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·Review·

Interaction and regulatory functions of μ- and δ-opioid receptors in nociceptive afferent neurons

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Abstract: μ-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 δ-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 Aδ- 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 μ- and δ-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.

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2 Expression of opioid receptors in nociceptive afferent neurons

Autoradiographic studies provide evidence showing the presence of opioid receptors in afferent Aδ- and C-fibers in laminae I and II of the spinal cord, including the binding sites for DOR and MOR agonists[1-5]. 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 Aδ-fibers is inhibited by the activa-

tion of DORs[6,7]. 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[8-12]. 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*)[13-15].

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[10,11,15,16]. However, the proper dilutions of the same antibodies are required to immunostain DORs specifically in the DRGs and laminae I and II[10]. 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[10,17]. It is possible that the antibodies recognize DORs in different states of activation, conformation, glycosylation and/or palmitoylation[18-21]. To exclude the possibility of non-specific immunostaining[22], it is important to carefully assess the antibody specificity and comprehensively analyze the distribution of DORs with multiple experimental approaches[10].

Based on the findings of DOR- and MOR-mediated inhibitory effects on both the Ca^{2+} currents in small DRG neurons and the release of substance P from C- and Aδ-afferents, as well as the results of immunostaining[7,16,17,23-29], 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[22]. 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[22]. 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[30].

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[10,11,31-33]. Importantly, using antibodies that recognize DOR/MOR heteromers, Gupta *et al.*[34] 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[35-37].

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[10,17,38,39] (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[10] (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[10,17,24,38-41]. Moreover, the LDCV localization of DORs was found to be disrupted in the small DRG neurons of protachykinin 1 gene (*Tac1*)-knockout mice[10,17,41,42] (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[17,43-47]. 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[26,33,34,38,48-51].

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[10,25,41]. 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[34]. 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[10,22,52]. It remains largely unknown why the DORs expressed in large DRG neurons cannot be efficiently transported to the terminals of Aβ-afferent fibers in the deep laminae of the spinal cord.

Using liquid chromatography-mass spectrometry combined with immunoblotting of subcellular fractions, Zhao *et al.*[39] 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, β_2-adrenergic receptor, $G_{\alpha i2}$, voltage-gated calcium channel α2δ1 subunit and P2X purinoceptor 2 are localized in substance P-positive LDCVs in small DRG neurons, whereas β_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 β2 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[53-58]. In addition, DORs and MORs may interact with β_2- and α_{2A}-adrenergic receptors that are expressed in DRG neurons, respectively[39,59-61]. 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[62]. 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[63,64].

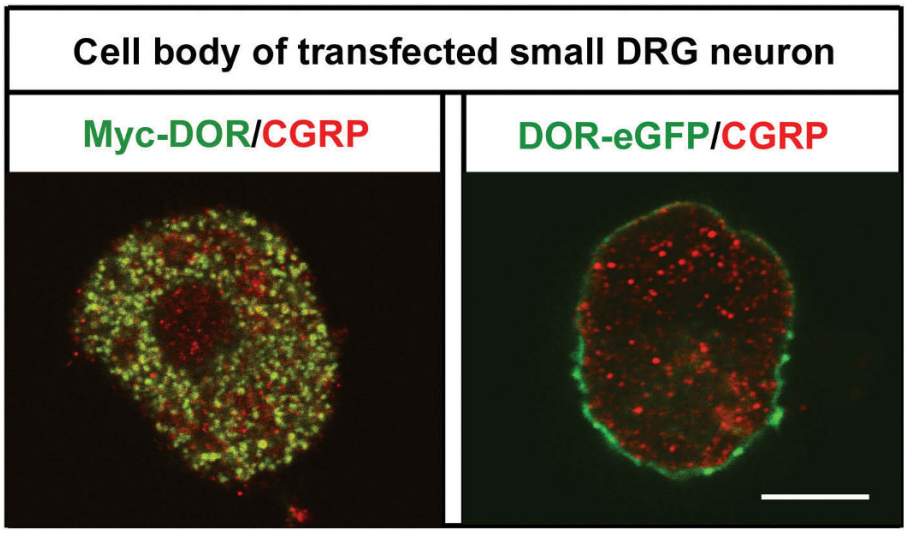

Fig. 1. Myc-δ-opioid receptor (DOR), but not DOR-enhanced green fluorescent protein (eGFP), can be sorted into large dense-core vesicles (LDCVs) in small dorsal root ganglion (DRG) neurons. Dissociated DRG neurons were transfected with plasmid expressing Myc-DOR or DOR-eGFP by electroporation. Double-immunostaining 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 μm. Images unpublished. See more details in Wang *et al.,* Proc Natl Acad Sci U S A, 2010[10].

