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REVIEW

Ligand-directed signalling within the opioid receptor family

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The classic model of GPCR activation proposed that all agonists induce the same active receptor conformation. However, research over the last decade has shown that GPCRs exist in multiple conformations, and that agonists can stabilize different active states. The distinct receptor conformations induced by ligands result in distinct receptor–effector complexes, which produce varying levels of activation or inhibition of subsequent signalling cascades. This concept, referred to as ligand-directed signalling or biased agonism has important biological and therapeutic implications. Opioid receptors are $G_{i/o}$ GPCRs and regulate a number of important physiological functions, including pain, reward, mood, stress, gastrointestinal transport and respiration. A number of *in vitro* studies have shown biased agonism at the three opioid receptors (μ, δ and κ); however, *in vivo* consequences of this phenomenon have only recently been demonstrated. For the μ and δ opioid receptors, the majority of reported ligand selective behavioural effects are observed as differential adaptations to repeated drug administration. In terms of the κ opioid receptor, clear links between ligand-selective signalling events and specific *in vivo* responses have been recently characterized. Drugs for all three receptors are either already used or are being developed for clinical applications. There is clearly a need to better characterize the specific events that occur following agonist stimulation and how these relate to *in vivo* responses. This understanding could eventually lead to the development of tailor-made pharmacotherapies where advantageous drug effects can be selectively targeted over adverse effects.

Abbreviations

ARM390, *N*,*N*-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; BRET, bioluminescence resonance energy transfer; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; DMR, dynamic mass redistribution; DPDPE, [D-Pen^{2,5}]enkephalin, [D-Pen^2,D-Pen^5]enkephalin; ESCRT, endosomal sorting complex required for transport; FRAP, fluorescence recovery after photobleaching; GRKs, GPCR kinases; JDTic, (3*R*)-7-hydroxy-*N*-((1*S*)-1-([(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]methyl)-2-methylpropyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; norBNI, norbinaltorphimine; SNC80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethyl benzamide; U50,488, trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide

Introduction

GPCRs are the most abundant receptor class in the human genome (Lagerstrom and Schioth, 2008), and as such, these receptors regulate diverse biological functions. Given their importance in physiological processes, they are the most commonly targeted receptor class for pharmacological therapies (Ma and Zemmel, 2002). Classical receptor theory had postulated that GPCRs existed in equilibrium between an inactive (R) and an active (R*) state, and that upon binding, all agonists equally promoted the same subsequent receptor regulation and signalling cascades (for review, see Kenakin, 2004). However, in the past 15 years, numerous studies have challenged this idea, and the current view is that the receptor

can exist in multiple states, and that agonists can initiate selective receptor conformations, which in turn engage distinct signalling and receptor regulatory responses (Kenakin, 2011; Reiter *et al.*, 2012). This concept has been referred to in a number of ways including: ligand-directed signalling, functional selectivity, biased agonism, ligand-biased efficacy, collateral efficacy and stimulus trafficking (Galandrin *et al.*, 2007; Rajagopal *et al.*, 2010; Vaidehi and Kenakin, 2010).

Divergent functional responses from ligand-bound GPCRs can be modulated at a number of different levels. Traditionally, it was thought that activation of G-proteins was the primary way by which GPCRs signalled. This G-protein-dependent signalling is mediated through Gα and Gβγ subunits, and includes regulation of adenylate cyclase, phospholipases, multiple kinases and ion channels. More recently, it has been shown that GPCRs can also mediate G-protein-independent signalling, through proteins such as β-arrestins and PDZ-containing proteins (Magalhaes *et al.*, 2012). β-Arrestin regulated responses are by far the best characterized, and it appears that not only do β-arrestins mediate receptor trafficking, but they also act as scaffolding molecules on which a number of signalling cascades are initiated (for review, see Sorkin and von Zastrow, 2009; Rajagopal *et al.*, 2010). Thus, functional selectivity may be observed by ligands promoting G-protein-dependent or -independent signalling or both. The concept of ligand-directed signalling has important implications from a therapeutic perspective and holds the promise of designing drugs to selectively avoid undesirable biological effects targeted by receptor activation. This review will focus on ligand-directed signalling within the family of opioid receptors as an example of GPCRs with diverse structural and functional ligands and high therapeutic importance.

