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Prostaglandin Levels, Vaginal Innervation, and Cyst Innervation as Peripheral Contributors to Endometriosis-Associated Vaginal Hyperalgesia in Rodents

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Abstract

Endometriosis is a painful condition characterized by growth of endometrial cysts outside the uterus. Here, we tested the hypothesis that peripheral innervation and prostaglandin levels contribute to endometriosis-associated pain. Female Sprague-Dawley rats (n=16) were surgically instrumented by transplanting uterine tissue onto mesenteric arteries within the pelvic cavity to create a model of endometriosis which forms extra-uterine endometrial cysts and **vaginal hyperalgesia**. Our results describe a significant positive correlation between endometriosis-induced vaginal hyperalgesia and cyst innervation density (sensory, $r=0.70$, $p=0.003$; sympathetic, $r=0.55$, $p=0.03$), vaginal canal sympathetic innervation density ($r=0.80$, $p=0.003$), and peritoneal fluid levels of the prostaglandins PGE2 ($r=0.65$, $p=0.01$) and PGF2 α ($r=0.63$, $p=0.02$). These results support the involvement of cyst innervation and prostaglandins in endometriosis-associated pain. We also describe how sympathetic innervation density of the vaginal canal is an important predictor of vaginal hyperalgesia.

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Author contributions

S. McAllister performed behavioral experiments, immunohistochemistry, analyzed and interpreted data, and wrote the manuscript. E. Faircloth and B. Giourgas helped process and analyze tissue samples and edited the manuscript. E. Leishman and H. Bradshaw analyzed peritoneal fluid samples with mass spectrometry and edited the manuscript. E. Gross contributed significantly to the writing of the manuscript.

Conflict of interest statement

There are no conflicts of interest to report.

Keywords

endometriosis; cyst; innervation; prostaglandin; vaginal hyperalgesia; pelvic pain

1. Introduction

Endometriosis is an estrogen-dependent condition characterized by extrauterine endometrial growths known as cysts. This condition affects approximately 10% of premenopausal women. Of those affected, 30-50% have severe pelvic pain (Giudice and Kao, 2004; Rogers et al., 2009). Painful symptoms include dyspareunia (vaginal hyperalgesia), dysmenorrhea (menstrual pain), dyschezia (pain on defecation), and chronic pelvic visceral and muscle pain; all which greatly reduce quality of life. Within the pelvic cavity, where endometriosis primarily occurs (Giudice and Kao, 2004; Stegmann et al., 2009), endometriosis can be subdivided into three categories: superficial peritoneal endometriosis, deeply infiltrating endometriosis, and ovarian (cystic) endometriosis (Ferrero et al., 2015; Raffi and Amer, 2011). In carefully documented studies, the location and amount of ectopic growth does not correlate with the presence or severity of pain symptoms except for that of deep infiltrating endometriosis (Fauconnier and Chapron, 2005; Stratton and Berkley, 2011; Vercellini et al., 2007). Hence, endometriosis is an “enigma” since how painful symptoms become associated with the condition is unclear. Due to this lack of knowledge, available pain treatments often only provide temporary relief, produce unwanted side effects, or are ineffective.

Further, similar to women, the amount of ectopic growth in endometriosis rats fails to correlate with symptom presence and pain severity; however, ectopic growth excision can provide long-term relief of painful symptoms (Alvarez et al., 2014; McAllister et al., 2009) suggesting **some** aspect of the growth contributes to the pain. One possibility is that the growth's sprouted sensory and sympathetic nerve supply opens a two-way line of communication between the growths and CNS that is capable of generating pain (Stratton and Berkley, 2011). Studies in the rodents (Alvarez et al., 2012; McAllister et al., 2009, 2012) and a few clinical studies support this hypothesis (Di Spiezio Sardo et al., 2015; Mechsner et al., 2009; Wang et al., 2010).

In women, the most commonly used drugs for the treatment of endometriosis-associated pain are nonsteroidal anti-inflammatory drugs (NSAIDs) hypothesized to work by suppressing prostaglandin levels via inhibition of cyclooxygenases (COX-1 and COX-2) that oxidize arachidonic acid (AA) (Streuli et al., 2013; Wu et al., 2010). However, NSAIDs must be used with caution due to their ability to produce significant adverse side effects including gastrointestinal bleeding, impaired renal function and interactions with other medications, making this a sub-optimal treatment systemically (Dhasmana et al., 2014). In women with endometriosis, peritoneal fluid levels of prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2α) are significantly greater than in women without the condition (Dawood et al., 1984; Karck et al., 1996; Wu et al., 2010). These prostaglandins are potent nociceptive activators (Ray et al., 2015; Smith, 2006) key mediators in pain and nociception (Lousse et al., 2012), and likely influence cyst innervation directly or indirectly to contribute to endometriosis-associated pain.

