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Post-translational modifications of Opioid Receptors

Mariana Lemos Duarte[1], **Lakshmi A. Devi**[1,*]

[1]Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

Post-translational modifications (PTM) are key events in signal transduction since they affect protein function by regulating their abundance and/or activity. PTMs involve the covalent attachment of functional groups to specific amino acids. Since they tend to be generally reversible, PTMs serve as regulators of signal transduction pathways. GPCRs are major signaling proteins that undergo multiple types of PTMs. In this Review, we focus on the opioid receptors, members of family A GPCRs, and highlight recent advances in the field that have underscored the importance of PTMs in the functional regulation of these receptors. Since opioid receptor activity plays a central role in the development of tolerance and addiction to morphine and other drugs of abuse, understanding the molecular mechanisms regulating receptor activity is of fundamental importance.

Keywords

Receptor desensitization; phosphorylation; glycosylation; palmitoylation; ubiquitination; morphine; drug addiction

Relevance of Opioid Receptor post-translational modifications

The opioid receptor family:

Opioids were being used as pain relievers long before we began to understand the biochemical properties of opioid receptors. Opioids have been used for more than 4,000 years, and the analgesic effects of morphine have been explored since it was isolated in 1805. Although the chronic usage of opioids leads to the development of tolerance (see Glossary), physical dependence, and addiction, morphine remains the drug of choice for acute pain.

The opioid receptors, members of the family A G protein-coupled receptors (GPCRs), consist of μ-Opioid Receptor (MOR), δ-Opioid Receptor (DOR), κ-Opioid Receptor (KOR),

*Corresponding Author: lakshmi.devi@mssm.edu (L.A. Devi).

and the Nociceptin/orphanin FQ receptor (NOP) [1]. Homology analysis shows that these receptors exhibit 49%−58% primary sequence identity with each other [1]. The ORs also exhibit high structural similarity with each other, which reinforces the notion that they all belong to the same family [2]. ORs appear to share a common evolutionary history, dating to ~450 millions of years ago [3].

Even though all ORs play a role in the modulation of pain, it is only in the past few decades that we have been able to clarify additional roles in behaviors and processes such as food intake, anxiety, depression, and immunomodulation [4,5]. MOR agonists are used in the treatment of pain but they are accompanied by undesirable side-effects such as respiratory depression and constipation [6,7]. KOR agonists produce dysphoric, hallucinogenic and psychotomimetic effects [8]. Interestingly, although DOR agonists have been shown to produce seizures [9], they have also been shown to have antidepressant and anxiolytic effects [10,11]. Finally, NOP agonists have been reported to cause somnolence and decrease blood pressure [12], although small molecule agonists have shown potential as anxiolytics, substance abuse medications, and antitussives [5]. Understanding the differences between side-effects and beneficial effects produced by OR agonists will be crucial to the development of safe analgesic drugs. The molecular mechanisms of ORs signaling are described in Box 1.

Post-translational modification (PTM) of GPCRs:

Receptor PTMs play a critical and important role in modulating signal transduction. Indeed, they represent the fine-tuning of receptor signaling. Among the PTMs that play key roles in GPCR signaling, glycosylation (sugar-linkage) [13,14], palmitoylation (palmitoyl-linkage) [15–18], phosphorylation (phosphate-linkage) [19–21] and ubiquitination (ubiquitin-linkage) [22–25], are the most frequently studied ones.

Glycosylation, which mainly takes place in the endoplasmic reticulum (ER) and Golgi apparatus, has been described as a quality control mechanism for GPCR synthesis, and has been shown to serve as a tag that directs the receptor to the plasma membrane [13,14]. Palmitoylation has been shown to play roles in receptor localization to lipid rafts on the plasma membrane, and in modifying protein-protein interactions including receptor dimerization [15–18]. Phosphorylation of GPCRs has been intensely studied, and results show that it plays a key role in regulating receptor activity including receptor desensitization and internalization [19,20]. Ubiquitination of GPCRs has been shown to affect receptor degradation, and in some cases this PTM serves as a regulator of the magnitude and the duration of GPCR signaling [22–25].

In this review, we focus on PTMs of opioid receptors. A thorough investigation of opioid receptor PTMs could be critical to understand the molecular mechanisms leading to the development of tolerance, as well as opioid-mediated side effects. This review is divided into three sections that are based on the location of the PTM on the receptor: extracellular (glycosylation), transmembrane (palmitoylation), and cytoplasmic (phosphorylation and ubiquitination) (Fig. 1). We mention the enzymes responsible for these PTMs in opioid receptors (ORs) and describe their biological relevance for opioid signaling (Fig. 2).

Glycosylation

Glycosylation consists of the attachment of sugar molecules to proteins. There are mainly two types of Glycosylation: N-linked and O-linked glycosylation. N-linked glycosylation consists of linking a sugar molecule to the nitrogen of an Asparagine (N) residue in a protein. The putative N-glycosylation motif is characterized by Asparagine-Xaa-Threonine/Serine (N-X-S/T) where Xaa can be any of the 20 natural amino acids except Proline, whereas O-linked glycosylation refers to linking a sugar molecule to the oxygen of Serine (S) or Threonine (T) residues in a protein [26,27]. During glycosylation, monosaccharide units such as galactose (Gal), mannose (Man), fucose (Fuc), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and sialic acids are covalently attached in linear or branching chains [28]. The initial step of glycosylation occurs co-translationally in the ER and this is followed by the formation of complex glycans in the Golgi apparatus. There are innumerable variations in monosaccharide composition, glycosidic linkages, and glycan branches, which leads to an incredibly diverse glycan repertoire [28]. Glycosylation has been described as a post-translational event that affects the maturation process of all secreted and transmembrane proteins. In the case of GPCRs, the correlation between receptor glycosylation, maturation and trafficking have been extensively studied (reviewed in [29–31]).

Functional significance of MOR glycosylation:

The initial evidence that ORs could be glycosylated came from studies with purified receptors [32–34]. Treatment with glycosidases such as Endo H, PNGase F, and O-glycosidase, or inhibition of GlcNac phosphotransferase with tunicamycin, led to changes in the molecular weight of the protein indicating N-linked and/or O-linked glycosylation of MOR [35–37]. The N-glycosylation motif is repeated 5 times in the amino-terminal extracellular portion of human MOR (hMOR) [38]. Among these, N-glycosylation at residue N^{40} in hMOR has been extensively studied [39–42] (Fig. 1; Table 1).

