In oculo transplants of myometrium from postpartum guinea pigs fail to support sympathetic reinnervation

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ABSTRACT

Sympathetic nerves to the enlarged fetus-containing region of the uterus undergo degenerative changes during late pregnancy and show slow regrowth after parturition. It is not known whether this unusual response of sympathetic nerves to smooth muscle hypertrophy is due to the sensitivity of short adrenergic neurons to hormonal changes, or whether the nerves respond to changes in the neurotrophic capacity of the target. We have investigated this question using in oculo transplantation. Small pieces of myometrium from the uterine horn of virgin guinea pigs, or from the region previously occupied by the placenta and fetus in postpartum guinea pigs, were transplanted into the anterior eye chamber. After 3 wk in oculo, the pattern of reinnervation of the transplants was assessed on whole mount stretch preparations stained for tyrosine hydroxylase. The histology of the transplants was examined in toluidine blue-stained semithin sections. Myometrial transplants from virgin donors and uterine artery transplants from both virgin and postpartum donors became organotypically reinnervated by sympathetic fibres from the host iris. In contrast, sympathetic nerves did not reinnervate myometrial transplants from postpartum donors, although they approached the transplants and became distributed in the surrounding connective tissue. All transplanted tissues showed a normal histological appearance. Both the myometrium and uterine artery from postpartum donors retained a hypertrophic appearance after 3 wk in oculo. We interpret these results to indicate that the degeneration of sympathetic nerves in late pregnancy, as well as their slow regrowth to the uterus after delivery, may be due to changes in uterine smooth muscle rather than a particular sensitivity of short adrenergic neurons to hormonal changes.

Key words: Uterus; myometrium; sympathetic nervous system; autonomic nervous system; pregnancy.

INTRODUCTION

The guinea pig uterus is supplied by an extensive plexus of adrenergic nerves that innervate blood vessels and myometrial smooth muscle (Marshall, 1970; Thorbert et al. 1977; Thorbert, 1978; Owman & Stjernquist, 1988). The adrenergic nerve supply to the uterus is composed of the classic long postganglionic fibres, whose cell bodies are in the lumbar and mesenteric ganglia and short postganglionic fibres originating in the paracervical ganglia located in the parametrial tissue outside the uterovaginal junction (Owman & Sjöberg, 1966). The long adrenergic fibres are thought to innervate predominantly the blood vessels (including intrauterine vessels) whereas short fibres are primarily associated with the myometrial smooth muscle (Marshall, 1981; Arkinstall & Jones, 1985).

Short adrenergic fibres to the female genital organs show characteristics different from those seen in other sympathetically innervated organs. For instance, they are thought to be particularly sensitive to sex steroids (Falck et al. 1974; Thorbert et al. 1978; Thorbert, 1978; Owman & Stjernquist, 1988). Even more remarkable is the pregnancy-induced depletion of noradrenaline (NA) in uterine nerves and its relationship with the location of the fetus (Thorbert, 1978; Alm et al. 1979; Owman, 1981; Arkinstall &

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Jones, 1985; Bell & Malcolm, 1988; Owman & Stjernquist, 1988). The most dramatic pregnancy-induced changes are observed in the areas of the uterus surrounding the fetus where a complete degeneration of myometrial and intramural perivascular adrenergic nerves and their surrounding Schwann cells is observed at term (Sporrong et al. 1981; Lundberg et al. 1987). A similar degree of reduction in NA levels is observed in areas of the uterus distant from the fetus, such as the cervix and the empty horn in unilateral pregnancies, but probably as a result of different mechanisms. In the cervix, it has been suggested that the low level of transmitter is caused by neuronal hyperactivity whereas in the empty horn it may be related to an inactivation of the NA synthesis (Owman & Stjernquist, 1988).

In late pregnancy, the density of noradrenergic nerves associated with the guinea pig uterine artery is also markedly reduced and there is a significant decrease in NA levels (Mione et al. 1990). These changes, however, are not related to axon degeneration as in the case of the myometrium but with a reduction in the density of axons detectable by the noradrenaline-fluorescence histochemical methods. Indeed, electron microscopical studies have shown that nerve fibres increase in number during pregnancy, matching the hypertrophy of the artery (Mione & Gabella, 1991).

