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Endothelin-converting enzyme-1 regulates trafficking and signalling of the neurokinin 1 receptor in endosomes of myenteric neurones

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Non-technical summary The movement of receptors to and from the surface of nerve cells determines whether nerves can detect and respond to stimulants in the extracellular fluid. It is well established that stimulated receptors move from the surface of nerves to intracellular compartments, but the processes that control movement of internalized receptors back to the surface are poorly understood. We show that an intracellular enzyme, endothelin-converting enzyme-1, controls the translocation of receptors back to the surface of neurones of the intestinal tract that control the normal process of digestion. This process is likely to regulate digestion, and abnormalities in the mechanism could cause digestive diseases.

Abstract Neuropeptide signalling at the plasma membrane is terminated by neuropeptide degradation by cell-surface peptidases, and by β-arrestin-dependent receptor desensitization and endocytosis. However, receptors continue to signal from endosomes by β-arrestin-dependent processes, and endosomal sorting mediates recycling and resensitization of plasma membrane signalling. The mechanisms that control signalling and trafficking of receptors in endosomes are poorly defined. We report a major role for endothelin-converting enzyme-1 (ECE-1) in controlling substance P (SP) and the neurokinin 1 receptor (NK_1R) in endosomes of myenteric neurones. ECE-1 mRNA and protein were expressed by myenteric neurones of rat and mouse intestine. SP (10 nM, 10 min) induced interaction of NK_1R and β-arrestin at the plasma membrane, and the SP–NK_1R–β-arrestin signalosome complex trafficked by a dynamin-mediated mechanism to ECE-1-containing early endosomes, where ECE-1 can degrade SP. After 120 min, NK_1R recycled from endosomes to the plasma membrane. ECE-1 inhibitors (SM-19712, PD-069185) and the vacuolar H^+ATPase inhibitor bafilomycin A_1, which prevent endosomal SP degradation, suppressed NK_1R recycling by >50%. Preincubation of neurones with SP (10 nM, 5 min) desensitized Ca^{2+} transients to a second SP challenge after 10 min, and SP signals resensitized after 60 min. SM-19712 inhibited NK_1R resensitization by >90%. ECE-1 inhibitors also caused sustained SP-induced activation of extracellular signal-regulated kinases, consistent with stabilization of the SP–NK_1R–β-arrestin signalosome. By degrading SP and destabilizing endosomal signalosomes, ECE-1 has a dual role in controlling endocytic signalling and trafficking of the NK_1R: promoting resensitization of G protein-mediated plasma membrane signalling, and terminating β-arrestin-mediated endosomal signalling.

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Abbreviations 7TMD, 7 transmembrane domain; DMEM, Dulbecco's modified Eagle's medium; ECE-1, endothelin-converting enzyme-1; EEA1, early endosomal antigen 1; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution; IR, immunoreactivity; MAPK, mitogen-activated protein kinase; ME-MP, muscularis externa myenteric plexus; NK_1R, neurokinin 1 receptor; NHS, normal horse serum; PDBu, phorbol 12,13-dibutyrate; PLA, proximity ligation assay; PSS, physiological salt solution; SP, substance P.

Introduction

Peptidergic neurotransmission requires the presence of receptors at the plasma membrane, where they can interact with neuropeptides in the extracellular fluid. The mechanisms that regulate signalling and trafficking of seven transmembrane domain (7TMD) neuropeptide receptors at the plasma membrane have been thoroughly investigated (reviewed in Gainetdinov *et al.* 2004). Mechanisms that terminate plasma membrane signalling include neuropeptide degradation by cell-surface peptidases such as neprilysin, which inactivates substance P (SP) and bradykinin and attenuates their proinflammatory actions (Okamoto *et al.* 1994; Lu *et al.* 1997; Sturiale *et al.* 1999), and receptor phosphorylation by G protein-coupled receptor kinases. Phosphorylation increases the affinity of 7TMD receptors for β-arrestins, which translocate from the cytosol to interact with agonist-occupied receptors at the plasma membrane. β-Arrestins uncouple receptors from heterotrimeric G proteins and thereby desensitize G protein-dependent signalling. β-Arrestins also couple receptors to the clathrin-mediated endocytic machinery, which depletes receptors from the cell surface and consequently diminishes cellular responsiveness to extracellular agonists. In addition, β-arrestins are adaptor molecules that interact with diverse signalling proteins. By recruiting receptors and signalling partners such as mitogen-activated protein kinases (MAPKs) to multi-protein signalling complexes (signalosomes), β-arrestins can transduce a second wave of G protein-*independent* signalling from the endosomal network (Murphy *et al.* 2009). Thus, β-arrestins have a dual role in regulating 7TMD receptors, terminating G protein-mediated signalling from plasma membranes, and initiating β-arrestin-mediated signalling from endosomal membranes. However, β-arrestin-mediated endosomal signals are distinct from G protein-mediated plasma membrane signals, in terms of mechanism, subcellular location, duration and outcome.

Compared to these mechanisms that regulate neuropeptide receptor signalling and trafficking at the plasma membrane, little is known about the processes that control neuropeptide receptor functions in endosomes. In particular, the processes that terminate β-arrestin-mediated signalling of 7TMD receptors in endosomes of neurones are unexplored, but are likely to involve destabilization of signalosomes, which induces dissociation of the neuropeptide–receptor–β-arrestin complex. This dissociation allows the receptor, freed from β-arrestins, to recycle, which is necessary for resensitization of G protein-mediated signalling at the plasma membrane (Oakley *et al.* 1999).

We recently identified a proteolytic mechanism for controlling signalling and trafficking of neuropeptide receptors in endosomes that is analogous to well-defined mechanisms that control receptors at the plasma membrane (Padilla *et al.* 2007; Roosterman *et al.* 2007; Cottrell *et al.* 2009). Endothelin-converting enzyme-1 (ECE-1) is a neprilysin-related metallo-endopeptidase that is present at the plasma membrane and in endosomes. In the acidic endosomal environment, ECE-1 degrades neuropeptides and thereby destabilizes peptide–receptor–β-arrestin–MAPK signalosomes, allowing receptors to recycle and re-engage G protein-dependent signalling at the plasma membrane, and terminating β-arrestin-mediated MAPK signalling from endosomes. This mechanism regulates neuropeptides that are ECE-1 substrates at acidic endosomal pH and controls receptors that form stable interactions with β-arrestins in endosomes, including SP and the neurokinin 1 receptor (NK_1R), and calcitonin gene-related peptide and the calcitonin receptor-like receptor. However, this mechanism has been studied thus far only in cell lines. Nothing is known about the role of ECE-1 in controlling endosomal signalling and trafficking of neuropeptide receptors in neurones.

We examined whether ECE-1 regulates endosomal signalling and trafficking of the NK_1R in myenteric neurones of the intestine. The NK_1R is expressed by several classes of myenteric neurones and mediates ascending contraction of the peristaltic reflex (Sternini *et al.* 1995; Grider, 2003). Although the contractile effects of SP in the intestine desensitize and resensitize (Gaddum, 1953), and SP-induces endocytosis and recycling of the NK_1R in myenteric neurones (Grady *et al.* 1996*b*; McConalogue *et al.* 1998), the mechanisms underlying these regulatory processes are not defined. We report that ECE-1, which is widely expressed in enteric neurones, promotes recycling and resensitization of plasma membrane G protein signalling, and terminates endosomal β-arrestin-mediated signalling of the NK_1R.

Methods

Ethical approval

The Institutional Animal Care and Use Committee of the University of California, San Francisco approved all procedures on experimental animals.

Animals

C57/BL6 mice (6–8 weeks, 20–25 g, male and female) and Sprague–Dawley rats (6 weeks, 200–250 g, male) were from Charles River Laboratories (Hollister, CA, USA). Animals were maintained under temperature- ($22 \pm 4^{\circ}$C) and light- (12 h light–dark cycle) controlled conditions with free access to food and water. Animals were anaesthetized with sodium pentobarbital (200 mg kg^{-1} I.P.) and killed by bilateral thoracotomy before tissue collection.

Reagents

SP was from Bachem (Torrance, CA, USA). The ECE-1 inhibitor PD-069185 (Ahn *et al.* 1998) was a gift from Pfizer Pharmaceuticals (Pfizer, UK). The NK_1R antagonist RP-67580 (Garret *et al.* 1992) was from Tocris Bioscience (Ellisville, MO, USA). Alexa Fluor 594 protein labelling kit, Fura 2-AM and Prolong Gold containing DAPI were from Invitrogen (Carlsbad, CA, USA). SP was labelled with Alexa Fluor 594 (Alexa-SP) and purified as described (Bunnett *et al.* 1995). Duolink Proximity Ligation Assay (PLA) was from Olink Biosciences (Uppsala, Sweden). Other reagents were from Sigma-Aldrich (St Louis, MO, USA).

Antibodies

Rabbit anti-NK_1R (94168) antibody has been described (Grady *et al.* 1996*a*). Mouse anti-β-arrestin1/2 (610551) and mouse anti-early endosomal antigen 1 (EEA1, 610457) antibodies were from BD Biosciences (San Jose, CA, USA). Goat anti-human ECE-1 (AF1784) antibody was from R&D Systems, Inc. (Minneapolis, MN, USA). Rabbit antibody to the N terminus of human ECE-1 that recognizes ECE-1b/d isoforms (Padilla *et al.* 2007) was from Invitrogen. Rabbit anti-ERK1/2 (9102) and mouse anti-phospho (p)-ERK1/2 (9106) antibodies were from Cell Signalling Technology (Danvers, MA, USA). Chicken anti-PGP9.5 (72910) was from Abcam (Cambridge, UK). Donkey anti-chicken, -rabbit, -mouse and -goat IgG conjugated to rhodamine red X, fluorescein isothiocyanate or cyanine 5 were from Jackson Immunoresearch (West Grove, PA, USA).

ECE-1 and NK_1R RT-PCR

The muscularis externa and myenteric plexus (ME-MP) of mouse ileum was obtained by sharp dissection. Total RNA was extracted, treated with DNase I and reverse transcribed to amplify ECE-1 isoforms and NK_1R. The following primers were used to detect mouse ECE-1 isoforms: ECE-1a forward: 5′-CCCTGGTCTCATGGTCTCGCT-3′; ECE-1b forward: 5′-GGTGCAGCATGCGGACCGTGT-3′; ECE-1c1 forward: 5′-GAGCCTTAGCGGGAGGTGCAT-3′; ECE-1c2 forward: 5′-GCAGCCTGCCCACCAGGGTAA-3′; ECE-1d forward: 5′-GCAATGGAGACGCTGAGGGAGT-3′; ECE-1 reverse: 5′-CGTAGCTGAAGAAGTCCTGGCA-3′. The following primers were used to detect the mouse NK_1R: forward: 5′-GTGCAACCTACCTGGCAAAT-3′; reverse: 5′-CTACTTCCTGCCTCTGCTGGT-3′. Products were separated by electrophoresis and sequenced.

ECE-1 Western blotting

Membranes were prepared from ME-MP of the mouse ileum and from HEK cells as described previously (Cottrell *et al.* 2009). Proteins (15 μg) were separated by SDS-PAGE and ECE-1 was detected by Western blotting (Cottrell *et al.* 2009).

