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Acute and chronic estrogen supplementation decreases uterine sympathetic innervation in ovariectomized adult virgin rats

E.V. Zoubina[1], A.L. Mize[2], R.H. Alper[2] and P.G. Smith[1]

[1]Department of Molecular and Integrative Physiology, and R.L. Smith Mental Retardation Research Center and
[2]Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas, USA

Summary. Uterine innervation undergoes substantial reorganization associated with changes in reproductive status. Nerves innervating the uterus are decreased in pregnancy and puberty, and even the normal rodent estrous cycle is characterized by fluctuations in numbers of myometrial nerve fibers. During the follicular (proestrus/estrous) phase of the estrous cycle, intact nerves are rapidly depleted and then return over the next 2-3 days in the luteal (metestrus/diestrus) phase. We hypothesize that uterine nerve depletion is initiated by increased circulating estrogen in the follicular phase. However, studies have not shown whether estrogen can reduce uterine innervation and, if so, whether the time course is compatible with the rapid changes observed in the estrous cycle. These questions were addressed in the present study. Mature ovariectomized virgin rats received 17-ß-estradiol as a single injection (10 μg/kg s.c.) or chronically from timed-release pellets (0.1 μg/pellet for 3 weeks sustained release). Total (protein gene-product 9.5-immunoreactive) and sympathetic (dopamine ß-hydroxylase-immunoreactive) uterine innervation was assessed quantitatively. Both total and sympathetic innervation was abundant in uterine longitudinal smooth muscle of ovariectomized rats. However, following acute or chronic estrogen administration, total and sympathetic fiber numbers were markedly decreased. This was not due to altered uterine size, as reductions persisted after correcting for size differences. Our results indicate that sympathetic nerves are lost from uterine smooth muscle after estradiol treatment in a manner similar to that seen in the intact animal during estrus and pregnancy. This suggests that the rise in estradiol prior to estrus is sufficient to deplete uterine sympathetic innervation.

Key words: Neuroplasticity, Uterus, Estrous cycle, Adrenergic, Autonomic

Offprint requests to: Peter G. Smith, Ph.D., Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KS, 66160-7401. Fax: (913) 588-5677. e-mail: psmith@kumc.edu

Introduction

Sympathetic innervation of the mammalian uterus is characterized by a high capacity for plasticity. From birth through puberty, uterine sympathetic nerves density increases progressively. However, at the time of the first ovulation, the number of nerves displaying noradrenergic markers decreases markedly (Brauer et al., 1992). Similarly, pregnancy is associated with reductions in uterine sympathetic axon markers, including norepinephrine and its biosynthetic enzymes (Sjoberg, 1968). This is due to an actual reduction of nerve numbers in pregnancy, as confirmed by immunostaining for the pan-neuronal marker, protein gene product (PGP) 9.5 (Lundberg et al., 1988; Haase et al., 1997) and by electron microscopic ultrastructural analysis (Owman, 1981; Sporrong et al., 1981). Therefore, uterine innervation is altered markedly as a function of development and pregnancy.

Although the mechanisms governing these alterations are unclear, changes in levels of ovarian steroids have been implicated. In the pregnant guinea pig uterus, implantation of porcelain pellets to mimic concepta can elicit uterine nerve degeneration if conducted in the presence of high levels of endogenous (Lundberg et al., 1989) or exogenous (Owman, 1981) ovarian sex steroids. Intra-uterine implantation of progesterone-containing pellets in guinea pigs can induce a local loss of uterine sympathetic nerve fluorescence, possibly as a result of inhibition of noradrenaline biosynthesis (Bell and Malcolm, 1978, 1988). Estrogen is also implicated in sympathetic nerve remodeling, as its administration to prepubertal rats prevents the normal developmental increase in uterine norepinephrine content (Brauer et al., 1995, 1999a,b). Moreover, because chronic estrogen also prevents the developmental increase PGP 9.5-immunoreactive (ir) nerve density, it has been suggested that this hormone may inhibit normal developmental increases in nerve density or possibly induce degeneration (Brauer et al., 1999a,b). Accordingly, ovarian steroid hormones are strongly implicated as determinants of uterine nerve

status in pregnancy and development.

In addition to puberty and pregnancy, uterine innervation also varies as a function of the normal sexual cycle. In the rat, fertility is governed by the estrous cycle, which is similar to the menstrual cycle of primates but lasts only 4-5 days. The proestrus and estrus phases, which correspond to the follicular phase of the human menstrual cycle, are characterized by reductions in uterine sympathetic dopamine ß-hydroxylase (DBH)-ir nerves, as well as total PGP 9.5-ir innervation (Zoubina et al., 1998). These depletions coincide with dramatic reductions in structurally intact myometrial axons and increases in degenerating axon remnants. The presence 2-3 days later of growth cones and increased fiber numbers indicates rapid regeneration of uterine innervation during metestrus and diestrus (Zoubina and Smith, 2000). These findings therefore indicate that cyclical degenerative and regenerative processes occur normally to sympathetic innervation during the rodent estrous cycle.

Estrogen may be important for the cyclical changes in innervation seen during the estrous cycle. Degeneration during proestrus and estrus is preceded by increasing plasma estrogen, which begins in late diestrus (Freeman, 1988). However, it has not been determined whether either acute or chronic estrogen administration can elicit depletion of innervation of the adult virgin uterus. Moreover, unlike pregnancy and puberty where the decrease in nerve density apparently occurs over the course of at least several days (Owman et al., 1986; Brauer et al., 1992), degeneration of sympathetic nerves during the estrous cycle apparently begins within 24 hours of the rise in plasma estrogen, and it is not clear whether this hormone can induce such dramatic effects on uterine innervation within such a short time frame.