A major point of the clinical relevance of N^{40} glycosylation in MOR is related to the A118G polymorphism (SNP database [dsSNP] Accession No rs1799971). The frequency of this polymorphism is ~22% in all the 2,504 individuals sequenced in the 1000 Genome Project [43,44]. When translated, this polymorphism leads to an exchange of asparagine (N) for aspartic acid (D) at position 40 resulting in D^{40} and, consequently, the loss of this glycosylation site [39]. Patients carrying the A118G allele (N40D amino acid substitution) are reported to have lower pain thresholds [45], and to require higher opioid doses to get an analgesic response [46,47]. A118G has also been reported to be associated with increased dependence on alcohol [48] and opiates such as heroin [49,50].

A number of studies have explored the molecular consequences of N40D substitution on receptor activity. A recent study investigated the impact of N40D variant using induced inhibitory neuronal cells (iNs) generated from induced pluripotent stem (iPS) cell lines from human subjects carrying the homozygous D^{40} polymorphism [40]. The D^{40} iNs exhibit stronger suppression of spontaneous inhibitory postsynaptic currents in comparison to N^{40}. Electrophysiological analyses of cultured neurons indicated that D^{40} iNs cells also exhibit altered sensitivity to the MOR agonist, DAMGO [40].

Studies examining the overexpression of D^{40} have reported an increase in the potency of MOR agonists [49,51,52]. Interestingly, the extent of glycosylation at N^{40} has also been reported to affect the affinity of some MOR agonists for the receptor in a cell- and agonist-dependent manner. In AV-12 and HEK293 cells, overexpression of the D^{40} hMOR isoform increased the binding affinity of β-endorphin in comparison to wild-type (N^{40}) receptors, but not of other opioid peptides and alkaloids tested [49,53]. However, these results were not reproducible in COS cells [42]; this could be due to variation of the glycan profile between different cell lines [54]. The extent of N-glycosylation of MOR also differs among mouse brain regions; for example, the level of glycosylation is higher in the thalamus in comparison to the striatum [55]. Taken together, as in the case of other GPCRs, OR glycosylation has been described to modulates the dynamics of the steady-state levels of the receptor at the cell surface and consequently, of protein abundance [37,39,56,57]. The mechanisms of how variation in glycosylation affects MOR function in a brain region-specific manner remains to be investigated.

Glycosylation of other opioid receptor members:

There is evidence that N^{18}, N^{33}, S^{6}, S^{25} and S^{29} at the N-terminal of DOR, and N^{25} and N^{29} of KOR are also glycosylated, [37,56,58,59] (Table 1). The glycosylation of DOR and KOR has been reported to affect receptor function by enabling receptor folding and cell surface localization [37,56]. O-glycosylation of hDOR was found to enhance ligand-binding and agonist-mediated inhibition of cAMP accumulation [59]. However, N-glycosylation of hDOR (and hKOR) do not affect diprenorphine (OR antagonist) binding [37,58]. However, studies with receptor mutants that could not be glycosylated (hDOR-N^{18}Q/N^{33}Q and hKOR-N^{25}Q/N^{39}Q) have reported increased rate of receptor internalization compared to wild-type receptors [37,56]. Furthermore, the N^{25}Q/N^{39}Q mutants in hKOR have been reported to exhibit increased agonist-induced receptor phosphorylation, internalization and desensitization [37]. Together, these studies suggest that glycosylation is able to affect receptor signaling in addition to facilitating receptor maturation.

Lipidation – Palmitoylation:

Lipid modification occurs through the covalent binding of lipids such as glycosylphosphatidylinositol (GPI) anchors, and fatty acids to distinct regions on the protein. There are three types of fatty acid modifications: N-myristoylation, palmitoylation, and isoprenylation (attachment of farnesyl or geranylgeranyl) [60]. Among these, palmitoylation has been most extensively studied in the case of GPCRs [15].

Palmitoylation is classically understood to involve the attachment of palmitate to one or more cysteine residues via a thioester bond (S-palmitate). Note that the attachment of palmitate is not exclusive to thioester bonds; recent studies have described palmitate linkage to an amide group (N-palmitate) in Gαs proteins [61]. The enzymes responsible for this PTM are the palmitoyl acyltransferases, members of the DHHC-CRD (Asp-His-His-Cys-cysteine-rich domain) family, and Rasp, a member of the membrane-bound O-acyltransferase (MBOAT) family [60]. Palmitoylation is a highly dynamic event and the balancing activity of palmitoyl acyltransferases (which add palmitate), and palmitoyl thioesterases (which remove palmitate) determine the stoichiometry of protein

palmitoylation at steady state. There are three classes of depalmitoylating thioesterase enzymes: acyl protein thioesterases, α/β hydrolase domain-containing 17 proteins (ABHD17s), and palmitoyl-protein thioesterases (PPTs) [62].

Palmitoylation of GPCRs has been shown to play a major role in membrane anchoring. Furthermore, crystal structure analysis has shown cholesterol-palmitoyl interaction in the case of the β2-adrenergic receptor [63]. The role of GPCR palmitoylation and its functional implication has been reviewed elsewhere [23,64,65].

Functional significance of MOR palmitoylation:

The first evidence that MOR could be palmitoylated came from studies using a recombinant system where CHO cells overexpressing rMOR were labeled with [^{3}H]palmitate [66]. Unlike other GPCRs that are palmitoylated on the C-terminal residues, the residue described to be palmitoylated, cysteine 170 (C^{170}) of rMOR was shown to be in the intracellular loop of MOR [18,66] (Fig. 1, Table 1).

Studies have reported that palmitate at the residue C^{170} of MOR interacts with the cholesterol-enriched lipid raft microdomains at the plasma membrane and this is thought to facilitate receptor homodimerization and G protein coupling/activation [18] (Box 2). Furthermore, expression of the rMOR mutant ($C^{170}A$) with impaired palmitoylation was reported to affect signaling (decrease ERK phosphorylation and modulate levels of cAMP) while this mutant was not found to affect the binding of morphine, naloxone or CTOP [18].

Taken together, the primary role of palmitoylation appears to be facilitation of protein anchoring to the cell membrane and protein-protein interactions. Thus, palmitoylation of OR leads to pleiotropic effects involving plasma membrane distribution, subcellular localization, endocytosis, and recycling [15].

Palmitoylation of other opioid receptors:

Similar to rMOR, palmitoylation in hDOR was initially described after labeling with [^{3}H]palmitate [67]. The C^{328} and C^{333} residues on the C-terminal region of mDOR have been described as potential palmitoylation sites [68] (Fig. 1; Table 1). In hDOR, palmitoylation plays a role in promoting protein trafficking from the ER to the membrane since lack of palmitoylation was found to decrease the expression of DOR at the cell surface [67]. The palmitoylation process occurs both in the ER and at the plasma membrane [67]. Receptor activation appears to modulate the extent of palmitoylation at the plasma membrane. For instance, leucine-enkephalin (LE) treatment was found to increase the incorporation of [^{3}H]palmitate in hDOR. Interestingly, DOR palmitoylation mediated by the agonist does not appear to require G protein coupling or receptor internalization or recycling [67].

