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TITLE: Receptors in Endosomes Mediate Chronic Pain Associated with Trauma and Stress: Nonopioid Targets for Pain

PRINCIPAL INVESTIGATOR: Nigel Bunnett, PhD

CONTRACTING ORGANIZATION: New York University, New York, NY

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14. ABSTRACT The grant seeks to determine whether G protein-coupled receptors (GPCRs) in endosomes, rather than at the plasma membrane, are mediators and therapeutic targets for chronic pain. Aim 1 examines whether GPCRs in endosomes control activity of ion channels and expression of genes that induce sustained neuronal excitation. Aim 2 determines whether endosomally-targeted antagonists inhibit channel activity, gene expression and hyperexcitability of neurons. Aim 3 evaluates the therapeutic potential of endosomally-targeted GPCR antagonists in trauma- and stress-induced pain relevant to military personnel. Progress has been made in all aims. Neuropeptides (substance P, calcitonin gene-related peptide) and proteases (trypsin) stimulated GPCR endocytosis and evoked sustained signals and hyperexcitability in pain-sensing neurons. Dynamin and clathrin inhibitors suppressed endocytosis, signaling and hyperexcitability. Therapies have been developed to block endosomal signaling and evaluated in preclinical models of trauma- and stress-induced pain. Therapies include: dynamin and clathrin inhibitors; lipid-conjugated antagonists that accumulate in endosomes; antagonists encapsulated into nanoparticles that disassemble in acidic endosomes. These antagonists effectively reversed nociception in preclinical models of visceral pain, migraine pain, and nerve injury pain. They were more effective than conventional antagonists of plasma membrane GPCRs. Thus, endosomal GPCRs have been identified as major mediators and therapeutic targets for chronic pain.					
15. SUBJECT TERMS Chronic pain; neuropathic pain; head injury pain; migraine pain; irritable bowel syndrome pain; G protein coupled receptors; endosomes; analgesics.					
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1. INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest class of transmembrane signaling proteins. They control most physiological and pathological processes, including pain, and are the target of over one third of FDA-approved drugs. GPCRs are traditionally considered to function at the plasma membrane, allowing cells to detect extracellular ligands. However, plasma membrane signaling is transient, and activated GPCRs usually undergo clathrin- and dynamin-mediated endocytosis. Endosomes were once considered merely conduits for GPCR trafficking to recycling or degradatory pathways. This grant investigates the novel concept that endosomes are a vital site for continued GPCR signaling in pain-sensing neurons that mediates sustained neuronal activity and pain. Thus, selective antagonists of endosomal GPCRs might provide superior relief from chronic pain than conventional drugs that are designed to target GPCRs at the plasma membrane. The inability of such drugs to effectively engage GPCRs in acidic endosomes might explain their lack of efficacy in clinical trials of chronic pain. The application focuses on receptors for neuropeptides (substance P [SP] neurokinin 1 receptor [NK₁R]; calcitonin gene-related peptide [CGRP] calcitonin like receptor [CLR]) and proteases (protease-activated receptor-2 [PAR₂]). These receptors have been implicated in nerve injury pain, migraine pain, and colonic pain of irritable bowel syndrome (IBS).

2. KEYWORDS

Chronic pain; neuropathic pain; head injury pain; migraine pain; irritable bowel syndrome pain; G protein-coupled receptors; endosomes; analgesics

3. ACCOMPLISHMENTS:

Goals of Project. The table indicates approved Statement of Work tasks, date of completed tasks, and percentage of tasks accomplished by those dates.

Abbreviations: GPCR, G Protein-Coupled Receptor; DRG, Dorsal Root Ganglia; PAR₂, Protease-Activated Receptor-2; NK₁R, Neurokinin 1 Receptor; CLR, Calcitonin Receptor-like Receptor; TRP, Transient Receptor Potential (ion channel); muGFP, monomeric ultrastable Green Fluorescent Protein; IBS-D, Irritable Bowel Syndrome, Diarrhea-Predominant; HC, Healthy Control; ABP, Activity-Based Probe; PTH, Post Traumatic Headache; FRET, Förster Resonance Energy Transfer; BRET, Bioluminescence Resonance Energy Transfer; RNA-Seq, RNA Sequencing

Aim 1: To determine whether GPCRs in endosomes of pain-sensing neurons control the activity of ion channels and the transcription of genes that induce sustained neuronal excitation.	Date	%
Major Task 1: Determine whether endosomal PAR ₂ in DRG neurons generates compartmentalized signals that regulate channel activity and sensitization		
<u>Subtask 1:</u> Establish breeding colonies of PAR ₂ -muGFP mice.	11/08/22	100
<u>Subtask 2:</u> Analyze endocytosis-dependent compartmentalized signaling in DRG neurons	11/08/22	100
<u>Subtask 3:</u> Assess PAR ₂ -mediated sensitization and TRP channel activation in DRG neurons	11/08/22	100
Major Task 2: Determine whether proteases in human colon biopsy specimens activate endosomal PAR ₂ in DRG neurons to generate signals that regulate channel activity and sensitization		
<u>Subtask 1:</u> Collect and process human colon biopsies from IBS-D (N=50) and HC (N=50) patients	11/08/22	100
<u>Subtask 2:</u> Determine whether proteases in human IBS-D biopsies sensitize DRG neurons and activate TRP channel through endosomal PAR ₂ .	11/08/22	100
<u>Subtask 3:</u> Profile activated serine and cysteine proteases in supernatants of IBS-D and HC biopsies using ABPs	11/08/22	75
Major Task 3: Determine whether endosomal PAR ₂ , NK ₁ R and CLR signals regulate transcription in pain-sensing neurons		
<u>Subtask 1:</u> Determine whether endosomal PAR ₂ , NK ₁ R and CLR signaling regulates transcription in DRG and spinal neurons	11/08/22	50

Aim 2: To determine whether endosomally-targeted antagonists inhibit endosomal GPCR signaling, channel activation, gene transcription and sustained hyperexcitability of pain-sensing neurons.		
<u>Subtask 1:</u> Generate small molecule tripartite PAR ₂ , NK ₁ R and CLR antagonists; generate small molecule quadripartite NK ₁ R and CLR antagonists; generate fluorescent probes	11/08/22	100
Major Task 1: Determine whether tripartite and quadripartite antagonists disrupt endosomal PAR ₂ , NK ₁ R and CLR signaling in HEK293 cells		
<u>Subtask 1:</u> Determine whether lipidated antagonists disrupt ligand/receptor interactions in endosomes	11/08/22	100
<u>Subtask 2:</u> Determine whether lipidated antagonists inhibit endosomal signaling of PAR ₂ , NK ₁ R and CLR in HEK293 cells	11/08/22	100
Major Task 2: Determine whether tripartite and quadripartite antagonists target PAR ₂ in endosomes of DRG neurons and NK ₁ R and CLR in endosomes of spinal neurons, and inhibit endosomal signaling		
<u>Subtask 1:</u> Determine whether tripartite and quadripartite antagonists colocalize with PAR ₂ , NK ₁ R and CLR on endosomes of pain-sensing neurons	11/08/22	100
<u>Subtask 2:</u> Determine whether tripartite and quadripartite antagonists inhibit endosomal signaling of PAR ₂ , NK ₁ R and CLR in pain-sensing neurons	11/08/22	100
Major Task 3: Determine whether tripartite and quadripartite antagonists disrupt PAR ₂ -induced sensitization and transcription in DRG neurons and NK ₁ R- and CLR-induced sensitization and transcription in spinal neurons		
<u>Subtask 1:</u> Determine whether tripartite and quadripartite antagonists inhibit sensitization and TRP activation in neurons	11/08/22	100
<u>Subtask 2:</u> Examine whether antagonists suppress gene expression	11/08/22	50
Aim 3: To investigate the therapeutic potential of endosomally-targeted GPCR antagonists in trauma- and stress-induced pain that is relevant to disorders of military personnel and veterans.		
Major Task 1: Conduct studies in preclinical model of migraine headache to determine feasibility of using tripartite CLR antagonists for migraine pain.		
<u>Subtask 1:</u> Generate tripartite antagonists of PAR ₂ , NK ₁ R and CLR; generate quadripartite probes for NK ₁ R and CLR antagonists, similar to Aim 2 above	11/08/22	100
<u>Subtask 2:</u> Develop a GTN-evoked model of migraine headache pain in C57BL/6 mice	11/08/22	100
<u>Subtask 3:</u> Determine efficacy of tripartite or non-lipidated CLR antagonists	11/08/22	100
Major Task 2: Conduct studies in preclinical model of nerve injury pain to determine feasibility of using quadripartite NK ₁ R/CLR antagonists for nerve injury pain.		
<u>Subtask 1:</u> Develop a spared nerve injury model of neuropathic pain in C57BL/6 mice	11/08/22	100
<u>Subtask 2:</u> Determine efficacy of quadripartite NK ₁ R/CLR antagonists and non-lipidated antagonists	11/08/22	100
Major Task 3: Conduct studies in preclinical model of IBS pain to determine feasibility of using tripartite PAR ₂ antagonists for post-inflammatory IBS pain.		
<u>Subtask 1:</u> Generate an IBS mouse model in C57BL/6 mice	11/08/22	100
<u>Subtask 2:</u> Training of electrophysiologist in Dr. Schmidt's laboratory by Dr. Bunnett's team on visceromotor responses following graded colorectal distention	11/08/22	75
<u>Subtask 3:</u> Determine efficacy of tripartite PAR ₂ antagonists and non-lipidated antagonists	11/08/22	100

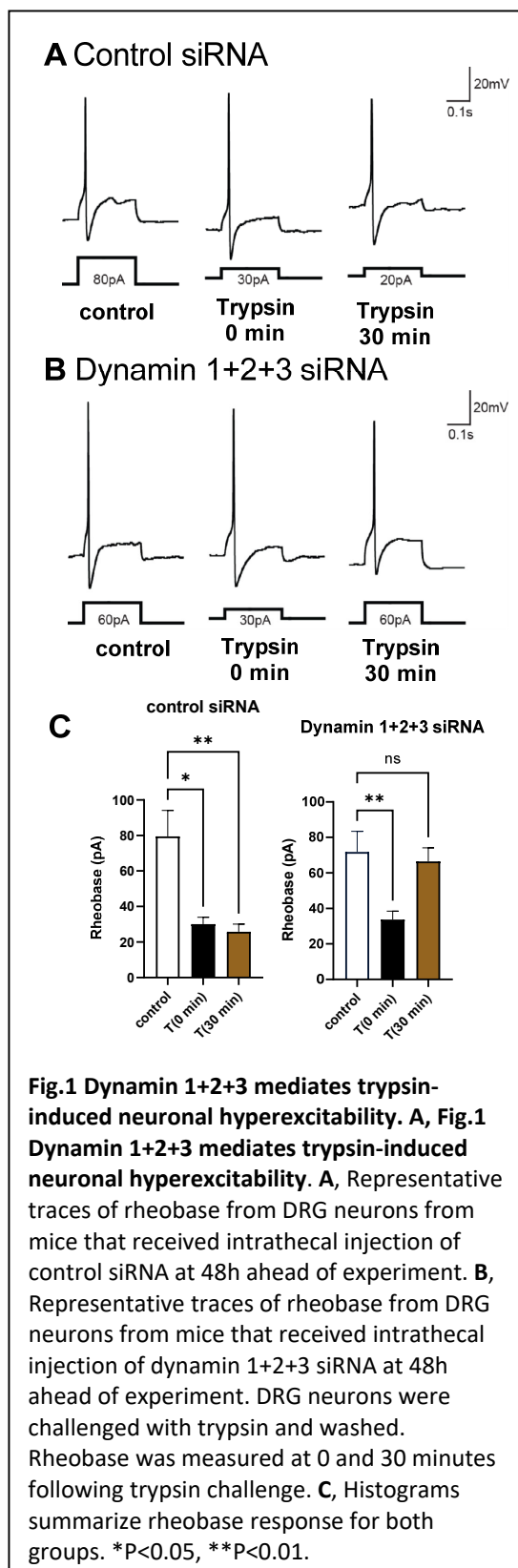


Fig.1 Dynamin 1+2+3 mediates trypsin-induced neuronal hyperexcitability. A, Fig.1 Dynamin 1+2+3 mediates trypsin-induced neuronal hyperexcitability. A, Representative traces of rheobase from DRG neurons from mice that received intrathecal injection of control siRNA at 48h ahead of experiment. B, Representative traces of rheobase from DRG neurons from mice that received intrathecal injection of dynamin 1+2+3 siRNA at 48h ahead of experiment. DRG neurons were challenged with trypsin and washed. Rheobase was measured at 0 and 30 minutes following trypsin challenge. C, Histograms summarize rheobase response for both groups. *P<0.05, **P<0.01.

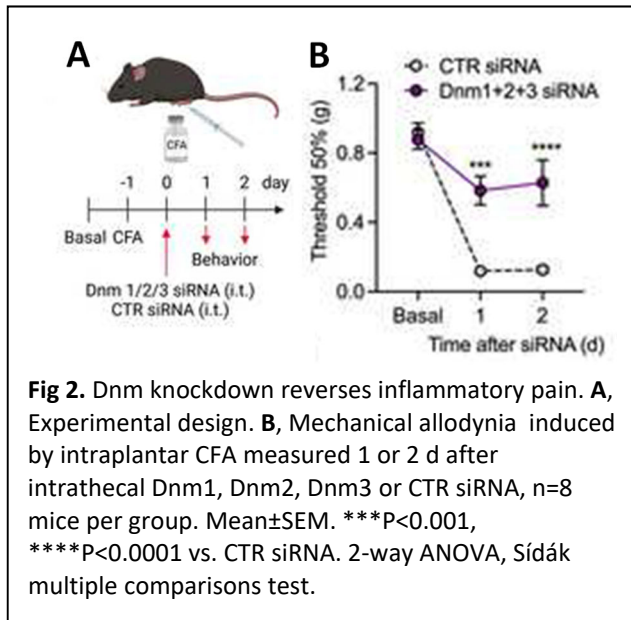
Research Progress

We have investigated the mechanisms by which G protein-coupled receptors (GPCRs) signal pain that is prevalent in military personnel and veterans including post-traumatic headache, nerve injury pain and irritable bowel syndrome pain. Previous drug discovery efforts, which have been largely unsuccessful, focus on targeting plasma membrane GPCRs. However, once activated GPCRs undergo endocytosis and intracellular trafficking. We investigated the hypothesis that GPCRs in endosomes (**eGPCRs**) generate sustained signals that mediate neuronal hypersensitivity and chronic pain. Thus, chronic pain results from sustained agonist release and eGPCR signaling. Antagonists designed to target plasma membrane GPCRs may not engage eGPCRs due to the acidic endosomal pH and eGPCR association with multi-protein signaling complexes. We have made progress in targeting eGPCRs for efficacious and sustained relief from headache, nerve injury pain and irritable bowel syndrome pain including, but not limited to, the accomplishments described below.

The endocytic mediator dynamin mediates nociceptor excitability and inflammatory pain and is a target for the treatment of pain. Chronic pain is common, poorly understood and difficult to treat. The analgesic properties of μ -opioid agonists dwindle with continued use (i.e., tolerance), and their usefulness is limited by on-target side effects of respiratory depression, constipation and addiction. Antagonists of pronociceptive receptors and channels are often developed to treat chronic pain. However, the redundancy of pain signaling pathways, where multiple receptors (for example, protease-activated receptor-2 (PAR₂), and calcitonin-like receptor (CLR), which, in combination with receptor activity modifying protein 1 (RAMP1), serves as the receptor for calcitonin gene related peptide (CGRP)) and channels can activate the same neurons, may limit the effectiveness of selective agents for the treatment of complex multi-modal forms of pain. Moreover, continued signaling from the receptors and channels following endocytosis, which is not antagonized by drugs limited by the plasma membrane, contributes to chronic pain.

To address the role of endocytosis, and a key molecular mediator, dynamin, in nociceptor excitability and pain the genes (*Dnm*) responsible for dynamin were manipulated. Small interfering RNA (siRNA) were used to knock down the genes responsible for dynamin expression. Dynamin is a GTPase that mediates fission of clathrin-coated synaptic vesicles and is also required for endocytosis. Of the three *Dnm* isoforms (*Dnm1*, *Dnm2*, *Dnm3* genes) in mammals, *Dnm1* and *Dnm3* are expressed in the nervous system. *Dnm1* deletion in mice impairs activity-dependent endocytosis of synaptic vesicles at nerve terminals and thereby disrupts neurotransmission during intense stimulation.

To evaluate the contribution of endocytosis to protease-induced hyperexcitability of nociceptors, the rheobase (the minimal input current required to fire one action potential) of small diameter neurons of mouse DRG was measured by patch-clamp recording of DRG neurons. *Dnm* siRNA did not affect the capacity of trypsin to cause initial hyperexcitability (0 min, from 72 ± 12 pA to 34 ± 5 pA) while abolished the persistent effects of trypsin (68 ± 8 pA) (**Fig. 1**).



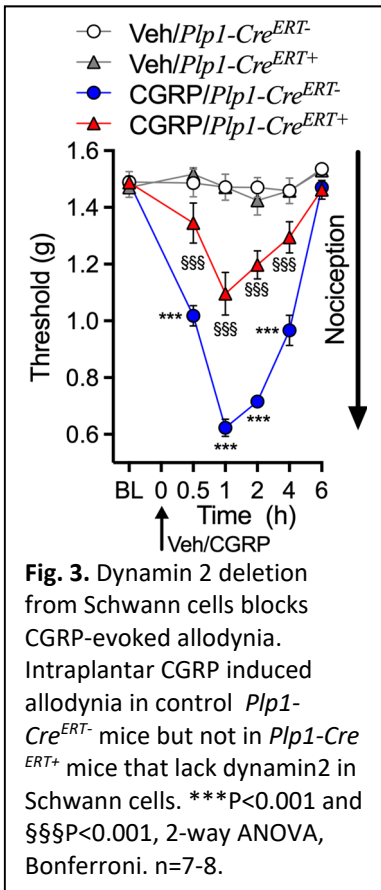
Chronic inflammatory pain was induced by intraplantar (i.pl.) injection of Complete Freund's Adjuvant (CFA) or vehicle (control) into the left hindpaw (Experimental design, **Fig. 2A**). Dnm1, Dnm2, Dnm3 or control siRNA was administered by intrathecal injection 24 hours after CFA. Withdrawal responses of the CFA-injected (left, ipsilateral) and non-injected (right, contralateral) hindpaws to stimulation with von Frey filaments was assessed daily for 48 h to evaluate mechanical allodynia. CFA-induced inflammation reduced the withdrawal threshold to von Frey filaments, consistent with mechanical allodynia (**Fig. 2d**). Dnm1, Dnm2 or Dnm3 siRNAs reversed mechanical allodynia with a larger effect at 48 h when compared to control siRNA (**Fig. 2d**).

The findings demonstrated with Dnm siRNA were confirmed with pharmacologic antagonists and in a model of neuropathic pain (data not shown). Normal functions were not affected. The inhibition of synaptic transmission in lamina I and II (substantia gelatinosa) of the spinal cord

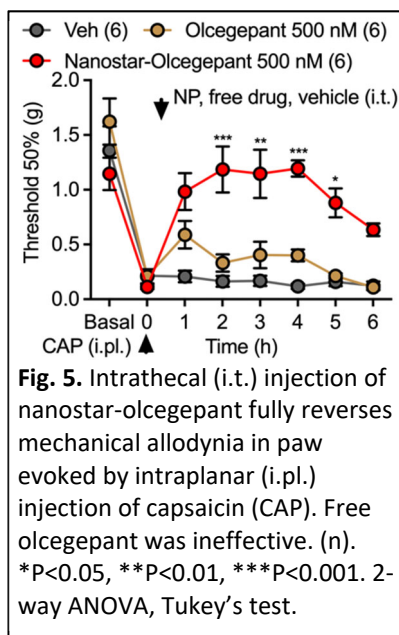
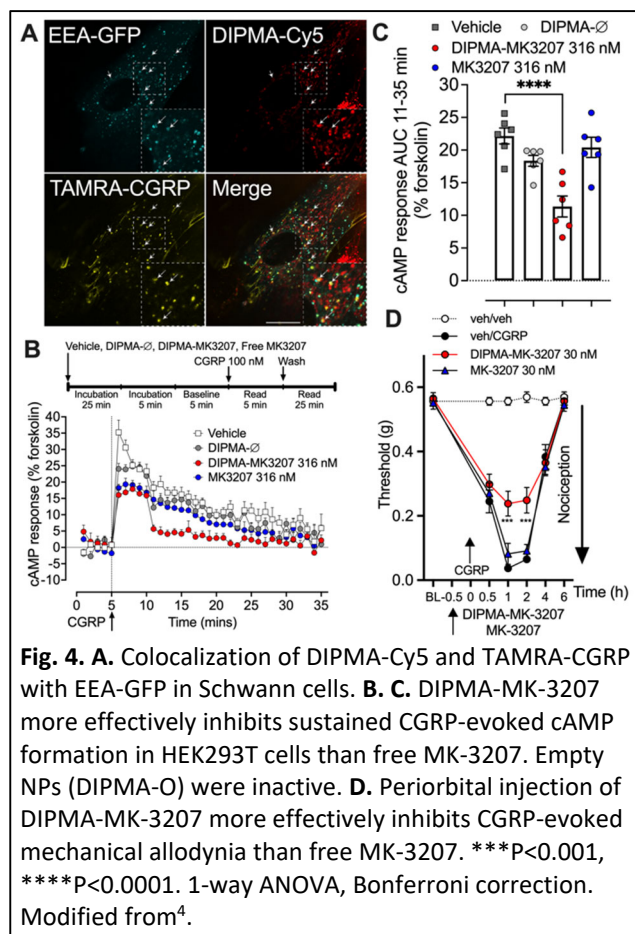
with genetic and pharmacologic inhibition of endocytosis offer a therapeutic approach for pain treatment. This work has been accepted by *PAIN*.

Endosomal signaling of CLR/RAMP1 in Schwann cells mediates migraine pain. Endosomal GPCR signaling in neurons and Schwann cells has a role in chronic pain. An injection of CGRP induces allodynia, which is a model of migraine pain. Periorbital injection of the endosomal inhibitors (Dyngo4a to antagonize dynamin or PitStop2 to antagonize clathrin) prevents CGRP-evoked periorbital mechanical allodynia in mice, implicating endosomal signaling in the nociceptive response to CGRP. To corroborate these results using a genetic approach and to address the role of dynamin on Schwann cells, dynamin2 (major isoform of Schwann cells) was deleted using an adenoviral vector. AAV2-EF1-mCherry-SICO-mDNM2-shRNA (5 μ l, 1×10^{13} GC/ml) was injected into the sciatic nerve of *Plp1-Cre^{ERT-}* and *Plp1-Cre^{ERT+}* mice. The nociceptive response to intraplantar CGRP (5 μ g/10 μ l) was assessed. In control mice (*Plp1-Cre^{ERT-}*), CGRP caused mechanical allodynia (**Fig. 3**). Dynamin2 knockdown in Schwann cells (*Plp1-Cre^{ERT+}* mice) abrogated CGRP-induced nociception; dynamin2 mRNA knockdown was confirmed (not shown). Thus, endosomal CLR signaling in Schwann cells mediates nociception.

Nanoparticles (NPs) loaded with a CGRP antagonist inhibit signaling and migraine-like pain. The identification of drug targets within endosomes (e.g., CLR/RAMP1 - a pain-related GPCR downstream from PAR₂) provides opportunities for harnessing pH-sensitive materials to chaperone drugs to endosomal targets. pH-responsive soft polymeric NPs were developed with a shell of hydrophilic monomers and a core of hydrophobic monomers including 2-[N,N-(diisopropylamino)ethyl] methacrylate (DIPMA, pKa 6.1). At endosomal pH (<6.0), DIPMA is protonated, leading to charge repulsion, disassembly and cargo release. DIPMA NPs encapsulating a CLR antagonist (MK-3207) provide



more effective inhibition of CGRP-mediated craniofacial pain than free MK-3207. CLR is also expressed by Schwann cells that ensheath trigeminal nociceptors that contribute to CGRP-dependent craniofacial pain. To examine uptake of DIPMA-NPs into endosomes containing CLR/RAMP1, Schwann cells expressing early endosomal antigen1-GFP (EEA-GFP) were incubated with DIPMA-Cy5 and TAMRA-CGRP. DIPMA-Cy5 and TAMRA-CGRP colocalized with EEA-GFP (**Fig. 4A**). The capacity of CLR antagonists to inhibit CLR/RAMP1 signaling was examined in HEK293T cells using a CAMYEL cAMP biosensor. CGRP stimulated a sustained



increase in cAMP (**Fig. 4B, C**). DIPMA-MK-3207 attenuated the sustained response whereas free MK-3207 was minimally effective. When injected into the facial region of mice, DIPMA-MK-3207 caused a large and sustained inhibition of CGRP (**Fig. 4D**) and capsaicin-evoked (not shown) mechanical allodynia, whereas free MK-3207 had a minor effect.

To examine therapeutic efficacy in mice, nanostar-olcegepant (CLR antagonist), free olcegepant (both 500 nM, 5 μ l), empty nanostar or vehicle was injected intrathecally 30 min after intraplantar injection of capsaicin. After intrathecal vehicle or empty nanostar, capsaicin caused sustained mechanical allodynia of the paw (**Fig. 5**). Nanostar-olcegepant strongly inhibited allodynia whereas free olcegepant was inactive. Thus nanomaterials can be loaded with antagonists to target eGPCRs in neurons or Schwann cells to treat chronic pain.

Training and Professional Development

The NYU Department of Molecular Pathobiology provides opportunities for mentorship, research training and professional development. Post-doctoral fellows have a faculty mentoring committee. Trainees learn laboratory skills from experienced investigators, and receive mentorship from the PI on literature reviews, experimental design, data analysis, scientific writing and presentation. They attend weekly laboratory meetings and journal clubs, and attend symposia and seminars at NYU. A team science training program of bimonthly seminars provides training in ethical conduct of research, rigor and reproducibility, experimental design, and career development.

Dissemination of Results

The Bunnett and Schmidt laboratories meet as a group each week to evaluate progress, provide feedback on technical issues and results, arrange transfer of mice and chemical resources, and trouble-shoot problems. Technical methods specific to the project are discussed. Member of the laboratory including students, postdoctoral fellows, scientists and principal investigators attend this joint laboratory meeting. Drs. Nguyen and Inoue presented their work on CGRP and chronic pain at the 2022 annual Society for Neuroscience meeting.

4. IMPACT

Impact on principal discipline. This work has identified new targets for the treatment of chronic pain, including pathologies that disproportionately affect military personnel such as migraine. We have discovered that painful stimuli cause the translocation of receptors from the cell surface to endosomes of neurons and Schwann cells that sense and transmit pain. Receptors in endosomes are primarily responsible for signaling persistent pain. Drugs that are designed and incorporated into nanomaterials to target these endosomal receptors provide more effective relief from chronic pain than conventional drugs that target receptors at the surface of cells. The failure of conventional drugs in clinical trials of chronic pain may be due to their inability to inhibit receptors in endosomes.

Impact on other disciplines. GPCRs mediate many diseases beyond pain, including cardiovascular disease, inflammatory diseases and cancer. Antagonists and agonists of GPCRs represent the single largest class of

drugs; more than one third of FDA-approved drugs target GPCRs. The concept that GPCRs in endosomes generate sustained signals that may underlie disease processes raises the prospect that GPCRs in endosomes might be the optimal target for the treatment of many chronic diseases.

Impact on technology transfer. The PI (Nigel Bunnett) is a scientific founder of Endosome Therapeutics, a start-up company that seeks to develop and commercialize drugs that target endosomal GPCRs. The National Center for Advancing Translational Science (NCATS) has initiated a collaboration to develop nanoparticle-encapsulated antagonists of GPCRs for the treatment of chronic pain.

Impact on society. The development of non-opioid treatments for chronic pain has the potential to lessen the opioid crisis, which is a major cause of mortality and morbidity in the USA.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change. Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them. Nothing to report.

Changes that had a significant impact on expenditures. Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. Nothing to report.

Significant changes in use or care of human subjects. Nothing to report.

Significant changes in use or care of vertebrate animals. Nothing to report.

Significant changes in use of biohazards and/or select agents. Nothing to report.

6. PRODUCTS

Journal publications

1. Scheff NN, Wall IM, Nicholson S, Williams H, Chen E, Tu NH, Dolan JC, Liu CZ, Janal MN, Bunnett NW, Schmidt BL. Oral cancer induced TRPV1 sensitization is mediated by PAR2 signaling in primary afferent neurons innervating the cancer microenvironment. *Sci Rep*, 2022;12(1):4121. PMCID: PMC8904826.
2. De Logu F, Nassini R, Hegron A, Landini L, Jensen DD, Latorre R, Ding J, Marini M, Souza Monteiro de Araujo D, Ramírez-García P, Whittaker M, Retamal J, Titiz M, Innocenti A, Davis TP, Veldhuis N, Schmidt BL, Bunnett NW, Geppetti P. Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice. *Nat Commun*, 2022;13(1):646. PMCID: PMC8813987.
3. Jaramillo-Polanco J, Lopez-Lopez C, Yu Y, Neary E, Hegron A, Canals M, Bunnett NW, Reed DE, Lomax AE, Vanner SJ. Opioid-Induced Pronociceptive Signaling in the Gastrointestinal Tract Is Mediated by Delta-Opioid Receptor Signaling. *J Neurosci*, 2022;42(16):3316-28. PMCID: PMC9034783.
4. Keely SJ, Urso A, Ilyaskin AV, Korbmacher C, Bunnett NW, Poole DP, Carbone SE. Contributions of bile acids to gastrointestinal physiology as receptor agonists and modifiers of ion channels. *Am J Physiol Gastrointest Liver Physiol*, 2022;322(2):G201-g22. PMCID: PMC8782647.
5. Latorre R, Hegron A, Peach CJ, Teng S, Tonello R, Retamal JS, Klein-Cloud R, Bok D, Jensen DD, Gottesman-Katz L, Rientjes J, Veldhuis NA, Poole DP, Schmidt BL, Pothoulakis CH, Rankin C, Xie Y, Koon HW, Bunnett NW. Mice expressing fluorescent PAR(2) reveal that endocytosis mediates colonic inflammation and pain. *Proc Natl Acad Sci U S A*, 2022;119(6). PMCID: PMC8833192.

Books. Nothing to report.

Other. Nothing to report.

Websites. Bunnett Laboratory Website: <https://dental.nyu.edu/faculty/molecular-pathobiology/bunnett-lab.html>

Technologies and techniques. Nothing to report.

Inventions and patents. Nothing to report.

Other products. Nothing to report.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Individuals who have worked on project

NEW YORK UNIVERSITY

Name:	Nigel Bunnett, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-3367-0644
Nearest person month worked:	2.04 calendar
Contribution to Project:	Project conception; experimental design; interpreting results; writing manuscripts.
Funding Support:	5R01NS102722; 5R01DE026806; 5R01DK118971; 1R01DE029951; R01DK130293; R01DK128142; Heptares; Takeda-NYU-Columbia Alliance

Name:	Rocco Latorre, PhD
Project Role:	Associate Research Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0003-2737-8233
Nearest person month worked:	5.28 calendar
Contribution to Project:	Analysis of the role of endosomal signaling in preclinical models of pain.
Funding Support:	5R01DK118971; R01DK130293; 1R01NS125413; Takeda-NYU-Columbia Alliance

Name:	Alan Hegron, PhD
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	0000-0002-7588-9209
Nearest person month worked:	3.31 calendar
Contribution to Project:	Analysis of the pathways of endosomal signaling and gene transcription.
Funding Support:	5R01NS102722; 5R01DK118971; 1R01NS116758; R01DK128142; Heptares

Name:	Raquel Tonello, PhD
Project Role:	Assistant Research Scientist
Researcher Identifier (e.g. ORCID ID):	0000-0002-2175-9804
Nearest person month worked:	4.74 calendar
Contribution to Project:	Analysis of the pathways of endosomal signaling and gene transcription.
Funding Support:	5R01NS102722; R01DE026806 5R01DK118971; 1R01DE029951

Name:	Chloe Peach, PhD
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	0000-0001-6315-1170
Nearest person month worked:	3.44 calendar
Contribution to Project:	Analysis of receptor signaling mechanisms in endosomes
Funding Support:	5R01NS102722; R01DE026806 1R01DE029951; Leon Levy Foundation

Change in support since last reporting period

Nigel Bunnett, PhD (PI)

NEW SUPPORT

W81XWH-22-1-0238

Bunnett (Lead PI)

09/01/2022 – 08/31/2025

Receptors in Endosomes Mediate Chronic Pain Associated with Trauma, Inflammation, and Stress: Non-Opioid Targets for Sustained Relief of Pain

R01DK128142

Koon

04/01/2022 – 02/28/2023 (Subcontract Period of Performance)

Oral elafin formulation against intestinal fibrosis

3R01DE029951-01S2

Bunnett/Schmidt

09/01/2022-08/31/2023

Targeting Endosomal Receptors for Treatment of Chronic Pain (Research Supplement)

8. SPECIAL REPORTING REQUIREMENTS

This is a collaborative award between Dr. N.W. Bunnett (New York University) and B.L. Schmidt (New York University).

9. APPENDICES

PDF of published manuscripts.



OPEN

Oral cancer induced TRPV1 sensitization is mediated by PAR₂ signaling in primary afferent neurons innervating the cancer microenvironment

Nicole N. Scheff¹, Ian M. Wall², Sam Nicholson², Hannah Williams², Elyssa Chen², Nguyen H. Tu², John C. Dolan², Cheng Z. Liu³, Malvin N. Janal⁴, Nigel W. Bunnett^{5,6} & Brian L. Schmidt^{1,5}✉

Oral cancer patients report sensitivity to spicy foods and liquids. The mechanism responsible for chemosensitivity induced by oral cancer is not known. We simulate oral cancer-induced chemosensitivity in a xenograft oral cancer mouse model using two-bottle choice drinking and conditioned place aversion assays. An anatomic basis of chemosensitivity is shown in increased expression of TRPV1 in anatomically relevant trigeminal ganglion (TG) neurons in both the xenograft and a carcinogen (4-nitroquinoline 1-oxide)-induced oral cancer mouse models. The percent of retrograde labeled TG neurons that respond to TRPV1 agonist, capsaicin, is increased along with the magnitude of response as measured by calcium influx, in neurons from the cancer models. To address the possible mechanism of TRPV1 sensitivity in tongue afferents, we study the role of PAR₂, which can sensitize the TRPV1 channel. We show co-expression of TRPV1 and PAR₂ on tongue afferents and using a conditioned place aversion assay, demonstrate that PAR₂ mediates oral cancer-induced, TRPV1-evoked sensitivity in an oral cancer mouse model. The findings provide insight into oral cancer-mediated chemosensitivity.

Oral cancer patients endure severe pain while undertaking routine oral functions that mechanically agitate soft tissue in the oral cavity such as eating and talking^{1,2}. On the other hand, these same patients report low levels of spontaneous pain. Using xenograft and carcinogen-induced oral cancer mouse models and orofacial assays, we have modeled and studied the functional/mechanical pain observed in oral cancer patients³. Anecdotal clinical evidence reveals that some oral cancer patients also suffer from chemosensitivity, especially to spicy foods and liquids. We have not studied this symptom to date. Substances within spicy food activate the transient receptor vanilloid channel 1 (TRPV1). While afferent neurons throughout the different heterosegmental regions express TRPV1, the oral cavity is an ideal site to study TRPV1 sensitization in the setting of cancer. Exposure of the soft tissue of the oral cavity to capsaicin is consistent with the eating habits of numerous cultures. Accordingly, application of capsaicin to the tongue in a rodent oral cancer model mirrors the experience of oral cancer patients who eat or drink spicy ingredients. A model that utilizes subcutaneous injection of capsaicin does not accurately recapitulate the topical exposure to spicy substances that patients experience.

Little is known regarding cancer-induced chemosensitivity. However, various cancer pain models involving several species reveal that TRPV1 is upregulated on sensory neurons that innervate oral cancers. Moreover, TRPV1 mediates oral cancer pain^{4–8}. For example, in an oral cancer pain model generated by inoculating squamous cell carcinoma into the gingiva of a rat, TRPV1 is overexpressed in the associated trigeminal ganglion

¹Department of Neurobiology and Hillman Cancer Research Center, University of Pittsburgh, Pittsburgh, USA. ²Department of Oral and Maxillofacial Surgery, Bluestone Center for Clinical Research, New York University (NYU) College of Dentistry, New York, USA. ³Pathology Department, NYU Langone Health, New York, USA. ⁴Department of Epidemiology and Health Promotion, NYU College of Dentistry, New York, USA. ⁵Department of Molecular Pathobiology, NYU College of Dentistry, New York, USA. ⁶Department of Neuroscience and Physiology, Neuroscience Institute, NYU Langone Health Neuroscience Institute, NYU Langone Health, New York, USA. ✉email: bls322@nyu.edu

(TG); mechanical allodynia and thermal hyperalgesia are observed in this model⁹. Agonists in the cancer micro-environment, including an elevated proton concentration, could activate TRPV1^{10–13}. Rhabdomyosarcoma and osteosarcoma secrete lipophilic substances that activate TRPV1 on TG neurons¹⁴. In one of the most promising studies to date, selective ablation of TRPV1 fibers with intrathecal resiniferatoxin reversed cancer pain and restored function in a canine bone cancer model¹⁵.

TRPV1 sensitization, rather than activation, is consistent with oral cancer pain during routine oral function, but not with spontaneous pain. TRPV1 sensitization occurs through phosphorylation of intracellular residues on the ion channel, a process that is modality specific¹⁶. Protease-activated receptor-2 (PAR₂), which has a prominent role in oral cancer pain^{3,17}, activates protein kinase C and A and leads to ion channel phosphorylation and sensitization of TRPV1¹⁸. The aim of our study was to determine whether oral cancer leads to TRPV1-mediated chemosensitivity, and whether the mechanism involves PAR₂, which is unknown in the setting of oral cancer. To study upregulation of TRPV1 on anatomically-relevant sensory neurons innervating the tongue, which we identified with the retrograde tracer DiI, in the setting of oral cancer, we used two oral cancer mouse models, the xenograft model with athymic nude mice and a carcinogen-induced oral cancer mouse model with wildtype C57BL/6 mice. We used PAR₂ knockout mice and conditioned place aversion (CPA) to assess the role of PAR₂ in oral cancer-induced TRPV1 sensitization. A better understanding of TRPV1 sensitization in the setting of cancer could provide insight into how TRPV1-mediated cancer nociception could be alleviated or preempted.

Results

Tongue primary afferents express TRPV1 protein that mediate aversion to capsaicin in the drinking water.

A significant portion of sensory nerve innervation to the tongue arises from neurons in the V3 region of the TG. We determined the percentage of retrograde labeled tongue afferents that express TRPV1 in order to gauge the functional impact of TRPV1 activation on aversion behavior. Using a retrograde tracer, the subpopulation of TRPV1-positive TG neurons that innervate the tongue was $32.95 \pm 3.5\%$ ($n = 3/\text{sex}/\text{group}$, Fig. 1A,B). We found no positive staining in the TRPV1 KO mouse (Fig. 1A,B). A two-bottle drinking aversion test was used to measure TRPV1 mediated oral sensitivity. Two bottles, one that contained water with vehicle (0.1% ethanol) and the other containing water with 1 μM capsaicin, were available for 5 days to single sex socially housed male and female mice ($n = 3/\text{cage}/\text{sex}$). TRPV1 KO mice were used as a comparison. Total water consumption (mL) was the same across sex and genotype (sex by genotype: $F(1,16) = 0.013$, $p = 0.909$; independent samples t-test with pooled sexes: $t(16) = 0.152$, $p = 0.881$). However, capsaicin water was avoided in wildtype mice, where both male and female C57BL/6 mice drank less than half the volume of water containing capsaicin compared to TRPV1 KO mice (Fig. 1C, $t(16) = 7.023$, $p < 0.001$).

TRPV1-mediated aversion to capsaicin during oral cancer progression. TRPV1 activation is thought to be involved in cancer pain^{19,20}; however, the effect of oral cancer on neuronal TRPV1 expression and sensitization is unclear. We have previously demonstrated a sex difference in pain secondary to oral cancer; male mice demonstrate significantly less oral cancer-induced nociceptive behavior due to a neutrophil-mediated endogenous opioid mechanism^{21,22}. Thus, due to the potential confounding effects of the immune system on oral chemosensitivity, male mice were analyzed separately for behavioral experiments. We used the oral cancer tongue xenograft model in tandem with the two-bottle choice assay to determine oral chemosensitivity during tumor growth. Naïve athymic nude mice were housed 3 per cage and baseline consumption (vehicle water) was measured for 7 days followed by HSC-3 inoculation or sham injection. On post inoculation (PID) 3, one vehicle water was replaced with 1 μM capsaicin water and consumption was measured by weight every 7 days for 4 weeks (Fig. 2A). We found an interaction between time and treatment in female mice only ($F(3,12) = 10.22$, $p = 0.0013$). Tumor-bearing female athymic nude mice demonstrated greater aversion to 1 μM capsaicin in the drinking water compared to sham at 3 ($p = 0.0343$) and 4 ($p = 0.0001$) weeks post inoculation (Fig. 2B). Total water consumption was similar over levels of time and treatment (Fig. 2C; $F(3,12) = 1.389$, $p = 0.294$). Consistent with our previous studies, we found no interaction between time and treatment in tumor-bearing male mice (Suppl Fig. 1, $F(3,12) = 0.522$, $p = 0.675$). In addition to oral sensitivity, we tested the hypothesis that capsaicin is driving pain in the oral cavity secondary to oral cancer by assaying CPA in mice that were conditioned with oral swabbing of vehicle (0.1% EtOH) followed by low dose 500 nM capsaicin (Fig. 2D). Pre-conditioning (baseline) times did not differ between the vehicle-paired chamber and the capsaicin-paired chamber ($p = 0.39$). Tumor-bearing female mice treated with oral capsaicin displayed CPA (Fig. 2E, $F(1,8) = 14.13$, $p = 0.006$); they spent less time in the capsaicin-paired chamber compared with either the pre-conditioning baseline ($p = 0.019$) or post-conditioning vehicle-paired chamber ($p = 0.001$; Fig. 2E). Oral capsaicin had no effect on sham female mice (Fig. 2F; $F(1,8) = 3.02$, $p = 0.121$) or in tumor-bearing male mice (Suppl Fig. 1, $F(1,8) = 0.063$, $p = 0.808$) or sham male mice (Suppl Fig. 1, $F(1,8) = 1.187$, $p = 0.308$).

Oral cancer-induced increase in TRPV1 protein expression in tongue primary afferents. Previous findings in a bone cancer mouse model found increased TRPV1 expression in dorsal root ganglia (DRG) neurons innervating the bone²⁰. We hypothesized that the increase in capsaicin-induced aversion behavior was due to an increase in TRPV1 protein expression in tongue trigeminal afferents. We analyzed TRPV1 protein expression in the xenograft and 4NQO-induced oral cancer models, as well as the associated controls (sham or Matrigel only and propylene glycol, respectively). Representative hematoxylin and eosin stained images of the cancer models and control tongues are shown in Fig. 3A. We used retrograde labeling from the tongue using DiI and immunohistochemistry in TG sections from tumor-bearing mice ($n = 4/\text{sex}$) compared to sham control ($n = 4/\text{sex}$). There was no interaction between sex and treatment ($F(1,12) = 1.744$, $p = 0.2112$), therefore, sexes were pooled. DiI + TRPV1 + neurons increased only in tumor-bearing mice (Fig. 3B,C; $t(14) = 2.187$,

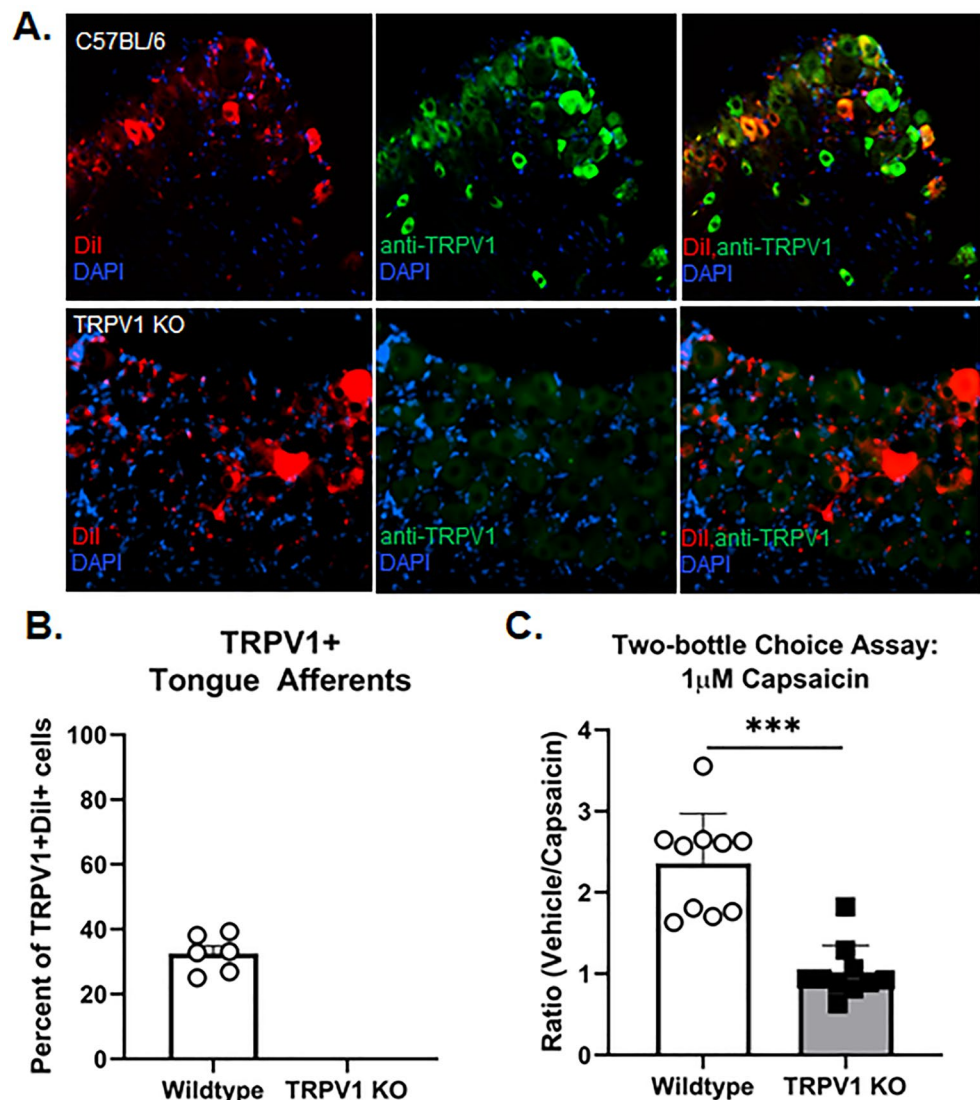


Figure 1. Tongue primary afferents express TRPV1 protein that mediates aversion to capsaicin in the drinking water. (A) Representative images of TRPV1-like immunoreactivity in TG sections from a retrograde labeled (Dil) female naïve C57BL/6 or female TRPV1 knockout (KO) mouse. (B) The pooled data indicate the percent of Dil + (red) neurons that were co-labeled with TRPV1 (green) from male and female C57BL/6 mice compared to TRPV1 KO mice ($n = 3/\text{sex}$). (C) There was a genotypic difference in TRPV1-mediated aversion to 1 μ M capsaicin; wildtype C57BL/6 male and female mice ($n = 5/\text{sex}$) demonstrate an aversion to capsaicin in the drinking water compared to TRPV1 KO mice which demonstrated no preference. *** $p < 0.001$.

$p = 0.046$). We have previously demonstrated that the immune system plays an important role in oral cancer pain²³. To determine if the lack of an intact immune system in athymic nude mice contributes to cancer-induced changes in TRPV1 expression, we measured TRPV1 expression in wildtype mice with 4NQO-induced oral SCC (oSCC, $n = 4$ female, 6 male) and vehicle treated mice ($n = 4$ female, 6 male). There was no interaction between sex and treatment ($F(1,16) = 0.918$, $p = 0.352$), therefore, sexes were pooled. Similar to the xenograft model, Dil + TRPV1 + cells increased only in tumor-bearing mice treated with 4NQO; Fig. 3D, $t(18) = 1.495$, $p = 0.0005$).

Oral cancer-induced increase in TRPV1 function. Next, we sought to determine cancer-induced changes in neuronal TRPV1 function. In dissociated retrograde labeled trigeminal neurons from mice, we quantified the percentage of retrograde labeled neurons that elicited a Ca^{2+} transient in response to 500 nM capsaicin as well as measured the magnitude of the capsaicin-evoked Ca^{2+} transient (Fig. 4A,B). We used neurons from mice with HSC-3 xenograft tumors ($n = 6/\text{sex}$) as well as 4NQO-treated mice with oSCC ($n = 11$ male, 12 female). Sham athymic mice ($n = 6/\text{sex}$) and vehicle-treated C57BL/6 mice ($n = 6/\text{sex}$) respectively were used as controls. There was no interaction between sex and treatment for either model (xenograft: $F(1,19) = 2.066$, $p = 0.167$; 4NQO: $F(1,31) = 1.400$, $p = 0.246$), therefore, sexes were pooled. Oral cancer evoked an increase in TRPV1 function and magnitude of response compared to controls. The percent of Dil + neurons in which capsaicin evoked

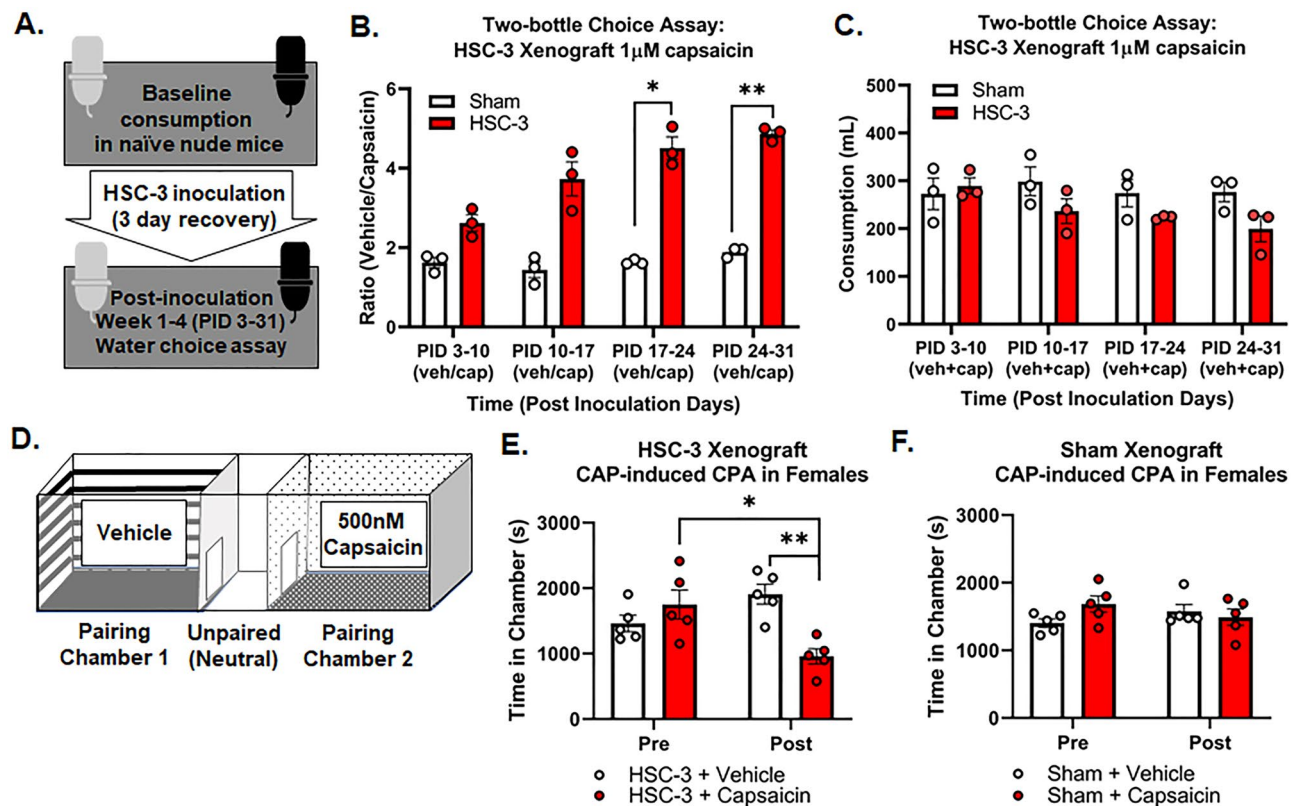


Figure 2. TRPV1-mediated aversion to capsaicin during oral cancer progression (A) Timeline for the two-bottle drinking assay using the oral cancer xenograft mouse model. Baseline water consumption using vehicle water (0.01% EtOH) was recorded prior to cancer cell injection into the tongue. (B) Tumor-bearing female athymic nude mice demonstrate greater aversion to 1 µM capsaicin in the drinking water compared to sham at 3 and 4 weeks post inoculation. We measured water consumed by cage; each cage housed 3 mice; each dot represents a cage of three female mice. Time x treatment, $p = 0.0013$. * $p < 0.05$, ** $p < 0.01$ (C) There was a similar reduction in total consumption with cancer progression in tumor-bearing mice regardless of time and treatment. $p > 0.05$. (D) Standard CPA box in which mice were conditioned with either saline or 500 nM capsaicin oral swabbing on post inoculation days 22–24. Place aversion was measured on day 25 and CPA was assessed as the difference in time spent in the capsaicin-paired chamber compared to saline-paired chamber. (E,F) Tumor-bearing female mice (white and red bars) spent less time in the capsaicin-paired chamber compared to baseline. Time x treatment $p = 0.0056$, * $p < 0.05$, ** $p < 0.01$. Capsaicin treatment had no effect in sham female mice. Time x treatment $p = 0.1207$.

a Ca^{2+} transient $> 20\%$ of baseline was increased in neurons from mice with HSC-3 xenograft ($t(21) = 6.829$, $p < 0.0001$ Fig. 4C) as well as 4NQO-treated mice with oSCC ($t(33) = 7.296$, $p < 0.0001$, Fig. 4D). The magnitude of the capsaicin-evoked Ca^{2+} transient, defined as the peak Ca^{2+} response minus baseline Ca^{2+} concentration, increased in mice with HSC-3 xenograft ($t(21) = 4.716$, $p = 0.0001$ Fig. 4C), as well as 4NQO-treated mice with oSCC ($t(33) = 5.508$, $p < 0.0001$; Fig. 4D) but not in their respective controls.

PAR₂-induced sensitization of the capsaicin-evoked Ca^{2+} transient. The tumor microenvironment is rich in protease and proteolytic peptides that directly activate PAR₂ on sensory neurons. PAR₂ activation has been shown to modulate TRPV1 activity^{18,24}. To determine whether PAR₂ and TRPV1 are co-expressed in tongue trigeminal neurons, we localized the proteins by immunofluorescence in tissue sections and measured the capsaicin-evoked Ca^{2+} transients in TG cultures using selective PAR₂ pharmacology. Immunoreactive PAR₂ and TRPV1 were colocalized in retrograde labeled tongue afferents in sections from TG from adult C57BL/6 mice and PAR₂ KO mice (Fig. 5A); $33.8 \pm 1.5\%$ of retrograde labeled tongue afferents co-expressed PAR₂ and TRPV1 protein ($n = 3$ mice). No PAR₂ and TRPV1 immunoreactive overlap was identified in PAR₂ KO mice ($n = 3$ mice). Next, we examined whether PAR₂ activation sensitizes TRPV1-mediated Ca^{2+} transients in dissociated TG neurons. Capsaicin evoked a Ca^{2+} transient $> 20\%$ of baseline in $28.6 \pm 2.5\%$ of DiI + neurons responding to 30 mM KCl ($n = 105$ neurons total) in both wildtype and PAR₂KO mice; there was no difference in the number of capsaicin-responsive neurons between genotype. The magnitude of the Ca^{2+} transient evoked by capsaicin varied with the interaction of drug treatment and genotype ($F(2.54) = 5.952$, $p = 0.005$). Pre-treatment with the specific PAR₂ ligand, 2-F (100 nM), potentiated the response to capsaicin applied 5 min later by $123.52 \pm 23.2\%$ ($n = 5/\text{sex}$) in neurons from wild-type mice compared to neurons pre-treated with vehicle ($p < 0.0001$; Fig. 5B,C). Furthermore, pre-treatment with the PAR₂ antagonist, GB88 (10 µM), inhibited the 2-F-induced potentiation

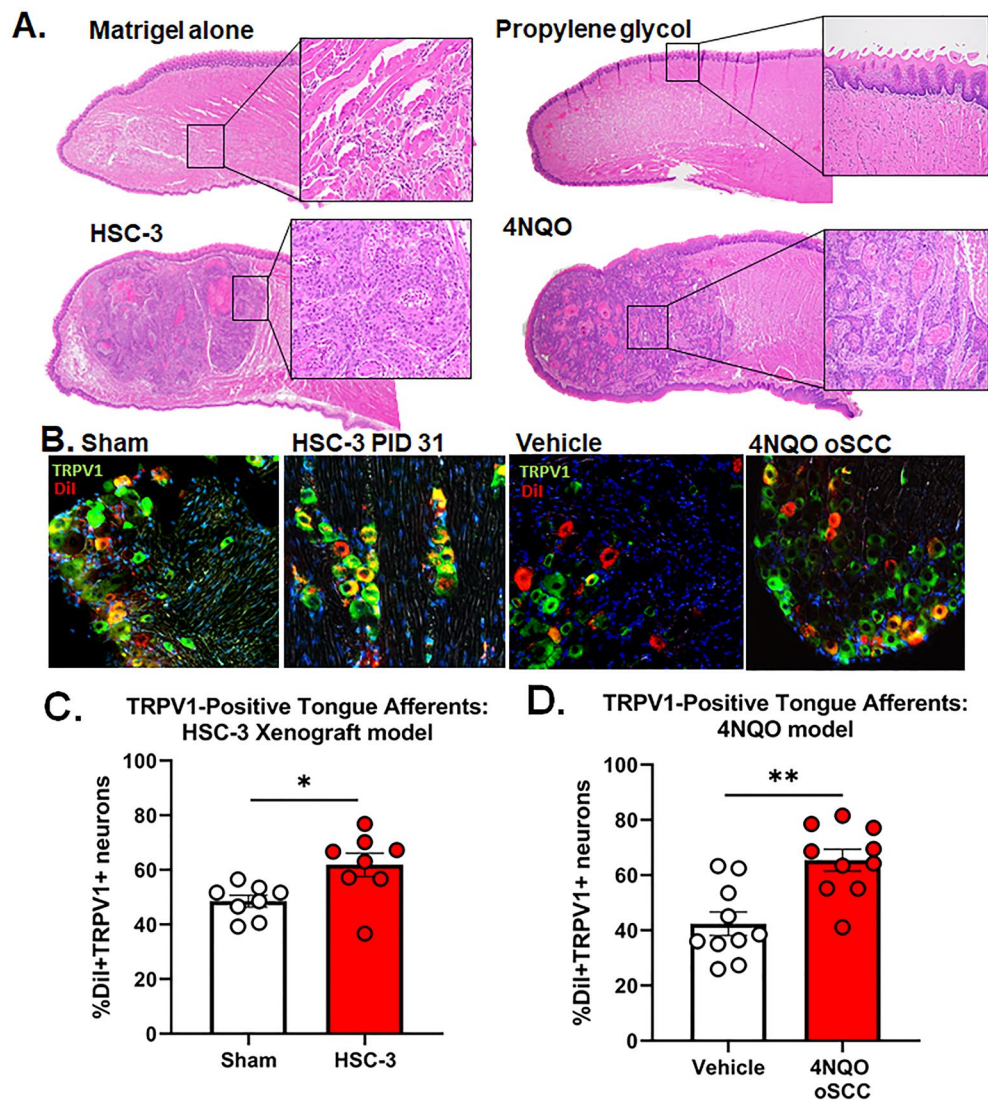


Figure 3. Oral cancer-induced increase in TRPV1 protein expression in tongue primary afferents. (A) Representative images of 5 μ m tongue sections stained with hematoxylin and eosin from sham (*i.e.*, Matrigel alone) and HSC-3-tumor bearing mice at PID 31, as well as propylene glycol- and 4NQO-treated mice at week 28. (B) Representative images of TRPV1-like immunoreactivity in retrograde labeled (DiI) tongue primary afferent neurons from a section from female sham and HSC-3 xenograft at PID 31 as well as a vehicle treated and 4NQO-treated female mouse with oSCC, 10 $\times$ magnification. (C) Pooled data from male and female mice indicates an increased percentage of DiI + neurons co-labeled with TRPV1 (green) from HSC-3 xenograft ($n = 4/\text{sex}$) but not sham ($n = 4/\text{sex}$) mice at PID 31. $*p < 0.05$. (D) Pooled data from male and female mice indicates an increased percentage of DiI + neurons co-labeled with TRPV1 in 4NQO-induced oSCC ($n = 4$ female, 6 male) mice, but not vehicle treated mice ($n = 4$ female, 6 male). $**p < 0.01$.

of the capsaicin-evoked transient by $41.98 \pm 17.7\%$ only in neurons from wildtype mice ($n = 5/\text{sex}$; $p < 0.0001$). There was no effect of 2-F ($p = 0.160$) or GB88 ($p = 0.119$) on the capsaicin-evoked transient in PAR₂KO mice ($n = 5/\text{group}/\text{sex}$, Fig. 5B,C).

TRPV1-induced aversion behavior in the presence of oral cancer is mediated by PAR₂. Lastly, we sought to determine if PAR₂ mediates the TRPV1-evoked nociception secondary to oral cancer. We used CPA assay in tandem with the acute oral cancer pain model to test the hypothesis that PAR₂ activation is required for capsaicin-induced aversion behavior in the presence of oral cancer. The acute supernatant model was used to quantify oral cancer-induced nociceptive behavior in the absence of tumor burden and illness associated with carcinogenesis. We first tested whether HSC-3 cell line supernatant alone evokes aversion behavior in wildtype C57BL/6 and PAR₂KO female mice. Based on previous findings using hindpaw von Frey, HSC-3 supernatant-induced sensitization of primary afferents lasts for about 24 h³. While a “cell culture media only” injection group is the more appropriate for supernatant injection, multiple injections into the tongue per day

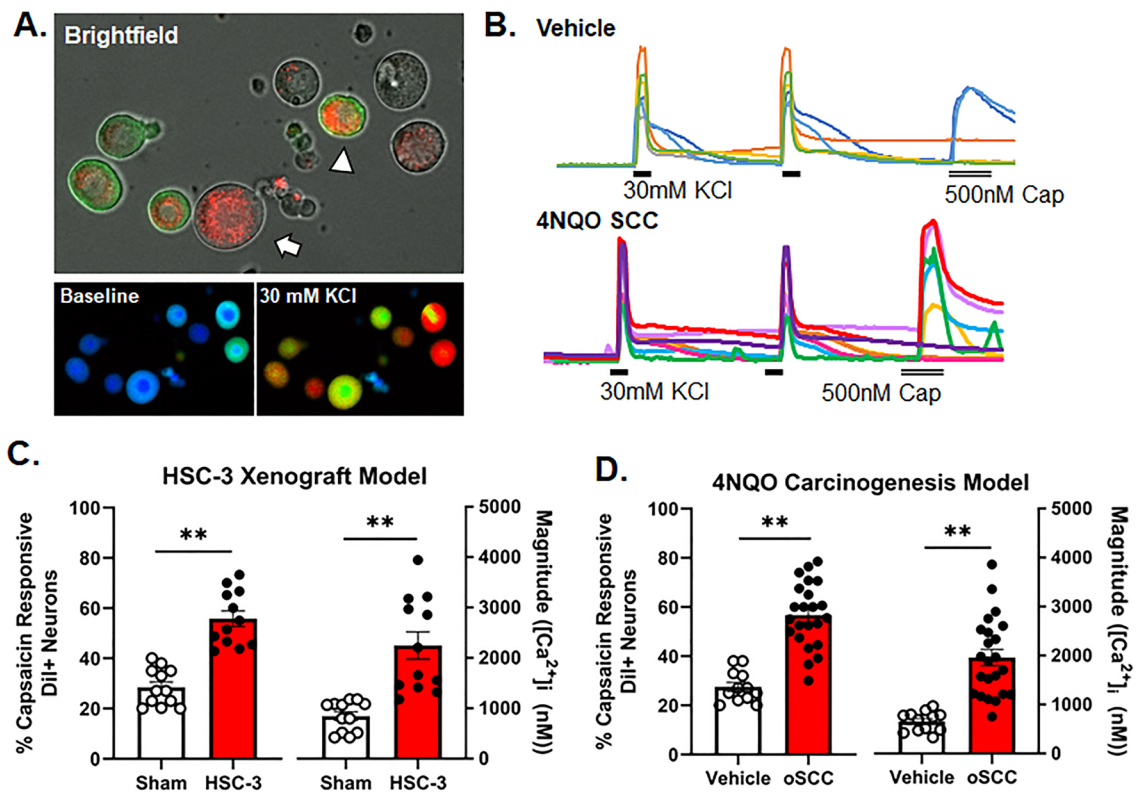


Figure 4. Oral cancer-induced increase in TRPV1 function (A) Image of dissociated TG neurons (bright field); a subset is retrograde labeled tongue afferents (DiI, white arrow) and IB4 nonpeptidergic (arrow head). (B) Traces of 30 mM KCl and 500 nM CAP-evoked transients from tumor bearing female mice. Ca^{2+} imaging was done at 20X magnification in fields containing ≥ 5 neurons and at least 2 coverslips were tested per mouse. For (C,D), which represent the HSC-3 xenograft and 4NQO models, respectively, each dot represents the average number of responders or the average Ca^{2+} transient magnitude of neurons tested from an individual mouse. The percentage of DiI + neurons that responded to CAP and the transient magnitude (peak-baseline) was increased in HSC-3 cancer-bearing mice ($n = 12$; 6/sex) compared to sham ($n = 12$; 6 mice/sex). $**p < 0.01$. $\Delta[\text{Ca}^{2+}]_i$ = change in intracellular Ca^{2+} concentration. The percentage of DiI + neurons that responded to CAP and the transient magnitude (peak-baseline) in CAP-evoked transients was increased in mice with 4NQO induced oSCC ($n = 22$; 10 male, 12 female) compared to vehicle-treated mice ($n = 12$; 6/sex). $**p < 0.01$.

over three days confounded the aversion behavior induced by the cancer supernatant. Therefore, for these CPA experiments, mice ($n = 10/\text{group}$) were conditioned with light anesthesia (2–2.5%) and no injection followed by either submucosal injection of cell line media or HSC-3 cell line supernatant (30 μl) into the tongue. Two-way ANOVA analyses showed that injection with cell line media does not evoke aversive behavior in either C57BL/6 ($F(1,18) = 0.581$, $p = 0.456$) or PAR_2KO ($F(1,18) = 0.175$, $p = 0.681$) mice (Fig. 6A,B). On the contrary, conditioning with HSC-3 cell culture supernatant evokes mild aversion behavior in C57BL/6 mice (Fig. 6C; interaction: $F(1,18) = 9.893$, $p = 0.006$; Holm Sidak: $p = 0.051$), but not in PAR_2KO mice (Fig. 6D; ($F(1,18) = 2.103$, $p = 0.164$)). Oral cancer supernatant contains proteases which can activate neuronal PAR_2 ^{3,17}. We hypothesize that low dose capsaicin swabbing potentiates oral cancer-induced aversion behavior in a PAR_2 dependent manner. To test this, mice were conditioned with no injection plus an oral swabbing of vehicle (0.01% EtOH) followed by HSC-3 cell line supernatant injection into the tongue plus, 1 h after injection, an oral swabbing of 500 nM capsaicin. Wildtype C57BL/6 mice demonstrated aversion to the chamber paired with HSC-3 supernatant plus capsaicin (Fig. 6E; interaction: $F(1,18) = 10.78$, $p = 0.004$; Holm Sidak: $p = 0.001$) whereas PAR_2KO mice did not (Fig. 6F; ($F(1,18) = 3.203$, $p = 0.090$)). Furthermore, the percent time spent in the treatment chamber varied with the interaction of treatment and genotype (Fig. 6G; $F(2,54) = 4.401$, $p = 0.017$); C57BL/6 mice spent less time than PAR_2KO only in the HSC-3 supernatant plus capsaicin chamber ($p = 0.033$).

Discussion

We report that oral cancer sensitizes TRPV1 through a PAR_2 -mediated mechanism. We propose that proteases cleave and activate PAR_2 on primary afferent neurons in the oral cancer microenvironment, which sensitizes TRPV1 on the same neurons. Back labeled, anatomically relevant oral nociceptors respond to oral cancer by overexpressing TRPV1. We study expression of TRPV1 in two oral cancer mouse models. The xenograft model is created with a human oral cancer cell line which has implications for the study of human oral cancer. The carcinogen-induced oral cancer mouse model involves transformation of oral keratinocytes to malignant oral keratinocytes, similar to what would occur in oral cancer patients, and can be generated in mice with an intact

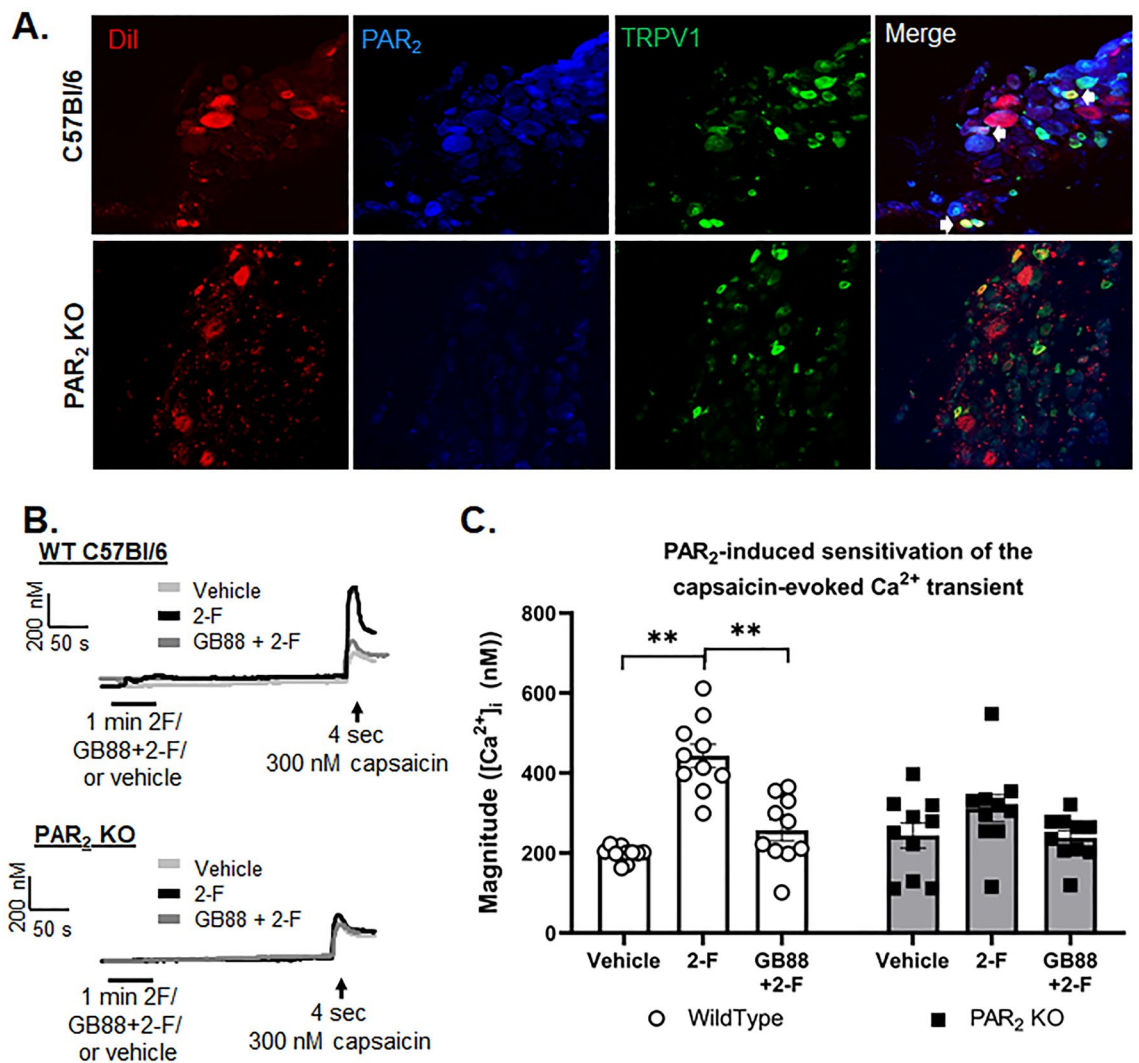


Figure 5. PAR₂-induced sensitization of the capsaicin-evoked Ca²⁺ transient. (A) Triple labeling of PAR₂ + TRPV1 + tongue primary afferent neurons using fast blue as retrograde tracer. (B) Representative traces from retrograde labeled tongue trigeminal neurons from (C) female wildtype C57BL/6 mouse or PAR₂ global knockout mouse. TGs were dissociated and neurons were loaded with Fura-2AM for Ca²⁺ imaging. After 30 s of baseline recording, neurons were treated for 1 min with either vehicle (normal bath, light gray), PAR₂ agonist, 2-F (100 nM, black) or pre-treatment with PAR₂ antagonist, GB88 (10 μM), followed by 2-F (dark gray). The drug was washed out for 5 min and the neuron was challenged with capsaicin (300 nM, 4 s). (C) Treatment with 2-F resulted in increased capsaicin-evoked Ca²⁺ transient amplitude (peak–baseline) in tongue neurons from male and female wildtype mice (n = 10; 5/sex). There was no effect of 2-F treatment in tongue neurons from PAR₂ KO mice (n = 10; 5/sex). **p < 0.01.

immune system. Pharmacologic activation of PAR₂ on oral nociceptors prior to the application of capsaicin enhances calcium influx. We employed two operant assays—drinking aversion and CPA—to confirm capsaicin sensitivity in oral cancer models; we used the former assay to confirm that PAR₂ mediates oral cancer-induced capsaicin sensitivity.

