

## A quantitative study of rat uterine sympathetic innervation during pregnancy and post partum

R. Chávez-Genaro<sup>A,D</sup>, P. Lombide<sup>A,B</sup> and G. Anesetti<sup>A,C</sup>

<sup>A</sup>Departamento de Histología y Embriología, Facultad de Medicina, General Flores 2125, CP 11800, Montevideo, Uruguay.

<sup>B</sup>Sección Biología Celular, Departamento de Biología Celular y Molecular, Instituto de Biología, Facultad de Ciencias, Iguá 4245, CP 11400, Montevideo, Uruguay.

<sup>C</sup>Area de Histología y Embriología, Facultad de Veterinaria, Las Places 1550, CP 11600, Montevideo, Uruguay.

<sup>D</sup>Corresponding author. Email: rchavez@fmed.edu.uy

**Abstract.** In mammals, pregnancy induces a transient and extensive degeneration of uterine sympathetic innervation. We used the models of unilateral oviduct ligation and *in oculo* myometrium transplant in pregnant rats to address the role of stretching forces and/or hormone milieu in the loss of sympathetic innervation. The sympathetic fibres of the uterine horn and *in oculo* myometrial transplants were quantified on tissue sections processed by the glyoxylic acid technique. In normal pregnant rats, the density of uterine horn innervation was significantly reduced at late pregnancy and recovery took place during post partum. The empty horn of pregnant rats showed no significant changes in density of myometrial innervation during pregnancy or post partum. *In oculo* myometrial transplants were organotypically reinnervated in virgin animals. When the transplants were exposed to gestational hormonal milieu, few or no fibres were observed to the end of pregnancy; however, a significant increase at post partum was observed. Results showed that both the effects of stretching and the hormone milieu derived from the fetus–placenta complex play a role as inductors of changes on sympathetic myometrial innervation during pregnancy and support the idea that immature muscular uterine fibres are more susceptible to the effects of pregnancy than those originating from adult animals.

*Extra keywords:* sympathetic nerves, transplant, uterus.

### Introduction

Sympathetic uterine myometrial innervation in mammals is organised in abundant perivascular fibres and muscular plexus. In the rat, uterine sympathetic nerve development takes place during the first 4 weeks of postnatal life. Full-grown innervation occurs at the end of the prepubertal period, showing a myometrial differential cephalic–caudal distribution (Brauer *et al.* 1992). These nerves are susceptible to changes in the endocrine environment or in response to experimental alterations in the circulating levels of sex hormones (Marshall 1981; Owman and Stjernquist 1988; Brauer *et al.* 1995, 1999; Zoubina *et al.* 2001; Chávez-Genaro *et al.* 2002). In a previous report, we showed that the magnitude of the response of sympathetic nerves to oestrogen treatment could be influenced by the maturation stage of sympathetic fibres, the age of the neurones and/or the developmental stage of the uterus (Chávez-Genaro *et al.* 2002). Changes in sympathetic innervation have also been shown in pregnant animals. In the rat, the sympathetic innervation of uterine tissue surrounding the conceptus

shows a complete degeneration of myometrial and intramural perivascular adrenergic nerves (Bell and Malcolm 1978; Thorbert 1978; Owman and Stjernquist 1988; Yamada 1988; Haase *et al.* 1997). The loss of uterine horn innervation in the pregnant rat has been associated with a dilution of trophic factors in the enlarged tissue (Haase *et al.* 1997) as well as the endocrine milieu. Uterine denervation during this physiological condition is thought to underlie the suppressed motor function of the organ, which is essential for the normal development of the fetus and the prevention of pre-term labour (Varol *et al.* 2000). After delivery of the fetus, a regenerative process of reinnervation occurs in a variable period as well as a hyperinnervation of the uterus has been reported (Haase *et al.* 1997). In the guinea-pig, the loss of catecholaminergic innervation during pregnancy has been associated with two processes: (1) direct mechanical damage due to stretching of the uterine tissue by the fetus; and (2) the hormonal influence of oestrogen and progesterone secreted by the fetus–placenta complex (Bell and Malcolm 1978; Owman 1981; Sumida and Pasqualini 1981). Other studies using *in oculo* transplant

