Estrous changes in vaginal nociception in a rat model of endometriosis

Angie M. Cason, Chad L. Samuelsen, and Karen J. Berkley*

Program in Neuroscience, Florida State University, Tallahassee, FL 32306-1270, USA

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Abstract

A rat model of endometriosis, in which pieces of uterine horn (versus fat in controls) are autotransplanted into the abdomen where they form cysts, reduces fecundity and produces vaginal hyperalgesia. The cysts gradually enlarge over a 2-month period postsurgically and then plateau. Cysts regress with low estrogen levels and reappear when they rise. Based on the hypothesis that the vaginal hyperalgesia depends upon the cysts, this study tested two predictions: that (1) the hyperalgesia would develop postsurgically in parallel with the cysts, and (2) the hyperalgesia would vary with estrous, being greatest when estrogen levels are high (proestrus) and least when low (estrus). In rats trained to escape vaginal distention, percentage escape responses to different distention volumes were measured across the rat’s 4-day estrous cycle for 2.5 months before and up to 4 months after autotransplantation of uterus (n = 9) or fat (n = 6) in abdominal sites. Vaginal pressures were also measured. In rats with uterine but not fat autotransplants, escape percentages increased postsurgically over a 2-month period and then plateaued. The increase was greatest in proestrus and failed to occur in estrus. Vaginal pressures were unchanged in all groups. These results strongly support the hypothesis that the vaginal hyperalgesia depends upon the cysts. Because the cysts were located in sites remote from the vagina, the hyperalgesia involves viscero-visceral interactions and is likely centrally mediated, whereas the estrous modulation could involve hormonal actions either on the cysts or, more likely, on vaginal afferent fibers, and/or on central neurons.

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Endometriosis is a common clinical disorder in which abnormal growths of viable endometrial tissue occur outside the uterus. Symptoms include reduced fertility and pelvic visceral pain, specifically dyspareunia (coital pain, vaginal allodynia, and hyperalgesia), severe dysmenorrhea (menstrual pain), dyschezia (pain on defecation), and chronic pelvic pain (Yoshinaga and Parrott, 2002). The ectopic growths produce or provoke abnormal production in nearby tissues of numerous substances, many of which are thought to play some role in the pathology and associated signs and symptoms of endometriosis. Such substances include steroid hormones, prostaglandins, matrix metalloproteinases, and numerous cytokines and growth factors (Anaf et al., 2002; Gazvani and Templeton, 2001; Yoshinaga and Parrott, 2002).

Because endometriosis occurs primarily in menstruating women, regresses postmenopausally and during pregnancy, recurs in postmenopausal women who are receiving estrogen replacement therapy, and is relieved by treatment with GnRH agonists, endometriosis is commonly considered an “estrogen-dependent” condition (Barbieri and Gordon, 1991; Goh and Hall, 1992; Chinegwundoh et al., 1995; Hurst et al., 2000; Yoshinaga and Parrott, 2002).

A rat model of surgically induced endometriosis was developed nearly 20 years ago (Vernon and Wilson, 1985; Rajkumar et al., 1990; Sharpe-Timms, 2002). The surgery involves partial hysterectomy of about one-third of one uterine horn and autotransplantation on blood vessels within the abdomen of small pieces of the removed uterus. The control surgical procedure includes the same partial hysterectomy, but pieces of fat tissue that were attached to the removed portion of the uterus are autotransplanted instead of uterine tissue. The uterine, but not the fat transplants, form cysts that grow rapidly during the first 30 days and then more slowly, stabilizing in size by ~2 months and remaining viable for at least 10 months (Vernon and Wilson, 1985).
The uterine autotransplantation reduces the rat’s fecundity (Vernon and Wilson, 1985; Sharpe-Timms, 2002). The cysts are responsive to steroid treatment; i.e., they disappear during pregnancy and after ovariectomy, and recur following estrogen replacement (Vernon and Wilson, 1985; Rajkumar et al., 1990). In addition, the cysts synthesize or induce synthesis by peritoneal cells (in culture) of many of the same substances found in the endometrial implants of women (Sharpe-Timms, 2002). Thus, surgically transplanted uterine tissue in the rat mimics the condition of endometriosis in women.

Despite considerable study of the influence of this surgical model on mechanisms of reduced fertility, it was unknown whether the surgical manipulation would alter pelvic nociception in rats. Recently, using a behavioral method for assessing vaginal nociception (Berkley et al., 1995, 2001; Bradshaw et al., 1999), we found that, similar to the situation in women with endometriosis, vaginal nociception was increased in rats with endometrial cysts, but not in rats whose transplants did not form cysts (Berkley et al., 2001). These results encouraged further use of this model to study mechanisms of the endometriosis-induced vaginal hyperalgesia.

