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PERIPHERAL NEURAL SPROUTING CONTRIBUTES TO ENDO-INDUCED VAGINAL HYPERALGESIA IN A RAT MODEL OF ENDOMETRIOSIS

By

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP	adenosine-5'-triphosphate
BDNF	Brain Derived Neurotrophic Factor
CGRP	Calcitonin Gene Related Peptide
CR	Cyst-Removal
CB	Cyst Burden
CNS	Central Nervous System
CV	Coefficient of variation
DAB	3, 3' diaminobenzidine
ENDO	Endometriosis
GnRH	gonadotropin-releasing hormone
NDMA	*N*-methyl-D-aspartate
NGS	Normal Goat Serum
NHS	Normal Horse Serum
PF	Peritoneal Fluid
PGE2	Prostaglandin E2
SCR	Sham-Cyst-Removal
shamENDO	sham-endometriosis
SP	Substance P
TH	Tyrosine Hydroxylase
TNFα	Tumor Necrosis Factor alpha
Trk A	Track A
VEGF	Vascular Endothelial Growth Factor
VMAT2	Vesicular Monoamine Transporter 2

ABSTRACT

Endometriosis, defined by ectopic growths of uterine tissue, is considered an enigma because it is unknown how or even if these abnormal growths contribute to the painful conditions including dysmenorrhea, dyspareunia, and chronic pelvic pain that often accompany the disease. Many clinicians and biomedical scientists assume that the amount of ectopic growth (cysts) predicts the presence or severity of pain symptoms, even though considerable evidence suggests that this assumption is unwarranted. Studies from our laboratory using a rat model of surgically-induced endometriosis (ENDO) demonstrated for the first time that the cysts develop a sensory and sympathetic nerve supply. This discovery gave rise to the hypothesis that this ***newly-sprouted innervation of the cysts is a significant contributor to the development (i.e., generation) and maintenance of painful symptoms. One of these common symptoms, studied here, is vaginal hyperalgesia*** (often called dyspareunia in women). The purpose of this dissertation was to use a combination of immunohistochemical, physiological, and behavioral methods to test various aspects of this hypothesis.

In the first study, the developmental time course of cyst innervation (sensory and sympathetic) and ENDO-induced vaginal hyperalgesia was examined over a 10 week period post-ENDO. It was found that rudimentary innervation **appears** within the cysts at 2 weeks post-ENDO, and becomes **active** at 3 weeks post-ENDO. Between 4 and 5 weeks post-ENDO, vaginal hyperalgesia becomes significant, but is highly variable as the innervation increases and approaches maturity. By 8 to 10 weeks post-ENDO the cyst innervation and hyperalgesia have both matured completely, plateaued and stabilized. Based on these findings, the developmental timeline was divided into three phases: *INITIAL* (1-2 weeks post-ENDO), *TRANSITIONAL* (4-6 weeks post-ENDO), and *ESTABLISHED* (8-10 weeks post-ENDO). In each phase, characteristics of the cyst innervation and vaginal hyperalgesia were found to be as follows: *INITIAL*, no innervation and no vaginal hyperalgesia; *TRANSITIONAL*, **immature** but **active** innervation and significant but highly variable hyperalgesia; *ESTABLISHED*, **mature** innervation and stabilized hyperalgesia both of which varied with the estrous cycle.

Then, in each of the three phases, the contribution of the cysts (and their innervation) to ENDO-induced vaginal hyperalgesia was tested, by removing the cysts and assessing the effect on the development and maintenance of the vaginal hyperalgesia. In the *TRANSITIONAL* phase,

the relationship between the severity of ENDO-induced vaginal hyperalgesia and the innervation of the cysts, eutopic uterus, and vaginal canal was also assessed.

The effect of cyst removal on ENDO-induced vaginal hyperalgesia in the *INITIAL* phase **prevented** the development of vaginal hyperalgesia. In the *TRANSITIONAL* phase, cyst removal did not significantly **alleviate** the vaginal hyperalgesia developed prior to cyst-removal, but, **prevented** its future development. In the *ESTABLISHED* phase, cyst removal completely **alleviate**d the vaginal hyperalgesia. Further, in the *TRANSITIONAL* phase, innervation of the cysts (sensory and sympathetic) and innervation of the vaginal canal (sympathetic only) significantly correlated with severity of ENDO-induced vaginal hyperalgesia.

Overall, results from these studies strongly support the general hypothesis that the innervation of the cysts contributes to ENDO-induced vaginal hyperalgesia. Specifically, the cyst innervation likely contributes to the development, severity, and maintenance of ENDO-vaginal hyperalgesia. Importantly however, the varying effects of cyst removal suggest that mechanisms by which the innervation operates to contribute to the vaginal hyperalgesia change during its progression through the three phases from peripheral sensitization to peripherally-independent then peripherally-dependent, hormonally-modulated central sensitization. Thus changes, which emerge most clearly in the *TRANSITIONAL* phase, could help explain the poorly-understood, clinically-challenging issue on how pain transitions from an acute to a chronic problem, not only in endometriosis but also in other chronic pain conditions.

CHAPTER ONE
GENERAL INTRODUCTION

Endometriosis is an estrogen-dependent disease characterized by extrauteral endometrial growths (also referred to as ectopic growths, lesions, and cysts). The disease affects ~ 10% of all premenopausal women and 30-50% of those who exhibit severe pelvic pain symptoms (Rogers *et al.,* 2009). These painful symptoms include dyspareunia (vaginal hyperalgesia), dysmenorrhea (pain with menstruation), dyschezia (pain on defecation), and chronic pelvic visceral and muscle pain which can greatly reduce quality of life. The amount of ectopic growth (i.e., the sign of the condition) does not correlate with the presence or severity of the pain symptoms (i.e., the symptoms). Endometriosis often co-occurs with other painful disorders including interstitial cystitis/painful bladder syndrome, irritable bowel syndrome, migraine headache, and is associated with subfertility (Stratton & Berkley, 2011). The condition is considered an "enigma" because how painful symptoms become associated with the condition is not known. Due to this lack of knowledge, available pain treatments often provide relief only temporarily, produce unwanted side effects, or are ineffective (Stratton & Berkley, 2011). Hence, additional research is necessary to advance the understanding of mechanisms underlying endometriosis-associated pain.

In a surgical rat model of endometriosis (ENDO), pieces of uterus are auto-transplanted onto the cascade mesenteric arteries. Over time, these transplants produce **signs** similar to those in women; e.g., innervated and vascularized cysts (Berkley *et al.,* 2004, 2005; Stratton & Berkley, 2011). The rat model also produces **symptoms** similar to those of women, including vaginal hyperalgesia (dyspareunia), referred abdominal muscle hyperalgesia, and bladder hyperactivity (Berkley *et al.,* 2004, 1995; Giamberardino *et al.,* 1995; Cason *et al.,* 2003; Nagabukuro & Berkley, 2007; McAllister *et al.,* 2009; Stratton & Berkley, 2011). Further, again similar to women, the amount of ectopic growth in ENDO rats fails to correlate with symptom presence and pain severity (Morrison *et al.,* 2006, Nagabukuro & Berkley *et al.,* 2007) and the rat's fecundity is reduced as in women (Vernon & Wilson, 1985; Stratton & Berkley, 2011).

Although there is a lack of correlation between the amount of growth and symptom presence or severity in both women with endometriosis and the rat ENDO model (Morrison *et al.* 2006; Nagabukuro & Berkley, 2007),excision of the ectopic growths in some women, can provide long-term relief of painful symptoms associated with condition (Coccia *et al.,* 2011).

This finding suggests that some aspect of the growths contributes to the painful symptoms. One likely aspect is the sensory and sympathetic innervation that sprouts into the growths. This sprouted innervation, which was discovered in our laboratory, revealed a two-way line of communication between the growths and the CNS (*see Fig. 25*), a potential source for both the initial development and later the maintenance of endometriosis-associated painful symptoms (Berkley *et al.,* 2004; 2005; Stratton & Berkley, 2011).

Results from another study from our laboratory (Zhang *et al,* 2008) provide further support for a role for the cysts' innervation in ENDO-induced vaginal hyperalgesia. Here, it was found that the cysts' sympathetic innervation density and growth factor contents change in parallel with estrous-dependent changes in hyperalgesic severity. Specifically, during the *ESTABLISHED* phase of development, after ENDO-induced vaginal hyperalgesia has plateaued and stabilized (become **mature**), the severity of vaginal hyperalgesia was found to change with estrous stage; significantly decreasing during the shift from proestrus (estradiol levels high) to estrus (estradiol levels near zero) (Cason *et al.,* 2004). It was further found that this decrease in hyperalgesia is paralleled by a significant decrease in the density of sympathetic innervation of the cysts as well as a significant decrease in the cysts' level of the growth factors VEGF and NGF (Zhang *et al.,* 2008). The growth factors are important because they potentially affect neural activity of both the sensory and sympathetic innervation of the cysts (Sofroniew *et al.,* 2001; Lazarovici *et al.,* 2006). In support of this suggestion, our laboratory also found that sensory fibers that sprout to innervate the cysts become sensitized, as indicated by an increase in their electrical activity (Berkley & Dmitrieva, 2013).

In human studies, evidence is also accumulating suggesting that the innervation of the ectopic growths, rather than the growths themselves, is the most significant predictive factor for endometriosis-associated pain in women (Howard, 2009; Stratton & Berkley, 2011). However, thus far, these studies are indirect due to inherent methodological issues. Specifically, it is difficult to assess the relationship between the growths and the painful symptoms in women directly due to problems in identifying, removing, and analyzing all growths and carrying out appropriate pain assessments and experimental manipulations in the clinic. Thus, the rat model of endometriosis can provide answers unobtainable in patients. Specifically, using ENDO rats, the relationship between the ectopic growths and the symptom of vaginal hyperalgesia (~ dyspareunia, the painful symptom in women with the condition) can be studied comprehensively.

In this model, all growths can be easily found by locating the suture used during the transplant surgery. The cysts can then be removed, measured, and their innervation analyzed using immunohistochemical methods. Further, in the rat model, the amount of vaginal hyperalgesia that develops post-ENDO can be assessed behaviorally to quantify a measure of hyperalgesic severity for comparison with innervation of the cysts and other potentially relevant structures (McAllister *et al.,* 2009).

Given this background information, the first series of experiments comprising this dissertation were designed to test the general hypothesis that the cysts, together with their sprouted sensory and sympathetic innervation, contribute to ENDO-induced vaginal hyperalgesia. The first study of the series analyzed the developmental time course of both the cysts' innervation (sensory and sympathetic) and ENDO-induced vaginal hyperalgesia. Results from this study allowed the developmental time course of both aspects to be divided into three phases as follows: *INITIAL*, *TRANSITIONAL,* and *ESTABLISHED.* The second study focused on the *ESTABLISHED* phase of development and assessed the contribution of the cyst and their **mature** innervation to the maintenance of stabilized ENDO-induced vaginal hyperalgesia. Then, in the third study, during the *INITIAL* phase of development, the contribution of the cysts to the development of ENDO-induced vaginal hyperalgesia was assessed. In a fourth study, during the TRANSITIONAL phase, the contribution of the cysts and their **immature** but **active** innervation, to significant but variable vaginal hyperalgesia was assessed. In a fifth study, also during the *TRANSITIONAL* phase, the relationship between the innervation (sensory and sympathetic) of the cysts, eutopic (normally located healthy) uterus, and vaginal canal and the severity of ENDO-induced vaginal hyperalgesia was assessed. In this same study, the innervation of the eutopic uterus was included as a potential contributor to the hyperalgesia based on findings in clinical studies, in women with endometriosis, suggesting that changes in innervation of their otherwise healthy eutopic uterus could contribute to their pain (Tokushige *et al*., 2007; Bokor *et al*., 2009; Al-Jefout *et al*., 2009; Stratton & Berkley, 2011). The last potential contributor included in this fifth study, the innervation of the vaginal canal, was a logical consideration given that the studies of this dissertation are focused on the symptom of vaginal hyperalgesia.

CHAPTER TWO
GENERAL METHODS

Ethics Statement The studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Florida State University's Animal Care and Use Committee as protocols # 9028, #1212, and #0913. All recovery surgery was performed under ketamine/xylazine anesthesia. Urethane anesthesia was used for terminal procedures.

Subjects Ninety four female adult virgin Sprague-Dawley rats obtained from Charles River (Wilmington, MA; Raleigh NC facility), weighing 175–225g at the beginning of the study were used for all studies combined. Rats 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, and maintained on a 12-h light/dark cycle, with lights on at 07:00 AM.

Estrous stage determination Estrous stage was determined by daily vaginal lavage performed approximately 2 h after lights on for all rats. Traditional nomenclature was used for the four estrous stages of proestrus, estrus, metestrus, and diestrus (Becker *et al.,* 2005). All rats maintained regular four-day estrous cycles throughout the study.

Anesthesia The rats were anesthetized with an intraperitoneal injection of a combination of ketamine hydrochloride (7.3 mg/kg) and xylazine (8.8 mg/kg) for survival procedures. For terminal procedures, they were first anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) then either euthanized via intracardiac overdose of urethane or transcardiac exsanguination (see animal sacrifice procedures section below).

Surgically-induced endometriosis (ENDO) or shamendometriosis (shamENDO) *(Fig. 1)* The ENDO and shamENDO surgeries were done following aseptic precautions and using a protocol originally developed by Vernon and Wilson (1985). Rats in metestrus or 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 x 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. Briefly, after surgery, rats were kept warm on a heating pad under a warming light and monitored continuously until she could move around in the cage to eat food and drink water. She was then returned to her home cage and monitored at least twice/day and weighed once/daily for one week. If sutures did not fall out on their own accord, they were removed ~7 days after surgery. Rats were observed for any signs of pain or distress (writhing, irritability, piloerection, poor grooming, extreme lethargy, hunched position). No rats showed any signs of pain or distress. Regular estrous cycling resumed in all rats within 3 days.

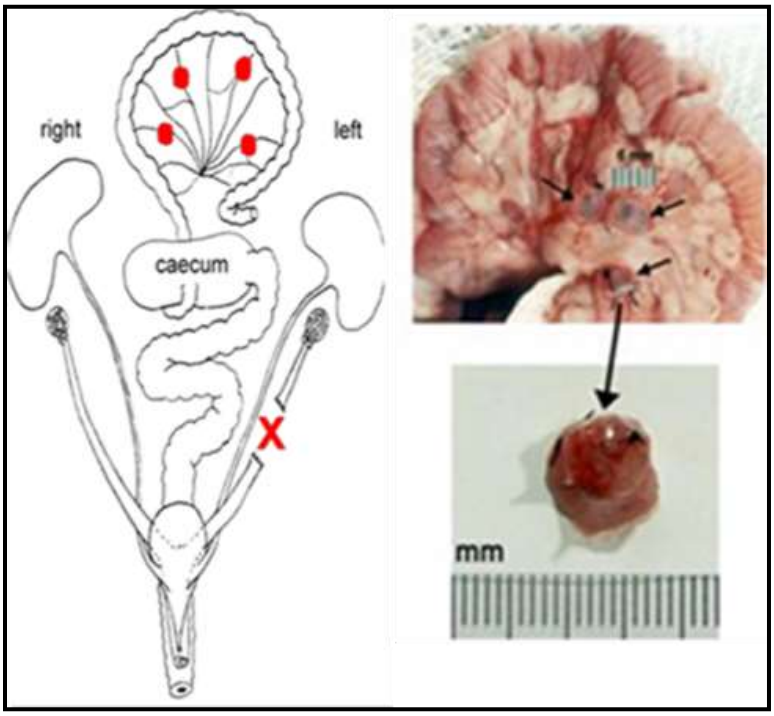

Figure 1. Surgical rat model of endometriosis (ENDO). Diagram (left): red X denotes site where a piece of uterine horn is removed for transplantation, red circles denote locations of cysts on mesenteric arteries after uterine transplant. Photos (right), top-to-bottom: cysts attached to mesentery (small arrows), a single excised cyst (long arrow points to it).

Surgical cyst-removal (CR) or sham-cyst-removal (SCR) The rats were anesthetized and treated during and after these surgeries 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 found, carefully freed from surrounding fat and connective tissue, and then measured. For sham-cyst-removal surgery, no additional manipulation of the cysts was done. For cyst-removal surgery, the sutures were carefully untied and removed with fine-tipped forceps, and the cysts were cut out using a Castroviejo dissecting scissors and a fine-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 as was done after ENDO and shamENDO surgery (see above). Regular estrous cycling resumed in all rats within 3 days.

Behavioral testing procedures Behavioral training and testing procedures were identical to those described in detail previously (Cason *et al.,* 2003; Berkley *et al.,* 2007; McAllister *et al.,* 2009; McAllister *et al.,* 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 in random order at intervals of ~60 s, and the percent escape response to each volume was calculated (*Fig 3*).

Behavioral apparatus and stimulator (*Fig. 2)* 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 photo sensor 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. This behavior, breaking the light beam, was defined as 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 (~10 mm long x 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 (for detailed information about pressures refer to Bradshaw *et al.,* 1999).

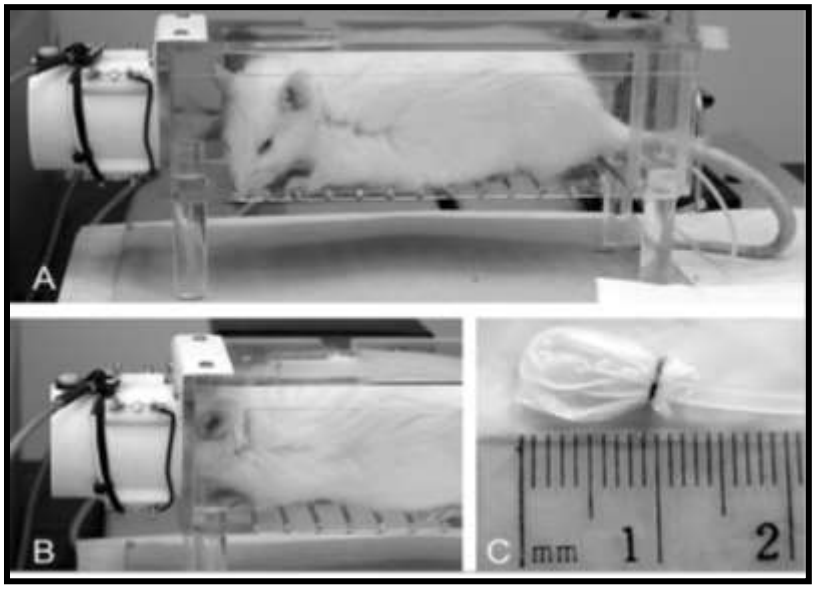

Figure 2. Behavioral testing apparatus, procedure, and stimulator. (A) Rat resting in testing chamber. (B) Rat escaping from vaginal distention by inserting nose in white tube. (C) Close-up of vaginal distention device (balloon). Figure adapted from Bradshaw & Berkley (2002) with permission.

Behavioral training During training, rats were first adapted to the testing chamber by being placed in the chamber for 10 min daily for 3–4 days (and being fed small amounts of peanut butter on a wood stick). Then training sessions were done to shape the required escape response. To train this response, the trainer gently pinched a rat's tail with a padded forceps using release of the pinch to gradually "reward" behaviors in which the rat approached the tube (rather than try to turn around to get at the forceps), and then extended its head into the tube (that behavior in actual testing situations would interrupt a light beam). Training sessions of 10 pinches delivered at ~1 min-intervals were run 3/week on non-consecutive days. Training was usually completed (>80% escape behavior) in 4–8 sessions. Rats were next trained to make identical escape responses, this time to deflate vaginal distention stimuli. 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. The vaginal training sessions were run 3/week on non-consecutive days for a total of 3–5 sessions. Ten large distention volumes (0.80–1.0 ml, inflation rate 1 ml/s) were delivered for a maximum of 15 s at ~1-min intervals. All rats learned the escape response, that is, responding 100% of the time, within 2–4 sessions. Once trained, actual testing sessions, began within a few days.

Behavioral testing sessions Testing sessions were run 3–4 times/week 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 15s elapsed, when the balloon rapidly deflated (0.5 ml/s). Eight different distention volumes, including a near zero level control volume of 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 as a non-response. The experimenter was blind to the volumes being delivered to the rat. After the escape trials were run, the rat was given a small amount of peanut butter on a wood stick, allowed to eat it in the chamber, and then returned to her home cage.

Data analyses of behavioral results: calculation of vaginal nociception Percent escape responses and vaginal pressures as a function of distention volume were assessed in each session. For each rat, the escape percentages and vaginal pressures from all sessions within each testing period were combined and mean values calculated. For example, in chapter 4 (*Fig. 8*) there were 3 testing periods as follows: baseline, middle, and late period. For each experimental testing period, an equal number of sessions were run in each of the four estrous stages. The averages from each of the rats were combined by group (when applicable) and entered into spreadsheets. This procedure produced a graph of the percent escape response as a function of distention volume for each testing period. 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 different testing period conditions.

Data analyses of behavioral results: calculation of area-under-the-curve (AUC) (Fig. 3) Using standard trapezoid rule methods (Yeh, 2002), AUC calculations were made on the data from each rat. This calculation yielded a single value of "AUC units" that provided an estimate of vaginal nociception during a specific testing period. When studies consisted of more than one group, two overall analyses were initially performed. First, a one-way ANOVA was done to assure that baselines did not differ between groups. Second, a two-way repeated measure ANOVA was done to determine whether the data differed across groups and testing period. Following these initial analyses, differences in the AUC between baseline and other testing periods were calculated for each rat, and then combined by group. Statistical differences between the two sets of AUCs were assessed by Student t-tests. All statistical analyses were done using Statistical Package for the Social Sciences software, version 15 or 17 (SPSS, Chicago, IL). Significance was set at $p \leq 0.05$.

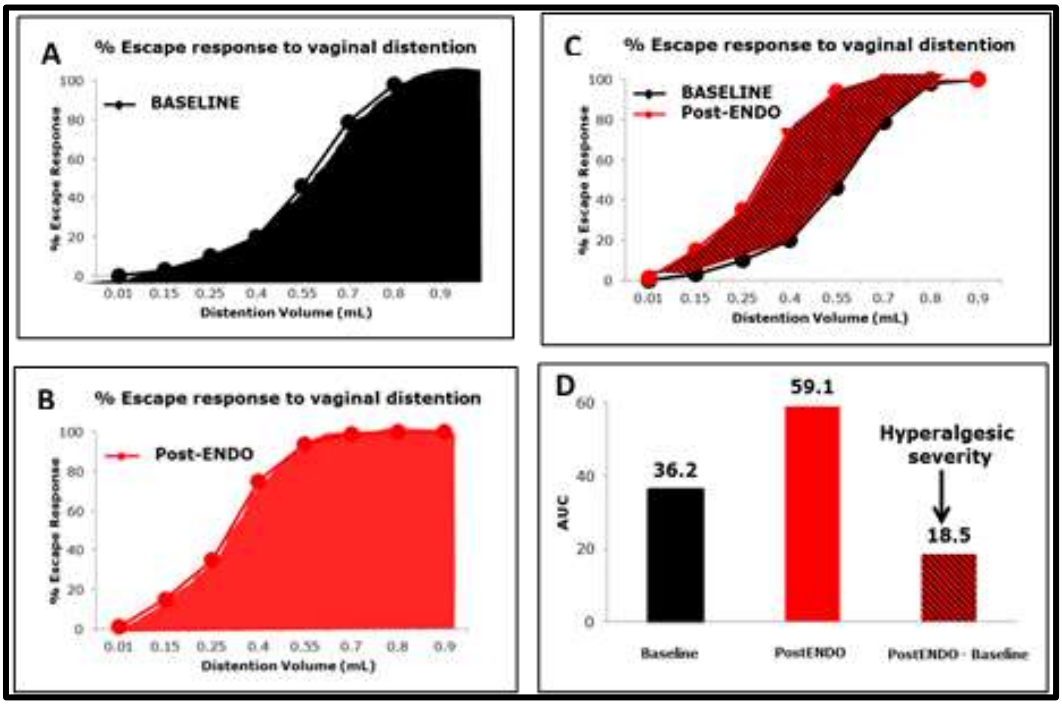

Figure 3. Calculation of hyperalgesic severity. Vaginal hyperalgesia is defined as a significant increase in vaginal nociception. Hyperalgesic severity is calculated by subtracting the (A) Baseline AUC (area-under-the-curve) value (black) from the (B) Post-ENDO AUC (solid red) which yields (C) the severity of hyperalgesia (black stripes on top of the solid red background). (D) Bar chart representing AUC for the baseline, Post-ENDO, and Post-ENDO minus baseline (hyperalgesic severity).

