Title
Trafficking of δ-opioid receptors and other G-protein-coupled receptors: implications for pain and analgesia

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A cell can regulate how it interacts with its external environment by controlling the number of plasma membrane receptors that are accessible for ligand stimulation. G-protein-coupled receptors (GPCRs) are the largest superfamily of cell surface receptors and have a significant role in physiological and pathological processes. Much research effort is now focused on understanding how GPCRs are delivered to the cell surface to enhance the number of ‘bioavailable’ receptors accessible for activation. Knowing how such processes are triggered or modified following induction of various pathological states will inevitably identify new therapeutic strategies for treating various diseases, including chronic pain. Here, we highlight recent advances in this field, and provide examples of the importance of such trafficking events in pain.

G-protein-coupled receptors and pain
G-protein-coupled receptors (GPCRs) have a significant role in normal physiological processes and can contribute to pathological states when such processes are disrupted [1,2]. Indeed, drugs that either directly or indirectly modulate GPCR function have proved to be effective therapeutics for the treatment of many disease states, and as many as 50% of marketed drugs target GPCRs [2]. GPCRs have also been implicated in either the suppression or generation of states symptomatic of chronic pathological pain including hyperalgesia (exaggerated response to a normally painful stimulus), allodynia (pain in response to a normally innocuous stimulus) and paroxysmal or spontaneous pain (Table 1). Chronic pain is thought to affect 17–31% of the population in North America – Canadian Pain Coalition (http://www.canadianpaincoalition.ca/). In addition to the physical and psychological consequences and the deleterious effects on quality of life of a sufferer, chronic pain has a tremendous economic impact and is associated with costs estimated to be over US$150 billion annually in the USA through healthcare expenses, disability and other expenditures. Considering the impact that chronic pain has on our society, a crucial need exists for the development of more effective pharmacotherapies due to the vast degree of unmet medical needs in this area.

Some GPCRs, such as cannabinoid (CB) and opioid receptors, have validated therapeutic value for pain management (Table 1), and continued exploitation of these receptor families has yielded more selective, potent analgesics with favorable side-effect profiles (for recent review, see Ref. [3]). Various institutions have mandated the identification and characterization of orphan GPCRs to discover novel receptor targets that have potential for treating chronic pain. This strategy led to the discovery of sensory neuron-specific receptors (SNSRs) [4], which seem to have discrete, appropriate anatomical localization and physiological properties consistent with a role in pain processing and thus are a feasible target for drug development to treat chronic pain. Nevertheless, we need not rely solely on the discovery or deorphanization of GPCRs for novel pain targets, because modifying the cell surface density of a specific GPCR can result in altered functional responses. Investigation of such events, and ways in which to exploit them to modulate cellular responses, is at an early stage. Certainly, one of the most intriguing prospects offered by controlling or regulating cell surface receptor density could be the treatment of pain.

Trafficking of GPCRs
The density of GPCRs at the plasma membrane is dynamic and is regulated by several processes that seek to adjust cellular responsiveness to external stimuli. Much of the research to date on the trafficking of GPCRs has concentrated on the events elicited after the application of agonist. Following agonist binding and the induced conformational change in the receptor, the ‘activated’ receptor is phosphorylated by G protein-receptor kinases recruited from the cytosol (reviewed in Refs [5–8]). This phosphorylation event and the ensuing recruitment of one of the arrestins results in rapid ‘desensitization’ of the receptor (reviewed in Refs [5,6,8–10]). The subsequent internalization of the ligand–receptor complex (also known as receptor-mediated endocytosis) reduces the density of receptors at the cell surface but does not necessarily lead to a decrease in the overall number of receptors (receptor downregulation). The internalized receptor can be recycled back to the cell surface or can be directed to the lysosomes for receptor degradation leading to ‘long-term desensitization’ of a receptor...
Several processes are thus implicated in regulating receptor density after the application of an agonist. Events modulating the intracellular trafficking or routing of receptors to the plasma membrane before agonist stimulation can also have profound consequences on receptor function and cellular responsiveness (Figure 2). GPCRs must undergo a continual process of maturation, where proteins are exocytosed from the endoplasmic reticulum (ER) to the plasma membrane by greatly