The present study was conducted to determine if exogenous estrogen modulates uterine nerve density of adult virgin rats deprived of endogenous estrogen by ovariectomy (OVX). In addition, short-term and long-term estrogen supplementations were compared to determine whether the actions of this steroid are consistent with changes in the intact animal during the estrous cycle, pregnancy, and puberty.

Materials and methods

Animals and surgical ovariectomy

Female virgin 2-3 month old Sprague-Dawley (Harlan) rats were housed 2-3 per cage in a climate-controlled room with a 12h light-dark cycle starting at 8 a.m. and received food and water *ad libitum*. A total of 22 ovariectomized rats were included in these experiments.

Animals were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (27.5 mg/kg, Sanofi Winthrop, New York, NY, USA), xylazine hydrochloride (2.5 mg/kg, Rompun, Miles, Shawnee Mission, KS, USA) and atropine sulfate (0.24 mg/kg, Vedco, St. Joseph, MO, USA). Dorsal bilateral incisions were made aseptically midway between lower ribs and crest of ileum, and both ovaries were isolated and removed. The incision was closed with suture clips (Fine Science Tools Inc., Foster City, CA, USA) and rats were allowed to recover before being returned to the animal facility. All experimental protocols adhered to NIH guidelines for laboratory animal care and were approved by the University of Kansas Medical Center Animal Care and Use Committee.

Estrogen treatments

For long-term estrogen treatment, rats were assigned randomly to receive 17-ß-estradiol or placebo (7 rats per group). One week after OVX, rats were anesthetized as described above and a small incision was made in the nape of the neck. Pellets containing either placebo or 0.1 mg 17-ß-estradiol (Innovative Research of America, Toledo, OH) were implanted subcutaneously. These pellets provide sustained release for 3 weeks, achieving plasma estradiol levels comparable to those during the later stages of pregnancy (Bridges, 1984; Conrad et al., 1994). Incisions were closed with suture and rats were allowed to recover before being returned to the animal facility. Two weeks later animals were sacrificed by decapitation and uterine tissues were harvested.

In the acute treatment protocol, 4 rats each were randomly assigned to treatment or control groups. Seven days after OVX, animals received a single subcutaneous injection of either 17-ß-estradiol (10 μg/kg, Sigma, St. Louis, MO, USA) or sesame oil vehicle alone. This dose of estradiol in OVX rats produces plasma estradiol levels similar to those during normal estrus (Medlock et al., 1991; Viau and Meaney, 1991). Rats were killed by urethane overdose 24 hours following the injection and uterine tissues were collected.

Serum estradiol determination

In the chronic treatment group, trunk blood was collected for serum estradiol determination at the time of sacrifice. In the acute group, blood was collected from femoral vein under inhalation anesthesia with isoflurane at 30 minutes and 1 hour after injection of 17-ß-estradiol or vehicle.

Blood samples were collected in Eppendorf tubes and immediately centrifuged at 10,000 rpm for 5 minutes. Serum was transferred to separate tubes and stored at -20 °C for 1-2 weeks prior to assay. A solid-phase ^{125}I radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) was used to measure serum estradiol. This assay had a lower limit of reliable detection of 38 pg/ml and shows negligible cross-reactivity to other hormones. Each sample was assayed in duplicate.

Tissue processing and immunofluorescence

The uterine horns were exposed and rapidly excised. In all groups, tissue was fixed by immersion in

Zamboni's solution (Stefanini et al., 1967) for 3 hours, washed for 1 week in phosphate-buffered saline at 4 °C, embedded in gelatin-albumin mixture and frozen. Fresh tissue was also obtained from rats receiving estradiol or vehicle injection 1 week after OVX. Fifteen μm transverse sections were cut from the ovarian region of the uterus, since previous studies have shown that this is where changes in innervation during the normal estrous cycle are most prominent (Zoubina et al., 1998; Zoubina and Smith, 2000). Sections were thaw-mounted onto Colorfrost Plus microscope slides (Fisher), air-dried and fixed for 5 minutes in 100% methanol at -20 °C.

Slides were rinsed in phosphate-buffered saline containing 0.3% Triton X-100, blocked for 20 minutes with 5% normal goat serum and 1% bovine serum albumin, and incubated overnight at 4 °C with primary rabbit polyclonal antisera to PGP 9.5 (1:400, Biogenesis) or DBH (1:400, Eugene Tech). Both fixation methods yield satisfactory DBH immunostaining though the signal is stronger in fresh-frozen tissue with methanol fixation. Antibodies were previously characterized in this laboratory (Simons and Smith, 1994; Smith and Fan, 1996) and specificity controls included omission of primary antisera and antigen preadsorption. Primary antibodies were visualized with goat anti-rabbit IgG conjugated to CY3 (1:400, Jackson ImmunoResearch). Slides were coverslipped with Fluoromount G and viewed with epifluorescence microscopy. Images were captured digitally with a DAGE 72 CCD camera and NIH Image software.

Quantitative analysis of innervation

PGP 9.5-ir and DBH-ir innervation was measured in longitudinal smooth muscle from transverse sections through the entire uterine horn 5-7 mm from the tubo-uterine junction, where cyclical changes are most prominent (Zoubina et al., 1998). Slides were coded and evaluated blindly. In each section, 4 quadrants located equidistant from each other and including the mesometrial, antimesometrial and intermesometrial regions were selected under brightfield illumination. Under epifluorescence illumination, images of these selected regions were captured digitally, and a stereology grid with an area corresponding to 0.25 mm^2 and line intersects at 20 μm intervals (NIH Image software) was superimposed randomly over the selected muscle field. The apparent percentage area of PGP 9.5-ir or DBH-ir nerves within the captured images was estimated by dividing all grid intersects overlying fluorescent nerve profiles by total grid intersects over the longitudinal smooth muscle, thus providing an index of nerve density. To determine the extent to which target size varied as a result of OVX and hormone treatments, smooth muscle area was measured planimetrically by tracing around the perimeters of the entire longitudinal muscle profile in brightfield images in the sections from which nerve density was determined. All values for target size presented refer to measurements from immersion-fixed tissue, which were slightly but not significantly lower than the fresh-frozen samples. To normalize innervation measurements for changes in target size, nerve density (i.e. nerve area percentage measured above) was multiplied by the target area to provide an index of the total innervation. Data were compared using one-way analysis of variance with post hoc analysis by Student Newman-Keuls test. A probability of <0.05 was taken to indicate a statistically significant difference.

