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REVIEW

Recent advances on the δ opioid receptor: from trafficking to function

Louis Gendron[1], Nitish Mittal[2], Hélène Beaudry[1] and Wendy Walwyn[2]

[1]*Département de physiologie et biophysique, Institut de pharmacologie de Sherbrooke, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, QC, Canada,* [2]*Hatos Center for Neuropharmacology, David Geffen School of Medicine, University of California in Los Angeles, Los Angeles, CA, USA*

Correspondence
Louis Gendron, Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, Sherbrooke, QC J1H 5H4, Canada. E-mail: Louis.Gendron@USherbrooke.ca or Wendy Walwyn, Hatos Center for Neuropharmacology, David Geffen School of Medicine, University of California in Los Angeles, MRL Building, 675 Charles Young Drive, Los Angeles, CA 90095, USA. E-mail: wwalwyn@ucla.edu

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Within the opioid family of receptors, δ (DOPrs) and μ opioid receptors (MOPrs) are typical GPCRs that activate canonical second-messenger signalling cascades to influence diverse cellular functions in neuronal and non-neuronal cell types. These receptors activate well-known pathways to influence ion channel function and pathways such as the map kinase cascade, AC and PI3K. In addition new information regarding opioid receptor-interacting proteins, downstream signalling pathways and resultant functional effects has recently come to light. In this review, we will examine these novel findings focusing on the DOPr and, in doing so, will contrast and compare DOPrs with MOPrs in terms of differences and similarities in function, signalling pathways, distribution and interactions. We will also discuss and clarify issues that have recently surfaced regarding the expression and function of DOPrs in different cell types and analgesia.

LINKED ARTICLES

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Abbreviations[1]

DOPr, δ opioid receptor; DPDPE, [D-Pen^{2,5}]enkephalin, [D-Pen^{2},D-Pen^{5}]enkephalin; KOPr, κ opioid receptor; LIMK, Lim domain kinase; MOPr, μ opioid receptor; ROCK, Rho-associated coiled-coil containing protein kinase; SNC80, 4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N*,*N*-diethylbenzamide

[1]Please note that drug or target nomenclature is not only in accordance with BJPs Concise Guide to Pharmacology (Alexander *et al.*, 2013) but also with the recent review of the opioid receptor nomenclature (Cox *et al.*, 2015).

Introduction

Of the opioid family of receptors, the μ opioid receptor (MOPr) is the most well known. In binding with morphine and other semi-synthetic opioids, MOPrs are a well-studied clinical target. Unfortunately, MOPr agonists also induce a number of unwanted effects such as constipation, respiratory depression, analgesic tolerance, dependence and euphoria, which limit medical use and may lead to non-medical abuse.

Another member of the opioid receptor family, the δ opioid receptor (DOPr), has high sequence similarity to the MOPr, yet has different physiological and pharmacological properties and is not selectively targeted by an approved pharmaceutical product. Our knowledge of how this receptor functions in different cell types and under different pathological conditions is rapidly evolving. We will present recent evidence of the roles that this receptor may play under different conditions and in different cell types, and discuss how trafficking of this receptor influences DOPr function.

The concept that the location of a GPCR such as the DOPr, either intracellular or in different cell types, plays an important role in how the receptor functions is not novel. However, the location, and hence the function, of the DOPr has recently been the subject of some debate. This has resulted in some confusion as to the role of the DOPr under normal or physiological conditions. We will discuss these issues and describe recent findings of where DOPrs are localized and how this receptor functions. Novel interactions, pathways and physiological effects of DOPr activation will also be described suggestive of possible clinical roles of this receptor.

Part I. An overview of DOPr localization, trafficking and function

In the following section, we will first explore the anatomical and cellular localization of DOPrs. This will be followed by an assessment of our current knowledge of the intracellular localization and trafficking of DOPrs. We will then examine recent insights into how DOPrs regulate physiological and pathological states. An underlying theme of how DOPr localization, whether at the regional, cellular or intracellular levels, influences DOPr function will be developed throughout. Where possible and where relevant, we will also compare and contrast DOPrs with MOPrs so as to further our understanding and functional relevance of these GPCRs as distinct receptors or MOPr–DOPr heteromers.

Anatomical localization of DOPrs in the mammalian nervous system. In the CNS, MOPr and DOPr differ in their anatomical location. Although MOPrs are distributed throughout the CNS with highest densities in the thalamus, striatum, interpeduncular complex, medial habenular nucleus, cortex, superior and inferior colliculi, and in the superficial layers of the spinal cord (Mansour *et al.*, 1994b; Le Merrer *et al.*, 2009), DOPrs are discretely expressed in specific regions of the brain with high densities of the receptor found in the olfactory bulb, cortex, striatum and amygdala. Along the pain pathways, DOPrs are also expressed in several structures involved in the perception (peripheral nerve endings), transmission (dorsal root ganglia neurons and grey matter of the spinal cord) and integration of painful stimuli (parabrachial nucleus, amygdala, hypothalamus, thalamus, cerebral cortex, periaqueductal grey area and rostroventral medulla) as well as in areas involved in the regulation of mood (Mansour *et al.*, 1994a; 1995; Cahill *et al.*, 2001a; Mennicken *et al.*, 2003). More recently, DOPrs were also shown to be expressed in peripheral NF200-positive axons surrounding hair follicles and other mechanosensory organs so likely regulates cutaneous mechanical hypersensitivity (Bardoni *et al.*, 2014).

Significant differences in DOPr expression exist across species. A good example of this is the progressive specialization of DOPr localization within the nociceptive pathway across the phylogenetic tree. In rodent dorsal root ganglia neurons, DOPr expression is dispersed across different cell types whereas in primates, DOPr mRNA is primarily detected in small- and medium-sized dorsal root ganglion cells and DOPr-binding sites are concentrated in laminae I–II of the spinal cord (Mennicken *et al.*, 2003). Furthermore, pharmacological (Pasquini *et al.*, 1992) and immunogold labelling of DOPr has revealed that this receptor is mainly localized in the cytoplasm of cells (Cheng *et al.*, 1995; Elde *et al.*, 1995; Zhang *et al.*, 1998; Cahill *et al.*, 2001a; Gendron *et al.*, 2006), suggesting that DOPrs are one of the few GPCRs that are sorted to the cell surface via the regulated secretory pathway (Guan *et al.*, 2005; Cahill *et al.*, 2007; Zhang *et al.*, 2010; Zhao *et al.*, 2011). Consistent with the high level of MOPrs on the cell membrane in nervous tissues, like most other GPCRs, MOPrs are delivered to the cell surface by the constitutive secretory pathway (Hamel and Beaudet, 1984; Van Bockstaele *et al.*, 1996). Furthermore, as MOPrs may be recycled (Yu *et al.*, 2010; Roman-Vendrell *et al.*, 2012), it is possible that those present on the cell membrane may be from either newly synthesized or recycled receptor pools.

