

# Facilitation of $\mu$ -Opioid Receptor Activity by Preventing $\delta$ -Opioid Receptor-Mediated Codegradation

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## SUMMARY

$\delta$ -opioid receptors (DORs) form heteromers with  $\mu$ -opioid receptors (MORs) and negatively regulate MOR-mediated spinal analgesia. However, the underlying mechanism remains largely unclear. The present study shows that the activity of MORs can be enhanced by preventing MORs from DOR-mediated codegradation. Treatment with DOR-specific agonists led to endocytosis of both DORs and MORs. These receptors were further processed for ubiquitination and lysosomal degradation, resulting in a reduction of surface MORs. Such effects were attenuated by treatment with an interfering peptide containing the first transmembrane domain of MOR (MOR<sup>TM1</sup>), which interacted with DORs and disrupted the MOR/DOR interaction. Furthermore, the systemically applied fusion protein consisting of MOR<sup>TM1</sup> and TAT at the C terminus could disrupt the MOR/DOR interaction in the mouse spinal cord, enhance the morphine analgesia, and reduce the antinociceptive tolerance to morphine. Thus, dissociation of MORs from DORs in the cell membrane is a potential strategy to improve opioid analgesic therapies.

## INTRODUCTION

G protein-coupled receptors (GPCRs) are known to form heteromers that may modulate the physiological and pharmacological functions of GPCRs (Gurevich and Gurevich, 2008). Functional association between  $\mu$ - and  $\delta$ -opioid receptors (MORs and DORs), two members of the GPCR superfamily, was first suggested by pharmacological studies showing that MOR activity could be modulated by DOR ligands (Lee et al., 1980; Schiller et al., 1999). The heteromers of MORs and DORs were identified in both cotransfected cells and membranes prepared from the spinal cord (Daniels et al., 2005; Fan et al., 2005; Gomes et al.,

2004; Jordan and Devi, 1999). In the lamina I–II of spinal cord, the agonist-binding sites and immunoreactivity of DORs are located in the afferent fibers of small dorsal root ganglion (DRG) neurons, and these presynaptic DORs mediate the inhibitory effects of opioid peptides released from spinal dorsal horn neurons (Besse et al., 1992; Cesselin et al., 1989; Mennicken et al., 2003; Minami et al., 1995; Zhang et al., 1998a). MORs, which are a major target of opioid analgesics such as morphine, are also expressed in small DRG neurons and might be coexpressed with DORs (Ji et al., 1995; Rau et al., 2005). Interestingly, MOR-mediated analgesia can be enhanced by pharmacologically blocking DORs, preventing DOR phosphorylation, and genetically deleting either the exon 2 of DOR1 gene (*Oprd1*) or the preproenkephalin gene (Chefer and Shippenberg, 2009; Gomes et al., 2004; Nitsche et al., 2002; Schiller et al., 1999; Standifer et al., 1994; Xie et al., 2009; Zhu et al., 1999). Therefore, DORs could negatively regulate MOR activity in the spinal cord and may be involved in  $\mu$ -opioid antinociceptive tolerance.

The cellular basis for the opioid receptor interaction was questioned because a DOR1 protein fused with the enhanced green fluorescence protein (DOR1-EGFP) could not be detected in MOR-containing small DRG neurons (Scherrer et al., 2009). Recently, the coexistence of MORs and DORs in small DRG neurons has been shown using multiple approaches, such as single-cell PCR, in situ hybridization combined with immunostaining and electrophysiological recording (Wang et al., 2010). Additionally, Gupta et al. (2010) detected the receptor heteromers in DRG neurons using antibodies that recognize MOR/DOR heteromers. Given that the receptor coexistence is a cellular basis for the modulation of opioid analgesia, the mechanisms underlying the DOR-mediated modulation of the MOR activity in the pain pathway remain largely unclear.

Cell biological studies have shown that GPCRs activated by selective agonists are often internalized and processed in either the recycling pathway for resensitization or the degradation pathway that leads to receptor downregulation (Trapaidze et al., 2000; Tsao and von Zastrow, 2000). It has been proposed that heteromerized GPCRs may traffic via a postinternalization pathway that is different to the one used when they are expressed alone. Therefore, receptor heteromerization may serve as a regulatory mechanism for controlling receptor availability

(Jordan et al., 2001; Pfeiffer et al., 2003; van Rijn et al., 2010). It has been shown that internalized MORs and DORs are differentially processed in postendocytotic pathways. Internalized MORs are mainly recycled to the cell surface and re-sensitized after treatment with a MOR-specific agonist, [D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin (DAMGO) (Arden et al., 1995; Finn and Whistler, 2001; Law et al., 2000). In contrast, internalized DORs were mainly found to be located in the lysosomal compartments for degradation after treatment with DOR agonists (Hislop et al., 2009; Trapaidze et al., 1996; Tsao and von Zastrow, 2000). Agonist-induced receptor phosphorylation and ubiquitination (Hicke, 1997; Katzmann et al., 2002) were reported to be involved in the endocytosis and downregulation of opioid receptors (Finn and Whistler, 2001; Hislop et al., 2009). MOR/DOR heteromers were also reported to recruit  $\beta$ -arrestin, which modified the dynamics of opioid-mediated extracellular signal-regulated kinase (ERK) activation (Rozenfeld and Devi, 2007). However, whether postendocytotic trafficking of MORs can be modulated by DORs remains to be examined. Furthermore, if DORs and MORs were colocalized in sensory afferent fibers, it would be interesting to explore the physical interaction and functional correlation between these two types of opioid receptors *in vivo*.

The aim of the present study was to investigate the postendocytotic process of the MOR/DOR complex after agonist stimulation and its correlation with the DOR-mediated negative regulation of MOR-mediated spinal analgesia. We found that the activation of DORs in the MOR/DOR complex could target MORs into the postendocytotic degradation pathway, resulting in MOR desensitization. Furthermore, morphine analgesia could be facilitated by disrupting the MOR/DOR interaction with an interfering peptide that corresponds to the first transmembrane domain (TM1) of MOR fused with the TAT peptide, which is the cell membrane transduction domain of the human immunodeficiency virus and used as a cell-penetrating vector to deliver small cargos or large molecules (Schwarze et al., 1999). Therefore, physical disassociation of MORs from DORs could be a strategy to enhance MOR-mediated analgesia.

## RESULTS

### Cointernalization of MORs and DORs following Agonist Treatment

To assess whether MOR trafficking could be modulated by activation of DORs, we examined the distribution and translocation of MORs and DORs in human embryonic kidney 293 (HEK293) cells that were cotransfected with plasmids expressing MOR with an N-terminal hemagglutinin (HA) tag (HA-MOR) and DOR with an N-terminal Myc tag (Myc-DOR). Because HA and Myc were tagged at the N termini of MOR and DOR, respectively, and exposed to the extracellular space following insertion of the receptors into the plasma membrane, HA-MOR and Myc-DOR on the cell surface of living cells could be pre-labeled using rabbit anti-HA and mouse anti-Myc antibodies. Under control conditions, the pre-labeled DORs and MORs were mainly present on the surface of the double-transfected HEK293 cells (Figure 1A). Interestingly, after a 30 min treatment with the selective DOR agonists deltorphin (Delt) I, Delt II, or (+)-4-[( $\alpha$ R)- $\alpha$ -(2S,5R)-4-Allyl-2,5-dimethyl-1-piperaziny]-3-me-

thoxybenzyl]-N,N-diethylbenzamide (SNC80) (1  $\mu$ M), the pre-labeled DORs and MORs were cointernalized and colocalized in the same vesicular structures (Figure 1A). When DAMGO (1  $\mu$ M), a selective MOR agonist, was applied for 30 min, the cointernalization of pre-labeled MORs and DORs was also observed in the double-transfected HEK293 cells (Figure 1A). The reaction induced by Delt I or DAMGO could be abolished using the DOR antagonist naltrindole (NTI) or the MOR antagonists naloxone and D-Phe-Cys-Tyr-D-Trp-Om-Thr-Pen-Thr-NH<sub>2</sub> (CTOP) (Figure 1A), indicating that the receptor cointernalization is induced in a receptor-specific manner.

