Trends in Pharmacological Sciences

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Review

Pain Signaling by GPCRs and RTKs

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Chronic pain is common and debilitating, yet is inadequately treated by current therapies, which can have life-threatening side effects. Treatments targeting G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs), key pain mediators, often fail in clinical trials for unknown reasons. Here, we discuss the recent evidence that GPCRs and RTKs generate sustained signals from multiprotein signaling complexes or signalosomes in intracellular compartments to control chronic pain. We evaluate the evidence that selective antagonism of these intracellular signals provides more efficacious and long-lasting pain relief than antagonism of receptors at the surface of cells. We highlight how the identification of coreceptors and molecular scaffolds that underpin pain signaling by multiple receptors has identified new therapeutic targets for chronic pain, surmounting the redundancy of the pain signaling pathway.

Highlights

G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) are key pain mediators, yet therapies designed to target these receptors at the plasma membrane often fail in clinical trials.

Accumulating recent evidence suggests that GPCRs and RTKs generate sustained signals from multiprotein signaling complexes or signalosomes in intracellular compartments of nociceptors and associated Schwann cells that control chronic pain. This realization raises new challenges and opportunities for improved analgesia.

Therapies designed to target GPCR signalosomes in endosomes of nociceptors and Schwann cells provide more efficacious and long-lasting pain relief than conventional antagonists of plasma membrane receptors.

The discovery of coreceptors and molecular scaffolds that underpin pain signaling by several RTKs has identified new therapeutic targets for chronic pain, surmounting the redundancy of the pain signaling pathway.

Chronic pain: mechanisms, challenges, and therapeutic advances

Chronic pain (see Glossary) is common, debilitating, and poorly understood [1,2]. Existing therapies are often ineffective and have life-threatening or fatal side effects. This review highlights recent advances in our understanding of the mechanisms by which receptors signal chronic pain and discusses how this deepened knowledge can lead to more effective and safer treatments for chronic pain.

Chronic pain is a hallmark of disease (cancer, diabetes, autoimmune disorders, and migraine), a consequence of injury and infection (nerve injury, and viral and bacterial infections), and a side effect of therapy (chemotherapy-induced peripheral neuropathy) [2]. Despite afflicting 30% of the global population, the mechanisms of chronic pain are poorly understood and existing therapies are inadequate. The analgesic efficacy of opioids, used for millennia to treat pain, wanes with use (tolerance), whereas their side effects of respiratory depression, sedation, and constipation worsen due to dose escalation and addiction, accounting for >80 000 deaths in the USA in 2023 [3]. By inhibiting the synthesis of prostaglandins (PGs), nonsteroidal anti-inflammatory drugs, with 30 billion annual doses in the USA, provide relief from inflammatory pain but delay its resolution and have life-threatening adverse actions on the digestive, cardiovascular, and renal systems [4]. The redundancy of the receptors that control pain may limit the efficacy of selective agents.

GPCRs and **RTKs** function cooperatively to control pain and are therapeutic targets for pain [5,6] (Figure 1). GPCRs are seven transmembrane domain proteins that interact with diverse extracellular ligands, ranging from ions to proteases, and couple to heterotrimeric G proteins. RTKs are single transmembrane domain proteins with intrinsic tyrosine kinase activity that mediate the actions of growth factors and are therapeutic targets for cancer. GPCRs and RTKs that are expressed by neurons of the pain pathway regulate excitability and the transmission of painful signals. There is a growing appreciation that receptors on Schwann cells that ensheathe nerves also control pain transmission (Box 1). However, although GPCRs and RTKs are mediators and therapeutic targets for chronic pain, most therapies fail in clinical trials. Inadequate knowledge of the

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mechanisms of sustained GPCR and RTK signaling that underlies chronic pain coupled with the inherent redundancy of pain mechanisms may contribute to this failure.

Here, we discuss recent advances in our understanding of how GPCRs and RTKs generate long-lasting signals from multiprotein complexes or **signalosomes** to control chronic pain. We discuss the evidence that therapeutic targeting of receptors in signalosomes can enhance analgesic efficacy of failed drug candidates while reducing side effects and surmounting the redundancy of pain signaling mechanisms.

Mechanisms and therapeutic targeting of GPCR pain signaling

Mechanisms of GPCR pain signaling

Agonist-bound GPCRs at the plasma membrane activate membrane-tethered G proteins, resulting in activation or inhibition of adenylyl cyclase (AC) or phospholipase C-β (PLC-β), which control levels of second messengers. GPCR signaling at the plasma membrane is tightly controlled and often transient. **GPCR kinases** phosphorylate activated receptors, increasing their affinity for **β-arrestins** [7]. β-Arrestins terminate plasma membrane signaling by disrupting GPCR/G protein association and by coupling GPCRs to the **clathrin endocytic machinery** (Figure 2). Several GPCRs that control pain internalize when activated, including neurokinin 1 receptor (NK_1R) for substance P (SP) [8], calcitonin-like receptor (CLR) for calcitonin gene-related peptide (CGRP) [9], the δ-opioid receptor, DOR) [10], neurotensin-1 receptor [11], and protease-activated receptor-2 (PAR_2) [12,13]. GPCR endocytosis occurs in conscious animals in response to endogenous agonists during pain and inflammation and is thus a physiological process [8,12,14]. Although β-arrestins mediate clathrin-dependent endocytosis of many receptors, some GPCRs internalize by β-arrestin and clathrin-independent mechanisms [15]. The dopamine D_2 receptor, which modulates pain, interacts with the PDZ adaptor protein GAIP/RGS19-interacting protein 1 (GIPC1), which couples the receptor to the myosin VI molecular motor to trigger β-arrestin-independent endocytosis [16]. Myosin-VI-mediated endocytosis dampens G protein signaling at the plasma membrane while activating extracellular signal regulated kinase1/2 (ERK1/2) signaling in **endosomes**. Not all pain-sensing GPCRs internalize. The prostaglandin E2 (PGE_2) EP2 receptor signals from the plasma membrane of Schwann cells, where AC generates a local cAMP signal that activates protein kinase (PK)A to sustain inflammatory pain [17]. The μ-opioid receptor (MOR) in neurons interacts the adaptor Tubby-like protein 3, which localizes the receptor to primary cilia, microdomains extending from the plasma membrane [18].

