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Revolution in GPCR signalling: opioid receptor heteromers as novel therapeutic targets: IUPHAR Review 10

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This article, written by a member of the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) subcommittee for the opioid receptors, confirms the existing nomenclature for these receptors and reviews our current understanding of their structure, pharmacology and functions and their likely physiological roles in health and disease. More information on this receptor family can be found in the Concise Guide to PHARMACOLOGY (http://onlinelibrary.wiley.com/doi/10.1111/bph.12445/abstract) and for each member of the family in the corresponding database (http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=50&familyType=GPCR).

GPCRs can interact with each other to form homomers or heteromers. Homomers involve interactions with the same receptor type while heteromers involve interactions between two different GPCRs. These receptor–receptor interactions modulate not only the binding but also the signalling and trafficking properties of individual receptors. Opioid receptor heteromerization has been extensively investigated with the objective of identifying novel therapeutic targets that are as potent as morphine but without the side effects associated with chronic morphine use. In this context, studies have described heteromerization between the different types of opioid receptors and between opioid receptors and a wide range of GPCRs including adrenoceptors, cannabinoid, 5-HT, metabotropic glutamate and sensory neuron-specific receptors. Recent advances in the field involving the generation of heteromer-specific reagents (antibodies or ligands) or of membrane-permeable peptides that disrupt the heteromer interaction are helping to elucidate the physiological role of opioid receptor heteromers and the contribution of the partner receptor to the side effects associated with opioid use. For example, studies using membrane-permeable peptides targeting the heteromer interface have implicated μ and δ receptor heteromers in the development of tolerance to morphine, and heteromers of μ and gastrin-releasing peptide receptors in morphine-induced itch. In addition, a number of ligands that selectively target opioid receptor heteromers exhibit potent antinociception with a decrease in the side effects commonly associated with morphine use. In this review, we summarize the latest findings regarding the biological and functional characteristics of opioid receptor heteromers both *in vitro* and *in vivo*.

Abbreviations

6′-GNTI, 6′-guanidinonaltrindole; AC, adenylyl cyclase; APJ, apelin receptor; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; DPDPE, D-penicillamine(2,5)-enkephalin; GRP, gastrin-releasing peptide; MPEP, 2-methyl-6-(phenylethynyl) pyridine; NNTA, *N*-naphthoyl-β-naltrexamine; RTP4, receptor transport protein 4; SNSR4, sensory neuron-specific receptor 4; YFP, yellow fluorescent protein

Links to online information in the IUPHAR/BPS Guide to PHARMACOLOGY

TARGETS	LIGANDS
5-HT1$_A$ receptor	5′-guanidinonaltrindole, GNTI
α2A-adrenoceptor	5-HT
Apelin receptor, APJ	CCL4 (MIP-1β)
β2-adrenoceptor	CCL5 (RANTES)
Cannabinoid CB1 receptor	CGRP
Chemokine CCR5 receptor	Clonidine
Chemokine CXCR4 receptor	DAMGO
δ receptor, DOP	DPDPE
Dopamine D1 receptor	DSLET
ERK1/2	dynorphin A
Gastrin-releasing peptide receptor (BB$_2$ receptor)	L-779,976
κ receptor, KOP	[Leu]enkephalin
MAPK	LPS
mGlu5 receptor	MPEP
μ receptor	M-MPEP
NK1 receptor	Naltriben
NOP receptor	Nor-binaltorphimine, nor-BNI
protein kinase C (PKC)	Substance P
SNSR4 (MRGPRX1)	U 50488
phospholipase C (PLC)	U 69593
Somatostatin sst$_2$ receptor	

This Table lists protein targets and ligands which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

Introduction

It is becoming generally accepted that GPCRs including opioid receptors interact with each other to form homomers and heteromers, opening new therapeutic possibilities for identifying drugs targeting GPCRs.

The physical interaction between opioid receptors was first reported in 1997 using δ receptors, and these studies showed that agonist treatment modulated the level of receptor homodimers (Cvejic and Devi, 1997). Similar studies also showed that the κ receptors existed as detergent-insensitive dimers (Jordan and Devi, 1999) and μ receptors existed in interacting complexes with distinct trafficking properties (He *et al.*, 2002). The most recent X-ray crystallization analysis of μ receptors (Manglik *et al.*, 2012) has revealed a twofold symmetrical dimer through a four-helix bundle motif formed by transmembrane segments 5 and 6. Although this dimeric arrangement of μ receptors could be due to the conditions used to crystallize the receptor, these structural findings are exciting as they enable the development of structure-based approaches to complement the more conventional drug discovery programmes, in addition to providing novel insights into the roles of oligomerization in GPCR function.

Over the last decade, an increasing number of studies have explored the ability of GPCRs including opioid receptors to heteromerize (either with members of the same family or related families). In the case of opioid receptors, early 'indirect' evidence for the presence of heteromers was provided by radioligand binding and electrophysiological studies that suggested interactions between μ and δ receptors (Zieglgänsberger *et al.*, 1982; Rothman *et al.*, 1985; 1988; Metcalf *et al.*, 2012; Akgun *et al.*, 2013). Moreover, studies

examining the effect of pretreatment with leucine-enkephalin on morphine-mediated analgesia (Vaught and Takemori, 1979a,b), and of δ receptor antagonists on the development of morphine tolerance and dependence (Abdelhamid and Takemori, 1991; Zhu *et al.*, 1999) further supported a functional interaction between μ and δ receptors. Recent studies using heteromer-selective reagents, such as antibodies (Gupta *et al.*, 2010), ligands (Daniels *et al.*, 2005; Waldhoer *et al.*, 2005; Gomes *et al.*, 2013b) or agents that disrupt the heteromer *in vivo* (He *et al.*, 2011), have begun to provide 'direct' evidence for opioid receptor heteromerization.

In addition to heteromers between opioid receptor types, heteromers involving opioid receptors and other GPCRs including adrenoceptors, metabotropic glutamate receptors, sensory neuron-specific receptors, have been described (Gomes *et al.*, 2013a). In this review, we describe *in vitro* and *in vivo* evidence for heteromers involving opioid receptors, the development of heteromer-selective ligands and the therapeutic potential of GPCR heteromers as target molecules for novel drug development.

Heteromers between opioid receptor types

δ and μ receptor heteromers

The δ and μ receptor heteromer (δ-μ heteromer) is the most extensively studied opioid receptor heteromer. The 'direct' interaction between δ and μ receptors was first demonstrated by co-immunoprecipitation studies (George *et al.*, 2000; Gomes *et al.*, 2000) where Flag-tagged μ receptors were found to form interacting complexes (~150 kDa) with *myc*-tagged δ receptors in HEK-293 cells coexpressing both receptors (Gomes *et al.*, 2000). Endogenous δ-μ complexes could also be detected by co-immunoprecipitation studies with the spinal cord membranes from wild-type, but not δ receptor knockout mice (Gomes *et al.*, 2004). Furthermore, BRET assays demonstrated that both receptors exist within 100Å of each other in live cells, which is close enough to allow for direct receptor–receptor interactions (Gomes *et al.*, 2004) (Table 1).

Examination of the ligand-binding properties of the δ-μ heteromers showed that the binding affinity of agonists to individual protomers was decreased in δ-μ heteromers when compared with individual receptors (George *et al.*, 2000) (Table 1). Interestingly, this was increased in the presence of agonists or antagonists selective for the partner protomer (Gomes *et al.*, 2000; 2004; 2011). Further analysis revealed that this was due to allosteric modulation; the occupancy of one protomer allosterically modulated ligand binding to the partner protomer by affecting the rate of dissociation of the ligand (Gomes *et al.*, 2011). These changes in the pharmacological properties of δ-μ heteromers compared with δ or μ homomers suggested possible differences in signalling between heteromers and homomers.

Comparison of the intracellular signalling between the δ-μ heteromers and δ or μ homomers showed interesting differences. For example, occupancy of one of the protomers in the δ-μ heteromer enhanced signalling mediated via the activation of the partner protomer (Gomes *et al.*, 2000). In addition, while δ or μ homomers are coupled to and signal via $G\alpha_{i/o}$ proteins, studies have reported that the δ-μ heteromers could be associated with a *Pertussis* toxin-insensitive G-protein such as Gz (George *et al.*, 2000; Fan *et al.*, 2005; Hasbi *et al.*, 2007) and/or with β-arrestin 2 (Rozenfeld and Devi, 2007). The latter study showed that activation of a protomer in δ-μ heteromers leads to β-arrestin 2-mediated signalling. This is characterized by the presence of a second phase of ERK activation that is PKC-independent and can be blocked by small interfering RNA to β-arrestin 2 (Rozenfeld and Devi, 2007) (Table 1). Moreover, δ-μ heteromer-mediated β-arrestin 2 signalling leads to changes in the spatiotemporal dynamics of ERK1/2 phosphorylation. For example, in cells expressing the δ-μ heteromer, ERK1/2 which was phosphorylated following treatment with the μ receptor agonist, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO), was not translocated to the nucleus (as seen with cells expressing only μ receptors). This leads to the phosphorylation of the cytoplasmic and not the nuclear ERK1/2 substrates and ultimately differential activation of transcription factors (Rozenfeld and Devi, 2007). Finally, a study showed that in GH3 cells expressing δ-μ heteromers, the μ receptor agonist, DAMGO, stimulated Ca^{+2}-mediated signalling instead of $G\alpha_{i/o}$-mediated signalling (Charles *et al.*, 2003). Taken together, these studies show that heteromerization increases the repertoire of signalling of μ and δ receptors.