In this study, using a rat model of endometriosis where a piece of uterine horn (~1 cm) is removed and cut into four equal pieces (~2mm × 2mm) followed by transplantation onto cascading mesenteric arteries within the rat peritoneal cavity (Vernon and Wilson, 1985). These uterine transplants over time produce innervated and vascularized cysts (Berkley et al., 2004; 2005) and symptoms similar to women including vaginal hyperalgesia (Berkley et al., 2007; McAllister et al., 2009, 2012) referred abdominal muscle hyperalgesia (Giamberardino et al., 2002; Nagabukuro and Berkley, 2007), and bladder hyperactivity (Morrison et al., 2006). Therefore, we examined the relationships between vaginal hyperalgesia, cyst sensory and sympathetic innervation density, and peritoneal fluid levels of PGE2 and PGF2 α 4-6wks after surgery when individual differences in hyperalgesic severity are greatest (McAllister et al., 2012). Further, we also analyzed the innervation density of the eutopic uterus as well as the vaginal canal. Since endometriosis-induced vaginal hyperalgesia was previously shown to be estrous-stage dependent we also examined how our results are influenced by estrous stage during development of endometriosis (Cason et al., 2003).

2. Materials and Methods

2.1. General description of rodents

Sixteen adult virgin female Sprague Dawley rats (175-200 g at arrival; Charles River, Raleigh, NC) were used in this experiment. Animals were housed individually in plastic cages lined with chip bedding and *ad libitum* access to rat chow and water. Housing was in environmentally controlled conditions (room temp temperature ~22 °C; 12-h light/dark cycle, with lights on at 07:00). This study conformed to the NIH Guidelines for the Care and Use of Laboratory Animals. The Florida State University Institutional Animal Care and Use Committee approved the experimental protocols of this study as #9028, #1212, and #0913.

In all rats, reproductive status was assessed daily by vaginal lavage ~2h after lights on. Traditional nomenclature was used for the four rat estrous stages of proestrus, estrus, metestrus, and diestrus (McLean et al., 2012). All rats maintained normal four-day estrous cycles throughout the study. All behavioral assessments (training and testing) were done ~3-8 h after lights on.

2.2. Surgical induction of endometriosis (endometriosis)

Endometriosis was induced following the protocol develop by Vernon and Wilson (1985). Briefly, using aseptic techniques, rats in diestrus were anesthetized intraperitoneally with a mixture of ketamine hydrochloride (73 mg/kg) and xylazine (8.8 mg/kg) and placed on a heating pad to maintain body temperature ~37°C. A midline abdominal incision was made to expose the uterus and a ~1-cm segment of the left uterine horn and associated fat tissue were removed and placed in warm sterile saline. The uterine horn, including the endometrium and myometrium, was then cut into 4 equal pieces (~ 2 mm × 2 mm) and sewn (4.0 nylon suture) onto cascading mesenteric arteries within the pelvic cavity that supply the caudal small intestine beginning at the cecum. The incision was closed in layers. Rats were monitored closely after surgery for potential complications. The postoperative recovery

period was uneventful, regular estrous cyclicity resumed within a few days, and assessment of vaginal nociception resumed ~1 wk after surgery.

2.3. Behavioral assessment of vaginal nociception

The behavioral training, testing, and assessment procedures were identical to those described in detail previously (McAllister et al., 2009, 2012). Rats were trained to perform an escape response to terminate vaginal distention produced by an inflatable latex balloon. During each testing session, eight different distention volumes were delivered three times each at random ~ 60 sec apart and the percent escape response to each volume given was assessed.

2.3.1. Behavioral apparatus and stimulator—The training and testing apparatus was grill-floored Plexiglas® chamber allowing movement but prevent the rat from turning around. In the front of the chamber, a hollow tube is extended containing a light-emitting diode and photo sensor. When a rat extended her nose into this tube, a light beam is broken and the stimulus is terminated. In other words, the rat breaking the light beam constituted an escape response. An opening in the rear of the chamber allowed the catheter (attached to the vaginal stimulator) to be connected to the computer-controlled stimulus-delivery device.

The vaginal stimulator consisted of a small latex balloon (~ 10mm long × 1.5 mm wide when uninflated) tied to a thin catheter with silk suture. Immediately prior to the training or testing session, the uninflated balloon was lubricated with K-Y® jelly, inserted into the midvaginal canal, and located to ensure the cervix was not touched (even when inflated). The vaginal canal was then distended by the delivery (computer-controlled) of different volumes of water to the balloon. A small-volume Cobe pressure transducer measured the pressures produced by the volumes of distention (corrected for compliance characteristics of the balloon).

2.3.2. Behavioral training—Rats were first adapted to the testing chamber by being placed in the box for 10 min daily for 3-4 days. Then, rats were then trained to perform an “escape response” which involves the rat extending her head into the hollow tube to interrupt the light beam. To do this, the trainer pinches the rat's tail with padded forceps and used a forcep release to shape the required “escape response.” These training sessions, done 3/week on non-consecutive days, consisted of 10 tail “pinches” delivered at ~1min-intervals. This “tail pinch” training was completed (>80% escape behavior) in 3-5 sessions.

Rats were then trained to make an identical escape response to terminate vaginal distention stimuli (the balloon). In these sessions, ten large distention volumes (0.80 ml – 1.0 ml, inflation rate 1 ml/s) were delivered for a maximum of 15 s at ~1-min intervals. All rats showed some behavioral response to these stimuli, which allowed the experimenter to use balloon deflation to shape the rat's escape response. This “balloon training” was done 3/wk on non-consecutive days and completed in 3-5 sessions. Once trained, baseline assessment of vaginal nociception began.

