Persistent genital hyperinnervation following progesterone administration to adolescent female rats

Running title: Adolescent progesterone & vaginal hyperinnervation

Summary sentence: High dose progesterone administration during adolescence, which strongly predisposes women to genital hyperinnervation and pelvic pain (vulvar vestibulodynia), produces persistently increased vaginal sensory and sympathetic innervation in a rat model

Keywords: Vulvodynia; oral contraceptives; progesterone; rat model; sensory nociceptor; sympathetic; parasympathetic; axons; vagina; cell culture; tissue explant culture.

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1Funding was provided by NIH RO1HD049615 (P.G.S.) with core support from NICHD P30HD002528.
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ABSTRACT
Provoked vestibulodynia, a female pelvic pain syndrome affecting substantial numbers of women, is characterized by genital hypersensitivity and sensory hyperinnervation. Previous studies have shown that the risk of developing provoked vestibulodynia is markedly elevated following adolescent use of oral contraceptives with high progesterone content. We hypothesized that progesterone, a steroid hormone with known neurotropic properties, may alter genital innervation through direct or indirect actions. Female Sprague Dawley rats received progesterone (20 mg/kg sc) from days 20-27; tissue was removed for analysis in some rats on Day 28, while others were ovariectomized on Day 43 and infused for 7 days with vehicle or 17beta estradiol. Progesterone resulted in overall increases in vaginal innervation at both Day 28 and 50 due to proliferation of peptidergic sensory and sympathetic (but not parasympathetic) axons. Estradiol reduced innervation in progesterone-treated and untreated groups. To assess mechanisms of sensory hyperinnervation, we cultured dissociated dorsal root ganglion neurons and found that progesterone increases neurite outgrowth by small unmyelinated (but not myelinated) sensory neurons, was receptor-mediated, and was non-additive with NGF. Pre-treatment of ganglion with progesterone also increased neurite outgrowth in response to vaginal target explants. However, pre-treatment of
vaginal target with progesterone did not improve outgrowth. We conclude that adolescent progesterone exposure may contribute to provoked vestibulodynia by eliciting persistent genital hyperinnervation via a direct effect on unmyelinated sensory nociceptor neurons, and that estradiol, a well-documented therapeutic, may alleviate symptoms in part by reducing progesterone-induced sensory hyperinnervation.

INTRODUCTION

Provoked vestibulodynia (PVD), formerly called vulvar vestibulitis syndrome, is a vulvodynia pain syndrome with a reported lifetime prevalence of approximately 16%, concurrently affecting up to 6% of adult females [1]. PVD can affect women of all ages and markedly compromises quality of life. PVD is characterized histologically by the presence of inflammatory cells and marked proliferation of vestibular epithelial and subepithelial axons [2-8]. While some pain relief is afforded by topical estrogen as well as neuroleptics and other centrally acting drugs [9], surgical excision may provide better outcomes; some 80% of patients are reported to experience relief when the hyperinnervated region is surgically excised [10], consistent with a peripheral origin of the pain. PVD is a major female health problem that demands a better understanding of its etiology and strategies for prevention.

Mechanisms responsible for PVD are unknown and multiple factors are implicated. These include repeated vaginal infections [11], co-morbid painful conditions [12], and childhood physical or sexual abuse [13]. Some studies have linked PVD risk to use of oral contraceptives. Oral contraceptive use is reported to increase overall relative risk of developing PVD; however risk was most pronounced in females receiving formulations containing high amounts of the female gonadal steroid hormone, progesterone. Relative risk was greatest when first use occurred between 10-15 years of age, with these individuals showing more than a 9-fold increase [1, 14-16], strongly implicating adolescent progesterone exposure as a factor in some cases of PVD.

Although the role of oral contraceptives in PVD remains controversial [17], progesterone should be given careful consideration given its potent effects on the nervous system. Progesterone is reported to be neuroprotective, to promote myelination, and to enhance axon outgrowth [18-23]. Further, vaginal tissues are well imbued with abundant progesterone receptors [24]. Therefore, progesterone acting directly on neurons or indirectly via vaginally derived neuroactive proteins could contribute to neural changes observed in PVD.