It is well known that following continued exposure to agonists, μ or δ receptors are phosphorylated; this leads to the recruitment of β-arrestin, receptor endocytosis to acidic endosomal compartments and termination of $G\alpha_{i/o}$ protein-mediated signalling. In the endosomes, the internalized receptors are either dephosphorylated and recycled back to the cell surface to undergo another round of signalling or are targeted to lysosomes for degradation. Very few studies have investigated how heteromerization modulates the endocytosis of the δ-μ heteromer. While one study found that each protomer in the δ-μ heteromer internalized independently from the other protomer, other studies found that the heteromer as a whole could be endocytosed by some selective agonists (DAMGO, deltorphin II, methadone) but not others, such as D-penicillamine(2,5)-enkephalin (DPDPE) or [(D-Ser^2, Leu^5]enkephalin-Thr^6 (Law *et al.*, 2005; Hasbi *et al.*, 2007; Milan-Lobo and Whistler, 2011). Moreover, methadone, a μ receptor agonist that induces homomer internalization and recycling, induced endocytosis of the δ-μ heteromer leading to its degradation and this effect could be blocked by co-treatment with naltriben, a δ receptor antagonist (Milan-Lobo and Whistler, 2011). Another study found that a bivalent ligand selective for the δ-μ heteromer, MDAN-21 (comprising a δ receptor antagonist pharmacophore, DN-20, separated by a 21-atom spacer from the μ receptor agonist pharmacophore, MA-19) (Table 4), did not induce heteromer internalization (Yekkirala *et al.*, 2013). As co-administration of the individual monovalent pharmacophores (DN-20 and MA-19) induced internalization of δ-μ heteromers, it has been suggested that the spacer arm in MDAN-21 by bridging both protomers in the δ-μ heteromer, immobilizes the latter thereby preventing its endocytosis (Table 1). In addition to modulation of receptor internalization and endocytosis, heteromerization appears to modulate the maturation and cell surface expression of the δ-μ heteromer. In cells coexpressing

Table 1

Heteromers between opioid receptor types[a]

Heteromer pair	*In vitro* heteromer properties (binding, signalling, trafficking)	*In vivo* effects of reagents targeting heteromers	References
δ-μ[b,c]	*Detection* Co-IP, BRET *Binding* ↓ in affinity for receptor-selective agonists. Allosteric modulation of μ receptor binding by δ receptor ligands and vice versa. *Signalling* Potentiation of μ receptor signalling by δ receptor ligands and vice versa. Switch from $G\alpha_{i/o}$ to either $G\alpha_z$ or $G\alpha_q$ signalling. Coupling to and signalling via β-arrestin 2. *Trafficking* Protomers internalize independently from each other. Some selective agonists can induce heteromer internalization. Methadone internalizes the δ-μ heteromer and targets it for degradation. MDAN-21 immobilizes the heteromer and prevents its endocytosis. Heteromer expression at the cell surface requires RTP4.	*δ-μ heteromer selective antibody* Antibody detects ↑ in heteromer levels in various brain regions after chronic morphine administration. *Bivalent ligand (MDAN21)* Antinociception (i.t, i.c.v. and s.c.) with MDAN21 is more potent than morphine without development of tolerance revealed and it is less rewarding than morphine. *Biased agonist (CYM51010)* Potent antinociception with reduced development of tolerance. *TAT-fusion protein (μTM1-TAT)* μTM1-TAT disrupts δ-μ heteromers; ↑ in morphine antinociception with ↓ in development of tolerance.	George *et al.*, 2000; Gomes *et al.*, 2000; 2004; 2011; 2013b; Charles *et al.*, 2003; Daniels *et al.*, 2005; Fan *et al.*, 2005; Law *et al.*, 2005; Hasbi *et al.*, 2007; Lenard *et al.*, 2007; Rozenfeld and Devi, 2007; Decaillot *et al.*, 2008; Gupta *et al.*, 2010; He *et al.*, 2011; Milan-Lobo and Whistler, 2011; Yekkirala *et al.*, 2013
δ-κ[b]	*Detection* Co-IP, BRET *Binding* ↓ in affinity for δ or κ receptor ligands. Binding co-operativity with δ + κ receptor ligands. *Signalling* ↑ in signalling with a combination of δ + κ receptor agonists. *Trafficking* ↓ in δ receptor internalization in cells expressing the δ-κ heteromer.	*δ-κ heteromer selective antibody* Antibody detects δ-κ heteromers in peripheral sensory neurons following thermal allodynia. Antibody enhances anti-allodynic effects of a δ agonist. *Bivalent ligand (KDN-21)* No antinociception following i.t administration. *Heteromer targeting agonist (6′-GNTI)*[d] Antinociception following i.t. but not i.c.v. administration. Antinociception to 6′-GNTI is more potent than that of U50,488.	Jordan and Devi, 1999; Ramsay *et al.*, 2002; Bhushan *et al.*, 2004; Waldhoer *et al.*, 2005; Berg *et al.*, 2012
μ-κ[b]	*Detection* Co-IP, BRET *Binding* ↓ in affinity for μ receptor-selective agonists. No change in affinity for κ agonists. *Signalling* Remains to be determined. *Trafficking* Remains to be determined.	*Heteromer targeting agonist (NNTA)* Antinociception (i.v., i.t., i.c.v.); NNTA exhibits more potent antinociception than morphine.	Jordan and Devi, 1999; Wang *et al.*, 2005a; Chakrabarti *et al.*, 2010; Yekkirala *et al.*, 2011
μ-NOP[b]	*Detection* Co-IP *Binding* ↑ in affinity for μ receptor agonist. *Signalling* ↓ in potency of μ receptor agonists. Cross-desensitization of μ receptor-mediated signalling by NOP agonist. *Trafficking* Remains to be determined.	*Heteromer targeting ligand ($IBN_{tx}A$)* $IBN_{tx}A$ exhibits more potent antinociceptive effect than morphine determined with reduced side effects.	Pan *et al.*, 2002; Wang *et al.*, 2005b; Majumdar *et al.*, 2011

[a]Modified from (Gomes *et al.*, 2013a).
[b,c]These heteromers are useful therapeutic targets, based on the known roles of one or both protomers. Therapeutic targets: [b]Antinociception; [c]Antinociceptive tolerance.
[d]A recent report shows that 6′-GNTI exhibits biased agonistic properties at κ opioid receptors (Rives *et al.*, 2012).

μ and δ receptors, the heteromer is localized to the Golgi apparatus and its cell surface expression requires a chaperone named receptor transport protein 4 (RTP4) (Decaillot *et al.*, 2008). RTP4 protects the receptor heteromer during folding and maturation from ubiquitination and proteasomal degradation (Decaillot *et al.*, 2008). Further studies are needed to examine if RTP4 affects ligand binding and signalling by the δ-μ heteromer.

In order to understand the role of δ-μ heteromers *in vivo*, heteromer-selective reagents (ligands, antibodies) as well as agents that selectively disrupt the heteromer have been developed. Studies with these reagents suggest a possible involvement of δ-μ heteromers in the development of tolerance to morphine. For example, δ-μ heteromer-selective antibodies detect increased heteromer levels in discrete brain regions following chronic morphine administration (Gupta *et al.*, 2010). Moreover, administration of a membrane permeable peptide TAT peptide (YGRKKRRQRRR) fused to the peptide representing transmembrane domain 1 of μ receptors disrupts the δ-μ heteromer and leads to an increase in morphine-mediated antinociception and a decrease in the development of tolerance to morphine (He *et al.*, 2011) (Table 1). These results suggest that the δ-μ heteromer could be a target for the development of antinociceptive therapeutics as potent as morphine but with lesser side effects such as antinociceptive tolerance and dependence. This is supported by studies with MDANs that differ in the length of the spacer arm between pharmacophores and selectively target the δ-μ heteromer. Of these, MDAN-21 was found to exhibit 100 times more potent antinociception and to be less rewarding than morphine (Daniels *et al.*, 2005; Lenard *et al.*, 2007). Moreover, chronic administration of MDAN-21 did not lead to the development of tolerance or dependence (Daniels *et al.*, 2005). In addition to MDANs, bivalent ligands comprising of a high-affinity μ receptor agonist (oxymorphone) joined by a spacer arm to a low affinity δ receptor antagonist (ENTI) or of a high-affinity μ receptor antagonist (naltrexone) joined by a spacer arm to a low affinity δ receptor agonist (DM-SNC80) have been generated (Harvey *et al.*, 2012) (Table 4). However, the antinociceptive effects of these ligands and their side effects have not been evaluated. More recently, a high throughput screening of a small molecule library for a δ-μ heteromer-selective ligand led to the identification of CYM51010 as a biased δ-μ heteromer agonist (Table 4); this study also showed that the compound exhibited potent antinociception with reduced antinociceptive tolerance (Gomes *et al.*, 2013b). The antinociceptive effect of CYM51010 was significantly blocked by δ-μ heteromer-selective antibodies (Gomes *et al.*, 2013b), which would suggest that, *in vivo*, this heteromer plays an important role in pain regulation (Table 1). Taken together, these studies support the δ-μ heteromer as a novel therapeutic target for pain attenuation with reduced side effects.

δ and κ opioid receptor heteromers

The δ and κ receptor heteromer (δ-κ heteromer) was the first opioid receptor heteromer to be reported. Co-immunoprecipitation studies using κ receptors tagged with a *myc* epitope and δ receptors tagged with a Flag epitope detected the presence of interacting complexes only in cells coexpressing both receptors (Jordan and Devi, 1999). In addition, BRET assays showed that the two receptors existed in close proximity and could directly interact in live cells (Ramsay *et al.*, 2002) (Table 1).

Examination of the binding, signalling and trafficking properties of the δ-κ heteromer showed that they were distinct from δ or κ homomers. For example, the binding affinities for δ or κ receptor agonists were lower at δ-κ heteromers compared with the respective receptor homomers (Jordan and Devi, 1999). An increase not only in the binding affinity but also in intracellular signalling was observed when the δ-κ heteromer was treated with a combination of δ and κ receptor agonists (Jordan and Devi, 1999) (Table 1). An increase in the binding affinity was also observed when the δ-κ heteromer was treated with a combination of δ or κ receptor antagonists (Jordan and Devi, 1999). These findings suggest the possibility of allosteric interactions between δ and κ receptors. With regard to the trafficking properties of the δ-κ heteromer, studies with etorphine, a potent non-selective opioid agonist that binds to both κ and δ receptors, show that it does not induce δ receptor internalization in cells expressing the δ-κ heteromer, while it induces receptor internalization in cells expressing only δ receptors (Jordan and Devi, 1999). These findings suggest that δ-κ heteromerization alters the trafficking of properties of δ receptors (Table 1).

In order to understand the role of δ-κ heteromers *in vivo*, heteromer-selective reagents such as antibodies have been generated. A recent study detected the presence of δ-κ heteromers in peripheral sensory neurons following thermal allodynia (Berg *et al.*, 2012). Moreover, the study found that a δ-κ heteromer-selective antibody could enhance the anti-allodynic effects of the δ receptor agonist, DPDPE; this suggests a role for δ-κ heteromers in modulation of thermal allodynia (Berg *et al.*, 2012) (Table 1). Ligands targeting the δ-κ heteromer include a bivalent ligand named KDN-21 and 6′-guanidinonaltrindole (6′-GNTI) (Table 4). KDN-21 comprises a κ receptor-selective antagonist pharmacophore, 5′-guanidinonaltrindole, which is tethered through a 21-atom spacer arm to the δ receptor-selective antagonist pharmacophore, naltrindole. KDN-21 exhibits selective δ1 receptor and κ2 receptor antagonistic activity and does not induce antinociception (Bhushan *et al.*, 2004) (Table 1). In contrast, 6′-GNTI functions as an agonist that induces ~50-times more potent antinociception than the selective κ receptor agonist U50488H when administered intrathecally but not intracerebroventricularly (Waldhoer *et al.*, 2005) (Table 1). However, a recent study reported that 6′-GNTI also exhibits biased agonistic properties at κ receptors (Rives *et al.*, 2012); this would suggest that the behavioural outcomes from studies with 6′-GNTI could be due to its activity at either κ receptors alone or δ-κ heteromers. Taken together, these studies suggest that δ-κ heteromers form a distinct functional signalling unit that could provide a target for the development of tissue-selective opiate analgesics.

