Research Paper

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Neonatal vaginal irritation results in long-term visceral and somatic hypersensitivity and increased hypothalamic–pituitary–adrenal axis output in female mice

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Abstract

Experiencing early life stress or injury increases a woman's likelihood of developing vulvodynia and concomitant dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis. To investigate the outcome of neonatal vaginal irritation (NVI), female mouse pups were administered intravaginal zymosan on postnatal days 8 and 10 and were assessed as adults for vaginal hypersensitivity by measuring the visceromotor response to vaginal balloon distension (VBD). Western blotting and calcium imaging were performed to measure transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) in the vagina and innervating primary sensory neurons. Serum corticosterone (CORT), mast cell degranulation, and corticotropin-releasing factor receptor 1 (CRF_1) expression were measured as indicators of peripheral HPA axis activation. Colorectal and hind paw sensitivity were measured to determine cross-sensitization resulting from NVI. Adult NVI mice had significantly larger visceromotor response during VBD than naive mice. TRPA1 protein expression was significantly elevated in the vagina, and calcium transients evoked by mustard oil (TRPA1 ligand) or capsaicin (TRPV1 ligand) were significantly decreased in dorsal root ganglion from NVI mice, despite displaying increased depolarization-evoked calcium transients. Serum CORT, vaginal mast cell degranulation, and CRF_1 protein expression were all significantly increased in NVI mice, as were colorectal and hind paw mechanical and thermal sensitivity. Neonatal treatment with a CRF_1 antagonist, NBI 35965, immediately before zymosan administration largely attenuated many of the effects of NVI. These results suggest that NVI produces chronic hypersensitivity of the vagina, as well as of adjacent visceral and distant somatic structures, driven in part by increased HPA axis activation.

Keywords: Neonatal insult, Vulvodynia, DRG, HPA axis, Comorbidity, TRP channels

1. Introduction

Chronic pelvic pain is the most common indication for referral to women's health specialists, encompassing 20% of all secondary care appointments and costing $881.5 million per year in outpatient management in the United States alone.[39] Specifically, vulvodynia affects 4% of women in the United States with a lifetime prevalence of 16%.[34,60] Like other idiopathic pain disorders, vulvodynia is proposed to be of a multifactorial nature, and treatment options include nonspecific lifestyle interventions, topical or oral medications, injections, physical therapy, and surgical procedures.[68] Clinically, vulvodynia has been associated with other chronic pelvic pain syndromes, most often interstitial cystitis and irritable bowel syndrome (IBS), and increased rates of mood disorders such as depression or anxiety.[4,9,23,24,29,46,48,52]

Strong evidence has linked early life adverse events with an increased likelihood of vulvodynia in adulthood, which has been attributed to dysfunctional regulation of the hypothalamic–pituitary–adrenal (HPA) axis.[4,25,32,50] The HPA axis regulates the response to stress and influences the perception of pain, largely through release of glucocorticoids initiated by corticotropin-releasing factor (CRF).[6] Indeed, vulvodynia patients demonstrate blunted serum cortisol cycles,[25] and acute stress exposure has been shown to increase symptom severity.[31] Biopsies from vulvodynia patients show increased infiltration and degranulation of mast cells,[30,40] which express receptors for CRF,[6] and acute stress has been shown to trigger mast cell degranulation and increase CRF peptide content in the surrounding milieu.[57,63] We recently published evidence that early life stress in mice is sufficient to induce increased vaginal sensitivity with a concomitant dysregulation of the HPA axis.[49] Other animal models of neonatal stress or injury in the gastrointestinal[2,5,13,18,59,71] and urinary[20,51,56] systems have demonstrated permanently enhanced sensitivity of the affected organ, increased numbers of dorsal horn neurons responsive to organ distension, a wider expression pattern of the transient receptor potential channels TRPA1 and TRPV1 among dorsal root ganglion (DRG) neurons innervating the affected organ, enhanced peptide content and vascular permeability of the affected organ, and disruption of the descending opioid inhibitory system (reviewed in Ref. 14). Increased colorectal sensitivity has also

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been observed in adult rats that underwent either neonatal bladder irritation[44] or repeated gastric suctioning during the first 2 postnatal weeks[59] and the latter in an HPA-dependent manner, suggesting that neonatal insult, as well as neonatal stress, is capable of invoking changes within the stress response system that may ultimately affect how noxious input is perceived.

Given the strong clinical evidence linking early life adverse events with an increased likelihood of developing vulvodynia in adulthood,[4,25,32,50] we conducted the following study to determine whether neonatal vaginal irritation (NVI) leads to permanent vaginal hypersensitivity, as well as comorbid sensitivities, in mice. We investigated the long-term impact of neonatal intravaginal zymosan on primary sensory neurons innervating the vagina and surrounding skin and peripheral HPA axis output that may contribute towards comorbid visceral hyperalgesia. We also tested the efficacy of antagonizing the CRF receptor, CRF_1, before NVI.

2. Materials and methods

2.1. Animals

Experiments were performed on female C57Bl/6 mice (Charles River, Wilmington, MA) born and housed in the Research Support Facility at the University of Kansas Medical Center. Mice received water and food ad libitum. All research performed conformed to NIH guidelines in accordance with the guidelines specified by the University of Kansas Medical Center Institutional Animal Care and Use Committee and the Committee for Research and Ethical Issues of IASP.

2.2. Vaginal irritation and corticotropin-releasing factor receptor 1 antagonist (NBI 35965) treatment

To induce NVI, female mice received an intravaginal instillation of 10 μL of 5% zymosan (Sigma, St. Louis, MO) in saline through a 26-gauge feeding needle attached to a Hamilton syringe on postnatal days 8 and 10. To determine the effect of the disruption of the vaginal hymen, which occurs during zymosan instillation on postnatal day 8, a separate cohort was treated intravaginally with 10 μL saline on postnatal days 8 and 10. A water-based lubricant (KY Jelly; Johnson & Johnson, New Brunswick, NJ) was liberally applied to the perivaginal region before instillation of zymosan or saline to avoid sensitization of surrounding somatic tissues. Mice were held briefly (less than 5 minutes) in a secondary container and observed for any adverse effects (eg, vaginal bleeding) before being returned to their home cages. All female pups from a given litter were assigned to the same treatment group to avoid any cross-exposure between pups. All mice remained undisturbed until weaning on postnatal day 22.

To block activation of the HPA axis during NVI, a separate group of mice was treated with saline or a CRF_1 antagonist, NBI 35965 (Tocris, Bristol, United Kingdom), before zymosan instillation. Female pups received either saline (10 μL) or NBI 35965 (20 mg/kg in saline; approximately 10 μL) intraperitoneally 20 minutes before zymosan instillation on both postnatal day 8 and 10. Naive mice were similarly treated with saline or NBI 35965 and held for 20 minutes without zymosan instillation. Again, all mice remained undisturbed until weaning on postnatal day 22.

