BJP British Journal of Pharmacology

DOI:10.1111/bph.12833
www.brjpharmacol.org

Themed Section: Opioids: New Pathways to Functional Selectivity

RESEARCH PAPER

Endothelin-converting enzyme 2 differentially regulates opioid receptor activity

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Received
15 November 2013
Revised
17 June 2014
Accepted
24 June 2014

BACKGROUND AND PURPOSE

Opioid receptor function is modulated by post-activation events such as receptor endocytosis, recycling and/or degradation. While it is generally understood that the peptide ligand gets co-endocytosed with the receptor, relatively few studies have investigated the role of the endocytosed peptide and peptide processing enzymes in regulating receptor function. In this study, we focused on endothelin-converting enzyme 2 (ECE2), a member of the neprilysin family of metallopeptidases that exhibits an acidic pH optimum, localizes to an intracellular compartment and selectively processes neuropeptides including opioid peptides *in vitro*, and examined its role in modulating μ receptor recycling and resensitization.

EXPERIMENTAL APPROACH

The effect of ECE2 inhibition on hydrolysis of the endocytosed peptide was examined using thin-layer chromatography and on μ opioid receptor trafficking using either ELISA or microscopy. The effect of ECE2 inhibition on receptor signalling was measured using a cAMP assay and, *in vivo*, on antinociception induced by intrathecally administered opioids by the tail-flick assay.

KEY RESULTS

The highly selective ECE2 inhibitor, S136492, significantly impaired μ receptor recycling and signalling by only those ligands that are ECE2 substrates and this was seen both in heterologous cells and in cells endogenously co-expressing μ receptors with ECE2. We also found that ECE2 inhibition attenuated antinociception mediated only by opioid peptides that are ECE2 substrates.

CONCLUSIONS AND IMPLICATIONS

These results suggest that ECE2, by selectively processing endogenous opioid peptides in the endocytic compartment, plays a role in modulating opioid receptor activity.

LINKED ARTICLES

This article is part of a themed section on Opioids: New Pathways to Functional Selectivity. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-2

Abbreviations

BAM22, Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly; DAMGO, Tyr-D-Ala-Gly-NMe-Phe-Gly-ol; DOP receptor, δ opioid receptor; dynorphin B, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr; ECE1, endothelin-converting enzyme-1; ECE2, endothelin-converting enzyme 2; EEA1, early endosomal antigen 1; HA, haemagglutinin; KOP receptor, κ opioid receptor; [Leu]enkephalin, leucine-enkephalin, Tyr-Gly-Gly-Phe-Leu; McaBk2, (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys-(2,4-dinitrophenyl); MOP receptor, μ opioid receptor

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Table of Links

TARGETS	LIGANDS
Angiotensin-converting enzyme	cAMP
ECE1	Captopril
ECE2	Chloroquine
Neprilysin	Cycloheximide
δ receptor	DAMGO
μ receptor	[^{3}H]-diprenorphine
κ receptor	Dynorphin B
Somatostatin receptors	Fentanyl
	GDP
	GTPγS
	[Leu]enkephalin
	Thiorphan

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

Introduction

Opioid peptides serve as endogenous ligands for the three types of opioid receptors, μ, δ and κ (aka MOP, DOP and KOP receptors) (Cox *et al.*, 2015). The endogenous opioid peptides are derived from post-translational processing of three major precursors: pro-enkephalin, prodynorphin and pro-opiomelanocortin (Gomes *et al.*, 2013c). Among them, pro-enkephalin-derived peptides are the most abundant (Fricker, 2012) and thought to primarily activate δ and μ opioid receptors (Pasternak, 2011) while prodynorphin-derived peptides are less abundant and thought to selectively bind and activate κ receptors (Pasternak, 2011). However, mounting evidence suggests that dynorphin peptides can also bind and activate μ and δ receptors (Chavkin *et al.*, 1985; Mansour *et al.*, 1995; Alt *et al.*, 1998). Binding of opioid peptides to their cognate receptors leads to receptor activation followed by internalization, recycling and/or degradation (von Zastrow and Williams, 2012). Many studies have investigated the mechanism underlying receptor internalization and desensitization and shown a role for receptor phosphorylation, arrestin recruitment and clathrin-coated pit-mediated endocytosis in the desensitization process (Hanyaloglu and von Zastrow, 2008). Relatively, fewer studies have explored the mechanisms underlying receptor recycling and resensitization. This study explores a role for the endocytosed ligand by examining the effect of its processing on receptor recycling and function.

It is generally thought that the receptor, endocytosed along with its agonistic ligand, dissociates from the latter in an acidic endocytic compartment leading to dephosphorylation and recycling of the receptor during acute agonist treatment, and/or trafficking of the receptor to a degradative compartment in the continued presence of the drug (Williams *et al.*, 2013). It is also thought that if the agonist endocytosed along with the receptor is a peptide, the latter is selectively processed in an endocytic compartment, thus modulating the rate and extent of receptor recycling and/or degradation (Mellman *et al.*, 1986). Support for such a notion comes from early studies with peptidic agonists of opioid receptors that showed that a significant portion of the endocytosed peptides accumulate in an acidic intracellular compartment (Gaudriault *et al.*, 1997; Trapaidze *et al.*, 2000). These results suggest an acidic endocytic compartment, such as recycling endosomes or late endosomes, as the major compartment for processing of peptide ligands.

In this study, we examined a role for peptide processing enzymes in opioid peptide hydrolysis in an acidic endocytic compartment and its effect on modulation of μ receptor trafficking. For this, we focused on ECE2, a member of the neprilysin family that has previously been shown to selectively process short neuropeptides and exhibit endocytic localization, and activity at acidic pH (Devi and Miller, 2013). We used an inhibitor of ECE2 that was identified by a combination of homology modelling based on the crystal structure of neprilysin, *in silico* screening by docking a 130 000 compound library and biochemical screening of the 30 predicted hits (Gagnidze *et al.*, 2008). This led to the identification of two compounds that exhibited significant efficacy (Gagnidze *et al.*, 2008); among their analogues, S136492 was selected for further studies because it exhibited the highest potency. We characterized the inhibitory properties of S136492 and find that it exhibits high selectivity for ECE2 (and does not inhibit the closely related ECE1 even at a high concentration of 100 μM). In this study, we showed that ECE2 co-localizes with μ receptors in endocytic vesicles and significantly enhances the rate of receptor recycling. Inhibition of ECE2 activity slowed down the rate of recycling and this was seen only for peptide agonists that are ECE2 substrates. In dorsal root ganglion (DRG)-derived cells that endogenously express ECE2

and μ receptors, we found enhanced co-localization of ECE2 and μ receptors in an intracellular compartment upon agonist treatment and that recycling of receptors endocytosed in response to ECE2 substrates was significantly impaired by the ECE2 inhibitor. Under these conditions, there was a significant decrease in the extent of receptor signalling. Finally, we demonstrated that antinociception mediated by intrathecal (i.t.) administration of peptide agonists that are ECE2 substrates, but not that mediated by non-substrates of EC2, was attenuated by the ECE2 inhibitor. Taken together, these studies support the involvement of ECE2 in the modulation of μ receptor function by endocytic processing.

Methods

Cells and transfection

CHO cells stably expressing N-terminally Flag-epitope tagged μ receptors (CHO-μ) were grown in F12 media containing 10% FBS, streptomycin-penicillin and 500 $\mu g \cdot mL^{-1}$ geneticin (G418). CHO-μ cells were transfected with N-terminally HA-epitope-tagged human ECE2 using lipofectamine as per the manufacturer's protocol and colonies with stable expression (CHO-μ-ECE2 cells) were selected in media containing 500 $\mu g \cdot mL^{-1}$ geneticin and 250 $\mu g \cdot mL^{-1}$ hygromycin B.

Receptor trafficking

Receptor trafficking studies were carried out as described previously (Trapaidze *et al.*, 2000). Briefly, CHO-μ, CHO-μ-ECE2 or F11 cells (2×10^5) were seeded into each well of a 24-well polylysine coated plate. The following day cells were treated with 100 nM or 1 μM of ligands for 30 min (to induce μ receptor internalization). Cells were washed (to remove the agonist) and incubated with media without the agonist (to induce μ receptor recycling) for various time periods or for 60 min at 37°C in the absence or presence of S136492 (20 μM final concentration), phosphoramidon (10 μM), thiorphan (10 μM), captopril (10 μM), cycloheximide (100 nM), bafilomycin (100 nM) or chloroquine (100 μM). These concentrations of reagents were selected based on their ability to completely inhibit enzymatic activity (Schulz *et al.*, 1991; Kukkola *et al.*, 1995; Shirotani *et al.*, 2001; Vermeirssen *et al.*, 2002; Gagnidze *et al.*, 2008), or to prevent endosomal acidification (Trapaidze *et al.*, 1996; Ippoliti *et al.*, 1998; Law *et al.*, 2012), or to inhibit protein synthesis (Law *et al.*, 2012). At the end of the incubation, medium was removed, cells chilled to 4°C, and briefly fixed with 4% paraformaldehyde for 3 min; this fixation protocol allows detection of only cell surface receptors but not intracellular receptors (Gupta *et al.*, 2014). The cells were processed for ELISA as described previously (Gupta *et al.*, 2008; Gomes *et al.*, 2013b) using 1:1000 dilution of anti-Flag M1 mouse monoclonal antibody or 1:500 dilution of anti-μ receptor rat polyclonal antibody (Gupta *et al.*, 2007) as primary antibodies and 1:1000 dilution of anti-mouse IgG or 1:500 dilution of anti-rat IgG coupled to HRP as secondary antibodies. Total cell surface receptors before agonist treatment for each independent experiment were taken as 100%. The % cell surface receptors following agonist-mediated internalization (taken as $t = 0$) were then subtracted from each individual time point to obtain % recycled receptors for that time point. In experiments examining the effects of different inhibitors on receptor recycling, the number of receptors recycled in the absence of inhibitor (control) was taken as 100%.