Results

Ovariectomized animals

Plasma estrogen levels at one and three weeks after OVX were below the limit of reliable detection. At one week following OVX, uteri appeared pale and small. Cross-sectional area of immersion-fixed longitudinal smooth muscle was 1.08±0.07 mm^2. At three weeks post-OVX, the morphological appearance and size of the uterus were similar to those at one week post-OVX. Longitudinal smooth muscle cross-sectional area was 1.17 ± 0.04 mm^2 (not significant versus 1 week post-OVX).

Nerve fibers immunoreactive for PGP 9.5 were most abundant within the longitudinal smooth muscle and around uterine blood vessels of OVX rats (Fig. 1a). In longitudinal smooth muscle, they occupied 15.1±1.4% of the smooth muscle sectional area at one week post-OVX and 13.9±1.4% at three weeks post-OVX (difference not significant). Total nerve area, as determined by multiplying longitudinal muscle cross-sectional area by percentage occupied by PGP-ir fibers, was 0.16±0.01 mm^2 at one week post-OVX, and 0.17±0.02 mm^2 at three weeks (not significantly different).

DBH-ir fibers were distributed similarly to PGP 9.5-ir fibers. These nerves occupied 9.8±0.5% of the smooth muscle area at one week post-OVX (Fig. 1d), and 5.3±0.4% three weeks post-OVX. Total area of DBH-ir profiles in the longitudinal smooth muscle was 0.13±0.01 mm^2 at one week post-OVX, and 0.06±0.01 mm^2 at three weeks post-OVX. No statistical comparisons were made between groups because of differences in fixation methods.

Acute estradiol treatment

In rats 1 week after OVX, a single subcutaneous injection of 10 μg/kg 17-ß-estradiol raised serum levels to 738 ±151 pg/ml at 30 minutes and 749±75 pg/ml at 1 hour after injection. At 24 hours after injection, uteri were enlarged and hyperemic, and longitudinal smooth muscle cross-sectional area in Zamboni-fixed tissue had increased to 1.74±0.15 mm^2 ($p<0.001$ vs. vehicle).

PGP 9.5-ir nerve density of longitudinal smooth muscle at this time was reduced by 72% relative to vehicle injection (Figs. 1b, 2a, $p<0.001$) and total PGP-ir nerve area was decreased by 56% relative to placebo-

treated animals (Fig.2b, p<0.001). There was no discernible change in numbers of perivascular nerves relative to untreated ovariectomized specimens.

Changes in longitudinal muscle innervation were even more pronounced for DBH-ir fibers. In rats treated acutely with estrogen, DBH-ir fiber density was 2.2±0.6%, which represents an 85% reduction relative to that of placebo-injected controls (Fig. 1e, 2a, p<0.001). Total DBH-ir nerve area was reduced by 69% (0.04±0.01 mm^2) relative to placebo-injected controls (Fig. 2b, p=0.002). Perivascular DBH-ir innervation appeared largely to be preserved.

Chronic estradiol treatment

In rats receiving estradiol-releasing pellets, serum estradiol was 220±33 pg/ml 2 weeks after the implantation. Uteri of these animals were enlarged and hyperemic, and longitudinal smooth muscle cross-sectional area had increased to 2.48±0.21 mm^2 (p=0.001 vs. OVX with placebo pellets and p=0.004 vs. acute estradiol).

Density of PGP-ir fibers in longitudinal smooth muscle after 2 weeks of sustained estrogen administration was 1.7±0.7%, or 12% of density in rats receiving placebo pellets (Figs. 1c, 3a, p<0.001). Total area of PGP-immunoreactive nerve profiles was reduced by 76% to 0.04±0.01 mm^2 (Fig. 3b, p<0.001). There was no significant difference in total PGP-ir area between the two treatment protocols. In contrast to the longitudinal muscle, vascular innervation density appeared to be essentially unchanged.

DBH-ir uterine nerves after chronic estrogen treatment occupied 0.9±0.2% of the longitudinal smooth muscle, which represents an 83% decrease relative to placebo-treated controls (Figs.1f, 3a, p<0.001). After normalizing for changes in the muscle size, total area of DBH-ir was decreased by 67% relative to vehicle-treated rats to 0.022±0.004 mm^2 (Fig. 3b, p=0.002). Perivascular DBH-ir nerves did not appear to be significantly reduced relative to placebo-implanted OVX rats.

Discussion

Uterine innervation after ovariectomy

Ovariectomy for one to three weeks rendered serum estradiol levels to below the lower limit of reliable detection by our assay. Under these conditions, the uterus had undergone appreciable atrophy, as typically occurs following withdrawal of steroid support (Johnson and Everitt, 1988). Because longitudinal smooth muscle cross-sectional area was similar at one and three weeks, maximal atrophy apparently is achieved within one week of steroid withdrawal, with little or no additional change for at least 2 weeks thereafter.

