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Sex differences in pain-related behaviors and clinical progression of disease in mouse models of colonic pain

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Abstract

Previous studies have reported sex differences in patients with irritable bowel syndrome and inflammatory bowel disease, including differences in visceral pain perception. Despite this, sex differences in behavioral manifestations of visceral pain and underlying pathology of the gastrointestinal tract have been largely understudied in preclinical research. In this study, we evaluated potential sex differences in spontaneous nociceptive responses, referred abdominal hypersensitivity, disease progression, and bowel pathology in mouse models of acute and persistent colon inflammation. Our experiments show that females exhibit more nociceptive responses and referred abdominal hypersensitivity than males in the context of acute but not persistent colon inflammation. We further demonstrate that, after acute and persistent colon inflammation, pain-related behavioral responses in females and males are distinct, with increases in licking of the abdomen only observed in females and increases in abdominal contractions only seen in males. During persistent colon inflammation, males exhibit worse disease progression than females, which is manifested as worse physical appearance and higher weight loss. However, no measurable sex differences were observed in persistent inflammation-induced bowel pathology, stool consistency, or fecal blood. Overall, our findings demonstrate sex differences in pain-related behaviors and disease progression in the context of acute and persistent colon inflammation, highlighting the importance of considering sex as a biological variable in future mechanistic studies of visceral pain as well as in the development of diagnostics and therapeutic options for chronic gastrointestinal diseases.

Keywords: Visceral pain, Sex differences, Colitis, Dextran sulfate sodium, Intracolonic capsaicin, Referred abdominal hypersensitivity, Chronic gastrointestinal diseases, Colonic pain

1. Introduction

Patients with irritable bowel syndrome (IBS), inflammatory bowel disease (IBD), and other chronic gastrointestinal diseases often manifest altered visceral pain perception that has been related to the development of visceral hypersensitivity.[10,38,51] Importantly, clinical studies have shown that women with IBS exhibit higher sensitivity to repetitive rectal distension and report more severe abdominal pain or discomfort than men, suggesting sex differences in visceral pain perception in IBS.[3,4,48] Sex differences have also been reported in the clinical manifestation, disease course, complications, psychiatric comorbidities, central processing of visceral pain, and pathophysiology of IBS and other chronic gastrointestinal disorders.[14,28,41,47] By contrast, a recent study found no sex differences in visceral pain thresholds to rectal balloon distensions in healthy young women and men,[20] suggesting that baseline visceral sensitivity is not dependent on sex. Evaluation of the mechanisms underlying sex differences in visceral sensitivity in human subjects is limited by methodological and experimental challenges which are thus not completely understood.[1,12,17] Preclinical studies in rodents offer a valuable alternative to evaluate sex-specific factors in chronic gastrointestinal diseases in a more controlled setting, providing insights towards the development of more effective diagnostic and treatment options for patients.

Preclinical studies evaluating sex differences in visceral sensitivity in rodents have predominantly measured visceromotor responses to colorectal distension in rats. These studies have shown that visceral sensitivity in rodents is sex-dependent, with females exhibiting higher visceromotor responses to colonic distension than males at baseline and after acute inflammation of the colon.[24,49] Sex differences in visceromotor responses to colonic distension have also been reported in the context of

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stress, where female rats exhibit a stronger relationship between early-life adversity and the development of stress-induced visceral hypersensitivity.[45] Separate studies further show that sex hormones contribute to sex differences in stress-induced visceral hypersensitivity, with estradiol facilitating and testosterone decreasing stress effects on visceral responses.[22] Despite this knowledge, sex differences in visceral sensitivity after acute and persistent inflammation of the bowel remains poorly understood. Evaluating and characterizing potential sex differences in visceral pain-related responses in mice under pathological conditions is essential for the evaluation of mechanisms driving sex differences in visceral pain.

In this study, we systematically evaluated potential sex differences in pain-related responses using 2 well-characterized mouse models of chemically induced colonic hypersensitivity: intracolonic capsaicin, which elicits transient neurogenic inflammation,[30] spontaneous nociceptive responses, and referred abdominal hyperalgesia[31,46]; and the dextran sulfate sodium (DSS) model of colitis, which elicits prolonged inflammation in the bowel as well as visceral and referred abdominal hypersensitivity.[2,6,11,21] Parallel experiments evaluated potential sex differences in disease progression of DSS-induced colitis and associated bowel pathology. Our experiments revealed sex differences in pain-related behaviors after acute colonic irritation as well as in the clinical progression of colitis. Pain-related behaviors and histopathology after persistent inflammation of the colon, however, were similar in males and females. Collectively, these findings set a foundation for future mechanistic studies of sex differences in visceral pain.

2. Materials and methods

2.1. Subjects

All animal procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH) and were approved by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke, and the National Institute on Deafness and other Communication Disorders. Adult male and female Swiss-Webster mice (Taconic Farms) between 8 and 13 weeks old were used for all experiments. Before experiment, littermates of the same sex were group-housed (up to 5 mice per cage) under reversed light/dark cycle, with 12 hours of white light (3.8 lux; 9 PM to 9 AM) alternated with 12 hours of complete darkness (9 AM to 9 PM). Red light (0.4 lux) was used as needed when experimenters or caretakers entered the room. One week before experiments, mice were randomly assigned to experimental groups. Two littermates of the same sex and treatment were housed in clean home cages, with the 2 mice separated from each other by a perforated Plexiglas divider. This housing strategy was selected to avoid potential tissue injury or stress because of social aggression, minimize possible effects on gut microbiota because of grouped housed bedding,[33] and avoid potential effects of social isolation because of single housing.[35] Food and water were provided ad libitum. Before all behavioral experiments, mice were handled as previously described for at least 5 days to minimize potential stress effects associated with handling.[19] Mice handling (5-10 lux), disease activity index (DAI) measurements (5-10 lux), and behavioral testing (25-50 lux) were performed under red light between 10 AM and 7 PM. Male and female mice were never tested simultaneously in the same behavior room. All experimental procedures were performed by an observer blind to experimental treatment and replicated at least 3 times.

2.2. Dextran sulfate sodium induced model of colitis

Male and female mice were randomly assigned to drinking water (control group) or 2.5% (wt/vol) DSS salt (reagent-grade, mol. wt. 36-50 kDa; MP Biomedicals, #16011050) dissolved in drinking water to induce colitis (experimental group), ad libitum for 7 days. On day 7, mice were switched to regular water ad libitum for the duration of the experiment. The next day, intracolonic capsaicin, referred abdominal sensitivity, and bowel sample collection experiments were performed. Each day, along with the handling of the mouse, the DAI was logged by an investigator blind to experimental treatment to evaluate and score disease progression as previously described.[7,26] The DAI is a composite of scores for weight loss, appearance, stool consistency, and blood presence in the stool using a Hemoccult card (Beckman Coulter; **Table 1**). Individual experimental timelines are included in each figure.

Drinking bottles containing an initial volume of 250 mL water or 2.5% DSS were weighted throughout the experiment. We used differences in bottle weight, water density (1 g/mL), and calculated density of 2.5% DSS (1 g/mL) to determine fluid consumption. Fluid intake over 4 to 7 days was averaged and adjusted by body weight (mL/g BW) for each animal. Fluid loss because of potential bottle dripping was calculated using water and 2.5% DSS bottles on empty cages that were manipulated identically to experimental cages, and the average amount of fluid lost because of dripping (1 mL) was subtracted from the fluid intake measured for all mice.

2.3. Intracolonic capsaicin

Male or female mice were habituated (for 1 hour) and tested on an elevated mesh platform in individual 11 × 11 × 13 cm ventilated opaque white Plexiglas boxes. A mirror was placed at 45° angle under the mesh platform to allow full visualization of the animals. Intracolonic injections were performed as previously described.[44]

Table 1

Disease activity index.

Score	Weight loss (%)	Stool consistency	Fecal blood	Appearance
0	None or gain	Formed and hard	Absence	Normal
1	1-5	Formed and soft		Scruffy
2	5-10	Loose stools/semiformed	Presence/hemoccult-positive	Hunched and scruffy
3	10-15	Mild diarrhea (watery)		Nonmotile, hunched, and scruffy
4	15 or more	Gross diarrhea	Gross bleeding	

Disease activity index score is the sum of the following components: the percentage of weight loss, stool consistency, fecal blood, and appearance. Each individual component was scored according to the descriptions in the table.

