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Themed Section: Opioids: New Pathways to Functional Selectivity

REVIEW

Opioid receptor trafficking and interaction in nociceptors

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Opiate analgesics such as morphine are often used for pain therapy. However, antinociceptive tolerance and dependence may develop with long-term use of these drugs. It was found that μ-opioid receptors can interact with δ-opioid receptors, and morphine antinociceptive tolerance can be reduced by blocking δ-opioid receptors. Recent studies have shown that μ- and δ-opioid receptors are co-expressed in a considerable number of small neurons in the dorsal root ganglion. The interaction of μ-opioid receptors with δ-opioid receptors in the nociceptive afferents is facilitated by the stimulus-induced cell-surface expression of δ-opioid receptors, and contributes to morphine tolerance. Further analysis of the molecular, cellular and neural circuit mechanisms that regulate the trafficking and interaction of opioid receptors and related signalling molecules in the pain pathway would help to elucidate the mechanism of opiate analgesia and improve pain therapy.

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Abbreviations
CGRP, calcitonin gene-related peptide; DOPr-eGFP, δ-opioid receptors inserted with the enhanced green fluorescent protein at the C-terminus; DAMGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; DRG, dorsal root ganglion; HA, haemagglutinin; IB4, isolectin B4; LDCV, large dense-core vesicle; PC12 cell, phaeochromocytoma cell; PM, plasma membrane

Background

Morphine is widely used for pain therapy. However, its clinical applications are often limited by the development of antinociceptive tolerance. That is, when a dose of morphine is given repeatedly and selectively for a single condition, it gradually loses its antinociceptive potency (Fields, 2004; 2011; Manchikanti and Singh, 2008). Studies over the past few decades have demonstrated the presence of many types of opioid receptor in the nociceptive sensory neurons and their Aδ- and C-fibre terminals in the superficial dorsal horn of the spinal cord (Fields *et al.*, 1980; Moskowitz and Goodman, 1984; Gouarderes *et al.*, 1991; Besse *et al.*, 1992; Mennicken *et al.*, 2003). The μ- and δ-opioid receptors are predominantly found to be expressed in small-diameter neurons of the dorsal root ganglion (DRG). These small DRG neurons convey the signals from peripheral nociceptors, thermoreceptors and sensitive mechanoreceptors to the superficial dorsal horn of spinal cord, and cause the release of the excitatory neurotransmitter glutamate, as well as the neuropeptides substance P and calcitonin gene-related peptide (CGRP), from the afferent terminals. This excitatory neurotransmission can be presynaptically inhibited by activating μ- or δ-opioid receptors (Ueda *et al.*, 1995; Zachariou and Goldstein, 1996; Beaudry *et al.*, 2011). Therefore, it is of interest to explore the molecular and cellular mechanisms that regulate opioid analgesia and tolerance. The present review discusses the expression, intracellular trafficking and interaction of opioid receptors in the pain pathway with a focus on small DRG neurons and the role of opioid receptor interactions in the modulation of opiate analgesia and antinociceptive tolerance.

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Co-expression of opioid receptors in nociceptive afferent neurons

Early autoradiographic studies showed that many opioid receptors and the binding sites for μ- and δ-opioid receptor agonists, are present in nociceptive afferent Aδ- and C-fibres terminating in the superficial dorsal horn of the spinal cord (Fields *et al.*, 1980; Moskowitz and Goodman, 1984; Gouarderes *et al.*, 1991; Besse *et al.*, 1992; Mennicken *et al.*, 2003). Moreover, the release of the excitatory neurotransmitter glutamate, the neuropeptides substance P and CGRP from afferent C- and Aδ-fibres could be inhibited by activating δ-opioid receptors with several δ-opioid receptor agonists (Ueda *et al.*, 1995; Zachariou and Goldstein, 1996; Beaudry *et al.*, 2011; Normandin *et al.*, 2013), suggesting the presynaptic localization of δ-opioid receptors on nociceptive afferents. This notion was supported by the finding that δ-opioid receptor mRNA is present in about 70% of DRG neurons, including both peptidergic [isolectin B4 (IB4)-negative] and non-peptidergic (IB4-positive) subsets of small neurons and mechanoreceptive large neurons, while μ-opioid receptors were expressed in the subsets of small DRG neurons and some large DRG neurons (Arvidsson *et al.*, 1995a; Minami *et al.*, 1995a; Wang and Wessendorf, 2001; Wang *et al.*, 2010; Gaveriaux-Ruff *et al.*, 2011; He *et al.*, 2011). The level of δ-opioid receptor mRNA in small DRG neurons is generally lower than that in large DRG neurons (Wang *et al.*, 2010). About one-third of δ-opioid receptor-expressing DRG neurons contain neuropeptides, such as substance P and CGRP (Wang *et al.*, 2010). Co-expression of μ- and δ-opioid receptors was found in a substantial population of peptidergic small DRG neurons by using single-cell PCR, *in situ* double hybridization and other approaches (Joseph and Levine, 2010; van Rijn *et al.*, 2010; Wang *et al.*, 2010; Beaudry *et al.*, 2011; He *et al.*, 2011). μ- and δ-opioid receptors are also co-expressed in small DRG neurons which do not contain substance P (Wang *et al.*, 2010).