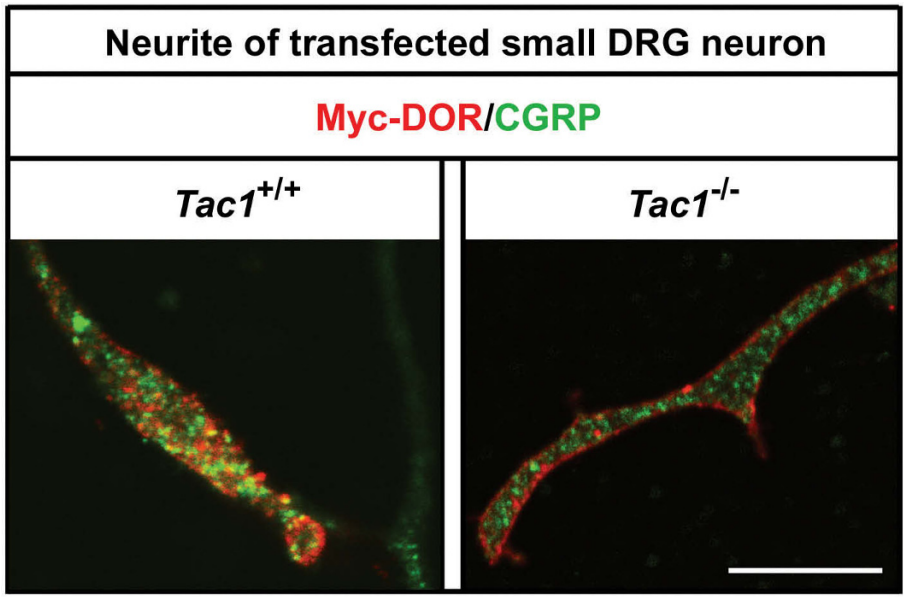

Fig. 2. Protachykinin-dependent large dense-core vesicle (LDCV)-localization and transport of δ-opioid receptors (DORs). Dissociated dorsal root ganglion (DRG) neurons were transfected with Myc-DOR by electroporation. *Tac1* encodes protachykinin, which is a precursor protein of the neuropeptide substance 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*$^{+/+}$). 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*$^{-/-}$). Scale bar, 8 μm. Images unpublished. See more details in Wang *et al.,* Proc Natl Acad Sci U S A, 2010[10].

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[10,11,27,29,31-34], the functional analysis of MOR/DOR heteromers would be critical for understanding opioid physiology and pharmacology[35-37]. 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[11,65]. The receptor complexes internalized by DOR agonists are ubiquitinated for lysosomal degradation, leading to a reduction of surface MORs[11]. In addition, a basal level of co-internalization and co-degradation of DORs and MORs occurs in the transfected cells[11,65]. 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[11,66]. The co-degradation of these opioid receptors is enhanced by treatment with exogenously applied DOR agonists[11] 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[65]. MOR/DOR heteromers could recruit β-arrestin, while DORs but not MORs are normally coupled with β-arrestin[67,68]. 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[35-37,69-71].

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[22]. 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[11,12,31,33,72], 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 Ca^{2+} current in small DRG neurons is enhanced after 10-Hz electrical stimulation[10]. Opioid receptor ligands are known to bind to opioid receptor subtypes with various affinities[73,74]. 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, ~10-fold lower affinity for MORs, and very low affinity for κ-opioid receptors (KORs); β-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 ~1 000-fold higher affinity for MORs over DORs. Deltorphin II binds to DORs with ~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[15,22,33,75-78]. 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[79]. However, after treatment with the TRP agonist icilin, the presynaptic inhibition induced by the DOR agonist increases and is blocked by the DOR antagonist[79], 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[80-82]. Early pharmacological studies showed that blockade of DORs often results in enhanced morphine analgesia and reduced tolerance[83-86], suggesting that DORs interact with MORs in the pain pathway[87-90]. Further studies revealed that morphine tolerance can be reduced by preventing DOR phosphorylation, deleting either *Oprd1* or *Penk1*, or deleting *Tac1* that reduces DOR transport to the spinal dorsal horn via LDCVs[14,15,17,91].

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[11]. 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[39] (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[11,65,93-95].

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.

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