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The oestrogenized rat myometrium inhibits organotypic sympathetic reinnervation

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Abstract

Chronic administration of oestrogen to rats during the infantile/prepubertal period provokes, at 28 days of age, complete loss of noradrenaline-labelled intrauterine sympathetic nerves. It is not known whether oestrogen inhibits the growth or causes the degeneration of developing uterine sympathetic nerves, or whether the uterus recovers its innervation following cessation of infantile/prepubertal oestrogen treatment. In the present study, we analysed the time-course of the effects of oestrogen on the development of uterine sympathetic nerves in the rat, using histochemical methods. In addition, the pattern of sympathetic reinnervation of the uterus of intact and ovariectomised females was assessed 3 and 6 months after cessation of chronic oestrogen treatment. The ability of sympathetic nerves to reinnervate the oestrogenized uterine tissue was assessed in intraocular transplants of uterine myometrium into ovariectomised host rats. Early exposure to oestrogen did not inhibit the approach of sympathetic nerves to the uterus, but prevented the normal growth and maturation of intrauterine sympathetic fibres and abolished the innervation that reached the organ before initiation of treatment. Three or six months following cessation of oestrogen treatment, most of the sympathetic nerves were restricted to the mesometrium and mesometrial entrance, whereas intrauterine innervation remained persistently depressed as a consequence of a sustained oestrous-like state provoked by ovarian dysfunction (polycystic ovary). An organotypic regrowth of uterine sympathetic nerves was observed in ovariectomised infantile/prepubertal oestrogen-treated animals. After 5 weeks in oculo, the innervation of oestrogenized myometrial transplants was reduced by 50%, and substantial changes in the pattern of reinnervation were observed. In control transplants, 86% of the nerves were terminal varicose myometrial and perivascular nerve fibres, whereas 14% were preterminal nerve bundles. In oestrogenized myometrial transplants, 83% of the noradrenaline-labelled intercepting nerves were enlarged preterminal bundles and only 17% were terminal fibres. These results indicate that the oestrogenized myometrium is unattractive for sympathetic nerves and inhibits organotypic sympathetic reinnervation.

Keywords: Uterus; Sympathetic nerves; Oestrogen; Plasticity; Regeneration; Transplantation

1. Introduction

Studies carried out in different mammalian species have shown that the uterus is supplied by sympathetic nerves which innervate blood vessels and myometrial smooth muscle. Sympathetic nerves to the uterus are susceptible to changes in the endocrine environment and exhibit dynamic changes in response to physiological and experimental alterations in the circulating levels of sex hormones (Marshall, 1981; Owman and Stjernquist, 1988). Uterine sympathetic nerves undergo a complete degeneration during pregnancy (Thorbert, 1978; Yamada, 1988; Owman and Stjernquist, 1988; Haase et al., 1997) and show 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) which only affects the myometrial-associated sympathetic innervation.

Previous studies by our group have shown that immature uterine sympathetic nerves are particularly susceptible to oestrogens (Brauer et al., 1995; Chávez-Genaro et al., 2002).

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Chronic exposure to oestrogen in rats during the infantile/prepubertal period leads to a complete loss of noradrenaline-labelled intrauterine sympathetic nerves, comparable to that observed at late pregnancy. More recently, using the in oculo transplantation method, we have observed that this unusual response of uterine sympathetic nerves to oestrogen is influenced by changes in the neuron–target interactions (Brauer et al., 2000; Chávez-Genaro et al., 2002).

In spite of these advances, there are several aspects of oestrogen action that still remain uncertain. For instance, it is not known whether infantile/prepubertal chronic oestrogen treatment inhibits the growth or causes the degeneration of developing uterine sympathetic fibres. Similarly, it is unknown whether these oestrogen-induced changes are long-lasting or if the uterus recovers an organotypic innervation following cessation of oestrogen treatment. Finally, the ability of sympathetic nerves to reinnervate the oestrogenized myometrium has not been investigated. To address these questions, in the present study we analysed the time-course of oestrogen activity on developing sympathetic nerves in the rat uterus. In addition, we assessed the pattern of sympathetic reinnervation of the uterus of intact and ovariectomised rat females 3 and 6 months after cessation of infantile/prepubertal chronic oestrogen treatment. Finally, the ability of sympathetic nerves to reinnervate the oestrogenized myometrium was assessed using the anterior eye chamber transplantation approach. Changes in the sympathetic innervation of the uterine horn and of in oculo myometrial transplants were assessed on cryostat tissue sections processed by the glyoxylic acid technique. In order to evaluate the reproductive status of the animals circulating levels of oestrogen and progesterone were measured by a competitive immunoassay and the appearance of the ovaries evaluated on histological sections at different times following cessation of chronic oestrogen treatment.

2. Materials and methods

2.1. Animals and chronic oestrogen treatment

Female Wistar-derived albino rats from the breeding colony held at the Instituto de Investigaciones Biológicas Clemente Estable (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. Chronic oestrogen treatment was performed with β-oestradiol 17-cypionate (Laboratorios Köning, Argentina), diluted to appropriate doses with peanut oil (Sigma, USA) to a final volume of 0.1 ml per dose. Females were injected subcutaneously with four doses of 10 μg oestrogen on days 10, 15, 20 and 25 of postnatal development (Brauer et al., 1995).

