

AWARD NUMBER: W81XWH-18-1-0432

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

PRINCIPAL INVESTIGATOR: Brian L. Schmidt, DDS, MD, PhD

CONTRACTING ORGANIZATION: New York University  
New York, NY 10010

REPORT DATE: Oct 2020

TYPE OF REPORT: Annual Progress Report

PREPARED FOR: U.S. Army Medical Research and Development Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                 |                                  |                                                   | Form Approved<br>OMB No. 0704-0188          |                                              |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|----------------------------------|---------------------------------------------------|---------------------------------------------|----------------------------------------------|
| Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                 |                                  |                                                   |                                             |                                              |
| 1. REPORT DATE<br>Oct 2020                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                 | 2. REPORT TYPE<br>Annual         |                                                   | 3. DATES COVERED<br>01Sep2019-31Aug2020     |                                              |
| 4. TITLE AND SUBTITLE<br>Receptors in Endosomes Mediate Chronic Pain Associated<br><br>with Trauma and Stress: Nonopioid Targets for Pain                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                 |                                  |                                                   | 5a. CONTRACT NUMBER<br>W81XWH-18-1-043      |                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  |                                                   | 5b. GRANT NUMBER<br>PR170507P2              |                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  |                                                   | 5c. PROGRAM ELEMENT NUMBER                  |                                              |
| 6. AUTHOR(S)<br><br>Brian L. Schmidt<br>E-Mail: bls322@nyu.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                 |                                  |                                                   | 5d. PROJECT NUMBER<br>0011176409            |                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  |                                                   | 5e. TASK NUMBER                             |                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  |                                                   | 5f. WORK UNIT NUMBER                        |                                              |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>NEW YORK UNIVERSITY<br>70 Washington Square S<br>New York, NY 10010                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                                 |                                  |                                                   | 8. PERFORMING ORGANIZATION REPORT<br>NUMBER |                                              |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)lth<br><br>U.S. Army Medical Research and Development Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                                 |                                  |                                                   | 10. SPONSOR/MONITOR'S ACRONYM(S)            |                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  |                                                   | 11. SPONSOR/MONITOR'S REPORT<br>NUMBER(S)   |                                              |
| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                 |                                  |                                                   |                                             |                                              |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |                                 |                                  |                                                   |                                             |                                              |
| 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<br>None listed.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                                 |                                  |                                                   |                                             |                                              |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |                                 |                                  | 17. LIMITATION<br>OF ABSTRACT<br><br>Unclassified | 18. NUMBER<br>OF PAGES<br><br>90            | 19a. NAME OF RESPONSIBLE PERSON<br>USAMRMC   |
| a. REPORT<br><br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | b. ABSTRACT<br><br>Unclassified | c. THIS PAGE<br><br>Unclassified |                                                   |                                             | 19b. TELEPHONE NUMBER (include area<br>code) |

## TABLE OF CONTENTS

|                                                     | <u>Page</u> |
|-----------------------------------------------------|-------------|
| 1. Introduction                                     | 1           |
| 2. Keywords                                         | 2           |
| 3. Accomplishments                                  | 2           |
| 4. Impact                                           | 7           |
| 5. Changes/Problems                                 | 5           |
| 6. Products                                         | 8           |
| 7. Participants & Other Collaborating Organizations | 8           |
| 8. Special Reporting Requirements                   | 9           |
| 9. Appendices                                       | 10          |

## 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 [NK<sub>1</sub>R]; calcitonin gene-related peptide [CGRP] calcitonin like receptor [CLR]) and proteases (protease-activated receptor-2 [PAR<sub>2</sub>]). 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; PAR<sub>2</sub>, Protease-Activated Receptor-2; NK<sub>1</sub>R, 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

| <b>Aim 1:</b> 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.             | <b>Date</b> | <b>%</b> |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|----------|
| <b>Major Task 1:</b> Determine whether endosomal PAR <sub>2</sub> in DRG neurons generates compartmentalized signals that regulate channel activity and sensitization                                    |             |          |
| <u>Subtask 1:</u> Establish breeding colonies of PAR <sub>2</sub> -muGFP mice.                                                                                                                           | 9/29/20     | 100      |
| <u>Subtask 2:</u> Analyze endocytosis-dependent compartmentalized signaling in DRG neurons                                                                                                               | 9/29/20     | 100      |
| <u>Subtask 3:</u> Assess PAR <sub>2</sub> -mediated sensitization and TRP channel activation in DRG neurons                                                                                              | 9/29/20     | 75       |
| <b>Major Task 2:</b> Determine whether proteases in human colon biopsy specimens activate endosomal PAR <sub>2</sub> 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                                                                                                      | 9/29/20     | 100      |
| <u>Subtask 2:</u> Determine whether proteases in human IBS-D biopsies sensitize DRG neurons and activate TRP channel through endosomal PAR <sub>2</sub> .                                                | 9/29/20     | 25       |
| <u>Subtask 3:</u> Profile activated serine and cysteine proteases in supernatants of IBS-D and HC biopsies using ABPs                                                                                    | 9/29/20     | 25       |
| <b>Major Task 3:</b> Determine whether endosomal PAR <sub>2</sub> , NK <sub>1</sub> R and CLR signals regulate transcription in pain-sensing neurons                                                     |             |          |

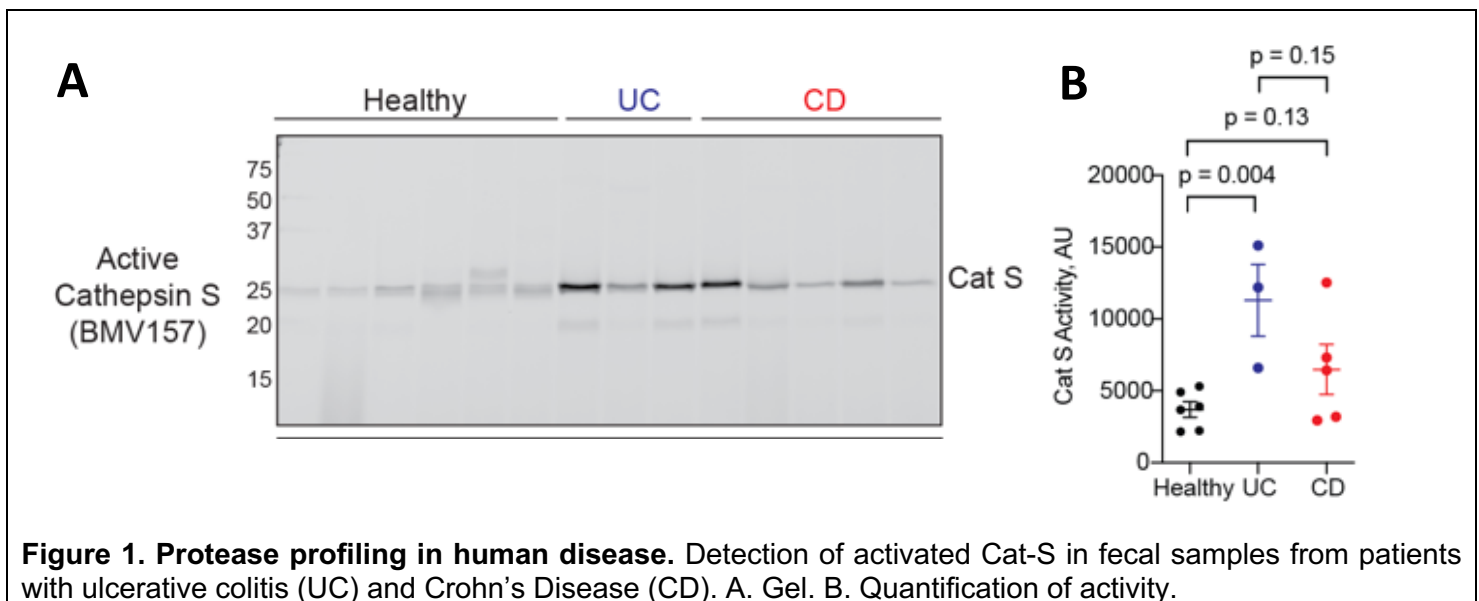
|                                                                                                                                                                                                                                                           |         |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|-----|
| <u>Subtask 1:</u> Determine whether endosomal PAR <sub>2</sub> , NK <sub>1</sub> R and CLR signaling regulates transcription in DRG and spinal neurons                                                                                                    | 9/29/20 | 10  |
| <b>Aim 2:</b> 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 <sub>2</sub> , NK <sub>1</sub> R and CLR antagonists; generate small molecule quadripartite NK <sub>1</sub> R and CLR antagonists; generate fluorescent probes                                   | 9/29/20 | 100 |
| <b>Major Task 1:</b> Determine whether tripartite and quadripartite antagonists disrupt endosomal PAR <sub>2</sub> , NK <sub>1</sub> R and CLR signaling in HEK293 cells                                                                                  |         |     |
| <u>Subtask 1:</u> Determine whether lipidated antagonists disrupt ligand/receptor interactions in endosomes                                                                                                                                               | 9/29/20 | 50  |
| <u>Subtask 2:</u> Determine whether lipidated antagonists inhibit endosomal signaling of PAR <sub>2</sub> , NK <sub>1</sub> R and CLR in HEK293 cells                                                                                                     | 9/29/20 | 100 |
| <b>Major Task 2:</b> Determine whether tripartite and quadripartite antagonists target PAR <sub>2</sub> in endosomes of DRG neurons and NK <sub>1</sub> 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 <sub>2</sub> , NK <sub>1</sub> R and CLR on endosomes of pain-sensing neurons                                                                            | 9/29/20 | 100 |
| <u>Subtask 2:</u> Determine whether tripartite and quadripartite antagonists inhibit endosomal signaling of PAR <sub>2</sub> , NK <sub>1</sub> R and CLR in pain-sensing neurons                                                                          | 9/29/20 | 100 |
| <b>Major Task 3:</b> Determine whether tripartite and quadripartite antagonists disrupt PAR <sub>2</sub> -induced sensitization and transcription in DRG neurons and NK <sub>1</sub> 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                                                                                                                          | 9/29/20 | 75  |
| <u>Subtask 2:</u> Examine whether antagonists suppress gene expression                                                                                                                                                                                    | 9/29/20 | 0   |
| <b>Aim 3:</b> 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.                                                      |         |     |
| <b>Major Task 1:</b> 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 <sub>2</sub> , NK <sub>1</sub> R and CLR; generate quadripartite probes for NK <sub>1</sub> R and CLR antagonists, similar to Aim 2 above                                                        | 9/29/20 | 100 |
| <u>Subtask 2:</u> Develop a GTN-evoked model of migraine headache pain in C57BL/6 mice                                                                                                                                                                    | 9/29/20 | 100 |
| <u>Subtask 3:</u> Determine efficacy of tripartite or non-lipidated CLR antagonists                                                                                                                                                                       | 9/29/20 | 100 |
| <b>Major Task 2:</b> Conduct studies in preclinical model of nerve injury pain to determine feasibility of using quadripartite NK <sub>1</sub> R/CLR antagonists for nerve injury pain.                                                                   |         |     |
| <u>Subtask 1:</u> Develop a spared nerve injury model of neuropathic pain in C57BL/6 mice                                                                                                                                                                 | 9/29/20 | 100 |
| <u>Subtask 2:</u> Determine efficacy of quadripartite NK <sub>1</sub> R/CLR antagonists and non-lipidated antagonists                                                                                                                                     | 9/29/20 | 100 |
| <b>Major Task 3:</b> Conduct studies in preclinical model of IBS pain to determine feasibility of using tripartite PAR <sub>2</sub> antagonists for post-inflammatory IBS pain.                                                                           |         |     |
| <u>Subtask 1:</u> Generate an IBS mouse model in C57BL/6 mice                                                                                                                                                                                             | 9/29/20 | 75  |
| <u>Subtask 2:</u> Training of electrophysiologist in Dr. Schmidt's laboratory by Dr. Bunnett's team on visceromotor responses following graded colorectal distention                                                                                      | 9/29/20 | 75  |
| <u>Subtask 3:</u> Determine efficacy of tripartite PAR <sub>2</sub> antagonists and non-lipidated antagonists                                                                                                                                             | 9/29/20 | 75  |

## Accomplishments

The general hypothesis of our DoD project is that activated G protein-coupled receptors (GPCRs) traffic to endosomes of nociceptors (*i.e.*, pain sensing neurons) and second order spinal neurons (*i.e.*, pain transmitting neurons) during injury and inflammation, including nerve-injury pain, irritable bowel syndrome (IBS) pain, and migraine/post-traumatic headache pain. GPCRs continue to signal from endosomes to control persistent hyperexcitability and chronic pain. Antagonists designed to target GPCRs in endosomes thus offer superior relief from pain.

**a) Profiling proteases in diseased human tissues.** We sought to identify proteases that are activated in painful diseases in patients and to determine the mechanisms by which they evoke pain. Through our collaboration with the Salisbury VA, we have collected biopsies of colonic mucosa from veterans: patients with the painful disorder IBS and healthy controls. We are now analyzing biopsy supernatants for protease activity using a panel of activity-based probes (ABPs) for serine and cysteine proteases.

We have published the development of two novel ABPs for neutrophil elastase and cathepsin X, and have used these probes to profile activated proteases in tissues from mice and patients with inflammatory bowel disease (IBD). Cathepsin S has previously been implicated in IBD pain. Using broad-spectrum and specific ABPs, we investigated cathepsin S activation in human and mouse colitis. We observed a significant increase in fecal cathepsin S in patients with ulcerative colitis compared to healthy controls (**Fig. 1A, B**). Mice with experimental colitis exhibited a modest increase in mucosal activity of both cathepsin S and X compared to naïve mice. Luminal secretion of cathepsin S was increased upon colitis induction. Using confocal microscopy, we determined the source of cathepsin S to be CD68<sup>+</sup> macrophages in the mucosa. To investigate the contribution of cathepsin S and cathepsin X to colitis, we induced colitis in cathepsin-deficient mice. Cathepsin X-deficient mice exhibited no clear differences in disease indicators compared to wild-type mice, while cathepsin S-deficient mice exhibited less rectal bleeding, less splenomegaly and improved histological scores. To determine whether pharmacologic inhibition of cathepsin S activity would likewise ameliorate symptoms of colitis, a reversible inhibitor LY3000328 was administered to mice with colitis. LY3000328 provoked a clear upregulation of cathepsin S and L activity, likely through a compensatory mechanism or accumulation of undegraded cathepsins. This increase in protease activity was associated with exacerbated histological scores and splenomegaly. Collectively, these results suggest that cathepsin S, but not cathepsin X, may promote symptoms of experimental colitis. This work is under review in *Am. J. Physiol.*

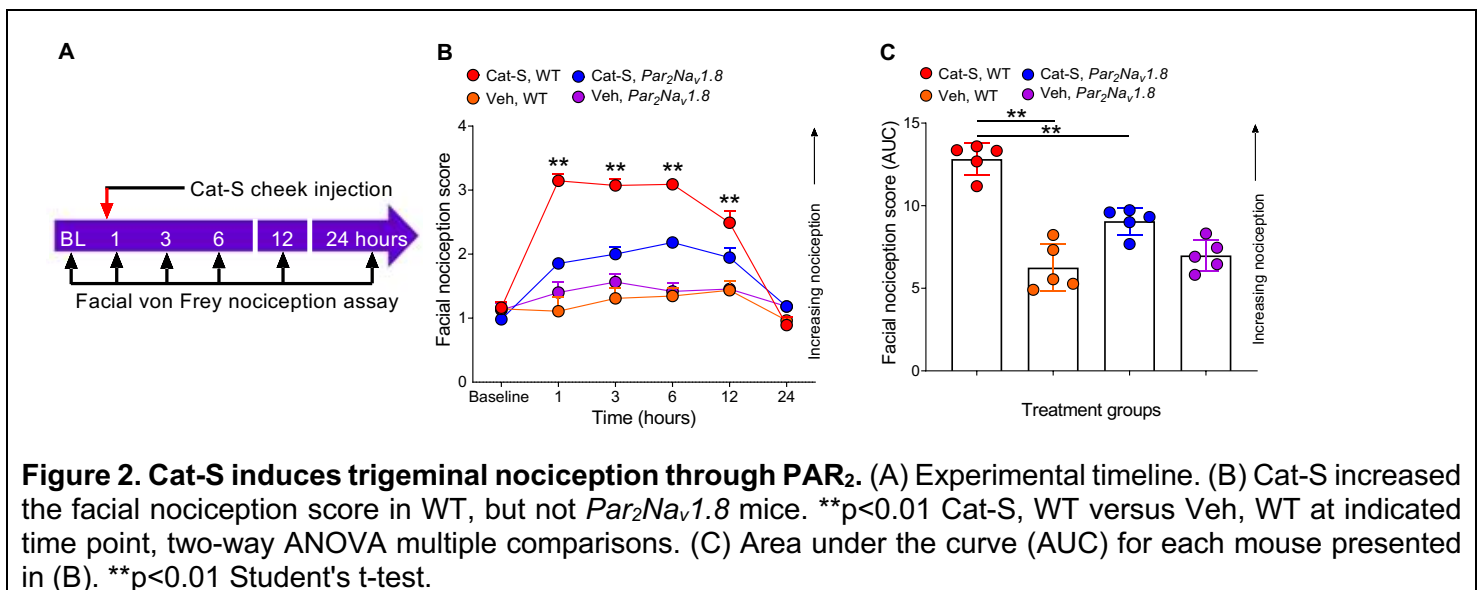


**b) Generation and characterization of knockin mice expressing PAR<sub>2</sub> fused to muGFP.** To specifically localize protease-activated receptor-2 (PAR<sub>2</sub>) in tissues and to assess PAR<sub>2</sub> endocytosis during disease, we generated knock-in mice expressing PAR<sub>2</sub> fused to monomeric ultrastable GFP (muGFP). Breeding colonies have been established and mice have been fully characterized. Analysis of gastrointestinal tissues and DRG by Q-PCR, revealed similar levels of expression of PAR<sub>2</sub> and PAR<sub>2</sub>-muGFP mRNA in wild type and knockin mice, respectively. Intraplanar administration of trypsin to wild type and knockin mice caused identical allodynia, confirming normal function of PAR<sub>2</sub>-muGFP *in vivo*. We mapped the distribution of PAR<sub>2</sub> in the intestinal tract

and in dorsal root ganglia (DRG) using antibodies to GFP, to amplify the signal, and RNAScope using probes to PAR<sub>2</sub> and GFP. PAR<sub>2</sub>-muGFP was robustly expressed by enterocytes and colonocytes, determined by immunofluorescence and RNAScope. RNAScope analysis revealed expression of PAR<sub>2</sub> in a small subset of DRG neurons. In mice with colitis, PAR<sub>2</sub>-muGFP was redistributed from the basolateral membranes of colonocytes to endosomes, consistent with the generation of proinflammatory proteases that activate PAR<sub>2</sub>. Exposure of isolated segments of colon to trypsin and a PAR<sub>2</sub>-selective agonist also evoked endocytosis of PAR<sub>2</sub> in colonocytes. Inhibitors of dynamin and clathrin blunted endocytosis, suggesting that endocytosis of PAR<sub>2</sub> proceeds by a dynamin and clathrin-mediated mechanism. To ascertain the importance of PAR<sub>2</sub> endocytosis and endosomal signaling to colonic pain, we administered a PAR<sub>2</sub> agonist into the colon and measured nociception by stimulating the abdomen with calibrated von Frey filaments. The PAR<sub>2</sub> agonist caused robust visceral nociception. We are currently determining whether intracolonic administration of dynamin and clathrin inhibitors attenuates PAR<sub>2</sub>-evoked nociception. We expect to submit this work for publication by December 2020.

**c) Endosomal signaling of proteases and PAR<sub>2</sub> in IBS pain.** In the previous reporting period, we published a study supporting a major role for proteases and PAR<sub>2</sub> in IBS pain. We found that colonic mucosa from patients with IBS releases proteases that evoke endocytosis of PAR<sub>2</sub> in nociceptors from mice. PAR<sub>2</sub> signals from endosomes of nociceptors to induce hyperexcitability and colonic pain. We developed a lipidated antagonist of PAR<sub>2</sub> that attenuated endosomal signaling and prevented IBS protease-evoked sensitization of nociceptors. In the current reporting period we have extended these findings by studying the capacity of proteases released from colonic biopsies from veterans with IBS to excite nociceptors.

**d) Proteases, PAR<sub>2</sub> and trigeminal pain.** For the migraine and post-traumatic headache models, we measure the withdrawal response to stimulation of the facial (*i.e.*, trigeminal) region with calibrated von Frey fibers. Two protease activators of PAR<sub>2</sub> that have not been tested for their capacity to induce trigeminal nociception are legumain (Lgmn) and cathepsin-S (Cat-S). Lgmn is a cysteine protease that requires an acidic pH (for example, inflamed or injured tissue) for activation. Cat-S is a cysteine lysosomal protease that is secreted by macrophages, in the periphery, and microglia in the brain. Since submission of the previous progress report, we tested the role of Lgmn and Cat-S in trigeminal nociception. The role of PAR<sub>2</sub> in Lgmn- and Cat-S-mediated trigeminal nociception was tested using *Par2Na<sub>v</sub>1.8* mice, which lack PAR<sub>2</sub> in Na<sub>v</sub>1.8-positive neurons such as nociceptors. We measured withdrawal responses to mechanical stimulation of the left cheek with von Frey filaments. After measurement of baseline responses, Lgmn or Cat-S was injected subcutaneously to the left cheek. Nociception was then tested at various times after protease injection. Lgmn and Cat-S induced facial nociception in wild type but not *Par2Na<sub>v</sub>1.8* mice (Figure 2, A-C). Our findings demonstrate that Lgmn and Cat-S produce nociception in the facial region through activation of PAR<sub>2</sub> on trigeminal afferent neurons, which are responsible for migraine and post-traumatic headache. Future studies will test whether the protease activators contribute to nociceptive behavior in the migraine and post-traumatic headache models.



**e) Tripartite antagonists of endosomal GPCR signaling.** GPCRs can continue to signal from endosomes to control important pathophysiological processes. We previously reported that sustained endosomal signaling of

the substance P (SP) neurokinin 1 receptor (NK<sub>1</sub>R) in spinal neurons mediates nociception. An NK<sub>1</sub>R antagonist, Spantide I, conjugated to cholestanol (Span-Chol) accumulates in endosomes, inhibits endosomal NK<sub>1</sub>R signaling, and causes prolonged antinociception in mice. We have now characterized in detail the mechanisms underlying the antinociceptive actions of Span-Chol. We used fluorescent correlation spectroscopy and biosensors targeted to subcellular compartments to determine the long-term location and activity of lipid-anchored probes. The cholestanol-anchor increased in the local concentration of probe at the plasma membrane. This localized enrichment corresponded with an increase in NK<sub>1</sub>R binding affinity over time, and increased potency for inhibition of NK<sub>1</sub>R calcium signaling. Span-Chol, but not Span, caused a persistent (>4 h) decrease in NK<sub>1</sub>R recruitment of  $\beta$ -arrestin and receptor internalization to early endosomes. Using targeted biosensors, we mapped the relative inhibition of NK<sub>1</sub>R signaling as the receptor moved into the cell. Span selectively inhibited cell surface signaling whereas Span-Chol selectively inhibited endosomal signaling. In a preclinical model of pain, Span-Chol caused prolonged antinociception (>9 h), which is attributable to increased local concentration at membranes, decreased NK<sub>1</sub>R endocytosis, and inhibition of endosomal signaling. Identifying the mechanisms that contribute to the increased preclinical efficacy of lipid-anchored NK<sub>1</sub>R antagonists is an important step toward understanding how we can effectively target intracellular GPCRs in disease. This manuscript is in revision for J. Biol. Chem.

**f) Nanoparticle-encapsulated antagonists of endosomal GPCR signaling.** We have developed nanoparticle-encapsulated antagonists of endosomal GPCRs for the treatment of pain. Nanoparticle encapsulation improves drug efficacy by enhancing the stability, tolerability, delivery and retention in diseased tissues. Nanoparticle-mediated drug delivery is especially useful for targets within endosomes because of the endosomal transport mechanisms of many nanomedicines within cells. We reported the design of a pH-responsive, soft polymeric nanoparticle for the targeting of acidified endosomes to precisely inhibit endosomal signalling events leading to chronic pain. In chronic pain, the SP NK<sub>1</sub>R redistributes from the plasma membrane to acidified endosomes, where it signals to maintain pain. Therefore, the NK<sub>1</sub>R in endosomes provides an important target for pain relief. The pH-responsive nanoparticles entered cells by clathrin- and dynamin-dependent endocytosis and accumulated in NK<sub>1</sub>R-containing endosomes. Following intrathecal injection into rodents, the nanoparticles, containing the FDA-approved NK<sub>1</sub>R antagonist aprepitant, inhibited SP-induced activation of spinal neurons and thus prevent pain transmission. Treatment with the nanoparticles led to complete and persistent relief from nociceptive, inflammatory and neuropathic nociception. This approach offers a much-needed nonopioid treatment option for chronic pain. We have expanded this work to examine the efficacy of nanoparticle-encapsulated and free aprepitant in neuropathic pain in mice. In the sural nerve spared model of neuropathic pain, nanoparticle-encapsulated aprepitant provided a more efficacious and sustained relief from mechanical hyperalgesia and cold allodynia than free aprepitant, at all doses tested (**Figure 3**). The intrathecal injection of inhibitors of endocytosis also inhibited nociception, reinforcing a role for endosomal signaling in neuropathic pain.

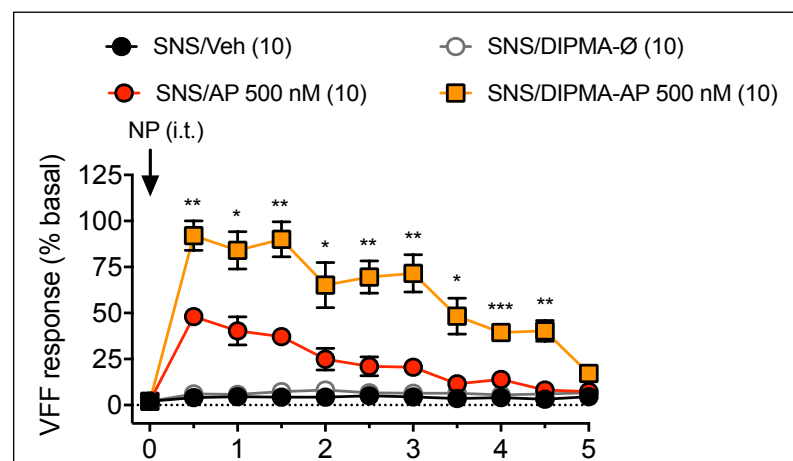
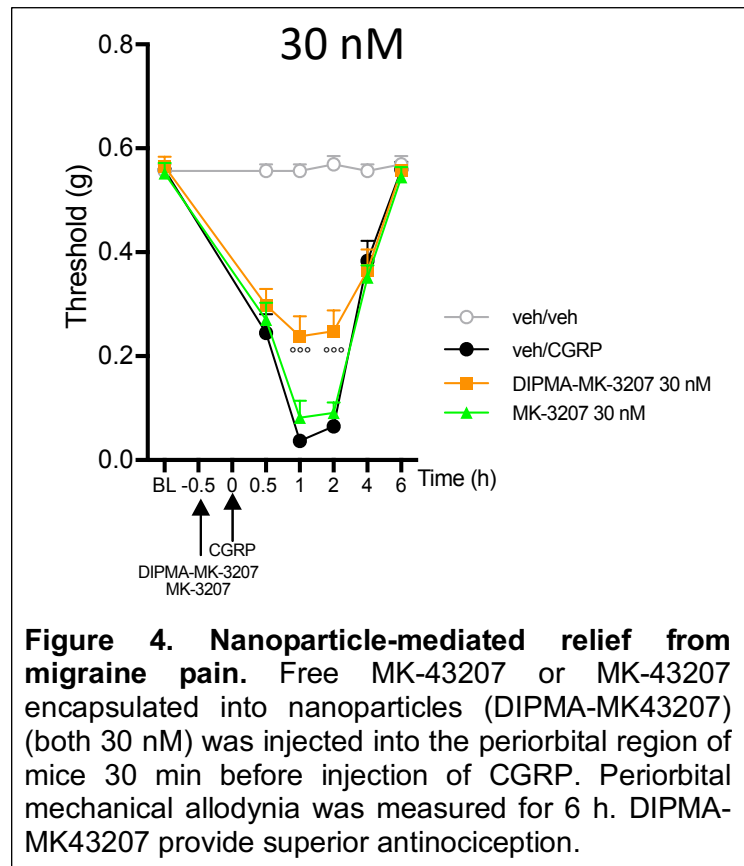


**Figure 3. Nanoparticle-mediated relief from nerve injury pain.** The sural nerve spared (SNS) model of neuropathic pain was studied in mice. After 10 days, free aprepitant (AP) or nanoparticle-encapsulated aprepitant (DIPMA-AP) was injected intrathecally (5  $\mu$ l, 500 nM). DIPMA-AP provided more complete and sustained relief from pain.

We have further refined nanoparticle-mediated drug delivery by developing nanoparticles to target endosomes of pain sensing and transmitting neurons. We reported that opioids from the inflamed colon activate  $\delta$ -opioid receptors (DOPr) in endosomes of nociceptors. Biopsies of inflamed colonic mucosa from patients and mice with colitis released opioids that activated DOPr on nociceptors to cause a sustained decrease in excitability. DOPr agonists inhibited mechanically-sensitive colonic nociceptors. DOPr endocytosis and endosomal signaling by protein kinase C (PKC) and extracellular signal regulated kinase (ERK) pathways mediated the sustained inhibitory actions of endogenous opioids and DOPr agonists. DOPr agonists stimulated recruitment of  $G\alpha_{i/o}$  and  $\beta$ -arrestin1/2 to endosomes. Analysis of compartmentalized signaling revealed a requirement of DOPr endocytosis for activation of PKC at the plasma membrane and

in the cytosol and ERK in the nucleus. We explored a nanoparticle delivery strategy to evaluate whether endosomal DOPr might be a therapeutic target for pain. The DOPr agonist DADLE was coupled to a liposome shell for targeting DOPr-positive nociceptors and incorporated into a mesoporous silica core for release in the acidic and reducing endosomal environment. Nanoparticles activated DOPr at the plasma membrane, were preferentially endocytosed by DOPr-expressing cells, and were delivered to DOPr-positive early endosomes. Nanoparticles caused a long-lasting activation of DOPr in endosomes, which provided sustained inhibition of nociceptor excitability and relief from inflammatory pain. Conversely, nanoparticles containing a DOPr antagonist abolished the sustained inhibitory effects of DADLE. Thus, DOPr in endosomes is an endogenous mechanism and a therapeutic target for relief from chronic inflammatory pain.

**g) Endosomal signaling of CGRP/CLR in Schwann cells and migraine pain.** We have reported a major role for Schwann cells in neuropathic pain. In the current reporting period, we have discovered a key role for Schwann cells and endosomal signaling in migraine pain. We observed that a human Schwann cell line and primary mouse Schwann cells express mRNA encoding calcitonin-like receptor (CLR) and receptor activity modifying protein 1



(RAMP1), the receptors for calcitonin-gene related peptide (CGRP). CGRP and CLR are therapeutic targets for chronic migraine in patients. In preclinical models of migraine pain in mice, selective deletion of CGRP receptors from Schwann cells abrogated supraorbital allodynia evoked by administration of CGRP and trinitroglycerin. The local injection of inhibitors of clathrin and dynamin also attenuated nociception. A small molecule CLR antagonist, MK-43207, incorporated into soft polymer nanoparticles, more effectively attenuated nociception than free MK-43207 (**Figure 4**). These results suggest that endosomal CGRP/CLR signaling in Schwann cells underlies migraine pain in mice. We studied the pathway by which endosomal CLR signals pain in isolated Schwann cells. CGRP evoked CLR endocytosis and activated adenylyl cyclase and protein kinase A, leading to generation of reactive oxygen species, which in turn activated the transient receptor potential ankyrin 1 (TRPA1) channel on nociceptors. Thus, CGRP/CLR signaling in endosomes of Schwann cells provides a peripheral mechanism for signaling migraine pain in mice. Endosomal CLR is a new target for the treatment of migraine 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 proves 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.

Dr. Schmidt has presented findings at the following meetings and seminar series:

## 2019

- University of Minnesota
- University of Miami
- University of Alabama
- Society of Neuroscience, Chicago, IL
- Royal College of Surgeons Ireland, Dublin, Ireland

## 2020

- New York University

### Plans for Next Reporting Period

Priorities for the next reporting period include:

Analysis of the mechanisms by which GPCRs in endosomes regulate TRP channel activity at the plasma membrane and gene expression in the nucleus of neurons that sense and transmit pain.

Analysis of proteases in biopsies of human colonic mucosa using activity-based probes, including newly developed and existing probes for serine and cysteine proteases. Investigation of whether activate proteases cause hyperexcitability of nociceptors *via* endosomal PAR<sub>2</sub>.

### 4. IMPACT:

**Impact on principal discipline.** This work has identified new targets for the treatment of chronic pain. We have discovered that painful stimuli cause the translocation of receptors from the cell surface to endosomes of neurons that sense and transmit pain. Receptors in endosomes are primarily responsible for signaling persistent pain. Drugs that target these 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 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.**

PI Dr. N.W. Bunnett has accepted the Chair of the Department of Molecular Pathobiology at New York University, effective 10/1/2019. This relocation has facilitated collaborations with Dr. B.L. Schmidt, the partnering PI.

The COVID19 crisis closed laboratories from March to July 2020, which affected productivity but provided opportunities for writing. Laboratories are now fully operational.

**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:

\* Papers included in appendix

### Journal publications

1. \*Anderson, B.M., Poole, D.P., Aurelio, L., Ng, G.Z., Fleischmann, M., Kasperkiewicz, P., Morissette, C., Drag, M., van Driel, I.R., Schmidt, B.L., Vanner, S.J., Bunnett, N.W. & Edgington-Mitchell, L.E. Application of a chemical probe to detect neutrophil elastase activation during inflammatory bowel disease. *Sci Rep* **9**, 13295, 2019. PMC6746801. Federal support: Yes
2. \*Ramirez-Garcia, P.D., Retamal, J.S., Shenoy, P., Imlach, W., Sykes, M., Truong, N., Constandil, L., Pelissier, T., Nowell, C.J., Khor, S.Y., Layani, L.M., Lumb, C., Poole, D.P., Lieu, T., Stewart, G.D., Mai, Q.N., Jensen, D.D., Latorre, R., Scheff, N.N., Schmidt, B.L., Quinn, J.F., Whittaker, M.R., Veldhuis, N.A., Davis, T.P. & Bunnett, N.W. A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain. *Nat Nanotechnol* **14**, 1150-1159, 2019. Federal support: Yes
3. \*Jimenez-Vargas, N.N., Gong, J., Wisdom, M.J., Jensen, D.D., Latorre, R., Hegron, A., Teng, S., DiCello, J.J., Rajasekhar, P., Veldhuis, N.A., Carbone, S.E., Yu, Y., Lopez-Lopez, C., Jaramillo-Polanco, J., Canals, M., Reed, D.E., Lomax, A.E., Schmidt, B.L., Leong, K.W., Vanner, S.J., Halls, M.L., Bunnett, N.W. & Poole, D.P. 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. PMC7334524. Federal support: Yes
4. Zhao, P., Pattison, L.A., Jensen, D.D., Jimenez-Vargas, N.N., Latorre, R., Lieu, T., Jaramillo, J.O., Lopez-Lopez, C., Poole, D.P., Vanner, S.J., Schmidt, B.L. & Bunnett, N.W. Protein kinase D and Gbetagamma mediate sustained nociceptive signaling by biased agonists of protease-activated receptor-2. *J Biol Chem* **294**, 10649-10662, 2019. PMC6615677. Federal support: Yes

**Books.** Nothing to report.

**Other.** Nothing to report.

**Websites.** Nothing to report.

**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:                                  | Brian Schmidt, DDS, MD, PhD                                                                                |
| Project Role:                          | PI                                                                                                         |
| Researcher Identifier (e.g. ORCID ID): | 0000-0002-2670-8774                                                                                        |
| Nearest person month worked:           | 1.43 calendar                                                                                              |
| Contribution to Project:               | Project conception; experimental design; provide human samples; interpreting results; writing manuscripts. |

|                  |                                                                                                                 |
|------------------|-----------------------------------------------------------------------------------------------------------------|
| Funding Support: | R01DE026806; R01DK118971;<br>U01DE022939; R21DE026964;<br>R01CA196263; R01CA204264;<br>R01CA228525; R01DE029951 |
|------------------|-----------------------------------------------------------------------------------------------------------------|

|                                        |                                                                                                                                                              |
|----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Name:                                  | Malvin Janal                                                                                                                                                 |
| Project Role:                          | Biostatistician                                                                                                                                              |
| Researcher Identifier (e.g. ORCID ID): | 0000-0003-1098-9687                                                                                                                                          |
| Nearest person month worked:           | 0.60 calendar                                                                                                                                                |
| Contribution to Project:               | Provide guidance on data interpretation and analysis; perform statistical analysis                                                                           |
| Funding Support:                       | R01DE029951; R01DE029493;<br>R01CA228525; R01DE026806;<br>R01DK118971; R01CA196263;<br>R01DE025992; R01DE026772;<br>R01DE026279; R21DE026964; PCS-1609-36824 |

|                                        |                                                                                |
|----------------------------------------|--------------------------------------------------------------------------------|
| Name:                                  | Elyssa Chen, PhD                                                               |
| Project Role:                          | Assistant Research Scientist                                                   |
| Researcher Identifier (e.g. ORCID ID): | N/A                                                                            |
| Nearest person month worked:           | 1.1 calendar                                                                   |
| Contribution to Project:               | Analysis of electrophysiologic response of neurons to proteases and inhibitors |
| Funding Support:                       | R01DE026806; R01DK118971                                                       |

|                                        |                                          |
|----------------------------------------|------------------------------------------|
| Name:                                  | Tu Nguyen, PhD                           |
| Project Role:                          | Assistant Research Scientist             |
| Researcher Identifier (e.g. ORCID ID): | N/A                                      |
| Nearest person month worked:           | 1.65 calendar                            |
| Contribution to Project:               | Analysis of trigeminal nociception       |
| Funding Support:                       | R01CA196263; R01DE026806;<br>R01CA228525 |

#### Change in support since last reporting period

##### **Brian Schmidt, DDS, MD, PhD (PI)**

##### **NEW SUPPORT**

1R01DE029951-01                      Bunnett/Schmidt (MPI)                      9/15/2020-8/31/2024  
Targeting Endosomal Receptors for Treatment of Chronic Pain

##### **COMPLETED SUPPORT**

Nothing to report

#### **Other organizations. Nothing to report**

#### **8. SPECIAL REPORTING REQUIREMENTS:**

This is a collaborative award between Dr. N.W. Bunnett (New York University) and B.L. Schmidt (New York University). Dr. Schmidt will submit his report for the 10/01/20 deadline.

## **9. APPENDICES:**

PDF of most relevant published manuscripts and manuscripts under revision and re-review.

OPEN

# Application of a chemical probe to detect neutrophil elastase activation during inflammatory bowel disease

Bethany M. Anderson<sup>1,2</sup>, Daniel P. Poole<sup>2</sup>, Luigi Aurelio<sup>3</sup>, Garrett Z. Ng<sup>1</sup>, Markus Fleischmann<sup>1,4</sup>, Paulina Kasperkiewicz<sup>5</sup>, Celine Morissette<sup>6</sup>, Marcin Drag<sup>5</sup>, Ian R. van Driel<sup>1</sup>, Brian L. Schmidt<sup>7</sup>, Stephen J. Vanner<sup>6</sup>, Nigel W. Bunnett<sup>2,8,9</sup> & Laura E. Edgington-Mitchell<sup>1,2,7</sup>

Neutrophil elastase is a serine protease that has been implicated in the pathogenesis of inflammatory bowel disease. Due to post-translational control of its activation and high expression of its inhibitors in the gut, measurements of total expression poorly reflect the pool of active, functional neutrophil elastase. Fluorogenic substrate probes have been used to measure neutrophil elastase activity, though these tools lack specificity and traceability. PK105 is a recently described fluorescent activity-based probe, which binds to neutrophil elastase in an activity-dependent manner. The irreversible nature of this probe allows for accurate identification of its targets in complex protein mixtures. We describe the reactivity profile of PK105b, a new analogue of PK105, against recombinant serine proteases and in tissue extracts from healthy mice and from models of inflammation induced by oral cancer and *Legionella pneumophila* infection. We apply PK105b to measure neutrophil elastase activation in an acute model of experimental colitis. Neutrophil elastase activity is detected in inflamed, but not healthy, colons. We corroborate this finding in mucosal biopsies from patients with ulcerative colitis. Thus, PK105b facilitates detection of neutrophil elastase activity in tissue lysates, and we have applied it to demonstrate that this protease is unequivocally activated during colitis.

Neutrophil elastase (NE) is a serine protease found within azurophilic granules of neutrophils<sup>1</sup>. During infection, active NE contributes to killing of pathogens, and mice lacking NE are more susceptible to bacterial and fungal infections<sup>1</sup>. NE also mediates inflammation by processing cytokines, chemokines and growth factors<sup>1</sup>. Furthermore, NE cleaves the extracellular N termini of protease-activated receptors (PARs), a family of G protein-coupled receptors (GPCRs), to initiate cellular signaling events that lead to inflammation and pain<sup>2–4</sup>.

NE has recently been implicated in the pathogenesis of inflammatory bowel diseases (IBD), which are characterized by chronic and relapsing inflammation in the gastrointestinal tract<sup>5</sup>. IBD comprises ulcerative colitis (UC) and Crohn's disease (CD), both of which are associated with diarrhea, rectal bleeding, increased urgency and pain. Mice lacking one copy of NE and a closely related neutrophil serine protease, proteinase 3 (PR3), exhibit improved symptoms in mouse models of colitis<sup>6</sup>. Enforced expression of elafin, an endogenous serine protease

<sup>1</sup>Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC, Australia. <sup>2</sup>Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia. <sup>3</sup>Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia. <sup>4</sup>Department of Cellular Immunology, Institute of Experimental Immunology, University Hospital Bonn, Bonn, Germany. <sup>5</sup>Department of Bioorganic Chemistry, Wrocław University of Technology, Wrocław, Poland. <sup>6</sup>Gastrointestinal Diseases Research Unit, Kingston General Hospital, Queen's University, Kingston, ON, Canada. <sup>7</sup>Department of Oral and Maxillofacial Surgery, New York University College of Dentistry, Bluestone Center for Clinical Research, New York, New York, USA. <sup>8</sup>Departments of Surgery and Pharmacology, Columbia University, New York, New York, USA. <sup>9</sup>Department of Pharmacology and Experimental Therapeutics, The University of Melbourne, Parkville, VIC, Australia. Correspondence and requests for materials should be addressed to L.E.E.-M. (email: [laura.edgingtonmitchell@unimelb.edu.au](mailto:laura.edgingtonmitchell@unimelb.edu.au))

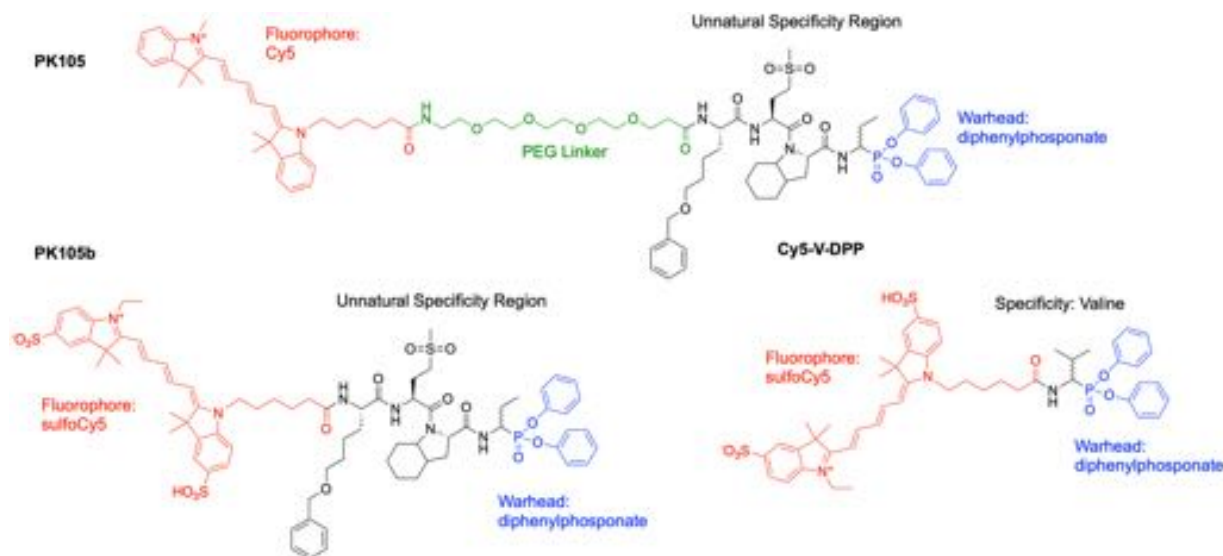


**Figure 1.** Structures of PK105<sup>16</sup>, PK105b, and Cy5-V-DPP<sup>13</sup>.

inhibitor, either by intracolonic administration of adenoviral vectors or introduction of elafin-expressing lactic acid bacteria, resulted in attenuation of symptoms in mouse models of colitis<sup>6</sup>. Treatment with a NE-selective inhibitor also reduced colitis symptoms<sup>7</sup>.

Colonic mucosal biopsies from patients with IBD exhibit elevated NE expression compared to healthy controls at both mRNA and protein levels<sup>8,9</sup>. Because NE is expressed as an inactive zymogen and can be tightly controlled by endogenous inhibitors once activated, measures of mRNA or total protein expression rarely reflect the pool of active functional enzyme<sup>10</sup>. Thus, tools to measure the specific activity of NE are required.

Commercially available chromogenic and fluorogenic substrate probes, including AAPV-p-nitroanilide and BODIPY-FL-elastin, respectively, have indicated an increase in elastase-like activity in biopsies from UC and CD patients and in mouse models of IBD<sup>6,7,11,12</sup>. However, these probes lack specificity and can be cleaved by multiple proteases<sup>5,10</sup>. Because the probes do not bind directly to the proteases upon cleavage, it is difficult to estimate the proportion of cleavage that is dependent on NE. Thus, improved tools are required to specifically measure NE activation and to improve our understanding of its functions during IBD pathogenesis.

We previously applied a fluorescent activity-based probe (ABP) for NE, Cy5-V-DPP, to track NE activation during colitis<sup>13</sup>. This probe contained a sulfonated cyanine 5 (Cy5) fluorophore and a P1 valine residue coupled to a diphenylphosphonate electrophile (DPP; 'warhead') that reacts with the active site serine of active NE in a covalent, irreversible manner (Fig. 1). While Cy5-V-DPP efficiently labeled recombinant NE and NE in cells with high expression (*e.g.*, bone marrow), we had little success in detecting NE activity in colitis tissues, presumably due to a lack of sensitivity. Use of hybrid combinatorial substrate libraries allowed for optimization of the NE substrate recognition sequence, which was coupled to Cy5 and the DPP warhead to generate the ABP, PK105<sup>14–17</sup>. While PK105 has been extensively characterized in purified neutrophils, its efficacy and specificity for NE in more complex tissue lysates has not been explored until now.

Herein, we characterized the reactivity profile of PK105b, an analogue of PK105, and explored its efficacy in complex tissue lysates. We applied PK105b in a mouse model of acute colitis and in human mucosal biopsies from patients with IBD to clearly measure activation of NE in inflamed tissue.

## Results

**Selectivity and potency of PK105b against purified serine proteases.** We synthesized an analogue of our previously published PK105 probe<sup>16</sup>, referred to as PK105b, in which the polyethylene glycol (PEG) linker was omitted and sulfoCy5 was used in place of the unsulfonated version (Fig. 1). This change allowed for a more direct comparison to our Cy5-V-DPP probe<sup>13</sup>, which included sulfoCy5 and no PEG. The only difference between the two probes compared in this study is the specificity region: Cy5-V-DPP contains a single P1 valine residue, while PK105b contains a tetrapeptide consisting of non-natural amino acids (Nle(OBzl)-Met(O)2-Oic-Abu) (Fig. 1).

We tested the reactivity of the PK105b against recombinant serine proteases, and compared its potency with Cy5-V-DPP. After incubation of proteases with increasing concentrations of probes for 30 minutes, the proteins were resolved by SDS-PAGE and binding was detected by in-gel fluorescence. Both probes clearly labeled NE and PR-3 in a concentration-dependent manner (Fig. 2A), though PK105b was more potent than Cy5-V-DPP. PK105b also clearly labeled trypsin, another serine protease, while trypsin binding by Cy5-V-DPP was negligible (Fig. 2A).

Using a commercially available fluorogenic substrate probe, AAPV-AMC, we compared the ability of the two probes to inhibit recombinant NE. Like Sivelestat, a commonly used NE inhibitor, PK105b immediately inhibited rNE activity at 0.1  $\mu$ M while Cy5-V-DPP only partially inhibited rNE activity at 1 and 10  $\mu$ M (Fig. 2B). These results indicate that PK105b binds to rNE more rapidly and more potently than Cy5-V-DPP. As Cy5-V-DPP and

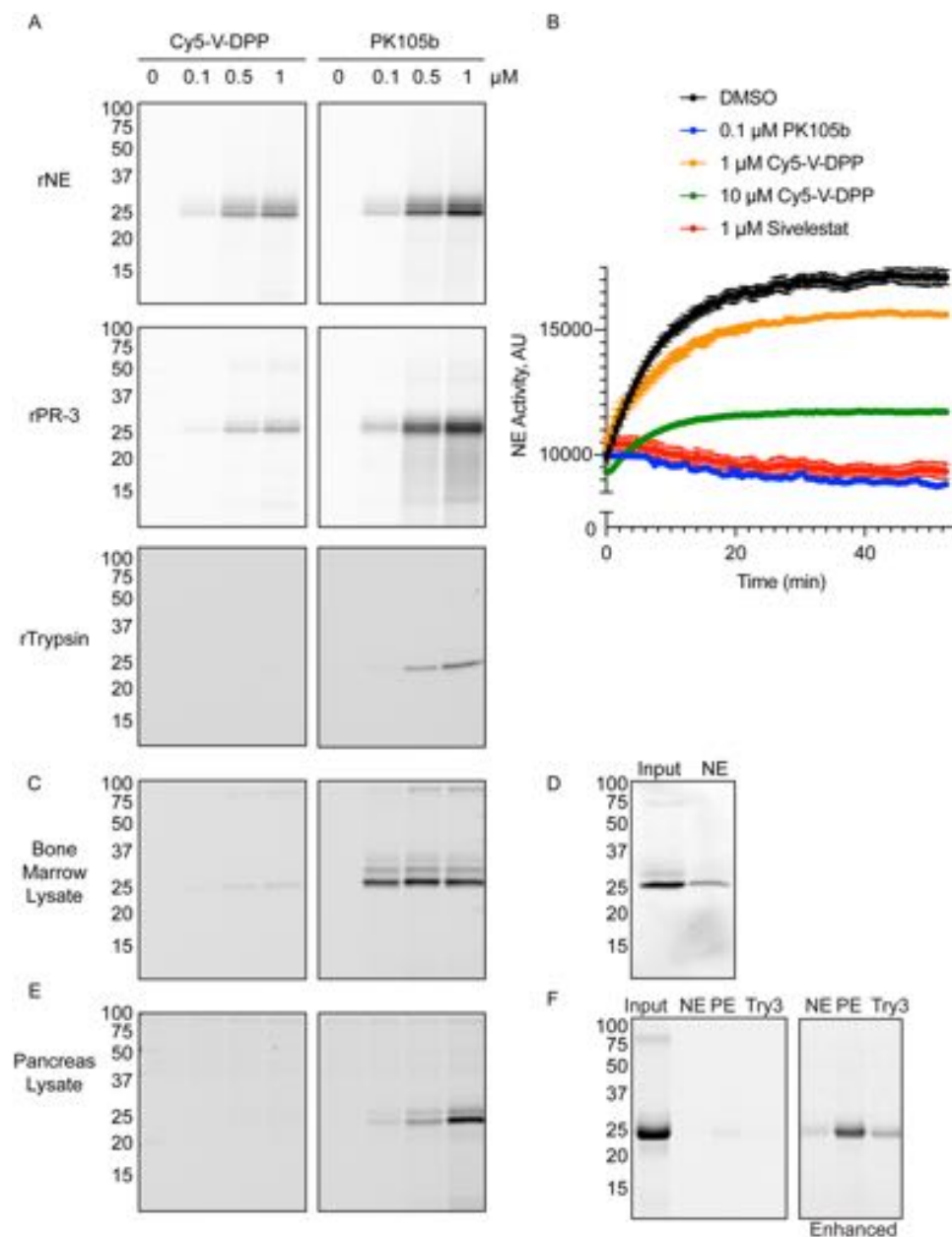


**Figure 2.** Comparison of selectivity profile for PK105b and Cy5-V-DPP. (A) Recombinant serine proteases (neutrophil elastase, proteinase 3, or trypsin) were incubated with increasing concentrations of Cy5-V-DPP or PK105b and binding was assessed by in-gel fluorescence. (B) Kinetic measurements of recombinant neutrophil elastase activity using the fluorogenic substrate probe, AAPV-AMC in the presence of DMSO (vehicle control) or the indicated concentration of PK105b, Cy5-V-DPP or Sivelestat. Error bars are shown as mean  $\pm$  SEM. (C,E) Lysates from mouse bone marrow or pancreas were incubated with increasing concentrations of Cy5-V-DPP or PK105b and binding was assessed by in-gel fluorescence. (D,F) PK105b-labeled lysates were immunoprecipitated with antibodies for neutrophil elastase, pancreatic elastase (PE) or trypsin 3 (Try3), as indicated. In (F), gain settings for pull-downs were enhanced in order to observe faint bands (right panel). Note: Cy5-V-DPP and PK105b labeling are depicted at equal gain settings, set independently for each sample type.

PK105b have identical DPP warheads, linkers, and sulfoCy5 fluorophores, we can attribute the improved potency and kinetics of PK105b to its extended non-natural specificity region, which likely fits better within the NE active site than valine alone.

**Selectivity and potency of PK105b in normal tissue lysates.** We compared the ability of PK105b and Cy5-V-DPP to detect protease activity in lysates prepared from mouse bone marrow. Two proteases were labeled by PK105b (Fig. 2C), and the 25-kDa protein was confirmed to be NE by immunoprecipitation with an NE-specific antibody (Fig. 2D). NE was specifically labeled by Cy5-V-DPP, but with a much lower potency than PK105b. We also examined the reactivity of the probes in lysates prepared from mouse pancreas, a tissue rich in

serine proteases. With PK105b, we observed strong labeling of 25-kDa proteins that was much more apparent than labeling by Cy5-V-DPP (Fig. 2E). Immunoprecipitation of PK105b-labeled lysates revealed that the target proteases consisted of a combination of NE, pancreatic elastase (PE), and trypsin 3 (Try3, also known as PRSS3 or mesotrypsin; Fig. 2F). Thus, PK105b exhibits a dramatic improvement in labeling efficiency in protein lysates over Cy5-V-DPP. In addition to the improved binding kinetics demonstrated above, the improved labeling profile of PK105b may also be due in part to enhanced stability of the compound afforded by the non-natural specificity region, which may be less prone to degradation than Cy5-V-DPP.