μ and κ receptor heteromers

A few studies have investigated possible heteromerization between μ and κ receptors (μ-κ heteromers). Early co-immunoprecipitation studies using antibodies to the epitope tags on the receptors were unable to detect the presence of interacting complexes between μ and κ receptors in heterologous cells (Jordan and Devi, 1999). However, a study used antibodies to endogenous μ and κ receptors to detect the

presence of μ and κ interacting complexes in spinal cord membranes from female but not male rats (Chakrabarti *et al.*, 2010) (Table 1). This study reported that the levels of μ-κ interacting complexes were dependent upon the stage of the oestrous cycle (Chakrabarti *et al.*, 2010). These results suggest that the detection of μ-κ interacting complexes may be susceptible to the detergent conditions used in co-immunoprecipitation studies or may depend on the tissue used. In addition, it is possible that in cells/tissues expressing μ and κ receptors the μ-κ interacting complexes may be inducible only under certain physiological conditions or may be under the regulation of sex hormones. Further support for the probable formation of μ-κ heteromers came from BRET assays showing that the two receptors are close enough to directly interact in live cells (Wang *et al.*, 2005a). Thus it appears that μ and κ receptors can form physiologically relevant heteromers and studies to explore the functional role of this heteromer in biological systems are needed.

Examination of the properties of the μ-κ heteromer using radioligand binding and [^{35}S]GTPγS assays revealed that the binding affinity of μ receptor agonists, such as DAMGO and endomorphin-1, is lower in cells coexpressing μ and κ receptors compared with cells expressing only μ receptors. In the case of the κ receptor agonists, U69593 and U50488H, no differences were observed between cells expressing μ-κ heteromers or κ receptors (Wang *et al.*, 2005a) (Table 1). These findings indicate that heteromerization with κ receptors alters the binding properties of μ receptors.

A few studies have focused on identifying μ-κ heteromer-selective ligands (Chakrabarti *et al.*, 2010; Yekkirala *et al.*, 2011). Although strong evidence for the role of dynorphin 1–17 at μ-κ heteromers is not available, it has been reported that *N*-naphthoyl-β-naltrexamine (NNTA) selectively activates μ-κ heteromers (Table 4), and it is 50 times more potent than morphine, as an antinociceptive agent (Yekkirala *et al.*, 2011) (Table 1). Moreover, intrathecal administration of NNTA leads to greater antinociceptive effect (~two orders of magnitude) than intracerebroventricular administration, an effect not seen in μ receptor-knockout mice, which would suggest a higher degree of functional coupling between μ and κ receptors in the spinal cord (Yekkirala *et al.*, 2011). In addition, the administration of NNTA does not lead to the development of physical dependence while antinociceptive tolerance to NNTA is low upon chronic intracerebroventricular administration and not observed upon chronic intrathecal administration (Yekkirala *et al.*, 2011) (Table 1). This suggests that μ-κ heteromers play important roles in pain regulation and that they may be viable targets for the development of analgesics devoid of the unwanted side effects associated with chronic morphine administration.

μ and nociceptin/orphanin FQ receptor heteromers

Studies showing that ligands for the nociceptin/orphanin F/Q (NOP) receptor modulated the antinociceptive effects of morphine (King *et al.*, 1998; Rizzi *et al.*, 2000) led to investigations on whether μ receptors and NOP receptors formed heteromers. Co-immunoprecipitation studies showed that μ receptors formed interacting complexes with NOP receptors in heterologous cells coexpressing both receptors and in adult rat dorsal root ganglions (Pan *et al.*, 2002; Wang *et al.*, 2005b; Evans *et al.*, 2010). In addition, studies using C-terminal deletion mutants suggested an involvement of the intracellular C-terminal region in the formation of these μ-NOP receptor complexes (Wang *et al.*, 2005b) (Table 1).

Examination of the pharmacological properties of μ-NOP complexes showed an increase in the binding affinity of μ receptor agonists including DAMGO and fentanyl, compared with cells expressing only μ receptors (Pan *et al.*, 2002) (Table 1). In addition, signalling assays showed that while the EC_{50} for inhibition of adenylyl cyclase (AC) activity and for activation of ERK1/2 phosphorylation by DAMGO was significantly increased in cells coexpressing μ and NOP receptors, compared with cells expressing only μ receptors, the EC_{50} for the NOP receptor agonist was not affected (Wang *et al.*, 2005b). These findings taken together with the data from co-immunoprecipitation studies suggest that μ and NOP receptors form heteromers that selectively impair the potency of μ-induced signal transduction pathways. In addition, while pretreatment with the NOP receptor agonist caused desensitization of not only NOP- but also DAMGO-induced inhibition of AC activity, pretreatment with DAMGO did not affect desensitization of NOP receptor-mediated pathways (Wang *et al.*, 2005b) (Table 1). These results suggest that μ-NOP heteromerization selectively causes cross-desensitization of μ receptor-mediated signal transduction.

A naltrexone derivative, $IBN_{tx}A$, has been identified as a putative μ-NOP heteromer-selective ligand based on the high-affinity binding of radiolabelled $IBN_{tx}A$ in cells coexpressing μ1G receptors (a μ receptor with mutations in the sixth transmembrane region) and NOP receptors (Majumdar *et al.*, 2011) (Table 4). This compound was 10 times more potent as an antinociceptive agent than morphine and did not display side effects, such as respiratory depression, physical dependence and appreciable constipation. Moreover, $IBN_{tx}A$ did not appear to be either rewarding or aversive in conditioned place preference studies (Majumdar *et al.*, 2011) (Table 1). Taken together, these results suggest that targeting the μ-NOP heteromer could provide a major advance in the design and development of new highly potent opiate analgesics, without many side effects.

Heteromers between opioid and cannabinoid receptors

δ opioid and cannabinoid CB_1 receptor heteromers

Several lines of evidence have suggested interactions between δ-opioid receptors and CB_1 receptors. These included studies showing that (i) δ receptor agonists decrease CB_1 receptor signalling, (ii) δ receptor antagonists attenuate CB_1 receptor-mediated anxiolytic effects, (iii) CB_1 receptor levels and signalling increase in the substantia nigra of δ receptor-knockout mice and (iv) δ receptor activity increases in the caudate putamen of CB_1 receptor-knockout mice (Shapira *et al.*, 1998; Berrendero and Maldonado, 2002; Berrendero *et al.*, 2003; Uriguen *et al.*, 2005). Direct interactions between δ and CB_1 receptors were suggested by BRET assays carried out in heterologous cells coexpressing luciferase-tagged CB_1

receptors and yellow fluorescent protein (YFP)-tagged δ receptors showing both receptors in close proximity in live cells (Rios *et al.*, 2006). This was further supported by co-immunoprecipitation studies using epitope-tagged receptors and by immunofluorescence studies showing colocalization of δ receptors with CB_1 receptors in cortical neurons (Rozenfeld *et al.*, 2012) (Table 2).

Heteromerization with δ receptors modulates the subcellular localization of CB_1 receptors as the latter receptor exhibits an intracellular localization when expressed alone and when coexpressed with δ receptors, it was found on the cell surface (Rozenfeld *et al.*, 2012). This differential localization requires the association of CB_1 receptors with the adaptor protein-2 whereas, in the absence of δ receptors, CB_1 receptors associate with adaptor protein-3 (Rozenfeld *et al.*, 2012). Signalling assays showed that the δ receptor and CB_1 receptor heteromer (δ-CB_1 heteromer) exhibited signalling distinct from that of receptor homomers. For example, the signalling potency of a CB_1 receptor agonist was decreased in cells expressing the heteromer compared with the CB_1 receptor homomer and this decrease in potency was not seen following knockdown of δ receptors (Rozenfeld *et al.*, 2012). Also, while δ or CB_1 receptor homomers signal via activation of $G\alpha_{i/o}$ proteins, δ-CB_1 heteromer-mediated signalling involves PLC-mediated recruitment of β-arrestin 3 and the activation of signalling pathways that promote cell survival (Rozenfeld *et al.*, 2012) (Table 2). Taken together, these findings show that δ-CB_1 heteromerization expands the signalling repertoire of individual receptors.

The observation that δ-CB_1 heteromer levels are altered under pathophysiological conditions is of great importance. In a rodent model of neuropathic pain where animals exhibit mechanical allodynia, δ-CB_1 heteromer levels (detected using a δ-CB_1 heteromer-selective monoclonal antibody) are significantly elevated in cortex, hypothalamus and midbrain (Bushlin *et al.*, 2012). Moreover, in this model of neuropathic pain, the activation of G-protein mediated signalling by a CB_1 receptor-selective agonist was increased while that of a δ receptor-selective agonist was decreased; however, the latter was restored in the presence of a CB_1 receptor-selective agonist (Bushlin *et al.*, 2012). These findings suggest that the pharmacological effects of δ and CB_1 receptors could be altered under conditions of neuropathic pain and that heteromer formation might be involved in these changes (Bushlin *et al.*, 2012) (Table 2). Taken together, these studies suggest that the δ-CB_1 heteromer could be a target for the development of novel therapeutics to treat neuropathic pain.

μ opioid and CB_1 receptor heteromers

Possible formation of heteromers between μ receptors and CB_1 receptors was suggested by studies showing functional interactions between these receptors. For example, antinociceptive synergy was reported when using a combination of morphine and a CB_1 receptor agonist (Cichewicz, 2004). Moreover, μ receptor-knockout mice do not exhibit conditioned place preference for CB_1 receptor agonists (Ghozland *et al.*, 2002) while the reinforcing effects of morphine as well as the severity of withdrawal symptoms from this drug are absent in CB_1 receptor-knockout mice (Ledent *et al.*, 1999). Direct evidence for the formation of μ and CB_1 receptor heteromers (μ-CB_1 heteromer) came from proximity-based assays such as BRET and FRET that showed these two receptors were close enough to directly interact in live cells (Rios *et al.*, 2006; Hojo *et al.*, 2008). Furthermore, co-immunoprecipitation studies demonstrated the presence of interacting μ-CB_1 receptor complexes in heterologous cells coexpressing both receptors (Hojo *et al.*, 2008).

Examination of the properties of the μ-CB_1 heteromer in heterologous and endogenous systems shows that while agonists to individual protomers can activate G-protein-mediated signalling and ERK phosphorylation, a combination of agonists to both protomers causes a decrease in signalling (Rios *et al.*, 2006). Moreover, a study used receptors fused to chimeric G-proteins to show that the agonist for either protomer in the μ-CB_1 heteromer induces signalling by activating the same G-protein (Hojo *et al.*, 2008). Heteromerization between μ and CB_1 receptors is of physiological relevance given that while agonists to either protomer cause an increase in neurite outgrowth in Neuro 2A cells, a combination of agonists to both receptors reduces neurite outgrowth by decreasing the phosphorylation of Src and STAT3 (Rios *et al.*, 2006) (Table 2). Taken together, these studies show that μ-CB_1 heteromerization leads to modulation of individual protomer signalling and that this heteromer may have a physiological role.