For developmental studies, oestrogen-treated animals were killed by cervical dislocation 2 days after each oestrogen dose (12, 17, 22 and 27 days of age; $n=6$ for each group). An equal number of females from matched litters were given peanut oil, killed at the same developmental stages and used as controls. To analyse the long-term effects oestrogen, animals were killed by decapitation 3 and 6 months after cessation of treatment ($n=8$ for each group). An additional group of females ($n=8$) was treated with oestrogen as before, submitted to bilateral ovariectomy at 28 days of age and killed by decapitation 3 months later. Surgical procedures were conducted in accordance with the international guidelines for animal care approved by the IIBCE. The vaginal cytology of intact adult animals (Long and Evans, 1922) was checked periodically and for three consecutive weeks before sacrifice.

2.2. In oculo transplantation

2.2.1. Donors

Donor rats were treated with four doses of 10 μg oestrogen ($n=2$) or vehicle ($n=2$) on days 10, 15, 20 and 25 of postnatal development and killed at 28 days of age. The uterus was removed under aseptic conditions and placed in sterile ice-cold Hank's balanced salt solution (Sigma) for dissection. The uterine horns were opened longitudinally and pinned on Sylgard (Dow Corning, UK) using 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 0.5 mm wide and 1.0 mm long.

2.3. Hosts

Ten prepubertal rats were ovariectomised bilaterally at 4 weeks of age under ether anaesthesia and used as host recipients between 1.5 and 2 months later (body weight: 230–250 g). Hosts were anaesthetised with 40 mg kg^{-1} of sodium pentobarbital administered intraperitoneally, followed by local administration of 0.5% proparacaine hydrochloride solution (Anestalcon, Alcon, Argentina). Mydriasis was achieved by application of a drop of 10 mg ml^{-1} atropine sulphate to the cornea (Olson and Malmfors, 1970). Control and oestrogen-pretreated myometrial transplants were inserted into the left and right eye, respectively, through a small slit in the pupillary region of the cornea made with a microsurgical blade (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, 2000). The pupil was not obstructed by the transplants and visually guided behaviour of the hosts was not impaired. Hosts were killed by cervical dislocation at 5 weeks following transplantation.

2.4. Histochemical demonstration of noradrenaline-labelled sympathetic nerves

The uterine horns were quickly removed, placed in cold Hank's balanced salt solution, cleaned from fat

connective tissue and weighed on a precision balance. Noradrenaline-labelled nerves were demonstrated in the cephalic, medial and caudal portions of uterine horn and in intraocular transplants by the glyoxylic acid method performed on cryostat tissue sections as previously described (de la Torre and Surgeon, 1976; Brauer et al., 1992, 1995). Considering that ovariectomy could damage the sympathetic innervation of the cephalic and middle parts of the uterine horn which travels in the superior ovarian nerve and ovarian nerve plexus (Houdeau et al., 1998), studies on the innervation in ovariectomised animals was restricted to the caudal region 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.

2.5. Nerve diameter and density measurements in intraocular transplants

Under the fluorescence microscope and using a 20× objective lens the image of three cryostat sections per transplant were captured digitally using a CoolSNAP-Pro Monochrome Digital Kit (Media Cybernetics, LP, USA) and the diameter of noradrenaline-labelled nerve bundles and fibres was measured using the Image-Pro Plus software (Media Cybernetics). An average of 50 measurements per transplant section was performed.

In order to obtain an estimation of the density of innervation a stereological grid with an area of 0.25 mm^2 and line intersects at 20-μm intervals was superimposed over these transplant images, and all the grid transects overlying nerve bundle and fibre profiles were counted. The resulting number was multiplied by 100 and divided by the total number of grid squares occupied by the image of the transplant (Chávez-Genaro et al., 2002). Since the different plane of sectioning occasionally affected discrimination between perivascular and myometrial-associated fibres, all nerves present in the transplants were included for the estimation of the intercept density. Results are given as percentage of area occupied by noradrenaline-labelled nerves and as the number of intercepting fibres and bundles per transplant. Due to the almost complete absence of myometrial-associated fibres in the uterine horn of developing and adult oestrogen-treated animals no quantitative estimations of nerve density were attempted.

2.6. Histology of the ovaries and intraocular myometrial transplants

For histological examination, the ovaries were fixed in Bouin's solution, embedded in paraffin, sectioned at 10 μm and stained with haematoxylin and eosin. Transplants with attached irises were fixed in 4% buffered paraformaldehyde for 2 h at 4 °C, dehydrated and embedded in Durcupan ACM (Fluka). Semi-thin sections of 0.5 μm were cut with a Sorvall MT2 ultramicrotome, stained with 0.1% toluidine blue and examined under the light microscope. Micrographs were taken with Ilford PANF 50 ASA film. For comparative purposes the histology of the uterine myometrium was examined at 3 and 6 months after cessation of oestrogen treatment.

2.7. Determination of oestrogen and progesterone plasma levels

Oestrogen and progesterone plasma levels were measured in oestrogen-treated rats at 3 days and 3 and 6 months after cessation of treatment. For comparative purposes hormone levels were assayed in a group of five adult females at oestrus. Animals were killed by decapitation and the blood collected into plastic Falcon tubes. Blood was centrifuged for 10 min at 5000 rpm and the recovered plasma stored at −20 °C until assay. Oestrogen and progesterone levels were determined, respectively, using the Immulite Estradiol and Immulite Progesterone kits (Diagnostic Products, USA). These kits have a lower detection limit of 15 pg ml^{-1} for oestrogen and 0.2 ng ml^{-1} for progesterone. Each sample was assayed in duplicate.

