Title: Neurokinin 1 Receptor Signaling in Endosomes Mediates Sustained Nociception and is Viable Therapeutic Target for Prolonged Pain Relief

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One Sentence Summary: The neurokinin 1 receptor signals from endosomes to induce sustained nociception, and antagonists that selectively target the receptor in endosomes provide more efficacious relief from pain.

Abstract:

G protein-coupled receptors (GPCRs) are conventionally considered cell-surface sensors of extracellular signals. They control most pathophysiological processes and are the target of thirty percent of therapeutic drugs. Activated receptors redistribute to endosomes, but whether endosomal receptors generate signals that control complex processes *in vivo* and are viable therapeutic targets is unexplored. We report that the substance P (SP) neurokinin 1 receptor (NK_1R) signals from endosomes to induce sustained excitation of spinal neurons and pain transmission, and that specific antagonism of the NK_1R in endosomes with membrane-anchored drug conjugates provides more effective and sustained pain relief. Pharmacological and genetic disruption of clathrin, dynamin and βarr blocked SP-induced NK_1R endocytosis, and prevented SP-stimulated activation of cytosolic protein kinase C, nuclear extracellular signal regulated, and transcription. Endocytic inhibitors prevented sustained SP-induced excitation of neurons in spinal cord slices *in vitro*, and attenuated nociception *in vivo*. NK_1R antagonists, when conjugated to cholestanol to promote endosomal targeting and retention, selectively inhibited endosomal signaling and sustained neuronal excitation. Cholestanol conjugation amplified and prolonged the anti-nociceptive actions of NK_1R antagonists. These results reveal a critical role for endosomal signaling of the NK_1R in the complex pathophysiology of pain, and demonstrate the utility of endosomally-targeted GPCR antagonists.

Main Text:

Introduction

Whereas acute pain allows avoidance of injury and is essential for survival, chronic pain accompanies disease (*e.g.*, inflammatory diseases, neuropathies) and therapy (*e.g.*, chemotherapy), afflicts twenty percent of individuals at some point of their lives, and is a major cause of suffering (*1*). The mechanisms that underlie the transition between acute (physiological) and chronic (pathological) pain and that sustain chronic pain are unknown. Current therapies for chronic pain are often ineffective or produce unacceptable side effects. The opioid epidemic, a leading cause of medication-induced death, highlights the need for improved pain therapy (*2*).

With almost 1,000 members in humans, G protein-coupled receptors (GPCRs) are the largest family of receptors, participate in most physiological and pathophysiological processes, and are the target of approximately thirty percent of therapeutic drugs (*3*). GPCRs control all steps of pain transmission (*1, 4*). GPCRs at the peripheral terminals of primary sensory neurons detect ligands from inflamed and injured tissues, and GPCRs control the activity of second order spinal neurons that transmit pain signals centrally. Although GPCRs are a major therapeutic target for chronic pain, most GPCR-targeted drugs fail in clinical trials, often for unknown reasons (*4, 5*).

GPCRs are conventionally viewed as cell-surface receptors that detect extracellular ligands and couple to heterotrimeric G proteins, which trigger plasma membrane-delimited signaling events (*e.g.*, second messenger formation, growth factor receptor transactivation, ion channel regulation). Activated GPCRs associate with β-arrestins (βarrs), which uncouple receptors from G proteins and terminate plasma membrane signaling. βarrs also couple receptors

to clathrin and adaptor protein-2 and convey receptors and ligands to endosomes (*6*). Once considered merely a conduit for GPCR trafficking, endosomes are a vital site of signaling (*4, 7, 8*). βarrs recruit GPCRs and mitogen-activated protein kinases to endosomes and thereby mediate endosomal GPCR signaling (*9, 10*). Some GPCRs elicit $G\alpha_s$-dependent signals from endosomes (*11, 12*). GPCR/G protein/βarr complexes also contribute to sustained signaling by internalized receptors (*13*). Although a growing number of GPCRs can signal from endosomes, the mechanisms and outcomes of endosomal signaling are incompletely understood, and its relevance to complex pathophysiological processes *in vivo* is unexplored. Drug discovery programs aim to identify ligands for cell-surface GPCRs, and whether endosomal GPCRs are a therapeutic target remains to be determined.

We examined the contribution of endocytosis of the neurokinin 1 receptor (NK_1R) to substance P (SP) -mediated nociception. Painful stimuli release SP from the central projections of primary sensory neurons in the dorsal horn of the spinal cord, where SP induces endocytosis of the NK_1R in second order neurons (*5, 14*). The NK_1R may also be internalized in pain sensing regions of the brain of patients with chronic pain (*5, 15*). We hypothesized that endosomal signaling is a critical but unappreciated contributor to pain transmission, and that targeting NK_1R antagonists to sites of endosomal signaling may provide a novel and effective route to pain relief. Thus, the clinical failure of conventional antagonists (*5*) may relate to their inability to target and antagonize the NK_1R within multi-protein signalosomes of acidified endosomes.

Results

Clathrin, Dynamin and βarrs Mediate NK_1R Endocytosis

To quantify NK_1R endocytosis, we used Bioluminescence Resonance Energy Transfer (BRET) to assess NK_1R proximity to βarrs and resident proteins of plasma (KRas) and early endosomal (Rab5a) membranes in HEK293 cells (fig. S1A). SP (1, 10 nM) increased NK_1R-RLuc8/βarr1/2-YFP BRET (fig. S1B,C), which is consistent with βarr-mediated NK_1R endocytosis (*16*). SP decreased NK_1R-RLuc8/KRas-Venus BRET and concomitantly increased NK_1R-RLuc8/Rab5a-Venus BRET (fig. S1D-G), indicating NK_1R endocytosis. The dynamin inhibitor Dyngo™-4a (Dy4) (*17*), the clathrin inhibitor Pitstop®-2 (PS2) (*18*), and dominant-negative dynamin K44E (*19*) inhibited NK_1R endocytosis, whereas inactive analogues (Dy4 inact, PS2 inact), and wild-type (WT) dynamin had no effect. Dynamin K44E increased the NK_1R-RLuc8/βarr1/2-YFP BRET, suggesting that endocytosis initiates NK_1R/βarr dissociation (fig. S1H). Dy4 and PS2 also inhibited endocytosis of fluorescent Alexa568-SP in HEK-NK_1R cells, causing retention in punctate structures (fig. S1I). These structures may represent ligand/receptor clusters in invaginated pits in cells treated with Dy4, or at the plasma membrane in cells treated with PS2. Thus, βarrs, clathrin and dynamin mediate SP-induced NK_1R endocytosis.

