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REVIEW

Different mechanisms of homologous and heterologous μ-opioid receptor phosphorylation

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The efficiency of μ-opioid receptor signalling is tightly regulated and ultimately limited by the coordinated phosphorylation of intracellular serine and threonine residues. Here, we review and discuss recent progress in the generation and application of phosphosite-specific μ-opioid receptor antibodies, which have proved to be excellent tools for monitoring the spatial and temporal dynamics of receptor phosphorylation and dephosphorylation. Agonist-induced phosphorylation of μ-opioid receptors occurs at a conserved 10 residue sequence ^{370}TREHPSTANT^{379} in the receptor's carboxyl-terminal cytoplasmic tail. Diverse opioids induce receptor phosphorylation at S375, present in the middle of this sequence, but only high-efficacy opioids have the ability to drive higher order phosphorylation on flanking residues (T370, T376 and T379). S375 is the initiating residue in a hierarchical phosphorylation cascade. In contrast, agonist-independent heterologous μ-opioid receptor phosphorylation occurs primarily at T370. The combination of phosphosite-specific antibodies and siRNA knockdown screening also facilitated the identification of relevant kinases and phosphatases. In fact, morphine induces a selective S375 phosphorylation that is predominantly catalysed by GPCR kinase 5 (GRK5), whereas multisite phosphorylation induced by high-efficacy opioids specifically requires GRK2/3. By contrast, T370 phosphorylation stimulated by phorbol esters or heterologous activation of G_q-coupled receptors is mediated by PKCα. Rapid μ-opioid receptor dephosphorylation occurs at or near the plasma membrane and is catalysed by protein phosphatase 1γ (PP1γ). These findings suggest that there are distinct phosphorylation motifs for homologous and heterologous regulation of μ-opioid receptor phosphorylation. However, it remains to be seen to what extent different μ-opioid receptor phosphorylation patterns contribute to the development of tolerance and dependence *in vivo*.

LINKED ARTICLES

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Abbreviations

AtT20 cells, mouse pituitary tumour cells; CaMKII, calcium-calmodulin kinase II; DAMGO, [D-Ala^2,N-MePhe^4,Gly-ol]-enkephalin; GRK, GPCR kinase; PNS, peripheral nervous system; PP, protein phosphatase; SP, substance P

Introduction

The prototypical opioid is morphine, an alkaloid derived from the opium poppy. In the early 19th century, morphine was isolated from opium. Today, morphine is still among the most potent clinically used analgesics. Morphine mediates all of its therapeutic effects via the μ-opioid receptor (Alexander *et al.*, 2013). However, its adverse effects such as respiratory depression, obstipation, tolerance and addiction limit the clinical use of the drug (Nestler, 1996; Nestler and Aghajanian, 1997; Koob *et al.*, 1998). Huge efforts in medicinal chemistry have led to thousands of morphine analogues and structurally distinct opioids (Corbett *et al.*, 2006). Nevertheless, at present there are no opioid analgesics without euphoriant and addictive effects available. Heroin was introduced in 1898 as one of the first morphine derivates with more pronounced addictive properties than morphine. The long-lasting actions of methadone and buprenorphine favoured these drugs for use in treating chronic pain and management of opioid dependence (Pergolizzi *et al.*, 2008). Fentanyl is rapidly taken up into body fat limiting its action which makes this drug advantageous in the operating room and certain outpatient procedures (Peng and Sandler, 1999). Etonitazene is one of the most potent opioids but is not used in clinical practice because it causes a strong respiratory depression. The drug of choice for various clinical indications and the safety of various opioids is influenced by significant differences among opioids in potency and efficacy. Buprenorphine is safer than methadone for management of opioid addicts because of its lower intrinsic efficacy at μ-opioid receptors that produces less respiratory depression. All μ-opioid receptor agonists produce adverse effects that include the development of tolerance after long-term use (Williams *et al.*, 2001; von Zastrow *et al.*, 2003; Connor *et al.*, 2004; Waldhoer *et al.*, 2004; Bailey and Connor, 2005; Christie, 2008; Koch and Hollt, 2008; Morgan and Christie, 2011).