2.8. Statistical analysis

Results are expressed as the mean ± S.E.M. Data were compared using the Kruskal–Wallis nonparametric ANOVA test, followed by the Dunn's multiple comparison test or the two-sided Mann–Whitney nonparametric test for unpaired data. Values of $p \leq 0.05$ were considered statistically significant.

Table 1
Wet weight of the isolated uterine horn, plasma levels of oestrogen and progesterone in adult cyclic rats at oestrus (AO) and in infantile/prepubertal chronic oestrogen-treated rats killed at 3 days (3D), 3 months (3M) and 6 months (6M) after cessation of treatment, and rats treated with oestrogen, ovariectomised at 28 days and killed 3 months later (Ovx/3M)

	Uterine horn (mg)	Oestrogen (pg ml^{-1})	Progesterone (ng ml^{-1})
AO	213 ± 16 (5)	69 ± 5	8.2 ± 0.8
3D	86 ± 4 (6)[a]	864 ± 379[a]	1.4 ± 0.3[a]
3M	161 ± 11 (8)[a,b]	86 ± 20[b]	4.4 ± 0.7
6M	175 ± 11 (8)[b]	85 ± 14[b]	12.5 ± 1.8[b,c]
Ovx/3M	56 ± 6 (8)[a,c,d]	Not detectable	Not detectable

Results are expressed as the mean ± S.E.M. (*n*). Data were compared using the Kruskal–Wallis nonparametric ANOVA test, followed by the Dunn's multiple comparison test. Values of $p \leq 0.05$ were considered statistically significant.

[a] Significant difference with AO.
[b] Significant difference with 3D.
[c] Significant difference with 3M.
[d] Significant difference with 6M.

