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Localization of estrogen receptor ERα, ERβ and GPR30 on myenteric neurons of the gastrointestinal tract and their role in motility

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ABSTRACT

Estrogen is well known to have a modulatory role on gastrointestinal tract, particularly through its interaction with nuclear estrogen receptors (ERs), alpha and beta (ERα/β). Recent functional studies also indicate that estrogen can activate a G-protein coupled estrogen receptor, GPR30, or GPER1. The present study was designed to identify either the presence or absence of nuclear ERs and GPR30 in the myenteric plexus of the stomach, duodenum, jejunum, ileum and colon of female and male mice. Immunofluorescence staining revealed a high expression of GPR30 in the cytoplasm but not within the nucleus of enteric neurons in female and male mice. ERβ localization was similar to GPR30, where it was expressed in cytoplasm of enteric neurons, but was absent from nuclei, opening up the possibility that ERβ and GPR30 might work together to manifest estrogenic effects. Comparatively, ERα was mainly located in the nuclei of enteric neurons. ERα, ERβ and GPR30 were also expressed in the cytoplasm of glial cells in the stomach and small intestine, but levels were lower in the colon. The expression nuclear:cytoplasm ratio of ERα was higher in male than female mice, which might relate to sex-dependent translocation of ERα from cytoplasm to nucleus in response to known plasma levels of estrogen. A functional study using isolated ileal segments showed that ERα, ERβ and GPR30 are involved in the neuronal-mediated contractions in female tissues, but only ERα was involved in male tissues. This may indicate although expression level was similar between males and females, the downstream mechanisms of ERβ and GPR30 could be different between sexes. The present study provides a rationale for the action of estrogen to modulate gastrointestinal function in health and disease in different sexes.

1. Introduction

Motility disorders of gastrointestinal tract are common from a variety of causes (Bharucha et al., 2016). Generally, the incidence of gastrointestinal disorders appears higher in females than males. Changes in motility and visceral sensitivity are affected by menstrual cycle, pregnancy and menopause (Baron et al., 1993; Palomba et al., 2011). This suggests estrogen plays an important role in the regulation of motor and sensory function of gastrointestinal tract, possibly through direct and/or indirect actions on neuronal, immune and endocrine pathways, as well as possibly through interactions with microbiota (Mulak et al., 2014). However, the precise role of estrogen and its receptors in the gastrointestinal tract in health and disease is not completely understood.

Estrogen is best known as ovarian hormone but can also be synthesized in a variety of tissues including the placenta, brain, adipose, liver, heart and brain. Synthesis is from cholesterol and/or testosterone via dehydroepiandrosterone and aromatase enzymes (Cui et al., 2013). There are three major forms of estrogen: estrone, estradiol (17β-estradiol; the major premenopausal estrogen) and estriol (Cui et al., 2013).

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERs, estrogen receptors; G-1, (±)-1-[(3aR*,4S*,9bS*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]- ethanone, a GPR30 agonist; G-15, 3aS/,4R/,9bR/)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H cyclopenta[c]quinolone; GPR30 or GPER1, G-protein coupled estrogen receptor; ICR, institute of cancer research; LMMP, longitudinal muscle myenteric plexus; MPP, methyl-piperidino-pyrazole, a ERα antagonist; MPTP, 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; PHTPP, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol, a ERβ antagonist; SERMs, selective estrogen receptor modulators; TTX, tetrodotoxin

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Table 1
Primary antibodies used in the study.

Primary antibodies	Company name, catalog no.	Dilution
Rabbit anti-choline acetyltransferase (ChAT)	*Abcam*, ab68779	1:300
Rabbit anti-neuronal nitric oxide synthase (nNOS)	*Cell signaling*, #4234	1:300
Rabbit anti estrogen receptor alpha (ERα)	*Santa Cruz*, sc-1616	1:300
Rabbit anti estrogen receptor beta (ERβ)	*Enzo*, ALX-210-132-C100	1:500
Rabbit anti GPR30	*Santa Cruz*, sc-48525-R	1:300
Mouse anti Glial fibrillary acidic protein (GFAP)-Cy3 conjugated	*Sigma*, c9205	1:1000

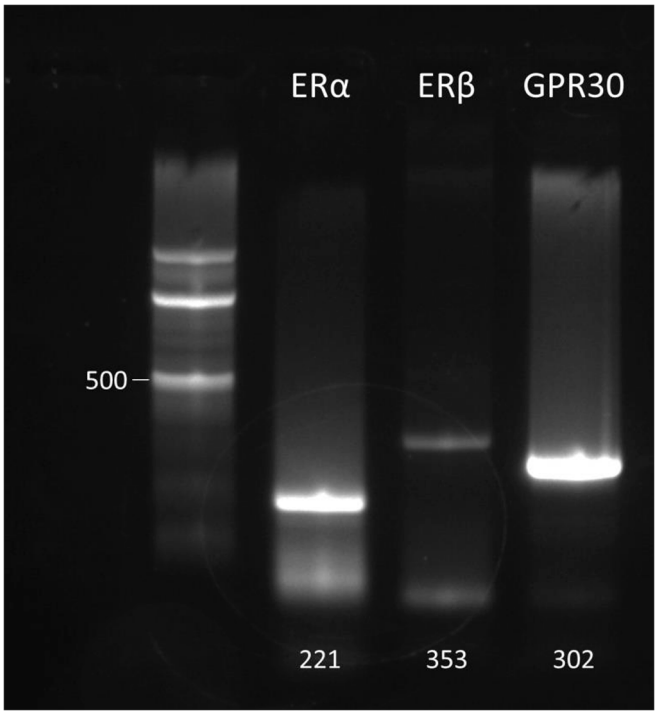

Fig. 1. PCR amplification product of ERα, ERβ and GPR30 expression in the female mouse ileum. Specific single bands for each gene with correct band size of 221 bp, 353 bp and 302 base pair respectively were confirmed through gel electrophoresis.

These estrogens can activate classical nuclear estrogen receptors (ERs) type alpha and beta (ERα and ERβ), and a relatively novel membrane bound/intracellular G protein coupled receptor, GPR30, also known as GPER1 (Cui et al., 2013). GPR30 transmembrane spanning receptors that are positively coupled to adenylate cyclase and can also be activated by estrogens and also several nuclear selective estrogen receptor modulators (SERMs) (Prossnitz and Barton, 2014). Whilst ICI 182,780 had been conventionally used as an ER antagonist (Diaz et al., 2004), it is now known to act as a GPR30 agonist (Filardo et al., 2000).

Recently, evidence has been presented showing involvement of GPR30 in mechanisms of enteric neurodegeneration induced by the administration of 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a common neurotoxin used to model Parkinson's disease, where constipation is a comorbidity (Cote et al., 2015; Poirier et al., 2016). It was shown activators of GPR30, including 17β-estradiol, G-1 and raloxifene, could reduce neuronal loss possibly through direct and indirect mechanisms involving the innate immune system (Cote et al., 2015). ERα, ERβ and GPR30 are expressed in the myenteric plexus of gastrointestinal tract (Campbell-Thompson et al., 2001a; Kawano et al., 2004; Zielinska et al., 2017). Nonetheless, there are no studies fully documenting the expression level and localization of the three ERs on enteric neurons. This present study investigated ERα, ERβ and GPR30 expression in the myenteric plexus of both male and female mice using immunohistochemistry and standard polymerase chain reaction (PCR) techniques; we compared our findings to the relative distribution of ER subtypes in the hippocampus. An organ bath study using isolated tissues was performed to evaluate the potential involvement of ERs to modulate electrical field stimulation (EFS)-induced contractions of the ileum using 17β-estradiol, the most potent and abundant naturally occurring form of estrogen, and a series of selective antagonists (Cui et al., 2013). We envisaged this study would yield information towards understanding the rationale behind the neuroprotective potential of ER ligands and their involvement in GI motility.

2. Materials and methods

2.1. Animals

Two-month old male and female (both between 20 and 25 g) out bred ICR mice were obtained from the Chinese University of Hong Kong. Mice were segregated into different sexes and housed in a temperature-controlled room (24 ± 1 °C) in plastic cages (1–5 mice per cage). Artificial lighting was provided between 06:00 and 18:00 h. Relative humidity was maintained at 50 ± 5%. Water and dry pelleted food chow (Teklad Global 19% Protein Extruded Rodent Diet, Sterilized, 2019S, *Harlan*, Madison, WI) were given *ad libitum*. All experiments were conducted under licence from the Government of the Hong Kong SAR and with permission from the Animal Experimentation Ethics Committee, The Chinese University of Hong Kong.