NK_1R Endocytosis Mediates SP Signaling in Subcellular Compartments

To study the link between GPCR endocytosis and signaling in subcellular compartments with high spatiotemporal fidelity, we expressed in HEK293 cells the NK_1R and Förster Resonance Energy Transfer (FRET) biosensors for cytosolic (CytoEKAR) or nuclear (NucEKAR) extracellular signal-regulated kinase (ERK) activity, plasma membrane (pmCKAR) or cytosolic (CytoCKAR) protein kinase C (PKC) activity, and plasma membrane (pmEpac2) or cytosolic (CytoEpac2) cAMP (fig. S2A) (*20*). SP (1 nM) induced a gradual and sustained

activation of nuclear ERK (Fig. 1A-C), and a rapid and sustained activation of cytosolic PKC (Fig. 1D-F) and cAMP (Fig. 1G-I). SP rapidly and transiently activated cytosolic ERK (fig. S2B, C), did not affect plasma membrane PKC (fig. S2D, E), and increased plasma membrane cAMP (fig. S2F, G). Inhibitors of clathrin (PS2) and dynamin (Dy4) abolished SP stimulation of nuclear ERK, cytosolic PKC and cytosolic cAMP, indicating a requirement for endocytosis. In contrast, PS2 and Dy4 did not affect SP activation of cytosolic ERK or plasma membrane cAMP, which do not require endocytosis, but amplified plasma membrane PKC activity. Expression of dynamin K44E, but not dynamin WT, prevented SP stimulation of nuclear ERK (Fig. 1J-L). Dynamin K44E did not prevent SP stimulation of cytosolic ERK, but caused the response to become sustained when compared to dynamin WT (fig. S2H-J). Knockdown of dynamin-1 and clathrin heavy chain with siRNA (fig. S2K, L) prevented SP activation of nuclear ERK (Fig. 1M, N).

Transcription is a major endpoint of GPCR signaling, including activation of nuclear ERK, and the β_2-adrenergic receptor signals from endosomes to regulate transcription (*21*). To investigate the contribution of NK_1R endocytosis to SP-stimulated transcription, we expressed in HEK-NK_1R cells a reporter encoding secreted alkaline phosphatase (SEAP) under control of the serum response element (SRE) transcription factor. SP (10 nM) stimulated SRE-SEAP secretion after 4 and 24 h, indicating stimulated transcription (Fig. 1O). Dynamin K44E abolished SP-stimulated transcription at both times. Dynamin K44E reduced the efficacy but not the potency of SP-induced transcription, measured after 24 h (fig. S2M). Thus, NK_1R endocytosis is required for SP stimulation of transcription.

We have previously shown that βarrs mediate NK_1R endosomal signaling and nuclear ERK activation (*9, 22, 23*). To examine the contribution of G proteins to endosomal NK_1R

signaling, we used BRET to study SP-induced trafficking of $G\alpha_q$ to Rab5a-positive early endosomes. SP (0.1-10 nM) decreased NK_1R-RLuc8/KRas-Venus and increased NK_1R-RLuc8/Rab5a-Venus BRET, demonstrating endocytosis, and decreased $G\alpha_q$-RLuc8/$G\gamma_2$-Venus BRET, consistent with G protein activation (Fig. 2A-C; fig. S3A-C). SP increased $G\alpha_q$-RLuc8/Rab5a-Venus BRET, which indicates $G\alpha_q$ translocation to early endosomes containing NK_1R (Fig. 2D; fig. S3D). We used immunofluorescence and super-resolution microscopy to localize NK_1R-immunoreactivity (IR), $G\alpha_q$-IR and early endosomal antigen 1 (EEA1)-IR. When cells were treated with SP at 4°C, NK_1R-IR and $G\alpha_q$-IR colocalized at the plasma membrane but were not detected in endosomes (Fig. 2E). After incubation with SP at 37°C, NK_1R-IR and $G\alpha_q$-IR colocalized with EEA1-IR in endosomes (Fig. 2E,F).

The $G\alpha_q$ inhibitor UBO-QIC prevented SP activation of nuclear ERK (Fig. 2G; fig. S3E), which also depends on βarrs and PKC but not epidermal growth factor receptor transactivation (*9, 22, 23*). UBO-QIC, the phospholipase C (PLC) inhibitor U73122 and the Ca^{2+} chelator EGTA prevented activation of cytosolic PKC (Fig. 2H; fig. S3F), consistent with a $G\alpha_q$, PLC and Ca^{2+}-dependent PKC pathway. UBO-QIC, the PKC inhibitor GF109203X and EGTA, but not the $G\alpha_s$ inhibitor NF449, prevented SP generation of cytosolic cAMP (Fig. 2I; fig. S3G), supporting a role for $G\alpha_q$-mediated activation of Ca^{2+}-dependent PKC in the generation of cAMP. UBO-QIC did not affect NK_1R endocytosis (fig. S3H). In addition to inhibiting PKCα (4% control), GF109203X (Bis-1) also inhibits other kinases (*24*), which may also contribute to SP signaling.

These results support the hypothesis that SP and the NK_1R signal from endosomes by $G\alpha_q$-mediated mechanisms to activate nuclear ERK and cytosolic PKC and cAMP.

Endocytosis Mediates Sustained SP-evoked Excitation of Spinal Neurons

The NK_1R mediates nociceptive transmission in second order spinal neurons, where painful stimuli induce SP release, NK_1R endocytosis and ERK activation (*5, 14, 25*). SP causes persistent NK_1R-dependent excitation of spinal neurons by unknown mechanisms (*26*). To evaluate whether NK_1R endosomal signaling mediates this sustained excitation, we made cell-attached patch clamp recordings from NK_1R-positive neurons in lamina I of the dorsal horn in slices of rat spinal cord. SP (1 μM, 5 min) stimulated NK_1R-IR endocytosis in spinal neurons (Fig. 3A,B). Brief exposure to SP (1 μM, 2 min) triggered rapid onset action potential firing that was sustained after washout (Fig. 3C-E). Dy4 but not Dy4 inact inhibited NK_1R endocytosis. Dy4 did not affect the initial onset of SP-induced firing, but prevented the sustained response, reducing both the firing rate and firing time, whereas Dy4 inact had no effect. The SP-induced firing rate (2 min normalized) was (events.2 min^{-1}) Dy4, 342.1±120.7 and Dy4 inact, 569.0±187.6 (P<0.05).

To define the signaling pathway that mediates SP-evoked excitation of spinal neurons, slices were preincubated with inhibitors of MEK (U0126) or PKC (GF109203X) or vehicle (control). U0126 inhibited SP-induced firing time of lamina I neurons by 67.5±8.3% (control, 10.01±1.8 min, n=10 cells, 8 rats; U0126, 3.2±0.8 min, n=6 cells, 6 rats; P<0.05) (Fig. 3F-H). GF109203X reduced SP-induced firing time of lamina I neurons by 56.8±8.2% (control, 10.01±1.8 min, n=10 cell, 8 rats; GF109203X, 4.33±0.82 min, n=7 cells, 4 rats; P<0.05). U0126 and GF109203X reduced the number of SP-stimulated action potentials by 84±5% and 61±15% respectively, compared to controls.

In addition to their role in endocytosis of GPCRs, dynamin and clathrin regulate vesicular transport and exocytosis, and thereby can control multiple components of synaptic transmission (*27, 28*). However, Dy4 did not affect the generation of excitatory post-synaptic currents

(EPSCs) in lamina I/II$_o$ neurons in response to primary afferent stimulation (Fig. 3I,J). PS2 and Dy4 did not affect capsaicin-stimulated release of SP or calcitonin gene-related peptide (CGRP) from segments of mouse dorsal spinal cord (Fig. 3K,L).