**Validation of PK105b in inflamed tissues.** To determine the effectiveness of PK105b to measure NE activation in inflamed tissues, we used a mouse model of *Legionella pneumophila* infection. *L. pneumophila* infection may result in Legionnaire's disease, a common cause of community or hospital-acquired pneumonia, and is associated with high neutrophil infiltration in the lung<sup>18,19</sup>. PK105b labeling was significantly increased in lysates prepared from infected lung tissues compared to uninfected lungs (Fig. 3A,C). The identity of the major 25-kDa species was confirmed to be NE by immunoblotting (Fig. 3B,D,E) and immunoprecipitation (Fig. 3F) with an NE-specific antibody. We also confirmed that PK105b binding was mediated by the DPP warhead and the specificity region, as the labelling could be competed by pre-treatment with PK101<sup>14,15</sup>, a biotinylated (non-fluorescent) analogue of PK105 (Fig. 3G). As neutrophils are the predominant source of NE during *L. pneumophila* infection, we also examined PK105b labelling in neutrophils from infected-lungs, which were sorted by flow cytometry at >97% purity (Fig. S1). Within this population of cells, we observed specific labeling of NE by PK105b (Fig. 3H).

We next determined the utility of PK105b to detect NE activation in a cancer setting, which is also rich in neutrophils<sup>20–22</sup>. Specifically, we utilized a mouse orthotopic xenograft model of oral squamous cell carcinoma in which human cancer cells (HSC-3) were injected into the tongue<sup>23</sup>. In this context, we observed clear labeling of a 25-kDa species in tumor tissues, but not normal tongue tissues (Fig. 4A,C). This species coincided with the size of mature NE as determined by immunoblot (Fig. 4B,D,E) and immunoprecipitation (Fig. 4F) with an NE-specific antibody. Several other high-molecular weight species were abundantly labeled by PK105b in these lysates (Fig. 4A), but they have not yet been identified. Nonetheless, they are likely to be binding to PK105b through the DPP and specificity region, and not Cy5, as binding could largely be competed with PK101, the non-fluorescent PK105 analogue (Fig. 4G).

**Application of PK105b to measure NE activation in experimental colitis.** Having validated PK105b in inflamed mouse tissues, we next applied this probe to investigate NE activation during acute experimental colitis induced by trinitrobenzenesulfonate (TNBS). As expected, mice exhibited loose stools, delayed defecation, weight loss (Fig. S2A), and colon shortening (Fig. S2B). We also observed damage to the mucosa by histological evaluation, as well as edema and inflammatory infiltrate (Fig. S2C). We analyzed colon lysates for NE activation by PK105b labeling and measurement of in-gel fluorescence. In proximal colons from healthy and inflamed mice, we observed little PK105b labeling. By contrast, in the distal region of inflamed colons, which is most affected in the TNBS model, we observed clear labeling of a 25-kDa protein (Fig. 5A). This band was virtually absent in distal colons of healthy mice that received vehicle instead of TNBS (Fig. 5A). We confirmed the identity of the protease to be NE by immunoprecipitation with an NE-specific antibody (Fig. 5D). All of the PK105b bands could be competed by PK101, the non-fluorescent PK105 analogue (Fig. 5E).

We also transferred the fluorescent gels to nitrocellulose membranes in order to immunoblot the samples for total NE expression. In all proximal colons and in healthy distal colons, we observed bands at 37 and 25 kDa (Fig. 5B). In the TNBS-treated distal colons, a new band appeared just below the 25-kDa protein. Only the lower species was labeled by PK105b, as revealed by overlay of the Cy5 fluorescence (Fig. 5C) and immunoprecipitation (Fig. 5D). To verify that the appearance of this smaller NE species was not an artefact of probe labeling, we immunoblotted inflamed distal colon samples in the presence and absence of PK105b. The smaller species was detected regardless of the presence of PK105b (Fig. 5F). Taken together, these data suggest that NE is subject to trimming in inflamed regions of the colon that permits its activation and thus its reaction with the PK105b probe.

For comparison, we also tested our previous NE probe, Cy5-V-DPP, in distal colon lysates and labeling of the 25-kDa species was barely distinguishable from the background (Fig. 5G). Thus, PK105b is clearly superior to Cy5-V-DPP for its ability to detect NE activity in tissue lysates. Both probes exhibit binding to several species in the 50–75-kDa range (Fig. 5A), and future proteomics assays will be required to determine their identity. Furthermore, we investigated secreted proteases found in the lumen of the colon (either luminal flush or in fecal pellets) with PK105b (Fig. 6A). In both samples, we observed two labeled proteases at 25 kDa. Immunoprecipitation confirmed low levels of NE in these samples, with pancreatic elastase and trypsin 3 being the predominant species (Fig. 6C). Nonetheless, NE activity could be clearly delineated by PK105b in lysates from colon tissues.

**Application of PK105b to measure NE activation in mucosal biopsies from IBD patients.** To translate our findings in mouse colitis to human disease, we examined PK105b labeling in mucosal biopsies from patients (Table 1). As in mice, we observed a significant increase in labeling in samples from patients with active UC compared to healthy individuals brought in for routine colonoscopy screening (Fig. 7A,D). In contrast to mice, where we observed a single 25-kDa species at labeled by PK105b, three species were labeled in human mucosal lysates, with the smallest form having the most activity. The banding pattern resembled that which was observed with recombinant human NE (Fig. 2A and in refs<sup>24,25</sup>). We confirmed these bands to be NE by immunoprecipitation with an NE-specific antibody (Fig. 7G), and they could be competed with PK101 (Fig. 7H).

Furthermore, when the same samples were immunoblotted for total NE expression, we observed NE bands in the healthy tissue at 37 and 25 kDa (Fig. 7B). UC tissues, however, displayed an additional doublet that was smaller than the 25-kDa species. The most active species, as indicated by PK105b labeling, corresponded to these

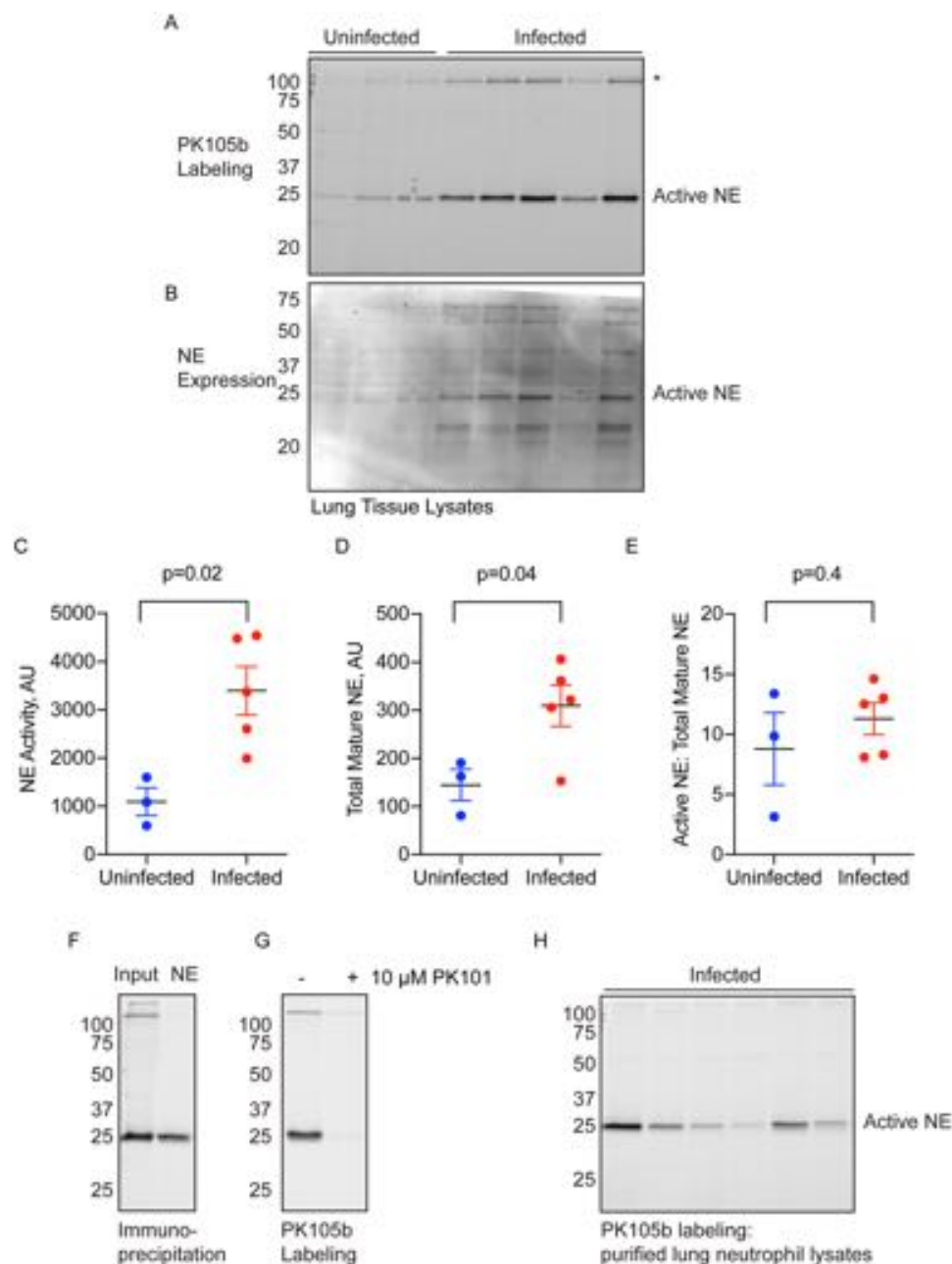


**Figure 3.** Detection of NE activity in *L. pneumophila*-infected lung tissue. (A) Lysates prepared from uninfected or *L. pneumophila*-infected lungs were labeled with 1  $\mu$ M PK105b and analyzed by in-gel fluorescence ( $n = 3-5$ ). \*Indicates high-molecular weight species of unknown identity. (B) Total NE expression in the samples in (A) was measured by immunoblotting with an NE-specific antibody. (C,D) Densitometry analysis of active NE in (A) and total mature NE in (B), respectively. (E) Ratios of active to total mature NE in the samples. (F) Labeling of NE by PK105b in infected lung tissue was confirmed by immunoprecipitation with an NE-specific antibody. (G) Competition of PK105b labeling in an infected lung lysate with PK101. (H) PK105b labeling in neutrophils isolated from infected lung tissue, as analyzed by in-gel fluorescence (refer to Fig. S1 for sorting strategy). Error bars are shown as mean  $\pm$  SEM.

smaller species (Fig. 7C,G). Thus, as we observed in mouse colitis, NE undergoes trimming during human UC that permits its activation and binding to PK105b.

Patient #13 was originally diagnosed with UC and we initially considered this biopsy to be an outlier given the low levels of NE activation. Upon follow-up studies, however, this diagnosis was re-evaluated and the patient was found to have an ileal stricture. The biopsies analyzed in this study were taken from normal tissue regions. These data therefore highlight the potential utility of using NE activity as a diagnostic marker for ulcerative colitis.

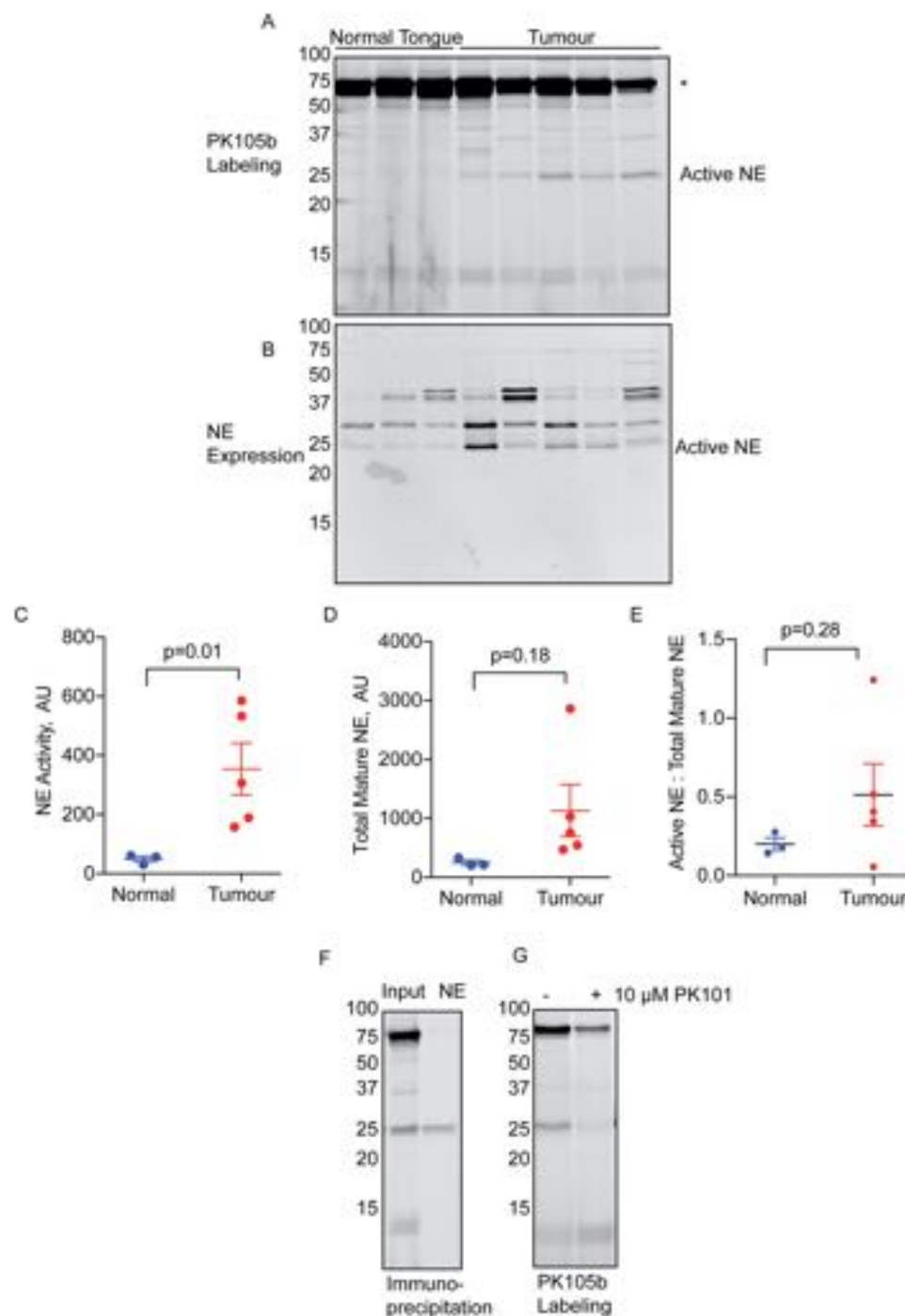


**Figure 4.** Detection of NE activity in oral cancer tissues. (A) Lysates prepared from normal mouse tongues or HSC-3 oral cancer tissues were labeled with 1  $\mu$ M PK105b and analyzed by in-gel fluorescence ( $n = 3-5$ ). \*Indicates high-molecular weight species of unknown identity. (B) Total NE expression in the samples in (A) was measured by immunoblotting with an NE-specific antibody. (C,D) Densitometry analysis of active NE in (A) and total mature NE in (B), respectively. (E) Ratio of active to total mature NE in the samples. Error bars are shown as mean  $\pm$  SEM. (F) NE labelling in cancer tissue was confirmed by immunoprecipitation with an NE-specific antibody. (G) Competition of PK105b labeling in oral cancer tissue lysate with PK101.

## Discussion

While PK105 was selective for NE in purified neutrophils, its ability to label NE in tissue lysates, which contain many cell types and proteases, had not been established. We tested PK105b, a close analogue of PK105, in bone marrow, pancreas, inflamed lungs, oral cancer, and human and mouse colitis tissues and fecal samples. Due to the covalent nature of PK105b, we could verify that NE was clearly targeted by this probe. We also observed that PK105b exhibited cross-reactivity with other serine proteases such as pancreatic elastase, proteinase-3, trypsin 3

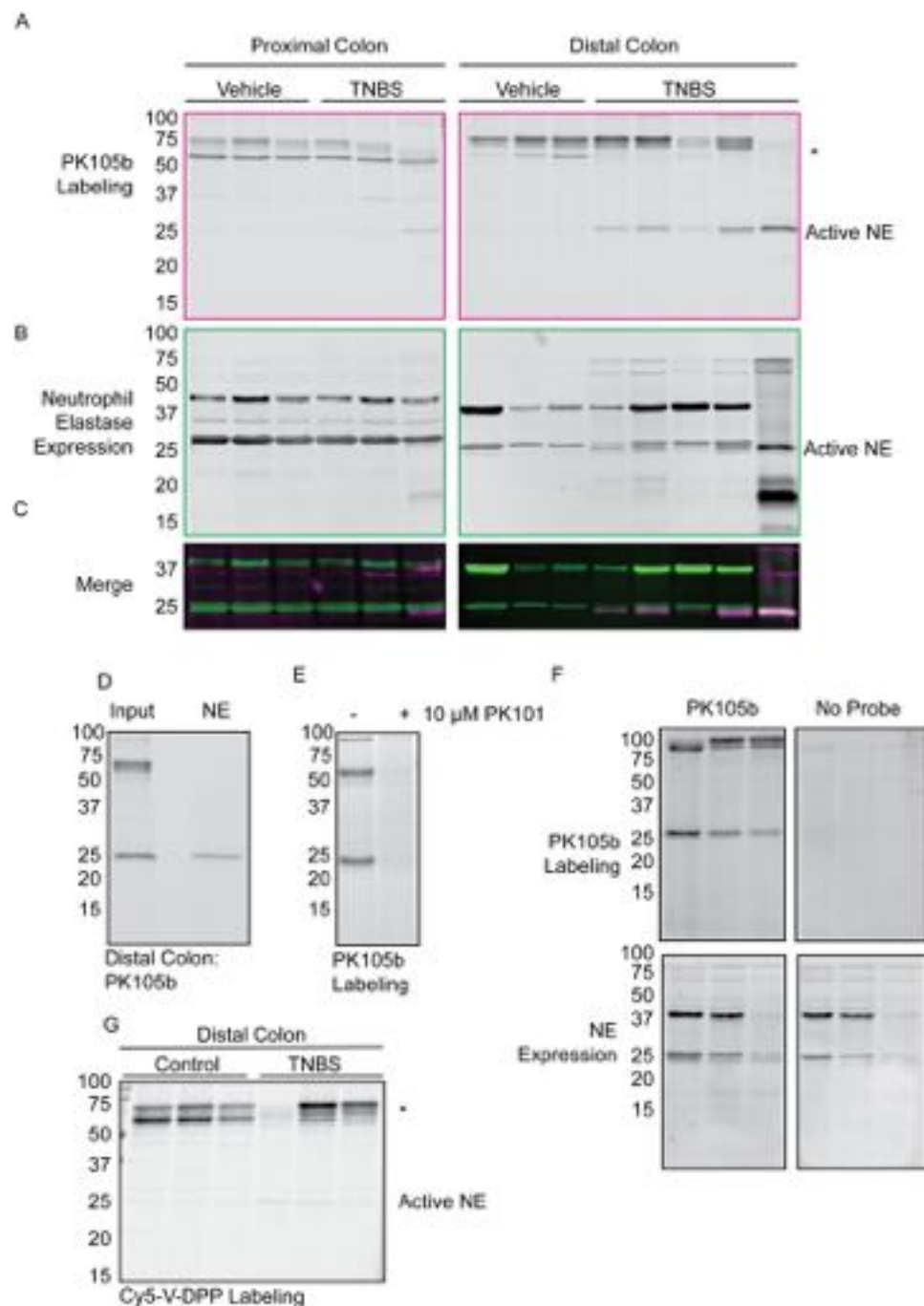


**Figure 5.** Application of PK105b to measure NE activation in acute experimental colitis. (A) Distal or proximal colons were excised from mice with acute colitis induced by TNBS and labeled *ex vivo* with 1  $\mu$ M PK105b. Probe binding was detected by in-gel fluorescence ( $n = 3-5$ ). \*Indicates high-molecular weight species of unknown identity. (B) Immunoblotting of samples in (A) with an NE-specific antibody to reveal total NE expression. (C) Merged image of PK105b labeling (active NE; magenta) and immunoblot (total NE expression; green). (D) Immunoprecipitation of PK105b-labeled inflamed distal colon lysate with a NE-specific antibody. (E) Competition of PK105b labeling in inflamed distal colon lysates with PK101. (F) In-gel fluorescence and NE immunoblot of distal colon lysates with or without PK105b labeling. (G) Distal colon lysates from A labeled with Cy5-V-DPP.

and other unknown proteases. This cross-reactivity was dependent on the tissues examined, with greater selectivity observed in purified lung neutrophils, lung tissue, and human colon biopsies and more cross-reactivity in pancreas, fecal samples, and cancer tissue. We confirmed that all PK105b binding was mediated by the DPP warhead in combination with the specificity region, and not due to non-specific binding via the Cy5 fluorophore, as all labeling could be blocked by pre-treatment with PK101, a non-fluorescent analogue of PK105.

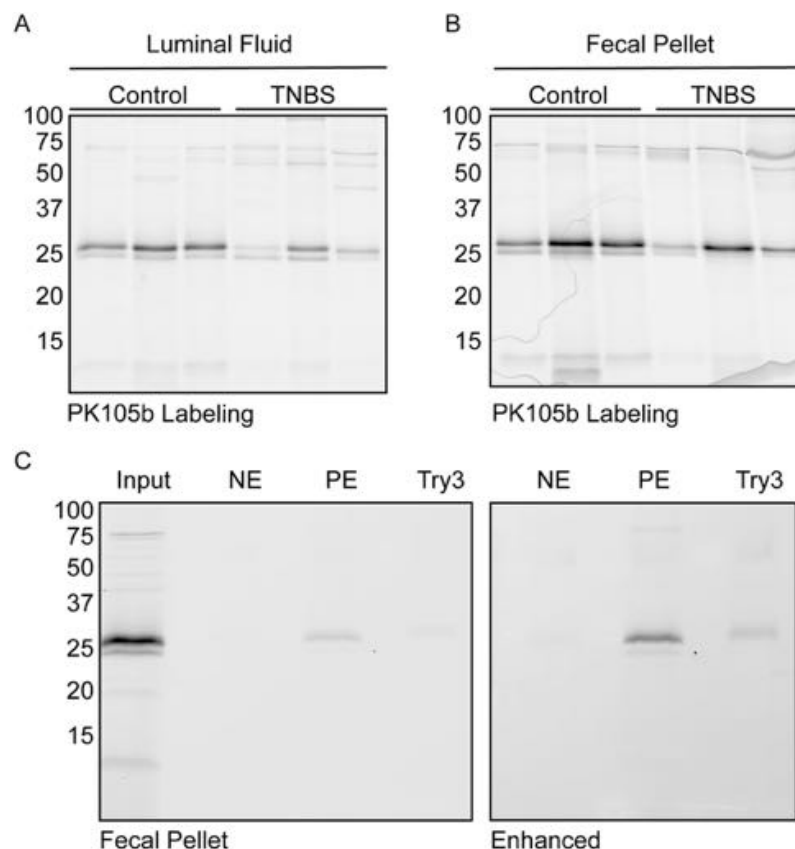


**Figure 6.** Luminal protease activity in acute experimental colitis. **(A,B)** Labeling of luminal fluids or fecal pellets from control or TNBS-treated mice with PK105b. **(C)** Immunoprecipitation of PK105b-labeled fecal samples with NE, PE, or trypsin 3-specific antibodies. Gain settings for the pull-downs were enhanced in order to observe faint bands (right panel).

While PK105b clearly has limitations, it is advantageous over traditional substrate probes such as AAPV-p-nitroanilide and BODIPY-FL-elastin, as these tools do not bind their targets covalently and the extent of their cross-reactivity in various contexts cannot readily be determined. Additionally, PK105b was advantageous over our previous diphenylphosphonate probe, Cy5-V-DPP<sup>13</sup>. In competition assays with the fluorogenic substrate, AAPV-AMC, PK105b reacted with rNE much more rapidly and potently than Cy5-V-DPP. This is most likely mediated by the improved non-natural specificity region in PK105b, which may bind more tightly within the NE active site. PK105b may also have enhanced sensitivity in tissue lysates due to increased stability, as its non-natural peptide sequence may be more resistant to degradation by other proteases than valine. PK105b is therefore one of the most effective tools available to study NE activation.

We applied PK105b to investigate NE activation during mouse colitis. In inflamed colons, we observed labeling of active NE, which was significantly increased compared to control tissue. Probe-labeled NE corresponded to the most mature species of NE detected by immunoblotting, which was not present in healthy tissue. We corroborated these findings in human colitis using mucosal biopsies from patients with UC, unequivocally demonstrating for the first time that NE is activated in IBD. Thus, PK105b is well suited for detection of both mouse and human NE during IBD and will be a valuable resource for future investigation of NE function. Furthermore, it may have utility as a diagnostic agent for IBD and for validating target engagement and efficacy of neutrophil elastase inhibitors in preclinical development as therapeutic agents.

## Experimental Procedures

**Probe synthesis and characterization.** Refer to Supplemental Materials for information on the synthesis of PK105b. Cy5-V-DPP<sup>13</sup> and PK101<sup>14</sup> were synthesized in house as described previously.

**Recombinant protease labeling.** Recombinant proteases (500 ng) were diluted in 20  $\mu$ l of phosphate-buffered saline (PBS): human neutrophil elastase (Elastin Products Company), porcine pancreatic trypsin type II-S (beta trypsin; Sigma), and human proteinase-3 (Sigma). PK105b or Cy5-V-DPP (0, 0.1, 0.5 or 1  $\mu$ M) was added from a 100x DMSO stock, and reaction was carried out at 37  $^{\circ}$ C for 30 minutes. Proteins were solubilized in 4x sample buffer (40% glycerol, 200 mM Tris-Cl [pH 6.8], 8% SDS, 0.04% bromophenol blue, 5% beta-mercaptoethanol), boiled and resolved on a 15% SDS-PAGE gel under reducing conditions. Probe labeling was detected by scanning the gel for Cy5 fluorescence on a Typhoon 5 flatbed laser scanner (GE Healthcare). Detailed protocols for ABP application are available in ref.<sup>26</sup>.

| Pt # | Symptoms                 | Medication           | Pathology                                                    | Endoscopy                             |
|------|--------------------------|----------------------|--------------------------------------------------------------|---------------------------------------|
| 1    | none                     | none                 | normal tissue                                                | none                                  |
| 2    | none                     | none                 | normal tissue                                                | none                                  |
| 3    | none                     | none                 | normal tissue                                                | none                                  |
| 4    | none                     | none                 | normal tissue                                                | none                                  |
| 5    | flare-up, 15–20 bm/d     | Steroids, biologic   | chronic inflammation, severe activity                        | pancolitis, Mayo 3 distal, 2 proximal |
| 6    | flare-up, 10 bm/d        | none                 | chronic inflammation, moderate to severe activity            | pancolitis, Mayo 2                    |
| 7    | chronic active, 4 bm/d   | none                 | chronic inflammation, mild activity                          | proctitis, Mayo 1                     |
| 8    | chronic active, 2–3 bm/d | 5-ASA                | chronic inflammation, marked activity                        | pancolitis, Mayo 3 distal, 2 proximal |
| 9    | new onset, 6–8 bm/d      | none                 | chronic inflammation, moderate activity                      | pancolitis, Mayo 2                    |
| 10   | chronic active, 12 bm/d* | Steroid enema, 5-ASA | chronic inflammation, mild activity                          | proctitis, Mayo 2                     |
| 11   | flare-up, 6–8 bm/d       | Imuran               | acute, chronic inflammation, deep ulcers                     | ileocolitis, deep ulcers              |
| 12   | flare-up, 4–5 bm/d       | 5-ASA                | chronic inflammation, severe activity                        | colitis                               |
| 13   | flare-up, pain, +2 bm/d  | 5-ASA                | normal tissue; chronic stricture with no active inflammation | ileal stricture, blind biopsies       |
| 14   | flare-up, 2–5 bm/day     | none                 | chronic inflammation, moderate activity                      | pancolitis, Mayo 1–2                  |

**Table 1.** Profiles of patients from which mucosal biopsies were collected. 5-ASA = 5 aminosalicylic acid; \*mucous, but infrequent stool; \*\*suspected flare up initially but with further imaging dx. with chronic stricture with no active inflammation; bm = bowel movement, most were bloody.

**Fluorogenic substrate assay.** Recombinant neutrophil elastase (20 nM) was diluted in PBS, and MeOSuc-Ala-Ala-Pro-Val-AMC (AAPV-AMC; 20  $\mu$ M; Bachem) was diluted in PBS containing PK105b (0.2  $\mu$ M), Cy5-V-DPP (2 or 20  $\mu$ M), sivelestat (2  $\mu$ M; Sigma) or 2% DMSO (vehicle control). The two solutions were mixed 1:1 in an opaque 96-well plate (100  $\mu$ l final volume), and probe-dependent fluorescence (355 nm excitation/460 nm emission) was immediately measured at 30 s intervals over the course of 1 h on a FLUOstar Omega plate reader (BMG Labtech).

**Animal ethics.** All animal experiments were conducted in accordance with the guidelines for the use of laboratory animals in research and approved protocols. Experiments involving healthy mice and colitis experiments were approved by the Monash University Animal Ethics Committee. Oral cancer experiments were approved by the Committee on Animal Research at New York University. Legionella experiments were approved by the University of Melbourne Animal Ethics Committee.

**Ex vivo tissue labeling.** Bone marrow was obtained by flushing tibias and femurs from healthy C57BL/6J mice with PBS. Cells were washed and resuspended in PBS prior to sonication on ice. Tissues were lysed by sonication on ice in PBS (10  $\mu$ l/mg tissue), and supernatants were cleared by centrifugation at 21,000 g for 10 min at 4 °C. Total protein (60  $\mu$ g, as measured by BCA assay, Pierce) was aliquoted in a total volume of 20  $\mu$ l PBS, and probe labeling and SDS-PAGE was carried out as above. Where indicated, PK101 (10  $\mu$ M) was added for 10 minutes prior to the addition of PK105b.

**Western blotting.** Fluorescent gels were transferred to nitrocellulose membranes and blotted using the Turbo Blot system (BioRad). Membranes were blocked using Li-Cor Odyssey blocking buffer diluted by 50% with PBS containing 0.05% Tween 20. Sheep anti-mouse neutrophil elastase/ELA2 (1:1000; R&D AF4517) was incubated overnight at 4 °C. Secondary antibody (goat-IR800, 1:5000; LiCor) was incubated for one hour at room temperature. Binding was detected by scanning with the IRLong filter on the Typhoon 5.

**Immunoprecipitation.** PK105b-labeled lysates (boiled in sample buffer) were divided into input and immunoprecipitation (IP) samples (100  $\mu$ g each). The IP samples were diluted in 500  $\mu$ l IP buffer (PBS [pH 7.4], 1 mM EDTA, 0.5% NP-40) along with 10  $\mu$ l antibody: Sheep anti-neutrophil elastase/ELA2 (R&D AF4517); rabbit anti-PRSS3 (Abcam ab105123); rabbit anti-pancreatic elastase (Abcam ab21593). Protein A/G beads (40  $\mu$ l slurry; Santa Cruz) were washed with IP buffer and then added to the sample. Tubes were rocked overnight at 4 °C. Beads were washed four times with IP buffer and once with 0.9% sodium chloride. After the last wash, all buffer was removed and beads were boiled in 2x sample buffer (20  $\mu$ l). Supernatants were then analyzed, alongside the input sample, by fluorescent SDS-PAGE as above.

**Legionella pneumophila infection model.** C57BL/6 mice were infected by intranasal inoculation with  $2.5 \times 10^6$  *L. pneumophila* 130b  $\Delta$ flaA in 50  $\mu$ l of PBS. Three days after infection, lungs were collected, minced and digested in 4 mL RPMI-1640 (Gibco) containing 3% FCS, 1 mg/mL DNaseI (Sigma-Aldrich) and 1 mg/mL Collagenase III (Worthington). Cells were collected by filtration through a 70- $\mu$ m filter, centrifuged and red blood cells lysed by resuspension in buffer containing 150 mM ammonium chloride and 50 mM Tris-HCl (pH 7.5) for 5 min. After washing with PBS containing 0.1% BSA and 2 mM EDTA, cells were stained with anti-Ly6G-FITC (1A8, BD Pharmingen), anti-CD11c-PE (N418, eBioscience), anti-Siglec-F-BV421 (E50-2440, BD Horizon), anti-CD64-Alexa 647 (X54-5/7.1, BD Pharmingen) and anti-Fc $\gamma$ II/III (2.4G2, WEHI monoclonal facility) for

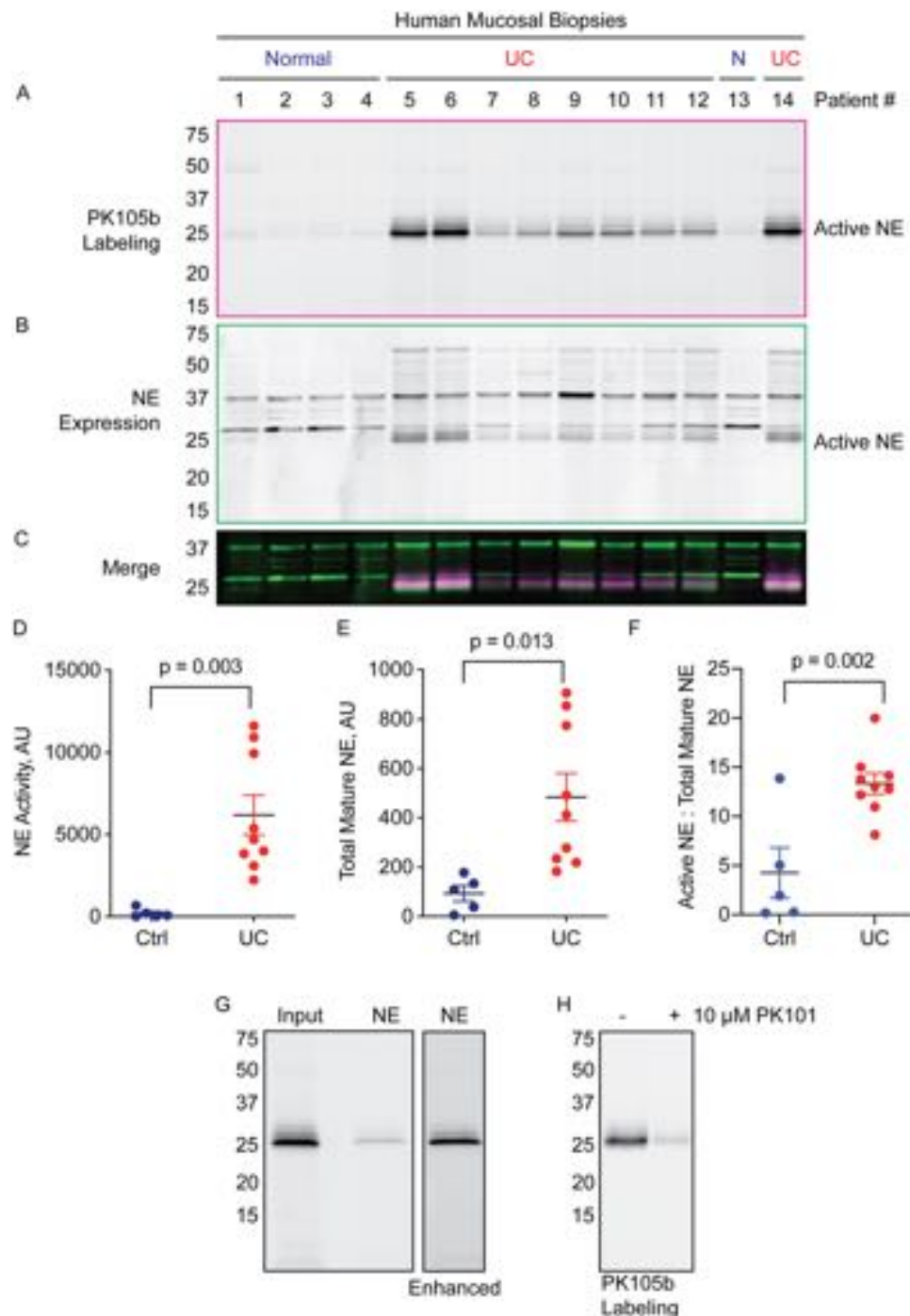


**Figure 7.** Application of PK105b to measure NE activation in mucosal biopsies from patients with IBD. **(A)** Mucosal biopsies from healthy controls ( $n = 5$ ) or patients with active UC ( $n = 9$ ) were labeled *ex vivo* with 1  $\mu$ M PK105b. Probe binding was assessed by in-gel fluorescence. **(B)** Samples in **(A)** were immunoblotted for total NE expression. **(C)** Merged image of PK105b labeling (active NE; magenta) and immunoblot (total NE expression; green). **(D,E)** Densitometry of active NE in **(A)** and total mature NE in **(B)**, respectively. **(F)** Ratio of active to total mature NE in the biopsy lysates. Error bars are shown as mean  $\pm$  SEM. **(G)** Immunoprecipitation of PK105b-labeled UC biopsy lysates with an NE-specific antibody. Gain settings for pulldown were enhanced in order to view faint bands (right panel). **(H)** Competition of PK105b labeling in inflamed colon lysates with PK101.

30 min at 4 °C. Cells were washed and resuspended in FACS buffer with 0.25  $\mu$ g/mL 7-AAD. Neutrophils were isolated using a Beckman Coulter MoFlo Astrios cell sorter into FCS and pellets of  $10^6$  cells snap frozen at  $-80^{\circ}\text{C}$ . Purities were assessed post-sorting using the same gating strategy. Purified neutrophils were lysed on ice in PBS with 0.1% Triton X-100, and supernatants were cleared by centrifugation. Alternatively, lung tissues from the same mice were snap frozen at the time of harvest and processed as above.

**Oral cancer model.** Female BALB/c nude mice (6–8 weeks old, Charles River Laboratories) were injected in the left lateral tongue under anesthesia ( $3 \times 10^5$  HSC-3 human oral squamous cell carcinoma cells suspended in 50  $\mu$ l vehicle [1:2 mixture of DMEM and Matrigel; Becton Dickinson], or vehicle alone). After two weeks, the resulting xenografted tumors and vehicle-injected tongues were excised, snap frozen, and analyzed as above.

**Colitis model.** Mice were purchased from the Monash University in-house colony. Colitis was induced in 10-week old male C57BL/6J mice by intracolonic infusion of picrylsulfonic acid solution (2,4,6-Trinitrobenzenesulfonic acid solution, TNBS; Sigma; 2.5 mg dissolved in 50% ethanol). Body weight and symptoms were recorded daily, and mice were humanely killed after three days. Upon colon extraction, luminal fluids were collected by flushing colons with PBS. Solids were removed by centrifugation and supernatant was concentrated using a 3-kDa cut-off centrifugal filter (Amgen). Pieces of proximal and distal colon were frozen for protease analysis or fixed in 4% paraformaldehyde overnight, paraffin embedded, sectioned, and stained with haematoxylin and eosin.

**Human mucosal biopsies.** Human mucosal biopsies were obtained from individuals during colonoscopy procedures at Hotel Dieu Hospital in Kingston, Ontario, Canada. Informed written and verbal consent was obtained prior to enrolment and all protocols were approved by and carried out in accordance with the guidelines and regulations of the Queen's University Human Ethics Committee. Patients were well-characterized individuals with active UC or healthy individuals undergoing routine colonoscopy for cancer screening (Table 1). For UC patients, biopsies were obtained from sites of active inflammation. Fresh biopsies were washed in PBS and then snap frozen for protease analysis as above.

**Statistical analysis.** All experiments were performed with at least three biological replicates. Data are reported as means  $\pm$  SEM. Statistical significance was determined by comparing two groups using a Student's t test, and p values of less than 0.05 were considered significant.

## Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

## References

- Korkmaz, B., Horwitz, M. S., Jenne, D. E. & Gauthier, F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. *Pharmacol. Rev.* **62**, 726–759 (2010).
- Zhao, P. *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).
- Lieu, T. *et al.* Antagonism of the proinflammatory and pronociceptive actions of canonical and biased agonists of protease-activated receptor-2. *Brit. J. Pharmacol.* **173**, 2752–2765 (2016).
- Jimenez-Vargas, N. N. *et al.* Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc. Nat. Acad. Sci.* **115**, E7438–E7447 (2018).
- Edgington-Mitchell, L. E. Pathophysiological roles of proteases in gastrointestinal disease. *Am. J. Physiol. Gastrointest. Liver Physiol.* **310**, G234–239 (2015).
- Motta, J. P. *et al.* Modifying the Protease, Antiprotease Pattern by Elafin Overexpression Protects Mice From Colitis. *Gastroenterol.* **140**, 1272–1282 (2011).
- Morohoshi, Y. *et al.* Inhibition of neutrophil elastase prevents the development of murine dextran sulfate sodium-induced colitis. *J. Gastroenterol.* **41**, 318–324 (2006).
- Uchiyama, K. *et al.* Serpin B1 protects colonic epithelial cell via blockage of neutrophil elastase activity and its expression is enhanced in patients with ulcerative colitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **302**, G1163–G1170 (2012).
- Kuno, Y. *et al.* Possible involvement of neutrophil elastase in impaired mucosal repair in patients with ulcerative colitis. *J. Gastroenterol.* **37**, 22–32 (2002).
- Edgington, L. E., Verdoes, M. & Bogoy, M. Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. *Cur. Op. Chem. Biol.* **15**, 798–805 (2011).
- Gecse, K. *et al.* Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic luminal factor impairing colonic permeability and sensitivity. *Gut* **57**, 591–599 (2008).
- Motta, J. P. *et al.* Food-Grade Bacteria Expressing Elafin Protect Against Inflammation and Restore Colon Homeostasis. *Sci. Translat. Med.* **4**, 158ra144 (2012).
- Edgington-Mitchell, L. E. *et al.* Fluorescent diphenylphosphonate-based probes for detection of serine protease activity during inflammation. *Bioorg. Med. Chem. Lett.* **27**, 254–260 (2017).
- Kasperkiewicz, P. *et al.* Design of ultrasensitive probes for human neutrophil elastase through hybrid combinatorial substrate library profiling. *Proc. Nat. Acad. Sci.* **111**, 2518–2523 (2014).
- Lechtenberg, B. C., Kasperkiewicz, P., Robinson, H., Drag, M. & Riedl, S. J. The Elastase-PK101 Structure: Mechanism of an Ultrasensitive Activity-based Probe Revealed. *ACS Chem. Biol.* **10**, 945–951 (2015).
- Kasperkiewicz, P., Altman, Y., D'Angelo, M., Salvesen, G. S. & Drag, M. Toolbox of Fluorescent Probes for Parallel Imaging Reveals Uneven Location of Serine Proteases in Neutrophils. *J. Am. Chem. Soc.* **139**, 10115–10125 (2017).
- Kasperkiewicz, P., Poreba, M., Groborz, K. & Drag, M. Emerging challenges in the design of selective protease substrates, inhibitors and activity-based probes for indistinguishable proteases. *FEBS J.* **284**, 1518–1539 (2017).
- Brown, A. S. *et al.* Cooperation between Monocyte-Derived Cells and Lymphoid Cells in the Acute Response to a Bacterial Lung Pathogen. *PLoS Pathog.* **12**, e1005691–20 (2016).
- Brown, A. S., Yang, C., Hartland, E. L. & van Driel, I. R. The regulation of acute immune responses to the bacterial lung pathogen *Legionella pneumophila*. *J. Leukocyte Biol.* **101**, 875–886 (2017).
- Lerman, I. & Hammes, S. R. Neutrophil elastase in the tumor microenvironment. *Steroids* **133**, 96–101 (2018).
- Kistowski, M. *et al.* A Strong Neutrophil Elastase Proteolytic Fingerprint Marks the Carcinoma Tumor Proteome. *Mol. Cell Proteomics* **16**, 213–227 (2017).
- 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 (2017).

23. 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 (2012).
24. Dau, T., Sarker, R. S. J., Yildirim, A. O., Eickelberg, O. & Jenne, D. E. Autoprocessing of neutrophil elastase near its active site reduces the efficiency of natural and synthetic elastase inhibitors. *Nat. Comm.* **6**, 1–8 (2015).
25. Schulz-Fincke, A.-C., Blaut, M., Braune, A. & Gütschow, M. A BODIPY-Tagged Phosphono Peptide as Activity-Based Probe for Human Leukocyte Elastase. *ACS Med. Chem. Lett.* **9**, 345–350 (2018).
26. Edgington, L. E. & Bogoy, M. *In vivo* imaging and biochemical characterization of protease function using fluorescent activity-based probes. *Curr. Protoc. Chem. Biol.* **5**, 25–44 (2013).

## Acknowledgements

We thank Cameron Nowell for maintaining the imaging facilities at the Monash Institute of Pharmaceutical Sciences, the Melbourne Histology Platform, and The Peter Doherty Institute ImmunoID flow cytometry facility. L.E.M. was supported by an Early Career Fellowship from the National Health and Medical Research Council of Australia (NHMRC, GNT1091636), a Grimwade Fellowship from the Russell and Mab Grimwade Miegunyah Fund at The University of Melbourne, a DECRA Fellowship from the Australian Research Council (ARC, DE180100418) and seed grants from Monash University. N.W.B. was supported by grants from the National Institutes of Health (NS102722, DE026806, DK118971) and the United States Department of Defense (W81XWH1810431). I.R.v.D., G.Z.N., and M.F. were supported by NHMRC grants (GNT1145244). M.F. was also supported by a fellowship by the German Research Council (DFG GRK 2168) and a Graduate Research Scholarship from The University of Melbourne. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

## Author Contributions

L.E.M. conceived study, planned all experiments, analyzed data, contributed funding, coordinated all collaborators, and wrote the manuscript. P.K. and M.D. produced the PK105 probe and contributed intermediates towards the synthesis of PK105b, which was made by L.A. B.M.A. obtained the biological data. G.Z.N., M.F., and I.R.v.D. provided samples for the *L. pneumophila* experiments. B.L.S. provided the oral cancer tissues. D.P.P. and N.B. contributed funding and intellectual input. S.V. and C.M. provided the human mucosal biopsies.

## Additional Information

**Supplementary information** accompanies this paper at <https://doi.org/10.1038/s41598-019-49840-4>.

**Competing Interests:** N.W.B. is a founding scientist of Endosome Therapeutics Inc. Research in N.W.B. and DP's laboratories is supported in part by Takeda Pharmaceuticals, Inc.

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019

# nature nanotechnology

**A non-opioid treatment option  
for chronic pain**



## BIOSENSING

The fouling problem is solved

## NANOREACTORS

Site-selective catalysis

## CANCER NANOTECHNOLOGY

All eyes on the microbiome

# A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain

Paulina D. Ramírez-García<sup>1,2</sup>, Jeffri S. Retamal<sup>1,2</sup>, Priyank Shenoy<sup>1,2</sup>, Wendy Imlach<sup>3</sup>, Matthew Sykes<sup>3</sup>, Nghia Truong<sup>1,2</sup>, Luis Constandil<sup>4</sup>, Teresa Pelissier<sup>4</sup>, Cameron J. Nowell<sup>1</sup>, Song Y. Khor<sup>1,2</sup>, Louis M. Layani<sup>1,2</sup>, Chris Lumb<sup>1,2</sup>, Daniel P. Poole<sup>1,2</sup>, TinaMarie Lieu<sup>1,2</sup>, Gregory D. Stewart<sup>1</sup>, Quynh N. Mai<sup>1,2</sup>, Dane D. Jensen<sup>5</sup>, Rocco Latorre<sup>5</sup>, Nicole N. Scheff<sup>6</sup>, Brian L. Schmidt<sup>6</sup>, John F. Quinn<sup>1,2</sup>, Michael R. Whittaker<sup>1,2</sup>, Nicholas A. Veldhuis<sup>1,2\*</sup>, Thomas P. Davis<sup>1,2,7\*</sup> and Nigel W. Bunnett<sup>1,2,5,8\*</sup>

**Nanoparticle-mediated drug delivery is especially useful for targets within endosomes because of the endosomal transport mechanisms of many nanomedicines within cells. Here, we report the design of a pH-responsive, soft polymeric nanoparticle for the targeting of acidified endosomes to precisely inhibit endosomal signalling events leading to chronic pain. In chronic pain, the substance P (SP) neurokinin 1 receptor (NK<sub>1</sub>R) redistributes from the plasma membrane to acidified endosomes, where it signals to maintain pain. Therefore, the NK<sub>1</sub>R in endosomes provides an important target for pain relief. The pH-responsive nanoparticles enter cells by clathrin- and dynamin-dependent endocytosis and accumulate in NK<sub>1</sub>R-containing endosomes. Following intrathecal injection into rodents, the nanoparticles, containing the FDA-approved NK<sub>1</sub>R antagonist aprepitant, inhibit SP-induced activation of spinal neurons and thus prevent pain transmission. Treatment with the nanoparticles leads to complete and persistent relief from nociceptive, inflammatory and neuropathic nociception and offers a much-needed non-opioid treatment option for chronic pain.**

Nanoparticle encapsulation improves drug efficacy by enhancing the stability, tolerability, delivery and retention in diseased tissues<sup>1–3</sup>. Interest in using nanoparticles to deliver anticancer drugs is perpetuated by the prospect of targeted delivery to tumour cells, and by the leaky vasculature and poor lymphatic drainage of tumours, which promote nanoparticle accumulation and uptake<sup>4</sup>. Stimulus-responsive nanoparticles can enhance targeted delivery and avoid undesirable exposure, further improving efficacy<sup>5</sup>. Triggers for nanoparticle disassembly and drug release include acidity, protease activity and redox imbalance within tumours. Inflammation and infection also acidify extracellular microenvironments<sup>6–9</sup>. However, few nanoparticle-based chemotherapeutics have been tested in patients, and the rationale has been questioned<sup>10</sup>.

Acidification of intracellular compartments, including endosomes and lysosomes, can be exploited for intracellular drug delivery<sup>11</sup>. For applications that require therapeutics to reach cytoplasmic or nuclear targets, the necessity and challenges of endosomal escape limit the usefulness of nanoparticle-mediated endosomal delivery<sup>12</sup>. The identification of drug targets within endosomes provides opportunities for harnessing pH-sensitive materials to chaperone

drugs to intracellular targets. The realization that G protein-coupled receptors (GPCRs) can signal from endosomes has created opportunities to improve drug efficacy and repurpose medicines<sup>13</sup>.

GPCRs are a large (>800) family of seven transmembrane proteins that control most physiological and pathological processes, and such GPCRs are the target of more than 30% of therapeutic drugs<sup>14</sup>. GPCR signalling is not confined to the plasma membrane, but also occurs within endosomes<sup>15,16</sup>. Location-biased compounds favour interactions with GPCRs in subcellular locations, leading to distinct signals<sup>17,18</sup>. Endosomal signalling of the substance P (SP) neurokinin 1 receptor (NK<sub>1</sub>R), calcitonin receptor-like receptor and protease-activated receptor-2 in primary sensory and spinal neurons mediates nociception<sup>19–21</sup>. Inhibitors of endocytosis and lipid-conjugated antagonists that target these receptors in endosomes provide effective anti-nociception<sup>19–21</sup>. Because these compounds are unlikely to be drug candidates, there remains the need to explore endosomal delivery of existing medicines. Endosomal delivery of GPCR ligands could enhance the treatment of many disorders<sup>13</sup>.

Herein, we demonstrate that soft polymeric pH-responsive nanoparticles alter the distribution and efficacy of an FDA-approved NK<sub>1</sub>R antagonist, aprepitant, which is used to treat emesis but has

<sup>1</sup>Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia. <sup>2</sup>Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville, Victoria, Australia. <sup>3</sup>Monash Biomedicine Discovery Institute, Monash University, Melbourne, Victoria, Australia. <sup>4</sup>Laboratory of Neurobiology, Center for the Development of Nanoscience and Nanotechnology (CEDENNA), University of Santiago de Chile, Santiago, Chile. <sup>5</sup>Departments of Surgery and Pharmacology, Columbia University Vagelos College of Physicians and Surgeons, Columbia University in the City of New York, New York, NY, USA. <sup>6</sup>Bluestone Centre for Clinical Research, New York University College of Dentistry, New York, NY, USA. <sup>7</sup>Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Brisbane, Queensland, Australia. <sup>8</sup>Department of Pharmacology and Therapeutics, University of Melbourne, Parkville, Victoria, Australia. \*e-mail: [nicholas.veldhuis@monash.edu](mailto:nicholas.veldhuis@monash.edu); [thomas.p.davis@monash.edu](mailto:thomas.p.davis@monash.edu); [nwb2@nyu.edu](mailto:nwb2@nyu.edu)

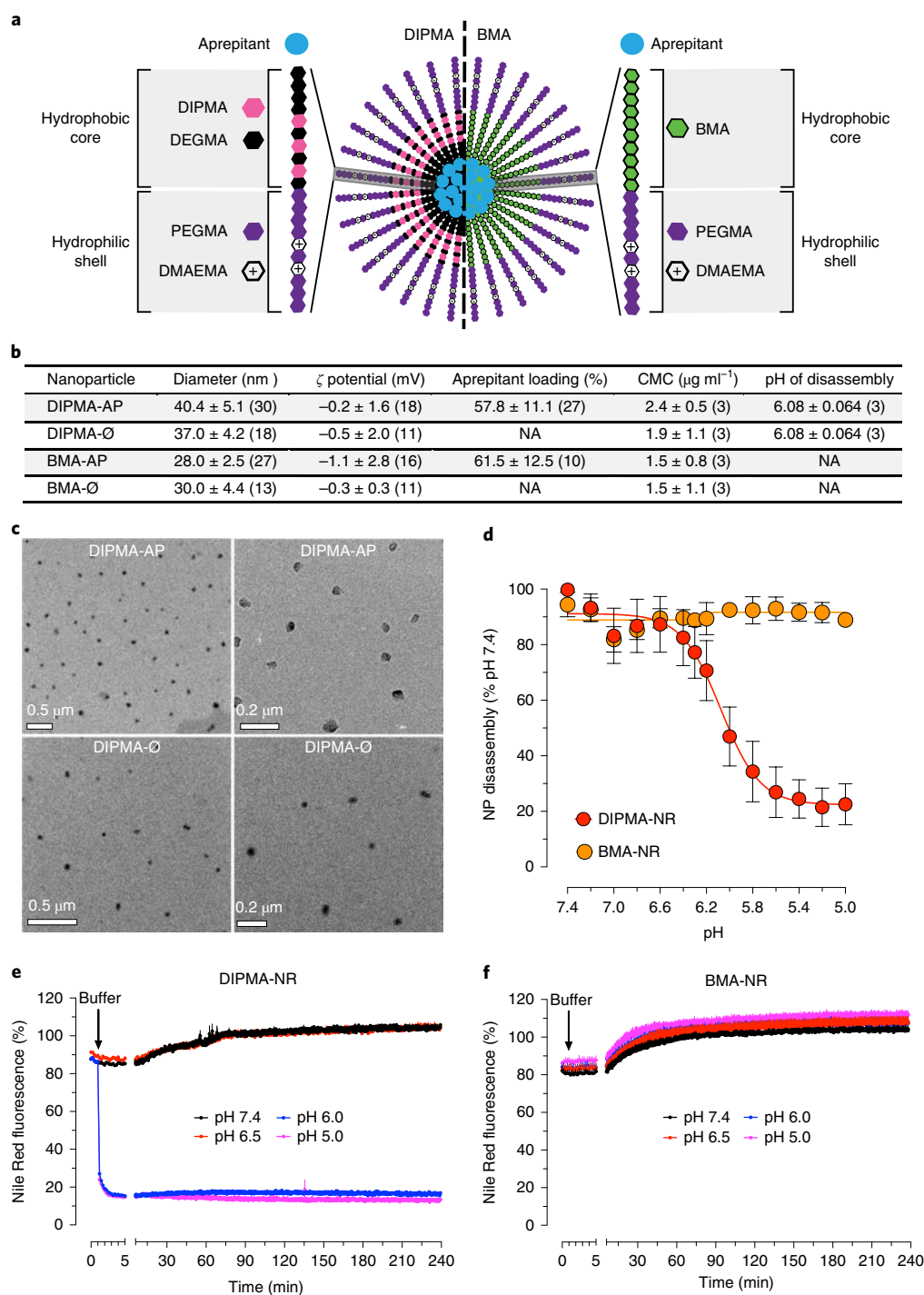


**Fig. 1 | Characterization of DIPMA and BMA nanoparticles.** **a**, Structure of pH-responsive (DIPMA) and pH-non-responsive (BMA) nanoparticles. Nanoparticles share the same hydrophilic shell of P(PEGMA-co-DMAEMA) but have different hydrophobic cores: P(DIPMA-co-DEGMA) or BMA. **b**, Properties of DIPMA- $\emptyset$ , BMA- $\emptyset$ , DIPMA-AP and BMA-AP (100 nM aprepitant) nanoparticles.  $\emptyset$ , empty; AP, aprepitant. Data are presented as mean  $\pm$  s.d. Values in parenthesis indicate the number ( $n$ ) of independent experimental replicates. Aprepitant incorporation for nanoparticles containing lower aprepitant concentrations (% initial aprepitant (mean  $\pm$  s.d.)): DIPMA-AP, 50 nM,  $58.4 \pm 7.7$ ,  $n = 9$  experiments; 25 nM,  $62.6 \pm 16.3$ ,  $n = 9$ ; BMA-AP, 50 nM,  $62.4 \pm 11.7$ ,  $n = 9$ ; 25 nM,  $65.2 \pm 16.2$ ,  $n = 9$ . CMC, critical micellar concentration. NA, not applicable. **c**, Transmission electron microscopy images of DIPMA-AP (100 nM aprepitant) and DIPMA- $\emptyset$  nanoparticles. Representative images of  $n = 2$  independent experiments are shown. **d**, pH-dependent Nile Red (NR) quenching of DIPMA-NR and BMA-NR nanoparticles in vitro, indicative of nanoparticle disassembly. Data are presented as mean  $\pm$  s.e.m.,  $n = 3$  independent experiments, triplicate observations. **e,f**, Time course of NR quenching of DIPMA-NR (**e**) and BMA-NR (**f**) nanoparticles in vitro and pH levels of 7.4, 6.5, 6.0 and 5.0. Data are presented as mean  $\pm$  s.e.m.,  $n = 3$  independent experiments, triplicate observations.

failed in trials for other indications<sup>22–24</sup>. Nanoparticles delivered aprepitant to endosomes containing activated NK<sub>1</sub>R, and induced a more complete and sustained anti-nociception in preclinical models

than conventional therapies, including opioids. Nanoparticle delivery minimized the dose of aprepitant required for anti-nociception, which might avoid off-target effects. Thus, nanoparticles have

potential beyond bulk drug delivery for cancer therapy and in fields where, to date, their applicability has been unrecognized. The use of nanoparticles to direct drugs to subcellular compartments from which GPCRs generate disease-relevant signals has broad applicability. The discovery that nanoparticle encapsulation enhances and prolongs analgesia provides opportunities for developing much-needed non-opioid therapies for pain.