Our results accord with those from two other studies that support the role of TRPV1 in oral cancer pain^{19,25}. Ruparel and colleagues reported that three oral cancer cell lines, including HSC-3, a cell line we used in our study, secrete lipids that produce nocifensive behavior and thermal hypersensitivity through TRPV1¹⁹. The role of TRPV1 in bone cancer pain was demonstrated several years earlier when Mantyh and colleagues used pharmacologic antagonism and genetic disruption of TRPV1 to reverse spontaneous and functional pain in early and late stages of cancer progression⁴. Similar to our findings with tongue cancer and TG neurons, cancer-induced over expression of TRPV1 occurs in the DRG in a bone cancer pain model²⁰. Cancer induces TRPV1-mediated functional changes as well. We demonstrated that back labeled neurons in two separate oral cancer models exhibit an elevated response to capsaicin. This effect is similar to the elevated response to capsaicin exhibited by neurons co-cultured with fibrosarcoma cells²⁶.

An understanding of the mechanism, and the second messengers that mediate TRPV1 sensitization, following PAR₂ activation might suggest a method for pharmacologic antagonism and treatment for oral cancer

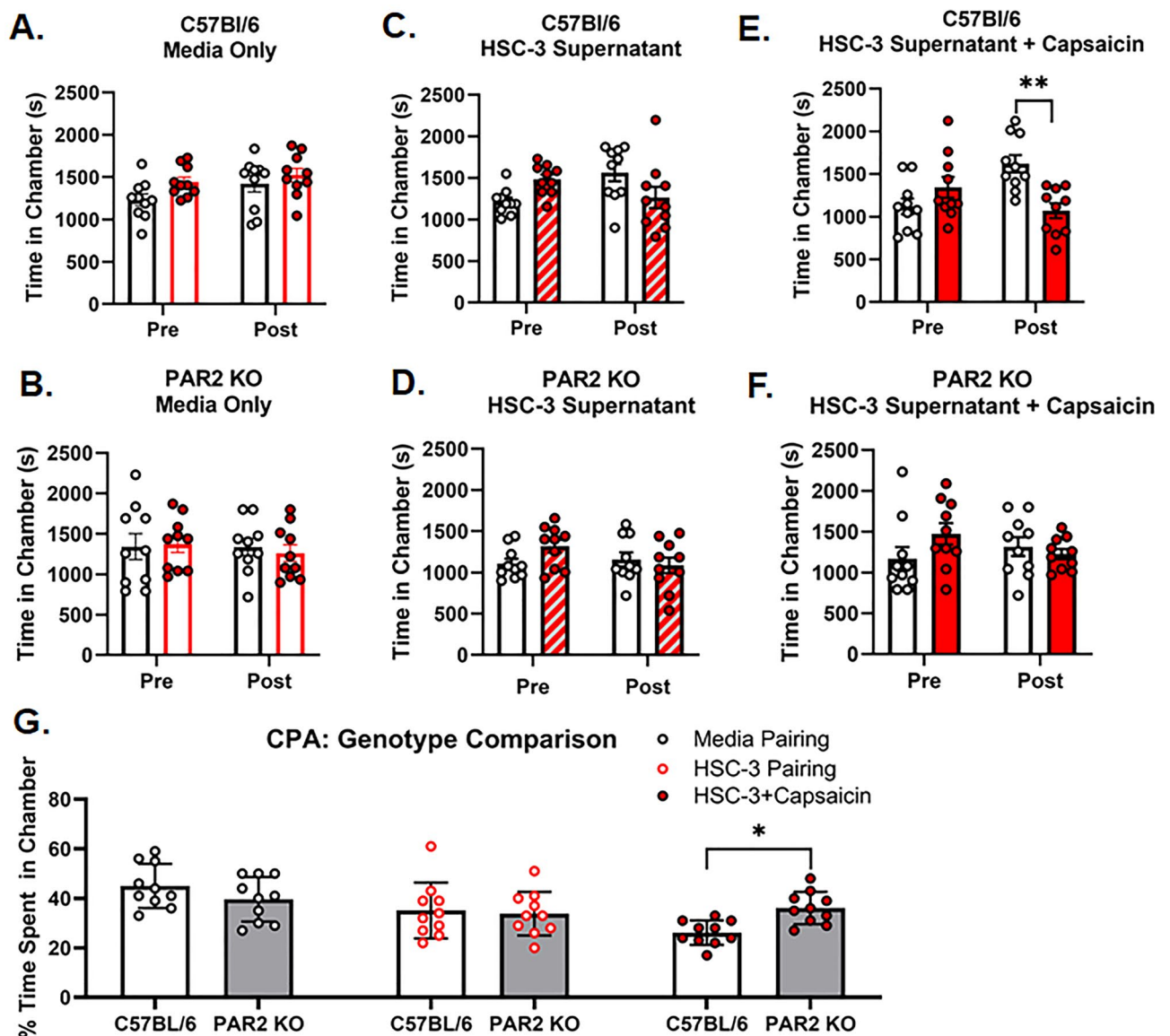


Figure 6. TRPV1-induced aversion behavior in the presence of oral cancer is mediated by PAR₂. C57BL/6 or PAR₂KO female mice were conditioned with either no injection or one of the following: saline injection, oral cancer supernatant injection into the tongue, or oral cancer supernatant followed by oral swabbing of 500 nM capsaicin 1 h post injection. The percent time spent in chamber was calculated as (time spent in the paired chamber / total time spent in both chambers post conditioning * 100). (A–F) Conditioned place aversion was evident in C57BL/6 mice treated with HSC-3 supernatant + 500 nM capsaicin but not in other treatment types or PAR₂KO mice. * $p < 0.05$, ** $p < 0.01$. (G) Conditioned place aversion was evident only in C57BL/6 mice in the chamber paired with HSC-3 supernatant + 500 nM capsaicin (genotype by treatment interaction, $p = 0.0170$).

pain. While the mechanism of PAR₂-mediated TRPV1 sensitization on tongue afferent neurons in the setting of oral cancer is not known, the mechanisms described for other conditions and neurons provide insight. PAR₂ sensitization of TRPV1 occurs in TG, DRG and nodose/jugular complex sensory ganglia^{18,27,28}. Sensitization of TRPV1 involves phosphorylation by PKC and PKA, which enhances ion channel activity^{29,30}. PKC mediates neuronal processing following cleavage of PAR₂³¹. PAR₂ activates PKC by producing diacylglycerol through phospholipase C following recruitment and activation of Gq proteins. Oral cancers secrete proteases that activate PAR₂, including legumain. In previous work we found that legumain cleaves PAR₂ and activates PKA; this cascade can subsequently phosphorylate and sensitize TRPV1^{17,32}. PAR₂ also increases the open probability (P_o) of the capsaicin-induced single channel in vagal pulmonary sensory neurons²⁸. PAR₂ activation increases the excitability of rat pulmonary capsaicin-induced chemoreflex in the lungs of rats²⁸. Trypsin, which cleaves PAR₂, enhances the capsaicin-induced pulmonary chemoreflex response and potentiates the whole-cell inward current induced by capsaicin in isolated nodose/jugular small-diameter neurons that innervate the lungs³³. Similar to our approach, Gu et al., studied anatomically, back labeled relevant sensory neurons. The in vivo effects of PAR₂ activation on the TRPV1-mediated tussive response have also been demonstrated in guinea pigs²⁴.

There are other mechanisms, in addition to PAR₂, by which oral cancer could sensitize TRPV1. Tumor necrosis factor alpha (TNFα), a pro-tumor inflammatory cytokine, is well known to potentiate TRPV1 activity leading to allodynia and thermal hyperalgesia in DRG³⁴ and Ca²⁺ influx in TG³⁵. We have recently demonstrated that TNFα is overexpressed in human oral cancer tissues³⁶, secreted from human oral cancer cell lines and upregulated in cancer tongue tissues collected from mice treated with 4NQO²³. Nerve growth factor (NGF) and adenosine triphosphate (ATP) are also secreted by oral cancer and can sensitize TRPV1^{37,38}. TRPV1 can be sensitized through increased expression and/or receptor phosphorylation at key intracellular residues. NGF activates p38, which increases TRPV1 translation in the cell body and leads to subsequent anterograde transportation of the ion channel to nerve terminals in the periphery³⁹. ATP sensitizes TRPV1 on TG neurons through phosphorylation of serine residues²⁷. These TRPV1 sensitization mechanisms (PAR₂, ATP, NGF) likely overlap as it has been shown that PAR₂ activation by trypsin increases ATP secretion through TRPV1 sensitization⁴⁰.

Limitations of our study include the control used for the CPA experiments, our use of global knockout mice, and single sex behavioral experimental design. Because generation of the HSC-3 xenograft cancer model requires a nude athymic mouse, and we used PAR₂ knockout mice on a C57BL/6 background to study the role of PAR₂, we used the HSC-3 supernatant model³. With regard to our CPA control, our previous work demonstrates that HSC-3 supernatant-induced sensitization of primary afferents lasts for about 24 h³. While a media only injection group is a more appropriate control for the CPA experiments, multiple injections per day over three days would likely confound the aversive behavior induced by the cancer supernatant. To address this injection-related concern, we demonstrated in a separate experiment that a saline injection does not induce aversive behavior. To obviate the potential confound of multiple injections, we employed CPA using anesthesia only condition pairing. From our demonstration that back labeled tongue afferent fibers co-express PAR₂ and TRPV1, along with our demonstration that PAR₂ activation enhances the calcium influx induced by TRPV1, we infer that PAR₂ and TRPV1 on sensory neurons mediate the capsaicin sensitivity observed in our cancer model. However, there is a possibility that PAR₂ expression on non-neuronal cells could mediate capsaicin sensitivity. We did not use a conditional PAR₂ knockout selective for afferent fibers; for the current study, we used a global knockout mouse. Now that we have PAR₂-Na_v1.8 mice, we plan to use these mice in our future oral cancer and capsaicin sensitivity studies. Lastly, we used female mice only for the behavioral experiments to determine if TRPV1-induced aversion behavior in the presence of oral cancer is mediated by PAR₂. Including the present study, we have now demonstrated a sex difference in pain and chemosensitivity secondary to oral cancer. We hypothesize that male mice demonstrate significantly less oral cancer-induced nociceptive behavior due to a neutrophil-mediated endogenous opioid mechanism^{21,22} and such, pre-treatment with naloxone in male mice may reveal the oral cancer-induced chemosensitivity during tumorigenesis.

In naïve mice, we found that 36% of lingual afferent fibers express TRPV1. Previous work by Elitt et al. and Wu et al. revealed TRPV1 expression in lingual TG neurons (25% and 17%, respectively)^{41,42}. Capsaicin sensitivity might be greater in humans with oral cancer than in our mouse oral cancer model. A recent study revealed that TRPV1 expression is higher in human DRG than in mouse DRG; approximately 75% in the former and 32% in the latter⁴³. Our study provides support for chemosensitivity testing in oral cancer patients to gain insight into neuronal plasticity in the context of oral cancer.

Methods

Animals. Male and female adult (6–20 weeks) athymic nude mice (NU/J, *Foxn1*^{nu}, stock #002019, Jackson Labs, Bar Harbor, ME, USA), and C57BL/6 mice (stock #027, Charles River, Wilmington, MA, USA), TRPV1 KO mice (B6.129X1-*Trpv1*^{tm1^{ju}}/J, stock #003770) and PAR₂KO mice (B6.Cg-*F2rl1*^{tm1^{mslb}}/J, stock #004993, Jackson Labs) were used. Mice were maintained on a 12:12 h light cycle and were housed in group cages in temperature-controlled rooms with access to food and water. Researchers were trained under the Animal Welfare Assurance Program. Experimental procedures were approved by the New York University Institutional Animal Care and Use Committee and were conducted in line with the National Institutes of Health guidelines for the use of laboratory animals in research.

Cell culture. Human oral cancer cell line, HSC-3 (human tongue oral SCC cell line, cell number JCRB0623, from the Japanese Collection of Research Bioresources Cell Bank), was utilized to produce the human oral cancer xenograft model in immunocompromised nude mice. Cells were maintained and cultured as previously described⁴⁴. HSC-3 cells were cultured in 10 cm² cell culture dishes at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin (50 U/mL). When cells reached 70% confluency, the culture medium was removed, and cells were washed with 5 mL PBS without Ca²⁺ and Mg²⁺. Cells were collected and resuspended in DMEM:matrigel (1:1; 1 × 10⁵ cells/20 μL HSC-3) for tongue inoculation; HSC-3 cells were collected from passage 7. Supernatant was collected as previously described for the conditioned place aversion (CPA) nociceptive behavior assay²³. Briefly, cells were cultured to 70% confluency, washed twice with 5 mL PBS without Ca²⁺ and Mg²⁺ and media was replaced with 3 mL of serum and antibiotic free culture media (DMEM for HSC-3). After 48 h, supernatant was removed and spun at 300 × g for 4 min to remove debris and injected into the lateral anterior portion of the tongue within 20 min.

Oral cancer xenograft model. A murine xenograft tongue cancer model was used to determine TRPV1 sensitization during oral carcinogenesis using the drinking choice behavior assay. Mice were inoculated with 1 × 10⁵ HSC-3 cells prepared in DMEM:Matrigel (1:1, 30 μL) into the anterior lateral portion of the tongue, as previously described²¹. HSC-3 is a human tongue squamous cell carcinoma; the use of this cell line and the study of the xenograft model has the advantage of increased translational relevance. Cancer growth and associated

nociceptive behavior develops in approximately 10 days to 2 weeks following inoculation^{21,25,37}. On post inoculation day (PID) 31, all mice were anesthetized with isoflurane followed by transcardial perfusion with cold Ca^{2+} /Mg²⁺ free Hank balanced salt solution (HBSS, Invitrogen) for TG harvest; harvested TG neurons were placed on ice cold HBSS for dissociation and acute culture. To prepare tissues for immunohistochemistry, mice were anesthetized with isoflurane followed by transcardial perfusion with ice cold PBS followed by 4% paraformaldehyde (PFA). The experimenters conducting the behavioral tests were blinded to the treatment groups; the allocation keys were held by NNS.

4-nitroquinoline-1-Oxide (4NQO) oral carcinogenesis model. To determine changes in TRPV1 expression and function during carcinogenesis in an animal with an intact immune system, which is an advantage of the carcinogen-induced oral cancer model, mice were offered carcinogen 4-nitroquinoline-1-oxide (4NQO; 100 µg/mL; Sigma Aldrich, St. Louis, MO, USA) in the drinking water or the equivalent dilution of the vehicle, propylene glycol for 16 weeks, which is required to induce transformation of oral keratinocytes to malignant keratinocytes²⁵. The mice were then monitored weekly under light anesthesia for body weight, tumor incidence, location, and size for an additional 12 weeks. If mice lost greater than 20% body weight prior to week 28 following the start of exposure to 4NQO, they were euthanized and TG and tongue tissue were collected. By week 28, all mice were euthanized regardless of clinical evaluation and health status. Euthanasia, perfusion and TG and tongue tissue preparation was performed as described for the oral cancer xenograft model. Tongue tissue was harvested by using bone scissors to bilaterally cut the jaw and spring-loaded scissors were used to free the tongue muscle from the floor of mouth. The tongue was dissected back to the oropharyngeal region, fixed in 10% formalin, and processed for paraffin embedding and slide preparation. Four 5 µm hematoxylin & eosin-stained tongue sections separated by 100 µm were evaluated for the presence of papillary and invasive SCC^{45,46}. Histopathologic analysis was performed by an oral and maxillofacial pathologist blinded to group identity; allocation keys were held by NNS. While calcium microfluorimetry and immunohistochemical evaluation was completed on all samples, only mice with histologically confirmed invasive lesions were included in subsequent data analyses.

Drinking choice behavior assay. Mice were tested for TRPV1-mediated oral aversion to capsaicin using a paired-preference drinking aversion assay. A total of 36 athymic nude mice (18 males and 18 females) were used for these experiments. Mice were housed by sex in groups of three mice per cage. Cages were fitted with two water bottles, and positions of the bottles were swapped daily. Mice were acclimatized by ad libitum access to water with 0.1% ethanol vehicle in both bottles for one week. After acclimatization, mice were inoculated with HSC-3 cells freshly prepared in DMEM:Matrigel (1:1, 20 µL) or DMEM:Matrigel (1:1, 20 µL) alone in the sham group. On PID 3, each cage was fitted with one bottle that contained vehicle (0.1% ethanol) and another bottle that contained 1 µM capsaicin (Sigma-Aldrich, St. Louis, MO). After seven days, the volume consumed from each bottle was measured by weight (1 mL water = 1 g). Fluid consumption was measured weekly for four weeks. An index measuring aversion to capsaicin was calculated individually for treatment group (sham/tumor-bearing) and sex as previously described⁴⁷. Solutions and cages were replaced with fresh solutions following each measurement. Oral aversion to capsaicin was determined by two-way ANOVA (time by treatment).

Conditioned place aversion assay. CPA occurs by pairing a painful experience with a distinct context resulting in subsequent avoidance of the same contextual cues^{48,49}. We use the CPA assay to measure aversive behaviors in response to three treatment groups, (1) injection of supernatant from oral cancer cell line culture, (2) low-dose capsaicin (500 nM, oral swabbing), and (3) supernatant injection followed by oral capsaicin swabbing. We performed a single trial CPA protocol. The 3-chamber CPA apparatus consists of two conditioning chambers with distinct tactile, visual, and olfactory cues, connected by a smaller neutral chamber that was brightly lit. The visual cues were horizontal stripe and dot wallpaper. The tactile cues were smooth and rough flooring. The olfactory cues were strawberry and mint. White noise was played to provide background noise and block out any extraneous sounds. On the first day (preconditioning) of the experiment, mice were introduced to the neutral chamber and allowed to explore all three chambers for 1 h. Baseline time spent in the chambers was measured using ANY-maze tracking software (Braintree Scientific, Braintree, MA, USA). Exclusion criteria included mice spending <20% or >80% time in a chamber. Drug-chamber pairings for conditioning were counterbalanced across subjects⁵⁰. On the second, third and fourth days, conditioning treatment varied based on experimental group. All mice underwent light anesthesia (2–2.5% isoflurane) prior to all treatments. For the supernatant-induced aversion, mice received anesthesia only followed by confinement into the appropriate pairing chamber for 1 h, following which they were returned to their home cage. Four hours later, mice received either submucosal injection of cell line media or HSC-3 cell line supernatant (30 µl) into the anterior lateral tongue followed by confinement into the opposite pairing chamber for 1 h. For the capsaicin-induced aversion, mice received an oral swabbing of vehicle (0.1% EtOH) followed by confinement into the appropriate pairing chamber for 1 h, following which they were returned to their home cage. Four hours later, mice received oral swabbing of 500 nM capsaicin followed by confinement into the opposite pairing chamber for 1 h. Low dose 500 nM capsaicin was chosen as it was found to be non-aversive in naïve athymic nude mice during the two-bottle choice assay (data not shown). For supernatant/capsaicin-induced aversion, mice received an oral swabbing of vehicle (0.01% EtOH) followed by confinement into the appropriate pairing chamber for 1 h, following which they were returned to their home cage. Three hours later, mice received submucosal injection of HSC-3 cell line supernatant (30 µl) into the anterior lateral tongue. One-hour later the mice received oral swabbing of 500 nM capsaicin followed by confinement into the opposite pairing chamber for 1 h. On the fifth day, mice were once again allowed to freely explore the apparatus for 1 h. Time spent in each chamber was recorded by

ANY-maze. The experimenter conducting the behavioral tests (IMW) was blinded to the treatment groups; the allocation keys were held by NNS.

Primary culture. Bilateral TGs were isolated by removing the skull cap and brain to expose the TGs readily visible in the base of the cranium. Using spring-loaded scissors the TGs were dissected and placed into cold HBSS and dissociated based on the previously described protocol⁵¹. Briefly, TG were minced and incubated in HBSS containing collagenase type II (Gibco) and dispase type II (Gibco) at 37 °C for 20 min and mechanically dissociated using a fire polished glass Pasteur pipette. A 12.5% by 28% percoll gradient was used to separate myelin and nerve debris from TG neurons. Cells were then plated in DMEM/F12 (Gibco) containing 5% fetal bovine serum and antibiotics (penicillin/streptomycin, 50 U/mL) on 5 mm coverslips. Coverslips were flooded 2 h later with Leibovitz L-15 media (Gibco) containing 10% fetal bovine serum, 5 mM HEPES and 5 mM glucose, and used at room temperature. Experiments were performed within 8 h of tissue harvest.

Calcium imaging. TG neurons plated on 5 mm coverslips were incubated with 5 μ M Ca^{2+} indicator fura-2 AM (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min in a physiological salt buffer (normal bath, 130 NaCl, 3 KCl, 2.5 CaCl_2 , 0.6 MgCl_2 , 10 HEPES, 10 Glucose, pH=7.4, 325 mOsm). Coverslips were placed in a recording chamber and continuously infused with normal bath at room temperature. Imaging was done at 20X magnification in fields containing ≥ 5 neurons and at least 2 coverslips were tested per mouse. To measure the neuronal Ca^{2+} response to TRPV1 activation, capsaicin was applied for 4 s. To measure the neuronal Ca^{2+} response to TRPV1 activation following PAR_2 agonist 2-Furoyl-LIGRLO-amide (2-F), or PAR_2 antagonist GB88, 2-F or 2-F+GB88 was applied for 1 min followed by a 5 min wash with normal bath and then capsaicin was applied for 4 s. To test for cell viability, 30 mM KCl was applied for 4 s at the end of each experiment. Drugs and KCl were applied with a fast-step perfusion system (switching time < 20 ms; Warner Instrument Co, Model SF-77B). A change in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) $\geq 30\%$ of baseline was considered as a response to treatment. The magnitude of the response was calculated as (Peak $[\text{Ca}^{2+}]_i$) – (Baseline $[\text{Ca}^{2+}]_i$). To account for biological variance, percentage of DiI + neurons that responded to capsaicin and the average magnitude of response across DiI + capsaicin-responsive neurons were calculated and the data for each mouse was reported. Fluorescence data was acquired by a Nikon Eclipse Ti microscope at 340 nm and 380 nm excitation wavelengths and analyzed with the TI Element Software (Nikon, Melville, NY, USA). $[\text{Ca}^{2+}]_i$ was determined from fura-2AM ratio. Calibration of imaging software to convert Fura-2 ratio following in situ calibration experiments was performed as previously described^{52,53}.

Immunohistochemistry. To determine changes in protein expression in TG ganglia neurons innervating the tongue, retrograde tracers were injected into the tongue 10 days prior to inoculation or administration of 4NQO or vehicle. Retrograde tracer 1,19-dioctadecyl-3,3,39,39 tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA) was dissolved to 170 mg/mL in DMSO, diluted 1:10 in 0.9% sterile saline, and injected bilaterally using a 30 g needle for a total volume of 5–7 μ L per tongue of adult male and female mice to label tongue afferents. Retrograde tracer, Fast Blue (Polysciences, Inc, Warrington, PA, USA), was dissolved using Milli-Q water to a final concentration of 2.0% and injected bilaterally using a 30 g needle for a total volume of 5–7 μ L per tongue of adult male and female C57Bl/6 mice to label tongue afferents. At least 10 days following tongue tracer injection, animals were euthanized through an overdose of inhaled isoflurane and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (Sigma Aldrich). TG were dissected, postfixed for 1 h in paraformaldehyde, and cryoprotected in 30% sucrose PBS at 4 °C. TG were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), sectioned (8 μ m), and mounted on Superfrost Plus slides (Fisher Scientific Company, Pittsburgh, PA). Sections were blocked for 1 h in 3% goat serum and incubated in primary antibody in PBS containing 1% bovine serum albumin overnight at room temperature. The primary antibodies used were as follows: guinea pig anti-TRPV1 (1:500, Alomone AGP-118, lot#ANO125), rabbit anti- PAR_2 (1:250, Thermo Scientific PA5-77685, lot#VJ3105322). Slides were extensively washed in PBS and incubated in goat anti-rabbit or guinea pig secondary antibodies conjugated to cyanine 2 or 3 (Jackson ImmunoResearch, West Grove, PA) in PBS at 1:250 for 2.5 h, extensively washed, and cover-slipped with FluoroGel II containing DAPI (Electron Microscopy Sciences). Sections were photographed using NIS Elements software and a Nikon Eclipse Ti microscope. For quantification of TRPV1-positive neurons across treatment groups as well as triple labeling of retrograde tracer (DiI), TRPV1-positive and PAR_2 -positive neurons, TG neurons with distinct nuclei within the V3 region were counted in every fifth section across three different sections per animal from 3 individual animals with a 20 $\times$ objective and identical laser gain settings.

Statistical analysis. An independent samples t-test or analysis of variance (ANOVA) was employed to evaluate the difference between groups defined by genotype and treatment, and mixed models ANOVA analysis was used when repeated measures over time were also included in the analysis. To adjust for multiple comparisons, the post hoc Holm-Sidak test statistic was employed. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using Prism (version 8) statistical software (GraphPad Software Inc., San Diego, CA, USA). Results were presented as mean and standard error of the mean in box/scatter configuration to show the biological variability. Our study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

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References

1. Connelly, S. T. & Schmidt, B. L. Evaluation of pain in patients with oral squamous cell carcinoma. *J. Pain* **5**, 505–510. <https://doi.org/10.1016/j.jpain.2004.09.002> (2004).
2. Kolokythas, A., Connelly, S. T. & Schmidt, B. L. Validation of the university of California San Francisco oral cancer pain questionnaire. *J. Pain* **8**, 950–953. <https://doi.org/10.1016/j.jpain.2007.06.012> (2007).
3. Lam, D. K., Dang, D., Zhang, J., Dolan, J. C. & Schmidt, B. L. Novel animal models of acute and chronic cancer pain: a pivotal role for PAR2. *J. Neurosci.* **32**, 14178–14183. <https://doi.org/10.1523/JNEUROSCI.2399-12.2012> (2012).
4. Ghilardi, J. R. *et al.* Selective blockade of the capsaicin receptor TRPV1 attenuates bone cancer pain. *J. Neurosci.* **25**, 3126–3131. <https://doi.org/10.1523/JNEUROSCI.3815-04.2005> (2005).
5. Honore, P. *et al.* Repeated dosing of ABT-102, a potent and selective TRPV1 antagonist, enhances TRPV1-mediated analgesic activity in rodents, but attenuates antagonist-induced hyperthermia. *Pain* **142**, 27–35. <https://doi.org/10.1016/j.pain.2008.11.004> (2009).
6. Karai, L. J., Russell, J. T., Iadarola, M. J. & Olah, Z. Vanilloid receptor 1 regulates multiple calcium compartments and contributes to Ca²⁺-induced Ca²⁺ release in sensory neurons. *J. Biol. Chem.* **279**, 16377–16387. <https://doi.org/10.1074/jbc.M310891200> (2004).
7. Niyama, Y., Kawamata, T., Yamamoto, J., Furuse, S. & Namiki, A. SB366791, a TRPV1 antagonist, potentiates analgesic effects of systemic morphine in a murine model of bone cancer pain. *Br. J. Anaesth.* **102**, 251–258. <https://doi.org/10.1093/bja/aen347> (2009).
8. Shinoda, M., Feng, B. & Gebhart, G. F. Peripheral and central P2X receptor contributions to colon mechanosensitivity and hypersensitivity in the mouse. *Gastroenterology* **137**, 2096–2104. <https://doi.org/10.1053/j.gastro.2009.06.048> (2009).
9. Nagamine, K. *et al.* Mechanical allodynia and thermal hyperalgesia induced by experimental squamous cell carcinoma of the lower gingiva in rats. *J. Pain* **7**, 659–670. <https://doi.org/10.1016/j.jpain.2006.02.013> (2006).
10. Meyer, K. A. Cancer of the stomach and colon. *Ky. Med. J.* **46**, 125–127 (1948).
11. Vaupel, P. W., Frinak, S. & Bicher, H. I. Heterogeneous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. *Cancer Res.* **41**, 2008–2013 (1981).
12. Newell, K., Franchi, A., Pouyssegur, J. & Tannock, I. Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1127–1131. <https://doi.org/10.1073/pnas.90.3.1127> (1993).
13. Gillies, R. J., Liu, Z. & Bhujwalla, Z. 31P-MRS measurements of extracellular pH of tumors using 3-aminopropylphosphonate. *Am. J. Physiol.* **267**, C195–203. <https://doi.org/10.1152/ajpcell.1994.267.1.C195> (1994).
14. Lautner, M. A., Ruparel, S. B., Patil, M. J. & Hargreaves, K. M. In vitro sarcoma cells release a lipophilic substance that activates the pain transduction system via TRPV1. *Ann. Surg. Oncol.* **18**, 866–871. <https://doi.org/10.1245/s10434-010-1328-1> (2011).
15. Brown, D. C., Agnello, K. & Iadarola, M. J. Intrathecal resiniferatoxin in a dog model: efficacy in bone cancer pain. *Pain* **156**, 1018–1024. <https://doi.org/10.1097/j.pain.000000000000115> (2015).
16. Joseph, J. *et al.* Phosphorylation of TRPV1 S801 contributes to modality-specific hyperalgesia in mice. *J. Neurosci.* **39**, 9954–9966. <https://doi.org/10.1523/JNEUROSCI.1064-19.2019> (2019).
17. Tu, N. H. *et al.* Legumain induces oral cancer pain by biased agonism of protease-activated receptor-2. *J. Neurosci.* **41**, 193–210. <https://doi.org/10.1523/JNEUROSCI.1211-20.2020> (2021).
18. Amadesi, S. *et al.* Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cε- and A-dependent mechanisms in rats and mice. *J. Physiol.* **575**, 555–571. <https://doi.org/10.1113/jphysiol.2006.111534> (2006).
19. Ruparel, S., Bende, M., Wallace, A. & Green, D. Released lipids regulate transient receptor potential channel (TRP)-dependent oral cancer pain. *Mol. Pain* **11**, 30. <https://doi.org/10.1186/s12990-015-0016-3> (2015).
20. Niyama, Y., Kawamata, T., Yamamoto, J., Omote, K. & Namiki, A. Bone cancer increases transient receptor potential vanilloid subfamily 1 expression within distinct subpopulations of dorsal root ganglion neurons. *Neuroscience* **148**, 560–572. <https://doi.org/10.1016/j.neuroscience.2007.05.049> (2007).
21. Scheff, N. N. *et al.* Granulocyte-colony stimulating factor-induced neutrophil recruitment provides opioid-mediated endogenous anti-nociception in female mice with oral squamous cell carcinoma. *Front. Mol. Neurosci.* **12**, 217. <https://doi.org/10.3389/fnmol.2019.00217> (2019).
22. Scheff, N. N. *et al.* Neutrophil-mediated endogenous analgesia contributes to sex differences in oral cancer pain. *Front. Integr. Neurosci.* **12**, 52. <https://doi.org/10.3389/fnint.2018.00052> (2018).
23. Scheff, N. N. *et al.* Tumor necrosis factor alpha secreted from oral squamous cell carcinoma contributes to cancer pain and associated inflammation. *Pain* **158**, 2396–2409. <https://doi.org/10.1097/j.pain.0000000000001044> (2017).
24. Gatti, R. *et al.* Protease-activated receptor-2 activation exaggerates TRPV1-mediated cough in guinea pigs. *J. Appl. Physiol.* **1985**(101), 506–511. <https://doi.org/10.1152/japplphysiol.01558.2005> (2006).
25. Ye, Y. *et al.* Adenosine triphosphate drives head and neck cancer pain through P2X2/3 heterotrimers. *Acta Neuropathol. Commun.* **2**, 62. <https://doi.org/10.1186/2051-5960-2-62> (2014).
26. Khasabova, I. A. *et al.* Chemical interactions between fibrosarcoma cancer cells and sensory neurons contribute to cancer pain. *J. Neurosci.* **27**, 10289–10298. <https://doi.org/10.1523/JNEUROSCI.2851-07.2007> (2007).
27. Saloman, J. L., Chung, M. K. & Ro, J. Y. P2X(3) and TRPV1 functionally interact and mediate sensitization of trigeminal sensory neurons. *Neuroscience* **232**, 226–238. <https://doi.org/10.1016/j.neuroscience.2012.11.015> (2013).
28. Gu, X. F., Lee, J. H., Yoo, S. B., Moon, Y. W. & Jahng, J. W. Intra-oral pre-treatment with capsaicin increases consumption of sweet solutions in rats. *Nutr. Neurosci.* **12**, 149–154. <https://doi.org/10.1179/147683009X423319> (2009).
29. Lopshire, J. C. & Nicol, G. D. The cAMP transduction cascade mediates the prostaglandin E2 enhancement of the capsaicin-elicited current in rat sensory neurons: whole-cell and single-channel studies. *J. Neurosci.* **18**, 6081–6092 (1998).
30. Premkumar, L. S. & Ahern, G. P. Induction of vanilloid receptor channel activity by protein kinase C. *Nature* **408**, 985–990. <https://doi.org/10.1038/35050121> (2000).
31. Kawabata, A., Kinoshita, M., Kuroda, R. & Kakehi, K. Capsazepine partially inhibits neurally mediated gastric mucus secretion following activation of protease-activated receptor 2. *Clin. Exp. Pharmacol. Physiol.* **29**, 360–361. <https://doi.org/10.1046/j.1440-1681.2002.03641.x> (2002).
32. Rathee, P. K. *et al.* PKA/AKAP/VR-1 module: A common link of Gs-mediated signaling to thermal hyperalgesia. *J. Neurosci.* **22**, 4740–4745 (2002).
33. Gu, Q. & Lee, L. Y. Hypersensitivity of pulmonary chemosensitive neurons induced by activation of protease-activated receptor-2 in rats. *J. Physiol.* **574**, 867–876. <https://doi.org/10.1113/jphysiol.2006.110312> (2006).
34. Constantin, C. E. *et al.* Endogenous tumor necrosis factor alpha (TNFα) requires TNF receptor type 2 to generate heat hyperalgesia in a mouse cancer model. *J. Neurosci.* **28**, 5072–5081. <https://doi.org/10.1523/JNEUROSCI.4476-07.2008> (2008).
35. Rozas, P. *et al.* Targeted overexpression of tumor necrosis factor-α increases cyclin-dependent kinase 5 activity and TRPV1-dependent Ca²⁺ influx in trigeminal neurons. *Pain* **157**, 1346–1362. <https://doi.org/10.1097/j.pain.0000000000000527> (2016).

36. Salvo, E. *et al.* TNF α promotes oral cancer growth, pain, and Schwann cell activation. *Sci. Rep.* **11**, 1840. <https://doi.org/10.1038/s41598-021-81500-4> (2021).
37. Ye, Y. *et al.* Nerve growth factor links oral cancer progression, pain, and cachexia. *Mol. Cancer Ther.* **10**, 1667–1676. <https://doi.org/10.1158/1535-7163.MCT-11-0123> (2011).
38. Ye, Y., Dang, D., Viet, C. T., Dolan, J. C. & Schmidt, B. L. Analgesia targeting IB4-positive neurons in cancer-induced mechanical hypersensitivity. *J. Pain* **13**, 524–531. <https://doi.org/10.1016/j.jpain.2012.01.006> (2012).
39. Ji, R. R., Samad, T. A., Jin, S. X., Schmoll, R. & Woolf, C. J. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* **36**, 57–68. [https://doi.org/10.1016/s0896-6273\(02\)00908-x](https://doi.org/10.1016/s0896-6273(02)00908-x) (2002).
40. Wu, L. *et al.* PAR-2 activation enhances weak acid-induced ATP release through TRPV1 and ASIC sensitization in human esophageal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **309**, G695–702. <https://doi.org/10.1152/ajpgi.00162.2015> (2015).
41. Elitt, C. M., Malin, S. A., Koerber, H. R., Davis, B. M. & Albers, K. M. Overexpression of artemin in the tongue increases expression of TRPV1 and TRPA1 in trigeminal afferents and causes oral sensitivity to capsaicin and mustard oil. *Brain Res.* **1230**, 80–90. <https://doi.org/10.1016/j.brainres.2008.06.119> (2008).
42. Wu, P., Arris, D., Grayson, M., Hung, C. N. & Ruparel, S. Characterization of sensory neuronal subtypes innervating mouse tongue. *PLoS ONE* **13**, e0207069. <https://doi.org/10.1371/journal.pone.0207069> (2018).
43. Shiers, S., Klein, R. M. & Price, T. J. Quantitative differences in neuronal subpopulations between mouse and human dorsal root ganglia demonstrated with RNAscope in situ hybridization. *Pain* **161**, 2410–2424. <https://doi.org/10.1097/j.pain.0000000000001973> (2020).
44. Ye, Y. *et al.* Anti-cancer and analgesic effects of resolvin D2 in oral squamous cell carcinoma. *Neuropharmacology* **139**, 182–193. <https://doi.org/10.1016/j.neuropharm.2018.07.016> (2018).
45. Naik, K. *et al.* The histopathology of oral cancer pain in a mouse model and a human cohort. *J. Dent. Res.* **100**, 194–200. <https://doi.org/10.1177/0022034520961020> (2021).
46. Sagheer, S. H. *et al.* 4NQO induced carcinogenesis: A mouse model for oral squamous cell carcinoma. *Methods Cell. Biol.* **163**, 93–111. <https://doi.org/10.1016/bs.mcb.2021.01.001> (2021).
47. Simons, C. T., Dessirier, J. M., Jinks, S. L. & Carstens, E. An animal model to assess aversion to intra-oral capsaicin: increased threshold in mice lacking substance p. *Chem. Senses* **26**, 491–497. <https://doi.org/10.1093/chemse/26.5.491> (2001).
48. Johansen, J. P., Fields, H. L. & Manning, B. H. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 8077–8082. <https://doi.org/10.1073/pnas.141218998> (2001).
49. Johansen, J. P. & Fields, H. L. Glutamatergic activation of anterior cingulate cortex produces an aversive teaching signal. *Nat. Neurosci.* **7**, 398–403. <https://doi.org/10.1038/nn1207> (2004).
50. King, T. *et al.* Contribution of afferent pathways to nerve injury-induced spontaneous pain and evoked hypersensitivity. *Pain* **152**, 1997–2005. <https://doi.org/10.1016/j.pain.2011.04.020> (2011).
51. Malin, S. A., Davis, B. M. & Molliver, D. C. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat. Protoc.* **2**, 152–160. <https://doi.org/10.1038/nprot.2006.461> (2007).
52. Scheff, N. N., Lu, S. G. & Gold, M. S. Contribution of endoplasmic reticulum Ca²⁺ regulatory mechanisms to the inflammation-induced increase in the evoked Ca²⁺ transient in rat cutaneous dorsal root ganglion neurons. *Cell Calcium* **54**, 46–56. <https://doi.org/10.1016/j.ceca.2013.04.002> (2013).
53. Appel, C. K., Scheff, N. N., Viet, C. T., Schmidt, B. L. & Heegaard, A. M. Decitabine attenuates nociceptive behavior in a murine model of bone cancer pain. *Pain* **160**, 619–631. <https://doi.org/10.1097/j.pain.0000000000001442> (2019).

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Author contributions

All authors listed contributed substantially to the work. N.N.S.; designed the research, conducted experiments, performed data analyses, and wrote the manuscript. I.M.W., S.N., and H.W. conducted behavioral and molecular experiments and provided technical support. C.Z.L. reviewed and interpreted histopathology. E.C. and N.H.T. assisted in research design, conducted experiments, and provided technical support. N.W.B. assisted in research design and provided technical assistance. M.N.J. performed data analysis and edited the manuscript. J.C.D. edited the manuscript. B.L.S. assisted in research design and wrote and edited the manuscript.

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Competing interests

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Additional information

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Correspondence and requests for materials should be addressed to B.L.S.

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OPEN

Schwann cell endosome CGRP signals elicit periorbital mechanical allodynia in mice

Francesco De Logu^{1,10}, Romina Nassini^{1,2,10}, Alan Hegron^{3,10}, Lorenzo Landini¹, Dane D. Jensen^{3,4}, Rocco Latorre³, Julia Ding⁵, Matilde Marini¹, Daniel Souza Monteiro de Araujo¹, Paulina Ramírez-García⁶, Michael Whittaker⁶, Jeffri Retamal⁶, Mustafa Titiz¹, Alessandro Innocenti⁷, Thomas P. Davis^{6,8}, Nicholas Veldhuis⁶, Brian L. Schmidt^{3,4,9}, Nigel W. Bunnett^{3,9}✉ & Pierangelo Geppetti^{1,2}✉

Efficacy of monoclonal antibodies against calcitonin gene-related peptide (CGRP) or its receptor (calcitonin receptor-like receptor/receptor activity modifying protein-1, CLR/RAMP1) implicates peripherally-released CGRP in migraine pain. However, the site and mechanism of CGRP-evoked peripheral pain remain unclear. By cell-selective RAMP1 gene deletion, we reveal that CGRP released from mouse cutaneous trigeminal fibers targets CLR/RAMP1 on surrounding Schwann cells to evoke periorbital mechanical allodynia. CLR/RAMP1 activation in human and mouse Schwann cells generates long-lasting signals from endosomes that evoke cAMP-dependent formation of NO. NO, by gating Schwann cell transient receptor potential ankyrin 1 (TRPA1), releases ROS, which in a feed-forward manner sustain allodynia via nociceptor TRPA1. When encapsulated into nanoparticles that release cargo in acidified endosomes, a CLR/RAMP1 antagonist provides superior inhibition of CGRP signaling and allodynia in mice. Our data suggest that the CGRP-mediated neuronal/Schwann cell pathway mediates allodynia associated with neurogenic inflammation, contributing to the algescic action of CGRP in mice.

¹Department of Health Sciences, Clinical Pharmacology and Oncology Section, University of Florence, Florence 50139, Italy. ²Headache Center, Careggi University Hospital, Florence 50139, Italy. ³Department of Molecular Pathobiology, College of Dentistry, New York University, New York, NY 10010, USA. ⁴Bluestone Center for Clinical Research, New York University College of Dentistry, New York, NY 10010, USA. ⁵Department of Anesthesiology, Columbia University, New York, NY 10010, USA. ⁶Drug Discovery Biology Theme and Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia. ⁷Plastic and Reconstructive Microsurgery - Careggi University Hospital, Florence 50139, Italy. ⁸Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, QLD 4072, Australia. ⁹Department of Neuroscience and Physiology and Neuroscience Institute, School of Medicine, New York University, New York, NY 10010, USA. ¹⁰These authors contributed equally: Francesco De Logu, Romina Nassini, Alan Hegron. ✉email: nwb2@nyu.edu; pierangelo.geppetti@unifi.it

For almost a century it has been known that cutaneous tissue injury elicits a local vascular response, referred to as neurogenic inflammation, that is associated to a wider area of increased sensitivity to mechanical stimuli¹. A subset of C-fiber primary afferents, which mediate neurogenic inflammation, is the main source of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP)^{2,3}. In rodents, noxious stimuli such as capsaicin, a pungent agonist of the transient receptor potential vanilloid 1 (TRPV1) channel⁴, evoke the peripheral release of CGRP which induces arteriolar vasodilatation² and of SP which elicits plasma protein extravasation⁵, and produce sensory responses, which encompasses acute nociception and prolonged mechanical allodynia⁶. Capsaicin administration to the human skin elicits a similar pattern of responses, consisting of local cutaneous vasodilatation and focal and transient burning pain (min) associated with widespread, sustained mechanical hypersensitivity (hrs)⁷. While CGRP has been identified as the mediator of neurogenic vasodilatation in rodents² and humans⁸, the cellular and molecular mechanisms underlying mechanical allodynia associated with neurogenic inflammation are unknown.

Mechanistic studies in animal models and humans have highlighted the role of CGRP in migraine pain⁹. Thus, small molecule antagonists of the CGRP receptor and monoclonal antibodies against CGRP or its receptor can relieve migraine pain¹⁰. The poor blood-brain barrier penetration of some small-molecule antagonists^{11,12} and of monoclonal antibodies^{13,14} suggests a peripheral contribution to CGRP-mediated migraine pain. However, little is known about the proalgesic actions of CGRP in the periphery. In mice, intraplantar injection of CGRP evokes mechanical allodynia¹⁵ and systemic CGRP causes facial grimace¹⁶. Periorbital CGRP injection, while failing to evoke spontaneous nociceptive behavior, produces sustained (~4 h) periorbital mechanical allodynia (PMA)¹⁷. CGRP released from trigeminal peripheral terminals mediates PMA in mice¹⁸ evoked by systemic (intraperitoneal) administration of the pro-headache agent glyceryl trinitrate (GTN)¹⁹. Facial cutaneous allodynia is one component of the migraine attack^{20,21}. Although the process that initiates migraine pain may originate in the central nervous system (CNS)^{22,23}, the cell type and signaling pathway by which CGRP acts in the periphery to cause pain are unknown.

The CGRP receptor is a heterodimer of calcitonin receptor-like receptor (CLR), a G protein-coupled receptor (GPCR), and receptor activity-modifying protein 1 (RAMP1), a single transmembrane domain CLR chaperone²⁴. These two components coexist in cells that mediate the actions of CGRP, for example, vascular myocytes². Satellite glial cells and Schwann cells express CLR/RAMP1 and are closely associated with peptidergic sensory neurons²⁵. While the extracellular space between the soma of trigeminal neurons and satellite glial cells is not a recognized locus for neurotransmission, the varicosities of C-fibers and the ensheathing Schwann cells are sites where neuropeptides, including CGRP²⁶, are normally released. Schwann cells from rat sciatic nerve respond to CGRP by increasing intracellular cAMP levels²⁷ and CLR/RAMP1 are expressed by Schwann cells that wrap CGRP + ve terminals of rat nociceptors^{25,28,29}. Schwann cells mediate mechanical allodynia in mouse models of neuropathic and cancer pain^{30,31}. Cutaneous Schwann cells can also directly activate sensory nerves to promote mechanical nociception³². Although GPCRs are usually considered to signal principally from the plasma membrane, GPCR kinases and β -arrestins (β ARRs) rapidly terminate this signaling. Persistent endosomal signaling of GPCRs, including CLR/RAMP1, underlies sustained neuronal activation and nociception in the CNS^{33–35}.

Herein, we hypothesized that mechanical allodynia associated with neurogenic inflammation is mediated by CGRP which

targets CLR/RAMP1 in Schwann cells ensheathing peripheral endings of nociceptors. By selective RAMP1 gene deletion in Schwann cells, we reveal that CGRP released from trigeminal terminals causes PMA by paracrine signaling to the surrounding Schwann cells. We also hypothesized that persistent CGRP/CLR/RAMP1 signaling from endosomes in Schwann cells underlies sustained PMA. By using inhibitors of clathrin- and dynamin-mediated endocytosis and stimulus-responsive nanoparticles designed to release CLR/RAMP1 antagonists in acidified endosomes, we found that CLR/RAMP1 endosomal signaling results in a cAMP-dependent release of nitric oxide (NO), which activates transient receptor potential ankyrin 1 (TRPA1), a proalgesic channel and sensor of oxidative stress³⁶.

Results

CGRP evokes PMA by activating Schwann cell CLR/RAMP1.

We detected CLR and RAMP1 mRNA and immunoreactivity in primary cultures of human Schwann cells (HSCs) or mouse Schwann cells (MSCs) taken from the sciatic or trigeminal nerve (Fig. 1a, Supplementary Fig. 1a). The S100 + ve mouse Schwann cell line (IMS32) recapitulated features of primary MSCs, including expression of CLR and RAMP1 mRNA and immunoreactivity (Supplementary Fig. 1a, b) and TRPA1-dependent Ca^{2+} response to allyl isothiocyanate (Supplementary Fig. 1c). Immunoreactive CLR and RAMP1 were also detected in S100 + ve Schwann cells in nerve bundles in biopsies of human abdominal and mouse periorbital skin (Fig. 1b).

In C57BL/6J male mice, periorbital CGRP elicited PMA (Supplementary Fig. 1d). Periorbital CGRP also evoked PMA in female mice (Supplementary Fig. 1d). Periorbital CGRP did not elicit allodynia in the hind paw (Supplementary Fig. 1e). Unless otherwise specified, all drugs were administered by subcutaneous periorbital injection (Supplementary Table 1). To assess the role of Schwann cell CLR/RAMP1 in CGRP-evoked PMA, Schwann cell-specific Cre mice (*Plp-Cre^{ERT}*) were crossed with RAMP1 floxed mice (*Ramp1^{fl/fl}*) to generate *Plp-Cre^{ERT+};Ramp1^{fl/fl}* and *Plp-Cre^{ERT+};Ramp1^{fl/fl}* (Control) mice. For selective deletion of RAMP1 in Schwann cells of the periorbital region, 4-hydroxytamoxifen (4-OHT) was administered daily for 3 days to *Plp-Cre^{ERT+};Ramp1^{fl/fl}* and Control mice. CGRP elicited PMA in both male and female Control mice that was similarly attenuated in *Plp-Cre^{ERT+};Ramp1^{fl/fl}* mice (Fig. 1c). In *Plp-Cre^{ERT+};Ramp1^{fl/fl}* mice 4-OHT treatment down-regulated RAMP1 immunoreactivity in S100 + ve cells surrounding trigeminal but not sciatic nerve fibers (Fig. 1d) and did not prevent paw mechanical allodynia evoked by intraplantar CGRP, which was prevented by intraplantar 4-OHT (Supplementary Fig. 1f).

Intravenous CGRP provokes delayed headache attacks in patients³⁷. Intraperitoneal CGRP caused PMA and paw allodynia in male and female C57BL/6J mice without gender difference (Fig. 1e, Supplementary Fig. 1g). In *Plp-Cre^{ERT+};Ramp1^{fl/fl}* mice treated with periorbital 4-OHT PMA, but not paw allodynia, was similarly reduced in males and females in response to intraperitoneal CGRP (Fig. 1f, g and Supplementary Fig. 1h, i). Systemic (intraperitoneal) 4-OHT reduced both PMA and paw allodynia by intraperitoneal CGRP (Supplementary Fig. 1j, k). These results reveal an essential role for CLR/RAMP1 of Schwann cells surrounding periorbital trigeminal endings in PMA elicited by local and systemic CGRP.

Stimulation of peptidergic trigeminal neurons evokes PMA by activating Schwann cell CLR/RAMP1.

To explore the ability of endogenous CGRP to elicit PMA, we administered capsaicin, which activates TRPV1⁴ thus releasing CGRP and SP from peptidergic nociceptors³. Periorbital capsaicin elicited acute

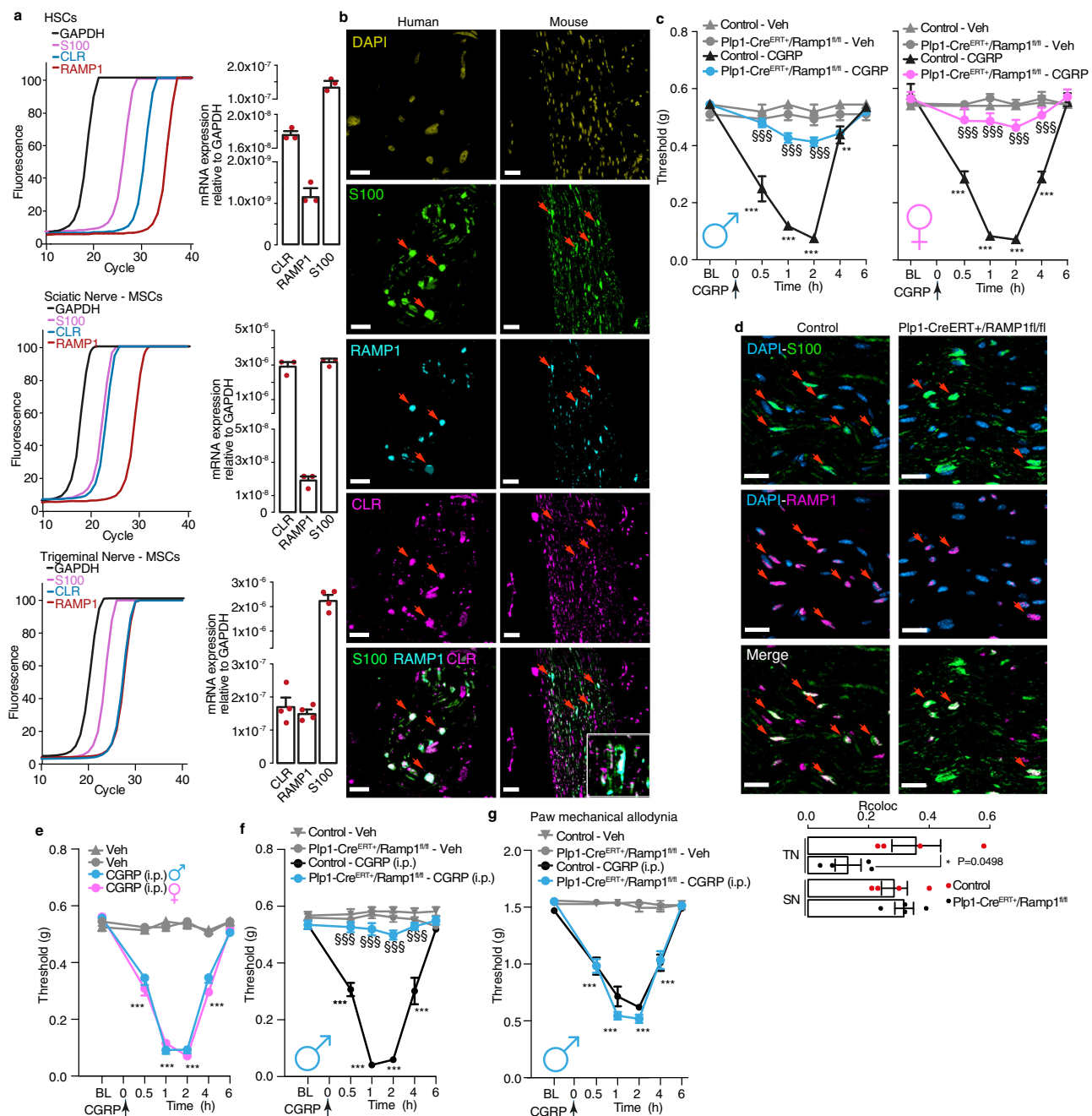
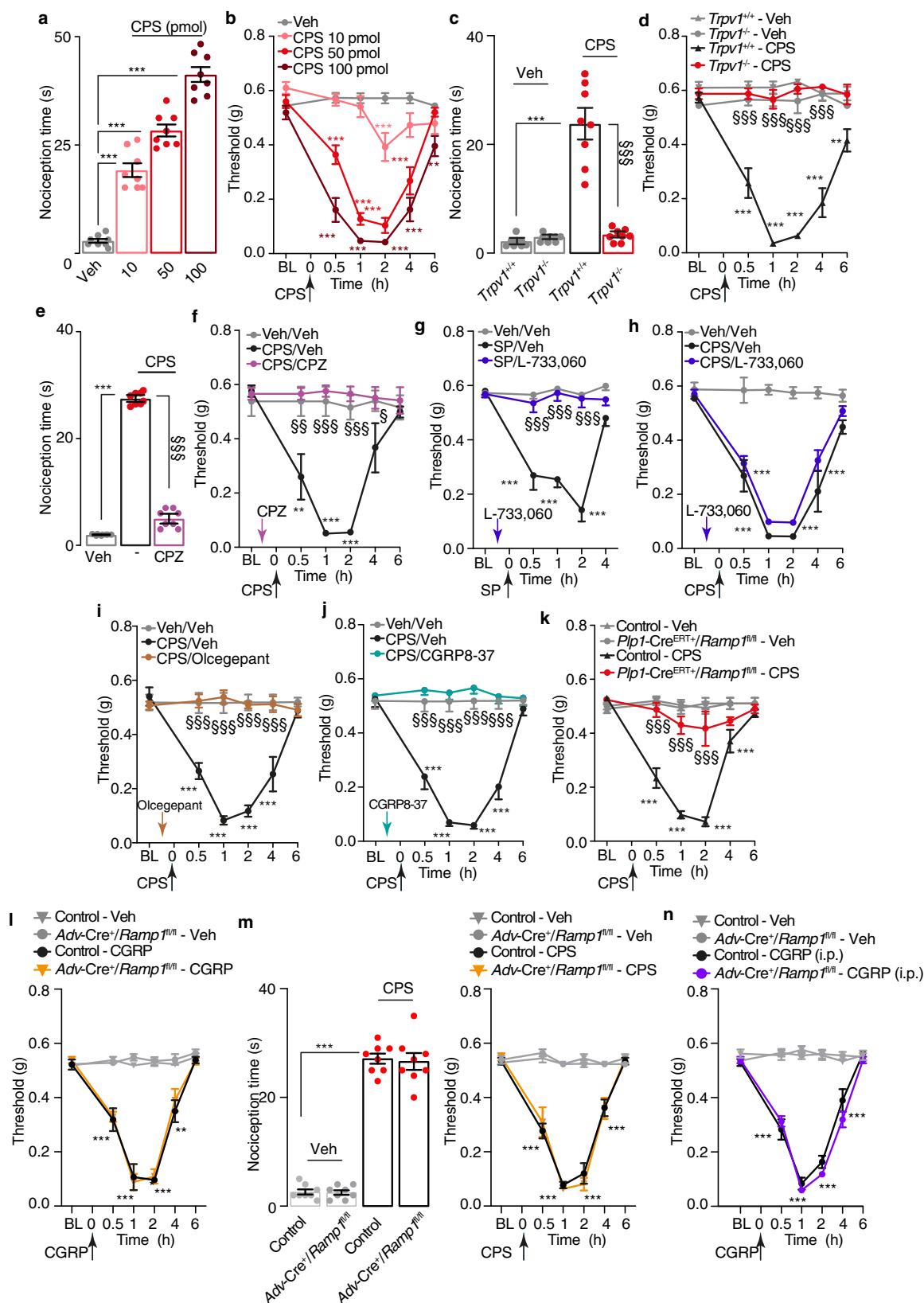


Fig. 1 Schwann cell RAMP1 mediates PMA evoked by CGRP. **a** Representative real-time PCR plot and cumulative data for GAPDH, S100, CLR and RAMP1 mRNA in HSCs ($n = 3$ independent experiments) and MSCs from trigeminal or sciatic nerve (MSCs from sciatic nerve $n = 3$ independent experiments; MSCs from trigeminal nerve, $n = 4$ independent experiments). **b** Representative images of DAPI and immunoreactive S100, RAMP1 and CLR in human and mouse cutaneous nerve bundles (scale, 10 μ m human, 50 μ m mouse) ($n = 3$ subjects). **c** PMA induced by CGRP (1.5 nmol) or vehicle in male and female *Plp1-Cre^{ERT}/Ramp1^{fl/fl}* and Control mice treated with periocular 4-OHT ($n = 8$ mice per group). **d** Representative images and colocalization value (Rcoloc) of S100 and RAMP1 in periorbital nerve and sciatic nerve trunks from *Plp1-Cre^{ERT}/Ramp1^{fl/fl}* and Control mice (scale, 20 μ m) ($n = 4$ replicates). TN (trigeminal nerve), SN (sciatic nerve). **e** PMA induced by intraperitoneal (i.p.) CGRP (0.1 mg/kg) or vehicle in male and female C57BL/6 J mice ($n = 8$ mice per group). **f** PMA and **(g)** paw mechanical allodynia induced by intraperitoneal (i.p.) CGRP (0.1 mg/kg) or vehicle in male *Plp1-Cre^{ERT}/Ramp1^{fl/fl}* and Control mice ($n = 8$ mice per group) treated with periocular 4-OHT. Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Veh, Control-Veh, and TN-Control, §§§ $P < 0.001$ vs. Control-CGRP. 2-way (**c**, **e**, **f**, **g**) or 1-way (**d**) ANOVA, Bonferroni correction. Source data are provided as a Source Data file.

(~10 min) nociceptive behavior (Fig. 2a) and, like CGRP, caused prolonged (~4 h) PMA (Fig. 2b). Allodynia was detected in the periorbital area but not in the hind paw (Supplementary Fig. 2a), indicating a local action. TRPV1 deletion (*Trpv1*^{-/-} mice) (Fig. 2c, d) or pretreatment with the TRPV1 antagonist capsazepine (Fig. 2e, f), prevented acute nociception and PMA. An antagonist (L-733,060) of the SP neurokinin 1 (NK₁) receptor,

which prevented SP-evoked PMA (Fig. 2g), failed to diminish capsaicin-evoked acute nociception and PMA (Fig. 2h, Supplementary Fig. 2b). The histamine H₁ receptor antagonist, astemizole, inhibited SP-evoked PMA (Supplementary Fig. 2c) but did not affect CGRP- or capsaicin-evoked PMA (Supplementary Fig. 2d, e). Pretreatment with the CLR/RAMP1 antagonists, CGRP8-37 or olcegepant, prevented PMA (Fig. 2i, j) but not acute



nociception evoked by capsaicin (Supplementary Fig. 2b). Importantly, 4-OHT markedly inhibited capsaicin-evoked PMA (Fig. 2k) but not acute nociception (Supplementary Fig. 2f) in *Plp1-Cre^{ERT+};Ramp1^{fl/fl}* mice observed in Control mice. CGRP-evoked PMA or capsaicin-evoked acute nociception and PMA were similar in mice with selective deletion of RAMP1 in

nociceptors (*Advillin-Cre⁺;Ramp1^{fl/fl}*, *Adv-Cre⁺;Ramp1^{fl/fl}*) and Control mice (Fig. 2l, m). Both PMA and paw allodynia were similar in *Adv-Cre⁺;Ramp1^{fl/fl}* and Control mice after intraperitoneal CGRP (Fig. 2n and Supplementary Fig. 3a). Thus, CGRP but not SP mediates allodynia resulting from excitation of TRPV1 + ve peptidergic nociceptors, and PMA evoked by both

Fig. 2 Capsaicin induces PMA via CGRP and CLR/RAMP1 in Schwann cells. **a** Acute nociception and **(b)** PMA after periorbital injection of capsaicin (CPS) or vehicle in C57BL/6 J mice. **c** Acute nociception and **(d)** PMA after CPS (50 pmol) or vehicle in *Trpv1*^{+/+} and *Trpv1*^{-/-} mice. **e** Acute nociception and **(f)** PMA after CPS (50 pmol) or vehicle in C57BL/6 J mice pretreated with capsazepine (CPZ, 100 pmol) or vehicle. **g, h** PMA after periorbital SP (3.5 nmol), CPS (50 pmol) or vehicle in C57BL/6 J mice pretreated (0.5 h) with L-733,060 (20 nmol) or vehicle. **i, j** PMA after CPS (50 pmol) or vehicle in C57BL/6 J mice pretreated (0.5 h) with olcegepant (1 nmol) or CGRP8-37 (10 nmol) or vehicle. **k** PMA after CPS (50 pmol) or vehicle in *Plp1-Cre*^{ERT+/+}/*Ramp1*^{fl/fl} or Control mice treated with periorbital 4-OHT or vehicle. **l** PMA after periorbital CGRP (1.5 nmol) or vehicle and **(m)** acute nociceptive response and PMA after periorbital CPS (50 pmol) or vehicle in *Adv-Cre*^{+/+}/*Ramp1*^{fl/fl} or Control mice. **n** PMA induced by intraperitoneal (i.p.) CGRP (0.1 mg/kg) or vehicle in *Adv-Cre*^{+/+}/*Ramp1*^{fl/fl} or Control mice. Mean±SEM, *n* = 8 mice per group. ****P* < 0.01, *****P* < 0.001 vs. Veh/Veh, *Trpv1*^{+/+}-Veh and Control-Veh; §*P* < 0.05, §§*P* < 0.01, §§§*P* < 0.001 vs. *Trpv1*^{+/+}-CPS, CPS/Veh, SP/Veh, Control-CPS, Control-CGRP. 1-way (**a, c, e** and **m** left panel) or 2-way (**b, d, f–l, m** right panel and **n**) ANOVA, Bonferroni correction. Source data are provided as a Source Data file.

endogenous and exogenous CGRP depends on CLR/RAMP1 of Schwann cells surrounding peripheral terminals of nociceptors, while the receptor of sensory nerve fibers is not implicated.

Schwann cell CLR/RAMP1 mediates the CGRP-dependent PMA evoked by GTN. Systemic GTN administration provokes sustained headaches in humans¹⁹. In mice intraperitoneal GTN elicits PMA¹⁸ that is, in part mediated by CGRP release from periorbital trigeminal terminals¹⁸. Here, systemic GTN elicited PMA (Fig. 3a) and paw allodynia (Fig. 3b) that were similar in Control and *Adv-Cre*^{+/+}/*Ramp1*^{fl/fl} mice. Olcegepant transiently and partially inhibited PMA (Fig. 3a), while did not affect GTN-evoked paw allodynia (Fig. 3b) in both mouse strains. RAMP1 deletion from trigeminal Schwann cells (*Plp1-Cre*^{ERT+/+}/*Ramp1*^{fl/fl} mice) partially inhibited PMA (Fig. 3c), but not paw allodynia (Fig. 3d). Importantly, treatment with olcegepant reduced GTN-evoked PMA in Control mice, but failed to further inhibit the response in *Plp1-Cre*^{ERT+/+}/*Ramp1*^{fl/fl} mice (Fig. 3c). Paw allodynia was unchanged by olcegepant (Fig. 3d). Together, the findings suggest that Schwann cell CLR/RAMP1 mediates the CGRP-dependent component of PMA in a mouse headache model.

Clathrin- and dynamin-mediated endocytosis of CLR/RAMP1 in Schwann cells mediates PMA. We investigated the CLR/RAMP1 signaling pathway in Schwann cells that mediates CGRP-evoked PMA. The CGRP-stimulated cAMP formation was measured in HSCs using a virally encoded cAMP cADDis reporter. CGRP stimulated a prompt concentration-dependent increase in cAMP formation in HSCs that was sustained for >300 s (Fig. 4a, b; Supplementary Movie 1). The CLR/RAMP1 antagonist olcegepant caused a concentration-dependent inhibition of CGRP-stimulated cAMP formation (Fig. 4c–e).

Endosomal signaling of GPCRs, including CLR/RAMP1, controls nociception^{33–35}. To assess CLR/RAMP1 endocytosis in Schwann cells, we incubated HSCs expressing the early endosome marker Rab5a-GFP with TAMRA-CGRP. In vehicle-treated cells, live-cell imaging revealed uptake of TAMRA-CGRP into Rab5a-GFP + ve early endosomes within 10 min that continued for 30 min (Fig. 4f, Supplementary Movie 2). Inhibitors of clathrin (PitStop2, PS2) or dynamin (Dynogo4a, Dy4) prevented the translocation of TAMRA-CGRP to endosomes causing retention of weak TAMRA-CGRP fluorescence at the cell surface (Fig. 4e, f; Supplementary Movies 3 and 4). Quantification of TAMRA-CGRP fluorescence intensity in Rab5a-GFP + ve endosomes or the proportion of endosomes containing TAMRA-CGRP confirmed that PS2 and Dy4 inhibited endocytosis of TAMRA-CGRP (Fig. 4g, h). Inactive analogs had no effect. Hypertonic sucrose (0.45 M) inhibits clathrin-mediated endocytosis, including agonist-stimulated endocytosis of GPCRs³⁸. Hypertonic sucrose also inhibited the uptake of TAMRA-CGRP in HSCs (Fig. 4g, h, Supplementary Movie 5). Injection of PS2 or

Dy4, but not their inactive analogs, prevented CGRP-evoked PMA, both in male and female mice (Fig. 4i, j and Supplementary Fig. 3b, c). PS2 or Dy4 also reversed capsaicin-evoked PMA (Fig. 4k, l). Thus, CGRP stimulates clathrin- and dynamin-mediated endocytosis of CLR/RAMP1 in Schwann cells, which sustains CGRP-evoked PMA.

CLR activates G_{α_s} , G_{α_q} , and G_{α_i} and recruits β ARR2 to the plasma membrane and endosomes. GPCRs, including CLR/RAMP1, can signal from endosomes by G_{α_s} , G_{α_q} , and β ARR-mediated mechanisms^{33–35}. We used enhanced bystander bioluminescence resonance energy transfer (EbbRET) to study the activation of Ga and recruitment of β ARR to the plasma membrane and early endosomes of HEK293T cells expressing human (h) CLR and RAMP1 (HEK-hCLR/RAMP1). CGRP-dependent activation of G_{α_s} , $G_{\alpha_{sq}}$, and $G_{\alpha_{si}}$ was assessed using an EbbRET assay that detects recruitment of mini (m) Ga coupled to *Renilla* (R)luc8 to the plasma membrane marker CAAX coupled to RGFP or the early endosome marker Rab5a coupled to tandem (td) RGFP. mGa proteins are N-terminally truncated Ga proteins that freely diffuse throughout the cytoplasm and bind to active conformations of GPCRs. Their translocation to GPCRs reflects Ga activation. m $G_{\alpha_{sq}}$ and m $G_{\alpha_{si}}$ were developed by mutating m G_{α_s} residues to equivalent G_{α_q} and G_{α_i} residues. Recruitment of β ARR was assessed by measuring EbbRET between Rluc2- β ARR2 and RGFP-CAAX or tdRGFP-Rab5a. CGRP induced a rapid increase in EbbRET between Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$, and Rluc2- β ARR2 with RGFP-CAAX, which was maximal at ~300 s and declined over 1000 s (Fig. 5a, b). CGRP increased EbbRET between Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$ and Rluc2- β ARR2 with tdRGFP-Rab5a that was fully sustained for 1300 s (Fig. 5c, d). EbbRET was similarly used to study the activation of Ga and recruitment of β ARR2 to the plasma membrane and endosomes of HSCs transfected with hCLR/RAMP1 (CLR/RAMP1 overexpression was required to amplify BRET signals). In HSCs, CGRP increased EbbRET between Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$ and Rluc2- β ARR2 with RGFP-CAAX and tdRGFP-Rab5a (Fig. 5e–h). EbbRET signals were sustained for 1300 s.