Many ligand-bound GPCRs generate sustained signals from **early endosomes (EEs)** that persist after plasma membrane signals fade [19] (Figure 2). Evidence that GPCRs signal in EEs derives from the use of genetically encoded biosensors that detect active conformations of GPCR and G proteins (nanobodies, mini-Gα biosensors), activated G proteins (G protein effector membrane translocation biosensors), and signaling effectors (β-arrestins) in subcellular compartments using microscopy and **bioluminescence resonance energy transfer (BRET)** assays. Investigations of the effects of endocytosis inhibitors on second messenger formation and kinase activity, measured using **Förster resonance energy transfer (FRET)** biosensors, provides evidence that GPCRs in endosomes activate effectors and second messengers in subcellular compartments. GPCRs in EEs were initially considered to signal principally *via* β-arrestins, which **scaffold** components of the mitogen-activated protein kinase pathway to activated GPCRs [20]. Recent work provides evidence for $G\alpha_s$-, $G\alpha_i$-, and $G\alpha_q$-mediated signaling of GPCRs in endosomes, including the NK_1R [8], CLR [9], PAR_2 [12–14], MOR and DOR [10,21,22]. Limitations to the use of biosensors include the requirement to overexpress receptors and biosensors, often in cell lines, the finding that different biosensors can yield disparate results [23], and the report that overexpression of mini-G proteins can disrupt GPCR trafficking and

Glossary

β-Arrestins: proteins that interact with phosphorylated GPCRs and desensitize plasma membrane signaling, mediate endocytosis, and recruit and organize signaling effectors.
BRET assay: detects the proximity of proteins in living cells that is based on the transfer of energy from a light-emitting protein (usually tagged with luciferase) to a light-sensitive molecule (usually a tagged with a fluorescent protein).
Chronic pain: persistent pain lasting >3 months.
Clathrin-mediated endocytosis: mechanism for internalizing GPCRs and RTKs from the plasma membrane and delivering cargo to endosomes.
Early endosomes (EEs): organelles that receive cargo, including GPCRs, RTKs, and their ligands from the plasma membrane. Early endosomes sort cargo to recycling or degradatory pathways.
Endosomes: membrane-bound organelles that derive from clathrin and dynamin-mediated internalization of GPCRs and RTKs from the plasma membrane.
FRET assay: detects second messengers and kinases in living cells that is based on the transfer of energy between light-sensitive molecules, from a donor to an acceptor fluorophore.
GPCR kinases: phosphorylate intracellular serine and threonine residues of activated GPCRs and thereby increase affinity for β-arrestins.
G protein-coupled receptors (GPCRs): seven transmembrane domain receptors that interact with many hormones and receptors to mediate homeostasis and detect photons, odorant and teste molecules to mediate environmental sensing. GPCRs couple to heterotrimeric G proteins and are the single largest target of therapeutic drugs.
Nanodomain: nanometer-sized assembly of proteins (i.e., signalosomes, see later) associated with plasma and endosomal membranes that are sites of GPCR and RTK signaling.
Nanoparticles (NPs): small carriers, often <200 nm, which can be used to encapsulate drug molecules and facilitate their selected delivery to diseased tissues, cells and organelles.
Nociceptors: sensory neurons that are specialized to detect harmful chemical, thermal, and mechanical stimuli and convey information to the central nervous system.

signaling [24]. Studies of signaling of endogenous GPCRs in primary cells and intact animals will be required to determine the physiological relevance of GPCR signaling in subcellular compartments to pain.

Signaling requires the coincidence of GPCRs, G proteins and effectors in the same membrane compartment. Although GPCR signaling pathway at the plasma membrane is well understood, less is known about the mechanisms by which GPCRs activate G proteins, AC, and PLC-β in EEs. For some GPCRs, endosomal signaling is a continuation of plasma membrane signaling, whereby GPCRs, G proteins and effectors traffic from the plasma membrane to endosomes. Several GPCRs activate $G\alpha_s$ in endosomes. Agonists of the $G\alpha_s$-coupled β_2 adrenergic receptor stimulate dynamin-dependent endocytosis of receptors and AC9 [25]. While β-arrestins mediate receptor endocytosis, AC9 traffics to endosomes by a β-arrestin-independent process. In striatal neurons, the activated D_1 receptor and AC9 are found in endosomes that form an intertwined network with Golgi-associated PKA in a juxtanuclear region [26]. This signaling complex preferentially activates protein kinase A in the nucleus, linking endosomal signaling to transcription. MOR, which couples to $G\alpha_i$, increases endosomal G protein activity in a different manner [21]. MOR activation transiently increases the active state of $G\alpha_{i/o}$ at the plasma membrane, followed by a sustained increase in activity in endosomes. In contrast to $G\alpha_s$-coupled GPCR, the MOR-induced

Receptor tyrosine kinases (RTKs): single transmembrane domain receptors for peptides and growth factors with intracellular tyrosine kinase domains. RTKs regulate metabolism, cell growth, and differentiation, and are validated therapeutic targets for the treatment of cancer.
Scaffold protein: recruits and organizes components of a signalosome, thereby enhancing the efficiency of signal transduction.
Signalosome: large protein complex comprising GPCRs or RTKs, signaling effectors and scaffolding proteins. Signalosomes mediate receptor signals from subcellular nanodomains at plasma and endosomal membranes.

