Classification: Biological Sciences; Pharmacology

Endosomal Signaling of the Receptor for Calcitonin Gene-Related Peptide Mediates Pain Transmission

Short Title: Endosomal pathways for the signaling of pain transmission

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ABSTRACT

G protein-coupled receptors (GPCRs) are considered to function primarily at the plasma membrane, where they interact with extracellular ligands and couple to G proteins that transmit intracellular signals. Consequently, therapeutic drugs are designed to target GPCRs at the plasma membrane. Activated GPCRs undergo clathrin-dependent endocytosis. Whether GPCRs in endosomes control pathophysiological processes *in vivo* and are therapeutic targets remains uncertain. We investigated the contribution of endosomal signaling of the calcitonin receptor-like receptor (CLR) to pain transmission. Calcitonin gene-related peptide (CGRP) stimulated CLR endocytosis, and activated protein kinase C (PKC) in the cytosol and extracellular signal regulated kinase (ERK) in the cytosol and nucleus. Inhibitors of clathrin and dynamin prevented CLR endocytosis and activation of cytosolic PKC and nuclear ERK, which derive from endosomal CLR. A cholestanol-conjugated antagonist, $CGRP_{8-37}$, accumulated in CLR-containing endosomes, and selectively inhibited CLR signaling in endosomes. CGRP caused sustained excitation of neurons in slices of rat spinal cord. Inhibitors of dynamin, ERK and PKC suppressed persistent neuronal excitation. $CGRP_{8-37}$-cholestanol, but not unconjugated $CGRP_{8-37}$, prevented sustained neuronal excitation. When injected intrathecally to mice, $CGRP_{8-37}$-cholestanol inhibited nociceptive responses to intraplantar injection of capsaicin, formalin or complete Freund's adjuvant more effectively than unconjugated $CGRP_{8-37}$. Our results show that CLR signals from endosomes to control pain transmission, and identify CLR in endosomes as a therapeutic target for pain. Thus, GPCRs function not only at the plasma membrane but also in endosomes to control complex processes *in vivo*. Endosomal GPCRs are a drug target that deserve further attention.

SIGNIFICANCE STATEMENT

GPCRs have long been considered to function primarily at the plasma membrane. Consequently, most drugs are designed to target GPCRs at the cell-surface. Ligand-bound GPCRs undergo clathrin- and dynamin-dependent endocytosis. It is uncertain whether GPCRs in endosomes control complex pathophysiological processes *in vivo* and are a viable therapeutic target. We report that the CGRP receptor signals from endosomes to regulate activity of pain-transmitting neurons in the spinal cord. Lipid-conjugated CGRP receptor antagonists accumulate in endosomes, selectively inhibit endosomal signals, and block sustained excitation of spinal neurons and persistent nociception. The results suggest that GPCRs in endosomes, in addition to those at the cell-surface, control ongoing pathophysiological processes *in vivo*, and identify GPCRs in endosomes as a new target for therapy.

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INTRODUCTION

G protein-coupled receptors (GPCRs) have long been considered to function primarily at the plasma membrane, where they interact with ligands in the extracellular fluid and couple to heterotrimeric G proteins that convey signals within the cell. Consequently, most therapeutic drugs are designed to target GPCRs at the cell-surface. Ligand-bound GPCRs interact with β-arrestins (βARRs), which desensitize G protein signaling, mediate receptor endocytosis, and thereby rapidly terminate plasma membrane signaling (1). The conventional view that GPCRs signal only from the plasma membrane has been challenged by reports that GPCRs can continue to signal from endosomes by G protein- and βARR-mediated mechanisms (2-9). However, the contribution of endosomal signaling of GPCRs to the control of complex pathophysiological processes *in vivo* is uncertain, and whether endosomal GPCRs are a viable therapeutic target is far from clear.

GPCRs in endosomes can generate signals in subcellular compartments (2, 6-8). Compartmentalized signaling involves GPCR association with signaling and regulatory proteins that determine the subcellular location of signals. By these mechanisms, a large number of different GPCRs can specifically control cellular functions using a small number of effectors. The importance of GPCR compartmentalized signaling for integrated responses requires further investigation.