To determine the amount of δ-opioid receptor protein, immunoblot analyses were carried out with δ-opioid receptor antibodies; these specifically detected δ-opioid receptors in the DRGs and the dorsal spinal cord of wild-type mice, but not in δ-opioid receptor-deficient mice (Wang *et al.*, 2010; He *et al.*, 2011; Zhao *et al.*, 2011). It is notable that the correct dilutions of the same antibodies should be used to determine the specific immunostaining of δ-opioid receptors in the DRGs and spinal cord (Wang *et al.*, 2010; Zhang and Bao, 2012). Although some antibodies specifically labelled δ-opioid receptors in the peptidergic small DRG neurons and large DRG neurons (Arvidsson *et al.*, 1995a; Guan *et al.*, 2005; Wang *et al.*, 2010; He *et al.*, 2011), these antibodies could not simultaneously label the δ-opioid receptors expressed in all subsets of DRG neurons and the receptors in all afferent terminals in the spinal cord. Thus, the immunostaining patterns in the spinal cord only partially represent the autoradiographic patterns of the binding sites of δ-opioid receptor agonists. For instance, the antibodies that stain δ-opioid receptors in small DRG neurons could detect the receptors accumulated in the afferent terminals in the superficial dorsal horn of spinal cord, but not the receptors in the spinal cord neurons. However, double immunostaining can be used to demonstrate the co-expression of δ- and μ-opioid receptors in small DRG neurons and their afferent terminals. Importantly, Gupta *et al.* have developed antibodies that recognize the δ-/μ-opioid receptor heteromer, and showed the presence of this opioid receptor heteromer in both small and large DRG neurons (Gupta *et al.*, 2010), supporting the notion that δ- and μ-opioid receptors are co-expressed in these neurons.

In addition to the above approaches, the expression of opioid receptors fused with various fluorescent proteins or epitope tags in animals may help to study the distribution of opioid receptors. However, in the mouse the expression of δ-opioid receptors inserted with the enhanced green fluorescent protein at the C-terminus (DOPr-eGFP) was only found in ~17% of DRG neurons by immunostaining with the antibody against GFP and most of these neurons were large neurons (Scherrer *et al.*, 2009). Moreover, DOPr-eGFP were not detected in μ-opioid receptor-containing small DRG neurons, suggesting that the δ-opioid receptor might not coexist with the μ-opioid receptor in nociceptive DRG neurons. These data do not accord with the results obtained using other multiple approaches, and could be due to a reduced expression of DOPr-eGFP or a degradation of the fusion protein which could not correctly move into the secretory pathway and get transported to the afferent axons (Guan *et al.*, 2005; Wang *et al.*, 2008; 2010). A recent study was able to show the presence of DOPr-eGFP in a few substance P-containing small DRG neurons, and coexistence of DOPr-eGFP and μ-opioid receptor in a small population of large DRG neurons that contained CGRP (Bardoni *et al.*, 2014). Therefore, it is also possible that the methods used in these studies are still not sensitive enough to detect the receptor expressed at low levels in various types of neurons. The distribution of DOPr-eGFP cannot fully represent the endogenous δ-opioid receptor in all subsets of DRG neurons (Zhang and Bao, 2012). It is still not known whether the *in vivo* expression of δ-opioid receptors inserted with a small tag, such as haemagglutinin (HA), Myc or Flag, might be better than GFP for showing their distribution in all subsets of DRG neurons.

The co-expression of δ- and μ-opioid receptors in peptidergic small DRG neurons is also supported by the finding that both of these receptors mediated inhibitory effects on the Ca^{2+} currents in the same small DRG neurons and the release of substance P from C- and Aδ-afferents (Arvidsson *et al.*, 1995a,b; Ji *et al.*, 1995; Zachariou and Goldstein, 1996; Zhang *et al.*, 1998a,b; Wu *et al.*, 2004; Guan *et al.*, 2005; Rau *et al.*, 2005; Walwyn *et al.*, 2005; Beaudry *et al.*, 2011; Kouchek *et al.*, 2013; Normandin *et al.*, 2013). Taken together, these results suggest that the co-expression of δ- and μ-opioid receptors in certain populations of small DRG neurons is the cellular basis for opioid receptor interactions in the pain pathway.

Distinct subcellular distribution of opioid receptors

Newly synthesized receptors are usually processed in the Golgi complex and assemble in various microvesicles in the constitutive secretory pathway to be transported and inserted into the plasma membrane (PM) spontaneously,

Table 1

Subcellular distribution of δ-opioid receptor (DOPr) and μ-opioid receptor (MOPr) in DRG neurons, PC12 cells and HEK293 cells