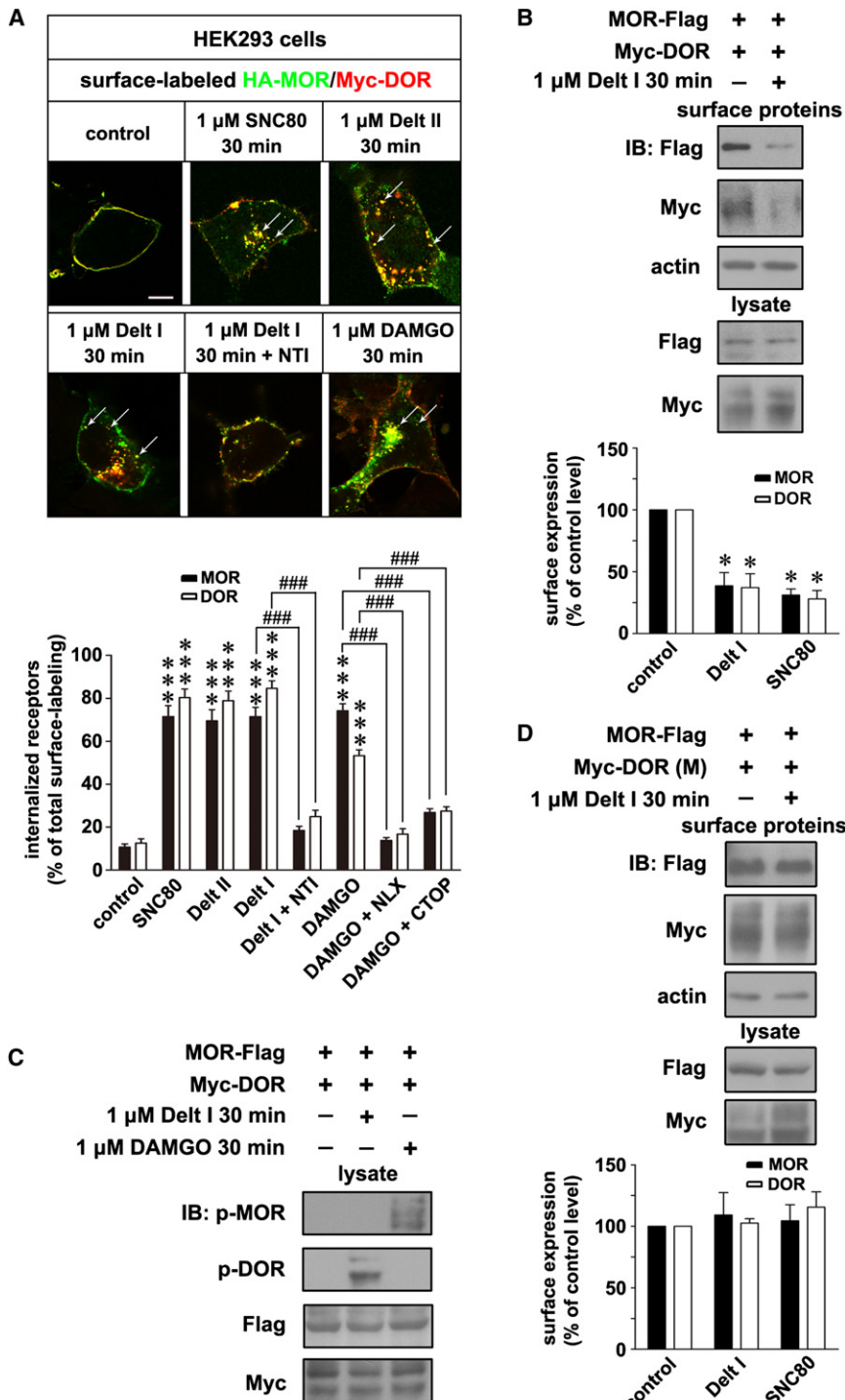
DOR-mediated cointernalization of DORs and MORs was further confirmed by direct measurement of the amount of receptors present on the cell surface using immunoblotting. After agonist treatment, the proteins that remained on the surface of HEK293 cells cotransfected with the plasmids expressing Myc-DOR and MOR fused with a Flag tag at the C terminus (MOR-Flag) were biotinylated and precipitated with immobilized streptavidin. Treatment with Delt I or SNC80 led to a marked reduction of both MORs and DORs on the cell surface (Figure 1B and see Figure S1A available online). These results indicate that a receptor-selective agonist can induce the cointernalization of both types of opioid receptors.

Receptor phosphorylation is involved in  $\delta$ -opioid peptide-induced DOR internalization and DAMGO-induced MOR internalization (Pak et al., 1999; Whistler et al., 2001). We observed that receptor-specific phosphorylation was involved in the agonist-induced cointernalization of MORs and DORs. In HEK293 cells coexpressing Myc-DOR and MOR-Flag, immunoblotting showed that treatment with Delt I (1  $\mu$ M) or SNC80 (5  $\mu$ M) for 30 min selectively enhanced DOR phosphorylation, while DAMGO (1  $\mu$ M) selectively increased MOR phosphorylation (Figures 1C and S1B). Thus, receptor-selective agonists specifically induce phosphorylation of the corresponding type of opioid receptor. This result also suggests that the DOR agonist-induced cointernalization of MORs and DORs is not due to a cross-reaction of the agonist or to a transphosphorylation of MORs by activation of DORs.

The role of the phosphorylation and internalization of DORs in the cointernalization of MORs was further evaluated by coexpressing MOR-Flag with a Myc-tagged, phosphorylation-deficient DOR mutant [Myc-DOR (M)] in which all serine and threonine residues (T352, T353, T358, T361, and S363) in the C terminus were mutated to alanine residues (Whistler et al., 2001). In Myc-DOR (M) and MOR-expressing HEK293 cells, neither surface Myc-DOR (M) nor surface MORs were internalized following a Delt I or SNC80 (1  $\mu$ M) treatment for 30 min (Figures 1D and S1C). These results confirm that activated DORs are required for cointernalization of MORs.

### Targeting Surface MORs into the Degradation Pathway by Activation of DORs

Next, we determined the postendocytotic fate of MORs cointernalized with DORs. Using triple-immunofluorescence staining in MOR- and DOR-expressing HEK293 cells, we observed that a 90 min treatment with Delt I (1  $\mu$ M), but not DAMGO (1  $\mu$ M), significantly increased the localization of MORs in lysosome-like compartments that were labeled using a LysoTracker probe



**Figure 1. Receptor-Specific Agonist Induces Cointernalization of MORs and DORs**

(A) Plasmids expressing HA-MOR and Myc-DOR were cotransfected into HEK293 cells. The HA-MORs and Myc-DORs present on the cell surface were labeled with antibodies against HA (in green) or Myc (in red), respectively. In the control cells, the prelabeled MORs and DORs were mainly localized on the cell surface. After a 30 min treatment with 1  $\mu$ M SNC-80, Delt I, Delt II, or DAMGO, the prelabeled MORs and DORs were internalized and colocalized in vesicular structures (arrows). The Delt I- and DAMGO-induced cointernalization of DORs and MORs could be attenuated by 5  $\mu$ M NTI, naloxone (NLX), or CTOP, respectively. The quantitative data show the ratio of the staining for internalized MORs or DORs versus the total surface immunostaining. Results are presented as mean  $\pm$  SEM. \*\*\*p < 0.001 versus the control; ###p < 0.001 versus indicated group. Scale bar, 8  $\mu$ m.

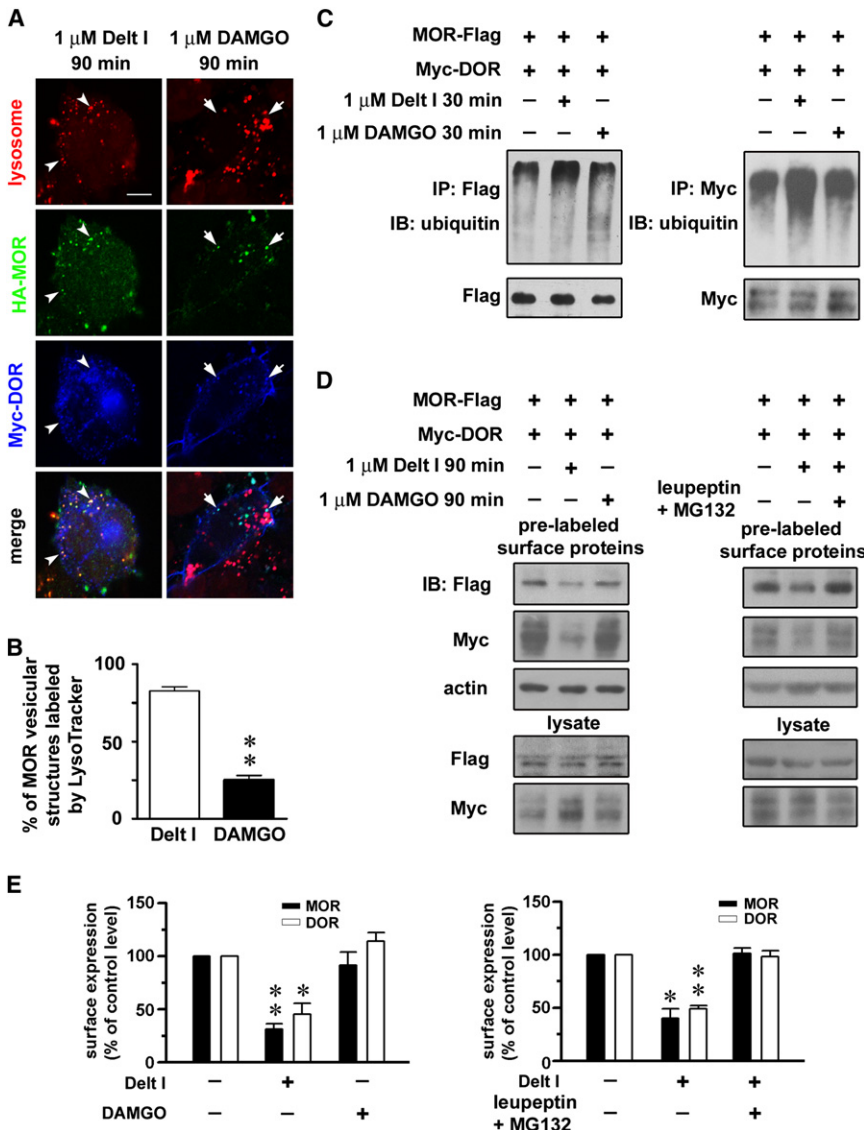
(B and C) The immunoblotting (IB) shows that in HEK293 cells coexpressing Myc-DOR and MOR-Flag, the levels of MORs and DORs on the cell surface were reduced after a 30 min treatment with 1  $\mu$ M Delt I or SNC80 (B). A 30 min treatment with 1  $\mu$ M Delt I increases the phosphorylation of DORs but does not affect that of MORs. Treatment with 1  $\mu$ M DAMGO only increased the phosphorylation of MORs (C). Three independent experiments showed similar results. Results are presented as mean  $\pm$  SEM. \*p < 0.05 versus the control.