Despite their small size, uteri of OVX animals were well endowed with nerves. Dopamine ß-hydroxylase, a key enzyme in norepinephrine biosynthesis, is a

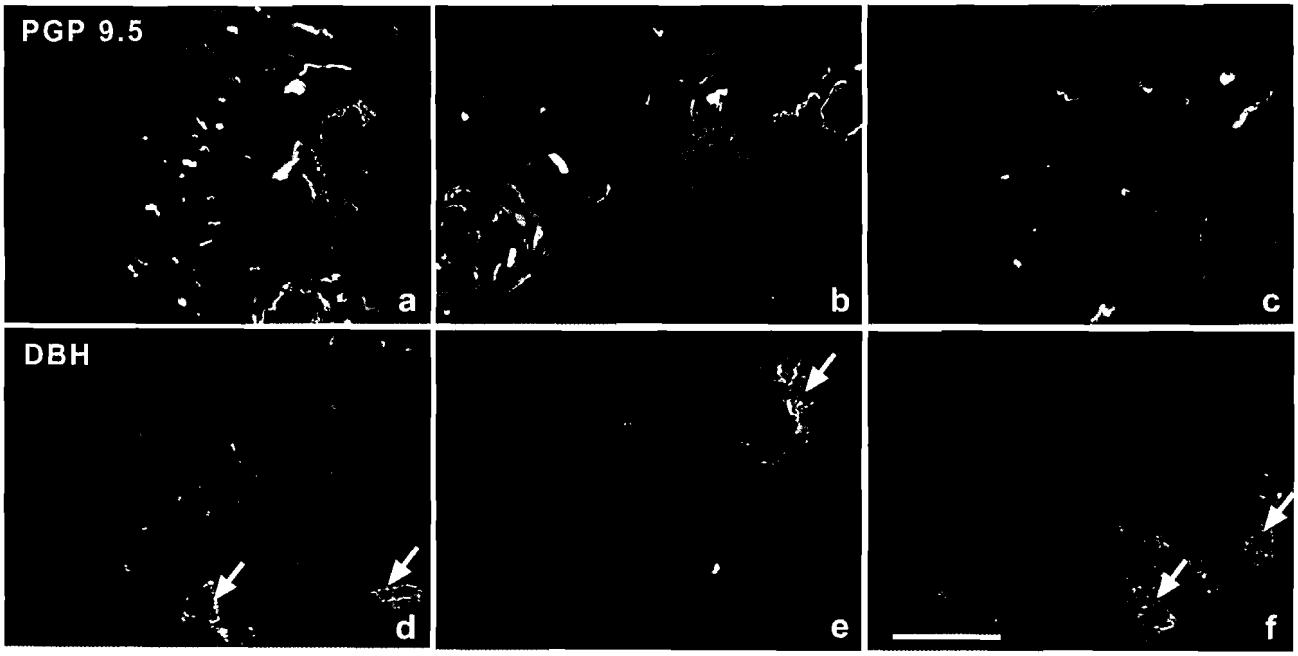

Fig. 1. Uterine innervation expressing immunoreactivity for the pan-neuronal marker Protein Gene Product (PGP) 9.5-ir **(a-c)** and the sympathetic neuronal marker dopamine ß-hydroxylase (DBH, **d-f**). Staining was performed on transverse sections of uteri from rats 1 week following ovariectomy **(a and d)**, 1 week ovariectomy plus a single injection of 17-ß-estradiol 24 hours prior to harvesting tissue **(b and e)** and 3 weeks following ovariectomy and 2 weeks of chronic estrogen treatment **(c and f)**. Arrows in b, d, and f indicate DBH-ir staining of perivascular nerve fibers. Bar: 50 μm.

selective and reliable marker commonly used to identify sympathetic innervation (Smith and Fan, 1996), and an abundance of fibers displaying this marker implies high numbers of sympathetic nerves and robust enzyme expression. On the other hand, PGP 9.5, a ubiquitin carboxyl-terminal hydrolase present in essentially all neural and neuroendocrine cells (Wilkinson et al., 1989), is expressed in all structurally intact axons (Lundberg et al., 1988; Haase et al., 1997; Li et al., 1997). Since the density and area of DBH-ir nerves at 1 week post-OVX was nearly 80% of that of PGP 9.5-ir nerves, it appears that the vast majority of nerves innervating the ovarian region of the uterine horn under low estrogen conditions derives from sympathetic neurons. It is also pertinent to note that the total uterine area occupied by nerves following ovariectomy for 1 week (0.16±0.01 mm^2) or 3 weeks (0.17±0.02 mm^2) are essentially identical to that measured previously in intact rats at diestrus (0.15±0.03 mm^2) (Zoubina et al., 1998) when plasma estrogen is at its nadir in the estrous cycle. This suggests that the reduced estrogen level during diestrus is sufficient to induce maximal myometrial innervation which is not further increased by prolonged estrogen deprivation. It also implies that the surgical procedure for ovariectomy did not substantially or persistently interrupt major neural pathways traveling to this portion of the uterus.

Effect of chronic estradiol treatment upon uterine innervation

Estrogen supplementation by continuous release pellets increased plasma estradiol levels to values similar to those reported for mature rats during estrus (Cheng and Johnson, 1974; Viau and Meaney, 1991) and pregnancy (Bridges, 1984; Conrad et al., 1994). The efficacy of this treatment regimen was verified by observations that uteri were enlarged and hyperemic, and longitudinal smooth muscle cross-sectional area was increased.

