Cell Tissue Res (2002) 308:61–73
DOI 10.1007/s00441-002-0521-3

REGULAR ARTICLE

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Differential effects of oestrogen on developing and mature uterine sympathetic nerves

Received: 10 August 2001 / Accepted: 20 December 2001 / Published online: 8 March 2002
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Abstract Oestrogen is a key factor in the remodelling of uterine sympathetic nerves during puberty and the oestrous cycle; these nerves are influenced by changes in their target uterine tissue. The magnitude of oestrogen-induced responses might however be influenced by the maturation stage of sympathetic nerve fibres, the age of the neurons and/or the developmental state of the uterus. We have therefore compared the sympathetic innervation of the uterus following chronic oestrogen treatment of infantile/prepubertal and young adult intact and ovariectomised rats. Treatment of infantile/prepubertal rats resulted in the complete loss of intrauterine noradrenaline (NA)-labelled sympathetic nerves and a marked reduction in the total NA content in the uterine horn. Chronic treatment of young adult rats had little effect. To examine whether the age of the neurons or the degree of development of the uterus determined responsiveness of nerves to oestrogen, we assessed the effects of oestrogen on the sympathetic reinnervation of intraocular transplants of young adult uterine myometrium into ovariectomised adult host rats. Early treatment (10 days post-transplantation) resulted in less sympathetic innervation than late treatment (30 days post-transplantation). Measurements of nerve growth factor (NGF) levels in the uterine horn of control rats before and after puberty and following infantile/prepubertal chronic oestrogen treatment and acute oestrogen treatment of young adult rats revealed a coordinated increase between the growth of the uterus and NGF protein levels. Thus, developing and recently regrown sympathetic nerves are more susceptible to oestrogen-induced changes in the uterus than mature nerves, differential susceptibility is not related to the age of the neurons or the developmental state of the uterus and changes in NGF protein do not account for the differential susceptibility of developing and mature uterine sympathetic nerve fibres to oestrogen. Growing sympathetic fibres are more vulnerable to oestrogen than mature fibres and nerve fibres that have been in contact for longer periods with their target become less susceptible to oestrogen.

Keywords Uterus · Sympathetic nerves · Plasticity · Oestrogen · Nerve growth factor · Rat (female Wistar-derived albino)

This work was supported by The Wellcome Trust, UK (CRIG Grant 058122/Z/99/Z/JC/KO) and PEDECIBA, Universidad de la República, Montevideo, Uruguay

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Introduction

The sympathetic innervation of the uterus is remarkable in that it exhibits dynamic changes in response to physiological and experimental changes in the circulating levels of sex hormones (Owman and Stjernquist 1988). Sex-hormone induced plasticity of uterine sympathetic nerves includes a complete degeneration of perivascular and myometrial innervation during pregnancy (Thorbert 1978; Yamada 1988; Owman and Stjernquist 1988; Haase et al. 1997). It is thought that pregnancy-induced changes involve the participation of both oestrogen and progesterone, although to our knowledge, attempts to mimic the dramatic effects of pregnancy by systemic

administration of sex hormones have been unsuccessful (Bell and Malcolm 1978, 1988). In addition to pregnancy-induced changes, the sympathetic innervation of the uterus undergoes a partial remodelling during puberty (Brauer et al. 1992) and the oestrous cycle (Adham and Schenk 1969; Marshall 1981; Melo and Machado 1993; Zoubina et al. 1998) but this only involves the myometrial-associated sympathetic innervation. Although the contribution of progesterone to these changes remains uncertain, there is increasing evidence that oestrogen is a key factor in the remodelling of uterine sympathetic nerves in the non-pregnant female (Brauer et al. 1995, 1999; Zoubina et al. 2001). In addition, recent studies indicate that oestrogen-induced responses in the sympathetic innervation are mediated by changes in the target uterine tissue (Brauer et al. 2000a; Krizsan-Agbas and Smith 2000).

In marked contrast to the changes observed during natural puberty and the oestrous cycle, chronic administration of oestrogen to rats during the infantile/prepubertal period (10–25 days after birth) provokes, at 28 days of age, a complete loss of noradrenaline-labelled (NA-L) intrauterine myometrial and perivascular sympathetic nerves, comparable to that observed at late pregnancy (Brauer et al. 1995). Although this differential response could result from differences in the physiological and experimental levels of oestrogen, it is possible that they could be related to differences in the maturational stage of the nerve fibres, the age of the sympathetic neurons, and/or the developmental state of the uterus. To address this question, we have evaluated the sympathetic innervation of the uterus following chronic oestrogen treatment of infantile/prepubertal and young adult intact and ovariectomised rats. To examine whether the age of the neurons or the degree of development of the uterus determine the responsiveness of nerves to oestrogen, we have assessed the effects of oestrogen on the sympathetic reinnervation of intraocular transplants of young adult uterine myometrium into ovariectomised adult host rats (Brauer et al. 2000a).

The selection of ages for the initiation of treatments (2, 10 and 30 days after birth and 10 and 30 days after transplantation) was based on previous data showing that development of sympathetic nerves to the rat uterus takes place during the first 4 weeks of postnatal life and fully grown innervation is attained by the end of the prepubertal period (about 4 weeks after birth; Brauer et al. 1992). In order to avoid the effects of circulating endogenous oestrogen in peripubertal animals, one group of females was bilaterally ovariectomised prior to the initiation of treatment (oestrogen or vehicle administration) 30 days after birth. Similarly, transplant recipients were bilaterally ovariectomised. Changes in the pattern and density of the sympathetic innervation of the uterine horn and of in oculo myometrial transplants were assessed in cryostat tissue sections processed by the glyoxylic acid technique. In addition, tissue levels of NA were measured in the uterine horn by high performance liquid chromatography with electrochemical detection (HPLC-ED). For comparative purposes, NA levels were measured in the uterine horn following puberty and acute oestrogen treatment.

To assess the possible role of altered neurotrophic support in this plasticity, we used a two-site enzyme-linked immunosorbent assay (ELISA) to measure NGF protein levels in the uterine horn of control rats before and after puberty and following infantile/prepubertal chronic oestrogen treatment and acute oestrogen treatment of young adult rats. Some of these results have been published in abstract form (Brauer et al. 2000c).

Materials and methods

Animals and oestrogen treatment

Female Wistar-derived albino rats from the breeding colony held at IIBCE (Montevideo, Uruguay) were used for this study. Animals were sexed at birth, weaned at 3 weeks and maintained under controlled conditions of temperature and illumination, with food and water *ad libitum*.

Oestrogen treatment was performed with β-oestradiol 17-cypionate (Laboratorios Köning, Argentina), diluted to appropriate doses with peanut oil (Sigma, St. Louis, Mo.) and administrated subcutaneously in a final volume of 0.1 ml per dose. Control animals from matched litters were injected with 0.1 ml peanut oil per dose. In all cases, animals were terminally anaesthetised with ether and killed by cervical dislocation. For acute oestrogen treatment, females were injected with a single dose of 40 µg oestrogen on day 25 after birth and killed at 28 days. For early chronic oestrogen treatment (ECOT), animals were injected with two doses of 5 µg oestrogen on days 2 and 7 of life, followed by three doses of 10 µg on days 14, 20 and 25 (total dose: 40 µg) and killed at 28 days of age. For medium chronic oestrogen treatment (MCOT), animals were given four doses of 10 µg oestrogen on days 10, 15, 20 and 25 of life (total dose: 40 µg) and killed at 28 days of age. For late chronic oestrogen treatment, animals were injected on days 30, 35, 40 and 45 days of age with either 10 µg (LCOT/10) or 15 µg (LCOT/15) oestrogen (total dose: 40 µg and 60 µg, respectively) and killed at 48 days of age. Additional females were ovariectomised bilaterally at 25 days of age under ether anaesthesia and injected with oil (OVX/OIL) or four doses of 10 µg oestrogen (OVX-LCOT/10) on days 30, 35, 40 and 45 (total dose: 40 µg) and killed at 48 days of age. Surgical procedures were conducted in accordance with the International Guidelines for Animal Care approved by the IIBCE. Finally, a group of control rats was killed at the first oestrus following vaginal canalisation.