Phosphorylation

Protein phosphorylation is one of the most frequent PTM events. It has been estimated that 30% of all cellular proteins are phosphorylated on at least one residue [21]. During phosphorylation, the γ-phosphate from ATP is transferred to the hydroxyl oxygen of serine

(S), threonine (T) or tyrosine (Y) residues of the target protein [21]. In GPCRs, phosphorylation affects the temporal dynamics of receptor signaling. Kinases, the enzymes that perform the phosphorylation reaction, represent a full 2% of the genome [21]. For the past few decades the kinases, G protein-coupled receptor kinases (GRKs), Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), proto-oncogene tyrosine-protein kinase (Src) and protein kinase C (PKC), have been among the most intensely investigated kinases that modulate GPCR phosphorylation [69–72]. Given the importance of phosphorylation in affecting the dynamics of receptor signaling and trafficking, GPCR phosphorylation has been extensively studied (reviewed in [73,74]).

Functional significance of MOR phosphorylation:

Initial studies of MOR phosphorylation were directly motivated by the classical concept that GPCR phosphorylation by GRKs leads to β-arrestin recruitment and receptor internalization (Box 1) [75]. These early discoveries highlighted the complexity of opioid signaling by connecting phosphorylation of MOR to agonist-induced receptor internalization, desensitization, recycling, analgesia and tolerance [76–79].

There are ~20 predicted phosphorylation sites in MOR (Fig. 3) that are conserved between mouse and rat (reviewed in [79]). These residues are located at the intracellular loop, as well as at the C-terminal region of MOR (Fig. 1, and Fig. 3). Mass spectrometry analysis and/or studies with phospho-specific antibodies have identified Y^{106}, Y^{166} and S^{266} in the intracellular loop [80,81]; and S^{363} and eight S/T residues within two cassettes ($T^{354}S^{355}S^{356}T^{357}$ and $T^{370}REHPS^{375}T^{376}ANT^{379}$) in the C-terminal tail to be phosphorylated [69,72,79,82–87]. These are seen in both cultured cells and in mouse brain tissue. [69,85,86].

The kinases responsible for phosphorylating specific MOR residues and their phosphorylation sites are shown in Table 1 and Fig. 3. The kinases are: Src for residue Y^{336} [80]; tyrosine kinases for residues Y^{106} and Y^{166} [81]; GRKs for residues S^{355}, T^{357}, T^{370}, S^{375}, T^{376} and T^{379} [69,85,87–90], PKC for residues S^{363} and T^{370} [69,91,92], and CAMKII for the residues S^{366} and T^{370} [69,93]. The exact molecular mechanism of how agonist-binding triggers the activation of these kinases and consequent receptor phosphorylation remains unclear. Studies have described that the only residues that are found to be phosphorylated under basal conditions are S^{363} and T^{370}, all the others are thought to be phosphorylated following receptor activation [69,82,86].

Most studies that aim at understanding the role of MOR phosphorylation focus on the S/T residues T^{354}, S^{355}, S^{356}, T^{357}, S^{363}, T^{370}, S^{375}, T^{376}, T^{379}, T^{383} and T^{394} at the C-terminal tail (Fig. 3, Table 1) [76,82,94,95]. These residues have been described to play an important role in the modulation of receptor desensitization, recycling, opioid analgesia and tolerance [78,94–96]. Recent studies in animals harboring mutations of phosphorylation residues described above (phosphorylation deficient mice) reported an increase in agonist-mediated analgesic effects with no changes (or exacerbation) of side effects such as respiratory depression and constipation [76]. These recent findings have raised a renewed interest in exploring the implications of MOR phosphorylation to the biological effects of opioids (Box 3).

The role of different agonists in mediating MOR phosphorylation, the kinases responsible for phosphorylation, and the biological relevance of this PTM are described in detail below (Fig. 3; Table 1). Since these phosphorylation events have been described to be involved in different processes of MOR signaling, we have structured the section of MOR phosphorylation by focusing on the correlation between this PTM and the distinct cell biological/pharmacological effects, including internalization, recycling, desensitization, antinociception and tolerance (Fig. 3; Table 1).

Effect of phosphorylation on MOR internalization: Early studies reported that different OR agonists differentially phosphorylate MOR and the extent of phosphorylation could be correlated to the extent of receptor internalization [79]. The mechanisms underlying the differences in agonist activation of MOR and its internalization were found to be directly related to the phosphorylation of specific residues by distinct GRKs isoforms [70,72,87,97]. Furthermore these studies reported that treatment with high-efficacy opioids such DAMGO ([D-Ala^2-MePhe^4-Gly-ol]), fentanyl, and etonitazene facilitated robust internalization [72,87,97,98] whereas, a partial agonist such as morphine did not [72,83,99]. Correlating this with MOR phosphorylation, studies have reported that high-efficacy opioids initially induce phosphorylation of S^{375}, followed by phosphorylation of T^{370}, T^{376} and T^{379} [72,83]. This process is mediated by GRK2/3, and serves to facilitate MOR diffusion in the plasma membrane [100] and subsequent β-arrestin recruitment and receptor internalization [72,87,97,98]. Supporting these findings, mutation of residues $S^{375}T^{376}ANT^{379}$ to $A^{375}A^{376}ANA^{379}$ decreased β-arrestin recruitment and internalization after treatment with Met-Enk [78,85]. Notably, mutation of residues $T^{354}S^{355}S^{356}T^{357}$ (which are not involved in the aforementioned hierarchical phosphorylation of MOR by GRK2/3) to $A^{354}A^{355}A^{356}A^{357}$ did not affect receptor internalization [85,94–96] (Fig. 3).

In contrast, morphine treatment was found to induce robust phosphorylation of S^{375}, but only a weak phosphorylation of T^{370}, T^{376}, and T^{379} [87]. Recent studies indicate that MOR phosphorylation at S^{375} is primarily by the kinase GRK5 [90,97] and to a lesser extent by GRK2/3 [87,90]. Increasing the expression of GRK2/3 led to enhanced mMOR internalization in response to morphine treatment supporting the idea that MOR phosphorylation at T^{370}, T^{376}, and T^{379} contribute to MOR internalization. [83,97].

Effect of phosphorylation on MOR recycling: The field has only recently begun to investigate the role of MOR phosphorylation on receptor recycling [77,101]. One of the strategies to explore MOR recycling is via the overexpression of MOR N-terminally tagged with a pH-sensitive GFP, which produces a fluorescence signal during vesicle fusion with the plasma membrane [77,101]. Studies have suggested a role for PKC in receptor recycling since treatment with a PKC inhibitor was found to reduce DAMGO-mediated MOR recycling [77]. Furthermore, mutation of S^{363} and T^{370} residues of mMOR (known PKC phosphorylated residues) to alanine led to impaired MOR recycling [101] (Fig. 3).