Animal sacrifice, tissue collection (fixed and fresh), cutting, & mounting At the time of sacrifice, rats were first anesthetized with urethane (1.2 g/kg), then the abdominal and pelvic cavities and organs opened, examined, and autopsy notes taken. These notes included measurements of cyst size (if present), and the appearance of pelvic and abdominal organ tissues. Cysts (and additional tissues such as the vaginal canal, bladder, parts of the colon and small intestine) were then collected. For fresh tissue collection, rats were then euthanized via intracardiac overdose of urethane. For fixed tissue collection, rats underwent transcardiac exsanguination, first with saline and then followed by perfusion with 4% paraformaldehyde and then their tissues harvested, post-fixed in 4% paraformaldehyde for 1 h, and then incubated in 30% sucrose overnight. For fresh tissue collection, tissue was carefully removed from the anesthetized rat, immediately frozen in dry ice, and then stored at -80 °C. Both fixed and fresh tissues were then 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).

Immunohistochemistry and analysis (fluorescence) Adjacent sections from each cyst were respectively single-immunolabeled using markers for sensory C-fibers and sympathetic fibers, as follows. Sections were treated with 5% normal goat serum (NGS) for 1 h, then were immunolabeled with one of the two rabbit primary antibodies: calcitonin gene related peptide (CGRP, sensory C-fibers, 1:4,000; Chemicon, CA) or for sympathetic fibers, vesicular monoamine transporter 2 (VMAT2, 1:1,000; Chemicon, CA) including 2% NGS at 4uC overnight. Sections were then incubated for 1.5 h with goat anti-rabbit Cy2-conjugated secondary antibody (1:300, Jackson ImmunoResearch lab, PA). Negative controls were routinely performed for each immunostaining run; they included omission of the primary or the secondary antibody or both. All immunostained sections were carefully examined using an epifocal microscope, and fiber labeling, if any, noted and qualitatively characterized.

Immunohistochemistry (3, 3' Diaminobenzidine) Adjacent sections for the same cysts, eutopic uterus, or vaginal canal were processed. Fresh sections were first briefly (10 min) post fixed with ice-cold acetone. Then tissue sections, fresh or fixed, were quenched with 0.3% H_2O_2 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 then immunostained with one of the following: rabbit anti-vesicular monoamine transporter 2 (VMAT2; 1:10,000; Chemicon, Temecula, CA), goat anti-tyrosine hydroxylase (TH; 1:800, Millipore, Temecula, CA), 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 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 Labs). Staining was visualized with 3, 3' diaminobenzidine (DAB kit, Vector Laboratories). For each antibody, the final dilution used (as specified above) had been determined in test sections that yielded the maximum labeling of neurites with minimal background. To minimize staining variability due to processing, sections from tissues in different groups were processed simultaneously with the same antibody. 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.

Quantification of Innervation (DAB) After processing, tissues were assessed microscopically for evidence of positive labeling. Initially, the density of nerve fibers was quantified as previously published (Zhang *et al.,* 2008). Briefly, in the cysts, VMAT2-, TH-, CGRP-, or SP -labeled sections from each rat were examined to identify a single section in that cyst that was cut through its center and the area within that section containing the densest labeling. (This area was usually the **hylus**, *see Fig. 4 below*) Care was taken so that the same area from the CGRP-, VMAT2-, and TH-stained sections from each cyst was analyzed. Each chosen section was photographed and exported to the Stereo Investigator program (MicroBrightField, VT) and coded. The appropriate area was outlined and # fibers within the area counted, so that the outcome measure was # fibers/mm2. Counts from the cysts from the same rats were averaged to produce an average fiber density for that rat for each marker.

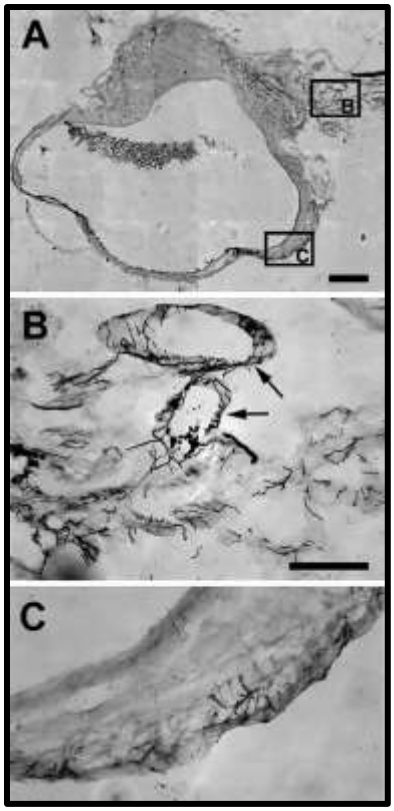

Figure 4. Photomicrographs of a cyst section showing the hylus region, myometrium, and epithelial lining. (A) Low-power view of area where photomicrographs in B and C were taken. (B) at the entrance to the cyst, neurites, shown here, the sympathetic (VMAT2-positive) fibers, are very dense and associated mainly with blood vessels (arrows) as they enter the wall of the cyst in the **hylus** region. (C) Further into the cyst, these neurites (labeled with VMAT2) become less dense but also extend into the myometrium and epithelial lining. Calibration bar: 500 μm for A; 100 μm for B and C. Figure adapted from Zhang *et al.* (2008).

Because this original quantification methodology proved laborious and overly time consuming (~2 hours/section/investigator), a different assessment method was established for use in later experiments. Briefly, at least two experimenters from a group of five, blinded to estrous stage and condition, but not blinded to marker, established a Likert-type scale of 0–4, where 0 indicated no fiber labeling and 4 indicated the densest possible labeling for each of the markers. To use this scale, all sections from each cyst stained with one of the four markers (i.e., VMAT2, TH, CGRP, or SP) were carefully scrutinized microscopically several times by each experimenter so that the range of 0–4 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. Overall, the scores of the five experimenters, at least two of whom assessed labeling for each

experiment, were highly correlated, with r > 0.9 for all scores. The average scores from the 2 or more assessors for each experiment were used to assess significance of differences between groups with the Wilcoxon Signed Ranks Test when applicable.

To justify the use of this more efficient, Likert-like method, its validity was tested by comparing the two assessment performed on the same sections through five cysts that had been immunostained for sensory or sympathetic nerve fibers. Assessment values correlated highly, with r values > 0.9.

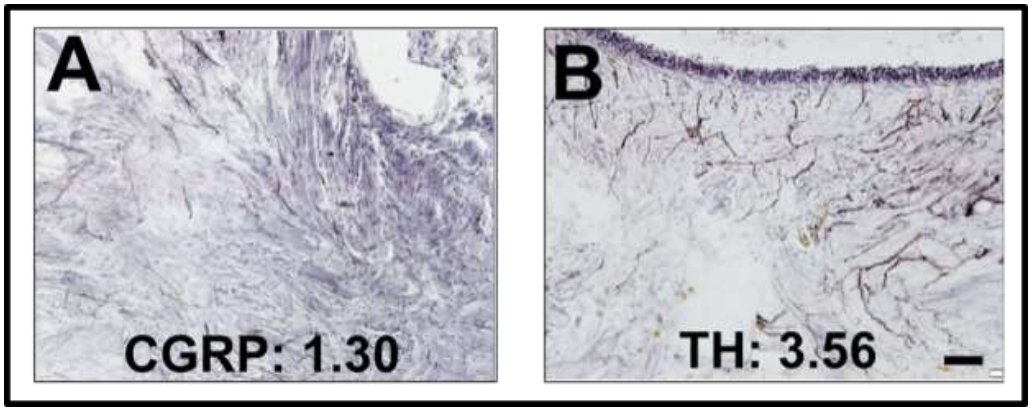

Figure 5. Examples of sensory and sympathetic fiber labeling within the cyst. (A) Sensory (GCRP) labeling, mean score = 1.30 (rat #4581). (B) Sympathetic (TH) labeling, mean score = 3.56 (rat # 4445). Calibration bar in B is 50 µm.

Calculation of Cyst Burden (CB) When cysts were located at the time of sacrifice (or at the time of sham-cyst removal), they were freed from surrounding fat and connective tissue, and their largest and smallest diameter measured to allow calculation of that 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 & Berkley, 2007). For example, suppose the four cysts from a rat had the following large and small diameters: 4 x 2mm=8; 3 x 1mm=3; 5 x 2 mm = 10; 6 x 2mm=12. Adding the multiplicands of 8+3+10+12 yields a cyst burden for this rat of 33. If no cysts were found, the cyst burden was a zero for that rat. Pearson correlation coefficient statistics were then used to assess correlations between the cyst burden and hyperalgesic severity.

Evans Blue dye (EB) extravasation The Evans Blue dye (EB) extravasation method was used in intact and denervated (splanchnic nerve and coeliac ganglion) cysts in rats to assess neurogenic sensitization indirectly. Rats were first anesthetized with urethane (1.2 g/kg) and placed on a warm heating pad (maintained as close as possible to 37° C). In some rats, cysts were denervated before EB was delivered. In those rats, a laparotomy was performed to expose the splanchnic nerve and coeliac ganglion. The splanchnic nerve was identified and cut rostral to the coeliac ganglion, and the ganglion itself was carefully denervated by cutting all nerves associated with it. The abdominal muscles and skin were sutured, after which EB was injected, as follows. The jugular vein was exposed through an incision and catheterized. EB (50 mg/kg in saline) was then delivered through the catheter. Thirty min later, excess dye was rinsed out of blood vessels by delivering 200 ml of saline through the catheter. Cysts were harvested, weighed, and incubated in formamide at 60° C for 48 h. Optical density of the sample and standard solutions was measured spectrophotometrically (l = 620 nm, UV: 1601; Shimadzu, Columbia, MD). The amount of EB extracted from each sample was calculated as mg EB/g of tissue.

CHAPTER THREE

SENSORY AND SYMPATHETIC INNERVATION OF ENDOMETRIAL CYSTS AND ENDO-INDUCED VAGINAL HYPERALGESIA OCCURS IN THREE PHASES: *INITIAL, TRANSITIONAL, AND ESTABLISHED*

McAllister SL, Dmitrieva N, Berkley KJ. Sprouted innervation into uterine transplants contributes to the development of hyperalgesia in a rat model of endometriosis. PLoS ONE 2012; 7:e31758.

3.1 Introduction

The main goal of this dissertation was to test the general hypothesis that the cysts and their innervation contribute to ENDO-induced vaginal hyperalgesia. However, first, the developmental time course of both the sensory and sympathetic innervation of the cysts and ENDO-induced vaginal hyperalgesia had to be determined.

From a previous study in our laboratory, we knew that when baseline (pre-ENDO) vaginal nociception, was compared with nociception at intervals after surgery (post-ENDO), that vaginal nociception increased in the first month post-ENDO period, but did not become significantly greater than baseline (i.e., hyperalgesia did not become significant) until 5 weeks post-ENDO, then stabilizing later (Cason *et al.,* 2003). Thus, overall, the results suggested that post-ENDO, vaginal hyperalgesia appears in the first month, becomes significant in the second month, and then stabilizes.

Additional studies from our laboratory, showed that after ENDO, sensory and sympathetic innervation sprouts to supply the cysts, and when examined at 8 and 11 weeks post-ENDO, the innervation is **mature**. Once **mature**, the innervation is robust and densest at the region of the hylus (*Fig. 4*), the area where blood vessels enter the cyst. This innervation density progressively decreases as the distance from the hylus region increases. Further, as this innervation develops, it accompanies the blood vessels as they enter the cyst hylus. Together, the blood vessels and innervation travel through the cyst wall, and then, small bundles and individual fibers, extend first into the myometrium, then the endometrial stroma, and finally the epithelium lining the lumen (*Fig. 4*) (Berkley *et al.,* 2004, 2005; Zhang *et al.*, 2008).

Here, this first study was designed to assess, in detail, the developmental time course, of the cyst innervation (sensory and sympathetic) and the ENDO-induced vaginal hyperalgesia. This assessment was done over a 10 week period post-ENDO, based on limited background information that, both the cyst innervation and vaginal hyperalgesia appears within the first month post-ENDO, and are then **mature** and stable, respectively, by around 2 months post-ENDO. This assessment also included determining when the innervation becomes **active**.

If the innervation of the cysts is contributing to the development of ENDO-induced vaginal hyperalgesia, then the innervation should sprout to supply the cysts *prior* to the appearance of significant vaginal hyperalgesia. Furthermore, this innervation should become **activ**e; that is, become able to activate neurons in the central nervous system (CNS), *prior* to the appearance of significant vaginal hyperalgesia (Berkley & Stratton, 2011). If the cyst innervation is contributing to both the development and maintenance of vaginal hyperalgesia, then, once the innervation and significant vaginal hyperalgesia appear Post-ENDO, the two should develop and stabilize in parallel, over the remainder of the 10 week time period.

Once the detailed developmental time course of the cyst innervation and ENDO-induced vaginal hyperalgesia was determined, then subsequent studies were designed to test the general hypothesis that the cysts and their innervation contribute to ENDO-induced vaginal hyperalgesia.

3.2 Methods

Subjects Forty-four female virgin Sprague-Dawley rats, weighing 175-225g at the beginning of the study were used. Rat housing and Estrous stage determination was as described in *General Methods*. Rat housing and Estrous stage determination was as described in *General Methods*.

Anesthesia All rats were anesthetized as described in *General Methods*.

Surgically-induced endometriosis (ENDO) The ENDO surgeries were performed and post-operative recovery monitored as described in *General Methods*.

Behavioral procedures Behavioral training, behavioral testing procedures, and testing sessions were completed as described in detail in *General Methods*.

Behavioral training and testing Behavioral training and testing as described in *General Methods* was done to assess vaginal nociception in rats (n=8). Baseline data was collected for 6–8 weeks before surgery, then again after ENDO surgery (post-ENDO) for 10 weeks. Testing

sessions in each of the two assessment periods included at least 3 days in each estrous stage. Data presented in *Fig.6* were obtained during proestrus, the stage when hyperalgesic severity is greatest post-ENDO (Cason *et al.,* 2003).

Data analyses of behavioral results Assessment of vaginal nociception and hyperalgesia were calculated using area-under-the-curve (AUC) methods as described in *General Methods*. For every rat, in every behavioral testing session, the percent escape response to eight distention volumes was assessed. The percent escape responses for each volume was then averaged within each testing period (i.e. baseline, and 2, 3, 4, 5, 6, 7, 8, 9, 10 weeks post-ENDO). These averages were then graphed as percent escape response as a function of distention volume. For each graph, an area-under-the-curve (AUC) calculation was then performed yielding a single value describing vaginal nociception for each testing period as described in *General Methods*. Statistical assessment included a one-way ANOVA, which was significant ($F_{8, 64} = 4.83$, $p < 0.001$), and therefore followed by post-hoc LSD tests, with significance set at $p \leq 0.05$).

Immunohistochemistry (fluorescence): sacrifice, tissue collection (fixed), cutting & mounting Rats in proestrus at 2, 3, 4, 6, or 10 weeks post-ENDO (n = 5 rats at each survival time) were perfused and their cysts collected (fixed), processed, and slide mounted as described in *General Methods.*

Immunohistochemistry (fluorescence): protocol and analysis Adjacent sections from the same cysts were then processed via immunofluorescence with a marker for sensory C- fibers, calcitonin gene related peptide (CGRP), or for sympathetic fibers, vesicular monoamine transporter 2 (VMAT2) and fiber labeling qualitatively characterized as described in *General Methods*.

Evans Blue dye (EB) extravasation Neurogenic activity was assessed by comparing EB dye extravasation in intact and denervated cysts from rats at 1, 2, 3, 4, 6, or 10 weeks after ENDO surgery (n=36 rats total, with n=3 rats for each survival time and each innervation class; i.e., intact or denervated) using the Evans Blue dye (EB) (*see General Methods*). Within each survival time and each innervation class (intact, denervated) 10 cysts were assessed.

Rats in the 1, 2, 3, 4, and 6 week groups were in proestrus on the day of the experiment. For the 10 week group we only had data from rats in metestrus on the day of the experiment. We chose to include these data because the severity of hyperalgesia in rats in metestrus is nearly as severe as it is in proestrus (Cason *et al.,* 2004); including this 10 week group would allow a more complete comparison to be made with the behavioral data shown in *Fig 6*. Because a two-way ANOVA was significant ($F_{11,120}$ =2.44, p =0.009), data were analyzed by post-hoc unpaired t-tests, with significance set at $p \leq 0.05$.

3.3 Results

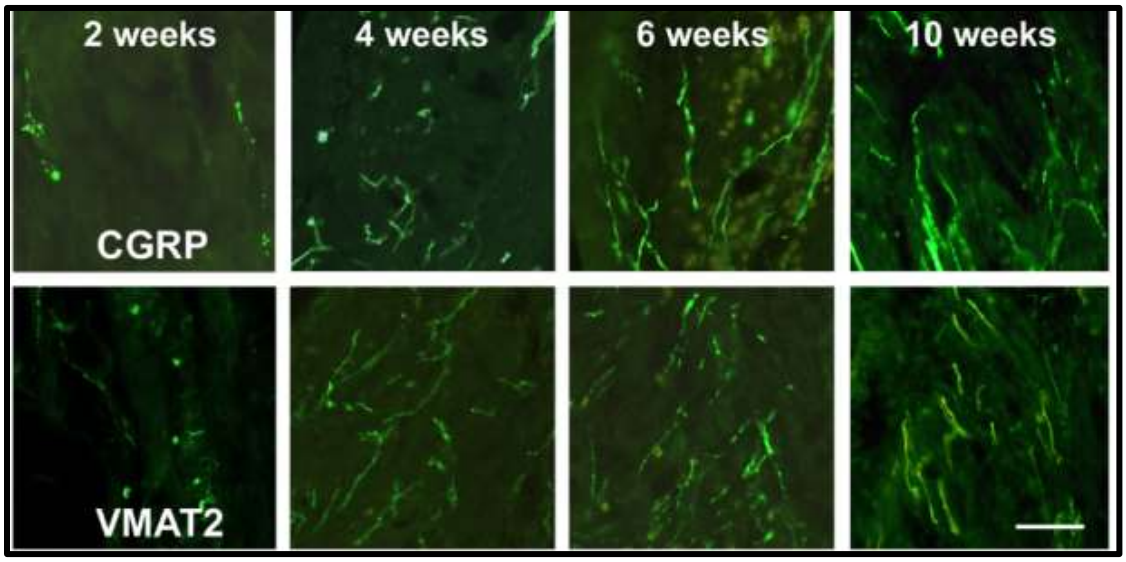

Figure 6. Time course of the development of innervation within the cysts after ENDO surgery. Photomicrographs of sensory (CGRP-positive) and sympathetic (VMAT2-positive) fibers in the cysts labeled at 2, 4, 6, and 10 weeks after ENDO surgery (n = 5 rats/survival time). Calibration bar is 50 μm. Sensory and sympathetic innervation appears within the cysts by 2 weeks, increase in number and density over the next ~6 weeks, and then attain a **mature** appearance by 10 weeks post-ENDO. Figure adapted from McAllister *et al.* (2012).

*Sensory and sympathetic innervation appeared within the cysts by 2 weeks post-ENDO and attained a **mature** appearance by 10 weeks post-ENDO (Fig.6)* Immunofluorescence staining with antibodies to CGRP (marker for sensory-fibers) and VMAT2 (marker for sympathetic fibers) revealed that both sensory and sympathetic neurites began to appear within the cysts at 2 weeks post-ENDO (i.e., no neurites were observed inside cysts harvested one week post-ENDO).

Further, at this 2 week post-ENDO time period, the neurites had a rudimentary, mostly punctate appearance and were located around the hylus (*Fig. 4*), the area where blood vessels are clearly seen to enter the cyst. Later, at 4 weeks post-ENDO, the characteristics of both sensory and sympathetic neurites changed their appearance so that they were no longer punctate, but instead branched. The density of both fibers at 4 weeks also dramatically increased, populating the entire wall of the cysts including the myometrial and endothelial layers, and remaining densest in the hylus area of the cysts. At 6 weeks post-ENDO, the sensory and sympathetic nerve fiber appearance further increase in density within the cysts. At 10 weeks post-ENDO, the density of both fibers had increased even further and were virtually identical to those previously observed in **mature** cysts 8 weeks post-ENDO (Berkley *et al.,* 2004) and 11 weeks post-ENDO (Zhang *et al.,* 2008).

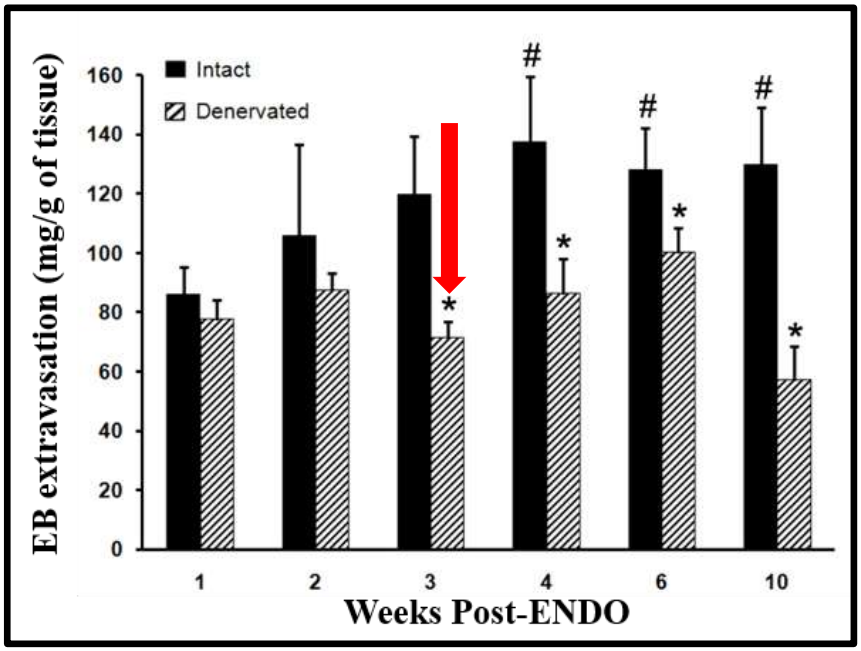

Figure 7. Time course of the effect of cyst denervation on Evans Blue dye extravasation after ENDO surgery. EB dye extravasation into *intact* cysts became significantly different from the 1 week time period at 4 weeks post-ENDO, #, ($p \leq 0.05$). The differences between *intact* and denervated cysts became significant at 3 weeks Post-ENDO, *, $p \leq 0.05$. Error bars are ± SEM (n=36 rats total; n = 3 rats per group with 10 cysts analyzed per group). Red arrow: note that the sensory fibers became functionally **active** (produced neurogenic activity) ~ 3 weeks after ENDO surgery, which is immediately following the appearance of the innervation within the cysts (*Fig. 6)*. Figure adapted from McAllister *et al.* (2012).

Cyst innervation became functionally active at 3 weeks post-ENDO *(Fig. 7)* EB dye extravasation into intact cysts increased progressively after ENDO ($F_{5,67} = 2.90$, $p = 0.02$) and became significantly different at 4 weeks post-ENDO from 1 week post-ENDO ($p \leq .05$). To estimate when neurogenic activity contributed to this extravasation, the extravasation in intact cysts was compared with the extravasation in cysts that had been denervated before the dye was injected. In other words, the neurogenic component could be assessed by the denervation-associated reduction in extravasation. Of importance is that, whereas there was a gradual increase in dye extravasation in the intact cysts, there were no significant differences over time after ENDO surgery in the denervated cysts ($F_{5,65} = 0.77$, $p = 0.57$). The differences between the intact and denervated cysts became significant 3 weeks Post-ENDO surgery, and remained that way through 10 weeks post-ENDO. In other words, sensory fibers became abnormally active (produced neurogenic activity) approximately 3 weeks after ENDO surgery.