To examine the effect of the ECE2 inhibitor, S136492, specifically on receptor internalization, CHO-μ-ECE2 cells (2×10^5 per well) were seeded into a 24-well polylysine coated plate. The following day, the plate was kept on ice and cells were incubated at 4°C for 1 h with 1:1000 anti-FLAG antibody in media to label cell surface μ receptors. Cells were washed three times and then treated with 1 μM DAMGO or 100 nM dynorphin B for 30 min at 37°C without or with 20 μM S136492. At the end of the incubation period, cells were briefly fixed (3 min) with 4% paraformaldehyde followed by three washes (5 min each) with PBS. Receptors present at the cell surface were determined using 1:1000 dilution (in PBS containing 1% BSA) of anti-mouse IgG coupled to HRP (Vector Laboratories) as described previously (Gupta *et al.*, 2008; Gomes *et al.*, 2013b). The % internalized receptors was calculated by taking total cell surface receptors before agonist treatment for each individual experiment as 100% and subtracting % surface receptors following 30 min agonist treatment.

Enzyme activity assays

Purified recombinant human ECE2 (32.5 ng) with a specific activity of 12 $pmol \cdot min^{-1} \cdot \mu g^{-1}$ protein was generated and characterized as described previously (Mzhavia *et al.*, 2003). Purified recombinant mouse ECE1 (30 ng) with a specific activity of 750 $pmol \cdot min^{-1} \cdot \mu g^{-1}$ protein was generated and characterized using a protocol similar to that used for ECE2 (Mzhavia *et al.*, 2003). Midbrain membranes (10 μg) from wild-type or ECE2 knockout mice were prepared as described previously (Ouimet *et al.*, 2010). Enzymatic activity was assayed using 0.2 M sodium acetate buffer, pH 5.5, or 50 mM Tris-Cl buffer, pH 7.4, in the absence or presence of the ECE2 inhibitor, S136492, for 10 min using the synthetic quenched fluorescent substrate, McaBk2 (10 μM), as described previously (Gagnidze *et al.*, 2008; Ouimet *et al.*, 2010). Relative fluorescence units before the addition of substrate (i.e. $t = 0$) were taken as 100%.

Degradation of [^{3}H]-DAMGO by ECE2

A total of 10 nM [^{3}H]-DAMGO was incubated at 37°C without or with purified ECE2 (32.5 ng) in 0.2 M sodium acetate buffer, pH 5.5, for 30 min in the absence or presence of 20 μM S136492. The tubes were placed on ice and contents were subjected to thin-layer chromatography using n-butanol : acetic acid : water (3:1:1 by volume), ~ 3 mm fractions were cut, and the radioactivity in each of the fractions was measured using a scintillation counter.

CHO-μ-ECE2 cells (2×10^5 cells per well) were incubated with 10 nM [^{3}H]-DAMGO for 30 min at 37°C without or with either 20 μM S136492, 10 μM captopril or 100 μM chloroquine. The cells were chilled to 4°C, washed three times in ice-cold 0.2 M sodium acetate buffer, pH 4.8, containing 500 mM sodium chloride to remove surface bound radioligand, followed by cell lyses and thin-layer chromatography of cell lysates as described above.

Binding assays

Membranes (50 μg) from cells expressing either μ or δ receptors were incubated with 10 nM [^{3}H]-diprenorphine in the absence or presence of 0–10 μM DAMGO, dynorphin B or [Leu]enkephalin (for μ receptors), or BAM22 (for δ receptors) in 50 mM HEPES buffer containing protease inhibitor cocktail at pH 7.4 or pH 5.5 and receptor binding estimated as described previously (Gomes *et al.*, 2003).

[^{35}S]-GTPγS binding

Membranes (50 μg) expressing μ receptors were subjected to [^{35}S]-GTPγS binding in the absence or presence of 0–10 μM DAMGO, dynorphin B or [Leu]enkephalin in 50 mM HEPES buffer containing protease inhibitor cocktail at pH 7.4 or pH 5.5 as described previously (Gomes *et al.*, 2003). Basal binding observed in the presence of GDP and in the absence of the agonist was taken as 100%.

Determination of cAMP levels

cAMP levels were determined as described previously (Cvejic *et al.*, 1996; Gomes *et al.*, 2003; 2004) with minor modifications. Briefly, CHO-μ-ECE2 or F11 cells (2×10^5 per well) were seeded into a 24-well polylysine coated plate. Cells were incubated with 1 μM DAMGO, fentanyl, or 100 nM dynorphin B for 30 min at 37°C in media containing 10 μM forskolin and 100 μM IBMX. The cells were washed and incubated without or with 20 μM ECE2 inhibitor (S136492) for 60 min, and the levels of cAMP in response to a second pulse (5 min treatment) of DAMGO, fentanyl or dynorphin B were determined using the HitHunter cAMP HS chemiluminescence detection kit from DiscoveRx (Gomes *et al.*, 2013a).

RT-PCR

Total RNA was isolated from F11 cells as described previously (Gomes *et al.*, 2013a). Single small and large dorsal ganglion (DRG) neurons were a gift from Dr Wendy Wawlyn (University of California, Los Angeles). cDNA from single DRG neurons was synthesized using MessageBOOSTER cDNA Synthesis from Cell Lysates Kit (MBCL90310; Epicentre, Madison, WI, USA) according to the manufacturer's protocol. Real-time PCR was performed as described previously (Gomes *et al.*, 2013a) using the Power SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers for μ receptors (forward: ACCCGAAAAGTCTGAGTGCT; reverse: GAGCTAAGGGGTCTGAGCAG), ECE2 (forward: AATGAAA TCGTCTTCC; reverse: GTCAGTGACTCATTCT) and GAPDH, a housekeeping gene used as an internal control (forward: TCAAGAAGGTGGTGAAGCAG; reverse: AGGTGGAAGAATG GGAGTTG). The data were analysed using the detection system software (version 2.2.1; Applied Biosystems) that generates a baseline subtracted amplification plot of normalized reporter values (ΔRn) versus cycle number. The amplification threshold was set at 6–7 of ΔRn linear dynamic range (50–60% of maximum ΔRn). The fractional cycle at which the intersection of amplification threshold and the plot occurs is defined as the threshold cycle (Ct value) for the plot.

Immunocytochemistry and confocal microscopy

Immunocytochemical staining and confocal microscopy were carried out as described previously with some modifications (Rozenfeld and Devi, 2008). Briefly, CHO cells (5000 cells) expressing HA-ECE2, or co-expressing FLAG μ receptors and HA-ECE2, or F11 cells (5000 cells) were plated on polylysine coated coverslips in 12 well dishes (Corning, Corning, NY, USA). Following different treatment conditions, cells were fixed using 4% paraformaldehyde in PBS for 15 min. Following fixation, cells were rinsed repeatedly with PBS and then permeabilized with 0.1% Triton X-100 in PBS. Cells were then blocked using 4% BSA in PBS for 1 h. Following initial blocking, cells were incubated with primary antibodies to either the epitope tags (1:500 in 4% BSA in PBS of anti-FLAG or anti-HA antibodies), μ receptors (1:1000 in 4% BSA in PBS), ECE2 (1:500 in 4% BSA in PBS) or EEA1 (1:500 in 4% BSA in PBS) for 1 h. Following extensive rinsing with PBS, cells were incubated with anti-mouse Alexa-594 or anti-rabbit or anti-rat Alexa-488 conjugated secondary antibodies (1:1000 in 4% BSA in PBS, Invitrogen) for 1 h, rinsed thoroughly and fixed using Prolong Gold anti-fade reagent containing DAPI (Invitrogen). Images were taken using a Leica TCS SP5 DM confocal fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany), and analysed using ImageJ software (http://rsbweb.nih.gov/ij/).

Animal studies

Male C57BL/6 mice (25–35 g; 6–12 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were maintained on a 12 h light/dark cycle with rodent chow and water available *ad libitum*, and they were housed in groups of five until testing. Animal studies were carried out according to protocols approved by the Icahn School of Medicine at Mount Sinai Animal Care and Use Committee. Mice (5–8 per group) were injected i.t. with either vehicle [6% DMSO, 5% Tween 80 containing 1X protease inhibitor cocktail (Cat. No. P2714; Sigma-Aldrich) and 1 nmol thiorphan in saline], dynorphin B (10 nmol), fentanyl (0.3 nmol) or [Leu]enkephalin (10 nmol) in vehicle without or with S136492 (3 nmol in vehicle), and analgesia was measured using the tail-flick assay (Gomes *et al.*, 2013d). The intensity of the heat source was set to 10 (this results in a basal tail-flick latency of 5–7 s for most animals) and the tail-flick latency was recorded at the indicated time period (0–90 min) after vehicle or drug administration. Cut-off latency was set at 20 s to minimize tissue damage. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Statistical analysis

GraphPad Prism 4.0 (GraphPad, La Jolla, CA, USA) was used for data analysis. The average of the replicates of each individual experiment was used for calculation of mean ± SEM; statistical significance was determined using mean ± SEM (n = independent experiments) and either Student's t-test or one-way ANOVA (Bonferroni's multiple comparison test) where applicable.