The exact role of PKC in MOR recycling is still unknown. Preliminary studies suggest that PKC phosphorylation of MOR occurs at multiple cellular compartments [77,101]. Although additional studies are necessary, the following evidence corroborate this hypothesis: (i) S^{363} and T^{370} residues of mMOR appear to be phosphorylated by PKC in the basal state

[69,82,86,92] and the phosphorylation level is not increased after agonist treatment [85,86], (ii) DAMGO treatment does not lead to PKC activation [102]; however, PKC inhibition affects MOR recycling following receptor internalization by DAMGO [77]. These findings support a role for PKC-mediated receptor phosphorylation in multiple compartments (at cell membrane in the basal state and in an intracellular compartment following agonist treatment-mediated receptor internalization).

Effect of phosphorylation on MOR desensitization: A hallmark of MOR is the rapid desensitization following acute treatment with an opioid Opioid-induced acute MOR desensitization has been studied electrophysiologically in neurons of the thalamus or locus coeruleus of mice or rats expressing different MOR mutants. This method has allowed the investigation of receptor desensitization at different time points and opioid concentrations [79]. It has been hypothesized that acute desensitization requires receptor phosphorylation, followed by β-arrestin recruitment and/or receptor internalization. Therefore, similar to the receptor internalization, the mechanism that drives the desensitization process is probe specific [70,72,76,78,85].

Electrophysiological studies showed that mutation of 10 S/T C-terminal residues (T^{354}, S^{355}, S^{356}, T^{357}, S^{363}, T^{370}, S^{375}, T^{376}, T^{379}, and T^{383}) significantly reduced the acute desensitization mediated by Met-Enk in locus coeruleus neurons [76,78,94]. Attenuation of Met-Enk-mediated acute desensitization was also observed in mice with mutations of both phosphorylation casettes i.e. $S^{375}T^{376}ANT^{379}$ and $T^{354}S^{355}S^{356}T^{357}$ [96]. However, this attenuation was not seen when the $S^{375}T^{376}ANT^{379}$ cassette alone was mutated [94,96]. Together, these data point towards the contribution of phosphorylation of specific C-terminal residues in the acute desensitization to Met-Enk.

Studies comparing acute desensitization by Met-Enk to that by morphine showed that mutation of the 11 S/T C-terminal residues to alanine (T^{393} in addition to the 10 S/T residues described earlier) did not abolish morphine-mediated receptor desensitization [95]. One potential explanation is that a different set of kinases is involved in morphine-mediated desensitization. For example, morphine-mediated PKC activation has been described to play a role in MOR desensitization [71,95,103–106]. Also, morphine-induced desensitization (but not Met-Enk) was reduced by PKC inhibition [95]. The molecular mechanism of how PKC modulates desensitization in a probe specific manner remains unclear. Recent proteomic analyses have reported that PKC, activated by acute treatment with morphine, can subsequently interact with regulators of MOR signaling [107]. Among these regulators, Raf kinase inhibitory protein (RKIP) is an interesting substrate since it has been found to inhibit GRK, and RKIP/GRK interactions have been found to enable Gαβγ-binding and signaling [107–109].

Effect of phosphorylation on opioid-mediated antinociception and tolerance: Studies have used transgenic animals to correlate MOR phosphorylation with antinociception and development of tolerance to opioids [76,78,94,110,111]. Studies using mice expressing MOR with alanine mutations in 10 S/T C-terminal phosphorylation sites (T^{354}, S^{355}, S^{356}, T^{357}, S^{363}, T^{370}, S^{375}, T^{376}, T^{379} and T^{383}) show that these animals exhibit enhanced antinociception compared to wild-type mice in response to fentanyl or

morphine [76]; this suggests that these phosphorylatable residues play a role in modulating opioid-induced analgesia [76].

Chronic opioid administration induces antinociceptive tolerance [76,78,94]. The related changes in MOR activation can be evaluated using classical tests of pain response in animals such as the hot-plate test [76,110,111]. Such studies show that tolerance to fentanyl and morphine is abrogated in mice with alanine mutations of 11 S/T C-terminal phosphorylation sites (or in 10 S/T C-terminal phosphorylation sites except for T^{394}) [76].

The role of specific residues in the C-terminal of MOR (listed above) that mediate opioid tolerance has also been investigated [76,110,111]. Interestingly, phosphorylation of S^{375} has been described to regulate tolerance to high-efficacy opioid agonists such as fentanyl, DAMGO, Met-Enk, or etonitazene, but not morphine [76,110]. In addition, mice expressing MOR with a T^{394}A mutation did not exhibit acute tolerance to either morphine or etorphine [111].

Studies examining specific enzymes responsible for receptor phosphorylation have implicated a number of kinases and phosphatases in mediating opioid tolerance [78,104,112,113]. For instance, PKC inhibition [104,114,115] and GRK3 knock-down was found to reverse tolerance induced by chronic morphine [113]. In contrast, inhibition of protein phosphatase PP2A, was found to enhance morphine antinociceptive tolerance [104,112].

Taken together, several studies point to the relevance of MOR C-terminal tail phosphorylation on the control of opioid analgesia and tolerance. The mechanisms responsible for opioid tolerance are not completely understood and require additional studies.

Phosphorylation of other opioids receptor: Phosphorylation of other ORs has been shown to play roles in receptor desensitization and internalization [109,116–121]. For instance, agonist-induced mDOR desensitization and internalization is regulated by residues T^{358} and S^{363} [117], in a mechanism potentially mediated by GRK2/3 and β-arrestin recruitment [120,122]. Interestingly, DOR phosphorylation at S^{344} is mediated by PKC in an agonist-independent manner [123].

Studies with mKOR reported agonist-mediated phosphorylation at S^{356}, T^{357}, T^{363} and S^{369}. Notably, the extent of phosphorylation at these residues is higher after U50,488H treatment as compared to etorphine treatment [119,124]. Agonist-induced KOR phosphorylation involves a mechanism mediated by GRK2/3 and GRK5/6 activation [121,124]. Similarly to other opioid receptors, PKC has also been implicated in agonist-independent KOR phosphorylation [124].

The activation of NOP by its endogenous ligand, nociceptin/orphanin FQ, has been reported to lead to the activation of kinases such as PKC, extracellular signal–regulated kinase 1 (ERK1) and ERK2, p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) [125–127]. Agonist-induced mNOP activation recruits GRK2/3 [116], leading to activation of the latter and consequently receptor phosphorylation at S^{346}, followed by

S^{351}, T^{362} and S^{363}. These events suggest that NOP phosphorylation facilitates receptor desensitization and internalization [116,128].