Calcitonin gene-related peptide (CGRP) is expressed throughout the nervous system (10). The CGRP receptor comprises calcitonin receptor-like receptor (CLR), a GPCR, and receptor activity modifying protein 1 (RAMP1), a single transmembrane protein that imparts ligand specificity and ensures CLR targeting to the cell-surface. Noxious stimuli evoke CGRP release from the terminals of primary sensory neurons in the dorsal horn of the spinal cord and in peripheral tissues. CGRP activates CLR/RAMP1 on spinal neurons to induce nociception, and on

peripheral arterioles to cause neurogenic inflammation. CGRP and CLR are targets for migraine pain (10). Although CGRP stimulates endocytosis of CLR/RAMP1 (11), the contribution of CLR/RAMP1 endocytosis to pain transmission is uncertain, and whether CLR in endosomes is a therapeutic target for pain is unknown. We have recently found that the neurokinin 1 receptor (NK_1R) signals from endosomes to mediate substance P (SP)-induced nociception (12). We now describe a major role for endosomal CLR in nociception, and identify CLR in endosomes as a therapeutic target.

RESULTS

CGRP stimulates clathrin- and dynamin-dependent endocytosis of CLR

We used Bioluminescence Resonance Energy Transfer (BRET) to quantify the proximity of CLR to βARR2, an adaptor for clathrin-mediated endocytosis, and to resident proteins of the plasma membrane (KRas), early endosomes (Rab5a), and recycling endosomes (Rab11) of HEK293 cells co-expressing CLR and RAMP1 (12, 13). CGRP (100 nM, continuous) increased CLR-RLuc/βARR2-YFP BRET (EC_{50} 2 nM, pEC_{50} 8.74±0.18) (Fig. 1A, Fig. S1A). CGRP decreased CLR-RLuc/KRas-Venus BRET, and increased CLR-RLuc/Rab5a-Venus BRET (Fig. 1B). After incubation with CGRP (15 min) and washout, there was an increase in CLR-RLuc/Rab11-Venus BRET (Fig. 1C). Dominant negative dynamin K44E (DynK44E), the dynamin inhibitor Dyngo4a (Dy4a, 30 μM) (14), and the clathrin inhibitor PitStop2 (PS2, 30 μM) (15) prevented CLR removal from the plasma membrane and inhibited trafficking to early endosomes (Fig. 1D). There was no effect of inactive Dy4a or PS2 analogs. We confirmed these results using two other assays of endocytosis. Cell-surface ELISA using antibodies to extracellular epitopes (HA-CLR, myc-RAMP1) showed that CGRP (100 nM, continuous) induced rapid removal of both receptor components from the plasma membrane (Fig. S1B). DynK44E, Dy4a and PS2, but not inactive analogs, inhibited CLR removal (Fig. 1E). CGRP

(100 nM, 15 min) induced CLR trafficking from the plasma membrane to early endosomes identified by early endosome antigen 1 (EEA1) immunoreactivity (IR) (Fig. 1F, Fig. S1C). Dy4a and DynK44E inhibited endocytosis. Expression of Rab5a-Venus did not appreciably alter the appearance of EEA1-positive early endosomes, supporting the suitability of the BRET approach to study CLR endocytosis (Fig. S1D).

CLR endocytosis mediates a subset of signals in subcellular compartments

To study CGRP signaling in subcellular compartments, we expressed in HEK cells HA-CLR/myc-RAMP1 and genetically encoded Förster Resonance Energy Transfer (FRET) biosensors for plasma membrane and cytosolic cAMP (pmEpac2, cytoEpac2, respectively), plasma membrane and cytosolic protein kinase C (PKC, pmCKAR, cytoCKAR), and cytosolic and nuclear extracellular signal regulated kinase (ERK, cytoEKAR, nucEKAR) (12, 13, 16). Biosensors are targeted to subcellular compartments and are reversibly modified by second messengers, kinases and phosphatases. Single cell high-content imaging was used to study signaling kinetics in subcellular compartments of living cells.

CGRP (1 nM, continuous) induced a rapid and sustained increase in plasma membrane and cytosolic cAMP (Fig. S2A-C), in agreement with CLR coupling to $G\alpha_s$ and adenylyl cyclase (10). CGRP induced a rapid and sustained activation of PKC in the cytosol but not at the plasma membrane (Fig. S2D-F), and a gradual and sustained activation of cytosolic and nuclear ERK (EC_{50} 50-70 nM) (Fig. S2G-I, Fig. S3A-D). DynK44E or Dy4a, but not wild-type (WT) dynamin or inactive Dy4a, abolished CGRP-induced activation of cytosolic PKC and nuclear ERK, but not cytosolic ERK (Fig. 2A-F).