## Results

**Synthesis and pH-dependent disassembly of nanoparticles.** Diblock copolymers were synthesized with the same hydrophilic shell of P(PEGMA-co-DMAEMA), but with different hydrophobic cores of P(DIPMA-co-DEGMA) to form pH-responsive nanoparticles or P(BMA) to form non-pH-responsive nanoparticles (DIPMA or BMA nanoparticles, respectively; Fig. 1a and Supplementary Fig. 1a,b). Gel permeation chromatography and  $^1\text{H}$ -NMR confirmed the molecular weight and composition of the nanoparticles (Supplementary Fig. 1c,d).

Nanoparticles were self-assembled with aprepitant (MK-869<sup>22</sup>), a hydrophobic NK<sub>1</sub>R antagonist, forming DIPMA-aprepitant (DIPMA-AP) and BMA-aprepitant (BMA-AP) (Fig. 1b). To generate nanoparticles for delivery of graded concentrations of aprepitant (25, 50 or 100 nM) but a constant concentration of polymer, nanoparticles were self-assembled with graded amounts of aprepitant and a fixed amount of polymer. For most studies, nanoparticles containing 100 nM aprepitant were used, with similar incorporation efficiency (Fig. 1b).

Nanoparticles are dynamic structures that remain assembled when the concentration of polymer exceeds the critical micelle concentration (Supplementary Fig. 2). The critical micellar concentrations of DIPMA-empty (DIPMA- $\emptyset$ ), BMA- $\emptyset$ , DIPMA-AP and BMA-AP were comparable (Fig. 1b). Nanoparticles were uniformly spherical, with similar diameters and  $\zeta$  potentials (Fig. 1b).

To examine pH-dependent disassembly, nanoparticles were loaded with Nile Red (NR), which fluoresces only in the hydrophobic core. Fluorescence quenching in aqueous solutions of graded pH was used to evaluate nanoparticle disassembly. DIPMA-NR fluorescence declined with increasing acidity (50% decrease,  $\text{pH } 6.08 \pm 0.06$ ; Fig. 1d), consistent with the protonation of the DIPMA tertiary amine ( $\text{p}K_{\text{a}} = 6.1$ ), charge repulsion and disassembly (Supplementary Fig. 2). BMA-NR fluorescence was unaffected by acidification (Fig. 1d). DIPMA-NR fluorescence declined to minimum levels within 4 min at pH 6.0 or 5.0 (Fig. 1e) whereas BMA-NR did not decline in acidic buffers (Fig. 1f). There was a small unexplainable increase in DIPMA-NR fluorescence at pH 6.5 or 7.4 and in BMA-NR fluorescence at pH 7.4, 6.5, 6.0 or 5.0.

**Uptake and disassembly of nanoparticles in cells.** Cellular uptake and trafficking of DIPMA nanoparticles labelled with cyanine 5 (DIPMA-Cy5) were examined by confocal microscopy in HEK-293 cells. NK<sub>1</sub>R endosomal trafficking and signalling are similar in HEK-293 cells and spinal neurons<sup>19</sup>. After incubation for 30 or 60 min, DIPMA-Cy5 nanoparticles were localized to early and late endosomes (Fig. 2a, Supplementary Fig. 3a and Supplementary Videos 1 and 2). HEK-293 cells expressing rat (r)NK<sub>1</sub>R-GFP were treated with SP to evoke NK<sub>1</sub>R endocytosis. At 30 and 60 min after SP, DIPMA-Cy5 nanoparticles co-localized with rNK<sub>1</sub>R-GFP in endosomes (Fig. 2b, Supplementary Fig. 3b and Supplementary Video 3). Determination of the Manders overlap coefficient<sup>25</sup> confirmed DIPMA-Cy5 co-localization with rNK<sub>1</sub>R-GFP, Rab5a-GFP and Rab7a-GFP (Fig. 2c).

The uptake and disassembly of DIPMA nanoparticles loaded with Coumarin 153 (DIPMA-CO), which fluoresces in an aqueous environment but not in the hydrophobic core, were examined by confocal microscopy and high content imaging. When DIPMA-CO nanoparticles were incubated with HEK-293 cells, there was an

increase in intracellular fluorescence from 1 to 10 min that continued for 30 min (Fig. 2e,f). Inhibitors of clathrin (PitStop2)<sup>26</sup>, dynamin (Dyno4a)<sup>27</sup> and endosomal acidification (Bafilomycin A1, which inhibits the vacuolar H<sup>+</sup>ATPase; NH<sub>4</sub>Cl, a lysosomotropic weak base) attenuated cellular fluorescence (Fig. 2c–f). These results are consistent with clathrin- and dynamin-dependent endocytosis, and pH-dependent disassembly of DIPMA nanoparticles in acidified endosomes. When non-pH-disassembling BMA-Cy5 nanoparticles were incubated with HEK-293 cells, there was a smaller increase in fluorescence from 1 to 10 min (Fig. 2d,e). Although PitStop2 and Dyno4a suppressed the fluorescence, Bafilomycin A1 and NH<sub>4</sub>Cl had no effect (Fig. 2d–f). BMA nanoparticles also enter cells by clathrin- and dynamin-dependent endocytosis, and release cargo by mechanisms that do not require endosomal acidification.

**Biodistribution and delivery of nanoparticle cargo.** To examine nanoparticle distribution in vivo, DIPMA-Cy5 or BMA-Cy5 nanoparticles were injected intrathecally (L4/L5), which delivers NK<sub>1</sub>R antagonists to spinal neurons<sup>19</sup>. Non-invasive imaging revealed that Cy5 fluorescence, which might be incorporated within nanoparticles or disassembled fluorophore, remained within the injection site for up to 24 h (Fig. 3a,b). Confocal imaging showed that DIPMA-Cy5 and BMA-Cy5 nanoparticles accumulated in a perinuclear region in cells throughout laminae I, II and III of the dorsal horn (Fig. 3c and Supplementary Videos 4 and 5). DIPMA-Cy5 nanoparticles were present in neurons, identified by co-localization with the neuronal marker PGP9.5, although detailed analysis of the cellular distribution was not possible due to loss of nanoparticle fluorescence during immunostaining.

To evaluate the usefulness of nanoparticles for drug delivery, free aprepitant, DIPMA-AP or BMA-AP was injected intrathecally to mice, then liquid chromatography–mass spectrometry (LC-MS) was used to quantify aprepitant in the spinal cord. At 1 h after injection, the spinal aprepitant concentration was approximately twofold higher for DIPMA-AP than for BMA-AP and approximately fourfold higher than for free aprepitant (Fig. 3d). At 4 h, spinal aprepitant was similar for DIPMA-AP and BMA-AP, and almost undetectable for free aprepitant. Thus, nanoparticle encapsulation causes retention of aprepitant within the spinal cord.

**Effects of nanoparticles on nociception.** To examine the hypothesis that incorporation into nanoparticles enhances the anti-nociceptive actions of aprepitant due to delivery to spinal neurons, the efficacy of free or nanoparticle-encapsulated aprepitant was evaluated in preclinical models of nociceptive, inflammatory and neuropathic pain (Fig. 4d). Nanoparticles, free aprepitant or vehicle was injected intrathecally before or after intraplantar injection of algogens or nerve injury. Mechanical nociception was studied in mice by measuring withdrawal responses to stimulation of the plantar surface of the hindpaw with calibrated von Frey filaments (VFFs) and in rats by measuring the pressure that induced withdrawal of the hindpaw (Randall–Selitto test).

Assessment of nociception requires normal motor coordination, which allows paw withdrawal from painful stimuli. The latency to fall from a rotarod was the same in mice after intrathecal injection of vehicle, DIPMA-AP, BMA-AP or DIPMA- $\emptyset$  (Supplementary Fig. 4). Nanoparticles do not interfere with motor coordination.

**Capsaicin.** Intraplantar capsaicin activates transient receptor potential-1 on primary sensory neurons to release SP in the dorsal horn, which evokes NK<sub>1</sub>R endocytosis in spinal neurons and allodynia<sup>19,28</sup>. In mice pretreated with intrathecal vehicle or DIPMA- $\emptyset$ , capsaicin decreased the VFF threshold from 0.5 to 4 h, which returned to baseline after 24 h (Fig. 4b,c). Free aprepitant (100 nM) and DIPMA- $\emptyset$  mixed with free aprepitant (100 nM) caused a modest anti-nociception after 1 h ( $16 \pm 4$  and  $15 \pm 3\%$  inhibition, respectively). BMA-AP

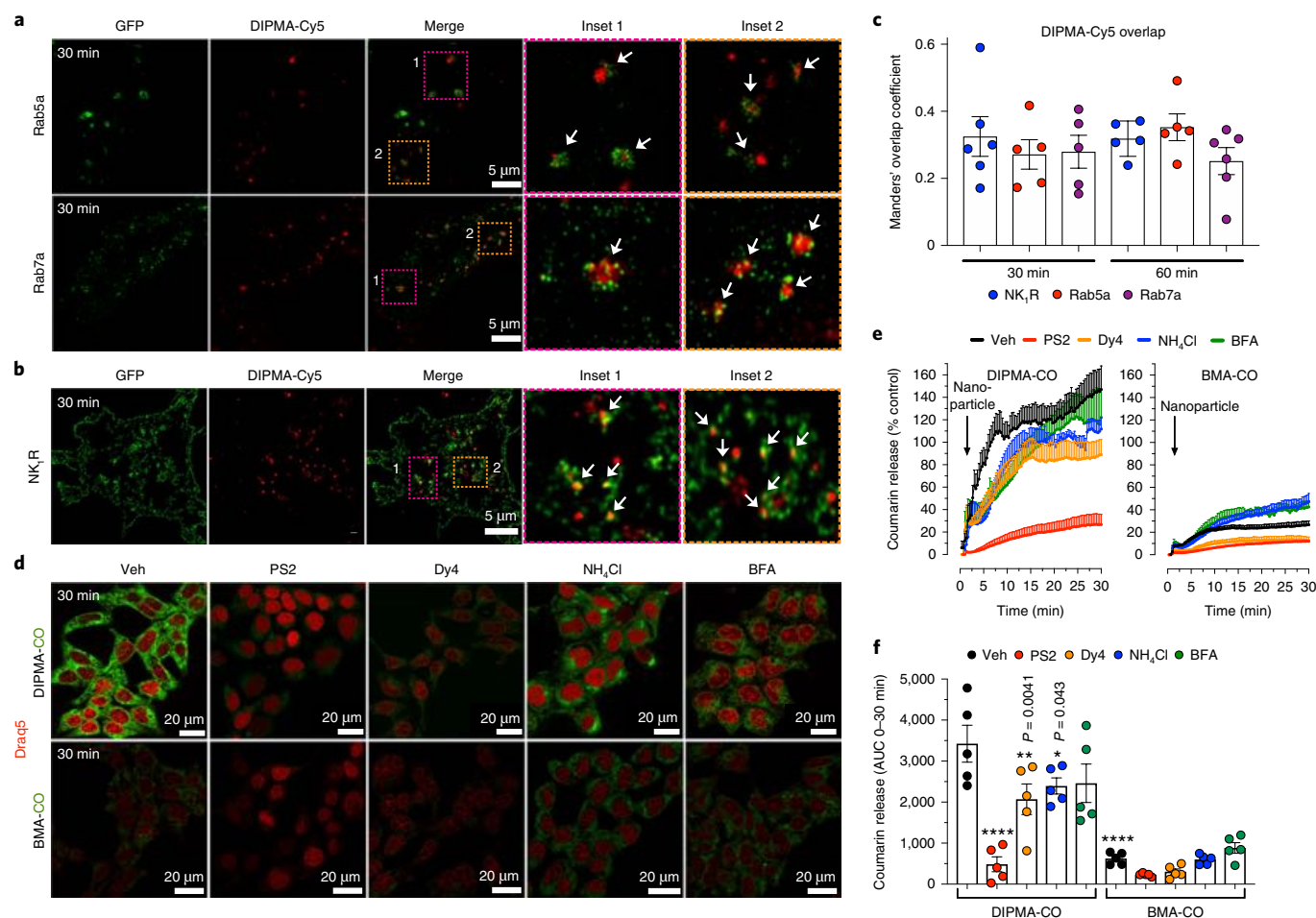


**Fig. 2 | Uptake and disassembly of DIPMA and BMA nanoparticles in HEK-293 cells.** **a**, To examine trafficking to endosomes, DIPMA-Cy5 nanoparticles were incubated with HEK-293 cells expressing Rab5a-GFP, which identifies early endosomes, or Rab7a-GFP, which marks late endosomes. Images show localization of DIPMA-Cy5 nanoparticles in Rab5a-GFP-positive early endosomes and Rab7a-GFP-positive late endosomes after incubation with HEK-293 cells for 30 min. Representative images of  $n = 5$  independent experiments are shown. **b**, To determine whether nanoparticles traffic to endosomes containing the NK<sub>1</sub>R, DIPMA-Cy5 nanoparticles were incubated with HEK-293 cells transfected with rNK<sub>1</sub>R-GFP; after 30 min, cells were challenged with 10 nM SP to promote internalization of rNK<sub>1</sub>R-GFP. The images show co-localization of DIPMA-Cy5 nanoparticles and NK<sub>1</sub>R-GFP in HEK-rNK<sub>1</sub>R cells at 30 min after stimulation with SP to induce NK<sub>1</sub>R endocytosis. Representative images of  $n = 5$  independent experiments are shown. **c**, Manders overlap coefficient to assess the degree of co-localization of DIPMA-Cy5 with NK<sub>1</sub>R-GFP, Rab5a-GFP and Rab7a-GFP at 30 min and 60 min. Data are presented as mean  $\pm$  s.e.m.,  $n = 5$  independent experiments. **d-f**, Uptake of DIPMA-CO and BMA-CO nanoparticles into HEK-293 cells. **d**, Representative images of  $n = 5$  independent experiments at 30 min after addition of DIPMA-CO or BMA-CO nanoparticles; Coumarin 153 is green, Draq5 nuclear stain is red. **e, f**, Kinetic analysis and quantification of uptake and disassembly of DIPMA-CO and BMA-CO nanoparticles assessed by Coumarin 153 fluorescence. In cells treated with DIPMA-CO, Coumarin 153 rapidly accumulated in the cytosol, indicative of rapid nanoparticle disassembly. PitStop2 (PS2), Dyngo4a (Dy4), Bafilomycin A1 (BFA) and NH<sub>4</sub>Cl inhibited the appearance of Coumarin 153 and nanoparticle disassembly. In cells treated with BMA-CO, Coumarin 153 slowly accumulated in the cytosol, indicative of minimal nanoparticle disassembly. PitStop2 and Dyngo4a, but not Bafilomycin A1 and NH<sub>4</sub>Cl, inhibited appearance of Coumarin 153 and nanoparticle disassembly. Data are presented as mean  $\pm$  s.e.m.,  $n = 5$  (**e, f**) independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$  compared to DIPMA-CO treated with vehicle. Two-way analysis of variance (ANOVA), Dunnett's post-hoc test.

(100 nM aprepitant) had a similar effect after 0.5–1 h, although the effect was sustained for 2 h. DIPMA-AP (100 nM aprepitant) caused marked anti-nociception at 0.5–1 h (1 h,  $34 \pm 3\%$  inhibition) that was sustained for 4 h ( $35 \pm 2\%$  inhibition).

**Complete Freund's adjuvant.** Intraplantar complete Freund's adjuvant (CFA) causes sustained mechanical allodynia and NK<sub>1</sub>R endocytosis in spinal neurons<sup>19,29</sup>, which allowed examination of the capacity of nanoparticle-encapsulated aprepitant to reverse inflammatory nociception. By 48 h after CFA injection, there was a marked decrease in VFF threshold (Fig. 4d–f). Intrathecal vehicle did not affect mechanical hyperalgesia, which persisted for 24 h. Aprepitant (100 and 300 nM) dose-dependently reversed hyperalgesia for 2–3 h

(1.5 h, % inhibition: 100 nM,  $30 \pm 6$ ; 300 nM,  $47 \pm 3\%$ ). BMA-AP (100 nM aprepitant) was as effective as free aprepitant (300 nM). DIPMA-AP (100 nM aprepitant) produced a larger inhibition of allodynia than the same dose of free aprepitant (1.5 h, % inhibition:  $54 \pm 4\%$ ), and the inhibition was maintained for 6 h, when other treatments were ineffective. Although systemic morphine (3 mg kg<sup>-1</sup>, intraperitoneal) fully reversed the mechanical allodynia after 0.5 h, the effect waned after 3 h.

**Nerve injury.** The sural nerve spared (SNS) model produces a mechanical hyperalgesia in rats for >50 days<sup>30,31</sup>, which permitted examination of the efficacy of nanoparticle-encapsulated aprepitant to relieve chronic neuropathic nociception in another species.

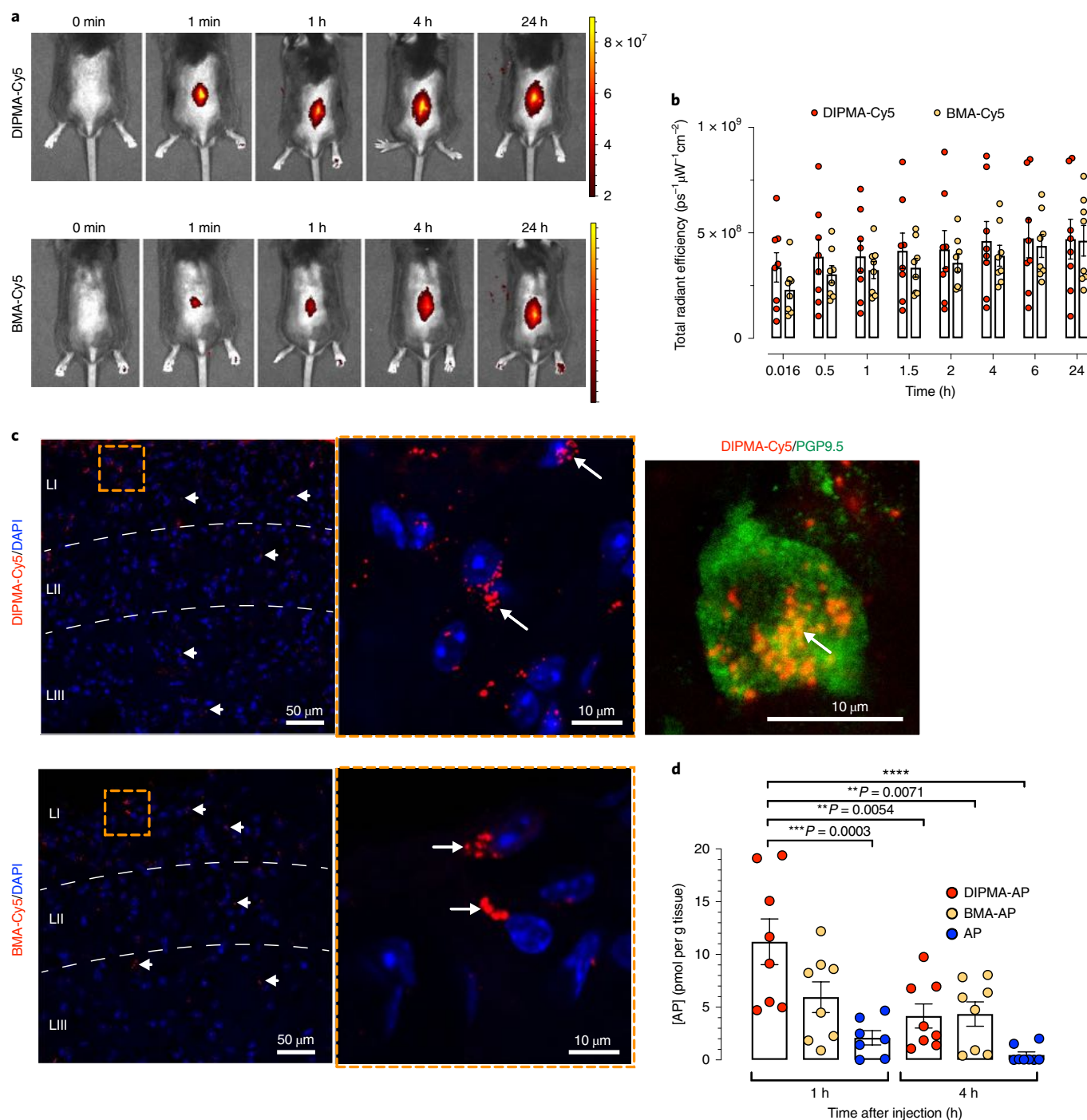


**Fig. 3 | Biodistribution and cellular uptake of nanoparticles and aprepitant delivery.** **a**, Distribution of DIPMA-Cy5 and BMA-Cy5 nanoparticles at various times after intrathecal injection of mice. Representative images of experiments on  $n=8$  mice are shown. Scale bar, Cy5 fluorescence intensity measured as radiant efficiency with units  $\text{ps}^{-1}\mu\text{W}^{-1}\text{cm}^{-2}$ . p, photons. s, seconds. W, watts. **b**, Quantification of the distribution of DIPMA-Cy5 and BMA-Cy5-nanoparticles at various times after intrathecal injection of mice assessed as the radiant efficiency of the images. Data are presented as mean  $\pm$  s.e.m.,  $n=8$  mice. **c**, Localization of DIPMA-Cy5 and BMA-Cy5 nanoparticles in the dorsal horn (laminae, LI–LIII) 1 h after intrathecal injection. The inset to the right shows accumulation of DIPMA-Cy5 nanoparticles in a perinuclear region of a spinal neuron, as confirmed by labelling with anti-PGP9.5. Representative images from  $n=5$  mice. **d**, Aprepitant concentrations in the spinal cord measured 1 h and 4 h after intrathecal injection of DIPMA-AP, BMA-AP or free aprepitant (100 nM). Data are presented as mean  $\pm$  s.e.m.;  $n=7$  mice for aprepitant at 1 h and  $n=8$  mice for aprepitant at 4 h (and DIPMA-AP and BMA-AP at 1 h and 4 h). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . Two-way ANOVA, Tukey's post-hoc test.

To confirm activation of the SP/NK<sub>1</sub>R system, we localized NK<sub>1</sub>R immunoreactivity (IR) in spinal neurons at 10 days after sham or SNS surgery by immunofluorescence. In sham rats, NK<sub>1</sub>R-IR was confined to the plasma membrane of the soma and neurites of

lamina I neurons (Supplementary Fig. 5a and Supplementary Video 6). In SNS rats, NK<sub>1</sub>R-IR was detected in endosomes of ipsilateral lamina I neurons but was localized to the plasma membrane of contralateral lamina I neurons (Supplementary Fig. 5a and Supplementary

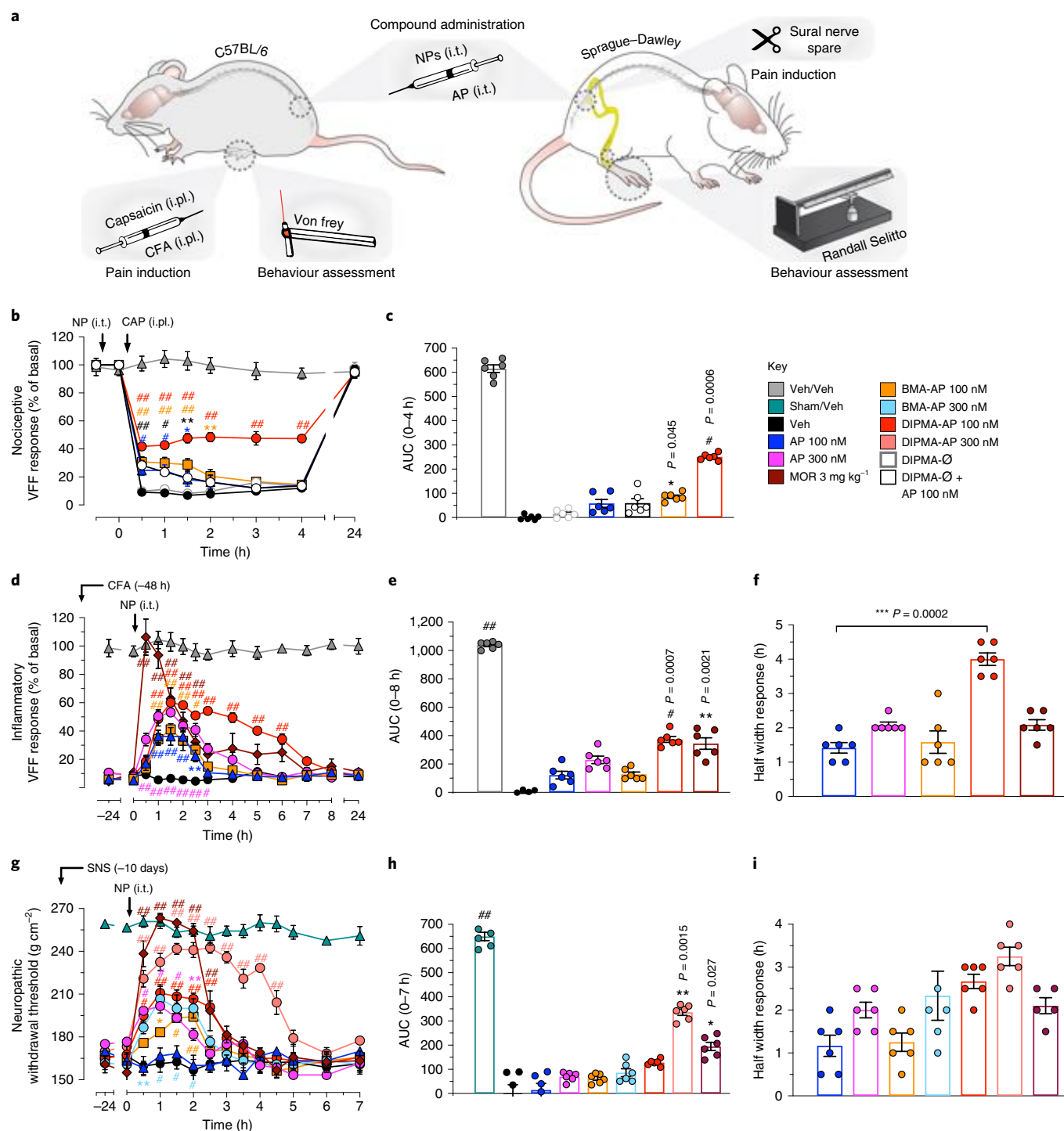


**Fig. 4 | Effects of nanoparticles on nociceptive, inflammatory and neuropathic nociception.** **a**, Preclinical models of nociceptive, inflammatory and neuropathic pain. In the capsaicin-evoked model of acute nociceptive pain in mice, AP, nanoparticles (NPs) or vehicle (Veh) (5  $\mu$ l) was injected intrathecally (i.t.) 30 min before intraplantar (i.p.) injection of capsaicin (CAP) or Veh. Withdrawal responses were measured to stimulation of the plantar surface of the injected hindpaw with VFF. In the complete Freund's adjuvant (CFA)-evoked model of sustained inflammatory nociception in mice, CFA or Veh was administered by i.p. injection; after 48 h, AP, NP or Veh was administered by i.t. injection (5  $\mu$ l). Withdrawal responses were measured to VFF stimulation of the plantar surface of the injected hindpaw. In the sural nerve spared (SNS) model, rats underwent SNS or sham surgery; after 10 days, AP, NP or Veh (10  $\mu$ l) was injected i.t. Withdrawal responses were assessed using the Randall-Selitto test. **b,c**, Capsaicin-induced mechanical allodynia in mice: kinetic VFF response (**b**) and integrated response as area under the curve (AUC) (**c**). **d-f**, CFA-evoked mechanical hyperalgesia in mice: VFF response (**d**), AUC (**e**) and half width response (**f**). **g-i**, SNS-evoked mechanical hyperalgesia in rats: neuropathic withdrawal threshold response (**g**), AUC (**h**) and half width response (**i**). Data are presented as mean  $\pm$  s.e.m.,  $n = 6$  animals for all experiments. \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.001$ , ### $P < 0.0001$  compared to vehicle. Two-way ANOVA, Dunnett's post-hoc test (**b,d,g**); one-way ANOVA, Dunn's post-hoc test (**c,e,f,h,i**).

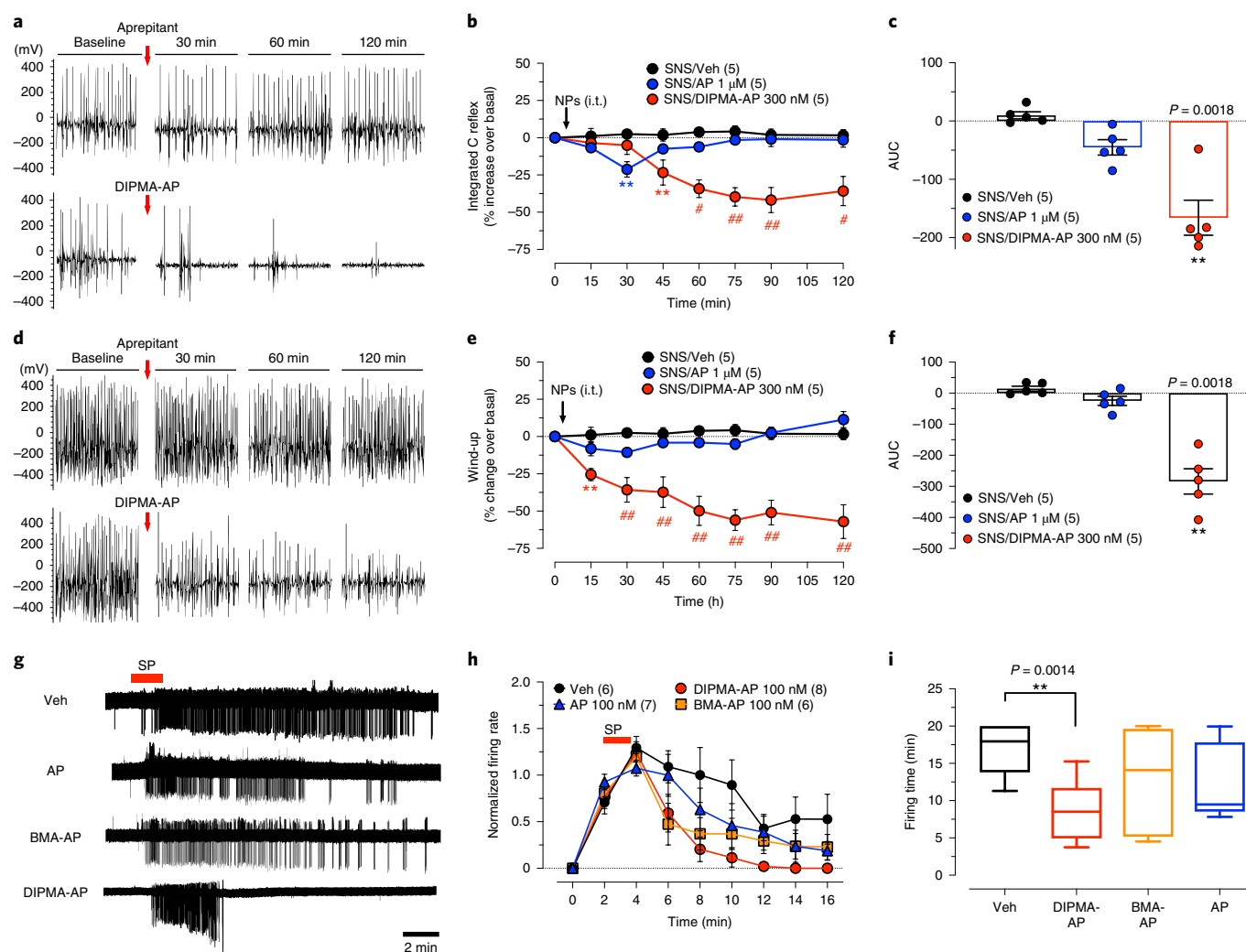


**Fig. 5 | Sensitization and activation of nociceptive transmission.** **a–f**, C-fibre reflex and wind-up in SNS rats. C-fibre reflexes (**a–c**) and wind-up (**d–f**) were measured at 10 days after SNS. AP, DIPMA-AP NP or Veh was administered by i.t. injection (10  $\mu$ l). **a,d**, Representative recordings comparing AP and DIPMA-AP. **b,e**, Time course of effects. Data are presented as mean  $\pm$  s.e.m.,  $n=5$  rats per group (in parentheses). \*\* $P < 0.005$ , # $P < 0.001$ , ### $P < 0.0001$  compared to vehicle. Two-way ANOVA, Dunn's post-hoc test. **c,f**, Integrated responses (AUC,  $n=5$  rats). \*\* $P < 0.005$ , vehicle compared to DIPMA-AP, one-way ANOVA, Dunn's post-hoc test. **g–i**, Cell-attached patch-clamp recordings of SP-induced excitation of lamina I neurons in slices of rat spinal cord. Tissues were preincubated with AP, NP or Veh, and then superfused with SP (1  $\mu$ M, 2 min). Action potential firing was measured: representative traces (**g**); normalized firing rate (**h**); firing time (**i**). Data are presented as mean  $\pm$  s.e.m.,  $n=6$  for rats for Veh,  $n=7$  rats for AP,  $n=8$  rats for DIPMA-AP and  $n=6$  rats for BMA-AP. \*\* $P=0.005$ , vehicle compared to DIPMA-AP. Unpaired  $t$ -test (two-sided).

Video 7). Quantification confirmed NK<sub>1</sub>R endocytosis. These results suggest activation of the SP/NK<sub>1</sub>R system, and are consistent with NK<sub>1</sub>R upregulation in the dorsal horn during neuropathic pain<sup>32</sup>.

At 10 days, SNS reduced the pressure that induced withdrawal of the hindpaw when compared to sham-operated rats, indicating mechanical hyperalgesia (Fig. 4g–i). Intrathecal vehicle did not affect mechanical hyperalgesia, which persisted for 7 h. Although low doses of aprepitant (100 nM) did not modify the withdrawal threshold, higher doses (300 nM) inhibited withdrawal thresholds after 0.5 h to a maximum of  $40 \pm 2\%$  inhibition after 1 h, with return to baseline after 2.5 h. Aprepitant (1  $\mu$ M) almost fully reversed hyperalgesia after 1 h ( $75 \pm 4\%$  inhibition), although hyperalgesia returned to baseline after 3 h (Supplementary Fig. 4). BMA-AP (100 and 300 nM aprepitant) inhibited hyperalgesia to a similar degree as free aprepitant (300 nM). DIPMA-AP (100 and 300 nM aprepitant) strongly reversed hyperalgesia, with almost complete inhibition after 1.5 h (300 nM,  $80 \pm 4\%$  inhibition) and maintenance for

4.5 h, when none of the other treatments were effective. DIPMA-AP (500 nM) provided complete relief from hyperalgesia for 4.5 h (Supplementary Fig. 6). Although morphine fully reversed hyperalgesia for 2 h, the effect was absent after 2.5 h.

The enhanced effects of DIPMA-AP could be related to delivery and retention of aprepitant in endosomes of spinal neurons containing activated NK<sub>1</sub>R, and the continued release of aprepitant as nanoparticles encounter increasingly acidified endosomal compartments. The anti-nociceptive actions of BMA-AP might be due to non-pH-responsive aprepitant release by unknown mechanisms.

**Effects of nanoparticles on neuronal activity.** Nociceptor C-fibres transmit painful stimuli centrally by releasing SP, calcitonin gene related peptide and glutamate in the dorsal horn<sup>33</sup>. Central sensitization (that is, amplified nociceptive transmission, decreased nociceptive threshold) is a hallmark of chronic pain. To examine sensitization, we measured the threshold current required to activate C-fibre reflexes, and assessed wind-up, a frequency-dependent

increase in the excitability of spinal cord neurons induced by electrical stimulation of C-fibres<sup>31</sup>. The threshold current required for activation of the C-fibre-mediated reflexes in the ipsilateral biceps femoris muscle was reduced in SNS rats compared to sham controls (SNS,  $3.2 \pm 2.8$  mA; sham,  $10.3 \pm 1.2$  mA,  $P < 0.05$ ), consistent with sensitization. Repeated 0.1 Hz electrical stimuli caused a constant and stable C-reflex activity over time, while repeated 1.0 Hz stimuli evoked a progressive increase in C-reflex frequency or wind-up (Fig. 5a–f). In SNS rats, intrathecal aprepitant (1  $\mu$ M) decreased the C-reflex only at 30 min, but did not affect wind-up. In contrast, DIPMA-AP (300 nM aprepitant) decreased C-reflex within 45 min and wind-up activity within 15 min, and inhibited responses for the duration of observations (120 min).

The effectiveness of DIPMA-AP to suppress nociception could be due to antagonism of sustained SP-induced excitation of spinal neurons, which requires NK<sub>1</sub>R signalling from endosomes<sup>19</sup>. To examine this possibility, we made cell-attached patch-clamp recordings from lamina I neurons in slices of rat spinal cord. In vehicle-treated slices, SP (1  $\mu$ M, 2 min) caused a rapid onset in action potential firing that persisted for 16 min after washout (Fig. 5g–i). Aprepitant (100 nM) or BMA-AP (100 nM aprepitant) had minimal effect on the onset, rate or duration of SP-induced firing. DIPMA-AP (100 nM) did not affect the initial onset of SP-evoked firing, but inhibited the rate of discharge after washout and the duration of excitation. When delivered in pH-responsive nanoparticles, aprepitant antagonizes endosomal NK<sub>1</sub>R signals that drive sustained excitation of spinal neurons.

**Effects of nanoparticles on endosomal signalling.** Endosomal NK<sub>1</sub>R signalling in HEK-293 cells activates nuclear extracellular signal-regulated kinase (ERK), which mediates SP-induced excitation of spinal neurons<sup>19</sup>. Painful stimuli (capsaicin) evoke phosphorylation of ERK (pERK) in spinal neurons, which requires NK<sub>1</sub>R endocytosis<sup>19</sup>. We examined whether nanoparticle-encapsulated aprepitant prevents capsaicin-evoked ERK activation in spinal neurons in vivo. Capsaicin induced a 3.9-fold increase in the number of pERK-IR expressing neurons in laminae I, II and III of the ipsilateral but not contralateral dorsal horn (Fig. 6a,b). Free aprepitant caused a 43% reduction, BMA-AP a 63% reduction and DIPMA-AP an 81% reduction in pERK-IR neurons. The more complete inhibitory action of DIPMA-AP on ERK signalling concurs with its enhanced anti-nociceptive actions.

ERK signalling in vitro was studied in primary cultures of mouse striatal neurons. SP increased  $[Ca^{2+}]_i$  in striatal neurons; pretreatment with aprepitant abolished responses, which are NK<sub>1</sub>R-dependent (Supplementary Fig. 7a,b). After neurons were incubated

with DIPMA-Cy5 nanoparticles and SP (100 nM) for 30 min, nanoparticles were detected in close proximity to endosomes containing NK<sub>1</sub>R-IR (Fig. 6c). SP (100 nM) and phorbol 12,13-dibutyrate (positive control, 10  $\mu$ M) stimulated nuclear pERK in striatal neurons (Fig. 6d and Supplementary Fig. 7c). DIPMA-AP reduced responses to basal levels, whereas free aprepitant was ineffective.

Förster resonance energy transfer (FRET) biosensors allow analysis of signalling in living cells with high spatiotemporal fidelity<sup>34</sup>. To examine activation of nuclear ERK, HEK-293 cells expressing human (h) NK<sub>1</sub>R were transfected with NucEKAR (nuclear ERK biosensor). SP (100 pM–1  $\mu$ M) activated nuclear ERK (half-maximal effective concentration, EC<sub>50</sub>, of 5.9 nM) (Supplementary Fig. 8a,b). Aprepitant inhibited the response to 5 nM SP ( $\sim$ EC<sub>50</sub>), but only at high concentrations (0.1, 1 and 10  $\mu$ M; half-maximal inhibitory concentration, IC<sub>50</sub>, of 45 nM) (Supplementary Fig. 8c,d). To determine the requirement for NK<sub>1</sub>R endosomal signalling, we transfected HEK-hNK<sub>1</sub>R cells with wild-type dynamin or dominant negative dynamin K44E, which inhibits NK<sub>1</sub>R endocytosis<sup>19</sup>. Compared to cells expressing wild-type dynamin, dynamin K44E attenuated ERK responses to all concentrations of SP, abolished the response to 10 nM SP, and reduced the potency of SP by approximately twofold and the efficacy by  $\sim$ 30% (Supplementary Fig. 8e–g).

DIPMA-Ø or BMA-Ø nanoparticles (10, 20 and 30  $\mu$ g ml<sup>−1</sup>, 30 min) did not activate nuclear ERK in HEK-293 cells (Supplementary Fig. 8h). DIPMA-Ø nanoparticles had no effect on SP (5 nM) stimulated activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells, although 30  $\mu$ g ml<sup>−1</sup> BMA had a small inhibitory effect (Supplementary Fig. 8i). DIPMA-Ø or BMA-Ø nanoparticles (1–100  $\mu$ g ml<sup>−1</sup>, 24 or 48 h) did not affect the viability of HEK-293 cells (Supplementary Fig. 8j).

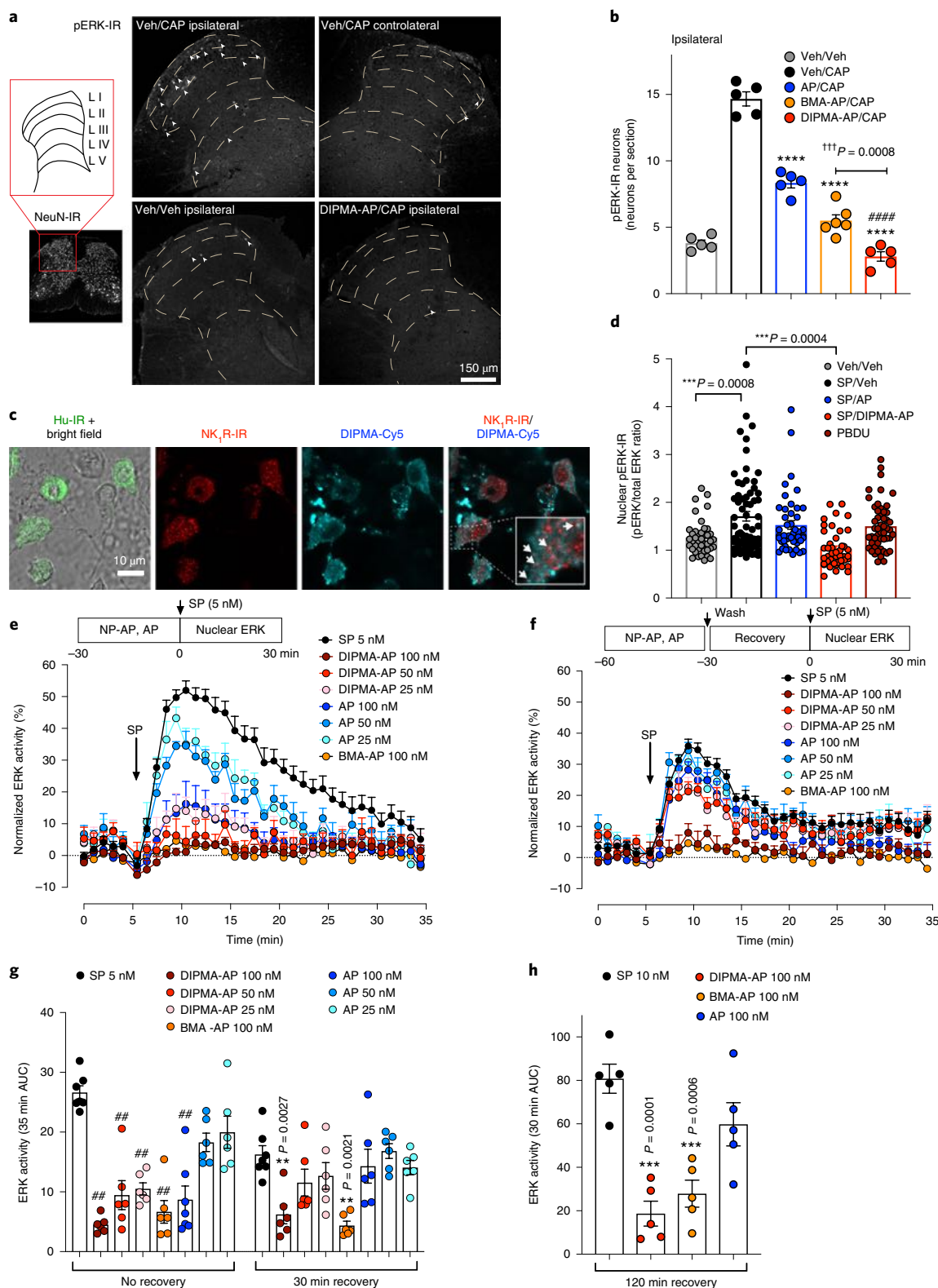
To compare the capacity of free aprepitant and nanoparticle-encapsulated aprepitant to antagonize the NK<sub>1</sub>R in endosomes, we measured SP-induced activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells. Cells were preincubated with vehicle, free aprepitant or DIPMA-AP (25, 50 and 100 nM aprepitant) for 30 min, and were challenged with SP (5 nM). In vehicle-treated cells, SP stimulated a rapid and sustained activation of nuclear ERK (Fig. 6e,g). At all concentrations, DIPMA-AP more completely inhibited this response than free aprepitant. To compare sustained antagonism of endosomal NK<sub>1</sub>R, cells were preincubated with vehicle, aprepitant or DIPMA-AP (100 nM) for 30 min, washed, recovered in medium without antagonist for 30 or 120 min, and then challenged with SP. Free aprepitant was now inactive, whereas DIPMA-AP (100 nM) abolished SP-induced activation of nuclear ERK (Fig. 6f–h). Although BMA-AP was less efficacious than DIPMA-AP in assays of nociception and ERK activity in spi-

**Fig. 6 | Antagonism of NK<sub>1</sub>R signalling in endosomes.** **a,b**, Localization of pERK in the spinal cord: representative images (a) and the number of pERK-IR neurons per section (b). AP, BMA-AP, DIPMA-AP or Veh was injected i.t. into mice. After 30 min, CAP or Veh was administered by i.p. injection. After 4 h, the spinal cord was collected for localization of pERK-IR or NeuN-IR (pan-neuronal marker). Data are presented as mean  $\pm$  s.e.m.,  $n = 5$  mice for Veh/Veh, Veh/CAP, AP/CAP, DIPMA-AP/CAP groups or  $n = 6$  mice for BMA-AP/CAP groups. \*\*\*\* $P < 0.0001$  compared to Veh/CAP; ##### $P < 0.0001$  compared to AP/CAP; ††† $P < 0.001$  compared to BMA-AP/CAP. One-way ANOVA, Bonferroni post-hoc test. **c**, Uptake of DIPMA-Cy5 nanoparticles in proximity to NK<sub>1</sub>R-IR endosomes in cultured striatal neurons stimulated with 100 nM SP for 30 min. Representative images from  $n = 4$  independent experiments are shown. **d**, Nuclear ERK signalling in primary cultures of mouse striatal neurons. Neurons were preincubated with Veh, free AP or DIPMA-AP (100 nM, 30 min), washed and recovered for 30 min. Neurons were challenged with SP (100 nM) or phorbol 12,13-dibutyrate (positive control, 10  $\mu$ M) for 30 min. Nuclear pERK-IR and total ERK-IR were detected by immunofluorescence and confocal imaging, and expressed as the ratio of phospho-ERK1/2 (Thr202/Tyr204) to total ERK. Data are presented as mean  $\pm$  s.e.m.,  $n = 41$  neurons for Veh/Veh, 68 neurons for SP/Veh, 46 neurons for SP/AP, 43 neurons for SP/DIPMA-AP, 52 neurons for SP/BMA-AP and 51 neurons for phorbol 12, 13-dibutyrate (PBDU, positive control), from four experiments (Veh, SP/AP, SP/DIPMA, SP/BMA) or six experiments (SP/Veh, PBDU) tested with independent nanoparticle preparations. \*\*\* $P < 0.001$ . One-way ANOVA, Tukey's post-hoc test. **e–h**, Effects of free AP, DIPMA-AP and BMA-AP on SP-induced activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells. Cells were preincubated with Veh, AP or DIPMA-AP for 30 min; they were either challenged with SP (no recovery, **e**), or were washed, recovered in antagonist-free medium for 30 or 120 min and then challenged with SP (30 min recovery, **f**). **g,h**, AUC of ERK assays. Results are expressed as normalized values by the maximum nuclear ERK response to 1  $\mu$ M phorbol 12,13-dibutyrate. Data are presented as mean  $\pm$  s.e.m.,  $n = 7$  independent experiments for SP (no recovery and 30 min recovery) and AP (100 nM, no recovery),  $n = 5$  independent experiments for 120 min recovery and  $n = 6$  independent experiments for all other data points; observations are in triplicate. \*\* $P < 0.005$ , \*\*\* $P < 0.001$ , ### $P < 0.0001$  compared to vehicle. One-way ANOVA, Tukey's post-hoc test.

nal neurons in vivo, BMA-AP inhibited SP-induced activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells to a similar degree as DIPMA-AP (Fig. 6e–h). Intracellular disassembly of BMA-AP nanoparticles by unknown mechanisms might release sufficient quantities of aprepitant to effectively antagonize the NK<sub>1</sub>R in endosomes. Non-selective effects of BMA on SP-stimulated nuclear ERK (Supplementary Fig. 8i) could also contribute.

## Discussion

The rationale for the current study is that painful stimuli evoke NK<sub>1</sub>R endocytosis in spinal neurons<sup>24,28</sup>, where NK<sub>1</sub>R endosomal signals mediate excitation and nociception<sup>19</sup>. Clathrin and dynamin inhibitors and lipid-conjugated antagonists that target the NK<sub>1</sub>R in endosomes inhibit nociception<sup>19</sup>. Considerable effort will be required to advance these compounds to the clinic. Dynamin



and clathrin inhibitors disrupt trafficking of many receptors and channels that control nociception. Lipid-conjugated antagonists can lose potency. Because lipidated antagonists incorporate into plasma and endosomal membranes, they cannot exclusively target endosomal signalling. pH-responsive nanoparticles deliver aprepitant to endosomes, without loss of potency. Nanoparticle encapsulation enhanced the anti-nociceptive actions of aprepitant in preclinical models of pain. These findings are consistent with the improved capacity of nanoparticle-encapsulated aprepitant to inhibit SP-induced excitation of spinal neurons and to cause a sustained inhibition of endosomal signalling. Nanoparticle uptake and sustained release of aprepitant in acidic endosomes containing the activated NK<sub>1</sub>R could account for these enhanced and persistent anti-nociceptive effects.

Further studies are necessary before nanoparticle-encapsulated analgesics can be advanced to clinical trials. They include toxicology, pharmacokinetic and pharmacodynamic studies in disease-relevant preclinical models. Therapeutic efficacy could be improved by combining into the same nanoparticles antagonists of different GPCRs that co-mediate pain transmission (for example, NK<sub>1</sub>R, calcitonin receptor-like receptor)<sup>19,21</sup>. By incorporating targeting groups into the nanoparticle shell, it might be possible to deliver drugs selectively to pain-transmitting neurons. Limitations of our study include the following: analysis of nociception rather than the perception of pain, which requires human studies; study of evoked rather than spontaneous nociception; examination of nanoparticle actions in cell lines or primary striatal neurons, rather than the spinal neurons that are the target of nanoparticle-encapsulated aprepitant.

Nanoparticle encapsulation could improve the therapeutic efficacy of antagonists and agonists of many GPCRs that signal from endosomes<sup>13,15,35</sup>. Although GPCRs are the target of most clinically approved drugs, many drugs fail during development for unknown reasons. Nanoparticle encapsulation could advance the development of drugs to treat multiple diseases by altering their intracellular distribution to fine-tune signalling processes of pathophysiological importance.

## Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41565-019-0568-x>.