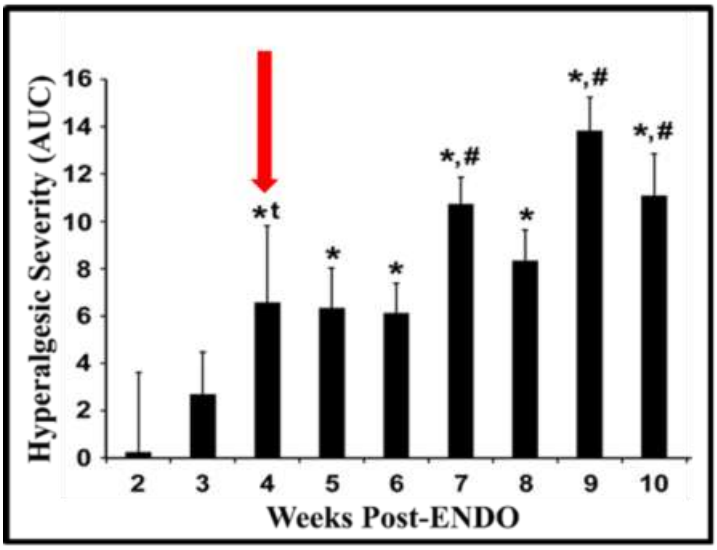

Figure 8. Time course of the effect of ENDO surgery on vaginal nociception and the development of vaginal hyperalgesia. Hyperalgesic severity (Baseline AUC subtracted from the post-ENDO AUC) at different times after ENDO surgery in the same rats. Error bars are ± SEM ($n = 8$). *, differs from 2 weeks; #, differs from 6 weeks, $p \leq 0.05$. For the 4 week time point, $*t = 0.059$. ENDO induces a vaginal hyperalgesia that appears at 4 weeks, becomes significant at 5 weeks, increases, plateaus, and then stabilizes by 10 weeks post-ENDO. Red arrow: note that at the 4 week time point vaginal hyperalgesia is approaching significance, which is immediately following when the cysts' sensory innervation becomes functionally **active** (*Fig. 7*). Figure adapted from McAllister *et al.* (2012).

ENDO-induced vaginal hyperalgesia reached significance at 5 weeks post-ENDO, increased and plateaued, and then stabilized by 10 weeks post-ENDO *(Fig. 8)* At 2 weeks post-ENDO, no changes in vaginal nociception occurred. At 3 weeks post-ENDO, vaginal hyperalgesia began to appear; i.e. there was an increase in the probability of escape responses to vaginal distention relative to baseline responses that was not significant. At 4 weeks post-ENDO, vaginal hyperalgesia increased further and had a notable amount of inter-rat variability relative to other time periods. By 5 weeks post-ENDO, vaginal hyperalgesia on average became significant. Over the next few weeks, hyperalgesia further increased, plateaued, and then finally stabilized by 10 weeks post-ENDO.

3.4 Discussion

Overall, this first study demonstrated that a progression of events occur post-ENDO. First, by 2 weeks post-ENDO, a rudimentary sensory and sympathetic innervation that appears punctate appears in the cysts. A week later, at 3 weeks post-ENDO, this innervation becomes functional (can induce neurogenic extravasation). At 4 weeks post-ENDO, the cysts are more densely populated by sensory and sympathetic nerve fibers that are no longer punctate, and vaginal hyperalgesia appears but is not yet significant. By 5 weeks post-ENDO, the vaginal hyperalgesia reaches significance. After this time point, both the hyperalgesia and the sensory and sympathetic innervation of the cysts continue to increase over time in parallel, plateau, and then stabilize by 10 weeks post-ENDO and are considered **mature**.

Importantly, two important findings of this study on the progression of innervation and hyperalgesia support the hypothesis that the cyst innervation contributes to the ENDO-induced vaginal hyperalgesia. First, the time point that the innervation infiltrates the cyst and then becomes **active**, 2 weeks and 3 weeks respectively, is immediately *prior* to the appearance of significant vaginal hyperalgesia (between 4-5 weeks post-ENDO), suggesting that the innervation contributes to the development of the hyperalgesia. Second, the fact that once the innervation and hyperalgesia both appear, they continue to increase in parallel and then stabilize at 8 to 10 weeks post-ENDO, suggests that the innervation contributes to the continued development and then later the maintenance of vaginal hyperalgesia.

Based on the overall results from this study, the developmental time course of the innervation of the cysts and the vaginal hyperalgesia was together separated into 3

distinguishable phases as follows: *INITIAL, TRANSITIONAL* and *ESTABLISHED* (*Table 1)*. In the *INITIAL* phase of development (1-2wks post-ENDO), innervation has not yet sprouted into the cysts and vaginal hyperalgesia has not yet appeared. In the *TRANSITIONAL* phase (4-6wks post-ENDO), **active** innervation has infiltrated the cysts and vaginal hyperalgesia has become significant overall, but remains variable between rats (CV: coefficient of variation = 1.67). In the *ESTABLISHED* phase (8-10wks post-ENDO), cyst innervation and vaginal hyperalgesia have already increased, plateaued, and now stabilized in parallel and are **mature**. During this *ESTABLISHED* phase, hyperalgesic severity is now less variable between rats (CV = 0.28).

Once these 3 developmental phases were defined, in the next study, cysts were removed during each phase (in different groups of rats) to assess the contribution of the cysts and their innervation to ENDO-induced vaginal hyperalgesia. Thus, in these studies, described in chapters 4, 5, and 6, cysts were removed in the *ESTABLISHED*, *INITIAL*, and then *TRANSITIONAL* phase, respectively.

Table 1. Phases of Development: *INITIAL, TRANSITIONAL,* and *ESTABLISHED* phases.

Developmental Phase	Cyst Innervation	Vaginal Hyperalgesia	Weeks Post-ENDO
INITIAL	**absent**	**absent**	**1-2 wks**
TRANSITIONAL	**immature but active**	**highly variable between rats, but overall significant**	**4-6 wks**
ESTABLISHED	**mature**	**stabilized**	**8-10 wks**

CHAPTER FOUR

ESTABLISHED PHASE: CONTRIBUTION OF THE CYSTS AND THEIR INNERVATION TO ENDO-INDUCED VAGINAL HYPERALGESIA.

McAllister SL, McGinty KA, Resuehr, D, Berkley KJ. Endometriosis-Induced vaginal hyperalgesia in the rat: role of the ectopic growths and their innervation. Pain 2009; 147:255-264. Copyright permission request to reproduce: Full Article Dec 2009 15; 147(1-3): 255-64 granted by the International Association for the Study of Pain® (IASP) reference #: 14-02231.

4.1 Introduction

Table 2. Phases of Development: *ESTABLISHED* phase.

Developmental Phase	Cyst Innervation	Vaginal Hyperalgesia	Weeks Post-ENDO
INITIAL	absent	absent	1-2 wks
TRANSITIONAL	immature but active	highly variable between rats, but overall significant	4-6 wks
ESTABLISHED	**mature**	**stabilized**	**8-10 wks**

As in women with endometriosis, the severity of painful symptoms in rats with ENDO fails to correlate with the *amount* of ectopic endometrial growth or "cyst burden" (Berkley & Nagabukuro, 2007; McAllister *et al.,* 2009). However, the relationship between the painful symptoms and the actual *presence* of the growths is unclear. In other words, it is unknown if the presence of the cysts is necessary for the maintenance of painful symptoms. Relevant to this question, is that in woman experiencing endometriosis-associated pain, surgical removal of the cysts can sometimes be effective in reducing painful symptoms (Abbott *et al.,* 2003; Vercellini *et al.,* 2003; Coccia *et al.,* 2011; Healey *et al.,* 2014). However, in these women, in whom surgical removal is effective, the probability of pain recurrence is between 20-40% (Vercellini *et al.,* 2009). Further, 20% of women who experience the recurrence of painful symptoms have no visible endometriotic lesions (Abbott *et al.,* 2003). These findings suggest that the presence of

the cysts, or some aspect of the cysts, contributes to the maintenance of painful symptoms in *some* women with endometriosis but that other factors are also contributing.

In the rat model of ENDO, the effect of surgical removal of the cysts on pain symptoms has not been assessed. Hence, the first logical step here was to remove the cysts during the *ESTABLISHED* phase, when the cyst innervation and vaginal hyperalgesia are **mature** (have increased, plateaued, and are stable), and assess the effect on the hyperalgesia. If, when the cysts are removed, vaginal hyperalgesia is reduced or eliminated, this supports the hypothesis that the presence of the cysts, which, importantly, includes their innervation, contributes to ENDO-induced vaginal hyperalgesia in the *ESTABLISHED* phase.

4.2 Methods

Subjects Twenty-two adult female virgin Sprague-Dawley rats, weighing 175-225g at the beginning of the study were used. Rat housing and estrous stage determination was as described in *General Methods*.

Anesthesia All rats were anesthetized as described in *General Methods*.

Surgically-induced endometriosis (ENDO) or shamendometriosis (shamENDO) The ENDO and shamENDO surgeries were performed and post-operative recovery monitored as described in *General Methods*.

Surgical cyst-removal (CR) or sham-cyst-removal (SCR) The cyst-removal and sham-cyst-removal surgeries were performed and post-operative recovery monitored as described in *General Methods.*

Behavioral procedures Behavioral training, behavioral testing procedures, and testing sessions were completed as described in detail in *General Methods*.

Behavioral Groups There were 5 main groups, as summarized in *Fig. 9* below. *Group 1*, the experimental group, consisted of rats that underwent ENDO surgery followed by complete cyst-

removal surgery (n = 6) during the *ESTABLISHED* phase of development. In *Group 2*, a control for a second surgery after ENDO, rats underwent ENDO surgery followed by a sham-cyst-removal surgery (n = 4) during the *ESTABLISHED* phase. In *Group 3*, a control for surgery after ENDO, rats underwent ENDO surgery and then were tested afterwards with no subsequent surgery for the same duration as Groups 1 and 2 (n = 4). In *Group 4*, a control for two successive surgeries, rats underwent shamENDO surgery followed by a second surgery during the *ESTABLISHED* phase. This second surgery, a sham-cyst-removal, mimicked cyst-removal surgery (n = 3). In *Group 5*, a full control for any surgery as well as for duration of vaginal nociceptive testing, vaginal nociception was assessed for a total time period similar to that in Groups 1–4, but with no surgery ever being performed in these rats (n = 3). Two additional rats, originally part of *Group 1* (ENDO surgery followed by cyst-removal surgery) were described separately because at autopsy either unusual pathology or an ectopic cyst was found (details provided below). As shown in *Fig. 9* below data from all five groups were compared between three chronological testing periods: (i) an initial baseline period of 6-8 weeks, (ii) a middle period of 8-10 weeks (post-ENDO, post-shamENDO, post ENDO period 1), and finally, (iii) a late period of ~ 8 wks (post-cyst-removal, post-sham-removal, or post-ENDO period 2). Importantly, it was at the end of the middle-testing period of ~8-10 weeks, in the experimental group (*Group 1*), that cysts were removed in *ESTABLISHED* phase to assess the effect on the post-ENDO vaginal hyperalgesia that had developed and stabilized.

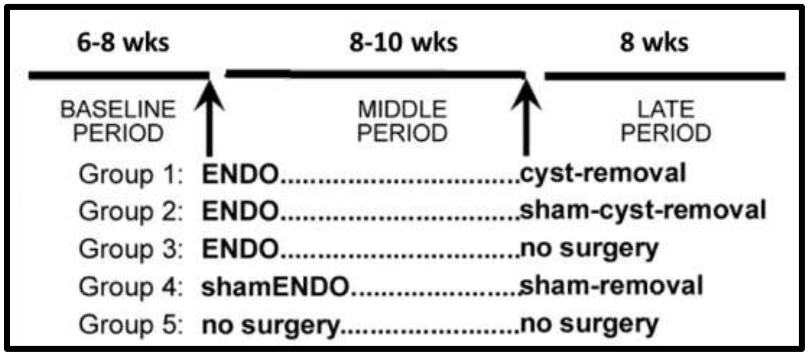

Figure 9. Summary and timeline of the three periods of assessment of vaginal nociception for the five groups in Chapter 4. *Group 1*: rats that underwent ENDO surgery followed by complete-cyst-removal surgery during the *ESTABLISHED* phase. *Group 2*: rats that underwent ENDO surgery followed by sham-cyst-removal surgery during the *ESTABLISHED* phase,

Figure 9 – continued

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 6-8 week period comprised the initial assessment of vaginal nociception. Middle period: this 8-10 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 8 weeks. In one rat in *Group 1*, however, the assessment period continued longer so that the total duration was ~6 months

Data analyses of behavioral results: vaginal nociception Percent escape responses and vaginal pressures as a function of distention volume were measured in each session as described in *General Methods*.

Data analysis of behavioral results: area-under-the-curve (AUC) calculations AUC calculations for all rats, using standard trapezoid rule methods (Yeh, 2002) were done as described in *General 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 periods. 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 ($F_{8, 45} = 4.12$, $p = 0.001$).

Additional analyses of behavioral data over time after surgery and by estrous stage
Previously, we reported that ENDO-induced hyperalgesia develops over time, not becoming significant until >1 month after the surgery (Cason *et al.,* 2003). Here, we wished to determine how quickly changes in hyperalgesia after cyst-removal in *Group1* or sham-cyst-removal in *Group 2* occurred. This analysis was done by calculating AUCs at baseline and at 2-week intervals after the surgery, then subtracting the baseline AUC from the post-surgery AUCs. This analysis assessed 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 (Cason *et al.,* 2003). Here, we wished to determine if the increase in ENDO-induced hyperalgesia that was produced by the sham-cyst-removal surgery in *Group 2* varied by estrous stage. Accordingly, the escape response data from this group were separated into two groups, proestrus versus all other stages (estrus, metestrus, and diestrus). AUC values for severity of hyperalgesia were calculated and analyzed statistically as described in *General Methods.*

Cyst Collection Cysts were harvested from two groups of rats: following post-ENDO testing in *Group 1* at their cyst-removal surgery performed during the *ESTABLISHED* phase of development (at the end of the middle period), or following post-sham-cyst-removal testing in *Group 2* (at the end of the late period). 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, and diestrus). At the time of cyst collection in all rats, the area where auto-transplants were sewn was thoroughly investigated, cysts were freed from surrounding fat, and the rat's "cyst burden" calculated as described in *General Methods*. For the cyst-removal group, it was possible at the time of sacrifice to determine if all cyst-removal surgeries (*Group 1*) had been successful; that is, all cysts had been completely removed. If not, remaining cyst growths were freed from fat and collected. All cysts were harvested fresh and immediately frozen in dry ice and stored at -80C.

Sacrifice At the end of all behavioral testing, all groups of rats were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg) , tissues collected, and then the rats euthanized via an intracardiac overdose of urethane (as described in *General methods*).

Immunohistochemistry and analysis (DAB) Cysts were processed and immunostained (DAB) 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 as described in *General Methods*. Further, cysts were analyzed using a Likert-type scale as described in *General Methods*, generating density scores that were then used to assess significance of differences between groups with the Wilcoxon Signed Ranks Test.

4.3 Results

Complete-cyst-removal in ENDO rats (Group 1) during the ESTABLISHED phase of development significantly decreased vaginal hyperalgesia. Results from *Group 1*, whose averaged data are shown in *Fig. 10A* above, and whose individual data are shown in *Figs. 11A-F* above, were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; post-ENDO surgery, and post-cyst-removal surgery (performed during the *ESTABLISHED* phase).

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 ($F_{14, 105} = 7.80$, $p < 0.001$).

Thus, percent escape responses (*Fig. 10A*) to some of the distention volumes, were influenced by the condition of the rat. Post-hoc tests showed that escape responses increased significantly post-ENDO ($p < 0.001$). After the cyst-removal surgery performed in the *ESTABLISHED* phase, 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 ($p = 0.80$).

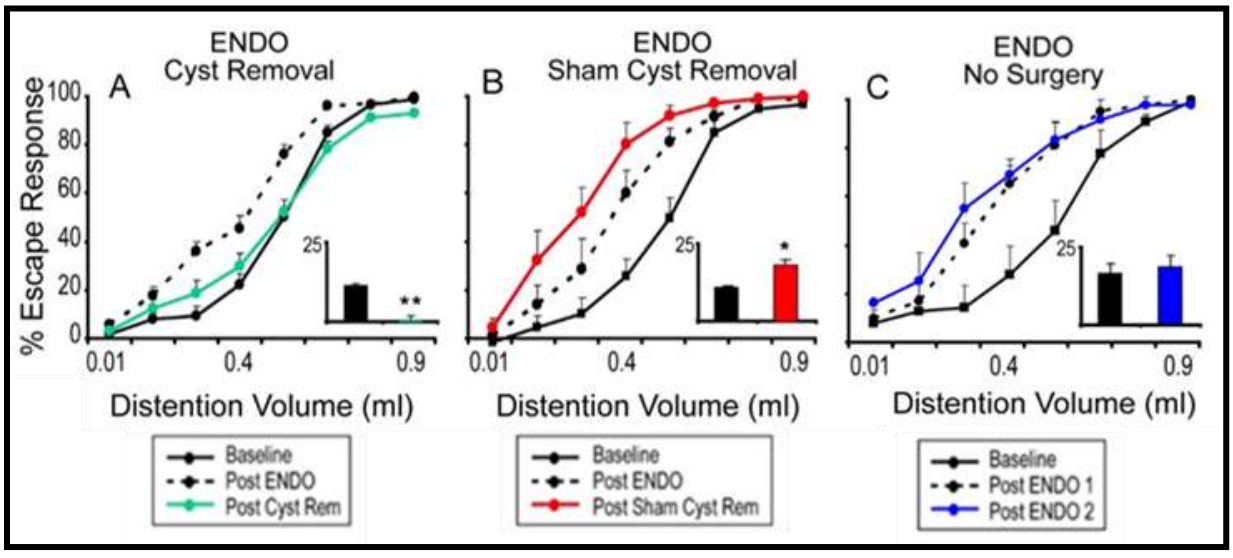

Figure 10. Effect of (A) cyst-removal (B) sham-cyst-removal, and (C) no additional surgery performed during the *ESTABLISHED* phase on ENDO-induced vaginal hyperalgesia.

Figure 10 – continued

Bar graphs inset into (A, B, C) show differences in the AUC between baseline and the middle period, and between baseline and the late period for each group. Asterisks indicate that the two AUCs of that group differed significantly. *$p < 0.05$; **$p = 0.001$. Error bars are ± SEM. Cyst-removal, sham-cyst-removal, and no additional surgery after the innervation and hyperalgesia are **mature** (8-10 weeks post-ENDO) alleviates, exacerbates, or has no effect, respectively, on **stabilized** vaginal hyperalgesia. Figure adapted from McAllister *et al.* (2009) with permission.

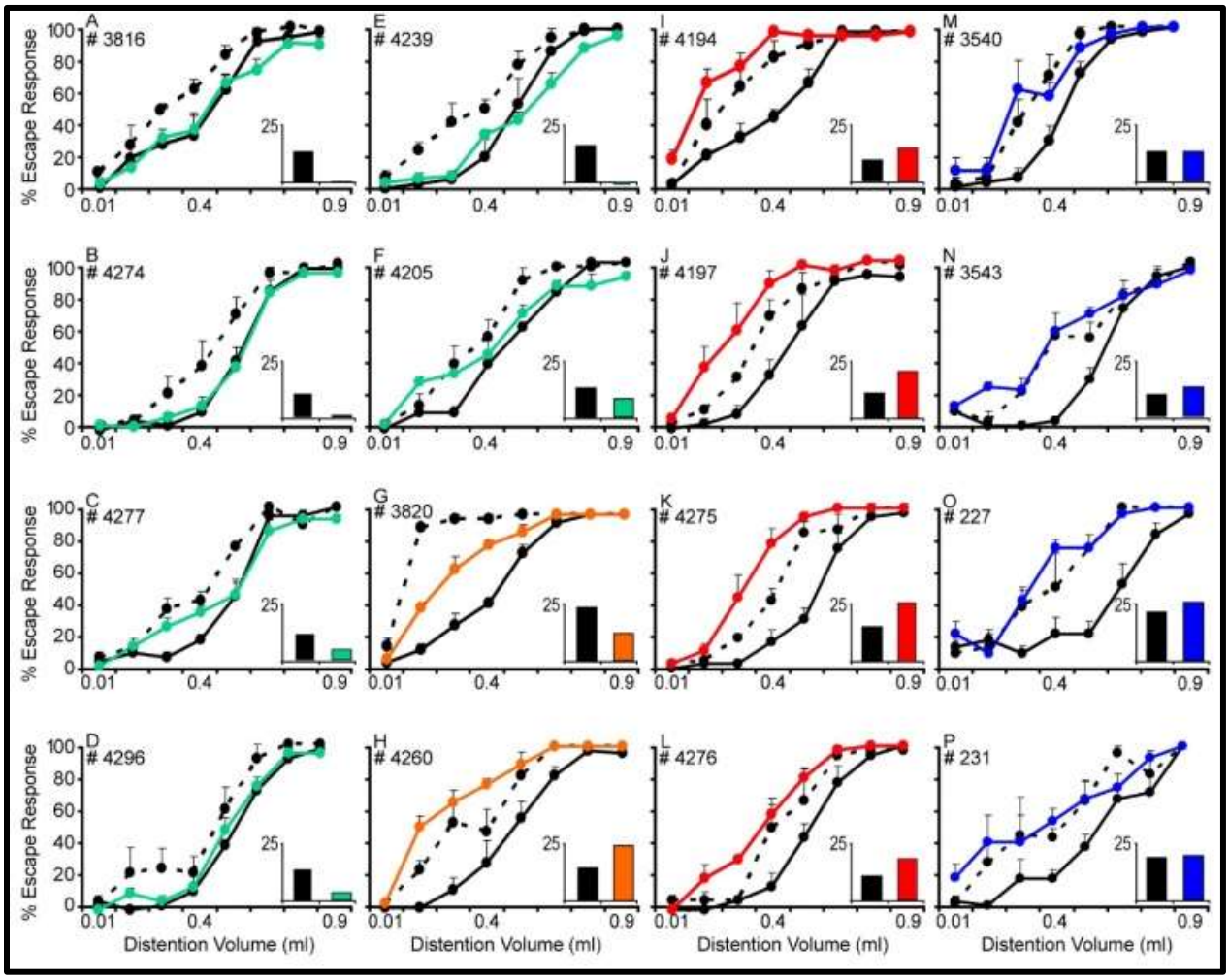

Figure 11. Effect of (A-F) cyst-removal (G-H) "extra-pathology" (originally in cyst-removal group but later excluded) (I-L) sham-cyst-removal, and (C) no additional surgery performed during the *ESTABLISHED* phase on ENDO-induced vaginal hyperalgesia, individual rat data. Each inset bar graph shows differences in the AUC between baseline and middle period, and between baseline and late period for that rat. Figure adapted from McAllister *et al.* (2009) with permission.

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 *Figs. 10A and 11A-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 (*Figs. 11A-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. 10A*), 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 during the *ESTABLISHED* phase.

Partial cyst-removal or ‘‘extra” pathology during the ESTABLISHED phase increased ENDO-induced vaginal hyperalgesia. Eight rats had initially been assigned to *Group 1*, in which complete cyst-removal was to have been performed. At autopsy, however, it was found that not all of the cyst tissue had been removed in two of the eight rats; i.e., the cyst-removal had been incomplete. Data from these two rats were therefore excluded from the ‘‘complete-cyst-removal” group and are presented separately here.

In the rat with partial cyst-removal, rat #4260 (data shown in *Fig. 11H*), the severity of the ENDO-induced vaginal hyperalgesia was similar to that in other rats with ENDO (*Fig. 10A-C*). 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 (5x5 mm). In this rat (*Fig. 11H*), with incomplete cyst-removal, the severity of her ENDO-induced vaginal hyperalgesia increased, unlike those with complete removal of their cysts (*Fig. 10A*), but similar to rats that had a sham-cyst-removal surgery (*Fig. 10B*).