In order to understand the physiological role of μ-CB_1 heteromers, bivalent ligands comprising a selective μ receptor agonist connected to a CB_1 receptor-selective antagonist/inverse agonist, via spacers of different lengths, have been generated (Le Naour *et al.*, 2013). Among these, the ligand having a 20-atom spacer was found to bridge both receptors in the μ-CB_1 heteromer (Table 4) and this compound exhibited potent antinociceptive effects without development of antinociceptive tolerance (Le Naour *et al.*, 2013) (Table 2). Because the development of antinociceptive tolerance is a key side effect of opiates and cannabinoids that limits their clinical use, the μ-CB_1 heteromer is a potential target for the development of analgesics with reduced side effects.

Heteromers involving opioid and catecholamine receptors

δ opioid and α_{2A} adrenoceptor heteromers

Functional interactions between δ and α_{2A} adrenoceptors suggested possible heteromerization between these two receptors. This included studies showing that attenuation of substance P-mediated antinociception was potentiated by a combination of α_{2A} adrenoceptor and δ receptor agonists (Fairbanks *et al.*, 2000), and that synergistic antinociceptive interactions between these two receptors were observed in μ receptor-knockout but not in α_{2A} adrenoceptor-knockout mice (Stone *et al.*, 1997; Fairbanks *et al.*, 2002; Guo *et al.*, 2003). Colocalization studies suggested that δ and α_{2A} adrenoceptors could form interacting complexes as these receptors were extensively colocalized to the same cells in the terminals of capsaicin-sensitive substance P-expressing primary afferent neurons (Riedl *et al.*, 2009). In addition, BRET assays show that these two receptors were close enough to directly interact in live cells (Rios *et al.*, 2004). Furthermore, co-immunoprecipitation studies using epitope-tagged receptors

Table 2

Heteromers involving opioid and cannabinoid/catecholamine receptors[a]

Heteromer pair	*In vitro* heteromer properties (binding, signalling, trafficking)	*In vivo* effects of reagents targeting heteromers	References
δ-CB_1[b]	*Detection* Colocalization, Co-IP, BRET *Binding* Remains to be determined. *Signalling* ↓ potency of CB_1 receptor agonist. Heteromer signalling involves PLC-mediated recruitment of β-arrestin. *Trafficking* ↑ in CB_1 receptor cell surface localization.	*δ-CB_1 heteromer-selective antibody* Antibody detects ↑ in heteromer levels in cortex in neuropathic pain model (L5 spinal nerve ligation/transection). Antibody blocks CB_1 receptor-mediated enhancement of δ receptor activity.	Rios *et al.*, 2006; Bushlin *et al.*, 2012; Rozenfeld *et al.*, 2012
μ-CB_1R[c]	*Detection* Co-IP, BRET, FRET *Binding* Remains to be determined. *Signalling* ↓ in signalling with μ + CB_1 receptor agonists (ERK1/2, Src, STAT3 phosphorylation). *Trafficking* CB_1 receptor agonist or longer spacer bivalent ligand does not induce internalization, but μ agonist or mixture of both monovalent agonists induces heteromer internalization.	*Bivalent ligand (μ agonist joined to CB_1 receptor antagonist by a spacer arm)* Antinociception (i.t. and i.c.v.) without development of tolerance.	Rios *et al.*, 2006; Hojo *et al.*, 2008; Le Naour *et al.*, 2013
δ-α_{2A}	*Detection* Colocalization, Co-IP, BRET *Binding* Remains to be determined. *Signalling* Remains to be determined. *Trafficking* Remains to be determined.	*Combination of δ receptor agonist and α_{2A} receptor agonist* Synergy in antinociceptive effect (i.t.). ↑ in δ receptor-mediated neurite outgrowth.	Rios *et al.*, 2004; Overland *et al.*, 2009; Riedl *et al.*, 2009
δ-β_2	*Detection* Co-IP, BRET *Binding* No change. *Signalling* No change. *Trafficking* Internalized by δ receptor agonists and vice versa.		Jordan *et al.*, 2001; Ramsay *et al.*, 2002
κ-β_2	*Detection* Co-IP, BRET *Binding* No change. *Signalling* No change in β_2 adrenoceptor-mediated G-protein signalling but ↓ in β_2 adrenoceptor-mediated ERK1/2 phosphorylation. *Trafficking* Suppression of β_2 adrenoceptor agonist-mediated β_2 adrenoceptor internalization.		Jordan *et al.*, 2001; Ramsay *et al.*, 2002
μ-α_{2A}	*Detection* Colocalization, Co-IP, BRET, FRET *Binding* Remains to be determined. *Signalling* ↑ in μ receptor signalling in the presence of α_{2A} adrenoceptor. ↓ in μ receptor signalling in the presence of α_{2A} adrenoceptor ligands. *Trafficking* Controversial. One study showed no internalization of the heteromer but another study showed internalization of the heteromer and cross-desensitization by α_2 adrenoceptor or μ agonist via p38 MAPK.		Jordan *et al.*, 2003; Zhang and Limbird, 2004; Vilardaga *et al.*, 2008; Tan *et al.*, 2009

[a]Modified from (Gomes *et al.*, 2013a).
[b,c]These heteromers are useful therapeutic targets, based on the known roles of one or both protomers. Therapeutic targets: [b]Neuropathic pain; [c]Antinociception.

show that the two receptors form interacting complexes (Rios *et al.*, 2004) (Table 2). Although a combination of colocalization, co-immunoprecipitation and BRET studies suggest the formation of δ-α_{2A} heteromers, not much is known about how interactions between these two receptors modulate the binding, signalling and trafficking properties of individual protomers. Such information is necessary in order to consider this receptor pair as a bona fide heteromer.

The physiological relevance of probable δ-α_{2A} heteromerization was examined by investigating agonist-mediated neurite outgrowth in Neuro 2A cells. These studies showed that coexpression of α_{2A} adrenoceptors could increase deltorphin II-(a δ receptor agonist) mediated neurite outgrowth in Neuro 2A cells. This suggests that α_{2A} adrenoceptors could allosterically modulate δ-receptor function (Rios *et al.*, 2004). Another study examined the effects of δ receptor and α_{2A} adrenoceptor agonists on antinociception and found that a combination of agonists to the two receptors resulted in ~30-fold increase in antinociceptive potency compared with administration of individual receptor agonists (Overland *et al.*, 2009). Moreover, this increase in antinociceptive potency by a combination of δ receptor and α_{2A} adrenoceptor agonists was blocked by a PKC and not by a PKA inhibitor, whereas when each agonist was individually administered its potency was blocked by a PLC and not by a PKC inhibitor (Overland *et al.*, 2009). The PKC isoform involved in these interactions between δ and α_{2A} adrenoceptor agonists has been recently identified as PKCε (Schuster *et al.*, 2013). The antinociceptive synergy observed with a combination of δ and α_{2A} adrenoceptor agonists is thought to be due to the synergistic inhibition of the release of calcitonin gene-related peptide (CGRP) from the terminals of primary afferent neurons in the spinal cord (Overland *et al.*, 2009) (Table 2). Together, these studies suggest a role for δ-α2A interacting receptor complexes in pain modulation but additional studies showing that disruption of the heteromer pair leads to changes in associating complexes and pain modulation would further support this point.

δ opioid and β_2 adrenoceptor heteromers

Studies showing that [Leu]enkephalin (a δ-receptor agonist) modulates β_2 adrenoceptor signalling and contraction in the heart suggested possible heteromerization between these two receptors (Pepe *et al.*, 1997; Xiao *et al.*, 1997). Co-immunoprecipitation studies showed that these receptors form interacting complexes at the cell surface of HEK-293 cells (Jordan *et al.*, 2001). In addition, BRET studies showed that δ receptors and β_2 adrenoceptors were in close proximity and could directly interact in live cells. Moreover, the BRET signal was not changed in the absence or presence of agonists to both receptors leading to the suggestion that δ-β_2 heteromers are constitutively formed in cells coexpressing both receptors (Ramsay *et al.*, 2002) (Table 2).

Examination of the ligand-binding properties of the δ-β_2 heteromer shows that heteromerization between these two receptors does not lead to alterations in the pharmacological properties of individual protomers (Jordan *et al.*, 2001) (Table 2). In addition, the signalling properties of δ receptor-agonists (inhibition of AC activity) are similar in cells expressing either δ receptors or the δ-β_2 heteromer (Jordan *et al.*, 2001). Similarly, the signalling properties of β_2 adrenoceptor agonists (stimulation of AC activity) are similar in cells expressing either β_2 adrenoceptors or the δ-β_2 heteromer (Jordan *et al.*, 2001). Moreover, the activation of MAPK induced by δ receptor or β_2 adrenoceptor agonists in δ-β_2 heteromer-expressing cells was similar to that observed in cells expressing individual receptors (Jordan *et al.*, 2001) (Table 2). These results indicate that heteromerization of δ receptors with β_2 adrenoceptors does not significantly affect signalling by agonists to individual protomers (Table 2).

Examination of the trafficking properties of the δ-β_2 heteromer shows that treatment with either δ receptor or β_2 adrenoceptor agonists induces heteromer internalization (Jordan *et al.*, 2001), while in cells expressing individual receptors, δ receptor agonists do not induce β_2 adrenoceptor endocytosis and β_2 adrenoceptor agonists do not induce δ-receptor endocytosis (Jordan *et al.*, 2001). These findings suggest that heteromerization between δ receptors and β_2 adrenoceptors leads to alterations in the trafficking of individual receptors (Table 2).

Very little 'direct evidence' is available about the physiological role of δ-β_2 heteromers. Studies suggest that interactions between δ receptors and β_2 adrenoceptors may play a role in pathological conditions such as myocardial ischaemia (Huang *et al.*, 2007). Thus further studies, using either heteromer-selective ligands/antibodies or agents that disrupt the heteromer, are needed to elucidate the role of the δ-β_2 heteromer during normal physiology and pathology.

κ-opioid receptor and β_2 adrenoceptor heteromers

Studies showing that κ receptors and β_2 adrenoceptors are present in the heart, and that a β_2 adrenoceptor agonist modulates radiolabelled ligand binding to κ receptors suggested possible heteromerization between these two receptors (Ventura *et al.*, 1989; Tai *et al.*, 1991). Co-immunoprecipitation studies carried out in HEK-293 cells coexpressing *myc*-tagged κ receptors and Flag-tagged β_2 adrenoceptors detected the presence of interacting complexes at the cell surface (Jordan *et al.*, 2001). Furthermore, BRET assays show that the two receptors are close enough to directly interact in live cells (Ramsay *et al.*, 2002) (Table 2). Taken together, these studies indicate that κ receptors and β_2 adrenoceptors can form heteromers in cells coexpressing both receptors.

Examination of the ligand-binding properties of κ-β_2 heteromers shows that there are no changes in binding affinity of individual receptor ligands, such as U-69593, norbinaltorphimine and isoprenaline, when comparing with cells expressing either κ receptors or β_2 adrenoceptors (Jordan *et al.*, 2001); this suggests that heteromerization between these two receptors does not lead to alterations in the pharmacological properties of individual receptor protomers (Table 2).

