

Estrogen Rapidly Modulates 5-Hydroxytryptophan-Induced Visceral Hypersensitivity via GPR30 in Rats

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BACKGROUND & AIMS: Sex hormones have been reported to modulate visceral hypersensitivity (VH). Estrogen regulates neurons not only by binding to estrogen receptors (ER α and ER β) to initiate transcription but also via the G-protein coupled receptor GPR30, which binds and rapidly mediates actions of estrogen. We examined the role of sex hormones in a VH model without colonic inflammation. **METHODS:** 5-Hydroxytryptophan (5HTP) was injected subcutaneously into awake female rats to induce VH; the 5HT₃ antagonist (granisetron) or saline (control) were injected 30 minutes later. Immunohistochemistry was used to quantify calcitonin gene-related peptide-immunoreactive (CGRP-IR) neurons in the dorsal root ganglion (DRG). 5HTP-induced VH was evaluated in ovariectomized rats injected with 17 β -estradiol, progesterone, or both. ER α/β agonist, GPR30 agonist, ER antagonist (ICI-182,780) or GPR30 antisense oligodeoxynucleotide were given to 5HTP-primed, estrogen-treated ovariectomized rats. **RESULTS:** Rats given 5HTP had increased VH that was inhibited by granisetron, accompanied by a decrease in CGRP-IR in the DRG. Ovariectomy eliminated 5HTP-induced VH, whereas estrogen and the combination of estrogen and progesterone, but not progesterone alone, restored the VH. The GPR30 agonist, but not the ER β agonist, rapidly restored VH. VH was preserved by coadministration of ICI-182,780 and estrogen but was absent after administration of the GPR30 antisense oligodeoxynucleotide. GPR30 colocalized with 5HT₃ in DRG neurons; no significant inflammation occurred in colonic mucosa. **CONCLUSIONS:** In the absence of mucosal inflammation, estrogen can rapidly modulate 5HTP-induced VH. Loss of gonad hormones suppresses VH, whereas estrogen replacement restores it. Estrogen-mediated VH appears to act through GPR30.

reports were mainly secondary to severe colonic mucosal inflammation,^{1,2} which is not observed in IBS patients. Therefore, the role of estrogen in the VH model in the absence of mucosal inflammation should be examined. To achieve this, subcutaneous injection of 5-hydroxytryptophan (5HTP, a serotonin precursor) to awake rats is used to induce VH without mucosal inflammation.³ Additionally, serotonin (5HT) is a critical molecule in mediating gut-to-brain signaling. Much of the pharmaceutical efforts over the past 2 decades has been focused on serotonin and 2 of its receptors: 5HT₃ and 5HT₄.⁴ Therapeutics targeting 5HT₃ and 5HT₄ receptors is effective in the treatment of IBS, particularly in female patients.^{5,6} Thus, it would be beneficial to understand the role of estrogen in 5HTP-induced VH.

Estrogen signals neurons by binding to estrogen receptors (ERs), which then interact with estrogen response elements to initiate transcription, ie, the classic “genomic” mechanism of steroid action. This effect is usually delayed at onset (within several hours to days) and prolonged in duration.⁷ Two nuclear ERs, ER α and ER β , have been previously cloned.⁸ In addition to these classical ERs, emerging data suggest that other estrogen receptors can also function as a cytoplasmic signaling molecule. This rapid action of estrogen can occur within seconds to minutes and does not involve transcriptional regulation.⁷ For example, we have shown that estrogen can restore mustard oil-induced VH in ovariectomized (OVX) rats in 90 minutes.² Others have demonstrated that a single injection of estrogen can rapidly phosphorylate CREB in gonadotropin-releasing neurons in OVX mice within only 15 minutes, and this effect can persist

Abbreviations used in this paper: 5HT, serotonin; 5HTP, 5-hydroxytryptophan; CGRP, calcitonin gene related peptide; CRD, colorectal distension; DPN, diethylpropionitrile; DRG, dorsal root ganglion; EMG, electromyogram; ER, estrogen receptor; GPCR, G-protein-coupled receptor; IR, immunoreactive; ODN, oligodeoxynucleotide; OVX, ovariectomized; PPT, propylpyrazole-triol; VH, visceral hypersensitivity; VMR, visceral motor response.

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Sex hormones are reported to be important in animal models in the modulation of “visceral hypersensitivity” (VH), a defining factor in pathogenesis of irritable bowel syndrome (IBS). However, the VH models in these

for 4 hours.⁹ A G-protein-coupled receptor, GPR30 (an alternative to the classical ERs), has recently been identified to be involved in the rapid action of estrogen through its direct association with estrogen.¹⁰ Therefore, we aimed to test the following hypotheses: (1) sex hormones can rapidly modulate 5HTP-induced VH in the absence of colonic inflammation, and (2) estrogen-dependent 5HTP-induced VH acts through the GPR30 receptor rather than through the traditional ER α and β pathway.

Materials and Methods

Animal Preparation, Electrode Implantation, and Intrathecal Catheterization

This study used female Sprague–Dawley rats (220–330 g), which were maintained on a 12-hour light-dark cycle with standard laboratory chow and tap water administered ad libitum. For rats undergoing visceral pain studies, electrodes were implanted using the following procedure. Under ether anesthesia, electromyogram (EMG) electrodes made from Teflon-coated stainless steel wire (7 strand) (A-M Systems, Inc, Carlsborg, WA) were implanted in the rat's abdominal external oblique muscle at least 7 days prior to experimentation. Electrodes were exteriorized onto the back of the neck. For the antisense oligonucleotide (ODN) study, rats were prepared with both electrode implantation and intrathecal catheterization. After sodium pentobarbital (65 mg/kg, intraperitoneal [IP]) anesthesia, the rat's occipital magnus from the dorsal side of the neck was opened, and the dura mater was incised. A catheter (PE-10 polyethylene tubing) was inserted intrathecally and advanced caudally to the lumbar enlargement. Catheter placements were verified by visual inspection after the animal was killed.

