Cell. Mol. Life Sci. (2009) 66:2093–2108
DOI 10.1007/s00018-009-0008-4

Cellular and Molecular Life Sciences

REVIEW

Membrane functional organisation and dynamic of μ-opioid receptors

André Lopez · Laurence Salomé

Received: 29 December 2008 / Revised: 17 February 2009 / Accepted: 18 February 2009 / Published online: 20 March 2009

Abstract The activation and signalling activity of the membrane μ-opioid receptor (MOP-R) involve interactions among the receptor, G-proteins, effectors and many other membrane or cytosolic proteins. Decades of investigation have led to identification of the main biochemical processes, but the mechanisms governing the successive protein–protein interactions have yet to be established. We will need to unravel the dynamic membrane organisation of this complex and multifaceted molecular machinery if we are to understand these mechanisms. Here, we review and discuss advances in our understanding of the signalling mechanism of MOP-R resulting from biochemical or biophysical studies of the organisation of this receptor in the plasma membrane.

Keywords Signal transduction · Protein interactions · Membrane functional domains · G-protein-coupled receptors

Introduction

Opioid receptors are the primary site of action of morphine and the opiate alkaloids used in the treatment of pain, and it is clear that analgesia is mediated principally by activation of the MOP receptor (MOP-R) [1]. This receptor has many other effects, such as respiratory depression, inhibition of gastrointestinal transit and feeding, euphoria, effects on anxiety and the release of hormones [2, 3]. The MOP-R, a class A G protein-coupled receptor (GPCR), is found principally in the central nervous system (CNS) [4], and CNS MOP-R genes have been cloned from more than eight different mammals, fish and amphibians [1]. As for all GPCRs, signalling via MOP-R is far more complicated than the classical model in which the receptor is activated by the binding of an agonist and then binds to a heterotrimeric G protein, subsequently interacting with effectors. Further direct interactions of these protagonists with a number of cytosolic proteins influence the membrane organisation of the signalling complex and regulate GPCRs pharmacology [5–7]. Several additional events must also be taken into account for a complete description of the mechanism associated with the initial stages of the signalling process. Various repeated couplings between similar or different G-proteins may occur [8], and successive couplings are also orchestrated between the activated G-protein subunits and their effectors. These sequential interactions suggest that the complex formed by the activated GPCR and the heterotrimeric G-proteins dissociates after its activation to initiate the signalling process [9]. Conflicting results have been obtained for several GPCRs, with the description of a single, stable GPCR-G-protein-effector complex throughout the entire process of signal transduction [5]. However, the dependence of GPCR functional efficiency on membrane composition demonstrates the importance of the mobility of the GPCR, G-proteins and effectors within the membrane for determining encounters between these molecules [10]. Thus, the overall biochemical mechanisms have been identified in terms of individual chemical reactions, but the sequences,

A. Lopez (✉) · L. Salomé (✉)
CNRS, IPBS (Institut de Pharmacologie et de Biologie Structurale), 205 route de Narbonne, 31077 Toulouse, France
e-mail: Andre.Lopez@ipbs.fr

L. Salomé
e-mail: Laurence.Salome@ipbs.fr

A. Lopez · L. Salomé
Université de Toulouse, UPS, IPBS, 31077 Toulouse, France

chronological order and molecular organisation of the signalling complex and surrounding molecules required for the effective encounters governing these successive protein–protein interactions remain to be established.

This review will first provide a recap of the basic characteristics of the structure of MOP-R before and after its activation by ligands. It will then focus on diverse aspects of the relationship between MOP-R function and the biophysical characteristics of the surrounding membrane. We provide an overview of the abundant publications on MOP-R, presenting and discussing the essential results and progress towards an understanding of the function of this receptor, its modulation by cell membrane organisation and its relationship with the receptor diffusion in the membrane.

Structural characteristics of MOP-R before and after activation

MOP-R structure and ligand binding

In the absence of experimental structures of opioid receptors, theoretical modelling, based on the structure of rhodopsin or β_2-adrenergic receptor (β_2AR) [11, 12], remains an important tool for the MOP-R structure–function analysis. MOP-R consists of an extracellular N-terminal domain, seven transmembrane helical domains (7TM) connected by three extracellular and three intracellular loops and an intracellular C-terminal tail, which is predicted to form a fourth intracellular loop after palmitoylation [13]. In addition, two cysteine residues, in the first and second extracellular loops, probably form an external disulphide bridge. Usually the 7TMs are arranged in a counter-clockwise fashion (viewed from the extracellular side), forming a tight helical bundle. MOP-R also undergoes post-transcriptional modifications (addition of Asn-linked glycosyl chains) at several sites in the N-terminal domain [1] (Fig. 1).

Taking into account MOP-R mutational analysis [14] and chimeric MOP receptor studies, computational modelling [15, 16] suggested that MOP-R has a binding cavity located in an inner inter-helical region constituted by TM helices 3, 4, 5, 6 and 7 and partially covered by dynamic extracellular loops, generating a hydrophobic cluster between extracellular loop 3 and the N-terminal region, allowing ligand selectivity (Fig. 1). Upon agonist entrance in the binding cavity, the internal conformation of the receptor would change, disrupting the basal hydrophobic and hydrophilic interactions within TM helices 1–5 and 6–7, bringing together these two sets of transmembrane segments. It is widely accepted that morphine-like agonists interact with residues in the bottom of the hydrophobic

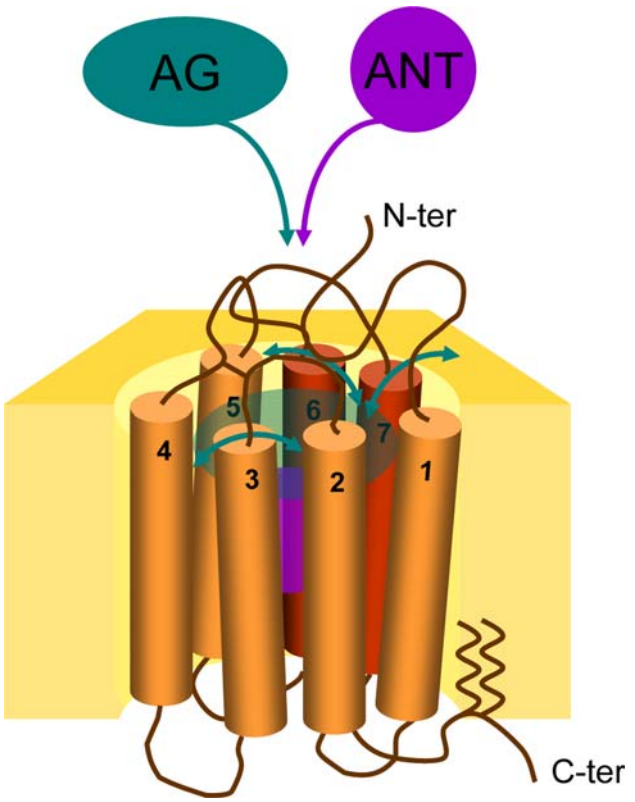

Fig. 1 Schematic structure of MOP-R in the membrane and the main changes consecutive to an agonist or an antagonist binding. The *green arrows* indicate the TMs whose movements induced by an agonist binding modify the internal receptor conformation. The relative positioning of agonists and antagonists inside the receptor's cavity is illustrated

cavity, whereas antagonists, such as naloxone, interact at a slightly deeper site in this cavity than agonists, causing sterical hindrance between TM3 and TM7 and preventing the receptor from adopting its active conformation. [16]. The conformational modifications following agonist binding involve relative displacements of TM helices 3, 6 and 7 within the receptor. These changes modify the cytoplasmic interactions of the receptor, resulting in the exposure of intracellular receptor domain loops and permitting G-protein activation. The key determinant of receptor coupling specificity to G proteins is the I3 intracellular loop, with the I2 intracellular loop and the C_{term} tail thought more likely to be involved in determining the efficiency of G-protein activation [16, 17].

The thickness of the hydrophobic transmembrane part of the receptor is also altered. Indeed, Alves et al. [18] demonstrated, by plasmon-waveguide resonance spectroscopy, an increase of about 10% in membrane thickness following activation of the δ opioid receptor (DOP-R) and rhodopsin [19]. Le Guyader et al. [20] recently confirmed these specific changes to membrane thickness, using the

fluorescence properties of a pyrene-labelled cholesterol and agonist stimulation of DOP-R in living HEK cells over-expressing this receptor. No direct observation of a similar pattern of behaviour has yet been obtained for MOP-R, but, as discussed later (paragraph 3-2), these receptors seem to have two different pharmacological profiles, depending on the membrane environment and corresponding to conformations with different thicknesses of the hydrophobic part of the receptor.

