Published in final edited form as:

Pain. 2009 December 15; 147(1-3): 255–264. doi:10.1016/j.pain.2009.09.022.

Endometriosis-Induced Vaginal Hyperalgesia in the Rat: Role of the Ectopic Growths and their Innervation

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Abstract

Endometriosis is a painful disorder defined by extrauterine endometrial growths whose contribution to pain symptoms is poorly understood. Endometriosis is created in rats by autotransplanting on abdominal arteries pieces of either uterus (ENDO), which form cysts, or fat (shamENDO), which do not form cysts. ENDO, but not shamENDO induces vaginal hyperalgesia. We tested the hypothesis that the cysts are necessary to maintain vaginal hyperalgesia by assessing the effect of surgically removing them. Complete cyst removal eliminated ENDO-induced vaginal hyperalgesia up to four months post-operatively. Sham-cyst-removal in ENDO rats, in which cysts were not removed, or partial cyst-removal increased the ENDO-induced hyperalgesia. The decreases and increases both took three to six weeks to develop. Changes in ENDO-induced hyperalgesia did not occur in a control group of ENDO rats who had no surgery after ENDO. In a double-surgery control group, neither shamENDO surgery nor a subsequent sham surgery that mimicked “removal” of non-existent cysts influenced vaginal nociception. In a no-surgery control group, vaginal nociception remained stable for > six months. The increases in ENDO-induced hyperalgesia produced by the sham-cyst-removal surgery were smaller in proestrus than in other estrous stages. During the other stages (but not during proestrus), sympathetic innervation of the cysts increased. These results suggest that maintenance of ENDO-induced vaginal hyperalgesia requires continued presence of at least some ectopic endometrial tissue, and that surgical treatment that fails to remove ectopic endometrial tissue can exacerbate the hyperalgesia, possibly due in part to an increase in the cysts’ sympathetic innervation.

Keywords

gynecological pain; vulvodynia; sympathetic fibers; estrous cycle; sensitization; neural sprouting

1. Introduction

Endometriosis is a poorly-understood condition defined by extrauterine endometrial growths (variously called ectopic growths, lesions, cysts, implants). Symptoms include subfertility, severe dysmenorrhea, dyspareunia (vaginal hyperalgesia), dyschezia, and chronic pelvic/abdominal and muscle pains. Endometriosis can also co-occur with other painful conditions, such as interstitial cystitis/painful bladder syndrome, irritable bowel syndrome, migraine

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Conflict of interest

There are no conflicts of interest associated with this study.

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headache, temporomandibular joint disorder, vulvodynia, and more [9,19]. A major clinical problem is that pain symptoms fail to correlate with signs [41]. Some women with extensive ectopic growths report minor or no symptoms; other women with few or no growths report widespread distressing pains. In general, although supportable theories concerning the pathogenesis of the ectopic growths exist, much less is known about how these growths come to be associated with pain symptoms in different individuals [19]. Neither the type, site, temporal pattern, nor, importantly, the severity of pain symptoms correlates meaningfully with the amount or site of ectopic growths [41]. Recently, however, it was found that the ectopic growths in both women and the rat model described below develop their own sensory and sympathetic nerve supply [7,8], suggesting that it is the growths' nerve supply that may be most relevant to the pains [3,45].

Endometriosis is surgically induced in rats by autotransplanting pieces of uterine horn (ENDO surgery), or, in controls, fat on abdominal arteries (shamENDO surgery [42]). The uterine but not fat transplants develop over a period of weeks into cysts with characteristics similar to ectopic growths in women [38]. Furthermore, like women with endometriosis, rats with ENDO are subfertile [38] and exhibit vaginal and abdominal muscle hyperalgesia and bladder hyperactivity [13,33,34]. Moreover, like women with endometriosis, the severity of symptoms in rats with ENDO fails to correlate with the amount of ectopic endometrial growth ("cyst burden" [34]).

Regardless of the extent of the ectopic growths, it still remains uncertain whether their presence is necessary to maintain symptoms. Of relevance to this issue is that one treatment for the pains, performed only if medical hormonal therapy fails, involves surgical removal of the growths. Although surgery in selected patients can relieve pain, it often recurs without necessarily being accompanied by return of ectopic endometrial growth [1,37,39].

The initial purpose of the present study was to test the hypothesis that the cysts are necessary for maintenance of ENDO-induced vaginal hyperalgesia by surgically removing the cysts in rats with ENDO. In one control group, rats with ENDO were subjected to a "sham-cyst-removal" surgery. Because this surgery significantly increased the ENDO-induced hyperalgesia, an additional purpose of the present study was to test the hypothesis that the cysts' innervation contributed to the increased hyperalgesia. This second hypothesis was tested by assessing the cysts' sensory and sympathetic innervation using immunocytochemistry.

2. Methods

2.1. Subjects and vaginal cytology

Subjects were 22 adult virgin female Sprague-Dawley rats obtained from Charles River (Wilmington, MA; Raleigh NC facility). They weighed 175–225 g at the start of the study and were housed individually in a temperature-controlled room (22.2 °C) in plastic cages lined with chip bedding, with *ad libitum* access to rat chow and water. They were maintained on a 12-h light/dark cycle, with lights on at 07:00.

Reproductive status was determined by daily vaginal lavage performed ~2h after lights on for all rats [5]. Traditional nomenclature was used for the four estrous stages of proestrus, estrus, metestrus, and diestrus [5]. All rats maintained normal four-day estrous cycles throughout training and testing. All behavioral training and testing was done ~3–8 h after lights on. The study and procedures were approved by Florida State University's Animal Care and Use Committee as protocol # 9028. Data for rats in control groups 3 and 5 were retrieved and reanalyzed from earlier studies [6,12,13].