Colorectal Distension Procedure

Rats were placed in plastic tunnels (6-cm diameter, 25-cm length) for the described experiments. During the 3 days preceding the experiments, the rats were trained to the experimental conditions by placing them singly in the tunnel for 3 hours per day. The colorectal distension (CRD) balloon was composed of a latex glove finger (7 cm long) attached to a rectal catheter (Medtronic, Skovlunde, Denmark). The balloon was inflated and left overnight to help equilibrate the tension in its wall. The inflatable device was introduced through the anal canal completely into the rectum in conscious rats and secured to the tail base. The tube was then connected to a barostat (Medtronic). The colon was distended by inflating the balloon to the desired pressure (20, 40, or 60 mm Hg) for 10-second intervals with 30-second intervals between distensions. Distensions were repeated 4 times for each experimental protocol with 5-minute intervals between each series.²

Ovariectomy Procedure

An ovariectomy was performed on experimental animals as previously described.² Briefly, ovaries were excised utilizing forceps through a 1-cm incision over both flanks while the rat was sedated under light ether anesthesia. A ligature was placed below the ovary, and the ovary was removed. Ovaries in sham-OVX rats were externalized from the abdominal cavity and then replaced without being excised. Rats were allowed 1-week recovery prior to experimentation. Implantation of EMG electrodes was conducted during ovary surgery in several rat groups.

Experiment Protocol

All experiments were performed at the same time of day (between 9:00 AM and 12:00 PM [noon]) to minimize the influence of circadian rhythms. The first experiment was designed to verify the 5HTP-induced VH in awake female rats and to explore its potential mechanism. The rats underwent 2 rounds of CRD during the proestrous stage. These 2 sessions were separated by 4–5 days, which corresponds to the rat ovarian cycle. During session 1 in the experimental group ($n = 7$), distilled water (vehicle) was injected subcutaneously 30 minutes prior to CRD; whereas in session 2, 5HTP (10 mg/kg; Sigma–Aldrich, St Louis, MO) was injected subcutaneously prior to the CRD. In the control group ($n = 5$), a subcutaneous distilled water injection was administered during both sessions. The CRD procedure with simultaneous EMG recording was performed as described. The EMG was recorded using a CED 1401 instrument and analyzed using Spike 2 software for Windows (Cambridge Electronic Design, Cambridge, UK). The raw EMG signal was rectified off-line, and the area under the curve (AUC) for baseline activity in each session was subtracted from the AUC for the rectified response to CRD to obtain an AUC difference.² In each session, the EMG values from individual distensions were averaged. We further tried to confirm the role of 5HT₃ receptors in 5HTP-induced VH³ and to identify the potential site of sensitization. Thirty minutes after 5HTP priming, subcutaneous injection of 5-HT₃ receptor antagonist (granisetron, 10 μ g/kg, $n = 6$, Sigma–Aldrich) or saline ($n = 6$) were applied. CRD with simultaneous EMG recording as described was then performed. Thereafter, the rats were anesthetized with sodium pentobarbital (65 mg/kg, IP) and perfused transcardially with normal saline followed by 4% paraformaldehyde in ice-cold phosphate-buffered saline (PBS). For quantifying the calcitonin gene-related peptide immunoreactive (CGRP-IR) neurons, dorsal root ganglions (DRGs) at L6 level were excised and postfixed in the same fixative at 4°C for 4 hours. All specimens were cryoprotected in 30% (wt/vol) sucrose, and sections were collected in PBS. The immunohistochemical study was performed as described.² Briefly, after washing in PBS (pH 7.4), free-floating sections were treated with

0.2% H₂O₂ until bubbles disappeared. Nonspecific binding was blocked by 3% normal goat serum plus 2% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 (PBST) for 1 hour. Primary antibody of anti-CGRP (1:2000; a rabbit polyclonal, Chemicon International, Temecula, CA) was then applied. After overnight shaking at 4°C, sections were incubated for 1 hour with goat anti-rabbit biotinylated secondary antibody (1:1000) (Pierce, Rockford, IL), followed by an avidin-biotin-horseradish peroxidase complex (Pierce) for 1 hour with intervening washes of PBST. Antigen was visualized by combining equal volumes of an ammonium nickel sulfate solution (30 mg/mL in 0.1 mol/L sodium acetate, pH 6.0) in the presence of 0.03% hydrogen peroxide. Following washing and dehydration through a graded series of ethanol solutions, sections were coverslipped with the mounting medium Permount (Merck, Darmstadt, Germany). To quantify the DRG neurons expressing CGRP, DRGs were sectioned at 30 μm. Three sections, 60 μm apart, were selected for quantification to avoid counting the same cells twice. Cells were counted on screen from the images of whole sections. Only neurons with clearly visible nuclei were counted.

The second experiment investigated the effects of OVX on 5HTP-induced VH. Similar to the protocol used in the first experiment, the abdominal muscle responses to CRD of the OVX and sham-OVX rats (n = 6 in each group) were recorded following subcutaneous injection of distilled water (session 1) or 5HTP (session 2). The 2 sessions were separated by 4–5 days, and the experiments were conducted in the proestrous stage in sham-OVX rats.

The third experiment examined the effects of ovarian steroid replacement on 5HTP-induced VH in OVX rats. To study this relationship, we administered subcutaneous injections of vehicle (sesame oil, n = 4), 17β-estradiol (n = 6, 10 μg/kg), progesterone (n = 4, 20 mg/kg), or both 17β-estradiol and progesterone (n = 6) 1 hour prior to distilled water or 5HTP injection in the OVX rats. In our previous experiments, sex hormone plasma levels peaked at 1.5 hours following the injection, and there was a 30-minute time lag between 5HTP injection and CRD.² As a result, we chose to administer the hormone injection 1 hour prior to 5HTP administration. Hormone or vehicle was injected twice, 4 days apart (for each of the 2 tests), to match the 4-day cycle in intact female rats. All experimental groups underwent CRD after injection of distilled water or 5 HTP in 2 sessions (separated by 4 days).

Because our experimental results indicated that 5HTP-induced VH is estrogen dependent, the fourth experiment was designed to investigate which ERs were involved in the pathogenesis of the observed phenomenon. Female rats underwent subcutaneous injection of a traditional ER agonist (the ERα agonist propylpyrazole-triol [PPT; 1, 10, and 100 μg/kg, n = 4 in each dose, Tocris

