Cervix remodeling and parturition in the rat: lack of a role for hypogastric innervation

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Abstract

The hypogastric nerve is a major pathway innervating the uterine cervix, yet its contribution to the processes of cervical ripening and parturition is not known. The main objective of this study was to determine the effect of hypogastric nerve transection on remodeling of the cervix and timing of birth. As an initial goal, processes associated with remodeling of the peripartum cervix were studied. The cervix was obtained from time-dated pregnant rats on days 15, 19, 21, and 21.5 of pregnancy, and *post partum* on the day of birth (day 22). The cervix was excised, post-fixed overnight, and sections stained to evaluate collagen content and structure or processed by immunohistochemistry to identify macrophages or nerve fibers. The census of macrophages and density of nerve fibers in the cervix peaked on day 21, the day before birth, and then declined *post partum*. These results replicate in time course and magnitude previous studies in mice. To address the main objective, the hypogastric nerve was bilaterally transected on day 15 post-breeding; sham-operated rats served as controls. Pups were born in both groups at normal term. Transection of the hypogastric nerves did not affect remodeling of collagen or the census of macrophages or the density of nerve fibers in the cervix. These findings support the contention that enhanced innervation and immigration of immune cells are associated with remodeling of the cervix and parturition, but that a neural pathway other than the hypogastric nerve may participate in the process of cervical ripening.

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Introduction

The cervix functions as a gatekeeper to protect the developing fetus, as well as contents of the pregnant uterus, from external vaginal ecology. After an appropriate duration of pregnancy, the cervix undergoes a remodeling process and birth occurs at a precise time of day in rodents and, to an extent, in humans (Longo & Yellon 1988). Recent findings indicate that the density of nerve fibers in the cervix is increased by the day before birth in mice (Kirby *et al.* 2005, Yellon *et al.* 2008). Since ripening of the cervix resembles a neuroimmune-mediated inflammatory response (Mackler *et al.* 1999, Richardson & Vasko 2002, Yellon *et al.* 2003), our attention has focused on the role that nerves may serve in processes associated with remodeling of the cervix at the conclusion of pregnancy.

The lower reproductive tract in females is highly innervated in non-pregnant (NP) humans and rodents. Innervation of the uterine cervix involves a topography of nerve fibers that, depending upon species, project from the lower thoracic, lumbar, and upper sacral spinal cord (Papka *et al.* 1987, Berkley *et al.* 1993, Sullivan *et al.* 1994, Houdeau *et al.* 1998). In the rat, spinal cord segments from thoracic 13 to lumbar 3 have afferent

connections via the hypogastric nerve and inferior mesenteric ganglion to caudal portions of the uterine horns and the uterine cervix (Baljet & Drukker 1980, Peters *et al.* 1987, Lee & Erskine 2000). The hypogastric nerve makes a significant contribution to the total innervation of the cervix in NP females (Owman 1981, Steinman *et al.* 1992). Hypogastric nerve fibers in the rat cervix are reported to regulate neurogenic motor responses of smooth muscle (Owman 1981, Stjernquist & Owman 1987, Papka & Traurig 1988, Melo & Machado 1993) and are components of a sensory afferent pathway that mediates nociception (Cunningham *et al.* 1991, Sandner-Kiesling *et al.* 2002). Transection of the hypogastric nerve is found to attenuate the increase in pain threshold during pregnancy (Gintzler *et al.* 1983). Moreover, hypogastric neurectomy abolishes behavioral response to uterine horn distention (Temple *et al.* 1999) and is reported to eliminate the perception of intense mechanical stimulation of the uterine horns (Berkley *et al.* 1993). In contrast to evidence that suggests a role for the pelvic nerve in receptivity to vaginocervical stimulation, ovulation, and the process of parturition (Carlson & De Feo 1965, Martinez-Gomez *et al.* 1998),

little is known about the importance of the hypogastric nerve in pregnancy or its contribution to processes associated with remodeling of the cervix and the process of birth. Thus, the dual focus of the present study was to establish the time course of characteristics for cervical ripening associated with parturition in the rat and to test the hypothesis that transection of the hypogastric nerve eliminates a critical pathway that regulates remodeling of the cervix and timing of birth.