In oculo transplantation

Donors

Four-week-old prepubertal rats (body weight: 50–60 g) were killed and the uterus was removed under aseptic conditions and placed in sterile ice-cold Hanks' balanced salt solution (Sigma) for dissection. The uterine horns were opened longitudinally and pinned on Sylgard (Dow Corning, UK) with micropins. The endometrium and most of the circular muscle layer were carefully removed and the longitudinal myometrial layer with its attached serosa was cut into strips of 1 mm width and 1.5 mm length.

Hosts and chronic oestrogen treatment

Prepubertal rats (4 weeks old) were ovariectomised bilaterally under ether anaesthesia and used as host recipients 1.5–2 months later (body weight: 230–250 g). Host recipients were anaesthetised with 40 mg/kg sodium pentobarbital administered intraperitone-

ally, followed by local administration of 0.5% proparacaine hydrochloride solution (Anestalcon, Alcon-Argentina). Mydryasis was achieved by application of a drop of 10 mg/ml atropine sulphate to the cornea (Olson and Malmfors 1970). Transplants were inserted through a small slit made with a microsurgical blade in the pupillary region of the cornea (Becton Dickinson, USA) and manipulated by gentle pressure on the cornea into the posterior irido-corneal angle of the eye (Olson and Malmfors 1970; Brauer et al. 1998, 2000a). The pupil was not obstructed by the transplants and visually guided behaviour of the hosts was not impaired. One group of the host recipients was treated with five doses of 50 µg oestrogen on days 10, 15, 20, 25 and 30 following transplantation and killed 3 days after the last injection (ECOT–33 days in oculo, 33dio). Another group was injected with five does of 50 µg oestrogen on days 30, 35, 40, 45 and 50 following transplantation and killed on day 53 (LCOT–53 days in oculo, 53dio). Control hosts were treated only with the vehicle.

Histochemical demonstration of sympathetic nerves

Sympathetic nerves were visualised by their content of NA by using the glyoxylic acid technique (de la Torre and Surgeon 1976). NA-L sympathetic nerves were demonstrated in cryostat tissue sections of intraocular transplants and in the cephalic, middle and lower parts of the uterine horn. Preparations were examined under a Nikon Eclipse 800 microscope equipped with epifluorescence and fitted with the appropriate filters. Micrographs were taken with Ilford HP5, 400 ASA film.

Quantitation of nerve fibres

In the uterine horn, the density of NA-L myometrial fibres was assessed in the longitudinal myometrial layer by using the method reported by Zoubina et al. (1998) with minor modifications. Nerve counting was carried out on two transverse cryostat section of the cephalic, middle and caudal region of the uterine horn per animal. Because ovariectomy could have damaged the sympathetic innervation of the cephalic and middle parts of the uterine horn, in the superior ovarian nerve and ovarian nerve plexus (Houdeau et al. 1998), assessment of nerve density in ovariectomised animals (OVX/OIL and OVX-LCOT/10) was restricted to the caudal region of the uterine horn.

Under the fluorescence microscope and by using a 10× objective lens, five different myometrial areas were captured digitally by means of the CoolSNAP-Pro Monochrome Digital Kit (Media Cybernetics, USA). Because of the uneven distribution of sympathetic nerves in the longitudinal muscle layer, particularly at the middle and caudal region of the uterine horn (see Results), areas selected for nerve density estimations included the antimesometrial border (one area), the mesometrial border (two areas) and the intermesometrial border (two areas). In order to obtain an estimate of the percentage area occupied by nerve fibres, a stereological grid with an area of 0.25 mm^2 and line intersects at 20-µm intervals was superimposed over these myometrial areas and all the grid transects overlying nerve profiles were counted. The resulting number was multiplied by 100 and divided by the total number of grid squares occupied by the smooth muscle. Because oestrogen treatment provokes major changes in the size of the uterus, corrections for changes in the size of the target were carried out in order to obtain the total nerve area. To this aim, the percentage of area occupied by nerves was multiplied by the total area of the longitudinal myometrial layer and divided by 100. Estimations of the longitudinal myometrial layer area were carried out on adjacent frozen tissue sections, mounted on gelatine-subbed glass slides, fixed by immersion in 4% paraformaldehyde and stained with haematoxylin and eosin.

A similar approach was used to estimate nerve density of the in oculo myometrial transplants. However, since discrimination between perivascular and myometrial-associated fibres was occasionally affected by the different plane of sectioning, all nerves present in the transplants were included for the estimation of the percentage area occupied by nerves. Similarly, since the plane of sectioning made it difficult to estimate the size of the transplants, no correction in the innervation density for changes in the size of the target was attempted.

Biochemical assay for NA

Isolated whole uterine horns were weighed and homogenised in 0.1 M perchloric acid (PCA; 10 mg wet weight tissue/40 µl PCA) containing 50 µl internal standard (3,4-dihydroxy-benzylamine, DHBA). After centrifugation at 20,000 *g* for 1 h at 4°C, supernatants were mixed with 10 mg alumina and 0.5 M TRIS (pH 8.6) was added to give a final volume of 1.5 ml (Keller et al. 1976). No changes in the final pH resulted from the addition of various volumes of TRIS. After two washing steps, catecholamines were eluted from the alumina with 100 µl 0.1 M perchloric acid. Following centrifugation at 15,000 *g* for 10 min at 4°C, a 50-µl aliquot of supernatant was injected into the HPLC-ED. The HPLC-ED system was equipped with a C18 reverse phase column (ODS 220×4.6 mm, BIOPHASE, USA), an amperometric detector (BAS LC-4C) and a Gilson-307 pump. The mobile phase (pH 3, flow rate 1 ml/min) was composed of 0.15 M citric acid, 0.6 mM sodium octyl sulphate with 2% acetonitrile and 1.7% of tetrahydrofuran. Detector sensitivity was set at 5–20 nA and the oxidation potential was fixed at 850 mV. In view of the small size of the transplants and the impossibility of completely removing the attached iris, determinations of NA on transplants were not attempted.

ELISA for NGF

Tissue levels of NGF were measured by using a two-site ELISA as previously described (Brauer et al. 2000b). The ELISA was carried out by binding either polyclonal goat anti-mouse NGF (1:1000) or preimmune serum (1:1163; Hazelton Research Products, Denver, Pa.) in 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) to parallel wells of a 96-well plate (Nunc Immunoplate I, Vanguard International, N.J.). After overnight incubation, plates were rinsed with 10 mM phosphate-buffered saline (PBS, ×4, pH 7.4), containing 0.05% Tween 20 and blocked with 1% fetal calf serum (Sigma) for a minimum of 1 h.

Samples to be analysed for NGF were diluted in 450 µl 10 mM PBS containing the protease inhibitors aprotinin (0.0001%), 0.1 mM benzethonium chloride and 0.1 mM phenylmethane sulphonylfluoride, 5% bovine serum albumin (Sigma) with 0.1% Tween 20 and homogenised for 30 s on ice. NGF purified from male submaxillary glands was used as a standard and was applied to the plate in concentrations ranging from 15 pg/ml to 1 ng/ml. Following an overnight incubation, duplicate samples and standards were removed and washed. The second primary antibody, monoclonal anti-mouse NGF 1G3, was added at 1:200 and incubated overnight. The plates were washed and then incubated for 6 h in biotinylated goat anti-rat IgG (Sigma; 1:625). All antibody incubations were carried out on a rotator at 4°C. The plates were washed and streptavidin-horseradish peroxidase (Zymed; 1:2000) was added at room temperature for 1 h. After a final wash, *o*-phenylenediamine was added in citrate buffer (pH 5.0) and the plates were placed on a rotator at room temperature in the dark for 10 min until colour development was complete. An aliquot (50 µl) 2.5 N H_2SO_4 was added to stop the reaction and optical densities were read at 492 nm on a BioRad plate reader interfaced to a Macintosh computer with MacReader 2.0 software. Data were calculated from the standard curves generated for each plate and expressed as the mean picograms of NGF per milligram of wet weight tissue. Results were expressed as total NGF per uterine horn and concentration of NGF per milligram of wet weigh tissue.