2.2. Tissue collection and preparation

Mice were sacrificed via carbon dioxide asphyxiation, with whole gastrointestinal tract was removed. The stomach, duodenum, jejunum, ileum and colon were dissected, and then flushed using pre-gassed (95% O_2 and 5% CO_2) room temperature Krebs' solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO4, 1.2 mM $MgSO_4$ $7H_2O$, 2.5 mM $CaCl_2$ $2H_2O$, 25 mM $NaHCO_3$ and 10 mM glucose) to remove faecal contents. Each segment was then cleaned of connective tissue and blood vessels before transecting along the mesenteric line to reveal the lumen. The mucosal and submucosal layers were removed by gentle rubbing using cotton wool swab, leaving longitudinal muscle with the myenteric plexus intact. The resultant longitudinal muscle myenteric plexus (LMMP) preparation was then spread flat on a petri dish, longitudinal muscle-side down, before fixing in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 1 h. In addition, the brain of each animal was also removed and sectioned into 300 μm thick slices using a tissue chopper (U0800, *Mc Ilwain*). Brain slices containing the hippocampus were transferred to 4% PFA in PBS and allowed to fix overnight at 4 °C.

2.3. Immunohistochemistry and confocal imaging

After fixing, brain slices and LMMP were rinsed three times for approximately 5 min in ice cold PBS. Tissues were then blocked with 1% bovine serum albumin (BSA) in 0.25% Triton X-100 in PBS for 1 h. After washing in PBS, tissues were stained with 4′,6-diamidino-2-phenylindole (DAPI), *Invitrogen*, D1306, 1:5000, for 2 h at room temperature to identify nuclei. Samples were then incubated with primary

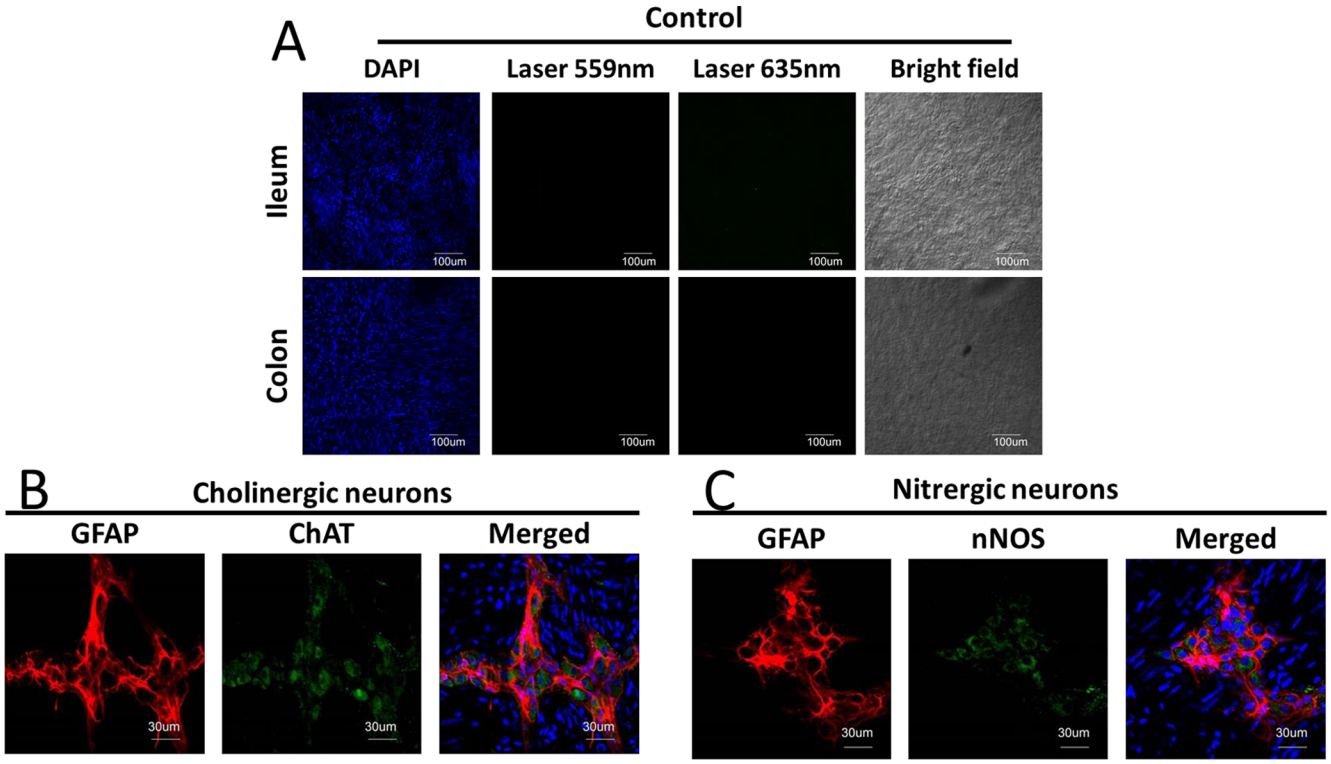

Fig. 2. Cholinergic and nitrergic neurons in the female mouse colon. (A) Negative control staining without incubation with primary antibodies or the Cy3-conjugated mouse anti-glial fibrillary acidic protein (GFAP), but only with DAPI and secondary antibodies goat anti-rabbit IgG (H + L) highly cross-adsorbed antibody, Alexa Fluor 647. The same control applies to staining of the estrogen receptors. (B) Photos were captured in grey-scale, and artificial colour was set using software: ChAT and nNOS stained with secondary antibodies linked with Alexa Fluor 647 are displayed in green. Neurons were counter stained with DAPI to identify nuclei in blue, and Cy3-linked glial fibrillary acidic protein (GFAP) in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Localization of ERα along the LMMP of both female (F) and male (M) ICR mice.

GI section	Gender	Neuronal Cytoplasm	Neuronal Nucleus	Neurites	Glial Cytoplasm	Glial Nucleus
Stomach	F	+ + +	+ + +	+ + +	+ +	−
	M	+	+ + + + +	+ + + +	+ +	−
Duodenum	F	+ +	+	+	+	−
	M	+ +	+ +	+ + +	+	−
Jejunum	F	+ +	+ +	+ +	+	−
	M	+ +	+ + +	+ + +	+	−
Ileum	F	+ +	+ +	+ +	+ +	−
	M	+	+ + +	+ + +	+	−
Colon	F	+ +	+ +	+	+	−
	M	+	+ + +	+ +	+	−

Relative expression of ERα indicated as + + + + +, + and − depicts maximum positive expression, minimum positive expression and negative or with similar intensity to the smooth muscle background respectively.

Table 3
Localization of ERβ along the LMMP of both female (F) and male (M) ICR mice.

GI section	Gender	Neuronal Cytoplasm	Neuronal Nucleus	Neurites	Glial Cytoplasm	Glial Nucleus
Stomach	F	+ +	−	+ + +	+	−
	M	+ + + +	−	+ + + +	+ +	−
Duodenum	F	+ +	−	+ + +	+ +	−
	M	+ + +	−	+ + +	+	−
Jejunum	F	+ + + +	−	+	+ +	−
	M	+ + +	−	+ + +	+	−
Ileum	F	+ +	−	+ +	+	−
	M	+ + +	−	+ +	+	−
Colon	F	+ + + + +	−	+	+	−
	M	+ + +	−	+ +	+	−

Relative expression of ERα indicated as + + + + +, + and − depicts maximum positive expression, minimum positive expression and negative or with similar intensity to the smooth muscle background respectively.

antibodies diluted with 3% BSA 0.25% Triton X-100 in PBS overnight at 4 °C in a cold room in the dark, with gentle shaking. Primary antibodies used are listed in Table 1. A single rabbit origin primary antibody was co-stained with the mouse origin glial fibrillary acidic protein (GFAP)-Cy3 conjugated primary antibody. The next day, samples were washed with PBS 3 times for 15 min each, to rinse away free primary antibodies. Sections were then immersed in secondary goat anti-rabbit IgG (H + L) highly cross-adsorbed antibody, Alexa Fluor 647 (*Thermo Fisher*, A21245, 1:2000 diluted with 3% BSA 0.25% Triton X-100) in PBS and then incubated for 2 h at room temperature in the dark with gentle shaking. Unbound antibodies were then washed away with PBS, 3 times, each wash lasting 15 min. Tissues were then mounted. Briefly, brain slices or LMMP were spread carefully on microscopic slides. One drop of Vectorshield® mounting medium (H-1000) was added, followed by a coverslip sealed with transparent nail polish. Slides were stored at 4 °C in the dark before observing (randomly chosen fields, three per tissue) using an *Olympus FV1000-ZCD Laser Scanning Confocal System,* or *Olympus FV1200-ZDC SIM Confocal System*, equipped with lasers set at 405 nm, 559 nm and 635 nm for excitation of DAPI, Cy3 and Alexa Flour 647, respectively. Under the microscope, areas chosen for photo taking were aided only with glial cell stained with GFAP-Cy3 to localize the myenteric plexus, without bias, from the Alexa 647 fluorescent signals for estrogen receptors. Photos were taken in grey scale. Artificial colour, blue, red and green, were assigned for DAPI, Cy3 (near infra-

Table 4
Localization of GPR30 along the LMMP of both female (F) and male (M) ICR mice.