Results

Experiment 1: remodeling in the peripartum cervix

As pregnancy neared term, hypertrophy was evident in the thickness of the luminal epithelium and reduced the numbers of cell nuclei per volume of tissue. More space surrounded stromal cells and nuclei, while smooth muscle cells appeared elongated in pregnant compared with NP cervixes. Mean numbers of cell nuclei ($\pm$ s.e.m.)/ $\mu\text{m}^3 \times 10^4$ were for NP: 11.07 ± 0.26 , D15:

8.24 ± 0.38 , D19: 6.87 ± 0.38 , D21: 6.17 ± 0.35 , D21.5: 5.51 ± 0.37 , and PP: 6.61 ± 0.42 respectively ($P < 0.05$, ANOVA $F = 45.22$, $df = 5$; cell nuclei density for NP controls $>$ all other groups; D15 $>$ D19, D21, D21.5, and PP groups; and D19 $>$ D21.5 group). Based upon the morphology of cells in the cervix and vaginal epithelium, NP rats were in the diestrus phase of their reproductive cycle. Thus, compared with NP controls, cells in stromal and smooth muscle areas of cervix appeared to undergo a twofold increase in size with pregnancy and, during the last 6 days before birth, further hypertrophy by more than 30%.

Collagen structure in the cervix varied with respect to reproductive status. Bright red stained collagen fibers were densely packed and regularly arranged in the smooth muscle dense region between luminal epithelium and stroma in NP rat cervix (Fig. 1). On day 15 of pregnancy, collagen structure was well organized but thinner and less dense compared with those in NP cervix. By day 19 of pregnancy, the density of birefringence was markedly reduced; spaces were evident

Figure 1 Top panels. Photomicrographs of picosirius-stained collagen in the cervix from rats that were non-pregnant (NP), day 15 pregnant (D15, 7 days before birth) or prepartum (D21.5, day before birth). Bright birefringence was evident as intense orange on a light yellow background. Scale bar = 50 μm . Bottom panel. Data are the mean optical density of birefringence from picosirius red-stained sections of cervix (OD) $\pm$ s.e.m. from pregnant rats on day 15, 19, 21 or 21.5 post-breeding (D15, D19, D21 or D21.5) and *post partum* (PP; day 22 post-breeding) and were normalized to total cell nuclei density in NP rats ($n = 3$ sections from each of 5–6 rats per group). Color photomicrographs were converted to grayscale images; regions of dense collagen appear as dark areas, i.e., high birefringence, while whiter areas represent reduced collagen and scattered fibrils (details in Materials and Methods). Thus, higher OD values represent reduced birefringence, indicative of reduced collagen and diffuse structure. Letter symbols indicate $P < 0.05$: 'a' versus NP, 'b' versus D15, and 'c' versus D19 ($P < 0.05$; ANOVA; $F = 20.48$, $df = 5$).

between fibrils. After birth (day 22 post-breeding), this pale red stain, an indication of sparse collagen or lack of structure, was intermixed with areas of more aligned and closely packed collagen. Optical density reflected these changes in birefringence of polarized light from sections of peripartum cervix. Typical of that found in cervix from NP mice, optical density measures were low when birefringence was high, an indication of dense collagen content and complex structure. As corrected for hypertrophy of tissue with pregnancy, mean optical density increased by day 15 of pregnancy relative to that in NP controls ($P < 0.05$, ANOVA). The peak optical density by day 21.5 of pregnancy indicated that collagen content and structure was maximally altered by the day before birth. In postpartum rats, optical density remained increased relative to that in cervixes from NP or day 15 pregnant rats. Thus, collagen structure had yet to return to that found in un-rimpened cervix.