NK_1R trafficking in organotypic preparations of myenteric plexus

Terminal ileum or distal colon from rats and mice were placed in ice-cold Hank's balanced salt solution (HBSS) containing nicardipine (1 μM) and tetrodotoxin (100 nM). Segments were opened, pinned mucosa-side down onto silicone elastomer-lined dishes, and incubated in physiological salt solution (PSS, mM: NaCl 118; KCl 4.8; $NaHCO_3$ 25; NaH_2PO_4 1.0; $MgSO_4$ 1.2; D-glucose 11.1; $CaCl_2$ 2.5). Tissues were incubated with SP (10 nM, 10 min, 37°C) or vehicle (H_2O), washed and recovered in SP-free PSS for 0–120 min at 37°C. To examine endocytosis of fluorescent SP, tissues were incubated with Alexa-SP (100 nM, 60 min, 4°C), washed and recovered in SP-free PSS for 0–120 min at 37°C. To induce release of endogenous SP from nerve fibres, preparations were depolarized with KCl (50 mM, 5 min, 37°C), washed and recovered in PSS for 0–60 min, all in the presence of peptidase inhibitors (thiorphan (100 nM), phosphoramidon, captopril and bestatin (all 10 μM)) (Grady *et al.* 1996*b*). Preparations were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4, overnight, 4°C), and whole-mounts were prepared of the longitudinal muscle and myenteric plexus (Poole *et al.* 2002).

NK_1R trafficking in cultured myenteric neurones

Myenteric neurones were isolated and cultured from the small intestine of rats and mice as described

previously (Grady *et al.* 1996*b*; McConalogue *et al.* 1998). Intestinal segments were placed in ice-cold HBSS containing nicardipine (1 μM) and antibiotic/antimycotic solution (penicillin G, 100 U ml^{-1}; streptomycin sulphate, 10 mg ml^{-1}; amphotericin B, 25 mg ml^{-1}). Segments were opened, pinned flat and the ME-MP was dissected. Tissue was digested with collagenase type IA (1 mg ml^{-1}) and DNase IV (0.1 mg ml^{-1}, 150 kU) in Dulbecco's modified Eagle's medium (DMEM) (1 h, 37°C) with agitation. Tissue was then digested with trypsin (0.25% solution with EDTA) (15 min, 37°C). Digestion was stopped by adding 20% fetal bovine serum (FBS) in DMEM containing soybean trypsin inhibitor (10 mg ml^{-1}) followed by centrifugation. A single cell suspension was achieved by sequential trituration. Neurones were plated onto poly-L-lysine/laminin-coated glass coverslips, and were cultured in DMEM supplemented with normal horse serum (NHS) (5%), FBS (5%), L-glutamine (2 mM), antibiotic/antimycotic and N1 neuronal supplement (1:100). Cytosine arabinoside (1 μM) was included in the culture media from day 2 onwards to inhibit proliferation of non-neuronal cells. Neurones were studied after 5–7 days. Cultured neurones were incubated in DMEM (0.1% BSA without phenol red, 5% CO_2–95% O_2) and were stimulated with SP (10 nM, 10 min, 37°C) or vehicle (H_2O). Cultures were washed and recovered in SP-free DMEM for 0–120 min. Neurones were fixed in 4% paraformaldehyde (20 min, 4°C) and processed for immunofluorescence or for examination of NK_1R and β-arrestin1/2 or total ERK1/2 and pERK1/2 interactions by proximity ligation assay (PLA).

Immunofluorescence

Whole-mounts and cultures were incubated in blocking buffer (10% NHS, 0.1% Triton X-100, 0.1 M PBS) for 30 min at room temperature, and then incubated with the following primary antibodies in blocking buffer: NK_1R (1:500), β-arrestin1/2 (1:100), ECE-1 (1:100), EEA1 (1:200) and PGP9.5 (1:100) (all 48 h, 4°C). Slides were washed and incubated with secondary antibodies in PBS (1:200, 2 h, room temperature). Slides were mounted in Prolong and examined by confocal microscopy.

Proximity ligation assay

Interactions between NK_1R and β-arrestin1/2 or total ERK1/2 and pERK1/2 were examined using *in situ* PLA (Soderberg *et al.* 2008) with anti-mouse PLA probe plus, anti-rabbit PLA probe minus and detection kit 563, according to the manufacturer's instructions. Neurones were permeabilized with 0.1% Triton X-100 in PBS for 4 min, blocked with 1% normal goat serum in PBS (blocking buffer) for 1 h at room temperature, and incubated with pairs of primary antibodies: NK_1R (1:500) plus β-arrestin1/2 (1:100), or total ERK1/2 (1:100) plus pERK1/2 (1:200) (overnight, 4°C). Neurones were washed in PBS and then incubated with PLA probes in a humidified chamber for 2 h at 37°C. Neurones were incubated with hybridization mixture for 15 min, washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), and incubated with ligation mixture for 15 min. Neurones were washed with TBS-T, incubated with amplification mixture for 90 min, washed with TBS-T and incubated with detection probes for 60 min. Finally, neurones were washed with 2× saline-sodium citrate buffer (SSC), 1× SSC, 0.2× SSC, 0.02× SSC and 70% ethanol. Coverslips were air-dried and mounted in Prolong. Images of PLA signals were collected using the 543 nm laser and five optical sections were taken (63× objective) at intervals of 0.32 μm. Collection settings were optimized for stimulated preparations and all subsequent images were taken using these settings. The number of PLA signals per neurone ('blobs') was counted using the BlobFinder software using the cell-average analysis mode (Allalou & Wahlby, 2009) (http://www.cb.uu.se/~amin/BlobFinder/).

Confocal microscopy

Preparations were observed using a Zeiss Axiovert microscope with a Zeiss 510 Meta confocal system, with Plan Apochromat ×100 (NA 1.4), Fluar Plan Apochromat ×63 (NA 1.4) and ×40 Plan-Neofluar (NA 1.3) objectives. The subcellular distribution of NK_1R was analysed from captured images using Image J (http://rsbweb.nih.gov/ij/) as described previously (Poole *et al.* 2007). Single optical sections of the neuronal soma, including the nucleus, were analysed. The threshold of individual images was defined as the fluorescence pixel intensity of the nucleus, which takes advantage of the absence of nuclear NK_1R-IR. Positive staining (i.e. NK_1R-IR above background) was set to 255 and negative staining was set to 0. A region of interest was manually drawn around the soma of NK_1R-IR neurones and the total positive pixels of the soma were measured. A second region of interest was drawn immediately beneath the plasma membrane (readily detected by autofluorescence) and the intracellular NK_1R-IR positive pixels were measured. Any positive nuclear staining (e.g. non-specific high-intensity spots) was subtracted from both values. The membrane and intracellular NK_1R pixels were then expressed as the relative percentages of the total cellular pixels (100%).

Ratiometric Ca^{2+} imaging of cultured myenteric neurones

$[Ca^{2+}]_i$ was measured in cultured myenteric neurones by ratiometric imaging of Fura 2-AM as described

previously (McConalogue *et al.* 1998). Cultured neurones were incubated in HBSS (containing 0.1% BSA and 20 mM Hepes, pH 7.4) and loaded with Fura 2-AM (5 μM, 45 min, 37°C). Coverslips were washed and mounted on a temperature-controlled open chamber at 37°C. Fluorescence of individual neurones was measured at 340 nm and 380 nm excitation, and 510 nm emission. Test substances were directly added to the chamber (50 μl injection). To assess desensitization and resensitization of the NK_1R, neurones were stimulated with SP (10 nM, 5 min), washed, recovered in SP-free medium for 10, 30 or 60 min, and then re-challenged with SP (10 nM). Responses of the same neurones to both SP challenges were examined. Finally, neurones were stimulated with carbachol (1 μM) and KCl (50 mM). Neurone identity was confirmed based on size, morphology and responsiveness to KCl. The magnitude of the change in emission ratio at 340/380 nm excitation, which is proportional to $[Ca^{2+}]_i$, and the proportion of the total number of KCl-responsive neurones that responded to SP challenge were determined. The extent of resensitization was calculated as the percentage of all KCl-responsive neurones that responded to the second SP challenge compared to the first SP challenge (normalized to 100%).

Drug treatments

Organotypic whole-mounts or cultured neurones were preincubated with the following drugs: ECE-1 inhibitors SM-19712 (Matsumura *et al.* 2000) (10 μM) or PD-069185 (Ahn *et al.* 1998) (30 μM) (60 min); H^+ATPase inhibitor bafilomycin A_1 (1 μM, 30 min); NK_1R antagonist RP-67580 (Garret *et al.* 1992) (100 nM, 5 min); dynamin GTPase inhibitor Dynasore (Macia *et al.* 2006) (80 μM, 10 min); or vehicle (control). Inhibitors were included throughout the experiments. As a positive control for the ERK1/2.pERK1/2 PLA, neurones were stimulated with phorbol 12,13-dibutyrate (PDBu, 100 nM, 10 min).

Statistical analysis

Data are presented as mean ± SEM of $n \geq 5$ experiments or animals. Results were compared by Student's t test (2 comparisons) or by ANOVA and Student–Newman–Keuls test (multiple comparisons). $P < 0.05$ was considered to be significantly different at the 95% confidence level.

Results

ECE-1 is expressed in myenteric neurones

We examined ECE-1 expression in ME-MP by RT-PCR and Western blotting. Transcripts corresponding to ECE-1a (293 bp), ECE-1b (390 bp), ECE-1c1 (387 bp), ECE-1c2 (473 bp) and NK_1R (557 bp), but not ECE-1d, were amplified from ME-MP of mouse ileum and identified by sequencing (Fig. 1*A*). ECE-1a, ECE-1b, ECE-1c and ECE-1d mRNA have been similarly identified in ME-MP of the rat ileum (Cottrell *et al.* 2009). ECE-1 immunoreactivity (IR) was detected by Western blotting of mouse ileum ME-MP with an apparent molecular mass of ~118 kDa, similar to that detected in HEK cells, which naturally express ECE-1 (Padilla *et al.* 2007) (Fig. 1*B*). Smaller bands are likely to represent ECE-1 degradation products, although this possibility remains to be investigated. We localized ECE-1-IR in myenteric plexus whole-mounts of rat ileum by immunofluorescence using an antibody to ECE-1b and d isoforms (Roosterman *et al.* 2007). This antibody was unsuitable for detection of ECE-1 in mice. ECE-1-IR was localized to endosomes but