Release of signalling cascades: G-protein effector activation

Agonist-activated MOP-R couples predominantly with pertussis toxin-sensitive $G_{\alpha i}/G_{\alpha o}$ (particularly $G_{\alpha o1}$, $G_{\alpha o2}$, $G_{\alpha i2}$, $G_{\alpha i3}$, and, to a lesser extent, $G_{\alpha i1}$ [21–24]), although coupling with pertussis toxin-insensitive G proteins, such as as $G_{\alpha z}$ [25] or $G_{\alpha q}$ ($G_{\alpha 16}$) [26], has been reported. In their resting state, when the α-subunit is bound to GDP, G-proteins exist as heterotrimers of α, β, γ subunits, and a $G_{\alpha}^{(GDP)}{}_{\beta\gamma}$ complex can associate with MOP-R. Agonist activation of MOP-R stimulates the catalytic GDP-GTP exchange on G_{α}. This requires dissociation of the G-protein MOP-R complex into $G_{\alpha}^{(GTP)}$ and $G_{\beta\gamma}$, leading independently to the activation or inhibition of multiple downstream MOP-R effectors. Indeed, $G_{\alpha i}$ inhibits adenylyl cyclases, whereas $G_{\beta\gamma}$ stimulates certain isoforms of adenylyl cyclases [27, 28]. By contrast, $G_{\alpha 16}$ and $G_{\beta\gamma}$ concomitantly activate phospholipase C [26, 29, 30]. Phospholipase A_2 is indirectly activated by $G_{\alpha o}$ after the stimulation of G protein-activated inwardly rectifying K^+ channels (GIRKs) and voltage-gated Ca^{2+} channels [31]. The complexity of cell signalling through MOP-R, as for all GPCR, results not only from the possible simultaneous (or successive) coupling of a receptor with different G-proteins, but also from the lifetime of the $G_{\alpha}^{(GTP)}$ complex, preventing the restoration of a $G_{\alpha}^{(GDP)}{}_{\beta\gamma}$ heterotrimer as long as the GPCR is activated. In addition to the inherent GTPase enzymatic activity leading to re-association of G_{α} and $G_{\beta\gamma}$, regulators of G protein signalling proteins (RGS) regulate the signal response by changing the intrinsic GTPase activity [32] and adjusting the temporal coupling of a GPCR and a $G_{\alpha}^{(GDP)}{}_{\beta\gamma}$ to satisfy cell requirements. The description of this signalling mechanism would be incomplete if we did not consider the role of many other intracellular proteins serving as scaffolding proteins, organising the partners involved in the signalling cascade [33]. These accessory proteins, such as activators of G-protein signalling (AGS), regulate the strength, efficiency and specificity of signal transfer upon MOP-R activation, directing the appropriate G-protein to the receptor in response to cell regulation [34]. They activate G proteins independently of the receptor with specificity for G_{α} subunits, accelerating hydrolysis and terminating signal transduction. All these mechanisms constitute alternative means of regulating the G_{α} subunit.

The data reported here support strongly the view that GPCRs, G-proteins and effectors are not randomly distributed in the plasma membrane and that GPCR signalling involves the concerted membrane displacements of molecules that must be located close together. This view underlies attempts to analyse the membrane environment and the constraints it imposes on GPCR signalling.

Effects of the lipid environment on MOP-R

Assuming that the thickness of the hydrophobic part of the receptor is modified after its activation, the biophysical characteristics of the membrane should have repercussions for the efficiency of receptor function. Certain lipids, such as cholesterol, are known to modify lipid fluidity and membrane thickness, and may therefore affect the activity of many transmembrane proteins [35–39]. In this context, extrapolating from the results obtained for DOP-R, changes in MOP-R conformation associated with agonist activation may be considered to generate differences between lipid and receptor hydrophobic membrane widths (lipid/protein hydrophobic mismatch) (1), driving activated MOP-R towards existing thicker zones of the membrane, such as raft nanodomains (2) establishing new raft zones, or (3) resulting in a loss of MOP-R activity due to a hydrophobic mismatch between the activated conformation of the receptor and the surrounding lipids in cases in which activated MOP-R is not driven towards existing raft domains and no new raft domains are created. These effects were clearly demonstrated for rhodopsin, the most extensively studied GPCR, after its reconstitution in model membranes, in which the modulation of active conformation was correlated with cholesterol content, fatty acid chain length and the degree of chain saturation [40–42]. These findings were consistent with there being an optimal thickness of the receptor for efficient functioning that is negatively modulated when a strong hydrophobic mismatch is generated by an inappropriate lipid composition of the surrounding membrane. For other GPCRs, problems with purification have resulted in most studies of the effect of lipids being carried out on native membranes. In all studies of galanin [43], cholecystokinin (CCK-R) [44], $serotonin_{1A}$ [45] and oxytocin receptors [46, 47], the lipid composition of the membrane has been shown to affect GPCR pharmacology [48]. In this context, several groups have attempted to determine the lipid requirements for optimal MOP-R function.

Modulation of MOP-R function by the nature of the surrounding lipids

MOP-R is systematically found to be inactive after solubilisation, and its pharmacological properties after reconstitution in model membranes have not been investigated. Farahbakhsh et al. [49] were the first to show that the pharmacological activity of the MOP receptor was dependent on cell membrane lipid composition.

It has been shown with enzymatic methods that an increase in phosphatidylcholine (PC) levels, accompanied by a decrease in cholesterol concentration, decreases the affinity of the receptor for opiate ligands, with normal levels of affinity recovered following the restoration of membrane composition. The phospholipid head group has been shown to be involved in this effect, but the greatest effect on the pharmacological properties of MOP-R was obtained by modifying cholesterol content [49].

Two modes of action of cholesterol on GPCRs have been described. The first involves direct, or even specific molecular interactions of cholesterol with a GPCR [10, 43]. The second is related to the classical effect of cholesterol on the biophysical properties of a lipid membrane. However, it is difficult to distinguish between these two modes of action. As cholesterol is present in considerable excess over the receptor, the molar ratio of the remaining cholesterol to receptor cannot fall below 1,000 for a large range of the normal levels of expression of these receptors. This holds for cholesterol depletion ratios as high as 99%, with the classical treatment of isolated cell membranes with MβCD removing 60% of the cholesterol present at most [50]. Thus, the experimental conditions it is actually possible to create are far from suitable for determining whether an interaction between a receptor and cholesterol is specific.

A thorough analysis of published data revealed only two studies providing evidence of a specific interaction of cholesterol with a GPCR, neither of which demonstrated the molecular specificity of this interaction. The first study reported an effect of cholesterol content on oxytocin receptor function [51]. The second showed that the human β_2-adrenergic receptor (hβ_2-AR) could be crystallised in an active form only in cholesterol-rich environments [12]. This suggests that the structural requirements for the functioning of this GPCR required the proximity of cholesterol. These two findings demonstrate that the presence of an appropriate lipid mixture around receptors optimises receptor function, but the question of the specificity of such interactions remains open.

No specific interaction between cholesterol and MOP-R has ever been demonstrated. Lagane et al. [52] replaced ergosterol, the sterol naturally present in yeast *Saccharomyces cerevisiae* (*SC*) membranes, with cholesterol and found that this substitution restored the binding properties of MOP-R expressed in *SC*. This suggests that the interaction between the receptor and cholesterol is specific, because these two sterols have opposite effects on ligand binding by MOP-R. Ergosterol constrains the receptor in an inactive state in yeast plasma membranes and cannot replace cholesterol in agonist activation. In a more recent study, these authors demonstrated recovery of the basal pharmacological profile of hMOP-R in cholesterol-depleted CHO cell membranes treated with a cholesterol, but not in such cells treated with ergosterol [53]. Given the large proportion of cholesterol retained in the membranes after depletion, as discussed above, these findings do not conclusively prove that a specific interaction occurs between cholesterol and MOP-R. However, these findings do raise questions about the changes in membrane microviscosity induced by these two sterols. For MOP-R, Emmerson et al. [54] suggested that membrane microviscosity modulated the "agonist-sensitive conformational change" of hMOP-R. It increased the agonist affinity constant after membrane rigidification by cholesteryl hemisuccinate (CHS) and decreased it after subsequent membrane fluidification by *cis*-vaccenic acid treatment [55]. However, the binding of antagonists or partial agonists, such as nalbuphine, is unaffected by CHS addition [54]. Gaibelet et al. [52, 53], using fluorescence polarisation measurements, showed that cholesterol depletion in CHO cell membranes effectively fluidified membranes and that this effect was reversed by complementation with either cholesterol or ergosterol. As different pharmacological profiles of MOP-R were observed in the complemented membranes, "global viscosity" does not appear to be a relevant membrane parameter for correlating membrane characteristics and MOP-R activity. We hypothesise that lateral pressure profiles through the bilayer would be different for the two sterols [56, 57] and induce distinct constraints to the changes of the receptor conformation consecutive to the agonist binding.

