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REVIEW

In vivo opioid receptor heteromerization: where do we stand?

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Opioid receptors are highly homologous GPCRs that modulate brain function at all levels of neural integration, including autonomous, sensory, emotional and cognitive processing. Opioid receptors functionally interact *in vivo*, but the underlying mechanisms involving direct receptor–receptor interactions, affecting signalling pathways or engaging different neuronal circuits, remain unsolved. Heteromer formation through direct physical interaction between two opioid receptors or between an opioid receptor and a non-opioid one has been postulated and can be characterized by specific ligand binding, receptor signalling and trafficking properties. However, despite numerous studies in heterologous systems, evidence for physical proximity *in vivo* is only available for a limited number of opioid heteromers, and their physiopathological implication remains largely unknown mostly due to the lack of appropriate tools. Nonetheless, data collected so far using endogenous receptors point to a crucial role for opioid heteromers as a molecular entity that could underlie human pathologies such as alcoholism, acute or chronic pain as well as psychiatric disorders. Opioid heteromers therefore stand as new therapeutic targets for the drug discovery field.

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Abbreviations
DOP, δ opioid receptor; DPDPE, [D-Pen^{2,5}]enkephalin, [D-Pen^{2},D-Pen^{5}]enkephalin; DRG, dorsal root ganglion; GNTI, guanidinonaltrindole; GRP, gastrin-releasing peptide; HIV, human immunodeficiency virus; IHC, immunohistochemistry; ISH, *in situ* hybridization; i.t., intrathecal; KDOP, κ opioid receptor δ opioid receptor agonist antagonist; KOP, κ opioid receptor; MDAN, μ opioid receptor δ opioid receptor agonist antagonist; MOP, μ opioid receptor; NNTA, N-naphthoyl-β-naltrexamine; norBNI, nor-binaltorphimine; pbFRET, photobleaching FRET; TAT, transactivating transcriptional activator

The opioid system

The opioid system is composed of three families of endogenous peptides, the enkephalins, dynorphins and β-endorphin, and three homologous GPCRs, the μ opioid receptor (MOP), δ opioid receptor (DOP) and κ opioid receptor (KOP) (Filizola and Devi, 2013; Cox *et al.*, 2015; receptor nomenclature follows Alexander *et al.*, 2013a). Opioid receptors and endogenous opioid peptides are expressed throughout the nervous system (Le Merrer *et al.*, 2009). The opioid system plays a key role in reward and motivation, and regulates emotional responses and cognition. The system also modulates nociception, neuroendocrine physiology and autonomic functions (see Walwyn *et al.*, 2010; Feng *et al.*, 2012). The involvement of the three opioid receptors in pain control, drug abuse and mood disorders has been extensively studied and has been the focus of recent reviews (Bruchas *et al.*, 2010; Gaveriaux-Ruff and Kieffer, 2011; Pradhan *et al.*, 2011; Raehal *et al.*, 2011; Lutz and Kieffer, 2012; 2013; Nadal *et al.*, 2013; Charbogne *et al.*, 2014).

Several decades of opioid pharmacology have uncovered the complexity of the opioid system physiology. In particular,

the analysis of the effects of opioid drugs *in vivo* has revealed functional interactions across receptors, particularly documented for MOP and DOP receptors. However, whether *in vivo* receptor interactions occur at circuit, cellular or molecular level remains highly debated. In this context, formation of a new molecular entity with specific signalling and/or trafficking promoted by physical interaction between two receptors has been proposed. Such heteromers would constitute the molecular determinant of the integrated changes observed at system level. Numerous studies have reported heteromerization of all three opioid receptors between themselves or with non-opioid receptors in transfected cells, but data related to endogenous receptors are necessary to accurately describe the impact of *in vivo* heteromerization.

This review briefly covers the concept of receptor heteromers and the criteria required to validate their existence *in vivo*. Our current view of the structural determinants involved in opioid receptor association is presented. Techniques available to determine physical proximity between two endogenous receptors are then listed with emphasis on their limitations, and the functional outcome of heteromer formation between endogenous receptors with known *in vivo* physical interactions is reviewed. Finally, new tools for *in vivo* studies are presented and their expected effects on drug discovery discussed.

Opioid heteromers: the genesis

Receptor heteromers: a definition

GPCRs have long been considered to function as monomers, but this view has now been disputed for several years. The first breach arose from the observation that an obligatory association between two different monomers is required for $GABA_B$ receptor signalling, (Jones *et al.*, 1998). The observation has now been extended to other class C GPCRs (Rondard *et al.*, 2011). It then led to question whether such heterodimerization was restricted to class C GPCRs or could also be extended to class A GPCRs whose prototype is rhodopsin and to which opioid receptors belong. In particular, could direct physical interactions between two receptor types promote conformational changes and modulate ligand binding or receptor signalling properties? A large number of studies were initiated to address physical association between class A GPCRs and to investigate the functional impact in heterologous systems where the receptors are not naturally produced (Birdsall, 2010; Rozenfeld and Devi, 2011). This generated a long list of potential candidates to form pairs named heteromers. This term has been proposed in an attempt to provide a nomenclature that clarifies associations between receptors (Ferre *et al.*, 2009). According to this classification, heteromeric receptors are defined as the minimal functional unit composed of two or more different subunits that are not functional on their own. Class C $GABA_B$ receptors fall into this category. On the other hand, heteromers refer to macromolecular complexes composed of at least two functional receptor units that show specific biochemical properties different from those of the individual components. This definition applies to a large number of the reported associations between class A receptors and will be adopted here.

How can opioid receptors form heteromers?

Recent crystallographic structures of the inactive forms of the four members of the opioid receptor family have brought to light interesting features (Granier *et al.*, 2012; Manglik *et al.*, 2012; Thompson *et al.*, 2012; Wu *et al.*, 2012). They revealed many common features in the four binding pockets with selective determinants essentially located on the extracellular surface (Filizola and Devi, 2013), therefore confirming the concept of message-address proposed some years ago (Décaillot *et al.*, 2003; Rosenbaum *et al.*, 2009). Interestingly, MOP receptors crystallized in a parallel dimeric form with a tight interface involving 28 amino acids of the transmembrane domains TM 5 and 6 (Manglik *et al.*, 2012). It is thus tempting to conclude that MOP receptors exist as homodimers. However, the arrangement in dimers may also result from crystallization conditions and/or from the modifications introduced in the structure to make them amenable to crystal formation. Interestingly, an additional interface involving TM 1, TM 2 and helix H8 is present that can also be seen in the KOP receptor crystal (Wu *et al.*, 2012*).

Using a subtractive correlated mutation method, *in silico* modelling predicted that the most likely receptor interface in MOP/DOP receptor heteromers would involve MOP TM 1 and DOP TM 4, 5 and 6 (Filizola *et al.*, 2002*). In this approach, mutation analysis was correlated to the structural information contained in three-dimensional molecular models of the transmembrane regions using the rhodopsin crystal structure as a template (Filizola *et al.*, 2002). Molecular dynamics on full-length, 3D models of MOP and DOP receptors also designated MOP TM 1 and 7, and DOP TM 4 and 5 as the most likely interface between the two receptors with an emphasis on MOP TM 1 and DOP TM 4 (Liu *et al.*, 2009*). TM 4 and 5 were also proposed as forming the interface in DOP receptor homodimers with TM 4/TM 4 associations being the most stable (Johnston *et al.*, 2011*).