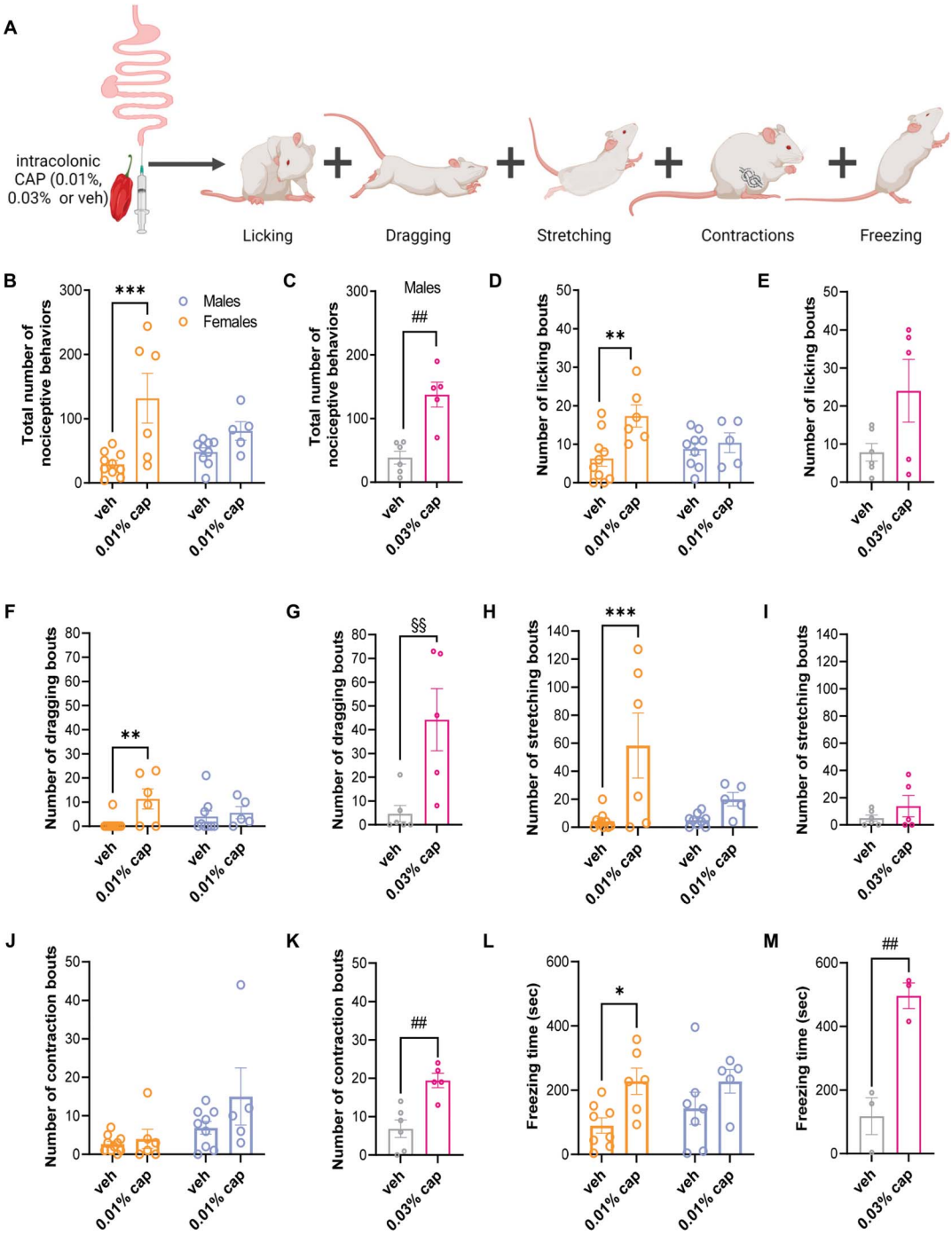

Figure 1. Capsaicin-induced nociceptive behaviors are higher in female than male mice and the behavioral manifestation is sex-dependent. (A) Timeline of behavioral experiments. (B-M) Male and female mice were injected with intracolonic capsaicin (0.01% or 0.03%) or control vehicle and spontaneous nociceptive responses, defined as licking, stretching, dragging, contractions of the abdomen, and freezing behaviors, were recorded. (B and C) Total number of pain-related behaviors. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 26) = 15.02, $P = 0.0006$; effect of sex, F (1, 26) = 0.8271, $P = 0.3715$; interaction, F (1, 26) = 3.984, $P = 0.0565$ (B); unpaired *t* test (2-tailed): t = 4.755, df = 9; $\eta^2 = 0.7153$ (C). (D and E) Licking bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 26) = 7.884, $P = 0.0093$; effect of sex, F (1, 26) = 0.9772, $P = 0.3320$; interaction, F (1, 26) = 4.360, $P = 0.0467$ (D); unpaired *t* test (2-tailed): t = 2.055; df = 9; $\eta^2 = 0.3194$ (E). (F and G) Dragging bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 26) = 5.842, $P = 0.0230$; effect of sex, F (1, 26) = 0.2798, $P = 0.6013$; interaction, F (1, 26) = 3.148, $P = 0.0877$ (F); Mann–Whitney *U* test; U = 1 (G). (H and I) Stretching bouts. (H) Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 26) = 12.53, $P = 0.0015$; effect of sex, F (1, 26) = 3.745, $P = 0.0639$; interaction, F (1, 26) = 4.034, $P = 0.0551$ (H); unpaired *t* test (2-tailed): t = 1.180, df = 9; $\eta^2 = 0.1340$ (I). (J and K) Contraction bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 26) = 2.614, $P = 0.1180$; effect of sex, F (1, 26) = 6.808, $P = 0.0149$; interaction, F (1, 26) = 1.369, $P = 0.2526$ (J); unpaired *t* test (2-tailed): t = 4.160, df = 9; $\eta^2 = 0.6578$ (K). (L and M) Time spent in freezing behavior. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, F (1, 22) = 7.971, $P = 0.0099$; effect of sex, F (1, 22) = 0.4598, $P = 0.5048$; interaction, F (1, 22) = 0.4621, $P = 0.5037$ (L); unpaired *t* test (2-tailed): t = 5.374; df = 4; $\eta^2 = 0.8784$ (M). Data are presented as mean ± SEM. n = 6 to 10 females and 3 to 9 males. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for veh vs 0.01% cap; $\#\#P < 0.01$ for veh vs 0.03% cap; §§$P < 0.01$ for veh vs 0.03% cap. ANOVA, analysis of variance; veh, vehicle.

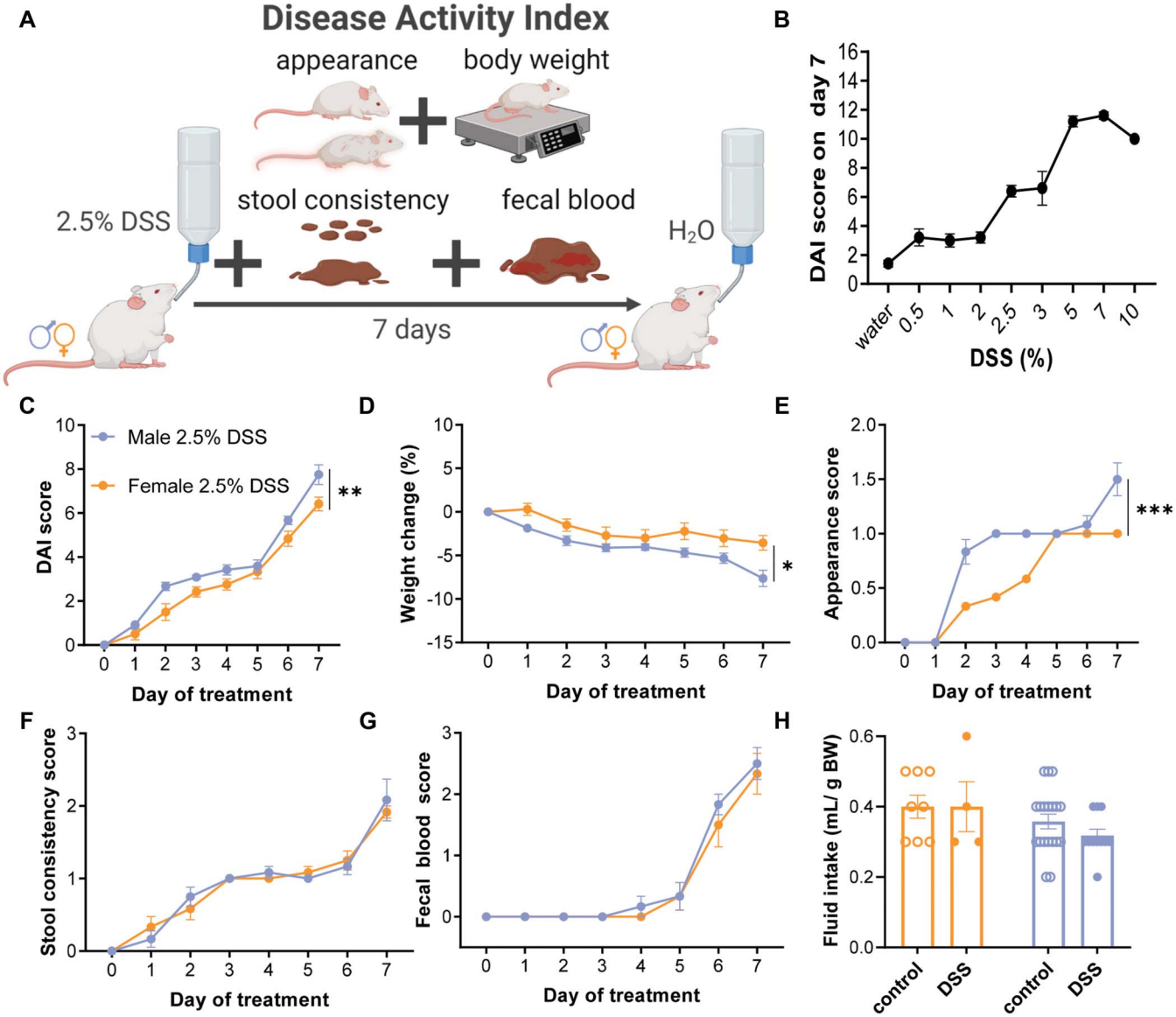

Figure 2. Clinical progression of disease in a DSS-induced mouse model of colitis is sexually dimorphic. (A) Timeline for the induction of experimental colitis. Male and female mice were treated with 2.5% DSS ad libitum for 7 days. Disease Activity Index (DAI) cumulative score was obtained daily to monitor the progression of DSS-induced colitis. (B) DAI score was dependent on the dose of DSS administered. Ordinary one-way ANOVA: F (8, 42) = 62.04; $P < 0.0001$ (n = 1-5 DSS and 15 control). (C-G) Cumulative DAI scores and its individual components, defined as percentage of weight change, appearance, stool consistency, and fecal blood, were analyzed. (C) Cumulative DAI scores. Two-way RM ANOVA: time = F (3.731, 82.09) = 193.4, $P < 0.0001$; sex = F (1, 22) = 11.07, $^{**}P = 0.0031$; time × sex = F (7, 154) = 1.828, $P = 0.0855$. (D) Weight change. Two-way RM ANOVA: time = F (3.946, 86.81) = 28.14, $P < 0.0001$; sex = F (1, 22) = 6.37, $^{*}P = 0.0193$; time × sex = F (7, 154) = 3.044, $P = 0.0050$. (E) Appearance. Two-way RM ANOVA: time = F (3.066, 67.45) = 74.72, $P < 0.0001$; sex = F (1, 22) = 20.77, $^{***}P = 0.0002$; time × sex = F (7, 154) = 5.936, $P < 0.0001$. (F) Stool consistency. Two-way RM ANOVA: time = F (3.039, 66.85) = 65.36, $P < 0.0001$; sex = F (1, 22) = 0.01996, $P = 0.8889$; time × sex = F (7, 154) = 0.6388, $P = 0.7232$. (G) Fecal blood. Two-way RM ANOVA: time = F (2.729, 60.04) = 69.09, $P < 0.0001$; sex = F (1, 22) = 0.5269, $P = 0.4755$; time × sex = F (7, 154) = 0.3093, $P = 0.9489$. (H) Average fluid intake adjusted by body weight of control and 2.5% DSS-treated male and female mice. Two-way ANOVA: effect of treatment, F (1, 38) = 0.3818, $P = 0.5403$; effect of sex, F (1, 38) = 3.718, $P = 0.0613$; interaction, F (1, 38) = 0.3818, $P = 0.5403$. Data are presented as mean ± SEM. n = 4 to 12 females and 11 to 12 males. See also Figure S1, available as supplemental digital content at http://links.lww.com/PAIN/B649. ANOVA, analysis of variance; DSS, Dextran sulfate sodium; RM, repeated-measures.

Briefly, mice were anesthetized with 1% isoflurane in an induction chamber, and then kept lightly anesthetized with 0.5% to 1% isoflurane at a flow rate of 0.5 L/min for the duration of the intracolonic injection procedure. A light layer of petroleum jelly (Vaseline) was applied to the perianal area and tubing to avoid the stimulation of somatic areas and ease tube insertion. Capsaicin (0.01% or 0.03%) was prepared using a stock solution of capsaicin (Sigma Aldrich, St. Louis, MO) diluted in ethanol, Tween 80, and saline (10/10/80, respectively). 50 μL of capsaicin (0.01% or 0.03%) or vehicle control (10% ethanol, 10% Tween 80, and 80% saline) was slowly injected via PE-10 nontoxic, sterile polyethylene tubing (0.28 mm ID/0.61 mm OD; Daigger Scientific, Vernon Hills , IL) with a rounded tip connected to a blunted 30 G × 1/2 needle (BD PrecisionGlide, WestNet, Canton, MA) and 1-cc syringe (Terumo, Fisher Scientific, Waltham, MA) that was gently introduced 4 cm into the colon via the anus. Once animals fully recovered from anesthesia, spontaneous nociceptive responses to intracolonic capsaicin (or vehicle control) were measured for 20 minutes. Full recovery from anesthesia was observed 1 to 5 minutes after completion of the intracolonic injection procedure

