Arresting inflammation: contributions of plasma membrane and endosomal signalling to neuropeptide-driven inflammatory disease

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Abstract

GPCR (G-protein-coupled receptor) signalling at the plasma membrane is under tight control. In the case of neuropeptides such as SP (substance P), plasma membrane signalling is regulated by cell-surface endopeptidases (e.g. neprilysin) that degrade extracellular neuropeptides, and receptor interaction with β-arrestins, which uncouple receptors from heterotrimeric G-proteins and mediate receptor endocytosis. By recruiting GPCRs, kinases and phosphatases to endocytosed GPCRs, β-arrestins assemble signalosomes that can mediate a second wave of signalling by internalized receptors. Endosomal peptidases, such as ECE-1 (endothelin-converting enzyme-1), can degrade SP in acidified endosomes, which destabilizes signalosomes and allows receptors, freed from β-arrestins, to recycle and resensitize. By disassembling signalosomes, ECE-1 terminates β-arrestin-mediated endosomal signalling. These mechanisms have been studied in model cell systems, and the relative importance of plasma membrane and endosomal signalling to complex pathophysiological processes, such as inflammation, pain and proliferation, is unclear. However, deletion or inhibition of metalloendopeptidases that control neuropeptide signalling at the plasma membrane and in endosomes has marked effects on inflammation. Neprilysin deletion exacerbates inflammation because of diminished degradation of pro-inflammatory SP. Conversely, inhibition of ECE-1 attenuates inflammation by preventing receptor recycling/resensitization, which is required for sustained pro-inflammatory signals from the plasma membrane. β-Arrestin deletion also affects inflammation because of the involvement of β-arrestins in pro-inflammatory signalling and migration of inflammatory cells. Knowledge of GPCR signalling in specific subcellular locations provides insights into pathophysiological processes, and can provide new opportunities for therapy. Selective targeting of β-arrestin-mediated endosomal signalling or of mechanisms of receptor recycling/resensitization may offer more effective and selective treatments than global targeting of cell-surface signalling.

Introduction

With almost 1000 members, the heptahelical GPCRs (G-protein-coupled receptors) comprise the largest family of cell-surface receptors. They allow cells to detect and respond to extraordinarily diverse environmental and endogenous stimuli, and they participate in most aspects of pathophysiological control. GPCR agonists are usually present in the extracellular environment, where they engage cell-surface receptors at the surface of cells to initiate plasma-membrane-delimited signalling events that have been investigated extensively and which are under tight physiological control. Alterations in the trafficking of receptors to and from the plasma membrane have marked effects on signal transduction and cellular responsiveness: removal from the plasma membrane would be expected to diminish responsiveness, whereas the replenishment of cell-surface receptors enhances the effects of extracellular agonists. Upon activation, many GPCRs traffic to endosomes, a large and dynamic tubulovesicular network that extends throughout the cytoplasm. Endocytosed receptors then redistribute to lysosomes, where degradation terminates signalling, or traffic back to the plasma membrane, where recycling mediates resensitization. The view that endosomes are simply a conduit for receptor trafficking to degradatory or recycling pathways has been revised by the appreciation that receptors can continue to signal from endosomes by processes that are distinct from those operating at the plasma membrane, in terms of mechanism, regulation and physiological outcome [1]. However, compared with our understanding of plasma membrane signalling, far less is known about the intracellular mechanisms of GPCR signalling.

Most information about the relationship between GPCR trafficking and signalling derives from studies of model systems, usually transfected cell lines. These studies have identified molecular mechanism that control the trafficking
and signalling of GPCRs at the plasma membrane and in endosomes, and have characterized the signalling mechanisms that arise from receptors at these different locations. Although much of the information derived from these approaches is applicable to highly differentiated cell types such as neurons, little is known about the importance of GPCR trafficking for complex pathophysiological processes, such as inflammatory signalling, pain transmission and proliferation. The present review summarizes our understanding of the relationship between GPCR trafficking and signalling, and seeks to assess the importance of these processes in complex and integrated pathophysiological processes. The present review concerns the trafficking and signalling of receptors for neuropeptides, and discusses contributions of β-arrestins and metalloendopeptidases to inflammatory signalling of neuropeptide receptors.