Physical contact between MOP and DOP receptors through MOP TM 1 is also supported by experimental data. Expression of the MOP TM 1 fused to the transactivating transcriptional activator (TAT) sequence of the cell transduction domain of the human immunodeficiency virus (HIV) indeed interfered with endogenous MOP/DOP receptor co-immunoprecipitation (He *et al.*, 2011*).

On the other hand, experimental approaches pointed to an involvement of the receptor C-termini in heteromer formation. Decreased MOP/DOP receptor co-immunoprecipitation was observed in HEK 293 cells upon expression of the DOP C-terminus fused to the TAT peptide (Kabli *et al.*, 2010*). Similarly, reduced co-immunoprecipitation of MOP/DOP heteromers, but not DOP/DOP or MOP/MOP homomers, was observed upon truncation of the C-terminus of either receptors in transfected COS cells (Fan *et al.*, 2005*). Decreased co-immunoprecipitation was also observed when the sequence specific for the MOP1D splice variant (RNEEPSS) was fused to the TAT sequence and expressed in the mouse spinal cord suggesting a role for the C-termini in MOP1D/GRP heteromer formation (Liu *et al.*, 2011b*). The distal part of the C-terminus, although involved in the formation of those heteromers, is not the only determinant as co-immunoprecipitation was only reduced and not abolished upon receptor truncation. MOP and NOP receptor

co-immunoprecipitation was no longer detected in transfected HEK 293 cells upon truncation of the distal C-terminal part of either receptors suggesting that C-termini are also involved in their physical association (Wang *et al.*, 2005*). Finally, direct interaction between MOP and the NMDA NR1 subunit (see Alexander *et al.*, 2013b for nomenclature) involved the C-termini of the two proteins, and the extent of association was modulated by PKC (Rodriguez-Munoz *et al.*, 2012*).

Interestingly, a peptide corresponding to the DOP receptor second intracellular loop fused to the TAT sequence reduced cell surface expression of this receptor in dorsal root ganglia (DRG) possibly through disruption of MOP/DOP heteromers as this peptide reduced MOP/DOP co-immunoprecipitation in transfected NG 108-15 cells (Xie *et al.*, 2009*).

Altogether, structural, biochemical and *in silico* data suggest that MOP TM 1 and DOP TM 4/TM 5 participate in the receptor interface in opioid heteromers and that additional interactions may occur between the C-termini.

Whether receptor pairs associate as early as the endoplasmic reticulum or whether opioid heteromer formation takes place at the plasma membrane remains unsolved. Some studies suggest that the receptors are exported together to the cell surface in heterologous system (Hasbi *et al.*, 2007; Décaillot *et al.*, 2008) or *in vivo* (Xie *et al.*, 2009). Also, the increase in cell surface expression of DOP receptors observed upon chronic morphine treatment was dependent on MOP receptors (Cahill *et al.*, 2001; Morinville *et al.*, 2003*). This observation is compatible with physical association taking place during export but can also reflect interactions forming at the cell surface. Interestingly, heteromerization between constitutively expressed MOP and DOP receptors expressed with an ecdysone-inducible system exclusively occurred at the surface of HEK 293 cells. In this system, cell surface expression of mutants unable to reach the cell surface on their own was not rescued by physical interaction with wild-type MOP and DOP receptor proteins (Law *et al.*, 2005*).

Minimal functional entity: monomer or dimer?

Despite growing evidence pointing to the existence of dimers, and possibly multimers between class A GPCRs, it is still strongly debated whether the minimal functional unit required to activate G proteins is a receptor monomer or dimer. It should be noted that the natural propensity of a given GPCR to form heteromers might be receptor-dependent. It is conceivable that some receptors do not require association with others to initiate intracellular cascades or only transiently interact to modulate downstream signalling as suggested for the dopamine D_2 receptors (Fonseca and Lambert, 2009*). Receptor-Gα fusion proteins that exhibit a fixed 1:1 receptor : G protein ratio were used in an attempt to better understand how opioid heteromers signal. Upon co-expression of receptor-Gα fusion proteins in transfected cells, physical association between DOP and MOP receptors (Snook *et al.*, 2006), or between DOP receptors and the chemokine receptor CXCR2 (Parenty *et al.*, 2008*) was revealed by co-immunoprecipitation. Functional data suggested that activation of one receptor is sufficient to initiate G protein signalling though the underlying mechanism was dependent on the amount of available Gα subunits. G protein activation was attributed to allosteric modulation when Gα accessibility was not a limiting factor (Parenty *et al.*, 2008; Snook *et al.*, 2008*). Alternatively, promiscuous contact between the functional receptor and the G protein was postulated when the two receptors were competing for a limited pool of Gα subunits (Molinari *et al.*, 2003; Snook *et al.*, 2006*). In the latter case, ligand binding is affected, and the observed changes can be mistaken for ligand-binding cooperativity, leading to erroneous assumptions of the existence of functional heteromers (Chabre *et al.*, 2009*).

Opioid heteromers in heterologous systems

Devi's group was the first to report heteromer formation involving opioid receptors. Upon cell co-transfection with tagged KOP and DOP receptors, close physical vicinity was detected by co-immunoprecipitation and was accompanied by changes in ligand-binding and receptor-signalling properties (Jordan and Devi, 1999*). Numerous studies in heterologous systems reported heteromerization of all three opioid receptors between themselves or with non-opioid partners. The alterations in ligand binding, receptor signalling or trafficking observed using epitope-tagged receptors in heterologous systems were attributed to specific properties elicited by physical association of the two transfected receptors (see Massotte, 2010; van Rijn *et al.*, 2010b; Rozenfeld and Devi, 2011; Stockton and Devi, 2012). The results were not always congruent, and it was then argued that alterations in signalling and trafficking properties could be artificially created as a result of enforced interactions between receptors that were overexpressed in a non-native environment. In particular, the high level of expression that largely exceeds, by at least 10-fold, the endogenous level, may introduce bias in the interpretation of the results. Indeed, allosteric modulation of ligand binding was associated with MOP/DOP heteromerization (Gomes *et al.*, 2011*; Gomes *et al.*, 2004). However, the amount of GTP available during the *in vitro* pharmacological experiments can affect the binding affinities of agonists to the monomers. Therefore, alterations in ligand-binding properties do not necessarily imply heteromer formation (Chabre *et al.*, 2009*).

With time, the physiological relevance of the reported heteromers became an increasing subject of concern. In a number of cases, there was no evidence supporting *in vivo* co-expression of the two partners within the same cell. In addition, it is widely acknowledged that the cellular content varies among cell lines and primary cultures (von Zastrow, 2010*). Although fundamental processes are conserved across cell types, substantial differences may exist between transfected and naturally expressing cells. For example, the MOP receptor agonist morphine does not induce receptor internalization in several transfected cell lines (Borgland *et al.*, 2003; Keith *et al.*, 1996*) or in neurons from the mouse locus coeruleus (Arttamangkul *et al.*, 2008*) but does induce sequestration upon MOP receptor transfection in rat striatal neurons (Haberstock-Debic *et al.*, 2005*). In the brain, MOP receptors can also respond differently to a stimulus when located in the soma or dendrites (Haberstock-Debic *et al.*, 2003*). In addition, the opioid pharmacology is complex, and *in vitro* ligand selectivities sometimes greatly differ from those demonstrable *in vivo*. For example,

[D-Pen^{2,5}]enkephalin, (DPDPE) is described *in vitro* as a DOP receptor selective agonist, but *in vivo* its analgesic effect is mainly mediated by MOP receptors (Scherrer *et al.*, 2004*). Also, the KOP receptor antagonist nor-binaltorphimine (norBNI) only exhibits moderate selectivity towards KOP receptors *in vivo* (Spanagel *et al.*, 1994*). All these observations underline the difficulty of extrapolating results obtained in transfected cells and the need to collect data using endogenous receptors.