of myometrium from post partum guinea-pigs showed the failure of sympathetic innervation observed in transplants of virgin animals. In this case, the results have been interpreted as due to changes in uterine muscle rather than a particular sensitivity of adrenergic neurones to hormonal changes provoked by gestation (Brauer *et al.* 1998, 2000b). In the rat, this issue has not been thoroughly quantified.

The aim of the present investigation was to quantify the number of sympathetic nerves during the pregnancy and post partum using the models of unilateral horn ligation and intraocular myometrial transplantation to observe the responsiveness of myometrial tissue without the influence of stretching forces.

## Materials and methods

### *Animals and treatments*

Female Wistar-derived albino rats from the breeding colony held at the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE, Montevideo, Uruguay) with a 10-h light : 14-h dark cycle and food and tap water *ad libitum* were used for this study. All experimental protocols used were in accordance with the Universidad de la República guidelines of laboratory animal care. Animals were assigned to one of the following two experimental groups:

1. *Intact*: Female virgin adult rats (180–200 g) were used to obtain daily vaginal smears. Only animals exhibiting at least two consecutive 4-day cycles were used. Rats were deeply anaesthetised with intraperitoneal injection of sodium pentobarbital (50 mg kg<sup>-1</sup>, Sigma, St Louis, MO, USA) and killed by cervical dislocation either on dioestrus 1 (D1) ( $n = 6$ ) or oestrus (E) ( $n = 6$ ).
2. *Pregnancy*: Twenty virgin cyclic adult rats at proestrus were mated with fertile males. The next morning, vaginal smears were checked for the presence of spermatozoa and considered as Day 1 of gestation. Pregnant animals were caged without males and killed as previously indicated on Days 7, 14 or 19 of pregnancy or 3–5 days following parturition.

### *Unilateral pregnancy*

Twelve virgin cyclic adult rats were anaesthetised with sodium pentobarbital (40 mg kg<sup>-1</sup>) and the oviduct (right or left) was exposed and ligated with seal. One week later, females at proestrus were mated. Pregnant animals were killed in the same way as the previous group.

### *In oculo myometrium transplant and pregnancy*

*Donors.* Prepubertal 4-week-old rats (50–60 g) were deeply anaesthetised and killed by cervical dislocation. The uterus was quickly removed under aseptic conditions and placed in sterile ice-cold Hanks balanced salt solution (Irvine Scientific, Santa Ana, CA, USA) for dissection. The uterine horns were opened longitudinally and pinned on Sylgard (Dow Corning, Northumberland, UK) using micropins. The endometrium and most of the circular smooth muscle layer were carefully removed and the longitudinal myometrial layer with its attached serosa was cut into strips (approximately 1.0 × 1.5 mm).

*Hosts.* Adult female virgin rats were anaesthetised with 40 mg kg<sup>-1</sup> of sodium pentobarbital administered intraperitoneally, followed by local administration of 0.5% proparacaine hydrochloride solution (Anestalcon; Alcon-Argentina, Buenos Aires, Argentina). Mydriasis was achieved by application of a drop of 10 mg mL<sup>-1</sup> atropine sulfate to the cornea. Myometrial transplants were inserted into the eye through a small slit in the pupillary region of the cornea made with a microsurgical blade (Becton Dickinson, Franklin Lakes,

NJ, USA) and manipulated by gentle pressure on the cornea into the posterior iridocorneal angle of the eye (Olson and Malmfors 1970; Brauer *et al.* 1998; Chávez-Genaro *et al.* 2002). The transplants did not obstruct the pupil, and visually guided behaviour of the hosts was not impaired. One week later, female hosts were divided into two groups: virgin and mated rats. Pregnant rats were killed at similar intervals as well as Group 2, and virgin animals were killed 25–30 days after transplant.