In the present study, we assessed progesterone’s ability to induce genital neuroplasticity. We treated sexually immature adolescent rats with progesterone and quantified vaginal innervation immediately after treatment and at sexual maturity. Because another gonadal steroid, estrogen (17β-estradiol, E2), is known to alter vaginal innervation [25-28] we assessed progesterone’s effects under low- E2 conditions (i.e in the adolescent rat at d28 prior to reproductive hormonal cycling [29] and after ovariectomy [OVX] in the sexually mature rat at 50d [30]). We also examined E2’s ability to modulate innervation after progesterone treatment in the sexually mature rat following OVX to eliminate the suppressive effect of this hormone on vaginal innervation density [25, 28]. In addition, we investigated progesterone’s mechanism of action in dissociated dorsal root ganglion (DRG) sensory neuron cultures and in vaginal and DRG explant co-culture experiments.
MATERIALS AND METHODS

In vivo experimental preparations

Beginning on postnatal day 20, thirty-three female Sprague Dawley rats (Harlan Breeding Laboratories, Indianapolis, Indiana) received 7 daily s.c. injections of vehicle (10% ethanol + 90% sesame oil, n=16) or progesterone (Sigma, St. Louis, MO, n=17) at a dose of 20 mg/kg which has been shown to be effective in altering peripheral innervation [31].

On Day 28, 6 rats receiving progesterone and 6 receiving vehicle were euthanized (pentobarbital, 100mg/kg, i.p.) and vaginal tissue harvested. The remaining 21 rats on day 43 received bilateral OVX [25, 30] under isoflurane anesthesia, and were implanted with a pellet containing 17β-estradiol (0.1mg/pellet, 21day release, Innovative Research of America, Sarasota, Florida, n=5 progesterone and n=5 controls) which raises serum E2 to levels approximating term pregnancy [30, 32] or placebo (Innovative Research of America, n=6 progesterone and n=5 controls). On day 50, rats were euthanized as above and vaginal tissue harvested. This experimental design allowed us to compare tissue histology under low-E2 conditions (i.e., day 28 intact [29] and day 50 OVX [30]) and to determine whether E2 is effective in reducing innervation in the adult after adolescent progesterone treatment.

All experimental protocols complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the University of Kansas Medical Center Animal Care and Use Committee.

Analysis of vaginal innervation

Vaginas were removed through a ventral midline lower abdominal incision by separating it from loosely adherent fascia and transecting it at the cervical junction. Tissue was fixed in Zamboni’s solution for 24 h at 4°C, washed daily for 7 days with 0.01 M PBS (pH 7.4), and transferred to 20% sucrose in PBS and at 4°C overnight. Tissue was cut transversely into four segments of equal length, snap-frozen in isopentane precooled in liquid nitrogen, and 10-micron frozen serial sections were cut perpendicular to the longitudinal axis of the vagina from the two central quarterns. Sections were thaw mounted onto Superfrost/Plus precleaned slides (Fisher Scientific, Pittsburgh, PA) and air-dried.

Sections were rinsed in PBS containing 0.3% Triton X-100 (PBST) and blocked for 1 h at room temperature in PBST containing 5% normal goat or donkey serum and 1% bovine serum albumin. Sections were incubated overnight at room temperature with rabbit polyclonal antisera against protein gene product 9.5 (PGP9.5, 1:1200; AbD Serotec, Raleigh, NC), a pan-neuronal marker [33]; tyrosine hydroxylase (TH, 1:200; Chemicon, Billerica, MA), a sympathetic neuronal marker protein [34]; and anti-calcitonin gene-related peptide (CGRP, 1:800; Chemicon), a marker of peptidergic nociceptors [35, 36]; or goat polyclonal antiserum against vesicular acetylcholine transporter (VACHT, 1:1000; Chemicon), which identifies vaginal cholingergic parasympathetic neurons [37]. Sections were washed three times in PBST followed by a 1h room temperature incubation with goat anti-rabbit IgG conjugated to Cy3, 1:200, for PGP9.5, TH and CGRP (Jackson ImmunoResearch, West Grove, PA) or donkey
anti-goat IgG conjugated to Cy3, 1:200 for VACHT (Jackson ImmunoResearch). Slides were coverslipped with Fluoromount G (Southern Biotech, Birmingham, AL). All antibodies used have been characterized previously and specificity confirmed by antigen preabsorption and primary antisera omission [25, 38].