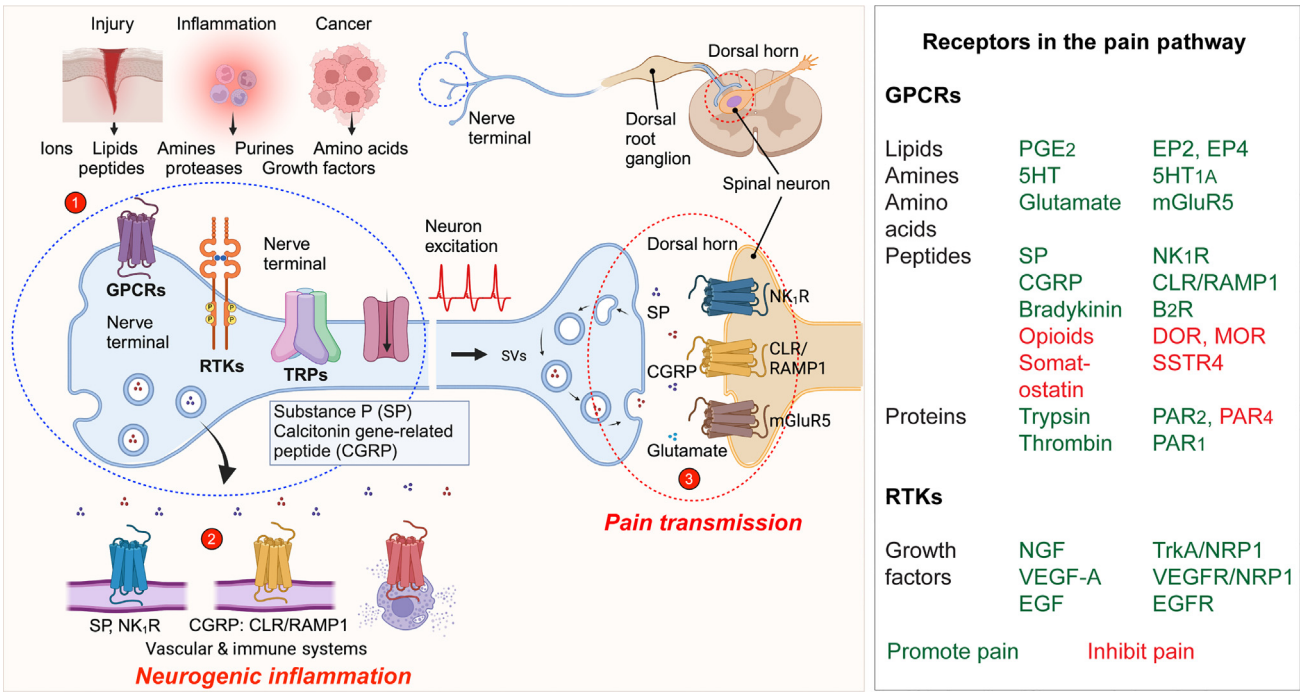

Figure 1. GPCRs and RTKs in the pain pathway. (1). Agonists released from diseased and damaged tissues activate GPCRs and RTKs expressed by the peripheral terminals of nociceptors with cell bodies in dorsal root ganglia. (2). SP and CGRP, which are released from the peripheral terminals of nociceptors, activate GPCRs in the vasculature and on immune cells to evoke plasma extravasation, vasodilation, and immune cell infiltration and activation, leading to neurogenic inflammation. (3). SP, CGRP, and glutamate released from synaptic vesicles (SVs) in the central projections of nociceptors in the dorsal horn of the spinal cord activate GPCRs on second order spinal neurons to trigger the central transmission of painful signals. The table shows selected GPCRs and RTKs that stimulate (green) or inhibit (red) pain. Abbreviations: 5HT, 5-hydroxytryptamine; B_2R, bradykinin 2 receptor; CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; DOR, δ-opioid receptor; EGF, epidermal growth factor; EGFR, EGF receptor; GPCR, G protein-coupled receptor; MOR, μ-opioid receptor; NGF, nerve growth factor; NK_1R, neurokinin 1 receptor; NRP1, neuropilin 1; PAR, protease-activated receptor; PGE_2, prostaglandin E_2; RTK, receptor tyrosine kinase; SP, substance P; TRP, transient receptor potential; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Box 1. Pain signaling in Schwann cells

- Although primary sensory neurons are indispensable for pain signaling, a proalgesic role of peripheral glial cells, including Schwann cells that surround nociceptors, is emerging. Schwann cells accompanying cutaneous nerve fibers to their epidermal terminals have been proposed as a subset of glial cells that are specialized to signal mechanical hypersensitivity [75,76]. Many receptors and ion channels that control pain signaling in neurons are also expressed by Schwann cells, where they indirectly regulate the activity of neurons to control pain.
- Global deletion of the wasabi receptor, the transient receptor potential ankyrin 1 (TRPA1) ion channel, not only attenuates pain but also reduces neuroinflammation, illustrated by effects on influx of macrophages and oxidative stress in a mouse model of neuropathic pain [77]. Selective silencing of TRPA1 in Schwann cells attenuates mechanical allodynia and neuroinflammation, while neuronal TRPA1 silencing reduces allodynia but not neuroinflammation. A feed-forward mechanism driven by Schwann cell TRPA1 has been proposed, which amplifies oxidative stress that sustains neuroinflammation and ensuing mechanical allodynia. Schwann cell TRPA1 has also been implicated in cancer pain [78] and in the painful alcoholic neuropathy that is associated with formation of acetaldehyde, a TRPA1 agonist [79].
- Schwann cells also contribute to CGRP-mediated migraine-like pain [9]. Deletion of the CGRP receptor, which comprises CLR (a GPCR) and receptor activity-modifying protein 1 (a chaperone), from Schwann cells ensheathing trigeminal nociceptors blocks CGRP-evoked preorbital mechanical allodynia in mice. CGRP stimulates CLR endocytosis and endosomal signaling in Schwann cells that generates cAMP to evoke mechanical allodynia. Inhibitors of **clathrin-mediated endocytosis** and NPs encapsulating a CLR antagonist block endosomal CGRP signaling and suppress CGRP-evoked allodynia, with relevance to therapies for migraine pain [9].
- Recent work has identified a key role for Schwann cells in PGE_2-evoked inflammatory pain in mice [17]. Selective silencing of the PGE_2 EP2 receptor (a GPCR) in Schwann cells and optogenetic activation of AC or phosphodiesterase in Schwann cells have provided evidence that EP2 receptor signals from plasma membrane nanodomains to mediate PG-evoked inflammatory pain. Inhibitors of clathrin-mediated endocytosis do not affect EP2 pain signaling, which originates from plasma membrane signalosomes. Antagonism of the EP2 receptor in Schwann cells ameliorates PGE_2-stimulated inflammatory pain, avoiding the detrimental consequences of suppressing PG synthesis with nonsteroidal anti-inflammatory drugs that inhibit cyclooxygenase enzymes [17].
- Thus, cAMP signals in different subcellular compartments of Schwann cells contribute to pain signaling. While CGRP-mediated migraine-like pain depends on CLR and cAMP signaling in endosomes [9], PG-mediated inflammatory pain requires EP2 and cAMP signaling from plasma membrane nanodomains [17].

increase of active-state $G\alpha_{i/o}$ in endosomes occurs independently of MOR internalization or MOR activation in endosomes. Agonists of angiotensin II type 1, bradykinin B_2, oxytocin, thromboxane A2α, and muscarinic M_3 receptors activate $G\alpha_{q/11}$ in endosomes by receptor endocytosis-dependent and -independent mechanisms [27]. Analysis of the distribution of endogenous G proteins in cell lines found constitutive endocytosis sufficient to supply nascent endocytic vesicles with 20–30% of the plasma membrane G protein density [28]. G proteins were detected in EEs, late and recycling endosomes, and lysosomes.

EEs are not the sole intracellular site of GPCR signaling. Membrane-permeant opioids (morphine) activate opioid receptors (ORs) in the Golgi apparatus by mechanisms distinct from those operating at the plasma membrane. MOR and DOR are phosphorylated and couple to $G\alpha_i$ in the Golgi apparatus but do not recruit β-arrestins in contrast to plasma membrane receptors [29]. Differences in the lipid composition between the plasma membrane and the Golgi apparatus may underlie these patterns of signaling that lead to differential effects on transcription and protein phosphorylation. The functional relevance of GPCR activation in the Golgi apparatus is unclear, where signaling may be hindered by the low levels of G proteins [28]. Gβγ signaling in the Golgi apparatus has been implicated with mobilization of PAR_2 stores, which sustains the pronociceptive actions of extracellular proteases [30].