Statistical analysis

Results from histochemical and biochemical studies are expressed as the mean ± SEM. Data were compared by using a one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. Values of $P \leq 0.05$ were considered statistically significant.

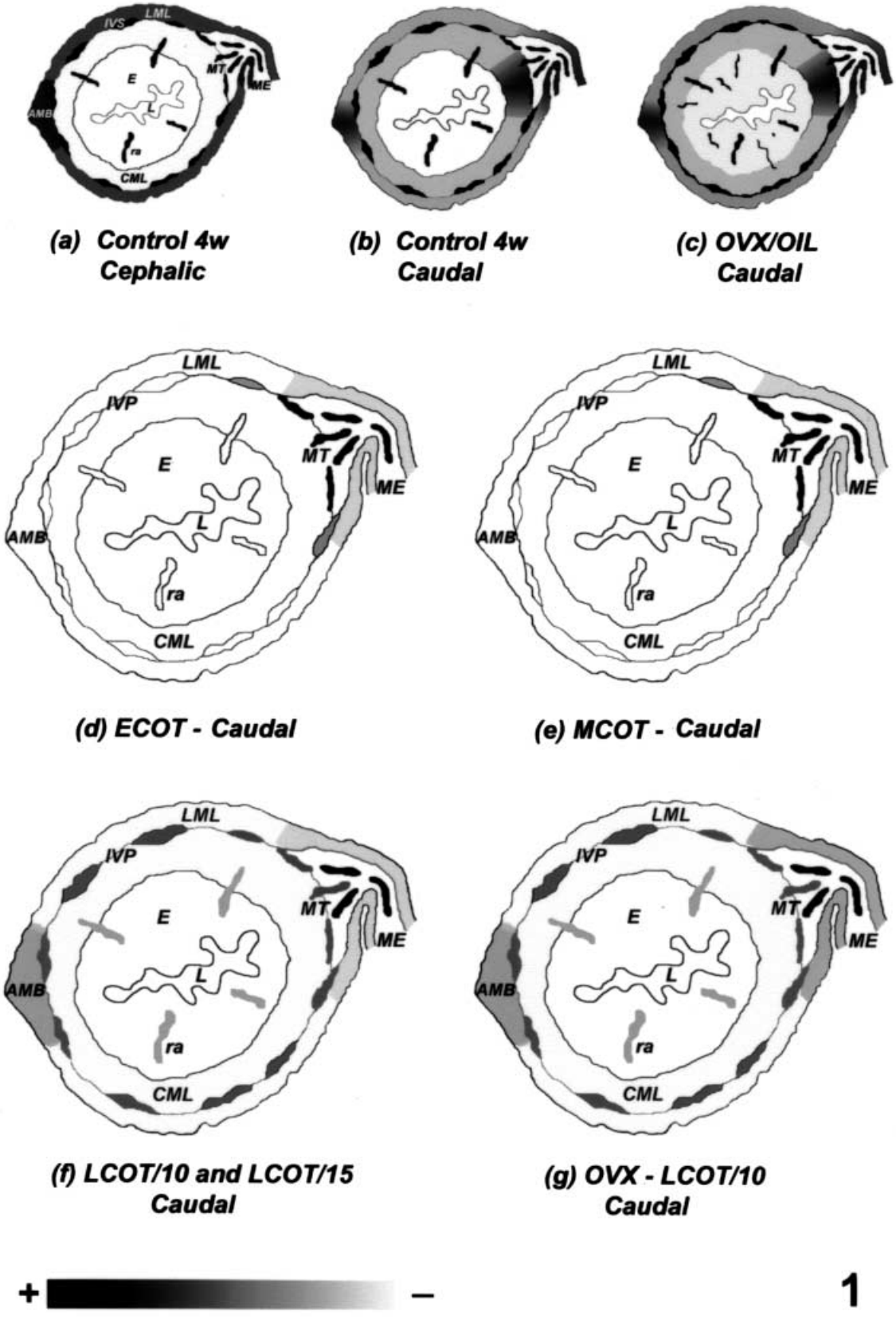


Fig. 1a–g. Schematic drawings illustrating the pattern and density of noaradrenaline-labelled sympathetic nerves in the rat uterus. **a, b** Differential cephalo-caudal distribution of sympathetic nerves in the prepubertal rat uterus. **c** Effects of prepubertal ovariectomy (*OVX/OIL*) on the sympathetic innervation of the caudal region of the uterine horn. **d, e** Effects of early (*ECOT*) and medium (*MCOT*) chronic oestrogen treatment, respectively. **f, g** Effects of late chronic oestrogen treatment to intact (*LCOT/10* and *LCOT/15*) and ovariecomised (*OVX-LCOT/10*). *AMB* Antimesometrial border, *LML* longitudinal myometrial layer, *CML* circular myometrial layer, *IVP* intramyometrial vascular space, *E* endometrium, *ra* radial arteries, *MT* mesometrial triangle, *ME* mesometrium, *L* lumen