To investigate the contribution of endocytosis to the activation of Ga proteins and β ARRs in endosomes, we preincubated cells with hypertonic sucrose. CGRP increased EbbRET between hCLR-Rluc8 and tdRGFP-Rab5a in HEK-hCLR/RAMP1 cells, consistent with CLR endocytosis (Fig. 5i). Hypertonic sucrose inhibited these changes, which indicates an inhibition of endocytosis (Fig. 5i). Hypertonic sucrose caused a delayed yet more sustained activation of Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$, and Rluc2- β ARR2 at the plasma membrane (Supplementary Fig. 4a–f), and an almost complete inhibition of activation of Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$ and Rluc2- β ARR2 in endosomes (Fig. 5j, k). Sucrose similarly delayed CGRP-induced recruitment of Rluc8-m G_{α_s} , Rluc8-m $G_{\alpha_{sq}}$, Rluc8-m $G_{\alpha_{si}}$ and

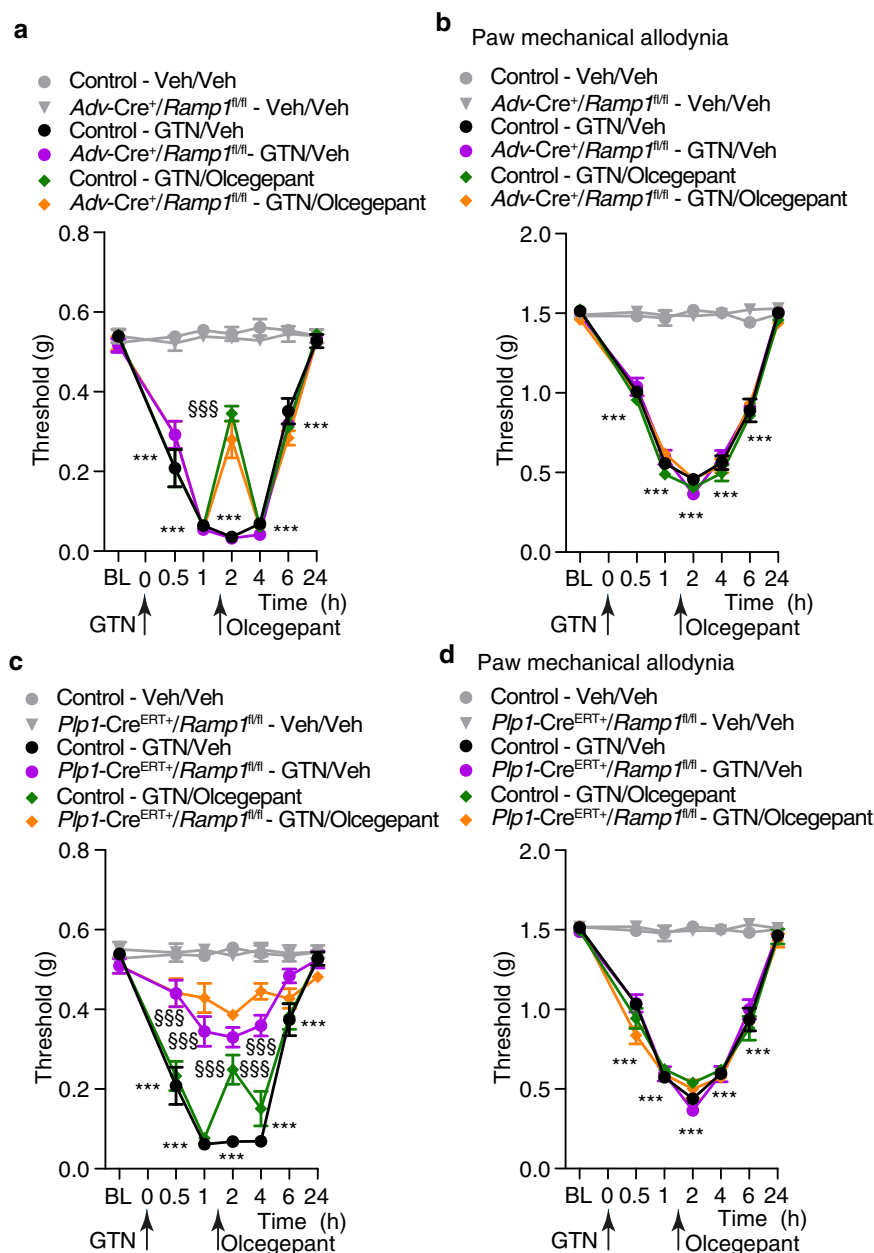


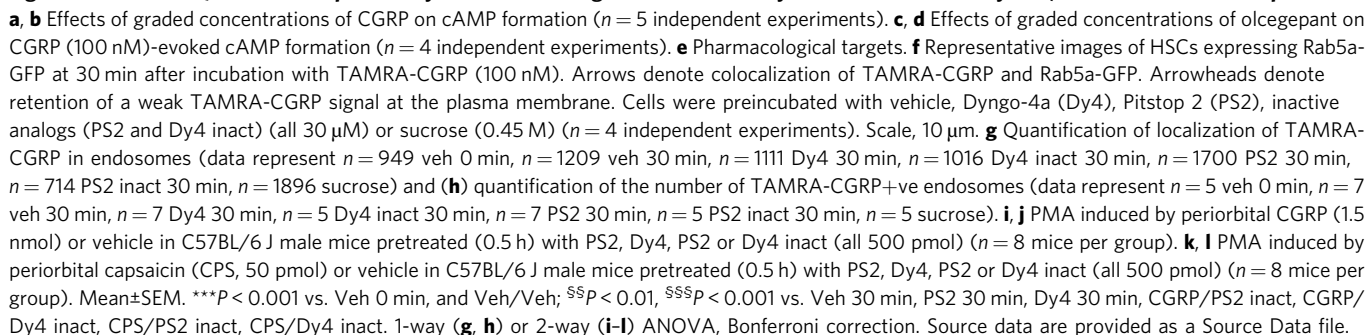
Fig. 3 GTN induces PMA via CGRP released from periorbital trigeminal terminals and, CLR/RAMP1 in Schwann cells. **a** PMA and **(b)** paw mechanical allodynia induced by intraperitoneal (i.p.) GTN (10 mg/kg) or vehicle in *Adv-Cre⁺/Ramp1^{fl/fl}* or Control mice post-treated (1.5 hs after GTN) with olcegepant (1 nmol) or vehicle. **(c)** PMA and **(d)** paw mechanical allodynia induced by GTN (10 mg/kg, i.p.) or vehicle in *Plp1-Cre^{ERT+}/Ramp1^{fl/fl}* or Control (treated with periorbital 4-OHT or vehicle) post-treated (1.5 hs after GTN) with olcegepant (1 nmol) or vehicle. Mean $\pm$ SEM, $n = 8$ mice per group. *** $P < 0.001$ vs. Control-Veh/Veh; §§§ $P < 0.001$ vs. Control-GTN/Veh. 2-way ANOVA, Bonferroni correction. Source data are provided as a Source Data file.

Rluc2- β ARR2 to the plasma membrane (Supplementary Fig. 4g–l) and almost completely inhibited activation of $G_{\alpha_{s,sg,si}}$ and β ARR2 in endosomes of HSCs expressing hCLR/RAMP1 (Fig. 5l, m).

To examine the contribution of endosomal CLR/RAMP1 signaling to CGRP-induced cAMP formation, we preincubated HSCs expressing the cADDis cAMP reporter with sucrose or vehicle. In vehicle-treated cells, CGRP stimulated a rapid (1 min) increase in cAMP formation that was sustained for 30 min (Fig. 5n, o). Sucrose reduced but did not abolish the initial response, yet strongly inhibited the sustained phase of CGRP-stimulated cAMP formation (Fig. 5n, o). Thus, CGRP initially activates G_{α} and β ARR at the plasma membrane, which is followed by sustained activation of G_{α} and β ARR in early endosomes. Endocytosis is necessary for the

recruitment of G_{α} and β ARR to endosomes. G_{α_s} continues to signal in endosomes, leading to sustained cAMP formation.

CLR/RAMP1 activation in Schwann cells releases NO, which initiates but does not sustain PMA. We investigated the mechanisms that sustain PMA following CLR/RAMP1 activation and endocytosis in Schwann cells. Pre- but not post-treatment (60 min after CGRP or capsaicin) with CLR/RAMP1 antagonists, olcegepant or CGRP8-37, attenuated PMA evoked by capsaicin and in accordance with previous studies^{17,18} PMA evoked by CGRP (Supplementary Fig. 5a–f). Similarly, inhibitors of clathrin- and dynamin-mediated endocytosis had no effect when administered



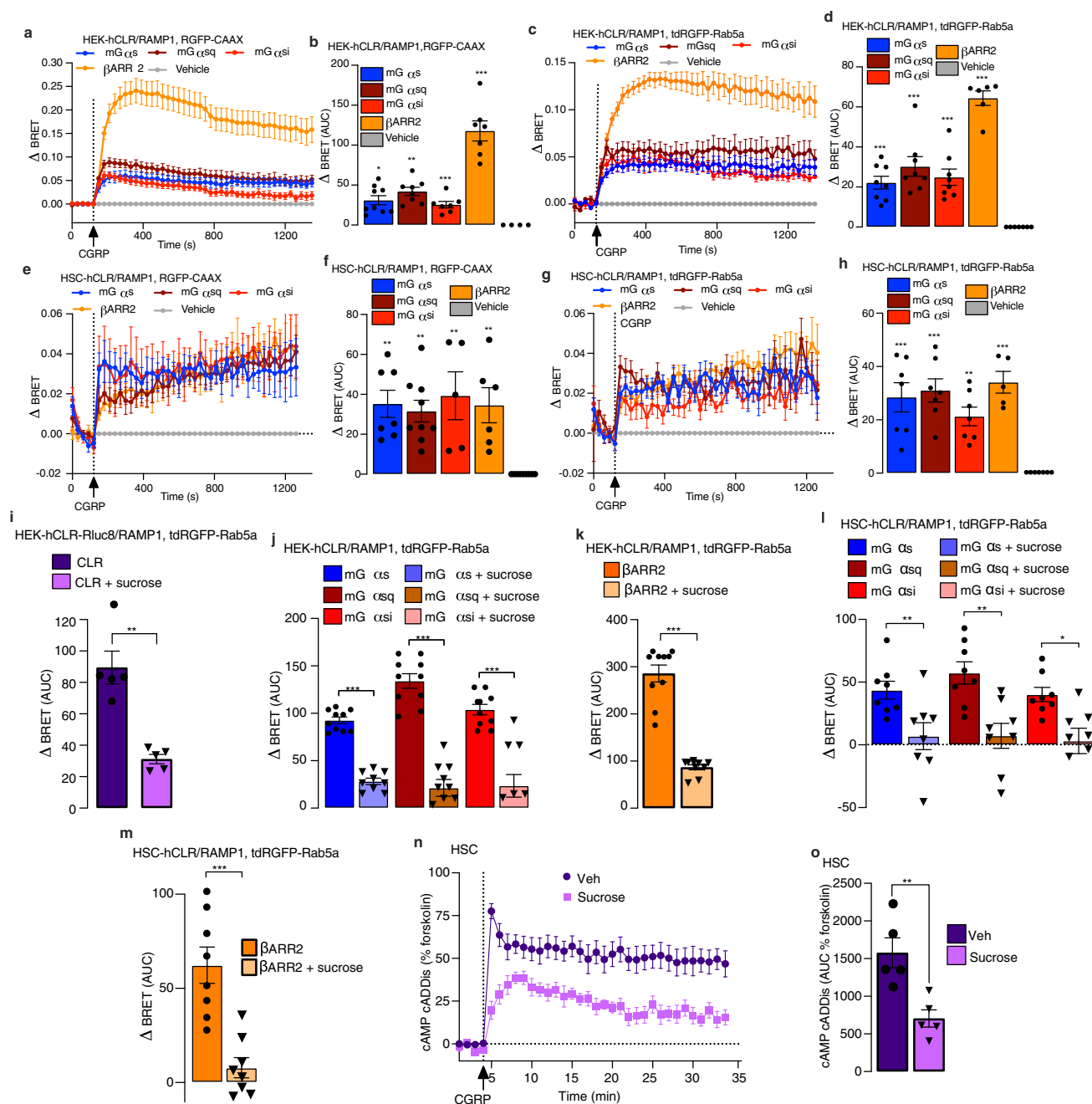


Fig. 5 CGRP leads to $G\alpha$ protein activation and β ARR2 recruitment at the plasma membrane and in early endosomes in HEK-hCLR/RAMP1 cells and HSCs-hCLR/RAMP1. Endosomal signaling generates sustained formation of cAMP in HSCs. (**a–d**) CGRP (100 nM) increased EbBRET between Rluc8-mG α_s , Rluc8-mG α_{sq} , Rluc8-mG α_{si} , and Rluc2- β ARR2 with RGFP-CAAX (**a, b**) and tdRGFP-Rab5a (**c, d**) in HEK-hCLR/RAMP1 cells. **a, c** time course. **b, d** area under curve (AUC) (**a, b** $n = 10$ mG α_s , $n = 8$ mG α_{sq} , $n = 7$ mG α_{si} , $n = 7$ β ARR2, $n = 9$ veh; **c, d** $n = 8$ mG α_s , $n = 8$ mG α_{sq} , $n = 8$ mG α_{si} , $n = 6$ β ARR2, $n = 9$ veh). **e–h** CGRP (100 nM) increased EbBRET between Rluc8-mG α_s , Rluc8-mG α_{sq} , Rluc8-mG α_{si} , and Rluc2- β ARR2 with RGFP-CAAX (**e, f**) and tdRGFP-Rab5a (**g, h**) in HSC-hCLR/RAMP1 cells. **e, g** time course. **f, h** AUC (**e, f** $n = 7$ mG α_s , $n = 9$ mG α_{sq} , $n = 5$ mG α_{si} , $n = 6$ β ARR2, $n = 9$ veh; **g, h** $n = 7$ mG α_s , $n = 7$ mG α_{sq} , $n = 7$ mG α_{si} , $n = 5$ β ARR2, $n = 7$ veh). (**i**) Hypertonic sucrose (0.45 M) inhibited CGRP (100 nM)-stimulated EbBRET between hCLR-Rluc8 and tdRGFP-Rab5a in HEK-hCLR/RAMP1 cells ($n = 5$ independent experiments). (**j, k**) Hypertonic sucrose (0.45 M) inhibited CGRP (100 nM)-stimulated EbBRET between Rluc8-mG α_s , Rluc8-mG α_{sq} , Rluc8-mG α_{si} and Rluc2- β ARR2 with tdRGFP-Rab5a in HEK-hCLR/RAMP1 cells (**j, k** $n = 10$ independent experiments). (**l, m**) Sucrose (0.45 M) inhibited CGRP (100 nM)-stimulated EbBRET between Rluc8-mG α_s , Rluc8-mG α_{sq} , Rluc8-mG α_{si} , and Rluc2- β ARR2 with tdRGFP-Rab5a in HSC-hCLR/RAMP1 cells. ($n = 8$ independent experiments). (**n, o**) Sucrose (0.45 M) inhibited CGRP (100 nM)-stimulated formation of cAMP in HSCs. **n** time course. **o**, AUC ($n = 5$ independent experiments). Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. Veh. 1-way ANOVA, Dunnett's correction (**b, d, f, h**) or parametric unpaired t test (**i–m, o**). Source data are provided as a Source Data file.

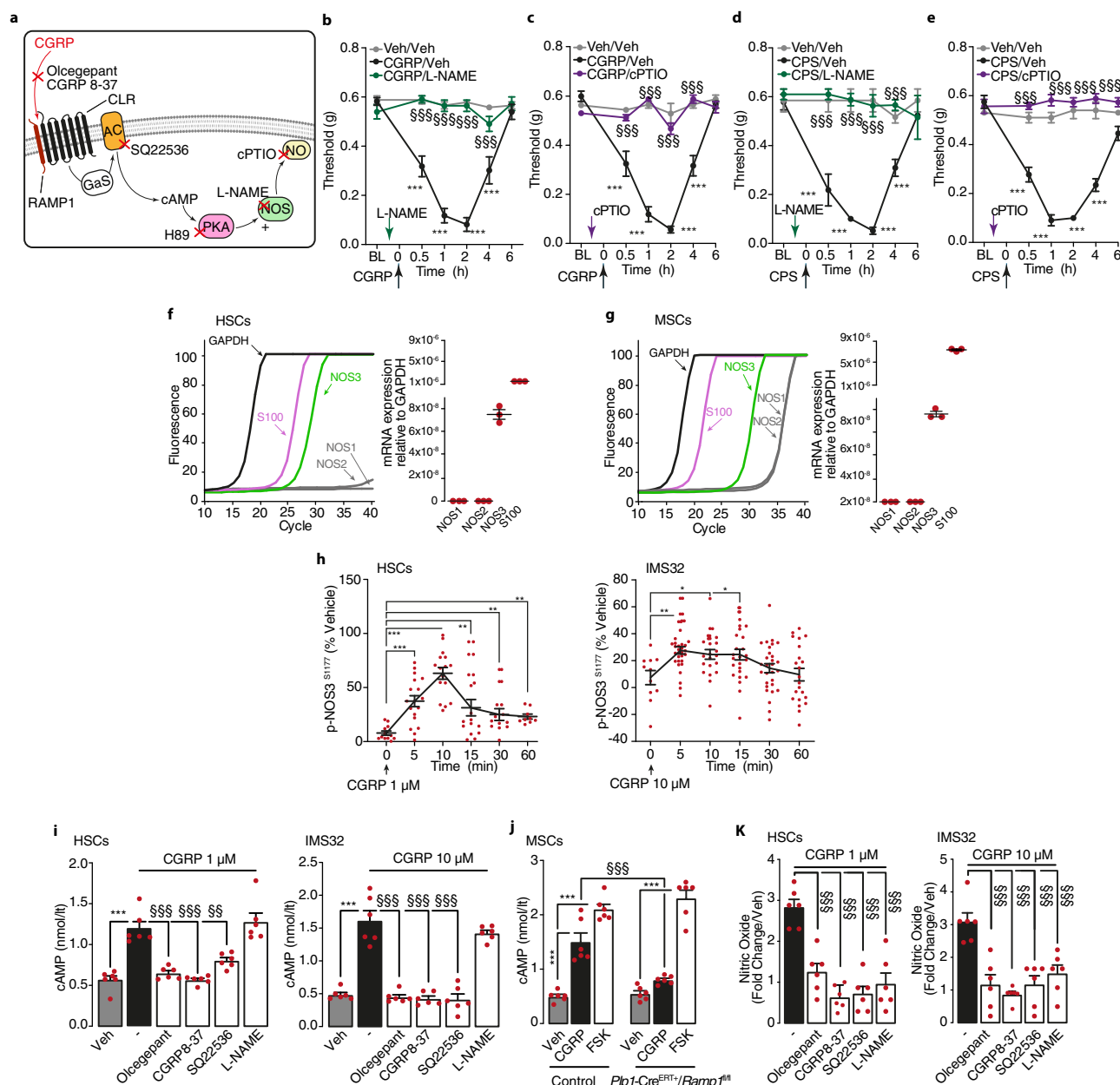


Fig. 6 Endogenous and exogenous CGRP induces PMA via NO production. **a** Pharmacological targets. **b–e** PMA after periorbital injection of CGRP (1.5 nmol), capsaicin (CPS, 50 pmol) or vehicle in C57BL/6 J mice pretreated (0.5 h) with L-NAME (1 μ M) and cPTIO (200 nmol) or vehicle ($n = 8$ mice per group). **f, g** Real-time PCR plot and cumulative data for GAPDH, S100, NOS1, NOS2 and NOS3 in primary HSCs and MSCs ($n = 3$ independent experiments). **h** In-cell p-NOS3^{S1177} ELISA in HSCs and IMS32 cells before (time 0, $n = 11$ independent experiments in both HSCs and IMS32) or after CGRP (time 5, $n = 18$ and $n = 31$, time 10, $n = 16$ and $n = 21$, time 15, $n = 15$ and $n = 25$, time 30, $n = 12$ and $n = 29$, time 60, $n = 9$ and $n = 22$ independent experiments in HSCs and IMS32, respectively). **i** cAMP assay in (i) HSCs, IMS32 and (j) MSCs from *Plp1-Cre^{ERT2}/Ramp1^{fl/fl}* or Control mice treated with intraperitoneal 4-OHT, **k** nitric oxide assay in HSCs and IMS32 cells treated with CGRP or vehicle ($n = 6$ independent experiments). Some cells were treated with olcegepant (1 μ M), CGRP8-37 (1 μ M), SQ22536 (100 μ M), L-NAME (100 μ M) or vehicle ($n = 6$ independent experiments). Mean $\pm$ SEM. (–) represents the combination of different vehicles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Veh/Veh, time 0 (min); \$\$\$ $P < 0.001$ vs. CGRP/Veh, CGRP 1 μ M and 10 μ M. 1-way (h–k) or 2-way (b–e) ANOVA, Bonferroni correction. Source data are provided as a Source Data file.

60 min after CGRP or capsaicin (Supplementary Fig. 5g–j). Thus, once induced by CGRP, CLR/RAMP1 antagonists or inhibitors of CLR/RAMP1 internalization are unable to attenuate PMA. Pre- but not post-treatment with the protein kinase A (PKA) inhibitor, H89, reduced PMA by CGRP and capsaicin (Supplementary Fig. 5k–n). NO has been implicated in CGRP-mediated vascular responses². Although NO can release CGRP with proalgesic functions, the contribution of NO to CGRP-evoked allodynia is uncertain. Pre-treatment with an NO synthase (NOS) inhibitor (L-NAME) or an

NO scavenger (cPTIO) (Fig. 6a), abrogated CGRP-evoked PMA (Fig. 6b, c). L-NAME and cPTIO pretreatment also attenuated capsaicin-evoked PMA (Fig. 6d, e). However, L-NAME and cPTIO did not affect PMA when administered 60 min after CGRP (Supplementary Fig. 5o, p) or capsaicin (Supplementary Fig. 5q, r). Thus, PKA-dependent NO release³⁹ is necessary to initiate, but is not sufficient to sustain, CGRP-evoked allodynia.

In vitro findings recapitulated in vivo results. HSCs, MSCs, and IMS32 cells predominantly expressed NOS3 (eNOS) mRNA, with

little or no expression of NOS1 and NOS2 (nNOS and iNOS, respectively) mRNA (Fig. 6f, g; Supplementary Fig. 5s). In both HSCs and IMS32 cells CGRP elicited a transient increase in NOS3 phosphorylation (*i.e.*, activation), consistent with NO generation, which peaked at 5–10 min and declined within 30–60 min (Fig. 6h), and a cAMP increase that was prevented by olcegepant, CGRP8-37 and an adenylyl cyclase inhibitor (SQ22536), but not by L-NAME (Fig. 6i). The increase in cAMP evoked by CGRP but not that elicited by forskolin was reduced in cultured MSCs obtained from *Plp-Cre^{ERT+};Ramp1^{fl/fl}* mice as compared to Control mice treated with intraperitoneal 4-OHT (Fig. 6j). In contrast, the CGRP-evoked increase in NO was attenuated by all these interventions, including NOS inhibition (Fig. 6k). The cAMP increase evoked by forskolin was unaffected by CLR/RAMP1 antagonism and NOS inhibition, and olcegepant failed to inhibit NO release by the NO donor NONOate (Supplementary Fig. 5t, u), indicating selectivity. NO release evoked by CGRP, but not that evoked by NONOate was inhibited by PS2 and Dy4, but not their inactive analogs (Supplementary Fig. 5v), further supporting selectivity. These results suggest that clathrin- and dynamin-dependent endocytosis and endosomal CLR/RAMP1 signaling evoke NOS activation and NO generation in Schwann cells.

Schwann cell TRPA1 mediates CGRP-evoked PMA. NO belongs to a series of reactive oxygen species (ROS) that target TRPA1⁴⁰. TRPA1 is coexpressed with TRPV1 and CGRP in a subpopulation of primary sensory neurons⁴¹. TRPA1 is expressed in Schwann cells of nerve bundles of human skin and mouse sciatic nerve, where it mediates mechanical allodynia in rodent models of pain^{30,42}. Immunoreactive TRPA1 was coexpressed with RAMP1 in S100 + ve Schwann cells in human abdominal and mouse periorbital cutaneous nerves bundles (Fig. 7a). Thus, CLR/RAMP1 might engage signaling pathways that activate TRPA1 in trigeminal Schwann cells to initiate allodynia (Fig. 7b). This hypothesis was supported by the observation that both CGRP- and capsaicin-evoked PMA were reduced in *Trpa1^{-/-}* mice and in mice with sensory neuron-specific deletion of TRPA1 (*Adv-Cre⁺;Trpa1^{fl/fl}*) (Fig. 7c, d and Supplementary Fig. 6a, b).

We next investigated the signaling pathway by which the CLR/RAMP1 activates TRPA1. In HSCs and IMS32 cells, CGRP stimulated a slowly developing yet sustained increase in Ca^{2+} response (Fig. 7e, f) and increased H_2O_2 levels (Supplementary Fig. 6c). Olcegepant, CGRP8-37, SQ22536, H89, L-NAME, Ca^{2+} -free medium, a ROS scavenger (PBN) or a NOX1 inhibitor (ML171) attenuated Ca^{2+} responses (Fig. 7e, f) and H_2O_2 levels (Supplementary Fig. 6c). A TRPA1 antagonist (A967079) inhibited CGRP-stimulated Ca^{2+} (Fig. 7e, f) and H_2O_2 responses (Supplementary Fig. 6c) but did not affect CGRP-stimulated NO formation (Fig. 7g). CGRP-evoked Ca^{2+} responses were reduced in Schwann cells from *Trpa1^{-/-}* mice (Supplementary Fig. 6d). These results support the hypothesis that CGRP liberates NO, which activates Schwann cell TRPA1; activated TRPA1 promotes a Ca^{2+} -dependent H_2O_2 generation that sustains a feed-forward mechanism comprising TRPA1 channel engagement and ROS release.

In vivo results corroborated this hypothesis. Whereas CLR/RAMP1 antagonists or NO inhibitors attenuated PMA only if given before CGRP or capsaicin, both pre- and post-treatment with a TRPA1 antagonist, a ROS scavenger and a NOX1 inhibitor reduced PMA (Fig. 7h–j; Supplementary Fig. 6e–g). Although pretreatment with TRPA1 or ROS inhibitors did not affect the acute nociception, they inhibited capsaicin-evoked PMA (Supplementary Fig. 6h–j). Post-treatment also attenuated capsaicin-evoked PMA (Supplementary Fig. 6h–j). These findings highlight the mechanistic differences between acute nociception and

delayed PMA. After an initial and transient NO-dependent phase, PMA is sustained by persistent ROS liberation, which targets TRPA1 in Schwann cells. This hypothesis is robustly supported by the observation that PMA evoked by CGRP or capsaicin was markedly attenuated in mice with selective deletion of TRPA1 in Schwann cells (*Plp-Cre^{ERT+};Trpa1^{fl/fl}*) (Fig. 7k; Supplementary Fig. 6k).

Targeting endosomal CGRP signaling provides superior relief of CGRP- and capsaicin-evoked PMA. The finding that persistent GPCR signaling from endosomes mediates pain transmission suggests that GPCRs in endosomes rather than at the plasma membrane are a valid and perhaps superior target for the treatment of pain^{33–35}. Nanoparticles have been used to deliver chemotherapeutics to tumor, where endocytosis and endosomal escape are necessary for drug delivery to cytosolic and nuclear targets⁴³. The realization that GPCRs within endosomes are a therapeutic target, raises the possibility of exploiting the acid microenvironment of endosomes as a stimulus for nanoparticle disassembly and release of antagonist cargo³⁴.

To target CLR in endosomes, we generated self-assembling soft polymer nanoparticles containing a CLR antagonist. Diblock copolymers were synthesized with a hydrophilic shell of P(PEGMA-co-DMAEMA) and a hydrophobic core of P(DIPMA-co-DEGMA) (Fig. 8a). Gel permeation chromatography and ^1H -nuclear magnetic resonance (^1H -NMR) confirmed the molecular weight and composition of nanoparticles (Supplementary Fig. 7a–d). Nanoparticles were self-assembled with MK-3207, a potent hydrophobic antagonist of human CLR/RAMP1, forming DIPMA-MK-3207 (Fig. 8a). Empty nanoparticles (DIPMA-Ø) were used as a control. Nanoparticles were uniformly spherical, with the similar diameter (30–35 nm) and ζ -potential (−0.4–1.3 mV) (Fig. 8b, c). DIPMA nanoparticles demonstrate a pH-dependent cargo release at pH < 6.5, consistent with the protonation of the DIPMA tertiary amine (pK_a 6.1), charge repulsion, and disassembly³⁴. DIPMA nanoparticles enter cells by clathrin- and dynamin-mediated endocytosis and disassemble in acidic early endosomes³⁴. To determine whether DIPMA nanoparticles target endosomes containing CLR/RAMP1, HSCs expressing early endosomal antigen-1-GFP (EEA1-GFP) were incubated with DIPMA-Cy5 for 30 min to allow the accumulation in EEA1-GFP + ve endosomes (Fig. 7d, Supplementary Movie 6). Cells were then incubated with TAMRA-CGRP, which was detected in endosomes containing Cy5-DIPMA within 5–10 min (Fig. 8d, Supplementary Movie 6). Thus, DIPMA nanoparticles accumulate with CLR/RAMP1 in early endosomes of Schwann cells.

To determine whether DIPMA-MK-3207 can antagonize CLR in endosomes, we measured CGRP-stimulated cAMP formation using the CAMYEL cAMP BRET sensor, which detects total cellular cAMP. HEK293T cells expressing rat CLR/RAMP1 (HEK-rCLR/RAMP1) were preincubated with graded concentrations of DIPMA-MK-3207 or free MK-3207, DIPMA-Ø or vehicle (control) for 30 min. Beginning at 0 min, baseline BRET was measured for 5 min, and cells were then challenged with CGRP. At 10 min, cells were washed to remove extracellular CGRP, and BRET was measured up to 35 min. In vehicle-treated cells, CGRP stimulated a prompt increase in cAMP formation (1st phase, 6–10 min) that gradually declined after agonist removal from the extracellular fluid (2nd phase, 11–35 min) (Fig. 8e). DIPMA-Ø did not affect this response. Free MK-3207 and DIPMA-MK-3207 (100, 316 nM) both inhibited CGRP-evoked cAMP in the 1st phase to a similar extent (Fig. 8f). During the 2nd phase, free MK-3207 was inactive at all concentrations whereas DIPMA-MK3207 (31.6, 100, 316 nM) strongly inhibited responses (Fig. 8g). The results suggest that

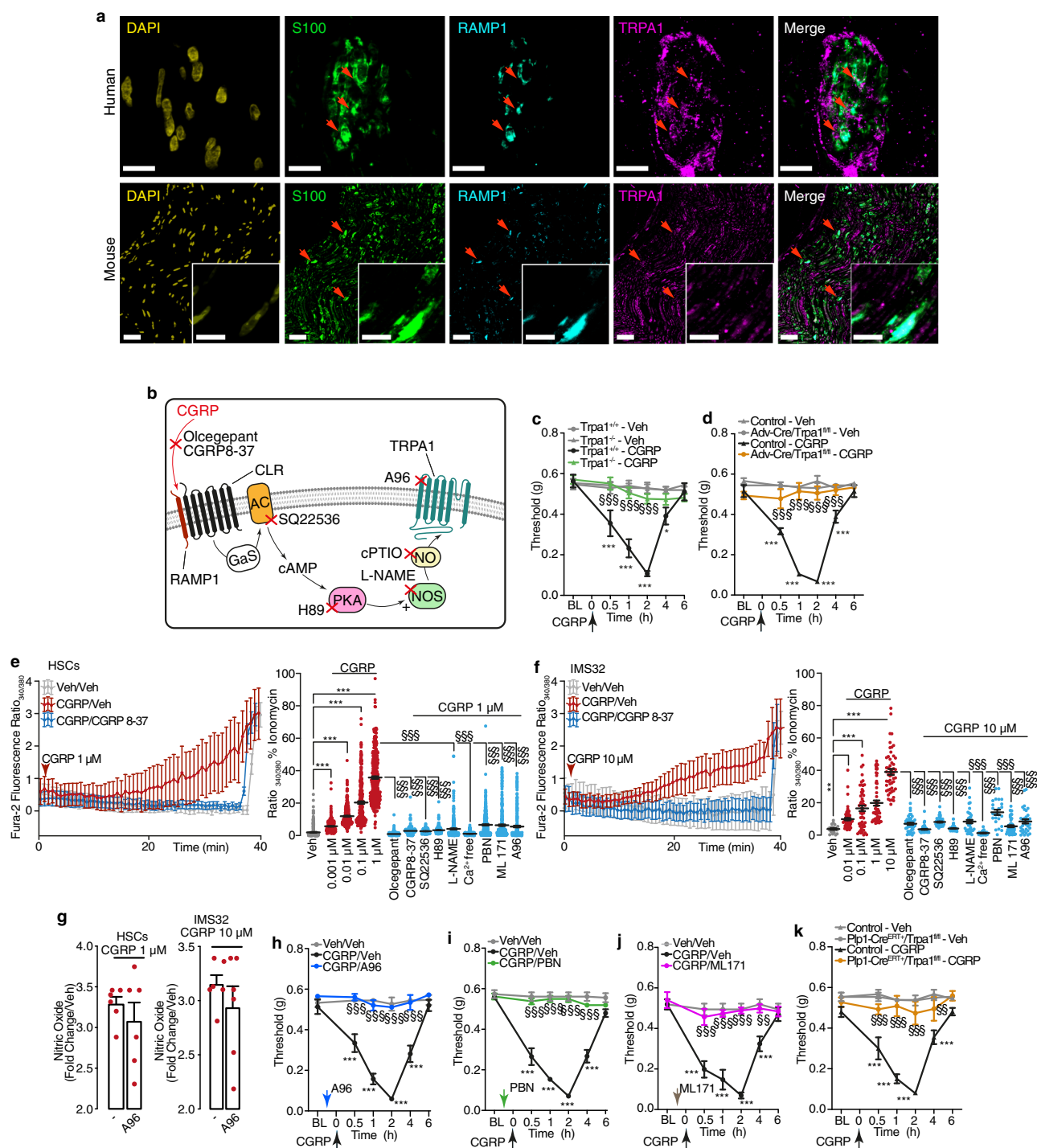
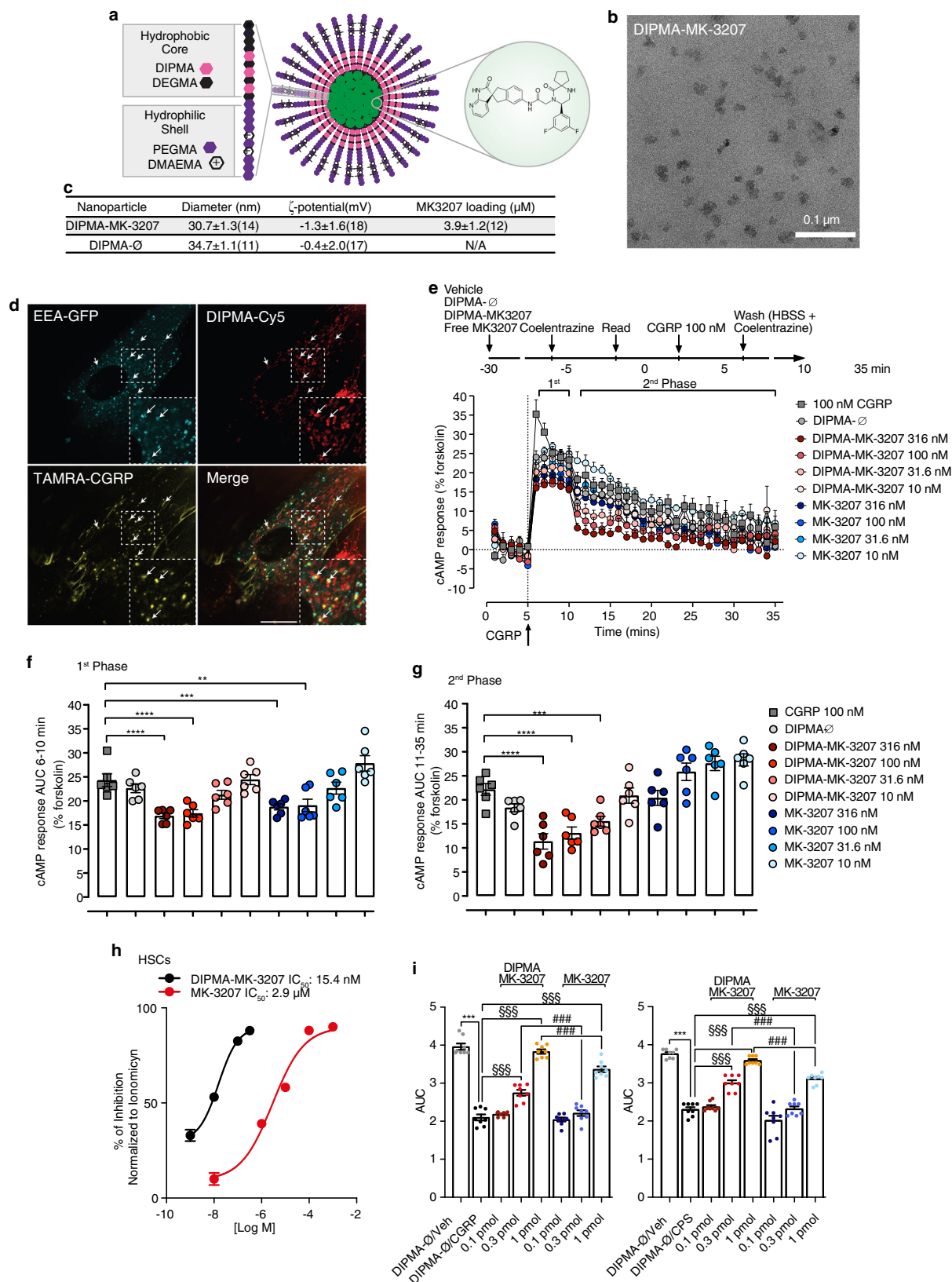


Fig. 7 CGRP induces ROS release via Schwann cell TRPA1 activation. **a** Representative images of localization of immunoreactive DAPI, S100, RAMP1 and TRPA1 in human abdominal and mouse periorbital cutaneous nerve bundles (Scale: 10 μ m human, 50 μ m mouse, inset 10 μ m) ($n = 3$ subjects).

b Pharmacological targets. **c, d** PMA after periorbital injection of CGRP (1.5 nmol) or vehicle in **(c)** *Trpa1*^{+/+} and *Trpa1*^{-/-} mice and in **(d)** *Adv-Cre*^{+/+}*Trpa1*^{fl/fl} or Control mice ($n = 8$ mice per group). **e, f** Ca^{2+} response in HSCs and IMS32 cells exposed to CGRP (0.001 μ M, HSCs $n = 284$; 0.01 μ M, HSCs $n = 291$, IMS32 $n = 62$; 0.1 μ M, HSCs $n = 297$, IMS32 $n = 53$; 1 μ M, HSCs $n = 362$, IMS32 $n = 57$; 10 μ M, IMS32 $n = 53$ cells) in the presence of olcegepant (100 nM, HSCs $n = 291$, IMS32 $n = 44$ cells), CGRP8-37 (100 nM, HSCs $n = 359$, IMS32 $n = 55$ cells), SQ22536 (100 μ M, HSCs $n = 225$, IMS32 $n = 47$ cells), H89 (1 μ M, HSCs $n = 292$, IMS32 $n = 58$ cells), L-NAME (10 μ M HSCs $n = 285$, IMS32 $n = 38$), Ca^{2+} -free medium (HSCs $n = 290$, IMS32 $n = 28$ cells), PBN (50 μ M, HSCs $n = 309$, IMS32 $n = 24$ cells), ML171 (1 μ M, HSCs $n = 320$, IMS32 $n = 49$ cells), A967079 (A96, 50 μ M, HSCs $n = 276$, IMS32 $n = 533$ cells) or vehicle (HSCs $n = 297$, IMS32 $n = 41$ cells). **g** Nitric oxide release in HSCs and IMS32 cells exposed to CGRP (1 or 10 μ M) in the presence of A96 (50 μ M) or vehicle ($n = 6$ independent experiments). **(h-j)** PMA after CGRP (1.5 nmol) or vehicle in C57BL/6 J male mice pre-treated (0.5 h) with **h** A96 (300 nmol), **i** PBN (670 nmol) or **(j)** ML171 (250 nmol) or vehicle. **k** PMA after CGRP (1.5 nmol) or vehicle in *Plp1-Cre*^{ERT+/+}*Trpa1*^{fl/fl} or Control mice treated with 4-OHT (**h-k** $n = 8$ mice per group). Mean $\pm$ SEM. (—) represents the combination of different vehicles. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. *Trpa1*^{+/+}-Veh, Control-Veh, Veh and Veh/Veh; \$\$\$ $P < 0.01$, \$\$\$\$ $P < 0.001$ vs. *Trpa1*^{+/+}-CGRP, Control-CGRP, CGRP 1 μ M and 1 μ M, CGRP/Veh. 2-way (**c, d, h-k**) or 1-way (**e, f**) ANOVA, Bonferroni correction. Source data are provided as a Source Data file.



DIPMA-MK3207 can antagonize the sustained phase of CGRP-stimulated formation of cAMP, which is attributable to endosomal CLR/RAMP1 signaling.

To assess the antagonism of the pain signaling pathway in HSCs, we measured CGRP-evoked changes in Ca^{2+} response, which depend on endosomal CGRP signaling and activation of

TRPA1. HSCs were preincubated with graded concentrations of DIPMA-MK-3207 or MK-3207 for 20 min to allow the accumulation in endosomes, and washed to remove extracellular compounds. At 10 min after washing, cells were challenged with CGRP and Ca^{2+} response was measured as an index of TRPA1 activity. DIPMA-MK-3207 inhibited CGRP-evoked increase in Ca^{2+}

Fig. 8 DIPMA-MK-3207 nanoparticles target endosomal CLR/RAMP1 signaling and provide superior relief from CGRP-evoked PMA. **a** pH-responsive DIPMA-MK-3207. **b** Transmission electron micrograph image of DIPMA-MK-3207 (Scale: 0.1 μ m), from two different nanoparticle preparations (3 images captured per sample). **c** Physicochemical properties of DIPMA-MK-3207 and DIPMA- $\emptyset$. **d** Uptake of DIPMA-Cy5 into HSCs expressing EEA1-GFP. Cells were preincubated with DIPMA-Cy5 (40–60 ng/ml) for 30 min and were then incubated with TAMRA-CGRP (100 nM) for 30 min. Arrows denote accumulation of TAMRA-CGRP in early endosomes containing DIPMA-Cy5. Representative images from $n = 5$ independent experiments (Scale: 10 μ m). **e–g** Effects of DIPMA-MK-3207, MK-3207, DIPMA- $\emptyset$ or vehicle on CGRP- (100 nM) stimulated cAMP formation in HEK-rCLR/RAMP1 cells. **e** Time course and **f, g** integrated response (AUC) before (1st phase) and after (2nd phase) washing to remove extracellular CGRP ($n = 6$ independent experiments). **h** Concentration-response curves of the inhibition by DIPMA-MK-3207 or free MK-3207 on the Ca^{2+} response to CGRP in HSCs (DIPMA-MK-3207: -9M , $n = 145$; -8M , $n = 361$; -7M , $n = 213$; -6.5M , $n = 150$; or free MK-3207: -8M , $n = 83$; -6M , $n = 106$; -5M , $n = 87$; -4M , $n = 127$; -3M , $n = 127$ cells). **i** PMA, expressed as AUC, after periorbital injection of CGRP (1.5 nmol), capsaicin (CPS, 50 pmol) or vehicles in C57BL/6 J male mice pre-treated (0.5 h) with DIPMA-MK-3207, MK-3207 (0.1, 0.3, 1 pmol), DIPMA- $\emptyset$ or vehicle ($n = 8$ mice per group). Mean $\pm$ SEM. *** $P < 0.001$ vs. DIPMA- $\emptyset$ /Veh, ### $P < 0.001$ vs. MK-3207 0.3 pmol and MK-3207 1 pmol. **f, g, i** 1-way ANOVA, Bonferroni correction. Source data are provided as a Source Data file.

response (IC_{50} 15.4 nM, 95% confidence interval, 10.9–21.0 nM) more potently than free MK-3207 (IC_{50} 2.9 μ M, 95% confidence interval, 1.9–4.2 μ M, $P < 0.0001$) (Fig. 8h). To assess antinociception, DIPMA-MK-3207 or free MK-3207 (0.1, 0.3, 1.0 pmol) was injected into the periorbital region 30 min before periorbital injection of CGRP or capsaicin. DIPMA-MK-3207 (0.3, 1.0 pmol) more effectively inhibited PMA than the same doses of free MK-3207 (Fig. 8i). DIPMA- $\emptyset$ had no effect. Thus, endosomal targeting enhances the efficacy of a CLR/RAMP1 antagonist in a preclinical model of migraine pain.

Discussion

The major findings of the present study are that CGRP causes PMA by activating CLR/RAMP1 of Schwann cells, CLR/RAMP1 signals from endosomes of Schwann cells to activate pain pathways, and endosomal CLR/RAMP1 can be targeted using nanoparticles and endocytosis inhibitors to relieve CGRP-evoked PMA. CLR/RAMP1 stimulation and trafficking to endosomes results in a persistent cAMP-dependent NOS activation and generation of NO, a mediator of migraine pain¹⁹. The role of NO in PMA is crucial, yet transient, as it is temporally limited to the engagement of TRPA1/NOX1, which releases ROS with a dual function. On one hand, ROS target TRPA1/NOX1 of Schwann cells to maintain ROS generation by a feed-forward mechanism. On the other hand, as suggested by experiments with selective TRPA1 deletion in primary sensory neurons, ROS target TRPA1 on nociceptors to signal allodynia to the CNS.

Periorbital capsaicin injection elicited acute nociception mediated by TRPV1 excitation and ensuing afferent discharge, which signals pain to the CNS. In a larger cutaneous area, capsaicin evoked delayed and prolonged PMA. While the acute pain response is most likely dependent on ion influx associated with TRPV1 activation, the mechanism underlying mechanical hypersensitivity^{7,44} has remained elusive. Our findings support the existence of a paracrine mechanism that underlies PMA associated with neurogenic inflammation. We suggest that capsaicin locally activates TRPV1 + ve nerve fibers to generate action potentials that propagate antidromically into collateral fibers which release CGRP in a broader area, thus eliciting widespread PMA. PMA depends on the interaction between peptidergic nerve fibers, surrounding Schwann cells and nociceptive neurons that convey allodynic signals to the CNS. CGRP liberated from the varicosities of trigeminal TRPV1 + ve nerve fibers binds to CLR/RAMP1 of adjacent Schwann cells.

CNS perturbations may target the trigeminovascular system and initiate the migraine attack^{22,23}. These central mechanisms may underlie the delayed facial allodynia associated with migraine^{20,21}. However, the beneficial effect of anti-CGRP medicines that do not cross the blood-brain barrier suggests that CGRP acts in the periphery to elicit pain. The peripheral site of the algic action of CGRP released from peptidergic C-fibers has

been proposed as the CLR/RAMP1 on adjacent non-peptidergic A δ -fibers⁴⁵ and more precisely at the level of the node of Ranvier²⁸. The present results in *Adv-Cre⁺;Ramp1^{fl/fl}* mice suggest that CGRP does not act on trigeminal nociceptors to cause PMA in mice. This is consistent with failure of CGRP administration to elicit any itch, pain or axon reflex responses in humans⁴⁶. Instead, our results support the hypothesis that CGRP released from trigeminal nociceptors targets CLR/RAMP1 on Schwann cells that wrap their terminals to evoke PMA. A limitation of our study is that we only assessed PMA that mimics one component of migraine pain. We cannot exclude the possibility that central mechanisms contribute to other pain symptoms of migraine, and that some of the locally administered antagonists used in the present study penetrated the CNS, where they could also influence pain transmission. Although human Schwann cells express CLR/RAMP1 and show functional responses to CGRP that can account for allodynia in mice, further work is needed to understand whether similar mechanisms account for migraine pain in humans.

Another limitation of the present study is that we cannot pinpoint which type of neuron conveys the signals that underlie mechanical allodynia in the trigeminal region. Specifically, we were unable to distinguish between TRPV1-expressing nerve fibers that release CGRP and TRPA1-expressing nerve fibers that are targeted by Schwann cell ROS and convey allodynic signals centrally, since TRPV1 and TRPA1 may coexist in the same population of CGRP-expressing A δ - or C-fiber primary sensory neurons^{2,41}. Most Schwann cells in Remak bundles contain multiple unmyelinated axons from C-fiber nociceptors, including CGRP + ve fibers, which release the bulk of CGRP², and non-peptidergic isolectin B4 + ve fibers⁴⁷. Thus, CGRP-evoked release of ROS from Schwann cells could induce allodynia by targeting TRPA1 on three neuronal subtypes, including the same A δ - or C-fiber that releases CGRP, a different C-fiber of the same Remak bundle, or a different adjacent A δ -fiber. The observation that both C-fiber and A δ -fiber nociceptors contribute to capsaicin-evoked hypersensitivity in humans⁴⁸ supports the hypothesis that both types of neurons^{28,45} are implicated in CGRP-mediated allodynia, thus highlighting the complex neural transmission of mechanical allodynia associated with neurogenic inflammation.

CLR/RAMP1 signals from endosomes by G-protein-mediated mechanisms that activate a subset of compartmentalized signals, including cytosolic protein kinase C and nuclear extracellular signal-regulated kinase; these kinases regulate excitation of spinal neurons and pain transmission³⁵. Our results show that CLR/RAMP1 activates G_{α_s} , G_{α_q} and G_{α_i} and recruits β ARRs in endosomes of Schwann cells, determined by EeBRET. Inhibitors of clathrin- and dynamin-mediated endocytosis blocked the recruitment of CLR/RAMP1, G_{α} and β ARR to endosomes, which presumably requires CLR/RAMP1 endocytosis. GPCR/ G_{α} signaling complexes have also been detected in endosomes by using

conformationally selective nanobodies⁴⁹. The observation that endocytosis inhibitors attenuated CGRP-stimulated cAMP formation and activation of NOS and TRPA1 reveals a central role for CLR/RAMP1 signaling in endosomes of Schwann cells in CGRP-evoked periorbital pain. Endocytosis of other Gs-coupled GPCRs is also necessary for the full repertoire of cAMP-mediated signaling outcomes, which entails endosomal recruitment of adenylyl cyclase^{9,50} and assembly of metastable accumulations of PKA⁵¹. We found that a nanoparticle-encapsulated CLR/RAMP1 antagonist, which targeted CLR/RAMP1 in endosomes and released cargo in the acidified endosomal microenvironment³⁴, also attenuated CGRP-stimulated cAMP formation and blunted TRPA1 activation.

The observation that periorbital injection of inhibitors of clathrin and dynamin and of DIPMA-MK-3207 prevented CGRP- and capsaicin-evoked PMA provides the evidence for a prominent role of endosomal CGRP signaling of pain from a peripheral site. The finding that nanoparticle encapsulation enhanced the potency of a CGRP antagonist for inhibition of endosomal signaling and resultant nociception supports the hypothesis that CLR/RAMP1 in endosomes mediates facial allodynia which contributes to migraine pain. Nanoparticle encapsulation similarly boosts the efficacy of an NK₁ receptor antagonist in pre-clinical models of inflammatory and neuropathic pain³⁴. An antagonist of CLR/RAMP1 conjugated to a membrane lipid cholestanol also accumulates in endosomes and provides superior relief from pain³⁵, which reinforces the importance of CLR/RAMP1 endosomal signaling for pain transmission.

Limitations of the present study include uncertainty about the nature of the CLR/RAMP1 signaling complex in endosomes of Schwann cells, which warrants further investigation by proteomic approaches. Although some of the pharmacological inhibitors used to dissect the signaling pathway can have non-specific actions, we bolstered confidence in selectivity by using inhibitors of the same pathway and by genetic deletion of GPCRs and TRP channels. Our findings reveal a prominent role for CLR/RAMP1 in Schwann cells for CGRP-evoked periorbital pain. Future studies will investigate the role of this pathway in pre-clinical models of migraine pain.

Monoclonal antibodies to CGRP, although beneficial, are not effective in all patients¹⁰. While non-CGRP-dependent mechanisms might explain this failure⁵², monoclonal antibodies likely do not inhibit CGRP signaling in endosomes. The small molecule CLR/RAMP1 antagonist, rimegepant, was found to resolve migraine attacks in patients treated with the anti-CLR/RAMP1 monoclonal antibody, erenumab⁵³. This unexpected result was interpreted by the inherent membrane permeability of the lipophilic antagonist rimegepant⁵⁴ that might favor inhibition of CGRP signaling in endosomes⁵³, while neither receptor-targeted nor ligand-targeted monoclonal antibodies internalized with CLR/RAMP1 activated by CGRP⁵⁵. Our results showing a superior inhibition of CGRP signaling in Schwann cells and of PMA by DIPMA-MK-3207, which selectively targets receptor activity in endosomes, reveal a better approach to control allodynia.

In 1936, Sir Thomas Lewis postulated¹ that in human skin action potentials are carried antidromically from the injured nerve terminal to collateral branches from where a chemical substance is released that produces the flare and increases the sensitivity of other fibers responsible for pain. CGRP has been previously identified as the mediator of neurogenic vasodilatation in rodents², and in humans⁸. Herein, we propose that CGRP is the 'chemical substance' that, via the essential role of endosomal CLR/RAMP1, TRPA1/NOX1 and oxidative stress of surrounding Schwann cells, sustains the enhanced sensitivity of primary sensory neurons associated with neurogenic inflammation (Fig. 9).

The present results suggest that peripherally acting anti-CGRP medicines reduce migraine pain in part by targeting the facial allodynia that originates from CGRP-mediated endosomal signaling in Schwann cells.

Methods

The research conducted complies with all relevant ethical regulations. Animal experiments were carried out in accordance with European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. The study was approved by the National Committee for the Protection of Animals used for Scientific Purposes of the Italian Ministry of Health (research permits #383/2019-PR and #765/2019-PR). The use of formalin-fixed paraffin-embedded (FFPE) sections of human abdominal cutaneous tissues was approved by the Local Ethics Committee of the Florence University Hospital (Area Vasta Toscana Centro) (18271_bio/2020), according to the Helsinki Declaration, and all patients gave their informed consent. Participants did not receive any form of compensation. Biopsies of human abdominal skin analyzed derived from three different patients [female, median age 58 years (range 56–61 years)].

Experimental model and subject details

Animals. Male and female mice were used throughout (25–30 g, 5–8 weeks). The following strains of mice were used C57BL/6 J mice (Charles River, RRI-D:IMSR_JAX:000664); wild-type (*Trpa1*^{+/+}) and TRPA1-deficient (*Trpa1*^{-/-}; B6129P-*Trpa1*^{tm1Kyk}/J; RRID:IMSR_JAX:006401, Jackson Laboratory) mice;⁵⁶ wild-type (*Trpv1*^{+/+}) and TRPV1-deficient (*Trpv1*^{-/-}; B6129X1-*Trpv1*^{tm1Jnl}/J; RRID:IMSR_JAX:003770, Jackson Laboratory) mice. Genetically modified mice were maintained as heterozygotes on a C57BL/6 J background.

To generate mice in which the *Trpa1* and *Ramp1* genes were conditionally silenced in Schwann cells/oligodendrocytes, homozygous 129S-*Trpa1*^{tm2Kyk}/J (floxed *Trpa1*, *Trpa1*^{fl/fl}; RRID:IMSR_JAX:008649 Jackson Laboratory) and C57BL/6N-*Ramp1*^{tm1c(EUCOMM)Wtsi}/H (floxed *Ramp1*, *Ramp1*^{fl/fl}; Stock No: EM:07401, MRC HARWELL Mary Lyon Center)⁵⁷ were crossed with hemizygous B6.Cg-Tg(*Plp1-Cre*^{ERT})3Pop/J mice (*Plp1-Cre*^{ERT}; RRID:IMSR_JAX:005975 Jackson Laboratory), expressing a tamoxifen-inducible Cre in myelinating cells (*Plp1*, proteolipid protein myelin 1)³⁰. The progeny (*Plp1-Cre*^{ERT}; *Trpa1*^{fl/fl} and *Ramp1-Cre*^{ERT}; *Trpa1*^{fl/fl}) was genotyped by PCR for *Trpa1*, *Ramp1* and *Plp1-Cre*^{ERT}. Mice negative for *Plp1-Cre*^{ERT} (*Plp1-Cre*^{ERT}; *Trpa1*^{fl/fl} and *Plp1-Cre*^{ERT}; *Ramp1*^{fl/fl}) were used as control. Both positive and negative mice to *Cre*^{ERT} and homozygous for floxed *Trpa1* (*Plp1-Cre*^{ERT}; *Trpa1*^{fl/fl} and *Plp1-Cre*^{ERT}; *Trpa1*^{fl/fl}, respectively) and floxed *Ramp1* (*Plp1-Cre*^{ERT}; *Ramp1*^{fl/fl} and *Plp1-Cre*^{ERT}; *Ramp1*^{fl/fl}) mice were treated with 4-hydroxytamoxifen (4-OHT) by subcutaneous periorbital (p.orb.) injection (0.02 mg/10 µl in corn oil once a day for 3 consecutive days). Some *Plp1-Cre*^{ERT}; *Ramp1*^{fl/fl} and *Plp1-Cre*^{ERT}; *Ramp1*^{fl/fl} mice were treated with intraperitoneal (i.p.) or intraplantar (i.pl.) 4-OHT (1 mg/100 µl or 0.02 mg/10 µl in corn oil once a day for 3 consecutive days, respectively).

Treatments resulted in Cre-mediated ablation of *Trpa1* and *Ramp1* in PLP-expressing Schwann cells/oligodendrocytes. To selectively delete the *Trpa1* and *Ramp1* gene in primary sensory neurons, *Trpa1*^{fl/fl} and *Ramp1*^{fl/fl} mice were crossed with hemizygous *Advillin-Cre* mice (*Adv-Cre*)^{30,58,59}. Both positive and negative mice to *Cre*^{ERT} and homozygous for floxed *Trpa1* (*Adv-Cre*⁺; *Trpa1*^{fl/fl} and *Adv-Cre*⁻; *Trpa1*^{fl/fl}, respectively) and floxed *Ramp1* (*Adv-Cre*⁺; *Ramp1*^{fl/fl} and *Adv-Cre*⁻; *Ramp1*^{fl/fl}) were used.

The group size of *n* = 8 animals for behavioral experiments was determined by sample size estimation using G*Power (v3.1)⁶⁰ to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Mice were allocated to vehicle or treatment groups using a randomization procedure (<http://www.randomizer.org/>). Four independent and blinded investigators performed the treatments, behavioral experiments, genotyping and data analysis, respectively. No animals were excluded from experiments.

The behavioral studies followed the animal research reporting in vivo experiment (ARRIVE) guidelines⁶¹. Mice were housed in a temperature (20 ± 2 °C) and humidity (50 ± 10%) controlled vivarium (12 h dark/light cycle, free access to food and water, five animals per cage). At least 1 h before behavioral experiments, mice were acclimatized to the experimental room and behavior was evaluated between 9:00 am and 5:00 pm. All the procedures were conducted following the current guidelines for laboratory animal care and the ethical guidelines for investigations of experimental pain in conscious animals set by the International Association for the Study of Pain⁶². Animals were anesthetized with a mixture of ketamine and xylazine (90 mg/kg and 3 mg/kg, respectively, i.p.) and euthanized with inhaled CO₂ plus 10–50% O₂.

Cell lines. Primary cultures of human Schwann cells (HSCs, #1700, ScienCell Research Laboratories) were grown and maintained in Schwann cell medium (#1701, ScienCell Research Laboratories) at 37 °C in 5% CO₂ and 95% O₂. Cells were passaged at 90% confluency and discarded after 12 passages. HEK293T (#CRL-3216™, American Type Culture Collection) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS, 10%), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37 °C in 5% CO₂ and 95% O₂. The mouse Schwann cell

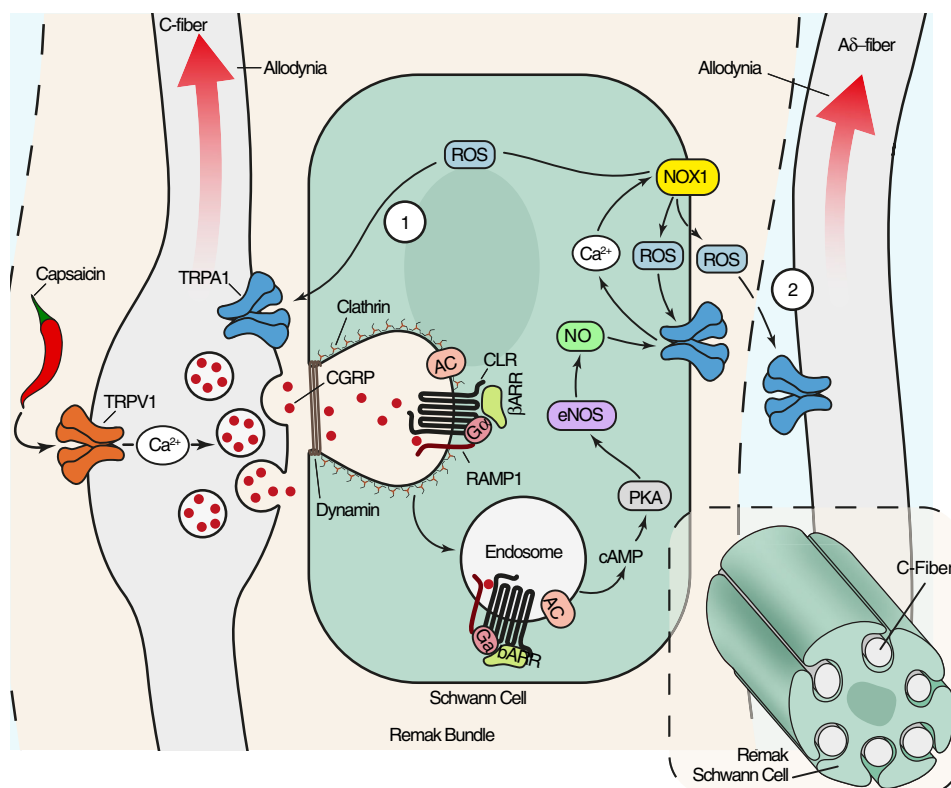


Fig. 9 Schematic representation of the pathway that signal prolonged cutaneous allodynia elicited by CGRP released and associated with neurogenic inflammation. The pro-migraine neuropeptide, CGRP, released from trigeminal cutaneous afferents, activates CLR/RAMP1 on Schwann cells. CLR/RAMP1 traffics to endosomes, where sustained G protein signaling increases cAMP and stimulates PKA that results in nitric oxide synthase activation. The ensuing release of nitric oxide targets the oxidant-sensitive channel, TRPA1, in Schwann cells, which elicits persistent ROS generation. ROS triggers TRPA1 on adjacent C- (1) or A δ -fiber (2) afferents resulting in periorbital allodynia, a hallmark of migraine pain. The inset shows several unmyelinated axons invaginated into a Schwann cell forming a Remak bundle.

line (IMS32 cells, #PMC-SWN-IMS32-COS, Cosmo Bio USA) was grown and maintained in Schwann cell medium (#PMC-SWN-MM-COS, Cosmo Bio USA) at 37 °C in 5% CO₂ and 95% O₂^{63,64}. All cells were used when received without further authentication.

Pharmacological reagents. Supplementary Table 1 and 2 provide doses, routes of administration and concentrations and of all pharmacological reagents.

Behavioral experiments

Treatment protocol. Subcutaneous injections were made in the periorbital area 2–3 mm from the external eyelid corner¹⁷. Briefly, the mouse was lifted by the base of the tail and placed on a solid surface with one hand and the tail was pulled back. Then, it was quickly and firmly picked up by the scruff of the neck with the thumb and index finger of the other hand. The injection was made rapidly by a single operator with minimal animal restraint. Mice received unilateral (right side) injections (10 μ l/site) of CGRP (1.5 nmol in 0.9% NaCl), SP (3.5 nmol in 0.9% NaCl), capsaicin (10, 50, 100 pmol in 0.1% dimethyl sulfoxide, DMSO), or vehicles (control). Mice received bilateral injections (10 μ l/site, right side same site as stimulus, left side symmetrical to right side) of antagonists and inhibitors. CGRP (1.5 nmol in 0.9% NaCl) or vehicle was also administered by intraplantar (i.pl., 20 μ l/site) or systemic (0.1 mg/kg, i.p.) injection. GTN was administered at 10 mg/kg, i.p. injection.

Acute nociception. Immediately after the p.orb. injection, mice were placed inside a plexiglass chamber and spontaneous nociception was assessed for 10 min by measuring the time (s) that the animal spent rubbing the injected area of the face with its paws^{17,65}.

Periorbital mechanical allodynia. PMA was assessed using the up-down paradigm^{66,67} in the same mice in which acute nociceptive responses were monitored. Briefly, mice were placed in a restraint apparatus designed for the evaluation of periorbital mechanical thresholds¹⁷. One day before the first behavioral observation, mice were habituated to the apparatus. PMA was evaluated in the periorbital region over the rostral portion of the eye (i.e., the area of the periorbital region facing the sphenoidal rostrum)⁶⁸ before (basal threshold) and after (0.5, 1, 2,

4, 6, 8 h) treatments. On the day of the experiment, after 20 min of adaptation inside the chamber, a series of 7 von Frey filaments in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 0.6, and 1.0 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 s to elicit a positive response. Mechanical stimuli were applied homolaterally outside the periorbital area at a distance of 6–8 mm from the site where stimuli were injected. The response was considered positive by the following criteria: mouse vigorously stroked its face with the forepaw, head withdrawal from the stimulus, or head shaking. Mechanical stimulation started with the 0.16 g filament. The absence of response after 5 s led to the use of a filament with increased force, whereas a positive response led to the use of a weaker (i.e. lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated from these scores by using a δ value of 0.205, previously determined.

Paw mechanical allodynia. Paw mechanical allodynia was evaluated by measuring the paw withdrawal threshold by using the up-down paradigm^{66,67}. Mice were acclimatized (1 h) in individual clear plexiglass boxes on an elevated wire mesh platform, to allow for access to the plantar surfaces of the hind paws. von Frey filaments of increasing stiffness (0.07, 0.16, 0.4, 0.6, and 1.0, 1.4 and 2 g) were applied to the hind paw plantar surfaces of mice with enough pressure to bend the filament. The absence of a paw being lifted after 5 s led to the use of the next filament with an increased force, whereas a lifted paw indicated a positive response, leading to the use of a subsequently weaker filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated.

Primary culture of mouse Schwann cells. Mouse Schwann cells (MSC) were isolated from sciatic or trigeminal nerves of C57BL/6 J, and from sciatic nerve of *Trpa1*^{+/+} and *Trpa1*^{-/-}, *Plp1-Cre*^{ERT+}; *Ramp1*^{fl/fl} and *Plp1-Cre*^{ERT+}; *Ramp1*^{fl/fl} mice^{30,69}. The epineurium was removed, and nerve explants were divided into 1 mm segments and dissociated enzymatically using collagenase (0.05%) and hyaluronidase (0.1%) in Hank's Balanced Salt Solution (HBSS, 2 hr, 37 °C). Cells were collected by centrifugation (150xg, 10 min, room temperature) and the pellet was resuspended

and cultured in DMEM containing fetal calf serum (10%), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), neuregulin (10 nM) and forskolin (2 μ M). Three days later, cytosine arabinoside (Ara-C, 10 mM) was added to remove fibroblasts. Cells were cultured at 37 °C in 5% CO₂ and 95% O₂. The culture medium was replaced every 3 days and cells were used after 15 days of culture.

qRT-PCR. Total RNA was extracted from HSCs, IMS32 and sciatic or trigeminal MSCs cells using the RNeasy Mini kit (Qiagen SpA), according to the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically by measuring the absorbance at 260 nm and 280 nm. RNA was reverse transcribed with the Qiagen QuantiTect Reverse Transcription Kit (Qiagen SpA) following the manufacturer's protocol. For mRNA relative quantification, rt-PCR was performed on Rotor Gene® Q (Qiagen SpA, Rotor-Gene® Q- Software Version 2.3.1.49). The relative abundance of mRNA transcripts was calculated using the delta CT method and normalized to GAPDH levels. The sets of primers for human and mouse cells are listed in the Supplementary Table 3.

Calcium imaging. HSCs, IMS32 and sciatic nerve MSCs cells were plated on poly-L-lysine-coated (8.3 μ M) 35 mm glass coverslips and maintained at 37 °C in 5% CO₂ and 95% O₂ for 24 h. Cells were loaded (40 min) with Fura-2 AM-ester (5 μ M) added to the buffer solution (37 °C) containing (in mM) 2 CaCl₂, 5.4 KCl; 0.4 MgSO₄; 135 NaCl; 10 D-glucose; 10 HEPES and bovine serum albumin (BSA, 0.1%) at pH 7.4. Cells were washed and transferred to a chamber on the stage of a fluorescent microscope for recording (Olympus IX 81) and exposed to CGRP (0.01–10 μ M) or vehicle (0.9% NaCl) and the Ca²⁺ response was monitored for approximately 40 min. In another set of experiments, IMS32 cells were exposed to AITC (30 μ M) or vehicle (0.03% DMSO). The Ca²⁺ response to CGRP was monitored in the presence of CGRP8-37 (100 nM), olcegepant (100 nM), SQ22536 (100 μ M), L-NAME (10 μ M), A967079 (50 μ M), PBN (50 μ M), H89 (1 μ M), ML171 (1 μ M) or vehicle (0.1 % DMSO), and in the presence of DIPMA-MK-3207 (1–1000 nM) and MK-3207 free drug (0.01–1000 μ M) or DIPMA-empty. Cells were preincubated with DIPMA-MK-3207 or MK-3207 for 20 min at room temperature to allow nanoparticle accumulation in endosomes. Some experiments used Ca²⁺-free buffer solution containing EDTA (1 mM). Results were expressed as percent increase in ratio_{340/380} over baseline normalized to the maximum effect induced by ionomycin (5 μ M) added at the end of each experiment.