NF449 (10 μM, $G\alpha_s$ inhibitor) suppressed activation of nuclear ERK but not cytosolic PKC, whereas NF023 (10 μM, $G\alpha_i$ inhibitor) had no effect (Fig. 2G, H). UBO-QIC (100 nM, $G\alpha_q$ inhibitor) blocked activation of cytosolic PKC but not nuclear ERK. U73122 (1 μM, phospholipase Cβ inhibitor), but not EGTA (100 μM, Ca^{2+} chelator), also inhibited activation of

cytosolic PKC. βARR1+2 siRNA, which we have shown inhibits endosomal NK_1R signaling (12, 13), did not affect either signal.

Thus, dynamin-dependent endocytosis of CLR mediates activation of nuclear ERK and cytosolic PKC, but not cytosolic ERK. Nuclear ERK activation requires $G\alpha_s$, whereas cytosolic PKC activation depends on $G\alpha_q$ but not Ca^{2+} mobilization.

A cholestanol-conjugated CLR antagonist inhibits CGRP signaling in endosomes

Conjugation to the membrane lipid cholestanol promotes endosomal delivery and retention of peptidase inhibitors and NK_1R antagonists, which enhances therapeutic efficacy (12, 17). We used a similar approach to deliver a membrane-impermeant CLR antagonist, $CGRP_{8-37}$, to CLR in endosomes. Tripartite probes were synthesized comprising: cholestanol, which promotes membrane insertion and anchoring, or ethyl ester (control), which does not insert into membranes; a flexible polyethylene glycol-12 (PEG) linker to promote antagonist presentation in an aqueous environment; and a cargo of either cyanine 5 (Cy5) for localization or $CGRP_{8-37}$ (Fig. 3A, Fig. S4).

To examine probe delivery to endosomes containing CLR, HEK-HA-CLR/myc-RAMP1 cells were incubated with Cy5-cholestanol (Cy5-Chol) or Cy5-Ethyl Ester (60 min, 37°C), washed, and incubated with Alexa488-anti-HA antibody (40 min) to label cell-surface CLR. Live cells were imaged by confocal microscopy (37°C). To induce CLR endocytosis, cells were stimulated with CGRP (50 nM) 3 h after initial exposure to probes. Before exposure to CGRP, Cy5-Chol was concentrated in endosomes, CLR was at the cell-surface (Fig. 3B), and Cy5-Ethyl Ester remained extracellular (Fig. 3C). Alexa488-anti-HA antibody did not bind to untransfected HEK cells, confirming specificity (Fig. 3D). After incubation with CGRP (30, 60 min), CLR and Cy5-Chol were colocalized in endosomes with overlapping pixel intensities (Fig. 3B, SI Video 1). The CLR and Cy5-Chol overlap coefficient significantly increased after incubation with CGRP (Fig. 3E). Thus, cholestanol conjugation delivers probes to endosomes containing CLR.

$CGRP_{8-37}$-Chol inhibited CGRP (1 nM)-induced cAMP formation in HEK-HA-CLR/myc-RAMP1 cells with an identical potency to unconjugated $CGRP_{8-37}$ (pEC_{50}: $CGRP_{8-37}$, 6.17±0.22; $CGRP_{8-37}$-Chol, 6.36±0.14; Fig. S5A).

To examine the capacity of $CGRP_{8-37}$-Chol and $CGRP_{8-37}$ to inhibit CLR signaling at the plasma membrane and in endosomes, we incubated HEK-HA-CLR/myc-RAMP1 cells with antagonists for 30 min, washed, and examined CGRP signaling immediately after washing (when cholestanol-conjugated probes were at the plasma membrane) or after 4 h (when probes were in endosomes). CGRP stimulation of cytosolic ERK (derived from plasma membrane CLR) or nuclear ERK (derived from endosomal CLR) was measured.

