[Frontiers in Bioscience 14, 3594-3607, January 1, 2009]

Modulation of opioid receptor function by protein-protein interactions

Konstantinos Alfaras-Melainis[1,2], Ivone Gomes[1], Raphael Rozenfeld[1], Venetia Zachariou[2], Lakshmi Devi[1]

[1]Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA, [2]Department of Pharmacology, University of Crete, Heraklion, Crete, Greece

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Proteins involved in biogenesis/trafficking of opioid receptors
4. Proteins that modulate opioid receptor signaling
    4.1. Regulators of G-protein signaling
    4.2. G-protein-coupled receptor kinases
    4.3. beta-arrestins
    4.4. Other proteins
5. Proteins involved in endocytosis and degradation
    5.1. Protein interactions that modulate receptor endocytosis
    5.2. Proteins that modulate receptor degradation
6. Interactions with other GPCRs that modulate receptor function
7. Summary and Perspectives
8. Acknowledgements
9.References

1. ABSTRACT

Opioid receptors, MORP, DORP and KORP, belong to the family A of G protein coupled receptors (GPCR), and have been found to modulate a large number of physiological functions, including mood, stress, appetite, nociception and immune responses. Exogenously applied opioid alkaloids produce analgesia, hedonia and addiction. Addiction is linked to alterations in function and responsiveness of all three opioid receptors in the brain. Over the last few years, a large number of studies identified protein-protein interactions that play an essential role in opioid receptor function and responsiveness. Here, we summarize interactions shown to affect receptor biogenesis and trafficking, as well as those affecting signal transduction events following receptor activation. This article also examines protein interactions modulating the rate of receptor endocytosis and degradation, events that play a major role in opiate analgesia. Like several other GPCRs, opioid receptors may form homo or heterodimers. The last part of this review summarizes recent knowledge on proteins known to affect opioid receptor dimerization.

2. INTRODUCTION

Drug addiction is a complex disease, resulting from repeated exposure to a number of substances, including opiates, psychostimulants, nicotine and alcohol (1-3). Addiction involves several brain circuits, neurotransmitter systems, ion channels, and signal transduction molecules. For example, the spinal cord and periaqueductal grey neurons control analgesic responses, AMPA and dopamine receptors in mesocorticolimbic sites play a critical role in drug reward and craving, several nuclei in the amygdala modulate reward and relapse, while the noradrenergic nuclei of the locus coeruleus are highly activated during opiate withdrawal (1,4-6). Considerable effort has been put towards understanding the neuroanatomical and neurochemical mechanisms of addiction however the cell specific events that lead to addiction are not fully understood.

Among the most abused drugs, the opioid alkaloids heroin and morphine, are known to cause euphoria, dependence, analgesia and respiratory depression

Table 1. DOPR interacting proteins and receptors

Interacting protein	Function	Reference
alpha$_{2A}$AR, beta$_2$AR, sensory neuron specific receptor, CXCR4	Modulation of signaling and trafficking of individual receptors	(126-128)
beta-arrestin 1 and beta-arrestin 2	Mediators of receptor signaling	(64)
Calnexin	Receptor transport out of the ER and cell expression	(12, 13)
GASP-1	Trafficking and cell surface expression of the receptor	(116)
glycoprotein M6A	Receptor endocytosis and recycling	(114)
GRK2	Receptor phosphorylation and endocytosis	(53,54)
GRK3	Receptor phosphorylation and endocytosis	(51)
GRK5	Receptor phosphorylation and endocytosis	(55)
MORP and KOPR	new receptor complexes	(76, 125, 126, 128)
NSF, SNX1 and GASP	Post-endocytotic sorting of the receptor to lysosomes	(115)
PKC	Receptor endocytosis	(108)
Protachykinin	Receptor trafficking	(17)
Ubiquitin	Receptor degradation	(10,11)

Table 2. KOPR interacting proteins and receptors

Interacting protein	Function	Reference
beta$_2$AR	Affects the trafficking properties of beta$_2$AR	(126, 127)
beta-arrestin 1 and beta-arrestin 2	Mediators of receptor signaling	(65)
DOPR	Novel receptor complexes	(126,127)
EBP50/NHERF	Increase receptor recycling	(123)
GEC-1	Enhance cell surface expression	(19)
GRK2	Receptor phosphorylation and endocytosis	(56)
GRK3	Receptor phosphorylation and endocytosis	(57)
Ubiquitin	Receptor endocytosis and degradation	(125)

Table 3. MOPR interacting proteins and receptors

Interacting protein	Function	Reference
alpha$_{2A}$AR, CB1, ORL1, NK1, SS2A or CCR 5 receptors	New receptor complexes	(127)
beta-arrestin 1 and beta-arrestin 2	Mediators of signaling and endocytosis	(67-70)
Calmodulin	Interferes with Galpha coupling/signaling	(81,82)
Filamin A	Scaffold for cytoplasmic and signaling proteins-affects endocytosis/resensitization	(111-113)
GASP-1	Targeting receptor to lysosomes for degradation	(90, 118, 119)
Glycoprotein M6A	Receptor endocytosis and recycling	(114)
GRK2	Receptor phosphorylation and endocytosis	(48,50)
GRK3	Receptor phosphorylation and endocytosis	(51,52)
GRK6	Receptor phosphorylation and endocytosis	(52)
Phospholipase D2	Receptor endocytosis	(91,92)
RGS9-2	Modulates signal transduction and endocytosis	(41)
Spinophilin	Receptor signaling and endocytosis	(85)
Synaptophysin	Interacts with dynamin, regulates resensitization	(78)
Tamalin	Modulates receptor localization	(88)
Ubiquitin	Receptor degradation	(118,122)

7). The use of opioid alkaloids as analgesics in the treatment of chronic pain is often limited by the serious side effects and the development of analgesic tolerance. Endogenous opiate peptides modulate mood, appetite and pain, by activating particular G protein coupled receptors, namely, mu (MOPR), delta (DOPR), and kappa (KOPR) (7). These receptors differ in tissue distribution and selectivity for endogenous ligands, and could differentially modulate the physiologic effects of opioid receptor activation (7). For example, the rewarding and analgesic effects of opioid alkaloids, are mostly mediated via MOPR, although DOPR and KOPR are also implicated in several opiate actions. Over the past decade, a number of *in vitro* and *in vivo* studies pointed to the importance of adaptive changes downstream of the receptors in the long-term effects of opioids (reviewed in 8). These adaptations may involve a switch in receptor signal transduction pathways, changes in receptor trafficking and recycling as well as changes in transcriptional activity. Emerging evidence suggests that dysfunctions of particular signal transduction molecules or molecules necessary for receptor localization and deactivation, are often the underlying causes of addictive behaviors (9). These same signal transduction molecules may offer new therapeutic targets for addiction and analgesia. In this review, we summarize recent reports on proteins that appear to modulate opiate receptor responsiveness via actions in receptor biosynthesis, localization, trafficking, signaling and deactivation (Tables 1-3).

3. PROTEINS INVOLVED IN BIOGENESIS/TRAFFICKING OF OPIOID RECEPTORS

While substantial effort has been put towards exploring the mechanisms modulating G protein-coupled receptor endocytosis and degradation (reviewed later in this article) relatively less effort has been put towards

examining the mechanisms modulating receptor biogenesis and cell surface expression. Among the opioid receptor types, the plasma membrane expression of DOPR has been extensively studied. In recombinant systems, only a fraction of the newly synthesized receptor is ultimately transported to the cell surface (10), the rest being retained in the endoplasmic reticulum (ER) from where it is retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome (11). In the ER, DOPR interacts with the chaperone protein, calnexin (12). This interaction can be regulated by membrane-permeable opioid ligands that bind to the newly synthesized receptor (in the ER) causing it to dissociate from calnexin (12). This facilitates DOPR transport out of the ER and expression at the plasma membrane which, in turn, decreases the proportion of receptors that would otherwise be targeted for degradation (13). These compounds, that facilitate proper folding of the receptor and their escape from the ER quality control system, are called "pharmacological chaperones".