Ubiquitination

Ubiquitination is the formation of a covalent bond between ubiquitin, a 76-amino acid protein, and the protein substrate [25]. This bond is usually formed between the C-terminal glycine (G^{76}) of ubiquitin and the epsilon amino group of the lysine of the target substrate, but in some cases, the ubiquitin can be attached to the amino group at the N-terminus of the substrate [129]. Ubiquitin has eight potential attachment points, allowing for the formation of polyubiquitin chains with a high structural diversity (reviewed in [130]). The ubiquitination process is mediated by three enzymatic reactions. In the first reaction, ubiquitin is activated at its C-terminus in an ATP-dependent manner by an E1-activating enzyme. Next, the activated ubiquitin is transferred to a cysteine residue on an E2-conjugating enzyme. Finally, the E2-ubiquitin intermediate interacts with an E3-ubiquitin ligase, which transfers the ubiquitin to the lysine residue on the substrate [23]. The E3-ubiquitin ligases are categorized into two families: the E6AP C terminus (HECT), which possess inherent catalytic activity, and the Really Interesting New Gene (RING), which facilitate the interaction between the substrate and the E2 enzymes (reviewed in [131,132]). Recent studies indicate that β-arrestin can function as an adaptor protein that facilitates the ubiquitination process by interacting with E3-ubiquitin ligases [22,98].

Ubiquitination is a transient PTM that is reversed by deubiquitinating enzymes (reviewed in [131,132]). Deubiquitinating enzymes are divided into five families: the ubiquitin carboxy-terminal hydrolases, ubiquitin–specific proteases, ovarian tumor-related proteases, Machado-Joseph disease protein domain proteases, and jab1/MPN domain-associated metalloisopeptidases (JAMM) (reviewed in [131,132]). The role of ubiquitination in regulating receptor levels and trafficking has been extensively studied in the case of GPCRs such as β2-adrenergic and vasopressin V2 receptors (reviewed in [132,133]).

Initially, ubiquitin was characterized as a degradation-tag that directs proteins towards the proteasome pathway. Indeed, in the context of GPCRs, the ubiquitination process is generally related to degradation of internalized receptors, in a mechanism linked to long-term desensitization of transmembrane signaling (reviewed in [22,130]). In addition, GPCRs can undergo agonist-mediated ubiquitination, and a few exhibit constitutive ubiquitination that modulates correct receptor trafficking to and from the plasma membrane [131,134]. While agonist-mediated ubiquitination occurs at the plasma membrane, requires receptor phosphorylation and promotes GPCR internalization and down-regulation, constitutively ubiquitinated receptors undergo reversible agonist-mediated deubiquitination at the plasma membrane [131,134]. In addition, some newly synthetized proteins require deubiquitination to translocate to the cell surface [135]. Depending of the ubiquitin lysine-linkage on the modified substrate different pathways can be activated [130]. For instance, poly-ubiquitin chains with lysine-48 (K^{48}) linkage have been implicated in substrate degradation [136], while ubiquitin-chains with lysine-63 (K^{63}) linkage have been associated with vesicular trafficking or kinase activation [137]. More comprehensive studies are needed to fully elucidate how ubiquitination regulates GPCR turnover and activity.

Functional significance of MOR ubiquitination:

Ubiquitination of ORs regulates their endocytosis and degradation [98,138,139]. In addition, misfolding of ORs in the ER can also activate the ubiquitination process and induce OR degradation [138,139] (Fig. 2). Interestingly, MOR ubiquitination is ligand specific [98,140], suggesting a possible role for this PTM in biased signaling (Box 3). Different MOR agonists can differentially activate ubiquitin attachment, in a process mediated by β-arrestin [132]. For instance, treatment with DAMGO, but not morphine, leads to increased receptor ubiquitination; β-arrestin-1 appears to play a role in this process since the increase in ubiquitination was abrogated in β-arrestin KO cells treated with DAMGO [98]. An interesting study reported that DADLE ([D-Ala^2, D-Leu^5]-Enkephalin) triggers ubiquitination of the first intracellular loop of MOR in a process mediated by β-arrrestin-2, which results in receptor down-regulation by lysosomal proteolysis [140,141] (Fig. 1; Table 1). Inhibition of MOR ubiquitination leads to a delay of the endocytic process via scission of clathrin-coated vesicles [141].

Ubiquitination of other opioids receptors: The ubiquitination of DOR has been described in different subcellular compartments. During biosynthesis, DOR is ubiquitinated in a process that works as a quality control that targets the misfolded receptor and labels it for degradation by the proteasome [139,142]. Interestingly, treatment with DOR agonists (Deltorphin I and II) leads to DOR endocytosis, followed by ubiquitination and lysosomal degradation [143].

KOR ubiquitination is enhanced by treatment with the agonists U50,488H and Dyn A [144]. The residue K^{63} of hKOR is polyubiquitinated in a process that takes place after receptor phosphorylation. Polyubiquitination contributes to changes in KOR expression, and this is an agonist-dependent process [144,145] (Table 1).

Finally, studies examining the maturation of MOR-DOR interacting complexes have found that the expression of DOR protects MOR from ubiquitination and degradation [146] and this, in turn, leads to increased cell surface expression of the MOR-DOR heteromer.

Concluding remarks and future directions

The binding of different agonists to ORs leads to different sets of molecular changes, which contributes to the complex pharmacological profile for opioids. The regulation of this complex process relies on the combination of all the PTMs described in this review: (i) the glycosylation of OR modulates the steady-state levels of the receptor at the cell surface, (ii) the hierarchical phosphorylation process affects receptor internalization/recycling as well as desensitization/tolerance, (iii) the palmitoylation process influences receptor distribution at the plasma membrane, and (iv) the ubiquitination process regulates receptor abundance (Fig. 2). In future studies it will be critical to investigate the crosstalk between PTM events, agonist-specificity and spatio-temporal dynamics of receptor signaling regulated by these PTMs (see Outstanding Questions).