Many mechanisms have evolved for the regulation of signal transduction. Decreased responsiveness to agonists has been correlated with desensitization and internalization of GPCRs (Pitcher *et al.*, 1998). At the level of the receptor, G proteins are uncoupled from the receptor as a consequence of receptor phosphorylation, which leads to the termination of signal transduction. This process is referred to as desensitization (Ferguson, 2001). The required intracellular phosphorylation of the receptor can be induced both homologously (agonist dependent) and heterologously (agonist independent) (Sibley and Lefkowitz, 1985). For this regulatory mechanism, two major protein families are primarily involved: GPCR kinases (GRKs) and second-messenger kinases such as PKA or PKC (Ferguson *et al.*, 1996; Ferguson and Caron, 1998; Krupnick and Benovic, 1998; Liu and Anand, 2001; Koch and Hollt, 2008). Desensitization and cellular tolerance can be induced by both homologous and heterologous phosphorylation of μ-opioid receptors.

Homologous μ-opioid receptor phosphorylation

Within the intracellular regions of the μ-opioid receptor, there are about 20 serine, threonine and tyrosine residues, which can potentially contribute to receptor desensitization and endocytosis (Connor *et al.*, 2004; Koch and Hollt, 2008). In this review, we will focus on the carboxyl-terminal phosphorylation sites of the μ-opioid receptor. Opioid neuropeptides can increase the basal phosphorylation of μ-opioid receptors. There are differences in the opioid drug-mediated phosphorylation of the μ-opioid receptor. An increased phosphorylation generally correlates with their ability to induce internalization (Zhang *et al.*, 1998; McPherson *et al.*, 2010). Earlier studies have used whole cell phosphorylation assays to elucidate agonist-induced μ-opioid receptor phosphorylation. From these studies, it became evident that the μ-opioid receptor can undergo homologous agonist-driven as well as heterologous PKC-mediated phosphorylation (Zhang *et al.*, 1996). Differential phosphorylation and desensitization of the μ-opioid receptor can be induced by agonists and phorbol esters (Zhang *et al.*, 1996; El Kouhen *et al.*, 2001; Pfeiffer *et al.*, 2003; Schulz *et al.*, 2004). Analysis of site-directed mutants first identified S375 as one major site of agonist-induced phosphorylation and S363 as a site of basal phosphorylation (El Kouhen *et al.*, 2001; Schulz *et al.*, 2004). More recent studies using mass spectrometry revealed T370, T376 and T379 as additional sites of agonist-driven phosphorylation (Lau *et al.*, 2011; Mouledous *et al.*, 2012; Chen *et al.*, 2013). However, neither whole cell phosphorylation assays nor mass spectrometry allows the examination of the spatial and temporal dynamics of agonist-driven phosphorylation of individual phosphate acceptor sites in the carboxyl-terminal cytoplasmic tail of the μ-opioid receptor.

The recent generation of phosphosite-specific antibodies confirmed that homologous agonist-induced phosphorylation of the μ-opioid receptor occurs primarily at a conserved 10 residue sequence ^{370}TREHPSTANT^{379} within the carboxyl-terminal cytoplasmic tail of the receptor, namely T370, S375, T376 and T379 (Figure 1) (El Kouhen *et al.*, 2001; Schulz *et al.*, 2004; Doll *et al.*, 2011; 2012; Lau *et al.*, 2011; Just *et al.*, 2013). This multisite phosphorylation is a hierarchical process with S375 representing the initiating site required for subsequent phosphorylation at T370, T376 and T379. High-efficacy agonists like DAMGO induce a robust phosphorylation at S375 within 20 s after exposure followed by phosphorylation at T370, while phosphorylation at T376 and T379 requires more extended periods. Interestingly, a single point mutation of S375 (S375A) is sufficient to promote a strong reduction of DAMGO-induced phosphorylation of T370, T376 and T379. A variety of structurally diverse agonists induce receptor phosphorylation at S375, but only high-efficacy agonists can cause higher order phosphorylation including T370, T376 and T379 in cell lines (Yu *et al.*, 1997; Zhang *et al.*, 1998; Whistler *et al.*, 1999; Schulz *et al.*, 2004; Johnson *et al.*, 2006; Doll *et al.*, 2011; 2012; Lau *et al.*, 2011; Just *et al.*, 2013). Lower efficacy agonists like morphine only promote S375 phosphorylation in HEK293 cells (Doll *et al.*, 2011).