Gaibelet et al. [53] also observed that whereas cholesterol depletion alone did not affect the binding of antagonists, the additional inhibition of G-protein coupling markedly decreased high-affinity agonist binding to receptors by 30%. This effect was abolished by the restoration of cholesterol content. These results are consistent with previous findings for *S. cerevisiae* membranes [52] and suggest that cholesterol alone, independently of G-proteins, can maintain MOP-R in a high-affinity agonist-binding state for a given population of hMOP-R. There may therefore be different conformational states of MOP-R affected differently in terms of agonist binding and G-protein coupling by variations of membrane cholesterol content. It is possible that the conformational state of the

receptor is correlated with its location in a particular domain of the membrane.

Changes in the membrane surroundings of MOP-R during signalling

It has been suggested that "rafts"—microdomains in the outer layer of cell membranes highly enriched in cholesterol and sphingolipids [58]—may act as signalling platforms, optimising receptor activation and signalling regulation [59]. Two types of lipid microdomains rich in cholesterol have been distinguished: "planar lipid rafts" and caveolae, which correspond to flask-shaped invaginations enriched in caveolins [60]. Caveolae are relatively stable, because their structure is organised and stabilised by caveolins. Planar rafts are considered to be unstable, with a short lifetime; they are small and continually exchange their component molecules with their immediate environment. As shown in Table 1, two experimental approaches can be used to isolate these raft lipid microdomains as the lighter membrane fraction floating in an isopycnic gradient. The first method is based on the solubilisation of cell membrane fractions with mild non-ionic detergents and generates DRM (detergent-resistant membrane) fractions associated with pre-existing "planar lipid rafts" and containing various amounts of caveolin [61]. In the second approach, sodium carbonate (Na_2CO_3) is used for the specific separation of caveolae as membrane domains enriched in caveolin and cholesterol [62].

No clear consensus concerning the distribution of MOP-R in the membrane emerges from studies based on these invasive approaches (Table 1). Zhao et al. [63] found that, in the basal state and after agonist activation, MOP-R was mostly present in the lipid rafts/caveolae of HEK293 cells. This distribution was disrupted by MβCD membrane treatment and restored by cholesterol complementation. Huang et al. [64] found that rMOP-R was mostly found in low-density fractions of total membranes of CHO cells or rat brain tissues treated with sodium carbonate, with neurons having no caveolae. In addition, after cholesterol depletion by MβCD treatment, which was assumed to disrupt rafts, rMOP-R was shifted to higher density fractions in both CHO and brain tissue membranes. Head et al. [65] tested the hypothesis that the GPCR involved in regulating adenylyl cyclase was located in caveolae, by analysing the distribution of rMOP-R in membrane fractions of cardiac myocytes treated with 1% Triton X-100 or Na_2CO_3 buffer. It was found that rMOP-R was equally partitioned between buoyant and heavier fractions, without taking into account the pellet. Conversely, Mouledous et al. [66] compared the membrane raft localisation of MOP-R in SH-SY5Y neuroblastoma cells by the two procedures and found that the receptor was undetectable in rafts after detergent treatment and massively concentrated in rafts in the detergent-free approach. Finally, Gaibelet et al. [53] using the detergent approach with CHO cell membrane fractions enriched in plasma membrane, found that less than 2% of hMOP-R was present in the basal state in DRM fractions, with most of the receptors (>90%) present in heavier, soluble non-DRM fractions. Antagonist treatments did not change the distribution of MOP-R, but an increase in the proportion of hMOP-R in DRM ($\sim$12.5%), inhibited by cholesterol depletion or the addition of Gpp(NH)p, was observed after agonist treatment. As 50% of hMOP-R have a high affinity for agonists, the DRM fraction represents 25% of all hMOP-R with a high affinity for agonist. This agonist-activated MOP-R population corresponds to a receptor conformation requiring a particular lipid environment, with a high cholesterol level and, probably, G-protein coupling. An analysis of the lipids present in the various fractions further confirmed that membrane thickness was greater in DRM fractions. However, it was not possible to determine whether these two conformations were related to different patterns of pharmacological behaviour.

The lack of a unifying view of the distribution of receptors in membrane domains undoubtedly stems from the drawbacks of these biochemical approaches. Firstly, different methods are used to prepare membrane extracts or fractions, resulting in diversity in the level of enrichment [67]. Furthermore, as suggested in many reports [68], the rate of solubilisation of membrane fractions depends on the nature of the detergent [69] and its concentration [70]. Discussions of the data obtained often fail to take into account the fraction of receptors remaining in the pellet of the gradient tube, even though this fraction may correspond to a large proportion of the receptors.

Finally, cholesterol has been shown to be required for stabilisation of the MOP-R conformation with the highest affinity for agonist [53]. This should be seen in the light of the recent suggestion that cholesterol may regulate interactions between β_2-AR [12] and G proteins, by generating a multitude of different receptor conformations responsible for consecutive coupling to different G proteins [12]. All these hypotheses require experimental verification.

Modulations of MOP-R signalling by protein interactions

Although the mechanism by which G protein-coupled receptors (GPCRs) translate extracellular signals in cells was initially thought to follow a simple linear model, a plethora of interactions with scaffolding and accessory or chaperone proteins has recently been proposed, even including the homo- or heterodimerisation of GPCRs.

Table 1 Raft and N-raft membrane distribution of MOP-R

Cell type and expression level	Membrane characteristic: (caveolae)	Raft/N-raft extraction methods	MOP-R detection	Rate of MOP-R basal distribution in ldf	MOP-R basal distribution in ldf after cholesterol depletion	Cholesterol depletion treatment and MOP-R pharmacology	References
Rat caudate putamen rMOP-R	(+)	Detergent-free	Anti-μC[a]	~70%	ND	+MBCD 2% −38% of agonist Bmax	[64, 119]
Rat thalamus rMOP-R$^+$	(−)	Detergent-free	Anti-μC[a]	~45%	ND	+MBCD 2% −26% of agonist Bmax	[64, 119]
HEK 293-rMOP-R (6.9 pmol/mg of memb. prot.)	(+)	Detergent-free	Anti-haemagglutinin	~>55%	Mainly located in non-raft domains	+MBCD 100 μM −100% Reversed after Chol. complementation	[63]
Rat cardiac myocytes rMOP-R$^+$	(+)	Detergent-free	Anti-μC[a]	~50%	ND	ND	[65]
Rat cardiac myocytes rMOP-R$^+$	(+)	1% TX100	Anti-μC[a]	~50%	ND	ND	[65]
CHO-hMOP-R-T7 (8.5 pmol/mg of memb. prot.)	(+)	0.25% TX100	Anti-T7	3% +DAMGO 12.5%	0.3% in rafts, and without raft relocation after agonist activation	+[5 mM] MBCD −50% of agonist Bmax, Reversed after Chol. complementation	[53]
CHO-HA-rMOP-R (1.9 pmol/mg memb. prot)	(+)	Detergent-free	Anti-μC[a]	80–90%	ND	+MBCD 2% ~+50% of agonist Bmax,	[64, 119]
SH-SY5Y	(−)	Detergent-free	Anti-GFP (YFP)	>90%	~50%	5.4-fold increase in EC_{50} after depletion	[66]
SH-SY5Y	(−)	0.2% TX100	Anti-GFP (YFP)	Undetectable	ND	ND	[66]

ldf low-density fraction, *TX100* Triton X-100, *ND* not determined

[a] Polyclonal antibody generated against a synthetic peptide corresponding to the last 16aa of the C-terminal domain of rMOP-R; MOP-R natural expression

Table 2 Cytoplasmic molecules interacting directly with MOP-R

Receptor state	Type of protein	MOP-R localisation of the interaction	Interaction effect	References
Basal	Calmodulin (CaM)	Intracellular with the I3 loop	Inhibition of constitutive activity, competition with G-proteins	[73, 74]
	Spinophilin	Not determined	After MOP-R agonist activation, promotes in the striatum the formation of the scaffolding GPCR signalling network, which may contain protein phosphatase 1, RGS protein and Gβ subunits	[75]
After ligand activation	G-proteins. Preferentially: G_{o1} & G_{o2}, G_{i1} & G_{i2}, G_z. G-protein coupling demonstrated but functional significance unclear: G_{i1}. Modest coupling demonstrated: G_{15}, G_{16}	I3 loop and carboxyl tail	Main route of GPCR signalling Direct fast activation of ERKs, inducing nuclear translocation of extracellular signal-regulated kinases (ERKs)	[24]
	β-arrestin	Carboxyl tail via adaptor proteins	GRK phosphorylation of MOP-R (at the start) Slow activation of ERKs, inducing cytosolic retention of the phosphorylated ERKs	[14, 82–84]
	Filamin A (FLA)(member of the family of actin cytoskeleton proteins)	Carboxyl tail	Coupling of MOP-R to actin after GRK phosphorylation, inducing receptor endocytosis via clathrin-coated pits	[85, 89, 120]
	G protein-coupled receptor kinases (GRKs)		Phosphorylation of MOP-R	[80, 81]
	Other kinases: -Protein kinase A (PKA), -Protein kinase C (PKC), -Ca^{2+}/calmodulin-dependent protein kinase II	MOP-R can also be tyrosine-phosphorylated (Tyr96, Tyr 106, Tyr166, and Tyr 336)		[90, 121]
	Extracellular signal-regulated kinases (ERKs)	Direct interaction between MOP-R and ERK not proven	See G proteins and β-arrestin	[90]
	Regulators of G protein signalling (RGSs)	Not determined	Sequestration of RGSZ1 (RGS17) and RGSZ2 (RGS20), leading to an effector antagonist effect	[91]

Thus, there may be different processes responsible for specific molecular collisions and accounting for differences in the signalling pathways and cellular trafficking of GPCRs as a function of the ligand. MOP-R is phosphorylated and internalised via clathrin-coated pits, much like other GPCRs, but MOP-R has a number of unusual features and only a few proteins interacting with this receptor have been identified to date. Many GPCRs (such as the β_2-adrenergic receptor) have a sequence of four amino acids, the DSLL motif, that interacts with EBP50 (Ezrin-Radixin-Moesin binding phosphoprotein 50), a protein mediating recycling [71]. The last 17 amino acids of MOP-R are sufficient and necessary for its specific recycling mechanism, which is not dependent on the actin cytoskeleton and does not involve EBP50. Thus, for MOP-R, recycling seems to depend on other as yet unidentified factors or signals [72]. We have restricted our discussion (Table 2) to proteins for which interactions with MOP-R have been clearly established.