Cookson, Ellisville, MO], the ERβ agonist diarylpropionitrile [DPN; 1, 10 or 100 μg/kg, n = 4 in each dose, Tocris Cookson]), the new GPR30 receptor agonist G1 (1, 10, and 50 μg/kg, n = 5 in each dose, kindly provided by Prof. Oprea), or vehicle (10% DMSO, n = 4, J. T. Baker Chemical Co. Phillipsburg, NJ) 1 hour prior to 5HTP administration to the OVX rats. The doses of PPT and DPN were based on studies from Chaudry's group. With their models, a single injection of PPT or DPN at a dose as low as 5 μg/kg can inhibit the injury of liver, lung, and heart from hemorrhagic shock, which is also accompanied by some biochemical alternations such as changes in cardiac heat shock proteins (heart), lactose dehydrogenase (LDH) activity, nitrate/nitrite, and interleukin (IL)-6 (lung), and protein kinase A (PKA)-dependent signaling (liver).^{11–13} Because G1 was able to restore 5HTP-induced VH in OVX rats, dosages at 0.01, 0.1, and 100 μg/kg (n = 5 at each dose) were further tested. In addition, the ER antagonist ICI-182,780 (1, 3, and 10 mg/kg, n = 5 in each dose, Tocris Cookson) or vehicle (10% DMSO, n = 5) together with 17β-estradiol was given to OVX rats and followed by 5HTP sensitization and CRD treatment. ICI-182,780 was selected because it is known to bind to both the ERα and the ERβ isoforms and maintains an antagonistic profile with both receptor types.¹⁴ Currently, selective GPR30 antagonist is not available. As an alternative, we administered the GPR30 antisense ODN sequence 5'-TTGGGAAGTCACATCCAT-3' (Eurogenetec Ait, Singapore) via an intrathecal injection (n = 8).¹⁵ A random ODN sequence, 5'-GATCTCAGCACGGCAAAT-3', and distilled water served as controls (n = 8 in each group).¹⁵ All ODNs were dissolved in water to a final concentration of 2 μg/μL. For each intrathecal injection, 20 μg of ODNs was used followed by a 20-μL normal saline flush. OVX rats were intrathecally injected with vehicle, random control, or antisense ODNs twice daily for 3 consecutive days. On the morning of the fourth day after intrathecal injection, 5HTP-induced VH was evaluated following replacement of 17β-estradiol (10 μg/kg) using the same method as described in experiment 4. After the EMG recording, the rats were killed and their DRGs removed from the L6 and S1 levels. The effects of antisense ODNs on inhibition of GPR30 expression in the DRG were tested at the protein level by both Western blotting and immunohistochemistry. For Western blotting, protein lysates from pooled DRG were prepared in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL) with a 1:100 dilution of a protease inhibition cocktail (Sigma-Aldrich). Proteins (20 μg) were fractionated on 12% polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, MA) at 100 V for 1 hour at 4°C, blocked for 2 hours in PBS containing 5% BSA, and incubated with the primary antiserum GPR30 (1:500; Abcam, Cambridge, UK) for 2 hours in PBS containing 1% BSA overnight at 4°C. After washing in PBS, mem-

branes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Jackson ImmunoResearch, West Grove, PA) in PBS containing 1% BSA for 2 hours at room temperature. Bands were visualized using the Western Lighting Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA) and appropriate exposure to Kodak x-ray (Kodak, Rochester, NY) film. Membranes were subsequently stripped and re probed for actin (anti- β -actin, 1:10,000; Abfrontier, Seoul, Korea). Films were scanned and band intensities measured using a Luminescence Imaging System (Fujinon LAS-4000, Fujifilm Corp, Tokyo, Japan), and quantification was performed using Multi-Gauge software (V 3.0). For immunohistochemical staining for GPR30 and CGRP, DRG from rats treated with distilled water, random, and antisense ($n = 3$ in each group) ODN rats were processed as described in experiment 1. The concentration for the primary antibody of anti-GPR30 is 1:500 (a rabbit polyclonal, Phoenix, Burlingame, CA).

In experiments 4 and 1, we found that GPR30 and 5HT3 receptor were involved in the 5HTP-induced VH, which was also accompanied by a decrease CGRP-IR neurons in DRG after 5HT3 antagonist. Thus, a close relation between GPR30 and 5HT3 receptors was suggested. In experiment 5, we then examined the colocalization of GPR30 and 5HT3 receptors in DRG. For double immunohistochemistry study, animals and their DRGs (L6/S1) were treated in a similar way as described in experiment 1, except sections were incubated with a mixture of goat polyclonal 5HT3 (1:100; Everest Biotech, Oxfordshire, UK) and rabbit polyclonal GPR30 antibodies (1:100; Phoenix) diluted in PBST containing 3% normal donkey serum overnight at 4°C. Next, free-floating sections were rinsed in PBST at room temperature and incubated with the appropriate Alexaconjugated (Invitrogen, Carlsbad, CA) secondary antibodies at a dilution of 1:200 for 1 hour at room temperature. Sections were

washed in PBST, rinsed in 0.1 mol/L phosphate buffer (PB; pH 7.4), and mounted with VECTASHIELD mounting medium (Vector, Burlingame, CA). As a control for immunolabeling specificity, primary antibody was excluded from the immunostaining procedure for a set of samples. Images were captured with a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan) consisting of an Olympus IX81 microscope. Images were processed with Olympus FV10-ASW 1.3 software. Neurons expressing GPR30 and 5HT3 were photographed and analyzed.

In the sixth experiment, we tried to verify that colonic inflammation was not found in the current model. Intact rats in the proestrus phase were pretreated with distilled water in session 1 and 5HTP in session 2 ($n = 3$ in each group). After sham or CRD treatment, the distal 7 cm of the descending colon and rectum were removed, fixed in 10% formalin, and examined histologically. Inflammation present in the colonic mucosa (none, mild, moderate, or severe) was graded after blind examination by a gastrointestinal pathologist.

Statistical Analysis

Differences between experimental groups were analyzed by Student *t* test; 1-way ANOVA; and post hoc by Tukey test, 2-way repeated-measures ANOVA, and post hoc with paired *t* test using Bonferroni correction for multiple comparison or 2-way mixed-design ANOVA followed by the Tukey post hoc test. Values are expressed as mean \pm SEM. Differences were considered to be statistically significant at $P < .05$.

Results

5HTP-Induced VH in Intact Female Rats via 5HT3 Receptor

Figure 1 shows an example of 5HTP-induced VH in an intact rat. The visceral motor response (VMR) is