### *Histochemical demonstration of noradrenalin-labelled sympathetic nerves*

The uterine horns were quickly removed, placed in cold Hanks balanced salt solution and cleaned of fat connective tissue. A piece of the middle region was selected owing to the fetus distribution and because the tubaric end did not show changes in the content of catecholamines during pregnancy (Thorbert 1978). Noradrenergic nerves were demonstrated in the middle region of the uterine horn and in intraocular transplants by the glyoxylic acid method (de la Torre and Surgeon 1976) performed on cryostat tissue sections as previously described (Brauer *et al.* 1992, 1995; Chávez-Genaro *et al.* 2002). Preparations were examined under an Eclipse 800 microscope (Nikon, Tokyo, Japan) equipped with epifluorescence and fitted with the appropriate filters. Micrographs were taken with Ilford HP5, 400 ASA film (Cheshire, UK).

### *Nerve density measurements*

In the uterine horn, the density of noradrenalin-labelled (NA-L) myometrial fibres was assessed in the longitudinal smooth muscle layer from transverse sections as reported by Zoubina *et al.* (1998), with minor modifications (Chávez-Genaro *et al.* 2002). Six to twelve transverse cryostat sections of each animal were analysed. Briefly, to estimate the density of innervation in each transverse section, five different myometrial areas were photographed including the mesometrial border (two areas), antimesometrial border (one area) and intermesometrial border (two areas). Then, a stereological grid with an area of 0.25 mm<sup>2</sup> and line intersects at 20- $\mu$ m intervals was superimposed over each tissue image 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 longitudinal smooth muscle layer (Chávez-Genaro *et al.* 2002). Results are given as percentage of area occupied by NA-L nerves (density of innervation). Because variations in plasma levels of oestrogen during normal cycle and pregnancy provoke changes in the size of the uterus, corrections were carried out in order to obtain the total nerve area. Measurements of myometrial longitudinal area were made on tissue preparations stained with haematoxylin and eosin. The images of entire uterine horn were captured at low magnification, and the area between the outer and inner perimeter of the longitudinal myometrial layer were traced and measured using the Image Pro Plus Program (Media Cybernetics, Silver Spring, MD, USA). The density of innervation was multiplied by the total area of the longitudinal myometrial layer, then divided by 100 and expressed as total nerve area.

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

### *Statistical analysis*

Results are expressed as the mean  $\pm$  s.e.m. Data were compared using an ANOVA test, followed by the Dunn's multiple comparison test or the two-sided Mann–Whitney non-parametric test for unpaired data. Values of  $P \leq 0.05$  were considered statistically significant.

## Results

### *Myometrial sympathetic innervation in cyclic rats*

Cyclic female adult rats showed a moderate sympathetic myometrial innervation in the circular and longitudinal smooth muscle layers, with an abundant distribution in the vascular zone separating both myometrial layers. Sparse non-vascular fibres were also observed (Fig. 1a). Myometrial longitudinal cross-sectional area of oestrous rats was statistically greater than for dioestrous animals ( $0.92 \pm 0.08$  v.  $0.59 \pm 0.10$  mm<sup>2</sup>,  $P < 0.05$ ). However, the density of innervation was lower in oestrous compared with dioestrous rats ( $4.80 \pm 0.69$  v.  $8.92 \pm 1$ ,  $P < 0.05$ ). When the density of sympathetic innervation was correlated with the longitudinal myometrial area, no significant changes in total nerve area were observed (oestrus:  $0.045 \pm 0.004$  v. dioestrus:  $0.053 \pm 0.005$ , ns). In spite of the results obtained and the similarity of hormonal milieu, the data of pregnant animals were compared with those of animals killed on oestrus day.