Determination of the structure of a GPCR, Gα, and β-arrestin megaplex by cryoelectron microscopy provides an understanding of GPCR signaling in endosomes at even higher resolution [31]. The activated GPCR can simultaneously interact with Gα, which engages the receptor core, and β-arrestin, which associates with the phosphorylated receptor C-tail, providing a structural basis for sustained GPCR endosome signaling [32].

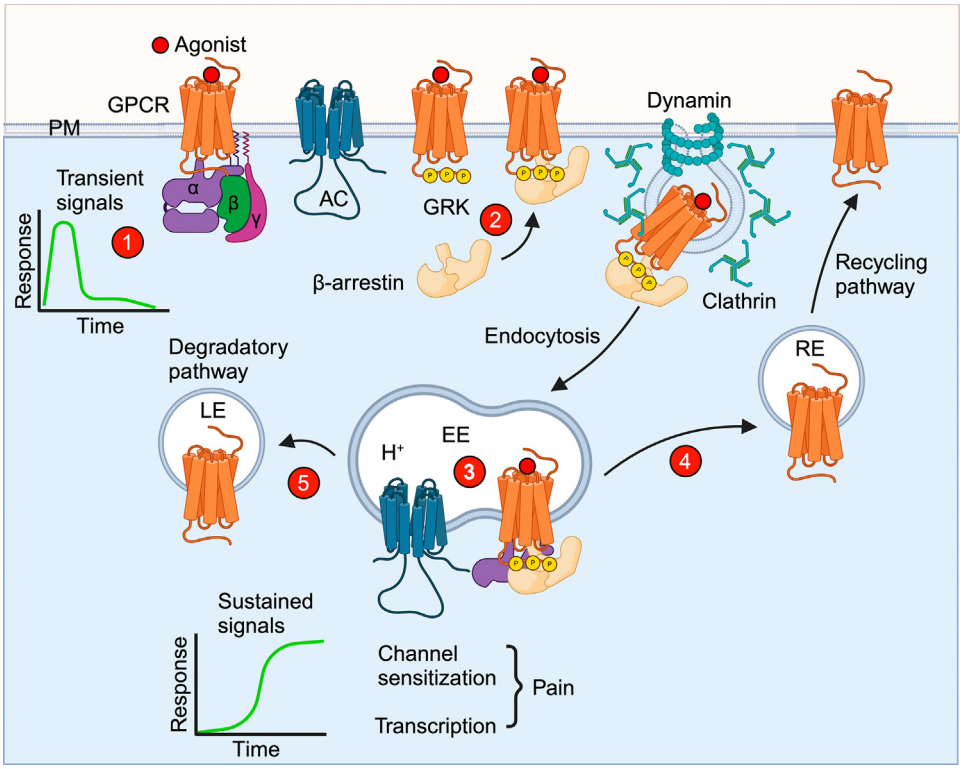

Figure 2. Mechanisms of G protein-coupled receptor (GPCR) signaling of pain from plasma membrane and early endosome (EE) nanodomains. (1). Agonist-bound GPCRs at the plasma membrane (PM) couple to heterotrimeric G proteins that activate effectors, including adenylyl cyclase (AC) in the case of $G\alpha_s$-coupled receptors. (2). GPCR kinases (GRKs) phosphorylate activated GPCRs, increasing their affinity for β-arrestins, which mediate G protein uncoupling and receptor endocytosis. These processes rapidly terminate plasma membrane signaling. (3). (4 and 5). GPCRs traffic back to the plasma membrane via recycling endosomes (REs) or to lysosomes for degradation via late endosomes (LEs). In EEs, GPCR, Gα, and β-arrestin signalosomes or megaplexuses activate effectors and second messengers in subcellular compartments, leading to expression of genes and sensitization of channels that underlie sustained pain.

The contributions of GPCR signaling from plasma membrane and EE to nociception have been determined using endocytosis inhibitors and endosome-targeted antagonists (described later). Clathrin and dynamin inhibitors prevent SP-stimulated NK_1R endocytosis and suppress nuclear ERK activity and cytosolic PKC activity and cAMP levels [8]. Endocytosis and ERK1/2 inhibitors prevent SP-induced activation of spinal neurons and, when injected intrathecally, endocytosis inhibitors blunt nociception in rodents [8]. Endocytosis inhibitors also prevent the recycling of synaptic vesicles in presynaptic terminals of **nociceptors**, which is required for the maintenance of neurotransmission in nociceptive circuits of the spinal cord [33]. Mice expressing C-terminally truncated human NK_1R, corresponding to a natural variant with aberrant signaling and trafficking, display attenuated SP-evoked excitation of spinal neurons and diminished nociceptive responses to SP, providing a link between NK_1R endocytosis and pain signaling [34]. Endocytosis inhibitors similarly block CGRP pain signaling in spinal neurons [35] and Schwann cells ensheathing trigeminal nociceptors [9], and suppress trypsin-evoked sensitization of nociceptors and mechanical allodynia in mice, which is attributable to PAR_2 endosomal signaling [12,14].

Endocytosis inhibitors do not block the pronociceptive actions of all GPCRs. Cathepsin S and elastase cleave PAR_2 at different sites from trypsin and activate the receptor by biased

mechanisms that neither recruit β-arrestins nor evoke PAR_2 endocytosis. Accordingly, endocytosis inhibitors do not affect cathepsin S or elastase-evoked nociception [14]. Although endocytosis inhibitors block the pronociceptive actions of PGE_2 EP4 receptors, which internalize when activated, they do not blunt pronociceptive responses to EP2 receptors, which signals pain from the plasma membrane of Schwann cells [17].

Therapeutic targeting of GPCR pain signaling

The realization that persistent GPCR signaling from EEs underlies pain raises challenges and opportunities for the improved analgesia. One challenge in developing endosomal-targeted therapies is achieving sufficient antagonist penetration of endosomes without inducing off-target effects in other tissues. Continued agonist production during chronic pain evokes the redistribution of GPCRs from the plasma membrane to EEs [8,10,12]. Reversal of GPCR endosomal signals requires that antagonists penetrate plasma and endosomal membranes and engage with conformations of GPCRs within multiprotein signaling complexes of acidified endosomes. The failure of antagonists, routinely characterized by their ability to bind and inhibit plasma membrane GPCRs, in clinical trials of chronic pain may relate to their ability to engage with internalized GPCRs. The preferential delivery of antagonists to endosomes creates an opportunity for improved treatment of pain without the side effects associated with the systemic delivery of antagonists. Several approaches, described later, have been devised to antagonize intracellular GPCR signaling of pain (Figure 3).