Materials

CHO cells were from American Type Culture Collection (Manassas, VA, USA). DMEM, F12 medium, penicillin-streptomycin, geneticin, hygromycin and lipofectamine were

from Invitrogen (Carlsbad, CA, USA). F11 cells (DRG-derived cells), S136492 (ChemSpider ID 22902217), phosphoramidon, thiorphan, protease inhibitor cocktail and anti-Flag M1 mouse monoclonal antibody were from Sigma-Aldrich (St. Louis, MO, USA). Anti-haemagglutinin (HA) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA) and anti-EEA1 antibodies were from BD Biosciences (San Jose, CA, USA). DAMGO (Tyr-D-Ala-Gly-NMe-Phe-Gly-ol), fentanyl, cycloheximide, chloroquine, captopril, dynorphin B (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr), leucine-enkephalin ([Leu]enkephalin) (Tyr-Gly-Gly-Phe-Leu) and BAM22 (Tyr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-Tyr-Gly) were from Tocris Bioscience (Minneapolis, MN, USA).

Results

ECE2 is a metallopeptidase that exhibits an acidic pH optimum (Emoto and Yanagisawa, 1995; Turner and Murphy, 1996; Yanagisawa *et al.*, 2000; Devi and Miller, 2013; Pacheco-Quinto and Eckman, 2013), localizes to an intracellular endocytic compartment (Devi and Miller, 2013; Pacheco-Quinto and Eckman, 2013) and selectively processes neuropeptides including opioid peptides *in vitro* (Mzhavia *et al.*, 2003). In this study, we explored the idea that ECE2, by processing endocytosed peptides, affects receptor trafficking and modulates receptor activity. For this, we used opioid peptides and their receptors (μ receptors) as a model. To test the role of ECE2 in receptor trafficking, we focused on receptor recycling and compared the rate and extent of recycling in cells co-expressing ECE2 and μ receptors with cells expressing μ receptors alone. DAMGO, a μ receptor-selective synthetic peptide, and dynorphin B, an endogenous opioid peptide with μ receptor activity (Chavkin *et al.*, 1985; Mansour *et al.*, 1995; Alt *et al.*, 1998), were used as agonists. We found that the expression of ECE2 significantly increased ($P < 0.001$) the extent of recycling in response to either agonist (Figure 1). The rate of receptor recycling in cells with ECE2 was much faster than in cells without ECE2; the $t_{1/2}$ of recycling with ECE2 was ~5–10 min faster than the $t_{1/2}$ of recycling without ECE2 (Figure 1B). This increase in rate and extent of recycling suggests that ECE2 facilitates trafficking via a rapid recycling pathway and this could be due to specific association with distinct endocytic proteins and/or direct association with the receptor as seen in the case of ECE1 and somatostatin receptors (Zhao *et al.*, 2013).

Because ECE2 is a peptide processing enzyme, we examined if the enzymatic activity played a role in receptor trafficking. For this, we used the selective inhibitor of ECE2, S136492, that was previously shown to be selective and potent (Gagnidze *et al.*, 2008). Firstly, we confirmed the selectivity of S136492 for ECE2 by comparing its ability to inhibit the closely related enzyme ECE1 (Figure 2). We found that S136492 (20 μM) completely inhibited ECE2 activity when assayed at pH 5.5 (Figure 2A) and exhibited an IC_{50} of 1.6 ± 0.1 μM (Figure 2C); ECE2 did not exhibit detectable activity at neutral pH. S136492 (20 μM) had no effect on ECE1 activity either at its optimal pH of 7.4 or at the acidic pH of 5.5 (Figure 2B). Next, we examined the selectivity of S136492 using tissue from wild-type animals (because they contain a repertoire of enzymes with properties similar to ECE2) and compared it to tissue from animals lacking ECE2 (that should have all other enzymes except ECE2). In wild-type membranes, the activity at low pH, but not that at neutral pH, was completely inhibited by 20 μM S136492 (Figure 2D). In contrast, in membranes from animals lacking ECE2, neither the activity at low pH nor that at neutral pH was affected by 20 μM S136492 (Figure 2E). It should be noted that we did observe activity at low pH in animals lacking ECE2; this suggests that the absence of ECE2 leads to a compensatory increase in the expression of related enzymes with peptidase activity at acidic pH; the fact that this activity was not affected by S136492 indicates that this inhibitor is highly selective for ECE2 and hence suitable for studies exploring the biological role of ECE2 activity in regulating receptor trafficking.

As the presence of ECE2 affected receptor recycling (Figure 1), we wondered if ECE2 activity affected receptor

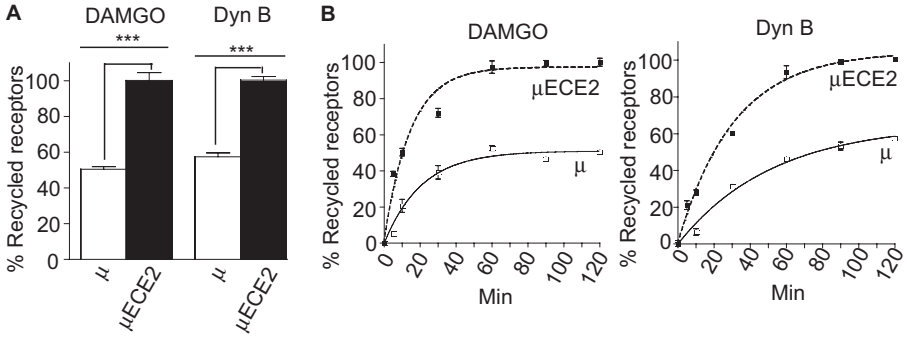
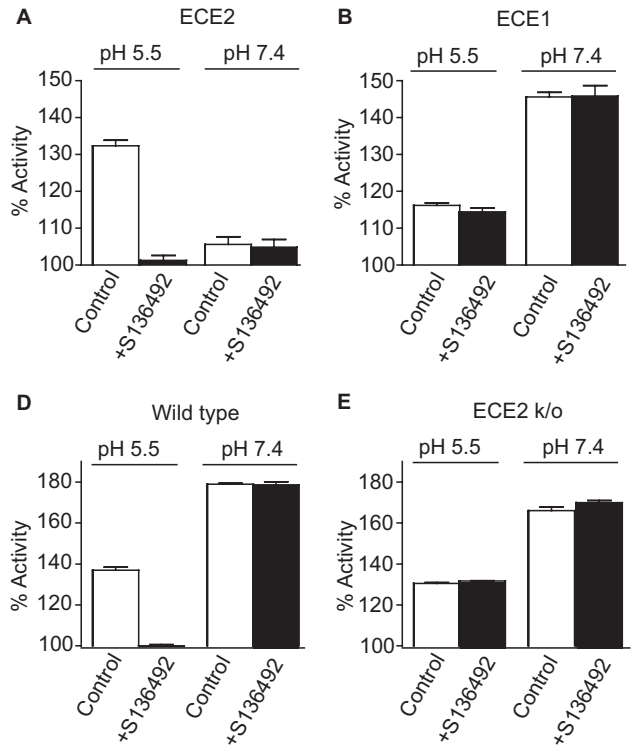


Figure 1

ECE2 expression leads to enhanced recycling of μ opioid receptors. CHO-μ or CHO-μ-ECE2 cells were incubated with 1 μM DAMGO or 100 nM dynorphin B (Dyn B) for 30 min ($t = 0$); cells were washed and incubated for either 120 min (A) or the indicated time periods (B) without the agonist. Cell surface receptors were quantified by ELISA as described in Methods. Levels of cell surface receptors before agonist treatment were taken as 100% for each individual experiment. % recycled receptors were calculated by subtracting surface receptors at $t = 0$ (30 min internalization) from each recycling time point. The data represent mean ± SEM from four to five independent experiments carried out in quadruplicate. $^{***}P < 0.001$, Student's *t*-test.



C

Enzyme	IC_{50} (μM)		E_{max} (% inhibition)	
	pH 5.5	pH 7.4	pH 5.5	pH 7.4
ECE2	1.6 ± 0.1	>100	94 ± 2	<1
ECE1	>100	>100	<10	<10

Figure 2

Selectivity of the inhibitor S136492 for ECE2. (A, B) Purified ECE2 (A) or ECE1 (B) was assayed for 10 min in the absence or presence of S136492 (20 μM) at pH 5.5 or pH 7.4 using the synthetic quenched fluorescent substrate, McaBk2, as described previously (Gagnidze *et al.*, 2008). Relative fluorescence units before the addition of substrate (i.e. $t = 0$) were taken as 100%. (C) The IC_{50} values were derived by carrying out enzymatic assays in the absence or presence of SM136492 (0–50 μM) at pH 5.5 or pH 7.4 as described previously (Gagnidze *et al.*, 2008). Data represent mean ± SEM of three independent experiments in triplicate. (D, E) Solubilized midbrain preparations from wild-type (D) and ECE2 knockout mice (ECE2 k/o) (E) were assayed for their enzymatic activity for 10 min using the synthetic quenched fluorescent substrate, McaBk2, in the absence or presence of S136492 (20 μM) at pH 5.5 or pH 7.4 as described previously (Gagnidze *et al.*, 2008). Relative fluorescence units before the addition of substrate (i.e. $t = 0$) were taken as 100%. Data represent mean ± SEM from three animals per group in triplicate.

internalization as well. The extent of receptor internalization was measured by the loss of antibody-labelled cell surface receptors upon treatment with DAMGO or dynorphin B. Inhibiting ECE2 did not lead to significant changes in the extent of receptor internalization mediated by either agonist (Figure 3A). In contrast, when the extent of receptor recycling was measured by quantifying the reappearance of cell surface receptors upon agonist removal for 60 min, we found that this is significantly diminished ($P < 0.001$) by the inhibitor (Figure 3B). The potency of S136492 to inhibit recycling in response to DAMGO and dynorphin B was in the low micromolar range (Figure 3C). Together, these results support the idea that ECE2 activity significantly modulates μ receptor trafficking by affecting recycling but not internalization.