The apparent percentage area occupied by axons immunoreactive for PGP9.5, TH, VACHT, and CGRP was assessed to provide an index of vaginal innervation density according to methods described previously [25, 27, 28]. Digital microscopic images (Nikon Eclipse TE300, Nikon Corp., Tokyo, Japan, Optronics MagnaFire camera, Optronics, Goleta, CA) were captured from two randomly selected sections from each of the two blocks containing the middle quarters of the vagina. In each section, six randomly selected fields containing submucosal muscular/connective tissue were captured. In each field, images were obtained using illumination for Cy3 fluorescence. The total area of the submucosal tissue compartment was measured planimetrically (analySIS v. 3.2; Soft Imaging System Corp., Lakewood, CO). The apparent percentage area occupied by immunoreactive nerves was determined by threshold discrimination, and divided by the total area of the sampled field to provide an index of innervation density. To control for changes that could arise secondary to alterations in the vaginal tissue size [25, 27], percentage area was multiplied by submucosal tissue area and innervation expressed as area of immunoreactive axons (mm²) per section. Values for the two central quarters of the vagina for each animal were averaged.

**Dissociated neuronal cultures**

Neurons from T13-S2 DRGs, which include vagina-projecting sensory neurons [39], were collected bilaterally from Sprague Dawley rats at postnatal days 0-2. DRGs were digested by incubation with 0.1% collagenase (Sigma, St. Louis, MO) at 37 °C for 30 min in serum free L-15 (Invitrogen, Carlsbad, CA) containing 3% glucose, followed by 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA) at 37°C for 25 min. DRG neurons were dispersed by trituration with a fire-polished Pasteur pipette, the cell suspension filtered through a 40µm cell strainer, and centrifuged at 300 g for 7 min. The cell pellet was resuspended in DMEM/F12 medium containing 1% Insulin-Transferrin-Selenium-X (ITS), 1% charcoal stripped (i.e., steroid-hormone depleted) FBS (Invitrogen, Carlsbad, CA), and uridine and FrdU (20µM, Sigma, St. Louis, MO). Neurons were plated on poly-D-lysine/laminin-coated 24 well tissue culture plates (BD Biosciences, San Jose, CA) and maintained at 37°C in 5% CO₂. Cultures contained either 20nM progesterone (Sigma, St. Louis, MO), 1µl/ml ethanol vehicle, or 20nM progesterone plus 20nM RU486 (Tocris, Bristol, UK), which is sufficient to block progesterone’s effects on cultured neurons [40]. Neurons were also grown in media containing 50ng/ml mouse NGF (Alomone Labs, Jerusalem, Israel) alone or in combination with 20nM progesterone. Following 72h in culture, neurons were fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) for 1 h at room temperature, rinsed with PBST for 10min 3 times, and blocked with normal goat serum for 1h at room temperature. Cultures were incubated overnight at room temperature with rabbit anti-peripherin polyclonal antibody (Chemicon, 1:500) and mouse monoclonal antibody to neurofilament 200 (NFH, Sigma, 1:400), followed by 3 washes in PBST for 10min each. The cultures were then incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:400) and Alexa Fluor 488 conjugated goat anti-mouse IgG.
(Invitrogen, 1:1000) at room temperature for 1h and washed 3 times in PBST for 10min each. Co-staining for peripherin and NFH allowed us to distinguish in culture the subclass of DRG neurons that typically provides vaginal innervation (unmyelinated peripherin-positive neurons) from the larger myelinated (NFH-positive) neurons that do not [25]. A preliminary analysis failed to show any differences in neuronal numbers in the presence or absence of progesterone.

To quantify neurite outgrowth, ten fields in each well were selected randomly and digital images captured and neurite area measured according to methods used previously [28, 41, 42]. In brief, a stereological grid (AnalySis version 3.2; Soft Imaging System Corp., Lakewood, CO) was superimposed over each image, and the number of line intersections overlying stained neurites was divided by total intersections within the field and multiplied by total field area. This was divided by the number of viable neurons with at least one neurite in the field, averaged for each well and expressed as neurite area (µm²) per neuron.

**Explant co-cultures**

L3-L4 DRG and vaginal tissue were harvested from 20 day old female rats. Ganglia were trimmed of rootlets, incubated in 0.1% collagenase in L-15 medium containing 3% glucose for 15 min at 37°, and cut into 3-4 pieces. Vaginas were stripped of adventitial connective tissue and cut into pieces approximately 1mm³.

Prior to co-culture, pieces of ganglia or vaginal tissue were pre-incubated at 37°C overnight and with 5% CO₂ in DMEM/F12 with 1% ITS and 1% charcoal stripped FBS medium containing either 20nM progesterone or 0.1% ethanol vehicle. Ganglion explants were rinsed 3 times with sterile PBS and then embedded alone or with a vaginal explant placed 1 mm away in a matrix containing 0.75mg/ml collagen I (Invitrogen, Carlsbad, CA) and 2.83mg/ml growth factor reduced matrigel (BD Biosciences, San Jose, CA) in a 48-well culture plate. Following matrix solidification, 200 µl of medium consisting of DMEM/F12, 1% ITS, and 1% charcoal stripped FBS was gently added to each well and the cultures were incubated at 37°C in 5% CO₂ for 48 hours.