Received: 22 October 2018; Accepted: 2 October 2019;

Published online: 04 November 2019

## References

- De Jong, W. H. & Borm, P. Drug delivery and nanoparticles: applications and hazards. *Int. J. Nanomed.* **3**, 133–149 (2008).
- Farokhzad, O. C. & Langer, R. Impact of nanotechnology on drug delivery. *ACS Nano* **3**, 16–20 (2009).
- Uhrich, K. E., Cannizzaro, S. M., Langer, R. S. & Shakesheff, K. M. Polymeric systems for controlled drug release. *Chem. Rev.* **99**, 3181–3198 (1999).
- Maeda, H. et al. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control Rel.* **65**, 271–284 (2000).
- Such, G. K., Yan, Y., Johnston, A. P. R., Gunawan, S. T. & Caruso, F. Interfacing materials science and biology for drug carrier design. *Adv. Mater.* **27**, 2278–2297 (2015).
- Chan, J. M., Farokhzad, O. C. & Gao, W. pH-responsive nanoparticles for drug delivery. *Mol. Pharm.* **7**, 1913–1920 (2010).
- Lynn, D. M., Amiji, M. M. & Langer, R. pH-responsive polymer microspheres: rapid release of encapsulated material within the range of intracellular pH. *Angew. Chem. Int. Ed.* **40**, 1707–1710 (2001).
- Mura, S., Nicolas, J. & Couvreur, P. Stimuli-responsive nanocarriers for drug delivery. *Nat. Mater.* **12**, 991–1003 (2013).
- Schmaljohann, D. Thermo- and pH-responsive polymers in drug delivery. *Adv. Drug Deliv. Rev.* **58**, 1655–1670 (2006).
- Wilhelm, S. et al. Analysis of nanoparticle delivery to tumours. *Nat. Rev. Mater.* **1**, 16014 (2016).
- Zhou, K. et al. Tunable, ultrasensitive pH-responsive nanoparticles targeting specific endocytic organelles in living cells. *Angew. Chem. Int. Ed.* **50**, 6109–6114 (2011).
- Nelson, C. E. et al. Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time and bioactivity in vivo. *ACS Nano* **7**, 8870–8880 (2013).
- Thomsen, A. R. B., Jensen, D. D., Hicks, G. A. & Bunnett, N. W. Therapeutic targeting of endosomal G protein-coupled receptors. *Trends Pharmacol. Sci.* **39**, 879–891 (2018).
- Hausser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B. & Gloriam, D. E. Trends in GPCR drug discovery: new agents, targets and indications. *Nat. Rev. Drug Discov.* **16**, 829–842 (2017).
- Murphy, J. E., Padilla, B. E., Hasdemir, B., Cottrell, G. S. & Bunnett, N. W. Endosomes: a legitimate platform for the signaling train. *Proc. Natl Acad. Sci. USA* **106**, 17615–17622 (2009).
- Vilardaga, J. P., Jean-Alphonse, F. G. & Gardella, T. J. Endosomal generation of cAMP in GPCR signaling. *Nat. Chem. Biol.* **10**, 700–706 (2014).
- Irannejad, R. et al. Functional selectivity of GPCR-directed drug action through location bias. *Nat. Chem. Biol.* **13**, 799–806 (2017).
- Stoeber, M. et al. A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* **98**, 963–976 (2018).
- 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**, eaa3447 (2017).
- Jimenez-Vargas, N. N. et al. Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc. Natl Acad. Sci. USA* **115**, E7438–E7447 (2018).
- 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).
- Kramer, M. S. et al. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. *Science* **281**, 1640–1645 (1998).
- Quartara, L., Altamura, M., Evangelista, S. & Maggi, C. A. Tachykinin receptor antagonists in clinical trials. *Expert Opin. Investig. Drugs* **18**, 1843–1864 (2009).
- 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).
- Manders, E. M. M., Verbeek, F. J. & Aten, J. A. Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* **169**, 375–382 (1993).
- Robertson, M. J. et al. Synthesis of the PitStop family of clathrin inhibitors. *Nat. Protoc.* **9**, 1592–1606 (2014).
- Robertson, M. J., Deane, F. M., Robinson, P. J. & McCluskey, A. Synthesis of Dynole 34-2, Dynole 2-24 and Dyno 4a for investigating dynamin GTPase. *Nat. Protoc.* **9**, 851–870 (2014).
- Mantyh, P. W. et al. Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation. *Science* **268**, 1629–1632 (1995).
- Stein, C., Millan, M. J. & Herz, A. Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alterations in behavior and nociceptive thresholds. *Pharmacol. Biochem. Behav.* **31**, 445–451 (1988).
- Decosterd, I. & Woolf, C. J. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. *Pain* **87**, 149–158 (2000).
- Bravo, D. et al. Pannexin 1: a novel participant in neuropathic pain signaling in the rat spinal cord. *Pain* **155**, 2108–2115 (2014).
- Abbadie, C., Brown, J. L., Mantyh, P. W. & Basbaum, A. I. Spinal cord substance P receptor immunoreactivity increases in both inflammatory and nerve injury models of persistent pain. *Neuroscience* **70**, 201–209 (1996).
- Geppetti, P., Veldhuis, N. A., Lieu, T. & Bunnett, N. W. G protein-coupled receptors: dynamic machines for signaling pain and itch. *Neuron* **88**, 635–649 (2015).
- Halls, M. L. & Canals, M. Genetically encoded FRET biosensors to illuminate compartmentalised GPCR signalling. *Trends Pharmacol. Sci.* **39**, 148–157 (2018).
- Irannejad, R. & von Zastrow, M. GPCR signaling along the endocytic pathway. *Curr. Opin. Cell Biol.* **27**, 109–116 (2014).

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2019

## Methods

**Materials.** Reagents were purchased from Sigma-Aldrich unless otherwise specified.

**Diblock copolymers and nanoparticles.** Polymer synthesis and characterization and nanoparticle assembly, pH-dependent disassembly in vitro and characterization are described in the Supplementary Methods.

**Cell lines.** The human (h) NK<sub>1</sub>R long isoform open reading frame with a CD8 signal sequence and N-terminal FLAG-tag was cloned into pcDNA5 FRT/TO between KpnI and NotI restriction sites using Gibson assembly (NEB). A stable cell line expressing hNK<sub>1</sub>R (HEK-hNK<sub>1</sub>R) was produced by co-transfecting FlpIn HEK-293 cells with hNK<sub>1</sub>R vector and pOG44 (0.5 µg and 4 µg, respectively), using polyethylenimine (PEI, Polysciences) at a 1:6 DNA:PEI ratio. Cells (~0.7 × 10<sup>6</sup>) were seeded into T-25 tissue culture flasks (Perkin Elmer) in Dulbecco's modified Eagle medium (DMEM) supplemented with penicillin (50 U ml<sup>-1</sup>) and streptomycin (50 U ml<sup>-1</sup>) (DMEM/pen/strep) and incubated for 24 h (37 °C, 5% CO<sub>2</sub>). Culture medium was changed to fresh DMEM/pen/strep prior to transfection and incubated for 24 h (37 °C, 5% CO<sub>2</sub>). The medium was changed to DMEM supplemented with 10% (vol/vol) fetal bovine serum (FBS) and hygromycin B (200 µg ml<sup>-1</sup>, Thermo Fisher Scientific) for stable cell line selection. Cell lines were tested and confirmed free of mycoplasma.

**Nanoparticle trafficking in HEK-293 cells.** HEK-293 cells were plated on poly-D-lysine coated chambers (ibidi, Germany) in DMEM supplemented with 10% (vol/vol) FBS (DMEM/FBS). After 24 h, cells were transfected with 300 ng of rat (r) NK<sub>1</sub>R-GFP per chamber and cultured for 48 h. To identify endosomal compartments, HEK-293 cells were infected with Rab5a-GFP (resident in early endosomes) or Rab7a-GFP (late endosomes) CellLight BacMam2.0 (Thermo Fisher Scientific) for 16 h. To examine localization of nanoparticles, cells were incubated in Leibovitz's L-15 medium with DIPMA-Cy5 nanoparticles (20 µg ml<sup>-1</sup>, 30 min, 37 °C) or vehicle, followed by addition of SP (10 nM). Cells were imaged at 30 and 60 min post-SP addition using a Leica SP8 confocal microscope equipped with HCX PL APO ×40 (NA 1.30) and HCX PL APO ×63 (NA 1.40) oil objectives. Images were analysed using Fiji<sup>36</sup> and deconvolved with Huygens Professional version 18.04 (Scientific Volume Imaging, <http://svi.nl>), using the CMLE algorithm with a signal-to-noise ratio of 10 and 100 iterations. Co-localization was evaluated by determination of the Manders overlap coefficient<sup>25</sup>.

**Uptake and disassembly of nanoparticles in HEK-293 cells.** Nanoparticles were self-assembled with 0.5 mg of Coumarin 153 per mg of DIPMA or BMA polymer (DIPMA-CO, BMA-CO). HEK-293 cells were preincubated for 30 min with vehicle (Hank's balanced salt solution, HBSS), dynamin inhibitor (Dyngo4a, 30 µM)<sup>27</sup>, clathrin inhibitor (PitStop2, 30 µM)<sup>26</sup>, vacuolar H<sup>+</sup>ATPase inhibitor (Bafilomycin A1, 1 µM) or NH<sub>4</sub>Cl (20 mM), which acts as a lysosomotropic weak base. Nuclei were stained using Drag5. Images were obtained with a Leica SP8 confocal microscope using an HCX PL APO ×63 (NA 2.0) oil objective. Images were taken every 10 s for 30 min, where the first five readings correspond to baseline images before the addition of DIPMA-CO nanoparticles (20 µg ml<sup>-1</sup>). All images were analysed using Fiji<sup>36</sup>. Kinetic data were normalized to the fluorescence of free Coumarin 153 (5 µg ml<sup>-1</sup>) at 30 min.

**Animals.** Male C57BL/6 mice (6–10 weeks) and pregnant Asmu:Swiss mice were sourced from the Monash Animal Research Platform. Male Sprague–Dawley rats (225–250 g) were obtained from the Faculty of Medicine of the University of Chile. Animals were housed in groups of four, maintained at a temperature of 22 ± 4 °C in a humidity-controlled environment with a 12 h light/dark cycle. Food and water were available ad libitum. For behavioural tests, investigators were blinded to the treatment groups and animals were randomly assigned to treatments and studied during the light cycle. Animals were euthanized by anaesthetic overdose and thoracotomy. Studies on animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and adhered to the ethical guidelines of the International Association for the Study of Pain<sup>37</sup>. Studies were approved by the Animal Ethics Committee of Monash Institute of Pharmaceutical Sciences, Monash University and the Bioethics Committee of the University of Santiago of Chile.

**Drug administration.** *Mice.* The following drugs were administered by intrathecal injection (5 µl) into the intervertebral space (L4/L5) of conscious mice: apreptant (100 and 300 nM), nanoparticles delivering an equivalent dose of apreptant (DIPMA-AP, BMA-AP, 10 µg ml<sup>-1</sup> 100 nM apreptant, 30 µg ml<sup>-1</sup> 300 nM apreptant), controls (10 µg ml<sup>-1</sup> of DIPMA-Ø and a mixture of 10 µg ml<sup>-1</sup> of DIPMA-Ø and apreptant 100 nM) or vehicle (artificial cerebrospinal fluid, aCSF). Treatments were administered 30 min before rotarod experiments and the induction of acute nociceptive pain or 48 h after the establishment of inflammatory nociception. For biodistribution studies, nanoparticles (50 µg ml<sup>-1</sup>) were administered intrathecally immediately after obtaining control images. For localization of nanoparticles in the spinal cord, nanoparticles (50 µg ml<sup>-1</sup>) were administered intrathecally 30 min after the induction of acute nociception with

capsaicin (see below). Morphine (3 mg kg<sup>-1</sup> intraperitoneal) was administered 48 h after induction of inflammatory nociception.

*Rats.* Drugs were administered by intrathecal injection (10 µl) into the intervertebral space (L4/L5) of conscious rats: apreptant (100 nM, 300 nM, 1 µM), nanoparticles loaded with apreptant (DIPMA-AP, BMA-AP, 10 µg ml<sup>-1</sup> 100 nM apreptant, 30 µg ml<sup>-1</sup> 300 nM apreptant, 50 µg ml<sup>-1</sup> 500 nM apreptant), DIPMA-Ø nanoparticles (10, 30 and 50 µg ml<sup>-1</sup>) or vehicle (aCSF). Treatments were administered 10 days after sural nerve transection or sham surgery. For electrophysiological studies, drugs were administered by intrathecal injection under anaesthesia (isoflurane 1.2–1.5%): apreptant (1 µM) or nanoparticles (30 µg ml<sup>-1</sup> 300 nM apreptant). Morphine (3 mg kg<sup>-1</sup>, intraperitoneal) was administered 10 days after sural nerve transection.

**Biodistribution of nanoparticles in the spinal cord.** Mice were sedated (2% isoflurane) and placed in an in vivo imaging system (IVIS spectrum Lumina II, Perkin Elmer). Posterior images were obtained using the Perkin Elmer Living Image software v4.3.1. After collection of a baseline image, nanoparticles (50 µg ml<sup>-1</sup>) were administered intrathecally (5 µl). Images were collected at 0.5, 1, 1.5, 2, 4, 8 and 24 h post DIPMA-Cy5 or BMA-Cy5 administration.

**Uptake of nanoparticles in the spinal cord.** Cy5-labelled nanoparticles were administered to mice (intrathecal). After 30 min, capsaicin (5 µg) was administered by subcutaneous intraplantar injection (10 µl) into the left hindpaw under sedation (2% isoflurane). This approach was used to mimic the therapeutic situation where nanoparticle-encapsulated drugs might be used to treat pain. At 1 h after nanoparticle administration, mice were transcardially perfused with 50 ml of PBS followed by 50 ml of ice-cold 4% paraformaldehyde (PFA). The spinal cord was removed, immersion fixed in 4% PFA (2 h, 4 °C) and cryoprotected in PBS containing 30% sucrose (24 h, 4 °C). The spinal cord (L3–L6) was embedded in tissue freezing medium (TFM, General Data), and 30 µm serial coronal sections were cut and mounted on Colorfrost Plus microscope slides (Fisher Scientific). Sections were washed twice in PBS, counter-stained with DAPI (5 µg ml<sup>-1</sup>, 5 min) and cover-slipped with ProLong Glass mounting medium (Thermo Fisher Scientific). Some sections were processed to detect neurons. Sections were blocked in PBS containing 0.2% Triton X-100 and 10% normal horse serum (NHS; 30 min, room temperature). Sections were incubated with rabbit anti-PGP9.5 (1:500, Abcam ab27053) in PBS containing 0.2% Triton X-100 and 3% NHS (60 min, room temperature). Sections were washed four times in PBS and incubated with donkey anti-rabbit Alexa488 (1:1,000, Thermo Fisher Scientific; 30 min, room temperature). Sections were imaged on a Leica SP8 confocal microscope with HC PLAPO ×40 or ×63 oil objectives.

**Determination of apreptant concentration in the spinal cord.** Apreptant (100 nM) or nanoparticles delivering an equivalent dose of apreptant (10 µg ml<sup>-1</sup> 100 nM apreptant) was administered by intrathecal injection to conscious mice. Mice were killed 1 h and 4 h post-treatment. The spinal cord (L2–L6) was removed for determination of the tissue concentration of apreptant by LC-MS, as described in the Supplementary Methods.

**Acute and inflammatory nociception in mice.** *Nociceptive pain.* Capsaicin (5 µg) or vehicle (0.9% NaCl) was administered by intraplantar injection (10 µl) into the left hindpaw of sedated mice (2% isoflurane) 30 min after intrathecal injection of drugs<sup>19</sup>.

*Inflammatory pain.* CFA (0.5 mg ml<sup>-1</sup>) or vehicle (0.9% NaCl) was administered by intraplantar injection (10 µl) into the left hindpaw of sedated mice (2% isoflurane)<sup>19,29</sup>. Drugs were administered by intrathecal injection 48 h after CFA.

*Mechanical allodynia.* Mechanical nociception was assessed by measuring withdrawal thresholds to stimulation of the plantar surfaces of the ipsilateral and contralateral hindpaws with calibrated VFFs<sup>19</sup>. Before experiments, mice were acclimatized to the experimental apparatus and environment for 2 h on two successive days. VFF withdrawal thresholds were measured in triplicate to establish a baseline for each mouse. For the capsaicin model, VFF withdrawal thresholds were measured at 30 min intervals for the first 2 h after drug administration, then at 60 min intervals for the next 2 h, and finally after 24 h. For the CFA model, VFF withdrawal thresholds were measured every 30 min for the first 3 h after drug administration, then at 60 min intervals for the next 5 h, and finally after 24 h. Results were normalized to the baseline withdrawal thresholds of each mouse. Results are expressed as a percentage of baseline, as AUC and as the half width response (the duration of the effect of each treatment calculated as the time to attain 50% of the maximal analgesic response).

**pERK localization in mouse spinal cord.** Vehicle (control), free apreptant and nanoparticles (all 100 nM apreptant) were administered by intrathecal injection to mice as described above. After 30 min, mice were sedated (2% isoflurane) and vehicle (0.9% NaCl) or capsaicin (5 µg) was administered by intraplantar injection (10 µl) into the left hindpaw. After 4 h, sections of spinal cord (L3–L6) were prepared as described above. Fixation, staining and imaging of sections are described in detail in the Supplementary Methods.

**Rotarod test.** Motor coordination was assessed in mice by a rotarod test as described in the Supplementary Methods.

**Neuropathic nociception in rats.** *Neuropathic pain.* Neuropathic nociception was induced in rats using a variation of the SNS injury model, which induces rapid onset and sustained mechanical and thermal hyperalgesia<sup>30</sup>. Under anaesthesia (2% isoflurane), the three terminal distal branches of the sciatic nerve (tibial, common peroneal and sural nerves) were identified and the sural nerve was transected<sup>31</sup>. For controls (sham), rats underwent a similar surgery but without transection of the sural nerve. After surgery, ketoprofen (3 mg kg<sup>-1</sup>) and enrofloxacin (5 mg kg<sup>-1</sup>) were administered subcutaneously for 2 days.

**Mechanical hyperalgesia.** Mechanical hyperalgesia was assessed in rats by measuring hindpaw withdrawal pressure thresholds using an algometer (Ugo Basile) with a cutoff value of 570 g to prevent injury<sup>38,39</sup>. Mechanical hyperalgesia was evaluated before (basal) and 5, 9 and 10 days after surgery. After evaluation at day 10, drugs were administered by intrathecal injection, and withdrawal thresholds were recorded every 30 min for 7 h. Results are expressed as the paw withdrawal pressure threshold (g cm<sup>-2</sup>), AUC and half-width response.

**Electrophysiological assessment of nociception in rats.** Nociceptive synaptic transmission was evaluated by measurement of electromyographic activity associated with the hind limb-flexion nociceptive reflex evoked by electrical activation of C-fibres of the sural nerve (C-reflex) as described previously<sup>40</sup> and in detail in the Supplementary Methods.

**Cell-attached patch-clamp recordings of rat spinal neurons.** Parasagittal slices (340 µm) were prepared from rat lumbar spinal cord as described in refs.<sup>19,41</sup>. Slices were transferred to a recording chamber and superfused with aCSF (2 ml min<sup>-1</sup>, 36 °C). Dodt-contrast optics were used to identify large (capacitance ≥20 pF), putative NK<sub>1</sub>R-positive neurons in lamina I based on their position, size and fusiform shape with dendrites that were restricted to lamina I. Spontaneous currents were recorded from NK<sub>1</sub>R-positive lamina I neurons in a cell-attached configuration in voltage clamp. Slices were preincubated in DIPMA-AP (10 µg ml<sup>-1</sup> 100 nM aprepitant), BMA-AP (10 µg ml<sup>-1</sup> 100 nM aprepitant) or aprepitant (100 nM) for 120 min, washed and incubated in antagonist-free aCSF for a further 30–60 min before recording. Slices were challenged with SP (1 µM, 2 min) and the firing rate for each cell was normalized to the response between the 2 and 4 min time points, which was not significantly different between groups. The firing time was determined as the duration of the response to the last action potential.

**NK<sub>1</sub>R localization in rat spinal cord.** At 10 days after sham or SNS surgery, rats were anaesthetized and transcardially perfused with 250 ml PBS followed by 250 ml 4% PFA. The spinal cord was removed, immersion fixed in 4% PFA (2 h, 4 °C) and cryoprotected in 30% sucrose in 0.1 M PBS (24 h, 4 °C). The spinal cord (L3–L6) was embedded in TFM (General Data) and 30 µm serial coronal sections were cut into 48-well plates containing PBS. Fixation, immunostaining, imaging and image analysis of spinal cord sections are described in the Supplementary Methods.

**Nanoparticle uptake and SP signalling in striatal neurons.** *Neuronal isolation and culture.* Primary striatal neurons were dissociated from E15–16 Asmu:Swiss mouse embryos as described in ref.<sup>42</sup>. Neurons (200,000 per well) were plated on poly-D-lysine-coated eight-well chamber slides (ibidi) in Neurobasal medium supplemented with B-27, 2 mM L-glutamine and penicillin/streptomycin.

**Nanoparticle uptake and NK<sub>1</sub>R localization.** At 5 days after isolation, neurons were equilibrated in HEPES-buffered saline (10 mM HEPES, 0.5% BSA, 10 mM D-glucose, 2.2 mM CaCl<sub>2</sub>·H<sub>2</sub>O, MgCl<sub>2</sub>·6H<sub>2</sub>O, 2.6 mM KCl, 150 mM NaCl, pH 7.4) for 30 min and then incubated with 50 µg ml<sup>-1</sup> DIPMA-Cy5 and 100 nM SP for 30 min. Neurons were fixed in 2% PFA and 1% sucrose in PBS (room temperature, 20 min) and blocked in PBS containing 0.3% Triton X-100 and 5% NHS for 24 h at 4 °C. Neurons were stained as described above for rat spinal cord slices, using rabbit anti-NK<sub>1</sub>R and mouse anti-Hu (HuC/HuD Monoclonal Antibody 16A11, Thermo Fisher Scientific; 24 h, 4 °C), washed in 4× PBS, and incubated with donkey anti-rabbit Alexa594 and donkey anti-mouse Alexa488 (1:500, Thermo Fisher Scientific; 24 h, 4 °C). Neurons were counter-stained with DAPI. Images were obtained on a Leica SP8 confocal microscope with HCX PL APO ×63 (NA 1.40) oil objective.

**Ca<sup>2+</sup> imaging.** At 5 days after isolation, neurons were incubated with Fura-2 AM ester (2 µM, 45 min, 37 °C, Thermo Fisher Scientific) in HEPES-buffered saline containing 4 mM probenecid and 0.05% pluronic F127. Neurons were recovered in fresh HEPES-buffered saline for 30 min before imaging on a Leica DMI-6000B microscope with HC PLAN APO 0.4 NA ×10 objective at 37 °C. Images were collected at 1.5 s intervals (excitation, 340 nm/380 nm; emission, 530 nm). To assess the functional expression of NK<sub>1</sub>R, neurons were preincubated with 300 nM aprepitant or vehicle (DMSO), and challenged with 100 nM SP and followed by 5 mM KCl.

**ERK activity.** At 8 days after isolation, neurons were equilibrated for 30 min in HEPES-buffered saline and then preincubated with DIPMA-AP (10 µg ml<sup>-1</sup>

100 nM aprepitant), aprepitant (100 nM) or vehicle (PBS) for 30 min. Neurons were washed, recovered for 30 min and challenged with SP (100 nM) or the positive control, phorbol 12,13-dibutyrate (10 µM), for 30 min. Neurons were fixed in 4% PFA (20 min at 4 °C) and blocked (0.3% Triton X-100 and 5% NHS; 24 h at 4 °C). Neurons were incubated with rabbit anti-phospho-p44/42 MAPK (ERK1/2 phospho-Thr202/Tyr204, 1:100, #4370, Cell Signalling Technology) and mouse anti-p44/42 MAPK (1:100, #4696, Cell Signalling Technology) (24 h at 4 °C). Neurons were washed four times in PBS and incubated with donkey anti-rabbit Alexa488 and donkey anti-mouse Alexa647 (1:500, Thermo Fisher Scientific; 2 h at room temperature). The nucleus was counter-stained with DAPI. Neurons were imaged using a Leica SP8 confocal microscope with an HCX PL APO ×63 (NA 1.40) oil objective. Nuclei of neurons were selected as regions of interest and the ratio of phospho-ERK to total ERK was calculated using mean fluorescence intensity values. The mean ERK ratio for all neurons within a single well was determined and the means of four experiments were compared for statistical analyses.

**FRET assays of endosomal NK<sub>1</sub>R signalling in HEK-293 cells.** HEK-hNK<sub>1</sub>R cells (~2 × 10<sup>6</sup>) were seeded into a 90 mm Petri dish (Corning) in DMEM/FBS/Hygro and incubated for 24 h (37 °C, 5% CO<sub>2</sub>). Before transfection, the medium was changed to fresh DMEM/FBS/Hygro and the nuclear ERK (nucERK) plasmid was transfected (2.5 µg DNA per dish) using PEI at a 1:6 ratio<sup>19</sup>. After 24 h, cells were plated in a poly-L-lysine-coated black 96-well CulturPlate (Perkin Elmer) and incubated for a further 24 h (37 °C, 5% CO<sub>2</sub>). On the day of the assay, cells were serum-starved for 6–8 h and then equilibrated in HBSS, supplemented with HEPES at 37 °C in a CO<sub>2</sub>-free incubator. FRET was assessed using a PHERAstar FS (BMG LABTECH) with optic module FI 430 530 480 and measurements were made every 1 min. Baseline was measured for 5 min followed by stimulation with SP, vehicle (HBSS) or phorbol 12,13-dibutyrate (1 µM), and further measurements for 30 min. For the SP concentration response curve, half logarithmic dilutions of SP were added (1 µM to 100 pM) and EC<sub>50</sub> was determined using the AUC after SP addition (30 min reading). For the aprepitant concentration response curve, logarithmic dilutions of aprepitant (10 µM to 1 pM) were added 30 min before baseline measurements, followed by the addition of 5 nM of SP. The IC<sub>50</sub> was determined for aprepitant as described. To assess the effect of nanoparticles on nuclear ERK signalling, DIPMA-Ø, DIPMA-AP, BMA-Ø or BMA-AP (30, 20 and 10 µg ml<sup>-1</sup>) was added 30 min before baseline measurements, followed by the addition of SP 5 nM or vehicle. Data were expressed as vehicle corrected values, normalized by the maximum response to the positive control.

**Cell viability assays.** Studies of the effects of nanoparticles on the viability of HEK-293 cells are described in the Supplementary Methods.

**Statistical analysis.** Data were analysed using GraphPad Prism 8 (GraphPad Software). Data are presented as mean ± s.e.m., unless noted otherwise. A two-tailed Student's *t*-test was used for two comparisons and exact *P* values are shown in the figures. For multiple comparisons, results were compared using one- or two-way ANOVA followed by post-hoc multiple comparison tests, as described in the figure legends. Exact adjusted *P* values are shown for bar graphs in figures when *P* ≥ 0.0001. *P* < 0.05 was considered significant.

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

## Data availability

All data generated or analysed during this study are available in this Article and its Supplementary Information or from the corresponding authors upon request.

## References

- Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).
- Zimmermann, M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* **16**, 109–110 (1983).
- Randall, L. O. & Selitto, J. J. A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn. Ther.* **111**, 409–419 (1957).
- Santos-Nogueira, E., Redondo Castro, E., Mancuso, R. & Navarro, X. Randall-Selitto test: a new approach for the detection of neuropathic pain after spinal cord injury. *J. Neurotrauma* **29**, 898–904 (2012).
- Retamal, J. et al. Burst-like subcutaneous electrical stimulation induces BDNF-mediated, cyclosporin B-sensitive central sensitization in rat spinal cord. *Front. Pharmacol.* **9**, 1143 (2018).
- Imlach, W. L., Bhola, R. F., May, L. T., Christopoulos, A. & Macdonald, J. C. A positive allosteric modulator of the adenosine α1 receptor selectively inhibits primary afferent synaptic transmission in a neuropathic pain model. *Mol. Pharmacol.* **88**, 460–468 (2015).
- Di Porzio, U., Daguet, M. C., Glowinski, J. & Prochiantz, A. Effect of striatal cells on in vitro maturation of mesencephalic dopaminergic neurones grown in serum-free conditions. *Nature* **288**, 370–373 (1980).

## Acknowledgements

This work was supported by the National Institutes of Health (NS102722, DE026806, DK118971), the Department of Defense (PR170507), the National Health and Medical Research Council (63303, 1049682, 1031886; N.W.B.), the Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (N.W.B. and T.P.D.), the Center for the Development of Nanoscience and Nanotechnology (CEDENNA, Fondecyt no. 1181622, L.C.) and Takeda Pharmaceuticals Inc. (N.W.B., N.A.V. and D.P.P.). We thank F. Chiu for mass spectrometry analysis of aprepitant loading and P. Zhao for advice about signalling assays.

## Author contributions

P.D.R.-G. prepared and characterized nanoparticles, examined nanoparticle uptake and disassembly, studied SP signalling in model cells and wrote the manuscript. J.S.R. studied the biodistribution and anti-nociceptive and in vivo electrophysiological actions of nanoparticles. P.S. studied the biodistribution and anti-nociceptive actions of nanoparticles. W.I. conceived and designed electrophysiological studies on spinal neurons. M.S. studied the excitation of spinal neurons, and N.T. prepared and characterized nanoparticles. L.C. conceived and designed neuropathic nociception and in vivo electrophysiological studies. T.P. conceived and designed neuropathic nociception. C.J.N. provided expertise in the analysis of confocal images and S.Y.K. obtained transmission electron microscopy images. L.M.L. characterized the critical micellar concentration and pH-disassembly of nanoparticles. C.L. studied SP signalling in model cells and D.P.P. studied nanoparticle uptake. T.M.L. studied anti-nociceptive actions of nanoparticles, G.D.S. prepared striatal neurons, and Q.N.M. prepared and

characterized nanoparticles. D.D.J. examined NK1R endocytosis, nanoparticle uptake into spinal neurons, and SP signalling in model cells and striatal neurons. R.L. examined NK<sub>1</sub>R endocytosis and nanoparticle uptake into spinal neurons. N.S.N. studied NK<sub>1</sub>R endocytosis in rats. B.L.S. designed experiments to examine NK<sub>1</sub>R endocytosis in rats. J.F.Q. designed nanoparticles and wrote the manuscript. M.R.W. designed nanoparticles. N.A.V. conceived experiments, studied SP signalling in neurons, interpreted the results and wrote the manuscript. T.P.D. conceived the experiments and designed the nanoparticles. N.W.B. conceived and designed the experiments, interpreted the results and wrote the manuscript.

## Competing interests

Research in N.A.V.'s, D.P.P.'s and N.W.B.'s laboratories is funded, in part, by Takeda Pharmaceuticals. N.W.B. is a founding scientist of Endosome Therapeutics.

## Additional information

**Supplementary information** is available for this paper at <https://doi.org/10.1038/s41565-019-0568-x>.

**Correspondence and requests for materials** should be addressed to N.A.V., T.P.D. or N.W.B.

**Peer review information** *Nature Nanotechnology* thanks Jean-Pierre Vilardaga and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

**Reprints and permissions information** is available at [www.nature.com/reprints](http://www.nature.com/reprints).

## Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see [Authors & Referees](#) and the [Editorial Policy Checklist](#).

### Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided  
*Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- ☐ ☒ A description of all covariates tested
- ☐ ☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g.  $F$ ,  $t$ ,  $r$ ) with confidence intervals, effect sizes, degrees of freedom and  $P$  value noted  
*Give  $P$  values as exact values whenever suitable.*
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's  $d$ , Pearson's  $r$ ), indicating how they were calculated

*Our web collection on [statistics for biologists](#) contains articles on many of the points above.*

### Software and code

Policy information about [availability of computer code](#)

#### Data collection

All nanoparticle imaging (Cy5-labelled or loaded with Coumarin) was acquired using Leica LAX software on a SP8 confocal system. Subsequent processing was performed using FIJI (ImageJ) version 1.0. For Cy5 particles live cell imaging, images were processed by deconvolution using Huygens Professional version 18.04 (Scientific Volume Imaging, The Netherlands, <http://svi.nl>). Animal imaging to assess Cy5-particle distribution following intrathecal injection were acquired on the Perkin Elmer IVIS Lumina II system using Living Image software, v4.3.1. FRET biosensor ERK signalling was collected on a Pherastar FS (BMG LABTECH).

#### Data analysis

For statistics, data were analysed using Graph Prism 8 (GraphPad Software, Inc., USA). For microscopy, images were analysed using Fiji. Colocalization was evaluated by determination of the Manders overlap coefficient.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

### Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All data generated or analysed during this study are available in this published article and its supplementary information files or from the corresponding author upon request.

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

|                 |                                                                                                                                                                                                                                                                                        |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample size     | No statistical method was used to determine sample size. The sample sizes for experiments on cells and animals were determined from our previously published investigations (Jensen et al, Science Trans Med, 2017, 9, eaal3447) and was sufficient to yield statistical significance. |
| Data exclusions | No data were excluded from the analyses.                                                                                                                                                                                                                                               |
| Replication     | For studies of cell lines and isolated cells, individual experiments (n) were replicated on different days with triplicate measurements for each individual experiment. For behavioral studies of nociception, experiments (n) were replicated on different individual animals.        |
| Randomization   | Cells from independent experiments were randomly selected for imaging and analysis. Animals were assigned at random to experimental treatments.                                                                                                                                        |
| Blinding        | For behavioural tests, investigators were blinded to the treatment groups.                                                                                                                                                                                                             |

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a                                 | Involved in the study                                           |
|-------------------------------------|-----------------------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Eukaryotic cell lines       |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology                          |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Human research participants            |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |

### Methods

| n/a                                 | Involved in the study                           |
|-------------------------------------|-------------------------------------------------|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq               |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Flow cytometry         |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging |

## Antibodies

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antibodies used | Primary antibodies: Rabbit NK1R antibody. Rabbit anti-PGP9.5 (1Abcam ab27053). Mouse anti-Hu (HuC/HuD Monoclonal Antibody 16A11, Thermo Fisher Scientific. Rabbit anti-phospho-p44/42 MAPK (ERK1/2 phospho-Thr202/Tyr204, #4370, Cell Signalling Technology). Mouse anti-p44/42 MAPK (#4696, Cell Signalling Technology).<br>Secondary antibodies: Donkey anti-rabbit Alexa594 and donkey anti-mouse Alexa488 or 647 (Thermo Fisher Scientific).                                                                                                                                                                                                                                                                                           |
| Validation      | NK1R antibody is characterized in: Grady, E.F., Baluk, P., Bohm, S., Gamp, P.D., Wong, H., Payan, D.G., Ansel, J., Portbury, A.L., Furness, J.B., McDonald, D.M. & Bunnett, N.W. Characterization of antisera specific to NK1, NK2, and NK3 neurokinin receptors and their utilization to localize receptors in the rat gastrointestinal tract. J Neurosci 16, 6975-6986 (1996).<br>Other primary and secondary antibodies are widely used and were characterized by the manufacturer and by controls (including omission of primary or secondary antibodies). Where possible, antibodies are validated as described in: Bron, R. & Bunnett, N.W. Antibodies: friend or foe? Am J Physiol Gastrointest Liver Physiol 309, G717-718 (2015). |

## Eukaryotic cell lines

Policy information about [cell lines](#)

|                          |                                                                  |
|--------------------------|------------------------------------------------------------------|
| Cell line source(s)      | HEK-293 cells were from ATCC.                                    |
| Authentication           | HEK-293 cells were authenticated by genomic sequencing.          |
| Mycoplasma contamination | HEK-293 cell lines were tested and confirmed free of mycoplasma. |

Commonly misidentified lines  
(See [ICLAC](#) register)

None.

## Animals and other organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research

|                         |                                                                                                                                                                                                                                                                                                                                                                                                                               |
|-------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Laboratory animals      | Male C57BL/6 mice (6-10 weeks) and pregnant Asmu:Swiss mice were from the Monash Animal Research Platform. Male Sprague-Dawley rats (225–250 g) were from the facilities of the Faculty of Medicine of the University of Chile.                                                                                                                                                                                               |
| Wild animals            | The study did not involve wild animals.                                                                                                                                                                                                                                                                                                                                                                                       |
| Field-collected samples | The study did not involve animals collected in the field.                                                                                                                                                                                                                                                                                                                                                                     |
| Ethics oversight        | Studies on animals were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and adhered to the ethical guidelines of the International Association for the Study of Pain. Studies were approved by the animal ethics committee of Monash Institute of Pharmaceutical Sciences, Monash University and the Bioethics Committee of the University of Santiago of Chile. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

In the format provided by the authors and unedited.

# A pH-responsive nanoparticle targets the neurokinin 1 receptor in endosomes to prevent chronic pain

Paulina D. Ramírez-García<sup>1,2</sup>, Jeffri S. Retamal<sup>1,2</sup>, Priyank Shenoy<sup>1,2</sup>, Wendy Imlach<sup>3</sup>, Matthew Sykes<sup>3</sup>, Nghia Truong<sup>1,2</sup>, Luis Constandil<sup>4</sup>, Teresa Pelissier<sup>4</sup>, Cameron J. Nowell<sup>1</sup>, Song Y. Khor<sup>1,2</sup>, Louis M. Layani<sup>1,2</sup>, Chris Lumb<sup>1,2</sup>, Daniel P. Poole<sup>1,2</sup>, TinaMarie Lieu<sup>1,2</sup>, Gregory D. Stewart<sup>1</sup>, Quynh N. Mai<sup>1,2</sup>, Dane D. Jensen<sup>5</sup>, Rocco Latorre<sup>5</sup>, Nicole N. Scheff<sup>6</sup>, Brian L. Schmidt<sup>6</sup>, John F. Quinn<sup>1,2</sup>, Michael R. Whittaker<sup>1,2</sup>, Nicholas A. Veldhuis<sup>1,2\*</sup>, Thomas P. Davis<sup>1,2,7\*</sup> and Nigel W. Bunnett<sup>1,2,5,8\*</sup>

<sup>1</sup>Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia. <sup>2</sup>Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville, Victoria, Australia. <sup>3</sup>Monash Biomedicine Discovery Institute, Monash University, Melbourne, Victoria, Australia. <sup>4</sup>Laboratory of Neurobiology, Center for the Development of Nanoscience and Nanotechnology (CEDENNA), University of Santiago de Chile, Santiago, Chile. <sup>5</sup>Departments of Surgery and Pharmacology, Columbia University Vagelos College of Physicians and Surgeons, Columbia University in the City of New York, New York, NY, USA. <sup>6</sup>Bluestone Centre for Clinical Research, New York University College of Dentistry, New York, NY, USA. <sup>7</sup>Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Brisbane, Queensland, Australia. <sup>8</sup>Department of Pharmacology and Therapeutics, University of Melbourne, Parkville, Victoria, Australia. \*e-mail: [nicholas.veldhuis@monash.edu](mailto:nicholas.veldhuis@monash.edu); [thomas.p.davis@monash.edu](mailto:thomas.p.davis@monash.edu); [nwb2@nyu.edu](mailto:nwb2@nyu.edu)

## **Supplementary Information**

### **A pH-Responsive Nanoparticle Targets the Neurokinin 1 Receptor in Endosomes to Prevent Chronic Pain**

Paulina D. Ramírez-García Jeffri S. Retamal, Priyank Shenoy, Wendy Imlach, Matthew Sykes, Nghia Truong, Luis Constandil, Teresa Pelissier, Cameron J. Nowell, Song Y. Khor, Louis M. Layani, Chris Lumb, Daniel P. Poole, TinaMarie Lieu, Gregory D. Stewart, Quynh N. Mai, Dane D. Jensen, Rocco Latorre, Nicole N. Scheff, Brian L. Schmidt, John F. Quinn, Michael R. Whittaker, Nicholas A. Veldhuis, Thomas P. Davis, Nigel W. Bunnett

## Table of Contents

|                                                             |           |
|-------------------------------------------------------------|-----------|
| <b>1. Supplementary methods</b>                             | <b>3</b>  |
| 1.1. Synthesis of diblock copolymers                        | 3         |
| 1.2. Analysis of diblock copolymers                         | 3         |
| 1.3. Self-assembly of nanoparticles                         | 4         |
| 1.4. Characterisation of nanoparticles                      | 4         |
| 1.5. Nanoparticle disassembly                               | 5         |
| 1.6. pERK localization in mouse spinal cord                 | 5         |
| 1.7. Rotarod test                                           | 5         |
| 1.8. Electrophysiological assessment of nociception in rats | 6         |
| 1.9. NK <sub>1</sub> R localization in rat spinal cord      | 6         |
| 1.10. Cell viability assays                                 | 6         |
| <b>Supplementary Figures</b>                                | <b>7</b>  |
| Supplementary Figure 1                                      | 7         |
| Supplementary Figure 2                                      | 9         |
| Supplementary Figure 3                                      | 10        |
| Supplementary Figure 4                                      | 11        |
| Supplementary Figure 5                                      | 12        |
| Supplementary Figure 6                                      | 14        |
| Supplementary Figure 7                                      | 15        |
| Supplementary Figure 8                                      | 17        |
| <b>Supplementary Videos</b>                                 | <b>19</b> |
| Supplementary Video 1                                       | 19        |
| Supplementary Video 2                                       | 19        |
| Supplementary Video 3                                       | 19        |
| Supplementary Video 4                                       | 19        |
| Supplementary Video 5                                       | 19        |
| Supplementary Video 6                                       | 19        |

|                          |    |
|--------------------------|----|
| Supplementary Video 7    | 19 |
| Supplementary References | 20 |

## 1. Supplementary Methods

**1.1. Synthesis of diblock copolymers.** Prior each synthesis, monomers were deinitiated using basic aluminium oxide. All polymers were synthesized via reversible addition fragmentation chain (RAFT) polymerization<sup>1</sup>. All reactions were carried out in toluene at 70°C and 400 RPM unless stated otherwise. Resulting polymers were purified by dialysis (molecular weight cut off, MWCO 3500, Membrane Filtration Products, USA) against acetone for 96 h. Residual solvent was evaporated and the final products were dried for 24 h in a vacuum oven at 37°C and 1000 mbar.

*P(PEGMA-co-DMAEMA) hydrophilic block copolymer.* The macromolecular chain transfer agent (macro-CTA), P(PEGMA-co-DMAEMA), was synthesized using 2-cyanoprop-2-yl dithiobenzoate (CPBD, 0.0736 g,  $3.34 \cdot 10^{-4}$  mol) as a RAFT agent and azobisisobutyronitrile (AIBN, 0.0054 g,  $3.34 \cdot 10^{-5}$  mol) as the initiator 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 added at a ratio of 10:1 and the mixture was left to react for 21 h.

*P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) diblock copolymer.* The chain extension reaction was initiated by AIBN (0.0017 g,  $1.033 \cdot 10^{-5}$  mol), using P(PEGMA-co-DMAEMA, 0.89 g,  $6.89 \cdot 10^{-5}$  mol) and the monomers 2-[N,N-(diisopropylamino)ethyl] methacrylate (DIPMA, 1.47 g,  $6.892 \cdot 10^{-3}$  mol) and di(ethylene glycol) methyl ether methacrylate (DEGMA, 0.1427 g,  $7.58 \cdot 10^{-4}$ ) at a ratio of 0.15:1:100:11. The mixture was left to react for 17.5 h.

*P(PEGMA-co-DMAEMA)-b-P(BMA) diblock copolymer.* Butyl methacrylate (BMA, 0.582 g, 0.0041 mol) was polymerized from the hydrophilic P(PEGMA-co-DMAEMA) block by a chain extension reaction in the presence of AIBN (0.0008 g,  $5.124 \cdot 10^{-6}$  mol) at a ratio of 120:1:0.2. The solution was left to react for 15 h.

*P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-Cy5) diblock copolymer.* The chain extension of P(PEGMA-co-DMAEMA, 0.5 g,  $3.85 \cdot 10^{-5}$  mol) was done by adding DIPMA (0.82 g,  $4.6 \cdot 10^{-3}$  mol) and 4,4-dimethyl-2-vinyl-2-oxazolin-5-one (VDM, 0.027 g,  $1.92 \cdot 10^{-4}$  mol) in the presence of AIBN (0.95 mg,  $5.77 \cdot 10^{-6}$  mol) at a ratio of 1:100:11:5:0.15. The mixture was left to react for 18 h. For the BMA diblock copolymer only BMA (0.66 g,  $3.87 \cdot 10^{-3}$  mol) was added and the ratios of macro-CTA:BMA:VDM:AIBN were 1:120:5:0.15 and the mixture was left to react for 19 h. Cy5 coupling was achieved by mixing 250 µL of the reaction with Cyanine 5 amine (Cy5, 0.008 g,  $1.20 \cdot 10^{-5}$  moles) and reacting at room temperature, 400 RPM for 72 h under dark conditions.

## 1.2. Analysis of diblock copolymers

*Gel permeation chromatography.* The molecular weights of polymers were determined by gel permeation chromatography using a Shimadzu (Kyoto, Japan) liquid chromatography system (Shimadzu, Japan) equipped with a (RID-10A) differential refractive index detector and SPD-20A ultraviolet-visible detector ( $\lambda = 633$  nm). 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 bead-size, 5000 Å pore size) in series at 40°C. The eluent used was N,N-dimethylacetamide (DMAC, HPLC grade, with 0.03% w/v LiBr) with a flow rate of 1 mL/min. A molecular weight calibration curve was produced using polystyrene standards with narrow molecular weights distribution ranging from

500 to  $2 \times 10^6$  Da.

**Proton-nuclear magnetic resonance ( $^1\text{H-NMR}$ ).** The conversion of polymers was assessed by  $^1\text{H-NMR}$  analysis using a Bruker Avance III 400 Ultrashield Plus spectrometer (USA) at 400 MHz running Topspin, version 1.3, using deuterated chloroform (chloroform- $d$ ) as solvent. Conversions (Conv%) and repeating monomer units ( $n$ ) were calculated by  $^1\text{H-NMR}$  using peak integrals ( $I$ ) where the subscript number indicates the location of the peak in ppm ( $I_x$ ). The Conv% and  $n$  for P(PEGMA-co-DMAEMA) were calculated using the  $^1\text{H-NMR}$  spectra (Supplementary Fig. 1D.i) with  $\text{Conv}\% = 100 \times \frac{(I_{4-4.6} - I_{5.7})}{I_{4-4.6}}$ ,  $n_{\text{PEGMA}} = \frac{I_{4.25} - I_{2.5}}{I_{7.8-7.9}}$  and  $n_{\text{DMAEMA}} = \frac{I_{2.5}}{I_{7.8-7.9}}$ . Conv% and  $n$  for P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA) were calculated using the  $^1\text{H-NMR}$  spectra (Supplementary Fig. 1D.ii) with  $\text{Conv}\% = 100 \times \frac{(I_{4-4.6} - I_{5.7})}{I_{4-4.6}}$ ,  $n_{\text{PEGMA}} = \frac{I_{4.25} - I_{2.5}}{I_{7.8-7.9}}$  and  $n_{\text{DMAEMA}} = \frac{I_{2.5}}{I_{7.8-7.9}}$ . Conv% and  $n$  for P(PEGMA-co-DMAEMA)-b-(BMA) were calculated using the  $^1\text{H-NMR}$  spectra (Supplementary Fig. 1D.iii) with  $\text{Conv}\% = 100 \times \frac{(I_{4-4.6} - I_{5.7})}{I_{4-4.6}}$ ,  $n_{\text{PEGMA}} = \frac{I_{4.25} - I_{2.5}}{I_{7.8-7.9}}$  and  $n_{\text{DMAEMA}} = \frac{I_{2.5}}{I_{7.8-7.9}}$ . Conv% and  $n$  for P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA-co-VDM) and P(PEGMA-co-DMAEMA)-b-(BMA-co-VDM) were calculated as described for P(PEGMA-co-DMAEMA)-b-(DIPMA-co-DEGMA) and P(PEGMA-co-DMAEMA)-b-(BMA) ( $^1\text{H-NMR}$  spectra not shown).

**1.3. Self-assembly of nanoparticles.** P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) was used as the diblock copolymer to self-assemble pH-responsive nanoparticles. P(PEGMA-co-DMAEMA)-b-P(BMA) was used to self-assemble control nanoparticles without pH responsive properties. For the self-assembly of nanoparticles loaded with aprepitant, a mixture of 5 mg of diblock copolymer and 53.5, 26.75 or 13.375  $\mu\text{g}$  of aprepitant was dissolved in 0.5 mL of tetrahydrofuran (THF). Empty ( $\emptyset$ ) nanoparticles were self-assembled without adding aprepitant. The mixture was then added into 4.5 mL of phosphate-buffered saline (PBS) under vigorous stirring at a flow rate of 1.2 mL/h, using a Harvard Apparatus syringe pump (USA) at room temperature. pH-responsive nanoparticles loaded with aprepitant (DIPMA-AP) and non-pH responsive nanoparticles loaded with aprepitant (BMA-AP) were dialyzed against PBS under nitrogen flow for 24 h (MWCO 3500). Assemblies without aprepitant (DIPMA- $\emptyset$  and BMA- $\emptyset$ ) were dialyzed using Slide-A-Lyzer mini dialysis devices MWCO 3.5K (Thermo Fisher Scientific, USA) for 24 h. The assembly of nanoparticles for live cell imaging and biodistribution studies was done as described for nanoparticles without aprepitant using P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5) and P(PEGMA-co-DMAEMA)-b-(BMA-co-Cy5), which include Cy5 on the hydrophobic portion, resulting in nanoparticles with Cy5 localized in the core (DIPMA-Cy5 and BMA-Cy5).

#### 1.4. Characterisation of nanoparticles

**Dynamic light scattering (DLS).** The size distribution of nanoparticles was determined by DLS using a Zetasizer Nano ZS ZEN3600 particle size analyser (Malvern, UK). DIPMA-AP, DIPMA- $\emptyset$  and BMA-AP (1 mg/mL) were added to polystyrene cuvettes. Light scattering was measured at 25°C and 173° backscatter angle.

**Ultra-performance liquid chromatography mass spectrometry (LC-MS).** Aprepitant loading 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-AP and BMA-AP (1 mg/mL) were dissolved in a 5:2 mixture of DMSO:0.1% formic acid in water. Samples were prepared for analysis by mixing an aliquot with internal standard solution (diazepam, 5  $\mu\text{g/mL}$ ) in a 5:2 ratio and making up to 500  $\mu\text{L}$  with the dilution solvent (1:1 mixture of 50% acetonitrile and 0.1% formic acid). Samples were fractionated on a Supelco Ascentis Express RP Amide column

(50 mm by 2.1 mm, 2.7  $\mu$ m particle size) equipped with a Phenomenex SecurityGuard precolumn fitted with a Synergi Polar cartridge. Aprepitant loading was quantified against aprepitant standards (0.016 to 20  $\mu$ M). The mobile phase consisted of 0.05% formic acid in water and acetonitrile and compounds were eluted under gradient conditions. Mass spectrometry was conducted in positive electrospray ionization conditions and elution of compounds monitored with multiple-reaction monitoring.

**Determination of critical micelle concentration.** The critical micellar concentration was determined by the pyrene  $I_1/I_3$  ratio<sup>2</sup>. A pyrene stock solution (50  $\mu$ M) was prepared in THF and 5  $\mu$ L of pyrene stock were added to 995  $\mu$ L of graded concentrations of nanoparticles (400 to 0.5  $\mu$ g/mL), obtained by diluting nanoparticle stock solutions in PBS. The mixture was stirred for 3 h at room temperature and the fluorescence spectrum of pyrene was recorded from 360 to 410 nm using an excitation wavelength of 336 nm in a RF5301PC Espectrofluorophotometer (Shimadzu, Japan). The emission intensities measured at 373 nm ( $I_1$ ) and 384 nm ( $I_3$ ) were used to calculate the pyrene  $I_1/I_3$  ratio.

**Transmission electron microscopy.** The morphology of nanoparticles was determined by transmission electron microscopy imaging using a Tecnai F20 transmission electron microscope at an accelerating voltage of 200 kV at ambient temperature. An aliquot (5  $\mu$ L) of 0.1 wt% nanoparticle solution (diluted with Milli-Q water) was deposited on a Formvar coated copper grid (GSCu100F-50, Proscitech, Australia) and was allowed to dry overnight in air and at room temperature.

**1.5. Nanoparticle disassembly.** Nile Red (NR) is a solvatochromic dye that fluoresces only in non-polar solvents, allowing determination of the pH of disassembly for the nanoparticles. Specifically, the pH of disassembly is identified by observing the loss of fluorescence of NR due to release of NR from the core of nanoparticles. Nanoparticles were self-assembled using 0.1 mg of NR per mg of polymer and dialyzed as previously described. pH-responsive nanoparticles loaded with Nile red (DIPMA-NR) and non-pH responsive nanoparticles loaded with NR (BMA-NR) were prepared at a concentration of 200  $\mu$ g/mL. For pH-dependent disassembly studies, nanoparticles were suspended in a mixture of 0.1 M citric acid and 0.2 M  $\text{Na}_2\text{HPO}_4$  buffer solutions with a pH range from 7.6 to 5.0. pH-dependent disassembly was assessed by measuring NR fluorescence (excitation/emission 552/636nm) using a FlexStation 3 (Molecular Devices, USA). The time course of nanoparticle disassembly was examined by measuring NR fluorescence at pH 7.4, 6.5, 6.0 and 5.0 over a 12 h period using a CLARIOstar (BMG LABTECH, Germany).

**1.6. pERK localization in mouse spinal cord.** Spinal cord sections were preincubated in 10% normal donkey serum (NDS) in 0.1 M PBS (1 h, room temperature), followed by rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:200; #4370; Cell Signalling Technology, Danvers, MA) and guinea-pig anti-NeuN (1:1000, Millipore, #abN90) in PBS containing 0.3% Triton X-100 and 5% NDS (overnight, 4°C). Sections were washed 4x in 0.1 M PBS containing 0.3% Triton X-100 and incubated with donkey anti-rabbit Alexa 488 and donkey anti-guinea-pig Alexa 568 (1:1000, Thermo Fisher Scientific) (45 min, room temperature). Sections were washed 5x in PBS, counter-stained with DAPI (10  $\mu$ g/mL, 5 min) and cover-slipped with ProLong Glass mounting medium (Thermo Fisher Scientific). Sections were imaged on Leica SP8 confocal microscope with HC PLAPO 40X for counting pERK-immunoreactive (IR) neurons or a 20X objective to collect representative images. For each experimental group, 6 sections of ipsilateral and contralateral dorsal horn were imaged. Only pERK-positive neurons co-labelled with NeuN were counted. To avoid re-counting the same neurons, the analysed sections were 100  $\mu$ m apart. The total number of neurons was averaged for each group.

**1.7. Rotarod test.** A rotarod test was used to assess whether the intrathecal injection of nanoparticles would affect normal motor function and thereby impede studies of nociception that

require examination of paw withdrawal from a painful stimulus. Prior to experiments, mice were acclimatized and trained on the rotarod apparatus for three consecutive runs on two successive days. On the day of the experiment, three baseline readings were recorded and a cut-off threshold of 120 second was pre-set. Nanoparticles (DIPMA-AP, BMA-AP, DIPMA-Ø, 10 µg/mL) or vehicle (aCSF) was injected intrathecally as described above. Subsequently, the latency of mice to fall (seconds) were recorded at 30, 60, 90, 120, 180 and 240 min post-injection<sup>3</sup>.

**1.8. Electrophysiological assessment of nociception in rats.** Rats were maintained under anaesthesia (1.2-1.5% isoflurane in oxygen using a diaphragm rodent facemask) and placed on a regulated thermal pad ( $37 \pm 0.5^{\circ}\text{C}$ ). EMG activity was measured using a pair of platinum stimulation electrodes inserted subcutaneously into the lateral part of the third and fourth toes, and recording electrodes inserted through the skin into the ipsilateral biceps femoris muscle<sup>4</sup>. The C-reflex corresponds to the integration of the reflex response into a 150-450 ms time window post-stimulus. Wind-up is a potentiation of the C-reflex response when the stimulating frequency is increased to 1 Hz. The wind-up score corresponds to the slope of the first seven consecutive C-reflex recordings obtained at 1 Hz stimulation. After recording to obtain a stable C-reflex response (~30 min), the threshold for C-reflex was estimated and the rats remained stimulated at 2X the threshold intensity for the duration of the experiment. The C-reflex was evaluated by the mean of 15 consecutive stimuli at 0.1 Hz while the next 7 stimuli at 1 Hz were used to evaluate wind-up. Recordings were made 10 days after surgery before (basal) and 30, 60, 90 and 120 min after intrathecal drug administration. The integrated C-reflex responses were expressed as a percentage of basal response.

**1.9. NK<sub>1</sub>R localization in rat spinal cord.** Free floating sections were blocked in PBS containing 0.3% Triton x-100 and 10% NDS (1 h, room temperature). Sections were incubated with rabbit anti-NK<sub>1</sub>R (1:1000, #94168) and guinea-pig anti-NeuN (1:1000, Millipore, abn90) in PBS containing 0.3% Triton X-100 and 3% NDS (overnight, 4°C). Sections were washed 4x in PBS and incubated with donkey anti-rabbit Alexa488 and donkey anti-guinea-pig Alexa568 (1:1000, Thermo Fisher Scientific) (2 h, room temperature). Sections were washed 5x in PBS, counter stained with DAPI (10 µg/ml, 5 min), and mounted onto ColorFrost Plus slides (VWR) with ProLong Glass mounting medium (Thermo Fisher Scientific). Sections were imaged on Leica SP8 confocal microscope with a HC PL APO 63x oil objective (NA 1.4). Z stacks of NK<sub>1</sub>R-positive neurons in lamina I of the dorsal horn were collected with a digital zoom of 5. To quantify NK<sub>1</sub>R endocytosis in lamina I neurons, the border of the cytoplasm of the soma was delineated by NeuN fluorescence. NK<sub>1</sub>R immunoreactivity within 5 pixels (0.5 µm) of the border was defined as plasma membrane receptor. The ratio of plasma membrane to cytosolic NK<sub>1</sub>R-IR fluorescence was determined in >6 lamina I neurons per condition.