After delivery, restoration of the normal pattern of innervation of the myometrium also shows considerable regional variation, taking several months in the horn, suggesting that regrowth of axons might be involved (Gardmark et al. 1971). In contrast, the time course of recovery in the cervix and empty horn is very much shorter than in the pregnant horn and histochemical as well as electron microscopical studies have shown that in these areas adrenergic nerves remain structurally intact during pregnancy (Lundberg et al. 1987; Owman & Stjernquist, 1988).

Since NA depletion occurs in parts of the uterus distant from the areas of distension provoked by the growing fetuses it has been suggested that hormones produced by the fetoplacental unit are involved. Attempts to mimic the effects of pregnancy by systemic administration of sex hormones have been unsuccessful. However, it has been shown that implantation of slow-release progesterone pellets into the lumen of the uterine horn produces an almost complete loss of fluorescence in the surrounding uterine nerves (Bell & Malcolm, 1978; Malcolm & Bell, 1984). Since the depleting effects of progesterone involve equally the myomotor (short) and vasomotor (long) adrenergic nerve fibres, it has been suggested that hormones do not affect only short adrenergic neurons. This view is supported by the observation that in the guinea pig, pregnancy provokes a generalised disappearance of nerves in the fetus-containing uterine horn, as demonstrated with the panneuronal marker protein gene product 9.5 (PGP 9.5), which therefore also involves the peptidergic innervation (Alm & Lundberg, 1988; Owman & Stjernquist, 1988). In the guinea pig, cholinergic nerves are present in the uterine artery wall (Bell, 1968) but they do not seem to penetrate into the uterus (Thorbert et al. 1977; Hammarström & Sjöstrand, 1979) and therefore are presumably not involved.

Because nerve changes in the pregnant uterus seem not to be the result of hormones acting directly on short adrenergic neurons, it is hypothesised that the innervation is affected by hormone-induced alterations in the neurotrophic capacity of uterine smooth muscle cells (Owman & Stjernquist, 1988; Corbacho et al. 1997). Similar concepts have been advanced to explain the growth of autonomic nerves in hypertrophic visceral organs (Gabella, 1984; Gabella et al. 1992), as well as the atrophic changes that affect autonomic neurons during ageing (Gavazzi & Cowen, 1996). As a first approach to test this hypothesis, we have transplanted myometrium from virgin and postpartum guinea pigs into the anterior eye chamber and compared the extent of reinnervation by host sympathetic nerves. For comparative purposes, explants from virgin and postpartum uterine arteries were also transplanted.

MATERIALS AND METHODS

Animals

Twenty-eight sexually mature virgin or 2–7 d postpartum albino guinea pigs (600–800 g body weight) were used.

In oculo transplantation

Three virgin (dioestrus) and 4 postpartum guinea pigs, of which 3 had been bilaterally pregnant and 1 unilaterally pregnant, were terminally anaesthetised with sodium pentobarbital administered intraperitoneally. The uterine horns and the uterine arteries were removed under aseptic conditions and placed in sterile ice-cold Hank's balanced salt solution (Gibco, UK) for dissection. In postpartum animals, only the periplacental region of the uterine horn was used for transplantation. This area was defined by large, clearly visible blood vessels in the endometrium and by the