Protein extraction and western immunoblot assay. HSCs and IMS32 cells were plated on 60 mm culture dish and maintained in 5% CO₂ and 95% O₂ (37 °C, until confluence). HSCs and IMS32 cells were homogenized in RIPA buffer [NaCl (150 mM), Tris-base (50 mM), EGTA (5 mM), Triton X-100 (1%), sodium deoxycholate (0.5%), sodium dodecyl sulfate (0.1%)] containing dithiothreitol (1 mM) and complete protease inhibitor cocktail. Lysates were centrifuged at 16,128x g at 4 °C for 10 min. Protein concentration in supernatants was determined using BCA protein assay (Thermo Scientific). Samples with equal amounts of proteins (20 μ g) were then separated by NuPAGE 4–12% bis-tris gel electrophoresis (Life Technologies), and the proteins were transferred to a nitrocellulose (Bio-Rad). Membranes were incubated with dry milk (5%) in Tris buffer [(TBST; Tris (20 mM) at pH 7.5, NaCl (150 mM)] containing Tween 20 (0.1%) for 1 h at room temperature, and incubated with the following primary antibodies: RAMP1 [ERP10867] (#ab156575, rabbit monoclonal, 1:1000, Abcam, Lot: GR3196403-5), CRLR (#NBPI-59073, rabbit polyclonal, 1:500, Novus Biological, Lot: 8312) or β -actin (#ab6276, mouse monoclonal, 1:5000, Abcam, Lot: GR 141-7) at 4 °C overnight. Membranes were then probed with goat anti-mouse or donkey antirabbit IgG conjugated with horseradish peroxidase (HRPO, 1:10,000, Bethyl Laboratories Inc., Cat#A90-516P and Cat#A120-208P) for 2 hr at room temperature. Finally, membranes were washed three times with TBST, and bound antibodies were detected using chemiluminescence reagents (Pierce™ ECL, Thermo Scientific), and revealed using an imaging system (ChemiDoc version 2.3.0.07 BioRad). The density of specific bands was measured using an image processing program (ImageJ 1.32J, National Institutes of Health) and normalized to β -actin.

In-cell ELISA assay. HSCs or IMS32 cells were plated in 96-well black wall clear bottom plates (Corning Life Sciences) (5 $\times$ 10⁵ cells/well) and maintained at 37 °C in 5% CO₂ and 95% O₂ for 24 h. HSCs and IMS32 cells were exposed to CGRP (1 and 10 μ M, respectively) or its vehicle (phosphate-buffered saline, PBS) for 5, 10, 15, 30 and 60 min, at 37 °C, then washed with DMEM pH 7.5 and fixed in 4% paraformaldehyde for 30 min. Cells were then washed with TBST (0.05%) and blocked with donkey serum (5%) for 4 h at room temperature and incubated overnight 4 °C with eNOSP^{S1177} (#ab184154, rabbit polyclonal, 1:100, Abcam, Lot: GR3257047-9). Cells were then washed and incubated with donkey anti-rabbit IgG conjugated with horseradish peroxidase (HRPO, 1:2000, Bethyl Laboratories Inc.) for 2 h at room temperature. Cells were then washed and stained using SIGMA-FAST OPD for 30 min protected from light. After the incubation period, the absorbance was measured at 450 nm. Change in NOS3 phosphorylation was calculated as a percentage of the signal in vehicle-treated cells.

cAMP ELISA assay. cAMP level was determined by the CatchPoint™ cyclic-AMP fluorescent assay kit (#R8088, Molecular Device) according to the manufacturer's

protocol. Briefly, HSCs or IMS32 cells were plated in 96-well black wall clear bottom plates (Corning Life Sciences) (5 $\times$ 10⁵ cells/well) and maintained in 5% CO₂ and 95% O₂ (24 h, 37 °C). The cultured medium was replaced with HBSS added with olcegepant (100 nM), CGRP8-37 (100 nM), SQ22536 (100 μ M), L-NAME (10 μ M) or vehicle (0.1% DMSO in HBSS) for 20 min at room temperature. HSCs or IMS32 cells were then stimulated with CGRP (1 and 10 μ M, respectively), forskolin (1 μ M, positive control) or their vehicles (HBSS) and maintained for 40 min at room temperature protected from light. Signal was detected 60 min after exposure to the stimuli. cAMP level was calculated using cAMP standards and expressed as nmol/l.

Nitric oxide assay. Nitric oxide was determined by using the fluorometric-orange assay kit (#ab219932, Abcam) according to the manufacturer's protocol. HSCs or IMS32 cells were plated 96-well black wall clear bottom plates (Corning Life Sciences) (5 $\times$ 10⁵ cells/well) and maintained in 5% CO₂ and 95% O₂ (24 h, 37 °C). The cultured medium was replaced with Hanks' balanced salt solution (HBSS) added with olcegepant (100 nM), CGRP8-37 (100 nM), SQ22536 (100 μ M), L-NAME (10 μ M), A967079 (50 μ M), PS2 or Dy4 (both 30 μ M), PS2 inact or Dy4 inact (30 μ M) or vehicle (0.1% DMSO in HBSS) for 20 min at room temperature. HSCs or IMS32 cells were then stimulated with CGRP (1 and 10 μ M, respectively), diethylamine NONOate (1 mM, positive control) or their vehicles (HBSS) and maintained for 40 min at room temperature protected from light. Signal was detected 60 min after exposure to the stimuli. Change in nitric oxide level was calculated as percentage of the vehicle.

H₂O₂ assay. H₂O₂ was determined by using the Amplex Red assay (#A12222, Thermo Fisher, Invitrogen). HSCs or IMS32 cells were plated in 96-well black wall clear bottom plates (Corning Life Sciences) (5 $\times$ 10⁵ cells/well) and maintained in 5% CO₂ and 95% O₂ (24 h, 37 °C). The cultured medium was replaced with Krebs-Ringer phosphate (KRP, composition in mM: 2 CaCl₂, 5.4 KCl; 0.4 MgSO₄; 135 NaCl; 10 D-glucose; 10 HEPES [pH 7.4]) added with olcegepant (100 nM), CGRP8-37 (100 nM), SQ22536 (100 μ M), L-NAME (10 μ M), A967079 (50 μ M), PBN (50 μ M) or vehicle (0.1% DMSO in KRP) for 20 min at room temperature. HSCs or IMS32 cells were then stimulated with CGRP (1 and 10 μ M, respectively) or its vehicle (KRP) added with Amplex red (50 μ M) and horseradish peroxidase (HRP, 1 U/ml) and maintained for 40 min at room temperature protected from light. Some experiments were performed in Ca²⁺-free KRP containing EDTA (1 mM). Signal was detected 60 min after exposure to the stimuli. H₂O₂ release was calculated using H₂O₂ standards and expressed as nmol/l.

Immunofluorescence. Anesthetized mice were transcardially perfused with PBS and 4% paraformaldehyde. Trigeminal and sciatic nerves were removed, postfixed for 24 h, and paraffin-embedded. Human and mouse formalin-fixed paraffin-embedded (FFPE) sections (5 μ m) were incubated with primary antibodies: TRPA1 (#ab58844, rabbit polyclonal, 1:400, Abcam, Lot: GR165165-21), S100 (#ab14849, mouse monoclonal [4B3], 1:300, Abcam, Lot: GR3233892-2), CLR (#NLS6731, rabbit polyclonal, 1:30, Novus Biologicals, Lot: QC58878-190422), RAMP1- Alexa Fluor 594 (#ab241335, rabbit polyclonal, 1:200, Abcam Lot: GR3247267-4), diluted in fresh blocking solution (PBS, pH 7.4, 2.5% normal goat serum, [NGS]). Sections were then incubated with the fluorescent polyclonal secondary antibodies Alexa Fluor 488 and 555 (#A32731, Lot: VA295501, #A32727, Lot: UL287768, 1:600; Invitrogen), and coverslipped using the mounting medium with DAPI (Abcam). Images were acquired by a Zeiss Axio Imager 2, Zeiss ZEN imaging 2020. The Pearson correlation (Rcoloc) value for RAMP1 and S100 in the colocalization studies were calculated using the colocalization Plugin of the ImageJ software (ImageJ 1.32J, National Institutes of Health).

cAMP cADDIS assay. HSCs were plated on poly-D-lysine-coated 96-well black wall clear bottom plates (Corning Life Sciences) (25 $\times$ 10³ cells/well) and incubated in 5% CO₂ and 95% O₂ for 4–6 h. HSCs were transduced with the baculovirus mediated Green Upward cADDIS cAMP reporter (25 μ l/well, Montana Molecular) following manufacturer's instructions, and cells were incubated in 5% CO₂ and 95% O₂ (48 h, 37 °C). HSCs were washed twice in HBSS plus HEPES (10 mM) pH 7.4. Cells were incubated in HBSS/HEPES with the CLR/RAMP1 antagonists olcegepant (100 pM–100 μ M) or vehicle (control) for 30 min. Plates were mounted in a FlexStation3 plate reader (Molecular Devices) and fluorescence (485–500 excitation, 515–530 emission with cutoff at 510) was monitored. The baseline was measured for 1 min, and cells were stimulated with human CGRP α (100 pM–10 μ M) or forskolin (10 μ M, positive control). For single-cell imaging, HSCs were plated on poly-D-lysine-coated 35 mm glass-bottom dishes (MatTek, Ashland) (40 $\times$ 10³ cells/dish) and incubated in 5% CO₂ and 95% O₂ (overnight, 37 °C). HSCs cells were transduced with Green Upward cADDIS and incubated for 48 h. HSCs were washed twice in HBSS/HBS and mounted on a Leica DMI8 microscope (Wetzlar, Germany). Fluorescence (470/40 excitation, 527/30 emission) was measured every 5 s. The baseline was measured for 30 s, and HSCs were challenged with human CGRP α (100 nM). Images were analyzed with ImageJ (NIH). To inhibit endocytosis, cells were incubated in HBSS containing 0.45 M sucrose or normal HBSS (control) for 30 min at 37 °C before cAMP assays.

CAMYEL BRET cAMP assay. HEK293T cells stably expressing the CAMYEL BRET sensor (~2 $\times$ 10⁶) were seeded into 90 mm Petri dish (Corning®, USA) in DMEM/FBS/

Geneticin and incubated in 5% CO₂ and 95% O₂ (24 h, 37 °C). Prior to the transfection, the medium was changed to fresh DMEM/FBS/Geneticin and rat CLR/RAMP1 was transfected (2.5 µg CLR/RAMP1 DNA/dish) using JetPEI (Polyplus Transfection, France) at a 1:6 ratio. After 24 h, cells were plated in poly-L-lysine coated black 96 well CulturPlate (Perkin Elmer, USA) and incubated in 5% CO₂ and 95% O₂ (24 h, 37 °C). BRET was assessed using a LUMistar (BMG LABTECH, Germany). On the day of the assay, cells were equilibrated in HBSS for 30 min, supplemented with 12 mM HEPES at 37 °C in CO₂-free incubator. DIPMA-MK-3207 or free MK-3207 was incubated for 25 min, followed by the addition of coelentrazine-h (50 µM) for 5 min. Baseline was then measured for 5 min, followed by stimulation with CGRP (100 nM, ~EC₅₀), vehicle (HBSS) or forskolin (1 µM, positive control), and further measurements for 5 min. Buffer was then replaced by HBSS with coelentrazine-h (50 µM) and measurements were resumed for further 25 min.

Synthesis of TAMRA-CGRP and endocytosis of TAMRA-CGRP and Cy5-DIPMA nanoparticles. TAMRA-CGRP was synthesized by JPT Peptide Technologies (Berlin, Germany). A lysine residue conjugated to TAMRA was added to the C-terminus of human CGRPα (AICDTATCVTHRLAGLLSRSGGVVKNFVPT NVGSKAF37-K-TAMRA). Peptide purity was > 94%. HSCs were plated on poly-D-lysine-coated 35 mm glass-bottom dishes (MatTek) (50 × 10³ cells/dish) and incubated in 5% CO₂ and 95% O₂ (overnight, 37 °C). HSCs were transduced with the BacMan CellLight Early Endosomes Rab5a-GFP marker (5 µl/dish) and incubated in 5% CO₂ and 95% O₂ (48 h, 37 °C). HSCs were washed twice in HBSS/HEPES. Cells were incubated with the endocytosis inhibitors PS2 or Dy4 (both 30 µM), PS2 inact or Dy4 inact (30 µM), sucrose (0.45 M) or vehicle (control) for 30 min. Cells were imaged using Leica SP8 confocal microscope (Wetzlar), LAS X imaging software. Images were captured every 15 s. Baseline fluorescence was recorded for 2 min. TAMRA-CGRP (1 µM) was added and cells were imaged for 30 min. TAMRA-CGRP internalization to early endosomes at 30 min was quantified using ImageJ (National Institutes of Health). Regions of interest (ROIs) were defined by thresholding the channel for early endosomes (GFP) and particles were analyzed for size (≥0.2 µm²) and for roundness (0.2–1). ROIs were then overlaid on the TAMRA-CGRP channel and mean fluorescence was quantified for each ROI. Data are presented as relative fluorescent units (RFUs) per endosome and percentage of CGRP- + ve endosomes (endosomes with TAMRA-CGRP fluorescence >350 RFU) to total endosomes. Imaging experiments were repeated 5–7 times per group and a minimum of 4 cells were imaged for each experiment. To assess the internalization nanoparticles, HSCs were plated as described and incubated with DIPMA-Cy5 nanoparticles (40–60 µg/ml) for 1 h. Cells were washed and imaged as stated.

EbbRET assays of G protein and βARR recruitment to the plasma membrane and endosomes cDNAs. Mini-G proteins coupled to Venus were from N. A. Lambert (Augusta University). Constructs were modified to replace Venus with Rluc8. Renilla (R) GFP-CAAX (prenylation CAAX box of KRas), tRGFP-Rab5a, Rluc2-βARR2 and human CLR-Rluc8 were from M. Bouvier (Université de Montréal).

Transfection. For EbbRET assays of trafficking of mini-G proteins to the plasma membrane or early endosomes, HEK293T cells were transfected using JetPEI (Polyplus Transfection, France) with bicistronic human myc-CLR-NanoBit/HA-RAMP1 (0.2 µg) (NanoBit: GVTGWRLCERILA) and Rluc8-mGα_{ai/s/sq} (0.1 µg)^{70,71} and either RGFP-CAAX (0.2 µg)⁷² for cell surface activation or tRGFP-Rab5a (0.15 µg) for activation in early endosomes. Schwann cells were transfected using lipofectamine 3000 with bicistronic human myc-CLR-NanoBit/HA-RAMP1 (1 µg) and Rluc8-mGα_{ai/s/sq} (2 µg) and either RGFP-CAAX (1.5 µg) or tRGFP-Rab5a (1.5 µg). For EbbRET assays of recruitment of βARR2⁷² to the plasma membrane or early endosomes, HEK293T cells were transfected with JetPEI with bicistronic human myc-CLR-NanoBit/HA-RAMP1 (0.2 µg) and Rluc2-βARR2 (0.1 µg) and RGFP-CAAX (0.2 µg) for plasma membrane recruitment or tRGFP-Rab5a (0.15 µg) for early endosome recruitment. Schwann cells were transfected using lipofectamine 3000 with bicistronic human myc-CLR-NanoBit/HA-RAMP1 (1 µg) and Rluc2-βARR2 (2 µg) and RGFP-CAAX (1.5 µg) or tRGFP-Rab5a (1.5 µg). For EbbRET assay of trafficking of CLR from the plasma membrane to early endosomes, HEK293T cells were transfected using JetPEI with human myc-CLR-Rluc8 (0.7 µg), human myc-RAMP1 (0.5 µg) and either plasma membrane marker RGFP-CAAX (0.2 µg) or early endosome marker tRGFP-Rab5a (0.15 µg).

BRET assays. HEK293T cells and HSCs were washed with HBSS buffer complemented with 10 mM HEPES at pH 7.4. Prolume Purple Coelenterazine (2.5 µM, NanoLight Technology) was added, and cells were incubated for 5 min at 37 °C. EbbRET was recorded for 22.5 min in a Synergy Neo2 Microplate reader (BioTek) (acceptor filter: 515 ± 30 nm; donor filter: 410 ± 80 nm). The baseline was measured for 2.5 min, and cells were challenged with human CGRPα (100 nM) or vehicle. ΔBRET represents the EbbRET signal in the presence of agonist subtracted by the EbbRET signal in the presence of the vehicle. To inhibit endocytosis, cells were incubated in HBSS containing 0.45 M sucrose or normal HBSS (control) for 30 min at 37 °C before EbbRET assays.

Synthesis and Characterization of Nanoparticles

Synthesis of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA). The polymer was synthesized in a two-step sequential polymerization by the reversible addition-

fragmentation chain (RAFT) method⁷³, as previously described³⁴. First, the hydrophilic block, P(PEGMA-co-DMAEMA), was synthesized using the RAFT agent, 2-cyanoprop-2-yl dithiobenzoate (CPBD, 0.0736 g, 3.34 × 10⁻⁴ mol) and the initiator azobisisobutyronitrile (AIBN, 0.0054 g, 3.34 × 10⁻⁵ mol) in a ratio of 1:0.1. The monomers poly(ethylene glycol) monomethyl ether methacrylate (PEGMA, 6 g, 0.02 mol) and 2-[N,N-(dimethylamino)ethyl] methacrylate (DMAEMA, 0.314 g, 0.001 mol) were used at a ratio of 10:1. The mixture, dissolved in toluene, was deoxygenated by sparging with nitrogen and left to react at 70 °C, 75x g for 21 h. The remaining monomers were removed by dialysis (MWCO 3500, Membrane Filtration Products) against acetone for 96 h and the final product was dried for 24 h in a vacuum oven at 37 °C and 1 mbar.

The chain extension reaction to incorporate the monomers 2-[N,N-(diisopropylamino)ethyl] methacrylate (DIPMA) and di(ethylene glycol) methyl ether methacrylate (DEGMA) was initiated by AIBN (0.0009 g, 5.35 × 10⁻⁶ mol), using the hydrophilic block P(PEGMA-co-DMAEMA), 0.4455 g, 3.56 × 10⁻⁵ mol) and the monomers DIPMA (0.7603 g, 3.56 × 10⁻³ mol) and DEGMA (0.0738 g, 3.92 × 10⁻⁴ mol), at a ratio of 0.15:1:100:11. The mixture was dissolved in toluene, deoxygenated and left to react at 70 °C, 75 × g for 17.5 h. The final product, P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA), was purified as described above.

Synthesis of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5). The chain extension of P(PEGMA-co-DMAEMA, 0.5 g, 3.85 × 10⁻⁵ mol) was done in toluene by adding DIPMA (0.82 g, 3.85 × 10⁻³ mol), DEGMA (0.080 g, 4.23 × 10⁻⁴ mol) and 4,4-dimethyl-2-vinyl-2-oxazolin-5-one (VDM, 0.027 g, 1.92 × 10⁻⁴ mol), in the presence of AIBN (1 mg, 5.77 × 10⁻⁶ mol) at a ratio of 1:100:11:5:0.15. The mixture was deoxygenated and left to react at 70 °C, 75 kg for 18 h. Cy5 coupling was performed by mixing 250 µl of the reaction with Cyanine5 amine (Cy5, 0.008 g, 1.20 × 10⁻⁵ mol). The mixture was left to react at room temperature, 75x g for 72 h under dark conditions and the final product was purified as described above.

Analysis of diblock copolymer

Gel permeation chromatography (GPC). The molecular weights of polymers were determined by GPC using a liquid chromatography system equipped with a (RID-10A) differential refractive index detector (λ = 633 nm) and SPD-20A ultraviolet detector (Shimadzu). Samples were fractionated using 5.0 µm bead-size guard column (50 × 7.8 mm) and three Shodex KF-805L columns (300 × 8 mm, 10 µm n = bead-size, 5000 Å pore size) in series at 40 °C and eluted in N,N-dimethylacetamide (DMAC, HPLC grade, with 0.03% w/v LiBr) at a flow rate of 1 ml/min. A molecular weight calibration curve was produced using polystyrene standards ranging from 500 to 2 × 10⁶ Da.

Proton-nuclear magnetic resonance (¹H-NMR). The conversion and composition of polymers were assessed by ¹H-NMR using a Bruker Avance III 400 Ultrashield Plus spectrometer (USA) at 400 MHz running Topspin, version 1.3 and deuterated chloroform (chloroform-d) as solvent. Conversions (Conv%) and repeating monomer units (n) were calculated using peak integrals (I) where the subscript number indicates the location of the peak in ppm (I_x). The Conv% were calculated using the ¹H-NMR spectra before starting the reactions (t = 0) and after the reactions were stopped (t = f) (¹H-NMR spectra not shown), with

$$\text{Conv}\% = \left(1 - \frac{I_{6.24(t=0)}}{I_{6.24(t=f)}}\right) \times 100.$$

For P(PEGMA-co-DMAEMA), n was calculated using the ¹H-NMR spectra with $n_{\text{PEGMA}} = \frac{I_{4.25} - I_{2.5}}{I_{7.8} - I_{7.9}}$ and $n_{\text{DMAEMA}} = \frac{I_{3.5}}{I_{7.8} - I_{7.9}}$. For P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA), n were calculated using the ¹H-NMR spectra with $n_{\text{DIPMA}} = \frac{I_3 + I_{2.6}}{2 \times I_{4.4}}$ × (n_{PEGMA} + n_{DMAEMA}) and $n_{\text{DEGMA}} = \frac{I_{3.396}}{I_{3.378}} \times n_{\text{PEGMA}}$ (Supplementary Fig. 6) For P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA-co-VDM), n was calculated as described for P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA) (¹H-NMR spectra not shown).

Self-assembly of nanoparticles (NPs). The diblock copolymer, P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) was used to self-assemble pH-responsive NPs. For the self-assembly of NPs loaded with MK-3207 (DIPMA- MK-3207) a mixture of 5 mg of the diblock copolymer and 53.5 µg of MK-3207 was dissolved in 0.5 ml of dimethylformamide (DMF). Empty NPs (DIPMA-Ø) were self-assembled without adding MK-3207. The mixture was then added into 4.5 ml of PBS under stirring at a flow rate of 1.2 ml/h, using a syringe pump (Harvard Apparatus). Assemblies of DIPMA-MK-3207 and DIPMA-Ø were dialyzed against PBS for 24 h (MWCO 3500, Membrane Filtration Products). The assembly of NPs for live-cell imaging and biodistribution studies was done as described for DIPMA-Ø using the diblock copolymer P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5), which couples Cy5 on the hydrophobic block, resulting in NPs with Cy5 incorporated in the core (DIPMA-Cy5).

Characterization of NPs

Dynamic light scattering (DLS). The size distribution of DIPMA-MK-3207 and DIPMA-Ø (1 mg/ml) was determined by DLS (Zetasizer Nano ZS ZEN3600 particle size analyser), using polystyrene cuvettes at 25 °C and 173° backscatter angle.

Ultra-performance liquid chromatography-mass spectrometry (LC-MS). MK-3207 loading into the core of NPs was assessed by LC-MS using a Waters Micromass Quattro Premier triple quadrupole mass spectrometer coupled to a Waters Acquity UPLC (USA). Freeze-dried DIPMA- MK-3207 (1 ml, 1 mg/ml) were dissolved in a mixture of DMSO and formic acid 0.1% (5:2). The samples were prepared for analysis by mixing an aliquot of each preparation with internal standard solution (diazepam, 5 µg/ml) in a 5:2 proportion and made up to 500 µl with the dilution solvent (acetonitrile 50%: formic acid 0.1%, 1:1). Samples were fractionated on a Supelco Ascentis Express RP Amide column (50 mm by 2.1 mm, 2.7 µm particle size) equipped with a Phenomenex SecurityGuard precolumn fitted with a Synergi Polar cartridge, maintained at 40 °C. MK-3207 loading was quantified against MK-3207 standards (0.016–20 µM). Compounds were eluted under gradient conditions with a mobile phase of formic acid (0.05%) and acetonitrile. Mass spectrometry was conducted in positive electrospray ionization conditions and elution of compounds were monitored with multiple-reaction monitoring.

Transmission electron microscopy (TEM). The morphology of NPs was determined by TEM imaging using a Tecnai F20 transmission electron microscope at an accelerating voltage of 120 kV at room temperature. Carbon-coated grids were prepared by plasma discharge (35 s). DIPMA-MK-3207 samples (5 µl, 1 mg/ml) were placed on the grid for 20 s. Samples were negatively stained with uranyl acetate (5 µl, 0.5 wt %, 25 s).

Statistical analysis. Results are expressed as mean ± standard error of the mean (SEM). For multiple comparisons, a one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni's test or Dunnett's test was used. Two groups were compared using Student's *t*-test. For behavioral experiments with repeated measures, the two-way mixed model ANOVA followed by the post-hoc Bonferroni's test was used. Statistical analyses were performed on raw data using Graph Pad Prism 8 (GraphPad Software Inc.). IC₅₀ values and confidence intervals were determined from non-linear regression models using Graph Pad Prism 8 (GraphPad Software Inc.). *P* values less than 0.05 (*P* < 0.05) were considered significant. Statistical tests used and the sample size for each analysis are listed in the Fig. legend.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data are provided with this paper.

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References

- Lewis, T. Experiments Relating to Cutaneous Hyperalgesia and Its Spread Through Somatic Nerves. *Clin. Sci.* **2**, 373–414 (1936).
- Russell, F. A., King, R., Smillie, S. J., Kodji, X. & Brain, S. D. Calcitonin gene-related peptide: physiology and pathophysiology. *Physiol. Rev.* **94**, 1099–1142 (2014).
- Geppetti P., Holzer P. Neurogenic Inflammation. Boca Raton (1996).
- Szallasi, A. & Blumberg, P. M. Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* **51**, 159–212 (1999).
- Steinhoff, M. S., von Mentzer, B., Geppetti, P., Pothoulakis, C. & Bunnett, N. W. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol. Rev.* **94**, 265–301 (2014).
- La, J. H. et al. Differential involvement of reactive oxygen species in a mouse model of capsaicin-induced secondary mechanical hyperalgesia and allodynia. *Mol. Pain.* **13**, 1744806917713907 (2017).
- Ali, Z., Meyer, R. A. & Campbell, J. N. Secondary hyperalgesia to mechanical but not heat stimuli following a capsaicin injection in hairy skin. *Pain* **68**, 401–411 (1996).
- Sinclair, S. R. et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). *Br. J. Clin. Pharmacol.* **69**, 15–22 (2010).
- Edvinsson, L., Haanes, K. A., Warfvinge, K. & Krause, D. N. CGRP as the target of new migraine therapies - successful translation from bench to clinic. *Nat. Rev. Neurol.* **14**, 338–350 (2018).
- Ashina, H. Migraine. *N. Engl. J. Med.* **383**, 10 (2020).
- Doods, H., Arndt, K., Rudolf, K. & Just, S. CGRP antagonists: unravelling the role of CGRP in migraine. *Trends Pharmacol. Sci.* **28**, 580–587 (2007).
- Hostetler, E. D. et al. In vivo quantification of calcitonin gene-related peptide receptor occupancy by telcagepant in rhesus monkey and human brain using the positron emission tomography tracer [11C]MK-4232. *J. Pharmacol. Exp. Ther.* **347**, 478–486 (2013).
- Noseda, R. et al. Fluorescently-labeled fremanezumab is distributed to sensory and autonomic ganglia and the dura but not to the brain of rats with uncompromised blood brain barrier. *Cephalalgia* **40**, 229–240 (2020).
- Johnson, K. W., Morin, S. M., Wroblewski, V. J. & Johnson, M. P. Peripheral and central nervous system distribution of the CGRP neutralizing antibody [(125)I] galcanezumab in male rats. *Cephalalgia* **39**, 1241–1248 (2019).
- Mogil, J. S. et al. Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc. Natl. Acad. Sci. USA* **102**, 12938–12943 (2005).
- Rea, B. J. et al. Peripherally administered calcitonin gene-related peptide induces spontaneous pain in mice: implications for migraine. *Pain* **159**, 2306–2317 (2018).
- De Logu, F. et al. Migraine-provoking substances evoke periorbital allodynia in mice. *J. Headache Pain* **20**, 18 (2019).
- Marone, I. M. et al. TRPA1/NOX in the soma of trigeminal ganglion neurons mediates migraine-related pain of glyceryl trinitrate in mice. *Brain* **141**, 2312–2328 (2018).
- Ashina, M., Hansen, J. M., BO, A. D. & Olesen, J. Human models of migraine - short-term pain for long-term gain. *Nat. Rev. Neurol.* **13**, 713–724 (2017).
- Lipton, R. B. et al. Cutaneous allodynia in the migraine population. *Ann. Neurol.* **63**, 148–158 (2008).
- Burstein, R., Yarnitsky, D., Goor-Aryeh, I., Ransil, B. J. & Bajwa, Z. H. An association between migraine and cutaneous allodynia. *Ann. Neurol.* **47**, 614–624 (2000).
- Goadsby, P. J. et al. Pathophysiology of Migraine: A Disorder of Sensory Processing. *Physiol. Rev.* **97**, 553–622 (2017).
- May, A. & Burstein, R. Hypothalamic regulation of headache and migraine. *Cephalalgia* **39**, 1710–1719 (2019).
- McLatchie, L. M. et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393**, 333–339 (1998).
- Lennerz, J. K. et al. Calcitonin receptor-like receptor (CLR), receptor activity-modifying protein 1 (RAMP1), and calcitonin gene-related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: differences between peripheral and central CGRP receptor distribution. *J. Comp. Neurol.* **507**, 1277–1299 (2008).
- Beckers, H. J., Klooster, J., Vrensen, G. F. & Lamers, W. P. Ultrastructural identification of trigeminal nerve terminals in the pterygopalatine ganglion of rats: an anterograde tracing and immunohistochemical study. *Brain Res.* **557**, 22–30 (1991).
- Cheng, L., Khan, M. & Mudge, A. W. Calcitonin gene-related peptide promotes Schwann cell proliferation. *J. Cell Biol.* **129**, 789–796 (1995).
- Edvinsson, J. C. A. et al. C-fibers may modulate adjacent Aδ-fibers through axon-axon CGRP signaling at nodes of Ranvier in the trigeminal system. *J. Headache Pain.* **20**, 105 (2019).
- Toth, C. C. et al. Locally synthesized calcitonin gene-related Peptide has a critical role in peripheral nerve regeneration. *J. Neuropathol. Exp. Neurol.* **68**, 326–337 (2009).
- De Logu, F. et al. Schwann cell TRPA1 mediates neuroinflammation that sustains macrophage-dependent neuropathic pain in mice. *Nat. Commun.* **8**, 1887 (2017).
- De Logu F., et al. Peripheral Nerve Resident Macrophages and Schwann Cells Mediate Cancer-induced Pain. *Cancer. Res.* **81**, 3387–3401 (2021).
- Abdo, H. et al. Specialized cutaneous Schwann cells initiate pain sensation. *Science* **365**, 695–699 (2019).
- Jensen D. D., et al. Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci. Transl. Med.* **9**, eaal3447 (2017).
- Ramirez-García, P. D. et al. A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain. *Nat. Nanotechnol.* **14**, 1150–1159 (2019).
- Yarwood, R. E. et al. Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc. Natl. Acad. Sci. USA* **114**, 12309–12314 (2017).
- Talavera, K. et al. Mammalian Transient Receptor Potential TRPA1 Channels: From Structure to Disease. *Physiol. Rev.* **100**, 725–803 (2020).
- Lassen, L. H. et al. CGRP may play a causative role in migraine. *Cephalalgia* **22**, 54–61 (2002).
- Guo, S. et al. Selectivity of commonly used inhibitors of clathrin-mediated and caveolae-dependent endocytosis of G protein-coupled receptors. *Biochimica et Biophysica Acta (BBA)*. *Biomembranes* **1848**, 2101–2110 (2015).
- Hosaka, K. et al. Calcitonin gene-related peptide activates different signaling pathways in mesenteric lymphatics of guinea pigs. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H813–H822 (2006).
- Miyamoto, T., Dubin, A. E., Petrus, M. J. & Patapoutian, A. TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS One* **4**, e7596 (2009).
- Bhattacharya, M. R. et al. Radial stretch reveals distinct populations of mechanosensitive mammalian somatosensory neurons. *Proc. Natl. Acad. Sci. USA* **105**, 20015–20020 (2008).

42. De Logu, F. et al. Schwann cells expressing nociceptive channel TRPA1 orchestrate ethanol-evoked neuropathic pain in mice. *J. Clin. Invest.* **129**, 5424–5441 (2019).
43. Farokhzad, O. C. & Langer, R. Impact of nanotechnology on drug delivery. *ACS nano* **3**, 16–20 (2009).
44. LaMotte, R. H., Shain, C. N., Simone, D. A. & Tsai, E. F. Neurogenic hyperalgesia: psychophysical studies of underlying mechanisms. *J. Neurophysiol.* **66**, 190–211 (1991).
45. Melo-Carrillo, A. et al. Fremanezumab-A Humanized Monoclonal Anti-CGRP Antibody-Inhibits Thinly Myelinated (Adelta) But Not Unmyelinated (C) Meningeal Nociceptors. *J. Neurosci.* **37**, 10587–10596 (2017).
46. Weidner, C. et al. Acute effects of substance P and calcitonin gene-related peptide in human skin—a microdialysis study. *J. Invest. Dermatol.* **115**, 1015–1020 (2000).
47. Murinson, B. B., Hoffman, P. N., Banihashemi, M. R., Meyer, R. A. & Griffin, J. W. C-fiber (Remak) bundles contain both isolectin B4-binding and calcitonin gene-related peptide-positive axons. *J. Comp. Neurol.* **484**, 392–402 (2005).
48. Ziegler, E. A., Magerl, W., Meyer, R. A. & Treede, R. D. Secondary hyperalgesia to punctate mechanical stimuli. Central sensitization to A-fibre nociceptor input. *Brain* **122**, 2245–2257 (1999).
49. Irannejad, R. et al. Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**, 534–538 (2013).
50. Lazar, A. M. et al. G protein-regulated endocytic trafficking of adenylyl cyclase type 9. *Elife* **9**, e58039 (2020).
51. Peng, G. E., Pessino, V., Huang, B., von Zastrow, M. Spatial decoding of endosomal cAMP signals by a metastable cytoplasmic PKA network. *Nat. Chem. Biol.* **17**, 558–566 (2021).
52. Olesen, J., Burstein, R., Ashina, M. & Tfelt-Hansen, P. Origin of pain in migraine: evidence for peripheral sensitisation. *Lancet. Neurol.* **8**, 679–690 (2009).
53. Mullin, K. et al. Potential for treatment benefit of small molecule CGRP receptor antagonist plus monoclonal antibody in migraine therapy. *Neurology* **94**, e2121–e2125 (2020).
54. Luo, G. et al. Discovery of (5S,6S,9R)-5-amino-6-(2,3-difluorophenyl)-6,7,8,9-tetrahydro-5H-cyclohepta[b]pyridine-9-yl 4-(2-oxo-2,3-dihydro-1H-imidazo[4,5-b]pyridin-1-yl)piperidine-1-carboxylate (BMS-927711): an oral calcitonin gene-related peptide (CGRP) antagonist in clinical trials for treating migraine. *J. Med. Chem.* **55**, 10644–10651 (2012).
55. Manoukian, R. et al. Effects of monoclonal antagonist antibodies on calcitonin gene-related peptide receptor function and trafficking. *J. Headache Pain* **20**, 44 (2019).
56. Kwan, K. Y. et al. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50**, 277–289 (2006).
57. Skarnes, W. C. et al. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337–342 (2011).
58. Guan, Z. et al. Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nat. Neurosci.* **19**, 94–101 (2016).
59. Zurborg, S. et al. Generation and characterization of an Advillin-Cre driver mouse line. *Mol. Pain* **7**, 66 (2011).
60. Faul, F., Erdfelder, E., Lang, A. G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175–191 (2007).
61. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M. & Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **8**, e1000412 (2010).
62. Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**, 109–110 (1983).
63. Watabe, K. et al. Spontaneously immortalized adult mouse Schwann cells secrete autocrine and paracrine growth-promoting activities. *J. Neurosci. Res.* **41**, 279–290 (1995).
64. Watabe, K. et al. Tissue culture methods to study neurological disorders: establishment of immortalized Schwann cells from murine disease models. *Neuropathology* **23**, 68–78 (2003).
65. Luccarini, P., Childeric, A., Gaydier, A. M., Voisin, D. & Dalle, R. The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception. *J. Pain* **7**, 908–914 (2006).
66. Dixon, W. J. Efficient analysis of experimental observations. *Annu. Rev. Pharmacol. Toxicol.* **20**, 441–462 (1980).
67. Chaplan, S. R., Bach, F. W., Pogrel, J. W., Chung, J. M. & Yaksh, T. L. Quantitative assessment of tactile allodynia in the rat paw. *J. Neurosci. Methods* **53**, 55–63 (1994).
68. Elliott, M. B., Oshinsky, M. L., Amenta, P. S., Awe, O. O. & Jallo, J. I. Nociceptive neuropeptide increases and periorbital allodynia in a model of traumatic brain injury. *Headache* **52**, 966–984 (2012).
69. Tao, Y. Isolation and culture of Schwann cells. *Methods Mol. Biol.* **1018**, 93–104 (2013).
70. Nehme, R. et al. Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PLoS One* **12**, e0175642 (2017).
71. Wan, Q. et al. Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. *J. Biol. Chem.* **293**, 7466–7473 (2018).
72. Namkung, Y. et al. Monitoring G protein-coupled receptor and beta-arrestin trafficking in live cells using enhanced bystander BRET. *Nat. Commun.* **7**, 12178 (2016).
73. Chiefari, J. et al. Living Free-Radical Polymerization by Reversible Addition—Fragmentation Chain Transfer: The RAFT Process. *Macromolecules* **31**, 5559–5562 (1998).

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Author contributions

F.D.L., R.N., A.H., L.L., D.D.J., R.L., J.D., M.M., D.S.M.d.A., P.R.-G., M.W., J.R., M.T., A.I., N.V., T.P.D., B.L.S., N.W.B., P.G., designed and conducted the experiments, performed the analysis, discussed the results, and wrote the paper.

Competing interests

N.W.B. is a founding scientist of Endosome Therapeutics Inc. P.G. is a member of the Scientific Advisory Board of Endosome Therapeutics Inc. The laboratory of N.V. and M.W. has been funded in-part by Endosome Therapeutics Inc. R.N., F.D.L. and P.G. are founding scientists of FloNext Srl. P.G. has been in advisory boards and/or received fees for lectures from Novartis, Amgen, TEVA, AbbVie. The remaining authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Nigel W. Bunnett or Pierangelo Geppetti.

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Opioid-Induced Pronociceptive Signaling in the Gastrointestinal Tract Is Mediated by Delta-Opioid Receptor Signaling

 Josue Jaramillo-Polanco,^{1*} Cintya Lopez-Lopez,^{1*} Yang Yu,¹ Emma Neary,¹ Alan Hegron,² Meritxell Canals,^{3,4} Nigel W. Bunnett,² David E. Reed,¹ Alan E. Lomax,¹ and Stephen J. Vanner¹

¹Gastrointestinal Diseases Research Unit, Kingston General Hospital, Queen's University, Kingston, Ontario K7L 2V7, Canada, ²Department of Molecular Pathobiology, Department of Neuroscience and Physiology, Neuroscience Institute, New York University, New York, New York 10010, ³Division of Physiology, Pharmacology and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, United Kingdom, and ⁴Centre of Membrane Protein and Receptors, Universities of Birmingham and Nottingham, Nottingham, NG7 2UH, United Kingdom

Opioid tolerance (OT) leads to dose escalation and serious side effects, including opioid-induced hyperalgesia (OIH). We sought to better understand the mechanisms underlying this event in the gastrointestinal tract. Chronic *in vivo* administration of morphine by intraperitoneal injection in male C57BL/6 mice evoked tolerance and evidence of OIH in an assay of colonic afferent nerve mechanosensitivity; this was inhibited by the δ -opioid receptor (DOPr) antagonist naltrindole when intraperitoneally injected in previous morphine administration. Patch-clamp studies of DRG neurons following overnight incubation with high concentrations of morphine, the μ -opioid receptors (MOPr) agonist [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO) or the DOPr agonist [D-Ala², D-Leu⁵]-Enkephalin evoked hyperexcitability. The pronociceptive actions of these opioids were blocked by the DOPr antagonist SDM25N but not the MOPr antagonist D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂. The hyperexcitability induced by DAMGO was reversed after a 1 h washout, but reapplication of low concentrations of DAMGO or [D-Ala², D-Leu⁵]-Enkephalin restored the hyperexcitability, an effect mediated by protein kinase C. DOPr-dependent DRG neuron hyperexcitability was blocked by the endocytosis inhibitor Pitstop 2, and the weakly internalizing DOPr agonist ARM390 did not cause hyperexcitability. Bioluminescence resonance energy transfer studies in HEK cells showed no evidence of switching of G-protein signaling from G_i to a G_s pathway in response to either high concentrations or overnight incubation of opioids. Thus, chronic high-dose opioid exposure leads to opioid tolerance and features of OIH in the colon. This action is mediated by DOPr signaling and is dependent on receptor endocytosis and downstream protein kinase C signaling.

Key words: colonic afferent nerves; delta-opioid receptor; dorsal root ganglia; mu-opioid receptor; opioid tolerance; opioid-induced hyperalgesia

Significance Statement

Opioids are effective in the treatment of abdominal pain, but escalating doses can lead to opioid tolerance and potentially opioid-induced hyperalgesia. We found that δ -opioid receptor (DOPr) plays a central role in the development of opioid tolerance and opioid-induced hyperalgesia in colonic afferent nociceptors following prolonged exposure to high concentrations of MOPr or DOPr agonists. Furthermore, the role of DOPr was dependent on OPr internalization and activation of a protein kinase C signaling pathway. Thus, targeting DOPr or key components of the downstream signaling pathway could mitigate adverse side effects by opioids.

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*J.J.-P. and C.L.-L. contributed equally to this work as co-first authors.

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Correspondence should be addressed to Stephen J. Vanner at stephen.vanner@kingstonhsc.ca.

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Introduction

Chronic abdominal pain is a debilitating symptom in gastrointestinal disorders, such as irritable bowel syndrome and inflammatory bowel disease. While opioid drugs are effective, they cause serious side effects (Bielefeldt et al., 2009; Targownik et al., 2014). Opioid tolerance (OT), where increasing opioid doses are required to maintain the same level of analgesia, underlies escalating opioid dosing and the resulting risk of serious side effects (Allouche et al., 2014; Cahill et al., 2016; Stein, 2016, 2018).

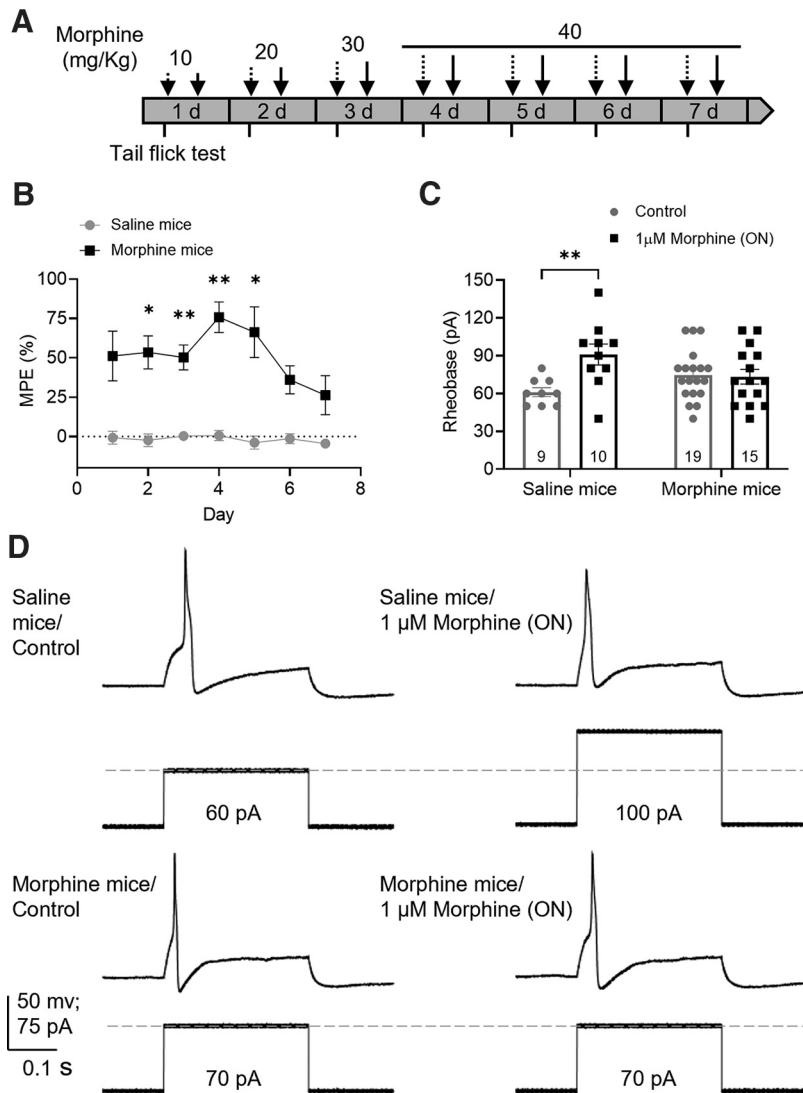


Figure 1. Chronic morphine treatment induces tolerance in mouse DRG neurons. **A**, Schematic protocol of chronic morphine treatment. Dashed arrows indicate morning i.p. injections. Solid arrows indicate afternoon i.p. injections. Tail-flick assay (short ticks) was performed before and after morning injections. **B**, *In vivo* tail-flick test showed that morphine had an antinociceptive effect compared with saline ($F_{(1,10)} = 117.5$, $p < 0.0001$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test); this effect declined after day 4 despite high doses suggesting OT. Results are expressed as the mean of % MPE $\pm$ SEM, saline mice = 6; morphine mice = 6. **C**, Effect of overnight 1 μ M morphine on rheobase of DRG neurons measured by patch clamp from saline and morphine-treated mice shows tolerance to morphine in neurons from morphine-treated mice ($F_{(1,49)} = 5.57$, $p = 0.022$, two-way ANOVA with Bonferroni's *post hoc* test). Number of cells appears in each bar. * $p \leq 0.05$. ** $p \leq 0.01$. **D**, Representative recordings of the rheobase (minimal current to elicit an action potential) of DRG neurons from saline and morphine-treated mice exposed to vehicle (control) or morphine overnight.

Moreover, there is evidence that increasing doses can lead to a paradoxical switch in signaling leading to enhanced pain (Mattioli et al., 2014), a condition called opioid-induced hyperalgesia (OIH), although this phenomenon is poorly understood.

Nociceptive signaling in the intestine is mediated by the small C-fiber and larger A δ -fiber DRG neurons (Robinson and Gebhart, 2008; Kyloh et al., 2011) whose distal axons innervate the intestine and central axons project to second-order neurons in the dorsal horn of the spinal cord. Opioid drugs and endogenous opioids bind to G-protein-coupled μ -, δ -, or κ -opioid receptors (MOPr, DOPr, KOPr) on the plasma membrane of these neurons (Stein, 2016; Spahn et al., 2018). In addition to conventional G-protein-mediated signaling, recent evidence also

revealed that β -arrestins (β arrests) couple receptors to clathrin and adaptor protein-2 and guide receptors and ligands to endosomes where further signaling occurs (Jensen et al., 2017; Jimenez-Vargas et al., 2018, 2020; Stoeber et al., 2018).

A large body of work has shown that the molecular mechanisms underlying OT are remarkably complex, and several mechanisms have been proposed, including opioid receptor interactions, receptor phosphorylation, G-protein uncoupling, and altered receptor internalization/recycling (Varga et al., 2004; Williams et al., 2013; Allouche et al., 2014; Galligan and Akbarali, 2014). Several studies have highlighted the importance of β arr2 in the development of morphine antinociceptive tolerance (Bohn et al., 1999; Raehal and Bohn, 2011; Kliewer et al., 2019; Grim et al., 2020; He et al., 2021). Interestingly, the mechanisms of tolerance may be tissue-dependent, further highlighting the need for studies in the intestine.

The analgesic actions of conventional opioid drugs in the intestine, such as morphine, are thought to be mediated primarily by MOPr activation, although DOPr signaling may also have important analgesic actions despite being less well studied (Pergolizzi et al., 2020). Recent clinical studies have shown that drugs targeting multiple opioid receptors, such as a novel agent with mixed effects (MOPr agonist/DOPr antagonist), in the GI tract may have sustained efficacy (Lacy, 2018), but their mechanism of action is poorly understood. To better understand the mechanisms of OT in nociceptive signaling in the intestine and the roles of MOPr and DOPr, we used a model of chronic morphine administration to induce OT and features of OIH. We found evidence of a significant functional MOPr–DOPr interaction underlying these phenomena and sought to understand the mechanisms involved.

Materials and Methods

Materials. SDM25N hydrochloride (SDM25N), D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP), naloxone hydrochloride (NLX), AR-M1000390 hydrochloride (ARM390), H 89 hydrochloride (H89), and GF 109203 $\times$ (GFX) were from Tocris Bioscience. Pitstop 2 (PS2) was from Abcam, and other reagents, including [D-Ala², D-Leu⁵]-Enkephalin (DADLE), and [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO), and naltrindole hydrochloride (NTI), were from Sigma Aldrich, unless stated otherwise.

Animals. All studies were approved by the Queen's University animal care committees in accordance with guidelines from their National Council of Animal Care. C57BL/6 mice (males and in a small series, females; 8–12 weeks, Charles River Laboratories) were maintained under controlled temperature ($22 \pm 1^\circ\text{C}$) and light (12 h light/dark cycle) with free access to food and water.

Chronic morphine treatment. Morphine (Sandoz) was administered by intraperitoneal (i.p.) injection twice daily (9:00 A.M. and 6:00 P.M.) for 7 d, at increasing dose (10 mg/kg first day, 20 mg/kg second day, 30 mg/kg third day, 40 mg/kg fourth to seventh day; see Fig. 1A) (Mattioli et al., 2014). Control mice were injected with saline solution

(NaCl 0.9% i.p.) twice daily for 7 d. On day 8, under general anesthetic (ketamine/xylazine), mice were killed, and the DRGs and colons were excised. Where indicated, mice were preinjected with 2.5 mg/kg i.p. NTI 20 min before morphine injections (Matthes et al., 1998). Mice weight was monitored each day during the protocol.

Tail-flick assay. The *in vivo* antinociceptive effect of chronic morphine treatment was tested using the tail-flick assay. Mice were gently placed head first into a restrainer with air holes with both legs inside the restrainer and tail exposed, and the distal one-third of the tail was immersed in a water bath at 52°C. The latency to tail-flick was video recorded before (to determine the baseline) and 30 min after the morning injection with a cutoff time of 10 s to prevent tissue damage. Each assay was performed in triplicate. The latency was measured later in videos starting from the immersion of the tail in the water until the first tail-flick. The investigator analyzing videos was blinded to treatments. Data are presented as percent of maximum possible effect (% MPE) = [(post-drug latency – baseline latency) ÷ (10 – baseline latency)] × 100 (Mattioli et al., 2014; Kang et al., 2017). Attenuation of the maximal antinociceptive effect of morphine overtime was considered as OT.

Retrograde labeling of colon-projecting DRG neurons. To identify colon-projecting DRG neurons, surgeries were performed to inject the retrograde tracer Fast Blue (17740-1, Polysciences) into the colon wall. Female mice were anesthetized via isoflurane inhalation (1.5%–2.5% isoflurane; oxygen flow: 1 L/min), placed on a heating pad, and subjected to midline laparotomy. The colon was carefully exposed, and Fast Blue (1.7% w/v in sterile water) was injected in small volumes (1–2 µl) into multiple sites on the colon wall. The gut was wiped down after each injection to remove excess dye. Bupivacaine (2 mg/kg intradermally) and tramadol (20 mg/kg subcutaneously) were given before surgery, and tramadol (20 mg/kg subcutaneously) was given daily for 3 d as a postoperative analgesic. Mice were killed 7–14 d after surgery, and DRGs (T9–T13) were acutely dissociated for patch-clamp recordings.

Isolation of DRG neurons. DRG neurons were acutely dissociated, as previously described (Ibeakanma et al., 2011). Briefly, mice were killed after ketamine/xylazine i.p. injection, and DRGs from T9–T13 were dissected and collected on Hanks' balanced salt solution. Neurons were enzymatically and mechanically dissociated in F-12 supplemented media (10% FBS, penicillin, streptomycin), plated on Poly-D lysine/laminin-coated coverslips, and placed at 37°C (95% O₂ and 5% CO₂) for 2 h before being incubated overnight with F-12 supplemented media. Electrophysiological recordings were performed the following day. Each treatment was tested on DRG neurons from 6–8 male mice, with exception of the recordings on retrogradely labeled DRG neurons that were obtained from 2 female mice.

Patch-clamp recordings. All experiments were performed at room temperature as previously described (Valdez-Morales et al., 2013). Patch pipettes were pulled (PC-100 Puller, Narishige) and fire-polished (3–5 MΩ) from borosilicate glass capillaries (Warner Instruments). Voltage-clamp recordings were performed by using Multiclamp 700B amplifier, digitized by Digidata 1440A AD converter, and recorded using pClamp 10.5 software (all by Molecular Devices). Dissociated DRG neurons were incubated acutely (30 min) or overnight at 37°C with morphine, the selective MOPr agonist DAMGO (Yekkirala et al., 2010) or the selective DOPr agonists DADLE (Raynor et al., 1994) or ARM390 (Marie et al., 2003). The OPr antagonists NLX (Wang et al., 2008), CTOP (Raynor et al., 1994), and SDM25N (McLamore et al., 2001), PKA inhibitor H89 (Davies et al., 2000), PKC inhibitor GFX (Toullec et al., 1991), or the selective inhibitor of clathrin-mediated endocytosis PS2 (Robertson et al., 2014) were incubated 30 min before the application of agonists. All cells with a membrane capacitance ≤30 pF and a resting membrane potential more negative than –40 mV were analyzed (Moore et al., 2002; Stewart et al., 2003). Where indicated, colon-projecting DRG neurons were identified by the Fast Blue fluorescence emitted from labeled cell bodies under short exposure to UV light with a 365 nm filter.

Perforated patch-clamp recordings were obtained with amphotericin B (0.24 mg/ml) (Ibeakanma et al., 2011), and neuronal excitability was assessed by measuring the rheobase and the action potential discharge at twice rheobase. The extracellular solution composition was as follows (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 D-glucose, pH

7.4. The pipette solution composition was as follows (mM): 110 K-glucuronate, 30 KCl, 10 HEPES, 1 MgCl₂, 2 CaCl₂, pH 7.25.

Ex vivo colonic afferent nerve recordings. Distal colon along with attached mesentery (containing the interior mesenteric ganglion) was removed intact and placed in an organ bath continuously superfused with carbonated (5% CO₂ and 95% O₂) Krebs buffer (composition, in mM as follows: NaCl, 118.4; NaHCO₃, 24.9; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose, 11.7; CaCl₂, 1.9) at 34°C. Krebs contained the L-type calcium channel blocker nifedipine (3 µM) and the muscarinic acetylcholine receptor antagonist atropine (5 µM) to suppress smooth muscle activity (Zar et al., 1990), as well as the cyclooxygenase inhibitor indomethacin (3 µM) to suppress potential inhibitory actions of endogenous prostaglandins (Peiris et al., 2017). The preparations were opened longitudinally along the mesenteric border and pinned flat with the mucosal side up. Lumbar splanchnic nerve was identified in the neurovascular bundle, teased apart into 5–6 fibers, and individually drawn into a glass suction electrode attached to a Neurolog headstage (NL100, Digitimer). Afferent nerve signals were amplified ×10,000 (NL104), filtered (150–800 Hz, NL125 band pass filter), and recorded on a computer via a Micro 1401 interface and Spike 2 software (version 7, Cambridge Electronic Design).

Receptive fields were identified by systematically stroking the mucosal surface or the mesenteric attachment with a brush. Once identified, receptive fields were tested with three distinct mechanical stimuli to allow classification: probing (with 1 g von Frey filament), mucosal stroking (0.4 g von Frey filament), and circumferential stretch. The circumferential stretch was applied manually by holding the gut wall with forceps and stretching gently. Vascular afferents that only respond to probing on the gut wall or the mesenteric attachment were included in this study since they are thought to be nociceptors (Brookes et al., 2013). Following a 30 min equilibration period, control probing responses (1 g) of each unit were examined by probing 3 times, each for 3 s with 10 s intervals between probings. Morphine (1 µM) was superfused into the organ bath for 15 min before reassessment of the probing response. Single-unit analysis was performed offline using the spike sorting function of Spike2 to discriminate the afferent nerve activity of individual units. The afferent nerve response to probing was assessed as average firing frequency during a 3 s period using a custom-made script in Spike2. Baseline activity was assessed as average firing frequency during a 120 s period after equilibration and reassessed in the presence of morphine. The investigator recording from afferent nerves was blinded to the treatments.

cDNAs. DOPr and MOPr were from GenCopoeia. GRK2 was from D. Jensen (Columbia University). RGFP-CAAX, Rluc2-*Gai/s/q*, GFP10-Gγ2, Gβ1, and Rluc2-βarrestin-2 were from M. Bouvier (Université de Montréal) (Gales et al., 2006; Namkung et al., 2016).

Cell culture and transfection. For bioluminescence resonance energy transfer (BRET) assays of G protein activation, HEK293T cells were cultured and transfected, as described previously (Jimenez-Vargas et al., 2020), using JetPEI (Polyplus Transfection) with DOPr and MOPr (0.2 µg), GRK2 (0.2 µg) and Rluc2-*Gai/s/q* (0.2 µg), and GFP10-Gγ2 (0.4 µg) with Gβ1 (0.1 µg). For ebBRET of βarr2 recruitment to the membrane, HEK293T cells were transfected with DOPr and MOPr (0.2 µg), GRK2 (0.2 µg), Rluc2-βarr2 (0.1 µg), and RGFP-CAAX (0.4 µg) for plasma membrane recruitment (Namkung et al., 2016).

BRET assays G protein activations and βarr recruitment to the plasma membrane. Transfected HEK293T cells were incubated overnight with vehicle, DADLE 10 µM, or DAMGO 10 µM. On the day of the assay, HEK293T cells were washed with Dulbecco's PBS, and then Tyrode's buffer was added, and cells were incubated for 30 min at 37°C. After the addition of Prolume Purple Coelenterazine (2.5 µM; NanoLight Technology), the cells were incubated for 5 min at 37°C. BRET was recorded for 22.5 min in a Synergy Neo2 Microplate reader (BioTek) (acceptor filter: 515 ± 30 nm; donor filter: 410 ± 80 nm). Cells were challenged with vehicle, DADLE 10 nM, DADLE 10 µM, DAMGO 10 nM, or DAMGO 10 µM after 2.5 min. Δ-BRET represents the BRET signal in the presence of agonist subtracted by the BRET signal in the presence of vehicle.

Statistical analyses. Data were analyzed using Prism 9 Software (GraphPad Software), and results are expressed as mean ± SEM. Normal distribution was assessed by parametric testing D'Agostino-

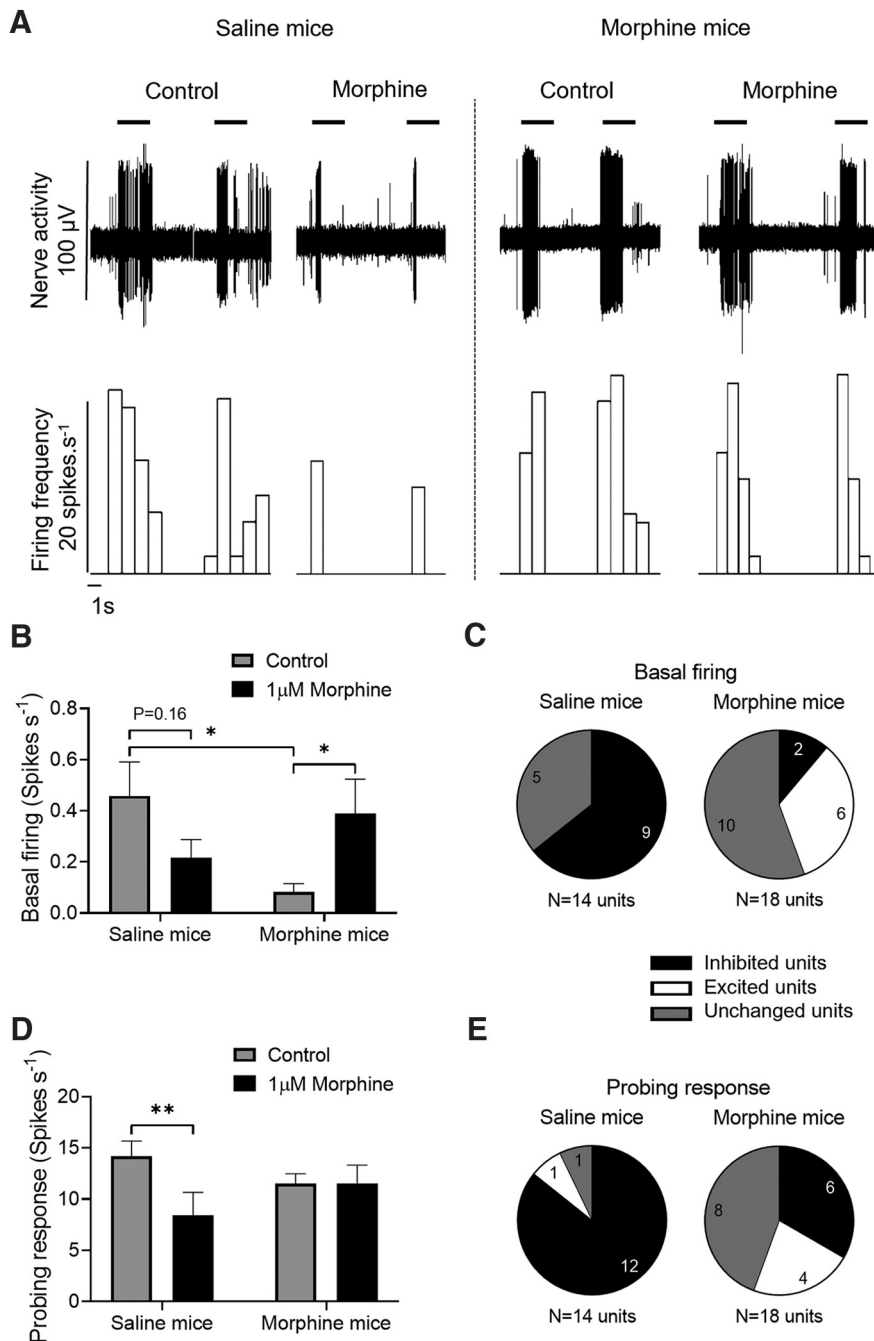


Figure 2. Chronic morphine treatment induces tolerance and OIH in mouse colon. Effect of acute application of 1 μ M morphine on the basal activity and probing response of colonic afferent axons from saline and morphine-treated mice. **A**, Representative recording of colonic nerve response to 1 g von Frey filament probing before and after administration of 1 μ M morphine. **B**, Acute morphine application did not affect the basal firing in afferent axons from saline-treated mice, while it increased firing in afferent axons from morphine-treated mice ($F_{(1,28)} = 10.06$, $p = 0.0037$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). **C**, Analysis of basal nerve activity showed an increased number of excited units on morphine-treated mice, whereas most of the units were inhibited in saline-treated mice (χ^2 , $df = 5.269$, 1 ; $p = 0.022$). **D**, Acute application of 1 μ M morphine inhibited the colonic afferent nerve response to probing in saline-treated mice but not in morphine-treated mice ($F_{(1,30)} = 6.35$, $p = 0.0173$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). **E**, Chronic exposure to morphine increased the number of units that were excited on probing response after acute morphine application (χ^2 , $df = 8.283$, 1 ; $p = 0.004$). **B**, **D**, Error bars indicate mean $\pm$ SEM. * $p \leq 0.05$. ** $p \leq 0.01$.

contingency table and then analyzed with the χ^2 test for trend. A p value ≤ 0.05 was considered significant.

Results

OT and colonic OIH

Mice were treated with morphine for 7 d, using an established model of OIH (Fig. 1A) (Ferrini et al., 2013; Mattioli et al., 2014). Tolerance of somatic nerves was assessed using a tail-flick test in conscious mice. Morphine's antinociceptive effect was maximum on day 4 and declined over the following days despite high opioid doses (Fig. 1B; $p = 0.002$, two-way repeated-measures ANOVA, $N = 6$ saline-, 6 morphine-treated mice), suggesting development of OT (Mattioli et al., 2014; Kang et al., 2017). Patch-clamp recordings from DRG neurons isolated from morphine- and saline-treated mice demonstrated that overnight application of morphine (1 μ M) inhibited neurons from saline-treated mice (rheobase increased by 49%; $p = 0.006$, two-way ANOVA, $n = 9$ control cells, 10 morphine-incubated cells/6 mice) but had no effect on neurons from mice treated with chronic morphine (Fig. 1C,D; $p > 0.99$, two-way ANOVA, $n = 15$ control cells, 19 morphine-incubated cells/6 mice).

To determine the effect of chronic morphine administration on excitability of afferent nerves innervating the colon, recordings were obtained from colons dissected from saline- and morphine-treated mice (Fig. 2A). Chronic morphine treatment decreased basal firing of afferent nerves (Fig. 2B; $p = 0.028$, two-way repeated-measures ANOVA, $n = 12$ units/6 saline-treated mice, 18 units/6 morphine-treated mice). However, while the acute application of morphine (1 μ M) reduced the basal firing activity of afferent nerves from saline-treated mice ($p = 0.16$, two-way repeated-measures ANOVA, $n = 12$ units/6 saline-treated mice), it evoked a paradoxical increase in basal firing in afferent nerves from morphine-treated mice (Fig. 2B; $p = 0.018$, two-way repeated-measures ANOVA, $n = 18$ units/6 morphine-treated mice). Similarly, morphine also inhibited the mechanosensitive response (von Frey filament, 1 g) in saline-treated mice but did not alter mechanosensitive responses in the morphine-treated mice (Fig. 2D; $p = 0.004$, two-way repeated-measures ANOVA, $n = 14$ units/6 saline-treated mice). Based on responses to the superfusion of morphine, units were classified in three groups: excited ($>20\%$ increase over baseline or control probing response), inhibited ($>20\%$

Pearson. Differences between two groups were examined using unpaired t test, whereas multiple groups were examined using one-way ANOVA with Dunnett's or Tukey's *post hoc* test as indicated, or two-way ANOVA with Bonferroni's *post hoc* test for multiple comparisons. A p value ≤ 0.05 was considered significant. After classification of unit responses, data were loaded into a

the morphine-treated mice (Fig. 2D; $p = 0.004$, two-way repeated-measures ANOVA, $n = 14$ units/6 saline-treated mice). Based on responses to the superfusion of morphine, units were classified in three groups: excited ($>20\%$ increase over baseline or control probing response), inhibited ($>20\%$

decrease from baseline or control probing response), and unchanged ($<20\%$ change) (Yu et al., 2019). Compared with saline-treated mice in which acute morphine application inhibited both basal firing (Fig. 2C; χ^2 , $df=5.269$, 1 ; $p=0.022$) and mechanosensitivity (Fig. 2E; χ^2 , $df=8.283$, 1 ; $p=0.004$) in most afferent units, morphine-treated mice had significantly fewer units with decreased firing and a greater number with increased or unchanged excitability.

Together, these studies demonstrate the development of OT in response to chronic morphine and provide evidence of a paradoxical increase in basal and mechanosensitive afferent nerve firing in response to the acute application of morphine, suggesting OIH.

Pronociceptive signaling evoked by morphine is mediated by DOPr

To examine mechanisms underlying the development of OT and morphine-induced hyperexcitability in colonic afferents neurons, DRG neurons from naive mice were treated with morphine at a low (10 nM) or a high (30 μ M) concentration either acutely (30 min) or overnight, and patch-clamp recordings were then obtained. Concentrations in the nanomolar range activate the classical G_i -mediated inhibitory pathway (Roedel et al., 2017), whereas concentrations in the micromolar to millimolar range have been used to induce a pronociceptive effect (Akaishi et al., 2000; Forster et al., 2009; Rowan et al., 2014). Acute application of low and high concentrations of morphine decreased neuron excitability compared with control; rheobase increased by 24% ($p=0.038$, one-way ANOVA, $n=15$ –21) and 30% ($p=0.004$, one-way ANOVA, $n=21$ on each group), respectively (Fig. 3A). Overnight incubation with high concentrations of morphine induced an increase in neuron excitability compared with control; rheobase decreased by 18% (Fig. 3B; $p=0.039$, one-way ANOVA, $n=22$ on each group). These data parallel the findings in afferent nerve recordings (Fig. 2), demonstrating that prolonged exposure to high concentration of opioids induces tolerance and a paradoxical increase in excitability of nociceptive neurons. The pronociceptive effect evoked by chronic morphine exposure was blocked by SDM25N (DOPr antagonist, 100 nM) (McLamore et al., 2001) but not by CTOP (MOPr antagonist, 100 nM) (Raynor et al., 1994), suggesting that the increased excitability induced by prolonged *in vitro* exposure to a high concentration of morphine is mediated by DOPr (Fig. 3C; for SDM25N: $p=0.019$; for CTOP: $p>0.99$, two-way ANOVA, $n=10$ –15).

