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**Opioid regulation of mu receptor internalisation: relevance to the development of tolerance and dependence**

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## Abstract

**Internalisation of the mu opioid receptor from the surface of cells is generally achieved by receptor occupancy with agonist ligands of high efficacy. However, in many situations the potent analgesic morphine fails to promote internalisation effectively and whether there is a direct link between this and the propensity for the sustained use of morphine to result in both tolerance and dependence has been studied intensely. Although frequently described as a partial agonist, this characteristic appears insufficient to explain the poor capacity of morphine to promote internalisation of the mu opioid receptor. Experiments performed using both transfected cell systems and *ex vivo/in vivo* models have provided evidence that when morphine can promote internalisation of the mu receptor there is a decrease in the development of tolerance and dependence. Although aspects of this model are controversial, such observations suggest a number of approaches to further enhance the use of morphine as an analgesic.**

**Keywords: morphine, tolerance, dependence, receptor internalisation, desensitisation**

## Introduction

Opiates and endogenous enkephalin neuropeptides perform their physiological actions by interacting with four different receptor subtypes, namely mu, delta, kappa and nociceptin receptors, belonging to the superfamily of G protein-coupled receptors [1]. G protein-coupled receptors or GPCRs are polypeptide proteins that cross the cellular lipidic bilayer via seven transmembrane domains enriched in hydrophobic amino acids. The central function of GPCRs is to transmit information from the extracellular face of the plasma membrane to within cells by coupling with heterotrimeric G proteins, another family of cell membrane proteins. The interaction between a receptor-G protein pair is promoted by the selective

binding of chemical compounds named agonists that generate specific conformational changes in the receptor. After coupling with a GPCR, Gα subunits of G proteins exchange GDP for GTP and may dissociate from their associated Gβγ dimeric subunits, initiating from each component diverse intracellular signalling cascades via activation of a variety of effectors including, amongst others, adenylyl cyclases, phospholipases and gated ion channel receptors.

Morphine, and chemical derivates of this alkaloid, are drugs widely known for their potent analgesic properties in clinical practice as well as for their recreational use. In general, these drugs bind preferentially to mu opioid receptors. In fact, the designation "mu" was originated when it was observed that morphine selectively discriminated this receptor from the other opioid receptor subtypes [1]. More recently, the development and phenotypic characterisation of genetically modified animals lacking expression of mu opioid receptors finally confirmed that morphine exerts both its analgesic and antinociceptive actions through this receptor in living organisms [2]. Although morphine is considered one of the most potent analgesic drugs, especially for the treatment of chronic or refractory pain, its use in clinical practice is limited by the occurrence of tolerance and dependence following prolonged treatment [3]. Tolerance is defined as a diminishment in the effect of a drug such that larger doses are necessary to produce the initial effect(s). This augmentation of the drug dose required for effect facilitates the development of undesired side effects that, in the case of morphine, may include constipation or respiratory depression. Dependence is related to neuronal changes at both the cellular level and in synaptic organisation that generate physical symptoms following withdrawal of the drug [4, 5]. The fact that not all opioid drugs generate the same degree of tolerance and dependence in experimental animals when they are administered chronically at equi-effective analgesic doses led to consideration that different effects must be produced at the molecular and cellular level when these agonists bind to mu receptors.

The development of recombinant cDNA methodology, combined with a wide range of molecular and cell biology techniques, made possible the expression of particular genes of interest in diverse host cells. Such heterologous expression systems have been extremely useful in investigations of events that occur at the cellular level when a GPCR is activated by a specific agonist. Although initially investigations were focussed predominantly on the $\beta_2$-adrenoceptor, many of the effects observed for this GPCR have been corroborated subsequently for other GPCR family members and have provided a series of paradigms that define key molecular process that occur following receptor activation [6]. Essentially these models postulate that the interaction of an agonist-occupied GPCR with a G protein is interrupted by the phosphorylation of the receptor by specific GPCR kinases or GRKs. This defines that the phosphorylated receptor is more prone to recruit and interact with arrestins than the native state of the receptor. As well as binding directly to GPCRs, the arrestins are cytosolic proteins that operate as scaffolding elements in different cell signalling processes and also facilitate the internalisation of GPCRs, as well as other classes of transmembrane receptors, by recruiting proteins involved in the endocytic machinery, such as clathrin and the AP-2 adapter complex, to form finally an endocytic vesicle. The participation of the GTPase dynamin allows the vesicle to be pinched off from the external membrane. The fate of such endosomes, containing the receptor, is non-uniform; in some cases endosomes merge to eventually form lysosomes that degrade proteins within them. By contrast, in other situations, endosomes are recycled to the plasma membrane, where dephosphorylated GPCRs are refreshed to a state able to interact productively yet again with agonist, re-initiating cellular signalling processes [7]. Regardless of the endocytic pathway followed by the endosomes, it can be considered as a general situation that continual stimulation of a receptor by constant

exposure to many agonist drugs results in a reduction of the receptor number in the plasma membrane and within the cell. These processes are known collectively as down-regulation.

In pharmacology, the term desensitisation refers to the loss of responsiveness of the assessed system to the continuing presence of a drug. From a molecular point of view, and in the case of GPCRs, this desensitisation takes place initially when the receptor is phosphorylated and, as a result of this and enhanced interaction with an arrestin, interaction with G-protein is interrupted. The desensitisation of a GPCR is designated as homologous or heterologous dependent on the nature of the protein kinase involved in the phosphorylation event(s) and whether the effect is manifest only on the receptor activated by the agonist [6]. Homologous desensitisation generally occurs when the receptor is activated by a specific agonist and a GRK is involved, whereas heterologous desensitisation refers to the participation of other kinases, e.g. Protein Kinase A or Protein Kinase C, that are activated by specific interacellular secondary messengers, not necessarily generated by the same receptor that becomes phosphorylated. In addition, down-regulation of the total cellular content of a receptor following endocytosis can be considered as another, longer term, mechanism of generating desensitisation. Equivalently, the process of recycling receptor-containing endosomes to the plasma membrane, where the receptor can be activated again by agonist, is known as resensitisation. Therefore, the concept of desensitisation in reference to receptor function may be equivalent to tolerance at the physiological level.

Morphine is distinct when compared to many other agonist drugs that bind to and activate the mu opioid receptor because of a noted accentuated capacity to generate tolerance and dependence when used in long-term treatments. The molecular and cellular basis of this particular feature has been the object of many studies during the last decade, which have

resulted in a plethora of concepts and hypotheses, and remains a matter of considerable conjecture and debate [8-13]. In this review we compile information on several of the approaches and studies that have provided some insight in the basis of this characteristic pharmacological event.