Next, we compared the specific requirement for ECE2 activity for receptor recycling by comparing the level of inhibition by the ECE2 inhibitor, S136492, to that induced by inhibitors of related enzymes. Furthermore, because ECE2 exhibited optimum activity at acidic pH, the effect of agents that inhibit acidification of intracellular organelles was examined (Figure 4). Inhibitors of ECE2 (S136492) or the ECE family (phosphoramidon) but not inhibitors of the related neprilysin (thiorphan) or angiotensin-converting enzyme (captopril) significantly affected the recycling of the receptors internalized in response to DAMGO or dynorphin B (Figure 4). Agents that inhibited the acidification of endocytic compartments also blocked receptor recycling (Figure 4A and C). Together, these results support a role for ECE2 activity and an acidic environment in modulating receptor recycling.

In order to play a role in receptor recycling, ECE2 would have to be located intracellularly in a compartment similar to that involved in receptor trafficking such as early and recycling endosomes. To examine this, we carried out co-localization studies with the early endosomal marker, EEA1, and found substantial co-localization (Figure 5A). ECE2 also exhibited substantial co-localization with μ receptors upon treatment with DAMGO (Figure 5B). Together, these results imply that ECE2 is co-localized with the internalized receptor in an endocytic compartment. Next, we examined if the ligand endocytosed with the receptor could be processed by ECE2. Firstly, the ability of purified ECE2 to process radiolabelled DAMGO was tested. For this, radiolabelled DAMGO was incubated with purified ECE2 for 30 min at pH 5.5 in the absence or presence of 20 μM of the ECE2 inhibitor, S136492. The extent of processing was determined by fractionating the

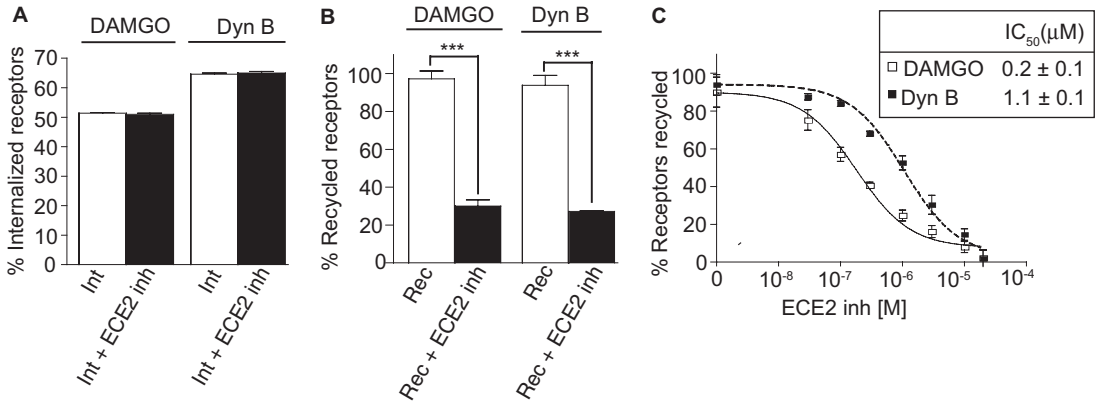


Figure 3

ECE2 activity is required for modulating the recycling but not the internalization of μ opioid receptors. (A) Receptors in CHO-μ-ECE2 cells were pre-labelled with anti-FLAG antibody (1 μg) for 60 min at 4°C (t = 0) as described in Methods. Pre-labelled cells were incubated with DAMGO (1 μM) or dynorphin B (Dyn B, 100 nM) for 30 min to allow for receptor internalization without (Int) or with 20 μM S136492 (Int + ECE2 inh). The level of surface receptors determined by ELISA at t = 0 for each individual experiment was taken as 100 % surface receptors. % internalized receptors were calculated as 100 – % surface receptors after agonist treatment. (B) CHO-μ-ECE2 cells were incubated with DAMGO (1 μM) or dynorphin B (100 nM) for 30 min (t = 0); cells were washed and incubated for 60 min in medium without (Rec) or with 20 μM S136492 (Rec + ECE2 inh). (C) CHO-μ-ECE2 cells were incubated with DAMGO (1 μM) or dynorphin B (100 nM) for 30 min (t = 0); cells were washed and incubated for 60 min in medium containing 0–50 μM S136492 (ECE2 inh). Cell surface receptors were quantified by ELISA as described in Methods. The level of surface receptors before agonist treatment for each individual experiment was taken as 100%. % recycled receptors (B, C) were calculated by subtracting % of surface receptors at t = 0 (30 min internalization) from each recycling time point. Data represent mean ± SEM of five independent experiments in triplicate. $^{***}P < 0.001$; Student's t-test.

reaction mixture by thin-layer chromatography to separate the substrate from the processed product(s). Radiolabelled DAMGO in the case of control (with no ECE2) or ECE2 in combination with the inhibitor was enriched in fraction #13, whereas the presence of ECE2 alone led to a leftward shift in this peak (to fraction #12), implying that DAMGO is processed by ECE2 (Figure 5C–E). When the co-endocytosed peptide was examined for its ability to be processed by ECE2, we found that more than 50% of the peptide was processed within 30 min of receptor endocytosis (Figure 5F). Pretreatment with the ECE2 inhibitor, S136492, or with chloroquine [an agent that increases the intracellular pH (Trapaidze *et al.*, 1996; Ippoliti *et al.*, 1998)] but not by captopril [an angiotensin-converting enzyme inhibitor (Vermeirssen *et al.*, 2002)] essentially completely blocked the processing of the endocytosed peptide (Figure 5F). Together, these results support the idea that endocytosed opioid peptides are processed by enzymes such as ECE2, and this process could affect receptor recycling.

If ECE2 activity is involved in modulating receptor recycling by processing the endocytosed peptide agonist, then receptor recycling mediated by non-peptide agonists (not hydrolysed by ECE2) should not be affected by the ECE2 inhibitor. We tested this hypothesis by comparing the rate and extent of recycling mediated by the peptidic agonists (DAMGO and dynorphin B) to that mediated by a non-peptidic agonist (fentanyl). Recycling in response to internalization by fentanyl was not impaired by S136492 whereas recycling in response to DAMGO or dynorphin B was significantly ($P < 0.001$) and robustly inhibited (Figure 6). These results together with the previous finding that ECE2 selectively processes opioid peptides (Mzhavia *et al.*, 2003) suggest that peptide processing by ECE2 affects the rate and extent of receptor recycling. To test this hypothesis further, we used additional opioid peptides (substrates and non-substrates of ECE-2). For example, BAM22, a pro-enkephalin-derived peptide, was found to be processed by ECE2, whereas [Leu]enkephalin was not (Mzhavia *et al.*, 2003). Both of these peptides bind to μ receptors with relatively high affinity (Mansour *et al.*, 1995). As with DAMGO and dynorphin B, recycling of receptors internalized by BAM22 was significantly ($P < 0.001$) impaired by S136492 while that of receptors internalized by [Leu]enkephalin or by fentanyl was not (Figure 7). Together, these results are consistent with the notion that opioid receptor recycling is differentially modulated by opioid peptides based on their ability to serve as substrates of ECE2.

Next, we examined the functional consequence of regulation of receptor recycling by ECE2 by measuring the effect of ECE2 inhibition on receptor signalling (decrease in intracellular cAMP). Cells co-expressing ECE2 and μ receptors were treated with agonists (DAMGO, dynorphin B or fentanyl) for 30 min and incubated in the absence of the agonist for 60 min (recycling) in the absence or presence of the inhibitor. The ability of the recycled receptors to signal was then quantified by measuring the extent of the decrease in intracellular cAMP in response to a second 5 min pulse with the respective agonists. A significant ($P < 0.001$) decrease in cAMP levels in the absence of the inhibitor was observed and this was not seen in the presence of the inhibitor in the case of DAMGO and dynorphin B (Figure 8A and B). In contrast, in the case of fentanyl (a non-peptide agonist), the significant decrease in cAMP levels seen in the absence of the inhibitor was not affected by its presence (Figure 8C); this supports the idea that the ECE2 inhibitor, by blocking the processing of internalized peptide substrates, significantly impairs receptor

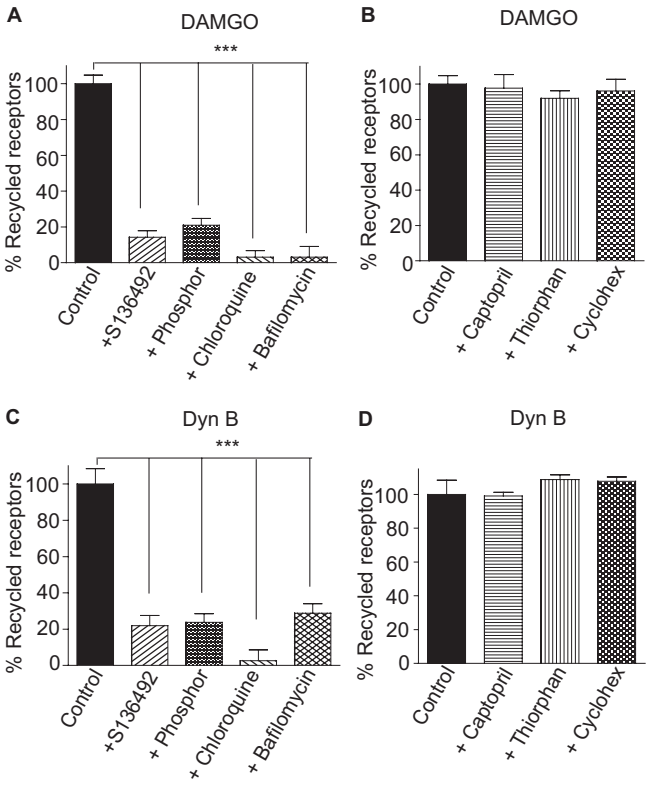


Figure 4

ECE2 and an acidic pH are required for modulation of μ opioid receptor recycling following endocytosis by agonists that are ECE2 substrates. CHO-μ-ECE2 cells were incubated with DAMGO (1 μM) (A, B) or dynorphin B (Dyn B, 100 nM) (C, D) for 30 min ($t = 0$); cells were washed and incubated for 60 min with medium without (control) or with 20 μM ECE2 inhibitor (+S136492), 10 μM phosphoramidon (+Phosphor), 100 μM chloroquine, 100 nM bafilomycin, 10 μM captopril, 10 μM thiorphan or 100 nM cycloheximide (+Cyclohex). Cell surface receptors were quantified by ELISA as described in Methods. % recycled receptors were calculated as described in legend to Figure 1 and % recycled receptors obtained with control were expressed as 100%. Data represent mean ± SEM of five to eight independent experiments in triplicate. $^{***}P < 0.001$; one-way ANOVA.

recycling and signalling. Together, these results support a role for ECE2 in modulating receptor signalling specifically by affecting the extent of resensitization.