Criteria to identify heteromers in native tissues

Limitations associated with heteromer identification in heterologous systems prompted efforts to delineate a framework that would encompass refined criteria. As a guideline, the International Union of Basic and Clinical Pharmacology therefore issued recommendations for the recognition and nomenclature of GPCR heteromers (Pin *et al.*, 2007*). According to these, receptor heteromers can only be accepted if their existence is unambiguously established in native tissues. Therefore, at least two of the following criteria should be met:

(1) There should be evidence for physical association in native tissues or primary cells. This can be obtained by co-immunoprecipitation, co-localization with antibody at electron microscopic level or energy transfer technologies using labelled ligands or knock-in animals.
(2) In native tissues, heteromerization must induce a specific functional property, either a new pharmacological profile or the activation of a specific transduction cascade, distinct from those induced by each receptor expressed alone.
(3) The use of knock-out animals or RNA_i technology must drastically alter the specific functional properties assigned to heteromers.

To date, *in vivo* physical association has only been validated for a limited number of heteromers identified in heterologous systems, often due to the lack of appropriate tools.

Tools and strategies to investigate physical proximity between endogenous receptors

In this section, technical approaches developed to provide evidence for physical association between endogenously expressed receptors are reviewed, and receptor pairs satisfying this criterion are listed (see Table 1).

mRNA

In the absence of selective antibodies, *in situ* hybridization (ISH) has often been used to map receptor expression in the brain. Although a good indication that the receptor may be produced in the cell, detection of mRNA transcript is no definite proof of its presence because mRNA may not be transcribed or protein synthesis may be restricted to specific conditions. Therefore, co-localization of two mRNAs represents a first hint but remains insufficient to establish *in vivo* receptor co-expression.

Approaches based on resonance energy transfer

FRET is based on the excitation of a donor fluorescent molecule that transfers its energy to a fluorescent acceptor that will re-emit with a lower energy. The efficiency of the transfer is proportional to the distance between the acceptor and the donor, and is also sensitive to their relative orientation (see Ishikawa-Ankerhold *et al.*, 2012*). Careful monitoring of several parameters such as the spectral overlap between the emission and the excitation spectra or the relative expression of the partners is required. Quantitative estimation of the monomer-dimer ratio by FRET thus remains difficult. In addition, the FRET signal that takes place between proteins located at the cell surface is difficult to isolate and can only be measured by sophisticated procedures such as time-resolved FRET (Maurel *et al.*, 2008; Cottet *et al.*, 2012*).

Photobleaching FRET (pbFRET) is based on the decrease of donor fluorescence intensity due to irreversible photochemical destruction of the excited state of the fluorophore during prolonged exposure to excitation light (see Ishikawa-Ankerhold *et al.*, 2012*). The decrease in donor fluorescence intensity is monitored in the absence and in the presence of the acceptor. Any slowdown of the photobleaching process upon addition of the energy acceptor would reflect additional donor deactivation by FRET and would suggest that energy acceptor and donor are in close proximity, hence dimerization. pbFRET has been used to monitor the dynamics of dopamine D_2 and somatostatin sst_2 heterodimers in transfected cells and in primary cultures of striatal neurons (Baragli *et al.*, 2007*). Using pbFRET, the authors showed agonist-promoted heteromerization in transfected cells but constitutive heteromerization in cultured striatal neurons that was abolished in the presence of the D_2 receptor antagonist eticlopride.

BRET is similar to FRET except that luciferase is used as the energy donor (Lohse *et al.*, 2012*; De *et al.*, 2013). One advantage over FRET is the absence of potential direct excitation of the energy acceptor. This generates a high signal to background ratio and simplifies controls of background levels. However, BRET is clearly not very sensitive and high-resolution; single cell imaging and analysis are difficult. Despite these limitations, a proof of principle for the use of BRET-based microscopy to image protein interactions with subcellular resolution in a living cell has been obtained (Coulon *et al.*, 2008*).

So far, identification of opioid heteromers by FRET (Hojo* *et al.*, 2008) or BRET (Ramsay *et al.*, 2002; Rios *et al.*, 2006; Juhasz* *et al.*, 2008) has been performed in heterologous systems in which engineered receptors can be expressed. FRET-based techniques should gain more interest for *in vivo* functional studies especially when combined with engineered knock-in mice expressing fluorescent versions of the opioid receptors (De *et al.*, 2013*; Cottet *et al.*, 2012; Aoki *et al.*, 2013; Erbs *et al.*, 2014).

Co-immunoprecipitation

To date, co-immunoprecipitation represents the most widely used approach to establish *in vivo* physical association. Immunoprecipitation is performed using an antibody selective for one receptor, and Western blot analysis is subsequently per-

Table 1

Opioid heteromers. Receptor pairs with reported *in vivo* close physical proximity

	In vivo physical proximity			
Receptor pair	**Tissue**	**Method**	***In vivo* functional impact**	**Reference***
DOP/KOP	Rat trigeminal ganglia	coIP	Allosteric interactions Thermal allodynia	Berg *et al.*, 2012
	Mouse	Bivalent ligand	Thermal analgesia KDAN-18, 6′-GNTI (i.t.)	Ansonoff *et al.*, 2010; Bhushan *et al.*, 2004; Waldhoer *et al.*, 2005
DOP/MOP	Rat DRG	coIP	Morphine tolerance (TAT-DOP-2 L expression)	Xie *et al.*, 2009
	Rat nucleus accumbens		↓Anxiety, depression	Kabli *et al.*, 2013
	Mouse spinal cord	coIP	Allosteric modulation of ligand binding Morphine thermal analgesia	Gomes *et al.*, 2000; Gomes *et al.*, 2004; Gomes *et al.*, 2011
	Mouse spinal cord	coIP	↓Morphine thermal analgesia Morphine tolerance	He *et al.*, 2011
	Mouse hippocampus	coIP		Erbs *et al.*, 2014
	Mouse brain	Selective antibody	CYM51010 analgesia	Gomes *et al.*, 2013b; Gupta *et al.*, 2010
	Mouse	Bivalent ligand	Morphine tolerance, dependence	Daniels *et al.*, 2005b
	Mouse		↑MOP signalling	Walwyn *et al.*, 2009
DOP/NOP	Rat DRG	coIP		Evans *et al.*, 2010
DOP/CB_1	Rat cortex	Selective antibody	DOP signalling (neuropathic pain)	Bushlin *et al.*, 2012
DOP/CXCR4	Human monocytes	coIP	Silent	Pello *et al.*, 2008
	Mouse brain Mouse glial culture	coIP	Silent	Burbassi *et al.*, 2010
KOP/MOP	Proestrous female rat Sc	coIP	KOP mediated thermal analgesia	Chakrabarti *et al.*, 2010; Liu *et al.*, 2011a
KOP/NOP	Rat DRG	coIP		Evans *et al.*, 2010
MOP/NOP	Rat DRG	coIP		Evans *et al.*, 2010
MOP/CB_1	Rat caude putamen	Electron microscopy		Rodriguez *et al.*, 2001
	SKSNH		Neuritogenesis	Rios *et al.*, 2006
MOP1D/GRP	Rat Sc	coIP	Morphine itch specific	Liu *et al.*, 2011b
MOP/NMDA NR1	Mouse PAG	coIP	Morphine tolerance	Rodriguez-Munoz *et al.*, 2012