GI section	Gender	Neuronal Cytoplasm	Neuronal Nucleus	Neurites	Glial Cytoplasm	Glial Nucleus
Stomach	F	+ + + +	−	+ +	+	−
	M	+ + + +	−	+ + + + +	+	−
Duodenum	F	+ + +	−	+ +	+	−
	M	+ + + +	−	+	+	−
Jejunum	F	+ +	−	+ +	+	−
	M	+ +	−	+ + +	+ +	−
Ileum	F	+ + +	−	+ + +	+ +	−
	M	+ +	−	+ +	+	−
Colon	F	+ + +	−	+ + +	+ +	−
	M	+ + + +	−	+	−	−

Relative expression of ERα indicated as + + + + +, + and − depicts maximum positive expression, minimum positive expression and negative or with similar intensity to the smooth muscle background respectively.

red) and Alexa 647 (far infra-red). Advantage of using near or far-infra-red fluorescent antibodies is that intestinal tissues show very low auto-fluorescent in the range of infra-red wavelengths. A scale bar was added using auto-alignment in the equipment-based software (FV10-ASW 1.7 Viewer, *Olympus*). A simple quantitative assay, performed by blinded experimenter, carefully looked at areas covered by glial cells and neuronal cells, and within the cell bodies. Areas within nucleus, cytoplasm and on neurites were compared to generate relative expression level from high to none with score "5″ to "0" for each area. Comparisons were performed for one or more cells (those showing clear boundaries between cytoplasm and nucleus) from pictures taken from 3 to 5 mice. Data were averaged and displayed as high to none using "+ + + + +" to "−".

2.4. Polymerase chain reaction (PCR) experiments

RNA was extracted from a small section of whole ileum using a *TaKaRa MiniBEST Universal RNA Extraction Kit*, #9767, while cDNA preparation was performed according to the protocol (*TaqMan® Reverse Transcription Reagents*, *Thermo Fisher*, N8080234 kit). Standard PCR was performed using *Taq* DNA Polymerase, native, *Thermo Fisher*, 18038018. Primer pairs were designed using nucleotide sequences downloaded from National Center for Biotechnology Information with sequence code gi320118648, 46877095, 254588033; these were used for designing the primers for ERα, ERβ and GPR30, respectively. Highly specific primer pairs were selected for PCR amplification. ERα: 5′-AAG CGT CAG AGA GAT GAC TTG GAA GG-3′ and 5′-GAG GCT TCA CTG AAG GGT CTA GAA GG-3′; ERβ: 5′-CTC CTC AAC TCC AGT ATG TAC CCC TT-3′ and 5′-TCT TTG CTC TTA CTG TCC TCT GTC GA-3′; GPR30: 5′-TGA TTG AGG TGT TCA ACC TGG ACG AG-3′ and 5′-CTC CCT GAC ATC AGC AAA GCA GAA GC-3'; with amplicon sizes of 221 bp, 353 bp and 302 bp, respectively.

2.5. Drugs

17β-estradiol (Sigma Aldrich, E2758); the ERα antagonist, methyl-piperidino-pyrazole (MPP) (Sigma Aldrich, M7068); the ERβ antagonist, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol (PHTPP) (Santa Cruz, CAS 805239-55-8); and the GPR30 antagonist, 3aS/,4R/,9bR/)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H cyclopenta[c]quinolone (G-15) (Tocris bioscience, 3678), were all prepared by dissolving in DMSO at 10 mM. Further dilutions were made from these stocks using Kreb's solution on the day of experiment. Tetrodotoxin (TTX) was purchased from Tocris Bioscience, catalog no. 1069, and was dissolved in distilled water. TTX (1 μM) was used as a sodium channel blocker to prevent neuronal depolarisations (Hoyle and Saffrey, 2012) at the end of each experiment.

2.6. Ex-vivo organ bath studies

Male (n = 3–4) and female (n = 3–4) mice were killed using carbon dioxide asphyxiation. The ileum was freshly dissected and equilibrated in Kreb's solution gassed with 5%/95% CO_2/O_2. Faecal contents were gently removed by flushing Kreb's solution through the lumen. A 1–1.5 cm segment of ileum was mounted between two stimulating electrodes under 0.5–1 g tension in a 10 ml organ bath containing Kreb's solution gassed with 5%/95% CO_2/O_2 at 37 °C. Changes in tension were recorded using an isometric force displacement transducer (Grass Instruments, Inc., Quincy, MA. USA). Signals were amplified (AD Instruments) and digitalized by analog-to-digital converter (Model ML870, PowerLab 8/30, AD Instruments), with recordings displayed and saved using LabChart 7 software (AD Instruments) running on a personal computer.

The ileum was stabilized for 30 min before the first administration of drugs or electrical field stimulation (EFS) at 90 V, 4 or 16 Hz with 5 s train duration, 1 ms pulse duration and with a 90 s time interval between each stimulation was done using a stimulator (Model S88, Grass Instruments, Inc., Quincy, MA. USA). 17β-estradiol (10 nM–100 μM) was added using a cumulative dosing schedule to the organ bath. The involvement of ERs was tested using ER antagonists: MPP (10 μM, ERα antagonists), PHTPP (10 μM, ERβ antagonists) and G-15 (10 μM, GPR30 antagonists) EFS was performed after 10 min incubation with the antagonists. Data were normalized to maximum contraction response induced by 120 mM potassium chloride (KCl).

2.7. Statistical analysis

Data are indicated as the mean ± S.E.M. Statistical analysis was performed using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were tested using a one-way ANOVA followed by Tukey's multiple comparison tests. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of ERα, ERβ and GPR30 in the gastrointestinal tract

Gel electrophoresis following PCR amplification of ERα, ERβ and GPR30 in the mouse ileum produced clear bands with sizes of 221 bp, 353 bp and 302 bp, respectively (Fig. 1). The stomach, duodenum, jejunum, ileum and colon LMMP were subjected to immunofluorescence staining using ERα, ERβ and GPR30 antibodies. Negative controls

