Effects of chronic oestrogen treatment are not selective for uterine noradrenaline-containing sympathetic nerves: a transplantation study

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ABSTRACT

Previous studies have shown that chronic administration of oestrogen during postnatal rat development dramatically reduces the total content of noradrenaline in the uterine horn, abolishes myometrial noradrenergic innervation and reduces noradrenaline-fluorescence intensity of intrauterine perivascular nerve fibres. In the present study we analysed if this response is due to a direct and selective effect of oestrogen on the uterine noradrenaline-containing sympathetic nerves, using the in oculo transplantation method. Small pieces of myometrium from prepubertal rats were transplanted into the anterior eye chamber of adult ovariectomised host rats. The effect of systemic chronic oestrogen treatment on the reinnervation of the transplants by noradrenaline-containing sympathetic fibres from the superior cervical ganglion was analysed on cryostat tissue sections processed by the glyoxylic acid technique. In addition, the innervation of the host iris was assessed histochemically and biochemically. The histology of the transplants and irises was examined in toluidine blue-stained semithin sections. These studies showed that after 5 wk in oculo, the overall size of the oestrogen-treated transplants was substantially larger than controls, and histology showed that this change was related to an increase in the size and number of smooth muscle cells within the transplant. Chronic oestrogen treatment did not provoke trophic changes in the irideal muscle. Histochemistry showed that control transplants had a rich noradrenergic innervation, associated with both myometrium and blood vessels. Conversely, in oestrogen-treated transplants only occasional fibres were recognised, showing a reduced NA fluorescence intensity. No changes in the pattern and density of innervation or in the total content of noradrenaline of the host irises were detected after chronic exposure to oestrogen. We interpreted these results to indicate that the effects of oestrogen on uterine noradrenaline-containing sympathetic nerves are neither selective or direct, but result from an interaction between sympathetic nerve fibres with the oestradiol-primed uterine tissue. A potential effect of oestrogen on the neurotrophic capacity of the uterus is discussed.

Key words: Autonomic nervous system; uterus; myometrium.

INTRODUCTION

The mammalian uterus is innervated by post-ganglionic noradrenaline-containing (NA-C) sympathetic nerves which innervate blood vessels and myometrial smooth muscle (Marshall, 1970; Owman & Stjernquist, 1988; Papka & Traurig, 1992). The sympathetic nerve supply to the uterus is described as composed of 'long' postganglionic fibres, whose cell bodies are in the inferior mesenteric and paravertebral sympathetic chain ganglia, and 'short' postganglionic fibres originating in the paracervical ganglia, located in the mesentery of the uterovaginal junction (Marshall, 1970; Kanerva et al. 1972; Owman & Stjernquist, 1988).

NA-C nerves to the uterus are susceptible to

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changes in the endocrine environment and demonstrate considerable plasticity under physiological conditions such as puberty (Brauer et al. 1992), oestrous cycle (Thorbert et al. 1978; Van Orden et al. 1980; Marshall, 1981; Owman & Stjernquist, 1988; Zoubina et al. 1998) and pregnancy (Thorbert, 1978; Marshall, 1981; Arkinstall & Jones, 1985; Bell & Malcolm, 1988; Owman & Stjernquist, 1988; Haase et al. 1997), as well as following manipulations such as ovariectomy and/or exogenous sex hormone administration (McKercher et al. 1973; Falck et al. 1974; Juorio et al. 1989).

Previous biochemical and histochemical studies (Brauer et al. 1995) showed that in the rat, hormone-induced plasticity in uterine sympathetic nerves is not restricted to the mature stage. Chronic administration of oestrogen during postnatal development (10–25 d after birth), led to a 70% reduction in total content of NA in the uterine horn, abolishes myometrial NA-C sympathetic nerves and reduces NA-fluorescence intensity of intrauterine perivascular nerve fibres.

Classically, hormone-induced changes in the sympathetic innervation of the uterus have been considered as a direct and selective effect of sex hormones on the system of *short* sympathetic fibres (Thorbert, 1978; Marshall, 1981; Owman, 1981; Owman & Stjernquist, 1988). The presence in the rat uterus of a substantial contribution of short sympathetic fibres remains controversial. Some authors have reported that one-third to a half of the neurons in the rat paracervical ganglia are noradrenergic (Kanerva et al. 1972; Inyama et al. 1985), whereas only a few or no noradrenergic neurons have been recognised by others (Garfield, 1986; Papka et al. 1987; Hondeau et al. 1995, 1998).