In order to determine the role of endogenous ECE2 activity in modulating recycling of native receptors, we used a DRG-derived cell line, F11, that has many features of DRG cells including native opioid receptors (Fan *et al.*, 1992; Jow *et al.*, 2006). First we ensured that F11 cells express native μ receptors and ECE2 by quantitative PCR analysis, and also that both small and large DRG neurons express ECE2 (Figure 9A). We then carried out immunocytochemical analysis to examine the localization of ECE2 and observed substantial co-localization with EEA1, a marker of early endosomes (Figure 9B). To see if the localization of ECE2 was enhanced upon receptor internalization, we used a rat polyclonal antibody against endogenous μ opioid receptors; this antibody exhibits μ receptor selectivity and recognizes μ receptors in wild-type tissue but not in tissues from animals lacking μ receptors (Gupta *et al.*, 2007). Treatment with DAMGO for 30 min enhanced the co-localization of the receptor with ECE2, and removal of the agonist for 60 min decreased the level of co-localization (Figure 9C). Importantly, this decrease was not seen in cells treated with the inhibitor during the recycling phase, indicating that inhibitor treatment leads to retention of the receptor and ECE2 in the same compartment.

Next, we examined the effect of the ECE2 inhibitor on the time course of recycling of native μ receptors in F11 cells internalized by either DAMGO or dynorphin B (substrates of ECE2). In both cases, S136492 significantly ($P < 0.001$) blocked the rate and extent of recycling (Figure 10A and B). Interestingly, while the inhibitor robustly impaired recycling in response to DAMGO or dynorphin B, it had no effect on the extent of recycling in response to [Leu]enkephalin (non-substrate of ECE-2, Figure 10). Together, these results imply that recycling by native μ receptors is modulated by endogenous ECE2, and that receptor trafficking can be differentially regulated by multiple opioid agonists based on their ability to be processed by ECE2, and this, in turn, would differentially affect receptor signalling.

To examine the effect of inhibition of endogenous ECE2 on the extent of signalling, native receptors internalized by DAMGO, dynorphin B or fentanyl were allowed to recycle in the absence or presence of the inhibitor, and the response of the cells (intracellular cAMP levels) to a second pulse of agonist treatment under these conditions was examined. As expected, F11 cells treated with DAMGO or dynorphin B without the inhibitor during the recycling phase responded to a second pulse of the agonist, while the presence of the inhibitor during the recycling phase blocked this effect (Figure 11A and B). Inhibitor treatment did not affect the level of response (cAMP levels) in the case of receptors internalized by fentanyl; these results are consistent with the idea that inhibition of ECE2 activity inhibits receptor recycling (by retaining receptors in an intracellular compartment), and this leads to decreases in surface receptor signalling resulting in receptor desensitization. While F11 cells are likely to contain other enzymes including ECE1 that are known to modulate receptor trafficking by processing peptides in an endosomal compartment, the selectivity of the ECE2 inhibitor used in this study (Figure 2) strongly supports a role for ECE2 in modulating μ receptor activity and function in this native system, and suggests that this inhibitor is suitable for exploring the role of ECE2 in *in vivo* studies.

Next, we examined a role for ECE2 activity in modulating opioid receptor function *in vivo*. For this, we selected dynorphin B and [Leu]enkephalin that represent an endogenous opioid peptide substrate and a non-substrate of ECE2 respectively; fentanyl (a synthetic μ receptor agonist) was used as a control. [Leu]enkephalin, dynorphin B or fentanyl was administered i.t. in the absence or presence of the ECE2 inhibitor to C57BL/6 mice, and the effect of ECE2 inhibition on antinociception over a period of 90 min was examined using the tail-flick assay (Gomes *et al.*, 2013d). It should be noted that the peptide agonists were used with a protease inhibitor cocktail (containing inhibitors of peptidases that hydrolyse short peptides) because exogenously administered peptides are prone to non-specific degradation that could affect receptor trafficking (Song and Marvizon, 2003). We also

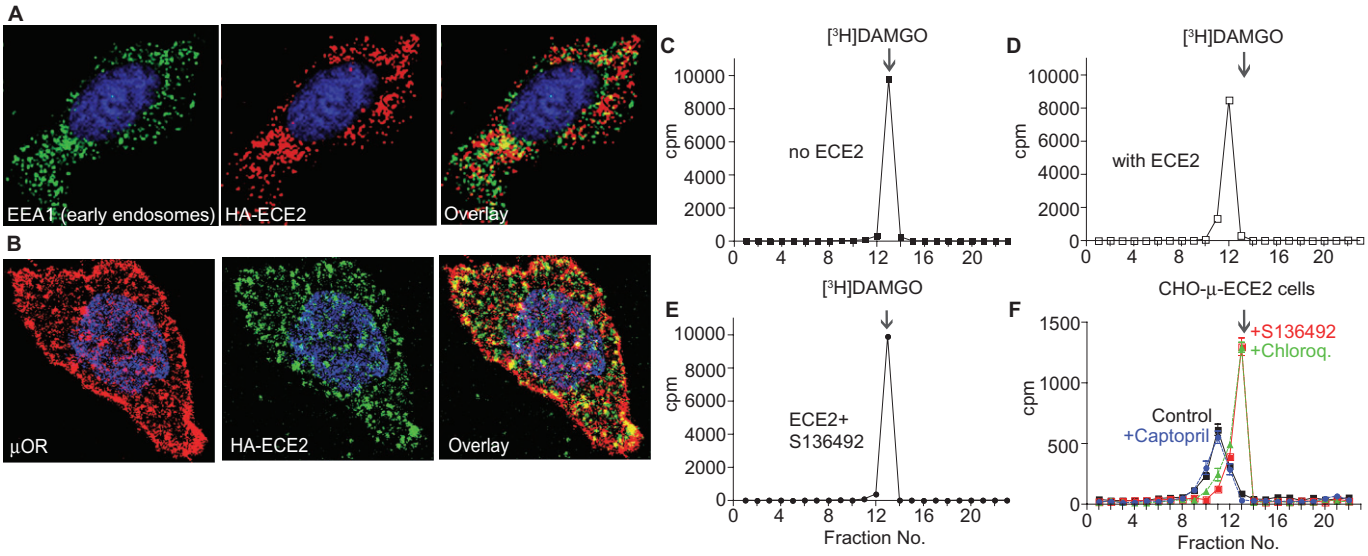


Figure 5

ECE2 co-localizes with μ opioid receptors in intracellular compartments and cleaves [^{3}H]-DAMGO at acidic pH. (A) Co-localization of ECE2 with EEA1, a marker for early endosomes. CHO-μ-ECE2 cells were stained with antibodies to the HA tag on ECE2 or to EEA1 and staining visualized by microscopy as described in Methods. (B) Co-localization of ECE2 with μ opioid receptors in intracellular compartments. CHO-μ-ECE2 cells were stained with antibodies to FLAG or HA tags and staining visualized by microscopy as described in Methods. (C) [^{3}H]-DAMGO (10 nM) was incubated without (C) or with purified ECE2 (D, E) at pH 5.5 for 30 min in the absence (D) or presence of 20 μM S136492 (E) followed by thin-layer chromatographic analysis as described in Methods. (F) CHO-μ-ECE2 cells were incubated with 10 nM [^{3}H]-DAMGO for 30 min at 37°C without or with either 20 μM S136492, 10 μM captopril or 100 μM chloroquine. The cells were subjected to acid wash to remove surface bound radiolabel, lysed, and subjected to thin-layer chromatographic analysis as described in Methods. The arrows indicate the position of full-length [^{3}H]-DAMGO. Data in (C–F) represent mean ± SEM of four to six independent experiments.