**1.10. Cell viability assays.** HEK-hNK<sub>1</sub>R cells were incubated with empty nanoparticles (1-100 µg/mL) for 24 and 48 h. Medium was replaced by phenol red-free DMEM, followed by incubation for 2 h (37°C, 5% CO<sub>2</sub>) with 10% (v/v) alamarBlue reagent (Thermo Fisher Scientific, USA). Fluorescence of the reduced active compound, resofurin, was measured (510/610nm exc/em) using a ClarioStar (BMG LABTECH, Germany).

## Supplementary Figures

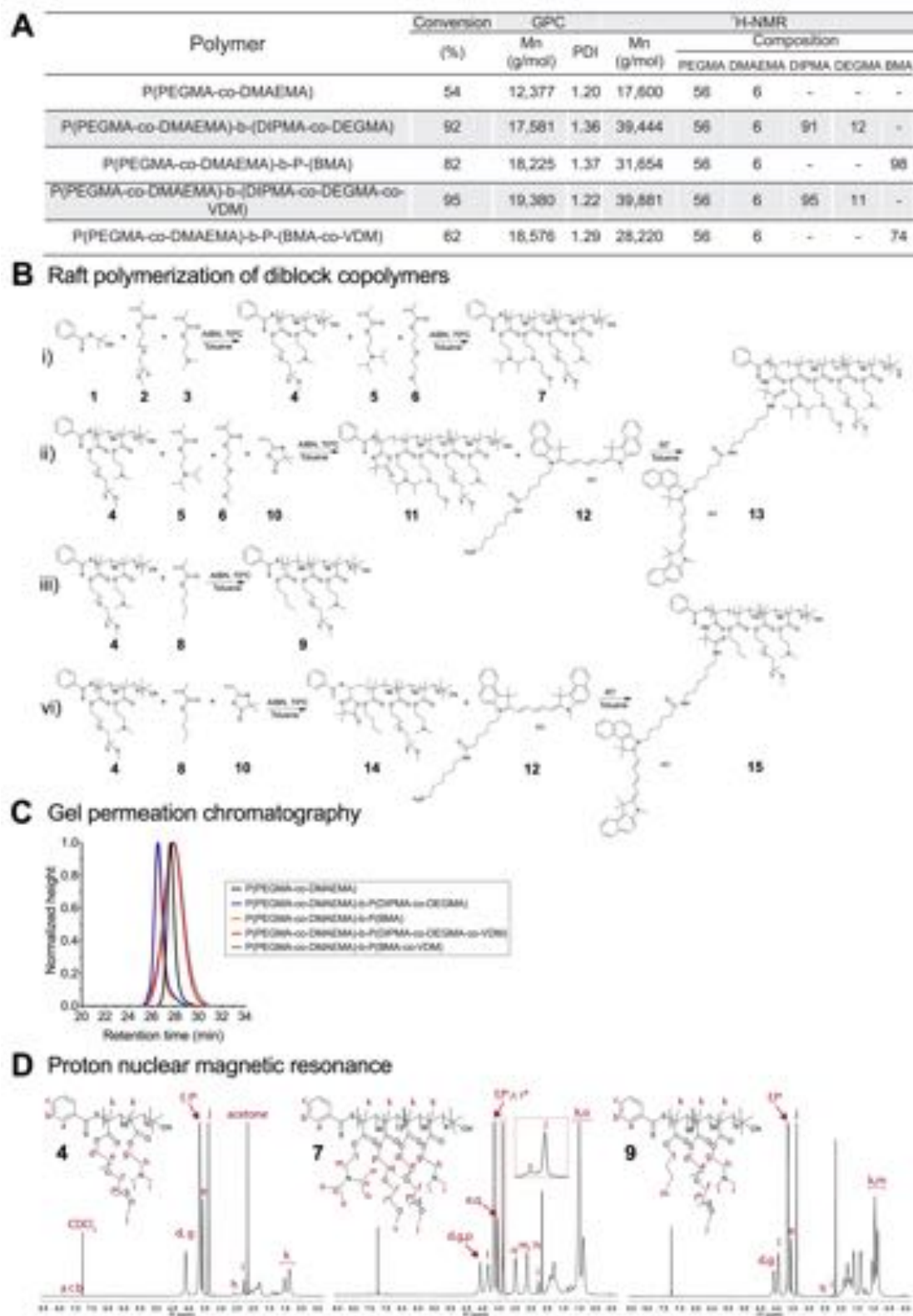


Figure S1. Synthesis and characterization of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-

**DEGMA) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-DEGMA) diblock copolymers. A.** Characterization of the hydrophilic block copolymers and the diblock copolymers. **B. i)** Sequential RAFT polymerization indicating synthesis of the hydrophilic block using (1) CPDB, (2) PEGMA and (3) DMAEMA to form (4) p(PEGMA-co-DMAEMA); synthesis of P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) by chain extension reaction, where addition of the pH-responsive monomer (5) DIPMA and the charge screening monomer (6) DEGMA to (4) the hydrophilic block forms (7) the diblock P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA); **ii)** Addition of (5) DIPMA, (6) DEGMA and (10) VDM to (4) the hydrophilic block to form the intermediate (11), followed by the addition of (12) Cy5 to form the final Cy5 conjugated polymer (13) P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA-co-Cy5). **iii)** Addition of (8) BMA to (4) the hydrophilic block forms (9) P(PEGMA-co-DMAEMA)-b-P(BMA). **iv)** Addition of (8) BMA and (10) VDM to (4) the hydrophilic block to form the intermediate (14), followed by the addition of (12) Cy5 to form the final Cy5 conjugated polymer (15) P(PEGMA-co-DMAEMA)-b-P(BMA-co-Cy5). **C.** Gel permeation chromatography traces showing a shift from P(PEGMA-co-DMAEMA) to higher molecular weight (*i.e.*, shorter retention time) after chain extension to form P(PEGMA-co-DMAEMA)-b-P(DIPMA-co-DEGMA) and P(PEGMA-co-DMAEMA)-b-P(BMA-co-DEGMA). **D.** <sup>1</sup>H-NMR spectra of the resulting polymers indicating the successful incorporation of the monomers. <sup>1</sup>H-NMR was used to estimate molecular weight since gel permeation chromatography was calibrated using polystyrene standards. **C** and **D** are from a single independent experiment because the polymer was made once and used throughout the project.

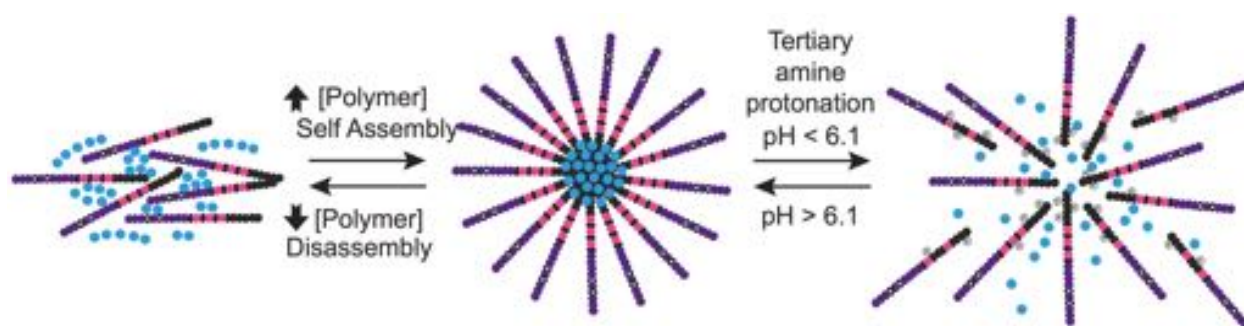


**Figure S2. Mechanism of concentration-dependent self-assembly and pH-dependent disassembly of DIPMA nanoparticles.** Increasing concentrations of polymer result in nanoparticle self-assembly in an aqueous solution. DIPMA nanoparticles possess a tertiary amine on the DIPMA units located in the core. At pH<6.1, protonation results in a change from neutral to positive charge on DIPMA that induce like-like charge repulsion, which destabilizes the nanoparticle core with the subsequent disassembly and release of cargo.

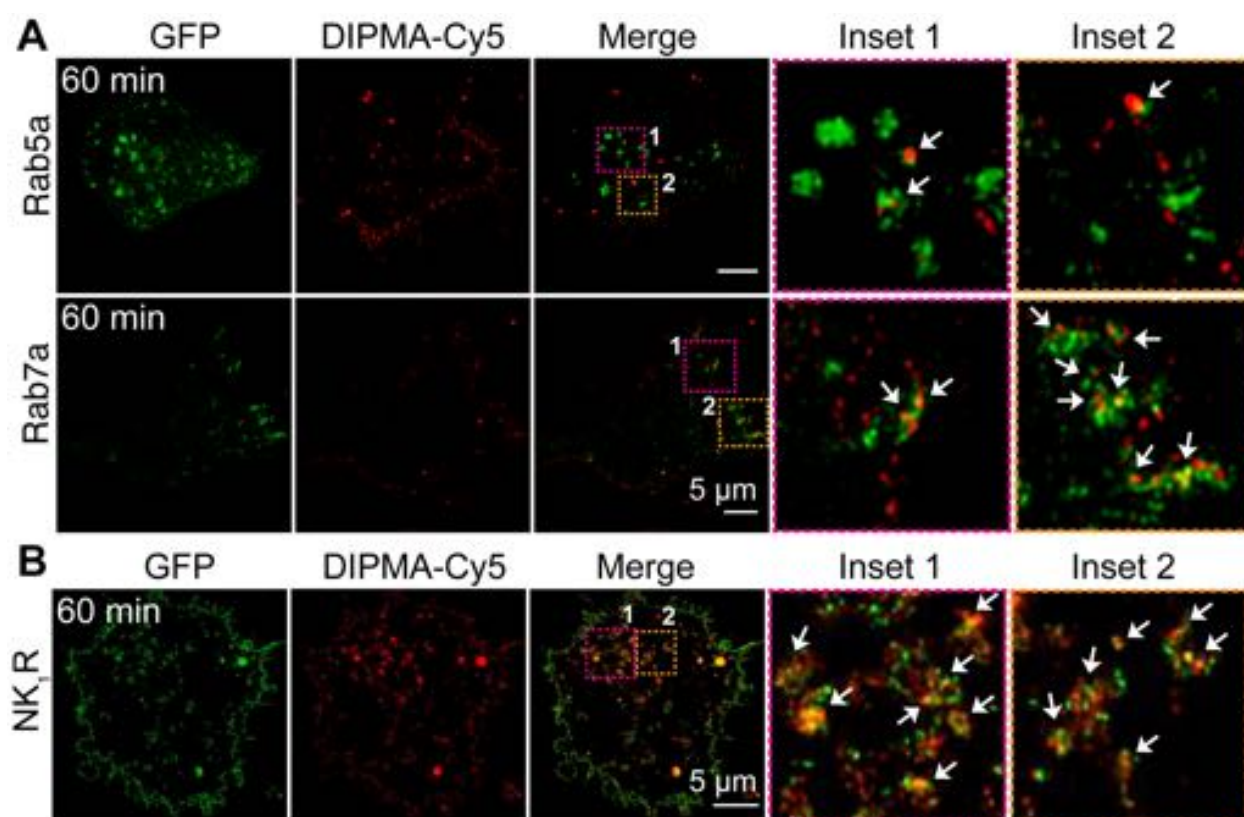


**Figure S3. Uptake of DIPMA-Cy5 nanoparticles in HEK-293 cells. A.** Localization of DIPMA-Cy5 nanoparticles in Rab5a-GFP early endosomes and Rab7a-GFP late endosomes after incubation with HEK-293 cells for 60 min. Representative results,  $n = 5$  independent experiments. **B.** Colocalization of DIPMA-Cy5 nanoparticles and NK<sub>1</sub>R-GFP in HEK-rNK<sub>1</sub>R cells at 60 min after stimulation with SP to induce NK<sub>1</sub>R endocytosis. Representative results,  $n = 5$  independent experiments.

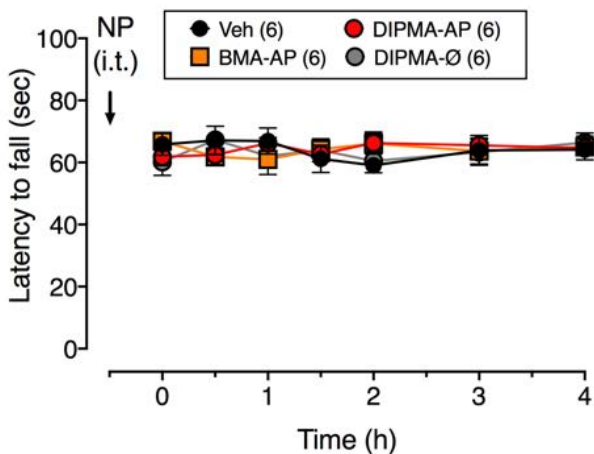


**Figure S4. Effects of nanoparticles on rotarod latency.** Effects of intrathecal (i.t.) injection of vehicle (Veh), DIPMA-Ø, BMA-AP or DIPMA-AP nanoparticles (NP) on latency to fall in mice, assessed using rotarod. Data are presented as mean  $\pm$  SEM from  $n = 6$  mice (numbers in parentheses) for each treatment group.

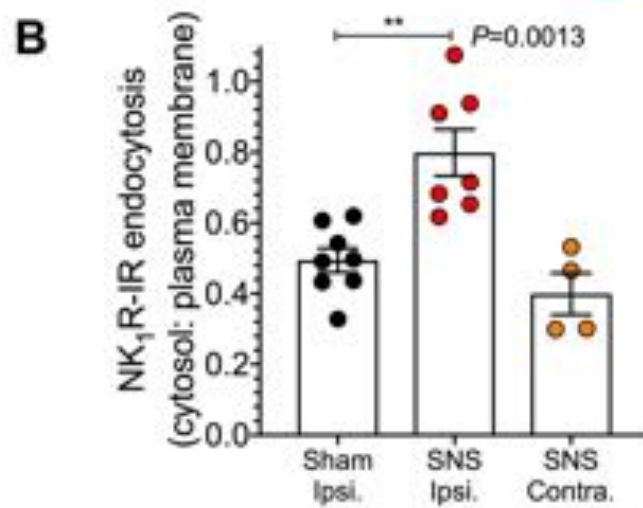
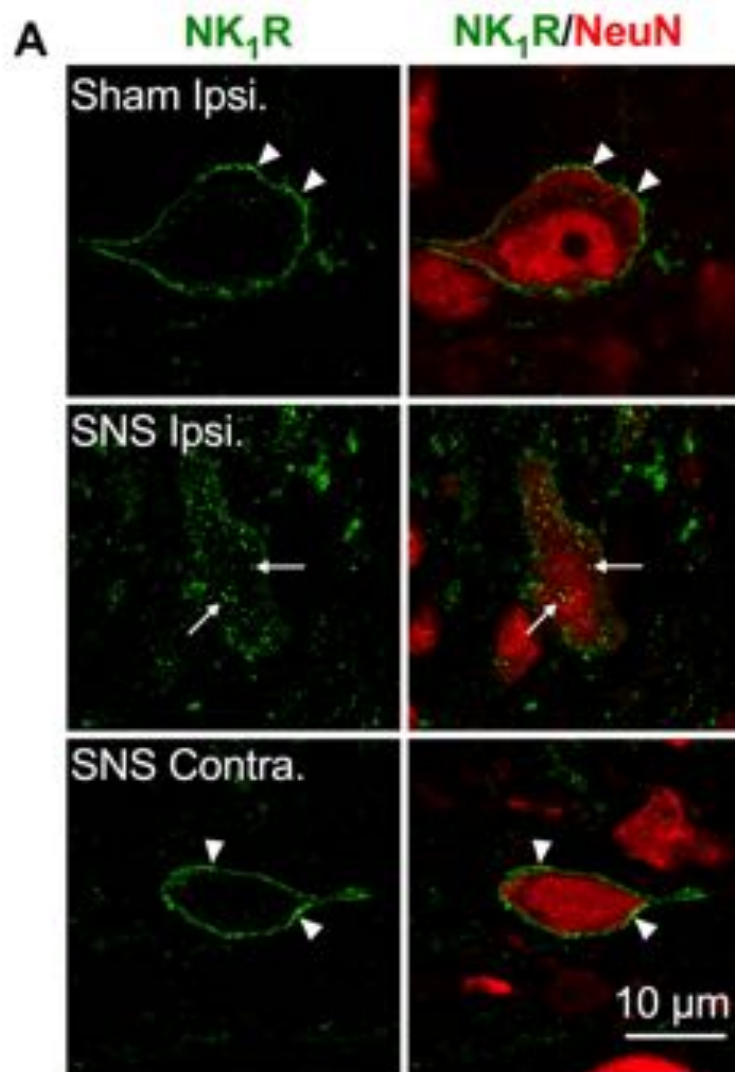


**Figure S5. NK<sub>1</sub>R endocytosis in chronic neuropathic nociception.** To confirm activation of the SP/NK<sub>1</sub>R system during chronic neuropathic nociception, the NK<sub>1</sub>R was localized in the dorsal

horn of rats 10 days after sural nerve spared (SNS) or sham surgery by immunofluorescence. **A.** Localization of NK<sub>1</sub>R-IR and NeuN-IR to the ipsilateral (Ipsi.) or contralateral (Contra.) lamina I dorsal horn of sham or SNS rats. Arrow heads denote plasma membrane. Arrows denote endosomes. **B.** Quantification of NK<sub>1</sub>R endocytosis, assessed as the cytosol:plasma membrane pixel intensity for NK<sub>1</sub>R-IR neurons. Data are presented as mean  $\pm$  SEM from sham ipsilateral ( $n = 8$  rats), SNS ipsilateral ( $n = 7$  rats) and SNS contralateral ( $n = 4$  rats) groups. 1-way ANOVA, non-parametric Tukey post-hoc test. \*\* $P < 0.01$ .

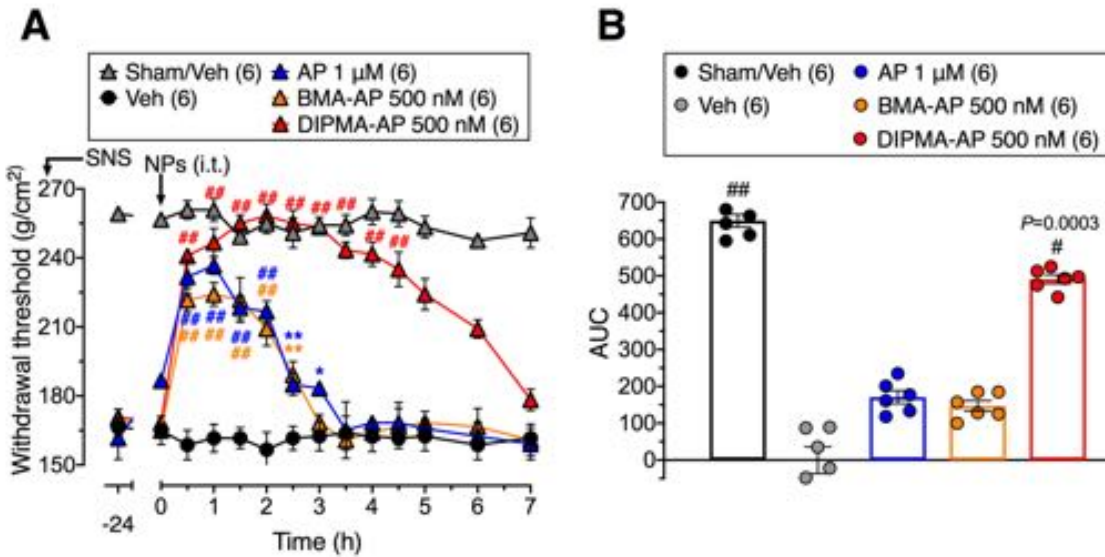


**Figure S6. Effects of nanoparticles on neuropathic nociception.** The sural nerve spared (SNS) model of chronic neuropathic pain was studied in rats. Vehicle (Veh), aprepitant (AP) or nanoparticle (NP) was administered by intrathecal injection 10 days after SNS or sham surgery. Paw withdrawal responses were assessed using the Randall-Selitto test. **A.** Time course of response. **B.** Area under curve (AUC) from 0-7 h. Data are presented as mean  $\pm$  SEM from  $n = 6$  rats (numbers in parentheses) for each treatment group. \* $P < 0.05$ , \*\* $P < 0.005$ , # $P < 0.001$ , ## $P < 0.0001$  compared to SNS vehicle. 1-way ANOVA, nonparametric Dunn's post-hoc test.

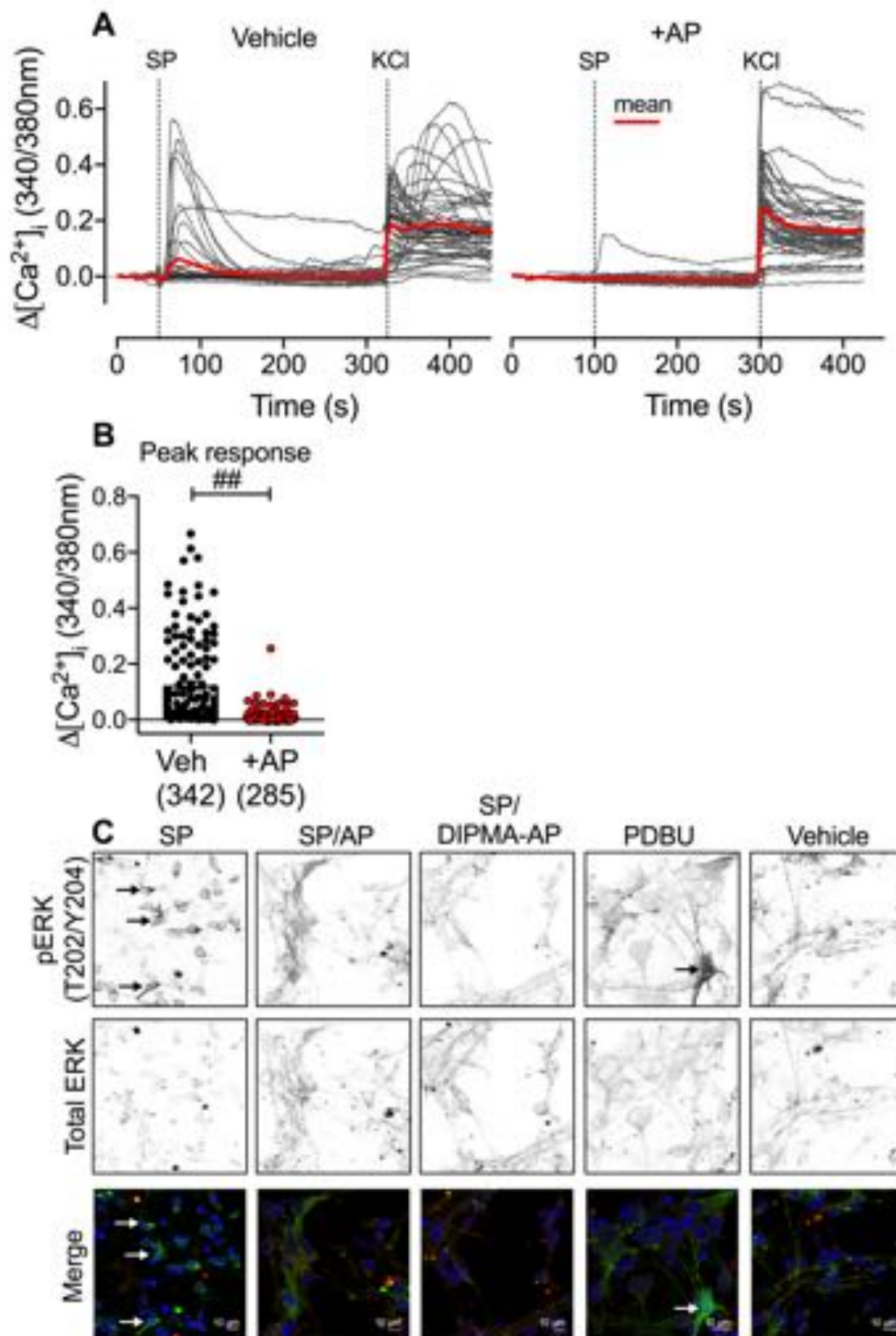


Fig. S7. SP signaling in mouse striatal neurons. SP (100 nM)-induced  $Ca^{2+}$  signaling in striatal

neurons pretreated with vehicle (Veh) or aprepitant (AP). **A.** Representative traces of  $[Ca^{2+}]_i$ . Grey lines show responses of individual neurons. Red lines show mean responses. **B.** Peak  $[Ca^{2+}]_i$  responses. Data are expressed as mean  $\pm$  SEM from  $n = 342$  neurons for SP plus vehicle or  $n = 285$  neurons for SP plus aprepitant (numbers in parentheses) from  $n = 3$  independent experiments for both treatment groups.  $###P < 0.0001$ , unpaired  $t$ -test (2-tailed). **C.** Representative images of phospho-ERK and total ERK immunostaining in cultured striatal neurons from  $n = 3$  independent experiments. Neurons were treated with SP, SP plus aprepitant (AP), SP plus DIPMA-AP, phorbol 12,13-dibutyrate (PDBU) or vehicle.

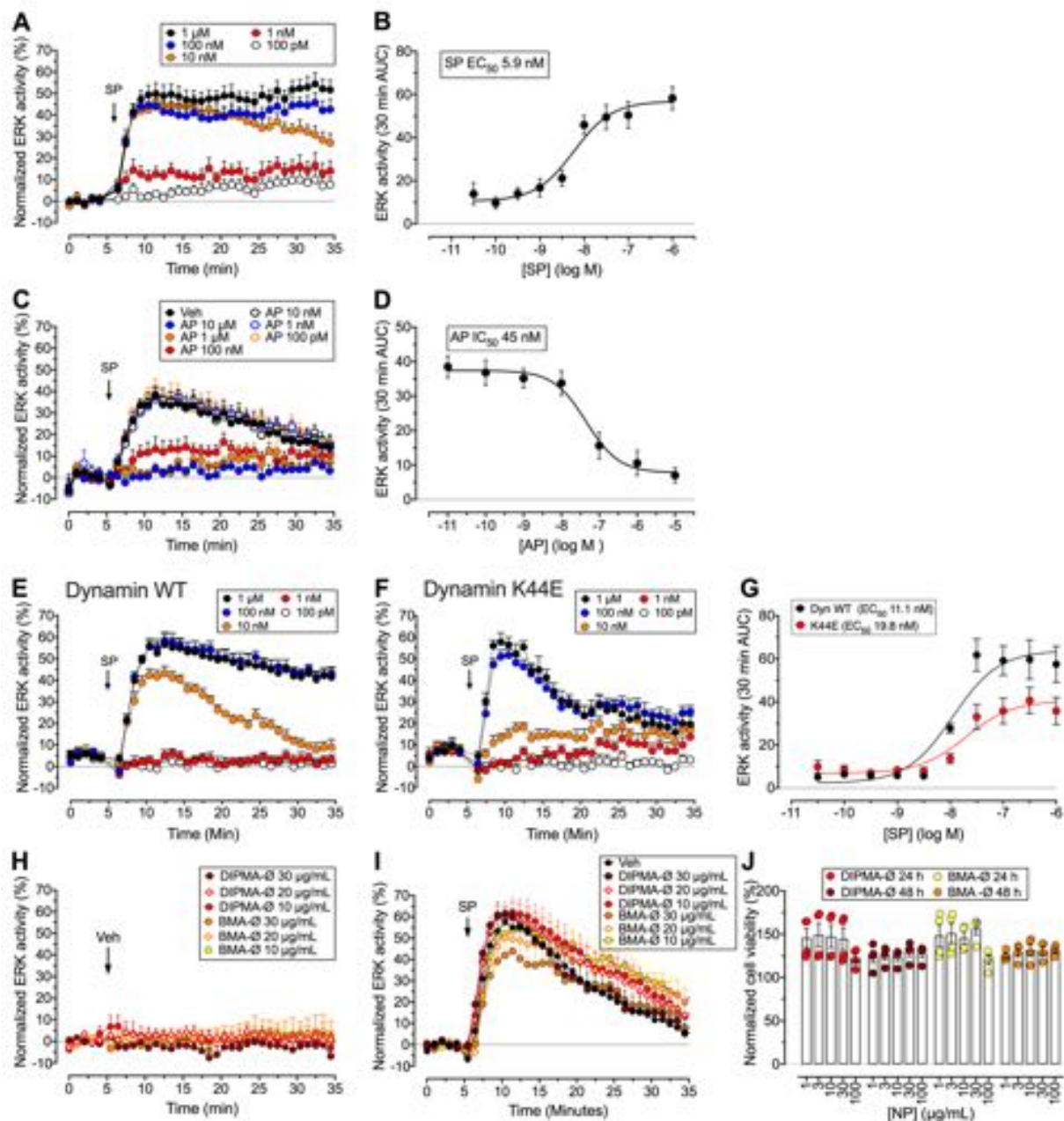


**Figure S8. Nuclear ERK signalling and toxicity assays in HEK-293 cells.** A-I. SP activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells. To examine activation of nuclear ERK, HEK-293 cells expressing hNK<sub>1</sub>R were transfected with NucEKAR (FRET biosensor for nuclear ERK). Data are expressed as mean  $\pm$  SEM, with triplicate observations made in each experiment. **A.** Effects of graded concentrations of SP on nuclear ERK activity;  $n = 8$  independent experiments. **B.** SP concentration-response curves;  $n = 8$  independent experiments. **C.** Effects of graded concentrations of aprepitant (AP) on nuclear ERK response to SP (5 nM);  $n = 7$  independent experiments. **D.** Aprepitant concentration-response curves;  $n = 7$  independent experiments. **E-G.** SP-induced activation of nuclear ERK in HEK-hNK<sub>1</sub>R cells expressing dynamin wildtype (Dyn WT, **E**) or dynamin K44E (Dyn K44E, **F**);  $n = 6$  independent experiments. **G.** SP concentration-response curves;  $n = 6$  independent experiments. **H, I.** Effects of DIPMA- $\emptyset$  and BMA- $\emptyset$  on basal nuclear ERK activity in HEK-293 cells (**H**) and on SP-stimulated nuclear ERK activity in HEK-

hNK<sub>1</sub>R cells (I) over 30 min;  $n = 7$  independent experiments. J. Effects DIPMA-Ø and BMA-Ø on viability of HEK-293 cells over 24 h and 48 h. Viability was examined using alamarBlue, which assess the capability of viable cells to reduce rezasurin to resofurin; triplicate observations,  $n = 4$  independent experiments.

## Supplementary Videos

**Video S1. Localization of DIPMA-Cy5 nanoparticles and Rab5a-GFP in HEK-293 cells.** The video shows trafficking of DIPMA-Cy5 nanoparticles (red) to Rab5a-GFP early endosomes (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 30 min.

**Video S2. Localization of DIPMA-Cy5 nanoparticles and Rab7a-GFP in HEK-293 cells.** The video shows trafficking of DIPMA-Cy5 nanoparticles (red) to Rab7a-GFP late endosomes (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 30 min.

**Video S3. Localization of DIPMA-Cy5 nanoparticles and NK<sub>1</sub>R-GFP in HEK-293 cells.** The video shows trafficking of DIPMA-Cy5 nanoparticles (red) and NK<sub>1</sub>R-GFP (green). Cells were incubated with DIPMA-Cy5 nanoparticles for 90 min and with SP for 60 min to induce NK<sub>1</sub>R endocytosis.

**Video S4. Localization of DIPMA-Cy5 nanoparticles in the mouse dorsal horn.** The video is a 3D projection of DIPMA-Cy5 nanoparticles in the perinuclear region of lamina I cells in the dorsal horn of the mouse spinal cord. The image was taken at 1 h after intrathecal injection of nanoparticles. Nuclei are stained with DAPI.

**Video S5. Localization of BMA-Cy5 nanoparticles in the mouse dorsal horn.** The video is a 3D projection of BMA-Cy5 nanoparticles in the perinuclear region of lamina I cells in the dorsal horn of the mouse spinal cord. The image was taken at 1 h after intrathecal injection of nanoparticles. Nuclei are stained with DAPI.

**Video S6. Localization of NK<sub>1</sub>R-IR in the rat dorsal horn after sham surgery.** The video is a 3D projection showing the subcellular localization of NK<sub>1</sub>R-IR (green) of lamina I spinal neuron (NeuN, red). The image was taken at 10 days after sham surgery.

**Video S7. Localization of NK<sub>1</sub>R-IR in the rat dorsal horn after SNS surgery.** The video is a 3D projection showing the subcellular localization of NK<sub>1</sub>R-IR (green) of lamina I spinal neuron (NeuN, red). The images were taken at 10 days after SNS surgery.

### Supplementary References

1. Chiefari, J., *et al.* Living free-radical polymerization by reversible addition–fragmentation chain transfer: The raft process. *Macromolecules* **31**, 5559-5562 (1998)
2. Aguiar, J., Carpena, P., Molina-Bolívar, J. A. & Carnero Ruiz, C. On the determination of the critical micelle concentration by the pyrene 1:3 ratio method. *J. Coll. Inter. Sci.* **258**, 116-122 (2003)
3. 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. Trans. Med.* **9**, eaal3447 (2017)
4. Bravo, D., *et al.* Pannexin 1: A novel participant in neuropathic pain signaling in the rat spinal cord. *Pain* **155**, 2108-2115 (2014)

# Endosomal signaling of delta opioid receptors is an endogenous mechanism and therapeutic target for relief from inflammatory pain

Nestor N. Jimenez-Vargas<sup>a</sup>, Jing Gong<sup>b</sup>, Matthew J. Wisdom<sup>c</sup>, Dane D. Jensen<sup>c,d</sup>, Rocco Latorre<sup>c</sup>, Alan Hegron<sup>c</sup>, Shavonne Teng<sup>c</sup>, Jesse J. DiCello<sup>e</sup>, Pradeep Rajasekhar<sup>e</sup>, Nicholas A. Veldhuis<sup>e,f</sup>, Simona E. Carbone<sup>e</sup>, Yang Yu<sup>a</sup>, Cintya Lopez-Lopez<sup>a</sup>, Josue Jaramillo-Polanco<sup>a</sup>, Meritxell Canals<sup>g</sup>, David E. Reed<sup>a</sup>, Alan E. Lomax<sup>a</sup>, Brian L. Schmidt<sup>d</sup>, Kam W. Leong<sup>b</sup>, Stephen J. Vanner<sup>a</sup>, Michelle L. Halls<sup>e,1</sup>, Nigel W. Bunnett<sup>c,1</sup>, and Daniel P. Poole<sup>e,f,1</sup>

<sup>a</sup>Gastrointestinal Diseases Research Unit, Division of Gastroenterology, Queen's University, Kingston, ON, Canada K7L 2V7; <sup>b</sup>Department of Biomedical Engineering, Fu Foundation School of Engineering and Applied Science, Columbia University, New York, NY 10032; <sup>c</sup>Department of Molecular Pathobiology, New York University College of Dentistry, New York, NY 10010; <sup>d</sup>Bluestone Center for Clinical Research, New York University College of Dentistry, New York, NY 10010; <sup>e</sup>Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia; <sup>f</sup>Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville, VIC 3052, Australia; and <sup>g</sup>Centre for Membrane Proteins and Receptors and Division of Physiology, Pharmacology, and Neuroscience, School of Life Sciences, Queen's Medical Centre, University of Nottingham, NG7 2RD Nottingham, United Kingdom

Edited by Robert J. Lefkowitz, Howard Hughes Medical Institute and Duke University Medical Center, Durham, NC, and approved May 18, 2020 (received for review January 9, 2020)

**Whether G protein-coupled receptors signal from endosomes to control important pathophysiological processes and are therapeutic targets is uncertain. We report that opioids from the inflamed colon activate  $\delta$ -opioid receptors (DOPr) in endosomes of nociceptors. Biopsy samples of inflamed colonic mucosa from patients and mice with colitis released opioids that activated DOPr on nociceptors to cause a sustained decrease in excitability. DOPr agonists inhibited mechanically sensitive colonic nociceptors. DOPr endocytosis and endosomal signaling by protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) pathways mediated the sustained inhibitory actions of endogenous opioids and DOPr agonists. DOPr agonists stimulated the recruitment of  $G\alpha_{i/o}$  and  $\beta$ -arrestin1/2 to endosomes. Analysis of compartmentalized signaling revealed a requirement of DOPr endocytosis for activation of PKC at the plasma membrane and in the cytosol and ERK in the nucleus. We explored a nanoparticle delivery strategy to evaluate whether endosomal DOPr might be a therapeutic target for pain. The DOPr agonist DADLE was coupled to a liposome shell for targeting DOPr-positive nociceptors and incorporated into a mesoporous silica core for release in the acidic and reducing endosomal environment. Nanoparticles activated DOPr at the plasma membrane, were preferentially endocytosed by DOPr-expressing cells, and were delivered to DOPr-positive early endosomes. Nanoparticles caused a long-lasting activation of DOPr in endosomes, which provided sustained inhibition of nociceptor excitability and relief from inflammatory pain. Conversely, nanoparticles containing a DOPr antagonist abolished the sustained inhibitory effects of DADLE. Thus, DOPr in endosomes is an endogenous mechanism and a therapeutic target for relief from chronic inflammatory pain.**

pain | inflammation | G protein-coupled receptors | signaling | nanomedicine

**G** protein-coupled receptors (GPCRs) control essential pathophysiological processes. One-third of Food and Drug Administration-approved drugs target GPCRs (1). GPCRs at the plasma membrane detect extracellular ligands and couple to heterotrimeric G proteins. Plasma membrane signaling is rapidly terminated. GPCR kinases phosphorylate activated GPCRs, which increases the affinity for  $\beta$ -arrestins ( $\beta$ ARRs) (2).  $\beta$ ARRs uncouple GPCRs from G proteins and desensitize signaling, and also couple GPCRs to the clathrin endocytic machinery (3).  $\beta$ ARRs also recruit GPCRs, G proteins, and mitogen-activated protein kinases to endosomes (4, 5). Endosomes are an important site of continued GPCR signaling (6–8).

GPCRs control multiple steps of pain transmission (9). Endosomal signaling of protease-activated receptor-2 in primary sensory neurons and of neurokinin 1 receptor (NK<sub>1</sub>R) and calcitonin-like receptor (CLR) in second-order neurons mediates neuronal excitation and pain transmission (10–13). The  $\delta$ -,  $\mu$ - and  $\kappa$ -opioid receptors (DOPr, MOPr, and KOPr) inhibit excitation of primary sensory, spinal, and supraspinal neurons and thereby induce analgesia (14). In patients with inflammatory bowel disease (IBD), infiltrating lymphocytes release opioids that activate opioid receptors on nociceptors to suppress excitability, providing an endogenous system of pain control (15–19). It is not known whether opioid receptors at the plasma

## Significance

G protein-coupled receptors are considered to function principally at the cell surface. We present evidence that the  $\delta$ -opioid receptor (DOPr) signals from endosomes to cause a sustained inhibition of pain. Opioids from the inflamed human and mouse colon, along with selective agonists that evoked DOPr internalization, inhibited the excitability of nociceptors by a mechanism requiring DOPr endocytosis. DOPr in endosomes generated a subset of signals in subcellular compartments that inhibited neuronal excitability. A DOPr agonist that was encapsulated into nanoparticles designed to selectively activate DOPr in endosomes of nociceptors caused a long-lasting inhibition of neuronal excitability and pain. Our results support the hypothesis that endosomal signaling of DOPr is an endogenous mechanism and therapeutic target for relief from inflammatory pain.

Author contributions: N.N.J.-V., J.G., D.D.J., R.L., A.H., J.J.D., P.R., N.A.V., S.E.C., Y.Y., C.L.-L., J.J.-P., M.C., D.E.R., A.E.L., B.L.S., K.W.L., S.J.V., M.L.H., N.W.B., and D.P.P. designed research; N.N.J.-V., J.G., M.J.W., D.D.J., R.L., A.H., S.T., J.J.D., P.R., N.A.V., S.E.C., Y.Y., C.L.-L., J.J.-P., D.E.R., and A.E.L. performed research; N.N.J.-V., J.G., M.J.W., D.D.J., R.L., A.H., J.J.D., P.R., N.A.V., S.E.C., Y.Y., C.L.-L., J.J.-P., M.C., D.E.R., A.E.L., B.L.S., K.W.L., S.J.V., M.L.H., N.W.B., and D.P.P. analyzed data; and N.N.J.-V., A.H., B.L.S., K.W.L., S.J.V., M.L.H., N.W.B., and D.P.P. wrote the paper.

Competing interest statement: N.W.B. is a founding scientist of Endosome Therapeutics Inc. Research in the laboratories of N.A.V., N.W.B., and D.P.P. is funded in part by Takeda Pharmaceuticals International.

This article is a PNAS Direct Submission.

Published under the PNAS license.

<sup>1</sup>To whom correspondence may be addressed. Email: michelle.halls@monash.edu, nwb2@nyu.edu, or daniel.poole@monash.edu.

This article contains supporting information online at <https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2000500117/-DCSupplemental>.

First published June 16, 2020.

membrane or in endosomes mediate this endogenous analgesic pathway and are the optimal target for treatment of inflammatory pain.

Here we investigated the hypothesis that opioids from the inflamed colon activate DOPr in endosomes of nociceptors to evoke signals that cause long-lasting inhibition of excitability and analgesia, and that DOPr in endosomes is a superior therapeutic target for inflammatory pain.

## Results

**DOPr Inhibits Inflammatory Pain.** We investigated whether opioids from the inflamed colon activate opioid receptors on nociceptors and decrease excitability. Segments of colon from healthy control (HC) mice and from mice with colitis induced by chronic administration of dextran sulfate sodium (cDSS) were incubated in culture medium for 24 h to allow opioid release into the supernatant (16–18). Mouse dorsal root ganglia (DRG) neurons were exposed to HC or cDSS supernatant for 60 min and then washed (Fig. 1A).

To assess sustained changes in excitability, the rheobase (minimum input current required to fire an action potential) of small-diameter neurons was measured by patch-clamp recordings at 30 min after washing ( $T = 30$  min) (11). The rheobase of neurons exposed to cDSS supernatant was  $29 \pm 6\%$  higher than that of neurons exposed to HC supernatant ( $P < 0.05$ ), consistent with decreased excitability (Fig. 1A and B). To examine whether these findings translate to IBD, supernatants were obtained from colonic biopsy specimens from HC patients and patients with chronic ulcerative colitis (cUC). The rheobase of neurons exposed to cUC supernatant was  $62 \pm 16\%$  higher than that of neurons exposed to

HC supernatant ( $P < 0.01$ ) (Fig. 1C and D). Preincubation of neurons with the DOPr antagonist SDM25N (100 nM, 60 min) abolished the sustained effects of cDSS supernatant on rheobase, whereas the MOPr antagonist CTOP (100 nM, 60 min) had no effect (Fig. 1E and F). Neither SDM25N nor CTOP affected the rheobase of neurons exposed to mouse HC supernatant (Fig. 1F). Thus, opioids from the inflamed colon cause a DOPr-mediated inhibition of nociceptors.

**Endosomal DOPr Inhibits Nociceptor Excitability.** To determine whether DOPr undergoes clathrin- and dynamin-mediated endocytosis in nociceptors, we isolated DRG neurons from knockin mice expressing DOPr fused to enhanced green fluorescent protein (DOPr-eGFP) (20). In vehicle-treated neurons, DOPr-eGFP was detected at the plasma membrane and in vesicles of the soma and neurites (Fig. 2A). The DOPr agonist DADLE (1  $\mu$ M, 30 min) induced depletion of DOPr-eGFP from the plasma membrane and redistribution to endosomes. Dyngo4a (Dy4; 30  $\mu$ M), which inhibits dynamin (21), and PitStop2 (PS2; 15  $\mu$ M), which inhibits clathrin-mediated endocytosis (22), prevented DADLE-evoked endocytosis of DOPr-eGFP, as confirmed by quantification of plasma membrane and cytosolic DOPr-eGFP (Fig. 2B).

To examine the contribution of DOPr endocytosis to the inhibitory effects of endogenous opioids, we pretreated neurons with Dy4 or PS2 and then challenged them with cDSS, cUC, or HC supernatant. Neurons were washed, and rheobase was measured after 30 min. Dy4 and PS2 prevented the sustained increase in rheobase of neurons exposed to cDSS and cUC supernatants (Fig. 2C and D). Inactive forms of Dy4 and PS2 do not affect the rheobase of nociceptors (11).

We similarly examined the contribution of endocytosis to the effects of DOPr- and MOPr-selective agonists on neuronal excitability. We exposed nociceptors to DOPr-selective agonists, including DADLE and SNC80 (10 nM, 15 min), which evoke  $\beta$ ARR recruitment and DOPr endocytosis, and a 10-fold higher concentration of ARM390 (100 nM, 15 min), a weakly internalizing agonist (23) (Fig. 2E). Neurons were washed, and rheobase was measured immediately ( $T = 0$  min) or 30 min ( $T = 30$  min) after washing. DADLE and SNC80 caused both immediate ( $T = 0$  min) and sustained ( $T = 30$  min) increases in rheobase (Fig. 2E and F). ARM390 increased rheobase at  $T = 0$  min but not at  $T = 30$  min (Fig. 2G). PS2 abolished the effects of DADLE and SNC80, but not of ARM390. The MOPr agonist DAMGO caused an immediate increase in rheobase that was not sustained and was inhibited by PS2 (Fig. 2H).

GPCRs in endosomes can activate protein kinase C (PKC) and extracellular signal-regulated kinases (ERKs), which control nociceptor excitability (11). To examine the role of these kinases in the sustained inhibitory actions of DOPr, we preincubated neurons with GF109203X (1  $\mu$ M, 30 min), which inhibits PKC (24), or with PD98059 (50  $\mu$ M, 30 min), which inhibits MEK1 (25). GF109203X and PD98059 abolished the sustained increase in rheobase ( $T = 30$  min) to DADLE (Fig. 2I and J).

To compare the chronic actions of DOPr agonists, neurons were incubated overnight with DADLE (100 nM) or ARM390 (300 nM). Neurons were washed, and rheobase was measured (Fig. 2K). DADLE caused both immediate ( $T = 0$  min) and sustained ( $T = 30$  min) increases in rheobase. PS2 blocked both phases (Fig. 2K). ARM390 caused an immediate increase ( $T = 0$  min), but not a sustained ( $T = 30$  min) increase, in rheobase, which was unaffected by PS2 (Fig. 2L). Thus, opioids from the inflamed colon and agonists that evoke DOPr endocytosis cause a sustained decrease in excitability of nociceptors that requires PKC and ERK signaling.

**Endosomal DOPr Inhibits Colonic Afferent Activity.** To assess whether endosomal DOPr signaling in the peripheral projections of colonic nociceptors mediates the inhibitory actions of opioids, we made extracellular recordings from lumbar splanchnic nerves innervating isolated segments of mouse distal colon (11). Nociceptors were identified by probing the colon or mesentery with von Frey filaments (VFF). Basal responses (1 g VFF, 100%) of each unit to repeated

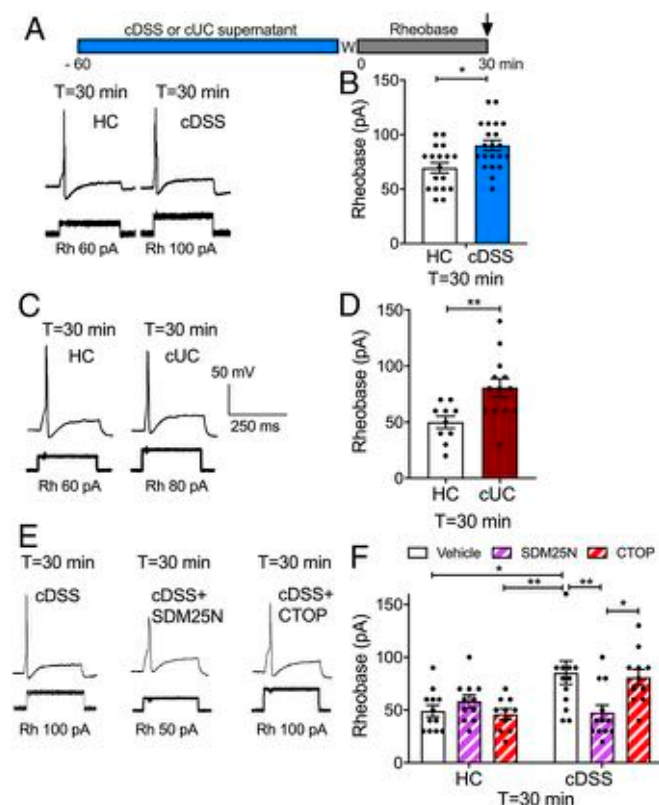


**Fig. 1.** Endogenous opioids and nociceptor excitability. Mouse DRG neurons were preincubated with supernatant from biopsies of HC, cDSS, or cUC colon and washed (W), and rheobase (Rh) was measured at 30 min after washing. Representative traces (A, C, and E) and pooled results (B, D, and F) of effects of supernatants from mouse (A, B, E, and F) and human (C and D) colonic biopsies. (E and F) Effects of antagonists of DOPr (SDM25N) or MOPr (CTOP) on responses to HC or cDSS supernatants. Data points indicate the number of studied neurons from  $n = 12$  to 16 mice in B, 6 mice in D, and 8 mice in F for each treatment (mean  $\pm$  SEM). \* $P < 0.05$ , \*\* $P < 0.001$ , two-way ANOVA with Tukey's post hoc test.

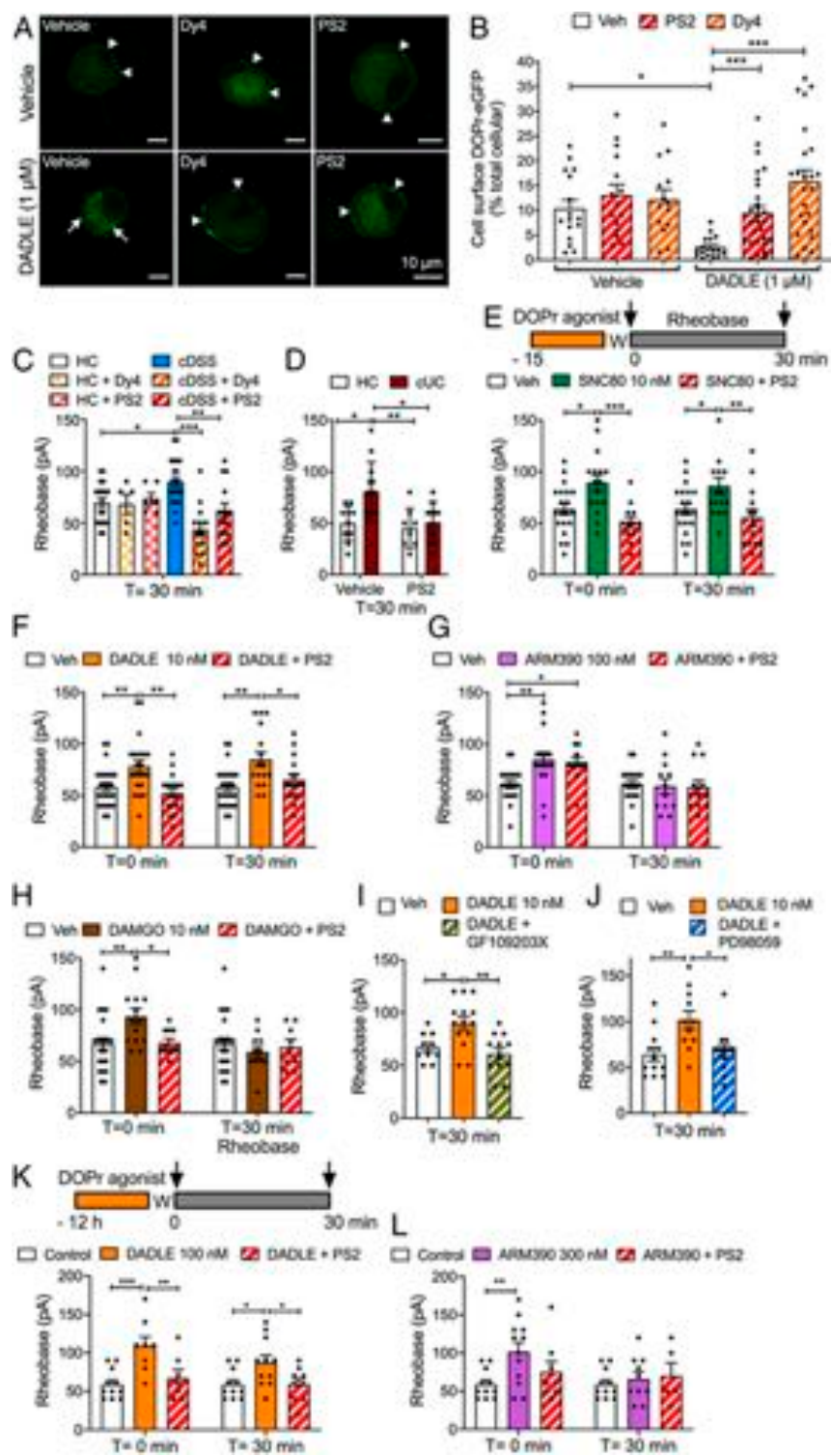


**Fig. 2.** Endosomal DOPr signaling and nociceptor excitability. (A and B) Endocytosis of DOPr-eGFP in DRG neurons from DOPr-eGFP mice. Neurons were incubated with vehicle (Veh) or DADLE (1  $\mu$ M, 30 min), and DOPr-eGFP was localized by immunofluorescence. Neurons were preincubated with vehicle, Dy4, or PS2. (A) Representative images from four independent experiments. Arrowheads denote plasma membrane; arrows, endosomal DOPr-eGFP. (B) Quantification of the proportion of total cellular DOPr-eGFP at the plasma membrane. Data points indicate the number of studied neurons (N). \* $P$  < 0.05, \*\*\* $P$  < 0.001, two-way ANOVA with Tukey's post hoc test. (C–L) Rheobase of mouse DRG neurons at 0 or 30 min after exposure to supernatant or DOPr agonists and washing. (C and D) Supernatant from cDSS, cUC, or HC biopsy specimens. (E–J) Neurons were incubated with the following agonists for 15 min and washed (W), and rheobase was measured at 0 or 30 min after washing: DOPr agonists SNC80 (E, 10 nM, internalizing), DADLE (F, 10 nM, internalizing) or ARM390 (G, 100 nM, weakly internalizing), and MOPr agonist DAMGO (H, 10 nM). In C–H, neurons were preincubated with Dy4, PS2, or vehicle. In I and J, neurons were preincubated with PKC inhibitor GF10923X or MEK1 inhibitor PD98059 before DADLE. (K and L) Neurons were incubated with the following agonists overnight and washed, and rheobase was measured at 0 or 30 min after washing: DADLE (K, 100 nM, internalizing) or ARM390 (L, 300 nM, weakly internalizing). Data points indicate the number of studied neurons from 12 to 16 mice in C, 6 mice in D, 10 to 15 mice in E–J, and 6 mice in K and L for each treatment (mean  $\pm$  SEM). \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, one-way or two-way ANOVA with Tukey's post hoc test.

stimulation (three times for 3 s) were recorded (Fig. 3A). Agonists of DOPr (SNC80 and ARM390) or MOPr (DAMGO) (all 100 nM) were superfused into the organ bath for 15 min. Tissues were washed, and responses to VFF probing were reassessed every 15 min for 1 h. Compared with basal responses, DAMGO and ARM390 transiently inhibited the activity of colonic nociceptors, whereas SNC80 had a persistent inhibitory effect (Fig. 3B and C). DAMGO maximally inhibited activity after 15 min of perfusion (i.e., 0 min,

53  $\pm$  10% inhibition). ARM390 (weakly internalizing) inhibited activity only at 0 min (29  $\pm$  4% inhibition). SNC80 (strongly internalizing) maximally inhibited activity at 30 min (33  $\pm$  9% inhibition), which persisted for 60 min. PS2 (50  $\mu$ M, 15 min) prevented the sustained inhibitory action of SNC80 (Fig. 3D). Thus, DOPr endosomal signaling within the peripheral projections of colonic nociceptors may induce a sustained inhibition of mechanical sensitivity.