The signalling properties of the κ-β_2 heteromer were investigated using the AC and the MAPK phosphorylation assays. In the case of the AC assay, in cells expressing the κ-β_2 heteromer, there were no changes in the ability of κ receptor agonists to inhibit and of β_2 adrenoceptor agonists to stimulate enzyme activity compared with cells expressing individual receptors (Jordan *et al.*, 2001). In the case of agonist-mediated MAPK activation, it was observed that in

cells expressing the κ-β_2 heteromer, the κ receptor agonist but not the β_2 adrenoceptor agonist could induce ERK1/2 phosphorylation. These findings suggest that while heteromerization with κ receptors does not significantly affect the functional G-protein coupling properties of β_2 adrenoceptors, it may promote biased signalling at β_2 adrenoceptors by preserving G-protein-mediated signalling (i.e. AC activity) but impairing MAPK signalling (Jordan *et al.*, 2001) (Table 2).

Examination of trafficking properties show that treatment with a β_2 adrenoceptor agonist, that induces β_2 adrenoceptor internalization in cells expressing only this receptor, does not induce β_2 adrenoceptor internalization in cells expressing the κ-β_2 heteromer (Jordan *et al.*, 2001) (Table 2). This suggests that heteromerization between κ receptors and β_2 adrenoceptors modulates the trafficking properties of the β_2 adrenoceptors.

Very little is known about the physiological relevance of κ-β_2 heteromers. Thus further studies are needed to evaluate the role of this heteromer pair in normal physiology and pathology and particularly in cardiac pathology, given the presence of both receptors in the heart.

μ opioid and α_{2A} adrenoceptor heteromers

Studies showing functional interactions between μ receptors and α_{2A} adrenoceptors suggested that these two receptors could form heteromers. These included studies showing that a combination of μ receptor and α_{2A} adrenoceptor agonists resulted in antinociceptive synergy and that the potency of morphine-mediated antinociception is decreased in mice lacking α_{2A} adrenoceptors (Drasner and Fields, 1988; Ossipov *et al.*, 1997; Stone *et al.*, 1997). Several lines of evidence support heteromerization between μ receptors and α_{2A} adrenoceptors. For example, immunohistochemical analysis using Flag-tagged μ and hemagglutinin-tagged α_{2A} adrenoceptors demonstrate colocalization of both receptors not only at the plasma membrane but also within intracellular vesicles (Jordan *et al.*, 2003). In addition, co-immunoprecipitation studies detect the presence of interacting μ-α_{2A} complexes in heterologous cells and in primary hippocampal neurons, and BRET and FRET assays show that the two receptors are close enough to form interacting complexes in live cells (Jordan *et al.*, 2003; Zhang and Limbird, 2004; Vilardaga *et al.*, 2008). Moreover, FRET analysis revealed that binding of an agonist to μ receptors suppressed the α_{2A} adrenoceptor agonist (norepinephrine)-induced FRET signal probably through a conformational change transmitted from the μ receptors to the α_{2A} adrenoceptors (Vilardaga *et al.*, 2008) (Table 2). Interestingly, while treatment with agonists to individual receptors, such as morphine (μ receptor) or clonidine (α_{2A} adrenoceptor), leads to an apparent increase in the levels of μ-α_{2A} complexes, a combination of agonists to the two receptors leads to a decrease to below the basal levels (Jordan *et al.*, 2003). This suggests that either co-occupancy of both protomers disrupts heteromer formation or makes the latter more susceptible to the effects of detergents used for cell lysis.

Examination of G-protein and MAPK activation in cells expressing the μ-α_{2A} heteromer shows that the morphine-mediated signalling is enhanced compared with cells expressing only μ receptors; however, this enhancement of signalling is not seen when a combination of morphine with clonidine (α_2 adrenoceptor agonist) is used (Jordan *et al.*, 2003). Furthermore, the G-protein as well as MAPK activation by noradrenaline (α_{2A} adrenoceptor agonist) is decreased in the presence of morphine (Vilardaga *et al.*, 2008). Similar findings were made with spinal cord neurons (Jordan *et al.*, 2003), suggesting that the interactions between μ receptors and α_{2A} adrenoceptors also take place in endogenous systems and that the signalling of μ receptors can be modulated by α_{2A} adrenoceptors (Table 2). This is also supported by studies carried out in mouse dorsal root ganglion neurons which show that prolonged treatment with the μ receptor agonist, DAMGO, or the α_2 adrenoceptor agonist, clonidine, induced cross-desensitization between μ and α_{2A} receptor-mediated inhibition of voltage-gated Ca^{+2} current and this was associated with the co-internalization of μ and α_{2A} adrenoceptors (Tan *et al.*, 2009).

Although studies have shown that the analgesic potency of spinally administered morphine is decreased in mice lacking α_{2A} adrenoceptors (Stone *et al.*, 1997), very little is known about the physiological relevance of μ-α_{2A} heteromers. Thus further studies, using either heteromer-selective ligands/antibodies or agents that disrupt the heteromer, are needed to elucidate the role of the μ-α_{2A} heteromer during normal physiology and pathology in particular during pain attenuation.

Other heteromers involving opioid receptors

δ opioid and chemokine receptor heteromers

The formation of heteromers between δ opioid receptors and the chemokine receptor CXCR4 (δ-CXCR4 heteromer) is suggested by FRET and co-immunoprecipitation studies carried out using heterologous cells and primary monocytes from healthy donors (Pello *et al.*, 2008) (Table 3). The FRET signal and the level of interacting δ-CXCR4 complexes did not change in the presence or absence of receptor-selective agonists such as DPDPE (δ receptor agonist) or CXCL12 (CXCR4 agonist), suggesting that these heteromers are constitutively formed (Pello *et al.*, 2008).

Examination of intracellular signalling shows that although selective agonists (DPDPE or CXCL12) lead to $G\alpha_{i/o}$-protein activation in cells that coexpress both receptors, a combination of these two agonists inhibits receptor association with $G\alpha_{i/o}$ protein (Pello *et al.*, 2008). These observations suggest that while δ-CXCR4 heteromers are fully functional when activated by agonists to either protomer, a combination of agonists to both protomers inactivates the heteromer. Furthermore, CXCR12-induced phosphorylation of CXCR4 (or desensitization of CXCR4) was not altered by cotreatment with DPDPE (Pello *et al.*, 2008) (Table 3), suggesting that the simultaneous activation of both protomers in δ-CXCR4 heteromers does not promote heterologous desensitization.

Very little is known about the physiological role of δ-CXCR4 heteromers. However, both δ receptors and CXCR4 are widely distributed in brain tissues and immune cells, and play key roles in inflammation processes and in pain sensation. As activation of both protomers in the δ-CXCR4 heteromer appears to result in a 'silent' receptor complex, further studies to evaluate the role of this heteromer *in vivo*, particularly under inflammatory conditions are needed.

Table 3

Heteromers involving opioid receptors and GPCRs other than cannabinoid or catecholamine receptors[a]

Heteromer pair	*In vitro* heteromer properties (binding, signalling, trafficking)	*In vivo* effects of reagents targeting heteromers	References
δ-CXCR4	*Detection* Co-IP, FRET *Binding* No change. *Signalling* Inactivated by δ + CXCR4 agonists. No association with G-proteins in the presence of δ + CXCR4 agonists. *Trafficking* No change.		Pello *et al.*, 2008
δ-SNSR-4	*Detection* BRET *Binding* Remains to be determined *Signalling* Preferential $G\alpha_q$ signalling and attenuation of $G\alpha_i$ signalling. *Trafficking* ↓ in δ receptor endocytosis by BAM22 in the presence of SNSR-4.		Breit *et al.*, 2006
κ-APJ	*Detection* Colocalization, Co-IP, BRET *Binding* Remains to be determined. *Signalling* ↑ in heteromer-mediated PKC signalling. *Trafficking* Remains to be determined.		Li *et al.*, 2012
μ-CCR5[b]	*Detection* Co-IP *Binding* Binding affinity for a ligand to one protomer not changed in the presence of ligand to partner protomer. *Signalling* ↓ in μ receptor -mediated G-protein activation by CCR5 receptor agonist and vice versa. *Trafficking* μ receptor internalization by μ and not by CCR5 receptor agonists and vice versa.	*Bivalent ligand (Bivalent ligand 1)* More potent inhibition of viral entry compared with naltrexone + maraviroc in antiviral activity assay.	Suzuki *et al.*, 2002; Chen *et al.*, 2004; Yuan *et al.*, 2012; 2013
μ1D-GRPR[c]	*Detection* Co-IP *Binding* Not reported. *Signalling* μ receptor-mediated Ca^{+2} signalling only in cells expressing the heteromer. *Trafficking* ↑ in μ receptor -mediated GRPR internalization.	*TAT-fusion protein (TAT-μ1D$_{CT}$)* TAT-μ1D$_{CT}$ disrupts μ1D-GRP receptor heteromers and blocks morphine-induced scratching without affecting analgesia.	Liu *et al.*, 2011
μ-mGlu$_5$[d]	*Detection* Co-IP *Binding* No change in binding affinity for μ receptor agonist. ↑ in binding affinity for mGlu$_5$ receptor antagonist. *Signalling* No change in μ receptor agonist-mediated inhibition of adenylate cyclase activity. mGlu$_5$ receptor antagonist ↓ μ receptor agonist-mediated phosphorylation and desensitization of μ receptors. *Trafficking* mGlu$_5$ receptor antagonist ↓ μ receptor agonist-mediated internalization of μ receptors.	*Bivalent ligand (MMG22)* MMG22 antinociception (i.t. and i.c.v.) is equipotent to morphine in naïve mice, and more potent in LPS-treated mice with less tolerance and respiratory depression. MMG22 shows antinociception (i.t.) in CFA-induced inflammatory pain or bone cancer pain model.	Schroder *et al.*, 2009; Akgun *et al.*, 2013