In the rat with “extra pathology”, rat #3820 (data shown in *Fig. 11G*), ENDO-induced an unusually severe vaginal hyperalgesia. During cyst-removal 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 x 13 mm). Although a slight swelling of this part of the left uterine horn as noted in most rats, no other rat exhibited such extensive pathology. In this rat with additional pathology, the complete-cyst-removal surgery increased the hyperalgesia (*Fig. 11G)*, 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. 11A-F)*.

Sham-cyst-removal in ENDO rats (Group 2) during the ESTABLISHED phase of development significantly increased vaginal hyperalgesia. Results from this group, whose averaged data are shown in *Fig. 10B*, and whose individual data are shown in *Figs. 11I-L* were analyzed by repeated measures ANOVA as a function of three different conditions: baseline, after ENDO surgery (post-ENDO), and 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 ($F_{14, 63} = 4.62$, $p < 0.001$). Thus, percent escape responses (*Fig. 9C*) to some of the distention 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 in the *ESTABLISHED* phase, 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 *Figs.10B and 11I-L*, compare the effects of the manipulations on the severity of vaginal hyperalgesia. Consistently in all rats (*Figs. 11I-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 in the *ESTABLISHED* phase. Overall (*Fig. 10B)*, 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 during the *ESTABLISHED* phase.

The significant decrease in vaginal hyperalgesia after complete-cyst-removal (Group 1) during the ESTABLISHED phase did not occur until 4 weeks after surgery. As shown in *Fig. 12A* below, there was a 3-4 week delay after cyst-removal surgery before hyperalgesia was significantly decreased.

The significant increase in vaginal hyperalgesia after sham-cyst-removal (Group 2) in the ESTABLISHED phase did not occur until 6 weeks after surgery. As shown in *Fig. 12B*, there was a 5-6-week delay after sham-cyst-removal before hyperalgesia was significantly increased.

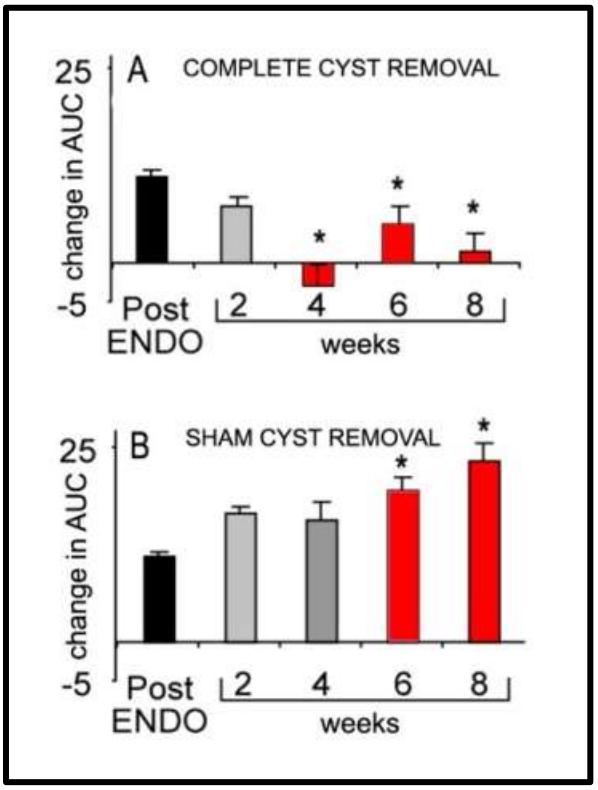

Figure 12. Time course of the effect of (A) complete-cyst-removal or (B) sham-cyst-removal during the *ESTABLISHED* phase on ENDO-induced vaginal hyperalgesia. Each bar shows differences in AUC from baseline for each time point. *Difference from post-ENDO, $p < 0.05$. The significant decreases (cyst-removal) and increases (sham-cyst-removal) in hyperalgesia are not immediate, but rather develop over weeks (cyst removal: 4 weeks, sham-cyst-removal: 6 weeks). Figure adapted from McAllister *et al.* (2009) with permission.

There were no changes in vaginal hyperalgesia over time (i.e. no significant increase in Endo-induced vaginal hyperalgesia occurred from post-ENDO period 1 and post-ENDO period 2: Group 3). Results from this group, whose averaged data are shown in *Fig. 10C*, and whose individual data are shown in *Figs. 11M-P*, were analyzed by repeated measures ANOVA as a function of three different conditions: baseline, post-ENDO period 1: between 1 and 10 weeks after ENDO surgery, and post-ENDO period 2: between 11 and 20 weeks after ENDO surgery.

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume ($F_{7, 63} = 139.09$, $p < 0.001$), surgical condition ($F_{2, 9} = 16.89$, $p = 0.001$), and there was a significant interaction between volume and surgical condition ($F_{14, 63} = 3.19$, $p = 0.001$).

Thus, percent escape responses (*Fig. 10C*) to some of the distention volumes 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 *Figs. 10C* and *11M-P,* compare the effects of the manipulations on the severity of vaginal hyperalgesia. Consistently, in all rats (*Figs 11M-P)*), there was a large increase in the AUC between baseline and the post-ENDO period 1 that did not differ significantly from the increase between baseline and the post-ENDO period 2 ($p = 0.122$). In other words, ENDO surgery induced a vaginal hyperalgesia whose severity stabilized by ~10 weeks post-ENDO in accordance with previous studies (Cason *et al.,* 2004; *Fig. 8*).

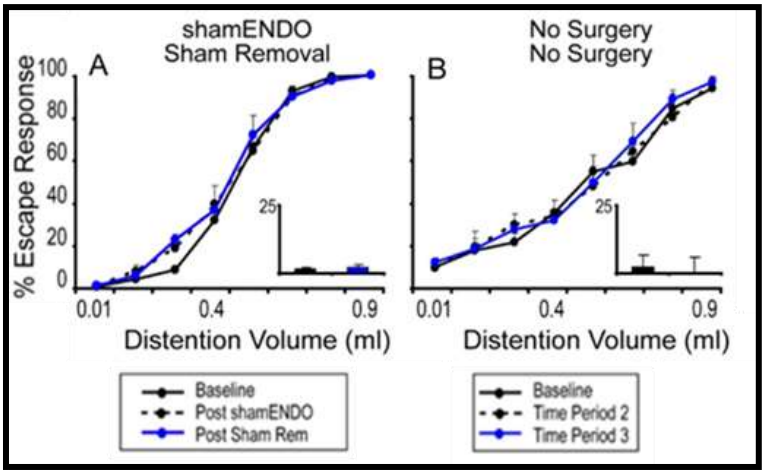

Figure 13. Effect of (A) shamENDO followed by a second sham surgery during the *ESTABLISHED* phase or (B) no surgery on vaginal nociception. Bar graphs inset into (A, B)

Figure 13 – continued

show differences in the AUC between baseline and the middle period, and between baseline and the late period for both groups. The two AUCs of both groups did not differ significantly. Error bars are ± SEM. ShamENDO followed by a sham-removal or no surgery produced no changes in vaginal nociception. Figure adapted from McAllister *et al.* (2009) with permission.

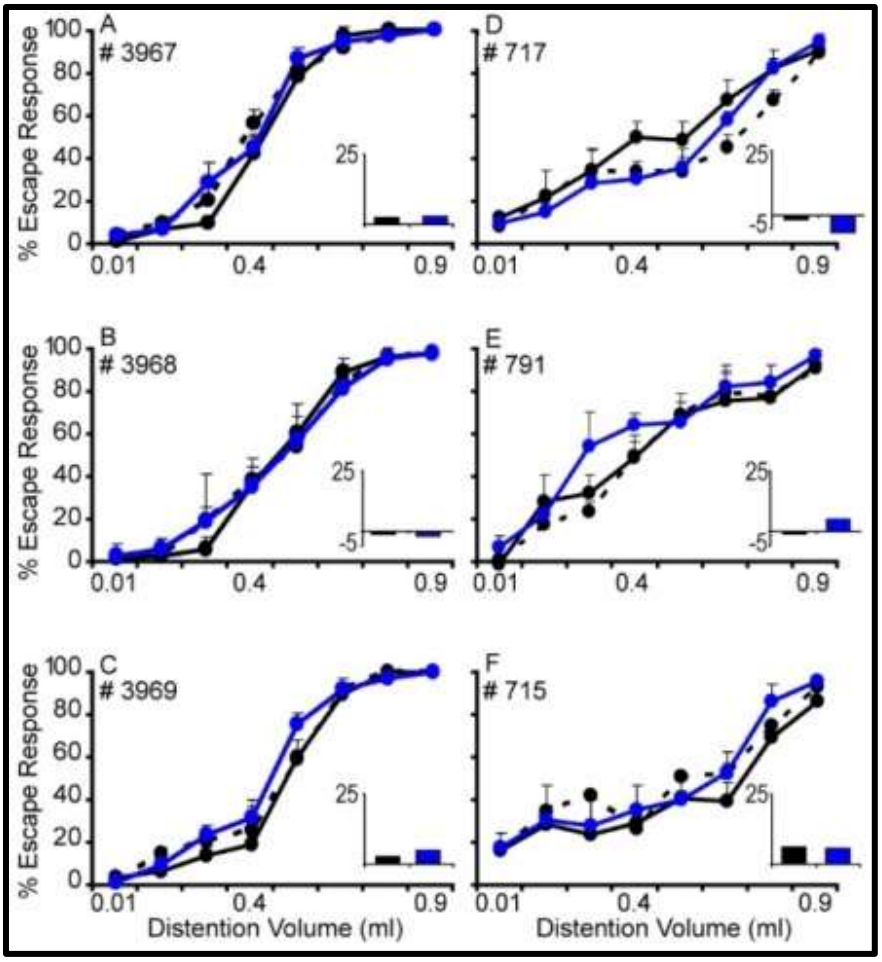

Figure 14. Effect of (A-C) shamENDO followed by a second sham surgery during the *ESTABLISHED* phase or (D-F) no surgery on vaginal nociception, individual rat data. Each inset bar graph shows differences in the AUC between baseline and middle period, and between baseline and late period for that rat. Figure adapted from McAllister *et al.* (2009) with permission.

ShamENDO followed by sham ''removal" (Group 4) in the ESTABLISHED phase produced no change in vaginal nociception Results from this group, whose averaged data are shown in *Fig. 13A*, and whose individual data are shown in *Figs. 14A-C*, were analyzed by repeated measures

ANOVA as a function of three different conditions: baseline; post-shamENDO surgery, and post sham-removal surgery performed in the *ESTABLISHED* phase.

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 ($F_{14, 42} = 0.81$, $p = 0.656$).

AUC calculations for escape responses, located in the insets within the graphs in *Fig*s. *13A and 14A-C* compare the effects of the manipulations on the severity of vaginal hyperalgesia. In the three rats (*Fig.14A-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. 13A)*, 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 performed in the *ESTABLISHED* phase failed to evoke vaginal hyperalgesia.

Healthy rats with no surgery (Group 5) had no changes in vaginal nociception over time

Results from this group, whose averaged data are shown in *Figs. 13B*, and whose individual data are shown in *Fig. 14D-F* were analyzed by repeated measures ANOVA as a function of three different conditions: baseline; a middle period; and a late 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 ($F_{14, 42} = 0.270$, $p = 0.995$).

AUC calculations for escape responses, located in the insets within the graphs in *Figs. 13B and 14D-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 the course of a 6-month period of testing.

The increase in vaginal hyperalgesia produced by sham-cyst-removal performed during the ESTABLISHED phase in ENDO rats (Group 2) occurred during estrus, metestrus, and diestrus but not during proestrus 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. 15A* 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 increased significantly ($p < 0.001$). In other words, what had been a cyclical change in vaginal hyperalgesia had been exacerbated by the additional surgery to become a chronic, steady level of hyperalgesia.

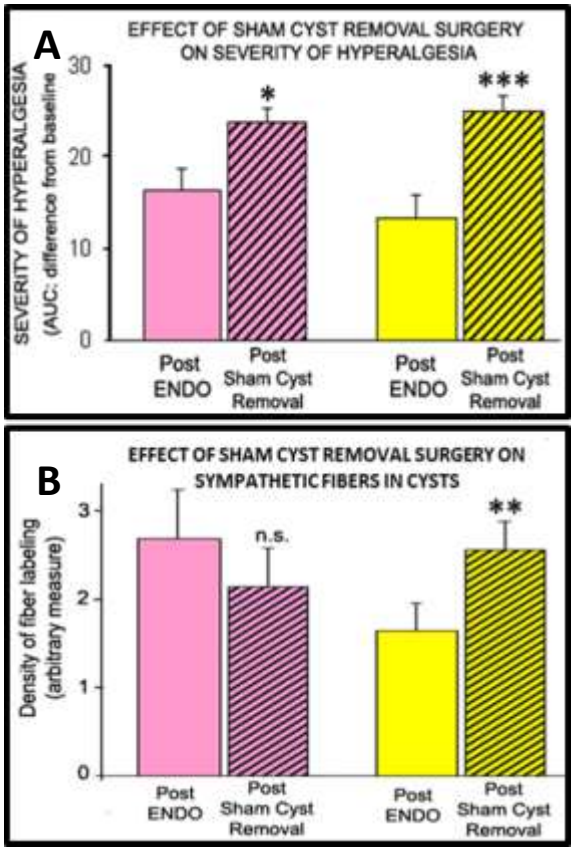

Figure 15. Effect of sham-cyst-removal performed during the *ESTABLISHED* phase on vaginal hyperalgesia and cyst sympathetic fiber density. (A) The severity of vaginal hyperalgesia that was produced by ENDO (solid bars) compared with the severity of vaginal hyperalgesia that was produced by sham-cyst-removal (hatched bars) evaluated in proestrus (left

Figure 15 – continued

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). **, $p < 0.05$; ***, $p < 0.001$. Sham-cyst-removal performed during the *ESTABLISHED* phase produces a significant increase in vaginal hyperalgesia and cyst sympathetic fiber density that occurs during estrus, metestrus, and diestrus but not during proestrus. Figure adapted from McAllister *et al.* (2009) with permission

The significant increase in sympathetic innervation of the cysts produced by sham-cyst-removal performed during the ESTABLISHED phase in ENDO rats (Group 2) occurred in the combined stages of estrus, metestrus, and diestrus but not proestrus As shown in *Fig. 15B*, the amount of sympathetic fiber labeling in cysts taken after post-ENDO testing (*Group 1*) compared with labeling in cysts taken after post-sham-cyst-removal testing (*Group 2*) did not differ when the two groups were compared in proestrus. In contrast, when the two groups were compared in the other combined stages 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 (*Fig. 15B*) in a manner similar to the increases in vaginal hyperalgesia (*Fig. 15A*). There were no significant differences in the amount of sensory fiber labeling across groups for cysts. *Fig. 5* provides example photomicrographs of sympathetic and sensory labeling within the cysts.

There was no significant correlation between cyst burden and ENDO-induced vaginal hyperalgesia at the end of the middle period. Cyst burdens calculated in ENDO rats at the end of the middle period (i.e., at the time of cyst-removal in *Group 1* or sham-cyst-removal in *Group 2*) ranged from 8 to 222 mm². This range is consistent with those previously-reported ranges (Morrison *et al.,* 2006; Nagabukuro & Berkley, 2007). Again consistent with our previous reports, there were no significant correlations between the cyst burdens in the 10 rats ($r = 0.024$, $p = 0.93$) and their vaginal hyperalgesia (assessed by the difference between the baseline AUC and post-ENDO AUC) during the *ESTABLISHED* phase.

There was no significant correlation between cyst burden and ENDO-induced vaginal hyperalgesia at the end of the late period. The cyst burdens calculated from cysts removed at the

end of the late period (i.e., at the time of sacrifice in both *Group 2* and *Group 3*) ranged from 0 to 195 mm². This range was again consistent with those reported previously and above (Morrison *et al.,* 2006, Nagabukuro & Berkley, 2007). There was no significant correlation between the cyst burdens and vaginal hyperalgesia in these 7 rats ($r = 0.21$, $p = 0.62$).

4.4 Discussion

These results demonstrate that when all cysts are completely removed during the *ESTABLISHED* phase (*Group 1*), vaginal hyperalgesia is fully **alleviated** for up to 6 months post-operatively. This finding supports the hypothesis that the cysts along with their innervation contribute to the maintenance of ENDO-induced vaginal hyperalgesia during this phase. Further, unexpected results from this study show that when surgery fails to remove all of the ectopic tissue in the *ESTABLISHED* phase (*Group 2*), ENDO-induced vaginal hyperalgesia is **exacerbated**. 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 (*Group 1*) and increases (*Group 2*) in hyperalgesia are not immediate, but rather develop over a period of 3-6 weeks (*Fig. 12A, B*). Sub-analysis by estrus stage of the increased hyperalgesia induced by sham-cyst-removal surgery (*Group 2*) showed that most of the increase occurred in estrous stages other than proestrus (*Fig. 15A*) during which the cysts' sympathetic innervation was elevated further (*Fig. 15B*) suggesting a role for the cyst innervation in the hyperalgesia.

Effects of cyst-removal When cysts were completely removed during the *ESTABLISHED* phase, ENDO-induced vaginal hyperalgesia was eliminated, suggesting that the **mature** cysts, or some characteristic of these cysts, contribute to the maintenance of the hyperalgesia. However, this study, in accordance with other studies, suggests the characteristic of the cysts contributing to the hyperalgesia is not the amount of cyst growth (cyst burden: CB). Thus, in this study, cyst burden (CB) fails to correlate with hyperalgesic severity as it did in previous studies (Morrison *et al.,* 2006, Nagabukuro & Berkley, 2007). Furthermore, partial-cyst-removal (which reduces cyst burden) failed to reduce hyperalgesia. A likely alternative to the cyst growth, per se, being the main contributing factor to vaginal hyperalgesia in the *ESTABLISHED* phase, is, as previously suggested, the sensory and sympathetic fibers that sprout to innervate the cysts (Berkley *et al*., 2004, 2005).

Again, studies from our lab support this hypothesis indirectly. The first study showed that the severity of ENDO-induced vaginal hyperalgesia in the *ESTABLISHED* phase was greatest in proestrus when estradiol levels are highest and least the next day in estrus when estradiol levels are lowest (Cason *et al.,* 2003). In another study from our lab, it was found that in the *ESTABLISHED* phase, as the rats transition from proestrus to estrus, there is a significant decrease in both the sympathetic fiber density innervating the cysts and also the cysts' contents of nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) (Zhang *et al.,* 2008). 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 (Zhang *et al.,* 2008). 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 in the *ESTABLISHED* phase.

Effect of sham-cyst-removal Once vaginal hyperalgesia stabilizes in the ESTABLISHED phase, no significant increases or decreases occur thereafter (*Fig. 6, Fig. 10C*). However, sham-cyst-removal increased this otherwise stabilized hyperalgesia (*Fig. 10B*). This increase is not likely be due to inflammation associated with a second surgery after ENDO because, because in rats with two sham surgeries, performed at similar times as the ENDO surgery and sham-cyst-removal surgery, had no change in vaginal nociception (*Fig. 14A*). Further, rats with cyst-removal had a complete alleviation of ENDO-induced vaginal hyperalgesia (Fig. 10A). 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 sham-cyst-surgery contribute to this increase.

One effect triggered immediately by the injury of any open abdominal surgery, is an increase in the abdomen of inflammatory mediators (Jesch *et al*., 2006). It is possible, therefore, that these agents increased activity of the cysts' afferent innervation (McMahon *et al*., 2005). The 5–6 week delay in the increased hyperalgesia (*Fig. 12B*) suggests, however, that longer-term processes were more important, triggered by, surgically-induced increases in inflammatory mediators. Examples of triggered processes include induction of nerve sprouting and increased central sensitization (discussed at length in Chapter 8 and Fig. 25's legend), 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 (Almarestani et al., 2008). 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 (DeLeo *et al*., 2004, McMahon *et al*., 2005; Wallace *et al*., 2007).

Additional clues for potential peripheral mechanisms that might underlie 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 (Cason *et al*., 2004), which parallels estrous changes in density of the cysts' sympathetic innervation (Zhang *et al*., 2008). 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, possibly inflammatory mediators.

CHAPTER FIVE

INITIAL PHASE: CONTRIBUTION OF THE CYSTS TO ENDO-INDUCED VAGINAL HYPERALGESIA

McAllister SL, Dmitrieva N, Berkley KJ. Sprouted innervation into uterine transplants contributes to the development of hyperalgesia in a rat model of endometriosis. PloS ONE 2012; 7:e31758. Reproduced as follows: open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

5.1 Introduction

Table 3. Phases of Development: *INITIAL* phase.

Developmental Phase	Cyst Innervation	Vaginal Hyperalgesia	Weeks Post-ENDO
INITIAL	**absent**	**absent**	**1-2 wks**
TRANSITIONAL	immature but active	highly variable between rats, but overall significant	4-6 wks
ESTABLISHED	mature	stabilized	8-10 wks

Our group discovered that ectopic growths harvested from ENDO rats and women with endometriosis develop their own C-fiber (sensory afferent) and sympathetic (autonomic efferent) nerve supply. These nerve fibers sprout from preexisting fibers innervating nearby territories to supply the growths (Berkley *et al.,* 2004; 2005). This discovery suggested that the ectopic growth's own innervation is a major contributor to pain associated with endometriosis (Howard, 2009), specifically the symptom of vaginal hyperalgesia. The previous studies in this dissertation tested this hypothesis by assessing ENDO-induced vaginal hyperalgesia in the *ESTABLISHED* phase. Here, in this study, this hypothesis was tested further by assessing the effects of removing the ectopic growths during the *INITIAL* phase (*prior* to the innervation sprouting and becoming **active** within the cysts) on the development of ENDO-induced vaginal hyperalgesia. If, this hypothesis is correct, then removal of the cysts should **prevent** or **reduce** the amount of vaginal hyperalgesia that develops (relative to the amount of

hyperalgesia that would have developed if cyst left intact). In other words, without the presence of the cysts, innervation cannot sprout and establish a two-way line of communication between the cysts and the CNS to evoke pain symptoms (*Fig. 25*), here, the symptom of vaginal hyperalgesia (Stratton & Berkley, 2011).

5.2 Methods

Subjects Eight female virgin Sprague-Dawley rats, weighing 175-225g at the beginning of the study were used. Rat housing and Estrous stage determination was as described in *General Methods*.

Anesthesia All rats were anesthetized as described in *General Methods*.

Surgically-induced endometriosis (ENDO) The ENDO surgeries were performed and post-operative recovery monitored as described in *General Methods*.

Surgical cyst-removal (CR) or sham-cyst-removal (SCR) The cyst-removal and sham-cyst-removal surgeries were performed and post-operative recovery monitored as described in *General Methods.*

Behavioral procedures Behavioral training, behavioral testing procedures, and testing sessions were completed as described in detail in *General Methods*.

Behavioral Groups There were 2 main groups, as shown in *Fig. 16* below. The experimental group, *Group 1*, consisted of rats that underwent ENDO surgery followed by complete cyst-removal during the *INITIAL* phase (n=5). The surgery control group, *Group 2*, underwent ENDO surgery followed by a sham-cyst-removal during the *INITIAL* phase (n=2). Vaginal nociception data in both groups were collected and compared between two chronological testing periods: (i) an initial baseline period of 6-8wks, and then a (ii) post period (after cyst-removal or sham-cyst-removal surgery), for an additional 8 weeks. Nociception was not assessed in the rats 1-2 weeks after ENDO (time between the baseline and post period) because this time was a recovery period for the rats from the ENDO surgery.

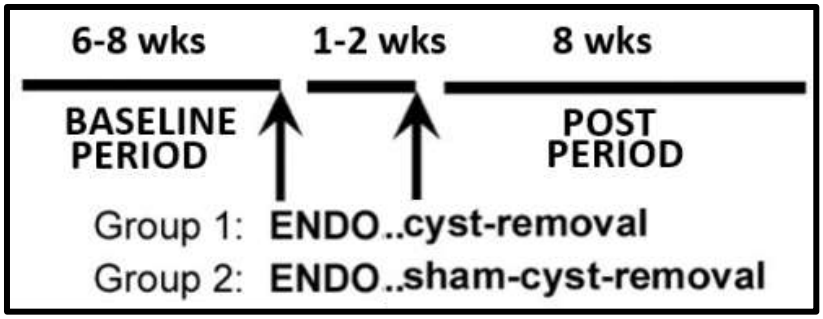

Figure 16. Summary and timeline of the two periods of assessment of vaginal nociception for the two groups in Chapter 5. *Group 1*: rats that underwent ENDO surgery followed by complete-cyst-removal surgery during the *INITIAL* phase. *Group 2*: rats that underwent ENDO surgery followed by sham-cyst-removal surgery during the *INITIAL* phase. Baseline period: this 6-8 week period comprised the initial assessment of vaginal nociception. Post period: this 8 week period comprised the second assessment of vaginal nociception.