![Diagram](image-url)
Figure 2. Principles of cell surface GPCR expression as a determinant of functional competence. The effects produced by a GPCR will be dictated by the cell surface expression of the receptor. (a) Whereas scant cell surface expression would produce a minimal response to application of endogenous or exogenous agonist, increased cell surface expression of a GPCR will elicit an enhanced response (b). (c) In terms of drug-induced effects, this principle predicts a change in (i) potency, as demonstrated by a leftward shift in the dose–response curve, or (ii) efficacy, as produced by an increase in the maximal response following activation of the GPCR, or possibly both (i) and (ii).

conserved mechanisms (reviewed in Refs [11,12]). Only successfully folded proteins are exported from the ER to the Golgi complex where they can undergo posttranslational modifications such as glycosylation. Upon exiting from the trans Golgi network, proteins are sorted to the constitutive or the regulated vesicular pathway. In the constitutive pathway, vesicles containing proteins are constantly exported to the plasma membrane, whereas in the regulated pathway, vesicles are exported to the plasma membrane in response to a particular signal. Although the information on this topic is scarce, GPCRs are generally believed to be exported from the trans Golgi network to the plasma membrane through the constitutive pathway, although exceptions have been reported.

Formation and trafficking of functional receptors leading to cell surface expression and activity have also been demonstrated to occur by means of multiple regulatory proteins (for recent review, see Refs [13–16]). Chaperone molecules, such as the receptor-activity-modifying proteins (RAMPs; for review, see Ref. [17]) have been implicated in the proper folding or exocytosis (or both) of some GPCRs to the cell membrane. Chemicals have also been reported to rescue intracellularly retained mutant proteins; for example, 4-phenylbutyric acid led to the secretion of the intra-cellularly trapped α1-antitrypsin both in vitro and in vivo [18]. In contrast to the nonspecific actions of chemical chaperones, cell-permeable opioid ligands (‘pharmacological chaperones’) promoted the maturation of immature δ-opioid (DOP) receptors present in the ER in HEK293S cells, leading to enhanced DOP receptor plasma membrane density [19]. In fact, pharmacological chaperones might account for the paradoxical augmentation of opioid-induced analgesia and attenuation of morphine tolerance by ultra-low doses of opioid receptor antagonists [20], whereby the opioid antagonists act as chaperones for the maturation of DOP receptors to retain morphine-induced analgesia. [Such a hypothesis assumes that DOP receptor trafficking modulates mechanisms responsible for μ-opioid (MOP) receptor desensitization or tolerance.]

Recently, it has been reported that some GPCRs are localized within intracellular compartments and seem to be fully functional, but are awaiting a certain stimulus to be targeted to the cell surface. In vitro studies have proposed that homologous (the same receptor) or heterologous (different receptors) cell surface recruitment could be one of the mechanisms responsible for regulating plasma membrane receptor density. In one example of homologous recruitment, stimulation with dopamine D1 agonists for 1–15 min led to targeting of intracellular D1 receptors to the cell surface of renal epithelial cells [21]. Heterologous recruitment has also been reported where atrial natriuretic peptide induced the trafficking of D1 receptors to plasma membranes in a renal epithelial cell line and in kidney cells [22]. Additionally, neuropeptide Y causes recruitment of cell surface α2-adrenoceptors in a renal epithelial cell line [22]. Thus, agonist treatment of one receptor can potentially affect the cell surface expression of either the same protein, or proteins from the same or different receptor classes.

The focus of the current review is to summarize, in the context of pain, research aimed at assessing the events modulating the density of GPCRs at the plasma membrane before the application of a ligand. Other comprehensive review articles on the regulation of GPCR trafficking, including receptor maturation processes, are available [7,23]. DOP receptors will be used as a model system because much research aimed at investigating GPCR trafficking to the cell surface before agonist application in the context of pain and analgesia has studied this receptor. Examples from other GPCRs will also be discussed, with an emphasis on findings with potential applications to relieve pain.