6′-GNTI, 6′-guanidinonaltrindole; coIP, co-immunoprecipitation; DRG, dorsal root ganglia; GRP, gastrin-releasing peptide receptor; i.t., intrathecal; KDAN, KOP receptor DOP receptor agonist antagonist; PAG, periaqueducal grey matter; Sc, spinal cord; TAT, transactivating transcriptional activator. *These references have been amended after first publication to correct misaligned citations (2 September 2014).

formed with an antibody selective for the other receptor. Detection of the second receptor by immunoblotting indicates that it is associated with the immunoprecipitated partner during the immunoprecipitation step, hence the co-immunoprecipitation denomination. Given the natural propensity of GPCRs to aggregate, proper controls need to be included to rule out the possibility of promiscuous receptor-receptor associations or the possibility of high-order aggregates that would artificially form during the solubilization step (Salim *et al.*, 2002; De Harrison and van der Graaf, 2006*). This is usually achieved by mixing membranes expressing one GPCR only before the immunoprecipitation step to ensure that detection of the two receptors by subsequent immunoblotting results from co-immunoprecipitation of physically close receptors and is not due to non-specific aggregation taking place during sample preparation. Such control membranes can be prepared from knock-out animals for one receptor or from tissues expressing only one of the two receptors as identified by ISH or by immunohistochemistry (IHC). Importantly, co-immunoprecipitation experiments depend on the selectivity of the antibodies. Therefore, the latter needs to first be validated using native tissues that do not express the target receptor. Another limitation is the requirement of a rather large quantity of tissue that may preclude the use of small brain areas. In addition, this approach does not provide cellular resolution but only

indicates close physical proximity of two receptors in a given compartment. If a particular cellular population is of interest, target neurons must be isolated before receptor co-immunoprecipitation experiments. Finally, close proximity does not necessarily imply that changes in cellular responses result from heteromer formation rather than being merely due to downstream modulation of the signalling cascades initiated independently by each receptor.

Immunohistochemical and immunocytochemical co-localization by fluorescence or electron microscopy

Raising selective antibodies against GPCRs often proves to be very difficult. Most class A GPCRs exhibit low immunogenicity because the transmembrane domains constitute the greater part of the protein with usually short loops, and N- or C-termini. In addition, the N-terminus often bears N-glycosylation sites that mask potential epitopes. The selectivity of the antibodies therefore needs to be carefully checked using native tissues that do not express the target receptor. Co-localization using electron microscopy unambiguously establishes whether two receptors are close to each other within the same cell. However, the low expression levels together with a likely limited abundance of heteromers render this approach complicated. Importantly, neuronal co-localization identified by fluorescence microscopy does not necessarily translate into physical proximity. Indeed, two fluorescent signals can be identified at the cell surface with the two receptors present in separate domains of the plasma membrane (Kivell *et al.*, 2004*). Therefore, neuronal co-localization of the fluorescent signals on its own is not sufficient to assess heteromer formation that requires additional approaches such as co-immunoprecipitation.

Recently, heteromer-specific monoclonal antibodies were generated by subtractive immunization. In this strategy, mice are first made tolerant to unwanted epitopes, before being immunized with membranes from the same cell type that co-express the two receptors of interest. Clones specific for heteromers are identified from screens with membranes that do not express the receptors, express only one of them or co-express the two (Sleister and Rao, 2002*; Gomes *et al.*, 2013a). Antibodies generated according to this procedure were used to identify opioid heteromers and to probe their specific signalling (Gupta *et al.*, 2010*; Berg *et al.*, 2012; Bushlin *et al.*, 2012) (see below Functional outcome of heteromerization between endogenous receptors).

Bivalent ligands

Bivalent ligands are compounds composed of two ligands each selective for one receptor type and linked together by a spacer of defined length (Daniels *et al.*, 2005b*; Schiller, 2010). Bivalent ligands called MDAN (MOP DOP receptor agonist antagonist) were generated by linking together the MOP agonist oxymorphone and DOP antagonist naltrindole with spacers of various lengths (Daniels *et al.*, 2005b). Efficient binding only occurred for ligand moieties about 22 Å apart, corresponding to a 19-atom spacer, which is consistent with the distance between the binding pockets of two GPCRs making contact (Daniels *et al.*, 2005b). Similarly, a series of bivalent ligands were generated to target KOP and DOP receptors. Efficient binding was observed for KDN21 with a 21-atom spacer between the κ receptor antagonist 5′-guanidinonaltrindole (GNTI) and the δ receptor antagonist naltrindole (Bhushan *et al.*, 2004*) and for KDAN-18 (KOP DOP receptor agonist antagonist) with an 18-atom spacer between the KOP agonist ICI-199, 441 and DOP antagonist naltrindole (Ansonoff *et al.*, 2010*). Therefore, bivalent ligands are able to bridge two adjacent receptors and can reveal their physical proximity and thus heteromer formation. The main limitation of this approach is the selectivity of each moiety. Naltrindole, for example, only shows moderate selectivity for DOP receptors with a 10- to 100-fold higher affinity for DOP compared with MOP receptors, depending on species and assay conditions. Therefore, binding of MDAN-19 to two physically close MOP receptors exerting antagonistic effects cannot be ruled out (Harvey *et al.*, 2012*). Designing bivalent ligands able to bridge two moieties that would each exhibit high *in vivo* selectivity for one receptor type is therefore essential because of the structural similarities existing between the opioid receptor binding pockets (see below Opioid heteromers: the genesis) (Filizola *et al.*, 2013*).

Opioid heteromers with identified physical proximity between endogenous receptors

Clear indication of close physical proximity has only been reported for a limited number of endogenous receptor pairs (see Table 1). However, more heteromers will undoubtedly be discovered in a near future with the arrival of new tools to efficiently tackle *in vivo* proximity. It is however essential to bear in mind that opioid heteromers have so far been identified in discrete regions of the nervous system, mostly the spinal cord and DRGs (Gomes *et al.*, 2004; van Rijn and Whistler, 2009; Xie *et al.*, 2009; Gupta *et al.*, 2010; He *et al.*, 2011*), and in one case appeared hormone-dependent (Liu *et al.*, 2011a*). Although current data unambiguously establish their existence *in vivo*, heteromer formation has to be validated for each region of interest.