In dorsal root ganglia and spinal cord neurons, DOPR is often found associated with substance P/calcitonin gene-related peptide (CGRP) positive neurons more specifically, in the regulated secretory pathway where it is sorted into large dense-core vesicles (14, 15). It is thought that in unstimulated axons, DOPR is principally retained in intracellular vesicular compartments. Upon stimulation, DOPR becomes incorporated into the membrane during exocytotic release of transmitters or peptide modulators (16). More recently, the mechanism underlying the sorting/retention of DOPR in the regulated secretory pathway has been uncovered (17). The 3rd luminal domain of DOPR interacts with protachykinin, the precursor molecule of the substance P neuropeptide, and this interaction is responsible for sorting DOPR to the large dense-core vesicles. Following stimulation, substance P is released from the central terminals of sensory neurons and this process leads to cell surface expression of DOPR (17). Compared to the proteins involved in the maturation of DOPR, relatively little is known about the maturation of other opioid receptors or their heterodimers. A study examining the ability of opiates to regulate the maturation of mutant MOPR that gets sequestered in an intracellular compartment (and degraded) supported the notion that opiates can function as pharmacological chaperones of MOPR. These studies suggest that similar to DOPR, MOPR may also associate with endogenous chaperones and this would help in receptor maturation and cell surface expression (18). In the case of KOPR, studies show that interaction with GEC-1, a member of the microtubule associated protein family, leads to an increase in KOPR expressed at the cell surface (19). GEC-1 interacts directly with the C-tail of KOPR in the ER/Golgi and this interaction could involve N-ethylmaleimide sensitive factor (NSF) regulated vesicle fusion dynamics (19). In addition, it has been shown that some opioid ligands like naloxone or naltrexone function as pharmacological chaperones in the ER promoting KOPR folding and maturation and enhancing cell surface expression of the receptor (20,21). In the case of opioid receptor heterodimers, it has been proposed that the MOR-DOR heterodimer could interact with Galphaz in the ER early after biosynthesis (22). However, a role for this interaction in receptor maturation has not been examined.

4. PROTEINS THAT MODULATE OPIOID RECEPTOR SIGNALING

A number of studies have shown that following receptor activation the dynamics of receptor signaling can be modulated either by interaction with proteins regulating G-protein mediated signaling or by promoting receptor desensitization/resensitization. In the following sections we will describe some of the proteins that have been shown to modulate opioid receptor signaling.

4.1. Regulators of G-protein signaling

RGS (Regulators of G protein signaling) proteins are among the most important modulators of receptor signaling (23,24). RGS proteins were first identified in yeast and nematode worms (25,26). Evidence for a role of RGS-like proteins in regulation of GPCR signaling came from studies on the sst2 and egl-10 genes (26-28). These studies revealed that RGS proteins act on GTP-bound Galphai and/or Galphaq proteins via a 120 amino acid domain (RGS domain), and regulate the duration of signaling by increasing the speed of GTP hydrolysis (23,24). The mammalian RGS family consists of about forty proteins categorized into nine subfamilies (23, 24). Beyond the common RGS domain, members of the RGS family vary in length, structure, regional and cellular localization (23,24). Growing evidence indicates that RGS proteins may have a more complex function, which involves interactions with scaffolding proteins, G protein subunits, and effector molecules (28).

Evidence for a role of RGS proteins in modulation of MOPR function comes from a number of *in vitro* and *in vivo* studies (24,29). Studies in a C6 glioma cell line stably expressing MOPR, have shown that endogenous RGS proteins have profound effects on maximal agonist response in a number of functional assays like inhibition of adenylyl cyclase activity, phosphorylation of mitogen-activated protein kinase, Akt phosphorylation and inhibition of calcium channels (30-33). However, these studies did not elucidate if specific RGS proteins were involved in the modulation of MOPR signaling. Interestingly, RGS4 a member of the B/R4 subfamily is thought to play a role in modulating MOPR activity *in vivo*. This is based on findings that suggest that RGS4 appears to be dynamically regulated at the transcriptional and translational level by morphine (34-36). In addition, RGS4 is thought to play a role in the development of opiate dependence, since over-expression of this protein decreases electrophysiological responses to morphine in locus coeruleus neurons (30). Co-immunoprecipitation assays indicate the presence of RGS17 (also known as RGSZ2) and RGS20 (also known as RGSZ1), members of the A/RZ subfamily, in MOPR immunoprecipitates from endogenous tissue (37). These observations suggest that RGS17 and RGS20 could play a role in modulating MOPR function.

The striatal enriched protein, RGS9-2, appears to potently modulate MOPR signaling. RGS9-2 belongs to the C/R7 family of RGS proteins characterized by the presence of at least two functional regions that facilitate interactions with GPCRs: The GGL (Ggamma-like) and the DEP (Disheveled, EGL-10, Pleckstrin) domains. The GGL domain forms a stable complex with the brain enriched G□eta5 protein. Cellular localization of C/R7 RGS proteins is mediated via their DEP domain and the DEP domain-associated syntaxin-like proteins called R7BP (RGS7 binding protein) and R9AP (RGS9 anchor protein) (38). Reports from genetic mouse models indicate that RGS9-2 is a major determinant of the sensitivity to the rewarding actions of psychostimulant and opioid drugs (35,39,40). Co-immunoprecipitation studies demonstrate that morphine treatment promotes the formation of complexes between RGS9-2, MOPR and beta-arrestin 2 (41). In addition, RGS9-2 functions as a negative modulator of MOR internalization and prevents opioid-induced phosphorylation of ERK1/2 (41). Interestingly, ablation of the RGS9 gene enhances the analgesic actions of morphine but does not affect the onset or duration of analgesia (41).

Most studies examining the interaction of RGS proteins with opioid receptors have focused on MOPR mainly because of the latter's role in morphine-mediated antinociception. There is little information on the interactions between RGS proteins and DOPR or KOPR. Recent findings suggest that in HEK-293 cells MOPR mediated inhibition of adenylyl cyclase activity is significantly reduced following co-expression of either RGS1, RGS2, RGS4, RGS9, RGS10 or RGS19 (also known as Galpha interacting protein, GAIP) (42). However, DOPR mediated inhibition of adenylyl cyclase activity is decreased only upon co-expression of RGS9 (42). In the case of KOPR, studies show that co-expression of KOPR with RGS4 in Xenopus oocytes leads to a decrease in the basal levels of GIRK1/2 currents and to an increase in agonist-mediated K^+-conductance (43). Another report has shown that treatment of PC12 cells with the KOPR agonist, U69,593, leads to an up-regulation on RGS4 mRNA levels, and this is blocked by the antagonist, norbinaltorphimine, or by pre-treatment with pertussis toxin (44). Therefore, further studies are needed to characterize the interactions of different RGS proteins with opioid receptors, to examine the functional consequences of these interactions, and to determine whether these interactions are cell- and/or tissue-specific.

Previous work documented that interactions between RGS proteins and effector molecules like adenylyl cyclase, GIRK channels, cGMP phosphodiesterase, guanylyl cyclase, phospholipase C, and calcium channels may control their function, localization and association with other signaling molecules (29, 45, 46). These studies suggest an important role for RGS proteins in modulating receptor activity perhaps by direct association; this needs to be further explored.

4.2. G protein-coupled receptor kinases

G protein-coupled receptor kinases (GRKs) play a critical role in attenuating receptor signaling leading to a desensitized state of the receptor upon continued exposure to the agonist (for review see 47). Specific GRKs phosphorylate the C-terminal tail of the receptors, which then associate with beta-arrestin. The binding of beta-arrestin to the phosphorylated receptor prevents the latter's association with heterotrimeric G-proteins resulting in the disruption of G-protein mediated signal transduction (48).

To date seven GRKs have been identified in humans that are divided into three classes: GRK1-, GRK2- and GRK4-like. GRK1 (rhodopsin kinase) and the closely related GRK7 (iodopsin kinase) are found primarily in the retina where they regulate the function of opsin. GRK2 and the closely related GRK3 are widely expressed and share a C-terminal pleckstrin homology domain that binds PIP2 (phosphatidylinositol bisphosphate) and the Gbetagamma subunit. GRK4, GRK5 and GRK6 lack this Gbetagamma subunit binding domain but use direct PIP_2 binding and/or covalent lipid modification with palmitate to reside primarily at the plasma membrane (49). Although there is no current evidence indicating the specificity of GRKs for opioid receptor subtypes, a number of studies in heterologous expression systems have shown that MOPR can be phosphorylated by GRK2 (48, 50), GRK3 (51,52) and GRK6 (52), DOPR can be phosphorylated by GRK2 (53, 54), GRK3 (51), and GRK5 (55) and KOPR by GRK2 (56) or GRK3 (57). However, in the case of KOPR, a study showed that agonist mediated phosphorylation of human KOPR was blocked by expression of a dominant negative GRK2 mutant, whereas rat KOPR was not phosphorylated by the same agonist even in the presence of GRK2 or GRK3 (58). These results suggest that there could be species differences regarding receptor phosphorylation by GRKs.

Few studies have looked at the *in vivo* consequences of the interactions between GRKs and opioid receptors. For example, studies examining the levels of MOPR in the brains of addicts that died due to opiate overdose observed a decrease in the levels of MOPR as well as GRK3, GRK6 and beta arrestin-2 (59). It addition it has been shown that a chronic infusion, in mice, of the non-selective opioid antagonist, naloxone, caused a dose-dependent increase in the levels of MOPR and a decrease in the levels of GRK2 (60). Although these studies demonstrate altered levels of receptors and GRKs, the functional implications of these changes have not been investigated. Interestingly in the case of KOPR, studies using mice lacking GRK3 indicated that the development of analgesic tolerance to kappa agonists could be due to prolonged receptor phosphorylation by GRK3 (61).