DOPr trafficking and function. Both MOPrs and DOPrs are $G_{i/o}$-coupled receptors, agonists of which activate canonical GPCR signalling cascades to reduce nociception, enhance euphoria or reduce anxiety, among other effects and recently described in several reviews (Al-Hasani and Bruchas, 2011; Williams *et al.*, 2013; Charbogne *et al.*, 2014). Novel trafficking and protein interactions, particularly of the DOPr, have recently come to light that may influence receptor signalling and are presented here.

Pre-assembled signalling complexes. GPCRs are often portrayed as single molecules present on the cell membrane. Upon binding to an agonist, these receptors recruit proteins to different regions of the receptor to activate downstream effector cascades. However, GPCRs have also been found as pre-assembled, receptor-specific protein complexes that are activated once on the cell membrane. For example, DOPrs may exist as a pre-assembled signalosome containing STAT5B, cSrc, Gα and Gβγ, so allowing enhanced STAT5 transcription in a cSrc and G-protein-dependent manner (Georganta *et al.*, 2010). Both DOPrs and MOPrs may also be constitutively associated with spinophilin, an actin-associated and dendritic spine-enriched protein (Fourla *et al.*, 2012). In a recombinant cell line setting, spinophilin is central to an agonist-specific complex consisting of a regulator of G-protein signalling (RGS) molecule, different Gα subunits and Gβγ subunits. This specificity could explain the

ability of spinophilin to reduce DOPr, but not necessarily MOPr, induced inhibition of AC and ERK phosphorylation, but enhance receptor internalization (Fourla *et al.*, 2012; Stratinaki *et al.*, 2013). The role of members of the RGS family, RGS4, 9 and 10, in altering opioid receptor function in rodent models of opioid tolerance, analgesia and dependence is currently under examination (Leontiadis *et al.*, 2009; Psifogeorgou *et al.*, 2011; Georgoussi *et al.*, 2012; Lamberts *et al.*, 2013; Stratinaki *et al.*, 2013). Furthermore, the reduced expression of RGS4 or 10 in the prefrontal cortex of opiate addicts suggests that these proteins may be involved in the human condition of opiate abuse (Rivero *et al.*, 2012).

Protein interactions that influence DOPr and MOPr biosynthetic pathways. The export of DOPrs to the cell membrane appears to be a critical step in regulating DOPr function. In transfected cells, DOPrs undergo extensive post-translational sorting in the endoplasmic reticulum (ER) where up to 50% of the immature receptor may be degraded (Petaja-Repo *et al.*, 2000; 2001). The remaining receptor forms a ternary complex with calnexin and a Ca^{2+} sensing ATPase to regulate receptor maturation in a Ca^{2+} and receptor-dependent manner (Petaja-Repo *et al.*, 2002; Leskela *et al.*, 2007; Tuusa *et al.*, 2010).

In contrast to DOPrs, much less is known of proteins that influence MOPr biosynthesis, possibly a result of the constitutive release of MOPrs to the cell membrane in a comparatively unregulated manner. Some insight into this process has recently been provided by Law and colleagues who, in using a targeted proteomic approach, identified a role for ribophorin I as a chaperone for MOPrs to the cell membrane (Ge *et al.*, 2009). Ribophorin I is one of two subunits of oligosaccharide transferase. This membrane protein complex is found in the rough ER and forms part of a quality control mechanism targeting misfolded proteins to a degradative fate. An interesting finding with respect to MOPr–DOPr interactions is that DOPrs and MOPrs may dimerize within the biosynthetic pathway (Hasbi *et al.*, 2007; Decaillot *et al.*, 2008), and that this is required to achieve full MOPr inhibitory coupling of voltage-gated ion channels in dorsal root ganglia neurons (Walwyn *et al.*, 2009).

Agonist-induced receptor trafficking alters receptor function. Similar to many GPCRs, ligand-activated DOPrs and MOPrs are phosphorylated by kinases such as G-protein receptor kinase (GRK) 2, 3 or 5, to recruit β-arrestin 1 or 2 and initiate internalization. After activation by an agonist, GRK-mediated phosphorylation of the carboxy-terminal tail (Thr358, Thr361 and Ser363 residues) of DOPr is rapidly observed (Pei *et al.*, 1995; Kramer *et al.*, 2000; Law *et al.*, 2000; Lowe *et al.*, 2002; Navratilova *et al.*, 2005; Zhang *et al.*, 2005). This leads to the recruitment of β-arrestin 1 and 2 (Kovoor *et al.*, 1999; Cen *et al.*, 2001a,b; Whistler *et al.*, 2001; Navratilova *et al.*, 2005; Zhang *et al.*, 2005), which in turn results in receptor desensitization and internalization of the ligand–receptor complex in clathrin-coated vesicles via a dynamin-dependent mechanism (Keith *et al.*, 1996; Chu *et al.*, 1997; Gaudriault *et al.*, 1997; Ko *et al.*, 1999; Law *et al.*, 1999; Hasbi *et al.*, 2000).

Removing a GPCR from the cell membrane has traditionally been equated with receptor desensitization and subsequent resensitization or degradation and down-regulation (Pippig *et al.*, 1993; 1995). However, recent studies of MOPr function suggest that this may not always be the case. Several investigators have shown that inhibition of receptor phosphorylation, β-arrestin 2 recruitment or internalization enhances receptor resensitization (Arttamangkul *et al.*, 2006; Dang *et al.*, 2011; Doll *et al.*, 2011; Quillinan *et al.*, 2011; and reviewed by Dang and Christie, 2012; Williams *et al.*, 2013). These findings suggest that MOPr internalization slows receptor resensitization, possibly by increasing the relative proportion of desensitized receptors on the cell membrane. An interesting interpretation of this finding is that morphine tolerance may not be equated with the relatively poor efficacy of morphine to induce receptor internalization.

Ligand-induced trafficking of endogenous and overexpressed DOPrs has also been shown to regulate receptor function. Mice expressing DOPr–eGFP at the DOPr locus were used to demonstrate that the efficacy of 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N*,*N*-diethylbenzamide (SNC80), a selective DOPr agonist, to induce hyperlocomotion was reduced if more receptors were internalized (Pradhan *et al.*, 2009). In transfected cells, Audet and colleagues used BRET to assess the inter-relationship between agonist, arrestin recruitment, internalization, recycling and signalling. They showed that the binding of peptidergic agonists such as [D-Pen^{2,5}]enkephalin, [D-Pen^{2},D-Pen^{5}]enkephalin (DPDPE) to DOPrs moved the carboxy (*C*)-terminal tail away from Gβγ to resulting in transient β-arrestin 2 recruitment. This led to receptor recycling and sustained analgesia. In contrast, SNC80, a non-peptidergic agonist, was found to alter the *C*-terminal folding to bring it closer to the amino terminal domain of Gγ2, allowing for sustained β-arrestin 2 recruitment, prolonged Gβγ association and ultimately prolonged receptor desensitization with minimal recycling (Audet *et al.*, 2012). This results in acute analgesic tolerance to repeated SNC80 but not DPDPE (Audet *et al.*, 2012). GPCR-associated sorting protein-1-bound DOPrs targeted for degradation are then actively transferred into lysosomes in an ubiquitin-dependent process (Whistler *et al.*, 2002; Henry *et al.*, 2010). Together these *in vitro* and *in vivo* data suggest that DOPrs, in contrast to MOPrs, may fit the traditional model of GPCR desensitization and trafficking, whereby internalization leads to enhanced receptor resensitization in an agonist-specific manner.