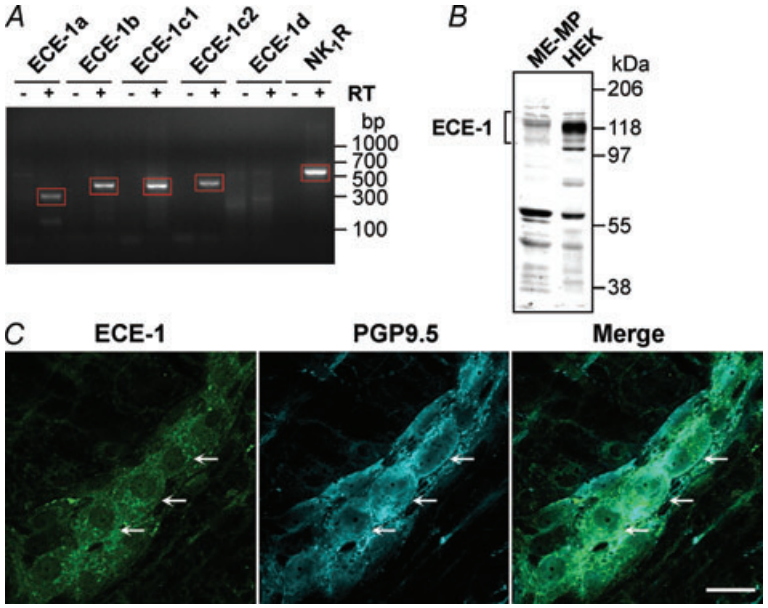

Figure 1. Expression and localization of ECE-1
A, RT-PCR amplification of mRNA from isolated ME-MP of the mouse ileum encoding ECE-1a (293 bp), ECE-1b (390 bp), ECE-1c1 (387 bp), ECE-1c2 (473 bp) and NK_1R (557 bp). ECE-1d was not detected. RT, reverse transcriptase. *B*, Western blotting for ECE-1 in ME-MP of mouse ileum and in HEK cells (which naturally express ECE-1). *C*, co-localization of ECE-1-IR and PGP9.5-IR in whole-mount of rat myenteric plexus, showing vesicular localization of ECE-1-IR in neurones. Arrows denote ECE-1-expressing neurones. Scale, 20 μm.

not to the plasma membrane of myenteric neurones that were identified by PGP9.5-IR (Fig. 1*C*), consistent with the predominant endosomal localization of ECE-1b/d isoforms (Padilla *et al.* 2007; Roosterman *et al.* 2007). ECE-1 was also localized to interstitial cells of Cajal, but was not detected in the longitudinal or circular muscle cells (not shown). Pre-adsorption of the antibody with ECE-1 abolished staining, as we have previously reported (Cattaruzza *et al.* 2009; Cottrell *et al.* 2009), indicating specific detection of endosomal ECE-1 in enteric neurones.

SP induces NK_1R and β-arrestin interactions at the plasma membrane and in endosomes

Despite the established importance of β-arrestins in regulating signalling and trafficking of GPCRs (Gainetdinov *et al.* 2004), including the NK_1R expressed in cell lines (McConalogue *et al.* 1999; DeFea *et al.* 2000), little is known about their ability to regulate endogenous receptors in neurones. We examined SP-induced trafficking of NK_1R and β-arrestins in organotypic preparations of myenteric plexus from rat ileum, since β-arrestin antibodies were raised in mice and were unsuitable for use in that species. In unstimulated myenteric neurones, NK_1R-IR was at the plasma membrane and β-arrestin-IR was cytosolic (Fig. 2*A*). SP (10 nM, 10 min, wash, 10 min recovery, 37°C) induced the redistribution of the NK_1R-IR and β-arrestin-IR to the same endosomes in close proximity to the plasma membrane (Fig. 2*A*). To examine SP-induced interactions between NK_1R and β-arrestins, we used an *in situ* PLA, which localizes protein–protein interactions at the cellular level (Soderberg *et al.* 2008). If two proteins are in close proximity (<40 nm), short DNA strands attached to secondary antibodies are ligated, and the DNA can be amplified by rolling circle amplification and detected using fluorescent oligonucleotides. NK_1R–β-arrestin interactions were examined in rat myenteric neurones in culture, since high levels of non-specific PLA signals were detected in whole-mounts. SP (10 nM) induced a >4-fold increase in the number of NK_1R–β-arrestin interactions within 10 min, and interactions persisted for at least 30 min (Fig. 2*B* and *C*). Omission of either of the primary antibodies prevented PLA signals, indicating specificity. These results indicate that SP stimulates NK_1R–β-arrestin interactions in endosomes of myenteric neurones.

Dynamin mediates SP-induced endocytosis of the NK_1R in myenteric neurones

SP stimulates NK_1R endocytosis in neurones by unknown mechanisms (Mantyh *et al.* 1995; Grady *et al.* 1996*b*; McConalogue *et al.* 1998). We used Dynasore, a selective

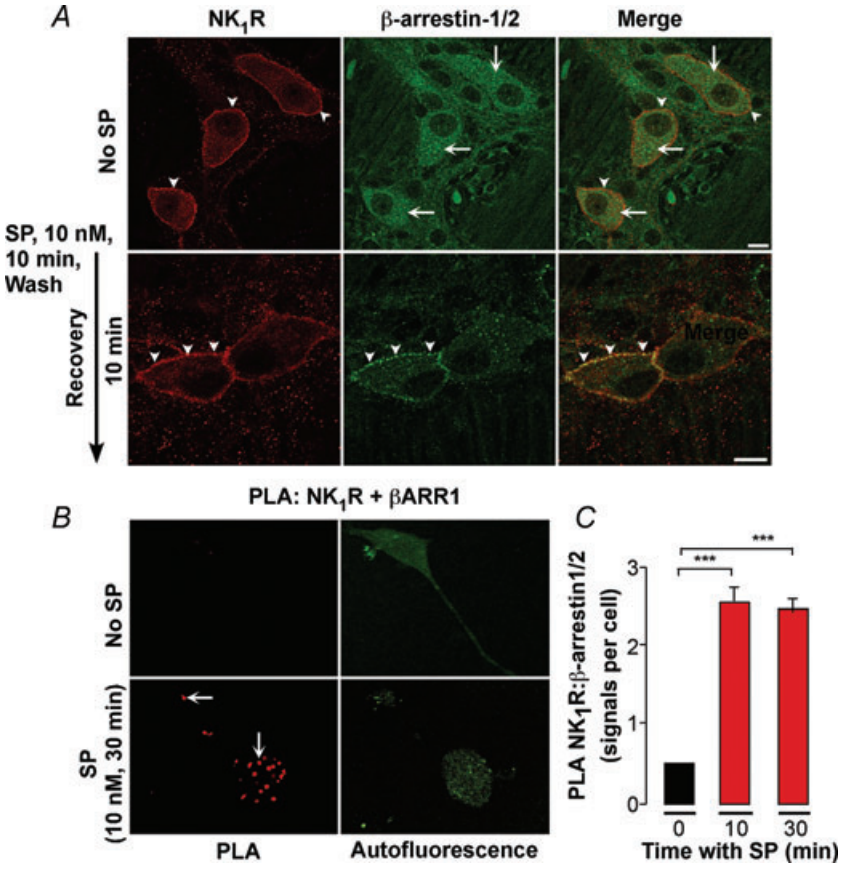

Figure 2. SP-induced trafficking and interaction of NK_1R and β-arrestins in myenteric neurones
A, organotypic preparations of rat ileum were unstimulated or incubated with SP (10 nM, 10 min), washed and recovered for 10 min. NK_1R and β-arrestin1/2 were detected in whole-mount preparations by immunofluorescence. In unstimulated neurones, NK_1R-IR was at the plasma membrane (arrowheads) and β-arrestin-IR was cytosolic (arrows). SP induced translocation of NK_1R-IR and β-arrestin-IR to the same endosomes (arrowheads). Scale, 10 μm. *B* and *C*, PLA for NK_1R and β-arrestin in rat myenteric neurones in culture. PLA signals are in red, and green is autofluorescence showing the localization of the neurones. In the absence of SP, PLA signals were minimal. SP (10 nM, 10 or 30 min) induced PLA signals (arrows) that denote NK_1R/β-arrestin interactions. ***$P < 0.001$. $n > 3$ experiments (i.e. neuronal cultures from 1 rat per preparation).

inhibitor of dynamin GTPase (Macia *et al.* 2006), to determine the role of dynamin in SP-induced endocytosis of the NK_1R in myenteric neurones. Organotypic preparations of mouse colon were incubated with Dynasore or vehicle (control), and were stimulated with SP (10 nM, 10 min, 37°C), washed and recovered for 30 min. The NK_1R was localized in whole-mounts of myenteric plexus by immunofluorescence. Dynasore alone did not alter the subcellular localization of NK_1R-IR, which remained at the plasma membrane of the soma and neurites of myenteric neurones (Fig. 3*A*). SP stimulated NK_1R endocytosis in the soma and in neurites, and Dynasore abolished endocytosis. Quantitative analysis of total cellular NK_1R-IR pixels indicated that $13.3 \pm 3.8\%$ were in endosomes of unstimulated neurones, $37.2 \pm 2.1\%$ were in endosomes of SP-stimulated neurones, and $15.1 \pm 1.1\%$ were in endosomes of Dynasore-treated SP-stimulated neurones (Fig. 3*B*; $P < 0.001$, Dynasore compared to vehicle). Thus, dynamin mediates SP-induced endocytosis of the NK_1R in myenteric neurones.

ECE-1 mediates SP-induced recycling of the NK_1R in myenteric neurones

When heterologously expressed, all ECE-1 isoforms localize to early endosomes, although ECE-1a and ECE-1c are also present at the plasma membrane (Padilla *et al.* 2007; Roosterman *et al.* 2007). SP and the NK_1R traffic to early endosomes containing ECE-1 in cells that heterologously express NK_1R and ECE-1 isoforms. To determine whether SP traffics with the NK_1R to ECE-1-containing endosomes of myenteric neurones, organotypic preparations of the rat ileum were incubated with Alexa-SP (100 nM, 60 min, 4°C), washed and recovered in SP-free medium for 10 or 30 min at 37°C. NK_1R, ECE-1 and EEA-1 were localized in whole-mounts of myenteric plexus by immunofluorescence. At 4°C, Alexa-SP was confined to the plasma membrane (not shown). After incubation at 37°C, Alexa-SP was detected in vesicles throughout the soma of myenteric neurones that also contained EEA1-IR (Fig. 4*A*), NK_1R-IR and ECE-1-IR (Fig. 4*B*).

To examine the post-endocytic sorting of NK_1R and ECE-1 in myenteric neurones, organotypic preparations of rat ileum were incubated with SP (10 nM, 10 min, 37°C), washed and recovered in SP-free medium for 30–120 min. NK_1R and ECE-1 were localized in whole-mounts of myenteric plexus by immunofluorescence. In unstimulated neurones, NK_1R-IR was confined to the plasma membrane of the soma and neurites, and ECE-1-IR was detected in endosomes (Fig. 5). At 30 min recovery, NK_1R-IR and ECE-1-IR colocalized in endosomes, and after 60 and 120 min recovery, NK_1R-IR was recycled to the plasma membrane. Quantitative analysis indicated that $77.7 \pm 1.8\%$ of total cellular NK_1R-IR pixels were at the plasma membrane of untreated neurones, $49.8 \pm 1.4\%$ were at the plasma membrane after 30 min recovery, and $75.9 \pm 1.4\%$ were at the plasma membrane after 120 min recovery (Fig. 6*A*; $P < 0.005$, 30 min recovery compared to unstimulated neurones). Thus, SP and the NK_1R initially traffic to early endosomes of myenteric neurones that also contain ECE-1. The NK_1R then recycles to the plasma membrane and ECE-1 remains in endosomes.