A case in point: modifying DOP receptor cell surface density to improve analgesic potency

Substantial interest has existed for several decades in developing selective DOP receptor ligands for the treatment of chronic pain because DOP receptor ligands are believed to have a much lower abuse potential than MOP receptor agonists such as morphine [24–26] in addition to reduced respiratory [27–29], cognitive [30,31] and gastrointestinal [32,33] impairments. Preclinical studies have demonstrated that δ-selective agonists elicit antinociception in various persistent and chronic pain models including inflammatory [34–38], neuropathic [26,39,40] and cancer [41] pains. Furthermore, spinal administration of
DADLE, a DOP receptor peptide agonist, was shown to produce analgesia in humans [42], although it is noted this peptide possesses activity at MOP receptors. Despite this promise, DOP receptors remain an unexploited pharmacological target for pain management.

Subcellular localization studies of DOP receptors by electron microscopy have been important in understanding DOP receptor function. Under normal, homeostatic conditions, only a small subset of DOP receptors is found in association with neuronal plasma membranes, with the majority of DOP receptors localized predominantly to intracellular sites within neurons [43–47]. This small number of plasma membrane-bound receptors is consistent with the fact that DOP receptor agonists have modest behavioral effects in acute-phase pain-testing paradigms [48].

It was demonstrated by us and others that prolonged stimulation of MOP receptors produced targeting of DOP receptors to plasma membranes in vivo [49–54]. The change in the subcellular distribution of DOP receptors was accompanied by increased antinociceptive potency of DOP receptor agonists in acute (tail-flick and hot-plate) and tonic (formalin) pain tests in rodents [49,52,55,56] (Box 1). Indeed, the trafficking of DOP receptors was not correlated with a change in DOP receptor radioligand binding or expression of mRNA or protein levels [50], confirming that targeting of existing intracellular DOP receptors to the plasma membrane probably accounts for the observed augmented functional competence of DOP receptors rather than a change in protein synthesis. Box 1 highlights mechanisms involved in the translocation of DOP receptors and ensuing functional consequences subsequent to chronic morphine treatment (Table 2).

Translocation of DOP receptors from intracellular compartments to neuronal plasma membranes could also account for the enhanced antinociceptive effect and intracellular signaling of δ-selective agonists in chronic pain states. Indeed, chronic inflammatory pain induced by intraplantar injection of complete Freund’s adjuvant (CFA) induced an increase in the cell surface expression of DOP receptors in postsynaptic [38,51] and presynaptic [54] sites in the dorsal spinal cord ipsilateral to the site of injury. The enhanced translocation of DOP receptors correlated with a leftward shift in the dose-dependent reversal of thermal hyperalgesia following spinal administration of a selective DOP receptor agonist [38]. Thus, events that alter DOP receptor subcellular localization have profound consequences for receptor function, and have implications for pain management.

The lessons learned from trafficking of DOP receptors to the plasma membrane before agonist application might not be directly applicable to other GPCRs. However, they do suggest that, in general, trafficking of GPCRs to the plasma membrane might be a regulated process that could be exploited pharmacologically, as was illustrated above with MOP receptor agonist treatments and DOP receptor cell surface recruitment.

**Mechanisms underlying trafficking events of GPCRs involved in pain**

**DOP receptor**

In addition to mechanisms cited earlier, enhanced plasma membrane expression of DOP receptors was also shown to occur in cultured dorsal root ganglion (DRG) neurons following brief depolarization by capsaicin, elevated extracellular potassium or ATP [57,58]. These latter studies have demonstrated that such activity-dependent trafficking events were mediated through a regulated pathway rather than the constitutive pathway because DOP receptors were inserted into large dense-core vesicles for transport to neuronal plasma membranes (for review, see Ref. [59]). Although such results have not been consistently reported [54,60,61], activity-dependent translocation of DOP receptors in DRG neurons following in vivo administration of capsaicin or induction of chronic inflammation has been demonstrated [54]. In addition, the population of DRG neurons exhibiting cell surface DOP receptor targeting was dependent on the type of stimulus, suggesting that modality-specific activity regulates receptor trafficking [54]. Indeed, there exist multiple pathways for regulated receptor translocation, in addition to evidence for receptor trafficking to distinct membrane compartments [62]. Figure 3 illustrates the various mechanisms proposed to trigger DOP receptor trafficking to neuronal plasma membranes.