The cloning of cDNA encoding for species orthologues of the mu and other opioid receptors in the early 1990s [1] permitted these receptors to be expressed either transiently or stably in heterologous systems, including transformed mammalian cell lines. As noted earlier, such experimental cell models have been extremely useful to complete the pharmacological characterisation, in terms of drug selectivity as well as agonist efficacies and potencies, of each opioid receptor subtype when expressed individually. Furthermore, such experimental models have allowed genetically modified receptors, for example containing a short amino acid sequence as an epitope that is recognised specifically by an antibody, to be expressed and studied. The use of such epitope-tagged receptors has provided means to employ immunocytochemical and biochemical techniques to facilitate the cellular and molecular studies of these GPCRs. Using such approaches, it was initially described that the rat mu opioid receptor, when stably expressed in HEK293 cells, does not internalise upon treatment with morphine, unlike the effects of enkephalin analogues such as [D-Ala$^2$, N-MePhe$^4$, Gly-ol$^5$]-enkephalin (DAMGO) [14]. Similarly, it was observed that the alkaloid etorphine and enkephalin peptides promoted rapid endocytosis of the murine mu opioid and delta opioid receptors expressed in HEK293 cells, whereas morphine did not facilitate the internalisation of these opioid receptors, even at concentrations that strongly inhibited adenylyl cyclase activity [15]. These reports were the first to demonstrate that rapid internalisation of the mu opioid receptor is facilitated by agonist activation, as had been described earlier for the $\beta_2$-adrenoceptor. However, occupancy and activation of the mu opioid receptor by certain

agonists, such as morphine, was insufficient to trigger the endocytic process. This feature of morphine seems not to be related to its chemical class because other alkaloid opioids such as etorphine and methadone do promote mu opioid receptor internalisation [15]. This agonist-dependency of mu opioid receptor internalisation is not a feature limited to heterologous expression systems. Simultaneous studies employing guinea pig myenteric motor neurons reported the first demonstration of rapid and agonist-selective endocytosis in cells that express mu opioid receptors endogenously. As in HEK293 cells, morphine, unlike etorphine, was completely ineffective in inducing mu opioid receptor internalisation [16]. Since these initial investigations, subsequent research on the inability of morphine to initiate internalisation of the mu opioid receptor has been focussed predominantly on the following questions: 1) Is there a correlation between agonist efficacy to activate the mu opioid receptor and capacity to facilitate receptor endocytosis? 2) What are the defining elements of the receptor protein structure that makes it reluctant to internalise in response to morphine? 3) Which proteins of the GPCR signalling machinery participate in mu receptor endocytosis and how might they facilitate endocytosis to morphine? 4) Is there any relationship between the lack of mu receptor internalisation and the development of tolerance and dependence after chronic morphine treatment? These issues will be considered in turn.

**1. Agonist efficacy and mu opioid receptor endocytosis**

Mu opioid receptors exert their actions by coupling preferentially to pertussis toxin-sensitive, heterotrimeric $G_i/G_o$ G proteins. After their dissociation, the Gα and Gβγ protein subunits regulate distinct effector systems, resulting in the inhibition of adenylyl cyclases, inhibition of voltage-gated calcium channels, stimulation of G protein-activated inwardly rectifying potassium channels (GIRKs) and stimulation of phospholipase Cβ (PLCβ) [1]. The initial characterisation of mu opioid receptors expressed in HEK293 cells [14, 15] showed that

effective inhibition of forskolin-stimulated adenylyl cyclase activity by morphine was not accompanied by receptor internalisation. These results led to the hypothesis that cell signalling and the endocytosis of mu opioid receptors in response to morphine were two independent processes. Similarly, observations obtained with a mutant of the rat mu opioid receptor, in which much of the intracellular C-terminal tail was truncated prior to stable expression in HEK293 cells [17] indicated that this form of the receptor was able to internalise constitutively (i.e. in the absence of an agonist ligand) and to recycle. However, this was not associated with G protein activation as no increase in the binding of the nucleotide [$^{35}$S]GTPγS was produced. A further, more comprehensive, study was performed in both living animals and in HEK293 cells stably expressing the mu opioid receptor [18]. Brain tissue from animals treated with effective analgesic doses of etorphine and morphine were analysed by microscopy and specific antibodies against mu opioid receptors. Receptors in neuronal cells from animals treated with etorphine were distributed mainly in intracellular pools, whereas in cells from animals treated with morphine the receptor was not internalised. In addition, the same authors assessed a range of different opioid drugs used in clinical practise in a cell line stably expressing the mouse mu opioid receptor and examined internalisation and adenylyl cyclase inhibition. They observed that effects on internalisation of individual ligands were not correlated with their potencies for receptor activation [18]. Another study performed in cultures of rat hippocampal neurons expressing a transfected, modified version of mu opioid receptor tagged at the C-terminus with the fluorescent protein YFP compared the internalisation promoted by three different agonists, DAMGO, methadone and morphine, with their capacity to induce tolerance in blocking inhibitory transmission and inhibiting voltage gated calcium channels [19]. DAMGO and methadone facilitated the internalisation of this receptor construct in cultured neurons, whereas morphine produced internalisation much more slowly, even after over-expressing β-arrestin-2 in the same cells.

By contrast, the three opioid agonists were equi-effective in inducing tolerance, demonstrating that morphine-induced tolerance in this experimental model was not necessarily accompanied by receptor endocytosis. Other investigations performed in AtT20 cells stably expressing the mouse mu opioid receptor compared DAMGO, morphine, methadone and pentazocine in regard of their efficacy to activate G proteins by monitoring inhibition of calcium channel currents and in their production of rapid, homologous desensitisation to inhibit these calcium channels. In addition, these functional parameters were compared with the ability of the agonists to promote receptor internalisation [20]. A similar rank order of these agonists was observed for their relative efficacies to inhibit calcium channel currents and to generate rapid desensitisation. However, the rank order for the same agonists for internalising the receptor was different. In this experimental model, the efficacy of opioids to produce activation of G proteins and rapid desensitisation was distinct from their capacity to internalise mu opioid receptors. Another important result from this study was that morphine, despite not internalising mu opioid receptors, produced rapid homologous desensitisation as assessed via inhibition of calcium channel currents. This dis-association between the capacity of opioid agonists to desensitise and internalise mu opioid receptor has not always been observed, however. Work conducted in rat locus coeruleus neurons and in HEK293 cells stably expressing rat mu opioid receptors resulted in opposite observations and conclusions [21]. Herein, activation of potassium channels was assessed for either peptide or alkaloid opioid agonists. The compounds presented distinct rank-order when comparing the magnitude of hyperpolarisation and the ability to cause homologous desensitisation. Moreover, the capacity of these agonists to cause receptor endocytosis in HEK293 cells correlated with the degree of desensitisation in locus coeruleus, suggesting that these processes might be linked and that agonist efficacy was not a predictor of the capacity of a particular agonist to cause mu opioid receptor desensitisation and/or internalisation. The

basis of the discrepancies between these two reports remain unclear but may be due to the differences in the experimental models used, in terms of the functional assay considered (activation of GIRKs versus inhibition of calcium channel currents), the host cell lines (HEK293 versus AtT20 cells) or the mu opioid receptor orthologue studied (rat versus mouse). Further investigations conducted in AtT20 cells expressing a GFP-tagged version of rat mu opioid receptor demonstrated that receptor uncoupling from G proteins is a GRK-dependent process and requires the threonine residue at position 180 [22]. Substitution of threonine 180 by alanine, however, did not inhibit receptor internalisation, suggesting that endocytosis was controlled by a non GRK-dependent mechanism in AtT20 cells. There is further evidence supporting the hypothesis that mu opioid receptor rapid desensitisation and internalisation are independent processes. For example, in a study performed in primary cultured neurons from mouse locus coeruleus, where fluorescent peptides were used to assess mu opioid receptor activation, desensitisation and internalisation, it was observed that desensitisation and recovery were not altered after blocking receptor internalisation using concavalin A [23].