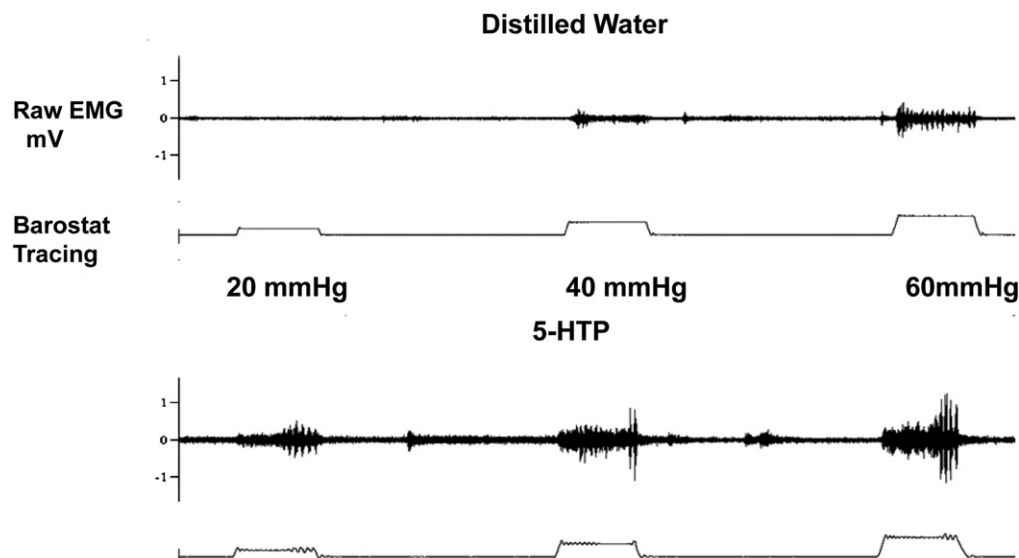


Figure 1. The visceromotor reflex (VMR) in response to CRD after distilled water or 5HTP subcutaneous injection. Enhanced electromyographic signals were noted after 5HTP injection.

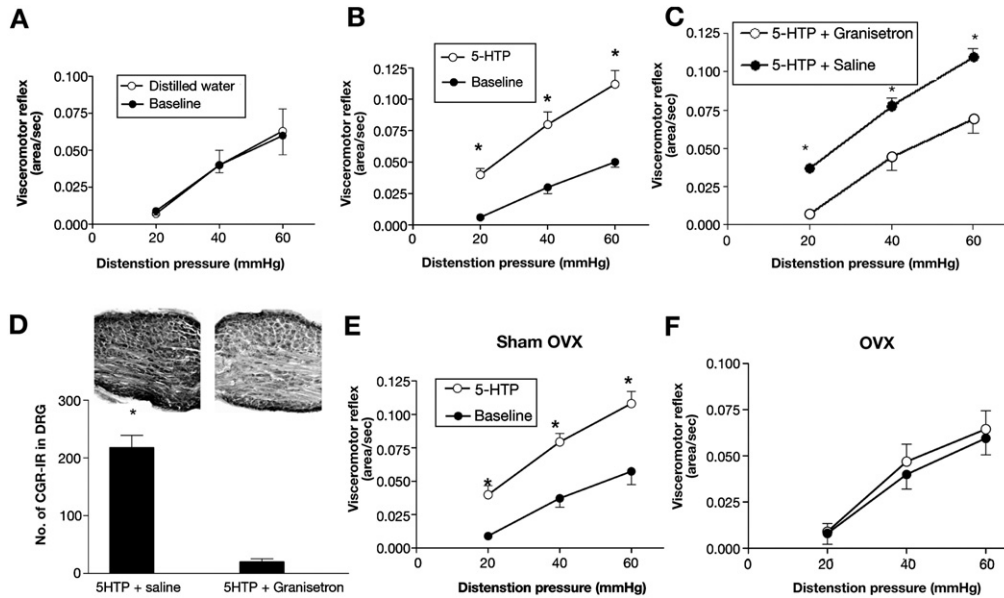


Figure 2. (A) The VMR following distilled water subcutaneous injection is similar to baseline in conscious, intact female rats. (B) In contrast, the VMR is significantly higher after 5HTP subcutaneous injection as compared with baseline (distilled water injection) ($P = .001$, repeated measures 2-way ANOVA). (C) Granisetron (5HT3 antagonist) inhibited the enhanced VMR upon 5HTP treatment ($P < .01$, mixed-design 2-way ANOVA). (D) Granisetron decreased the numbers of CGRP-IR DRG neurons upon 5HTP treatment. ($P < .001$, Student t test); (E and F) The effects of 5HTP on sham-OVX and OVX rats. The VMR after 5HTP treatment in sham-OVX rat was significantly higher than baseline ($P < .01$, repeated measures 2-way ANOVA). OVX rats did not exhibit abdominal muscle hyper-responsiveness to CRD after administration of 5HTP. $*P < .05$, Student t test or paired t test with Bonferroni correction.

reflected by the raw EMG signal and increases gradually in a pressure-dependent manner in both sessions 1 (distilled water) and 2 (5HTP). Subcutaneous injection of 5HTP, but not distilled water, significantly increased the

EMG signal measured in the external oblique abdominal muscle in response to CRD relative to the baseline signal in intact rats ($P = .001$, Figure 2A and B). The 5HTP-induced VH was inhibited by granisetron, which was

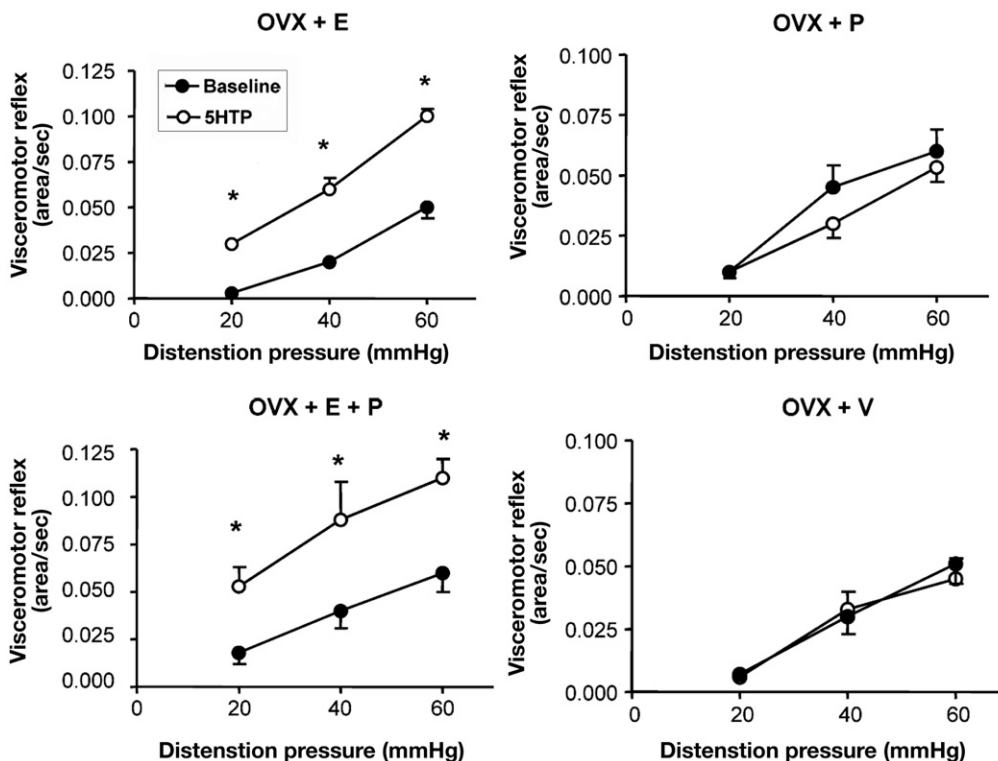


Figure 3. Effect of pretreatment with 17β -estradiol (E), progesterone (P), both E and P (E+P), or vehicle (V; sesame oil) on the VMR to CRD in OVX rats upon distilled water (baseline) or 5HTP subcutaneous injection. The OVX rats can rapidly restore 5HTP-induced visceral hypersensitivity (VH) within 90 minutes following a single-dose injection of estrogen, either with ($P < .01$, repeated measures 2-way ANOVA) or without progesterone ($P < .01$, repeated measures 2-way ANOVA). $*P < .05$, paired t test with Bonferroni correction.