Oestrogen receptors were demonstrated in an unidentified subpopulation of neurons in the rat paracervical ganglion (Papka et al. 1997) but, to our knowledge, there is no direct evidence for the presence of sex hormone receptors on the uterine-projecting sympathetic neurons. The possibility that uterine sympathetic transmission may be regulated by sex hormones at the central level appears to be supported by the observation of oestrogen receptors in the intermediolateral column of the rat spinal cord (Williams & Papka, 1996; Williams et al. 1997).

An alternative explanation is that sex hormones affect the neurotrophic capacity of the uterus, i.e. the primary target of sex hormone activity is the uterine tissue rather than short sympathetic neurons. Recent studies by our group, using in oculo transplantation, have provided novel evidence in support of this concept by showing that in the guinea pig, pregnancy induces alterations in the uterine tissue that indirectly affect the innervating neurons (Brauer et al. 1998*a*).

In order to extend this concept, in the present study we have transplanted small pieces of myometrium from prepubertal rats into the anterior eye chamber of adult ovariectomised host rats and assessed the effects of systemic chronic oestrogen treatment on the reinnervation of the transplants by the long adrenergic fibres from the superior cervical ganglion. For comparative purposes the innervation of the host iris was analysed. Part of these results have been published in abstract form (Brauer et al. 1998*b*).

MATERIALS AND METHODS

Animals

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

In oculo transplantation

Donors. Six 4-wk-old prepubertal rats (body weight 50–60 g) were anaesthetised with ether and killed by cervical dislocation. The uterus was removed under aseptic conditions and placed in sterile ice-cold Hanks' balanced salt solution (Irvine Scientific, USA) 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 1 mm width and 1.5 mm long.

Hosts. Twelve prepubertal rats (4-wk-old) were ovariectomised bilaterally under ether anaesthesia and used as host recipients between 1.5 and 2 mo later (body weight 230–250 g). Bilateral transplants were made into the anterior eye chamber. Host animals were anaesthetised with 40 mg/kg of sodium pentobarbital administered intraperitoneally, 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 & Malmfors, 1970). Transplants were inserted, 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 iridocorneal angle of the eye (Olson &

Malmfors, 1970; Brauer et al. 1998*a*). In most of the animals the pupil was not obstructed by the transplants and visually guided behaviour of the hosts was not impaired. Transplants were left in oculo for 5 wk after which host animals were terminally anaesthetised with ether and transplants were removed attached to the host irises.

Oestrogen treatment

Host rats were injected with 5 doses of 50 μg oestradiol on d 10, 15, 20, 25 and 30 following transplantation and killed 3 d after the last injection. Oestrogen treatment was given subcutaneously as β-oestradiol 17-cypionate (Laboratorios Köning, Argentina), diluted to appropriate doses with peanut oil (Sigma, USA). Control animals were injected with the vehicle alone (Brauer et al. 1995). An additional group of rats was injected with oestrogen (n = 4) or vehicle (n = 3) and the whole irises removed and stored at −70 °C for biochemical assays.

Histochemical demonstration and quantitation of NA-C nerves

NA-C nerves were demonstrated in transplants by the glyoxylic acid method performed on cryostat sections (de la Torre & Surgeon, 1976). After removal of the transplants, the remaining irises were processed as whole mounts for the histochemical demonstration of NA-C nerve fibres (Furness & Costa, 1975). Preparations were examined under a Nikon Microphot FX microscope equipped with epifluorescence and fitted with the appropriate filters. Micrographs were taken with Ilford XP2, 400 ASA film.

The density of irideal NA-C nerve fibres was estimated using a ×40 objective lens and an ocular scale set at different directions through the whole mount preparations (Brauer et al. 1992). The number of nerves (bundles and isolated fibres) that intersected the ocular scale was counted in 10 areas per iris. The density of innervation was expressed as the mean number of nerves per millimetre of tissue (nerves/mm).