These changes in uterine morphology were accompanied by altered innervation. Estrogen supplementation for 2 weeks led to a substantial reduction in longitudinal smooth muscle DBH-ir sympathetic fibers. This reduction is consistent with

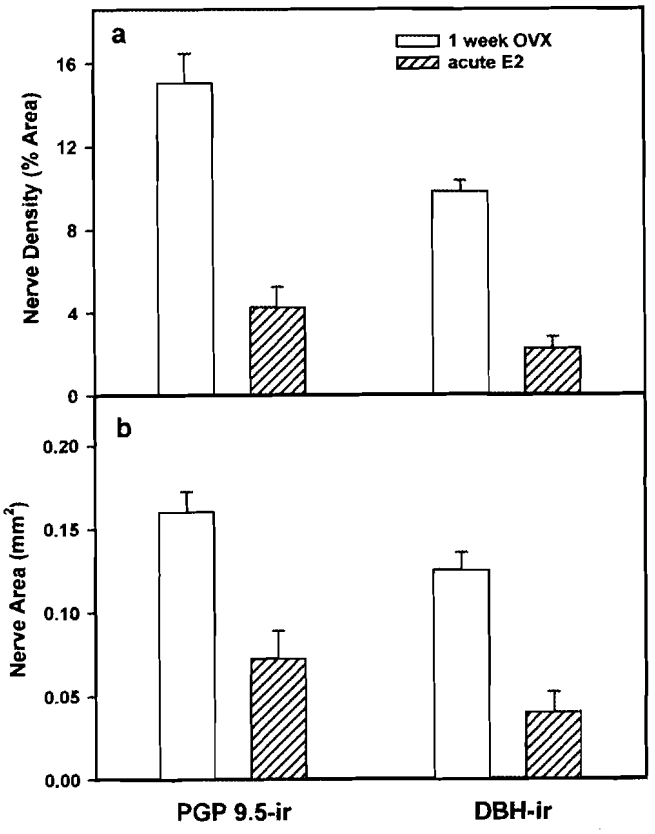

Fig. 2. Quantitative analysis of uterine innervation in uteri from rats 1 week following ovariectomy (OVX, N=4) and following a single injection of 17-β-estradiol 24 hours (acute E2, N=4) prior to harvesting tissue. PGP 9.5-ir and DBH-ir innervation is quantified as the percentage of sectional area occupied by immunoreactive fibers (Nerve Density) in **panel a**. In **panel b**, nerve density was multiplied by the total sectional area (mm^2) to yield an estimate of total Nerve Area.

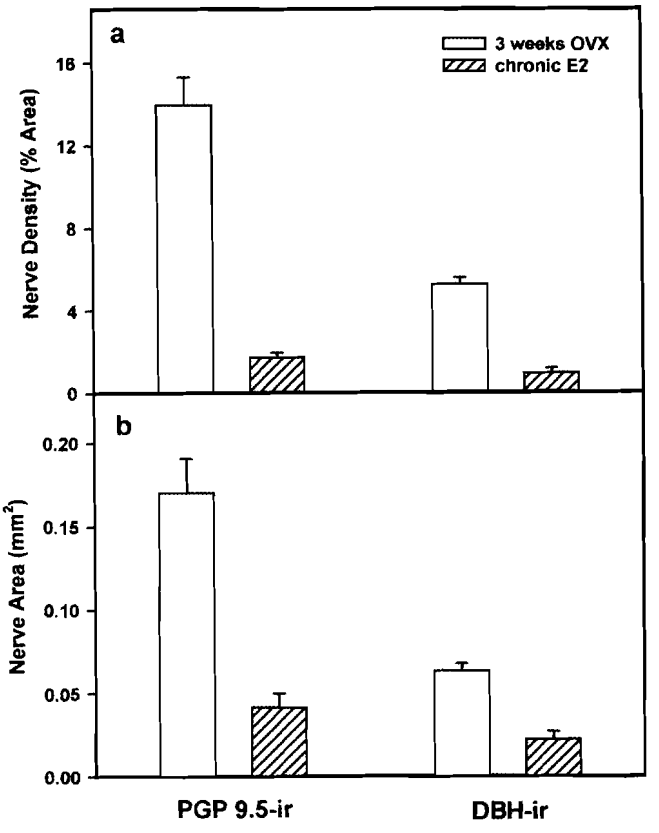

Fig. 3. Quantitative analysis of uterine innervation in uteri from rats at 3 weeks following ovariectomy (OVX, N=7) and following 2 weeks of 17-β-estradiol supplementation (chronic E2, N=7) prior to harvesting tissue. PGP 9.5-ir and DBH-ir innervation was quantified as the percentage of sectional area occupied by immunoreactive fibers (Nerve Density) in **panel a**. Nerve density was multiplied by the total sectional area (mm^2) to yield an estimate of total Nerve Area in **panel b**.

declines reported for norepinephrine and its biosynthetic enzymes after chronic estrogen treatment of prepubertal rats (Brauer et al., 1999a,b), and is reminiscent of the depletion of noradrenergic fibers seen in the pregnant uterus (Thorbert, 1978; Sporrong et al., 1981; Haase et al., 1997). In contrast to myometrial innervation, perivascular DBH-ir fibers seemed largely to be spared, a finding consistent with previous observations (Corbacho et al., 1997; Brauer et al., 1999a,b).

The lower number of myometrial DBH-ir fibers in OVX rats receiving chronic estrogen replacement is attributable to an actual reduction in nerves rather than simply enzyme depletion. Hence, the abundant total innervation revealed by PGP 9.5 immunostaining at 1 week post-OVX was dramatically reduced by 2 weeks of estrogen supplementation, and this decrease cannot be accounted for simply by increased uterine size. The present study did not investigate the cellular mechanisms leading to the loss of intact nerve fibers, but it is reasonable to suggest that terminal axonal degeneration must have played a role in the loss of these fibers, as occurs in pregnancy (Sporrong et al., 1978; Thorbert et al., 1978) and during the estrous cycle (Zoubina et al., 1998). Accordingly, while multiple factors including elevated local progesterone levels (Bell and Malcolm, 1978, 1988) and mechanical stretch (Lundberg et al., 1989) are likely to contribute to uterine nerve degeneration in pregnancy, our observations support the notion that rising estrogen levels may also play a critical role in this process. They also suggest estrogen-induced reductions in uterine nerve density in prepubertal rats may be due, at least in part, to degenerative changes (Brauer et al., 1999a,b). The present findings therefore implicate estrogen as a mediator of the relatively long-term reductions in uterine sympathetic innervation seen in pregnancy and pubescence.