Receptor subtype	Subcellular localization	Cell type	Method	Reference
DOPr	PM, LDCV, MV, endosome, TGN	Small DRG neuron	IFM, IEM, IB	Cheng *et al.*, 1995; Zhang *et al.*, 1998a; Bao *et al.*, 2003; Guan *et al.*, 2005; Wang *et al.*, 2010; Gupta *et al.*, 2010; Zhao *et al.*, 2011
	PM, GN	Large DRG neuron	IFM	Wang *et al.*, 2010; Gupta *et al.*, 2010
	PM, LDCV, MV, TGN	PC12 cell	IFM, IEM, IB	Bao *et al.*, 2003; Guan *et al.*, 2005; Wang *et al.*, 2008
	PM, MV, TGN	HEK293 cell (Tg)	IFM, IEM	Guan *et al.*, 2005; Wang *et al.*, 2010
DOPr-HA (or -Myc, -FLAG)	PM, LDCV, TGN	Small DRG neuron (Tg)	IFM	Wang *et al.*, 2010
	PM, GN	Large DRG neuron (Tg)	IFM	Wang *et al.*, 2010
	PM, LDCV, MV, TGN	PC12 cell (Tg)	IFM, IEM	Bao *et al.*, 2003; Guan *et al.*, 2005; Wang *et al.*, 2008; Wang *et al.*, 2010
	PM, MV, TGN	HEK293 cell (Tg)	IFM, IEM	Whistler *et al.*, 2001; Whistler *et al.*, 2002; Guan *et al.*, 2005; Rozenfeld and Devi, 2007; Wang *et al.*, 2010; Milan-Lobo and Whistler, 2011; He *et al.*, 2011
DOPr-GFP (or -RFP)	PM, GN	Small DRG neuron (Tg)		Wang *et al.*, 2010; Pradhan *et al.*, 2010; Pettinger *et al.*, 2013
	PM, GN	Large DRG neuron (Tg)		Pradhan *et al.*, 2009; Wang *et al.*, 2010; Pradhan *et al.*, 2010
	PM, GN	PC12 cell (Tg)		Wang *et al.*, 2008
	PM, GN	HEK293 cell (Tg)		Kabli *et al.*, 2010
MOPr	PM, MV, TGN	Small DRG neuron	IFM, IEM	Zhang *et al.*, 1998b
	PM, GN	HEK293 cell (Tg)	IFM	Wang *et al.*, 2010
MOPr-HA (or -Myc, -FLAG)	PM, GN	PC12 cell (Tg)	IFM	Guan *et al.*, 2005
	PM, GN	HEK293 cell (Tg)	IFM	Whistler *et al.*, 1999; Whistler *et al.*, 2002; He *et al.*, 2002; Pfeiffer *et al.*, 2002; Rozenfeld and Devi, 2007; Wang *et al.*, 2010; Milan-Lobo and Whistler, 2011
MOPr-GFP	PM, GN	HEK293 cell (Tg)		Celver *et al.*, 2004; Kabli *et al.*, 2010

MV, microvesicle; TGN, trans-Golgi network; Tg, transgene; IFM, immunofluorescent microscopy; IEM, immuno-electron microscopy; IB, immunoblotting.

while secretory polypeptides and proteins are collected in large dense-core vesicles (LDCVs) in the regulated secretory pathway to be stored in the cytoplasm and released at the PM in response to stimuli that increase the intracellular levels of Ca^{2+}. Immunostaining with antibodies against δ-opioid receptors or epitope-tag HA and Myc shows that both the endogenous δ-opioid receptor and exogenously expressed HA- and Myc-δ-opioid receptors are mainly located intracellularly and often associated with LDCVs in both peptidergic small DRG neurons and phaeochromocytoma (PC12) cells, whereas HA- or Myc-δ-opioid receptors are mostly present on the cell surface of large DRG neurons and HEK293 cells, which do not contain LDCVs and neuropeptides (Table 1; Cheng *et al.*, 1995; Bao *et al.*, 2003; Guan *et al.*, 2005; Wang *et al.*, 2010; Zhao *et al.*, 2011; Zhang and Bao, 2012). In addition, the scattered distribution of δ-opioid receptors in the cytoplasm indicates the receptors in the constitutive pathway. In contrast to the tagged δ-opioid receptors, HA- and Myc-μ-opioid receptors often appear on the cell surface, consistent with the localization of μ-opioid receptors shown using μ-opioid receptor antibodies (Table 1; Zhang *et al.*, 1998b; 2010; Wang *et al.*, 2010). Thus, in the steady state, there are two pools of opioid receptors in nociceptive afferent neurons, the surface pool containing mostly μ-opioid receptors and an intracellular pool of δ-opioid receptors (Figure 1).

It is of interest to elucidate the mechanisms that regulate receptor trafficking in different cell types. The HA- or Myc-tagged δ-opioid receptors present in the LDCV of transfected small DRG neurons could be shifted to the PM in the absence of a protachykinin that interacts with δ-opioid receptors, consistent with the reduction in δ-opioid receptors located in the LDCV of small DRG neurons observed in protachykinin gene-knockout mice (Guan *et al.*, 2005; Ma *et al.*, 2008; Wang *et al.*, 2010; Zhang *et al.*, 2010). Moreover, numerous δ-opioid receptors underwent degradation in the protachykinin-deficient small DRG neurons (Guan *et al.*, 2005). Thus, the δ-opioid receptor/protachykinin interaction is essential for the transporting of δ-opioid receptors into LDCVs in nociceptive sensory neurons. However, the mechanisms for the