Explants were fixed overnight at 4°C in 4% paraformaldehyde (Sigma, St. Louis, MO), rinsed 3 times with PBST for 1h, blocked with normal goat serum overnight at 4°C. Explant cultures were incubated for 48h at 4°C with rabbit anti-peripherin polyclonal antibody (Chemicon, 1:1200), followed by 5 washes for 1 h each. Explant cultures were then incubated with Cy3-conjugated goat anti rabbit IgG (Jackson ImmunoResearch Laboratories, 1:600 at 4°C for 24 h), and washed 3 times in PBST for 1h each.

Numbers of neurites crossing a line parallel to and 200µm distant to the surface of the ganglion explant facing the vaginal explant, or the face with maximal outgrowth for DRG explants alone, were counted using a Nikon Eclipse (TE300) inverted microscope with an eyepiece reticle. Neurites were visualized by focusing through the entire depth of the explant. To normalize for differences in ganglion size, neurite count was divided by the length of the sectioned face of the ganglia and expressed as neurites per mm.

**Statistical analysis**

All values are presented as the mean ± SEM. The effect of progesterone on vaginal innervation under low E2 conditions (28d and 50d OVX) was assessed using 2-way
anova with post-hoc comparisons by Student-Newman-Keuls test. Comparisons of the effect of E2 at 50d were made using 1-way anova with Student-Newman-Keuls post-hoc testing. Neuronal cultures and explants were evaluated using 1-way anova with Student-Newman-Keuls or Bonferonni testing. Differences were considered significant at $p \leq 0.05$.

RESULTS

**Vaginal innervation in vehicle-injected rats**

The distribution of PGP9.5-immunoreactive (-ir) vaginal nerve fibers in vehicle-injected 28d rats was similar to that described previously [25, 27, 43] with fibers localized within the connective tissue and muscular layer (Fig. 1a), although the density appeared less than that of the adult. By d 50, OVX rats receiving vehicle appeared to have slightly higher PGP-9.5-ir axon density (Fig. 1b) although differences were not significant (Fig. 1g). OVX rats receiving 7d of sustained E2 elevation showed lower PGP9.5-ir innervation density than those with low-E2 status (Fig. 1c, g, $p<0.001$).

PGP9.5-ir vaginal axons comprise multiple populations including presumptive sympathetic fibers immunoreactive for TH [34], paracervical ganglionic cholinergic parasympathetic fibers containing VACHT [37], and peptidergic sensory nociceptor axons immunoreactive for CGRP [44]. TH-ir fibers were localized predominantly to blood vessels and the muscularis in 28d vehicle-injected rats (Fig. 2a) and their distribution and frequency remained comparable at 50 d in OVX rats (Fig. 2b, g) and their distribution and frequency remained comparable at 50 d in OVX rats (Fig. 2b, g). E2 treatment decreased TH-ir axon density relative to the low-E2 state (Fig. 2c, g, $p<0.001$). VACHT-ir fibers in 28d control rats were associated primarily with the vasculature and nonvascular smooth muscle (Fig. 3a), were increased at 50d in OVX rats (Fig. 3b, g, $P<0.001$), and decreased by E2 treatment (Fig. 3.c, g, $p<0.001$). CGRP-ir fibers were distributed within connective tissue, blood vessels, and smooth muscle (Fig. 4a and showed no change at 50d in OVX rats (Fig. 4b, g). E2 treatment reduced their density (Fig. 4c, g, $p<0.001$).

**Progesterone increases vaginal innervation**

Distributions of PGP9.5-ir fibers in 28d rats treated for 7d with progesterone were comparable to those of vehicle injected rats, but density was increased by 45% (Fig. 1d, g, $p<0.001$). Axon density was comparably elevated at 50d in OVX rats following progesterone administration from d20-27 (Fig. 1e, g). In 50d progesterone-treated rats, density was reduced by E2 for 7 d (Fig. 1f, g, $p<0.001$) but was greater than that of E2-treated rats not receiving progesterone ($p<0.05$).

Progesterone treatment increased TH-ir fiber density in 28d rats by 38% ($p<0.01$, Fig. 2d, g). At 50d, TH-ir innervation density was also increased in progesterone-treated OVX rats at 50d ($p<0.01$) relative to vehicle-injected controls (Fig. 2e, g). Progesterone treatment did not attenuate the reduction in innervation density in 50 d OVX rats elicited by E2 (Fig. 2f, g, $p<0.001$).