accompanied by a decrease of EMG signal ($P < .01$, Figure 2C) and CGRP-IR neurons in the L6 DRG ($P < .001$, Figure 2D).

5HTP-Induced VH in Female Rats With Sham OVX or OVX

After 5HTP subcutaneous injection, sham OVX rats showed significantly increased VMR over baseline ($P < .01$), whereas OVX rats showed a loss of 5HTP-induced abdominal muscle hyper-responsiveness to CRD (Figure 2E and F).

Hormone Treatment and VH

In 5HTP-treated OVX rats, replacement with 17β -estradiol alone ($P < .01$) or in combination with progesterone ($P < .01$) had significantly higher VMR when compared with the baseline, whereas the rats with progesterone or vehicle pretreatment showed no evidence of

VH (Figure 3). This result suggested that estrogen replacement restored 5HTP-induced VH in OVX rats.

Effects of $ER\alpha/\beta$ Agonist, GPR30 Agonist, ER Antagonist (ICI-182,780), and GPR30 Antisense ODN on 5HTP-Induced VH

Neither PPT nor DPN at the dosages tested restored 5HTP-induced VH in OVX rats ($P > .05$ at all doses; Figure 4). The vehicle (10% DMSO) also showed no significant difference between baseline and 5HTP session in OVX rats. On the contrary, pretreatment of OVX rats with selective GPR30 agonist G1 at dosages from 1 to 50 $\mu\text{g}/\text{kg}$ significantly increased EMG levels following 5HTP sensitization in OVX rats ($P < .01$ for all 3 dosages; Figure 5A). Figure 5B shows that a sharp dose-response curve of G1 administration correlates with 5HTP-induced VH. G1 showed no effects on VH at low doses (0.1 or 0.01 $\mu\text{g}/\text{kg}$), and it reached a plateau after 1 $\mu\text{g}/\text{kg}$.

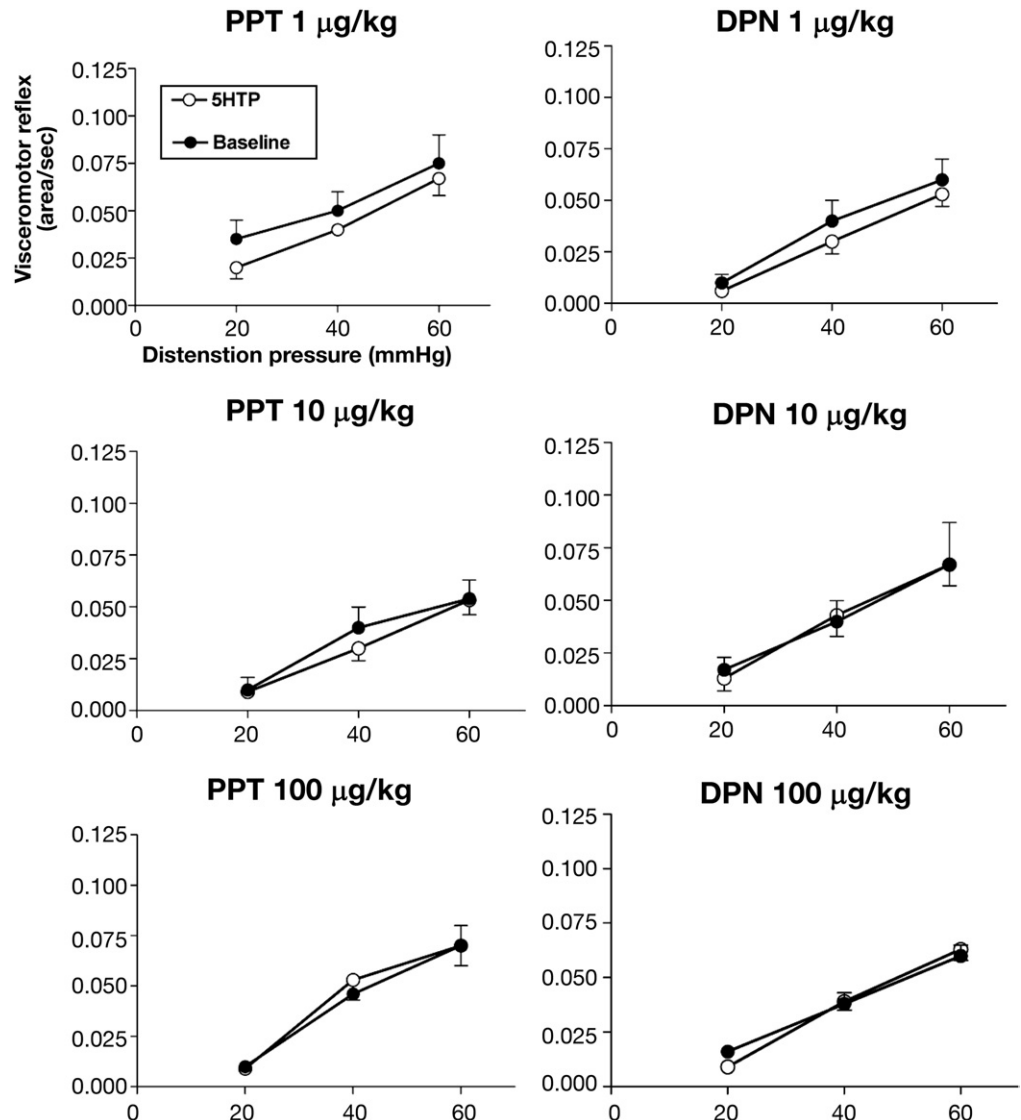


Figure 4. Effect of pretreatment with vehicle, an estrogen receptor α ($ER\alpha$) agonist (propylpyrazole-triol, PPT, at 1, 10, and 100 $\mu\text{g}/\text{kg}$) or an $ER\beta$ agonist (diarylpropionitrile, DPN at 1, 10 or 100 $\mu\text{g}/\text{kg}$) on the VMR to CRD in OVX rats following distilled water (baseline) or 5HTP subcutaneous injection. VH cannot be restored by either PPT or DPN treatment ($P > .05$ at all dosages, repeated measures 2-way ANOVA).