2.2. Experimental groups and overview of experimental protocols

There were five main groups, summarized in Fig. 1: Group 1 included rats that underwent ENDO surgery followed by complete-cyst-removal surgery (n = 6). Group 2 included rats that underwent ENDO surgery followed by a sham-cyst-removal surgery (n = 4). Control groups included the following: Group 3 was a control for surgery after ENDO. In this group, rats underwent ENDO surgery and then were tested afterwards, with no subsequent surgery, for the same duration as Groups 1 and 2 (n = 4). Group 4 was a control for two-surgeries. In this group, rats underwent shamENDO surgery followed by a second sham surgery that mimicked cyst-removal surgery (n = 3). Group 5 was a full control for surgery as well as for duration of vaginal nociceptive testing. In this group, vaginal nociception was assessed for a total period of time similar to that in Groups 1–4, but with no surgery ever being performed (n = 3). Two additional rats, originally part of Group 1 (ENDO surgery followed by cyst-removal surgery) were separated from this group and described separately, because at autopsy either unusual pathology or an ectopic cyst was found (details provided below).

Throughout the entire testing period for all rats, escape responses to vaginal distention were assessed in ~1h-testing sessions 3–4 times/wk. As shown in Figs. 1–4, data from all groups were compared between three chronological testing periods: (i) an initial, baseline period, (ii) a post-ENDO or post-shamENDO or middle-testing period, and finally (iii) a post-cyst removal or post-sham removal or late-testing period.

At the end of all testing, each rat was anesthetized with urethane (1.2 g/kg, i.p.). The abdominal and pelvic cavities were opened. First, the uterine horns, ovary, and other pelvic organs were examined. Next, the area where the autotransplants had been sewn was thoroughly investigated to determine if all cysts had been completely removed in the cyst-removal groups, and to identify and measure the cysts *in situ* in the sham-cyst-removal group. When cysts were found, they were freed from surrounding fat and connective tissue, and their largest and smallest diameter measured (most cysts have an ovoid shape [42]) to allow calculation of that rat's "cyst burden." Cyst burden was calculated by multiplying the largest and smallest diameters of each cyst and then adding those values together to obtain a total value [34]. The cysts were harvested, immediately frozen in dry ice, and stored at –80°C. After harvesting the tissue, the rats were sacrificed. As described in detail below, cysts were later immunostained with markers specific for sensory and sympathetic nerve fibers (calcitonin gene related peptide [CGRP] or Substance P [SP] for sensory fibers, and vesicular monoamine transporter 2 [VMAT2] for sympathetic fibers, respectively; [35,44,45]).

2.3. ENDO, shamENDO, cyst-removal, and sham-cyst-removal surgeries

The ENDO and shamENDO surgeries were done using aseptic precautions and following a protocol originally developed by Vernon and Wilson [42]. 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. Four pieces of uterine horn (~2 mm × 2 mm) or, for shamENDO, four similarly-sized pieces of fat were cut from this segment. These pieces (uterine for ENDO, or fat for shamENDO) were sewn around alternate cascade mesenteric arteries that supply the caudal small intestine starting from the caecum using 4.0 nylon sutures. After making sure there was no bleeding in the abdominal cavity, the wound was closed in layers. Rats were closely observed during the postsurgical period for potential complications (none occurred). Postoperative recovery was uneventful, and regular estrous cyclicity resumed in all rats within a few days.

For the cyst-removal and sham-cyst-removal surgeries, the rats were anesthetized and treated during and after surgery in the same way as during and after the ENDO and shamENDO surgeries. An off-midline incision was made to expose the area where the autotransplants had been sewn, and the cysts were located and carefully freed from surrounding fat and connective tissue and measured. For sham cyst-removal surgery, no other manipulation of the cysts was done. For cyst-removal surgery, using fine-tipped forceps, the sutures were carefully untied and removed, and the cysts were cut out using a combination of iris scissors and a small-tipped cautery. Care was taken, using absorbent sterile gauze, to make sure that none of the contents of the cysts leaked into the cavity and that bleeding was contained. In all cases, bleeding was minimal. After assuring that all bleeding was stopped, the wound was closed in layers. Rats were closely observed during the postsurgical period for potential complications (none occurred), and behavioral testing resumed one week later. Regular estrous cyclicity resumed in all rats within a few days, and retesting began ~1 wk after each surgery.

2.4. Behavioral testing procedures

Behavioral training and testing procedures were identical to those described in detail previously [6,10,11,12,13]. 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 in random order at intervals of ~ 60 sec, and percent escape response to each volume was assessed.

2.5. Behavioral apparatus and stimulator

The training and testing apparatus was a small rectangular, grill-floored Plexiglas® chamber designed to contain the rat just enough to prevent her from turning around. A hollow tube containing light-emitting diodes and a photosensor extended from the front of the chamber. If the rat extended its nose into this tube, a light beam was broken that terminated the stimulus. In other words, the rat's 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 and automated stimulus-delivery device.

The vaginal stimulator was 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 and inserted into the mid-vaginal canal, located so that it would not touch the cervix even when inflated. Inflating the balloon with different volumes of water using a computer-controlled pump distended the vaginal canal. The pressure produced by each volume of distention (corrected for compliance characteristics of the balloon) was measured through a small-volume Cobe pressure transducer.

