

# Interaction and regulatory functions of $\mu$ - and $\delta$ -opioid receptors in nociceptive afferent neurons

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**Abstract:**  $\mu$ -opioid receptor (MOR) agonists such as morphine are powerful analgesics used for pain therapy. However, the use of these drugs is limited by their side-effects, which include antinociceptive tolerance and dependence. Earlier studies reported that MOR analgesic tolerance is reduced by blockade of  $\delta$ -opioid receptors (DORs) that interact with MORs. Recent studies show that the MOR/DOR interaction in nociceptive afferent neurons in the dorsal root ganglion may contribute to morphine analgesic tolerance. Further analysis of the mechanisms for regulating the trafficking of receptors, ion channels and signaling molecules in nociceptive afferent neurons would help to understand the nociceptive mechanisms and improve pain therapy.

**Keywords:** peripheral nervous system; opioid receptor; nociceptive pathways

## 1 Introduction

Small-diameter neurons in the dorsal root ganglia (DRGs) convey signals from nociceptors, thermoreceptors and sensitive mechanoreceptors to the dorsal horn of the spinal cord through afferent A $\delta$ - and C-fibers that terminate in laminae I and II. In response to peripheral noxious stimulation, the excitatory neurotransmitter glutamate is released from these afferent terminals in the superficial dorsal horn. Studies over the past decades showed that this excitatory neurotransmission is negatively regulated by inhibitory regulators released from local neurons, such as opioid peptides acting on the presynaptic  $\mu$ - and  $\delta$ -opioid receptors (MORs and DORs). Accumulating evidence

suggests that the DOR/MOR interaction in nociceptive afferent neurons is a mechanism for morphine analgesic tolerance. In the present review, we summarize the recent studies on the expression and interaction of opioid receptors in primary sensory neurons and their functional impact on pain modulation, and discuss their potential roles in the pain therapy.

## 2 Expression of opioid receptors in nociceptive afferent neurons

Autoradiographic studies provide evidence showing the presence of opioid receptors in afferent A $\delta$ - and C-fibers in laminae I and II of the spinal cord, including the binding sites for DOR and MOR agonists<sup>[1–5]</sup>. The presence of DORs on nociceptive afferents is supported by findings that release of the excitatory neurotransmitters glutamate, substance P and calcitonin gene-related peptide (CGRP) from afferent C- and A $\delta$ -fibers is inhibited by the activa-

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Article ID: 1673-7067(2012)02-0121-10  
Received date: 2012-01-04; Accepted date: 2012-02-13

tion of DORs<sup>[6,7]</sup>. These early findings suggested the presence of DORs on nociceptive afferent neurons, including the peptidergic subset of small DRG neurons.

Following the cloning of opioid receptor genes, DOR1 mRNA is found in ~70% of DRG neurons, including both peptidergic [isolectin B4 (IB4)-negative] and non-peptidergic (IB4-positive) subsets of small neurons and large neurons, while MOR1 mRNA is mainly present in peptidergic small neurons<sup>[8-12]</sup>. DOR-mediated spinal analgesia is attenuated by the intrathecally applied antisense oligodeoxynucleotide of the DOR1 gene (*Oprd1*), and the deletion of *Oprd1* or the preproenkephalin gene (*Penk1*)<sup>[13-15]</sup>.

Endogenous DORs can be detected specifically by immunoblotting with DOR antibodies, including commercially available antibodies, in the DRGs and the dorsal spinal cord of wild-type but not DOR-deficient mice<sup>[10,11,15,16]</sup>. However, the proper dilutions of the same antibodies are required to immunostain DORs specifically in the DRGs and laminae I and II<sup>[10]</sup>. This is an example for testing the specificity of receptor antibodies, though it is well-known that all antibodies must be used at appropriate concentrations for specific immunoblotting and immunostaining in various tissues. So far, no antibodies are available to simultaneously stain the endogenous DORs in all subsets of DRG neurons and their afferent terminals. Individual antibodies can be used to immunostain DORs in peptidergic small DRG neurons and large DRG neurons but not DORs expressed in IB4-positive small DRG neurons<sup>[10,17]</sup>. It is possible that the antibodies recognize DORs in different states of activation, conformation, glycosylation and/or palmitoylation<sup>[18-21]</sup>. To exclude the possibility of non-specific immunostaining<sup>[22]</sup>, it is important to carefully assess the antibody specificity and comprehensively analyze the distribution of DORs with multiple experimental approaches<sup>[10]</sup>.

Based on the findings of DOR- and MOR-mediated inhibitory effects on both the Ca<sup>2+</sup> currents in small DRG neurons and the release of substance P from C- and Aδ- afferents, as well as the results of immunostaining<sup>[7,16,17,23-29]</sup>, DORs and MORs are suggested to be co-expressed in peptidergic small DRG neurons. However, a study in the

mouse expressing DOR with insertion of enhanced green fluorescent protein (eGFP) at the C-terminus showed that only ~17% of DRG neurons are immunostained for DOR-eGFP<sup>[22]</sup>. Besides, most of these immunostained neurons are large and NF200-positive, while DOR-eGFP is rarely detected in peptidergic small DRG neurons that express MORs. In the dorsal spinal cord, the DOR-eGFP-positive structures overlap with PKCγ-expressing neurons in inner lamina II<sup>[22]</sup>. Nevertheless, it remains unclear whether these eGFP-positive structures are Aβ-afferents from the DOR-eGFP-expressing large DRG neurons or IB4-positive C-afferents, or due to ectopic expression of eGFP in local neurons. In fact, the mechanosensitive Aβ-afferents of large DRG neurons primarily project to spinal laminae III and IV in rodents<sup>[30]</sup>.

To evaluate these conflicting results, several laboratories have recently used multiple approaches, including single-cell PCR, *in situ* double-hybridization, electrophysiological recording, biochemical analysis and pharmacological approaches, to analyze the expressional correlation and functional interaction of DORs with MORs. Their results showed that DORs and MORs are co-expressed in peptidergic small DRG neurons<sup>[10,11,31-33]</sup>. Importantly, using antibodies that recognize DOR/MOR heteromers, Gupta *et al.*<sup>[34]</sup> were able to demonstrate the presence of the opioid receptor heteromer in DRG neurons. Thus, DORs and MORs are co-expressed in a considerable population of peptidergic small DRG neurons, and form heteromers that are involved in pain modulation<sup>[35-37]</sup>.