Opium has been used for many centuries for its medicinal and euphoric properties, and the use and abuse of this plant ultimately led to the discovery of the endogenous opioid system. One of the first breakthroughs in understanding the unique pharmacology of opium occurred in 1806, when Friedrich Wilhelm Serturner isolated the primary active ingredient in opium and called it morphine, after Morpheus, the god of dreams (Scott, 1969). The elucidation of the alkaloid structure of morphine led to the development of the synthetic opioid heroin, which was found to be more potent than morphine and even more problematic for triggering addictive behaviours. Many other opioid agonists have since been characterized, but to date, there are still no commercially available opioid therapeutics that are both effective analgesics and free from abuse liability.

The opioid receptor family includes three members: the μ, δ and κ opioid receptors. The existence of opioid receptors was discovered in 1973 by three separate groups, all using opioid radioligand binding in brain homogenates (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973), and genes encoding μ, δ and κ receptors were subsequently cloned in the early 1990s (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Chen *et al.*, 1993; Minami *et al.*, 1993). The μ, δ, and κ opioid receptors are encoded by *Oprm1*, *Oprd1* and *Oprk1* genes, respectively. Opioid receptors are activated by a family of naturally occuring endogenous peptides, the first of which was discovered in 1975, and genes identified in the early 1980s. These neuropeptides, which include the enkephalins, endorphins and dynorphins, are processed from larger precursor proteins encoded by *Penk*, *Pdyn* and *Pomc* genes. Opioid receptors are located throughout the body, and regulate a number of important behaviours such as reward, pain, stress, gastrointestinal transport and mood through receptors in both the central and peripheral nervous systems (for recent reviews, see Al Hasani and Bruchas, 2011; Sauriyal *et al.*, 2011).

The three opioid receptors show a high degree of sequence homology, and a common opioid receptor binding pocket within the helical transmembrane core has been postulated based upon modelling and structure activity studies (Metzger and Ferguson, 1995; Paterlini, 2005). The greatest divergence in sequence between the receptors occurs at extracellular domains and *in vitro* studies using mutant receptors have identified these regions as important for ligand selectivity (Kane *et al.*, 2006). Likewise, these studies have identified helical domain-mediated mechanisms for opioid receptor activation within the membrane core receptor domain (Decaillot *et al.*, 2003), which is highly similar across the three receptors. Very early on, *in vitro* receptor expression in transfected cells identified the first example of opioid ligand-directed trafficking (Arden *et al.*, 1995; Keith *et al.*, 1996), and site-directed mutagenesis experiments also provided indirect evidence for the existence of multiple active receptor conformations (Befort *et al.*, 1996), preparing the ground for biased agonism at opioid receptors. As may be inferred from the breadth of structural diversity of the peptide and alkaloid agonists that bind to opioid receptors, not all ligands interact with the same components of the receptor protein. Numerous structure–activity studies have identified key amino acids in the opioid receptors that selectively disrupt binding and signalling of some but not all agonists (Kane *et al.*, 2006). The ligand diversity and therapeutic importance of opioid drugs makes the opioid receptors excellent model GPCRs to understand the basis of ligand-directed signalling.

Ligand-directed signalling at the μ opioid receptor

Ligand directed signalling via the μ opioid receptor has important implications given the wide use of μ opioid receptor targeting drugs such as morphine, fentanyl, oxycodone and heroin both as analgesics and drugs of abuse. In mice null for the μ opioid receptor, morphine loses both its analgesic efficacy and rewarding properties (Matthes *et al.*, 1996; Contet *et al.*, 2004), as well as many other well-described biological activities (for review, see Gaveriaux-Ruff and Kieffer, 2002), demonstrating that this receptor mediates multiple effects of the prototypic opiate drug throughout brain circuits. Given their therapeutic importance, agonists that selectively induce discrete μ opioid receptor signalling complexes could be critical in developing pharmacotherapies that dissociate μ agonist-induced pain relief from reward and constipation, or distinguish adaptations to exogenous opioids such as tolerance and hyperalgesia (Kieffer and Evans, 2002; Evans, 2004).