2.6. Behavioral training

After the rat was first adapted to the testing chamber by placing her in it for 10 min daily for 3–4 days (and feeding her small amounts of peanut butter on a wood stick), training sessions began in which the trainer pinched the rat's tail with a padded forceps, using its release to shape a required "escape response," which involved the rat extending her head into the tube to interrupt the light beam. Training sessions of 10 pinches delivered at ~1min-intervals were run 3/week on non-consecutive days. Training was usually completed (>80% escape behavior) in 4–8 sessions.

The rat was next trained to make identical escape responses to deflate vaginal distention stimuli. These sessions were run 3/wk on non-consecutive days for a total of 3–5 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 deflation of the vaginal balloon to shape the rat's escape

responses. All rats learned the escape response within 2–4 sessions. Once trained, testing sessions began.

2.7. Behavioral testing

Testing sessions were run 3 to 4 times/wk on non-consecutive days. Each testing session included a series of 24 computer-controlled escape trials that were run at ~1-min intervals (range 50 – 70 s). Each trial consisted of rapid inflation of the balloon (1 ml/s) to a fixed volume, where it remained until the rat made an escape response or 15 s elapsed, when the balloon rapidly deflated (0.5 ml/s). Eight different distention volumes, including a control volume (0.01 ml), were delivered three times each in random order. The computer recorded stimulus volume, stimulus pressure, and response latency for each trial. The maximum latency of 15 s was considered to be no response. The experimenter was blind to the volumes being delivered to the rat. After the escape trials were run, the rat was given a amount of peanut butter on a wood stick and removed from the testing chamber.

2.8. Data analyses of behavioral results

Percent escape responses and vaginal pressures as a function of distention volume were measured in each session. For each rat, the escape percentages and vaginal pressures from all sessions for each testing period (at least 12 sessions/testing period) were combined, and the mean values for each testing period calculated. For each testing period, sessions included an equal number of sessions in each of the four estrous stages.

We previously found that escape responses after ENDO surgery do not become significantly greater than baseline until one month post-surgery (i.e., the hyperalgesia develops in parallel with the growth of the cysts, which grow most rapidly during the first month, slowing, then stabilizing thereafter [13]). Accordingly, as previously reported [6], data analyzed for testing period 2 (post-ENDO, or post-shamENDO, or middle-testing period) included sessions run beginning 4 wks after ENDO or shamENDO surgery (or no surgery) had been performed (i.e., responses from the first post-operative 4 wks are not included; Fig. 1).

The averages from each of the rats were combined by group and entered into spread sheets. (Data from the two rats with unusual pathology are presented separately.) Statistical analyses were performed using repeated measure ANOVAs followed, if significant, by post-hoc Fishers LSD tests. If conditions differed significantly, one-way ANOVAs were used to determine the significance of differences for each distention volume between the three testing period conditions.

Area-under-the-curve (AUC) calculations were also performed for each rat, using standard trapezoid rule methods. Two overall analyses were initially performed. First, a one-way ANOVA was done to assure that baselines did not differ between groups. They did not ($P=0.429$). Second, a two-way repeated measure ANOVA was done to determine whether the data differed across groups and testing period. This analysis was significant [$F(14,45) = 6.21$, $P < 0.001$], and showed that the AUC values differed significantly by group [$F(4,45) = 5.12$, $P = 0.002$], testing period [$F(2,45) = 13.95$, $P < 0.001$], with a significant group by testing period interaction [$F(8,45) = 4.12$, $P = 0.001$]. Following these initial analyses, differences in the AUC between baseline and testing period 2, and between baseline and testing period 3 were calculated for each rat, then combined by group. Statistical differences between the two sets of AUCs were assessed by Student *t*-tests. Pearson correlation coefficient statistics were done to assess correlations between the cyst burden and AUCs. All statistical analyses were done using Statistical Package for the Social Sciences software, version 15 or 17 (SPSS, Chicago, IL). Significance was set at $P \leq 0.05$.

2.9. Additional analyses of behavioral data over time after surgery and by estrous stage

Previously, we reported that ENDO-induced hyperalgesia develops over a period of weeks, not becoming significant until >1 month after the surgery [13]. Here, we wished to determine how quickly changes in hyperalgesia after cyst-removal in Group 1 or sham-cyst-removal in Group 2 occurred. This analysis was done by calculating AUCs at baseline and at two-week intervals after the surgery, then subtracting the baseline AUC from the post-surgery AUCs to assess the severity of hyperalgesia over time after the cyst-removal or sham-removal surgeries. One-way ANOVAs were performed for each group (which were significant in both cases) and then followed by post-hoc LSD tests to determine when the decreases or increases became significant.

Previously, we reported that the severity of ENDO-induced hyperalgesia was greatest in proestrus compared to the other stages [13]. Here, we wished to determine if the increase in ENDO-induced hyperalgesia that was produced by the sham-cyst-removal surgery in Group 2 (Fig. 2C) varied by estrous stage. Accordingly, the escape response data from this group were separated into two groups, proestrus versus all other stages (estrus, metestrus, diestrus). AUC values for severity of hyperalgesia were calculated and analyzed statistically as described above.