To determine the impact of intravaginal zymosan on vaginal sensitivity in adult female mice, 8-week-old female mice were anesthetized with inhaled isoflurane (4% induction, 2.5% maintenance) and secured on a platform that elevated the pelvic region approximately 5 cm above the working surface. A water-based lubricant (KY Jelly) was liberally applied to the perivaginal region to avoid sensitization of surrounding somatic tissues. Mice received an intravaginal instillation of 50 μL of 5% zymosan (Sigma) in saline through a 26-gauge feeding needle attached to a Hamilton syringe. Mice remained in the elevated position for 5 minutes to prevent leakage of zymosan. On recovery from anesthesia, mice were returned to their home cages. The entire procedure was repeated 2 days later to replicate the NVI procedure. After the second zymosan treatment, the mice remained undisturbed outside of routine animal husbandry for an additional 8 weeks.

2.3. Experimental design

Naive and NVI mice that did not receive saline or NBI 35965 as neonates were assessed for thermal and mechanical hind paw sensitivity before vaginal balloon distension (VBD) or colorectal distension (CRD) and killed 3 weeks after experimentation was completed (**Table 1**). Mice that received saline or NBI 35965 as neonates were assessed for either hind paw or pelvic organ sensitivity; however, only mice that underwent hind paw assessment were used for further in vitro analysis (**Table 1**).

2.4. Electromyographic electrode implantation and organ distension

The visceromotor response (VMR) to either VBD or CRD was evaluated in separate groups of adult (at least 8-week old) mice. Electrode implantation was performed as previously described.[15] Under inhaled isoflurane (4% induction, 2.5% maintenance) and aseptic conditions, the bare ends of 2 Teflon-coated stainless steel wires (3 mm; Grass Technologies, West Warwick, RI) were inserted into the right lateral abdominal musculature, secured through 5-0 prolene sutures, tunneled subcutaneously to a small incision at the nape of the neck, and externalized for access during testing. Skin incisions were closed using 5-0 silk suture. After recovery from anesthesia, mice were housed singly and allowed to recover for a minimum of 4 days before undergoing testing.

Table 1

Age of mice at experimental time points.

	Thermal hind paw	Mechanical hind paw	Vaginal balloon distension	Colorectal distension	Sacrifice
Naive/NVI					
VBD group	6	7	10		13
CRD group	6	7		10	13
Saline/NBI 35965-treated					
Hind paw	6	7			9
VBD/CRD			10	12	

Naive and NVI mice underwent behavioral and/or physiological testing at the above noted ages (in weeks). In the initial study, mice were assessed for thermal and mechanical hind paw sensitivity before vaginal balloon distension (VBD) or colorectal distension (CRD) and killed 3 weeks after experimentation was completed. In the NBI 35965 study, mice were assessed for either hind paw or pelvic organ sensitivity; however, only mice that underwent hind paw assessment were used for further in vitro analysis.

2.4.1. Vaginal balloon distension

To facilitate balloon insertion and obtain proper restraint during VBD, mice were briefly sedated with inhaled isoflurane, and a custom-made latex balloon (1 cm in length) was inserted into the vagina and secured to the base of the tail with tape. The mouse was then placed into a Broome rodent holder (Kent Scientific, Torrington, CT), the free ends of the electrode wires were attached to a differential amplifier (Model 1700; A-M Systems, Sequim, WA), and the mice were allowed to recover from anesthesia for 30 minutes. The balloon was inflated with air from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator (Matheson-Linweld, Kansas City, MO), and a separate pressure monitor (World Precision Instruments, Sarasota, FL) was used to regulate the intraballoon pressure. Each pressure (40, 60, 80, 100, and 120 mmHg) was applied 3 times for 20 seconds with intervening 4-minute rest periods.

2.4.2. Colorectal distension

To facilitate balloon insertion and obtain proper restraint during CRD, mice were briefly sedated with inhaled isoflurane and a custom-made polyethylene plastic cylinder (1.5 cm in length and 0.7 cm diameter) was inserted through the anus until the proximal end of the balloon was 0.5 cm from the anal verge (total balloon insertion, 2 cm) and secured to the base of the tail with tape. The mouse was then restrained and allowed to recover as described above. The balloon was inflated and monitored as described above, and each pressure (15, 30, 45, 60, and 75 mmHg) was applied 3 times for 20 seconds with intervening 4-minute rest periods.

A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow through the system. Electromyographic (EMG) electrode activity was amplified, filtered, and recorded on a personal computer with Spike 2v7 software (Cambridge Electronic Design, Cambridge, United Kingdom) for off-line analysis. The VMR was quantified by measuring the area under the curve for the entire distension period divided by the duration of the distension and expressed as a percent of baseline activity (before each distension).

2.5. Behavioral analyses

Mice underwent a 30-minute acclimatization period within the testing room on the day before each behavioral test. Mice were allowed to acclimate to each apparatus for 30 minutes before testing, and the experimenter was blinded to the treatment status of the mice. Mice were tested for thermal sensitivity by measuring paw withdrawal latency to radiant heat[33] and for mechanical sensitivity using calibrated monofilaments. For thermal sensitivity, mice were placed in individual clear plastic chambers (11 × 5 × 3.5 cm) on the 30°C heated glass surface of a thermal analgesiometer (UARDG; Department of Anesthesiology, University of California San Diego, La Jolla, CA). Hind paws were tested for a total of 3 times per side with a minimum of 5 minutes between applications, and the latency to hind paw withdrawal from the stimulus was automatically recorded within 0.01 second. The stimulus terminated automatically at 20 seconds to avoid tissue damage. Individual responses were averaged per mouse, and group mean values were determined as previously described.[17] For mechanical sensitivity, mice were placed into individual clear plastic chambers (11 × 5 × 3.5 cm) on a wire mesh screen elevated 55 cm above a table. The up–down method was performed to test mechanical sensitivity using a standard set of monofilaments (1.65, 2.36, 2.83, 3.22, 3.61, 4.08, 4.31, 4.74 g; Stoelting, Wood Dale, IL).[22] Beginning with the 3.22 g monofilament, mice received a single application of the monofilament to the plantar surface of the right hind paw. A negative response was followed by the next larger filament, and a positive response (considered a brisk withdrawal of the paw) was followed by the next smaller gram filament. The experimenter continued to move up or down the series, depending on the previously elicited response, for an additional 4 applications after the first positive response was observed for a minimum of 5 or a maximum of 9 total monofilament applications. The value in $\log_{10}$ units of the final monofilament applied in the trial series was used to calculate a 50% g threshold for each mouse, and group mean values were determined as previously described.[12]

2.6. Retrograde labeling of vaginal and perivaginal neurons

All surgical procedures were performed under aseptic conditions in a designated animal surgery area. Mice were anesthetized by inhaled isoflurane (4% induction, 2.5% maintenance), and using a Hamilton microsyringe (33-gauge needle), multiple 1.5-2 μL injections of Alexa Fluor 488-conjugated cholera toxin-β (CTB, 2 mg/mL in saline, 8 μL total; Invitrogen, Grand Island, NY) were made circumferentially within the distal region of the vagina, approximately 3-5 mm from the external opening. Using a separate Hamilton microsyringe (33-gauge needle), 2-3 μL injections of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; 2% in DMSO, 5 μL total; Sigma) were made on the right and left side, subcutaneously, at the junction between the hairy skin and the opening of the vagina. Mice were returned to their home cages and allowed to recover from anesthesia under observation.