As with most GPCRs, ligand binding to the μ opioid receptor can induce receptor internalization, a complex regu-

latory process that can lead to diminished receptor activation despite the continued presence of ligand [for review see (Evans, 2004; Kelly *et al*., 2008)]. μ Receptor internalization is followed by receptor recycling back to the cell surface, leading to restoration of receptor function (Koch *et al*., 2005). Early evidence for agonist-selective trafficking was revealed by the differential effects of morphine, DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin), and fentanyl on receptor trafficking in transfected cells, with morphine inducing poor internalization compared with DAMGO and fentanyl. Further early work indicated differential phosphorylation upon agonist activation, whereby morphine appeared to induce little receptor phosphorylation compared to DAMGO and fentanyl. Subsequent studies revealed differential agonist-dependent signalling and desensitization, suggesting biased responses involving GPCR kinases (GRKs), PKC and β-arrestins (for review, see Evans, 2004; Kelly *et al*., 2008).

Recent technological developments have started to further characterize ligand-directed signalling at the μ opioid receptor. A new approach, fluorescence recovery after photobleaching (FRAP), has revealed agonist-receptor-specific biophysical events at the level of the plasma membrane (Sauliere-Nzeh *et al*., 2010). In these studies, morphine triggered diffusion of the fluorescent recombinant receptor in neuroblastoma cells, which was pertussis toxin-sensitive. In contrast, DAMGO induced a sucrose-dependent aggregation to small isolated domains for half the receptors, and free long-range receptor diffusion for the other half. Another recent approach developed to investigate ligand-directed signalling at the μ opioid receptor measured dynamic mass redistribution (DMR) of the receptor upon agonist binding. This promising approach provides real-time optical fingerprints of GPCR signalling in living cells, and a first heat map based on the numerical analysis of DMR parameters for about 50 μ ligands under 13 experimental conditions was recently reported (Morse *et al*., 2011). This study revealed a number of novel pharmacological properties for several commonly used opioid ligands; including differences in opioid receptor affinity for specific G-proteins and activation of divergent signalling cascades (Morse *et al*., 2011). Although these studies may not definitively prove the existence of ligand-directed signalling, they do provide the basis for further investigation.

Agonist-directed signalling at the μ opioid receptor is also currently being characterized within native cell systems. In the case of ERK phosphorylation, morphine was shown to activate ERK pathways in the cytosol via PKCε leading to ribosomal S6 kinase stimulation. In contrast, etorphine triggered phospho-ERK translocation into the nucleus through a β-arrestin pathway, modulating Elk-1 and gene expression. First observed in HEK293 cells (Zheng *et al*., 2008), this differential signalling event was later linked to spine stability and morphology of hippocampal neurons, where morphine decreased dendritic spine volume while etorphine, fentanyl and DAMGO did not (Zheng *et al*., 2011). In addition, biased agonism has been observed in primary cultures of dorsal root ganglia cells where DAMGO but not morphine-activated P38 MAPK. Pharmacological blockade of P38 MAPK disrupted desensitization of DAMGO but not morphine signalling to calcium channels (Tan *et al*., 2009), and this supports previous data showing DAMGO-induced P38 activation regulates endocytosis via the early endosomal antigen 1 and Rabenosyn5, both components of the early endosome (Mace *et al*., 2005). Interestingly, in primary cultures from dorsal root ganglia, DAMGO and clonidine, but not morphine, induced cross-desensitization and co-internalization of both μ receptors and α_{2A}-adrenoceptors. The cross-desensitization of adrenoceptors and opioid receptors was disrupted both by the absence of β-arrestin 2 and the blockade of P38 MAPK, suggesting that biased agonism can also be important in regulating signalling cascades initiated by other GPCRs (Tan *et al*., 2009). In the case of morphine, desensitization of the μ opioid receptor in dorsal root ganglia was dependent on β-arrestin 2, which was not observed for other higher efficacy agonists (Mittal *et al*., 2012). Furthermore, whole cell patch clamp experiments in locus coeruleus slices found that desensitization of the μ opioid receptor by morphine was mediated by PKCα, while DAMGO used a GRK2-dependent mechanism (Bailey *et al*., 2009).

A recent behavioural study demonstrated that JNK signalling is selectively involved in morphine but not fentanyl analgesic tolerance (Melief *et al*., 2010). Thus, inhibition of JNK signalling either genetically or pharmacologically prevented acute tolerance to morphine but not fentanyl. Conversely, GRK3 knockout mice maintained acute tolerance to morphine but did not show acute tolerance to fentanyl (Terman *et al*., 2004). Together, these data demonstrate the existence of μ agonist-specific signalling mechanisms in the development of analgesic tolerance, with JNK and GRK3 kinases mediating distinct forms of tolerance. Effector systems associated with μ opioid receptors and the potential distinct signalling complexes that probably operate *in vivo* are schematized in Figure 1.