### 3 Regulation of the plasma membrane insertion of opioid receptors

In both peptidergic small DRG neurons and PC12 cells, immunostaining with antibody against epitope-tag hemagglutinin (HA) or Myc shows that the exogenously expressed HA- and Myc-DORs are mainly intracellularly distributed and often associated with large dense-core vesicles (LDCVs) which contain neuropeptides<sup>[10,17,38,39]</sup> (Fig. 1). In contrast, HA- and Myc-DORs expressed in large DRG neurons are present on the cell surface, suggesting that the trafficking of DORs is regulated by distinct

mechanisms in different neurons. It is clear that DOR-eGFP cannot be sorted into LDCVs to be transported effectively to the afferent terminals for storage and membrane insertion in response to stimulation, but can be transported via the constitutive secretory pathway for delivery to the cell surface without any special stimulation<sup>[10]</sup> (Fig. 1). Therefore, eGFP is not a proper tag for labeling receptors to study the trafficking of newly synthesized receptors, although it remains a good tag for analyzing the internalization of receptors on the cell surface.

The subcellular distribution of HA- or Myc-tagged DORs expressed in DRG neurons is consistent with the endogenous distribution pattern shown by immunostaining with DOR antibodies<sup>[10,17,24,38-41]</sup>. Moreover, the LDCV localization of DORs was found to be disrupted in the small DRG neurons of protachykinin 1 gene (*Tac1*)-knockout mice<sup>[10,17,41,42]</sup> (Fig. 2), indicating an essential role of the DOR/protachykinin interaction in sorting DORs into LDCVs. The third extracellular domain of DOR mediates the agonist-binding and the interaction with protachykinin<sup>[17,43-47]</sup>. Intracellular DORs are inserted into the plasma membrane following a variety of chemical and behavioral stimuli, including sustained pain conditions and prolonged treatment with morphine or ethanol<sup>[26,33,34,38,48-51]</sup>.

In peptidergic small DRG neurons and PC12 cells, both endogenous MORs and exogenously expressed tagged MORs can be inserted into the plasma membrane without stimulation<sup>[10,25,41]</sup>. Therefore, the interaction between MORs and DORs could be enhanced by stimuli that induce the membrane insertion of DORs, although opioid receptor heteromers could also be present in the cytoplasm<sup>[34]</sup>. In large DRG neurons that do not contain neuropeptides and LDCVs, immunostaining of DORs can be present on the surface of cell bodies, but is mostly absent from their afferent terminals in spinal laminae III and IV, consistent with the receptor autoradiographic results<sup>[10,22,52]</sup>. It remains largely unknown why the DORs expressed in large DRG neurons cannot be efficiently transported to the terminals of A $\beta$ -afferent fibers in the deep laminae of the spinal cord.

Using liquid chromatography-mass spectrometry combined with immunoblotting of subcellular fractions,

Zhao *et al.*<sup>[39]</sup> identified 298 proteins in LDCV membranes purified from the dorsal spinal cord, including G-protein-coupled receptors, G-proteins and other signaling molecules, as well as ion channels. Interestingly, DOR,  $\beta_2$ -adrenergic receptor,  $G_{\alpha i2}$ , voltage-gated calcium channel  $\alpha 2\delta 1$  subunit and P2X purinoceptor 2 are localized in substance P-positive LDCVs in small DRG neurons, whereas  $\beta_1$ -adrenergic receptor, Wnt receptor frizzled 8 and dishevelled 1 are present in substance P-negative LDCVs. Furthermore, DOR1/ $G_{\alpha i2}$ / $G_{\beta 1\gamma 5}$ /phospholipase C  $\beta 2$  complexes are found to associate with LDCVs. Thus, the plasma membrane properties of nociceptive afferent neurons can be rapidly modified in response to noxious stimulation, acute or chronic inflammation and drug treatments. In fact, DOR-mediated functions are involved in the DOR interaction with many membrane proteins such as  $Ca^{2+}$  channels and  $Na^+, K^+$ -ATPase that are expressed in small DRG neurons<sup>[53-58]</sup>. In addition, DORs and MORs may interact with  $\beta_2$ - and  $\alpha_{2A}$ -adrenergic receptors that are expressed in DRG neurons, respectively<sup>[39,59-61]</sup>. Although the functional consequences of the stimulus-induced co-insertion of LDCV-associated molecules remain largely unclear, one could expect that such a reaction would cause a "phenotypic" modification of the plasma membrane, enabling a shift of the sensitivity of nociceptive afferent neurons to many neurotransmitters, neuromodulators and applied drugs.

In the nervous system, DORs expressed in different types of neurons may have subcellular distribution patterns distinct from that in DRG neurons. Moreover, only 40% of DORs expressed in transfected HEK cells are transported to the cell surface, while the rest are retained in the endoplasmic reticulum<sup>[62]</sup>. Such intracellular retention of DORs may also be present in many neurons. It would be interesting to reveal the mechanisms for retaining DORs intracellularly and releasing these receptors from the retention pool. Although cell biological analysis of the mechanisms of DOR trafficking in the brain is very limited, several studies showed that nerve growth factor triggers the cell surface expression of DORs, and DOR activation can induce the plasma membrane insertion of GABA $_A$  receptors<sup>[63,64]</sup>.