To date, co-immunoprecipitation experiments revealed close proximity between all members of the opioid family (MOP-DOP, MOP-KOP, KOP-DOP) and the closely related opioid receptor-like NOP (NOP-MOP/DOP/KOP) (see Table 1). Co-immunoprecipitation experiments also uncovered physical proximity between DOP receptors and the chemokine CXCR4 receptor (Burbassi *et al.*, 2010*). In the case of cannabinoid CB_1 receptors, physical proximity with MOP receptors was visualized by electron microscopy (Rodriguez *et al.*, 2001*) and close proximity with DOP receptors inferred from heteromer-specific antibodies (Bushlin *et al.*, 2012*). Finally co-immunoprecipitation of the MOP1D splice variant with the gastrin-releasing peptide receptor (GRP) was observed in the mouse spinal cord (Liu *et al.*, 2011b*). Co-immunoprecipitation has also been observed in the synaptosomal fraction from different brain areas between MOP receptors and the NR1 subunit of the ionotropic receptor NMDA (Rodriguez-Munoz *et al.*, 2012*).

Receptor pairs with identified neuronal co-localization

In vivo co-localization in the same neuron has been reported for a few receptor pairs though physical proximity remains to

Table 2

Receptor pairs with reported neuronal co-localization

Receptor pair	Tissue	Method	Reference*
DOP/α_{2A} adrenergic	Rat spinal cord	IHC	Riedl *et al.*, 2009
DOP/α_{2C} adrenergic	Rat spinal cord	IHC	Riedl *et al.*, 2009
MOP/α_{2A} adrenergic	Rat spinal cord Rat locus coeruleus	IHC electrophysiology	Riedl *et al.*, 2009; Illes and Norenberg, 1990
MOP/α_{2C} adrenergic	Rat spinal cord	IHC	Riedl *et al.*, 2009
MOP/dopamine D1	Rat striatum,cortex	IHC	Juhasz *et al.*, 2008
MOP/serotonin $5HT_{2A}$	Rat brain	ISH	Lopez-Gimenez *et al.*, 2008
MOP/CXCR4	Rat brain	IHC	Heinisch *et al.*, 2011
MOP/CX_3CR1	Rat brain	IHC	Heinisch *et al.*, 2011

DOP, delta opioid receptor; IHC, immunohistochemistry; MOP, mu opioid receptor. *These references have been amended after first publication to correct misaligned citations (2 September 2014).

be established, and therefore, heteromer formation remains hypothetical (see Table 2). MOP and $5HT_{2A}$ receptors were co-localized in several rat brain areas at the mRNA level (Lopez-Gimenez *et al.*, 2008*). MOP and dopamine D_1 receptors were co-localized in the rat cortex and striatum (Juhasz *et al.*, 2008*). DOP receptors were co-localized with α_{2A} adrenoceptors in substance P neurons from the rat spinal cord by IHC (Riedl *et al.*, 2009*) and to a small extent with α_{2C} adrenoceptors (Riedl *et al.*, 2009). Limited co-localization in the rat spinal cord was also observed by IHC between MOP and α_{2A} or α_{2C} adrenoceptors (Riedl *et al.*, 2009). Electrophysiological recordings indicated additional neuronal co-localization between MOP receptors and α_{2A} adrenoceptors in the locus coeruleus (Illes and Norenberg, 1990*). MOP receptors were also co-localized by IHC with the chemokine receptors CXCR4 or CX_3CR1 in the hippocampus, cingulate cortex, periaqueductal grey, nucleus accumbens, ventral tegmental area and globus pallidus (Heinisch *et al.*, 2011*). In addition, co-expression in periaqueductal grey neurons was confirmed by electrophysiological recording (Heinisch *et al.*, 2011). For these receptor pairs, indication of physical proximity is still lacking. Therefore, functional outcome cannot yet be attributed to heteromer formation as it may rather reflect interactions at the level of the signalling cascades as for MOP receptors and CXCR4 (Patel *et al.*, 2006; Sengupta *et al.*, 2009*), or MOP and α_{2A} adrenoceptors in the locus coeruleus (Stone and Wilcox, 2004*).

Functional outcome of heteromerization between endogenous receptors

In vivo physical proximity is a prerequisite to postulate the existence of heteromers. Although necessary, this requirement is however not sufficient. Indeed, evidence has to be brought that functional interactions directly result from physical association and are not merely produced by cross-talk between downstream signalling. This section reviews our current knowledge of the functional changes observed upon opioid heteromer formation between endogenous receptors.

MOP/DOP heteromers

MOP and DOP receptors are crucial modulators of both nociception and opiate analgesia (see Raehal *et al.*, 2011*; Gaveriaux-Ruff and Kieffer, 2011; Pradhan *et al.*, 2011). In particular, the contribution of DOP receptors has been the subject of much interest because of its potential therapeutic impact (Bailey and Connor, 2005; Cahill *et al.*, 2007; Zhang *et al.*, 2006*) because of the proposal that DOP receptors play a key role in opiate analgesia and morphine tolerance, possibly through formation of MOP/DOP heteromers.

In transfected cells, MOP/DOP receptor specific antibodies blocked ligand binding (Gupta *et al.*, 2010*) and β-arrestin recruitment (Gomes *et al.*, 2013b*). In addition, they affected G protein-dependent signalling in membranes from both transfected cells and mouse brain by significantly blocking the potentiation of MOP receptor agonist-mediated cAMP by DOP receptor antagonists (Gupta *et al.*, 2010*). Changes in signalling properties upon MOP/DOP receptor co-expression were attributed to a switch from *Pertussis*-sensitive to *Pertussis*-insensitive G protein coupling (George *et al.*, 2000; Fan *et al.*, 2005; Hasbi *et al.*, 2007*) or to coupling to a new pathway (Rozenfeld and Devi, 2007*), but this remains to be confirmed *in vivo*.

Treatment with a low dose of naltriben pharmacologically stabilized MOP/DOP receptors at the cell membrane in transfected HEK 293 cells by blocking methadone-promoted MOP/DOP receptor internalization without affecting MOP/DOP signalling (Milan-Lobo and Whistler, 2011*). *In vivo*, chronic administration of the MOP receptor selective agonist methadone with a low dose of the DOP receptor selective antagonist naltriben resulted in reduced thermal analgesia that could be restored by promoting internalization with methadone or by blocking signalling with the antagonist naltrindole (Milan-Lobo *et al.*, 2013*).

In SK-NS-H cells endogenously expressing the two receptors, enhanced MOP receptor agonist binding and signalling

were observed in the presence of agonists, antagonists or inverse agonists for DOP receptors (Gomes *et al.*, 2000; 2011; Gomes *et al.*, 2004*). This suggests that occupancy of DOP receptors by any type of ligand acts as an allosteric modulator and triggers conformational changes in MOP receptors that potentiate ligand binding and receptor function.

Surface expression of MOP receptors was reduced in DRGs from DOP receptor knock-out mice, whereas the MOP receptor agonists, DAMGO and morphine, induced less inhibition of voltage-dependent calcium channels (Walwyn *et al.*, 2009*). Also, chronic morphine administration increased DOP receptor surface expression in neurons and was MOP receptor-dependent (Cahill *et al.*, 2007*). In addition, reduced tolerance was observed on pharmacological blockade of DOP receptors (Abdelhamid *et al.*, 1991; Zhu *et al.*, 1999*) or in DOP receptor knock-out animals (Zhu *et al.*, 1999; Nitsche *et al.*, 2002*). Finally, i.c.v. injection of the bivalent ligand MDAN-19 reduced dependence and tolerance in mice, however, as mentioned earlier (see Tools and strategies to investigate physical proximity between endogenous receptors); selectivity towards MOP/DOP heteromers remains questionable (Harvey *et al.*, 2012*).