Administration of progesterone to developing rats did not increase density of VACHT-ir nerves at 28 or 50 d (Fig. 3d, e, g). E2 reduced VACHT-ir innervation density in 50d rats ($p<0.001$) treated with progesterone in a manner similar to that of vehicle-injected rats (Fig. 3 f, g).
CGRP-ir innervation of 28d rats was increased relative to vehicle injection (Fig. 4 d, g, p<0.001). This elevation was also observed at day 50 (Fig. 4 e, g, p<0.001). Pre-pubertal progesterone treatment did not attenuate the decrease in CGRP-ir axon density induced by adult E2 administration (Fig. 4f, g).

**Progesterone directly promotes sensory neuritogenesis**

We cultured T13-S2 DRG neurons in the presence or absence of progesterone in cultures where charcoal stripping of FBS ensured that our addition of progesterone to the defined media represented the only source of this hormone. Immunostaining for the intermediate filament protein peripherin, a selective marker for unmyelinated nociceptors which comprise the bulk of CGRP-ir sensory neurons, revealed many neurons with moderate neurite outgrowth after 72h (Fig. 5a) as seen previously [28, 41, 42]. The addition of progesterone to the medium at the time of plating increased neurite outgrowth by 27% relative to addition of vehicle (Fig. 5b, d, p<0.01,). Addition of the progesterone receptor antagonist RU486 prevented progesterone-induced outgrowth (Fig. 5c,d, p<0.05,).

**Progesterone promotes sprouting of unmyelinated but not myelinated DRG neurons**

We assessed whether progesterone also elicits sprouting of myelinated sensory neurons by staining for NFH, a marker of large DRG neurons. NFH-ir neurons were frequently encountered in cultures and readily produced neurites in the defined media (Fig. 6a). Addition of progesterone had no effect on neurite outgrowth by myelinated neurons (Fig. 6b; 2321±196 vs 2666±158 µm² for vehicle and progesterone, respectively).

Progesterone is reported to affect expression of neurotrophin receptors expressed by DRG neurons [45]. To assess whether neurotrophin signaling modulation may contribute to progesterone’s neuritogenic effects, we cultured DRG neurons in the absence (Fig. 6c) or presence (Fig. 6d) of progesterone, confirming our prior finding of increased neuritogenesis (Fig. 6g). To these cultures we also added NGF, the preferred neurotrophin for peptidergic neurons, either alone (Fig. 6e) or in combination with progesterone (Fig. 6f). NGF increased outgrowth of peripherin-ir fibers relative to controls (Fig. 6g, p<0.001), and the neuritogenic effect of NGF was greater than that of progesterone (Fig. 6g, p<0.05). Addition of progesterone and NGF in combination also elicited neurite outgrowth that was greater than control cultures (p<0.01), but the effect was comparable to that of NGF alone (Fig. 6g).

**Effect of progesterone on ganglion-vaginal explant co-cultures**

Our dissociated cell cultures and previous explant culture studies [23] provide evidence for a direct effect of progesterone on DRG sensory neurons that is non-additive to NGF-induced outgrowth. However, target tissues are capable of synthesizing and releasing multiple factors that can either promote or repel axon outgrowth [28, 46-49]. To assess whether progesterone may modulate sensory neurite outgrowth via actions on the vaginal target tissue, we pre-cultured pieces of DRG or vagina in the presence or absence of progesterone, and then cultured the DRGs in defined, progesterone-free medium with or without vaginal explants; this approach
ensures that progesterone’s effects are restricted to only one tissue or the other, and excludes contributions of other hormonal factors that may be modulated by progesterone in vivo. Neurite quantitation was performed in peripherin-immunostained specimens since our dissociated cell culture studies showed this to be the affected population.

DRG explants cultured without progesterone pretreatment and in the absence of vaginal explants elaborated moderate numbers of peripherin-ir neurites (Fig. 7a). DRG explants cultured in the presence of vaginal tissue without progesterone pretreatment extended neurites (Fig. 7b) to a degree comparable to that of DRG explants cultured alone (Fig. 7g).

Pretreatment of DRG explants with progesterone (Fig. 7c) increased neurite outgrowth beyond that seen with untreated DRG explants (Fig. 7g, p<0.05). Co-culture of P-pretreated DRG explants with vaginal tissue (Fig. 7d, e) did not increase outgrowth beyond that of progesterone-treated DRG explants cultured alone (Fig. 7g). Neurite outgrowth was not increased by pretreating vaginal explants with progesterone (Fig. 7e, f, g).