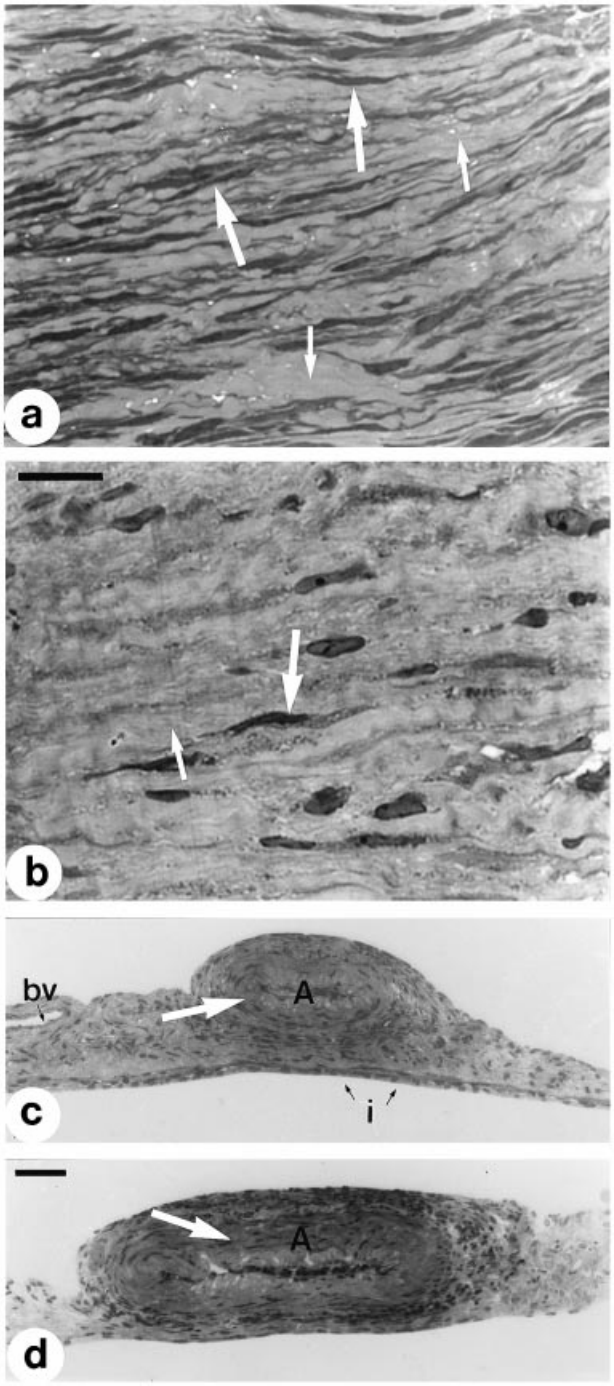

Fig. 1. Toluidine blue stained semithin sections of the guinea pig myometrium and uterine artery transplants after 3 wk in oculo. Transplanted virgin (*a*) and postpartum (*b*) myometrium, both at same magnification. The large arrow in *b* indicates the approximate position of the adventitial-medial border, with the adventitia occupying the lower third of the micrograph. Note the hypertrophic appearance of the smooth muscle cells (large arrows). Small arrows, connective tissue stroma. Bar, 10 μm. Transplanted virgin (*c*) and postpartum (*d*) uterine arteries, both at same magnification. i, host iris; A, uterine artery transplant; bv, host blood vessel; arrows, medial layer of arteries. Bar, 30 μm.

local expansion of the uterus associated with the fetus. Middle segments of the uterine horn were opened longitudinally and pinned on Sylgard (Dow Corning, UK) using micropins. The endometrium and the circular muscle layer were carefully removed and the longitudinal myometrial layer with its attached serosa was cut into strips 2 mm wide and 4 mm long. Middle segments of the uterine artery were cleaned of fat and connective tissue and cut in 5 mm long pieces. Some preparations were processed directly for immunohistochemistry without transplantation for comparison with the transplanted samples.

Bilateral transplants of explants of 10 virgin and 12 postpartum myometrium, and of 9 virgin and 11 postpartum uterine arteries were made into the anterior eye chamber of sexually mature female guinea pigs at dioestrus. Host animals were anaesthetised with halothane and 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; Burnstock, 1974; Gavazzi et al. 1992). The pupil was not obstructed by the transplants and visually guided behaviour of the hosts was not impaired. Transplants were left in oculo for 3 wk after which host animals were terminally anaesthetised with sodium pentobarbital and transplants were removed attached to the host irides.

Immunohistochemistry

Irides with the attached transplants, as well as samples of nontransplanted tissues, were pinned on pieces of Sylgard with micropins and fixed for 2 h in buffered 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at room temperature, washed in 0.05 M Hepes buffer, pH 7.4, and incubated for 1.5 h in 0.4% Triton X100 plus 3% swine serum in Hepes buffer. Tissues were incubated overnight at room temperature in a humid chamber with a rabbit antibody against tyrosine hydroxylase (TH) (1:50, Eugene Tech, USA). At the end of incubation, tissues were washed in PBS and incubated with swine antirabbit fluorescein isothiocyanate (FITC; 1:40; Dako, UK) for 1.5 h at room temperature. Tissues were counterstained with 0.05% pontamine sky blue (BDH Chemicals, UK) diluted in PBS in order to reduce background autofluorescence (Cowen et al. 1985) and mounted in antifade mountant (Citifluor, UK).

Transplanted and nontransplanted tissues were examined with a ×20 oil objective on a Bio-Rad MRC 600 confocal microscope. The aperture and gain settings were set to maximise contrast for each preparation. Kalman filtering was used to remove background noise. Series of optical sections, each

4 μm in depth, were captured by the confocal microscope and stored on optical disc, creating files that were used to generate images showing the full depth of the nerve plexus in a 2-dimensional projection image.