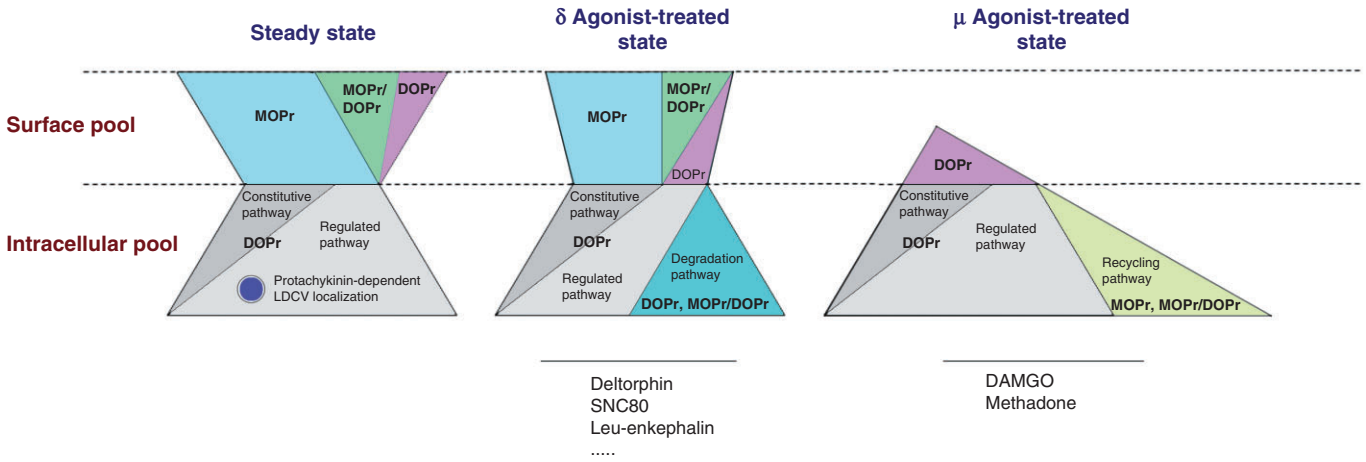

Figure 1

Proposed models of opioid receptor trafficking and interaction in nociceptive afferent neurons in response to the acute treatment with opioid agonists. There are two pools of δ-opioid receptors (DOPr), namely the surface pool and the intracellular pool, in peptidergic small DRG neurons, whereas the μ-opioid receptor (MOPr) is mainly present on the cell surface. In the steady state, the intracellular δ-opioid receptor can be transported via the LDCVs in the regulated secretory pathway, in addition to the constitutive transport of δ-opioid receptors via microvesicles. A limited number of surface δ-opioid receptors interact with μ-opioid receptors and form μ-/δ-opioid receptor heteromers. Both δ-opioid receptors and the heteromers are internalized following treatment with a δ-opioid receptor agonist, and processed for degradation. At the same time, the δ-agonists may induce a slow but lasting cell-surface expression of δ-opioid receptors that maintains the neuronal sensitivity to δ-agonists. Both μ-opioid receptors and the heteromers are internalized by some μ-opioid receptor agonists such as DAMGO, and processed for recycling.

transfer of δ-opioid receptors into secretory vesicles could be significantly different in the various types of neurons and cells.

The distributions of exogenous HA- or Myc-δ-opioid receptors and tagged μ-opioid receptors in DRG neurons are consistent with the distribution patterns of endogenous δ- and μ-opioid receptors shown with the δ- and μ-opioid receptor-specific antibodies (Cheng *et al.*, 1995; Zhang *et al.*, 1998a,b; 2010; Bao *et al.*, 2003; Guan *et al.*, 2005; Wang *et al.*, 2010; Zhao *et al.*, 2011). However, in small DRG neurons and PC12 cells transfected with the plasmids expressing DOPr-eGFP, the DOPr-eGFP cannot be sorted into LDCVs, but is transported via the constitutive secretory pathway to be inserted spontaneously into the PM (Table 1; Wang *et al.*, 2008; 2010; Zhang and Bao, 2012). Therefore, eGFP insertion may alter the intracellular trafficking of the newly synthesized receptors. It was also noticed that the δ-opioid receptor antibodies could not simultaneously label the δ-opioid receptors in LDCVs or in the PM of neurons, suggesting that the antibodies might preferentially recognize the δ-opioid receptors in different conformational or folding states. Interestingly, DOPr-eGFP synthesized in large DRG neurons could be transported to the peripheral terminals of Aβ-fibers in the skin (Bardoni *et al.*, 2014), but not to the central terminals of Aβ-fibres in the lamina III-V of spinal cord. This polarized transport of the receptor could be true, because neither autoradiography nor immunostaining could show the enrichment of δ-opioid receptors in Aβ-fibre terminals in the deep dorsal horn (Mennicken *et al.*, 2003; Wang *et al.*, 2010; see Zhang and Bao, 2012). However, both methods could detect the δ-opioid receptors stored in C- and Aδ-fibre terminals in the spinal lamina I–II, although the expression level of the receptor in small DRG neurons is much lower than that in large DRG neurons. Accumulating evidence suggest that the protein levels of δ-opioid receptors in the central or peripheral terminals are not always match to the levels of receptor mRNA in the DRG neurons, due to the differential processing of the synthesized receptors for transport in different types of neurons. Therefore, the mechanism for regulating the opioid receptor trafficking is an attractive research direction for the cell biology of neurons.