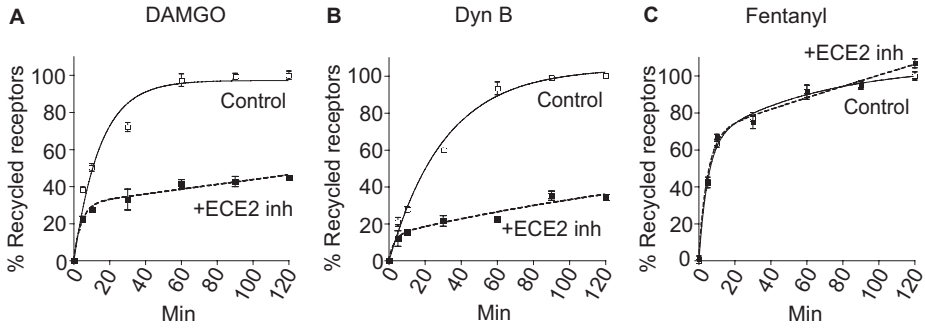


Figure 6

Recycling of receptors endocytosed by a peptidic agonist (and not by a non-peptidic agonist) is blocked by ECE2 inhibition. CHO-μ-ECE2 cells were incubated with DAMGO (1 μM) (A), dynorphin B (Dyn B, 100 nM) (B) or fentanyl (1 μM) (C) for 30 min (t = 0); cells were washed and incubated for various time periods in medium without (control) or with 20 μM S136492 (+ECE2 inh). Cell surface receptors were quantified by ELISA as described in Methods. Levels of cell surface receptors before agonist treatment for each individual experiment were taken as 100%. % recycled receptors were calculated by subtracting surface receptors at t = 0 (30 min internalization) from each recycling time point. Data represent mean ± SEM of five to eight independent experiments in triplicate.

included thiorphan (1 nmol), an enkephalinase inhibitor in the cocktail, because this has been shown to protect opioid peptides from degradation and to enhance opioid peptide-mediated analgesia (Chipkin *et al.*, 1982; Stevens *et al.*, 1995). Administration of the inhibitor S136492 alone had a small, but not significant effect on tail-flick latency (Figure 12). Fentanyl administration, in the absence or presence of S136492, led to a rapid increase in tail-flick latency by 3 min and returned to basal levels by 30 min (Figure 12A). Similarly, [Leu]enkephalin administration, in the absence or presence of the inhibitor, led to a rapid increase in tail-flick latency within 3 min that returned to basal levels by about 10 min (Figure 12B). In contrast, while dynorphin B administration in the absence of the inhibitor led to a rapid increase in tail-flick latency, that is, as robust as the latency elicited by [Leu]enkephalin and fentanyl, the presence of the inhibitor

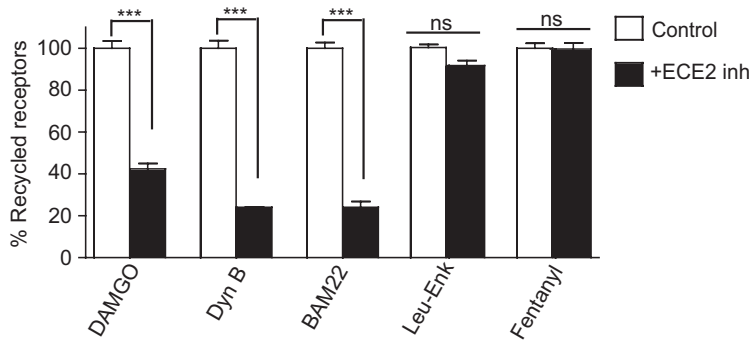


Figure 7

ECE2 inhibition prevents recycling of μ opioid receptors endocytosed following exposure to endogenous peptides that are the substrates of ECE2. CHO-μ-ECE2 cells were incubated with either DAMGO (1 μM), dynorphin B (Dyn B, 100 nM), BAM22 (100 nM), [Leu]enkephalin (100 nM) or fentanyl (1 μM) for 30 min ($t = 0$); cells were washed and incubated for various time periods in medium without (control) or with 20 μM S136492 (+ECE2 inh). Cell surface receptors were quantified by ELISA as described in Methods. Levels of cell surface receptors before agonist treatment for each individual experiment were taken as 100%. % recycled receptors were calculated by subtracting surface receptors at t = 0 (30 min internalization) from each recycling time point and expressing % recycled receptors obtained with control as 100. Data represent mean ± SEM of five independent experiments in triplicate. $^{***}P < 0.001$; ns, not significant; Student's t-test.

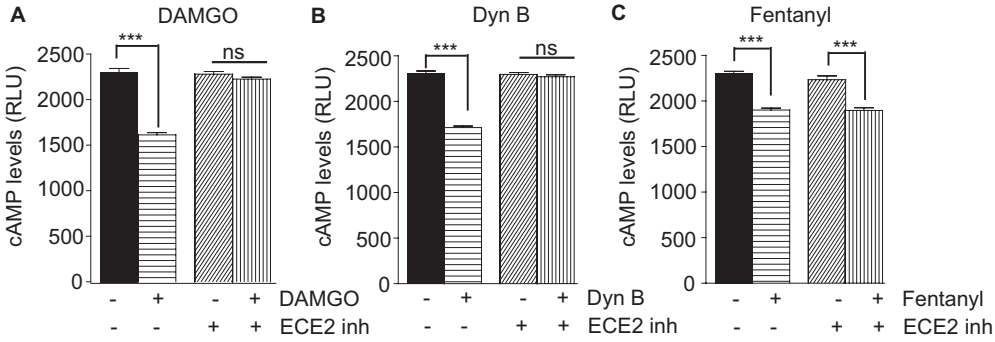


Figure 8

Inhibition of ECE2 activity affects μ opioid receptor resensitization. CHO-μ-ECE2 cells were incubated with DAMGO (1 μM) (A), dynorphin B (Dyn B, 100 nM) (B) or fentanyl (1 μM) (C) along with 10 μM forskolin for 20 min; cells were then washed and incubated for 60 min (recycling phase) in medium without or with 20 μM S136492 (ECE2 inh). Cells were washed and cAMP levels in response to a second 5 min treatment with DAMGO, dynorphin B or fentanyl were determined using the DiscoveRx kit as described in Methods. Data represent mean ± SEM of five independent experiments in sixtuplicate. RLU, relative luminescence units; $^{***}P < 0.001$; ns, not significant; Student's t-test.

led to a significant ($P < 0.05$) attenuation of the dynorphin B-mediated antinociception (Figure 12C). These results showing that the antinociceptive activity of dynorphin B (substrate) but not of fentanyl or [Leu]enkephalin (non-substrates) was attenuated by the ECE2 inhibitor suggest that the ability of the peptides to be processed by ECE2 contributes to opioid peptide-mediated antinociception *in vivo*.

Discussion

Neuropeptide receptor functions are modulated by multiple mechanisms including receptor internalization/desensitization and recycling/resensitization. While several studies have focused on the endocytosed receptor, few studies have focused on the co-endocytosed neuropeptide and examined its role in modulating receptor function. Although it is generally accepted that peptidases in the endocytic compartment selectively process the endocytosed peptide, little is known about the specific peptidases involved. We previously reported that ECE2, a metalloendopeptidase, selectively hydrolyses neuropeptides including opioid peptides at the endocytic pH *in vitro* (Devi and Miller, 2013). In this study, we examined the involvement of ECE2 in modulating μ opioid receptor function. Using μ receptor agonists that are ECE2 substrates, we observed that the presence of ECE2 protein was sufficient to enhance receptor trafficking, and that the inhibition of ECE2 activity blocked receptor recycling and resensitization (and had no effect on receptor internalization). Receptor recycling was also blocked by agents that prevent endosomal acidification, such as chloroquine or bafilomycin, and this is consistent with a requirement for acidic pH in this process (Mellman *et al.*, 1986). Taken together with the acidic pH preference of ECE2, these findings strongly suggest that