**GPCR trafficking during pathophysiological conditions**

Investigation of the trafficking of GPCRs in *vivo* is hampered by the low levels of receptor expression and the difficulty in the specific detection of receptors using antibodies. However, the availability of highly selective antibodies to the SP (substance P) NK1R (neurokinin 1 receptor) has enabled detailed studies of trafficking of endogenously expressed receptors in primary cells and intact animals. SP is prominently expressed by primary spinal afferent neurons and enteric neurons, and, when released, activates the NK1R on diverse cell types to induce neurogenic inflammation and pain. Under basal conditions, the NK1R is mostly present at the plasma membrane of multiple cell types, including endothelial cells of post-capillary venules of the airways [2], myenteric neurons of the intestine [3] and neurons in the dorsal horn of the spinal cord [4,5]. However, within minutes of administration of exogenous SP or after activation of reflexes that release endogenous SP, the NK1R undergoes a dramatic alteration in its subcellular location. Thus the intravenous injection of SP to rats stimulates NK1R endocytosis in endothelial cells of post-capillary venules [2]. Similarly, mechanical stimulation of the intestinal mucosa, which evokes the reflex release of SP from enteric neurons, and the intraplantar injection of capsaicin, which activates nociceptive neurons of the dorsal root ganglia, induce NK1R endocytosis in enteric and spinal neurons respectively [4–6]. Diseases that are associated with SP release and NK1R endocytosis in enteric and spinal neurons respectively [4–6]. SP-conjugated toxins have been used to selectively ablate NK1R-expressing neurons in spinal cord, which revealed a major role for these neurons in pain transmission [8].

Other neuropeptides also internalize after stimulation with exogenous and endogenous agonists and during pathophysiological states. The administration of certain opioids and opiates triggers a robust internalization of μ-opioid receptors in neurons in the brain and the spinal cord, and endogenously released opioids also cause endocytosis, although to a more limited degree [9,10]. Transgenic mice expressing the δ-opioid receptor tagged with GFP (green fluorescent protein) have been invaluable for examining receptor trafficking in neurons in response to endogenous and exogenous opioids and opiates, thereby avoiding the use of antibodies of sometimes questionable specificity [11,12].

**β-Arrestin-dependent regulation of receptors at the plasma membrane**

The arrestin family of proteins include visual arrestins, arrestin-1 and arrestin-4, which are primarily localized in photoreceptors of the visual sensory tissue, and non-visual arrestin-2 and arrestin-3, respectively known as β-arrestin-1 and β-arrestin-2, which are ubiquitously expressed (reviewed in [13]). β-Arrestins were named for their capacity to arrest

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**Figure 1** | Redistribution of the NK1R in myenteric neurons of the inflamed colon

Whole mounts containing the myenteric plexus were prepared from the colons of IL-10−/− mice with or without piroxicam treatment (‘colitis’ and ‘control’ respectively). Tissues were collected in Krebs buffer containing the NK1R antagonist RP67580 (10−5 M) and tetrodotoxin (10−7 M) to reduce NK1R internalization during harvest. NK1R immunoreactivity was detected using established methods [45]. In controls, NK1R was detected at the cell surface of the soma and neurites, with low levels of intracellular staining. In contrast, NK1R was redistributed from the cell surface to endosomes in both locations in inflamed tissues. Scale bars, 10 μm.
signalling of β-adrenergic receptors, although they regulate many GPCRs, including receptors for neuropeptides. The α-arrestins comprise an ancient and larger family of proteins that share the arrestin-fold structure, although the functions of α-arrestins are still largely unknown.