To determine whether DOPr activation is also necessary for morphine-induced hyperalgesia *in vivo*, we examined the effect of the DOPr antagonist NTI (2.5 mg/kg) (Matthes et al., 1998). The DOPr antagonist was administered daily 20 min before morphine injections (Fig. 4A), and results from these mice were compared with those treated with morphine alone. The tail-flick test showed that NTI reduced the antinociceptive effect (MPE) of morphine at day 3, suggesting the involvement of DOPr in morphine-induced analgesia (Fig. 4B; $p=0.031$, two-way repeated-

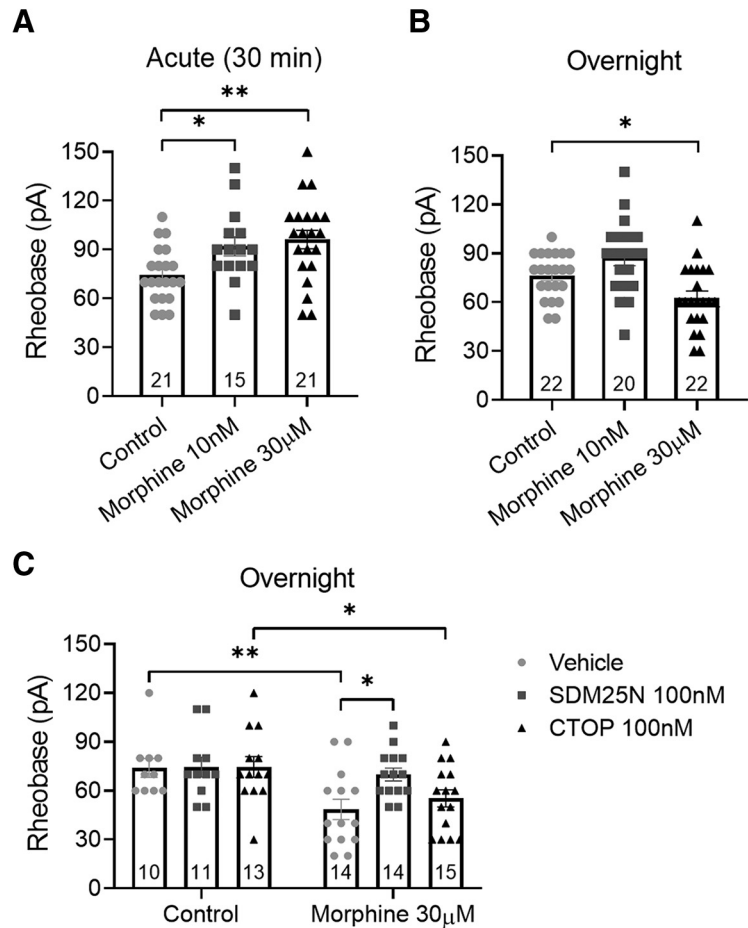


Figure 3. Overnight incubation with high concentrations of morphine has a pronociceptive effect on naive DRG neurons mediated by DOPr. **A**, Effect of the acute incubation of morphine on the excitability of DRG neurons measured by perforated patch clamp. Both low and high concentrations of morphine increased neuron rheobase compared with control ($F_{(2,54)} = 5.79$, $p=0.0053$, one-way ANOVA with Dunnett's *post hoc* test). **B**, Overnight incubation of low-concentration morphine had no effect on rheobase, while high concentration decreased neuron rheobase compared with control ($F_{(2,61)} = 8.93$, $p=0.0004$, one-way ANOVA with Dunnett's *post hoc* test). **C**, DOPr antagonist SDM25N but not MOPr antagonist CTOP prevented the pronociceptive effect induced by high-concentration morphine ($F_{(1,71)} = 12.45$, $p=0.0007$, two-way ANOVA with Bonferroni's *post hoc* test). Number of cells appears in each bar. Error bars indicate mean $\pm$ SEM. $*p \leq 0.05$. $**p \leq 0.01$.

measures ANOVA, $N=7$ morphine-, 7 NTI + morphine-treated mice). Importantly, the analgesic effect of morphine was reduced on day 7 in morphine-treated mice ($p=0.003$, two-way repeated-measures ANOVA, $N=7$ mice), whereas NTI prevented this tolerance ($p=0.15$, two-way repeated-measures ANOVA, $N=7$ mice). In patch-clamp studies of DRG neurons, acute morphine application increased the excitability of nociceptive neurons from morphine-treated mice (rheobase decreased 26%, $p=0.049$, two-way ANOVA, $n=15$ control cells, 16 morphine-incubated cells/7 mice), but it did not affect the excitability of neurons from NTI + morphine mice ($p>0.99$, two-way ANOVA, $n=17$ control cells, 15 morphine-incubated cells/7 mice), demonstrating that blocking DOPr with NTI *in vivo* prevented the increase in DRG neuron excitability induced by chronic morphine (Fig. 4C). *Ex vivo* afferent nerve recordings in colons from mice receiving morphine alone showed that basal firing and mechanosensitivity were unaffected by the acute application of morphine (i.e., exhibited tolerance) (Fig. 4D,F; $p>0.99$ and 0.25 , respectively, two-way repeated-measures ANOVA, $n=15$ units/7 morphine-treated mice), whereas in colons from NTI + morphine mice, the acute morphine application inhibited mechanosensitivity

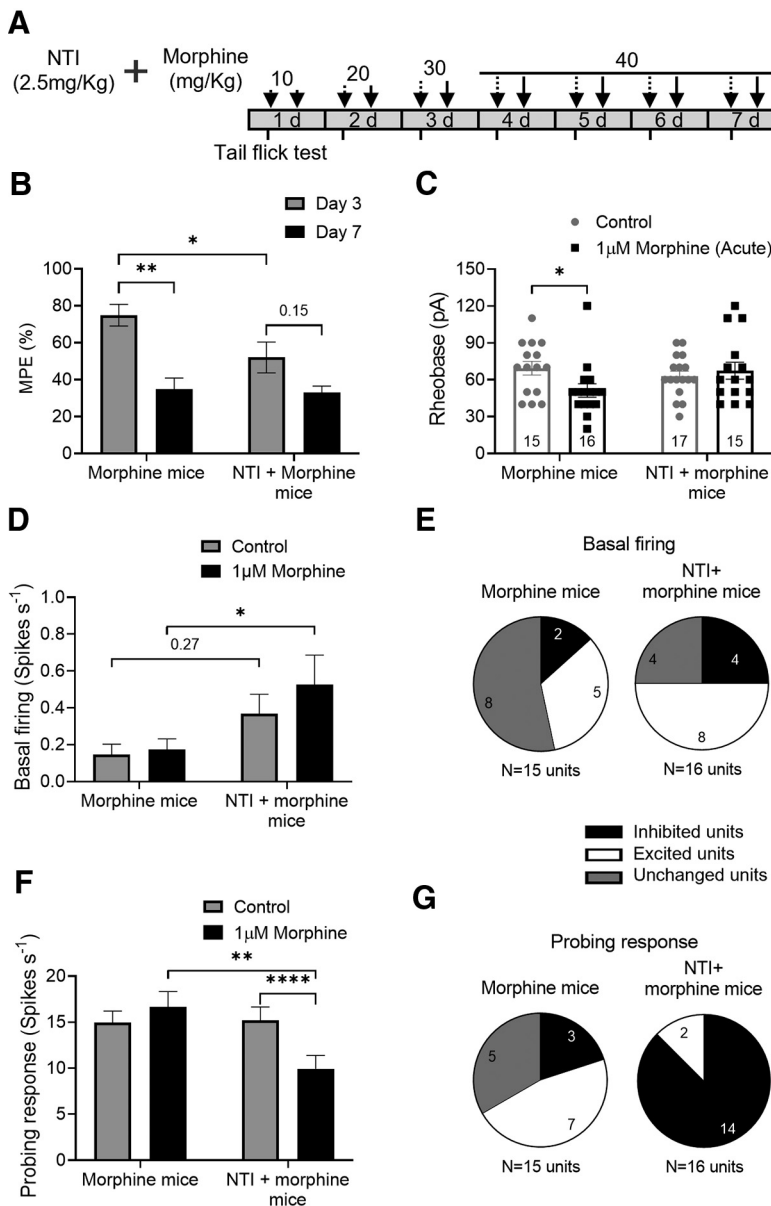


Figure 4. The DOPr inhibitor NTI prevents tolerance and the pronociceptive effect induced by chronic morphine. **A**, Schematic protocol of chronic morphine treatment in the presence of DOPr antagonist NTI. **B**, *In vivo* tail-flick test on NTI + morphine-treated mice showed an inhibition on the maximum antinociceptive effect reached on day 3 by morphine-treated mice ($F_{(1,12)} = 5.52$, $p = 0.037$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). This maximum antinociceptive effect decreased on day 7 only in morphine-treated mice ($F_{(1,12)} = 17.67$, $p = 0.0012$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). Results are expressed as mean of % MPE $\pm$ SEM, morphine mice = 7; NTI + morphine mice = 7. **C**, Acute morphine incubation decreased rheobase of neurons from morphine-treated mice but did not have any effect on neurons from NTI + morphine-treated mice ($F_{(1,59)} = 4.163$, $p = 0.046$, two-way ANOVA with Bonferroni's *post hoc* test). Number of cells appears in each bar. **D–G**, Effect of acute application of 1 μ M morphine on the basal activity and probing response of colonic afferent axons from morphine (6 mice) and NTI + morphine-treated mice (5 mice). **D**, No changes were observed on basal firing in afferent nerves from morphine- or NTI + morphine-treated mice ($F_{(1,29)} = 1.53$, $p = 0.226$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). **E**, Quantification of units showed no difference between groups in terms of basal activity (χ^2 , $df = 2.28$, $p = 0.131$). **F**, Acute application of morphine inhibited the afferent response to probing only in NTI + morphine mice ($F_{(1,29)} = 21.44$, $p < 0.0001$, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test). **G**, Most of the units were inhibited in response to probing after acute morphine exposure on NTI + morphine mice compared with those from morphine-treated mice, which showed a higher number of excited units (χ^2 , $df = 14.06$, $p = 0.0002$). Error bars indicate mean $\pm$ SEM. * $p \leq 0.05$. ** $p \leq 0.01$. **** $p \leq 0.0001$.

(Fig. 4F; $p < 0.0001$, two-way repeated-measures ANOVA, $n = 16$ units/7 NTI + morphine-treated mice). The number of afferent units with different responses to acute morphine application in terms of basal firing between morphine mice and NTI + morphine mice was similar (Fig. 4E; χ^2 , $df = 2.280$, 1 ;

$p = 0.13$). However, acute morphine decreased the number of excited mechanosensitive units and increased the number of inhibited units in NTI + morphine mice (Fig. 4G; χ^2 , $df = 14.06$, 1 ; $p = 0.0002$), suggesting that blocking DOPr activation significantly reduced OIH in morphine-treated mice.

The role of MOPr and DOPr signaling in the paradoxical increased DRG neuron excitability induced by opioids

We have previously shown that MOPr and DOPr are functionally expressed on colonic DRG neurons and coexpressed on a significant proportion of these neurons (Guerrero-Alba et al., 2018). To examine their specific roles in the development of opioid-induced neuronal hyperexcitability, the effects of the MOPr agonist DAMGO and the DOPr agonist DADLE were examined using acute application (30 min) and overnight incubation (Figs. 5 and 6, respectively) with low (10 nM) and high (10 μ M) agonist concentrations. DAMGO and DADLE have similar binding affinities for their receptors ($IC_{50} \sim 2$ nM), evoking inhibition at nanomolar concentrations (Raynor et al., 1994; Guerrero-Alba et al., 2018), and we sought to determine whether higher concentrations can evoke an excitatory effect. Following acute application of 10 nM or 10 μ M DAMGO, neuronal excitability was decreased compared with control (Fig. 5A; $p = 0.002$ and 0.009 , respectively, two-way ANOVA, $n = 28$ –30). This decreased excitability was selectively blocked by 100 nM CTOP (Fig. 5A; $p = 0.0012$ and 0.02 , respectively, two-way ANOVA, $n = 18$ –30), but not by 100 nM SDM25N (Fig. 5B; $p > 0.99$, two-way ANOVA, $n = 20$ –30). Acute application of 10 nM DADLE also decreased neuron excitability, whereas 10 μ M DADLE increased neuron excitability (Fig. 5C; $p = 0.026$ and 0.018 , two-way ANOVA, $n = 23$ –26). Both effects were blocked by SDM25N (Fig. 5C; $p = 0.003$ and 0.046 , two-way ANOVA, $n = 23$ –26), whereas CTOP failed to block the excitatory effect by 10 μ M DADLE (Fig. 5D; $p > 0.99$, two-way ANOVA, $n = 14$ on each group).

After overnight exposure, 10 nM DAMGO decreased neuron excitability compared with control, whereas 10 μ M DAMGO increased excitability (Fig. 6A; $p < 0.0001$ and 0.03 , respectively, two-way ANOVA, $n = 23$ –25). Importantly, this excitatory effect of DAMGO was blocked by the DOPr antagonist SDM25N (Fig. 6B; $p < 0.0001$, two-way ANOVA, $n = 10$ –13) but not by the MOPr antagonist CTOP (Fig. 6A; $p > 0.99$, two-way ANOVA, $n = 22$ –23); 10 nM DADLE had no

effect on the mean rheobase suggesting desensitization, whereas 10 μ M DADLE increased neuron excitability (Fig. 6C; $p = 0.296$ and 0.004, respectively, two-way ANOVA, $n = 23$ –28). This pronociceptive effect was also blocked by SDM25N (Fig. 6C; $p = 0.006$, two-way ANOVA, $n = 23$ –33) but also by CTOP (Fig. 6D; $p = 0.008$, two-way ANOVA, $n = 19$ on each group), an unexpected finding given that CTOP did not block the pronociceptive action evoked by DADLE (Fig. 5D). Together, our data suggest that DOPr is involved in the paradoxical increased neuronal excitability induced by MOPr and DOPr agonists.

To confirm our fundamental observation in gut specific neurons, we performed patch-clamp recordings on colon-projecting DRG neurons (Fast Blue-labeled) from female mice. The DOPr agonist DADLE 10 μ M overnight incubated increased DRG neuron excitability compared with vehicle (Fig. 6E; $p = 0.0035$, unpaired t test, $n = 8$ on each group).

The DOPr-mediated pronociceptive effect is endocytosis-dependent

To examine the intracellular signaling underlying hyperexcitability evoked by overnight incubation with high concentrations of DAMGO and DADLE, we used the opioid receptor antagonist NLX and the inhibitor of endocytosis, PS2. NLX is an alkaloid that can permeate the plasma membrane and also affect the intracellular signaling of opioid receptors (Wang et al., 2008), whereas PS2 is an inhibitor of clathrin-mediated endocytosis (Robertson et al., 2014). The pronociceptive effects of DAMGO and DADLE were blocked by 1 μ M NLX (Fig. 7A; $p < 0.0001$ and 0.002 respectively, two-way ANOVA, $n = 12$ –16) and also by 20 μ M PS2 (Fig. 7B; $p = 0.027$ and 0.016 respectively, two-way ANOVA, $n = 14$ –19). To further characterize the involvement of endocytic pathways in the increased neuronal excitability evoked by high concentrations of opioid agonists and mediated by DOPr, DRG neurons were incubated acutely (30 min) and overnight with high concentration of a weakly internalizing DOPr agonist ARM390 (10 μ M) (Marie et al., 2003; Pradhan et al., 2009). We previously showed that DADLE and SNC80 but not ARM390 (100 nM) induced β arr recruitment as well as DOPr internalization (Jimenez-Vargas et al., 2020). Acute incubation with ARM390 did not alter the mean rheobase of DRG neurons, whereas overnight incubation decreased neuron excitability despite the high concentration (Fig. 7C; $p = 0.039$, one-way ANOVA, $n = 20$ or 21). Together, these data suggest that the neuronal hyperexcitability induced by opioids is dependent on DOPr internalization.

Overnight exposure to a high concentration of opioids switches MOPr and DOPr signaling in response to acute opioid re-exposure to a pronociceptive pathway

To further examine the mechanisms underlying the switch of opioid signaling to a pronociceptive pathway, we studied the response to low-concentration (10 nM) MOPr and DOPr agonists following overnight incubation of DRG neurons with high

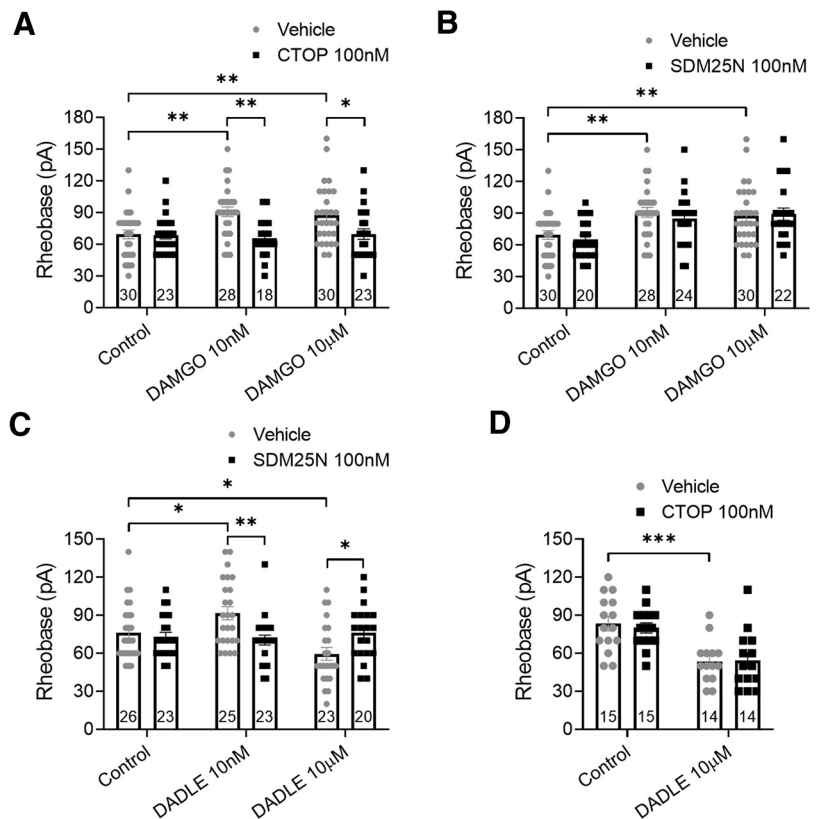


Figure 5. Acute application of MOPr and DOPr agonists shows that pronociceptive actions are mediated by DOPr. **A**, **B**, Effect of low-concentration (10 nM) and high-concentration (10 μ M) DAMGO applied acutely (30 min) on neuron excitability. Patch-clamp recordings show that low and high concentrations of DAMGO increased neuron rheobase. This antinociceptive effect was inhibited by MOPr antagonist CTOP (**A**: $F_{(2,146)} = 3.655$, $p = 0.028$) but not by DOPr antagonist SDM25N (**B**: $F_{(2,148)} = 0.339$, $p = 0.713$). **C**, **D**, A low concentration of DADLE increased neuron rheobase, whereas a high concentration decreased neuron rheobase. DOPr antagonist SDM25N blocked both effects induced by DADLE (**C**: $F_{(1,134)} = 8.35$, $p = 0.0004$), whereas CTOP was unable to block the pronociceptive effect evoked by high-concentration DADLE (**D**: $F_{(1,54)} = 0.1555$, $p = 0.695$). Number of cells appears in each bar. Error bars indicate mean $\pm$ SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; two-way ANOVA with Bonferroni's *post hoc* test.

concentrations (10 μ M) of DAMGO or DADLE (Fig. 8A). As shown in Figure 6, sustained exposure to 10 μ M DADLE increased neuron excitability. However, after 1 h washout of DADLE, neuron excitability recovered to control levels (Fig. 8B). Neurons were then re-exposed to acute application (30 min) of 10 nM of either a DOPr or MOPr agonist. DADLE 10 nM but not DAMGO increased neuron excitability rather than decreasing it ($p = 0.002$ and $p > 0.99$, respectively, one-way ANOVA, $n = 16$ or 17), as previously observed in naive neurons (Fig. 5), suggesting a switch in receptor downstream signaling pathways as a result of the prior overnight exposure to DADLE (Fig. 8B). Interestingly, following overnight incubation with 10 μ M DAMGO, re-exposure to both 10 nM DAMGO or DADLE increased neuron excitability (Fig. 8C; $p = 0.003$ on both cases, one-way ANOVA, $n = 10$ –12).

PKC mediates the pronociceptive effect by opioids after overnight exposure to 10 μ M DADLE

To further understand the intracellular mechanism underlying neuronal hyperexcitability after opioid re-exposure, we explored the role of PKA and PKC signaling. Opioid inhibition of neurons occurs via G_i -protein linked to PKA pathway, whereas PKC ϵ signaling has been implicated in pronociceptive responses induced by opioids (Joseph et al., 2010). Following washout of the

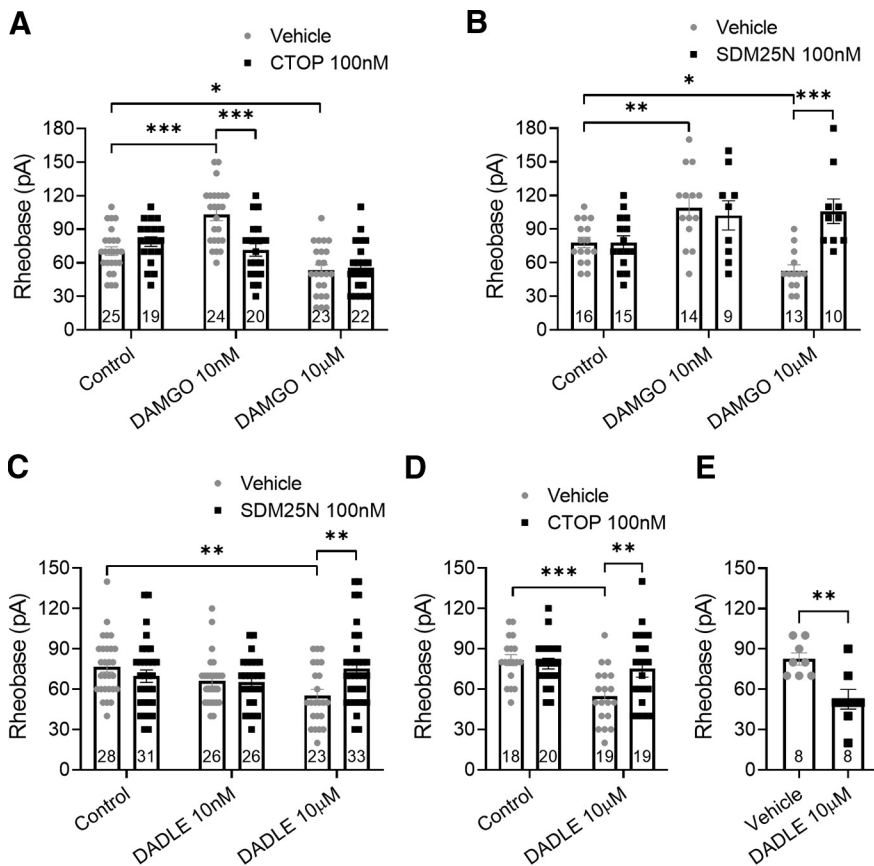


Figure 6. DOPr mediates the pronociceptive effect induced by high concentrations of MOPr and DOPr agonists applied overnight. **A, B,** Effect of low-concentration (10 nM) and high-concentration (10 μM) DAMGO overnight incubated on DRG neuron excitability. DRG neuron rheobase increased with low-concentration but decreased with high-concentration DAMGO. **A,** CTOP prevented the antinociceptive effect by 10 nM DAMGO but had no effect on the pronociceptive effect by 10 μM DAMGO ($F_{(2,127)} = 9.898$, $p < 0.0001$). **B,** SDM25N inhibited only the pronociceptive effect by 10 μM DAMGO ($F_{(2,71)} = 8.144$, $p = 0.0007$). **C, D,** Low-concentration DADLE had no effect on neuron rheobase, while high concentrations decreased neuron rheobase; this pronociceptive effect by high-concentration DADLE was inhibited by both SDM25N (**C**: $F_{(2,161)} = 5.022$, $p = 0.0077$) and CTOP (**D**: $F_{(1,72)} = 5.605$, $p = 0.0206$). **E,** Effect of 10 μM DADLE on DRG neurons innervating the colon of female mice. High-concentration DADLE decreased the rheobase of Fast Blue-labeled neurons ($t_{(14)} = 3.506$, $p = 0.0035$, unpaired t test). Number of cells appears in each bar. Error bars indicate mean $\pm$ SEM. **A–D,** $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; two-way ANOVA with Bonferroni's *post hoc* test.

excitatory effect of overnight 10 μM DADLE or DAMGO incubation, PKA or PKC inhibitors were applied to DRG neurons 30 min before acute incubation with 10 nM opioids (Fig. 8D). H89

1 μM failed to block the pronociceptive effect of 10 nM DADLE re-exposure (Fig. 8E,G; rheobase decreased compared with H89 control; $p = 0.02$ and $p = 0.012$ respectively, one-way ANOVA, $n = 12$ –16), but blocked the pronociceptive effect of 10 nM DAMGO re-exposure (Fig. 8G; rheobase not different compared with H89 control, $p = 0.15$, one-way ANOVA, $n = 16$ or 17). GFX 1 μM blocked the pronociceptive effect of 10 nM DADLE re-exposure following either overnight 10 μM DADLE or DAMGO incubation (Fig. 8F,H; rheobase not different from GFX control, $p = 0.63$ and $p = 0.09$, respectively, one-way ANOVA, $n = 12$ –18), suggesting that PKC signaling plays a role in the pronociceptive responses mediated by DOPr.

Gα_i signaling and βarr2 recruitment are lost after sustained exposure to opioids

BRET assays were performed in HEK293T cells to measure the activation of Gα_i, Gα_s, and Gα_q proteins following acute opioid

agonists re-exposure. Acute application of DADLE and DAMGO to cells not previously exposed to opioids activated Gα_i subunits but minimal Gα_q and not Gα_s proteins. DADLE evoked a larger response than DAMGO (Fig. 9). Gα_i subunit activation by acute opioids decreased when cells were previously exposed to a high concentration of DADLE (10 μM) or DAMGO (10 μM) (Fig. 9A–D; one-way ANOVA), suggesting possible desensitization of Gα_i signaling after prolonged opioid exposure. We did not find evidence of Gα_s or Gα_q protein activation after opioid re-exposure (Fig. 9E–L; one-way ANOVA).

βarr2 recruitment to the plasma membrane was also measured in HEK293T cells. Acute application of DADLE (Fig. 9M,N) or high concentration of DAMGO (Fig. 9P) evoked significant recruitment of βarr2 to the plasma membrane. This recruitment was absent when cells were re-exposed to opioids after overnight incubation with high concentration of DADLE or DAMGO (Fig. 9M–P; one-way ANOVA).

Discussion

The opioid crisis in the developed world has fueled ongoing interest in strategies to mitigate the serious and potentially lethal side effects of opioid drugs. These side effects are driven by escalating dosing as a result of OT and possibly the less well-described phenomenon of OIH, where opioids are thought to paradoxically increase nociceptive signaling following chronic exposure to high doses (Farmer et al., 2017). An important site of analgesia derived from systemic opioids is the peripheral nociceptive DRG neuron, such as those innervating the intestine (Guerrero-Alba et al., 2018). Here we focus on peripheral nociceptive signaling mechanisms

in the colon using *in vivo* models of OT and corresponding *in vitro* models of OPr desensitization. We provide evidence of decreased antinociceptive and enhanced pronociceptive signaling from afferent nerves following chronic morphine exposure and corresponding changes in *in vitro* studies of isolated DRG neurons. Moreover, we found evidence of a functional MOPr–DOPr interaction where these events were driven by activation of DOPr in response to high doses of either MOPr or DOPr agonists.

Morphine is a common analgesic used to manage pain over days to weeks in multiple clinical settings. In our *in vivo* studies with morphine exposure for only 7 d, we observed decreased analgesia and enhanced pronociceptive signaling in colonic afferent nerves, when the nerves were challenged with an acute application of morphine. These findings were evident after quantification of the number of individual units differentially altered by acute morphine in basal afferent nerve firing and mechanical probing responses, suggesting that both basal firing and mechanosensitive properties are affected. In parallel, patch-clamp

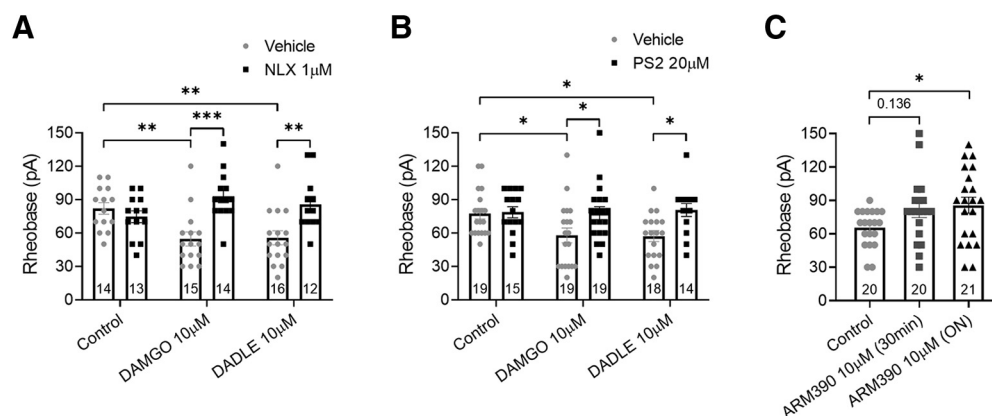


Figure 7. Opioid-induced DRG neuronal hyperexcitability is dependent on receptor endocytosis. **A**, Patch-clamp recordings show that the OPr antagonist naloxone (NLX) prevented the decrease in the rheobase evoked by high-concentration DAMGO and DADLE applied overnight ($F_{(2,78)} = 8.143$, $p = 0.0006$, two-way ANOVA with Bonferroni's *post hoc* test). **B**, The clathrin inhibitor PS2 also prevented the decrease on the rheobase evoked by high-concentration opioids ($F_{(1,98)} = 10.63$, $p = 0.0015$, two-way ANOVA with Bonferroni's *post hoc* test). **C**, Acute application of a weakly internalizing DOPr agonist ARM390 at equally high concentration did not alter neuron rheobase, whereas overnight incubation increased neuron rheobase ($F_{(2,58)} = 3.064$, $p = 0.054$, one-way ANOVA with Dunnett's *post hoc* test). Number of cells appears in each bar. Error bars indicate mean $\pm$ SEM. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

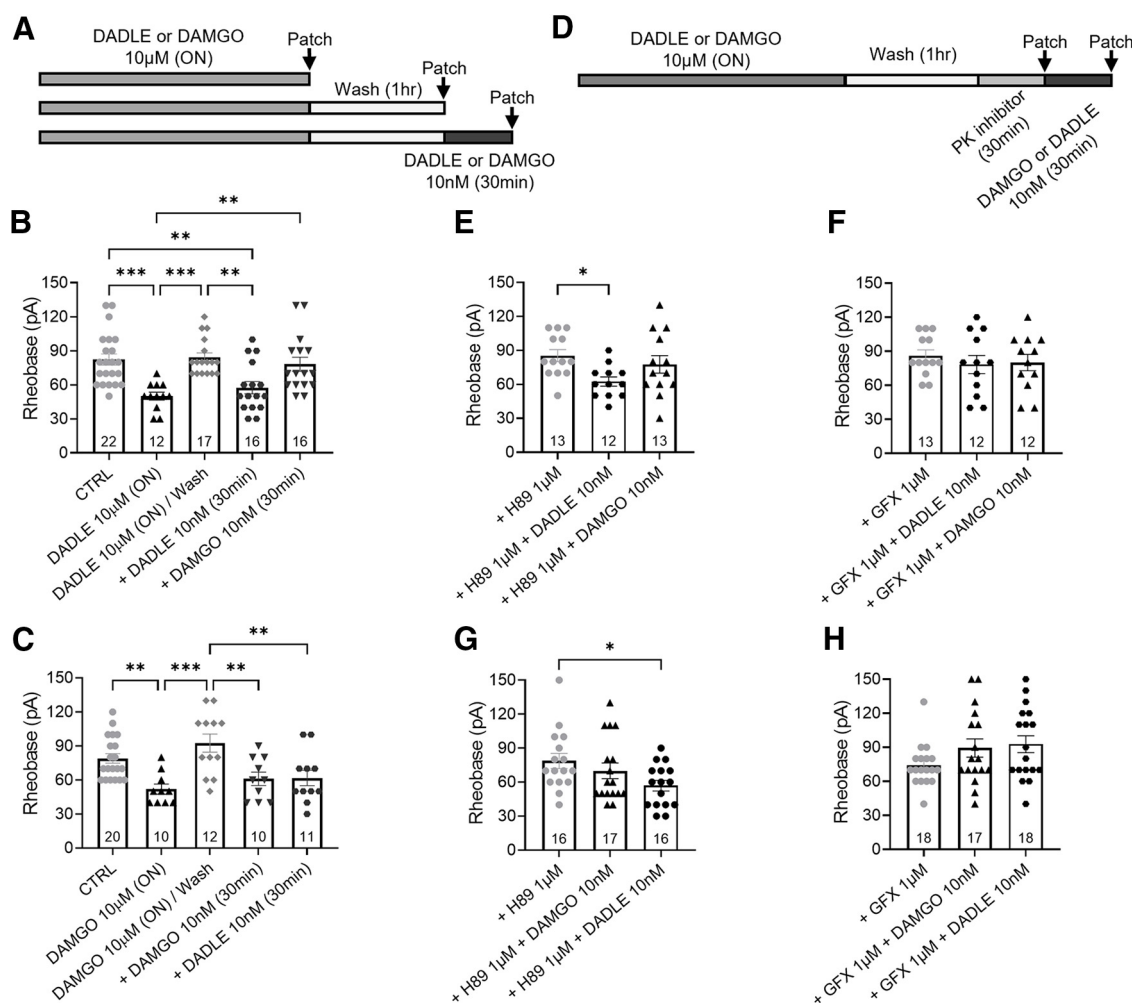


Figure 8. Overnight exposure to high-concentration opioids switches low-concentration DOPr and MOPr agonist responses to a PKC-dependent pronociceptive pathway. **A**, Schematic protocol showing the paradigm used in **B** and **C**. Arrows indicate perforated patch-clamp recordings at different treatment points. **B**, The pronociceptive effect induced by high-concentration DADLE is lost after 1 h washout with control media, re-exposure to a low-concentration DADLE paradoxically decreased neuron rheobase ($F_{(4,78)} = 8.474$, $p < 0.0001$). **C**, After washout of high-concentration DAMGO overnight, acute re-exposure to DAMGO or DADLE paradoxically decreased neuron rheobase ($F_{(4,58)} = 7.111$, $p < 0.0001$). **D**, Schematic protocol showing the paradigm used in **E–H**. Arrows indicate perforated patch-clamp recordings after incubation with PKA or PKC inhibitors with or without opioid re-exposure. **E**, **F**, Following the washout of overnight high-concentration DADLE, PKC inhibitor GFX ($F_{(2,34)} = 0.382$, $p = 0.686$) but not PKA inhibitor H89 ($F_{(2,35)} = 3.681$, $p = 0.035$) prevented the paradoxical increased excitability induced by acute re-exposure to low-concentration DADLE. **G**, **H**, Following washout of overnight high-concentration DAMGO, PKA inhibitor H89 ($F_{(2,53)} = 4.1$, $p = 0.022$) only prevented the pronociceptive effect induced by acute re-exposure to low-concentration DAMGO while PKC inhibitor GFX ($F_{(2,50)} = 2.246$, $p = 0.116$) prevented the increased excitability induced by acute re-exposure to both low-concentration DAMGO or DADLE. Number of cells appears in each bar. Error bars indicate mean $\pm$ SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; one-way ANOVA with Dunnett's *post hoc* test.

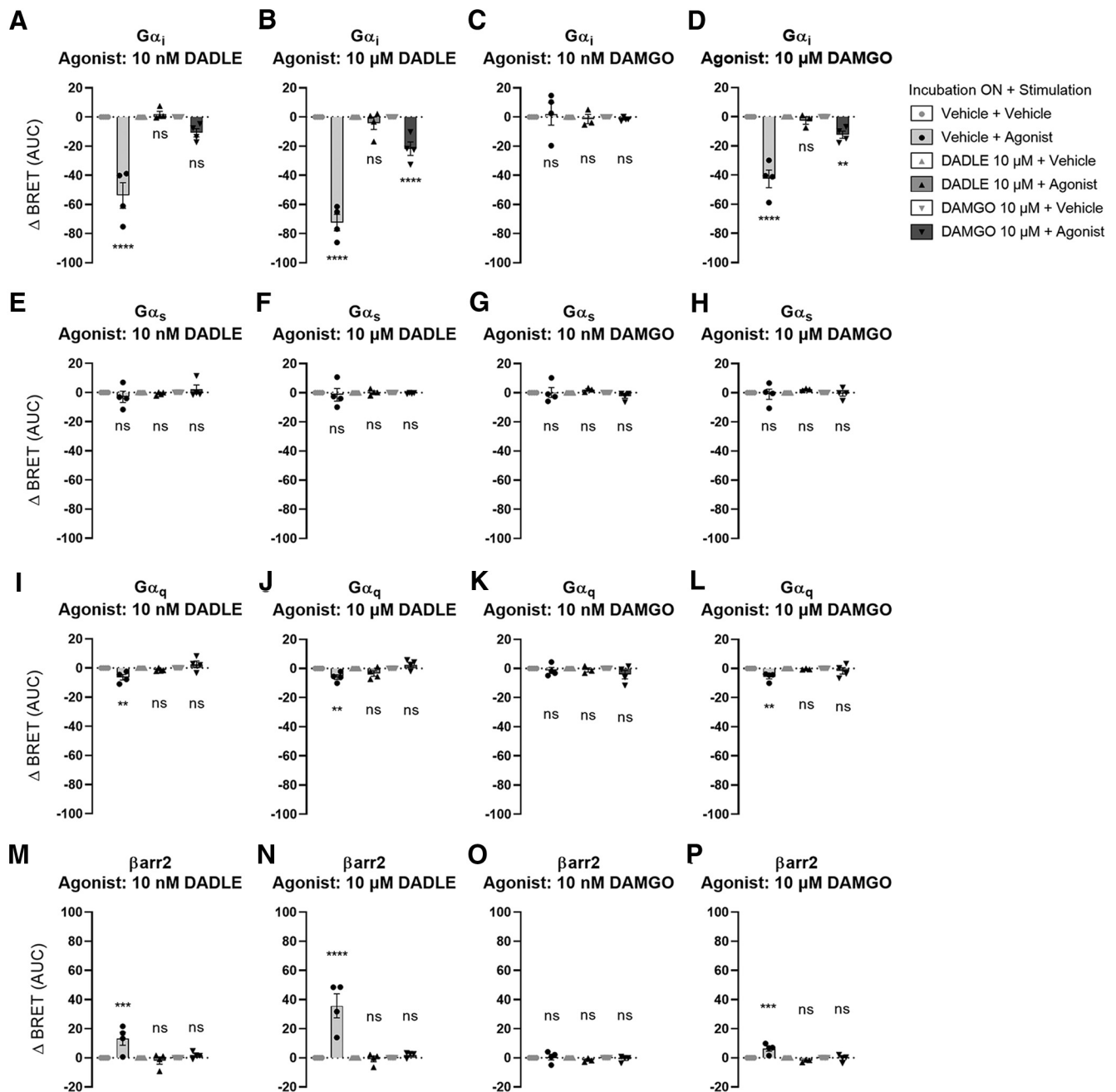


Figure 9. Overnight incubation with high-concentration opioids decreased acute $G\alpha_i$ activation and β arr2 recruitment. Transfected HEK293T cells were treated overnight with vehicle, high-concentration (10 μ M) DADLE or DAMGO, washed for 30 min, and then stimulated with vehicle, or agonist with low-concentration (10 nM) DAMGO or DADLE or high-concentration (10 μ M) DAMGO or DADLE. BRET was recorded over time to measure activation of $G\alpha_i$ (A: $F_{(5,24)} = 45.25$, $p < 0.0001$; B: $F_{(5,24)} = 99.2$, $p < 0.0001$; C: $F_{(5,23)} = 0.1771$, $p = 0.9685$; D: $F_{(5,23)} = 51.16$, $p < 0.0001$), $G\alpha_s$ (E: $F_{(5,24)} = 0.8942$, $p = 0.5007$; F: $F_{(5,24)} = 0.1545$, $p = 0.9766$; G: $F_{(5,23)} = 0.9256$, $p = 0.4824$; H: $F_{(5,23)} = 0.477$, $p = 0.7896$), and $G\alpha_q$ (I: $F_{(5,24)} = 7.611$, $p < 0.0002$; J: $F_{(5,24)} = 8.63$, $p < 0.0001$; K: $F_{(5,23)} = 1.63$, $p = 0.1919$; L: $F_{(5,23)} = 6.034$, $p = 0.001$) by dissociation of Rluc2- $G\alpha$ with GFP10-G- γ 2 after agonist stimulation. Consequently, a decrease of BRET reflects an activation of $G\alpha$ protein. Following the same cell treatment, recruitment of Rluc2- β arr2 to the plasma membrane (rGFP-CAAX) was also measured using ebBRET (M: $F_{(5,24)} = 8.966$, $p < 0.0001$; N: $F_{(5,24)} = 24.1$, $p < 0.0001$; O: $F_{(5,23)} = 0.7804$, $p = 0.574$; P: $F_{(5,23)} = 13.28$, $p < 0.0001$), an increase of ebBRET reflecting the recruitment of β arr2 to the plasma membrane. Data were collected from 3–7 independent experiments. Bars represent mean of the area under the curve $\pm$ SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$; one-way ANOVA with multiple comparisons and Tukey's *post hoc* test.

studies of individual DRG neurons from the same animals demonstrated receptor desensitization in response to the acute application of morphine. Furthermore, chronic incubation (overnight) with high-dose morphine recapitulates the switch from antinociceptive to pronociceptive signaling following an acute morphine challenge (Fig. 3C), a phenomenon observed by others (Khomula et al., 2019).

The analgesic actions of morphine and related compounds are mediated largely by MOPrs. Our studies, however, identified

that the switch to pronociceptive pathways is mediated by DOPr signaling, based on several findings. Our *in vitro* studies of isolated DRG neurons demonstrated that the selective DOPr antagonists blocked the pronociceptive actions of overnight morphine incubation, whereas the MOPr antagonist had no effect. We observed a similar phenomenon *in vivo* where the pronociceptive actions of acute application of morphine following chronic exposure were blocked by the selective DOPr antagonist NTI, suggesting that DOPr plays a significant role in the development of

hyperalgesia. We then used selective MOPr and DOPr agonists and their respective antagonists to recapitulate these findings. The higher concentrations we used may have exceeded concentrations reported to be selective (Raynor et al., 1994). Previous studies suggest that morphine and DAMGO at high concentrations can activate DOPr (Jordan et al., 2000; Zhao et al., 2003). Nonetheless, we found that the classical inhibition evoked by acute application at these high concentrations was blocked by their respective antagonists (Fig. 5).

Our studies may suggest a functional MOPr–DOPr interaction following sustained application of high-dose agonists. We previously showed that MOPr and DOPr are coexpressed on a significant proportion of nociceptive DRG neurons (Guerreiro-Alba et al., 2018). The concept of MOPr–DOPr interaction underlying OPr-mediated inhibition is well described in previous studies (DiCello et al., 2020), although the mechanisms are poorly understood and may be multifactorial. Additionally, selectivity studies found that MOPr agonists morphine and DAMGO showed higher affinity to cells coexpressing MOPr–DOPr receptors compared with cells expressing only MOPr (Yekkirala et al., 2010). In our studies, where neurons were exposed to high concentrations of DADLE and DAMGO overnight and then excitability allowed to recover during a washout, we found diverging effects when we re-exposed these neurons to low concentrations of the agonists. DAMGO overnight exposure primed the neurons to a pronociceptive action in response to a subsequent challenge with both the MOPr and DOPr agonists, whereas overnight DADLE only resulted in a pronociceptive response to subsequent DADLE, but not DAMGO. These findings further support the concept that the pronociception evoked high-dose opioids are selectively mediated by activation of DOPr by either MOPr or DOPr agonists.

DOPr agonists are being developed for the treatment of chronic pain, as well as psychiatric disorders (Gendron et al., 2015). Like other GPCRs, DOPrs exhibit agonist-selective recruitment of arrestins, resulting in functional selectivity between agonists. Arrestins are multifaceted proteins that play an important role in DOPr trafficking and signaling. Recent studies suggest that weakly internalizing DOPr agonists have sustained signaling from the plasma membrane and exhibit sustained antinociception, whereas those that externalize and recruit arrestins exhibit rapid tolerance. OPr internalization has received considerable attention in studies of DOPr antinociceptive signaling and have shown that tolerance is induced by high internalizing DOPr agonist through β arr recruitment (Pradhan et al., 2010, 2016; Vicente-Sanchez et al., 2018), but the cellular mechanisms underlying DOPr hyperalgesia have not been studied. We found that the weakly internalizing DOPr agonist ARM390 did not evoke neuronal hyperexcitability, unlike DADLE, and that the actions of DADLE were blocked by the clathrin inhibitor PS2. Thus, our studies suggest that the DOPr-mediated pronociceptive signaling is also dependent on receptor internalization.

Opioid inhibitory effects are classically linked to $G\alpha_i$ and $G\beta\gamma$ protein signaling, and downstream inhibition of adenylate cyclase and PKA activity, although other signaling pathways have been implicated (Al-Hasani and Bruchas, 2011). We did not find a switch in $G\alpha_i$ to $G\alpha_s$ or $G\alpha_q$ signaling; however, our results suggest that DOPr-mediated pronociceptive signaling is PKC-dependent, which is similar to findings in other studies using MOPr selective agonists (Joseph et al., 2010). It has been reported that PKC activation can be downstream of $G\alpha_i$ pathway via $G\beta\gamma$ subunits or calcium entry (Camps et al., 1992;

Nishizuka, 1992). Further studies are needed to determine the role of PKC in the mechanisms of phosphorylation and internalization of OPr that might lead to OT and OIH.

There are several potential limitations of the current study. All opioid agonists are analgesic, yet selective activation of MOPr and DOPr exhibits different behavioral effects. DOPr agonists have anxiolytic and antidepressant activity (Valentino and Volkow, 2018), and MOPr agonists induce euphoria and stress coping. And while such actions could alter the outcome of behavioral studies, there is little evidence they have impacted the current study. Our electrophysiological measurements of tolerance and OIH were obtained using *ex vivo* peripheral afferent nerve preparations and *in vitro* patch-clamp studies of isolated neurons following chronic treatment and thus would not be directly influenced by the psychological impact of DOPr or MOPr signaling. While the tail flick test measures of tolerance could be influenced by psychological changes, our electrophysiological studies showed parallel correlates of tolerance and OIH in the same animals. Another potential limitation is the lack of *in vivo* dose ranging studies and correlation with the concentrations used in the *in vitro* studies. Further studies are needed in this regard, but the current studies show evidence of the observed *in vitro* correlates of tolerance and OIH in the *in vivo* studies, and the doses used were well tolerated by the animals and thus are likely comparable with those used in humans. A final issue relates to the identity of the DRG neurons studied. While the studies conducted using afferent recordings from the colon and patch-clamp studies of neurons retrogradely labeled from the colon of male and female mice demonstrate the pronociceptive actions of DOPr in gut-specific neurons, most of the patch-clamp studies were conducted on nonlabeled neurons; thus, many of these would be somatosensory neurons. Thus, it is likely that the findings described in this study relate not only to visceral neurons but also to the peripheral somatosensory system.

In conclusion, DOPr plays a major role in the development of OT and OIH in colonic afferent nociceptors. The development of OIH requires prolonged exposure to high opioid concentrations, leading to activation of mechanisms, such as OPr internalization and activation of PKC signaling pathway. Targeting key components on these identified pathways could mitigate the development of OT and OIH in the colon.

References

- Akaishi T, Saito H, Ito Y, Ishige K, Ikegaya Y (2000) Morphine augments excitatory synaptic transmission in the dentate gyrus through GABAergic disinhibition. *Neurosci Res* 38:357–363.
- Al-Hasani R, Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 115:1363–1381.
- Allouche S, Noble F, Marie N (2014) Opioid receptor desensitization: mechanisms and its link to tolerance. *Front Pharmacol* 5:280.
- Bielefeldt K, Davis B, Binion DG (2009) Pain and inflammatory bowel disease. *Inflamm Bowel Dis* 15:778–788.
- Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT (1999) Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286:2495–2498.
- Brookes SJ, Spencer NJ, Costa M, Zagorodnyuk VP (2013) Extrinsic primary afferent signalling in the gut. *Nat Rev Gastroenterol Hepatol* 10:286–296.
- Cahill CM, Walwyn W, Taylor AM, Pradhan AA, Evans CJ (2016) Allostatic mechanisms of opioid tolerance beyond desensitization and downregulation. *Trends Pharmacol Sci* 37:963–976.
- Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ, Gierschik P (1992) Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. *Nature* 360:684–686.

- Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105.
- DiCello JJ, Carbone SE, Saito A, Rajasekhar P, Ceredig RA, Pham V, Valant C, Christopoulos A, Veldhuis NA, Canals M, Massotte D, Poole DP (2020) Mu and delta opioid receptors are coexpressed and functionally interact in the enteric nervous system of the mouse colon. *Cell Mol Gastroenterol Hepatol* 9:465–483.
- Farmer AD, Gallagher J, Bruckner-Holt C, Aziz Q (2017) Narcotic bowel syndrome. *Lancet Gastroenterol Hepatol* 2:361–368.
- Ferrini F, Trang T, Mattioli TA, Laffray S, DelGuidice T, Lorenzo LE, Castonguay A, Doyon N, Zhang WB, Godin AG, Mohr D, Beggs S, Vandal K, Beaulieu JM, Cahill CM, Salter MW, De Koninck Y (2013) Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. *Nat Neurosci* 16:183–192.
- Forster AB, Reeh PW, Messlinger K, Fischer MJ (2009) High concentrations of morphine sensitize and activate mouse dorsal root ganglia via TRPV1 and TRPA1 receptors. *Mol Pain* 5:17.
- Gales C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat Struct Mol Biol* 13:778–786.
- Galligan JJ, Akbarali HI (2014) Molecular physiology of enteric opioid receptors. *Am J Gastroenterol Suppl* 2:17–21.
- Gendron L, Mittal N, Beaudry H, Walwyn W (2015) Recent advances on the delta opioid receptor: from trafficking to function. *Br J Pharmacol* 172:403–419.
- Grim TW, Schmid CL, Stahl EL, Pantouli F, Ho JH, Acevedo-Canabal A, Kennedy NM, Cameron MD, Bannister TD, Bohn LM (2020) A G protein signaling-biased agonist at the mu-opioid receptor reverses morphine tolerance while preventing morphine withdrawal. *Neuropsychopharmacology* 45:416–425.
- Guerrero-Alba R, Valdez-Morales EE, Jimenez-Vargas NN, Bron R, Poole D, Reed D, Castro J, Campaniello M, Hughes PA, Brierley SM, Bunnett N, Lomax AE, Vanner S (2018) Coexpression of mu and delta opioid receptors by mouse colonic nociceptors. *Br J Pharmacol* 175:2622–2634.
- He L, Gooding SW, Lewis E, Felth LC, Gaur A, Whistler JL (2021) Pharmacological and genetic manipulations at the micro-opioid receptor reveal arrestin-3 engagement limits analgesic tolerance and does not exacerbate respiratory depression in mice. *Neuropsychopharmacology* 46:2241–2249.
- Ibeaknma C, Ochoa-Cortes F, Miranda-Morales M, McDonald T, Spreadbury I, Cenac N, Cattaruzza F, Hurlbut D, Vanner S, Bunnett N, Vergnolle N, Vanner S (2011) Brain-gut interactions increase peripheral nociceptive signaling in mice with postinfectious irritable bowel syndrome. *Gastroenterology* 141:2098–2108.e5.
- Jensen DD, Lieu TM, Halls ML, Veldhuis NA, Imlach WL, Mai QN, Poole DP, Quach T, Aurelio L, Conner J, Klein Herenbrink C, Barlow N, Simpson JS, Scanlon MJ, Graham B, McCluskey A, Robinson PJ, Escrivou V, Nassini R, Materazzi S, et al. (2017) Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci Transl Med* 9:eal3447.
- Jimenez-Vargas NN, Pattison LA, Zhao P, Lieu TM, Latorre R, Jensen DD, Castro J, Aurelio L, Le GT, Flynn B, Klein Herenbrink C, Yeatman HR, Edgington-Mitchell L, Porter CJ, Halls ML, Canals M, Veldhuis NA, Poole DP, McLean P, Hicks GA, et al. (2018) Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc Natl Acad Sci USA* 115:E7438–E7447.
- Jimenez-Vargas NN, Gong J, Wisdom MJ, Jensen DD, Latorre R, Hegron A, Teng S, DiCello JJ, Rajasekhar P, Veldhuis NA, Carbone SE, Yu Y, Lopez-Lopez C, Jaramillo-Polanco J, Canals M, Reed DE, Lomax AE, Schmidt BL, Leong KW, Vanner SJ, et al. (2020) Endosomal signaling of delta opioid receptors is an endogenous mechanism and therapeutic target for relief from inflammatory pain. *Proc Natl Acad Sci USA* 117:15281–15292.
- Jordan BA, Cvejic S, Devi LA (2000) Opioids and their complicated receptor complexes. *Neuropsychopharmacology* 23:S5–S18.
- Joseph EK, Reichling DB, Levine JD (2010) Shared mechanisms for opioid tolerance and a transition to chronic pain. *J Neurosci* 30:4660–4666.
- Kang M, Mischel RA, Bhavé S, Komla E, Cho A, Huang C, Dewey WL, Akbarali HI (2017) The effect of gut microbiome on tolerance to morphine mediated antinociception in mice. *Sci Rep* 7:42658.
- Khomula EV, Araldi D, Levine JD (2019) In vitro nociceptor neuroplasticity associated with in vivo opioid-induced hyperalgesia. *J Neurosci* 39:7061–7073.
- Kliwer A, Schmiedel F, Sianati S, Bailey A, Bateman JT, Levitt ES, Williams JT, Christie MJ, Schulz S (2019) Phosphorylation-deficient G-protein-biased mu-opioid receptors improve analgesia and diminish tolerance but worsen opioid side effects. *Nat Commun* 10:367.
- Kyloh M, Nicholas S, Zagorodnyuk VP, Brookes SJ, Spencer NJ (2011) Identification of the visceral pain pathway activated by noxious colorectal distension in mice. *Front Neurosci-Switz* 5:16.
- Lacy BE (2018) Review article: an analysis of safety profiles of treatments for diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 48:817–830.
- Marie N, Landemore G, Debout C, Jauzac P, Allouche S (2003) Pharmacological characterization of AR-M1000390 at human delta opioid receptors. *Life Sci* 73:1691–1704.
- Matthes HW, Smadja C, Valverde O, Vonesch JL, Foutz AS, Boudinot E, Denavit-Saubie M, Severini C, Negri L, Roques BP, Maldonado R, Kieffer BL (1998) Activity of the delta-opioid receptor is partially reduced, whereas activity of the kappa-receptor is maintained in mice lacking the mu-receptor. *J Neurosci* 18:7285–7295.
- Mattioli TA, Leduc-Pessah H, Skelhorne-Gross G, Nicol CJ, Milne B, Trang T, Cahill CM (2014) Toll-like receptor 4 mutant and null mice retain morphine-induced tolerance, hyperalgesia, and physical dependence. *PLoS One* 9:e97361.
- McLamore S, Ullrich T, Rothman RB, Xu H, Dersch C, Coop A, Davis P, Porreca F, Jacobson AE, Rice KC (2001) Effect of N-alkyl and N-alkenyl substituents in noroxymorphindole, 17-substituted-6,7-dehydro-4,5alpha-epoxy-3,14-dihydroxy-6,7,2',3'-indolomorphina ns, on opioid receptor affinity, selectivity, and efficacy. *J Med Chem* 44:1471–1474.
- Moore BA, Stewart TM, Hill C, Vanner SJ (2002) TNBS ileitis evokes hyperexcitability and changes in ionic membrane properties of nociceptive DRG neurons. *Am J Physiol Gastrointest Liver Physiol* 282:G1045–G1051.
- Namkung Y, Le Gouill C, Lukashova V, Kobayashi H, Hogue M, Khoury E, Song M, Bouvier M, Laporte SA (2016) Monitoring G protein-coupled receptor and beta-arrestin trafficking in live cells using enhanced bystander BRET. *Nat Commun* 7:12178.
- Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607–614.
- Peiris M, Hockley JR, Reed DE, Smith ES, Bulmer DC, Blackshaw LA (2017) Peripheral KV7 channels regulate visceral sensory function in mouse and human colon. *Mol Pain* 13:1744806917709371.
- Pergolizzi JV Jr, Raffa RB, Rosenblatt MH (2020) Opioid withdrawal symptoms, a consequence of chronic opioid use and opioid use disorder: current understanding and approaches to management. *J Clin Pharm Ther* 45:892–903.
- Pradhan AA, Walwyn W, Nozaki C, Filliol D, Erbs E, Matifas A, Evans C, Kieffer BL (2010) Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance. *J Neurosci* 30:16459–16468.
- Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gaveriaux-Ruff C, Kieffer BL (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* 4:e5425.
- Pradhan AA, Perroy J, Walwyn WM, Smith ML, Vicente-Sanchez A, Segura L, Bana A, Kieffer BL, Evans CJ (2016) Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J Neurosci* 36:3541–3551.
- Raehal KM, Bohn LM (2011) The role of beta-arrestin2 in the severity of antinociceptive tolerance and physical dependence induced by different opioid pain therapeutics. *Neuropharmacology* 60:58–65.
- Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, Reisine T (1994) Pharmacological characterization of the cloned kappa-, delta-, and mu-opioid receptors. *Mol Pharmacol* 45:330–334.
- Robertson MJ, Deane FM, Stahlschmidt W, von Kleist L, Haucke V, Robinson PJ, McCluskey A (2014) Synthesis of the Pitstop family of clathrin inhibitors. *Nat Protoc* 9:1592–1606.
- Robinson DR, Gebhart GF (2008) Inside information: the unique features of visceral sensation. *Mol Interv* 8:242–253.
- Roeckel LA, Utard V, Reiss D, Mouheiche J, Maurin H, Robe A, Audouard E, Wood JN, Goumon Y, Simonin F, Gaveriaux-Ruff C (2017)

- Morphine-induced hyperalgesia involves mu opioid receptors and the metabolite morphine-3-glucuronide. *Sci Rep* 7:10406.
- Rowan MP, Bierbower SM, Eskander MA, Sztajn K, Por ED, Gomez R, Veldhuis N, Bunnett NW, Jeske NA (2014) Activation of mu opioid receptors sensitizes transient receptor potential vanilloid type 1 (TRPV1) via beta-arrestin-2-mediated cross-talk. *PLoS One* 9:e93688.
- Spahn V, Del Vecchio G, Rodriguez-Gaztelumendi A, Temp J, Labuz D, Kloner M, Reidelbach M, Machelska H, Weber M, Stein C (2018) Opioid receptor signaling, analgesic and side effects induced by a computationally designed pH-dependent agonist. *Sci Rep* 8:8965.
- Stein C (2016) Opioid receptors. *Annu Rev Med* 67:433–451.
- Stein C (2018) New concepts in opioid analgesia. *Expert Opin Investig Drugs* 27:765–775.
- Stewart T, Beyak MJ, Vanner S (2003) Ileitis modulates potassium and sodium currents in guinea pig dorsal root ganglia sensory neurons. *J Physiol* 552:797–807.
- Stoeber M, Jullie D, Lobingier BT, Laeremans T, Steyaert J, Schiller PW, Manglik A, von Zastrow M (2018) A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* 98:963–976.e965.
- Targownik LE, Nugent Z, Singh H, Bugden S, Bernstein CN (2014) The prevalence and predictors of opioid use in inflammatory bowel disease: a population-based analysis. *Am J Gastroenterol* 109:1613–1620.
- Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 266:15771–15781.
- Valdez-Morales E, Guerrero-Alba R, Ochoa-Cortes F, Benson J, Spreadbury I, Hurlbut D, Miranda-Morales M, Lomax AE, Vanner S (2013) Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. *Neurogastroenterol Motil* 25:39–46.e4.
- Valentino RJ, Volkow ND (2018) Untangling the complexity of opioid receptor function. *Neuropsychopharmacology* 43:2514–2520.
- Varga EV, Navratilova E, Stropova D, Jambrosic J, Roeske WR, Yamamura HI (2004) Agonist-specific regulation of the delta-opioid receptor. *Life Sci* 76:599–612.
- Vicente-Sanchez A, Dripps JJ, Tipton AF, Akbari H, Akbari A, Jutkiewicz EM, Pradhan AA (2018) Tolerance to high-internalizing delta opioid receptor agonist is critically mediated by arrestin 2. *Br J Pharmacol* 175:3050–3059.
- Wang HY, Frankfurt M, Burns LH (2008) High-affinity naloxone binding to filamin A prevents mu opioid receptor-Gs coupling underlying opioid tolerance and dependence. *PLoS One* 3:e1554.
- Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, Christie MJ (2013) Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* 65:223–254.
- Yekkiala AS, Kalyuzhny AE, Portoghese PS (2010) Standard opioid agonists activate heteromeric opioid receptors: evidence for morphine and [d-Ala (2)-MePhe(4)-Glyol(5)]enkephalin as selective mu-delta agonists. *ACS Chem Neurosci* 1:146–154.
- Yu Y, Villalobos-Hernandez EC, Pradhananga S, Baker CC, Keating C, Grundy D, Lomax AE, Reed DE (2019) Deoxycholic acid activates colonic afferent nerves via 5-HT3 receptor-dependent and -independent mechanisms. *Am J Physiol Gastrointest Liver Physiol* 317:G275–G284.
- Zar MA, Iravani MM, Luheshi GN (1990) Effect of nifedipine on the contractile responses of the isolated rat bladder. *J Urol* 143:835–839.
- Zhao GM, Qian X, Schiller PW, Szeto HH (2003) Comparison of [Dmt1] DALDA and DAMGO in binding and G protein activation at mu, delta, and kappa opioid receptors. *J Pharmacol Exp Ther* 307:947–954.

MINI-REVIEW

Contributions of bile acids to gastrointestinal physiology as receptor agonists and modifiers of ion channels

Stephen J. Keely,^{1*} Andreacarola Urso,^{2,3*} Alexandr V. Ilyaskin,⁴  Christoph Korbmacher,⁴ Nigel W. Bunnett,^{5,6}  Daniel P. Poole,^{7,8} and  Simona E. Carbone^{7,8}

¹Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland; ²Department of Surgery, Vagelos College of Physicians and Surgeons, Columbia University, New York, New York; ³Department of Pharmacology, Columbia University, New York, New York; ⁴Institute of Cellular and Molecular Physiology, Friedrich-Alexander University Erlangen-Nürnberg, Bavaria, Germany; ⁵Department of Molecular Pathobiology, Neuroscience Institute, New York University, New York, New York; ⁶Department of Neuroscience and Physiology, Neuroscience Institute, New York University, New York, New York; ⁷Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia; and ⁸Australian Research Council, Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia

Abstract

Bile acids (BAs) are known to be important regulators of intestinal motility and epithelial fluid and electrolyte transport. Over the past two decades, significant advances in identifying and characterizing the receptors, transporters, and ion channels targeted by BAs have led to exciting new insights into the molecular mechanisms involved in these processes. Our appreciation of BAs, their receptors, and BA-modulated ion channels as potential targets for the development of new approaches to treat intestinal motility and transport disorders is increasing. In the current review, we aim to summarize recent advances in our knowledge of the different BA receptors and BA-modulated ion channels present in the gastrointestinal system. We discuss how they regulate motility and epithelial transport, their roles in pathogenesis, and their therapeutic potential in a range of gastrointestinal diseases.

bile acid; FXR; motility; secretion; TGR5

INTRODUCTION

Bile acids (BAs) are amphipathic steroidal molecules found as a major component of bile. BAs are synthesized in hepatocytes, stored in the gall bladder, and released into the small intestine in response to the ingestion of a meal. Classically, BAs are known for their roles in digestion through facilitating the solubilization of fatty acids, stimulating bile flow and intestinal motility, regulating glucose levels, and promoting cholesterol secretion and catabolism (1). BAs modulate their own synthesis, transport, and detoxification and aid in the maintenance of a healthy gut microbiome. Primary BAs, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized in the liver and the vast majority are conjugated (e.g., tauro-CA or glyco-CA) before their release into the ileum. In the terminal ileum, ~95% of released BAs are reabsorbed through the ileal BA transporter, IBAT (also known as apical sodium-dependent BA transporter, ASBT). BAs that are not reabsorbed in the ileum enter the colon where they undergo structural modifications catalyzed by anaerobic bacterial enzymes, such as 7 α -dehydroxylase, to

become secondary BAs (e.g., deoxycholic acid, DCA and lithocholic acid, LCA) (2).

Normally, the BAs are reabsorbed in the terminal ileum and are recycled back to the liver via the enterohepatic circulation. However, disruptions to the enterohepatic circulation, whether due to increased BA synthesis or defective reuptake in the small intestine, results in BA accumulation in the colon. When present at physiological levels, BAs act as important signaling molecules that exert their actions through specific receptors located on the cell surface and in the nucleus. However, given their amphipathic nature and detergent-like properties, increased levels of BAs in the colon can have deleterious effects on the lipid composition of cellular membranes in this region and exert cytotoxic and membranolytic effects (3, 4). Given the detergent-like properties of BAs, caution must be taken when interpreting studies where high concentrations of exogenous BAs have been used. Unless specifically assessed, any observed effects may be attributable to deleterious effects of BAs on the lipid composition of cellular membranes, receptor-mediated effects, or a combination of the two. In this review, we aim to summarize recent advances in the field of BA signaling in the gut

* S. J. Keely and A. Urso contributed equally to this work.

Correspondence: S. E. Carbone (Simona.Carbone@monash.edu).

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with a particular emphasis on how they regulate transport function and motility, and consideration of the effects of physiological versus pathophysiological concentrations of BAs.

BILE ACID RECEPTORS

The discovery that BAs can act as transmitters and mediate cellular signaling processes has been a significant advancement to our understanding of how they exert their functional effects. BAs selectively activate a broad range of G protein-coupled receptors (GPCRs), nuclear receptors, and ion channels. Strategies that indirectly alter their actions at these sites are already used to treat a range of conditions. For example, modulation of luminal BA levels, either through sequestration or altered transport, is currently the main therapeutic approach to treat BA-associated disorders, such as cholestatic itch or gallstones. However, the potential for directly targeting BA-activated receptors and ion channels now offers additional opportunities for therapy. This review provides a summary of GPCRs, nuclear receptors, and ion channels that are influenced by BAs. It aims to contextualize how the distribution, functions, and regulation of BA receptors relate to gastrointestinal (GI) physiology and how their dysregulation is implicated in digestive disease and disorders.

BILE ACID INTERACTION WITH G PROTEIN-COUPLED RECEPTORS

GPCRs are dynamic signaling proteins that are mainly located on the cell surface. They enable cells to detect and respond to a diverse array of extracellular ligands and stimuli. It is now known that BAs can activate several distinct GPCRs, including established bona fide BA receptors (Takeda G protein-coupled receptor 5, TGR5), receptors that interact with BAs in addition to their primary orthosteric agonists (e.g., muscarinic receptors), and putative BA receptors (e.g., Mrgprs). A summary of the current findings is provided in Table 1, with a more detailed description listed in the following sections.

Takeda G Protein-Coupled Receptor 5

TGR5, also known as bile acid receptor, GPBA, GpBAR1, or M-BAR1 is activated by both primary and secondary BAs. TGR5 is G_s-coupled meaning activation of the receptor stimulates adenylyl cyclase to produce cAMP. TGR5 is expressed by a diverse range of cell types in tissues including, but not limited to, gastrointestinal, liver, brown adipose, skeletal muscle, and placental. Within the gastrointestinal tract, TGR5 is expressed by epithelia, enteroendocrine cell subsets, enteric neurons, enteric glia, smooth muscle, monocytes/macrophages, and extrinsic afferents (6, 27). The roles of TGR5 in intestinal motility and secretion will be discussed in detail throughout this review. TGR5 has additional signaling roles in many other gastrointestinal processes that have not been discussed here including inflammation and the development of cancers, and visceral pain (6, 28).

Mas-Related G Protein-Coupled Receptor X4

Mrgprs are a family of orphan GPCRs consisting of more than 50 members. There are several subfamilies, with mouse

and human receptors characterized separately. For example, sensory neurons in the dorsal root ganglia (DRG) of mice express MRGPRA1, whereas the ortholog Mas-related G protein-coupled receptor X4 (MRGPRX4) is expressed in human tissues (29). Their contributions to sensation have not been fully characterized, but roles in nociception and itch have been identified. In patients with the gall bladder condition, primary biliary cholangitis, elevated levels of blood BAs are known to cause the symptom of cholestatic itch and BA sequestrants or IBAT inhibitors are used to treat the condition. However, when studying BA activation of the MRGPR family of receptors, interpretation of results may be complicated by differences in the responses of the mouse and human orthologs. For example, recent evidence from in vivo studies in transgenic mice indicates that BA-induced itch is not dependent on the expression of several Mrgprs. Mice lacking a cluster of Mrgpr genes (Mrgpr-cluster^{-/-}) exhibit itch in response to DCA in a similar manner to control mice (30). HEK cells transfected with the mouse ortholog MRGPRA1 do not respond to BAs as measured by Ca²⁺ signaling. In contrast, cells expressing the human MRGPRX4 are activated by BAs, and this is the only human receptor to respond (11). Similarly, mice expressing the humanized receptor demonstrate a greater itch response following acute injection of DCA or ursodeoxycholic acid (UDCA) compared with control mice. This same study showed that control mice responded comparably to both BAs, and this is evidence that a Mrgpr-mediated response may not involve activation of TGR5, since UDCA is a poor agonist for the latter receptor (11). It should be noted that longer administration of UDCA is used to treat pruritus associated with cholestasis, with suggested benefits such as stimulation of biliary BA excretion, replacement of the toxic endogenous BA pool, and stabilization of plasma membranes and cytoprotection of cholangiocytes (31).