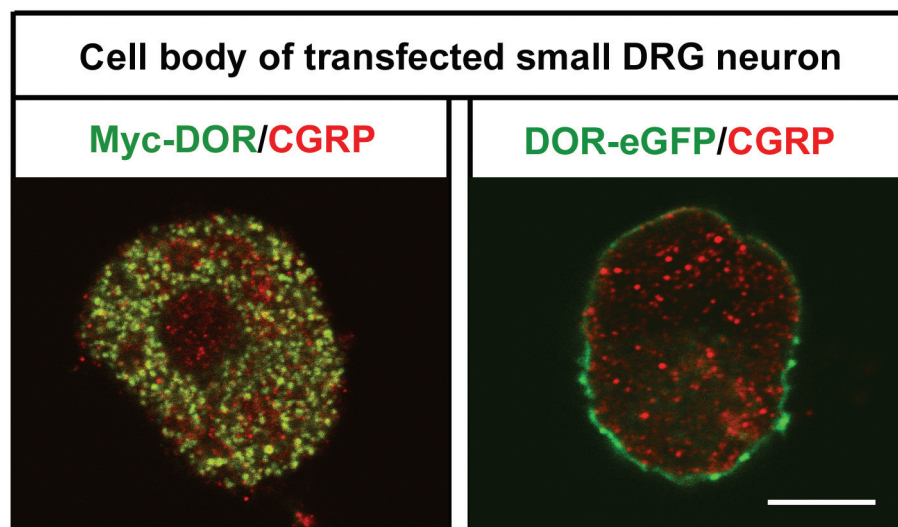


Fig. 1. Myc- $\delta$ -opioid receptor (DOR), but not DOR-enhanced green fluorescent protein (eGFP), can be sorted into large dense-core vesicles (LDCVs) in small dorsal root ganglion (DRG) neurons. Dissociated DRG neurons were transfected with plasmid expressing Myc-DOR or DOR-eGFP by electroporation. Double-immunostaining with antibodies against Myc (green) or neuropeptide calcitonin gene-related peptide (CGRP) (red) showed that Myc-DOR is localized in CGRP-containing LDCVs in small DRG neurons. However, immunostaining with CGRP antibody showed that DOR-eGFP (green) does not localize in CGRP-positive LDCVs (red), but is distributed on the cell surface of small DRG neurons. Scale bar, 8  $\mu$ m. Images unpublished. See more details in Wang *et al.*, Proc Natl Acad Sci U S A, 2010<sup>[10]</sup>.

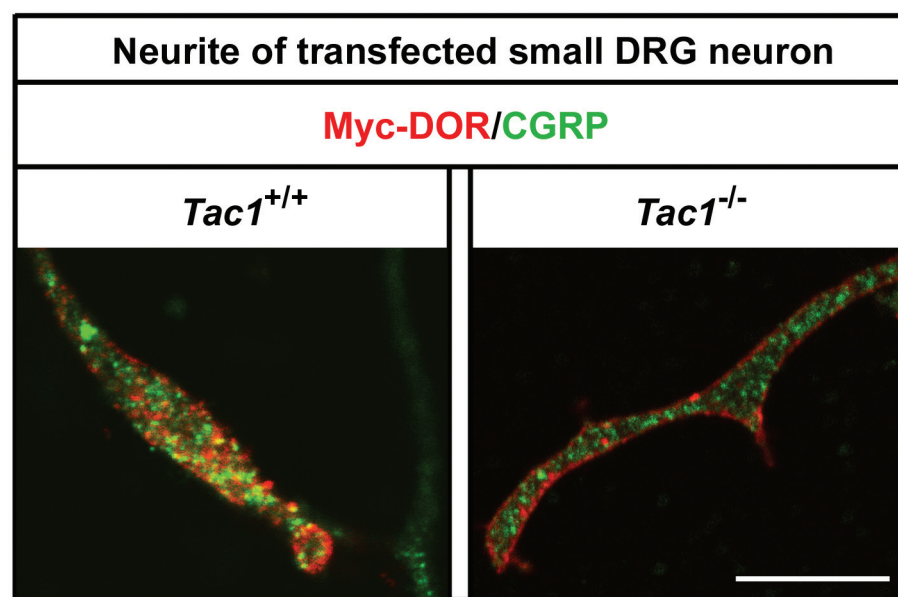


Fig. 2. Protachykinin-dependent large dense-core vesicle (LDCV)-localization and transport of  $\delta$ -opioid receptors (DORs). Dissociated dorsal root ganglion (DRG) neurons were transfected with Myc-DOR by electroporation. *Tac1* encodes protachykinin, which is a precursor protein of the neuropeptide substance P. Substance P and calcitonin gene-related peptide (CGRP) are often co-localized in LDCVs in the small DRG neurons of normal mice. Double-immunostaining with antibodies against Myc or the neuropeptide CGRP showed that CGRP (green) and exogenously expressed Myc-DOR (red) are co-localized in LDCVs in the distal part of the neurites of small DRG neurons cultured from wild-type mice (*Tac1*<sup>+/+</sup>). In contrast, Myc-DOR is absent from CGRP-containing LDCVs, but is localized on the surface of the neurites of small DRG neurons of *Tac1*-knockout mice (*Tac1*<sup>-/-</sup>). Scale bar, 8  $\mu$ m. Images unpublished. See more details in Wang *et al.*, Proc Natl Acad Sci U S A, 2010<sup>[10]</sup>.

## 4 Opioid receptor interaction in nociceptive afferent neurons

Given that co-expression of DORs and MORs in peptidergic small DRG neurons could be a cellular basis for opioid receptor interaction in the pain pathway<sup>[10,11,27,29,31-34]</sup>, the functional analysis of MOR/DOR heteromers would be critical for understanding opioid physiology and pharmacology<sup>[35-37]</sup>. Recent studies show that treatment with either DOR agonists or the MOR agonist Tyr-*D*-Ala-Gly-MePhe-Gly-ol (DAMGO) and methadone but not morphine results in endocytosis of DOR/MOR heteromers in transfected HEK293 cells<sup>[11,65]</sup>. The receptor complexes internalized by DOR agonists are ubiquitinated for lysosomal degradation, leading to a reduction of surface MORs<sup>[11]</sup>. In addition, a basal level of co-internalization and co-degradation of DORs and MORs occurs in the transfected cells<sup>[11,65]</sup>. The mechanism of such a reaction in the cells remains unknown. However, the basal level of co-degradation of MOR/DOR heteromers in the spinal cord may be caused by opioid peptide enkephalin released from dorsal horn neurons<sup>[11,66]</sup>. The co-degradation of these opioid receptors is enhanced by treatment with exogenously applied DOR agonists<sup>[11]</sup> or persistent release of endogenous opioid peptides with a high affinity for DORs. It is interesting that DOR antagonists attenuate the methadone-induced co-internalization of MOR/DOR heteromers in transfected HEK293 cells<sup>[65]</sup>. MOR/DOR heteromers could recruit  $\beta$ -arrestin, while DORs but not MORs are normally coupled with  $\beta$ -arrestin<sup>[67,68]</sup>. Taken together, the interaction between DORs and MORs plays an important role in regulating receptor trafficking, signaling, functioning and metabolism, and is involved in the mechanisms of pain modulation and brain disorders<sup>[35-37,69-71]</sup>.