Thus, NK_1R endocytosis and resultant ERK and PKC signaling mediate sustained SP-induced firing of spinal neurons. The effects of dynamin and clathrin inhibitors in the spinal cord are unrelated to changes in glutaminergic-mediated fast synaptic transmission or the exocytosis of neuropeptides.

Clathrin, Dynamin, and βarrs Mediate NK_1R Endocytosis and Nociception in Vivo

To determine the involvement of dynamin and clathrin in NK_1R endocytosis *in vivo*, we injected Dy4, PS2, inactive analogues or vehicle intrathecally (L3/L4) to rats. After 30 min, vehicle or capsaicin was administered by intraplantar injection. The spinal cord was removed 10 min later, and the NK_1R was localized by immunofluorescence and confocal microscopy. In vehicle-treated control rats, the NK_1R-IR was mostly at the plasma membrane of lamina I neurons (% NK_1R-IR within 0.5 μm of plasma membrane: 80.7±1.6; n=3 rats, 6 neurons analyzed per rat; Fig. 4A,C, Movie S1). Intraplantar injection of capsaicin stimulated NK_1R endocytosis (42.1±5.6; P=0.0027 to control (t-test); Movie S2). Intrathecal injection of Dy4 or PS2, but not inactive analogues, inhibited capsaicin-stimulated NK_1R endocytosis (Dy4 59.6±0.2 vs. Dy4 inact 49.9±0.8, P=0.0004; PS2 69.0±1.1 vs. PS2 inact 51.9±1.3, P=0.0135; Movies S3, S4; Fig. 4A,C). Painful peripheral stimuli activate ERK in NK_1R-expressing spinal neurons, which contributes to hyperalgesia (*25*). Intraplantar capsaicin stimulated ERK phosphorylation in lamina I/II dorsal horn neurons (Fig. 4B,D). Dy4 or PS2 prevented capsaicin-stimulated ERK

activation in spinal neurons. Thus, painful stimuli induce clathrin- and dynamin-dependent NK_1R endocytosis in spinal neurons, which is required for ERK signaling.

Does NK_1R endocytosis in spinal neurons mediate pain transmission? To evaluate the importance of the NK_1R, clathrin and dynamin for nociception, we injected vehicle, NK_1R antagonist SR140,333 (*29*), Dy4, PS2 or inactive analogues intrathecally (L3/L4) to mice. After 30 min, vehicle or capsaicin was administered by intraplantar injection into one hindpaw. Withdrawal responses were measured to stimulation of the plantar surface of the ipsilateral (injected) and contralateral (non-injected) hindpaws with von Frey filaments, and edema was assessed by measuring thickness of the ipsilateral paw. In vehicle (intrathecal)-treated mice, capsaicin caused mechanical allodynia and edema for 4 h. SR140,333 caused a partial and transient inhibition of capsaicin-induced allodynia, whereas Dy4 and PS2, but not inactive analogues, caused a large and sustained inhibition of allodynia (Fig. 4E, fig. S4A). Edema was unaffected, confirming local action in the spinal cord (fig. S4B).

Given the role of clathrin and dynamin in vesicular transport and synaptic transmission (*27, 28*), the antinociceptive actions of Dy4 and PS2 could be due to impaired motor responses rather than nociceptive transmission. However, Dy4 and PS2 did not affect withdrawal responses of the contralateral paw or rotarod latency, suggesting normal motor behavior (Fig. 4F,G). Intrathecal Dy4 also inhibited capsaicin-evoked mechanical allodynia in rat, which supports a role for dynamin in nociception in different species (fig. S4C).

We used genetic approaches to disrupt endocytosis in view of possible off-target actions of dynamin inhibitors (*30*). Intrathecal injection of dynamin-1 siRNA knocked down dynamin-1-IR (fig. S4D) and inhibited capsaicin-evoked allodynia after 24 h and 48 h in mice (Fig. 4H, fig. S4E). Intrathecal βarr1+2 siRNA knocked down βarr1+2 mRNA (fig. S4F) and inhibited

capsaicin-evoked allodynia at 36 h (Fig. 4I). siRNAs did not affect withdrawal responses of the contralateral paw (fig. S4G,H), consistent with normal motor function.

Endocytosis and subsequent recycling mediate resensitization and sustained signaling of several GPCRs, including the NK_1R (*31*). Thus, the antinociceptive actions of endocytic inhibitors could be due to disrupted resensitization of plasma membrane signaling rather than impaired endosomal signaling. Endothelin-converting enzyme-1, which is coexpressed with the NK_1R in spinal neurons (*22*), degrades SP in endosomes and thereby promotes recycling and resensitization of the NK_1R (*32*). However, intrathecal injection of SM-19712, an inhibitor of endothelin-converting enzyme-1 that prevents NK_1R recycling and resensitization (*32*), had no effect on capsaicin-induced allodynia (Fig. 4J). These results suggest that the analgesic actions of endocytic inhibitors are unrelated to disrupted resensitization. Consistent with a role for NK_1R endocytosis and βarrs in SP-evoked nuclear ERK signaling (*9*), intrathecal MEK inhibitor U0126 inhibited capsaicin-evoked allodynia (*25*) (Fig. 4K).

The effects of inhibitors of dynamin and clathrin on non-inflammatory and inflammatory pain were examined. Intrathecal injection of Dy4 and PS2 blunted both the early (non-inflammatory) and late (inflammatory) phases of the nocifensive response to intraplantar formalin (Fig. 4L). When injected intrathecally 36 h after intraplantar injection of complete Freund's adjuvant (CFA), which causes sustained inflammatory pain, inhibitors of dynamin and clathrin reversed pre-existing mechanical hyperalgesia (Fig. 4M). The NK_1R was robustly internalized in spinal neurons of mice after intraplantar injection of capsaicin, formalin and CFA (fig. S5A-D). Intrathecal injection of Dy4 prevented capsaicin- and formalin-induced NK_1R endocytosis, and reversed CFA-induced NK_1R endocytosis.

These results suggest that clathrin and dynamin mediate pain-evoked endocytosis of NK_1R in spinal neurons, which is required for nociception.

Disruption of NK_1R/βarr Interactions Inhibits NK_1R Endocytosis and Nociception in Vivo. To substantiate involvement of NK_1R endocytosis in nociception, we devised a pharmacological approach to inhibit NK_1R/βarr interactions and NK_1R endocytosis. G protein receptor kinases (GRKs) phosphorylate S/T-rich regions in the C-terminus of GPCRs, which interact with βarrs (*33*). A deletion mutant NK_1Rδ311 lacks the C-terminus and corresponds to a naturally occurring NK_1R variant (*5*) (Fig. 5A). NK_1Rδ311 was normally expressed at the plasma membrane of HEK293 cells, but did not associate with βarrs or internalize (Fig. 5B,C, fig. S6A-C). In HEK-NK_1Rδ311 cells, SP stimulated cytosolic but not nuclear ERK, and did not affect transcription activity, consistent with endocytosis-dependent nuclear ERK signaling and transcription (Fig. 5D,E). Peptides corresponding to predicted phosphorylation sites in the C-terminus of mouse NK_1R were conjugated to membrane-penetrating Tat peptide (Fig. 5A). A combination of three peptides inhibited SP-induced NK_1R-RLuc8/βarr2-YFP BRET and prevented SP-induced NK_1R endocytosis, compared to a control peptide, suggesting effective disruption of NK_1R/βarr interactions (Fig. 5F,G). When injected intrathecally, inhibitors of NK_1R/βarr interactions suppressed capsaicin-evoked allodynia and formalin-induced nociceptive behavior, and reversed CFA-induced hyperalgesia (Fig. 5H-J). Together, these results support a role for βarr-mediated NK_1R endocytosis and endosomal signaling in nociception.