Based on their pharmacological profile, MOP/DOP heteromers were postulated as the molecular entity corresponding to the δ 1 subtype (van Rijn and Whistler, 2009*). TAN-67, identified as a MOP/DOP receptor selective agonist using co-transfected HEK 293 cells, decreased ethanol but not sucrose consumption in wild type but not DOP receptor knock-out mice (van Rijn and Whistler, 2009). This suggests that MOP/DOP receptor selective targeting may represent a more efficacious treatment of alcohol dependence. Recently, CYM51010 was reported as a biased MOP/DOP receptor agonist whose *in vivo* activity was blocked by MOP/DOP heteromer selective antibodies (Gomes *et al.*, 2013b*). Systemic administration of CYM51010 induced thermal acute analgesia similar to morphine, whereas chronic administration of CYM51010 produced lesser antinociceptive tolerance compared with morphine. CYM51010 thus appears as the precursor of new class of analgesics drugs.

In the nervous system, MOP/DOP receptors are mainly co-localized in the nociceptive pathway [virtual brain atlas at https://mordor.ics-mci.fr/ (Erbs *et al.*, 2014*)] where MOP/DOP receptor physical proximity was established in several areas (see Table 1 and references therein). Accordingly, interfering with MOP/DOP receptor physical interaction *in vivo* modified the functional outcome. Expression of the MOP TM1 fused to the TAT sequence not only blocked endogenous MOP/DOP co-immunoprecipitation but also MOP/DOP degradation in the lysosomal compartment (He *et al.*, 2011*). Also, expression of the fusion construct in the spinal cord increased morphine thermal analgesia and decreased morphine tolerance (He *et al.*, 2011). Similarly, expression of the DOP receptor C-terminus fused to the TAT sequence decreased co-immunoprecipitation and reduced anxiolytic and antidepressive effects induced by UFP 512, described as selective for MOP/DOP heteromers, when expressed in the rat nucleus accumbens (Kabli *et al.*, 2010; Kabli *et al.*, 2013*). Finally, a peptide corresponding to the DOP second intracellular loop fused to the TAT sequence reduced morphine tolerance in rat and reduced cell surface expression of DOP receptors in DRGs (Xie *et al.*, 2009*).

DOP/KOP heteromers

Co-immunoprecipitation experiments performed on rat trigeminal ganglia cultures identified close proximity between DOP and KOP receptors in peripheral sensory neurons (Berg *et al.*, 2012*). On the functional point of view, the putative DOP/KOP heteromer-selective agonist 6′-GNTI, which was originally described as a KOP receptor agonist (Waldhoer *et al.*, 2005) inhibited PGE_2-stimulated cAMP accumulation *in vitro* and elicited a strong antinociceptive response *in vivo*. Either a DOP or a KOP receptor antagonist (Berg *et al.*, 2012) blocked both of them. 6′-GNTI also exhibited high potency and efficacy in activating DOP/KOP heteromers in transfected cells and mediated thermal analgesia when injected at the spinal level but not upon i.c.v injection (Waldhoer *et al.*, 2005*). Similarly, the bivalent ligand KDN-21 composed of the KOP receptor antagonist 5′-GNTI and DOP receptor antagonist naltrindole presented a different selectivity profile upon intrathecal (i.t.) or i.c.v. administration (Bhushan *et al.*, 2004*). This suggests that DOP/KOP heteromers may only be present in the spinal cord with no functional analgesic DOP/KOP heteromers in the brain (Waldhoer *et al.*, 2005*). Alternatively, this could indicate that DOP and KOP receptors associate with different partners at the spinal and supraspinal levels therefore yielding distinct pharmacological and signalling profiles (Bhushan *et al.*, 2004*). Indeed, based on the binding and signalling properties of KDN-21, the pharmacologically defined δ 1 subtype was attributed to DOP/KOP heteromers (Bhushan *et al.*, 2004; Xie *et al.*, 2005*). However, this subtype was also attributed to MOP/DOP heteromer formation using the selective agonist TAN-67 (see above) (van Rijn and Whistler, 2009*). Once more, this illustrates the difficulty of interpreting *in vivo* pharmacological data from bivalent ligands. More recently, 6′-GNTI and KDAN-18 composed of the KOP agonist ICI-199, 441 and the DOP antagonist naltrindole linked by an 18-atom long spacer were tested on knock-out animals. Upon i.t. injection in DOP or KOP receptor knock-out mice, the two compounds showed reduced potency and efficacy in thermal analgesia, suggesting selective DOP/KOP receptor bridging (Daniels *et al.*, 2005a; Ansonoff *et al.*, 2010*).

Using a subtractive strategy, Devi's group also generated monoclonal antibodies directed against DOP/KOP heteromers (Berg *et al.*, 2012*). *In vivo*, the anti-allodynic effect of the DOP receptor agonist DPDPE was increased by local injection of DOP/KOP heteromer selective antibodies in the plantar test suggesting that within DOP/KOP heteromers, KOP receptor antagonists can act as allosteric modulators of responses to DOP receptor agonists (Berg *et al.*, 2012).

MOP/KOP heteromers

The spinal cord is a region of the CNS in which components of opioid analgesic pathways manifest sexual dimorphism in rodents. In males, spinal morphine antinociception results from the exclusive activation of spinal MOP receptors, whereas in females, spinal morphine antinociception requires the concomitant activation of spinal MOP and KOP receptors (Liu *et al.*, 2007; Liu *et al.*, 2011a*). Spinal KOP receptor density is indeed significantly greater in proestrous female rats (Liu *et al.*, 2007*). Accordingly, co-immunoprecipitation of MOP and KOP receptors was by far more abundant in the spinal cord of proestrous female rats

compared with male or diestrous female rats (Chakrabarti *et al.*, 2010*). In addition, endogenous spinal dynorphin 1–17 appeared to bind spinal MOP/KOP heteromers because morphine analgesia was reduced by the KOP receptor antagonist norBNI and the anti dynorphin A antibody in proestrous but not diestrous female rats (Chakrabarti *et al.*, 2010). The authors therefore postulated that MOP/KOP heteromers could be the molecular transducer for the female-specific dynorphin/KOP receptor component of spinal morphine antinociception. Estrogen would control the amount of MOP/KOP heteromers that would be predominant in female spinal analgesia and could represent a new target for pain management in females (Liu *et al.*, 2011a*).

The monovalent ligand N-naphthoyl-β-naltrexamine (NNTA) selectively activated MOP/KOP heteromers in transfected HEK 293 cells (Yekkirala *et al.*, 2011*). *In vivo*, NNTA i.t. injection induced thermal analgesia in the mouse tail-flick test that was abolished in MOP receptor knock-out mice and was decreased by the KOP receptor antagonist norBNI (Yekkirala *et al.*, 2011). In addition, NNTA (i.t.) did not induce tolerance or physical dependence although it presented aversive effects. NNTA i.c.v. injection only produced weak analgesia suggesting low functional MOP/KOP receptor expression in the brain and pointing to the potential interest in more detailed functional studies of MOP/KOP receptors *in vivo*.

OP/NOP heteromers

The opioid receptor-like NOP co-immunoprecipitated with MOP, DOP and KOP receptors in rat DRGs suggesting heteromer formation with each of them (Evans *et al.*, 2010*).