The combination of phosphosite-specific antibodies and siRNA knockdown screening has led to the identification of relevant kinases mediating these markedly drug-selective phosphorylation patterns (Table 1). The initiating residue S375 in the hierarchical phosphorylation cascade can be phosphorylated by GRK2/3 as well as GRK5 isoforms in an agonist-dependent manner (Schulz *et al.*, 2004; McPherson

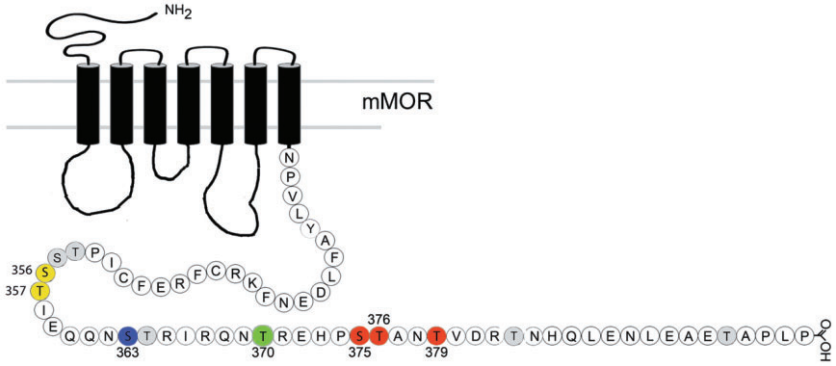

Figure 1

Schematic representation of the mouse μ-opioid receptor (mMOR) with putative phosphorylation sites in the C-terminus. Key: yellow: S356/T357 no antibodies available at the moment; blue: S363 constitutive phosphorylation; green: T370 homologous and heterologous phosphorylation site; orange: S375, T376 and T379 only homologous phosphorylation; grey: no phosphorylation detectable; these sites were targeted for the generation of phosphosite-specific antibodies (Doll *et al.*, 2011; 2012; Just *et al.*, 2013).

Table 1

Summary of agonist-induced phosphorylation site specific phosphorylation in which kinases are involved

Phosphorylation site	Agonist	Kinase	Cell type
S363	Constitutive	PKC	HEK293 cells[1,2]/brain tissue[2]
	PMA (heterologous)	PKC	CHO cells[3]
T370	DAMGO (homologous)	GRK2/3	HEK293 cells[4]
	PMA (heterologous)	PKCα	HEK293 cells[2]
S375	Morphine (homologous)	GRK5	HEK293 cells[4]/brain tissue[4]
	DAMGO (homologous)	GRK2/3	HEK293 cells[4]
T376	DAMGO (homologous)	GRK2/3	HEK293 cells[5]
T379	DAMGO (homologous)	GRK2/3	HEK293 cells[5]

[1]Doll *et al.* (2011), [2]Illing *et al.* (2013), [3]Feng *et al.* (2011a), [4]Doll *et al.* (2012), [5]Just *et al.* (2013).

et al., 2010; Doll *et al.*, 2012). However, phosphorylation at each of the other residues within the ^{370}TREHPSTANT^{379} phosphorylation motif specifically necessitates GRK2/3 isoforms. Thus, the morphine-induced S375 phosphorylation is predominantly catalysed by GRK5. Multisite phosphorylation induced by high-efficacy opioids is catalysed by GRK2/3.

In transfected HEK293 cells and in primary neuronal cultures, DAMGO induces a robust phosphorylation whereas morphine stimulates μ-opioid receptor phosphorylation only weakly. Overexpression of GRK2 or GRK3 strongly enhances phosphorylation and facilitates morphine-induced internalization (Schulz *et al.*, 2004). So far, in *in vivo* intact mouse brain, only S375 phosphorylation has been shown to occur during agonist stimulation (Grecksch *et al.*, 2011). Interestingly, GRK5-deficient mice exhibit reduced levels of morphine-induced S375 phosphorylation, suggesting that phosphorylation of this residue in brain tissue is mediated in part by GRK5 (Doll *et al.*, 2012).

