versus corresponding control group. (f) Representative immunoblotting showed that morphine injection (5 mg/kg, s.c.) significantly increased the ubiquitination levels of MORs and DORs in mouse DRG tissues after 60 min. Three independent experiments displayed similar results.
MOR: μ-opioid receptor; DOR: δ-opioid receptor; TfR: transferrin receptor; IP: immunoprecipitation.

We further compared the tolerance development of other clinical MOR-targeting opioids. The antinociceptive effects of 0.2 mg/kg fentanyl and 10 mg/kg methadone were found to be reduced in mice three days after daily subcutaneous injection of drugs similar to that of morphine, while 100 mg/kg tramadol only produced weak analgesic tolerance (Figure 7(c)). Interestingly, the morphine-induced antinociception was also decreased in mice treated with chronic tramadol, methadone, or fentanyl (Figure 7(d)), suggesting that the analgesic tolerance induced by morphine and other MOR-targeting opioids displays cross-tolerance. The cross-tolerance of methadone and morphine was strongest, while the effect between tramadol and morphine was relatively weak that maybe due to the slight decline of tramadol antinociception during chronic administration (Figure 7(d)). Taken together, MOR-targeting

opioids display drug tolerance correlated with the level of drug-induced MOR co-internalization with DORs.

## Discussion

Opioid receptors are the major targets of clinical opioid analgesics, including morphine, fentanyl, methadone, and tramadol. The present study reported the morphine-induced MOR co-internalization and co-degradation with DORs in cells expressing the MOR/DOR heteromers. Fentanyl and methadone also caused significant MOR co-internalization with DORs in cells expressing MOR/DOR heteromers similar to morphine. However, tramadol hardly induced this effect. Moreover, daily application of morphine, fentanyl, and methadone resulted in dramatic drug tolerance in mice, while daily administration of tramadol could
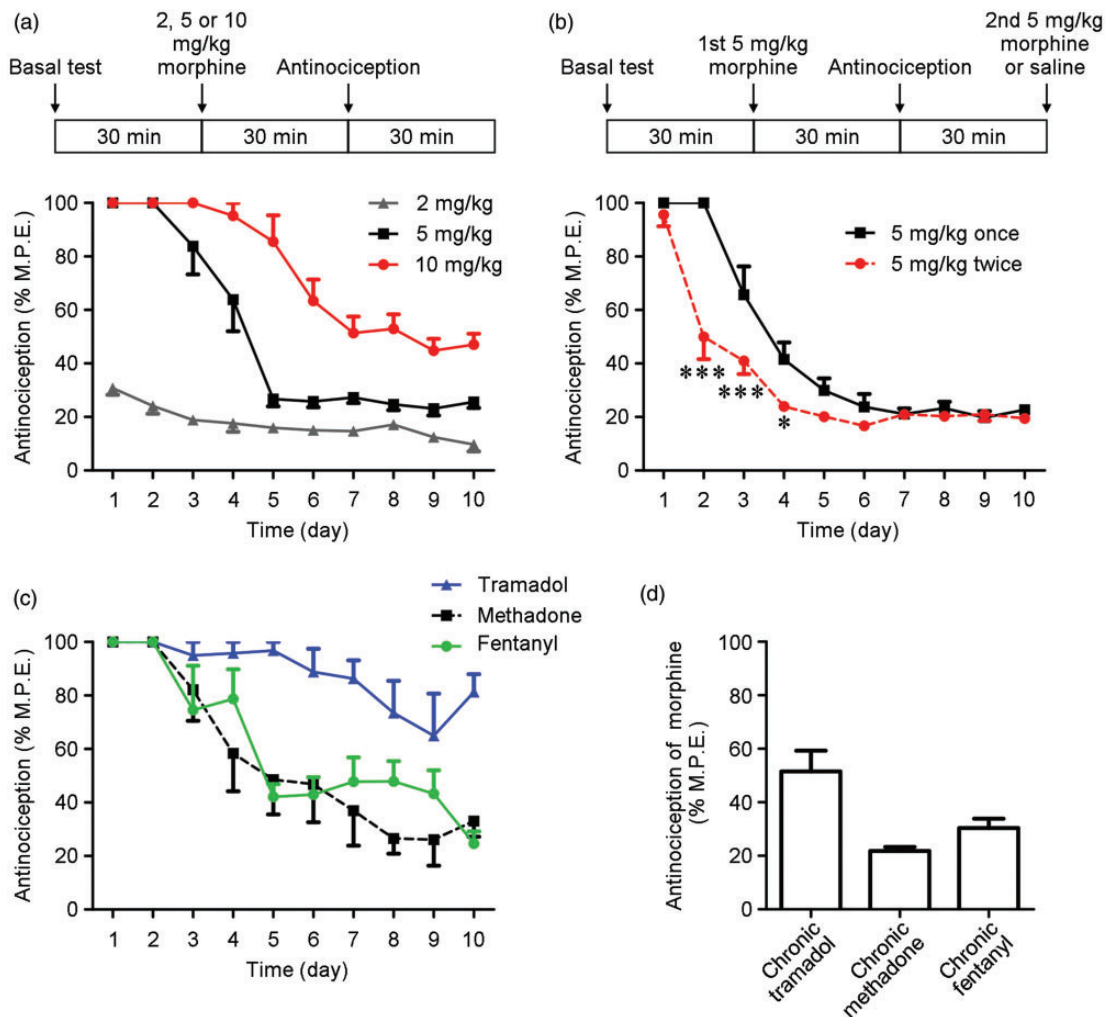
**Figure 7.** Clinical MOR-targeting opioids induce analgesic tolerance. Adult male mice were administrated daily with opioids (s.c.) for 10 days, and the antinociception was detected 30 min after daily injection through tail immersion test at 52°C. (a) During 10-day administration of daily 2 mg/kg (n = 6), 5 mg/kg (n = 6), or 10 mg/kg (n = 7) morphine (single injection), the antinociceptive effect was reduced and the analgesic tolerance was induced. (b) Application of daily 5 mg/kg morphine twice (n = 11) caused analgesic tolerance earlier than that induced by the treatment with daily 5 mg/kg morphine once (n = 11). The antinociceptive effect was detected 30 min after first injection. *$p < 0.05$ and ***$p < 0.001$ versus corresponding group of the treatment with daily 5 mg/kg once (two-way ANOVA). (c) Administration of daily 10 mg/kg methadone (n = 6) or 0.2 mg/kg fentanyl (n = 6) also caused analgesic tolerance similar to morphine, but the treatment with daily 100 mg/kg tramadol (n = 6) only produced weak analgesic tolerance. (d) The morphine-induced antinociception (5 mg/kg) was also attenuated in mice pre-treated with tramadol (n = 6), methadone (n = 6), or fentanyl (n = 5) for 10 days (chronic). The cross-tolerance between methadone and morphine was strongest, while tramadol showed the weakest cross-tolerance with morphine. The results are presented as the mean ± SEM.