It is a dogma in the GPCR field that receptor phosphorylation ultimately leads to its endocytosis via recruitment of beta-arrestins. Studies demonstrating that interactions between opioid receptors and GRKs lead to endocytosis are described in section 5.

4.3. Beta-arrestins

Opioid receptor phosphorylation by GRKs is thought to lead to the recruitment and binding of beta-arrestin to the phosphorylated C-terminal tail. This then

leads to the attenuation of G-protein mediated signaling and receptor endocytosis. Studies describing the involvement of beta-arrestins in opioid receptor endocytosis are described in section 5. A number of studies have shown that beta-arrestins can play an important role in modulating receptor signaling. In the following paragraphs we will describe evidence for the involvement of beta-arrestins in the modulation of opioid receptor signaling.

To date four functional arrestin gene family members have been cloned. Two of these are expressed only in the retina (visual arrestin and cone arrestin) and regulate photoreceptor function. The non-visual arrestins, beta-arrestin 1 and beta-arrestin 2 are expressed ubiquitously in all cells and tissues and function in the desensitization of most GPCRs except rhodopsin. Beta-arrestin 1 and beta-arrestin 2 exhibit 78% homology and contain binding motifs for clathrin and the $beta_2$-adaptin subunit of the AP-2 complex in their C-terminal tail which allows them to function as adaptor proteins and target GPCRs to clathrin coated pits for endocytosis (62, 63). The different opioid receptor subtypes exhibit different requirements for binding beta-arrestin 1 and beta-arrestin 2 (64) that could lead to different signaling outcomes (65, 66). GST pull down assays show that the 3rd intracellular loop and the C-terminal tail of DOPR and only the C-terminal tail of KOPR can interact with beta-arrestin 1 or beta-arrestin 2 (64). These studies did not observe any interaction between beta-arrestin 1 or beta-arrestin 2 and MOPR (64). However, studies in HEK-293 cells using beta-arrestin 2 tagged to GFP or in striatal neurons using dominant negative beta-arrestin 2 show that agonist activated MOPR can recruit beta-arrestin 2 (67, 68) although the efficacy of recruitment is agonist dependent (69). Interestingly, mice lacking beta-arrestin 2 exhibit a potentiation and increased duration of the analgesic effect of morphine underscoring the importance of beta-arrestin 2 in mediating MOPR function (70). In the case of DOPR, a BRET assay suggested that receptor phosphorylation promoted receptor selectivity for beta-arrestin 2 over beta-arrestin 1 without affecting the stability of the receptor-beta-arrestin complex (71). However, another study used fluorescence and co-immunoprecipitation to show that agonist treated DOPR bound and recruited beta-arrestin 1 and beta-arrestin 2 to the plasma membrane (72). In addition, it has been shown that over expression of beta-arrestin 1 leads to an attenuation of DOPR and KOPR but not MOPR mediated activation of G-proteins and inhibition of cAMP levels (73).

In addition to being involved in the attenuation of G protein mediated signaling, studies have shown that beta-arrestins can induce a sustained ERK phosphorylation that is distinct from the transient G-protein mediated ERK phosphorylation (74). A recent study showed that MOPR ligands such as etorphine and fentanyl, but not morphine or methadone, induced ERK phosphorylation via a beta-arrestin dependent pathway. This led to the translocation of phosphorylated ERK to the nucleus leading to an increase in the activity of Elk-1 and in the transcription of GRK2 and beta-arrestin 2 (75). More recently, a study showed that heterodimerization between MOPR and DOPR promotes the recruitment of beta-arrestin 2 to the plasma membrane leading to changes in the spatio-temporal dynamics of ERK-mediated signaling that are quite distinct from those observed with the MOPR homodimer (76). Taken together, these studies show that beta-arrestins play an important role in mediating opioid receptor signaling by serving as a switch between G protein dependent and independent signaling mechanisms.

4.4. Other proteins

In addition to RGSs, GRKs and beta-arrestins several other proteins have been shown to modulate opioid receptor signaling. MOPR has been shown to interact with actin and the intermediate filament-binding protein, periplakin. This interaction occurs between the fourth intracellular loop/helix VIII of the receptor and the C-terminal rod and linker region of periplakin. Periplakin reduces the coupling between MOPR and a Galphai fusion protein (77), suggesting a possible modulatory role of periplakin on MOPR function. MOPR function can also be regulated by interaction with synaptophysin. This complex facilitates MOPR endocytosis. This in turn accelerates the rate of receptor resensitization (78).

Another protein that has been shown to affect MOPR signaling is calmodulin (CAM). CAM is a ubiquitous Ca^{+2} sensitive regulatory protein, implicated in the regulation of a number of cytoplasmic enzymes including adenylyl cyclases, Ca^{2+}/CAM-dependent kinases and phosphatases, ion channels, Ca^{+2}-ATPases among others (79, 80). Several new studies show that calmodulin interacts directly with the 3rd intracellular loop of MOPR which results in reduced basal G protein coupling and attenuation of agonist mediated increase in GTPgammaS binding (81, 82).

There is also evidence that the scaffolding protein, spinophilin, interacts with $alpha_2$ adrenergic $alpha_2$AR), D2 dopamine and MOPR (83-85). Spinophilin contains a putative F-actin-binding domain at the amino terminus, a single PDZ domain, and a region predicted to form a coiled-coil structure at the carboxyl terminal (85, 86). A GPCR interacting domain lies between the actin binding and the PDZ domain (83, 84). In addition, spinophilin targets protein phosphatase 1 to specific substrates (83). Spinophilin competes with beta-arrestin 2 following activation of $alpah_2$AR, and plays a critical role in $alpha_2$AR stabilization at the cell surface. Recent data indicate that spinophilin is part of the signaling complexes involved in the modulation of MOPR signaling and endocytosis (85). Interestingly, spinophilin knockout mice exhibit reduced sensitivity to the analgesic effects of morphine, early development of tolerance but higher degree of physical dependence and increased sensitivity to the rewarding actions of the drug (85).

Another scaffold protein shown to interact with opioid receptors is Tamalin. This protein consists of multiple interacting domains, including PDZ, a leucine zipper region and a carboxy terminal PDZ binding motif (87). Tamalin interacts with PSD-95, PSD-95-associated proteins, ARFs (ADP-ribosylation factors), cytohesins, and

Mint-2 and is required for neuritic localization of mGluR1a (87). In the nucleus accumbens, Tamalin appears to be part of signaling complexes that modulate the analgesic actions of morphine via association with MOPR (88).

A direct association with the plasma membrane localized phospholipid-specific phosphodiesterase, phospholipase D2 (PLD2), appears to modulate opioid receptor expression at the cell surface (89, 90). This interaction is mediated via the Phox homology domain in the N terminal region of PLD2 (91). In general, PLD2 regulates agonist dependent and agonist-independent endocytosis of MOPR, DOPR as well as CB_1 cannabinoid receptors (90, 92). Taken together, these studies show that protein-protein interactions can play an important role in modulating opioid receptor signaling.

5. PROTEINS INVOLVED IN ENDOCYTOSYS AND DEGRADATION

Receptor endocytosis is required for agonist induced turnover and resensitization of opioid receptors. As mentioned in the previous section following receptor activation by agonists the C-terminal tail of the receptors is phosphorylated by specific GRKs and this leads to the recruitment of beta-arrestin to the phosphorylated receptors leading to the disruption of G-protein mediated signal transduction (48). In addition, beta-arrestins tether the receptors to clathrin-coated pits (93, 94) leading to receptor endocytosis. Following endocytosis, opioid receptors are either recycled back to the cell surface to undergo another round of signaling and/or they are targeted to lysosomes for degradation. In the following section we will describe the proteins that have been implicated in the endocytosis and degradation of opioid receptors.

5.1. Protein interactions that modulate receptor endocytosis

A number of studies show that the phosphorylation of specific residues in the C-terminal tail of opioid receptors is required for receptor internalization via clathrin-coated pits (56, 95-97). Present knowledge indicates that GPCR kinases (GRKs) are involved in the phosphorylation of activated opioid receptors. Confocal microscopy studies with enhanced green fluorescent protein (GFP) tagged opioid receptors and red fluorescent protein (RFP) tagged GRKs show that activated DOPR co-internalizes with GRK 2 and 3 while MOPR endocytosis is not accompanied by GRK endocytosis (55). In the case of KOPR, laser scanning microscopy using GFP tagged human KOPR and RFP tagged GRK2 or GRK3 suggests that GRK3 is better able to induce KOPR internalization than GRK2 (57).