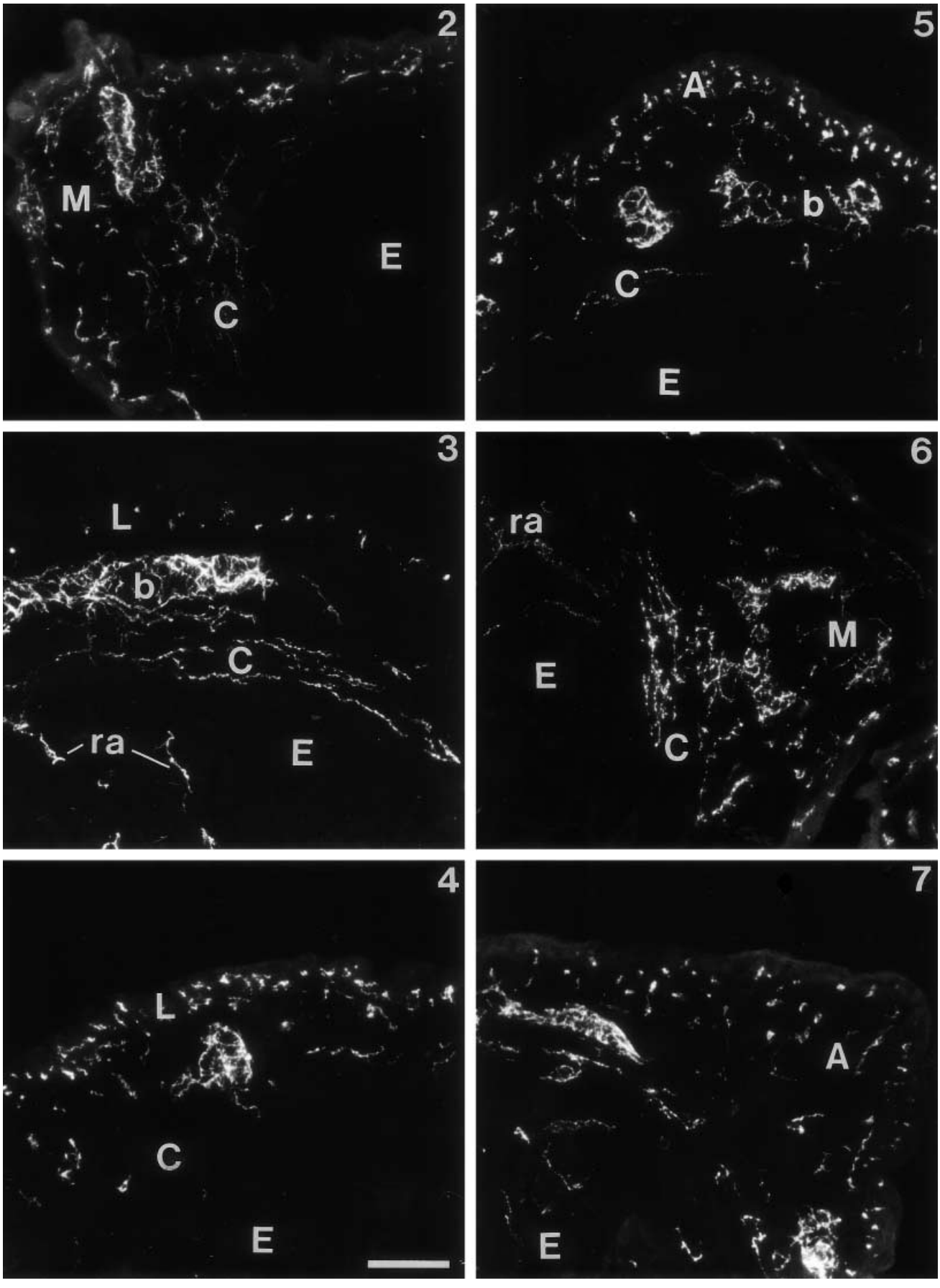


Fig. 2–7 Legend see page 66

Results

Effects of chronic oestrogen treatment on the growth-rate and ovulation of the animals

The growth rate of the animals was unaffected by the various protocols of chronic oestrogen treatment. All oestrogen-treated animals showed an open vagina and were acyclic with persistent cornified cells in the vaginal smear (Long and Evans 1922). Animals at puberty showed ova in their Fallopian tubes.

Effects of oestrogen on NA-L uterine sympathetic nerves

In prepubertal 4-week-old controls, NA-L sympathetic fibres penetrated the uterus through the mesometrium, accompanying blood vessels or as free bundles and isolated fibres (Figs. 1a, b, 2). Within the uterus, NA-L nerves were distributed in both the circular and longitudinal myometrial layer and around blood vessels located in the intramyometrial vascular space. Some nerve fibres penetrated into the endometrium, mainly being associated with the radial arteries (Fig. 1a, b). This general pattern of innervation showed, however, considerable regional variation. Most of the fibres associated with the circular myometrial layer were seen in the caudal region of the uterine horn (Figs. 1b, 3), whereas they were extremely sparse in the cephalic region (Figs. 1a, 4). In the caudal region, the highest density of innervation of the circular layer was seen facing the mesometrium (Fig. 1b). In the longitudinal myometrial layer, quantitative studies showed that the percentage area occupied by NA-L nerves was higher in the cephalic region of the uterine horn than in the caudal region (Table 1) and this difference persisted after correction for the size of the muscle layer (Table 2). In all uterine regions analysed, NA-L fibres associated with the longitudinal myometri-

Table 1 Quantitative assessment of the effects of ovariectomy and various protocols of late chronic oestrogen administration on the percentage area occupied by noradrenaline-labelled sympathetic nerves in the longitudinal myometrial layer of the rat uterine horn. In intact animals, quantification was carried out in the cephalic, middle and caudal regions of the uterine horn, whereas in ovariectomised animals, analysis was restricted to the caudal region. The percentage area occupied by nerves is expressed as the mean ± SEM (n=6). Data were compared by a one-way analysis of variance followed by the Tukey-Kramer multiple comparison test. Values of $P \leq 0.05$ were considered statistically significant

Treatment	Cephalic	Middle	Caudal
CONTROL 4w	52.0±1.1	35.7±0.8[a]	32.2±2.1[a]
OVX/OIL	–	–	39.6±2.3[b]
LCOT/10	11.4±0.6[b]	10.8±1.1[b]	9.0±0.7[b, c]
LCOT/15	11.0±0.3[b]	9.0±0.5[b]	7.2±0.3[b, c]
OVX + LCOT/10	–	–	8.7±0.8[b, c]

[a] Significant difference with the cephalic region with comparable treatments
[b] Significant difference with control 4w in comparable uterine areas
[c] Significant difference with OVX/OIL in comparable uterine areas

◀ **Fig. 2** Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn in prepubertal four-week old controls. Mesometrial (*M*) innervation (*C* circular myometrial layer, *E* endometrium). *Bar* (in Fig. 4) 100 µm

Fig. 3 As in Fig. 2. Pattern of distribution of NA-L nerves in the intermesometrial region of the caudal region of the prepubertal control uterine horn (*b* blood vessels, *C* circular myometrial layer, *E* endometrium, *L* longitudinal myometrial layer, *ra* radial arteries). *Bar* (in Fig. 4) 100 µm

Fig. 4 As in Fig. 2. Pattern of distribution of NA-L nerves in the intermesometrial region of the cephalic region of the prepubertal control uterine horn (*C* circular myometrial layer, *E* endometrium, *L* longitudinal myometrial layer). *Bar* 100 µm

Fig. 5 As in Fig. 2. Innervation of the antimesometrial border (A) in the caudal region of the uterine horn of prepubertal controls (*b* blood vessels, *C* circular myometrial layer, *E* endometrium). *Bar* (in Fig. 4) 100 µm

Fig. 6 Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn in ovariectomised animals at 48 days of age. Mesometrial (*M*) innervation (*C* circular myometrial layer, *E* endometrium, *ra* radial arteries). *Bar* (in Fig. 4) 100 µm

Fig. 7 As in Fig. 6. Innervation of the antimesometrial border (A) in the caudal region of the uterine horn of ovariectomised rats. *Bar* (in Fig. 4) 100 µm

Fig. 8 Noradrenaline-labelled sympathetic fibres demonstrated by ▶ the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn. Effects of early and medium chronic oestrogen treatment. Mesometrial (M) innervation. *Bar* (in Fig. 15) 100 µm

Fig. 9 As in Fig. 8. Complete absence of noradrenaline-labelled nerves in the intermesometrial region of the uterine horn (*b* blood vessles, *C* circular myometrial layer, *L* longitudinal myometrial layer). *Bar* (in Fig. 15) 100 µm

Fig. 10 As in Fig. 8. Sympathetic fibres associated with blood vessels (*b*) located close the mesometrial entrance. *Bar* (in Fig. 15) 100 µm

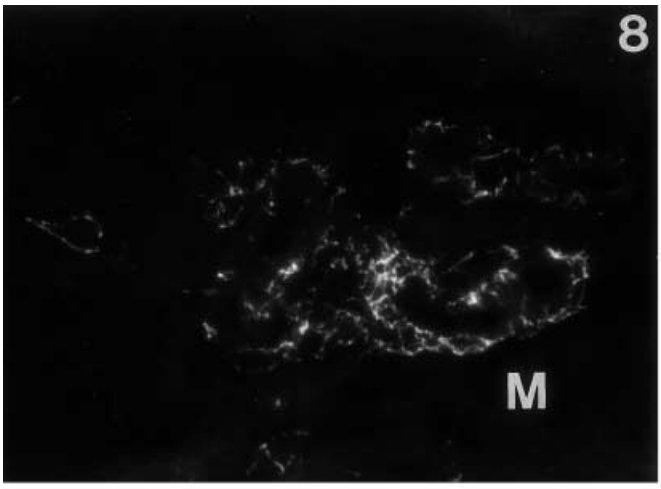
Fig. 11 As in Fig. 8. Sympathetic fibres associated with blood vessels (*b*) located close the mesometrial entrance. Note that nerves show a reduced fluorescence intensity and intensely fluorescent enlargements (*large arrow*). *Bar* (in Fig. 15) 100 µm