Finally, knock-in mice expressing a mutant μ opioid receptor that is able to internalize and recycle in response to morphine showed increased analgesia and reward, and reduced tolerance, dependence and addictive behaviour (Kim *et al*., 2008; Berger and Whistler, 2011). Importantly, morphine but not methadone produced enhanced analgesia and low tolerance with methadone exhibiting similar effects in knock-in and wild-type animals (Kim *et al*., 2008). These observations show that facilitated receptor internalization improved the drug profile specifically for the low-internalizing agonist (morphine) and suggest that the high-internalizing properties of methadone contribute to optimal analgesic efficacy and duration. μ opioid receptor internalizing agonists and the associated signalling complexes therefore represent valuable molecular targets for more effective analgesics (Berger and Whistler, 2010).

Together, these data demonstrate that agonist-biased signalling has behavioural consequences. These findings also strongly suggest that morphine preferably recruits β-arrestin 2-mediated pathways *in vivo*, and recent evidence suggests that other μ agonists (methadone, fentanyl) may recruit both β-arrestin 1 and 2 signalling (Groer *et al*., 2011). It will be interesting to examine *in vivo* properties of novel compounds such as herkinorin that, in contrast to all the known μ agonists including morphine, do not recruit β-arrestin 2 (Tidgewell *et al*., 2008). Indeed, very recent work suggests that herkinorin, as compared with morphine, shows attenuated tolerance following chronic use (Lamb *et al*., 2012).

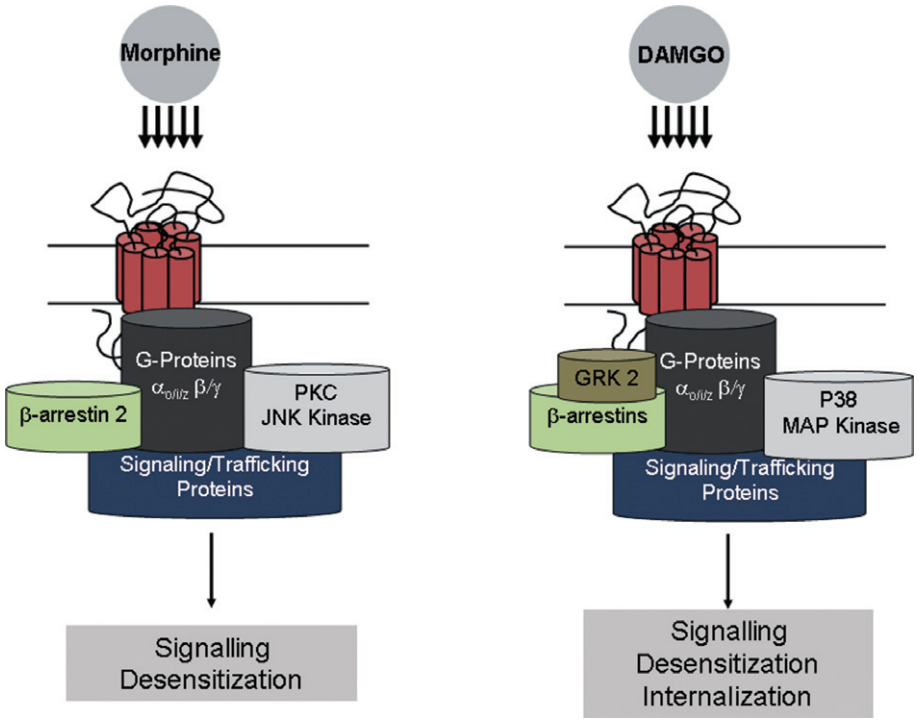

Figure 1

Ligand-specific signalling complexes at the μ opioid receptor. Treatment with morphine or DAMGO elicits differential signalling and trafficking of the μ opioid receptor. Activation of the μ opioid receptor by the low-internalizing agonist morphine is thought to result in receptor desensitization via a β-arrestin and PKC-dependent pathway. In contrast, the high-internalizing agonist DAMGO desensitizes the receptor in a GRK2-dependent manner and recruits P38 MAPK, which appears to be critical for μ opioid receptor desensitization and internalization. Such differences could feasibly be explained by different receptor conformations that allow similar G-protein activation but different kinase recruitment and hence desensitization. Further work is needed to explain how these ligand-specific complexes relate to tolerance.