By degrading SP in acidified endosomes of model cell lines, ECE-1 destabilizes the SP–NK_1R–β-arrestin complex and promotes NK_1R recycling (Roosterman *et al.* 2007; Cottrell *et al.* 2009). To evaluate whether ECE-1 similarly controls post-endocytic trafficking of the NK_1R in myenteric neurones, organotypic preparations of rat

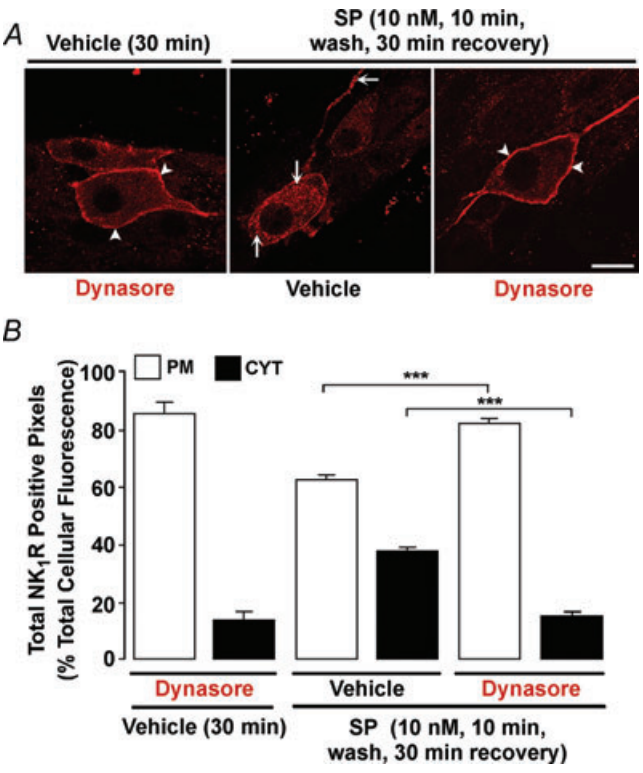

Figure 3. Dynamin-mediated NK_1R endocytosis in myenteric neurones
Organotypic preparations of mouse ileum were incubated with Dynasore or vehicle, and were unstimulated or stimulated with SP (10 nM, 10 min, wash, 30 min recovery). NK_1R was localized in whole-mounts of myenteric plexus by immunofluorescence (*A*), and the pixel intensity at the plasma membrane (PM) and in the cytosol (CYT) was determined (*B*). Dynasore alone for 30 min did not affect the distribution of NK_1R-IR, which remained at the plasma membrane (arrowheads). In vehicle-treated neurones, SP induced NK_1R-IR translocation to endosomes (arrows). Dynasore abolished SP-induced endocytosis of the NK_1R-IR, causing receptor retention at the plasma membrane (arrowheads). Scale, 10 μm. $^{***}P < 0.001$. 12–91 neurones per group, $n = 3$ mice.

ileum were treated with the ECE-1 selective inhibitors SM-19712 (Umekawa *et al.* 2000) or PD-069185 (Ahn *et al.* 1998). These are highly selective ECE-1 inhibitors that do not suppress the activity of other proteases that degrade SP, such as neprilysin. Inhibition of ECE-1 did not affect SP-induced endocytosis of NK_1R-IR, which translocated from the plasma membrane to early endosomes that also contained ECE-1-IR (Fig. 6*A* and *B*). However, in marked contrast to neurones with active ECE-1, in which NK_1R recycled after 60 and 120 min recovery (Fig. 5, Fig. 6*A*), in neurones treated with either ECE-1 inhibitor, NK_1R-IR was retained in endosomes containing ECE-1 at both time points (Fig. 7*A* and *B*). Quantitative analysis of NK_1R-IR localization at the plasma membrane and endosomes confirmed these observations. In vehicle-treated control preparations, the proportion of NK_1R-IR pixels that were detected in endosomes was $21.7 \pm 1.8\%$ before SP treatment, $49.0 \pm 1.4\%$ after 30 min recovery post-SP, and $23.3 \pm 1.4\%$ at 120 min (Fig. 6*A*; $P < 0.005$, 30 min recovery compared to unstimulated neurones), indicative of NK_1R endocytosis and recycling. In SM-19712-treated neurones, these proportions were $25.6 \pm 1.4\%$ before treatment, $47.2 \pm 1.3\%$ after 30 min recovery, and $48.3 \pm 1.4\%$ at 120 min recovery (Fig. 6*B*; $P < 0.005$, 120 min recovery, SM-19712 compared to vehicle), and in PD-069185-treated neurones, the proportions were $19.4 \pm 2.0\%$ before treatment, $50.8 \pm 2.1\%$ after 30 min recovery post-SP, and $53.0 \pm 1.9\%$ at 120 min (Fig. 6*C*; $P < 0.005$, 120 min recovery, PD-069185 compared to vehicle). Thus, SM-19712 caused a 2-fold (48.2/23.3%) and PD-069185 caused a 2.3-fold (52.9/23.3%) increase in levels of NK_1R-IR in endosomes at 120 min, when compared to controls.

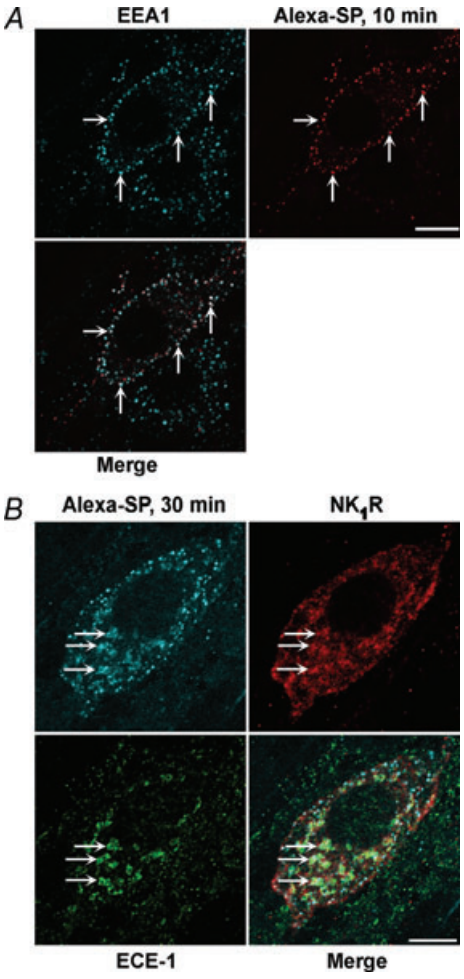

Figure 4. Translocation of SP to early endosomes containing ECE-1 in myenteric neurones
Organotypic preparations of rat ileum were incubated with Alexa-SP (100 nM, 60 min, 4°C), washed and recovered in SP-free medium for 10 min (*A*) or 30 min (*B*) at 37°C. Alexa-SP was simultaneously localized in whole-mounts of myenteric plexus with EEA1 (*A*) and with NK_1R and ECE-1 (*B*), which were detected by immunofluorescence. Arrows show colocalization of Alexa-SP with EEA1-IR, NK_1R-IR and ECE-1-IR. Scale, 10 μm.

We similarly determined whether active ECE-1 is required for SP-induced endocytosis and recycling of the NK_1R in myenteric neurones of the mouse colon. Organotypic preparations from mouse colon were incubated with SP (10 nM, 10 min, 37°C), washed and recovered in SP-free medium for 30–120 min. In neurones with active ECE-1, SP induced trafficking of NK_1R-IR to endosomes after 30 min recovery, and by 60 min NK_1R-IR was completely recycled (Fig. 8*A* and *B*). SM-19712 did not affect NK_1R endocytosis, but caused retention of NK_1R-IR in endosomes at 60 min recovery (Fig. 8*A* and *C*). The proportion of NK_1R-IR pixels in endosomes at 60 min recovery was $14.5 \pm 1.2\%$ in vehicle-treated neurones and $63.6 \pm 2.7\%$ in SM-19712-treated neurones ($P < 0.005$, 60 min recovery, SM-19712 compared to vehicle). Thus, SM-19712 caused a 4.4-fold (63.3/14.47%) increase in levels of NK_1R in endosomes at 60 min, when compared to controls. Bafilomycin A_1, an inhibitor of vacuolar H^+ATPase, prevents endosomal acidification and thereby suppresses ECE-1-mediated degradation of SP in acidified endosomes (Roosterman *et al.* 2007). Bafilomycin A_1 also caused marked retention of NK_1R-IR in endosomes at 60 min recovery (Fig. 8*A*).

To evaluate whether ECE-1 regulates NK_1R recycling after stimulation of neurones with physiologically relevant concentrations of SP, organotypic preparations of mouse colon were incubated with KCl (50 mM, 5 min, 37°C), which stimulates the release of endogenous SP (Grady *et al.* 1996*b*), or were unstimulated. Preparations were then washed, recovered in SP-free medium, and the NK_1R was localized in whole-mounts of myenteric plexus. In unstimulated neurones, NK_1R-IR was confined to the plasma membrane ($89.8 \pm 1.1\%$ of total NK_1R-IR pixels) (Fig. 9*A* and *B*). KCl induced trafficking of NK_1R to endosomes at 30 min ($37.2 \pm 2.1\%$ of total NK_1R-IR pixels) and depletion from the plasma membrane. Preincubation of tissue with the NK_1R antagonist

RP-67580 abolished KCl-induced endocytosis of the NK_1R (Fig. 9*A*), which is thus attributable to release of SP and activation of the NK_1R. After 60 min recovery, the NK_1R was detected at the plasma membrane ($85.5 \pm 1.2\%$ of total NK_1R-IR pixels), indicating recycling. SM-19712 did not affect NK_1R endocytosis at 30 min, but caused retention of NK_1R-IR in endosomes at 60 min. The proportion of NK_1R-IR pixels in endosomes at 60 min recovery was $14.4 \pm 1.2\%$ in vehicle-treated neurones and $47.7\% \pm 3.6\%$ in SM-19712-treated neurones (Fig. 9*A* and *B*; $P < 0.005$, 60 min recovery, SM-19712 compared to vehicle).

These results indicate that endocytic sorting of the NK_1R to the plasma membrane requires ECE-1 activity in acidified endosomes, where ECE-1 can degrade SP to promote signalosome disassembly.