Our initial findings suggested that the increased vaginal nociception depended upon the cysts. If so, then vaginal nociception should increase postsurgically in parallel with the growth of the cysts, that is, over a ~2-month time period. Furthermore, given the putative estrogen dependency of the condition, the increased nociception should be greatest when estrogen levels are highest. Accordingly, using the same behavioral methods as our previous study, the present study investigated how vaginal nociception changed with time after surgical induction of endometriosis and how it varied with the rat’s ovarian cycle.

Methods

The behavioral methods and the surgical procedures used in this study were virtually identical to those that have been described in detail and illustrated in previous publications (Berkley et al., 1995, 2001; Bradshaw et al., 1999; Giamberardino et al., 2002). The text below abbreviates those descriptions. The study was approved by Florida State University’s Animal Care and Use Committee (Protocol 9028) and complied with the NIH Guide for Care and Use of Laboratory Animals and guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Animals

Fifteen adult virgin female Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA) were used. Some of the data from seven of these rats have been reported previously (Berkley et al., 2001). The rats weighed ~150 g at the beginning and ~325 g at the end of the study. They were housed individually in plastic cages with chip bedding, and ad libitum access to rat chow and water. They were maintained on a 12:12 light:dark cycle (lights on at 07:00 AM). Estrous stage was monitored daily by vaginal lavage 2 h after lights on. Rats were trained and tested only when they maintained regular 4-day estrous cycles and at about the same time of day (3–6 h after lights on).

Behavioral apparatus and distention stimulus

The training and testing apparatus was a small rectangular Plexiglas chamber designed to fit the rat snugly in order to prevent her from turning around. A hollow tube containing light-emitting diodes and a photosensor extended from the front of the chamber. The rat could extend her nose into this tube, thus breaking the light beam and terminating the stimulus (i.e., escape response, see below). An opening in the rear of the chamber allowed the catheter (attached to the vaginal stimulator) to be connected to the automated stimulus apparatus.

The vaginal stimulator was a small latex balloon (~10 mm 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 (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 pressure transducer.

Training procedure

After 3–4 days of 10-min acclimation sessions in the chamber, 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. This response involved the rat extending her head into the tube, thereby interrupting the light beam. Training sessions of 10 pinches delivered at ~1-min intervals were run 3/week on nonconsecutive days. Training was usually completed in 4–8 sessions.

The rat was next trained to make identical escape responses to deflate vaginal distention stimuli. These sessions were run 3/week on nonconsecutive days for a total of 3–5 sessions. Ten large stimuli (0.80–1.0 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 the larger 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.
Testing procedures

During each computer-controlled, 1-h testing session, two behaviors were assessed consecutively: (1) detection thresholds, and (2) escape responses. Testing sessions were run 3/week on nonconsecutive days in a manner to ensure that approximately equal numbers of sessions were run in each of the four estrous stages. At the beginning of each testing session, after inserting the lubricated stimulator into the vaginal canal, placing the rat in the testing apparatus, and delivering 3 tail pinches, a series of 5 detection trials were run, followed by 24 escape trials. Testing sessions were run ~2.5 months before surgery (baseline) until data from 3 to 4 sessions in each estrous stage had been obtained. Following recovery from the endometriosis or sham surgery, sessions were then run for up to 4 more months.

Detection thresholds

The detection trials, run at intervals of ~1 min, were randomly programmed to be “real” trials 70% of the time and “sham” trials 30% of the time. The experimenter was unaware of whether the trial was a real or sham trial. During each detection trial, whether real or sham, a timer was started and the balloon was, respectively, either inflated at a slow steady rate (0.05 ml/s) or not inflated while the experimenter carefully observed the rat’s behavior. If the rat exhibited any “new behavior,” the trial was terminated and the rat’s behavior recorded. New behaviors included grooming, repositioning the hind paws, and stretching. The maximum time for both trial types was at which the distention volume reached (or, for sham trials, would have reached) a preset maximum value of 1.0 ml or 20 s. For each trial, the computer recorded the elapsed time (latency) and the distention volume (either the real volume, or, for sham trials, what that volume would have been if the balloon had been inflated). If the rat failed to exhibit any new behaviors during this period, the stimulus, whether real or sham, was considered to have been undetected. The volume at which the rat exhibited any new behavior was considered to be the detection threshold. As discussed and explained at length in Bradshaw et al. (1999), this volume was an overestimate because the distention stimulus continued to increase during the time the rat was responding. The threshold was accordingly corrected by subtracting 0.015 ml from the recorded volume.