Effect of acute estradiol treatment on uterine innervation

While long-term estrogen administration leads to changes in uterine sympathetic innervation similar to those of pregnancy and puberty, the estrous cycle is characterized by much more rapid variations in uterine innervation. Marked axon degeneration and loss of sympathetic nerves occur over the course of 1-2 days (Zoubina et al., 1998; Zoubina and Smith, 2000) and is preceded by a relatively brief increase in plasma estrogen (Freeman, 1988). If the short-term increase in estrogen during the estrous cycle is responsible for nerve degeneration, it should be possible to initiate a similar decrease in sympathetic innervation using a treatment regimen that simulates the normal physiological rise during proestrus. Our measurements confirmed that a single injection of 10 μg/kg estradiol increases plasma estrogen within 30 minutes to levels approximating those seen in the estrous cycle (Medlock et al., 1991; Viau and Meaney, 1991), and studies by others have shown that this peak is followed by a substantial decline at 6 hours and a return to baseline within 24 hours (Cheng and Johnson, 1974; Medlock et al., 1991).

This treatment regimen produced rapid changes in uterine size, confirming that effects on uterine morphology are manifest within 24 hours. Moreover, at this time numbers of both PGP 9.5-ir and DBH-ir nerves per uterine section were reduced by 56% and 69%, respectively, to a level comparable to that measured previously during estrus (Zoubina et al., 1998). These findings therefore confirm that an acute rise in estrogen induces myometrial nerve depletion with a timeframe similar to that occurring normally during the estrous cycle (Zoubina et al., 1998; Zoubina and Smith, 2000) and in the early stages of pregnancy (Sporrong et al., 1981).

In addition to showing that estrogen produces uterine nerve degeneration with a rapid time course, these findings argue against a role of several other hormones that also vary as a function of the estrous cycle. The estrous cycle rise in estrogen is followed by increased levels of luteinizing hormone, follicle stimulating hormone, progesterone and prolactin in late proestrus and estrus, and these might also act to decrease innervation density during estrus. However, in contrast to diestrus, ovariectomy is characterized by markedly elevated levels of luteinizing hormone and follicle stimulating hormone (Johnson and Everitt, 1988), indicating that high uterine nerve density is maintained despite increased levels of these hormones. Similarly, while prolactin and progesterone plasma levels are low after ovariectomy, acute estradiol treatment similar to that employed in this study is not accompanied by significant increases in these hormones (Johnson and Everitt, 1988; Viau and Meaney, 1991; Conrad et al., 1994). Therefore, our findings support the idea that circulating estrogen is the primary determinant of variations in uterine sympathetic innervation during the estrous cycle.

Estrogen as a neurodegenerative agent

The findings of the present study provide evidence that estrogen induces degeneration of terminal sympathetic fibers within the myometrium. This conclusion stands somewhat in contrast to literature showing that estrogen can protect central neurons from injury (Behl et al., 1997; Weaver et al., 1997; Sawada et al., 1998; Culmsee et al., 1999; Singer et al., 1999; Pike, 1999) and promote axonal outgrowth (Kow and Pfaff, 1973; Matsumoto and Arai, 1981; McEwen et al., 1991; Chowen et al., 1992; Honjo et al., 1992; Cambiasso et al., 1995; Tanzer and Jones, 1997; Toran-Allerand et al., 1999). However, estrogen does induce death of some central neurons (Brawer et al., 1993), and in findings reminiscent of the present study, DBH-ir fibers in the primate prefrontal cortex are increased by ovariectomy and decreased by exogenous estrogen (Kritzer and Kohama, 1999). Therefore, estrogen can exert varied effects on different neuronal populations. Moreover, because estrogen causes myometrial sympathetic

innervation to degenerate while uterine perivascular innervation is spared, the effects of this hormone may differ with respect to homologous neurons projecting to different targets.

These varied effects may be ascribed to estrogen's manifold actions, particularly with regard to neurotrophic factors and their receptors. Sympathetic nerves depend upon neurotrophic factors for survival and outgrowth, and it is well established that estrogen can modulate neurotrophin synthesis (Siminoski et al., 1987; Sohrabji et al., 1995; Gibbs, 1998), although the extent to which this occurs in the uterus during pregnancy is at present uncertain (Naves et al., 1998; Brauer et al., 2000; Varol et al., 2000). Importantly, estrogen may also affect neuronal neurotrophin receptor expression (Sohrabji et al., 1994), thereby altering neuronal responsiveness to neurotrophins independent of changes in their absolute target levels. Consistent with this concept, neurotrophin receptor expression has been shown to decrease in humans during pregnancy (Naves et al., 1998). Although the mechanism by which estrogen can induce selective degeneration of myometrial sympathetic innervation remains unresolved, hormone-induced modifications of neurotrophic proteins and their neuronal receptors would seem to be plausible candidates.

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References

Bell C. and Malcolm S.J. (1978). Observations on the loss of catecholamine fluorescence from intrauterine adrenergic nerves during pregnancy in the guinea-pig. J. Reprod. Fertil. 53, 51-58

Bell C. and Malcolm S.J. (1988). Neurochemistry of the sympathetic innervation to the uterus. Clin. Exp. Pharmacol. Physiol. 15, 667-674

Behl C., Skutella T., Lezoualc'h F., Post A., Widmann M., Newton C.J. and Holsboer F. (1997). Neuroprotection against oxidative stress by estrogens: structure-activity relationship. Mol. Pharmacol. 51, 535-541.