ECE-1 promotes NK_1R resensitization in myenteric neurones

Although ECE-1-induced recycling of the NK_1R is a major mechanism of resensitization of responses to SP in cell lines (Roosterman *et al.* 2007), the role of ECE-1 in NK_1R resensitization in neurones is unknown. To assess desensitization and resensitization, myenteric neurones cultured from the mouse intestine were repeatedly challenged with SP (10 nM) and $[Ca^{2+}]_i$ was measured by ratiometric imaging. Both the magnitude of the increase in $[Ca^{2+}]_i$ and the proportion of the KCl-responsive neurones that also responded to SP were determined. SP (10 nM, 5 min) caused a rapid and transient increase in $[Ca^{2+}]_i$ in $54 \pm 6.4\%$ of myenteric neurones (Fig. 10*A*). When the same neurones were re-challenged with SP (10 nM) 10 min after the initial receptor stimulation, SP signals were undetectable (Fig. 10*A* and *C*), indicating complete desensitization of the NK_1R. These neurones maintained responsiveness to carbachol. Responses were partially recovered when the interval between SP challenges was 30 min (not shown). Both the magnitude of the response and the proportion of neurones with a detectable response were almost fully recovered when the interval between SP challenges was 60 min, indicating a high degree of resensitization (Fig. 10*A* and *C*). This resensitization coincided with recycling of NK_1R-IR to the plasma membrane of cultured myenteric neurones (not shown). The ECE-1 inhibitor SM-19712 did not affect the initial increase in $[Ca^{2+}]_i$ in response to SP, which was detected in $60.2 \pm 6.1\%$ of myenteric neurones, nor the subsequent desensitization of this response to a second SP challenge at 10 min (Fig. 10*B* and *C*). However, SM-19712 strongly inhibited resensitization after 60 min (% resensitized neurones compared to initial SP response (100%), vehicle control, $74 \pm 20\%$; SM-19712, $10.8 \pm 3.8\%$; $P < 0.05$). SM-19712 also inhibited recycling of NK_1R-IR in cultured myenteric neurones (not shown). These data are consistent with a major role of ECE-1 in regulating resensitization of NK_1R-mediated Ca^{2+} signalling in myenteric neurones. The NK_1R antagonist RP-67580 (Garret *et al.* 1992) markedly inhibited responses to SP, which were detectable in only $7.9 \pm 3.6\%$ of cultured neurones ($P < 0.005$ compared to vehicle), indicating that SP responses are attributable to activation of the NK_1R (Fig. 10*D* and *E*).

To examine whether SP selectively desensitizes the NK_1R and not other receptors, we examined the

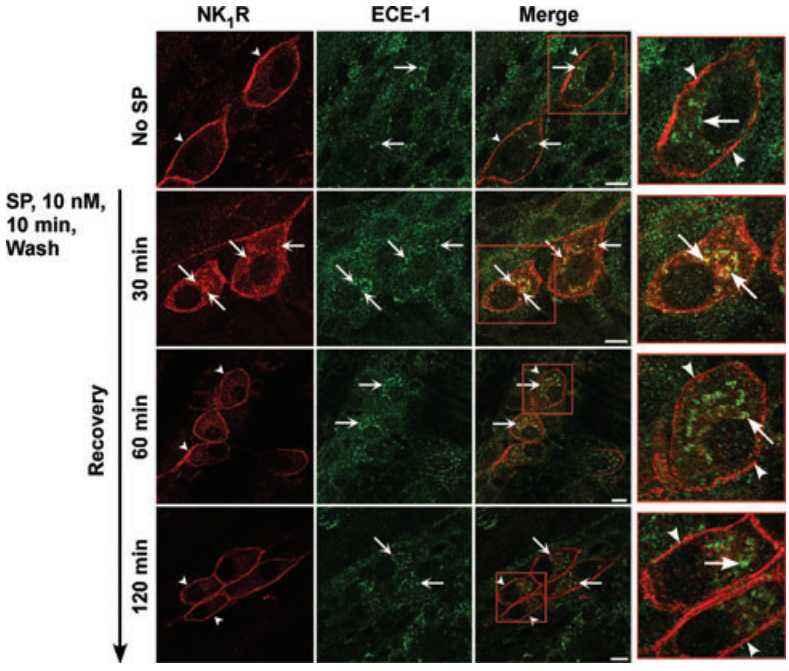

Figure 5. SP-induced endocytosis and recycling of the NK_1R in myenteric neurones
Organotypic preparations of rat ileum were unstimulated or stimulated with SP (10 nM, 10 min), washed, and recovered for 30–120 min. NK_1R and ECE-1 were localized in whole-mounts of myenteric plexus by immunofluorescence. In unstimulated neurones, NK_1R-IR was at the plasma membrane (arrowheads) and ECE-1-IR was in endosomes (arrows). At 30 min recovery, NK_1R-IR colocalized with ECE-1-IR in endosomes (arrows). At 60 and 120 min recovery, NK_1R-IR had recycled to the plasma membrane (arrowheads). Scale, 10 μm.

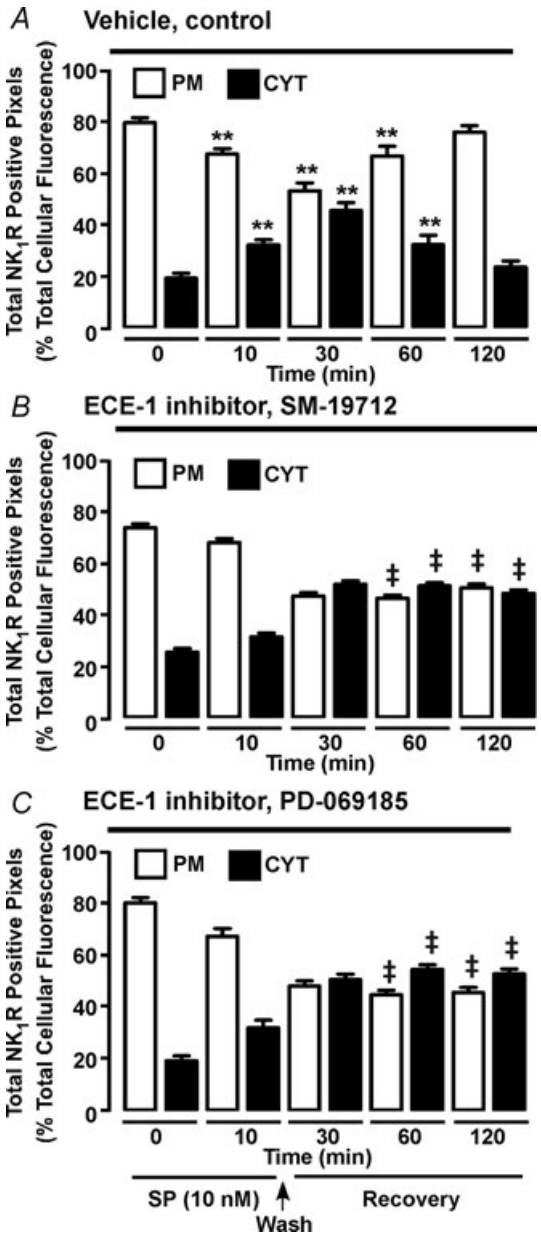

Figure 6. Quantitative analysis of ECE-1 regulation of NK_1R recycling in myenteric neurones
Organotypic preparations of rat ileum were incubated with vehicle (*A*) or the ECE-1 inhibitors SM-19712 (*B*) or PD-069185 (*C*). Neurones were unstimulated or stimulated with SP (10 nM, 10 min), washed and recovered for 30–120 min. NK_1R was localized in whole-mounts of myenteric plexus by immunofluorescence and pixel intensity at the plasma membrane (PM) and in the cytosol (CYT) was determined. In vehicle-treated preparations before SP stimulation (0 min), NK_1R-IR was predominantly at the plasma membrane. SP reduced plasma membrane intensity and increased endosomal intensity at 10 and 30 min, indicating endocytosis. At 60–120 min recovery, plasma membrane intensity was increased and endosomal intensity was decreased, indicating recycling. SM-19712 and PD-069185 did not affect NK_1R-IR endocytosis, but caused NK_1R-IR retention in endosomes at 60 and 120 min, indicating inhibition of recycling. $^{**}P < 0.005$ to 0 min vehicle control. $^{‡}P < 0.005$ to vehicle control. 25–88 neurones per group from $n = 6$ rats.

responses of neurones to carbachol. Carbachol (1 μM) increased $[Ca^{2+}]_i$ in myenteric neurones (Fig. 11*A* and *B*). The magnitude of the response to carbachol was unaffected by preincubation of neurones with SP (10 nM, 5 min), both in neurones that responded to SP and thus expressed the NK_1R, and in non-SP responsive neurones that lacked detectable NK_1R. In contrast, preincubation with SP caused marked desensitization of the NK_1R at the same time point (Fig. 10). The response to carbachol was unaffected by SM-19712 (10 μM, continuous) compared to vehicle (control, Fig. 11*A* and *B*). Thus, preincubation with SP selectively desensitizes the NK_1R but does not heterologously desensitize muscarinic receptors in myenteric neurones. ECE-1 does not regulate the responses of neurones to carbachol.

ECE-1 attenuates NK_1R-mediated ERK1/2 activation in myenteric neurones

β-Arrestins assemble a SP–NK_1R–β-arrestin–Src signalosome in endosomes that mediates G protein-independent activation of ERK1/2 (DeFea *et al.* 2000). The mechanisms that terminate β-arrestin-mediated MAPK signalling in neuronal endosomes are unknown. We examined whether ECE-1, by degrading SP and destabilizing the signalosome, could attenuate MAPK signalling. To enable sensitive detection of ERK1/2 activation at the cellular level, we used a PLA with primary antibodies to ERK1/2 and pERK1/2. Rat myenteric neurones in culture were stimulated with PDBu (100 nM) or SP (10 nM) and pERK1/2:ERK1/2 PLA signals were quantified. In unstimulated neurones there were few signals by PLA. PDBu (positive control) stimulated a 4-fold increase in PLA signals within the cytosol and nucleus within 10 min, indicating formation of a pERK1/2 and ERK1/2 complex and ERK1/2 activation (Fig. 12*A* and *B*). SP also stimulated a 4-fold increase in PLA signals within 10–30 min that declined after 120 min (Fig. 12*A* and *B*). The ECE-1 inhibitor SM-19712 did not affect the magnitude of the early phases of SP-induced ERK1/2 activation, but at 120 min, the response was completely maintained and >2-fold higher than in vehicle-treated preparation (Fig. 12*A* and *B*). Omission of primary antibodies abolished PLA signals (not shown). These results are consistent with SM-19712 causing endosomal retention of NK_1R and suggest that endosomal ECE-1, by destabilizing the β-arrestin signalosome, attenuates SP-induced ERK1/2 activation.

Discussion

In contrast to the extensively studied mechanisms that control G protein signalling and trafficking of neuropeptide receptors at the plasma membrane, very little

is known about the control of receptor signalling and trafficking in endosomes. Cell-surface peptidases that degrade neuropeptides in the extracellular fluid, and β-arrestins, which uncouple receptors from G proteins and couple receptors to the endocytic machinery, rapidly attenuate plasma membrane signalling. Our results suggest that an analogous proteolytic mechanism regulates the association of the NK_1R with β-arrestins in endosomes, and thereby controls the post-endocytic trafficking and signalling of the NK_1R in enteric neurones. We observed that neurones of the myenteric plexus of the intestine that express the NK_1R also express ECE-1 isoforms within endosomes. SP and the NK_1R traffic to endosomes containing ECE-1, where we have previously reported that ECE-1 can degrade neuropeptides, including SP, in the acidic endosomal environment (Padilla *et al.* 2007; Roosterman *et al.* 2007, 2008). Inhibition of ECE-1 caused retention of the NK_1R in endosomes, preventing recycling back to the plasma membrane and inhibiting resensitization of plasma membrane signalling. ECE-1 inhibition also resulted in markedly sustained activation of ERK1/2, presumably by a β-arrestin-mediated, endosomal mechanism. These observations reveal a new proteolytic mechanism that controls endosomal signalling and trafficking of the NK_1R in myenteric neurones.