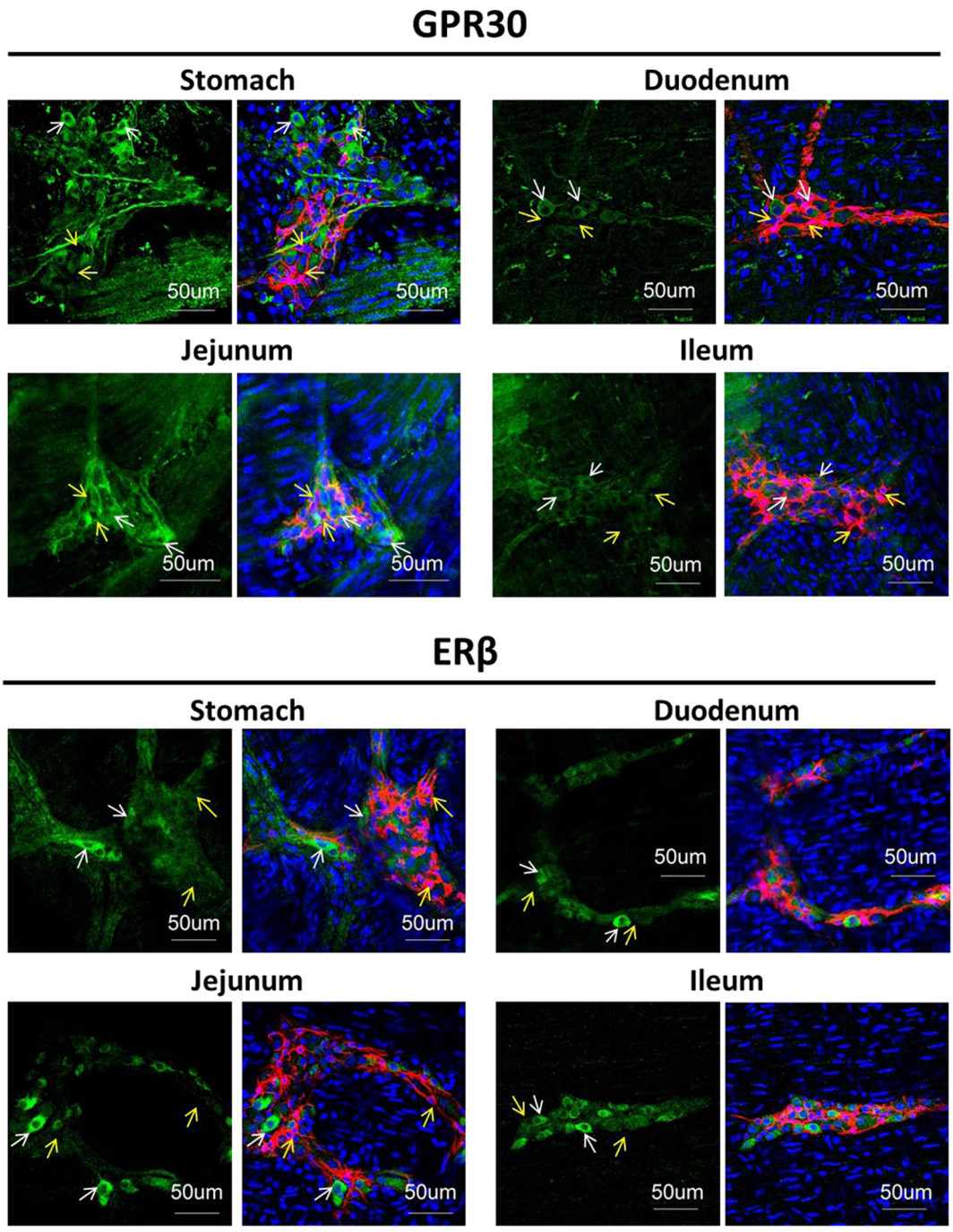

Fig. 3. Expression of ERβ and GPR30 in the stomach, duodenum, jejunum and ileum in the female mouse. ERβ and GPR30 stained with secondary antibodies linked with Alexa Fluor 647 are displayed in green. Receptors were counter stained with nuclei in blue, and Cy3-linked glial fibrillary acidic protein (GFAP) in red. Glial cells had a smaller nucleus (yellow arrows), and ERs-expressed cells had a large nucleus with few observable nucleolides within the nucleus (white arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stained with only DAPI and secondary antibodies showed no fluorescence signals under laser wavelengths of 559 nm and 635 nm (Fig. 2A). Relative expression levels of ERα, ERβ and GPR30 in each gastrointestinal section is summarized in Tables 2–4, respectively; the number of animals used and number of cells counted is summarized in Table 6. Cell bodies of enteric neuronal and glial cells were distinguished by the sizes of nuclei. Neuronal cells were identified as having larger nuclei with dots of nucleosome, whilst glial cells were identified as having smaller and denser nuclei, with GFAP staining. Neuronal cells were separately labelled using immunofluorescence staining for choline acetyltransferase and neuronal nitric oxide synthase, where they were characterised as having larger nuclei with dots of nucleosome, as shown in Fig. 2B, 2C. The expression patterns of ERβ and GPR30 were similar in both male (n = 3) and female (n = 4–5) mice. ERβ and GPR30 were located in the cytoplasm of neuronal cell bodies, but not within nucleus as shown in Fig. 3. The expression of ERβ and GPR30 was along neurites, either neuronal or glial neurites, and this was seen in the stomach, small intestine and colon. In the colon, expression of ERβ and

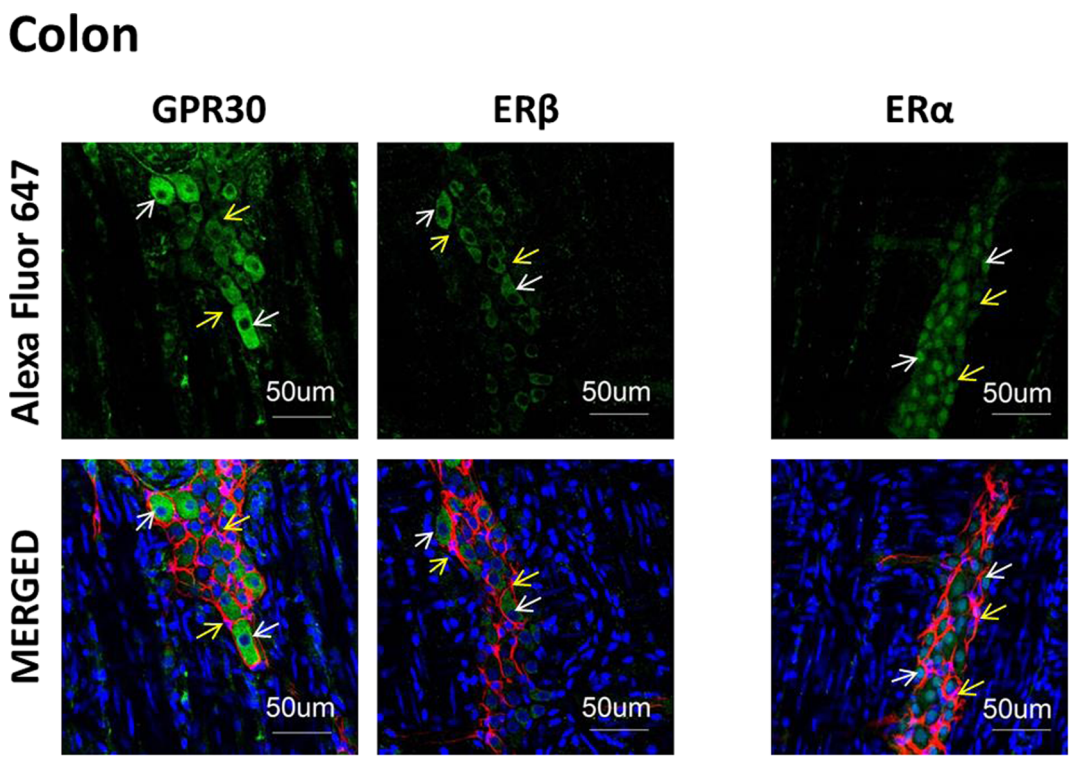

Fig. 4. Expression of ERα, ERβ and GPR30 in the colon of male mice. ERα, ERβ and GPR30 stained with secondary antibodies linked with Alexa Fluor 647 are displayed in green. Receptors were counter stained with nuclei in blue, and Cy3-linked glial fibrillary acidic protein (GFAP) in red. ERα were highly expressed within the neuronal nucleus, but not ERβ and GPR30. Glial cells had a smaller nucleus (yellow arrows), and ERs-expressed cells had a large nucleus with few observable nucleolides within the nucleus (white arrows). N = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

GPR30 was highly localized within the neuronal cell bodies (Fig. 4). In contrast, ERα was highly expressed inside the nuclei of enteric neurons, but curtailed in the cytoplasm (Fig. 4). The expression pattern of ERα was different between males and females. Thus, the ratio of nuclear:cytoplasm ERα expression appeared higher in males than females in the stomach, small intestine and colon (Fig. 5, Table 2), while the expression level and patterns of ERβ and GPR30 appeared to be similar between males and females. (Tables 3 and 4) However, all three types of ERs were absent from the nuclei of glial cells, which were labelled using yellow arrows in Figs. 3–5.

3.2. Expression pattern of ERα, ERβ and GPR30 in the hippocampus

Hippocampal neurons were examined for cellular localization comparing with enteric myenteric neurons. The expression of ERα was evident in the nuclei of hippocampal pyramidal and granule cells. ERβ expression was observed within a thin layer of cytoplasm surrounding the nucleus of hippocampal neurons, but was lower in the nucleus. ERβ was also expressed in the cytoplasm of glial cells, but was absent within the nucleus. GPR30 were seen in the cytoplasm of the hippocampal neurons and glial cells, but were absent from the nucleus (Fig. 6). The expression pattern of ERα, ERβ and GPR30 in hippocampal neurons of the female and male ICR mouse (n = 3–6) are summarized in Table 5. There were no significant differences in the expression localization and pattern in the hippocampus between female and male animals.

3.3. 17β-estradiol inhibits EFS-induced contraction in mouse ileum

17β-estradiol (10 nM–100 μM) induced an approximate 25% maximal inhibition of baseline of the mouse ileum at 100 μM (Fig. 7A–C). 17β-estradiol (30 μM–100 μM) also significantly induced an inhibition in EFS-induced contractions of the ileum; the approximate maximum inhibition was 25% at 4 Hz stimulation and 69% at 16 Hz stimulation, at 100 μM. Perfusing Kerbs' solution for 8 min (flow rate approximately 5 ml/min) into the chamber partially reversed the inhibition induced by 17β-estradiol (indicated by "wash" in Fig. 7). The addition of TTX (1 μM, sodium ions channels blocker) completely blocked the EFS-induced contractions indicating the response was mediated via neurones (Fig. 7). Incubation of tissues with 10 μM MPP (ERα antagonist) (Fig. 8) prevented the 10 μM 17β-estradiol-induced inhibition of EFS-induced contractions in both female and male tissues. Incubation with 10 μM G-15 (GPR30 antagonist) (Fig. 9) partially blocked the 10 μM 17β-estradiol-induced inhibition of EFS-induced contractions of tissue from females only. Administration of 10 μM PHTPP (ERβ-antagonist) (Fig. 10) alone significantly inhibited the EFS-induced contractions and this effect was irreversible by washing only in male. In female tissues, 10 μM PHTPP incubation did not cause this inhibition, and had similar effects to 10 μM MPP in preventing 10 μM 17β-estradiol-induced inhibition of EFS-induced contractions. In summary, ERα, ERβ and GPR30 are involved in neuronal induced contraction in ileum of females, but only ERα is involved in male tissues. PHTPP also exerts an inhibitory effect only in ileum of males.