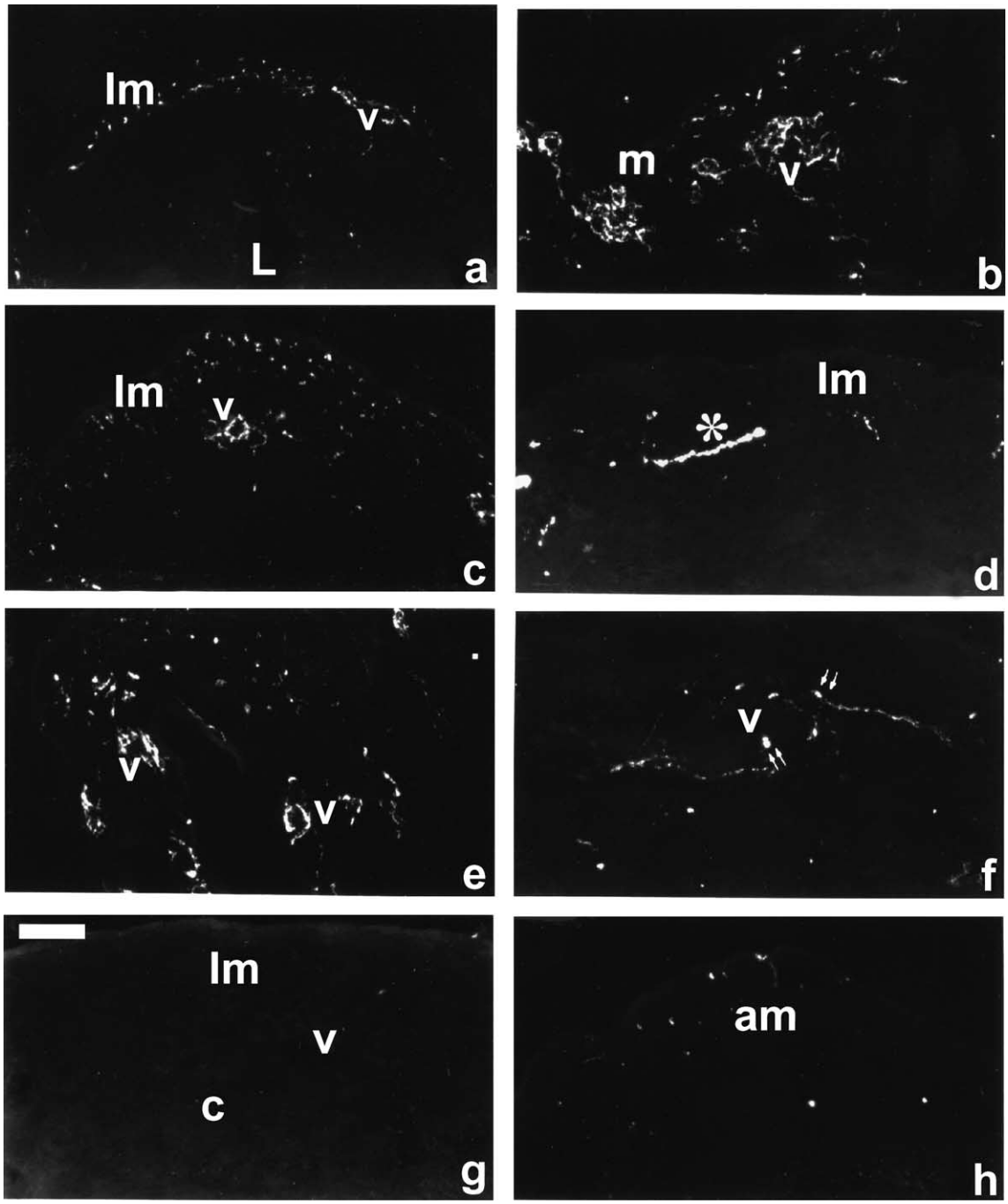

Fig. 1. Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn during postnatal development (a–c), and at different stages following infantile/prepubertal oestrogen treatment (d–h). In panel (a), the intrauterine innervation of a 12-day-old control is illustrated. Panels (b) and (c) show, respectively, the innervation of the mesometrium (m) and uterine horn in a 22-day-old control. Panel (d) shows the intrauterine innervation following administration of the first oestrogen dose at 12 days of age. A nerve bundle (*) showing intensely fluorescent enlargements is illustrated. Panels (e)–(h) show, respectively, the sympathetic innervation of different uterine regions 2 days after the third dose of oestrogen (22 days of age). Panel (e) illustrates the mesometrium (m). In panel (b), blood vessels (v) located close to the mesometrial entrance are shown. Note the presence of nerve fibres exhibiting intensely fluorescent enlargements (small double arrows). Panels (g) and (h) show, respectively, the appearance of intermesometrial and antimesometrial (am) aspects of the uterine horn. c—circular myometrial layer; lm—longitudinal myometrial layer; L—uterine lumen. Bar (in g) = 100 μm.

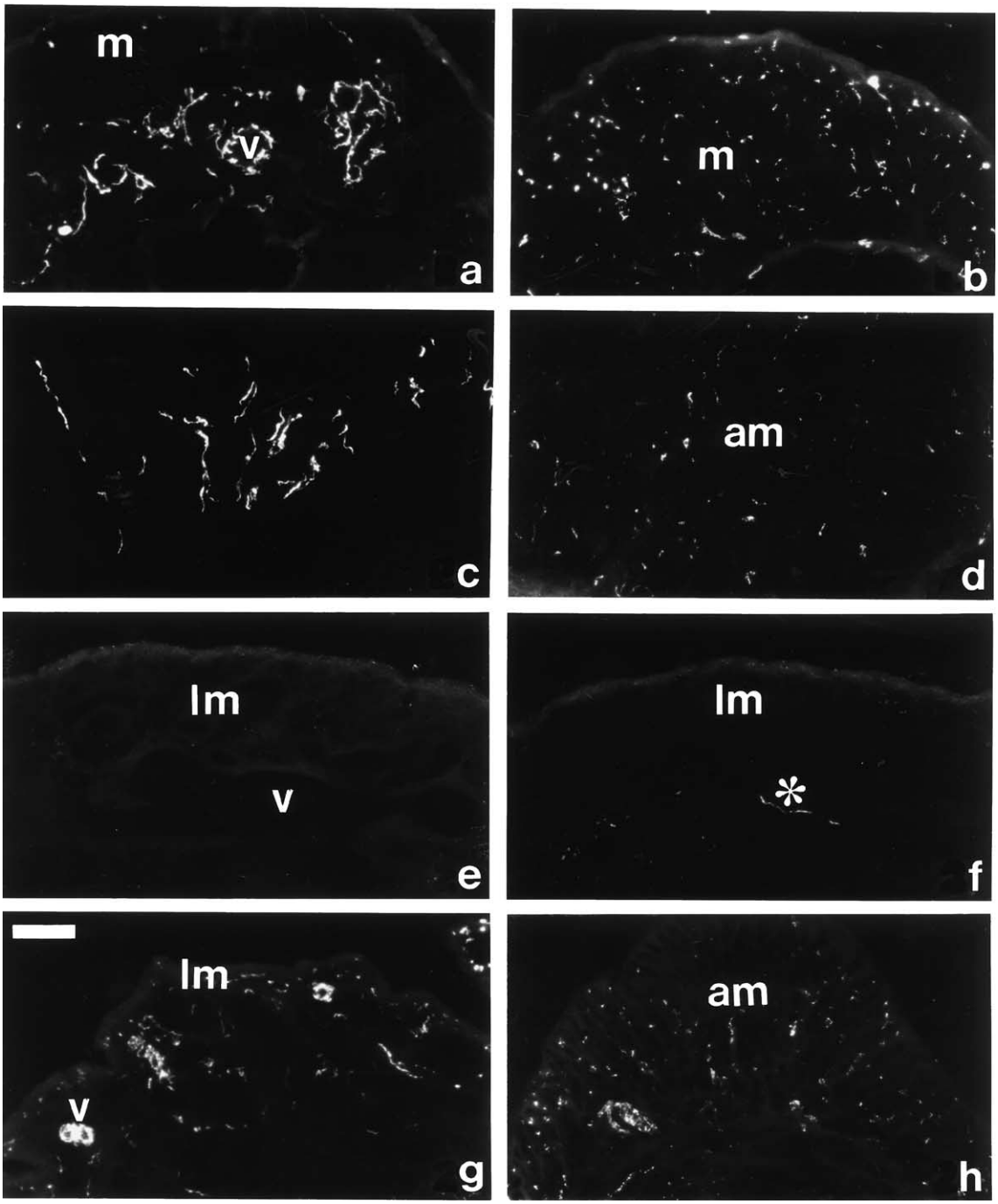

Fig. 2. Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the rat uterine horn. Panels (a), (c) and (e) illustrate, respectively, the innervation of intact rats at 3 months after cessation of oestrogen treatment. In (a), the mesometrial (m) and perivascular (v) innervation is shown. In (b), thick nerve bundles in the mesometrial entrance are seen. Panel (e) shows the absence of intrauterine sympathetic nerves. Panels (b), (d) and (f) illustrate, respectively, the innervation of intact rats at 6 months after cessation of oestrogen treatment. Panels (b) and (d) show, respectively, the innervation associated with the mesometrial (m) and antimesometrial (am) smooth muscle. In (f), an isolated intrauterine preterminal fibre (*) close to the mesometrial entrance is seen. Panels (g) and (h) illustrate, respectively, the innervation of the intermesometrial and antimesometrial (am) aspects of the uterine horn of rats treated with oestrogen, ovariectomised at 28 days and killed 3 months later. lm—longitudinal myometrial layer. Bar (in g) = 100 μm.