(D) In HEK293 cells expressing MOR-Flag and the phosphorylation-deficient DOR mutant (Myc-DOR (M)), the presence of receptors on the cell surface was not reduced after Delt I or SNC80 treatment. Results are presented as mean  $\pm$  SEM. See also Figure S1.

We further examined whether the cointernalized MORs were degraded. The surface proteins of transfected HEK293 cells were biotinylated before drug treatment. DORs and MORs that remained intact in the biotinylated proteins were examined 90 min after treatment with 1  $\mu$ M of Delt I or DAMGO. We observed that the amount of biotinylated DORs and MORs was significantly reduced following the Delt I treatment, but no significant changes were observed following the DAMGO treatment (Figures 2D and 2E).

(Figures 2A and 2B). An immunoprecipitation (IP) experiment showed that, in HEK293 cells coexpressing MOR-Flag and Myc-DOR, a 30 min treatment with Delt I (1  $\mu$ M) resulted in a marked increase in the ubiquitination of both MORs and DORs, whereas DAMGO (1  $\mu$ M) did not noticeably change the ubiquitination level of both MORs and DORs (Figure 2C).

DOR agonist-induced receptor degradation is known to be sensitive to inhibitors of lysosomal proteolysis (Tsao and von Zastrow, 2000) and MG132 (Tanowitz and von Zastrow, 2002), a compound that inhibits a number of proteasome-associated proteases and potently suppresses the effect of various cysteine proteases and cathepsins. We did not observe any



**Figure 2. Activation of DORs Targets Surface MORs into Degradation Pathway**

(A and B) The HA-MOR and Myc-DOR present on the cell surface of cotransfected HEK293 cells were pre-labeled with antibodies against HA (in green) or Myc (in blue), and the lysosome-like compartments were labeled using LysoTracker (in red). After a 90 min treatment with 1  $\mu$ M Delt I, the internalized DORs and MORs were mainly colocalized in the LysoTracker-labeled compartments (arrowheads). After a 90 min treatment with 1  $\mu$ M DAMGO, the most vesicular structures containing the internalized DORs and MORs were not labeled by LysoTracker (arrows) (A). The quantitative data show the percentage of MOR-positive vesicular structures labeled by LysoTracker (B). Results are presented as mean  $\pm$  SEM. \*\* $p < 0.01$ ,  $n = 41$ . Scale bar, 8  $\mu$ m. (C) The immunoprecipitation (IP) experiment showed that ubiquitination of MORs and DORs in HEK293 cells cotransfected with plasmids expressing MOR-Flag and Myc-DOR was enhanced by a 30 min treatment with 1  $\mu$ M Delt I. However, it was not enhanced by 1  $\mu$ M DAMGO. (D and E) HEK293 cells expressing Myc-DOR and MOR-Flag were surface-biotinylated before drug treatment. Immunoblotting showed that the amount of biotinylated DORs and MORs was significantly reduced after 90 min treatment with 1  $\mu$ M Delt I (D). Incubation of the cells with 100  $\mu$ M leupeptin and 10  $\mu$ M MG132 prevented the Delt I-induced MOR/DOR degradation (D). The quantitative data are plotted as a percentage of the control (E). Results are presented as mean  $\pm$  SEM. \*\* $p < 0.01$  and \* $p < 0.05$  versus the untreated cells ( $n = 3$ ).

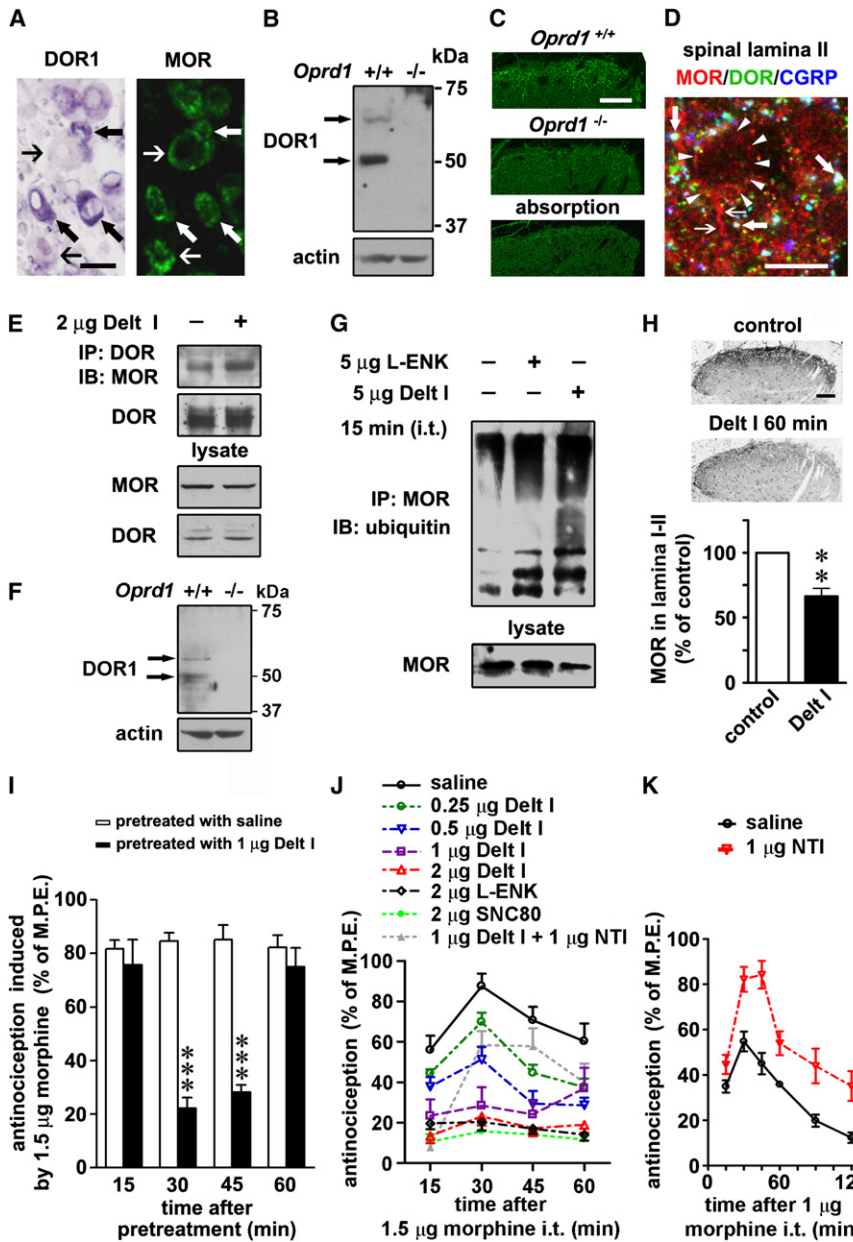
Delt I-induced reduction of DORs and MORs when using a 4 hr pretreatment with a mixture of MG132 (10  $\mu$ M) and leupeptin (100  $\mu$ M), a lysosomal protease inhibitor (Figures 2D and 2E). Our results indicate that the cointernalized MORs and DORs are targeted to lysosomes for degradation.