2.3.3. Behavioral testing procedures—Assessment of vaginal nociception was performed in 1 hr long testing sessions in which 24 computer-controlled escape trials were run at ~1-min intervals (range 50 – 70 s). Briefly, eight different distention volumes,

including a control volume (0.01 ml), were delivered to the balloon (in the rat's vaginal canal) three times each in random order. Each volume delivery rapidly inflated the balloon (1 ml/s) until the rat made an escape response or the maximum latency of 15 s was reached. Then, the balloon rapidly deflated (0.5 ml/s). The computer recorded stimulus volume, stimulus pressure, and response latency for each trial. The maximum latency of 15 s was considered no response. The experimenter was blinded to the volumes being delivered to the rat vaginal canal. Testing sessions were run 3 to 4 times/wk on non-consecutive days.

2.4. Data analyses of behavioral results

2.4.1. Calculation of vaginal nociception—Vaginal nociception was measured by percent escape response as a function of distention volume in each testing session. For each rat, 12 testing sessions were performed both during the baseline period and the post-endometriosis period for a total of 24 testing sessions. Escape percentages from all sessions within a testing period (baseline or post-endometriosis) were combined and the mean values calculated and entered into a spreadsheet. This procedure produced a graph of the percent escape response as a function of distention volume for each testing period. These data were then used in the calculation of the area-under-the-curve (AUC) (see below).

2.4.2 Calculation of area-under-the-curve (AUC) and severity of hyperalgesia—

Area-under-the-curve (AUC) calculations were carried out using standard, trapezoid rule methods (Yeh, 2002). This calculation yielded a single value of “AUC units” that provided an estimate of vaginal nociception during both testing periods. Differences in the AUC between baseline and post-endometriosis testing periods were then calculated for each rat, creating a single number for change in nociception reflecting the “severity of hyperalgesia”. Baseline and post-endometriosis AUC data was then combined for the group and the statistical differences between AUCs were assessed by Student *t*-tests.

2.4.3. Additional analyses of vaginal nociception by estrous stage—The escape response data from this group was also analyzed by estrous stage (proestrus, estrus, metestrus, and diestrus) and the AUCs (baseline and post-endometriosis) calculated for each stage. Within each rat's 12 testing sessions during baseline and 12 testing sessions post-endometriosis, 3 testing sessions were performed in each of the 4 estrous stages. For each rat, the estrous stage average was found at baseline and then post-endometriosis. Then, these averages were combined and averaged for the group.

2.5 Biochemical Analysis

2.5.1 Immunohistochemistry—At the time of sacrifice, in proestrus, rats were first anesthetized with urethane (1.2 g/kg), then underwent trans-cardiac exsanguination, first with saline and then followed by perfusion with 4% paraformaldehyde. The ectopic uterus (cysts), eutopic (uninjured uterus), and vaginal mid-canal (region stimulated by vaginal balloon) were then harvested and post-fixed in 4% paraformaldehyde for 1 h then incubated in 30% sucrose overnight. Tissue was embedded in Histo Prep freezing medium (Fisher Scientific), frozen, and cut serially in 20µm-thick sections using a cryostat, and mounted on slides in 10 sets of sections (i.e., sections on a slide were separated by 200 µm). Adjacent sections for the same cysts, eutopic uterus, or vaginal canal were processed. The fixed tissue

sections were quenched with 0.3% H₂O₂ in phosphate buffered saline (PBS) for 1 h and then blocked in 0.3% Triton X-100 in PBS with 5% horse serum (HS) for 1 h. Sections were immunostained with one of the following: goat anti-tyrosine hydroxylase (TH; 1:800, Millipore, Temecula, CA), rabbit anti-calcitonin gene related peptide (CGRP; 1:10,000; Chemicon) in 0.3% Triton X-100 in PBS including 2% HS for 2 h at room temperature (RT) followed by 4 °C overnight. The next day, sections were washed in PBS and incubated in biotinylated goat anti-rabbit (or horse anti-goat) IgG (Vector Labs) at RT followed by incubation in ABC (Vector Laboratories). Staining was visualized with 3, 3'-diaminobenzidine (DAB kit, Vector Laboratories). For each antibody, the final dilution used (as specified above) was previously determined in test sections that yielded the maximum labeling of neurites with minimal background. Controls included omission of the primary antibody, omission of the secondary antibody, and omission of both the primary and secondary. There was no labeling in any of the control sections.

2.5.2. Quantification of nerve fiber density—After processing, the cyst, uterine, and vaginal samples were examined microscopically for evidence of positive antibody labeling. A Likert-type scale of 0–4 was established to quantify the labeling density, with 0 indicating no fiber labeling and 4 indicating the densest possible labeling. To establish what constituted the 0–4 range, all cyst sections stained with the TH marker were first examined by three experimenters (SM, BF, BG). Then, sections through each sample were assigned an overall score from 0 to 4, using 0.5 intervals. Scores were then assigned to the CGRP-labeled cyst sections using the scale established with the TH marker.