Escape trials

Following the detection trials, a series of 24 computer-controlled escape trials 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 an escape response had been made or 15 s had elapsed, when it was then rapidly deflated. Eight different distention volumes, including a control (0.01 ml), were delivered three times each in random order. The computer recorded stimulus volume and stimulus pressure for each trial.

Surgical procedures

Following the baseline (presurgical) behavioral assessments, rats were anesthetized and subjected to either experimental endometriosis (n = 9; ENDO) or sham endometriosis (n = 6; SHAM) surgery. Rats were anesthetized ip with a mixture of ketamine hydrochloride (73 mg/kg) and xylazine (8.8 mg/kg). 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 saline. Five pieces of uterine horn (~2 × 2 mm) or, for the sham procedure, five similarly sized pieces of fat were cut from this segment. These pieces were sewn around blood vessels in various structures using 4.0 nylon sutures. In most rats (6 in the ENDO group; 5 in the SHAM group), one piece was sewn on the left ovary, three on alternate cascade mesenteric arteries that supply the caudal small intestine starting from the caecum, and the fifth on the right abdominal wall artery at approximately the level of the L2 dermatome (Fig. 1). In the rest of the rats (3 ENDO, 1 SHAM), all pieces were sewn on alternate mesenteric arteries. Rats were closely observed during the postsurgical period for potential complications. Postoperative recovery was uneventful, and regular estrous cyclicity resumed within ~1 week. The state of autotransplants was assessed at autopsy by locating each of the sutures that had been used to tie the uterine or fat autotransplants and then examining and measuring any cysts that were found at each site.

Data analysis

The following measures were available from each session: From the detection trials, data included the probability
of a detection response and the volume (or potential volume) at which the detection response was made during each of the real (and sham) trials. From the escape trials, data included measures of the percentage escape response to different volumes of distention, and the pressures produced by each of those volumes. Detection thresholds were analyzed using a series of one-way ANOVAs to compare values across estrous stage during the presurgical period and to compare pre- and postsurgical detection volumes across estrous stages. Differences in escape responses and pressures produced by each distention volume during the pre- and postsurgical periods, across time, and across estrous stages were analyzed using repeated-measures ANOVAs, followed by post hoc Fisher’s least-significant difference comparisons, with alpha levels set at 0.05. Postsurgical data were divided into three groups: (1) 1 week–1 month following surgical manipulation, (2) 1 month–2 months following surgical manipulation, and (3) greater than 2 months after surgery. Only data from the 1- to 2-month and >2-month period were used for subsequent estrous stage analysis. One-way ANOVAs were used to determine the volumes at which escape response percentages and vaginal pressures became significantly greater than control volumes within groups.

Results

Autopsy

At autopsy, all ENDO rats exhibited fully formed cysts at least 2 mm in diameter in at least 3 of the 5 transplant sites. The transplants that most often failed to form cysts and were the smallest were those on the abdominal wall. No cysts were found in any of the SHAM rats.

General influence of surgery

Consistent with previous analyses of the rats behaviors obtained from videotapes made continuously for 2–3 weeks postsurgery (Giamberardino et al., 2002) and from our previous study (Berkley et al., 2001), none of the rats showed any signs of unusual or pain behaviors after recovery from surgery. They continued to eat, drink, gain weight, and respond to the vaginal smearing procedures in the same way they had before surgery.

Escape responses

Escape response percentages increased as a function of distention volume in all rats in all groups. Overall, escape responding increased significantly after surgery in the ENDO group \([F(1,16) = 6.091, P = 0.025]\), but not in the SHAM group. As shown in Fig. 2, these increases became greater over time \([F(3,29) = 3.797, P = 0.021]\) in the ENDO group. There were no changes in the SHAM group.

When compared with presurgical percentages, postsurgical escape percentages did not become significantly greater than presurgical levels until the 1- to 2-month \((P = 0.017)\) and the >2-month \((P = 0.004)\) postsurgical periods, with no difference between these two postsurgical periods \((P = 0.408)\).

In order to evaluate the influence of estrous stage, data from the 1- to 2-month and >2-month postsurgical periods were combined. The results are shown in Fig. 3. Escape percentages varied significantly with estrous stage in the ENDO \([F(7,228) = 6.278, P < 0.001]\), but not the SHAM group. Responses in the ENDO group were significantly greater than presurgical levels during proestrus \((P < 0.001)\), metestrus \((P = 0.002)\), and diestrus \((P = 0.003)\), but not during estrus \((P = 0.173)\).