The density of NA-C nerve fibres in transplants (bundles and isolated fibres) was estimated using a ×10 objective lens and an ocular scale set at 6 different positions on main diameter transplant sections. Discrimination between vascular and non-vascular nerve fibres was not attempted. The density of NA-C nerves was expressed as the mean total number of fibres per transplant section.

Biochemical assay for NA

The total content of NA in the isolated whole iris was measured by high performance chromatography liquid with electrochemical detection (HPLC-ED), as previously reported (Brauer et al. 1995). Briefly, tissues were suspended in 0.1 M $HClO_4$ (50 μl/mg of tissue), homogenised by sonication, and centrifuged at 10000 *g* for 15 min at 4 °C. Fifty microlitres of the supernatant were injected in the HPLC-ED system. Results are expressed as total content of NA per iris. In view of the small size of the transplants and the impossibility of removing completely small pieces of attached iris, biochemical studies on these tissues were not attempted.

Statistical analysis

Results of morphometric and biochemical studies are given as the mean ± S.E.M. Mean values were compared by the 2-tailed Student's *t* test. A level of probability of $P \leqslant 0.05$ was taken to indicate statistical significance.

Histology

Transplants with attached irises were fixed in 4% buffered paraformaldehyde for 2 h at 4 °C, dehydrated and embedded in Durcupan ACM (Fluka). Semithin sections (0.50 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.

RESULTS

Histology

At the time of removal transplants were attached to the host irises by 2–4 connective tissue bridges and fully revascularised. The overall size of control transplants was reduced after the transplantation period, whereas it was substantially increased in those treated with oestrogen. Histology showed that smooth muscle cells in control transplants (Fig. 1*A*, *B*) were immature, relatively small and showed poor development of the basal lamina. Muscle bundles were difficult to identify, due to the poor development of the surrounding extracellular matrix (Fig. 1*B*). Following chronic oestrogen treatment (Fig. 1*C*, *D*), smooth muscle cells were larger than in controls and showed a fully mature appearance. Individual muscle