Stimulus-induced cell-surface expression of δ-opioid receptors and G-protein complex

It could be expected that the LDCV-localized δ-opioid receptors would be inserted into the PM when the exocytosis of LDCVs occurs in response to various stimuli, such as membrane depolarization and treatment with capsaicin or ATP, which elevate the intracellular levels of Ca^{2+} (Bao *et al.*, 2003; Wang *et al.*, 2010; Zhao *et al.*, 2011). The rate of δ-opioid receptor insertion is dependent on the pattern of Ca^{2+} elevation. The δ-opioid receptor insertion following treatment with δ-opioid receptor agonists, which induces a low level but long-lasting Ca^{2+} elevation, is more pronounced than that induced by K^+-induced membrane depolarization which induces a fast and high Ca^{2+} influx (Bao *et al.*, 2003; Figure 1). In fact, a number of chemical and behavioural stimuli, including sustained pain conditions and prolonged treatment of morphine or ethanol could induce δ-opioid receptor insertion (Cahill *et al.*, 2001; Bao *et al.*, 2003; Patwardhan *et al.*, 2005; Walwyn *et al.*, 2005; Gendron *et al.*, 2006; Ma *et al.*, 2006; Gupta *et al.*, 2010; van Rijn *et al.*, 2012; Pettinger

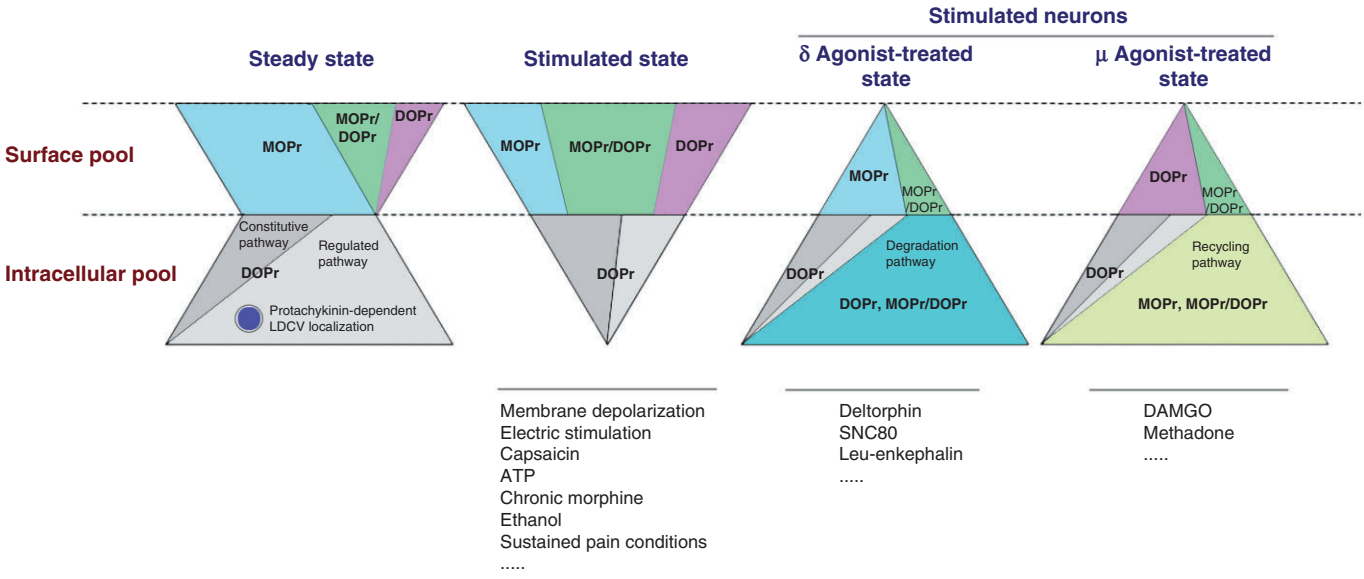

Figure 2

Proposed models of the stimulus-induced opioid receptor trafficking and interaction in nociceptive afferent neurons and the subsequent effects of agonist treatments. In the stimulated state induced by membrane depolarization and other stimuli, the δ-opioid receptor (DOPr) in the regulated pathway could be delivered to the surface pool and, therefore, increase the number of μ-/δ-opioid receptor heteromers as well as δ-opioid receptor monomers and homomers. When the stimulated neurons are further treated with δ-opioid receptor agonists, the heteromers, δ-opioid receptor monomers and homomers are internalized and processed for degradation. The reduction of μ-opioid receptors (MOPr) on the cell surface could be significant due to the increased number of μ-/δ-opioid receptor heteromers. In contrast, the heteromers, μ-opioid receptor monomers and homomers internalized by μ-opioid receptor agonists, such as DAMGO, could be recycled to the cell surface.

et al., 2013) (Figure 2). It is likely that in addition to δ-opioid receptors in the regulated secretory pathway, δ-opioid receptors in the constitutive secretory pathway could be also available for the stimulus-induced membrane insertion, since morphine-induced surface expression of δ-opioid receptors was shown in both small DRG neurons and the spinal dorsal horn neurons with the antibodies that seldom detected the LDCV-localized δ-opioid receptors (Cahill *et al.*, 2001; Gendron *et al.*, 2006). In contrast to the δ-opioid receptor, μ-opioid receptors are expressed on the cell surface without stimulation (Zhang *et al.*, 1998b; 2010; Wang *et al.*, 2010). Therefore, the μ-/δ-opioid receptor interaction is enhanced by the stimulus-induced δ-opioid receptor insertion, although the opioid receptor heteromers can also be present in the cytoplasm (Gupta *et al.*, 2010). In the stimulated state, the number of μ-/δ-opioid receptor heteromers in the surface pool is increased in nociceptive afferent neurons (Figure 2).