The functional effects of DOPr agonists. In the following section we will examine the ability of DOPrs to alter diverse physiological and pathological states.

Analgesia. The role of MOPr and DOPr in the control of pain has been thoroughly described (for reviews, see Gaveriaux-Ruff and Kieffer, 2011; Bodnar, 2013). Although these receptors share common roles in nociceptive pathways, at the spinal level MOPr and DOPr agonists were recently shown to inhibit distinct types of pain (Scherrer *et al.*, 2009). Indeed, it was found that MOPr agonists specifically alleviate thermal pain while DOPr agonists inhibit mechanical pain. These findings opposed numerous studies in which the spinal MOPr agonist DAMGO was shown to efficiently alleviate both heat (Porreca *et al.*, 1984; Malmberg and Yaksh, 1992; Nagasaka and Yaksh, 1995; Kondo *et al.*, 2005; Scherrer *et al.*,

2009; van Rijn *et al.*, 2012; Normandin *et al.*, 2013) and mechanically induced nociception (Nichols *et al.*, 1995; Sluka *et al.*, 2002; Kondo *et al.*, 2005; Chen and Pan, 2006; Joseph and Levine, 2010; van Rijn *et al.*, 2012; Normandin *et al.*, 2013). Similarly, the activation of spinal DOPr by selective agonists was shown to equally relieve heat (Stewart and Hammond, 1994; Tseng *et al.*, 1997; Qiu *et al.*, 2000; Cahill *et al.*, 2001b; 2003; Morinville *et al.*, 2003; Gendron *et al.*, 2007a,b; Beaudry *et al.*, 2009; Dubois and Gendron, 2010; Normandin *et al.*, 2013) and mechanical hyperalgesia (Miaskowski *et al.*, 1990; 1991; Sutters *et al.*, 1990; Holdridge and Cahill, 2007; Scherrer *et al.*, 2009; Joseph and Levine, 2010; Otis *et al.*, 2011; Normandin *et al.*, 2013). More recently, using an *in vivo* electrophysiological approach to measure the activation of the diffuse nociceptive inhibitory controls, we demonstrated that spinal MOPr- and DOPr-selective agonists equally attenuate thermal and mechanically induced nociception (Normandin *et al.*, 2013). In addition, the conditional deletion of either MOPr or DOPr in NaV1.8-positive primary afferent neurons respectively reduced MOPr- and DOPr-mediated peripheral analgesia (Gaveriaux-Ruff *et al.*, 2011; Weibel *et al.*, 2013). The latter studies not only support a similar role for MOPr and DOPr in pain control but also challenge the recent views that the distinction between pain modalities occurs at the level of primary afferents (Abrahamsen *et al.*, 2008; Cavanaugh *et al.*, 2009; Scherrer *et al.*, 2009) rather than at the spinal and/or supraspinal levels (Perl, 2007).

Anxiety, stress and depression. DOPr activation can also reduce depression, possibly as a result of the ability of DOPrs to relieve stress or anxiety, as recently reviewed in Le Merrer *et al.* (2009) and Pradhan *et al.* (2011). This has been shown by a reduction in the immobility induced by the forced swim test (Jutkiewicz *et al.*, 2003; 2005b) or of the conditioned suppression of locomotor activity following foot-shock (Saitoh *et al.*, 2004; Nieto *et al.*, 2005) in rodents. High levels of DOPr expression in the central nucleus of the amygdala may play an important role in this effect (Randall-Thompson *et al.*, 2010). Based on these preclinical data, a phase II clinical trial was initiated to examine the effects of a DOPr agonist, AZD2327, on major depressive disorders. This small trial of 22 participants, 14 of which received AZD2327, failed, but some symptoms of depression were reduced in patients with co-morbid anxiety, reflective of preclinical findings in rodents (http://clinicaltrials.gov/show/NCT00759395).

Addiction. DOPr expression in different limbic and corticolimbic regions suggests that this receptor could alter euphoric states. In contrast with MOPrs, there has been little evidence that DOPr agonists result in overt drug-seeking behaviours. There is, however, evidence that DOPrs may influence drug-seeking behaviours induced by psychostimulants such as cocaine or amphetamine (Dikshtein *et al.*, 2013; Bosse *et al.*, 2014). In examining the persistence of cocaine seeking in self-administering rats, β-endorphin reduced cocaine reinstatement after forced abstinence by activating DOPrs in the nucleus accumbens (NAcc) (Dikshtein *et al.*, 2013). This contrasts with the findings of Simmons and Self (2009) who showed that β-endorphin, acting on MOPrs, but not DOPrs, reinstates previously extinguished cocaine-seeking behaviours. Interestingly, these differences could have resulted from the fact that forced abstinence (Dikshtein *et al.*, 2013) or extinction (Simmons and Self, 2009) could induce different cellular responses. In addition, DOPr activation by deltorphin II-based peptides has also been shown to enhance the locomotor sensitization to cocaine in a dose-dependent manner (Kotlinska *et al.*, 2010). This role of DOPrs could be linked to a particular aspect of addiction-related behaviours: the cognitive control of decision making (Laurent *et al.*, 2012; Bertran-Gonzalez *et al.*, 2013). Epidemiological evidence of the association of a single nucleotide polymorphism in OPRD1 with cocaine addiction in some human populations (Crist *et al.*, 2013) complements these preclinical studies in rodents. DOPrs may also play a role in the profile of morphine-induced addiction; DOPr inhibition or a lack of functional DOPs in rodents reduces the rewarding properties of morphine (Chefer and Shippenberg, 2009; Shippenberg *et al.*, 2009; Billa *et al.*, 2010; Le Merrer *et al.*, 2011), possibly mediated by DOPr regulation of spatial and contextual cues (Le Merrer *et al.*, 2012). There has also been evidence of DOPrs playing a role in the addiction profile induced by alcohol where behavioural responding to ethanol increases DOPr function in several regions. This suggests that DOPrs may play a protective role in chronic alcohol disorders and is being further explored (Margolis *et al.*, 2008; Mitchell *et al.*, 2012; Nielsen *et al.*, 2012; van Rijn *et al.*, 2012). It is tempting to suggest that the influence of DOPrs on the addiction profile of these compounds may not be a direct result of DOPr signalling within the effected cells or pathways but rather an indirect, and concurrent, anxiolytic action of DOPrs (Lutz and Kieffer, 2013; Charbogne *et al.*, 2014).