keep most of drug efficiency. Different MOR-targeting opioids displayed cross-tolerance. This study provides a correlation of MOR/DOR co-internalization with drug tolerance caused by clinical MOR-targeting opioids.

## Clinical MOR-targeting opioids induce MOR co-internalization and co-degradation with DORs

Previous studies report that DAMGO induces robust MOR internalization.[29,41] However, the morphine-induced MOR internalization remains controversy. Application of 5 mg/kg morphine (s.c.) led to a pronounced increase of intracellular puncta in the processes of rat nucleus accumbens neurons.[27] Inconsistently, in layer II neurons of the rat parietal cortex, injection of 40 mg/kg morphine (s.c.) did not cause MOR translocation from cell surface through permeable immunostaining of brain slice.[28] In HEK293 cells stably expressing epitope-tagged MORs, immunostaining detected unchanged distribution of total MORs after a 10-min treatment with 10 μM morphine.[29] In the present

study, a 30-min treatment with morphine induced the internalization of pre-labeled cell-surface MORs in HEK293 cells expressing MOR alone or MOR/DOR in a dose-dependent manner. Co-expression of DORs enhanced the internalization of cell-surface MORs induced by morphine in HEK293 cells. This phenomenon was also observed in primary sensory neurons co-expressing MOR/DOR. These data provide evidences to support that morphine could also induce MOR internalization especially remarkable in cells and neurons co-expressing MOR/DOR. The DOR interaction with MORs may change the conformation of MORs vulnerable to be internalized; therefore, morphine causes more MOR internalization in MOR/DOR heteromers than MORs alone. Controversy results of morphine-induced internalization among different studies may result from the differences of experimental conditions including treatment duration and cell situation, and the sensitivity of detection methods. Pre-labeling of cell-surface receptor in living cells provides more sensitive detection for the MOR internalization induced by morphine.

After endocytosis, MORs and DORs generally choose different trafficking pathways: internalized MORs activated by DAMGO can be resensitized and recycled back to cell membrane; DADLE, a specific DOR agonist, induces DOR internalization and further entry to lysosomes for degradation.[17,42] These works were performed in the condition of MOR or DOR expression alone, but in vivo MOR/DOR co-expression exists extensively. Previous study of our laboratory had found that when co-expressing MORs and DORs, Delt I (1 μM) could cause the MOR/DOR co-degradation but DAMGO (1 μM) could not induce such an effect.[21] So we further investigated the post-endocytic trafficking of the co-internalized MORs and DORs induced by 10 μM morphine. In HEK293 cells co-expressing MOR/DOR, morphine-caused internalized MOR/DOR heteromers were partially sorted into lysosomes and significantly reduced subsequently, indicating receptor entry to the degradation pathway, while DAMGO-induced internalized MORs more likely recycled back to cell membrane, which might result from the difference of receptor conformation change and recruited downstream effectors after agonist stimulation. Surprisingly, treatment of 10 μM DAMGO also led to a degree of surface MOR/DOR decrease which might be due to high drug concentration. Downregulation of MORs certainly results in a reduction of receptor function.