Fig. 12 Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn. Effects of late chronic oestrogen treatment to intact and ovariectomised animals. Mesometrial (M) innervation (*b* blood vessels, *L* longitudinal myometrial layer). *Bar* (in Fig. 15) 100 µm

Fig. 13 As in Fig. 12. Innervation of the antimesometrial (*A*) area of the uterine horn. Note that although most of the nerve fibres appear to be well preserved, some intensely fluorescent enlargements are seen in fibres associated with the myometrium (*arrow*). *Bar* (in Fig. 15) 100 µm

Fig. 14 As in Fig. 12. Innervation of the intermesometrial area of the uterine horn (*b* blood vessels, *L* longitudinal myometrial layer). *Bar* (in Fig. 15) 100 µm

Fig. 15 As in Fig. 12. Note that although most of the nerve fibres appear to be well preserved, some intensely fluorescent enlargements are seen in fibres associated with the myometrium blood vessels (*arrow*). *Bar* 100 µm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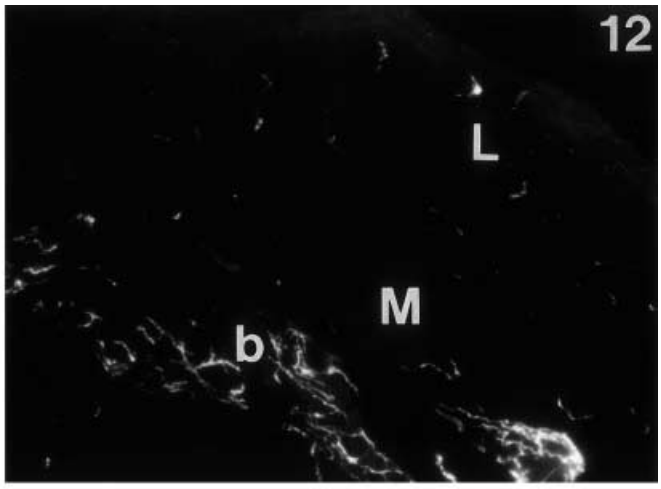
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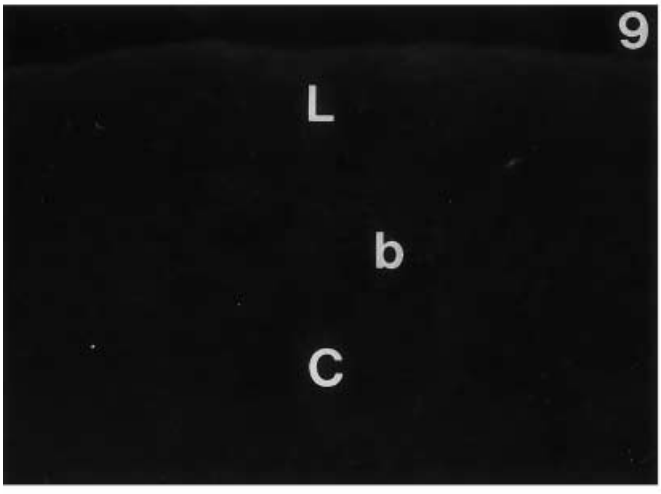
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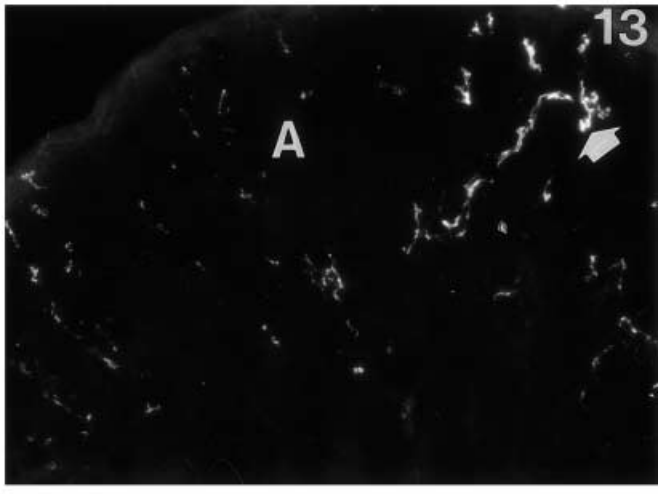
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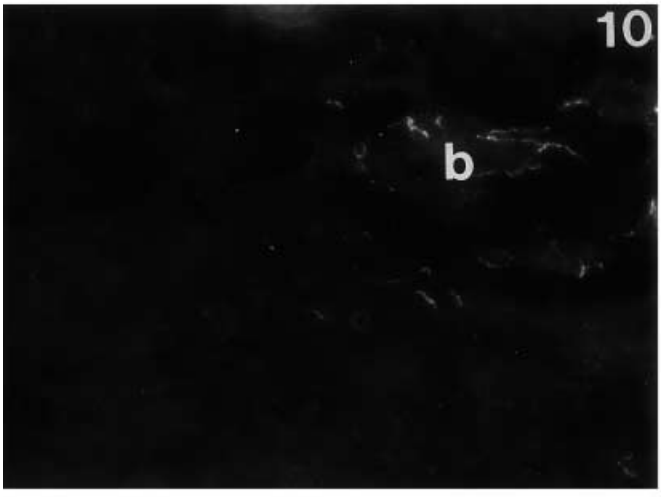
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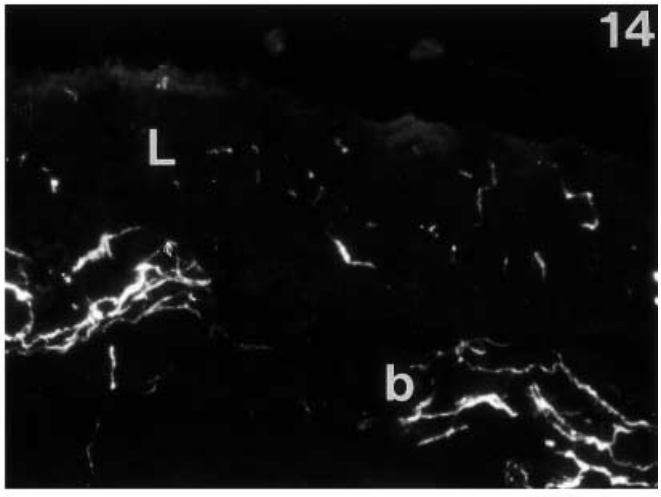
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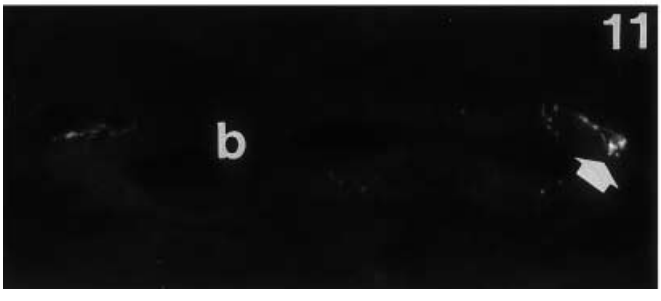
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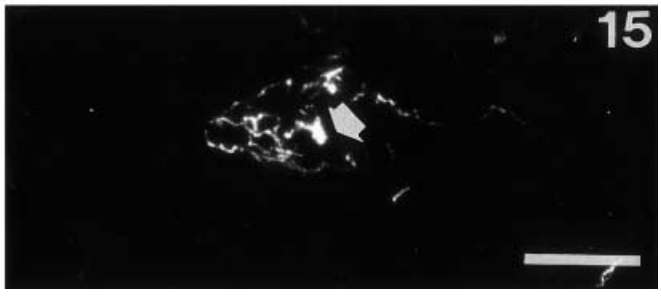
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Table 2 Quantitative assessment of the effects of ovariectomy and various protocols of late chronic oestrogen administration on the total nerve area (mm^2) occupied by noradrenaline-labelled sympathetic nerves in the longitudinal myometrial layer of the rat uterine horn after correction for changes in the size of the uterus. In intact animals, quantification was carried out in the cephalic, middle and caudal regions of the uterine horn, whereas in ovariectomised animals, analysis was restricted to the caudal region of the uterine horn. The total nerve area in the longitudinal myometrial layer is expressed as the mean ± SEM (n=6; other statistical details as in Table 1)