2.7. Cell culture and calcium imaging

Neurons were retrogradely labeled from the vagina and perivaginal skin in naive and NVI mice as described above. After a recovery period of 7 to 10 days, mice were overdosed with inhaled isoflurane (>5%) and perfused with ice-cold Ca^{+2}/Mg^{+2}-free Hanks' balanced salt solution (HBSS; Invitrogen) and lumbosacral (LS; L5-S1) DRG were dissected and prepared for culture as previously described.[43] Dissociated cells were resuspended in F12 media (Invitrogen) containing 10% FBS and penicillin/streptomycin (50 U/mL) and plated onto 12-mm poly-D-lysine/laminin precoated coverslips (BD Biosciences, Franklin Lakes, NJ). Cells were incubated overnight at 37°C and imaged the following day as previously described.[13] Before imaging, the cells were incubated in HBSS containing 5 mg/mL BSA (Sigma) and 2 μM fura-2-acetoxymethyl ester (Invitrogen) for 30 minutes at 37°C. Coverslips were placed in a QE-1 quick change platform (Warner Instruments, Hamden, CT) and mounted on an inverted Nikon TiE microscope stage (Melville, NY) with HBSS buffer flowing at 5 mL/min, controlled by a gravity flow system (Warner Instruments). Perfusate temperature was maintained at 30°C using a dual bipolar temperature controller and a 6-line solution heater (Warner Instruments). Chemicals were delivered using a gravity-feed pinch valve control perfusion system (Warner Instruments). Firmly attached, CTB- and DiI-positive neurons were identified and chosen as regions of interest using Nikon Elements Advanced Research Imaging software (Nikon). Unlabeled adjacent cells were also identified and imaged. Absorbance data at 340 and 380 nm were collected at 1 Hz during drug application using a Photometrics HQ2 dual-mode

cooled CCD camera (Boyce Scientific, Gray Summitt, MO). Responses were measured as the ratio of 340/380 nM excitation and 510 nM emission ($\Delta F_{340/380}$) controlled by a high-speed Fura/widefield Xenon illuminated filter wheel (Boyce Scientific). All fields were first tested with a brief application (4s) of 30 mM K^+ (high K^+) to ensure that cells were healthy and responsive. After a 5-minute recovery period, 1 μM capsaicin (Sigma) or 100 μM mustard oil (MO; Sigma) was applied for 5 or 10 seconds, respectively. A concentration of 10 mM capsaicin in 1-methyl-2-pyrrolidinone was used as a stock solution; 1 μM capsaicin was made fresh daily in HBSS. A concentration of 100 mM MO in 1-methyl-2-pyrrolidinone was made fresh daily and diluted to 100 μM using HBSS. Peak responses >0.1 $\Delta F_{340/380}$ were included in the analysis and were easily distinguishable from optical noise (<0.02 $\Delta F_{340/380}$). The prevalence of capsaicin- or MO-responsive vaginal, perivaginal skin, and unlabeled afferents was determined as a percentage of total healthy (high K^+-responsive) CTB-, DiI-positive, and dye-free cells, respectively. Any cell with significantly diminished Fura-2 signal over the duration of the experiment or that did not recover to baseline before agonist application was not included in the analysis. Peak Ca^{2+} influx, total Ca^{2+} influx, and time to 50% of the peak (T50) were calculated using Microsoft Excel.

2.8. Western blot

Total proteins were isolated from approximately 50 mg of snap-frozen DRG, vagina, and colon tissue samples using cell extraction buffer (Invitrogen) containing Halt protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA) and Na_3VO_4 (Sigma). Protein concentrations were determined using a D_C protein assay (Thermo Fisher). Samples were reduced by heating to 95°C for 5 minutes in the presence of 2-mercaptoethanol, subjected to SDS-PAGE (Criterion 4% to 12% Bis-Tris gels; Bio-Rad, Hercules, CA), and transferred to a nitrocellulose transfer membrane (Whatman GmbH, Dassel, Germany) by Criterion Blotter wet transfer (Bio-Rad). The membranes were blocked for 1 hour at room temperature in 5% milk in Tris-buffered saline with Tween-20 (TBST) and incubated overnight at 4°C with antisera to CRF_1 (1:500; Millipore, Billerica, MA), TRPV1 (1:1000; Alomone Labs, Jerusalem, Israel), or TRPA1 (1:1000; Aviva Systems Biology, San Diego, CA) and GAPDH (1:2000; Cell Signaling Technology, Danvers, MA) diluted in 5% milk in TBST. Membranes were then washed with TBST and incubated for 1 hour with anti-rabbit secondary antibody (1:10,000; Cell Signaling). Densitometry was performed using Quantity One 4.6.9 software (Bio-Rad).

2.9. Serum corticosterone

After decapitation, trunk blood was collected in 1.5 mL tubes, allowed to clot on ice for 1 hour, and spun at 10,000 rpm at 4°C for 10 minutes. Serum (clear supernatant) was collected and an enzymatic immunoassay kit (Enzo Life Sciences, Farmingdale, NY) was used to quantify serum corticosterone (CORT) content according to the manufacturer's instructions.

2.10. Toluidine blue staining of mast cells

Mice were transcardially perfused with ice-cold 4% paraformaldehyde, and the entire length of the vagina (minus the cervix) was removed, postfixed, and cryoprotected in 30% sucrose overnight. Tissue was flash-frozen in ice-cold heptane, mounted in Tissue-Tek OCT mounting medium (Sakura Finetek, Torrance, CA), and cryosections were cut at 10-μm thickness. Nonserial sections spanning the length of the tissue were stained with acidified toluidine blue and examined with light microscopy (Nikon eclipse 90i), for cells exhibiting metachromatic color change. Digital images were captured using a Photometrics HQ2 dual-mode cooled CCD camera (Boyce Scientific), and the total number of nondegranulated mast cells (dense metachromasia with no or faint nuclear outline and/or no granular extrusion around the cell) and degranulated mast cells (less-intense metachromasia and obvious clear outline of the nucleus and/or free granules within the cytoplasm), as described in Ref. 28, were counted in at least 8 separate sections. The ratio of degranulated/total mast cell infiltrates and total infiltrates per section were calculated for each mouse.

2.11. Statistics

Calculations were made using Microsoft Excel, and statistical analyses were performed using Student *t* test or 2-way analysis of variance (ANOVA) followed by Bonferroni's or Fisher's least significant difference posttest (GraphPad Prism 6; GraphPad Software, La Jolla, CA) as indicated in the article. All data are expressed as the mean ± SEM. A P value of <0.05 was considered significant.