Both MORs and DORs have been known for decades to inhibit nociceptive transmission in the spinal cord. However, a study published in 2009 suggested that DORs and MORs function in segregated spinal sensory circuits mediating the inhibitory effect on mechanical or thermal hyperalgesia respectively, due to the absence of DOR-eGFP in the MOR-expressing peptidergic small DRG

neurons and the presence of DOR-eGFP in large DRG neurons<sup>[22]</sup>. However, during the past two years, many studies have demonstrated that DOR agonists and MOR agonists induce analgesic effects on both thermal and mechanical hyperalgesia through activating these opioid receptors co-expressed in nociceptive afferent neurons<sup>[11,12,31,33,72]</sup>, consistent with the finding of coexistent DORs and MORs in peptidergic small DRG neurons. Thus, accumulating evidence shows that DORs and MORs can interact and function in the same nociceptive sensory circuit.

It has been noted that the translocalization of DORs and the opioid receptor interaction in nociceptive sensory neurons may enable modulation of the pharmacological effects of opioid agonists. The inhibitory effect of a DOR agonist on the  $\text{Ca}^{2+}$  current in small DRG neurons is enhanced after 10-Hz electrical stimulation<sup>[10]</sup>. Opioid receptor ligands are known to bind to opioid receptor subtypes with various affinities<sup>[73,74]</sup>. The opioid agonists targeting preferentially to one type of opioid receptor often also bind to the other two types at low affinities. Endogenous opioid peptide enkephalins have the highest affinity for DORs,  $\sim 10$ -fold lower affinity for MORs, and very low affinity for  $\kappa$ -opioid receptors (KORs);  $\beta$ -endorphin binds to MORs and DORs with high affinity, but has little affinity for KORs; dynorphin has preferential affinity for KORs, but also binds to MORs and DORs with high affinity. DAMGO has  $\sim 1\,000$ -fold higher affinity for MORs over DORs. Deltorphin II binds to DORs with  $\sim 3\,000$ -fold higher affinity over MORs. The selectivity of opioid receptor agonists and antagonists is a concern in the interpretation of experimental data.

In the resting state, only a limited number of DORs is present on the cell surface of nociceptive afferent neurons while MORs are abundant. MORs might be activated when a high dose of DOR agonist is used, whereas a low dose of DOR agonist could be sufficient to induce a DOR-specific effect when a large number of intracellular DORs are inserted into the plasma membrane in response to various stimuli. This idea may explain some seemingly conflicting findings that DOR agonist-induced antinociception is mediated by MORs under normal circumstance, but

mainly by DORs following physiological or pathological stimulation<sup>[15,22,33,75-78]</sup>. Under basal conditions, presynaptic inhibition in laminae I–II of the spinal cord is induced by a high concentration of a DOR agonist, and this effect is attenuated by a MOR antagonist<sup>[79]</sup>. However, after treatment with the TRP agonist icilin, the presynaptic inhibition induced by the DOR agonist increases and is blocked by the DOR antagonist<sup>[79]</sup>, suggesting that the TRP agonist-induced surface expression of DORs is important for producing a DOR-selective inhibitory effect. Therefore, the ratio of DOR *versus* MOR in the plasma membrane and the formation of DOR/MOR heteromers appear to be important factors that regulate the pharmacological properties of opioid ligands *in vivo*.

## 5 Contribution of opioid receptor interaction to the mechanism of morphine antinociceptive tolerance

Opioid analgesics (e.g. morphine) with high affinity for MORs are still the most powerful analgesics available for pain relief. However, their chronic use may lead to the development of antinociceptive tolerance and dependence<sup>[80-82]</sup>. Early pharmacological studies showed that blockade of DORs often results in enhanced morphine analgesia and reduced tolerance<sup>[83-86]</sup>, suggesting that DORs interact with MORs in the pain pathway<sup>[87-90]</sup>. Further studies revealed that morphine tolerance can be reduced by preventing DOR phosphorylation, deleting either *Oprdl* or *Penk1*, or deleting *Tac1* that reduces DOR transport to the spinal dorsal horn via LDCVs<sup>[14,15,17,91]</sup>.

A recent study showed that the DOR agonist-induced co-degradation of MORs may contribute to morphine antinociceptive tolerance, and morphine tolerance can be attenuated by treatment with an interfering molecule containing the first transmembrane domain of MOR that interacts with DOR and disrupts the MOR/DOR interaction<sup>[11]</sup>. Thus, dissociation of MORs from DOR-mediated co-degradation in nociceptive afferents may be a potential strategy to improve opioid analgesic therapies. Further studies are needed to more comprehensively understand the mechanisms of morphine antinociceptive tolerance,

because the processes of internalization of MOR/DOR heteromers might involve many receptors, ion channels, pumps, G-proteins and other signaling molecules that interact with these opioid receptors<sup>[39]</sup> (see also references 37 and 92). Moreover, it would be interesting to further study the regulatory mechanisms for post-endocytic trafficking of the MOR/DOR heteromers, including mechanisms for degradation and recycling, following different MOR agonists such as DAMGO, methadone and other opioid analgesics<sup>[11,65,93-95]</sup>.