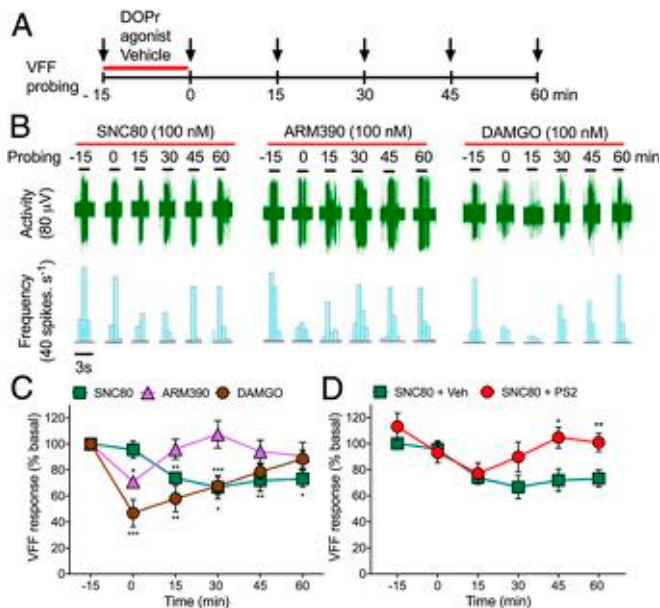


**Fig. 3.** MOPr and DOPr inhibition of colonic nociceptors. (A) Experimental protocol to examine MOPr and DOPr regulation of responses of colonic nociceptors to VFF probing. (B) Representative responses to agonists of DOPr (SNC80 and ARM390, 100 nM) and MOPr (DAMGO, 100 nM). (C and D) Time course of responses. In D, tissue was preincubated with PS2 or vehicle (Veh) before SNC80.  $n = 5$  mice for each treatment. Data are mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , two-way ANOVA with Tukey's post hoc test.

**DOPr Agonist Differentially Activate G Proteins, Recruit  $\beta$ ARRs, and Stimulate Endocytosis.** We characterized the differential effects of DOPr agonists on receptor signaling and trafficking using bioluminescence resonance energy transfer (BRET) (26). HEK293 cells were transiently transfected with  $G\alpha$ -Rluc8 subtypes plus  $G\gamma$ 2-Venus,  $G\beta$ 1, and DOPr.  $G\alpha$ -Rluc8/ $G\gamma$ 2-Venus BRET was measured to assess G protein dissociation (activation). SNC80, DADLE, and ARM390 (100 nM) decreased  $G\alpha_{i1}$ -Rluc8/ $G\gamma$ 2-Venus and  $G\alpha_o$ -Rluc8/ $G\gamma$ 2-Venus BRET, indicative of  $G\alpha_{i/o}$  and  $G\beta\gamma$  activation (SI Appendix, Fig. S1 A and B). SNC80, DADLE, and ARM390 had no effect on  $G\alpha_s$ -Rluc8/ $G\gamma$ 2-Venus BRET or  $G\alpha_q$ -Rluc8/ $G\gamma$ 2-Venus BRET (SI Appendix, Fig. S1 C and D). SNC80, DADLE, and ARM390 decreased  $G\alpha_{1,2,3,o}$ -Rluc8/ $G\gamma$ 2-Venus BRET with similar efficacy and an order of potency of DADLE > SNC80 > ARM390 (SI Appendix, Fig. S1 E–G).

To investigate  $\beta$ ARR recruitment, HEK293 cells were transfected with DOPr-Rluc and  $\beta$ ARR1/2-YFP. SNC80 and DADLE, but not ARM390 (all 100 nM), increased DOPr-Rluc/ $\beta$ ARR1/2-YFP BRET (SI Appendix, Fig. S1 H and I). ARM390 increased BRET only at high concentrations (>1  $\mu$ M). The order of potency for  $\beta$ ARR recruitment was DADLE > SNC80 > ARM390 (SI Appendix, Fig. S1 J and K).

Thus, SNC80, DADLE, and ARM390 induce DOPr coupling to  $G\alpha_{i/o}$ , and SNC80 and DADLE, but not ARM390, stimulate DOPr coupling to  $\beta$ ARR1/2. These results are consistent with the capacity of SNC80, but not of ARM390, to promote DOPr-eGFP phosphorylation, which is required for  $\beta$ ARR recruitment (27).

To assess DOPr trafficking, we measured bystander BRET between DOPr-Rluc and Venus-tagged proteins resident of the plasma membrane (HRas-Venus, lipid rich; KRas-Venus, non-lipid-rich) and endosomes (Rab5a, early; Rab7a, late; Rab11a, recycling) (26). SNC80 and DADLE (100 nM) decreased BRET between DOPr-Rluc, HRas-Venus, and KRas-Venus (SI Appendix, Fig. S1 L and M). These changes were mirrored by an increase in BRET between DOPr-Rluc and Rab5a-Venus (SI Appendix, Fig. S1 N). SNC80 stimulated BRET between DOPr-Rluc and Rab7a-Venus (SI Appendix, Fig. S1 O). ARM390 (100 nM) did not affect BRET between

DOPr and plasma membrane or endosomal proteins. SNC80, DADLE, or ARM390 did not affect BRET between DOPr-Rluc and Rab11a-Venus (SI Appendix, Fig. S1 P). Thus, SNC80 and DADLE cause DOPr internalization to early endosomes, whereas ARM390 does not. Internalized DOPr traffics to degradatory pathways in neurons (28).

**DOPr Agonists Differentially Activate G Proteins and  $\beta$ ARRs at the Plasma Membrane and in Endosomes.** To assess activation of G proteins at the plasma membrane and in endosomes of HEK293 cells, we measured enhanced bystander (eb) BRET between mini-G proteins (Rluc8-m $G\alpha_{si/o/sq}$ ) (29, 30) and *Renilla* (R) GFP-CAAX (prenylation CAAX box of KRas) (31) for plasma membrane activation or tandem (td) RGFP-Rab5a for early endosome activation. Whereas  $G\alpha$  proteins associate with  $G\beta\gamma$  subunits and GPCRs in the plasma membrane, mini- $G\alpha$  proteins are N-terminally truncated and freely diffuse throughout the cytoplasm. Mini- $G\alpha$  proteins can translocate to active GPCRs at the plasma membrane or in organelles. Mini- $G\alpha_{si}$  and  $G\alpha_{sq}$  proteins were developed by mutating m $G\alpha_s$  residues to equivalent  $G\alpha_q$  and  $G\alpha_i$  residues. Recruitment of  $\beta$ ARRs was assessed by measuring ebBRET between Rluc8- $\beta$ ARR1 (32) or Rluc2- $\beta$ ARR2 (31) and RGFP-CAAX or tdRGFP-Rab5a. Rab5a was localized to endosomes (SI Appendix, Fig. S2 A). SNC80, DADLE, and ARM390 (100 nM) increased Rluc8-m $G\alpha_{si/o}$ /RGFP-CAAX ebBRET (SI Appendix, Fig. S2 B–E) but did not affect Rluc8-m $G\alpha_{sq}$ /RGFP-CAAX ebBRET (SI Appendix, Fig. S2 F and G). SNC80 and DADLE, but not ARM390, increased Rluc8- $\beta$ ARR1 or Rluc2- $\beta$ ARR2/RGFP-CAAX ebBRET (SI Appendix, Fig. S2 H–K). SNC80 and DADLE, but not ARM390, increased Rluc8-m $G\alpha_{si/o}$ /tdRGFP-Rab5a ebBRET (SI Appendix, Fig. S2 L–O). These agonists did not affect Rluc8-m $G\alpha_{sq}$ /tdRGFP-Rab5a ebBRET (SI Appendix, Fig. S2 P and Q). SNC80 and DADLE, but not ARM390, increased Rluc8- $\beta$ ARR1 or Rluc2- $\beta$ ARR2/tdRGFP-Rab5a ebBRET (SI Appendix, Fig. S2 R–U). Pertussis toxin blunted  $\beta$ ARR recruitment to the plasma membrane (SI Appendix, Fig. S2 H–K) and endosomes (SI Appendix, Fig. S2 R–U), indicating involvement of  $G\alpha_{i/o}$  signaling.

The foregoing results suggest that SNC80, DADLE, and ARM390 activate  $G\alpha_{i/o}$  at the plasma membrane. Only agonists that strongly internalize DOPr (SNC80 and DADLE) activate  $G\alpha_{i/o}$  in endosomes and recruit  $\beta$ ARR1/2 to the plasma membrane and endosomes.

**Endosomal DOPr Activates a Subset of Compartmentalized Signals.** To examine DOPr signaling in subcellular compartments, we expressed DOPr and Förster resonance energy transfer (FRET) biosensors targeted to the plasma membrane, cytosol, or nucleus in HEK293 cells (10, 33). FRET biosensors included pmCKAR (plasma membrane PKC), cytoCKAR (cytosolic PKC), cytoEKAR (cytosolic ERK), and nucEKAR (nuclear ERK) (Fig. 4). To probe the link between endocytosis and compartmentalized signaling, we compared the effects of strongly internalizing (SNC80 and DADLE) and weakly internalizing (ARM390) DOPr agonists and used inhibitors of clathrin and dynamin.

SNC80 and DADLE (100 nM) stimulated a sustained increase in plasma membrane and cytosolic PKC activity (Fig. 4 A–C). ARM390 (100 nM) did not affect plasma membrane or cytosolic PKC activity. All three agonists stimulated a sustained increase in cytosolic ERK activity (Fig. 4 D and F). SNC80 and DADLE, but not ARM390, caused sustained activation of nuclear ERK (Fig. 4 E and F). These results suggest that DOPr signals from endosomes to activate plasma membrane and cytosolic PKC and nuclear ERK.

To assess the importance of endocytosis for compartmentalized signaling, we expressed wild-type (WT) dynamin or K44E dominant negative mutant dynamin (K44E dynamin) (34), or treated cells with PS2 or the inactive analog PS2 inactive. In control experiments with WT dynamin and PS2 inactive, SNC80 and DADLE induced rapid and sustained increases in PKC activity at the plasma membrane and in the cytosol (Fig. 5 A–F). Dynamin K44E and PS2 abolished SNC80 and DADLE stimulation of PKC at the plasma membrane and in the cytosol (Fig. 5 A–F and SI Appendix, Fig. S3 A and B). SNC80 and DADLE induced a gradual and sustained increase in

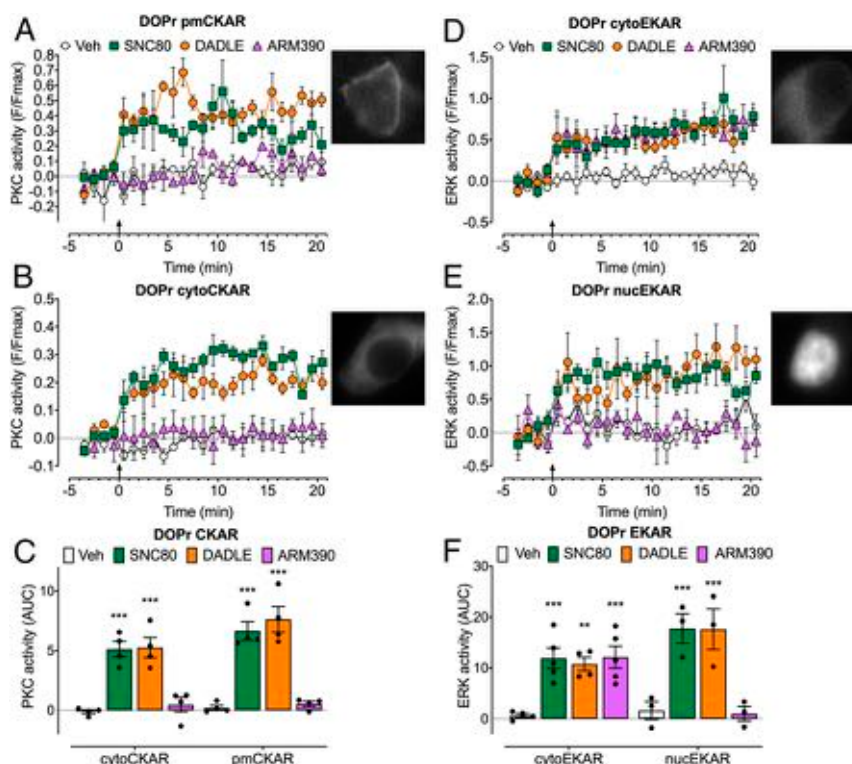


**Fig. 4.** DOPr-mediated PKC and ERK signaling in subcellular compartments of HEK293 cells. FRET biosensors for pmCKAR and cytoCKAR or cytoEKAR and nucEKAR were coexpressed with DOPr. *Insets* show cellular localization of FRET biosensors. Agonists (all 100 nM) or vehicle (Veh) were administered at the arrows. (A and B) Time course of plasma membrane (A) and cytosolic (B) PKC. (C) Integrated responses of plasma membrane and cytosolic PKC over 20 min (area under the curve [AUC]). (D and E) Time course of activation of cytosolic (D) and nuclear (E) ERK. (F) Integrated responses of cytosolic and nuclear ERK over 20 min (AUC). Data points show results of individual experiments.  $n = 4$  (A–C),  $n = 5$  cytoEKAR,  $n = 3$  nucEKAR (D–F) independent experiments. Data are mean  $\pm$  SEM.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  ligand to vehicle, one-way ANOVA with Tukey's post hoc test.

ERK activity in the cytosol and nucleus (Fig. 5 G–L). Dynamin K44E and PS2 did not affect SNC80- and DADLE-induced cytosolic ERK activity but abolished SNC80- and DADLE-induced nuclear ERK activity (Fig. 5 G–L and *SI Appendix*, Fig. S3 C and D). The contribution of  $\beta$ ARR1/2 to signaling was examined by siRNA knockdown (10, 26).  $\beta$ ARR1/2 siRNA, but not scrambled siRNA (control), inhibited SNC80-induced activation of nuclear, but not cytosolic, ERK (Fig. 5 M–O).

To evaluate DOPr compartmentalized signaling in nociceptors, we expressed FRET biosensors in DRG neurons from DOPr-eGFP mice. SNC80 and DADLE stimulated sustained activation of PKC at the plasma membrane and in the cytosol (Fig. 6 A–C) and of ERK in the cytosol and nucleus (Fig. 6 D–F). ARM390 stimulated a sustained activation of cytosolic ERK but did not affect plasma membrane PKC or nuclear ERK activity. Dy4 abolished SNC80-stimulated activation of nuclear ERK, whereas cytosolic ERK activity was unaffected (Fig. 6 G–J). These results suggest that DOPr endocytosis in HEK293 cells and primary nociceptors mediates activation of plasma membrane and cytosolic PKC and nuclear ERK, but not of cytosolic ERK.

**Nanoparticle-Encapsulated Agonists Target Endosomal DOPr.** The realization that endosomal DOPr signaling mediates the inhibitory actions of opioids on nociceptor excitability suggests that agonists that activate DOPr in endosomes might provide effective relief from inflammatory pain. Nanoparticles can be used to deliver an NK<sub>1</sub>R antagonist into endosomes of spinal neurons, where acidification triggers nanoparticle disassembly and antagonist release, leading to sustained antinociception (12). We incorporated DADLE into mesoporous silica nanoparticles (MSNs) designed to dissolve and release cargo in the acidic and reducing endosomal environment (35, 36) (Fig. 7A). For selective

targeting of DOPr-expressing neurons, we cloaked MSNs with PEGylated liposome covalently linked to DADLE.

Empty nanoparticles (LipoMSN), DADLE-coated nanoparticles (DADLE-LipoMSN), and nanoparticles with a DADLE coat and core (DADLE-LipoMSN-DADLE) were spherical with a hydrodynamic diameter of 140 to 210 nm, a surface charge of +28 to 36 mV, and a polydispersity index of 0.24 to 0.27 (Fig. 7 B and C). The loading efficiency of DADLE into the MSN core was  $57 \pm 6\%$ . To examine MSN disassembly and cargo release, MSNs loaded with DADLE-Alexa647 (MSN-DADLE-Alexa647) were incubated in buffers at pH 7.2 or 5.2 or with or without 10 mM glutathione to mimic the acidic and reducing conditions of endosomes. The release of DADLE-Alexa647 into buffer was faster and more complete at pH 5.2 and in the presence of glutathione, and it continued for 24 h (Fig. 7 D and E).

To determine whether a DADLE-Lipo shell could facilitate selective uptake by DOPr-expressing cells, MSNs loaded with Alexa Fluor 647 and coated with DADLE-Lipo were incubated with untransfected HEK293 cells or HEK-DOPr cells for 2 h. The number of cells containing Alexa647 was determined by flow cytometry. DADLE-LipoMSN-Alexa647 was internalized into  $66 \pm 7\%$  of HEK-DOPr cells, compared with  $22 \pm 1\%$  of untransfected HEK293 cells ( $P < 0.05$ ,  $t$  test), indicating preferential delivery to cells expressing DOPr (Fig. 7F). Dy4 and PS2, but not inactive analogs, inhibited DADLE-LipoMSN-Alexa647 uptake by HEK-DOPr cells, consistent with clathrin- and dynamin-mediated endocytosis (Fig. 7G).

To determine whether nanoparticles target DOPr in endosomes, HEK-HA-DOPr cells expressing Rab5a-GFP were incubated with HA antibodies to label surface DOPr. Cells were incubated with DADLE-LipoMSN-Alexa647 (20  $\mu$ M DADLE, 200  $\mu$ g/mL LipoMSN) and imaged by confocal microscopy. DADLE-LipoMSN-Alexa647 accumulated at the plasma membrane, stimulated endocytosis of HA-DOPr, and colocalized with HA-DOPr in early endosomes at 30 min (Fig. 7H and

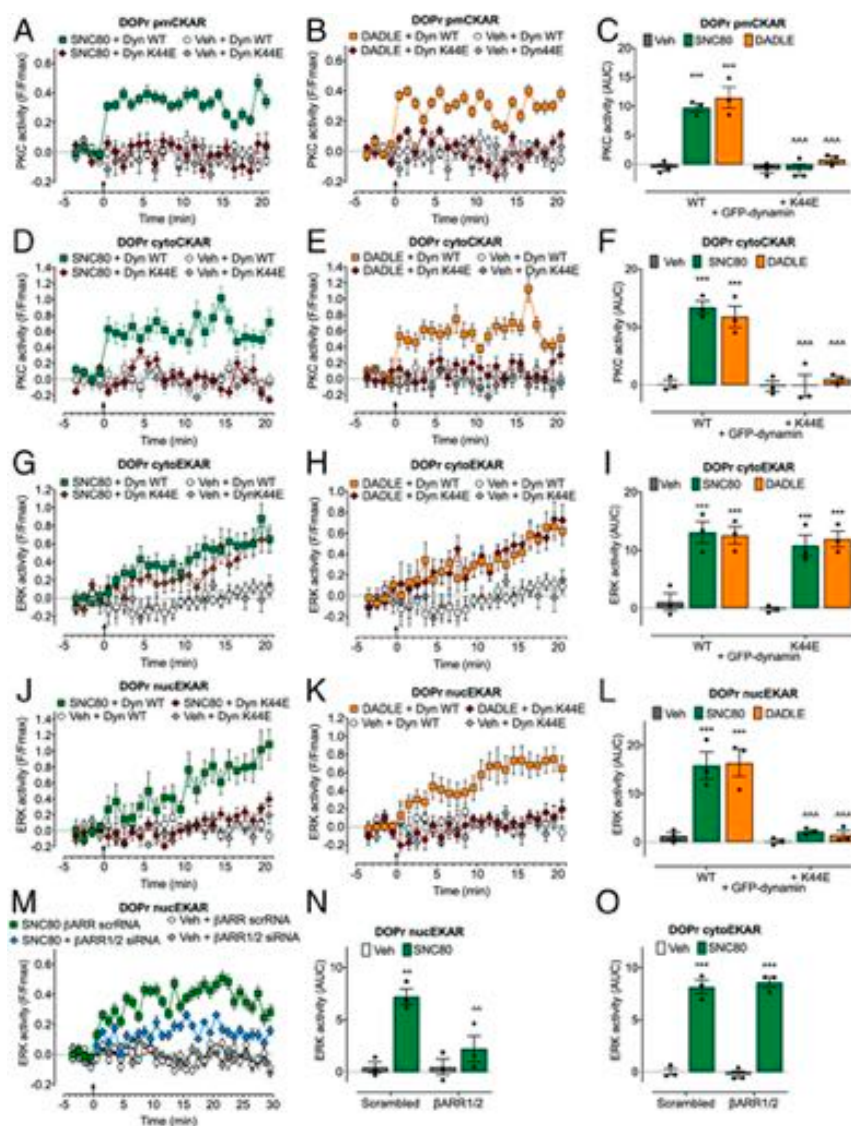


**Fig. 5.** Endosomal DOPr-mediated PKC and ERK signaling in subcellular compartments of HEK293 cells. FRET biosensors for pmCKAR and cytoCKAR or cytoEKAR and nucEKAR were coexpressed with DOPr and either dynamin WT (Dyn WT) or dominant negative dynamin K44E (Dyn K44E) (A–L) or with  $\beta$ ARR1+2 siRNA or scrambled (scr) siRNA (control) (M–O). Agonists (all 100 nM) or vehicle (Veh) were administered at the arrows. (A–C) Plasma membrane PKC activity. (D–F) Cytosolic PKC activity. (G–I and O) Cytosolic ERK activity. (J–N) Nuclear ERK activity. (A, B, D, E, G, H, J, K, and M) Time course of responses. (C, F, I, L, N, and O) Integrated responses over 20 or 30 min (AUC). Data points show results of individual experiments.  $n = 3$  independent experiments. Data are mean  $\pm$  SEM. \*\*\* $P < 0.01$ , \*\*\*\* $P < 0.001$  ligand to vehicle; ^ $P < 0.01$ , ^^ $P < 0.001$  inhibitors to control; two-way ANOVA with Tukey's post hoc test.

*SI Appendix, Fig. S44*). Live cell imaging, which avoided the loss of nanoparticle fluorescence during immunostaining, revealed DADLE-LipoMSN-Alexa647 binding to the plasma membrane and uptake into Rab5a-GFP endosomes within 30 min (*Movie S1*). Control LipoMSN-Alexa647, lacking the DADLE targeting group, showed diminished uptake (*Movie S2*).

We examined whether DADLE nanoparticles activate DOPr signaling at the plasma membrane (inhibition of cAMP,  $\beta$ ARR1 recruitment) and in endosomes (nuclear ERK). Compared with Lipo-MSN or vehicle, DADLE (100 nM), DADLE-LipoMSN (20  $\mu$ M DADLE), and DADLE-LipoMSN-DADLE (20  $\mu$ M DADLE) inhibited forskolin (10  $\mu$ M)-stimulated formation of cAMP in HEK-DOPr cells but not in untransfected HEK293 cells (Fig. 7I and *SI Appendix, Fig. S4 B and C*). DADLE and DADLE-LipoMSN-DADLE increased DOPr-Rluc8/ $\beta$ ARR1-YFP BRET (Fig. 7J and *SI Appendix, Fig. S4D*). DADLE, DADLE-LipoMSN, and DADLE-LipoMSN-DADLE activated nuclear ERK, which was particularly sustained for DADLE-LipoMSN and DADLE-LipoMSN-DADLE (Fig. 7K and *SI Appendix, Fig. S4E*). These results suggest that DADLE coupled to the liposome shell

can activate DOPr at the plasma membrane and stimulate DOPr endocytosis. DADLE released from the MSN core in endosomes might activate DOPr to stimulate nuclear ERK activity.

Primary cultures of DRG neurons from DOPr-eGFP knockin mice were studied to assess nanoparticle targeting and uptake into neurons. Neurons were incubated with DADLE, LipoMSN-Alexa647 (control), or DADLE-LipoMSN-Alexa647 (1  $\mu$ M, 60 min, 37  $^{\circ}$ C) and fixed. GFP and the neuronal marker Hu were localized by immunofluorescence. DADLE evoked endocytosis of DOPr-eGFP in neurons (*SI Appendix, Fig. S5A*). LipoMSN-Alexa647 was detected at the surface of some neurons but did not promote DOPr-eGFP endocytosis (Fig. 8A). DADLE-LipoMSN-Alexa647 evoked DOPr-eGFP internalization and colocalized in endosomes with DOPr-eGFP.

**A Nanoparticle-Encapsulated DOPr Agonist Provides Long-Lasting Antinociception.** To assess antinociception, DRG neurons were incubated with DADLE, DADLE-LipoMSN, or DADLE-LipoMSN-DADLE (100 nM DADLE) for 30 min and washed, and then rheobase was

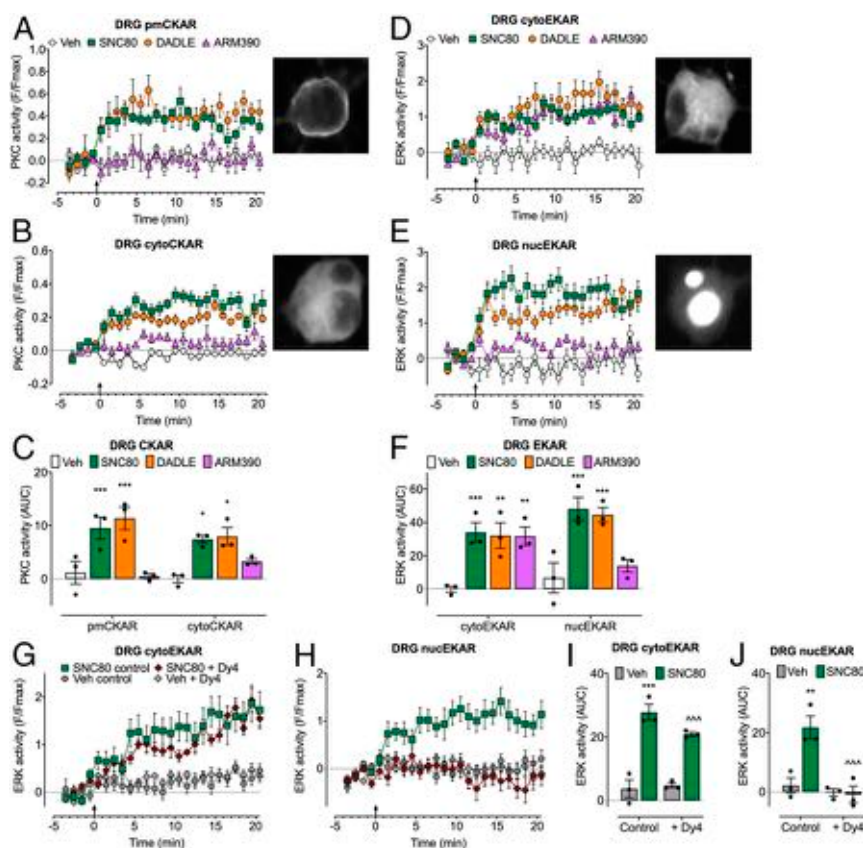


**Fig. 6.** Endosomal DOPr-mediated PKC and ERK signaling in subcellular compartments of DRG neurons. FRET biosensors for pmCKAR and cytoCKAR or cytoEAKAR and nucEAKAR were expressed in DRG neurons from DOPr-eGFP mice. Insets show localization of FRET biosensors. Agonists (all 100 nM) or vehicle (Veh) were administered at arrow. (A and B) Time course of plasma membrane (A) and cytosolic (B) PKC. (C) Effects of agonist treatments on PKC over 20 min (AUC). (D and E) Time course of cytosolic ERK (D) and nuclear ERK (E). (F) Effects of agonists on ERK activity over 20 min (AUC). (G and H) Time course of effects of dynamin inhibitor (Dy4) on cytosolic (G) and nuclear (H) ERK activity. (I and J) Effects of Dy4 treatments on ERK over 20 min (AUC). Data points show results of individual experiments.  $n = 3$  independent experiments. Data are mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  ligand to vehicle; ^^^ $P < 0.001$  inhibitor to control; one-way ANOVA with Tukey's post hoc test.

measured at 0, 90, 120, or 180 min after washing. DADLE and DADLE-LipoMSN increased rheobase only at 0 min (Fig. 8B and C). DADLE-LipoMSN-DADLE increased rheobase at 0, 90, and 120 min ( $26 \pm 6\%$  at 120 min). PS2 prevented the sustained inhibitory actions of DADLE-LipoMSN-DADLE (Fig. 8B and C).

To assess the activity of peripheral colonic nociceptors, extracellular recordings were made from colonic afferents. DADLE-LipoMSN-DADLE (100 nM DADLE) was superfused into the organ bath for 30 min. Responses to VFF probing were assessed at 60 min and 120 min after washing. DADLE-LipoMSN-DADLE inhibited the activity of colonic nociceptors for at least 120 min ( $54 \pm 13\%$  inhibition) (Fig. 8D). PS2 prevented the sustained inhibitory action of DADLE-LipoMSN-DADLE.

To assess inflammatory nociception, complete Freund's adjuvant (CFA) was administered to mice by intraplantar injection, and withdrawal responses to stimulation of the plantar surface of the ipsilateral paw with VFFs were measured after 48 h. When administered by intrathecal injection to target DOPr in the central projections of nociceptors and on spinal neurons, DADLE (100 nM, 5  $\mu$ L) had a moderate and transient antinociceptive action, whereas DADLE-LipoMSN-DADLE (100 nM DADLE, 5  $\mu$ L) had a strong antinociceptive action that was sustained for 6 h (Fig. 8E). LipoMSN (1  $\mu$ g/mL, 5  $\mu$ L) had no effect. Nanoparticles did not affect withdrawal responses of the contralateral paw (SI Appendix, Fig. S5B). Thus, neuronal-targeted stimulus-responsive nanoparticles provide long-lasting antinociception.

**A Nanoparticle-Encapsulated DOPr Antagonist Prevents the Sustained Antinociceptive Actions of DOPr.** To provide evidence that DOPr endosomal signaling underlies sustained inhibition of neuronal

excitability, we encapsulated the DOPr antagonist SDM25N into nanoparticles with a liposome shell (LipoMSN-SDM25N). LipoMSN-SDM25N had a hydrodynamic diameter of  $176.5 \pm 0.6$  nm, a surface charge of  $+32 \pm 3$  mV, and a polydispersity index of  $0.15 \pm 0.02$ . SDM25N loading efficiency was  $73.5 \pm 0.8\%$ . To assess the uptake of nanoparticles lacking the DADLE targeting group, LipoMSN-Alexa647 nanoparticles were incubated with HEK293 cells (0 to 4 h, 37 °C). After 120 min, LipoMSN-Alexa647 was detected in Rab5a-positive early endosomes (Fig. 8F). LipoMSN-Alexa647 was internalized in  $67.7 \pm 1.3\%$  of HEK293 cells after 120 min, as assessed by flow cytometry (Fig. 8G). To determine whether an endosomally targeted DOPr antagonist can block nociception, DRG neurons were incubated with LipoMSN-SDM25N (100 nM SDM25N, 100  $\mu$ g/mL LipoMSN) or LipoMSN (100  $\mu$ g/mL, control) (120 min, 37 °C) (Fig. 8H), then washed, incubated with DADLE (10 nM, 15 min) and washed again. Rheobase was measured at 0 and 30 min after washing. In LipoMSN-treated neurons, DADLE increased rheobase at 0 min ( $53.44 \pm 17.1\%$ ) and 30 min ( $55.56 \pm 10.07\%$ ) compared with control. LipoMSN-SDM25N had no effect on the rheobase at 0 min ( $52.18 \pm 13.78\%$ ) but abolished the inhibitory effect of DADLE at 30 min. These results support the hypothesis that DOPr signals from endosomes to cause persistent antinociception.

## Discussion

Our results support the hypothesis that DOPr in endosomes is as a key component of an endogenous mechanism of pain control, and

that endosomal DOPr is a viable therapeutic target for chronic inflammatory pain.

**Antinociceptive Signaling of Endosomal DOPr.** Several observations suggest that DOPr signaling in endosomes mediates the sustained antinociceptive actions of endogenous opioids and certain DOPr-selective agonists (*SI Appendix, Fig. S6*). Biopsy specimens of inflamed human and mouse colon released opioids that caused a sustained inhibition of the excitability of nociceptors, as revealed by increased rheobase. These effects are attributable to DOPr, because a selective antagonist prevented inhibition. Colitis evokes endocytosis of DOPr-eGFP in myenteric neurons, consistent with opioid release and DOPr activation (37). Our findings support reports of an opioid-mediated mechanism of antinociception in inflamed colon (15–19). DOPr agonists that stimulated robust receptor endocytosis (DADLE and SNC80) caused a persistent inhibition of nociceptor excitability, whereas a weakly internalizing DOPr agonist (ARM390) had only a transient inhibitory action. Inhibitors of clathrin and dynamin prevented agonist-evoked endocytosis of DOPr-eGFP in nociceptors and blocked the sustained inhibitory actions of endogenous opioids and internalizing DOPr agonists.

These results support a role for endosomal signaling of DOPr in regulating sustained excitability of the soma, which was examined by patch clamp recordings. Similar mechanisms may control the excitability of nerve endings in the colon, since SNC80 caused a long-lasting inhibition of mechanically sensitive nociceptors, whereas weakly internalizing ARM390 did not. A clathrin inhibitor blocked the effects of SNC80, which require endosomal signaling. DOPr endocytosis has also been linked to analgesic tolerance (23, 27, 38, 39). A DOPr antagonist (SDM25N) incorporated into nanoparticles designed to deliver and release cargo in endosomes prevented the sustained inhibitory actions of DADLE on nociceptor excitability. These findings suggest that DADLE continues to activate DOPr in endosomes to inhibit nociception.

Our results do not exclude a role for plasma membrane signaling of DOPr in antinociception. Inhibitors of endocytosis and nanoparticle-encapsulated SDM25N did not affect the short-term inhibitory effects of DOPr agonists on excitability. Thus, DOPr signaling at the plasma membrane and in endosomes mediates antinociception, but with different time courses.

Our results reveal spatial and temporal differences in the way in which DOPr and MOPr regulate the excitability of nociceptors. A MOPr antagonist did not prevent the inhibitory actions of colonic supernatants on neuronal excitability, suggesting that MOPr does not contribute antinociception during colitis. Although the MOPr agonist DAMGO transiently decreased the excitability of DRG neurons and colonic afferents, these effects were not sustained. A clathrin inhibitor prevented the transient inhibitory actions of DAMGO, which likely require endosomal signaling of MOPr. These results are in agreement with studies in which a conformationally selective nanobody was used to detect activated MOPr in subcellular compartments (40).

Biophysical approaches were used to examine DOPr trafficking and signaling in HEK-DOPr cells and nociceptors, with consistent results. All DOPr agonists (DADLE, SNC80, and ARM390) activated  $G\alpha_{i/o}$  with similar efficacy. Only strongly internalizing agonists (DADLE and SNC80) potentially recruited  $\beta$ ARR1/2 and stimulated DOPr depletion from the plasma membrane and accumulation and retention in early endosomes. The results confirm reported differences in the ability of DADLE, SNC80, and ARM390 to promote DOPr internalization (23). These differences are attributable to GRK-induced DOPr phosphorylation; SNC80 induces DOPr phosphorylation at Ser<sup>363</sup>, whereas ARM390 does not (27).

The use of FRET biosensors targeted to the plasma membrane, cytosol, or nucleus revealed that DOPr endocytosis is necessary for a subset of signals in subcellular compartments. Our results suggest that DOPr signaling from the plasma membrane activates ERK in the cytosol, whereas DOPr signaling in endosomes activates PKC at the plasma membrane and in the cytosol and activates ERK in the nucleus but not in the cytosol. Support for these conclusions derives from the observation that internalizing agonists alone activated

plasma membrane and cytosolic PKC and nuclear ERK. Inhibitors of clathrin- and dynamin-mediated endocytosis, dominant negative dynamin, and  $\beta$ ARR1/2 knockdown selectively suppressed these signals. Other GPCRs also signal from endosomes to regulate subsets of compartmentalized signals (10, 11, 13). Inhibitors of PKC and MEK1 prevented the sustained inhibitory actions of DADLE on neuronal excitability, providing a link between endosomal DOPr signaling and antinociception. PKC is a critical regulator of DOPr-mediated signaling and antinociception (41). DOPr endocytosis is also required for ERK activation and trafficking to perinuclear and nuclear locations (42).

$G\alpha_{i/o}$  and  $\beta$ ARRs may mediate endosomal DOPr signaling, since internalizing, but not weakly internalizing, DOPr agonists stimulated the recruitment of mini- $G\alpha_{i/o}$  and  $\beta$ ARR1/2 to early endosomes, as determined by BRET.  $\beta$ ARR1/2 knockdown inhibited SNC80-stimulated nuclear ERK activation, possibly due to inhibition of DOPr endocytosis and endosomal signaling. Further studies are needed to determine the contribution of  $\beta$ ARRs and  $G\alpha_{i/o}$  to endosomal DOPr signaling.

**Therapeutic Targeting of Endosomal DOPr.** The realization that GPCRs can signal from endosomes to mediate pain has revealed endosomal GPCRs as a viable therapeutic target (8). Conjugation to transmembrane lipids or encapsulation into pH-tunable nanoparticles delivers antagonists of pronociceptive GPCRs to endosomes (10–13). Endosomally targeted antagonists preferentially inhibit endosomal signaling and provide enhanced antinociception compared with conventional antagonists. The present study shows that endosomally targeted agonists of antinociceptive GPCRs also provide long-lasting pain relief. DADLE-LipoMSN-DADLE inhibited nociceptor excitability for at least 3 h after washout, in contrast to the transient inhibitory action of free DADLE. DADLE-LipoMSN-DADLE caused a long-lasting inhibition of mechanically evoked activation of colonic nociceptors and effectively reversed inflammatory nociception. One component of the enhanced antinociceptive properties of nanoparticles might relate to the selective delivery of primary sensory neurons to endosomes. Targeted delivery to DOPr-expressing neurons was accomplished by cloaking MSNs with PEGylated liposomes covalently linked to DADLE. DADLE-LipoMSNs retained the ability to activate DOPr in HEK-DOPr cells, as assessed by inhibition of cAMP, recruitment of  $\beta$ ARR1, stimulation of DOPr endocytosis, and activation of nuclear ERK (*SI Appendix, Fig. S6*). Uptake of DADLE-LipoMSNs by HEK-DOPr cells was threefold greater than that by untransfected HEK cells, suggesting preferential targeting. DADLE-LipoMSNs entered cells by clathrin-mediated endocytosis and were delivered to DOPr-positive early endosomes. Another component of enhanced antinociception could be the sustained activation of DOPr in endosomes, which was attained by incorporating DADLE into the MSN core. DADLE release was accelerated in the acidic and reducing endosomal environment and continued for 24 h. The finding that a clathrin inhibitor abrogated the antinociceptive actions of DADLE-LipoMSN-DADLE indicates a requirement for nanoparticle endocytosis.

More direct evidence for a role of endosomal DOPr in antinociception was provided by incorporating the DOPr antagonist SDM25N into nanoparticles. When preincubated with neurons to allow for endosomal accumulation, followed by extensive washing to remove extracellular antagonist, LipoMSN-SDM25N prevented the sustained inhibitory actions of DADLE on nociceptor excitability, supporting endosomal signaling. The immediate inhibitory actions of DADLE were unaffected and likely arose from plasma membrane DOPr.

Incorporation into nanoparticles can enhance the stability and delivery of drugs, thereby improving efficacy (43–45). Stimulus-responsive nanoparticles deliver combinations of chemotherapeutics to tumors, where increased vascular permeability and extracellular acidification promote delivery and cargo release (46, 47). Although nanoparticles are often endocytosed, endosomal disruption is necessary for drug delivery to cytosolic and nuclear targets, which can compromise efficacy (48). The discovery of GPCRs in endosomes as therapeutic targets provides an opportunity to use nanoparticles to deliver treatments for pain (8). Our results

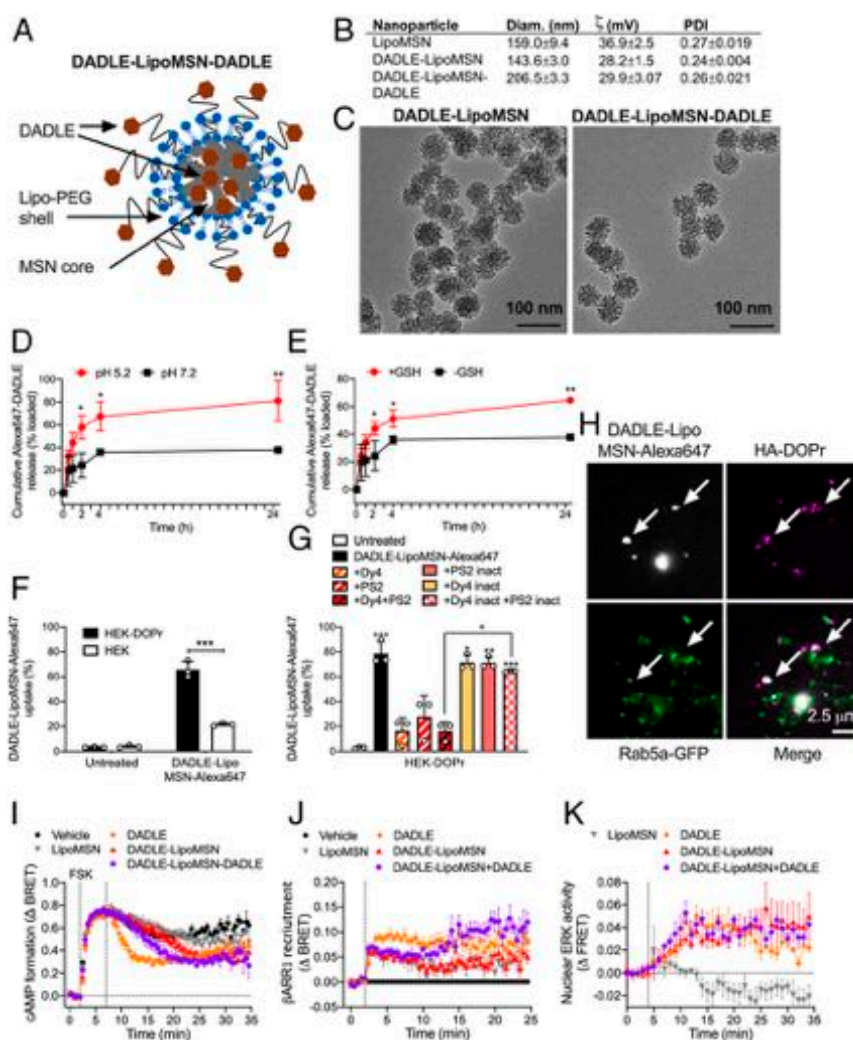


**Fig. 7.** Characterization of nanoparticles. (A) Structure of DADLE-LipoMSN-DADLE. (B) Physical properties of nanoparticles.  $n = 4$  experiments. (C) Transmission electron micrographs of DADLE-LipoMSN and DADLE-LipoMSN-DADLE. Representative images,  $n = 3$  independent experiments. (D and E) Time course of in vitro release of DADLE-Alexa647 from MSN-DADLE-Alexa647 at graded pH (D) and glutathione concentrations (E).  $n = 3$  independent experiments.  $*P < 0.05$ ,  $**P < 0.01$ ,  $t$  test with Holm-Sidak correction. (F and G) Uptake of DADLE-LipoMSN-DADLE-Alexa647 into HEK293 control and HEK-DOPr cells determined by flow cytometry. (F) Uptake into HEK293 control and HEK-DOPr cells after 2 h.  $***P < 0.001$ ,  $t$  test with Holm-Sidak correction. (G) Effects of inhibitors of clathrin and dynamin and inactive analogs on uptake into HEK-DOPr cells after 2 h.  $n = 3$  independent experiments.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  compared with untreated cells, one-way ANOVA with Tukey's post hoc test. (H) Uptake of DADLE-LipoMSN-DADLE-Alexa647 into HEK-HA-DOPr cells after 30 min. Arrows show colocalization of DADLE-LipoMSN-DADLE-Alexa647 with DOPr in Rab5a-positive early endosomes. Representative images from four independent experiments. (I–K) Effects of DADLE (100 nM), DADLE-LipoMSN (20  $\mu$ M), and DADLE-LipoMSN-DADLE (20  $\mu$ M) on forskolin (FSK; 10  $\mu$ M)-stimulated cAMP formation (I),  $\beta$ ARR1 recruitment (J), and activation of nuclear ERK (K).  $n = 5$  independent experiments. All results are mean  $\pm$  SEM.

demonstrate the feasibility of using nanoparticles to target nociceptors with consequent reductions in dose. Nanoparticles might allow the simultaneous delivery to endosomes of agonists or antagonists of several endosomal GPCRs involved in pain. Since multiple GPCRs control pain transmission (9), the ability to target multiple receptors in pain-transmitting neurons for prolonged periods might provide effective and long-lasting antinociception.

Nanoparticle-encapsulated GPCR ligands may have utility beyond the treatment of pain. GPCRs control many pathophysiological processes and are the targets of more than one-third of Food and Drug Administration-approved drugs (1). Many GPCRs internalize when activated and likely continue to signal from endosomes. The use of stimulus-responsive nanoparticles for delivery of drugs to endosomes of targeted cells might enhance efficacy with reduced doses and fewer side effects.

**Limitations.** This study has several limitations. We cannot exclude a possible role for plasma membrane signaling even in the sustained

inhibitory actions of opioids. The relative contributions of plasma membrane and endosomal signaling likely depend on the nature and concentration of the ligand and the time at which nociception is assessed. The differential effects of DOPr agonists that strongly (SNC80 and DADLE) or weakly (ARM390) promote endocytosis support a role for endosomal DOPr signaling for sustained antinociception. ARM390 is a partial agonist for  $\beta$ ARR recruitment, which may explain its inability to cause long-lasting antinociception. We were unable to determine whether DOPr endosomal signaling involves G proteins and  $\beta$ ARRs, which mediate endosomal signaling of other GPCRs (4, 5, 10, 11, 49, 50). Although dynamin and clathrin inhibitors blocked a subset of DOPr signals and inhibited sustained antinociception, these inhibitors also have nonspecific actions (51). Dominant negative dynamin and  $\beta$ ARR knockdown replicated some effects of endocytosis inhibitors but could affect other functions as well. We cannot exclude the possibility that DOPr signals from intracellular compartments other than endosomes, since MOPr can signal from different compartments depending on the membrane

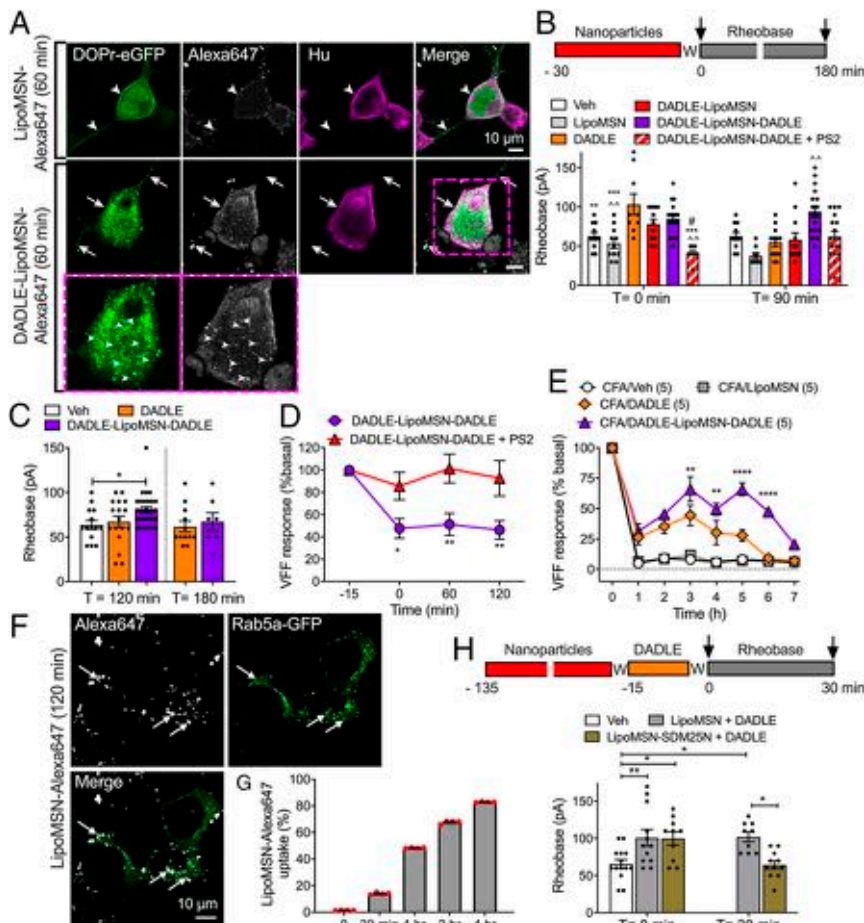


**Fig. 8.** Effects of nanoparticle-encapsulated DOPr ligands on nociceptors. (A) Uptake of LipoMSN-Alexa647 (control) or DADLE-LipoMSN-Alexa647 into primary cultures of DRG neurons from DOPr-eGFP mice. Neurons were incubated with nanoparticles for 60 min. Representative images from two experiments, from four mice. (B and C) Rheobase of mouse DRG neurons at 0, 90, 120, or 180 min after exposure to DADLE, DADLE-LipoMSN-DADLE, DADLE-LipoMSN (all 100 nM), LipoMSN (control), or vehicle (control) and washing. Some neurons were exposed to PS2 and DADLE-LipoMSN-DADLE. Data points indicate the number of studied neurons from  $n = 6$  to 12 mice in B and C for each treatment. Compared with \*DADLE,  $\Delta$ DADLE-LipoMSN-DADLE, and #DADLE-LipoMSN;  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , one-way (B) or two-way (C) ANOVA with Tukey's post hoc test. (D) Colonic afferent activity at 0, 60, or 120 min after exposure of tissues to DADLE-LipoMSN-DADLE (100 nM). Some preparations were exposed to PS2 and DADLE-LipoMSN-DADLE.  $n = 5$  mice per group.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ , two-way (\*) ANOVA with Sidak's post hoc test. (E) Ipsilateral paw withdrawal responses in mice. DADLE, DADLE-LipoMSN-DADLE (both 100 nM DADLE), LipoMSN, or vehicle (Veh) was injected intrathecally at 48 h after intraplantar CFA.  $n = 5$  mice per group.  $^{**}P < 0.01$ ,  $^{****}P < 0.0001$  DADLE compared with DADLE-LipoMSN-DADLE, two-way ANOVA with Tukey's multiple comparison post hoc test. (F) Uptake of LipoMSN-Alexa647 into endosomes of HEK293 cells expressing Rab5a-GFP after 120 min. (G) Time course of uptake of LipoMSN-Alexa647 into HEK293 cells.  $n = 3$  independent experiments. (H) Rheobase of mouse DRG neurons. Neurons were incubated with LipoMSN-SDM25N (100 nM) or LipoMSN (control) for 120 min, washed (W), incubated with DADLE (10 nM, 15 min), and washed again. Rheobase was measured at 0 or 30 min after washing. Data points indicate the number of studied neurons from four mice for each treatment.  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ , two-way ANOVA with Tukey's post hoc test. All results are mean  $\pm$  SEM.

permeability of the agonist (40). Although our results show that PKC and ERK mediate the inhibitory actions of endosomal DOPr on nociceptor excitability, the targets of these kinases and how they inhibit nociception remain to be defined. Toxicologic analysis of nanoparticle constituents, pharmacokinetic studies of nanoparticle cargo, and pharmacodynamic studies in preclinical models of pain will be necessary before this approach can be advanced to patients.

## Materials and Methods

**Animal Subjects.** Institutional Ethics Committees approved the mouse studies.

**Human Subjects.** The Queen's University Human Ethics Committee approved the human studies. Patients undergoing colonoscopy for routine clinical care gave informed consent for biopsy specimens of the mucosa to be obtained from the descending colon during colonoscopy and for their data to be recorded for research purposes. Biopsy specimens of mucosa were collected from the descending colon of three patients with active cUC and three healthy control patients. Disease severity was evaluated using the endoscopy

component of the Mayo Clinic score for ulcerative colitis (SI Appendix, Table S1).

**Colon Supernatants.** Mice were treated for three cycles with 2% DSS in drinking water to induce chronic colitis or with water (control). Segments of whole colon were incubated in medium (24 h) to obtain supernatants (16–18). Biopsy specimens of colonic mucosa from cUC patients and controls (SI Appendix, Table S1) were incubated in medium to obtain supernatants (11, 52).

**Patch Clamp Recording.** Patch clamp recordings were made from mouse DRG neurons (11, 16, 18, 52). Neurons were preincubated for 60 min with supernatants and then washed. Neurons were stimulated for 15 min with DADLE (10 nM), SNC80 (10 nM), ARM390 (100 nM), DAMGO (10 nM), or vehicle (control) and then washed. Neurons were also incubated overnight (12 to 16 h) with DADLE (100 nM) or ARM390 (300 nM) and then washed. In some experiments, neurons were preincubated for 30 min with SDM25N (100 nM), CTOP (100 nM), Dy4 (30  $\mu$ M), PS2 (15  $\mu$ M), GF109203X (1  $\mu$ M), PD98059 (50  $\mu$ M), or vehicle. Rheobase was measured after agonist treatment and washing.

**Extracellular Recording.** Extracellular recordings were made from the lumbar splanchnic nerve innervating isolated mouse distal colon (11, 53, 54). SNC80, ARM390, or DAMGO (all 100 nM) was superfused into the organ bath for 15 min. In some studies, colon was preincubated for 15 min with PS2 (50  $\mu$ M) before SNC80.

**cdNAs, Cell Culture, and Transfection.** Details are provided in *SI Appendix, Materials and Methods*.

**Dissociation of DRG Neurons.** DRG neurons were dispersed from DOPr-eGFP mice (55).

**BRET Assays.** BRET was measured in HEK293 cells (10, 26).

**FRET Assays.** FRET was measured in HEK293 cells and DRG neurons from DOPr-eGFP mice (10, 55). After FRET imaging, DOPr-eGFP was localized by immunofluorescence. FRET was measured in neurons expressing DOPr-eGFP.

**DOPr-eGFP Trafficking.** DRG neurons from DOPr-eGFP mice were exposed to vehicle, DADLE (1  $\mu$ M), DADLE-LipoMSN-Alexa647 (1  $\mu$ M DADLE), or LipoMSN-Alexa647 (10  $\mu$ g/mL LipoMSN) (30 or 60 min, 37 °C). In some experiments, neurons were preincubated with Dy4 (30  $\mu$ M) or PS2 (15  $\mu$ M) (30 min). DOPr-eGFP in neurons was localized by immunofluorescence.

**Preparation and Physicochemical Characterization of Nanoparticles.** Details are provided in *SI Appendix, Materials and Methods*.

**Cellular Targeting of LipoMSNs.** HEK293-HA-DOPr or untransfected cells were incubated with DADLE-LipoMSN-Alexa647 or LipoMSN-Alexa647 (40  $\mu$ g/mL). Uptake of nanoparticles was quantified by flow cytometry. In some experiments, cells were preincubated with Dy4, PS2, or inactive analogs (10  $\mu$ g/mL, 30 min). For imaging studies, cells were transfected with Rab5a-GFP. After 24 h, cells were preincubated with rat anti-HA. Cells were washed and incubated with DADLE-LipoMSN-Alexa647 or LipoMSN-Alexa647 (20  $\mu$ M DADLE, 200  $\mu$ g/mL LipoMSN). HA-DOPr was localized by

immunofluorescence. In some experiments, LipoMSN-Alexa647 uptake was examined by live cell imaging.