Basal interactions

The interaction of calmodulin (CaM) with MOP-R abolishes basal G-protein coupling and attenuates agonist-stimulated G-protein coupling of the receptor through direct interaction with the third intracellular (I3) loop of the receptor. As a result, CaM inhibits the constitutive activity of the receptor [73]. Following stimulation of the MOP-R with morphine, CaM is rapidly translocated to the nucleus, and an influx of Ca^{2+} into the cells is observed in MOP-R-transfected HEK-293 cells and in SH-SY5Y human neuroblastoma cells [74]. These results suggest that MOP-R uses a Ca^{2+}/CaM signalling pathway to regulate transcriptional activity.

Charlton et al. [75] recently demonstrated that spinophilin, a ubiquitous protein, co-immunoprecipitates from mouse striatum with MOP-R in basal conditions. This interaction is greatly enhanced by agonist activation of the MOP-R with morphine or fentanyl. Spinophilin is known to bind to several elements of the GPCR signalling network, including protein phosphatase 1 and RGS protein. Together with MOP-R, it may form a multi-protein complex with a putative role in functional regulation.

Protein interactions after MOP-R activation

Interactions with G-proteins (Table 2), the first and main signalling partner of MOP-R, will not be discussed further here because they have already been described in detail elsewhere [76, 77]. Instead, we prefer to highlight various processes altering the functional coupling of a receptor to its G-proteins and of the G-protein subunits to their second messengers. Recent evidences indicate that β-arrestins 1 and 2 are common multifunctional adaptor and transducer molecules that regulate GPCR intracellular signalling pathways [78]. They are also implicated in the MOP-R signalling efficiency and in its endocytosis [79]. Endocytosis involves MOP-R phosphorylation by G protein-coupled receptor kinases (GRKs), such as GRK2 in particular [80, 81], followed by the uncoupling of G-proteins after a recruitment of cytosolic β-arrestin [14, 82–84]. This process is rapid, occurring within 1 min of MOP-R activation, and is abolished in cells lacking filamin A [85]. This suggests that actin may play a role in the MOP-R trafficking mediated by filamin A, which has been shown to bind the carboxyl tail of the human MOP-R via its carboxy-terminal region [85].

Phosphorylated receptors are generally internalised through endocytosis via clathrin-coated pits. For MOP-R, G-protein coupling is not required, as prior treatment of cells with pertussis toxin does not abolish the endocytosis of the receptor [86], and a recycling process brings the receptors back to the plasma membrane. This process is activated by endogenous peptide ligands or several opioid drugs promoting a massive and rapid receptor endocytosis via clathrin-coated pits [87]. In contrast, morphine, which causes weak GRK phosphorylation and little β-arrestin recruitment, has a limited efficacy to induce internalisation [88]. MOP-R down-regulation was revealed to be mediated by two G-protein independent distinct cellular signal transduction pathways [89, 90]. One is GRK-dependent and proceeds through phosphorylation of residues in the C-terminal region of MOP-R, whereas the other is tyrosine kinase-dependent and involves phosphorylation of residues in the second intracellular loop of MOP-R. Thus, the amount of MOP-R at the plasma membrane is controlled by many factors, including phosphorylation, dephosphorylation, endocytosis, recycling, degradation and de novo synthesis.

Many other cellular protein factors are thought to interact with the agonist-MOP-R complex. The role of the Map kinases ERK 1 and ERK2 (extracellular signal-regulated kinases), which are proline-directed protein kinase, has been investigated [84]. Generally, two partners—G proteins and β-arrestins—induce the activation of ERKs, through different effects: the nuclear translocation of ERK and its cytosolic retention, respectively. For MOP-R, Zheng et al. [84] observed deviations from this established ERK-mediated cellular pathway as a function of the agonist ligand. While morphine and methadone activate ERKs via a PKC- but not β-arrestin- dependent pathway, etorphine and fentanyl activate ERKs in a β-arrestin-dependent manner [84].

A direct GPCR-RGS interaction has clearly been demonstrated for RGS proteins. In membranes from periaqueductal grey matter (PAG), both RGSZ2 and the related

protein RGSZ1 co-precipitate with MOP-R. However, only Gαz subunits co-precipitate with RGSZ2. The RGSZ1 and RGSZ2 proteins influence MOP-R signalling by sequestering Gα subunits, thereby playing the role of effector antagonists [91].

Homo- and hetero-oligomerisation of MOP-R with other GPCR

The oligomerisation of GPCRs is believed to modulate the response to agonist binding, with hetero-oligomerisation regulating the cross-talk between different pathways. It is difficult to determine whether a GPCR exists as an oligomer. This is particularly true for homo-oligomers. However, analyses of the pharmacology of pairs of co-expressed MOP-R bearing different inactive mutations [92] and measurements of bioluminescence resonance energy transfer (BRET) combined with quantitative radioligand binding assays [93] have provided convincing demonstrations of MOP-R homo-dimerisation.

Studies indicating the existence of heterodimers are more numerous. Table 3 lists the various heterodimers, the method by which they were identified and the changes to MOP-R pharmacology they triggered. An opioid receptor, the κ opioid receptor (KOP-R), was shown in a single BRET-based study to form oligomers with MOP-R [93], whereas a number of studies with a large panel of approaches suggested that MOP-R may oligomerise with the δ opioid receptor (DOP-R) [33, 93–99]. These studies revealed that the binding of both agonists and antagonists to DOP-R had a synergic effect on MOP-R, increasing the number of MOP-Rs in a high-affinity state. About one tenth of all known GPCRs have been shown to form hetero-oligomers with MOP-R (Table 3), with different effects, depending on the pharmacology of MOP-R. The activation of α2A adrenergic receptors increases G-protein signalling [100], whereas the activation of nociceptin [101], CCR5 chemokine [102, 103], CXCR4 chemokine [104], β_2 adrenergic [105], NPFF [106] and somatostatine sst_{2a} [107] receptors decreases the efficiency of MOP-R efficacy.

These findings raise questions about the role of direct receptor-receptor interactions in modulation of the response of MOP-R by another GPCR. As discussed in the next section, evidence supporting this hypothesis has been obtained from analyses of the membrane diffusion of MOP-R [106].

MOP-R membrane confinement and dynamics

A survey of the data obtained with static approaches shows that: (1) the pharmacological function of MOP-R is modulated by the surrounding membrane environment; (2) several active conformations of MOP-R with different membrane thicknesses co-exist during signalling; (3) a single receptor can induce different cascades of molecular events; (4) a multitude of accessory proteins, including GPCRs, intervene at different stages of receptor function. These observations raise many questions that can only be answered by dynamics studies. What type of membrane organisation can meet such constraints? Are receptors and other molecules confined to the membrane? What changes accompany signalling? Is the modulation of pharmacological response by lipid membrane composition associated with changes in the membrane organisation of MOP-R?