### *Myometrial sympathetic innervation in pregnant rats*

In 7-days-pregnant animals with both uterine horn bearing fetuses, the longitudinal myometrial area was similar to observed in virgin animals killed on oestrus day ( $0.84 \pm 0.03$  v.  $0.92 \pm 0.08$  mm<sup>2</sup>, ns). Neither the arrangement of fibres (Fig. 1b) nor the density of innervation or the total nerve area were significantly different (Fig. 2a). As pregnancy advanced, the uterine horn size increased and the amount of sympathetic fibres with specific fluorescence decreased, disappearing completely at 19 days of pregnancy (Figs 1c, 2a). Three to five days post partum, the longitudinal myometrial layer remained hypertrophic with respect to 7-days-pregnant animals ( $2.13 \pm 0.16$  v.  $0.84 \pm 0.03$  mm<sup>2</sup>,  $P < 0.05$ ). However, the density of innervation and the total nerve area of sympathetic fibres reached similar values as observed at early pregnancy (Figs 1d, 2a).

### *Unilateral pregnancy*

#### *Horn with fetus*

The myometrial longitudinal layer in the occupied horn of rats with unilateral pregnancy showed a pattern of distribution and density of sympathetic fibres similar to that observed in animals carrying fetuses in both horns (data not shown).

#### *Empty horn*

The longitudinal myometrial area of empty horn in unilateral 7-days-pregnant animals ( $0.84 \pm 0.03$  mm<sup>2</sup>) was similar to that observed in animals with occupied horn. At Day 19 of the gestational period, the longitudinal myometrial area increased ( $1.49 \pm 0.01$  mm<sup>2</sup>,  $P < 0.05$ ). However, neither the density of innervation nor the total nerve area showed significant differences during gestational or post partum periods with respect to virgin animals (Fig. 2b).