The most well-defined function of β-arrestins is to regulate the signalling and trafficking of GPCRs at the plasma membrane [13] (Figure 2). Many activated receptors undergo rapid serine/threonine phosphorylation by G-protein receptor kinases. Phosphorylation serves to increase the affinity of receptors for β-arrestins, which translocate from the cytosol to interact with receptors at the plasma membrane. This interaction sterically hinders GPCR coupling to heterotrimeric G-proteins and thereby mediates receptor desensitization. β-Arrestins also couple GPCRs to the endocytic machinery, including clathrin and AP2 (adaptor protein 2). By mediating desensitization and endocytosis, β-arrestins act in general to terminate G-protein-dependent signalling of receptors at the plasma membrane. In this manner, β-arrestins control signalling of many GPCRs, including the NK₁R [14,15].

GPCRs fall into two classes on the basis of the affinity of their interactions with β-arrestins, which depends on the presence of phosphorylated serine/threonine residues within the receptor C-terminus and third intracellular loop. ‘Class A’ GPCRs (e.g. neurokinin 3 receptor, β₂-adrenergic receptor, bradykinin B₂ receptor and μ-opioid receptor) have few phosphorylation sites and interact with β-arrestin-2 with low affinity and transiently at or close to the plasma membrane. ‘Class B’ GPCRs (e.g. NK₁R, neurotensin receptor 1, vasopressin V₂ receptor and angiotensin ₁A receptor) are highly phosphorylated, and interact with both β-arrestin-1 and β-arrestin-2 with high affinity for prolonged periods in endosomes. The affinity of these interactions markedly influences agonist-mediated trafficking of GPCRs. Whereas class A GPCRs rapidly dissociate from β-arrestins and quickly recycle and resensitize, class B GPCRs slowly dissociate from β-arrestins in endosomes, and therefore exhibit delayed recycling and resensitization. Thus the NK₁R slowly recycles and resensitizes, whereas the NK₁R recycles and resensitizes more rapidly [16]. By sequestering β-arrestins in endosomes, class B GPCRs can impede the association of class A GPCRs with β-arrestins and thereby affect their trafficking and signalling. For example, the activated NK₁R sequesters β-arrestins in endosomes and thereby impedes the β-arrestin-dependent desensitization and endocytosis of the NK₁R [17]. Although these mechanisms were first identified in model cell
systems, they have been replicated in neurons. Thus NK1R activation blocks β-arrestin-mediated endocytosis of the NK1R in enteric neurons [17], and of the μ- opioid receptor in neurons of the amygdala and locus coeruleus [18]. Since most cells express multiple GPCRs, this mechanism of β-arrestin-mediated regulation of receptors is likely to be widespread, particularly in cell types or subcellular regions, such as nerve terminals, with limited expression of β-arrestins.

Although β-arrestins are best known for terminating plasma membrane signalling of GPCRs, they also contribute to resensitization of plasma membrane signalling. An established mechanism of β-arrestin-dependent resensitization involves receptor endocytosis and intracellular processing, which may entail the dissociation of ligand in acidified endosomes and receptor dephosphorylation by endosomal phosphatases. However, upon agonist exposure, a substantial portion of desensitized receptors remain at the cell surface and can resensitize by a β-arrestin-dependent process [19]. In the case of the NK1R, β-arrestins recruit PP2A (protein phosphatase 2A) to desensitized NK1R at the plasma membrane, where PP2A dephosphorylates the NK1R and thereby resensitizes plasma membrane signalling.