Local (intrathecal, periorbital, and intracolonic) administration of inhibitors of dynamin-1,2,3 or adaptor-associated kinase-1 ameliorates nociception in preclinical models of inflammatory, neuropathic and migraine pain by blunting GPCR endosomal signaling and inhibiting endocytosis of

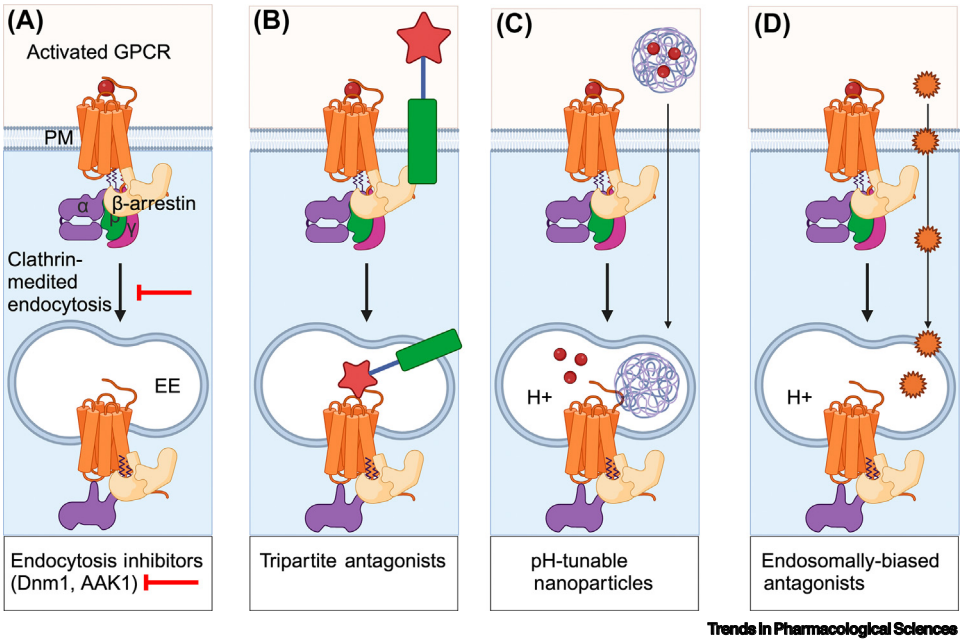

Figure 3. Therapeutic targeting of G protein-coupled receptor (GPCR) signaling of pain in early endosomes (EEs). Therapeutic strategies to blunt GPCR signaling of pain in EEs include: (A) inhibitors of clathrin-mediated endocytosis; (B) tripartite antagonists, where a transmembrane lipid promotes incorporation and retention in endosome membranes; (C) pH-tunable nanoparticles (NPs) designed to enter and accumulate in endosomes, where acidification evokes NP disassembly and antagonist release; and (D) endosomally biased antagonists with altered pKa and lipophilicity to promote endosomal targeting and retention. Abbreviations: EE, early endosome; PM, plasma membrane.

synaptic vesicles [8,9,12,33] (Figure 3A). Adaptor-associated kinase-1 inhibitors are being developed for disorders, including pain [36].

Conjugation of GPCR antagonists to transmembrane lipids enhances delivery to EEs (Figure 3B). Tripartite probes, comprising an antagonist, a linker, and cholestanol, accumulate in EEs [8,14,35,37]. Tripartite NK_1R, CLR, and PAR_2 antagonists cause a long-lasting inhibition of endosomal signaling and activation of nociceptors and spinal neurons, and have more persistent antinociceptive actions than non-lipidated counterparts.

The propensity of **nanoparticles (NPs)** to enter cells by endocytosis has been leveraged to deliver antagonists to GPCRs in endosomes for treatment of pain [38–40] (Figure 3C). NPs have been used to deliver anticancer drugs, where surface modifications to enhance tumor targeting and incorporation of features that promote NP disassembly in the cancer microenvironment reduce dosing, minimizing systemic exposure and side effects [41]. For chemotherapeutics against extra-endosomal targets, the necessity and challenges of endosomal escape complicate NP-mediated delivery. The identification of GPCR targets in EEs obviates this need and allows development of NP formulations for treatment of pain. pH-tunable NPs engineered to disassemble and release the hydrophobic NK_1R antagonist, aprepitant, in acidic EEs cause long-lasting inhibition of SP-evoked endosomal signaling and activation of spinal neurons [42,43]. When injected intrathecally, NP-aprepitant provides efficacious and sustained reversal of pain in rodents, whereas unencapsulated aprepitant is minimally efficacious. Similar NP formulations of the CLR antagonist, MK-43207, provide long-lasting inhibition of CGRP-stimulated endosomal signaling in Schwann cells and inhibit CGRP-induced periorbital mechanical allodynia, relevant to migraine [9]. Limitations of pH-tunable NPs include premature disassembly in the acidified extracellular fluid of diseased tissues, and the immediate release of cargo in acidic organelles, which may limit the duration of action. Dendrimer and core-shell polymeric NPs have been developed to circumvent these limitations by releasing antagonist for days in a non-pH-dependent fashion. These NP formulations of the negative allosteric modulator of PAR_2, AZ3451, reverse activation of PAR_2, $G\alpha_q$, and β-arrestins in EEs, and provide effective and long-lasting reversal of inflammatory pain of the colon after luminal administration, whereas unencapsulated AZ3451 is largely ineffective [13]. NP-AZ3451 more effectively relieves oral cancer pain than unencapsulated antagonist [44]. NPs have been refined by surface conjugation of groups that target NPs to specific cell types. Mesoporous silica NPs coated with liposomes conjugated to a DOR agonist are preferentially endocytosed by DOR-expressing cells and when injected intrathecally effectively relieve inflammatory pain in mice [10].

The criteria for the rationale design of endosomally biased ligands that would engage GPCRs in signalosomes of acidic endosomes are not fully defined (Figure 3D). A clue has been provided by the development of N-(3-fluoro-1-phenethylpiperidin-4-yl)-N-phenyl propionamide (NFEPP), a fluorinated fentanyl derivative with an acidic pKa [45]. NFEPP preferentially binds to MOR at acidic pH and inhibits pain signaling that emanates from the acidified microenvironment of diseased tissues without the side effects caused by activating MOR in healthy tissues with normal extracellular pH [46–48]. Analogs of the NK_1R antagonist, netupitant, designed to penetrate membranes and persist in acidic endosomes through altered lipophilicity and pKa cause sustained inhibition of endosomal signals and provide more potent, efficacious and sustained antinociceptive effects than conventional antagonists [34].