**LipoMSNs and DOPr Signaling.** Details are provided in *SI Appendix, Materials and Methods*.

**LipoMSNs and Electrophysiology.** Rheobase was measured at 0 to 180 min after exposure to DADLE or SDM25N nanoparticles and washing. Colonic afferent responses were assessed at 0 to 120 min after exposure to nanoparticles and washing.

**LipoMSNs and Inflammatory Pain.** The investigator was blinded to the treatments. Mice were assigned at random to treatments and acclimatized. CFA (0.5 mg/mL) was administered by intraplantar injection (10  $\mu$ L) into the left hindpaw. DADLE (100 nM), DADLE-LipoMSN-DADLE (100 nM DADLE, 0.8  $\mu$ g/mL LipoMSN), LipoMSN (1  $\mu$ g/mL LipoMSN, control), or vehicle (control) was injected intrathecally (5  $\mu$ L) at 48 h after CFA. Paw withdrawal to VFF was determined (10, 12).

**Statistics.** Results were analyzed and graphs prepared using Prism 8. Results are expressed as mean  $\pm$  SEM. Statistical significance was assessed using *t* tests or one-way or two-way ANOVA with Tukey's or Sidak's post hoc test (*SI Appendix, Table S2*).

**Data Availability.** All of the data and protocols are provided in the main text or the *SI Appendix*.

**ACKNOWLEDGMENTS.** We thank Lih En Tiah for technical support and Dr. Malvin Janal for statistical support. The tdRGFP-Rab5a plasmid was designed and validated by Christian LeGouill and Lucas Tabajara Parreiras e Silva from the laboratory of Dr. Michel Bouvier. RGFP-CAAX and  $\beta$ -arrestin2-Rluc2 were provided by the Bouvier laboratory. This work was supported by grants from the NIH (NS102722, DE026806, and DK118971 to B.L.S. and N.W.B.), Department of Defense (W81XWH1810431, to B.L.S. and N.W.B.), Crohn's Colitis Canada (D.E.R., A.E.L., and S.J.V.), National Health and Medical Research Council (63303, 1049682, 1031886, 1049730, 1121029, and 1083480 to M.C., M.L.H., N.W.B., and D.P.P.), and Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology (N.W.B.).

1. A. S. Hauser, M. M. Attwood, M. Rask-Andersen, H. B. Schiöth, D. E. Gloriam, Trends in GPCR drug discovery: New agents, targets and indications. *Nat. Rev. Drug Discov.* **16**, 829–842 (2017).
2. K. E. Komolov, J. L. Benovic, G protein-coupled receptor kinases: Past, present and future. *Cell. Signal.* **41**, 17–24 (2018).
3. Y. K. Peterson, L. M. Luttrell, The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol. Rev.* **69**, 256–297 (2017).
4. K. A. DeFea *et al.*, The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta-arrestin-dependent scaffolding complex. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11086–11091 (2000).
5. 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).
6. R. Irannejad, M. von Zastrow, GPCR signaling along the endocytic pathway. *Curr. Opin. Cell Biol.* **27**, 109–116 (2014).
7. J. E. Murphy, B. E. Padilla, B. Hasdemir, G. S. Cottrell, N. W. Bunnett, Endosomes: A legitimate platform for the signaling train. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 17615–17622 (2009).
8. 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).
9. P. Geppetti, N. A. Veldhuis, T. Lieu, N. W. Bunnett, G. Protein-Coupled Receptors, G protein-coupled receptors: Dynamic machines for signaling pain and itch. *Neuron* **88**, 635–649 (2015).
10. 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**, eaal3447 (2017).
11. 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).
12. 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).
13. 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).
14. G. Corder, D. C. Castro, M. R. Bruchas, G. Scherrer, Endogenous and exogenous opioids in pain. *Annu. Rev. Neurosci.* **41**, 453–473 (2018).
15. J. Boué *et al.*, Endogenous regulation of visceral pain via production of opioids by colitogenic CD4(+) T cells in mice. *Gastroenterology* **146**, 166–175 (2014).
16. R. Guerrero-Alba *et al.*, Co-expression of  $\mu$  and  $\delta$  opioid receptors by mouse colonic nociceptors. *Br. J. Pharmacol.* **175**, 2622–2634 (2018).
17. R. Guerrero-Alba *et al.*, Stress activates pronociceptive endogenous opioid signalling in DRG neurons during chronic colitis. *Gut* **66**, 2121–2131 (2017).
18. E. Valdez-Morales *et al.*, Release of endogenous opioids during a chronic IBD model suppresses the excitability of colonic DRG neurons. *Neurogastroenterol. Motil.* **25**, 39–46.e4 (2013).
19. M. Verma-Gandhu *et al.*, CD4<sup>+</sup> T-cell modulation of visceral nociception in mice. *Gastroenterology* **130**, 1721–1728 (2006).
20. 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).
21. M. J. Robertson, F. M. Deane, P. J. Robinson, A. McCluskey, Synthesis of dynole 34-2, dynole 2-24 and dyngo 4a for investigating dynamin GTPase. *Nat. Protoc.* **9**, 851–870 (2014).
22. M. J. Robertson *et al.*, Synthesis of the Pitstop family of clathrin inhibitors. *Nat. Protoc.* **9**, 1592–1606 (2014).
23. A. A. Pradhan *et al.*, Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J. Neurosci.* **36**, 3541–3551 (2016).
24. D. Toulec *et al.*, The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* **266**, 15771–15781 (1991).
25. D. T. Dudley, L. Pang, S. J. Decker, A. J. Bridges, A. R. Saltiel, A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686–7689 (1995).
26. D. D. Jensen *et al.*, Endothelin-converting enzyme 1 and  $\beta$ -arrestins exert spatio-temporal control of substance P-induced inflammatory signals. *J. Biol. Chem.* **289**, 20283–20294 (2014).
27. A. A. Pradhan *et al.*, In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* **4**, e5425 (2009).

28. D. P. Poole *et al.*, Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice. *Gastroenterology* **141**, 982–991.e18 (2011).
29. Q. Wan *et al.*, Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. *J. Biol. Chem.* **293**, 7466–7473 (2018).
30. R. Nehmé *et al.*, Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PLoS One* **12**, e0175642 (2017).
31. Y. Namkung *et al.*, Monitoring G protein-coupled receptor and  $\beta$ -arrestin trafficking in live cells using enhanced bystander BRET. *Nat. Commun.* **7**, 12178 (2016).
32. P. Donthamsetti, J. R. Quejada, J. A. Javitch, V. V. Gurevich, N. A. Lambert, Using bioluminescence resonance energy transfer (BRET) to characterize agonist-induced arrestin recruitment to modified and unmodified G protein-coupled receptors. *Curr. Protoc. Pharmacol.* **70**, 2 14 11–12 14 14 (2015).
33. M. L. Halls, M. Canals, Genetically encoded FRET biosensors to illuminate compartmentalised GPCR signalling. *Trends Pharmacol. Sci.* **39**, 148–157 (2018).
34. J. S. Herskovits, C. C. Burgess, R. A. Obar, R. B. Vallee, Effects of mutant rat dynamin on endocytosis. *J. Cell Biol.* **122**, 565–578 (1993).
35. C. Pinese *et al.*, Sustained delivery of siRNA/mesoporous silica nanoparticle complexes from nanofiber scaffolds for long-term gene silencing. *Acta Biomater.* **76**, 164–177 (2018).
36. D. Shao *et al.*, Bioinspired diselenide-bridged mesoporous silica nanoparticles for dual-responsive protein delivery. *Adv. Mater.*, e1801198 (2018).
37. J. J. DiCello *et al.*, Inflammation-associated changes in DOR expression and function in the mouse colon. *Am. J. Physiol. Gastrointest. Liver Physiol.* **315**, G544–G559 (2018).
38. C. M. Cahill, S. V. Holdridge, A. Morinville, Trafficking of delta-opioid receptors and other G-protein-coupled receptors: Implications for pain and analgesia. *Trends Pharmacol. Sci.* **28**, 23–31 (2007).
39. A. A. Pradhan *et al.*, Ligand-directed trafficking of the  $\delta$ -opioid receptor in vivo: Two paths toward analgesic tolerance. *J. Neurosci.* **30**, 16459–16468 (2010).
40. M. Stoeber *et al.*, A genetically encoded biosensor reveals location bias of opioid drug action. *Neuron* **98**, 963–976.e5 (2018).
41. M. Narita, H. Mizoguchi, J. P. Kampine, L. F. Tseng, Role of protein kinase C in desensitization of spinal delta-opioid-mediated antinociception in the mouse. *Br. J. Pharmacol.* **118**, 1829–1835 (1996).
42. E. G. Ignatova, M. M. Belcheva, L. M. Bohn, M. C. Neuman, C. J. Coscia, Requirement of receptor internalization for opioid stimulation of mitogen-activated protein kinase: Biochemical and immunofluorescence confocal microscopic evidence. *J. Neurosci.* **19**, 56–63 (1999).
43. W. H. De Jong, P. J. Borm, Drug delivery and nanoparticles: Applications and hazards. *Int. J. Nanomedicine* **3**, 133–149 (2008).
44. O. C. Farokhzad, R. Langer, Impact of nanotechnology on drug delivery. *ACS Nano* **3**, 16–20 (2009).
45. K. E. Uhrich, S. M. Cannizzaro, R. S. Langer, K. M. Shakesheff, Polymeric systems for controlled drug release. *Chem. Rev.* **99**, 3181–3198 (1999).
46. H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. *J. Control. Release* **65**, 271–284 (2000).
47. G. K. Such, Y. Yan, A. P. Johnston, S. T. Gunawan, F. Caruso, Interfacing materials science and biology for drug carrier design. *Adv. Mater.* **27**, 2278–2297 (2015).
48. C. E. Nelson *et al.*, Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo. *ACS Nano* **7**, 8870–8880 (2013).
49. D. Calebiro *et al.*, Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol.* **7**, e1000172 (2009).
50. R. Irannejad *et al.*, Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**, 534–538 (2013).
51. R. J. Park *et al.*, Dynamin triple knockout cells reveal off target effects of commonly used dynamin inhibitors. *J. Cell Sci.* **126**, 5305–5312 (2013).
52. E. E. Valdez-Morales *et al.*, Sensitization of peripheral sensory nerves by mediators from colonic biopsies of diarrhea-predominant irritable bowel syndrome patients: A role for PAR2. *Am. J. Gastroenterol.* **108**, 1634–1643 (2013).
53. S. M. Brierley, R. C. Jones, 3rd, G. F. Gebhart, L. A. Blackshaw, Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* **127**, 166–178 (2004).
54. P. A. Hughes *et al.*, Post-inflammatory colonic afferent sensitisation: Different subtypes, different pathways and different time courses. *Gut* **58**, 1333–1341 (2009).
55. M. L. Halls, D. P. Poole, A. M. Ellisdon, C. J. Nowell, M. Canals, Detection and quantification of intracellular signaling using FRET-based biosensors and high content imaging. *Methods Mol. Biol.* **1335**, 131–161 (2015).

# Protein kinase D and G $\beta\gamma$ mediate sustained nociceptive signaling by biased agonists of protease-activated receptor-2

Received for publication, November 30, 2018, and in revised form, May 23, 2019 Published, Papers in Press, May 29, 2019, DOI 10.1074/jbc.RA118.006935

Peishen Zhao<sup>‡</sup>, Luke A. Pattison<sup>‡</sup>, Dane D. Jensen<sup>§</sup>, Nestor N. Jimenez-Vargas<sup>¶</sup>, Rocco Latorre<sup>§</sup>, TinaMarie Lieu<sup>‡</sup>, Josue O. Jaramillo<sup>¶</sup>, Cintya Lopez-Lopez<sup>¶</sup>, Daniel P. Poole<sup>‡</sup>, Stephen J. Vanner<sup>¶</sup>, Brian L. Schmidt<sup>||</sup>, and Nigel W. Bunnett<sup>§1</sup>

From the <sup>‡</sup>Monash Institute of Pharmaceutical Sciences and Australian Research Council Centre of Excellence in Convergent Bio-Nano Science and Technology, Monash University, Parkville, Victoria 3052, Australia, the <sup>§</sup>Departments of Surgery and Pharmacology, Columbia University Vagelos College of Physicians and Surgeons, Columbia University, New York, New York 10032, the <sup>¶</sup>Gastrointestinal Diseases Research Unit, Division of Gastroenterology, Queen's University, Kingston, Ontario K7L 3N6, Canada, and the <sup>||</sup>Bluestone Center for Clinical Research, New York University College of Dentistry, New York, New York 10010

Edited by Henrik G. Dohlman

Proteases sustain hyperexcitability and pain by cleaving protease-activated receptor-2 (PAR<sub>2</sub>) on nociceptors through distinct mechanisms. Whereas trypsin induces PAR<sub>2</sub> coupling to G $\alpha_q$ , G $\alpha_s$ , and  $\beta$ -arrestins, cathepsin-S (CS) and neutrophil elastase (NE) cleave PAR<sub>2</sub> at distinct sites and activate it by biased mechanisms that induce coupling to G $\alpha_s$ , but not to G $\alpha_q$  or  $\beta$ -arrestins. Because proteases activate PAR<sub>2</sub> by irreversible cleavage, and activated PAR<sub>2</sub> is degraded in lysosomes, sustained extracellular protease-mediated signaling requires mobilization of intact PAR<sub>2</sub> from the Golgi apparatus or *de novo* synthesis of new receptors by incompletely understood mechanisms. We found here that trypsin, CS, and NE stimulate PAR<sub>2</sub>-dependent activation of protein kinase D (PKD) in the Golgi of HEK293 cells, in which PKD regulates protein trafficking. The proteases stimulated translocation of the PKD activator G $\beta\gamma$  to the Golgi, coinciding with PAR<sub>2</sub> mobilization from the Golgi. Proteases also induced translocation of a photo-converted PAR<sub>2</sub>-Kaede fusion protein from the Golgi to the plasma membrane of KNRK cells. After incubation of HEK293 cells and dorsal root ganglia neurons with CS, NE, or trypsin, PAR<sub>2</sub> responsiveness initially declined, consistent with PAR<sub>2</sub> cleavage and desensitization, and then gradually recovered. Inhibitors of PKD, G $\beta\gamma$ , and protein translation inhibited recovery of PAR<sub>2</sub> responsiveness. PKD and G $\beta\gamma$  inhibitors also attenuated protease-evoked mechanical allodynia in mice. We conclude that proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms stimulate PKD in the Golgi; PAR<sub>2</sub> mobilization and *de novo* synthesis repopulate the cell surface with intact receptors and sustain nociceptive signaling by extracellular proteases.

Sustained signaling by membrane-impermeant agonists in the extracellular environment requires the presence of functional receptors at the surface of cells. The level of functional receptors at the plasma membrane is a dynamic balance between processes that inactivate or remove receptors and those that reactivate or replenish receptors (1). Desensitization and endocytosis inactivate and remove functional receptors from the plasma membrane and thereby diminish cellular responsiveness. Recovery of responsiveness requires repopulation of the plasma membrane with functional receptors by mechanisms that include resensitization of receptors or membrane insertion of fresh receptors. However, in contrast to the mechanisms of receptor desensitization and endocytosis, which have been extensively studied (2), the mechanisms that repopulate the plasma membrane with functional receptors are not fully understood (1).

G protein-coupled receptors (GPCRs)<sup>2</sup> comprise the largest family of transmembrane receptors and are the target of one-third of therapeutic drugs (3). The mechanisms that attenuate plasma membrane signaling of GPCRs have been extensively studied. G protein-coupled receptor kinases and second messenger kinases can phosphorylate activated receptors, which then associate with  $\beta$ -arrestins. By uncoupling GPCRs from G proteins and coupling GPCRs to clathrin and adaptor protein 2,  $\beta$ -arrestins mediate receptor desensitization and endocytosis, which together attenuate plasma membrane signaling (4). Some endocytosed receptors, including the substance P neurokinin 1 receptor (5) and the  $\mu$ -opioid receptor (6), recycle back to the plasma membrane, which restores their function at the cell surface. Other receptors, exemplified by chemokine receptor type 4 (CXCR4), traffic to lysosomes (7). For these receptors, *de novo* protein synthesis or translocation of intact receptor from a preexisting pool is required for replenishment of the plasma membrane with functioning receptors.

This work was supported by National Institutes of Health Grants NS102722, DE026806, and DK118971; Department of Defense Grant W81XWH1810431 (to N. W. B. and B. L. S.); and National Health and Medical Research Council Grants 63303, 1049682, and 1031886 (to N. W. B.). Nigel Bunnett is a founding scientist of Endosome Therapeutics Inc. Research in Nigel Bunnett's laboratory was funded in part by Takeda Pharmaceuticals Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

<sup>1</sup> To whom correspondence should be addressed: Columbia University Vagelos College of Physicians and Surgeons, 21 Audubon Av., Rm. 209, New York City, NY 10032. Tel.: 212-305-4480; E-mail: nb2733@cumc.columbia.edu.

<sup>2</sup> The abbreviations used are: GPCR, G protein-coupled receptor; PAR, protease-activated receptor; NE, neutrophil elastase; CS, cathepsin-S; PKC, protein kinase C; PKA, protein kinase A; PKD, protein kinase D; PAR, protease-activated receptor; hPAR, human PAR; BRET, bioluminescence resonance energy transfer; DRG, dorsal root ganglia; VFF, von Frey filament(s); HBSS, Hanks' balanced salt solution; AUC, area under the curve; HA, hemagglutinin; ANOVA, analysis of variance.

PARs are a small family of GPCRs that are activated by proteolytic cleavage within the extracellular N-terminal region (8). Several serine and cysteine proteases can cleave and activate PAR<sub>2</sub>, including trypsins (9, 10), mast cell tryptase (11), kallikreins (12), NE (13, 14) and CS, a cysteine protease from antigen-presenting cells (15). Trypsin, tryptase, and kallikreins activate PAR<sub>2</sub> by a canonical mechanism. Trypsin cleaves PAR<sub>2</sub> at R<sup>36</sup> ↓ S<sup>37</sup>, which reveals the tethered ligand domain (S<sup>37</sup>LIGKV for human PAR<sub>2</sub>). This cleavage results in PAR<sub>2</sub> coupling to G<sub>α<sub>q</sub></sub>, G<sub>α<sub>s</sub></sub>, and β-arrestins, leading to mobilization of Ca<sup>2+</sup>, generation of cAMP, and activation of protein kinase C (PKC) and A (PKA) and of extracellular signal-regulated kinases (16, 17). β-Arrestins mediate desensitization and endocytosis of PAR<sub>2</sub>, which then traffics to lysosomes and is degraded (17). Given the irreversible mechanism of proteolytic activation, cleaved PAR<sub>2</sub> cannot be reactivated by proteolysis (16). Recovery of cell surface PAR<sub>2</sub> signaling involves mobilization of intact receptors from a preexisting Golgi pool as well as synthesis of fresh receptors (16). NE and CS are activated and released from inflammatory cells at sites of injury and inflammation, retain activity in extracellular fluid, and can cause PAR<sub>2</sub>-dependent inflammation and pain (14, 15, 18). However, CS and NE activate biased pathways of PAR<sub>2</sub> signaling and trafficking. CS cleaves human PAR<sub>2</sub> at E<sup>56</sup> ↓ T<sup>57</sup> to expose the tethered ligand (T<sup>57</sup>VFSVDEFS<sub>A</sub>), which promotes PAR<sub>2</sub> coupling to G<sub>α<sub>s</sub></sub> (15). NE cleaves at A<sup>66</sup> ↓ S<sup>67</sup> and S<sup>67</sup> ↓ V<sup>68</sup>, adjacent to the first transmembrane domain, which activates PAR<sub>2</sub> by a nontethered ligand mechanism and induces coupling to G<sub>α<sub>s</sub></sub> and G<sub>α<sub>12/13</sub></sub> (13, 14). CS- and NE-activated PAR<sub>2</sub> fails to couple to G<sub>α<sub>q</sub></sub> and neither recruits β-arrestins nor internalizes. We recently reported that trypsin cleavage of PAR<sub>2</sub> at the plasma membrane induces translocation of Gβγ to the Golgi apparatus, where Gβγ activates PKD (19). PKD mediates the mobilization of PAR<sub>2</sub> stores from the Golgi apparatus, which replenishes the plasma membrane with fresh receptors that are necessary for sustained trypsin signaling (19). In the present study, we investigated the mechanisms that underlie sustained signaling of proteases that activate PAR<sub>2</sub> by biased mechanisms.

## Results

### Proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms induce PAR<sub>2</sub>-dependent PKD activation

By using immunoblotting and immunofluorescence, we have previously reported that trypsin activation of PAR<sub>2</sub> leads to PKD phosphorylation (activation) in the Golgi apparatus (19). To quantitatively assess PKD activation in live cells with high spatial and temporal fidelity, we expressed in human embryonic kidney (HEK293) cells genetically encoded FRET biosensors for PKD that are targeted to the cytosol (Cyto-DKAR) or Golgi apparatus (Golgi-DKAR) (20). We examined whether proteases that activate PAR<sub>2</sub> by canonical or biased mechanisms can stimulate PKD activity in the cytosol or plasma membrane of HEK293 cells transiently expressing human PAR<sub>2</sub> (hPAR<sub>2</sub>). To confirm that alterations in FRET were attributable to PKD activation, we expressed a T/A mutated PKD sensor (DKAR-T/A) in which the PKD phosphorylation site in the substrate domain is mutated.

We first confirmed the expected subcellular localization of PKD FRET biosensors expressed in HEK293 cells by confocal microscopy. Cyto-DKAR was uniformly distributed throughout the cytosol, whereas Golgi-DKAR colocalized exclusively with immunoreactive TGN58K, a marker of the Golgi apparatus (Fig. 1A).

Trypsin (10 nM), CS (100 nM), NE (100 nM), and the PAR<sub>2</sub>-selective agonist 2-furoyl-LIGRLO-NH<sub>2</sub>, an analogue of the tethered ligand (10 μM), increased PKD activity in the cytosol and Golgi apparatus within 2 min, which was sustained for at least 20 min (Fig. 1, B–E). The PAR<sub>2</sub> antagonist I-343 (18) (10 μM) inhibited trypsin-, CS-, and NE-induced PKD activation (Fig. 1, F–H). I-343 did not inhibit activation of PKD in the Golgi apparatus in response to phorbol 12,13-dibutyrate (200 nM) or thrombin (30 units/ml), a PAR<sub>1</sub> agonist, and I-343 alone had no effect on PKD activity (Fig. 1I). These results show that proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms activate PKD in the cytosol and Golgi apparatus of HEK293 cells. These effects are mediated by PAR<sub>2</sub>. There was no effect of trypsin or 2-furoyl-LIGRLO-NH<sub>2</sub> on DKAR-T/A FRET (Fig. 1, B and E), although CS and NE caused a minor increase in DKAR-T/A FRET (Fig. 1, C and D). One possibility is that kinases other than PKD might phosphorylate DKAR-T/A, such as PKA, which is robustly activated by NE (13, 14) and CS (15). Further studies are necessary to address this question.

### Proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms induce Gβγ translocation to Golgi

Gβγ subunits can activate PKD in the Golgi apparatus (21). Trypsin activation of PAR<sub>2</sub> at the cell surface induces translocation of Gβγ subunits to the Golgi apparatus, where Gβγ activates PKD (19). We used bioluminescence resonance energy transfer (BRET) to examine whether CS and NE, which activate PAR<sub>2</sub> by biased mechanisms, also promote Gβγ trafficking to the Golgi apparatus. We transiently expressed in HEK293 cells Gγ2-Venus and Giantin-RLuc8, a Golgi-resident protein (22), which permitted measurement of the proximity between Gγ2-Venus and Giantin-RLuc8 using bystander BRET. We also expressed hPAR<sub>2</sub>, Gβ1, and either G<sub>α<sub>q</sub></sub> or G<sub>α<sub>s</sub></sub>, which allowed assessment of the requirement of different Gα subunits.

We confirmed the expected subcellular localization of BRET biosensors expressed in HEK293 cells by confocal microscopy. In unstimulated cells, Gγ2-Venus was uniformly cytosolic, whereas Giantin-Venus colocalized exclusively with immunoreactive TGN58K in the Golgi apparatus (Fig. 2A). We were unable to localize Giantin-RLuc8 because of the lack of a suitable epitope tag or antibody, but we expect that it would have the identical localization as Giantin-Venus.

Trypsin increased Gγ2-Venus/Giantin-RLuc8 BRET in the presence of either G<sub>α<sub>q</sub></sub> or G<sub>α<sub>s</sub></sub> (Fig. 2, B–D). However, trypsin increased Gγ2-Venus/Giantin-RLuc8 BRET with higher potency in cells expressing G<sub>α<sub>q</sub></sub> (pEC<sub>50</sub> (negative logarithm of the EC<sub>50</sub>) = 12.74 ± 0.36) compared with G<sub>α<sub>s</sub></sub> (pEC<sub>50</sub> = 9.75 ± 0.65). CS also increased Gγ2-Venus/Giantin-RLuc8 BRET in the presence of either G<sub>α<sub>q</sub></sub> or G<sub>α<sub>s</sub></sub> (Fig. 2, E–G). In contrast to trypsin, CS increased BRET with higher potency in cells expressing G<sub>α<sub>s</sub></sub> (pEC<sub>50</sub> = 11.90 ± 0.35) than G<sub>α<sub>q</sub></sub> (pEC<sub>50</sub> =

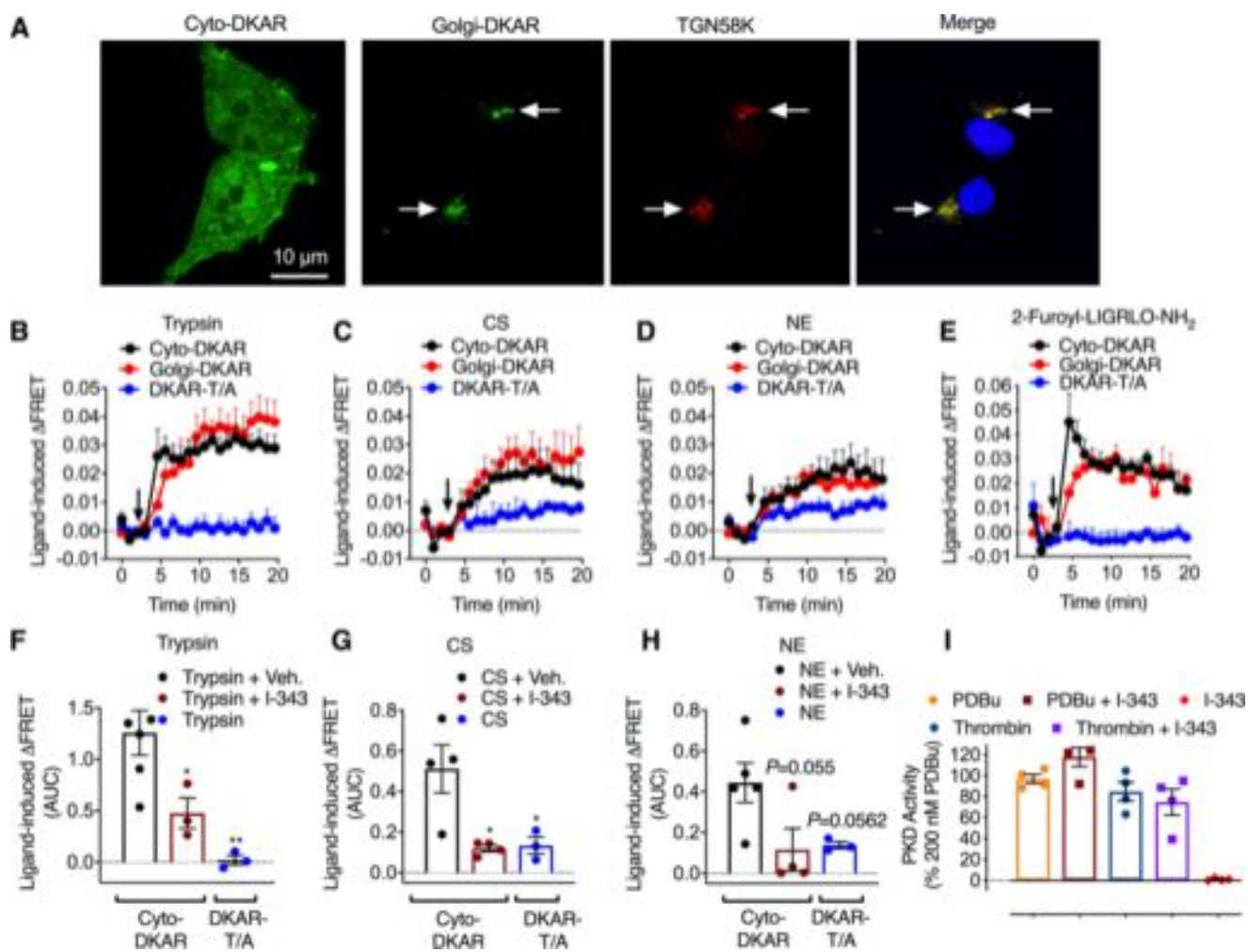


**Figure 1. Protease-induced activation of PKD.** A, localization of FRET PKD biosensors Cyto-DKAR and Golgi-DKAR with TGN58K in HEK293 cells. B–E, time course of trypsin-induced (B, 10 nM), CS-induced (C, 100 nM), NE-induced (D, 100 nM), and 2-furoyl-LIGRLO-NH<sub>2</sub>-induced (E, 10 μM) PKD activity in HEK293 cells expressing FRET biosensors for PKD in the cytosol (Cyto-DKAR) and Golgi apparatus (Golgi-DKAR). DKAR-T/A is a PKD control sensor. Agonists were added at the arrow. F–H, AUC for Cyto-DKAR and DKAR-T/A in HEK293 cells treated with trypsin (F), CS (G), or NE (H) and either vehicle or PAR<sub>2</sub> antagonist (I-343). I, effects of I-343 on PKD activity in the Golgi apparatus stimulated by phorbol 12,13-dibutyrate (PDBu) or thrombin. *n* = 3–5 experimental replicates, triplicate observations. \*, *p* < 0.05; \*\*, *p* < 0.01 to vehicle (one-way ANOVA, Bonferroni multiple comparisons). Error bars, S.E.

10.35 ± 0.81). NE increased Gγ2-Venus/Giantin-RLuc8 BRET only in the presence of Gα<sub>s</sub> (pEC<sub>50</sub> = 11.84 ± 0.45), not Gα<sub>q</sub> (Fig. 2, H–J). Thus, proteases that activate PAR<sub>2</sub> at the cell surface by canonical and biased mechanisms may evoke the rapid translocation of Gβγ to the Golgi apparatus. Although Gβγ subunits could originate from the plasma membrane, it is also possible that PAR<sub>2</sub> activation evokes translocation of Gβγ from the cytosol to the Golgi apparatus or may reveal Gβγ subunits already present in the Golgi apparatus. The effects of overexpression of Gα subunits on Gγ2-Venus/Giantin-RLuc8 BRET are consistent with the capacity of trypsin-activated PAR<sub>2</sub> to couple to Gα<sub>q</sub> or Gα<sub>s</sub> and for CS- and NE-activated PAR<sub>2</sub> to couple preferentially to Gα<sub>s</sub> (13–15). Studies with selective Gα inhibitors or with Gα-deficient HEK293 cells will be required to determine the absolute requirement of particular Gα subunits for protease-stimulated Gγ2-Venus/Giantin-RLuc8 BRET.

#### Proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms mobilize PAR<sub>2</sub> from the Golgi apparatus

To quantitatively examine whether PAR<sub>2</sub> activation at the cell surface mobilizes receptors from the Golgi apparatus, we

examined the proximity between PAR<sub>2</sub>-RLuc8 and TGN38-Venus, a Golgi-resident protein (23). PAR<sub>2</sub>-RLuc8 was prominently localized to the plasma membrane, and TGN38-Venus was confined to the Golgi apparatus, as confirmed by colocalization with immunoreactive TGN58K (Fig. 3A). Thus, BRET sensors have the expected subcellular localization.

Trypsin (10 and 100 nM), CS (100 nM), and NE (100 nM) all induced a rapid decrease in PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET within 2 min that was sustained for 20 min (Fig. 3, B–D). Gallein, which inhibits Gβγ activity (24), suppressed the decrease in PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET in cells exposed to trypsin, CS, and NE (Fig. 3, E–J). To confirm involvement of Gβγ, we treated cells with a peptide corresponding to the C terminus of GPCR kinase 2 (GRK2i), which inhibits Gβγ activity (25). GRK2i (100 μM) attenuated the decrease in PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET in cells exposed to trypsin, CS, and NE to a similar degree as gallein (Fig. 3, H–J).

We then evaluated the role of kinases in mobilization of PAR<sub>2</sub> from the Golgi apparatus. Surprisingly, in view of the known role of PKD in protein sorting within the Golgi apparatus (19, 21, 26, 27), the PKD inhibitor CRT0066101 (100 nM)

## PKD and G $\beta\gamma$ sustain PAR<sub>2</sub> nociceptive signaling

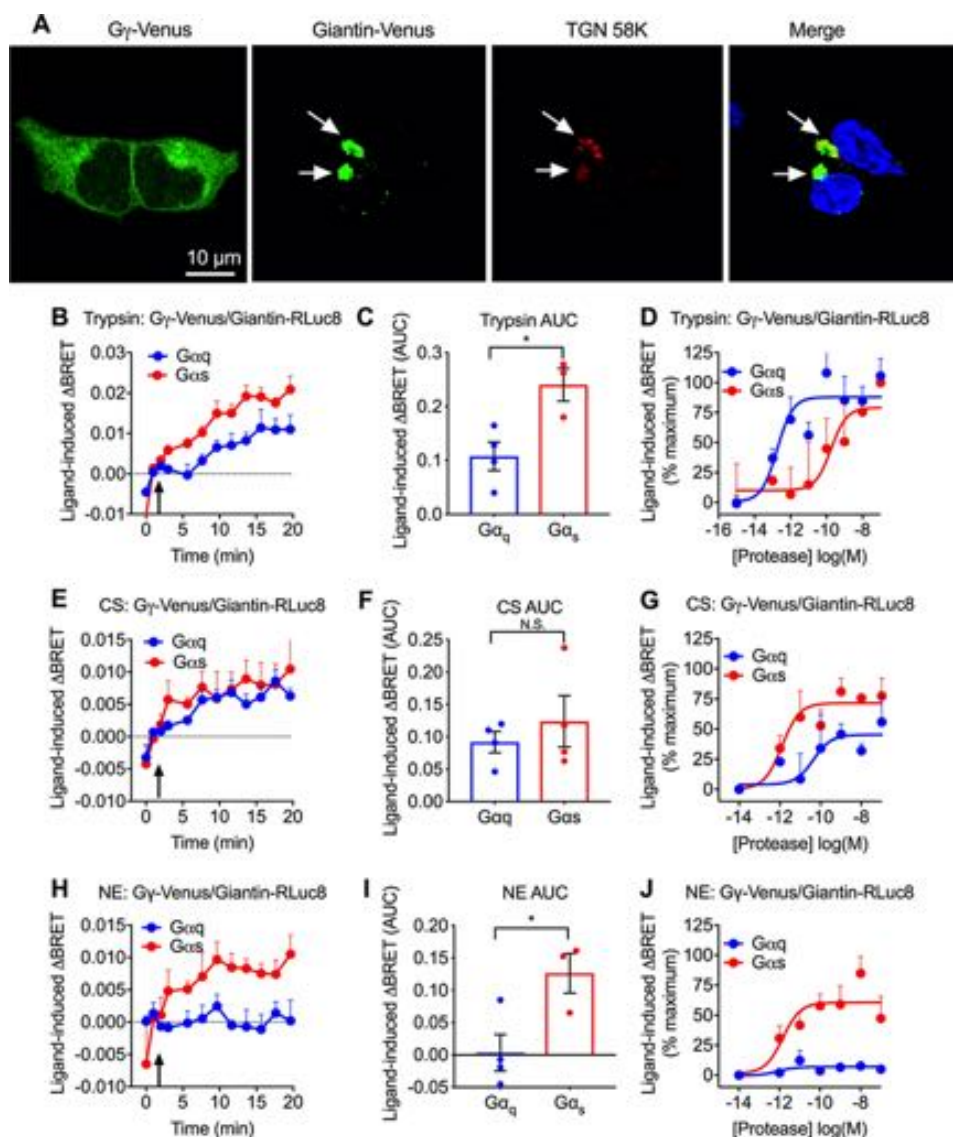


**Figure 2. Protease-induced translocation of G $\gamma$  to Golgi apparatus.** A, localization of BRET biosensors G $\gamma$ -Venus and Giantin-Venus with TGN 58K in HEK293 cells. B–J, protease-evoked BRET. Effects of trypsin (B–D), CS (E–G), and NE (H–J) on G $\gamma$ -Venus/Giantin-RLuc8 BRET in HEK293 cells. B, E, and H, time course of trypsin-induced (10 nM), CS-induced (100 nM), and NE-induced (100 nM) BRET. Agonists were added at the arrow. C, F, and I, AUC of BRET response. D, G, J, concentration–response analysis. G $\beta$  was coexpressed with either G $\alpha_q$  or G $\alpha_s$ .  $n = 3$ –6 experimental replicates, triplicate observations. \*,  $p < 0.05$  (Student's  $t$  test). Error bars, S.E.

(28) did not affect the decrease in PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET (Fig. 3, E–J). A G $\alpha_q$ /Ca<sup>2+</sup>/PKC pathway has been reported to control Golgi organization and secretion (29). Gö6983 (1  $\mu$ M), a nonselective PKC inhibitor (30), did not significantly inhibit trypsin-, CS-, or NE-evoked mobilization of PAR<sub>2</sub> from the Golgi apparatus (Fig. 3, E–J). PKA has been shown to be required in the formation of vesicles from the Golgi (31). Given the capacity of CS- and NE-activated PAR<sub>2</sub> to couple to G $\alpha_s$ , adenylyl cyclase, cAMP, and PKA, we also evaluated the contribution of PKA to PAR<sub>2</sub> mobilization from the Golgi apparatus. H-89 (1  $\mu$ M), which inhibits PKA and other kinases (32, 33), had no effect on trypsin-, CS-, or NE-induced mobilization of PAR<sub>2</sub> (Fig. 3, H–J).

We confirmed that the PKD and PKC inhibitors blocked kinase activity in HEK293 cells by expressing FRET biosensors for cytosolic PKD (Cyto-DKAR) and PKC (Cyto-CKAR).

CRT0066101 (100 nM) abolished trypsin (10 nM)-evoked activation of PKD (Fig. 4, A and B), and Gö6983 (1  $\mu$ M) abolished trypsin-evoked activation of PKC (Fig. 4, C and D). We have previously shown that H-89 inhibits CS-mediated PAR<sub>2</sub> signaling events (15).

These results indicate a major role for G $\beta\gamma$  subunits in mediating the capacity of trypsin, CS, and NE to mobilize PAR<sub>2</sub> from the Golgi apparatus of HEK293 cells. Further studies are necessary to understand the unexpected finding that PKD inhibitors did not affect protease-evoked liberation of PAR<sub>2</sub> from the Golgi apparatus as determined by measurements of PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET. However, because TGN38 can shuttle between the Golgi apparatus and plasma membrane, it is possible that proximity between TGN38 and PAR<sub>2</sub> in the exocytic and endocytic pathways may confound the results (23).

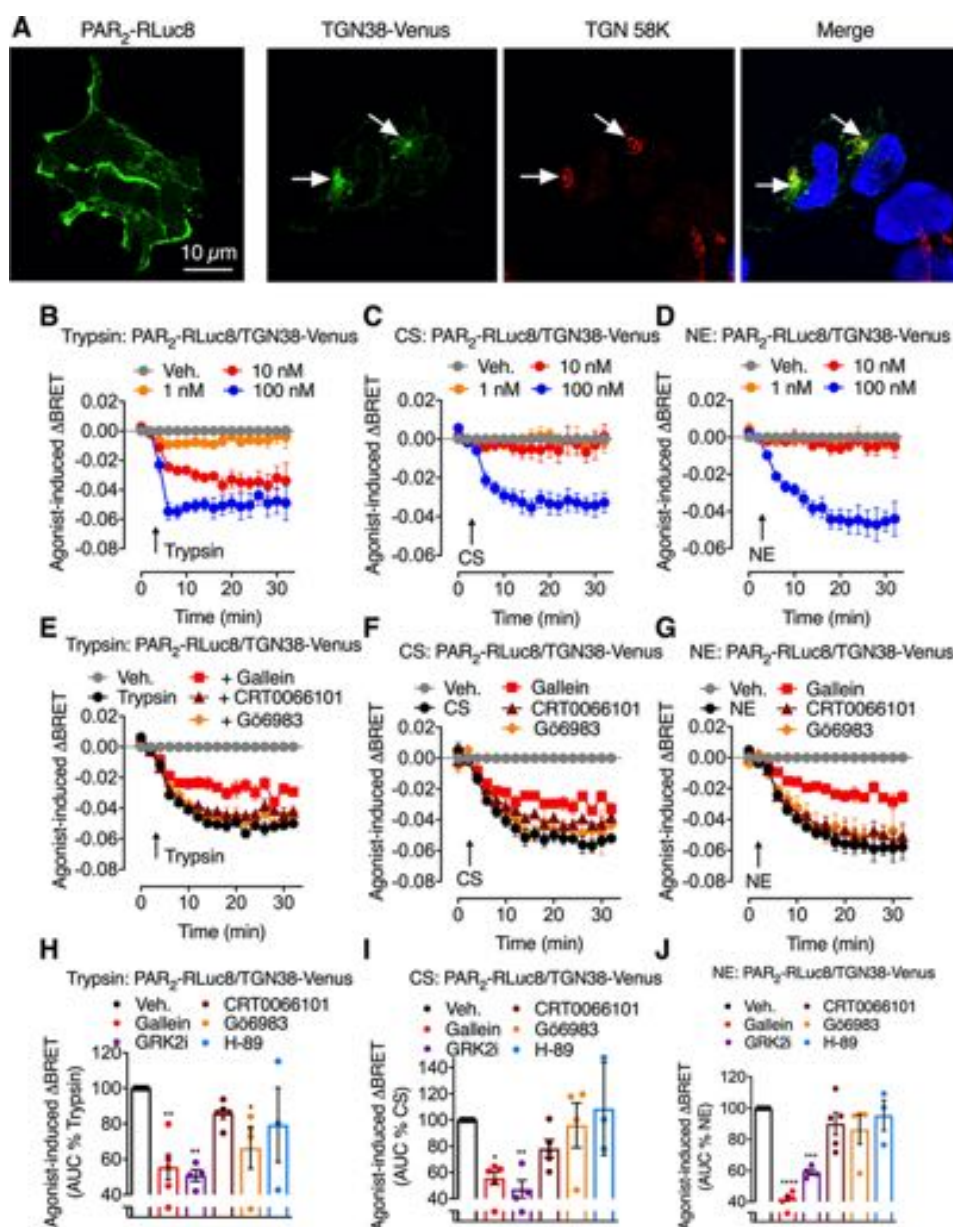


**Figure 3. Protease-induced translocation of PAR<sub>2</sub> from the Golgi apparatus.** A, localization of BRET biosensors PAR<sub>2</sub>-RLuc8 and TGN38-Venus with TGN 58K in HEK293 cells. B–D, protease-evoked BRET. B–D, time course of trypsin-, CS-, and NE-induced BRET between PAR<sub>2</sub>-RLuc8 and TGN38-Venus in HEK293 cells. E–J, effects of inhibitors of G $\beta\gamma$  (gallein), PKD (CRT0066101), PKC (Gö6983), and PKA (H-89) on trypsin-induced (10 nM), CS-induced (100 nM), and NE-induced (100 nM) BRET between PAR<sub>2</sub>-RLuc8 and TGN38-Venus. E–G, time course. H–J, AUC. *n* = 3 (A–C) or 3–5 (D–J) experimental replicates, triplicate observations. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*\*, *p* < 0.0001 (one-way ANOVA, Bonferroni multiple comparisons). Error bars, S.E.

### Proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms induce G $\beta\gamma$ and PKD-dependent translocation of PAR<sub>2</sub> to the plasma membrane

In light of the paradoxical inhibition of PAR<sub>2</sub> mobilization from the Golgi apparatus by G $\beta\gamma$  inhibitors but not by PKD inhibitors, as determined by PAR<sub>2</sub>-RLuc8/TGN38-Venus BRET, we sought an alternative approach to study depletion of PAR<sub>2</sub> from the Golgi apparatus. To localize the redistribution of PAR<sub>2</sub> from the Golgi apparatus to the plasma membrane, we expressed in rat kidney epithelial (KNRK) cells PAR<sub>2</sub> fused at the C terminus to Kaede, a photoconvertible protein. We have previously shown that PAR<sub>2</sub>-Kaede is functional and suitable for analysis of intracellular trafficking of PAR<sub>2</sub> (19). We selected KNRK cells for these studies rather than HEK293 cells because

the Golgi pool of PAR<sub>2</sub> is particularly distinct in KNRK cells compared with HEK293 cells, where it is diffuse and more challenging to localize (16). Trypsin induces  $\beta$ -arrestin-mediated endocytosis and endosomal signaling of PAR<sub>2</sub> of PAR<sub>2</sub> in KNRK cells (17, 34). The prominent Golgi store of PAR<sub>2</sub>-Kaede was photoconverted from green to red using a confocal microscope laser. Cells were then exposed to proteases and monitored to assess depletion of PAR<sub>2</sub>-Kaede from the Golgi apparatus and insertion of mobilized PAR<sub>2</sub>-Kaede into the plasma membrane. Before photoconversion, PAR<sub>2</sub>-Kaede (green) was detected at the plasma membrane and in the Golgi apparatus (Fig. 5, A and B). Confocal illumination of the Golgi resulted in rapid green to red photoconversion of PAR<sub>2</sub>-Kaede in the Golgi apparatus but not the plasma membrane. NE (100 nM, 30 min)

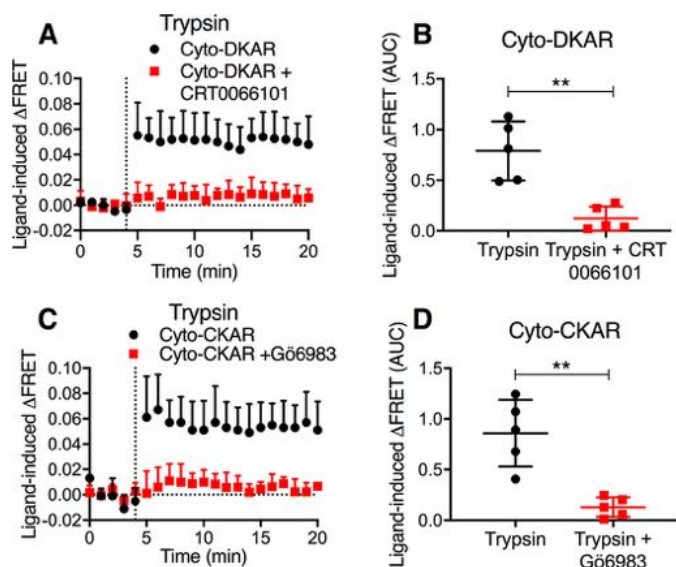


**Figure 4. Antagonism of PKD and PKC.** Time course (A and C) and AUC (B and D) of trypsin-stimulated activation of cytosolic PKD (A and B) and cytosolic PKC (C and D). The effects of inhibitors of PKD (CRT0066101) and PKC (Gö6983) are shown.  $n = 5$  experimental replicates, triplicate observations. \*\*,  $p < 0.01$  (Student's  $t$  test). Error bars, S.E.

induced a significant depletion of PAR<sub>2</sub>-Kaede red from the Golgi apparatus and a corresponding increase in PAR<sub>2</sub>-Kaede red at the plasma membrane (Fig. 5A). CRT0066101 (Fig. 5, C and D) and gallein (Fig. 5, E and F) both prevented the depletion of PAR<sub>2</sub>-Kaede red from the Golgi apparatus and insertion into the plasma membrane. Similarly, CS (100 nM, 30 min) induced a significant depletion of PAR<sub>2</sub>-Kaede red from the Golgi apparatus and a nonsignificant increase in PAR<sub>2</sub>-Kaede red at the plasma membrane (Fig. 6A). CRT0066101 (Fig. 6B) and gallein (Fig. 6C) both prevented the depletion of PAR<sub>2</sub>-Kaede red from the Golgi apparatus. These results suggest that NE and CS activate PAR<sub>2</sub> at the plasma membrane to induce Gβγ- and PKD-mediated recruitment of intact PAR<sub>2</sub> from the Golgi apparatus to the plasma membrane.

#### PKD and Gβγ mediate recovery of plasma membrane PAR<sub>2</sub> signaling

CS and NE cleave PAR<sub>2</sub> distal to the trypsin cleavage site and the trypsin-exposed tethered ligand (14, 15). By removing these domains, CS and NE prevent the capacity of trypsin to activate PAR<sub>2</sub>. Recovery of responsiveness to trypsin thus requires repopulation of the plasma membrane with intact PAR<sub>2</sub>. We made use of this paradigm to determine whether Gβγ- and PKD-induced mobilization of intact PAR<sub>2</sub> from the Golgi apparatus mediates the recovery of trypsin-responsiveness in HEK293 cells.

HEK293 cells were first exposed to trypsin (10 nM), CS (100 nM), NE (100 nM), or vehicle (control) for 10 min (Fig. 7A). Cells were then washed, recovered for 110 min at 37 °C, and challenged with trypsin (10 nM) at 120 min after the first challenge with proteases.  $[Ca^{2+}]_i$  was monitored to assess PAR<sub>2</sub> activation. We have previously reported that exposure to CS and NE (100 nM, 10 min) prevents the capacity of trypsin to mobilize  $Ca^{2+}$ , which indicates that CS and NE can initially cleave and disarm the receptor under these conditions (14, 15). In some

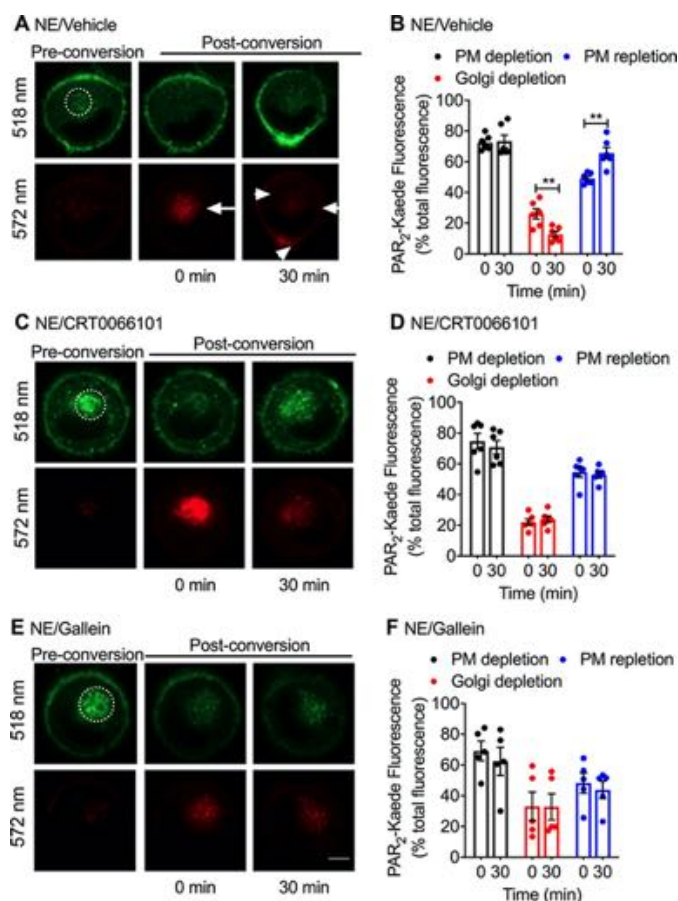


**Figure 5. NE-induced trafficking of PAR<sub>2</sub>-Kaede from the Golgi apparatus to the plasma membrane.** PAR<sub>2</sub>-Kaede located in the region of the perinuclear Golgi apparatus of KNRK-PAR<sub>2</sub>-Kaede cells was green/red photoconverted using a confocal laser (dashed circle, illuminated region). Cells were then incubated with NE (100 nM) for 0 or 30 min. PAR<sub>2</sub>-Kaede fluorescence was measured at 518 and 572 nm at the cell surface or Golgi. Cells were pre-incubated with vehicle (A and B), PKD inhibitor (CRT0066101, 100 nM) (C and D), or Gβγ inhibitor (gallein, 10 μM) (E and F). A, C, and E, representative images before and after photoconversion and at 0 or 30 min after protease challenge. B, D, and F, quantification of PAR<sub>2</sub>-Kaede (518 nm, green) at the plasma membrane and of photoconverted PAR<sub>2</sub>-Kaede (572 nm, red) in the Golgi region and at the plasma membrane.  $n = 5$ –6 experiments, >30 cells analyzed per condition. \*\*,  $p < 0.01$  (Student's  $t$  test). Scale bar, 10 μm. Error bars, S.E.

experiments, cells were pre-incubated with CRT0066101, gallein, or vehicle to assess the mechanism of recovery. The first challenge with trypsin caused a prompt increase in  $[Ca^{2+}]_i$ , and this response was unaffected by CRT0066101 or gallein (Figs. 7, B–D and 8A). In contrast, CS and NE did not affect  $[Ca^{2+}]_i$ , which is in accordance with the inability of CS- and NE-activated PAR<sub>2</sub> to couple to Gα<sub>q</sub> and mobilize intracellular  $Ca^{2+}$  ions (14, 15). When cells were challenged with trypsin at 120 min after the first exposure to trypsin, CS, or NE, the secondary responses to trypsin were almost fully recovered relative to those of cells challenged initially with vehicle (Figs. 7, C and D and 8). These results are consistent with replenishment of the plasma membrane with intact PAR<sub>2</sub>. CRT0066101 or gallein inhibited recovery of trypsin responsiveness in cells first challenged with trypsin, CS, or NE (Figs. 7, C and D and 8, B–F). The combination of both CRT0066101 and gallein had a further inhibitory effect, which might suggest parallel PKD and Gβγ pathways (Figs. 7, C and D and 8, D, G, and J). These data

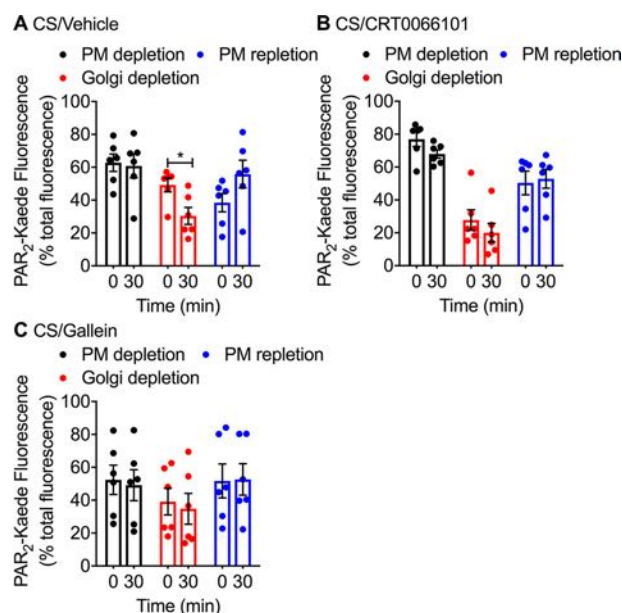


**Figure 6. CS-induced trafficking of PAR<sub>2</sub>-Kaede from the Golgi apparatus to the plasma membrane.** PAR<sub>2</sub>-Kaede located in the region of the perinuclear Golgi apparatus of KNRK-PAR<sub>2</sub>-Kaede cells was green/red photoconverted. Cells were then incubated with CS (100 nM) for 0 or 30 min. PAR<sub>2</sub>-Kaede fluorescence was measured at the cell surface or Golgi. Cells were pre-incubated with vehicle (A), PKD inhibitor (CRT0066101, 100 nM) (B), or Gβγ inhibitor (gallein, 10 μM) (C). Quantification of PAR<sub>2</sub>-Kaede (518 nm, green) at the plasma membrane and of photoconverted PAR<sub>2</sub>-Kaede (572 nm, red) in the Golgi region and at the plasma membrane is shown. *n* = 6 experiments, >30 cells analyzed per condition. \*, *p* < 0.05 (Student's *t* test). Error bars, S.E.

suggest that Gβγ- and PKD-mediated recruitment of intact PAR<sub>2</sub> from the Golgi apparatus to the plasma membrane is required for recovery of trypsin responses in HEK293 cells.