### β-Arrestin-dependent regulation of receptors in endosomes

In addition to interacting with multiple GPCRs, β-arrestins are scaffolding proteins that associate with functionally and structurally diverse proteins, including endocytic proteins, kinases, phosphatases, growth factor receptors and ion channels, at the plasma membrane and in intracellular locations. By recruiting some of these proteins to internalized GPCRs, β-arrestins can assemble multiprotein signalosomes that mediate a second wave of G-protein-independent, but β-arrestin-dependent, signalling from internalized receptors [13] (Figure 2). Examples of β-arrestin-mediated signalling events include: ERK (extracellular-signal-regulated kinase)-dependent regulation of apoptosis, survival, translation and proliferation [14,20–22]; PP2A-mediated phosphorylation of Akt, which leads to activation of glycogen synthase kinase 3 and dopaminergic behaviour [23]; PI3K (phosphoinositide 3-kinase)-mediated phospholipase A2 activation and vasodilatation [24]; and inhibition of NF-κB (nuclear factor κB) gene expression through stabilization of IκB [25]. Although some β-arrestin-mediated signalling events emanate from GPCRs at the plasma membrane and in endosomes, the precise subcellular location of β-arrestin signals is not always well defined.

The list of signalling systems that utilize β-arrestins keeps growing, and now includes receptors that are atypical GPCRs, such as Smoothened and Frizzled receptors, or that do not belong to the GPCR family, including the type III transforming growth factor-β receptor and cytokine receptors [13]. These findings strongly suggest that the functional roles of β-arrestins are much broader than currently understood. Of note for inflammation and pain, β-arrestin-1 interacts with the TRPV4 (transient receptor potential vanilloid 4) ion channel to promote channel ubiquitination and functional down-regulation [26]. Since the activation of this channel promotes inflammation and pain, the interaction with β-arrestin-1 could terminate inflammatory and nociceptive signalling.

### β-Arrestin-dependent inflammatory signalling

β-Arrestins are ubiquitous and interact with multiple GPCRs, as well as other receptors, channels and downstream signalling proteins that control inflammatory signalling. Whereas β-arrestins terminate plasma membrane signalling, they can transmit distinct signals by internalized receptors. In the light of this complexity, it is difficult to predict whether β-arrestins initiate or terminate inflammation, and the overall contribution of β-arrestins to such a complex pathophysiological process may depend on the receptors and β-arrestin isoform involved and the exact stage of the process. However, studies of mice lacking β-arrestin-1 or β-arrestin-2 have revealed their roles in inflammation.

β-Arrestins have an established role in the recruitment and activation of immune cells at sites of inflammation and injury. β-Arrestins have been implicated in chemokine-receptor-induced granule release from neutrophils [27]. β-Arrestin-2 deletion enhances neutrophil recruitment and wound healing, which suggests that β-arrestin-2 is a negative regulator of the chemokine receptor CXCR2 [28]. However, T-cells from β-arrestin-2-knockout mice exhibit impaired migration, and in a model of allergic inflammation of the airways, β-arrestin-2 deletion suppresses migration of Th2 cells and inhibits production of cytokines and mucin [29,30]. Conversely, β-arrestin-2 deletion does not affect leucocyte recruitment and cytokine production induced by PAR2 (protease-activated receptor 2), which has protective anti-constrictor effects through β-arrestin-2-independent mechanisms [29]. β-Arrestins have also been implicated in inflammation of the colon, where PAR2 agonists induce the disassembly of tight junctions and increase paracellular epithelial permeability of colonocytes by a mechanism that requires β-arrestin-dependent activation of ERK1/2 [31]. This mechanism promotes the paracellular transit of macromolecules and bacteria from the colonic lumen, which would be expected to promote colonic inflammation. Consistent with these pro-inflammatory effects, β-arrestin-2 deletion is protective in sepsis [32]. However, in a model of anti-collagen antibody-induced arthritis in mice, β-arrestin-2-knockout mice exhibited a more severe inflammation, suggesting a protective role for β-arrestin-2 [33].