Lipid Conjugation Delivers NK_1R Antagonists to Endosomes and Selectively Blocks Sustained Endosomal Signals

We observed that clathrin, dynamin and βarr inhibitors and siRNA, including selective inhibitors of NK_1R/βarr interactions, suppress SP-induced NK_1R endocytosis, compartmentalized signaling, transcription and neuronal excitability, and have anti-nociceptive actions. These findings support the hypothesis that endosomal NK_1R signaling underlies sustained neuronal excitation and nociception. Thus, selective antagonism of endosomal receptors could be an effective treatment for pain. To investigate this possibility, and to provide direct evidence for the importance of endosomal signaling for nociception, we devised a novel approach to deliver and concentrate GPCR antagonists in early endosomes.

Lipid conjugation anchors drugs at membrane surfaces and promotes endosomal delivery (*34*). We synthesized tripartite probes comprising cholestanol (Chol, promotes membrane insertion, anchoring) or ethyl ester (control, no membrane anchoring), a flexible polyethylene glycol (PEG) linker, and a cargo of either cyanine 5 (Cy5) for localization, or spantide I (Span), a peptidic membrane impermeant NK_1R antagonist (*35*) (Fig. 6A). Some probes were labeled with Span and cyanine 5. When incubated with HEK293 cells, Cy5-Chol inserted into the plasma membrane within 5 min, whereas Cy5-Ethyl ester remained entirely extracellular (Fig. 6B, Movies S5, S6). After 4 h of continuous incubation, Cy5-Chol was concentrated in Rab5a-positive early endosomes, although Cy5-Chol was also detected at the plasma membrane (Fig. 6C). When incubated with HEK-NK_1R-GFP cells for 4 h, Cy5-Chol also colocalized with NK_1R-GFP in endosomes (cells were stimulated with SP to induced NK_1R endocytosis) (Fig. 6C). When HEK-NK_1R-GFP cells were pulse-incubated with Cy5-Chol for 30 or 60 min, washed, and allowed to recover for 4 h, Cy5-Chol was gradually removed from the plasma membrane and accumulated in NK_1R-GFP-positive endosomes, although some probe remained at the plasma membrane (fig. S7A, C). Cy5-Ethy ester was not taken up by cells after pulse-incubation (fig.

S7B). Quantification of Cy5-Chol uptake after 30 min pulse-incubation, indicated that 69% of cell-bound probe was internalized at 4 h and 79% at 8 h after washing (fig. S7D). After pulse incubation, Cy5-Span-Chol trafficked to NK_1R-GFP-positive endosomes (Fig. 6C). Dy4 inhibited uptake of Chol-conjugated tripartite probes, consistent with constitutive dynamin-mediated endocytosis (fig. S7E).

We used FRET to quantify association of tripartite probes with the NK_1R in endosomes. NK_1R with extracellular N-terminal SNAP-Tag® was expressed in HEK293 cells and cell-surface NK_1R was labeled with membrane impermeant SNAP-Surface™-549. SP (10 nM, 30 min) evoked translocation of SNAP-549-NK_1R to endosomes (Fig. 6D). Cells were treated with Cy5-Chol, and FRET between SNAP-549-NK_1R and Cy5-Chol was measured in regions of interest within the cytosol. Cy5-Chol/SNAP-549-NK_1R FRET was detected after 5 min and increased for 60 min (Fig. 6D, E, Movie S7). FRET was not detected in control cells lacking NK_1R (Fig. 6E).

Span-Chol antagonized SP (3 nM, EC_{80})-stimulated Ca^{2+} signaling in HEK-NK_1R cells (pIC_{50} Span, 8.23±0.21; Span-Chol 8.44±0.29) and thus retains activity. Since the tripartite probe was concentrated in endosomes after 4 h, we examined NK_1R endosomal signaling 4 h after preincubation with antagonists. When HEK-NK_1R cells were preincubated with Span-Chol, Span or SR140333 for 30 min, and then immediately challenged with SP, all antagonists blocked nuclear ERK (Fig. 6F, H) and cytosolic ERK (fig. S8A, C) activity, indicating effective antagonism of cell-surface NK_1R. When cells were pulse-incubated with antagonists for 30 min, washed, and stimulated with SP 4 h later (to allow lipidated antagonists to concentrate in endosomes), Span-Chol alone inhibited nuclear ERK (derives from endosomal NK_1R) (Fig. 6G, H) and no antagonist inhibited cytosolic ERK (derives from plasma membrane NK_1R) (fig. S8B, C). Span-Chol also prevented SP-induced transcription. HEK-NK_1R cells were incubated with

Span or Span-Chol for 30 min, washed, recovered for 4 h, and then stimulated with SP for 20 h. Span-Chol abolished SP-stimulated SRE-SEAP secretion (derives from endosomal NK_1R), whereas unconjugated Span was ineffective (Fig. 6I). However, when continuously incubated with antagonists, both Span-Chol and Span inhibited transcription. Span-Chol did not affect isoprenaline-induced activation of nuclear ERK, which is mediated by the endogenous β_2-adrenergic receptor (fig. S8D). Thus, the effects of tripartite antagonists are not mediated by a non-specific disruption of endosomal signaling.

The results show that lipid conjugation promotes the effective delivery and retention of antagonists to endosomes containing the NK_1R. After pulse incubation, Span-Chol causes sustained and selective antagonism of endosomal not plasma membrane NK_1R. Unconjugated Span and SR140,333, a potent small molecule antagonist, are unable to effectively inhibit persistent NK_1R signaling in endosomes.

Endosomally Targeted NK_1R Antagonists Block Nociception

To assess whether antagonism of the endosomal NK_1R blocks sustained SP-induced excitation of spinal neurons, we incubated slices of rat spinal cord with Span-Chol or Span for 60 min, washed, and challenged with SP 60 min later. In vehicle- or Span-treated slices, SP caused brisk firing that was sustained after washout (Fig. 7A-C). As observed with endocytic inhibitors, Span-Chol did not suppress the initial excitation, but prevented sustained excitation. The SP-induced firing rate (2 min normalized) was (events.2 min^{-1}) Span-Chol, 196.6±81.6, and Span, 242.6±95.9 (P<0.05).