4. Discussion

Estrogen is a key female reproductive hormone, being intimately involved in ovulation (Cui et al., 2013). Estrogen also has well-known actions in the brain to affect behaviour (Hadjimarkou and Vasudevan, 2017). In addition, several studies have revealed the importance of estrogen in neuroprotection, through ERβ, but not ERα (Baron et al.,

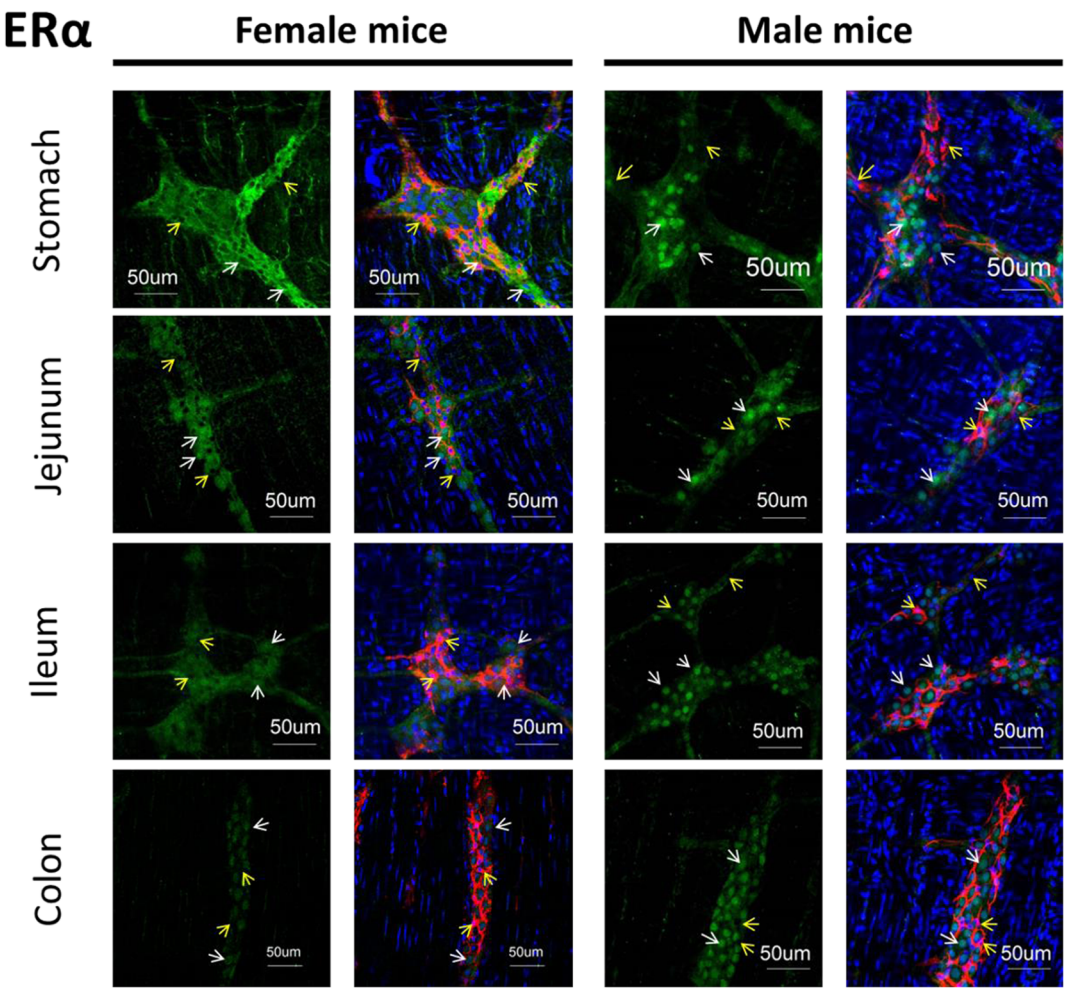

Fig. 5. Expression of ERα in the stomach, jejunum, ileum and colon of female and male mice ERα stained with secondary antibodies linked with Alexa Fluor 647 are displayed in green. Receptors were counter stained with nuclei in blue, and Cy3-linked glial fibrillary acidic protein (GFAP) in red. Glial cells had a smaller nucleus (yellow arrows), and ERs-expressed cells had a large nucleus with few observable nucleolides within the nucleus (white arrows). N = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1993; Walf et al., 2008; Zhao et al., 2013). Also, GPR30 in the brain provides an additional mechanism whereby estrogen might affect neuronal and endocrine functions, and neuroprotection (Lu and Herndon, 2017). Since neurons in the central nervous system highly resemble those found in the enteric nervous system, it might be possible to hypothesize similar differential roles of ER in physiological processing and neuroprotection in the periphery.

The action of estrogen to affect cognitive function is through mechanism involving kinase signalling through ERs in the hippocampus (Kumar et al., 2015). Our studies found ERα was expressed in the nucleus of various sub-regions of the hippocampus, with lower levels present in the cytoplasm and neurites. Conversely, both ERβ and GPR30 were mostly absent from nuclei, and were localised in the cytoplasm of the hippocampus. GPR30 was highly expressed in neurites of hippocampus. ERβ was highly expressed in cytoplasm of glial cells within the hippocampus. This expression pattern was similar between male and female brains. Thus, our data are in agreement with previous reports (Mitra et al., 2003).

The major finding of our study was to add to the evidence of the expression of GPR30 on various elements in the gastrointestinal tract of both male and female animals. GPR30 was present in the cytoplasm of myenteric neurons, neurites, and glial cells of the stomach, duodenum, jejunum, ileum and colon. However, GPR30 was absent from nuclei in all areas of the gastrointestinal tract, which is comparable with its known cellular distribution in structures in the brain and is consistent with a recent published study (Zielinska et al., 2017). The distribution of GPR30 between males and females in the neuronal cytoplasm were similar. Highly expressed GPR30 in the gastrointestinal tract could be an important therapeutic target in health and diseases. Studies had suggested GPR30 as a potential therapeutic target in irritable bowel disease, where G-1, an agonist at GPR30, reduced stress-induced colonic hypermotility and reduced mustard-oil induced pain behaviour (Zielinska et al., 2017); benefits are also seen in clinical studies (Qin et al., 2014).

The distribution of ERα in our studies was consistent with known literature, where it was found in both nucleus and cytoplasm of myenteric neurons, with a comparatively higher level seen in the nucleus (Campbell-Thompson et al., 2001b; Kawano et al., 2004). The localization of ERβ was different when using antibodies specify for the C or N-terminus of ERβ. ERβ was found in the nucleus when antibodies against the N-terminus were used (Campbell-Thompson et al., 2001b). The antibodies we used had specificity for the C-terminus of ERβ and we did not see ERβ in the nucleus of myenteric neurons, but it was highly expressed in the cytoplasm, which is consistent with the