Analysis of point mutations of these residues indicates that multiple phosphorylations within the ^{370}TREHPSTANT^{379} sequence are necessary for the internalization-promoting activity of opioids (Schulz *et al.*, 2004; Doll *et al.*, 2011; 2012; Just *et al.*, 2013). Mutation of all potential phosphate acceptor sites including T370, S375, T376 and T379 is required for a complete inhibition of agonist-induced μ-opioid receptor internalization (Just *et al.*, 2013). Opioid drugs that cannot induce higher order phosphorylation involving these residues, whether or not they are able to induce phosphorylation at S375, are inefficient at promoting internalization of μ-opioid receptor. The same phosphorylation sites also control the opioid receptor internalization in relevant neurons (Just *et al.*, 2013).

In addition to the ^{370}TREHPSTANT^{379} motif, agonist-dependent phosphorylation of S356 and T357 within the ^{354}TSST^{357} cluster was identified by mass spectrometry (Chen *et al.*, 2013). At present, phosphosite-specific antibodies for pS356 and pT357 are not available for a more detailed analysis. Nevertheless, phosphorylation of the ^{354}TSST^{357} cluster is not required for μ-opioid receptor endocytosis because a TSST-4A mutant μ-opioid receptor was still internalized to the same extent as the wild-type receptor (Lau *et al.*, 2011).

Heterologous μ-opioid receptor phosphorylation

There is considerable interest in the involvement of PKC phosphorylation in the regulation of μ-opioid receptors by morphine (Kelly *et al.*, 2008). In native locus coeruleus neurons, activation of PKC leads to an increased desensitization of μ-opioid receptor signalling by morphine (Bailey *et al.*, 2004; 2009). Inhibition of PKC in HEK293 cells reduces the morphine-induced but not the DAMGO-induced phosphorylation and desensitization of the μ-opioid receptor (Johnson *et al.*, 2006). However, it is not known whether PKC can phosphorylate μ-opioid receptors directly or indirectly.

Recent evidence using phosphosite-specific antibodies suggests that activation of PKC by PMA leads to a selective and concentration-dependent phosphorylation of T370 in HEK293 cells (Doll *et al.*, 2011). In addition, Feng *et al.* (2011a) showed a PMA-induced phosphorylation of S363 in CHO cells. HEK293 cells express a wide range of PKC isoforms (Atwood *et al.*, 2011). PMA activates both the classical (α, β1, β2, γ) and the new PKC isoforms (ε, δ, η, θ) (Nishizuka, 1992). A limited siRNA knockdown screen revealed that PKCα is specifically required for PMA-driven T370 phosphorylation of μ-opioid receptors. The PMA-induced heterologous T370 phosphorylation is considerably slower than the DAMGO-induced homologous phosphorylation of the same residue. Under conditions when chemical PKC inhibitors blocked PMA-mediated T370 phosphorylation, DAMGO still facilitated T370 phosphorylation to the same extent, suggesting that homologous phosphorylation occurred independently of PKC. Heterologous PKC-driven phosphorylation also facilitated internalization of the wild-type μ-opioid receptor but not of the T370A mutant (Illing *et al.*, 2013).

Interestingly, a similar PKC-dependent T370 phosphorylation also occurs after heterologous activation of G_q protein-coupled receptors co-expressed with μ-opioid receptors in the cell. Illing *et al.* observed a selective and concentration-dependent phosphorylation of T370 after stimulation of neurokinin-1 receptors with substance P (SP). siRNA knockdown revealed that the SP-driven T370 phosphorylation was also mediated by PKCα in HEK293 cells. SP-induced μ-opioid receptor phosphorylation and internalization were completely blocked in the presence of chemical PKC inhibitors. In addition, several other studies reported heterologous-induced phosphorylation of the μ-opioid receptor after activation of other GPCRs (Pfeiffer *et al.*, 2002; Chen *et al.*, 2004; Mouledous *et al.*, 2012).