Data analyses of behavioral results Behavioral nociception data calculations were as described in *General Methods* and identical to those used in previous studies (Cason *et al.,* 2004; Berkley *et al.,* 2007). For each rat, escape percentages (as a function of volume) from all sessions for each testing period were combined and the mean values calculated for each testing period. The averages from each of the rats were combined by group and entered into a spread sheet. Statistical analyses were performed using repeated measure ANOVA followed, if significant, by post-hoc Fishers LSD test.

Area-under-the-curve (AUC) calculations For each rat, an area-under-the-curve (AUC) calculation was performed as described in *General Methods*. Differences in the baseline AUC and post period AUC (*Group 1* post-cyst-removal, *Group 2* post-sham-cyst-removal) were calculated for each rat and then combined by group.

Sacrifice and cyst collection Cysts were harvested and measured following post- ENDO testing in experimental group (*Group 1*) during their cyst-removal surgery, or in the control group (*Group 2*), at sacrifice following post-sham-cyst-removal testing. At the end of all testing in both groups, each rat was anesthetized with urethane and the area where the auto-transplants had been sewn was thoroughly investigated to assure 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. The cysts were harvested fresh, immediately frozen in dry ice, and stored at -80 °C. After harvesting the tissue, the rats were sacrificed.

5.3 Results

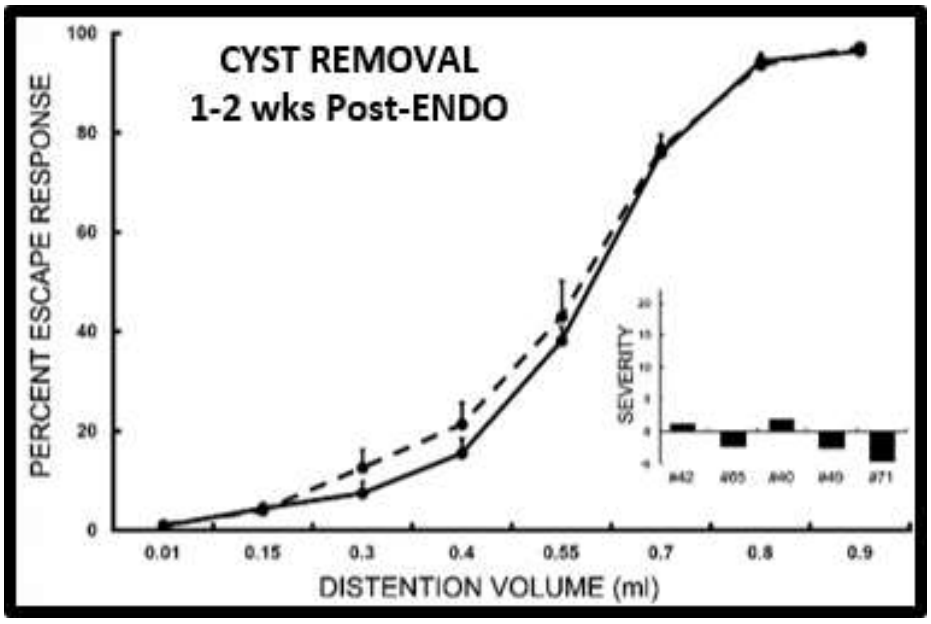

Figure 17. Effect of cyst-removal performed during the *INITIAL* phase on the development of ENDO-induced vaginal hyperalgesia. This graph shows percent escape response as a function of vaginal distention volume before (baseline, solid line) and after cyst-removal surgery (dashed line). The inset bar graphs show the difference in AUC between baseline and the post period for each rat in the group (n=5). Error bars are ± SEM. Cyst-removal prior to the cysts acquiring innervation (1-2 weeks post-ENDO) **prevents** the future development of vaginal hyperalgesia. Figure adapted from McAllister *et al.* (2012).

Complete-cyst-removal in ENDO rats (Group 1) during the INITIAL phase prevented the subsequent development of significant vaginal hyperalgesia). Results from this group (n=5), whose averaged data are shown in *Fig. 17*, were analyzed by repeated measures ANOVA as a function of two conditions: baseline and post period (after cyst-removal surgery).

Percent escape responses to different volumes of vaginal distention in this group of rats differed significantly as a function of volume ($F_{7, 56} = 385.7$, $p < 0.001$), but there was no significant effects of surgical condition ($F_{1, 8} = 0.908$, $p = 0.368$), nor was there a significant interaction between volume and surgical condition ($F_{7, 56} = 0.436$, $p = 0.875$).

For the five rats in *Group 1*, AUC calculations, inset within graph *Fig. 17*, show the effect of cyst- removal on the development of vaginal hyperalgesia. Consistently in all rats, there was no significant increase in the AUC between baseline and the post period. In other words, cyst-removal surgery during the *INITIAL* phase **prevented** the subsequent development of vaginal hyperalgesia.

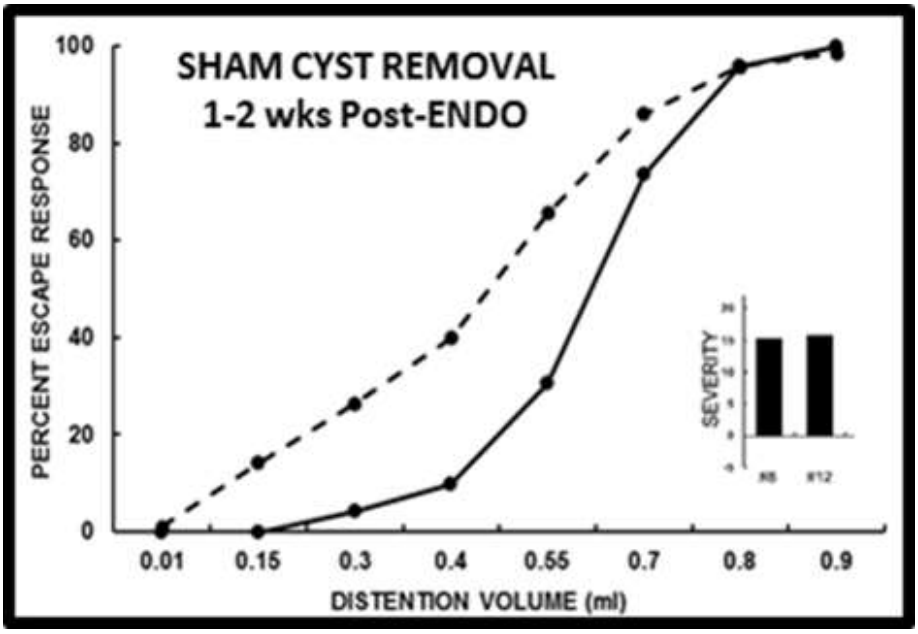

Figure 18. Effect of sham-cyst-removal performed during the *INITIAL* phase on the development of ENDO-vaginal hyperalgesia. This graph shows percent escape response as a function of vaginal distention volume before (baseline, solid line) and after sham-cyst-removal surgery (dashed line). The inset bar graphs show the difference in AUC between baseline and the post period for both rats in the group (n=2). Sham-cyst-removal prior to the cysts acquiring innervation (1-2 weeks post-ENDO) does not **prevent** the future development of additional vaginal hyperalgesia. Figure adapted from McAllister *et al.* (2012).

Sham-cyst-removal in ENDO rats (Group 2) during the INITIAL phase does not prevent subsequent vaginal hyperalgesia from developing (*Fig. 18*) In contrast, when a sham-cyst-removal surgery was performed during the *INITIAL* phase, vaginal hyperalgesia did develop. Inset within *Fig. 18,* AUC calculations show the effect of sham-cyst removal on the development of vaginal hyperalgesia. In both rats, there was a large increase in the AUC between baseline and the post period (after sham-cyst-removal). In other words, sham-cyst-removal surgery during the *INITIAL* phase did not **prevent** the development of vaginal hyperalgesia.

5.4 Discussion

This study demonstrates that when the cysts are removed during the *INITIAL* phase (*Group 1*), that is, before innervation sprouts the infiltrate the cysts, the development of vaginal hyperalgesia is **prevented.** Sham-cyst-removal does not prevent this development. These findings suggest that the presence of the cysts and their innervation are necessary for the development of ENDO-induced vaginal hyperalgesia to occur.

In the previous study, in chapter 4, the removal of **mature** cysts during the *ESTABLISHED* phase once hyperalgesia was stabilized **alleviated** the vaginal hyperalgesia. That study suggested the cysts *and* their innervation contribute to the maintenance of ENDO-induced vaginal hyperalgesia. Thus, together these two studies provide support for the *general* hypothesis of this dissertation that the cysts (and their innervation) contribute to both the development and maintenance of ENDO-induced vaginal hyperalgesia. Mechanisms underlying these two processes are discussed at length in Chapter 8.

CHAPTER SIX

TRANSITIONAL PHASE: CONTRIBUTION OF THE CYSTS AND THEIR ACTIVE INNERVATION TO ENDO-INDUCED VAGINAL HYPERALGESIA

McAllister SL, Dmitrieva N, Berkley KJ. Sprouted innervation into uterine transplants contributes to the development of hyperalgesia in a rat model of endometriosis. PLoS ONE 2012; 7:e31758.

6.1 Introduction

Table 4. Phases of Development: *TRANSITIONAL* phase (A).

Developmental Phase	Cyst Innervation	Vaginal Hyperalgesia	Weeks Post-ENDO
INITIAL	absent	absent	1-2 wks
TRANSITIONAL	**immature but active**	**highly variable between rats, but overall significant**	**4-6 wks**
ESTABLISHED	mature	stabilized	8-10 wks

The previous two studies, in chapter 4 and 5, provide evidence that the cysts (and their innervation) contribute to the development (*INITIAL* phase) and maintenance (*ESTABLISHED* phase) of ENDO-induced vaginal hyperalgesia. In this study, the contribution of the cysts and their innervation to ENDO-induced vaginal hyperalgesia in the *TRANSITIONAL* phase was assessed. Here, the cysts were removed when innervation was **immature** but **active** and when vaginal hyperalgesia was approaching significance, and then the effect of this removal on hyperalgesia assessed. Without manipulation during this phase, innervation and hyperalgesia would continue to increase over time until both were **mature** and stabilized in the *ESTABLISHED* phase. If the general hypothesis is correct, that the cysts and their innervation contribute to ENDO-induced vaginal hyperalgesia, then cyst-removal during this phase should

eliminate or **reduce** the amount of vaginal hyperalgesia developed prior to cyst-removal and/or **prevent** or **reduce** the subsequent amount of vaginal hyperalgesia that develops post-cyst-removal.

6.2 Methods

Subjects Six female virgin Sprague-Dawley rats, weighing 175-225g at the beginning of the study were used. Rat housing and Estrous stage determination was as described in *General Methods*.

Anesthesia All rats were anesthetized as described in *General Methods*.

Surgically-induced endometriosis (ENDO) The ENDO surgeries were performed and post-operative recovery monitored as described in *General Methods*.

Surgical cyst-removal (CR) or sham-cyst-removal (SCR) The cyst-removal and sham-cyst-removal surgeries were performed and post-operative recovery monitored as described in *General Methods.*

Behavioral procedures Behavioral training, behavioral testing procedures, and testing sessions were completed as described in detail in *General Methods*.

Behavioral Groups There were 2 main groups, as shown in *Fig. 19 below*. The experimental group, *Group 1*, consisted of rats that underwent ENDO surgery followed by complete cyst-removal during the *TRANSITIONAL* phase (n=4). The surgery control group, *Group 2*, underwent ENDO surgery followed by a sham-cyst-removal during the *TRANSITIONAL* phase (n=2). Vaginal nociception data in both groups was collected and compared between three chronological testing periods: (i) an initial baseline period of 6-8wks, a (ii) post period 1 (post-ENDO period of 4-6wks), and then a (iii) post period 2 (after cyst-removal or sham-cyst-removal surgery), for an additional 8 weeks.

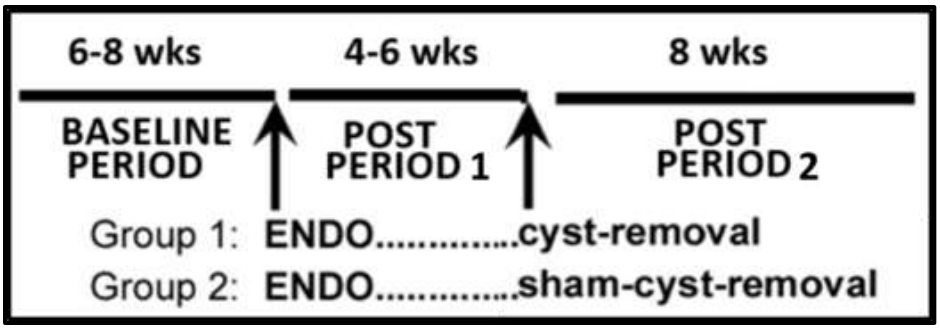

Figure 19. Summary and timeline of the three periods of assessment of vaginal nociception for the two groups in Chapter 6. *Group 1*: rats that underwent ENDO surgery followed by complete-cyst-removal surgery during the *TRANSITIONAL* phase. *Group 2*: rats that underwent ENDO surgery followed by sham-cyst-removal surgery during the *TRANSITIONAL* phase. Baseline period: this 6-8 week period comprised the initial assessment of vaginal nociception. Post period 1: this 4-6 week period comprised the second assessment of vaginal nociception. Post period 2: this 8 week period comprised the third assessment of vaginal nociception.

Data analyses of behavioral results Behavioral nociception data calculations were as described in *General Methods* and identical to those used in previous studies (Cason *et al.,* 2004, Berkley *et al.,* 2007). For each rat, escape percentages (as a function of volume) from all sessions for each testing period were combined and the mean values calculated for each testing period. The averages from each of the rats were combined by group and entered into a spread sheet. Statistical analyses were performed using repeated measure ANOVA followed, if significant, by post-hoc Fishers LSD test.

Area-under-the-curve (AUC) calculations For each rat, an area-under-the-curve (AUC) calculation was performed as described in *General Methods*. Differences in the baseline AUC and post period 1 (post-ENDO period) AUC, and between baseline and post period 2 (*Group 1* post-cyst-removal, *Group 2* post-sham-cyst-removal) were calculated for each rat, and then combined by group. When appropriate, differences between the two sets of AUCs were assessed by Student t-tests.

Sacrifice and cyst collection Cysts were harvested and measured following post- ENDO testing in experimental group (*Group 1*) during their cyst-removal surgery, or in the control group (*Group 2*), following post-sham-cyst-removal testing at sacrifice. At the end of all testing, each

rat was anesthetized with urethane, and the area where the auto-transplants had been sewn was thoroughly investigated to assure 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. The cysts were harvested fresh, immediately frozen in dry ice, and stored at -80 °C. After harvesting the tissue, the rats were sacrificed.

Calculation of Cyst Burden (CB) At the time of cyst-removal, here in the *TRANSITIONAL* phase, cysts were measured and their "cyst burden" calculated as described in *General Methods*. Pearson correlation coefficient statistics were then done to assess correlations between the cyst burden and severity of vaginal hyperalgesia.

6.3 Results

Cyst-removal during the TRANSITIONAL phase did not alleviate the vaginal hyperalgesia that had developed prior to cyst-removal but prevented the subsequent increase in vaginal hyperalgesia As shown in *Fig. 20* and *Fig. 21*, removal of the cysts during the *TRANSITIONAL* phase (n=4), when sensory and sympathetic innervation have infiltrated the cysts and become **active** and vaginal hyperalgesia has or is becoming significant, did **not alleviate** or **reduce** the significant amount of vaginal hyperalgesia that had developed prior to cyst-removal. However, this cyst-removal did **prevent** subsequent vaginal hyperalgesia from developing in three of the four rats in the group (*Fig 21*). Results from this group (n=4), whose averaged data are shown in *Fig. 20*, were analyzed by repeated measures ANOVA as a function of three conditions: baseline, post period 1 (post-ENDO), and post period 2 (post-cyst-removal surgery).

For the four rats in *Group 1*, AUC calculations for escape responses, inset within graph (*Fig. 21A-D*) shows the effects of the surgical manipulations (ENDO surgery and cyst-removal surgery) on the severity of vaginal hyperalgesia for each rat. The inset bar graphs show the change in AUC between the baseline and post period 1 (post-ENDO) compared to the change in AUC between the baseline and post period 2 (post-cyst-removal). Overall, as a group (*Fig. 20*),

the difference between the baseline and post period 1 (post-ENDO) compared with the difference between the post period 2 (post-cyst-removal) was not significant ($p = 0.627$).

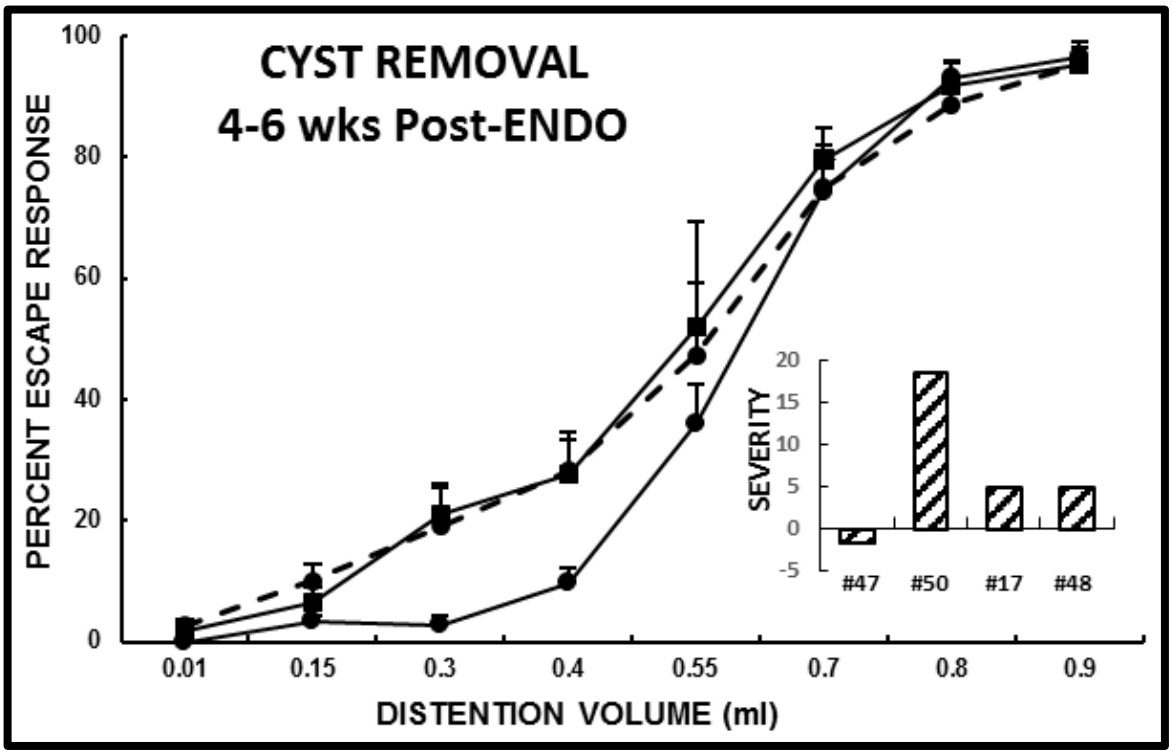

Figure 20. Effect of cyst-removal performed during the *TRANSITIONAL* phase on ENDO-induced vaginal hyperalgesia. The graph shows percent escape response as a function of vaginal distention volume before (baseline, solid line with circle markers), after ENDO surgery (solid line with square markers), and after cyst-removal surgery (dashed line with circle markers). The inset bar graphs show the difference in AUC between baseline and the post period 2 for each rat in the group (n=4), Error bars are ± SEM. Cyst-removal when the cysts are acquiring **active** innervation, but before the innervation is **mature** (4 to 6 weeks post-ENDO) does not **alleviate** the vaginal hyperalgesia that developed prior to cyst-removal but **prevents** the future development of additional vaginal hyperalgesia in three of the four rats. Figure adapted from McAllister *et al.* (2012).

Cyst burden did not correlate with the severity *of ENDO-induced vaginal hyperalgesia.* Cyst burdens in this group ranged from 7 to 167 mm^2. Consistent with previous findings (Morrison *et al.,* 2006; Nagabukuro & Berkley, 2007; McAllister *et al.,* 2009), there was no significant correlation between the rat cyst burden and severity of vaginal hyperalgesia. ($r = -0.65$, $p = 0.35$).

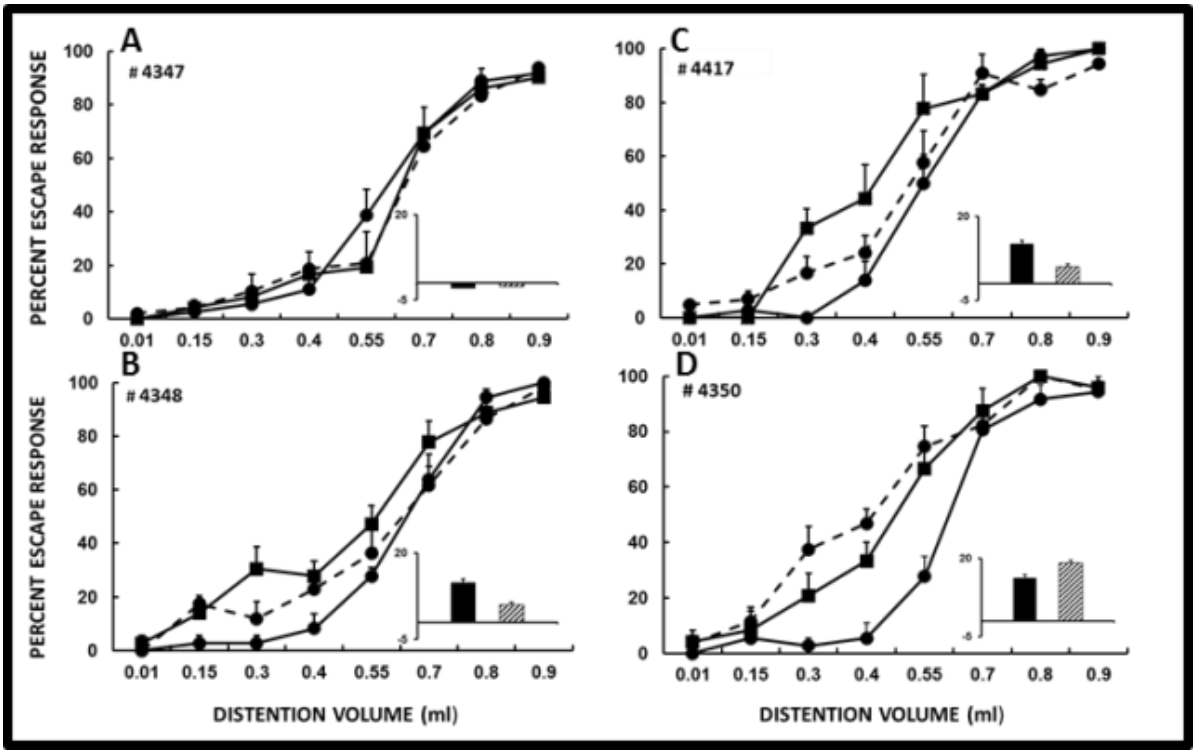

Figure 21. Effect of (A-D) cyst-removal performed during the TRANSITIONAL phase on ENDO-induced vaginal hyperalgesia, individual rat data. Each graph shows percent escape response as a function of vaginal distention volume before (baseline, solid line with circle markers), after ENDO surgery (solid line with square markers), and after cyst-removal surgery (dashed line with circle markers) for an individual rat. Error bars are ± SEM. The inset bar graph depicts the change in AUC (severity of hyperalgesia) between baseline and post period 1 (solid bar) and between baseline and post period 2 (hatched bar) for that rat.

