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Molecular Pharmacology of δ-Opioid Receptors

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ABBREVIATIONS: AA, arachidonic acid; AC, adenylate cyclase; ADL5747, *N*,*N*-diethyl-3-hydroxy-4-spiro[chromene-2,4′-piperidine]-4-ylbenzamide; hydrochloride; ADL5859, *N*,*N*-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl)benzamide; hydrochloride; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPK, AMP-activated protein kinase; AP, adaptor protein; AR, adrenergic receptor; ARD-353, 4-[[(2*R*,5*S*)-4-[(*R*)-[4-(diethylcarbamoyl)phenyl]-(3-hydroxyphenyl)methyl]-2,5-dimethylpiperazin-1-yl]methyl]benzoic acid; ARF, ADP ribosylation factor; ARM100390, *N*,*N*-diethyl-4-(phenylpiperidin-4-ylidene-methyl)-benzamide; AZD2327, 4-[(*R*)-(3-aminophenyl)-[4-[(4-fluorophenyl)methyl]-piperazin-1-yl]methyl]-*N*,*N*-diethylbenzamide; βarr, β-arrestin; BDNF, brain-derived neurotrophic factor; Bid, 1*H*-benzimidazole-2-yl; BK, bradykinin; BNTX, 7-benzylidenenaltrexone; BRET, bioluminescence resonance energy transfer; BU-48, N-Cyclopropylmethyl-[7alpha,8alpha,2′, 3′]-cyclohexano-1′[S]-hydroxy-6,14-endo-ethenotetrahydronororip avine; BU72, 17-methyl-3-hydroxy-[5β,7β,3′,5′]-pyrrolidino-2′[*S*]-phenyl-7α-methyl-6,14-endoethenomorphinan; BUBUC, H-Tyr-D-Cys(*t*Bu)-Gly-Phe-Leu-Thr(*t*Bu)-OH; BW373U86, 4-[(*R*)-[(2*S*,5*R*)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-*N*,*N*-diethylbenzamide; CaMKII, calcium calmodulin-dependent protein kinase II; CB, cannabinoid receptor; CCR, CC chemokine receptor; CGRP, calcitonin gene–related peptide; Cha, cyclohexylalanine; CHO, Chinese hamster ovary; CXCR, CXC chemokine receptor; DADLE, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH; DAG, diacylglycerol; DALCE, [D-Ala^2,Leu^5,Cys^6]enkephalin; DIPP, H-Dmt-Tic-Phe-Phe-OH; DIPP-NH_2, H-Dmt-Tic-Phe-Phe-NH_2; DIPP-NH_2[Ψ], H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2; Dmt, 2′,6′-dimethyltyrosine; [Dmt^1]DALDA, H-Dmt-D-Arg-Phe-Lys-NH_2; DOPr, δ-opioid receptor; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]; DPLPE, H-Tyr-c[D-Pen-Gly-Phe-Pen]; DRG, dorsal root ganglion; DSLET, H-Tyr- D-Ser-Gly-Phe-Leu-Thr-OH; DTLET, H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH; ECL, extracellular loop; eGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; EPSC, excitatory postsynaptic current; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FRET, fluorescence resonance energy transfer; GASP, G protein–coupled receptor–associated sorting protein; GFP, green fluorescent protein; GPCR, G protein–coupled receptor; GRK, G protein–coupled receptor kinase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[35S]thio)triphosphate; HEK, human embryonic kidney; HPETE, hydroperoxyeicosatetraenoic acid; ICI 154129, *N*,*N*-diallyl-Tyr-Gly-Ψ-(CH_2S)-Phe-Leu-OH; ICI 174864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; ICL, intracellular loop; IL, interleukin; IP_3, inositol trisphosphate; IPSC, inhibitory postsynaptic current; JNJ-20788560, 9-[(1*R*,5*S*)-8-azabicyclo[3.2.1]octan-3-ylidene]-*N*, *N*-diethylxanthene-3-carboxamide; JNK, c-Jun N-terminal kinase; JOM-13, H-Tyr-c[D-Cys-Phe-D-Pen]OH; (+)-KF4, (+)-5-(3-hydroxyphenyl)-4-methyl-2-(3-phenylpropyl)-2-azabicyclo[3.3.1]non-7-yl-(1-phenyl-1-cyclopentane)carboxamide; KNT-127, 6,7-Didehydro-17-methylquinolino[2′,6′:6,7] morphinan-3,14β-diol; KOPr, κ-opioid receptor; KSK-103, H-Dmt-c(SCH_2CH_2S)[D-Cys-Aic-D-Pen]OH; M6G, morphine-6-glucoronide; MAPK, mitogen-activated protein kinase; mcp, 4′(N-methylcarboxamido)phenylalanine; mcpTIPP, H-mcp-Tic-Phe-Phe-OH; (2*S*)-Mdp, (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; MOPr, μ-opioid receptor; MVB, multivesicular body; 2-Ncp, 4′-[*N*-(2-(naphthalene-2-yl)ethyl)-carboxamido]phenylalanine; NGF, nerve growth factor; NHERF, Na^+/H^+ exchange regulatory factor; NMDA, *N*-methyl-D-aspartate; NRM, nucleus raphe magnus; NTB, naltriben; NTI, naltrindole; *p*-F, *p*-fluorophenylalanine; PA, phosphatidic acid; PAG, periaqueductal gray; PAR, protease-activated receptor; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PPTA, preprotachykinin A; PTX, pertussis toxin; RGS, regulator of G signaling protein; RTK, receptor tyrosine kinase; SB-235863, [8*R*-(4*bS**,8α,8α,8α, 12*b*β)]7,10-dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl-5,6,7,8,12,12*b*-hexahydro-(9*H*)-4,8-methanobenzofuro[3,2-*e*]pyrrolo[2,3-*g*]isoquinoline hydrochloride; SNC80, 4-[(*R*)-[(2*R*,5*S*)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-methoxyphenyl)methyl]-*N*,*N*-diethylbenzamide; SoRI 20411, 5′(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylpyrido [2′,3′:6,7]morphinan; SoRI 22138, 5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-(3-phenylpropoxy) pyrido[2′,3′:6,7]morphinan; STAT, signal transducer and activator of transcription; $t_{1/2}$, terminal half-life; SYK-153, 6,7-Didehydro-17-methylquinolino[2′,3′:6,7]morphinan-3,8′,14β-triol; TAN-67, 3-[(4*aS*,12*aR*)-2-methyl-1,3,4,5,12,12*a*-hexahydropyrido[3,4-*b*]acridin-4*a*-yl]phenol; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TICP[Ψ], H-Tyr-TicΨ[CH_2NH]Cha-Phe-OH; TIPP, H-Tyr-Tic-Phe-Phe-OH; TIPP[Ψ], H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH; TM, transmembrane; TMH, transmembrane helix; Tmp, 2′,4′,6′-trimethylphenylalanine; Trk, tyrosine receptor kinase; TRK-850, (5*R*,9*R*,13*S*,14*S*)-17-cyclopropylmethyl-6,7-didehydro-4,5-epoxy-5′,6′-dihydro-3-methoxy-4′*H*-pyrrolo[3,2,1-*ij*]quinolino[2′,1′:6,7]morphinan-14-ol(1*b*) methanesulfonate; TRK-851, (5*R*,9*R*,13*S*,14*S*)-17-cyclopropylmethyl-6,7-didehydro-4,5-epoxy-8′-fluoro-5′,6′-dihydro-4′*H*-pyrrolo[3,2,1-*ij*]quinolino[2′,1′:6,7]morphinan-3,14-diol(1*c*) methanesulfonate; TRPV1, transient receptor potential cation channel subfamily V member 1; UMB 425, 4α,9-Dihydroxy-7α-(hydroxymethyl)-3-methyl-2,3,4,4α,5,6-hexahydro-1H-4,12-methanobenzofuro [3,2-e]isoquinolin-7(7αH)-one; UPF-512, Dmt-Tic-NH-CH(CH_2COOH)-Bid; VTA, ventral tegmental area.

***Abstract*——Opioids are among the most effective analgesics available and are the first choice in the treatment of acute severe pain. However, partial efficacy, a tendency to produce tolerance, and a host of ill-tolerated side effects make clinically available opioids less effective in the management of chronic pain syndromes. Given that most therapeutic opioids produce their actions via μ-opioid receptors (MOPrs), other targets are constantly being explored, among which δ-opioid receptors (DOPrs) are being increasingly considered as promising alternatives. This review addresses DOPrs from the perspective of cellular and molecular determinants of their pharmacological diversity. Thus, DOPr ligands are examined in terms of structural and functional variety, DOPrs' capacity to engage a multiplicity of canonical and noncanonical G protein–dependent responses is surveyed, and evidence supporting ligand-specific signaling and regulation is analyzed. Pharmacological DOPr subtypes are examined in light of the ability of DOPr to organize into multimeric arrays and to adopt multiple active conformations as well as differences in ligand kinetics. Current knowledge on DOPr targeting to the membrane is examined as a means of understanding how these receptors are especially active in chronic pain management. Insight into cellular and molecular mechanisms of pharmacological diversity should guide the rational design of more effective, longer-lasting, and better-tolerated opioid analgesics for chronic pain management.**

I. Introduction

Opioids have been used in pain management since ancient times and are still preferred in the treatment of acute severe pain. However, prolonged use of opioids is problematic not only because of their partial analgesic efficacy for management of chronic pain syndromes (Ballantyne and Shin, 2008; Franklin, 2014) but also because of their tendency to produce ill-tolerated gastrointestinal effects, frequent induction of tolerance, potential for abuse and fear, and risk of respiratory depression (Morgan and Christie, 2011).

The use of transgenic mice models has now established that desired and undesired effects of clinically available opioids are mediated via μ-opioid receptors (MOPrs) (Charbogne et al., 2014). This has stimulated research on δ-opioid receptors (DOPrs) and κ-opioid receptors (KOPrs) as alternative targets for the rational development of novel, better-tolerated analgesics. Both of these receptor types evoke effective analgesia (Kieffer and Gavériaux-Ruff, 2002; Chavkin, 2011; Gavériaux-Ruff and Kieffer, 2011), but stress and dysphoric responses associated with KOPr activation (Bruchas and Chavkin, 2010; Van't Veer and Carlezon, 2013) make DOPrs a more attractive alternative for analgesic drug development. In fact, DOPr agonists instead possess anxiolytic- and antidepressant-like actions (Chu Sin Chung and Kieffer, 2013). Their ability to evoke these emotional responses is highly desirable not only in terms of novel therapeutic applications but also because of the frequent association of anxiety and mood disorders with chronic pain (Goldenberg, 2010a,b). Together with this advantageous psychopharmacological profile, DOPr agonists have demonstrated analgesic efficacy in animal models of chronic pain (Kieffer and

Gavériaux-Ruff, 2002) and their side effect profile is milder than that of MOPr agonists, particularly concerning respiratory depression (Cheng et al., 1993; Gallantine and Meert, 2005), gastrointestinal transit (Gallantine and Meert, 2005; Feng et al., 2006), and physical dependence (Cowan et al., 1988; Codd et al., 2009). DOPr participation in reward responses is also considerably less than that of MOPrs, being mostly associated with learning of physiologic rewards (Laurent et al., 2012; Charbogne et al., 2014). Consequently, DOPr activation does not facilitate intracranial self-stimulation (Do Carmo et al., 2009), their agonists are not discriminated as morphine substitutes (Gallantine and Meert, 2005), and DOPrs do not display reinforcing properties (Banks et al., 2011). Despite these advantages, DOPr agonists display considerable potential for tolerance (Pradhan et al., 2010; Audet et al., 2012) and may increase forebrain excitability by reducing the threshold for seizures (Jutkiewicz et al., 2005, 2006; Chu Sin Chung and Kieffer, 2013). Both of these are still outstanding issues in the search for safer and more effective opioid analgesics.

Importantly, the magnitude of undesired effects is not the same for all DOPr analgesics, suggesting that rational design of novel agonists may realistically improve their therapeutic profile. To advance this goal, an in-depth understanding of the molecular and cellular determinants of DOPr ligand signaling diversity is essential and constitutes the focus of this review. Thus, DOPr ligands were examined in terms of their structural assortment and modes of interaction with the receptor. Different signaling cascades activated by DOPr were identified, and evidence supporting ligand-specific signaling and regulation was analyzed. Reports of multiple DOPr subtypes were also surveyed in light of novel insight on the ability of DOPr to organize into multimeric arrays and to adopt multiple active conformations. Finally, current understanding of mechanisms targeting DOPr to the membrane were also addressed, because this regulated cellular process seems to underlie the unique efficacy of DOPr agonists in the treatment of chronic pain syndromes.

II. Genes Codifying δ-Opioid Receptors and Regulation of Expression

Using a random primed expression cDNA library from NG108-15 cells, Kieffer et al. (1992) isolated DNA codifying for a 372–amino acid protein with the pharmacological selectivity profile of DOPrs (Kieffer et al., 1992). Using an alternative strategy, Evans et al. (1992) concomitantly cloned a similar protein that was able to bind enkephalins. Building on these sequences, it was possible to subsequently locate the murine DOPr gene (*Oprd*) to the distal region of chromosome 4, to chromosome 5 in rats, and to the short arm of chromosome 1 in humans (Bzdega et al., 1993; Befort et al., 1994; Kaufman et al., 1994). In all species, the coding region of the DOPr gene is interrupted by two introns of 26 kb and 3 kb located after transmembrane (TM) domains 1 and 4, respectively (Simonin et al., 1994). Despite the fact that pharmacologically distinct DOPr subtypes have been reported, no alternative splicing of the gene has been described thus far, suggesting that the gene encodes only one protein (but see section VII). In mice, the DOPr gene spans over 32 kb, with transcription initiation sites between 390 and 140 nucleotides upstream of the ATG translation start codon and the polyadenylation site being situated 1.24 kb downstream of the translation stop codon (Augustin et al., 1995). A series of studies, performed mainly on cells of murine origin, revealed that the DOPr gene is highly regulated (Wei and Loh, 2011) (Fig. 1). Thus, an analysis of the 1.3-kb 5′ receptor–flanking sequence in this species revealed that the promoter region of the DOPr gene lacks a classic TATA box or any consensus initiator but instead contains a G + C–rich region and a GC box known to bind members of the specificity protein 1 transcription factor family (Augustin et al., 1995; Liu et al., 1999; Smirnov et al., 2001). An E box that binds upstream stimulatory factors has also been described in the mouse DOPr promoter (Liu et al., 1999). In mouse NS20Y cells and in the mouse brain, the transcription factor E twenty-six–1 binds to an E twenty-six–1 binding site overlapping the E box and acts as a transactivator for DOPr expression (Sun and Loh, 2001). In mouse neuroblastoma × rat glioma hybrid NG108-15 cells, regulation of the DOPr gene by adaptor protein (AP)-1 and AP-2 transcription factors has also been described through their respective binding to elements located 355 bp and 157 bp upstream of the start codon (Wöltje et al., 2000). In rat pheochromocytoma PC12 cells, an interaction of the DOPr promoter with transcription factor nuclear factor-κB and its partner p300 was also observed and the interaction would be responsible for nerve growth factor (NGF)–induced expression of DOPr (Chen et al., 2006a, 2007, 2010). In immune cells, transcription of the mouse DOPr gene is also controlled by Ikaros and Ikaros-2 (Sun and Loh, 2002, 2003). An interleukin (IL)-4 responsive element was also found in the mouse promoter and in cells of human or mouse origin; this responsive element binds signal transducer and activator of transcription (STAT) 6 and is strongly inducible by IL-4 (Börner et al., 2004). Finally, DNA methylation of the mouse DOPr gene also suggests that it is epigenetically regulated (Wang et al., 2003, 2005b).

III. Characterization of δ-Opioid Receptor Structure

A. Primary and Secondary Structures of δ-Opioid Receptors

As mentioned above, mouse, rat, and human DOPr genes encode a protein of 372 amino acid residues with

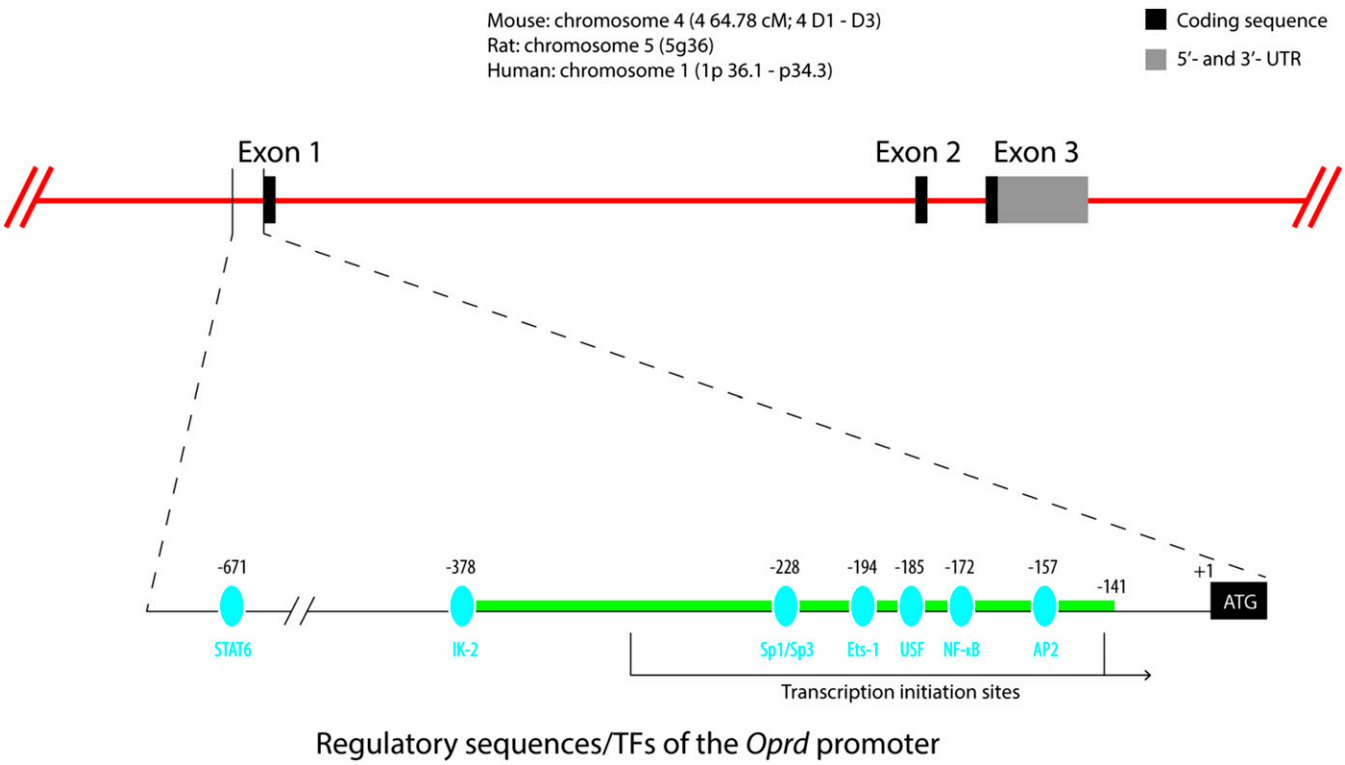

Fig. 1. Organization of the *Oprd* gene and its regulatory elements. In all species, the DOPr gene (*Oprd*) occupies approximately 32 kb on the chromosome. The upper panel illustrates the coding region interrupted by two introns (26 kb and 3 kb) located after TM domains 1 and 4. Regulatory elements and transcription factors are illustrated in the lower panel. Note that most of these findings have been described upon studying the mouse *Oprd*. Numbers above the map correspond to the 5′ ends of the transcription factor binding sites (blue circles) in relation to the initiation codon (designated by +1). Ets, E twenty-six; Ik, Ikaros; NF-κB, nuclear factor κB; Sp1, specificity protein 1; Sp3, specificity protein 3; TF, transcription factor; USF, upstream stimulatory factor; UTR, untranslated region. This figure is adapted from Wei and Loh (2011).

7 TM-spanning domains (Fig. 2A) (Evans et al., 1992; Kieffer et al., 1992). The primary amino acid sequence of DOPr is highly conserved among these species, with more than 90% homology (Fig. 2B). Besides phosphorylation sites (described in section V.A), a sequence analysis revealed that DOPrs can be otherwise modified on different residues. At its N termini, DOPr possesses two putative *N*-glycosylation sites (residues Asn18 and Asn33; Fig. 2A) that play an important role in receptor folding and its exit from the endoplasmic reticulum (ER) (Petaja-Repo et al., 2000). *O*-Glycosylation of DOPrs has also been described (Petaja-Repo et al., 2000) but, as opposed to heavily *O*-glycosylated proteins (e.g., low-density lipoprotein receptors), extracellular domains of DOPrs do not display putative acceptor motifs containing serine and threonine residues. The role of this putative *O*-linked glycan remains unknown at this time. DOPr also possesses a disulfide bond linking Cys121 in the first extracellular loop (ECL) to Cys198 in the second ECL. The disulfide bond is thought to stabilize the conformation of both loops and could be involved in the closure of the ligand binding pore delimited by the helixes (Brandt et al., 1999). Finally, DOPr is palmitoylated on Cys333. This post-translational modification of the receptor plays an essential role in its membrane expression by promoting its export from the ER (Petäjä-Repo et al., 2006). Interestingly, palmitoylation was also shown to happen at the membrane via an activation-dependent mechanism, where it likely regulates DOPr signaling (Petäjä-Repo et al., 2006).

Similar to other class A G protein–coupled receptors (GPCRs), highly conserved amino acid residues are found within each TM domain of DOPr. In particular, the strictly conserved Asn67 and Asp95 are part of a highly conserved network of polar residues likely involved in stabilizing the protein. In DOPr, this network is completed by residues Asn131, Ser135, Asn310, Ser311, and Asn314, which were shown to be involved in the allosteric control of signaling by the sodium ion (Fenalti et al., 2014). Among other conserved residues are the DRY motif [Asp145-Arg146-Tyr147], located at the intracellular end of DOPr TM3; the CWxP [Cys273-Trp274-Ala275-Pro276], located within TM6; and the $NP(x)_2Y(x)_6F$ [Asn314-Pro315-$(x)_2$-Tyr318-$(x)_6$-Phe325] at the end of TM7. The effect of a mutation within the DRY motif is receptor dependent (Rovati et al., 2007). To our knowledge, the effect of a mutation within the DRY motif of DOPr has not been reported. However, in MOPr, mutation of the aspartate residue alters the activity and the G protein coupling of the receptor (Li et al., 2001). Although nothing has been specifically done for DOPrs to date, the CWxP motif was shown to play an important role for class A GPCRs. The role of the highly conserved cysteine residue is unclear, but it is speculated that it

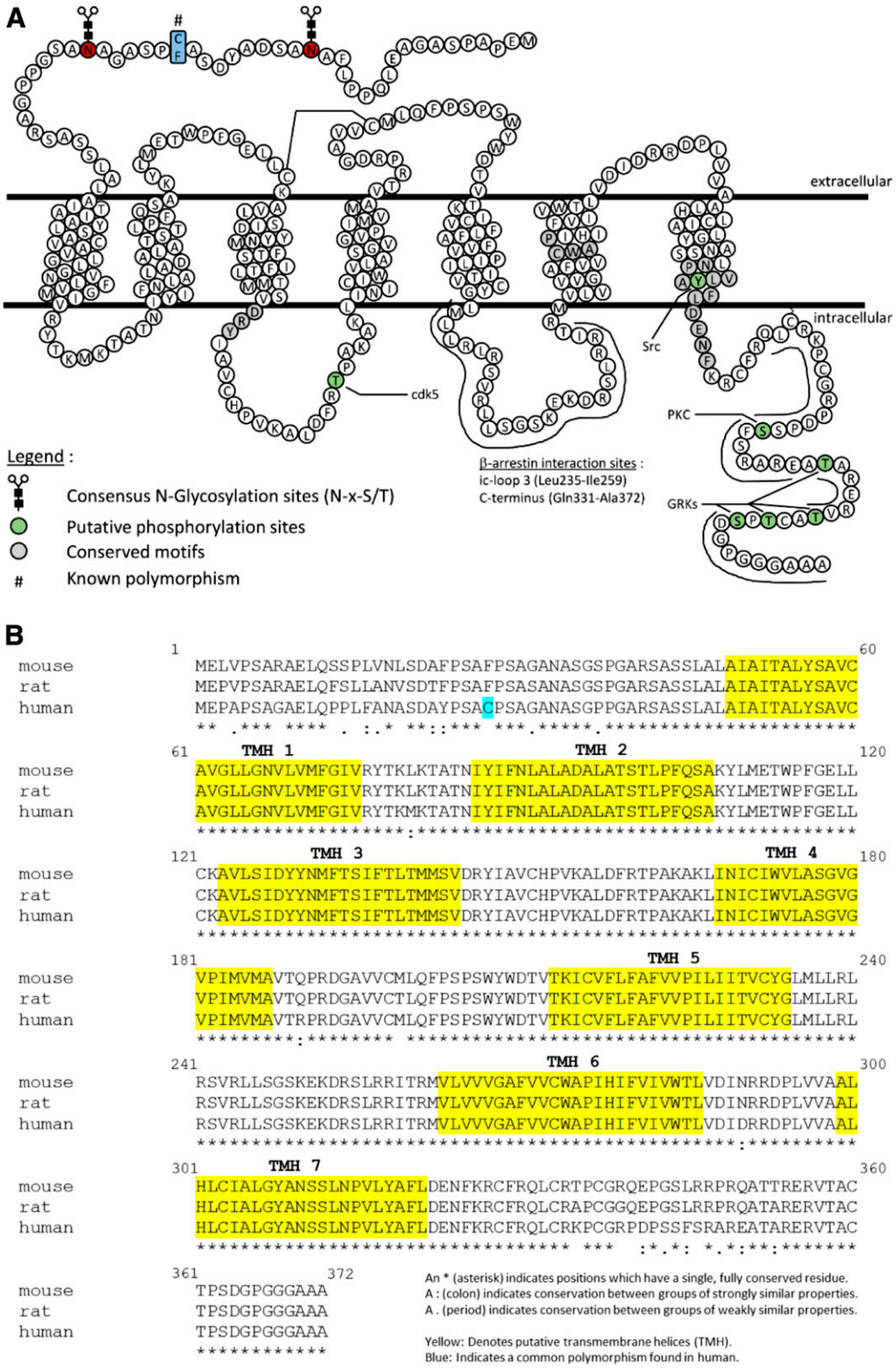

Fig. 2. Primary and secondary amino acid structures of the DOPr and its conserved motifs. (A) The human DOPr in the serpentine format is shown. In all species, the DOPr contains 372 amino acid residues arranged into 7 TM-spanning domains. Motifs that are highly conserved within the rhodopsin-like GPCRs appear in gray, putative phosphorylation sites are in green, and consensus *N*-glycosylation sites are in red. The βarr binding sites are also shown. (B) The primary amino acid sequence alignment reveals a >90% homology of mouse, rat, and human DOPrs. The amino acid sequences forming the putative TMHs are highlighted in yellow. The most common human polymorphism (Phe27/Cys27) is highlighted in light blue.

participates in the rearrangement of the TM6 and TM7 interface after activation of the receptor (Olivella et al., 2013). The proline residue would produce a movement of TM6 away from TM3, an essential step for G protein activation (Moreira, 2014). Within the CWxP motif, the tryptophan residue is certainly the most documented.

This residue is referred to as the "tryptophan switch" or the "aromatic lock." The replacement of this residue usually impairs the activity of the receptor (Ahuja and Smith, 2009; Holst et al., 2010). Finally, the $NP(x)_2Y(x)_6F$ motif of DOPr includes residue Asn314, which, as mentioned above, is part of the conserved polar network inside the receptor and therefore plays an important role in stabilizing the protein. The $NP(x)_2Y(x)_6F$ motif has also been involved in the agonist-induced receptor internalization and extracellular signal-regulated kinase (ERK) signaling, with phosphorylation of Tyr318 being an essential step in mediating these effects (Kramer et al., 2000b) (further discussed in section V.A).

B. Alterations to δ-Opioid Receptor Primary Structure: Polymorphisms

The three exons encoding the human DOPr protein display only two known polymorphisms (Simonin et al., 1994; Wei and Loh, 2011). One of them is found in exon 3, where a silent C for T exchange affects codon 307 (Gly307Gly) (Mayer et al., 1997). The other, found in exon 1, corresponds to a nonsynonymous modification (G80T) resulting in Cys for Phe transversion at position 27 (Phe27Cys; Fig. 2) (Gelernter and Kranzler, 2000). Both alleles of the nonsynonymous mutations display similar pharmacological and signaling properties (Leskelä et al., 2009), but their maturation and ligand-independent trafficking differ (Leskelä et al., 2009, 2012; Sarajärvi et al., 2011). Thus, the least frequent variant Cys27 (Gelernter and Kranzler, 2000) displays greater precursor retention in the ER and enhanced turnover of mature surface receptors compared with the more common Phe27 allele (Leskelä et al., 2009). These differences have been interpreted as manifestation of a gain-of-function phenotype with possible pathophysiological consequences (Leskelä et al., 2009), a hypothesis that was verified by showing that human neuronal cell lines that expressed the Cys27 variant displayed altered endocytic trafficking and abnormal processing of amyloid precursor protein (Sarajärvi et al., 2011).

C. Tertiary Structure: Crystallization Studies

A 3.4-Å resolution structure of the naltrindole (NTI)–bound mouse DOPr fused to the T4 lysozyme at intracellular loop (ICL) 3 was first published by Granier et al. (2012). It shows the typical seven-pass transmembrane helix (TMH) structure, similar to the overall backbone structure of MOPr (Manglik et al., 2012) and KOPr (Wu et al., 2012). NTI occupies an exposed binding pocket similar in shape to the binding pockets of MOPr and KOPr. Despite low sequence homology, the ECL2 β-hairpin structure is similar to that in the ECL2s of MOPr and KOPr. The morphinan part of NTI (see section IV.B.2 for details on ligand structure) is inserted deep into the binding pocket, with its positively charged nitrogen atom forming the expected salt bridge with D128 in the third TMH. Comparison of antagonist-bound DOPr , MOPr, and KOPr structures confirms the validity of the "message-address" concept first proposed by Schwyzer (1977) to explain structure-activity relationships of adrenocorticotropic hormone and related peptide hormones. Thus, the lower portion of the binding pocket is conserved among DOPr, MOPr, and KOPr and binds the morphinan group ("message segment") of the ligand, which is responsible for its efficacy, whereas the upper part is divergent among the three receptors and its interaction with the distinct "address" segment of the ligand is responsible for receptor selectivity. In the case of the DOPr-NTI complex, the indole moiety of the ligand engages in a hydrophobic interaction with the leucine residue in position 300. The DOPr selectivity of NTI is a result of this interaction, because corresponding residues in MOPr (W318) and KOPr (Y312) would produce a steric clash with NTI's indole group.

Subsequently, a 1.8-Å high-resolution structure of the human DOPr with an amino-terminal b_{562}RIL fusion protein [b_{562}RIL-DOPr(ΔN/ΔC)] was published (Fenalti et al., 2014). Overall, this structure is very similar to the 3.4-Å resolution structure of the mouse DOPr construct (root-mean-square deviation of 0.91 Å over all structurally characterized Cα atoms). The "closed," inactive conformation of ICL3 was clearly defined in this structure, which also revealed the key role of R291 in stabilizing the ECL3 conformation (Fig. 3). Importantly, the structure showed details of the sodium allosteric binding site with the coordination of the sodium ion by five oxygen atoms (D95, S135, N131, and two water molecules). This structural information prompted a study on sodium-dependent allosteric modulation through mutation of key sodium site residues. N131 mutation to alanine or valine enhanced constitutive activity for the β-arrestin (βarr) pathway, while abolishing G protein signaling. When D95, N310, and N314 were mutated to alanine, antagonists at the wild-type DOPr, such as NTI, acted as βarr-biased agonists at these receptor mutants. The authors concluded that the sodium coordinating residues act as "efficacy switches" at the DOPr. Overall, the high-resolution structure in conjunction with the performed mutation studies provided important insight into allosteric regulation, biased signaling, and water solvent networks of DOPr. As is the case with other GPCR-ligand interactions, a number of water-mediated NTI contacts with DOPr are evident. The involvement of water molecules in ligand binding complicates structure-based drug design.

The crystal structure of the human DOPr in complex with the mixed MOPr agonist/DOPr antagonist H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2; Dmt is 2′,6′-dimethyltyrosine and Tic is 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) (Schiller et al., 1999a) (described in section IV.D.1) at 2.7-Å resolution was recently determined by serial femtosecond crystallography using an X-ray free electron laser (Fenalti et al., 2015). Thus, for the first time, the crystal structures of a GPCR bound to a peptide ligand and to a

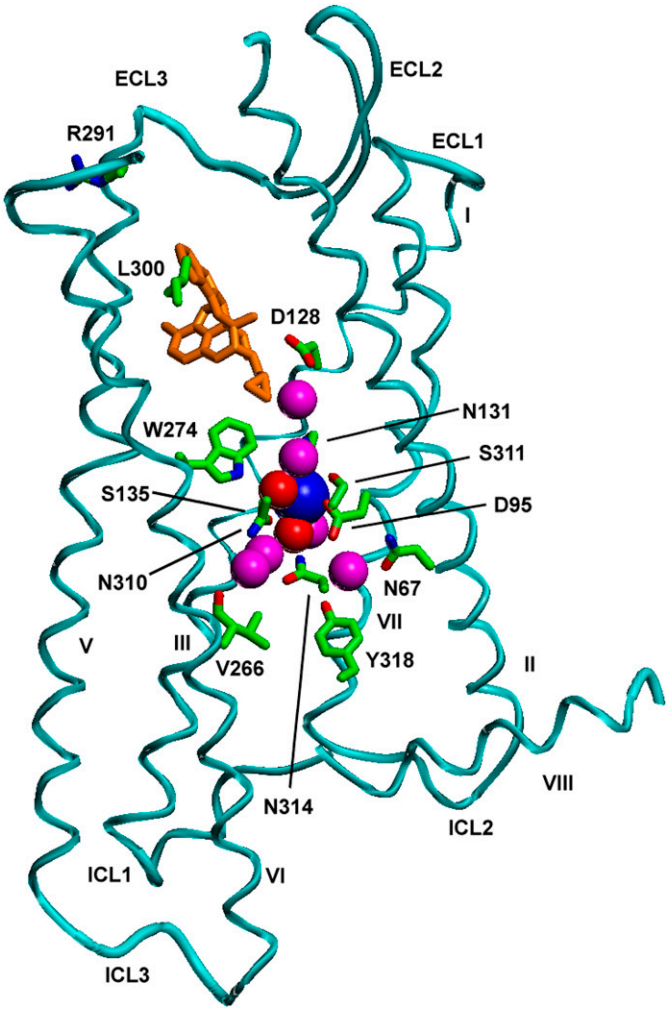

Fig. 3. DOPr-NTI crystal structure. The DOPr structure is shown in blue and residues around the allosteric sodium site appear as green sticks. Sodium is shown as a blue sphere; waters in the first and second coordination shells are shown as red and magenta spheres, respectively. NTI is shown as orange sticks. This figure is adapted from Fig. 1A in Fenalti et al. (2014), generated by using coordinates deposited in the Protein Data Bank under accession code 4NH6.

nonpeptide ligand are now known. Overall, the structure of the DOPr-DIPP-NH_2 complex is similar to the 1.8-Å resolution structure of the DOPr-NTI complex (Fenalti et al., 2015) (Fig. 4). DIPP-NH_2 and NTI bind to the same orthosteric binding site cavity with partial overlap of the pharmacophores. However, because of its larger molecular size, DIPP-NH_2 induces an expansion of the orthosteric binding site compared with the DOPr- NTI complex, resulting in a small outward movement of the extracellular parts of TMHs II and VI and an outward movement of 2 Å of ECL2.

As shown in Fig. 4, when DOPr structures in complex with DIPP-NH_2 or with NTI are superimposed, the Dmt phenol moiety of the peptide overlaps with the phenol moiety of NTI but is tilted by approximately 30°. As expected, the positively charged N-terminal amino group of DIPP-NH_2 forms a salt bridge with D128 in the third TMH, thus playing the same role as the positively charged nitrogen of NTI in analogous salt bridge formation. The Tyr^1-Tic^2 amide bond of DIPP-NH_2 has the *cis* configuration and the Tic residue overlaps with the benzene moiety of the indole ring of DOPr-bound NTI. The side chains of Phe^3 and Phe^4 interact with receptor residues outside the NTI-occupied binding pocket. An attempt was made to identify structural details implicated in the bifunctional profile of DIPP-NH_2 through superimposition of the crystal structure of MOPr in the inactive state (Manglik et al., 2012) with the DOPr-DIPP-NH_2 structure. Very recently, the crystal structure of MOPr bound to the morphinan agonist BU72 (17-methyl-3-hydroxy-[5β,7β,3′,5′]-pyrrolidino-2′[*S*]-phenyl-7α-methyl-6,14-endoethenomorphinan) and a G protein mimetic camelid antibody fragment was solved (Huang et al., 2015). The availability of the crystal structures of MOPr in the active state and of DOPr in the inactive state should now permit the structure-based design of MOPr agonists/DOPr antagonists, a promising class of compounds expected to induce analgesia with reduced unwanted effects (see section IV.D.1).