#### NE-induced activation of PKD in the Golgi apparatus of nociceptors mediates sustained hyperexcitability

We have recently reported that trypsin, CS, and NE induce sustained nociception in mice by activating PAR<sub>2</sub> expressed by a subpopulation of primary sensory neurons (18). Trypsin, CS, and NE also cause a PAR<sub>2</sub>-dependent hyperexcitability of nociceptors, which is a hallmark of chronic pain (18). PKD activity is necessary for maintaining trypsin-evoked hyperexcitability of nociceptors, consistent with PAR<sub>2</sub> mobilization from Golgi stores (19). We examined whether PKD and Gβγ contribute to the maintenance of protease-evoked hyperexcitability. The excitability of nociceptors of mouse dorsal root ganglia (DRG) was determined by measurement of the rheobase (minimum current required to fire a single action potential) using patch-clamp recordings. Neurons were exposed to NE (7.8 nM, 0.2 units/ml, 20 min) or vehicle (control) and washed, and rheobase was measured at 0, 30, or 150 min after washing (Fig. 9A). Immediately after washing (0 min), there was a 32% decrease in rheobase of NE-treated neurons, when compared with vehicle (*p* < 0.001), consistent with hyperexcitability (Fig. 8B). Hyperexcitability was maintained at 30 min after washing (25% decrease in rheobase compared with vehicle, *p* < 0.05), but after 150 min, hyperexcitability had declined to control levels (Fig. 9B). To determine whether neurons had recovered their capacity to respond to PAR<sub>2</sub> agonists at 150 min after exposure to NE

and washing, they were challenged with trypsin (50 nM, 10 min) and washed, and rheobase was immediately measured. In NE-exposed neurons, trypsin induced a 35% decrease in rheobase when compared with vehicle-treated controls (*p* < 0.01) (Fig. 9B). These results show that NE causes an initial hyperexcitability of nociceptors, which is sustained for 30 min and declines after 150 min. At this time, neurons have regained their capacity to respond to trypsin, which may require mobilization of intact PAR<sub>2</sub>.

To determine whether PKD, Gβγ, or new protein synthesis mediates this recovery of hyperexcitability, neurons were pre-incubated with CRT0066101 (10 nM, PKD inhibitor), gallein (10 μM, Gβγ inhibitor), cycloheximide (10 μg/ml, protein synthesis inhibitor), or vehicle 30 min before NE exposure; inhibitors were present throughout the experiment. NE- and trypsin-evoked hyperexcitability was determined. CRT0066101 and gallein had no effect on the capacity of NE to cause an immediate hyperexcitability (0 min after NE) (Fig. 9C). However, both the PKD and Gβγ inhibitors prevented trypsin-induced hyperexcitability at 150 min after NE exposure (Fig. 9D). Similarly, cycloheximide did not affect an initial response to trypsin (0 min), but abolished the recovery of trypsin-induced hyperexcitability at 150 min after NE (Fig. 9D). Thus, after exposure to NE, PKD, Gβγ, and new protein synthesis are necessary for the restoration of protease-evoked hyperexcitability. This result is consistent with the role of PKD and Gβγ in mobilization of newly synthesized PAR<sub>2</sub> from the Golgi apparatus and replenishment of the plasma membrane with fresh receptors that are required for recovery of protease signaling.

#### PKD and Gβγ are required for sustained nociception to proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms

The local (intraplantar) injection of trypsin, CS, and NE causes persistent mechanical allodynia in mice by activating PAR<sub>2</sub> on nociceptors (18). Whereas PAR<sub>2</sub> signaling from endosomes mediates trypsin-induced allodynia, PAR<sub>2</sub> signaling from the plasma membrane mediates CS- and NE-induced allodynia. Because cleaved PAR<sub>2</sub> cannot be reactivated by proteases and is eventually degraded, the mobilization of fresh PAR<sub>2</sub> may be necessary for the continuation of protease-evoked pain. To examine this possibility, we administered to mice inhibitors of PKD and Gβγ. The PKD inhibitor CRT0066101 (80 mg/kg, orally), the Gβγ inhibitor gallein (100 mg/kg, intraperitoneally), or vehicle (control) was administered to mice. After 2 h (CRT0066101) or 30 min (gallein), mice received an intraplantar injection of trypsin (140 nM, 0.04 units/μl), CS (2.5 μM, 0.06 units/μl), or NE (1.18 μM, 0.03 units/μl) (10 μl). Paw withdrawal responses to stimulation of the plantar surface with calibrated von Frey filaments (VFF) were measured every hour for 4 h after protease injection. In vehicle-treated mice, trypsin, CS, and NE caused mechanical allodynia of the ipsilateral (protease-injected) paw after 1 h that was sustained for 4 h (Fig. 10). CRT0066101 significantly blunted responses to trypsin, CS, and NE from 1 to 2 h (Fig. 10, A–C). Gallein also inhibited trypsin-, CS-, and NE-induced allodynia (Fig. 9, E–G). CRT0066101 and gallein did not affect withdrawal responses of the contralateral (noninjected) paw (Fig. 10, D and H). These

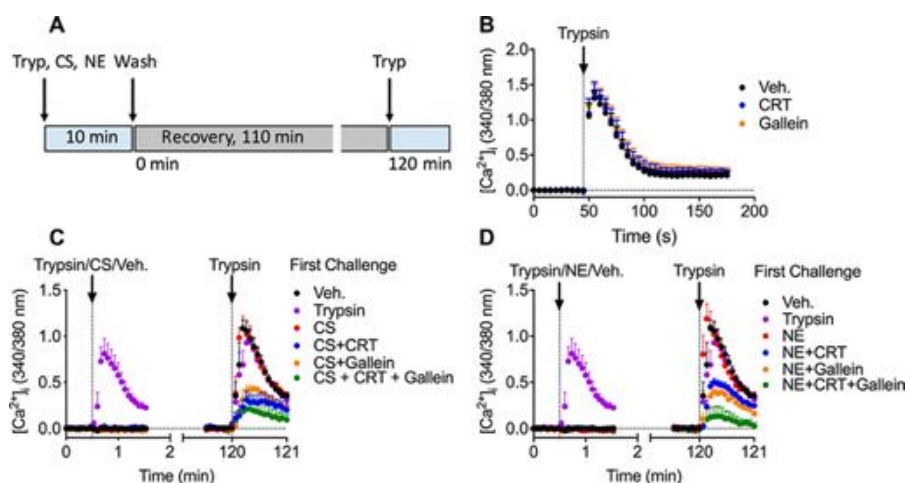


**Figure 7. Recovery of PAR<sub>2</sub>-mediated signaling.** A, experimental design. HEK293 cells were first challenged with trypsin (10 nM), CS (100 nM), NE (100 nM), or vehicle for 10 min. Cells were then washed, recovered for 110 min, and then challenged with trypsin (10 nM) at 120 min after the first challenge with proteases. B–D, measurement of  $[Ca^{2+}]_i$  in HEK293 cells. B, effects of PKD inhibitor (CRT0066101, CRT, 100 nM) or Gβγ inhibitor (gallein, 10 μM) on responses to a single challenge with trypsin. C and D, responses to a first challenge with trypsin, CS, or vehicle (C) or with trypsin, NE, or vehicle (D) and a second challenge with trypsin after 120 min. *n* = 4 (B) or 5 (C and D) experimental replicates from triplicate observations. Error bars, S.E.

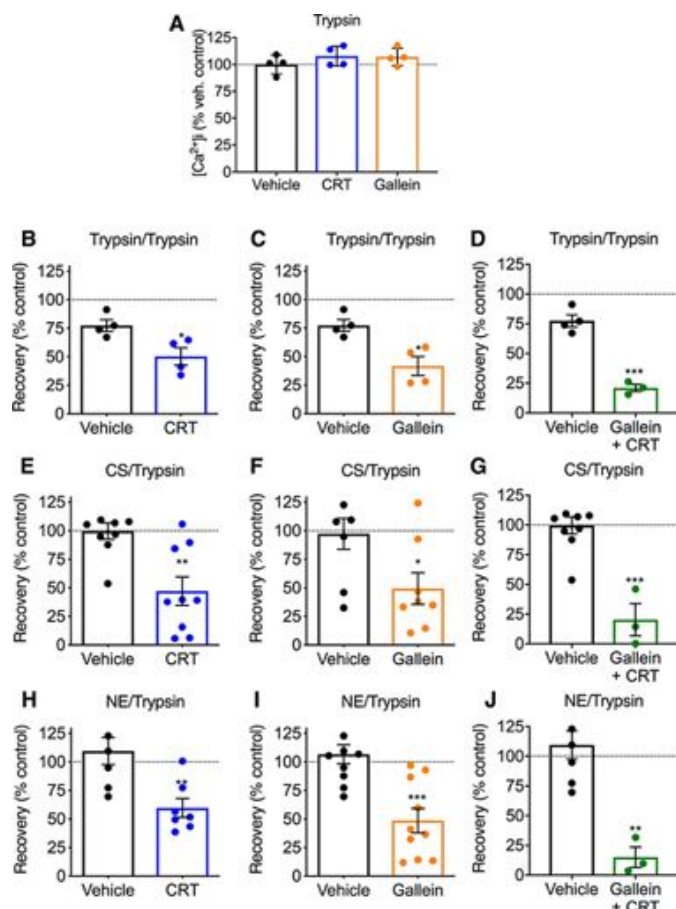


**Figure 8. Recovery of PAR<sub>2</sub>-mediated signaling.** A, effects of PKD inhibitor (CRT0066101 (CRT), 100 nM) or Gβγ inhibitor (gallein, 10 μM) on responses of HEK293 cells to a single challenge with trypsin. B–J, recovery of  $Ca^{2+}$  signaling in HEK293 cells. Cells were first challenged with trypsin (10 nM) (B–D), CS (100 nM) (E–G) or NE (H–J). Cells were then washed, recovered for 120 min, and then challenged with trypsin (10 nM). In some experiments, cells were pre-incubated with PKD inhibitor (CRT0066101) (B, E, and H) or Gβγ inhibitor (gallein) (C, F, and I) or both PKD and Gβγ inhibitor (D, G, and J). *n* = 3–10 experimental replicates from triplicate observations. \*, *p* < 0.05; \*\*, *p* < 0.01; \*\*\*, *p* < 0.001 (Student's *t* test). Error bars, S.E.

results reveal a role for PKD and Gβγ in sustained protease-mediated mechanical allodynia in mice.

## Discussion

We have recently investigated the mechanisms by which trypsin, CS, and NE initiate pain (18). After intraplantar injection in mice, trypsin, CS and NE cause mechanical allodynia by activating PAR<sub>2</sub> on Na<sub>v</sub>1.8-positive nociceptors. These proteases also evoke hyperexcitability of nociceptors, a characteristic of chronic pain, but by distinct mechanisms (18). Trypsin induces an initial hyperexcitability of nociceptors by a mechanism that entails PAR<sub>2</sub> signaling at the plasma and activation of PKC. PAR<sub>2</sub> then internalizes and signals from endosomes by β-arrestin- and Gα<sub>q</sub>-mediated mechanisms that activate extracellular signal-regulated kinase, which contributes to sustained hyperexcitability of nociceptors. In contrast, CS and NE evoke hyperexcitability by PAR<sub>2</sub> signaling at the plasma membrane, which activates PKA (14, 15, 18). Accordingly, whereas inhibitors of clathrin- and dynamin-mediated endocytosis block trypsin-evoked allodynia, they have no effect on nociceptive responses to CS and NE, which do not cause PAR<sub>2</sub> endocytosis (18). Regardless of the mechanism of PAR<sub>2</sub> activation and initial hyperexcitability, the maintenance of pain evoked by extracellular proteases requires mobilization of intracellular stores or *de novo* synthesis of PAR<sub>2</sub>. These processes are necessary because trypsin-activated PAR<sub>2</sub> traffics to lysosomes and is degraded. Even though CS or NE do not cause endocytosis of PAR<sub>2</sub>, once cleaved, this receptor cannot be reactivated by a protease (14, 15).

Our current results show that proteases that activate PAR<sub>2</sub> by biased mechanisms evoke Gβγ translocation to the Golgi apparatus of HEK293 cells. They also indicate that these proteases activate PKD within the Golgi apparatus. Gβγ and PKD promote mobilization of intact PAR<sub>2</sub> from the Golgi apparatus, which replenishes the plasma membrane with intact receptors that allow for a recovery of cellular responsiveness to extracellular proteases. These mechanisms also appear to operate in another cell line (KNRK) as well as in primary sensory neurons,

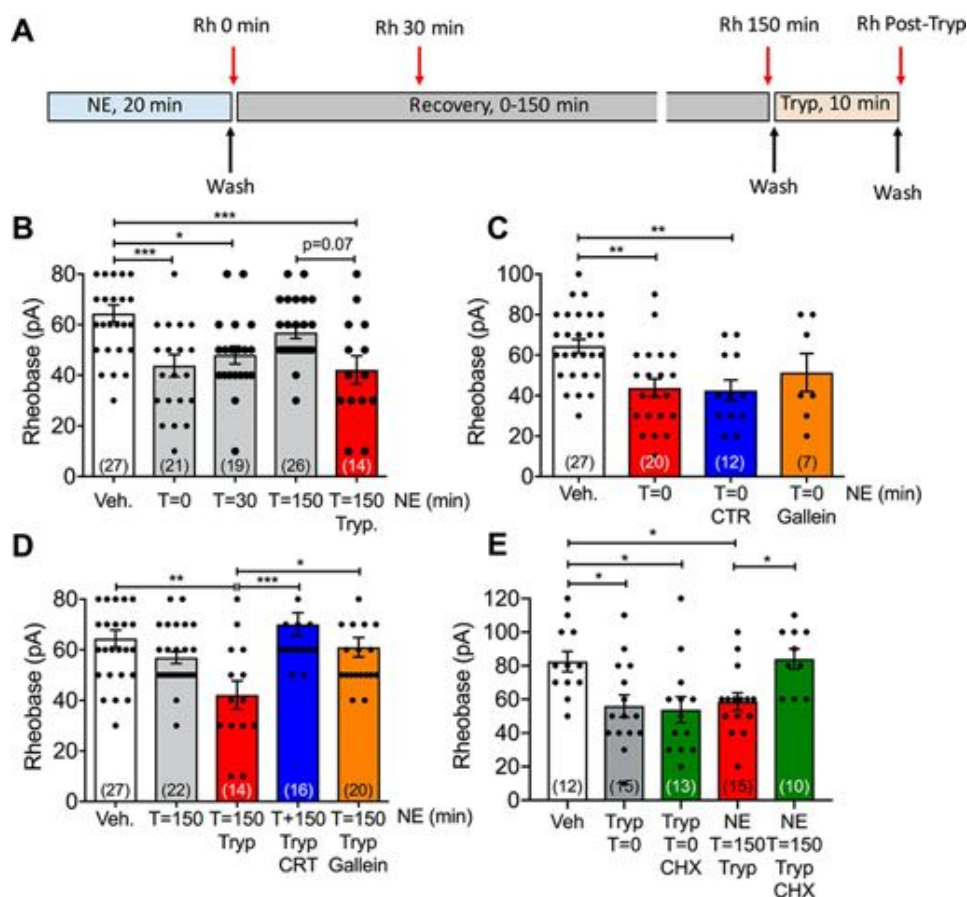


**Figure 9. Recovery of  $PAR_2$ -mediated hyperexcitability of nociceptors.** A, experimental design. DRG neurons from mice were exposed to NE (7.8 nM, 0.2 units/ml) for 20 min, washed, and recovered for up to 150 min. Changes in neuronal excitability were assessed at 0, 30, or 150 min after washing by measurement of rheobase. To assess recovery of excitability, some neurons were challenged with trypsin (Tryp, 50 nM, 10 min). At 150 min after NE exposure, neurons were washed, and rheobase was immediately measured. To examine the mechanism of recovery, neurons were pre-incubated with PKD inhibitor (CRT0066101 (CRT), 10 nM),  $G\beta\gamma$  inhibitor (gallein, 10  $\mu$ M), protein synthesis inhibitor (cycloheximide (CHX), 10  $\mu$ g/ml), or vehicle for 30 min before NE (inhibitors were included throughout). B–E, measurements of rheobase in protease-treated DRG neurons. B, time course of responses at time 0 min ( $T = 0$ ), 30 min ( $T = 30$ ), and 150 min ( $T = 150$ ) after NE and recovery of trypsin response at 150 min after NE. C, NE responses at time 0 min after NE. D, effects of CRT and gallein on responses to trypsin at 150 min after NE. E, effects of cycloheximide on initial response to trypsin (0 min) and on responses to trypsin at 150 min after NE. Numbers in parentheses indicate numbers of neurons studied from 6–13 mice. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  (one-way ANOVA, Bonferroni multiple comparisons). Error bars, S.E.

where inhibitors of  $G\beta\gamma$  and PKD prevented the recovery of trypsin-evoked hyperexcitability of NE-treated neurons. They may also function in intact mice, because  $G\beta\gamma$  and PKD inhibitors blunted trypsin-, CS-, and NE-evoked mechanical allodynia. These results agree with our report that  $G\beta\gamma$  and PKD mediate trypsin-evoked mobilization of  $PAR_2$  from the Golgi apparatus (19). They also support the role for  $G\beta\gamma$  in activating PKD within the Golgi apparatus (21), where PKD controls protein trafficking to secretory pathways (26, 27).

The use of FRET biosensors for PKD that are targeted to the cytosol or Golgi apparatus enabled analysis of PKD activity with high spatial and temporal fidelity and revealed that trypsin, CS, and NE can all activate PKD in the cytosol and Golgi apparatus. These responses are likely mediated by  $PAR_2$  because the  $PAR_2$ -selective agonist 2-furoyl-LIGRLO-NH<sub>2</sub> also activated PKD in the Golgi apparatus, and I-343, a  $PAR_2$  antagonist (18), abolished responses to trypsin, CS, and NE. Although I-343 can also inhibit  $PAR_1$ , which is expressed in HEK cells, I-343 had no effect on thrombin-activated PKD.

We observed that trypsin, CS, and NE all stimulated an increase in bystander BRET between  $G\gamma$ -Venus and Giantin-

RLuc8, which is consistent with translocation of  $G\gamma$  to the Golgi apparatus. Whereas the overexpression of  $G\alpha_q$  was required for trypsin-evoked translocation of  $G\gamma$ -Venus, overexpression of  $G\alpha_s$  was necessary for maximal CS- and NE-evoked translocation. These results are in accordance with the mechanisms by which these proteases activate  $PAR_2$ . Thus, the canonical agonist trypsin induces  $PAR_2$  coupling to  $G\alpha_q$ , and the biased agonists CS and NE evoke  $PAR_2$  coupling to  $G\alpha_s$  (14, 15).

Our studies of the contribution of PKD to mobilization of  $PAR_2$  from the Golgi apparatus and sustained signaling by extracellular proteases were not always consistent between different cell types and experimental approaches. In HEK293 cells, we found that trypsin, CS, and NE all activated PKD in the Golgi apparatus, assessed using a Golgi-targeted FRET biosensor. Trypsin, CS, and NE caused a decrease in bystander BRET between  $PAR_2$ -RLuc8 and TGN38-Venus, which is consistent with mobilization of  $PAR_2$  from the Golgi apparatus. By selective photoconversion of  $PAR_2$ -Kaede within the Golgi apparatus of KNRK cells, we were able to observe that CS and NE stimulated removal of  $PAR_2$  from the Golgi apparatus and insertion into the plasma membrane. Inhibitors of PKD and

## PKD and G $\beta\gamma$ sustain PAR<sub>2</sub> nociceptive signaling

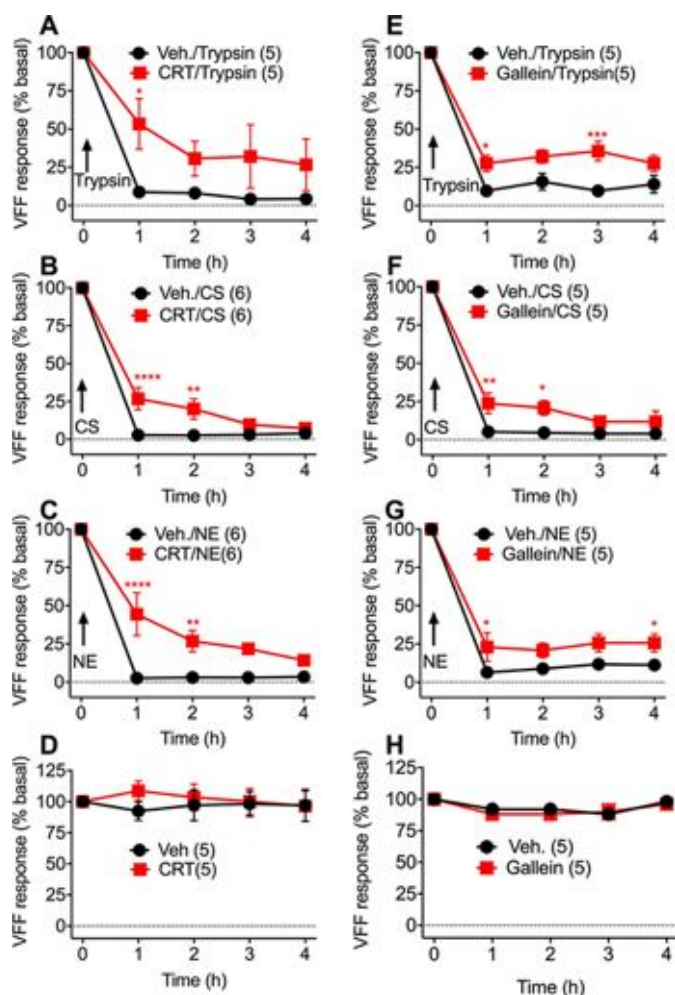


**Figure 10. PKD and G $\beta\gamma$ -dependent mechanical hyperalgesia.** Mice were pretreated with CRT0066101 (80 mg/kg, orally), gallein (100 mg/kg, intraperitoneally), or vehicle (orally or intraperitoneally) for 2 h (CRT0066101) or 30 min (gallein) before intraplantar injection of trypsin (140 nM) (A, E, D, and H), CS (2.5  $\mu$ M) (B and F), or NE (1.18  $\mu$ M) (C and G) (all 10  $\mu$ l). A–C and E–G, VFF withdrawal responses of the ipsilateral protease-injected paws. D and H, VFF withdrawal responses of the contralateral noninjected paws. Numbers in parentheses indicate mouse numbers. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$  (two-way ANOVA, Bonferroni multiple comparisons). Error bars, S.E.

G $\beta\gamma$  attenuated the mobilization of PAR<sub>2</sub>-Kaede from the Golgi apparatus of KNRK cells (confocal imaging). They also suppressed recovery of sustained PAR<sub>2</sub> signaling in HEK293 cells (Ca<sup>2+</sup> assays) and neurons (rheobase assays). Although a PKD inhibitor suppressed removal of PAR<sub>2</sub>-Kaede from the Golgi apparatus of KNRK cells, it did not affect the decrease in bystander BRET between PAR<sub>2</sub>-RLuc8 and TGN38-Venus in HEK293 cells. We have no explanation for this discrepancy. One possibility is that PKD promotes mobilization of PAR<sub>2</sub> from the Golgi apparatus of KNRK cells but not HEK293 cells. This discrepancy could also be related to differences in assay sensitivity, where an effect of the PKD inhibitor could be assessed in assays of PAR<sub>2</sub>-Kaede mobilization in individual KNRK cells but not in BRET assays of PAR<sub>2</sub>-RLuc8 and TGN38-Venus proximity in populations of HEK293 cells. Another explanation could be that TGN38 is not confined to the Golgi apparatus. Although at steady state TGN38 is principally localized to the Golgi apparatus, it constitutively shuttles

between the Golgi apparatus and plasma membrane by the exocytic and endocytic pathways (23). It is therefore possible that proximity between TGN38 and PAR<sub>2</sub> in these pathways may confound the results. It is also possible that multiple kinases, in addition to PKD, regulate PAR<sub>2</sub> trafficking from the Golgi apparatus or that PKD differentially controls trafficking of newly synthesized but not stored PAR<sub>2</sub>. Further experiments are required to examine these possibilities. However, the observation that G $\beta\gamma$  and PKD inhibitors disrupt the recovery of PAR<sub>2</sub> Ca<sup>2+</sup> signaling in HEK293 cells pretreated with trypsin, CS, or NE supports their role in maintaining the capacity of extracellular proteases to signal via PAR<sub>2</sub>.

It is increasingly appreciated that GPCRs can generate signals in subcellular compartments. Compartmentalization of signaling can regulate specific physiological processes, including pain transmission (18, 35, 36). GPCRs can also activate PKD in distinct subcellular compartments, often by different mechanisms. Thus, PAR<sub>2</sub> agonists induce PKC-dependent activation of PKD at the plasma membrane, where PKD may regulate the activity of transient receptor potential ion channels (37). Vasopressin, bombesin, and neurokinin 3 receptors rapidly activate PKD by a PKC-dependent mechanism (38–40). Canonical and biased agonists of PAR<sub>2</sub> also cause G $\beta\gamma$ -dependent activation of PKD in the Golgi apparatus, where PKD can control PAR<sub>2</sub> mobilization (19). G $\alpha_q$  subunits can also control PKD activity and regulate trafficking from the Golgi apparatus to the plasma membrane (29, 41).

Biased agonism describes the phenomenon whereby the binding of different ligands to the same receptor in an identical cellular background results in differential activation of signaling pathways (42). Proteases, which are activated during injury and inflammation, that cleave PAR<sub>2</sub> at different sites activate very distinct pathways of receptor signaling and trafficking (14, 15, 18). Our results reveal a common mechanism of recovery of PAR<sub>2</sub> signaling capacity at the plasma membrane, regardless of the mechanism of activation. Thus, after activation of PAR<sub>2</sub> by trypsin (PAR<sub>2</sub> coupling to G $\alpha_q$ , G $\alpha_s$ ,  $\beta$ -arrestins, PAR<sub>2</sub> endocytosis), CS, or NE (PAR<sub>2</sub> coupling to G $\alpha_s$ , retention at cell surface), G $\beta\gamma$ -mediated activation of PKD within the Golgi apparatus mediates the mobilization and plasma membrane trafficking of fresh PAR<sub>2</sub> that sustains signaling by extracellular proteases. Other GPCR family members that couple to different G $\alpha$  subunits also evoke trafficking of G $\beta\gamma$  to the Golgi apparatus. For example, agonists of muscarinic M2 receptors (which couple to G $\alpha_{i/o}$ ) and M3 receptors (which couple to G $\alpha_q$ ) both evoke Golgi translocation of G $\beta\gamma$  (43). This may be a general mechanism by which activation of GPCRs at the plasma membrane repopulates the cell surface with nondesensitized receptors.

Our results also provide information about the maintenance of pain. We observed that brief exposure of nociceptors to NE induced an initial period of hyperexcitability that gradually recovered to baseline levels, when neurons recovered their capacity to respond to trypsin. Inhibitors of PKD and G $\beta\gamma$  both impeded this recovery. An inhibitor of protein synthesis also impeded the recovery of trypsin-evoked hyperexcitability of NE-treated neurons. The systemic administration of PKD and G $\beta\gamma$  inhibitors to intact mice also blunted nociception evoked

by intraplantar administration of trypsin, CS, or NE. These results highlight the importance of GPCR trafficking for pain transmission. Endocytosis of GPCRs, including PAR<sub>2</sub>, is important for the initiation and maintenance of pain transmission (18, 35, 36). However, in the case of nonrecycling GPCRs, notably PAR<sub>2</sub>, synthesis of new receptors and mobilization and exocytosis of intact receptors is necessary for the maintenance of nociceptive signaling by extracellular ligands. These findings have implications for therapy. Inhibitors of clathrin- and dynamin-mediated endocytosis and lipid-conjugated antagonists that target GPCRs in endosomes can provide effective pain relief (18, 35, 36). Inhibitors of exocytosis, including Gβγ and PKD inhibitors, may also provide pain relief.

Limitations to the current study include the use of model HEK293 and KNRK cells, which may not replicate findings in nociceptive neurons and intact animals, possible confounding effects of tagging PAR<sub>2</sub> with luciferase or Kaede, and the use of pharmacological inhibitors that can lack selectivity (32, 33). However, we observed that inhibitors of different mediators of Golgi-to-plasma membrane trafficking (PKD and Gβγ) gave consistent results in experiments with model cell lines, primary sensory neurons in short-term culture, and intact mice. Moreover, fusion to Kaede does not affect the function of PAR<sub>2</sub> (19).

In summary, this study provides evidence that proteases that activate PAR<sub>2</sub> by canonical and biased mechanisms stimulate PKD in the Golgi; PAR<sub>2</sub> mobilization and *de novo* synthesis repopulate the cell surface with intact receptors and sustain nociceptive signaling by extracellular proteases. Antagonism of these pathways may offer another approach to treat chronic pain.

## Experimental procedures

### Animals

Animal Ethics Committees of Monash University and Queen's University approved the procedures using mice. C57BL/6 mice (8–12 weeks, male) were studied. Mice were maintained in a light-controlled (12-h light/dark cycle) temperature-controlled (22 ± 4 °C) environment with free access to food and water.

### Cell culture

HEK293 cells and KNRK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and containing 1% penicillin and streptomycin (5% CO<sub>2</sub>, 37 °C). HEK293 and KNRK cells expressing human PAR<sub>2</sub> with N-terminal FLAG and C-terminal HA11 epitopes (FLAG-PAR<sub>2</sub>-HA) have been described previously (19).

### FRET PKD and PKC activation assays

HEK293 cells were plated at a density of 4 × 10<sup>6</sup> cells/10-cm dish. Cells were transiently transfected with the hPAR<sub>2</sub> (1 μg) plus FRET PKD biosensors Cyto-DKAR, Golgi-DKAR, or DKAR-T/A (2.5 or 5 μg) or PKC sensor Cyto-CKAR (5 μg) using polyethyleneimine (20). At 24 h after transfection, cells were seeded on poly-D-lysine-coated OptiPlate-96 96-well plates (PerkinElmer Life Sciences). FRET was assessed 72 h post-transfection, following overnight serum restriction. Cells

were equilibrated with HBSS, 10 mM HEPES, pH 7.4, for 30 min. Cyan fluorescent protein/yellow fluorescent protein FRET was measured using a PHERAstar plate reader (BMG LabTech) with excitation wavelength 425 ± 5 nm, and emission wavelength 550 ± 25 nm/490 ± 10 nm (18, 35, 36). Cells were challenged with trypsin (10 nM), CS (100 nM), or NE (100 nM), and measurements continued for 20 min at 1-min intervals. In some experiments, cells were pre-incubated with the PAR<sub>2</sub>-antagonist I-343 (10 μM) for 30 min, and antagonist was present throughout the experiments. For kinetic measurements, data were corrected to baseline and vehicle controls. The area under the curve (AUC, 20 min) was determined to quantify the effects of inhibitors.

### BRET Gγ translocation assays

HEK293 cells were plated at a density of 4 × 10<sup>6</sup> cells/10-cm dish. Cells were transiently transfected with hPAR<sub>2</sub> (0.9 μg), Gα<sub>q</sub> (1.33 μg) or Gα<sub>s</sub> (1.33 μg), Gβ1 (1.33 μg), Gγ2-Venus (2 μg), and Giantin-RLuc8 (0.5 μg) using polyethyleneimine. At 24 h after transfection, cells were seeded on poly-D-lysine-coated OptiPlate-96 96-well plates (PerkinElmer Life Sciences), and cultured overnight. Cells were equilibrated with HBSS, 10 mM HEPES, pH 7.4, for 30 min before the assay. Coelenterazine h (5 μM, Nanolight Technologies) was added to the cells for 5 min before measurements. RLuc8 luminescence (480 nm) and Venus fluorescence (530 nm) were measured using a LumiSTAR Omega Luminometer (BMG LabTech) before and after exposure to proteases: trypsin, 10<sup>-13</sup> to 10<sup>-7</sup> M; NE, 10<sup>-12</sup> to 10<sup>-7</sup> M; CS, 10<sup>-12</sup> to 10<sup>-7</sup> M; or vehicle (control) (18, 35, 36). Data were corrected to baseline and vehicle-treated conditions to generate the ligand-induced signal, which was normalized to the maximum trypsin response.

### BRET PAR<sub>2</sub> Golgi trafficking assays

HEK293 cells were transiently transfected with hPAR<sub>2</sub>-RLuc8 (1 μg) and TGN38-Venus (4 μg). BRET was measured as described above.

### [Ca<sup>2+</sup>]<sub>i</sub> assays

HEK293 cells were plated (30,000 cells/well) in poly-D-lysine-coated ViewPlate-96 96-well plates (PerkinElmer Life Sciences). Cells were loaded with Fura-2/AM (1 μM; Invitrogen) for 1 h at 37 °C in assay buffer (150 mM NaCl, 2.6 mM KCl, 0.1 mM CaCl<sub>2</sub>, 1.18 mM MgCl<sub>2</sub>, 10 mM D-glucose, 10 mM HEPES, pH 7.4) containing 4 mM probenecid and 0.5% BSA. Fluorescence was measured at 340- and 380-nm excitation and 530-nm emission using a FlexStation III Microplate Reader (Molecular Devices) (19). The 340- and 380-nm ratio was measured as an indication of [Ca<sup>2+</sup>]<sub>i</sub>. To measure loss and recovery of PAR<sub>2</sub>-mediated changes in [Ca<sup>2+</sup>]<sub>i</sub>, cells were first incubated with trypsin (10 nM), CS (100 nM), NE (100 nM), or vehicle for 10 min, washed three times with assay buffer, recovered at 37 °C for 110 min, and then rechallenged with trypsin (10 nM) at 120 min after the first challenge. For quantification of the recovery of Ca<sup>2+</sup> signaling, the maximal responses to the second trypsin challenge were normalized to the vehicle-pretreated control.

## Localization of FRET and BRET biosensors

HEK293 cells were transfected as stated with  $PAR_2$ -HA-RLuc8, Cyto-DKAR, Golgi-DKAR, Giantin-Venus, TGN38-Venus, or  $G\gamma$ -Venus (2.5  $\mu$ g). Cells were plated on 12-mm glass coverslips, and 48 h after transfection, cells were fixed in 4% paraformaldehyde (20 min, 4 °C). Cells were washed with PBS and incubated with blocking buffer (PBS + 0.3% saponin + 3% horse serum) (1 h, room temperature).  $PAR_2$ -HA-RLuc8 was detected using immunofluorescence with HA antibody; other sensors were detected with fluorescent tags. Cells were incubated with rat anti-HA (1:1000; Roche Applied Science, clone 3F10), and mouse anti-TGN58K (1:200; Abcam) in PBS + 0.2% saponin + 1% horse serum (1 h, room temperature). Cells were washed three times in PBS and incubated with goat anti-rat Alexa 488 and donkey anti-mouse Alexa 568 IgG (1:1000; Invitrogen) (1 h, room temperature). Slides were washed three times in PBS, counterstained with 4',6-diamidino-2-phenylindole (1  $\mu$ g/ml), and mounted with ProLong Glass antifade mounting medium (Invitrogen). Cells were imaged on a Leica SP8 confocal microscope with a  $\times 63$  (numerical aperture 1.4) objective with a digital zoom of 2.5. Images were processed with ImageJ (National Institutes of Health).

## Live-cell imaging and quantification of $PAR_2$ -Kaede trafficking

KNRK- $PAR_2$ -Kaede cells were plated onto glass 8-well chamber slides at a density of 100,000 cells/well and cultured overnight. Cells were equilibrated in HBSS, 10 mM HEPES, pH 7.4, at 37 °C for 30 min before the assay. Live-cell images were collected using a Leica TCS SP8 laser-scanning confocal microscope with a Leica HCX PL APO  $\times 63$  oil immersion objective.

The Leica FRAP wizard was used to green/red photoconvert  $PAR_2$ -Kaede within the perinuclear region of interest with the laser settings of pre-photoconversion image at 400 Hz, UV laser at 10% power, and two passes at 400 Hz, as we have described previously (19). Images were collected for 5 min, cells were challenged with CS (100 nM) or NE (100 nM), and images were collected at 5-min intervals for another 30 min. FIJI software (National Institutes of Health) was used to determine the intensity of  $PAR_2$ -Kaede signal at the plasma membrane, in the perinuclear region, and throughout the cell. To quantify the depletion of  $PAR_2$  from the plasma membrane, the intensities of Kaede green at the plasma membrane and throughout the entire cell were determined and expressed as a ratio. To quantify the depletion of  $PAR_2$  from the Golgi apparatus and repletion of the plasma membrane with mobilized  $PAR_2$ , the intensities of Kaede red were determined at the plasma membrane, in the perinuclear region of the cell, and throughout the cell and were expressed as ratios.

## Inhibitors

Cells were pre-incubated with CRT0066101 (100 nM; Tocris Bioscience), gallein (10  $\mu$ M; Tocris Bioscience), Gö6983 (1  $\mu$ M; Sigma-Aldrich), H-89 (1  $\mu$ M; Sigma-Aldrich), GRK2i (100  $\mu$ M; Tocris), or vehicle (control) for 30 min, and the inhibitors were present throughout the experiments.

## Patch-clamp studies of nociceptors

To obtain dispersed neurons, mouse DRG (T9–T13) were incubated in collagenase (1 mg/ml; Worthington) and dispase (4 mg/ml; Roche Applied Science) (10 min, 37 °C) and triturated with a fire-polished Pasteur pipette. Dispersed cells were seeded onto laminin-coated (0.017 mg/ml) and poly-D-lysine-coated (2 mg/ml) glass coverslips in 24-well plates. Neurons were maintained in F12 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum and containing penicillin and streptomycin (5% CO<sub>2</sub>, 37 °C) for 16 h. Neurons were pre-incubated for 20 min with NE (7.8 nM, 0.2 units/ml) or vehicle (control) and washed three times with F12 medium. Changes in neuronal excitability were assayed immediately (0 min) or at 30 and 150 min after NE application. After 150 min, some cells were challenged with trypsin (50 nM, 10 min) and washed. To evaluate the role of PKD,  $G\beta\gamma$ , and new protein synthesis on NE-evoked neural excitability, some cells were pre-incubated with CRT0066101 (10 nM), gallein (10  $\mu$ M), cycloheximide (10  $\mu$ g/ml), or vehicle (control) 30 min before NE application, and inhibitors were included throughout the study. The excitability of small-diameter neurons (<30-picoFarad capacitance) was assessed using perforated patch-clamp with amphotericin B (240  $\mu$ g/ml) in current clamp mode at room temperature, as we have described (18, 19). Only those neurons with resting membrane potentials more negative than –40 mV were analyzed. Changes in excitability were quantified by measuring the rheobase. Recordings were made using Axopatch 200B amplifiers, digitized by Digidata 1322A, and stored and processed using pClamp 10.1 software (Molecular Devices). The recording chamber was continuously perfused (2 ml/min) with external solution comprising 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM D-glucose, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, pH to 7.4 with 3 M NaOH. The pipette solution was as follows: 110 mM potassium gluconate, 30 mM KCl, 10 mM HEPES, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, pH 7.25, with 1 M KOH.

## Mechanical hyperalgesia

Mechanical hyperalgesia was assessed by measuring paw withdrawal responses to stimulation of the plantar surface of the mouse paw with calibrated VFF (14, 15, 18). CRT0066101 (Tocris Bioscience; 80 mg/kg) or vehicle (control, 0.9% NaCl) was administered by gavage (200  $\mu$ l) 2 h before intraplantar injections of proteases. Gallein (100 mg/kg) or vehicle (control, 0.5% DMSO in 0.9% NaCl) was administered intraperitoneally (200  $\mu$ l) 30 min before intraplantar injection of proteases. For intraplantar injections, mice were sedated with 5% isoflurane. Trypsin (140 nM, 0.04 units/ $\mu$ l), CS (2.5  $\mu$ M, 0.06 units/ $\mu$ l), NE (1.18  $\mu$ M, 0.03 units/ $\mu$ l), or vehicle was injected subcutaneously into the plantar surface of the left hind paw (10  $\mu$ l). Withdrawal responses to stimulation of the plantar surface of the ipsilateral (injected) and contralateral (noninjected) hind paws with calibrated VFF were measured hourly for 4 h. Investigators were blinded to the experimental treatments, and mice were randomly assigned to treatment groups.

## Statistics

Data are presented as mean  $\pm$  S.E. For studies of cells, triplicate observations from  $n > 3$  experiments were made for each

treatment group. For experiments with isolated neurons,  $n = 7$ –27 DRG neurons from 6–13 mice were studied per treatment. For studies of nociceptive behavior,  $n = 5$ –6 mice were studied per treatment. Student's  $t$  tests were used to assess differences between two groups. Differences between multiple groups were assessed by one-way (FRET, BRET, electrophysiology) or two-way (mouse behavior) ANOVAs followed by Bonferroni's correction for multiple comparisons.  $p < 0.05$  was considered significant at the 95% confidence level.

**Author contributions**—P. Z., D. P. P., S. J. V., B. L. S., and N. W. B. conceptualization; P. Z., L. A. P., D. D. J., N. N. J.-V., R. L., T. L., J. O. J., and C. L.-L. data curation; P. Z., L. A. P., D. D. J., N. N. J.-V., R. L., and T. L. formal analysis; P. Z., D. P. P., S. J. V., B. L. S., and N. W. B. supervision; P. Z., L. A. P., D. D. J., N. N. J.-V., R. L., T. L., S. J. V., B. L. S., and N. W. B. investigation; P. Z., D. D. J., N. N. J.-V., and R. L. visualization; P. Z., L. A. P., D. D. J., N. N. J.-V., R. L., T. L., and N. W. B. methodology; P. Z., L. A. P., D. D. J., R. L., and N. W. B. writing—original draft; D. D. J., N. N. J.-V., R. L., and T. L. validation; D. D. J., D. P. P., S. J. V., B. L. S., and N. W. B. project administration; D. P. P., S. J. V., B. L. S., and N. W. B. funding acquisition; D. P. P., S. J. V., B. L. S., and N. W. B. writing—review and editing; S. J. V., B. L. S., and N. W. B. resources.

## References

- Dong, C., Filipeanu, C. M., Duvernay, M. T., and Wu, G. (2007) Regulation of G protein-coupled receptor export trafficking. *Biochim. Biophys. Acta* **1768**, 853–870 [CrossRef Medline](#)
- Smith, J. S., and Rajagopal, S. (2016) The  $\beta$ -arrestins: multifunctional regulators of G protein-coupled receptors. *J. Biol. Chem.* **291**, 8969–8977 [CrossRef Medline](#)
- Hauser, A. S., Attwood, M. M., Rask-Andersen, M., Schiöth, H. B., and Gloriam, D. E. (2017) Trends in GPCR drug discovery: new agents, targets and indications. *Nat. Rev. Drug Discov.* **16**, 829–842 [CrossRef Medline](#)
- Kang, D. S., Tian, X., and Benovic, J. L. (2014) Role of  $\beta$ -arrestins and arrestin domain-containing proteins in G protein-coupled receptor trafficking. *Curr. Opin. Cell Biol.* **27**, 63–71 [CrossRef Medline](#)
- Grady, E. F., Garland, A. M., Gamp, P. D., Lovett, M., Payan, D. G., and Bunnett, N. W. (1995) Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol. Biol. Cell* **6**, 509–524 [CrossRef Medline](#)
- von Zastrow, M., Svingos, A., Haberstock-Debic, H., and Evans, C. (2003) Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. *Curr. Opin. Neurobiol.* **13**, 348–353 [CrossRef Medline](#)
- Marchese, A., and Benovic, J. L. (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J. Biol. Chem.* **276**, 45509–45512 [CrossRef Medline](#)
- Ossovskaya, V. S., and Bunnett, N. W. (2004) Protease-activated receptors: contribution to physiology and disease. *Physiol. Rev.* **84**, 579–621 [CrossRef Medline](#)
- Bohm, S. K., Kong, W., Bromme, D., Smeekens, S. P., Anderson, D. C., Connolly, A., Kahn, M., Nelken, N. A., Coughlin, S. R., Payan, D. G., and Bunnett, N. W. (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem. J.* **314**, 1009–1016 [CrossRef Medline](#)
- Cottrell, G. S., Amadesi, S., Grady, E. F., and Bunnett, N. W. (2004) Trypsin IV, a novel agonist of protease-activated receptors 2 and 4. *J. Biol. Chem.* **279**, 13532–13539 [CrossRef Medline](#)
- Corvera, C. U., Déry, O., McConalogue, K., Böhm, S. K., Khitin, L. M., Caughey, G. H., Payan, D. G., and Bunnett, N. W. (1997) Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J. Clin. Invest.* **100**, 1383–1393 [CrossRef Medline](#)
- Oikonomopoulou, K., Hansen, K. K., Saifeddine, M., Tea, I., Blaber, M., Blaber, S. I., Scarisbrick, L., Andrade-Gordon, P., Cottrell, G. S., Bunnett, N. W., Diamandis, E. P., and Hollenberg, M. D. (2006) Proteinase-activated receptors, targets for kallikrein signaling. *J. Biol. Chem.* **281**, 32095–32112 [CrossRef Medline](#)
- Ramachandran, R., Mihara, K., Chung, H., Renaux, B., Lau, C. S., Muruve, D. A., DeFea, K. A., Bouvier, M., and Hollenberg, M. D. (2011) Neutrophil elastase acts as a biased agonist for proteinase-activated receptor-2 (PAR2). *J. Biol. Chem.* **286**, 24638–24648 [CrossRef Medline](#)
- Zhao, P., Lieu, T., Barlow, N., Sostegni, S., Haerteis, S., Korbmacher, C., Liedtke, W., Jimenez-Vargas, N. N., Vanner, S. J., and Bunnett, N. W. (2015) 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 [CrossRef Medline](#)
- Zhao, P., Lieu, T., Barlow, N., Metcalf, M., Veldhuis, N. A., Jensen, D. D., Kocan, M., Sostegni, S., Haerteis, S., Baraznenok, V., Henderson, I., Lindström, E., Guerrero-Alba, R., Valdez-Morales, E. E., Liedtke, W., et al. (2014) Cathepsin S causes inflammatory pain via biased agonism of PAR2 and TRPV4. *J. Biol. Chem.* **289**, 27215–27234 [CrossRef Medline](#)
- Böhm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G., and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J. Biol. Chem.* **271**, 22003–22016 [CrossRef Medline](#)
- Déry, O., Thoma, M. S., Wong, H., Grady, E. F., and Bunnett, N. W. (1999) Trafficking of proteinase-activated receptor-2 and  $\beta$ -arrestin-1 tagged with green fluorescent protein:  $\beta$ -arrestin-dependent endocytosis of a proteinase receptor. *J. Biol. Chem.* **274**, 18524–18535 [CrossRef Medline](#)
- Jimenez-Vargas, N. N., Pattison, L. A., Zhao, P., Lieu, T., Latorre, R., Jensen, D. D., Castro, J., Aurelio, L., Le, G. T., Flynn, B., Herenbrink, C. K., Yeaman, H. R., Edgington-Mitchell, L., Porter, C. J. H., Halls, M. L., et al. (2018) Protease-activated receptor-2 in endosomes signals persistent pain of irritable bowel syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7438–E7447 [CrossRef Medline](#)
- Jensen, D. D., Zhao, P., Jimenez-Vargas, N. N., Lieu, T., Gerges, M., Yeaman, H. R., Canals, M., Vanner, S. J., Poole, D. P., and Bunnett, N. W. (2016) Protein kinase D and Gβγ subunits mediate agonist-evoked translocation of protease-activated receptor-2 from the Golgi apparatus to the plasma membrane. *J. Biol. Chem.* **291**, 11285–11299 [CrossRef Medline](#)
- Kunkel, M. T., Toker, A., Tsien, R. Y., and Newton, A. C. (2007) Calcium-dependent regulation of protein kinase D revealed by a genetically encoded kinase activity reporter. *J. Biol. Chem.* **282**, 6733–6742 [CrossRef Medline](#)
- Jamora, C., Yamanouye, N., Van Lint, J., Laudenslager, J., Vandenheede, J. R., Faulkner, D. J., and Malhotra, V. (1999) Gβγ-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell* **98**, 59–68 [CrossRef Medline](#)
- Linstedt, A. D., and Hauri, H. P. (1993) Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679–693 [CrossRef Medline](#)
- Banting, G., and Ponnambalam, S. (1997) TGN38 and its orthologues: roles in post-TGN vesicle formation and maintenance of TGN morphology. *Biochim. Biophys. Acta* **1355**, 209–217 [CrossRef Medline](#)
- Bonacci, T. M., Mathews, J. L., Yuan, C., Lehmann, D. M., Malik, S., Wu, D., Font, J. L., Bidlack, J. M., and Smrcka, A. V. (2006) Differential targeting of Gβγ-subunit signaling with small molecules. *Science* **312**, 443–446 [CrossRef Medline](#)
- Koch, W. J., Hawes, B. E., Inglese, J., Luttrell, L. M., and Lefkowitz, R. J. (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates Gβγ-mediated signaling. *J. Biol. Chem.* **269**, 6193–6197 [Medline](#)
- Liljedahl, M., Maeda, Y., Colanzi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell* **104**, 409–420 [CrossRef Medline](#)
- Yeaman, C., Ayala, M. I., Wright, J. R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W. J., and Malhotra, V. (2004) Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network. *Nat. Cell Biol.* **6**, 106–112 [CrossRef Medline](#)

28. Harikumar, K. B., Kunnumakkara, A. B., Ochi, N., Tong, Z., Deorukhkar, A., Sung, B., Kelland, L., Jamieson, S., Sutherland, R., Raynham, T., Charles, M., Bagherazadeh, A., Foxton, C., Boakes, A., Farooq, M., *et al.* (2010) A novel small-molecule inhibitor of protein kinase D blocks pancreatic cancer growth *in vitro* and *in vivo*. *Mol. Cancer Ther.* **9**, 1136–1146 [CrossRef Medline](#)
29. Coria, A. S., Masseroni, M. L., and Díaz Añel, A. M. (2014) Regulation of PKD1-mediated Golgi to cell surface trafficking by Gα<sub>q</sub> subunits. *Biol. Cell* **106**, 30–43 [CrossRef Medline](#)
30. Gschwendt, M., Dieterich, S., Rennecke, J., Kittstein, W., Mueller, H.-J., and Johannes, F.-J. (1996) Inhibition of protein kinase C μ by various inhibitors. Inhibition from protein kinase C isoenzymes. *FEBS Lett.* **392**, 77–80 [CrossRef Medline](#)
31. Muñoz, M., Martín, M. E., Hidalgo, J., and Velasco, A. (1997) Protein kinase A activity is required for the budding of constitutive transport vesicles from the trans-Golgi network. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14461–14466 [CrossRef Medline](#)
32. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alessi, D. R., and Cohen, P. (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem. J.* **408**, 297–315 [CrossRef Medline](#)
33. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105 [CrossRef Medline](#)
34. DeFea, K. A., Zalevsky, J., Thoma, M. S., Déry, O., Mullins, R. D., and Bunnett, N. W. (2000) β-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J. Cell Biol.* **148**, 1267–1281 [CrossRef Medline](#)
35. Jensen, D. D., Lieu, T., Halls, M. L., Veldhuis, N. A., Imlach, W. L., Mai, Q. N., Poole, D. P., Quach, T., Aurelio, L., Conner, J., Herenbrink, C. K., Barlow, N., Simpson, J. S., Scanlon, M. J., Graham, B., *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**, eaa13447 [CrossRef Medline](#)
36. Yarwood, R. E., Imlach, W. L., Lieu, T., Veldhuis, N. A., Jensen, D. D., Klein Herenbrink, C., Aurelio, L., Cai, Z., Christie, M. J., Poole, D. P., Porter, C. J. H., McLean, P., Hicks, G. A., Geppetti, P., Halls, M. L., *et al.* (2017) Endosomal signaling of the receptor for calcitonin gene-related peptide mediates pain transmission. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 12309–12314 [CrossRef Medline](#)
37. Amadesi, S., Grant, A. D., Cottrell, G. S., Vaksman, N., Poole, D. P., Rozengurt, E., and Bunnett, N. W. (2009) Protein kinase D isoforms are expressed in rat and mouse primary sensory neurons and are activated by agonists of protease-activated receptor 2. *J. Comp. Neurol.* **516**, 141–156 [CrossRef Medline](#)
38. Jacamo, R., Sinnott-Smith, J., Rey, O., Waldron, R. T., and Rozengurt, E. (2008) Sequential protein kinase C (PKC)-dependent and PKC-independent protein kinase D catalytic activation via G<sub>q</sub>-coupled receptors: differential regulation of activation loop Ser<sup>744</sup> and Ser<sup>748</sup> phosphorylation. *J. Biol. Chem.* **283**, 12877–12887 [CrossRef Medline](#)
39. Poole, D. P., Amadesi, S., Rozengurt, E., Thacker, M., Bunnett, N. W., and Furness, J. B. (2008) Stimulation of the neurokinin 3 receptor activates protein kinase C epsilon and protein kinase D in enteric neurons. *Am. J. Physiol. Gastrointest. Liver Physiol.* **294**, G1245–G1256 [CrossRef Medline](#)
40. Sinnott-Smith, J., Jacamo, R., Kui, R., Wang, Y. M., Young, S. H., Rey, O., Waldron, R. T., and Rozengurt, E. (2009) Protein kinase D mediates mitogenic signaling by G<sub>q</sub>-coupled receptors through protein kinase C-independent regulation of activation loop Ser<sup>744</sup> and Ser<sup>748</sup> phosphorylation. *J. Biol. Chem.* **284**, 13434–13445 [CrossRef Medline](#)
41. Waldron, R. T., Innamorati, G., Torres-Marquez, M. E., Sinnott-Smith, J., and Rozengurt, E. (2012) Differential PKC-dependent and -independent PKD activation by G protein α subunits of the G<sub>q</sub> family: selective stimulation of PKD Ser<sup>748</sup> autophosphorylation by Gα<sub>q</sub>. *Cell. Signal.* **24**, 914–921 [CrossRef Medline](#)
42. Kenakin, T. (2011) Functional selectivity and biased receptor signaling. *J. Pharmacol. Exp. Ther.* **336**, 296–302 [CrossRef Medline](#)
43. Akgoz, M., Kalyanaraman, V., and Gautam, N. (2004) Receptor-mediated reversible translocation of the G protein βγ complex from the plasma membrane to the Golgi complex. *J. Biol. Chem.* **279**, 51541–51544 [CrossRef Medline](#)

**Protein kinase D and G $\beta\gamma$  mediate sustained nociceptive signaling by biased agonists of protease-activated receptor-2**

Peishen Zhao, Luke A. Pattison, Dane D. Jensen, Nestor N. Jimenez-Vargas, Rocco Latorre, TinaMarie Lieu, Josue O. Jaramillo, Cintya Lopez-Lopez, Daniel P. Poole, Stephen J. Vanner, Brian L. Schmidt and Nigel W. Bunnett

*J. Biol. Chem.* 2019, 294:10649-10662.

doi: 10.1074/jbc.RA118.006935 originally published online May 29, 2019

---

Access the most updated version of this article at doi: [10.1074/jbc.RA118.006935](https://doi.org/10.1074/jbc.RA118.006935)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 43 references, 28 of which can be accessed free at <http://www.jbc.org/content/294/27/10649.full.html#ref-list-1>