Quantification of nerve fibres

The density of innervation by TH-immunoreactive (TH-I) nerve fibres of transplanted virgin and postpartum uterine arteries was assessed quantitatively after 3 wk in oculo using established techniques of image analysis (Gavazzi et al. 1992, 1995). A minimum of 3 fields were analysed per specimen. Nerve density was expressed as intercept density (ID/mm; number of nerve bundles per mm) and as area % (percentage of frame area occupied by specific fluorescence). The diameter of the flattened vessels was assessed directly from the stretch preparations under the light microscope. Results are expressed as mean ±S.E.M. Mean values were compared using 2-tailed Student's t test. Values of $P < 0.05$ were considered statistically significant.

Histology

After examination under the confocal microscope, transplants were removed from glass slides and processed for histological studies. Tissues were refixed in 4% PFA for 2 h at room temperature, washed in PBS, dehydrated in alcohol and acetone and embedded in Durcupan ACM (Fluka). Semithin sections (0.25–0.50 μm) were cut with a Sorvall MT2 ultramicrotome, stained with 0.1% toluidine blue and examined in the light microscope. Micrographs were taken on 25 ASA Agfapan film.

RESULTS

At the time of removal all transplants were attached to the iris. For myometrial transplants, attachment was by 2–5 connective tissue bridges (Olson & Malmfors, 1970). After 1 wk in oculo transplants had become revascularised. The overall size of transplants was reduced after the transplantation period. Histological examination showed that transplants were partially surrounded by a thin layer of connective-like tissue which was continuous with the connecting bridges. Smooth muscle cells in both virgin (Fig. 1*a*) and postpartum (Fig. 1*b*) myometrial transplants showed a normal appearance, although those from postpartum donors were clearly hypertrophic. Transplanted uterine arteries (Fig. 1*c*, *d*) showed the characteristic trilaminar structure composed of tunica intima, tunica media and tunica adventitia. Like the myometrium, the postpartum uterine artery was markedly hypertrophic (Fig. 1*d*).

Table. *Quantitative assessment of vessel diameter and innervation density of tyrosine hydroxylase-immunorective nerve fibres in transplants of the virgin and postpartum guinea pig uterine artery after* 3 *wk in oculo*

		Nerve density	
	Diameter (μm)	Area (%)	Intercept density
Virgin	137±12 (7)	13.17±1.47 (4)	48.37±4.86 (4)
Postpartum	251±24 (8)*	7.45±0.57 (6)*	33.26±1.67 (6)*

Nerve density is expressed as Area % (percentage of frame area occupied by specific fluorescence) and Intercept density (nerve fibres per mm). Results are given as mean ±S.E.M. Comparisons between virgin and postpartum data were performed using 2-tailed Student's t test. Values of $P < 0.05$ were considered statistically significant (*). n for each determination is given in brackets.

Immunohistochemical methods showed that TH-I fibres approached the myometrial transplants through the connecting bridges that attached the transplant to the host iris. In virgin transplants, TH-I fibres initially penetrated into the surrounding connective-like sheath and in some points penetrated deeply into the smooth muscle. The pattern of reinnervation of virgin myometrial transplants by host TH-I nerves (Fig. 2*a*) closely resembled that seen in vivo (Fig. 3*a*). Smooth and varicose TH-I fibres formed parallel arrays, probably following the direction of orientation of smooth muscle cells. Occasionally, blood vessel-related TH-I fibres were seen in the transplant (Fig. 2*a*).

In myometrial transplants from postpartum donors (Fig. 2*b*), several smooth TH-I fibres were seen running in the connecting bridge and in the surrounding connective sheath, but they did not penetrate into the transplant. In the connective sheath some of the fibres were varicose whereas others were thicker and more intensely fluorescent than those seen in the bridges. Occasionally, curved fibres were observed travelling back from the sheath towards the connecting bridge (Fig. 2*b*). Even when transplants were revascularised, we failed to observe TH-I nerves around the blood vessels. In nontransplanted postpartum myometrium, no TH-I fibres were observed in the outer muscle layer or the associated blood vessels (Fig. 3*b*).