Two complementary methods have been used to assess the lateral mobility of MOP-R. Fluorescence recovery after photobleaching (FRAP) provides average measurements over all tagged molecules and single particle tracking (SPT) analyses single receptors. For a critical comparison of these two methods, readers should refer to a recent review [108]. Both methods have already provided relevant information about the relationship between the dynamic organisation and function of MOP-R.

hMOP-R and vrFRAP analysis

The acquisition of fluorescence recovery at variable spot radii (vrFRAP) can be used to identify and characterise microdomains in a membrane, making it possible to determine whether membrane confinements are connected or isolated and to evaluate their permeability [109]. MOP-R diffusion has been extensively analysed by vrFRAP at the surface of SHSY-5Y cells (Table 4) [106, 110]. In the basal state, the diffusion of MOP-R is confined to connected semi-permeable domains [106, 110] not related to DRM domains [111]. The addition of an antagonist had no effect, whereas agonists did affect this diffusion. The dynamic changes observed differ between agonists. After DAMGO binding, the receptor population displays slow diffusion restricted to isolated smaller domains corresponding to receptors undergoing internalisation. By contrast, after morphine binding, all receptors display slower diffusion in smaller, connected, semi-permeable domains than in the basal state, probably due to the interaction of MOP-R with G proteins. Finally, these results demonstrate the existence of two different patterns of membrane compartmentalisation for MOP-R, each correlated with one of the major events occurring at the plasma membrane after receptor activation [111]. Direct physical interaction has been shown to be involved in the cross-talk between MOP-R and NPFF2 receptors [106]. Indeed, the addition of an agonist of NPFF2 receptors induces a major modification of MOP-R, which undergoes long-range free diffusion [106]. These results suggest that receptor

Table 3 Homo- or hetero-oligomerisation of MOP-R with GPCR

GPCR	Cell model (type of transfection)	Method of identification	Changes in GPCR pharmacology	References
MOP-R	HEK 293	Recovery of MOP-R pharmacology by co-expression of two non-functional MOP-R-Gα mutants		[92]
	HEK 293 (TT)	Quantitative BRET analysis combined with quantitative radio-ligand binding assays		[93]
δ opioid receptor (DOP-R)	COS-7, CHO-K1 (TT)	Co-IP	Decrease in agonist binding affinity	[94]
	HEK293 (TT), CHO (ST), SKNSH (NE)	Co-IP	Agonist or antagonist activation of DOP-R, synergy with MOP-R (increase in number of high-affinity sites)	[33, 95]
	GH3, GH3MOP-R, GH3MOP-R-DOP-R	Patch-clamp technique and imaging of $[Ca^{2+}]$ flux	Change in the inhibition of $[Ca^{2+}]$-related mechanism with DOP-R is co-expressed: New Gq signalling pathway proposed	[96]
			Potentiation of MOP-R by DOP-R ligands (agonists, antagonists or inverse-agonists)	[122]
	HEK293 (TT)	BRET + Co-IP		[93]
	HEK293 (TT)	Quantitative BRET analysis combined with quantitative radioligand binding assays		
	HEK293 (ST of EcR293, which stably expressed the heterotrimeric ecdysone receptor and the retinoid X receptor)	Quantitative BRET		[97]
	HEK293 (TT) of MOP-R and DOP-R fused with the pertussis toxin-insensitive Giα	Co-IP and ligand binding analysis	Increase in MOP-R signalling requires an active conformation of DOP-R	[99, 123]
	CHO, HEK293, SKNSH	Co-IP, phospho-ERH assays, β-arrestin localisation, cell fractionation	MOP-R DOP-R heterodimers in the naïve state are in a conformation conducive to β-arrestin-mediated signalling. These associations are altered by treatment with MOP-R and DOP-R agonists	[98]
κ opioid receptor (KOP-R)	HEK 293 (TT)	Quantitative BRET analysis combined with quantitative radioligand binding assays		[93]
Nociceptin receptor (NOC-R)	CHO (TT)	Co-IP	Increased by a factor of up to 250 the affinity of opioid ligands by NOC-R	[101]

Table 3 continued

GPCR	Cell model (type of transfection)	Method of identification	Changes in GPCR pharmacology	References
Neuropeptide FF receptor (NPFF2)	SH-SY5Y (ST)	FRET, FRAP	Neuropeptide FF (NPFF) modulates the opioid system by exerting functional anti-opioid activity on neurons	[106]
α_{2A} adrenergic receptor	HEK293, MDCK, primary neurons	Co-IP, BRET, G coupling and MAP kinase assays	Treatment of MOP-R with α_{2A} agonists increases receptor signalling	[100]
	HEK293	FRET	Heterodimer allowed a cross-conformational switch permitting the direct inhibition of one receptor by the other with subsecond kinetics	[124]
sst_{2A} somatostatin receptor	HEK293 (ST)	Co-IP	Heterodimerisation did not affect the ligand binding or coupling properties of receptors but promoted the cross-modulation of phosphorylation, internalisation and desensitisation of these receptors	[107]
Dopamine D1 receptor	HEK293 (TT)	Nuclear translocation pathway		[125]
CCR5 chemokine receptor	CEM174 (NE)	Co-IP, crosslinking of membrane proteins	Morphine suppresses the inhibitory effect of MIP-1β	[126]
	CHO (TT)	Co-IP, binding and internalisation assays	The activation of either receptor affected the G-protein coupling of the other, probably due to enhanced phosphorylation of the receptor	[102]
CXCR4 chemokine receptor	Cortical neurons (NE)	Analytical pharmacology	DAMGO abolished the neuroprotective effect of CXCL12 (the specific CXCR4 ligand) in NMDA neurotoxicity studies	[104]

TT transient transfection, *ST* stable transfection, *NE* natural expression, *Co-IP* co-immunoprecipitation

Table 4 Diffusion behaviour of MOP-R in living cell membranes

Type of MOP-R and tag	Cell type	Techniques	Conditions	Diffusion type	Confinement size [l(μm)]	Behaviour origin	References
hMOP-R (eGFP)*	SH-SY5Y	vrFRAP 20 or 37°C	Free	Confined	0.7 ± 0.1	–	[111]
hMOP-R (eGFP)*	SH-SY5Y	vrFRAP (14°C)	Free DAMGO	Confined	1.0 ± 0.4	Actin interaction	[111]
			morphine	Confined + Free	0.6 ± 0.3	G-protein interaction	
				Confined	0.6 ± 0.2		
hMOP-R (YFP)*	SH-SY5Y	vrFRAP	Free	Confined	0.02–0.07 $\mu m^2/s$	Change in basal interaction	[106]
		vrFRAP	+1DMe**	80% free	1.03 ± 0.26 μm	Activation of NPFF2 receptors modifies hMOP-R diffusion	
				20% confined	0.2 $\mu m^2/s$		
					Very small confinements		
hMOP-R (T7)*	NRK	SPT	Free	Walking confined diffusion	0.2	Protein-protein interaction	[114]
MOP-R (T7)*	NRK	SPT	Free cytoskeleton drug	Hop diffusion	0.21–07	Actin filament	[112]
					0.32		

* Labelled molecule, + cells expressing wild-type NPFF2 receptors in addition to tagged MOP receptors, ** 1DMe, D-Tyr-Leu-(NMe)Phe-Gln-Pro-Gln-Arg-Phe-NH_2

compartmentalisation may be a prerequisite for subsequent efficient signalling.

hMOP-R and SPT analysis

The conclusions drawn from FRAP analysis are corroborated by two independent studies of hMOP-R diffusion at the surface of NRK cells by SPT providing direct evidences for receptor confinement [112–114]. However, the origin of the observed confinements remains unclear.

In both SPT studies, two different types of complex movements could be clearly distinguished among the hMOP-R trajectories. A minor population of hMOP-R displayed a "directed diffusion", i.e., a slow diffusion combined to an oriented displacement (Fig. 2). This population having a weight of the order of the membrane receptor recycling rate and increasing from 10 to 50% for agonist-bound receptors, it was attributed to receptors entering the internalisation pathway [114]. The large majority of MOP-R (90%) exhibited a combination of a short-term confined diffusion with a long-term random walk, which were interpreted differently in the two studies. Daumas et al. [114, 115] showed, by a thorough statistical analysis of their data, that the physical picture underlying this behaviour is a so-called "walking confined diffusion", i.e., a diffusion of hMOP-R confined to a domain

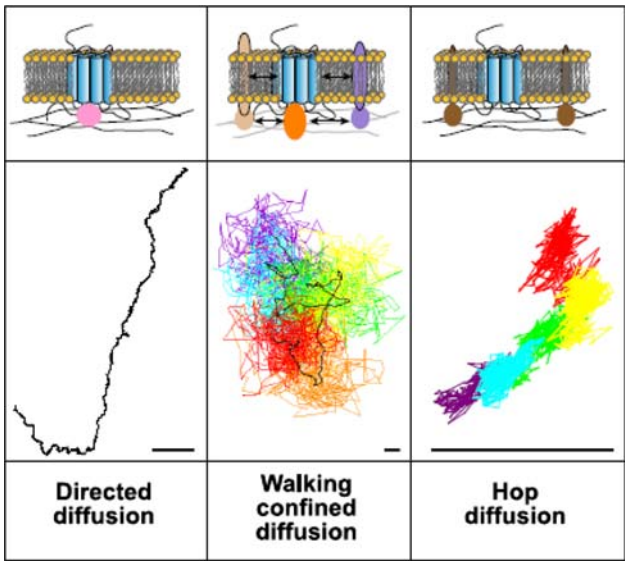

Fig. 2 Different membrane dynamic organisations of hMOP-R as inferred from trajectories obtained by single particle tracking experiments with 40-nm gold colloids (scale bar 100 nm). Link to actin filaments (entrance of the receptors in the internalisation pathway) is associated to a "directed diffusion" [114]. Involvement in protein-protein attractive interactions leads to the confined diffusion of the receptor into a domain that itself diffuses randomly. "Walking confined diffusion" (the *continuous black line* shows the displacement of the domain) [114]. Restriction of the receptor's diffusion by the membrane skeleton filaments and anchored proteins gives rise to a diffusion proceeding by successive jumps across barriers "hop diffusion" [112]

that itself diffuses (Fig. 2). They furthermore argued that attractive interprotein interactions are at the origin of the confinement and proposed a simple theoretical model explaining the observed quadratic increase of the domain size (found to vary between 30 and 550 nm) with the short-term diffusion coefficient [114]. Suzuki et al. [112] interpreted instead that the receptors undergo rapid "hop diffusion" over a network with two pore sizes (about 200 and 700 nm), according to the "membrane skeleton picket and fence model" [116] (Fig. 2).