IV. δ-Opioid Receptor Ligands

A. δ-Opioid Receptor Agonists

1. Peptide δ-Opioid Receptor Agonists. Naturally occurring peptide DOPr agonists are the enkephalins [Met^5]-enkephalin and [Leu^5]enkephalin (Hughes et al., 1975) and the deltorphins dermenkephalin (Kreil et al., 1989), deltorphin I, and deltorphin II (Erspamer et al., 1989)

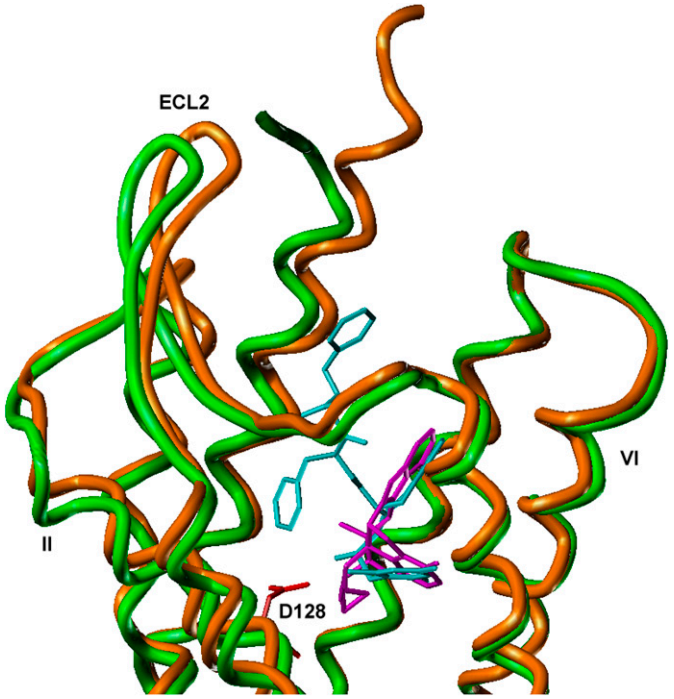

Fig. 4. DOPr structure (green) bound to DIPP-NH_2 (blue sticks) superimposed on a DOPr structure (orange) bound to NTI (magenta sticks). This figure is adapted from Fig. 2D in Fenalti et al. (2015), generated by using coordinates deposited in the Protein Data Bank under accession code 4RWD.

H-Tyr-Gly-Gly-Phe-Met-OH	[Met^5]enkephalin
H-Tyr-Gly-Gly-Phe-Leu-OH	[Leu^5]enkephalin
H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	Dermenkephalin
H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	Deltorphin I
H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	Deltorphin II

Fig. 5. Naturally occurring peptide DOPr agonists.

(Fig. 5). The enkephalins are only moderately DOPr selective and are subject to rapid enzymatic degradation, whereas the deltorphins have high DOPr selectivity and are enzymatically more stable.

Linear enkephalin analogs with enhanced DOPr selectivity include H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE) (Beddell et al., 1977), H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH (DSLET) (Gacel et al., 1980), H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET) (Zajac et al., 1983), and H-Tyr-D-Cys(*t*Bu)-Gly-Phe-Leu-Thr(*t*Bu)-OH (BUBUC) (Gacel et al., 1990) (Fig. 6). The conformationally constrained, cyclic enkephalin analogs H-Tyr-c[D-Pen-Gly-Phe-D-Pen] (DPDPE) and H-Tyr-c[D-Pen-Gly-Phe-Pen] (DPLPE) are highly DOPr selective (Mosberg et al., 1983), and DPDPE has become a widely used pharmacological tool. Substitution of *p*-fluorophenylalanine [Phe(*p*-F)] for Phe^4 in DPLPE and C-terminal extension with Phe led to a compound, H-Tyr-c[D-Pen-Gly-Phe(*p*-F)- L-Pen]-Phe-OH, with further improved DOPr selectivity (Hruby et al., 1997). A des-Gly analog of DPDPE, H-Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13), also turned out to be a potent and selective DOPr agonist (Mosberg et al., 1988). Among various prepared cyclic lanthionine enkephalin analogs, H-Tyr-c[D-Val$_L$-Gly-Phe-D-Ala$_L$]OH displayed high DOPr agonist potency and selectivity (Rew et al., 2002) and was shown to attenuate cancer-related bone pain with systemic administration (Brainin-Mattos et al., 2006). Structural modifications of the already potent and very selective deltorphins resulted in compounds with further improved DOPr binding affinity and selectivity (Sasaki et al., 1991; Sasaki and Chiba, 1995; Bryant et al., 1997).

A different class of DOPr agonists was discovered through structural modification of Tyr-Tic-Phe-Phe (TIPP; Tic is 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) (see section IV.B.1). Replacement of Tyr^1 in TIPP with 4′[*N*-((4′-phenyl)phenethyl)carboxamido]phenylalanine (Bcp) or 4′-[*N*-(2-(naphthalene-2-yl)ethyl)carboxamido]-phenylalanine (2-Ncp) led to potent and selective DOPr agonists (Berezowska et al., 2009, 2012). In a direct comparison with DPDPE, [2-Ncp^1]TIPP showed 8-fold higher DOPr binding affinity, comparable DOPr versus MOPr selectivity, and 14-fold higher DOPr agonist potency in the guanosine 5′-*O*-(3-[35S]thio)triphosphate ([^{35}S]GTPγS) binding assay. Since opioid peptides lacking the Tyr^1 hydroxyl group are known to have very weak opioid activity, this result indicates that the large naphthylethyl group of 2-Ncp interacts with an accessory binding site to strengthen the binding interaction. Therefore, this compound has a distinct DOPr binding mode; given this difference, it may be worthwhile to examine its signaling profile across different responses to unveil possible functional selectivity. A series of C-terminally substituted H-Tyr-Tic-NH_2 analogs with DOPr agonist properties was reported in 1999 (Schiller et al., 1999c). Among these compounds, one of the isomers of H-Tyr-Tic-NH-CH_2-CH(Ph)COOEt was identified as a DOPr agonist with subnanomolar potency and high DOPr binding selectivity. The structurally related dipeptide analog Dmt-Tic-NH-CH(CH_2COOH)-Bid (UPF-512; Bid is 1*H*-benzimidazole-2-yl) is also a potent DOPr agonist (Balboni et al., 2002b). This compound displayed partial efficacy to inhibit cAMP production in human embryonic kidney (HEK) cells and to induce internalization in rat cortical neurons (Charfi et al., 2014) with anxiolytic- and antidepressant-like activities when administered peripherally to mice (Vergura et al., 2008).

2. Nonpeptide δ-Opioid Receptor Agonists. The first reported nonpeptide DOPr agonist BW373U86 (4-[(*R*)-[(2*S*,5*R*)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-hydroxyphenyl)methyl]-*N*,*N*-diethylbenzamide), a racemic compound (Fig. 7), showed high DOPr binding affinity (Chang et al., 1993) but induced convulsions in mice (Comer et al., 1993). SNC80 (4-[(*R*)-[(2*R*,5*S*)-2,5-dimethyl-4-prop-2-enylpiperazin-1-yl]-(3-methoxyphenyl)-methyl]-*N*,*N*-diethylbenzamide), an analog of one enantiomer of BW373U86 with high DOPr selectivity (Calderon et al., 1994), produced antinociceptive effects with systemic administration (Bilsky et al., 1995). In addition, a structurally related DOPr agonist, ARD-353 (4-[[(2*R*,5*S*)-4-[(*R*)-[4-(diethylcarbamoyl)phenyl]-(3-hydroxyphenyl)methyl]-2,5-dimethylpiperazin-1-yl]methyl]benzoic acid), was shown to reduce

H-Tyr-D-Ala-Gly-Phe-D-Leu-OH	(DADLE)
H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH	(DSLET)
H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH	(DTLET)
H-Tyr-D-Cys(*t*Bu)-Gly-Phe-Leu-Thr(*t*Bu)-OH	(BUBUC)
H-Tyr-c[D-Pen-Gly-Phe-D(or L)-Pen]OH	(DPDPE, DPLPE)
H-Tyr-c[D-Pen-Gly-Phe(*p*-F)-L-Pen]-Phe-OH	
H-Tyr-c[D-Cys-Phe-D-Pen]OH	(JOM-13)
H-Tyr-c[D-Val$_L$-Gly-Phe-D-Ala$_L$]OH	
H-2-Ncp-Tic-Phe-Phe-OH	
H-Dmt-Tic-NH-CH(CH_2COOH)-Bid	(UFP-512)

Fig. 6. Peptide DOPr agonists.

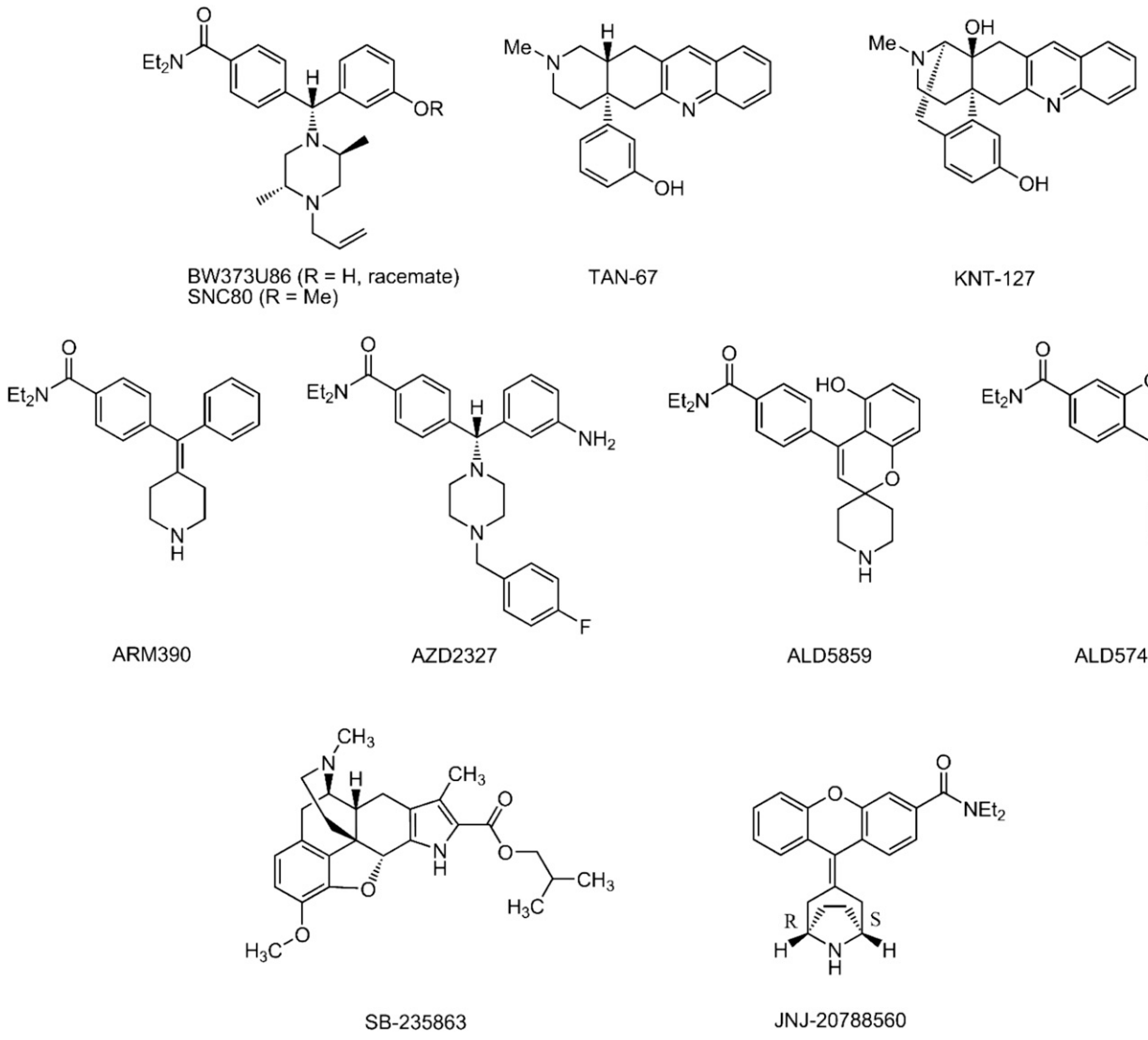

Fig. 7. Nonpeptide DOPr agonists.

myocardial infarct size (Watson et al., 2006). Whereas SNC80 also produces convulsions, ARD-353 does not. TAN-67 (3-[(4*aS*,12*aR*)-2-methyl-1,3,4,5,12,12*a*-hexahydropyrido[3,4-*b*]acridin-4*a*-yl]phenol), a heterocycle-fused octahydroisoquinoline derivative, was reported to be a potent and selective DOPr agonist, capable of producing an antinociceptive effect with subcutaneous administration in an acetic acid and abdominal constriction assay (Nagase et al., 1998). A TAN-67 analog, KNT-127 (6,7-Didehydro-17-methylquinolino[2′,3′:6,7] morphinan-3,14β-diol), showed 7-fold higher DOPr binding affinity and 26-fold higher antinociceptive potency compared with its parent (Nagase et al., 2010). Structural modification of KNT-127 led to a DOPr agonist (SYK-153 [6,7-Didehydro-17-methylquinolino [2′,3′:6,7]morphinan-3,8′,14β-triol]) with a further improved in vitro activity profile (Ida et al., 2012). ARM100390 [*N*,*N*-diethyl-4-(phenylpiperidin-4-ylidene-methyl)-benzamide] (also known as ARM390), a compound structurally derived from SNC80, is a potent, highly selective, and stable DOPr agonist (Wei et al., 2000). ARM100390 and SNC80 produced comparable antinociception and distinct types of tolerance in inflammatory pain models (Pradhan et al., 2010). In the case of SNC80-treated mice, analgesic tolerance was linked to strong DOPr downregulation, whereas with ARM100390 it was due to abolition of DOPr-regulated Ca^{+} channel inhibition. Unlike SNC80, ARM100390 did not induce tolerance to locomotor and anxiolytic effects. AZD2327 (4-[(*R*)-(3-aminophenyl)-[4-[(4-fluorophenyl)methyl] piperazin-1-yl]methyl]-*N*,*N*-diethylbenzamide), a potent and selective DOPr agonist structurally derived from SNC80, showed promising antidepressant and anxiolytic activities in a number of animal models (Hudzik et al., 2011). The spirocyclic DOPr agonists ADL5859 [*N*,*N*-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl)benzamide;hydrochloride] (Le Bourdonnec et al., 2008) and ADL5747 [*N*,*N*-diethyl-3-hydroxy-4-spiro [chromene-2,4′-piperidine]-4-ylbenzamide; hydrochloride] (Le Bourdonnec et al., 2009) are potent, selective, and

orally available DOPr agonists, with ADL5747 being approximately 50-fold more potent than ADL5859 in an animal model of inflammatory pain. Neither compound produced convulsions. A subsequent study revealed that these two compounds were also effective in a neuropathic pain model and, unlike SNC80, did not produce receptor internalization (Nozaki et al., 2012).

The pyrrolomorphinan type DOPr agonist SB-235863 ([8*R*-(4*bS**,8*aα*,8*aβ*, 12*bβ*)]7,10-dimethyl-1-methoxy-11-(2-methylpropyl)oxycarbonyl-5,6,7,8,12,12*b*-hexahydro-(9*H*)-4,8-methanobenzofuro[3,2-*e*]pyrrolo[2,3-*g*]isoquinoline hydrochloride) was inactive in acute pain models but reversed thermal hyperalgesia in inflammatory and neuropathic pain models with oral administration (Petrillo et al., 2003). The compound did not slow gastrointestinal transit, did not affect motor coordination, and lacked proconvulsant activity. The selective DOPr agonist JNJ-20788560 (9-[(1*R*,5*S*)-8-azabicyclo[3.2.1]octan-3-ylidene]-*N*,*N*-diethylxanthene-3-carboxamide) given orally was antihyperalgesic in inflammatory pain models without producing tolerance but was quite inactive in an uninflamed radiant heat test (Codd et al., 2009). It did not produce side effects seen with commonly used opioid analgesics, such as inhibition of gastrointestinal transit, respiratory depression, abuse potential, and proconvulsant activity.

B. δ-Opioid Receptor Antagonists

1. Peptide δ-Opioid Receptor Antagonists. Structural modifications of enkephalins at the N terminus resulted in a number of DOPr antagonists. An early example was ICI 174864 (*N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH), which has moderate DOPr antagonist potency (Cotton et al., 1984) (Fig. 8) and has been a useful tool in opioid research for many years. An interesting discovery was that elimination of the N-terminal positive charge in combination with 2′,6′-dimethylation of the Tyr^1 aromatic ring is a generally applicable structural modification to convert opioid peptide agonists into antagonists (Schiller et al., 2003). This can be achieved by the replacement of Tyr with (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (2*S*-Mdp). Indeed, substitution of (2*S*)-Mdp for Tyr^1 in the potent and highly DOPr-selective cyclic enkephalin analog H-Tyr-c[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH (Hruby et al., 1997) resulted in a highly selective DOPr antagonist, (2*S*)-Mdp-c[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH, with subnanomolar DOPr antagonist activity (Schiller et al., 2003). This compound was the first DOPr antagonist with a cyclic enkephalin-derived structure. Importantly, (2*S*)-Mdp^1 analogs of opioid peptides lack the ability to form a salt bridge with the key Asp residue in the third TMH of opioid receptors. It is possible that the lack of salt bridge formation may result in a distinct receptor conformation with possible functional consequences.

N,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174864)

(2*S*)-Mdp-c[D-Pen-Gly-Phe(*p*-F)-Pen]-Phe-OH

H-Tyr-Tic-Phe-Phe-OH (TIPP)

H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH (TIPP[Ψ])

H-Dmt-Tic-Phe-Phe-OH (DIPP)

H-Tyr-TicΨ[CH_2NH]Cha-Phe-OH (TICP[Ψ])

H-Dmt-Tic-OH

N,*N*-Me_2Dmt-Tic-OH

H-Tyr-D-Ala-Gly-Phe-Leu-Cys-OH (DALCE)

Fig. 8. Peptide DOPr antagonists.

Peptides of the TIPP family are the most potent and selective peptide-based DOPr antagonists (Schiller et al., 1999b). TIPP (Schiller et al., 1992) and its pseudopeptide analog H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH (TIPP[Ψ]) (Schiller et al., 1993) are highly potent and selective DOPr antagonists. In particular, TIPP[Ψ] displayed subnanomolar DOPr binding affinity and extraordinary DOPr selectivity (K_i^{μ}/K_i^{δ} = 10,500, being >500 times more DOPr selective than the nonpeptide DOPr antagonist NTI; see section IV.B.2). A TIPP analog containing Dmt in place of Tyr^1, DIPP, showed 25-fold increased δ antagonist activity and still high DOPr selectivity (Schiller et al., 1999b). The Cha^3 analogs of TIPP and TIPP[Ψ], H-Tyr-Tic-Cha-Phe-OH and H-Tyr-TicΨ[CH_2NH]Cha-Phe-OH (TICP[Ψ]) (Cha is cyclohexylalanine), also turned out to be highly selective DOPr ligands with subnanomolar antagonist activity in the mouse deferens assay (Schiller et al., 1996, 1999b). The DIPP-related dipeptide analogs H-Dmt-Tic-OH (Salvadori et al., 1995) and *N*,*N*-Me_2Dmt-Tic-OH (Salvadori et al., 1997) are selective DOPr antagonists with somewhat lower antagonist potency compared with the most potent tetrapeptide antagonists of the TIPP family (Schiller et al., 1999b).

The hexapeptide [D-Ala^2,Leu^5,Cys^6]enkephalin (DALCE) was reported to be a selective, irreversible DOPr antagonist, binding covalently to the receptor by a thiol-disulfide exchange mechanism (Bowen et al., 1987). The use of DALCE in an in vivo study produced evidence for the existence of two types of DOPrs (DOPr1 and DOPr2), because it acted as a long-lasting antagonist of the antinociceptive effect of DPDPE (DOPr1 agonist) but not of deltorphin II (DOPr2 agonist) (Jiang et al., 1991). As discussed in section IX, the nature of the DOPr1 and DOPr2 subtypes remains to be clarified.

2. Nonpeptide δ-Opioid Receptor Antagonists. NTI was the first nonpeptidic DOPr antagonist reported

(Portoghese et al., 1988) and NTI has been a very useful pharmacological tool for many years. It shows marked DOPr versus MOPr selectivity but is less selective than the best peptide DOPr antagonists. At least two reports identified NTI as a DOPr neutral antagonist (Neilan et al., 1999; Tryoen-Tóth et al., 2005). The benzofuran analog of NTI, naltriben (NTB) (Fig. 9), is also a potent DOPr antagonist (Portoghese et al., 1991) but acts as a KOPr agonist at higher doses, thereby diminishing the antagonist effect at DOPr (Stewart et al., 1994). In an antinociceptive assay, NTI and NTB showed differential antagonism of the DOPr agonists DSLET and DPDPE, which was interpreted in terms of DOPr heterogeneity (Sofuoglu et al., 1991). The 5-isocyanate analog of NTI, 5′-NTII, was the first irreversible nonpeptide DOPr antagonist (Portoghese et al., 1990), which produced long-lasting antagonism of the antinociceptive effects of the DOPr2 agonists DSLET and deltorphin II but not of that of the DOPr1 agonist DPDPE (Jiang et al., 1991; Vanderah et al., 1994). The NTI derivative TRK-850 [(5*R*,9*R*,13*S*,14*S*)-17-cyclopropylmethyl-6,7-didehydro-4,5-epoxy-5′,6′-dihydro-3-methoxy-4′*H*-pyrrolo[3,2,1-*ij*]quinolino[2′,1′:6,7]morphinan-14-ol(1*b*) methanesulfonate] showed moderate DOPr binding affinity and DOPr partial agonist activity (Sakami et al., 2008b). A TRK-850 analog, TRK-851 [(5*R*,9*R*,13*S*,14*S*)-17-cyclopropylmethyl-6,7-didehydro-4,5-epoxy-8′-fluoro-5′,6′-dihydro-4′*H*-pyrrolo[3,2,1-*ij*]quinolino[2′,1′:6,7]morphinan-3,14-diol(1*c*) methanesulfonate], also showed DOPr antagonist properties and was metabolically more stable (Sakami et al., 2008a). Both compounds were shown to be orally active antitussive agents.

The naltrexone derivative 7-benzylidenenaltrexone (BNTX) showed 100-fold higher binding affinity for [^{3}H]DPDPE binding sites (DOPr1) than for [^{3}H]DSLET binding sites (DOPr2), which was taken as evidence to indicate that BNTX is a DOPr1-selective antagonist (Portoghese et al., 1992).

(+)-KF4 [(+)-5-(3-hydroxyphenyl)-4-methyl-2-(3-phenylpropyl)-2-azabicyclo[3.3.1]non-7-yl-(1-phenyl-1-cy-clopentane)carboxamide] was the first reported DOPr antagonist from the 5-phenylmorphan class of opioids (Carroll et al., 2004) and the (+)-KF4 analog, delmorphan A, showed subnanomolar potency and improved DOPr selectivity (Thomas et al., 2006).

C. δ-Opioid Receptor Constitutive Activity and Inverse Agonists

Most of the antagonists described in the previous section were identified using the classic mouse vas deferens assay, which does not provide the conditions for monitoring constitutive activity of the receptor. Monitoring spontaneous activity is an essential condition for revealing inverse agonism, since the behavior can be observed when stabilization of an inactive state of the receptor depletes spontaneously active, signaling conformation(s) (Kenakin, 2004a). Inverse agonism was first observed by Costa and Herz (1989) using DOPr as a model. In their landmark study, the authors showed that the peptide ICI 174864 could display negative intrinsic efficacy, causing a reduction in spontaneous GTPase activity similar to uncoupling agents such as pertussis toxin (PTX) or *N*-ethylmaleimide. Moreover, when experiments were carried out in the presence of K^+ instead of that of uncoupling Na^+ ions, basal activity was increased, as was inverse efficacy for the peptide (Costa and Herz, 1989). Since ICI 174864 produced a similar reduction in basal signaling as PTX in the presence of K^+ ions, it was considered a full inverse agonist. ICI 154129 [*N*,*N*-diallyl-Tyr-Gly-Ψ-(CH_2S)-Phe-Leu-OH] and naloxone produced submaximal but significant inhibition and were therefore considered partial inverse agonists (Costa and Herz, 1989). Because inverse agonists preferentially recognize and stabilize an inactive uncoupled state of DOPr (Piñeyro et al., 2001), receptors stabilized by these ligands interact poorly with G proteins in lipid bilayers (Alves et al., 2003), and their binding affinity for membrane receptors increases in the presence of uncoupling agents such as PTX, Na^+, and/or guanine nucleotides (Costa and Herz, 1989; Neilan et al., 1999; Piñeyro et al., 2001).

After the initial description of this signaling modality, numerous other peptidic and nonpeptidic ligands were shown to display inverse agonist behavior in G protein activation and cAMP accumulation assays (summarized in Table 1). Like those of agonists, their responses were blocked by drugs such as TIPP, naloxone, and NTI, which frequently behave as antagonists (Costa and Herz, 1989; Chiu et al., 1996; Mullaney et al., 1996; Szekeres and Traynor, 1997; Labarre et al., 2000). However, it is important to keep in mind that a drug's

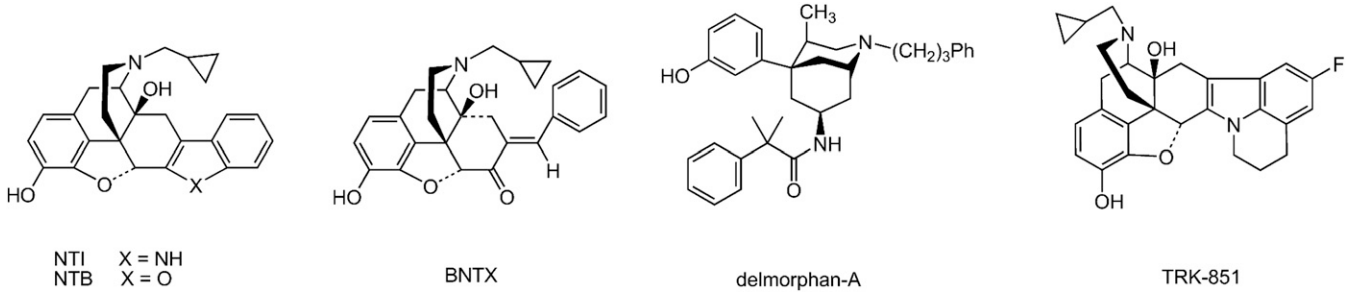

Fig. 9. Nonpeptide DOPr antagonists.

TABLE 1
DOPr inverse agonists and systems in which inverse activity has been evidenced

Type of System	DOR Species	Cell Type	Assay	Drug	Observations	Reference
Native systems	mDOR	NG108-15 hybrid cell	GTPase assay (membranes, high K)	ICI 174864, naloxone, ICI 154129	Inhibition of basal activity ICI 174864 > ICI 154129 > NLX	Costa and Herz (1989)
			GTPase assay (intact cells)	ICI 174866	Inhibition of basal activity	
			Binding affinity ± PTX (membranes)	ICI 174866	Increased affinity (presence PTX)	
			GTPγS binding (membranes)	ICI 174864	Inhibition of basal activity	Szekeres and Traynor (1997)
				NTI, BNTX, TIPP, NTB	No effect on basal activity	
Overexpression systems	mDOR	Rat 1 fibroblasts	GTPase assays (membranes)	ICI 174864	Inhibition of basal activity	Mullaney et al. (1996)
		HEK293 cells		Naloxone	No effect on basal activity	
				ICI 174864	Inhibition of basal activity of WT DOPr, D128N, and D128A; not Y129A	Befort et al. (1999)
		HEK293 cells	cAMP accumulation (intact cells)	ICI 174866	Potentiation of cAMP, production by FSK	Chiu et al. (1996)
				Naloxone, NTI	No effect on FSK stimulated cAMP production	
	Rat DOR	C6 glioma	GTPγS binding absence Na^+ ions (membranes)	ICI 174864, clocinnamox (C-CAM), BNTX, NTB	Inhibition of basal activity NTB ≥ BNTX ≥ C-CAM ≥ ICI 174864	Neilan et al. (1999)
				Naloxone, NTI	No effect on basal activity	
			Binding affinity ± Na^+/GppNHp	ICI 174864, C-CAM	Leftward shift with Na+/GppNHp	
				BNTX, NTB	No effect of Na^+/GppNHp	
	hDOR	HEK-293 cells	GTPγS binding (membranes)	TICP[Ψ]	Inhibition of basal activity of WT DOPr and of constitutively active DOPr (M262T)	Tryoen-Tóth et al. (2005)
				TICP[Ψ]	No effect on basal activity of WT DOPr, Inhibition of basal activity of constitutively active DOPr (M262T)	
				Naloxone, NTB, BNTX, ICI 174864, H-Dmt-Tic-OH *N*, *N*$(CH_3)_2$-Dmt-Tic-NH_2	No effect on basal activity of WT DOPr or constitutively active DOPr (M262T)	
			Reporter gene assay (intact cells)	*N*,*N*$(CH_3)_2$-Dmt-Tic-NH_2	Inhibition of basal activity of WT DOPr and constitutively active DOPr (M262T)	
				Naloxone, NTB, BNTX	No effect on basal activity of WT DOPr, Inhibition of basal activity of constitutively active DOPr (M262T)	
			GTPγS binding (membranes)	ICI 174864, *N*,*N*$(CH_3)_2$-Dmt-Tic-NH_2, NTI	Inhibition of basal activity ICI 174864 ≥ *N*,*N*$(CH_3)_2$-Dmt-Tic-NH_2 >>> NTI	Labarre et al. (2000)
			GTPγS binding (membranes)	ICI 174864, TICP[Ψ], naloxone	Inhibition of basal activity TICP[Ψ] ≥ ICI 174864 ≥ NLX	Piñeyro et al. (2005), Audet et al. (2008)
			cAMP accumulation	ICI 174864, TICP[Ψ]	Inhibition of basal activity TICP[Ψ] ≥ ICI 174864	
	hDOR	CHO cells	GTPγS binding (membranes)	ICI 174864, (2*S*,3*R*)TMT-L-Tic-OH	Inhibition of basal activity TMT-L-Tic-OH compound is more potent than ICI 174864	Hosohata et al. (1999)
				ICI 174864, 17-fluoroethyl NTI derivatives	Inhibition of basal activity ICI 174864 > 17-fluoroethyl NTI derivatives	Nemoto et al. (2015)
		GH3 cells	GTPγS binding (membranes)	ICI 174864	Inhibition of basal activity	Liu and Prather (2002), Martin et al. (2002)
				TIPP, naltriben, naloxone, ICI 154129	No effect on basal activity	
			Binding affinity ± Na^+/GppNHp	ICI 174864 TIPP	Leftward shift of affinity for ICI 174864 and TIPP, ICI 174864 > TIPP	
			cAMP accumulation (intact cells)	ICI 174864 TIPP	ICI 174864 inhibits basal activity TIPP stimulates basal activity	

FSK, forskolin ICI 174866, N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH; NLX, naloxone; WT, wild type.

phenotypic behavior as an agonist, antagonist, or inverse agonist is influenced by the assays, cell types, and receptor species that are used to test the ligands. A quick inspection of Table 1 shows that naloxone behaved as an inverse agonist when tested for its effect on GTPase activity in mDOPrs/NG108-15 cells and membranes (Costa and Herz, 1989). On the other hand, it was an antagonist in almost all of the other systems tested (Chiu et al., 1996; Mullaney et al., 1996; Neilan et al., 1999; Piñeyro et al., 2005; Tryoen-Tóth et al., 2005), except for two reports in which it behaved as a weak partial agonist both in guanosine 5′-3-*O*-(thio)-triphosphate (GTPγS) and cAMP accumulation assays (Liu and Prather, 2002; Piñeyro et al., 2005). NTI consistently behaved as an antagonist in all systems tested (Chiu et al., 1996; Szekeres and Traynor, 1997; Neilan et al., 1999; Labarre et al., 2000), but its fluoroethyl derivatives displayed partial inverse responses in relation to ICI 174864 (Nemoto et al., 2015). NTB was without efficacy in GTPγS binding assays carried out in mDOPrs/NG108-15 cells (Szekeres and Traynor, 1997) and human DOPrs/HEK membranes (Tryoen-Tóth et al., 2005). However, it reduced basal GTPγS binding in membranes from rat DOPr/C6 glioma cells (Neilan et al., 1999). NTB similarly behaved as an inverse agonist in HEK cells expressing a constitutively active state (M262T) of human DOPr (Tryoen-Tóth et al., 2005), and a similar pattern of inverse agonist responses as NTB was observed for naltrexone derivative BNTX (Neilan et al., 1999; Tryoen-Tóth et al., 2005).

The observed variations in drug behavior are associated with system-related differences in the propensity of DOPr to isomerize between active and inactive conformation(s) and in the ability of existing G proteins to stabilize the active state(s) (Kenakin, 2004b). This is exemplified by changes in ligand responses after interventions that modify such variables. First, greater membrane availability of G proteins increased basal GTPγS binding and turned naloxone from a weak partial agonist into a weak inverse agonist (Piñeyro et al., 2005). Second, DOPr desensitization and its consequent uncoupling from the G protein was accompanied by NTB changing from a partial agonist to a partial inverse agonist (Liu and Prather, 2002). Third, TICPΨ turned from being an antagonist when tested in wild-type human DOPr to an inverse agonist when tested in constitutively active human DOPr (Y308H mutant) (Tryoen-Tóth et al., 2005). It is interesting to note that despite similar levels of constitutive activity, TICPΨ displayed inverse agonist behavior in the Y308H but not the M262T mutant (Tryoen-Tóth et al., 2005). This observation implies that one of these active conformations cannot be depleted by TICPΨ, arguing that in addition to having spontaneous activity, the receptor must be in a "permissive" active conformation that allows destabilization in favor of a less active state.

D. Mixed μ-Opioid Receptor Agonists/δ-Opioid Receptor Antagonists

Selective blockade with a DOPr antagonist greatly reduced the development of morphine tolerance and dependence, suggesting synergistic contribution of both receptors to these side effects (Abdelhamid et al., 1991; Fundytus et al., 1995; Billa et al., 2010; Beaudry et al., 2015a). Development of tolerance and dependence after chronic morphine administration was similarly reduced by antisense oligodeoxynucleotides to DOPr (Kest et al., 1996), whereas analgesic activity was retained without induction of tolerance in DOPr knockout mice chronically treated with morphine (Zhu et al., 1999). Whether the reduction in tolerance and dependence associated with the coadministration of a MOPr agonist and a DOPr antagonist is due to interactions with distinct, noninteracting MOPr and DOPr (interaction at the systems level) or to their association with the two receptors in a complex (MOPr/DOPr heterodimer) is still a matter of investigation. Regardless of the mechanism, these various observations provide a strong rationale for the development of mixed MOPr agonists/-DOPr antagonists as analgesics with expected low propensity to produce analgesic tolerance and physical dependence. Two types of mixed MOPr agonists/DOPr antagonists have been described. With one type, no clear distinction can be made between molecular moieties that are responsible for MOPr agonist and DOPr antagonist behavior (integrated pharmacophores), and such compounds are usually discovered by chance. In another type of ligand, distinct MOPr agonist and DOPr antagonist components are connected to each other directly or via a linker. A review of MOPr agonists/DOPr antagonists reported until 2006 was previously published (Ananthan, 2006).