A number of studies have demonstrated that beta-arrestin recruitment is required for opioid receptor endocytosis. In the case of MOPR, studies show that among most MOPR agonists morphine does not cause receptor endocytosis in heterologous expression systems; however, enhanced expression of recombinant beta-arrestin 2 and GRKs has been shown to increase morphine mediated MOPR internalization (50, 98). Interestingly, morphine can induce MOPR internalization in neurons, which is inhibited by dominant negative beta-arrestin 2 (68, 99, 100). In the case of DOPR, studies indicate that beta-arrestin 1 is required for phosphorylation dependent DOPR internalization while beta-arrestin 2 is required for phosphorylation–independent DOPR internalization (72). In the case of KOPR, selective peptide agonists (but not non-peptide agonists) were shown to induce endocytosis of rat KOPR (101). However, human KOPR has been shown to undergo endocytosis in response to the non-peptide agonist, U50,488H, but not etorphine by a mechanism involving GRKs, beta-arrestin 2 and dynamin I (102).

In addition to GRKs and beta-arrestins, a number of other proteins have been implicated in opioid receptor endocytosis. Studies show that RGS9-2, a protein that is enriched in striatum, delays agonist induced HA-MOPR internalization in transiently transfected PC12 cells (41). Coimmunoprecipitation assays reveal that morphine treatment enhances the interaction between RGS9-2 and MOPR (41). Another RGS, RGS14, has been found to associate with MOPR in the periaqueductal gray matter (PAG) neurons. Silencing of RGS14 leads to increased phosphorylation of S375 in the C-terminal tail of MOPR by morphine subsequently leading to increased receptor internalization and recycling to the membrane (103).

In the case of DOPR, a study has implicated RGS19 in its endocytosis. Immunofluorescence labeling and deconvolution analysis show that in the absence of agonist RGS19 is spatially segregated from Galphai3 and DOPR in clathrin-coated domains of the cell membrane while the Galphai3-YFP and DOPR are located in non-clathrin-coated microdomains of the plasma membrane (104). Upon receptor activation, Galphai3 partially colocalizes with RGS19 in clathrin-coated pits. Blocking of endocytosis with a dynamin mutant leads to a striking overlap in the distribution of DOPR, Galphai3-YFP and RGS19 in clathrin-coated pits (104). This suggests a mechanism where after agonist treatment DOPR and Galphai3 move together into clathrin-coated pits where Galphai3 and RGS19 meet and turn off G protein signaling. Subsequently Galphai3 returns to non-clathrin coated microdomains, RGS19 remains associated with clathrin-coated pits and DOPR is internalized via clathrin-coated vesicles (104).

Another protein implicated in MOPR endocytosis is p38MAPK. It has been shown that phosphorylation of 38MAPK is sufficient to trigger the constitutive internalization of MOPR even in the absence of agonist (105) since its inhibitor, SB203580, strongly impairs MOPR endocytosis. p38MAPK can phosphorylate the Rab5 effectors, EEA1 and Rabenosyn-5, on Thr-1392 and Ser-215 respectively and these phosphorylation events regulate the recruitment of the phosphorylated molecules to the plasma membrane ultimately leading to MOPR endocytosis (105). Interestingly, a phosphomimetic mutation of Thr-1392 in EEA1 can bypass the requirement for p38MAPK alphaisoform in MOPR endocytosis (105).

Another molecule implicated in GPCR endocytosis is phospholipase D, an enzyme that hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid. Phospholipase D has been implicated in signal transduction, membrane trafficking and cytoskeletal reorganization (for review see 106). Two mammalian isoforms of this enzyme have been identified: phospholipase D1, present mostly in intracellular membranes, and phospholipase D2, associated mostly with the plasma membrane (107). Studies show that phospholipase D2, which has been implicated in the formation of endocytotic vesicles, plays a role in MOPR endocytosis. Yeast two-hybrid and coimmunoprecipitation assays demonstrate interactions between the C-terminal tail of MOPR and phospholipase D2. In addition, MOPR endocytosis can be blocked by a dominant negative mutant of phospholipase D2 and, by inhibition of phospholipase D2-mediated phosphatidic acid production (91). Agonist induced and ARF-dependent phospholipase D2 activation have been reported to be a prerequisite for MOPR endocytosis and recycling (92). Interestingly, endogenous opioids as well as DAMGO, but not morphine, have been shown to promote phospholipase D2 activation and rapid MOPR recycling (92). Receptor mediated phospholipase D2 activation has been shown to be also required for the endocytosis of DOPR (92).

Protein kinase C (PKC) has been implicated in agonist-independent endocytosis of DOPR (108). Activation of PKC by either PMA (phorbol 12-myristate 13 acetate), an $alpha_{1A}$AR agonist or by ionomycin can lead to DOPR endocytosis that requires the phosphorylation of S344. This PKC mediated DOPR internalization involves a beta-arrestin and clathrin-dependent mechanism and does not require receptor phosphorylation by GRKs (108). A study showed that MOPR can also be endocytosed by a G-protein independent but tyrosine kinase-dependent mechanism, which can be blocked by genistein treatment (109).

Another protein that has been implicated in opioid receptor endocytosis is synaptophysin. Synaptophysin is an acidic Ca^{+2}-binding glycoprotein of ~38kDa that is present mostly in synaptic vesicles. It has 4 transmembrane regions and is reported to be the major cholesterol binding protein in synaptic vesicles (110). Coimmunoprecipitation and BRET studies show that in HEK cells co-expressing MOPR and synaptophysin both proteins are constitutively associated and this leads to increased MOPR endocytosis. The authors postulated that this was due to the ability of synaptophysin to recruit dynamin to the plasma membrane thus facilitating the fission of clathrin-coated vesicles. This was supported by the observation that a synaptophysin mutant that no longer interacted with dynamin prevented agonist mediated MOPR endocytosis (78).

Another report showed that the C-terminal tail of human MOPR binds to the carboxyl terminal region of human filamin A (111). Filamin A is a homodimeric F-actin cross-linker with a high molecular weight (~280 kDa) that organizes actin filaments into three-dimensional arrays linking them to the cell membrane. In addition, Filamin A anchors a variety of transmembrane proteins to the actin cytoskeleton and provides a scaffold for many cytoplasmic and signaling molecules (112). Confocal microscopy studies revealed that MOPR internalization was greatly reduced in the absence of filamin A (113). Interestingly, chronic morphine treatment was found to upregulate MOPR levels in cells lacking filamin A although the mechanisms are not clearly understood (113).

MOPR also interacts with glycoprotein M6A (114). M6a is a member of the proteolipid protein family of tetraspan membrane proteins and is mainly expressed in neurons. The transmembrane domains 4, 5, and 6 of MOPR and the protein stretch/domain including transmembrane domains 3 and 4 of M6a are important regions for the MOPR-M6a interaction. This interaction leads to enhanced MOPR endocytosis and recycling, whereas a dominant negative M6a (truncated mutant) prevents agonist-induced MOPR internalization. M6A has been shown to also interact with DOPR and thus may act as a scaffold molecule in the regulation of opioid receptor endocytosis (114).

5.2. Proteins that modulate receptor degradation

Following endocytosis receptors can either be dephosphorylated and recycled back to the cell surface or be targeted to lysosomes for degradation. An interesting study screened a library of C-terminal tails to 59 GPCRs fused to glutathione S- transferase for their ability to interact with 4 adapter proteins proposed to be involved in post-endocytotic sorting of receptors: ERM (ezrin-radixin-moesin)-binding phosphoprotein EB50, N-ethylmaleimide-sensitive factor, sorting nexin 1 (SNX1) and GASP. This study observed a strong interaction of DOPR with NSF (an adapter protein suggested to be involved in receptor recycling), SNX1 and GASP (adapter proteins suggested to be involved in lysosomal targeting of the receptors) (115).

Association with the G protein-coupled receptor associating protein (GASP-1) is thought to be involved in the trafficking and cell surface expression of DOPR (116). GASP-1 is found throughout the CNS and has been implicated in the sorting of a number of native GPCRs to the lysosome after endocytosis. GASP-1 interacts with the C terminal of several GPCRs from class A and class B subfamilies (117). The highest levels of binding for GASP-1 were observed with MOPR and with $beta_1$AR (90,118,119). In fact, mutant forms of the MOPR with increased affinity to GASP show enhanced post endocytic receptor degradation (90, 117,118).