Table 3

Continued

Heteromer pair	*In vitro* heteromer properties (binding, signalling, trafficking)	*In vivo* effects of reagents targeting heteromers	References
μ-5-HT_{1A}	*Detection* Colocalization, Co-IP, BRET *Binding* Remains to be determined. *Signalling* Transactivation of G-protein fused to 5-HT_{1A} receptor by μ receptor agonist. Activation of ERK1/2 by μ receptor agonist is blocked by 5-HT_{1A} receptor agonist pretreatment. *Trafficking* No co-internalization by protomer-selective agonists.		Daval *et al.*, 1987; Pompeiano *et al.*, 1992; Wang *et al.*, 1998; Zhang *et al.*, 2000; Kishimoto *et al.*, 2001; Cussac *et al.*, 2012
μ-$NK1^{d}$	*Detection* Colocalization, Co-IP, BRET *Binding* ↑ in affinity for μ receptor agonist. No change in affinity for NK_1 receptor agonist. *Signalling* Pre-incubation with μ agonist ↓ NK_1 receptor-mediated ERK phosphorylation and vice versa. *Trafficking* Co-internalization by protomer-selective agonists.	*Bivalent peptide* Assay shows that the peptide exhibits μ agonist and NK_1 receptor antagonist activity. *Small molecule ligands* Assay shows that the ligands exhibit μ agonist and NK_1 receptor antagonist activity. *Multifunctional μ/δ agonist/NK_1 receptor antagonist compound (TY027)* TY027 exhibits antinociception (i.c.v., i.t.) in naïve mice. TY027 exhibits antinociception (i.t., i.v.) against spinal nerve ligation-induced hyperalgesia. TY027 produced antinociception with low tolerance, dependence or rewarding effects and was not accompanied by opioid-related emesis or constipation.	Aicher *et al.*, 2000a,b; Pfeiffer *et al.*, 2003; Yamamoto *et al.*, 2007; Vardanyan *et al.*, 2011; Largent-Milnes *et al.*, 2013
μ-sst_{2A}	*Detection* Colocalization, Co-IP *Binding* ↓ in binding affinity for sst_2 receptor agonists. No change in binding affinity for μ receptor agonist. *Signalling* ↑ inhibition of adenylate cyclase activity. No change in ERK1/2 activation. Pretreatment with the protomer agonist causes cross-desensitization. *Trafficking* sst_2 receptor agonist induces heteromer internalization. μ agonist internalizes μ receptors but not sst_{2A} receptors.		Pfeiffer *et al.*, 2002

[a]Modified from (Gomes *et al.*, 2013a).
[b,c,d]These heteromers are useful therapeutic targets, based on the known roles of one or both protomers. Therapeutic targets: [b]Antiviral activity; [c]Morphine-induced itch; [d]Antinociception.

δ opioid and sensory neuron-specific receptor (SNSR4) heteromers

The sensory neuron-specific receptor 4 is a GPCR with many names including SNSR3, SNSR4 and the official name of MRGPRX1 receptor; for brevity here it will be referred to as SNSR4. Heteromerization between δ receptors and SNSR4 was investigated based on studies showing that both receptors are present in dorsal root ganglia, and are activated by the bovine medulla adrenal peptide 22 (BAM22; a cleavage product of proenkephalin) although δ receptors mediate antinociceptive responses while SNSR4 mediates nociceptive responses (Lembo *et al.*, 2002; Grazzini *et al.*, 2004). BRET assays carried out in heterologous cells coexpressing δ and SNSR4 show that both receptors are in close proximity to one another and

could directly interact in live cells (Breit *et al.*, 2006) (Table 3). While δ receptor-selective agonists activate $G\alpha_{i/o}$-mediated signalling, SNSR4-selective agonists activate $G\alpha_q$-mediated signalling in cells expressing either δ receptors or SNSR4 or in cells coexpressing both receptors; this suggests that each receptor in the heteromeric complex acts as an independent signalling unit (Breit *et al.*, 2006). Interestingly, naltrexone, an opioid receptor antagonist, can block BAM22-mediated $G\alpha_q$ activation (Breit *et al.*, 2006) suggesting transinhibition of SNSR4 signalling by δ receptors within the δ-SNSR4 heteromer. Furthermore, costimulation of both protomers in the δ-SNSR4 heteromer leads to preferential activation of $G\alpha_q$-mediated signalling (PLC activation) and inhibition of $G\alpha_{i/o}$-mediated signalling (Breit *et al.*, 2006) (Table 3). This regulatory influence of SNSR4 on δ receptor signalling is not due to the PKC-mediated δ receptor desensitization (Breit *et al.*, 2006). Similar observations were made with cultured dorsal root ganglia from rat embryos (Breit *et al.*, 2006), indicating that these changes in δ receptor coupling and signalling because of heteromerization with SNSR4 also occur *in vivo*.

As both δ and SNSR4 contribute to the regulation of pain sensation (antinociception and nociception, respectively), further investigation on the role of the δ-SNSR4 heteromer in normal physiology and pathology is needed.

κ opioid and apelin receptor (APJ) heteromers

Studies have suggested the formation of heteromers between κ receptors and APJ receptors. Immunocytochemical studies in heterologous cells coexpressing both receptors show that both are colocalized predominantly at the plasma membrane (Li *et al.*, 2012). Co-immunoprecipitation studies show that both receptors form interacting complexes and BRET assays show that they are in close enough proximity to directly interact in live cells (Li *et al.*, 2012). Interestingly, treatment with receptor-specific agonists such as dynorphin A1–13 (for κ receptors) and apelin-13 (for APJ receptors) increased the BRET ratio, indicating that either the heteromerization between these two receptors was facilitated by receptor occupancy or that the latter induced conformational changes that decreased the distance between the epitope tags on individual protomers (i.e. between luciferase and YFP tags) (Li *et al.*, 2012) (Table 3).

Signalling by the κ-APJ heteromer was examined in heterologous cells and in cells that endogenously express both receptors. These studies show that treatment with an agonist to either receptor induces a PKC-dependent ERK1/2 activation that is two- to threefold higher in cells coexpressing both receptors compared with cells expressing individual receptors (Li *et al.*, 2012). In addition, heteromerization between κ and APJ receptors leads to an increase in PKC-mediated signalling and a decrease in PKA-mediated signalling compared with cells expressing individual receptors (Li *et al.*, 2012) (Table 3). Taken together, these studies show that heteromerization between κ and APJ receptors leads to modulation of signalling by individual protomers.

The functional consequence of κ-APJ heteromerization was observed at the level of cell proliferation where treatment with either dynorphin A1–13 or apelin-13 significantly increased the proliferation of cells expressing the heteromer compared with cells expressing individual receptors (Li *et al.*, 2012). Although not much is known about the role of the κ-APJ heteromer *in vivo* either in normal physiology or during pathology, the distribution of the dynorphin/κ receptor system and of the apelin/APJ system in the nuclei of the hypothalamus involved in regulation of arginine vasopressin release as well in the cardiovascular system (Sherman *et al.*, 1986; Tsushima *et al.*, 1993; Reaux *et al.*, 2001), suggests a potential role for κ-APJ heteromers in cardiovascular regulation.

μ opioid and chemokine receptor CCR5 heteromers

Studies showing that μ-opioid receptors are present in immune cells and that morphine treatment increased the expression of the chemokine receptor CCR5 in lymphocytes led to investigations on heteromerization between these two receptors (Chuang *et al.*, 1995; Miyagi *et al.*, 2000). Co-immunoprecipitation studies show that CCR5 forms an interacting complex with μ receptors in cell lines that coexpress both receptors and that this is not modulated by treatment with receptor-selective ligands (Suzuki *et al.*, 2002; Chen *et al.*, 2004) (Table 3).

Examination of the pharmacological properties of the μ-CCR5 heteromer shows that pretreatment with the μ receptor agonist, DAMGO, did not change the binding of radiolabelled CCL4 (MIP-1β) to CCR5. Similarly, CCL5, another CCR5 ligand, did not change the binding properties of radiolabelled ligands to μ receptors (Chen *et al.*, 2004) (Table 3). Interestingly, pretreatment with either DAMGO or CCL5 reduced CCL5- or DAMGO-mediated [^{35}S]GTPγS binding respectively (Chen *et al.*, 2004). These results indicate that pretreatment with agonist to one protomer in the heteromeric complex reduces the ability of the partner receptor to activate G-proteins.

Examination of the trafficking properties of μ-CCR5 heteromers shows that the μ agonist, DAMGO, induced internalization of μ receptors and not of CCR5 while the CCR5 agonist, CCL5, induced internalization of CCR5 and not of μ receptors (Chen *et al.*, 2004) (Table 3) suggesting that agonists selective for one receptor do not affect internalization of the other receptor in μ-CCR5 heteromers. Interestingly, pretreatment with either DAMGO or CCL5 can enhance the phosphorylation of both receptors in the heteromer, suggesting heterologous desensitization or cross-desensitization (Chen *et al.*, 2004). In this context, activation of PKCζ has been reported to be involved in the cross-desensitization between μ and CCL5 (Song *et al.*, 2011) (Table 3). The cross-desensitization between μ receptors and CCR5 within the heteromeric complex may modulate the physiological effects of opioids and chemokines in pathological conditions such as HIV infection or opiate addiction (Table 3).

A bivalent ligand targeting the μ-CCR5 heteromer that comprises a μ-selective antagonist pharmacophore, naltrexone, tethered through a 21-atom spacer to the CCR5-selective antagonist pharmacophore, maraviroc, has been developed (Yuan *et al.*, 2012) (Table 4). This bivalent ligand is reported to be twice as potent as an inhibitor of viral entry, as a mixture of both antagonists *in vitro* (Yuan *et al.*, 2013) (Table 3), suggesting a possible clinical usefulness of bivalent ligands targeting the μ-CCR5 heteromer against infection by HIV.

Table 4

List of ligands targeting opioid receptor heteromers

Target Heteromer Pair	Ligands	Pharmacophores	Spacer length	References
δ-μ	Bivalent ligand MDAN21	δ antagonist: DN-21 μ agonist: MA-19	21-atom	(Daniels *et al.*, 2005; Gomes *et al.*, 2013b; Harvey *et al.*, 2012)
	Bivalent ligand L2	δ antagonist:ENTI μ agonist: oxymorphone	optimized for the heteromer(19–22-atom)	
	Bivalent ligand L4	δ agonist: DM-SNC80 μ antagonist: naltrexone	(not exactly mentioned)	
	Biased agonist CYM51010	δ-μ agonist: CYM51010	Not applicable (N/A)	
δ-κ	Bivalent ligand KDN-21	δ antagonist: naltrindole (NTI) κ antagonist: 5′-guanidinonaltrindole (5′-GNTI)	21-atom	(Bhushan *et al.*, 2004; Waldhoer *et al.*, 2005)
	Heteromer targeting agonist 6′-GNTI*	δ-κ agonist: 6′-guanidinonaltrindole (6′-GNTI)	N/A	
μ-κ	Heteromer targeting agonist NNTA	μ-κ agonist: N-naphthoyl-β-naltrexamine (NNTA)	N/A	(Yekkirala *et al.*, 2011)
μ1G-NOP	Heteromer targeting ligand $IBN_{tx}A$	μ1G-NOP agonist: iodobenzoylnaltrexamide ($IBN_{tx}A$)	N/A	(Majumdar *et al.*, 2011)
μ-CB_1R	Bivalent ligand	μ agonist: α-oxymorphamine CB1 antagonist: SR141716	20-atom	(Le Naour *et al.*, 2013)
μ-CCR5	Bivalent ligand Bivalent ligand 1	μ antagonist: naltrexone CCR5 antagonist: maraviroc	21-atom	(Yuan *et al.*, 2012)
μ-mGluR5	Bivalent ligand MMG22	μ agonist: oxymorphone mGluR5 antagonist: m-methoxy-2-methyl-6-(phenylethynyl) pyridine (M-MPEP)	22-atom	(Akgun *et al.*, 2013)
μ-NK1	Bivalent peptides	Opioid agonist: H-Tyr-D-Ala-Gly-Phe NK1 antagonist: Pro-Leu-Trp-O-3,5-$Bzl(CF_3)_2$	N/A	(Largent-Milnes *et al.*, 2013; Vardanyan *et al.*, 2011; Yamamoto *et al.*, 2007)
	Bivalent ligands small molecule	μ agonist: fentanyl NK1 antagonist: L732138	N/A	
	Multifunctional μ/δ agonist/NK1 antagonist compound TY027	μ-NK1 agonist: H-Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-3,5-$Bn(CF_3)_2$ (TY027)	N/A	

*A recent report shows that 6′-GNTI exhibits biased agonistic properties at κ receptors (Rives *et al.*, 2012); N/A, not available

μ opioid 1D (μ1D) receptor and gastrin-releasing peptide (GRP) receptor heteromers

μ1D is a μ receptor isoform comprising exons 1–3 and 8–9 of the *Oprm* gene and μ1D receptors colocalize with GRP receptors (also known as bombesin BB_2 receptors) in the dorsal horn of the spinal cord (Liu *et al.*, 2011). Co-immunoprecipitation studies with heterologous cells coexpressing both receptors or with spinal cord membranes show that μ1D and GRP receptors form interacting complexes (Liu *et al.*, 2011) (Table 3).