One of the most elegant demonstrations of the lack of association between agonist efficacy and capacity to induce receptor endocytosis is the experiments reported by Whistler and collaborators using a chimeric mutant of the mu opioid receptor [24]. These studies were performed in HEK293 cells stably expressing either the wild type murine mu opioid receptor or a mu-delta opioid receptor chimera. This receptor mutant, described initially by Afify and collaborators [25], was generated by exchanging the C-terminal tail between mu and delta opioid receptors. Morphine, unlike other alkaloid agonists and enkephalin analogues, failed to promote either mu or delta opioid receptor endocytosis. However, the chimeric form of the mu opioid receptor containing the C-terminus of the delta opioid receptor, whilst conserving the pharmacological profile of morphine in terms of potency and efficacy, demonstrated

strong internalisation upon exposure to morphine. After transient expression of GIRK channels in the same cells, different agonists were assessed for stimulation of potassium currents in parallel with internalisation experiments, explored using both immunocytochemistry and a biotinylation protection assay. Morphine and DAMGO were equi-effective in stimulating potassium currents through either mu or mu-delta opioid receptors whereas, as described earlier, morphine failed to internalise mu opioid receptors. These studies indicate a key role for the receptor C-terminal tail in defining internalisation (see also section 2) but not the relative functional activity of the agonists. Similar internalisation data were generated in cultured neurons transfected with the same chimeric receptor [24], thus excluding the possibility of artifactual consequences reflecting the use of HEK293 cells. Although it has been suggested that the poor capacity in many settings of morphine to cause internalisation of the mu receptor might simply reflect the partial agonist nature of this ligand, this is clearly too simple a view to sustain further support.

This lack of correlation between agonist activity/efficacy and capacity to promote receptor endocytosis inspired Whistler and colleagues to propose the RAVE hypothesis [24]. RAVE is the acronym for Receptor Activity Versus Endocytosis, and is a ratio that considers for a particular drug agonist efficacy in relation to its capability to induce receptor endocytosis. For instance, if we consider DAMGO and morphine acting at the mu opioid receptor, both agonists display similar efficacy to stimulate signalling processes in many settings. However, the facilitation of receptor endocytosis by DAMGO is much more extensive than morphine. This defines that the "RAVE index" for DAMGO is lower than for morphine. The same is true for other alkaloid agonists that promote mu receptor internalisation, such as methadone and etorphine. Mu opioid receptors, as many other GPCRs, generally recycle to the plasma membrane after endocytosis [7], where they will be ready again to be activated by the agonist. The absence of receptor internalisation after agonist binding might, therefore, be anticipated

to cause a deficiency in receptor desensitisation-resensitisation processes and, as a result, aberrant continuous signalling, resulting in extensive and diverse cellular alterations. The development of tolerance and dependence observed for some opioid drugs, such as morphine, following chronic treatment could be the physiological consequence of these cellular alterations. According to this hypothesis, it could be predicted that opioid agonists presenting a high "RAVE index" would generate stronger levels of tolerance and dependence than those with a lower ratio and several recent investigations have attempted to validate this hypothesis in physiological models of opioid analgesia (see section 4).

**2. Key elements of mu opioid receptor structure implicated in endocytosis**

As detailed earlier [24], it appears that the C-terminal tail of the mu receptor is a key component of its primary structure involved in receptor endocytosis. Initial experiments conducted in HEK293 cells described a mutant form of the rat mu receptor lacking most of the intracellular C-terminal tail that displayed constitutive internalisation and recycling [17]. Splice variation in the C-terminal tail of mu receptors is common, and the range and extent of variation is species dependent [26-28]. Characterisation in HEK293 cells of two splice isoforms of the rat mu receptor that vary only in the length of the C-terminal tail, indicated that the shorter isoform desensitised at a slower rate and resensitised more rapidly than the longer isoform when inhibition of intracellular levels of cAMP after stimulation with DAMGO was assessed [29]. Receptor internalisation studies also revealed that the shorter isoform endocytosed faster and recycled more rapidly than the longer isoform [29]. Similar studies have been performed with C-terminal splice variants of the mouse mu receptor stably expressed in HEK293 cells [30]. The MOR1 and MOR1C isoforms were phosphorylated, internalised and down-regulated when stimulated with DAMGO but not in response to morphine. By contrast, the MOR1D and MOR1E splice variants showed similar levels of

phosphorylation, internalisation and down-regulation in response to both DAMGO and morphine whilst functional assays measuring inhibition of intracellular cAMP accumulation showed the same desensitisation and resensitisation ratio for DAMGO for each of the four variants, whilst MOR1 and MOR1C desensitised more rapidly and did not resensitise after treatment with morphine. Splice variants of the rat mu receptor have been quantified by RT-PCR in different brain areas [26], and the three most abundant, i.e. MOR1, MOR1A and MOR1B, were expressed in HEK293 cells together with GIRK channels to assess their regulation. Morphine induced rapid desensitisation for each of these three isoforms whereas DAMGO produced a slower rate of desensitisation via MOR1B. Furthermore, over-expression of a dominant negative mutant of GRK2 demonstrated that the rate of desensitisation of MOR1 and MOR1A was independent of the agonist employed, whilst for MOR1B this was not true. Another comprehensive study generated point mutations, individually or in combination, of 12 Ser/Thr residues to Ala in the C-terminal tail of the rat mu receptor [31]. Three of these residues ($Ser^{363}$, $Thr^{370}$ and $Ser^{375}$) were identified as phosphorylation sites and the internalisation of these mutants in response to DAMGO was explored. The $Ser^{375}$Ala substitution displayed a reduced extent of internalisation, whilst the $Ser^{363}$Ala and $Thr^{370}$Ala substitutions showed significant increases in receptor internalisation kinetics. Although all of the above studies were performed in heterologous expression systems, neurons from mice treated intracerebroventricularly with DAMGO and morphine [32] have been examined by microscopy using antibodies reportedly selective for two MOP receptor splice variants (MOR-1 and MOR-1C). The MOR-1 variant internalised in response to DAMGO but not to morphine whereas MOR-1C internalised in response to both ligands. Regardless of whether receptor desensitisation and internalisation are correlated or independent processes, the C-terminal intracellular domain is considered to be the region of receptor structure essential for these cellular events. This is not surprising as the receptor C-