The morphology and anatomical distribution of ED-1-stained macrophages was comparable with that in previous reports of macrophages that reside in the murine cervix. ED-1-stained cells were evident in the stroma, around blood vessels, and in the submucosal

epithelium (Fig. 2). Dark-stained cells were associated with counterstained cell nuclei. More macrophages were present in stroma and smooth muscle regions of the peripartum cervix compared with that earlier in pregnancy or in NP rats. Macrophage numbers in the cervix varied with respect to stage of pregnancy. The peak number of stained cells occurred on day 21.5 of pregnancy and remained elevated in the postpartum group compared with that in rats on day 15 of pregnancy or in NP controls.

Nerve fibers, as identified by an antibody to neurofilament protein peripherin, were stained brown and morphologically punctate, likely fibers in cross-section, or extended lengths of thin individual or interwoven fibrils. Occasionally, fibers had beaded varicosities. Nerve fibers were predominantly found within the subepithelial stroma, interspersed between smooth muscle bundles in the perimetrium, and sometimes associated with blood vessels (Fig. 3). With hypertrophy of cervix during pregnancy, nerve fibers were increasingly present in stroma and regions of smooth muscle. The area of cervix that contained nerve fibers was significantly increased by day 15 of pregnancy compared

Figure 2 Top panels. Photomicrographs of ED-1-stained macrophages in the rat cervix. Macrophages were darkly stained and sections counterstained with hematoxylin (under the microscope, cells were brown with violet counterstain of nuclei). Scale bar = 50 μ m. Bottom panel. Mean number of macrophages ($\pm$ S.E.M.; $n = 5-6$ rats/group) in the cervix of rats normalized to cell nuclei relative to that in the non-pregnant group. Mean numbers of macrophages in NP rats were $3.30 \text{ cells}/\mu\text{m}^2 \times 10^2$ nucleus area in cervix. See Materials and Methods for details of the cell-counting procedure. Group designations and letter symbols of statistical analysis are the same as in Fig. 1 ($P < 0.05$; ANOVA; $F = 9.39$, $df = 5$).

Figure 3 Top panels. Photomicrographs of peripherin-stained nerve fibers in the rat cervix. Nerve fibers were darkly stained and sections were counterstained with hematoxylin (under the microscope, fibers were brown with violet counterstain of nuclei). Scale bar = 50 μm . Bottom panel. Under brightfield microscopy, boxes in an eyepiece reticle grid (10 $\times$ 10 boxes) that contained immunoreactive nerve fibers were counted, multiplied by the area of the grid (2401 $\mu\text{m}^2/\text{box} \times 10 \mu\text{m}$ section thickness), then normalized to average cell nuclei counts in the respective group and to cell nuclei counts in non-pregnant rats to account for hypertrophy of cervix with pregnancy and for variability related to individual tissue sections. Data are the mean fiber density/area of cervix ($\pm$ S.E.M.; $n=5-6$ rats/group) in at least 21 non-overlapping vertical and horizontal grid placements in a total of two sections from each cervix. Group designations and letter symbols of statistical analysis are the same as in Fig. 1 ($P<0.05$; ANOVA; $F=8.74$, $df=5$).

with that in NP rats. Further increases in the density of nerve fibers were evident by day 19 of pregnancy with peak area of innervation found by the day before birth, day 21.5 of pregnancy. After birth, the density and distribution of nerve fibers was not significantly different from values on days 15 and 19 of pregnancy.