Transplanted uterine arteries from both virgin (Fig. 2*c*) and postpartum (Fig. 2*d*) donors had become reinnervated by host TH-I nerves after 3 wk in oculo. The pattern of innervation closely resembled that

observed in nontransplanted vessels (Fig. 3*c*, *d*). The density of reinnervation of the transplanted postpartum uterine arteries was lower than in the virgin vessel (Table 1). However, because of the hypertrophy of the vessel wall during pregnancy (Table 1), it is likely that the extent of growth of host nerves on the larger postpartum arteries was comparable to or even greater than that on virgin vessels.

DISCUSSION

Classically, it has been proposed that the atrophy of uterine sympathetic nerves which occurs in the last stages of pregnancy, as well as the slow regrowth of these nerves after delivery, is associated with the direct effects of sex hormones on the short adrenergic neurons (Thorbert, 1978; Marshall, 1981; Owman, 1981; Sporrong et al. 1981; Lundberg et al. 1988; Owman & Stjernquist). Hypertrophic target tissues, including muscular visceral organs, have in general been found to induce coordinated changes in their innervating neurons (Gabella, 1984; Steers et al. 1990). An alternative hypothesis to explain the atrophy of uterine nerves is therefore that pregnancy induces alterations in the myometrium that indirectly affect the innervating neurons, i.e. the primary target of hormonal activity is uterine smooth muscle rather than short adrenergic neurons.

The in oculo transplantation model (Olson & Malmfors, 1970) has been widely used to study trophic interactions between neurons and their targets. Using this approach, different visceral organs have been shown to exert a profound influence over the organotypic pattern and density of their innervation (Burnstock, 1974; Gavazzi et al. 1992) and over neurotransmitter expression (Stevens & Landis, 1990; Cowen et al. 1996) of innervating neurons. For example, transplants of the sympathetically innervated submandibular gland became well reinnervated by host sympathetic nerves whereas the normally noninnervated sublingual gland was not reinnervated (Olson & Malmfors, 1970). In the latter tissue, fluorescent nerves from the host iris were seen encircling the transplant but failed to penetrate into it. Similarly, dynamic changes in nerve fibre density over particular tissues, such as those occurring during development and ageing (Burnstock, 1974; Thrasivoulou & Cowen, 1995; Cowen et al. 1996), have been shown by the in oculo transplantation approach to be regulated by changes in the target. We have therefore used this model to study the possible influence of myometrial muscle over its sympathetic innervation.

The present study has confirmed the influence of muscle targets over their innervation by showing that the uterine myometrium from virgin donors, normally innervated by the system of short adrenergic fibres arising from the paracervical ganglia, became organotypically reinnervated by long adrenergic fibres from the superior cervical ganglion. Similarly, both the virgin and postpartum uterine arteries were successfully reinnervated by host sympathetic nerves. The areal density of innervation of the postpartum uterine artery was lower than in the virgin vessel. However, this difference could be related to the marked hypertrophy observed in the postpartum vessel, i.e. the nerves became more spread out over the vessel wall. Indeed, there is evidence of nerve growth matching the hypertrophy of the uterine artery during pregnancy in guinea pigs (Mione & Gabella, 1991) and in rats, from our own observations of nontransplanted vessels (data not shown). These results confirm the concept that nerves become matched to their targets as a result of epigenetic influences associated with the target tissue.

The loss of nerves in the postpartum myometrium (Thorbert, 1978; Owman, 1981; Owman & Stjernquist, 1988) also appears to be strongly influenced by alterations in the uterine muscle. Host noradrenergic fibres were able to reach the connective tissue sheath surrounding the transplants of postpartum myometrium, but failed to reinnervate the muscle or the intramuscular blood vessels. The absence of ingrowing nerves does not seem to be related to degeneration of smooth muscle cells in the transplant, since histological studies showed the presence of normal hypertrophic smooth muscle cells, similar to those observed in vivo at 4 wk postpartum (Brauer, unpublished observations). Thus not only does the target influence the pattern of innervation in myometrium and uterine artery, but changes in targets affect dynamic regulation of nerve growth and atrophy in particular tissues (Gavazzi & Cowen, 1996). These results also suggest that the slow regrowth of uterine sympathetic nerves after parturition is not related to a particular vulnerability of short adrenergic neurons to hormonal changes.