## 6 Conclusion

In addition to the renewed concept of the coexistence of DORs and MORs in peptidergic small DRG neurons, there are several emerging concepts related to the stimulus- or activity-dependent dynamic distribution of opioid receptors, their interacting membrane proteins, and signaling molecules in nociceptive sensory circuits. Typical examples could be the stimulus-induced cell surface expression of the intracellularly stored DORs and the formation and trafficking of DOR/MOR heteromers. These dynamic changes in presynaptic opioid receptor trafficking would modify the sensitivity of nociceptive afferents to the opioid analgesics, and could be involved in morphine antinociceptive tolerance and other side-effects. Taking advantage of the advanced approaches of molecular cell biology, researchers can further explore the mechanisms for controlling the trafficking of opioid receptors in different subsets of DRG neurons. Such studies would contribute not only to the pain mechanism and therapies but also to the molecular cell biology and physiology of neurons.

**Acknowledgements:** This work was supported by the grants from the National Natural Science Foundation of China (31130066) and the National Basic Research Development Program of China (2009CB522005 and 2011CBA00400).

## References:

- [1] Mennicken F, Zhang J, Hoffert C, Ahmad S, Beaudet A, O'Donnell

- D. Phylogenetic changes in the expression of delta opioid receptors in spinal cord and dorsal root ganglia. *J Comp Neurol* 2003, 465: 349–360.
- [2] Besse D, Lombard MC, Perrot S, Besson JM. Regulation of opioid binding sites in the superficial dorsal horn of the rat spinal cord following loose ligation of the sciatic nerve: comparison with sciatic nerve section and lumbar dorsal rhizotomy. *Neuroscience* 1992, 50: 921–933.
- [3] Moskowitz AS, Goodman RR. Light microscopic autoradiographic localization of  $\mu$  and  $\delta$  opioid binding sites in the mouse central nervous system. *J Neurosci* 1984, 4: 1331–1342.
- [4] Gouarderes C, Beaudet A, Zajac JM, Cros J, Quirion R. High resolution radioautographic localization of [ $^{125}$ I]FK-33-824-labelled mu opioid receptors in the spinal cord of normal and deafferented rats. *Neuroscience* 1991, 43: 197–209.
- [5] Fields HL, Emson PC, Leigh BK, Gilbert RF, Iversen LL. Multiple opiate receptor sites on primary afferent fibres. *Nature* 1980, 284: 351–353.
- [6] Ueda M, Sugimoto K, Oyama T, Kuraishi Y, Satoh M. Opioidergic inhibition of capsaicin-evoked release of glutamate from rat spinal dorsal horn slices. *Neuropharmacology* 1995, 34: 303–308.
- [7] Zachariou V, Goldstein BD.  $\delta$ -Opioid receptor modulation of the release of substance P-like immunoreactivity in the dorsal horn of the rat following mechanical or thermal noxious stimulation. *Brain Res* 1996, 736: 305–314.
- [8] Minami M, Maekawa K, Yabuuchi K, Satoh M. Double *in situ* hybridization study on coexistence of  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptor mRNAs with preprotachykinin A mRNA in the rat dorsal root ganglia. *Mol Brain Res* 1995, 30: 203–210.
- [9] Wang H, Wessendorf MW. Equal proportions of small and large DRG neurons express opioid receptor mRNAs. *J Comp Neurol* 2001, 429: 590–600.
- [10] Wang HB, Zhao B, Zhong YQ, Li KC, Li ZY, Wang Q, *et al.* Co-expression of  $\delta$ - and  $\mu$ -opioid receptors in nociceptive sensory neurons. *Proc Natl Acad Sci U S A* 2010, 107: 13117–13122.
- [11] He SQ, Zhang ZN, Guan JS, Liu HR, Zhao B, Wang HB, *et al.* Facilitation of  $\mu$ -opioid receptor activity by preventing  $\delta$ -opioid receptor-mediated codegradation. *Neuron* 2011, 69: 120–131.
- [12] Gaveriaux-Ruff C, Nozaki C, Nadal X, Hever XC, Weibel R, Matifas A, *et al.* Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain* 2011, 152: 1238–1248.
- [13] Standifer KM, Chien CC, Wahlestedt C, Brown GP, Pasternak GW. Selective loss of delta opioid analgesia and binding by antisense oligodeoxynucleotides to a delta opioid receptor. *Neuron* 1994, 12: 805–810.
- [14] Nitsche JF, Schuller AG, King MA, Zeng M, Pasternak GW, Pintar JE. Genetic dissociation of opiate tolerance and physical dependence in delta-opioid receptor-1 and preproenkephalin knockout mice. *J Neurosci* 2002, 22: 10906–10913.
- [15] Zhu Y, King MA, Schuller AG, Nitsche JF, Reidl M, Elde RP, *et al.* Retention of supraspinal  $\delta$ -like analgesia and loss of morphine tolerance in  $\delta$  opioid receptor knockout mice. *Neuron* 1999, 24: 243–252.
- [16] Arvidsson U, Dado RJ, Riedl M, Lee JH, Law PY, Loh HH, *et al.*  $\delta$ -opioid receptor immunoreactivity: distribution in brainstem and spinal cord, and relationship to biogenic amines and enkephalin. *J Neurosci* 1995, 15: 1215–1235.
- [17] Guan JS, Xu ZZ, Gao H, He SQ, Ma GQ, Sun T, *et al.* Interaction with vesicle luminal protachykinin regulates surface expression of  $\delta$ -opioid receptors and opioid analgesia. *Cell* 2005, 122: 619–631.
- [18] Gupta A, Decaillet FM, Gomes I, Tkalych O, Heimann AS, Ferro ES, *et al.* Conformation state-sensitive antibodies to G-protein-coupled receptors. *J Biol Chem* 2007, 282: 5116–5124.
- [19] Gupta A, Rozenfeld R, Gomes I, Raehal KM, Decaillet FM, Bohn LM, *et al.* Post-activation-mediated changes in opioid receptors detected by N-terminal antibodies. *J Biol Chem* 2008, 283: 10735–10744.
- [20] Micovic V, Ivanovic MD, Dosen-Micovic L. Docking studies suggest ligand-specific delta-opioid receptor conformations. *J Mol Model* 2009, 15: 267–280.
- [21] Petäjä-Repo UE, Hogue M, Leskelä TT, Markkanen PM, Tuusa JT, Bouvier M. Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human  $\delta$  opioid receptor. *J Biol Chem* 2006, 281: 15780–15789.
- [22] Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, *et al.* Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell* 2009, 137: 1148–1159.
- [23] Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano AH, Dado RJ, *et al.* Distribution and targeting of a  $\mu$  opioid receptor (MOR1) in brain and spinal cord. *J Neurosci* 1995, 15: 3328–3341.
- [24] Zhang X, Bao L, Arvidsson U, Elde R, Hökfelt T. Localization and regulation of the delta-opioid receptor in dorsal root ganglia and spinal cord of the rat and monkey: evidence for association with the membrane of large dense-core vesicles. *Neuroscience* 1998, 82: 1225–1242.
- [25] Zhang X, Bao L, Shi TJ, Ju G, Elde R, Hökfelt T. Down-regulation of mu-opioid receptors in rat and monkey dorsal root ganglion neurons and spinal cord after peripheral axotomy. *Neuroscience* 1998, 82: 223–240.
- [26] Walwyn W, Maidment NT, Sanders M, Evans CJ, Kieffer BL, Hales TG. Induction of  $\delta$  opioid receptor function by up-regulation of membrane receptors in mouse primary afferent neurons. *Mol Pharmacol* 2005, 68: 1688–1698.
- [27] Rau KK, Caudle RM, Cooper BY, Johnson RD. Diverse immunocytochemical expression of opioid receptors in electrophysiological