When assayed immediately after 30 min pre-incubation, both $CGRP_{8-37}$ and $CGRP_{8-37}$-Chol inhibited CGRP-stimulated activation of cytosolic and nuclear ERK (Fig. 4A, B, E, F). When cells were pulse-incubated with antagonists for 30 min, washed and then stimulated with CGRP 4 h later, only $CGRP_{8-37}$-Chol was capable of inhibiting nuclear ERK (Fig. 4C, D, E, F). Using the population-based FRET assay, we demonstrated that while $CGRP_{8-37}$-Chol had similar potency in inhibiting nuclear and cytosolic ERK after 30 min pre-incubation (pIC_{50}: cytosolic ERK, 5.57±0.37; nuclear ERK, 6.23±0.23), $CGRP_{8-37}$-Chol more potently inhibited nuclear ERK (pIC_{50}: 6.24±0.34) than cytosolic ERK (pIC_{50}: < 5) when the cells were pulse-incubated with the antagonists (Fig. 4G-H, Fig. S6A-H). A probe lacking $CGRP_{8-37}$ (PEG-Biotin-Chol) had no effect on ERK activation, which excludes non-specific disruption of signaling by cholestanol or PEG. In cells that were pulse-incubated with $CGRP_{8-37}$-Chol, CGRP still stimulated CLR endocytosis 4 h later, as shown by the decrease in CLR-RLuc/KRas-Venus BRET and the increase in CLR-RLuc/Rab5a-Venus BRET (Fig. S5B). The results show that cholestanol conjugation provides a mechanism for selective and sustained antagonism of endosomal CLR signaling.

CLR signaling in endosomes mediates nociceptive transmission

To determine whether spinal neurons express functional CLR, we examined CGRP signaling in neurons isolated from the dorsal horn of the rat spinal cord. CGRP increased $[Ca^{2+}]_i$ in 52±17% (942 neurons, 15 rats) of neurons (Fig. S7A). Preincubation with $CGRP_{8-37}$ (1 μM, 30 min) abolished CGRP signals, which confirms expression of CLR (Fig. S7B). Neuronal excitation was examined by cell-attached patch clamp recordings from lamina I neurons in slices of rat spinal cord. Transient exposure to CGRP (1 μM, 2 min) stimulated firing of action potentials that was sustained for at least 20 min after washout (Fig. 5A-C). CGRP-responsive neurons also responded to SP (1 μM), supporting co-expression of CLR and NK_1R in second-order spinal neurons.

To determine whether CLR signaling from endosomes contributes to CGRP-induced excitation, we incubated spinal cord slices with Dy4a or inactive Dy4a (30 μM) or vehicle 10 min before CGRP challenge. Dy4a did not affect the immediate CGRP-induced excitation, but prevented the sustained response (Fig. 5A-C). We preincubated tissue with U0126 (MEK inhibitor), GF109203X (PKC inhibitor) (1 μM, 30-45 min) to examine the underlying signaling mechanisms. U0126 reduced the CGRP-stimulated firing time of lamina I neurons by 72.4±5.1% (U0126, 17.37±1.2 min; control, 4.8±0.9 min; $P<0.0001$, N=neurons for U0126, N=5 neurons for control, 7 rats), and reduced the average number of spontaneous action potentials by 86.6±11.6% compared to controls (Fig. 5D-F). GF109203X reduced CGRP-stimulated firing time by 76.5±5.3% (GF109203X, 17.37±1.2 min; control 4.07±0.9 min; $P<0.0001$, N=8 neurons for GF109203X, N=5 neurons for control, 9 rats), and reduced the average number of CGRP-induced action potentials by 98.8±0.4% compared to controls. CGRP (1 μM, 5 min) induced endocytosis of CLR-IR in spinal neurons (Fig. 5G, H). Dy4a inhibited CGRP-induced endocytosis of CLR-IR.

To obtain direct support for the concept that CLR signaling in endosomes mediates sustained excitation of spinal neurons, we preincubated spinal cord slices with vehicle, $CGRP_{8-37}$

or $CGRP_{8-37}$-Chol (1 μM, 60 min), washed, and challenged with CGRP 60 min later. In vehicle-treated slices, CGRP caused rapid onset action potential discharge that was sustained after washout (Fig. 6A-C). $CGRP_{8-37}$ did not affect this response. However, as observed with a dynamin inhibitor, $CGRP_{8-37}$-Chol abolished sustained CGRP-evoked firing without affecting initial responses.

The results support the hypothesis that endosomal CLR signaling mediates persistent excitation of spinal neurons. CLR in endosomes activates PKC and ERK, which control neuronal excitation.