Ligand-directed signalling at the δ opioid receptor

Compared with the more clinically relevant μ opioid receptor – at least at the time of writing – δ opioid receptors have been relatively understudied. However, recent advances in the pharmacological and genetic tools used to study this receptor have revealed its importance in a number of physiological processes (Pradhan *et al.*, 2011). Stimulation of δ opioid receptors does not result in many of the adverse effects associated with μ agonists, including addictive liability (Stevenson *et al.*, 2005; Codd *et al.*, 2009), respiratory depression (Takita *et al.*, 1997; Codd *et al.*, 2009) and constipation (Petrillo *et al.*, 2003; Codd *et al.*, 2009). Although δ agonists are poor analgesics in acute pain (Gallantine and Meert, 2005), they are highly effective in animal models of chronic inflammatory and neuropathic pain (Fraser *et al.*, 2000; Hurley and Hammond, 2000; Cahill *et al.*, 2003; Nadal *et al.*, 2006; Gaveriaux-Ruff *et al.*, 2008). Interestingly, δ opioid receptors also modulate emotional state. Genetic deletion of either the δ opioid receptor or its endogenous ligand, enkephalin, results in anxiogenic and depressive-like behaviours (Konig *et al.*, 1996; Filliol *et al.*, 2000), and δ opioid receptor agonists produce anxiolytic and anti-depressant effects (Broom *et al.*, 2002a; Saitoh *et al.*, 2004; Perrine *et al.*, 2006).

Agonist activation of the δ opioid receptor can initiate both G-protein-dependent and -independent signalling pathways. Agonist-induced activation of the δ opioid receptor leads to receptor desensitization, which for some agonists is attributed to receptor internalization. δ Opioid receptor internalization has been observed following binding of endogenous opioids (leu and met-enkephalin), peptides ([D-Pen^{2,5}]enkephalin, [D-Pen^{2},D-Pen^{5}]enkephalin (DPDPE), deltorphin) and small molecules (SNC80) (Bradbury *et al.*, 2009; Pradhan *et al.*, 2009). Unlike internalization of the μ opioid receptor, which results in rapid redistribution of most receptors back to the cell membrane, δ opioid receptors are predominantly targeted for degradation through the endosomal sorting complex required for transport (ESCRT) machinery (Henry *et al.*, 2011).

Convergent evidence from a number of different *in vitro* studies reveals ligand-directed signalling and trafficking of the δ opioid receptor. In bioluminescence resonance energy transfer (BRET) studies, δ opioid receptor ligands did not equally engage β-arrestin 2. Most ligands were agonists for inducing G-protein coupling to the receptor but showed variable efficacy for arrestin-receptor interactions. In addition, ligands that induced strong physical interactions with G-proteins but weak or no β-arrestin 2 interaction acted as competitive antagonists for arrestin binding (Molinari *et al.*, 2010). This subset of ligands could be explained as partial

agonists for β-arrrestin 2 recruitment, and presumably arrestin-mediated signalling; however, they did show full agonist activity in recruiting G-protein subunits. In addition, it was found that δ opioid receptors were in constitutive complexes with G-proteins, and that each δ ligand induced a specific conformation resulting in divergent activation of second messenger transducers (Audet *et al.*, 2008). Evidence from BRET studies may more closely reveal true ligand directed signalling, as this technique directly measures protein–protein interactions that can reflect specific ligand-induced receptor conformations.

Cellular studies examining ligand-specific desensitization and receptor trafficking have also found biased agonism at the δ opioid receptor. Differential desensitization of the δ opioid receptor was observed following stimulation with either peptide (DPDPE and deltorphin II) or alkaloid (etorphine) agonists (Allouche *et al.*, 1999). Furthermore, each class of ligand differentially activated kinases (Marie *et al.*, 2008) and distinctly mediated β-arrestin 1 recruitment (Aguila *et al.*, 2012) in order to initiate receptor desensitization. In addition, agonists differentially regulated sorting of the δ opioid receptor following internalization. In SK-N-BE cells expressing Flag-tagged human δ opioid receptor, the peptides DPDPE and deltorphin, and SNC80 appeared to promote receptor degradation while the endogenous enkephalins and etorphine promoted receptor recycling (Marie *et al.*, 2003b; Lecoq *et al.*, 2004). Taken together, these *in vitro* studies indicate that the δ opioid receptor exists in multiple active conformation states.