### DOR-Mediated Downregulation of MORs in the Spinal Cord and Attenuation of Morphine Analgesia

Both DOR binding sites and immunoreactivity were found to be located in the afferent fibers of the lamina I-II of the spinal cord (Besse et al., 1992; Mennicken et al., 2003; Zhang et al., 1998a), which is enriched in MOR-containing afferent fibers and local neurons (Zhang et al., 1998b). Using in situ double-hybridization, we found that a large fraction of MOR-positive small DRG neurons (79%,  $n = 643$ ) expressed DOR1 (Figure 3A). This result is consistent with our recent report (Wang et al., 2010). DOR1<sup>3-17</sup> antiserum primarily recognizes DORs, as demon-

strated by the detection of Myc-DOR1 expressed in HEK293 cells (Figure S2A) and the lack of DOR-immunoblots in extracts of spinal cords from *Oprd1* exon 1-deleted mice (Figure 3B). DOR1 could be detected in the spinal cord of wild-type mice. Moreover, the DOR-immunostaining pattern in the lamina I-II of the mouse spinal cord could be abolished in *Oprd1* exon 1-deleted mice and after antiserum preabsorption with the immunogenic peptide (10<sup>-6</sup> M) (Figure 3C). Triple-immunofluorescence staining showed that MOR/DOR-containing nerve terminals were frequently found in the lamina I-II of the spinal cord and that many of them immunostained for the calcitonin gene-related peptide (CGRP) (Figure 3D), which is a marker of peptidergic afferent fibers. In addition, a number of MOR-positive neurons and dendrites were found in the spinal lamina II (Figure 3D). Thus, coexistence of MORs and DORs in sensory afferent fibers provides a cellular basis for the MOR/DOR interaction in the dorsal spinal cord.

Coimmunoprecipitation (coIP) showed that the MOR/DOR interaction occurred in the spinal dorsal horn of mice and that it was enhanced by intrathecal injection (i.t.) of Delt I (2  $\mu$ g) for 15 min (215.2%  $\pm$  23.0% of control,  $p < 0.01$ ,  $n = 5$ ) (Figure 3E). The specificity of the antibodies against DOR1<sup>1-60</sup> used for IP was

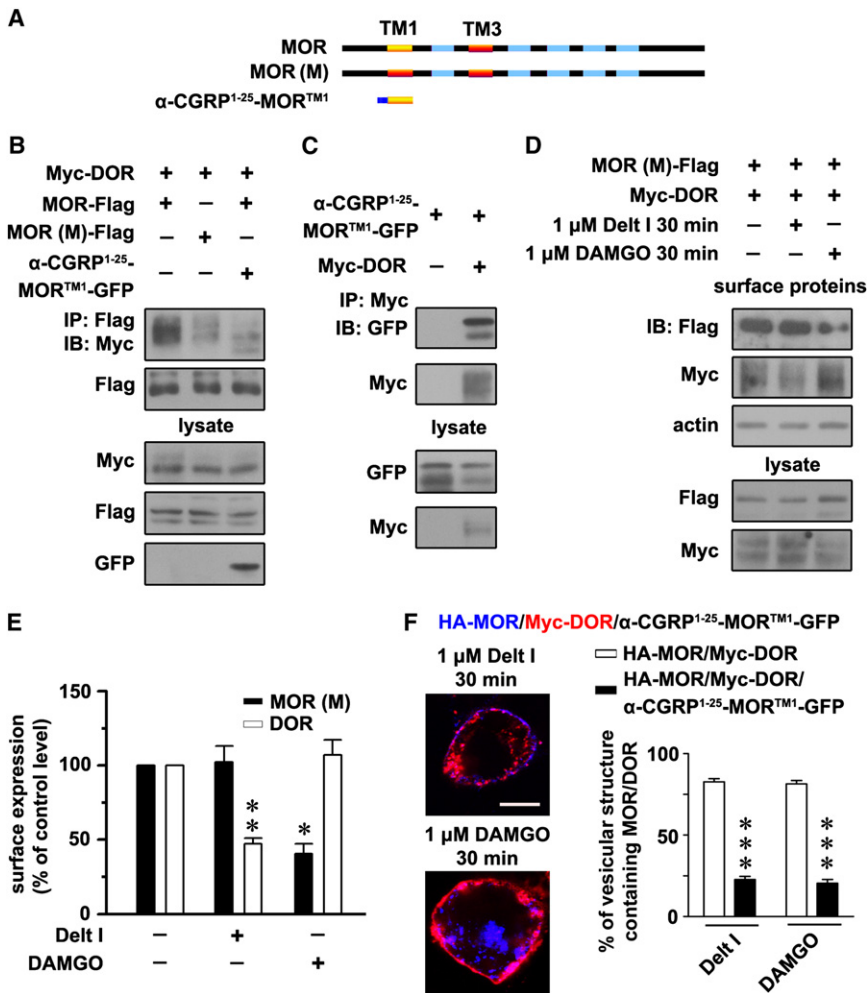


15 min,  $n$  = 6 at 30 min,  $n$  = 8 at 45 min, and  $n$  = 6 at 60 min. Results are presented as mean  $\pm$  SEM. \*\*\* $p$  < 0.001 versus the saline-pretreated mice. Morphine analgesia is dose-dependently reduced by Delt I applied 30 min prior to the morphine treatment. This effect is blocked by cotreatment with NTI (i.t.) (J). Results are presented as mean  $\pm$  SEM.  $p$  < 0.01 for 0.25  $\mu$ g,  $p$  < 0.001 for 0.5  $\mu$ g,  $p$  < 0.01 for 1  $\mu$ g, and  $p$  < 0.001 for 2  $\mu$ g (ANOVA),  $n$  = 6~7 mice/group. A similar effect was induced by pretreatment (i.t.) with L-ENK ( $p$  < 0.001,  $n$  = 7) or SNC80 ( $p$  < 0.001,  $n$  = 8) (J). Cotreatment with NTI (i.t.) increased the morphine (i.t.)-induced analgesia (K). Results are presented as mean  $\pm$  SEM.  $p$  < 0.001 (ANOVA),  $n$  = 11 and 8 mice for saline and NTI treatment, respectively. See also Table S1 (J) and Table S2 (K).

See also Figure S2.

confirmed by the loss of immunoblot and IP signals in the spinal cord of *Oprd1* exon 1-deleted mice (Figures 3F and S2B). A 45 min treatment with Delt I or SNC80 or leucine-enkephalin (L-ENK) (5  $\mu$ g, twice at an interval of 15 min, i.t.), an endogenous DOR agonist expressed in dorsal horn neurons (Cesselin et al., 1989), increased the ubiquitination of MORs (Figures 3G and

S2C). Immunohistochemistry showed that the intensity of MOR-immunostaining in the spinal lamina I-II was significantly reduced in mice after a 1 hr treatment with Delt I (2  $\mu$ g/15 min, i.t.) (Figure 3H). These results suggest that the activation of DORs leads to a downregulation of MORs in afferent fibers of the spinal cord.



**Figure 4. The TM1 Domain of MOR Mediates MOR Interaction with DOR**

(A) The diagram shows wild-type MORs, mutated MORs (MOR(M)) with a substitution of MOR<sup>63-93</sup> which contains the TM1 domain (MOR<sup>TM1</sup>) with MOR<sup>144-163</sup> which contains the TM3 (MOR<sup>TM3</sup>), and MOR<sup>TM1</sup> fused with  $\alpha$ -CGRP<sup>1-25</sup> at the N terminus and GFP at the C terminus.

(B and C) CoIP experiments showed that Myc-DOR interacted with MOR-Flag in transfected HEK293 cells; it only weakly interacted with MOR (M)-Flag (B). The MOR/DOR interaction was markedly reduced in the presence of MOR<sup>TM1</sup>-GFP (B), which could directly interact with Myc-DOR (C). Each immunoblot represents three independent experiments.

(D and E) In HEK293 cells coexpressing Myc-DOR with MOR(M)-Flag, treatment with 1  $\mu$ M Delt I for 30 min reduced the surface expression of DORs; it did not affect the surface expression of MORs (M). Treatment with 1  $\mu$ M DAMGO induced the internalization of MOR(M). Results are presented as mean  $\pm$  SEM. \* $p$  < 0.05, \*\* $p$  < 0.01 versus untreated cells ( $n$  = 3).

(F) In triple-transfected HEK293 cells, the agonist-induced cointernalization of HA-MOR and Myc-DOR was attenuated by coexpressed  $\alpha$ -CGRP<sup>1-25</sup>-MOR<sup>TM1</sup>-GFP. GFP-positive cells were analyzed. Results are presented as mean  $\pm$  SEM. \*\*\* $p$  < 0.001 versus the cells only expressing HA-MOR and Myc-DOR ( $n$  = 39). Delt I ( $n$  = 42) and DAMGO ( $n$  = 41)-treated cells were analyzed. Scale bar, 4  $\mu$ m.