Loss of Schwann cells may also contribute to the slow regrowth of uterine nerves after parturition (Lundberg et al. 1988). Pregnancy-induced degeneration of sympathetic nerves in the uterine horn, possibly due to mechanical damage from overdistension, involves loss of Schwann cells, whereas in the empty horn and cervix the number of Schwann cells appears to remain unchanged. In the postpartum uterus, the absence of the guidance pathways and

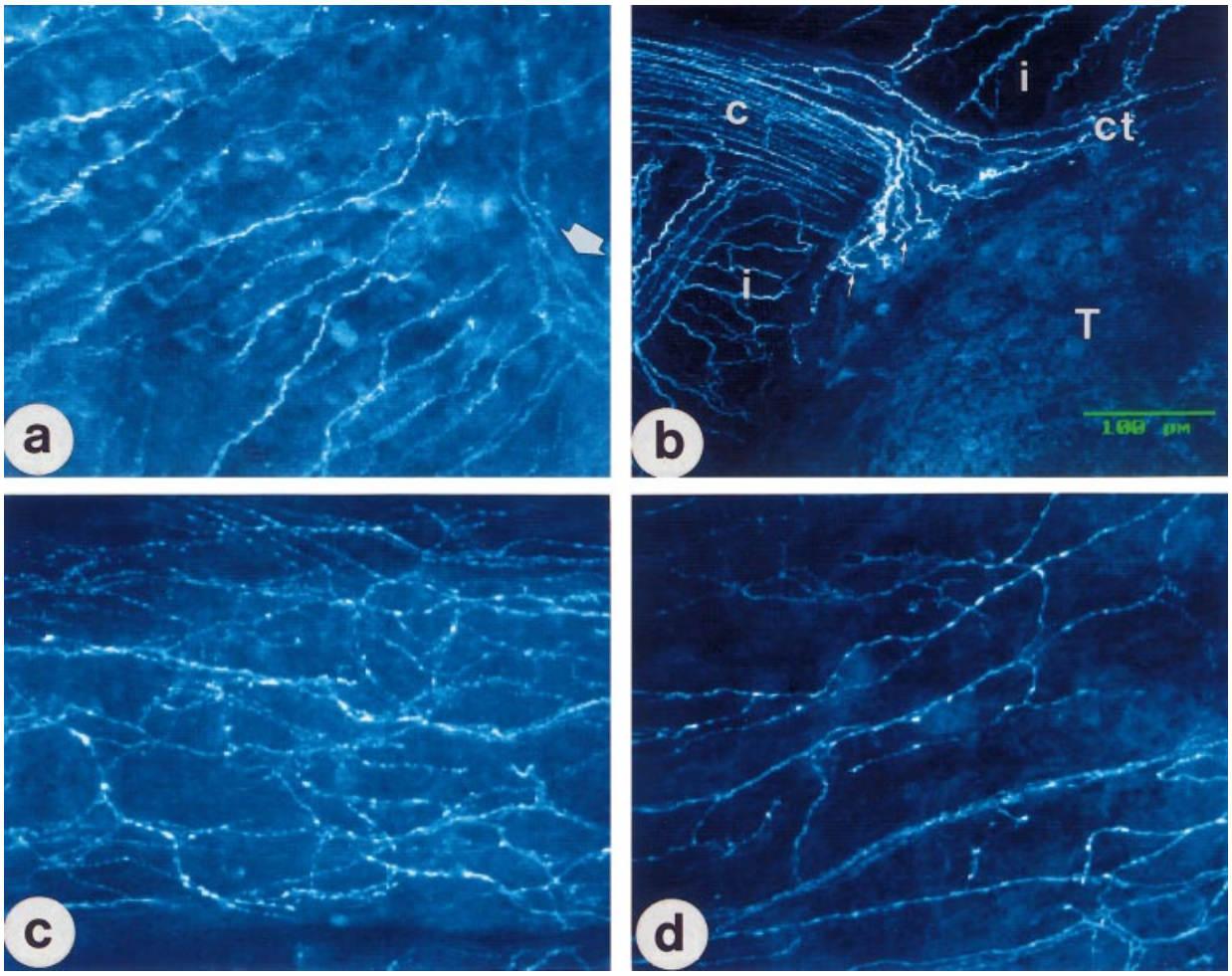

Fig. 2. Confocal projection images of the guinea pig myometrium and uterine artery transplants after 3 wk in oculo, showing the pattern of reinnervation by host tyrosine hydroxylase-immunoreactive (TH-I) nerve fibres. (*a*) Transplanted virgin myometrium. Parallel arrays of preterminal and terminal TH-I nerve fibres are seen within the transplant. Arrow, blood vessel; (*b*) transplanted postpartum myometrium. Several TH-I fibres are seen travelling into the connecting bridge (*c*) and penetrating into the connective tissue (ct) surrounding the transplant. No nerve fibres are seen within the transplant (T). i, iris; small arrows, curved fibres. (*c*) Transplanted virgin and (*d*) postpartum uterine arteries. Note the relatively low density of TH-I nerve fibres in the postpartum compared with the virgin artery. Bar, 100 µm.