3. Results

3.1. Effects of infantile/prepubertal chronic oestrogen treatment on the reproductive state of the animals

Intact adult oestrogen-treated animals, killed 3 and 6 months after cessation of treatment, were acyclic and showed a persistent presence of cornified cells in the vaginal smear. None of these animals showed ova shed at the time of sacrifice. The weight of the uterus was increased in all intact oestrogen-treated animals and showed a marked reduction following ovariectomy (Table 1). Histological examination of the ovaries (not illustrated) showed the presence of numerous growing follicles but no signs of ovulation in animals killed immediately after cessation of oestrogen treatment. Three months later, several growing follicles with an engrossed theca were seen in the ovaries and the interstitial gland was markedly enlarged. No corpora lutea were seen in these animals. Six months after cessation of treatment, several growing follicles showing signs of luteinization were recognised. Hormone assays (Table 1) showed that in animals killed immediately after cessation of treatment, oestrogen levels were 12 times higher than in adult controls at oestrus, whereas progesterone levels were significantly lower. Three and six months after cessation of treatment, oestrogen levels reached similar values to those seen in adult animals at oestrus. A progressive increase in progesterone levels was observed between the third and sixth months.

3.2. Developmental effects of infantile/prepubertal chronic oestrogen treatment on the sympathetic innervation of the uterus

In 12-day-old controls, noradrenaline-labelled sympathetic fibres penetrated into the uterus through the mesometrium, accompanying blood vessels or as delicate free bundles and isolated fibres. Within the uterus, noradrenaline-labelled nerves were distributed around blood vessels located in the intramyometrial vascular space and associated with both the circular and longitudinal myometrial layers (Fig. 1a). Nerve fibres were thin, varicose and exhibit a moderate fluorescence intensity. The pattern of distribution attained at 12 days of age remained unchanged at the following postnatal stages; however, a generalised increase in the innervation density as well as in the thickness and fluorescence intensity of mesometrial (Fig. 1b) and intrauterine (Fig. 1c) nerve fibres was observed.

Two days after the first dose of oestrogen (12 days of age), the pattern of innervation of the mesometrium remained unchanged (not shown). Within the uterine horn, most of the innervation was associated with blood vessels and thick bundles showing intensely fluorescent enlargements (Fig. 1d) were frequently observed in both myometrial and perivascular locations. Following the second and third doses of oestrogen (17 and 22 days of age), most of the noradrenaline-labelled sympathetic fibres were seen in the mesometrial blood vessels and smooth muscle (Fig. 1e). Within the uterus, the only fibres recognised were those associated with the blood vessels located near the mesometrial and antimesometrial border (Fig. 1f), whereas blood vessels located between these areas showed no innervation (Fig. 1g). Some perivascular fibres showed intensely fluo-

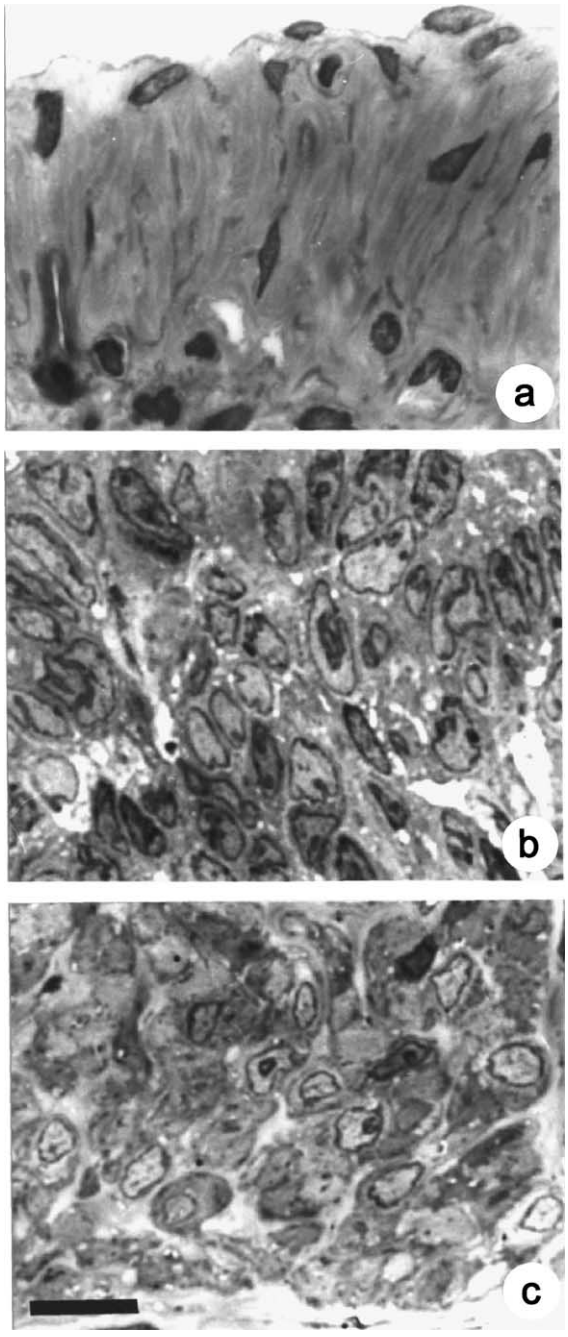

Fig. 3. Toluidine blue-stained semi-thin sections. Panel (a) shows a control myometrial transplant after 5 weeks in oculo. In panel (b), an oestrogen-pretreated myometrial transplant at 5 weeks following transplantation is illustrated. Panel (c) shows myometrial cells in the longitudinal muscle of an intact rat 3 months after cessation of infantile/prepubertal chronic oestrogen treatment. Bar (in c) = 25 μm.