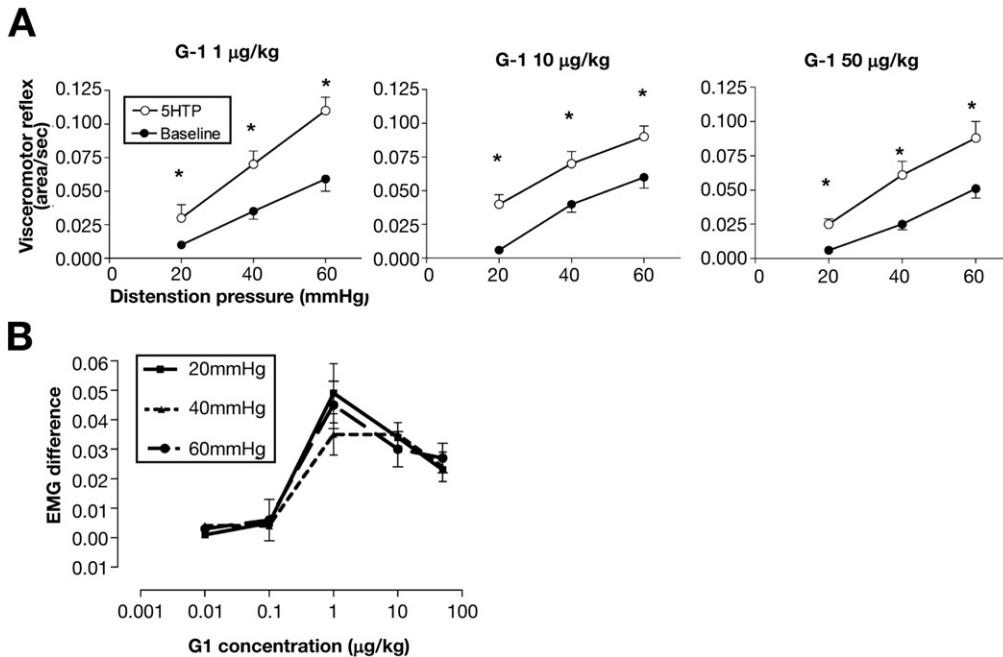


Figure 5. (A) Effect of pretreatment with a GPR30 agonist (G1 at 1, 10, and 50 $\mu\text{g}/\text{kg}$) on the VMR to CRD in OVX rats following distilled water (baseline) or 5HTP subcutaneous injection. G1, similar to the effect of 17β -estradiol, can rapidly restore 5HTP-induced VH at the testing dosage ($P < .01$, repeated measures 2-way ANOVA); $*P < .05$, paired t test with Bonferroni correction. (B) The dose-response curve of G1 on the VMR to CRD at 20 to 60 mm Hg in OVX rats upon 5HTP sensitization.

5HTP-induced VH in 17β -estradiol-treated OVX rats can still be observed with the administration of vehicle or the ER antagonist ICI-182,780 ($P < .01$ for all 3 dosages; Figure 6A). Figure 6B demonstrates that 5HTP-induced VMR in estrogen-treated OVX rats intrathecally injected with random ODN or distilled water is significantly higher than in rats treated with the GPR30 antisense ODN ($P < .01$). This result suggested that 5HTP-induced VH in estrogen-treated OVX rats was attenuated by knockdown of GPR30 using an antisense ODN.

Effects of the antisense ODN in inhibiting GPR30 expression in DRG were confirmed at the protein level by Western blotting and immunohistochemistry. In a Western blot analysis, GPR30 expression was markedly attenuated after intrathecal injection of the antisense ODN as compared with random ODN and vehicle groups ($P < .01$; Figure 7A). In immunohistochemical studies, the number of GPR30 immunoreactive (GPR30-IR) DRG neurons was found to be significantly reduced after antisense ODN injection but was maintained at baseline levels in the rats treated with random ODN or distilled water (Figure 7B). Furthermore, the number of CGRP-IR DRG neurons remained constant, and the cells demonstrated intact morphology (Figure 7B). This data further indicated that DRG neurons were not damaged by 3-day treatment with antisense ODN and that the knockdown was gene specific.

Colocalization of GPR30 and 5HT3 Receptors in DRG

GPR30 and 5HT3 expression were examined in the DRG. Ninety-four percent (221/235) of all GPR30-IR neurons also showed 5HT3-IR (Figure 8). Conversely, 90%

(221/244) of 5HT3-IR neurons demonstrated GPR30-IR (Figure 8).

Effects of 5HTP on Colonic Inflammation

Histopathologic examination of colonic sections demonstrated no or very mild mucosal inflammation in samples collected from 5HTP-treated rats, after both sham and CRD stimulation.

Discussion

In this study, we first showed that the 5HTP-induced VH in awake female rats is estrogen dependent, suggesting that the sex hormone can affect visceral nociceptive processing in the absence of colonic inflammation. This rapid 5HTP-induced and estrogen-dependent VH may act through the non-genomic pathway (GPR30 receptor) and not the traditional $\text{ER}\alpha/\beta$.

Two previous studies have shown that mustard oil-induced VH displayed different patterns between intact and OVX rats.^{1,2} However, marked colonic mucosal inflammation was noted after the mustard oil instillation, where a majority of IBS patients exhibit chronic visceral hypersensitivity without overt inflammation. Consequently, mustard oil-induced colonic inflammation may provide a good model for VH but not appropriate for IBS. With the current rat model, we demonstrated that estrogen, in the absence of colonic inflammation, can modulate 5HTP-induced VH.