trophic molecules supplied by Schwann cells may contribute to the slow regrowth of sympathetic nerves after delivery (Lundberg et al. 1988). It is therefore feasible that pregnancy-induced changes in Schwann cells contribute to the absence of ingrowing nerves in the transplanted postpartum myometrium after 3 wk in oculo.

Influences of targets over their innervating neurons include target-derived neurotrophic factors (Levi-Montalcini, 1987). These trophic factors include nerve growth factor (NGF) and related neurotrophins, as well as cytokines (Gavazzi & Cowen, 1996). In general, it has been shown that the level of NGF correlates with the density of sympathetic and sensory innervation in the target tissue (Korsching & Thoenen, 1983). For instance, it has been shown that transgenic mice over-expressing NGF in the skin exhibit hyperinnervation by sympathetic and sensory fibres (Albers et al. 1994). Similarly, it has been demonstrated that the blood vessels of genetically hypertensive rats exhibit increased density of perivascular sympathetic nerves and higher levels of NGF compared with normotensive ones (Spitsbergen et al. 1995).

Target size is thought to influence neuronal size and the extent of axonal and dendritic arborisation in the innervating neurons. When an organ undergoes hypertrophy the autonomic innervation adapts so that an appropriate neuroeffector relationship is maintained. For instance, it has been shown that partial occlusion of the urethra causes a marked hypertrophy of the bladder which is accompanied by a parallel and reversible hypertrophy in the innervating pelvic ganglion neurons (Gabella et al. 1992). Similarly, chronic oestrogen treatment to prepubertal