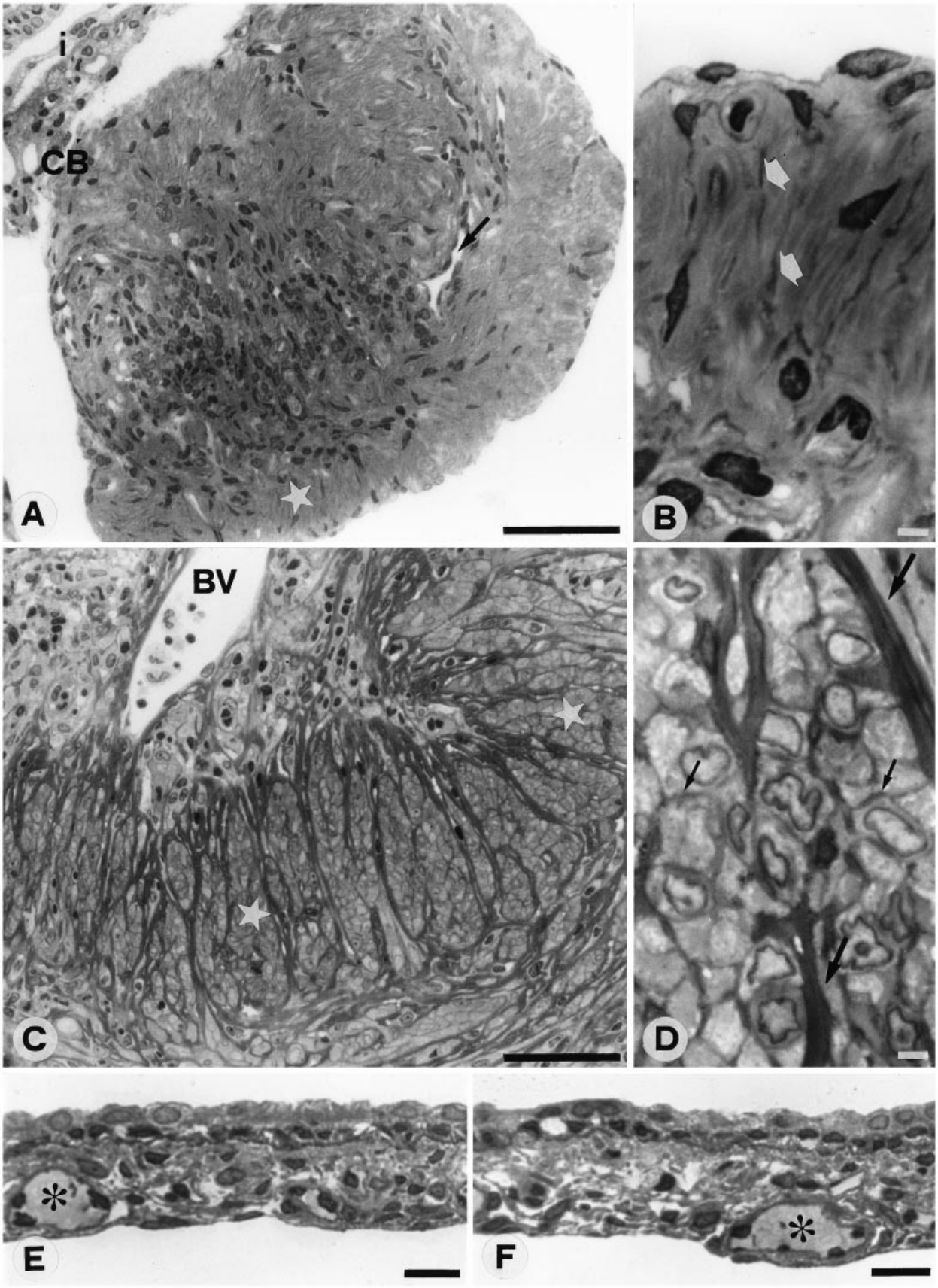

Fig. 1. Toluidine blue-stained semithin sections of rat myometrial transplants after 5 wk in oculo and host irises in control (*A*, *B*, *E*) and following chronic oestrogen treatment (*C*, *D*, *F*). (*A*) Control transplant. i, host iris; CB, connecting bridge; ★, myometrial smooth muscle; arrow, blood vessel. (*B*) Higher magnification showing the immature appearance smooth muscle cells in control myometrial transplants.