2.10. Immunocytochemical processing and analyses

Cysts were harvested from two groups of rats: following post-ENDO testing in group 1 during their cyst-removal surgery, and following post-sham-cyst-removal testing in group 2 at sacrifice. Cysts were harvested from half the rats when they were in proestrus, and from the other half when they were in one of the other three stages (estrus, metestrus, diestrus). In all cases, the cysts were carefully removed from the anesthetized rat, immediately frozen in dry ice, and stored at -80°C . For processing, each cyst was cut serially on a cryostat in 20 μm -thick sections and thaw-mounted at 200- μm intervals (i.e., 10 sets of slides/cyst). Sections were briefly (10 min) postfixed with ice-cold acetone and quenched with 0.3% H_2O_2 in phosphate buffered saline (PBS) for 1h and then blocked in 0.3% Triton X-100 in PBS with 5% horse serum (HS) for 1 h. Sections were then immunostained with rabbit anti-vesicular monoamine transporter 2 (VMAT2; 1:10,000; Chemicon, Temecula, CA), or rabbit anti-calcitonin gene related peptide (CGRP; 1:10,000; Chemicon), or rabbit-anti-substance P (SP; 1:10,000; Bachem, Torrance, CA) in 0.3% Triton X-100 in PBS including 2% HS for two hours at room temperature (RT) followed by 4°C overnight. The next day, sections were washed in PBS and incubated at RT for 1h in ImmPRESSTM Reagent (MP-7401, Vector Laboratories, Burlingame, CA). Staining was visualized with 3,3'-diaminobenzidine (DAB kit, Vector Laboratories). For each antibody, the dilution used was the one that in test sections produced maximum labeling of neurites with minimal background. To minimize staining variability due to processing, sections from cysts in different groups were processed simultaneously with the same antibody. Controls included omission of the primary antibody, and omission of ImmPRESSTM Reagent. There was no labeling in any of the control sections.

After processing, cysts were examined microscopically for evidence of positive labeling. Because the cysts had been harvested unfixed from anesthetized rats, the immunostaining was not amenable to assessment by counting nerve fibers (as previously performed [45]). We therefore developed a different assessment strategy, carried out by two experimenters (DR and KM) blinded to estrous stage and condition (i.e., post-ENDO *versus* post-sham-cyst-removal), but not to marker. A scale of 0 to 4 was established, where 0 indicated no fiber labeling and 4 indicated the densest possible labeling for each of the three markers. To use this scale, all sections from each cyst that had been stained with one of the three markers (i.e., VMAT2, CGRP, or SP) were carefully scrutinized microscopically several times by each experimenter so that the range of 0 to 4 for that specific marker was clearly understood. Next, sections through

each cyst were assigned an overall score from 0 to 4, using 0.5 intervals. The scores of the two experimenters were highly correlated, with $r > 0.9$ for all scores. These scores were used to assess significance of differences between groups with the Wilcoxon Signed Ranks Test.

3. Results

3.1. Effect of complete-cyst-removal in ENDO rats

Results from this group, whose averaged data are shown in Fig. 2A, B, and whose individual data are shown in Fig. 3A–F, were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; after ENDO surgery; post cyst-removal surgery.

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume [$F(7,105) = 507.42$, $P < 0.001$], condition [$F(2,15) = 16.84$, $P < 0.001$], and there was a significant interaction between volume and surgical condition [$F(14,105) = 7.80$, $P < 0.001$]. Analysis of pressures showed that they differed significantly as a function of volume [$F(7,105) = 193.77$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,15) = 0.425$, $P = 0.66$], nor was there a significant interaction between volume and surgical condition [$F(14,105) = 0.885$, $P = 0.58$].

Thus, percent escape responses (Fig. 2A) to some of the distention volumes, but not the pressures produced by those volumes (Fig. 2B), were influenced by the condition of the rat. Post-hoc tests showed that escape responses increased significantly after the ENDO surgery ($P < 0.001$). After cyst-removal surgery, escape responses returned to baseline levels, so that post-cyst-removal escape percentages were significantly lower than those during the post-ENDO period ($P < 0.001$), and did not differ significantly from baseline responses ($P = 0.80$).

AUC calculations for escape responses compare the effects of the surgical manipulations on the severity of vaginal hyperalgesia. These bar graphs, located in the insets within the graphs in Fig. 2A and Fig. 3A–F, display the change in AUC between the baseline and post-ENDO periods compared with the change in AUC between the baseline and post-cyst-removal periods. Consistently in all rats (Fig. 3A–F), there was a large increase in the AUC between baseline and post-ENDO, but no difference (or small increases) between baseline and post-cyst-removal surgery. Overall (Fig. 2A), the difference between the baseline and post-ENDO periods compared with the difference between the baseline and post-cyst-removal periods was significant ($P = 0.001$). In other words, ENDO surgery induced a vaginal hyperalgesia that was eliminated by surgical removal of all four of the cysts.

3.2. Effects of sham-cyst removal in ENDO rats (Group 2)

Results from this group, whose averaged data are shown in Fig. 2C, D, and whose individual data are shown in Fig. 3I – L, were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; after ENDO surgery; post sham-cyst-removal surgery.

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume [$F(7,63) = 182.36$, $P < 0.001$], surgical condition [$F(2,9) = 7.91$, $P = 0.01$], and there was a significant interaction between volume and surgical condition [$F(14,63) = 4.62$, $P < 0.001$]. Analysis of pressures showed that they differed significantly as a function of volume [$F(7,63) = 93.48$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,9) = 0.325$, $P = 0.73$], nor was there a significant interaction between volume and surgical condition [$F(14,63) = 0.891$, $P = 0.57$].

Thus, percent escape responses (Fig. 2C) to some of the distention volumes, but not the pressures (Fig. 2D) produced by those volumes, were influenced by the condition of the rat. Post-hoc tests showed that escape responses increased significantly after the ENDO surgery

($P = 0.05$). After sham-cyst-removal surgery, escape responses tended to increase even further, so that escape percentages after sham-cyst-removal surgery tended to be elevated compared to the post-ENDO period ($P = 0.12$) and differed at a greater level of significance from baseline responses ($P = 0.003$) than during the post-ENDO period ($P = 0.05$).