CLR signaling in endosomes mediates nociception

Does endosomal delivery enhance the antinociceptive efficacy of CLR antagonists? To evaluate this possibility, we administered vehicle, $CGRP_{8-37}$ or $CGRP_{8-37}$-Chol (5 μl, 10 μM) to mice by intrathecal injection 3 h before intraplantar injection of capsaicin. This time allows accumulation of tripartite probes in endosomes of spinal neurons (12). We examined mechanical nociception by stimulation of the plantar surface of the paw using von Frey filaments. In vehicle-treated mice, capsaicin caused mechanical allodynia of the ipsilateral paw that was sustained for 4 h (Fig. 7A). Intrathecal $CGRP_{8-37}$ had a transient anti-nociceptive effect at 1 h, whereas $CGRP_{8-37}$-Chol induced a larger anti-nociceptive effect that was sustained for 4 h. $CGRP_{8-37}$ and $CGRP_{8-37}$-Chol did not affect withdrawal responses of the contralateral (non-injected) paw (Fig. 7B). $CGRP_{8-37}$-Chol blunted the non-inflammatory (first phase) and inflammatory (second phase) of the nocifensive response to intraplantar formalin more effectively than $CGRP_{8-37}$ (Fig. 7C, D). When injected 36 h after intraplantar injection of complete Freund's adjuvant (CFA), which causes a long-lasting inflammatory hyperalgesia, $CGRP_{8-37}$-Chol but not $CGRP_{8-37}$ reversed the mechanical hyperalgesia (Fig. 7E).

Painful stimuli also induce the release of SP from the central terminals of primary sensory neurons in the dorsal horn, where SP induces NK_1R endocytosis in second-order neurons

and central transmission (12). To target the endosomal NK_1R, we conjugated the NK_1R antagonist spantide (Span) to cholestanol. When co-administered, Span-Chol (5 µl, 50 µM) and $CGRP_{8-37}$-Chol (5 µl, 10 µM) caused a marked (~75%) reversal of CFA-induced mechanical hyperalgesia, whereas the combination of unconjugated Span and $CGRP_{8-37}$ had no effect (Fig. 7F).

DISCUSSION

Our results support the hypothesis that complex pathophysiological events, such as nociceptive transmission in the spinal cord, are not solely mediated by activation of GPCRs at the plasma membrane. We propose that GPCRs in endosomes generate sustained signals in subcellular compartments that underlie complex pathophysiological processes *in vivo*, and that endosomal receptors are a valid but neglected therapeutic target.

By using FRET biosensors targeted to subcellular compartments, we found that CGRP stimulates cAMP formation in the cytosol and plasma membrane, activates cytosolic and nuclear ERK, and activates PKC only in the cytosol. These signals were maintained in the continued presence of CGRP, suggesting sustained CLR activation. The observation that inhibitors of endocytosis prevent activation of cytosolic PKC and nuclear ERK suggest that these signals arise from persistent endosomal CLR signaling. In contrast, endocytic inhibitors did not affect CGRP-induced activation of cytosolic ERK, which likely originates from cell-surface CLR (Fig. S8). Cytosolic PKC activation depends on $G\alpha_q$ and is independent of Ca^{2+} mobilization. In contrast, nuclear ERK activation requires activation of $G\alpha_s$. Thus, both $G\alpha_q$ and $G\alpha_s$ mediate CLR signaling from endosomes (Fig. S8). These findings support reports that $G\alpha_q$ and $G\alpha_s$ mediate endosomal signaling of other GPCRs (2, 5, 12, 18).

Protease-activated receptor-2 (4), NK_1R (3, 12), β_2-adrenergic receptor (5), parathyroid receptor-1 (2), PAC1 receptor (7), dopamine D_1 receptor (19), and receptors for glucagon-like

peptide 1 (20), luteinizing hormone (21), and thyroid stimulating hormone (18) can signal from endosomes. Evidence of endosomal signaling derives from studies of model cells treated with inhibitors of endocytosis. These approaches provide mechanistic information but offer limited insight into the contribution of endosomal GPCR signaling for control of complex processes in intact tissues or animals. The observation that inhibitors of dynamin, ERK and PKC suppress CGRP-induced excitation of spinal neurons suggests that endosomal CLR signaling is necessary for nociceptive transmission. Inhibitors of endocytosis also inhibit SP-induced excitation of spinal neurons and PACAP-induced excitability of cardiac neurons, and can suppress nociception (7, 12). Thus, endocytosis of several GPCRs may be required for their actions on neuronal function.