To evaluate whether endosomal targeting improves the efficacy and duration of action of NK_1R antagonists for the treatment of pain, we administered cholestanol-conjugated or

conventional antagonists by intrathecal injection 3 h before intraplantar injection of capsaicin. This time was selected to allow endosomal accumulation of lipidated antagonists. When Cy5-Chol was injected intrathecally, probe was detected in laminae I-III neurons after 3-6 h, confirming delivery and retention in pain-transmitting neurons (Fig. 7D). Cy5-Chol did not affect nociception, which excludes non-specific actions of cholestanol (Fig. 7E). Span-Chol, but not Span or SR140333, inhibited capsaicin-evoked mechanical allodynia (Fig. 7E). When administered 30 min after capsaicin, intrathecal Span was transiently antinociceptive, whereas Span-Chol caused a delayed (3 h), persistent (6 h) and substantial (>50%) antinociception (Fig. 7F).

The small molecule NK_1R antagonist L-733,060 (*36*) conjugated to Chol antagonized SP (3 nM, EC_{80})-stimulated Ca^{2+} signaling in HEK-NK_1R cells (% inhibition, 1 nM SP: 10 nM L-733,060, 40.8±8.9; 10 nM L-733,060-Chol, 71.1±9.2) and thus retained activity. When injected intrathecally 3 h before intraplantar capsaicin, L-733,060-Chol was antinociceptive from 1-4 h, whereas L-733,060 was antinociceptive only at 1 h (Fig. 7G).

When injected intrathecally 3 h before intraplantar formalin, Span-Chol inhibited both phases of nocifensive behavior more completely than Span or SR140333 (Fig. 7H). When injected intrathecally 36 h after intraplantar CFA, Span-Chol inhibited mechanical hyperalgesia from 1-6 h, whereas the antinociceptive actions of Span and SR140333 were minor and transient for Span (Fig. 7I, J).

The enhanced potency and duration of action of lipidated antagonists could be due to improved metabolic stability rather than appropriate targeting of endosomal NK_1R. However, Span and Span-Chol were similarly stable in human cerebrospinal fluid (Fig. 7K). Thus,

enhanced stability does not account for the sustained antinociceptive actions of cholestanol-conjugated antagonists.

Collectively, our results reveal, for the first time, the importance of endosomes as platforms for GPCR compartmentalized signaling that underlies sustained excitation of spinal neurons and nociception, and show that targeting endosomal GPCR signaling provides a novel alternative for sustained pain relief.

Discussion

Our results support a reinterpretation of the notion that the primary physiological actions of GPCRs *in vivo* are mediated by cell-surface receptors. By studying the NK_1R as a prototypical GPCR that traffics to endosomes, we show that endosomal receptors convey sustained signals that underlie excitation and nociceptive transmission in spinal neurons, and that targeting these receptors in endosomes is required for optimal pharmacological intervention.

We report that endosomal GPCRs generate a spectrum of signals in subcellular compartments. Clathrin and dynamin disruption prevented NK_1R endocytosis, and inhibited activation of nuclear ERK, cytosolic PKC and cytosolic cAMP. Dynamin inhibitors also blocked SP-induced transcription, which is likely mediated by nuclear ERK. A C-terminally truncated mutant, $NK_1R\delta311$, was also unable to internalize, activate nuclear ERK, or stimulate transcription. $G\alpha_q$ inhibition blocked NK_1R endosomal signals, and endosomes contained both activated NK_1R and $G\alpha_q$. Our results are consistent with the hypothesis that the NK_1R in endosomes signals by $G\alpha_q$-dependent processes that activate nuclear ERK, cytosolic PKC and cytosolic cAMP to cause nociception (Fig. 8A, Movie S8). By delivering activated NK_1R to endosomes and serving as a scaffold for signaling complexes, βarrs facilitate these signals (*9, 22,*

23). Our findings add to the growing number of GPCRs, including β_2-adrenergic and thyroid stimulating hormone receptors (*11, 12*), known to signal from endosomes by G protein-dependent processes, and provides the first *in vivo* evidence, to our knowledge, that this endosomal mechanism is physiologically relevant.

Pharmacological and genetic disruption of clathrin, dynamin and βarrs blocked SP-induced excitation of spinal neurons, attenuated pain-evoked NK_1R endocytosis and ERK activation in spinal neurons, and suppressed nociception. Inhibitors of MEK and PKC suppressed sustained SP-induced excitation of spinal neurons, which likely requires compartmentalized ERK and PKC signaling. A MEK inhibitor also blocked nociception. These findings suggest that endosomal NK_1R signaling is necessary for sustained excitation of spinal neurons and nociceptive transmission in the spinal cord, and reveal, for the first time, a vital link between endosomal signaling and nociception.

Our results provide new information about the contribution of clathrin and dynamin to SP-induced excitation of spinal neurons and nociceptive transmission (Fig. 8B). Although the effects of endocytic inhibitors on NK_1R internalization and nociception are consistent with a role for NK_1R endocytosis in pain, clathrin and dynamin mediate endocytosis and exocytosis of many GPCRs, ion channels and neuropeptides that control pain transmission (*27, 28*). However, the findings that selective disruption of NK_1R-βarr interactions using membrane-permeant peptides, and specific antagonism of endosomal NK_1R with lipidated antagonists, effectively suppress neuronal excitation and nociception in several models, provide direct support for a major contribution of the endosomal NK_1R to pain. Clathrin and dynamin inhibitors did not affect fast synaptic transmission in the spinal cord or capsaicin-evoked neuropeptide release from spinal

terminals of nociceptors, and had no effect on motor coordination *in vivo*. These results suggest that synaptic transmission and vesicular transport were unaffected.

Our discovery that endosomes are platforms for compartmentalized GPCR signaling that underlies pathophysiologically important processes *in vivo* has therapeutic implications. Delivery of antagonists to endosomes may facilitate the disruption of sustained signals from endosomal GPCRs that underlie disease, and could provide enhanced efficacy and selectivity (Fig. 8C). The accumulation of tripartite probes in NK_1R-positive endosomes demonstrates the feasibility of endosomal delivery. The capacity of Span-Chol, L-733,060-Chol, but not unconjugated antagonists, to specifically antagonize endosomal NK_1R signaling and sustained excitation of spinal neurons, and to cause prolonged and more effective antinociception demonstrates the importance of endosomal signaling for pain, and illustrates the therapeutic utility of endosomally-directed drugs. Lipidated antagonists were able to both prevent and reverse nociception, demonstrating therapeutic efficacy. The NK_1R redistributes from the plasma membrane to endosomes in chronic inflammatory and neurological diseases that are associated with persistent SP release (*5*). Conventional antagonists are designed to target GPCRs at the cell-surface. We propose that the inability of conventional antagonists to effectively target the NK_1R in endosomes, where the receptor assembles a multi-protein signalosome in an acidic environment, contributes to their lack of clinical success (*5*).

Herein we report that endosomal GPCR signaling is a critical mediator and a viable therapeutic target for pain. Given that many activated GPCRs internalize, and that GPCRs participate in most pathophysiological processes and are the target of one third of approved drugs, our study suggests that therapeutic targeting of endosomal GPCRs is a new paradigm of drug delivery that offers more effective and selective treatments for disease.

Materials and Methods

Supplementary Materials and Methods provide complete methods.

Animals. Institutional Animal Care and Use Committees approved all studies. Animals were maintained in a temperature-controlled environment with a 12 h light/dark cycle and free access to food and water.