AUC calculations, located in the insets within the graphs in Fig. 2C and Fig. 3I–L, compare the effects of the manipulations on the severity of vaginal hyperalgesia. Consistently in all rats (Fig. 3I–L), there was a large increase in the AUC between baseline and post-ENDO, and an even larger increase between baseline and post-sham-cyst-removal surgery. Overall (Fig. 2C), the difference between the baseline and post-ENDO periods compared with the difference between the baseline and post-sham-removal periods was significant ($P = 0.034$), indicating an increase in vaginal hyperalgesia after the sham-cyst-removal surgery. In other words, ENDO surgery induced a vaginal hyperalgesia whose severity was increased by a sham surgical procedure similar to cyst-removal surgery.

3.3. Timing of the effects of complete-cyst removal and sham-cyst removal on ENDO-induced hyperalgesia

As shown in Fig. 5A, the severity of hyperalgesia after cyst-removal surgery was not significantly decreased until 3–4 wks after the surgery. Similarly, as shown in Fig. 5B, hyperalgesic severity after sham-cyst-removal surgery was not significantly increased until 5–6 wks after the surgery.

3.4 Effects over time after ENDO surgery (i.e., no post-ENDO surgery; Group 3)

Results from this group, whose averaged data are shown in Fig. 2E, F, and whose individual data are shown in Fig. 3M – P, were reanalyzed (from earlier studies) by repeated measures ANOVA as a function of three different conditions: baseline; between 5 and 12 wks after ENDO surgery (post period #1); between 13 and 20 wks after ENDO surgery (post period #2).

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume [$F(7,63) = 139.09$, $P < 0.001$], surgical condition [$F(2,9) = 16.89$, $P = 0.001$], and there was a significant interaction between volume and surgical condition [$F(14,63) = 3.19$, $P = 0.001$]. Analysis of pressures showed that they differed significantly as a function of volume [$F(7,63) = 181.38$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,9) = 0.72$, $P = 0.51$], nor was there a significant interaction between volume and surgical condition [$F(14,63) = 1.21$, $P = 0.29$].

Thus, percent escape responses (Fig. 2E) to some of the distention volumes, but not the pressures produced by those volumes (Fig. 2F), were influenced by the condition of the rat. Post-hoc tests showed that escape responses increased significantly after the ENDO surgery ($P = 0.002$). There were no changes, however, over time between the two post-ENDO assessment periods ($P = 0.345$).

AUC calculations, located in the insets within the graphs in Fig. 2E and Fig. 3M – P, compare the effects of the manipulations on the severity of vaginal hyperalgesia. Consistently, in all rats (Fig. 3M – P), there was a large increase in the AUC between baseline and the post-ENDO period that did not differ significantly from the increase between baseline and the second post-ENDO period ($P = 0.122$). In other words, ENDO surgery induced a vaginal hyperalgesia whose severity did not change between 5 – 12wks after ENDO and 13 – 20wks after ENDO surgery.

3.5. Effects of double sham surgeries: shamENDO followed by sham “removal” (Group 4)

Results from this group, whose averaged data are shown in Fig. 2G, H, and whose individual data are shown in Fig. 4A–C, were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; post shamENDO surgery, post sham-removal surgery.

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume [$F(7,42) = 400.63$, $P < 0.001$], but there was no significant effect of surgical condition [$F(2,6) = 0.558$, $P = 0.600$] nor was there a significant interaction between volume and surgical condition [$F(14,42) = 0.81$, $P = 0.656$]. Analysis of pressures showed that they differed significantly as a function of volume [$F(7,42) = 91.43$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,6) = 1.54$, $P = 0.29$], nor was there a significant interaction between volume and surgical condition [$F(14,42) = 0.652$, $P = 0.81$].

AUC calculations for escape responses, located in the insets within the graphs in Fig. 2G and Fig. 4A–C, compare the effects of the manipulations on the severity of vaginal hyperalgesia. In the three rats (Fig. 4A–C), there were inconsistent and small changes in the AUC between baseline and post-shamENDO, and between baseline and post-sham-removal surgery. Overall (Fig. 2G), the small difference between the baseline and post-shamENDO periods compared with the slightly larger difference between the baseline and post-sham-removal periods was not significant ($P = 0.206$). In other words, shamENDO surgery failed to evoke vaginal hyperalgesia, and a subsequent surgery similar to cyst-removal surgery again failed to evoke vaginal hyperalgesia.

3.6. Effects of no surgery in healthy rats: vaginal nociception over time (Group 5)

Results from this group, whose averaged data are shown in Figs. 2I, J, and whose individual data are shown in Fig. 4D–F were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; a middle testing period; and a late testing period. These periods were chronologically identical to the three periods for the other groups.

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume [$F(7,42) = 67.67$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,6) = 0.170$, $P = 0.848$], nor was there a significant interaction between volume and surgical condition [$F(14,42) = 0.270$, $P = 0.995$]. Analysis of pressures showed that they differed significantly as a function of volume [$F(7,42) = 301.91$, $P < 0.001$], but there were no significant effects of surgical condition [$F(2,6) = 0.118$, $P = 0.890$], nor was there a significant interaction between volume and surgical condition [$F(14,42) = 0.459$, $P = 0.942$].