Brauer M.M., Lincoln J., Blundell D. and Corbacho A. (1992). Postnatal development of noradrenaline-containing nerves of the rat uterus. J. Auton. Nerv. Syst. 39, 37-50.

Brauer M.M., Corbacho A.M. and Burnstock G. (1995). Effects of chronic and acute oestrogen treatment on the developing noradrenaline-containing nerves of the rat uterus. Int. J. Dev. Neurosci. 13, 791-798.

Brauer M.M., Llodra J., Scorza M.C., Chavez R., Burnstock G., Thrasivoulu C. and Cowen T. (1999a). Differential effects of prepubertal chronic oestrogen treatment on the synthesis of noradrenaline in uterine myometrial and perivascular sympathetic nerves. Int. J. Dev. Neurosci. 17, 295-303.

Brauer M.M., Llodrá J., Scorza M.C., Chávez R., Burnstock G., Thrasivoulu C. and Cowen T. (1999b). Differential effects of prepubertal chronic oestrogen treatment on the synthesis of noradrenaline in uterine myometrial and perivascular sympathetic nerves. Int. J. Dev. Neurosci. 17, 295-303.

Brauer M.M., Shockley K.P., Chavez R., Richeri A., Cowen T. and Crutcher K.A. (2000). The role of NGF in pregnancy-induced degeneration and regeneration of sympathetic nerves in the guinea pig uterus. J. Auton. Nerv. Syst. 79, 19-27.

Brawer J.R., Beaudet A., Desjardins G.C. and Schipper H.M. (1993). Pathologic effect of estradiol on the hypothalamus. Biol. Reprod. 49, 647-652

Bridges R.S. (1984). A quantitative analysis of the roles of dosage, sequence, and duration of estradiol and progesterone exposure in the regulation of maternal behavior in the rat. Endocrinology 114, 930-940.

Cambiasso M.J., Diaz H., Caceres A. and Carrer H.F. (1995). Neuritogenic effect of estradiol on rat ventromedial hypothalamic neurons co-cultured with homotopic or heterotopic glia. J. Neurosci. Res. 42, 700-709.

Cheng H.C. and Johnson D.C. (1974). Temporal changes in serum estradiol and prolactin in immature female rats given a single injection of estradiol benzoate. Steroids 24, 657-664.

Chowen J.A., Torres-Aleman I. and Garcia-Segura L.M. (1992). Trophic effects of estradiol on fetal rat hypothalamic neurons. Neuroendocrinology 56, 895-901.

Conrad K.P., Mosher M.D., Brinck-Johnsen T. and Colpoys M.C. (1994). Effects of 17 beta-estradiol and progesterone on pressor responses in conscious ovariectomized rats. Am. J. Physiol. 266, R1267-R1272.

Corbacho A.M., Brauer M.M. and Perez T. (1997). Development and maturation of noradrenaline-containing nerves of the rat uterine artery. Effects of acute and chronic oestrogen treatment. Int. J. Dev. Neurosci. 15, 363-371.

Culmsee C., Vedder H., Ravati A., Junker V., Otto D., Ahlemeyer B., Krieg J.C. and Krieglstein J. (1999). Neuroprotection by estrogens in a mouse model of focal cerebral ischemia and in cultured neurons: evidence for a receptor-independent antioxidative mechanism. J. Cereb. Blood Flow Metab. 19, 1263-1269.

Freeman M.E. (1988). The ovarian cycle of the rat. In: The physiology of reproduction. Knobil E. and Neil J. (eds). Raven Press. New York. pp 1893-1928.

Gibbs R.B. (1998). Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. Brain Res. 787, 259-268.

Haase E.B., Buchman J., Tietz A.E. and Schramm L.P. (1997). Pregnancy-induced uterine neuronal degeneration in the rat. Cell Tissue Res. 288, 293-306.

Honjo H., Tamura T., Matsumoto Y., Kawata M., Ogino Y., Tanaka K., Yamamoto T., Ueda S. and Okada H. (1992). Estrogen as a growth factor to central nervous cells. Estrogen treatment promotes development of acetylcholinesterase-positive basal forebrain neurons transplanted in the anterior eye chamber. J. Steroid Biochem. Mol. Biol 41, 633-635.

Johnson M.H. and Everitt B.J. (1988). Anonymous. Essential reproduction. Blackwell Scientific Publications. Oxford.

Kow L.M. and Pfaff D.W. (1973). Effects of estrogen treatment on the size of receptive field and response threshold of pudendal nerve in the female rat. Neuroendocrinology 13, 299-313.

Kritzer M.F. and Kohama S.G. (1999). Ovarian hormones differentially influence immunoreactivity for dopamine beta-hydroxylase, choline acetyltransferase, and serotonin in the dorsolateral prefrontal cortex

of adult rhesus monkeys. J. Comp. Neurol. 409, 438-451.

Li G.L., Farooque M., Holtz A. and Olsson Y. (1997). Expression of the ubiquitin carboxyl-terminal hydrolase PGP 9.5 in axons following spinal cord compression trauma. An immunohistochemical study in the rat. APMIS 105, 384-390.

Lundberg L.M., Alm P. and Thorbert G. (1989). Local mechanical effects and humoral factors evoke degeneration of guinea pig uterine innervation. Acta Obstet. Gynecol. Scand. 68, 487-496.

Lundberg L.M., Alm P., Wharton J. and Polak J.M. (1988). Protein gene product 9.5 (PGP 9.5). A new neuronal marker visualizing the whole uterine innervation and pregnancy-induced and developmental changes in the guinea pig. Histochemistry 90, 9-17.