1. Peptide μ-Opioid Receptor Agonists/δ-Opioid Receptor Antagonists. Examples of the integrated pharmacophore type are DIPP-NH_2 and the pseudopeptide H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[Ψ]) (Schiller et al., 1999b) (Fig. 10). The crystal structure of the DIPP-NH_2–bound DOPr was presented in section III. C. DIPP-NH_2[Ψ] was the first reported compound with balanced MOPr agonist/DOPr antagonist properties and subnanomolar binding affinities for both receptors (Schiller et al., 1995, 1999a). As expected, DIPP-NH_2[Ψ] given intracerebroventricularly produced a potent antinociceptive effect, no physical dependence, and less tolerance than morphine. The dipeptide analog H-Dmt-Tic-NH-$(CH_2)_3$-Ph is also a potent MOPr agonist/DOPr antagonist with subnanomolar MOPr and DOPr binding affinities. Subsequently, the dipeptide derivative Dmt(NMe_2)-Tic-NH-1-adamantane (Salvadori et al., 1999) and the tripeptide analog H-Dmt-Tic-Gly-NH-CH_2-Ph (Balboni et al., 2002a) were also reported to be potent MOPr agonists/DOPr antagonists.

H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2)

H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[Ψ])

Dmt(*N*Me_2)-Tic-NH-1-adamantane

H-Dmt-c[D-Orn-2-Nal-D-Pro-Gly-]

H-Tyr-c(SS)[D-Cys-1-Nal-Nle-Cys]X (X = NH_2 or OH)

H-Dmt-c(SCH_2CH_2S)[D-Cys-Aic-D-Pen]Ser(Glc)-NH_2

H-Dmt-Pro-Phe-NH-C_2H_4-Ph

H-Dmt-Pro-Trp-D-1-Nal-NH_2

H-Dmt-Pro-Tmp-Phe-NH_2

H-Dmt→D-Arg→Phe→Lys-NH-CH_2-CH_2-NH-Phe←Cha[$NHCH_2$]ΨTic←Tyr-H

([Dmt^1]DALDA→CH_2CH_2NH←TICP[Ψ])

H-Tyr→Pro→Phe→Phe→NH-CH_2-CH_2-NH←Tic←Dmt-H

Fig. 10. Peptide MOPr agonists/DOPr antagonists.

The cyclic β-casomorphin analog H-Dmt-c[D-Orn-2-Nal-D-Pro-Gly] showed a balanced MOPr agonist/DOPr antagonist profile with subnanomolar MOPr and DOPr binding affinities. The cyclic pentapeptides H-Tyr-c(SS)[D-Cys-1-Nal-Nle-Cys]X (X is NH_2 or OH) are mixed MOPr/DOPr ligands with potent full agonist activity at the MOPr and low efficacy at the DOPr (Anand et al., 2012). The JOM-13–derived cyclic peptide H-Dmt-c(SCH_2CH_2S)[D-Cys-Aic-D-Pen]OH (KSK-103) showed MOPr partial agonist/DOPr antagonist activity in [^{35}S]GTPγS and cAMP accumulation assays with low nanomolar binding affinities at both receptors (Purington et al., 2011). C-terminal extension of KSK-103 with a β-glucosylserine residue resulted in a compound, H-Dmt-c(SCH_2CH_2S)[D-Cys-Aic-D-Pen]Ser(Glc)-NH_2, with a similar in vitro activity profile and with improved bioavailability (Mosberg et al., 2014). This compound given intraperitoneally showed antinociceptive potency similar to that of morphine and did not produce acute tolerance.

Several analogs of the endomorphins with a mixed MOPr agonist/DOPr antagonist profile have also been described. The endomorphin-2 analog H-Dmt-Pro-Phe-NH-C_2H_4-Ph (Fujita et al., 2004) and the endomorphin-1 analog H-Dmt-Pro-Trp-D-1-Nal-NH_2 (Fichna et al., 2007) both showed high MOPr agonist potency and moderate DOPr antagonist activity in vitro. The endomorphin-2 analog H-Dmt-Pro-Tmp-Phe-NH_2 (Tmp is 2′,4′,6′-trimethylphenylalanine) was reported to be a potent MOPr agonist/DOPr antagonist with high binding affinity for both receptors (Li et al., 2007).

The first MOPr agonist/DOPr antagonist of the distinct pharmacophore type contained the MOPr agonist component H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA) (Schiller et al., 2000) and the DOPr antagonist component TICP[Ψ] connected "tail to tail" via a short linker (Weltrowska et al., 2004). In this bifunctional compound, the [Dmt^1]DALDA segment plays a dual role as a potent MOPr agonist and as a vector capable of carrying the MOPr/DOPr ligand construct across the blood–brain barrier. The resulting compound, [Dmt^1]DALDA→CH_2CH_2NH←TICP[Ψ], was designed to interact with MOPr and DOPr in a monovalent fashion. In vitro, the compound showed the expected μ agonist/δ antagonist profile with MOPr and DOPr binding affinities in the low nanomolar range. In the mouse tail-flick test, this compound given subcutaneously produced a long-lasting antinociceptive effect with a potency similar to that of morphine and with low propensity to induce analgesic tolerance (Schiller, 2010). Using the same design principle, the bifunctional peptide H-Tyr-Pro-Phe-Phe→$NHCH_2CH_2$←Tic-Dmt, containing the MOPr agonist component endomorphin-2 and the DOPr antagonist component H-Dmt-Tic, was prepared later by Salvadori et al. (2007).

2. Nonpeptide μ-Opioid Receptor Agonist/δ-Opioid Receptor Antagonists. The hydroxymorphinan-derived pyridomorphinan SoRI 20411 [5′-(4-Chlorophenyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-17-methylpyrido[2′,3′:6,7]morphinan] is a MOPr agonist/DOPr antagonist with approximately 10-fold lower antinociceptive potency than morphine (intracerebroventricular administration) and with low propensity to produce analgesic tolerance (Ananthan et al., 2004). Compared with the latter compound, the 14-alkoxypyridomorphinan SoRI 22138 [5′-(4-Chlorophenyl)-17-(cyclopropylmethyl)-6,7-didehydro-4,5α-epoxy-3-hydroxy-14-(3-phenylpropoxy)pyrido[2′,3′:6,7]morphinan] is a more potent and more balanced MOPr agonist/DOPr antagonist (Ananthan et al., 2012) (Fig. 11). It did not produce tolerance and dependence in cells expressing MOPr and DOPr. In the mouse tail-flick assay, SoRI 22138 given intracerebroventricularly displayed similar analgesic potency and reduced potential for tolerance compared with morphine. The 5,14-bridged morphinan-based compound UMB 425 [4α,9-Dihydroxy-7α-(hydroxymethyl)-3-methyl-2,3,4,4α,5,6-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinolin-7(7αH)-one] has nanomolar binding affinity and efficacy similar to morphine at the MOPr and moderate DOPr antagonist activity (Healy et al., 2013). The results of the in vivo testing (subcutaneous administration) indicated that this compound had similar antinociceptive potency as morphine but a lower propensity to induce analgesic tolerance. The compound eluxadoline is a balanced MOPr agonist/DOPr antagonist with binding affinities of approximately 1 nM at the two receptors (Breslin et al., 2012). It is peripherally acting and is in phase III clinical development for treatment of diarrhea-predominant irritable bowel syndrome. Finally, 4-substituted piperazines with MOPr partial agonist/DOPr antagonist properties were recently reported (Bender et al., 2014).

The bifunctional MOPr agonists/DOPr antagonists described above interact in a monovalent fashion with

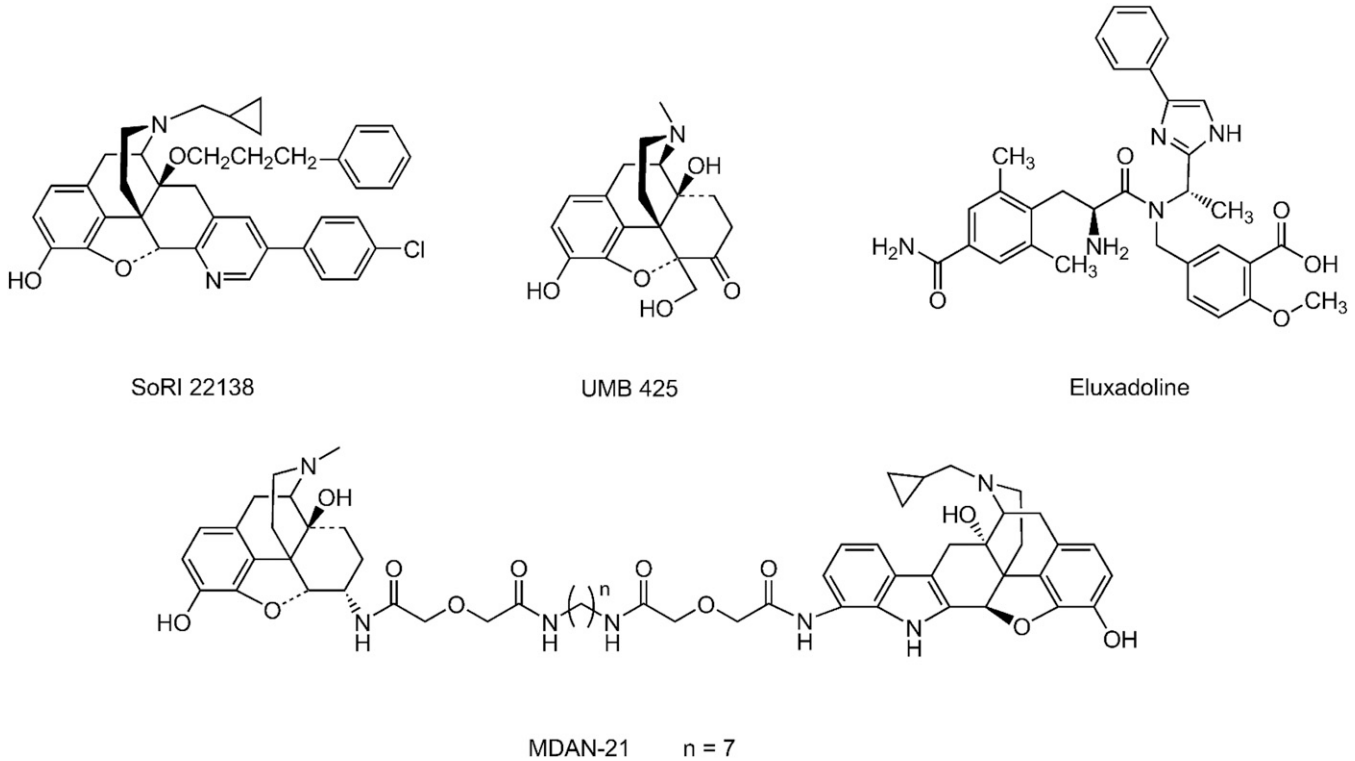

Fig. 11. Nonpeptide MOPr agonists/DOPr antagonists.

MOPrs and DOPrs. On the basis of a different concept, compounds containing the MOPr agonist component oxymorphone and the DOPr antagonist component NTI connected via a linker of varying length [μ-δ agonist-antagonist (MDAN) series] were designed with the expectation that such bivalent ligands would simultaneously interact with MOPr and DOPr binding sites in a MOPr/DOPr heterodimer (Daniels et al., 2005). Within the series of compounds prepared, MDAN-21 (Fig. 11) given intracerebroventricularly showed the highest antinociceptive potency in the mouse tail-flick test but was less potent than a monovalent ligand containing oxymorphone only attached to the linker. The authors' explanation that the decreased potency of MDAN-21 may be due to negative allosteric cooperativity at the MOPr/DOPr heterodimer is in conflict with observations of positive allosterism seen with a MOPr agonist and a DOPr antagonist interacting with the MOPr/DOPr heterodimer (Gomes et al., 2004, 2011). MDAN-21 produced no analgesic tolerance and no physical dependence after chronic administration. The activity profile of MDAN-21 could also be due to its interaction with both the orthosteric binding site and an accessory (allosteric) site at MOPr, as an alternative to the proposed bivalent MOPr/DOPr heterodimer binding mode. Compounds with this binding mode at GPCRs are referred to as bitopic ligands (Lane et al., 2013). In vitro studies might clarify this issue.

In summary, development of both peptide and nonpeptide agonists with high selectivity for DOPr has been very successful, allowing characterization of DOPr responses. Selective DOPr antagonists have been generated, with the nonpeptide antagonists reported to date being somewhat less selective than several of the highly selective peptide antagonists. Peptide DOPr antagonists are particularly useful as tools in molecular pharmacology studies, whereas nonpeptide DOPr antagonists are preferred for in vivo studies because of their better bioavailability. DOPr ligands with a mixed MOPr agonist/DOPr antagonist profile have been developed as analgesics with demonstrated low propensity to produce analgesic tolerance and physical dependence, but their drug-like properties still need to be improved. The use of many of these compounds as pharmacological tools is detailed in the following sections.

Finally, an exciting new development is the identification of DOPr positive allosteric modulators (Burford et al., 2015). It is anticipated that major efforts will be made in the years to come to examine the potential of these novel types of DOPr ligands as therapeutic agents with reduced side effects.

V. Synthesis and Membrane Targeting of δ-Opioid Receptors

A. Trafficking of δ-Opioid Receptors to the Plasma Membrane

The density of GPCRs at the plasma membrane is a dynamic and regulated process. Events modulating the membrane trafficking or routing of receptors to the cell membrane have profound consequences on receptor

function and cellular responsiveness. GPCRs must undergo a continual process of maturation, in which proteins are exocytosed from the ER to the Golgi complex and are trafficked to the plasma membrane. Only successfully folded proteins are exported from the ER to the Golgi complex, where they can undergo post-translational modifications such as glycosylation. The ER provides a quality-control step in GPCR maturation but it is the Golgi apparatus that provides chaperone proteins to escort receptors to the plasma membrane. Upon exiting from the trans-Golgi network, proteins are sorted to the constitutive or the regulated vesicular pathway. In the constitutive pathway, vesicles containing receptors are constantly directed to the plasma membrane along microtubules of the cytoskeleton. However, membrane trafficking of receptors may also occur via a regulated pathway, where specialized secretory vesicles are exported to the plasma membrane in response to a particular signal (e.g., a pronociceptive stimulus). There is evidence that DOPrs are targeted to neuronal plasma membranes via both constitutive and regulated pathways (Fig. 12).

Ultrastructural localization using electron microscopy immunogold, photoaffinity-labeled receptors with [^{125}I]-DTLET and biochemical subcellular fractionation techniques reveal that the majority of DOPrs are localized predominantly to intracellular sites, with only a small subset of DOPrs found in association with neuronal plasma membranes throughout the central and peripheral nervous systems (Pasquini et al., 1992; Zerari et al., 1994; Arvidsson et al., 1995; Cheng et al., 1995, 1997; Elde et al., 1995; Zhang et al., 1998; Petaja-Repo et al., 2000; Cahill et al., 2001b; Commons et al., 2001; Wang and Pickel, 2001; Petäjä-Repo et al., 2002, 2006; Commons, 2003; Guan et al., 2005; Lucido et al., 2005; Gendron et al., 2006). The small number of plasma membrane-bound receptors is consistent with the observation that DOPr agonists have modest effects in modulating nociception and reward (Cahill et al., 2007; Pradhan et al., 2011); however, systemic administration of DOPr agonists such as SNC80 produces locomotor hyperactivity, anxiolytic effects, antidepressant effects, and absence seizures (Pradhan et al., 2011; Chu Sin Chung and Kieffer, 2013; Gendron et al., 2015). The fact that a low number of membrane-bound receptors are required for certain cellular functions, and that the majority of DOPrs are reserve receptors awaiting targeting to the plasma membrane, suggests that DOPrs are primarily engaged after specific physiologic stressors. Notably, this predominant localization to intracellular compartments is similar to other type 1 GPCRs that are targeted to lysosomal degradation, rather than recycling/resensitization pathways, after agonist-induced internalization. For example, opioid receptor-like 1 (nocicpetin), dopamine type 1, neurotensin type 2, and protease-activated receptor (PAR) 2 receptors are primarily localized to intracellular compartments. Moreover, like DOPrs, they can be recruited from intracellular sites to the plasma membrane under specific conditions (Hein et al., 1994; Brismar et al., 1998; Perron et al., 2006; Mittal et al., 2013).

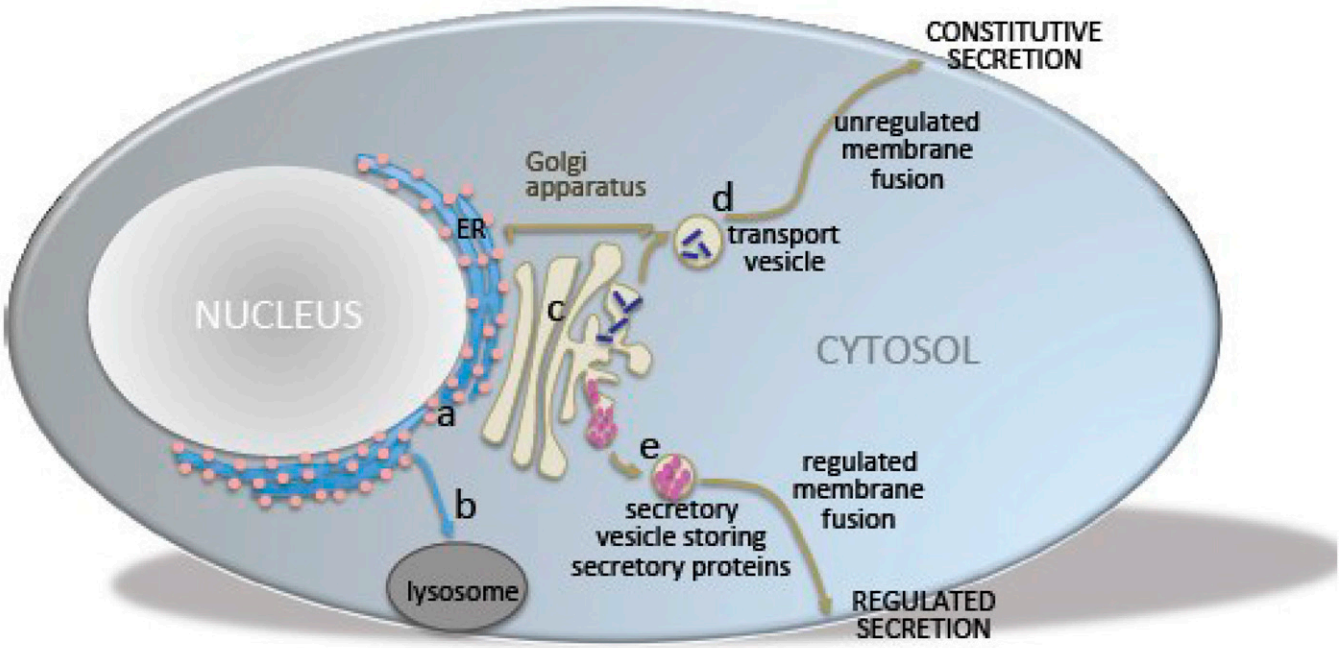

Fig. 12. Trafficking of DOPr. Two pathways have been proposed: regulated (secretory) and constitutive. (A) DOPrs are synthesized in ribosomes within the ER. (B) A large majority of DOPrs are targeted to lysosomes for degradation. (C) Only successfully folded proteins reach the Golgi apparatus, where they mature and undergo glycosylation. (D and E) Mature DOPrs are trafficked to the plasma membrane via either a constitutive pathway involving cytoskeletal proteins (including cofilin and βarr-1) (D) and/or a regulated (secretory) pathway (E). See Table 2 for a summary of evidence.

Although there is strong evidence that DOPr subcellular localization is primarily localized to intracellular compartments, controversy regarding this dogma has surfaced (Table 2). The creation of DOPr–enhanced green fluorescent protein (eGFP) mice allowed visualization of fluorescently tagged receptors within neurons from different brain structures in live tissue. Use of these mice demonstrated that DOPr in sensory neurons had a predominant cell surface expression (Scherrer et al., 2006, 2009). This result has questioned the validity of classic approaches in which it was proposed that use of nonspecific reagents led to inaccurate results of receptor cellular and subcellular localization. Further fueling the controversy was a high-profile communication suggesting that DOPr antibodies are nonspecific because they produce immunolabeling in DOPr knockout mice (Scherrer et al., 2009). Despite the conflicting reports on DOPr subcellular localization, the cellular distribution of the fluorescently tagged DOPr was in accordance with studies using DOPr antibodies (immunohistochemistry) (Cahill et al., 2001a) and autoradiography approaches (Goody et al., 2002); in fact, these latter studies were used to validate the DOPr-eGFP transgenic mouse. To address the argument, one must consider the parameters that alter antibody specificity, such as differences in fixation, antibody incubation conditions, antibody concentration, and whether amplification protocols such as avidin-biotin are used. To our knowledge, no study has yet systematically reproduced protocol conditions in DOPr knockout mice to elucidate whether all studies using DOPr antibodies are all nonspecific. In fact, we know that many of these antibodies must recognize DOPr, because the subcellular distribution changes with agonist treatment (i.e., there is an increase in receptor internalization that can be detected with DOPr antibodies) (Gastard, 2000; Hasbi et al., 2000; Ong and Cahill, 2015; Ong et al., 2015). The fact that the rather large eGFP tag (26.9 kDa) was added at the receptor C terminus in DOPr-eGFP knock-in mice raised much concern, given the interaction of the receptor with signaling, sorting, trafficking, and regulatory proteins essentially within the C-terminal domain of the receptor. There is no doubt that the use of genetically encoded fluorescent proteins has revolutionized cell biology, allowing the study of receptor dynamics in live tissue; however, an appreciation of their potential limitation and caveats must be realized (Costantini and Snapp, 2013; Lajoie et al., 2014). For example, eGFP can form covalent oligomers in oxidizing environments, such as the ER, causing misfolding and rendering them nonfluorescent (Aronson et al., 2011). Thus, although eGFP can clearly fold and form fluorescent molecules in the ER (Kaether and Gerdes, 1995; Subramanian and Meyer, 1997; Dayel et al., 1999), anti-GFP immunoblots of nonreducing SDS-PAGE gels reveal that up to 50% of total ER eGFP is incorporated into disulfide bonded oligomers (Jain et al., 2001). Accordingly, a substantial proportion of intracellular DOPr in DOPr-eGFP knock-in mice may not be visible due to misfolded (nonfluorescent) eGFP molecules that affect not only the visualization of fluorescent green fluorescent protein (GFP) but also the binding of GFP antibodies. Thus, relying on eGFP visualization or antibodies to GFP to quantify subcellular distribution is confounded (Aronson et al., 2011; Costantini and Snapp, 2013). Moreover, one study noted that even immunolabeling GFP is not necessarily sufficient to detect low levels of DOPr-eGFP expression. Poole et al. (2011) pretreated animals with SNC80 prior to tissue collection to induce pooling of DOPr in endosomes to subsequently detect GFP immunoreactivity (Poole et al., 2011). This suggests that DOPr-eGFP surface expression is commonly extant and below the detection threshold of GFP immunolabeling. Consistent with this possibility, fusion of various tags to DOPr reportedly induces distinct subcellular distribution patterns, whereby eGFP fused at the N or C terminus caused DOPr to be primarily localized to the cell surface compared with Myc- or hemagglutinin-fused proteins (Wang et al., 2008). The altered trafficking of DOPr by fusion with eGFP is not unique, in that studies have demonstrated that the addition of an eGFP molecule on other GPCRs such as cannabinoid 1 receptors (McDonald et al., 2007), β-adrenergic receptors (ARs)

TABLE 2
Strengths and weaknesses of approaches used to visualize subcellular DOPr localization

Controversy: DOPr Subcellular Localization Intracellular vs Plasma Membrane	
High plasma membrane localization • Visualization of GFP in DOPr transgenic mice	Low plasma membrane localization • Electron microscopy using immunogold techniques • Electron microscopy using radioligands • Internalization of Fluo-DOPr ligands
Strength: • Direct visualization • Not reliant on DOPr antibody (although is reliant on GFP antibody)	Strength: • Direct visualization • Active/functional receptors
Weakness: • GFP causes formation of oligomers that do not fluoresce (thereby decreasing visualization of intracellular receptors) • GFP alters GPCR membrane trafficking (e.g., CB1, βAR, and M4) • DOPr is overexpressed in DOPr-GFP mice	Weakness: • Antibody specificity questioned due to evidence of labeling in DOPr KO mice (note: cellular localization of DOPr via antibodies and DOPr-GFP mice is virtually identical) • Ligand specificity for DOPr

(McLean and Milligan, 2000), and the muscarinic M4 receptor (Madziva and Edwardson, 2001) can also alter receptor trafficking and processing. Nevertheless, stimulated DOPr membrane trafficking has been identified in transgenic knock-in DOPr-eGFP mice (Bertran-Gonzalez et al., 2013) and these mice can be used reliably to study ligand-induced intracellular redistribution of receptors (Pradhan et al., 2009, 2010, 2015; Faget et al., 2012). In addition, despite the altered trafficking and/or visualization of receptors with the eGFP tag, this fusion protein was not found to significantly alter the physiologic effects produced by DOPr activation via exogenous agonists (Pradhan et al., 2010; Bertran-Gonzalez et al., 2013; Pettinger et al., 2013; Bardoni et al., 2014).

Table 2 summarizes the evidence and the strengths and weaknesses of the approaches that have been used to identify subcellular localization of DOPr. Given that such a large molecule (GFP) is attached to the C-terminal tail of DOPr, which is important for trafficking and the confounds of oligomerization of GFP molecules affecting visualization of fluorescence, relying solely on DOPr-eGFP mice for ultrastructural analysis is unrealistic. That said, there are also concerns that simple tissue isolation of slices for electrophysiological or neurochemical release studies or cell culture of isolated dorsal root ganglions (DRGs) and other neurons from wild-type (unmanipulated) animals may be a sufficient stimulus to modulate DOPr trafficking, because low potassium-induced depolarization or stress is sufficient to induce an increase in DOPr function and membrane trafficking. One of the biggest downfalls that has fed the controversy in this area is the overinterpretation of results and lack of consideration of alterative explanations for the observations.

Evidence that presynaptic DOPrs are targeted to neuronal plasma membranes via the regulated pathway arises from studies on sensory neurons. Various studies reported that DOPrs were colocalized with substance P–containing primary afferent neurons (Elde et al., 1995; Guan et al., 2005; Wang et al., 2010; He et al., 2011) and on large dense core vesicles (Arvidsson et al., 1995; Cheng et al., 1995; Zhang et al., 1998; Zhao et al., 2011), although this was not consistently reported (Cahill et al., 2001a); the work from Scherrer et al. (2009) incorrectly cited this latter work as supportive of DOPr localization in large dense core vesicles. It was postulated that intracellular retention of DOPr was maintained by DOPr binding to large dense core vesicles containing the neuropeptide substance P (Guan et al., 2005). Furthermore, this latter study also demonstrated that deletion of the preprotachykinin A (PPTA) gene, which encodes for substance P, reduced stimulus-induced surface insertion of DOPr, as evidenced by a decrease in total DOPr protein, loss of DOPr localization within large dense core vesicles without changing surface DOPr expression, and reduced [^{3}H]deltorphin autoradiographic binding in the dorsal superficial spinal cord. However, many studies do not support these results, and again, more controversy and debate in DOPr pharmacology arose regarding the validity of the importance of the secretory pathway in DOPr membrane translocation. First, general suspicion of DOPr antibody nonspecific labeling was suggested, because many of these earlier studies used DOPr antibodies that had crossreactivity with substance P (Elde et al., 1995) and immunolabeling of DOPr with commercially available antibodies was reportedly absent in PPTA knockout mice (Scherrer et al., 2009; Bardoni et al., 2014). Second, colocalization of substance P and DOPr in sensory neurons was absent in DOPr-eGFP knock-in mice (Scherrer et al., 2009). Third, the enhanced antihyperalgesic effects of DOPr agonists in a model of chronic inflammatory pain compared with pain-naïve controls was not altered by genetic deletion of substance P in PPTA knockout mice (Dubois and Gendron, 2010). Finally, an insufficient population of DOPrs is present in substance P–containing neurons to produce a physiologic effect (Bardoni et al., 2014). Nevertheless, DOPr immunoreactivity was identified in substance P neurons within the myenteric plexus of the small intestine of DOPr-eGFP knock-in mice (Poole et al., 2011), demonstrating that DOPrs can be coexpressed with substance P. Moreover, DOPr activation also inhibits substance P release from primary afferents (Beaudry et al., 2011; Kouchek et al., 2013; Normandin et al., 2013). The dispute over whether DOPrs are present in substance P–containing neurons remains unresolved, because in situ hybridization and single-cell polymerase chain reaction studies report different results (Scherrer et al., 2009; Wang et al., 2010). However, again, we should caution reliance on eGFP-tagged DOPr knock-in mice for visualization of DOPr distribution, because intermolecular disulfide bonded eGFP that is nonfluorescent was shown to confound quantification of total levels of GFP in secretory pathways (including with use of GFP antibodies) (Jokitalo et al., 2001; Aronson et al., 2011). Similarly, studies also report conflicting results over whether DOPr expression exists in calcitonin gene–related peptide (CGRP) sensory neurons (Scherrer et al., 2009; Pettinger et al., 2013). However, electrophysiological data with patch-clamp experiments and CGRP release assays strongly suggest significant coexpression of DOPr with this neuropeptide (Pettinger et al., 2013). Other evidence that DOPrs are targeted to the plasma membrane via the regulated secretory pathway arises from studies demonstrating that a painful (noxious) stimulus causes membrane trafficking of the receptor. For example, Patwardhan et al. (2005) demonstrated that DOPr membrane trafficking was produced by the inflammatory mediator bradykinin (BK) (Patwardhan et al., 2005), a finding replicated by others (Pettinger et al., 2013). The

regulated trafficking of DOPr via BK was demonstrated by total internal reflection fluorescence microscopy of trigeminal and dorsal root sensory neurons transfected with DOPr fused with eGFP (Pettinger et al., 2013). Table 3 presents a summary of the evidence for DOPr trafficking being regulated by the secretory pathway and the problems or limitations with such evidence. It is very hard to argue with physiologic data showing that DOPr function is modulated by neuropeptide release, supporting a role for both constitutive and regulated pathways being involved in DOPr trafficking (at least in sensory neurons). The reliance on visualization of DOPr in specific cell populations using DOPr-eGFP mice should also be cautioned. Initial studies demonstrated that DOPr was not colocalized in MOPr sensory neurons, suggesting that these opioid receptors modulate different sensory modalities (Scherrer et al., 2009). However, subsequent studies published by the same research group and colleagues demonstrated that indeed DOPrs and MOPrs are coexpressed in CGRP-expressing sensory neurons and a discrete population of small-, medium-, and large-diameter DRGs, as well as coexpression in many brain regions when the DOPr-eGFP mouse was bred with a mCherry-tagged MOPr (Bardoni et al., 2014; Erbs et al., 2015).

Many studies have identified that DOPr maturation and membrane trafficking, as well as enhanced function, is facilitated or augmented by various stimuli. For example, prolonged exposure to drugs of abuse, physiologic stressors, and learning-related plasticity all promote DOPr membrane translocation. DOPrs have a critical role in adaptive responses and neuronal plasticity in various brain regions important for learning, reward, analgesia, and mood. However, few studies have examined mechanisms responsible for constitutive sorting of DOPr. It is well acknowledged that the mechanism of membrane trafficking of GPCRs from the Golgi apparatus to the plasma membrane is a dynamic process involving actin cytoskeletal proteins. A specific protein identified to be involved in facilitating or inhibiting the release of proteins from the Golgi to the cell membrane is cofilin, an actin-severing protein and a potent regulator of actin filament dynamics (Heimann et al., 1999; Egea et al., 2006; Salvarezza et al., 2009). The ability of cofilin to bind and depolymerize actin is abolished by phosphorylation of a serine residue by LIM-kinase 1, a serine/threonine kinase containing the LIM and PDZ domains (Yang et al., 1998). Since β-arrs bind to cofilin and associated regulatory proteins, they can thereby regulate GPCR functions via interaction with these cytoskeletal proteins (Zoudilova et al., 2007, 2010). A recent study demonstrated that such processes are involved in DOPr membrane trafficking. It was identified that activation of DOPr and the nociception receptor [opioid receptor-like 1 (nocicpetin)] in peripheral sensory neurons can activate cofilin to regulate actin polymerization (Mittal et al., 2013), thus controlling receptor cell surface expression. This process involved recruitment of Rho-associated coiled-coil–containing protein kinase, LIM-kinase, and βarr-1 (but not βarr-2). Importantly, the activation of cofilin was not involved in receptor desensitization or agonist-induced receptor internalization, thus providing a strong argument for the involvement of this protein in receptor cell surface trafficking. In this latter study, βarr-1 knockout mice exhibited enhanced DOPr function and, importantly, the enhanced effectiveness of DOPr agonists in attenuating pain associated with an inflammatory insult also recruited this pathway, because the DOPr antihyperalgesic effect was blocked by a Rho-associated coiled-coil–containing protein kinase inhibitor (Mittal et al., 2013). Although it remains unclear whether both constitutive and regulated pathways of DOPr trafficking engage such processes, it is

TABLE 3
Support and confounds for proposed DOPr trafficking pathways

Controversy: Regulated (Secretory) vs Constitutive Trafficking: Does Secretory Pathway Exist?	
Secretory	Constitutive
Support: • Colocalization with substance P in large dense core vesicles (light and electron microscopy) • Ephys supports co-localization of CGRP and DOPr • PPTA KO shows decreased DOPr in plasma membrane • PPTA KO shows decreased DOPr radioligand binding • Enhanced DOPr agonist analgesia is absent in PPTA KO mice	Support: • All literature agree in constitutive trafficking of DOPr • Evidence that it involves cytoskeletal proteins including cofilin and beta-arrestin-1
Confounds: • DOPr antibody labeling is decreased in PPTA KO mice (may show cross reactivity with SP) • SP and DOPr co-localization are not present in DRG of DOPr-GFP mice, but is present in myenteric plexus • In situ studies show both co-localization of SP and DOPr and no co-localization • GFP visualization in secretory pathways is confounded by oligomerization. • Tissue isolation (itself) for ex vivo and in vitro studies may trigger DOPr trafficking	Confounds: • None (to our knowledge)

tempting to speculate that the enhanced DOPr trafficking produced by inflammatory pain may be partially due to the engagement of βarrs by other GPCRs. That is, if βarr-1 is recruited to other GPCRs after their activation (e.g., activation of BK receptors), its inhibitory tone on DOPr trafficking may no longer be sufficient to suppress DOPr export from the Golgi apparatus to the cell membrane. Such a competition process may explain why DOPr agonists administered into the hindpaw do not alleviate prostaglandin E2–induced thermal allodynia but will if they are primed by prior administration of BK (Rowan et al., 2009). Similarly, in the trigeminal nucleus, DOPr agonists do not inhibit neuropeptide release or inhibit adenylate cyclase (AC) unless primed with other Gq-coupled receptors such as BK and PAR2 receptor agonists (Patwardhan et al., 2005, 2006).

NGF via activation of phospholipase C (PLC) and calcium calmodulin-dependent protein kinase II (CAMKII) was identified to be necessary for chronic morphine-induced DOPr membrane trafficking in the nucleus raphe magnus (NRM) (Bie et al., 2010). This mechanism was proposed to be part of the secretory regulated pathway. Brainstem slices incubated with a tyrosine receptor kinase (Trk) A antagonist blocked the morphine-induced increase in DOPr function, as measured by deltorphin-induced inhibition of electrically evoked excitatory postsynaptic currents (EPSCs) or inhibitory postsynaptic currents (IPSCs) in NRM neurons (Bie et al., 2010). The enhanced DOPr function was also prevented by incubating brainstem slices with a PLCγ antagonist, a phosphoinositide 3-kinase (PI3K) antagonist, a CaMKII inhibitor, and depletion of intracellular calcium stores with thapsigargin. However, DOPr trafficking was not modulated by either a mitogen-activated protein kinase (MAPK) inhibitor or a protein kinase C (PKC) inhibitor (Bie et al., 2010). This latter study also implicated recruitment of a scaffolding protein Na^+/H^+ exchange regulatory factor (NHERF)-1, which was shown to be involved in the sorting of internalized β-2ARs and KOPrs to recycling pathways for resensitization (Huang et al., 2004; Weinman et al., 2006; Hanyaloglu and von Zastrow, 2008). Finally, the appearance of increased functional DOPr induced by chronic morphine was blocked by an inhibitor of protein transport from the ER to the Golgi apparatus (Bie et al., 2010), suggesting that morphine modulates the maturation and sorting of newly synthesized DOPrs. However, increased membrane trafficking, but not changes in total DOPr protein, was observed in the NRM and spinal cord after chronic morphine (Cahill et al., 2001b; Ma et al., 2006).