- cally defined cells of rat dorsal root ganglia. *J Chem Neuroanatomy* 2005, 29: 255–264.
- [28] Wu ZZ, Chen SR, Pan HL. Differential sensitivity of N- and P/Q-type  $\text{Ca}^{2+}$  channel currents to a  $\mu$  opioid in isolectin B4-positive and -negative dorsal root ganglion neurons. *J Pharmacol Exp Ther* 2004, 311: 939–947.
- [29] Ji RR, Zhang Q, Law PY, Low HH, Elde R, Hökfelt T. Expression of  $\mu$ -,  $\delta$ -, and  $\kappa$ -opioid receptor-like immunoreactivities in rat dorsal root ganglia after carrageenan-induced inflammation. *J Neurosci* 1995, 15: 8156–8166.
- [30] Bao L, Wang HF, Cai HJ, Tong YG, Jin SX, Lu YJ, *et al.* Peripheral axotomy induces only very limited sprouting of coarse myelinated afferents into inner lamina II of rat spinal cord. *Eur J Neurosci* 2002, 16: 175–185.
- [31] Joseph EK, Levine JD. Mu and delta opioid receptors on nociceptors attenuate mechanical hyperalgesia in rat. *Neuroscience* 2010, 171: 344–350.
- [32] Beaudry H, Dubois D, Gendron L. Activation of spinal  $\mu$ - and  $\delta$ -opioid receptors potently inhibits substance P release induced by peripheral noxious stimuli. *J Neurosci* 2011, 31: 13068–13077.
- [33] van Rijn RM, Brissett DI, Whistler JL. Emergence of functional spinal delta opioid receptors after chronic ethanol exposure. *Biol Psychiatry* 2012, 71: 232–238.
- [34] Gupta A, Mulder J, Gomes I, Rozenfeld R, Bushlin I, Ong E, *et al.* Increased abundance of opioid receptor heteromers after chronic morphine administration. *Sci Signal* 2010, 3: ra54.
- [35] Zhang X, Bao L, Guan JS. Role of delivery and trafficking of  $\delta$ -opioid peptide receptors in opioid analgesia and tolerance. *Trends Pharmacol Sci* 2006, 27: 324–329.
- [36] van Rijn RM, Whistler JL, Waldhoer M. Opioid-receptor-heteromer-specific trafficking and pharmacology. *Curr Opin Pharmacol* 2010, 10: 73–79.
- [37] Stockton SD Jr, Devi LA. Functional relevance of  $\mu$ - $\delta$  opioid receptor heteromerization: A Role in novel signaling and implications for the treatment of addiction disorders. *Drug Alcohol Depend* 2012, 121(3): 167–172.
- [38] Bao L, Jin SX, Zhang C, Wang LH, Xu ZZ, Zhang FX, *et al.* Activation of delta opioid receptors induces receptor insertion and neuropeptide secretion. *Neuron* 2003, 37: 121–133.
- [39] Zhao B, Wang HB, Lu YJ, Hu JW, Bao L, Zhang X. Transport of receptors, receptor signaling complexes and ion channels via neuropeptide-secretory vesicles. *Cell Res* 2011, 21: 741–753.
- [40] Cheng PY, Svingos AL, Wang H, Clarke CL, Jenab S, Beczkowska IW, *et al.* Ultrastructural immunolabeling shows prominent pre-synaptic vesicular localization of delta-opioid receptor within both enkephalin- and nonenkephalin-containing axon terminals in the superficial layers of the rat cervical spinal cord. *J Neurosci* 1995, 15: 5976–5988.
- [41] Zhang X, Bao L, Ma GQ. Sorting of neuropeptides and neuropeptide receptors into secretory pathways. *Prog Neurobiol* 2010, 90: 276–283.
- [42] Ma GQ, Wang B, Wang HB, Wang Q, Bao L. Short elements with charged amino acids form clusters to sort protachykinin into large dense-core vesicles. *Traffic* 2008, 9: 2165–2179.
- [43] Decaillot FM, Befort K, Filliol D, Yue S, Walker P, Kieffer BL. Opioid receptor random mutagenesis reveals a mechanism for G protein-coupled receptor activation. *Nat Struct Biol* 2003, 10: 629–636.
- [44] Meng F, Ueda Y, Hoversten MT, Thompson RC, Taylor L, Watson SJ, *et al.* Mapping the receptor domains critical for the binding selectivity of delta-opioid receptor ligands. *Eur J Pharmacol* 1996, 311: 285–292.
- [45] Pepin MC, Yue SY, Roberts E, Wahlestedt C, Walker P. Novel "restoration of function" mutagenesis strategy to identify amino acids of the delta-opioid receptor involved in ligand binding. *J Biol Chem* 1997, 272: 9260–9267.
- [46] Varga EV, Li X, Stropova D, Zalewska T, Landsman RS, Knapp RJ, *et al.* The third extracellular loop of the human delta-opioid receptor determines the selectivity of delta-opioid agonists. *Mol Pharmacol* 1996, 50: 1619–1624.
- [47] Wang WW, Shahrestanifar M, Jin J, Howells RD. Studies on mu and delta opioid receptor selectivity utilizing chimeric and site-mutagenized receptors. *Proc Natl Acad Sci U S A* 1995, 92: 12436–12440.
- [48] Gendron L, Lucido AL, Mennicken F, O'Donnell D, Vincent JP, Stroh T, *et al.* Morphine and pain-related stimuli enhance cell surface availability of somatic  $\delta$ -opioid receptors in rat dorsal root ganglia. *J Neurosci* 2006, 26: 953–962.
- [49] Ma J, Zhang Y, Kalyuzhny AE, Pan ZZ. Emergence of functional delta-opioid receptors induced by long-term treatment with morphine. *Mol Pharmacol* 2006, 69: 1137–1145.
- [50] Cahill CM, Morinville A, Lee MC, Vincent JP, Collier B, Beaudet A. Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. *J Neurosci* 2001, 21: 7598–7607.
- [51] Patwardhan AM, Berg KA, Akopain AN, Jeske NA, Gamper N, Clarke WP, *et al.* Bradykinin-induced functional competence and trafficking of the  $\delta$ -opioid receptor in trigeminal nociceptors. *J Neurosci* 2005, 25: 8825–8832.
- [52] Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Laustriat D, Cao YQ, *et al.* Knockin mice expressing fluorescent  $\delta$ -opioid receptors uncover G protein-coupled receptor dynamics *in vivo*. *Proc Natl Acad Sci U S A* 2006, 103: 9691–9696.
- [53] Li KC, Zhang FX, Li CL, Wang F, Yu MY, Zhong YQ, *et al.* Follistatin-like 1 suppresses sensory afferent transmission by activating  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. *Neuron* 2011, 69: 974–987.