terminus is a domain where many amino acids susceptible to be phosphorylated by different protein kinases are located. Particularly for the rat mu receptor, $Ser^{375}$ has been described as a key residue homologously phosphorylated after receptor stimulation with either DAMGO or morphine [33]. Interestingly, DAMGO-stimulated receptors dephosphorylated and then resensitised more rapidly, compared to morphine-occupied receptors, which persisted in the plasma membrane in the phosphorylated state for a longer period of time. Moreover, although not absolutely required for some specific receptors [6], the interaction of phosphorylated GPCRs with arrestins to initiate the endocytic process often occurs through the receptor C-terminal intracellular domain [34]. Nevertheless, a recent report [35] describes mu receptor variants outwith the C-terminus that display differences in signalling and internalisation. These modifications are naturally occurring mutations or single nucleotide polymorphisms (SNP) of the human mu receptor. Two different mu receptors SNPs, containing either $Leu^{85}Ile$ or $Arg^{181}Cys$ variants, were expressed in HEK293 cells and characterised by functional and internalisation assays. Both variants displayed distinct behaviours from the wild type receptor. The $Leu^{85}Ile$ mu receptor was endocytosed in response to morphine as robustly as with DAMGO, whereas the $Arg^{181}Cys$ variant lacked both signalling and internalisation in response to DAMGO.

## 3. The role of other proteins in mu receptor endocytosis in response to opioid agonists

There are many proteins of the cell endocytic machinery involved in the internalisation of GPCRs after their stimulation by an agonist [6]. As noted earlier, following agonist binding, conformational changes in the receptor promote interaction with GRKs. There are seven GRK subtypes, differentiated by their cell type expression as well as their intracellular distribution [36]. Some, such as GRK2 and GRK3, are cytosolic proteins that migrate to the plasma membrane upon GPCR activation. GRK-mediated phosphorylation and subsequent

interactions with arrestins abrogates receptor coupling to G proteins and constitutes, at the molecular and cellular level, the desensitisation process of GPCRs [37]. In addition, arrestins trigger endocytosis by recruiting other proteins, such as clathrin and AP-2, which facilitate the formation of clathrin-coated vesicles. Finally, the GTPase dynamin completes the formation of vesicles and hence sequesters receptors inside endosomes [7].

Initial experiments in HEK293 cells expressing the murine mu receptor demonstrated that internalisation promoted by etorphine and DAMGO was a dynamin-dependent process [38]. As noted earlier, morphine failed to induce MOP receptor endocytosis. However, following over-expression of a β-arrestin or GRK2 in these cells, morphine promoted rapid endocytosis of mu receptors. In addition, over-expression of β-arrestin facilitated the uncoupling of mu receptors from G proteins in response to morphine. Similar studies using rat mu receptor, expressed transiently in HEK293 cells, noted that over-expression of GRK2 and β-arrestin-1 enhanced receptor internalisation by etorphine and promoted receptor internalisation by morphine [39]. However, over-expression of β-arrestin-1 did not facilitate morphine-mediated internalisation of mu receptor whereas over-expression of GRK2 or β-arrestin 1 plus GRK2 facilitated receptor endocytosis to morphine. Additionally, over-expression of GRK2 enhanced mu receptor phosphorylation in response to morphine. Equivalent results were observed in the neuronal cell model, NG108-15, transfected to transiently express rat mu receptor tagged with the fluorescent protein eGFP [40]. By contrast, over-expression of phosducin, a regulator of G protein signalling, blocked the internalisation of mu receptor in response to etorphine, an effect that was reversed by the simultaneous co-expression of phosducin and β-arrestin-1. As in other cell lines, morphine failed to internalise mu receptors expressed in NG108-15 cells. However, in agreement with the above, over-expression of β-arrestin-1 facilitated mu receptor internalisation by morphine in these cells. In more native systems, including enteric neurons of the guinea pig [41], it was observed that endocytosis of

endogenous mu receptors by DAMGO and etorphine was clathrin-mediated and that the receptor recycled following endosomal acidification. Following recycling, the mu receptors were again functional.

Overall, it is reasonable to conclude that mu receptor internalisation is dynamin-dependent and both GRKs and arrestins are cytosolic protein determinants of the endocytic process. Nevertheless, it is important to note that, in the majority of cases, conclusions have been derived via either over-expression of these proteins or of equivalent dominant negative mutants. Further studies, using for example interference mRNA technologies to silence the endogenous expression of these proteins, would assist in corroborating their role in mu receptor endocytosis. An important issue to consider is why morphine does not cause internalisation of mu receptors in the same cells in which other opioid agonists do and why this deficiency in receptor internalisation in response to morphine may be compensated by overexpression of GRKs and/or arrestins. One possible reason is that the conformational change promoted in the mu receptor by morphine is not sufficient to activate and recruit GRKs and arrestins in the surrounding plasma membrane area where the stimulated receptors are located. Over-expression of these cytosolic proteins would provide a higher probability of their interaction with morphine-occupied mu receptors. In such a situation, morphine would be expected to produce a specific homologous desensitisation of mu receptors because it would be dependent on the level of GRK and/or arrestin expressions in cells. However, this is not the case for all opioid agonists that fail to cause internalisation of mu receptor. It has been reported recently that a chemical derivate of salvinorin A named herkinorin is a novel, selective agonist for the mu receptor [42]. Experiments using the mouse mu receptor stably expressed in HEK293 cells resulted in an absence of both receptor internalisation and recruitment of β-arrestin 2 to the plasma membrane after stimulation with herkinorin. Over-expression of GRK2 facilitated mu receptor endocytosis and β-arrestin 2 mobilisation by

morphine, but herkinorin still failed to produce substantial changes in the trafficking properties of the receptor. This did not reflect the lack of agonist activity of herkinorin because ERK1/2 phosphorylation experiments resulted in similar maximal responses to DAMGO, morphine and herkinorin. The results with herkinorin suggest that hypotheses to explain the absence of mu receptor internalisation by morphine cannot be directly extrapolated to other opioid agonists. Nevertheless, other investigations have corroborated this hypothesis for morphine in more native models. For example in nucleus accumbens transfected by injection of a viral gene transfer vector encoding either the wild type mu receptor or the chimeric mu-delta receptor [43]. Interestingly, there can also be differences in effects of morphine on endogenous mu receptors in distinct compartments of the same cell. In cell bodies of the nucleus accumbens morphine failed to internalise mu receptors, whereas in neuronal processes morphine produced a rapid and prominent effect on the distribution of mu receptor [43]. This may reflect heterogeneous intracellular distribution of other proteins involved in the endocytic process, such as GRKs and arrestins, but further work is required to assess this directly. In a similar study by the same team using primary cultures of rat striatal neurons [44], morphine induced the redistribution of both endogenous and recombinant mu receptors. Morphine and DAMGO internalised the receptors to the same extent and in both cases these effects were inhibited by the over-expression of a dominant negative mutant of β-arrestin 2. Although endogenous expression levels of β-arrestin1/2, as assessed by immunoblotting, were equivalent in cultured striatal neurons and HEK293 cells, substantial differences were found in GRK2 expression, with GRK2 being expressed at substantially higher levels in striatal neurons than in HEK293 cells.