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Glossary

Post-translational modification
any modification that a protein undergoes after its translation, such as phosphorylation, glycosylation, palmitoylation or ubiquitination

Glycosylation
the covalent linkage of a sugar to an amino acid residue on secreted or membrane-bound proteins. Amino acid residues that are primarily glycosylated are asparagine, serine, and threonine

Phosphorylation
the covalent attachment of a phosphate group to a serine, tyrosine or threonine residue on a substrate in eukaryotes

Palmitoylation
lipid modification characterized by the addition of a palmitate (16-carbon saturated fatty acid) to a cysteine residue via a thioester linkage

Ubiquitination
attachment of one or more ubiquitin proteins (8.6 kDa) to a lysine residue on the substrate

Efficacy
ability of an agonist to effectively activate the receptor once it is bound to it; determines how efficient an agonist is at producing a desired effect

Opioid-induced desensitization
the decrease in signaling response produced by the receptor after acute exposure to opioid agonists. This effect takes place in seconds to minutes after exposure to the opioid agonist and is reversible upon removal of opioid agonist from the system

Tolerance
the reduced effect of a drug after chronic use. In contrast to opioid-induced desensitization, tolerance develops over hours in cellular contexts, and days to weeks in animal models. Tolerance is characterized by the need to increase the dose of the drug in order to maintain the desired effect, leading to a rightward shift in the dose-response curve. One of the hallmarks of tolerance is a prolonged recovery period after desensitization

Opioid addiction
a chronic disease characterized by a compulsive and continuous need to use opioid drugs

Withdrawal

symptoms that occur upon discontinuing usage of a drug. In mouse models, the opioid withdrawal symptoms are jumping, “wet-dog” shakes, excessive grooming, weight loss and diarrhea

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BOX 1:

Steps of agonist-mediated opioid receptor signaling

For the sake of simplicity, a linear cascade of events following receptor activation is described below. Opioid receptors are GPCRs that couple to inhibitory G proteins (Gi proteins). G proteins form a heterotrimeric complex comprised of α, β and γ subunits. GPCR activation induces the exchange of GDP for GTP at the α-subunit of the associated heterotrimeric G protein, and this in turn leads to the dissociation of the α-subunit from the βγ-subunit complex (reviewed in [79]). This results in the activation of downstream signaling cascades, including the modulation of calcium and potassium channels, the activation of phospholipase C, inhibition of adenylyl cyclase activity, enhancement of phosphorylation of protein kinases such as mitogen-activated protein kinase (MAPK), and activation of kinases such as PKC and GRKs [147]. Opioid receptor-mediated activation of inwardly rectifying potassium channels and inhibition of voltage-gated calcium channels leads to a decrease in neurotransmitter release (reviewed in [107]).

One of the mechanisms responsible for termination of this intracellular signaling is restoration of the heterotrimeric G-protein complex. This involves the hydrolysis of GTP bound to the Gα subunit that is facilitated by the regulator of G-protein signaling (RGS) [148,149]. Another mechanism involves receptor phosphorylation by GRKs followed by β-arrestin recruitment, and receptor internalization [70,87,132]. Interestingly, β-arrestin has also been shown to function as a multifunctional adaptor protein that in addition to mediating endocytosis, induces a second wave of signaling [141]. Following internalization, the receptor is either recycled back to the cell surface to initiate another wave of signaling, or targeted for degradation [77,101,131].

It is increasingly becoming apparent that GPCR signaling events are not hierarchical, in that multiple signaling pathways can be activated from the receptor at the cell surface as well as from different subcellular compartments (reviewed in [150]). Furthermore, GPCR activation by different agonists can lead to differential and at times ‘biased’ signaling (reviewed in [150]).

In summary, the classical steps of opioid receptor signaling are 1) agonist binding, 2) a G protein mediated signaling response, 3) receptor phosphorylation mediated by GRKs and internalization mediated by β-arrestin recruitment, followed by 4) recycling or degradation of the receptor (Fig. 2). In this review we discuss the relevance of different post translational modifications (PTM) in the regulation of these steps.

BOX 2:

Relevance of PTM events for opioid dimerization

Post-translational modifications have been thought to affect receptor dimerization [18,146,151,152]. For instance, μ-opioid receptor (MOR) palmitoylation at the second intracellular loop (C^{170}) has been described to facilitate receptor homodimerization, via cholesterol-palmitoyl interactions [18]. In addition, δ-opioid receptor (DOR) phosphorylation at the second intracellular loop (T^{161}) has been described to facilitate MOR-DOR heterodimerization [151]. This phosphorylation process is mediated by cyclin-dependent kinase 5 (Cdk5) [151]. Finally, studies have shown that the expression and cell trafficking of MOR-DOR heteromers are regulated by ubiquitination and by receptor transporter protein 4 (RTP4), a Golgi chaperone that protects the heteromer from ubiquitination and degradation leading to enhanced cell surface expression [146,152]. Taken together, these observations suggest that a combination of PTM events contribute to the regulation of levels and function of MOR-DOR heteromers.

BOX 3:

The role of MOR phosphorylation and β-arrestin recruitment in regulating opioid effects

Recent findings highlight the importance of receptor phosphorylation and β-arrestin recruitment in regulating opioid receptor function. These include studies that show that, (i) mutations of C-terminal MOR phosphorylation residues (to alanine) impair interactions with β-arrestin, enhance analgesia, diminish analgesic tolerance, but do not suppress respiratory depression, constipation and opioid withdrawal symptoms [76,87]; (ii) phosphorylation of MOR upon morphine or DAMGO treatment is not impaired in *β*-arrestin 1/2 KO cells [98]; (iii) the lack of β-arrestin-2 suppresses the lethal side effects of opioid treatment without affecting the analgesic response [98,114,153,154]. Therefore, the role of β-arrestin needs to be systematically explored considering its relevance to different aspects of MOR PTM events.

Outstanding question box:

- What roles do PTMs play in the spatio-temporal dynamics of OR signaling?
- What are the effects of the glycan profile of the host cells in OR glycosylation and binding of opioid ligands?
- What is the contribution of receptor palmitoylation to the extent of phosphorylation and how does it affect agonist-mediated receptor distribution and internalization?
- What is the role of phosphorylation at non-C-terminal sites (i.e. intracellular loops) in modulating receptor activity?
- What is the role of β-arrestin recruitment in receptor ubiquitination, and how is this connected to differences in agonist-mediated receptor activation?

Highlights

- Post-translational modifications in G-protein coupled receptors are responsible for fine-tuning receptor signaling. Receptor subcellular localization, membrane distribution dynamics, protein-protein interactions, and receptor signaling are all events mediated by post translational modifications on specific amino acid residues.
- Glycosylation of the opioid receptor plays a role in modulating the steady-state levels at the cell surface. In human μ-Opioid Receptor, asparagine 40 (N^{40}) is one residue that has been described to be glycosylated, and $N^{40}D$ polymorphism has been implicated in pain sensitivity and dependence on alcohol and heroin.
- Palmitoylation and phosphorylation modulate the dynamics of movement of μ-Opioid Receptor at the plasma membrane and specifically, diffusion into lipid rafts. The crosstalk between these two post-translational modification events regulates agonist-mediated receptor distribution and internalization.
- Phosphorylation of the C-terminus of μ-Opioid Receptor is associated with agonist-induced receptor internalization, recycling, desensitization, as well as analgesia and development of tolerance.
- Ubiquitination of the μ-Opioid Receptor affects receptor degradation in a process dependent on β-arrestin recruitment.