The volume at which the escape percentages become significantly greater than the escape percentages to the con-
control volume (to 0.01 ml) can be considered as a threshold of nociceptive sensitivity (Bradshaw et al., 1999). Any decreases in this threshold would be an indication of allodynia.

This value ranged from 0.2 to 0.35 ml before surgery. After surgery, this value did not decrease in the SHAM group, but did decrease during all stages in the ENDO group, with the
Pressure

Vaginal pressures increased as a function of distention volume during all four estrous stages, becoming significantly greater than control percentages at volumes ≥0.10 ml. As previously found (Bradshaw et al., 1999), the pressures produced in the vagina by different volumes of distention varied with estrous stage during the presurgical period \( F(3,56) = 10.745, P < 0.001; \) data taken from all 15 rats, regardless of surgical group. Vaginal pressures were significantly greater during estrus \( (P < 0.05) \) than the other three estrous stages and significantly lower in diestrus than either proestrus \( (P < 0.01) \) or estrus \( (P < 0.001) \).

Vaginal pressures did not change significantly in either the ENDO or the SHAM groups either as a function of time following surgery (data not shown) or with estrous stage (Fig. 4).

Detection

All rats exhibited more detection responses to real vaginal stimuli (99.3%) than to sham stimuli (23.7%). The mean detection threshold for real stimuli was ~0.27 ml and neither varied significantly with estrous stage nor changed significantly after surgery in either group.

Discussion

The probability of escape responses to vaginal distention increased after the ENDO, but not the SHAM surgery, an indication of vaginal hyperalgesia. In addition, the distention volume at which the escape responses became significantly greater than control volumes (nociceptive sensitivity) was significantly reduced after the ENDO but not the SHAM surgery, an indication of vaginal allodynia. These results confirm and extend previous findings (Berkley et al., 2001) indicating that surgical induction of endometriosis in the rat, in a manner that reduces fecundity, increases vaginal nociception.

Potential mechanisms of increased vaginal nociception

The vaginal hyperalgesia developed gradually over a 2-month period before stabilizing. This time course is nearly identical to the growth of the cysts, which, as they grow, have been shown to become increasingly vascularized, surrounded by deposits of fat, and embedded in and associated with adhesions to adjacent tissues (Vernon and Wilson, 1985). This parallel time course, together with the failure of the SHAM surgery to increase vaginal nociception, provides strong support for concluding not only that it is the cysts and not the surgical manipulation that gives rise to the increased nociception but also that the amount of increase relates to some aspect of the cysts’ growth. This conclusion supports clinical findings indicating that the intensity of pain is related to the depth of infiltration of the ectopic endometrial tissue (Koninckx et al., 1991).

There are several mechanisms by which the cysts could increase vaginal nociception. One possibility involves the many substances that are abnormally produced or misexpressed either by the cysts themselves or nearby tissues. Such substances include steroid hormones, prostaglandins, matrix metalloproteinases, and numerous cytokines and growth factors, including interleukins 1, 6, 8, 10, and 13; tumor necrosis factor alpha; intercellular adhesion molecule 1; monocyte chemotactic protein; vascular endothelial growth factor; insulin-like growth factor; nerve growth factor; platelet-derived growth factor; epidermal growth factor; basic fibroblast growth factor; macrophage-colony stimulating factor; transforming growth factor beta; and hepatocyte growth factor, and their receptors (Anaf et al., 2002; Gazvani and Templeton, 2001; Yoshinaga and Parrott, 2002).

How any or combinations of these substances might influence sensory input from the vaginal canal is, however, unknown. One way they could act is by somehow reducing vaginal compliance so that the pressures produced by vaginal distention would be increased. This possibility is unlikely because vaginal compliance was unchanged by the surgical induction of endometriosis in the ENDOS and SHAM groups.

Another way they might act is to sensitize vaginal afferent fibers, but the means by which such sensitization could occur is problematic. In humans, when endometriosis is associated with deep dyspareunia, the ectopic endometrial growths are most likely to be found on the uterosacral ligament (Fauconnier et al., 2002; Porpora et al., 1999). The uterosacral ligament is located near the vaginal canal and shares the same inferior hypogastric plexus innervation (Rogers, 1998). In the present study, however, the active cysts in most cases were located in the upper abdomen and sometimes on the ovary. These areas are remote from the vagina and do not share the same innervation. Regions where the active cysts were located are innervated by splanchnic nerve fibers traveling via the superior mesenteric, inferior mesenteric, and celiac ganglia to lower thoracic/lumbar segments (Jänig and Morrison, 1986; Papka and Traurig, 1993; Traurig and Papka, 1993), whereas the mid-vaginal canal is innervated by the pelvic nerve whose afferent fibers enter the spinal cord much further caudally via the L6, S1, and S2 dorsal roots (Berkley et al., 1993).