Ubiquitin, a 76 amino acid polypeptide, has been shown to be involved in the degradation of GPCRs. It attaches covalently to the epsilon-amino group of lysine residues present in GPCRs and targets the latter for proteasomal degradation (via lysine48-linked ubiquitination) or for receptor down-regulation in lysosomes (via lysine63-linked ubiquitination). In the case of DOPR, a majority of synthesized receptors are transported to the cytoplasmic site of the ER membrane via the Sec61 translocon, where they are deglycosylated and

conjugated with ubiquitinin prior to degradation by the cytoplasmic 26 S proteasomes (10,11). There is some controversy regarding the involvement of ubiquitination in the degradation of DOPR endocytosed from the plasma membrane. A study showed that ubiquitination was not required for either the ligand-induced endocytosis of DOPR or for its post-endocytic trafficking to lysosomes since treatment with an inhibitor of proteasomal degradation or mutation of all cytoplasmic lysine residues in DOPR did not inhibit ligand-induced receptor endocytosis or the proteolytic degradation of endocytosed receptors (119). Another study showed that DOPR endocytosis was not affected by lysosomal protease inhibitors but was significantly attenuated by proteasome inhibitors leading to the accumulation of polyubiquitinated receptors (120). Interestingly, association with GASP1 has been shown to be able to modulate the trafficking of DOPR but not MOPR to lysosomes in the absence of ubiquitination (116). This ubiquitin-independent trafficking of DOPR to lysosomes utilizes some (Vps4 and Hrs) but not all (Tsg101) of the vacuolar protein sorting machinery that is needed for lysosomal sorting of ubiquitinated receptors (121). In the case of MOPR, although the endocytosed receptors can be sorted into lysosomes, most of them are thought to recycle rapidly to the plasma membrane due to the presence of a specific 17-amino acid sequence in the C-terminal tail that specifically promotes the sorting of receptors into a rapid recycling pathway (122). In addition, deletion of the "recycling signal" from the cytoplasmic tail of MOPR enhances its interaction with GASP leading to receptor degradation after endocytosis (118).

In the case of KOPR, there is evidence for interactions with Ezrin-radixin-moesin (ERM)-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF), a PDZ domain-containing phosphoprotein. This interaction occurs between the PDZ domain I of EBP-50 and the C- terminal tail of KOPR and appears to block agonist-induced KOPR down-regulation by increasing the recycling rate of internalized receptors (123). Interestingly, a study showed that agonist-mediated endocytosis of human KOPR could target the receptors to lysosomes and proteasomes for degradation by a process requiring GRK2, beta-arrestin 2, dynamin I, and rab5 (124). In a later study the authors went on to show that the targeting of human KOPR to lysosomes for degradation also required the ubiquitination of three lysine residues in the C-terminal tail of KOPR via Lys 63-linked polyubiquitination (125).

6. INTERACTIONS WITH OTHER GPCRS THAT MODULATE RECEPTOR FUNCTION

Another level of protein-protein interaction leading to the modulation of receptor function is provided by receptor heterodimerization. In this case the receptor associates with another receptor type leading to the modulation of binding, signaling or trafficking properties. In the case of opioid receptors studies show that DOPR can heterodimerize with KOPR or MOPR leading to the formation of new receptor complexes that exhibit pharmacological and signaling properties that are distinct from each individual receptor (reviewed in 126,127). In addition, a number of studies have shown that opioid receptors can also heterodimerize with non-opioid receptors. For example studies show that MOPR can heterodimerize with either $alpha_{2A}$AR, CB1 cannabinoid, ORL1, NK1, somatostatin 2A or chemokine 5 receptors leading to a modulation in the binding, signaling, trafficking or a combination of these (reviewed in 127). Also DOPR has been shown to heterodimerize with $alpha_{2A}$AR, $beta_2$AR, sensory neuron specific receptor or CXCR4 leading to modulation in the signaling or trafficking properties of individual receptors (reviewed in 127-128). In the case of KOPR, besides its heterodimerization with DOPR as mentioned above, it has been shown to heterodimerize with $beta_2$AR and this drastically affects the trafficking properties of $beta_2$AR (reviewed in 126, 127). Finally, recent studies support a role for dimerization in the maturation and trafficking of a variety of GPCRs including MOPR and DOPR (128).

7. SUMMARY AND PERSPECTIVES

The biosynthesis, cellular localization, trafficking, signaling and degradation of opioid receptors can be modulated by interactions with proteins as diverse as cytoskeletal proteins, signaling molecules, enzymes, kinases, etc. This would, in turn, modulate the responsiveness of opioid receptors following exposure to agonists. Although a great deal is known about proteins that modulate the trafficking of opioid receptors much less is known about proteins that interact with the receptors during biogenesis and help target them to the cell surface. Another area of research would involve the elucidation of the machinery involved in opioid receptor degradation. This would, in turn, contribute to our understanding of opioid receptor function and could provide insights into the development of tolerance to opiates.

In the last decade it has become increasingly apparent that opioid receptor function can be modulated by heterodimerization with other receptors. However, very little is known about the machinery involved in the biogenesis, trafficking and degradation of opioid receptor heterodimers. These studies would help us in understanding the role and regulation of opioid receptor heterodimerization in health and disease states as well as help in the design of novel heterodimer-specific reagents (ligands, allosteric modulators and antibodies).

8. ACKNOWLEDGEMENTS

This work was supported by NIDA grants DA008863 and DA019521 (L.A.D.) and by the Greek Secretariat for Research and Technology-PENED03 (V.Z.)

9. REFERENCES

1. G.F. Koob. The neurobiology of addiction: a neuroadaptational view relevant for diagnosis. *Addiction* 101, 23-30 (2006)

2. S.E. Hyma., R.C. Malenka. Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat Rev Neurosci* 2, 695-703 (2001)

3. D.W. Self, E.J. Nestler. Relapse to drug-seeking: neural and molecular mechanisms. *Drug and Alcohol Dependence* 51, 49-60 (1998)

4. J. Bossert, U. Ghitza, L. Lu, D. Epstein, Y. Shaham. Neurobiology of relapse to heroin and cocaine seeking: An update and clinical implications. *Eur J Pharmacol* 526, 36-50 (2005)

5. E.J. Nestler, G.K. Aghaganian. Molecular and cellular basis of addiction. *Science* 278, 58-63 (1997)

6. C.E. Inturrisi. Clinical pharmacology of opioids for pain. *Clin J Pain* 18, S3-13 (2002)

7. B.L. Kieffer. Opioids: first lessons from knockout mice. *Trends Pharmacol Sci* 20, 19-26 (1999)

8. C. Contet, B.L. Kieffer, K. Befort. Mu opioid receptor, a gateway to drug addiction. *Curr Opin Neurobiol* 14, 370-378 (2004)

9. M.J. Christie. Cellular adaptations to chronic opioids:tolerance, withdrawal and addiction. *Br J Pharmacol* 154, 384-396 (2008)

10. U.E. Petaja-Repo, M. Hogue, A. Laperriere, P. Walker, M. Bouvier. Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. *J Biol Chem* 275, 13727-13736 (2000)

11. U.E. Petaja-Repo, M. Hogue, A. Laperriere, S. Bhalla, P. Walker, M. Bouvier. Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. *J Biol Chem* 276, 4416-4423 (2001)

12. T.T. Leskelä, P.M. Markkanen, E.M. Pietilä, J.T. Tuusa, U.E. Petäjä-Repo. Opioid receptor pharmacological chaperones act by binding and stabilizing newly synthesized receptors in the endoplasmic reticulum. *J Biol Chem* 282, 23171-23183 (2007)

13. U.E. Petäjä-Repo, M. Hogue, S. Bhalla, A. Laperrière, J.P. Morello, M. Bouvier. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. *EMBO J* 21, 1628-1637 (2002)

14. P.Y. Cheng, A.L. Svingos, H. Wang, C.L. Clarke, S. Jenab, I.W. Beczkowska, C.E. Inturrisi, V.M. Pickel. Ultrastructural immunolabeling shows prominent presynaptic vesicular localization of delta-opioid receptor within both enkephalin- and nonenkephalin-containing axon terminals in the superficial layers of the rat cervical spinal cord. *J Neurosci* 15, 5976-5988 (1995)

15. X. Zhang, L. Bao, U. Arvidsson, R. Elde, T. Hökfelt. Localization and regulation of the delta-opioid receptor in dorsal root ganglia and spinal cord of the rat and monkey: evidence for association with the membrane of large dense-core vesicles. *Neuroscience* 82, 1225-1242 (1998)

16. P.Y. Cheng, L.Y. Liu-Chen, V.M. Pickel. Dual ultrastructural immunocytochemical labeling of mu and delta opioid receptors in the superficial layers of the rat cervical spinal cord. *Brain Res* 778,367-380 (1997)

17. J.S. Guan, Z. Z. Xu, H. Gao, S. Q. He, G. Q. Ma, T. Sun, L. H. Wang, Z. N. Zhang, I. Lena, I. Kitchen, R. Elde, A. Zimmer, C. He, G. Pei, L. Bao, and X. Zhang. Interaction with vesicle luminal protachykinin regulates surface expression of delta-opioid receptors and opioid analgesia. *Cell* 122, 619-631 (2005)