Sham-cyst-removal in the TRANSITIONAL phase does not prevent subsequent vaginal hyperalgesia from developing When a sham-cyst-removal surgery was performed during the *TRANSITIONAL* phase, significant vaginal hyperalgesia continued to develop in both rats (*Fig. 22*).

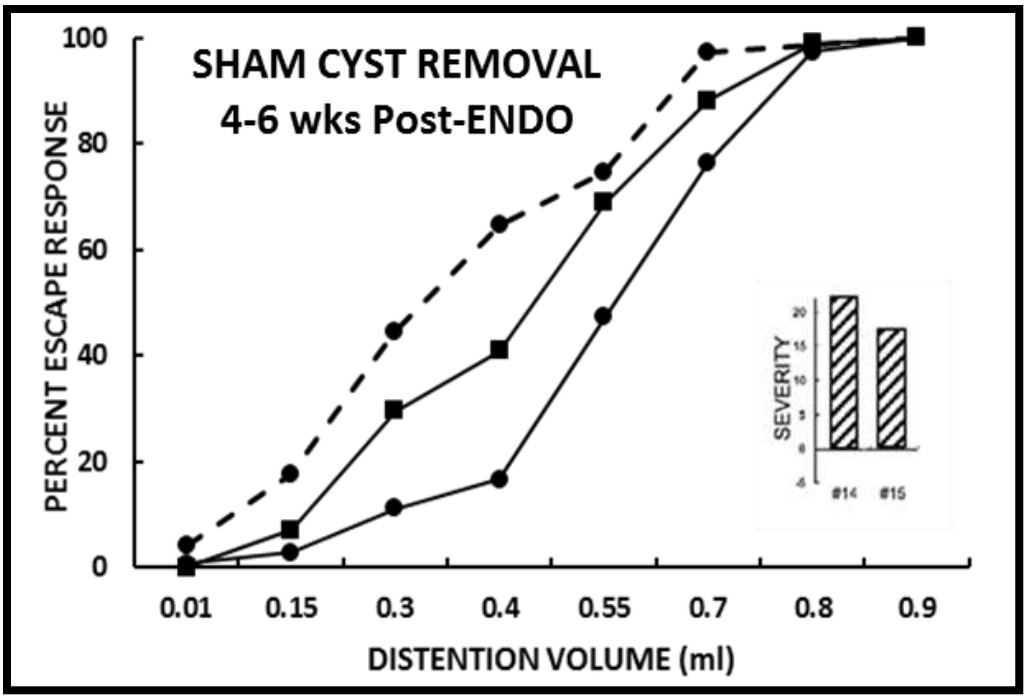

Figure 22. Effect of sham-cyst-removal during the TRANSITIONAL phase on ENDO-induced vaginal hyperalgesia. The graph shows percent escape response as a function of vaginal distention volume before (baseline, solid line with circle markers), after ENDO surgery (solid line with square markers), and after sham-cyst-removal surgery (dashed line with circle markers). The inset bar graphs show the difference in AUC between baseline and the post period 2 for both rats in the group (n=2). Sham-cyst-removal when the cysts are acquiring **active** innervation, but before the innervation is **mature** (4 or 6 weeks post-ENDO) does not **prevent** the future development of additional vaginal hyperalgesia. Figure adapted from McAllister *et al.* (2012).

6.4 Discussion

Results from this study demonstrate that when the cysts are removed during the *TRANSITIONAL* phase (*Group 1*), when sprouted innervation has infiltrated the cysts and become **active**, the development of subsequent vaginal hyperalgesia is **prevented**. This finding supports the hypothesis that some aspect of the cysts, likely the cysts' innervation, contributes to the development of ENDO-induced vaginal hyperalgesia, because removal of the cysts (and their **immature** but **active** innervation) **prevents** the progressive increase in vaginal hyperalgesia that would otherwise occur in ENDO rats with intact cysts.

On the other hand, the vaginal hyperalgesia that developed prior to the cyst-removal is **neither alleviated** nor **reduced**. Further, this study suggests that other factors, independent of

the presence of the cysts and their developing innervation, maintain the hyperalgesia during this phase. In other words, if, once the cysts and their innervation are removed, the previously developed hyperalgesia is maintained, other factors must be involved in the maintenance.

Importantly, the findings of this study, in accordance with previous study findings continue to suggest a changing role for the innervation of the cysts as the ENDO-induced vaginal hyperalgesia progresses through the three phases. These potential changing roles are discussed at length in Chapter 8 and illustrated in *Fig. 25*.

CHAPTER SEVEN

TRANSITIONAL PHASE: THE RELATIONSHIP BETWEEN THE INNERVATION OF CYSTS, VAGINAL CANAL, AND EUTOPIC UTERUS AND THE SEVERITY OF ENDO-INDUCED VAGINAL HYPERALGESIA

7.1 Introduction

Table 5. Phases of Development: *TRANSITIONAL* phase (B).

Developmental Phase	Cyst Innervation	Vaginal Hyperalgesia	Weeks Post-ENDO
INITIAL	absent	absent	1-2 wks
TRANSITIONAL	**immature but active**	**highly variable between rats, but overall significant**	**4-6 wks**
ESTABLISHED	mature	stabilized	8-10 wks

Some evidence suggests that, in addition to cyst innervation, other peripheral neural factors might contribute to endometriosis-associated pain. These factors include changes in innervation of other pelvic organs, specifically the eutopic (normally located healthy) uterus (Al-Jefout et al., 2009; Bokor, 2009; Stratton & Berkley, 2011) and the vaginal canal. Clinically, some studies have reported that the appearance of, or an increase in innervation of the eutopic (normally-located healthy) uterus plays a role in endometriosis-associated pain; but, the evidence is questionable due to study limitations (Al-Jefout *et al*., 2009; Bokor *et al*., 2009; Stratton & Berkley, 2011). Because vaginal nociception (i.e., vaginal hyperalgesia) is being studied here, another logical potential contributor to the hyperalgesia is the innervation of the vaginal canal.

Results from the previous study, in which the cysts were removed during the *TRANSITIONAL* phase, suggest that the cysts and their innervation contribute to the development of ENDO-induced vaginal hyperalgesia. But, as previously suggested, additional factors are likely contributing to the hyperalgesia during this phase because cyst-removal at this time does **not eliminate** or **reduce** previously developed vaginal hyperalgesia.

In this study, density of innervation of the vaginal canal and eutopic uterus was assessed in relation to the severity of ENDO-induced vaginal hyperalgesia. The innervation of the cysts

was also assessed in relation to hyperalgesic severity, further investigating the role of cyst innervation in ENDO-induced vaginal hyperalgesia during this phase.

If the innervation of the cysts is contributing to the severity of ENDO-induced vaginal hyperalgesia during the *TRANSITIONAL* phase, then the density of innervation (sensory and/or sympathetic) of the cysts should significantly correlate with the severity of hyperalgesia. Further, if the innervation of either vaginal canal or eutopic uterus contributes to the vaginal-hyperalgesia in this phase then their innervation density (sensory and/or sympathetic) should significantly correlate with the vaginal hyperalgesia.

7.2 Methods

Subjects Fourteen adult female virgin Sprague-Dawley rats, weighing 175-225g at the beginning of the study were used. Rat housing and Estrous stage determination was as described in *General Methods*.

Anesthesia The rats were anesthetized as described in *General Methods*.

Surgically-induced endometriosis (ENDO) The ENDO surgeries were performed and post-operative recovery monitored as described in *General Methods*.

Behavioral procedures Behavioral training, behavioral testing procedures, and testing sessions were completed as described in detail in *General Methods*.

Behavioral Groups All rats in this study underwent ENDO surgery. As shown in *Fig. 23*, vaginal nociception data was collected and compared between two chronological testing periods: (i) an initial baseline period and then a (ii) post period (post-ENDO) of ~4-6wks.

Data analyses of behavioral results: vaginal nociception and area-under-the curve (AUC) calculations Percent escape responses as a function of distention volume was measured in each session as described in *General Methods*. The averages from each of the rats were combined by group and entered into spreadsheets. AUC calculations of hyperalgesic severity for all rats were then done as described in *General Methods*.

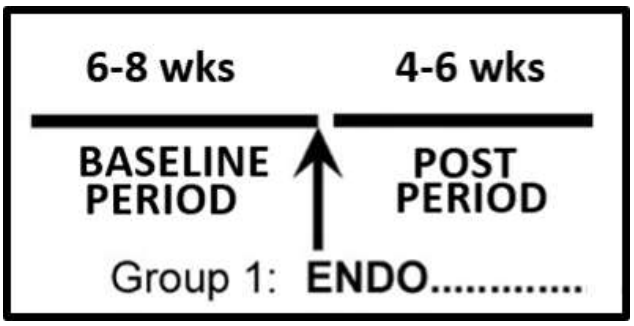

Figure 23. Summary and timeline of the two periods of assessment of vaginal nociception for the one group in Chapter 7. *Group 1*: rats that underwent ENDO surgery and then were sacrificed during the *TRANSITIONAL* phase. Baseline period: this 6-8 week period comprised the initial assessment of vaginal nociception. Post period: this 4-6 week period comprised the second assessment of vaginal nociception.

Sacrifice and Tissue Collection At the end of all testing, ~4-6wks after ENDO surgery, rats were sacrificed in proestrus as described in *General Methods*. The cysts, eutopic uterus, and vaginal canal were collected in accordance with the fixed tissue collection protocol described in *General Methods*. The tissues were then prepared for immunohistochemistry as described in *General Methods*.

Immunohistochemistry and Analysis (DAB) Cysts, eutopic uterus, and vaginal canal were immunostained with rabbit anti-calcitonin gene related peptide (CGRP; 1:10,000; Millipore) or goat anti-Tyrosine Hydroxylase (TH, 1:800, Millipore) and analyzed as described in *General Methods*. This analysis provided a sensory (CGRP) and sympathetic (TH) innervation density score for each rat's cysts, eutopic uterus, and vaginal canal. For the cysts, density of innervation scores for all cysts from the same rats were averaged to produce one score/rat. Pearson product correlation analyses were then used to assess the significance of the correlations between sensory or sympathetic innervation density (in each tissue) and hyperalgesic severity (as defined by AUC calculation).

Calculation of Cyst Burden (CB) At the time of sacrifice, after the 4-6 weeks post-ENDO period of data collection, cysts were measured and their "cyst burden" calculated as described in *General Methods*. Pearson correlation coefficient statistics were then done to assess correlations between the cyst burden and severity of vaginal hyperalgesia.

7.3 Results

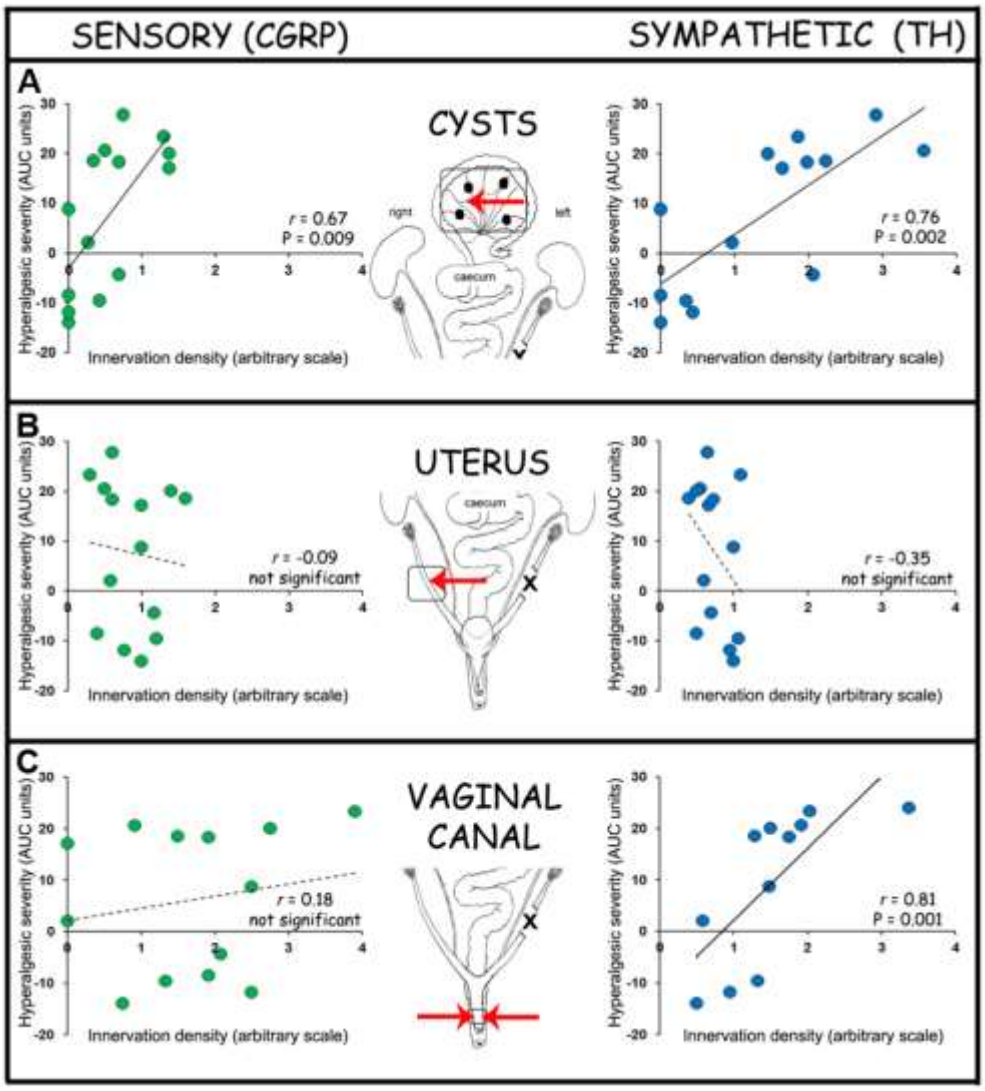

Figure 24. Correlation between the severity of hyperalgesia and the innervation density of the cysts, uterus, and mid-vaginal canal during the *TRANSITIONAL* phase. These correlations were all from data collected from the same rats. The left column represents the density of sensory innervation and the right column the density of sympathetic innervation for each representative tissue analyzed. (A) The correlation between hyperalgesic severity and innervation density in the cysts. (B) The correlation between hyperalgesic severity and the innervation density of the mid-uterine horn (harvested from the uninjured uterine horn). (C) The correlation between hyperalgesic severity and innervation of the vaginal canal (harvested from mid-canal, the region that had been stimulated). The hyperalgesic severity, when the cysts are acquiring **active** innervation, but before the innervation is **mature** (4 to 6 weeks post-ENDO), correlates significantly with the innervation density of the cysts (sympathetic and sensory) and vaginal canal (sympathetic only).

Cysts: hyperalgesic severity *correlated significantly with the density of both CGRP+ (sensory) nerve fibers and TH+ (sympathetic) nerve fibers.* (*Fig. 24A*) The correlation between vaginal hyperalgesia and CGRP+ density was r = 0.67, $p = 0.009$. The correlation between vaginal hyperalgesia and TH+ density was r = 0.76, $p = 0.002$.

Eutopic uterus: hyperalgesic severity *did not correlate with either the density of CGRP+ (sensory) nerve fibers or the density of TH+ (sympathetic) nerve fibers.* (*Fig. 24B*) There was no correlation between hyperalgesic severity and density of CGRP+ and TH+ nerve fibers within the eutopic uterus (r = -0.09, and r = -0.35, respectively).

Vaginal canal: hyperalgesic severity *correlated with the density of TH+ (sympathetic) nerve fibers but not with the density of CGRP+ (sensory) nerve fibers. (Fig. 24C)* For the vaginal canal, the density of TH+ but not CGRP+ fibers correlated significantly with hyperalgesic severity (r = 0.81, $p = 0.001$ and r = 0.18, respectively).

Cyst burden did not correlate with the severity *of ENDO-induced vaginal hyperalgesia.* Cyst burdens ranged from 0 to 81 mm². Consistent with previous findings (Morrison *et al.,* 06, Nagabukuro & Berkley 07, McAllister *et al.,* 09), there was no significant correlation between the rat cyst burden and severity of vaginal hyperalgesia. The overall correlation was r = 0.26.

7.4 Discussion

This study, carried out during the *TRANSITONAL* phase, found a significant positive correlation between cyst innervation density (both sensory and sympathetic) and the severity of vaginal hyperalgesia. Further, this study found a significant positive correlation between vaginal canal innervation density (sympathetic only) and the severity of vaginal hyperalgesia. These findings provide additional supporting evidence for the involvement of the innervation of the cysts (sensory and sympathetic) in ENDO-induced vaginal hyperalgesia, here, as a potential modulator of hyperalgesic severity in the *TRANSITIONAL* phase. These findings also suggest an additional contributor to hyperalgesic severity during this phase, the sympathetic innervation of the vaginal canal.

Overall, the findings of this study, when compared with those of the previous studies, continue to suggest a changing role for the innervation of the cysts in ENDO-induced vaginal hyperalgesia. Mechanisms underlying these changing roles are discussed at length in Chapter 8.

CHAPTER EIGHT

GENERAL RESULTS, DISCUSSION, AND CONCLUSIONS

Table 6. Results Summary.

Developmental Phase		*INITIAL*	*TRANSITIONAL*	*ESTABLISHED*
Weeks post-ENDO		1-2 weeks Post-ENDO	4-6 weeks Post-ENDO	8-10 weeks Post-ENDO
Vaginal hyperalgesia		Absent	Significant or reaching significance, highly variable	Plateaued and now stabilized
Cyst innervation relative to vaginal hyperalgesia	**Sensory**	N/A	Significant positive correlation	No significant differences between proestrus and estrus (Zhang *et al.,* 2008)
	Sympathetic	N/A	Significant positive correlation	Significant decrease of both measures from proestrus to estrus (Zhang *et al.,* 2008)
Effect of cyst-removal on vaginal hyperalgesia		Prevents future development	Does not alleviate previously developed hyperalgesia but prevents subsequent development (3/4 rats)	Alleviates
Effect of sham-cyst-removal on vaginal hyperalgesia		Increases	Increases	Increases
Eutopic uterus innervation relative to vaginal hyperalgesia	**Sensory**	N/A	No correlation	No significant differences between ENDO, ShamENDO, or naïve (McAllister *et al.,* 2014)
	Sympathetic	N/A	No correlation	No significant differences between ENDO, ShamENDO, or naïve (McAllister *et al.,* 2014)
Vaginal canal innervation	**Sensory**	N/A	No correlation with vaginal hyperalgesia	No significant differences between ENDO, ShamENDO, or naïve (McAllister *et al.,* 2014)
	Sympathetic	N/A	Significant positive correlation with vaginal hyperalgesia	No significant differences between ENDO, ShamENDO, or naïve (McAllister *et al.,* 2014).

Table 6 – continued

Developmental Phase	*INITIAL*	*TRANSITIONAL*	*ESTABLISHED*
Cyst burden (CB) relative to vaginal hyperalgesia	N/A	No correlation	No correlation
Relationship between estrous stage, vaginal hyperalgesia, & cyst innervation	N/A	No correlation	Significant parallel decrease in vaginal hyperalgesia and sympathetic innervation of the cysts from proestrus to estrus (Zhang *et al.,* 2008)
Relationship between PF growth factors, vaginal hyperalgesia, & cyst innervation	N/A	?	NGF & VEGF levels Significantly greater in proestrus then estrus potentially influencing the sensory innervation of the cysts and contributing to hyperalgesia (Zhang *et al.,* 2008)

In the first study, the developmental timelines of vaginal hyperalgesia and innervation of the cysts (sensory and sympathetic) were examined over a 10-week period post-ENDO. It was found that rudimentary innervation appears within the cysts at 2 weeks post-ENDO and becomes **active** at 3 weeks post-ENDO (*Fig. 7*). Between 4 and 5 weeks post-ENDO, vaginal hyperalgesia becomes significant, but is highly variable as the innervation increases and approaches maturity. By 8 to 10 weeks post-ENDO the innervation and hyperalgesia have plateaued and stabilized (*Fig. 5, Fig. 8*). Based on these findings, the developmental timeline was divided into three phases (*Table 1*): *INITIAL* (1-2 weeks post-ENDO), *TRANSITIONAL* (4-6 weeks post-ENDO), and *ESTABLISHED* (8-10 weeks post-ENDO). The contribution of the cysts (and their innervation) was then tested in each phase. The tests were done by removing the cysts in the *INITIAL*, *TRANSITIONAL*, and *ESTABLISHED* phases and assessing the effect on the development (*INITIAL* and *TRANSITIONAL* phases) and maintenance (*TRANSITIONAL* and *ESTABLISHED* phases) of ENDO-induced vaginal hyperalgesia. Additionally, the relationship

between the severity of ENDO-induced vaginal hyperalgesia and the innervation of the cysts, eutopic uterus, and vaginal canal was assessed during the *TRANSITIONAL* phase.

Removing the cysts in the *INITIAL* phase of development, when cyst innervation and vaginal hyperalgesia are both absent, prevented the subsequent development of hyperalgesia, which suggests that the cysts, or some aspects of the cysts, play an important role in the early development of ENDO-induced vaginal hyperalgesia (*Fig. 17*). Removing the cysts in the *TRANSITIONAL* phase, when **immature** sensory and sympathetic innervation has sprouted into the cysts and become **active** and the vaginal hyperalgesia is highly variable between rats, did not overall significantly **alleviate** or reduce the vaginal hyperalgesia that had developed prior to cyst-removal (*Fig. 20*). However, cyst-removal did prevent the development of subsequent vaginal hyperalgesia (*Fig. 20*).

Results from the *TRANSITIONAL* phase support the hypothesis that the cysts and their innervation contribute to the development of ENDO-induced vaginal hyperalgesia, because significant vaginal hyperalgesia had developed at this phase, and, because once the cysts were removed no additional vaginal hyperalgesia developed. However, once the cysts were removed, vaginal hyperalgesia was maintained, suggesting that the mechanism responsible for maintenance did not depend on the physical presence of the cysts and their innervation. The last study in the *TRANSITIONAL* phase, found that the innervation of the cysts (sensory and sympathetic) and the innervation of the vaginal canal (sympathetic only) significantly correlated with the severity of ENDO-induced vaginal hyperalgesia (*Fig. 24*). These findings provide further support for the hypothesis that cyst innervation influences vaginal hyperalgesia and suggest that innervation of the vaginal canal also contributes.

Removal of the cysts in the *ESTABLISHED* phase, which included their **mature** innervation, eliminated stabilized ENDO-induced vaginal hyperalgesia. This finding supports the conclusion that the cysts and their innervation contribute to maintenance of the stabilized hyperalgesia during this phase (*Fig. 10A, Fig. 11A-F*).

Overall, the results of these five studies provide strong support for the general hypothesis that the innervation of the cysts contributes to ENDO-induced vaginal hyperalgesia. Specifically, the cyst innervation likely contributes to the development, severity, and maintenance of ENDO-induced vaginal hyperalgesia. Importantly, however, the results also suggest that mechanisms by which the innervation operates to contribute to the hyperalgesia

change during its progression through the three phases. Specifically, the fact that cyst removal did not **alleviate** vaginal hyperalgesia in the *TRANSITIONAL* phase, but did **alleviate** vaginal hyperalgesia in the *ESTABLISHED* phase, suggests different mechanisms underlie the development and then continued maintenance of vaginal hyperalgesia. This difference, which emerges in the *TRANSITIONAL* phase, could help explain how endometriosis-associated pain transitions from an acute to a chronic pain condition. A discussion of this transition from acute pain--defined as "of sudden onset and expected to last a short time" (Institute on Medicine, 2011), to chronic--defined as "pain which has persisted beyond normal tissue healing time" (IASP) follows below.