Estrogen commonly acts through intracellular receptors, ie, $\text{ER}\alpha/\beta$, which bind to the ligand and then translocate to the nucleus upon activation, thus acting as ligand-dependent transcription factors.⁷ The down-

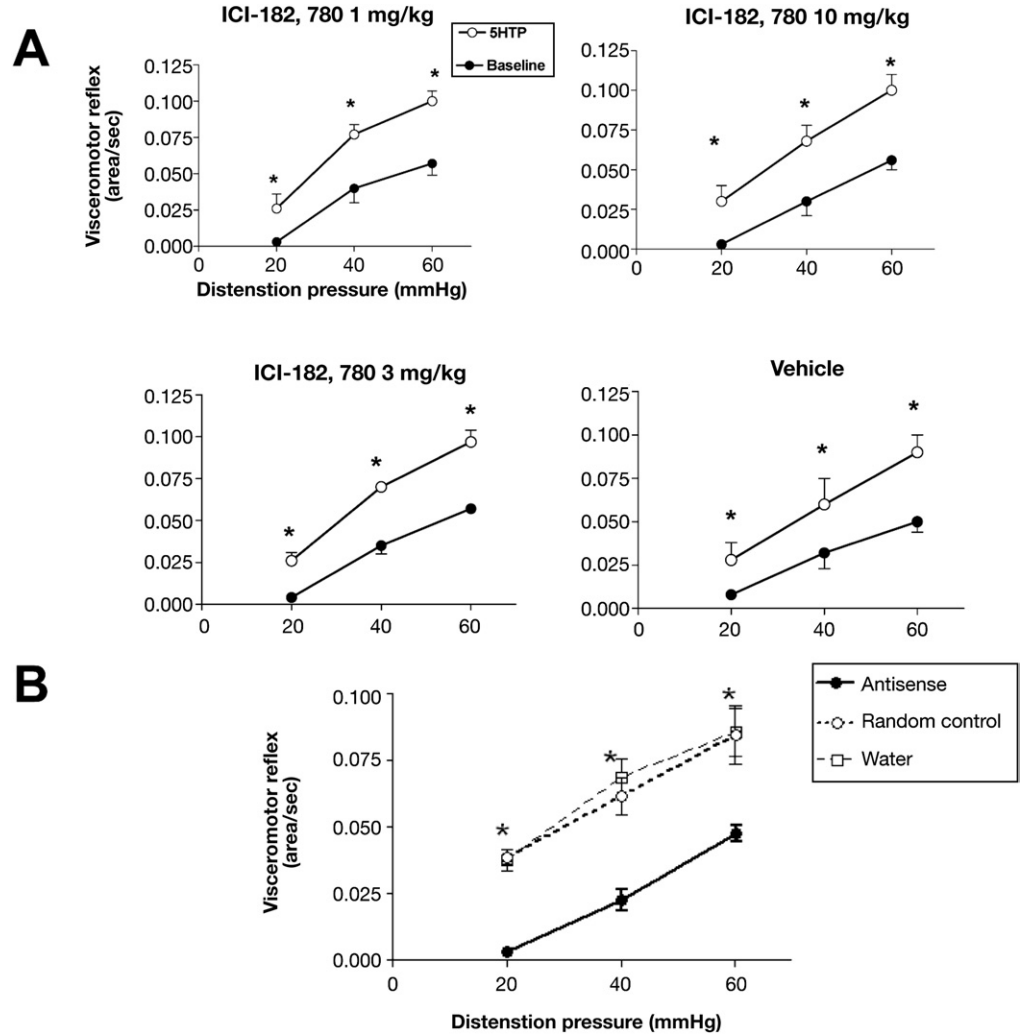


Figure 6. (A) Effect of pretreatment with an ER antagonist (ICI-182,780 at 1, 3, and 10 mg/kg) or vehicle (10% DMSO) together with 17 β -estradiol on the VMR to CRD in OVX rats upon distilled water (baseline) or 5HTP subcutaneous injection. 5HTP-induced VH was still observed after both ICI-182,780 and vehicle administration ($P < .01$, repeated measures 2-way ANOVA); $*P < .05$, paired t test with Bonferroni correction. (B) After 3 consecutive days of injection of antisense ODN for GPR30 (20 μ g, twice a day), 5HTP-induced VH was significantly suppressed when compared with OVX rats treated with either random ODN or distilled water ($P < .01$, mixed-design 2 way ANOVA); $*P < .05$, 1-way ANOVA followed by the Tukey post hoc test.

stream effects of this signaling mechanism are usually delayed (within hours to days) and long acting.⁷ However, rapid actions of estrogen associated with its receptor in the plasma membrane have also been identified.⁷ These rapid, “non-genomic” estrogen-dependent signals have been reported in correlation with somatic and visceral pain/sensation.^{2,9,16,17} It is suggested that rapid non-genomic estrogen-mediated signaling may involve a transmembrane G-protein-coupled receptor (GPCR), GPR30.^{10,18} In our study, estrogen can restore VH in OVX rats in 90 minutes, which is rapid and suggested a non-genomic estrogen-signaling pathway. We demonstrated that G1, a selective GPR30 agonist, but not PPT or DPN (ER α/β agonists), can restore 5HTP-induced VH in OVX rats. Furthermore, the VH in OVX rats is still preserved under the treatment of nuclear ER antagonist ICI-182,780. Although ICI-182,780 is widely used as an ER antagonist, it has been shown to activate GPR30.¹⁹ Thus, this maintenance of the VH induced by 5-HTP in rats treated by 17 β -estradiol and ICI-182,780 could be interpreted as either the result of ER antagonism and action of E2 on GPR30 or as direct effect of ICI-182,780

on GPR30. Because using the ODN antisense for GPR30 produced a significant decrease in 5-HTP-induced VH, the possibility of the latter explanation is less likely. Taken together, our findings support the hypothesis that estrogen-dependent, 5HTP-induced VH acts through the GPR30 receptor.

Serotonin-related receptors may also promote the estrogen-dependent, 5HTP-induced VH. 5HT3 receptors are present in the spinal afferents originating in the DRG, and serotonin-evoked excitation of extrinsic sensory neurons is primarily mediated by 5-HT3 receptors.²⁰ Blockade of 5HT3 receptor-mediated activation of spinal afferents by alosetron depresses the afferent signaling of CRD in the rat.²¹ We also showed the effect of 5HT3 antagonist in the inhibition of 5HTP-induced VH, which was accompanied by a decrease of CGRP-IR neurons in DRG. The codistribution of 5HT3 and GPR30 receptors shown in DRG neurons further suggested that both receptors may be active in DRG.