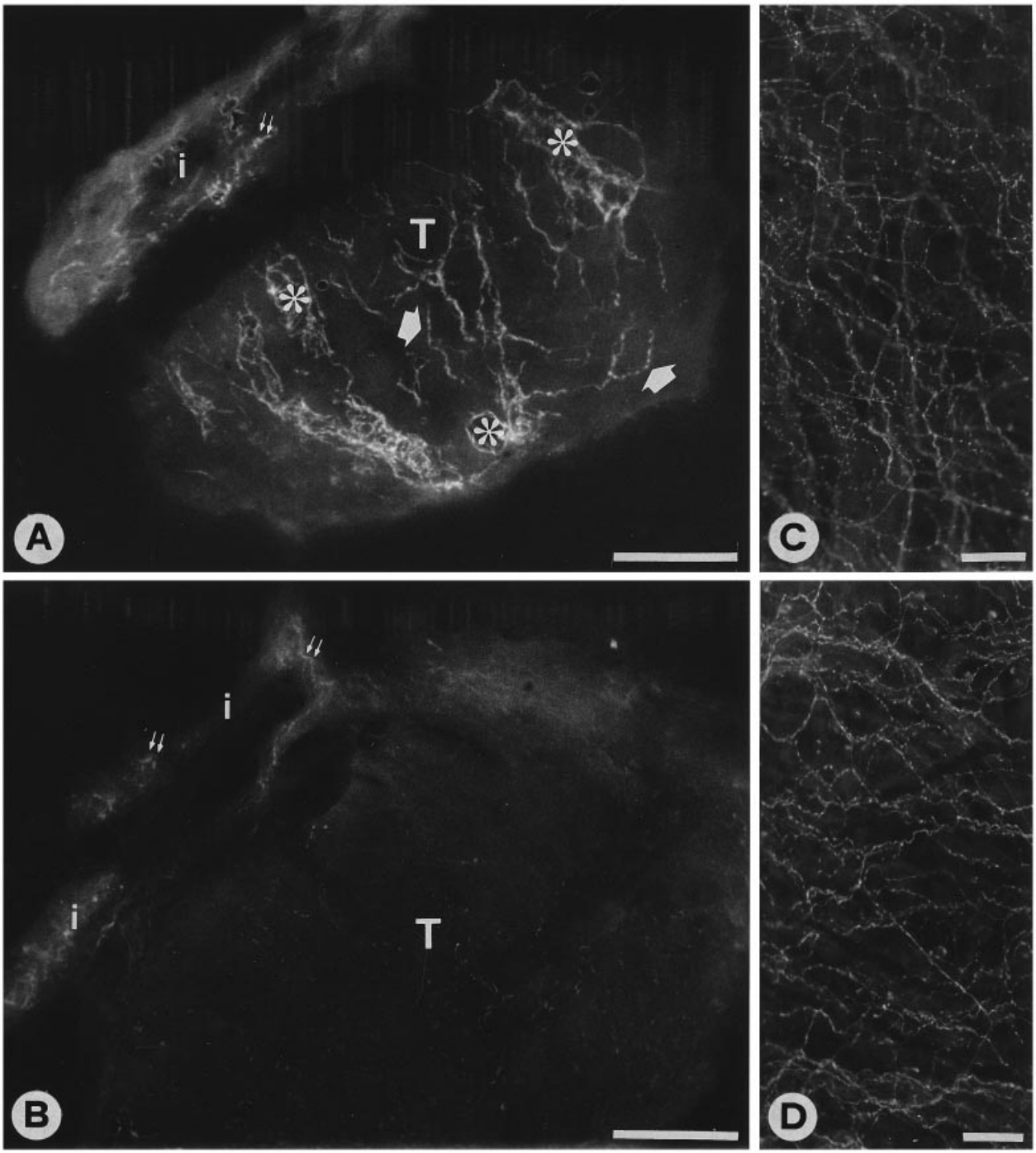

Fig. 2. (*A*, *B*) Noradrenaline-containing sympathetic fibres demonstrated by the glyoxylic acid technique on cryostat sections of control (*A*) and oestrogen-treated (*B*) myometrial transplants (T) after 5 wk in oculo. Several preterminal and terminal noradrenaline-containing nerve fibres are seen within the control transplant. ✲, perivascular fibres; thick arrows, myometrial associated fibres. Note the absence of nerve fibres in the myometrial transplant after chronic oestrogen treatment. i, host iris; double small arrows, irideal noradrenaline-containing sympathetic fibres. (*C*, *D*) Noradrenaline-containing nerve fibres of control (*C*) and oestrogen-treated (*D*) host irises demonstrated by the glyoxylic acid technique on whole mount stretch preparations. Bar, 100 μm.

cells were surrounded by a well developed basal lamina and a thick layer of extracellular matrix enclosed smooth muscle bundles (Fig. 1*D*). No changes in the histological structure of the iris were observed after chronic oestrogen treatment (Fig. 1*E*, *F*).

Arrows, extracellular matrix. (*C*) Partial view of a myometrial transplant after chronic oestrogen treatment. BV, blood vessel in the connecting bridge; ★, myometrial smooth muscle. (*D*) Higher magnification showing the mature appearance myometrial smooth muscle cells in oestrogen-treated transplants. Small arrows, smooth muscle basal lamina; large arrows, extracellular matrix surrounding smooth muscle bundles. (*E*, *F*) Host iris in control rats (*E*) and following chronic oestrogen treatment (*F*). ✲, irideal blood vessel. Bar, 50 μm.

Table. *Quantitative assessment of innervation density of noradrenaline-containing nerve fibres in myometrial transplants and host iris and biochemical assessment of NA in host iris in control rats and following chronic oestrogen treatment*

	Control	Oestrogen-treated
Myometrial transplants		
Intercept density	12.0 ± 0.9 (8)	0.48 ± 0.14 (10)*
Iris		
Intercept density	12.94 ± 0.35 (8)	12.85 ± 0.27 (10) ns
NA total content/iris (ng)	1.98 ± 0.10 (3)	2.04 ± 0.28 (4) ns

Nerve density is expressed as intercept density (nerve fibres/mm). Morphometric and biochemical results are given as the mean ± S.E.M. (n). Mean values were compared by the 2 tailed Student's *t* test. Values of $P \leqslant 0.05$ were considered statistically significant (*); ns, nonsignificant.