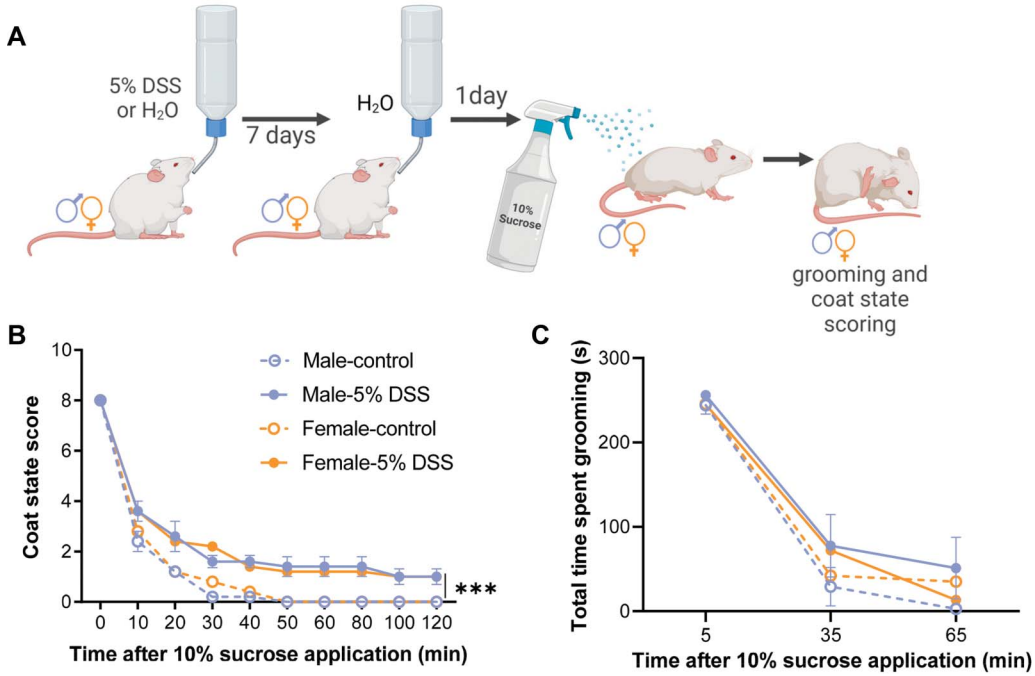

Figure 3. Motivation to groom is comparable in both sexes after DSS-induced colitis. (A) Timeline for splash test experiment. (B and C) 10% sucrose solution was applied to the dorsal coat of control and DSS-treated male and female mice. Coat state score and total time spent grooming were recorded. (B) Coat state score. Three-way RM ANOVA: effect of time = F (1.752, 28.03) = 534.6, $P < 0.0001$; effect of sex = F (1, 16) = 0.03101, $P = 0.8624$; effect of treatment = F (1, 16) = 22.60, ***$P = 0.0002$; time × sex = F (9, 144) = 1.165, $P = 0.3219$; time × treatment = F (9, 144) = 4.110, $P = 0.0001$; sex × treatment = F (1, 16) = 0.1240, $P = 0.7293$; time × sex × treatment = F (9, 144) = 0.1538, $P = 0.9978$. (C) Total time spent grooming. Three-way RM ANOVA: effect of time = F (1.921, 30.73) = 140.1, $P < 0.0001$; effect of sex = F (1, 16) = 0.007390, $P = 0.9326$; effect of treatment = F (1, 16) = 1.945, $P = 0.1822$; time × sex = F (2, 32) = 0.04756, $P = 0.9536$; time × treatment = F (2, 32) = 0.7362, $P = 0.4869$; sex × treatment = F (1, 16) = 1.472, $P = 0.2427$; time × sex × treatment = F (2, 32) = 0.6018, $P = 0.5539$. Data are presented as mean ± SEM. n = 5 females and 5 males. ANOVA, analysis of variance; DSS, Dextran sulfate sodium; RM, repeated-measures.

and was defined as maintaining upright posture and walking normally about the cage. Spontaneous nociceptive responses were defined as licking of abdomen, stretching of abdomen, dragging, and abdominal contractions. The time spent freezing after the injection was also measured during the 20-minute test period.

In this study, intracolonic injections were performed in 3 of the 6 experiments presented. In the first experiment, shown in **Figure 1**, intracolonic injections were performed in naive male and female mice. The mice for the experiments described in **Figures 2 and 3** did not receive intracolonic injection. For the experiments in **Figures 4 and 5**, male and female mice were treated with 2.5% DSS for 7 days, followed by regular water for 1 day and intracolonic injections on day 8. Finally, no intracolonic injection was performed in the experiments described in **Figure 6**.

2.4. Referred abdominal hypersensitivity

Referred abdominal hypersensitivity was evaluated 45 minutes after capsaicin (or vehicle control) intracolonic injection as described previously.[44] The abdominal hair of all test mice was removed (24 hours) before testing with a depilatory (Nair). Intracolonic injections were performed as described in the section above. A 0.16-g von Frey filament (North Coast Medical, Inc, San Jose, CA) was applied to the abdomen for approximately 1 to 2 seconds, with a stimulus interval of 15 seconds. Positive responses were defined as a rapid withdrawal, jumping, licking, or abdominal contractions immediately after application of the von Frey filament to the abdomen. A total of 10 trials were performed, and the number of positive responses per animal was recorded and reported as response frequency.

2.5. Splash test

Control and DSS-treated male and female mice were individually transferred to a new home cage with regular bedding and were habituated in them for at least 1 hour. Approximately 2 mL of 10% sucrose solution was applied to the animal's coat on the dorsal surface of the body, excluding the head and neck, using a spray bottle (Qorpak; PLC-03438). Immediately after sucrose solution application, the coat state of the animals was scored every 10 minutes for 1 hour and every 20 minutes starting at the 1-hour time point until the 2-hour time point from the sucrose solution application. The coat state score was obtained using the following system: 8 = wet, soaked dorsal coat; 4 = scruffy and humid dorsal coat; 2 = dry and smooth lower back coat but upper back coat is still scruffy and/or humid; 1 = mostly dry and smooth dorsal coat with a few scruffy patches; and 0 = completely dry and smooth dorsal coat. The time spent in grooming behavior was measured during a 5-minute period immediately after sucrose solution application as well as 35 and 65 minutes after sucrose solution application. The observer was always blind to experimental treatment.

2.6. Bowel sample collection and histopathological analysis

On experiment day 8, control and DSS-treated males and females were anesthetized with 1% isoflurane in an induction chamber and euthanized by cervical dislocation. Bowels were dissected and the colon was carefully removed to measure its length. The colon was subsequently divided in proximal, medial, medial/distal, and distal 1-cm sections, fixed in 10% neutral buffered formaldehyde (Azer Scientific, Morgantown, PA) for 24

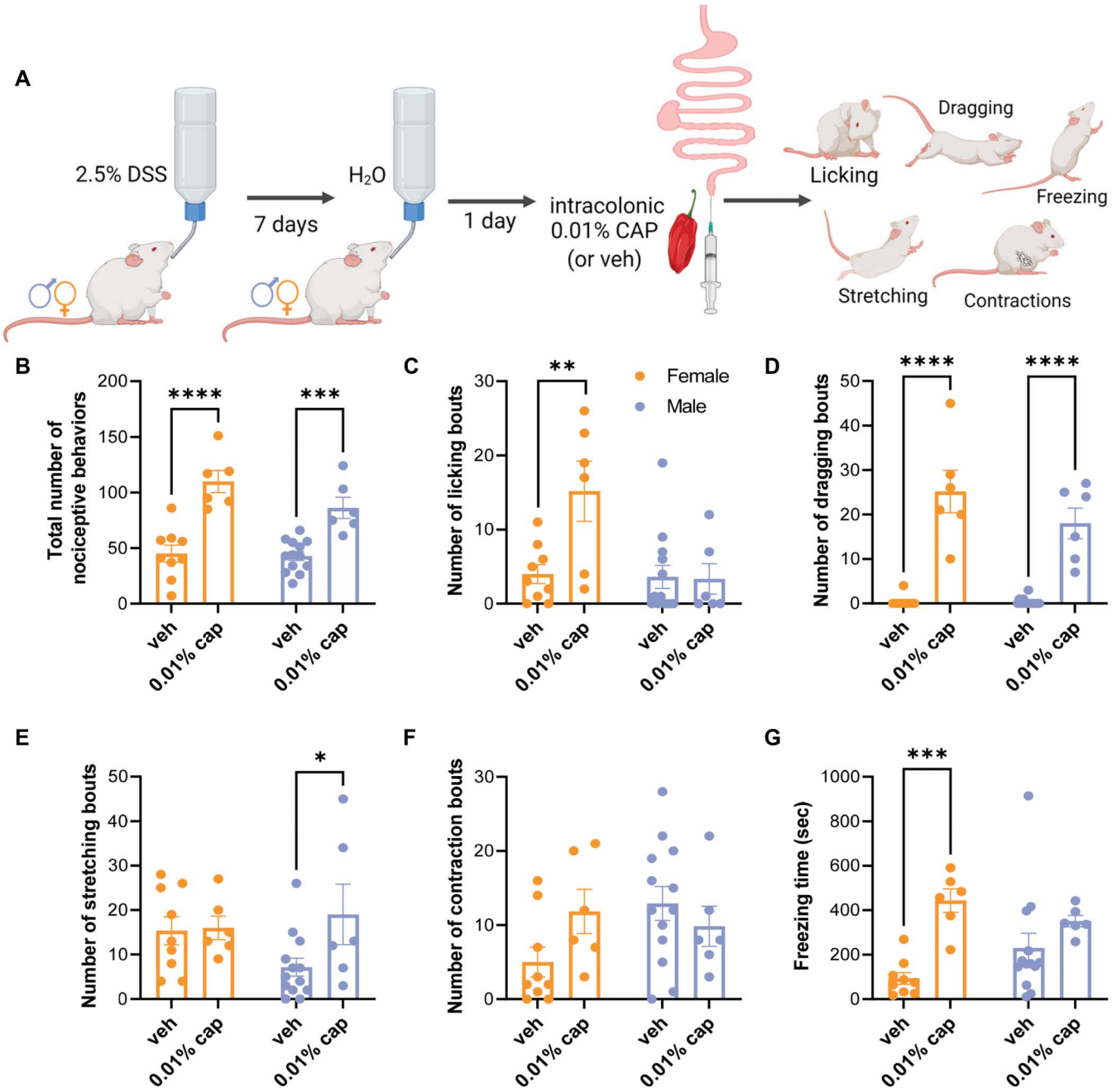

Figure 4. Capsaicin-induced hypersensitivity is comparable in male and female mice after DSS-induced colitis, but the behavioral manifestation is sex-dependent. (A) Timeline of behavioral experiments. DSS-treated male and female mice were injected with intracolonic 0.01% capsaicin or control vehicle and spontaneous nociceptive responses, defined as licking, stretching, dragging, and contractions of the abdomen, and freezing behaviors, were recorded. (B) Total number of pain-related behaviors. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 54.58$; $P < 0.0001$; effect of sex, $F(1, 30) = 3.092$; $P = 0.0889$; interaction, $F(1, 30) = 2.159$; $P = 0.1522$. (C) Licking bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 6.124$, $P = 0.0192$; effect of sex, $F(1, 30) = 7.716$, $P = 0.0093$; interaction, $F(1, 30) = 6.775$, $P = 0.0142$. (D) Dragging bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 96.74$, $P < 0.0001$; effect of sex, $F(1, 30) = 2.819$, $P = 0.1036$; interaction, $F(1, 30) = 2.726$, $P = 0.1092$. (E) Stretching bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 3.048$, $P = 0.0911$; effect of sex, $F(1, 30) = 0.5223$, $P = 0.4755$; interaction, $F(1, 30) = 2.433$, $P = 0.1293$. (F) Contraction bouts. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 0.5044$, $P = 0.4830$; effect of sex, $F(1, 30) = 1.263$, $P = 0.2700$; interaction, $F(1, 30) = 3.544$, $P = 0.0695$. (G) Time spent in freezing behavior. Two-way ANOVA followed by Šídák multiple comparisons test: effect of treatment, $F(1, 30) = 15.24$, $P = 0.0005$; effect of sex, $F(1, 30) = 0.1353$, $P = 0.7156$; interaction, $F(1, 30) = 3.614$, $P = 0.0669$. Data are presented as mean ± SEM. n = 6 to 9 females and 6 to 13 males. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ for veh vs 0.01% cap. ANOVA, analysis of variance; DSS, Dextran sulfate sodium; veh, vehicle.

hours and stored in 70% ethanol until processing. Tissue samples were embedded in paraffin blocks, cut in 5-μm sections, fixed to glass slides, and stained with hematoxylin and eosin (H&E) by Histoserv (Germantown, MD). Histopathological analysis of the tissue samples was performed as previously described.[9,27] Tissue damage was assessed using a cumulative histology score of glands-mucosa, inflammation, and % of colonic tissue affected. The score for glands-mucosa was as follows: 0 = no changes in glands-mucosa structure; 1 = loss of up to 1/3 of gland, crypts lifted off muscularis mucosae; 2 = loss of 2/3 of