Treatment	Cephalic	Middle	Caudal
CONTROL 4w	0.09±0.002	0.07±0.002	0.05±0.003[a]
OVX/OIL	–	–	0.06±0.003
LCOT/10	0.11±0.006	0.10±0.009	0.08±0.006
LCOT/15	0.10±0.002	0.08±0.005	0.07±0.008
OVX + LCOT/10	–	–	0.07±0.008

[a] Significant difference with the cephalic region with comparable treatments

Table 3 Quantitative assessment of the effects of ovariectomy and various protocols of late chronic oestrogen administration on the percentage area occupied by noradrenaline-labelled sympathetic nerves in the antimesometrial border of the rat uterine horn. In intact animals, quantification was carried out in the cephalic, middle and caudal regions of the uterine horn, whereas in ovariectomised animals, analysis was restricted to the caudal region. The percentage of area occupied by nerves is expressed as the mean ± SEM (n=6; other statistical details as in Table 1)

Treatment	Cephalic	Middle	Caudal
CONTROL 4w	58.6±5.0	48.6±1.6	46.0±3.9[a]
OVX/OIL	–	–	43.8±4.6
LCOT/10	16.6±1.2[b]	16.0±2.0[b]	13.9±1.3[b, c]
LCOT/15	16.6±0.5[b]	13.5±1.0[b]	11.0±0.5[b, c]
OVX + LCOT/10	–	–	12.4±1.7[b, c]

[a] Significant difference with the cephalic region with comparable treatments
[b] Significant difference with control 4w in comparable uterine areas
[c] Significant difference with OVX/OIL

um were particularly concentrated in the antimesometrial border (Figs. 1a, b, 5, Table 3), whereas the innervation of the mesometrial and intermesometrial areas was sparser and less uniformly distributed, particularly in the middle and caudal regions of the uterine horn (Fig. 3).

Prepubertal ovariectomy did not alter the distribution of NA-L sympathetic nerves but led to a generalised increase in the innervation density, including that associated with mesometrial, intramyometrial and endometrial blood vessels (Figs. 1c, 6, 7). In the longitudinal myometrial layer, a significant increase in the percentage area occupied by nerves was observed (Table 1) but this increase did not reach statistical significance in the antimesometrial border (Table 3). After correcting for the total area of the longitudinal muscle layer, no significant changes in the total nerve area were observed (Table 2).

Both early and medium chronic oestrogen treatment (ECOT and MCOT, respectively) led to a similar pattern of changes (Fig. 1d, e). The only fibres were in the mesometrium (Fig. 8); no nerve fibres were seen within the uterus (Fig. 9). On occasion, some nerve fibres were observed around blood vessels located close to the mesometrium (Fig. 10). Generally, these fibres were less intensely fluorescent than those in controls and some of them showed intensely fluorescent enlargements that resemble images of nerve fibre degeneration (Fig. 11).

Following late chronic oestrogen treatment of intact (LCOT/10 and LCOT/15; Fig. 1e) and ovariectomised animals (OVX/LCOT10; Fig. 1f) the pattern of innervation of the various uterine regions resembled that observed in prepubertal intact and ovariectomised controls. However, the innervation was considerably sparser (Figs. 12, 13, 14). Although intramyometrial blood vessels appeared well innervated, NA-L nerve fibres were absent from radial arteries and only a few nerve fibres were seen in the circular myometrial layer of the caudal region of the horn. Most of the NA-L nerve fibres appeared to be well preserved but occasional fibres showing intensely fluorescent enlargements were seen at both myometrial (Fig. 13) and perivascular locations (Fig. 15).Quantitative studies performed on the longitudinal myometrial layer (Tables 1, 3) showed that the percentage area occupied by NA-L nerves was substantially reduced in all groups of late chronic oestrogen-treated animals (LCOT/10, LCOT/15 and OVX/LCOT-10) when compared with both prepubertal 4-week-old controls and ovariectomised controls of 48 days of age. This reduction, however, did not persist when values were corrected for the area of the longitudinal muscle layer and accordingly no significant changes in the total nerve area were observed (Table 2).

Effects of oestrogen on uterine levels of NA

The results of the biochemical studies are summarised in Table 4. The weight of the uterine horn was slightly reduced by prepubertal ovariectomy and showed a marked increase following puberty and after all protocols of oestrogen administration. Both early and medium chronic oestrogen treatment (ECOT and MCOT, respectively) resulted in a marked decrease in the total content of NA in the uterine horn (83% and 69%, respectively). The total content of NA was unchanged at puberty, however, since the weight of the uterus was markedly increased; a fourfold decrease in the concentration per milligram of wet weight tissue was observed (control: 2.61±0.42 ng/mg NA; after puberty: 0.66±0.05 ng/mg NA). Acute (AOT) and late administration of oestrogen to intact animals (LCOT/10 and LCOT/15) reduced uterine NA total content to a similar extent (38–39%), but this difference did not reach statistical significance when compared with 4-week-old prepubertal controls. Total NA content showed a 67% increase following ovariectomy. Ovariectomised animals treated with oestrogen (OVX-LCOT/10) showed a 69% decrease with respect to ovariectomised controls (OVX-OIL).