Learning and memory. Radioligand binding and DOPr–eGFP mice show intense DOPr expression in the hippocampus (Crain *et al.*, 1986; Erbs *et al.*, 2012) where these receptors are found on interneurons and act presynaptically to inhibit GABA release (Rezai *et al.*, 2012; Piskorowski and Chevaleyre, 2013). Further electrophysiological studies demonstrate that DOPrs are required to induce long-term depression of parvalbumin-expressing neurons within CA2 (Piskorowski and Chevaleyre, 2013) and inhibit the excitatory temporoammonic pathway from the entorhinal cortex to CA1 (Rezai *et al.*, 2013). DOPrs are also critical for the induction of long-term potentiation in dentate granule cells (Xie and Lewis, 1995). At the behavioural level, mice lacking DOPrs show impaired hippocampal and striatal-based learning and motor tasks (Le Merrer *et al.*, 2013). Another measure of cognition, the ability to make a decision based on past experience, has recently shown to be mediated by DOPr trafficking and hence function in the cholinergic interneurons of the shell of the NAcc (Laurent *et al.*, 2012; Bertran-Gonzalez *et al.*, 2013).

Hypoxia. The up-regulation of DOPrs during hypoxic preconditioning may induce neuronal, cardiac and retinal protection to subsequent hypoxic events (Gao *et al.*, 2012; Husain *et al.*, 2012; Maslov *et al.*, 2013). The underlying mechanism remains unclear but may be mediated by increased BDNF–TrkB signalling (Tian *et al.*, 2013), modification of micro-RNA expression (He *et al.*, 2013; Yang *et al.*, 2013), and altered mitochondrial and ion channel function (Fischbach *et al.*, 2003). A similar protective effect of DOPr

agonists in maintaining cellular integrity has been seen during mammalian hibernation, a state of low-energy stores and oxygen depletion. Indeed circulating opioid peptides are considered a 'trigger of hibernation' (Oeltgen *et al.*, 1988) and may play an important role in cell proliferation, scar formation and wound healing in hibernating black bears (Iaizzo *et al.*, 2012).

Immune function. DOPr expression on astroglia and in T cells may explain the reported immunomodulatory roles of DOPr ligands. DOPr forms a heterodimer with CXCR4, a co-receptor for CD4s and an important target receptor for HIV virions. These heterodimers have also been found on astrocytes and neurons where activation by either ligand silences activity of both receptors (Pello *et al.*, 2008). DOPr expression and up-regulation has more recently been found in hepatocellular carcinoma and is associated with enhanced tumour formation (Tang *et al.*, 2013). DOPrs are also expressed on dendritic cells and may trigger chemotaxis *in vitro* and dendritic cell migration *in vivo* (Benard *et al.*, 2008).

Other physiological and pathological effects of DOPr signalling. Aside its role in analgesia, the expression of DOPrs in mechanoreceptors in the skin suggests that it also regulates touch. Indeed, DOPr-positive axons have been found surrounding hair follicle endings and the base of Merkel cells in mice (Bardoni *et al.*, 2014). *In vitro* and *in vivo* studies also suggest a role for DOPrs in development. The DOPr antagonist ICI 174,864 inhibits embryogenesis (Gallego *et al.*, 2009), and DOPr agonists favour proliferation over neuronal differentiation (Hauser *et al.*, 2000; Persson *et al.*, 2003). Interestingly, studies in rodents and/or non-human primates suggest that DOPr agonists may improve the clinical outlook of Parkinson's disease (Hille *et al.*, 2001; Mabrouk *et al.*, 2009) and of migraine (Pradhan *et al.*, 2014).

Convulsions. In contrast to these many beneficial effects of DOPr activation, some DOPr agonists have a proconvulsant effect that could be a major drawback to any clinical use of DOPr agonists (Comer *et al.*, 1993; Negus *et al.*, 1994; Jutkiewicz *et al.*, 2006). These convulsions are mediated by nitric oxide, tend to be short lived (Khavandgar *et al.*, 2002) and are subject to tolerance (Jutkiewicz *et al.*, 2005a). Importantly, as convulsions may be separable from other functional effects of DOPr agonism (Broom *et al.*, 2002a,b; Jutkiewicz *et al.*, 2005b) and are agonist and dose specific (Hudzik *et al.*, 2011; Saitoh *et al.*, 2011), this drawback could be overcome.

Part II. Novel aspects of DOPr function

The first section of the review discussed the localization of DOPrs, new insights into DOPr interacting and signalling partners, and an update on known functional effects of DOPr activation, suggesting that DOPrs may be a promising target for diverse pathological conditions. This sets the stage for the second part of this review in which two critical aspects of DOPr function will be described in more detail: the role of DOPrs in cells that express MOPrs and the ability of DOPrs to be functionally up-regulated by different stimuli. This next section will thus examine several contentious issues that have recently come to light regarding DOPr function.

DOPr function in MOPr-expressing cells. The activation of MOPr by chronic morphine treatments or other MOPr agonists *in vivo* was shown to increase the effects of DOPr agonists, that is, DOPr function (Cahill *et al.*, 2001b; Morinville *et al.*, 2003; Hack *et al.*, 2005; Ma *et al.*, 2006; Gendron *et al.*, 2007a). In a similar way, DOPr functions are increased in inflammatory pain models (Hylden *et al.*, 1991; Hurley and Hammond, 2000; Cahill *et al.*, 2003; Patwardhan *et al.*, 2005; Gendron *et al.*, 2006; 2007a; Pettinger *et al.*, 2013; Pradhan *et al.*, 2013), an effect abolished in MOPr knockout mice (Gendron *et al.*, 2007b). Indeed, under various conditions it has been shown that the expression of MOPr is essential for DOPr to be fully functional (Sora *et al.*, 1997a,b; Loh *et al.*, 1998; Matthes *et al.*, 1998; Hosohata *et al.*, 2000; Guo *et al.*, 2003; Morinville *et al.*, 2003; 2004a; Gendron *et al.*, 2007b). Although the exact mechanism by which MOPr can regulate DOPr's functions remains unknown, several lines of evidence point towards direct interactions between MOPr and DOPr and between their signalling cascades.