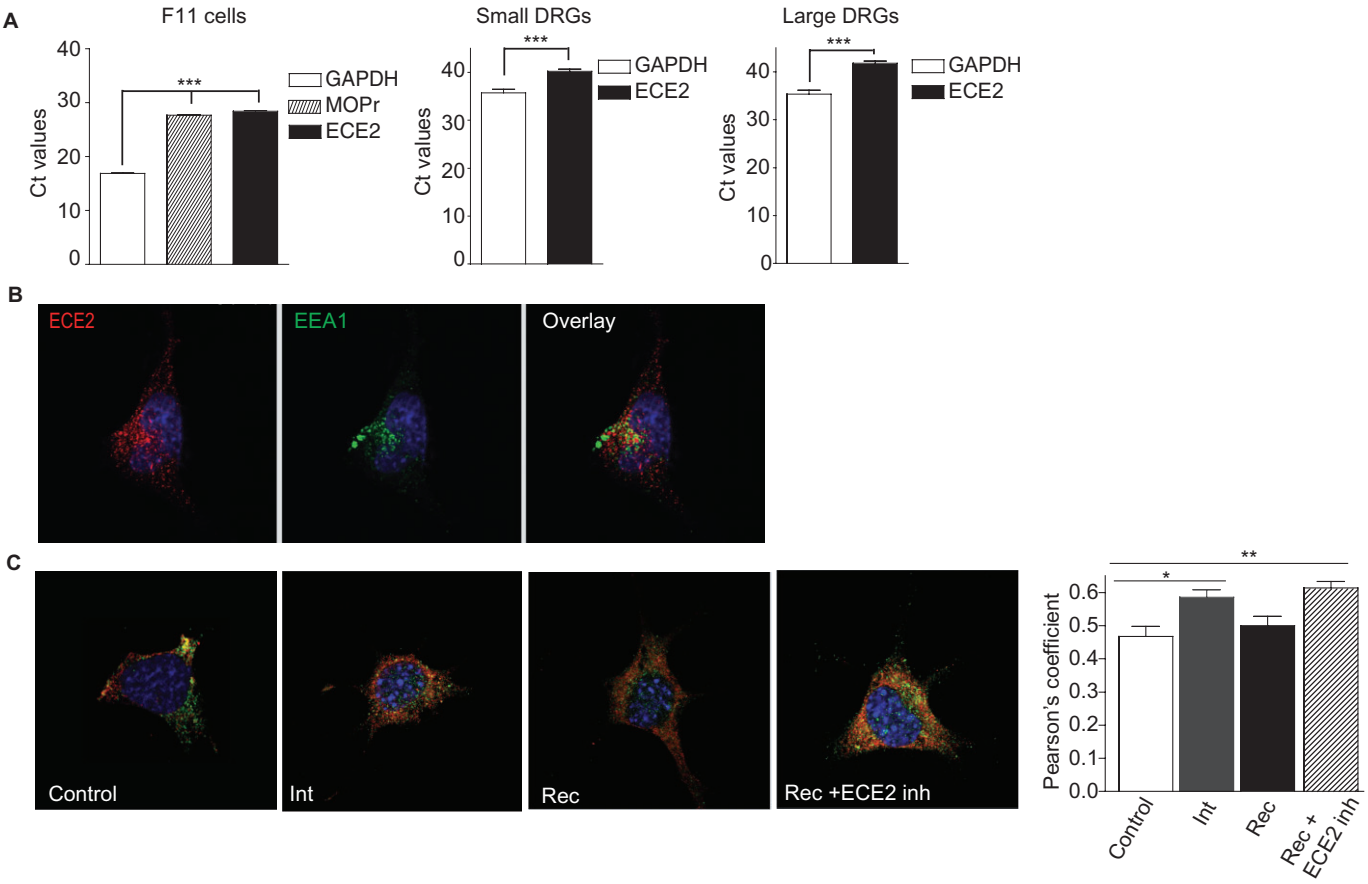


Figure 9

Co-localization of ECE2 and μ opioid receptors in F11 cells and DRGs. (A) qRT-PCR analysis shows that F11 cells, small and large DRGs, express ECE2 mRNA. Data represent mean ± SEM (n = 3–6). ***P < 0.001. (B) Co-localization of ECE2 with EEA1, a marker for early endosomes. F11 cells were stained with antibodies to ECE2 or EEA1 and staining was visualized by microscopy as described in Methods. There is substantial co-localization of ECE2 (red) with EEA1 (green). (C) Co-localization of ECE2 with μ opioid receptors in intracellular compartments. F11 cells were stained with antibodies to μ receptors and to ECE2, and staining was visualized by microscopy as described Methods. Pearson's coefficient shows that during receptor internalization (Int; agonist treatment for 30 min), there is an increase in co-localization of μ opioid receptors (green) with ECE2 (red), which is reduced during recycling with media (Rec) but not following inhibition of ECE2 activity during recycling (Rec + ECE2 inh). n = 10 fields (10–15 cells/field). *P < 0.05; **P < 0.01; ***P < 0.001; Student's t-test.

ECE2 activity in an endocytic compartment is involved in modulating μ receptor recycling and resensitization.

Subcellular localization studies revealed that both exogenously expressed ECE2 and native ECE2 co-localize with endosomal markers, and this localization of ECE2 confirms the finding that ECE2 localizes to early and recycling endosomes in a human neuroblastoma cell line (Pacheco-Quinto and Eckman, 2013). The latter study also showed that intracellular ECE2 is able to degrade amyloid peptides and an acidic pH environment is required for this process. In the present study, we found that ECE2 processes other small bioactive peptides, such as opioid peptides that are co-endocytosed with the receptor (Figure 5). Several reports with a related enzyme, ECE1, using a variety of bioactive peptides, including substance P (Roosterman *et al.*, 2007; Cottrell *et al.*, 2009), neurokinin A (Cattaruzza *et al.*, 2009), corticotrophin releasing factor (Hasdemir *et al.*, 2012) and calcitonin gene-related peptide (Padilla *et al.*, 2007), have demonstrated the involvement of ECE1 in modulating cognate receptor function by processing the co-internalized peptides in an endocytic compartment. To date, the ability of ECE1 to process opioid peptides and modulate opioid receptor function has not been reported. Our finding that ECE2 processes opioid peptides and is able to modulate opioid receptor recycling supports a novel role for this family of enzymes in modulating peptide receptor function by affecting its endocytic processing.

An interesting observation made in the present study is that the presence of ECE2 speeded up the recycling of μ receptors in a ligand-dependent manner. It is possible that the presence of ECE2 facilitates a switch from a 'long or slow' recycling pathway to a 'short or fast' recycling pathway, and the rate of recycling is further modulated when peptide agonists that are its substrates are used as receptor ligands. The existence of such 'short' and 'long' recycling pathways has been documented in the case of 5-HT_{1A} receptors that were found to recycle via the Rab4 positive 'short' and Rab11 positive 'long' endosomal recycling pathways (Fichter *et al.*,

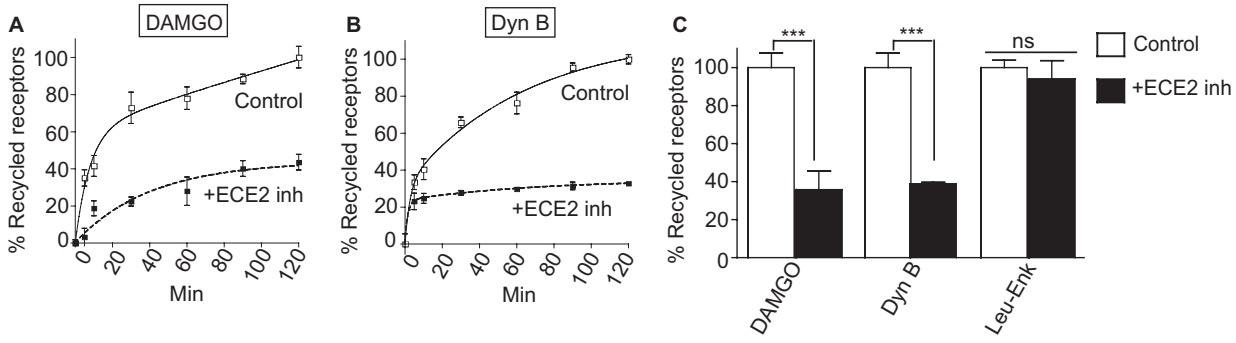


Figure 10

Inhibition of endogenous ECE2 activity in F11 DRG-derived cell line impairs recycling of native μ opioid receptors. F11 cells were incubated with 1 μM DAMGO (A) or 100 nM dynorphin B (B) for 30 min ($t = 0$); cells were washed and incubated (0–120 min) in medium without (control) or with 20 μM S136492 (+ECE2 inh). (C) F11 cells were incubated with 1 μM DAMGO, 100 nM of either dynorphin B, or [Leu]enkephalin, for 30 min ($t = 0$); cells were washed and incubated for 60 min in medium without (control) or with 20 μM S136492 (+ECE2 inh). Cell surface receptors were quantified by ELISA as described in Methods. Levels of cell surface receptors before agonist treatment for each individual experiment were taken as 100%. % recycled receptors were calculated by subtracting surface receptors at $t = 0$ (30 min internalization) from each recycling time point. For (C), % recycled receptors obtained with control were taken as 100. The data represent mean ± SEM of five to eight independent experiments in triplicate. $^{***}P < 0.001$; ns, not significant; Student's *t*-test.

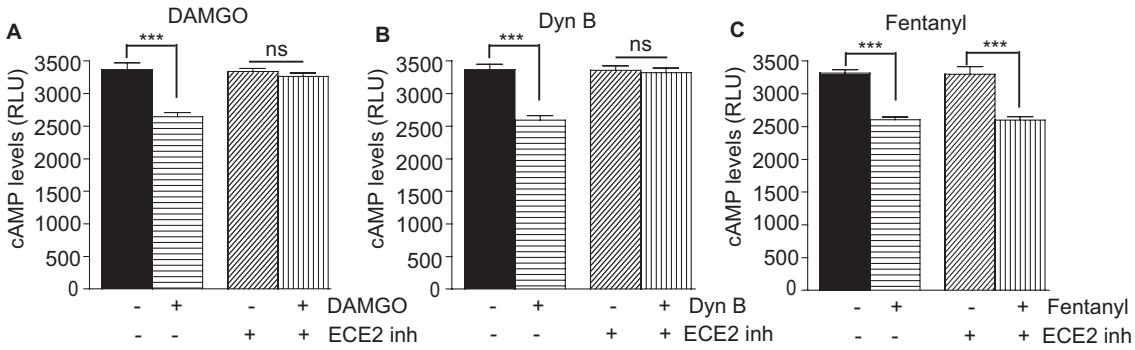


Figure 11

Inhibition of endogenous ECE2 activity affects native μ opioid receptor resensitization in F11 cells. F11 cells were incubated with DAMGO (1 μM) (A), dynorphin B (Dyn B, 100 nM) (B) or fentanyl (1 μM) (C), along with 10 μM forskolin for 20 min; cells were then washed and incubated for 60 min in medium without or with 20 μM S136492 (ECE2 inh). Cells were washed and cAMP levels in response to a second 5 min treatment with DAMGO, dynorphin B or fentanyl were determined using the DiscoveRx kit as described in Methods. Data represent mean ± SEM of four independent experiments in sixtuplicate. RLU, relative luminescence units; $^{***}P < 0.001$; ns, non-significant; Student's *t*-test.

2010). Interestingly, both Rab4 and Rab11 have been shown to be involved in μ receptor recycling (Roman-Vendrell *et al.*, 2012). It will be interesting to see if ECE2 switches the pathway of receptor recycling from a Rab11 positive 'long' to a Rab4 positive 'short' pathway, and if this affects the type and extent of signalling by the endocytosed receptors.

In a recent study, we reported ECE2-mediated regulation of δ opioid receptors (Gupta *et al.*, 2014). We showed that the presence of ECE2 hastens the rate of δ receptor recycling. Furthermore, ECE2 activity was found to play an important role in regulating receptor recycling and resensitization, and this is also observed in primary neurons expressing endogenous receptors and ECE2. As seen with μ opioid receptors (Figures 7 and 10), δ receptor recycling is also differentially affected by ligands that are substrates of ECE2 in that recycling and resensitization of the receptor by substrates such as BAM22, but not by non-substrates such as [Leu]enkephalin, are decreased by ECE2 inhibition (Gupta *et al.*, 2014). It is likely that in the endocytic compartment, the internalized peptide is processed by ECE2 giving rise to peptides with differential signalling (at the same receptor or other receptors either in the endosomes or following release at the cell surface). For example, processing of BAM22 yields BAM12 (Mzhavia *et al.*, 2003); these peptides differentially bind and activate different opioid receptors (Mizuno *et al.*, 1980a,b; Davis *et al.*, 1990). It is yet to be seen if these two peptides exhibit 'biased' signalling at δ receptors and/or other opioid receptors.