AUC calculations for escape responses, located in the insets within the graphs in Fig. 2I and Fig. 4D–F, compare changes in nociceptive sensitivity between the three time periods. These small changes were not significant ($P = 0.918$). In other words, vaginal nociception in healthy rats was stable over a 6-month period of testing.

3.7. Effect of partial cyst removal or “extra” pathology in two ENDO rats

Eight rats had initially been assigned to group 1, the post-ENDO - complete cyst-removal group. At autopsy, however, we found that cystic tissue remained in two of the eight rats. These two rats were therefore excluded from the “complete-cyst-removal” group and are presented separately here.

3.7.1. Unusual pathology—In the rat whose data are shown in Fig. 3G (#3820), ENDO induced an unusually severe vaginal hyperalgesia. During cyst-removal surgery, performed 12

wks after the ENDO surgery, although all four cysts and their sutures were located and completely removed, there was an unusually large amount of bleeding during the surgery that did not occur in any other rat. Furthermore, at autopsy, the rostral remnant of the left uterine horn (i.e., between the area removed for ENDO surgery and the ovary) had swollen into what resembled a very large cyst (22 mm × 13 mm). Although a slight swelling of this part of the left uterine horn was noted in most rats, no other rat exhibited such extensive pathology. As can be seen in Fig. 3G, in contrast to the virtually complete alleviation of the ENDO-induced vaginal hyperalgesia produced by complete-cyst removal in all six rats without extensive additional pathology (Fig. 3A–F), in this rat with additional pathology, the complete-cyst-removal surgery only partially alleviated the hyperalgesia.

3.7.2. Incomplete cyst removal—The severity of the ENDO-induced vaginal hyperalgesia for the rat whose data are shown in Fig. 3H (#4260), was similar to that in other rats with ENDO (Fig. 3A–F, I–P). However, during cyst-removal surgery in this rat, whereas three of the four original transplants (cysts) and sutures were located and removed, the fourth transplant and suture could not be found. Because extensive surgery would have been required to locate this cyst, it was allowed to remain. At autopsy, this cyst was found buried in fat and scar tissue near the caecum (5 mm × 5 mm). In this rat (Fig. 3H), unlike those with complete removal of their cysts (Fig. 3A–F), but similar to rats that had a sham-cyst-removal surgery (Fig. 3I–L), partial cyst-removal *increased* the severity of her ENDO-induced vaginal hyperalgesia.

3.8. Correlation between cyst burden and ENDO-induced vaginal hyperalgesia (AUC)

Cyst burdens ranged from 8 to 222 mm². This range is consistent with those reported previously [33]. Again consistent with our previous report, there were no significant correlations either within each group or overall between the cyst burdens in the 14 ENDO rats and their vaginal hyperalgesia (assessed by the difference between the baseline AUC and post-ENDO AUC). The overall correlation was $r = 0.024$ ($P = 0.93$).

3.9. Estrous stage variations in vaginal hyperalgesia in the sham-cyst-removal group (Group 2)

The severity of hyperalgesia as assessed by AUC in the sham-cyst-removal group (Group 2) after ENDO and next after sham-cyst-removal are shown in Fig. 6A for values assessed in proestrus compared with the three other stages combined. The two-way ANOVA was significant ($[F(3,28) = 8.191, P < 0.001]$) and effects differed significantly by condition (post-ENDO versus post-sham-cyst-removal; $[F(1,28) = 13.87, P = 0.001]$). Whereas, in proestrus, the severity of ENDO-induced hyperalgesia did not change significantly after sham-cyst-removal surgery, in the three other stages combined, hyperalgesic severity did increase significantly ($P < 0.001$).

3.10. Innervation of cysts harvested from rats in Groups 1 and 2

As shown in Fig. 6B, the amount of sympathetic fiber labeling in cysts taken after post-ENDO testing (from Group 1 rats) compared with labeling in cysts taken after post-sham-cyst-removal testing (from Group 2 rats) did not differ when the two groups were compared in proestrus. In contrast, when the two groups were compared in the other stages, the density of sympathetic fiber labeling was significantly greater in cysts from rats after post-sham-cyst removal than in cysts from rats after post-ENDO testing ($P = 0.036$). In other words, sympathetic fiber labeling differed between groups in a manner similar to the increases in vaginal hyperalgesia shown in Fig. 6A. There were no significant differences in the amount of sensory fiber labeling across groups for cysts immunostained with either CGRP or SP. Fig. 6C provides example photomicrographs of neurites immunopositively-labeled with VMAT2, CGRP, and SP.

4. Discussion

These results demonstrate that when ectopic endometrial cystic tissue is completely removed, ENDO-induced vaginal hyperalgesia is fully alleviated for up to four months postoperatively. The results also show that surgery that fails to remove all the ectopic tissue exacerbates ENDO-induced vaginal hyperalgesia. This exacerbation is not due simply to surgery (Groups 3 and 4), nor is it due to lengthy vaginal nociceptive testing (Group 5). The decreases and increases in hyperalgesia are not immediate, developing over a period of 3 – 6 weeks. Sub-analyses by estrous stage of the increased hyperalgesia induced by sham-cyst-removal surgery showed that most of the increase occurred in estrous stages other than proestrus during which the cysts' sympathetic innervation was elevated. These findings suggest that increased sympathetic innervation of the cysts contributes to the exacerbation of ENDO-induced hyperalgesia produced by sham-cyst-removal surgery.