**DISCUSSION**

The vagina is richly innervated by autonomic and sensory nerves that regulate vaginal smooth muscle tone [43] and respond to noxious stimuli [50]. An important feature of female reproductive tract innervation is the remarkable degree of physiological plasticity that occurs as a function of variations in ovarian steroid levels. For example, term pregnancy is accompanied by massive uterine sympathetic axon degeneration [51], and repetitive sympathetic pruning occurs throughout the rat estrous cycle as well [52]. Uterine sympathetic degeneration occurs within 24h of a single injection of E2 [30], and mice lacking functional ERα show uterine sympathetic hyperinnervation that is not reduced by endogenous or exogenous E2 [53].

Vaginal innervation is also hormonally responsive, with degeneration of autonomic and sensory nerves occurring at term pregnancy [27] and following sustained administration of exogenous E2 [25]. Accordingly, it is reasonable to speculate that progesterone, with its well-recognized neurotrophic effects [18-23], might also impact vaginal innervation. In particular, DRG explants show rapid progesterone-induced increases growth cone motility [23], consistent with our findings in dissociated DRG neuron cultures of direct, progesterone receptor mediated sensory neurite outgrowth. Progesterone is also known to affect DRG CGRP content in vivo [54]. Therefore, evidence supports the idea that progesterone could affect vaginal innervation.

Indeed, this study shows that progesterone does promote vaginal axon outgrowth in vivo. Adolescent rats showed increased overall PGP9.5-ir vaginal innervation density following 7d of progesterone administration. Consistent with effects on DRG explants [23] and peptide content [54], DRG-derived CGRP-ir vaginal fibers are increased, thus contributing to the increase in overall innervation. In addition, an increase in TH-ir sympathetic fibers also contributes to increased innervation density after progesterone. To our knowledge, this is the first demonstration that progesterone can influence sympathetic innervation of target tissues, and underscores a need for further research concerning the mechanism by which this reproductive steroid exerts its effect.
While both sympathetic and sensory peptidergic neurons appear to respond to progesterone with increased terminal outgrowth, this response is selective. Hence, density of VACHT-ir parasympathetic axons in the vaginal tissue was unchanged and, similarly, its effect on DRG sensory neurons is restricted to the smaller diameter nociceptive component, as the larger-diameter myelinated NFH-ir neurons showed no change in the presence of progesterone. This is somewhat unexpected given evidence in other systems that progesterone promotes regeneration and remyelination of larger-diameter fibers [18, 19]. It is not clear why different populations of sensory and autonomic neurons respond differently, but such selectivity could be attributable to differential expression of progesterone receptors or variations in down-stream signaling.

A striking feature of progesterone-induced hyperinnervation is its persistence. The elevated levels of sensory and sympathetic innervation observed immediately following progesterone treatment persisted in low-E2 rats observed 22d later. This is impressive given that this is the period during which rats reach full sexual maturity (typically around 35d, based on vaginal opening and hormonal cycling [55, 56]). It is not clear what accounts for this persistence. One possibility is that, while changes to the target tissue may not be necessary for progesterone-mediated outgrowth, the hormone modifies vaginal tissue in a manner conducive to maintaining hyperinnervation. Consistent with this idea, progesterone is reported to increase levels of some neurotrophic factors in tissues [57] including the female reproductive tract [46]. However, the absence of an effect of progesterone treatment on vaginal explants argues against this. A more likely explanation may be that progesterone administration in a critical developmental period alters the set-point for terminal arborization such that increased target innervation becomes a persistent feature.

E2 and progesterone are often viewed as having antagonistic actions in reproductive tissues (for example see [58]), and our findings suggest that this is the case regarding vaginal innervation as well. Hence, while progesterone persistently increased vaginal innervation under low E2 conditions, a sustained rise in E2 similar to that of pregnancy resulted in a marked reduction in innervation that was not prevented by prior progesterone exposure. The reduction observed in the progesterone-treated rats was similar to that observed previously during exogenous administration of E2 [25] and at term pregnancy [27]; estrogen-induced depletion of vaginal innervation is thought to be an adaptive process that facilitates parturition by reducing vaginal smooth muscle tone and transmission of pain sensations [27]. Accordingly, while progesterone may persistently elevate vaginal innervation density under low E2, this can be overcome (at least temporarily) when E2 is elevated.