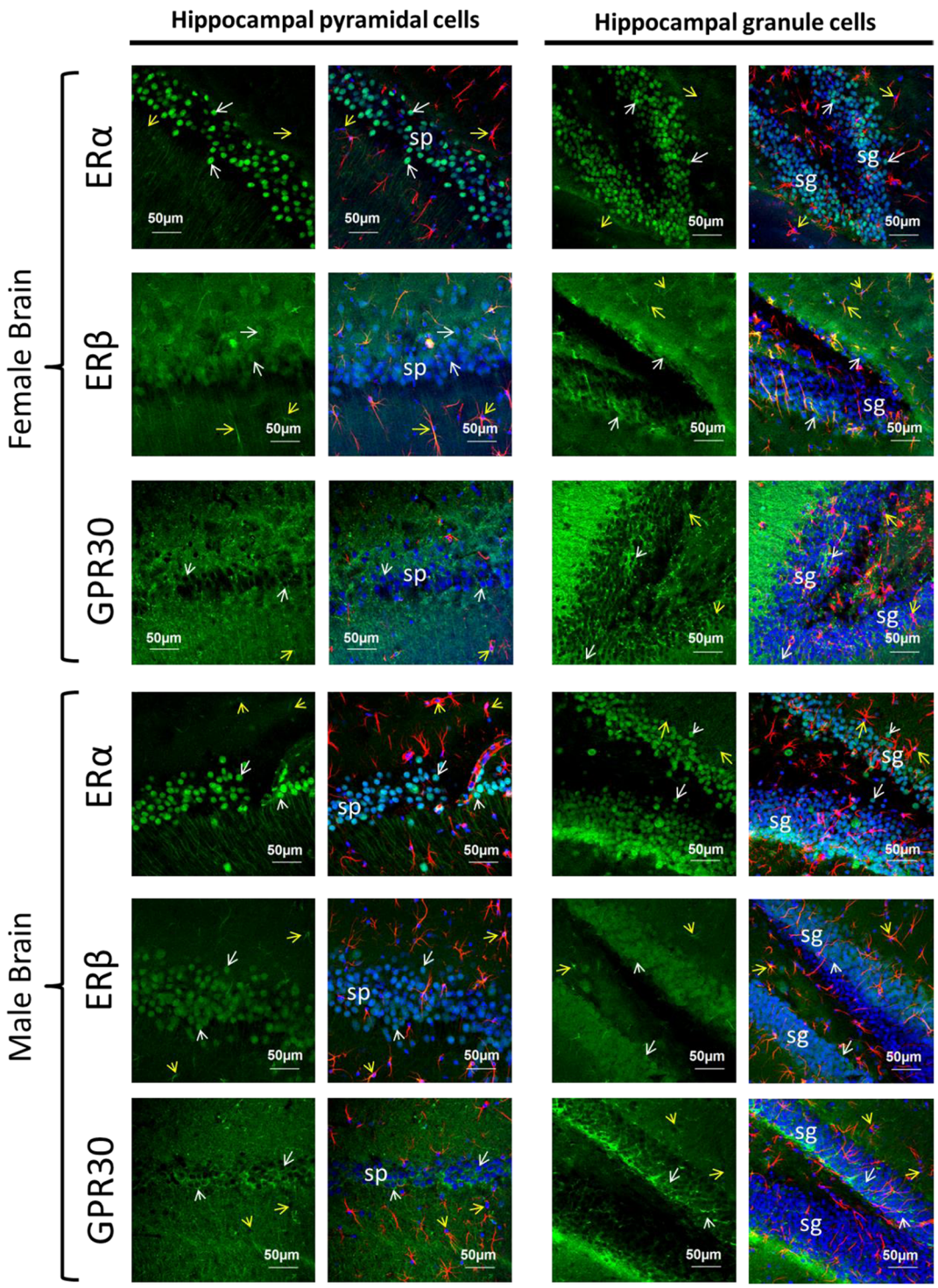

Fig. 6. ERα, ERβ and GPR30 expression in the hippocampus of female and male mice. Nuclei in blue, and Cy3-linked glial fibrillary acidic protein (GFAP) in red, ERα, ERβ and GPR30 in green. Granule cells or pyramidal cells in the hippocampus are illustrated with (yellow arrows), and astrocytes with (white arrows), N = 3–6. sp: hippocampal pyramidal cell layers; sg: hippocampal granule cell layers. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Localization of ERs in the hippocampus.

Section	Receptors	Neuronal Cytoplasm	Neuronal Nucleus	Neurites
Female mice				
Pyramidal cells	ERα	−	+ + + +	+
	ERβ	+ +	+	+
	GPR30	+ +	−	+ +
Granule cells	ERα	−	+ + + +	−
	ERβ	+ +	+	+
	GPR30	+ +	−	+ +
Glial cells	ERα	−	−	−
	ERβ	+ +	−	+ +
	GPR30	+	−	+
Male mice				
Pyramidal cells	ERα	−	+ + + +	+ +
	ERβ	+ + +	+ +	−
	GPR30	+ + +	−	+
Granule cells	ERα	−	+ + + +	−
	ERβ	+ +	+ +	+ +
	GPR30	+ + +	−	+
Glial cells	ERα	−	−	−
	ERβ	+ + +	−	+
	GPR30	−	−	+ +

Relative expression of ERα indicated as + + + + +, + and − depicts maximum positive expression, minimum positive expression and negative or with similar intensity to the background respectively.

Table 6
Number of cells counted and animals used.

Subtype	Gender		Stomach	Duodenum	Jejunum	Ileum	Colon
ERα	Male (n = 3)	Neuron	6	26	22	27	32
		Glial	6	23	14	25	22
	Female (n = 4–5)	Neuron	6	8	9	6	18
		Glial	6	8	9	6	15
ERβ	Male (n = 3)	Neuron	6	21	19	32	37
		Glial	6	22	28	22	29
	Female (n = 4–5)	Neuron	18	6	6	13	6
		Glial	18	6	6	12	6
GPR30	Male (n = 3)	Neuron	6	13	10	9	37
		Glial	6	9	9	6	17
	Female (n = 4–5)	Neuron	9	12	20	6	16
		Glial	9	12	18	6	18

Female hippocampus (n = 6)	ERα	ERβ	GPR30
Pyramidal cell	30	30	30
Granule cell	30	30	30
Glial cell	30	30	30
Female hippocampus (n = 3)	ERα	ERβ	GPR30
Pyramidal cell	15	15	15
Granule cell	15	15	15
Glial cell	15	15	15

published literature. Although estrogen was known to affect the contractile state of gastrointestinal tract through ERα and ERβ, there are also recent reports showing a role of these receptors and GPR30 in neuroprotection and the induction of nitric oxide (Cote et al., 2015; Li et al., 2016; Poirier et al., 2016). A similar expression pattern for ERβ and GPR30 between male and female mice in the gastrointestinal tract might indicate sex-independent functions of these receptors, whereas other studies in the brain reveal importance of ERβ, but not ERα in neuroprotection (Walf et al., 2008; Zhao et al., 2013). Another recent paper showed that ERβ activation repairs damaged myenteric plexi in adult mice (D'Errico et al., 2018). However, they were unable to detect ERα expression in murine cultured myenteric ganglia, which is different from what we found, where we identified ERα expression by PCR assays and immunofluorescent staining. However, direct comparison is difficult as *in-vitro* cell culturing could have significantly altered gene expression.

The level of ERα in the neuronal nuclei in males was higher than in females in the stomach, jejunum to colon, but not the duodenum. This was matched by a comparative lower level in the cytoplasm. This could have been due to ER translocation events in enteric neurons, which might be sex-dependent. Expectedly, nuclear translocation events are higher in females because of the higher level of estrogen in blood. It might be possible that enteric neurons in males are more sensitive to translocation, and/or ERα might stay longer within the nucleus after translocation, whereas in females, ERα might be recycled or degraded at a relatively faster rate. It is also possible that there are unknown functions of ERα in males. In females, cytoplasmic ERα is believed to initiate sex-dependent functions through established signalling pathways. This might explain why women have a higher incidence of irritable bowel diseases, or menstrual-related GI motility disorders than men (Baron et al., 1993; Palomba et al., 2011). However, studies have only focussed on ERβ in irritable bowel disease in relation to the function of mast- but not neuronal-cells (Cao et al., 2012; Pierdominici et al., 2015). Further experiments are required to deduce the significance of ERα in the enteric nervous system of both male and female.

Other previous studies demonstrated the number of ERα-positive cells in the large intestine changes with respect to the stage of the estrus cycle in mice. In addition, ERα-positive cells can be induced by administering estradiol (Kawano et al., 2004). It is possible that responsiveness of enteric neurons through ERα and ERα expression changes in response to blood levels of estrogen which then manifests as gastric dysthymia, diarrhoea and irritable bowel syndrome especially in females. Although this is an attractive hypothesis, the specific role of ER receptor subtypes has not been fully elucidated.

In the isolated tissue experiments, the effect of 17β-estradiol to inhibit EFS-induced contraction of the ileum was consistent with a recent study using female mouse colon (Zielinska et al., 2017). They also found that MPP (ERα antagonist) partially reversed the inhibitory effects of 17β-estradiol, but not PHTPP (ERβ antagonist) or G15 (GPR30 antagonist). These finding are inconsistent with our data using mouse ileum, where we found that all MPP, PHTPP and G15 partially reversed the inhibitory effects of 17β-estradiol in females. However, when we compare the data using male ileum, we found that only MPP reversed the inhibitory effects of 17β-estradiol, but not G-15. These data may indicate that 17β-estradiol exerts inhibitory effects on ileal contractions via ERα in both sexes, and through GPR30 and ERβ only in females. Interestingly, the expression localization studies only revealed ERα ratio differences between males and females, but no apparent differences in the expression of GPR30 and ERβ. It is possible that whilst expression levels of GPR30 and ERβ are similar between males and females, the active state and sensitivity of receptors, or downstream pathways are different. We also found that PHTPP alone could induce inhibitory effects on EFS-induced contractions only in the ileum from males, but not females. This may indicate that the downstream mechanism of ERβ to affect GI motility is different between males and females. Further investigations are required to understand the mechanisms involved.