Until recently, very little was known about the *in vivo* consequences of agonist-induced δ opioid receptor trafficking. The development of a knock-in mouse model expressing fluorescent δ opioid receptor (DOR-eGFP) (Scherrer *et al.*, 2006; 2009) has opened the possibility to correlate ligand-induced receptor trafficking with receptor function *in vivo*. These animals express functional δ opioid receptors at physiological levels, which are directly visible *in vivo*. At behaviourally relevant doses, the prototypic agonist SNC80 (Bilsky *et al.*, 1995) produces internalization of DOR-eGFP throughout the peripheral and central nervous systems of these animals (Scherrer *et al.*, 2006; Pradhan *et al.*, 2009; Poole *et al.*, 2011). Acute administration of SNC80 also reversed hyperalgesia in a model of inflammatory pain. However, this initial treatment with SNC80 also produced robust receptor internalization and G-protein uncoupling resulting in acute behavioural desensitization (Pradhan *et al.*, 2009). Subsequently, chronic treatment with SNC80 resulted in extensive receptor degradation, as was predicted from *in vitro* studies, leading to generalized behavioural tolerance to all agonist effects (Pradhan *et al.*, 2010) and Figure 2). Contrary to SNC80, the δ agonist ARM390 did not produce detectable receptor phosphorylation or internalization following agonist binding, although efficacy and potency for G-protein activation (Marie *et al.*, 2003a; Pradhan *et al.*, 2009), and analgesic properties (Pradhan *et al.*, 2010) were similar. The different receptor trafficking properties of ARM390 had remarkable consequences *in vivo*. Acute treatment with ARM390 did not produce behavioural desensitization, and δ opioid receptors remained located on the cell surface and coupled to G proteins. However, chronic treatment ultimately led to analgesic tolerance, despite unchanged δ opioid receptor number, G-protein coupling and cell membrane localization. Unlike the generalized tolerance produced by SNC80, ARM390 produced an analgesic tolerance, probably due to changes in second messenger responses in pain-specific pathways (Pradhan *et al.*, 2010 and Figure 2). These data indicate that ligand-specific trafficking of the δ opioid receptor can produce two distinct types of behavioural tolerance. From a therapeutic perspective, these findings imply that non-internalizing δ receptor agonists may be more efficacious for the treatment of diseases unrelated to pain, such as anxiety and depression.

In other studies examining tolerance following chronic administration of systemically available δ opioid receptor agonists, analgesic tolerance was not observed in models of chronic pain (Petrillo *et al.*, 2003; Jutkiewicz *et al.*, 2005; Beaudry *et al.*, 2009; Codd *et al.*, 2009). To date, very few δ agonists have been characterized at the level of *in vivo* receptor trafficking, and in order to truly determine the relationship between δ opioid receptor internalization and tolerance, a thorough study examining a number of different ligands under experimentally controlled conditions needs to be performed.

A major caveat to the development of δ agonists is that some, but not all, δ agonists also produce convulsions, an effect that is specific to the activation of the δ opioid receptor (Broom *et al.*, 2002b; Scherrer *et al.*, 2006). Currently, it is impossible to screen for this ligand-specific behavioural effect *in vitro*. Understanding which signalling pathways mediate this agonist-selective response would allow for the development of *in vitro* screening tools for novel agonists, thus saving time, money and the necessary behavioural studies. Importantly, this type of screen would further encourage the development of δ opioid receptor agonists for clinical use.

Ligand-directed signalling at the κ opioid receptor

κ Opioid receptors have been implicated in a number of physiological responses, including nociception, stress, mood, feeding, gut motility and diuresis. Therapeutically, κ opioid receptor agonists are being explored as alternatives to μ analgesics, as they have low abuse potential and produce minimal effects on gastrointestinal transit and respiration. In addition, κ agonists may relieve or prevent hyperalgesia produced by chronic use of μ opioid receptor therapies (for review, see Kivell and Prisinzano, 2010; Vanderah, 2010). However, the clinical relevance of κ agonists is limited as central activation of κ opioid receptors produces dose-dependent dysphoria and some agonists such as salvinorin A produce hallucinations (Gonzalez *et al.*, 2006). The endogenous κ ligand, dynorphin, can be released during stress and produce behavioural correlates of dysphoria, depression and anxiety, effects that have been linked to pro-addictive behaviours and drug relapse (for review, see Bruchas *et al.*, 2010).