rescent enlargements (Fig. 1f). Some remaining varicose noradrenaline-labelled nerve fibres were present at the antimesometrial border of the longitudinal myometrial layer (Fig. 1h). No nerve fibres were seen in other regions of the longitudinal and circular myometrial layer (Fig. 1g). After the last dose of oestrogen (28 days of age), sympathetic nerves were restricted to the mesometrium and blood vessels located close to the mesometrial entrance, but no nerve fibres were seen within the uterus (not shown).

3.3. Long-term effects of infantile/prepubertal chronic oestrogen on the sympathetic innervation of the uterus

Three months after cessation of treatment, most of the noradrenaline-labelled sympathetic fibres were seen in the mesometrium in association with blood vessels and mesometrial smooth muscle (Fig. 2a). These fibres were thicker and more intensely fluorescent than in control and oestrogen-treated developing animals (Fig. 1b,e, respectively). Within the uterus, several thick noradrenaline-labelled preterminal bundles were recognized at the mesometrial entrance (Fig. 2c). No perivascular or myometrial fibres were seen in any region of the uterus (Fig. 2e).

Six months after cessation of oestrogen treatment, a well-developed plexus of nerve fibres and bundles were seen in association with blood vessels and mesometrial smooth muscle (Fig. 2b). Both perivascular and parametrial fibres were intensely fluorescent and showed varicosities. Within the uterine horn, a modest regrowth of nerves was seen in the antimesometrial border of the longitudinal myometrial

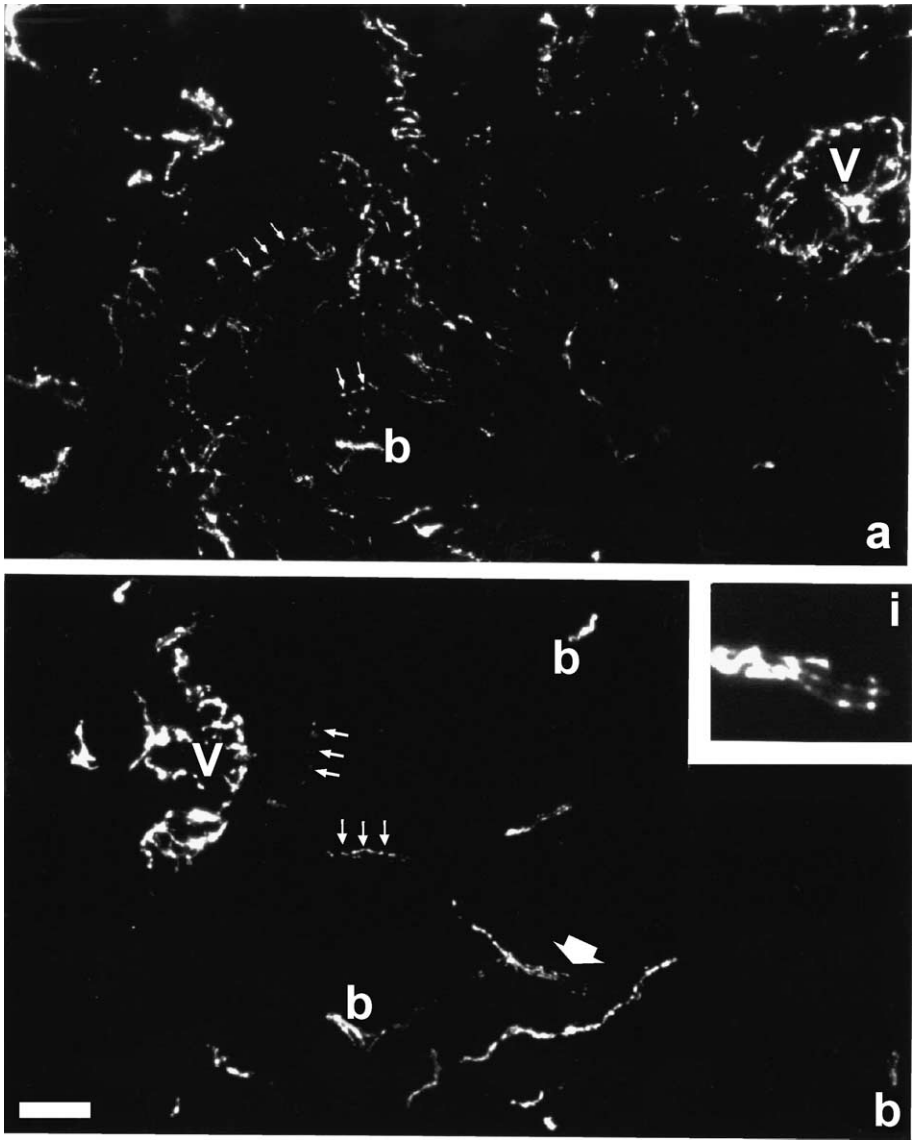

Fig. 4. Noradrenaline-labelled sympathetic fibres demonstrated by the glyoxylic acid technique in control (a) and oestrogen pretreated myometrial transplants after 5 weeks in oculo. Note the difference in the relative contribution of varicose fibres (small arrows) and nerve bundles (b) to the total innervation of both transplant types. The large arrow in (b) and inset (i) show delicate fibres arising from a nerve bundle. V—blood vessels. For panels (a) and (b), bar = 25 μm. For the inset, bar = 100 μm.