Histochemistry

Five weeks after transplantation, control transplants were reinnervated by NA-C sympathetic fibres which appeared associated with blood vessels and myometrial smooth muscle (Fig. 2*A*). Following chronic oestrogen treatment, only occasionally isolated fibres, showing a reduced NA-fluorescence intensity could be visualised (Fig. 2*B*). Accordingly, a significant reduction in the innervation density was observed (Table). Whole mount stretch preparations of the host iris showed no changes in the pattern and density of innervation after chronic exposure to oestrogen (Fig. 2*C*, *D*, Table).

Biochemistry

Biochemical studies (Table) showed that the total content of NA of the iris was unchanged by chronic oestrogen treatment.

DISCUSSION

For more than 3 decades sex-hormone and pregnancy-induced plasticity in uterine sympathetic nerves has been interpreted as a direct and selective effect of ovarian/placental hormones on the system of short sympathetic neurons innervating the female genital organs (Marshall, 1970, 1981; Thorbert, 1978; Owman, 1981; Owman & Stjernquist, 1988). More recently, the possibility that the uterus could regulate its state of innervation through hormonal-induced variations in the local production of neurotrophic factors has been suggested (Owman & Stjernquist, 1988; Corbacho et al. 1997; Haase et al. 1997; Zoubina et al. 1998). However, this hypothesis has only recently begun to be tested experimentally (Brauer et al. 1998*a*, *b*; Duchemin et al. 1998).

In a previous study, we analysed the possibility that pregnancy-induced degeneration of guinea pig uterine sympathetic nerves is related to changes in the neurotrophic capacity of the uterus, using the in oculo transplantation approach (Brauer et al. 1998*a*). This study showed that uterine myometrial transplants from virgin guinea pig donors became organotypically reinnervated by sympathetic fibres from the host superior cervical ganglion. Conversely, host sympathetic fibres failed to reinnervate myometrial transplants from early postpartum donors (2–7 d after delivery). These observations were interpreted as indicating that degeneration of uterine sympathetic nerves during pregnancy and their slow regrowth after parturition are initiated by alterations in the target rather than resulting from a particular vulnerability of short adrenergic neurons to pregnancy-induced hormonal changes.

Our current transplantation study in the rat supports this concept by showing a similar pattern of oestrogen-induced changes in the reinnervation of myometrial transplants by the long sympathetic fibres from superior cervical ganglia. Unexpectedly, chronic exposure to oestrogen affected equally both the myometrial and perivascular innervation of transplants. This result is not easy to explain since in previous studies we showed that chronic administration of oestrogen during postnatal development (10–25 d after birth) selectively affected myometrial innervation (Brauer et al. 1999) and only reduced NA-fluorescence intensity of intrauterine perivascular nerve fibres (Brauer et al. 1995) and had no effect on the innervation of the uterine artery (Corbacho et al. 1997). A possible explanation for this difference could be the degree of maturity of blood vessels and their associated NA-C sympathetic nerves at the time when oestrogen treatment was started. In our previous studies, chronic administration of oestrogen was commenced at 10 d after birth, when blood vessels and their associated sympathetic nerves were almost fully mature, and probably less susceptible to changes in the endocrine environment. In our current study, oestrogen treatment was initiated at 10 d after transplantation, when blood vessels and their

associated nerves were probably immature and still growing into the transplant.