4.1. Effects of cyst removal

The fact that completely removing the cysts eliminated ENDO-induced vaginal hyperalgesia suggests that the cysts are necessary to maintain it, raising the question of what cyst characteristics are relevant. The amount of cystic growth does not appear important, both because cyst burden fails to correlate with hyperalgesic severity (here and previously [34]), and because partial cyst removal fails to reduce the hyperalgesia. Instead, what may be more important are C-fiber sensory and sympathetic fibers that sprout to innervate the cysts [7,8].

Recent findings support this hypothesis indirectly. We showed [13] that the severity of ENDO-induced vaginal hyperalgesia was greatest in proestrus when estradiol levels are highest [17] and least the next day in estrus when estradiol levels are lowest [17]. We also found [45] that, from proestrus to estrus, there was a significant decrease in density of sympathetic fibers innervating the cysts and in the cysts' contents of nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). Furthermore, TrkA, the receptor for NGF, was located on both sensory and sympathetic fibers, suggesting that proestrous-to-estrous changes in NGF in the cysts influences functioning of both their sensory and sympathetic fibers [16,28,31]. Together, these results support the hypothesis that proestrous-to-estrous changes in functioning of both sympathetic and sensory innervation of the cysts contributes to changes in ENDO-induced vaginal hyperalgesic severity.

Previously ([8,13], we argued that central sensitization could be a major factor underlying ENDO-induced vaginal hyperalgesia not only because afferent fibers supplying the cysts appear to be sensitized [15], which would induce central sensitization [23], but also because spinal segments associated with the cysts (mid-thoracic) are remote from spinal segments receiving input from mid-vagina (L6/S1). If such central sensitization was maintained by peripheral sensitization, then removal of the putatively-sensitized peripheral afferents via cyst removal should eliminate the hyperalgesia, which it did.

An important effect of cyst removal on ENDO-induced hyperalgesia, however, was that this elimination did not occur immediately; i.e., the hyperalgesia took 3–4 weeks to disappear (Fig. 5A). This delay supports the hypothesis that while peripherally-maintained central sensitization could contribute to ENDO-induced hyperalgesia, other central factors may be involved. Examples include peripherally-independent central sensitization [30,36] or effects of stress on sympathetic output to the area previously occupied by the cysts, which could continue to activate afferents there [18,21,25]. Of relevance to this conclusion is that we previously found that cyst removal during the rats' transition into estropause failed to prevent subsequent increases in severity of ENDO-induced hyperalgesia that paralleled steady increase in estradiol levels [6]. We attributed this failure to peripherally-independent central sensitization that remains sensitive to high steady levels of estradiol.

4.2. Effect of sham-cyst removal

The increase in severity of ENDO-induced vaginal hyperalgesia after sham-cyst-removal surgery could have been due to failure of the surgery to prevent a progressive increase in the effects of ENDO over time. This possibility is unlikely because there were no changes in severity of the hyperalgesia assessed during a similar time period in rats that had not had a second surgery (Fig. 2E). The increased severity could also have been due only to inflammatory factors associated with the second surgery. This possibility is also unlikely, for two reasons. First, complete cyst removal completely alleviated the hyperalgesia. Thus, the cysts' presence was required for the increase to occur. Secondly, shamENDO surgery followed by sham-removal surgery had no influence on vaginal nociception. It appears, therefore, that the surgical procedure that mimicked cyst removal (i.e., sham-cyst removal) somehow increased further the cysts' exacerbating influence on vaginal nociception. The question then arises as to what aspects of the cyst-surgery interaction contribute to this increase.

One effect triggered immediately by the injury of open abdominal surgery is an increase in abdominal cytokines and growth factors [22]. It is possible, therefore, that these agents increased activity of the cysts' afferent innervation [29]. The 5–6 week delay in the increased hyperalgesia (Fig. 5B) suggests that a cascade of time-consuming factor(s) either triggered by or independent of surgically-induced increases in cytokine/growth factors were responsible. Examples of the former include induction of nerve sprouting and increased central sensitization, both of which would need further experimental study. Regarding nerve sprouting, inflammation-induced sympathetic sprouting can take weeks to develop and this sprouting induces hyperalgesia [2]. Regarding central sensitization, increased peripheral inflammation (such as that added by surgery to the cysts' own inflammation) can enhance central sensitization via several processes such as central neuroimmune activation and glial activation, both of which can take days/weeks to develop [14,29,43].

Additional clues for potential peripheral mechanisms underlying the increases of ENDO-induced hyperalgesia induced by sham-cyst removal come from estrous/estradiol-dependent differences in the increases. As discussed above, severity of ENDO-induced hyperalgesia correlates positively with estradiol levels during the estrous cycle [13], which parallels estrous changes in density of the cysts' sympathetic innervation [45]. In contrast, the additional increases in hyperalgesia after sham-cyst removal occur mainly in stages other than proestrus. The end result is that hyperalgesic severity is the same across all estrous stages. In other words, sham-cyst-removal surgery appears to abolish estradiol's control of hyperalgesic severity during the estrous cycle. What accompanies this loss is that, after sham-cyst removal, the proestrous-to-estrous decrease in sympathetic innervation no longer occurs. In other words, estradiol no longer appears to be required for dense sympathetic innervation of the cysts, suggesting the innervation becomes maintained by other factors.