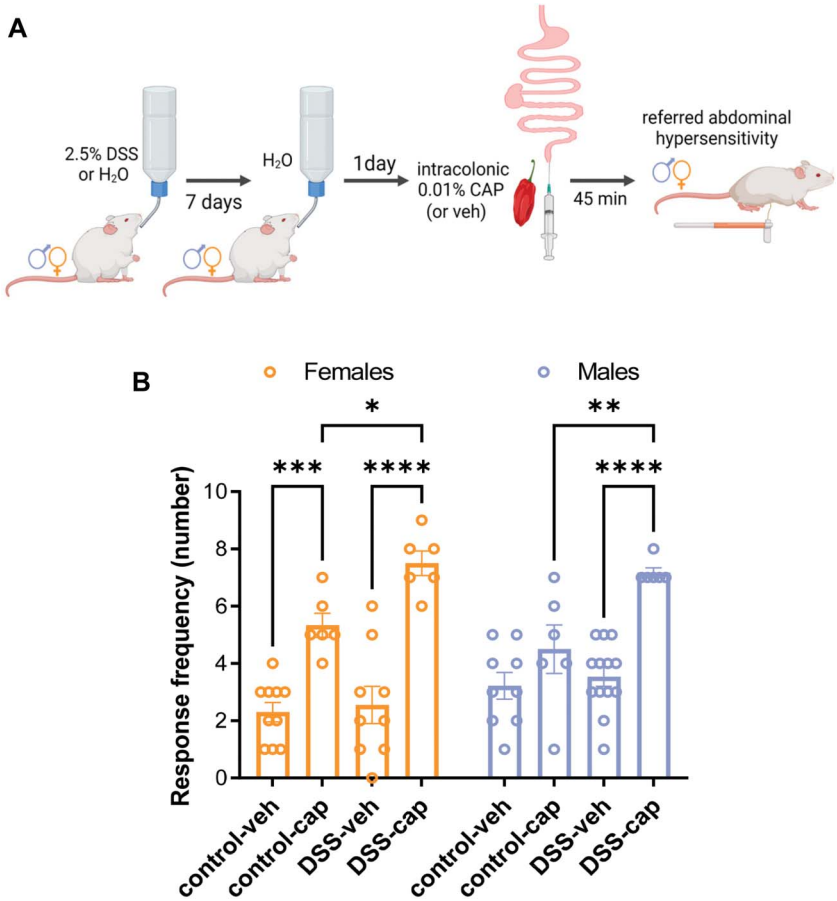

Figure 5. Referred abdominal hypersensitivity is sex-dependent after acute but not persistent colon inflammation. (A) Timeline for von Frey behavioral test in the context of acute and persistent colon irritation. Control and DSS-treated male and female mice were injected with intracolonic 0.01% capsaicin or vehicle control, and abdominal sensitivity to tactile stimulation was measured. (B) Quantification of response frequency to tactile stimulation of the abdomen in control and DSS-treated male and female mice after intracolonic injection of 0.01% capsaicin or vehicle control. Two-way ANOVA followed by Tukey multiple comparisons test: effect of treatment, F (3, 57) = 34.15, $P < 0.0001$; effect of sex, F (1, 57) = 0.2744, $P = 0.6024$; interaction, F (3, 57) = 1.657, $P = 0.1864$. Data are presented as mean ± SEM. n = 6 to 13 males and 6 to 10 females. *$P < 0.05$ and **$P < 0.01$ for control-cap vs DSS-cap; ***$P < 0.001$ for control-veh vs control-cap; ****$P < 0.0001$ for DSS-veh vs DSS-cap. ANOVA, analysis of variance; DSS, Dextran sulfate sodium; veh, vehicle.

gland, crypts lifted off muscularis mucosae, little inflammation, thinning and loss of epithelial cells in the *intact* glands, cryptitis; 3 = loss of all the glands but the superficial epithelium is intact, mild infiltrate; and 4 = erosion, ulceration, mild—moderate infiltrate. Inflammation score was defined as 0 = none to few leucocytes; 1 = mild, some increase in leucocytes at tips of crypts; 2 = moderate; and 3 = severe, dense infiltrate throughout, transmural. Percent of colonic tissue affected was scored as 1 = 1% to 25%; 2 = 25% to 50%; 3 = 50% to 75%; and 4 = 75% to 100%. Analyses of the histology score and its individual components were initially performed separately per anatomical section of the colon collected (ie, proximal, medial, medial/distal, and distal). This analysis showed that DSS-induced histopathology is comparable between all colon sections evaluated within bowels from mice of the same sex and dose of DSS administered. Based on these results, we calculated the average score of all 4 colonic sections per mouse for each parameter and used these values for subsequent graphs and analyses.

2.7. Statistics

The results are expressed as mean ± SEM. Analysis was performed using either unpaired *t*-tests (without Welch correction for variance), Mann–Whitney *U* tests, 2-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests, or by Šidák multiple comparison. Normality was assessed using Kolmogorov–Smirnov test. All analyses were performed using GraphPad Prism (v. 9), and *P* values lower than 0.05 were considered significant and are reported in figure legends. **Table 2** offers a complete report of the statistical analysis performed.

3. Results

3.1. Female mice display more capsaicin-induced spontaneous pain-related behaviors than male mice

The intracolonic capsaicin model was used to evaluate potential sex differences in spontaneous pain-related behaviors during acute colon inflammation (**Fig. 1A**). Spontaneous capsaicin-induced nociceptive behaviors were defined as licking, stretching, and dragging of the abdomen as well as abdominal contractions. Consistent with previous reports,[30,31] intracolonic application of capsaicin (0.01%) elicited robust pain-related behaviors that were significantly ($P < 0.0001$) higher than those observed after an intracolonic injection of vehicle in female mice (**Fig. 1B**). In marked contrast, however, spontaneous pain-related behaviors were indistinguishable in male mice after the

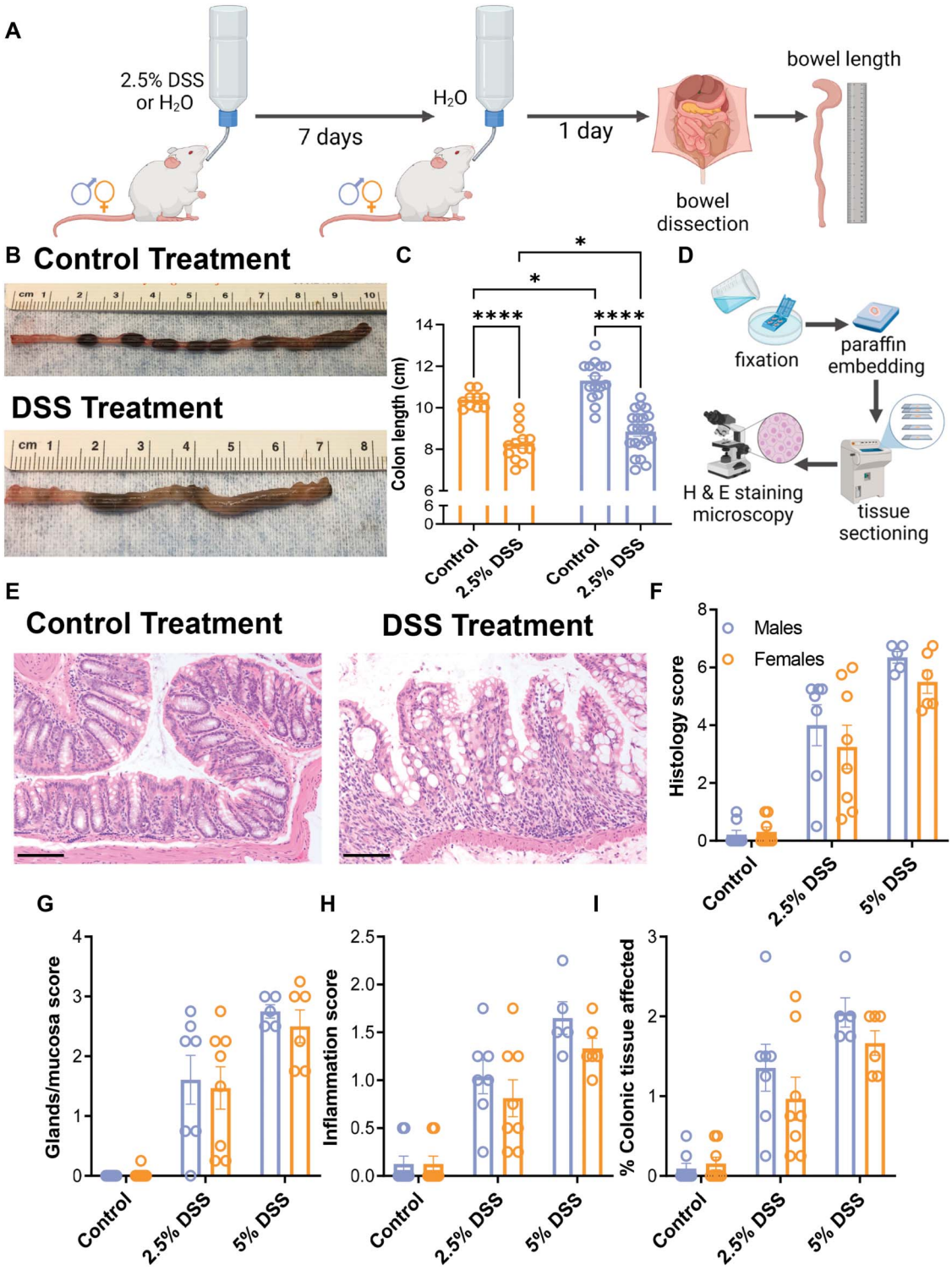

Figure 6. DSS-induced bowel pathology is comparable in males and females. (A) Timeline for bowel pathology analysis. Bowels of control and DSS-treated male and female mice were dissected, colon length was measured 1 day after the end of DSS treatment, and colonic samples were collected for histopathological analysis. (B) Representative images of control (top) and DSS-treated (bottom) colon samples. (C) Colon length in control and DSS-treated male and female mice. Two-way ANOVA followed by Tukey multiple comparisons test: effect of treatment, F (1, 56) = 101.7, $P < 0.0001$; effect of sex, F (1, 56) = 9.351, $P = 0.0034$. (D) Timeline for histological analysis of DSS-induced colon pathology. (E) Representative images of colon sections from control (left) and DSS-treated (right) mice stained with hematoxylin and eosin. Scale bar = 100 μm. (F-I) Cumulative histology score and its individual components, defined as glands/mucosa score, inflammation score, and % colonic tissue affected in bowels from control and DSS-treated male and female mice. (F) Cumulative histology score. Two-way ANOVA: effect of treatment, F (2, 36) = 64.15, $P < 0.0001$; effect of sex, F (1, 36) = 1.505, $P = 0.2278$; interaction, F (2, 36) = 0.5736, $P = 0.5686$. (G) Glands/mucosa score. Two-way ANOVA: effect of treatment, F (2, 36) = 50.16, $P < 0.0001$; effect of sex, F (1, 36) = 0.3129, $P = 0.5794$; interaction F (2, 36) = 0.1472, $P = 0.8636$. (H) Inflammation score. Two-way ANOVA: effect of treatment, F (2, 36) = 45.19, $P < 0.0001$; effect of sex, F (1, 36) = 2.346, $P = 0.1343$; interaction F (2, 36) = 0.6558, $P = 0.5251$. (I) % Colonic tissue affected. Two-way ANOVA: effect of treatment, F (2, 36) = 38.35, $P < 0.0001$; effect of sex, F (1, 36) = 2.120, $P = 0.1540$; interaction F (2, 36) = 0.9276, $P = 0.4047$. Data are presented as mean ± SEM. n = 6 to 13 females and 5 to 21 males. ****$P < 0.0001$ for control vs 2.5% DSS; *$P < 0.05$ for females vs males. ANOVA, analysis of variance; DSS, Dextran sulfate sodium.

Table 2

Statistical analyses.