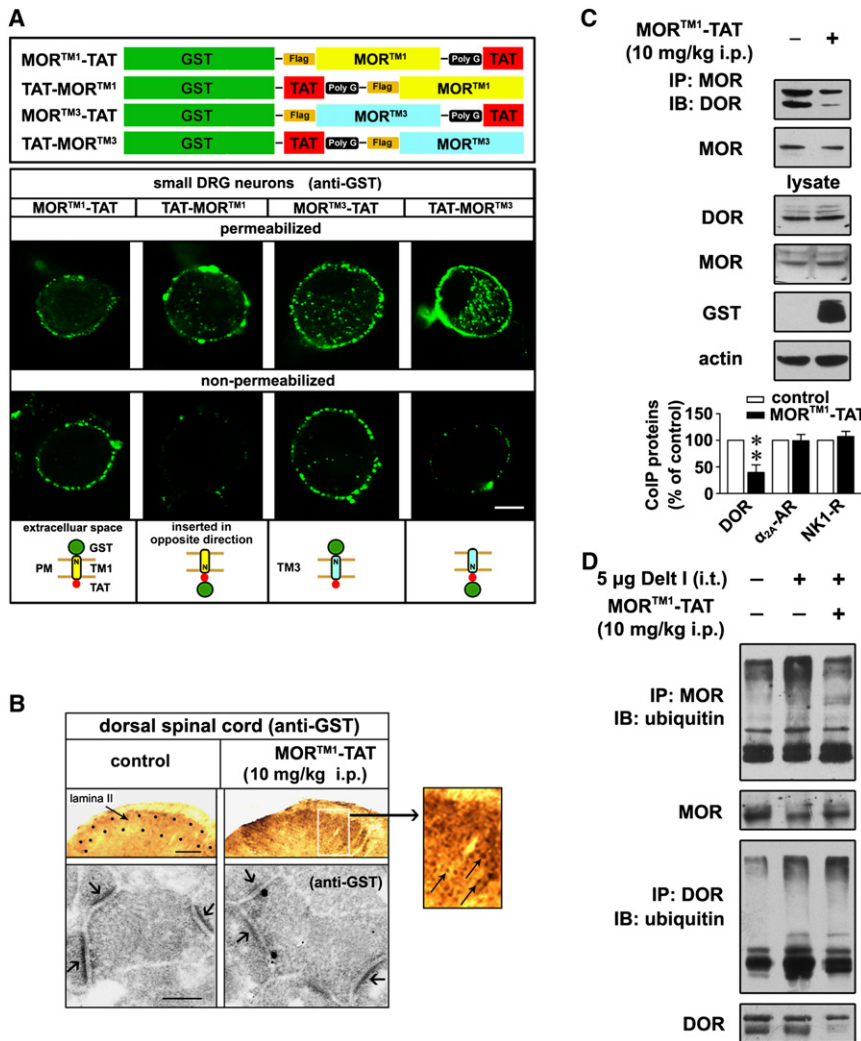
We have further found that the activation of DORs attenuated morphine analgesia. Using a tail-immersion test at 52°C, we found that morphine-induced spinal antinociception was markedly attenuated when mice were pretreated with Delt I (1  $\mu$ g, i.t.) 30–45 min prior to the morphine treatment (1.5  $\mu$ g, i.t.) (Figure 3I). We also found that Delt I inhibited the morphine effect in a dose-dependent manner when Delt I or SNC80 was applied 30 min prior to the morphine treatment (Figure 3J; Table S1). A similar effect was induced by pretreatment with L-ENK (2  $\mu$ g, i.t.) (Figure 3J; Table S1). The Delt I-induced inhibition of morphine antinociception was blocked by cotreatment with NTI (Figure 3J; Table S1). Furthermore, NTI treatment (1  $\mu$ g, i.t.) facilitated morphine-induced spinal antinociception (Figure 3K; Table S2). This result is consistent with previous findings (Gomes et al., 2004). These data suggest that the DOR-mediated down-regulation of MORs in the dorsal spinal cord leads to a reduction in MOR-mediated analgesia.

**First Transmembrane Domain of MOR Mediates MOR Interaction with DOR**

To fully evaluate the role of the MOR/DOR interaction in the negative regulation of the MOR activity, we searched for the

domain of MOR that mediates its interaction with DOR. Using the computational analysis, Filizola and colleagues (2002) predicted the TM1 domain of MOR as the most likely binding interface with DOR. We constructed a mutated MOR (MOR(M)) in which MOR<sup>63-93</sup> containing the predicted TM1 (MOR<sup>TM1</sup>) was substituted by MOR<sup>144-163</sup> containing the predicted TM3 (MOR<sup>TM3</sup>) (Figure 4A). CoIP experiments showed that, while DOR interacted with MOR, it did not interact with MOR(M) in cotransfected HEK293 cells (Figure 4B). We then constructed a plasmid expressing a chimera protein that contained TM1 with the signal peptide of  $\alpha$ -CGRP fused at the N terminus and a GFP fused at the C terminus ( $\alpha$ -CGRP<sup>1-25</sup>-MOR<sup>TM1</sup>-GFP). The signal peptide of  $\alpha$ -CGRP was used to sort the fusion protein into the endoplasmic reticulum. It is then removed by a signal peptidase, and the resulting GFP-tagged MOR<sup>TM1</sup> is threaded through the membrane of the endoplasmic reticulum. CoIP experiments showed that the MOR<sup>TM1</sup> peptide interacted with coexpressed DORs in cotransfected HEK293 cells (Figure 4C), indicating that the TM1 domain of MOR mediates the MOR interaction with DORs.

Using MOR(M) and  $\alpha$ -CGRP<sup>1-25</sup>-MOR<sup>TM1</sup> as tools, we demonstrated that a physical interaction was essential for a cointernalization of MORs and DORs. Treatment with Delt I (1  $\mu$ M) did not lead to a cointernalization of MOR(M) and DORs (Figures 4D and 4E). This is not due to a problematic internalization of MOR(M), as



**Figure 5. Spinal MOR/DOR Interaction Can Be Disrupted by MOR<sup>TM1</sup>-TAT**

(A) Cultured mouse DRG neurons were treated with four types of fusion proteins containing the TM1 or TM3 with the GST tag at the N terminus and TAT at either the N or C terminus. Permeabilized immunostaining with GST antibodies showed that all fusion proteins were mainly localized on the cell surface of small DRG neurons. Nonpermeabilized immunostaining, which detects the GST tag on the extracellular side of the plasma membrane, showed that both MOR<sup>TM1</sup>-TAT and MOR<sup>TM3</sup>-TAT, but not TAT-MOR<sup>TM1</sup> and TAT-MOR<sup>TM3</sup>, were inserted into the plasma membrane of small DRG neurons in the same direction as that of TMs in the native MOR. Scale bar, 8  $\mu$ m.

(B) Mice treated with MOR<sup>TM1</sup>-TAT (i.p., three injections within 2.5 hr, 10 mg/kg/injection) were subjected to pre-embedding immunogold-silver labeling. Light microscopy analysis (upper panel) showed MOR<sup>TM1</sup>-TAT-labeling in the nerve terminals in the dorsal horn of the lumbar spinal cord, with a high intensity of immunostaining in the lamina I-II. The boxed area shows the sample at a high magnification. At the ultrastructural level (lower panel), MOR<sup>TM1</sup>-TAT-labeling was associated with the plasma membrane of the afferent terminals of the glomerulus in the lamina II. Arrows point to the postsynaptic zone. Scale bars, 100  $\mu$ m for light micrographs and 200 nm for electron micrographs.

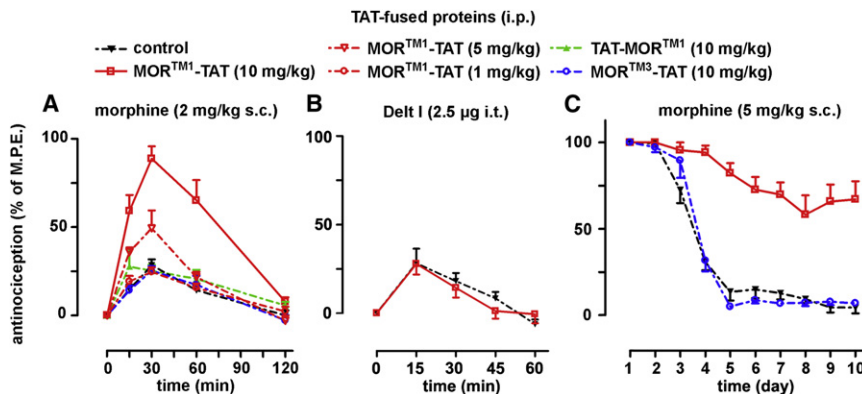
(C and D) CoIP experiments showed that the basal MOR/DOR interaction in the mouse spinal cord was reduced after a 2.5 hr pretreatment with MOR<sup>TM1</sup>-TAT (i.p., three injections, 10 mg/kg/injection); the interactions between MORs and  $\alpha_{2A}$ -AR or NK1-R were unaffected (C). The same pretreatment also reduced the MOR ubiquitination induced by treatment with 5  $\mu$ g Delt I but did not affect that of DORs (D). Three independent experiments were performed. Results are presented as mean  $\pm$  SEM. \*\*p < 0.01 versus control mice. See also Figure S3.