3. Results

To determine the impact of NVI on vaginal sensitivity, we measured the EMG activity of the abdominal musculature (termed VMR) to VBD. All groups of mice displayed a stepwise and significant increase in VMR as higher intraballoon pressures were administered ($P < 0.0001$, 2-way repeated-measures ANOVA; **Fig. 1A**). The NVI mice displayed significantly higher VMR than naive mice over the entire series, particularly at the highest 2 pressures applied (**Fig. 1**). To control for the impact of rupturing the vaginal hymen during the initial application of zymosan on P8, a separate cohort of female mice was treated intravaginally with saline alone on P8 and 10. Saline-treated mice were not significantly different from naive mice and had a significantly lower VMR at the highest distension pressure compared with NVI mice (**Fig. 1A**). To determine whether intravaginal zymosan administration during adulthood would also produce prolonged vaginal hypersensitivity, we treated 8-week-old female mice with intravaginal zymosan on 2 nonconsecutive days and measured the VMR during VBD 8 weeks later. Mice treated with intravaginal zymosan did not have significantly different VMR compared with age-matched control mice (**Fig. 1B**).

Western blotting and calcium imaging were performed to determine the potential contribution of altered TRP channel protein expression and function, respectively, towards the development of vaginal hypersensitivity in NVI mice. Western blotting revealed a significant increase in TRPA1 protein in the vagina of NVI mice, compared with naive mice (**Fig. 2A**). No significant change in TRPA1 or TRPV1 protein levels was observed in LS (L6-S1) DRG from NVI mice compared with naive mice (**Fig. 2A, B**). Calcium transients in response to 30 mM K^+ (high K^+), 100 μM MO (TRPA1-responsive), or 1 μM capsaicin (TRPV1-responsive) were measured in dissociated LS DRG retrogradely labeled from the vagina and perivaginal skin, as well as in adjacent unlabeled cells. Functional TRPA1 expression was largely unchanged as no significant effect of NVI or target organ on the percentage of MO-responsive neurons was observed (**Fig. 2C**). Functional TRPV1 expression was also not affected by NVI; however, in naive mice, the percentage of capsaicin-responsive

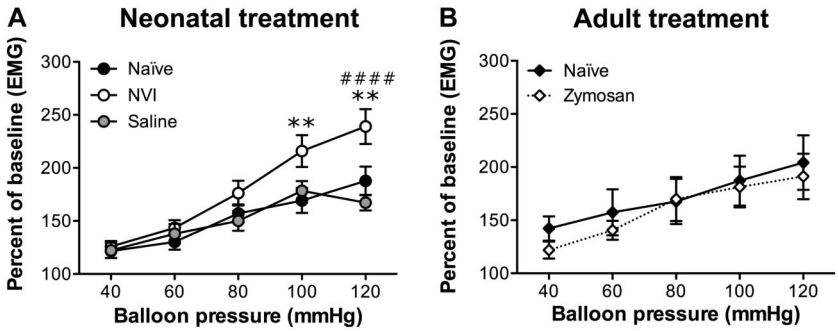

Figure 1. Neonatal vaginal irritation (NVI) significantly increased vaginal sensitivity. (A) The visceromotor response (VMR) was measured by recording the EMG activity of the abdominal musculature during graded balloon distension of the vagina (VBD). NVI mice (n = 11) had a significantly higher VMR during VBD than either naive (n = 23) or saline-treated (n = 18) mice over the entire distension series ($P < 0.05$), as well as at the highest intraballoon pressures applied. (B) Eight-week-old female mice received intravaginal zymosan (zymosan, n = 7) or remained undisturbed (naive, n = 8), and the VMR was measured during VBD 8 weeks later. No significant difference in VMR was observed between mice that received zymosan and those that did not. $^{**}P < 0.01$ vs naive, $\#\#\#\#P < 0.0001$ vs saline; 2-way repeated-measures ANOVA and Bonferroni's posttest.

neurons was significantly higher in vagina-specific DRG neurons compared with either perivaginal skin–specific or unlabeled neurons (**Fig. 2D**). When compared across both MO and capsaicin, NVI significantly reduced the percentage of agonist-responsive vagina-specific neurons ($P < 0.05$, 2-way ANOVA), but NVI had no effect on either perivaginal skin–specific or unlabeled neurons. Analysis of calcium transients revealed a significant impact of both NVI and peripheral target on