This same protocol was followed for the uterine and vaginal canal samples. Scores from all investigators were averaged for each tissue. For all cysts found in each rat at the time of sacrifice (this number varied), three sections were analyzed per cyst sample and a TH and CGRP score assigned. The three section scores per cyst sample were then averaged. The average for all cysts within a rat were then combined and averaged to produce a mean score for that rat. For the eutopic uterine horn and vaginal canal, one sample was collected per rat, with the experimenter being careful to consistently collect the same “middle” region of the tissue to control for changes in innervation density present in the proximal and distal portions of these tissues. For each uterine horn and vaginal canal sample, three sections were analyzed, scored, and then averaged for each rat. Three investigators assessed all sections of each sample. Overall, the scores of the three experimenters were highly correlated, with $r > 0.9$ for all scores.

2.5.3. Calculation of the cyst burden—When cysts were located at the time of sacrifice, they were freed from surrounding fat and connective tissue, and the largest and smallest diameter measured to allow calculation of the rat's “cyst burden.” This calculation involved multiplying the largest and smallest diameters of each cyst and then adding the values from each cyst to obtain a total number (Nagabukuro and Berkley, 2007). For example, if a rat had four cysts with the following large and small diameters: $4 \times 2\text{mm}=8$; $3 \times 1\text{mm}=3$; $5 \times 2\text{mm}=10$; $6 \times 2\text{mm}=12$, adding the multiplicands of $8+3+10+12$ together yielded a total cyst burden of 33 for that rat.

2.5.4. Peritoneal fluid collection and (Tandem) Mass Spectrometry—Lipid extraction was performed on peritoneal fluid samples as previously described (Leishman 2016a,b). In brief, samples were placed in 5 mL of HPLC-grade methanol (Avantor Performance Materials, Center Valley, PA, USA) then spiked with 500 picomoles deuterium-labeled *N*-arachidonoyl ethanolamine (d8AEA; Cayman Chemical, Ann Arbor, MI, USA), as an internal standard. Samples were placed on ice in darkness for 2 hours and were then centrifuged at 19,000g for 20 minutes at 20°C. Supernatants were decanted and diluted with HPLC water (purified in house) to make a 75:25 water to supernatant solution. Partial purification was achieved using C-18 solid phase extraction columns (Agilent, Palo Alto, CA, USA). A series of 4 elutions with 1.5 mL of 60%, 75%, 85%, and 100% methanol were collected for analysis. Samples were analyzed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer with electrospray ionization (Foster City, CA, USA). 20 µL from each elution were chromatographed using XDB-C18 reversed phase HPLC analytical column (Agilent) and optimized mobile phase gradients. Mobile phase A: 20% / 80% (v / v) methanol / water and 1 mM ammonium acetate (Sigma, St. Louis, MO, USA). Mobile phase B: 100% methanol, 1 mM ammonium acetate. Two Shimadzu 10ADvp pumps (Columbia, MD, USA) provided the pressure for gradient elution.

Samples were screened for the lipids PGE2 and PGF2α. Levels of both compounds were determined by running each sample using a multiple reactions monitoring (MRM) method as previously described (Leishman et al., 2016 a,b). Analysis of the HPLC/MS/MS data was performed using Analyst software (Applied Biosystems, Framingham, MA, USA) as previously describe. Lipid concentrations measured in picomoles per gram were adjusted for the extraction's percent recovery.

2.5.5. Statistical Analysis—For correlational analysis, Spearman's rank correlation coefficient (Spearman's rho) was used to assess the relationship between the following: severity of endometriosis-induced vaginal hyperalgesia (in proestrus), number of cysts, total cyst burden (CB), density of sensory and sympathetic innervation of the ectopic cysts, eutopic uterus, and vaginal canal, and the peritoneal fluid levels of PGE2 and PGF2α. For all other statistical analysis, Student t-tests and 1-way or 2-way (with or without repeated measures) analysis of variance (ANOVA) followed by Bonferroni post-test was performed using Statistical Package for the Social Sciences software, version 19 (SPSS, Chicago, IL) or GraphPad Prism (La Jolla, CA), as denoted in the manuscript. These data are expressed as mean ± SEM. Significance was set at $p < 0.05$.

3. Results

3.1. Analysis of endometriosis-induced vaginal hyperalgesia

When vaginal nociception was analyzed by group (n=16) with all estrous stages combined, we found that significant vaginal hyperalgesia developed post-endometriosis when compared to baseline (**Fig. 1A**). When the group data was further analyzed by estrous stage; for example, when baseline vaginal nociception during proestrus was compared with vaginal nociception post-endometriosis during proestrus, no significant differences were found (**Fig. 1B**). This was true for all of the four estrous stages (**Fig. 1B**). No significant variability in

baseline vaginal nociception was found within the group ($p = 0.82$) that could later predict an increased response to vaginal distention after endometriosis surgery.

3.2. Lack of correlation between endometriosis-induced vaginal hyperalgesia and cyst burden

There was no correlation between the number of cysts present within the peritoneal cavity and the severity of vaginal hyperalgesia (**Fig. 2A**, $r = 0.39$, $p = 0.13$). Further, consistent with previous findings, there was no correlation found between total cyst burden and the severity of vaginal hyperalgesia (**Fig. 2B**, $r = 0.19$, $p = 0.47$) (McAllister et al., 2009, 2012; Nagabukuro and Berkley, 2007).