Examination of the signalling properties of the μ1D-GRPR heteromer shows activation of the PLC-mediated Ca^{2+} signalling pathway by either morphine or GRP in cells coexpressing μ1D and GRP receptors (Liu *et al.*, 2011). Moreover, morphine or GRP receptor-induced calcium spikes are blocked by the GRP receptor antagonist or by naloxone, indicating that morphine cross-activates GRP receptors through μ1D receptors (Liu *et al.*, 2011) (Table 3). The μ1D-GRPR heteromer exhibits unique trafficking properties in that morphine treatment induces GRP receptor internalization in cells coexpressing both receptors but not in cells expressing only GRP receptors while a GRP receptor agonist did not induce μ1D receptor internalization in cells coexpressing both receptors (Liu *et al.*, 2011). Taken together, these results suggest that heteromerization leads to modulation of protomer signalling and trafficking properties.

In order to elucidate the physiological roles of μ1D-GRPR heteromers, a membrane-permeable peptide consisting of TAT fused to $μ1D_{CT}$ (TAT-$μ1D_{CT}$), that disrupts heteromer formation, has been developed. Intrathecal administration of TAT-$μ1D_{CT}$ specifically blocks morphine-induced scratching without affecting morphine-induced analgesia (Liu *et al.*, 2011) (Table 3). This suggests that the μ1D-GRPR heteromer may play a role in morphine-induced scratching. Moreover, the uncoupling of morphine-induced analgesia and

morphine-induced scratching by the TAT-μ1D$_{CT}$ peptide underscores the necessity for elucidating the function of individual μ receptor isoforms, which could be useful in the development of novel analgesics without side effects.

μ opioid and metabotropic glutamate mGlu$_5$ receptor heteromers

Co-immunoprecipitation studies show that μ and mGlu$_5$ receptors can form interacting complexes in HEK-293 cells coexpressing both receptors (Schroder *et al.*, 2009). Interestingly, treatment with 2-methyl-6-(phenylethynyl) pyridine (MPEP), a mGlu$_5$ receptor antagonist, increases the levels of interacting complexes (Schroder *et al.*, 2009); this suggests that occupancy of mGlu$_5$ receptors by MPEP affects the conformation of μ receptors which either facilitates the formation of μ-mGlu$_5$ heteromers or stabilizes the heteromer under the conditions used for receptor solubilization.

Examination of the pharmacological properties of μ-mGlu$_5$ heteromers shows that the binding affinity of the μ receptor agonist, DAMGO, is not changed when compared with cells expressing only μ receptors, while the binding affinity of the mGlu$_5$ receptor specific antagonist is increased when compared with cells expressing only mGlu$_5$ receptors (Schroder *et al.*, 2009). In addition, while the presence of mGlu$_5$ receptors does not affect binding and signalling by μ receptors, occupancy of mGlu$_5$ receptors with the inhibitor MPEP causes a decrease in DAMGO-mediated phosphorylation, internalization and desensitization of μ receptors (Schroder *et al.*, 2009). These findings suggest that a change in the conformation of mGlu$_5$ receptors by MPEP might allosterically regulate μ receptor function.

Given the wide expression of μ receptors and mGlu$_5$ receptors in the CNS and their role in regulation of pain transmission, opioid analgesia, dependence and withdrawal, ligands targeting this heteromer pair could play a role in pain regulation. In this context, a bivalent ligand targeting the μ-mGlu$_5$ heteromer has been developed. This ligand, MMG22, comprises a μ receptor agonist pharmacophore, oxymorphone, and a mGlu$_5$ receptor antagonist pharmacophore, *m*-methoxy-MPEP linked via a 22-atom spacer arm (Table 4). MMG22 exhibits antinociception similar to morphine in naïve mice (Akgun *et al.*, 2013). However, it exhibits 4000 times more potent antinociception and less tolerance and respiratory depression compared with morphine in LPS-treated mice, an inflammatory pain model (Akgun *et al.*, 2013). Taken together, these results suggest that MMG22 may be useful as a pharmacological tool to investigate μ-mGlu$_5$ heteromers *in vivo*, and in the development of novel drugs to treat inflammatory pain (Table 3).

μ opioid and 5-HT$_{1A}$ receptor heteromers

Heteromerization between μ receptors and 5-HT$_{1A}$ receptors was examined based on studies showing that (i) acute treatment with morphine increased 5-HT synthesis in different brain regions (Sastre-Coll *et al.*, 2002), (ii) the inhibition of 5-HT synthesis by 5-HT$_{1A}$ receptor agonists was enhanced in morphine-dependent animals (Sastre-Coll *et al.*, 2002), (iii) chronic administration of a 5-HT$_{1A}$ receptor agonist to the dorsal raphe nucleus delayed the development of tolerance to morphine (Nayebi and Charkhpour, 2006), (iv) chronic morphine administration increased 5-HT$_{1A}$ receptor activity in the medial prefrontal cortex, and decreased 5-HT1A receptor activity in the dorsal raphe nucleus (Lutz *et al.*, 2011) and (v) μ and 5-HT$_{1A}$ receptors colocalize in discrete brain regions (Daval *et al.*, 1987; Pompeiano *et al.*, 1992; Wang *et al.*, 1998; Zhang *et al.*, 2000; Kishimoto *et al.*, 2001). Co-immunoprecipitation and proximity-based assays carried out in cells coexpressing both receptors suggest that μ and 5-HT$_{1A}$ receptors could form heteromers (μ-5-HT$_{1A}$) (Cussac *et al.*, 2012) (Table 3).

Not much is known about the pharmacological properties of the μ-5-HT$_{1A}$ heteromer. Examination of the signalling properties showed that the μ receptor agonist could activate a Gα$_o$ protein that was covalently fused to 5-HT$_{1A}$ receptors only in cells coexpressing both receptors (Cussac *et al.*, 2012). In addition, phosphorylation of ERK1/2 induced following activation of μ receptors was blocked in the presence of the 5-HT$_{1A}$ receptor agonist (Cussac *et al.*, 2012). Examination of the trafficking properties of this heteromer shows that treatment with the agonist to one protomer did not induce internalization of the partner protomer (Cussac *et al.*, 2012). Although these studies indicate that μ-5-HT$_{1A}$ heteromerization may modulate the signalling properties of the μ receptor, further studies are needed to not only characterize the pharmacological properties of the μ-5-HT$_{1A}$ heteromer but also its role during normal physiology and pathology.

μ opioid and substance P receptor heteromers

Electron microscopy studies demonstrating colocalization of μ receptors with the substance P NK$_1$ receptor, in the dendrites of the dorsal horn, together with co-immunoprecipitation and proximity-based assays showing that μ and NK$_1$ receptors form interacting complexes provide evidence for μ-NK$_1$ heteromerization (Aicher *et al.*, 2000a,b; Pfeiffer *et al.*, 2003) (Table 3).

Examination of the pharmacological properties of the μ-NK$_1$ heteromer shows that the μ receptor agonist, DAMGO, exhibits ~threefold higher affinity compared with cells expressing only μ receptors, while substance P showed similar affinity as cells expressing only NK$_1$ receptors (Pfeiffer *et al.*, 2003). Competition-binding assays showed that DAMGO or substance P did not compete with [^{3}H]substance P or [^{3}H]DAMGO, respectively, in μ-NK$_1$ heteromer expressing cells (Pfeiffer *et al.*, 2003) (Table 3). However, both receptors in the μ-NK$_1$ heteromer can be cross-phosphorylated and co-internalized into the same endosomal compartment by protomer-selective agonists. This involves the recruitment of β-arrestin and formation of stable β-arrestin receptor complexes that are co-internalized (Pfeiffer *et al.*, 2003) (Table 3). Taken together, these results indicate that although μ-NK$_1$ heteromerization does not affect the binding properties of the individual receptor protomers, it modulates their trafficking properties.

Activation of the μ-NK$_1$ heteromer by either DAMGO or substance P leads to rapid and transient MAPK activation (ERK1/2 phosphorylation) that is comparable with cells expressing individual receptors (Pfeiffer *et al.*, 2003). However, pre-incubation with either DAMGO or substance P significantly attenuates either NK$_1$ or μ receptor-dependent ERK1/2 phosphorylation, respectively, suggesting that the μ-NK$_1$ heteromer undergoes homologous cross-desensitization (Pfeiffer *et al.*, 2003). Moreover, the resensitization of

μ receptor-mediated MAPK signalling is severely delayed in cells expressing μ-NK_1 heteromers as compared with cells expressing only μ receptors (Pfeiffer *et al.*, 2003) (Table 3). These results indicate that the formation of heteromers between μ and NK_1 receptors influences the kinetics of signalling by individual protomers.