Analysis of MOR signaling and regulation can provide insights into the limitations of opioids, including on-target side effects and tolerance. To minimize side effects while retaining analgesic properties, biased agonists of MOR were developed that favor G protein signaling mediating

analgesia and minimize β-arrestin signaling mediating respiratory depression and constipation. One such biased agonist gained approval for treatment of moderate pain, but retained the side effects of opioids [49]. Recent studies have questioned whether β-arrestin signaling mediates the adverse actions of opioids and propose that the low intrinsic efficacy for G protein activation accounts for improved side effect profiles of new opioid agonists [50,51]. The clustering of MOR ligands based on their G protein and β-arrestin-signaling profiles and side effects has the potential to identify features that underlie beneficial and detrimental properties [52]. Examination of long-term opioid tolerance in cultured neurons suggest that presynaptic tolerance is mediated by a depletion of receptors from the surface by cycles of receptor endocytosis and recycling that depend on GPCR kinase 2 phosphorylation of the MOR C tail [53]. Analysis of the proteome associated with MOR provided insights into how opioids developed to treat pain affect the MOR-associated proteome [54].

Mechanisms and therapeutic targeting of RTK pain signaling

Mechanisms of RTK pain signaling

There has been intense interest in how RTKs signal, spurred by their role in cancer [55]. Ligand binding to plasma membrane RTKs induces receptor dimerization, activation of the intracellular tyrosine kinase domain, and trans-autophosphorylation of receptor tyrosine residues, which serve as docking platforms for scaffold proteins containing Src homology-2 (SH2) and phospho-tyrosine-binding (PTB) domains. Lacking catalytic activity, scaffold proteins organize signaling complexes including mitogen-activated protein kinase/p38, phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B, phospholipase Cγ, Ras-GTPase-activating protein, Janus kinase/signal transducer and activator of transcription, proto-oncogene c-Src, and focal adhesion kinase signaling cascades. Several RTKs are implicated in pain, including tropomyosin

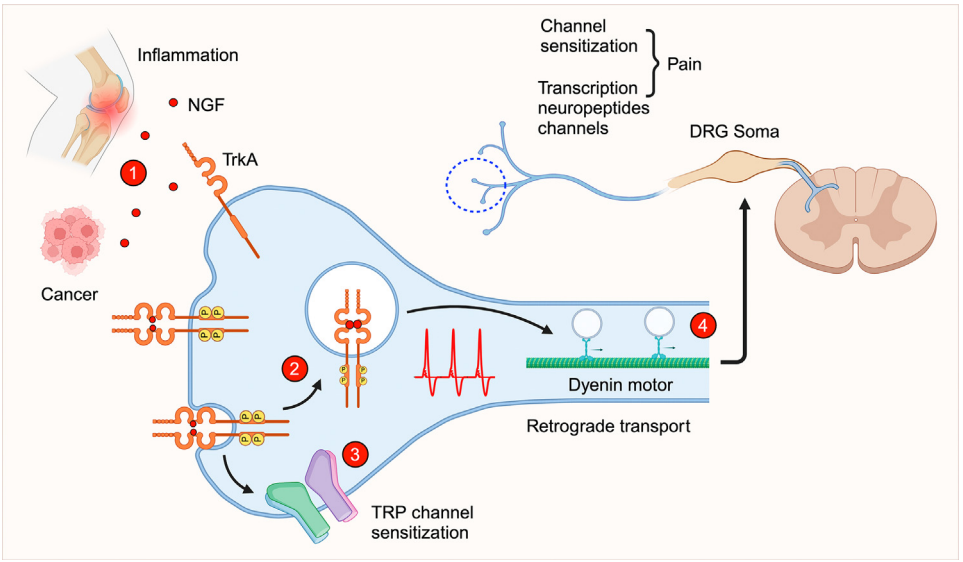

Figure 4. Mechanisms of nerve growth factor (NGF) and tropomyosin receptor kinase A (TrkA) signaling of pain from plasma membrane (PM) and early endosome (EE) nanodomains. (1). NGF released from diseased tissues binds to TrkA at the PM, which causes TrkA dimerization, autophosphorylation, and recruitment of effectors. (2). The NGF/TrkA complex undergoes clathrin-dependent and independent endocytosis. (3). NGF/TrkA activates PM ion channels, including transient receptor potential (TRP) channels, leading to sensitization and pain. (4). NGF/TrkA in signaling endosomes undergo dynein-meditated retrograde transport to the distant soma to regulate transcriptional events that underlie pain. Abbreviation: DRG, dorsal root ganglion.

receptor kinase A (TrkA), a receptor for nerve growth factor (NGF), the epidermal growth factor receptor (EGFR), and the vascular endothelial growth factor receptor (VEGFR). The mechanisms by which these RTKs signal pain are not fully understood.

NGF/TrkA signaling sensitizes sodium, calcium, and transient receptor potential vanilloid 1 channels of rodent and human nociceptors, causing allodynia and hyperalgesia [56,57] (Figure 4). The NGF/TrkA complex is endocytosed and continues to signal, representing one of the most thoroughly characterized endosomal signaling complexes [58]. Retrograde transport of NGF/TrkA complexes from peripheral terminals to the distant soma by microtubule motor-mediated transport activates ERK5, leading to phosphorylation of the transcription factor cAMP response element-binding protein, which controls the neurotrophic actions of NGF. Dynein motor-mediated transport of TrkA from axon terminals to the distant soma promotes neuronal survival, which may explain why dynein mutations are associated with neurodegenerative diseases [59]. NGF signals pain from multiprotein signalosomes comprising receptors, coreceptors, and molecular scaffolds (Figure 5). Neuropilin-1 (NRP1) is a type I transmembrane protein that functions as a coreceptor for proteins with a basic C-end rule (CendR) motif (R/KXXR/K), which interacts with extracellular NRP1 domains [60]. NGF contains two CendR motif and interacts with NRP1 with nanomolar affinity [57]. NRP1 also associates with TrkA, serving as a molecular chaperone that escorts TrkA from the biosynthetic pathway to the plasma membrane and then to signaling endosomes. The adaptor protein GIPC1, which interacts with NRP1 and TrkA and couples to