Figure	Type of test	Sample size	Statistical data
Figure 1			
1B (total # nociceptive behaviors, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 10 veh and 6 cap males = 9 veh and 5 cap	Two-way ANOVA Interaction: F (1, 26) = 3.984; $P = 0.0565$ Treatment: F (1, 26) = 15.02; $P = 0.0006$ Sex: F (1, 26) = 0.8271; $P = 0.3715$ *Post hoc*: Šídák multiple comparisons test Females: $P = 0.0004$ for veh vs 0.01% cap
1C (total # nociceptive behaviors, 0.03% cap, males only)	Unpaired *t* test (2-tailed)	males = 6 veh and 5 cap	t = 4.755, df = 9; $P = 0.0010$; $\eta^2 = 0.7153$
1D (licking, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 10 veh and 6 cap males = 9 veh and 5 cap	Two-way ANOVA Interaction: F (1, 26) = 4.360; $P = 0.0467$ Treatment: F (1, 26) = 7.884; $P = 0.0093$ Sex: F (1, 26) = 0.9772; $P = 0.3320$ *Post hoc*: Šídák multiple comparisons test Females: $P = 0.0026$ for veh vs 0.01% cap
1E (licking, 0.03% cap, males only)	Unpaired *t* test (2-tailed)	males = 6 veh and 5 cap	t = 2.055; df = 9; $P = 0.0700$; $\eta^2 = 0.3194$
1F (dragging, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 10 veh and 6 cap males = 9 veh and 5 cap	Two-way ANOVA Interaction: F (1, 26) = 3.148; $P = 0.0877$ Treatment: F (1, 26) = 5.842; $P = 0.0230$ Sex: F (1, 26) = 0.2798; $P = 0.6013$ *Post hoc*: Šídák multiple comparisons test Females: $P = 0.0096$ for veh vs 0.01% cap
1G (dragging, 0.03% cap, males only)	Mann–Whitney *U* test	males = 6 veh and 5 cap	$P = 0.0087$ (2-tailed); U = 1
1H (stretching, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 10 veh and 6 cap males = 9 veh and 5 cap	Two-way ANOVA Interaction: F (1, 26) = 4.034; $P = 0.0551$ Treatment: F (1, 26) = 12.53; $P = 0.0015$ Sex: F (1, 26) = 3.745; $P = 0.0639$ *Post hoc*: Šídák multiple comparisons test Females: $P = 0.0008$ for veh vs 0.01% cap
1I (stretching, 0.03% cap, males only)	Unpaired *t* test (2-tailed)	males = 6 veh and 5 cap	t = 1.180, df = 9; $P = 0.2682$; $\eta^2 = 0.1340$
1J (contractions, 0.01% cap)	Two-way ANOVA	females = 10 veh and 6 cap males = 9 veh and 5 cap	Two-way ANOVA Interaction: F (1, 26) = 1.369; $P = 0.2526$ Treatment: F (1, 26) = 2.614; $P = 0.1180$ Sex: F (1, 26) = 6.808; $P = 0.0149$
1K (contractions, 0.03% cap, males only)	Unpaired *t* test (2-tailed)	males = 6 veh and 5 cap	t = 4.160, df = 9; $P = 0.0024$; $\eta^2 = 0.6578$
1L (freezing, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 8 veh and 6 cap males = 7 veh and 5 cap	Two-way ANOVA Interaction: F (1, 22) = 0.4621; $P = 0.5037$ Treatment: F (1, 22) = 7.971; $P = 0.0099$ Sex: F (1, 22) = 0.4598; $P = 0.5048$ *Post hoc*: Šídák multiple comparisons test Females: $P = 0.0336$ for veh vs 0.01% cap
1M (freezing, 0.03% cap, males only)	Unpaired *t* test (2-tailed)	males = 3 veh and 3 cap	t = 5.374, df = 4; $P = 0.0058$; $\eta^2 = 0.8784$
Figure 2			
2B (DSS dose-response)	Ordinary one-way ANOVA	Control = 15; 0.5, 1, 2, 2.5, 3,5, 7% DSS = 5; 10% DSS = 1	Ordinary one-way ANOVA F (8, 42) = 62.04; $P < 0.0001$
2C (DAI, 2.5% DSS)	Two-way RM ANOVA	females = 12 DSS; males = 12 DSS	Two-way RM ANOVA Time × sex: F (7, 154) = 1.828; $P = 0.0855$ Time: F (3.731, 82.09) = 193.4; $P < 0.0001$ Sex: F (1, 22) = 11.07; $P = 0.0031$
2D (% weight change, 2.5% DSS)	Two-way RM ANOVA	females = 12 DSS; males = 12 DSS	Two-way RM ANOVA

(*continued on next page*)

Table 2 (*continued*)

Figure	Type of test	Sample size	Statistical data
			Time × sex: F (7, 154) = 3.044; $P = 0.0050$ Time: F (3.946, 86.81) = 28.14; $P < 0.0001$ Sex: F (1, 22) = 6.372; $P = 0.0193$
2E (appearance, 2.5% DSS)	Two-way RM ANOVA	females = 12 DSS; males = 12 DSS	Two-way RM ANOVA Time × sex: F (7, 154) = 5.936; $P < 0.0001$ Time: F (3.066, 67.45) = 74.72; $P < 0.0001$ Sex: F (1, 22) = 20.77; $P = 0.0002$
2F (stool consistency, 2.5% DSS)	Two-way RM ANOVA	females = 12 DSS; males = 12 DSS	Two-way RM ANOVA Time × sex: F (7, 154) = 0.6388; $P = 0.7232$ Time: F (3.039, 66.85) = 65.36; $P < 0.0001$ Sex: F (1, 22) = 0.01996; $P = 0.8889$
2G (fecal blood, 2.5% DSS)	Two-way RM ANOVA	females = 12 DSS; males = 12 DSS	Two-way RM ANOVA Time × sex: F (7, 154) = 0.3093; $P = 0.9489$ Time: F (2.729, 60.04) = 69.09; $P < 0.0001$ Sex: F (1, 22) = 0.5269; $P = 0.4755$
2H (fluid intake, 2.5% DSS)	Two-way ANOVA	females = 8 control and 4 DSS males = 19 control and 11 DSS	Two-way ANOVA Interaction: F (1, 38) = 0.3818; $P = 0.5403$ Treatment: F (1, 38) = 0.3818; $P = 0.5403$ Sex: F (1, 38) = 3.718; $P = 0.0613$
Figure 3			
3B (coat state time course, 5% DSS)	Three-way RM ANOVA	females = 5 control and 5 DSS males = 5 control and 5 DSS	Three-way RM ANOVA Time × treatment: F (9, 144) = 4.110; $P = 0.0001$ Time × sex: F (9, 144) = 1.165; $P = 0.3219$ Sex × treatment: F (1, 16) = 0.1240; $P = 0.7293$ Time × sex × treatment: F (9, 144) = 0.1538; $P = 0.9978$ Time: F (1.752, 28.03) = 534.6; $P < 0.0001$ Sex: F (1, 16) = 0.03101; $P = 0.8624$ Treatment: F (1, 16) = 22.60; $P = 0.0002$
3C (time spent grooming time course, 5% DSS)	Three-way RM ANOVA	females = 5 control and 5 DSS males = 5 control and 5 DSS	Three-way RM ANOVA Time × treatment: F (2, 32) = 0.7362; $P = 0.4869$ Time × sex: F (2, 32) = 0.04756; $P = 0.9536$ Sex × treatment: F (1, 16) = 1.472; $P = 0.2427$ Time × sex × treatment: F (2, 32) = 0.6018; $P = 0.5539$ Time: F (1.921, 30.73) = 140.1; $P < 0.0001$ Sex: F (1, 16) = 0.007390; $P = 0.9326$ Treatment: F (1, 16) = 1.945; $P = 0.1822$
Figure 4			
4B (total # nociceptive behaviors in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 9 veh and 6 cap males = 13 veh and 6 cap	Two-way ANOVA Interaction: F (1, 30) = 2.159; $P = 0.1522$ Treatment: F (1, 30) = 54.58; $P < 0.0001$ Sex: F (1, 30) = 3.092; $P = 0.0889$ *Post hoc*: Šídák multiple comparisons test Females: $P < 0.0001$ for veh vs 0.01% cap Males: $P = 0.0003$ for veh vs 0.01% cap Two-way ANOVA

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Table 2 (*continued*)

Figure	Type of test	Sample size	Statistical data
4C (licking in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 9 veh and 6 cap males = 13 veh and 6 cap	Interaction: F (1, 30) = 6.775; P= 0.0142 Treatment: F (1, 30) = 6.124; P= 0.0192 Sex: F (1, 30) = 7.716; P = 0.0093 *Post hoc*: Šídák multiple comparisons test Females: P = 0.0031 for veh vs 0.01% cap
4D (dragging in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 9 veh and 6 cap males = 13 veh and 6 cap	Two-way ANOVA Interaction: F (1, 30) = 2.726; P= 0.1092 Treatment: F (1, 30) = 96.74; P< 0.0001 Sex: F (1, 30) = 2.819; P = 0.1036 *Post hoc*: Šídák multiple comparisons test Females: P< 0.0001 for veh vs 0.01% cap Males: P< 0.0001 for veh vs 0.01% cap
4E (stretching in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 9 veh and 6 cap males = 13 veh and 6 cap	Two-way ANOVA Interaction: F (1, 30) = 2.433; P= 0.1293 Treatment: F (1, 30) = 3.048; P= 0.0911 Sex: F (1, 30) = 0.5223; P = 0.4755 *Post hoc*: Šídák multiple comparisons test Males: P = 0.0433 for veh vs 0.01% cap
4F (contractions in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA	females = 9 veh and 6 cap males = 13 veh and 6 cap	Two-way ANOVA Interaction: F (1, 30) = 3.544; P= 0.0695 Treatment: F (1, 30) = 0.5044; P = 0.4830 Sex: F (1, 30) = 1.263; P = 0.2700
4G (freezing in 2.5% DSS-induced colitis, 0.01% cap)	Two-way ANOVA followed by Šídák multiple comparisons test	females = 9 veh and 6 cap males = 13 veh and 6 cap	Two-way ANOVA Interaction: F (1, 30) = 3.614; P= 0.0669 Treatment: F (1, 30) = 15.24; P= 0.0005 Sex: F (1, 30) = 0.1353; P = 0.7156 *Post hoc*: Šídák multiple comparisons test Females: P = 0.0008 for veh vs 0.01% cap
Figure 5			
5B (von Frey, 2.5% DSS, 0.01% cap)	Two-way ANOVA followed by Tukey multiple comparisons test	females = 6 control-cap, 6 DSS-cap, 9 DSS-veh, 10 control-veh males = 6 control-cap, 6 DSS-cap, 9 control-veh, 13 DSS-veh	Two-way ANOVA Interaction: F (3, 57) = 1.657; P= 0.1864 Treatment: F (3, 57) = 34.15; P< 0.0001 Sex: F (1, 57) = 0.2744; P = 0.6024 *Post hoc*: Tukey multiple comparisons test Females: P = 0.0004 for control-veh vs Control-cap; P< 0.0001 for DSS-veh vs DSS-cap; P = 0.0389 for control-cap vs DSS-cap Males: P< 0.0001 for DSS-veh vs DSS-cap; P = 0.0069 for control-cap vs DSS-cap
Figure 6			
6C (bowel length, 2.5% DSS)	Two-way ANOVA followed by Tukey multiple comparisons test	females = 10 control and 13 DSS males = 15 control and 21 DSS	Two-way ANOVA Treatment: F (1, 56) = 101.7; P< 0.0001 Sex: F (1, 56) = 9.351; P = 0.0034 *Post hoc*: Tukey multiple comparisons test Females: P< 0.0001 for control vs 2.5% DSS Males: P< 0.0001 for control vs 2.5% DSS Control: P = 0.0175 for females vs males 2.5% DSS: P = 0.0175 for females vs males
6F (histology)	Two-way ANOVA	females = 8 control, 8 (2.5% DSS), 6 (5% DSS) males = 8 control, 7 (2.5% DSS), 5 (5% DSS)	Two-way ANOVA Interaction: F (2, 36) = 0.5736; P = 0.5686 Treatment: F (2, 36) = 64.15; P< 0.0001 Sex: F (1, 36) = 1.505; P = 0.2278
6G (glands/mucosa)	Two-way ANOVA	females = 8 control, 8 (2.5% DSS), 6 (5% DSS) males = 8 control, 7 (2.5% DSS), 5 (5% DSS)	Two-way ANOVA Interaction: F (2, 36) = 0.1472; P = 0.8636 Treatment: F (2, 36) = 50.16; P< 0.0001

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Table 2 (*continued*)