A recent study in mice examining dysphoria induced by κ opioid receptor activation has identified the signalling pathway through which this behaviour is regulated. Conditioned place aversion to the κ agonist U50,488 and stress-induced reinstatement of drug seeking (probably a result of

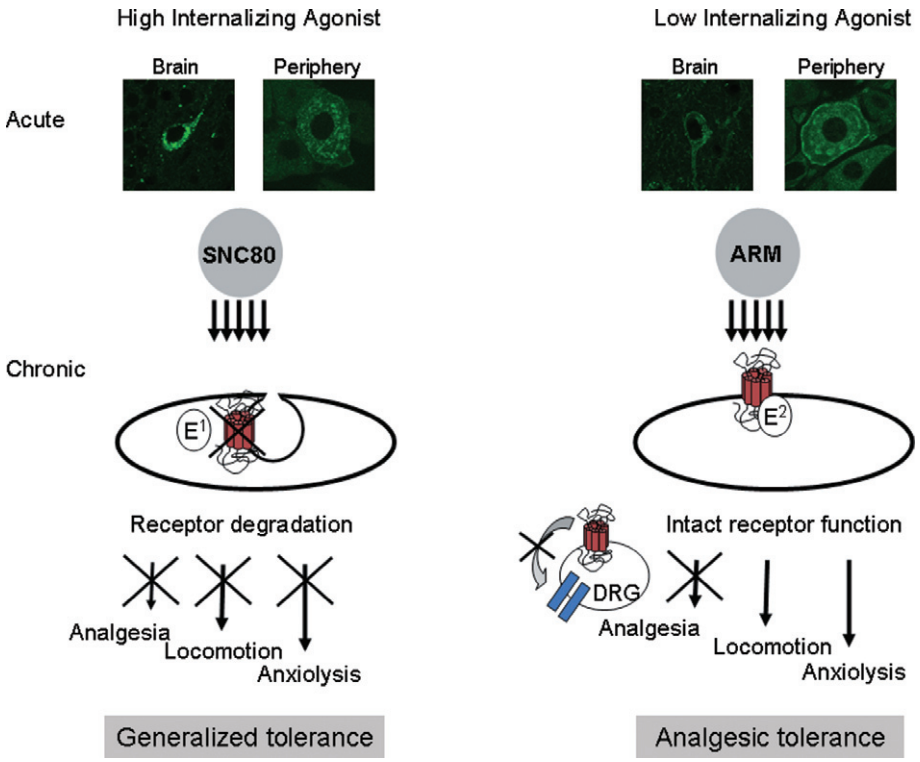

Figure 2

The behavioural consequences of functional selectivity at the δ opioid receptor. SNC80 and ARM390 (ARM) have comparable selectivity and potencies for the δ opioid receptor, but highly distinct internalization properties. Systemic SNC80, but not ARM390, produces clear receptor internalization *in vivo* as shown in slices from DOR-eGFP knock-in mice (representative images from the hippocampus and dorsal root ganglia) (Pradhan *et al.*, 2009). Chronic administration of either agonist produces two distinct forms of tolerance. Repeated administration of SNC80 produces widespread receptor down-regulation, thus resulting in a generalized tolerance where all δ agonist-induced behaviours are affected. In contrast, chronic administration of the low-internalizing agonist, ARM390, appears to affect δ opioid receptors only at the level of the dorsal root ganglia, thus producing tolerance at the level of pain processing (Pradhan *et al.*, 2010).

dynorphin release) was mediated by the specific activation of P38 MAPK by κ opioid receptors in the dorsal raphe nucleus (Land *et al.*, 2009). In addition, κ-opioid receptor activation of the P38 MAPK pathway in glia also appeared to be important for the development of hyperalgesia following peripheral neuropathy (Xu *et al.*, 2007). κ Opioid receptors activate the P38 MAPK pathway through G-protein-independent signalling via GRK3 and β-arrestins (Bruchas *et al.*, 2006). These results suggest that the development of κ ligands that only activate G-protein-dependent events may produce analgesia and circumvent the dysphoric effects. Although no such ligands presently exist, this is a clear example of how the characterization of the signalling pathways that mediate specific behaviours may ultimately be used to tailor drug development.