Both μ and NK_1 receptors play important roles in modulation of nociceptive responses. Therefore, targeting the μ-NK_1 heteromer could lead to the development of novel therapeutics to treat pain. In this context, attempts have been made to develop ligands that selectively target μ-NK_1 heteromers. These include bivalent peptides designed to possess the peptide sequences for both μ/δ receptor agonist and NK_1 receptor antagonist (Table 4). These bivalent peptides exhibited potent binding affinity and G-protein activation similar to that of either DPDPE or DAMGO alone (Yamamoto *et al.*, 2007). Furthermore, the bivalent peptides exhibited agonistic activity for opioid receptors and antagonistic activity for NK_1 receptors in the guinea pig isolated ileum assay (Yamamoto *et al.*, 2007). Further studies are needed to elucidate the effects of these peptides on pain regulation *in vivo*. Other ligands developed to selectively target the μ-NK_1 heteromer include small molecules that represent variations of combinations of structures of the opioid agonist fentanyl and the NK_1 receptor antagonist pharmacophore L732138 (Vardanyan *et al.*, 2011); however, very little is known about the role of these small molecules in pain modulation. More recently, TY027, a multifunctional μ/δ receptor agonist/ NK_1 receptor antagonist compound, has been shown to have a preclinical profile of excellent antinociceptive efficacy, low abuse liability and no opioid-related emesis or constipation (Largent-Milnes *et al.*, 2013). TY027 exhibited antinociceptive efficacy in both non-injured and spinal nerve-ligated animals (Largent-Milnes *et al.*, 2013). In non-injured animals, the antinociceptive effect of TY027 was similar to that of morphine in the tail-flick test (Largent-Milnes *et al.*, 2013). Moreover, repeated administration of TY027 did not lead to development of antinociceptive tolerance, dependence or reward (Largent-Milnes *et al.*, 2013). Taken together, compounds targeting μ-NK_1 heteromers could be a promising therapeutic approach in treating patients who suffer from acute and chronic pain (Table 3).

μ opioid and somatostatin sst_{2A} receptor heteromers

The somatostatin sst_{2A} receptor and the μ receptor are closely related GPCRs that share ~38% sequence homology (Pfeiffer *et al.*, 2002). Heteromerization between these two GPCRs was suggested by immunohistochemical studies showing colocalization in the locus coeruleus (Pfeiffer *et al.*, 2002). Furthermore, co-immunoprecipitation studies showed that μ and sst_{2A} receptors formed interacting complexes (Pfeiffer *et al.*, 2002) (Table 3).

Pharmacological studies with μ-sst_{2A} heteromers showed that the binding affinities for the sst_{2A} receptor-selective agonist, L-779,976, were twofold lower than in sst_{2A} receptor homomers, while those for the μ receptor agonist, DAMGO, were similar to that of μ receptor homomers (Pfeiffer *et al.*, 2002) (Table 3). Signalling studies showed that the ability of L-779,976 or DAMGO to inhibit AC activity was higher in cells coexpressing both receptors compared with cells expressing either sst_{2A} or μ receptors; however, no differences were observed for agonist-mediated ERK1/2 phosphorylation between cells expressing receptor heteromers and those expressing individual homomers (Pfeiffer *et al.*, 2002). In addition, pretreatment with the agonist to one protomer attenuated signalling by the agonist to the partner protomer (Pfeiffer *et al.*, 2002); this suggests cross-desensitization of signalling in μ-sst_{2A} heteromers. Furthermore, cross-phosphorylation of the receptors was also observed after treatment with receptor-selective agonists in cells coexpressing μ-sst_{2A} heteromers (Pfeiffer *et al.*, 2002) (Table 3). Taken together, these results indicate that heteromerization between μ and sst_{2A} receptors leads to alterations in the signalling mediated via individual protomers.

Examination of the trafficking properties of the μ-sst_{2A} heteromer shows that while sst_{2A} receptor activation causes heteromer internalization, μ receptor activation induced only endocytosis of μ, but not of sst_{2A} receptors (Pfeiffer *et al.*, 2002). These results suggest that μ-sst_{2A} heteromerization influences the trafficking properties of individual receptors (Table 3).

μ receptors and sst_{2A} receptors coexist and functionally interact in pain-processing pathways (Schulz *et al.*, 1998), and some studies have described extensive cross-talk between opioid- and somatostatin-mediated analgesic responses (Betoin *et al.*, 1994). However, further studies are needed to elucidate the *in vivo* role of μ-sst_{2A} heteromers in antinociception.

Concerns and limitations of techniques used to detect and characterize receptor heteromers

As described above, over the last decade, an increasing number of heteromers involving opioid receptors have been described. Research for the existence of most of these heteromers was brought about by evidence showing functional interactions between two receptor protomers. This gives rise to the question of whether a reported heteromer is truly a heteromeric complex where occupancy/activation of one protomer modifies binding at or signalling of the other protomer or whether the interactions between two receptors expressed in the same cells are the result of cross-talk because of activation of down-stream signalling cascades. A case in point involves δ opioid and dopamine D_1 receptors where studies show functional interactions between these two receptors (George and Kertesz, 1987; Daunais and McGinty, 1994; Unterwald and Cuntapay, 2000; Ito *et al.*, 2006). Immunoelectron microscopic studies showing colocalization of both these receptors in the cytosol as well as in the plasma membrane of neurons in the striatum (Ambrose *et al.*, 2006) is not sufficient to establish that the two receptors heteromerize as it does not demonstrate that the receptors are directly associated with each other. Therefore, further evidence for the formation of heteromers between δ and dopamine D_1 receptors, such as co-immunoprecipitation studies and proximity-based assays, are needed.

Most of the heteromers pairs described in this review were determined through the use of epitope-tagged receptors and

a combination of co-immunoprecipitation studies and proximity-based assays such as BRET or FRET. It is to be noted that there are limitations to these techniques. For example, in the case of co-immunoprecipitation studies, the inherent hydrophobic nature of GPCRs could lead to the artifactual formation of interacting complexes. Thus when carrying out these studies, appropriate controls are needed such as (i) the use of tissue from animals lacking one of the receptors being investigated, (ii) using cells that express one of the two receptors, (iii) mixing cells that individually express each receptor prior to solubilization and immunoprecipitation, (iv) using different detergents for solubilization and (v) cross-linking cell surface proteins prior to solubilization and immunoprecipitation. Moreover, co-immunoprecipitation studies show that two receptors form interacting complexes but do not provide evidence for direct association between two receptors. Proximity-based assays, such as BRET or FRET, where a positive signal with/without ligand treatment would indicate that the two receptors are less than 100Å apart and therefore are directly associated with each other, have recently been used to provide support for direct association. However, a positive signal in these proximity-based assays could also be obtained because of overexpression of the receptors or because receptor occupancy leads to a movement of the acceptor and donor tags towards each other. Thus while carrying out proximity-based assays using epitope-tagged receptors, care should be taken to express both receptors at physiological levels and/or express different ratios of both receptors.

The limitations in the techniques used to ascertain whether two receptors form heteromers requires that a set of rules be applied to determine whether two receptors heteromerize. For *in vitro* studies, this would include the requirement that (i) the two receptors are present not only in the same cells but also in the same subcellular compartment; (ii) interacting receptor complexes can be isolated from cells that coexpress both receptors (but not from cells expressing only one receptor) using antibodies selective to the epitope tags on the receptors (and appropriate controls); (iii) proximity-based assays demonstrate that the donor or acceptor protein-tagged receptors exhibit energy transfer only in cells coexpressing receptors that are shown to interact by immunoprecipitation but not with receptors that have been shown not to interact; (iv) the heteromer exhibits a unique 'signalling profile' that is pharmacological, signalling or trafficking properties and that (v) the unique properties of the heteromer are blocked by agents (antibodies, ligands, peptides) that selectively recognize the heteromer. In a recent review, a set of criteria has been proposed to establish receptor heteromers in native tissue (Ferré *et al.*, 2009). These include that: (i) the two receptors should localize not only in the same cells in a tissue but also in the same subcellular compartment; (ii) interacting receptor complexes should be immuno-isolated from wild-type animal tissue but not from animals lacking one of the receptors using receptor-selective antibodies; (iii) the signalling profile described for the heteromer pair in heterologous cells should match with that observed with native tissue; (iv) selective probes, such as antibodies or labelled ligands, should detect the presence of the heteromer in tissue sections; (iv) disruption of the heteromer through the use of agents that disrupt the heteromeric interactions should lead to reduced association and accompanying alterations of the signalling profile of the heteromer in native tissues.

A point to be kept in mind is that varying the ratio of individual receptor protomers would affect the level of receptor heteromers. Studies using proximity-based assays, such as BRET, have examined this point in detail and found that increasing the concentration of the YFP-tagged protomer while keeping the concentration of the luciferase-tagged protomer constant leads to a saturation of the detected BRET signal (this is an hyperbolic curve). These studies found that receptor heteromerization occurs at relatively low expression levels, leads to saturation, and that for optimal receptor heteromerization the two individual protomers have to be present at relatively equal levels (Canals *et al.*, 2003; Terrillon *et al.*, 2003; Breit *et al.*, 2004). In our investigations of δ-μ receptor heteromerization, we found that increasing amounts of μ receptors into cells stably expressing δ receptors led to an initial increase followed by a decrease in δ-μ receptor interacting complexes as examined by co-immunoprecipitation (Rozenfeld and Devi, 2007). Similarly, increasing the levels of δ receptors (compared with μ) led to an initial increase and then a decrease in heteromer levels as examined by ligand-binding studies (Gomes *et al.*, 2011). These results indicate that GPCR heteromerization is expression-dependent and that the two receptors need to be expressed at approximately equal levels for optimal heteromeric association.

The majority of the above criteria are met by only a handful of heteromers; this is due to the lack of tools available to probe the presence or regulation of heteromers in native tissue. The recent development of reagents, such as heteromer-selective antibodies (Gupta *et al.*, 2010; Berg *et al.*, 2012; Bushlin *et al.*, 2012), TAT peptides that disrupt heteromers (He *et al.*, 2011; Liu *et al.*, 2011) or ligands targeting the heteromer (Bhushan *et al.*, 2004; Daniels *et al.*, 2005; Majumdar *et al.*, 2011; Yekkirala *et al.*, 2011; Yuan *et al.*, 2012; Akgun *et al.*, 2013; Gomes *et al.*, 2013b; Le Naour *et al.*, 2013), show promise that such studies are likely to move the field forward towards elucidating a role for GPCR heteromers in normal cell function and in pathology.

Conclusion

In the last decade, the formation of heteromers between opioid receptors and various GPCRs has been demonstrated and their unique intracellular signalling elucidated. Most studies used analytical techniques such as co-immunoprecipitation, colocalization, BRET and FRET assays in heterologous cells coexpressing epitope-tagged receptors to demonstrate the presence of receptor heteromers. Furthermore, recent GPCR crystal structural analysis provides support for the possibility that some GPCRs can form heteromers. Although these valuable techniques allow us to view the heteromers *in vitro*, when considering the physiological roles of heteromers *in vivo*, additional analytical tools are needed. As shown in this review, such *in vivo* analytical tools including selective ligands, antibodies and TAT-fusion proteins targeting selected opioid receptor heteromers are being developed and are providing clues to the physiological roles of these heteromers. For example, heteromer-selective antibodies help us ascertain the distribution of the heteromer in

endogenous tissues and to detect changes in protein heteromer levels under specific pathological/therapeutical conditions such as neuropathic pain state or chronic drug treatment. Of great interest is the fact that many of the heteromer-selective ligands described in this review exhibited more or equipotent antinociceptive effects when compared with morphine but with lesser side effects such as development of tolerance or dependence after chronic administration. These findings strongly support the idea of using heteromers involving opioid receptors as targets for the development of novel therapeutic agents to treat pain.

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Conflict of interest

The authors declare no conflict of interest.

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