3.3. Correlation between endometriosis-induced vaginal hyperalgesia and innervation density of the ectopic uterus (cyst), eutopic uterus, and vaginal canal

Representative examples of histological staining from the endometrial cysts are shown for both TH and CGRP-stained cysts (**Fig. 3**). A significant positive correlation was found between the severity of endometriosis-induced vaginal hyperalgesia and the density of the sensory innervation of the cysts (**Fig. 4A**, top panel; $r = 0.70$, $p = 0.003$), sympathetic innervation of the cysts (**Fig. 4A**, bottom panel; $r = 0.55$, $p = 0.03$), and the sympathetic innervation of the vaginal canal (**Fig. 4C**, bottom panel; $r = 0.80$, $p = 0.0003$). This was in the presence of no correlation between severity of endometriosis-induced vaginal hyperalgesia and sensory innervation of the eutopic uterus (**Fig. 4B**, top panel), sympathetic innervation of the eutopic uterus (**Fig. 4B**, bottom panel), or the density of the sensory innervation of the vaginal canal (**Fig. 4C**, top panel).

3.4. Correlation between endometriosis-induced vaginal hyperalgesia and peritoneal fluid levels of PGE2 & PGF2 α

We next analyzed whether prostaglandin levels correlated with the severity of vaginal hyperalgesia. There was a significant positive correlation between severity of vaginal hyperalgesia and peritoneal levels of PGE2 (**Fig. 5A**; $r = 0.65$, $p = 0.01$) and PGF2 α (**Fig. 5B**; $r = 0.63$, $p = 0.02$).

3.5. Correlation between peritoneal fluid levels of PGE2 & PGF2 α and innervation density of the ectopic uterus (cyst), eutopic uterus, and vaginal canal

We next analyzed prostaglandin levels in the peritoneal fluid of these rodents, including PGE2 and PGF2 α . We found a strong positive correlation between the density of the sensory innervation of the cysts and the peritoneal fluid levels of PGE2 (**Fig. 6A**, top panel; $r = 0.55$, $p = 0.05$) but not PGF2 α (**Fig. 6B**, top panel; $r = 0.36$, $p = 0.22$). There was no correlation between the density of the sympathetic innervation of the cysts and peritoneal fluid levels of PGE2 (**Fig. 6A**, bottom panel; $r = 0.31$, $p = 0.31$) or PGF2 α (**Fig. 6B**, bottom panel; $r = 0.24$, $p = 0.42$).

4. Discussion

Together, these findings support the contribution of the sensory and sympathetic innervation of the endometrial cysts and sympathetic innervation of the vaginal canal to the development

of endometriosis-induced vaginal hyperalgesia. This is independent of estrous stage or innervation of the eutopic uterus. Increased levels of PGE and PGF2 α in the peritoneal fluid were also associated with increased vaginal hyperalgesia and PGE was also found to correlate with the sensory innervation of the endometrial cysts. Further, these findings are the first to show that the vaginal canal sympathetic innervation is significantly altered by a rodent model of endometriosis.

4.1 Ectopic uterus (cyst) innervation and endometriosis-induced vaginal hyperalgesia

Previous studies show that, after endometriosis surgery, cyst innervation appears within the cysts at 2 weeks but does not become active until 3 weeks. This is important because once active, the innervation is capable of generating the significant vaginal hyperalgesia that occurs one week later at 4 weeks (McAllister et al., 2009, 2012). The innervation and hyperalgesia then further increase in parallel, peak, and become established by 8 weeks after endometriosis surgery (McAllister et al., 2009, 2012). If cysts are removed prior to appearance of the innervation within the cysts, then the development of hyperalgesia is prevented; whereas, cyst removal after innervation and hyperalgesia are established, eliminates hyperalgesia (McAllister et al., 2009, 2012). Further, once cyst innervation and hyperalgesia are established and do not further increase, estrous cycle dependent fluctuations do occur in vaginal hyperalgesia and cyst innervation. For example, there is a significant decrease in vaginal hyperalgesia from proestrus to estrus that is paralleled by a significant decrease in sympathetic innervation of the cysts (Zhang et al., 2008). Further, growth factors such as nerve growth factor capable of influencing the cyst sensory innervation also significantly decrease from proestrus to estrus (Zhang et al., 2008). Together, the previous findings, which suggest cyst innervation contributes to endometriosis-induced vaginal hyperalgesia, are further supported by results in this study, in which the density of both the sensory and sympathetic innervation of the cysts strongly correlate with hyperalgesia during development.

In women with endometriosis, the relationship between ectopic growth innervation and endometriosis-associated pain is unclear. Studies have shown that women with endometriosis and pain have growths with greater nerve fiber density than woman with endometriosis without pain (Di Spiezio Sardo et al., 2015; Yao et al., 2010). In another study, a positive correlation was found between pain severity and cyst nerve fiber density (Mechsner et al., 2009). Specific support for cyst sensory and sympathetic innervation in endometriosis-associated generalized hyperalgesia has also been found (Chen et al., 2014). Our findings in the rat are similar to these clinical studies; however, other clinical studies have found no correlation between endometrial ectopic growth innervation and endometriosis-associated pain (Al-Fozan et al., 2004; Tulandi et al., 1998).