In addition to the migration of inflammatory cells, β-arrestins control the migration of tumour cells. Metastatic breast cancer cells can secrete a trypsin-like proteases that promotes cell migration by autocrine activation of PAR2 and assembly of a PAR2–β-arrestin-2–ERK1/2 complex that is required for reorganization of the cytoskeleton, extension of pseudopodia and chemotaxis [34]. β-Arrestin-2 deletion promotes tumour growth and angiogenesis in a heterotopic
murine model of lung cancer by regulating inflammation via activation of CXCR2 and NF-κB [35]. In another murine model of endotoxiaemia induced by lipopolysaccharide, β-arrestin-1 and β-arrestin-2 have been shown to regulate cytokine secretion in a cell-type-specific manner where both β-arrestins have overlapping, but non-redundant, roles in regulating inflammatory cytokine production by a MAPK (mitogen-activated protein kinase)-independent mechanism [36]. In particular, deletion of β-arrestin-2 decreased production of KC (keratinocyte chemotactant) (IL-8 murine analogue) and of PGE₂ (prostaglandin E₂).

**Metalloendopeptidase-dependent regulation of receptors at the plasma membrane**

The degradation of neuropeptides in the extracellular fluid by cell-surface metalloendopeptidases provides a second mechanism, in addition to β-arrestin-mediated receptor desensitization, that can terminate plasma membrane signalling of neuropeptides (Figure 2). For example, by degrading SP at the cell surface, the membrane metalloendopeptidase neprilysin restricts activation of the NK₁R [37]. By similar mechanisms, neprilysin and other cell-surface peptidases, such as angiotensin-converting enzyme and ECE (endothelin-converting enzyme) can attenuate the biological actions of neuropeptides in multiple systems.

**Metalloendopeptidase-dependent regulation of receptors in endosomes**

ECE-1 is a neprilysin-related membrane metalloendopeptidase that is best known for its role in converting and activating the precursor big endothelin into endothelin 1, a major regulator of systemic blood pressure and sodium homeostasis. However, in a manner that is analogous to the role of neprilysin at the plasma membrane, ECE-1 regulates the trafficking and signalling of neuropeptide receptors in endosomes (Figure 2). ECE-1 exists as four isoforms (a–d) that share a catalytic domain but have distinct, but overlapping, subcellular distributions. Although all forms are found in early endosomes, the a and c isoforms are located predominantly at the plasma membrane, and the b and d isoforms are mainly located in the endosomes. All isoforms are generated from a single gene through different promoters that regulate the expression of their unique N-terminus, which specifies their subcellular distribution. Of note, ECE-1 preferentially degrades certain neuropeptides at an acidic pH, similar to endosomal pH, including SP, CGRP (calcitonin gene-related peptide), somatostatin-14, neurotensin and bradykinin [38]. By degrading neuropeptides in endosomes, ECE-1 can regulate the trafficking and signalling of internalized GPCRs. Thus ECE-1 can degrade SP, CGRP, somatostatin-14 and neurotensin in acidified early endosomes, which promotes disassembly of the endosomal peptide–receptor–β-arrestin and MAPK signalosomes, allowing β-arrestins to return to the cytosol and initiating receptor recycling and resensitization of plasma membrane signalling [39–43]. By the same process of signalosome disassembly, ECE-1 can terminate β-arrestin-mediated endosomal MAPK signalling of the NK₁R [39]. Although this regulatory mechanism was initially defined in model cell systems, it also operates in primary neurons and endothelial cells that naturally express ECE-1 and the GPCRs for neuropeptides [44,45]. However, the mechanism is selective for those neuropeptides that are ECE-1 substrates at endosomal pH, and for those receptors that exhibit sustained and high-affinity interactions with β-arrestins in endosomes (class B GPCRs).

**Metalloendopeptidase-dependent inflammatory signalling**

Some of the neuropeptides that are regulated by neprilysin at the plasma membrane and ECE-1 in endosomes play a causative role in inflammation, including SP, CGRP and neurotensin. Thus metalloendopeptidases may regulate inflammatory signalling of neuropeptides.