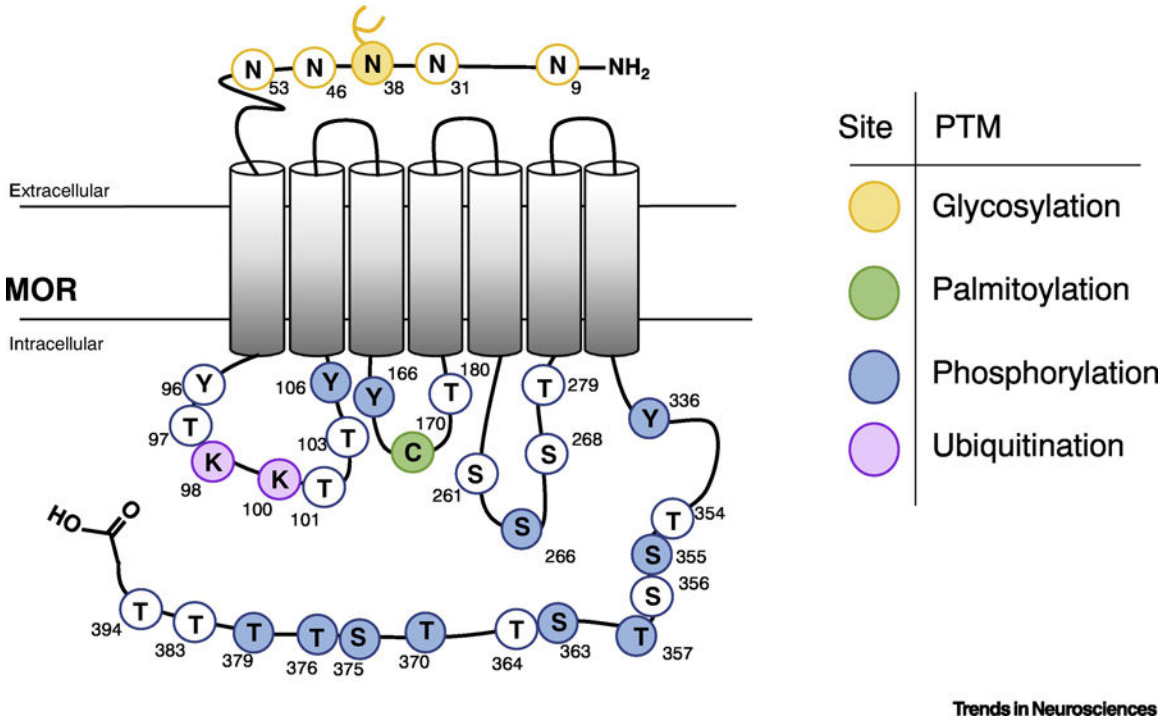

Figure 1: Post-translational modification of μ-Opioid Receptor.

A schematic representation of rat μ-Opioid Receptor showing putative residues that could be post-translationally modified. Each post-translational modification category is represented by a different color. Filled circles represent known PTMs and white circles represent putative PTMs. The residue numbers in rat MOR are indicated above each reside. Since the N^{40} has been shown to be glycosylated, it is indicated with a branched chain.

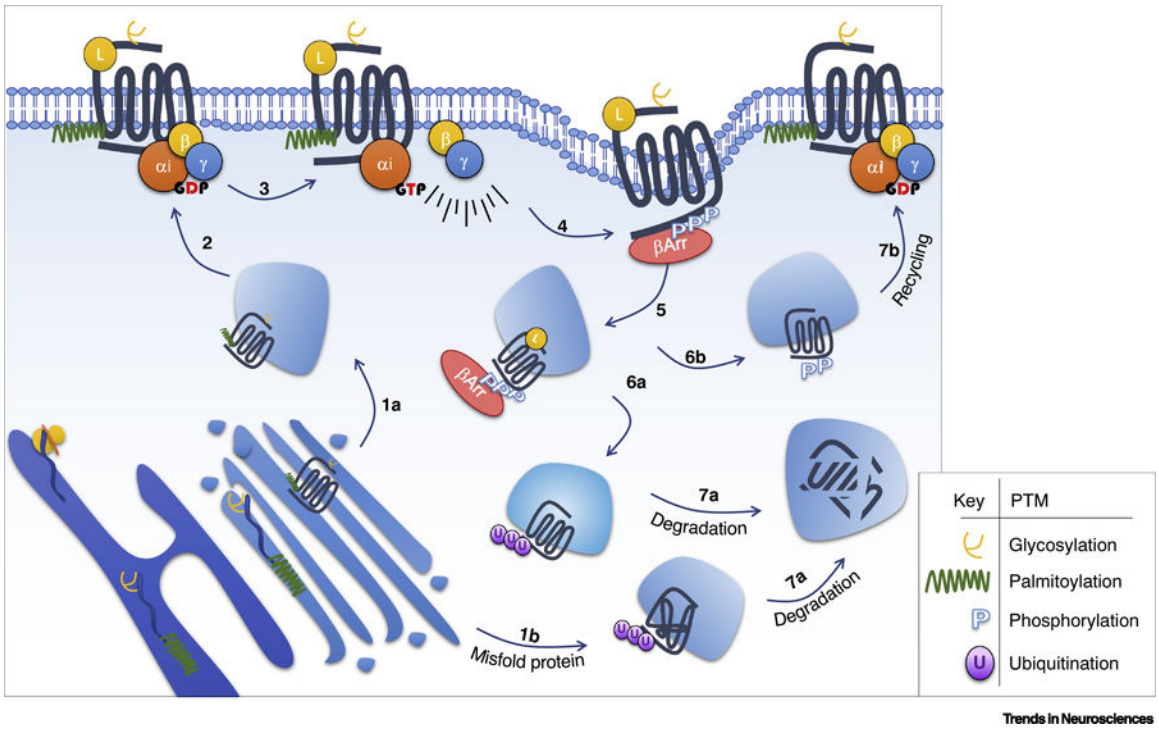

Figure 2: Schematic summary of the major PTM events in μ-Opioid Receptor signaling.

The opioid receptor undergoes glycosylation and palmitoylation in the endoplasmic reticulum and Golgi apparatus during maturation, which affect receptor's cell surface expression (1a, 2). At the cell surface, agonist exposure leads to activation of G heterotrimeric proteins (3), followed by phosphorylation of the receptor (4), leading to β-arrestin recruitment (5) and receptor endocytosis. In the endosomal compartment the receptor can be ubiquitinated (6a) and degraded (7a), or it can be recycled (6b; 7b). Misfolded proteins are ubiquitinated (1b) and subsequently degraded (7a). Please note that the numbers do not reflect hierarchical events.