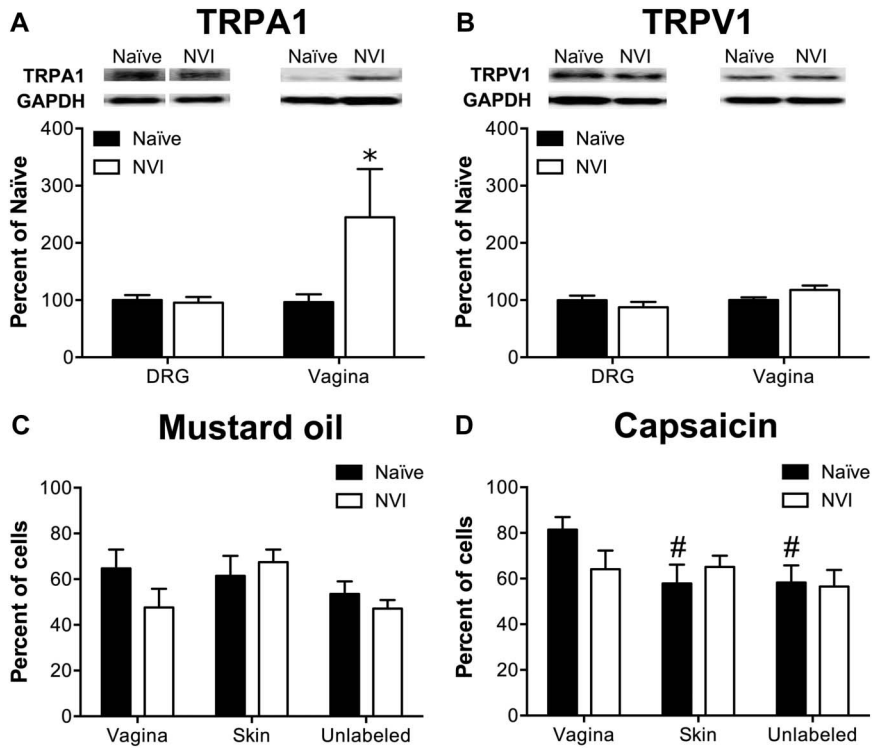

Figure 2. NVI increased TRPA1 protein expression in the vagina but not in primary sensory neurons innervating the vagina. (A) Representative Western blots are shown for TRPA1 and corresponding GAPDH protein expression with bands at 127 and 35 kD, respectively, in both DRG and vagina from naive and NVI mice. TRPA1 protein expression was significantly increased in vagina, but not DRG, from NVI mice compared with naive mice. (B) Representative Western blots are shown for TRPV1 and corresponding GAPDH protein expression with bands at 85 and 35 kD, respectively, in both DRG and vagina from naive and NVI mice. TRPV1 protein expression was not significantly different in NVI DRG or vagina compared with naive counterparts. Calcium imaging was performed on lumbosacral (L5-S1) DRG neurons retrogradely labeled by injection of Alexa Fluor–conjugated cholera toxin-β (CTB) into the distal vagina, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) into the perivaginal skin, and adjacent unlabeled DRG to measure responses to 100 μM mustard oil (MO; C) and 1 μM capsaicin (D). (C) No significant difference in functional TRPA1 expression, measured as the percentage of MO-responsive DRG neurons, was observed between naive and NVI mice for any population of DRG neurons tested. (D) Only in naive mice, DRG neurons back-labeled from the vagina were significantly more likely to respond to 1 μM capsaicin, suggesting greater functional TRPV1 expression, compared with those back-labeled from the perivaginal skin or unlabeled DRG. When compared across both agonists, vagina-specific DRG neurons from NVI mice had a significantly reduced percentage of responsive neurons compared with naive ($P < 0.05$; 2-way ANOVA). (A and B): naive, DRG: n = 10, vagina: n = 5; NVI, DRG: n = 8, vagina: n = 4; (C): naive, n = 8, NVI, n = 10; (D): naive, n = 8; NVI, n = 6. $^{*}P < 0.05$ vs naive, $\#P < 0.05$ vs vagina; 2-way ANOVA and Bonferroni's posttest.

MO-evoked peak Ca^{2+} influx and of peripheral target on total Ca^{2+} influx (**Table 2**). A significant impact of NVI on peak Ca^{2+} influx, total Ca^{2+} influx, and signal decay (T50; time to 50% of peak response) was observed in response to capsaicin, particularly on the population of unlabeled neurons (**Table 2**). In contrast to agonist-evoked responses, high K^+-evoked responses were significantly larger in DRG neurons from NVI mice than from naive, with high K^+ evoking a significantly larger total Ca^{2+} influx in vagina-specific neurons from NVI mice compared with vagina-specific neurons from naive mice or unlabeled DRG from NVI mice (**Table 2**). Time to decay after high K^+ application was also significantly longer in vagina-specific DRG from NVI mice compared with vagina-specific DRG from naive mice and skin-specific or unlabeled DRG from NVI mice (**Table 2**).

To determine whether NVI altered the peripheral output of the HPA axis, we assayed serum CORT levels and histologic evidence of extrusion of mast cell contents and CRF_1 protein expression in the vagina. Serum CORT was significantly higher in NVI mice compared with naive (**Fig. 3A**). Also, CRF_1 protein level (**Fig. 3B**) was significantly increased and the percentage of toluidine blue–stained mast cells showing evidence of extruded granules (**Fig. 3C-G**) was significantly higher in NVI compared with naive mice.

In light of the evidence of increased activity of the HPA axis and previous studies reporting cross-organ sensitization after neonatal organ irritation,[13,44,59] we assayed for hind paw sensitivity and VMR during CRD. Indeed, NVI mice displayed both a significantly lower withdrawal threshold to mechanical stimulation (**Fig. 4A**) and a significantly shorter withdrawal latency to thermal stimulation of the hind paw (**Fig. 4B**), compared with naive. A slight, yet significant, increase in VMR during CRD was observed in NVI mice, compared with naive mice, most prominently at the highest intraballoon pressure applied (**Fig. 4C**). Western blot analysis revealed that NVI diminished TRPV1 protein in the colon, whereas TRPA1 and CRF_1 protein were not affected in NVI mice, compared with naive (**Fig. 4D**).

To determine whether activation of the HPA axis is driving the changes after NVI, we treated neonatal mice with a CRF_1 antagonist, NBI 35965, 20 minutes before each zymosan instillation. NVI mice treated with NBI 35965 displayed a significantly lower VMR during VBD compared with saline-treated NVI mice (**Fig. 5A**). Treatment with NBI 35965 did not affect the VMR of naive mice during VBD (**Fig. 5A**) or CRD (**Fig. 5B**). The increase in VMR during CRD at 75 mmHg that was observed in the initial group of NVI mice was not detected in the saline-treated NVI mice, likewise the VMR of NVI mice treated with NBI 35965 did not differ from any other group during CRD (**Fig. 5B**). Treatment with NBI 35965 completely abolished both mechanical (**Fig. 5C**) and thermal (**Fig. 5D**) hind paw hypersensitivity in NVI mice and had no effect on naive mice. Vaginal CRF_1 protein level (**Fig. 6A**) and histologic evidence of mast cell degranulation (**Fig. 6C**) were significantly lower in NVI mice treated with NBI 35965, compared with saline-treated NVI mice. The level of CRF_1 protein in the

Table 2

Calcium imaging parameters.

	Peak Ca^{2+} influx ($\Delta F_{340/380}$)	Total Ca^{2+} influx ($\Delta F_{340/380}$)	T50 (s)
100 mM mustard oil			
Vagina			
Naive (27)	0.68 ± 0.067	124.5 ± 20.0	142.6 ± 22.1
NVI (41)	0.54 ± 0.060	96.6 ± 15.8	151.2 ± 18.4
Skin			
Naive (52)	0.68 ± 0.047	117.1 ± 13.9	130.5 ± 16.8
NVI (67)	0.62 ± 0.045	118.7 ± 13.6	153.0 ± 15.6
Unlabeled			
Naive (147)	0.55 ± 0.029	85.8 ± 8.15	118.8 ± 12.1
NVI (174)	0.50 ± 0.027	81.7 ± 7.09	130.1 ± 10.6
	*#	#	
1 mM capsaicin			
Vagina			
Naive (44)	0.88 ± 0.084	142.1 ± 18.9	136.0 ± 19.6
NVI (34)	0.86 ± 0.095	115.9 ± 20.3	116.4 ± 20.6
Skin			
Naive (32)	0.85 ± 0.078	149.4 ± 21.7	184.3 ± 25.2
NVI (25)	0.68 ± 0.103	88.9 ± 17.7	96.9 ± 12.0
Unlabeled			
Naive (138)	0.92 ± 0.041	172.6 ± 11.9	182.6 ± 13.4
NVI (128)	0.64 ± 0.044****	113.4 ± 10.9**	150.0 ± 12.6
	*	**	**
30 mM K^+			
Vagina			
Naive (88)	0.70 ± 0.036	38.9 ± 4.29	28.0 ± 5.09
NVI (96)	0.76 ± 0.048	64.1 ± 8.07**	60.3 ± 9.47****
Skin			
Naive (104)	0.63 ± 0.30	25.6 ± 3.74	11.2 ± 2.34
NVI (85)	0.78 ± 0.047	44.5 ± 7.28	23.6 ± 5.07####
Unlabeled			
Naive (490)	0.59 ± 0.015	24.3 ± 1.53	14.2 ± 1.21
NVI (374)	0.65 ± 0.021	33.3 ± 2.63####	24.4 ± 2.65*####
	**##	****####	****####+

Results are expressed as mean ± SEM. The number of cells used for quantification is indicated in parentheses. Data were analyzed using 2-way ANOVA: symbols at the bottom of each column set indicate significant impact of NVI (*, **, ****$P < 0.05, 0.01, 0.0001$), peripheral target (#, ##, ####$P < 0.05, 0.01, 0.0001$), or interaction effect (+$P < 0.05$); and Tukey's test for multiple comparisons: symbols within the table indicate *, **, ****$P < 0.05, 0.01, 0.0001$ vs naive, ####$P < 0.05$ vs vagina.