1. Acute Stressors. Brief depolarization increases the membrane expression of a number of GPCRs and channels, including transient receptor potential cation channel subfamily V member 1 (TRPV1) (Bao et al., 2003; Guan et al., 2005; Gendron et al., 2006), the purinergic receptor P2Y1 (Bao et al., 2003), PAR2 receptor (Patwardhan et al., 2006), and BK receptors (Patwardhan et al., 2005; Rowan et al., 2009; Cayla et al., 2012; Pettinger et al., 2013). Such activation also promotes DOPr membrane insertion in sensory neurons. DOPr membrane trafficking was revealed by various techniques including immunohistochemistry, internalization of fluorescent deltorphin, cell surface DOPr biotinylation, live images of neurons transfected with DOPr-eGFP, and real-time total internal reflection fluorescence microscopy (note the limitations of DOPr visualization highlighted in Table 2). Many of these studies demonstrate that the increase in DOPr cell surface expression positively correlates with an increase in DOPr receptor function. For example, a 4-hour NGF treatment of brain slices or systemic administration of NGF in vivo promotes DOPr translocation in NRM neurons (Bie et al., 2010). This is in contrast with the acute effects of NGF on cultured secretory PC12 cells, which promotes intracellular retention of DOPr (Kim and von Zastrow, 2003). A brief physiologic stressor, such as a forced swim stress (Commons, 2003) or water deprivation (Stein et al., 1992), also increases DOPr cell surface expression in the periaqueductal gray (PAG) (immunogold technique) and caudate and accumbens nuclei (radioligand binding). Furthermore, chronic stress induced by repeated foot shock increases DOPr function in the ventral tegmental area (VTA) (Margolis et al., 2011). This latter study reported that DOPr activation promoted postsynaptic $GABA_A$ receptor insertion into the plasma membrane of VTA neurons, including dopaminergic neurons. Finally, 7 days of hypoxic preconditioning in the asphyxial cardiac arrest model also increased functional DOPr in the hippocampus and cortex, which were associated with neuroprotective effects (Gao et al., 2012). Although various physiologic stressors were identified to promote DOPr membrane trafficking, there is specificity in that various other activators of cell signaling fail to mobilize DOPr transport. For example, prolonged treatment of brain slices (up to 5 hours) with the AC activator forskolin, protein kinase A (PKA) activator 8-bromo-cAMP, PKC activator phorbol 12-myristate 13-acetate, or incubation with neuropeptide cholecystokinin all fail to promote DOPr membrane translocation (Patwardhan et al., 2005; Bie et al., 2010).

2. Pain. Within the pain neuromatrix, DOPrs are strategically located to modulate nociceptive transmission. They are present on primary afferent terminals of sensory neurons as well as on the soma and dendrites of intrinsic neurons within the dorsal spinal cord (Besse et al., 1990; Mansour et al., 1994; Cheng et al., 1995; Minami et al., 1995; Cahill et al., 2001a). Tissue injury associated with chronic inflammation increases DOPr function, as evidenced by an enhanced antihyperalgesic effect and an increase in coupling to voltage-dependent

calcium channels. These changes in function were positively correlated with recruitment of DOPr from intracellular stores to the plasma membrane in the spinal cord, neocortex, and sensory neurons (Cahill et al., 2003; Lucido et al., 2005; Gendron et al., 2006; Pradhan et al., 2013). Membrane trafficking in the spinal cord and sensory neurons was also proposed to account for the enhanced potency or appearance of effects produced by DOPr agonists in various brain regions after unilateral hindpaw inflammation (Hylden et al., 1991; Fraser et al., 2000; Hurley and Hammond, 2000; Qiu et al., 2000; Cao et al., 2001). One mechanism implicated in the translocation of DOPr to the cell surface is the dependence on the presence of MOPr, because there was no effect in MOPr knockout mice (Morinville et al., 2003; Gendron et al., 2007). Interestingly, although βarr-2 regulates DOPr internalization and function (Qiu et al., 2007; Zhang et al., 2008), the absence of βarr-2 had no effect on the analgesic profile of DOPr agonists or on the enhanced coupling of the receptor to voltage-dependent calcium channels in a model of chronic inflammatory pain (Pradhan et al., 2013). This is consistent with the lack of βarr-2 involvement in the constitutive DOPr membrane trafficking described by Mittal et al. (2013).

3. Reward and Addiction. Although immunohistochemical (Cahill et al., 2001a) and in situ hybridization studies (Mansour et al., 1994), as well as studies with DOPr-eGFP knock-in mice (Faget et al., 2012; Scherrer et al., 2006), identify DOPr in mesolimbic brain structures important for reward and reinforcement, administration of DOPr agonists produced conflicting results in outcome measures of reward and addictive behaviors (Méndez and Morales-Mulia, 2008; Shippenberg et al., 2008; Rodríguez-Arias et al., 2010; Pradhan et al., 2011; Mitchell et al., 2014). Some studies report that DOPr agonists do not produce a place preference in otherwise drug-naïve animals (Hutcheson et al., 2001; Mitchell et al., 2014) but will after prolonged ethanol consumption (Mitchell et al., 2014). Similarly, negligible DOPr agonist abuse–related effects were evident in a monkey self-administration model (Negus et al., 1998) or in a rat model of intracranial self-stimulation (Do Carmo et al., 2009). Although other studies demonstrate that DOPr agonists will produce a conditioned place preference in otherwise naïve animals, these agonists fail to elicit dopamine release in the nucleus accumbens (Bals-Kubik et al., 1990; Longoni et al., 1998). Despite the dispute regarding whether DOPr activation produces rewarding effects in its own right, many studies consistently report that DOPr activation contributes to the rewarding and craving effects of various drugs of abuse, including morphine, heroin, cocaine, alcohol, methamphetamine, and 3,4-methylenedioxymethamphetamine, as measured by either conditioned place preference or self-administration paradigms (reviewed in Pradhan et al., 2011). However, the fact that the ability of predictive learning and influence of choice is absent in DOPr knockout mice (Le Merrer et al., 2011) should raise concerns about the validity of concluding a role of DOPr in reward-like behavior. Hence, knockout mice exhibit impaired learning to both appetitive and aversive stimuli, suggesting it is necessary contextual learning. Moreover, there is evidence that DOPr may also be important in memory retrieval, because the expression of place preference and opioid reinstatement was prevented by acute DOPr antagonist administration immediately prior to post–conditioning day testing, after conditioning to morphine (Bie et al., 2012) or ethanol (Bie et al., 2009a). Nevertheless, DOPr membrane translocation, as measured by immunogold electron microscopy techniques, was evident 48 hours after withdrawal from a 2-week binge cocaine protocol (Ambrose-Lanci et al., 2008). Other studies predicted that DOPr might be protective against drug-seeking and craving behavior. Hence, intra–nucleus accumbens infusions of the DOPr antagonist NTI prevented cocaine-seeking behavior, suggesting that an endogenous DOPr tone suppressed active lever presses after abstinence (Dikshtein et al., 2013). However, considering that active lever pressing after forced abstinence is influenced by stress, such data may be confounded because NTI might exacerbate stress-induced relapse. Prolonged exposure to ethanol also promotes an upregulation of functional DOPr in the spinal cord in pain-mediating circuits (van Rijn et al., 2012). Similarly, chronic ethanol was also shown to modulate DOPr membrane translocation in the VTA. DOPr activation with DPDPE injected into the VTA inhibited evoked, spontaneous, and miniature $GABA_A$ IPSCs in low-drinking animals, but it did not change evoked or spontaneous $GABA_A$ IPSCs in naïve or high-drinking animals (Margolis et al., 2008). This result was correlated with the observation that intra-VTA administration of DPDPE attenuated ethanol consumption. This latter study suggested that DOPr produces a neuroprotective effect, such that receptor activation will inhibit elevated alcohol consumption (produced in the low drinkers). This is consistent with the observation that DOPr knockout mice consume more ethanol than their wild-type littermates (Roberts et al., 2001). Subsequent studies reported that the neuroprotective effects of VTA DOPr in modulating ethanol consumption were positively correlated with the occurrence of affective-like behaviors and higher corticosterone blood levels prior to ethanol self-administration (Mitchell et al., 2012). Importantly, although much of the literature cited above (in this section) highlights changes in DOPr function, few studies have demonstrated that drugs of abuse regulate DOPr membrane trafficking. An elegant study by Bertran-Gonzalez et al. (2013) demonstrated that persistent, learning-related plasticity, using a pavlovian-instrumental transfer task, induced translocation of DOPr to the somatodendritic

compartment of cholinergic interneurons within the shell of the nucleus accumbens. This receptor translocation was induced by predictive learning and was important for expression of subsequent choice between goal-directed actions in tests assessing the influence of such learning. Interestingly, learning-related plasticity did not change in DOPr membrane translocation (as measured by fluorescent anti-GFP in DOPr-eGFP mice) in either the core of the nucleus accumbens or dorsomedial striatum (Bertran-Gonzalez et al., 2013). Subsequent studies by the same research group reported that increased DOPr membrane trafficking was associated with predictive learning on choice between actions, regardless of whether those predictions involve the presence or absence of reward (Laurent et al., 2015).

4. Chronic Morphine. Various paradigms of prolonged or chronic morphine administration promote cell surface trafficking of DOPr. For example, prolonged but not acute in vivo treatment with morphine, methadone, or etorphine for at least 48 hours was shown to increase plasmalemma expression of DOPr in somatodendritic profiles (as measured by immunogold techniques using DOPr antibodies) within the dorsal spinal cord (Cahill et al., 2001b; Morinville et al., 2004a) and primary sensory neurons (Gendron et al., 2006). Subsequent studies identified that a similar phenomenon occurs in other neurons within the peripheral and central nervous systems (Hack et al., 2005; Walwyn et al., 2005; Ma et al., 2006; Bie et al., 2009b; Chieng and Christie, 2009; Zhang and Pan, 2012). DOPr-mediated presynaptic inhibition of GABAergic synaptic currents in the PAG (a brain region important for descending inhibitory control of pain transmission) was only evident after prolonged, but not acute, morphine exposure (Hack et al., 2005). Chronic administration of morphine increased DOPr membrane trafficking (as measured by changes in DOPr agonist effectiveness) in neurons of the central nucleus of the amygdala that project to the PAG (Bie et al., 2009b; Chieng and Christie, 2009). The effects in the PAG and spinal cord were identified to require expression of MOPr, because the enhanced DOPr activity was absent in MOPr null mice (Morinville et al., 2004b; Hack et al., 2005). However, unlike the chronic inflammatory pain models (Pradhan et al., 2013) and DOPr constitutive transport (Mittal et al., 2013), chronic morphine treatment failed to induce DOPr function in βarr-2 null mice, suggesting that this protein plays a crucial role in the induction of surface expression of DOPr in the PAG (Hack et al., 2005). The reason for the mechanistic differences in DOPr trafficking is unknown; however, prolonged stimulation with a MOPr agonist may lead to the expression of gene product(s) or epigenetic changes involved in the folding and translocation of DOPr, perhaps as a consequence of βarr binding and trafficking. Indeed, NGF locally infused into the central amygdala mimicked morphine-induced DOPr translocation; since histone hyperacetylation induced reward sensitization through NGF signaling in the central amygdala that enhanced DOPr function, it was suggested that NGF in the central amygdala is one of the target genes activated epigenetically through histone modifications, and activation of this NGF signaling cascade may promote behaviors of opioid reward and drug sensitization (Bie et al., 2012). Another protein implicated in morphine-induced DOPr membrane trafficking is cyclin-dependent kinase 5 (Cdk5). Cdk5 phosphorylates Thr161 in the second loop of DOPr, which is required for normal cell surface expression of DOPr and the formation of DOPr-MOPr heterodimers (Xie et al., 2009). This latter study demonstrated that inhibition of Cdk5 activity or overexpression of a DOPr mutant lacking the Cdk5 phosphorylation site displayed relatively low cell surface DOPr expression. In morphine-treated animals, inhibition of Cdk5 reduced the analgesic effect of spinal deltorphin (Beaudry et al., 2015b). These studies demonstrate that chronic morphine treatment causes a functional upregulation of DOPr in areas of the central nervous system important for pain modulation, emotion, and anxiety.

B. Chaperones Play an Essential Role in Cell Surface Trafficking of δ-Opioid Receptors

A large percentage (over 60%) of newly synthesized DOPrs are primarily retained in the ER compartment and are modified by the addition of ubiquitin prior to ultimately being targeted for proteasomal degradation. Receptors are ultimately degraded by proteasomes, where export from the ER represents the rate-limiting step in the maturation and cell surface expression of DOPr (Petaja-Repo et al., 2000, 2001; Petäjä-Repo et al., 2002, 2006). The remaining receptors are targeted to intracellular sites, with only a paucity of functional receptors making it to the plasma membrane (Cahill et al., 2001b). Typically, GPCRs are folded and post-translationally modified within the ER before they are transported through the Golgi prior to expression at the plasma membrane. The proper folding and maturation of receptors within the ER is dependent on several molecular chaperones and folding catalysts. These processes are dependent on high calcium concentrations maintained by sarco(endo)plasmic reticulum calcium ATPase 2b (SERCA2b) (Brostrom and Brostrom, 2003). DOPr forms a ternary complex with two ER-resident proteins: the calcium pump SERCA2b and the molecular chaperone calnexin (Tuusa et al., 2007, 2010). The dynamic interactions between these proteins orchestrate DOPr biogenesis, where SERCA2b is responsible for integrating protein folding and maturation (Tuusa et al., 2010). Calnexin is a lectin ER chaperone that binds to newly synthesized, incompletely folded DOPr (Helenius and Aebi, 2004; Leskelä et al., 2007, 2009; Markkanen and Petäjä-Repo, 2008). Palmitoylation, a post-translational lipid modification, is also important in the ER export and trafficking of DOPr to the cell

surface (Petäjä-Repo et al., 2006). Hence, preventing palmitoylation inhibited DOPr transport to the cell surface, suggesting that palmitoylation is a required step in the maturation of DOPr and its successful release from the ER. An early estrogen-regulated protein belonging to a family of microtubule-associated proteins, GEC1, which increases the cell surface expression of KOPr, was shown not to be involved in the maturation and membrane trafficking of DOPrs (Chen et al., 2006b).

As described above, DOPrs (and other GPCRs) are folded and post-translationally modified within the ER membrane before they are transported through the Golgi to the plasma membrane. However, it was identified that DOPr precursors "stuck" in the ER prior to degradation are not irreversibly defective, represent fully competent folding intermediates, and are not permanently misfolded off-pathway products. Using metabolic pulse-chase labeling of DOPr expressed in HEK293 cells, Petäjä-Repo et al. (2002) demonstrated that an increase in plasma membrane expression could be induced by pharmacological (DOPr ligands) chaperones, whereby the treatment promoted maturation of the receptor within the ER and enhanced DOPr cell membrane density. This latter study identified several ligands that could act as pharmacological chaperones for the maturation and membrane trafficking of DOPr. Chaperone properties were independent of intrinsic signaling efficacy because both agonists (e.g., buprenorphine, TAN-67, SNC80) and antagonists (e.g., naltrexone, NTB, NTI) could promote receptor maturation, although agonist-promoted downregulation counters the benefits of chaperone actions. Of the compounds tested, the nonpeptidic (i.e., small molecule) ligands, but not the peptidic ligands, were able to act as DOPr chaperones. Thus, membrane permeability of the chaperone was considered a prerequisite for the enhanced maturation and export of DOPr from the ER. This mechanism may explain the effects of in vivo naloxone treatment on increasing plasma membrane expression of DOPr in spinal cord neurons (Cahill et al., 2001b). It is unknown why pharmacological compounds will act as receptor chaperones, but it is speculated that ligand binding assists in the proper folding of DOPr and thus in its continued maturation and export. Nonetheless, pharmacological chaperones for the maturation of GPCRs are not unique to DOPr. Ligand chaperones are being sought as novel pharmacological treatments for various genetic diseases caused by the misfolding of GPCRs, owing to their ability to increase the cell surface trafficking of proteins. For example, ligands may rescue cell surface expression and function of vasopressin receptors for treatment of nephrogenic diabetes insipidus (Morello et al., 2000; Bernier et al., 2004).

Immature DOPrs in the ER exist as both homomers and heteromers and it was proposed that the formation of these complexes may regulate the total number of receptors at the cell surface. This thesis is exemplified by $GABA_B$ receptors, in which heteromeric assembly between GABA receptor subunits is necessary for cell surface expression; $GABA_{B(1)}$ is responsible for binding of GABA, whereas $GABA_B$ (Staquicini et al., 2011) is necessary for surface trafficking and G protein coupling (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). $GABA_B$ receptor plasma membrane availability is controlled by mechanisms involving an ER retention motif and assembly-dependent ER export. Blocking proteasomal activity (mediated by ER-associated degradation) and inactivation of ubiquitination sites considerably enhances total and cell surface expression of $GABA_B$ receptors (Zemoura et al., 2013). Such mechanisms are not restricted to $GABA_B$ receptors. Indeed, similar to the dimerization-dependent expression known for class C receptors such as $GABA_B$ (Marshall et al., 1999), plasma membrane receptor expression is also enhanced by formation of dimers or heteromers of class A receptors such as β-ARs and α-ARs (Hague et al., 2004; Salahpour et al., 2004). DOPrs not only exist as monomers, but they also form homomers and heteromeric complexes with other opioid receptors and other GPCRs. As detailed in section IX.C.1, the existence of DOPr and KOPr as well as DOPr and MOPr heteromers has been demonstrated in the central and peripheral nervous systems using heteromer-specific opioid ligands and antibodies, respectively (Waldhoer et al., 2005; Gupta et al., 2010). Formation of these complexes may enhance their maturation and trafficking to neuronal plasma membranes. It was identified that a Golgi chaperone, receptor transport protein 4 (RTP4), is a regulator of MOPr-DOPr heteromer expression at the cell membrane. Hence, MOPrs, when complexed with DOPrs, are retained in the Golgi compartment, resulting in the decreased cell surface expression of both receptors. Coexpression of RTP4, a member of the receptor transport protein family that is known to participate in the export of odorant and taste receptors (Saito et al., 2004; Behrens et al., 2006), leads to enhanced cell surface expression as well as decreased ubiquitination of receptors (Décaillot et al., 2008). Thus, RTP4 regulates the proportion of MOPr and DOPr heterodimers, leading to changes in the extent of signaling by these receptors.

VI. δ-Opioid Receptor Signal Transduction

Like other opioid receptors, DOPrs signal predominantly via the activation of heterotrimeric G proteins. Early support for this notion was provided by the observation that guanine nucleotides could change the binding affinities of opioid agonists in neuroblastoma (Blume, 1978) and brain membranes (Childers and Snyder, 1978). Subsequently, the observation that opioids reduced cAMP production in a PTX-sensitive manner allowed researchers to specifically implicate

Gαi/o proteins as transducers of opioid receptor activation (Hildebrandt et al., 1983; Hsia et al., 1984). Since these early observations the repertoire of opioid-mediated signals has continuously grown, first with the discovery that βγ dimers derived from Gαi/o proteins could modulate membrane-delimited enzymes (e.g., ACs, PLCβ, and channels such as Cav2 and Kir3) and, more recently, with the finding that DOPrs also engage kinase signaling cascades. Concomitant with such multiplication of effectors, the availability of novel cell-based assays (Audet et al., 2008; Tudashki et al., 2014), Gα-specific antibodies (Garzón et al., 1997; Law and Reisine, 1997), antisense oligonucleotides (Standifer et al., 1996), and gene silencing/editing technologies (Zhang et al., 2003) enabled researchers to confirm that DOPrs pleiotropically couple to a multiplicity of G proteins beyond the classic $G\alpha i_{1,2,3/o}$ subtypes (reviewed in Piñeyro and Archer-Lahlou, 2007). This coupling diversity allows opioid receptors to trigger a great variety of signals (summarized in Table 4), whose contribution to the in vivo actions of DOPr ligands is the subject of active investigation. Indeed, better knowledge of which signals support desired and undesired in vivo actions should allow a more rational approach to the design of therapeutic DOPr ligands. An overview of signals modulated by DOPr is given below and, where available, information on cellular and in vivo responses supported by each pathway is also provided.

A. δ-Opioid Receptors and Adenylate Cyclase Signaling

It has long been accepted that DOPr modulates cAMP production by engaging different PTX-sensitive Gi/o protein subtypes (reviewed in Quock et al., 1999). In immortalized cell lines expressing either endogenous or recombinant DOPr, Gαi2 has been frequently associated with cAMP inhibition, although additional subtypes are also involved in this response (Table 4). For example, DOPrs expressed in Chinese hamster ovary (CHO), COS cells (George et al., 2000; Fan et al., 2005), and HEK293 cells (Tsu et al., 1997; Tso et al., 2000) activate Gαz and modulate AC activity in a PTX-insensitive manner. In COS and CHO cells, AC inhibition by DOPr agonists was only evident when these receptors were coexpressed with MOPrs (George et al., 2000); however, in HEK cells, DOPrs expressed by themselves were able to both inhibit (Tsu et al., 1997; Ho and Wong, 2000; Tso et al., 2000) or stimulate cAMP production via Gαz (Tsu et al., 1997; Ho et al., 2001). The reason why some cellular backgrounds but not others may allow DOPrs to activate Gz in the absence of MOPrs is not evident. Gαz has a very slow GTP hydrolysis rate (Casey et al., 1990), which can be selectively accelerated by regulator of G signaling protein (RGS) Z1 (Glick et al., 1998), greatly reducing Gαz-mediated inhibition of cAMP production (Mao et al., 2004). Differences in cellular complement of RGS proteins may explain why DOPr inhibition of cAMP production was seen in HEK cells but not in CHO or COS cells. However, if this is indeed the case, Gαz activation by the MOPr-DOPr dimer would imply that the heteromer is capable of inactivating RGSZ1/2.

Acute DOPr activation in the brain may also produce positive and negative modulation of cAMP levels. Thus, although activation of DOPrs in striatal membranes consistently inhibited cAMP production, their activation in the olfactory bulbs and frontal cortex produced both types of responses (Table 4). This distinct pattern of cAMP modulation by DOPr closely correlates with region-specific expression of ACs that are distinctively modulated by Gαi/o and Gβγ. Thus, AC5, which is activated by Gαs and inhibited by Gαi/o, is confined to the striatum, where it predominates (Matsuoka et al., 1997; Pavan et al., 2009; Pierre et al., 2009) and controls cAMP modulation by MOPr, DOPr, D1, and D2 receptors (Noble and Cox, 1997; Unterwald and Cuntapay, 2000; Kim et al., 2006; Kheirbek et al., 2009). The contribution of AC5 to DOPr-mediated behaviors is clearly illustrated by the observation that the infusion of DOPr agonists into the striatum in transgenic mice lacking this effector produces much less cAMP inhibition and fails to enhance locomotion as observed in wild-type mice (Kim et al., 2006). AC5 is highly expressed by medium spiny neurons as well as cholinergic interneurons (Mons et al., 1995), a distribution that matches postsynaptic expression of DOPr (Scherrer et al., 2006; Bertran-Gonzalez et al., 2013). Those in cholinergic interneurons were recently shown to traffic to the membrane during processing of environmental information that can guide choices of future behavioral outputs (Bertran-Gonzalez et al., 2013). Such information processing is critical for decision making and may be compromised in the context of addiction (Guitart-Masip et al., 2014) or during depression (Seymour and Dolan, 2008). Consistent with such deficits, chronic exposure to morphine, nicotine, and cocaine compromises DOPrs' ability to modulate striatal cAMP levels (Noble and Cox, 1997; Perrine et al., 2008; McCarthy et al., 2011). Furthermore, anxiety and depressive-like behaviors associated with cocaine withdrawal can be mitigated by SNC80 treatment (Perrine et al., 2008), suggesting a possible link between the affective component of withdrawal and reduced AC5 inhibition by DOPr. It is also tempting to speculate that antidepressant and/or anxiolytic actions of DOPr agonists (Saitoh et al., 2004; Jutkiewicz et al., 2005) could, at least in part, result from their ability to inhibit striatal AC5. In support of this notion is the observation that an increase in striatal cAMP response element binding activity promotes dynorphin upregulation and brain-derived neurotrophic factor (BDNF) production, both of which are mediators of depressive-like behaviors associated with drug addiction and/or social stress (Krishnan et al., 2007; Chartoff et al., 2009).

TABLE 4
Signaling pathways activated by DOPrs and putative cellular and/or in vivo functions they support

Effector	Observed Response after DOR Activation	Cell Type/Structure in Which Response Was Observed	Possible In Vivo Response or Established by the Study	G Protein/Reported Signaling Cascade Involved	Reference
G protein	GTP hydrolysis	HEK293 cells	—	Gαi1 is more effective turnover than Gαo	Moon et al. (2001)
	Conformational rearrangement within heterotrimeric G protein subunits	HEK293 cells	—	Gαi1β1γ2 activation	Audet et al. (2008)
	Photoaffinity labeling	NG108-15 hybrid; SK-N-BE cells	—	Gαi2, Gαi3, Gαo	Offermanns et al. (1991), Prather et al. (1994), Allouche et al. (2000)
	Immunoreactivity active G protein conformation	Murine PAG membranes	—	Gαi2, Gαz	Garzón et al. (1997)
	GTPγS^{35} incorporation	Rat striatum, rat hippocampus	Movement, reward, mood, anxiety, cognition	Gαi3, Gαz	Kabli et al. (2014)
	Loss of spinal analgesia by G protein knockdown	Intrathecal injection of antisense nucleotides and DOPr agonists (mice)	Analgesia[a] (thermal; tail-flick test)	Gαi1, Gαi2, Gαi3, Gαo, Gαx/z, Gαq	Standifer et al. (1996)
	Loss of supraspinal analgesia by G protein knockdown	Intracerebroventricular injection of antisense nucleotides and DOPr agonists (mice)	Analgesia[a] (thermal; tail-flick test)	Gαi2, Gαi3, Gαo, Gα11, Gαq, Gαz	Garzón and Sánchez-Blázquez (1995), Sánchez-Blázquez and Garzón (1998)
AC	cAMP inhibition	COS and CHO cells	—	Gαz, Gαi3	George et al. (2000), Fan et al. (2005)
	cAMP inhibition	HEK293 cells; NG108-15 and neuro 2A cells	—	Gαi2, Gαz	McKenzie and Milligan (1990), Tsu et al. (1997), Ho and Wong (2000), Megaritis et al. (2000), Zhang et al. (2006)
	cAMP inhibition superactivation cAMP production	HEK293 cells	Cellular tolerance	Gαi/o; Gαz Gαi/o (not Gαz)	Tso et al. (2000)
	cAMP inhibition	SK-N-BE	—	Gαi1, Gαi2, Gαi3, Gαo	Allouche et al. (1999)
	cAMP inhibition superactivation cAMP production	Neuro 2A cells	Cellular tolerance	Gαi2	Zhang et al. (2006)
	cAMP inhibition	Rat frontal cortex	—	Not assessed	Perrine et al. (2008)
	cAMP inhibition	Homogenates of rat olfactory bulb (external plexiform and granule cell layers)	Limbic circuit; emotions, depression	Not assessed	Onali and Olianas (2004)
	cAMP inhibition	Rat striatum and nucleaus accumbens membranes	Movement, reward, anxiety, predictive- associative learning/decision making	Gαi/o	Unterwald and Cuntapay (2000), Perrine et al. (2008)
	cAMP inhibition (striatopallidal and striatonigral pathway)	Rat caudate-putamen nucleaus and accumbens membranes	Movement, reward, anxiety, predictive-associative learning/decision making	Not assessed	Noble and Cox (1997)
	cAMP inhibition	Murine nucleus accumbens membranes	Movement, reward, anxiety, predictive-associative learning/decision making	Not assessed	McCarthy et al. (2011)
	cAMP inhibition	WT and AC5$^{-/-}$ mice striatal membranes	Psycholocomotion[a]	Gαi/o	Kim et al. (2006)
	cAMP inhibition	Murine striatal membranes	Movement, reward, anxiety, predictive- associative learning/ decision making	Gαi/o	Olianas et al. (2012)
	Inhibition DARP32 phosphorylation by adenosine (striatopallidal pathway)	Rat striatal homogenates	Movement, reward, anxiety, predictive- associative learning/ decision making	not assessed	Lindskog et al. (1999)

(*continued*)

TABLE 4—*Continued*

Effector	Observed Response after DOR Activation	Cell Type/Structure in Which Response Was Observed	Possible In Vivo Response or Established by the Study	G Protein/Reported Signaling Cascade Involved	Reference
	Superactivation cAMP production	CHO cells	Cellular tolerance	Gαi/o → G$\beta\gamma$ → PLCβ3 → IP_3 → PKC → Raf1 → ACV/VI	Rubenzik et al. (2001), Varga (2003)
	Increase cAMP production	HEK293 cells	—	Gαi2; Gαz → G$\beta\gamma$ → ACII	McKenzie and Milligan (1990), Tsu et al. (1997), Ho and Wong (2000), Megaritis et al. (2000), Zhang et al. (2006)
	Increased AC activity	Homogenates of rat olfactory bulb (granule cell layer)	Limbic circuit; emotions, depression	Not assessed	Onali and Olianas (2004)
	Increase cAMP production synergism D2R	Hippocampal neuron cultures newborn rats	—	Gαi/o → G$\beta\gamma$ → AC → PKA → CREB	Yao et al. (2003)
	Increase cAMP production synergism D1R	Murine PFC membranes	Reward-motivated attentional processing, mood, anxiety	Gαi/o → G$\beta\gamma$AC → PKA → 1) CREB; 2) pNR; 3) pGluR1	Olianas et al. (2012)
	Increase cAMP production synergism VIPR	Rat spinal cord (slices)	Visceral analgesia	Gαi/o... → PLCβ → IP_3 →... → AC	Liu and Gintzler (2003)
Kinase cascade ERK	ERK1/2 activation	CHO cells	—	1) Gαi/o → G$\beta\gamma$ → Src → Raf/MEK/ERK1/2; 2) βarr1/2 → Raf/MEK/ERK1/2	Xu et al. (2010)
	ERK1/2 activation	HEK293 cells	—	Gαi/o → PLC → Integrin a5b1 → PKCδ → EGFR → MEK/ERK1/2	Eisinger and Ammer (2008a, 2009)
	ERK1/2 activation	COS-7 cells	—	Gαi/o → G$\beta\gamma$ → Ras → Raf/MEK/ERK1/2	Belcheva et al. (1998)
	ERK1/2 activation	Jurkat human T lymphocyte cell line	Modulation cytokine secretion	Gαi/o → ?→ MEK/ERK1/2 (G$\beta\gamma$/PI3K independent)	Hedin et al. (1999)
	ERK1/2 activation	NG108-15 hybrid cells	—	Gαi/o → ? → MEK/ERK1/2 (G$\beta\gamma$/PI3K independent)	Zhang et al. (2003)
	ERK1/2 activation	NG108-15 hybrid cells	—	Gαi/o → PLC → Integrin a5b1 → PKCδ → TrkA → MEK/ERK1/2	Eisinger and Ammer (2008a,b)
	ERK1/2 activation evidence of neuroprotection	PC12 cells	Prosurvival/antiapoptotic effects upon serum deprivation[a] (measured as reduced LDH release and DNA fragmentation)	MEK → ERK1/2 → increased survival	Hayashi et al. (2002)
	ERK1/2 activation evidence of neuroprotection	Pluripotent murine stem cell line differentiated to neurons	Neuroprotection after exposure to ROS[a] (measured as reduced caspase 3 activation)	Trk → reduced caspase 3 immunoreactivity	Narita et al. (2006)
	ERK1/2 activation evidence of neuroprotection	Embryonic rat cortical rat neuron cultures	Prosurvival/antiapoptotic actions during severe hypoxia[a] (measured as reduced LDH release and DNA fragmentation); response requires hypoxic preconditioning	MEK → ERK1/2 → Bcl2 → increased survival	Ma et al. (2005)
	ERK1/2 activation evidence of neuroprotection	Embryonic rat cortical neuron cultures	Increased survival after oxygen glucose deprivation[a] (as measured by levels)	MEK → ERK1/2 → increased survival	Ke et al. (2009)
	ERK1/2 activation	Embryonic murine astrocyte-enriched cultures	Increased expression of EAAT1 and EAAT2 and of glutamate uptake[a]	MEK → ERK1/2 → increased EAAT1 and EAAT2 expression	Liang et al. (2014)
	ERK1/2 activation evidence of neuroprotection	Rat cortical slices	Prosurvival actions during glucose deprivation[a] (measured as reduced LDH release and DNA fragmentation)	MEK → ERK1/2 → Bcl2 → increased survival	Zheng et al. (2012)

(*continued*)

TABLE 4—*Continued*

Effector	Observed Response after DOR Activation	Cell Type/Structure in Which Response Was Observed	Possible In Vivo Response or Established by the Study	G Protein/Reported Signaling Cascade Involved	Reference
	ERK1/2 activation evidence of neuroprotection	Rat cortical and striatal homogenates	Downregulation of FADD[a]; possible offset of proapoptotic caspase 3/8 activity	MEK → ERK1/2 → reduced FADD	García-Fuster et al. (2007)
	ERK1/2 activation; evidence of cardioprotection	Neonatal rat cardiomyocytes	Ultrastructural features of apoptosis upon serum glucose deprivation	MEK → ERK1/2-PI3K → increased survivin expression → reduced apoptosis	Yao et al. (2007)
	ERK1/2 activation; evidence of cardioprotection	Neonatal rat cardiomyocytes	Prosurvival/antiapoptotic actions upon serum deprivation[a] (measured as reduced IDH release, caspase 3 activation and DNA fragmentation)	MEK → ERK1/2 → reduced apoptosis / increased survival	Shen et al. (2012)
	ERK1/2 activation; evidence of cardioprotection	Neonatal rat cardiomyocytes	Increased survival after serum deprivation[a] (as measured by MTT levels)	PKC/MEK → ERK1/2 → reduced apoptosis / increased survival	Suo et al. (2014)
	ERK1/2 activation; evidence of cardioprotection	Rat hearts	Reduction in infarct see at reperfusion after coronary ligation[a]	MEK → ERK1/2 activation and calcineurin suppression → reduced infarct size	Ikeda et al. (2006)
AKT	Akt activation	CHO cells	—	Gαi/o → Src/PDGFR/IGFR1/ AMPK → PI3K → Akt → GSK3 inhibition	Olianas et al. (2011)
	Akt activation; evidence of neuroprotection	NG108-15 hybrid cells	Caspase 3 and GSK3 inhibition[a] in serum deprived cells (prosurvival, antiapopototic actions)	Gαi/o → Trk → PI3K → Akt → caspase; GSK3 inhibition	Heiss et al. (2009)
	Akt and ERK1/2 activation; evidence of cardioprotection	Adult rabbit cardiomyocytes	Ischemic preconditioning (measured by mitochondrial membrane integrity/ROS production)	Metalloproteinase → Src/HB-EGF shedding → EGFR → PI3K/PKC → Akt/ERK1/2 → cardioprotection	Philipp et al. (2006), Cohen et al. (2007), Förster et al. (2007)
		Rabbit isolated hearts	Reduced infarct size upon reperfusion		
p38	p38 activation	NG108-15 hybrid cells	—	Gαi/o → p38	Zhang et al. (1999b)
	p38 activation evidence of cardioprotection	Murine hearts	Protection from functional consequences of ischemia/ reperfusion as evidenced by contractile recovery	Not assessed	Peart et al. (2007)
	p38 inhibition evidence of neuroprotection	Embryonic rat cortical neuron cultures	Prosurvival/antiapoptotic actions during severe hypoxia[a] (measured as reduced IDH release and DNA fragmentation) Response requires hypoxic preconditioning	MEK → ERK1/2 → p38 → increased survival	Ma et al. (2005), Hong et al. (2007)
	p38 inhibition evidence of neuroprotection	Embryonic rat DRG neuron cultures	Blocked increase in Nav.1 expression after exposure to hyperglycemic culture medium[a]	Not assessed	Chattopadhyay et al. (2008)
	p38 inhibition counters proinlfammatory response	Murine macrophage cell line (RAW 264.7)	Limited proinflammatory response[a] measured as reduced production of proinflammatory cytokine (TNFα) and of neutrophil attaracting chemokine (MIP-2) after exposure to lipopolysaccharide	Not assessed	Husted et al. (2005)