Figure	Type of test	Sample size	Statistical data
			Sex: F (1, 36) = 0.3129; $P = 0.5794$
6H (inflammation)	Two-way ANOVA	females = 8 control, 8 (2.5% DSS), 6 (5% DSS) males = 8 control, 7 (2.5% DSS), 5 (5% DSS)	Two-way ANOVA Interaction: F (2, 36) = 0.6558; $P = 0.5251$ Treatment: F (2, 36) = 45.19; $P < 0.0001$ Sex: F (1, 36) = 2.346; $P = 0.1343$
6I (% colonic tissue affected)	Two-way ANOVA	females = 8 control, 8 (2.5% DSS), 6 (5% DSS) males = 8 control, 7 (2.5% DSS), 5 (5% DSS)	Two-way ANOVA Interaction: F (2, 36) = 0.9276; $P = 0.4047$ Treatment: F (2, 36) = 38.35; $P < 0.0001$ Sex: F (1, 36) = 2.120; $P = 0.1540$
Supplemental Figure 1, http://links.lww.com/PAIN/B649			
S1B (DAI, water)	Two-way RM ANOVA	females = 6 control; males = 6 control	Two-way RM ANOVA Time × sex: F (7, 70) = 0.2977; $P = 0.9526$ time: F (3.197, 31.97) = 1.413; $P = 0.2562$ Sex: F (1, 10) = 0.3056; $P = 0.5925$
S1C (% weight change, water)	Two-way RM ANOVA	females = 6 control; males = 6 control	Two-way RM ANOVA Time × sex: F (7, 70) = 0.3933; $P = 0.9032$ Time: F (2.662, 26.62) = 6.179; $P = 0.0033$ Sex: F (1, 10) = 0.3333; $P = 0.5765$
S1D (appearance, water)	Two-way RM ANOVA	females = 8 control; males = 6 control	Two-way RM ANOVA Time × sex: F (7, 84) = 0.9351; $P = 0.4841$ Time: F (2.051, 24.61) = 0.9351; $P = 0.4082$ Sex: F (1, 12) = 2.571; $P = 0.1348$
S1E (stool consistency, water)	Two-way RM ANOVA	females = 8 control; males = 6 control	Two-way RM ANOVA Time × sex: F (7, 84) = 0.7011; $P = 0.6709$ Time: F (3.192, 38.30) = 2.094; $P = 0.1136$ Sex: F (1, 12) = 1.044; $P = 0.3271$
S1F (fecal blood, water)	Not applicable (all values are zero)	females = 6 control; males = 6 control	

Detailed information about statistical tests, sample sizes, and statistical results. $F_{(DFn,\ DFd)}$: degree of freedom for the numerator of the F ratio, for the denominator of the F ratio.
ANOVA, analysis of variance; DAI, disease activity index; df, degrees of freedom; DSS, Dextran sulfate sodium; RM, repeated-measures.

intracolonic administration of 0.01% capsaicin or vehicle control (**Fig. 1B**). To determine whether male mice can display capsaicin-induced hypersensitivity, we next injected mice with a higher dose of capsaicin (0.03%). As illustrated in **Figure 1C**, intracolonic administration of 0.03% capsaicin elicited significant ($P = 0.0010$) increases in spontaneous pain-related behaviors in males, compared with vehicle-injected mice. These results demonstrate that capsaicin-induced spontaneous nociceptive responses are sex-dependent, with higher doses of capsaicin required in males than in females to elicit comparable nociceptive behavioral responses.

The individual components contributing to the total number of capsaicin-induced nociceptive behaviors were analyzed to identify which specific behaviors contribute to the observed sex differences (**Figs. 1D–K**). Consistent with the results observed in the cumulative analysis, females injected with 0.01% capsaicin displayed significant increases in most behavioral parameters when compared with control vehicle-injected females. Thus, female mice injected with 0.01% capsaicin displayed a significant ($P < 0.01$) increase in the number of licking bouts (**Fig. 1D**), dragging bouts (**Fig. 1F**), and stretching bouts (**Fig. 1H**), compared with vehicle-injected females. The number of contraction bouts was the only parameter in females that was indistinguishable between capsaicin- and vehicle-injected mice (**Fig. 1J**). In marked contrast and consistent with the results observed in the cumulative behavioral analysis, all the parameters measured were indistinguishable between vehicle- and capsaicin-injected male mice (**Figs. 1D, F, H, J**), confirming the lack of measurable behavioral hypersensitivity in male mice after intracolonic injection of 0.01% capsaicin.

Evaluation of the individual behavioral components in male mice injected with a higher dose of capsaicin (0.03%) revealed that the capsaicin-induced increases in behavioral responses observed at this concentration were mainly driven by significant ($P < 0.01$) increases in abdominal dragging and contraction bouts (**Figs. 1G and K**) but not by changes in licking or stretching of the abdomen (**Figs. 1E and I**). Together, these results highlight that the behavioral manifestation of capsaicin-induced hypersensitivity is sexually dimorphic, with increases in licking and stretching of the abdomen observed in females only and increases in

Table 3

Onset of individual disease activity index components after dextran sulfate sodium treatment in male and female mice.

	Males		Females	
	Water (n = 6)	2.5% DSS (n = 12)	Water (n = 6)	2.5% DSS (n = 12)
Disease activity index				
Day 1	−0.2 ± 0.2	−0.9 ± 0.2**	−0.3 ± 0.2	−0.5 ± 0.3
Day 2	−0.2 ± 0.2	−2.7 ± 0.2****	−0.7 ± 0.3	−1.5 ± 0.4*
Day 3	−0.5± 0.3	−3.1 ± 0.1****	−0.7 ± 0.3	−2.4 ± 0.2****
Day 4	−0.7 ± 0.3	−3.4 ± 0.2****	−0.5 ± 0.2	−2.8 ± 0.3****
Day 5	−0.7 ± 0.3	−3.6 ± 0.3****	−0.7 ± 0.5	−3.3 ± 0.3****
Day 6	−0.3 ± 0.2	−5.7 ± 0.2****	−0.3 ± 0.2	−4.8 ± 0.3****
Day 7	−0.3 ± 0.2	−7.8 ± 0.4****	−0.5 ± 0.3	−6.4 ± 0.3****
Body weight loss (%)				
Day 1	−0.4 ± 0.4	1.9 ± 0.5*	0.9 ± 0.6	−0.3 ± 0.7
Day 2	−0.3 ± 0.9	3.3 ± 0.6***	1.0 ± 1.1	1.5 ± 0.7
Day 3	−0.3 ± 0.9	4.1 ± 0.5****	−0.1 ± 1.6	2.7 ± 1.0
Day 4	0.1 ± 1.2	4.0 ± 0.4****	−0.5 ± 1.5	3.0 ± 1.0
Day 5	−2.4 ± 1.1	4.7 ± 0.5****	−1.9 ± 1.3	2.2 ± 1.0
Day 6	−3.2 ± 0.8	5.3 ± 0.6****	−2.1 ± 1.0	3.0 ± 1.0
Day 7	−3.3 ± 1.0	7.7 ± 0.9****	−2.2 ± 1.3	3.6 ± 0.8**
Appearance score				
Day 1	0	0	0	0
Day 2	0	−0.8 ± 0.1****	0	−0.3 ± 0.1
Day 3	0	−1.0 ± 0.0****	0	−0.4 ±0.1
Day 4	0	−1.0 ± 0.0****	0	−0.6 ± 0.1*
Day 5	0	−1.0 ± 0.0****	−0.1 ± 0.1	−1.0 ± 0.0****
Day 6	0	−1.1± 0.1****	−0.3 ± 0.2	−1.0 ± 0.0****
Day 7	0	−1.5 ± 0.2****	−0.1 ± 0.1	−1.0 ± 0.0****
Stool consistency score				
Day 1	−0.2 ± 0.2	−0.2 ± 0.1	0	−0.3 ± 0.1
Day 2	0	−0.8 ± 0.1***	−0.3 ± 0.2	−0.6 ± 0.1*
Day 3	−0.3 ± 0.2	−1.0 ± 0.0****	−0.3 ± 0.2	−1.0 ± 0.0****
Day 4	−0.5 ± 0.2	−1.1 ± 0.1****	−0.3 ± 0.2	−1.0 ± 0.0****
Day 5	−0.5 ± 0.2	−1.0 ± 0.0****	−0.4 ± 0.2	−1.1 ± 0.1****
Day 6	−0.3± 0.2	−1.2 ± 0.1****	0	−1.3 ± 0.1****
Day 7	−0.3 ± 0.2	−2.1 ± 0.3***	−0.3 ± 0.2	−1.9 ± 0.1****
Fecal blood score				
Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0
Day 4	0	−0.2 ± 0.2	0	0
Day 5	0	−0.3 ± 0.2	0	−0.3 ± 0.2
Day 6	0	−1.8 ± 0.2*	0	−1.5 ± 0.4****
Day 7	0	−2.5 ± 0.3***	0	−2.3 ± 0.3****

Values are presented as the mean ± SEM difference from day 0 within the same treatment. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$; Repeated-measures 2-way ANOVA followed by Šídák multiple comparisons test compared with day 0.

DSS, Dextran sulfate sodium.

abdominal contractions seen solely in male mice. The only common behavioral manifestation observed between sexes after intracolonic capsaicin administration was increases in dragging of the abdomen.

Previous studies have indicated that intracolonic capsaicin elicits freezing behaviors in a dose-dependent manner.[7] Consistent with these findings, the time spent freezing was significantly ($P < 0.05$) higher in females after intracolonic injection of 0.01% capsaicin than in females after the injection of vehicle control (**Fig. 1L**). In line with the sex differences presented above, no measurable differences were observed in the time spent freezing in male mice injected with 0.01% capsaicin when compared with vehicle control (**Fig. 1L**). Time spent freezing, however, was significantly ($P < 0.01$) higher in males when a higher dose of capsaicin (0.03%) was used, compared with vehicle control (**Fig. 1M**). These results demonstrate that although both males and females exhibit capsaicin-induced freezing, males require a higher dose of capsaicin than females to display freezing behaviors, further confirming that the manifestation of capsaicin-induced nociceptive responses is sex-dependent and that females are more hypersensitive.

3.2. Clinical progression of disease in a Dextran sulfate sodium–induced mouse model of colitis is sexually dimorphic

The DSS model of colitis was used to evaluate sex differences in clinical progression of disease as well as pain-related responses and referred abdominal sensitivity in mice. Male and female mice were treated with either 2.5% DSS or water ad libitum for 7 days (**Fig. 2A**). Disease activity index, defined as a cumulative score based on the percentage of weight loss, stool consistency, presence of fecal blood, and physical appearance (**Table 1**), was