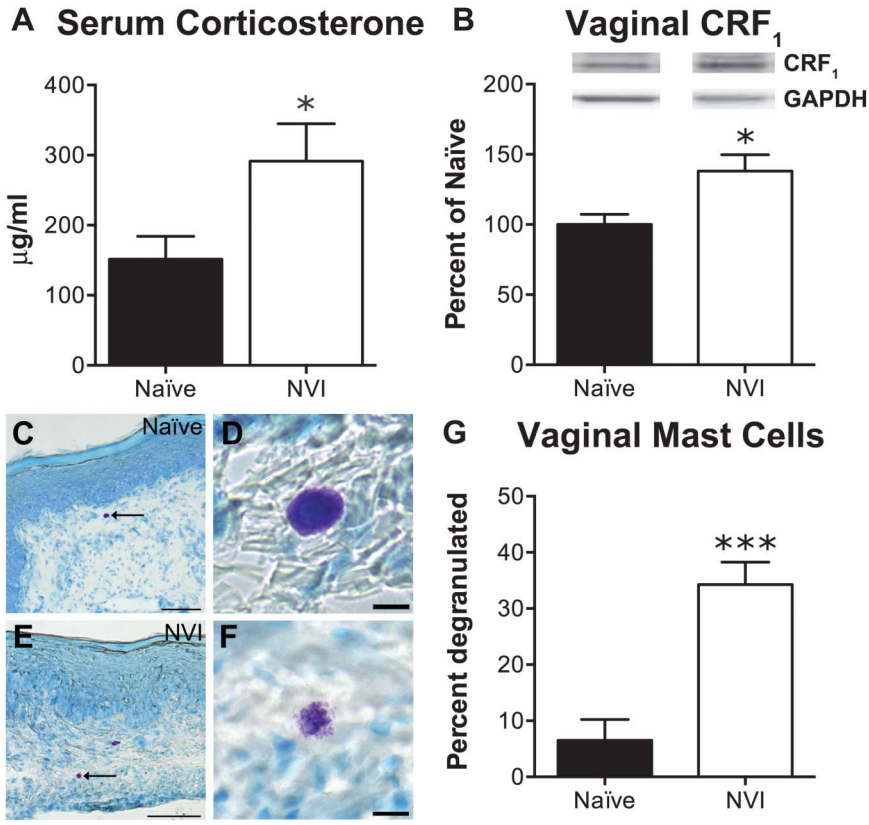

Figure 3. NVI mice display evidence of increased output from the hypothalamic–pituitary–adrenal (HPA) axis. (A) Total corticosterone measured in the serum of NVI mice (n = 7) at sacrifice was significantly higher than that of naive mice (n = 9). (B) Representative Western blots are shown for CRF_1 and corresponding GAPDH protein expression with bands at 55 and 35 kD, respectively, in the vagina of naive and NVI mice. CRF_1 protein expression was significantly increased in vagina from NVI mice (n = 4) compared with naive mice (n = 4). Mast cells, and evidence of degranulation, were identified by acidified toluidine blue staining in sections of vagina from naive (n = 3; C and D) and NVI mice (n = 3; E and F). High-magnification photomicrographs show examples of intact mast cells (D) and degranulated mast cells with extruded granules (F) in naive and NVI vagina, respectively. (G) The percentage of mast cells that exhibited evidence of extruded granules was significantly higher in NVI vagina than naive vagina. *, ***$P < 0.05$, 0.001 vs naive; Student *t* test. Scale bars equal 100 μm (C and E) and 10 μm (D and F).

colon was unaffected by NBI 35965 treatment in any of the groups (**Fig. 6B**).

4. Discussion

Experiencing adverse events early in life increases the risk of developing chronic pelvic pain disorders, including vulvodynia, during adolescence and adulthood. Here, we have investigated the outcomes of neonatal exposure to intravaginal zymosan, a cell wall component of yeast that has been shown to induce long-lasting hypersensitivity in the bladder of rats after neonatal intravesicular administration.[51] In this study, we have provided the first evidence that neonatal, but not adult, irritation of the vagina produces long-lasting sensitization of not just the vagina

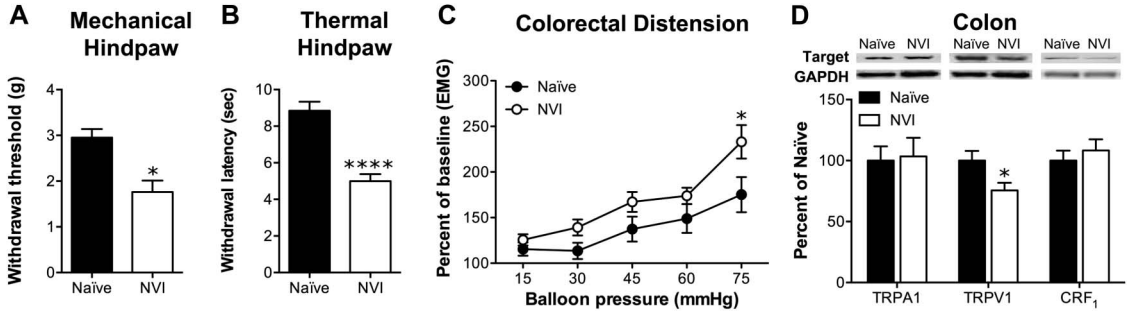

Figure 4. NVI mice display cross-sensitization in both hind paw and colon. (A) The withdrawal threshold of the hind paw to a mechanical stimulus was significantly lower in NVI mice (n = 8) compared with naive mice (n = 7). (B) NVI mice (n = 8) displayed a significantly shorter withdrawal latency to thermal stimulation of the hind paw than naive mice (n = 7). (C) NVI mice (n = 5) also displayed significantly higher VMR during CRD than naive mice (n = 5) over the entire dissension series ($P < 0.05$), as well as at the highest intraballoon pressure applied. (D) Representative Western blots are shown for TRPA1, TRPV1, CRF_1 and corresponding GAPDH protein expression with bands at 127, 85, 55, and 35 kD, respectively, in the colon of naive and NVI mice. Only TRPV1 protein expression in the colon was affected by NVI (n = 4), with a significant decrease compared with naive (n = 6). (A, B, and D): *, ****$P < 0.05$, 0.0001 vs naive; Student *t* test. (C): *$P < 0.05$ vs naive; 2-way repeated-measures ANOVA and Bonferroni's posttest.