Recently, at least 298 proteins have been identified in the LDCV membrane purified from the dorsal spinal cord, including GPCRs, G-proteins and other signalling molecules, and ion channels (Zhao *et al.*, 2011). In small DRG neurons, δ-opioid receptor/$G_{\alpha i2}$/$G_{\beta 1\gamma 5}$/PLC β2 complexes are localized in the substance P-containing LDCVs. Electrical stimulation at 10 Hz increases the cell-surface level of δ-opioid receptors and $G_{\alpha i2}$ in small DRG neurons. In contrast to $G_{\alpha i2}$, G_o is mostly associated with the PM (Campbell *et al.*, 1993; Menon-Johansson and Dolphin, 1993; Zhao *et al.*, 2011). G_o contributes to maximally efficient signalling and the antinociceptive effects of μ-opioid receptors (Lamberts *et al.*, 2011). Therefore, stimuli, such as membrane depolarization and capsaicin treatment, could induce the cell-surface expression of a preassembled δ-opioid receptor/G-protein complex, which may interact with the surface μ-opioid receptor and G_o (Figure 3). Furthermore, δ-opioid receptor-mediated functions may also be regulated by the δ-opioid receptor interaction with numerous membrane proteins such as Ca^{2+} channels and Na^+, K^+-ATPase that are expressed in small DRG neurons (Mata *et al.*, 1991; Hamada *et al.*, 2003; Deng *et al.*, 2009; Wu *et al.*, 2009; Heinke *et al.*, 2011; Li *et al.*, 2011). The δ- and μ-opioid receptors could interact with β_2- and α_{2A}-adrenoceptors in DRG neurons (Jordan *et al.*, 2001; 2003; Overland *et al.*, 2009; Zhao *et al.*, 2011; Schuster *et al.*, 2013). It is expected that the stimulus-induced insertion of δ-opioid receptors and related signalling complex would rapidly change the sensitivity of nociceptive afferent neurons to many neurotransmitters, neuromodulators and applied drugs.

Post-endocytic pathways for the opioid receptor complex

The GPCRs activated by selective agonists are often internalized and processed in either the recycling pathway for re-sensitization or the degradation pathway that leads to receptor down-regulation (Trapaidze *et al.*, 2000; Tsao and von Zastrow, 2000). Accumulated evidence has shown that internalized μ- and δ-opioid receptors are differentially processed in post-endocytotic pathways. Internalized μ-opioid

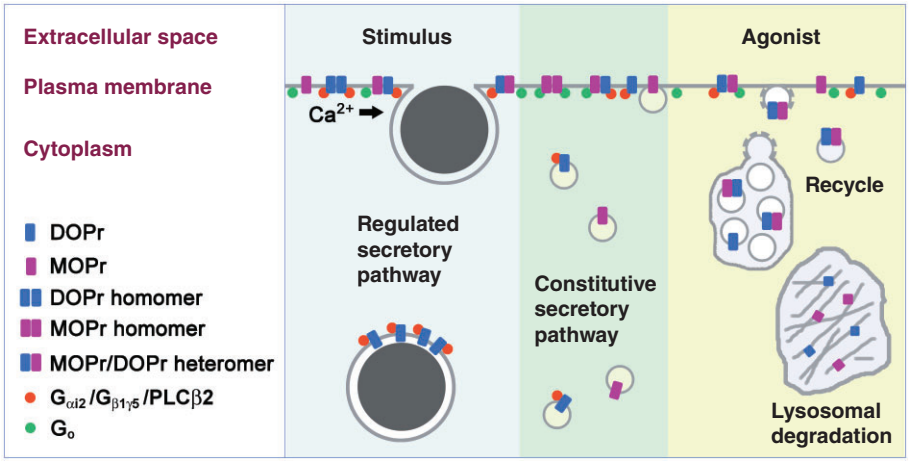

Figure 3

Subcellular translocation of δ-opioid receptors (DOPr) and related signalling molecules in nociceptive afferent neurons. Preassembled DOPr/$G_{\alpha i2}$/$G_{\beta 1\gamma 5}$/PLCβ2 complexes associated with the LDCV membrane are delivered to the cell surface in response to the Ca^{2+} influx. The receptor/G-protein complex might be also associated with the microvesicles in the constitutive secretory pathway to be delivered spontaneously. In the PM, the DOPr/$G_{\alpha i2}$/$G_{\beta 1}$/PLCβ2 complexes interact with μ-opioid receptors (MOPr) which may interact with G_o during MOPr agonist treatment. Following agonist treatment, the receptor heteromers are internalized, and processed for either recycling to the cell surface or degradation in the lysosomes.

receptors can be recycled to the PM and re-sensitized after treatment with a specific agonist, [D-Ala^2, N-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO; Arden *et al.*, 1995; Law *et al.*, 2000; Finn and Whistler, 2001). In contrast, internalized δ-opioid receptors can be processed in the lysosomal compartments for degradation after treatment with their agonists (Trapaidze *et al.*, 1996; Tsao and von Zastrow, 2000; Hislop *et al.*, 2009). Agonist-induced receptor phosphorylation and ubiquitination are involved in the endocytosis and down-regulation of opioid receptors (Finn and Whistler, 2001; Hislop *et al.*, 2009).