- [54] Hamada K, Matsuura H, Sanada M, Toyoda F, Omatsu-Kanbe M, Kashiwagi A, *et al.* Properties of the  $\text{Na}^+/\text{K}^+$  pump current in small neurons from adult rat dorsal root ganglia. *Br J Pharmacol* 2003, 138: 1517–1527.
- [55] Mata M, Siegel GJ, Hieber V, Beaty MW, Fink DJ. Differential distribution of  $\text{Na,K-ATPase}$  alpha isoform mRNAs in the peripheral nervous system. *Brain Res* 1991, 546: 47–54.
- [56] Wu ZZ, Cai YQ, Pan HL. A functional link between T-type calcium channels and  $\mu$ -opioid receptor expression in adult primary sensory neurons. *J Neurochem* 2009, 109: 867–878.
- [57] Deng H, Yang Z, Li Y, Bao G, Friedrich T, Gu Q, *et al.* Interactions of  $\text{Na}^+/\text{K}^+ \text{-ATPase}$  and co-expressed delta-opioid receptor. *Neurosci Res* 2009, 65: 222–227.
- [58] Heinke B, Gingl E, Sandkuhler J. Multiple targets of  $\mu$ -opioid receptor-mediated presynaptic inhibition at primary afferent A $\delta$ - and C-fibers. *J Neurosci* 2011, 31: 1313–1322.
- [59] Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA. Oligomerization of opioid receptors with  $\beta_2$ -adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. *Proc Natl Acad Sci U S A* 2001, 98: 343–348.
- [60] Jordan BA, Gomes I, Rios C, Filipovska J, Devi LA. Functional interactions between  $\mu$  opioid and  $\alpha_{2A}$ -adrenergic receptors. *Mol Pharmacol* 2003, 64: 1317–1324.
- [61] Overland AC, Kitto KF, Chabot-Dore AJ, Rothwell PE, Fairbanks CA, Stone LS, *et al.* Protein kinase C mediates the synergistic interaction between agonists acting at  $\alpha_2$ -adrenergic and delta-opioid receptors in spinal cord. *J Neurosci* 2009, 29: 13264–13273.
- [62] Petäjä-Repo UE, Hogue M, Bhalla S, Laperriere A, Morello JP, Bouvier M. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. *EMBO J* 2002, 21: 1628–1637.
- [63] Bie B, Zhang Z, Cai YQ, Zhu W, Zhang Y, Dai J, *et al.* Nerve growth factor-regulated emergence of functional  $\delta$ -opioid receptors. *J Neurosci* 2010, 30: 5617–5628.
- [64] Margolis EB, Mitchell JM, Hjelmstad GO, Fields HL. A novel  $\delta$  opioid receptor-mediated enhancement of GABA $_A$  receptor function induced by stress in ventral tegmental area neurons. *J Physiol* 2011, 589: 4229–4242.
- [65] Milan-Lobo L, Whistler JL. Heteromerization of the  $\mu$ - and  $\delta$ -opioid receptors produces ligand-biased antagonism and alters  $\mu$ -receptor trafficking. *J Pharmacol Exp Ther* 2011, 337: 868–875.
- [66] Cesselin F, Bourgoin S, Clot AM, Hamon M, Le Bars D. Segmental release of Met-enkephalin-like material from the spinal cord of rats, elicited by noxious thermal stimuli. *Brain Res* 1989, 484: 71–77.
- [67] Cheng ZJ, Yu QM, Wu YL, Ma L, Pei G. Selective interference of  $\beta$ -arrestin 1 with  $\kappa$  and  $\delta$  but not  $\mu$  opioid receptor/G protein coupling. *J Biol Chem* 1998, 273: 24328–24333.
- [68] Rozenfeld R, Devi LA. Receptor heterodimerization leads to a switch in signaling:  $\beta$ -arrestin2-mediated ERK activation by  $\mu$ - $\delta$  opioid receptor heterodimers. *FASEB J* 2007, 21: 2455–2465.
- [69] Chao D, Xia Y. Ionic storm in hypoxic/ischemic stress: can opioid receptors subside it? *Prog Neurobiol* 2010, 90: 439–470.
- [70] Pradhan AA, Befort K, Nozaki C, Gaveriaux-Ruff C, Kieffer BL. The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol Sci* 2011, 32: 581–590.
- [71] Berger AC, Whistler JL. How to design an opioid drug that causes reduced tolerance and dependence. *Ann Neurol* 2010, 67: 559–569.
- [72] Kim HJ, Seol TK, Lee HJ, Yaksh TL, Jun JH. The effect of intrathecal mu, delta, kappa, and alpha-2 agonists on thermal hyperalgesia induced by mild burn on hind paw in rats. *J Anesth* 2011, 25: 884–891.
- [73] Janecka A, Fichna J, Janecki T. Opioid receptors and their ligands. *Curr Top Med Chem* 2004, 4: 1–17.
- [74] Trescot AM, Datta S, Lee M, Hansen H. Opioid pharmacology. *Pain Physician* 2008, 11: S133–153.
- [75] Matthes HW, Smadja C, Valverde O, Vonesch JL, Foutz AS, Boudinot E, *et al.* Activity of the  $\delta$ -opioid receptor is partially reduced, whereas activity of the  $\kappa$ -receptor is maintained in mice lacking the  $\mu$ -receptor. *J Neurosci* 1998, 18: 7285–7295.
- [76] Scherrer G, Befort K, Contet C, Becker J, Matifas A, Kieffer BL. The delta agonists DPDPE and deltorphin II recruit predominantly mu receptors to produce thermal analgesia: a parallel study of mu, delta and combinatorial opioid receptor knockout mice. *Eur J Neurosci* 2004, 19: 2239–2248.
- [77] van Rijn RM, Whistler JL. The  $\delta 1$  opioid receptor is a heterodimer that opposes the actions of the  $\delta 2$  receptor on alcohol intake. *Biol Psychiatry* 2009, 66: 777–784.
- [78] Dubois D, Gendron L. Delta opioid receptor-mediated analgesia is not altered in preprotachykinin A knockout mice. *Eur J Neurosci* 2010, 32: 1921–1929.
- [79] Wrigley PJ, Jeong HJ, Vaughan CW. Dissociation of  $\mu$ - and  $\delta$ -opioid inhibition of glutamatergic synaptic transmission in superficial dorsal horn. *Mol Pain* 2010, 6: 71.
- [80] Manchikanti L, Singh A. Therapeutic opioids: a ten-year perspective on the complexities and complications of the escalating use, abuse, and nonmedical use of opioids. *Pain Physician* 2008, 11: S63–88.
- [81] Fields HL. The doctor's dilemma: opiate analgesics and chronic pain. *Neuron* 2011, 69: 591–594.
- [82] Fields H. State-dependent opioid control of pain. *Nat Rev Neurosci* 2004, 5: 565–575.
- [83] Abdelhamid EE, Sultana M, Portoghesi PS, Takemori AE. Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. *J Pharmacol Exp Ther* 1991, 258: 299–303.
- [84] Schiller PW. Bi- or multifunctional opioid peptide drugs. *Life Sci*