The activity-dependent translocation of GPCRs, such as DOP receptors, raises the question of whether neuronal responsiveness is dynamically regulated by electrical activity and what advantage it poses to GPCR responsiveness. One provocative possibility is that activity-dependent
control of agonist responsiveness at GPCRs might be part of a mechanism that controls or modulates synaptic plasticity, which is fundamental to the generation of various pain states. Using the DOP receptor as an example, we know that these receptors are localized to intracellular and plasma membranes that extend along the soma, axon, terminals and dendrites in various neuronal types within the peripheral and central nervous systems. Translocation to augment cell surface expression can be induced by various stimuli, including brief depolarization or noxious stimulation (Table 2), raising the question of whether DOP receptors could have an important role in modulating activity-dependent plasticity and thereby dampening or reversing mechanisms maintaining chronic pain states. A putative role for DOP receptors in activity-dependent synaptic plasticity has been reported in the hippocampus [63], but evidence of such effects remain absent in regions important for pain transmission. A more simplistic generalized view is that stimulus-evoked translocation of GPCRs to neuronal plasma membranes is an inherent mechanism that has evolved to control the transmission of nociceptive information to higher brain centers. However, whether regulation of GPCR trafficking is responsible for the modulation of synaptic events associated with various pain states has not been directly addressed.

Other GPCRs

For other receptors, association with several accessory proteins seems to be necessary for proper delivery to the plasma membrane and for functional activity (for recent reviews, see Refs [13–15]). For instance, in the case of GABA<sub>B</sub> receptors, heteromeric assembly between GABA receptor subunits was shown to be necessary for cell surface expression and receptor recognition characteristics in addition to coupling to intracellular signaling cascades [64–67]. GABA<sub>B</sub> receptors are known to control neuronal excitability and modulate synaptic neurotransmission; they have an important role in many physiological aspects of pain modulation.
activities and have been implicated in a variety of neurodegenerative and pathophysiological disorders including chronic pain.

Homer proteins participate in the regulation of metabotropic glutamate (mGlu) receptors. Because mGlu receptors can modulate nociceptive processing at various levels of the nervous system (spinal and supraspinal) and are crucially involved in both peripheral and central sensitization associated with prolonged or chronic morphine treatment, Homer proteins contain a PDZ-like domain that specifically binds to mGlu receptors, and these proteins are rapidly induced by excitatory synaptic activity in neurons [69]. Such proteins have been found to regulate the retention (Homer 1b) or maturation (Homer 1a) of mGlu receptors to be inserted into the plasma membrane [70] and are required for clustering (Homer 1c) of the mGlu receptors at the cell surface in neuronal dendrites [71]. Additionally, Homer 1a was previously shown to attenuate constitutive (agonist-independent) activity of type I mGlu receptors [72], demonstrating that this protein modulates not only trafficking, but also signaling. Conversely, long-form Homer proteins are not only involved in GPCR trafficking but are also important in the coupling of type I mGlu receptors to intracellular mitogen-activated protein kinases (MAPKs) [73], which are important messengers linking synaptic activity to nuclear transcriptional control of plasticity-related genes including those involved in chronic pain [74]. A recent study identified that Homer 1a operates in a negative feedback loop to regulate the excitability of the pain pathway in an activity-dependent manner in a model of chronic inflammatory pain [75]. In this study, preventing the activity-induced upregulation of Homer 1a exacerbated inflammatory pain, most probably as a result of the role of Homer 1a in uncoupling glutamate receptors (metabotropic and ionotropic) from intracellular signaling cascades, which in turn resulted in counteracting spinal cord sensitization. Thus, modulating the activity of Homer proteins, in turn resulting in alterations in mGlu receptor function, could be a new therapeutic avenue to alleviate chronic pain.
A RAMP protein was shown to be required for the transport of calcitonin receptor-like (CRL) receptors to the plasma membrane, but the RAMP protein associated with the receptor dictated the pharmacological profile: thus, RAMP1 association was necessary for a mature calcitonin gene-related peptide (CGRP) receptor, but RAMP2 produced an adrenomedullin receptor [76]. Furthermore, it was recently reported that Apg8L, a RAMP2 produced an adrenomedullin receptor [76]. with the receptor dictated the pharmacological profile: the plasma membrane, but the RAMP protein associated transport of calcitonin receptor-like (CRL) receptors to the plasmic reticulum [13]. This inadequate trafficking of receptors to neuronal plasma membranes consequently prevents arginine vasopressin from being able to elicit its antidiuretic effects. Likewise, retinitis pigmentosa might result from improper intracellular trafficking and localization of rhodopsin receptors (reviewed in Ref. [79]). Thus, aberrations in protein trafficking might underlie the pathophysiology of various diseases and could represent potential sites for pharmacological intervention.