18. V. Chaipatikul, L.J. Erickson-Herbrandson, H.H. Loh, P.Y. Law. Rescuing the traffic-deficient mutants of rat mu-opioid receptors with hydrophobic ligands. *Mol Pharmacol* 64, 32-41 (2003)

19. C. Chen, J. G. Li, Y. Chen, P. Huang, Y. Wang, and L. Y. Liu-Chen. GEC1 interacts with the kappa opioid receptor and enhances expression of the receptor. J Biol Chem 281, 7983-7993 (2006)

20. Y. Chen, C. Chen, Y. Wang, L.Y. Liu-Chen. Ligands regulate cell surface level of the human kappa opioid receptor by activation-induced down-regulation and pharmacological chaperone-mediated enhancement: differential effects of nonpeptide and peptide agonists. *J Pharmacol Exp Ther* 319,765-775 (2006)

21. K.M. Wannemacher, P.N. Yadav, R.D.Howells. A select set of opioid ligands induce upregulation by promoting the maturation and stability of the rat kappa opioid receptor in human embryonic kidney 293 cells. *J Pharmacol Exp Ther* 323, 614-625 (2007)

22. A. Hasbi, T. Nguyen, T. Fan, R. Cheng, A. Rashid, M. Alijaniaram, M.M. Rasenick, B.F. O'Dowd, S.R. George. Trafficking of preassembled opioid mu-delta heterooligomer-Gz signaling complexes to the plasma membrane: coregulation by agonists. *Biochemistry* 46, 12997-13009 (2007)

23. G.X. Xie, P.P Palmer. How regulators of G protein signaling achieve selective regulation. *J Mol Biol* 366, 349-365 (2007)

24. S.B. Hooks, K. Martemyanov, V. Zachariou. A role of RGS proteins in drug addiction. *Biochem Pharmacol* 75, 76-84 (2008)

25. H.G. Dohlman, J. Song, D. M. Apanovitch, P. R. DiBello, and K. M. Gillen. Regulation of G protein signaling in yeast. *Semin Cell Dev Biol* 9, 135-141 (1998)

26. M.R. Koelle, and H. R. Horvitz. EGL-10 regulates G protein signaling in the C. elegans nervous system and shares a conserved domain with many mammalian proteins. *Cell* 84, 115-125 (1996)

27. H.G. Dohlman, J. Song, D. Ma, W. E. Courchesne, and J. Thorner. 1996. Sst2, a negative regulator of pheromone signaling in the yeast Saccharomyces cerevisiae: expression, localization, and genetic interaction and physical association with Gpa1 (the G-protein alpha subunit) *Mol Cell Biol* 16,5194-5209 (1996)

28. S.A. Burchett. Regulators of G protein signaling: a bestiary of modular protein binding domains. *J Neurochem* 75, 1335-1351 (2000)

29. M. Abramow-Newerly, A. A. Roy, C. Nunn, and P. Chidiac. RGS proteins have a signalling complex: interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins. *Cell Signal* 18, 579-591 (2006)

30. M.J. Clark, C.A. Furman, T.D. Gilson, J.R. Traynor. 2006. Comparison of the relative efficacy and potency of mu-opioid agonists to activate Galphai/o containing a pertussis toxin-insensitive mutation. *J Pharmacol Exp Ther* 317, 858-864 (2006)

31. M.J. Clark, C. Harrison, H. Zhong, R.R. Neubig, J.R. Traynor. Endogenous RGS protein action modulates mu-opioid signalling through Galphao: effects on adenylyl cyclace, extracellular signal-regulated kinases and intracellular calcium pathways. *J Biol Chem* 278, 9418-9425 (2003)

32. M.J. Clark, J.R. Traynor. Modulation of adenylyl cyclace sensitization by PTX-insensitive GalphaoA, Galphai1, Galphai2 or Galphai3. *J Neurochem* 99, 1494-1504 (2006)

33. M.J. Clark, J.J. Linderman, J.R. Traynor. Endogenous regulators of G-protein signalling differentially modulate full and partial mu-opioid agonist at adenylyl cyclase as predicted by a collision coupling model. *Mol Pharmacol* 73, 1538-1548 (2008)

34. G.B. Bishop, W. E. Cullinan, E. Curran, and H. B. Gutstein. Abused drugs modulate RGS4 mRNA levels in rat brain: comparison between acute drug treatment and a drug challenge after chronic treatment. *Neurobiol Dis* 10, 334-43 (2002)

35. S.J. Gold, M. H. Han, A. E. Herman, Y. G. Ni, C. M. Pudiak, G. K. Aghajanian, R. J. Liu, B. W. Potts, S. M. Mumby, and E. J. Nestler. Regulation of RGS proteins by chronic morphine in rat locus coeruleus. *Eur J Neurosci* 17, 971-980 (2003)

36. M. Garnier, P.F. Zaratin, G. Ficalora, M. Valente, L. Fontanella, M.-H. Rheee, K.J. Blumer, M.A. Scheideler. Up-regulation of regulator of G protein signalling 4 expression in a model of neuropathic pain and insensitivity to morphine. *J Pharmacol Exp Ther* 304, 1299-1306 (2003)

37. J. Garzon, M. Rodriguez-Munoz, A. Lopez-Fando, P. Sanchez-Blasquez. The RGSZ2 protein exists in a complex with mu-opioid receptors and regulates the desensitization capacity of Gz proteins. *Neuropshychopharmacol* 30, 1632-1648 (2005)

38. K.A. Martemyanov, P. J. Yoo, N. P. Skiba, and V. Y. Arshavsky. R7BP, a novel neuronal protein interacting with RGS proteins of the R7 family. *J Biol Chem* 280, 5133-5136 (2005)

39. Z. Rahman, J. Schwarz, S. J. Gold, V. Zachariou, M. N. Wein, K. H. Choi, A. Kovoor, C. K. Chen, R. J. DiLeone, S. C. Schwarz, D. E. Selley, L. J. Sim-Selley, M. Barrot, R. R. Luedtke, D. Self, R. L. Neve, H. A. Lester, M. I. Simon, and E. J. Nestler. RGS9 modulates dopamine signaling in the basal ganglia. *Neuron* 38, 941-952 (2003)

40. V. Zachariou, V., D. Georgescu, N. Sanchez, Z. Rahman, R. DiLeone, O. Berton, R. L. Neve, L. J. Sim-Selley, D. E. Selley, S. J. Gold, and E. J. Nestler. Essential role for RGS9 in opiate action. *Proc Natl Aca. Sci U S A* 100, 13656-13661 (2003)

41. K. Psifogeorgou, P. Papakosta, S.J. Russo, R.L. Neve, D. Kardassis, S.J. Gold, V. Zachariou V.2007. RGS9-2 is a negative modulator of mu-opioid receptor function. *J Neurochem* 103, 617-625 (2007)

42. Z. Xie, Z. Li, L. Guo, C. Ye, J. Li, X. Yu, H. Yang, Y. Wang, C. Chen, D. Zhang, and L. Y. Liu-Chen. Regulator of G protein signaling proteins differentially modulate signaling of mu and delta opioid receptors. *Eur J Pharmacol* 565, 45-53 (2007)

43. C. Ulens, P. Daenens, J. Tytgat. Changes in GIRK1/GIRK2 deactivation kinetics and basal activity in the presence and absence of RGS4. *Life Sci* 67, 2305-2317 (2000)

44. T. Nakagawa, M. Minami, M. Satoh. Up-regulation of RGS4 mRNA by opioid receptor agonists in PC12 cells expressing cloned mu- or kappa-opioid receptors. *Eur J Pharmacol* 433, 29-36 (2001)

45. S.Hollinger, and J. R. Hepler. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev 54, 527-559 (2002)

46. E.L. Riddle, R. A. Schwartzman, M. Bond, and P. A. Insel. Multi-tasking RGS proteins in the heart: the next therapeutic target? *Circ Res* 96,401-411 (2005)

47. E. Kelley, C.P. Bailey, G. Henderson, G. Agonist-selective mechanisms of GPCR desensitization. *Br J Pharmacol* 153, S379-S388 (2008)

48. S.S.G. Ferguson. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* 53, 1-24 (2001)

49. R.T. Premont, R.R. Gainetdinov. Physiological roles of G protein-coupled receptor kinases and arrestins. *Annual Rev Physiol* 69, 511-534 (2007)

50. J. Zhang, S.S. Ferguson, L.S. Barak, S.R. Bodduluri, S.A. Laporte, P.Y., Law, M.G. Caron. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proc Natl Acad Sci U S A* 95, 7157-7162 (1998)

51. C.V. Carman, J.L. Benovic. 1998. G-protein-coupled receptors: turn-ons and turn-offs. *Curr Opin Neurobiol* 8, 335-344 (1998)

52. M.A. Hurlé MA. Changes in the expression of G protein-coupled receptor kinases and beta-arrestin 2 in rat brain during opioid tolerance and supersensitivity. *J Neurochem* 77, 486-492 (2001)