Experiment 2: effect of HnX on timing of birth

In Sham-operated controls, births occurred spontaneously at normal term (Table 1). The timing of birth was not significantly different in rats with hypogastric nerve transections. Most rats gave birth on day 22 of pregnancy; one of 6 HnX rats gave birth on day 23 post-breeding. Pups in both groups were found alive in the morning on the day of birth. Thus, transection of the hypogastric nerve did not interfere with the timing of birth or delivery of pups. Moreover, transection of the hypogastric nerve did not affect hypertrophy of the cervix with pregnancy. The cell nuclei per area of cervix was equivalent in groups of Sham and HnX rats, as well as

to the cell nuclei density in PP rats in Experiment 1 ($P>0.05$, Student's *t*-test).

Transection of the hypogastric nerves did not affect remodeling of the cervix at the conclusion of pregnancy. Birefringence of polarized light from picrosirius red-stained sections was similar in Sham and HnX rats; comparable with that seen in unoperated PP rats in Experiment 1. Collagen fibers were loosely and diffusely distributed in sections of cervix from both groups. Open pockets with sparse picrosirius red stain were evident in portions of most sections irrespective of treatment.

Table 1 Day of birth, pups/litter, and density of cell nuclei in the cervix of sham-operated controls (Sham) or hypogastric nerve transected rats (HnX).

Group	<i>n</i>	Day of birth	Pups/litter	Cell nuclei ($\#/\mu\text{m}^3 \times 10^{-4}$)
Sham	5	22	10 ± 3.4	4.15 ± 0.7
HnX	6	22.2 ± 0.4	9 ± 3.6	4.09 ± 1.4

Data are mean $\pm$ S.E.M.; n = number of rats/group.

Figure 4 Top panels. Photomicrographs of collagen fibers in the cervix of rats that were sham-operated (Sham) or had their hypogastric nerve transected (HnX) on day 14–15 of pregnancy. Graph indicates the mean optical density ($\pm$ S.E.M.; 3 sections/rat, $n=5-6$ rats in each group) of birefringence of polarized light normalized to cell nuclei density to account for variability due to hypertrophy of cervix with pregnancy, as well as among individual sections and mice. $P>0.05$ HnX versus Sham, Student's t -test, $F=3.11$). Middle panels. Photomicrographs of macrophages in the cervix of a Sham or HnX rat. Graph is the mean number of macrophages normalized to total cell nuclei density in cervix of each group ($\pm$ S.E.M., $n=5-6$; $P>0.05$, Student's t -test, $F=0.06$). Bottom panels. Photomicrographs of nerve fibers in the cervix of a Sham or HnX rat. Note spiral of thick nerve fiber bundle. Mean area $\pm$ S.E.M. of cervical tissue with nerve fibers normalized to total cell nuclei density ($n=5-6$ rats; $P>0.05$, Student's t -test, $F=0.04$). Scale bars=50 μ m.

Optical density was equivalent between Sham and HnX groups ($P=0.11$, $df=9$, Student's t -test; Fig. 4 top). In addition, the census of dark brown- ED-1-stained macrophages was not significantly different between Sham and HnX groups ($P=0.99$, $df=9$, Student's t -test; Fig. 4 middle). Finally, peripherin-stained nerve fibers were commonly found near blood vessels and as bundles in subepithelial regions of the cervix. The area of cervix that contained nerve fibers was the same in Sham controls and HnX rats ($P=0.86$, $df=9$,

Student's t -test; Fig. 4 bottom). Thus, collagen content and complexity of structure, as well as density of macrophages and innervation were similar in Sham and HnX rats.

Discussion

The present findings indicate that hypertrophy of cervix, reduced collagen content and structure, enhanced presence of immune cells, and increased local