In the presence of an agonist, receptors not entering into the internalisation pathway remain in the "walking confined diffusion" mode, but display a marked slowing down in strengthened domains [117]. This observation provides strong support for the involvement of inter-protein interactions in receptor confinement, which would be enhanced by changes in receptor conformation or the multitude of accessory proteins coming into play after agonist binding. The possible localisation of the receptors into rafts, already ruled out on the basis of biochemical analyses, is also not consistent with the dynamic behaviour observed, which is clearly different from associated transitory confinements, as demonstrated by Jacobson et al. [118].

Perspectives

Whereas many studies of MOP-R pharmacology have been carried out, driven by considerable interest in this receptor as a therapeutic molecule target, very few studies have analysed the diffusion of this receptor within the membrane. However, these studies have demonstrated the informative potential of such analyses for a detailed understanding of the molecular mechanisms of GPCR signal transduction. Two major modes of diffusion have been demonstrated for MOP-R: directed diffusion, probably involved in the redistribution of receptors to specific sites, and confined diffusion, presumably favouring interactions with signal transduction partners. Data for the partners of this receptor—G proteins, effectors and accessory proteins—are even more sparse, making it difficult to develop a picture of the dynamic organisation of this complex system. There is a need for simultaneous analyses of the diffusion of different signalling partners, based on emerging multicolour detection techniques with high levels of spatial and temporal resolution, to take up this challenge.

References

1. Waldhoer M, Bartlett SE, Whistler JL (2004) Opioid receptors. Annu Rev Biochem 73:953–990
2. Law PY, Loh HH, Wei LN (2004) Insights into the receptor transcription and signaling: implications in opioid tolerance and dependence. Neuropharmacology 47(Suppl 1):300–311
3. Pasternak GW (2004) Multiple opiate receptors: deja vu all over again. Neuropharmacology 47(Suppl 1):312–323
4. Mansour, A, Schafer, MKH, Newman, SW, Watson SJ (1991) Central distribution of opioid receptors: a cross-species comparison of the multiple opioid system of the basal ganglia. In: Almeida O, Shippenberg T (eds) Neurobiology of opioids. Springer, Berlin, pp 169–183
5. Hur EM, Kim KT (2002) G protein-coupled receptor signalling and cross-talk: achieving rapidity and specificity. Cell Signal 14:397–405
6. Becamel C, Gavarini S, Chanrion B, Alonso G, Galeotti N, Dumuis A, Bockaert J, Marin P (2004) The serotonin 5-HT2A and 5-HT2C receptors interact with specific sets of PDZ proteins. J Biol Chem 279:20257–20266
7. Bockaert J, Marin P, Dumuis A, Fagni L (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. FEBS Lett 546:65–72
8. Neubig RR (1994) Membrane organization in G-protein mechanisms. FASEB J 8:939–946
9. Digby GJ, Lober RM, Sethi PR, Lambert NA (2006) Some G protein heterotrimers physically dissociate in living cells. Proc Natl Acad Sci USA 103:17789–17794
10. Fahrenholz F, Klein U, Gimpl G (1995) Conversion of the myometrial oxytocin receptor from low to high affinity state by cholesterol. Adv Exp Med Biol 395:311–319
11. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. Science 289:739–745
12. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318:1258–1265
13. Chen C, Shahabi V, Xu W, Liu-Chen LY (1998) Palmitoylation of the rat mu opioid receptor. FEBS Lett 441:148–152
14. Chavkin C, McLaughlin JP, Celver JP (2001) Regulation of opioid receptor function by chronic agonist exposure: constitutive activity and desensitization. Mol Pharmacol 60:20–25
15. Filizola M, Laakkonen L, Loew GH (1999) 3D modeling, ligand binding and activation studies of the cloned mouse delta, mu; and kappa opioid receptors. Protein Eng 12:927–942
16. Pogozheva ID, Przydzial MJ, Mosberg HI (2005) Homology modeling of opioid receptor-ligand complexes using experimental constraints. AAPS J 7:E434–E448
17. Kobilka B, Schertler GF (2008) New G-protein-coupled receptor crystal structures: insights and limitations. Trends Pharmacol Sci 29:79–83
18. Alves ID, Cowell SM, Salamon Z, Devanathan S, Tollin G, Hruby VJ (2004) Different structural states of the proteolipid membrane are produced by ligand binding to the human delta-opioid receptor as shown by plasmon-waveguide resonance spectroscopy. Mol Pharmacol 65:1248–1257
19. Salamon Z, Wang Y, Soulages JL, Brown MF, Tollin G (1996) Surface plasmon resonance spectroscopy studies of membrane proteins: Transducin binding and activation by rhodopsin monitored in thin membrane films. Biophys J 71:283–294
20. Le Guyader L, Le Roux C, Mazeres S, Gaspard-Iloughmane H, Gornitzka H, Millot C, Mingotaud C, Lopez A (2007) Changes of the membrane lipid organization characterized by means of a new cholesterol-pyrene probe. Biophys J 93:4462–4473
21. Nikolaev VO, Boettcher C, Dees C, Bunemann M, Lohse MJ, Zenk MH (2007) Live cell monitoring of mu-opioid receptor-