There are conflicting data regarding the role of GPR30 as an estrogen receptor. For example, Pedram et al could not demonstrate cAMP or extracellular signal-regulated

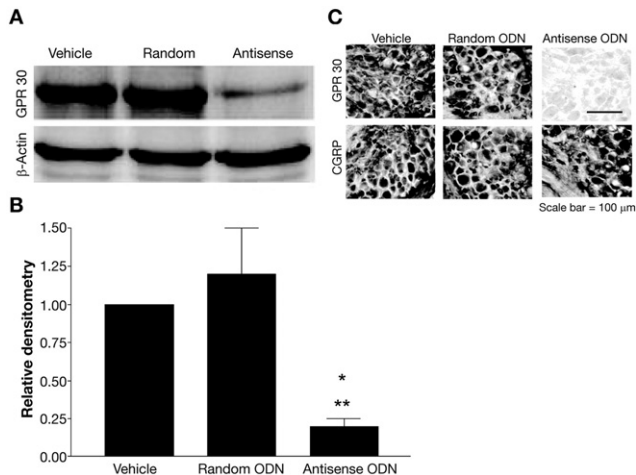


Figure 7. GPR30 expression in DRG neurons (L6/S1) is decreased by gene-specific antisense ODNs. (A) Western blot analysis showed that GPR30 expression was markedly attenuated in the antisense ODN-treated group when compared with the random ODN and vehicle (distilled water) groups; (B) Mean densitometry levels for GPR30 expression relative to vehicle control. GPR30 expression levels were significantly attenuated in the DRG neurons of antisense ODN-treated rats ($n = 8$) as compared with the random ODN and vehicle ($n = 8$, $P < .05$, vehicle vs antisense ODN; $**P < .01$ random ODN vs antisense ODN, 1-way ANOVA followed by post hoc with Tukey test). (C) As shown by immunohistochemical staining, GPR30-IR neurons were significantly decreased after antisense ODN injection but not after injection with random ODN or distilled water. CGRP-IR DRG neuron expression remained the same, with the cells demonstrating intact morphology among all 3 groups.

kinase activation in GPR30-positive, ER-negative breast cancer cells.²² Using very high expression of GPR30 in transfected COS-7 or CHO cell lines, Otto et al found that GPR30 were not responsive to estradiol.²³ Membrane bound receptor was also reported to be affected by traditional estrogen receptor antagonists in the isolated DRG neuron.²⁴ The reason for the discrepancy is not known. A possible explanation is that the expression of GPR30 or the intracellular signal mechanism is cell type specific. Similarly, the subcellular localization of GPR30 is a matter of debate. Although there is a general agreement that GPR30 is a non-nuclear estrogen receptor,^{10,25} the site at which GPR30 is concentrated, ie, plasma membrane vs cytoplasm, remains to be clearly resolved.^{10,26,27} We found that the GPR30 receptor appears to concentrate in the cytoplasm of DRG neurons, which is consistent with a recent report in which GPR30-IR is found to be distributed throughout the cytoplasm in the spinal neurons and sensory and autonomic ganglion cells of rats.²⁸ It is noteworthy that many GPCRs are expressed intracellularly in endogenous settings.^{29,30} We agree with Filardo et al's view that the abundant intracellular expression of GPR30 may be related to receptor trafficking patterns observed for other GPCRs.²⁷ Retention of GPCRs within the endoplasmic reticulum is a common feature of GPCRs' biogenesis as a consequence of multi-

ple regulatory events, such as carbohydrate processing,³¹ disulfide bond exchange,³² and proteolytic editing.³³

The effect of gonadal steroids on the nociceptive pathways in visceral pain may be exerted at various levels of the neuraxis. We and others have demonstrated a potential role for the "spinal" mechanism of estrogen-mediated VMR or visceral hypersensitivity.^{1,2} On the other hand, estrogen has been demonstrated to restore stress-induced visceral hypersensitivity in OVX rats, which supports the "supraspinal" role for estrogen in mediating visceral pain.¹⁶ In the current study, we found reduced GPR30 expression in the DRG neurons after intrathecal antisense ODN treatment and colocalization of 5HT3 and GPR30 receptors in the DRG neurons. The CGRP-IR neurons in DRG are decreased in our 5HTP-induced VH model. A recent paper showed that the selective GPR30 agonist G-1 causes nociceptor sensitization in DRG culture.³⁴ Therefore, we suggest that "peripheral" mechanism in DRG may be involved in our observed 5HTP-induced VH. Further in vitro experiments regarding the role of GPR30 in mediating VH in colon-innervated DRG neurons is necessary to prove our in vivo findings.

GPR30 is similarly distributed among male and female rats in the brain, spinal cord, adrenal gland, and autonomic and sensory ganglion.^{28,35} A similar distribution pattern of genomic estrogen receptors can also be observed in the central nervous system of male and female rats and mice.^{36,37} Although a sex difference is not apparent in the pattern of distribution of genomic and nongenomic estrogen receptors in the nervous system, the density or pharmacologic property of estrogen receptors may be substantially different in females compared

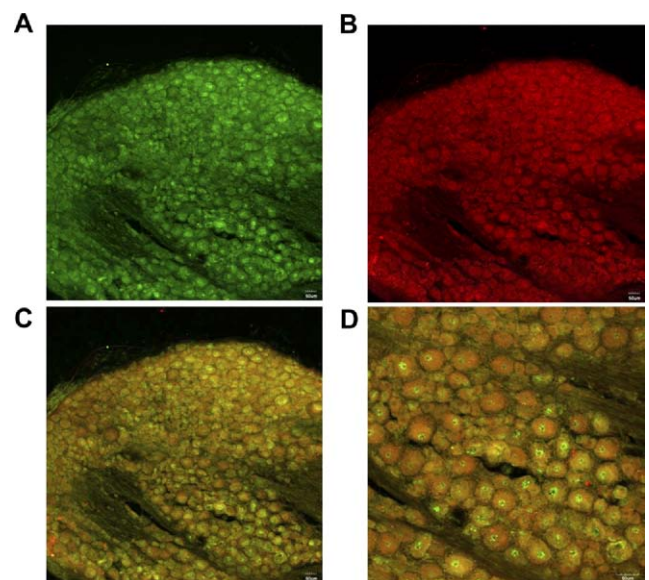


Figure 8. Double immunocytochemical staining of 5HT3-IR and GPR30-IR DRG neurons (L6). (A) An example of 5HT3-IR neurons (green); (B) GPR30-IR neurons (red); (C and D) merge of 5HT3-IR and GPR30-IR staining.

with males. In female rats, we have shown the evidences of GPR30 receptor in mediating the estrogen-dependent and 5HTP-induced VH. Conversely, a recent publication demonstrated that injection of estrogen or G1 into the hind paw of male rats can rapidly induce dose-dependent mechanical hyperalgesia.³⁴ Although the role of GPR30 receptor in the visceral pain of male rats remains to be fully resolved, available evidence shows that GPR30 is functionally active in male and female animals.

In conclusion, in the absence of colonic mucosal inflammation, estrogen can rapidly modulate VH induced by the peripheral injection of a serotonin precursor. The loss of gonadal hormone signaling suppresses VH, whereas estrogen replacement results in its restoration. Estrogen-mediated VH appears to act via the newly identified GPR30 receptor rather than through the classical ER α or ER β pathways.

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Conflicts of interest

The authors disclose no conflicts.

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