innervation are processes that characterize prepartum remodeling of the cervix in the rat. These findings correlate well with previous reports of parameters associated with cervical remodeling in several strains of mice (Mackler *et al.* 1999, Kirby *et al.* 2005, Yellon *et al.* 2008), as well as to replicate the time course for prepartum changes in collagen structure (Marx *et al.* 2006) using a different method of analysis, optical density versus luminosity. Consistent with (Bosquiazzo *et al.* 2005), the census of macrophages in the cervix peaked by the day before birth, 12 h or so before pups delivered in the present study. Collectively, the evidence supports the proposition that remodeling of the cervix involves an increased presence of macrophages and nerve fibers during the peripartum period. Recruitment of macrophages may be important for the remodeling process because these immune cells produce proinflammatory cytokines, contain leukocyte collagenases, and are a source for prostaglandins and nitric oxide (Junqueira *et al.* 1980, Hertelendy & Zakar 2004, Doherty & Broide 2007). Each of these factors promotes inflammatory processes that include collagen breakdown, edema, and vasodilation in other tissues. In addition, evidence in rabbits (Uchiyama *et al.* 1992) and women (Tanaka *et al.* 1998, Facchinetti *et al.* 2005, Huber *et al.* 2005, Tornblom *et al.* 2005) indicates that proinflammatory cytokines, i.e., message and/or protein concentrations, are increased in association with ripening of the cervix at term. The temporal correlation between increased immune cell-associated products and enhanced numbers of macrophages raise the possibility that immune cells may participate in processes of relaxation, remodeling, and ripening of the cervix in the preparation for parturition. These findings have implications in humans for similar processes associated with ripening of the cervix (Ekman-Ordeberg *et al.* 2003).

The importance of a peak in nerve fibers in the prepartum cervix has yet to be determined. After day 19, the distribution of nerve fibers during the period leading up to birth was relatively stable and did not keep pace with the significant hypertrophy of cells in the cervix. Neuropeptidergic nerve fibers, such as those that stain for calcitonin gene-related peptide (CGRP), densely innervate the rat cervix (Mowa *et al.* 2003) and, in the mouse, the density of CGRP-immunoreactive fibers increase by the day before birth (Kirby *et al.* 2005). Other neuropeptidergic fibers that are present within the cervix (Taurig *et al.* 1991, Papka & McNeill 1993) could also serve neuroeffector functions of sensory fibers to promote vasodilation or leukocyte immigration (Holzer 1988). The idea that neural secretions may mediate local immigration or activation of immune cells extends from studies in the cutaneous immune system where sensory neuropeptides promote inflammation (Collins *et al.* 1998, Kong *et al.* 1998, Richardson & Vasko 2002). Thus, neurogenic activation of an inflammatory immune

reaction in the cervix may be a crucial upstream component of the multiphasic mechanism described by Challis *et al.* (2002) for the transition from Phase 0 to 1 in the process of parturition.

Findings that support an increase in immune cells in the prepartum rodent cervix are based upon studies in which specific immunohistochemically stained cells were counted in a known volume of tissue (Mackler *et al.* 1999, Yellon *et al.* 2008). Although the cervix increases in mass with pregnancy compared with that when not pregnant, i.e., hypertrophy of the cervix reflects an increase in total number of cells and extracellular space, the actual numbers of cell nuclei per area of a microscopic field significantly decreases by the day before birth versus day 15 of pregnancy in rodents or compared with that in NP controls. As stroma and smooth muscle cells in the cervix increase in size with pregnancy, evident in photomicrographs in the present study and other reports (Mackler *et al.* 1999, Timmons & Mahendroo 2006, Yellon *et al.* 2008), intercellular spaces decrease in area of microscopic analysis. Even though the area within the extracellular matrix for immune cells to occupy was reduced, the findings in rodents, our past studies in mice and by Bosquiazzo *et al.* in Wistar rats, indicate that the census of immune cells in the cervix increase several fold relative to the total population of cells in the cervix. Greater numbers of immune cells are likely due to immigration into the peripartum cervix, since macrophages and neutrophils differentiate, but do not replicate in tissue. In mice and rats, the time course for immigration of macrophages is remarkably similar; peak numbers occur by the day before birth and remain elevated on the day of birth. This finding is consistent with evidence that leukocytes are increased in the cervix from women at term relative to that earlier in pregnancy (Bokstrom *et al.* 1997). Whether this increase occurs before or after the start of labor is controversial (Osman *et al.* 2003) and may depend upon the complex challenge to obtain cervical biopsies from non-laboring women at term.