Prior to this discussion, it is important to understand the concepts of peripheral and central sensitization. Sensitization, defined as "an increased responsiveness of nociceptive neurons to their normal input and/or recruitment of a response to normally subthreshold inputs" (Loeser & Treede, 2008) can be peripheral or central. Peripheral sensitization (*see Fig. 25*), an important neural mechanism underlying the development of acute pain, occurs when peripheral nociceptors (high-threshold primary sensory neurons) are exposed to injury, damaged tissue, and/or inflammatory mediators resulting in their increased excitability and lowered threshold (Latremoliere & Woolf, 2009). A simplified overview of peripheral sensitization in 3 steps is as follows: (1) injury and/or tissue damage generates the release of inflammatory mediators; (2) these mediators act on G-protein-coupled receptors (GPCRs) or tyrosine kinase receptors (TKRs) at nociceptor terminals; (3) this process activates intracellular signaling pathways that lead to the phosphorylation of different receptors and ion channels producing changes in nociceptor threshold and kinetics (Mifflin & Kerr, 2014). Acute pain associated with this process is usually short-lived, reverses as the injury heals, and is confined to the injury site (Mifflin & Kerr, 2014).

Central sensitization (*see Fig. 25*), involved in the development of chronic pain, is a "facilitated excitatory synaptic response and depressed inhibition causing amplified responses to noxious and innocuous inputs." (Woolf & Salter, 2000) In other words, the CNS undergoes neuronal plasticity that results in a "gain" (Woolf & Salter, 2000) in processing of peripheral noxious and non-noxious stimuli so that the same input from the periphery has a stronger CNS response (Deumen *et al*., 2013). In contrast to peripheral sensitization, central sensitization is associated with pain that is long-term and not confined to the initial injury site (Mifflin & Kerr, 2014). Instead, central sensitization is thought to be responsible for the expansion of pain

outside the initial injury site, the generation of secondary hyperalgesia (Mifflin & Kerr, 2014). For example, for ENDO-induced vaginal hyperalgesia, the site of the hyperalgesia (vagina, whose peripheral input to the CNS is at spinal segments L6/S1), is remote from the injury site (or uterine transplant (cyst) in the peritoneal cavity, associated with spinal segments at the mid-thoracic level) (*see Fig. 25*) (Stratton & Berkley, 2011). Briefly, central sensitization involves tissue injury, nerve damage, or intense/persistent peripheral noxious stimuli that initiate a series of events that ultimately alter CNS processing of nociceptive and non-nociceptive input, which in turn, can amplify pain responses (Mifflin & Kerr, 2014). For example, intense repeated noxious stimuli can activate peripheral nociceptors and generate a release of factors at central synapses such as glutamate, substance P (SP), calcitonin gene-related peptide (CGRP), and brain derived neurotrophic factor (BDNF). These factors can then bind with different receptors and activate various intracellular signaling pathways that lead to phosphorylation of membrane receptors and channels (i.e. NDMA and AMPA receptors for glutamate) involved in central sensitization (Pitcher *et al*., 2000; Afrah *et al*., 2002). This activation ultimately induces changes in spinal cord dorsal horn neurons, increasing their sensitivity, so that excitatory input that was subthreshold becomes capable of generating action potentials (Woolf & King, 1990; Simone & Ochoa, 1991). In summary, in central sensitization, CNS (i.e., spinal cord and brain) neuronal activity is now greater than it was originally in response to peripheral input (Deumens *et al*., 2013). Over time, this central sensitization is thought to play an important role in chronic pain (Woolf, 2011).

Importantly, central sensitization can be *dependent* or *independent* of peripheral sensitization. In peripherally-*dependent* central sensitization, continued input from the periphery is necessary and sufficient for both the <u>maintenance</u> and modulation of central sensitization (Gold & Gebhart, 2010; Baron *et al*., 2013). For example, peripheral sensitization that occurs after injury and likely generates acute pain may not subside after the initial pathology resolves; instead, irreversible or unresolved nociceptive sensitization may persist, thereby continually renewing central sensitization and, it is thought, a chronic pain state (Gold & Gebhart, 2010). In other words, a peripheral-central interaction occurs; in which ongoing nociceptive activity from the periphery persists and contributes to the <u>development</u> and <u>maintenance</u> of central sensitization (Baron *et al*., 2013). Hence, removing or blocking the peripheral input to the CNS

can **alleviate** peripherally-*dependent* central sensitization and its associated pain (Baron *et al*., 2013).

In contrast, peripherally-*independent* central sensitization, originally initiated by peripheral input, does not require additional or continuous peripheral input for its maintenance. Instead, long-lasting modifications in synaptic functioning occur to maintain the central sensitization (Woolf & Salter, 2000; Voscopoulos & Lema, 2010). These modifications include changes in the structure, connectivity, and survival of neurons (Voscopoulos & Lema, 2010). Thus, in peripherally-*independent* central sensitization, removal of the initial peripheral input source, will not eliminate central sensitization or **alleviate** chronic pain.

INITIAL PHASE: This phase can be considered in the context of acute pain associated with an "acute injury" from the autotransplantation surgery. Acute pain, which occurs suddenly and lasts for a limited amount of time, is associated with a specific probable cause, usually an injury, with the pain receding after the injury heals (Institute of Medicine, 2011). Here, the acute injury, surgical autotransplantation of uterine tissue, in essence represents a site of tissue damage/injury that generates the release of inflammatory mediators to promote healing/tissue regeneration. These inflammatory molecules, that potentially include among others, adenosine-5'-triphosphate (ATP), nerve growth factor (NGF), tumor necrosis factor-alpha (TNFα), bradykinin, prostaglandins, and serotonin which are released by epithelial cells, mast cells, and macrophages (Gold & Gebhart, 2010), can directly (or indirectly) activate the free nerve endings of peripheral nociceptors at the injury site. Supporting this suggestion is the finding that between 4-7 days after transplanting uterine tissue, there is a progressive infiltration in and around the area of transplant of mast cells and macrophages, which likely release the inflammatory mediators, ultimately leading to peripheral sensitization and the generation of primary hyperalgesia (Uchiide *et al*., 2002). Indeed, in other studies in rats with ENDO, where primary mechanical hyperalgesia was examined, it was found that by day 12 post-ENDO, there was a significant decrease in pain threshold (and increase in response) to mechanical stimulation (mechanical hyperalgesia) at the site of the implant (Alvarez *et al*., 2012). As expected, studies here in this dissertation during the *INITIAL* phase, found no development of vaginal hyperalgesia (i.e., there was no secondary hyperalgesia) prior to cyst-removal (McAllister *et al*., 2012). Significant ENDO-induced "secondary" (i.e., vaginal) hyperalgesia does not become evident until the *TRANSITIONAL* phase.

TRANSITIONAL PHASE: This phase can be considered in the context of a significant transition from acute pain associated with "neural sprouting" and peripheral sensitization, to a chronic pain maintained by peripherally-*independent* central sensitization. In this phase, the cysts have accrued a sensory and sympathetic nerve supply that quickly (within a week) becomes **active**. Importantly, this nerve supply, recently sprouted from pre-existing nerve fibers in the splanchnic nerve (Berkley *et al*., 2004), presents a new and/or additional potential source of acute pain, relative to the *INITIAL* phase, because peripheral nerve sprouting is known to increase background and evoke activity in these fibers (Gebhart, 1999; Janig *et al*., 1996).

Further, these newly-sprouted nerve fibers within the cysts, like fibers at the "injury site" in the *INITIAL* phase, can be stimulated by inflammatory factors released by both immune cells, and now, the endometrial growths. These factors can include, but are not limited to, prostaglandins, histamine, serotonin, bradykinin, interleukins, acetylcholine, VEGF, TNFα, and NGF (Bergqvist *et al*., 2001; Anaf e*t al*., 2002). Importantly, the cysts' sprouted fibers can also release other sensitizing agents, for example, the release of substance P and calcitonin-gene-related-peptide (CGRP) by sensory fibers, and prostaglandin E2 by sympathetic fibers (Mey *et al*., 2007; El Sawy et al., 2011, Zaidi & Matthews, 2013). These sensitizing agents further increase the activation of immune cells and cause vasodilation (Voscopoulos & Lema, 2010). Thus, overall, this combined additional molecular activity further increases total nociceptive input, and the peripherally-sensitized fibers, both within the cysts and possibly still at the injury site, persistently bombard the spinal cord neurons with peripheral input to induce central sensitization. This process represents the transition from acute to chronic pain. Therefore, significant vaginal hyperalgesia develops during this phase likely due to the initiation of central sensitization. However, removal of the cysts and their peripherally-sensitized fibers does not **alleviate** the significant vaginal hyperalgesia, but does prevent its further development. This finding suggests that the vaginal hyperalgesia is maintained by central sensitization that is *independent* of the peripherally-sensitized cyst fiber input (i.e. peripherally-*independent* central sensitization).

This transition, from acute to chronic pain, is extremely important clinically; yet, no clear explanation exists for its occurrence (Institute of Medicine, 2011). However, numerous theories have been proposed including: glial activation, neurodegeneration, loss of function of descending inhibitory pain fibers, and altered neuronal voltage-gated sodium and calcium channel function

(Voscopoulos & Lema, 2010; Kyranou & Puntillo, 2012; Mifflin & Kerr, 2014; Gerdle, 2014). For example, continuous noxious peripheral input, to the spinal cord from the cysts peripherally-sensitized fibers, could lead to inhibitory interneuron death and hence, a dysregulated transmission of ascending nociceptive input (Voscopoulos & Lema, 2010).

Other factors may also contribute to the ENDO-induced vaginal hyperalgesia during the *TRANSITIONAL* phase. First, studies here found a significant positive correlation between the sympathetic innervation density of the cysts and the severity of vaginal hyperalgesia, suggesting this innervation could play a role in the vaginal hyperalgesia. The main function of the vaginal sympathetic innervation, in non-pathological conditions, is to regulate vaginal blood flow by modulation of noradrenergic vasoconstriction (Munarriz *et al*., 2011). Hence, a decrease in sympathetic innervation yields an increase in vaginal blood flow (Kim *et al*., 2004). It has been suggested that an increase in blood flow, when associated with an abnormally-innervated region (i.e. vagina), can generate painful symptoms in that specific region, as a result of direct application of mechanical stimuli to the peripheral nerves made abnormally-responsive due to the blood vessel distention (Quinn, 2009). However, importantly, the correlation found in the *TRANSITIONAL* phase, involved only the density of the sympathetic innervation of the cysts, not an increase or decrease in the sympathetic innervation relative to "normal" conditions. Thus, the application of this finding is somewhat limited here.

Lastly, another possible contributor during the *TRANSITIONAL* phase, is "indirect coupling" between sensory and sympathetic fibers within the cysts. This theory proposes that sensory afferent fibers can be sensitized by compounds released by sympathetic terminals, such as the prostaglandins PGE and PGF2α (Michaelis & Janig, 1998; Khasar *et al*., 1999). Indeed, pilot studies done in our laboratory during the *TRANSITIONAL* phase, found that the hyperalgesic severity correlated significantly with the peritoneal fluid levels of PGE2 and PGF2 α ($r=0.64$, $p= <0.0001$; $r= 0.52$, $p=.004$, respectively) (data not shown). Further supporting this theory, in women with endometriosis, relative to healthy women, peritoneal microphages release significantly greater amounts of prostaglandins (Wu *et al*., 2002); specifically, PGE2 and PGF2α (Karck *et al*., 1996). Thus, it is possible that this "indirect coupling", sensitizes sensory fibers, further increasing peripheral nociceptive input.

ESTABLISHED PHASE: In this phase, cyst removal completely eliminates the vaginal hyperalgesia, suggesting the central sensitization that was peripherally-*independent* in the

TRANSITIONAL phase, now becomes peripherally-*dependent*. Also, in this phase, the sensory and sympathetic innervation of the cysts and the vaginal hyperalgesia are stable, and both vary with the estrous cycle, suggesting that hormonal modulation now begins to contribute to maintenance of the hyperalgesia (Cason *et al*., 2003; Zhang *et al*., 2008).

In women, modulation of endometriosis-associated pain by estrogen is a widely accepted concept (Stratton & Berkley, 2011). What supports this acceptance is that gonadotropin-releasing hormone (GnRH) agonists, that put a patient into a hypo-estrogenic state, is efficient in reducing pain symptoms such as dyspareunia (similar to vaginal hyperalgesia in the rat), dysmenorrhea, and non-menstrual pelvic pain (Olive, 2004; Batzer, 2006) . Further supporting estrogen's involvement in endometriosis-associated pain is that during menopause, when circulating estradiol levels drop, endometriosis-associated pain is alleviated, and the pain returns with estrogen replacement therapy (Bulun, 2011; Soliman & Hillard, 2006). However, how this modulation might occur is poorly understood. It could involve modification of peripheral sensory and sympathetic fibers as well as central neurons (Babichev, 2005; Chakraborti *et al*., 2007; Gillies & McArthur, 2010).

Regarding peripheral fibers, numerous studies provide support for estrogen's role in the modulation of sensory and sympathetic innervation and in the regulation, synthesis, and/or release of growth factors (Zoubina *et al*., 1998; Krizsan-Agbas *et al*., 2008; Richeri *et al*., 2005). In the *ESTABLISHED* phase here, estrogen also appears to modulate the sympathetic innervation of the cysts and growth factors, such as NGF and VEGF. Thus, in proestrus, when estrogen levels are greatest, the sympathetic innervation, NGF, and VEGF are significantly greater than in estrous, when estrogen levels are the lowest. Further, these growth factors, NGF and VEGF, have been shown to be involved in nerve fiber regulation, including fiber growth and survival (Hasan, 2005; Berbic & Fraser, 2011, Stratton & Berkley, 2011). Additional studies suggest that estrogen influences peripheral sensory neuron sensitivity and sympathetic nerve actions on primary neurons (Kaur *et al*., 2007; Stratton & Berkley, 2011).

The most parsimonious explanation for at least part of estrogen's putative modulatory role in ENDO-induced vaginal hyperalgesia, is that estrogen influences the density of the cysts' sympathetic innervation density, which in turn, influences the severity of hyperalgesia. In fact, it appears that the ectopic uterus (cysts), responds to changes in circulating estrogen levels in a manner similar to the eutopic uterus. Thus, the increase in estrogen associated with proestrus

produces a rapid degeneration of sympathetic innervation that is apparent in estrus (Zoubina & Smith, 1998; Zoubina & Smith, 2000; Zhang *et al*., 2008). It is likely that, these estrogen-dependent changes in the cysts' sympathetic innervation somehow influence the stable sensory innervation off the cysts, to alter nociceptive input, and generate changes in hyperalgesic severity.

Of relevance here is our laboratory's finding that, unlike the cyst's *sympathetic* innervation, the cyst's *sensory* innervation did not change with the estrous cycle (Zhang *et al*., 2008). It is possible, however that changes in the sympathetic innervation affected the functioning of the sensory innervation. One way for the sympathetic innervation of the cysts, modulated by estrogen, to influence the sensory innervation of the cysts and ultimately induce vaginal hyperalgesia, involves NGF. Thus, studies have found that sympathetic nerve terminals indirectly mediate NGF-induced sensitization of nociceptors. First, studies have shown, in rats and in humans, that systemic NGF injections lead to mechanical hyperalgesia (Lewin *et al*., 1994; Petty *et al*., 1994). Further, studies in rats, suggest NGF contributes to the development of hyperalgesia during inflammation; thus, experimental inflammation produces mechanical hyperalgesia paralleled by a significant increase in NGF of the inflamed tissue, and anti-NGF prevents the increase in both NGF and the hyperalgesia (Woolf *et al*., 1994). Potential mechanisms include direct sensitization of nociceptors by the binding of NGF to its receptor TrkA , and also, an increase in the synthesis of substance P and CGRP in afferent cell bodies, as a result of NGF taken up by the afferent terminal and transported to the cell body (McMahon, 1996; Woolf, 1996). Importantly, in rats, a local injection of NGF is followed by mechanical hyperalgesia that disappears or is significantly reduced when the rats sympathetic fibers have been removed chemically (Woolf, 1996). These data suggest that during inflammation the following occurs: inflammatory cells release NGF, NGF acts on TrkA receptors on sympathetic nerve terminals, these terminals generate the release of inflammatory mediators, resulting in nociceptor sensitization to mechanical stimuli (Janig & Habler, 2000). Taken together, these findings suggest that the proestrus-to-estrous (high-to-low estrogen level) changes in NGF with the cysts, which can influence the functioning of both the sensory and sensory innervation of the cysts, as NGF's receptor TrkA is found on both fiber types there, could explain the estrogen-dependent changes in vaginal hyperalgesia (Zhang *et al*., 2008). In other words, the elevated NGF levels in proestrus, likely act on the increased sympathetic innervation density of the cysts,

via its TrkA receptors, to generate the significant increase in hyperalgesia seen in proestrus relative to estrus. Elevated NGF levels likely also influence the sensory innervation of the cysts, generating an increase in substance P and CGRP, contributing to the significant hyperalgesia in proestrus.

Further supporting this idea, that NGF influences the innervation of the cyst to generate pain, is that in women with endometriosis, elevated levels of NGF (within the cysts and peritoneal fluid) are associated with pain (Barcena de Arellano *et al*., 2011). Also, recent studies, with anti-NGF therapy that blocks the NGF/TrkA pathway, and subsequently sensory and sympathetic sprouting in a rat model of arthritis, also reversed established hyperalgesia (Ghilardi *et al*., 2012). Further, the synthesis of NGF and its receptors (including TrkA) have been found to be up-regulated under the effect of estrogens, confirming a crucial link between estrogen, innervation, and hyperalgesia (Bjorling *et al*., 2002; Krizsan-Agbas *et al*., 2003; Hasan, 2005).

Another potential contributor to ENDO-induced vaginal hyperalgesia, in the *ESTABLISHED* phase, is VEGF, as VEGF levels within the cysts were found to be significantly greater in proestrus than estrus. VEGF is found in the eutopic endometrium, cysts, and peritoneal macrophages, and has been shown to play an important role in the maintenance of endometriosis (Gazvani & Templeton, 2002). Further, in women with endometriosis, VEGF levels are elevated in the peritoneal fluid and associated with a more advanced form of the disease (Gilabert-Estelles *et al*., 2007; Mahnke *et al*., 2000; Pupo-Nogueira *et al*., 2007). Important to results here, is that VEGF and its receptors have been shown to promote and maintain sympathetic innervation, suggesting it may do so here within the cysts (Marko & Damon, 2008).

Another finding, in the *ESTABLISHED* phase, supports the suggestion that other factors, in addition to peripherally-*dependent* central sensitization, contribute to ENDO-induced vaginal hyperalgesia. Thus, cyst-removal in this phase eliminated the vaginal hyperalgesia; but, the elimination was not immediate. Rather, the complete alleviation of vaginal hyperalgesia took 3-4weeks to disappear (*Fig. 12A*). This delay suggests that, although peripherally-*dependent* central sensitization likely contributes to ENDO-induced hyperalgesia, other central factors may also be involved. For example, peripherally-*independent* central sensitization as suggested in the *TRANSITIONAL* phase (Melzack *et al*., 2001; Ren & Dubner, 2008) or effects of stress on

sympathetic output to the area previously occupied by the cysts, which could continue to activate afferents there (Gibbs *et al*., 2008; Janig *et al*., 1996; Khasar *et al*., 2005).

CLINICAL CONSIDERATIONS. Overall, as discussed above, the transition from an acute event in the *INITIAL* phase progressing to a chronic pain in the *ESTABLISHED* phase is an important concept. How acute pain transitions to chronic pain is poorly understood, whether it be endometriosis-related or any other chronic pain. Understanding mechanisms that underlie this transition is of utmost importance, because as the pain becomes chronic it significantly reduces quality of life for ~ 100 million adults nationally (Gaskin & Richard, 2012) and comes with an economic burden of between $560 and $635 billion dollars annually (Larner, 2014). There is a dire need for additional research to increase the understanding of the transition from acute to chronic pain, in endometriosis and any other chronic pain condition. The model of endometriosis used in this dissertation may therefore provide a means to further our understanding.

The combined results from this dissertation and previous results from our lab indicate that there is a continuous and, importantly, a changing role of the sensory and sympathetic innervation of the cysts in the progression of ENDO-induced vaginal hyperalgesia. Initially, the innervation contributes to the development of ENDO-induced vaginal hyperalgesia. Later, after the innervation is established, the innervation contributes to modulation of its severity. Although there are no clinical data yet available that address the former component (neural sprouting into ectopic growths during early stages of pain development), evidence is accumulating in women to support the latter component (neural modulation of pain once endometriosis is established) (Howard *et al*., 2009; Mechsner *et al*., 2009).

Furthermore, the results highlight the importance of a necessary shift in biomedical research on endometriosis from that focused on treating the signs of the condition (i.e. targeted at reducing the size of the growths) to that focused on treating the pain associated with the condition (i.e. via increasing the understanding of the mechanisms underlying the pain, likely peripheral and central sensitization). A substantial amount of evidence exists, from studies in both rats and in women with endometriosis, that cyst size or amount of cyst growth (cyst burden) does not predict (or correlate with) the severity of the painful symptoms associated with endometriosis (Nagabukuro & Berkley, 2007; McAllister *et al*., 2009; Stratton & Berkley, 2011; McAllister *et al*., 2012). Yet, a significant amount of research continues focused on reducing

cyst size. A shift in focus is essential in order to find effective treatments for endometriosis-associated pain.

Other clinical implications of the results here relate to the clinical decision for surgical removal of the cysts to alleviate pain. The results suggest two important factors that should be taken into consideration: the ability to remove all ectopic growth, and the cyclical (menstrual) aspects of the preexisting pain. Thus, if it is known in advance that some ectopic tissue will be difficult to remove by either excision or ablation, and then it may prove to be the case that, like the rat model, surgery will be unsuccessful or even iatrogenic. However, if a patient's pains are strongly cyclical, it may increase the likelihood that successful surgical removal of the cysts will alleviate the endometriosis-associated pains. These factors warrant experimental investigation in women.

Finally, the results of this dissertation support the suggestion that painful endometriosis in women, already considered an inflammatory condition (Stratton & Berkley, 2011), would benefit from being additionally classified as a "neuropathic pain" condition. Neuropathic pain has recently been formally defined by the International Association for the Study of Pain as ''pain caused by a lesion or disease of the somatosensory nervous system'' (http://www.iasp-pain.org/AM/Template.cfm?Section = Pain_Defi…isplay.cfm&ContentID = 1728#Neuropathicpain, accessed October 12, 2014). Although a neuropathic pain classification is usually applied to painful skin or musculoskeletal conditions that involve direct damage to peripheral or central neural structures, studies here suggest that visceral C-fibers are peripherally-sensitized in association with the initial inflammation and ''may become a source of neuropathic pain'' (Sauer & Reeh, 2009). Thus, for example, a neuropathic classification has recently been effectively applied to pain associated with visceral disorders such as pancreatitis, in which abnormal nerve sprouting, like that associated with endometriosis, plays a substantial role in the development of pain (Demir *et al*., 2011).

The considerations above put a strong emphasis on prevention. There is an astonishingly high prevalence of up to 90% of adolescent girls who suffer from dysmenorrhea, with 15–20% reporting the pain as severe (Davis & Westhoff, 2001; Durain, 2004; Rapkin & Winer, 2009). A significant portion of this latter group is likely to have endometriosis (Stratton & Berkley, 2011). Recent brain imaging studies have shown that moderate-to-severe dysmenorrhea is associated with significant brain dysfunction and abnormalities in the hypothalamic–pituitary–adrenal

(HPA) axis (a reduction in cortisol levels) (Tu *et al*., 2009; Vincent *et al*., 2009; Tu *et al*., 2010; Berkley & McAllister, 2011; Stratton & Berkley, 2011). Furthermore, and importantly, these same studies also show that the longer the history of the pain, the more severe the effects on the brain and the HPA axis. Thus, one immediate consequence of reframing endometriosis as a neuropathic/inflammatory pain condition would be to reinforce the urgency of developing strategies for earlier diagnosis of endometriosis (Berkley & McAllister, 2011; May *et al*., 2011) as well as treatment of its associated pain (e.g., dysmenorrhea) before that pain can become chronic, as previously discussed, and other painful co-morbid conditions arise. Such classification would also bring under consideration how therapeutic strategies now in use or under development for neuropathic pain might be applied to painful or potentially painful endometriosis (O'Connor & Dworkin, 2009; de Leon-Casasola, 2011).