### *Sympathetic uterine myometrial innervation during pregnancy in in oculo transplants*

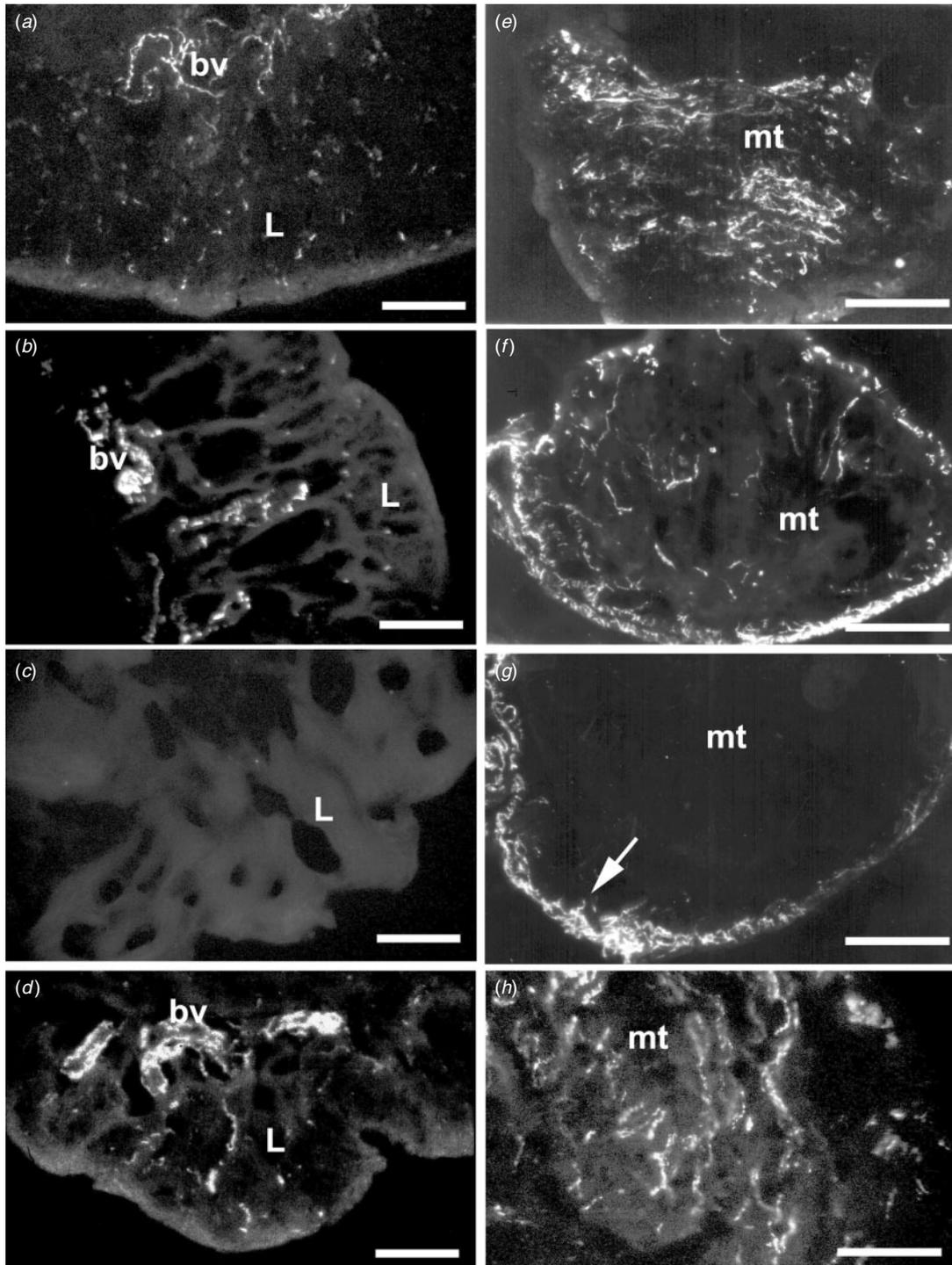
After 25 days *in oculo*, myometrial transplants from pre-pubertal donors to adult virgin rats were organotypically reinnervated by sympathetic nerves, although with a wide range of variability (Table 1). The sympathetic fibres appeared to be associated with blood vessels and myometrial smooth muscle (Fig. 1e). The transplant exposed to 7 or 14 days of gestational hormonal milieu showed a density of innervation similar to transplants in virgin animals (Table 1; Fig. 1f). Meanwhile, the transplants exposed to 19 days of pregnancy showed a significant decrease in the number of sympathetic fibres, with some having all sympathetic nerves eliminated (Table 1; Fig. 1g). Transplants in animals killed in the post partum period showed the richest innervation of all groups studied. The minor number of fibres in these transplant animals was around the highest value for the other experimental groups (Table 1; Fig. 1h).

## Discussion

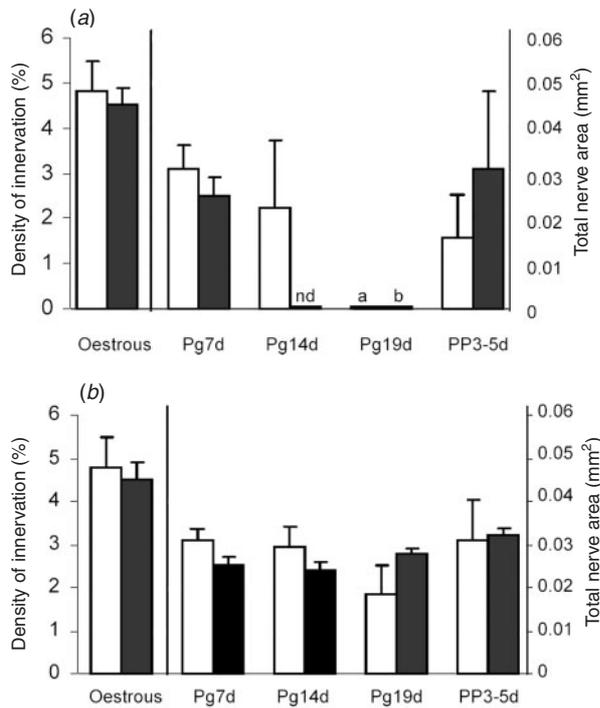
The results reported in the present study describe for the first time a quantitative estimation of the percentage of sympathetic innervation of the uterine horn in the rat during pregnancy and post partum using the models of unilateral horn ligation or intraocular myometrial transplantation in pregnant rats.