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TABLE 4—*Continued*

Effector	Observed Response after DOR Activation	Cell Type/Structure in Which Response Was Observed	Possible In Vivo Response or Established by the Study	G Protein/Reported Signaling Cascade Involved	Reference
JNK	JNK activation	COS-7 cells	—	G$\alpha i/o \rightarrow G\beta\gamma \rightarrow$ Src $\rightarrow$ Rac + Cdc42 $\rightarrow$ p-JNK	Kam et al. (2003)
	JNK activation ATF-2 activation	CD3-activated splenic T cells; Jurkat human T lymphocyte cell line	Cytokine gene transcription	JNK $\rightarrow$ pJNK $\rightarrow$ JNK-ATF-2 complex $\rightarrow$ p-ATF-2 $\rightarrow$ transcription	Shahabi et al. (2003)
	JNK activation c-Jun activation	CD3-activated splenic T cells	Cytokine gene transcription	G$\alpha i/o \rightarrow$ pJNK $\rightarrow$ PI3K $\rightarrow$ pAkt $\rightarrow$ p-c-Jun $\rightarrow$ transcription	Shahabi et al. (2006)
Phospholipase PLCβ	PLCβ1 activation	COS-7 cells	—	Gα14 $\rightarrow$ PLCβ1 $\rightarrow$ IP formation	Ho et al. (2001)
	PLCβ1 activation	COS-7 cells	—	Gα16 $\rightarrow$ PLCβ1 $\rightarrow$ IP formation	Lee et al. (1998), Chan (2003)
	PLCβ2/β3 activation mobilization intracellular Ca^{+2} stores	NG108-15 hybrid cells	—	G$\alpha i/o \rightarrow$ PLC$\beta \rightarrow IP_3 \rightarrow$ intracellular Ca^{+2} stores	Jin et al. (1994), Smart and Lambert (1996), Yoon et al. (1999)
	Mobilization intracellular Ca^{+2} stores	SH-SY5Y cells	—	G$\alpha i/o \rightarrow G\beta\gamma \rightarrow$ intracellular Ca^{+2} stores; requires concomitant Gq activation	Chen et al. (2000), Yeo et al. (2001)
	PLCβ2/β3 activation mobilization intracellular Ca^{+2} stores	Rabbit iris-ciliary body	Reduction in acqueous flow rate and intraocular pressure	G$\alpha i/o \rightarrow G\beta\gamma \rightarrow$ PLC$\beta \rightarrow IP_3 \rightarrow$ intracellular Ca^{+2} stores	Dortch-Carnes and Potter (2003)
	PLCβ3 activation muscle contraction	Dispersed intestinal muscle cells guinea pig intestine	Intestinal smooth muscle contraction	Gαi2/Gαo $\rightarrow G\beta\gamma \rightarrow$ PLCβ3 $\rightarrow IP_3 \rightarrow$ intracellular Ca^{+2} stores	Murthy and Mahklouf (1996)
	Synergistic peripheral analgesia	Hind paw injection of agonists (rats)	Analgesia[a] (thermal, paw withdrawl)	Priming by BK/PKC/AA necessary for DOPr analgesia	Rowan et al. (2009)
	Spinal analgesia inhibition NT release from primary afferents	Intrathecal injection of agonist (rats)	Analgesia[a] (thermal, tail-immersion)	Coactivation of PKC by α2ADR necessay for analgesic synergism; DOPr analgesia requires PKC activity	Overland et al. (2009)
	Spinal analgesia	Intrathecal injection of agonist (mice)	Analgesia[a] (thermal, tail-flick)	DOPr analgesia requires PIC activity; PKC inhibition enhances analgesia	Narita et al. (2000)
PLA2	Arachidonate release	CHO cells	—	G$\alpha i/o \rightarrow$ intracellular Ca^{+2} stores $\rightarrow$ PKC $\rightarrow$ ERK1/2 $\rightarrow$ PLA2 $\rightarrow$ AA	Fukuda et al. (1996)
	Inhibition GABAergic currents supraspinal analgesia	NRM slices (rats), intracerebral injection of agonist into NRM (rats)	AA release/HPETEs production in NRM; analgesia (thermal; tail-flick test)	PLA2 $\rightarrow$ AA $\rightarrow$ 12-LOX $\rightarrow$ HPETEs $\rightarrow$? $\rightarrow$ reduced GABA release	Zhang and Pan (2010, 2012)
PLD2	PLD2 activation	HEK293; SHSY-5Y cells	DOPr endocytosis	DOPr/ARF interaction (?) $\rightarrow$ PLD2 $\rightarrow$ PA $\rightarrow$ DAG $\rightarrow$ p38 $\rightarrow$ EEA-1	Koch et al. (2006), Yang et al. (2010)
Channel Kir3	Ba^{+2}-sensitive, K^+ current with inward rectification	Xenopus oocytes	—	G$\alpha i/o \rightarrow G\beta\gamma \rightarrow$ Kir3 channel	Ikeda et al. (1995), Kovoor et al. (1997), Kobayashi et al. (1998), Zhao et al. (2006)
	Spinal analgesia	Intrathecal injection	Analgesia[a] (thermal, tail-flick test)	Analgesia lost in Kir3.1 or Kir3.2 knockout mice or in mice that received tertiapin Q intrathecally	Marker et al. (2005)
	Peripheral analgesia	intra-masseter injection	Analgesia[a] (noxious mechanical, chemical stimuli)	Analgesia lost by intramasseter injection of tertiapin Q	Chung et al. (2014)
	Outward currents in local and projection neurons of RVM	Rostral-ventro-medial-medulla slices (rats)	Regulation of local and projection neurons pro or antinociceptive (?)	Outward current presumably Kir3-mediated	Marinelli et al. (2005)

(*continued*)

TABLE 4—*Continued*

Effector	Observed Response after DOR Activation	Cell Type/Structure in Which Response Was Observed	Possible In Vivo Response or Established by the Study	G Protein/Reported Signaling Cascade Involved	Reference
	Outward currents in local GABAergic neurons of RVM	Rostral-ventro-medial-medulla slices (mice)	Disinhibition of descending antinociceptive pathway	Outward current presumably Kir3-mediated	Pedersen et al. (2011)
	Outward (inhibitory) currents in PAG	Periaqueductal gray slices (mice)	Disinhibition of the descending antinociceptive pathway by inhibiting GABAergic interneurons	Current produced by inward rectifying channel	Vaughan et al. (2003)
	Outward (inhibitory) currents in low threshold bursting GABAergic neurons of central amygdala projecting to PAG	Amygdala slices (rats)	Inhibition of GABA release in PAG	Current produced by inward rectifying channel; effect remarkably increased by morphine pretreatment	Chieng and Christie (2009)
	Inhibition of IPSCs recorded from pyramidal hippocampal neurons (CA1)	Hipppocampal slices (mice)	Enhanced pyramidal neuron excitability via presynaptic desinhibition[a]; learning and memory	Not assessed; presumably Kir3	Rezai et al. (2013)
	Outward (inhibitory) currents in GABAergic interneurons stratum oriens (CA1)	Hipppocampal slices (rats)	Inhibition of GABAergic interneurons desinhibition of pyramidal cells[a]; learning and memory	Current is only partly due to inward rectifying channel; part is due to I_h inactivation	Svoboda and Lupica (1998), Svoboda et al. (1999)
Cav2	Inhibition of membrane currents mediated by voltage-operated Ca^{+2} channels	Human small-cell lung carcinoma cells	—	Gαi/o → VOCC undistinguished; revesible by hyperpolareation	Sher et al. (1996)
	Inhibition N-type Ca^{+2} channel currents	NG108-15 hybrid cells	—	PTX-sensitive, Cav2.2 modulation reversible by hyperpolaization	Morikawa et al. (1999), Toselli et al. (1999)
	Inhibition Cav2.2 channel currents	DRG small and medium neurons (rat 7–11 days old)	Inhibition neurotransmitter release[a]; analgesia	DOPrs also inhibited Cav2.1 channel currents	Acosta and López (1999)
	Inhibition of depolarization driven increase in intracellular Ca^{+2}; reduction in CGRP release	Dissociated DRG neurons (adult rats)	Inhibition CGRP release; analgesia	DOPrs also inhibited Cav2.1 channel permeability to Ca^{+2}	Khasabova et al. (2004)
	Inhibition Cav2.2 channel currents	DRG small neurons (rat 5 to 6 weeks old)	Inhibition neurotransmitter release; analgesia	DOPrs also inhibited Cav2.1 channel currents inhibition of all Cav2 channels was more effective in isolectin IB4 negative neurons	Wu et al. (2008)
	Inhibition Cav2.2 channel currents analgesia in chronic pain model	Medium-large neurons; DRGs from 8- to 14-week-old mice	Inhibition neurotransmitter release; analgesia[a] (mechanical allodynia)	Cav2.2 responses to DOPr agonists were observed in 33% of neurons from naïve mice and 100% of CFA-treated mice	Mittal et al. (2013), Pradhan et al. (2013)
	Inhibition Cav2.2 channel currents	Neurons in TG and DRGs from 7-day-old rats transfected with DOPr-GFP exposed or not to BK	Inhibition neurotransmitter release[a]; analgesia	Cav2.2 responses to DOPr agonists were observed in 23% of control neurons and 54% of neurons exposed to BK; greater CGRP release after BK	Pettinger et al. (2013)

ATF, activating transcription factor; CFA, complete-Freund's-adjuvant; CREB, cAMP response element binding; DARP32, dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa; D1R, dopamine 1 receptor; D2R, dopamine 2 receptor; EAAT, excitatory amino acid transporter; FADD, Fas-associated death domain; HB-EGF, heparin-binding EGF-like growth factor; IDH, isocitrate dehydrogenase; IGFR, insulin-like growth factor 1 receptor; LDH, lactic dehydrogenase; MEK, mitogen-activated protein kinase kinase; MIP, macrophage inflammatory protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NRM, nucleus raphé magnus; PDGFR, platelet-derived growth factor receptor; PFC, prefrontal cortex; ROS, reactive oxygen species; RVM, rostroventromedial medulla; TNF, tumor necrosis factor; VIPR, vasointestinal polypeptide receptor; VOCC, voltage operated calcium channel; WT, wild type.

[a]Established by the study.

In contrast with predominantly inhibitory responses evoked by striatal DOPr, stimulation of those present in the olfactory bulbs (Onali and Olianas, 2004), medial prefrontal cortex (Olianas et al., 2012), and primary cultures of hippocampal neurons (Yao et al., 2003) potentiate cAMP production by Gαs-coupled receptors. This opposing modulation of cAMP levels by striatal and hippocampal receptors is consistent with divergent effects of DOPr silencing upon behaviors controlled by these structures. In particular, DOPr knockout mice display enhanced striatum-dependent procedural learning but reduced performance in hippocampal-mediated memory tasks (Le Merrer et al., 2013), suggesting a scenario in which respective AC inhibition and activation by DOPr may act as a brake and trigger for striatal and hippocampal learning. cAMP increases associated with DOPr activation in these structures involved Gβγ subunits, suggesting the probable involvement of AC2 or AC4 since both are activated by the Gβγ dimer (Pavan et al., 2009). The distribution of AC2/AC4 expression in the brain parallels that of DOPr-positive cAMP responses, with both ACs being present in the olfactory bulbs, prefrontal cortex, and hippocampus (Matsuoka et al., 1997; Baker et al., 1999; Olianas et al., 2013). However, it is interesting to note that in the hippocampus, AC2 and AC4 are predominantly present in dendrites of granule and pyramidal cells (Baker et al., 1999), whereas DOPrs are highly expressed presynaptically, on GABAergic interneurons (Rezaï et al., 2012). This distribution would imply that the few DOPrs of pyramidal cells very effectively modulate these enzymes or that the modulation is indirect via a mechanism that remains to be elucidated. Together with AC2/AC4, DOPrs in the frontal cortex may also activate cAMP production via AC1 (Olianas et al., 2013; Ujcikova et al., 2014). Together with AC3 and AC8, AC1 belongs to the family of Ca^{+2}/calmodulin-dependent ACs (Pavan et al., 2009), which are activated via phosphorylation via Ca^{+2}-sensitive kinases after Gβγ-mediated stimulation of PLCβ and Ca^{+2} mobilization from intracellular stores. DOPr-mediated increases in cortical cAMP production via this pathway could support neuroadaptive changes including PKA-induced phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor 1 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (GluR1) subunits, as well as cAMP response element binding activation (Olianas et al., 2013). Such changes constitute the bases of synaptic plasticity, long-term memory formation, trophism, and survival, all of which counter cellular and molecular changes associated with depressive illness (Marsden, 2013). Thus, activation of cAMP production in forebrain cortical areas may constitute another putative mechanism for the antidepressant actions of DOPr agonists.

cAMP signaling by DOPr may also contribute to the analgesic effects of opioids, but the exact way in which these signals may influence nociception remains to be fully elucidated. The fact that acute opioid analgesia is reduced or increased in mice respectively lacking AC5 (Kim et al., 2006) or overexpressing AC7 (Yoshimura et al., 2000) argues in favor of AC involvement in acute opioid antinociception. Conversely, the presence of normal acute morphine analgesia in double AC1/AC8 knockout mice (Li et al., 2006) indicates minimal implication of Ca^{+2}/calmodulin-dependent kinases in this type of response. Failure of PKA inhibitors to modify acute morphine analgesia also supports the notion that cAMP effectors are minimally involved in the acute analgesic responses to opioids (Bernstein and Welch, 1999), as does the observation that phosphodiesterase inhibitors do not modify analgesia caused by intracerebroventricular or intrathecal administration of DPDPE (Suh et al., 1995). On the other hand, cAMP signaling has been consistently implicated in cellular adaptations that result from sustained DOPr stimulation and may contribute to analgesic tolerance (Tso et al., 2000; Varga, 2003; Zhang et al., 2006). These adaptations generally involve an increase in AC catalytic activity, although the mechanisms contributing to such an increase vary according to the type of AC involved (Avidor-Reiss et al., 1997; Varga, 2003). One of such mechanisms has been well characterized in CHO cells, in which sustained opioid activation leads to AC5/AC6 superactivation via AC phosphorylation by rapidly accelerated fibrosarcoma (Raf)-1 (Varga et al., 1999), whose activation is in turn induced by Rous sarcoma oncogene cellular homolog (Src), PKC, and/or calmodulin (Varga, 2003) via Gβγ- and PLCβ-dependent pathways (Rubenzik et al., 2001). As a consequence of Raf-1–dependent AC superactivation, cultured sensory neurons display increased PKA activity and enhanced release of CGRP (Yue et al., 2008), which may contribute to the development of analgesic tolerance (Tumati et al., 2010). Other PKA-dependent mechanisms contributing to opioid tolerance as well as pain sensitization include AMPA receptor phosphorylation and targeting of AMPA receptors to the membrane (Asiedu et al., 2011; Zhuo, 2012) as well as PKA-dependent phosphorylation of NMDA receptors (Qiu et al., 2013), which lead to increased neuronal excitability. Thus, in circumstances in which increased tissue levels of cAMP lead to excessive PKA activity and pain sensitization, acute AC inhibition by DOPr ligands may contribute to its analgesic actions (Zhang and Pan, 2010). At the same time, since adaptations of the AC pathway may lead to analgesic tolerance, ligand-directed modulation of this cascade seems a suboptimal strategy for the development of novel opioid analgesics.

B. δ-Opioid Receptors and Mitogen-Activated Protein Kinase Signaling

DOPrs are central to the adaptive/protective actions coordinated by the opioid system in response to stressful stimuli. Thus, in addition to the analgesic, anxiolytic,

and antidepressant actions (Ribeiro et al., 2005; Hsu et al., 2015) that were mentioned in previous sections, opioids also promote cell survival (Hayashi et al., 2002; Ma et al., 2005), cardioprotection (Ikeda et al., 2006; Tsutsumi et al., 2010; Headrick et al., 2015), neuroprotection (He et al., 2013b; Liu et al., 2015), modulation of the immune/inflammatory response (Neptune and Bourne, 1997; Hedin et al., 1999; Sharp, 2006; Wang et al., 2014), and wound healing (Bigliardi-Qi et al., 2006; Iaizzo et al., 2012; Bigliardi et al., 2015). At the cellular level, a majority of these responses are mediated by an evolutionarily conserved set of kinase cascades whose activation has been traditionally associated with receptor tyrosine kinases (RTKs) (Takeda et al., 2011). More recently it has become well accepted that these pathways are also engaged by GPCRs (O'Hayre et al., 2014), and DOPrs are no exception.

1. Extracellular Regulated Kinase Cascade. DOPr activation of the ERK1/2 cascade has documented implication in most of the stress responses just mentioned. With the exception of a report in which DOPr-induced phosphorylation of ERK1/2 was linked to βarr2 (Xu et al., 2010), activation of this MAPK has been found to be predominantly G protein mediated (Table 4) and involves the release of G$\beta\gamma$ dimers that relay the signal to Src or PLCβ (Belcheva et al., 1998; Hedin et al., 1999; Xu et al., 2010). PLC stimulation then leads to RTK transactivation via a mechanism involving integrins and PKCδ (Eisinger and Ammer, 2008a,b). The RTK engaged via this cascade may vary according to cellular background and ongoing signaling activity. Thus, although DOPrs in HEK cells induced transactivation of the epidermal growth factor receptor (EGFR), those in neuroblastoma $\times$ glioma NG108-15 hybrid cells recruited TrkA activity (Eisinger and Ammer, 2008a,b). In either case, the insulin-like growth factor-1 receptor could replace the prevailing RTK when it became desensitized (Eisinger and Ammer, 2011; but also see Kramer et al., 2002). At a cellular level, DOPr signaling via Trk receptors and the ERK1/2 pathway was associated with antiapoptotic actions in cortical neuron cultures (Ma et al., 2005), and DOPr activation of the ERK cascade in the brain supported neuronal survival during exposure to ischemic/metabolic insult. In such cases, the antiapoptotic factor B-cell lymphoma (Bcl)2 (Su et al., 2007) or downregulation of the Fas-associated death domain (García-Fuster et al., 2007) was necessary for neuroprotection to occur. TrkB receptors for BDNF are well known to participate in the response to classic antidepressants (Duman, 2014; Haase and Brown, 2015), and DOPr stimulation by endogenous and exogenous agonists produces antidepressant-like effects that are concomitant with BDNF and TrkB mRNA upregulation in different limbic areas (Torregrossa et al., 2004; Zhang et al., 2006). What is less clear is whether DOPr stimulation in the brain leads to TrkB receptor transactivation and, if so, what is the contribution of this signaling modality to the antidepressant actions of DOPr ligands.

ERK activation by DOPr was also described in keratinocyte cell lines. In these cells, proliferation, expression of transcription factors (POU domain, class 2, transcription factor 3), and expression of differentiation markers (keratin, type I cytoskeletal 10) that are normally associated with normal skin stratification and wound healing were repressed by phospho-ERK. Consistent with these observations, organotypic skin cultures containing DOPr-overexpressing keratinocytes displayed KRT10 repression and reduced epidermal thickness (Neumann et al., 2015). Conversely, DOPr-deficient mice exhibited markedly increased expression of KRT10, hypertrophic wound edges, and delayed wound healing (Bigliardi-Qi et al., 2006; Bigliardi et al., 2009). Although the therapeutic potential of this type of response is undeniable, DOPr effects in the human skin were opposite to those described just above (Neumann et al., 2015), underscoring the need for a better understanding of the human neuroendocrine skin system (Slominski, 2015) before any treatment strategy in wound healing can be envisioned.

2. p38 Mitogen-Activated Protein Kinase Cascade. DOPrs have been associated with stimulation or inhibition of the p38 cascade (Table 4). In the case in which stimulation was observed, it was PTX sensitive (Zhang et al., 1999b) and associated with cardioprotective effects of DOPr agonists in young mice. In aging animals, DOPr stimulation induced no p38 activation and no increase in contractility upon recovery from ischemia (Peart et al., 2007). DOPr agonists also resulted in p38 activation in a murine macrophage cell line (Husted et al., 2005) and in cultured astrocytes (Liang et al., 2014). In the former, p38 activity reduced proinflammatory cytokine production; in the latter, it contributed together with ERK1/2 to the upregulation of excitatory amino acid transporters (Liang et al., 2014). Because glutamate removal of the extracellular space may reduce excitotoxicity, the authors suggested a neuroprotective role for the activation of this cascade by astrocytic DOPr.

On the other hand, in prenatal cortical neuron cultures, DOPr activation was associated with inhibition of p38 activity. In this setting, p38 had deleterious effects on neuronal survival after ischemic insults and DOPr activation during preconditioning reduced subsequent activation of MAPK during ischemia, restoring Bcl2/cytochrome c levels and increasing survival (Ma et al., 2005; Hong et al., 2007). DOPr stimulation in cultured DRG neurons also inhibited p38 activation and the associated increase in Nav1.7 channel expression that was caused by increasing glucose concentrations in the incubation medium (Chattopadhyay et al., 2008). Similar observations were obtained in DRG neurons recovered from streptozotocin-diabetic mice that were injected with a viral vector codifying for proenkephalin.

Normalization of the levels of Nav1.7 channels upon viral injection was associated with reversal of the behavioral response to nociceptive stimuli. These biochemical and behavioral responses were both abolished by blocking DOPr with NTI (Chattopadhyay et al., 2008).

3. c-Jun N-Terminal Kinase Cascade. DOPrs activate c-Jun N-terminal kinase (JNK) in multiple cell types, although the signaling events that relay the information from the receptor to the MAPK itself differ across cellular backgrounds (Table 4). Thus, in T lymphocytes (Shahabi et al., 2006) where DOPr plays an important role in triggering the innate immune response, JNK activation involved the PI3K/Akt cascade (Sharp et al., 2001; Shahabi et al., 2003). In these cells, JNK stimulation by DOPr agonists activated activating transcription factor–2 and c-Jun promoting transcription of proinflammatory cytokines such as IL-2, γ-interferon, and tumor necrosis factor-α (Shahabi et al., 2003, 2006). In neuroblastoma × glioma or COS7 cells, a role for PI3K in JNK activation via DOPr was ruled out, with the relay of information being accomplished via Src family kinases and small G proteins of the Rho family (Kam et al., 2003). Neuronal responses associated with JNK activation by DOPr remain to be characterized.

4. Akt Pathway. In immortalized neuronal and non-neuronal cell lines, DOPr-mediated transactivation of RTKs may also lead to downstream activation of the PI3K-Akt pathway (Heiss et al., 2009; Olianas et al., 2011), caspase inhibition, cytoprotection (Heiss et al., 2009), and suppression of glycogen synthase kinase 3 activity (Heiss et al., 2009; Olianas et al., 2011). Cytoprotection and glycogen synthase kinase 3 activity are modulated by antidepressant and mood-stabilizing drugs (Beaulieu, 2012). Their regulation by DOPr agonists warrants further study in relation to the antidepressant actions of these ligands.

The Akt pathway also participates in DOPr-mediated cardioprotection, particularly during preconditioning prior to severe ischemia and myocardial infarction (Ikeda et al., 2006; Philipp et al., 2006). In rabbit cardiomyocytes, DOPr-mediated preconditioning involves stimulation of metalloproteases, shedding of heparin-binding EGF-like growth factor, and activation of EGFR, PI3K, and Akt, which lead to ERK1/2-dependent cardioprotection (Philipp et al., 2006; Cohen et al., 2007; Förster et al., 2007). In rats, activation of cardiac ERK1/2 by DOPr required PKC activity (Suo et al., 2014); in both species, ERK1/2 inhibition prevented the protective effect of DOPr agonists. At the organ level, DOPr-mediated cardioprotection was clearly evidenced by reduced myocardial infarct size (Ikeda et al., 2006; Philipp et al., 2006); at the cellular level, DOPr-dependent ERK1/2 activation mitigated apoptosis triggered by ischemic-like insults (Cohen et al., 2007; Yao et al., 2007) or by serum deprivation (Shen et al., 2012; Suo et al., 2014), reducing lactic dehydrogenase release, decreasing DNA fragmentation, and/or preserving metabolic activity.

AMP-activated protein kinase (AMPK) also helps control metabolic stress associated with hypoxia/ischemia and glucose deprivation. In CHO cells, AMPK is required for DOPr-stimulated Akt activity (Olianas et al., 2011) and DOPr-induced glucose uptake (Olianas et al., 2012). Endogenously expressed DOPrs in primary olfactory neurons also activate glucose uptake via AMPK, suggesting the involvement of this pathway in neuronal metabolism. In this cellular background, AMPK activation by DOPr involved Gβγ, Ca^{+2} mobilization, and calmodulin-dependent protein kinase kinase activity, whereas an increase in glucose uptake also required activation of Gαq (Olianas et al., 2012).

C. δ-Opioid Receptors and Phospholipase Signaling

Lipases that hydrolyze membrane phospholipids are also DOPr effectors.

1. Phospholipase C. Within this family of phosphoinositide-specific lipases, PLCβ1 is regulated by Gα, whereas Gβγ subunits released by heterotrimeric $G\alpha_{i/o}$ proteins modulate PLCβ2 and PLCβ3 (Kadamur and Ross, 2013). All PLC isoforms may be engaged by DOPr to metabolize phosphatidyl-inositol-4,5-biphosphate into diacylglycerol (DAG) and inositol trisphosphate (IP_3), but the path most frequently engaged by these receptors is Gβγ dependent. Thus, despite inducing IP_3 production via Gα16 (Lee et al., 1998; Chan et al., 2003) and Gα14 (Ho et al., 2001; Lo and Wong, 2004), the physiologic relevance of these DOPr responses remains to be explored. On the other hand, at endogenous levels of expression, IP_3 production by DOPr agonists is PTX sensitive and is blocked by Gβγ scavengers (Smart and Lambert, 1996; Dortch-Carnes and Potter, 2003), and it promotes Ca^{+2} release from intracellular stores and results in PKC stimulation (Jin et al., 1994; Yoon et al., 1999; Yeo et al., 2001). Moreover, consequences of DOPr signaling via the Gβγ cascade include physiologic responses such as modulation of aqueous humor dynamics and control of intraocular pressure (Dortch-Carnes and Potter, 2003), visceral smooth muscle contraction (Murthy and Makhlouf, 1996), and spinal (Ohsawa et al., 1998; Overland et al., 2009) and supraspinal modulation of nociception (Ohsawa et al., 1998; Sánchez-Blázquez and Garzón, 1998; Narita et al., 2000). Although DOPr fully activates PLCβ activity and Ca^{+2} mobilization in smooth muscle cells (Murthy et al., 1996), NG108-15 cells (Jin et al., 1994; Allouche et al., 1996; Yoon et al., 1999), and CHO cells (Yeo et al., 2001), DOPr signaling via this pathway is not as efficient in other cellular backgrounds such as SH-SY5Y and COS-7 cells, requiring coactivation either of Gαq (Yeo et al., 2001) or Gα16 (Chan et al., 2000) to produce a measurable PLCβ response. Because the sensitivity of all three PLCβ

isozymes to G$\beta\gamma$ stimulation is not the same (PLCβ3 > PLCβ2 >>> PLCβ1; Kadamur and Ross, 2013), cell-dependent differences in receptor ability to evoke PLCβ-mediated responses have been attributed to distinct isoforms prevailing in different systems (Yeo et al., 2001). This assumption is supported by the fact that in gastrointestinal smooth muscle where DOPrs induce PLCβ/Ca^{+2} signaling independent of other stimuli, the response is mediated by PLCβ3 (Murthy and Makhlouf, 1995, 1996; Murthy et al., 1996).

Synergistic interaction between DOPrs and other receptors that engage PKC activity via the DAG/IP_3/Ca^{+2} pathway has been observed in vivo. In particular, concomitant activation of PKC by DOPr and BK or DOPr and α_{2A} receptors led to enhanced peripheral (Rowan et al., 2009) and spinal (Overland et al., 2009) analgesia by DOPr agonists, respectively. However, together with a greater antinociceptive response to DOPr agonists, PKC activity also increased the development of analgesic tolerance, as indicated by potentiation of spinal antinociception by deltorphin II in animals that also received calphostin C (Narita et al., 1996, 2000). Although tolerance was associated to DOPr desensitization by the kinase (Narita et al., 1996), PKC's contribution to peripheral analgesia was related to its ability to activate phospholipase A2 (PLA2) (Kennedy et al., 1996). Indeed, arachidonic acid (AA) not only substituted for BK in potentiating peripheral analgesia by DOPr agonists (Rowan et al., 2009), but AA was also identified as the mediator of BK analgesic priming downstream of PKC (Berg et al., 2007; Sullivan et al., 2015). Together with rapid PKC-dependent potentiation of analgesia, BK promoted DOPr targeting to the membrane. However, unlike analgesic potentiation, BK-induced membrane targeting of DOPr was neither blocked nor reproduced by respective incubation with PKC blockers or activators (Patwardhan et al., 2005), indicating no active role for the kinase in this response. As discussed in section V.A, a putative mechanism by which coactivation of other GPCRs could address DOPr to the membrane may involve their sequestration of βarrs so that these are no longer available to suppress DOPr export from the Golgi apparatus (Mittal et al., 2013).

Mobilization of intracellular Ca^{+2} stores that results from DOPr activation of PLCβ may support peripheral analgesia via an alternative mechanism that involves CaMKII rather than PKC activity. In particular, it is known that release of intracellular Ca^{+2} stores by Gq-coupled receptors activates CaMKII, which stimulates neuronal nitric oxide synthase and nitric oxide release leading to cGMP production and protein kinase G–mediated activation of K_{ATP} channels; this then produces analgesia via hyperpolarization of primary afferents (Gong et al., 2015). A direct association of this cascade with antinociceptive actions of peripherally administered DOPr agonists is suggested by the fact by K_{ATP} blockers, neuronal nitric oxide synthase inhibitors, and inactive cGMP analogs all interfere with DOPr-mediated analgesia (Pacheco and Duarte, 2005; Saloman et al., 2011; Gutierrez et al., 2012).

2. Phospholipase A2. Cytosolic PLA2 is normally activated after its phosphorylation by ERK and PKC, whereas Ca^{+2} availability ensures its translocation to the membrane. There, PLA2 induces AA production that is subsequently converted into active eicosanoids by the action of cyclooxygenases, lipoxygenases, and epoxygenase/cytochrome P450 (Qiu et al., 1998; Gijón et al., 1999; Sun et al., 2005). In CHO cells, PKC and ERK both contributed to PLA2 stimulation by DOPr, although the role of PKC in this cascade was upstream of ERK and was not directly on the lipase (Fukuda et al., 1996). The type of eicosanoids produced downstream of AA by DOPr agonists was not characterized in CHO cells; however, in GABAergic interneurons of the NRM, AA produced by DOPr activation was metabolized to hydroperoxyeicosatetraenoic acids (HPETEs) by 12-lipoxygenase (Zhang and Pan, 2012). HPETEs produced in this fashion were involved in presynaptic inhibition of GABA release within the NRM and in analgesic effects of DOPr agonists infused into the nucleus of animals pre-exposed to morphine (Zhang and Pan, 2010, 2012). Eicosanoid-based control of GABA release and neuronal excitability was initially described within the PAG for MOPr agonists. Therein, HPETE production by morphine reduced local release of GABA, most plausibly through the modulation of presynaptic voltage-gated K^+ channels on GABAergic terminals (Vaughan et al., 1997; Lau and Vaughan, 2014). As a result of the reduction in extracellular GABA, PAG neurons projecting to the rostral medulla enhanced analgesia by morphine (Vaughan et al., 1997; Lau and Vaughan, 2014). Interestingly, unlike MOPr in the PAG and DOPr in the NRM (Zhang and Pan, 2010, 2012), activation of PAG DOPr was not associated with presynaptic modulation of K^+ conductance, instead inducing postsynaptic hyperpolarization of local somata via inward rectifying K^+ channels (Vaughan et al., 2003). The study did not determine whether this hyperpolarization corresponded to soma of local GABAergic neurons or to pain modulatory neurons projecting to the spinal cord, so it is difficult to speculate whether PAG DOPrs produce analgesic or pronociceptive actions. In any case, modulation of inward rectifying K^+ channels by DOPr is more likely mediated via direct G$\beta\gamma$ interaction (see below) than via PLA2 activity.

3. Phospholipase D2. Phospholipase D (PLD) is a phospholipid-specific diesterase that hydrolyzes phosphatidylcholine into phosphatidic acid (PA) and choline. Of the two known mammalian isoforms, PLD2 is the one exclusively present at the membrane where it is activated both by DOPr and MOPr (Koch et al., 2004). Both receptors rely on small G proteins of the ADP ribosylation factor (ARF) family to induce PLD2

activation (Koch et al., 2006; Yang et al., 2010), although the mechanism linking activation of the receptor to that of the small G protein remains to be fully elucidated. Studies carried out on MOPrs indicate a direct interaction between the receptor and the lipase that also associates with ARF (Koch et al., 2003). This supramolecular organization suggested the possibility that conformational rearrangements triggered by receptor activation may lead to downstream PLD2 stimulation (Koch et al., 2003). The fact that ARF activates PLD2 by binding to the NP*XX*Y motif of other GPCRs and not via the activation of heterotrimeric subunits, supports this reasoning (Mitchell et al., 1998). Whether DOPrs form a similar complex with PLD2 and ARF1/6 has not been assessed, but a similar mechanism of activation as MOPrs seems reasonable given the high conservation of the NP*XX*Y motif among these receptors (see Fig. 2). PA released by PLD2 is converted to DAG, which activates p38. The activated MAPK phosphorylates Rab5 effectors (Macé et al., 2005), which regulate the formation, trafficking, and fusion of clathrin-coated vesicles to early endosomes (Zerial and McBride, 2001), providing a plausible explanation of why inhibition of PLD2 interferes with DOPr and MOPr internalization (Yang et al., 2010).

D. δ-Opioid Receptors and Activation of G Protein–Coupled Inward Rectifier Potassium Channels

The ability of DOPrs to activate these G$\beta\gamma$ effectors has been demonstrated in vitro (Ikeda et al., 1995; Kovoor et al., 1997; Kobayashi et al., 1998) as well as in vivo (Svoboda and Lupica, 1998; Williams et al., 2001; Vaughan et al., 2003), and the role of Kir3 channels (or G protein–activated K^+ channels) in opioid analgesia has been clearly established using null mice for different channel subunits (Mitrovic et al., 2003; Marker et al., 2005). Moreover, Kir3 contribution to the clinical response of opioid analgesics has been confirmed in association studies showing that genetic variations of different Kir3 subunits influence opioid dose requirements for pain management (Lötsch et al., 2010; Bruehl et al., 2013). For DOPrs in particular, Kir3 contribution to peripheral (Chung et al., 2014) and spinal analgesia (Marker et al., 2005) has been clearly documented in preclinical models. Thus, peripheral injection of the Kir3 channel blocker tertiapin Q into the masseter muscle of capsaicin-treated rats prevented both analgesic effects of a peripheral DPDPE injection as well as the suppressive effects of this DOPr agonist on trigeminal nucleus activation (Chung et al., 2014). Moreover, immunocytochemistry analysis revealed that the majority of Kir3.1 and Kir3.2 subunits were present in nonpeptidergic small size afferents (Chung et al., 2014), which also express DOPr (Bardoni et al., 2014). The postsynaptic distribution of Kir3.1/3.2 subunits in lamina IIo of the spinal dorsal horn (Marker et al., 2005) is similarly consistent with DOPr expression, as visualized by autoradiography (Pradhan and Clarke, 2005) and in DOPr-GFP–expressing mice (Scherrer et al., 2009). In keeping with this distribution, analgesia by intrathecal administration of deltorphin II was reduced in tertiapin Q–treated mice and in mice lacking either Kir3.1 or Kir3.2 subunits (Marker et al., 2005).

DOPrs also activate Kir3 currents at sites of supraspinal nociceptive modulation, although the actual contribution of these signals to analgesia may vary across structures and, as for peripheral afferents, their contribution does not usually become evident without previous exposure to stimuli that target DOPr to the membrane (Hack et al., 2005; Morgan et al., 2009; Zhang and Pan, 2010, 2012) (see also section V). Thus, administration of DOPr agonists into the rostroventromedial medulla of morphine-treated animals produced inhibitory Kir3 currents in local GABAergic neurons (Marinelli et al., 2005; Pedersen et al., 2011). The disinhibition of antinociceptive descending neurons that results from reduced local GABAergic tone may promote analgesia (Thorat and Hammond, 1997). Approximately 25% of PAG neurons also display DOPr-induced Kir3 currents (Vaughan et al., 2003) but, unlike those in the rostroventromedial medulla, morphine preexposure does not increase the proportion of PAG neurons with DOPr responses. Moreover, intra-PAG administration of DOPr agonists does not produce analgesic effects but rather behavioral immobility (Morgan et al., 2009). The latter was associated with enhanced DOPr-dependent Kir3 currents on GABAergic afferents projecting to the PAG from the central amygdala (Chieng and Christie, 2009), suggesting that DOPr modulation of Kir3 channels may actually contribute to conditioned fear responses orchestrated by the amygdala and executed through the PAG.