In the context of the digestive system, the expression of Mrgprs has been described predominantly in primary afferent DRG neurons innervating the intestine. However, some studies have characterized mRNA expression in healthy or inflamed GI tissues. More specifically, expressions of MrgA4, MrgB2, MrgB8, MrgE, and MrgF have been demonstrated by immunohistochemistry in neurochemically defined subsets of enteric neurons (32–35). Activation of Mrgprs in cells such as mast cells could in turn activate closely associated primary afferent and sensory neurons through release of mediators including histamine and proteases. Mrgprs can interact with other cell surface receptors, such as transient receptor potential (TRP) channels to promote cellular responses (36). The relative importance of these interactions in the context of other BA-activated receptors and ion channels is not known. There are populations of primary afferent neurons innervating the gut wall that coexpress Mrgprs and TGR5. Given that both receptors are involved in histamine-independent itch pathways in the skin, this has led some researchers to postulate their function as a visceral “irritant sensing system,” suggesting their contribution to pain and symptomology in irritable bowel syndrome (IBS) (6). In a recent study in mice by Castro et al., chronic visceral hypersensitivity (CVH) responses to colorectal distention were induced by intraluminal application of DCA, and agonists of MRGPRA3 and MRGPC11. Whether DCA was acting on

Table 1. Effects of BAs on G protein-coupled receptors and nuclear receptors

Receptor	Bile Acid	Concentration	Tissue Preparation	Response	Effect	Solvent	Refs.
<i>G protein-coupled receptors</i>							
TGR5 (GPR130, GpBAR1)	DCA OA	1–100 μ M 100 μ M	Proximal colon from <i>tgr5</i> -WT and KO mice	$\uparrow$ WT KO only 100 μ M at DCA	5-HT and CGRP from EC cells to stimulate peri- staltic reflex	0.1% Ethanol, distilled water	(5)
	DCA TLCA CCDC	100 μ M	DRG neurons inner- vating colon (mouse)	$\uparrow$ $\uparrow$ $\uparrow$	Increased intracellular Ca^{2+} in subpopulations of neurons (%) DCA ~20%, TLCA ~ 25%, CCDC ~30%	NA	(6)
	CCDC	100 μ M, 100 μ L rectal enema	Spinal cord (mouse)	$\uparrow$	Phosphorylated MAP-ki- nase-ERK-1/2 immunore- activity (pERK-IR) enhanced in response to colorectal distention	Saline	
	INT-777	10–100 μ M	Proximal colon (mouse)	$\uparrow$	Increased transepithelial resistance and reduced short circuit current, reduced with TTX or neuron-free preparation	DMSO	(7)
	UDCA	3–60 μ M		-	Weak TGR5 agonist		
	LCA, DCA	10 and 30 μ M	Enteroendocrine cell line, STC-1	$\uparrow$	Promote GLP-1 secretion	DMSO	(8)
			Cells transfected with TGR5 siRNA	-	Reduced LCA responses at 10 and 30 μ M		
			Cells transfected with TGR5 cDNA	$\uparrow$	Increased LCA responses at 30 μ M		
	Range of conjugated & unconjugated BAs	Concentration curve	COS-7 monkey kid- ney cells express- ing human or rat TGR5	$\uparrow$	Increased cAMP production rank order of potency LCA>DCA>CDCA UDCA and CA ~20% efficacy at 10 μ M	Modified Krebs- Ringer buffer	(9)
	UDCA BA mixture	10 mM Total BA ~9 mM, 1 mM each	In vivo intraluminal <i>tgr5</i> -WT and KO mice	-UDCA BA mix: $\uparrow$ WT - KO	GLP-1 and PYY release	Isotonic saline	
MrgprX4	LCA RO5527239 (TGR5 agonist)	Concentration curve	CHO cells trans- fected with TGR5	$\uparrow$	Increased cAMP production EC_{50} = 457 and 3.6 nM E_{Max} ~110% both	NA	(10)
	DCA RO5527239	Concentration curve	STC-1	$\uparrow$	Increased cAMP production EC_{50} = 1.59 μ M and 321 nM GLP-1 release EC_{50} = 11 μ M and 321 nM		
	DCA, UDCA CDCA, CA, OA	2–100 μ M	HEK293 cells	$\uparrow$	Increased $[\text{Ca}^{2+}]_i$		(11)
	DCA UDCA	10 μ M 100 μ M	DRG from +X4 humanized mice	$\uparrow$	~5%–6% of +X4 sensory neurons activated by both	Water, DMSO, or 0.1 M NaOHNA	
Muscarinic M2	DCA, t-DCA, UDCA CDCA	1–2 mM	In vivo +X4 human- ized mice	$\uparrow$	Increased cholestatic itch		
	t-CDCA, g-DCA, t-DCA, t-CA	100 μ M	Ventricular neonatal rat myocytes	$\uparrow$	Reduced contraction (G_i coupled) (role in TGR5 mediated release of cAMP found to be inde- pendent of contraction response)	L-15 media	(12)
	TCA	0–100 μ M	Ventricular neonatal rat myocytes	Partial agonist	Radioligand binding, K_d = 17 μ M Reduced cAMP production (30% of carbachol response) Reduced contraction (0.2 and 1 mM)	DMSO HBSS	(13)

Continued

Table 1.— Continued

Receptor	Bile Acid	Concentration	Tissue Preparation	Response	Effect	Solvent	Refs.
Muscarinic M3	CDCA, LCA, g-LCA, t-LCA, g-DCA, t-DCA, UDCA	30 μ M	Ex vivo, precision-cut lung slices	↓	Inhibited acetylcholine contractile responses t-LCA IC ₅₀ = 3.2 μ M	NA	(14)
S1PR2	t-CA, t-DCA, g-DCA, g-CA, t-UDCA	50–100 μ M	Primary rat hepatocytes	↑	Activation of ERK1/2 and AKT	DMSO	(15)
	t-CA	100 μ M	MLE		Activation of ERK1/2 and AKT Cell proliferation and migration	PBS	(16)
FPR	CDCA	25–400 μ M	Human monocytes	↓	Inhibited monocyte chemotaxis to fMLP IC ₅₀ = 100 μ M	Ethanol	(17)
			Human monocytes		Inhibited monocyte Ca flux by fMLP		
			EFTR cell		Inhibited cell migration by fMLP IC ₅₀ = 125 μ M		
			EFTR cell		Inhibited Ca ²⁺ flux by fMLP IC ₅₀ = 50 μ M		
			Human monocytes EFTR cell		Radioligand fMLP binding IC ₅₀ = 140 μ M		
	DCA	25–200 μ M	Human monocytes and neutrophils	↓	Inhibited fMLP induced chemotaxis IC ₅₀ = 100 μ M	PBS	(18)
		25 μ M			Inhibited fMLP induced Ca ²⁺ mobilization		
		5–200 μ M			Radioligand fMLP binding		
<i>Nuclear receptors</i>							
	CDCA > DCA and LCA	50 μ M concentration curve for CDCA	CV-1 cells with rat FXR HepG2 cells with human FXR	↑	Transactivation of FXR with luciferase reporter CDCA EC ₅₀ = 50 μ M and 10 μ M rat and human FXR	Ethanol	(19)
	CDCA > DCA and LCA	100 μ M	CV-1 cells	↑	Reporter gene assay. Increased CAT activity	NA	(20)
	CDCA, g-CDCA, t-CDCA			↑	FRET ligand sensing assay Increased relative fluorescence CDCA EC ₅₀ = 4.5 μ M g- and t-CDCA EC ₅₀ = 10 μ M		
FXR	CA GW4062 (FXR agonist)	200 mg/kg 100 mg/kg	Mice treated in vivo 72 h before to tissue harvest	Ileum, jejunum and duodenum ↑ Liver-	FGF-15 mRNA present in ileum, jejunum and duodenum	NA	(21, 22)
	CDCA	Concentration curve	Human ileum and colon mucosal biopsies	Ileum ↑ Colon-	FGF19 expression EC ₅₀ = 20 μ M		
	CA	50 μ M	6-h incubation	Comparison with matched con. CDCA	80% of CDCA response		
	DCA LCA OCA	20 μ M			40% 4% 5 times greater than CDCA response		
PXR	LCA 3-keto-LCA	Concentration curve	CV-1 cells with human PXR	↑ ↑	Scintillation proximity binding assay IC ₅₀ = 9 and 15 μ M	NA	(23)
	LCA	100 μ M	CV-1 cells with human or mouse PXR and reporter plasmid	↑ (Human)	Reporter gene assay. Increased CAT activity		
	3-keto-LCA CDCA CA		(Cyp3a23)2-tk-CAT	↑ - -			

Continued

Table 1.— Continued

Receptor	Bile Acid	Concentration	Tissue Preparation	Response	Effect	Solvent	Refs.
VDR	DCA CDCA DCA LCA	100 μ M	CV-1 cells with human and rodent PXR and reporter plasmids	- $\uparrow$ $\uparrow$ $\uparrow$	Reporter gene activity and CYP3A4 reporter gene activity	NA	(24)
	LCA	8 mg/day for 4 days, oral gavage	PXR-WT and KO mice in vivo	$\uparrow$ WT -KO	Northern blot analysis CYP3A11 mRNA		
	LCA 3-keto-LCA	Concentration curve	HEK293 cells transfected with VDR	$\uparrow$	GAL4-receptor luciferase assay EC ₅₀ = 8 and 3 μ M	NA	(25)
	LCA 3-keto-LCA		Monkey kidney COS-7	$\uparrow$	Competitive binding assay [³ H]1,25(OH) ₂ D ₃ K _i = 29 and 8 μ M		
	CA, CDCA LCA	0.8 mmol/kg, oral gavage	Ileum tissue of Vdr WT and KO	$\uparrow$ WT -KO	Induces mRNA expression of Cyp24a1	Ethanol and corn oil	(26)

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid. 3-Keto-LCA is the major metabolite of LCA in rats. UDCA, ursodeoxycholic acid; t-, tauro-conjugated; g-, glyco-conjugated bile acid. Cell lines: rat basophil leukemia cell line transfected with FPR EFTR cell; mouse large cholangiocytes MLE; monkey kidney CV-1 cells; human hepatoma HepG2; enteroendocrine cell line, STC-1. Responses: activation, $\uparrow$; inhibition, $\downarrow$; no change, -. Solvents: NA, no information available; DMSO stock, dimethyl sulfoxide (DMSO).

Mrgpr receptors was not studied here. Although the expression profiles of TGR5, MRGPRA3, and MRGPC11 were identified in mouse sensory DRG neurons, expression of MRGPRA1 was not mentioned. The same study examined the response profiles of human DRG neurons. Although they found that cells responded to TGR5-prefering agonists they did not study receptor specificity for these responses and this may be due to limitations in the pharmacological tools currently available. The expression profiles of MRGPRA4 were also not mentioned (6).

Muscarinic Acetylcholine Receptors

Some BAs can interact with muscarinic receptors to influence cholinergic signaling. This is likely the result of structural similarities between some BAs and acetylcholine (ACh), as has been identified for lithocholic acid conjugates (37, 38). Secondary BAs, including tauroolithocholic acid (t-LCA), block cholinergic-induced contractions of mouse and human airway smooth muscle evoked by either exogenous ACh or by electrical stimulation of parasympathetic nerve terminals, possibly by acting as antagonists of M3 muscarinic receptors (14). Urso et al. found that this was not through specific activation of TGR5, as BAs continued to relax precontracted smooth muscle tissues from *tgr5*^{-/-} mice, and selective TGR5 small molecule agonists did not relax tissue from wild-type animals. A role for BAs in muscarinic signaling is further supported by studies in cardiomyocytes, where taurocholate inhibited cAMP-production in a pertussis toxin-sensitive manner to limit contraction. This response was prevented upon pharmacological inhibition or siRNA knockdown of the M2 muscarinic receptor (12, 13). Muscarinic receptors have prominent roles in gastrointestinal motility and secretion. Therefore, it is surprising that not more is known about the implications of BAs interacting with muscarinic receptors in these processes.

Sphingosine-1-Phosphate Receptor 2

Sphingosine-1-phosphate receptor 2 (S1PR2) is one of a series of receptors that respond to the bioactive lipid mediator, lysosphingolipid (S1P). S1PRs are ubiquitously expressed in

different tissues and this is reflected by their roles in many processes including angiogenesis, lymphangiogenesis, cell proliferation and motility, immune cell function, and lipid metabolism (39). S1PR2 also has a role as a BA receptor (40). In GPCR screening assays, rodent hepatocytes responded to BAs through extracellular signal-regulated kinase (ERK)1/2 and protein kinase B/Akt signaling. These BA-mediated responses were reduced by an S1PR2-selective antagonist, knockdown of the receptor, and using cells from S1PR2 knockout mice (15). Furthermore, the proliferation of cholangiocytes and onset of liver fibrosis in response to experimental bile duct ligation were attenuated in S1PR2-deficient mice, consistent with the proposed interaction of S1PR2 and BAs (16).

Formyl Peptide Receptors

Formyl peptide receptors (FPRs) are a group of GPCRs involved in innate immune responses. *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP), a major chemotactic factor, is an agonist with different binding affinities for the three FPRs. fMLP binds with high- and low affinities to formyl peptide receptor (FPR) and formyl peptide receptor-like type-1 (FPRL-1), respectively. However, formyl peptide receptor-like type-2 (FPRL-2) does not bind to *N*-formyl peptides. Roles for FPRs in BA-induced signaling have been demonstrated in studies on human monocytes and using human embryonic kidney cells (HEK-293) transfected with FPR and FPRL-1. In these cells, pretreatment with CDCA and DCA inhibited fMLP binding to its receptors and associated elevations in intracellular Ca²⁺ (17, 18). In these same studies, cell migration stimulated by fMLP was inhibited by CDCA and DCA treatment (17, 18). Although these studies did not assess whether TGR5 mediates BA-dependent inhibition of chemotaxis and calcium signaling in these cells, computational modeling experiments have demonstrated antagonist binding sites for DCA on the FPR (41), supporting the idea that direct inhibition of these BA receptors is involved. Intestinal microbiota are known to produce fMLP, and recent studies of interactions between the microbiome, FPRs, and gut motility suggest that FPR-expressing enteric neurons are

important mediators of probiotic-induced gut motility (42). It is therefore plausible that BAs could modulate gastrointestinal physiology through actions on neuronal FPRs. However, direct experimental evidence for such interactions is currently lacking. Furthermore, since FPRs are also expressed by intestinal neutrophils with different expression patterns noted for cells isolated from tissues of patients with Crohn's disease (43), BA/FPR interactions on intestinal immune cells could have important implications for pathogenesis of inflammatory bowel diseases (IBDs).

NUCLEAR RECEPTORS FOR BILE ACIDS

Nuclear receptors are a family of ligand-activated transcription factors, comprised of a ligand-binding domain and a DNA-binding domain to promote or suppress transcription. Activators for these receptors include steroid hormones such as estrogen and progesterone and lipid-soluble mediators such as retinoic acid and vitamin D. In this way they allow factors such as hormones or nutrients to modulate gene expression (44). BAs are ligands for several nuclear receptors with roles in glucose, lipid, and energy metabolism (45). These receptors regulate BA levels in the enterohepatic circulation and mediate processes in inflammation. They represent novel targets for a range of therapies for metabolic, cholestatic liver, and inflammatory bowel diseases (46, 47).

Farnesoid X Receptor

Farnesol was originally thought to be the cognate ligand for farnesoid X receptor (FXR), but this agonist only activates the receptor at supraphysiological concentrations (48). In contrast, conjugated and unconjugated BAs activate FXR at physiological levels with CDCA being one of the most potent agonists (19, 20, 49). FXR is highly expressed in liver and intestinal tissues and has significant roles in mediating both metabolic processes and BA homeostasis. Studies of FXR knockout mice provide evidence for the roles of FXR-dependent BA metabolism, as these mice have elevated levels of circulating BAs and they excrete fewer BAs in their fecal matter (50). In hepatocytes, FXR activation limits the production of primary BAs by various pathways that include downregulating the rate-limiting enzyme for BA synthesis. It also stimulates the secretion of BA from the liver into the gall bladder and limits hepatic reuptake. In intestinal cells, FXR activation promotes downstream pathways that lead to intracellular transport of BAs and reduce BA production in hepatocytes (45, 51). In human ileal enterocytes, BA-mediated activation of FXR stimulates transcription of the hormone fibroblast growth factor 19 (FGF-19, mouse ortholog FGF-15) (21, 22). FGF-19/FGF-15 then activates a downstream pathway in hepatocytes, ultimately inhibiting the production of BAs (45). Maintaining BA homeostasis is important for liver function but when improperly regulated can lead to a range of digestive diseases. For example, BA-diarrhea (BAD) is thought to be caused by alterations in enterohepatic circulation. Given the roles of FXR in stimulating these processes, FXR agonists have been successfully tested for their therapeutic potential to treat this condition (51, 52). The therapeutic potential may extend beyond regulating the enterohepatic circulation of BAs since activation of FXR has been

shown to promote antisecretory effects in colonic epithelial cells (53). These additional roles are discussed in more detail later in this review (see BILE ACIDS AND INTESTINAL TRANSPORT OF ELECTROLYTES AND FLUID). In the liver, FXR activation also limits glucogenesis and lipogenesis, therefore, targeting FXR has potential as a therapeutic strategy to treat a range of metabolic conditions, such as nonalcoholic steatohepatitis (54, 55).

Pregnane X Receptor

A range of lipophilic chemicals bind to Pregnane X receptor (PXR) including xenobiotics, endogenous hormones, and BAs (56). PXR is responsible for the metabolism, detoxification, and elimination of such agents. It is highly expressed in the liver and small intestine (24). PXR activation enhances the production of enzymes responsible for BA metabolism/detoxification, represses enzymes involved in BA synthesis, and alters the expression of BA transport proteins (23, 24, 57). LCA also activates PXR (24, 57). Along with FXR, targeting PXR has been proposed as a possible IBD treatment, particularly for patients with Crohn's disease associated with BA-diarrhea [BAD (58)]. In patients with BAD, the activities of enzymes that catalyze BA metabolism are reduced, enzymes that facilitate BA synthesis are elevated and hence the balance of BAs levels is dysregulated. These studies have postulated that PXR or FXR may be deactivated in these patients (59, 60). Dextran sulfate sodium (DSS)-induced colitis is more severe in PXR knockout mice when compared with the wild-type control, and activation of PXR can alleviate the development of inflammation in wild-type mice (61).

Vitamin D Receptor

Typically, vitamin D receptor (VDR) responds to the active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ 1,25(OH)₂D₃. The receptor is expressed in the intestine and kidneys where it mediates a range of processes such as calcium homeostasis, cellular differentiation, and immunity.

LCA is a metabolite of CDCA. Although the primary BAs, CDCA and CA, do not activate VDR, LCA is a potent VDR agonist (25). Oral administration of LCA to mice can induce the expression of some of the same target genes as VDR activation by 1,25(OH)₂D₃ (26). Although clarity on the physiological relevance for LCA activation of VDR is lacking, it is thought to have a protective role by mediating the elimination and breakdown of LCA, given the cytotoxic and pro-oncogenic nature of the BA (62, 63). Since, intestinal bacteria metabolize CDCA to LCA, it is hypothesized that this provides a mechanism for intestinal bacteria to regulate host VDR function. Recent evidence suggests that the LCA-VDR interactions may have protective roles for the intestinal epithelial barrier (64). This is supported by observations that VDR knockout mice develop dextran sulfate sodium (DSS) colitis more quickly and robustly than wild-type animals (65), and in patients with Crohn's disease or ulcerative colitis colonic epithelial VDR expression is reduced (66).

LCA acts on FXR, PXR, and VDR to modulate BA metabolism, and the downstream effects of these interactions regulate the activities of the cytochrome P450 (CYP) family of enzymes (45). These enzymes protect the body from

a variety of lipophilic chemicals, such as BAs, and their actions are controlled at a transcriptional level by many different signaling molecules. Activation of VDR, by either LCA or $1,25(\text{OH})_2\text{D}_3$, induces transcription of the CYP3A4 gene (25). The CYP3A4 enzyme assists in the detoxification of LCA and other BAs by catalyzing their hydroxylation. Hydroxylation of BAs means they are more hydrophilic, facilitating their excretion in fecal matter or urine. The importance of this interaction is demonstrated in VDR knockout mice, where intestinal, circulating, and fecal BA pools are all dysregulated (65, 67). Insertion of the CYP3A4 gene into these mutant mice improves the BA pool size so that the relative levels of BAs in liver and intestine homogenates return to levels comparable with those of wild-type mice. It also reduces the effects of LCA-induced toxicity by stimulating LCA metabolism (68).

Constitutive Androstane Receptor

Constitutive androstane receptor (CAR) is expressed in the liver and intestines where it mediates the detoxification of phenobarbital-like inducers (69). Although endogenous activators for CAR are not well characterized, both androstenol and androstanol have been identified as repressive endogenous ligands for constitutive activity. BAs are unlikely to directly activate CAR (70) but indirect activation has been suggested in LCA-induced liver toxicity models. CAR knockout mice develop more severe damage compared with wild-type controls, highlighting that LCA detoxification could not occur in these mice (71). CAR expression is reduced in the colon from mice with DSS-induced colitis. Similarly, mucosal intestinal biopsies from patients with Crohn's disease and ulcerative colitis also have reduced CAR expression when compared with healthy individuals (72).

FXR, PXR, and CAR regulate overlapping target genes in BA metabolism. To investigate how they coordinate their activities, several studies have used double knockout mice for different combinations of these receptors. They suggest that the receptors jointly protect against BA-induced liver toxicity (71, 73, 74). The gut microbiome regulates the function and expression of CAR and PXR receptors by stimulating the conversion of primary BAs to secondary BAs. Tissues from germ-free (GF) mice express significantly lower mRNA levels for CAR, PXR, and FXR as well as for a range of cytochrome P450 enzymes relative to mice housed under specific pathogen-free (SPF) conditions (75). In conventional mice, daily administration of CAR and PXR agonists changes the biodiversity of their microbiome by reducing two taxa in the *Bifidobacterium* genus (76). Again, this highlights the potential interplay between the gut microbiome, the regulation of BAs levels, the expression this group of receptors, and their activation by BAs.

BILE ACIDS AS MODIFIERS OF ION CHANNEL ACTIVITY

BAs can also exert their effects through interactions with ion channels. Specific ionotropic receptors for BAs have not been identified to date. However, there is good evidence that BAs can modulate the functions of several members of the epithelial sodium channel/degenerin (ENaC/DEG) family.

This interaction may be physiologically relevant in tissues where BAs have access to these channels, such as in the intestine and bile ducts. The regulation of these channels by hormones and local factors is highly complex and involves the regulation of channel expression, trafficking, and open probability. Several members of the ENaC/DEG family of ion channels can qualify as ligand-gated ion channels. These include the acid sensing ion channels (ASICs) and the FMRFamide-activated Na^+ channel (FaNaC) that are activated by protons and the tetrapeptide FMRFamide, respectively. For other members of this family, no specific activating ligands have been described with the possible exception of BA-sensitive ion channel (BASIC). A summary of the actions of BAs on ion channels and ionotropic receptors is provided in Table 2.

BA-Sensitive Ion Channel

BA-sensitive ion channel (BASIC) is a member of the ENaC/DEG ion channel family (previously known as intestinal Na^+ channel (INaC) in humans or brain-liver-intestine Na^+ channel (BLINaC) in rodents) (94, 95). BASIC is expressed on the apical membrane of rat cholangiocytes (77, 80). It shows very low basal activity in heterologous expression systems, but is strongly activated by submillimolar concentrations of BAs (77–82). Based on this observation, it is possible that BAs may act as endogenous ligands for the channel in tissues where they are present at high concentrations, such as the bile ducts and the small intestine. However, the stimulatory effect of BA on BASIC does not seem to be as potent and as specific as the stimulatory effect of ligands on classical ionotropic receptors. Moreover, a modulatory effect of BAs on ion channel activity does not seem to be limited to BASIC.

Epithelial Sodium Channel

Epithelial sodium channel (ENaC) is expressed in the apical membrane of epithelia in a range of tissues, including the distal colon and the biliary ducts (96). It mediates transepithelial sodium absorption and therefore regulates fluid volume (97–100). Recent evidence suggests that BA sensitivity is not a unique feature of BASIC but may be a common feature of channels belonging to the ENaC/DEG family. Similar or lower concentrations of BAs needed to activate BASIC can stimulate the rat, mouse, and human ENaC (83–85). Generally, BAs activate ENaC with varying abilities, but some also inhibit the channel (84–86).

It is likely that exposure of ENaC to changing levels of BAs in cholangiocytes and distal colon may modify the function of the channel under physiological conditions. In kidney and distal colon, ENaC plays an important role in mediating sodium absorption. In these tissues, ENaC is mainly stimulated by the mineralocorticoid aldosterone but can also be regulated by additional mediators and local mechanisms (101–104).

Despite the location of ENaC on the apical membrane, BAs may also affect the channel through interactions originating from the basal surface. Preliminary studies demonstrated that LCA applied to the serosal side of the mouse distal colon had a sustained stimulatory effect on ENaC-mediated transepithelial

Table 2. Effects of bile acids on ion channels and ionotropic receptors

Ion Channel	Bile Acid	Effect: Stimulation ↑ Inhibition ↓	Concentration Used or EC ₅₀ /IC ₅₀ , if Available	Solvent	Cells Expression System	Refs.		
Epithelial sodium channel/degenerin ion channel family								
BASIC ^{rat}	g-CDCA, t-CDCA g-HDCA, t-HDCA	↑↑↑	1–1.5 mM	NA	<i>X. laevis</i> oocytes	(77, 78)		
	t-CA, t-βMCA	↑	g-HDCA: EC ₅₀ ~ 2 mM					
	UDCA	↑↑↑	EC ₅₀ ~ 2.5 mM					
	t-UDCA, t-HDCA	↑↑↑	1–2 mM t-UDCA: EC ₅₀ ~ 2.7 mM	NA	HEK293 cells	(79)		
	t-CA, t-LCA t-DCA, t-CDCA	↑↑						
	t-CDCA, t-HDCA t-UDCA	↑↑					500 μM–1 mM	NA
	BASIC ^{human}	t-DCA	↑↑↑	500 μM–1 mM	Bath solution	<i>X. laevis</i> oocytes	(77, 81, 82)	
t-CDCA		↑↑↑	NA					
t-CA, t-HDCA t-DCA, t-CDCA		↑ ↑↑↑						
BASIC ^{mouse}		t-CA, t-UDCA t-HDCA, t-LCA	↑	1–2 mM	NA	HEK293 cells	(79)	
	t-CA, t-UDCA, t-HDCA t-LCA, t-DCA, t-CDCA	↑↑	1–2 mM t-UDCA: EC ₅₀ ~ 4.5 mM	NA	HEK293 cells	(79)		
	αβγENaC ^{rat}	CA	↑↑	1 mM t-DCA: EC ₅₀ ~ 260 μM t-CA: EC ₅₀ ~ 410 μM	DMSO stock	<i>X. laevis</i> oocytes	(83)	
t-DCA		↑↑↑						
g-CA, t-CA		↑↑						
t-CDCA, t-UDCA		↑						
t-βMCA, t-HDCA		No effect						
αβγENaC ^{human}	t-DCA	↑↑↑	250 μM	Bath solution	<i>X. laevis</i> oocytes	(84)		
	t-CA, t-CDCA	↑↑						
	CA, DCA	↑						
	CDCA	↓						
δβγENaC ^{human}	CA, t-CA, DCA, t-DCA CDCA, t-CDCA	↑↑↑						
	αβγENaC ^{mouse}	DCA, t-CA g-CA	↑↑↑ ↑↑	1 mM DCA: EC ₅₀ ~ 80 μM t-CA: EC ₅₀ ~ 150 μM	H ₂ O stock	<i>X. laevis</i> oocytes	(85)	
CA, t-LCA, UCA		↑↑	DMSO stock					
CDCA, t-CDCA 12βHICA		↑						
HDCA t-HDCA		↓ ↓↓	H ₂ O stock					
t-CA		↑↑	1 mM t-CA: EC ₅₀ ~ 200 μM		H ₂ O stock			mpkCCD14 cells
t-HDCA		↓						
ENaC ^{human}	UDCA	↓↓	500 μM	NA	NuLi-1 and CuFi-1 airway epithelial cells	(86)		
ASIC1a ^{human}	t-CDCA	↑↑↑	500 μM t-DCA: EC ₅₀ ~ 150 μM	Bath solution	<i>X. laevis</i> oocytes	(87)		
	t-DCA	↑↑						
	t-CA	↑						
Other ion channels and ionotropic receptors								
BK _{Ca} ^{rabbit}	CA, DCA, LCA, t-LCA	↑↑↑	100 μM	DMSO ethanol stock	Smooth muscle cells	(88)		

Continued

Na⁺ transport. This is reminiscent of the stimulatory effect of aldosterone (105). The delayed and sustained stimulatory effect of LCA on ENaC may be due to transcriptional regulation involving a nuclear receptor, such as FXR. A dual

stimulatory effect of BAs on ENaC from the apical and basolateral side may serve as a compensatory mechanism in the colon to limit fluid loss during diarrhea resulting from impaired ileal BA absorption (for a more detailed discussion

Table 2.— Continued

Ion Channel	Bile Acid	Effect: Stimulation ↑ Inhibition ↓	Concentration Used or EC ₅₀ /IC ₅₀ , if Available	Solvent	Cells Expression System	Refs.
NMDA ^{mouse}	CA, g-CA, t-CA		IC ₅₀ ~ 500-700 μM			
	DCA, t-DCA	↓↓↓	IC ₅₀ ~ 60-70 μM			
	CDCA, t-CDCA					
	g-CDCA		IC ₅₀ ~ 130 μM			
GABA _A ^{mouse}	CA, g-CA, t-CA		IC ₅₀ ~ 500-700 μM	NA	Hypothalamic neurons	(89, 90)
	DCA, t-DCA		IC ₅₀ ~ 150 μM			
	CDCA, g-CDCA	↓↓↓	IC ₅₀ ~ 70-80 μM			
	t-CDCA		IC ₅₀ ~ 70-100 μM			
GABA _A α1 ^{rat} β1 ^{rat} γ2L ^{mouse} α1 ^{rat} β2 ^{rat} γ2L ^{mouse} α1 ^{rat} β3 ^{human} γ2L ^{mouse} α1 ^{rat} β3 ^{human} β3 ^{human} β3 ^{human} γ2L ^{mouse}	UDCA	↓↓↓	IC ₅₀ ~ 70-100 μM	NA	HEK293 cells	(90)
			IC ₅₀ ~ 7 μM			
			IC ₅₀ ~ 200 μM			
NMDA GluN1 ^{rat} 2A ^{rat} GluN1 ^{rat} 2B ^{rat}	CA, g-CA, t-CA					
	CDCA, g-CDCA	↓	100 μM			
	t-CDCA					
GluN1 ^{rat} 2D ^{rat}	CA, g-CA, t-CA	↓		NA	<i>X. laevis</i> oocytes	(91)
	CDCA, g-CDCA	↓↓	100 μM			
	t-CDCA		t-CDCA: IC ₅₀ ~ 180 μM			
GluN1 ^{rat} 3B ^{mouse}	CA, g-CA, t-CA	↓	100 μM			
	CDCA, g-CDCA	↓↓↓	t-CDCA: IC ₅₀ ~ 40-80 μM			
	t-CDCA					
P2X4 ^{human}	CA, t-CA, CDCA	No effect		Bath solution	<i>X. laevis</i> oocytes	(92)
	t-CDCA	↓↓	250-500 μM			
	DCA, t-DCA	↓↓↓	t-DCA: IC ₅₀ ~ 160 μM			
	t-CA	No effect				
P2X2 ^{rat}	t-UDCA, t-DCA	↓				
	t-CDCA	↓↓	20 μM			
	t-LCA, HDCA	↓↓↓	t-HDCA: IC ₅₀ ~ 11 μM			
	t-HDCA, g-HDCA					
P2X1 ^{rat}	t-HDCA	No effect	20 μM	H ₂ O stock	<i>X. laevis</i> oocytes	(93)
	t-LCA	↓	200 μM			
P2X3 ^{rat}	t-HDCA	No effect	20 μM			
P2X7 ^{rat}	t-LCA		200 μM			
P2X4 ^{rat}	t-LCA	↑	200 μM			
	t-HDCA	No effect	20 μM			
P2X2 ^{human}	t-LCA	↑↑				
	t-HDCA	No effect	20 μM			

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; UCA, ursodeoxycholic acid; UDCA, ursodeoxycholic acid; 12βHICA, 12β-hydroxyisocholic acid; βMCA, β-muricholic acid; t-, tauro-conjugated; g-, glyco-conjugated bile acid. Solvents: NA, no information available; bath solution, bile acids/bile salts were directly dissolved in bath solution to obtain the indicated final concentrations; DMSO stock, dimethyl sulfoxide (DMSO) stock solutions were prepared with bile acids at a concentration of 100 mM; H₂O stock, aqueous stock solutions were prepared with bile acid concentrations ranging from 20 to 100 mM; DMSO/ethanol stock, concentrated dimethyl sulfoxide (DMSO) stock solutions were prepared with bile acids at a concentration of 333 mM and then further diluted 1/10 in 95% ethanol.

of the effects of BA on intestinal transport see BILE ACIDS AND INTESTINAL TRANSPORT OF ELECTROLYTES AND FLUID).

Human Acid-Sensing Ion Channel ASIC1a

ASIC1a is another member of the ENaC/DEG family of ion channels. Although BAs do not directly activate ASIC1a, they positively modulate proton-activated ASIC1a currents (87). BAs may not act as classical ligands on the orthosteric site of the channel, but rather modulate the function of already

active ASIC1a channels by interacting with a topographically distinct, allosteric site. Using site-directed mutagenesis and computer simulation it has been shown that BAs may directly interact with the transmembrane domains of ENaC/DEG ion channels in the so-called “degenerin region” known to be functionally important for channel gating (81, 84, 87). BA binding to the transmembrane domains of the channel probably stabilizes the channel open conformation leading to increased channel activity. Several ASICs are responsible

for distinct proton-gated currents in neurons regulating gastrointestinal function (106). Thus, it is tempting to speculate that modulation of ASICs by BAs may contribute to gastrointestinal physiology and pathophysiology.

Other Ion Channels and Ionotropic Receptors

BA may also modulate other ion channels and ionotropic receptors. For example, BAs have been shown to modify the function of large-conductance Ca^{2+} - and voltage-activated K^+ (BK) channels via distinct channel subunits and sites (88), they also inhibit currents mediated by ionotropic NMDA and GABA_A receptors (89–91). Recently, it has been demonstrated that the human purinergic receptor P2X₄, an ATP-gated non-selective cation channel, is inhibited by unconjugated or tauro-conjugated DCA (92). The inhibitory effect of BAs is not limited to P2X₄ and has also been reported for the related P2X₂ receptor (93). A comparison of the crystal structure of

P2X₄ revealed a high degree of similarity in topology and architecture with that of ASIC1 (107), suggesting structural similarities of their BA binding sites. Indeed, tauro-DCA probably interacts with the transmembrane domains of P2X₄ thereby stabilizing its closed state (92). P2X₄ is abundantly expressed in hepatocytes and cholangiocytes (108, 109) and P2X₂ is present in gall bladder and enteric neurons (110, 111). Therefore, BAs may potentially play a role as local modulators of purinoceptors in these systems.

BILE ACIDS AND INTESTINAL TRANSPORT OF ELECTROLYTES AND FLUID

Bile Acids Modulate Intestinal Fluid and Electrolyte Transport in Pathophysiology

Intestinal fluid absorption is critical for effective homeostatic control of whole body fluid and electrolyte balance.

Table 3. Effects of bile acids on gastrointestinal motility

GI Region/Application Method/ Measurement	Animal Species	Bile Acid	Concentration Used	Effect:	Refs.
Stomach and small intestine					
Stomach and small intestine/oral gavage/phenol red	Rat	t-DCA DCA	26–100 mM 5–26 mM	GE ↓ SIT ↓ GE ↓↓ IT ↓↓	(117)
Stomach-cecum/ileum infusion/environmental hydrogen analysis		t-CA DCA	10 mM 10 mM	SCTT ↑ SCTT ↓	(118)
Stomach/nasogastric/paracetamol absorption test	Human (healthy and type 2 diabetes)	CDCA	1.25 g in 100 mL water	GE ↓	(119)
Small intestine					
Jejunum and ileum/intraluminal infusion/lactulose and in vivo manometry	Human (healthy)	g-CDCA g-CA	15–30 mM 15 mM	SIT ↓ SIT ↓	(120, 121)
Terminal ileum/intraluminal infusion/ex vivo manometry	Rabbit	Rabbit bile t-DCA	0.1–10 mM	MA ↓ MA ↓ at 10 mM	(122)
Colon (in vivo)					
GI and colonic transit/oral/side effect profile	Human (cholesterol gallstone)	UDCA CDCA	7–8 and 14–15 mg/kg/day (3–12 mo)	BF – BF ↑	(123)
Proximal colon/rectal perfusion/in vivo manometry and GI sensation	Human (healthy)	CDCA	1 mM	Sensory threshold ↓ PS ↑	(124)
GI and colonic transit/oral capsule/scintigraphy method and bowel function	Human (healthy and IBS-C)	CDCA	500, 1,000 mg	CT ↑ BF ↑	(125) (126)
GI and colonic transit/rectal perfusion/ GI sensation	Human (healthy)	TCA	1,500 and 3,500 mg	Sensory threshold ↓ BF ↑	(127)
Colon (in vitro/ex vivo)					
Colonic motility/bath addition muscle exposure/contraction assay	Mouse <i>tgr5-wt</i> and <i>ko</i>	UDCA DCA	100 μM 1, 10, 100 μM	WT contraction – WT contraction ↓ KO contraction –	(128, 129)
Colonic motility/ bath addition mucosal exposure/ contraction assay	<i>tgr5-wt</i> and <i>ko</i>	UDCA DCA LCA OA	100 μM 1, 10, 100 μM 1, 10, 100 μM 100 μM	WT PR – WT PR ↑ KO – WT PR ↑ KO – WT PR ↑ KO –	(129)
Colonic smooth muscle/ bath addition muscle exposure/ contraction assay	Rat	DCA	1, 10, 100 μM	Contraction –	(130)
Colon					
Colon and rectum/intraluminal infusion/ex vivo manometry	Rabbit	t-CA g-CA	2–16 mM 8–24 mM	MA ↑ MA ↑	(131)
		g-CA DCA	3–30 mM	MA – MA ↑	(132)

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; t-, tauro-conjugated; g-, glyco-conjugated bile acid; Rank order of potency for TGR5 is t-LCA > LCA > DCA > CDCA, UDCA and OA are weak TGR5 agonists. Responses: stimulation, ↑; inhibition, ↓; no change, –. BF, bowel function; CT, colonic transit; GE, gastric emptying; IBS-C, irritable bowel syndrome-constipation predominate; MA, motor activity; PR, peristaltic reflex; PS, propagating sequences; SIT, small intestinal transit.

Na^+ absorption drives fluid uptake across small intestinal villi and surface cells of the colon, whereas Cl^- secretion drives fluid secretion occurring mainly from the crypts. Three main mechanisms drive Na^+ absorption along the gut as follows: while digestion is occurring, small intestinal Na^+ -nutrient co-transporters predominate; during the interdigestive period, Na^+/H^+ exchange predominates along the entire length of the intestine; and electrogenic Na^+ absorption is the primary mechanism driving fluid absorption in the distal colon (112). Depending on physiological versus pathophysiological conditions, BAs can activate both proabsorptive and prosecretory pathways to influence ion flux and water retention.

There has long been an association between increased luminal BA levels and the onset of diarrhea in humans. However, the roles of BAs in fluid dysregulation were not recognized until much later than their effects on intestinal motility (see BILE ACIDS AND INTESTINAL MOTILITY). The first preclinical demonstration of such actions came in 1966, where perfusion of DCA, at a concentration of 100 μM , to the rat jejunum inhibited intestinal fluid absorption (113). Based on observations in patients with ileitis and increased BA turnover, Hofmann (114) proposed that the increased levels of luminal BAs were causing the clinical symptoms of diarrhea. This hypothesis was subsequently supported by clinical studies showing that direct administration of dihydroxy BAs to the colorectum induced diarrhea in healthy individuals (115), whereas infusion of conjugated and unconjugated BAs inhibited jejunal water absorption (116).

Typically, only dihydroxy BAs (e.g., DCA and CDCA) alter epithelial transport processes and, in humans, diarrhea only occurs when BAs are present at pathophysiological levels (see Table 3). In the small intestine where their uptake to enterohepatic circulation can occur via IBAT, conjugates of DCA and CDCA exert similar actions to their nonconjugated counterparts but with lower potency. However, in the colon there is a lack of apical uptake pathways and therefore luminal conjugated BAs cannot enter the epithelium unless the barrier becomes compromised, as occurs in conditions of intestinal inflammation. Conjugated BAs can then gain access to the basolateral domain and enter the epithelial cell via basolateral transporters to stimulate increases in free cytosolic Ca^{2+} (for more details see *Molecular Mechanisms of Bile Acid-Induced Fluid Transport*), thereby activating intracellular mechanisms that promote fluid secretion and inhibit absorption (133, 134). Interestingly, unlike the other dihydroxy BAs, UDCA does not stimulate fluid secretion in the colon and appears to exert antisecretory actions when it is present at sufficiently high levels (135).

Molecular Mechanisms of Bile Acid-Induced Fluid Transport

BAs induce luminal fluid accumulation by inhibiting Na^+ absorption and promoting Cl^- secretion in both the small intestine and colon. This has been demonstrated in many animal models using in vitro and in vivo methods to assess fluid and ion transport in the ileum (136–139) and colon (140, 141). Studies using cultured intestinal epithelial cell lines and on isolated human colonic crypts have provided considerable

insights into mechanisms by which BAs induce luminal fluid accumulation and diarrhea. In Caco-2 cells, a model of absorptive epithelial cells, conjugated BAs inhibit Cl^-/OH^- exchange (142). Likewise, when present at high concentrations, CDCA inhibits both Na^+/H^+ exchange (NHE) activity and $\text{Cl}^-/\text{HCO}_3^-$ exchange in isolated human colonic crypts (143). As discussed in BILE ACIDS AS MODIFIERS OF ION CHANNEL ACTIVITY, BAs can also modify the function of ENaC, which plays a role in Na^+ absorption in the distal colon, particularly when plasma aldosterone levels are increased to minimize renal and intestinal sodium losses. High concentrations of BAs can rapidly induce Cl^- secretion from the crypts across both small intestinal and colonic epithelial cells. This effect appears to involve activation of basolateral K^+ channels, where K^+ leaving the cells provides the electrochemical driving force for Cl^- to exit through channels in the apical membrane. This most likely occurs through the cystic fibrosis transmembrane conductance regulator (CFTR) (144).

The actions of BAs on these transport proteins appear to be predominantly mediated by elevations in intracellular Ca^{2+} (133, 143, 145). BAs have been shown to induce increases in intracellular Ca^{2+} by both influx across the plasma membrane and release from the endoplasmic reticulum (143, 146, 147). However, the upstream mechanisms leading to increased intracellular Ca^{2+} are still not well defined. The fact that only dihydroxy BAs are active might point to the involvement of a receptor. However, neither TGR5, which appears to exert antisecretory actions (7, 148), nor the nuclear receptor, FXR, is a likely candidate. As noted previously in this review, BAs have detergent-like properties and by inducing perturbations in the cell membrane, this may lead to activation of downstream signaling pathways, thereby promoting their prosecretory and antiabsorptive actions (149, 150). It appears that BAs can also directly interact with endoplasmic reticulum membranes to increase cytosolic Ca^{2+} levels (151, 152).

It is clear from studies of isolated epithelial cells that BAs can exert direct actions on intestinal absorption and secretion. However, studies from ex vivo tissues demonstrate that BAs acting on other mucosal cells may also contribute to this dysregulation. As discussed in *Actions of Bile Acids on the Mucosa to Promote Colonic Motility*, luminal BAs can stimulate enterochromaffin cells (ECC) within the mucosa to release serotonin (5-HT). 5-HT can then activate enteric neural pathways, including those that regulate fluid secretion (153–155). Such neural pathways may provide a mechanism by which changes in intestinal fluid and electrolyte transport can be coordinated with changes in motility and permeability (5, 156–158). BAs can also activate mucosal mast cells to release their mediators (159), most notably histamine, and this may be important in mediating Cl^- secretory responses to CDCA (160). It is also important to note that mast cells and enteric nerves exist close to one another in the intestinal mucosa and that interactions between these two cell types are likely to be important in regulating epithelial transport responses to BAs (161, 162).

Roles of Bile Acids in Epithelial Fluid and Electrolyte Transport under Physiological Conditions

In contrast to their acute prosecretory and antiabsorptive effects at high concentrations, BAs have recently been

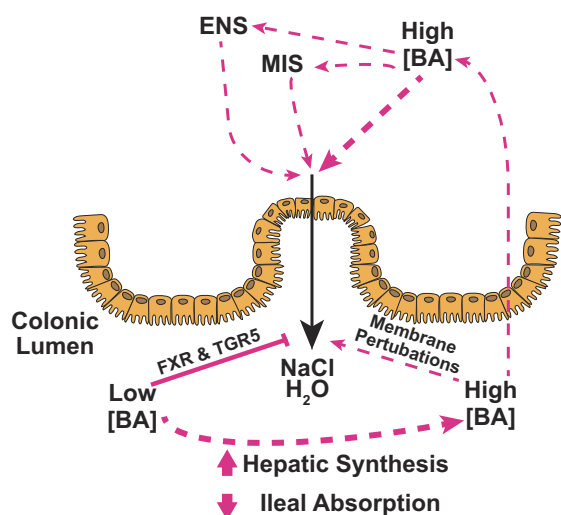


Figure 1. Bile acids (BAs) in regulation of colonic epithelial Cl^- secretion. BAs are normally present in the colon at relatively low concentrations where they act via BA receptors, such as Farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5), to inhibit salt and water secretion into the lumen (*left*). We propose that such actions serve to promote normal colonic absorptive function. In some pathological conditions, increased delivery of BAs to the colon may result from either increased synthesis of primary BAs by hepatocytes or reduced ileal absorption. The resulting high levels of luminal BAs induce perturbations in the epithelial cell membranes to promote Cl^- secretion and inhibit Na^+ absorption, thereby promoting luminal fluid accumulation (*right*). Such direct effects of BAs on the epithelium are amplified by indirect actions involving the release of neurotransmitters and mediators from the enteric nervous and mucosal immune systems (ENS and MIS, respectively). It is thought that, under such circumstances, increased colonic fluid secretion is a protective mechanism that serves to dilute the abnormally high levels of luminal BAs.

shown to exert antisecretory actions at more physiologically relevant levels (53, 105, 163). Such antisecretory effects are slow in onset (12–24 h) and for DCA occur at concentrations as low as 50 μM , a concentration that is well within the normal physiological range for this BA in the proximal colon (164). Such antisecretory actions of naturally occurring BAs are mimicked by selective FXR agonists and are associated with reduced expression and activity of basolateral Na^+/K^+ -ATPase pumps and apical CFTR Cl^- channels (53). We have proposed that under such physiological conditions, BAs may function to inhibit colonic fluid secretion, thereby promoting the normal absorptive function of the colon (summarized in Fig. 1). Thus, by virtue of their opposing actions at low versus high concentrations, dihydroxy BAs may have the capacity to dynamically control the fluidity of the luminal contents. In this context, bacterially mediated deconjugation of luminal BAs would be essential to permit their entry into the cell where FXR activation can occur to induce antisecretory actions. Thus, diet or disease-associated changes in the colonic microbiota that result in altered expression of bile salt hydrolase activity could have important implications for fluid and electrolyte transport (165–169).

Studies have also investigated the potential role for TGR5 in regulating colonic fluid and electrolyte transport. These revealed that TGR5 also exerts antisecretory effects, although its role appears to be distinct to that of FXR. Treatment of muscle-stripped, nerve-free, mucosal

preparations of rat colon with the TGR5-selective agonist, INT-777, rapidly and transiently reduced basal Cl^- secretion with a concomitant inhibition in the capacity of the tissue to evoke secretory responses to Ca^{2+} -dependent secretagogues (148). Furthermore, the effects of INT-777 were mimicked by LCA, an endogenous ligand for TGR5. Subsequent studies revealed that TGR5 activation may also dampen colonic Cl^- secretion by inhibiting submucosal neurons. Such actions are proposed to represent a regulatory mechanism that may counterbalance BA-induced fluid secretion (7). A schematic representation of the roles of BAs in regulating colonic intestinal fluid and electrolyte transport is depicted in Fig. 1.

BILE ACIDS AND INTESTINAL MOTILITY

There is strong, consistent evidence that oral delivery or rectal administration of BAs promotes colonic motility. Although early experimental evidence from *in vivo* studies in rabbit demonstrated that luminal infusion of secondary BAs promotes colonic motility (131, 132), the most compelling evidence comes from clinical studies (124–126). Rectal perfusion of CDCA to healthy individuals reduces the perceived defecatory urge and enhances the frequency of distension-evoked pressure waves or bowel movements (124, 127). Similarly, oral administration of CDCA significantly accelerates colonic transit, but not gastric emptying or colon filling (125). These effects are not limited to healthy patients since in individuals with constipation, oral administration of BAs increases stool frequency and decreases stool consistency (126, 170). This work highlights the therapeutic potential of targeting BA signaling to treat gastrointestinal motility disorders. A summary of the roles of BAs in GI motility and the relative concentrations required to stimulate these actions is presented in Table 3.

Mechanisms for the Prokinetic Actions of BA and the Case for TGR5

Early literature from clinical studies implicates the role of TGR5 in the prokinetic actions of BAs in the colon. Results from clinical trials testing the efficacy of UDCA and CDCA to dissolve and treat gallstones demonstrated that patients treated with CDCA, but not UDCA, experienced diarrhea as a side effect (123, 171, 172). Furthermore, constipation was relieved by treatment with CDCA, but not UDCA (123). This may be explained by the differing agonist activities of the BAs at TGR5, where UDCA has a lower efficacy and potency for TGR5 agonism than CDCA (9, 173). This aligns with evidence from candidate genotype analysis using PCR-based methods, where TGR5 genotype variations are associated with constipation and diarrhea in patients with IBS (174, 175).

Although selective TGR5 agonists are becoming available, the functional effects of TGR5-selective drugs on colonic motility in humans have not yet been investigated. However, studies in mice and dogs demonstrate that TGR5 is expressed throughout the gastrointestinal tract in enteric neurons, epithelial and enteroendocrine cells, and smooth muscle. TGR5 has been identified as a regulator of colonic motility in these species under normal conditions (5, 128, 176). Indeed, there are likely several mechanisms by which BAs can modulate

intestinal motility. Data from *in vitro* studies suggest that BAs exert prokinetic actions when exposure is limited to the mucosa (5), but exposure to the muscularis externa inhibits intestinal motility (128). In the latter case, BAs may have a direct action on the smooth muscle cells to impair contractile activity (130, 177), or may directly activate inhibitory enteric motor neurons that express TGR5 (128). Such inhibitory actions of BAs on the muscularis externa do not contradict the findings from clinical studies. The absence of transporters, such as ASBT, that permit the absorption of conjugated BAs in the colon limits their access to this tissue layer (178), and most clinical studies involve BA exposure to the gut lumen. Therefore, it is likely that the prokinetic actions of BAs are due to effects initiated in the mucosa. Direct effects of BAs on the muscularis externa may be more relevant in disease conditions, such as inflammation, where loss of barrier function and increased paracellular permeability allows both conjugated and unconjugated BAs to passively pass through the mucosa (179, 180).

Actions of Bile Acids on the Mucosa to Promote Colonic Motility

BAs promote the release of several bioactive mediators from the intestinal mucosa. For example, isolated human ECC release 5-HT following exposure to sodium deoxycholate (57), while mucosal application of BAs to rat jejunum (153) and mouse colon promotes 5-HT release possibly via a TGR5-dependent mechanism (5). This BA-mediated 5-HT release activates neural pathways to promote peristaltic contractions (5).

In addition to 5-HT, it is well documented that BAs also stimulate intestinal L cells to release the peptide hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) (119, 181–185). This involves TGR5-dependent and TGR5-independent mechanisms (8, 9, 186, 187). The release of GLP-1 and PYY is important for regulating appetite and metabolism. One mechanism of action of GLP-1 in metabolism is to promote insulin release, and hence GLP-1 receptor agonists are used clinically to treat diabetes mellitus. GLP-1 and PYY promote digestion and nutrient absorption by delaying gastric emptying and reducing intestinal motility. Infusion of BAs to the lumen of the stomach and small intestine *in vivo* reduces gastric emptying and intestinal transit in the rat, rabbit, and human (117, 118, 120, 121). The association between BAs, the release of neuropeptides, and delayed small intestinal motility is supported by studies in the rabbit ileum (122) and human stomach (119), where luminal perfusion of BAs increases the plasma concentrations of PYY or GLP-1, respectively, and this is associated with reduced GI motility in these regions.

BAs mediate opposing effects on motility of the small versus large intestines and it is unclear whether neuropeptide release alone can account for these differences. Rectal administration of tauro-CA in humans dose-dependently increases plasma GLP-1 and PYY levels (127, 182) and this is associated with increased defecation frequency (127). GLP-1 or PYY may directly regulate the BA-mediated changes in motility and this could explain the differential actions in the upper gut and colon where they are present at differing concentrations. *In vitro* studies show that application of GLP-1 inhibits the responses of intestinal muscle strips to electrical stimulation or exogenous application of ACh without influencing basal tone. This

inhibition is likely due to an action on enteric inhibitory neurons (188–190), but also involves actions on the smooth muscle cells themselves (189). Myenteric and submucosal neurons functionally express GLP-1 receptors. In studies on whole-mount preparations of rat colon, application of the highly selective agonist, exendin-4, stimulated increases in intracellular calcium in a proportion of neurons from both regions (191). *In vivo* motility studies indicate that regulation from the central nervous system may also be involved (191, 192). There is conflicting data as to whether PYY stimulates (193, 194) or inhibits GI smooth muscle contractility (195, 196) and these effects may be region-specific (197, 198). PYY is a 36-amino acid polypeptide that is endogenously expressed as either full-length PYY1-36 or as the truncated PYY3-36 form. These peptides have different affinities for the Y receptors that PYY targets (199). Therefore, the presence and activity of PYY variants may account for differences in the contractile responses of different regions to PYY. The cell surface enzyme, dipeptidyl peptidase IV (DPP-IV), both inactivates GLP-1 and converts PYY from its long to its truncated form. Thus, the activity of DPP-IV may play a significant role in shaping the contractile responses to PYY and GLP-1 following BA activation. GLP-1 containing L-cells are evenly distributed across the stomach, small intestine, and colon. In contrast, PYY containing L-cells typically populate the distal small intestine and colon only (195, 200, 201). Whether TGR5 is equivalently expressed by all L-cells regardless of if they contain PYY or GLP-1 remains to be determined, and how these profiles may also contribute to the generation of gut motility patterns is also unknown.

Differing results using taurine-conjugated versus unconjugated isoforms and the use of individual BAs versus mixtures of BAs provide better insight on how they influence motility (see Table 3). In a study by Brown et al. (118), perfusion of the ileum with taurocholic acid (tauro-CA) increased stomach to caecum transit time. In contrast, this transit time decreased with perfusion of DCA. Ullmer et al. found that taurine-conjugated and unconjugated BAs have an equal binding affinity for human-TGR5 in cell lines. However, their effectiveness to increase plasma PYY levels in animals depended on systemic versus luminal application of the agonists (10). The taurine-conjugated TGR5 agonist tauro-RO5527239 did not stimulate secretion of PYY from L-cells, whereas the unconjugated form did. Conjugated forms of BAs or TGR5 agonists have limited ability to access the receptor that is located on the basal side of epithelial cells (186). We speculate that this, in turn, would limit the release of factors, such as PYY, by these cells and thus potentially limit their influence on intestinal motility. Given the importance of the colonic microbiome in determining BA deconjugation, it is clear that changes in bacterial populations that express bile salt hydrolases are likely to significantly impact how BAs modulate fecal transit through the colon. (166–169). BA modulation by the colonic microbiome may therefore be influenced by factors such as diet modification or the onset of disease.

Bile Acids and Intestinal Motility Disorders

Enhanced delivery of BAs to the colon has long been associated with diarrhea (114, 202), with ~25%–33% of patients with chronic functional diarrhea having increased BA delivery to the colon (203). In some rare cases, BA-malabsorption

(BAM) can be due to loss-of-function mutations in intestinal bile acid transporters, such as ASBT and organic solute transporter β (Ost β) (5, 204–206). However, BAM is most commonly due to structural damage brought about by ileal inflammation from conditions such as Crohn's disease, or following surgical or radiation procedures (207–209). Idiopathic BAM is characterized by increased delivery of BAs in the stool in the absence of structural pathology. Alterations in BA synthesis (174, 210–212) or the enterohepatic circulation (213) are attributed as the causes of BAM and the resultant diarrhea in these conditions. Moreover, studies suggest that 10%–26% of patients with diarrhea predominant IBS (IBS-D) may have BAM as the underlying cause (214). Conversely, recent evidence suggests that some instances of constipation may result from reduced BA delivery to the colon. In a retrospective study, Vijayvargiya et al. (215) reported that 15% of patients with IBS-C had decreased fecal BA levels compared with healthy control patients (45 patients, 108 controls). This evidence for the important roles of BAs in a range of GI motility conditions has prompted research into the therapeutic potential of altering BA delivery to the colon or influencing BA receptor signaling.

More recent clinical studies have shifted away from the direct administration of BAs to patients, to investigating the therapeutic potential of modulating the delivery of endogenous BAs. This approach has produced results consistent with the effects of exogenous administration of BAs. One such strategy is to inhibit the reuptake of BAs in the terminal ileum, using drugs such as IBAT inhibitor, Elobixibat (A3309) (216–218). In vivo studies in constipated dogs (218) and clinical trials conducted in Europe, North America, and Japan have all demonstrated Elobixibat to effectively increase the number of spontaneous bowel movements and to improve stool consistency in patients with chronic constipation (219–222). Elobixibat is now an approved drug for the treatment of constipation in Japan (223). There is limited knowledge as to whether administration of ASBT inhibitors can promote GI motility under control conditions (i.e., non-constipated patients). However, in vivo experiments in dogs demonstrated that Elobixibat induced giant migrating contractions and increased defecation frequency and fecal wet weight within 10 h of administration (224).

Several other IBAT inhibitors, including volixibat, maralixibat, odeixibat (A4250), and GSK2330672 are in various stages of clinical trials to treat conditions such as nonalcoholic steatohepatitis (225), type 2 diabetes mellitus (226), and cholestatic pruritus in primary biliary cholangitis (227–231). In all of these studies, diarrhea and abdominal pain are cited as treatment-related adverse events, with an incidence ranging from 10% to 80%. Although it is unclear whether a patient subtype is more or less likely to develop these symptoms, the dosage of the drugs can be correlated with the number of reported incidences. Recently published results of a phase 2 clinical trial for GSK2330672 to treat cholestatic pruritus reported diarrhea as an adverse event in 38%, 65%, and 66% of patients receiving 20, 90, and 180 mg of the drug, respectively. This was compared with 11% of patients receiving the placebo (229). This highlights the importance of establishing efficacy, safety, and tolerability of these drugs to treat these conditions. As noted elsewhere, BAs will cause symptoms such as diarrhea when delivered at high,

pathophysiological concentrations; therefore, it would be interesting to know how the levels of fecal BAs correlated with symptom severity. In healthy volunteers, volixibat increased fecal BAs by 1.6–3.2 times compared with those that had received placebo. It is worth noting that there was no clear correlation between dosage of volixibat (0.5–5 mg) and excreted BAs. How these levels correlated with symptom severity was not reported. However, these results highlight that there may be variability in the ways in which patients respond to these drugs.

Another approach to modulating endogenous levels of BAs involves the use of BA sequestrants that have opposing effects to ASBT inhibitors and reduce free BA levels in the colon. Such drugs, which include cholestyramine and colesteslam, have been trialed to treat a range of diarrhea-associated conditions including idiopathic BA-diarrhea and BA-associated diarrhea in Crohn's disease and IBS-D (57, 125, 217, 232–234). As FXR activation leads to the inhibition of BA production in hepatocytes, FXR agonists have also been trialed to treat BA-induced diarrhea (52, 86, 235). Two small clinical trials conducted in the UK and the USA have demonstrated the benefits of the FXR agonists obeticholic acid and tropifexor. Daily tropifexor administration had a small but significant effect on slowing ascending colonic motility in patients with primary BA-diarrhea in a recent safety profiling study [60 μ g daily, $n = 8$ (235)]. Obeticholic acid given to patients with primary BA-diarrhea improved stool frequency and formation during a 2-wk treatment period [25 mg, daily, $n = 10$ (52)]. However, patients with chronic idiopathic diarrhea did not have improved symptoms ($n = 8$) indicating that neither FXR activation nor limiting physiological levels of BA production have roles in controlling colonic motility (52). This differs to patients with BA-diarrhea, where removing excess BAs reduces the symptoms of the disease. It is important to note that GI motility and secretion are interlinked, where increasing secretion will stimulate motility. FXR agonists limit intestinal fluid secretion in mouse models of secretory diarrhea (53). However, these studies did not specifically study direct GI motor indices.

When considering the potential for targeting BAs for therapeutic purposes, it is important to consider how the gut microbiome interacts with these molecules and their receptors to modulate intestinal motility (236). There is a growing appreciation for the roles of intestinal bacteria in a range of GI disorders. BAs increase intestinal permeability, providing greater opportunity for microbiota to access the gut wall. Although the BAs can modulate motility directly, the inflammation promoted by the infiltrating bacteria can also alter intestinal motility (237). This pairs with the proposed role of TGR5 in regulating intestinal inflammation, which may involve TGR5 expressing-circulating macrophages (179, 180). Recent studies in mice have highlighted that with age-related changes to the microbiome there is an increase in fecal deconjugated BAs. TGR5 activation by conjugated BAs in young mice stimulated an anti-inflammatory phenotype in muscularis macrophages (mMac) protecting normal motility. With aging, the shift of fecal BA content to deconjugated BAs led to reduced TGR5 activation. This resulted in a phenotypic switch of mMac to a more proinflammatory state, stimulating low-grade inflammation in these tissues and thus

delayed GI transit (238). The microbiome-BA balance and its role in intestinal motility seem an obvious consideration when trying to understand complex treatment strategies such as fecal transplants (236). This could have important implications for our understanding of the pathology of a range of GI motility disorders but may also provide novel therapeutic strategies for these conditions.

CONCLUSION

The BA receptor axis is a viable therapeutic target, as evidenced by the clinical use of BA sequestrants and more recently IBAT inhibitors. With the discovery of specific BA receptors, BA-modulated ion channels, and BA transporters, our understanding of the roles of BAs in physiological processes and disease has evolved. BAs have physiological actions as signaling molecules through binding and activation of both cell surface and nuclear receptors and through modifying ion channel function. They also drive pathophysiological changes in disease and may have mechanistic roles in GI disorders including IBS. As outlined in this review, there remains the need for greater understanding of the functional importance of BA receptors, particularly those which have only recently been defined.

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AUTHOR CONTRIBUTIONS

S.E.C. analyzed data; D.P.P. and S.E.C. prepared figures; S.J.K., A.U., A.V.I., C.K., N.W.B., D.P.P., and S.E.C. drafted manuscript; S.J.K., A.U., A.V.I., C.K., N.W.B., D.P.P., and S.E.C. edited and revised manuscript; S.J.K., A.U., A.V.I., C.K., N.W.B., D.P.P., and S.E.C. approved final version of manuscript.

REFERENCES

- de Aguiar Vallim TQ, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab* 17: 657–669, 2013. doi:10.1016/j.cmet.2013.03.013.
- Peleman C, Camilleri M, Busciglio I, Burton D, Donato L, Zinsmeister AR. Colonic transit and bile acid synthesis or excretion in patients with irritable bowel syndrome-diarrhea without bile acid malabsorption. *Clin Gastroenterol Hepatol* 15: 720–727.e1, 2017. doi:10.1016/j.cgh.2016.11.012.
- Mekhjian HS, Phillips SF, Hofmann AF. Colonic absorption of unconjugated bile acids: perfusion studies in man. *Dig Dis Sci* 24: 545–550, 1979. doi:10.1007/BF01489324.
- Mikov M, Fawcett JP, Kuhajda K, Kevresan S. Pharmacology of bile acids and their derivatives: absorption promoters and therapeutic agents. *Eur J Drug Metab Pharmacokinet* 31: 237–251, 2006. doi:10.1007/BF03190714.
- Alemi F, Poole DP, Chiu J, Schoonjans K, Cattaruzza F, Grider JR, Bunnett NW, Corvera CU. The receptor TGR5 mediates the prokinetic actions of intestinal bile acids and is required for normal defecation in mice. *Gastroenterology* 144: 145–154, 2013. doi:10.1053/j.gastro.2012.09.055.
- Castro J, Harrington AM, Lieu T, Garcia-Caraballo S, Maddern J, Schober G, O'Donnell T, Grundy L, Lumsden AL, Miller P, Ghetti A, Steinhoff MS, Poole DP, Dong X, Chang L, Bunnett NW, Brierley SM. Activation of pruritogenic TGR5, MrgprA3, and MrgprC11 on colon-innervating afferents induces visceral hypersensitivity. *JCI insight* 4: 332, 2019. doi:10.1172/jci.insight.131712.
- Duboc H, Tolstanova G, Yuan PQ, Wu V, Kaji I, Biraud M, Akiba Y, Kaunitz J, Million M, Tache Y, Larauche M. Reduction of epithelial secretion in male rat distal colonic mucosa by bile acid receptor TGR5 agonist, INT-777: role of submucosal neurons. *Neurogastroenterol Motil* 28: 1663–1676, 2016. doi:10.1111/nmo.12866.
- Katsuma S, Hirasawa A, Tsujimoto G. Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem Biophys Res Commun* 329: 386–390, 2005. doi:10.1016/j.bbrc.2005.01.139.
- Kuhre RE, Wewer Albrechtsen NJ, Larsen O, Jepsen SL, Balk-Møller E, Andersen DB, Deacon CF, Schoonjans K, Reimann F, Gribble FM, Albrechtsen R, Hartmann B, Rosenkilde MM, Holst JJ. Bile acids are important direct and indirect regulators of the secretion of appetite- and metabolism-regulating hormones from the gut and pancreas. *Mol Metab* 11: 84–95, 2018. doi:10.1016/j.molmet.2018.03.007.
- Ullmer C, Alvarez Sanchez R, Sprecher U, Raab S, Mattei P, Dehmlow H, Sewing S, Iglesias A, Beauchamp J, Conde-Knappe K. Systemic bile acid sensing by G protein-coupled bile acid receptor 1 (GPBAR1) promotes PYY and GLP-1 release. *Br J Pharmacol* 169: 671–684, 2013. doi:10.1111/bph.12158.
- Meixiong J, Vasavda C, Snyder SH, Dong X. MRGPRX4 is a G protein-coupled receptor activated by bile acids that may contribute to cholestatic pruritus. *Proc Natl Acad Sci USA* 116: 10525–10530, 2019. doi:10.1073/pnas.1903316116.
- Ibrahim E, Diakonov I, Arunthavarajah D, Swift T, Goodwin M, McIlvrde S, Nikolova V, Williamson C, Gorelik J. Bile acids and their respective conjugates elicit different responses in neonatal cardiomyocytes: role of Gi protein, muscarinic receptors and TGR5. *Sci Rep* 8: 7110–7112, 2018. doi:10.1038/s41598-018-25569-4.
- Sheikh Abdul Kadir SH, Miragoli M, Abu-Hayyeh S, Moshkov AV, Xie Q, Keitel V, Nikolaev VO, Williamson C, Gorelik J. Bile acid-induced arrhythmia is mediated by muscarinic M2 receptors in neonatal rat cardiomyocytes. *PLoS One* 5: e9689, 2010. doi:10.1371/journal.pone.0009689.
- Urso A, D'Ovidio F, Xu D, Emala CW, Bunnett NW, Perez-Zoghbi JF. Bile acids inhibit cholinergic constriction in proximal and peripheral airways from humans and rodents. *Am J Physiol Lung Cell Mol Physiol* 318: L264–L275, 2020. doi:10.1152/ajplung.00242.2019.
- Studer E, Zhou X, Zhao R, Wang Y, Takabe K, Nagahashi M, Pandak WM, Dent P, Spiegel S, Shi R, Xu W, Liu X, Bohdan P, Zhang L, Zhou H, Hylemon PB. Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. *Hepatology* 55: 267–276, 2012. doi:10.1002/hep.24681.
- Wang Y, Aoki H, Yang J, Peng K, Liu R, Li X, Qiang X, Sun L, Gurley EC, Lai G, Zhang L, Liang G, Nagahashi M, Takabe K, Pandak WM, Hylemon PB, Zhou H. The role of sphingosine 1-phosphate receptor 2 in bile-acid-induced cholangiocyte proliferation and cholestasis-induced liver injury in mice. *Hepatology* 65: 2005–2018, 2017. doi:10.1002/hep.29076.
- Chen X, Yang D, Shen W, Dong HF, Wang JM, Oppenheim JJ, Howard MZ. Characterization of chenodeoxycholic acid as an endogenous antagonist of the G-coupled formyl peptide receptors. *Inflamm Res* 49: 744–755, 2000. doi:10.1007/s000110050656.
- Chen X, Mellon RD, Yang L, Dong HF, Oppenheim JJ, Howard MZ. Regulatory effects of deoxycholic acid, a component of the anti-inflammatory traditional Chinese medicine Niu Huang, on human

- leukocyte response to chemoattractants. *Biochem Pharmacol* 63: 533–541, 2002. doi:10.1016/S0006-2952(01)00917-0.
19. Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B. Identification of a nuclear receptor for bile acids. *Science* 284: 1362–1365, 1999. doi:10.1126/science.284.5418.1362.
 20. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Klier SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM. Bile acids: natural ligands for an orphan nuclear receptor. *Science* 284: 1365–1368, 1999. doi:10.1126/science.284.5418.1365.
 21. Zhang JH, Nolan JD, Kennie SL, Johnston IM, Dew T, Dixon PH, Williamson C, Walters JRF. Potent stimulation of fibroblast growth factor 19 expression in the human ileum by bile acids. *Am J Physiol Gastrointest Liver Physiol* 304: G940–G948, 2013. doi:10.1152/ajpgi.00398.2012.
 22. Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, Gerard RD, Repa JJ, Mangelsdorf DJ, Klier SA. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab* 2: 217–225, 2005. doi:10.1016/j.cmet.2005.09.001.
 23. Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Klier SA. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* 98: 3369–3374, 2001. doi:10.1073/pnas.051551698.
 24. Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA* 98: 3375–3380, 2001. doi:10.1073/pnas.051014398.
 25. Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. Vitamin D receptor as an intestinal bile acid sensor. *Science* 296: 1313–1316, 2002. doi:10.1126/science.1070477.
 26. Ishizawa M, Akagi D, Makishima M. Lithocholic acid is a vitamin D receptor ligand that acts preferentially in the ileum. *Int J Mol Sci* 19: 1975, 2018. doi:10.3390/ijms19071975.
 27. Yoneno K, Hisamatsu T, Shimamura K, Kamada N, Ichikawa R, Kitazume MT, Mori M, Uo M, Namikawa Y, Matsuoka K, Sato T, Koganei K, Sugita A, Kanai T, Hibi T. TGR5 signalling inhibits the production of pro-inflammatory cytokines by in vitro differentiated inflammatory and intestinal macrophages in Crohn's disease. *Immunology* 139: 19–29, 2013. doi:10.1111/imm.12045.
 28. Jia W, Xie G, Jia W. Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat Rev Gastroenterol Hepatol* 15: 111–128, 2018. doi:10.1038/nrgastro.2017.119.
 29. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* 106: 619–632, 2001. doi:10.1016/S0092-8674(01)00483-4.
 30. Meixiong J, Vasavda C, Green D, Zheng Q, Qi L, Kwatra SG, Hamilton JP, Snyder SH, Dong X. Identification of a bilirubin receptor that may mediate a component of cholestatic itch. *eLife* 8: e44116, 2019. doi:10.7554/eLife.44116.
 31. Gulamhussein AF, Hirschfield GM. Primary biliary cholangitis: pathogenesis and therapeutic opportunities. *Nat Rev Gastroenterol Hepatol* 17: 93–110, 2020. doi:10.1038/s41575-019-0226-7.
 32. Van Remoortel S, Ceuleers H, Arora R, Van Nassauw L, De Man JG, Buckinx R, De Winter BY, Timmermans J-P. Mas-related G protein-coupled receptor C11 (Mrgprc11) induces visceral hypersensitivity in the mouse colon: a novel target in gut nociception? *Neurogastroenterol Motil* 31: e13623, 2019. doi:10.1111/nmo.13623.
 33. Avula LR, Buckinx R, Favoreel H, Cox E, Adriaensen D, Van Nassauw L, Timmermans JP. Expression and distribution patterns of Mas-related gene receptor subtypes A-H in the mouse intestine: inflammation-induced changes. *Histochem Cell Biol* 139: 639–658, 2013. doi:10.1007/s00418-013-1086-9.
 34. Avula LR, Buckinx R, Alpaerts K, Costagliola A, Adriaensen D, Van Nassauw L, Timmermans JP. The effect of inflammation on the expression and distribution of the MAS-related gene receptors MrgE and MrgF in the murine ileum. *Histochem Cell Biol* 136: 569–585, 2011. doi:10.1007/s00418-011-0862-7.
 35. Inclan-Rico JM, Kim BS, Abdus-Saboor I. Beyond somatosensation: Mrgprs in mucosal tissues. *Neurosci Lett* 748: 135689, 2021. doi:10.1016/j.neulet.2021.135689.
 36. Luo J, Feng J, Liu S, Walters ET, Hu H. Molecular and cellular mechanisms that initiate pain and itch. *Cell Mol Life Sci* 72: 3201–3223, 2015 [Erratum in *Cell Mol Life Sci* 72: 3587–3588, 2015]. doi:10.1007/s00018-015-1904-4.
 37. Cheng K, Chen Y, Zimniak P, Raufman JP, Xiao Y, Frucht H. Functional interaction of lithocholic acid conjugates with M3 muscarinic receptors on a human colon cancer cell line. *Biochim Biophys Acta* 1588: 48–55, 2002. doi:10.1016/S0925-4439(02)00115-1.
 38. Raufman J-P, Chen Y, Cheng K, Compadre C, Compadre L, Zimniak P. Selective interaction of bile acids with muscarinic receptors: a case of molecular mimicry. *Eur J Pharmacol* 457: 77–84, 2002. doi:10.1016/S0014-2999(02)02690-0.
 39. Nagahashi M, Yuza K, Hirose Y, Nakajima M, Ramanathan R, Hait NC, Hylemon PB, Zhou H, Takabe K, Wakai T. The roles of bile acids and sphingosine-1-phosphate signaling in the hepatobiliary diseases. *J Lipid Res* 57: 1636–1643, 2016. doi:10.1194/jlr.R069286.
 40. Keitel V, Stindt J, Häussinger D. Bile acid-activated receptors: GPBAR1 (TGR5) and other G protein-coupled receptors. In: *Bile Acids and Their Receptors*, edited by Fiorucci S, Distrutti E. Cham: Springer International Publishing, 2019, p. 19–49.
 41. Ferrari C, Macchiarulo A, Costantino G, Pellicciari R. Pharmacophore model for bile acids recognition by the FPR receptor. *J Comput Aided Mol Des* 20: 295–303, 2006. doi:10.1007/s10822-006-9055-1.
 42. Chandrasekharan B, Saeedi BJ, Alam A, Houser M, Srinivasan S, Tansey M, Jones R, Nusrat A, Neish AS. Interactions between commensal bacteria and enteric neurons, via fpr1 induction of ROS, increase gastrointestinal motility in mice. *Gastroenterology* 157: 179–194, 2019. doi:10.1053/j.gastro.2019.03.045.
 43. Anton PA, Targan SR, Shanahan F. Increased neutrophil receptors for and response to the proinflammatory bacterial peptide formyl-methionyl-leucyl-phenylalanine in Crohn's disease. *Gastroenterology* 97: 20–28, 1989. doi:10.1016/0016-5085(89)91410-8.
 44. Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. *Annu Rev Physiol* 65: 261–311, 2003. doi:10.1146/annurev.physiol.65.092101.142528.
 45. Li T, Chiang JY. Nuclear receptors in bile acid metabolism. *Drug Metab Rev* 45: 145–155, 2013. doi:10.3109/03602532.2012.740048.
 46. Li T, Chiang JYL. Bile acid signaling in metabolic disease and drug therapy. *Pharmacol Rev* 66: 948–983, 2014. doi:10.1124/pr.113.008201.
 47. Schaap FG, Trauner M, Jansen PL. Bile acid receptors as targets for drug development. *Nat Rev Gastroenterol Hepatol* 11: 55–67, 2014. doi:10.1038/nrgastro.2013.151.
 48. Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, Weinberger C. Identification of a nuclear receptor that is activated by farnesol metabolites. *Cell* 81: 687–693, 1995. doi:10.1016/0092-8674(95)90530-8.
 49. Wang H, Chen J, Hollister K, Sowers LC, Forman MB. Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* 3: 543–553, 1999. doi:10.1016/S1097-2765(00)80348-2.
 50. Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* 102: 731–744, 2000. doi:10.1016/S0092-8674(00)00062-3.
 51. Keely SJ, Walters JRF. The Farnesoid X receptor: good for bad. *Cell Mol Gastroenterol Hepatol* 2: 725–732, 2016. doi:10.1016/j.jcmgh.2016.08.004.
 52. Walters JRF, Johnston IM, Nolan JD, Vassie C, Pruzanski ME, Shapiro DA. The response of patients with bile acid diarrhoea to the farnesoid X receptor agonist obeticholic acid. *Aliment Pharmacol Ther* 41: 54–64, 2015. doi:10.1111/apt.12999.
 53. Mroz MS, Keating N, Ward JB, Sarker R, Amu S, Aviello G, Donowitz M, Fallon PG, Keely SJ. Farnesoid X receptor agonists attenuate colonic epithelial secretory function and prevent experimental diarrhoea in vivo. *Gut* 63: 808–817, 2014. doi:10.1136/gutjnl-2013-305088.
 54. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, Chalasani N, Dasarthy S, Diehl AM, Hameed B, Kowdley KV, McCullough A, Terrault N, Clark JM, Tonascia J, Brunt EM, Kleiner DE, Doo E; NASH Clinical Research Network. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre,