In the context of signalling by the endocytosed receptor complex, an important point to consider is if the concentration of peptides internalized into endocytic compartments is high enough to bind and signal in the acidic compartment.

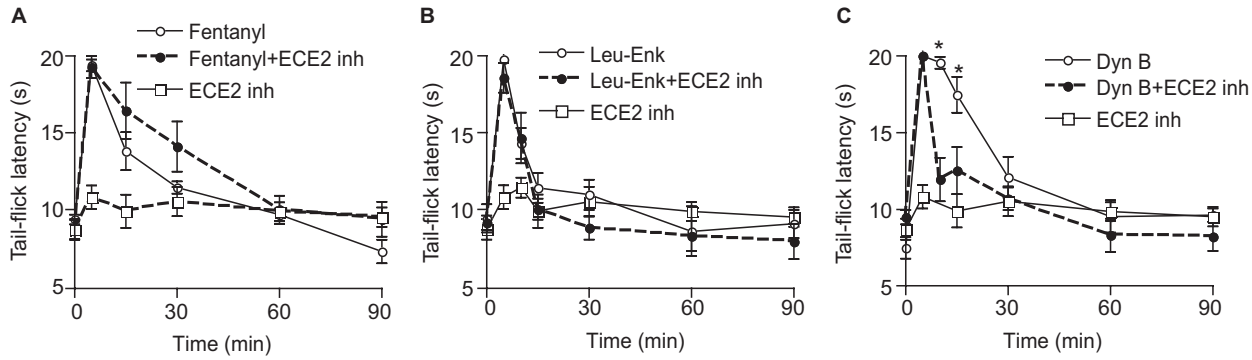


Figure 12

ECE2 plays a role in modulating opioid-mediated analgesia. C57BL/6 mice (5–8 per group) were injected (i.t.) with either vehicle (6% DMSO, 5% Tween 80 and protease inhibitor cocktail) or vehicle containing fentanyl (0.3 nmol) (A), [Leu]enkephalin (10 nmol) (B) or dynorphin B (10 nmol) (C) in the absence or presence of S136492 (ECE2 inh, 3 nmol), and analgesia was measured using the tail-flick assay at 5,10, 15, 30, 60 and 90 min. $^{*}P < 0.05$ as compared with the absence of the ECE2 inhibitor; Student's *t*-test.

With regard to the intravesicular concentration, a common misconception is that if a single peptide molecule is brought into an endosome with the receptor, the peptide's concentration would be too low to activate the receptor. However, a simple calculation reveals that this is far from the case. For example, the concentration of a single peptide in an endocytic vesicle with a diameter of 100 nm would be 3 μM; this is calculated using Avogadro's constant (6e + 23 molecules/mol or 1.7e – 24 mol/molecule) and the volume of a sphere with a diameter of 100 nm ($4/3\pi r^3$ or 5.2e-19 L). The diameter of early endocytic vesicles has been reported to be 50–100 nm (Hansen *et al.*, 1991; Stoorvogel *et al.*, 1996; Grunfelder *et al.*, 2003) whereas the diameter of recycling endosomes is reported to be around 250–500 nm (Jean-Alphonse *et al.*, 2014). Thus, the concentration of a single peptide in the endosome with a diameter of 500 nm would be 26 nM. This concentration would be higher if one considers that the number of μ receptors/endosomal vesicles has been estimated to be ~ 25–50 (Roman-Vendrell *et al.*, 2012); in such a case, the concentration of peptides co-endocytosed with the receptor in individual recycling endosomes would increase to 0.6–1.3 μM. Hence, the concentration of the agonist in the endosomes would be high enough to facilitate intracellular signalling even if the affinity of the peptide for the receptor is reduced at the low pH of endocytic compartments. In fact, when we directly examined binding of peptide ligands to opioid receptors at pH 5.5, we found that both [Leu]enkephalin and dynorphin B bind μ receptors with high affinity (~2 and 50 nM, respectively) and retained substantial binding at 1 μM (Tables 1 and 2). Given that the concentration of the ligand within the vesicle (~1 μM) is about 20- to 500-fold higher than the affinity at pH 5.5, the endocytosed peptide is likely to bind and activate receptors in the endosomal compartment. Direct examination of effect of pH on receptor signalling showed that these opioid peptide ligands elicit substantial signalling at acidic pH as seen by the retention of ~ 50% of the activity seen at pH 7.4 (Table 2). We also found that BAM22 has an IC_{50} for δ receptors of 7.4 nM at neutral pH and of 35 nM at acidic pH (using [^{3}H]-diprenorphine as the radioligand). Hence, as seen in the case of μ receptors,

Table 1

Affinity of peptide ligands for μ receptors at neutral and endosomal pH

	Binding IC_{50} (nM)	
Ligands	**pH 7.4**	**pH 5.5**
DAMGO	1.2 ± 0.1	22 ± 1.3
Dynorphin B	4.7 ± 0.1	52 ± 1.2
[Leu]enkephalin	1.4 ± 0.1	2.2 ± 0.1

Binding studies were carried out using membranes (50 μg) from cells expressing μ receptors as described in Methods. Data represent mean ± SEM (n = 3).

BAM22 co-endocytosed with δ receptors is likely to be at a high enough concentration to elicit intracellular signalling. Thus, peptides that are not substrates of ECE2 could continue to signal in the acidic pH, whereas those that are substrates either would stop signalling (if processing the peptide leads to the loss of receptor binding) or would continue to signal if the peptide is further processed to smaller bioactive peptides that are able to elicit signalling (serving as agonists). The fact that opioid peptides retain their ability to bind and signal at endosomal pH, taken together with reports of endocytic signalling by a variety of neuropeptides and bioactive peptides that are co-endocytosed with their cognate receptors (Padilla *et al.*, 2007; Roosterman *et al.*, 2007; Cattaruzza *et al.*, 2009; Cottrell *et al.*, 2009; Hasdemir *et al.*, 2012), indicates the importance of subcellular localization of the receptor in modulating the type and extent of signalling that can be further regulated by the presence or absence of endopeptidases. The fact that opioid receptor activation leads to activation of multiple signalling cascades, including those activated by Gαi, arrestin as well as cascades not involving arrestins (Mittal *et al.*, 2013), supports this idea of a complex regulation of receptor signalling from multiple compartments.

Table 2

Binding and signalling of peptidic μ receptor agonists at neutral and endosomal pH

	Binding		[^{35}S]-GTPγS	
	% specific binding at 1 μM		E_{max} (% basal) at 1 μM	
	pH 7.4	pH 5.5	pH 7.4	pH 5.5
DAMGO	72 ± 1	80 ± 1	139 ± 3	125 ± 1
Dynorphin B	54 ± 2	66 ± 1	146 ± 3	120 ± 1
[Leu]Enk	71 ± 1	78 ± 2	185 ± 9	140 ± 2

Binding and [^{35}S]-GTPγS studies were carried out using membranes (50 μg) from cells expressing μ receptors as described in Methods. For binding studies, [^{3}H]-diprenorphine binding in the absence of peptide agonists (i.e. total binding) was taken as 100 %. % specific binding at 1 μM was obtained by subtracting the % radiolabelled bound at 1 μM (i.e. non-specific binding) from the total binding. For [^{35}S]-GTPγS binding, basal binding observed in the absence of the peptidic agonist was taken as 100%. Data represent mean ± SEM (n = 3).

While classically, dynorphin peptides are thought to bind and activate κ receptors, several studies have also found that these peptides bind and efficiently activate μ opioid receptors (Quirion and Pert, 1981; Sanchez-Blazquez *et al.*, 1984; Schulz *et al.*, 1984; Chavkin *et al.*, 1985; Mansour *et al.*, 1995). Moreover, while studies show that dynorphin B and [Leu]enkephalin have similar affinities for μ receptors, that is, in the nanomolar range (this study and Mansour *et al.*, 1995), and are able to efficiently activate this receptor (Mansour *et al.*, 1995; Alt *et al.*, 1998), they differ in their ability to serve as ECE2 substrates. This differential processing is likely to regulate receptor recycling leading to differential signalling. Our *in vivo* studies support such a notion. We showed that ECE2 inhibition attenuated the antinociceptive activity of dynorphin B (substrate) but not of fentanyl or [Leu]enkephalin (non-substrate). While it is likely that the antinociception by dynorphin B and [Leu]enkephalin is mediated by other receptors in addition to μ receptors, studies with receptor-selective antagonists are needed to define the receptor type that is being modulated by ECE2 *in vivo*. Nonetheless, the data presented in this study strongly suggest a key role for ECE2 in modulating antinociception mediated by the opioid peptide/receptor system *in vivo*. Our previous finding that deletion of ECE2 produces a decrease in opiate-induced analgesia links ECE2 to the actions of μ receptors (Miller *et al.*, 2011) and supports such an idea. It is likely that in regions of the brain (periaqueductal grey, rostral ventral medulla, to name a few) and spinal cord, which are involved in pain processing and exhibit co-expression of both ECE2 and μ receptors, ECE2 modulates analgesia and other biological actions of opioid receptors.

Acknowledgements

We thank Dr Lloyd Fricker (AECOM) for many helpful discussions and Dr Wendy Wawlyn (UCLA) for small and large DRG neurons. We also thank Khatuna Gagnidze for characterization of ECE2 inhibitors and Lydia Miller for initial studies examining intracellular localization of ECE2. This work was supported in part by NIH grants (NS26880 and DA 019521 to L. A. D.).

Author contributions

A. G. carried out trafficking and signalling studies; W. F. carried out behavioural studies; I. G. carried out enzymatic and signalling studies, analysed data and helped in manuscript preparation; E. B. carried out behavioural and imaging studies; L. A. D. planned the experiments, helped with data analysis and interpretation, and wrote the manuscript.

Conflict of interest

None.

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