A prime illustration of functional selectivity is observed at the level of κ antagonists. Unlike antagonists for the μ and δ opioid receptors, certain κ opioid receptor antagonists (antagonist defined as ability to block κ agonist activity both *in vitro* and *in vivo*) have an extremely long duration of action. For example, a single injection of the κ selective antagonists, norbinaltorphimine (norBNI), guanidinonaltrindole or JDTic, maintains continual blockade of κ opioid receptors for up to 3 weeks (Horan *et al.*, 1992; Carroll *et al.*, 2004; Bruchas *et al.*, 2007). This duration of action is in sharp contrast to the pan-opioid receptor antagonist naloxone, which only lasts for several hours. Recent work has shown that this long duration of action is mediated by activation of JNK. Surprisingly, norBNI and other long acting antagonists have been found to activate JNK through the κ opioid receptor, and treatment with these antagonists was found to increase phospho-JNK levels in the brain and spinal cord in wild-type mice but not κ opioid receptor knockout mice (Bruchas *et al.*, 2007; Melief *et al.*, 2011). In addition, the administration of the JNK inhibitor SP600125 blocked the long lasting antagonism induced by norBNI on the analgesic effects of the κ agonist U50, 488 (Bruchas *et al.*, 2007). JNK1 in particular appears to mediate long-term antagonism, as norBNI and other long-lasting antagonists act as short duration competitive antagonists in JNK1 KO mice (Melief *et al.*, 2010; 2011). Interestingly, this is a case where a functional antagonist is not just a classical antagonist in blocking agonist binding but a 'collateral agonist' for the JNK signalling cascade, the activation of which produces long duration inactivation of κ receptor signalling via a mechanism yet to be elucidated (Bruchas *et al.*, 2007). Therapeutically, κ opioid receptor antagonists are being developed for the treatment of stress, anxiety and depression, and as an aid to curb drug relapse.

Understanding the mechanisms regulating the unique kinetics of κ opioid receptor antagonists has important implications for future drug design.

Perspectives

It should be recognized that beyond ligand-directed signalling, there are multiple mechanisms that could readily mediate differential *in vivo* activities of drugs targeting opioid receptors. Factors such as intrinsic drug efficacy, pharmacodynamics, drug selectivity and ligand accessibility to selective receptor populations probably explain the differential effects of many drugs. In addition, the ability of a ligand to differentially activate splice variants of receptors or selectively activate homo- or heterodimeric receptor complexes are also possibilities that may masquerade as ligand-directed signalling (reviewed by Evans, 2004). Overall, biased agonism is one of the many mechanisms by which opioid ligands could produce diverse physiological effects.

The concept of biased agonism has profound implications, both in terms of understanding the complexity of GPCR pharmacology and for facilitating drug development (Bosier and Hermans, 2007; Galandrin *et al.*, 2007). The very recent crystal structures of all three opioid receptors with ligands in place promises to visualize different receptor conformations as a result of different ligand interactions (Granier *et al.*, 2012; Manglik *et al.*, 2012; Wu *et al.*, 2012). The ability of ligands to discretely activate particular signalling pathways, which may in turn regulate specific *in vivo* responses, opens the possibility of separating desirable from adverse drug effects. However, the evidence for this phenomenon is primarily based on *in vitro* experiments using recombinant cell systems, with only a few studies demonstrating clear agonist-selective activity *in vivo*. A major challenge in GPCR research will be to demonstrate the physiological relevance of agonist-biased signalling and regulation. There is clearly a need for further studies to bridge the gap between *in vitro* findings showing differential agonist signalling cascades and the *in vivo* behavioural consequence of signalling specificity. In the opioid receptor field, the concept of biased agonism *in vivo* is emerging, and the promise of biased μ agonists that are better analgesics with less abuse liability, or δ and κ agonists with targeted and sustained efficacy in the treatment of pain and mood disorders has now been validated in animal models. Future drug development will be required to determine the clinical relevance of these findings.

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Conflict of interest

The authors declare no conflict of interest in the preparation of this manuscript.

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