- randomised, placebo-controlled trial. *Lancet* 385: 956–965, 2015 [Erratum in *Lancet* 385: 946, 2015]. doi:10.1016/S0140-6736(14)61933-4.
55. **Chávez-Talavera O, Tailleux A, Lefebvre P, Staels B.** Bile acid control of metabolism and inflammation in obesity, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease. *Gastroenterology* 152: 1679–1694.e.3, 2017. doi:10.1053/j.gastro.2017.01.055.
 56. **Cheng J, Ma X, Gonzalez FJ.** Pregnane X receptor- and CYP3A4-humanized mouse models and their applications. *Br J Pharmacol* 163: 461–468, 2011. doi:10.1111/j.1476-5381.2010.01129.x.
 57. **Kidd M, Modlin IM, Gustafsson BI, Drozdov I, Hauso O, Pfragner R.** Luminal regulation of normal and neoplastic human EC cell serotonin release is mediated by bile salts, amines, tastants, and olfactants. *Am J Physiol Gastrointest Liver Physiol* 295: G260–G272, 2008. doi:10.1152/ajpgi.00056.2008.
 58. **Fiorucci S, Carino A, Baldoni M, Santucci L, Costanzi E, Graziosi L, Distrutti E, Biagioli M.** Bile acid signaling in inflammatory bowel diseases. *Dig Dis Sci* 66: 674–693, 2021. doi:10.1007/s10620-020-06715-3.
 59. **Wilson A, Almousa A, Teft WA, Kim RB.** Attenuation of bile acid-mediated FXR and PXR activation in patients with Crohn's disease. *Sci Rep* 10: 1866, 2020. doi:10.1038/s41598-020-58644-w.
 60. **Iwamoto J, Saito Y, Honda A, Miyazaki T, Ikegami T, Matsuzaki Y.** Bile acid malabsorption deactivates pregnane X receptor in patients with Crohn's disease. *Inflamm Bowel Dis* 19: 1278–1284, 2013. doi:10.1097/MIB.0b013e318281f423.
 61. **Shah YM, Ma X, Morimura K, Kim I, Gonzalez FJ.** Pregnane X receptor activation ameliorates DSS-induced inflammatory bowel disease via inhibition of NF- κ B target gene expression. *Am J Physiol Gastrointest Liver Physiol* 292: G1114–G1122, 2007. doi:10.1152/ajpgi.00528.2006.
 62. **Owen RW, Dodo M, Thompson MH, Hill MJ.** Fecal steroids and colorectal cancer. *Nutr Cancer* 9: 73–80, 1987. doi:10.1080/01635588709513914.
 63. **Woolbright BL, Li F, Xie Y, Farhood A, Fickert P, Trauner M, Jaeschke H.** Lithocholic acid feeding results in direct hepatotoxicity independent of neutrophil function in mice. *Toxicol Lett* 228: 56–66, 2014. doi:10.1016/j.toxlet.2014.04.001.
 64. **Yao B, He J, Yin X, Shi Y, Wan J, Tian Z.** The protective effect of lithocholic acid on the intestinal epithelial barrier is mediated by the vitamin D receptor via a SIRT1/Nrf2 and NF- κ B dependent mechanism in Caco-2 cells. *Toxicol Lett* 316: 109–118, 2019. doi:10.1016/j.toxlet.2019.08.024.
 65. **Kim SD, Kwon S, Lee SK, Kook M, Lee HY, Song K-D, Lee H-K, Baek S-H, Park CB, Bae Y-S.** The immune-stimulating peptide WKYMVm has therapeutic effects against ulcerative colitis. *Exp Mol Med* 45: e40, 2013. doi:10.1038/emmm.2013.77.
 66. **Liu W, Chen Y, Golan MA, Annunziata ML, Du J, Dougherty U, Kong J, Musch M, Huang Y, Pekow J, Zheng C, Bissonnette M, Hanauer SB, Li YC.** Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest* 123: 3983–3996, 2013. doi:10.1172/JCI65842.
 67. **Schmidt DR, Holmstrom SR, Fon Tacer K, Bookout AL, Kliewer SA, Mangelsdorf DJ.** Regulation of bile acid synthesis by fat-soluble vitamins A and D. *J Biol Chem* 285: 14486–14494, 2010. doi:10.1074/jbc.M110.116004.
 68. **Cheng J, Fang Z-Z, Kim J-H, Krausz KW, Tanaka N, Chiang JL, Gonzalez FJ.** Intestinal CYP3A4 protects against lithocholic acid-induced hepatotoxicity in intestine-specific VDR-deficient mice. *J Lipid Res* 55: 455–465, 2014. doi:10.1194/jlr.M044420.
 69. **Wei P, Zhang J, Egan-Hafley M, Liang S, Moore DD.** The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* 407: 920–923, 2000. doi:10.1038/35038112.
 70. **Miao J, Fang S, Bae Y, Kemper JK.** Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolism is mediated by competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1 α . *J Biol Chem* 281: 14537–14546, 2006. doi:10.1074/jbc.M510713200.
 71. **Zhang J, Huang W, Qatanani M, Evans RM, Moore DD.** The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. *J Biol Chem* 279: 49517–49522, 2004. doi:10.1074/jbc.M409041200.
 72. **Hudson GM, Flannigan KL, Erickson SL, Vicentini FA, Zamponi A, Hirota CL, Alston L, Altier C, Ghosh S, Rioux KP, Mani S, Chang TK, Hirota SA.** Constitutive androstane receptor regulates the intestinal mucosal response to injury. *Br J Pharmacol* 174: 1857–1871, 2017. doi:10.1111/bph.13787.
 73. **Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB Jr, Kliewer SA, Gonzalez FJ, Sinal CJ.** Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* 278: 45062–45071, 2003. doi:10.1074/jbc.M307145200.
 74. **Stedman CAM, Liddle C, Coulter SA, Sonoda J, Alvarez JGA, Moore DD, Evans RM, Downes M.** Nuclear receptors constitutive androstane receptor and pregnane X receptor ameliorate cholestatic liver injury. *Proc Natl Acad Sci USA* 102: 2063–2068, 2005. doi:10.1073/pnas.0409794102.
 75. **Toda T, Saito N, Ikarashi N, Ito K, Yamamoto M, Ishige A, Watanabe K, Sugiyama K.** Intestinal flora induces the expression of Cyp3a in the mouse liver. *Xenobiotica* 39: 323–334, 2009. doi:10.1080/00498250802651984.
 76. **Dempsey JL, Wang D, Siginir G, Fei Q, Rafferty D, Gu H, Yue Cui J.** Pharmacological activation of PXR and CAR downregulates distinct bile acid-metabolizing intestinal bacteria and alters bile acid homeostasis. *Toxicol Sci* 168: 40–60, 2019. doi:10.1093/toxsci/kfy271.
 77. **Wiemuth D, Sahin H, Falkenburger BH, Lefevre CMT, Wasmuth HE, Gründer S.** BASIC-a bile acid-sensitive ion channel highly expressed in bile ducts. *FASEB J* 26: 4122–4130, 2012. doi:10.1096/fj.12-207043.
 78. **Wiemuth D, Sahin H, Lefevre CM, Wasmuth HE, Gründer S.** Strong activation of bile acid-sensitive ion channel (BASIC) by ursodeoxycholic acid. *Channels (Austin)* 7: 38–42, 2013. doi:10.4161/chan.22406.
 79. **Lenzig P, Wirtz M, Wiemuth D.** Comparative electrophysiological analysis of the bile acid-sensitive ion channel (BASIC) from different species suggests similar physiological functions. *Pflugers Arch* 471: 329–336, 2019. doi:10.1007/s00424-018-2223-z.
 80. **Wiegrefe S, Lohrer D, Wirtz M, Wiemuth D.** The bile acid-sensitive ion channel (BASIC) mediates bile acid-dependent currents in bile duct epithelial cells. *Pflugers Arch* 473: 1841–1850, 2021. doi:10.1007/s00424-021-02622-2.
 81. **Ilyaskin AV, Kirsch SA, Böckmann RA, Sticht H, Korbmacher C, Haerteis S, Diakov A.** The degenerin region of the human bile acid-sensitive ion channel (BASIC) is involved in channel inhibition by calcium and activation by bile acids. *Pflugers Arch* 470: 1087–1102, 2018. doi:10.1007/s00424-018-2142-z.
 82. **Lefevre CMT, Diakov A, Haerteis S, Korbmacher C, Gründer S, Wiemuth D.** Pharmacological and electrophysiological characterization of the human bile acid-sensitive ion channel (hBASIC). *Pflugers Arch* 466: 253–263, 2014. doi:10.1007/s00424-013-1310-4.
 83. **Wiemuth D, Lefevre CM, Heidtmann H, Gründer S.** Bile acids increase the activity of the epithelial Na⁺ channel. *Pflugers Arch* 466: 1725–1733, 2014. doi:10.1007/s00424-013-1403-0.
 84. **Ilyaskin AV, Diakov A, Korbmacher C, Haerteis S.** Activation of the human epithelial sodium channel (ENaC) by bile acids involves the degenerin site. *J Biol Chem* 291: 19835–19847, 2016. doi:10.1074/jbc.M116.726471.
 85. **Wang X-P, Im SJ, Balchak DM, Montalbetti N, Carattino MD, Ray EC, Kashlan OB.** Murine epithelial sodium (Na⁺) channel regulation by biliary factors. *J Biol Chem* 294: 10182–10193, 2019. doi:10.1074/jbc.RA119.007394.
 86. **Mroz MS, Harvey BJ.** Ursodeoxycholic acid inhibits ENaC and Na/K pump activity to restore airway surface liquid height in cystic fibrosis bronchial epithelial cells. *Steroids* 151: 108461, 2019. doi:10.1016/j.steroids.2019.108461.
 87. **Ilyaskin AV, Diakov A, Korbmacher C, Haerteis S.** Bile acids potentiate proton-activated currents in *Xenopus laevis* oocytes expressing human acid-sensing ion channel (ASIC1a). *Physiol Rep* 5: e13132, 2017. doi:10.14814/phy2.13132.
 88. **Dopico AM, Bukiya AN.** Regulation of Ca²⁺-sensitive K⁺ channels by cholesterol and bile acids via distinct channel subunits and sites. *Curr Top Membr* 80: 53–93, 2017. doi:10.1016/bs.ctm.2017.07.001.
 89. **Schubring SR, Fleischer W, Lin JS, Haas HL, Sergeeva OA.** The bile steroid chenodeoxycholate is a potent antagonist at NMDA and GABA(A) receptors. *Neurosci Lett* 506: 322–326, 2012. doi:10.1016/j.neulet.2011.11.036.

90. Yanovsky Y, Schubring SR, Yao QL, Zhao Y, Li S, May A, Haas HL, Lin JS, Sergeeva OA. Waking action of ursodeoxycholic acid (UDCA) involves histamine and GABA(A) receptor block. *Plos One* 7: e42512, 2012. doi:10.1371/journal.pone.0042512.
91. Koch A, Bonus M, Gohlke H, Klöcker N. Isoform-specific inhibition of N-methyl-D-aspartate receptors by bile salts. *Sci Rep* 9: 10068, 2019. doi:10.1038/s41598-019-46496-y.
92. Ilyaskin AV, Sure F, Nesterov V, Haerteis S, Korbmacher C. Bile acids inhibit human purinergic receptor P2X4 in a heterologous expression system. *J Gen Physiol* 151: 820–833, 2019. doi:10.1085/jgp.201812291.
93. Schmidt A, Jousen S, Hausmann R, Gründer S, Wiemuth D. Bile acids are potent inhibitors of rat P2X2 receptors. *Purinergic Signal* 15: 213–221, 2019. doi:10.1007/s11302-019-09657-2.
94. Sakai H, Lingueglia E, Champigny G, Mattei MG, Lazdunski M. Cloning and functional expression of a novel degenerin-like Na⁺ channel gene in mammals. *J Physiol* 519: 323–333, 1999. doi:10.1111/j.1469-7793.1999.0323m.x.
95. Schaefer L, Sakai H, Mattei MG, Lazdunski M, Lingueglia E. Molecular cloning, functional expression and chromosomal localization of an amiloride-sensitive Na⁺ channel from human small intestine. *FEBS Lett* 471: 205–210, 2000. doi:10.1016/S0014-5793(00)01403-4.
96. Li Q, Kresge C, Bugde A, Lamphere M, Park JY, Feranchak AP. Regulation of mechanosensitive biliary epithelial transport by the epithelial Na⁺ channel. *Hepatology* 63: 538–549, 2016. doi:10.1002/hep.28301.
97. Garty H, Palmer LG. Epithelial sodium channels: function, structure, and regulation. *Physiol Rev* 77: 359–396, 1997. doi:10.1152/physrev.1997.77.2.359.
98. Schild L. The epithelial sodium channel and the control of sodium balance. *Biochim Biophys Acta* 1802: 1159–1165, 2010. doi:10.1016/j.bbadis.2010.06.014.
99. Rossier BC. Epithelial sodium channel (ENaC) and the control of blood pressure. *Curr Opin Pharmacol* 15: 33–46, 2014. doi:10.1016/j.coph.2013.11.010.
100. Rotin D, Staub O. Function and regulation of the epithelial Na⁺ channel ENaC. *Compr Physiol* 11: 2017–2045, 2021. doi:10.1002/cphy.c200012.
101. Loffing J, Korbmacher C. Regulated sodium transport in the renal connecting tubule (CNT) via the epithelial sodium channel (ENaC). *Pflügers Arch* 458: 111–135, 2009. doi:10.1007/s00424-009-0656-0.
102. Rossier BC, Stutts MJ. Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annu Rev Physiol* 71: 361–379, 2009. doi:10.1146/annurev.physiol.010908.163108.
103. Kleyman TR, Kashlan OB, Hughey RP. Epithelial Na⁺ channel regulation by extracellular and intracellular factors. *Annu Rev Physiol* 80: 263–281, 2018. doi:10.1146/annurev-physiol-021317-121143.
104. Kleyman TR, Eaton DC. Regulating ENaC's gate. *Am J Physiol Cell Physiol* 318: C150–C162, 2020. doi:10.1152/ajpcell.00418.2019.
105. Bertog M, Reeh L, Bunnett NW, Korbmacher C. Lithocholic acid has a sustained stimulatory effect on the epithelial sodium channel (ENaC) in mouse late distal colon. *Acta Physiol* 219: 164, 2017. doi:10.1111/apha.12870.
106. Holzer P. Acid-sensing ion channels in gastrointestinal function. *Neuropharmacology* 94: 72–79, 2015. doi:10.1016/j.neuropharm.2014.12.009.
107. Gonzales EB, Kawate T, Gouaux E. Pore architecture and ion sites in acid-sensing ion channels and P2X receptors. *Nature* 460: 599–605, 2009. doi:10.1038/nature08218.
108. Doctor RB, Matzakos T, McWilliams R, Johnson S, Feranchak AP, Fitz JG. Purinergic regulation of cholangiocyte secretion: identification of a novel role for P2X receptors. *Am J Physiol Gastrointest Liver Physiol* 288: G779–G786, 2005. doi:10.1152/ajpgi.00325.2004.
109. Besnard A, Gautherot J, Julien B, Tebbi A, Garcin I, Doignon I, Péan N, Gonzales E, Cassio D, Grosse B, Liu BK, Safya H, Cauchois F, Humbert L, Rainteau D, Tordjmann T. The P2X4 purinergic receptor impacts liver regeneration after partial hepatectomy in mice through the regulation of biliary homeostasis. *Hepatology* 64: 941–953, 2016. doi:10.1002/hep.28675.
110. Ruan HZ, Burnstock G. P2X2 and P2X3 receptor expression in the gallbladder of the guinea pig. *Auton Neurosci* 111: 89–96, 2004. doi:10.1016/j.autneu.2004.03.002.
111. Castelucci P, Robbins HL, Poole DP, Furness JB. The distribution of purine P2X(2) receptors in the guinea-pig enteric nervous system. *Histochem Cell Biol* 117: 415–422, 2002. doi:10.1007/s00418-002-0404-4.
112. Barrett KE, Keely SJ. Electrolyte secretion and absorption in the small intestine and colon. In: *Textbook of Gastroenterology*, edited by Podolsky D, Camilleri M, Fitz J, Kalloo A, Shanahan F, Wang T. Hoboken, NJ: Wiley-Blackwell, 2015.
113. Forth W, Rummel W, Glasner H. [On the absorption inhibiting effect of bile acids]. *Naunyn Schmiedeberg's Arch Pharmacol Exp Pathol* 254: 364–380, 1966.
114. Hofmann AF. The syndrome of ileal disease and the broken enterohepatic circulation: choleheic enteropathy. *Gastroenterology* 52: 752–757, 1967.
115. Mekhjian HS, Phillips SF, Hofmann AF. Colonic secretion of water and electrolytes induced by bile acids: perfusion studies in man. *J Clin Invest* 50: 1569–1577, 1971. doi:10.1172/JCI106644.
116. Wingate DL, Krag E, Mekhjian HS, Phillips SF. Relationships between ion and water movement in the human jejunum, ileum and colon during perfusion with bile acids. *Clin Sci Mol Med* 45: 593–606, 1973. doi:10.1042/cs0450593.
117. Feldman S, Gibaldi M. Effect of bile salts on gastric emptying and intestinal transit in the rat. *Gastroenterology* 54: 918–921, 1968.
118. Brown NJ, Read NW, Richardson A, Rumsey RD, Bogtoft C. Characteristics of lipid substances activating the ileal brake in the rat. *Gut* 31: 1126–1129, 1990. doi:10.1136/gut.31.10.1126.
119. Hansen M, Scheltema MJ, Sonne DP, Hansen JS, Sperling M, Rehfeld JF, Holst JJ, Vilsbøll T, Knop FK. Effect of chenodeoxycholic acid and the bile acid sequestrant colestevam on glucagon-like peptide-1 secretion. *Diabetes Obes Metab* 18: 571–580, 2016. doi:10.1111/dom.12648.
120. Penagini R, Misiewicz JJ, Frost PG. Effect of jejunal infusion of bile acids on small bowel transit and fasting jejunal motility in man. *Gut* 29: 789–794, 1988. doi:10.1136/gut.29.6.789.
121. Penagini R, Spiller RC, Misiewicz JJ, Frost PG. Effect of ileal infusion of glycochenodeoxycholic acid on segmental transit, motility, and flow in the human jejunum and ileum. *Gut* 30: 609–617, 1989. doi:10.1136/gut.30.5.609.
122. Armstrong DN, Krenz HK, Modlin IM, Ballantyne GH. Bile salt inhibition of motility in the isolated perfused rabbit terminal ileum. *Gut* 34: 483–488, 1993. doi:10.1136/gut.34.4.483.
123. Roda E, Bazzoli F, Labate AM, Mazzella G, Roda A, Sama C, Festi D, Aldini R, Taroni F, Barbara L. Ursodeoxycholic acid vs. chenodeoxycholic acid as cholesterol gallstone-dissolving agents: a comparative randomized study. *Hepatology* 2: 804–810, 1982. doi:10.1002/hep.1840020611.
124. Bampton PA, Dinning PG, Kennedy ML, Lubowski DZ, Cook IJ. The proximal colonic motor response to rectal mechanical and chemical stimulation. *Am J Physiol Gastrointest Liver Physiol* 282: G443–G449, 2002. doi:10.1152/ajpgi.00194.2001.
125. Odunsi-Shiyanbade ST, Camilleri M, McKinzie S, Burton D, Carlson P, Busciglio IA, Lamsam J, Singh R, Zinsmeister AR. Effects of chenodeoxycholate and a bile acid sequestrant, colestevam, on intestinal transit and bowel function. *Clin Gastroenterol Hepatol* 8: 159–165, 2010. doi:10.1016/j.cgh.2009.10.020.
126. Rao AS, Wong BS, Camilleri M, Odunsi-Shiyanbade ST, McKinzie S, Ryks M, Burton D, Carlson P, Lamsam J, Singh R, Zinsmeister AR. Chenodeoxycholate in females with irritable bowel syndrome-constipation: a pharmacodynamic and pharmacogenetic analysis. *Gastroenterology* 139: 1549–1558, 2010. doi:10.1053/j.gastro.2010.07.052.
127. Wu T, Bound MJ, Standfield SD, Gedulin B, Jones KL, Horowitz M, Rayner CK. Effects of rectal administration of taurocholic acid on glucagon-like peptide-1 and peptide YY secretion in healthy humans. *Diabetes Obes Metab* 15: 474–477, 2013. doi:10.1111/dom.12043.
128. Poole DP, Godfrey C, Cattaruzza F, Cottrell GS, Kirkland JG, Pelayo JC, Bunnett NW, Corvera CU. Expression and function of the bile acid receptor GpBAR1 (TGR5) in the murine enteric nervous system. *Neurogastroenterol Motil* 22: 814–e228, 2010. doi:10.1111/j.1365-2982.2010.01487.x.
129. Alemi F, Kwon E, Poole DP, Lieu T, Lyo V, Cattaruzza F, Cevikbas F, Steinhoff M, Nassini R, Materazzi S, Guerrero-Alba R, Valdez-Morales E, Cottrell GS, Schoonjans K, Geppetti P, Vanner SJ,

- Bunnett NW, Corvera CU. The TGR5 receptor mediates bile acid-induced itch and analgesia. *J Clin Invest* 123: 1513–1530, 2013. doi:10.1172/JCI64551.
130. Hu LD, Yu BP, Yang B. Deoxycholic acid inhibits smooth muscle contraction via protein kinase C-dependent modulation of L-type Ca^{2+} channels in rat proximal colon. *Mol Med Rep* 6: 833–837, 2012. doi:10.3892/mmr.2012.984.
 131. Kirwan WO, Smith AN, Mitchell WD, Falconer JD, Eastwood MA. Bile acids and colonic motility in the rabbit and the human. *Gut* 16: 894–902, 1975. doi:10.1136/gut.16.11.894.
 132. Falconer JD, Smith AN, Eastwood MA. The effects of bile acids on colonic motility in the rabbit. *Q J Exp Physiol Cogn Med Sci* 65: 135–144, 1980. doi:10.1113/expphysiol.1980.sp002497.
 133. Dharmasathaphorn K, Huott PA, Vongkovit P, Beuerlein G, Pandolfi SJ, Ammon HV. Cl^- secretion induced by bile salts. A study of the mechanism of action based on a cultured colonic epithelial cell line. *J Clin Invest* 84: 945–953, 1989. doi:10.1172/JCI114257.
 134. Keely SJ, Scharl MM, Bertelsen LS, Hagey LR, Barrett KE, Hofmann AF. Bile acid-induced secretion in polarized monolayers of T84 colonic epithelial cells: structure-activity relationships. *Am J Physiol Gastrointest Liver Physiol* 292: G290–G297, 2007. doi:10.1152/ajpgi.00076.2006.
 135. Kelly OB, Mroz MS, Ward JB, Colliva C, Scharl M, Pellicciari R, Gilmer JF, Fallon PG, Hofmann AF, Roda A, Murray FE, Keely SJ. Ursodeoxycholic acid attenuates colonic epithelial secretory function. *J Physiol* 591: 2307–2318, 2013. doi:10.1113/jphysiol.2013.252544.
 136. Teem MV, Phillips SF. Perfusion of the hamster jejunum with conjugated and unconjugated bile acids: inhibition of water absorption and effects on morphology. *Gastroenterology* 62: 261–267, 1972.
 137. Gracey M, Papadimitriou J, Burke V, Thomas J, Bower G. Effects on small-intestinal function and structure induced by feeding a deconjugated bile salt. *Gut* 14: 519–528, 1973. doi:10.1136/gut.14.7.519.
 138. Volpe BT, Binder HJ. Bile salt alteration of ion transport across jejunal mucosa. *Biochim Biophys Acta* 394: 597–604, 1975. doi:10.1016/0005-2736(75)90145-5.
 139. Freel RW, Hatch M, Earnest DL, Goldner AM. Dihydroxy bile salt-induced alterations in NaCl transport across the rabbit colon. *Am J Physiol Gastrointest Liver Physiol* 245: G808–G815, 1983. doi:10.1152/ajpgi.1983.245.6.G808.
 140. Binder HJ, Rawlins CL. Effect of conjugated dihydroxy bile salts on electrolyte transport in rat colon. *J Clin Invest* 52: 1460–1466, 1973. doi:10.1172/JCI107320.
 141. Mauricio AC, Slawik M, Heitzmann D, von Hahn T, Warth R, Bleich M, Greger R. Deoxycholic acid (DOC) affects the transport properties of distal colon. *Pflügers Arch* 439: 532–540, 2000. doi:10.1007/s004249900226.
 142. Alrefai WA, Saksena S, Tyagi S, Gill RK, Ramaswamy K, Dudeja PK. Taurodeoxycholate modulates apical Cl^-/OH^- exchange activity in Caco2 cells. *Dig Dis Sci* 52: 1270–1278, 2007. doi:10.1007/s10620-006-9090-8.
 143. Pallagi-Kunstár É, Farkas K, Maléth J, Rakonczay Z Jr, Nagy F, Molnár T, Szepes Z, Venglovecz V, Lonovics J, Rázga Z, Wittmann T, Hegyi P. Bile acids inhibit Na^+/H^+ exchanger and $\text{Cl}^-/\text{HCO}_3^-$ exchanger activities via cellular energy breakdown and Ca^{2+} overload in human colonic crypts. *Pflügers Arch* 467: 1277–1290, 2015. doi:10.1007/s00424-014-1560-9.
 144. Moschetta A, Portincasa P, Debellis L, Petruzzelli M, Montelli R, Calamita G, Gustavsson P, Palasciano G. Basolateral Ca^{2+} -dependent K^+ -channels play a key role in Cl^- secretion induced by taurodeoxycholate from colon mucosa. *Biol Cell* 95: 115–122, 2003. doi:10.1016/s0248-4900(03)00011-x.
 145. Devor DC, Sekar MC, Frizzell RA, Duffey ME. Taurodeoxycholate activates potassium and chloride conductances via an IP_3 -mediated release of calcium from intracellular stores in a colonic cell line (T84). *J Clin Invest* 92: 2173–2181, 1993. doi:10.1172/JCI116819.
 146. Gerbino A, Ranieri M, Lupo S, Caroppo R, Debellis L, Maiellaro I, Caratozzolo MF, Lopez F, Colella M. Ca^{2+} -dependent K^+ efflux regulates deoxycholate-induced apoptosis of BHK-21 and Caco-2 cells. *Gastroenterology* 137: 955–964, 2009. doi:10.1053/j.gastro.2009.03.038.
 147. Chien JM, Chou CT, Liang WZ, Cheng JS, Chang HT, Tseng HW, Kuo SY, Kuo CC, Chen FA, Shieh P, Ho CM, Lin JR, Kuo DH, Jan CR. Effect of deoxycholic acid on Ca^{2+} movement, cell viability and apoptosis in human gastric cancer cells. *Toxicol Mech Methods* 25: 113–119, 2015. doi:10.3109/15376516.2014.990597.
 148. Ward JB, Mroz MS, Keely SJ. The bile acid receptor, TGR5, regulates basal and cholinergic-induced secretory responses in rat colon. *Neurogastroenterol Motil* 25: 708–711, 2013. doi:10.1111/nmo.12148.
 149. Akare S, Martinez JD. Bile acid induces hydrophobicity-dependent membrane alterations. *Biochim Biophys Acta* 1735: 59–67, 2005. doi:10.1016/j.bbalip.2005.04.006.
 150. Jean-Louis S, Akare S, Ali MA, Mash EA Jr, Meuillet E, Martinez JD. Deoxycholic acid induces intracellular signaling through membrane perturbations. *J Biol Chem* 281: 14948–14960, 2006. doi:10.1074/jbc.M506710200.
 151. Combettes L, Berthon B, Claret M, Erlinger S. Selective permeabilization of the endoplasmic reticulum by monohydroxylated bile acids in liver. *Hepatology* 9: 663–665, 1989. doi:10.1002/hep.1840090431.
 152. Combettes L, Dumont M, Berthon B, Erlinger S, Claret M. Release of calcium from the endoplasmic reticulum by bile acids in rat liver cells. *J Biol Chem* 263: 2299–2303, 1988.
 153. Peregrin AT, Ahlman H, Jodal M, Lundgren O. Involvement of serotonin and calcium channels in the intestinal fluid secretion evoked by bile salt and cholera toxin. *Br J Pharmacol* 127: 887–894, 1999. doi:10.1038/sj.bjp.0702615.
 154. Tantisira MH, Jodal M, Lundgren O. Intraluminal bile salt increases rate of firing in afferent fibers from the small intestine of the rat. *Experientia* 43: 168–169, 1987. doi:10.1007/BF01942837.
 155. Karlström L, Cassuto J, Jodal M, Lundgren O. The importance of the enteric nervous system for the bile-salt-induced secretion in the small intestine of the rat. *Scand J Gastroenterol* 18: 117–123, 1983. doi:10.3109/00365528309181570.
 156. Karlström L. Evidence of involvement of the enteric nervous system in the effects of sodium deoxycholate on small-intestinal transepithelial fluid transport and motility. *Scand J Gastroenterol* 21: 321–330, 1986. doi:10.3109/00365528609003082.
 157. Forsgård RA, Korpela R, Stenman LK, Osterlund P, Holma R. Deoxycholic acid induced changes in electrophysiological parameters and macromolecular permeability in murine small intestine with and without functional enteric nervous system plexuses. *Neurogastroenterol Motil* 26: 1179–1187, 2014. doi:10.1111/nmo.12383.
 158. Sun Y, Fihn BM, Sjövall H, Jodal M. Enteric neurones modulate the colonic permeability response to luminal bile acids in rat colon in vivo. *Gut* 53: 362–367, 2004. doi:10.1136/gut.2003.015867.
 159. Quist RG, Ton-Nu HT, Lillienau J, Hofmann AF, Barrett KE. Activation of mast cells by bile acids. *Gastroenterology* 101: 446–456, 1991. doi:10.1016/0016-5085(91)90024-f.
 160. Gelbmann CM, Schteingart CD, Thompson SM, Hofmann AF, Barrett KE. Mast cells and histamine contribute to bile acid-stimulated secretion in the mouse colon. *J Clin Invest* 95: 2831–2839, 1995. doi:10.1172/JCI117988.
 161. Arizono N, Matsuda S, Hattori T, Kojima Y, Maeda T, Galli SJ. Anatomical variation in mast cell nerve associations in the rat small intestine, heart, lung, and skin. Similarities of distances between neural processes and mast cells, eosinophils, or plasma cells in the jejunal lamina propria. *Lab Invest* 62: 626–634, 1990.
 162. Stead RH, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. *Gastroenterology* 97: 575–585, 1989. doi:10.1016/0016-5085(89)90627-6.
 163. Keating N, Mroz MS, Scharl MM, Marsh C, Ferguson G, Hofmann AF, Keely SJ. Physiological concentrations of bile acids down-regulate agonist induced secretion in colonic epithelial cells. *J Cell Mol Med* 13: 2293–2303, 2009. doi:10.1111/j.1582-4934.2009.00838.x.
 164. Hamilton JP, Xie G, Raufman JP, Hogan S, Griffin TL, Packard CA, Chatfield DA, Hagey LR, Steinbach JH, Hofmann AF. Human cecal bile acids: concentration and spectrum. *Am J Physiol Gastrointest Liver Physiol* 293: G256–G263, 2007. doi:10.1152/ajpgi.00027.2007.
 165. Wei W, Wang HF, Zhang Y, Zhang YL, Niu BY, Yao SK. Altered metabolism of bile acids correlates with clinical parameters and the gut microbiota in patients with diarrhea-predominant irritable bowel syndrome. *World J Gastroenterol* 26: 7153–7172, 2020. doi:10.3748/wjg.v26.i45.7153.

166. Zhan K, Zheng H, Li J, Wu H, Qin S, Luo L, Huang S. Gut microbiota-bile acid crosstalk in diarrhea-irritable bowel syndrome. *Biomed Res Int* 2020; 3828249, 2020. doi:10.1155/2020/3828249.
167. Sagar NM, Duboc H, Kay GL, Alam MT, Wicaksono AN, Covington JA, Quince C, Kokkorou M, Svolos V, Palmieri LJ, Gerasimidis K, Walters JRF, Arasaradnam RP. The pathophysiology of bile acid diarrhoea: differences in the colonic microbiome, metabolome and bile acids. *Sci Rep* 10: 20436, 2020. doi:10.1038/s41598-020-77374-7.
168. Camilleri M, Vijayvargiya P. The role of bile acids in chronic diarrhea. *Am J Gastroenterol* 115: 1596–1603, 2020. doi:10.14309/ajg.0000000000000696.
169. Jeffery IB, Das A, O'Herlihy E, Coughlan S, Cisek K, Moore M, Bradley F, Carty T, Pradhan M, Dwivedi C, Shanahan F, O'Toole PW. Differences in fecal microbiomes and metabolites of people with vs without irritable bowel syndrome and bile acid malabsorption. *Gastroenterology* 158: 1016–1028.e8, 2020. doi:10.1053/j.gastro.2019.11.301.
170. Bazzoli F, Malavolti M, Petronelli A, Barbara L, Roda E. Treatment of constipation with chenodeoxycholic acid. *J Int Med Res* 11: 120–123, 1983. doi:10.1177/030006058301100211.
171. Fromm H, Sarva RP, Ravitch MM, McJunkin B, Farivar S, Amin P. Effects of jejunoileal bypass on the enterohepatic circulation of bile acids, bacterial flora in the upper small intestine, and absorption of vitamin B12. *Metabolism* 32: 1133–1141, 1983. doi:10.1016/0026-0495(83)90060-4.
172. Sackmann M, Pauletzki J, Aydemir U, Holl J, Sauerbruch T, Hasford J, Paumgartner G. Efficacy and safety of ursodeoxycholic acid for dissolution of gallstone fragments: comparison with the combination of ursodeoxycholic acid and chenodeoxycholic acid. *Hepatology* 14: 1136–1141, 1991.
173. Iguchi Y, Nishimaki-Mogami T, Yamaguchi M, Teraoka F, Kaneko T, Une M. Effects of chemical modification of ursodeoxycholic acid on TGR5 activation. *Biol Pharm Bull* 34: 1–7, 2011. doi:10.1248/bpb.34.1.
174. Camilleri M, Klee EW, Shin A, Carlson P, Li Y, Grover M, Zinsmeister AR. Irritable bowel syndrome-diarrhea: characterization of genotype by exome sequencing, and phenotypes of bile acid synthesis and colonic transit. *Am J Physiol Gastrointest Liver Physiol* 306: G13–G26, 2014. doi:10.1152/ajpgi.00294.2013.
175. Camilleri M, Vazquez-Roque MI, Carlson P, Burton D, Wong BS, Zinsmeister AR. Association of bile acid receptor TGR5 variation and transit in health and lower functional gastrointestinal disorders. *Neurogastroenterol Motil* 23: 995–e458, 2011. doi:10.1111/j.1365-2982.2011.01772.x.
176. Giaretta PR, Suchodolski JS, Blick AK, Steiner JM, Lidbury JA, Rech RR. Distribution of bile acid receptor TGR5 in the gastrointestinal tract of dogs. *Histol Histopathol* 34: 69–79, 2019. doi:10.14670/HH-18-025.
177. Dopico AM, Walsh JV Jr, Singer JJ. Natural bile acids and synthetic analogues modulate large conductance Ca^{2+} -activated K^{+} (BKCa) channel activity in smooth muscle cells. *J Gen Physiol* 119: 251–273, 2002. doi:10.1085/jgp.20028537.
178. Meier Y, Eloranta JJ, Darimont J, Ismail MG, Hiller C, Fried M, Kullak-Ublick GA, Vavricka SR. Regional distribution of solute carrier mRNA expression along the human intestinal tract. *Drug Metab Dispos* 35: 590–594, 2007. doi:10.1124/dmd.106.013342.
179. Biagioli M, Carino A, Cipriani S, Francisci D, Marchiano S, Scarpelli P, Sorcini D, Zampella A, Fiorucci S. The bile acid receptor GPBAR1 regulates the M1/M2 phenotype of intestinal macrophages and activation of GPBAR1 rescues mice from murine colitis. *J Immunol* 199: 718–733, 2017. doi:10.4049/jimmunol.1700183.
180. Cipriani S, Mencarelli A, Chini MG, Distrutti E, Renga B, Bifulco G, Baldelli F, Donini A, Fiorucci S. The bile acid receptor GPBAR-1 (TGR5) modulates integrity of intestinal barrier and immune response to experimental colitis. *PLoS One* 6: e25637, 2011 [Erratum in *PLoS One* 8, 2013. doi:10.1371/journal.pone.0025637].
181. Adrian TE, Ballantyne GH, Longo WE, Bilchik AJ, Graham S, Basson MD, Tierney RP, Modlin IM. Deoxycholate is an important releaser of peptide YY and enteroglucagon from the human colon. *Gut* 34: 1219–1224, 1993. doi:10.1136/gut.34.9.1219.
182. Adrian TE, Gariballa S, Parekh KA, Thomas SA, Saadi H, Al Kaabi J, Nagelkerke N, Gedulin B, Young AA. Rectal taurocholate increases L cell and insulin secretion, and decreases blood glucose and food intake in obese type 2 diabetic volunteers. *Diabetologia* 55: 2343–2347, 2012. doi:10.1007/s00125-012-2593-2.
183. Dumoulin V, Moro F, Barcelo A, Dakka T, Cuber JC. Peptide YY, glucagon-like peptide-1, and neurotensin responses to luminal factors in the isolated vascularly perfused rat ileum. *Endocrinology* 139: 3780–3786, 1998. doi:10.1210/endo.139.9.6202.
184. Meyer-Gerspach AC, Steinert RE, Keller S, Malarski A, Schulte FH, Beglinger C. Effects of chenodeoxycholic acid on the secretion of gut peptides and fibroblast growth factors in healthy humans. *J Clin Endocrinol Metab* 98: 3351–3358, 2013. doi:10.1210/jc.2012-4109.
185. Plaisancié P, Dumoulin V, Chayvialle JA, Cuber JC. Luminal peptide YY-releasing factors in the isolated vascularly perfused rat colon. *J Endocrinol* 151: 421–429, 1996. doi:10.1677/joe.0.1510421.
186. Brighton CA, Rievaj J, Kuhre RE, Glass LL, Schoonjans K, Holst JJ, Gribble FM, Reimann F. Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G protein-coupled bile acid receptors. *Endocrinology* 156: 3961–3970, 2015. doi:10.1210/en.2015-1321.
187. Harach T, Pols TW, Nomura M, Maida A, Watanabe M, Auwerx J, Schoonjans K. TGR5 potentiates GLP-1 secretion in response to anionic exchange resins. *Sci Rep* 2: 430, 2012. doi:10.1038/srep00430.
188. Amato A, Baldassano S, Liotta R, Serio R, Mulè F. Exogenous glucagon-like peptide 1 reduces contractions in human colon circular muscle. *J Endocrinol* 221: 29–37, 2014. doi:10.1530/JOE-13-0525.
189. May AT, Crowe MS, Blakeney BA, Mahavadi S, Wang H, Grider JR, Murthy KS. Identification of expression and function of the glucagon-like peptide-1 receptor in colonic smooth muscle. *Peptides* 112: 48–55, 2019. doi:10.1016/j.peptides.2018.11.007.
190. Halim MA, Degerblad M, Sundbom M, Karlborn U, Holst JJ, Webb DL, Hellström PM. Glucagon-like peptide-1 inhibits prandial gastrointestinal motility through myenteric neuronal mechanisms in humans. *J Clin Endocrinol Metab* 103: 575–585, 2018. doi:10.1210/jc.2017-02006.
191. O'Brien R, O'Malley D. The Glucagon-like peptide-1 receptor agonist, exendin-4, ameliorated gastrointestinal dysfunction in the Wistar Kyoto rat model of Irritable Bowel Syndrome. *Neurogastroenterol Motil* 32: e13738, 2020. doi:10.1111/nmo.13738.
192. Tolessa T, Gutniak M, Holst JJ, Efendic S, Hellstrom PM. Glucagon-like peptide-1 retards gastric emptying and small bowel transit in the rat: effect mediated through central or enteric nervous mechanisms. *Dig Dis Sci* 43: 2284–2290, 1998. doi:10.1023/a:1026678925120.
193. Ferrier L, Segain JP, Pacaud P, Cherbut C, Loirand G, Galmiche JP, Blottière HM. Pathways and receptors involved in peptide YY induced contraction of rat proximal colonic muscle in vitro. *Gut* 46: 370–375, 2000. doi:10.1136/gut.46.3.370.
194. Fox-Threlkeld JA, Daniel EE, Christinck F, Woskowska Z, Cipris S, McDonald TJ. Peptide YY stimulates circular muscle contractions of the isolated perfused canine ileum by inhibiting nitric oxide release and enhancing acetylcholine release. *Peptides* 14: 1171–1178, 1993. doi:10.1016/0196-9781(93)90172-d.
195. Lundberg JM, Tatemoto K, Terenius L, Hellström PM, Mutt V, Hökfelt T, Hamberger B. Localization of peptide YY (PYY) in gastrointestinal endocrine cells and effects on intestinal blood flow and motility. *Proc Natl Acad Sci USA* 79: 4471–4475, 1982. doi:10.1073/pnas.79.14.4471.
196. Tough IR, Forbes S, Tolhurst R, Ellis M, Herzog H, Bornstein JC, Cox HM. Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y_1 and Y_2 receptors. *Br J Pharmacol* 164: 471–484, 2011. doi:10.1111/j.1476-5381.2011.01401.x.
197. Ferrier L, Segain JP, Bonnet C, Cherbut C, Lehur PA, Jarry A, Galmiche JP, Blottière HM. Functional mapping of NPY/PYY receptors in rat and human gastro-intestinal tract. *Peptides* 23: 1765–1771, 2002. doi:10.1016/s0196-9781(02)00133-x.
198. Krantis A, Potvin W, Harding RK. Peptide YY (PYY) stimulates intrinsic enteric motor neurones in the rat small intestine. *Naunyn Schmiedeberg Arch Pharmacol* 338: 287–292, 1988. doi:10.1007/BF00173402.
199. Larhammar D. Structural diversity of receptors for neuropeptide Y, peptide YY and pancreatic polypeptide. *Regul Pept* 65: 165–174, 1996. doi:10.1016/0167-0115(96)00110-3.
200. Cho HJ, Kosari S, Hunne B, Callaghan B, Rivera LR, Bravo DM, Furness JB. Differences in hormone localisation patterns of K and L

- type enteroendocrine cells in the mouse and pig small intestine and colon. *Cell Tissue Res* 359: 693–698, 2015. doi:10.1007/s00441-014-2033-3.
201. Svendsen B, Pedersen J, Albrechtsen NJ, Hartmann B, Toräng S, Rehfeld JF, Poulsen SS, Holst JJ. An analysis of cosecretion and coexpression of gut hormones from male rat proximal and distal small intestine. *Endocrinology* 156: 847–857, 2015. doi:10.1210/en.2014-1710.
 202. Austad WI, Lack L, Tyor MP. Importance of bile acids and of an intact distal small intestine for fat absorption. *Gastroenterology* 52: 638–646, 1967.
 203. Valentin N, Camilleri M, Altayar O, Vijayvargiya P, Acosta A, Nelson AD, Murad MH. Biomarkers for bile acid diarrhoea in functional bowel disorder with diarrhoea: a systematic review and meta-analysis. *Gut* 65: 1951–1959, 2016. doi:10.1136/gutjnl-2015-309889.
 204. Oelkers P, Kirby LC, Heubi JE, Dawson PA. Primary bile acid malabsorption caused by mutations in the ileal sodium-dependent bile acid transporter gene (SLC10A2). *J Clin Invest* 99: 1880–1887, 1997. doi:10.1172/JCI119355.
 205. Ho RH, Leake BF, Urquhart BL, Gregor JC, Dawson PA, Kim RB. Functional characterization of genetic variants in the apical sodium-dependent bile acid transporter (ASBT; SLC10A2). *J Gastroenterol Hepatol* 26: 1740–1748, 2011. doi:10.1111/j.1440-1746.2011.06805.x.
 206. Sultan M, Rao A, Elpeleg O, Vaz FM, Abu-Libdeh B, Karpen SJ, Dawson PA. Organic solute transporter- β (SLC51B) deficiency in two brothers with congenital diarrhea and features of cholestasis. *Hepatology* 68: 590–598, 2018. doi:10.1002/hep.29516.
 207. Arlow FL, Dekovich AA, Priest RJ, Behr WT. Bile acids in radiation-induced diarrhea. *South Med J* 80: 1259–1261, 1987. doi:10.1097/00007611-198710000-00015.
 208. Shin A, Camilleri M, Vijayvargiya P, Busciglio I, Burton D, Ryks M, Rhoten D, Lueke A, Saenger A, Girtman A, Zinsmeister AR. Bowel functions, fecal unconjugated primary and secondary bile acids, and colonic transit in patients with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 11: 1270–1275.e1, 2013. doi:10.1016/j.cgh.2013.04.020.
 209. Aldini R, Roda A, Festi D, Sama C, Mazzella G, Bazzoli F, Morselli AM, Roda E, Barbara L. Bile acid malabsorption and bile acid diarrhea in intestinal resection. *Dig Dis Sci* 27: 495–502, 1982. doi:10.1007/BF01296727.
 210. Camilleri M, Busciglio I, Acosta A, Shin A, Carlson P, Burton D, Ryks M, Rhoten D, Lamsam J, Lueke A, Donato LJ, Zinsmeister AR. Effect of increased bile acid synthesis or fecal excretion in irritable bowel syndrome-diarrhea. *Am J Gastroenterol* 109: 1621–1630, 2014. doi:10.1038/ajg.2014.215.
 211. Lee JM, Ong JR, Vergnes L, de Aguiar Vallim TQ, Nolan J, Cantor RM, Walters JRF, Reue K. Diet1, bile acid diarrhea, and FGF15/19: mouse model and human genetic variants. *J Lipid Res* 59: 429–438, 2018. doi:10.1194/jlr.M078279.
 212. Walters JR, Tasleem AM, Omer OS, Brydon WG, Dew T, Le Roux CW. A new mechanism for bile acid diarrhea: defective feedback inhibition of bile acid biosynthesis. *Clin Gastroenterol Hepatol* 7: 1189–1194, 2009. doi:10.1016/j.cgh.2009.04.024.
 213. Montagnani M, Love MW, Rössel P, Dawson PA, Qvist P. Absence of dysfunctional ileal sodium-bile acid cotransporter gene mutations in patients with adult-onset idiopathic bile acid malabsorption. *Scand J Gastroenterol* 36: 1077–1080, 2001. doi:10.1080/003655201750422693.
 214. Wedlake L, Thomas K, Lalji A, Anagnostopoulos C, Andreyev HJ. Effectiveness and tolerability of colessevelam hydrochloride for bile-acid malabsorption in patients with cancer: a retrospective chart review and patient questionnaire. *Clin Ther* 31: 2549–2558, 2009. doi:10.1016/j.clinthera.2009.11.027.
 215. Vijayvargiya P, Busciglio I, Burton D, Donato L, Lueke A, Camilleri M. Bile acid deficiency in a subgroup of patients with irritable bowel syndrome with constipation based on biomarkers in serum and fecal samples. *Clin Gastroenterol Hepatol* 16: 522–527, 2018. doi:10.1016/j.cgh.2017.06.039.
 216. Chedid V, Vijayvargiya P, Camilleri M. Elobixibat for the treatment of constipation. *Expert Rev Gastroenterol Hepatol* 12: 951–960, 2018. doi:10.1080/17474124.2018.1522248.
 217. Simrén M, Tack J. New treatments and therapeutic targets for IBS and other functional bowel disorders. *Nat Rev Gastroenterol Hepatol* 15: 589–605, 2018. doi:10.1038/s41575-018-0034-5.
 218. Gillberg P-G, Dahlström M, Starke I, Östlund-Lindqvist A-M. The IBAT inhibition by A3309 a potential mechanism for the treatment of constipation. *Gastroenterology* 138: S224, 2010. doi:10.1016/S0016-5085(10)61017-7.
 219. Chey WD, Camilleri M, Chang L, Rikner L, Graffner H. A randomized placebo-controlled phase IIb trial of a3309, a bile acid transporter inhibitor, for chronic idiopathic constipation. *Am J Gastroenterol* 106: 1803–1812, 2011. doi:10.1038/ajg.2011.162.
 220. Nakajima A, Seki M, Taniguchi S. Determining an optimal clinical dose of elobixibat, a novel inhibitor of the ileal bile acid transporter, in Japanese patients with chronic constipation: a phase II, multicenter, double-blind, placebo-controlled randomized clinical trial. *J Gastroenterol* 53: 525–534, 2018. doi:10.1007/s00535-017-1383-5.
 221. Nakajima A, Seki M, Taniguchi S, Ohta A, Gillberg PG, Mattsson JP, Camilleri M. Safety and efficacy of elobixibat for chronic constipation: results from a randomised, double-blind, placebo-controlled, phase 3 trial and an open-label, single-arm, phase 3 trial. *Lancet Gastroenterol Hepatol* 3: 537–547, 2018. doi:10.1016/S2468-1253(18)30123-7.
 222. Simrén M, Bajor A, Gillberg P-G, Rudling M, Abrahamsson H. Randomized clinical trial: the ileal bile acid transporter inhibitor A3309 vs. placebo in patients with chronic idiopathic constipation—a double-blind study. *Aliment Pharmacol Ther* 34: 41–50, 2011. doi:10.1111/j.1365-2036.2011.04675.x.
 223. Nakajima A, Taniguchi S, Kurosu S, Gillberg PG, Mattsson JP, Camilleri M. Efficacy, long-term safety, and impact on quality of life of elobixibat in more severe constipation: post hoc analyses of two phase 3 trials in Japan. *Neurogastroenterol Motil* 31: e13571, 2019. doi:10.1111/nmo.13571.
 224. Taniguchi S, Yano T, Imaizumi M, Manabe N. Elobixibat, an ileal bile acid transporter inhibitor, induces giant migrating contractions during natural defecation in conscious dogs. *Neurogastroenterol Motil* 30: e13448, 2018. doi:10.1111/nmo.13448.
 225. Newsome PN, Palmer M, Freilich B, Sheikh MY, Sheikh A, Saries H, Herring R, Mantry P, Kayali Z, Hassanein T, Lee H-M, Aithal GP; Volixibat in Adults study group. Volixibat in adults with non-alcoholic steatohepatitis: 24-week interim analysis from a randomized, phase II study. *J Hepatol* 73: 231–240, 2020. doi:10.1016/j.jhep.2020.03.024.
 226. Tiessen RG, Kennedy CA, Keller BT, Levin N, Acevedo L, Gedulin B, van Vliet AA, Dorenbaum A, Palmer M. Safety, tolerability and pharmacodynamics of apical sodium-dependent bile acid transporter inhibition with volixibat in healthy adults and patients with type 2 diabetes mellitus: a randomised placebo-controlled trial. *BMC Gastroenterol* 18: 3, 2018. doi:10.1186/s12876-017-0736-0.
 227. Mayo MJ, Pockros PJ, Jones D, Bowlus CL, Levy C, Patanwala I, Bacon B, Luketic V, Vuppalandi R, Medendorp S, Dorenbaum A, Kennedy C, Novak P, Gu J, Apostol G, Hirschfield GM. A randomized, controlled, phase 2 study of maralixibat in the treatment of itching associated with primary biliary cholangitis. *Hepatol Commun* 3: 365–381, 2019. doi:10.1002/hep4.1305.
 228. Schneider BL, Spino C, Kamath BM, Magee JC, Bass LM, Setchell KD, Miethke A, Molleston JP, Mack CL, Squires RH, Murray KF, Loomes KM, Rosenthal P, Karpen SJ, Leung DH, Guthery SL, Thomas D, Sherker AH, Sokol RJ; Childhood Liver Disease Research Network. Placebo-controlled randomized trial of an intestinal bile salt transport inhibitor for pruritus in alagille syndrome. *Hepatol Commun* 2: 1184–1198, 2018. doi:10.1002/hep4.1244.
 229. United States National Library of Medicine. Dose Response Study of GSK2330672 for the Treatment of Pruritus in Participants With Primary Biliary Cholangitis. 4th May 2021. <https://clinicaltrials.gov/show/NCT02966834>.
 230. Al-Dury S, Wahlström A, Wahlin S, Langedijk J, Elferink RO, Ståhlman M, Marschall HU. Pilot study with IBAT inhibitor A4250 for the treatment of cholestatic pruritus in primary biliary cholangitis. *Sci Rep* 8: 6658, 2018. doi:10.1038/s41598-018-25214-0.
 231. Hegade VS, Kendrick SFW, Dobbins RL, Miller SR, Thompson D, Richards D, Storey J, Dukes GE, Corrigan M, Elferink RPJO, Beuers U, Hirschfield GM, Jones DE. Effect of ileal bile acid transporter inhibitor GSK2330672 on pruritus in primary biliary cholangitis: a double-blind, randomised, placebo-controlled, crossover, phase 2a study. *Lancet* 389: 1114–1123, 2017. doi:10.1016/S0140-6736(17)30319-7.

232. **Beigel F, Teich N, Howaldt S, Lammert F, Maul J, Breitenreiter S, Rust C, Göke B, Brand S, Ochsenkühn T.** Colesevelam for the treatment of bile acid malabsorption-associated diarrhea in patients with Crohn's disease: a randomized, double-blind, placebo-controlled study. *J Crohns Colitis* 8: 1471–1479, 2014. doi:[10.1016/j.crohns.2014.05.009](https://doi.org/10.1016/j.crohns.2014.05.009).
233. **Money ME, Hofmann AF, Hagey LR, Walkowiak J, Talley NJ.** Treatment of irritable bowel syndrome-diarrhea with pancrealipase or colessevelam and association with steatorrhea. *Pancreas* 38: 232–233, 2009. doi:[10.1097/MPA.0b013e31817c1b36](https://doi.org/10.1097/MPA.0b013e31817c1b36).
234. **Camilleri M, Acosta A, Busciglio I, Boldingh A, Dyer RB, Zinsmeister AR, Lueke A, Gray A, Donato LJ.** Effect of colessevelam on faecal bile acids and bowel functions in diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 41: 438–448, 2015. doi:[10.1111/apt.13065](https://doi.org/10.1111/apt.13065).
235. **Camilleri M, Nord SL, Burton D, Odyebo I, Zhang Y, Chen J, Im K, Bhad P, Badman MK, Sanders DS, Walters JRF.** Randomised clinical trial: significant biochemical and colonic transit effects of the farnesoid X receptor agonist tropifexor in patients with primary bile acid diarrhoea. *Aliment Pharmacol Ther* 52: 808–820, 2020. doi:[10.1111/apt.15967](https://doi.org/10.1111/apt.15967).
236. **Ge X, Zhao W, Ding C, Tian H, Xu L, Wang H, Ni L, Jiang J, Gong J, Zhu W, Zhu M, Li N.** Potential role of fecal microbiota from patients with slow transit constipation in the regulation of gastrointestinal motility. *Sci Rep* 7: 441, 2017. doi:[10.1038/s41598-017-00612-y](https://doi.org/10.1038/s41598-017-00612-y).
237. **Munch A, Strom M, Soderholm JD.** Dihydroxy bile acids increase mucosal permeability and bacterial uptake in human colon biopsies. *Scand J Gastroenterol* 42: 1167–1174, 2007. doi:[10.1080/00365520701320463](https://doi.org/10.1080/00365520701320463).
238. **Becker L, Spear ET, Sinha SR, Haileselassie Y, Habtezion A.** Age-related changes in gut microbiota alter phenotype of muscularis macrophages and disrupt gastrointestinal motility. *Cell Mol Gastroenterol Hepatol* 7: 243–245.e2, 2019. doi:[10.1016/j.jcmgh.2018.09.001](https://doi.org/10.1016/j.jcmgh.2018.09.001).



Mice expressing fluorescent PAR₂ reveal that endocytosis mediates colonic inflammation and pain

Rocco Latorre^{a,b}, Alan Hegron^{a,b}, Chloe J. Peach^{a,b}, Shavonne Teng^{a,b}, Raquel Tonello^{a,b}, Jeffri S. Retamal^{a,b,c}, Rafael Klein-Cloud^{a,b}, Diana Bok^{a,b,c}, Dane D. Jensen^{a,b,c}, Lena Gottesman-Katz^{a,b}, Jeanette Rientjes^d, Nicholas A. Veldhuis^e, Daniel P. Poole^e, Alex R. B. Thomsen^f, Brian L. Schmidt^{a,b,c}, Charalabos H. Pothoulakis^g, Carl Rankin^g, Ying Xie^g, Hon Wai Koon^g, and Nigel W. Bunnett^{a,b,1}

^aDepartment of Molecular Pathobiology, Neuroscience Institute, New York University, New York, NY 10010; ^bDepartment of Neuroscience and Physiology, Neuroscience Institute, New York University, New York, NY 10010; ^cBluestone Center for Clinical Research, New York University College of Dentistry, New York, NY 10010; ^dGene Modification Platform, Monash University, Clayton, VIC 3168, Australia; ^eDrug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia; ^fDepartment of Molecular Pathobiology, Department of Neuroscience and Physiology, Neuroscience Institute, New York University, New York, NY 10010; and ^gVatche and Tamar Manoukian Division of Digestive Diseases, David Geffen School of Medicine, University of California, Los Angeles, CA 90095

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G protein-coupled receptors (GPCRs) regulate many pathophysiological processes and are major therapeutic targets. The impact of disease on the subcellular distribution and function of GPCRs is poorly understood. We investigated trafficking and signaling of protease-activated receptor 2 (PAR₂) in colitis. To localize PAR₂ and assess redistribution during disease, we generated knockin mice expressing PAR₂ fused to monomeric ultrastable green fluorescent protein (muGFP). PAR₂-muGFP signaled and trafficked normally. PAR₂ messenger RNA was detected at similar levels in *Par₂-muGFP* and wild-type mice. Immunostaining with a GFP antibody and RNAScope in situ hybridization using *F2rl1* (PAR₂) and *Gfp* probes revealed that PAR₂-muGFP was expressed in epithelial cells of the small and large intestine and in subsets of enteric and dorsal root ganglia neurons. In healthy mice, PAR₂-muGFP was prominently localized to the basolateral membrane of colonocytes. In mice with colitis, PAR₂-muGFP was depleted from the plasma membrane of colonocytes and redistributed to early endosomes, consistent with generation of proinflammatory proteases that activate PAR₂. PAR₂ agonists stimulated endocytosis of PAR₂ and recruitment of Gα_q, Gα_i, and β-arrestin to early endosomes of T84 colon carcinoma cells. PAR₂ agonists increased paracellular permeability of colonic epithelial cells, induced colonic inflammation and hyperalgesia in mice, and stimulated proinflammatory cytokine release from segments of human colon. Knockdown of dynamin-2 (*Dnm2*), the major colonocyte isoform, and Dnm inhibition attenuated PAR₂ endocytosis, signaling complex assembly and colonic inflammation and hyperalgesia. Thus, PAR₂ endocytosis sustains protease-evoked inflammation and nociception and PAR₂ in endosomes is a potential therapeutic target for colitis.

signaling | receptors | proteases | endocytosis | inflammation

G protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors, control most physiological and pathological processes, and are the target of >30% of approved drugs (1). Despite their medical importance, the effects of disease on the function and subcellular distribution of GPCRs are poorly understood. GPCRs at the plasma membrane interact with extracellular ligands and couple to intracellular heterotrimeric G proteins. However, GPCR signaling at the plasma membrane is often transient. GPCR kinases phosphorylate activated receptors, increasing their affinity for β-arrestins (βARRs) (2). βARRs uncouple GPCRs from G proteins, which mediates desensitization, and couple GPCRs to clathrin and adaptor protein 2, which mediate endocytosis. These processes terminate plasma membrane signaling. During disease, the continued generation of agonists could trigger the endocytosis of GPCRs. Although endosomes were once considered merely as a pathway for receptor trafficking, mounting evidence indicates that endosomes are a site of sustained GPCR

signaling by Gα- and βARR-mediated mechanisms (3–9). GPCRs can also continue to signal from other intracellular compartments, including the Golgi apparatus (10, 11). The contribution of intracellular GPCR signaling to disease is not fully understood.

We investigated the impact of colitis on endosomal signaling of protease-activated receptor 2 (PAR₂), which mediates the proinflammatory and pronociceptive actions of proteases (12). Trypsin (13), tryptase (14), coagulation factors VIIa and Xa (15), and kallikreins (16) cleave PAR₂ to reveal a tethered ligand that activates the receptor. Cathepsin S, legumain, and elastase cleave at different sites and activate biased mechanisms of PAR₂ signaling (17–19). During colitis, multiple proteases from the host and the microbiome activate PAR₂ (20–25). PAR₂ activation evokes the redistribution of ZO1 and occludin

Significance

Colonic epithelial cells protect against inflammation by forming a barrier against ingress of microbes, digestive enzymes, and metabolic waste from the lumen. Protease-activated receptor-2 (PAR₂) mediates the proinflammatory and pronociceptive actions of proteases. We found that the proteolytic environment of the inflamed mouse colon induces redistribution of PAR₂ from the basolateral plasma membrane to endosomes of colonocytes. Endocytosis allows the assembly of a PAR₂, Gα, and β-arrestin signaling complex that mediates sustained increases in colonocyte paracellular permeability and persistent inflammation and hyperalgesia of the colon. Thus, PAR₂ endocytosis and endosomal signaling disrupts the normal protective function of colonocytes and underlies colonic inflammation and pain. PAR₂ in endosomes is a potential therapeutic target for inflammatory and painful diseases of the colon.

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¹To whom correspondence may be addressed. Email: nwb2@nyu.edu.

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from tight junctions of colonocytes, leading to increased paracellular permeability, influx of proinflammatory macromolecules and bacteria into the mucosa, and inflammation (26, 27). PAR₂ activation on nociceptors stimulates the release of neuropeptides that cause neurogenic inflammation and pain in the skin (28, 29) and colon (7, 22, 30). Proteases and PAR₂ have been implicated in colonic diseases, including inflammatory bowel disease (IBD), irritable bowel syndrome, and cancer (12). However, the signaling mechanisms by which PAR₂ induces disease are incompletely understood.

Our understanding of the function of PAR₂ is hampered by an inadequate knowledge of its cellular and subcellular localization in health and disease states. Because GPCR antibodies can lack sensitivity and specificity and are unsuitable for studying receptor trafficking in living cells, we generated a knockin mouse expressing PAR₂ fused to monomeric ultrastable green fluorescent protein (muGFP). Colitis resulted in translocation of PAR₂-muGFP from the basolateral membrane of colonocytes to early endosomes. Endocytosis of PAR₂ led to the assembly of a signaling complex (signalosome) comprising PAR₂ and Gα_q, Gα_i, or βARR. Endocytosis of PAR₂ was necessary for sustained increases in paracellular permeability, release of proinflammatory cytokines and chemokines, and colonic inflammation and hyperalgesia.

Results

Generation of *Par₂-mugfp* Mice. To gain an understanding of the cellular and subcellular distribution of PAR₂ and how it might be altered during disease, we generated mice expressing mouse PAR₂ fused at the intracellular C terminus to muGFP. muGFP harbors stabilizing mutations (Q69L and N164Y), which improve hydrophobic packing in the core and facilitate hydrogen bonding, and a mutation at the dimer interface (F223D), which prevents dimerization (31). muGFP retains fluorescence after harsh fixation and resists undesirable dimerization. To verify functionality of PAR₂-muGFP, we compared signaling of PAR₂-muGFP and PAR₂ with N-terminal FLAG and C-terminal HA11 epitopes transiently expressed in KNRK cells, which express low levels of PAR₂ and have been used extensively to study PAR₂ functions (13). Trypsin and 2-furoyl-LIGRLO-NH₂ (2F), a PAR₂ agonist that is an analog of the tethered ligand domain, stimulated a concentration-dependent increase in IP-1 formation with similar potency and efficacy in cells expressing PAR₂-muGFP or PAR₂-HA11 (half maximal effective concentration of KNRK-PAR₂-muGFP: trypsin, 5.4×10^{-8} M; 2F, 9.1×10^{-8} M; half maximal effective concentration of KNRK-PAR₂-HA11: trypsin, 5.4×10^{-8} M; 2F, 7.8×10^{-8} M) (*SI Appendix, Fig. S1 A and B*). There were no responses to trypsin or 2F in cells expressing muGFP alone. Thus, PAR₂-muGFP signals similarly to PAR₂-HA11 in KNRK cells, suggesting that muGFP does not affect the normal functions of PAR₂.

To generate knockin mice, a targeting construct was generated comprising *Par₂-mugfp*, a floxed PGK neomycin cassette, and a downstream KpnI site for Southern screening (Fig. 1A). Five embryonic stem cell clones expressing the construct were identified by Southern blotting (1.25% incorporation); blotting at the 3' and 5' arms of homology confirmed correct targeting (Fig. 1B). Screening with an internal probe to the NEO cassette excluded random integration or insertion of a concatemer. The presence and the integrity of *loxP* sites and insertion of *Par₂-mugfp* were verified by sequencing. Two clones were injected into BALB/c mouse blastocysts. Chimeras (black and white) were generated from both clones and bred with wild-type C57BL/6J female mice to generate black F1 progeny, indicating germline transmission. Expression of *Par₂-mugfp* was confirmed by Southern blotting (Fig. 1C) and reverse-transcription polymerase chain reaction (RT-PCR) (Fig. 1D).

Characterization of *Par₂-mugfp* Mice. We compared the expression of *F2rl1*, the gene encoding for PAR₂, in *Par₂-mugfp* and wild-type mice using quantitative RT-PCR (qRT-PCR). *F2rl1* expression in the stomach, duodenum, ileum, colon, and dorsal root ganglia (DRG) was not different in *Par₂-mugfp* and wild-type mice (Fig. 1E). *F2rl1* was expressed at higher levels in the jejunum of *Par₂-mugfp* mice for unknown reasons. Thus, in most tissues, *F2rl1* is expressed at similar levels in *Par₂-mugfp* and wild-type mice.

Localization of PAR₂-muGFP Immunoreactivity and Messenger RNA.

We localized PAR₂-muGFP by indirect immunofluorescence and confocal microscopy using a GFP antibody to amplify the signal. This approach avoided use of GPCR antibodies, which can lack selectivity. In *Par₂-mugfp* mice, GFP immunoreactivity was prominently localized to the basolateral plasma membrane of epithelial cells of the villi and crypts in the duodenum, jejunum, ileum, and lining mucosal folds and crypts in the colon (Fig. 2A and B). There was no detectable GFP immunoreactivity in epithelial cells of the small or large intestine of wild-type mice, which confirms selectivity of the GFP antibody (Fig. 2A and B). PAR₂-muGFP immunoreactivity was detected at low levels in the enteric nervous system of the small and large intestines of *Par₂-mugfp* mice, but the cellular localization was not further studied.

We used RNAScope in situ hybridization with probes to *F2rl1* and *Gfp* to confirm PAR₂-muGFP localization in the colon. In *Par₂-mugfp* mice, *F2rl1* and *Gfp* were prominently detected in colonocytes (Fig. 2C). In wild-type mice, *F2rl1*, but not *Gfp*, was detected in colonocytes. RNAScope was used to detect PAR₂ expression with high sensitivity in DRG neurons. In *Par₂-mugfp* mice, *F2rl1*, and *Gfp* were coexpressed in a subpopulation of DRG neurons (Fig. 2D). *F2rl1*, but not *Gfp*, was expressed in DRG neurons of wild-type mice (Fig. 2E).

These results confirm the expression of PAR₂ in intestinal epithelial cells and a subpopulation of primary sensory neurons, and they validate the use of *Par₂-mugfp* mice for studies of the cellular localization of this receptor.