Regulators of G-protein signalling (RGS) is other family of proteins involved in GPCR function, including mu opioid receptors. Receptor signalling processes are modulated negatively by RGS proteins as these facilitate the hydrolysis of GTP associated with Gα

subunits and therefore interrupt activation of different cellular effectors. RGS9-2 is a brain-specific splice variant of the RGS9 subtype expressed in neural regions associated with nociception and where mu opioid receptors are expressed. RGS9-2 is particularly highly enriched in striatum and, although expressed at substantially lower levels in PAG and spinal cord, is present [45]. *In vitro* experiments performed with mouse PAG membranes showed that mu opioid receptor could be co-immunoprecipitated with α-subunits of $G_{i/o/z/q/11}$ proteins, $G\beta_{1/2}$ subunits as well as both RGS9-2 and its partner protein $G\beta_5$ [46]. These interactions were modulated by morphine since 30 minutes and 3 hours after drug administration the co-immunoprecipitation of mu receptor-Gα subunit was reduced up to 50% whilst the interaction Gα-RGS9-2 was conversely increased. Further studies have examined RGS9-2 involvement in mu opioid receptor endocytosis and signalling [47]. Mouse embryonic fibroblasts (MEF) from both wild type and RGS9-2 knock out animals were transiently transfected with mu receptor and receptor internalisation in response to morphine was explored. Receptors expressed in wild type MEF were internalised after 30 minutes of morphine treatment whereas in RGS-2 KO MEF receptor endocytosis occurred much more rapidly. Equivalent results were obtained in PC12 cells transiently expressing RGS9-2 and mu opioid receptors, where RGS9-2 over-expression delayed DAMGO-induced receptor internalisation. Furthermore, and in agreement with previous observations, morphine treatment enhanced interactions between mu receptor and RGS9-2 assessed by co-immunoprecipitation in PC12 cells and promoted the association of RGS9-2 with β-arrestin 2. Moreover, over-expression of RGS9-2 prevented phosphorylation of extracellular signal-regulated kinase (ERK) mediated by the mu opioid receptor. Overall, these results indicated that RGS9-2 negatively modulates mu opioid receptor signalling and the rate of receptor endocytosis [47]. Some aspects of these studies are surprising as, in general, the internalisation of many GPCRs does not require signal generation or even the expression of G protein. Thus, further work is required to define

if the reported effects of RGS9-2 in these studies is due to the regulation of the G protein GTPase cycle or might reflect that many RGS protein also have direct interactions with other signalling proteins, and in some cases, can interact directly with GPCRs. RGS14 is another RGS subtype that has been linked to mu opioid receptor function. Silencing of RGS14 expression in mouse PAG neurons enhanced mu receptor phosphorylation at $Ser^{375}$ in response to morphine [48]. Subsequently the receptors were internalised and recycled to the plasma membrane. Additionally, RGS14 prevented GRK-mediated phosphorylation of mu receptors activated by morphine and, consequently receptor endocytosis mediated by β-arrestin 2.

There are other proteins, such as phospholipase D2 (PLD2), that are able to modulate the endocytic process by interacting directly with mu receptors [49]. HEK293 cells stably expressing the rat MOR1 mu receptor and human PLD2 showed receptor internalisation accompanied by an increase in PLD2 activity after treatment with DAMGO. On the other hand, morphine failed either to induce PLD2 activation or to produce receptor endocytosis. However, phorbol ester activation of PLD2 facilitated the internalisation of mu receptor by both DAMGO and morphine. Furthermore, inhibition of PLD2 by 1-butanol or PLD2 dominant-negative mutants prevented agonist-mediated endocytosis of mu receptors, defining the participation of PLD2 in this process.

Not surprisingly, other GPCRs are able to modulate mu opioid receptor endocytosis. The first demonstration of a receptor facilitating mu receptor internalisation reflected a potential role for mu opioid receptor dimerisation or oligomerisation [50]. These authors demonstrated, firstly in HEK293 cells, that the chimeric mu-delta receptor construct previously described [24] promoted intracellular accumulation of wild type mu receptor after treatment with morphine when the two forms were co-expressed. Furthermore, equivalent results were obtained in cultured neurons transfected with the same mu opioid receptor constructs. Most

interestingly, sub-effective doses of DAMGO, i.e. doses that did not directly produce mu opioid receptor internalisation, promoted wild type mu receptor internalisation in response by morphine, again suggesting a potential involvement of receptor dimerisation/oligomerisation in this process although the molecular contribution of DAMGO to this process remains uncertain. These results were extended in studies performed in living animals by exploring the development of tolerance to chronic treatment with morphine. Sub-effective doses of DAMGO in combination with morphine resulted in a more attenuated development of tolerance than treatments using morphine alone. In addition, examination by immuno-histochemistry of brain cells revealed that animals treated with DAMGO plus morphine showed mu opioid receptors in intracellular compartments whilst the animals treated with morphine alone did not. Recently, modulation of mu opioid receptor internalisation by heteromerisation with other GPCRs, including the metabotropic mGluR5 has also been described [51]. When rat mu opioid and mGluR5 receptors were co-expressed in HEK293 cells, the non-competitive mGluR5 antagonist MPEP decreased DAMGO-induced mu receptor phosphorylation, internalisation and desensitisation, whereas non-selective, competitive mGluR5 agonists or antagonists had no effect on this process. Moreover, immunoprecipitation experiments demonstrated an increase in the extent of interaction between the receptors in samples from cells treated with MPEP. Collectively these results suggest a possible allosteric modulation of mGluR5 that affects agonist-induced MOP receptor signalling and regulation via receptor-receptor interactions [52]. Other studies report modulation of mu opioid receptor endocytosis by other GPCRs via mechanisms that do not involve receptor heteromerisation. This is the case of pharmacological interactions between co-expressed human serotonin 5-HT$_{2A}$ and mu opioid receptors [53]. By generating cell lines in which the 5-HT$_{2A}$ receptor was located at an inducible locus, whilst also constitutively expressing mu receptors, these authors demonstrated that treatment of cells expressing only