53. K. Schulz, S. Müller, G. Belke-Louis, R. Schulz. Rat beta-adrenergic receptor kinases 1 and 2 in mouse neuroblastoma X rat glioma NG 108-15 hybrid cells. *Biochem Pharmacol* 55,65-70 (1998)

54. J. Guo, Y. Wu, W. Zhang, J. Zhao, L.A. Devi, G. Pei, L. Ma L. Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation. *Mol Pharmacol* 58,1050-1056 (2000)

55. R. Schulz, A. Wehmeyer, K. Schulz K. Opioid receptor types selectively cointernalize with G protein-coupled receptor kinases 2 and 3. *J Pharmacol Exp Ther* 300, 376-384 (2002)

56. J.P.McLaughlin, M. Xu, K. Mackie, C. Chavkin. Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. *J Biol Chem* 278, 34631-34640 (2003)

57. R. Schulz, A. Wehmeyer, K. Schulz. Visualizing preference of G protein-coupled receptor kinase 3 for the process of kappa-opioid receptor sequestration. Mol Pharmacol 61, 1444-1452 (2002)

58. J. Li, J.-C. Li, C. Chen, F. Zhang, L.-Y. Liu-Chen. Molecular basis of differences in (-) (trans)-3-4-dichloro-N-methyl-N- (2- (1-pyrrolidiny)-cyclohexyl)benzeneacetamide-induced desensitization and phosphorylation between human and rat kappa opioid receptors expressed in Chinese Hamster ovary cells. *Mol Pharmacol* 61, 73-84 (2002)

59. M. Ferrer-Alcon, R. La Harpe, J.A. Garcia-Sevilla. Decreased immunodensities of micro-opioid receptors, receptor kinases GRK2/6 and beta-arrestin-2 in postmortem brains of opiate addicts. *Brain Res Mol Brain Res* 121 114-122 (2004)

60. V. Rajashekara, C.N. Patel, K. Patel, V. Purohit, B.C. Yoburn, B.C. Chronic opioid antagonist treatment dose-dependently regulates mu-opioid receptors and trafficking proteins *in vivo*. *Pharmacol Biochem Behav* 75,909-913 (2003)

61. J.P.McLaughlin, L.C. Myers, P.E. Zarek, M.G. Caron, R.J. Lefkowitz, T.A. Czyzyk, J.E. Pintar, C. Chavkin. Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J Biol Chem* 279, 1810-1818 (2004)

62. L.M. Luttrell. 2005. Regulators of CPCR activity: the arrestins In The G-protein coupled receptor handbook. Ed: L.A. Devi 159-198 (2005)

63. S.M. DeWire, A. Seungkirl, R.J. Lefkowwitz, S.K. Shenoy. Beta arrestins and cell signaling. *Annu Rev Physiol* 69, 483-510 (2007)

64. B. Cen, Y. Xiong, L. Ma, G. Pei. Direct and differential interaction of beta-arrestins with the intracellular domains of different opioid receptors. *Mol Pharmacol* 59, 758-764 (2001)

65. R.H. Oakley, S.A. Laporte, J.A. Holt, M.G. Caron, L.S. Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* 275, 17201-17210 (2000)

66. A. Tohgo, E.W. Choy, D. Gesty-Palmer, K.L. Pierce, S. Laporte, R.H. Oakley, M.G. Caron, R.J. Lefkowitz, L.M. Luttrell. The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem* 278,6258-6267 (2003)

67. L.M. Bohn, L.A. Dykstra, R.J. Lefkowitz, M.G. Caron, L.S. Barak. 2004. Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Mol Pharmacol* 66, 106-112 (2004)

68. H. Haberstock-Debic, K.A. Kim, Y.J. Yu, M. von Zastrow M. 2005. Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J Neurosci* 25, 7847-7857 (2005)

69. C.E. Groer, K. Tidgewell, R.A. Moyer, W.W. Harding, R.B. Rothman, T.E. Prisinzano, L.M. Bohn. An opioid agonist that does not induce micro-opioid receptor--arrestin interactions or receptor internalization. *Mol Pharmacol* 71, 549-557 (2007)

70. L.M. Bohn, R.J. Lefkowitz, R.R. Gainetdinov, K. Peppel, M.G. Caron, F.T. Lin. 1999. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286, 2495-2498. (1999)

71. Y. Qiu, H.H. Loh, P.Y. Law. 2007. Phosphorylation of the delta-opioid receptor regulates its beta-arrestins selectivity and subsequent receptor internalization and adenylyl cyclase desensitization. *J Biol Chem* 282, 22315-22323 (2007)

72. X. Zhang, F. Wang, X. Chen, J. Li, B. Xiang, Y.Q. Zhang, B.M. Li, L. Ma. 2005. Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. *J Neurochem* 95,169-178 (2005)

73. Z.J. Cheng, Q.M. Yu, Y.L. Wu, L. Ma, G. Pei. Selective interference of beta-arrestin 1 with kappa and delta but not mu opioid receptor/G protein coupling. *J Biol Chem* 273, 24328-243333 (1998)

74. S. Ahn, S.K. Shenoy, H. Wei, J.R. Lefkowitz. Differential kinetic and spatial patterns of beta-arrestin and G-protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279, 35518-35525 (2004)

75. H. Zheng, H.H. Loh, P.Y. Law. 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 (2008)

76. R. Rozenfeld, L.A. Devi. 2007. Receptor heterodimerization leads to a switch in signaling: beta-arrestin 2-mediated ERK activation by mu-delta opioid receptor heterodimers. *FASEB J* 21,2455-2465 (2007)

77. G.J. Feng, E. Kellett, C.A. Scorer, J. Wilde, J.H. White, G. Milligan. 2003. Selective interactions between helix VIII of the human mu-opioid receptors and the C terminus of periplakin disrupt G protein activation. *J Biol Chem* 278, 33400-33407 (2003)

78. Y.J. Liang, D.F. Wu, L.Q. Yang, V. Höllt, T. Koch. Interaction of the mu-opioid receptor with synaptophysin influences receptor trafficking and signaling. *Mol Pharmacol* 71, 123-131 (2007)

79. P. James, T. Vorherr, E. and Carafoli. Calmodulin-binding domains: just two faced or multi-faceted? *Trends Biochem Sci* 20, 38-42 (1995)

80. H. Schulman, P.I. Hanson, and T. Meyer. 1992. Decoding calcium signals by multifunctional CaM kinase. *Cell Calcium* 13, 401-411 (1992)

81. D. Wang, W. Sadee, J.M. Quillan. Calmodulin binding to G-protein-coupling domain of opioid receptors *J Biol Chem* 274, 22081-22088 (1999)

82. D. Wang, C.K. Surratt, W. Sadee. Calmodulin regulation of basal and agonist-stimulated Gprotein coupling by the mu opioid receptor (OP (3)) in morphine-pretreated cell *J Neurochem* 75, 763-771 (2000)

83. F.D. Smith, G. S. Oxford, and S. L. Milgram. Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem* 274, 19894-19900 (1999)

84. A.E. Brady, and L. E. Limbird. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. *Cell Signal* 14, 297-309 (2002)

85. J.J. Charlton, P.B. Allen, K. Psifogeorgou, S. Chakravarty, I. Gomes, R.L. Neve, L.A. Devi, P. Greengard, E.J. Nestler, V. Zachariou V. Multiple actions of spinophilin regulate mu opioid receptor function. *Neuron* 58,238-247 (2008)

86. N. Tilakaratne, and P. M. Sexton. G-Protein-coupled receptor-protein interactions: basis for new concepts on receptor structure and function. *Clin Exp Pharmacol Physiol* 32, 979-987 (2005)

87. J. Kitano, Y. Yamazaki, K. Kimura, T. Masukado, Y. Nakajima, and S. Nakanishi. Tamalin is a scaffold protein that interacts with multiple neuronal proteins in distinct modes of protein-protein association. *J Biol Chem* 278, 14762-14768 (2003)

88. M. Ogawa, T. Miyakawa, K. Nakamura, J. Kitano, K. Furushima, H. Kiyonari, R. Nakayama, K. Nakao, K. Moriyoshi, and S. Nakanishi. Altered sensitivities to morphine and cocaine in scaffold protein tamalin knockout mice. *Proc Natl Acad Sci U S A* 104, 14789-14794 (2007)

89. M. Bhattacharya, A. V. Babwah, and S. S. Ferguson. Small GTP-binding protein-coupled receptors. *Biochem Soc Trans* 32,1040-1044. (2004)

90. G. Milligan. Opioid receptors and their interacting proteins. *Neuromolecular Med* 7, 51-59 (2005)

91. T. Koch, L.O. Brandenburg, S. Schulz, Y. Liang, J. Klein, V. Hollt. 2003. ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. *J Biol Chem* 278, 9979-9985 (2003)