ECE-1 is expressed in the enteric nervous system

We amplified mRNA that encodes multiple isoforms of ECE-1 (ECE-1a, ECE-1b, ECE-1c1 and ECE-1c2) from ME-MP of the mouse intestine by RT-PCR, and confirmed ECE-1 expression and localization to endosomes using an antibody that detects ECE-1b/d isoforms. When expressed in cell lines, ECE-1b/d isoforms are most prominently

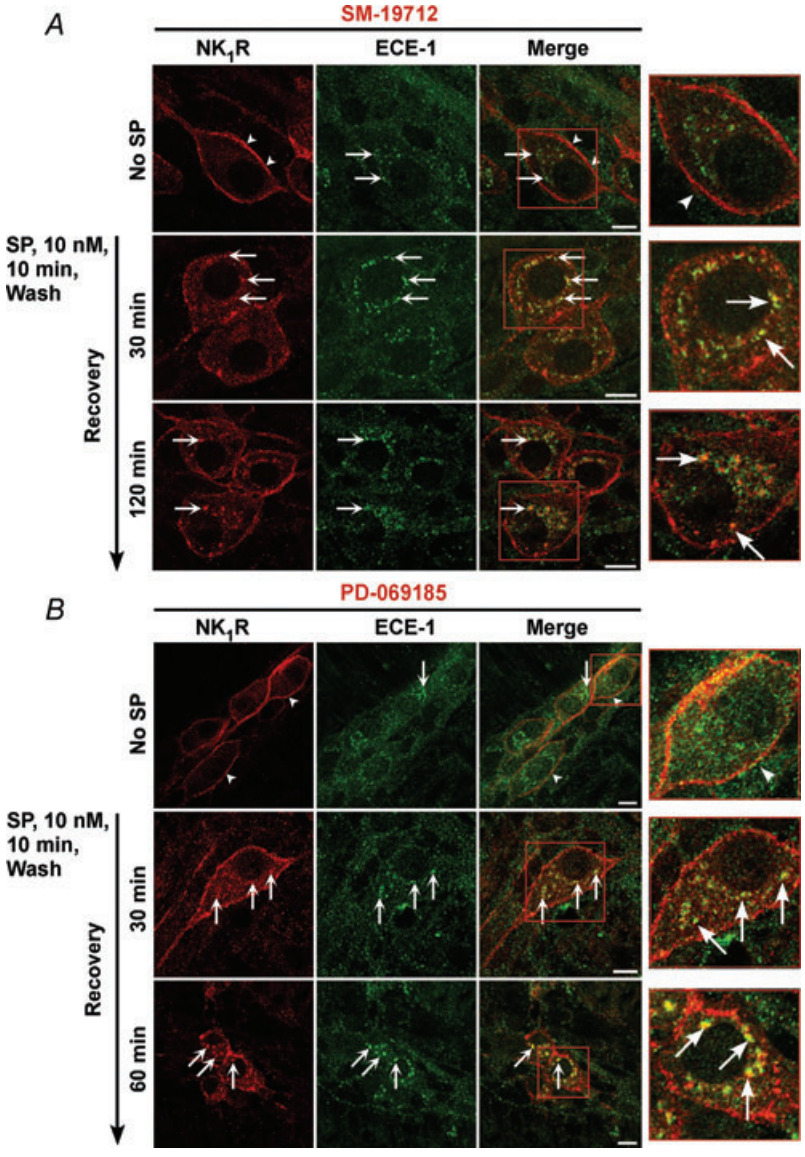

Figure 7. Effects of ECE-1 inhibition on recycling of the NK_1R
Organotypic preparations of rat ileum were incubated with the ECE-1 inhibitors SM-19712 (*A*) or PD-069185 (*B*), and were unstimulated or stimulated with SP (10 nM, 10 min), washed and recovered for 30–120 min. NK_1R and ECE-1 were localized in whole-mounts of myenteric plexus by immunofluorescence. In unstimulated neurones, NK_1R-IR was at the plasma membrane (arrowheads) and ECE-1-IR was in endosomes (arrows). At 30 min recovery, NK_1R-IR colocalized with ECE-1-IR in endosomes (arrows). At 60 and 120 min recovery, NK_1R-IR was prominently detected in endosomes (arrows). Scale, 10 μm.

localized to early endosomes and ECE-1a/c isoforms are most prominently present at the plasma membrane (Padilla *et al.* 2007; Roosterman *et al.* 2007). However, all isoforms are found in endosomes and constitutively internalize and possibly recycle via the endosomal network. These results support other reports of ECE-1 expression in myenteric neurones of rats (Cottrell *et al.* 2009), and are consistent with the detection of ECE-1 substrates (big-endothelin) and products (endothelin-1) in enteric neurones of humans (Escrig *et al.* 1992). ECE-1-IR has also been detected in endothelial, epithelial, smooth muscle and neuroendocrine cells of the intestine, and in neurones of sympathetic ganglia, indicating widespread expression (Hsu & Huang, 2003). Although we did not attempt to classify the subtypes of myenteric neurones that express ECE-1, ECE-1-IR was detected in neurones expressing the NK_1R, as well as other cell types that express this receptor, including interstitial cells of Cajal. Further studies will be necessary to identify those classes of enteric neurones that express ECE-1 by neurochemical coding analyses.

ECE-1 promotes recycling and resensitization of NK_1R signalling at the plasma membrane of myenteric neurones

Although several studies have reported that SP stimulates endocytosis and recycling of the NK_1R in enteric neurones (Grady *et al.* 1996*b*; McConalogue *et al.* 1998; Southwell *et al.* 1998; Mann *et al.* 1999), the mechanism and function of this trafficking have not been defined. We observed that brief exposure of neurones to SP resulted in translocation of β-arrestins to the plasma membrane and then to endosomes, where β-arrestins colocalized with the NK_1R. By using a PLA that is sensitive in detecting protein–protein interactions at the subcellular level, we observed interaction of the NK_1R and β-arrestins in SP-stimulated neurones. These results support the well-defined role of β-arrestins in mediating desensitization and endocytosis of the NK_1R in cell lines (McConalogue *et al.* 1999; DeFea *et al.* 2000). Moreover, NK_1R–β-arrestin interactions in myenteric neurones coincided with desensitization of

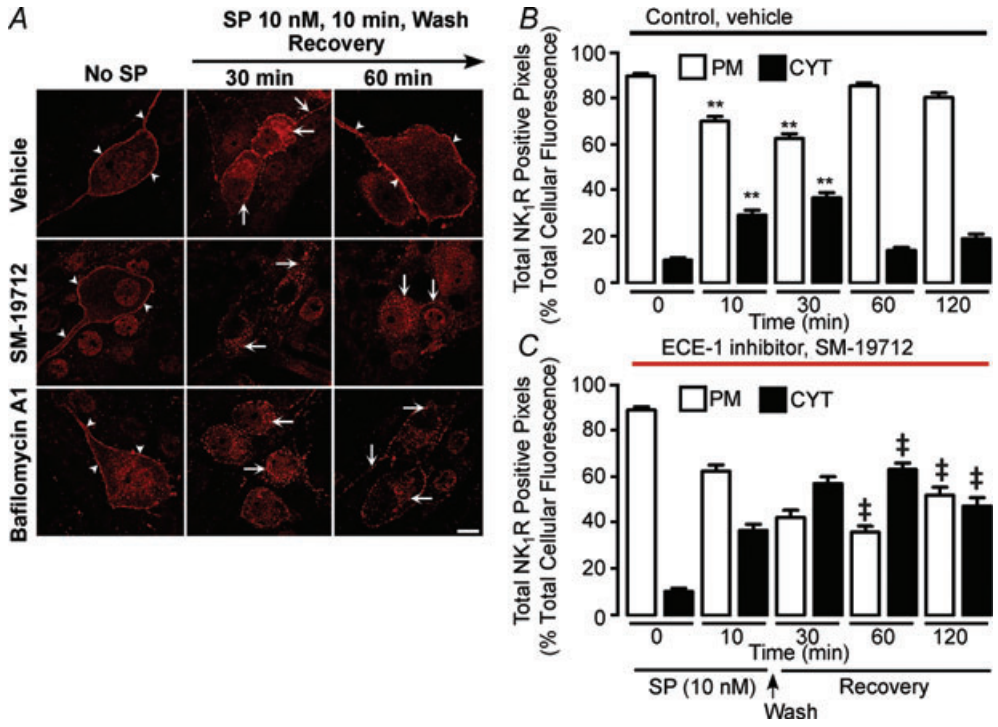

Figure 8. ECE-1-mediated recycling of the NK_1R in myenteric neurones
Organotypic preparations of mouse colon were unstimulated or stimulated with SP (10 nM, 10 min), washed and recovered for 30–120 min. NK_1R was localized in whole-mounts of myenteric plexus by immunofluorescence (*A*) and pixel intensity at the plasma membrane (PM) and in the cytosol (CYT) was determined (*B* and *C*). In unstimulated neurones, NK_1R-IR was at the plasma membrane (arrowheads). At 30 min recovery, NK_1R-IR was detected in endosomes (arrows), and after 60 min recovery, NK_1R-IR had recycled to the plasma membrane (arrowheads). SM-19712 did not affect endocytosis, but caused retention of the NK_1R-IR in endosomes at 60 and 120 min (arrows), indicating inhibition of recycling. Bafilomycin A1 also caused retention of the NK_1R in endosomes at 60 min recovery (arrows). Scale, 10 μm. $^{**}P < 0.005$ to 0 min. $^{\ddagger}P < 0.005$ to vehicle control. 25–84 neurones per group from $n = 3$ mice.

SP-mediated Ca^{2+} signalling, which is probably mediated by β-arrestins. The dynamin GTPase inhibitor Dynasore (Macia *et al.* 2006) abolished SP-stimulated endocytosis of the NK_1R in myenteric neurones, consistent with clathrin- and dynamin-mediated endocytosis (Grady *et al.* 1995, 1996*b*; Schmidlin *et al.* 2001).

In myenteric neurones with active ECE-1, the internalized NK_1R recycled from early endosomes to the plasma membrane, and recycling coincided with resensitization of G protein-mediated NK_1R signalling at the plasma membrane, assessed by measurement of SP-stimulated Ca^{2+} transients. The inhibition of endosomal ECE-1 by several approaches blocked NK_1R recycling and resensitization, indicating a major role for ECE-1 in these processes. Two distinct ECE-1 inhibitors, SM-19712 (Umekawa *et al.* 2000) and PD-069185 (Ahn *et al.* 1998), inhibited these events. Bafilomycin A_1, a vacuolar H^+ATPase inhibitor that prevents endosomal acidification and suppresses ECE-1 degradation of SP in endosomes, which only occurs at acidic endosomal pH (Roosterman *et al.* 2007), also inhibited recycling. A limitation of this approach is the reliance on pharmacological tools to block ECE-1 activity, since we could not efficiently transfect myenteric neurones with ECE-1 cDNA or siRNA. However, we have previously reported that whereas overexpression of ECE-1 accelerates receptor recycling and resensitization, ECE-1 knockdown has the opposite effects (Padilla *et al.* 2007; Roosterman *et al.* 2007). In the present study we observed that ECE-1 inhibition prevented NK_1R recycling after release of endogenous SP by K^+ depolarization, suggesting that this mechanism is physiologically relevant.