Although these other factors could involve pro-inflammatory agents, specific suggestions are difficult because little information exists on how peripheral pathology influences peripheral neuronal sensitivity to estradiol. There is, however, precedence for our findings. Thus, during the estrous cycle, sympathetic innervation of the healthy ovary and other organs reaches its peak when estradiol levels are highest [4,32]. In contrast, after the ovaries are surgically removed (OVX), estradiol replacement *reduces* both sympathetic innervation and NGF in reproductive and other tissues [24,26,40]. Although these results appear to provide conflicting evidence for estradiol's influence on sympathetic innervation, the results could also indicate that the trauma of the OVX surgery itself [20] affects estradiol's actions on sympathetic innervation. Another explanation, of course, is that the direction of estradiol's actions on sympathetic innervation differs depending on whether circulating ovarian hormones have been completely removed (by OVX) or not (cycling rat).

Of relevance is that, as discussed above, surgical cyst removal performed when rats transition into estropause does not prevent increases in ENDO-hyperalgesia associated with increases in estradiol levels later on during estropause [27]. Thus, while changes in estradiol-sensitivity of sympathetic innervation may contribute to the exacerbation of ENDO-induced hyperalgesia by sham-cyst-removal surgery, estradiol-sensitive central factors cannot be excluded.

4.3. Clinical relevance

In women with endometriosis, removal of ectopic endometrial growths can successfully reduce pain in up to 80% of carefully-selected patients, but the relief is temporary; in at least half of the patients pain returns sometimes worse than before surgery, and sometimes without recurrence of ectopic growths [1,37,39]. The results here in rats suggest that several factors warrant experimental investigation in women as well as possible inclusion in the clinical treatment decision-making process for surgery. These factors include: ability to remove all ectopic growth, and cyclical (menstrual) aspects of the preexisting pain. Thus, if it is known in advance that some ectopic tissue will likely be difficult to remove by either excision or ablation, and/or if the patient's pains are strongly cyclical, then it may prove to be the case that, like the rat model, surgery will be unsuccessful or even iatrogenic.

Acknowledgments

We thank Natalia Dmitrieva, Ph.D. for constructive discussions throughout the course of the study, Jenna McHenry for help with behavioral assessments, and John Chalcraft for help with figures. This study was supported by NIH grant RO1-NS11892.

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Figure 1.

Summary and timeline of the three periods of assessment of vaginal nociception for the five main experimental groups. **Group 1:** rats that underwent ENDO surgery followed by complete-cyst-removal surgery. **Group 2:** rats that underwent ENDO surgery followed by sham-cyst-removal surgery, **Group 3:** rats that underwent ENDO surgery with no additional surgery. **Group 4:** rats that underwent ShamENDO surgery followed by a sham-removal surgery.. **Group 5:** rats that underwent no surgery. **BASELINE PERIOD:** This five to six week period comprised the initial assessment of vaginal nociception. Although rats continued to be assessed during the four weeks after baseline (i.e., after ENDO, shamENDO or “no surgery”), these data were not included in analyses comparing the three assessment periods. **MIDDLE PERIOD:** This six to eight week period comprised the second assessment of vaginal nociception. **LATE PERIOD:** This period comprised the third assessment of vaginal nociception. For most rats, the duration of this period was ~ eight weeks. In one rat in Group 1, however, the assessment period continued longer so that the total duration was ~ six months.

Figure 2.

Percent escape response to different volumes of distention of the vaginal canal (A, C, E, G, I) and the resulting vaginal pressures (B, D, F, H, J) in each of the five experimental groups. Bar graphs inset into (A, C, E, G, I) show differences in the AUC between baseline and middle testing period, and between baseline and late testing period for each group. Asterisks indicate that the two AUCs of that group differed significantly. * $P < 0.05$; ** $P = 0.001$. Data in this and subsequent figures are shown as mean $\pm$ SEM.

Figure 3.

Percent escape response to different volumes of distention for individual rats. (A – F) show data from rats in Group 1 (ENDO surgery followed by complete-cyst-removal surgery). (G – H) show data from rats originally in Group 1, but excluded due to “extra-pathology” at time of autopsy (see Results). (I – L) show data from rats in Group 2 (ENDO surgery followed by a sham-cyst-removal surgery). (M – P) show data from rats in Group 3 (ENDO surgery only). Each inset bar graph shows differences in the AUC between baseline and middle testing period, and between baseline and late testing period for that rat.

Figure 4.

Percent escape response to volumes of distention for individual rats: (A – C) rats in Group 4 (shamENDO surgery followed by a second sham surgery) and (D – F) rats in Group 5 (no surgery). Each inset bar graph shows differences in the AUC between baseline and middle testing period, and between baseline and late testing period for that rat.

Figure 5.

Time course of the effect of (A) complete cyst removal or (B) sham-cyst removal on ENDO-induced vaginal hyperalgesia. Each bar shows differences in AUC from baseline for each time point. *, difference from post-ENDO, $P < 0.05$.

Figure 6.

(A) For rats in Group 2, the severity of vaginal hyperalgesia that was produced by ENDO (solid bars) compared with the severity of vaginal hyperalgesia that was produced by sham-cyst-removal (hatched bars) evaluated in proestrus (left pair of bars) compared with all other stages combined (right pair of bars). (B) The density of (VMAT2) sympathetic fiber labeling in cysts harvested post-ENDO (solid) compared with the density in cysts harvested after sham-cyst removal (hatched) in proestrus (left pair of bars) compared with other all other stages combined (right pair of bars). (C) Examples of immunolabeling in four cysts. Photomicrographs were adjusted for brightness and contrast. Top photomicrographs show VMAT2 (sympathetic fiber labeling) in two cysts, one harvested after completion of post-ENDO testing (left) and the other

after completion of sham-cyst-removal testing (right). The bottom photomicrographs are examples of CGRP and SP labeling. **, $P < 0.05$; ***, $P < 0.001$