the mu receptor did not result in desensitisation, internalisation or down-regulation whilst, as anticipated, DAMGO treatment produced robust effects on each of these parameters. However, after induction of 5-$HT_{2A}$ receptor expression, treatment of the cells with a combination of morphine plus serotonin, but not serotonin alone, caused MOP receptor internalisation to a similar extent as observed for DAMGO. The use of a 5-$HT_{2A}$ receptor antagonist (mianserin), a $G_q/_{11}$ uncoupling compound (YM254890) or a Protein Kinase C inhibitor (Ro318220), inhibited internalisation of the mu receptor promoted by the combination of morphine plus serotonin, demonstrating the involvement of 5-$HT_{2A}$ receptor signalling in this process. Although it could not be absolutely discarded, heteromerisation between 5-$HT_{2A}$ and mu receptors as a basis to explain these observations of facilitation was considered unlikely because the cellular distribution of the two receptors did not coincide; mu receptor was expressed predominantly at the plasma membrane whereas 5-$HT_{2A}$ receptors underwent constitutive internalisation and recycling and, at steady-state, were present predominantly in recycling endocytic vesicles [53]. Another recent report has described the regulation of mu receptor endocytosis and desensitisation by NK1 neurokinin receptors [54]. Mouse mu opioid and rat neurokinin 1 receptors were transiently expressed in both primary striatal and amygdala neurons and in the neuroblastoma 2A cell line. Each of these systems is unusual because morphine promotes endocytosis of mu receptors expressed alone. Following co-expression of mu and neurokinin 1 receptors, the activation of the neurokinin 1 receptor population with substance P inhibited mu receptor desensitisation and endocytosis. In addition, this heterologous pairing also resulted in a functionally significant attenuation of morphine-induced desensitisation of mu receptor signalling via adenylyl cyclases. Studies involving over-expression of β-arrestin 2-GFP and a neurokinin 1 receptor mutant unable to interact with β-arrestin2 indicated that the negative modulation of mu receptor endocytosis by

the neurokinin 1 receptor might be achieved by sequestering arrestins in endosomes and hence limited their availability to interact with the mu receptor [54].

## 4. Endocytosis of mu opioid receptors and development of morphine tolerance and dependence in living animals

Chronic treatment of experimental animals with morphine results in development of tolerance and dependence to this ligand. Morphine tolerance is manifest as the lost of effectiveness in nociception assays during the course of the sustained administration of an initially effective dose of the drug. In addition, chronic exposure to morphine also results in a dependence that is defined by the appearance of physical symptomatology after the abrupt withdrawal of the drug. Although both processes are the result of morphine action and consequently the result of mu opioid receptor activation, the cellular and molecular basis of each event is different. In physiological terms, tolerance may be considered the result of deficient receptor resensitisation at the cellular level, whereas dependence is more related to changes that occur in diverse elements of the cell signalling machinery involved in receptor activation. The mu opioid receptor couples preferentially to Gαo/i Gprotein subunits and these inhibit the activity of adenylyl cyclases and hence reduce intracellular levels of cAMP. It has been noted for many receptors that couple to inhibitory Gαo/i G proteins that persistent receptor stimulation results in a paradoxical enhancement of adenylyl cyclase activity that increases the levels of accumulated cAMP when the action of the inhibitory receptor is terminated [55]. This adenylyl cyclase superactivation or supersensitisation is considered to be an adaptive cellular response to compensate for chronic inhibitory input. Adenylyl cyclase superactivation has been observed routinely after chronic stimulation of a number of GPCRs (for review see [55]), and in the case of morphine and the mu opioid receptor it is considered as one of the cellular hallmarks of dependence and withdrawal. As mentioned earlier, the replacement of

the C-terminal tail of the mu opioid receptor with the C-terminal tail of the delta opioid receptor generates a chimeric receptor that is endocytosed in response to morphine [24]. Additional studies conducted by the same authors [56], evaluated the induction of adenylyl cyclase superactivation by mu opioid receptor mutants expressed in HEK293 cells following chronic treatment with morphine. Mutant receptors that internalised in response to morphine generated a lower cAMP superactivation than the wild-type receptor. Conversely, a mutant form of the receptor that failed to induce GRK-mediated phosphorylation, arrestin recruitment or endocytosis in response to methadone resulted in a higher cAMP superactivation than the wild-type receptor following chronic treatment with this drug. Therefore, there appears to be a negative correlation between agonist facilitation of mu opioid receptor endocytosis and the generation of adenylyl cyclase superactivation. Nevertheless, other reports point to opposite conclusions. Studies with the rat mu opioid receptor expressed in HEK293 cells in a inducible manner [57] described that the magnitude of cAMP superactivation was dependent on mu receptor density, regardless the agonist used, and experiments using dynamin dominant-negative mutants indicated that this process was not dependent on receptor internalisation. Furthermore, the specifics of cell surface location of mu opioid receptors may to be a determinant for adenylyl cyclase superactivation because after long term agonist treatments the mu receptor was reported to be located in lipid rafts. Similarly, experiments conducted in CHO cells stably expressing the human mu opioid receptor compared DAMGO (internalising ligand) with herkinorin (non-internalising ligand) in their ability to produce tolerance, desensitisation and up-regulation of the cAMP system [58]. That both agonists were equivalent in modulating these pharmacological parameters appears to exclude a direct relationship between mu receptor internalisation and the generation of cAMP superactivation. Overall, there is still considerable debate and controversy as to whether the production of adenylyl cyclase superactivation is a feature that distinguishes mu opioid agonists that are

poorly able to promote receptor internalisation. Many of the key findings relate to heterologous expression systems by means *in vitro* experimental approaches. However, numerous investigations have been conducted to attempt to elucidate whether conditions that facilitate mu opioid receptor endocytosis by morphine in cellular experimental models may be translated to a reduction of the development of morphine tolerance and dependence in *in vivo* physiological models. In this regard, experiments in rats to assess the development of tolerance using equi-effective doses of three different opioid agonists showed that etonitazene, an agonist that promotes mu opioid receptor internalisation in HEK293 cells, developed less tolerance and lower sensitisation to locomotor stimulants than morphine and buprenorphine, agonists that fail to promote receptor internalisation in heterologous expression systems [59]. Other investigations validated in living animals results obtained concurrently in heterologous expression systems. For example, the demonstration, as noted earlier, that mu opioid receptor endocytosis may be facilitated by receptor homo-oligomerisation. Sub-effective doses of DAMGO in combination with morphine also inhibit the development of tolerance following chronic treatments as assessed in tail-flick nociception studies. This reduction in tolerance was accompanied by mu opioid receptor internalisation in neuronal cells [50]. These studies have been extended. The combination of morphine with a small dose of methadone provided a "pharmacological cocktail" that preserved the full analgesic properties of morphine but were reported not to generate tolerance and dependence [60]. Initial experiments in HEK293 cells demonstrated that the mu opioid receptor internalised when treated with this drug combination and that this was accompanied by a reduction in cAMP superactivation compared to cells treated with morphine alone. Animals treated chronically with this "cocktail" presented considerably less tolerance in tail-flick nociception assays together with a predominantly intracellular mu receptor distribution in neurons. In addition, this reduction in tolerance and dependence was associated with