We did not directly determine the mechanism by which ECE-1 promotes recycling and resensitization of the NK_1R in myenteric neurones, due to difficulty in examining neuropeptide degradation in myenteric neurones. In cell lines, ECE-1 makes a major contribution to the degradation of internalized neuropeptides, including

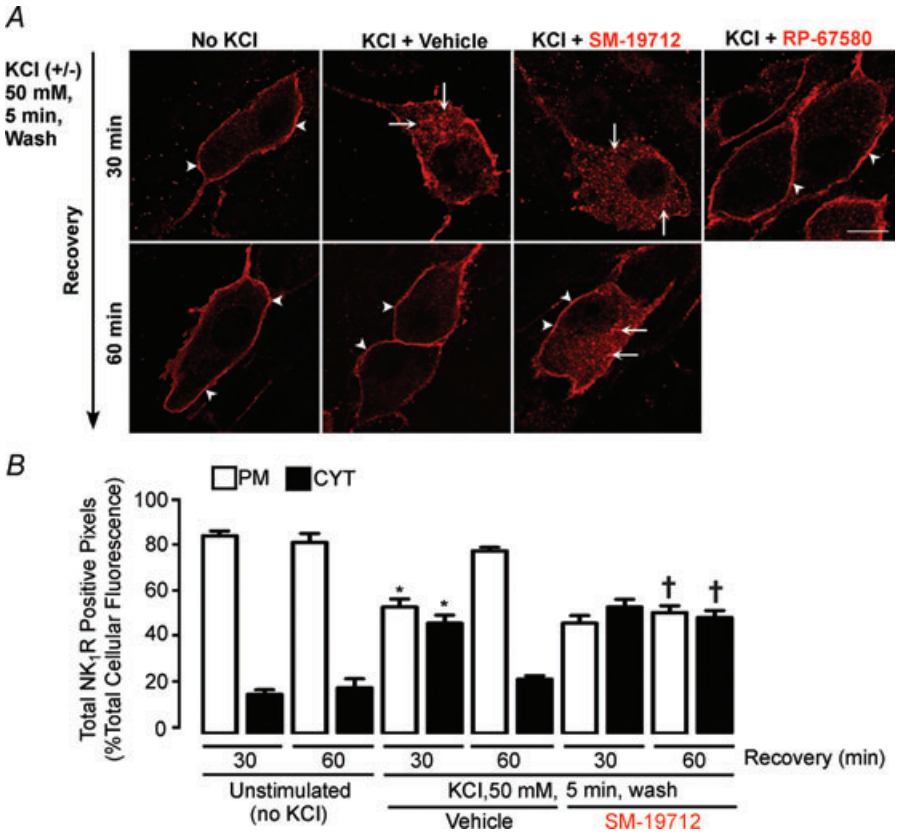

Figure 9. ECE-1-mediated recycling of the NK_1R in response to endogenous SP in myenteric neurones
Organotypic preparations of mouse colon were unstimulated or stimulated with KCl (50 mM, 5 min), washed and recovered for 30 or 60 min. NK_1R was localized in whole-mounts of myenteric plexus by immunofluorescence (*A*) and pixel intensity at the plasma membrane (PM) and within the cytosol (CYT) was determined (*B*). In unstimulated neurones, NK_1R-IR remained at the plasma membrane (arrowheads). KCl stimulated trafficking to endosomes at 30 min (arrows), and recycling to the plasma membrane at 60 min (arrowheads). The ECE-1 inhibitor SM-19712 did not affect endocytosis at 30 min, but caused NK_1R retention in endosomes (arrows), and thus suppressed recycling at 60 min. The NK_1R antagonist RP-67580 prevented KCl-induced endocytosis at 30 min. Scale, 10 μm. $^*P < 0.05$ compared to no KCl at 30 min; $^\dagger P < 0.05$ compared to KCl at 60 min. 12–39 neurones per group from $n = 3$ mice.

SP, calcitonin gene-related peptide and somatostatin-14, whereas ECE-1 inhibitors and bafilomycin A_1 prevent this degradation (Padilla *et al.* 2007; Roosterman *et al.* 2007, 2008). Moreover, ECE-1 degrades these neuropeptides only at endosomal pH 5.5 and not at extracellular pH 7.4. Thus, a likely explanation of the effects of ECE-1 inhibition on NK_1R recycling and resensitization is suppressed degradation of SP in endosomes, and consequent stabilization of the endosomal SP–NK_1R–β-arrestin signalosome, which traps the NK_1R in endosomes. Dissociation of β-arrestins and receptor recycling is necessary for resensitization of other 7TMD receptors (Oakley *et al.* 1999), including the NK_1R (Schmidlin *et al.* 2001; Roosterman *et al.* 2004).

We observed that the NK_1R is only partially internalized at a time when SP-induced Ca^{2+} signalling is fully desensitized (10 min). Thus, endocytosis does not mediate desensitization, which is likely to depend on NK_1R phosphorylation by G protein-coupled receptor kinases and interaction with β-arrestins. Although our results suggest that ECE-1-dependent NK_1R recycling is a major mechanism for resensitization, we cannot exclude the possibility that surface-retained receptors could resensitize without the requirement for internalization. In support of this possibility, we have recently reported a role for protein phosphatase 2A in resensitization of non-internalized NK_1R (Murphy *et al.* 2011). This mechanism proposes that some NK_1Rs are phosphorylated at the plasma membrane

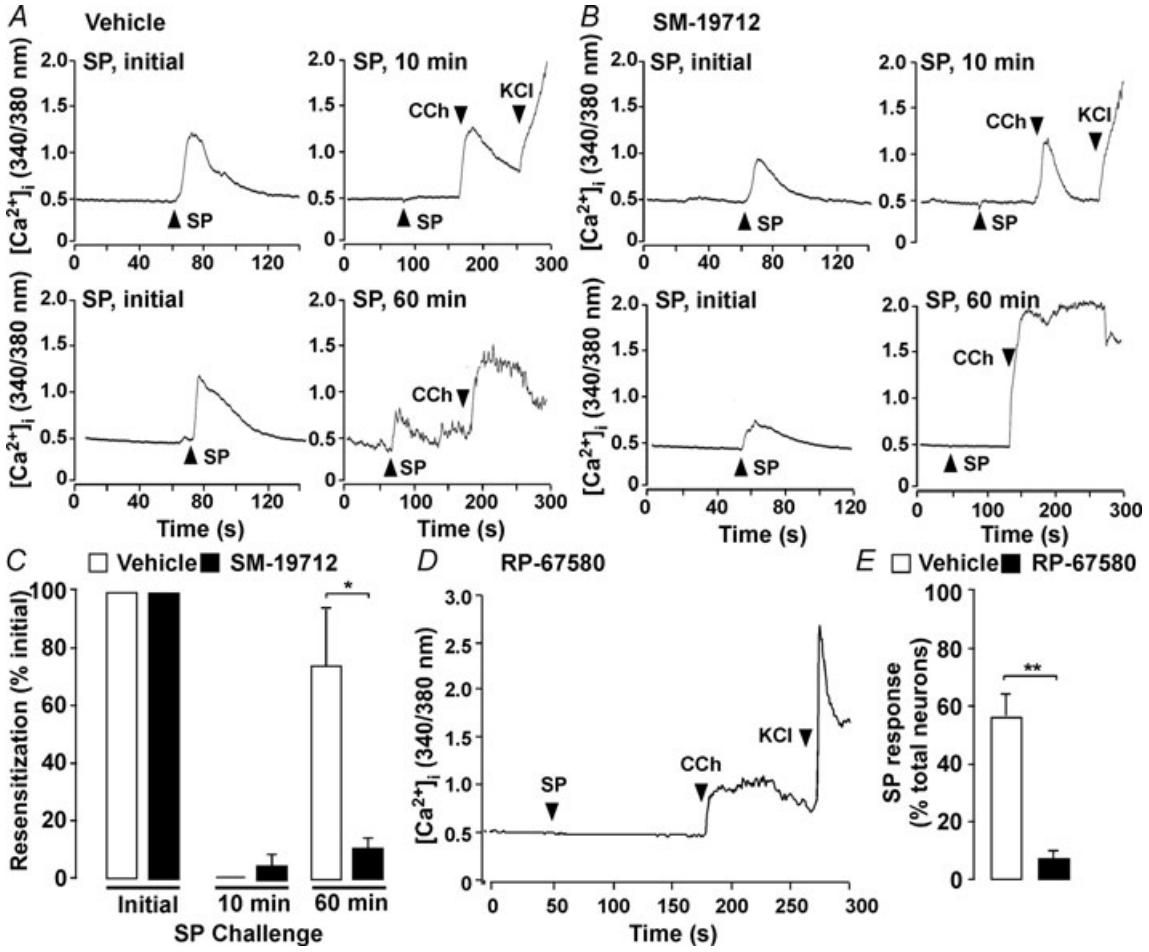

Figure 10. ECE-1-mediated resensitization of SP-induced Ca^{2+} transients in myenteric neurones
A–C, mouse myenteric neurones in culture were challenged with SP (initial, 10 nM, 5 min), washed, and the same neurones were re-challenged (10 nM SP) after 10 or 60 min recovery. After the final SP challenge, neurones were exposed to carbachol (CCh, 1 μM) and then KCl (50 mM). *A* and *B*, Ca^{2+} transients from the same neurones in each experiment (upper panel: 10 min re-challenge; lower panel: 60 min re-challenge) treated with vehicle (*A*) or SM-19712 (*B*). *C*, resensitization of neurones treated with vehicle or SM-19712. The proportion of neurones responsive to initial SP were recorded and are represented as 100% when compared to the proportion of the same neurones responsive to SP re-challenge. $^*P < 0.05$ to vehicle, 46–102 neurones per group, $n > 4$ experiments (i.e. neuronal cultures from 3–5 mice per preparation). *D* and *E*, mouse myenteric neurones in culture were incubated with vehicle or the NK_1R antagonist RP-67580, which suppressed detectable changes in $[Ca^{2+}]_i$. $^{**}P < 0.005$ compared to vehicle, 149–176 neurones per group, $n > 4$ mice.

and desensitized by a second messenger kinase such as protein kinase C. These receptors neither interact with β-arrestins nor internalize. Protein phosphatase 2A translocates to the plasma membrane and dephosphorylates the surface-retained NK_1Rs to mediate resensitization. Further studies are required to determine the contribution of protein phosphatase 2A to resensitization of the NK_1R in enteric neurones.