DAMGO (1  $\mu$ M) could efficiently induce the internalization of MOR(M) (Figures 4D and 4E). Consistently, a 30 min treatment with DAMGO (1  $\mu$ M) did not induce a cointernalization of DORs with MOR(M) (Figures 4D and 4E). It can thus be concluded that the MOR/DOR interaction is required for receptor cointernalization. Most importantly, the MOR/DOR interaction was reduced in HEK293 cells that were triple-transfected with plasmids expressing DOR, MOR and  $\alpha$ -CGRP<sup>1-25</sup>-MOR<sup>TM1</sup> (Figure 4B), indicating that the MOR<sup>TM1</sup> peptide can competitively disrupt the MOR/DOR interaction. Immunocytochemistry showed that the coexpression of MOR<sup>TM1</sup> attenuated both the Delt I- and DAMGO-induced MOR/DOR cointernalization (Figure 4F). Thus, the MOR<sup>TM1</sup> peptide can be used to disrupt the MOR/DOR interaction, and abolish the agonist-induced cointernalization.

#### TAT-Directed Membrane Insertion of the Transmembrane Domain of MOR

To evaluate the physiological relevance of receptor hetero-oligomerization, a method allowing the *in vivo* dissociation of GPCRs is required. Because the intracellular delivery of proteins

can be achieved by the fusion of proteins to the TAT peptide (YGRKKRRQRRR) (Schwarze et al., 1999), we tested whether this peptide could be used to insert fusion proteins into the plasma membrane. The MOR<sup>TM1</sup>-TAT protein was thus prepared by fusion of the TAT peptide at the C terminus of the MOR<sup>TM1</sup> peptide, and GST and Flag tags at the N terminus of the peptide (Figure 5A). To examine the localization of the MOR<sup>TM1</sup>-TAT protein, primary DRG neurons cultured from mice were incubated with the fusion protein and subjected to permeabilized or non-permeabilized immunostaining with antibodies against GST. Interestingly, we found that the MOR<sup>TM1</sup>-TAT protein was mainly located in the plasma membrane of 91% cultured small DRG neurons (n = 123). Some MOR<sup>TM1</sup>-TAT-containing vesicular structures were observed in the cytoplasm (Figure 5A). A lower number of cultured large DRG neurons was also stained (43%, n = 40). These results indicate that MOR<sup>TM1</sup>-TAT can be effectively inserted into the plasma membrane of small DRG neurons as a result of both the penetration capacity of the TAT peptide and the hydrophobic property of the transmembrane domain.



**Figure 6. MOR<sup>TM1</sup>-TAT Enhances Morphine Analgesia**

(A) The tail-immersion test at 52°C showed that morphine (2 mg/kg, s.c.)-induced analgesia was enhanced by a 2.5 hr pretreatment with MOR<sup>TM1</sup>-TAT (i.p., three injections, 10 mg/kg/injection, n = 10); it was not enhanced by pretreatment with TAT-MOR<sup>TM1</sup> (n = 9) or MOR<sup>TM3</sup>-TAT (n = 11) at the same dosage. The time course showed that the MOR<sup>TM1</sup>-TAT-enhancing effect of morphine lasted for at least 60 min (p < 0.001 at 15 min and 30 min and p < 0.01 at 60 min, versus control; n = 11 and 10 for control mice and mice treated with MOR<sup>TM1</sup>-TAT, respectively) and was the highest 30 min after injection of the morphine. MOR<sup>TM1</sup>-TAT enhanced the morphine effect in a dose-dependent manner (p < 0.01 at 30 min,

5 mg/kg/injection, n = 7; p > 0.05 at 30 min, 1 mg/kg/injection, n = 9). Results are presented as mean ± SEM. See also Table S3.

(B) Delt I-induced spinal analgesia was unaffected in mice treated with MOR<sup>TM1</sup>-TAT (n = 10), versus control mice (n = 11). Results are presented as mean ± SEM. See also Table S4.

(C) The tolerance to morphine was demonstrated by the loss of the analgesic response using a daily subcutaneous administration (s.c.) of 5 mg morphine/kg during 5 days (n = 21). The daily administration of MOR<sup>TM1</sup>-TAT (i.p., three injections within 2.5 hr/day, 10 mg/kg/injection) in mice (n = 11) resulted in a reduced tolerance, while treatment with MOR<sup>TM3</sup>-TAT (i.p., three injections within 2.5 hr/day, 10 mg/kg/injection, n = 10) had no effect. Analgesia was tested 30 min after administration of morphine with the tail-flick assay. Results are presented as mean ± SEM. See also Table S5.

Furthermore, we found that MOR<sup>TM1</sup>-TAT was inserted into the plasma membrane in the same direction as that of TM1 in the native MOR. Nonpermeabilized immunostaining with GST antibodies showed that the N terminus of the MOR<sup>TM1</sup> peptide was located on the extracellular side of the plasma membrane (Figure 5A). In contrast, when the TAT peptide was fused at the N terminus of the MOR<sup>TM1</sup> peptide (TAT-MOR<sup>TM1</sup>), the fusion protein was inserted into the plasma membrane in a direction opposite to that of TM1 in the native MOR, as evidenced by the fact that the GST fused at the N terminus of the MOR<sup>TM1</sup> peptide was largely undetectable by nonpermeabilized immunostaining (Figure 5A). The same patterns of membrane insertion were observed for the MOR<sup>TM3</sup>-TAT and TAT-MOR<sup>TM3</sup> proteins in cultured small DRG neurons (Figure 5A). It can thus be concluded that the TAT peptide serves as both a cell-penetrating element and a guiding signal that determines the membrane insertion direction in these fusion proteins.

### Disruption of the MOR/DOR Interaction in the Dorsal Spinal Cord

We decided to test whether MOR<sup>TM1</sup>-TAT could disrupt the MOR-DOR interaction in the dorsal horn of the spinal cord. MOR<sup>TM1</sup>-TAT was intraperitoneally infused (i.p., three injections within 2.5 hr, 10 mg/kg/injection) in mice. A pre-embedding immunogold-silver staining showed that MOR<sup>TM1</sup>-TAT could be transported to the lamina I-II of the mouse spinal cord and associated with the membrane of afferent terminals (Figure 5B). A quantitative analysis showed that 68.8% ± 7.9% of the immunogold-silver particles (n = 44) were associated with the plasma membrane of axon terminals in the lamina II of the mouse spinal cord. Immunoblotting further proved the presence of MOR<sup>TM1</sup>-TAT in the dorsal spinal cord after intraperitoneal infusion (Figure 5C). These results indicate that the systemically applied MOR<sup>TM1</sup>-TAT can be transported into the spinal cord and inserted into the plasma membrane of afferent terminals.

The systemically applied MOR<sup>TM1</sup>-TAT was found to reduce the DOR-mediated MOR ubiquitination in the spinal cord. CoIP experiments showed that the MOR/DOR interaction in the mouse spinal cord was significantly reduced by applying a 2.5 hr treatment with MOR<sup>TM1</sup>-TAT (i.p., three injections, 10 mg/kg/injection) (Figure 5C). The same treatment also reduced the Delt-induced ubiquitination of MORs in the mouse spinal cord. However, it did not reduce DOR ubiquitination (Figure 5D). MORs also interact with  $\alpha_{2A}$ -adrenergic receptors ( $\alpha_{2A}$ -ARs) (Jordan et al., 2003) and neurokinin 1 receptors (NK1-Rs) (Pfeiffer et al., 2003). It was found that MORs colocalize with  $\alpha_{2A}$ -ARs in primary sensory afferents (Overland et al., 2009) or NK1-Rs in some neurons in the spinal lamina I (Spike et al., 2002). CoIP experiments showed that MORs interacted with  $\alpha_{2A}$ -ARs and NK1-Rs in the mouse spinal cord (Figures 5C and S3). However, neither the MOR/ $\alpha_{2A}$ -AR interaction nor the MOR/NK1-R interaction was reduced by systemically applied MOR<sup>TM1</sup>-TAT (Figures 5C and S3). These results suggest that the membrane insertion of MOR<sup>TM1</sup>-TAT results in selective disruption of the MOR/DOR interaction.