It seems likely, therefore, that central neural mechanisms are involved in the increased vaginal nociception observed here. Neurons within the lower thoracic/lumbar and L6–S2 spinal segments respond to stimulation of the vagina (Berkley et al., 1993), and there is extensive communication between the two sets of segments (Wall et al., 1993). Thus, it is possible that sensitized afferents innervating regions surrounding the active cysts create a central sensitization...
within the lower thoracic/upper lumbar spinal cord that is referred to the vaginal canal via interaction with the L6–S2 segments. A similar process has been observed previously and called “viscero-visceral referred hyperalgesia” (Giamberardino et al., 2002).

**Influence of estrous stage**

The fact that escape responses in the SHAM group did not vary with estrous stage confirms previous findings in intact rats (Bradshaw et al., 1999). For the ENDO group, an...
important finding here was that the vaginal hyperalgesia and allodynia were greatest when the rat was in proestrus and the vaginal hyperalgesia failed to occur when the rat was in estrus. Rats were always tested 3–6 h after lights on. During this period in proestrus, serum estradiol levels have reached their peak, and progesterone levels are just beginning or have not yet begun to increase; during this period in estrus, serum levels of both hormones are very low (Freeman, 1994). In the other two stages, estradiol levels are climbing but are still much lower than they are in proestrus, and progesterone levels increase slightly only in metestrus (Freeman, 1994). Thus, the amount of vaginal hyperalgesia appears to be associated with estrogen levels; i.e., high estrogen, large increase; less estrogen, smaller increase; basal estrogen, no increase. This association strongly supports the clinical characterization of endometriosis as “estrogen dependent” (Yoshinaga and Parrott, 2002), but it does not rule out progesterone involvement (Chwalisz et al., 2002).

The question then arises as to how the putative estrogen modulation occurs. One possibility is that estrogen acts on the cysts themselves. This possibility is supported by the fact that the cysts regress following ovariotomy and reappear following estrogen replacement (Rajkumar et al., 1990). It is also supported by the fact that the cysts regress during pregnancy and reappear postpartum (Vernon and Wilson, 1985). During pregnancy, progesterone is the dominant hormone (Fajer and Barraclough, 1967), with estradiol level remaining relatively low until it surges just before parturition (Yoshinaga et al., 1969; Shaikh, 1971).

Despite this supportive evidence, it remains uncertain whether any features (molecular or structural) of the cysts vary with the rat’s estrous cycle. Vernon and Wilson remarked in their original study (1985) that all their autotransplants “grew into large cystic structures, irrespective of the stage of the reproductive cycle” (p. 691). Furthermore, in women, normal cyclical variations in the expression of matrix metalloproteinases and their inhibitors in eutopic endometrium are muted in ectopic endometrial tissue (Bruner-Tran et al., 2002).

It may therefore be the case that the estrous variation in vaginal hyperalgesia and allodynia produced by the ENDObiography surgery does not involve estrogen action on the cysts themselves. Estrogen receptor-α and -β immunoreactivity and mRNA have been found both in peripheral afferent neurons that supply the genital organs and in lumbo-sacral spinal dorsal horn (Papka et al., 2001) where neurons are responsive to genital stimulation (Berkley et al., 1993). Furthermore, progesterone receptors have been found in the spinal cord (Labombarda et al., 2000) that are estrogen-inducible and vary with the rat’s estrous cycle (Monks et al., 2001). Thus, regardless of whether the cysts are affected, it is also possible that the estrogen acts on peripheral and/or central neurons.

Implications for fertility

Whatever the mechanisms prove to be that underlie the estrous variations observed here, the fact that the increases in vaginal nociception produced by the ENDObiography surgery were greatest in proestrus could have implications for the mechanisms by which the ENDo surgery reduces fertility or fecundity. Because proestrus is the stage in which solicitation and mating behavior are peaking (Erskine, 1989), it would seem likely that the existence of significantly increased vaginal sensitivity during that stage would make mating less tolerable and, in so doing, negatively affect some aspect of mating. Such an influence could contribute to the reduction in viable embryos and pups at term that occurs in rats subjected to the ENDo surgery (Vernon and Wilson, 1985). Future support for this hypothesis from studies now underway would strongly encourage clinical investigation of this issue in women.

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