DOPr modulation of supraspinal Kir3 channels has also been associated with postsynaptic K^+ currents in parvalbumin-positive GABAergic interneurons in the hippocampus (Svoboda and Lupica, 1998; Svoboda et al., 1999; Rezai et al., 2013). In particular, DOPrs activate Kir3 channels and suppress hyperpolarization-activated cation currents (I_h), thus silencing GABA interneurons and enhancing pyramidal/granule cell excitability through disinhibition (Svoboda and Lupica, 1998; Drake et al., 2007). DOPrs located on pyramidal neurons may also directly enhance their activity by inhibiting the depolarization-limiting M current (Moore et al., 1994), although this effect is mediated not via Kir3 channels but most likely via phosphatidylinositol biphosphate consumption upon DOPr activation. The overall increase in hippocampal excitability that results from these different mechanisms may support long-term potentiation (Martinez et al., 2011) and favor learning/memory tasks modulated by endogenous opioids (Le Merrer et al., 2013). At the same time, they may also underlie the proconvulsant effects of exogenous DOPr agonists (Broom et al., 2002; Jutkiewicz et al., 2005, 2006).

Importantly, the potential to generate seizures is not the same for all DOPr agonists. This potential is associated with SNC80 (Broom et al., 2002; Jutkiewicz et al., 2005, 2006), BW373U86 (Jutkiewicz et al., 2006), and deltorphin II (De Sarro et al., 1992; Di Giannuario et al., 2001) but not by KNT-127 (Saitoh et al., 2011), ADL5859 (Le Bourdonnec et al., 2008), or ADL5747 (Le Bourdonnec et al., 2009). ADL compounds that were devoid of proconvulsive actions in preclinical models have also been tested for acute (ClinicalTrials.gov identifier NCT00993863) and chronic (ClinicalTrials.gov identifier NCT00979953) pain management in phase 2 clinical trials. Both studies have been completed and although no results were made available for acute pain outcomes, none of the compounds were more effective than placebo in patients suffering from osteoarthritic pain. These observations raise the question of whether the strategy used with ADL compounds to control proconvulsive actions may have also offset their analgesic efficacy in humans, which is a real possibility given that Kir3 channels contribute to both responses. Potential for generating seizures is an important limitation to the development of clinically useful DOPr ligands, and the fact that SNC80 fails to produce seizures in transgenic mice lacking DOPr in parvalbumin-positive GABAergic interneurons (Le Merrer et al., 2013) reasonably circumscribes the cause of this side effect to this specific neuronal population. However, we still ignore whether it is Kir3 activation and/or other signals that determine differential ligand tendency to induce seizures through these neurons. In this sense, TRV250, another DOPr analgesic devoid of proseizure activity, is thought to derive this desired profile from a signaling bias that favors G protein signaling over βarr2 recruitment (http://www.trevenainc.com/TRV250.php). Among all of the data analyzed in the previous paragraphs, βarr2-mediated signaling was reported only on one occasion (Xu et al., 2010), making it difficult to appreciate how βarr-dependent signaling may contribute to seizure activity. An alternative is that seizures might be associated with intracellular signaling such that βarr promotes these responses by facilitating DOPr internalization. In such cases, compounds with the most effective internalization could also be the ones most prone to seizures, as is the case for SNC80. The notion that analgesic properties and proconvulsing activity may be separated through drug design is supported by the fact that BU48 (N-Cyclopropylmethyl-[7alpha,8alpha,2′, 3′]-cyclohexano-1′[S]-hydroxy-6,14-endo-ethenotetrahydronororip avine), a buprenorphine-derived DOPr agonist, displays the latter but not the former (Broom et al., 2000). It is important to note that proconvulsive activity of compounds described above was not always evaluated using the same administration route; SNC80 was administered either intravenously or subcutaneously, ADL5859 and ADL5747 were given orally, deltorphin was microinjected in the dorsal hippocampus, and BU48 was administered subcutaneously. The influence of these differences is not trivial since the rate at which the drug reaches central DOPrs influences its proconvulsive activity (Jutkiewicz et al., 2005) and the former is directly influenced by the route of administration. Nonetheless, a systematic comparison of the signaling profiles of the nonanalgesic, proconvulsant ligand BU48 with that of DOPr analgesics reported to display distinct potential for inducing seizures could inform on the molecular determinants of proconvulsant activity and guide the rational development of DOPr analgesics free of these side effects.

E. δ-Opioid Receptors and Inhibition of Voltage-Dependent Cav2 Channels

Cav2 channels play a pivotal role in triggering neurotransmitter and hormone release. They are typically subject to voltage-dependent inhibition by direct binding of the $G\beta\gamma$ dimer released from heterotrimeric $G\alpha_{i/o}$ proteins to Cav2α1 channel subunits (Zamponi et al., 2015). Activation of DOPr endogenously expressed in small-cell lung carcinoma (Sher et al., 1996) and NG108-15 neuroblastoma $\times$ glioma hybrid cell lines (Morikawa et al., 1998) inhibited high-threshold voltage-dependent currents mediated by Ca^{+2} channels in a cAMP-independent but PTX-sensitive manner, and the effect was progressively attenuated at increasingly higher membrane potentials (Sher et al., 1996; Morikawa et al., 1998; Toselli et al., 1999). The use of Cav2 subtype–specific channel blockers allowed further characterization of the type of channel subject to DOPr modulation. In DRG sensory neurons, the most prominent inhibition by DOPr agonists was on N-type (Cav2.2) channels sensitive to ω-conotoxin GVIA, but additional occlusion by ω-agatoxin-IVA implied that Cav2.1 channels (P/Q) were also DOPr effectors (Acosta and López, 1999; Khasabova et al., 2004; Wu et al., 2008). The proportion of neurons in primary DRGs or trigeminal neuron cultures that were sensitive to Cav2 channel inhibition by DOPr varied between approximately 75% (Acosta and López, 1999) and none (Walwyn et al., 2005), depending on the agonist used, the recording conditions, the size of neurons, and pre-exposure to stimuli that target DOPr to the membrane (Khasabova et al., 2004; Mittal et al., 2013; Pettinger et al., 2013; Pradhan et al., 2013). Thus, in naïve animals, the highest levels of responsiveness were associated with the use of less selective ligands and the absence of tetraethylammonium in the bath, which was shown to mask the effect DOPr agonists on N-type channels (Wu et al., 2008). Concerning neuronal populations that display Cav2 modulation by DOPr, when responses from small versus medium-large size neurons were assessed in parallel, 30% of medium-large neurons versus $<$10% of small neurons displayed Ca^{+2} channel currents responsive to DOPr agonists (Acosta and López, 1999; Wu et al., 2008; Pradhan et al., 2013). Independent of these

considerations, responsiveness to DOPr agonists was enhanced in neurons obtained from animals with inflammatory pain (Pradhan et al., 2013) or neurons exposed to inflammatory mediators (Pettinger et al., 2013). Furthermore, only animals with an inflammatory response displayed analgesic responses to SNC80 injection (Pradhan et al., 2013); since the drug was administered systemically, it is difficult to ascertain the specific contribution of Cav2 channel modulation to this analgesic response. On the other hand, studies using intrathecal administration of SNC80 or deltorphin II indicated reduced substance P release in a formalin pain model but not in acute thermal escape (Beaudry et al., 2011; Kouchek et al., 2013), supporting the overall idea that inflammation potentiates DOPr inhibitory actions on sensory neurons.

VII. Regulation of δ-Opioid Receptor Signaling

A. δ-Opioid Receptor Phosphorylation

1. δ-Opioid Receptor Phosphorylation by G Protein–Coupled Receptor Kinases. Phosphorylation is usually considered the first step in ligand-dependent regulation of the receptor. In the case of DOPr, phosphorylation by G protein–coupled receptor kinases (GRKs) occurs with an approximate terminal half-life ($t_{1/2}$) of 1.5 minutes, reaching its maximum within 10 minutes, and persisting for as long as 1 hour with little or no degradation of the receptor (Pei et al., 1995; Zhao et al., 1997; Law et al., 2000; Tsao and von Zastrow, 2000; Navratilova et al., 2007). In contrast with desensitization, the rapid kinetics of phosphorylation are not influenced by receptor expression levels (Law et al., 2000). Truncation (Zhao et al., 1997; Murray et al., 1998) and mutagenesis studies (Guo et al., 2000; Kouhen et al., 2000; Law et al., 2000) have localized major phosphorylation sites to the DOPr C-terminal tail, since its removal abolished phosphorylation by endogenous or overexpressed GRK2 and GRK5 (Zhao et al., 1997). Of the seven Ser/Thr residues present in this region (Fig. 2), Thr358 and Ser363 were identified as actual GRK2 substrates (Guo et al., 2000; Zhang et al., 2008), the latter being the primary phosphorylation site (Kouhen et al., 2000). Thr361, on the other hand, functions as a site for kinase recognition but its presence is also needed for the adequate phosphorylation of the other two residues (Guo et al., 2000). Glu355 and Asp364 are also kinase interaction sites, their presence being necessary for optimal GRK2/GRK3/GRK5 recruitment and subsequent phosphorylation of the receptor at positions 358 and 363 (Li et al., 2003; Zhang et al., 2005). A ligand-induced association between DOPr and these kinases was first established by coimmunoprecipitation, which showed that DOPr-GRK interaction was time dependent and paralleled the time course of receptor phosphorylation (Li et al., 2003; Zhang et al., 2005). Thus, interaction is first revealed within the initial minute of stimulation, peaks at 3–5 minutes, and disappears after 10 minutes (Li et al., 2003), the time at which phosphorylation has reached its maximum (Pei et al., 1995; Law et al., 2000). Ligand-induced recruitment of GRK2/GRK3 to DOPr was also studied in live cells, revealing rapid colocalization between GRK2/3 and membrane DOPr (first minute). In this setting, agonist DOPr-GRK colocalization could be detected within endosomes at the end of 15-minute stimulation, suggesting that the kinase translocated with the receptor to clathrin-coated vesicles and presumably dissociated from the receptor as it continued its post-endocytic itinerary (Schulz et al., 2002). No colocalization was evidenced between DOPr and GRK6 or between MOPrs and GRK2 in this same study (Schulz et al., 2002), indicating certain specificity in kinase interaction with different opioid receptors. Translocation of GRK2 to the membrane and its physical interaction with DOPr required the presence of $G\beta\gamma$, which was recovered as a part of a coimmunoprecipitation complex together with the receptor and the kinase (Schulz et al., 2002; Li et al., 2003). By contrast, activity of the G protein did not appear an absolute requirement for DOPr phosphorylation since PTX did not influence the levels of pSer363 detected after exposure to different agonists (Bradbury et al., 2009). This observation could indicate that $G\beta\gamma$-independent GRKs (GRK5/6) may take over when those that rely on the dimer for membrane recruitment are not functional or are poorly expressed. Alternatively, GRK2/3 could be recruited in the proximity of the receptor even if heterotrimeric G proteins are inactive, an option that seems less plausible since several GRK2 binding residues on $G\beta\gamma$ are the same as those that interact with $G\alpha_{GDP}$ in the inactive heterotrimer (Ford et al., 1998).

2. Phosphorylation by Kinases Other than G Protein–Coupled Receptor Kinases. There is no evidence that agonist-dependent DOPr phosphorylation involves second-messenger–regulated kinases, but DOPrs undergo Src-mediated phosphorylation of Tyr residues upon activation by agonists. Thus, activation by DPDPE, DTLET, and etorphine, but not by morphine or TICP (Kramer et al., 2000a; Audet et al., 2005; Hong et al., 2009), increased pTyr levels within DOPr precipitates. Tyr phosphorylation was as fast ($t_{1/2}$ = 2 minutes) as that of Ser/Thr and reached its maximum within 10 minutes (Kramer et al., 2000a; Audet et al., 2005). Because TICP and DPDPE produced similar levels of Src activation but only DPDPE induced Tyr phosphorylation of the receptor, it was suggested that these ligands could stabilize DOPr into conformations that are distinctively recognized by the kinase (Audet et al., 2005). The fact that DPDPE and TICP stabilize different DOPr conformations was later corroborated in fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) assays (Audet et al., 2008), as detailed in section VIII.

C.1. DOPrs contain several cytosolic residues that may be targeted by tyrosine kinases. These are located in the first and second ICLs, as well as the NP*XX*Y motif at the cytosolic end of TMH7. Substitution of the latter by Phe (Y318F) reduced ligand-induced phosphorylation of the receptor by almost 50% (Kramer et al., 2000b). Importantly, it is worth noting that although this residue may be a critical Src substrate, pTyr-mediated mechanisms beyond the receptor may also contribute to the regulation of DOPr signaling. One of such mechanisms involves GRK2, which is activated after phosphorylation by Src (Sarnago et al., 1999; Fan et al., 2001). In particular, DOPr activation by DPDPE, but not TIPP or morphine, increased the overall pTyr content present in GRK2, resulting in the phosphorylation of DOPr at Ser363. Both Tyr phosphorylation of GRK2 and DOPr phosphorylation by this kinase were blocked by Src inhibitors (Hong et al., 2009), indicating tandem regulation of DOPr by Tyr phosphorylation of GRK2 followed by Ser/Thr phosphorylation of the receptor.

Activation of PKC by phorbol esters, mobilization of intracellular Ca^{+2}, or activation of Gαq-coupled receptors induces ^{32}P incorporation by DOPr (Pei et al., 1995; Xiang et al., 2001). This phosphorylation is also quick and reaches levels of ^{32}P incorporation within 5 minutes that are similar to those induced by direct DOPr activation. Joint or successive stimulation of PKC and DOPr does not induce ^{32}P incorporation beyond that produced by each stimulus alone (Xiang et al., 2001), and DOPr mutants mimicking GRK-mediated phosphorylation do not undergo as much PKC-dependent phosphorylation as wild types, suggesting that phosphorylation by GRKs may put a "brake" on phosphate incorporation by PKC (Xiang et al., 2001). Mutagenesis studies have identified Ser344 as the site of PKC-mediated ^{32}P incorporation, with minimal or no contribution by other C-terminal Ser/Thr residues (Xiang et al., 2001).

Finally, DOPrs are also substrates for Cdk5 (Xie et al., 2009; Chen et al., 2012). Cdk5 is a proline-directed serine/threonine kinase that requires proline in the +1 position and displays a preference for substrates bearing a basic residue in the +3 position relative to the substrate residue (Songyang et al., 1996). The only DOPr residue with these characteristics is Thr161, located in the second ICL. This residue accounts for a considerable portion of basal DOPr phosphorylation. Its substitution by Ala or the inhibition of Cdk5 reduces basal DOPr phosphorylation status both in immortalized cell lines and in DRG neurons (Xie et al., 2009). As detailed in section V.A, in its phosphorylated state, Thr161 promoted DOPr expression at the membrane and its heterodimerization with MOPrs (Xie et al., 2009; Chen et al., 2012).

B. δ-Opioid Receptor–β-Arrestin Interaction

DOPr activation (Whistler et al., 2001; Zhang et al., 2005; Qiu et al., 2007; Molinari et al., 2010; Audet et al., 2012) or its phosphorylation by PKC (Xiang et al., 2001) induces recruitment of βarr1/βarr2 to the receptor, as assessed by colocalization studies and direct protein-protein interaction assays. In vitro GST pull-down studies have mapped βarr1/2 interaction sites to ICL3 (Leu235-Ile259) and to the C-terminal end (Gln331-Ala372) of the receptor (Cen et al., 2001b), whereas surface plasmon resonance studies have further indicated that both regions of the receptor bind to non-superimposing sites on βarr1 (Cen et al., 2001a). The use of truncation mutants in coimmunoprecipitation assays has also validated a role for both regions in βarr1 binding. In the case of the C-terminal tail in particular, comparison of the wild-type DOPr with truncated mutants lacking the last 15 residues (Δ15) showed that the amount of βarr1 recovered with the truncated receptor was reduced but not abolished (Cen et al., 2001a), suggesting that βarr1 may interact with DOPr in the absence of C-terminal tail phosphorylation. This possibility was then corroborated in studies showing that receptors in which either all C-terminal Ser/Thr residues (Qiu et al., 2007) or in mutants in which specific GRK2 phosphorylation sites (Thr358, Thr363) and its interaction (Th361) sites (Zhang et al., 2005, 2008) were substituted by Ala still maintained their capacity to recruit βarr. Full-length DOPrs in which C-terminal Ser/Thr residues were substituted by Ala were also used to assess the influence of C-terminal tail phosphorylation on the recruitment of βarr, showing that the substitution abolished DOPr-βarr2 colocalization at the membrane (Whistler et al., 2001). This observation was interpreted as an indication that the nonphosphorylated C-terminal tail of DOPr could function as a "brake," antagonizing βarr recruitment and its associated functions (Whistler et al., 2001). More recent studies with similar Ala substitution mutants have shown that βarr2 may interact with DOPr in a phosphorylation-independent manner (Zhang et al., 2005; Qiu et al., 2007). The major difference between studies that unveiled βarr2 recruitment to the non-phosphorylated C terminus (Zhang et al., 2005; Qiu et al., 2007) and the one that did not (Whistler et al., 2001) was the way in which recruitment was assessed, using physical interaction readouts (coimmunoprecipitation or BRET) in the former and βarr/DOPr colocalization in the latter. When recruitment of βarr1/2 to wild-type DOPr and DOPr lacking all C-terminal Ser/Thr residues was compared side by side, it was determined that although phosphorylation is not essential for recruitment and it specifically enhances DOPr interaction with βarr2 (Qiu et al., 2007). The same study also showed that both βarrs similarly contributed to the internalization of wild-type DOPr and mutants, whereas βarr2 was primarily responsible for desensitization of wild-type DOPr (Qiu et al., 2007). By contrast, another study that compared βarr1/2 contribution to the internalization of DOPr specifically lacking Thr358/

Th361/Thr363 concluded that only βarr2 mediates internalization of the receptor without these phosphorylation sites (Zhang et al., 2005).

βarr1/2 association with phosphorylated and nonphosphorylated DOPr C-terminal tails has been correlated with distinct postendocytic itineraries; DOPrs lacking these phosphorylation sites were preferentially targeted for degradation by βarr2, whereas wild-type receptors were partly recycled to the membrane via interaction with either βarr (Zhang et al., 2008).

Several studies have shown that different ligands differ considerably in their ability to recruit βarr to DOPr (Keith et al., 1996; Molinari et al., 2010; Audet et al., 2012; Charfi et al., 2014). The factors contributing to these differences are multiple. To determine the relationship that exists between βarr recruitment and a ligand's ability to signal, Molinari et al. (2010) systematically compared intrinsic activities of DOPr agonists to induce both types of responses. Although the authors did not find a direct linear correlation between E_{max} values for G protein activation and βarr2 recruitment, they observed that these responses were not randomly independent (i.e., no ligand displayed greater intrinsic efficacy for βarr recruitment than for G protein activation, and partial G protein responses were always associated with reduced or no βarr recruitment) (Molinari et al., 2010). As discussed in section VIII.A, a possible interpretation for these observations is the existence of system bias determined by the fact that G proteins are readily available for DOPr interaction at the membrane, whereas βarrs are recruited from the cytosol. Alternatively, the distinct pattern of intrinsic efficacies for G protein activation and βarr recruitment may also indicate that structural requirements for DOPr–βarr association are more stringent than those required for G protein activation (Molinari et al., 2010). This interpretation is consistent with the fact that βarr interacts with GPCRs via successive steps that involve the recognition of phosphorylation sites on the receptor and the insertion of βarr's finger loop within the receptor core (see details below) (Shukla et al., 2013, 2014; Szczepek et al., 2014). It is also noteworthy that, in the case of GRK2/GRK3, poor phosphorylation of the receptor may be a direct consequence of low signaling efficacy since the ligand's ability to recruit (Li et al., 2003) and activate (Hong et al., 2009) this type of kinase is linked to G protein activation. Therefore, reduced capacity of partial agonists to activate the G protein may underlie low receptor phosphorylation (Kramer et al., 2000a; Audet et al., 2005; Navratilova et al., 2005; Hong et al., 2009) and poor βarr recruitment, at least in certain immortalized cell lines (Charfi et al., 2014). It is also worth noting that prolonged ERK1/2 stimulation by partial but not by highly efficacious agonists (Eisinger et al., 2002; Eisinger and Schulz, 2004; Audet et al., 2005) may additionally interfere with βarr recruitment. This is particularly true for βarr1, whose phosphorylation by MAPK results in its inactivation (Lin et al., 1997; Eisinger et al., 2002). The imbalance displayed by certain ligands in promoting signaling and βarr recruitment has also been attributed to the stabilization of ligand-specific states for opioid receptors (Martini and Whistler, 2007; Raehal et al., 2011). Kinetic studies of DOPr-βarr interaction have helped address this issue. The fact that full and partial agonists recruit βarr with a similar $t_{1/2}$ (Molinari et al., 2010) would argue against ligand-specific conformations. On the other hand, the observation that the time course of DOPr-βarr2 dissociation may differ among agonists (Audet et al., 2012) supports the notion that ligand-specific conformations of the receptor distinctively interact with βarrs. For example, DOPr-βarr2 dissociation follows different kinetics depending on whether the receptor had been stimulated by DPDPE or SNC80, a difference that could not be simply attributed to the ligand dissociating from the receptor at different rates (Audet et al., 2012). In this case, different βarr2 dissociation profiles correlated with divergent positioning of the receptor C terminus with respect to the Gβγ dimer, in which SNC80, the ligand that induced the more stable interaction between DOPr and βarr2, also promoted βarr2 association to Gβγ locking all interaction partners within a stable complex (Audet et al., 2012). Recent studies indicated that stable interaction between βarrs and opioid receptors may lead to the sensitization of TRPV1 signaling (Rowan et al., 2014a). The fact that this effect was associated with the administration of SNC80 but not ARM100390 (Rowan et al., 2014b) was interpreted as an indication that SNC80-occupied DOPrs could release TRPV1 signaling by acting as a scavengers for βarrs that are otherwise constitutively associated with the channel.

C. δ-Opioid Receptor Desensitization

1. δ-Opioid Receptor Desensitization by G Protein–Coupled Receptor Kinases. Phosphorylation by GRKs rapidly attenuates responsiveness of agonist-occupied receptors. Kinetic (Pei et al., 1995; Kouhen et al., 2000; Law et al., 2000; Lowe et al., 2002; Navratilova et al., 2007) and mutagenesis studies (Kovoor et al., 1997; Wang et al., 1998; Kouhen et al., 2000; Law et al., 2000; Lowe et al., 2002; Navratilova et al., 2007) have addressed the extent to which DOPr phosphorylation may contribute to its functional desensitization. The rationale guiding these studies was the reasoning that if desensitization is a direct consequence of DOPr phosphorylation, then both should occur within a similar time frame. Consistent with this notion, AC and Ca^{+2} channel responses induced by endogenously expressed DOPr (Cai et al., 1996; Morikawa et al., 1998; Willets and Kelly, 2001) displayed desensitization within the first 3–5 minutes of receptor activation, which is the time course of phosphorylation. Similar observations were obtained for Kir3-channel currents recorded from

oocytes in which the receptor was cotransfected with GRK2 and βarr2 (Kovoor et al., 1997; Lowe et al., 2002). On the other hand, if receptors were expressed at very high levels, if concentrations of the desensitizing agonist were submaximal, or if partial agonists were used, desensitization occurred at a much slower rate (Bot et al., 1997; Kouhen et al., 2000; Law et al., 2000; Marie et al., 2003a; Qiu et al., 2007), with half-lives more than 10-fold slower than those of phosphorylation (Navratilova et al., 2007). The fact that DOPr phosphorylation contributes to its rapid desensitization is also supported by reports showing that truncation of the receptor or Ala substitution of all C-terminal Ser/Thr residues reduced the rate and maximal desensitization of Kir3 (Kovoor et al., 1997) and AC responses (Qiu et al., 2007). Similar modifications of AC desensitization were observed when Ser363 was substituted by Ala, pointing to the crucial role of this residue in the rapid modulation of DOPr responsiveness to agonists such as deltorphin II (Kouhen et al., 2000; Navratilova et al., 2007) and DPDPE (Law et al., 2000). This residue was also phosphorylated in vivo in the brain and spinal cord of mice that had been treated with two consecutive injections of systemic SNC80 but not ARM100390 (Pradhan et al., 2009). The fact that ARM100390 also failed to produce rapid desensitization of signaling and tolerance points to the importance of Ser363 in the regulation of DOPr responses in vivo. Admittedly, the fact that SNC80 was administered intraperitoneally and ARM100390 was given orally makes it difficult to directly compare the effects of the two compounds. Indeed, when the C-terminal mutations mentioned just above were assessed in heterologous systems, desensitization was reduced but not abolished, suggesting that phosphorylation of the receptor C terminus is but one of the mechanisms underlying desensitization. In this sense, it is worth noting that a DOPr mutant in which all of the Ser residues in the third cytoplasmic loop were mutated to Ala desensitized at the same rate as wild-type DOPr, arguing against an essential contribution of this domain to functional regulation (Kovoor et al., 1997). At the same time, the presence of residual desensitization in C-terminal Ser/Thr mutants is consistent with the observation that both βarr1 and βarr2 can be recruited to receptors with no C-tail phosphorylation to induce their desensitization (Cheng et al., 1998; Zhang et al., 2005; Qiu et al., 2007). Finally, overexpression or silencing of GRK2/3 (Pei et al., 1995; Lowe et al., 2002) and GRK5/6 (Willets and Kelly, 2001) have identified both families of kinases as mediators of phosphorylation-dependent desensitization of DOPr.

Structural studies characterizing interactions of activated/phosphorylated GPCRs with βarr1 (Shukla et al., 2014) or with the finger loop of visual βarr (Szczepek et al., 2014) have provided insight as to how receptor phosphorylation and βarr recruitment may result in functional uncoupling of the receptor from the Gα subunit. In keeping with the classic multistep model of βarr activation (Gurevich and Benovic, 1993; Gurevich and Gurevich, 2014), structural studies indicate that βarrs engage the phosphorylated receptor in two steps. The first encompasses the association of the phosphorylated C terminus of the receptor to the N-terminal domain of βarr (Shukla et al., 2013, 2014) and the second involves the insertion of the finger loop of βarr within the receptor core, particularly the space between TM3, TM5, and TM6 (Shukla et al., 2014; Szczepek et al., 2014). Since this space also lodges the C-terminal end of activated Gα (Chung et al., 2011; Rasmussen et al., 2011), βarr would preclude classic GPCR signaling and induce desensitization by replacing the Gα C terminus with its finger loop. Conformational changes that result from its successive interaction steps with the receptor also modify N- and C-terminal domains of βarr (Zhuang et al., 2013), increasing its affinity for clathrin and AP-2, two main components of the endocytic machinery (Kang et al., 2014). Because recruitment to the receptor and conformational changes that allow βarr interaction with the endocytic machinery are concomitant, a great majority of ligands that promote functional desensitization also enhance receptor sequestration, and the frequent association of the two processes initially led to the notion that opioid receptor internalization and desensitization were causally linked (Whistler et al., 1999; Alvarez et al., 2002).

A more parsimonious interpretation is that the relationship between the two phenomena is complex. Two types of observations allow us to categorically conclude that internalization is not required for DOPr desensitization to take place: 1) sustained exposure (30 minutes to 3 hours) to ligands such as ARM100390 (Marie et al., 2003a; Pradhan et al., 2010) or TIPP (Hong et al., 2009) induces desensitization in systems in which they fail to induce internalization, and 2) replacement of Ser363 by Ala interferes with desensitization without affecting sequestration (Navratilova et al., 2007; but also see Law et al., 2000). On the other hand, as discussed in section VII.D on DOPr trafficking, the way in which internalization may influence the course of desensitization must be considered in relation to postendocytic routing and receptor targeting to recycling (Trapaidze et al., 2000; Archer-Lahlou et al., 2009; Audet et al., 2012; Gupta et al., 2014) and degradation (Tsao and von Zastrow, 2000; Whistler et al., 2002).

The idea that some receptors may continue to signal after internalization must also be considered in the context of desensitization (Mullershausen et al., 2009; Irannejad and von Zastrow, 2014; Tudashki et al., 2014). According to the traditional view, GPCRs effectively signal to G proteins only when they are at the plasma membrane and, aside from noncanonical βarr-mediated signaling (Shukla et al., 2011), internalized receptors have been classically considered inactive,

simply trafficking toward degradation or recycling (Hanyaloglu and von Zastrow, 2008). More recently, spectroscopic assays that allow real-time monitoring of GPCR signaling revealed that cAMP modulation by Gαs and Gαi/o-coupled receptors may take place after internalization has been completed (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009; Irannejad et al., 2013), questioning sequestration as a mechanism of signal termination at least for enzymatic effectors. For example, by monitoring the kinetics of internalization and of cAMP inhibition by DOPr ligands, Tudashki et al. (2014) showed that although sequestration may contribute to the initial rapid decay of acute cAMP responses, endocytosis does not control the magnitude or duration of cAMP inhibition over longer stimulation periods. For example, 60-minute exposure to agonists that induce either maximal internalization or no internalization at all produced similar cAMP inhibition, an observation that could not be attributed to differences in signaling efficiency (Tudashki et al., 2014). The idea that intracellular signaling could make the duration of opiate-mediated cAMP inhibition independent of internalization may call into question the hypothesis that noninternalizing ligands such as morphine induce analgesic tolerance by producing sustained inhibition of AC function (Martini and Whistler, 2007) and raises the possibility that analgesic tolerance could rather be related to distinct substrates being modulated by membrane and intracellular AC signaling.

2. δ-Opioid Receptor Desensitization by Kinases Other than G Protein–Coupled Receptor Kinases. Unlike GRKs, which recognize and phosphorylate agonist-occupied receptors, kinases controlled by cytosolic signals (second messengers, kinase cascades) do not distinguish between active, agonist-occupied receptors and inactive ones that are ligand free. Cascades that mediate the latter regulation may be activated either by DOPr themselves (Yoon et al., 1998) or by other receptors. For example, signaling by endogenous DOPr expressed in neuroblastoma (SK-N-BE) or neuroblastoma/glioma hybrid cells (SK-N-SH) undergoes desensitization after activation of dopamine D2 receptors or α2ARs (Namir et al., 1997) as well as ligand-gated ion channels such as AMPA or NMDA (Ben et al., 1999). DOPr desensitization induced by kainate (Ben et al., 1999) or by DOPr activation itself (Yoon et al., 1998; Marie et al., 2008) may be blocked by PKC inhibitors, which is consistent with the observation that DOPr may undergo PKC-dependent phosphorylation (Xiang et al., 2001). Other kinases such as PKA and ERK1/2, which have also been implicated in desensitization of DOPr signaling, did not induce DOPr phosphorylation or modified their number nor agonist affinity (Xu et al., 2013), suggesting that regulation by these kinases most likely takes place downstream of the receptor, at the G protein or effector level.

Src is another example of a kinase participating in downstream regulation of DOPr signaling. In particular, Src activation of ubiquitin ligase casitas B-lineage lymphoma was recently shown to drive degradation of EGFRs that support DOPr-mediated activation of ERK (Eisinger and Ammer, 2009).

D. δ-Opioid Receptor Trafficking

As discussed in preceding sections, GRK-mediated phosphorylation of DOPr in the cytoplasmic tail promotes receptor engagement of βarr, driving DOPr accumulation in clathrin-coated pits and its subsequent packaging into endocytic vesicles after scission from the membrane by dynamin (Chu et al., 1997; Zhang et al., 1999a; Whistler et al., 2001). Regulated endocytosis of DOPr was proposed many years ago to mediate DOPr downregulation by initiating the delivery of receptors to lysosomes for subsequent proteolytic destruction (Law et al., 1984). This hypothesis has been strongly supported over the years, both in studies of model cell systems and in native neurons (Tsao and von Zastrow, 2000; Scherrer et al., 2006). However, lysosomal downregulation is not the only postendocytic fate of DOPrs. Rather, as outlined briefly below, the endocytic fate of DOPr can vary depending on receptor engagement with discrete sorting machineries that specifically direct the downstream fate of internalized DOPr.

One protein complex engaged by internalized DOPr is the so-called endosomal sorting complex required for transport. This complex recognizes ubiquitinated receptors and drives their physical transfer to the lumen of multivesicular bodies (MVBs), which later fuse with lysosomes (Henne et al., 2011). For some signaling receptors, such as EGFR, ubiquitin-directed transfer to the endosome lumen is essential for receptor sorting to lysosomes. Indeed, if EGFR ubiquitination is prevented, internalized EGFRs aberrantly recycle to the plasma membrane with bulk membrane flux (Raiborg and Stenmark, 2009). DOPrs are also ubiquitinated after agonist-induced activation (in the first cytoplasmic loop by atrophin-interacting protein 4) and they traverse typical MVBs (Hislop et al., 2009; Henry et al., 2011). However, in marked contrast with EGFRs, neither DOPr ubiquitination nor transfer to the MVB lumen is essential for subsequent receptor delivery to lysosomes (Tanowitz and Von Zastrow, 2002; Hislop et al., 2009).

The reason for this is that DOPrs also engage other endosome-associated sorting machinery, called the G protein–coupled receptor–associated sorting protein (GASP) complex (Whistler et al., 2002; Marley and von Zastrow, 2010; He et al., 2013a). The GASP machinery effectively retains DOPrs in the endosome limiting membrane and inhibits their entry to the recycling pathway. Accordingly, DOPr engagement of the GASP complex promotes the subsequent delivery of internalized receptors to lysosomes, irrespective of DOPr

ubiquitination or transfer to the lumen of MVBs. This effectively provides additional flexibility in the endocytic trafficking itinerary of DOPrs, allowing receptors traversing the endocytic pathway to remain in the limiting membrane for a prolonged time period compared with EGFRs (Hislop and von Zastrow, 2011).

Accordingly, DOPrs are not simply "hard wired" for exclusive delivery to lysosomes after endocytosis. From the earliest studies, a fraction of the internalized DOPr was noted to recycle rapidly, and the degree of DOPr recycling can be quite large in some cases (Trapaidze et al., 2000; Tsao and von Zastrow, 2000). Typically, recycling is favored by shorter periods of agonist exposure and individual agonists differ in the degree to which they promote the sorting of internalized receptors into the recycling pathway or to lysosomes (Marie et al., 2003b; Audet et al., 2012). Ligand-specific differences in sorting DOPr for recycling versus lysosomal degradation have been attributed to distinct protein interactions established by DOPr when stabilized by different ligands (see section VII.B) or with ligand-specific sensitivity to endothelin-converting enzymes, which enhances DOPr recycling when occupied by ligands that act as convertase substrates (Gupta et al., 2014). Furthermore, although both exogenously administered drugs and endogenous opioids drive DOPr endocytosis in vivo, receptors accumulate in different endocytic compartments (Faget et al., 2012). Thus, the postendocytic trafficking fate of DOPr appears to be flexible and subject to differential control by natural ligands and drugs, both in model cell systems and in vivo.

Several lines of evidence suggest the possibility that DOPr engagement of multiple endosomal machineries has still more physiologic significance. First, the GASP machinery is subject to control by conventional signaling proteins such as heterotrimeric G proteins (Rosciglione et al., 2014), suggesting that this machinery may afford additional integration between receptor signaling and trafficking. Second, there is evidence that GASP controls various noncanonical signaling processes, such as through binding to specific transcription factors (Abu-Helo and Simonin, 2010) or regulating autophagy (He et al., 2013a). Third, by retaining receptors in endosomes without requiring transfer to the endosome lumen, the GASP machinery may also support more conventional G protein signaling, which is increasingly thought to be initiated from the endosome-limiting membrane as well as from the plasma membrane (Calebiro et al., 2009; Ferrandon et al., 2009; Irannejad et al., 2013; Irannejad and von Zastrow, 2014).

E. δ-Opioid Receptor Regulation by Regulators of G Protein Signaling

RGSs constitute a heterogeneous family of proteins that is characterized by the presence of a homologous RGS domain of approximately 120 amino acids in length. This domain binds to Gα subunits, accelerating their GTPase activity and therefore facilitating the switch of Gα from a GTP-bound active state to a GDP-bound inactive one. Thus, by arresting G protein activity, RGS proteins are key regulators of opioid receptor signaling (Xie and Palmer, 2005; Traynor, 2012). As mentioned in section IX.C.2.b, RGS4 directly interacts with DOPr to negatively modulate receptor signaling via cAMP and ERK pathways both in heterologous (Leontiadis et al., 2009) and endogenous (Wang et al., 2009) expression systems, an effect that is potentiated by spinophilin (Fourla et al., 2012). Since RGS4 and Gα subunits bind the receptor at common residues (Georgoussi et al., 2006), it is not yet clear whether RGS4 inhibition of DOPr signaling is due to normal acceleration of GTPase activity or to its competition with the G protein for binding to the receptor. Overexpression of RGS4 was also shown to accelerate DOPr internalization (Leontiadis et al., 2009), although the underlying mechanism also remains to be characterized. Independent of the mechanisms involved, the functional in vivo relevance of this modulator in regulating DOPr-mediated responses was recently unveiled using transgenic mice in which the lack of RGS4 almost doubled the magnitude of SNC80's antidepressant-like effects (Stratinaki et al., 2013).