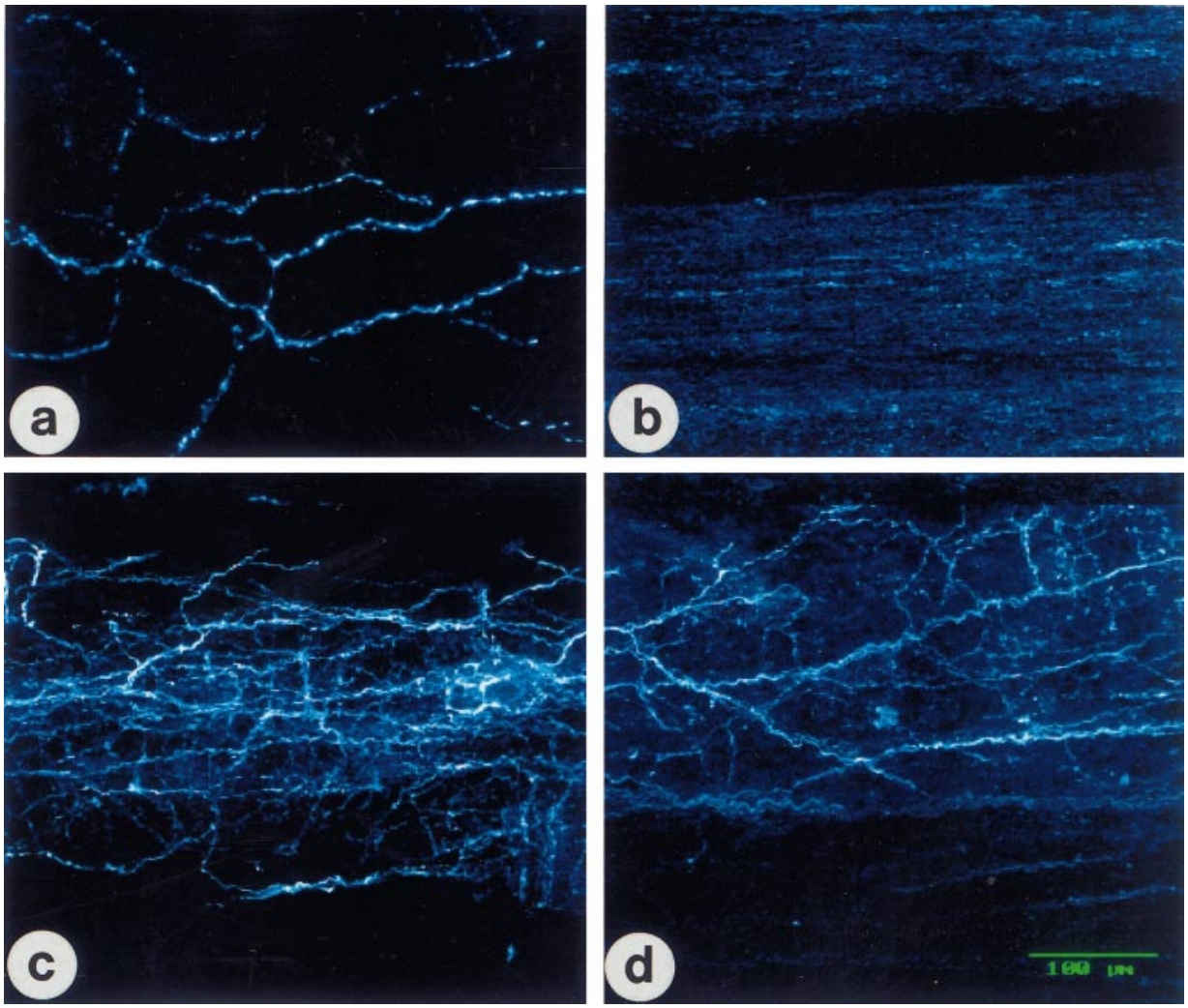

Fig. 3. Confocal projection images of the normal pattern of innervation of the guinea pig myometrium (outer longitudinal muscle layer) and uterine artery by tyrosine hydroxylase-immunoreactive (TH-I) nerve fibres. (*a*) Virgin and (*b*) postpartum myometrium; (*c*) virgin and (*d*) postpartum uterine arteries. Bar, 100 µm.

rats provokes a marked increase in the size of the bladder which is accompanied by a parallel increase in the tissue content of noradrenaline (Brauer et al. 1995). In marked contrast to these matching changes in visceral organs and their autonomic innervation, the massive growth of the uterus during pregnancy is accompanied by a dramatic decay in the sympathetic innervation, which is reflected initially in a functional impairment and at term, in the gravid horn, by the degeneration of adrenergic nerve terminals (Owman & Stjernquist, 1988). A decline in the density of noradrenaline-containing sympathetic nerves is observed in the rat uterus following puberty and prepubertal oestrogen treatment (Brauer et al. 1992, 1995). This change probably represents a 'dilution' of the nerve fibres which fail to keep pace with the growth of the uterine smooth muscle, rather than neurodegeneration.

In this context, it may be hypothesised that the pregnancy-induced changes in the sympathetic innervation of the uterus are related to a local reduction in the target production of neurotrophic factors such as NGF. This suggestion, based on the neurotrophic hypothesis, may explain the uneven, region-specific nature of the pregnancy-induced changes in the sympathetic innervation of the uterus, i.e. different regions of the uterus respond to hormonal changes with local variations in the production of neurotrophic substances. This concept would also explain the slow regrowth of nerves after delivery (Owman, 1981; Owman & Stjernquist, 1988).

In the pregnant guinea pig uterus, degeneration of sympathetic nerves is accompanied by a reduced density of sensory nerves containing substance P (SP) and calcitonin gene-related peptide (CGRP) (Lundberg et al. 1988). Changes in neurotrophic

factor production during pregnancy may affect peptidergic sensory, as well as sympathetic nerves, because both are sensitive to NGF (Kessler et al. 1983; Korsching & Thoenen, 1983). However, peptidergic nerves to the uterine artery are unaffected by pregnancy (Mione et al. 1988).

Alternative explanations for the absence of ingrowing sympathetic nerves in the postpartum myometrial transplant should be also considered. The first would involve changes in the production of extracellular matrix bound neurotrophic factors, such as laminin, which are known to influence axonal outgrowth and Schwann cell migration during normal development, regeneration and atrophy of peripheral nerves (de Curtis, 1991; Anton et al. 1994; Gavazzi & Cowen, 1996) and may act synergistically with NGF (Cowen et al. 1997).

Finally, the possibility that a repulsive factor is produced by the postpartum myometrium seems to be supported by the presence of fibres which approach the postpartum myometrial transplant via the connecting bridges and then apparently change direction and turn away. Further studies are needed to distinguish between these different possibilities.

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