4.2. Eutopic uterus innervation and endometriosis-induced vaginal hyperalgesia

In naïve rats, the sympathetic innervation of the eutopic uterus fluctuates during the estrous cycle with innervation degenerating as estrogen increases and regenerating as estrogen levels rise so that maximal fiber density is seen at diestrus and minimal density seen at estrus; whereas, sensory innervation remains unchanged (Zoubina and Smith, 2001). Here, in rats with endometriosis and developing hyperalgesia, no significant correlation was found

between the eutopic uterus sensory or sympathetic innervation density and hyperalgesia in proestrus. In rats with endometriosis and established hyperalgesia, the eutopic uterus sensory and sympathetic innervation density is not significantly different from that of naïve rats or rats with sham-endometriosis (McAllister et al., 2014) in proestrus. These findings in rats, suggest eutopic uterus innervation is not involved with endometriosis-induced vaginal hyperalgesia. However, here, when the difference in uterus innervation was analyzed between the eutopic and ectopic uterus (cysts) and was correlated with the severity of vaginal hyperalgesia during development, a significant positive correlation was found. This suggests that from the time of uterine auto-transplant to rat sacrifice, a change in innervation occurred that potentially contributed to the hyperalgesia.

Clinically, some studies have found an appearance of or an increase in the innervation of the eutopic uterus associated with endometriosis pain (Al-Jefout et al., 2009; Bokor et al., 2009; Quinn and Armstrong, 2004). However, in women with peritoneal endometriosis no correlation was found between the presence of nerve fibers within the eutopic uterus and symptoms (Leslie et al., 2013; Zevallos et al., 2015). In another study, no statistical differences were found between eutopic uterus innervation density in women with endometriosis and pain versus other conditions, such as adenomyosis or uterine fibroids and pain (Zhang et al., 2010). Although the contribution of the eutopic uterus innervation is not clear, agreeably the biochemical, functional, and genetic differences of the cells of the eutopic endometrium in women with endometriosis are increasingly gaining recognition for their role in endometriosis-associated pain (Carvalho et al., 2011).

4.3. Vaginal canal innervation and endometriosis-induced vaginal hyperalgesia

In naïve normal cycling rats and pre-menopausal women, the number and density of sensory and sympathetic fibers within the vagina are similar and estrous-stage dependent fluctuations in nerve fiber density do not occur, nor do changes in vaginal sensitivity (Liao and Smith, 2011; Ting et al., 2004). In rats with a sham-endometriosis (fat surrounding uterus auto-transplanted instead of the uterus) surgery, no changes in vaginal sensitivity occur either. However, in endometriosis rats, as previously mentioned, significant vaginal hyperalgesia develops and then stabilizes. When the sensory and sympathetic innervation of the vaginal canal was compared between rats with stabilized endometriosis-induced vaginal hyperalgesia, sham-endometriosis, or naïve rats no significant differences in innervation were seen (McAllister et al., 2014) suggesting the group differences in vaginal hypersensitivity could not be explained by innervation.

However, in this study, a strong positive correlation was found between vaginal canal sympathetic innervation density and endometriosis-induced vaginal hyperalgesia during development, when hyperalgesic severity is highly variable. Prior to this, no direct correlation between vaginal canal sympathetic innervation and endometriosis-associated hyperalgesia in women or rats was found.

In non-pathological conditions, the main function of the vagina's sympathetic innervation is regulation of vaginal blood flow by modulation of noradrenergic vasoconstriction (Munarritz et al., 2003); i.e., a decrease in sympathetic innervation yields an increase in vaginal blood flow and increased lubrication (Kim et al., 2004). However, if vaginal innervation is aberrant

it can become abnormally-responsive (Quinn, 2009). Thus, previously “normal” blood flow increases within blood vessels can now act as mechanical stimuli activating the aberrant innervation and generate region specific painful symptoms (Quinn, 2009).

In rats, surgical menopause or ovariectomy (OVX) was found to increase both the sensory and sympathetic innervation of the vagina; whereas, estrogen replacement returned vaginal innervation to pre-OVX levels (Griebing et al., 2012). Further, OVX rats with endometriosis-induced vaginal hyperalgesia that were then supplemented with estrogen systemically, had an alleviation in vaginal hyperalgesia that returned to baseline levels. Together, these findings suggest that the aforementioned return of vaginal innervation to pre-OVX levels after estrogen supplementation could be involved in the alleviation of vaginal hyperalgesia (Berkley et al., 2007).

Similarly, in post-menopausal women with vaginal discomfort including pain, the use of hormone therapy (both systemic and topical) reduces both sensory and sympathetic innervation and provides pain relief, also suggesting vaginal innervation contributes to vaginal pain (Griebing et al., 2012). These combined findings, in women and rats, suggest that estrogens are capable of influencing vaginal innervation thereby affecting endometriosis-induced vaginal hyperalgesia, but the mechanism is unclear, especially while the innervation and associated hyperalgesia are developing and not yet stable.