RGS19 (or Gα-interacting protein), which is predominantly localized to clathrin-coated pits/vesicles (De Vries et al., 1998), has also been associated with DOPr signaling. In this case, ligand-activated DOPrs and their associated Gαi3 proteins translocated to clathrin pits where Gαi3 met RGS19, which turned off DOPr signaling before internalization took place (Elenko et al., 2003).

Finally, DOPr signaling is also negatively modulated by RGS12, particularly in gastric smooth muscle cells where Gβγ-mediated signaling promotes contractility (Huang et al., 2006). In these cells, DOPr induces Gβγ-dependent stimulation of PI3K and Src activation. Active Src in turn phosphorylates Gαi1–3 subunits, promoting their recruitment of RGS12 via its phosphotyrosine binding domain. RGS12 subsequently blocks G protein signaling (Huang et al., 2014).

F. Membrane Microdomains and Regulation of δ-Opioid Receptor Signaling

In contrast with previous thought, membrane proteins do not freely partition in a "fluid mosaic" (Singer and Nicolson, 1972) but they instead organize within specialized domains of the plasma membrane (Neubig, 1994). A specific type of such domains is defined by enrichment of cholesterol and glycosphingolipids in the outer leaflet of the membrane's bilayer, and these are known as membrane rafts (Pike, 2003). Lipid-enriched rafts may or may not contain the cholesterol binding protein caveolin, whose presence defines membrane

invaginations known as caveolae (Patel et al., 2008; Head et al., 2014). Because lipid subdomains that contain caveolin or not have both been found to be enriched in GPCRs and downstream signaling partners (Toselli et al., 2001; Patel et al., 2008; Levitt et al., 2009), membrane rafts have been proposed as signaling platforms that help determine the speed, accuracy, and magnitude of GPCR response (Patel et al., 2008). However, their exact role remains to be fully elucidated because of the lack of universally accepted methods for microdomain isolation, as well as incomplete understanding of what determines the presence and the signaling capacity of GPCRs and their signaling partners in these specialized regions of the membrane across different cell types. For example, in myocytes (Patel et al., 2006) and in CHO cells (Huang et al., 2007), endogenous and overexpressed DOPrs localize to caveolae and coimmunoprecipitate with cav-3 or cav-1, respectively. On the other hand, DOPrs and caveolin in HEK cells partition separately, with DOPrs localizing within much denser fractions that also contain the transferrin receptor, a marker of clathrin-coated pits (Levitt et al., 2009). The functional consequences of DOPr localization to caveolae and of their association with caveolin are not universal either. Thus, the presence of cav-3 is required for DOPr signaling in myocytes (Patel et al., 2006; Tsutsumi et al., 2010), but overexpression of caveolin blocks DOPr inhibition of Cav2 channels in hybrid NG108-15 cells (Toselli et al., 2001) and caveolae disruption enhances Gα activation by DOPrs in CHO cells (Huang et al., 2007). Neurons, like NG108-15 cells, naturally express low to undetectable levels of caveolin. Despite of this, 70% of endogenously expressed caudate-putamen and NG108-15 DOPrs cosegregate with lipid-enriched membrane factions (Huang et al., 2007), indicating that DOPr confinement to lipid rafts does not require caveolin. Nonetheless, their presence in this specialized domain is necessary for signaling, since raft disruption by cholesterol depletion causes DOPrs to redistribute to higher-density fractions and interferes with GTPγS binding by DPDPE. The reduction in agonist response could be related to uncoupling of the receptor from the G protein, since DOPr affinity for the agonist was also reduced by loss of lipid rafts (Huang et al., 2007). The observation that 30-minute exposure to full agonists caused 25% of DOPrs to translocate to high-density fractions (Huang et al., 2007) is also consistent with the notion that outside lipid rafts DOPrs are uncoupled from the G protein, presumably in their way to clathrin-coated pits for internalization.

VIII. Signaling Bias of δ-Opioid Receptor Ligands

A. Conceptualization of Biased Signaling

The term *biased agonism* or *ligand bias* describes the ability of a drug to distinctively engage one signaling path over another (Urban et al., 2007). The distinct ability with which each ligand may engage different downstream signals is determined by the unique set of interactions that each ligand-receptor complex establishes with the effectors of these signals (Kenakin and Miller, 2010; Onaran and Costa, 2012; Onaran et al., 2014). Thus, ligand bias is part of the process of receptor activation and should be distinguished from system bias (Kenakin et al., 2012; Onaran and Costa, 2012; Kenakin and Christopoulos, 2013), which is determined by postactivation steps. Postactivation steps introduce differences among the responses controlled by different signaling/regulatory partners because these partners do not all translate the pharmacological stimulus imparted by the active ligand-receptor complex in the same way. Unlike biased agonism that is ligand specific, system bias systematically affects responses generated by all ligands. For example, Molinari et al. (2010) reported that a great majority of DOPr ligands systematically displayed greater relative intrinsic efficacies (E_{max}) in G protein activation than βarr recruitment assays. The need for βarr to diffuse from the cytosol to the membrane was identified as a possible factor that could systematically reduce the efficiency of this response compared with G proteins whose activation takes place directly at the membrane. Systematic confounders must be taken into account when interpreting experimental data since they may result in biased responses without there being any ligand-dependent bias.

B. Recognizing Ligand Bias from Experimental Data

1. Single Concentration Assays. The most frequently used experimental approach to identify biased agonism of opioid ligands has been the comparison of responses induced by a single, maximally effective concentration of different agonists (Alvarez et al., 2002; Pradhan et al., 2010; Raehal et al., 2011; Charfi et al., 2014). In this setting, the disproportion of maximal responses for different outcomes (e.g., high signaling E_{max} versus minimal internalization capacity) has been frequently interpreted as bias. However, as explained in the preceding section, systematic disproportion among maximal effects does not allow us to unambiguously conclude whether the imbalance is attributable to the ligand itself or to differences in the efficiency with which the activated receptor engages the postactivation steps supporting the response. Actually, the reversal in their rank order is the only criterion that allows us to unequivocally draw conclusions on the existence of ligand bias from maximal responses (Kenakin, 2007). This type of behavior cannot be simply explained by scalar system factors such as amplification and was one of the initial indications that GPCRs could adopt ligand-specific conformations (Meller et al., 1992; Spengler et al., 1993; Berg et al., 1998). Ligand-specific conformations are the molecular substrate of biased agonism

(Kenakin, 1994; Leff, 1996; Urban et al., 2007; Onaran and Costa, 2012) and their existence in the case of DOPr has been evidenced by unveiling the unique interactions established between the receptor activated by different ligands and its downstream signaling partners. For example, plasmon wave resonance assays revealed that each agonist-DOPr complex displays distinct affinities for different Gαi/o subunits (Alves et al., 2003, 2004). Similarly, BRET assays showed that the conformational rearrangements that take place at the interface of DOPr and heterotrimeric Gαi1β1γ2 subunits are not the same for all agonists (Audet et al., 2008, 2012).

2. Estimating Bias from Dose-Response Curves. An alternative way to recognize and measure ligand signaling bias is to estimate the efficiency with which it evokes each response of interest and then determine whether these efficiencies significantly differ from one another. The operational model developed by Black and Leff (1983) has been proposed as an analytical tool to estimate such a parameter in the form of the transduction coefficient (log)τ/K_A, where τ is the ligand's efficacy and K_A is its "functional affinity," corresponding to the theoretical affinity the ligand would have for the active state(s) of the receptor responsible for the response (Black and Leff, 1983; Rajagopal et al., 2011; Kenakin and Christopoulos, 2013; Onaran et al., 2014). A detailed account of the steps involved in obtaining these parameters from experimental data has been presented elsewhere, both from a general theoretical viewpoint (Kenakin et al., 2012; Kenakin, 2015) and within the context of DOPr signaling (Charfi et al., 2014, 2015; Nagi and Pineyro, 2016). Here, we will mention two aspects in relation to the quality of information provided by quantitative approaches compared with identification of ligand bias through comparison of single maximal responses. First, in quantitative methods as they have been published, there is a "built-in procedure" to systematically "dissect away" system confounders, which consists of normalizing the response produced by each ligand to a common standard. Since standards and ligands are similarly affected by the same system confounders, reference-weighed responses cancel out system bias. Second, dose-response curves provide much more information about a drug than a single reading at maximal concentration. Indeed, quantitative analysis of internalization and AC responses by DOPr agonists clearly revealed that simply relying on E_{max} values may be misleading in the identification of biased ligands (Charfi et al., 2014). Thus, despite producing similar DOPr internalization at maximal concentrations, internalization efficiency (τ/K_A) for SNC80 was 20-fold lower than that of DPDPE. This was because DPDPE's dose-response curve was considerably left-shifted with respect to that of SNC80, information that was available via the estimation of the K_A parameter (Charfi et al., 2014). Since SNC80 and DPDPE displayed similar efficiency in cAMP accumulation assays, it was concluded that SNC80 was biased toward cAMP inhibition rather than internalization, compared with DPDPE (Charfi et al., 2014).

C. Distinguishing Ligand Bias from Biased Responses of δ-Opioid Receptor Agonists

Considerable evidence indicates that DOPr activation by different ligands may result in biased responses, but an important question that must be addressed in each case is the extent to which these observations can be alternatively determined by ligand or system-dependent factors. As stated just above, bias quantification and/or reversal in the rank order of responses or evidence of ligand-specific conformations are valid criteria to distinguish between the two types of contributions.

1. Recognizing Ligand-Specific Signaling by δ-Opioid Receptors. DOPrs behave as pleiotropic receptors capable of stimulating different G proteins (Allouche et al., 1999; Alves et al., 2004; Piñeyro and Archer-Lahlou, 2007) and there is considerable evidence indicating their ligand-specific modulation. For example, studies in SK-N-BE neuroblastoma cells that endogenously express DOPr have shown that the rank order of maximal GTPγS binding across different Gα subtypes is not conserved for all ligands. In particular, a maximally effective concentration of deltorpin I induced activation of $G\alpha_o$ and $G\alpha_{i2}$ but not of $G\alpha_{i1/3}$ or $G\alpha_{PTXinsensitive}$ ($G\alpha o \geq G\alpha_{i2} >>> G\alpha_{i1/3}$ and $G\alpha_{PTXinsensitive}$); DPDPE activated $G\alpha_{i2,}$ $G\alpha_o$, and $G\alpha_{PTXinsensitive}$ without modifying GTP uptake by $G\alpha i_{1/3}$ ($G\alpha_{i2} > G\alpha_o \geq G\alpha_{PTXinsensitive} >>> G\alpha_{i1/3}$), whereas etorphine activated $G\alpha_{i2,}$ $G\alpha_{PTXinsensitive}$, and $G\alpha i_{1/3}$, leaving $G\alpha_o$ unchanged ($G\alpha_{i2} = G\alpha_{PTXinsensitive} > G\alpha i_{1/3} >>> G\alpha_o$) (Allouche et al., 1999). Since the SK-N-BE neuroblastoma cell line used in this study only expressed DOPr (Polastron et al., 1994), the distinct profile of Gα activation by any of these ligands cannot be attributed to concomitant activation of MOPrs, therefore demonstrating that ligand-specific DOPr responses may be observed at endogenous levels of expression. The idea that DOPr agonists distinctively modulate G protein activation is also supported by BRET data indicating that conformational rearrangements that take place at the receptor-Gα interface and among G$\alpha\beta\gamma$ subunits is ligand specific and associated with distinct downstream responses. For example, DOPr activation by SNC80, DPDPE, and morphine reduced the distance between position 60 of the $G\alpha_{i1}$ subunit and the N terminus of Gγ but the same regions were drawn apart by TICP[Ψ] (Audet et al., 2008). Although the former ligands inhibited cAMP production and promoted ERK activation, TICP[Ψ] behaved as an inverse agonist enhancing cAMP production but as an agonist in the MAPK cascade (Audet et al., 2005, 2008).

Mechanisms involved in ERK activation by DPDPE and TIPP, an analog of TICP[Ψ] (see section IV), also

differed. Although DPDPE promoted Gβγ-PLCβ3-cSrc association into a complex that led to MAPK activation via Raf-1, stimulation by TIPP relied on βarr1/2 (Xu et al., 2010). The authors interpreted these differences as evidence that DPDPE and TIPP displayed bias in their ability to engage the two pathways leading to ERK activation. However, some caution is warranted because no evidence is provided that differential Ser363 phosphorylation by the two ligands is ligand specific and is not simply related to magnitude of response. Mutating Ser for Ala at this position abolished phosphorylation of the receptor by DPDPE, precluding its ability to engage the PLCβ3/Src/Raf1 pathway and causing it to shift to the βarr1/2 pathway (Hong et al., 2009; Xu et al., 2010). Thus, given that pSer363 was necessary to engage the Gβγ/PLCβ3/cSrc/Raf-1 cascade and since the weakest agonist failed to induce its phosphorylation, we cannot exclude that differences between TIPP and DPDPE are efficacy related. A cellular background with a different complement of kinases could perhaps allow TIPP to induce Ser363 phosphorylation and engage the same pathway as DPDPE. For example, GRK2 was involved in DOPr internalization by morphine and TIPP in neurons but not in HEK293 cells (Charfi et al., 2014), indicating that βarr responses dependent on phosphorylation may indeed be influenced by cellular background.

2. Recognizing Ligand-Specific Regulation of δ-Opioid Receptors. DOPrs undergo different levels of phosphorylation depending on the ligand activating the receptor. Ser363 is phosphorylated by maximally effective concentrations of agonists such as DPDPE (Guo et al., 2000; Kouhen et al., 2000), SNC80 (Pradhan et al., 2009), (+)-BW373U86 (Bradbury et al., 2009), and deltorphin II (Marie et al., 2008). On the other hand, overall P^{32} incorporation by morphine was much less than that produced by deltorphin II (Navratilova et al., 2005), whereas TIPP or TAN-67 produced no visible change in pSer363 levels (Bradbury et al., 2009; Xu et al., 2010). These quantitative differences do not allow us to unequivocally conclude whether they are attributable to ligand-specific conformations or are simply determined by ligand efficacy. Indeed, since TIPP, morphine, and TAN-67 behave as partial agonists in G protein activation (Quock et al., 1997; Tudashki et al., 2014), their reduced capacity to phosphorylate the receptor is most likely due to the fact that as partial agonists, these drugs induce a marginal increase in Ser363 phosphorylation. Unlike these quantitative differences, mutagenesis studies have clearly established that different agonists may phosphorylate DOPrs at different intracellular domains, implying that receptors occupied by different ligands expose different phosphorylation sites to regulatory kinases (Varga et al., 2004; Liggett, 2011; Just et al., 2013). Thus, DPDPE and SNC80, which were shown to stabilize distinct DOPr conformations over the time course of desensitization (Audet et al., 2012), differed in their ability to phosphorylate Ser/Thr residues outside of the receptor's C tail, with SNC80 being the only one capable of phosphorylating the truncated DOPr mutant (Okura et al., 2003). Although the region and residues involved in the differential response to DPDPE and SNC80 remain to be determined, the third ICL could be a likely candidate, since it contains Ser/Thr residues that contribute to βarr binding (Cen et al., 2001a,b), a response that is also distinctively engaged by the different conformations stabilized by DPDPE and SNC80 (Audet et al., 2012).

Maximal internalization by DOPr agonists has been compared with maximal G protein or AC responses in heterologous expression systems (Bradbury et al., 2009; Charfi et al., 2014), cultured neurons (Charfi et al., 2014), and in vivo brain samples (Pradhan et al., 2009). All of these studies show that at least one of the following ligands displays minimal internalization with maximal or near maximal signaling: ARM100390, morphine, TIPP, mcpTIPP [H-mcp-Tic-Phe-Phe-OH; mcp is 4′-(N-methylcarboxamido)phenylalanine], and SB-235863. The observed imbalance has been repeatedly interpreted as ligand bias, but the disproportion could simply be due to different amplification of signaling and internalization responses. In this sense, it is worth noting that morphine, TIPP, mcpTIPP [H-mcp-Tic-Phe-Phe-OH; mcp is 4′-(methyl-carboxamido)phenylalanine], and SB-235863 have been tested in both cAMP and G protein activation assays, revealing full second-messenger inhibition but partial G protein stimulation (Charfi et al., 2014; Tudashki et al., 2014). The fact that the least amplified response reveals partial agonism for poorly internalizing ligands is consistent with the interpretation that low internalization profiles are not ligand specific but are efficacy related. This interpretation is supported by the observation that when assessed in neuronal cultures maximal DOPr internalization by morphine and TIPP was directly correlated with partial intrinsic activities of these ligands in G protein activation assays (Charfi et al., 2014; Tudashki et al., 2014). Finally, DOPr-eGFP mice have allowed us to compare in vivo DOPr internalization by different ligands (Pradhan et al., 2009, 2010). Comparison of SNC80 to ARM100390 showed that only SNC80 induced internalization and acute analgesic tolerance, prompting the notion that internalization bias could be predictive of this type of tolerance (Pradhan et al., 2009, 2010). However, when SNC80's internalization and signaling efficiencies [$\log(\tau/K_A)$] were estimated in a controlled, in vitro experimental environment, it displayed approximately 50-fold preference toward signaling compared with DPDPE (Charfi et al., 2014). In addition, the internalization-biased ligand (DPDPE) did not produce acute tolerance when tested in vivo, whereas SNC80 did (Audet et al., 2012), suggesting that internalization bias might not be an absolute criterion predictive of this side

effect. Moreover, ARM100390's internalization capacity may be cell dependent since it induced internalization of the Flag-DOPr coexpressed with Kir3 channels in cultured neurons, reaching approximately 75% of SNC80's response (Nagi et al., 2015). There are several differences among studies reporting divergent observations for ARM100390 internalization. First, in the in vivo study, DOPr-eGFP was knocked in to attain physiologic expression (Pradhan et al., 2009), whereas Flag-DOPrs transfected onto cortical neurons were overexpressed (Nagi et al., 2015). However, overexpression would be expected to saturate the internalization machinery and reduce, not enhance, internalization capacity. One possible explanation for the lack of DOPr-eGFP internalization by ARM100390 could be that the fluorophore at the C terminus interferes with βarr function. Alternatively, the presence of the channel effector might have stabilized βarr interaction with the signaling complex, allowing more efficient internalization by ARM100390.

IX. Pharmacological δ-Opioid Receptor Subtypes

A. Evidence for δ-Opioid Receptor Subtypes

Using classic in vivo behavioral pharmacology, early reports revealed the existence of multiple DOPr subtypes. Indeed, this approach revealed that analgesia produced by intracerebroventricular administration of the selective DOPr agonists DPDPE and deltorphin II was blocked by the antagonists DALCE and NTII, respectively (Jiang et al., 1991). Similarly, NTB was found to inhibit the analgesic effects of the DOPr agonists DSLET and deltorphin II but not of DPDPE (Sofuoglu et al., 1991, 1993). By contrast, the analgesia induced by DPDPE was found to be sensitive to the naltrexone derivative BNTX, which had no effect on DSLET or deltorphin II (Portoghese et al., 1992; Sofuoglu et al., 1993) (further details on these ligands are provided in section IV). Similar results were later confirmed by others in different species using various ligands and/or routes of administration (Stewart and Hammond, 1993, 1994; Ossipov et al., 1995). Absence of analgesic crosstolerance between DPDPE and deltorphin II was also observed (Mattia et al., 1991), adding to this line of evidence. Further supporting the existence of two DOPr subtypes, the analgesia induced by DPDPE was mediated by an ATP-sensitive potassium channel, whereas deltorphin II–induced analgesia was mediated by a potassium channel insensitive to ATP (Wild et al., 1991). Together, these findings were considered as the first evidence for the existence of pharmacologically distinct DOPr subtypes—one preferentially activated by DPDPE and blocked by DALCE and BNTX (subtype 1; DOPr1), and the other activated by deltorphin II and blocked by NTB and NTII (subtype 2; DOPr2). These observations raised the possibility that not only do structural differences exist among DOPr subtypes, but DOPr1 and DOPr2 may also differ in their signaling mechanisms.

At a molecular level, the inhibitory action of DPDPE on adenylyl cyclase activity was more efficiently blocked by BNTX than NTB, whereas the latter preferentially reduced deltorphin II–induced inhibition of cAMP production (Búzás et al., 1994; Noble and Cox, 1995). In the spinal cord, electrophysiological studies have also described two pharmacological DOPr subtypes, each having a distinct role. Electrically evoked EPSCs were indeed more efficiently inhibited by DPDPE than by deltorphin II, suggesting a primary role for DOPr1 (Glaum et al., 1994).

As previously mentioned (section II), DOPr cloning revealed that the receptor is encoded by a single gene containing three exons (Evans et al., 1992; Kieffer et al., 1992). Since no DOPr splice variants have been identified at this time, mechanisms other than distinct DOPr proteins may define pharmacological receptor subtypes. These determinants still remain elusive (Dietis et al., 2011). For example, radioligand binding experiments performed in peripheral tissues and brain membrane preparations have failed to clearly establish the existence of multiple DOPr subtypes (Birkas et al., 2011; see also Fowler and Fraser, 1994; Zaki et al., 1996), although certain observations point to some degree of DOPr heterogeneity. Indeed, [^{3}H]-deltorphin II binding was competed in a biphasic manner by DPDPE (Negri et al., 1991), [^{3}H]-NTI has multiple binding sites ([4′-Cl-Phe4]DPDPE and [D-Ala2,Glu4]deltorphin displaced approximately 50% of [^{3}H]-NTI) in the brain; Yamamura et al., 1992; Fang et al., 1994), and [D-Ala^2, (2*R*,3*S*)-cyclopropyl^{E}Phe^4, Leu^5]enkephalin has a lower agonist activity in the mouse vas deferens assay compared with its brain binding affinity (Shimohigashi et al., 1987). Similarly, in the human neuroblastoma cell line SK-N-BE, where only one DOPr mRNA was sequenced, binding experiments revealed two DOPr subtypes (Allouche et al., 2000). Moreover, although NTB failed to reveal DOPr subtype heterogeneity in the mouse vas deferens (Makó and Rónai, 2001), [^{3}H]-DPDPE and [^{3}H]-deltorphin II binding revealed two DOPr subtypes in membrane preparations from the human cerebral cortex (Kim et al., 2001). Furthermore, in the mouse caudate putamen, the effect of enkephalins on DOPr1 translated into inhibition of MOPr-mediated antinociception, whereas activation of DOPr2 potentiated this effect (Noble et al., 1996).

Although the molecular entities of DOPr1 and DOPr2 remain to be determined, new evidence continues to support distinct roles for these pharmacological subtypes in various functions. In particular, a role for DOPr2 was described in morphine sensitization (Shippenberg et al., 2009) and morphine-conditioned responses (Billa et al., 2010). It has also been proposed that chronic morphine treatment increases MOPr-DOPr heteromer formation, with morphine acting as a

pharmacochaperone bringing the dimer to the cell surface (Costantino et al., 2012). Interestingly, DOPr2, but not DOPr1 blockade, was shown to reduce the development of morphine tolerance (Beaudry et al., 2015a), further supporting the idea that DOPr2 and the MOPr-DOPr heteromer share a similar pharmacological profile. Recently, the DOPr2 pharmacological profile was also associated with cardioprotection (Shen et al., 2012) and anxiolytic effects of KNT-127 (Sugiyama et al., 2014). DOPr1, on the other hand, could rather be a target to prevent alcohol abuse (Mitchell et al., 2014). Both subtypes were shown to be valid targets for the treatment of neuropathic pain (Mika et al., 2001). Despite nearly 30 years after they were first described, one should admit that the entire concept of DOPr subtypes still relies solely on indirect pharmacological observations. In fact, the most recent data still suffer from these same limitations, because earlier results and the molecular substrates of DOPr's pharmacological diversity remain elusive.

B. Putative Mechanisms of δ-Opioid Receptor Pharmacological Diversity

Pharmacological and molecular studies disagree on the existence of DOPr subtypes. Since this diversity is only observed in vivo, it has sometimes been attributed to the lack of specificity of opioid ligands for the different opioid receptors. There are, however, alternative explanations for pharmacological diversity involving distinct interactions or the duration of association between the receptor and the ligand. The development of subtype-specific DOPr ligands has been suggested as a possible strategy to increase therapeutic efficacy and/or reduce side effects (van Rijn et al., 2013). Although this approach is attractive, its success necessarily involves a better understanding of the determinants of this diversity. In the sections below, we analyze ligand pharmacokinetics and allosteric properties of the receptor protein as possible substrates for pharmacologically defined DOPr subtypes. Although DOPr association with distinct signaling and regulatory partners are reviewed as a source of allosteric variation of the receptor, there is no evidence clearly linking any of these associations with the distinct DOPr1 and DOPr2 pharmacological profiles that have been described in vivo.

1. Contribution of Binding Kinetics to Pharmacological Diversity of δ-Opioid Receptors. Intriguingly, pharmacological DOPr subtypes have been clearly defined in vivo, but these profiles have not been successfully reproduced in vitro. In vitro and in vivo conditions are particularly distinct with respect to how the ligand is made available to the receptor. In vitro, ligand concentrations are homogeneous and constant over time, resembling a "closed system." In vivo the situation resembles an "open system," in which the ligand diffuses to and from the receptor as it is administered and gets depleted from the organism (Tummino and Copeland, 2008). Thus, although in vitro the drug is constantly available to form a ligand-receptor complex, in vivo the complex is subject to changes in drug availability. In the latter conditions, the lifetime of ligand-receptor association is determined by the speed at which the drug exits the compartment where the receptor is located and the rate at which it dissociates (K_{off}) from the protein. If the drug dissociates slowly, it could remain bound to the receptor even if surrounding concentrations have decreased. On the other hand, a drug that dissociates rapidly will also be rapidly washed away, such that the time the drug spends in contact with the receptor is mainly determined by its K_{off} (Copeland et al., 2006; Kenakin and Williams, 2014). DOPr ligands are expected to differ in their K_{off} values, but the types of differences have not been systematically compared for the agonists that define DOPr1 and DOPr2 subtypes. The duration of receptor-ligand interaction determines the lifetime of the active signaling complex; since the type of signal produced by a receptor may vary over time (e.g., G protein versus βarr-dependent pathways), ligands with different kinetics may elicit different responses. Moreover, a ligand with slow but not one with rapid dissociation kinetics, may remain associated with the receptor to promote intracellular signaling (Calebiro et al., 2009; Ferrandon et al., 2009; Irannejad et al., 2013; Irannejad and von Zastrow, 2014), and such difference may support distinct responses for two agonists. An interesting observation in this sense is the time course of cAMP inhibition by DPDPE and deltorphin II (i.e., although both ligands internalize DOPr with a similar $t_{1/2}$ of approximately 15 minutes, only deltorphin II produces measurable cAMP inhibition over a 2-hour period) (Tudashki et al., 2014). Downstream effectors of short and prolonged cAMP inhibition will not necessarily be the same, providing yet another time-related explanation for differences between DPDPE and deltorphin II responses.

Differences in dissociation kinetics may also explain the distinct susceptibility of agonist responses to blockade by antagonists, another of the criteria defining DOPr subtypes. One of such examples was recently reported in human subjects for naloxone blockade of the respiratory depression induced by morphine and morphine-6-glucoronide (M6G) (Olofsen et al., 2010). M6G's dissociation kinetics are much slower than those of morphine. Hence, when naloxone was administered to surmount respiratory depression by each of these agonists, its effect on morphine was rapid and maximally effective, whereas the effect on M6G was slow and partial and could not be improved by increasing the dose of naloxone (Olofsen et al., 2010). Hence, in the same way kinetic differences underlie differential blockade of in vivo morphine and M6G responses, they could also support differential sensitivity of DPDPE and deltorphin II–preferring sites for the different antagonists.

Nonetheless, even if kinetically determined, differences in agonist-induced responses and distinct susceptibility to antagonists could still be exploited to generate DOPr compounds with "kinetic functional selectivity" (Hoffmann et al., 2015), an avenue that might be worth exploring.

2. Allosteric Properties of the Receptor as a Source of Pharmacological Diversity. Being allosteric proteins, receptors are not conformationally unique but rather exist in a multiplicity of states that are partly determined by their interaction with natural allosteric modulators (e.g., Na^+ ions) (Fenalti et al., 2014) as well as other membrane (e.g., other receptors, effectors) and/or cytosolic proteins (e.g., signaling partners, regulatory proteins) (Kenakin and Miller, 2010). Based on such allosteric properties, interactions of the receptor with other cellular proteins were earlier considered as plausible explanations of the observed diversity, with the DOPr1 subtype being correlated with noncomplexed receptors, whereas DOPr2 was considered the result of DOPr association with other receptors of unknown type (Vaught et al., 1982; Schoffelmeer et al., 1988a,b; Rothman et al., 1992; Cha et al., 1995). More recently, the actual observation that DOPr dimerization with other receptors could change DOPr pharmacology (George et al., 2000; Gomes et al., 2001; Levac et al., 2002; Fan et al., 2005; Hasbi et al., 2007; Rozenfeld and Devi, 2007; Fujita et al., 2015) lent further support to this hypothesis (van Rijn et al., 2010, 2013). However, there is currently no consensus on whether the pharmacology of specific heteromers may correspond to DOPr1 (van Rijn and Whistler, 2009) or DOPr2 (Porreca et al., 1992; Xu et al., 1993). Moreover, there are also numerous nonreceptor proteins that also interact with the receptor and could also induce allosteric changes to support pharmacological DOPr subtypes. Both types of supramolecular complexes are described below.

C. Supramolecular Organization of δ-Opioid Receptors

1. δ-Opioid Receptor Interaction with Receptor Proteins. Although it is well known that GPCRs can function as monomers (Ernst et al., 2007; Whorton et al., 2007, 2008; Kuszak et al., 2009), the association between different receptor subtypes into homo- and hetero-oligomers has the potential to greatly affect the pharmacological and functional properties as well as the trafficking properties of either receptor. This was initially exemplified by the essential association between the class C GPCRs $GABA_{(1)}$ and GABA (Staquicini et al., 2011) subtypes to form a functional $GABA_B$ receptor (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999). The concept has now extended to rhodopsin-like class A GPCRs. As extensively reviewed recently, receptor hetero-oligomers are independent entities with distinct properties and regulation (Fujita et al., 2014, 2015; Gomes et al., 2016).

In this section, we will focus on DOPr homo- and hetero-oligomerization with other opioid and nonopioid membrane receptors and discuss their effect on the pharmacology, coupling to G proteins, internalization, and signaling properties of each protomer (in this context, a protomer is defined as a structural subunit of a larger structure). The observations made for each receptor pair are summarized in Table 5. Although examples of interaction between DOPrs and other receptors are multiple, one should keep in mind that for the existence of homo- or hetero-oligomers to be widely accepted, a series of criteria should be fulfilled (Pin et al., 2007; Ferré et al., 2009). In brief, it is essential that 1) in native tissue, both receptors (in the case of hetero-oligomers) can be detected in the same subcellular compartment within the cell; 2) close proximity between receptors can be demonstrated through the use of proximity ligation assays, FRET or BRET-based assays, or heteromer-selective probes such as antibodies that recognize the oligomeric receptor in tissue from wild types but not in tissue lacking one of the receptors; 3) receptors coimmunoprecipitated from wild-type tissue but not those lacking one of the receptors; 4) the heteromer pair exhibits a "biochemical fingerprint" in wild-type tissue that matches that seen in heterologous cells coexpressing both but not just one of the receptors; and 5) heteromer formation can be disrupted by agents such as TAT peptides, leading to alterations in the "biochemical fingerprint" to one that resembles that of individual receptor protomers. Since the hetero-oligomers as novel therapeutic targets are only relevant inasmuch as they exist in vivo, these criteria can be further formulated as three sine qua non conditions for native tissues (Gomes et al., 2016): 1) heteromer components should colocalize and physically interact, 2) heteromers should exhibit properties distinct from those of the protomers, and 3) heteromer disruption should lead to a loss of heteromer-specific properties.

a. Homo-oligomerization of δ-opioid receptors. In NG108-15 cells, early studies using target size analysis revealed that opioid agonists bind to a high molecular weight isoform of DOPr (McLawhon et al., 1983; Ott et al., 1986). Shortly thereafter, Simonds et al. (1985) provided further evidence suggesting that DOPr has a tendency to oligomerize by demonstrating that purified DOPr bound to [^{3}H]-fentanylisothiocyanate migrated at 58,000 Da (monomer) and 110,000 Da (dimer) in a denaturating polyacrylamide gel (Simonds et al., 1985). Molecular and structural modeling has since predicted that DOPr homodimers rapidly associate and dissociate through a symmetric interface involving the fourth and/or the fifth TMHs (Filizola and Weinstein, 2002; Provasi et al., 2010; Johnston et al., 2011). In transfected cells, an essential role for the

TABLE 5
DOPr oligomerization: functional and pharmacological consequences

Oligomer Name	Detection Technique	Detection In Vivo	Binding Properties	G Protein Coupling and Signaling	Internalization and Trafficking	Reference
DOPr-DOPr	Denaturating SDS-PAGE, coimmunoprecipitation, BRET	Not reported	Not changed	Not changed	Not changed	Simonds et al. (1985), Cvejic and Devi (1997), McVey et al. (2001), Ramsay et al. (2002), Sarkar et al., 2012
DOPr-KOPr	Coimmunoprecipitation, BRET	Not reported	Decreased binding affinity for DOPr or KOPr, cooperativity when DOPr and KOPr ligands are administered together	Potentiation of cAMP inhibition and the ERK1/2 activation	Decreased etorphine-induced internalization	Jordan and Devi (1999), Ramsay et al. (2002), Berg et al. (2012)
DOPr-MOPr	Coimmunoprecipitation, BRET, immunolocalization with a DOPr-MOPr heteromer-specific antibody, TAT peptide disruption	Coimmunoprecipitation, immunolocalization with a DOPr-MOPr heteromer-specific antibody	Decreased affinity for synthetic ligands, increased affinity for endogenous opioid peptides, allosteric modulation of DOPr and MOPr ligand binding	Shifts from Gαi to Gaz coupling, turns MOPr functions from inhibitory to excitatory, potentiation of cAMP inhibition and the ERK1/2 activation, shifts ERK1/2 activation from transient to sustained	Ligand-specific cointernalization of DOPr or MOPr	George et al. (2000), Gomes et al. (2000, 2004, 2011), Charles et al. (2003), Fan et al. (2005), Law et al. (2005), Rozenfeld and Devi (2007), Xie et al. (2009), Gupta et al. (2010); Kabli et al. (2010), He et al. (2011), Milan-Lobo and Whistler (2011), Sarkar et al. (2012), Erbs et al. (2015)
DOPr-β2AR	Coimmunoprecipitation, BRET, FRET	Not reported	Not changed	Not changed	Nonstimulated DOPr cointernalizes with β2AR	Jordan et al. (2001), McVey et al. (2001), Ramsay et al. (2002), Cao et al. (2005)
DOPr-$\alpha 2_A$AR	Coimmunoprecipitation, FRET	Not reported	Not reported	$\alpha 2_A$AR potentiates DOPr-mediated neurites outgrowth	Not reported	Rios et al. (2004)
DOPr-$\alpha 1_A$AR	BRET	Not reported	Not reported	Not reported	Not reported	Ramsay et al. (2004)
DOPr-CCR5	Coimmunoprecipitation, crosslinking agents	Not reported	Not reported	Not reported	Not reported	Suzuki et al. (2002)
DOPr-CXCR4	Coimmunoprecipitation, FRET	Not reported	Not changed	The DOPr-CXCR4 hetero-oligomer cannot be activated by agonists	Not changed	Pello et al. (2008)
DOPr-CXCR2	Coimmunoprecipitation, BRET, FRET	Not reported	Not changed	CXCR2 antagonist increased DOPr-mediated G protein activation	Not reported	Parenty et al. (2008)
DOPr-SNSR-4	BRET	Not reported	Not changed	SNSR-4 activation reduced DOPr- mediated signaling	Not reported	Breit et al. (2006)
DOPr-SSTR4	Coimmunoprecipitation, FRET	Coimmunoprecipitation, FRET	Not reported	Synergistic effect on receptor- mediated sgnaling	Activation of both receptors retained them at the cell surface	Somvanshi and Kumar (2014)
DOPr-CB1	Coimmunoprecipitation, BRET	Immunolocalization with a DOPr-, CB1 heteromer-specific antibody	Increased binding of DOPr ligands upon CB1 occupancy	Decreased CB1-mediated signaling, increased DOPr-mediated signaling	Altered CB1 localization	Bushlin et al. (2012), Rozenfeld et al. (2012)

SNSR-4, sensory neuron–specific receptor subtype 4; SSTR, somatostatin type 4 receptor.