Basal μ-opioid receptor phosphorylation

Earlier studies using whole cell phosphorylation assays and site-directed mutagenesis suggested that S363 is phosphorylated under basal conditions (El Kouhen *et al.*, 2001). Using mass spectrometry, Chen *et al.* (2013) showed that both S363 and T370 are phosphorylated under basal conditions. By using *in vitro* phosphorylation assays they also demonstrated that S363 is phosphorylated by PKC, but in contrast, that T370 is the substrate of calcium-calmodulin kinase II (CaMKII). Doll *et al.* (2011) generated phosphosite-specific antibodies for S363 and observed that S363 is constitutively phosphorylated under basal conditions in HEK293 cells. However, PKC-mediated S363 phosphorylation of the μ-opioid receptor appears to depend on the cellular environment. While S363 phosphorylation occurs constitutively in HEK293 cells (Doll *et al.*, 2011; Feng *et al.*, 2011b), it requires activation of PKC by phorbol esters in CHO cells (Doll *et al.*, 2011; Feng *et al.*, 2011b). Nevertheless, more recent evidence suggests that S363 is also constitutively phosphorylated in mouse brain *in vivo* (Illing *et al.*, 2013). S363 phosphorylation in HEK293 cells can be blocked by prolonged inhibition of PKC activity. Results obtained from siRNA knockdown studies suggest that both classical and new PKC isoforms may contribute to constitutive phosphorylation at S363 in HEK293 cells (Illing *et al.*, 2013).

Mechanisms of μ-opioid receptor dephosphorylation

Although the regulation of agonist-induced phosphorylation has been studied in detail for many GPCRs, the molecular mechanisms and functional consequences of receptor dephosphorylation are far from being understood. Complete dephosphorylation of the T370 and S375 of the μ-opioid receptor occurs within 3–5 min after agonist removal (Doll *et al.*, 2011). In HEK293 cells, the phosphatase activity required for this rapid dephosphorylation was inhibited in a concentration-dependent manner only by calyculin A but not by okadaic acid (Doll *et al.*, 2012). Both calyculin A and okadaic acid can effectively block protein phosphatase 2 (PP2), PP4 and PP5 activities. In contrast to okadaic acid, calyculin A is also a potent inhibitor of PP1 activity, suggesting that PP1 activity is required for dephosphorylation of the μ-opioid receptor. Three distinct catalytic subunits named α, β and γ are known for PP1. Selective inhibition of PP1α or PP1β expression had no effect on dephosphorylation. In contrast, inhibition of PP1γ expression resulted in a clearly delayed T370 and S375 dephosphorylation after agonist removal (Doll *et al.*, 2012). Inhibition of PP2α, PP2β, PP4 or PP5 expression did not alter the time course of μ-opioid receptor dephosphorylation. Thus, these findings identify PP1γ as the bona fide GPCR phosphatase for the ^{370}TREHPSTANT^{379} motif in HEK293 cells.

Inhibition of PP1γ expression facilitates detection of phosphorylated μ-opioid receptors at the plasma membrane already 5 min after agonist exposure (Doll *et al.*, 2012). This enhanced ability to detect phosphorylated μ-opioid receptors at the plasma membrane persists throughout extended treatment periods. These results strongly suggest that dephosphorylation of the μ-opioid receptor is initiated directly after receptor activation at or near the plasma membrane. Consistent with these observations, T370 and S375 dephosphorylation were not affected by treatment of acutely stimulated cells with concanavallin A, which largely prevents receptor internalization (Doll *et al.*, 2011). Nevertheless, PP1γ-mediated dephosphorylation facilitates μ-opioid receptor recycling (Doll *et al.*, 2012).

Outstanding issues and questions

- To what extent does the phosphorylation of the ^{370}TREHPSTANT^{379} motif and/or the phosphorylation of the ^{354}TSST^{357} cluster contribute to β-arrestin recruitment and desensitization of the μ-opioid receptor?
- What is the biological significance of PKC-mediated phosphorylation of the μ-opioid receptor? Does it contribute to morphine-induced desensitization?
- How is the PP1 complex assembled and recruited to phosphorylated μ-opioid receptors?
- How is μ-opioid receptor phosphorylation regulated in different brain regions *in vivo*?
- To what extent are opioid-mediated behaviours such as tolerance and dependence determined by distinct μ-opioid receptor phosphorylation patterns?