While these findings are intriguing, caution is necessary when extrapolating from animal research to humans. In particular, the relationship between progesterone and E2 in development is complex and a limitation of these studies is our inability to faithfully replicate hormonal interactions that occur when oral contraceptives, which typically contain combinations of hormones, are administered against the backdrop of a changing endogenous hormonal profile in development. Nonetheless these findings may provide important insight in understanding possible relationships between adolescent high-progesterone oral contraceptives and increased risk for PVD [1]. Although systematic histologic studies assessing genital innervation from women receiving high-progesterone oral contraceptives are not available, there is wide
agreement that a hallmark of PVD is the proliferation of perivaginal subepithelial innervation, which was clearly present in our progesterone-treated rats. Some of the fibers contributing to the hyperinnervation were immunoreactive for CGRP, a marker of the peptidergic family of nociceptors, and increases in these fibers have been noted in tissue from patients with PVD [7, 59]. While sympathetic fibers also contribute to progesterone-induced hyperinnervation in rats, this population has not been well investigated in human PVD and it is uncertain as to the extent to which sympathetic fibers contribute to hyperinnervation clinically. However, sympathetic axon proliferation has been noted in a murine model of PVD [60] and is implicated in other painful conditions [61]. Accordingly, the genital hyperinnervation observed after adolescent progesterone treatment is consistent with histological changes documented previously in localized pain syndromes and appears to be similar in some regards to that seen in PVD patients.

While adolescent progesterone oral contraceptives can strongly predispose individuals to PVD later in life, clearly this is a multifactorial disorder in which progesterone is likely to play a partial role. For example, another predisposing factor is a history of inflammation. The incidence of PVD is increased substantially in women experiencing frequent vaginal infections [62, 63], and repeated yeast infections induced in mice give rise to some features reminiscent of PVD [60]. In this regard it is noteworthy that inflammation also contributes to hyperinnervation [64, 65], raising the possibility that progesterone and inflammation could act synergistically to promote hyperinnervation and hypersensitivity.

The ability of E2 to reduce vaginal innervation also has relevance to human PVD. Sustained elevations in E2 can reduce vaginal innervation in both rats [25, 28] and humans [26], and topical E2 application has long been a mainstay in treating symptoms of PVD. Our findings that sustained E2 elevation is effective in reducing innervation density in both controls and progesterone-treated rats provide a biological rationale for continued use of E2 as a treatment strategy. It is relevant, however, that histologic analysis of tissue from patients with PVD has provided evidence that E2 receptor signaling may be reduced [66]. If so, this could clearly alter the ability of E2 to reduce innervation, and could further contribute to the hyperinnervation and related symptoms.

ACKNOWLEDGEMENTS
We thank Drs. Dora Krizsan-Agbas, Anuradha Chakrabarty and Aritra Bhattacherjee for technical guidance regarding the dissociated neuronal culture studies and the staff of the Kansas Intellectual and Developmental Disabilities Research Center.

REFERENCES

**Figure legends**

**Figure 1.** Vaginal innervation immunostained for the pan-neuronal marker, PGP9.5 in rats receiving injections of vehicle (V, a-c) or progesterone (P, d-f) on days 20-27. Tissue was obtained from intact rats at 28d (a, d), from 50d rats receiving ovariectomy (OVX) on d43 (b, e), and from 50d OVX rats receiving estrogen (E2) infusion from d43-50 (c, f). Axons are largely absent from the epithelial mucosa (E) but are present in the submucosal vascularized connective tissue (C) and the muscular layer (M). Quantitative analysis (g) showed that progesterone increased PGP9.5 axon density in 28d intact rats and 50d OVX rats (*P<0.05) as compared to vehicle injections. E2 reduced axon density in vehicle-injected and progesterone treated rats at 50d (#p<0.001), and innervation density in progesterone-treated rats was greater than vehicle-injected rats after E2 (*p<0.05). Numbers of experimental subjects in parentheses. Bar in f = 50µm for a-f.

**Figure 2.** Vaginal innervation immunostained for tyrosine hydroxylase (TH) in rats receiving vehicle injections (V, a-c) or progesterone (P, d-f) on days 20-27. Tissue was obtained from intact rats at 28d (a, d), from 50d rats receiving ovariectomy (OVX) on d43 (b, e), and from 50d OVX rats receiving estrogen (E2) infusion from d43-50 (c, f). Quantitative analysis (g) showed that progesterone increased TH-ir axon density in 28d
intact and 50d OVX rats (*p<0.01). E2 reduced axon density in vehicle- and progesterone -treated rats (# p<0.001) to comparable levels. Numbers of experimental subjects as in Fig. 1. Bar in f = 50µm for a-f.