In conclusion, the present study provides evidence of ERα, ERβ and GPR30 expression on enteric neurons and enteric glial cells. Both GPR30 and ERβ were localized in the cytoplasm within neuronal cell bodies, and along neurites, but not in the nucleus. This was different from the expression ERα in nuclei. In addition, ERα expression ratio in nucleus to cytoplasm was different between male and female mice. These findings, coupled with the involvement of ERα in the mechanism of estrogen to inhibit EFS-induced contractions of isolated ileal tissue in both sexes, but not via GPR30 and ERβ in males, may have relevance to the cyclical action of female hormones in health and gastrointestinal diseases.

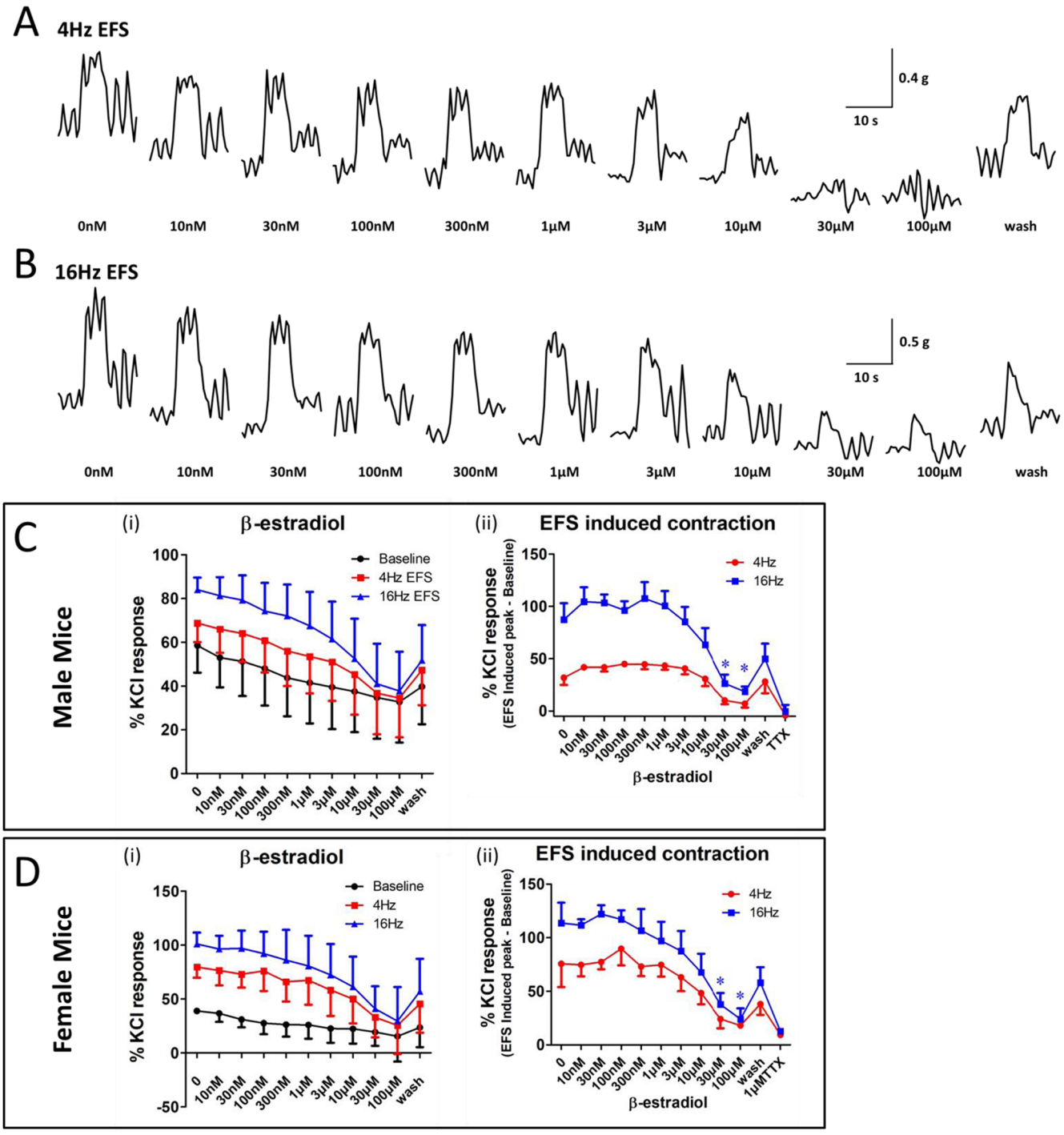

Fig. 7. The effect of 17β-estradiol on electrical field stimulation (EFS)-induced contractions of the ileum. Representative raw traces of contraction response under EFS with 5 s train duration, 1 ms pulse duration at 90 V of (A) 4 Hz and (B) 16 Hz stimulation. (C-D) Data represents mean ± S.E.M. (C) in male mice, N = 4, and (D) in female mice, N = 3. Significant differences relations are indicated as * $p < 0.05$ (one-way ANOVA followed by Tukey's multiple comparison tests). (i) EFS contraction peak normalized by maximum contraction induced by 120 mM potassium chloride (KCl) (ii) Normalized induced contraction calculated by (contraction peak – baseline).

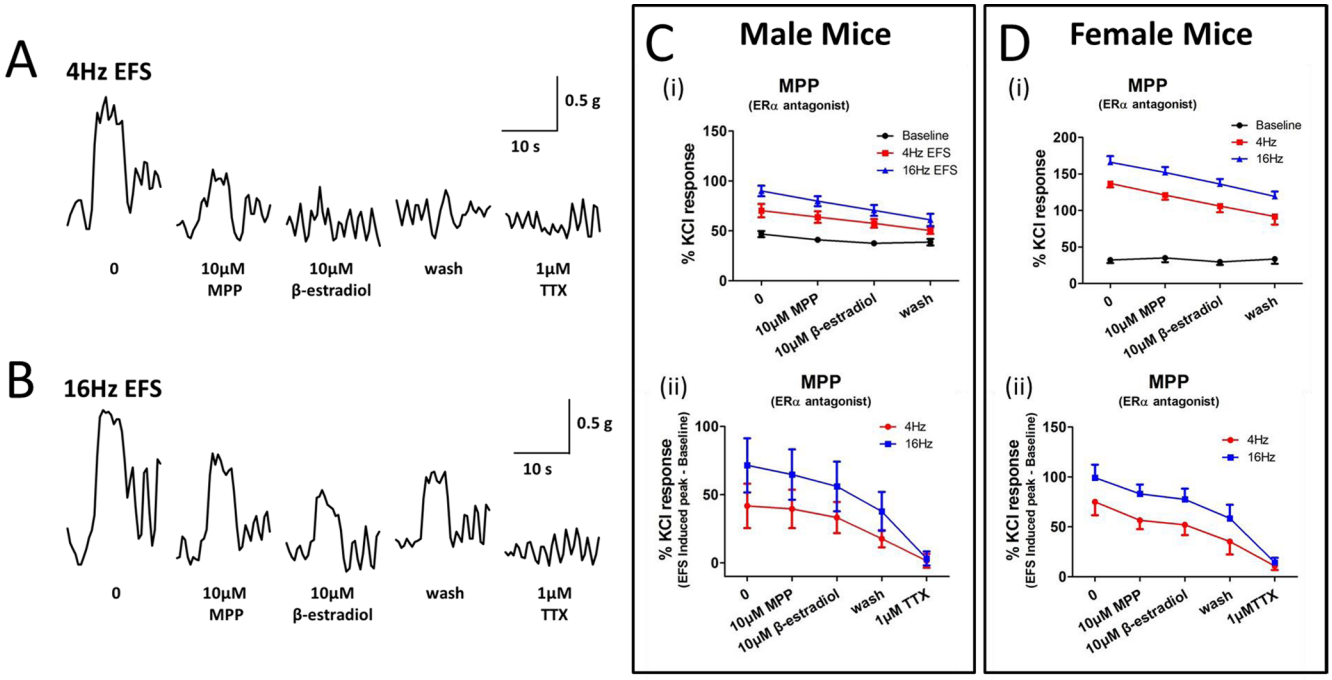

Fig. 8. Pre-treatment with methyl-piperidino-pyrazole (MPP), an ERα antagonist, blocked the inhibitory effect of 10 μM 17β-estradiol. Representative raw traces of contraction response under EFS with 5 s train duration, 1 ms pulse duration at 90 V of (A) 4 Hz and (B) 16 Hz stimulation. (C–D) Data represents mean ± S.E.M. (C) in male mice, N = 4, and (D) in female mice, N = 4. No significant differences were found ($p > 0.05$, one-way ANOVA followed by Tukey's multiple comparison tests) (i) EFS contraction peak normalized by maximum contraction induced by 120 mM potassium chloride (KCl) (ii) Normalized induced contraction calculated by (contraction peak – baseline).