Tripartite probes. Sulfonated cyanine 5 carboxylic acid, Span I and L-733,060 were conjugated to cholestanol or aspartate ethyl ester *via* a flexible PEG linker by standard Fmoc solid-phase peptide synthesis, and were analyzed by LCMS (fig. S9, fig. S10).

Alexa568-SP. SP was labeled with AlexaFluor568 NHS ester and purified by reverse-phase HPLC.

NK_1R-βarr inhibitors. Peptides were synthesized corresponding to predicted phosphorylation sites in the C-terminus of the mouse NK_1R ($S^{398}SSFYSNM^{405}$, $S^{390}NSKTMTE^{397}$, $L^{382}TSNGSSR^{389}$) or control peptide (MSNSYSFS) with N-terminal Tat sequence (YGRKKRRQRRR).

cDNAs. cDNAs have been described encoding sensors for BRET (*37, 38*) and FRET (*39-42*). cDNAs have been reported encoding full length and truncated δ311 rat HA-NK_1R (*43*), GFP-dynamin and GFP-dynamin K44E (*19*).

Cell lines, transfection. HEK293 cells stably expressing rat HA-NK_1R have been described (*32*). HEK293 cells were transiently transfected as described (*23*).

BRET. HEK293 cells were transfected with the BRET sensors, and BRET ratios were determined after 48 h, as described (*23*).

FRET biosensors of compartmentalized signaling. HEK293 cells were transfected with NK_1R, FRET biosensors, and siRNA, and FRET was assessed after 48 or 72 h, as described (*20, 23*).

FRET assays of endosomal NK_1R targeting. HEK293 cells were transfected with SNAP-Tagged® NK_1R. After 48 h, the cell-surface NK_1R was labeled with SNAP-Surface™ 549 photostable fluorescent substrate. Cells were washed, recovered for 30 min, and stimulated with SP to induce NK_1R endocytosis. Cells were incubated with Cy5-Chol. SNAP-549/Cy5 sensitized emission FRET was measured by confocal imaging.

Transcription assays. HEK293 cells were transfected with NK_1R or $NK_1R\delta311$ and SEAP reporter gene under the control of the SRE consensus sequence. Some cells were also transfected with dynamin WT, dynamin K44E or pcDNA3. After 48 h, cells were then stimulated with vehicle or SP for 4 or 20 h. SEAP activity was determined as described (*44*).

Ca^{2+} assays. $[Ca^{2+}]_i$ was measured as described (*23*).

Cell-surface ELISA. HEK293 cells transiently transfected with HA-NK_1R or HA-$NK_1R\delta311$ were fixed. Detergent permeabilized or non-permeabilized cells were incubated with anti-HA, followed by anti-mouse horseradish peroxidase-conjugated antibody, which was detected using a colorimetric assay.

NK_1R trafficking in cell lines. HEK-NK_1R cells were incubated with Alexa568-SP (100 nM, 20 min, 4°C), washed, incubated for 30 min at 37°C, and fixed. To examine NK_1R and $G\alpha_q$ trafficking, cells were incubated in HBSS with SP (100 nM, 15 min) or vehicle and fixed. NK_1R, $G\alpha_q$ and EEA1 were detected by immunofluorescence (*23*).

Inhibitors. HEK293 cells were preincubated for 30 min with 30 μM Dy4, Dy4 inactive (*17*), PS2, or PS2 inactive (*18*), 100 nM UBO-QIC, 1 μM U73122, 10 μM NF449, 1 μM GF109203X,

100 μM EGTA, 30 μM each of cell penetrating NK_1R peptides, 100 μM control peptide or vehicle.

Cy5 tripartite probe uptake. HEK293 cells were infected with CellLight® Rab5a-RFP or were transfected with rat NK_1R-GFP. Uptake of Cy5-Chol, Cy5-Ethyl ester or Cy5-Span-Chol was examined in live cells by confocal imaging.

Spinal cord slices. Parasagittal slices were prepared from rat lumbar spinal cord as described (*45*).

Electrophysiology. Spontaneous currents were recorded in NK_1R-positive lamina I neurons in cell-attached configuration using patch electrodes (*45*). Slices were preincubated with Dy4 or Dy4 inact (30 μM, 10 min) or with U0126 or GFX109203X (1 μM, 30-45 min) before recording. Slices were preincubated with Span-Chol or Span (1 μM, 60 min), washed and incubated in antagonist-free medium for a further 60 min. Slices were challenged with SP (1 μM, 2 min). The firing rate for each cell was normalized to the response at the 2 min time point and firing time was determined as the duration of the response to last action potential. To assess NK_1R endocytosis, spinal cord slices were incubated with SP (1 μM, 5 min), fixed and processed to localize NK_1R.

Neuropeptide release. Neuropeptide release was measured from superfused segments of mouse dorsal spinal cord as described (*46*). Tissues were superfused with Dy4, PS2, inactive analogues (30 μM) or vehicle (0.3% DMSO/saline) for 30 min. Tissues were then superfused with capsaicin (0.3 μM, 10 min) in the presence of inhibitors or controls. Superfusate was analyzed for SP and CGRP by ELISA.

NK_1R endocytosis in rat spinal neurons. Dy4, Dy4 inact, PS2, PS2 inact (50 μM), or vehicle (1% DMSO/saline) was injected intrathecally (10 μl, L3/L4) into conscious rats. After 30 min,

capsaicin (12.5 μg) or vehicle (20% ethanol, 10% Tween 80, 70% saline) was injected subcutaneously into the plantar surface of one hindpaw (25 μl). After 10 min, rats were transcardially perfused with fixative. NeuN, NK_1R and phospho-ERK were localized by immunofluorescence (*47, 48*).

Nociception in mice and rats, NK_1R endocytosis in spinal neurons in mice. Withdrawal responses to stimulation of the plantar surface of the hind-paw with graded von Frey filaments and paw edema were measured as described (*47, 48*). Capsaicin (5 μg), Complete Freund's Adjuvant (CFA, 2 $mg.ml^{-1}$), or vehicle (capsaicin, 20% ethanol, 10% Tween 80, 70% saline; CFA, saline) was injected into the plantar surface of the left hindpaw (10 μl). von Frey scores (left and right paws) and paw thickness (left paw) were measured for 30-240 min after capsaicin, and 36-40 h after injection of CFA. Results are expressed as percent pre-injected values. Formalin (4%, 10 μl) was injected into the plantar surface of the left hindpaw. Nocifensive behavior (flinching, licking, biting of the injected paw) was recorded for 60 min. At the end of experiments, mice were transcardially perfused with PBS and PFA, and the spinal cord was removed and processed to localize the NK_1R by immunofluorescence. Investigators were unaware of test agents.

Intrathecal injections in mice. Dy4, Dy4 inact, PS2, PS2 inact (all 50 μM), SR-140333 (15 μM), SM-19712 (8 mM), U0126 (100 μM), 30 μM each of cell penetrating NK_1R peptides, 100 μM control peptide, or vehicle (1% DMSO/saline) was injected intrathecally (5 μl, L3/L4) 30 min before intraplantar injection of capsaicin or formalin, or 36 h after CFA. Span (50 μM), Span-Chol (50 μM), L-733,060 (100 nM), L-733,060-Chol (100 nM), or Cy5-Chol (10 μM) was injected intrathecally 3 h before or 30 min after intraplantar injection of capsaicin, 3 h before formalin, or 36 h after CFA.