To address the second objective of the present study, the findings support the contention that the hypogastric nerve does not regulate cervical ripening or the process of parturition. In the peripartum cervix, collagen content and structure, macrophage census, and density of nerve fibers were the same as in rats that had bilateral transection of the hypogastric nerve as in sham-operated controls. These results raise the possibility that a neural pathway other than that involving the hypogastric nerve may affect cervical ripening. Transection of the pelvic nerve, the sensory viscerocutaneous branch in particular, is associated with delayed onset of birth, dystocia, prolonged duration of labor or prevention of delivery in a majority of rats (Burden *et al.* 1990, Martinez-Gomez *et al.* 1998). With the exception that collagen density was observed to increase in the cervix after pelvic neurectomy (Burden *et al.* 1984), the consequences of

pelvic nerve transection on morphological processes associated with ripening of the cervix are not known. Moreover, based upon evidence in NP rats, innervation by the vagus nerve may also be important to regulate cervical function (Cueva-Rolon *et al.* 1996, Collins *et al.* 1999, Guevara-Guzman *et al.* 2001). In conjunction with the lack of effect of hypogastric nerve transection on the timing of birth, the data clearly indicate that innervation of the lower uterus and cervix by the hypogastric nerve is unlikely to have a role in the process of parturition at normal term.

Other important physiological functions are mediated by the hypogastric innervation of the lower uterus and cervix. This nerve projection is reported to be involved in micturition, blood flow, and uterine contractility (de Groat & Theobald 1976, Sato *et al.* 1989, 1996, Vera *et al.* 1997, Dmitrieva *et al.* 2001). Although, the hypogastric nerve contains sympathetic and sensory neuropeptidergic fibers, the cervix contains few sympathetic fibers (Houdeau *et al.* 1998, Mowa & Papka 2004). By contrast, a variety of peptide-containing fibers, including substance P and vasoactive intestinal peptide are part of the hypogastric innervation of the cervix (Dalsgaard *et al.* 1983, Carvalho *et al.* 1986). The apparent absence of these projections following hypogastric nerve transection did not affect the processes of cervical remodeling or processes related to the normal timing of birth. All pups were born alive, on time, and without evidence of dystocia or forestalled labor. Activity by abdominal musculature to promote fetal expulsion (Higuchi *et al.* 1987) or smooth muscle 'ratcheting' to maintain intrauterine tension between contractions (Leppert 1995) may compensate for elimination of hypogastric innervation to ensure progression of labor and timely delivery of pups. Parturition could also progress without input from the hypogastric nerve if there was redundancy in neurotransmitter phenotypes innervating the cervix or if hypertrophy of nerve fibers from another nerve pathway compensates for loss of the hypogastric projection. For example, sympathetic fibers and projections from the paravertebral ganglia chain course with the splanchnic and pelvic nerves into the paracervical nerve plexus to innervate the cervix in NP rat (Hulsebosch & Coggeshall 1982, Houdeau *et al.* 1995, Komisaruk *et al.* 1996). In the present study, thick bundles of nerve fibers were commonly present in the cervix by the day before birth in rats lacking hypogastric innervation, but were absent in sham-controls. Thus, changes in morphology and possibly activity of remaining nerve fibers may reinforce input from the CN to regulate processes associated with remodeling of the cervix and timing of birth.

In summary, sympathetic and peptidergic components of the hypogastric nerve do not appear to influence the process of cervical ripening or parturition. Absence of evidence for an alteration in collagen remodeling and immigration of macrophages into the prepartum cervix

between rats following transection of the hypogastric nerve and sham controls suggest that cervical ripening progresses without hypogastric nerve innervation. Thus, the present study leads to the conclusion that other neural pathways contribute to the processes of cervical ripening and parturition.