Results reported in this paper suggest that the effects of oestrogen on uterine noradrenaline-containing sympathetic nerves are neither selective or direct, but result from an interaction between sympathetic fibres (either short or long) with the oestradiol-primed uterine tissue. The absence of histochemical and biochemical demonstrable changes in the innervation of the host iris argue against a generalised effect of the oestrogen on all sympathetic fibres arising from the superior cervical ganglia. It is not known, however, whether the marked reduction in the number of NA-C in oestrogen-treated transplants is due to the absence of sympathetic nerves or to reduced expression of NA in innervating nerves. In a recent study, using the panneural marker protein gene product 9.5 (PGP 9.5), we observed that chronic exposure to oestrogen led to a dramatic reduction in density of myometrial-associated PGP 9.5-immunoreactive nerve fibres, suggesting that oestrogen either inhibits development or causes degeneration of uterine sympathetic and nonsympathetic nerve fibres (Brauer et al. 1999). The first alternative would be supported by the observation that in the rat, development of myometrial-associated sympathetic nerves occurs during the first 4 wk of postnatal life (Brauer et al. 1992) thus overlapping the time-course of oestrogen treatment. The second possibility is supported by the observation of a hormone-induced remodelling in the sympathetic nerves of the rat uterus during the oestrous cycle, where the decline in the density of DβH-I nerve fibres observed at pro-oestrous and oestrus is accompanied by a reduction of PGP-I fibres at oestrus (Zoubina et al. 1998). These 2 explanations are not mutually exclusive and it is possible that oestrogen could either inhibit nerve development as well as cause atrophy of mature nerve fibres.

Histological results presented in this paper showed that following chronic exposure to oestrogen, myometrial transplants were substantially larger than controls and this growth was related to the increase in size and probably number of smooth muscle cells within the transplant. This trophic response, normally observed during puberty (Brauer et al. 1992) and following oestrogen treatment (Spratto & Miller, 1968; Brauer et al. 1995) is consistent with the presence of oestrogen receptors in the myometrium (Ennis & Stumpf, 1989). In contrast, no trophic changes were observed in the host irideal smooth muscle following chronic oestrogen treatment.

In this context, the present transplantation study supports the linked hypotheses that: (1) the primary target for oestrogen is the uterine tissue (smooth muscle and related nonneural tissues) rather than the system of uterine short adrenergic neurons and (2) oestrogen induces alterations in the neurotrophic capacity of the uterus.

Targets influence their innervating neurons via target-derived soluble neurotrophic factors, such as nerve growth factor (NGF) and related neurotrophins, as well as cytokines (Levi-Montalcini, 1987; Gavazzi & Cowen, 1996). During pre and postnatal development, soluble neurotrophic factors have been shown to influence growth, survival and transmitter expression of sympathetic neurons (Barde, 1989; Landis, 1990; Thoenen, 1995; Cowen et al. 1996; Gallo & Letourneau, 1998). In the mature animal, growth, neurotransmitter expression and probably other aspects of sympathetic neuronal plasticity remain strongly influenced by targets and associated neurotrophic factors (Ruit et al. 1990; Spitsbergen et al. 1995; Thrasivoulou & Cowen, 1995; Cowen & Gavazzi, 1998).

In early postnatal development neurotrophin levels correlate with the density of sympathetic innervation of the target tissue (Korsching & Thoenen, 1983; Alberts et al. 1994). As a consequence, hypoinnervation and neurodegeneration in adulthood have been predicted to coincide with neurotrophic deprivation (Cowen & Gavazzi, 1998). On these grounds, it may be hypothesised that oestrogen-induced changes in the sympathetic innervation of the uterus are related to a local reduction in the target production of soluble neurotrophic factors, such as NGF. This possibility is reinforced by the observation that in the rat, pregnancy-induced uterine denervation may be related to down-regulation of NGF expression (Duchemin et al. 1998).

In addition, taking into account the fact that chronic oestrogen treatment provokes a substantial development of the smooth muscle basal lamina and the extracellular matrix surrounding smooth muscle bundles, a possible contribution of extracellular matrix-bound neurotrophic factors should be considered (De Curtis et al. 1991). For instance, it has been shown that reduced laminin availability correlates with nerve fibre atrophy in the blood vessel wall of ageing rats (Gavazzi et al. 1995). Proteoglycans may also have positive or negative effects on nerve growth, by interacting with other ECM components and with growth factors (Braunewell et al. 1995; Landolt et al. 1995). It has been demonstrated that changes in hormonal balance may modify the expression of proteoglycans and other extracellular matrix components in the uterus (Cidadao et al. 1990; Rider

et al. 1992; Stewart et al. 1998). Work is in progress to distinguish between these different possibilities.

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