prevention of the up-regulation of NMDA receptor subunits in some brain areas. Similar findings were obtained in experiments conducted in rats injected intrathecally with opioids and assessed for nociception and that subsequently used their spinal cords for immunohistological analysis [61]. The co-administration of sub-analgesic doses of either DAMGO or fentanyl with morphine resulted in potentiation of morphine analgesic effects and facilitated mu opioid receptor internalisation. Another significant validation in living animals of previous *in vitro* results was the use of genetically engineered mice expressing the mu-delta opioid receptor chimera described earlier [24]. These animals were treated chronically with either morphine or methadone and then examined for tolerance to antinociceptive responses in the hot-plate latency test and for naloxone precipitated withdrawal in parallel with wild-type littermates [62]. Methadone treatment resulted in no significant differences between the genotypes. However, the knock-in mice showed substantially reduced antinociceptive tolerance to morphine, together with reduced physical dependence assessed by scoring standard withdrawal behaviours including jumping, "wet dog" shakes, paw licks and paw tremors. Further studies employing these knock-in mice have explored a number of changes at the physiological level that have been implicated previously in the development of morphine tolerance and dependence, including adenylyl cyclase superactivation, alterations in NMDA and glucocorticoid receptor levels and c-fos gene expression [63]. Chronic morphine treatment of wild type mice resulted in brain region-dependent superactivation of cAMP (striatum), reduction of NMDA subunits (NR1, NR2A and NR2B) in the PAG and up-regulation of NR2B in thalamus, increase in glucocorticoid receptor protein level in PAG and thalamus and increase of c-fos expression in striatum. Conversely, none of these parameters were altered in the knock-in mice following morphine treatment. As mentioned earlier, *in vitro* experiments performed in different cell models defined involvement of proteins including the arrestins, GRKs and RGS in receptor endocytosis. The ablation of expression of

several of these proteins in genetic modified animals has been achieved. Such knock-out (KO) animals have become an essential group of experimental tools to elucidate the possible participation of arrestin, GRKs and RGS in physiological processes following mu opioid receptor activation *in vivo*. For example, mice lacking expression of β-arrestin 2 exhibit a remarkable potentiation and prolongation of the analgesic effect of morphine, consistent with the concept that mu opioid receptor desensitisation was impaired in these animals [64]. This is despite radioligand binding assays showing no difference to wild type littermates in [$^{3}$H]naloxone binding sites in all the brain regions examined. By contrast, DAMGO stimulated greater binding of [$^{35}$S]GTPγS in membranes derived from β-arrestin 2 KO mice than those derived from wild-type littermates. Additional studies found that desensitisation of mu opioid receptors failed to occur after chronic morphine treatment of β-arrestin 2 KO mice [65] and these animals failed to develop antinociceptive tolerance. Nevertheless, chronic morphine-induced up regulation of adenylyl cyclase activity was not prevented. Interestingly, [$^{35}$S]GTPγS binding assays performed in membranes from brain of wild-type mice pre-treated chronically with morphine showed a reduction of DAMGO stimulation, whereas this was not observed in membranes from the β-arrestin 2 KO mice. Other alterations observed in β-arrestin 2 KO mice were attenuation of both respiratory depression and constipation caused by morphine treatment [66]. Unlike the case for β-arrestin 2, phenotypic characterisation of mice lacking individual GRK subtypes has not provided conclusive results in this regard. Studies conducted with GRK3 KO mice showed no differences in acute antinociceptive responses to either fentanyl or morphine [67]. However, *in vivo* experiments assessing electrophysiological response in hippocampus indicated that tolerance to fentanyl was blocked by GRK3 deletion. Another recent report describes the characterisation of GRK6 KO mice [68]. Over-expression of GRK6 in HEK293 cells transiently expressing mu opioid receptors facilitated morphine-induced β-arrestin 2 recruitment and mu receptor

internalisation, whilst acute morphine treatment of GRK6 KO mice induced greater locomotor activation but less constipation than in wild-type litter mates. Nevertheless, thermal antinociception, analgesic tolerance and physical dependence were not affected by ablation of the GRK6 gene. The important discrepancy found in this study between *in vitro* and *in vivo* results confirms the need for caution when interpreting and/or extrapolating observations from heterologous expression systems to physiological models. As mentioned earlier, RGS proteins play an important role in modulating mu opioid receptor signalling and endocytosis. Characterisation of RGS9 KO mice revealed that these animals displayed enhanced behavioural responses to acute and chronic morphine treatment [45]. These alterations included morphine analgesia with delayed tolerance that was accompanied by exacerbated signs of physical dependence and withdrawal [45]. Similarly, *in vivo* knock-down of RGS9-2 expression in mice prevents morphine from altering the association between mu opioid receptors and G-proteins as well as the absence of development of tolerance [46]. As such, these authors concluded that the development of morphine tolerance was caused by the stabilisation and retention of mu receptor-activated Gα subunit complexes by RGS9-2. Further studies have been performed in mice in which levels of RGS4 could be controlled. Systemic injections of morphine induced comparable antinociceptive effects in wild type and RGS4 KO animals in the tail flick test as well as in the first reaction of the hot-plate test. Additionally, no difference between mutant and wild type mice was observed for somatic signs of abstinence to opioids [69]. A recent study employed both constitutive RGS4 KO mice, conditional nucleus accumbens-targeted RGS4 KO mice and mice overexpressing RGS4 in the nucleus accumbens [70]. However, results were difficult to interpret in relation to the actions of morphine since it appeared that in nucleus accumbens RGS4 acted as a negative regulator of morphine reward whereas in locus coeruleus RGS4 opposed the development of physical dependence by morphine. Thus, although RGS4 can act as a positive

modulator of opiate analgesics such as methadone and fentanyl, it may not affect either morphine analgesia or tolerance.