Materials and Methods

NP and time-dated gravid Long Evans Rats were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). Rats were housed singly on 12 h light:12 h darkness cycle (lights off at 0700 h PST) with food and water *ad libitum*. Animals were cared for in accordance with National Institute of Health Office of Laboratory Animal Welfare policies and the protocol approved by the Institutional Animal Care and Use Committee.

Experiment 1: time course study of peripartum cervical remodelling

Rats that were NP or post-breeding day 15 (D15), day 19 (D19), day 21 (D21), day 21.5 (D21.5) or day 22 (*post partum* PP, estimated as 4–8 h after birth) were killed with CO₂ gas ($n=4-5$ rats/group). The lower reproductive tract including the cervix, attached end of vagina, and body of uterus where horns converged was immediately removed and immersed in 4% paraformaldehyde. Tissue was obtained soon after lights on except for the day 21.5 group in which the procedure occurred a few hours before lights off. After 24 h the cervix was processed and embedded in paraffin. Cervices were sectioned longitudinally at 10 μ m, mounted on positively charged glass slides, and processed as described below. Rats in this study served, in part, as unoperated controls to compare with sham-operated controls in Experiment 2.

Experiment 2: effects of hypogastric nerve transection on cervical remodeling and birth

Rats were anesthetized on day 14–15 post-breeding with an i.m. injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The protocol for transection of the hypogastric nerve followed previously described procedures (Cross & Glover 1958, Cunningham *et al.* 1991, Cueva-Rolon *et al.* 1996, Dmitrieva *et al.* 2001). Briefly, a midline abdominal incision and blunt dissection exposed the descending aorta. The small bowel was gently retracted and wrapped in saline-saturated gauze. The hypogastric nerve was identified to course bilaterally on the surface of aorta caudal to the branch of the ilio-lumbar vessels and distal to the inferior mesenteric nerve plexus. Methylene blue dye was applied topically to clearly identify these myelinated nerve fibers (Seif *et al.* 2004). Rats were then randomly assigned to either a sham control group (Sham; $n=5$), in which the small bowel was replaced, or proceeded to undergo hypogastric neurectomy (HnX; $n=6$). In the HnX group, the blue-tinged hypogastric nerves caudal to the inferior mesenteric ganglion were dissected free from fascia and the blood vessels and a bilateral 5 mm segment removed. In both

groups, the abdominal musculature and fascia were closed with a running suture stitch and the skin secured with surgical staples. One pregnant rat with five implantation sites, two reabsorptions, and two full fetuses, in the right uterine horn and one fetus in the left horn, was excluded from the study. Post-operatively, yohimbine was administered to accelerate recovery from anesthesia (10 mg/kg, i.m). External compression of the bladder was performed periodically to express urine given the potential side effect of a weakened urethral sphincter reflex (Dmitrieva *et al.* 2001, Yoshiyama & de Groat 2002). Near term, rats were observed at about 2–4 h intervals between 0800 h and 0700 h. The cervix was obtained as described for rats in Experiment 1, above.

To confirm that this procedure abolished the hypogastric nerve projection to the cervix, NP and day 15 pregnant rats were subjected to the sham or hypogastrectomy procedure described above ($n=2-3$ Sham or 2 HnX respectively for NP and pregnant groups). Before closing the abdomen, the cervix of each rat was injected in three separate locations with 4 μ l of 4% True Blue dye, a retrograde neural tract tracer. This method has been used to identify connections between the cervix and spinal cord (Berkley *et al.* 1993). After 7 days, rats were perfused with warm saline followed by 4% paraformaldehyde. The dorsal root ganglia in the thoracic (T10–T13) and lumbar (L1–L3) segments of the spinal cord was carefully excised and post-fixed overnight. Tissues were immersed in 30% sucrose, cryostat sectioned at 30 μ m, counterstained with mounting medium containing DAPI (Vector Labs, Burlingame, CA, USA), and fluorescence evaluated using an appropriate filter set (472–504 nm). True Blue fluorescence was only found in dorsal root ganglia from the lower thoracic segment of Sham rats (Fig. 5, pale grey-filled cells reflect green fluorescence). No fluorescent cells were present in dorsal root ganglia from thoracic segments of spinal cord in HnX rats or from segments of lumbar spinal cord in either group (data not shown). DAPI counterstain was present in all dorsal root ganglia (bright dots were seen as intense punctate violet-blue fluorescence).