layer (Fig. 2d). These fibres were thinner and less intensely fluorescent than those observed in the parametrial tissue, and some of them exhibited varicosities. No nerve fibres were seen in other regions of the longitudinal and the circular myometrial layer (Fig. 2f). The pattern of innervation of intrauterine blood vessels was heterogeneous. Blood vessels located near the mesometrium and the antimesometrial border were sparsely innervated (Fig. 2f) whereas most of the blood vessels located between these areas showed no innervation. These perivascular fibres did not shown the characteristic network pattern observed in mesometrial vessels but were mainly thick, straight and non-varicose. No nerve fibres were seen in the endometrium or in association with radial arteries. No regional variations were detected between the cephalic, middle and caudal aspects of the uterus in intact oestrogen-treated animals at 3 and 6 months following cessation of oestrogen treatment.

In rats ovariectomised immediately after cessation of chronic oestrogen treatment, a well-developed plexus of noradrenaline-labelled sympathetic nerves was observed around mesometrial and intrauterine blood vessels as well as in relation with both myometrial layers (Fig. 2g,h). Some fibres were seen in the endometrium in association with the radial arteries.

3.4. Effects of infantile/prepubertal chronic oestrogen pretreatment on the sympathetic reinnervation of intraocular myometrial transplants

After 5 weeks in oculo, both control and oestrogenized myometrial transplants were attached to the host iris and well revascularised. Histological examination showed that smooth muscle cells in both control (Fig. 3a) and oestrogenized transplants (Fig. 3b) had a normal appearance, although those pretreated with oestrogen were hypertrophic. These cells were not dissimilar to those observed in the uterine horn of intact oestrogen-treated rats 3 months after cessation of oestrogen treatment (Fig. 3c).

Table 2
Effects of chronic oestrogen pretreatment on the innervation density of noradrenaline-labelled sympathetic nerves in myometrial transplants after 5 weeks in oculo

	Control	Oestrogen (pretreated)
Area (%) occupied by noradrenaline-labelled nerves	35.9 ± 1.8	20.8 ± 2.4 *
Total number of intercepting nerves (bundles and fibres) per transplant cross-section	161 ± 10	81 ± 15 *
Total number of intercepting bundles per transplant cross-section	23 ± 3	67 ± 10 *
Total number of intercepting fibres per transplant cross-section	138 ± 9	14 ± 5 *

Results are expressed as the mean ± S.E.M. ($n = 6$). Data were compared by the two-sided Mann–Whitney nonparametric test.
* $p \leq 0.001$.

Table 3
Effects of chronic oestrogen pretreatment on the diameter (μm) of noradrenaline-labelled nerve bundles and fibres in intraocular myometrial transplants 5 weeks after transplantation

	Control	Oestrogen (pretreated)
Nerve bundles	2.95 ± 0.12	4.60 ± 0.13 *
Nerve fibres	0.85 ± 0.02	0.83 ± 0.03

Results are expressed as the mean ± S.E.M. ($n = 6$). Data were compared by the two-sided Mann–Whitney nonparametric test.
* $p \leq 0.001$.

Histochemistry showed that after 5 weeks in oculo, myometrial transplant from prepubertal controls were well reinnervated by noradrenaline-labelled sympathetic nerves which appeared associated with blood vessels and myometrial smooth muscle (Fig. 4a). After the same period, oestrogenized myometrial transplants (Fig. 4b) showed a substantially lower innervation density, measured as both the percentage area occupied by noradrenaline-labelled nerves or as the total number of intercepting nerves per transplant section (Table 2). In addition to changes in the innervation density, it was observed that in control transplants, most of the reinnervating sympathetic nerves were terminal varicose myometrial and perivascular fibres (86% of the total number of intercepting nerves, Table 2), whereas only a small amount (14%) of the intercepting nerves were nerve bundles. In contrast, in oestrogenized myometrial transplants, most of the noradrenaline-labelled nerves were preterminal free running and paravascular bundles (83%), and only occasional terminal fibres were observed (17%). On occasions, delicate fibres were seen emerging from isolated bundles (Fig. 3b, inset). Finally, the diameter of nerve bundles was increased by 56% in oestrogen pretreated transplants but no changes in the diameter of terminal fibres were observed (Table 3).

4. Discussion

The results reported in this paper confirm and extend our previous findings (Brauer et al., 1995) by showing that early exposure to oestrogen did not inhibit the approach of sympathetic fibres to the uterus; however, it prevented the normal growth and maturation of intrauterine sympathetic fibres (Brauer et al., 1992). During the time-course of oestrogen treatment, particularly following the administration of the first dose, several thick bundles showing intensely fluorescent enlargements were seen within the uterus. These structures could be interpreted as signs of retraction or degeneration of the immature intrauterine nerve fibres that reached the organ before initiation of treatment; however, electron microscope studies will be required to distinguish between these possibilities (Sporrong et al., 1981; Yamada, 1988; Alm et al., 1988; Zoubina and Smith, 2000). These effects of oestrogen appear, however, to be a local phenomenon because both the vascular and non-vascular innervation in the mesometrium were unaffected

by treatment. Similar differential local effects have been demonstrated in the extrinsic innervation of the guinea pig uterus at term pregnancy (Thorbert, 1978; Alm et al., 1988).