**Conclusions**

Although mu opioid receptors comply with general paradigms established for other GPCRs in terms of receptor desensitisation, internalisation and resensitisation, the initiation of these processes is dependent on the identity of the participating agonist. This has been examined most extensively for morphine, not least because of the widespread use of this drug as a remarkably effective analgesic. In contrast to many other opioid alkaloids and enkephalins, morphine is frequently reported to be unable to promoter effective receptor internalisation despite displaying high agonist efficacy in many (but not all) assays. Because receptor internalisation is believed to be required to allow desensitised GPCRs to resensitise, hypotheses have been developed that suggest that the inability of morphine to promote receptor internalisation is causally linked to the development of tolerance to morphine over time and, potentially, to the symptoms of physical dependence. However, in certain cells and tissues, morphine is able to stimulate mu receptor internalisation from the cell surface, either when used alone or in combination with other receptor ligands (**Table 1**) . **Figure 1** summarises situations in which morphine does promote internalisation of mu receptors expressed in heterologous systems. It is now widely accepted that GPCRs are organised as dimers or higher order oligomers. This structural feature has been promulgated as a means to explain the endocytosis of morphine-occupied mu receptors by sub-effective doses of other opioid agonists (**Figure 1A**). Other possibilities include structural changes in relation to the protomer or the primary structure of the receptor (**Figure 1B**), and such effects may be related to the expression pattern of particular splice variants or, potentially in man, to particular polymorphisms. Among the most compelling sets of observations to correlate the generally

poor ability of morphine to promote mu receptor internalisation with tolerance and/or physical dependence are studies that have employed a chimeric mu-delta receptor in which the C-terminal tail of the mu receptor was replaced with the equivalent region of the delta receptor. Initially in heterologous cells systems, and more recently in transgenic animals, this chimeric receptor has been used to link morphine tolerance with the lack of capacity of the mu receptor to internalise. Even in model systems, alterations in the expression level of other proteins, including GRKs, arrestins and RGS proteins, can have dramatic effects on the morphine-mediated regulation of mu receptor function (**Figure 1C**). Such observations require further analysis in animal models, but once again, transgenic and knock-out lines have begun to illuminate this issue. Finally, activation of signalling cascades by other GPCRs co-expressed with mu opioid receptors may also facilitate their internalisation after morphine activation (**Figure 1D**). If such studies are translated to animal models and validate the link between the capacity of morphine-occupied mu receptors to internalise and the development of tolerance and dependence, they also suggest simple pharmacological strategies to limit the development of tolerance. Certainly, results obtained *in vitro* related to the protein structure of mu receptors (see **Figure 1A and Figure 1B**), have been largely confirmed in living animals; where chronic treatment with morphine that results in receptor internalisation in neural cells is generally accompanied by a substantial reduction of the development of morphine tolerance and dependence, suggesting an association between, but remains far from defining a causal link between, the two pharmacological events. Further results from in *vivo* experiments with animals lacking expression of proteins involved in mu receptor internalisation have so far resulted in uncertain conclusions. Mice devoid of either β-arrestin 2 or RGS9 protein expression develop reduced tolerance to antinociception following chronic treatment with morphine compared to their corresponding wild type littermates. However, these mutant animals do not present any significant alteration in the development of morphine dependence

and withdrawal. The divergence between these physiological consequences of prolonged morphine treatment suggests that they may be independent phenomena. As such, the correlation between mu receptor internalisation and desensitisation/resensitisation events at the cellular level, and the development of physical dependence probably occurs as the result of more complex and interconnected alterations, comprising multiple modifications. Further research needs to be conducted to elucidate the neuronal basis of morphine dependence before extrapolating results from single cell models to living animals.

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**Abbreviations**

GPCR: G-protein coupled receptor; DAMGO: [D-Ala$^{2}$, N-MePhe$^{4}$, Gly-ol$^{5}$]-enkephalin; GTP: Guanosine-5'-triphosphate; GDP: Guanosine-5'-diphosphate; HEK: human embryonic kidney cells; GFP: green fluorescent protein; YFP: yellow fluorescent protein; GRK: G protein-coupled receptor kinase; GIRK: G protein-activated inward rectifier potassium channel; ERK: Extracellular signal-regulated kinase; RGS: Regulator of G protein signalling; PAG: periaqueductal grey; MEF: mouse embryonic fibroblasts; cAMP: cyclic adenosine monophosphate; CHO: chinese hamster ovary cells; NMDA: N-methyl D-aspartate glutamate receptor.

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Actions of Regulator of G Protein Signaling 4 Oppose Morphine Reward and Dependence but Promote Analgesia. *Biol Psychiatry,* **2009**.

**Table 1. Does morphine cause internalization of the mu opioid receptor?**

| Cellular model | Mu receptor | Endocytosis in response to morphine | References |
|---|---|---|---|
| HEK293 | rat, recombinant | NO | [14, 21, 39, 49] |
| HEK293 | mouse, recombinant | NO | [15, 24, 38, 42] |
| HEK293 | mouse MOR1, recombinant | NO | [30] |
| HEK293 | mouse MOR1C, recombinant | NO | [30] |
| HEK293 | mouse MOR1D, recombinant | YES | [30] |
| HEK293 | mouse MOR1E, recombinant | YES | [30] |
| HEK293 | human, recombinant | NO | [35] |
| HEK293 | human, YFP-tagged | NO | [53] |
| AtT20 | mouse, recombinant | NO | [20] |
| AtT20 | rat, recombinant | NO | [22] |
| NG108-15 | rat, GFP-tagged | NO | [40] |
| MEF | mouse, recombinant | YES | [47] |
| Neuroblastoma 2A | mouse, recombinant | YES | [54] |
| Myenteric motor neurons | guinea pig, endogenous | NO | [16] |
| Neurons from parietal cortex layer II | rat, endogenous | NO | [18] |
| Cultures of rat hippocampal neurons | rat, YFP-tagged | low rate | [19] |
| Cultures of rat hippocampal neurons | mouse, recombinant | NO | [24] |
| Neurons from lateral septum | mouse MOR1, endogenous | NO | [32] |
| Neurons from lateral septum | mouse MOR1C, endogenous | YES | [32] |
| Neurons from nucleus accumbens (cell bodies) | rat, endogenous | NO | [43] |
| Neurons from nucleus accumbens (neuronal processes) | rat, endogenous | YES | [43] |
| Cultures of rat striatal neurons | rat, recombinant and endogenous | YES | [44] |
| Neurons from PAG | mouse, endogenous | YES | [48] |
| Cultures of rat striatal neurons | mouse, recombinant | YES | [54] |

| | | | |
|---|---|---|---|
| Cultures of rat amygdala neurons | mouse, recombinant | YES | [54] |

## Figure legend

**Figure 1. Experimental conditions that result in mu opioid receptor internalization in response to morphine**

Although in many situations morphine is unable to cause internalisation of mu opioid receptors to a significant extent**,** this is not an invariant observation. Situations in which morphine is able to promote such internalisation are illustrated.

**A.** Combinations of morphine with sub-effective doses of agonists that at higher concentrations are themselves able to stimulate receptor internalisation. This has been suggested to reflect aspects of mu receptor oligomerisation. **B.** Variations in the receptor C-terminal tail of the mu receptor produced artificially by substitution with a distinct sequence (receptor chimeras) or in naturally occuring splice variants. **C.** Variation in the expression levels of polypeptides implicated in receptor signalling processes including arrestins, GRKs and RGS proteins. **D.** Activation of distinct signalling pathways by other receptors co-expressed in the same cell as mu receptor. See text for further details.

**Figure 1**