Clinical MOR-targeting opioids also include fentanyl, methadone, and tramadol. Previous study has detected the internalization of MORs induced by fentanyl and methadone in transfected cells expressing MOR alone.[43] In the present study, fentanyl and methadone were further detected to cause MOR internalization

in HEK293 cells and primary sensory neurons co-expressing MOR/DOR. The MOR internalization induced by fentanyl and methadone was more pronounced than that by morphine. Tramadol hardly resulted in MOR internalization in HEK293 cells and primary sensory neurons co-expressing MOR/DOR. Therefore, MOR-targeting opioids display different patterns of drug-induced MOR translocation.

The internalization of GPCR is initiated with phosphorylation of receptor by kinases and then processed to recruit arrestins.[16] Notably, the different ability of opioids to cause MOR internalization is related to the effect of drugs on receptor phosphorylation by GPCR kinase.[44] Meanwhile, the receptor phosphorylation and internalization induced by MOR-targeting opioids are both correlated with β-arrestin2 binding.[43] Recruitment of various kinases and arrestins by clinical MOR-targeting opioids may explain their difference on MOR internalization.

## Clinical MOR-targeting opioids cause drug tolerance

Morphine tolerance involves the modulation of opioid receptors through several levels of nociceptive circuits.[1,45] The previous study suggests that MORs expressed in nociceptive neurons are responsible for the morphine-induced hyperalgesia and tolerance.[46] The present study supports that co-expression of DORs enhances the MOR internalization induced by morphine.

Co-existence of MORs and DORs in DRG neurons was previously shown by immunostaining, single-cell PCR and electrophysiological methods.[3,40] Further analysis from our single-cell RNA-sequencing data[35] showed co-expression of *Oprm1* and *Oprd1* distributed dispersedly in various types of DRG neurons. The relatively small number of *Oprd1*-positive neurons in our sequencing data might be caused by the low expression levels of *Oprd1* and the relatively low efficiency of *Oprd1* amplification. Meanwhile, we found *Tac1*, previously representative of peptidergic, small DRG neurons, and *Nefh*, traditional marker of large DRG neurons, both exist in multiple types of primary sensory neurons. *Oprm1/Oprd1* co-expression in DRG neurons provides a prerequisite for MOR co-internalization with DORs after opioid treatment.

Drug tolerance has been explained by receptor downregulation, loss of receptor activity, and super-activation of downstream effectors.[17] The previous study assumes that morphine fails to promote MOR internalization, leading to prolonged MOR signaling that causes cAMP super-activation and cellular tolerance,[47] while the confirmation of morphine-induced MOR

internalization did not give a support for this hypothesis. Notably, the mutant D-MOR (degrading MOR, the C-terminal of MOR replaced by that of DOR) knock-in mice exhibit MOR down-regulation and a more rapid onset of morphine tolerance.[48] Similarly, the DOR interaction with MORs enhances the dynamic of MORs and guides opioid-induced internalization and degradation of MORs. Down-regulation of cell-surface MORs decreases receptor availability for drugs, leading to the reduction of morphine efficacy that is regarded as opioid tolerance. Unfortunately, the change in total MORs remains uncertain after chronic morphine treatment[2] and needs to be extensively investigated.

Clinical MOR-targeting opioids show different pharmacological properties including drug efficacy, drug metabolism, and drug interaction.[49] The behavior test in the present study showed that these opioids also displayed different levels of analgesic tolerance. Methadone and fentanyl induced drug tolerance similar to morphine after chronic drug treatment, and tramadol only produced weak analgesic tolerance. Accordingly, methadone and fentanyl caused MOR internalization similar to morphine after the drug treatment, but tramadol barely led to MOR internalization. Different effects of these opioids on MOR internalization may be linked to their analgesic tolerance. Tramadol did not induce MOR internalization but still produced weak tolerance, suggesting other pathway causing drug tolerance than receptor down-regulation. Furthermore, the cross-tolerance of morphine with other opioids infers that these drugs may share common mechanisms of tolerance.

## Authors' Contributions

FB performed the immunostaining, immunoblotting, and behavioral experiments. C-LL provided the single-cell RNA-sequencing data. X-QC and Y-JL helped to confirm the results of immunoblotting experiments. FB, LB, and XZ designed experiments and wrote the manuscript.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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