Although the contribution of progesterone to the pregnancy-induced degeneration of uterine sympathetic nerves still remains uncertain (Bell and Malcolm, 1978, 1988), our current sex hormone assays showed that progesterone levels were very low immediately after cessation of the infantile/prepubertal chronic oestrogen treatment, thus arguing against a substantial contribution of this hormone to degeneration of sympathetic nerves in the nonpregnant female (Zoubina et al., 2001). Three and six months after cessation of oestrogen treatment, circulating levels of oestrogen and progesterone reached similar values to those observed at oestrus in adult cyclic animals. Although oestrogen-treated animals were acyclic and showed no ova shed at the time of sacrifice, this oestrus-like hormonal profile could be explained by the presence of growing and luteinized follicles within the ovaries. Our results are consistent with those showing that administration of a single dose of oestradiol valerate to cycling rats resulted in loss of oestrous cyclicity, anovulation and an almost continuous presence of vaginal cornified cells (Yen, 1991; Dissen et al., 2000), a condition that resembles the human polycystic ovarian syndrome (Schulster et al., 1984).

Three and six months following cessation of oestrogen treatment, a well-developed sympathetic innervation was seen in the mesometrium and mesometrial entrance; however, no nerve fibres were seen within the uterus 3 months after cessation of oestrogen treatment. Even after 6 months, only a very modest and not organotypic regrowth of the intrauterine innervation was observed around some blood vessels and in association with the antimesometrial border of the longitudinal myometrial layer. Two possible and not necessary exclusive explanations could account for these observations. One is that oestrogen could affect intrauterine sympathetic regrowth by acting directly on sympathetic neurons (Krizsan-Agbas and Smith, 2000; Zoubina and Smith, in press). The other explanation is that oestrogen could impair sympathetic reinnervation indirectly, by changing the receptivity of the target uterine tissue to nerves (Brauer et al., 1998, 2000; Krizsan-Agbas and Smith, 2000; Chávez-Genaro et al., 2002). Both possibilities are supported by our current observation showing that 3 months after ovariectomy, sympathetic nerves organotypically reinnervate the uterus. This observation is consistent with the finding that ovariectomy increases the density of sympathetic nerves and noradrenaline levels in the rat uterus (Chávez-Genaro et al., 2002) and that oestrogen receptor alpha knock out mice exhibit uterine sympathetic hyperinnervation (Zoubina and Smith, 2001).

Our current transplantation experiments suggest that oestrogen affects the receptivity of the myometrium to sympathetic nerves. As previously shown (Brauer et al., 2000; Chávez-Genaro et al., 2002), myometrial transplants from prepubertal donors were organotypically reinnervated by sympathetic nerves after 5 weeks in oculo, and accordingly, a well-developed plexus of varicose perivascular and myometrial fibres was seen in these transplants. In contrast, reinnervation was partially inhibited in oestrogen pretreated transplants and even more relevant, enlarged thick nerve bundles composed most of the reinnervation. These results would indicate that the oestrogenized myometrial transplants enforce fasciculation of normally growing nerves but prevent axons from branching outside nerve bundles. These effects cannot be explained by a direct action of oestrogen on the reinnervating neurons from the superior cervical ganglia, because host recipients were ovariectomised prepubertally and they received no oestrogen supplementation during the transplantation period. It is therefore possible to postulate that differences in the pattern of reinnervation of control and oestrogenized myometrial transplants could be explained by differences in the target uterine tissue. This possibility is supported by the observation that even after 5 weeks in oculo, myometrial cells remained hypertrophic and were not dissimilar to those seen in the uterine horn of intact rats 3 months after cessation of oestrogen treatment. Taken together, our findings suggest that in addition to potential degenerative effects (Zoubina et al., 1998; Zoubina and Smith, 2000) oestrogen has growth-inhibitory effects on uterine sympathetic nerves and that the oestrogenized myometrium is unattractive for sympathetic nerves and inhibits the development of an organotypic terminal plexus.

Factors preventing the development of an organotypic terminal plexus into both the oestrogenized uterus and myometrial intraocular transplants still remain unclear. It is known that nerve growth factor (NGF) is a key signal mediating terminal sprouting (Levi-Montalcini, 1987; Terenghi, 1999; Gallo and Letourneau, 1998; Patel et al., 2000); however, previous studies by our group have shown that following oestrogen treatment, the total content of NGF increases in parallel with the weight of the uterus thus sustaining the concentration per unit of wet weight tissue (Chávez-Genaro et al., 2002). In this context, it is possible to speculate that the growth-promoting effects of NGF are overridden by other, possibly inhibitory, signals produced by the oestrogen-primed myometrium. For instance, it is known that in addition to soluble neurotrophic factors, neurite outgrowth and Schwann cell migration are affected by signals provided by the extracellular matrix (Anton et al., 1994; Tessier-Lavigne and Goodman, 1996; Cowen et al., 1997; Cowen and Gavazzi, 1998) and that repulsive factors also preclude neurite growth into selected targets (Giger et al., 1998; Reza et al., 1999). Further studies are needed to distinguish between these possibilities.

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