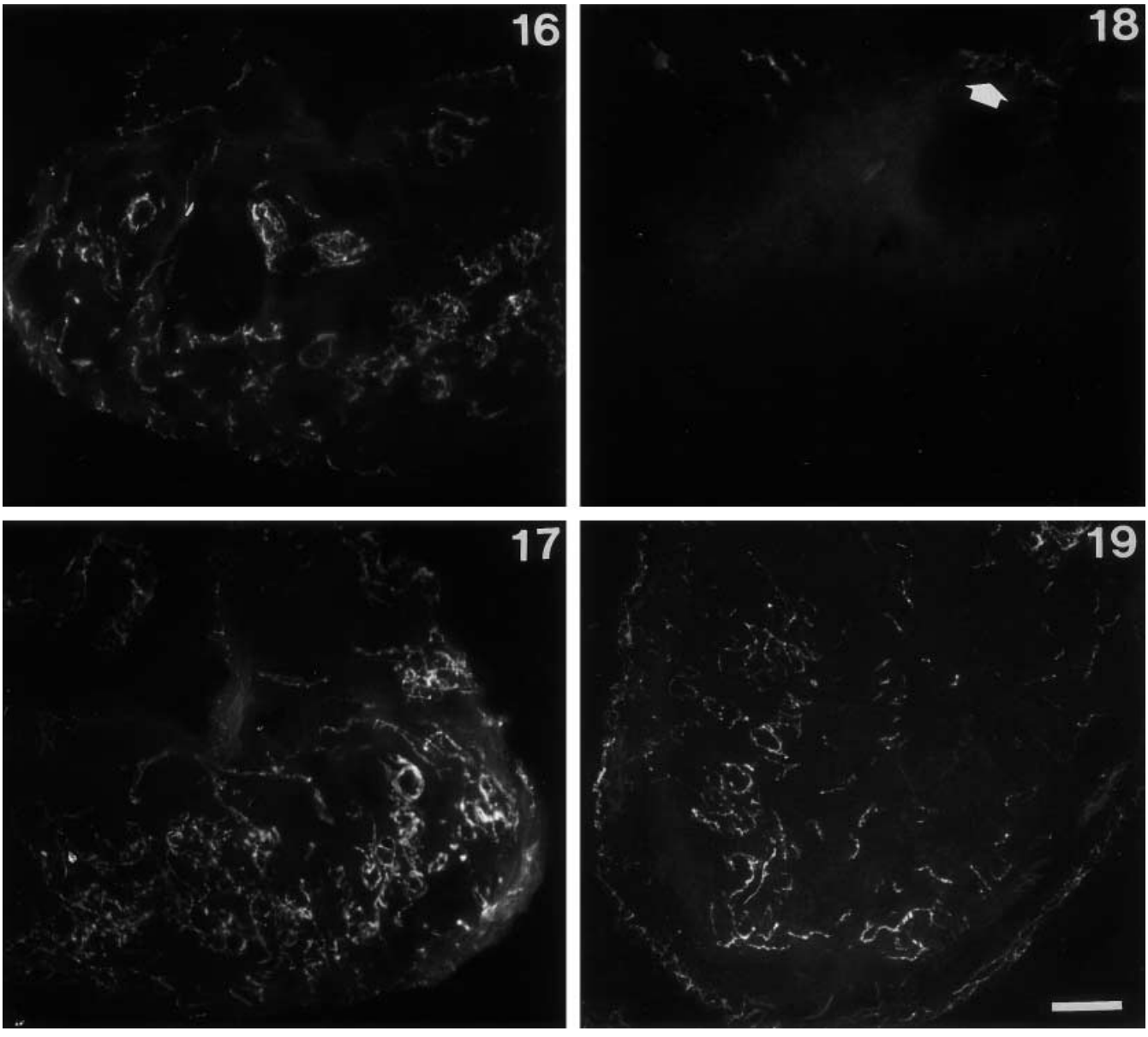

Fig. 16 Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on cryostat sections of in oculo myometrial transplants. Innervation of control myometrial transplants at 33 after transplantation. *Bar* (in Fig. 19) 100 µm

Fig. 17 As in Fig. 16. Innervation of control myometrial transplants at 53 days after transplantation. *Bar* (in Fig. 19) 100 µm

Fig. 18 As in Fig. 16. Almost complete absence of NA-L sympathetic nerves in the in oculo myometrial transplants following early chronic oestrogen treatment (ECOT-33dio). *Arrow* indicates fibres located in the periphery of the transplant. *Bar* (in Fig. 19) 100 µm

Fig. 19 Pattern of innervation of late chronic oestrogen-treated in oculo myometrial transplants (LCOT-53 dio). *Bar* 100 µm

Effects of oestrogen on the sympathetic reinnervation of in oculo myometrial transplants

After 33 days in oculo (33dio) control transplants were well reinnervated by NA-L sympathetic fibres, which appeared to be associated with blood vessels and myometrial smooth muscle (Fig. 16). Moreover, 53 days after transplantation (53dio), the density of innervation of control transplants was increased (Fig. 17) with respect to the 33dio group (Table 5). Chronic oestrogen treatment starting on day 10 after transplantation (ECOT-33dio) led to an almost complete loss of NA-L perivascular and myometrial-associated nerve fibres. Occasional isolated fibres, showing a reduced NA-fluorescence intensity, were visualised in the periphery of the transplants (Fig. 18). Several NA-L nerve fibres were recognised (Fig. 19) in late chronic oestrogen-treated transplants (LCOT-53dio). The percentage area occupied by NA-L

Table 4 Effects of natural puberty, prepubertal ovariectomy, acute oestrogen treatment (AOT) and various protocols of chronic oestrogen administration on wet weight and total content of noradrenaline (NA) of the rat uterine horn. Results are expressed as the mean ± SEM (*n*; other statistical details as in Table 1)

Treatment	Weight (mg)	NA total content (ng)
CONTROL 4w	16.5±0.7 (10)	41.4±6.0
ECOT	82.6±5.5 (8)[a]	12.7±1.2[a]
MCOT	84.3±3.1 (9)[a]	7.0±1.1[a]
PUBERTY	60.9±4.1 (6)[a, b, c]	39.7±4.0[a, b, c]
AOT	63.4±3.6 (6)[a,b, c]	25.6±1.5[c]
LCOT/10	108.5±5.6 (8)[a, b, c, d, e, h]	25.8±3.9[c, h]
LCOT/15	111.4±2.5 (8)[a, b, c, d, e, h]	25.4±3.0[c, h]
OVX/OIL	13.8±0.4 (8)[b, c, d, e, f, g]	69.1±7.3[a, b, c, d, f, g]
OVX+LCOT/10	105.4±5.0 (8)[a, b, c, d, e, h]	22.5±1.8[a, c, h]

[a] Significant difference with the control 4w; [b] Significant difference with ECOT
[c] Significant difference with MCOT; [d] Significant difference with puberty
[e] Significant difference with AOT; [f] Significant difference with LCOT/10
[g] Significant difference with LCOT/15; [h] Significant difference with OVX

Table 5 Quantitative assessment of the effects of the various protocols of chronic oestrogen administration on the percentage area occupied by noradrenaline-labelled perivascular and myometrial-associated sympathetic nerves of in oculo myometrial transplants. The apparent percentage area occupied by nerves is expressed as the mean ± SEM (*n*; other statistical details as in Table 1)

Control 33 days in oculo	Control 53 days in oculo	ECOT 33 days in oculo	LCOT 53 days in oculo
35.9±1.8 (10)	62.6±6.3 (8)[a]	4.6±1.5 (8)[a, b]	28.0±4.2 (8)[b, c]

[a] Significant difference from Control 33 days in oculo; [b] Significant difference with Control 53 days in oculo
[c] Significant difference between treatments

Table 6 Comparative assessment of the effects of natural puberty, acute oestrogen treatment (AOT) and medium chronic oestrogen treatment (MCOT) on the tissue weight (mg), NGF protein total content (pg) and NGF concentration (pg/mg wet weight tissue) of the rat uterine horn. Results are expressed as the mean ± SEM (*n*; other statistical details as in Table 1)

Treatment	Uterine horn weight	NGF total content	NGF concentration
CONTROL 4w	20.6±1.0 (4)	9.25±2.0	0.47±0.13
PUBERTY	61.0±11.2 (5)[a]	18.7±4.9	0.31±0.08
AOT	62.9±2.5 (6)[a]	18.2±6.0	0.28±0.09
MCOT	81.4±3.2 (7)[a]	38.5±6.2[a]	0.48±0.08

[a] Significant difference from CONTROL 4w

nerves of these transplants was significantly lower than their respective controls (53dio) but significantly higher than those submitted to early chronic oestrogen treatment (ECOT-33dio; Table 5).

Effects of puberty and oestrogen treatment on NGF protein levels

The results of the NGF protein assay of the different animal groups are summarized in Table 6. Following medium chronic oestrogen treatment, the total content of NGF protein in the uterine horn showed a four-fold increase. This increase matched the growth of the uterus and, accordingly, no changes in concentration were observed. Similarly, NGF total content showed a modest increase after puberty and following acute oestrogen treatment. This increase, however, was sufficient to maintain the concentration of NGF when expressed per unit of wet weight tissue.

Discussion

Effects of ovariectomy and oestrogen on uterine innervation and NA levels

The results reported in this paper regarding the distribution and regional variations of sympathetic nerves in the prepubertal rat uterus are in agreement with previous findings in the immature and cycling rat (Papka et al. 1985; Brauer et al. 1992; Melo and Machado 1993; Houdeau et al. 1998; Zoubina et al. 1998).

Prepubertal ovariectomy leads to a marked increase in the total content of NA in the uterine horn and this change is related to a generalised increase in the density of uterine NA-L sympathetic nerves. Previous studies have shown that the development of uterine sympathetic nerves takes place during the first 4 weeks of postnatal life and the adult pattern of innervation is reached immediately before puberty (Brauer et al. 1992). Following the first surge of oestrogen at puberty, a marked decrease in the density of uterine NA-L nerves and in NA concentration has been observed. In the light of the present results, it appears that

at 4 weeks of age, uterine-related sympathetic nerves remain capable of growing but their growth capacity is diminished by the increase in oestrogen levels accompanying puberty. This observation is reinforced by our current findings showing that the treatment of ovariectomised animals with oestrogen reduces the density of the innervation of NA-L sympathetic nerves. Consistent with these concepts, recent studies have demonstrated that oestrogen-receptor-alpha knock-out mice exhibit uterine sympathetic hyperinnervation (Zoubina and Smith 2001).