### Facilitation of Morphine Analgesia by MOR<sup>TM1</sup>-TAT

Finally, we examined whether a disruption of the MOR/DOR interaction in the spinal cord would lead to a modulation of morphine analgesia. We found that systemically applied MOR<sup>TM1</sup>-TAT protein reduced the DOR-mediated suppression of morphine analgesia. When the MOR<sup>TM1</sup>-TAT protein was applied 2.5 hr (i.p., three injections, 10 mg/kg/injection) prior to the morphine treatment (2 mg/kg, s.c.), the spinal analgesic effect of morphine was facilitated with 3-fold increase at the peak level (Figure 6A). The enhancement of the morphine effect lasted for at least 60 min (Figure 6A). Moreover, MOR<sup>TM1</sup>-TAT protein also increased the antinociceptive effect of morphine in a dose-dependent manner (Figure 6A; Table S3).

It was found that the MOR<sup>TM1</sup>-TAT-induced effect was specific. Indeed, neither TAT-MOR<sup>TM1</sup>, which was inserted in

the opposite direction, nor MOR<sup>TM3</sup>-TAT induced such an effect (Figure 6A; Table S3). The spinal analgesia induced by Delt I (2.5 µg, i.t.) was unaffected (n = 9) (Figure 6B; Table S4). These results strongly suggest that DORs normally suppress MOR activity in the spinal cord, and morphine analgesia can be increased by a physical dissociation of MORs and DORs.

Additionally, it was found that the infused MOR<sup>TM1</sup>-TAT reduced the tolerance to morphine. The analgesic effect of morphine was found to be reduced in mice 3 days after the morphine treatment (2 mg/kg/day, s.c.) (Figure 6C; Table S5). MOR<sup>TM1</sup>-TAT or MOR<sup>TM3</sup>-TAT was applied daily (i.p., three injections within 2.5 hr, 10 mg/kg/injection) prior to the daily subcutaneous administration of morphine (5 mg/kg, s.c.). In contrast to the untreated mice, the antinociceptive effect of morphine in MOR<sup>TM1</sup>-TAT-treated mice was largely intact for 3–4 days and was maintained at ~70% of the initial effectiveness for 9–10 days (Figure 6C; Table S5). These results suggest that disrupting the MOR/DOR interaction in the spinal cord with the MOR<sup>TM1</sup>-TAT protein can prevent morphine tolerance.

## DISCUSSION

The present study shows that the activation of DORs in MOR/DOR complexes on the cell surface leads to a cointernalization and codegradation of MORs and DORs. Based on the colocalization of MORs and DORs in nociceptive afferent fibers, it can be concluded that a DOR-mediated downregulation of MORs can also be induced in the spinal dorsal horn. This process can be attenuated by systemically applying MOR<sup>TM1</sup>-TAT to dissociate MORs from DORs in sensory afferents and improve morphine-induced spinal analgesia. The physical dissociation of MORs from DORs in the pain pathway could therefore be exploited to enhance MOR-mediated analgesia and reduce the associated side effects.

### DOR-Mediated Postendocytotic Processing of MORs

After receptor-selective agonist stimulation, DORs are internalized and often concentrated in lysosomal compartments for degradation (Bao et al., 2003; Gaudriault et al., 1997; Trapaidze et al., 1996; Tsao and von Zastrow, 2000), while MORs are internalized by agonists such as DAMGO and mainly processed in the recycling pathway for resensitization (Law et al., 2000; Qiu et al., 2003). The present study shows that MORs and DORs can be cointernalized by activating either DORs or MORs with a receptor-specific agonist. However, the postendocytotic pathway of MORs can be shifted to lysosomal degradation when DORs in the receptor complex are activated. This agonist-induced effect on the MOR/DOR trafficking is determined by distinct biochemical processes. The DOR- or MOR-selective agonist only induces the phosphorylation of the corresponding type of opioid receptor. Receptor cross-phosphorylation, which occurs between MORs and somatostatin receptors or NK1-Rs (Pfeiffer et al., 2003), was not observed between MORs and DORs.

Interestingly, treatment with a DOR agonist elevates the ubiquitination of both DORs and MORs, whereas the MOR agonist DAMGO does not change the constitutive ubiquitination of both receptors. These findings are consistent with the notion

that a receptor endocytosis can be carried out in a ubiquitin-dependent or ubiquitin-independent way (Holler and Dikic, 2004). Although ubiquitination might be unnecessary for DOR degradation (Tanowitz and von Zastrow, 2002), the correlation between such a modification and the MOR/DOR degradation provides a mechanism for the DOR-mediated modulation of the postendocytotic processing of MORs.

### The MOR/DOR Interaction in the Pain Pathway

In cotransfected cells, MORs and DORs form heteromers (Daniels et al., 2005; Fan et al., 2005; Gomes et al., 2004; Jordan and Devi, 1999). The occupancy of DORs by antagonists may enhance MOR binding and signaling activity (Gomes et al., 2004). Although MOR/DOR heteromers were found in a membrane obtained from the spinal cord (Gomes et al., 2004), reports on the coexpression of opioid receptors in DRG neurons have been controversial. The presence of DORs and MORs in the same neurons (Ji et al., 1995; Rau et al., 2005) and the absence of DOR1-EGFP in MOR-containing neurons (Scherrer et al., 2009) were both reported. However, the later finding could not exclude that the absence of DOR1-EGFP in small neurons might be due to transcriptional modifications during the knockin procedure or to the degradation of newly synthesized DOR1-EGFP because of its inability to adopt the conformation that is required for trafficking in secretory pathways. The above-mentioned *in situ* double-hybridization experiments have revealed the coexistence of DORs and MORs in a considerable population of small DRG neurons, consistent with results obtained with other approaches (Wang et al., 2010). These results, together with the recent finding of opioid receptor heteromers in DRG neurons (Gupta et al., 2010), suggest that the coexpression of MORs and DORs in nociceptive afferent neurons is a cellular basis for their interaction in the pain pathway.

Pharmacological and genetic data indicate that the MOR-mediated spinal analgesia is negatively regulated by activation of DORs and that the tolerance to morphine can be reduced by a pharmacological blocking or genetic deletion of DORs (Chefer and Shippenberg, 2009; Fan et al., 2005; Gallantine and Meert, 2005; Gomes et al., 2004; Nitsche et al., 2002; Schiller et al., 1999; Standifer et al., 1994; Xie et al., 2009; Zhu et al., 1999). Although the MOR-mediated analgesia was unaffected by the deletion of the *Oprd1* exon 1 in mice (Scherrer et al., 2009), it remains unclear whether this distinct phenotype is due to the truncated DOR1 protein that remained in the mutant mice (Wang et al., 2010). Our finding on the improvement of morphine-induced analgesia by disrupting the MOR/DOR interaction further supports the role of DORs in the regulation of MOR-mediated analgesia.

The DOR activation-induced reduction of the number of MORs on the cell surface could be important in the regulation of the neuronal sensitivity to  $\mu$ -opioids. The MOR/DOR interaction may be enhanced by opioid agonist stimulation and membrane depolarization that induce the surface expression of intracellular DORs in the pain pathway (Bao et al., 2003; Cahill et al., 2001; Ma et al., 2006; Patwardhan et al., 2005; Walwyn et al., 2005). Prolonged morphine treatments increase the cell surface expression of intracellular DORs (Gendron et al., 2006; Morinville et al., 2003) and the MOR/DOR heteromerization in DRG neurons

(Gupta et al., 2010). Although our immunostaining procedure may not be sensitive enough to detect low levels of DORs in the dorsal horn neurons, prolonged morphine treatments also induce a surface expression of DORs in spinal interneurons (Morinville et al., 2003). Therefore, chronic morphine treatments may enhance the DOR-mediated inhibitory effects on the MOR activity. It is also possible that surface-expressed DORs are accessible to opioid peptides, such as ENK, that are released from spinal interneurons (Cesselin et al., 1989) and would thus be involved in the regulation of MOR activity in afferent terminals.

### TAT Peptide Serving As a Guide for Oriented Membrane Insertion of Proteins

It is noteworthy that the TAT peptide can serve as a guiding signal in the MOR<sup>TM1</sup>-TAT protein, enabling the insertion of the exogenous TM1 peptide into the plasma membrane in the direction that is required for its function. This method provides an approach to analyze the functional roles of a receptor interaction in vivo by physically dissociating two types of GPCR in the plasma membrane, while maintaining the function of each type of GPCR. The identification of the heteromerization interface of GPCRs is required for designing a molecular probe that effectively disrupts the receptor interaction. The present study shows that the insertion direction of the transmembrane domain of a receptor can be determined by the fusion of the TAT peptide at either the C or N terminus. This determination is based on both the identification of the transmembrane domain specifically mediating the receptor interaction and the membrane penetration capacity of the TAT peptide. Using such an approach to specifically disrupt the physical interaction between receptors and/or ion channels in the plasma membrane is not only a tool for the functional analysis of the membrane protein interaction in vivo but also a potential strategy for medical intervention.