NOP physically interacts with N-type calcium channels in the brain and DRGs, as shown by co-immunoprecipitation (Beedle *et al.*, 2004*). In DRGs, this interaction mediates a tonic inhibition of calcium entry by an agonist-independent inhibition of N-type channels due to constitutive receptor activity (Altier *et al.*, 2006*). In addition, NOP receptors mediate N-type channel trafficking to and from the plasma membrane (Altier *et al.*, 2006). NOP and MOP receptors, on the other hand, form heteromers in DRGs (Evans *et al.*, 2010*). In addition, co-immunoprecipitation with DOP or KOP receptors points to a potential contribution of NOP receptors in the regulation of the nociceptive information that deserves proper investigation.

Interestingly, co-expression of opioid receptors with NOP in TsA 2 cells affected nociceptin-induced inhibition of N type channels (Evans *et al.*, 2010). Also, internalization of both NOP receptors and N type channels was promoted in these cells by the MOP receptor agonist DAMGO suggesting the existence of larger macromolecular complexes involving multiple receptor types and voltage-gated calcium channels (Evans *et al.*, 2010). If such signalling complexes exist *in vivo*, they add further complexity to the regulation of pain mechanisms.

Opioid/CB_1 receptor heteromers

Close vicinity between MOP receptors and cannabinoid CB_1 receptors was established in rat striatal membranes using electron microscopy (Rodriguez *et al.*, 2001*). Negative allosteric modulation of both opioid and cannabinoid signalling was observed in SK-SN-H cells that endogenously express the two receptors and a decrease in cannabinoid signalling by the MOP receptor selective agonist DAMGO was observed in striatal membranes (Rios *et al.*, 2006*). Hence, heteromer formation may account for some of the functional interactions that underlie analgesia or addiction as both receptors are targets for drugs of abuse. Recently, DOP/CB_1 heteromers were identified in membrane domains of mouse cortical primary neurons using heteromer-specific antibodies (Rozenfeld *et al.*, 2012*). In a model of neuropathic pain in rats, enhanced expression of DOP and CB_1 receptors was observed in cortical membranes 2 weeks after L5 nerve lesioning. Non-signalling doses of CB_1 receptor ligands allosterically enhanced DOP receptor activity, and this was blocked by the heteromer-specific antibody, suggesting that DOP/CB_1 heteromers represent a suitable target for blockade of negative mood states associated with neuropathic pain (Bushlin *et al.*, 2012*).

DOP/CXCR4 heteromers

Physical proximity between DOP and the chemokine CXCR4 receptor was detected in human monocytes (Pello *et al.*, 2008*) as well as in brain tissue and glial culture from MOP receptor knock-out mice (Burbassi *et al.*, 2010*). DOP/CXCR4 heteromers appeared to be silent in immune cells (Pello *et al.*, 2008*) and brain glia (Burbassi *et al.*, 2010*). Although the physiological significance remains unclear, this observation deserves further investigation as it may open new therapeutic perspectives, in particular in HIV pathology.

MOP1D/GRP heteromers

A pivotal role in opioid-induced itch was attributed to heteromer formation between the MOP1D splice variant and the GRP receptor, supported by receptor co-immunoprecipitation, in the mouse spinal cord (Liu *et al.*, 2011b*). Itch would result from the unidirectional transactivation of GRP receptors by the MOP agonist morphine, selectively activating MOP1D receptors at the level of the heteromer (Liu *et al.*, 2011b). *In vitro* experiments in transfected HEK 293 cells suggested that morphine activated GRP receptor downstream calcium signalling as the effect of morphine was abolished by blocking PLCβ or by antagonizing IP_3 receptors (Liu *et al.*, 2011b). Injection (i.t.) of the MOP1D-specific sequence as a TAT fusion protein blocked morphine-induced scratching but did not affect morphine-induced thermal antinociception or GRP-induced scratching (Liu *et al.*, 2011b). This study points to interesting directions for the design of novel agents with fewer side effects compared with morphine. It should however be noted that the expression of the MOP1D splice variant remains controversial as it could not be detected in rats (Oldfield *et al.*, 2008*), suggesting possible species specificity.

MOP/non-GPCR heteromers

Co-immunoprecipitation of MOP and the subunit NR1 of the ligand-gated channel NMDA was observed in the synaptosomal fraction prepared from different brain areas, supporting close physical proximity (Rodriguez-Munoz *et al.*, 2012*). Interestingly, morphine disrupted this complex by PKC-mediated phosphorylation of the NR1 C terminus. Morphine also potentiated the NMDA-CaMKII pathway implicated in

morphine tolerance. This finding opens very exciting perspectives regarding the involvement of functional complexes formed between opioid receptors, and more generally GPCRs, and ligand-gated channels in the regulation of neuronal activity. It also suggests the possibility of selectively targeting these receptors with bivalent ligands as therapeutic drugs.

Effects on drug discovery

Few opioid heteromers have been validated *in vivo*. Nevertheless, their implication in the development of a number of pathological states affecting the nervous system cannot be ignored, in particular for pain management. Opioid heteromers represent excellent molecular targets because of their relative abundance in the nociceptive pathways. Indeed, MOP/DOP, MOP/KOP, DOP/KOP, MOP/CB_1, MOP1D/GRP and (M-D-K)OP/NOP heteromers have been associated with antinociceptive responses. So far, *in vivo* data support specific ligand binding and signalling distinct from currently used opiate drugs such as morphine with less tolerance upon chronic use. Respiratory depression represents another major drawback associated with administration of high doses of morphine. Interestingly, MOP/DOP heteromers are not detected in the pre-Bötzinger complex that controls respiratory depression (Erbs *et al.*, 2014*). Selective ligands for MOP/DOP heteromers with no or low affinity for MOP receptors would therefore represent excellent candidates as the next generation of analgesic drugs. Similarly, selective targeting of MOP/DOP heteromers could provide new treatments for alcoholism (van Rijn *et al.*, 2010a*), and selective targeting of DOP/CB_1 heteromers could reduce anxiety and depressive states associated with chronic neuropathic pain (Bushlin *et al.*, 2012*).

One should however bear in mind that the observed behavioural modifications could reflect a change in the homeostasis of the system or could result from modifications in downstream signalling of the receptors without necessarily requiring their physical association or even their co-expression within the same cell. Although it is often difficult to unambiguously name heteromers as the molecular entity underlying a disease, identification of neuronal co-localization and establishment of physical proximity for an increasing number of receptor pairs will undoubtedly provide new potential targets for the pharmacological treatment of neurological disorders.

Future directions

Naturally expressing cell lines to identify functional heteromer-specific properties

As mentioned earlier, heteromer-specific properties identified in transfected cells often show limited predictive value when challenged *in vivo*. This is especially true when taking into account the complexity of the opioid receptor pharmacology that represents a significant limitation for the validation of new therapeutic drugs.

Whatever their rationale, new strategies exploring molecular and cellular aspects associated with heteromer formation should be tested only on cells endogenously expressing the receptors, either primary cultures derived from the tissue of interest or cell lines that closely resemble endogenous ones, such as immortalized primary cells or neuroblastoma. Embryonic and induced pluripotent stem cells on the other hand may offer a new development because of their capacity to differentiate into all types of somatic cells including neurons (Bibel *et al.*, 2004; Plachta *et al.*, 2004*). In-depth phenotyping indicated the ability of stem cells to give rise to specialized populations corresponding to region and/or neurotransmitter-specific neuronal and glial types (Liu and Zhang, 2011*; Peljto and Wichterle, 2011). This approach could be adapted to drug screening systems because it is reasonable to speculate that drugs identified in one of these cellular contexts will show the same pharmacological profile when administered in the intact organism. Another advantage of embryonic stem cells is the possibility to isolate them from genetically modified animals. Knock-out mice for one of the partner receptors, knock-in mice endogenously expressing GPCRs fused to fluorescent proteins and/or combinations of them represent powerful tools to assess *in vivo* the functional role of opioid heteromers.