C-terminal tail of DOPr for dimerization was also described (Cvejic and Devi, 1997).

The existence of DOPr homo-oligomers was subsequently confirmed using various techniques such as coimmunoprecipitation or BRET assays (Cvejic and Devi, 1997; McVey et al., 2001; Ramsay et al., 2002; Sarkar et al., 2012). A specific functional role for the homodimer has not as yet been established. In heterologous systems, the levels of DOPr homodimers are reduced upon activation of DOPr with selective agonists, but not by morphine (Cvejic and Devi, 1997), and their disruption was suggested to occur prior to its internalization (Cvejic and Devi, 1997). The homodimerization process is thought to involve the C-terminal tail of DOPr because a mutant-receptor bearing a 15–amino acid deletion in the C-terminal tail failed to dimerize and did not undergo agonist-induced internalization (Cvejic and Devi, 1997). The levels of DOPr homodimers were also shown to be increased by the selective blockage of MOPr in natural killer cells (Sarkar et al., 2012). Again, no specific functional role of the homodimer was revealed in these studies. Thus, based on current knowledge, DOPr-DOPr homo-oligomers do not seem to exhibit properties different from the monomer and, for this reason, targeting them in vivo appears to be of no benefit compared with the monomers.

b. Oligomerization with other receptors. As many other GPCRs, DOPr was shown to form receptor complexes with other opioid receptors. The DOPr-KOPr hetero-oligomer was not only the first opioid receptor complex to be described, but it was also the first demonstration of an association between two functional GPCR protomers (Jordan and Devi, 1999). First detected by coimmunoprecipitation (Jordan and Devi, 1999) and BRET studies in transfected cells (Ramsay et al., 2002), the existence of DOPr-KOPr hetero-oligomers was further supported by coimmunoprecipitation studies in rat peripheral sensory neurons (Berg et al., 2012). In transfected cells, the consequence of an association between DOPr and KOPr is the formation of a receptor complex with a unique pharmacology. Indeed, the DOPr-KOPr hetero-oligomer displays reduced binding affinities for isolated DOPr and KOPr ligands but affinity (for agonists and antagonists) may be increased when ligands for both receptors are used in combination (Jordan and Devi, 1999). In vivo, a KOPr antagonist enhanced the analgesic potency of DPDPE but decreased that of DADLE and SNC80 (Berg et al., 2012). The latter observations support the idea that these receptors, when organized within a complex, act on each other as allosteric regulators. Although the formation of a DOPr-KOPr hetero-oligomer reduced the potency of etorphine (a nonselective opioid agonist) to induce internalization of DOPr, cAMP inhibition and ERK1/2 activation were potentiated when cells expressing both receptors were simultaneously exposed to DOPr and KOPr agonists (Jordan and Devi, 1999). Therefore, the association of DOPr with KOPr provides a functional receptor, distinct from the protomers, which is synergistically activated by selective opioid ligands. The only missing evidence to confirm the existence of the DOPr-KOPr hetero-oligomer is therefore a demonstration that disruption of the heteromer leads to a loss of the heteromer-specific properties.

In the early 1990s, the noncompetitive binding interactions between MOPr- and DOPr-selective ligands in rat brain membrane preparations was interpreted as a receptor complex comprising MOPr and DOPr (Rothman et al., 1992). Using a similar approach, more recent experiments performed in CHO cells expressing either receptor alone or in combination have supported the existence of MOPr-DOPr hetero-oligomers (Rutherford et al., 2008). Since then, the association between DOPr and MOPr is certainly the most studied opioid receptor complex (Fujita et al., 2015). In transfected cells, many techniques were used to show that DOPr and MOPr can associate into a detergent-stable hetero-oligomer (George et al., 2000; Gomes et al., 2000, 2004; Wang et al., 2005a; Hasbi et al., 2007; Décaillot et al., 2008; Gupta et al., 2010; Kabli et al., 2010; Golebiewska et al., 2011). Most importantly, DOPr and MOPr have been successfully coimmunoprecipitated from spinal cord extract, suggesting that this hetero-oligomer constitutively exists in live animals (Gomes et al., 2004; Xie et al., 2009; He et al., 2011). Most recently, MOPr and DOPr were shown to be coexpressed in various neuronal tissues of mice expressing fluorescent versions of DOPr and MOPr (Erbs et al., 2015). In the hippocampus of double knock-in mice, Erbs et al. (2015) further showed that DOPr-eGFP and mCherry-MOPr coimmunoprecipitate (Erbs et al., 2015). In intact tissue, the existence of the DOPr-MOPr hetero-oligomer was also shown using an antibody directed against this oligomer (Gupta et al., 2010). Interestingly, the abundance of the DOPr-MOPr hetero-oligomer is increased by chronic treatment with morphine in neurons (Gupta et al., 2010) and by ethanol in natural killer cells (Sarkar et al., 2012). The subcellular compartment where MOPr and DOPr associate with each other remains a matter of investigation. Indeed, using BRET studies, Hasbi et al. (2007) suggested that the DOPr-MOPr hetero-oligomer exists as a preassembled signaling complex in the ER (Hasbi et al., 2007). By contrast, others showed that the hetero-oligomer rather associates at the cell surface (Law et al., 2005). Similarly, it is still unclear how the protomers physically interact. Molecular modeling and computational analysis of dynamic structures revealed that the most likely interface of the DOPr-MOPr hetero-oligomer involves TMH4 and TMH5 of DOPr and TMH1 and TMH7 of MOPr (Filizola and Weinstein, 2002; Liu et al., 2009). This was supported by a study from He et al. (2011), who showed that a fusion protein consisting of TMH1 of MOPr fused to the TAT peptide was able

to disrupt the interaction between DOPr and MOPr (He et al., 2011). More recently, Provasi et al. (2015) used coarse-grained molecular dynamics simulations to uncover the preferred supramolecular organization and dimer interfaces of opioid receptors in a cell membrane model. Using this approach, they not only confirmed that DOPr and MOPr can interact via their TM domains but also that the complex, once formed, does not readily dissociate (Provasi et al., 2015). Others also provided evidence for a role of the C-terminal tail (O'Dowd et al., 2012; Kabli et al., 2014) and the second (Xie et al., 2009) and third ICLs of DOPr (O'Dowd et al., 2012) in the heterodimerization process. In HEK293 and SK-N-SH cells, MOPr and DOPr were also shown to associate as morphine-sensitive tetramers (Golebiewska et al., 2011).

The pharmacological and functional consequences of DOPr-MOPr oligomerization have been thoroughly studied, and DOPr-MOPr hetero-oligomers were shown to display a distinct pharmacological profile compared with individually expressed receptors (George et al., 2000). Indeed, cells coexpressing DOPr and MOPr showed a decreased affinity for synthetic opioids but an increased affinity for endogenous opioid peptides (George et al., 2000). Incubation of cells expressing both receptors with a selective ligand of either MOPr or DOPr was further shown to allosterically increase the number of binding sites as well as the binding affinity of selective ligands of the other receptor (Gomes et al., 2000, 2004, 2011). The latter effect was mediated by a decrease in the rate of dissociation of DOPr ligands (Gomes et al., 2000, 2011). In natural killer cells, hetero-oligomerization was rather associated with a decrease in opioid binding (Sarkar et al., 2012).

The coexpression of DOPr and MOPr was shown to induce a shift in G protein coupling. Indeed, as opposed to the individual receptors that are preferentially coupled to the PTX-sensitive $G\alpha_i$ subunits, the DOPr-MOPr hetero-oligomer coupled to a PTX-insensitive $G\alpha_z$ protein (George et al., 2000; Fan et al., 2005). On the other hand, another study reported that the hetero-oligomer will increase Ca^{2+} signaling in a PLC- and PTX-dependent manner, turning MOPr signaling functions from inhibitory to excitatory (Charles et al., 2003). In addition to modifying G protein coupling, DOPr and MOPr oligomerization was shown to influence activation of the ERK1/2 cascade. In particular, DOPr- and MOPr-mediated activation of ERK1/2 could be increased by the occupancy of the other protomer with low agonist or antagonist concentrations (Gomes et al., 2000). The hetero-oligomerization of MOPr and DOPr is also associated with a shift from a rapid and transient activation of ERK1/2 by individual receptors to sustained phosphorylation of MAPK by the hetero-oligomer (Rozenfeld and Devi, 2007). Finally, hetero-oligomerization can also modify the trafficking of DOPr and MOPr. In cells coexpressing DOPr and MOPr, DOPr was shown to cointernalize with MOPr after the selective activation of either receptor (He et al., 2011; Milan-Lobo and Whistler, 2011). However, this is in sharp contrast with a previous study demonstrating that the DOPr-MOPr hetero-oligomer was not internalized after the activation of only one of the protomers (Law et al., 2005). This suggests that endocytosis of the DOPr-MOPr hetero-oligomer could be ligand-selective (Law et al., 2005; Hasbi et al., 2007; Kabli et al., 2010; He et al., 2011; Milan-Lobo and Whistler, 2011).

Admittedly, the available literature strongly supports the existence of DOPr-MOPr hetero-oligomers. All of the criteria established to confirm the existence of heteromers, in vitro and in vivo, have been fulfilled. This complex, therefore, represents a distinct target with specific roles and functions. Although more work is needed to fully understand the pharmacology and the signaling cascades of this new entity, it is already described as a novel therapeutic target for pain and various brain disorders (Fujita et al., 2015).

c. Oligomerization with nonopioid receptors. DOPr can also oligomerize with a number of nonopioid receptors (Fujita et al., 2015). Early studies provided evidence that DOPr can associate with various AR subtypes. In transfected cells, coimmunoprecipitation, BRET, and FRET studies revealed that DOPr can dimerize with β2ARs (Jordan et al., 2001; McVey et al., 2001; Ramsay et al., 2002). However, this interaction is barely present unless both receptors are expressed at very high levels (i.e., >250,000 receptors per cell), raising the possibility that interactions between these GPCRs of different families (namely, opioid and adrenergic) could be artifacts due to high levels of expression (Ramsay et al., 2002). In fact, no such interaction between DOPr and β2AR has been described in native tissue. Nonetheless, the binding properties of the DOPr-β2AR hetero-oligomer remained unchanged, since DOPr and β2AR ligands have similar affinities for their respective receptors regardless of whether they are expressed in the same or in distinct cells (Jordan et al., 2001). Interestingly, the amount of DOPr-β2AR hetero-oligomers can be increased after the activation of either receptor by selective DOPr and β2AR agonists (McVey et al., 2001). In cells coexpressing DOPr with β2AR, nonstimulated DOPr is cointernalized after stimulation of the β2AR with isoproterenol (Jordan et al., 2001). DOPr and β2AR are known to pursue distinct postendocytic trafficking (Tsao and von Zastrow, 2000), with the former being commonly targeted for degradation and the latter rapidly recycling to the plasma membrane. However, the oligomerization of DOPr with β2AR barely impaired recycling of the latter (Cao et al., 2005). Thus far, no association between DOPr and the other subtypes of βARs has been described.

In HEK cells, coimmunoprecipitation studies suggested that DOPr and α_{2A}AR form a receptor complex

(Rios et al., 2004). FRET studies in these cells further revealed that these receptors were within 100 Å of each other, supporting the possibility that they can associate and form hetero-oligomers (Rios et al., 2004). In Neuro2A cells, the fact that the expression of α_{2A}AR is sufficient to promote DOPr-mediated neurite outgrowth further suggests that α_{2A}AR potentiates the functional properties of DOPr (Rios et al., 2004). Unless a direct interaction between DOPr and α_{2A}AR can be demonstrated in native tissue, the existence of this hetero-oligomer will remain uncertain. For instance, these receptors were shown to colocalize in substance P–containing primary afferents and in the superficial laminae of the spinal cord (Riedl et al., 2009), where they synergize via a PKCε-dependent mechanism (Schuster et al., 2013). Among other possibilities (see section VI.C), putative oligomerization between DOPr and α_{2A}AR provides a mechanism to explain analgesic synergy of their agonists (see Chabot-Dore et al., 2015 for a review on α_{2A}AR and DOPr synergism). BRET studies also demonstrated a propensity of DOPr to associate with α_{1A}AR (Ramsay et al., 2004). The role and function of this hetero-oligomer is yet to be described.

In the periphery, opioid receptors are highly expressed in immune cells (Stein and Küchler, 2012), where DOPr has been shown to associate with different chemokine receptors. The first demonstration for an interaction between DOPr and chemokine receptors was provided by Suzuki et al. (2002), who showed that endogenous CC chemokine receptor CCR5 and DOPr can be coimmunoprecipitated in human and monkey lymphocytes. Indeed, the use of crosslinking agents suggests that these receptors coexist on the cell membrane within an intermolecular distance of 11.4 Å (Suzuki et al., 2002). Through the activation of MOPr, morphine was found to enhance the expression of CCR5, an effect further increased by the MOPr-CCR5 hetero-oligomer (Suzuki et al., 2002). Although the role of the DOPr-CCR5 has not yet been determined, one could argue that given the high homology between DOPr and MOPr, the interaction between DOPr and CCR5 may also provide a mechanism to regulate the expression of the latter.

Other chemokine receptors shown to associate with DOPr are CXC chemokine receptors CXCR2 and CXCR4. Interestingly, the interaction of DOPr with either receptor has an important effect on its own functional properties. Indeed, although the DOPr-CXCR4 hetero-oligomer expressed in immune cells binds CXCR4 and DOPr ligands, simultaneous application of agonists for both receptors does not promote activation of their signaling cascades (Pello et al., 2008). The CXCR2-DOPr hetero-oligomerization was shown in HEK293 cells using different resonance energy transfer techniques as well as coimmunoprecipitation. In this particular case, the blockade of CXCR2 with an antagonist increased DOPr-mediated G protein activation, without affecting the binding properties of opioid ligands (Parenty et al., 2008). Therefore, although CXCR4 provides a dominant negative effect and a suppression of the signaling of DOPr, CXCR2 rather acts as an allosteric regulator able to enhance the effect of DOPr agonists. DOPr was also shown to oligomerize with sensory neuron–specific receptor subtype 4, which similarly acts as a dominant negative regulator of DOPr signaling (Breit et al., 2006). However, the existence of these hetero-oligomers remains to be confirmed in vivo.

More recently, DOPr was shown to interact and synergize with the somatostatin type 4 receptor to control pain. A direct interaction between these receptors in the brain, spinal cord, and heterologous systems is supported by coimmunoprecipitation and FRET studies (Somvanshi and Kumar, 2014). Again, further work is needed to confirm that this hetero-oligomer possesses distinct characteristics and roles in native tissue.

Finally, DOPr was also shown to interact with the cannabinoid receptor CB1. The first evidence of DOPr-CB1 hetero-oligomerization comes from the demonstration that DOPr–yellow fluorescent protein and CB1-Luc, when coexpressed in HEK293 cells, produced a significant and specific BRET signal (Rios et al., 2006). The dimerization of DOPr with CB1 was further confirmed by coimmunoprecipitation studies. Indeed, in transfected Neuro2A cells, DOPr and CB1 were shown to coexist in a protein complex comprising both receptors together with AP-2 and AP-3 (Rozenfeld et al., 2012). The formation of DOPr-CB1 dimers was shown to influence the subcellular localization and signaling properties of CB1 (Rozenfeld et al., 2012). Although the effect of such an interaction on DOPr functions in transfected cells has not been determined, Bushlin et al. (2012) recently found that the levels of DOPr-CB1 hetero-oligomers are altered in a rat model of neuropathic pain (Bushlin et al., 2012). In particular, in cortical membranes, a DOPr-CB1–specific antibody revealed a significant increase in the levels of DOPr-CB1 hetero-oligomers during neuropathic pain. This effect was accompanied by increased binding and activity of DOPr upon occupancy of CB1 with an agonist or an antagonist (Bushlin et al., 2012). The latter observations suggest that the interaction with CB1 acts as an allosteric modulator of DOPr.

Although many interactions between DOPr and other receptors have been presented in this section, to our knowledge, only one receptor pair involving DOPr currently fulfills all criteria to be recognized as a functional entity (namely, the DOPr-MOPr hetero-oligomer). DOPr-KOPr and DOPr-CB1 hetero-oligomers fulfill most criteria and should soon be confirmed as functional hetero-oligomers with distinct roles and functions. However, further work is required before the other receptor pairs can be considered as existing hetero-oligomers.

2. δ-Opioid Receptor Interaction with Nonreceptor Proteins. In addition to interacting with receptors, DOPrs may associate with a variety of signaling and regulatory proteins. As already discussed above, these associations may theoretically define pharmacological DOPr subtypes, and they may also contribute to signaling specificity by DOPrs; both aspects are discussed in the following paragraphs with respect to canonical and noncanonical signaling and regulatory partners.

a. δ-Opioid receptor association with canonical signaling proteins. The diversity of signals that result from DOPr activation raises the question as to how each of them might be specifically engaged to support the distinct functions controlled by these receptors. Part of this functional specificity is ensured by specific expression of effectors within cell populations that support different functions. At the same time, a diversity of potential signaling partners is made available to the receptor within each cell, raising the question of how the receptor "chooses" the adequate effector for mediating the required type of response. Functional studies have addressed this question by assessing how DOPr signaling relates to that of other receptors that share downstream signaling partners. For example, DOPr and other Gi/o-coupled receptors endogenously expressed in neuroglioma cells do not compete with one another for G protein activation, instead displaying additive responses to agonist stimulation (Graeser and Neubig, 1993; Shapira et al., 2000). This type of behavior is consistent with an organization in which each receptor and its cognate G protein are either precoupled in the absence of agonist (Wreggett and De Léan, 1984; Tian et al., 1994) or are confined within microdomains in which the receptor and its downstream signaling partner are present in high enough concentrations to allow rapid, unrestricted, and almost instantaneous interactions (Gross and Lohse, 1991). In SK-N-SH neuroblastoma cells endogenously expressing MOPr and DOPr, the combined effects of DOPr and MOPr agonists were also additive, which is also consistent with activation of distinct pools of G proteins (Shapira et al., 2000). By contrast, in studies completed in transfected COS-7 cells and in SH-SY5Y neuroblastoma cells, DOPr and MOPr, as well as other GPCRs, compete for G protein activation and AC modulation, with no evidence of additive responses (Shapira et al., 2000; Levitt et al., 2011). This signaling output is consistent with a translocation-collision model in which different receptors travel laterally within the membrane to activate a shared pool of downstream effectors (Rimon et al., 1978).

Because of these mixed results, alternative approaches have used biochemical (coimmunoprecipitation) and biophysical (BRET) readouts to address the question of how receptors and downstream signaling partners associate with one another. Data obtained from these studies indicate that in CHO or HEK cells that were transfected with DOPr, endogenous Gαi1, Gαo, and Gβ1 subunits could be coimmunoprecipitated with nonstimulated receptors (Law and Reisine, 1997; Audet et al., 2008). BRET approaches similarly revealed that transfected DOPr and heterotrimeric G$\alpha\beta\gamma$ subunits constitutively associate both in membrane preparations (Molinari et al., 2010) and in live cells (Audet et al., 2008). However, an important limitation of these results is that they all involved some degree of overexpression, which could have influenced the tendency of receptors to spontaneously associate with heterotrimeric subunits. On the other hand, cellular mechanisms may warrant high concentrations of signaling partners in physiologic conditions and, at the same time, mass action is most likely only one of many factors influencing DOPr association into multimeric arrays. For example, although the spontaneous transfer of energy between BRET pairs consisting of DOPr and Gβ1 subunits was susceptible to modulation by guanine nucleotides, the interaction between overexpressed MOPrs and Gβ1 was not (Molinari et al., 2010), arguing that high constitutive activity and not only mass action may have contributed to the spontaneous association between DOPr and G proteins (Costa and Herz, 1989; Vezzi et al., 2013).

BRET and coimmunoprecipitation assays revealed that DOPr may additionally associate with downstream effectors such as Kir3 channels (Richard-Lalonde et al., 2013; Nagi et al., 2015). The idea that GPCRs and Kir3 channel association may take place beyond overexpression systems is supported by biochemical and functional observations obtained with native dopamine D_2 and $GABA_B$ receptors in brain membranes. Indeed, Kir3 subunits were coimmunoprecipitated from brain membranes with either of these receptors (Lavine et al., 2002; Ciruela et al., 2010). In addition, cocaine or amphetamine administration leads to cointernalization of $GABA_B$ and Kir3 subunits in the VTA and prelimbic cortex (Padgett et al., 2012; Hearing et al., 2013), further arguing in favor of an association between receptors and effectors, which supports their joint regulation. Although there is currently no direct evidence of DOPrs physically associating with any effector in the nervous system, the observation that DOPrs and Kir3 subunits cointernalize when transfected onto cortical neurons (Nagi et al., 2015) is reminiscent of the behavior displayed by native $GABA_B$/Kir3 complexes. Moreover, even if overexpression may have influenced the DOPr-Kir3 association as stated above, this consideration need not invalidate the resemblance between these complexes and native $GABA_B$/Kir3 assemblies. Indeed, similar to overexpressed proteins, $GABA_B$ receptors and Kir3 channels may attain considerably high densities in the synaptic/perisynaptic microdomains where they exert their physiologic function (Choquet and Triller, 2003). For example, postsynaptic density protein 95 and synapse-associated protein

97 directly interact with and restrict the mobility of Kir3.2c subunits, concentrating the channel within the synapse and warranting their activation by G proteins (Inanobe et al., 1999; Hibino et al., 2000; Nassirpour et al., 2010). Furthermore, in an effort to determine whether and how restricted mobility could influence Kir3 channel activation by opioid receptors, Lober et al. (2006) compared the rate of current generation by "freely moving" and immobilized MOPrs in cerebellar granule cells (Lober et al., 2006). Immobility made no difference, implying that free diffusion within the membrane was not a prerequisite for optimal channel activation. These observations differ from data obtained in locus coeruleus neurons, in which a translocation-collision paradigm with a shared population of downstream channels was the most appropriate model to explain Kir3 current modulation by MOPrs and α_2ARs (North and Williams, 1985). Active Kir3.2 homotetramers were recently cocrystallized with four Gβ1γ2 dimers, in which the active channel was the organizing center of the array (Whorton and MacKinnon, 2013). Taking this organization as a template and based on the observation that immobilized receptors may effectively activate Kir3 channel effectors (Lober et al., 2006), Nagi and Pineyro (2014) proposed a possible organization for DOPr-G$\alpha\beta\gamma$-Kir3 signaling arrays in which each of the tetrameric subunits of the channel associates with a corresponding receptor and a G$\alpha\beta\gamma$ trimer (Nagi and Pineyro, 2014). In this sense, it is also worth noting that in myocardial membranes, where Kir3 channels are the main effector of muscarinic receptors, only a model based on receptor/G protein tetramers could recreate agonist binding sensitivity to guanine nucleotides (Redka et al., 2014).

Independent of whether the constitutive association between receptors and downstream signaling partners is a common physiologic phenomenon or the product of overexpression, kinetic studies with resolution within the millisecond range agree on the fact that once the receptor has been activated, its association with G proteins is maintained beyond initiation of signaling (Galés et al., 2005, 2006; Hein et al., 2005, 2006). Consensus similarly exists with respect to the constitutive association between heterotrimeric G proteins and Kir3 channel effectors (Bünemann et al., 2003; Riven et al., 2006; Robitaille et al., 2009; Berlin et al., 2010, 2011; Richard-Lalonde et al., 2013). Thus, both consensual observations imply that the receptor, G protein, and effector may be part of a single array containing all signaling partners even if for a limited time period, as has been described in HEK cells transfected with DOPrs, Gαoβ1γ2 subunits, and Kir3.1/3.2 channels. As stated in the introductory paragraphs, allosteric properties (Kenakin and Miller, 2010) warrant that the conformation adopted by the receptor within an array will be determined by its unique interactions with signaling partners that function as natural allosteric modulators. Therefore, it is conceivable that DOPrs that couple to a given effector via one type of Gαi/o protein will adopt slightly different conformations than those that couple to another effector via an alternative G protein. These differences can be theoretically exploited to tailor orthosteric agonists that will preferentially activate one effector over another, even if these oligomers do not define DOPr subtypes identified thus far. Of course, the prospect of directing the pharmacological stimulus to specific signaling complexes will require not only their identification but also clear establishment of which types of arrays support the desired versus undesired responses triggered by DOPr ligands.

b. δ-Opioid receptor association with noncanonical signaling proteins. Although G proteins were the first DOPr interaction partners to be identified, a variety of other proteins from different functional categories also associate with the receptor (Georgoussi et al., 2012). These are summarized in Table 6, where it is possible to identify signaling modulators such as calmodulin or periplakin, both of which are thought to block G protein activation by competing for the same interaction site on the receptor (Wang et al., 1999; Feng et al., 2003). The interaction sites of both of these proteins to opioid receptor were initially mapped for MOPr (Wang et al., 1999; Feng et al., 2003). Evidence of direct periplakin interaction with the C tail of DOPr was then confirmed in a yeast two-hybrid system and the interaction site was mapped to DOPr residues 321 to 331 (LDENFKRCFRE) based on analogy with the MOPr association site (Fig. 2) (Feng et al., 2003). Evidence of calmodulin association with DOPr is indirect (inferred from functional assays), with its association with the receptor being presumably constitutive and released upon activation (Wang et al., 1999). It is not yet clear whether the release of calmodulin serves a signaling function per se, but its dissociation from the receptor allows effective coupling of the latter to the G protein. The third protein in this category is RGS4. GST pull-down assays showed that this regulator of G protein signaling interacts with the first 26 amino acids of the DOPr C-terminal tail, thus overlapping interaction sites for periplakin, Gα, and G$\beta\gamma$ (Merkouris et al., 1996; Georgoussi et al., 2006). Moreover, coimmunoprecipitation assays have shown that DOPr constitutively associates with RGS4 and agonist stimulation does not seem to modify the amount of modulator proteins that coprecipitate with the receptor (Georgoussi et al., 2006; Wang et al., 2009). On the other hand, activation of the receptor was also shown to cause RGS4 redistribution from the cytosol to the membrane (Leontiadis et al., 2009), suggesting agonist-induced recruitment; the reason for this discrepancy is not clear. Receptor activation also modified the association of DOPr-RGS4 complexes to different Gα subunits. Thus, although Gαi2 coprecipitated with the complex only after agonist exposure, Gαi1 and Gαi3 interaction with DOPr-RGS4

TABLE 6
DOPr association with noncanonical signaling and regulatory proteins

Protein Class Function	Protein Name	Function of Interacting Protein	Evidence and Site of Interaction	Reference
Signaling modulators	Calmodulin	Interferes with basal and agonist-induced G protein activation	Indirect evidence: calmodulin binds to ICL3 of MOPr; shown to compete with G protein activation by DOPr	Wang et al. (1999)
	Periplakin	Interferes with agonist-induced G protein activation	Evidence of direct interaction by yeast two-hybid involves helix 8 in DOPr C-terminal tail (residues 321–331)	Feng et al. (2003)
	RGS4	Interferes with DOPr signaling	Evidence of direct interaction in GST pull down involves residues 311–333	Georgoussi et al. (2006), Wang et al. (2009)
			Coimmunoprecipitation assays showed constitutive association not modified by agonist	
	Spinophilin	Ubiquitous multidomain scaffold that interact with cytoskeleton: enriched in dendritic spines	Evidence of direct interaction by GST pull down	Fourla et al. (2012)
			Interaction involves ICL3 and residues 311–333	
Signaling	STAT5B	Activator of transcription	Evidence of direct interaction established in vitro	Georganta et al. (2010)
		Its release from DOPrs promotes transcription activation of STAT5-dependant genes	Evidence of association in cells by coimmunoprecipitation	
			Involves interaction with YXXL motif	
			Association is ligand-independent and abolished by DOPr activation	
			Gα and Gβ subunits are also part of the complex	
Desensitization and trafficking	GRK2	Desensitization, internalization	Evidence of interaction by coimmunoprecipitation in cells	Li et al. (2003)
			Involves last 31 amino acids in C tail	
			Interaction require DOPr activation	
	Arrestins	Desensitization, internalization	Evidence of association by BRET	Zhang et al. (2005)
			Involves IL-3 (amino acids 235–259) and C terminus (amino acids 331–372)	Audet et al. (2012)
			Interaction requires DOPr activation	
	Glycoprotein M6a	Sorts DOPrs to recycling endosomes	Evidence of interaction by BRET DOPr/M6a	Liang et al. (2008)
			Presumably involves DOPr TM 4, 5, 6 (in analogy with MOPr)	Wu et al. (2007)
	GASP1	Commits DOPr to lysosomal degradation	Evidence of direct interaction by GST pull down, involves last 50 amino acids in C terminus (including helix 8), evidence also by coimmunoprecipitation	Whistler et al. (2002)
			Constitutive association	Simonin et al. (2004)
				Heydorn et al. (2004)
	NHERF	Mediates NGF-dependant membrane targeting of intracellular DOPrs	Evidence obtained by coimmunoprecipitation	Bie et al. (2010)
			Site not determined	
			Association is NGF dependent	
	SNX1 (retromer component)	Not determined	Evidence of direct interaction established in vitro	Heydorn et al. (2004)
			Involved helix 8 in DOPr C terminus	
Ubiquitination	Ubiquitin	Degradation of misfolded DOPrs, not essential for postendocytic sorting to lysosomes	Evidence obtained in cells by WB (ubiquitin detection)	Petaja-Repo et al. (2001)
			Cytosolic lysines	Tanowitz and Von Zastrow (2002)
				Hislop et al. (2009)
	AUP1	Promotes ubiquitination of misfolded proteins	Evidence obtained in cells by coimmunoprecipitation	Petko et al. (2013)
			IL-2 (established for MOPr spontaneous association)	Mueller et al. (2008)
	SIAH 1, SIAH 2	RING finger ubiquitin ligases	Evidence obtained in cells by coimmunoprecipitation	Petko et al. (2013)
			Site of interaction IL-2 (in analogy with MOPr)	
			Spontaneous association	Hu et al. (1997)
Biosynthetic pathway	Calnexin	Facilates proper folding of recepor in the ER	Evidence obtained in cells by coimmunoprecipitation	Petäjä-Repo et al. (2002)
			Site not determined	
			Spontaneous association	
	VAPA	Involved in ER to Golgi transport	Evidence obtained in cells by coimmunoprecipitation	Petko et al. (2013)
			Site of interactions is IL-2	
			Established in vitro for MOPrs	Peretti et al. (2008)

AUP1, ancient ubiquitous protein 1; RING, really interesting new gene; SIAH, seven in absentia homolog; SNX1, sorting nexin 1; VAPA, vesicule-associated protein A; WB, western blot.

was constitutive and reduced upon agonist treatment (Leontiadis et al., 2009). The same juxtamembrane region of the DOPr C-terminal domain that interacts with RGS4 and Gα subunits as well as the third ICL also binds spinophilin, a multidomain scaffold protein that is highly enriched in dendritic spines (Fourla et al., 2012). By simultaneously binding RGS4, the receptor, Gα and G$\beta\gamma$ subunits spinophilin would stabilize a multimeric signaling complex that enhances RGS4 modulation of DOPr signaling (Fourla et al., 2012).

The next category of DOPr-interacting proteins corresponds to those involved in receptor desensitization and trafficking. Some of these proteins, such as GRKs and βarrs, interact with the receptor after its stimulation (see sections VII.A and VII.B for details), whereas others like GASPs (Whistler et al., 2002; Simonin et al., 2004) and glycoprotein M6a (Wu et al., 2007; Liang et al., 2008) associate constitutively (Fig. 2). GASPs constitute a family of at least 10 members, most of which are highly expressed in the central nervous system. Of them, GASP-1 has been studied in greater detail, being highly expressed in the amygdala, striatum, hippocampus, and thalamus but absent from the spinal cord (Simonin et al., 2004). GASP-1 interacts with DOPr in the same C-terminal region as RGS4 and periplakin, but this is not the case for its closest homolog, GASP-2. DOPr interaction with the other GASP family members has not been assessed (Simonin et al., 2004). The role of GASP-1 as a sorting protein that commits DOPr to MVBs (Whistler et al., 2002; Marley and von Zastrow, 2010) is discussed in detail in section VII.D. Glycoprotein M6a is a membrane protein with four TMHs, two of which (the third and fourth) directly interact with TMH4, TMH5, and TMH6 of the receptor (Wu et al., 2007). M6a overexpression was shown to redirect DOPr to the recycling path (Liang et al., 2008), but it is not yet known whether it has this same effect at endogenous expression levels. NHERF also interacts with DOPr, although with much lower affinity than KOPr (Huang et al., 2004). However, in brainstem neurons, increases in extracellular levels of NGF induce NHERF expression and drive its interaction with DOPr, redirecting receptors from their intracellular stores to the membrane (Bie et al., 2010). Molecular determinants of this interaction remain to be unveiled, since DOPrs lack signals present in other NHERF-interacting GPCRs (Cao et al., 1999; Huang et al., 2004).

The third family of DOPr-interacting proteins involves ubiquitin, ubiquitin ligases seven in absentia homologs 1 and 2 (Petko et al., 2013), as well as ancient ubiquitous protein 1, a protein that participates in ER-associated degradation by interacting with the receptor as well as ubiquitin and ubiquitin ligases (Chen et al., 2006c). Ubiquitination is a means by which many internalized membrane proteins are sorted in MVBs and thereby directed to lysosomes. DOPrs are normally ubiquitinated and traverse MVBs but, in contrast with many receptors, can do so even when their ubiquitination is blocked (Tanowitz and Von Zastrow, 2002; Hislop et al., 2009). The reason, as discussed in section VII.D, is that DOPrs engage additional endosomal sorting machinery that does not require ubiquitination (Whistler et al., 2002; Henry et al., 2011). In addition to its role in postendocytic regulation, DOPr ubiquitination in the ER promotes proteosomal degradation of receptors that are misfolded during synthesis (Petaja-Repo et al., 2001). As previously discussed (section V), appropriate folding and maturation of DOPr is assisted by its direct association with the resident ER protein calnexin (Petäjä-Repo et al., 2002). Vesicle-associated membrane protein-associated protein A, the other partner in the biosynthetic pathway with which DOPr directly interacts, is known to facilitate cargo progression from the ER to the Golgi (Peretti et al., 2008; Petko et al., 2013).

STAT5B is classically known for its function as a transcription factor that mediates genomic actions of growth factors (Rotwein, 2012). More recently, it has been assigned a nongenomic role maintaining structure and function of the ER and adequate anterograde secretory function (Sehgal, 2013). STAT5B constitutively associates with the YXXL motif in the DOPr C terminus and is released upon receptor activation undergoing Src-dependent phosphorylation and transcriptional activation (Georganta et al., 2010). The physiologic significance of this path remains to be elucidated. "Nongenomic" actions of STAT5B do not require phosphorylation (Sehgal, 2013). Within this context, its constitutive association with DOPr could be interpreted as a means for the receptor to progress along the biosynthetic path.

X. Conclusions and Future Directions

DOPrs respond to a vast array of structurally diverse ligands and crystallization studies are starting to unveil the exact contacts established by specific ligand-receptor pairs. Biophysical in vitro (plasmon resonance) and in cellulo (spectroscopy) approaches further indicate that distinct binding modalities may result in the stabilization of ligand-specific conformations of the receptor, whereas real-time functional assays have confirmed that distinct receptor states support biased responses that add texture to DOPr signaling. Novel quantitative approaches are also becoming available that will make it actually possible to measure differences in ligand texture and correlate these with in vivo responses and with specific molecular interactions between the ligand and the receptor. With access to this knowledge, it should be possible to verify specific hypotheses concerning the type of signals that support desired and undesired actions of DOPr ligands and to fine-tune the structure so as to eventually attain the greatest analgesic efficacy with the best tolerated side

effect profile possible. The task should be further assisted by our increasing insight into the allosteric nature of these receptors, our growing knowledge of their interactions with other signaling and regulatory proteins, and access to transgenic animals that should allow a better characterization of these interactions in a more physiologic environment. Finally, increasing knowledge of how different pathologic conditions may affect DOPr expression at the membrane and an improved understanding of how membrane and intracellularly generated signals contribute to analgesic responses and/or undesired side effects should further contribute to the rational design of more effective, longer-lasting, and better-tolerated opioid analgesics for therapeutic use.

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