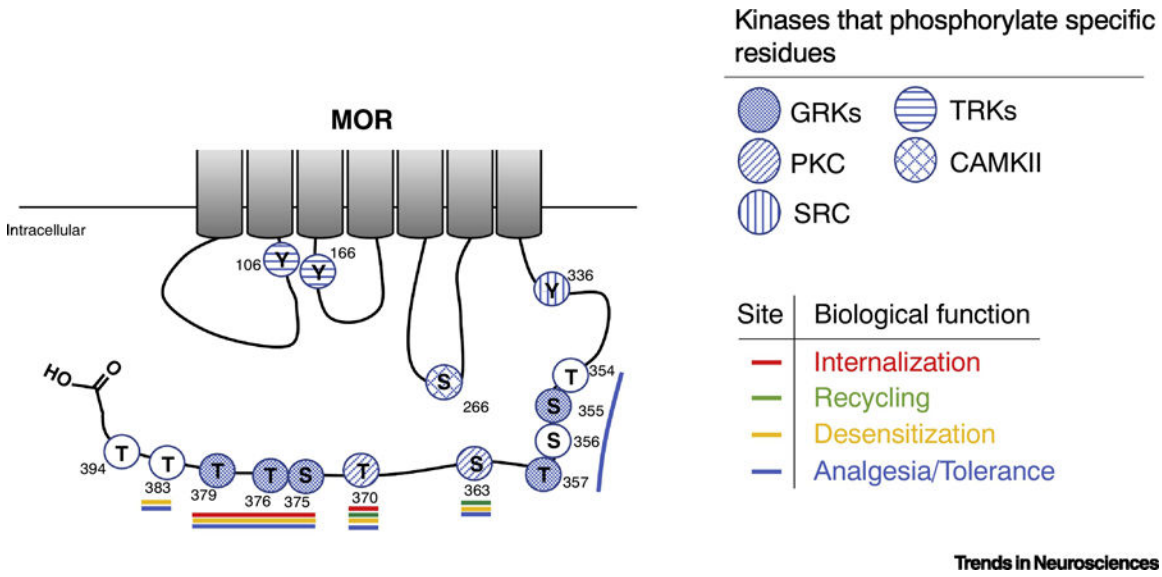

Figure 3: Biological relevance of μ-Opioid Receptor phosphorylation.

A schematic representation of the intracellular regions of rat μ-Opioid Receptor showing phosphorylatable residues and their biological relevance. Kinases that modify individual residues are indicated with filled hatches. The residue number is indicated next to each residue. Filled circles represent known phosphorylation sites and open circles represent putative phosphorylation residues. Residues that affect distinct biological activities are indicated with a colored line; in red are the residues described to play a role in MOR internalization; in green are the residues described to play a role in MOR recycling; in yellow are the residues described to play a role in MOR desensitization; and in blue are the residues described to play a role in opioid analgesia and tolerance. GRKs, G protein-coupled receptor kinases; RTKs, receptor tyrosine kinases; PKC, protein kinase C; CAMKII, Ca^{++}/calmodulin kinase II; Src, Src kinase.

Table 1:

Summary of the PTM events in Opioid Receptors.

PTM	Receptor	Enzyme	Residue	Occurrence	Role	Ref
Glycosylation	hMOR	Ribophorin I	N^{40}	Constitutive	Regulates levels of MOR at the cell surface; affects agonist-binding. $N^{40}D$ exhibits increased dependence to alcohol and opiates	[39,40,48,50,57]
	hDOR	N.D.	N^{18}; N^{33}	Constitutive	Regulates receptor trafficking.	[56]
		GalNac transferase	S^{6}; S^{25}; S^{29}	Constitutive	Enhances ligand-binding, and modulates cAMP signaling.	[59]
	hKOR	N.D.	N^{25}; N^{39}	Constitutive	Regulates receptor trafficking. Affects agonist-induced phosphorylation.	[37]
Palmitoylation	rMOR	PATs	C^{170}	Constitutive	Facilitates membrane association and modulates subcellular localization.	[15,18,66]
	rDOR	N.D.	C^{328}; C^{333}	Constitutive/ Induced	Modulates protein trafficking and subcellular localization. Palmitoylation is increased by agonist treatment.	[67,68]
Phosphorylation	m/rMOR	Src	Y^{336}	Induced	Chronic morphine or etorphine treatment leads to phosphorylation by Src; this switches MOR signaling response from inhibitory to stimulatory.	[80]
		Tyrosine Kinase	Y^{106}; Y^{166}	Induced	DAMGO-mediated tyrosine kinase phosphorylation regulates receptor-G protein coupling efficacy.	[81]
		GRKs	S^{355}; T^{357}; T^{370}; S^{375}; T^{376}; T^{379}	Induced	DAMGO, fentanyl, Met-Enk and etonitazene induce hierarchical phosphorylation of these residues via GRK2/3, facilitating β-arrestin recruitment, MOR internalization, desensitization and tolerance. Morphine induces phosphorylation of S^{375} and weak phosphorylation of T^{370}, T^{376} and T^{379}, via GRK5/6.	[69,76,78,85,87–90,100]
		PKC	S^{363}; T^{370}	Constitutive/ Induced	Modulates morphine-induced desensitization, and tolerance. PKC inhibition impairs DAMGO-mediated MOR recycling.	[69,77,91,92,101]
		CAMKII	T^{370}; S^{26}	Induced	S^{266} phosphorylation modulates DAMGO-induced desensitization.	[69,93]
	mDOR	GRK2	T^{358}; S^{363}	Induced	Hierarchical phosphorylation required for clathrin-mediated receptor internalization.	[117,118]
		PKC	S^{344}	Constitutive	Modulates heterologous regulation via β-Arrestin- and clathrin-mediated receptor internalization.	[123]
		Cdk5	T^{161}	Induced	Regulates cell surface expression of DOR and facilitates the formation of DOR-MOR heterodimers.	[151]
	mKOR	GRKs	S^{356}; T^{357}; T^{363}; S^{369}	Induced	U50,488H treatment leads to GRK-mediated phosphorylation, β-arrestin recruitment and internalization.	[119]
		PKC	S^{356}; T^{357}; T^{363}; S^{369}	Constitutive	Modulates agonist independent KOR internalization.	[124]
	mNOP	GRK2/3	S^{346}; S^{351}; T^{362}; S^{363}	Induced	Affects receptor desensitization and internalization.	[116,128]
Ubiquitination	mMOR	N.D.	K^{98}; K^{100}	Induced	Regulates internalization and degradation induced by DAMGO and DADLE treatment.	[98,140,141]

PTM	Receptor	Enzyme	Residue	Occurrence	Role	Ref
	hDOR	N.D.	N.D.	Induced	Induces receptor internalization and degradation.	[139,142]
	hKOR	N.D.	K^{63}	Induced	Modulates receptor expression.	[144,145].

The list includes (i) the PTM event, (ii) the enzyme responsible for the PTM (iii) the residues involved, (iv) whether the PTM event is agonist induced or constitutive and, (v) the role of the PTM in opioid signaling. N.D., non-described.