Estimates of the prevalence of mood disorders in patients with chronic pain indicate that a substantial proportion of these patients display debilitating depression. On the basis of a large-scale, population-based survey of pain and depression in the USA, Magni and colleagues found that 18% of people suffering from chronic pain could also be classified as depressed [80]. Moreover, another study reported that the prevalence of clinical depression in patients with chronic pain is as high as 30–54% [81]. Although comorbidity does not necessarily indicate commonality of underlying mechanisms, antidepressant drugs have been proven to be efficacious in alleviating neuropathic pain symptoms [82]. Interestingly, in addition to their analgesic effects in chronic pain, DOP receptors have also been implicated in mood disorders. DOP receptor-null mutant animals exhibit depressive-like behaviors, suggesting that an endogenous tone at this receptor site regulates mood [83]. Additionally, DOP receptor agonists and endogenous opioid peptides produce antidepressant effects in animal models of depression and anxiety [84–91]. Subjecting rats to a cold water swim test (which is similar to the forced swim test used in anxiety paradigms) has been shown to elicit trafficking of DOP receptors to neuronal plasma membranes [92]. In this latter study, under homeostatic conditions the DOP receptors were associated with large dense-core vesicles within GABA-containing neurons localized in the ventrolateral periaqueductal gray, whereas the stress stimulus produced an increase in plasma membrane-bound receptors. Hence, activity-dependent initiation of a regulated vesicular pathway was responsible for DOP receptor trafficking. Further studies will be required to determine whether regulating DOP receptor trafficking could be a viable treatment strategy for treating mood disorders such as depression.

Interestingly, a member of the s100 EF-hand protein family (p11) was shown recently to be necessary for the cell surface expression of 5-hydroxytryptamine (5-HT)1B receptors [93]. In this latter study, coexpression of p11 with 5-HT1B receptors enhanced the ability of this GPCR to counteract forskolin-stimulated cAMP formation. This discovery has relevant clinical implications with respect to neuropathic disorders such as obsessive compulsive disorder, depression, anxiety and aggression. Indeed, p11 expression was reduced in patients who suffered from unipolar depression, and antidepressant agents increased p11 expression [93]. It is tempting to speculate that the elevation of p11 by antidepressant drugs and consequential increase in functional, bioavailable 5-HT1B receptors accounts for at least part of the clinical efficacy of such drugs.

Concluding remarks

Taken together, alterations in the subcellular distribution of GPCRs can have dramatic physiological and potentially pathological consequences for cellular function. We have only just begun to investigate such events and ways in which to exploit them to modulate cellular responses. Certainly, a potential application of controlling or regulating cell surface receptor density could be the treatment of pain. Using the DOP receptor as an example, it is clear that modulating the number of cell surface receptors has tremendous potential for treatment of pain and other disease states such as mood disorders. However, we must be mindful that this is an evolving area of research and it is not yet known whether what we have learned from the DOP receptor can be extrapolated to other GPCRs. Additionally, many GPCRs seem to have various mechanisms, whether through oligomerization, heteromerization, chaperones or accessory proteins, for regulating export to the plasma membrane, casting doubt on the general belief that GPCRs are constitutively delivered to the plasma membrane.

It is predicted that extensive investigation of trafficking events for various GPCRs will be required before we can identify whether commonalities can be extrapolated to GPCRs within receptor classes or families. Nevertheless, as we elucidate how GPCRs are regulated in various pathological states, the potential for intervention to harness trafficking events could prove to be a valuable opportunity that enables better diagnostics and novel strategies for optimizing therapeutic action. It could be timely to explore the regulation of GPCR cell surface trafficking because these mechanisms have major potential in achieving desired clinical endpoints for various diseases.
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