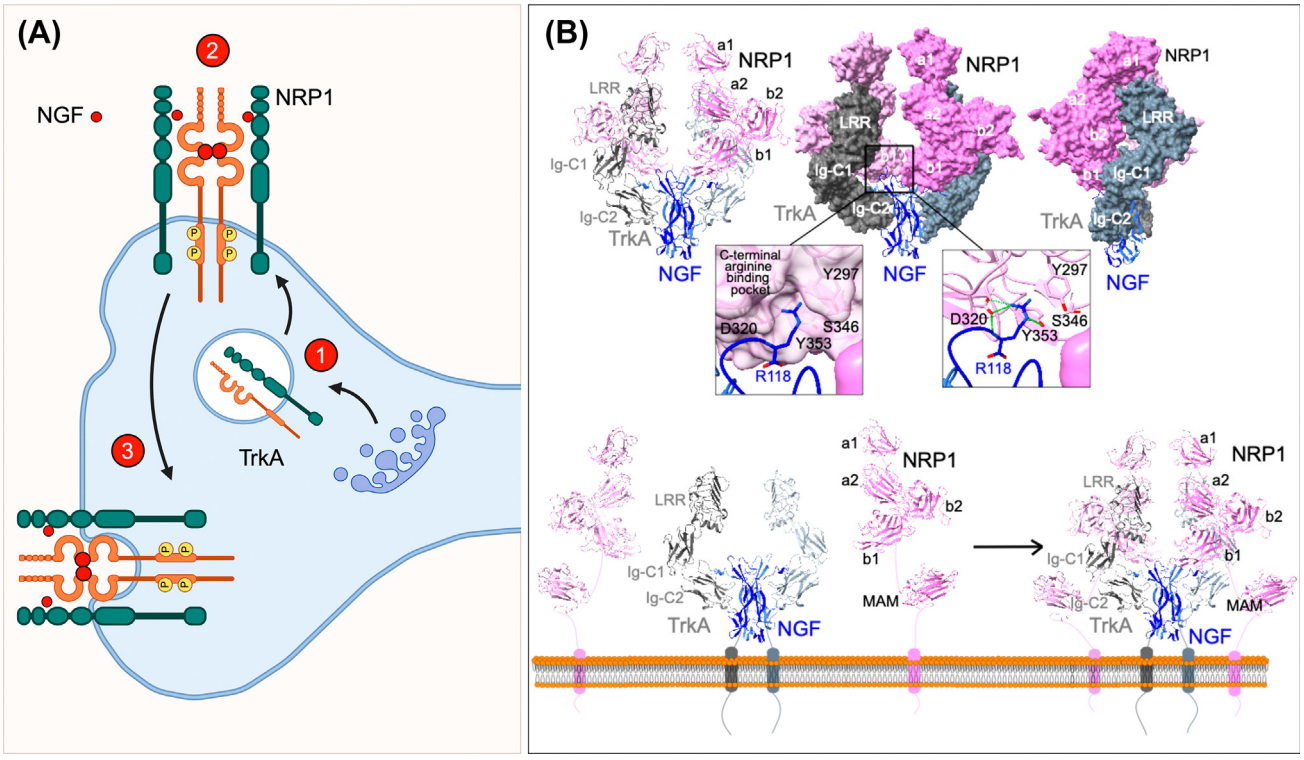

Figure 5. Contribution of neuropilin 1 (NRP1) to nerve growth factor (NGF) and tropomyosin receptor kinase A (TrkA)-evoked pain. (A) Mechanisms by which NRP1 contributes to NGF and TrkA pain signaling. (1). NRP1 chaperones TrkA from the biosynthetic pathway to the plasma membrane. (2). NRP1 binds to NGF and serves as a coreceptor that enhances TrkA activation and signaling. (3). The NGF/TrkA/NRP1 complex at the plasma membrane and in early endosome (EE) facilitates NGF signaling of pain. (B) Molecular model showing the assembly of an NGF, TrkA, and NRP1 complex at the plasma membrane with a 2:2:2 stoichiometry (reproduced, with permission, from [57]).

the myosin VI motor, mediates NRP1/TrkA association, trafficking and signaling [57]. Molecular modeling suggests that a C-terminal R/KXXR/K NGF motif interacts with extracellular 'b' NRP1 domain within a plasma membrane NGF/TrkA/NRP1 of 2:2:2 stoichiometry, thereby facilitating NGF pain signaling (Figure 5).

Multiple ligands interact with EGFR, with graded affinities. High affinity EGFR ligands include EGF, transforming growth factor α, betacellulin, and heparin-binding epidermal growth factor-like growth factor; low affinity ligands include amphiregulin, epiregulin, and epigen. After binding EGF, the EGFR undergoes endocytosis and is trafficked to lysosomes. The question as to whether EGFR continues to signal from the endosome under physiological conditions has been controversial, with results affected by cell type and agonist type and concentration. BRET has been used to monitor the recruitment of SH2-domain proteins to subcellular compartments of cell lines in response to different EGFR ligands [61]. Both EGF and epiregulin stimulated the recruitment of SH2 effectors to the plasma membrane, although epiregulin was less potent. Whereas EGF also stimulated translocation of SH2 effectors to EEs, epiregulin did not, consistent with its inability to trigger EGFR endocytosis. Activated EGFR interacts with the SH domain adaptor, growth factor receptor-bound protein 2, which mediates Ras signaling and endocytosis. Live imaging of HeLa cells stimulated with physiological concentrations of EGFR ligands provides evidence for prolonged localization and activity of EGFR-bound protein 2 complexes in endosomes, which correlates with sustained ERK1/2 activation [62]. This process may extend signaling of internalized EGFRs to compensate for rapid downregulation of surface EGFRs. EGFR mediates nociception in preclinical models and is strongly associated with pain in human genome studies. Heparin-binding EGF-like growth factor directly causes neuronal excitation and elicits pain-like behaviors in animals, whereas EGF and epiregulin require repeated injections or concurrent inflammation or injury to produce pain [63,64]. In preclinical models of inflammatory and neuropathic pain, the epiregulin–EGFR complex exacerbates pain by sensitizing transient receptor potential vanilloid 1.

VEGF-A is related to NGF since angiogenesis and neurogenesis parallel one other in development and cancer. VEGF-A induces vascularization and angiogenesis and is a mediator of cancer and neuropathic pain. NRP1 is a coreceptor for VEGF-A that facilitates activation of sodium and calcium currents and neuropathic pain [65,66]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein also binds to NRP1, and thereby impedes VEGF-A-evoked pain [66].

Therapeutic targeting of RTK pain signaling

Monoclonal antibodies to growth factors and their receptors are established therapies for cancer and emerging therapies for pain. Monoclonal antibodies targeting NGF demonstrate superior efficacy compared with naproxen or oxycodone for treatment of hip and knee osteoarthritis pain [67]. Despite these findings, some patients experienced rapidly progressive osteoarthritis, possibly due to dysfunctional innervation, which prevented regulatory approval. Therapies against nociceptor-enriched targets, including the NRP1-NGF complex and its GIPC1 scaffold, may surmount the detrimental effects of systemic NGF sequestration with monoclonal antibodies. Antagonists to NRP1 and GIPC1 prevent NGF-induced excitation of mouse and human nociceptors and ameliorate NGF-evoked mechanical allodynia, and might provide an alternative treatment to pain that is driven by NGF [57].