4.4. Peritoneal fluid prostaglandins and endometriosis-induced vaginal hyperalgesia

This study suggests a significant role for prostaglandins in the pathophysiology of endometriosis and its associated pain, similar to findings in other studies (Ray et al., 2015; Sacco et al., 2012; Wu et al., 2010). Here, a significant positive correlation was found between peritoneal fluid levels of PGE2 and PGF2 α and the severity of endometriosis-induced vaginal hyperalgesia during development. In women with endometriosis, a chronic pelvic inflammatory condition, there is an increased number, concentration, and activation of peritoneal macrophages within the peritoneal cavity (Karck et al., 1996) and accordingly, significantly elevated peritoneal fluid concentrations of prostaglandins (e.g. PGE2 and PGF2 α). This study also found a significant positive correlation between PGE2 and cyst sensory innervation density suggesting the two may influence one another to generate pain (Devor et al., 1994; Janig and Habler, 2000; Janig et al., 1996). Further supporting the role of prostaglandins in endometriosis-induced vaginal hyperalgesia, rats treated with Indomethacin, a non-selective COX inhibitor, had a reduction in stabilized endometriosis-induced vaginal hyperalgesia (Dmitrieva et al., 2012).

4.5 Analysis of endometriosis-induced vaginal hyperalgesia by estrous stage

Once stabilized, endometriosis-induced hyperalgesia is significantly greater in proestrus relative to other estrous stages (Cason et al., 2003). Therefore, we wanted to determine, if the vaginal hyperalgesia varied by estrous stage during development at ~4-6 wks post-endometriosis, when vaginal hyperalgesia is overall significant but highly variable (McAllister et al., 2012). In this study, no estrous stage dependent differences in endometriosis-induced vaginal hyperalgesia were found during development similar to the findings of other researchers studying primary mechanical hyperalgesia (Alvarez et al.,

2012). These combined findings suggest that estrogen (and potentially other hormones) primarily influence endometriosis-induced vaginal hyperalgesia once it has stabilized rather than during its development.

4.5. Study Limitations

A few limitations within our study should be noted, including that we studied only rats with endometriosis and did not have a naïve control group. However, it is known that naïve rats or rats with sham-endometriosis surgery (fat is auto-transplanted instead of uterus) do not have changes in vaginal nociception or develop vaginal hyperalgesia (Berkley et al., 2007). Further, in naïve rats, the sensory innervation of the vagina and uterus and the sympathetic innervation of the vagina do not fluctuate. However, the sympathetic innervation of the uterus does fluctuate with estrous cycle therefore, we collected all tissue for analysis in the same estrous stage (proestrus). Further, in another study, in which tissue innervation was analyzed at 8 wks post-endometriosis, when vaginal hyperalgesia has already peaked and become established, we found no significant differences between the vaginal or eutopic uterus innervation of the rats with endometriosis compared to rats that were naïve or had sham-endometriosis surgery. If differences in innervation were contributing to vaginal hyperalgesia, we would expect to see for example, an increased tissue innervation in the rats with endometriosis relative to the naïve and sham-endometriosis rats that do not develop hyperalgesia (McAllister et al., 2014).

It should also be noted, that in studying visceral pain in animals, the most commonly used method, is hollow organ distention with responses measured as electromyographic (EMG) activity of the abdominal muscles, labeled visceromotor response (VMR) (Christianson and Gebhardt, 2007). Instead, we used a well-established, robust, and reproducible behavioral method of quantifying stimulus– escape response patterns to vaginal distention (Cason et al., 2004) to provide valuable information in regards to pelvic pain. However, vaginal distention and VMR methods have previously been used in anesthetized rodents (Nagabukuro and Berkley, 2007) and more recently, in awake rodents (Dmitrieva et al., 2012; Pierce et al., 2015) and we may incorporate this method in future studies. We also did not measure the distribution of nerve fibers in the surrounding tissues. However, in light of these limitations, our data still provides valuable information in regards to the pathophysiology of endometriosis-associated pain.

4.6. Potential mechanisms underlying endometriosis-induced vaginal hyperalgesia: a changing role for innervation

If cyst innervation contributes to endometriosis-induced vaginal hyperalgesia, the question then becomes *how*? One likely possibility is that the elevated levels of inflammatory factors in the peritoneal fluid of both rats and women with endometriosis directly or indirectly activate and/or sensitize free nerve endings of peripheral nociceptors within the cysts. Therefore, NSAIDs that suppress prostaglandins likely alleviate endometriosis-associated pain by reducing or eliminating PG activation/sensitization of nerve fibers within the cysts. Findings here, that peritoneal fluid levels of PGE2 significantly correlate with the cyst's sensory innervation density and the severity of endometriosis-induced vaginal hyperalgesia during development, support this idea.

One possible mechanism by which the cyst's increased innervation density may generate pain is via a "coupling" between the sensory and sympathetic fibers (Devor et al., 1994; Janig and Habler, 2000; Janig et al., 1996). This idea proposes that sensory afferent fibers can be sensitized by compounds released by sympathetic terminals, such as prostaglandin PGE2 (Khasar et al., 1998; Michaelis and Janig, 1998). Hence, via "indirect coupling" the elevated levels of PGE2 in women and rats, potentially released by cyst sympathetic fibers, can sensitize sensory fibers and increase peripheral nociceptive input to generate painful symptoms. In fact, sympathetic fibers can produce and release prostaglandins upon norepinephrine (NE) binding to α_2 -adrenergic receptors on sympathetic fibers (Gonzales et al., 1989; Sherbourne et al., 1992; Tracey et al., 1995) and hyperalgesia induced by NE has been shown to be mediated by sympathetic terminal PG release (Levine et al., 1986).