Intrathecal siRNA in mice. Cationic liposome and adjuvant anionic polymer (*49*) were used to deliver siRNA targeting mouse dynamin-1 (*50*), mouse βarr1 plus mouse βarr2 or control siRNA. siRNA lipoplexes were administered by intrathecal injection (L1-L4, 5 µl). After behavioral testing (24-48 h), the spinal cord (L1-L4) was collected for analysis of dynamin-1 and βarr1 and βarr2 expression.

Assessment of siRNA knockdown. Knock-down of siRNA targets in cell lines and spinal cord was confirmed by Western blot and q-PCR.

Rotarod latency. Baseline time trials (cut-off 120 s) were recorded. Dy4, PS2, inactive analogues (50 µM), or vehicle was injected intrathecally (5 µl, L3/L4). After 30 min, mice were placed on the rotarod with accelerating velocity for up to 120 s. Latency time to fall was determined at 30, 90 and 120 min.

Confocal microscopy, image analysis. Samples were observed using a Leica SP8 confocal microscope. Z stacks were collected of NK_1R-positive neurons in lamina I of the dorsal horn. NK_1R endocytosis and pERK expression were quantified using ImageJ. The ratio of plasma membrane to cytosolic NK_1R-IR fluorescence was determined to assess endocytosis. To quantify ERK activation, the ratio of the number of pERK-IR neurons to total NeuN-N-positive neurons in lamina I/II was determined.

Super-resolution microscopy. Cells were observed using a Leica DMI6000 Ground State Depletion microscope. The number of EEA1-IR endosomes per cell containing NK_1-IR and Gα-IR in endosomes was determined.

Metabolic stability of tripartite probes. Span or Span-Chol (10 $\mu g.ml^{-1}$) was incubated in human cerebrospinal fluid (0-4 h, 37°C). Samples were analyzed by LC/MS. Peptides were quantified by comparison to calibration standards (50-50,000 $ng.ml^{-1}$).

Statistical analyses. Data are presented as mean±SEM, unless noted otherwise. Differences were assessed using Student's t test for two comparisons. For multiple comparisons, differences were assessed using one- or two-way ANOVA followed by Dunnett's multiple comparison test, Tukey's multiple comparison test, Sidak's multiple comparisons test, or Dunn's multiple comparisons test (table S1).

List of Supplementary Materials

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probes; L.A. synthesized and purified tripartite probes; J.C synthesized and purified tripartite probes; C. KH analyzed transcription; N.B. synthesized and purified fluorescent SP; J.S.S. designed tripartite probes; M.J.S. conceived and designed the studies to use tripartite probes to therapeutically target endosomal receptors; B.G. designed tripartite probes; A.MC. designed and synthesized inhibitors of endocytosis and inactive control analogues; P.J.R. designed and characterized inhibitors of endocytosis; V.E. designed and prepared cationic liposome and anionic polymer adjuvant for *in vivo* delivery of siRNA; R.N. measured neuropeptide release from spinal cord; S.M. measured neuropeptide release from spinal cord; P.G. conceived, designed and analyzed studies to examine neuropeptide release from nociceptors; M.J.C. conceived, designed and analyzed studies of excitation of spinal neurons; C.J.P. conceived and designed the studies to use tripartite probes to therapeutically target endosomal receptors; M.C. conceived, designed and completed all BRET analyses of subcellular NK_1R trafficking and G protein activation; N.W.B. conceived the studies, designed experiments, interpreted the results and wrote the manuscript. **Competing interests:** Work in N.W.B's laboratory is funded in part by Takeda Pharmaceuticals Inc. N.W.B. has filed a patent for use of lipidation to target GPCRs in endosomes.

Figure Legends

Fig. 1. NK_1R endocytosis-dependent compartmentalized signaling. A-I. Effect of inhibitors of dynamin (Dy4) and clathrin (PS2), and of inactive (inact) analogues, on SP-induced spatiotemporal signaling profiles for nuclear ERK (NucEKAR, A-C), cytosolic PKC (CytoCKAR, D-F), cytosolic cAMP (CytoEpac2, G-I) measured in HEK293 cells using FRET biosensors. **B, E, H.** Representative ratiometric images and sensor localization. Max: response to positive controls. **C, F, I**. Area under curve (AUC) of A, D, G. **J, K.** Effect of dynamin WT (**J**) or dominant negative K44E (**K**) overexpression on the spatiotemporal profile of SP-induced nuclear ERK. **L.** AUC of J, K. **M.** Effect of clathrin heavy chain and dynamin-1 siRNA on the spatiotemporal profile of SP-induced nuclear ERK. **N.** AUC of M. **O.** Effect of dynamin WT or K44E overexpression on the SP-induced SRE-SEAP. $^{**}P<0.01$, $^{***}P<0.001$ to vehicle (Veh); $^{\wedge\wedge}P<0.01$, $^{\wedge\wedge\wedge}P<0.001$ control to inhibitors. A-N, 30-354 cells, 3-5 experiments, O, n=3-5 experiments. ANOVA, Tukey's test (C, F, I, N); Sidak's test (L); Dunnett's test (O).

Fig. 2. G protein-dependent NK_1R signaling in endosomes. A-D. SP-induced BRET between NK_1R-RLuc8 and KRas-Venus or Rab5a-Venus, and between $G\alpha_q$-RLuc8/$G\gamma_2$-Venus or Rab5a-Venus in HEK293 cells. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ to baseline. Triplicate observations, n>3 experiments. **E, F.** NK_1R-IR (green), $G\alpha_q$-IR (cyan), EEA1-IR (red) localization by super-resolution microscopy. Blue boxes, plasma membrane; red boxes, endosomes. 60-66 cells per condition (20-22 cells from n=3 experiments). $^{****}P<0.0001$. **G-I.** Effect of inhibitors of $G\alpha_q$ (UBO-QIC) or PLC (U73122), Ca^{2+}-chelation (EGTA), or inhibitors of $G\alpha_s$ (NF449) or PKC (GF109203X, GFX) on SP-induced nuclear ERK, cytosolic PKC and cytosolic cAMP measured using FRET biosensors (AUC, area under the curve). $^{***}P<0.001$ SP to vehicle; $^{\wedge\wedge\wedge}P<0.001$

control to inhibitor. 35-67 cells, 3 experiments. ANOVA, Dunnett's test (A-D); Sidak's test (F, G); Tukey's test (H, I).