Processing and staining of the cervix

To visualize collagen structure and content, three 10 μ m cross-sections of cervix from each rat were stained with picosirius red as previously described (Leppert 1995, Yu *et al.* 1995, Luque *et al.* 1996, Kirby *et al.* 2005). A single black and white photograph, at 20 $\times$ objective, was obtained from each section of cervix. Images were acquired in areas of cervix that did not contain luminal epithelium, uterus, vaginal tissue or other vacant spaces. In each image, nine adjacent but non-overlapping grids were arranged in a cross pattern over the image. The optical density of polarized light in black and white photographs of each grid was analyzed by NIH Image J software with gray scale threshold calibrated using the Rodbard standard curve (<http://rsb.info.nih.gov/ij/>). Areas of high collagen content and complexity were bright red and, with conversion to black and white, became white regions of low optical density. By contrast, low collagen content and complexity were proportionally darker with higher optical density. Owing to the expected hypertrophy and hyperplasia of

Figure 5 Photomicrographs of True Blue fluorescence in a dorsal root ganglion from the lower thoracic segment of the spinal cord in non-pregnant (top panels) or pregnant rats that were sham-operated (Sham) or hypogastric nerve transected (HnX). Note individual and clustered green fluorescent-tagged cells along lower periphery of the dorsal root ganglion from Sham, but not HnX rats. DAPI counterstain is the bright blue-violet punctate fluorescence. True Blue was injected into the cervix of non-pregnant or day 15 pregnant rats after Sham or HnX surgery. Dorsal root ganglia were obtained 7 days later as described in detail in Materials and Methods. Scale bar=100 μ m.

the gravid versus non-gravid cervix, optical density data were normalized to the number of cell nuclei density for each rat. This method of analysis differs from the assessment of luminosity of collagen in the prepartum cervix by Marx *et al.* (2006) and allows direct comparisons with previous work in mice (Kirby *et al.* 2005, Yellon *et al.* 2008).

Macrophage and nerve fiber immunohistochemistry

Following previously described procedures (Mackler *et al.* 1999, Bosquiazzo *et al.* 2005, Kirby *et al.* 2005), slide-mounted sections were treated with proteinase K for 3 min, placed in a 3% hydrogen peroxide solution for 15 min, then for 30 min each incubated with a blocking solution and then primary antisera ED-1 to visualize macrophages (1:200 dilution, Serotec, Oxford, UK). Biotinylated anti-mouse IgG was used as a secondary antibody (1:400 dilution; Vector Labs). For nerve fibers, sections were similarly processed except a 1% hydrogen peroxide solution was used and the primary antisera was to peripherin, a neurofilament IV-specific antigen (1:500 dilution, Novus Biologicals, Littleton, CO, USA). Biotinylated donkey anti-rabbit IgG served as the secondary antibody (1:200 dilution, Fitzgerald/RDI, Concord, MA, USA). In each run, incubation of sections without the primary antibody served as a negative control for non-specific stain. Macrophage counts and area with nerve fibers were normalized to cell nuclei density in each section to account for hypertrophy and hyperplasia of cervix among rats and with respect to pregnancy.

Statistical analyses

Data were normally distributed ($P > 0.05$, Levene's test for homogeneity of variance). Individual comparisons were made by ANOVA (Experiment 1) or with a Student's *t*-test (Experiment 2) using SPSS statistics software (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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