**Figure 3.** Vaginal innervation immunostained for vesicular acetylcholine transporter (VACHT) in rats receiving vehicle injections (V, a-c) or progesterone (P, d-f) on days 20-27. Tissue was obtained from intact rats at 28d (a, d), from 50d rats receiving ovariectomy (OVX) on d43 (b, e), and from 50d OVX rats receiving estrogen (E2) infusion from d43-50 (c, f). Quantitative analysis (g) showed that although innervation increased between 28 and 50d (+ P<0.001), progesterone did not affect innervation at either age. E2 reduced axon density in vehicle-injected and progesterone-treated rats at 50d (# p<0.001), and levels after E2 were comparable. Numbers of experimental subjects as in Fig. 1. Bar in f = 50µm for a-f.

**Figure 4.** Vaginal innervation immunostained for calcitonin gene-related peptide (CGRP) in rats receiving vehicle injections (V, a-c) or progesterone (P, d-f) on days 20-27. Tissue was obtained from intact rats at 28d (a, d), from 50d rats receiving ovariectomy (OVX) on d43 (b, e), and from 50d OVX rats receiving estrogen (E2) infusion from d43-50 (c, f). Quantitative analysis (g) showed that progesterone increased CGRP-ir axon density at both ages (*P<0.001). E2 reduced axon density in vehicle-injected and progesterone-treated rats at 50d (#p<0.001), and levels after E2 were comparable. Numbers of experimental subjects as in Fig. 1. Bar in f = 50µm for a-f.

**Figure 5.** Neonatal dissociated DRG neurons were cultured for 72 hours in the presence of vehicle (V, a), progesterone (P, b) or progesterone in combination with the progesterone receptor antagonist RU486 (RU, c). Immunostain for peripherin, a marker of unmyelinated neurons, revealed neurons and neurites. Bar in c = 50µm for a-c. Quantitation (d) showed that progesterone increased neurite outgrowth (*p<0.01), and that RU486 reduced progesterone-induced neurite outgrowth (&p<0.05) to a level comparable to vehicle alone. Values represent averages of 10 randomly selected fields from each of 3 wells in 3 separate cultures in each group.

**Figure 6.** Neonatal dissociated DRG neurons cultured for 72 hours in the presence of vehicle (V, a, c), 20nM progesterone (P, b, d), or 50ng/ml nerve growth factor (NGF) alone (e) or in combination with progesterone (f). Cultures were immunostained for neurofilament H (NFH, a, b) to identify outgrowth by myelinated fibers. Other panels (c-f) show cultures stained for the unmyelinated axon marker, peripherin. Bar in f = 50µm for a-f. Quantitative analysis (g) shows increased neuritogenesis in progesterone vs vehicle treated cultures (*p<0.05). NGF increased outgrowth relative to vehicle controls and to progesterone treatment (%p<0.05). Outgrowth in cultures treated with both NGF and progesterone was greater than vehicle and progesterone treated cultures ($p<0.01) but comparable to NGF alone. Values represent averages of 10 randomly selected fields from each of 3 wells in 3 separate cultures in each group.
Figure 7. Explants of dorsal root ganglion (DRG) and vaginal tissue (VAG) pre-incubated with either vehicle (v) or 20nM progesterone (p) prior to culturing in matrigel plus collagen gel in defined media for 48 hours and immunostaining for peripherin. DRG explants cultured alone were pre-incubated with vehicle (a) or progesterone (c). Cocultures consisted of ganglion and vaginal explants with vehicle preincubation (b), progesterone-treated DRG and vehicle-treated vagina (d), vehicle-treated DRG and progesterone-treated vagina (e), and both DRG and vagina preincubated with progesterone (f). Bar in f = 50µm for a-f. Numbers of DRG neurites were counted (g). DRG pretreatment with progesterone increased outgrowth beyond that of vehicle alone (*p<0.05) while vaginal tissue did not affect DRG outgrowth. N = 7-8 explant cultures per treatment group.
Figure 1
Figure 2

(a) 28d V

(b) 50d OVX

(c) 50d E2

(d) TH

(e) 50d OVX

(f) 50d E2

(g) Bar graph: TH-ir axon area (mm²)

- Day 28 Intact
- Day 50 OVX
- Day 50 E2

Legend:
- Vehicle
- Progesterone

Significance:
- * indicates significant difference
- # indicates significant difference
Figure 3
Figure 4
Figure 6
Figure 7