### Increased Opioid Analgesia by Disruption of the MOR/DOR Interaction

The present study shows that a systemically applied MOR<sup>TM1</sup>-TAT protein disrupts the MOR/DOR interaction in the spinal cord and improves morphine analgesia. This result is consistent with findings on enhanced morphine analgesia obtained by other pharmacological or genetic approaches. The finding that morphine analgesia is strongly enhanced by disrupting the basal MOR/DOR interaction in the spinal cord with a membrane-inserted MOR<sup>TM1</sup>-TAT protein suggests that DORs in the MOR/DOR complexes can be activated by endogenous opioid-peptides, such as ENK, that are released from the dorsal horn neurons in response to nociceptive stimulation (Cesselin et al., 1989). Therefore, suppression of the MOR activity by endogenous  $\delta$ -opioid peptides could play a role in the homeostatic regulation of the spinal opioid system. The MOR<sup>TM1</sup>-TAT proteins that are present in the plasma membrane could competitively bind to DORs that are inserted into the plasma membrane during the nociceptive stimulation and chronic treatment with opioids (Bao et al., 2003; Cahill et al., 2001; Ma et al., 2006; Patwardhan et al., 2005; Walwyn et al., 2005), thereby attenuating the MOR/DOR interaction. Thus, MOR<sup>TM1</sup>-TAT enhances morphine analgesia and reduces the tolerance to morphine by reducing the DOR-mediated suppressive effect

on the MOR activity. This approach might benefit pain therapies by reducing the dosage and side effects of morphine.

## EXPERIMENTAL PROCEDURES

### Plasmid Construction

The procedures for the construction of plasmids expressing Myc-DOR, HA-MOR, MOR-Flag, MOR(M),  $\alpha$ -CGRP<sup>1-25</sup>-MOR<sup>TM1</sup>-GFP, GST-Flag-MOR<sup>TM1</sup>-TAT, and other TAT-fused proteins are provided in [Supplemental Information](#). All primers and oligonucleotides are listed in [Table S6](#).

### Cell Culture and Transfection

HEK293 cells were cultured in MEM containing 10% fetal bovine serum (Invitrogen). The cells were transfected with 1–2  $\mu$ g plasmid/35 mm dish or 2–3  $\mu$ g plasmid/60 mm dish using the calcium phosphate method and were cultured for 2–3 days.

### In Situ Hybridization

Detailed procedure is provided in [Supplemental Information](#). Briefly, the probe for DOR1 was labeled with digoxigenin, and the MOR1 probe was labeled with fluorescein. Sections of mouse DRGs were hybridized with two probes, and then processed for detection of fluorescein and digoxigenin signals.

### Immunostaining

HEK293 cells cotransfected with Myc-DOR and HA-MOR expression plasmids (see [Supplemental Information](#)) were preincubated with rabbit (Rb) anti-HA antibody (1:500, Clontech), mouse anti-Myc antibody (1:200, Developmental Studies Hybridoma Bank), and/or LysoTracker Red DND-99 (1:500, Molecular Probes) for 30 min at 37°C. Cells were then treated with 1  $\mu$ M SNC80, Delt I or II, or DAMGO for 30 or 90 min. Cells were pretreated with the antagonist for 30 min before agonist incubation. Cells were fixed with 4% paraformaldehyde and 0.2% picric acid and then immunostained. Cultured DRG neurons (Bao et al., 2003) were treated with 0.5 nM TAT-fused protein three times within 12 hr and preincubated with Rb anti-GST antibody (1:1,000, Proteintech Group) for 30 min at 37°C for nonpermeabilized staining. Cells were fixed and incubated with secondary antibodies conjugated with fluorescein. For permeabilized staining, cells were fixed and stained with anti-GST antibody.

Wild-type mice and *Oprd1* exon 1-deleted mice (Jackson Lab) were fixed with above fixative. Cryostat sections of L4–5 spinal segments were immunostained with Rb antiserum against DOR1<sup>3-17</sup> (1:50,000, gift from Dr. R. Elde), or a mixture of the DOR1<sup>3-17</sup> antiserum, guinea pig anti-MOR antibody (1:400, Neuromics) and mouse anti-CGRP antibody (1:400, Celltech). Some sections were processed for immunoperoxidase staining (Zhang et al., 1998b). For quantification, three sections from each mouse were analyzed. The specificity of the antibodies was tested by preabsorption with the corresponding immunogenic peptides ( $10^{-6}$  M). The specificity of the DOR1<sup>3-17</sup> antiserum was further examined in Myc-DOR1-expressing HEK293 cells and sections of the spinal cord from *Oprd1* exon 1-deleted mice.

### Electron Microscopy

Pre-embedding immunogold-silver labeling was processed as previously described (Zhang et al., 1998a). Briefly, mice were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde. Vibratome sections of the spinal cord were incubated with Rb anti-GST antibody (1:600) and labeled with the 1.4 nm gold particle-conjugated secondary antibody (1:200, Nanoprobes). Ultrathin sections were examined with an electron microscope.

### Cell Surface Biotinylation and Immunoblotting

Cell surface biotinylation was performed before or after treatment with 1  $\mu$ M Delt I or SNC80 for 30 min as previously described (Bao et al., 2003). The lysates were precipitated with streptavidin. For detection of the receptor phosphorylation, cells were treated with 1  $\mu$ M Delt I, SNC80, or DAMGO for 30 min. Cells were lysed in ice-cold RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mg/ml BSA). Samples were subjected to SDS-PAGE, transferred to membranes, probed with the indicated antibodies, and visualized with enhanced chemiluminescence. L4–5 spinal

segments of wild-type mice and *Oprd1* exon 1-deleted mice were prepared for immunoblotting. Antibodies against Flag (1:1,000, Sigma), Myc (1:2,000), DOR1<sup>3-17</sup> (1:5,000), DOR1<sup>1-60</sup> (1:1,000, Santa Cruz), phospho-DOR (Ser363) (1:1,000, Neuromics), phospho-MOR (Ser375) (1:1,000, Neuromics), and actin (1:10,000, Santa Cruz) were used. The specificity of the DOR1<sup>3-17</sup> antiserum was examined by using spinal cord extracts from *Oprd1* exon 1-deleted mice. Intensities of immunoreactive bands of the proteins versus actin were quantified.

#### Immunoprecipitation

Detailed procedure is provided in [Supplemental Information](#). Briefly, the suspended lysate of cells and tissues was precipitated with 0.5–2 µg of antibodies. For detection of the receptor ubiquitination, cells or tissues were lysed in 0.1 ml RIPA buffer with 10 mM N-ethylmaleimide and then mixed with 0.3 ml of 8 M urea. The lysate-urea suspension was diluted to reduce the urea concentration to 2 M and subjected to immunoprecipitation. Immunoprecipitates were processed for immunoblotting. The specificity of the DOR1<sup>1-60</sup> antiserum was tested using spinal cord extracts from *Oprd1* exon 1-deleted mice.

#### Preparation of GST- and TAT-Fused Proteins

GST- and TAT-fused proteins were expressed and purified. Briefly, the proteins were expressed in *Escherichia coli* BL21 (DE3). The bacteria were harvested by centrifugation, resuspended, and sonicated. The proteins were purified with glutathione-Sepharose beads, concentrated and quantitatively analyzed.

#### Drug Treatments and Behavior Tests

Experiments complied with the policy of the Society for Neuroscience (USA) on the use of animals. Adult male mice were maintained on a 12 hr light/dark cycle. Studies were conducted during the light phase of the cycle. The antinociceptive effect was assessed using the tail-flick test. The latency to the first sign of a rapid tail-flick was taken as the behavioral endpoint. Each mouse was tested for baseline latency by immersing one-third of its tail in 52°C water and recording the time to response. All drugs dissolved in 5 µl of distilled water were administered via lumbar puncture. Delt I or Delt I with NTI, SNC80, or L-ENK was administered i.t. 30 min before the morphine treatment (1.5 µg, i.t.). NTI (1 µg, i.t.) was administered together with morphine (1 µg, i.t.). TAT-fused proteins (1, 5, or 10 mg/kg) were applied (i.p.) 2.5 hr, 1.5 hr, or 30 min before the morphine treatment (2 mg/kg, s.c.). A maximum score was assigned (100%) to animals not responding within 10 s to avoid tissue damage. Antinociception was calculated by the following formula: % maximum possible effect (M.P.E) = 100 × (test latency – baseline latency)/(10 – baseline latency).

#### Statistics

Data are presented as mean ± SEM. Statistical analysis was performed using PRISM (GraphPad Software) with a two-tailed, paired or unpaired Student's t test. For behavioral tests, single-dose data were analyzed using one-way ANOVA, followed by a two-tailed, unpaired Student's t test for between group comparisons. Differences were considered significant at  $p < 0.05$ .

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at [doi:10.1016/j.neuron.2010.12.001](https://doi.org/10.1016/j.neuron.2010.12.001).

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