Double fluorescent knock in mice

As previously emphasized, neuronal co-localization is mandatory before any attempt to associate changes in ligand binding or in receptor signalling and trafficking with opioid heteromer formation. Still, this prerequisite is too often missing, mostly due to the lack of appropriate tools for *in vivo* investigation. In particular, antibodies against opioid receptors have very often proven to be non-selective, which hampered *in vivo* mapping. Recently, fluorescent knock-in mice expressing a functional DOP receptor in fusion with the eGFP (DOP-eGFP) were successfully generated and used to address receptor trafficking *in vivo* (Scherrer *et al.*, 2006; Pradhan *et al.*, 2010; Pradhan *et al.*, 2009*). These mice were crossed with animals expressing functional MOP receptors in fusion with the red fluorescent protein mcherry (MOP-mCherry) to give rise to double fluorescent knock-in mice. Fine mapping of MOP and DOP receptors in the mouse CNS identified brain areas in which neuronal co-localization was visible under basal conditions (see Figure 1) (Erbs *et al.*, 2014*). These regions were mainly located in the brainstem within neuronal circuits essential for survival (see Figure 2). A large portion was present in the nociceptive pathway, but co-localization was also visible in circuits related to food and water consumption or to sexual behaviour (Erbs *et al.*, 2014). Interestingly, MOP and DOP receptors appear essentially expressed on distinct neurons in the forebrain, suggesting a limited role of MOP/DOP heteromers in high-order processing.

Double knock-in mice therefore constitute an exceptional tool to address changes in MOP/DOP co-localization occurring at various stages of pathological conditions including chronic pain, psychiatric disorders or drug addiction. In addition, physical proximity can be addressed in regions of co-localization by co-immunoprecipitation with antibodies against the fluorescent proteins or by FRET providing solid ground for heteromer studies (see Tools and strategies to investigate physical proximity between endogenous receptors).

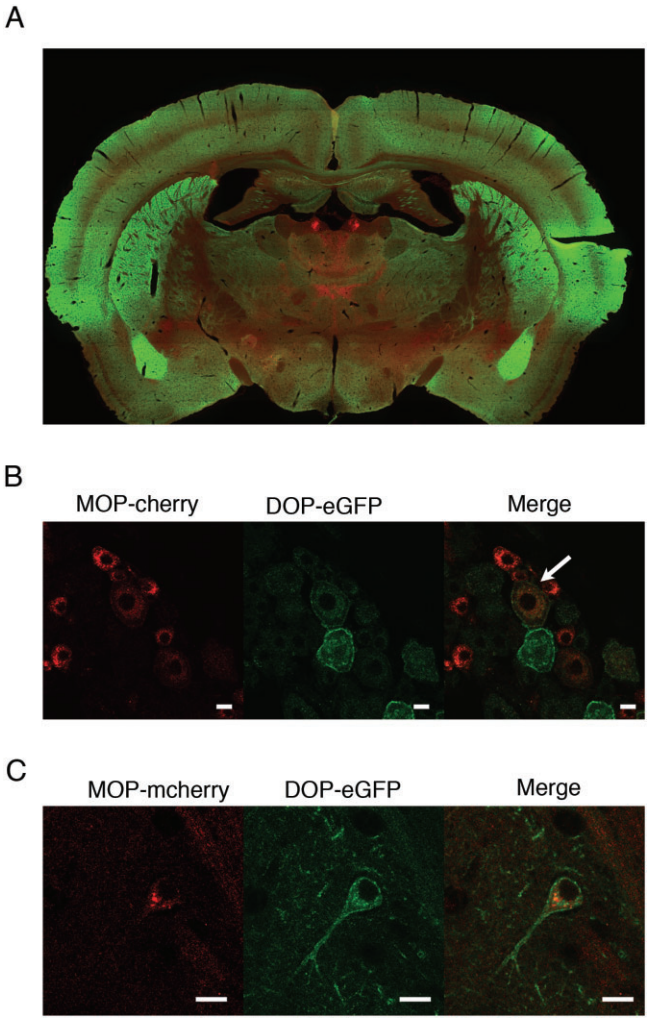

Figure 1

Double fluorescent knock in mice expressing DOP receptors fused to the GFP (green) and MOP receptors fused to the red fluorescent protein mcherry (red). (A) General view of a coronal brain section. (B) Neuronal co-localization of DOP and MOP receptors in dorsal root ganglia (white arrow). (C) Neuronal co-localization of DOP and MOP receptors in the hippocampus. Scale bars 10 μm.

Finally, double knock-in mice enable direct visualization of heteromer trafficking *in vivo* in response to a pharmacological stimulation or a physiological challenge promoting endogenous opioid peptide release. Comparing receptor trafficking between neurons co-expressing the two receptors and neurons expressing one receptor type only will allow the separation of specific MOP/DOP behavioural outcomes, thereby providing a way to address the contribution of MOP/DOP heteromers to physiological processes and pathological states.

Conclusions

Despite a lot of effort and numerous studies performed on heterologous systems, our current knowledge of the mechanisms by which GPCRs physically assemble and function *in vivo* remains very limited. The extremely small number of heteromers formed between endogenous receptors that have been unambiguously identified is striking and suggests that the role of such associations is likely to be underrated. In particular, the consequences of opioid heteromer formation on cellular signalling are still poorly understood, as is our view of its contribution to physiopathological states. Involvement of opioid heteromer formation has been proposed in human pathologies such as alcoholism, acute or chronic pain, as well as psychiatric disorders but deserves further investigation. Altogether, our appraisal of opioid heteromers, as fully identified therapeutic targets, is still in its infancy. No doubt that the development of more sophisticated biophysical, biochemical and pharmacological tools together with the use of native cells and genetically modified animals will open unsuspected perspectives in the drug discovery field.

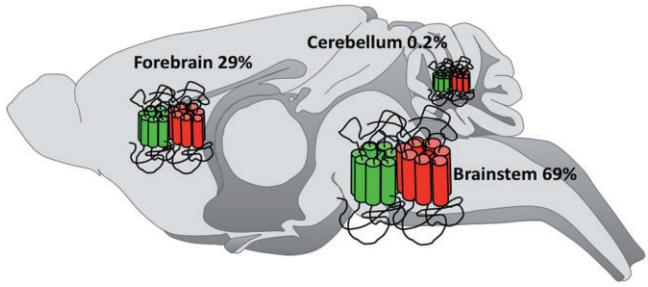

Figure 2

Relative distribution of neurons co-expressing DOP and MOP receptors in the mouse brain as determined using double fluorescent knock in mice expressing DOP receptors fused to the GFP and MOP receptors fused to the red fluorescent protein mcherry. Percentages of MOP/DOP receptor co-localization in the mouse brain are indicated.

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Conflict of interest

I have no conflict of interest to declare.

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