MOPr–DOPr localization. Despite a significant level of overlap of MOPr and DOPr expression in numerous structures of the CNS and the similar roles they play in pain control, the cellular distribution of these opioid receptors is controversial and highly debated. The controversy was initiated by two different findings: questionable selectivity of the available DOPr antibodies and the cellular and subcellular distribution of the DOPr tagged with a 238 amino acid fluorescent protein, eGFP, in genetically engineered mice (Scherrer *et al.*, 2006). Indeed, it has since been suggested that some DOPr antibodies are non-specific, labelling a protein still expressed in mice lacking DOPrs (Scherrer *et al.*, 2009; Bardoni *et al.*, 2014). These contentious issues have led to further studies, and most antibodies have now been shown to be specific, at least when used under proper conditions (Overland *et al.*, 2009; Riedl *et al.*, 2009; Xie *et al.*, 2009; Billa *et al.*, 2010; Wang *et al.*, 2010; Schuster *et al.*, 2013). More convincingly, Zhang and collaborators used three different commercially available antibodies and showed specific DOPr labelling in wild-type mouse dorsal root ganglia and spinal cords. In the same study, no DOPr labelling was observed with any of these antibodies in DOPr knockout mice (Wang *et al.*, 2010), helping to resolve the first point of contention. With respect to the co-expression of opioid receptors, immunolabelling of MOPrs in DOPr–eGFP mice suggested that DOPr and MOPr were rarely co-expressed in the same neurons. In primary afferents of these mice, DOPr–eGFP was shown to be expressed on Aδ and Aβ fibres, while MOPr-like immunostaining was mainly present on peptidergic nociceptors (Scherrer *et al.*, 2009). In this study, approximately 2% of nociceptive neurons were reported to co-express MOPr and DOPr. In a later study using the same DOPr–eGFP mouse line, the co-expression of MOPr and DOPr was reported at more than 5% of dorsal root ganglia neurons (Bardoni *et al.*, 2014). Using double knockin mice expressing mCherry–MOPr and DOPr–eGFP, Massotte and colleagues recently reported that more than 30% of dorsal root ganglia neurons of all types (i.e. small, medium and large) co-express MOPrs and DOPrs (Erbs *et al.*, 2014). The reasons for these different results from three studies that have used the same DOPr–eGFP knockin mouse line are unclear, but could result from differences in MOPr

and GFP immunolabelling technique and the settings or criteria used to define labelled from non-labelled cells.

There is now considerable biochemical evidence supporting that DOPr is expressed in peptidergic primary afferents. In sensory neurons DOPr was shown to interact with the substance P domain of protachykinin in large dense core vesicles (LDCVs) (Guan *et al.*, 2005). Although this phenomenon is not always required (Dubois and Gendron, 2010), the interaction with protachykinin was shown to participate in the sorting of DOPr into the LDCVs. This promotes DOPr insertion into the plasma membrane of peptidergic primary afferents and translates to an increased analgesic potency of DOPr agonists (Guan *et al.*, 2005). Single-cell RT-PCR also revealed the presence of both MOPr and DOPr mRNAs in substance P containing dorsal root ganglion cells (Wang *et al.*, 2010). Functional evidence for the expression of DOPr in these neurons also exists. In small peptidergic neurons, DOPr was indeed shown to be involved in the inhibition of glutamate, substance P and CGRP release (Ueda *et al.*, 1995; Zachariou and Goldstein, 1996; Beaudry *et al.*, 2009; Overland *et al.*, 2009; Kouchek *et al.*, 2013; Normandin *et al.*, 2013). DOPr was also found to synergize with α_{2A}-adrenergic receptors in peptidergic primary afferents via a PKC-dependent mechanism (Overland *et al.*, 2009; Riedl *et al.*, 2009; Schuster *et al.*, 2013). Altogether, these *in vivo* observations support the conclusions made with DOPr antibodies and therefore endorse the presence of DOPrs on substance P-containing afferent neurons. In a more recent study, Scherrer and collaborators found a higher level of MOPr and DOPr–eGFP co-expression in DOPr–eGFP mice than previously reported and with both receptors being expressed in a population of CGRP-expressing myelinated nociceptors, but not in substance P-containing nociceptors (Bardoni *et al.*, 2014).

Putative MOPr–DOPr heterodimers. The possibility that a MOPr–DOPr heteromer may exist *in vivo* opens a new era of research and represents an exciting opportunity to develop novel therapeutics with unique pharmacology. For instance, computational studies have described a potential interaction between $TM1^{MOPr}$ and $TM4^{DOPr}$ (Liu *et al.*, 2009). *In vitro*, overexpression of MOPr and DOPr in the same cells revealed that these receptors can indeed physically interact (George *et al.*, 2000; Gomes *et al.*, 2000; 2004; Hasbi *et al.*, 2007; Decaillot *et al.*, 2008; Gupta *et al.*, 2010; Kabli *et al.*, 2010; Golebiewska *et al.*, 2011). Indeed in heterologous systems, the use of BRET techniques demonstrated that MOPr and DOPr form homo- and hetero-oligomers (Wang *et al.*, 2005; Hasbi *et al.*, 2007). Using this technique, George and collaborators further observed that the heteromer constitutively interact in the ER before being targeted to the plasma membrane as a preassembled signalling complex (Hasbi *et al.*, 2007). This however contrasts with others who suggested that the MOPr–DOPr oligomer associates at the cell surface (Law *et al.*, 2005). *In vivo*, endogenous MOPr and DOPr were successfully co-immunoprecipitated from mouse spinal cord extracts, suggesting that they can physically associate and interact (Gomes *et al.*, 2004; Xie *et al.*, 2009; He *et al.*, 2011). In the double knockin mice, Massotte and collaborators were also able to co-immunoprecipitate DOPr–eGFP with mCherry–MOPr from the hippocampus (Erbs *et al.*, 2014). However, only few studies thus far revealed direct evidence for the presence of endogenous MOPr–DOPr heteromers in intact tissue. One such example comes from Devi's group who generated an antibody directed against the MOPr–DOPr heterodimer and showed that it is present in various brain areas and in dorsal root ganglion cells (Gupta *et al.*, 2010). In support of such an interaction, the DOPr agonist SNC80 was recently shown to produce antinociception by activating the MOPr–DOPr heteromer (Metcalf *et al.*, 2012). More recently, the high-throughput screening of a small-molecule library gave rise to the identification of the first MOPr–DOPr heteromer-selective biased agonist (Gomes *et al.*, 2013). The activity of the compound CYM51010 was indeed found to be specific to cells expressing both MOPr and DOPr as CYM51010-induced β-arrestin recruitment and ^{35}S-GTPγS binding were only present in cells overexpressing both receptors and were blocked by the MOPr–DOPr heteromer antibody (Gomes *et al.*, 2013).

Measures of MOPr–DOPr function. Because no DOPr splice variants have been identified so far, it has been suggested that the interaction between DOPr and MOPr could be responsible for the two postulated pharmacologically distinct DOPr subtypes, DOPr1 and DOPr2 (van Rijn *et al.*, 2010; 2013). In addition to their ability to physically interact, it has been shown that co-expression of κ opioid receptor (KOPr) or MOPr with DOPr leads to changes in DOPr pharmacology. Indeed, the interaction between KOPr and DOPr results in a new receptor that exhibits distinct ligand binding and functional properties (Jordan and Devi, 1999). In cells expressing both MOPr and DOPr, DPDPE displays a reduced affinity as compared with cells expressing DOPr alone (George *et al.*, 2000). MOPr and DOPr co-expression was also shown to modify the G-protein coupling of the receptors (George *et al.*, 2000; Hasbi *et al.*, 2007). Indeed, although DOPrs and MOPrs recruit the G-protein subunit $G_{\alpha i}$ when expressed separately, dimerization of MOPr with DOPr is associated with a shift in G-protein coupling from the $G_{\alpha i}$ to the $G_{\alpha z}$ subunit. In addition to changes in G-protein coupling, heteromerization of MOPr and DOPr is associated with changes in the kinetics of ERK activation (Rozenfeld and Devi, 2007). In fact, when DOPr is expressed alone it activates ERK in a rapid and transient manner whereas MOPr–DOPr heteromer activation leads to a sustained phosphorylation of ERK. Interestingly, DOPr's trafficking is also modified in cells expressing MOPrs. Indeed, DOPr is co-internalized with MOPr following activation with a MOPr agonist (He *et al.*, 2011; Milan-Lobo and Whistler, 2011). Similarly, MOPr is co-internalized with DOPr and targeted to lysosomal degradation after treatment with a DOPr agonist (He *et al.*, 2011). The latter observations therefore suggest that DOPr can also alter the functions and the trafficking of MOPr. This was further evidenced by the fact that DOPr activation was shown to increase the antinociceptive effects of spinal MOPr agonists (He and Lee, 1998) and that the expression of DOPr contributes to the full expression of MOPr's inhibitory effects on voltage-dependent Ca^{2+} channels in nociceptive neurons (Walwyn *et al.*, 2009). A direct role of MOPr–DOPr heterodimerization in this effect was supported by the fact that the expression of a dimerization-deficient DOPr mutant in DOPr knockout neurons failed to fully restore the inhibitory coupling of MOPr (Walwyn *et al.*, 2009).