Agonist-Evoked Trafficking of PAR₂-muGFP in the Colon. Upon activation, many GPCRs, including PAR₂, undergo endocytosis (3, 7). GPCR trafficking is usually studied in cell lines overexpressing receptors. Little is known about agonist-evoked trafficking of endogenous GPCRs in intact tissues or organisms, largely due to the inadequate specificity or sensitivity of GPCR antibodies. To examine agonist-evoked trafficking of PAR₂ in tissues, isolated segments of colon from *Par₂-mugfp* mice were incubated with trypsin (140 nM), 2F (100 μM), or vehicle (60 min, 37°C). In vehicle-treated colon, PAR₂-muGFP was principally localized to the basolateral plasma membrane of colonocytes (Fig. 3A and B, arrowheads). Trypsin or 2F stimulated depletion of PAR₂-muGFP from the plasma membrane and accumulation in intracellular vesicles (Fig. 3A and B, arrows). Vesicles containing PAR₂-muGFP were costained with an antibody to early endosomal antigen 1 (EEA1) and are thus early endosomes (Fig. 3C and D; *SI Appendix, Fig. S2A*, arrows). Some vesicles were costained with an antibody to Rab7a and are late endosomes (*SI Appendix, Fig. S2B*). Quantification of endocytosis, assessed by the cytosol-plasma membrane pixel intensity, confirmed extensive endocytosis of PAR₂-muGFP (Fig. 3E and F). Thus, agonists evoke robust endocytosis of endogenous PAR₂ in the intact colon.

Inflammation-Evoked Trafficking of PAR₂-muGFP in the Colon. Little is known about the impact of disease on the subcellular distribution of GPCRs. The continued generation of agonists during chronic disease could induce the redistribution of receptors from the plasma membrane to endosomes. Continued GPCR

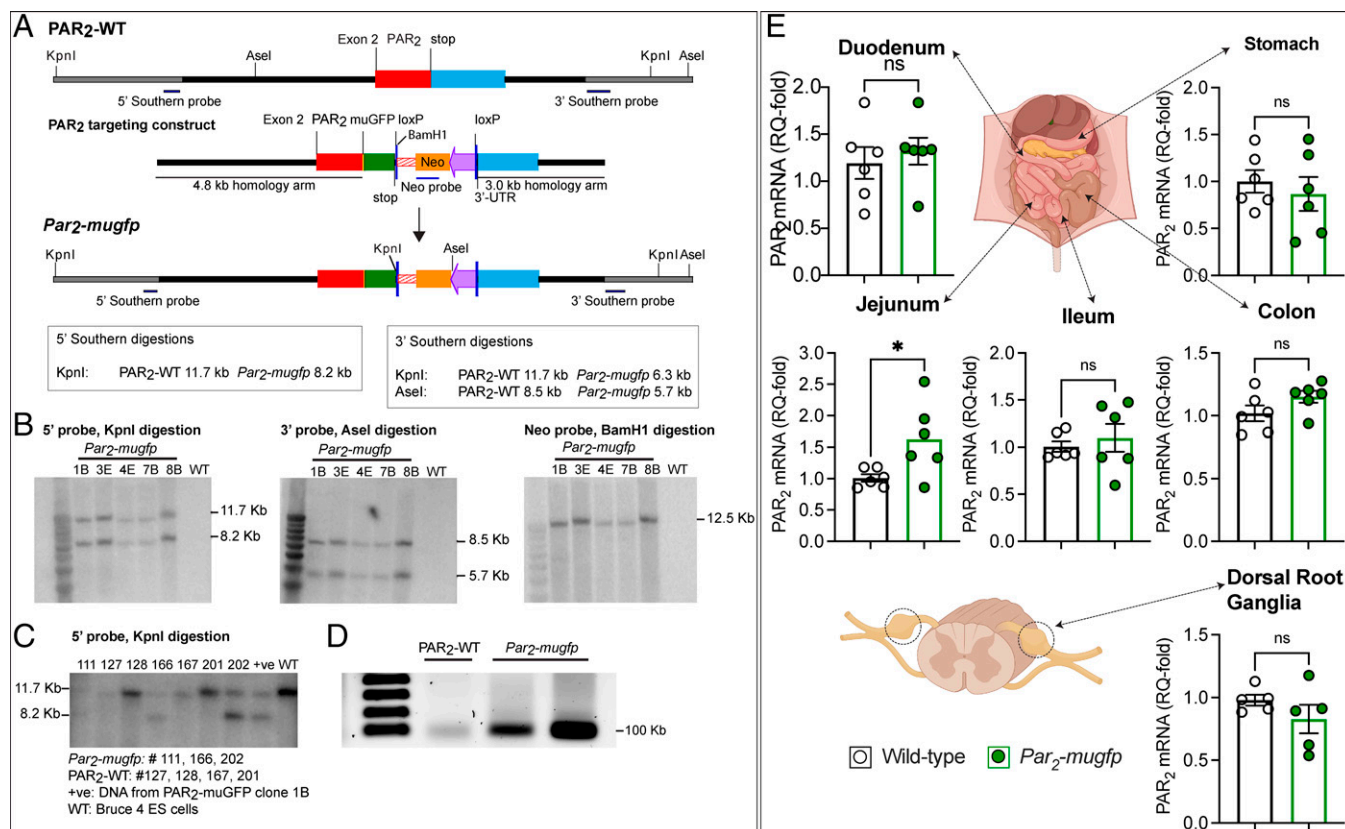


Fig. 1. Generation and characterization of *Par2-mugfp* mice. (A) *Par2-mugfp* targeting construct comprising a downstream phosphoglycerine kinase neomycin cassette flanked by *loxP* sites and a downstream *KpnI* site. (B) Southern blot confirming correct targeting. (C) Southern blot and (D) RT-PCR blot confirming *Par2-mugfp* expression in mice. WT, wild-type. (E) Expression of *F2r11* (*Par2*) mRNA in the digestive tract and DRG of *Par2-mugfp* and wild-type mice determined by qRT-PCR. *n* = 6 mice. Mean $\pm$ SEM; ns, nonsignificant. **P* < 0.05, Student's *t* test.

signaling from intracellular compartments (e.g., endosomes) underlies disease processes, including persistent pain (5–9). This has implications for therapy, because GPCRs in endosomes might be inaccessible to antagonists that fail to penetrate the plasma and endosomal membranes (e.g., monoclonal antibodies) or cannot effectively engage conformations of GPCRs within multiprotein signaling complexes in acidic endosomes (32). Thus, an understanding of disease-evoked trafficking of GPCRs is required for the design of optimal therapies. We investigated whether *PAR2* redistributes to endosomes of colonocytes during colitis, when multiple host and microbiome proteases are activated (20, 22, 23, 25).

Par2-mugfp mice were treated with 3% dextran sulfate sodium (DSS) in drinking water for 7 d to induce mucosal inflammation of the colon. Controls received plain water. DSS induced signs of colitis, including fecal blood, diarrhea, weight loss, and colon shortening (Fig. 4*A* and *B*). *PAR2*-muGFP was localized at the basolateral plasma membrane of colonocytes in control mice (Fig. 4*C*, arrowheads). At 24 h after DSS treatment, *PAR2*-muGFP was depleted from the plasma membrane and prominently detected in intracellular vesicles of colonocytes (Fig. 4*C*, arrows). *Par2-mugfp* mice were also treated with trinitrobenzene sulphonic acid (TNBS; 4 mg/mL in 0.9% NaCl and 50% ethanol, 150- μ L enema) to induce transmural inflammation of the colon. Control mice received vehicle (0.9% NaCl and 50% ethanol). After 72 h, TNBS-treated mice had extensive transmural inflammation, with mucosal and submucosal infiltration of neutrophils (Fig. 4*D* and *E*). *PAR2*-muGFP was prominently detected in intracellular vesicles (Fig. 4*F*, arrows) and also present at the plasma membrane (Fig. 4*F*, arrowheads) of colonocytes. In regions of marked inflammation and mucosal

disruption, *PAR2*-muGFP was detected in immune cells of the mucosa (Fig. 4*G*, arrowheads) and EEA1-positive early endosomes of colonocytes (Fig. 4*G*, arrows).

In two preclinical models of IBD, *PAR2*-muGFP redistributes from the plasma membrane to endosomes of colonocytes. This redistribution is likely attributable to the activation of proteases in the inflamed colon.

Mechanism and Pathway of *PAR2* Trafficking in Colonocytes. Activated *PAR2* undergoes clathrin-mediated endocytosis and traffics to early endosomes and lysosomes (13). *PAR2* ubiquitination is necessary for lysosomal targeting (33). Recovery of cellular responsiveness requires G $\beta\gamma$ -dependent activation of protein kinase D, which liberates newly synthesized *PAR2* from the Golgi apparatus and stimulates translocation to the plasma membrane (34, 35).

We used enhanced bystander bioluminescence resonance energy transfer (ebBRET), which capitalizes on the affinity of *Renilla*-tagged proteins, to study the intracellular trafficking of *PAR2* in T84 human colon carcinoma cells (26). To assess *PAR2* endocytosis, we coexpressed *PAR2* coupled to *Renilla* luciferase 8 (*Rluc8*) and the plasma membrane marker CAAX coupled to *Renilla* GFP (RGFP) or the early endosome marker Rab5a coupled to tandem (td) RGFP. 2F (100 μ M) decreased BRET (i.e., proximity) between *PAR2*-*Rluc8* and RGFP-CAAX and increased BRET between *PAR2*-*Rluc8* and tdRGFP-Rab5a, consistent with endocytosis (Fig. 5*B* and *C*). Hypertonic (0.45 M) sucrose, dominant-negative dynamin (Dnm) K44A, and Dnm2 siRNA inhibited endocytosis of *PAR2* (Fig. 5*D–G*). Dnm2 was the predominant Dnm isoform in T84 cells, which expressed Dnm1 and Dnm3 messenger RNA (mRNA) at lower

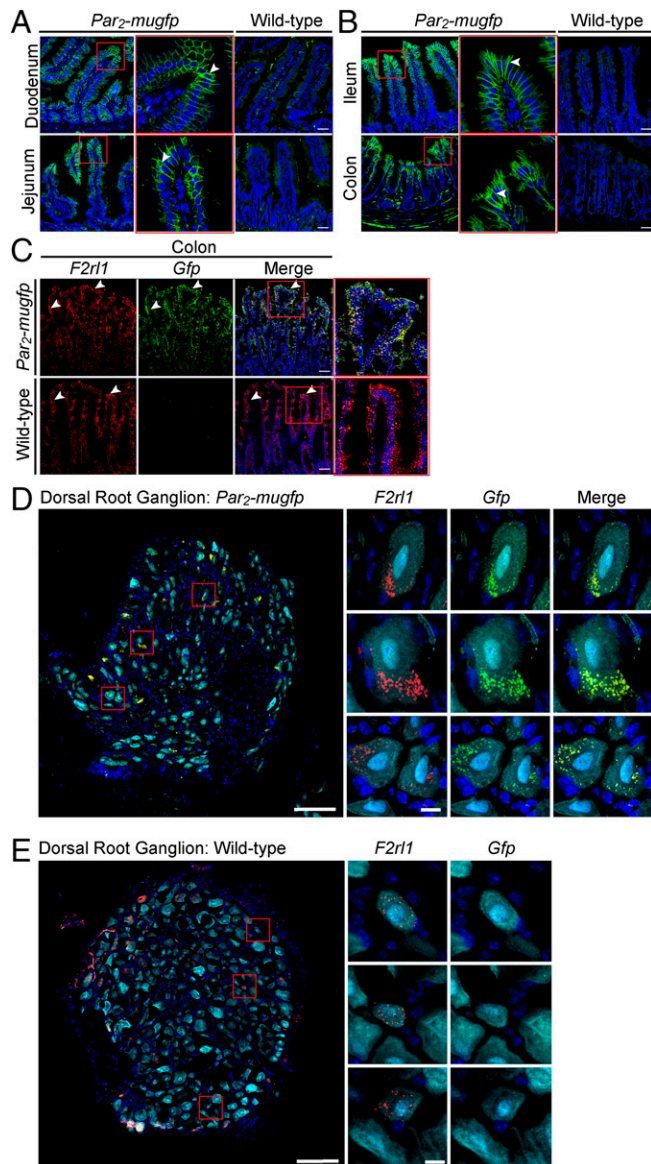


Fig. 2. Localization of PAR₂-muGFP in the intestine and DRG by immunofluorescence and RNAScope in situ hybridization. (A and B) Localization of GFP immunoreactivity in duodenum and jejunum (A) and ileum and colon (B) of *Par₂-mugfp* and wild-type mice. (C) RNAScope localization of *F2r11* and *Gfp* mRNA in colon of *Par₂-mugfp* and wild-type mice. Arrowheads indicate immunoreactivity at the basolateral membrane of epithelial cells and mRNA expression within epithelial cells. (D and E) RNAScope localization of *F2r11* and *Gfp* mRNA in DRG of *Par₂-mugfp* (D) and wild-type (E) mice. (Scale bars, 10 μ m.) Representative images, independent experiments. *n* = 5 mice.

levels (SI Appendix, Fig. S3A). siRNA knockdown of Dnm2 was verified by qRT-PCR (SI Appendix, Fig. S3B).

To study the intracellular trafficking of PAR₂, we expressed BRET1 sensors for PAR₂ and subcellular compartments in HEK293T cells (Fig. 5A), where BRET1 responses were larger. 2F (10 μ M) and trypsin (100 nM) decreased BRET between PAR₂-Rluc8 and Venus-KRas (plasma membrane) and increased BRET between PAR₂-Rluc8 and Venus-Rab5a (early endosome) (Fig. 5 H–K; SI Appendix, Fig. S4 A–D). BRET between PAR₂-Rluc8 and Venus-Rab7a (late endosome) was unaffected (Fig. 5 L and M; SI Appendix, Fig. S4 E and F). 2F increased BRET between PAR₂-Rluc8 and Venus-Giantin (*cis*-Golgi) and caused a transient decrease and then a sustained increase in BRET between PAR₂-Rluc8 and YFP-TGN-38

(*trans*-Golgi) (Fig. 5 N–Q). Trypsin did not affect BRET between PAR₂-Rluc8 and Venus-Giantin but caused a transient decrease in BRET between PAR₂-Rluc8 and YFP-TGN-38 (SI Appendix, Fig. S4 G–J). 2F and trypsin stimulated BRET between PAR₂-Rluc8 and Venus-Rab11a (recycling endosomes) (Fig. 5 R and S; SI Appendix, Fig. S4 K and L). DnmK44A inhibited the recruitment of PAR₂ to most compartments.

To determine whether fusion to muGFP affects PAR₂ trafficking, we expressed PAR₂-muGFP in HEK293T cells expressing markers of early and late endosomes and the Golgi apparatus tagged with red fluorescent protein (RFP) and localized PAR₂ and markers by confocal microscopy. 2F (10 μ M, 30 min) stimulated trafficking of PAR₂-muGFP from the plasma membrane to early endosomes (Fig. 5T; SI Appendix, Fig. S5). PAR₂-muGFP was not detected in late endosomes or the Golgi apparatus by microscopy (Fig. 5U; SI Appendix, Fig. S6).

Assembly of a PAR₂ Signaling Complex with G α or β ARR. GPCRs can assemble G α and β ARR signaling complexes in endosomes, which enable sustained signaling of internalized receptors (3, 4, 36, 37). Within endosomes, G α subunits can transduce signals and β ARRs serve as scaffolds that organize GPCRs and signaling enzymes. Because agonists stimulated trafficking of PAR₂ to early endosomes of T84 and HEK293T cells, we examined the assembly of signaling complexes in this compartment. The activation of G α_q , G α_i , and G α_s was studied using ebBRET to detect recruitment of mini-G α (mG α) coupled to Rluc8 to RGFP-CAAX or tdRGFP-Rab5a (Fig. 6A). mG α proteins are N-terminally truncated G α proteins that freely diffuse within the cytosol and bind to active conformations of GPCRs, which reflects G α activation. mG α_{sq} and mG α_{si} were developed by mutating mG α_s residues to equivalent G α_q and G α_i residues. Recruitment of β ARR was assessed by measuring ebBRET between Rluc2- β ARR2 and RGFP-CAAX or tdRGFP-Rab5a (Fig. 6A). In T84 cells, 2F (100 μ M) stimulated the rapid recruitment of mG α_{sq} , mG α_{si} , or β ARR2 to the plasma membrane and endosomes, although β ARR2 recruitment to endosomes was slower and more sustained than recruitment to the plasma membrane (Fig. 6 B–D). mG α_s was not recruited to the plasma membrane or endosomes.

We studied the mechanisms of assembly of signaling complexes in HEK293T cells. 2F (100 μ M) and trypsin (100 nM) stimulated the recruitment of mG α_{sq} , mG α_{si} , and β ARR2 to the plasma membrane and endosomes of HEK293T cells (SI Appendix, Fig. S7 A–F). Whereas the recruitment of mG α_{sq} , mG α_{si} and β ARR2 at the plasma membrane declined over 1,400 s, their recruitment to endosomes increased or was sustained. Hypertonic sucrose delayed the recruitment of mG α_{sq} , mG α_{si} , and β ARR2 to the plasma membrane, although ebBRET signals ultimately reached those of controls. In contrast, hypertonic sucrose abolished recruitment of mG α_{sq} , mG α_{si} , and β ARR2 to endosomes.

Conventional BRET is limited to measuring the proximity between two proteins. To simultaneously measure the proximity between PAR₂, an effector (mG α or β ARR), and a localization marker, we used NanoLuc Binary Technology (38), NanoBiT BRET (nbBRET) (Fig. 6E). A split luciferase assay was developed in which PAR₂ was tagged at the C terminus with the natural peptide fragment of NanoLuc (NP, a 13-residue NanoLuc fragment), and a plasma membrane or endosomal marker was tagged with the large BiT fragment of NanoLuc (LgBiT-CAAX or LgBiT-FYVE, respectively). Luminescence occurs from a complex between PAR₂-NP and LgBiT-CAAX or LgBiT-FYVE, which serves as an energy donor for fluorophore-tagged mG α or β ARR. In T84 colonocytes, 2F (100 μ M) increased nbBRET between PAR₂, mG α_{si} , mG α_{sq} , or β ARR1 and markers of the plasma membrane or endosome (Fig. 6 F–H). In HEK293T cells, 2F and trypsin also stimulated nbBRET

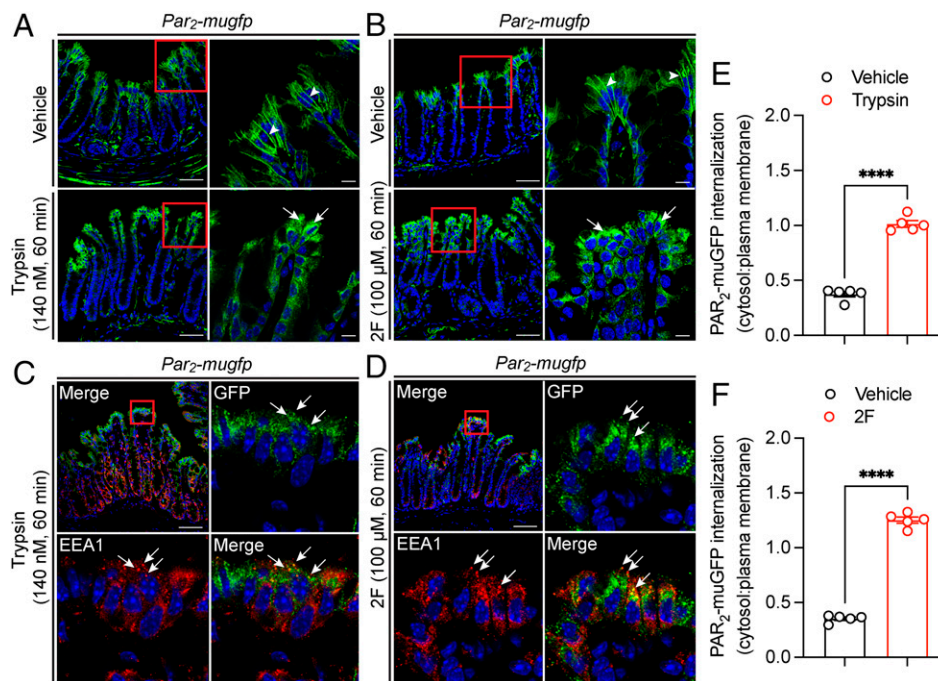


Fig. 3. Agonist-evoked endocytosis of PAR₂-muGFP in the colon. (A and B) Localization of GFP immunoreactivity in isolated segments of colon from *Par2-mugfp* mice incubated with vehicle, trypsin (A, 140 nM, 60 min) or 2F (B, 100 μM, 60 min). (C and D) Colocalization of GFP and EEA1 in segments treated with trypsin (C) or 2F (D). Arrowheads denote plasma membrane, and arrows denote endosomes. (Scale bar, 10 μm.) Representative images and independent experiments are shown ($n = 5$ mice). (E and F) Quantification of PAR₂-muGFP internalization after vehicle, trypsin (E), or 2F (F) (cytosol–plasma membrane pixel intensity). Mean $\pm$ SEM; $n = 5$ mice. **** $P < 0.0001$, Student's t test.

between PAR₂, mGα_{si}, mGα_{sq}, or βARR1 and CAAX or FYVE (Fig. 6 I–K; *SI Appendix, Fig. S8 A–H*). Recruitment of mGα and βARR to early endosomes lagged behind recruitment to the plasma membrane. nbBRET signals were maintained for at least 1,200 s, suggesting assembly of stable PAR₂/mGα or PAR₂/βARR complexes. Hypertonic sucrose inhibited agonist-stimulated assembly of PAR₂, mGα_{si}, mGα_{sq}, or βARR1 complexes in early endosomes (*SI Appendix, Fig. S8 A, B, F, and G*). DynK44A had similar inhibitory effects on signalosome assembly but to a lesser extent (*SI Appendix, Fig. S8 C, D, G, and H*). The PAR₂ antagonist GB88 prevented 2F and trypsin-stimulated signalosome assembly in endosomes of HEK293T cells (*SI Appendix, Fig. S8 I and J*). Immunofluorescence and confocal microscopy confirmed localization of HA-LgBiT-CAAX at the plasma membrane and HA-LgBiT-FYVE in endosomes (*SI Appendix, Fig. S8K*).

In unstimulated HEK293T cells, immunoreactive PAR₂ was localized to the plasma membrane, and immunoreactive Gα_q and YFP-βARR1/2 were cytosolic (Fig. 6L). In cells exposed to 2F (10 μM, 30 min), PAR₂, Gα_q, and YFP-βARR1/2 were colocalized in early endosomes.

Our results suggest that agonists induce assembly of a signal complex comprising PAR₂ and Gα_q, Gα_i, or βARR1/2 in early endosomes. This process requires Dnm-dependent endocytosis of PAR₂.

PAR₂ Endocytosis and the Colonic Epithelial Barrier. Tight junctions between colonocytes form a barrier that prevents the ingress of proinflammatory macromolecules and microbes from the lumen. Alterations in tight junction structure contribute to impaired barrier function and inflammation in IBD. PAR₂ agonists promote paracellular permeability of the colon (39) by a mechanism that involves βARR-dependent activation of ERK1/2 and redistribution of ZO1 and occludin from tight junctions (3, 26), suggesting a role for endosomal PAR₂ signaling.

Analysis of the expression of Dnm isoforms in the mouse colon by RNAScope in situ hybridization and RT-qPCR revealed prominent expression of *Dnm2* in colonocytes, whereas *Dnm1* and *Dnm3* mRNA were largely confined to the enteric nervous system (Fig. 7 A and B). When applied to the basolateral surface of T84 monolayers, trypsin (100 nM) and 2F (10 μM) decreased transepithelial electrical resistance (TEER) after 6 h, consistent with redistribution of tight junctional proteins and increased paracellular permeability (Fig. 7C). The PAR₂ antagonist GB88 prevented these effects, confirming a role for PAR₂. Dnm2 siRNA similarly prevented the effects of trypsin and 2F on TEER, implicating a requirement for PAR₂ endocytosis (Fig. 7 D and E).

PAR₂ Endocytosis, Colonic Inflammation, and Pain. To ascertain whether Dnm2 could contribute to colonic inflammation in vivo, we administered Dnm2 or control small hairpin RNA (shRNA) into the colon of *Par2-mugfp* mice. After 48 h, RNAScope in situ hybridization revealed ~50% knockdown of Dnm2 mRNA in colonocytes of mice treated with Dnm shRNA compared to control shRNA (Fig. 8 A and B). To assess the effects of Dnm2 knockdown on PAR₂-mediated colonic inflammation and pain, we administered 2F (100 μM) or vehicle into the colon by enema (150 μL). Endocytosis of PAR₂-muGFP, colonic levels of proinflammatory factors, and colonic nociception were assessed. In mice receiving control shRNA, 2F stimulated endocytosis of PAR₂-muGFP in colonocytes after 6 h; Dnm2 shRNA inhibited endocytosis (Fig. 8C). In control mice, 2F increased mRNA levels of tumor necrosis factor α (TNF-α), C-X-C motif chemokine ligand 1 (CXCL1), and interleukin-1β (IL-1β) in the colon (Fig. 8 D–F). Intracolonic 2F decreased withdrawal responses of the abdomen to calibrated von Frey filaments from 1 to 5 h, which indicates hyperalgesia (Fig. 8 G and H). These results are consistent with the known proinflammatory and pronociceptive actions of PAR₂ in the colon (7, 22, 30). Dnm2 shRNA inhibited

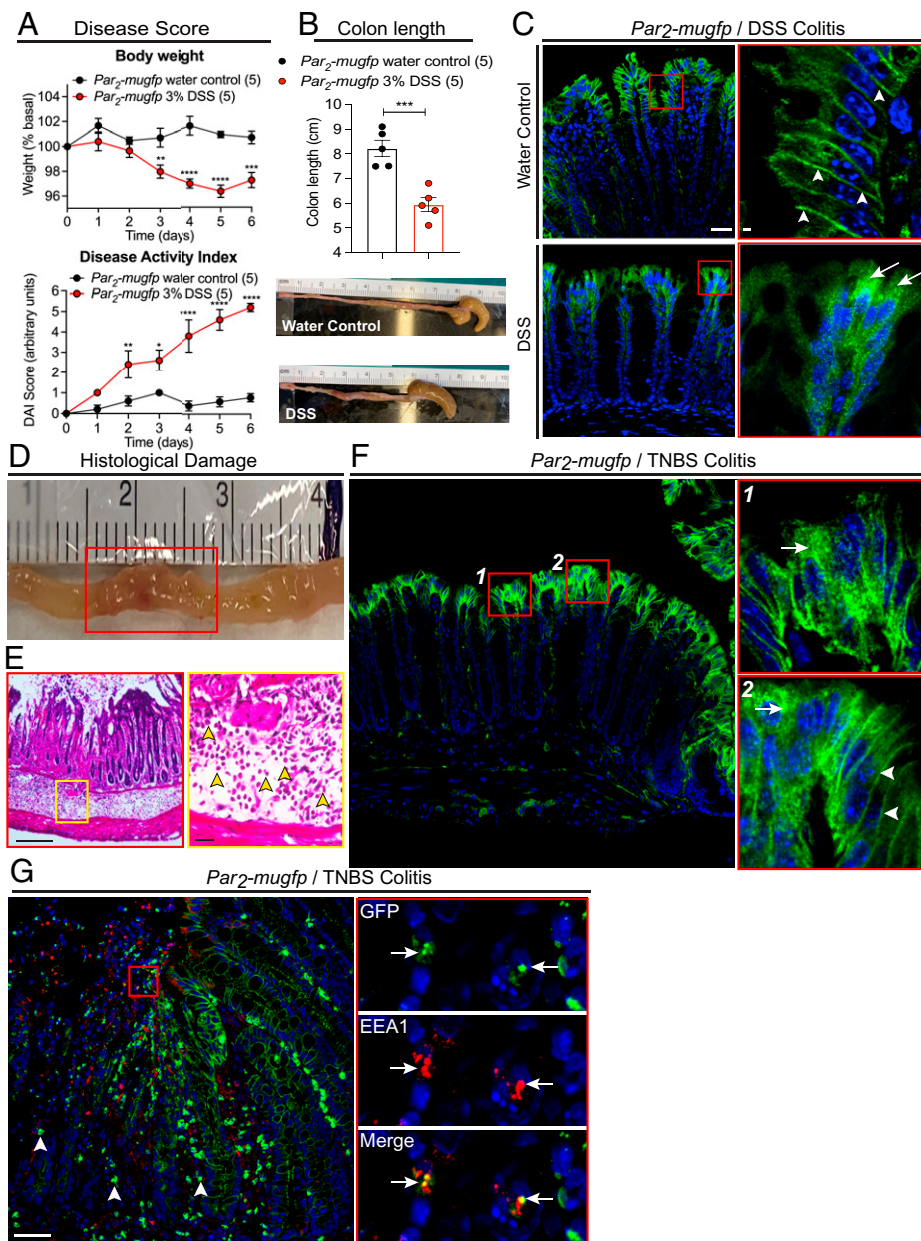


Fig. 4. Colitis-evoked endocytosis of PAR₂-muGFP. (A–C) DSS colitis in *Par₂-mugfp* mice. (A) Body weight and disease activity index (DAI) after DSS or water (control). (B) Colon length after DSS or water. Mean ± SEM, *n* = 5 mice. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 compared to water, two-way ANOVA, Tukey's test (A), and Student's *t* test (B). (C) Redistribution of PAR₂-muGFP from the plasma membrane (arrowheads) to endosomes (arrows) after DSS. (D–F) TNBS colitis in *Par₂-mugfp* mice. (D) Grossly inflamed colon. (E) Histological sections showing infiltration of neutrophils in the submucosa (yellow arrowheads). (F) Redistribution of PAR₂-muGFP from the plasma membrane to endosomes after TNBS. (G) Localization of PAR₂-muGFP in infiltrating immune cells (arrowheads) and colocalization of GFP and EEA1 in colonocytes (arrows). (Scale bars, 10 μm.) Representative images and independent experiments are shown (*n* = 5 mice).

2F-stimulated inflammation and nociception. The pronociceptive response to intracolonic 2F was similar in *Par₂-mugfp* and wild-type mice but was attenuated in *Par₂Na_v1.8* mice that lack PAR₂ in Na_v1.8-positive nociceptors (7) (Fig. 8 *I* and *J*). These results implicate PAR₂ on Na_v1.8-positive neurons in colonic pain and are in line with our report that proteases induce somatic nociception by activating PAR₂ on Na_v1.8 neurons (7). The intraplantar injection of trypsin (140 nM, 10 μL) induced mechanical allodynia to a similar degree in the ipsilateral paw but not contralateral paw of *Par₂-mugfp* and wild-type mice (SI Appendix, Fig. S9 *A* and *B*). Mechanical allodynia was significantly attenuated in *Par₂^{-/-}* mice, confirming a role for PAR₂.

IL-8 is proinflammatory cytokine that is released by colonocytes (40). To examine whether PAR₂ evokes inflammation of the human colon, we incubated explants of normal human colonic mucosa with 2F (10 μM, 4 h) and measured IL-8 release into culture medium. 2F stimulated IL-8 release (Fig. 8*K*). Preincubation of explants with dyngo4a (30 μM), a Dnm inhibitor, blocked PAR₂-mediated IL-8 release.

These results suggest that PAR₂ agonists increase paracellular permeability, stimulate cytokine and chemokine release from colonocytes, and sensitize colonic nociceptors. These proinflammatory and pronociceptive actions require Dnm-mediated endocytosis of PAR₂.

Discussion

Although proteases and PAR₂ are implicated in inflammatory diseases, the impact of inflammation on the subcellular distribution and function of PAR₂ has not been investigated. The use of knockin mice expressing PAR₂ fused to muGFP allowed examination of the effects of inflammation on the subcellular trafficking of PAR₂ with high resolution. We detected a major redistribution of PAR₂ from the basolateral membrane to early endosomes of colonocytes in two preclinical models of IBD, which is likely due to increased proteolytic activity in the inflamed colon. By using BRET to probe the formation and composition of PAR₂ signaling complexes, we found that agonists induce the assembly of signalosome comprising PAR₂ and Gα_i, Gα_q or βARR1/2 in colonic epithelial cells. Our results suggest that Dnm2-mediated endocytosis of PAR₂ in the colon is necessary for paracellular permeability, generation of proinflammatory cytokines and chemokines, and sensitization of colonic nociceptors (Fig. 7E).

PAR₂ Localization and Trafficking. Mice expressing fluorescent opioid receptors have been extensively used to analyze tissue distribution and the effects of endogenous agonists and drugs on receptor trafficking (41, 42). The use of knockin mice in which *Par₂* was replaced by *Par₂-mugfp* enabled specific and high-resolution analysis of the tissue distribution and subcellular trafficking of PAR₂ in inflamed tissues. Fusion of muGFP did not affect PAR₂ signaling and trafficking in cell lines, and mRNA encoding *Par₂-mugfp* was expressed at similar levels to endogenous *Par₂* in most regions of the digestive tract and sensory ganglia. PAR₂ agonists induced comparable somatic and visceral nociception in *Par₂-mugfp* and wild-type mice. These findings suggest that PAR₂-muGFP functions normally. Within the digestive tract, PAR₂-muGFP immunoreactivity was prominently localized to the basolateral plasma membrane of epithelial cells lining the small and large intestine. RNAScope using probes to *F2rl1* and *Gfp* confirmed expression by enterocytes and colonocytes. The lack of GFP immunoreactivity and mRNA in wild-type mice verified selectivity. The detection of PAR₂ in intestinal epithelial cells confirms reports using receptor antibodies and functional assays (27, 43).

Detection of PAR₂ in DRG neurons required the sensitivity of RNAScope in situ hybridization, which revealed expression in a subset of neurons, in agreement with other reports (7, 44). The activation of PAR₂ in DRG nociceptors stimulates the release of substance P and calcitonin gene-related peptide, which mediate neurogenic inflammation in peripheral tissues and the central transmission of pain (28, 29). Proteases may cause pain in the colon directly by activating PAR₂ on nociceptors or indirectly by activating PAR₂ on colonocytes and stimulating release of pronociceptive factors. The observation that deletion of PAR₂ from Na_v1.8 neurons blunts colonic nociception supports a primary role for neuronal PAR₂. Deletion of *Par₂* from Na_v1.8 neurons also attenuates protease-evoked somatic nociception (7, 44).

The identity of the proteases that activate PAR₂ and stimulate endocytosis was not addressed in the current study. Protease probes and proteomics have been used to identify serine and cysteine proteases that are activated in the colon of mice with colitis and patients with IBD (20–25). These include proteases from immune cells [e.g., neutrophil elastase and cathepsin S (20–22)], proteases from colonocytes [e.g., trypsins (45)], and proteases from the microbiome. An understanding of whether PAR₂ is principally expressed at the apical or basolateral membrane of intestinal epithelial cells has implications for mechanisms of activation. Luminal proteases from epithelial cells, exocrine glands, or colonic bacteria could activate apical PAR₂, whereas proteases from mucosal immune or epithelial cells could activate PAR₂ at the basolateral membrane. Our

finding of predominant basolateral expression is consistent with reports that basolateral, but not apical, application of PAR₂ agonists increases paracellular permeability (43), although PAR₂ has also been detected at the apical membrane (27).

PAR₂ Signaling Complexes. We studied the intracellular trafficking of PAR₂ in colon-derived epithelial cells (T84) and model cell lines (HEK293T) by using BRET to assess the proximity of PAR₂ to subcellular markers and confocal microscopy to localize PAR₂. Our results show that trypsin and 2F deplete PAR₂ from the plasma membrane and induce accumulation in early endosomes, which were identified using several markers (Rab5a, EEA1, FYVE, and CellLights RFP). Dominant-negative DnmK44A, knockdown of Dnm2 (the principal isoform of T84 cells and colonocytes), and hypertonic sucrose blunted endocytosis of PAR₂, suggesting a major role for Dnm2. We did not detect PAR₂ in Rab7a-positive late endosomes, possibly due to receptor degradation. 2F, but not trypsin, increased the BRET signal for PAR₂ in the *cis*-Golgi and stimulated a transient decrease and then a sustained increase in the BRET signal in the *trans*-Golgi. The differential effects of 2F and trypsin likely relate to their different mechanisms of PAR₂ activation. Agonist-evoked depletion of PAR₂ from the *trans*-Golgi is consistent with our studies showing that proteases induce Gβγ- and protein kinase D-dependent mobilization of PAR₂ from the Golgi apparatus to the plasma membrane (34, 35). Agonist-evoked translocation of PAR₂ from the plasma membrane to the Golgi apparatus was not prominent, because we detected PAR₂-muGFP in early endosomes, but not the Golgi apparatus.

Several observations support the hypothesis that PAR₂ agonists induce assembly of a PAR₂ signaling complex with Gα or βARR in early endosomes of T84 and HEK293T cells. eBRET revealed that 2F and trypsin stimulated the recruitment of Gα_q, Gα_i, and βARR2 to early endosomes. NbBRET, a split luciferase BRET assay that allows simultaneous measurements of proximity between PAR₂, an effector (Gα, βARR), and an early endosomal marker, confirmed agonist-evoked recruitment of receptor and effector to early endosomes. Dominant-negative DnmK44A, Dnm2 siRNA, and hypertonic sucrose inhibited the recruitment of PAR₂, Gα_q, Gα_i, and βARR1/2 to endosomes. We also detected PAR₂, full-length Gα_q, and βARR in endosomes by microscopy. Further experiments are needed to determine whether a PAR₂, Gα, and βARR megaplex forms in endosomes. It remains to be determined whether the complex traffics from the plasma membrane to endosomes or PAR₂ recruits Gα and βARR to endosomes. Because GPCRs can form megaplexes with Gα and βARR, the former possibility is more likely. A limitation of our work is that we did not investigate the signals that emanate from internalized PAR₂. We have previously reported that PAR₂ in endosomes regulates kinases (MEKK1 and ERK1/2) in certain subcellular compartments (3, 7). The endosomal complex was stable during the period of measurement (1,200 s), which might give rise to sustained signals in subcellular compartments that control critical functions of colonocytes, including tight junction assembly and release or proinflammatory and pronociceptive factors. Our results do not exclude the possibility that PAR₂ signals from other organelles, such as the Golgi apparatus, as is the case for thyroid-stimulating hormone receptors and opioid receptors (10, 11). Proteases of late endosomes, including cathepsin S and legumain, can activate PAR₂ even in acidic conditions, which could facilitate receptor activation in other subcellular compartments (17, 22). However, the most parsimonious explanation of our results is that signalosomes comprising PAR₂ and Gα_q, Gα_i, or βARR1/2 assemble predominantly in early endosomes.

Endosomal PAR₂ Signaling, Colonic Inflammation, and Pain. Our results suggest that endosomal signaling of PAR₂ in the colon increases paracellular permeability and evokes secretion of cytokines and chemokines, which would be expected to amplify

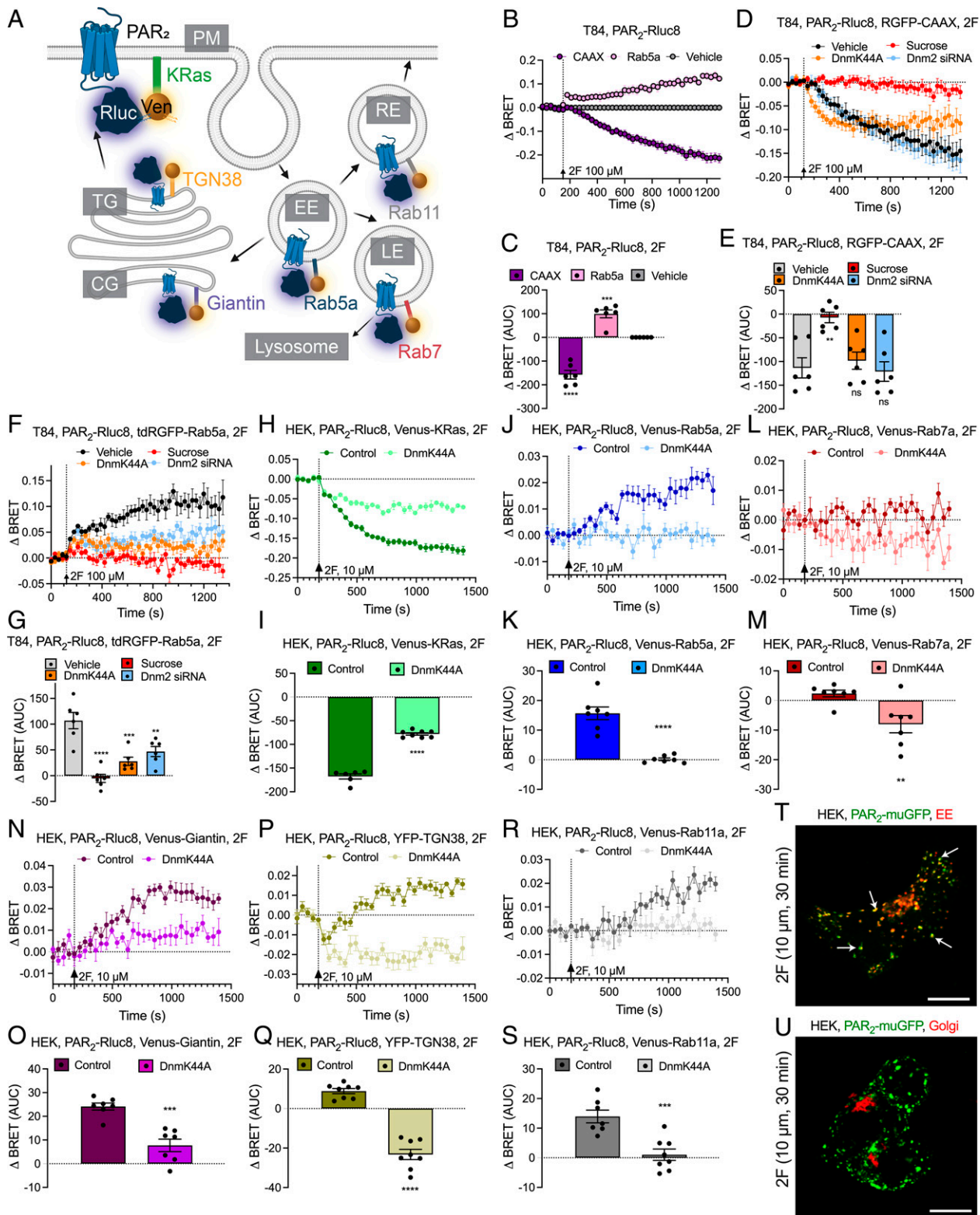


Fig. 5. PAR₂ trafficking to endosomes. (A) BRET assays of the proximity between PAR₂-Rluc8 and Venus- or YFP-tagged proteins resident to plasma membrane (PM; CAAX or K_{Ras}), early endosomes (EE; Rab5a), late endosomes (LE; Rab7a), recycling endosomes (RE; Rab11a), *cis*-Golgi (CG; giantin), or *trans*-Golgi (TG; TGN38). (B–G) Effects of 2F (100 μ M) on translocation of PAR₂-Rluc8 from the plasma membrane (RGFP-CAAX) to early endosomes (tdRGFP-Rab5a) of T84 cells treated with vehicle, hypertonic sucrose, or DnmK44A or Dnm2 siRNA. (H–S) Effects of 2F (10 μ M) on translocation of PAR₂-Rluc8 from the plasma membrane [K_{Ras}-Venus (H and I)] to early endosomes [Rab5a-Venus (J and K)], late endosomes [Rab7a-Venus (L and M)], *cis*-Golgi [giantin-Venus (N and O)], *trans*-Golgi [TGN38-YFP (P and Q)], and recycling endosomes [Rab11-Venus (R and S)] in HEK293T cells. Cells were transfected with DnmK44A or untransfected (control). AUC, area under the curve. Mean $\pm$ SEM; $n = 6$ –11 independent experiments. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$, one-way ANOVA or Tukey's test. (T and U) Localization of PAR₂-muGFP and CellLight markers of early endosomes (T) and the Golgi complex (U) in HEK293T cells. Arrows denote colocalization of PAR₂-muGFP and marker of early endosomes. Representative images of $n = 5$ experiments are shown. (Scale bars, 10 μ m.)

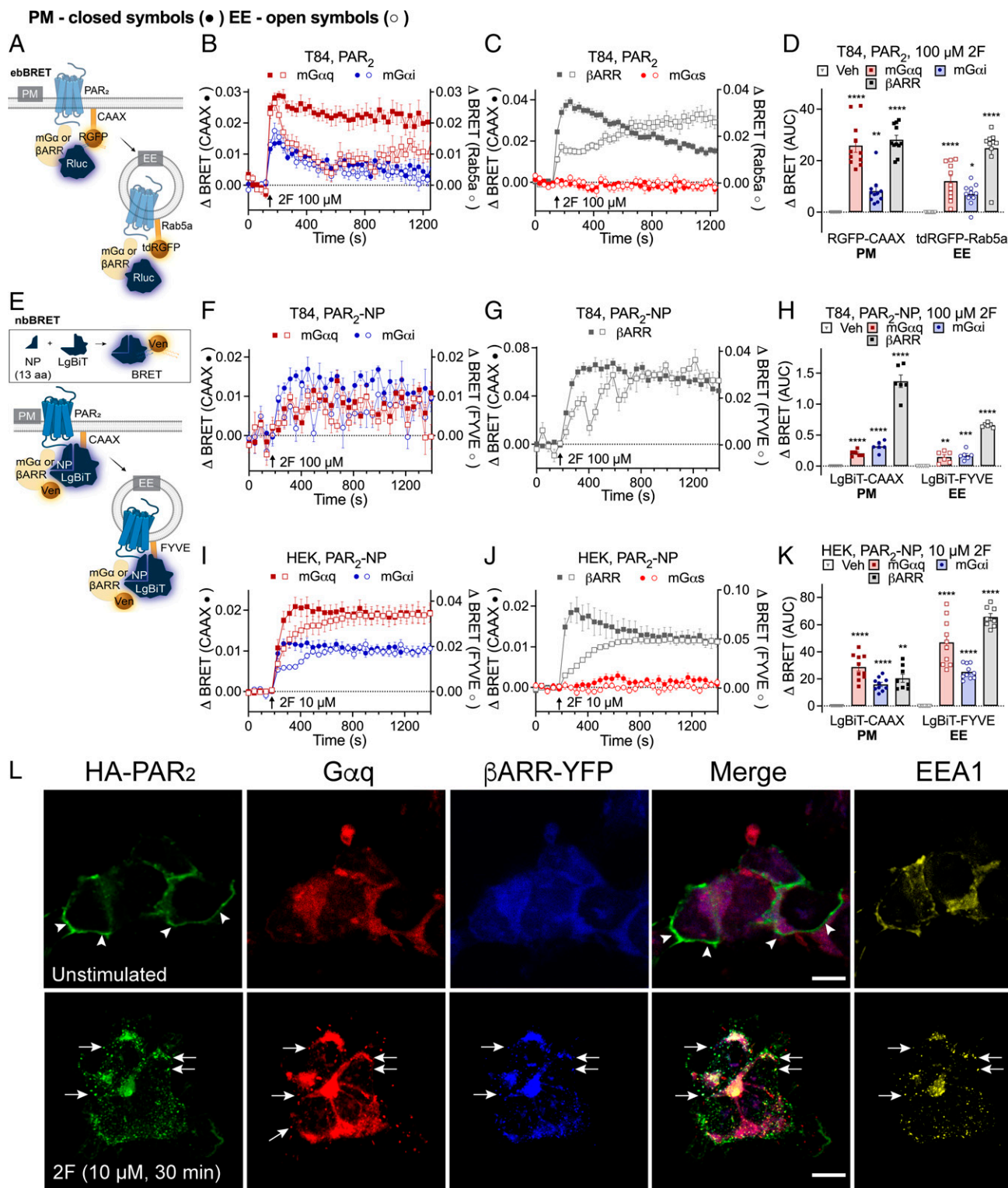


Fig. 6. PAR₂, Gα and βARR signalosome assembly in endosomes. (A) EbBRET assays of proximity between effector (Gα; βARR) and proteins resident to plasma membrane (PM; CAAX) or early endosomes (EE; Rab5a). (B–D) Effects of 2F (100 μM) on the recruitment of mGα_{sq}, mGα_{si}, (B) βARR2 or mGα_s negative control (C) to the plasma membrane (RGFP-CAAX, left axis, closed symbols) or early endosomes (tdRGFP-Rab5a, right axis, open symbols) of T84 cells, and (D) area under the curve (AUC). (E) NbBRET uses luciferase split into two fragments to detect BRET between receptor and effector (PAR₂ and mGα or βARR) for proteins resident to the PM (CAAX) or early endosomes (FYVE). (F–H) Effects of 2F (100 μM) on recruitment of mGα_{sq}, mGα_{si} (F) or βARR1 (G) to the plasma membrane (LgBiT-CAAX) or early endosomes (LgBiT-FYVE) of T84 cells, and (H) AUC. (I–K) Effects of 2F (10 μM) on recruitment of mGα_{sq}, mGα_{si} (I), βARR1 or mGα_s (J) to the PM (LgBiT-CAAX) or early endosomes (LgBiT-FYVE) of HEK293T cells. (K) AUC. Mean ± SEM. *n* = 6–11 independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. (D and K) One-way ANOVA with Dunnett's (D) or Holm–Šidák's (K) test compared to vehicle. (H) Unpaired *t* test. (L) Localization of immunoreactive HA-PAR₂, Gα_q and EEA1 plus YFP-βARR1 and YFP-βARR2 in HEK293T cells. Cells were unstimulated or incubated with 2F (10 μM, 30 min). Arrowheads, plasma membrane. Arrows, colocalization of HA-PAR₂, Gα_q and YFP-βARR1 + 2 in EEA1-positive early endosomes. (Scale bars, 10 μm.) Representative images from *n* = 5 experiments are shown.

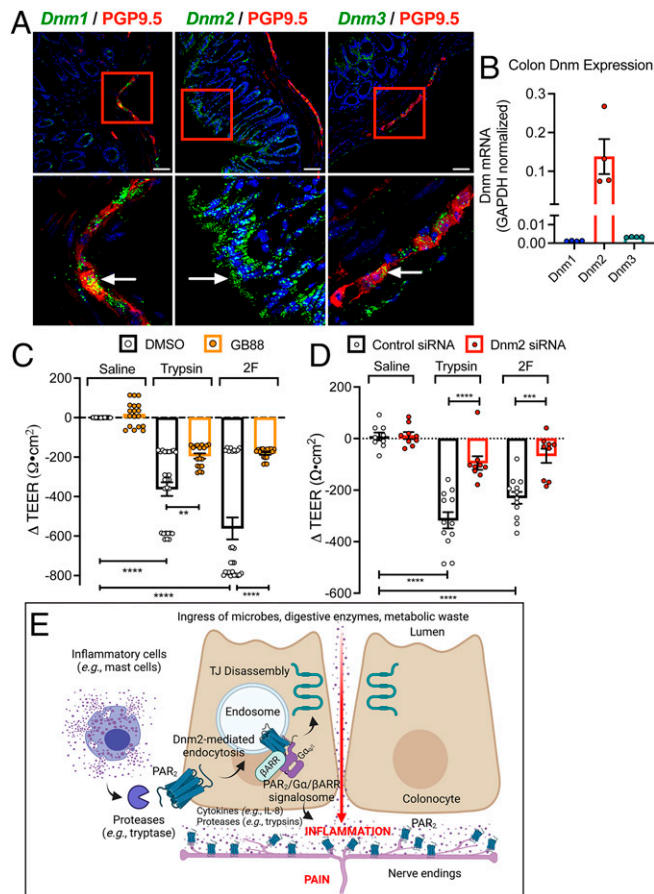


Fig. 7. PAR₂ endosomal signaling and colonic inflammation and pain. (A) Localization of *Dnm1-3* in mouse colon by RNAScope in situ hybridization. (Scale bars, 50 μm.) Representative images, independent experiments, *n* = 3 mice. (B) Expression of *Dnm1-3* in mouse colon by qRT-PCR. *n* = 4 mice. (C) Effects of trypsin (100 nM), 2F (10 μM), or vehicle (saline, control) on TEER of T84 cells after 6 h. Cells were preincubated with GB88 (PAR₂ antagonist) or vehicle (DMSO, control). (D) Effects of trypsin (100 nM), 2F (10 μM), or vehicle (saline, control) on TEER of T84 cells after 5 h. Cells were preincubated with *Dnm2* or control siRNA. Mean ± SEM. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 by one-way ANOVA, Tukey's test, or Student's *t* test. (E) Proposed mechanism by which proteases (e.g., trypsin) from mucosal inflammatory cells (e.g., mast cells) activate PAR₂ at the basolateral membrane of colonocytes to evoke PAR₂ endocytosis and assembly of a PAR₂, Gα, and βARR signalosome in endosomes. Endosomal signaling causes disassembly of tight junctions (TJ) and release of proinflammatory cytokines (e.g., IL-8) and possibly proteases (e.g., trypsin) from colonocytes. The ingress of microbes, proteases, and metabolites from the colonic lumen, and the release of cytokines and proteases from colonocytes, cause inflammation and pain. Proteases activate PAR₂ on nociceptive terminals in the colon to cause pain.

inflammation. In two preclinical mouse models of IBD, PAR₂ was depleted from the basolateral plasma membrane of colonocytes and prominently detected in cytosolic vesicles, including early endosomes. The proteolytic environment of the inflamed intestine is likely to activate PAR₂ and trigger endocytosis and sustained endosomal signaling. In health, tight junctions between colonocytes maintain a barrier that prevents the ingress of macromolecules and microbes from the colonic lumen into the mucosa, which protects against inflammation. Disruption of the barrier would be expected to promote inflammation. When applied to the basolateral surface, trypsin and 2F decreased electrical resistance of polarized monolayers of T84 cells, consistent with a loss of barrier function. The finding that a PAR₂ antagonist and *Dnm2* siRNA inhibited this effect suggests a major role for PAR₂ endocytosis and endosomal signaling in redistribution of tight junctional

proteins and loss of barrier function. We previously reported that PAR₂ agonists, including mast cell tryptase, stimulate a redistribution of ZO1 and occludin from tight junctions of T84 cells by a mechanism that involves βARRs, MEKK1, and ERK1/2 (26), in line with a role for endosomal signaling of PAR₂ in this process. The observations that *Dnm2* knockdown inhibited agonist-induced endocytosis of PAR₂-muGFP in colonocytes and prevented release or proinflammatory cytokines and chemokines *in vivo*, and that a *Dnm* inhibitor blocked PAR₂-stimulated secretion of IL-8 from biopsy specimens of human colon, further support a role for PAR₂ endocytosis in colitis. *Dnm2* knockdown also abrogated 2F-evoked colonic hyperalgesia, a finding that could be secondary to reduced release from colonocytes of cytokines or proteases that sensitize nociceptors. Because deletion of PAR₂ from Na_v1.8-positive neurons blunted 2F-evoked colonic hyperalgesia, the latter is likely. The identity of colonocyte-derived proteases remains to be determined, although colonocytes express isoforms of trypsin that activate PAR₂ (45). Further studies are required to ascertain whether endocytosis is necessary for other actions of PAR₂ in the intestine, such as inflammation associated with bacterial proteolysis and food allergies (46).

Our findings support the hypothesis that proteases in the inflamed colon evoke the assembly of a PAR₂ complex with Gα or βARR in endosomes (Fig. 7E). Signals that emanate from this complex disrupt tight junctions and promote release of proinflammatory factors that drive inflammation and pain. The influx of proteases from the colonic lumen could further amplify PAR₂-mediated inflammation and pain. Therapeutic targeting of PAR₂ in endosomes, which might be achieved using lipidated antagonists (7) or endosomally targeted nanoparticles (8), could restrict endosomal signaling and ameliorate IBD and other inflammatory and painful diseases associated with enhanced proteolysis and PAR₂ activation.

Materials and Methods

See [SI Appendix](#) for details.

Animals. The institutional animal care and use committees of New York University and Monash University approved studies of mice.

PAR₂-muGFP Mice. A segment of genomic DNA containing exon 2 of mouse *Par₂* (*F2rl1*) was subcloned from a genomic bacterial artificial chromosome (BAC). The *Par₂-muGFP* gene was synthesized by fusing muGFP to the 3' end of exon 2. The targeting construct comprised *Par₂-muGFP*, a phosphoglycerine kinase neomycin cassette flanked by loxP sites. The targeting vector was electroporated into C57BL/6J embryonic stem cells. Clones were screened by Southern blotting, sequenced, and injected into mouse blastocysts. Chimeras were bred with wild-type female mice to generate black F1 progeny. Expression of *Par₂-muGFP* was confirmed by Southern blotting and PCR.

qRT-PCR. cDNA was amplified with primers shown in [SI Appendix, Table S1](#).

Immunofluorescence. PAR₂-muGFP was localized using a GFP antibody. Endosomes were detected using EEA1 and Rab7a antibodies, and the plasma membrane was identified using E-cadherin antibodies. Neurons were identified using antibodies to NeuN or PGP9.5.

RNAscope In Situ Hybridization. Probes to *F2rl1*, *Gfp*, and *Dnm* isoforms were used for in situ hybridization ([SI Appendix, Table S1](#)).

Ex Vivo PAR₂ Trafficking. Colon segments were incubated with trypsin, 2F, or vehicle. PAR₂-muGFP was detected by GFP immunofluorescence.

Colitis-Induced PAR₂ Trafficking. Mice were treated with DSS or TNBS to evoke colitis.

Nociception. Investigators were blinded to treatments and genotypes. PAR₂ agonists were administered into the colon or paw. Abdominal or paw withdrawal thresholds von Frey filaments were assessed.

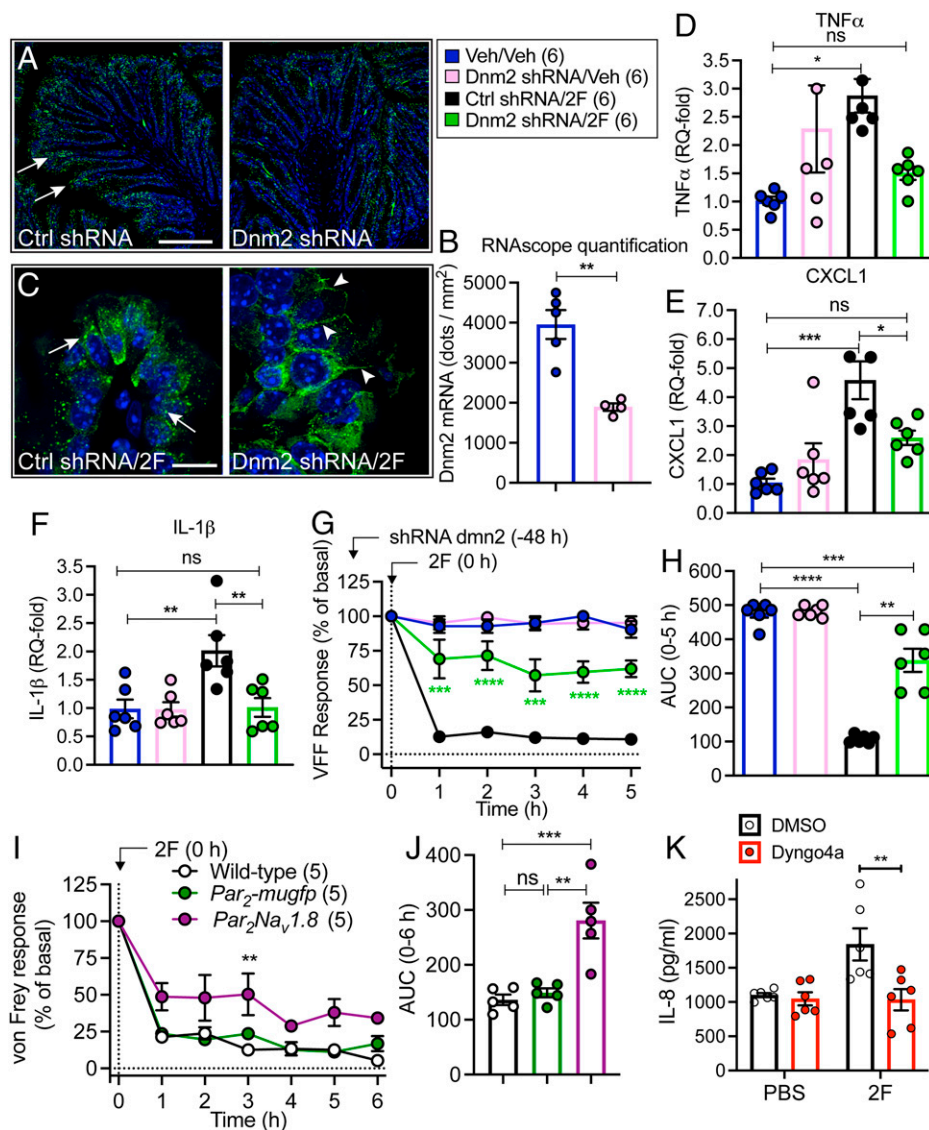


Fig. 8. Contribution of Dnm2 to PAR₂-evoked inflammation and pain in the colon. (A–H) Dnm2 or control (Ctrl) shRNA was administered to *Par2-mugfp* mice by intracolonic injection. Mice were studied after 48 h. (A) Localization of Dnm2 by RNA scope. Representative images are shown. (Scale bars, 100 μm.) (B) Quantification of Dnm2 in the colon. (C) Localization of GFP immunoreactivity in the colon at 5 h after intracolonic injection of 2F. Arrows denote endosomes; arrowheads denote cell surface. (Scale bars, 10 μm.) (A–C) *n* = 5 mice. (D–F) Levels of TNF-α (D), CXCL1 (E), and IL-1β (F) in the colon 5 h after intracolonic injection of 2F. (G and H) Abdominal von Frey filament withdrawal responses 1–5 h after intracolonic injection of 2F. (G) Time course. (H) Area under the curve (AUC) from 0 to 5 h. (D–H) *n* = 6 mice. (I and J) Abdominal von Frey filament withdrawal responses of wild-type, *Par2-mugfp*, and *Par2Na_v1.8* mice 1–6 h after intracolonic injection of 2F. (I) Time course. (J) Area under curve from 0 to 6 h. *n* = 5 mice. (K) Effects of 2F (10 μM) on IL-8 release from explants of human colonic mucosa. Explants were preincubated with dyngo4a or vehicle (DMSO, control). Mean ± SEM; *n* = 6. (B) ***P* < 0.01 by Student's *t* test. (D–J) **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001 by one- and two-way ANOVA or Tukey's test. (K) ***P* < 0.01 by Student's *t* test.

In Vivo shRNA Dnm2 Knockdown. Dnm2shRNA or control shRNA was administered into the colon. PAR₂ endocytosis, expression of cytokines and chemokines, and nociception were studied.

Cell Lines, cDNA, and Transfection. Complementary DNA (cDNA) encoding BRET biosensors for PAR₂, mGα, βARR1/2, markers of the plasma membrane, endosomes, and the Golgi apparatus were transfected into T84 cells using Lipofectamine 3000 and into HEK293T cells using PEI. Dominant-negative DnmK44A or Dnm2 or control siRNA (SI Appendix, Table S1) was similarly transfected.

PAR₂-mugFP Signaling. KNRK cells expressing PAR₂-mugFP or PAR₂-HA11 were stimulated with PAR₂ agonists, and IP-1 formation was measured.

PAR₂-mugFP Signaling. HEK293T cells expressing PAR₂-mugFP and CellLight organelle markers were challenged with PAR₂ agonists and examined by confocal microscopy.

Trafficking of PAR₂, βARR, and Gα_q. HEK293T cells stably expressing FLAG-PAR₂-HA were transfected with YFP-βARR1 plus YFP-βARR2. Cells were challenged with PAR₂ agonists, and then PAR₂, Gα_q, and EEA1 were localized by immunofluorescence and confocal microscopy.

BRET Assays. T84 or HEK293T cells were preincubated with luciferase substrates and challenged with PAR₂ agonists, and BRET was measured using a luminometer.

T84 TEER. T84 cells were cultured on Transwell inserts until TEER was >2,000 Ω. PAR₂ agonists were added to the basolateral compartment, and TEER was measured at 0 and 6 h. GB88 or vehicle was added to the basolateral and apical compartments 30 min before PAR₂ agonists. Dnm2 siRNA or control siRNA and Lipofectamine 3000 were added to both compartments overnight before PAR₂ agonists.

IL-8 Secretion from Human Colonic Mucosa. Institutional review board approval was waived since no patient-identifiable information was obtained. IL-8 secretion was measured by enzyme-linked immunosorbent assay (ELISA) from isolated segments of colonic mucosa collected from patients undergoing resection for treatment of colon cancer.

Statistics. Data are presented as mean $\pm$ SEM. Differences were assessed using Student's *t* test for two comparisons and one- or two-way ANOVA and Tukey's, Dunnett's, or Sidak's post hoc test for multiple comparisons. *P* < 0.05 was considered significant at the 95% confidence level.

Data Availability. All study data are included in the article and/or [SI Appendix](#).

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1. M. Canals, D. P. Poole, N. A. Veldhuis, B. L. Schmidt, N. W. Bunnett, G-protein-coupled receptors are dynamic regulators of digestion and targets for digestive diseases. *Gastroenterology* **156**, 1600–1616 (2019).
2. Y. K. Peterson, L. M. Luttrell, The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol. Rev.* **69**, 256–297 (2017).
3. K. A. DeFea *et al.*, Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J. Cell Biol.* **148**, 1267–1281 (2000).
4. R. Irannejad *et al.*, Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**, 534–538 (2013).
5. D. D. Jensen *et al.*, Neurokinin 1 receptor signaling in endosomes mediates sustained nociception and is a viable therapeutic target for prolonged pain relief. *Sci. Transl. Med.* **9**, eaa13447 (2017).
6. N. N. Jimenez-Vargas *et al.*, Endosomal signaling of delta opioid receptors is an endogenous mechanism and therapeutic target for relief from inflammatory pain. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 15281–15292 (2020).
7. N. N. Jimenez-Vargas *et al.*, Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7438–E7447 (2018).
8. P. D. Ramirez-Garcia *et al.*, A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain. *Nat. Nanotechnol.* **14**, 1150–1159 (2019).
9. R. E. Yarwood *et al.*, Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 12309–12314 (2017).
10. A. Godbole, S. Lyga, M. J. Lohse, D. Calebiro, Internalized TSH receptors en route to the TGN induce local G_s-protein signaling and gene transcription. *Nat. Commun.* **8**, 1–15 (2017).
11. M. Stoeber *et al.*, A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* **98**, 963–976 (2018).
12. V. S. Ossoskaya, N. W. Bunnett, Protease-activated receptors: Contribution to physiology and disease. *Physiol. Rev.* **84**, 579–621 (2004).
13. S. K. Bohm *et al.*, Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem. J.* **314**, 1009–1016 (1996).
14. C. U. Corvera *et al.*, Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J. Clin. Invest.* **100**, 1383–1393 (1997).
15. E. Camerer, W. Huang, S. R. Coughlin, Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5255–5260 (2000).
16. K. Oikonomopoulou *et al.*, Proteinase-activated receptors, targets for kallikrein signaling. *J. Biol. Chem.* **281**, 32095–32112 (2006).
17. N. H. Tu *et al.*, Legumain induces oral cancer pain by biased agonism of protease-activated receptor-2. *J. Neurosci.* **41**, 193–210 (2021).
18. P. Zhao *et al.*, Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. *J. Biol. Chem.* **289**, 27215–27234 (2014).
19. P. Zhao *et al.*, Neutrophil elastase activates protease-activated receptor-2 (PAR2) and transient receptor potential vanilloid 4 (TRPV4) to cause inflammation and pain. *J. Biol. Chem.* **290**, 13875–13887 (2015).
20. B. M. Anderson *et al.*, Application of a chemical probe to detect neutrophil elastase activation during inflammatory bowel disease. *Sci. Rep.* **9**, 13295 (2019).
21. N. Barlow *et al.*, Demonstration of elevated levels of active cathepsin S in dextran sulfate sodium colitis using a new activatable probe. *Neurogastroenterol. Motil.* **27**, 1675–1680 (2015).
22. F. Cattaruzza *et al.*, Cathepsin S is activated during colitis and causes visceral hyperalgesia by a PAR2-dependent mechanism in mice. *Gastroenterology* **141**, 1864–1874 (2011).
23. G. S. Cottrell *et al.*, Protease-activated receptor 2, dipeptidyl peptidase I, and proteases mediate Clostridium difficile toxin A enteritis. *Gastroenterology* **132**, 2422–2437 (2007).
24. A. Denadai-Souza *et al.*, Functional proteomic profiling of secreted serine proteases in health and inflammatory bowel disease. *Sci. Rep.* **8**, 7834 (2018).
25. K. K. Hansen *et al.*, A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8363–8368 (2005).
26. C. Jacob *et al.*, Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *J. Biol. Chem.* **280**, 31936–31948 (2005).
27. W. Kong *et al.*, Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8884–8889 (1997).
28. M. Steinhoff *et al.*, Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat. Med.* **6**, 151–158 (2000).
29. N. Vergnolle *et al.*, Proteinase-activated receptor-2 and hyperalgesia: a novel pain pathway. *Nat. Med.* **7**, 821–826 (2001).
30. N. Cenac *et al.*, Proteinase-activated receptor-2-induced colonic inflammation in mice: Possible involvement of afferent neurons, nitric oxide, and paracellular permeability. *J. Immunol.* **170**, 4296–4300 (2003).
31. D. J. Scott *et al.*, A novel ultra-stable, monomeric green fluorescent protein for direct volumetric imaging of whole organs using CLARITY. *Sci. Rep.* **8**, 667 (2018).
32. A. R. B. Thomsen, D. D. Jensen, G. A. Hicks, N. W. Bunnett, Therapeutic targeting of endosomal G-protein-coupled receptors. *Trends Pharmacol. Sci.* **39**, 879–891 (2018).
33. C. Jacob *et al.*, c-Cbl mediates ubiquitination, degradation, and down-regulation of human protease-activated receptor 2. *J. Biol. Chem.* **280**, 16076–16087 (2005).
34. D. D. Jensen *et al.*, Protein kinase D and G $\beta\gamma$ subunits mediate agonist-evoked translocation of Protease-activated Receptor-2 from the Golgi apparatus to the plasma membrane. *J. Biol. Chem.* **291**, 11285–11299 (2016).
35. P. Zhao *et al.*, Protein kinase D and G $\beta\gamma$ mediate sustained nociceptive signaling by biased agonists of Protease-Activated Receptor-2. *J. Biol. Chem.* **294**, 10649–10662 (2019).
36. A. H. Nguyen *et al.*, Structure of an endosomal signaling GPCR-G protein- β -arrestin mega-complex. *Nat. Struct. Mol. Biol.* **26**, 1123–1131 (2019).
37. A. R. B. Thomsen *et al.*, GPCR-G protein- β -arrestin super-complex mediates sustained G protein signaling. *Cell* **166**, 907–919 (2016).
38. A. S. Dixon *et al.*, NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–408 (2016).
39. N. Cenac *et al.*, Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am. J. Pathol.* **161**, 1903–1915 (2002).
40. D. D. Jensen *et al.*, Endothelin-converting enzyme 1 and β -arrestins exert spatiotemporal control of substance P-induced inflammatory signals. *J. Biol. Chem.* **289**, 20283–20294 (2014).
41. D. P. Poole *et al.*, Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice. *Gastroenterology* **141**, 982–991 (2011).
42. G. Scherrer *et al.*, Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 9691–9696 (2006).
43. N. Cenac *et al.*, PAR2 activation alters colonic paracellular permeability in mice via IFN-gamma-dependent and -independent pathways. *J. Physiol.* **558**, 913–925 (2004).
44. S. N. Hassler *et al.*, The cellular basis of protease-activated receptor 2-evoked mechanical and affective pain. *JCI Insight* **5**, e12793 (2020).
45. G. S. Cottrell, S. Amadesi, E. F. Grady, N. W. Bunnett, Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. *J. Biol. Chem.* **279**, 13532–13539 (2004).
46. A. Caminero *et al.*, Duodenal bacterial proteolytic activity determines sensitivity to dietary antigen through protease-activated receptor-2. *Nat. Commun.* **10**, 1–14 (2019).