- 2010, 86: 598–603.
- [85] Schiller PW, Fundytus ME, Merovitz L, Weltrowska G, Nguyen TM, Lemieux C, *et al.* The opioid  $\mu$  agonist/ $\delta$  antagonist DIPP-NH<sub>2</sub>[ $\Psi$ ] produces a potent analgesic effect, no physical dependence, and less tolerance than morphine in rats. *J Med Chem* 1999, 42: 3520–3526.
- [86] Schiller PW, Weltrowska G, Berezowska I, Nguyen TM, Wilkes BC, Lemieux C, *et al.* The TIPP opioid peptide family: development of  $\delta$  antagonists,  $\delta$  agonists, and mixed  $\mu$  agonist/ $\delta$  antagonists. *Biopolymers* 1999, 51: 411–425.
- [87] George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, *et al.* Oligomerization of  $\mu$ - and  $\delta$ -opioid receptors. Generation of novel functional properties. *J Biol Chem* 2000, 275: 26128–26135.
- [88] Jordan BA, Devi LA. G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 1999, 399: 697–700.
- [89] Law PY, Erickson-Herbrandson LJ, Zha QQ, Solberg J, Chu J, Sarre A, *et al.* Heterodimerization of  $\mu$ - and  $\delta$ -opioid receptors occurs at the cell surface only and requires receptor-G protein interactions. *J Biol Chem* 2005, 280: 11152–11164.
- [90] Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, Devi LA. A role for heterodimerization of  $\mu$  and  $\delta$  opiate receptors in enhancing morphine analgesia. *Proc Natl Acad Sci U S A* 2004, 101: 5135–5139.
- [91] Xie WY, He Y, Yang YR, Li YF, Kang K, Xing BM, *et al.* Disruption of Cdk5-associated phosphorylation of residue threonine-161 of the  $\delta$ -opioid receptor: impaired receptor function and attenuated morphine antinociceptive tolerance. *J Neurosci* 2009, 29: 3551–3564.
- [92] Minneman KP. Heterodimerization and surface localization of G protein coupled receptors. *Biochem Pharmacol* 2007, 73: 1043–1050.
- [93] He L, Fong J, von Zastrow M, Whistler JL. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. *Cell* 2002, 108: 271–282.
- [94] Yu YJ, Dhavan R, Chevalier MW, Yudowski GA, von Zastrow M. Rapid delivery of internalized signaling receptors to the somatodendritic surface by sequence-specific local insertion. *J Neurosci* 2010, 30: 11703–11714.
- [95] Puthenveedu MA, Lauffer B, Temkin P, Vistein R, Carlton P, Thorn K, *et al.* Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. *Cell* 2010, 143: 761–773.