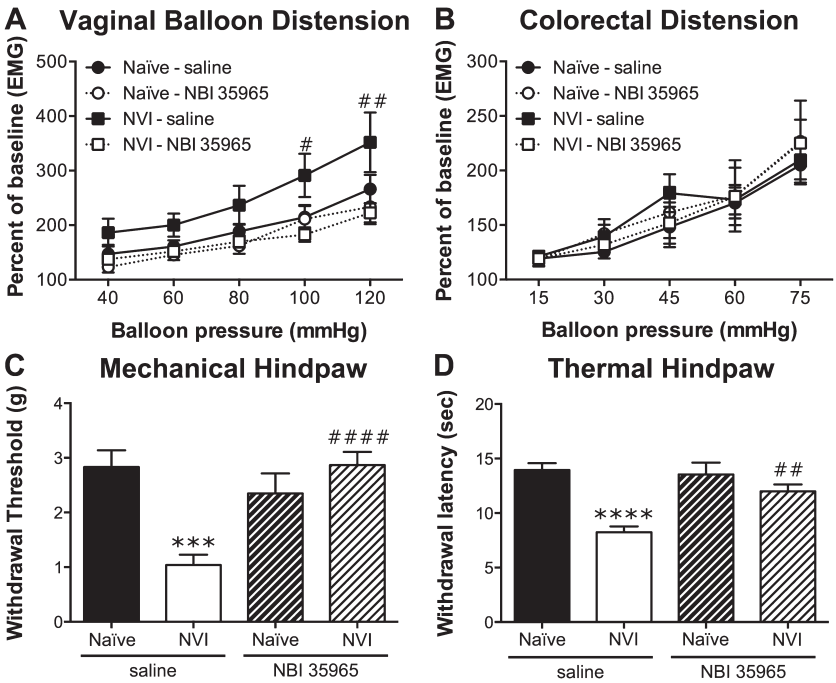

Figure 5. Neonatal treatment with NBI 35965 prevented vaginal and hind paw hypersensitivity in NVI mice. (A) The VMR during VBD was significantly lower in NBI 35965-treated NVI mice (n = 11) compared with saline-treated NVI mice (n = 8) over the entire distension period ($P < 0.05$), as well as at the 2 highest pressures applied. Naive mice treated with saline (n = 8) or NBI 35965 (n = 8) displayed similar VMR during VBD. (B) The VMR during CRD did not differ between any of the groups (n = 7-9 per group). Neonatal treatment with NBI 35965 significantly attenuated the decrease in mechanical withdrawal threshold (C) and thermal withdrawal latency (D) in NVI mice (n = 13) compared with saline-treated NVI mice (n = 11). Naive mice treated with saline (n = 10) or NBI 35965 (n = 8) had similar mechanical withdrawal thresholds (C) and thermal withdrawal latencies (D). (A and B): #, ##$P < 0.05$, 0.01 vs saline-treated; 2-way RM ANOVA and Bonferroni posttest; (C and D): ***, ****$P < 0.001$, 0.0001 vs naive, ##, ####$P < 0.01$, 0.0001 vs saline-treated; 2-way ANOVA and Bonferroni's posttest.

but also of the hind paw and possibly colon. Increased downstream output from the HPA axis, which was attenuated by neonatal treatment with a CRF_1 antagonist, NBI 35965, and changes in primary neuron signaling were also observed in NVI mice and likely contribute towards the increased visceral and somatic sensitivity.

Permanently heightened visceral sensitivity has been shown to arise after organ irritation or inflammation during the first 2 weeks of postnatal development. This has been shown extensively to occur in both the gastrointestinal[2,5,13,18,71] and urinary tracts,[19,51,56] and this study provides the first evidence that the reproductive system is also susceptible to neonatal irritation. Mice that received intravaginal zymosan on P8 and 10 displayed an overall increase in VMR during VBD, most prominently at the 2 highest intravaginal balloon pressures. This observation, as well as that of cross-sensitization of the colon only at the highest intraballoon pressure, is striking in that it more closely resembles changes in colorectal sensitivity after neonatal colon irritation[13] than it does the effect of neonatal maternal separation on vaginal sensitivity, which increased VMR across all pressures.[49] This dichotomy in the qualitative impact of early life insult vs stress on visceral sensitivity is also apparent in rat studies assessing either

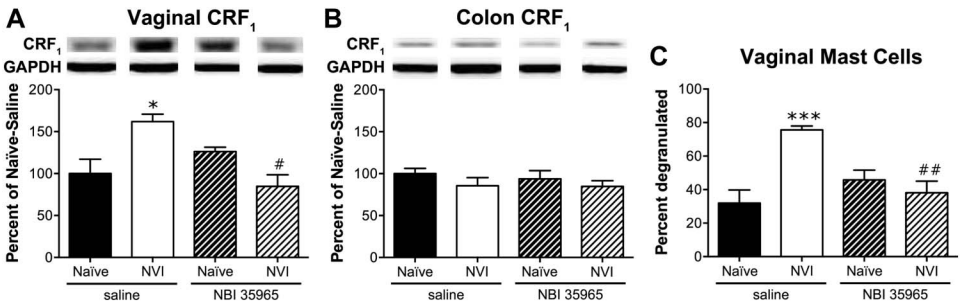

Figure 6. Neonatal treatment with NBI 35965 prevents increased vaginal CRF_1 protein level and mast cell degranulation after NVI. Representative Western blots are shown for CRF_1 and corresponding GAPDH protein expression with bands at 55 and 35 kD, respectively, in the vagina (A) and colon (B) of naive and NVI mice treated with saline or NBI 35965. (A) Neonatal treatment with NBI 35965 completely abolished the increase in CRF_1 protein level in vagina from NVI mice (n = 5), compared with saline-treated NVI mice (n = 5). Protein levels of CRF_1 in vagina were unaffected by neonatal NBI 35965 treatment in naive mice (n = 5), compared with saline treatment (n = 4). (B) The level of CRF_1 protein in colon from naive and NVI mice treated with neonatal saline (n = 4) or NBI 35965 (n = 5) did not differ. (C) The percentage of mast cells, identified by acidified toluidine blue staining, that demonstrated evidence of degranulation was significantly diminished in the vagina from NVI mice treated with NBI 35965 (n = 5) compared with saline (n = 5). The extent of mast cell degranulation in the vagina of naive mice treated with saline (n = 4) or NBI 35965 (n = 5) did not differ. *, ***$P < 0.05$, 0.001 vs naive, #, ##$P < 0.05$, 0.01 vs saline-treated; 2-way ANOVA and Bonferroni's posttest.

bladder or colorectal sensitivity after neonatal inflammation or maternal separation, with the former generating increased VMR only at presumed nociceptive pressures and the latter increasing VMR at both nonnoxious and noxious pressures.[2,18,19,45,51,71] In this study, instillation of intravaginal saline, which caused disruption of the vaginal hymen, was not sufficient to produce vaginal hypersensitivity suggesting that physical manipulation of the vagina alone is not sufficient to permanently sensitize the organ. Likewise, recapitulating the neonatal insult in adult female mice was not sufficient to produce a long-lasting vaginal hypersensitivity, indicating that the neonatal period represents a critical time for the induction of chronic pain, as has been illustrated in multiple other studies.[2,51,53]