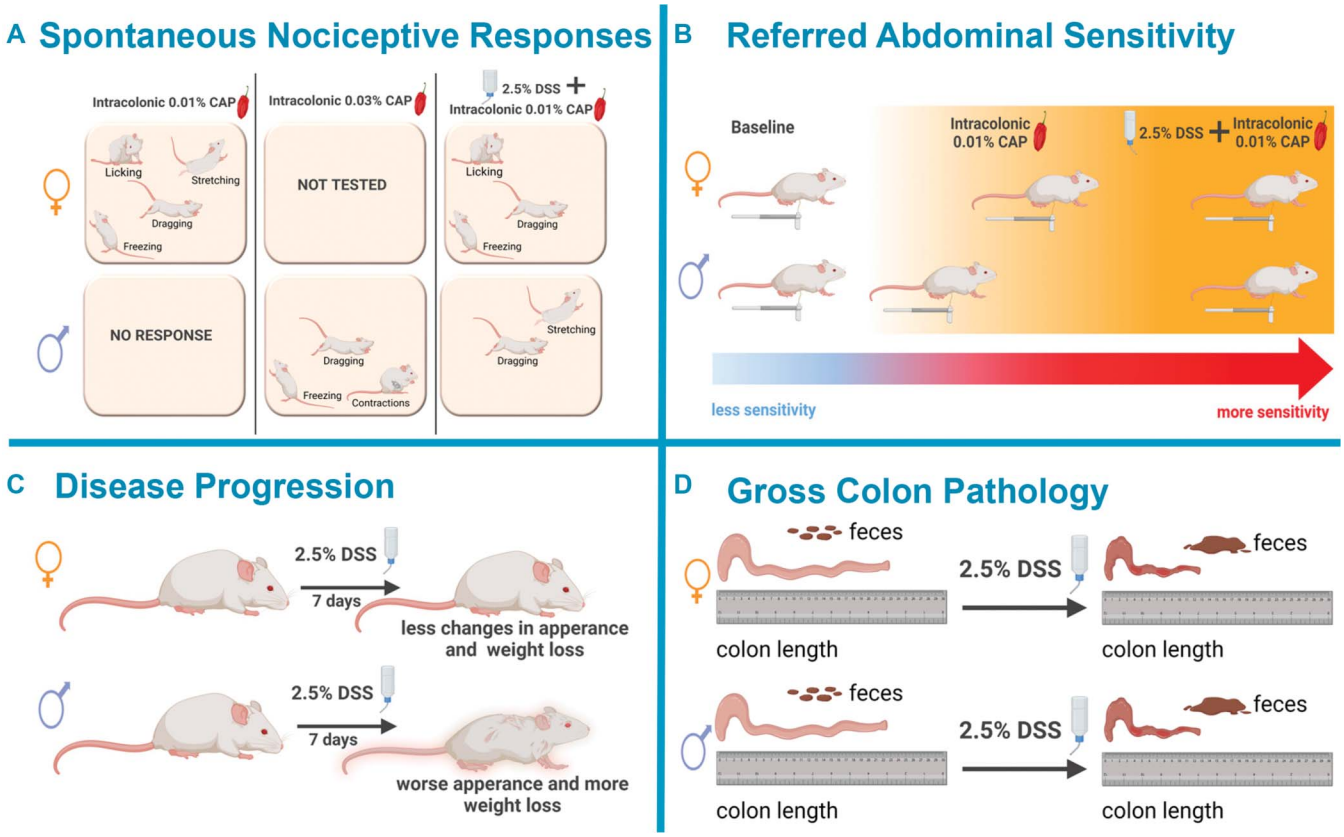

Figure 7. Summary of main findings. (A) Behavioral manifestation of capsaicin-induced spontaneous nociceptive responses is sex-dependent after acute and persistent colon inflammation. After intracolonic capsaicin, females exhibit more capsaicin-induced nociceptive behaviors than males. In the context of DSS-induced colitis, colonic hypersensitivity is comparable in both sexes, but the manifestation is distinct. (B) Referred abdominal hypersensitivity is higher in females after intracolonic capsaicin but is similar between sexes after persistent colon inflammation or at baseline. (C) Progression of DSS-induced colitis is sex-dependent. Worse physical appearance and greater weight loss is observed in males compared with females. (D) No measurable sex difference was observed in DSS-induced bowel shortening. DSS, Dextran sulfate sodium.

calculated daily to monitor the clinical progression of colitis. As illustrated in **Figure 2B**, the DAI score was dependent on the dose of DSS administered, with increasing percent of DSS resulting in higher DAI score. The rest of the experiments in this study were performed using 2.5% DSS, which consistently elicited disease in all mice but was not at ceiling level.

Consistent with previous studies,[2,11,43] 2.5% DSS treatment in drinking water resulted in increases in DAI scores in both males and females in a time-dependent manner, with higher DAI scores as the days of treatment progressed. Notably, DSS-treated male mice displayed significantly ($P = 0.0031$) higher DAI scores when compared with female mice, demonstrating that male mice have worse clinical progression of colitis than female mice (**Fig. 2C**).

To dissect out the components within the cumulative DAI scores that contribute to the observed sex differences in the progression of colitis, we analyzed and compared each disease parameter individually in both male and female mice (**Figs. 2D–G**). Similar to the composite DAI scores, the progression of the individual disease components was also time-dependent, with higher scores observed with treatment progression in both males and females. Although DSS-induced changes in body weight, physical appearance, and stool consistency were observed within a few days of treatment (**Figs. 2D, E and F**), the presence of blood in the stool was not observed until days 4 to 6 after treatment (**Fig. 2G**).

Comparison of the individual disease components in males and females further revealed that the sex differences observed in the composite DAI scores in DSS-treated mice arise from differences in the percentage of body weight loss and physical appearance scores but not from differences in the scores for stool consistency or fecal blood. Thus, as shown in **Figure 2D**, although both DSS-treated male and female mice lost weight during the days of treatment, the percentage of weight loss over time was significantly ($P = 0.0193$) higher in male than in female mice. Male mice also displayed worse DSS-induced changes in physical appearance than female mice, with a significantly ($P = 0.0002$) higher appearance score measured in male than in female mice (**Fig. 2E**). By contrast, stool consistency and fecal blood scores were comparable in male and female mice for the duration of the DSS treatment (**Figs. 2F and G**), demonstrating that these 2 disease components are not sex-dependent.

Evaluation of the time course for DSS-induced disease progression further revealed that the onset of symptoms associated with colitis is also sex-dependent (**Table 3**). Dextran sulfate sodium treated males, for example, exhibited significant ($P = 0.0042$) changes in cumulative DAI scores at treatment day 1, whereas female mice did not exhibit significant ($P = 0.0157$) changes until treatment day 2. Sex differences in symptom onset consistent with colitis were most pronounced in DSS-induced changes in body weight where males started to lose significant ($P = 0.0124$) weight from treatment day 1, whereas females did not display significant ($P = 0.0098$) DSS-induced weight loss until treatment day 7. Similarly, males start to exhibit significantly ($P < 0.0001$) worse appearance scores on day 2 of DSS treatment,

whereas the onset for significant ($P = 0.0165$) changes in appearance scores in females is on day 4. Consistent with the lack of sex differences in DSS-induced changes in stool consistency and presence of blood in the stool, analysis of the onset of changes in these 2 parameters was comparable in both sexes. Thus, significant ($P < 0.05$) DSS-induced changes in stool consistency scores were first observed on treatment day 2 and significant ($P < 0.05$) changes in fecal blood scores were observed on treatment day 6 in both male and female mice.

Cumulative DAI, appearance, stool consistency, and fecal blood scores were comparable in control males and female mice for the duration of the experiments (Figure S1, available as supplemental digital content at http://links.lww.com/PAIN/B649). As expected, control male and female mice displayed significant ($P = 0.0033$) weight gain during the course of the experiment (Figure S1C, available as supplemental digital content at http://links.lww.com/PAIN/B649). Analysis of fluid consumption during the DSS-induced colitis experiments further revealed that the average fluid intake was comparable in water- and DSS-treated mice independently of sex (**Fig. 2H**). These findings show that fluid intake does not contribute to the observed sex differences in disease progression of DSS-induced colitis. Altogether, these results demonstrate that although both male and female mice treated with 2.5% DSS develop a clinical profile consistent with colitis, marked sex differences are observed in both the severity and onset of symptoms, with males consistently exhibiting worse presentation of symptoms compared with females.

3.3. Motivation to groom is comparable in both sexes after Dextran sulfate sodium–induced colitis

The experiments described above revealed worse physical appearance in DSS-treated males compared with females (**Fig. 2E**). To assess whether differential motivation in self-grooming behavior contributes to sex differences in DSS-induced changes in physical appearance, we performed the splash test in control and DSS-treated male and female mice while simultaneously scoring coat states to monitor changes in physical appearance as a function of time (**Fig. 3A**). Immediately after sucrose solution application, all animals had a maximum coat state score independent of sex or DSS/water treatment (**Fig. 3B**). The coat state in control male and female mice returned to baseline 50 minutes after sucrose solution application. Consistent with the appearance results shown in **Figure 2E**, the coat state of DSS-treated male and female mice did not return to baseline, displaying a scruffy and humid physical appearance for the duration of the experiment. Analysis of the total time spent grooming revealed, however, that control and DSS-treated male and female mice spend comparable time grooming (**Fig. 3C**), suggesting that self-grooming motivation does not contribute to worse physical appearance in DSS-treated animals independently of sex.

3.4. Capsaicin-induced hypersensitivity is comparable in male and female mice after Dextran sulfate sodium–induced colitis, but the behavioral manifestation is sex-dependent

Altered pain perception in patients with IBS and other functional gastrointestinal disorders has been associated with the development of visceral pain hypersensitivity.[10,40] Thus, the model of intracolonic capsaicin was used to evaluate spontaneous pain-related behaviors in male and female mice treated with 2.5% DSS as a model of IBS, IBD, and other gastrointestinal disorders (**Fig. 4A**). Consistent with the results observed in naive female mice (**Fig. 1**), DSS-treated females displayed a significant ($P < 0.0001$) increase in the total number of spontaneous nociceptive behaviors upon intracolonic administration of 0.01% capsaicin compared with respective control mice injected with vehicle (**Fig. 4B**). Interestingly, unlike naive male mice (**Fig. 1**), DSS-treated males showed a significant ($P < 0.001$) increase in the total number of spontaneous nociceptive behaviors upon intracolonic administration of 0.01% capsaicin compared with those injected with vehicle control (**Fig. 4B**).

To identify the specific pain-related behaviors contributing to capsaicin-induced hypersensitivity observed in both sexes, the individual components that comprise the total number of pain-related behaviors were analyzed (**Figs. 4C–F**). Consistent with the results observed in naive female mice (**Fig. 1**), DSS-treated females showed a significant ($P < 0.01$) increase in the number of capsaicin-induced licking (**Fig. 4C**), dragging (**Fig. 4D**), and freezing responses (**Fig. 4G**) compared with vehicle-treated females, whereas the number of contraction bouts (**Fig. 4F**) were indistinguishable between capsaicin- and vehicle-treated females. By contrast, unlike the capsaicin-induced increases in stretching bouts seen in naive female mice (**Fig. 1**), capsaicin and vehicle-injected females displayed comparable stretching bouts after DSS treatment (**Fig. 4E**).

Our experiments in naive male mice showed that both the cumulative and individual spontaneous pain-related behaviors were comparable in mice injected with 0.01% capsaicin or vehicle control (**Fig. 1**). In contrast with these results, evaluation of the individual capsaicin-induced pain-related behaviors after DSS-induced colitis showed significant ($P < 0.05$) increases in the number of dragging (**Fig. 4D**) and stretching bouts (**Fig. 4E**) after intracolonic injection of 0.01% capsaicin, compared with intracolonic vehicle control injections. Consistent with the results observed in naive male mice, however, the number of licking (**Fig. 4C**) and contraction bouts (**Fig. 4F**) and time spent freezing (**Fig. 4G**) were indistinguishable in capsaicin and vehicle-injected DSS-treated males. Collectively, these results demonstrate that both sexes develop capsaicin-induced hypersensitivity to intracolonic application of 0.01% capsaicin after DSS-induced colitis. The manifestation of hypersensitivity, however, was sex-dependent, with both sexes displaying increases in dragging behavior (**Fig. 4D**) but only females showing increases in licking and freezing behaviors (**Figs. 4C and G**) and only males displaying increases in stretching behaviors (**Fig. 4E**).

3.5. Referred abdominal hypersensitivity is sex-dependent after acute but not persistent colon inflammation

Previous studies have shown that after intracolonic administration of capsaicin or DSS-induced colitis, mice display referred abdominal hypersensitivity, manifested as an increase in the frequency of pain-related responses to tactile stimulation of the abdomen during the von Frey test.[21,31] The next set of experiments aimed at evaluating potential sex differences in baseline responses to tactile stimulation of the abdomen using a 0.16-g von Frey filament as well as in referred abdominal hypersensitivity after acute and persistent colon inflammation, induced by intracolonic administration of 0.01% capsaicin or DSS in drinking water, respectively (**Fig. 5A**). Response frequency was indistinguishable between males and females in control conditions (**Fig. 5B**), demonstrating that baseline responses to tactile stimulation of the abdomen are not sex-dependent. Consistent with previous reports,[31] intracolonic injection of 0.01% capsaicin significantly ($P < 0.001$) increased response frequency to tactile

stimulation of the abdomen in female mice compared with intracolonic vehicle control injections (**Fig. 5B**). By contrast, response frequencies were indistinguishable in male mice after intracolonic administration of 0.01% capsaicin or vehicle control. These findings are consistent with the higher capsaicin-induced spontaneous nociceptive responses we observe in females (**Fig. 1**), further demonstrating that referred abdominal hypersensitivity is also sex-dependent in the context of acute colon inflammation.