Concluding remarks

GPCRs regulate a myriad of physiological processes. Termination of signalling of activated GPCRs is essential for maintenance of cellular homeostasis. Desensitization of GPCR signalling causes a reduction in receptor response to repeated or long-lasting stimuli. Agonist-induced phosphorylation allows binding of β-arrestin to the receptor that promotes desensitization of G protein signalling and induces receptor internalization. The classical paradigm of the GPCR life cycle dictates that receptors have to internalize into an acidic endosomal compartment to become dephosphorylated.

Emerging evidence suggests that distinct opioid analgesics induce strikingly different patterns of μ-opioid receptor phosphorylation that result in different spatial and temporal dynamics of β-arrestin trafficking, internalization and recycling. In fact, morphine induces a selective S375 phosphorylation that is predominantly catalysed by GRK5, whereas multisite phosphorylation induced by high-efficacy opioids is mediated by GRK2/3. Rapid dephosphorylation of the μ-opioid receptor is initiated at or near the plasma membrane and specifically requires PP1γ. So far, only agonist-induced S375 phosphorylation has been shown in mouse brain *in vivo*. It remains to be seen to what extent different μ-opioid receptor phosphorylation patterns contribute to the development of opioid tolerance and dependence.

Conflict of interest

None.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ and CGTP Collaborators (2013). The Concise Guide to PHARMACOLOGY 2013/14: G-Protein Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011). Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12: 14.

Bailey CP, Connor M (2005). Opioids: cellular mechanisms of tolerance and physical dependence. Curr Opin Pharmacol 5: 60–68.

Bailey CP, Kelly E, Henderson G (2004). Protein kinase C activation enhances morphine-induced rapid desensitization of mu-opioid receptors in mature rat locus ceruleus neurons. Mol Pharmacol 66: 1592–1598.

Bailey CP, Llorente J, Gabra BH, Smith FL, Dewey WL, Kelly E *et al.* (2009). Role of protein kinase C and mu-opioid receptor (MOPr) desensitization in tolerance to morphine in rat locus coeruleus neurons. Eur J Neurosci 29: 307–318.

Chen C, Li J, Bot G, Szabo I, Rogers TJ, Liu-Chen LY (2004). Heterodimerization and cross-desensitization between the mu-opioid receptor and the chemokine CCR5 receptor. Eur J Pharmacol 483: 175–186.

Chen YJ, Oldfield S, Butcher AJ, Tobin AB, Saxena K, Gurevich VV *et al.* (2013). Identification of phosphorylation sites in the COOH-terminal tail of the mu-opioid receptor. J Neurochem 124: 189–199.

Christie MJ (2008). Cellular neuroadaptations to chronic opioids: tolerance, withdrawal and addiction. Br J Pharmacol 154: 384–396.

Connor M, Osborne PB, Christie MJ (2004). Mu-opioid receptor desensitization: is morphine different? Br J Pharmacol 143: 685–696.

Corbett AD, Henderson G, McKnight AT, Paterson SJ (2006). 75 years of opioid research: the exciting but vain quest for the Holy Grail. Br J Pharmacol 147 (Suppl. 1): S153–S162.

Doll C, Konietzko J, Poll F, Koch T, Hollt V, Schulz S (2011). Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. Br J Pharmacol 164: 298–307.

Doll C, Poll F, Peuker K, Loktev A, Gluck L, Schulz S (2012). Deciphering micro-opioid receptor phosphorylation and dephosphorylation in HEK293 cells. Br J Pharmacol 167: 1259–1270.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH (2001). Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid receptor internalization. J Biol Chem 276: 12774–12780.

Feng B, Li Z, Wang JB (2011a). Protein kinase C-mediated phosphorylation of the mu-opioid receptor and its effects on receptor signaling. Mol Pharmacol 79: 768–775.

Feng B, Li Z, Wang JB (2011b). Protein kinase C-mediated phosphorylation of the mu-opioid receptor and its effects on receptor signaling. Mol Pharmacol 79: 768–775.

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

Ferguson SS, Caron MG (1998). G protein-coupled receptor adaptation mechanisms. Semin Cell Dev Biol 9: 119–127.

Ferguson SS, Downey WE 3rd, Colapietro AM, Barak LS, Menard L, Caron MG (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271: 363–366.