mediated G-protein activation reveals strong biological activity of close morphine biosynthetic precursors. J Biol Chem 282: 27126–27132
22. Gonzalez-Maeso J, Rodriguez-Puertas R, Meana JJ (2002) Quantitative stoichiometry of G-proteins activated by mu-opioid receptors in postmortem human brain. Eur J Pharmacol 452:21–33
23. Blanchet C, Sollini M, Luscher C (2003) Two distinct forms of desensitization of G-protein coupled inwardly rectifying potassium currents evoked by alkaloid and peptide mu-opioid receptor agonists. Mol Cell Neurosci 24:517–523
24. Connor M, Christie MD (1999) Opioid receptor signalling mechanisms. Clin Exp Pharmacol Physiol 26:493–499
25. Garzon J, Castro M, Sanchez-Blazquez P (1998) Influence of Gz and Gi2 transducer proteins in the affinity of opioid agonists to mu receptors. Eur J Neurosci 10:2557–2564
26. Lee JW, Joshi S, Chan JS, Wong YH (1998) Differential coupling of mu-, delta-, and kappa-opioid receptors to G alpha16-mediated stimulation of phospholipase C. J Neurochem 70:2203–2211
27. Diel S, Beyermann M, Llorens JM, Wittig B, Kleuss C (2008) Two interaction sites on mammalian adenylyl cyclase type I and II: modulation by calmodulin and G(betagamma). Biochem J 411:449–456
28. Chakrabarti S, Regec A, Gintzler AR (2005) Chronic morphine acts via a protein kinase Cgamma-G(beta)-adenylyl cyclase complex to augment phosphorylation of G(beta) and G(betagamma) stimulatory adenylyl cyclase signaling. Brain Res Mol Brain Res 138:94–103
29. Ikeda K, Kobayashi T, Kumanishi T, Yano R, Sora I, Niki H (2002) Molecular mechanisms of analgesia induced by opioids and ethanol: is the GIRK channel one of the keys? Neurosci Res 44:121–131
30. Williams JT, Christie MJ, Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 81:299–343
31. Clark MJ, Harrison C, Zhong H, Neubig RR, Traynor JR (2003) Endogenous RGS protein action modulates mu-opioid signaling through Galphao: effects on adenylyl cyclase, extracellular signal-regulated kinases, and intracellular calcium pathways. J Biol Chem 278:9418–9425
32. Xie Z, Li Z, Guo L, Ye C, Li J, Yu X, Yang H, Wang Y, Chen C, Zhang D, Liu-Chen LY (2007) Regulator of G protein signaling proteins differentially modulate signaling of mu and delta opioid receptors. Eur J Pharmacol 565:45–53
33. Brady AE, Limbird LE (2002) G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell Signal 14:297–309
34. Sato M, Blumer JB, Simon V, Lanier SM (2006) Accessory proteins for G proteins: partners in signaling. Annu Rev Pharmacol Toxicol 46:151–187
35. Gimpl G, Fahrenholz F (2002) Cholesterol as stabilizer of the oxytocin receptor. Biochim Biophys Acta 1564:384–392
36. Scanlon SM, Williams DC, Schloss P (2001) Membrane cholesterol modulates serotonin transporter activity. Biochemistry 40:10507–10513
37. Nunez MT, Glass J (1982) Reconstitution of the transferrin receptor in lipid vesicles: effect of cholesterol on the binding of transferrin. Biochemistry 21:4139–4143
38. Pike LJ, Casey L (2002) Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking. Biochemistry 41:10315–10322
39. Westover EJ, Covey DF, Brockman HL, Brown RE, Pike LJ (2003) Cholesterol depletion results in site-specific increases in epidermal growth factor receptor phosphorylation due to membrane level effects: studies with cholesterol enantiomers. J Biol Chem 278:51125–51133
40. Polozova A, Litman BJ (2000) Cholesterol dependent recruitment of di22:6-PC by a G protein-coupled receptor into lateral domains. Biophys J 79:2632–2643
41. Niu SL, Mitchell DC, Litman BJ (2002) Manipulation of cholesterol levels in rod disk membranes by methyl-beta-cyclodextrin: effects on receptor activation. J Biol Chem 277: 20139–20145
42. Niu SL, Mitchell DC, Lim SY, Wen ZM, Kim HY, Salem N Jr, Litman BJ (2004) Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to N-3 fatty acid deficiency. J Biol Chem 279:31098–31104
43. Pang L, Graziano M, Wang S (1999) Membrane cholesterol modulates galanin-GalR2 interaction. Biochemistry 38:12003–12011
44. Harikumar KG, Puri V, Singh RD, Hanada K, Pagano RE, Miller LJ (2005) Differential effects of modification of membrane cholesterol and sphingolipids on the conformation, function, and trafficking of the G protein-coupled cholecystokinin receptor. J Biol Chem 280:2176–2185
45. Pucadyil TJ, Chattopadhyay A (2004) Cholesterol modulates ligand binding and G-protein coupling to serotonin(1A) receptors from bovine hippocampus. Biochim Biophys Acta 1663: 188–200
46. Gimpl G, Klein U, Reilander H, Fahrenholz F (1995) Expression of the human oxytocin receptor in baculovirus-infected insect cells: high-affinity binding is induced by a cholesterol-cyclodextrin complex. Biochemistry 34:13794–13801
47. Klein U, Gimpl G, Fahrenholz F (1995) Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. Biochemistry 34:13784–13793
48. Lazar DF, Medzihradsky F (1992) Altered microviscosity at brain membrane surface induces distinct and reversible inhibition of opioid receptor binding. J Neurochem 59:1233–1240
49. Farahbakhsh ZT, Deamer DW, Lee NM, Loh HH (1986) Enzymatic reconstitution of brain membrane and membrane opiate receptors. J Neurochem 46:953–962
50. Huang P, Xu W, Yoon SI, Chen C, Chong PL, Liu-Chen LY (2007) Cholesterol reduction by methyl-beta-cyclodextrin attenuates the delta opioid receptor-mediated signaling in neuronal cells but enhances it in non-neuronal cells. Biochem Pharmacol 73:534–549
51. Gimpl G, Fahrenholz F (2000) Human oxytocin receptors in cholesterol-rich vs: cholesterol-poor microdomains of the plasma membrane. Eur J Biochem 267:2483–2497
52. Lagane B, Gaibelet G, Meilhoc E, Masson JM, Cezanne L, Lopez A (2000) Role of sterols in modulating the human mu-opioid receptor function in *Saccharomyces cerevisiae*. J Biol Chem 275:33197–33200
53. Gaibelet G, Millot C, Lebrun C, Ravault S, Saulière A, André A, Lagane B, Lopez A (2008) Cholesterol content drives distinct pharmacological behaviours of μ-opioid receptor in different microdomains of the CHO plasma membrane. Mol Memb Biol 25:423–435
54. Emmerson PJ, Clark MJ, Medzihradsky F, Remmers AE (1999) Membrane microviscosity modulates mu-opioid receptor conformational transitions and agonist efficacy. J Neurochem 73:289–300
55. Shinitzky M, Skornick Y, Haran-Ghera N (1979) Effective tumor immunization induced by cells of elevated membrane-lipid microviscosity. Proc Natl Acad Sci USA 76:5313–5316
56. Cantor RS (1999) The influence of membrane lateral pressures on simple geometric models of protein conformational equilibria. Chem Phys Lipids 101:45–56
57. Cantor RS (1999) Lipid composition and the lateral pressure profile in bilayers. Biophys J 76:2625–2639

58. Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 68:533–544
59. Chidiac P (1998) Rethinking receptor-G protein-effector interactions. Biochem Pharmacol 55:549–556
60. Kurzchalia TV, Parton RG (1999) Membrane microdomains and caveolae. Curr Opin Cell Biol 11:424–431
61. Harder T, Simons K (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr Opin Cell Biol 9:534–542
62. Kurzchalia TV, Dupree P, Parton RG, Kellner R, Virta H, Lehnert M, Simons K (1992) VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. J Cell Biol 118:1003–1014
63. Zhao H, Loh HH, Law PY (2006) Adenylyl cyclase superactivation induced by long-term treatment with opioid agonist is dependent on receptor localized within lipid rafts and is independent of receptor internalization. Mol Pharmacol 69:1421–1432
64. Huang P, Xu W, Yoon SI, Chen C, Chong PL, Unterwald EM, Liu-Chen LY (2007) Agonist treatment did not affect association of mu opioid receptors with lipid rafts and cholesterol reduction had opposite effects on the receptor-mediated signaling in rat brain and CHO cells. Brain Res 1184:46–56
65. Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA (2005) G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. J Biol Chem 280:31036–31044
66. Mouledous L, Merker S, Neasta J, Roux B, Zajac JM, Mollereau C (2008) Neuropeptide FF-sensitive confinement of mu opioid receptor does not involve lipid rafts in SH-SY5Y cells. Biochem Biophys Res Commun 373:80–84
67. Cezanne L, Navarro L, Tocanne JF (1992) Isolation of the plasma membrane and organelles from Chinese hamster ovary cells. Biochim Biophys Acta 1112:205–214
68. Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K (2003) Resistance of cell membranes to different detergents. Proc Natl Acad Sci USA 100:5795–5800
69. Babiychuk B, Draeger A (2006) Biochemical characterization of detergent-resistant membranes: a systematic approach. Biochem J 397:407–416
70. André A, Gaibelet G, Le Guyader L, Welby M, Lopez A, Lebrun C (2008) Membrane partitioning of various delta-opioid receptor forms before and after agonist activations: the effect of cholesterol. Biochim Biophys Acta 1778:1483–1492
71. Gage RM, Kim KA, Cao TT, von Zastrow M (2001) A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. J Biol Chem 276:44712–44720
72. Tanowitz M, von Zastrow M (2003) A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. J Biol Chem 278:45978–45986
73. Wang D, Surratt CK, Sadee W (2000) Calmodulin regulation of basal and agonist-stimulated G protein coupling by the mu-opioid receptor (OP(3)) in morphine-pretreated cell. J Neurochem 75:763–771
74. Wang D, Tolbert LM, Carlson KW, Sadee W (2000) Nuclear Ca^{2+}/calmodulin translocation activated by mu-opioid (OP3) receptor. J Neurochem 74:1418–1425
75. Charlton JJ, Allen PB, Psifogeorgou K, Chakravarty S, Gomes I, Neve RL, Devi LA, Greengard P, Nestler EJ, Zachariou V (2008) Multiple actions of spinophilin regulate mu opioid receptor function. Neuron 58:238–247
76. Kristiansen K (2004) Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. Pharmacol Ther 103:21–80
77. Holinstat M, Oldham WM, Hamm HE (2006) G-protein-coupled receptors: evolving views on physiological signalling: symposium on G-protein-coupled receptors: evolving concepts and new techniques. EMBO Rep 7:866–869
78. Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science 308:512–517
79. Marie N, Aguila B, Allouche S (2006) Tracking the opioid receptors on the way of desensitization. Cell Signal 18:1815–1833
80. Carman CV, Barak LS, Chen C, Liu-Chen LY, Onorato JJ, Kennedy SP, Caron MG, Benovic JL (2000) Mutational analysis of Gbetagamma and phospholipid interaction with G protein-coupled receptor kinase 2. J Biol Chem 275:10443–10452
81. Celver JP, Lowe J, Kovoor A, Gurevich VV, Chavkin C (2001) Threonine 180 is required for G-protein-coupled receptor kinase 3- and beta-arrestin 2-mediated desensitization of the mu-opioid receptor in Xenopus oocytes. J Biol Chem 276:4894–4900
82. Qiu Y, Law PY, Loh HH (2003) Mu-opioid receptor desensitization: role of receptor phosphorylation, internalization, and representation. J Biol Chem 278:36733–36739
83. Deng HB, Yu Y, Pak Y, O'Dowd BF, George SR, Surratt CK, Uhl GR, Wang JB (2000) Role for the C-terminus in agonist-induced mu opioid receptor phosphorylation and desensitization. Biochemistry 39:5492–5499
84. Zheng H, Loh HH, Law PY (2008) Beta-arrestin-dependent mu-opioid receptor-activated extracellular signal-regulated kinases (ERKs) translocate to nucleus in contrast to G protein-dependent ERK activation. Mol Pharmacol 73:178–190
85. Onoprishvili I, Andria ML, Kramer HK, Ancevska-Taneva N, Hiller JM, Simon EJ (2003) Interaction between the mu opioid receptor and filamin A is involved in receptor regulation and trafficking. Mol Pharmacol 64:1092–1100
86. Remmers AE, Clark MJ, Alt A, Medzihradsky F, Woods JH, Traynor JR (2000) Activation of G protein by opioid receptors: role of receptor number and G-protein concentration. Eur J Pharmacol 396:67–75
87. Sternini C, Spann M, Anton B, Keith DE Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC (1996) Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. Proc Natl Acad Sci USA 93:9241–9246
88. Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. J Biol Chem 271:19021–19024
89. Borgland SL, Connor M, Osborne PB, Furness JB, Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. J Biol Chem 278:18776–18784 Epub 2003 Mar 17
90. Pak Y, O'Dowd BF, Wang JB, George SR (1999) Agonist-induced, G protein-dependent and -independent down-regulation of the mu opioid receptor: the receptor is a direct substrate for protein-tyrosine kinase. J Biol Chem 274:27610–27616
91. Garzon J, Rodriguez-Munoz M, Lopez-Fando A, Sanchez-Blazquez P (2005) The RGSZ2 protein exists in a complex with mu-opioid receptors and regulates the desensitizing capacity of Gz proteins. Neuropsychopharmacol 30:1632–1648
92. Pascal G, Milligan G (2005) Functional complementation and the analysis of opioid receptor homodimerization. Mol Pharmacol 68:905–915
93. Wang D, Sun X, Bohn LM, Sadee W (2005) Opioid receptor homo- and heterodimerization in living cells by quantitative bioluminescence resonance energy transfer. Mol Pharmacol 67:2173–2184