92. T. Koch, D. F. Wu, L. Q. Yang, L. O. Brandenburg, and V. Hollt. Role of phospholipase D2 in the agonist-induced and constitutive endocytosis of G-protein coupled receptors. *J Neurochem* 97, 365-372 (2006)

93. A. Claing, S.A. Laporte, M.G. Caron, R.J. Lefkowitz. RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. *Prog Neurobiol* 66, 61-79, (2002)

94. O.B. Goodman Jr, J.G. Krupnick, F. Santini, V.V. Gurevich, R.B. Penn, A.W. Gagnon, J.H. Keen, J.L. Benovic. Role of arrestins in G-protein-coupled receptor endocytosis. *Adv Pharmacol* 42:429-433 (1998)

95. N. Trapaidze, D.E. Keith, S. Cvejic, C.J. Evans, L.A. Devi. Sequestration of the delta opioid receptor. Role of the C terminus in agonist-mediated internalization. *J Biol Chem* 271, 29279-29285 (1996)

96. R. El Kouhen, A.L. Burd, L.J. Erickson-Herbrandson, C.Y. Chang, P.Y. Law, H.H. Loh. Phosphorylation of

Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. *J Biol Chem* 276, 12774-12780 (2001)

97. S. Schulz, D. Mayer, M. Pfeiffer, R. Stumm, T. Koch, V. Höllt. V. Morphine induces terminal micro-opioid receptor desensitization by sustained phosphorylation of serine-375. *EMBO J* 23, 3282-3289 (2004)

98. J.L. Whistler, M. von Zastrow. Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci U S A* 95, 9914-9919. (1998)

99. H. Haberstock-Debic, M. Wein, M. Barrot, E.E. Colago, Z. Rahman, R.L. Neve, V.M. Pickel, E.J. Nestler, M. von Zastrow, A.L. Svingos. Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J Neurosci* 23, 4324-4332 (2003)

100. W.M. Walwyn, W. Wei, C.W. Xie, K. Chiu, B.L. Kieffer, C.J. Evans, N.T. Maidment. Mu opioid receptor-effector coupling and trafficking in dorsal root ganglia neurons. *Neuroscience* 142, 493-503 (2006)

101. B.A. Jordan, S. Cvejic, L.A. Devi. Kappa opioid receptor endocytosis by dynorphin peptides. *DNA Cell Biol* 19, 19-27 (2000)

102. J.G. Li, L.Y. Luo, J.G. Krupnick, J.L. Benovic, L.Y. Liu-Chen. U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. *J Biol Chem* 274, 12087-12094 (1999)

103. M. Rodriguez-Munoz, E. de la Torre-Madrid, G. Gaitan, P. Sanchez-Blazquez, and J. Garzon. RGS14 prevents morphine from internalizing Mu-opioid receptors in periaqueductal gray neurons. *Cell Signal* 19:2558-2571 (2007)

104. E. Elenko, T. Fischer, I. Niesman, T. Harding, T. McQuistan, M. Von Zastrow, M. G. Farquhar. Spatial regulation of Galphai protein signaling in clathrin-coated membrane microdomains containing GAIP. *Mol Pharmacol* 64, 11-20 (2003)

105. G. Macé, M. Miaczynska, M. Zerial, A.R. Nebreda. Phosphorylation of EEA1 by p38 MAP kinase regulates mu opioid receptor endocytosis. *EMBO J* 24, 3235-3246 (2005)

106. P. Huang, M.A. Frohman. The potential for phospholipase D as a new therapeutic agent. *Expert Opin Ther Targets* 11, 707-716 (2007)

107. C.W. Colley, T.C. Sung, R. Roll, J. Jenco, S.M. Hammond, Y. Altshuller, D. Bar-Sagi, A.J. Morris, M.A. Frohman. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr Biol* 7, 191–201 (1997)

108. B. Xiang, G.H. Yu, J. Guo, L. Chen, W. Hu, G. Pei, L. Ma. Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. *J Biol Chem* 276,4709-4716 (2001)

109. Y. Pak, B.F. O'Dowd, J.B. Wang, S.R. George. 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 (1999)

110. F. Valtorta, M. Pennuto, D. Bonanomi, F. Benfenati. Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? *Bioessays* 26, 445-453 (2004)

111. I. Onoprishvili, M.L. Andria, H.K. Kramer, N. Ancevska-Taneva, J.M. Hiller, E.J. Simon. Interaction between the mu opioid receptor and filamin A is involved in receptor regulation and trafficking. *Mol Pharmacol* 64, 1092-1100 (2003)

112. R. Uribe, D. Jay. D. A review of actin binding proteins: new perspectives. *Mol Biol Rep* 2007 Oct 16. epub ahead of print (2007)

113. I. Onoprishvili, E.J. Simon. Chronic morphine treatment up-regulates mu opioid receptor binding in cells lacking filamin A. *Brain Res* 1177, 9-18 (2007)

114. D.F. Wu, T. Koch, Y.J. Liang, R. Stumm, S. Schulz, H. Schröder, V. Höllt. Membrane glycoprotein M6a interacts with the micro-opioid receptor and facilitates receptor endocytosis and recycling. *J Biol Chem* 282, 22239-22247 (2007)

115. A. Heydorn, B.P. Søndergaard, B. Ersbøll, B. Holst, F.C. Nielsen, C.R. Haft, J. Whistler, T.W. Schwartz. A library of 7TM receptor C-terminal tails. Interactions with the proposed post-endocytic sorting proteins ERM-binding phosphoprotein 50 (EBP50), N-ethylmaleimide-sensitive factor (NSF), sorting nexin 1 (SNX1), and G protein-coupled receptor-associated sorting protein (GASP) *J Biol Chem* 279, 54291-54303 (2004)

116. J.L. Whistler, J. Enquist, A. Marley, J. Fong, F. Gladher, P. Tsuruda, S. R. Murray, M. Von Zastrow. Modulation of postendocytic sorting of G protein-coupled receptors. *Science* 297,615-620 (2002)

117. F. Simonin, P. Karcher, J. J. Boeuf, A. Matifas, B. L. Kieffer. Identification of a novel family of G protein-coupled receptor associated sorting proteins. *J Neurochem* 89, 766-775 (2004)

118. D. Thompson, M. Pusch, J. L. Whistler. Changes in G protein-coupled receptor sorting protein affinity regulate postendocytic targeting of G protein-coupled receptors. *J Biol Chem* 282:29178-85 (2007)

119. M. Tanowitz, M. Von Zastrow. M. Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. *J Biol Chem* 277, 50219-50222 (2002)

120. K. Chaturvedi, P. Bandari, N. Chinen, R.D. Howells. Proteasome involvement in agonist-induced down-regulation of mu and delta opioid receptors. *J Biol Chem* 276,12345-12355 (2001)

121. J.N. Hislop, A. Marley, M. Von Zastrow. Role of mammalian vacuolar protein-sorting proteins in endocytic trafficking of a non-ubiquitinated G protein-coupled receptor to lysosomes. *J Biol Chem* 279,22522-22531 (2004)

122. M. Tanowitz, M. von Zastrow. A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J Biol Chem* 278, 45978-45986 (2003)

123. J.G. Li, C. Chen, L.Y. Liu-Chen. Ezrin-radixin-moesin-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. *J Biol Chem* 277, 27545-27552 (2002)

124. J.G. Li, J.L. Benovic, L.Y. Liu-Chen. Mechanisms of agonist-induced down-regulation of the human kappa-opioid receptor: internalization is required for down-regulation. *Mol Pharmacol* 58,795-801. Erratum in: *Mol Pharmacol* 59,1355, 2001 (2000)

125. J.G. Li, D.S. Haines, L.Y. Liu-Chen. LY.2008. Agonist-promoted Lys63-linked polyubiquitination of the human kappa-opioid receptor is involved in receptor down-regulation. *Mol Pharmacol* 73, 1319-1330 (2008)

126. I. Gomes, B.A. Jordan, A. Gupta, C. Rios, N. Trapaidze, L.A. Devi. LA. 2001.G protein coupled receptor dimerization: implications in modulating receptor function. *J Mol Med* 79,226-242 (2001)

127. C.D. Rios, B.A. Jordan, I. Gomes, L.A. Devi. LA. G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* 92, 71-87 (2001)

128. R. Rozenfeld, N.S. Abul-Husn, I. Gomes, L.A. Devi. L.A. 2007. An emerging role for the delta opioid receptor in the regulation of mu opioid receptor function. *ScientificWorld Journal* 7, 64-73 (2007)

Key Words: Opioid Receptors, Opiates, Morphine, Review

Send correspondence to: Lakshmi A. Devi, Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, 19-84 Annenberg Building, One Gustave L. Levy Place, New York, NY 10029, Tel: 212-241-8345, Fax: 212-996-7214, E-mail: lakshmi.devi@mssm.edu