In vivo, the sustained activation of MOPr was shown to increase the level of MOPr–DOPr heteromers in various brain areas and in nociceptive neurons (Gupta *et al.*, 2010). When the formation of the MOPr–DOPr heteromers is prevented, the cell surface expression of DOPr was shown to be reduced and the antinociceptive effects of DOPr agonists decreased (Xie *et al.*, 2009). Disruption of MOPr–DOPr heteromers in the accumbens was also shown to abolish the antidepressant- and anxiolytic-like actions of DOPr agonists (Kabli *et al.*, 2013). Similarly, the heterodimerization of MOPr with DOPr was shown to have important consequences on MOPr functions. Indeed, in acute pain models the absence of DOPr attenuates the development of morphine-induced antinociceptive tolerance (Kest *et al.*, 1996; Zhu *et al.*, 1999; Chefer and Shippenberg, 2009; Walwyn *et al.*, 2009). The disruption of the MOPr–DOPr heteromer was also shown to increase morphine analgesia and decrease tolerance (Xie *et al.*, 2009; He *et al.*, 2011). Taken together, these results provide evidence for MOPr–DOPr heteromers as a distinct functional target for opioid ligands and represent a mechanism to regulate the functions of DOPr.

Functional up-regulation of DOPrs

A brief history. The ability of DOPrs to undergo a functional up-regulation, first described in the 1980s, was attributed to an increase in receptor function (Young *et al.*, 1982; 1983; Barg *et al.*, 1984) that could be influenced by chronic morphine and ethanol (Charness *et al.*, 1986; Danks *et al.*, 1988; Rothman *et al.*, 1989). Simantov and colleagues then found that the DOPr ligand, DPDPE, but not other ligands, increased the levels of Gα subunits in cultured cells (Vogel *et al.*, 1990), and Inturissi and colleagues found that an increase in DOPr sensitivity could not be explained by increased receptor expression (Jenab and Inturrisi, 1997). Studies from the late 1990s and 2000s have shown that even in different systems, cell types and under different pathological conditions such as chronic pain, cell division, hypoxia and scar formation, DOPr function could be enhanced (Chen *et al.*, 1997; Dickenson, 1997; Thorlin *et al.*, 1997; 1999; Cahill *et al.*, 2003; Morinville *et al.*, 2003; Ma *et al.*, 2005; Cheng *et al.*, 2008). The development of mutant mice lacking opioid receptors or ligands demonstrated how opioid receptor function can also be regulated by ligand availability (Brady *et al.*, 1999). The underlying mechanisms of DOPr up-regulation were then suggested to be a result of enhanced DOPr trafficking to the cell membrane (Cahill *et al.*, 2001b), making DOPr a promising analgesic target (Cahill *et al.*, 2007). During the past decade, up-regulation of endogenous DOPr has been shown in different models of pain (Cahill *et al.*, 2003; Morinville *et al.*, 2004b; Pradhan *et al.*, 2013), alcohol (van Rijn *et al.*, 2012), chronic morphine (Chieng and Christie, 2009; Morgan *et al.*, 2009), hypoxia (Peng *et al.*, 2009) and in the progression of cancer (Otis *et al.*, 2011; Tang *et al.*, 2013).

Cell surface receptor levels. Enhanced DOPr function is commonly defined by enhanced efficacy of a bound agonist in either a cellular or a behavioural context (Chieng and Christie, 2009; Pradhan *et al.*, 2013). A number of studies have shown that this increase in signalling results from an increase in the number of receptors on the cell membrane (Cahill *et al.*, 2001b; Scherrer *et al.*, 2006; Walwyn *et al.*, 2009). Conversely, removing receptors through internalization or degradation decreases the response to a subsequent agonist challenge (Scherrer *et al.*, 2006; Pradhan *et al.*, 2009). Together, this suggests that DOPr signalling, and hence functionality, is sensitive to the number of receptors on the cell membrane. This relationship between cell surface receptor levels and functionality could be influenced by the DOPr biosynthetic pathway (Petaja-Repo *et al.*, 2000), which regulates the number of receptors released to the cell membrane (Dong *et al.*, 2007; Achour *et al.*, 2008). Integral to this concept is that DOPrs are found in an intracellular location close to the cell membrane and can be readily and rapidly released to the cell membrane. As previously discussed, there have been a number of reports of endogenous DOPrs found within the cell either in association with the Golgi, with pre-synaptic vesicles or in the sub-plasmalemmal space. Furthermore, few receptors have been shown to be on the cell membrane (Arvidsson *et al.*, 1995; Cheng *et al.*, 1995; 1997; Zhang *et al.*, 1998; Cahill *et al.*, 2001a,b; Bao *et al.*, 2003; Lucido *et al.*, 2005; Fristad *et al.*, 2006; Gendron *et al.*, 2006; Wang *et al.*, 2008b). Many of these reports examined endogenous DOPr localization in paraformaldehyde-fixed tissue using an anti-DOPr antibody and electron microscopy to visualize the gold particles. Conversely, when imaged with an alternative technique, that is, by imaging dorsal root ganglia neurons from DOPr–eGFP knockin mice, eGFP-labelled receptors were primarily found on the cell membrane (Scherrer *et al.*, 2006; Bardoni *et al.*, 2014). This could be a result of the eGFP tag. Indeed Zhang and colleagues observed that both *N*- and *C*-terminal eGFP-tagged DOPrs are localized on the cell surface whereas DOPrs with smaller tags (e.g. Myc and haemagglutinin) show a vesicular localization (Wang *et al.*, 2008a). Although DOPrs were overexpressed in this study, the different localization of receptors with smaller versus larger tags suggests that the size of the tag may alter DOPr localization. When compared with wild-type mice, the eGFP tag also increased DOPr mRNA and binding levels, DOPr agonist-induced G-protein activation and Ca^{2+} channel inhibition (Scherrer *et al.*, 2009; Bardoni *et al.*, 2014). Together these data suggest that DOPr trafficking and function may be altered by a *C*-terminal eGFP fusion protein. Interestingly, DOPr would not be the first GPCR to show altered trafficking and function when fused to eGFP (McLean and Milligan, 2000; Madziva and Edwardson, 2001; McDonald *et al.*, 2007; Roy *et al.*, 2007).