Our biochemical studies have shown that after both early and medium chronic oestrogen treatment, the total NA content of the uterine horn is dramatically reduced, thus confirming previous findings (Brauer et al. 1995, 1999). The histochemical studies have revealed that this fall is related to a complete absence of myometrial and perivascular NA-labelled sympathetic nerve fibres. Different explanations could account for the absence of NA-L uterine sympathetic nerves following early and medium chronic oestrogen treatment. As the rat uterus is innervated by developing NA-L sympathetic fibres from birth, it is possible that early exposure to oestrogen could cause the degeneration of the developing uterine sympathetic plexus. This possibility is supported by the almost complete absence of tyrosine-hydroxylase-positive nerves in chronic oestrogen-treated animals (Brauer et al. 1999) and by our current observations showing intensely fluorescent enlargements not dissimilar to the images of nerve degeneration observed in the guinea pig uterus during early pregnancy (Thorbert 1978; Brauer et al. 2000b) and in the gut of streptozotozin diabetic rats (Lincoln et al. 1984). An alternative explanation is that the reduction in NA levels induced by early and medium chronic oestrogen treatment could make intact sympathetic axons no longer visible by fluorescence histochemistry, as shown for sympathetic nerves of the guinea pig vas deferens following chronic guanethidine treatment (Evans et al. 1973). Electron-microscopic studies are required to distinguish between these possibilities.

In marked contrast to the effects provoked by early and medium chronic oestrogen treatment, several myometrial and perivascular NA-L nerve fibres have been seen after late administration of oestrogen to intact and ovariectomised prepubertal animals. This difference does not seem to be related to differences in the circulating levels of oestrogen, since neither the increase in oestrogen dose nor removal of potential endogenous oestrogen by ovariectomy alters the pattern of late-oestrogen-induced changes. These results, therefore, indicate that mature uterine sympathetic fibres are less susceptible to the effects of oestrogen than developing ones.

Quantitative studies have shown a reduction in the apparent density of sympathetic nerve fibres in the longitudinal myometrial layer of late-oestrogen-treated animals but this change does not persist after correction for changes in the layer size. These results suggest that late exposure to oestrogen provokes a “dilution” of myometrial NA-L sympathetic fibres in the enlarged myometrium and prevents parallel growth between sympathetic nerves and its target tissue. A “dilution” of the myometrial sympathetic innervation that fails to keep pace with the size of the uterus has also been seen in the rat uterine horn following puberty (Brauer et al. 1992) and in the cervix and tubal end of the guinea-pig uterine horn at term pregnancy (Brauer et al. 2000b). This unusual behaviour of uterine sympathetic nerves clearly contrasts with that observed in other hypertrophic target tissues, including muscular visceral organs, where changes in target size induce coordinated changes in their innervating neurons (Gabella 1984; Steers et al. 1990).

In contrast to natural puberty, both acute and chronic exposure to oestrogen of intact and ovariectomised young adult rats leads to a modest reduction in the total content of NA in the uterine horn. It is probable that this difference can be explained by the high doses of oestrogen employed in our experimental approach. In our view, two possible explanations could account for this reduction. One is that oestrogen affects the metabolism, release or uptake of NA in uterine sympathetic fibres and the second is that oestrogen causes a partial degeneration of sympathetic nerve fibres. Our current observations show that most of the myometrial and perivascular NA-L nerve fibres are well preserved following late oestrogen treatment of intact and ovariectomised animals. However, an almost complete absence of NA-L fibres around radial arteries and in the circular myometrial layer has been found. In addition, intensely fluorescent enlargements suggesting nerve degeneration have occasionally been recognised in both myometrial and perivascular locations. In line with this concept, recent studies in the adult rat have shown that during the natural oestrous cycle, the density of dopamine-beta-hydroxylase-positive nerves decreases at proestrus and oestrus and increases at dioestrus (Zoubina et al. 1998). Electron-microscopic studies (Zoubina and Smith 2000) indicate that these changes are related to cycles of degeneration and regeneration of terminal axons. These observations suggest that some of the mature uterine sympathetic nerve fibres remain vulnerable to the effects of oestrogen with consequent effects on NA levels.

Effects of chronic oestrogen on the reinnervation of intraocular myometrial transplants

Our transplantation experiments have confirmed observations made in the uterus by showing that early administration of oestrogen results in an almost complete loss of NA-L sympathetic nerve fibres, whereas late administration only leads to a reduction in the apparent density of innervation. These results indicate that mature sympathetic fibres arising from the superior cervical ganglia are less vulnerable to oestrogen-induced changes in the target than immature fibres. In addition, taking into account that, in transplantation experiments, reinnervating neurons belong to adult hosts, our current results indicate that the differential response to oestrogen depends on the maturation state of the nerve fibres, although it does not

seem to be related to the age of the neuron. Finally, since the myometria used for all our transplantation experiments were taken from prepubertal donor animals, the present results argue against the possibility that the differential response to oestrogen observed in developing and prepubertal animals is associated with different developmental stages of the uterus.

Factors controlling oestrogen-induced plasticity of uterine sympathetic nerves

Our present results indicate that developing uterine-related sympathetic fibres are more vulnerable to oestrogen than mature fibres. Although the factors controlling this differential vulnerability are not clear at present, a potential involvement of neuronal oestrogen receptor expression could be postulated. Immunoreactivity and mRNA for alpha and beta oestrogen receptors have been shown in the rat uterine-projecting preganglionic and postganglionic parasympathetic and sensory neurons (Williams and Papka 1996; Williams et al. 1997; Papka et al. 1997, 2001). To our knowledge, there is only indirect evidence indicating the presence of oestrogen receptors in sympathetic neurons innervating the female genital organs (Shinohara et al. 2000; Ubuka et al. 2001). However, the possibility that oestrogen could affect sympathetic transmission at the preganglionic level cannot at present be disregarded.

An alternative explanation for this differential vulnerability is suggested by transplantation results showing that, as for uterine-related sympathetic fibres, reinnervating fibres from the superior cervical ganglia that have been in contact with the target uterine tissue for long periods become less susceptible to oestrogen. Although the nature of the signals produced by the sex-hormone-primed uterus is still unknown (Brauer et al. 2000b; Varol et al. 2000), it is possible that oestrogen could affect the neurotrophic capacity of the uterus. Because sympathetic nerves are susceptible to changes in the availability of NGF, we have assessed the possibility that oestrogen can reduce the levels of NGF in the rat uterus. These studies, however, have shown that early exposure to oestrogen, natural puberty and acute oestrogen treatment of prepubertal rats provoke coordinated changes between the growth of the uterus and the total content of NGF protein. The failure of oestrogen to reduce NGF protein levels in parallel with NA-L sympathetic nerves during postnatal development or during early adult life indicates that NGF deprivation is not the key factor underlying oestrogen-induced remodelling of uterine innervation. These findings do not exclude the possibility that oestrogen deprives sympathetic neurons of NGF through changes in neuronal receptivity (Toran-Allerand 2000) and retrograde transport (Thrasivoulou et al. 2000) or by limiting its availability to fibres through changes in the amount and composition of the uterine extracellular matrix (Millaruelo et al. 1988; de Curtis 1991; Cowen et al. 1997; Cowen and Gavazzi 1998).

Acknowledgements The technical assistance of Mrs. K. Shockley and N. Cuña is greatly appreciated. The authors would like to thank the Neurochemistry Division of the IIBCE for access to the HPLC facilities.

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