Grecksch G, Just S, Pierstorff C, Imhof AK, Gluck L, Doll C *et al.* (2011). Analgesic tolerance to high-efficacy agonists but not to morphine is diminished in phosphorylation-deficient S375A mu-opioid receptor knock-in mice. J Neurosci 31: 13890–13896.

Illing S, Mann A, Schulz S (2013). Heterologous regulation of agonist-independent μ-opioid receptor phosphorylation by protein kinase C. Br J Pharmacol 171: 1330–1340.

Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ *et al*. (2006). Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. Mol Pharmacol 70: 676–685.

Just S, Illing S, Trester-Zedlitz M, Lau EK, Kotowski SJ, Miess E *et al*. (2013). Differentiation of opioid drug effects by hierarchical multi-site phosphorylation. Mol Pharmacol 83: 633–639.

Kelly E, Bailey CP, Henderson G (2008). Agonist-selective mechanisms of GPCR desensitization. Br J Pharmacol 153 (Suppl. 1): S379–S388.

Koch T, Hollt V (2008). Role of receptor internalization in opioid tolerance and dependence. Pharmacol Ther 117: 199–206.

Koob GF, Sanna PP, Bloom FE (1998). Neuroscience of addiction. Neuron 21: 467–476.

Krupnick JG, Benovic JL (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 38: 289–319.

Lau EK, Trester-Zedlitz M, Trinidad JC, Kotowski SJ, Krutchinsky AN, Burlingame AL *et al*. (2011). Quantitative encoding of the effect of a partial agonist on individual opioid receptors by multisite phosphorylation and threshold detection. Sci Signal 4: ra52.

Liu JG, Anand KJ (2001). Protein kinases modulate the cellular adaptations associated with opioid tolerance and dependence. Brain Res Brain Res Rev 38: 1–19.

McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C *et al*. (2010). Mu-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. Mol Pharmacol 78: 756–766.

Morgan MM, Christie MJ (2011). Analysis of opioid efficacy, tolerance, addiction and dependence from cell culture to human. Br J Pharmacol 164: 1322–1334.

Mouledous L, Froment C, Dauvillier S, Burlet-Schiltz O, Zajac JM, Mollereau C (2012). GRK2 protein-mediated transphosphorylation contributes to loss of function of mu-opioid receptors induced by neuropeptide FF (NPFF2) receptors. J Biol Chem 287: 12736–12749.

Nestler EJ (1996). Under siege: the brain on opiates. Neuron 16: 897–900.

Nestler EJ, Aghajanian GK (1997). Molecular and cellular basis of addiction. Science 278: 58–63.

Nishizuka Y (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607–614.

Peng PW, Sandler AN (1999). A review of the use of fentanyl analgesia in the management of acute pain in adults. Anesthesiology 90: 576–599.

Pergolizzi J, Boger RH, Budd K, Dahan A, Erdine S, Hans G *et al*. (2008). Opioids and the management of chronic severe pain in the elderly: consensus statement of an International Expert Panel with focus on the six clinically most often used World Health Organization Step III opioids (buprenorphine, fentanyl, hydromorphone, methadone, morphine, oxycodone). Pain Pract 8: 287–313.

Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, Schulz S (2002). Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization. J Biol Chem 277: 19762–19772.

Pfeiffer M, Kirscht S, Stumm R, Koch T, Wu D, Laugsch M *et al*. (2003). Heterodimerization of substance P and mu-opioid receptors regulates receptor trafficking and resensitization. J Biol Chem 278: 51630–51637.

Pitcher JA, Freedman NJ, Lefkowitz RJ (1998). G protein-coupled receptor kinases. Annu Rev Biochem 67: 653–692.

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

Sibley DR, Lefkowitz RJ (1985). Molecular mechanisms of receptor desensitization using the beta-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 317: 124–129.

Waldhoer M, Bartlett SE, Whistler JL (2004). Opioid receptors. Annu Rev Biochem 73: 953–990.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M (1999). Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23: 737–746.

Williams JT, Christie MJ, Manzoni O (2001). Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 81: 299–343.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR, Wang JB (1997). Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 272: 28869–28874.

von Zastrow M, Svingos A, Haberstock-Debic H, Evans C (2003). Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. Curr Opin Neurobiol 13: 348–353.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY *et al*. (1998). 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.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, Wang JB (1996). Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 271: 11449–11454.