We used cholestanol-conjugated probes, which accumulated in early endosomes containing CLR, to specifically evaluate the function of CLR in endosomes. After transient incubation and recovery, $CGRP_{8-37}$-Chol prevented CGRP-induced activation of nuclear ERK, which originates from endosomal CLR, but did not affect activation of cytosolic ERK, which derives from CLR at the plasma membrane, or inhibit CLR endocytosis. Thus, $CGRP_{8-37}$-Chol selectively inhibits endosomal CLR signaling. $CGRP_{8-37}$-Chol inhibited CGRP-evoked excitation of spinal neurons, whereas unconjugated, membrane-impermeant $CGRP_{8-37}$ was inactive. After intrathecal injection, $CGRP_{8-37}$-Chol inhibited and reversed nociception more efficaciously than unconjugated antagonist. The capacity of $CGRP_{8-37}$-Chol to specifically antagonize endosomal CLR signaling and sustained excitation of spinal neurons, and to cause prolonged antinociception, reveals the importance of endosomal signaling for nociception, and illustrates the therapeutic utility of endosomally-directed drugs. Combined lipidated CLR and NK_1R antagonists could be especially effective antinociceptive drugs. The results also support a role for CGRP, released from the central projections of peptidergic nociceptors, and CLR/RAMP1 on second order spinal neurons in mechanical nociception (10).

There are several limitations to our study. We investigated CLR signaling in HEK293 cells. It will be necessary to determine whether CLR trafficking also regulates signaling in spinal neurons. The findings that inhibitors of dynamin and endosomal CGRP signaling ($CGRP_{8-37}$-Chol, MEK, PKC inhibitors) attenuate CGRP-induced excitation of neurons in spinal cord slices suggests that this is the case. However, the effects of endocytosis inhibitors on neuronal excitation require cautious interpretation because clathrin and dynamin mediate trafficking of many receptors and ion channels that regulate excitation of neurons (22, 23). Although we did not investigate the mechanisms by which endosomal CLR causes sustained excitation of spinal neurons, PKC and ERK may regulate the activity of ion channels and the transcription of genes that control excitation (12). PKC can also mediate CLR desensitization (24).

Our findings may be relevant to the development of CLR antagonists for the treatment of migraine (10). The vasodilator actions of CGRP are likely mediated by an adenylyl cyclase, cAMP and PKA pathway that operates at the plasma membrane to activate ATP-sensitive K^+ channels that lead to relaxation. Inhibiting this pathway could compromise blood supply to other organs, such as the heart. The specific targeting of endosomal signaling may provide a novel strategy to limit this side effect. Whether the efficacy of small molecule CLR antagonists depends on their capacity to antagonize endosomal CLR signaling is unknown. GPCRs are the largest class of signaling proteins, control many pathophysiological processes, and are the target of thirty percent of therapeutic drugs. Thus, our findings that GPCRs signal from endosomes *in vivo* and are targets for therapy may have far reaching consequences.

MATERIALS AND METHODS

SI provides detailed Materials and Methods.

cDNAs. CLR, RAMP1, βARR2, DynK44E, Dyn-WT, BRET and FRET cDNAs have been described (11-13).

Tripartite probes. Tripartite probes were synthesized on solid-phase (12).

Cell culture, transfection. HEK cells were transiently transfected and studied after 24-48 h.

FRET. Single cell FRET was measured by high content imaging (12, 13, 16). Population-based FRET was measured using a PHERAstar plate reader (BMG LabTech).

BRET. BRET was measured as described (12, 13).

Inhibitors. Cells were incubated with 30 μM Dy4a, Dy4a inactive, PS2, PS2 inactive, 10 μM NF449, 10 μM NF023, 100 nM UBO-QIC, 1 μM U73122, 100 μM EGTA, or vehicle (control) (30 min preincubation, inclusion throughout). Cells were preincubated with $CGRP_{8-37}$, $CGRP_{8-37}$-Chol, PEG-Biotin-Chol (30 nM-10 μM) or vehicle, washed, and challenged with CGRP (1 μM) at 0 or 4 h after washing.

Cell-surface ELISA. Cell-surface CLR and RAMP1 were quantified by ELISA using HA or myc antibodies (12).