The δ-/μ-opioid receptor interaction plays an important role in regulating the opioid receptor trafficking, signalling and metabolism (Zhang *et al.*, 2006; Berger and Whistler, 2010; Chao and Xia, 2010; van Rijn *et al.*, 2010; Pradhan *et al.*, 2011; Stockton and Devi, 2011). Given that the δ-opioid receptor interacts with the μ-opioid receptor and forms a heteromer (Gomes *et al.*, 2004; Law *et al.*, 2005), it would be interesting to know whether or how the receptor complexes are internalized and processed following agonist stimulation. In transfected cells, treatment with either δ-opioid receptor agonists or the μ-opioid receptor agonist DAMGO and methadone results in endocytosis of the μ-/δ-opioid receptor heteromers (He *et al.*, 2011; Milan-Lobo and Whistler, 2011). The δ-opioid receptor agonist-induced slow but constant membrane insertion of δ-opioid receptors could be an intrinsic mechanism for replenishing the loss of surface δ-opioid receptors and maintaining the neuronal sensitivity to the agonists (Bao *et al.*, 2003). β-Arrestin mediates the internalization of many GPCRs. The μ-/δ-opioid receptor heteromers could constitutively recruit β-arrestin, while the δ-opioid receptor but not the μ-opioid receptor is normally coupled with β-arrestin (Cheng *et al.*, 1998; Rozenfeld and Devi, 2007). Moreover, the heteromers internalized by δ-opioid receptor agonists are often processed for lysosomal degradation, resulting in a reduction in both surface δ- and μ-opioid receptors (He *et al.*, 2011; Figures 2 and 3). The basal level of co-degradation of μ-/δ-opioid receptor heteromers in the dorsal spinal cord may be caused by the opiate peptide enkephalin released from the local neurons (Cesselin *et al.*, 1989; He *et al.*, 2011). The receptor co-degradation was enhanced by exogenously applied δ-opioid receptor agonists (He *et al.*, 2011) or persistent release of endogenous opioid peptides.

The μ-/δ-opioid receptor heteromers internalized by DAMGO are not subjected to the lysosomal degradation pathway, but might be recycled (He *et al.*, 2011). Early studies showed that δ-opioid receptor agonists phosphorylated these receptors, leading to their degradation (Trapaidze *et al.*, 1996; Gaudriault *et al.*, 1997; Tsao and von Zastrow, 2000; Bao *et al.*, 2003; Hislop *et al.*, 2009). In contrast, the μ-opioid receptor, which is phosphorylated and internalized by DAMGO, is recycled to the cell surface and re-sensitized (Arden *et al.*, 1995; Law *et al.*, 2000; Finn and Whistler, 2001; Qiu *et al.*, 2003). In the μ-/δ-opioid receptor heteromers, the μ- and δ-opioid receptors could be phosphorylated by their respective agonists without cross-phosphorylation between the two receptors (He *et al.*, 2011). Such a segregated biochemical process in the receptor heteromers may lead to different fates for the internalized μ-/δ-opioid receptor heteromers following treatment with opiate ligands. We propose that the surface pool of μ-/δ-opioid receptor heteromers could be reduced by δ-opioid receptor agonists, but recycled by some μ-opioid receptor agonists such as DAMGO (Figure 2). However, it is not known whether the G-proteins might be involved in the internalization and post-endocytic trafficking of receptor heteromers. Moreover, it would be interesting to explore the differential regulation of μ-/δ-opioid receptor trafficking in nociceptive neurons following the application of various opioid receptor agonists.

Pharmacological significance of opioid receptor redistribution

Opioid receptor ligands are known to bind to opioid receptor subtypes with various affinities (Janecka *et al.*, 2004; Trescot *et al.*, 2008). The opioid agonists preferentially targeting one type of opioid receptor often also bind to two other opioid receptors with low affinities (Janecka *et al.*, 2004). For instance, endogenous Leu-enkephalin has only modest affinity and selectivity for δ-opioid receptors. Deltorphin II binds to δ-opioid receptors with ~3000-fold higher affinity than μ-opioid receptors. DAMGO has ~1000-fold higher affinity for μ- compared to δ-opioid receptors. HEK293 cells and other cell lines expressing one type of opioid receptor are often used to analyse the pharmacological properties and selectivity of opioid ligands. However, when such analyses are applied for *in vivo* analyses of pain modulation, the data interpretation sometimes appears to be complicated.