Fig. 3. NK_1R endocytosis and neuronal excitation in spinal cord slices. A. Effect of Dy4 and Dy4 inact on SP-induced endocytosis of NK_1R-IR in rat spinal neurons. Arrows, internalized, arrowheads, cell surface NK_1R. **B.** Quantification of endocytosis. ****P<0.0001. 18 neurons per group (6 neurons in slices from n=3 rats). **C-H.** Effects of Dy4, Dy4 inact, U0126 (MEK inhibitor) and GF109203X (PKC inhibitor) on SP-induced firing of rat spinal neurons. **C, F.** Representative traces. **D, G.** Firing rate normalized to 2 min. **E, H.** Firing duration to last action potential. 6-7 neurons per group from n=8-17 rats. *P<0.05, **P<0.01, ***P<0.001. **I, J.** Effect of Dy4 and Dy4 inact on excitatory post-synaptic currents (EPSC) in lamina I/II_o induced by primary afferent stimulation. n=11 neurons. **K, L.** Effects of Dy4, PS2 and inactive analogues on capsaicin-stimulated SP-IR (**K**) and CGRP-IR (**L**) release from segments of mouse spinal cord. n=6 experiments. ANOVA, Tukey's test (B); Sidak's test (D, G); Dunn's test (E, H).

Fig. 4. NK_1R endocytosis, ERK signaling and nociception *in vivo*. Effects of intrathecal (i.t.) injections of inhibitors or siRNA. **A-B.** Localization of NK_1R-IR (**A**) and pERK-IR (**B**) in rat spinal neurons 10 min after intraplantar (i.pl.) vehicle or capsaicin (Cap). L, lamina. In A, arrowheads show cell-surface and arrows show endosomal NK_1R. In B, arrows show pERK-IR (green) and red shows NeuN (neuronal marker). **C, D.** Quantification of NK_1R endocytosis (**C**) and pERK-expressing neurons (**D**). **P<0.01, ***P<0.001. Neuronal numbers: Veh, 54; capsaicin, 52; Dy4, 28; Dy4 inact, 18; PS2, 22; PS2 inact, 19 (>6 neurons in sections from n=3 rats). **E, F, H-K.** Nociception in mice after intrathecal injection of endocytic inhibitors (Dy4, PS2), NK_1R antagonist (SR140,333; SR), dynamin-1 or βarr1+2 siRNA, endothelin-converting enzyme-1 inhibitor (SM-19712; SM), or MEK inhibitor (U0126). von Frey withdrawal responses

of capsaicin-injected (**E, H-K**) or contralateral (**F**) paw are shown. **G.** Rotarod latency. **L.** Formalin (form) nocifensive behavior. **M.** von Frey withdrawal responses of the CFA-injected paw. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to control. (n) mouse number. Student's t-test (C, D); ANOVA, Dunnett's test (E-M).

Fig. 5. Disruption of NK_1R/βarr interactions. A. Mouse NK_1R C-terminus, indicating NK_1Rδ311 truncation and sequences of Tat-conjugated NK_1R and control peptides. **B, C.** SP-induced BRET between WT NK_1R-RLuc8 or NK_1Rδ311-RLuc8 and βarr2-YFP or Rab5a-Venus. Triplicate observations, n>3 experiments. **D**. SP-induced cytosolic ERK (CytoEKAR), nuclear ERK (NucEKAR) measured using FRET biosensors. *P<0.05. 49-99 cells, 3 experiments. **E.** Effect of SP on SRE-SEAP release from HEK-NK_1Rδ311 cells. **F, G.** Effect of control and 3 NK_1R peptides on SP-induced NK_1R-Rluc8/βarr2-YFP BRET (**F**) and NK_1R endocytosis (**G**). **H-I.** Effects of intrathecally administered control and NK_1R peptides on capsaicin-induced mechanical allodynia (**H**), formalin-evoked nocifensive behavior (**I**) and CFA-induced mechanical hyperalgesia (**J**) in mice. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to control. (n) mouse number. ANOVA, Sidak's test (D, G); Dunnett's test (H-J).

Fig. 6. Antagonism of endosomal NK_1R. A. Structure of tripartite probes. **B.** Cy5-Ethyl ester or Cy5-Chol uptake in HEK293 cells. **C.** Cy5-Chol or Cy5-Span-Chol (red) trafficking to Rab5a-RFP- (blue) and NK_1R-GFP-positive (green) endosomes. Asterisk, extracellular; arrowheads, plasma membrane; arrows, endosomes. **D, E.** Cy5-Chol:SNAP549-NK_1R FRET, indicating localization of SNAP549-NK_1R, Cy5-Chol and FRET signals (**D**), and time course of FRET in the whole cell and cytosol (**E**). *P<0.05, **P<0.001 to control; ^^^P<0.001 control to inhibitors. 6-9 cells, n=3 experiments. **F-H.** FRET assays of nuclear ERK activity (NucEKAR) immediately after (pre-incubation, **F**) or 4 h after (pre-pulse, **G**) a 30 min incubation with Span, Span-Chol or

SR140333 (SR). **H.** AUC of G, H. **P<0.01, ***P<0.001 to vehicle; ^^^P<0.001 to antagonists. 31-417 cells, n=3-5 experiments. **I.** Effects of Span or Span-Chol on SP-induced SRE-SEAP. ANOVA, Sidak's test (H).

Fig. 7. Antagonism of endosomal NK_1R in spinal cord slices and *in vivo*. A-C. Effects of tripartite antagonists on SP-induced firing of rat spinal neurons. **A.** Representative traces. **B.** Firing rate normalized to 2 min. **C.** Firing duration to last action potential. 6-7 neurons per group from n=5-18 rats. **D.** Localization of Cy5-Chol (arrows) in superficial laminae (L) 6 h after intrathecal injection in mouse. **E-J.** Effects of intrathecally administered Cy5-Chol, Span, Span-Chol, L-733,066 (L733) or L-733,0660-Chol on nociception in mice. **E-G.** Von Frey withdrawal responses of capsaicin-injected paw. **H.** Nocifensive behavior after intraplantar formalin. **I, J.** Von Frey withdrawal responses of CFA-injected paw. (n) mouse number. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 to control. **I.** Kinetics of degradation of Span and Span-Chol in human cerebrospinal fluid. n=2, mean ± SD. ANOVA, Sidak's test (B); Dunn's test (C); Dunnett's test (E-J).

Fig. 8. Endosomal platforms for signaling pain. A. Nociceptive signaling. NK_1R couples to $G\alpha_q$ (1), PLC-dependent Ca^{2+} mobilization (2), and A Disintegrin And Metalloproteinase-(ADAM)-dependent epidermal growth factor receptor (EGFR) transactivation, which stimulates cytosolic ERK (4). Ca^{2+} activates PKC, which stimulates adenylyl cyclase (AC) to produce plasma membrane cAMP (5). GRK-phosphorylated NK_1R interacts with βarrs (6), which scaffold clathrin and adaptor protein 2 (AP2), leading to SP/NK_1R endocytosis (7). Endosomal SP/NK_1R (8) stimulates cytosolic PKC activity, cytosolic cAMP, and nuclear ERK activity (9), which drive neuronal excitation and nociceptive transmission. **B. Antinociception, endocytic inhibitors.** Inhibition of dynamin (1), clathrin (2), or βarrs (3) prevents SP/NK_1R endocytosis, endosomal signaling and nociceptive transmission. **C. Antinociception, tripartite antagonists.**

Tripartite antagonists incorporate into the plasma membrane (1), traffic to endosomes (2), and suppress SP/NK_1R nociceptive signaling.