We, and others, have previously shown discrepancies in both immunohistochemical and functional TRP channel expression between viscera-, muscle-, and skin-specific DRG populations, with the former 2 populations having greater TRP channel expression than the latter population.[16,35,41] Accordingly, DRG neurons back-labeled from the vagina responded more widely to capsaicin than did unlabeled or perivaginal skin–specific DRG neurons. The percentage of perivaginal skin–specific DRG neurons that responded to MO or capsaicin in this study was larger than we have reported previously for cutaneous afferents labeled through injection of retrograde tracer into the saphenous nerve[16,41] and likely reflects differences in the neurochemical phenotype of sensory neurons innervating the ventral hind paw and the perivaginal area. Likewise, functional TRPA1 and TRPV1 expression was higher in unlabeled DRG neurons from both naive and NVI mice, compared with previous publications of unlabeled primary DRG cultures.[11,42] This is likely due to the relatively high percentage of visceral afferent neurons in our preparations, as only L5-S1 DRGs were used in this study and TRPV1 has previously been shown to be enhanced in LS regions due to the pelvic afferent contribution.[16] Unlike in previous studies of neonatal colon irritation,[13,71] functional expression of TRPA1 and TRPV1 was not increased in target-specific DRG neurons from NVI mice. Evidence of increased neuronal excitability, in the form of significantly larger high K^+-evoked calcium transients, was observed. Increased depolarization-evoked calcium transients have been reported in adult mouse models of inflammatory-induced interstitial cystitis[21] and chronic pancreatitis,[55] the latter of which was shown to be TRP channel dependent, suggesting that altered calcium regulation may be a common mechanism shared among visceral pain disorders.

Peripheral neuroinflammatory changes likely contribute towards enhanced vaginal sensitivity in NVI mice. Increased TRPA1 protein levels within the vagina were observed, despite no changes in protein or functional expression within the DRG. This observed increase could partially be attributed to enhanced expression on peripheral terminals within the tissue, as TRPA1 is exclusively expressed by TRPV1- and neuropeptide-expressing neurons,[26,61] which have been shown to be more prevalent in biopsies from patients with vulvodynia,[66,67] IBS,[1] and interstitial cystitis/painful bladder syndrome (IC/PBS).[65] Pharmacologic disruption of TRPA1 signaling has been shown to attenuate visceral hypersensitivity in rodent models of IBS,[69,72] IC/PBS,[21] and both acute and chronic pancreatitis,[54,55] indicating that TRPA1 plays a pivotal role in a wide range of visceral pain syndromes. Increased nerve–mast cell proximity has also been observed in biopsies from vulvodynia,[30,40] IC/PBS,[38,64] and IBS[62] patients and has been postulated to drive the increased sensitivity in the affected tissues.

A history of early-life adverse events has been routinely shown to increase HPA axis output in both human patients and rodent models.[47,70] In this study, increased serum CORT, histologic evidence of increased extruded mast cell contents, and enhanced CRF_1 protein expression in the vagina all indicate an increase in HPA axis output resulting from NVI. Indeed, antagonizing CRF_1 at the time of neonatal insult abolished both vaginal and hind paw hypersensitivity, as well as the increase in CRF_1 protein level and histologic evidence of mast cell degranulation in the vagina. The reversal of hind paw sensitivity by CRF_1 antagonism differs from a similar study that reported a reversal of colorectal sensitivity, but not hind paw thermal hyperalgesia, in adult rats that were treated with the CRF_1 antagonist, antalarmin, before neonatal gastric suctioning.[59] Considering the consistency with which we have observed comorbid hind paw hypersensitivity in mouse models of early insult or stress,[13,49] it will be important to determine how both potential convergence at the level of the spinal cord and HPA axis dysfunction may contribute towards the development of somatic functional pain disorders, such as fibromyalgia and temporomandibular joint disorder, that are commonly diagnosed alongside chronic pelvic pain disorders.[3,4,8] To preserve NVI-induced changes within the HPA axis, the mice used in this study were not subjected to daily vaginal smears to determine estrous cycle. Preliminary work in our laboratory (data not shown) and from others[10,44] has shown that experimental treatment intended to increase visceral sensitivity is sufficient to negate any effect of the estrous cycle.

Finally, many of the observations made in this study, and variations from previous studies of neonatal colon irritation, may be specific to the use of zymosan as the neonatal irritant. Zymosan was chosen for this study not only because of its use in neonatal bladder irritation studies[19,20,51] but also because it is a clinically relevant choice due to the recent rise in *Candida* infections in young children,[36] which has been attributed to both the increasing overuse of antibiotics[7] and infection after hospital-borne *Candida* exposure in the neonatal intensive care unit.[58] A study by Farmer et al.[27] reported that repeated (<6) weekly intravulvar injections of zymosan produced long-lasting vulvar hypersensitivity in the majority of treated mice, in a similar manner as repeated intravaginal inoculation with *Candida*. Previous studies of neonatal colon irritation used known TRP channel agonists, namely acetic acid and MO, to induce the neonatal irritation, and observed increased expression of the respective TRP channels in adult DRG.[13,71] Intracolonic zymosan has been shown to increase colorectal sensitivity when administered in adult mice and genetic deletion of TRPV1 diminished, but did not completely abolish, the increase in VMR,[37] suggesting that zymosan-induced visceral hypersensitivity may partially occur through a non-TRP receptor-mediated mechanism. Previous studies of neonatal bladder irritation using zymosan reported increased bladder weight, plasma extravasation, and neuropeptide content but no significant change in tissue histology, eosinophil infiltration, or mast cell infiltration or degranulation within either the bladder or colon of adult rats.[20,44] These results, along our observation of increased mast cell degranulation, suggest that intravaginal zymosan likely produced long-lasting neurogenic inflammation in the vaginas of NVI mice.

In conclusion, this study has provided evidence that, similar to the gastrointestinal and urinary systems, the reproductive system is vulnerable to early life insult. Both visceral and somatic secondary hypersensitivity was observed in NVI mice, suggesting that the consequences of NVI were not confined to the primarily affected organ. Depolarization-evoked calcium transients were increased in primary sensory neurons innervating the vagina, and, importantly, antagonizing CRF_1 at the time of insult prevented

both vaginal and hind paw hypersensitivity, as well as attenuated peripheral indicators of increased HPA axis output. Considering the extensive diagnostic overlap among chronic pelvic pain disorders, as well as with other somatic pain disorders, such as migraine and fibromyalgia, and mood disorders, taking inventory of a patient's early life history could provide valuable insight as to how best to treat comorbid symptoms arising from multiple diagnoses.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Supplemental Digital Content

A supplemental video to accompany this article is available online at http://links.lww.com/PAIN/A164.

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