In the resting state, only a limited number of δ-opioid receptors is present on the cell surface of nociceptive afferent neurons while μ-opioid receptors are present in abundance (Figure 2). A high dose of a δ-opioid receptor agonist might activate surface μ-opioid receptors, whereas a low dose of this agonist could only be enough to induce a δ-opioid receptor-specific effect when the number of δ-opioid receptors on the cell surface is markedly increased after various stimulations (Figure 2). This hypothesis may explain some seemingly conflicting observations suggesting that δ-opioid receptor agonist-induced antinociception is mediated by μ-opioid receptors under basal conditions, but mainly by δ-opioid receptors following physiological or pathological stimuli (Matthes *et al.*, 1998; Zhu *et al.*, 1999; Scherrer *et al.*, 2004; 2009; van Rijn and Whistler, 2009; Dubois and Gendron, 2010). This could also explain why the presynaptic inhibition of sensory afferents in the spinal cord by a high dose of a δ-opioid receptor agonist could be mediated by μ-opioid receptors in basal conditions, but by δ-opioid receptors after treatment with a TRPV1 agonist (Wrigley *et al.*, 2010), which may increase the cell-surface expression of δ-opioid receptors (Bao *et al.*, 2003; Zhang *et al.*, 2006). Therefore, changes in the number of opioid receptors and the ratio of δ- to μ-opioid receptors may contribute to the pharmacological properties of opioid ligands *in vivo*. The role the surface delivery of the δ-opioid receptor/$G_{\alpha i2}$/$G_{\beta 1}$/PLCβ2 complex and its interaction with μ-opioid receptors and G_o plays in the pharmacological effects of opiate analgesics remains to be investigated.

Role of opioid receptor interaction in morphine antinociceptive tolerance

Recent studies have shown that both thermal and mechanical hyperalgesia are inhibited by either δ- or μ-opioid receptor agonists through the activation of δ- or μ-opioid receptors, respectively, in nociceptive afferents (Joseph and Levine, 2010; Gaveriaux-Ruff *et al.*, 2011; He *et al.*, 2011; Kim *et al.*, 2011; Normandin *et al.*, 2013). These results are consistent with the coexistence of δ- and μ-opioid receptors in peptidergic small DRG neurons, and support the notion that δ- and μ-opioid receptors interact in the nociceptive sensory circuit. Such an interaction would affect many therapeutic aspects of opiate drugs.

Opioid analgesics (e.g. morphine) with high affinity for μ-opioid receptors are still the most powerful analgesics available for pain relief. However, their long-term use may lead to the development of antinociceptive tolerance and dependence (Fields, 2004; 2011; Manchikanti and Singh, 2008). Early studies showed that blockage of δ-opioid receptors enhanced morphine analgesia, and reduced analgesic tolerance (Abdelhamid *et al.*, 1991; Schiller *et al.*, 1999a,b; Schiller, 2010). Further studies revealed that morphine tolerance can be reduced by intrathecal application of the antisense oligodeoxynucleotide of the δ-opioid receptor gene (*Oprd1*), deleting either *Oprd1* or the preproenkephalin gene (*Penk1*), preventing δ-opioid receptor phosphorylation or deleting *Tac1*, which reduces the transport of δ-opioid receptors to the spinal dorsal horn via LDCVs (Standifer *et al.*, 1994; Zhu *et al.*, 1999; Nitsche *et al.*, 2002; Guan *et al.*, 2005; Xie *et al.*, 2009; Chen *et al.*, 2012).

Daniels *et al.* (2005) reported that the bivalent ligands targeting the δ-/μ-opioid receptor heterodimer with the spacer length between the two pharmacophores longer than 22 Å did not induce morphine tolerance and dependence, suggesting that this heterodimer could be a signalling unit mediating tolerance and dependence through specific signal transducers that recognize and coupled the heterodimer but not μ-opioid receptor monomers/homomers. Recently, δ-opioid receptor agonist-induced co-degradation of μ-opioid receptors was found to be one of the mechanisms for morphine antinociceptive tolerance, and the tolerance could be reduced by disrupting the δ-/μ-opioid receptor interaction in the PM of nociceptive afferents with a TAT- and glutathione S-transferase-fused first transmembrane domain of the μ-opioid receptor that mediates the interaction with δ-opioid receptors (Filizola *et al.*, 2002; He *et al.*, 2011). This is direct evidence that the physical dissociation of μ- from δ-opioid receptors in nociceptive afferents *in vivo* improves opioid analgesia. Although some mechanisms of receptor internalization have been studied (He *et al.*, 2002; 2011; Puthenveedu *et al.*, 2010; Yu *et al.*, 2010; Milan-Lobo and Whistler, 2011; Patierno *et al.*, 2011; Anselmi *et al.*, 2013), it would be interesting to further study the regulatory mechanisms for post-endocytic trafficking of the μ-/δ-opioid receptor heteromers following the application of different μ-opioid receptor agonists such as DAMGO, methadone and other opioid analgesics.

Conclusions

There is accumulating evidence that δ- and μ-opioid receptors are co-expressed in the nociceptive afferent neurons. The δ-opioid receptors can be distributed into both the constitutive and regulated secretory pathways. In contrast, μ-opioid receptors are mainly transported via the constitutive pathway. Thus, the δ-opioid receptor is often transferred into the PM in a stimulus-dependent manner, while the μ-opioid receptor moves there spontaneously. In the PM, these two types of opioid receptor interact and form heteromers to modulate the neuronal sensitivity to the opiate analgesics.

The δ-opioid receptor agonist-induced co-degradation of μ-opioid receptors could be one of the mechanisms of morphine antinociceptive tolerance. It would be interesting to further study the translocation and interaction of opioid receptors and related signalling molecules in the nociceptive afferents, and their contribution to the pharmacological mechanisms of opiate analgesia.

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Conflict of interest

The authors have no conflicts of interest.

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