It is also possible that "direct coupling" between the cyst's sensory and sympathetic fibers, in which the fibers are physically close enough in proximity to influence one another, is involved in endometriosis-associated pain. After peripheral nerve injury it has been shown that sensory C-fibers can begin expressing adrenoceptors that can directly be stimulated by NE released from sympathetic nerve terminals (O'Halloran and Perl, 1997; Sato and Perl, 1991). Further, during inflammation, C-fibers can become more sensitive to NE (Banik et al., 2001). Supporting the suggestion of "direct coupling" is the finding that sensory and adrenergic fibers co-localize within peritoneal ectopic endometrial growths of women with endometriosis allowing for interaction between the two fiber types (Tokushige et al., 2007). Once the cysts' nerve fibers are affected by these inflammatory mediators, the fibers themselves can also release other sensitizing agents, for example, the release of substance P and calcitonin-gene-related-peptide (CGRP) by sensory fibers and also prostaglandins by both sensory and sympathetic fibers (Gonzales et al., 1989; Zaidi and Matthews, 2013). These sensitizing agents further increase the activation of immune cells and cause vasodilation and plasma extravasation overall amplifying effects (Maggi, 1995).

5. Conclusions

Overall, these results implicate a changing role for innervation in endometriosis-associated pain, of which during development, prostaglandins may influence cyst innervation and/or vaginal canal sympathetic innervation. A "coupling" between the cyst sensory and sympathetic innervation involving prostaglandins may also contribute to pain during development. However, once endometriosis-induced vaginal hyperalgesia is stabilized, estradiol and growth factors primarily modulate/influence cyst innervation to induce vaginal hyperalgesia.

Clinically, these findings suggest that when women with experience endometriosis-associated painful symptoms that are not dependent on menstrual cycle (non-cyclic pain), endometriosis may be just beginning to develop and NSAIDs, that suppress prostaglandins, may provide optimal pain alleviation. Once endometriosis is established, and painful symptoms become dependent on the hormonal fluctuations within the menstrual cycle (cyclic pain), the most effective treatment for pain alleviation may be medications that put women in a hypoestrogenic state. Results from this study may also help explain why: cyst-removal surgery does not always alleviate endometriosis-associated pain, when cyst-removal

does provide relief it is often only temporary, and why pain can return without the return of the growths. For example, if cysts are surgically removed and pain is not alleviated, then aberrant vaginal canal sympathetic innervation may then be contributing to endometriosis-induced painful symptoms such as dyspareunia. Overall, these findings suggest that developmental stage of the condition should be considered when determining the best treatment strategy for endometriosis-associated pain and further, emphasize the clinical importance of individualized medicine for the treatment of endometriosis-associated pain.

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- PGE2 and PGF2 α levels correlate with endometriosis-associated vaginal hyperalgesia.
- Vaginal canal sympathetic innervation density correlates with vaginal hyperalgesia.
- Cyst innervation in endometriosis correlates with vaginal hyperalgesia.
- Sympathetic-sensory coupling potentially underlies endometriosis-associated pain.

Fig. 1.

For the group (n=16), Vaginal nociception (AUC units) at baseline (black bar) and 4-6wks post-endometriosis (striped bar) analyzed with (A) all estrous stages combined and then (B) divided and analyzed by estrous stage. With all estrous stages combined (A) significant vaginal hyperalgesia developed 4-6wks post-endometriosis compared to baseline. When analyzed by estrous stage (B) no significant differences were found between baseline and post-endometriosis within each estrous stage. ***p = 0.0001 vs. baseline; two-way RM ANOVA and Bonferroni post-test.

Fig. 2. Correlation between hyperalgesic severity and (A) number of cysts and (B) cyst burden at 4-6 wks post-endometriosis. Spearman's correlation found no correlation between hyperalgesic severity (AUC units) and number of cysts or cyst burden.

Fig. 3. Examples of nerve fiber labeling in the ectopic uterus (cyst) with their associated innervation density mean scores. The top panel shows CGRP (sensory) labeling and the bottom panel shows TH (sympathetic) labeling. Calibration bar is 50 μ m.

Fig. 4.

Correlation between vaginal hyperalgesia and CGRP-sensory (top panel) and TH-sympathetic (bottom panel) innervation density of the (A) ectopic uterus (cysts), (B) eutopic uterus, and (C) vaginal canal at 4-6 wks post-endometriosis. Spearman's correlation found a significant correlation between hyperalgesic severity (AUC units) and the innervation density of the cysts (sympathetic and sensory) and vaginal canal (sympathetic only). Asterisks indicate a significant correlation. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

Fig. 5.

Correlation between hyperalgesic severity and peritoneal fluid levels of (A) PGE2 and (B) PGF2α at 4-6 wks post-endometriosis. Spearman's correlation found a significant correlation between hyperalgesic severity (AUC units) and PGE2 (pmol/g) and PGF2α (pmol/g) concentrations. Asterisks indicate a significant correlation. * $p < 0.05$.

Fig. 6.

Correlation between peritoneal fluid levels of (A) PGE2 and (B) PGF2 α and CGRP- sensory (top panel) and TH-sympathetic (bottom panel) innervation density of the endometrial cysts at 4-6 wks post-endometriosis. Spearman's correlation found a significant correlation between PGE2 concentration (pmol/g) and cyst sensory innervation density. Asterisk indicates a significant correlation. *p = 0.05.