Cy5-Chol and CLR internalization. Cells were incubated with Cy5 probes (200 nM, 60 min, 37°C), washed, and incubated with Alexa Fluor® 488-labeled anti-HA monoclonal antibody (40 min, room temperature). Live cells were imaged by confocal microscopy. Cells were stimulated with CGRP (50 nM) 3 h after probe addition. Cy5-Chol and HA-CLR-IR colocalization was assessed (25).

Animals. Institutional Animal Care and Use Committees approved all studies. Rats (Sprague-Dawley, males, 3-8 weeks) and mice (C57BL/6, males, 6-10 weeks) were studied.

Electrophysiology. Parasagittal slices were prepared from rat lumbar spinal cord. Spontaneous currents were recorded from lamina I neurons by cell-attached patch electrodes (26). Slices were preincubated with Dy4a or Dy4a inact (30 μM, 10 min), or with $CGRP_{8-37}$-Chol or $CGRP_{8-37}$ (1 μM, 60 min; tissue was washed, and incubated in antagonist-free medium for 60 min before recording). Slices were challenged with CGRP (1 μM, 2 min) and then SP (1 μM, 2 min). The firing rate for each cell was normalized to the response between 2-4 min, which was not

significantly different between groups. The firing time was determined as the duration of the response to last action potential.

CLR localization. Spinal cord slices were incubated with CGRP (1 μM, 5 min) and processed to localize CLR-IR (27). The plasma membrane/cytosolic pixel intensity ratio was determined to assess CLR-IR endocytosis (12).

Nociception. Nociception was studied in mice (12). Capsaicin (5 μg), CFA (2 $mg.ml^{-1}$), or vehicle was injected subcutaneously into the plantar surface of the left hindpaw (10 μl). von Frey scores were measured for 1-4 h post-capsaicin, and 36-40 h post-CFA. Formalin (4%, 10 μl) was injected subcutaneously into the plantar surface of the left hindpaw. Nocifensive behavior was recorded for 60 min. $CGRP_{8-37}$ (10 μM), $CGRP_{8-37}$-Chol (10 μM), Span (50 μM) or Span-Chol (50 μM) was injected intrathecally (5 μl, L3/L4) 3 h before injection of capsaicin or formalin, or 36 h after CFA. Investigators were blinded to test agents.

Spinal neuron culture. Superficial (lamina I-III) dorsal horn neurons from 1-2 day old neonatal rats were cultured for 6-8 d before $[Ca^{2+}]_i$ assays (28).

Statistics. Results are mean±SEM. Differences were assessed using Student's t test (two comparisons) or one- or two-way ANOVA and Dunnett's (BRET, nociception), Tukey's (FRET), Sidak's (average firing rate of spinal neurons), or Dunn's (duration of firing response of spinal neurons) tests (multiple comparisons).

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FIGURE LEGENDS

Fig. 1. CLR endocytosis. A-D. BRET assays of CLR-RLuc8 and βARR2-YFP (**A**), Rab5a-Venus and KRas-Venus (**B, D**), and Rab11-Venus (**C**) proximity in HEK cells. **D.** CLR-RLuc8 and Rab5a-Venus or KRas-Venus BRET (100 nM CGRP, 15 min). **E.** Cell-surface HA-CLR ELISA in HEK cells (100 nM CGRP, 15 min). N=3 experiments. **F.** Confocal images of HA-CLR-IR and EEA1-IR in HEK cells. DynK44E, dominant negative dynamin; Dy4a, dynamin inhibitor; PS2, clathrin inhibitor; inact, inactive analog. N=3-6 experiments, **P*<0.05, ***P*<0.01, ****P*<0.005, *****P*<0.0001 to basal or control. ANOVA, Sidak's test.

Fig. 2. CLR compartmentalized signaling. FRET assays of CGRP-induced activation of cytosolic PKC (cytoCKAR, **A, B, G**), cytosolic ERK (cytoEKAR, **C, D**), and nuclear ERK (nucEKAR, **E, F, H**) in individual HEK-HA-CLR/myc-RAMP1 cells. **A, C, E**: kinetics; **B, D, F, G, H**: area under curve (AUC). Cells were treated with inhibitors of dynamin (DynK44E, Dy4a), βARR siRNA, $G\alpha_s$ (NF449), $G\alpha_i$ (NF023), $G\alpha_q$ (UBO-QIC), phospholipase Cβ (U73122), or with a Ca^{2+} chelator (EGTA). n=29-453 cells, N=3 experiments. ***P*<0.01, ****P*<0.001 to vehicle; ^^^*P*<0.001 to CGRP control. ANOVA, Tukey's test.