EGFR is a therapeutic target for cancer, including oral cancer, which commonly features aberrant EGFR signaling. Patients with lung or oral cancer report pain relief following EGFR-targeted cancer therapy [68], leading to increased interest in EGFR involvement in cancer pain. In addition to observations with cancer patients, EGFR signaling contributes to nociception in preclinical mouse

models of oral cancer [69]. RTK-targeted therapies are being explored for their analgesic potential in nononcological conditions. Treatment that combines opioids and RTK inhibitors could preclude or reduce the clinical challenge of opioid tolerance [5].

The therapeutic efficacy of monoclonal antibodies may be limited if the target is internalized, in which case inhibitors of endocytosis could improve efficacy by retaining targets at the plasma membrane. Poor responsiveness of cancer patients to EGFR antibodies may relate to endosomal sequestration of receptors, which would be inaccessible to monoclonal antibodies in the extracellular fluid. Prochlorperazine, which is used to treat emesis and psychosis, inhibits dynamin. Prochlorperazine relocalizes EGFR from endosomes to the plasma membrane and increases antibody-dependent cellular cytotoxicity induced by cetuximab, the EGFR monoclonal antibody [70,71]. Whether endocytosis inhibitors increase the efficacy of RTK monoclonal antibodies for treatment of chronic pain is unknown.

Allosteric inhibition is another promising approach to the treatment of RTK-mediated pain. Although NGF-targeting monoclonal antibodies are effective in rodents and humans, development of small molecule inhibitors of TrkA remains challenging. One difficulty is that the small molecules are pan-Trk inhibitors, affecting not only TrkA, but also TrkB and TrkC, which leads to side effects including dizziness, withdrawal pain, hyperphagia, and obesity [72]. A compound has been reported to selectively inhibit TrkA enzymatic phosphorylation [73]. The compound binds to the kinase domain of TrkA and not the ATP binding site and is therefore an allosteric inhibitor of TrkA [73]. The allosteric modulator reduces pain in a chronic osteoarthritis model that is produced with intra-articular injection of monoiodoacetate. The compound is a potent allosteric kinase inhibitor with increased selectivity for TrkA over TrkB. Allosteric inhibitors of EGFR overcome therapy-resistant EGFR mutations in the treatment of cancer [74]. The allosteric inhibitors for EGFR bind to sites that are different from the tyrosine kinase inhibitors that bind ATP. Small molecular allosteric inhibitors for RTKs are anticipated to emerge as potential analgesic targets.

Concluding remarks and future perspectives

Once activated at the cell surface, GPCRs and RTKs assemble multiprotein signalosomes, internalize and continue to signal. Evidence for signaling of internalized receptors derives from the use of biosensors that can detect activated receptors and signaling mediators in intracellular compartments of living cells. In contrast to plasma membrane signaling, which is often transient, intracellular signaling is sustained and may be relevant to disease processes, including pain. The observations that inhibitors of endocytosis abrogate intracellular signals and their downstream sequalae, such as excitation of nociceptors and induction of pain-like behavior, provides evidence that intracellular receptor signaling mediates pain and support the contention that intracellular receptors are relevant pain targets. The finding that endosomal targeting of antagonists can enhance analgesic efficacy supports this notion, and raises the possibility that the failure of antagonists in clinical trials of chronic pain – when receptors are likely internalized due to continuous agonist formation – may relate to their inability to engage receptors in endosomes. Antagonists that effectively reverse sustained receptor signaling in endosomes may thus provide efficacious relief of chronic pain. The selective delivery of antagonisms to **nanodomains** of specific cell types that mediate pain, possibly achievable using targeted NPs, may reduce dosing, minimizing the side effects from systemic antagonism.

Challenging questions remain to be tackled (see Outstanding questions). GPCR and RTK signaling is most conveniently studied in model cells in which receptors and biosensors are overexpressed, possibly leading to artefactual findings. Whether endogenous receptors and effectors operate similarly in functionally relevant human cells remains to be determined. Whether these

Outstanding questions

What are the mechanisms by which GPCRs and RTKs signal in primary cells from humans to cause pain? Most information derives from studies of model cell lines or of cells derived from experimental animals.

What are the mechanisms by which receptors and their effectors traffic to and signal from endosomes? Most information derives from analysis of signaling at the plasma membrane.

What are the criteria for designing antagonists of receptors within multiprotein signalosomes of acidified intracellular compartments that signal pain? Antagonists are usually characterized by their ability to target cell surface receptors.

Do therapies targeting coreceptors and scaffolding proteins that underpin pain signaling by several receptors provide more effective analgesia by surmounting the redundancy of pain signaling pathways? Because many GPCRs and RTKs evoke pain, antagonists of individual receptors may lack efficacy.

Do therapies characterized in rodents translate to patients? Analgesics are commonly tested in preclinical mouse models, where pain is assessed by measuring nociceptive responses to short-term noxious stimuli. A major challenge is to translate these fundings to the treatment of chronic pain in patients.

processes are perturbed during chronic disease is largely unknown. Compared with plasma membrane signaling, the mechanisms of endosomal signaling of GPCRs and RTKs are not fully understood. There is uncertainty relating to the mechanisms by which G proteins and their effectors traffic to endosomes and engage with activated GPCRs, and the pathways by which GPCRs in endosomes control the activity of ion channels at the plasma membrane and transcription of genes in the nucleus that control chronic pain are not fully understood.

Compared with RTK signaling of cancer, the mechanisms by which RTKs signal pain is less well understood. Although it is firmly established that NGF and TrkA signaling from endosomes controls neurodevelopment, the contribution of endosomal signaling of TrkA, EGFR, and VEGFR to pain are, to our knowledge, unknown. The criteria for design of GPCR and RTK ligands that effectively target receptors in nanodomains of specific cell types remain to be delineated. Pain mechanisms and treatments in laboratory settings are usually evaluated by analysis of behavioral responses of highly inbred rodents to noxious stimuli, which usually fail to capture the complex emotional and cognitive components of pain in patients. Whether preclinical models of chronic pain in rodents faithfully replicate poorly understood forms of chronic pain in patients is a major unanswered question.

The high redundancy of GPCRs and TRKs in pain signaling, often coexpressed in the same cells, likely limits the efficacy of therapies targeting individual receptors. Targeting processes that are shared by several receptors may overcome this redundancy. For example, inhibitors of endocytosis blunt pronociceptive signaling of several GPCRs and impede the endocytic recycling of synaptic vesicles in nociceptive spinal circuits that sustains pain transmission [8,14,33,35]. Inhibitors of the endocytic protein adaptor-associated kinase-1 have progressed to clinical trials for pain. The identification of NRP1 as a coreceptor for several growth factors that mediate pain suggests that antagonism could be broadly effective [57,65].

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Declaration of interests

N.W.B. is the founding scientist of Endosome Therapeutics. The remaining authors have no interests to declare.

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