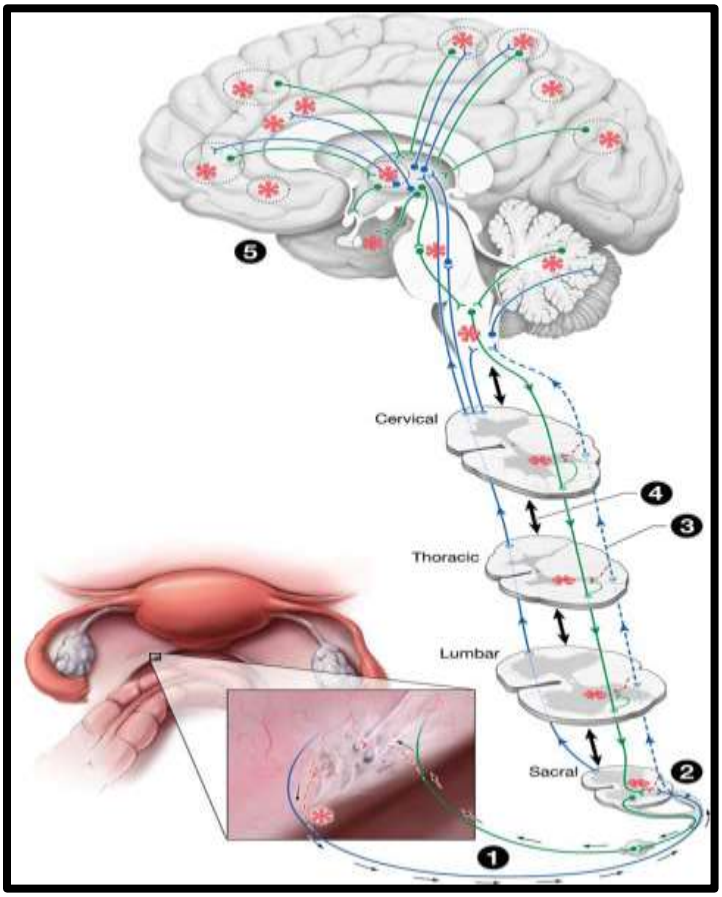

Figure 25. Illustration of how endometrial lesions (cysts) can engage the nervous system to give rise to different types of pain associated with endometriosis. Part ❶: Laparoscopic view

Figure 25 – continued

of pelvic organs with deeply infiltrating lesion expanded in the inset. Both sensory (blue) and sympathetic nerve fibers (green) sprout axon branches (red dashed lines) from nerve fibers that innervate nearby blood vessels to innervate this lesion. Fibers that sprouted new axons become sensitized (red asterisk). Part ❷: Two-way connection between innervated cysts and spinal cord (concentrated within sacral segments of the pelvic region). Sensitized peripheral nerve fibers, in turn, sensitize spinal sacral segment neurons. This 'central sensitization', shown by the red asterisk in the sacral segment, can become *independent* of and is modulated differently from peripheral sensitization. Part ❸: Input from peripheral afferent fibers to the spinal cord via their dorsal roots is concentrated in the sacral segments (associated with body part the fibers innervate), but fiber branches extend to other segments (blue dashed lines). Normally, these dorsal root branches have minimal impact on neurons in other segments unless the fibers become sensitized. If sensitized, they can then sensitize neurons in other segments (red dashed branches into the lumbar, thoracic and cervical spinal cord dorsal horn and the red asterisks at those levels). Part ❹: Normally, multiple intersegmental spinal connections exist to coordinate healthy bodily functions via excitatory and inhibitory synaptic connections (double-arrowed black lines). This intersegmental communication can influence how central sensitization modifies how neurons in remote segments process nociceptive and nonnociceptive sensory information ('remote central sensitization'), shown as red asterisks. Together, actions in Parts 3 and 4 can lead to increased nociception not only at sacral entry segments but also in any other segment. Part ❺: Multiple connections exist that ascend from every level of the spinal cord to the brain (blue lines) and descend from the brain to the spinal cord (green lines). Thus, in health, input from the spinal cord engages neurons throughout the brain that they are interconnected via complex ascending and descending inhibitory/excitatory synapses. Input from sensitized spinal neurons can affect activity throughout the neuroaxis, altering normal processing of nociceptive and non-nociceptive information. Some regions that can be influenced are depicted by red asterisks. Although asterisks are shown on the medial surface of the cortex, some influenced areas extend to parts of the lateral prefrontal, frontal, parietal lobes and within the temporal lobe (dotted black ellipses). These influences can become *independent* of peripheral sensitization associated with the cysts' innervation (Part 1). Such actions provide mechanisms for different types of endometriosis-associated pain. Figure adapted from Stratton & Berkley (2011) with permission.

APPENDIX A

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September 9, 2014

The Graduate School
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To Whom It May Concern:

Concerning the thesis/dissertation submitted to the Graduate School by:

Graduate Student:	**Stacy McAllister**
Thesis/Dissertation Title:	**Peripheral Neural Sprouting Contributes to Endo-Induced Vaginal Hyperalgesia in a Rat Model of endometriosis**
Department:	**Psychology**
Major Professor:	**Dr. Karen Berkley**

The above named graduate student has provided assurance to the FSU Animal Care and Use Committee that all animal procedures utilized in the work resulting in this thesis/dissertation are described in FSU ACUC Protocol(s):

Protocol Number	Title	Date ACUC Approval
9028	**Neural Mechanisms of Gynecological Pain**	April 13, 2004 and March 23, 2007
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The Animal Care and Use Committee has confirmed that this student was included as a project member during the period covering their thesis/dissertation work. This institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance Number is A3854-01.

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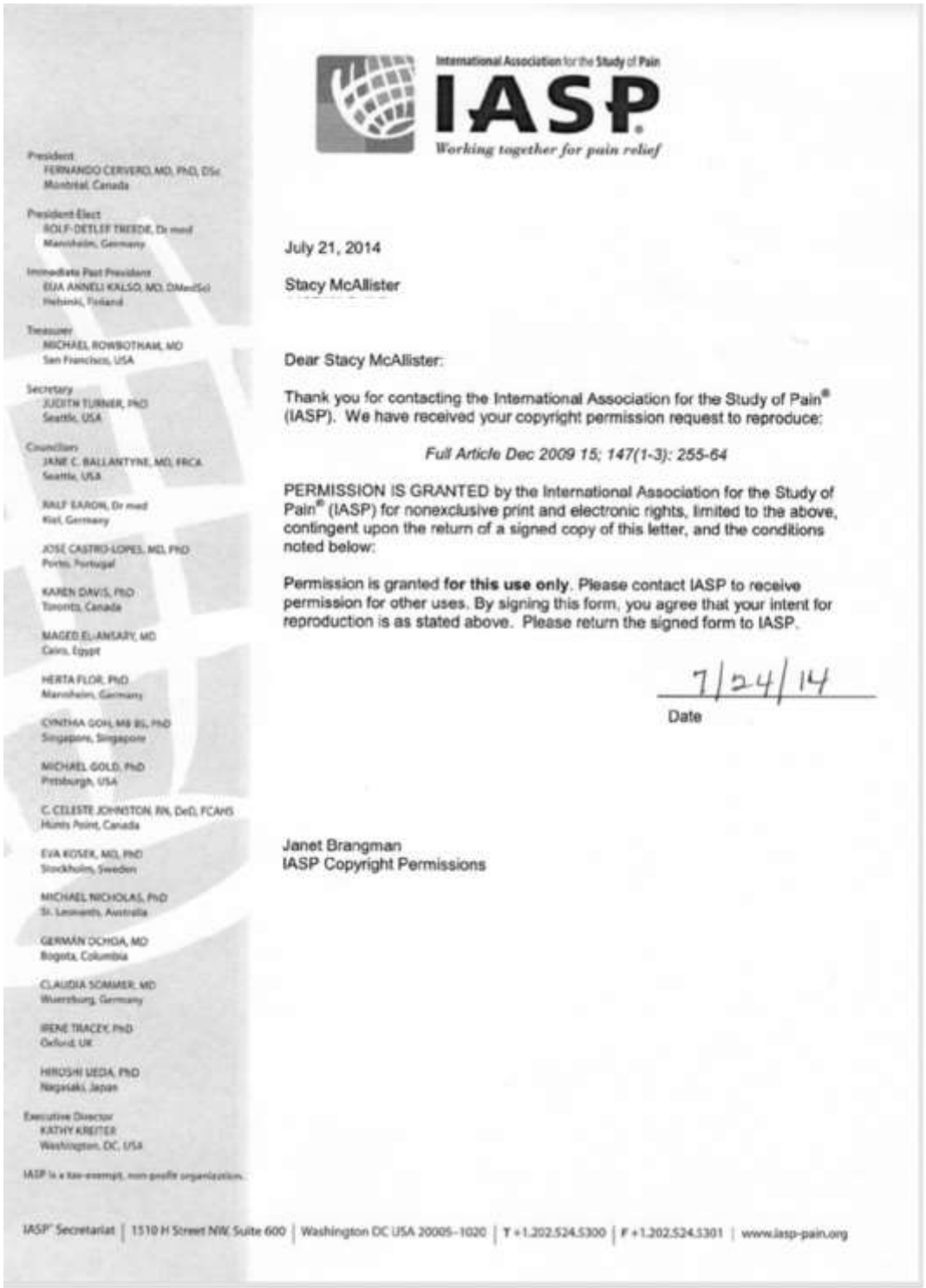

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Zaidi, Z. F., & Matthews, M. R. (2013). Source and origin of nerve fibres immunoreactive for substance P and calcitonin gene-related peptide in the normal and chronically denervated superior cervical sympathetic ganglion of the rat. *Autonomic Neuroscience : Basic & Clinical, 173*(1-2), 28-38.

Zhang, G., Dmitrieva, N., Liu, Y., McGinty, K. A., & Berkley, K. J. (2008). Endometriosis as a neurovascular condition: Estrous variations in innervation, vascularization, and growth factor content of ectopic endometrial cysts in the rat. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 294*(1), R162-71.

Zoubina, E. V., Fan, Q., & Smith, P. G. (1998). Variations in uterine innervation during the estrous cycle in rat. *The Journal of Comparative Neurology, 397*(4), 561-571.

Zoubina, E. V., & Smith, P. G. (2000). Axonal degeneration and regeneration in rat uterus during the estrous cycle. *Autonomic Neuroscience : Basic & Clinical, 84*(3), 176-185.

BIOGRAPHICAL SKETCH

Stacy L. McAllister, Ph.D.

Department of Psychology/Program in Neuroscience

Education:

Florida State University	Tallahassee, FL	Ph.D. Neuroscience *Advisor: Karen Berkley, Ph.D.*
Florida State University	Tallahassee, FL	completed 67 of 70 credit hours towards Bachelor of Science in Biology when I was accepted to/started PhD Program in Neuroscience (2009)
Florida State University	Tallahassee, FL	Bachelor of Science: Psychology 2004
Florida State University	Tallahassee, FL	Bachelor of Science: Sociology 2001

Research Experience:
Senior Laboratory Technician, Florida State University, Berkley Lab 2004- 2009

Teaching Experience:
Instructor of Record, PSB 2000: Brain and Behavior (~200 students), FSU, 2011
Supervised Teaching under Dr. Orenda Johnson-Lyons, PSB 2000: Brain and Behavior, FSU, 2010
Lecturer, FSU Brain Awareness Program, Brain Bee Preparation Session and Class Room visits, 2010-2013
Teaching Assistant for Dr. Friedrich Stephan, PSB 3004L: Physiological Psychology Lab, FSU, 2004

Other Experience and Professional Memberships:
Member, Society for Neuroscience, 2004-Present
Member, Society of Behavioral Neuroendocrinology, 2010-Present
Member, Association for Women in Science, 2010-Present
Ad hoc reviewer, Pain, 2011, 2012

Conference Presentation Grants:
Congress of Graduate Students (COGS) Conference Presentation Grant, FSU, 2009, 2010, 2011, 2012, 2014
Neuroscience-Psychology Department Presentation Travel Award, FSU, 2010, 2011 , 2012, 2014
South East Nerve Conference Presentation Grant, NSF, 2011

Neuroscience Program Involvement/Outreach:
Co-Coordinator, FSU Brain Awareness Program, Neuroscience Graduate Student Association Education Committee, FSU, Tallahassee, FL, 2009-2013
FSU Brain Fair, Co-founder, 2012
Lecturer, Friday Neuroscience Lecture & K-12 Brain Awareness Visits, 2009 – 2013
Member, FSU Brain Awareness Program (CNEC), FSU, Tallahassee, FL, 2014
Neuroscience Rep, FSU Psychology Dept. Graduate Advisory Committee (GAC), Tallahassee, FL 2009-2011
Treasurer, FSU Neuroscience Graduate Student Advisory Committee (GSAC), Tallahassee, FL, 2011-2012
Volunteer/Presenter, Godby High School Career Fair, Tallahassee, FL, 2010
Volunteer/Judge, DeSoto Trail Elementary School Science Fair, Tallahassee, FL, 2011
Volunteer/Judge, Capital Regional Science & Engineering Fair, 2012

Awards:
American Psychological Foundation (APF) and the Council of Graduate Departments of Psychology (COGDOP) Graduate Research Scholarship in Psychology, Nominee, 2011
Bryan Robinson Endowment for the Neurosciences, Tallahassee Memorial HealthCare, Honorable Mention, 2011

Lloyd M. Beidler Neuroscience Graduate Research Scholar award for outstanding research productivity and creativity, FSU, 2012

Publications:

Research Papers:

McAllister SL, Garcia C, Dmitrieva N, Berkley KJ**.** The influence of endocannabinoids system (ECS) on endometrial cyst innervation; in preparation.

McAllister SL, Berkley KJ. Endometriosis (ENDO)-induced vaginal hyperalgesia in the rat: different contributions of sensory and sympathetic innervation of the ectopic growths, vaginal canal, and eutopic uterus; in preparation.

McAllister SL, Dmitrieva N, Berkley KJ. Sprouted innervation into uterine transplants contributes to the development of hyperalgesia in a rat model of endometriosis. PLoS ONE 2012; 7:e31758. *Recommended by Faculty of 1000.*

Berkley KJ, **McAllister SL**. Don't dismiss dysmenorrhea! Pain 2011; 152:1940-1941.

Dmitrieva N, Nagabukuro H, Resuehr D, Zhang GH, **McAllister SL,** McGinty KA, Mackie K, Berkley KJ. Endocannabinoid involvement in endometriosis. Pain 2010; 151:703-710.

McAllister SL, McGinty KA, Resuehr, D, Berkley KJ. Endometriosis-Induced vaginal hyperalgesia in the rat: role of the ectopic growths and their innervation. Pain 2009; 147:255-264.

Berkley, KJ, **McAllister SL**, Accius BE, Winnard KP. Endometriosis-induced vaginal hyperalgesia in the rat: effect of estropause, ovariectomy, and estradiol replacement. Pain 2007; 132:150-159.

*Abstracts for Invited Talk/Oral/Poster Presentations (*Presenting Author):*

McAllister SL*, Giourgas BK, Berkley KJ. **(Nov 2014). .**Changes in sensory and sympathetic innervation of pelvic organs and ectopic uterine growths during the development and stabilization of endometriosis (ENDO)-induced vaginal hyperalgesia in the rat. Poster *to be presented* at the 44th annual meeting of the Society for Neuroscience, Washington DC.

McAllister SL*, Berkley KJ. **(Nov 2013).** Endometriosis (ENDO)-induced vaginal hyperalgesia in the rat: Different contributions of sensory and sympathetic innervation of the ectopic growths, vaginal canal, and eutopic uterus Poster presented at the 43rd annual meeting of the Society for Neuroscience, San Diego.

McAllister SL*, Pyatok SA, Faircloth EK, Giourgas BK, Berkley KJ. **(Oct 2012).** Endometriosis (ENDO)-induced vaginal hyperalgesia in the rat: sensory and sympathetic innervation of the ectopic growths but not eutopic uterus contributes to vaginal hyperalgesia. Poster presented at the 42nd annual meeting of the Society for Neuroscience, New Orleans.

McAllister SL*, Saland SK, Lieberworth C, Smith AS, Stathopoulos AM, Biggs L. **(Oct 2012).** Neuroscience graduate students at the Florida State University share their enthusiasm about the brain with the public Poster presented at the 42nd annual meeting of the Society for Neuroscience, New Orleans.

Pyatok SA, **McAllister SL**, Dmitrieva N, Giourgas BK, Faircloth EK, Berkley, KJ. **(Oct 2012).** Mechanisms of endometriosis-associated hyperalgesia in a rat model: Sensory-sympathetic coupling. Poster presented at the 42nd annual meeting of the Society for Neuroscience, New Orleans.

Giourgas BK, Nikonova L, Herzog AJ, **McAllister SL,** Dmitrieva N, Eckel LA, Berkley KJ. **(Oct 2012).** Endometriosis (ENDO)-induced vaginal hyperalgesia in the rat: sensory and sympathetic innervation of the ectopic growths but not eutopic uterus contributes to vaginal hyperalgesia. Poster presented at the 42nd annual meeting of the Society for Neuroscience, New Orleans.

McAllister SL*, Berkley, KJ. Endometriosis (ENDO)-induced vaginal hyperalgesia in the rat: sensory and sympathetic innervation of the ectopic growths but not eutopic uterus contributes to hyperalgesic severity. Poster presented at:

-- FSU Graduate Research Day, Tallahassee, FL. **(Apr 2012)**

--Rushton Lecture Series, Tallahassee, FL. **(Apr 2012)**

McAllister SL*, Herzog AJ, Faircloth EK, Giourgas BK, Dmitrieva N, Berkley KJ. Endometriosis-induced vaginal hyperalgesia in the rat: potential contribution of sensory-sympathetic coupling in the ectopic growths. *Poster presented at*:

--Life Sciences Symposium "Found in Translation", Tallahassee, FL, **(Jan 2012)**

--41st annual meeting of the Society for Neuroscience, Washington DC. **(Nov 2011)**

Giourgas BK, Herzog AJ, **McAllister SL**, McGinty KA, Berkley KJ. **(Nov 2011).** Endometriosis in the rat does not influence innervation of the rat's eutopic uterus. Poster presented at the 41st annual meeting of the Society for Neuroscience, Washington DC.

Dmitrieva N, Sacher F, Faircloth EK, **McAllister SL**, Berkley KJ. **(Nov 2011).** Endometriosis (ENDO) in the rat: telemetric assessment of ENDO-induced referred vaginal hyperalgesia and the effect of Indomethacin. Poster presented at the 41st annual meeting of the Society for Neuroscience, Washington DC.

Smith AS, Lieberwirth C, **McAllister SL**, Saland SK. **(Nov 2011).** Celebrating brain awareness week throughout the entire academic school year. Poster presented at the 41st annual meeting of the Society for Neuroscience, Washington DC.

McAllister SL*, Dmitrieva N, McGinty KA, Herzog AJ, Berkley KJ **(Apr 2011)**. Endometriosis (ENDO) in the rat: Sympathetic and sensitized sensory innervation of the ectopic growths develops in parallel with ENDO-induced vaginal hyperalgesia. Poster presented at FSU Graduate Research Day, Tallahassee, FL.

McAllister SL*, Herzog AJ, Dmitrieva N, Berkley KJ. Endometriosis (ENDO) in the rat: Individual differences in sensory and sympathetic innervation of the ectopic growths correlate with individual differences in the severity of hyperalgesia. *Poster presented at:*

--Rushton Lecture Series, Tallahassee, FL. **(Apr 2011)**
--Fowler Symposium, Tallahassee, FL. **(Mar 2011)**
--South East Nerve Net Conference, St. Augustine, FL. **(Mar 2011)**
--Evolutionary Medicine: Contributions to the Study of Disease and Immunity Lecture Series, Tallahassee, FL. **(Feb 2011)**
--Life Sciences Symposium "From Molecules to Medicine", Tallahassee, FL. **(Jan 2011)**
--40th annual meeting of the Society for Neuroscience, San Diego, CA. **(Nov 2010)**

Herzog AJ, McGinty KA, **McAllister SL**, Dmitrieva N, Berkley KJ **(Nov 2010)**. Endometriosis (ENDO) in the rat: upregulation of ERα in spinal cord but not afferent fibers innervating the ectopic growths contribute to estrous differences in the severity of ENDO-induced vaginal hyperalgesia. Poster presented at the 40th annual meeting of the Society for Neuroscience, San Diego, CA.

Dmitrieva N, Herzog AJ, McGinty KA, **McAllister SL**, Berkley KJ **(Nov 2010)**. Endometriosis (ENDO)-induced hyperalgesia in the rat: Contribution of nerve growth factor (NGF) and trkA in dorsal root ganglion (DRG) neurons innervating the ectopic growths. Poster presented at the 40th annual meeting of the Society for Neuroscience, San Diego, CA.

Lieberwirth C, Maffeo M, **McAllister SL**, Saland SK, Smith AS **(Nov 2010)**. Neuroscience educational outreach by the Florida State University. Poster presented at the 40th annual meeting of the Society for Neuroscience, San Diego, CA.

McAllister, SL* (Jul 2010). Individual differences in endometriosis-induced vaginal hyperalgesia. Talk presented at the FSU Neuroscience Summer Seminar, Tallahassee, FL.

McAllister SL*, McHenry JA, Berkley KJ. Endometriosis (ENDO) in the rat: Removing the ectopic growths prevents the development and alleviates ENDO-induced vaginal hyperalgesia. *Poster presented at:*

--FSU Graduate Research Day, Tallahassee, FL. **(Apr 2010)**
--FSU Rushton Lecture Series, Tallahassee, FL. **(Oct 2009)**
--39th annual meeting of the Society for Neuroscience, Chicago, IL. **(Oct 2009)**

McGinty KA, Zhang GH, **McAllister SL**, Herzog AJ, Crampton, LJ, Dmitrieva N, and Berkley KJ **(Oct 2009)**. Endometriosis (ENDO) and co-morbidity with bladder dysfunction: Influence of ENDO and shamENDO on spinal c-Fos expression induced by distention of the uninflamed and inflamed bladder. Poster presented at the 39th annual meeting of the Society for Neuroscience, Chicago, IL.

McAllister SL*, McGinty KA, McHenry JA, Berkley KJ **(Nov 2008)**. Endometriosis in the rat: effect of removing the ectopic growths on endometriosis-induced vaginal hyperalgesia. Poster presented at the 38th annual meeting of the Society for Neuroscience, Washington, DC.

McAllister SL*, Dmitrieva N, Zhang GH, Liu Y, McGinty KA, Mackie K, Resuehr D, and Berkley KJ **(Oct 2007)**. Endometriosis in the rat: potential involvement of the endocannabinoid system. Poster presented at the 37th annual meeting of the Society for Neuroscience, San Diego, CA.

Zhang GH, Dmitrieva N, McGinty KA, **McAllister SL**, Resuehr D, and Berkley KJ **(Oct 2007)**. Endometriosis in the rat: a neurovascular condition? Poster presented at the 37th annual meeting of the Society for Neuroscience, San Diego, CA.

McGinty KA, Zhang GH, Dmitrieva N, Liu Y, **McAllister SL**, Resuehr D, and Berkley KJ **(Oct 2007)**. Endometriosis in the rat: contribution of sympathetic innervation and VEGF and NGF levels in the ectopic growths to endometriosis-induced vaginal hyperalgesia. Poster presented at the 37th annual meeting of the Society for Neuroscience, San Diego, CA.

Resuehr D, Dmitrieva N, Zhang GH, Liu Y, McGinty KA, **McAllister SL**, Mackie, K, and Berkley KJ **(Oct 2007)**. Endometriosis in the rat: differences in estrous-dependent effects of long-term treatment with WIN 55212-2 on CB1 receptors and the growth factors VEGF and NGF Poster presented at the 37th annual meeting of the Society for Neuroscience, San Diego, CA.

McAllister SL*, Accius, BE, Winnard KP, and Berkley KJ **(Nov 2007)**. Endometriosis-induced vaginal hyperalgesia in the rat: effect of ovariectomy (OVX), OVX plus estradiol (OVX+E), and natural reproductive senescence. Poster presented at the 36th annual meeting of the Society for Neuroscience, Atlanta, GA.

McGinty, KA, Dmitrieva, N, Liu, Y, **McAllister, SL**, Accius, BE, Stack, A, Mackie, K, and Berkley, KJ **(Oct 2007)**. Endometriosis in the rat: estrous changes in size, vascularization, and innervation of the ectopic growths. Poster presented at the 36th annual meeting of the Society for Neuroscience, Atlanta, GA.