Fig. 3. Tripartite probes. A. Probe structure. **B-D.** Confocal images of live HEK cells. **B, C.** HEK-HA-CLR/myc-RAMP1 cells were incubated with Cy5-Chol (**B**) or Cy5-Ethyl Ester (**C**). CLR was labeled with HA-Alexa488 antibody. Cells were stimulated with CGRP (50 nM, 30 or 60 min) to induce endocytosis. Insets (white boxes) show magnified regions and co-localization (arrows). Traces (left) show relative overlap of pixel intensities for HA-CLR and Cy5-Chol along dashed lines. **D.** Untransfected HEK cells incubated with Cy5-Chol. **E.** Overlap coefficient for HA-CLR and Cy5-Chol or Cy5-Ethyl Ester. n=6-14 cells, N= 4 experiments. $^{**}P<0.01$ to 0 min. ANOVA, Dunnett's test.

Fig. 4. Tripartite antagonism of CLR. ERK activity was assessed in individual HEK-HA-CLR/myc-RAMP1 cells expressing FRET biosensors for cytosolic ERK (cytoEKAR, **A, C, E**) or nuclear (nucEKAR, **B, D, F**) ERK. Cells were preincubated with vehicle, $CGRP_{8-17}$, or $CGRP_{8-37}$-Chol for 30 min and washed. CGRP-stimulated ERK activity was assessed immediately after washing (**A, B**, 30 min preincubation) or 4 h after washing (**C, D**, 4 h pre-pulse). **A-D**: kinetics. **E, F**: area under curve (AUC). **G, H.** Effects of graded concentrations of $CGRP_{8-37}$-Chol on cytosolic (**G**) and nuclear (**H**) ERK signaling in populations of HEK-HA-CLR/myc-RAMP1 cells. A-F, n=159-417 cells, N=3 experiments; G-H, N=4-9 experiments. $^{***}P<0.001$ to vehicle; $^{\wedge\wedge\wedge}P<0.001$ to antagonist vehicle control. ANOVA, Tukey's test.

Fig. 5. CLR endocytosis and excitation of spinal neurons. A-F. CGRP-induced activation of lamina I neurons in rat spinal cord slices. Dy4a, dynamin inhibitor; GF109203X, PKC inhibitor; U0126, MEK inhibitor. **A, D.** Representative traces. **B, E.** Firing rate normalized to 2 min. **C, F.** Firing duration to last action potential. n=5-8 neurons per group, N=19 rats. $^{*}P<0.05$, $^{***}P<0.001$. ANOVA, Sidak's multiple comparisons test (firing rate), or Dunn's multiple comparisons test (firing time). **G.** Confocal images of CLR-IR. Arrow: endosomes. Arrow head: plasma membrane. **H.** Quantification of CLR endocytosis. n=6-8 neurons per group, N=3 rats. $^{*}P<0.05$, $^{***}P<0.001$. ANOVA, Tukey's test.

Fig. 6. Tripartite antagonism of CGRP-induced excitation of spinal neurons. Spinal cord slices were incubated with vehicle (Veh), $CGRP_{8-37}$ or $CGRP_{8-37}$-Chol for 60 min, washed, and challenged with CGRP 60 min later. **A.** Representative traces. **B.** Firing rate normalized to 2 min. **C.** Firing duration to last action potential. n=5-7 neurons per group, N=21 rats. **P*<0.05, ***P*<0.01. ANOVA, Sidak's test (firing rate), or Dunn's test (firing time).

Fig. 7. Tripartite antagonism of nociception. Antagonists were injected intrathecally 3 h before intraplantar injection of capsaicin (Cap, **A, B**) or formalin (Form, **C, D**) or 36 h after CFA (**E, F**). von Frey withdrawal responses to stimulation of the planar surface of the injected paws (**A, E, F**) or non-injected paws (**B**) and nocifensive behavior (**C, D**) were assessed. (N). **P*<0.05, ***P*<0.01, ****P*<0.001 to basal or vehicle control. ANOVA, Dunnett's test.