The contribution of neprilysin to regulating inflammation has been examined by studying neprilysin-deficient mice or by treating animals with neprilysin inhibitors. Neprilysin-knockout mice exhibit spontaneous inflammatory oedema that is caused by diminished degradation of the pro-inflammatory neuropeptides SP and bradykinin [46]. Neprilysin-knockout mice show markedly exacerbated colitis [47], ileitis [48] and dermatitis [49], and recombinant neprilysin ameliorates inflammation [47].

By degrading neuropeptides in endosomes and disassembling the β-arrestin signalosomes, ECE-1 promotes resensitization of plasma membrane signalling, yet terminates β-arrestin-mediated endosomal signalling. Thus the contribution of ECE-1 to inflammation will depend on the relative importance of plasma membrane compared with endosomal signalling to the inflammatory process. The role of ECE-1 in inflammation has been examined using ECE-1 inhibitors. ECE-1 inhibitors prevent resensitization of SP-induced plasma extravasation in rats, in line with the inhibition of recycling and resensitization of the NK₁R. Moreover, ECE-1 inhibitors suppress trinitrobenzenesulfonic acid-induced colitis in mice (N.W. Bunnett, unpublished work), which is mediated by SP and the NK₁R [47].

The protective role of ECE-1 inhibition in inflammation and tissue injury has been demonstrated also in another experimental model of colitis, induced by ingestion of dextran sodium sulfate [50]. Considered together, these results suggest that ECE-1 acts primarily to promote the resensitization of plasma membrane signalling of the NK₁R, which is necessary for the sustained pro-inflammatory actions of SP. However, it is also possible that an ECE-1 inhibitor blocks the activation of endothelin, and thereby improves colitis-induced vascular dysfunction and tissue injury.

Recent observations suggest that ECE-1 similarly controls inflammatory signalling of neurotensin. Neurotensin promotes inflammation and proliferation in the intestine.
by activating the high-affinity neurotensin 1 receptor. Neurotensin promotes endocytosis and recycling of the neurotensin 1 receptor in human colonocytes, and ECE-1 inhibition blocks receptor recycling [40]. Neurotensin stimulates pro-inflammatory signalling in colonocytes, including activation of ERK1/2, JNK (c-Jun N-terminal kinase) and NF-κB, and release of IL-8. ECE-1 inhibitors suppress the pro-inflammatory signalling of neurotensin by suppressing receptor recycling, which is necessary for the sustained neurotensin signalling that is required for inflammation. However, β-arrestins also contribute to inflammatory signalling, which is suppressed by selective knockdown of β-arrestin-1 or β-arrestin-2 [40].

Conclusions

Given its fundamental importance, the signalling of GPCRs at the plasma membrane is under tight physiological control and has been a subject of intensive investigation. However, it is now clear that many activated GPCRs internalize, and that internalized receptors can continue to signal by processes that are distinct from those that occur at the plasma membrane in terms of mechanism, regulation and physiological outcome. β-Arrestins have divergent roles in regulating signalling by cell-surface and internalized GPCRs. Whereas β-arrestins negatively regulate plasma membrane signalling by mediating receptor desensitization and endocytosis, β-arrestins positively transmit signals from internalized receptors. In the case of neuropeptide signalling, membrane metalloendoproteinases also differentially regulate signalling from the plasma membrane and endosomes. Thus nephilysin can terminate neuropeptide signalling at the cell surface, whereas endosomal ECE-1 can terminate endosomal signalling yet promote the resensitization of plasma membrane signalling.

There remains much to be learnt about the mechanisms and importance of GPCR signalling at specific subcellular locations. In particular, the relative importance of plasma membrane and intracellular GPCR signalling to complex processes such as inflammation, pain and proliferation/cancer is not well understood. However, an understanding of processes such as inflammation, pain and proliferation/cancer membrane and intracellular GPCR signalling to complex endosomal signalling yet promote the resensitization of internalized receptors. In the case of neuropeptide signalling,

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