Evaluation of DSS-induced referred abdominal hypersensitivity in males and females revealed that both male and female mice displayed significant ($P < 0.0001$) increases in response frequencies to tactile stimulation of the abdomen using a 0.16-g von Frey filament after intracolonic administration of 0.01% capsaicin when compared with animals injected with intracolonic vehicle control (**Fig. 5B**). Further analyses showed that, as expected, capsaicin-induced hypersensitivity to tactile stimulation is potentiated by DSS-induced persistent colon inflammation, with both sexes showing a significant ($P < 0.05$) increase in response frequencies to tactile stimulation of the abdomen after intracolonic administration of 0.01% capsaicin in the context of DSS-induced colitis when compared with control mice injected with 0.01% capsaicin. These combined results are consistent with our findings showing that spontaneous capsaicin-induced responses in the context of DSS-induced colitis is not sex-dependent (**Fig. 4**) and further demonstrate that referred abdominal hypersensitivity in the context of persistent colon inflammation is also not sex-dependent.

3.6. Dextran sulfate sodium induced bowel pathology is comparable in males and females

The results presented above show that clinical progression of disease and behavioral manifestation of hypersensitivity after DSS treatment are sexually dimorphic. To gain mechanistic insight into whether DSS-induced bowel pathology contributes to the observed sex differences in disease progression and behavioral hypersensitivity, colon lengths and histopathological analysis of the bowels of control and DSS-treated mice of both sexes were evaluated (**Fig. 6A**). Bowel length is commonly used to evaluate macroscopic manifestations of gastrointestinal disease in rodents, with shortening of the bowel reported in many gastrointestinal conditions, including DSS-induced colitis.[2,43] Consistent with previous reports, analysis of our experiments showed that both sexes exhibit significant ($P < 0.0001$) shortening of the bowel after DSS treatment when compared with their respective water controls (**Figs. 6B and C**). Further analysis revealed that female bowels are significantly ($P < 0.05$) shorter than male bowels in both control and DSS-treated mice. Comparison of the change in average bowel length in control and DSS-treated mice reveals, however, that both sexes display a bowel shortening of ~19% after DSS treatment, when compared with their respective water controls (**Fig. 6C**). These results demonstrate that the manifestation of DSS-induced colitis is comparable in males and female mice at the gross pathological level.

Histopathological analysis of the colon of both control and DSS-treated male and female mice was performed to assess bowel pathology at the microscopic level (**Figs. 6D–I**). As illustrated in the representative images of H&E-stained colon sections in **Figure 6E**, in control condition, the glands-mucosa structure is intact with healthy and organized colon crypts. By contrast, colon sections of DSS-treated mice show high infiltration of inflammatory cells, ulceration, and a highly damaged glands-mucosa structure. Analyses of the cumulative histology score, composed of glands-mucosa, inflammation, and % of colonic tissue affected scores, revealed that DSS induces increases in histology scores in bowels from both male and female mice in a dose-dependent manner, with higher histology scores associated with increasing dose of DSS administered (**Fig. 6F**). Consistent with our analysis of gross bowel pathology, analyses of histology scores showed comparable pathology at the microscopic level between DSS-treated male and female mice. Analyses of the individual components further demonstrated comparable damage of the colonic crypts and mucosa (**Fig. 6G**), inflammatory infiltration of leucocytes (**Fig. 6H**), and % of colonic tissue affected (**Fig. 6I**) in DSS-treated males and females. Altogether, these results show no measurable sex difference in DSS-induced gross and microscopic colon pathology.

4. Discussion

Chronic visceral pain is predominantly reported in women, and higher pain sensitivity has been shown in women patients.[50] Despite this, the majority of preclinical pain studies have focused only on males.[39] The limited inclusion of sex as a biological variable in preclinical studies results in an incomplete understanding of the biological processes underlying pain.[39] In this study, we evaluated and characterized sex differences in pain-related responses, disease progression of colitis, and colitis-induced colon pathology using the intracolonic capsaicin and DSS-induced colitis mouse models of chemically induced colonic hypersensitivity (**Fig. 7**). Our behavioral experiments show sex differences in pain-related behaviors, with females exhibiting more pain-related responses and referred abdominal hypersensitivity than males in the context of acute colon inflammation induced by intracolonic capsaicin (**Figs. 7A and B**). We further show that DSS-induced colonic hypersensitivity and referred abdominal hypersensitivity are similar between males and females, but the manifestation of capsaicin-induced behavioral responses is different between sexes. Although both sexes exhibited dragging, stretching, and freezing in response to intracolonic capsaicin, increases in licking of the abdomen were only observed in females and increases in abdominal contractions were only displayed by males (**Fig. 7A**). Consistent with other studies,[2,11,43] we also show that progression of DSS-induced colitis is sex-dependent, with males exhibiting worse clinical progression, manifested as worse appearance and higher weight loss, than in females (**Fig. 7C**). No measurable sex differences were observed, however, in colitis-induced colon pathology, stool consistency, or fecal blood (**Fig. 7D**). Together, these findings stress the importance of incorporating both sexes in preclinical and clinical studies of pain and further demonstrate that gross colon pathology and histopathology do not contribute to the observed sexual dimorphism in behaviors and colitis disease progression.

4.1. Behavioral manifestation of pain-related behaviors after colonic inflammation is sex-dependent

Although previous studies consistently show that DSS-induced colitis and intracolonic capsaicin produce colonic hypersensitivity and referred abdominal hyperalgesia in both sexes,[21,31] potential sex differences in pain-related behaviors in these 2 models of colonic pain have not been evaluated. In this study, we demonstrated that females display higher capsaicin-induced spontaneous nociceptive responses than males. We also show that the behavioral manifestation of pain-related responses to

intracolonic capsaicin is dependent on sex, with increases in licking of the abdomen observed only in females and increases in abdominal contractions only displayed by males (**Figs. 1 and 4**).

Sex differences in behavioral outputs have been described previously in other contexts such as fear and escape behaviors,[13,15,29] highlighting the importance of characterizing behavioral assays in both males and females. Previous studies have further shown that neural circuits driving distinct pain-related responses, such as paw withdrawal or licking in response to evoked tactile stimulation, are unique.[18] Our behavioral experiments showing that male and female mice exhibit distinct behavioral responses to intracolonic capsaicin suggest that recruitment of neural circuits in responses to a particular noxious stimulus might be sex-dependent.

4.2. Clinical progression of disease in a mouse model of colitis is sex-dependent

We show that DSS treatment induces a clinical profile consistent with colitis in both male and female mice, and that males exhibit a worse clinical progression of disease (**Fig. 2**). The DAI used to measure clinical progression of colitis is typically presented as a cumulative score of appearance, weight loss, stool consistency, and fecal blood.[2,7,11,27,36,43] Analysis of the individual DAI components in this study revealed that higher DAI scores in males are driven by greater weight loss and worse appearance but that DSS-induced changes in stool consistency and fecal blood are similar between sexes. The lack of measurable sex differences in stool consistency and fecal blood, together with comparable DSS-induced shortening of the bowel and histopathological analysis (**Fig. 6**), suggests that bowel pathology is not the driving force behind the sex differences in weight loss. Weight loss is a hallmark in animal models of experimental colitis and has been associated with decreases in food intake and/or changes in metabolic rates, locomotor activity, or body fat content.[37] Similarly, weight loss in patients with IBD has been related with decreased appetite, alterations in metabolic hormones, and/or malabsorption of nutrients.[8] Future studies to explore whether DSS induces sex-dependent changes in food consumption and/or metabolic alterations that contribute to colitis-induced weight loss are needed.

Despite the worse physical appearance seen in DSS-treated males (**Fig. 2E**), coat state and time spent grooming in the splash test was similar between sexes (**Fig. 3**). These results are consistent with findings from previous studies in males[21] and suggest that motivation to self-groom is not affected after DSS-induced colitis and it is not sex-dependent. When interpreting these findings, it is important to consider that sucrose solution application in this test creates an acute challenge that experimentally evokes grooming. Motivation to groom when presented with an acute challenge like sucrose application might be different to motivation to self-groom for daily upkeeping. Previous studies have shown that self-grooming is an evolutionary conserved innate behavior in mice that involves a complex sequencing pattern.[25] The worse physical appearance in DSS-treated males, combined with the lack of sex or treatment-dependent effect on time spent grooming in response to sucrose application, suggests that males are less efficient at grooming than females after the induction of colitis.

An important caveat of the experiments here is that the same percentage of DSS was used in males and females, despite males having greater initial body weight than females and the comparable fluid consumption levels measured in all experimental groups (**Fig. 2H**). Consequently, the DSS dose per body weight is lower in males than it is in females. Despite these differences in absolute DSS intake, males exhibit worse disease progression than females, further emphasizing sexual dimorphism in colitis disease progression.

4.3. Comparison with other studies and potential translational application

Our behavioral experiments show that female mice display more capsaicin-induced spontaneous pain-related responses than males (**Fig. 1**). These results are consistent with previous studies showing that female rats have higher visceromotor responses to noxious colorectal distension at baseline and after intracolonic injection of mustard oil than male rats.[16,23,24] Measurements of visceromotor responses to colorectal distension are typically performed in lightly anesthetized or loosely restrained rats, which may confound the behavioral outcomes measured and limit the interpretation of findings. The consistencies between our behavioral findings in freely behaving mice and those in previous studies using lightly anesthetized or loosely restrained rats demonstrate that sex differences in visceral pain-related behaviors are comparable and can be equally studied in freely behaving mice or in rats under light anesthesia or loosely restrained. Our results showing that female rodents display higher colonic sensitivity than males are also consistent with clinical studies in humans that also show higher visceral sensitivity in women than in men,[3,4,48] validating the use of rodent preclinical models to mechanistically study sex differences in visceral pain. Finally, the demonstration that sex differences in pain-related responses to colonic inflammation are recapitulated in mice is important as they offer a more enriched repertoire of molecular genetic tools to study behavior at circuit and mechanistic levels.

It is important to note that our experiments showed that hypersensitivity in the context of DSS-induced colitis is not sex-dependent (**Fig. 4**). These findings contrast with clinical studies that report that female patients with IBS exhibit higher visceral sensitivity and report more severe abdominal pain or discomfort than men.[3,4,48] As discussed above, however, our experiments also demonstrated that the behavioral manifestation of capsaicin-induced nociceptive responses in mice is sex-dependent (**Figs. 1 and 4**), suggesting that pain perception is differentially experienced by females and males. Given that pain is a multidimensional experience that comprises affective, cognitive, and somatosensory components, studies that evaluate these components in humans are needed to understand the mechanisms driving sex differences in visceral pain.

At a pathological level, consumption of DSS in drinking water has been shown to disturb the colonic lumen structure, eliciting an inflammatory response that emulates IBDs such as ulcerative colitis (UC) and Crohn disease (CD).[32] Consistent with previous reports,[43] we also show that DSS induces shortening and histopathological changes in the colon (**Fig. 6**) that resemble the shortening of the small bowel and histopathological features observed in patients with ulcerative colitis (UC) and Crohn disease (CD),[42] further validating the applicability of this model to study IBDs. Notably, however, while previous studies have reported sex differences in DSS-induced bowel shortening and histopathology,[2,11,34] our experiments showed that bowel pathology is comparable between sexes. These discrepancies could be due to differences in strain used, DSS supplier, or animal facility–specific factors that have been previously shown to strongly influence DSS experiments.[5,34]

A final important consideration is that the experiments in this study are focused on colonic pain mouse models. Whether our

findings can be generalized to other types of visceral pain and other species, including humans, remains unknown. Nonetheless, the observed sex differences in pain-related behaviors and clinical progression of colitis highlight the importance of considering sex as a biological variable in both preclinical and clinical pain studies.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Appendix A. Supplemental digital content

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