Despite the controversy described above regarding the specificity of antibodies, photoaffinity labelling of DOPrs in the rat striatum with [^{125}I]-azido-DTLET, performed before the widespread use of antibodies, had revealed that this receptor was principally expressed inside the cells (Pasquini *et al.*, 1992). Predominant membrane expression of DOPr has only been observed in the genetically engineered mice expressing DOPr–eGFP using standard confocal or light microscopy. In addition to the effect of the *C*-terminal tag on receptor trafficking, our ability to distinguish membrane receptors from those present near the plasma membrane may be limited by the resolution of standard confocal or light microscopy. Such microscopy is limited by the diffraction of light, a concept first defined by the German physicist Ernst Karl Abbe in the

1800s, and known as the Abbe diffraction limit. For the GFPe emission wavelength of 488 nm, this limit would be around ~175–250 nm when a high numerical (NA) objective lens (NA = 1.4) is used. Thus, confocal or light microscopy does not have the resolving power to differentiate DOPrs localized on the cell membrane from those that are 200 nm beneath the cell membrane. As the antibodies used in electron microscopic studies have now been shown to specifically label DOPrs (Xie *et al.*, 2009; Billa *et al.*, 2010; Wang *et al.*, 2010), and the membrane density of DOPr-like immunostaining and function of DOPr can be enhanced under different conditions (as described above), it is likely that the endogenous receptor has a predominant intracellular localization under normal conditions.

Physiological and pathological evidence of DOPr up-regulation. In light of the considerable doubt in the field whether DOPrs are exported to the cell membrane to enhance DOPr responding under either normal or pathological conditions, studies using a functional readout of DOPr signalling have surfaced. A recent example is from a study by Balleine and colleagues who have shown that pavlovian conditioning and pavlovian instrumental transfer, as measured by food reward, induce a translocation of DOPrs, assessed in DOPr–eGFP mice, to the cell membrane of striatal cholinergic interneurons (Bertran-Gonzalez *et al.*, 2013). This could explain the deficit in pavlovian transfer in mice lacking DOPrs (Laurent *et al.*, 2012). Interestingly in these neurons, DOPr–eGFP is described as having an intracellular location under normal conditions. This is in marked contrast with the description of DOPr–eGFPs in dorsal root ganglia neurons (Scherrer *et al.*, 2009; Bardoni *et al.*, 2014).

Another example is the analgesic effect of DOPrs in animal models of chronic pain. The ability of DOPr agonists to relieve acute mechanical pain is unremarkable (Pradhan *et al.*, 2013). However, chronic pain induced by inflammatory injury or neuropathic insult increases the analgesic efficacy of DOPr agonists (Kabli and Cahill, 2007; Pradhan *et al.*, 2013), suggesting that chronic pain up-regulates DOPrs. Factors associated with this pathological condition such as bradykinin and arachidonic acid (Patwardhan *et al.*, 2005) may 'prime' DOPrs and increase receptor function (Rowan *et al.*, 2009). Other pathological conditions such as chronic alcohol exposure (van Rijn *et al.*, 2012) and hypoxia (Gao *et al.*, 2012) have also been shown to enhance DOPr responding.

The role of β-arrestin 1 and the actin cytoskeleton in regulating DOPr trafficking and function. Dynamic remodelling of the cytoskeleton, particularly of the actin filaments, provides the network along which intracellular proteins may be trafficked as needed. This mechanism allows the Golgi apparatus to sort and traffic newly synthesized proteins to the cell membrane (Salvarezza *et al.*, 2009; Lowe, 2011). Both the actin severing protein, cofilin, and the upstream kinase, Lim domain kinase (LIMK), control the release of specific proteins from the Golgi to the cell membrane, demonstrating how dynamic cytoskeletal remodelling controls protein export (Heimann *et al.*, 1999; Egea *et al.*, 2006; Salvarezza *et al.*, 2009). A similar dynamic regulation of actin turnover to alter the leading and trailing edges of lymphocytes and allow directed cell migration outlines a role for β-arrestin 1 or 2 in cytoskeletal remodelling. This is likely a result of these arrestin subunits binding with cofilin, the inactivating phosphatase chronophin, and the activating kinase LIMK, resulting in a spatiotemporal regulation of actin turnover by these scaffolding proteins (DeFea, 2007; Zoudilova *et al.*, 2007; 2010; Xiao *et al.*, 2010). We have recently shown a similar role of β-arrestin 1, but not 2, in regulating LIMK and cofilin to affect actin turnover and regulate DOPr function in dorsal root ganglion neurons (Mittal *et al.*, 2013).

The described cellular and behavioural studies (Mittal *et al.*, 2013) allowed us to propose the following pathway: under normal or wild-type conditions, agonist binding to DOPrs activates the RhoA-ROCK (RhoA-associated coiled-coil containing protein kinase) LIMK pathway resulting in an enhanced but local activation of cofilin. This leads to a controlled export of DOPr-containing cargo vesicles to the cell membrane and allows a limited response to a DOPr agonist such as SNC80. This pathway can be enhanced by removing β-arrestin 1. In this scenario, SNC80 activates LIMK through the RhoA-ROCK pathway but cofilin dephosphorylation and activation does not occur. This leaves stable actin 'tracks' in place resulting in enhanced export of DOPrs from the Golgi to the plasma membrane, and enhanced DOPr function. This pathway can be blocked by inhibiting ROCK, the kinase responsible for phosphorylating LIMK and inactivating cofilin. In this scenario, agonist-induced activation of the pathway does not occur and additional receptors are not released to the cell membrane (see the schematic model in Figure 1).

Such regulated release of DOPrs in an agonist-dependent manner may be required to obtain an initial functional response to a DOPr agonist. Thereafter, the properties of DOPr ligands, receptor phosphorylation, β-arrestin 1 or 2 recruitment, the roles of other regulatory proteins such as PKC and bradykinin, and subsequent trafficking, internalization and resensitization may further regulate DOPr function.

Physiological relevance of the ROCK-LIMK–β-arrestin 1 pathway. We found that the behavioural effects of the DOPr agonist, SNC80, can be influenced by genetic deletion or pharmacological inhibition of different proteins within this pathway (Figure 1). In mice lacking β-arrestin 1, the hyperlocomotor and analgesic effects of SNC80 are enhanced; this can be blocked by the δ antagonist, naltrindole. Pharmacological inhibition of ROCK reduced both the hyperlocomotor and analgesic effects of SNC80. Furthermore, the enhanced efficacy of SNC80 in the complete Freund's adjuvant (CFA) model of chronic inflammatory pain (Pradhan *et al.*, 2013) was inhibited by ROCK (Mittal *et al.*, 2013).