Matsumoto A. and Arai Y. (1981). Neuronal plasticity in the deafferented hypothalamic arcuate nucleus of adult female rats and its enhancement by treatment with estrogen. J. Comp. Neurol. 197, 197-205.

McEwen B.S., Coirini H., Westlind-Danielsson A., Frankfurt M., Gould E., Schumacher M. and Woolley C. (1991). Steroid hormones as mediators of neural plasticity. J. Steroid Biochem. Mol. Biol. 39, 223-232.

Medlock K.L., Forrester T.M. and Sheehan D.M. (1991). Short-term effects of physiological and pharmacological doses of estradiol on estrogen receptor and uterine growth. J. Recept. Res. 11, 743-756.

Naves F.J., Vazquez M.T., San Jose I., Martinez-Almagro A. and Vega J.A. (1998). Pregnancy-induced denervation of the human uterine artery correlates with local decrease of NGF and TrkA. It. J. Anat. Embryol. 103, 279-290.

Owman C. (1981). Pregnancy induces degenerative and regenerative changes in the autonomic innervation of the female reproductive tract. In: CIBA Foundation symposium 83: Symposium on development of the autonomic nervous system. Elliott K. and Lawrenson G. (eds). Pitman Medical. London. pp 252-279.

Owman C., Alm P., Sjoberg N.O. and Stjernquist M. (1986). Structural, biochemical, and pharmacological aspects of the uterine autonomic innervation and its remodeling during pregnancy. In: The physiology and biochemistry of the uterus in pregnancy and labor. Huszar G. (ed). CRC Press. Boca Raton, Fla. pp 5-23.

Pike C.J. (1999). Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. J. Neurochem. 72, 1552-1563

Sawada H., Ibi M., Kihara T., Urushitani M., Akaike A. and Shimohama S. (1998). Estradiol protects mesencephalic dopaminergic neurons from oxidative stress-induced neuronal death. J. Neurosci. Res. 54, 707-719.

Siminoski K., Murphy R.A., Rennert P. and Heinrich G. (1987). Cortisone, testosterone, and aldosterone reduce levels of nerve growth factor messenger ribonucleic acid in L-929 fibroblasts. Endocrinology 121, 1432-1437.

Simons E.J. and Smith P.G. (1994). Sensory and autonomic innervation of the rat eyelid: origins and peptide phenotypes. J. Chem. Neuroanat. 7, 35-47.

Singer C.A., Figueroa-Masot X.A., Batchelor R.H. and Dorsa D.M. (1999). The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J. Neurosci. 19, 2455-2463.

Sjoberg N.O. (1968). Considerations of the cause of disappearance of the adrenergic transmitter in uterine nerves during pregnancy. Acta physiol. Scand. 72, 510-517.

Smith P.G. and Fan Q. (1996). Sympathetic nerve trajectories to rat orbital targets: role of connective tissue pathways. J. Comp. Neurol. 365, 69-78.

Sohrabji F., Miranda R.C. and Toran-Allerand C.D. (1994). Estrogen differentially regulates estrogen and nerve growth factor receptor mRNAs in adult sensory neurons. J. Neurosci. 14, 459-471.

Sohrabji F., Miranda R.C. and Toran-Allerand C.D. (1995). Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. Proc. Natl. Acad. Sci. USA 92, 11110-11114.

Sporrong B., Alm P., Owman C., Sjoberg N.O. and Thorbert G. (1978). Ultrastructural evidence for adrenergic nerve degeneration in the guinea pig uterus during pregnancy. Cell Tissue Res. 195, 189-193.

Sporrong B., Alm P., Owman C., Sjoberg N.O. and Thorbert G. (1981). Pregnancy is associated with extensive adrenergic nerve degeneration in the uterus. An electronmicroscopic study in the guinea-pig. Neuroscience 6, 1119-1126

Stefanini M., DeMartino C. and Zamboni L. (1967). Fixation of ejaculated spermatozoa for electron microscopy. Nature 216, 173-174

Tanzer L. and Jones K.J. (1997). Gonadal steroid regulation of hamster facial nerve regeneration: effects of dihydrotestosterone and estradiol. Exp. Neurol. 146, 258-264.

Thorbert G. (1978). Regional changes in structure and function of adrenergic nerves in guinea-pig uterus during pregnancy. Acta Obstet. Gynecol. Scand. 79, 1-32.

Thorbert G., Alm P., Owman C., Sjoberg N.O. and Sporrong B. (1978). Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea-pig, and their relationship to the localization of the conceptus. Acta Physiol. Scand. 103, 120-131.

Toran-Allerand C.D., Singh M. and Setalo G.J. (1999). Novel mechanisms of estrogen action in the brain: new players in an old story. Front. Neuroendocrinol. 20, 97-121.

Varol F.G., Duchemin A., Neff N.H. and Hadjiconstantinou M. (2000). Nerve growth factor (NGF) and NGF mRNA change in rat uterus during pregnancy. Neurosci. Lett. 294, 58-62.

Viau V. and Meaney M.J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. Endocrinology 129, 2503-2511.

Weaver C.E.J., Park-Chung M., Gibbs T.T. and Farb D.H. (1997). 17beta-Estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. Brain Res. 761, 338-341.

Wilkinson K.D., Lee K., Deshpande S., Duerksen-Hughes P., Boss J.M. and Pohl J. (1989). The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 246, 670-673.

Zoubina E.V. and Smith P.G. (2000). Axonal degeneration and regeneration in rat uterus during the estrous cycle. Auton. Neurosci. 84, 176-185.

Zoubina E.V., Fan Q. and Smith P.G. (1998). Variations in uterine innervation during the estrous cycle in rat. J. Comp. Neurol. 397, 561-571.

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