ECE-1 terminates endosomal signalling of the NK_1R in myenteric neurones

There is a growing realization that 7TMD receptors can continue to signal from endosomes by mechanisms that are distinct from those operating at the plasma membrane, and that β-arrestins play a critical role in these processes (Murphy *et al.* 2009). Whereas β-arrestins terminate G protein signalling at the plasma membrane, they

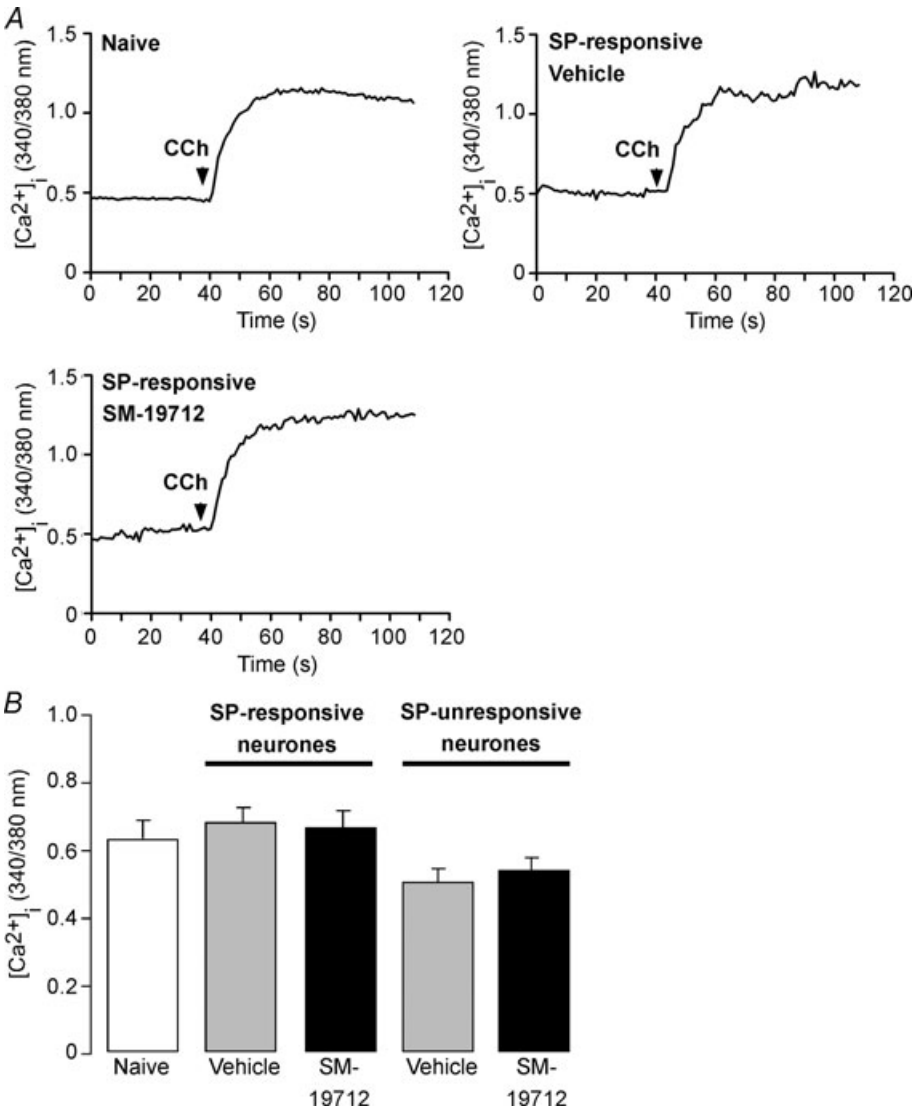

Figure 11. Responses of myenteric neurones to carbachol
Mouse myenteric neurones in culture were challenged with carbachol (CCh, 1 μM), and Ca^{2+} transients were measured. *A*, Ca^{2+} transients to carbachol in naïve neurones (i.e. no prior stimulation) and in SP-responsive neurones preincubated with SP (10 nM, 5 min). SP-responsive neurones preincubated with SM-19712 (10 μM, throughout experiment) or vehicle (control). *B*, mean responses to carbachol in naïve neurones, in SP-responsive neurones preincubated with SP (10 nM, 5 min), and in non-SP responsive neurones lacking detectable NK_1R. Both SP-responsive and non-responsive neurones preincubated with SM-19712 or vehicle control. 26–43 neurones per group, $n = 3$ experiments (i.e. neuronal cultures from 3–5 mice per preparation). Note that the response to carbachol was unaffected by preincubation with SP or SM-19712.

recruit receptors and signalling partners to signalosomes, and thereby transmit a second wave of G protein *independent* signals. SP interacts with Src and induces assembly of a SP–NK_1R–β-arrestin–Src signalosome that mediates sustained ERK1/2 signalling (DeFea *et al.* 2000). We observed that SP stimulated ERK1/2 activation in myenteric neurones, and that an ECE-1 inhibitor caused markedly sustained ERK1/2 signalling, probably by stabilizing the signalosome. A limitation of our approach is that we could not directly assess the contribution of β-arrestins to this signalling pathway due to difficulties in expressing β-arrestin siRNA in enteric neurones. However, disruption of β-arrestins in cell lines attenuates SP-stimulated ERK1/2 activation (DeFea *et al.* 2000), and ECE-1 inhibition with SM-19712 or bafilomycin A_1 stabilizes the signalosome and prolonged ERK1/2 signalling in model cell systems (Cottrell *et al.* 2009).

Cell-surface membrane metallo-endopeptidases, such as neprilysin, that degrade neuropeptides in the extracellular fluid, protect against sustained neuropeptide signalling. Neprilysin deletion or inhibition results in plasma extravasation and exacerbated inflammation due to the impaired degradation and amplified signalling of the proinflammatory neuropeptides SP and bradykinin (Lu *et al.* 1997; Sturiale *et al.* 1999). ECE-1 may similarly protect against uncontrolled signalling of receptors in endosomes. SP can induce neurotoxicity by β-arrestin-, Raf- and ERK1/2-mediated activation of Nur77, a mediator of cell death (Castro-Obregon *et al.* 2004). From studies of cell lines, we reported that ECE-1 inhibitors cause remarkably sustained SP-induced activation of Nur77, which results in cell death (Cottrell *et al.* 2009). By terminating SP signalling in endosomes and attenuating activation of ERK1/2, ECE-1 may also protect against the SP-induced neurotoxicity in the enteric nervous system.

Physiological functions of ECE-1 in the enteric nervous system

ECE-1 is best known for its role in the extracellular and intracellular conversion of big endothelin to endothelin-1, which plays a major role in regulating blood pressure and sodium homeostasis (Kohan *et al.* 2011). ECE-1 can also generate endothelins in the enteric nervous system, which may control motility and secretion (Escrig *et al.* 1992). The prominent detection of ECE-1 in endosomes, and the realization that ECE-1 can degrade certain neuropeptides in endosomes and thereby regulate receptor trafficking and signalling (Padilla *et al.* 2007; Roosterman *et al.* 2007, 2008; Cattaruzza *et al.* 2009; Cottrell *et al.* 2009), have revealed new functions for endosomal ECE-1. By promoting recycling and resensitization of the NK_1R in myenteric neurones, ECE-1 may allow the peristaltic reflex to be maintained. SP and the NK_1R mediate ascending contraction during peristalsis, when intestinal distension triggers the release of SP and orad contraction of smooth muscle (Grider, 2003). Given that the contractile effects of SP in the intestine rapidly desensitize to repeated challenge (Gaddum, 1953), resensitization, possibly mediated by ECE-1-dependent NK_1R recycling, may permit the maintenance of peristalsis, which is essential for normal digestion. It is also possible that ECE-1 protects against sustained β-arrestin-mediated signalling of the NK_1R, which could cause neurotoxicity (Castro-Obregon *et al.* 2004). The endothelin system plays an essential role in the development of neural crest-derived tissues, including enteric neurones. Enteric neurones are absent from mice deficient in ECE-1, recapitulating the effects of deletion of the endothelin B receptor or endothelin-3 (Yanagisawa *et al.* 1998). Although these findings indicate that ECE-1-mediated processing of endothelin and activation of endothelin B receptors is required for normal development of enteric neurones, the absence

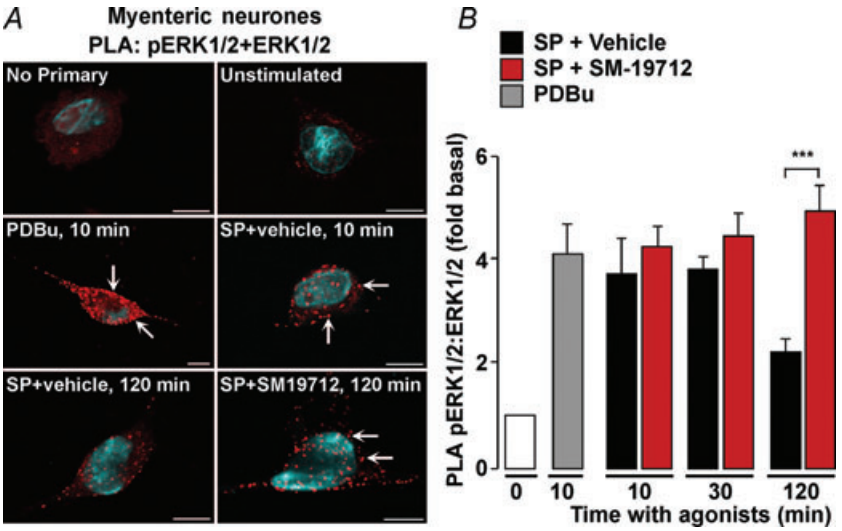

Figure 12. ECE-1 attenuation of SP-induced ERK1/2 signals in myenteric neurones
Rat myenteric neurones in culture were challenged with PDBu (100 nM) or SP (10 nM), and neurones were processed for PLA for ERK1/2 and pERK1/2. PDBu and SP strongly induced PLA signals (in red) within 10–30 min, indicative of activation of pERK1/2 (arrows). SP-induced PLA signals were attenuated after 120 min. The ECE-1 inhibitor SM-19712 did not affect the initial increase in PLA signals, but caused sustained signals at 120 min, indicative of prolonged activation of pERK1/2. The blue stain in *A* (DAPI) denotes the nucleus. ***$P < 0.001$. $n > 3$ experiments (i.e. neuronal cultures from 1 rat per preparation).

of ECE-1 may also reveal unexpected toxic effects of neuropeptides in the enteric nervous system.

In addition to SP, endosomal ECE-1 can regulate endosomal trafficking of receptors for other neuropeptides that are ECE-1 substrates at endosomal pH, including calcitonin gene-related peptide and somatostatin-14, which control numerous gastrointestinal functions, including peristalsis (Grider, 2003). Additional studies are required to delineate the other functions of ECE-1 in the enteric nervous system that may be related to the control of endocytic trafficking and signalling of various neuropeptide receptors. Whether ECE-1 deficiency contributes to abnormal functions of the enteric nervous system due to abnormal endosomal signalling remains to be determined.

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J.-C.P. and D.P.P. designed, performed and analysed experiments and wrote the manuscript; G.S.C. performed experiments and provided critical scientific input; M.S. provided critical scientific input; N.W.B. designed and supervised the study and wrote the manuscript. All authors approved the final version.

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