In these assays the DOPr agonists, SNC80 and DPDPE, were found to be the principle activators of this pathway. But it is also possible that other receptors or molecules may either initiate activation or are important intermediates. For example, bradykinin, arachidonic acid or perhaps DOPr auto-antibodies, but not endogenous opioids, may activate this pathway in the CFA model of chronic pain (Patwardhan *et al.*, 2005; Gendron *et al.*, 2007b; Ranganathan *et al.*, 2009; Rowan *et al.*, 2009; Pettinger *et al.*, 2013). Other receptors and kinases such as PAR_2 or PKC could also be involved in up-regulating DOPrs (Patwardhan *et al.*, 2005; Norcini *et al.*, 2009; Rowan *et al.*, 2009; Hagenacker *et al.*, 2010).

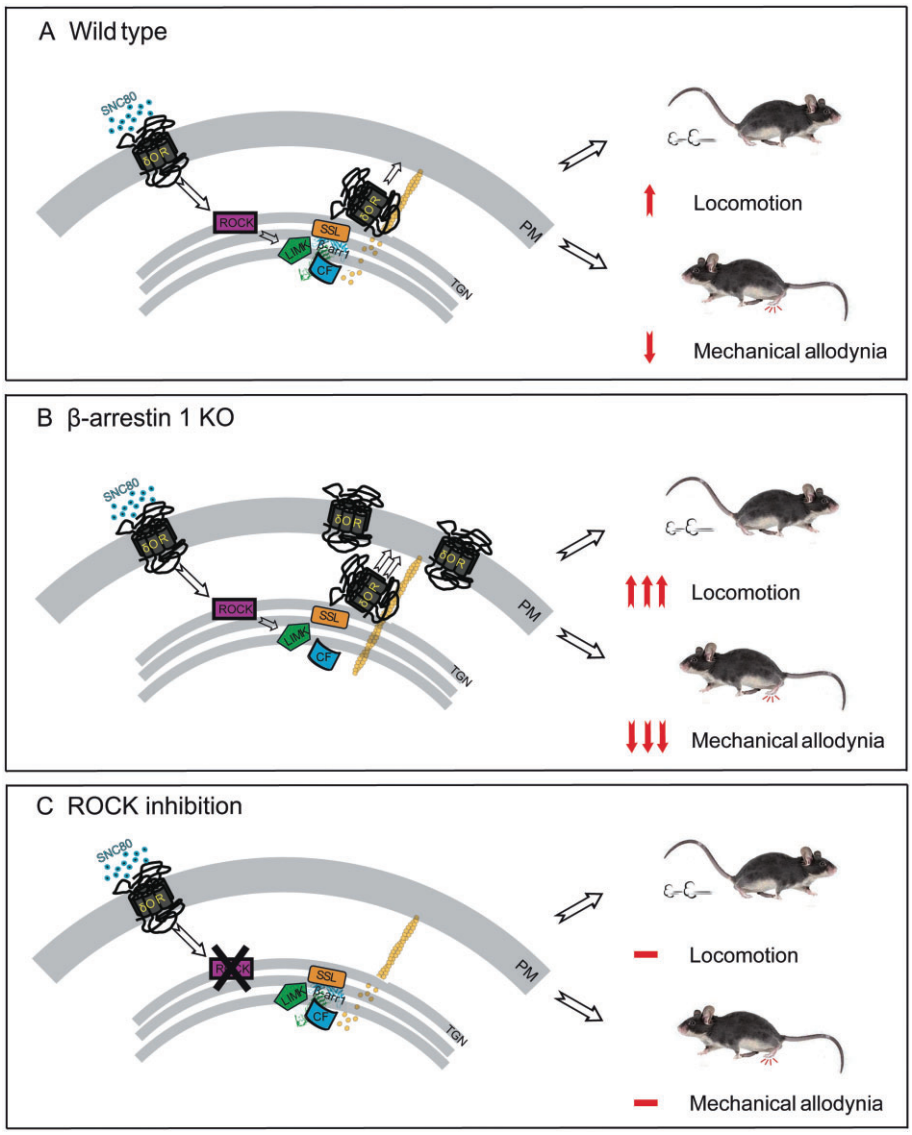

Figure 1

A schematic model of ROCK-LIMK–β-arrestin 1 dependent regulation of DOPr function. (A) The DOPr agonist, SNC80, binds with DOPrs to activate RhoA-ROCK. As β-arrestin 1 is associated with LIMK and one of the phosphatases, possibly slingshot (SSL), within the trans-Golgi network, cofilin is activated to increase actin filament severing and turnover. This allows a regulated release of DOPrs to the cell membrane to influence the functional effect of the DOPr agonist, SNC80. (B) In the absence of β-arrestin 1, LIMK phosphorylates and inactivates cofilin. This leaves stable actin 'tracks' in place to enhance DOPr release to the plasma membrane and increases SNC80-induced locomotion and the pain-relieving effects of SNC80 following a mechanical stimulus (C). Preventing ROCK phosphorylation of LIMK prevents DOPr activation of the pathway and agonist-induced DOPr release to the cell membrane blocking the locomotor and analgesic effects of SNC80 (modified from Mittal *et al.*, 2013).

Summary

Undoubtedly, MOPrs and DOPrs can interact to form heteromers in a heterologous system where the receptors are often overexpressed. Although of a particular interest for the regulation of these receptors and their downstream signalling cascades, the MOPr–DOPr dimer is only of clinical interest if demonstrated *in vivo*. We have recently witnessed the first *in vivo* evidence of the existence of MOPr–DOPr heteromer. Although much still needs to be carried out to describe the role of this receptor complex, we now have insights that this complex may play distinct physiological roles in the regulation of pain and depression. Concerns of DOPr antibody specificity have also cast some doubt whether DOPr functional up-regulation results from enhanced DOPr trafficking to the cell membrane. In assessing recent findings based on cellular and behavioural measures of DOPr function, it appears that DOPrs are indeed trafficked to the cell membrane in a regulated manner and that this could explain how DOPr signalling is enhanced under different physiological and pathological conditions.

Conclusion

The ability of MOPr and DOPr agonists or various pathological conditions to enhance DOPr function suggests that this receptor may represent a promising clinical target to treat different pathologies. As current findings suggest that DOPr agonists induce fewer side effects and have a reduced potential for abuse than MOPrs, DOPr agonists may indeed provide an alternate target for the treatment of chronic pain and other pathologies. Furthermore, the exciting possibility that DOPrs and MOPrs could form heteromers *in vivo* with distinct pharmacology and physiological effects represents an opportunity to develop novel classes of therapeutics. The discovery of the pathway by which DOPr function may be influenced by receptor trafficking to the cell membrane provides a new approach to manipulate receptor function. Together these recent advances in our understanding of DOPr function clarify current issues and provide new insight into possible clinical use of these opioid receptors.

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Conflict of interest

The authors declare no conflict of interest.

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