94. George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF (2000) Oligomerization of mu- and delta-opioid receptors: generation of novel functional properties. J Biol Chem 275:26128–26135
95. Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA (2000) Heterodimerization of mu and delta opioid receptors: a role in opiate synergy. J Neurosci 20:RC110(5)
96. Charles AC, Mostovskaya N, Asas K, Evans CJ, Dankovich ML, Hales TG (2003) Coexpression of delta-opioid receptors with micro receptors in GH3 cells changes the functional response to micro agonists from inhibitory to excitatory. Mol Pharmacol 63:89–95
97. Law PY, Erickson-Herbrandson LJ, Zha QQ, Solberg J, Chu J, Sarre A, Loh HH (2005) Heterodimerization of mu- and delta-opioid receptors occurs at the cell surface only and requires receptor-G protein interactions. J Biol Chem 280:11152–11164
98. Rozenfeld R, Devi LA (2007) Receptor heterodimerization leads to a switch in signaling: beta-arrestin2-mediated ERK activation by mu-delta opioid receptor heterodimers. FASEB J 21:2455–2465
99. Snook LA, Milligan G, Kieffer BL, Massotte D (2006) Mu-delta opioid receptor functional interaction: insight using receptor-G protein fusions. J Pharmacol Exp Ther 318:683–690
100. Jordan BA, Gomes I, Rios C, Filipovska J, Devi LA (2003) Functional interactions between mu opioid and alpha 2A-adrenergic receptors. Mol Pharmacol 64:1317–1324
101. Pan YX, Bolan E, Pasternak GW (2002) Dimerization of morphine and orphanin FQ/nociceptin receptors: generation of a novel opioid receptor subtype. Biochem Biophys Res Commun 297:659–663
102. Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen LY (2004) Heterodimerization and cross-desensitization between the mu-opioid receptor and the chemokine CCR5 receptor. Eur J Pharmacol 483:175–186
103. Zhang N, Oppenheim JJ (2005) Crosstalk between chemokines and neuronal receptors bridges immune and nervous systems. J Leukoc Biol 78:1210–1214
104. Patel JP, Sengupta R, Bardi G, Khan MZ, Mullen-Przeworski A, Meucci O (2006) Modulation of neuronal CXCR4 by the micro-opioid agonist DAMGO. J Neurovirol 12:492–500
105. Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci USA 98:343–348
106. Roumy M, Lorenzo C, Mazeres S, Bouchet S, Zajac JM, Mollereau C (2007) Physical association between neuropeptide FF and micro-opioid receptors as a possible molecular basis for anti-opioid activity. J Biol Chem 282:8332–8342
107. Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S (2002) Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 277:19762–19772
108. Baker A, Sauliere A, Dumas F, Millot C, Mazeres S, Lopez A, Salome L (2007) Functional membrane diffusion of G-protein coupled receptors. Eur Biophys J 36:849–860
109. Salome L, Cazeils JL, Lopez A, Tocanne JF (1998) Characterization of membrane domains by FRAP experiments at variable observation areas. Eur Biophys J 27:391–402
110. Sauliere A, Gaibelet G, Millot C, Mazeres S, Lopez A, Salome L (2006) Diffusion of the mu opioid receptor at the surface of human neuroblastoma SH-SY5Y cells is restricted to permeable domains. FEBS Lett 580:5227–5231
111. Saulière, A. (2007) Etapes membranaires de la transduction du signal par les recepteurs couplés aux protéines G: Organisation dynamique du récepteur μ aux opioïdes humain à la surface de neuroblastomes, in Thesis Biophysique. Université Paul Sabatier- Toulouse III: Toulouse. 200. Available after request to authors
112. Suzuki K, Ritchie K, Kajikawa E, Fujiwara T, Kusumi A (2005) Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques. Biophys J 88:3659–3680
113. Daumas F, Destainville N, Millot C, Lopez A, Dean D, Salome L (2003) Interprotein interactions are responsible for the confined diffusion of a G-protein-coupled receptor at the cell surface. Biochem Soc Trans 31:1001–1005
114. Daumas F, Destainville N, Millot C, Lopez A, Dean D, Salome L (2003) Confined diffusion without fences of a g-protein-coupled receptor as revealed by single particle tracking. Biophys J 84:356–366
115. Meilhac N, Le Guyader L, Salome L, Destainville N (2006) Detection of confinement and jumps in single-molecule membrane trajectories. Phys Rev E Stat Nonlin Soft Matter Phys 73:011915(4)
116. Fujiwara T, Ritchie K, Murakoshi H, Jacobson K, Kusumi A (2002) Phospholipids undergo hop diffusion in compartmentalized cell membrane. J Cell Biol 157:1071–1081
117. Daumas F (2002) Diffusion latérale du récepteur μ aux opioïdes analysée par suivi de particule unique à la surface de cellules vivantes: relation organisation dynamique-fonction, in Thesis Biophysique. Université Paul Sabatier-Toulouse III: Toulouse. 147. Available after request to authors
118. Jacobson K, Dietrich C (1999) Looking at lipid rafts? Trends Cell Biol 9:87–91
119. Huang P, Chen C, Xu W, Yoon SI, Unterwald EM, Pintar JE, Wang Y, Chong PL, Liu-Chen LY (2008) Brain region-specific N-glycosylation and lipid rafts association of the rat mu opioid receptor. Biochem Biophys Res Commun 365:82–88
120. Onoprishvili I, Ali S, Andria ML, Shpigel A, Simon EJ (2008) Filamin A mutant lacking actin-binding domain restores mu opioid receptor regulation in melanoma cells. Neurochem Res 33:2054–2061
121. El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH (2001) Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. J Biol Chem 276: 12774–12780
122. Gomes I, Gupta A, Filipovska J, Szeto HH, Pintar JE, Devi LA (2004) A role for heterodimerization of mu and delta opiate receptors in enhancing morphine analgesia. Proc Natl Acad Sci USA 101:5135–5139
123. Snook LA, Milligan G, Kieffer BL, Massotte D (2008) Co-expression of mu and delta opioid receptors as receptor-G protein fusions enhances both mu and delta signalling via distinct mechanisms. J Neurochem 105:865–873
124. Vilardaga JP, Nikolaev VO, Lorenz K, Ferrandon S, Zhuang Z, Lohse MJ (2008) Conformational cross-talk between alpha2A-adrenergic and mu-opioid receptors controls cell signaling. Nat Chem Biol 4:126–131
125. Juhasz JR, Hasbi A, Rashid AJ, So CH, George SR, O'Dowd BF (2008) Mu-opioid receptor heterooligomer formation with the dopamine D1 receptor as directly visualized in living cells. Eur J Pharmacol 581:235–243
126. Suzuki S, Chuang LF, Yau P, Doi RH, Chuang RY (2002) Interactions of opioid and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. Exp Cell Res 280:192–200