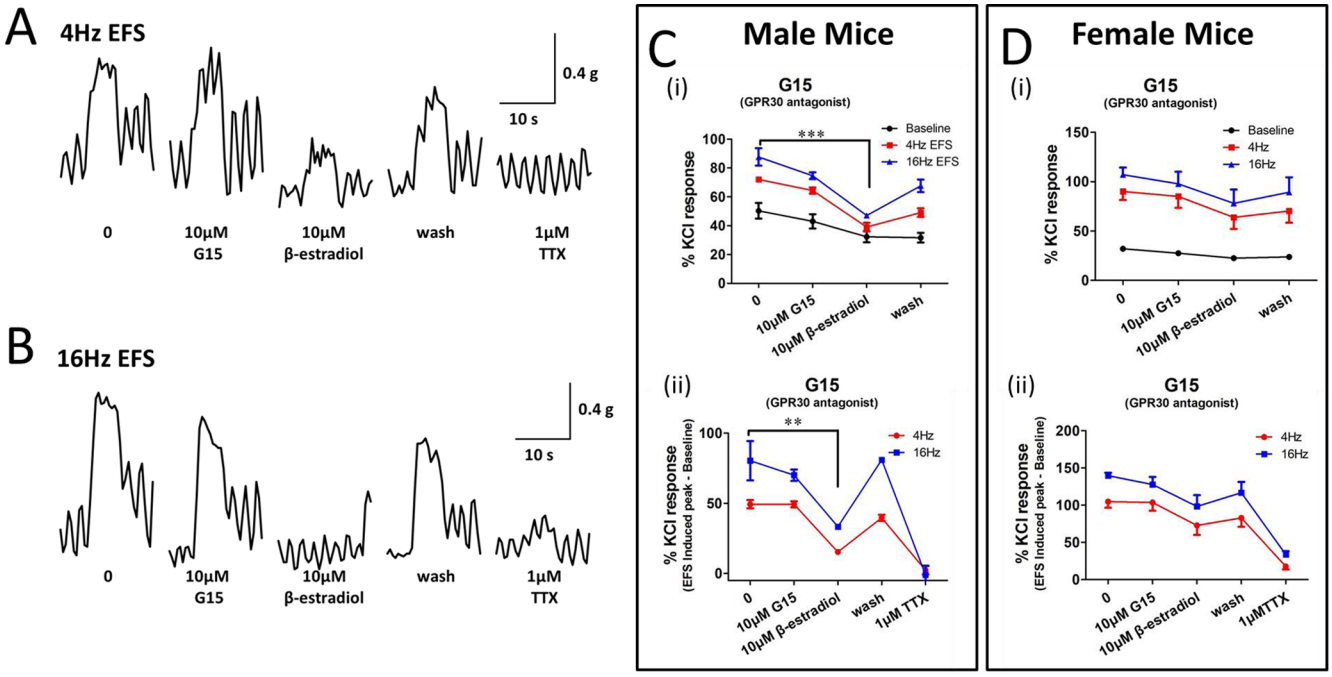

Fig. 9. Pre-treatment with G15, a GPR30 antagonist, failed to block the inhibitory effect of 10 μM 17β-estradiol. Representative raw traces of contraction response under EFS with 5 s train duration, 1 ms pulse duration at 90 V of (A) 4 Hz and (B) 16 Hz stimulation. (C–D) Data represents mean ± S.E.M. (C) in male mice, N = 3, and (D) in female mice, N = 4. Significant differences relations are indicated as ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA followed by Tukey's multiple comparison tests). (i) EFS contraction peak normalized by maximum contraction induced by 120 mM potassium chloride (KCl) (ii) Normalized induced contraction calculated by (contraction peak – baseline).

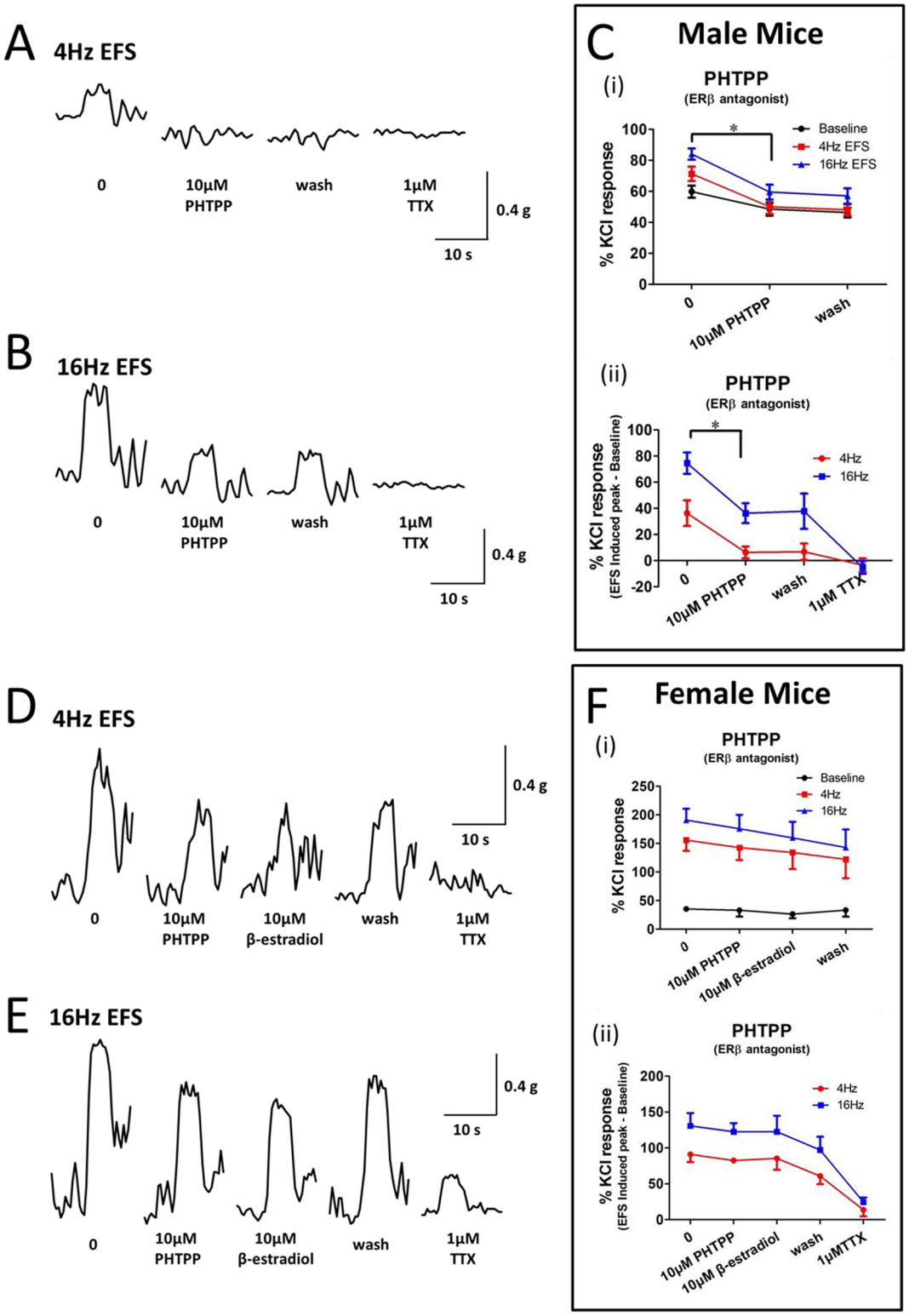

Fig. 10. Pre-treatment with 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol (PHTPP), an ERβ antagonist, led to an inhibitory effect similar to 10 μM 17β-estradiol. Representative raw traces of contraction response EFS with 5 s train duration, 1 ms pulse duration at 90 V of (A) 4 Hz and (B) 16 Hz stimulation in male mice and (D–E) in female mice. (C, F) Data represents mean ± S.E.M. of (C) in male mice, N = 4, and (F) in female mice, N = 3. Significant differences relations are indicated as $^{*}p < 0.05$ (one-way ANOVA followed by Tukey's multiple comparison tests). (i) EFS contraction peak normalized by maximum contraction induced by 120 mM potassium chloride (KCl) (ii) Normalized induced contraction calculated by (contraction peak – baseline).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.016.

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