In our strain of rats, the density of myometrial sympathetic innervation in the middle portion of the uterine horn showed a wide range of variability between the two phases of the oestrous cycle studied (oestrus v. dioestrus day), even though significant differences in oestrogen plasma levels are known (Lindzey and Korach 1998). However, this difference disappeared when corrections for variations in longitudinal muscle cross-sectioned area were performed, allowing us to suggest a spatial dilution of nerves on the tissue during oestrus rather than a disappearance of the sympathetic fibres. Either a large variability between individuals (Haase *et al.* 1997) or no changes in the density of innervation during the oestrous cycle have been reported by some authors (Papka *et al.* 1985; Melo and Machado 1993; Haase *et al.* 1997) and denied by others (Adham and Schenk 1969; Robbins *et al.* 1990; Zoubina and Smith 2000). The differences observed between experiments could be a result of differences between the strains of rats used as well as the variability of uterine innervation between animals. Another possibility to be considered is the uterine horn region used for estimations, since previous results demonstrated considerable regional variation in the density of innervation of the uterine horn (Chávez-Genaro *et al.* 2002).

Our results showed that the initial effects of hormonal pregnancy milieu (7 days) do not lead to a fast significant effect on the percentage of sympathetic fibres of the longitudinal myometrial layer. Biochemical measurements of



**Fig. 1.** Noradrenalin-labelled sympathetic fibres demonstrated by the glyoxylic acid technique on transverse cryostat sections of the antimesometrial area of the rat uterine horn (*a-d*) and *in oculo* myometrial transplant (*e-h*). Numerous fluorescent fibres are evident in the longitudinal myometrial layer (L) and associated at blood vessels (bv) in adult cyclic rats in (*a*) oestrous day and (*b*) pregnant animals at 7 days of gestation. (*c*) At the end of gestation (19 days) note the enlargement of the muscular layer and the absence of fluorescent fibres. (*d*) Three to 5 days post partum fluorescent fibres displayed a similar distribution pattern that showed in cyclic or early pregnant animals. *In oculo* myometrial transplants (mt) obtained from (*e*) virgin or (*f*) early pregnant rats (7 days) showed a dense to moderate innervation derived from iris. (*g*) In myometrial transplants of 19-days-pregnant rats the absence of fibres in the myometrial tissue was noted. However, in the periphery of the transplant, fibres could be detected. (*h*) Myometrial transplants obtained from post partum rats showed a rich innervation, greater than observed in control animals. Scale bar = 100  $\mu$ m.



**Fig. 2.** (a) Density of sympathetic myometrial innervation and total nerve area of oestrous, pregnant and post partum rats during normal pregnancy. A decline in the density of innervation and in total nerve area was observed at the end of the gestational period followed by recovery during the post partum period. (b) In the empty horn of unilateral pregnant animals both parameters were without significant changes in all stages studied. (a,b)  $P < 0.05$  v. 7, 14 days of pregnancy and post partum; nd, data not determined because of difficulties in the histological processing of the gravid uterus due to the thinness of the myometrium.

**Table 1. Sympathetic myometrial innervation in *in oculo* myometrial transplants**

Data show the mean  $\pm$  s.e.m. of the number of noradrenaline-labelled sympathetic fibres in *in oculo* uterine myometrial transplant of virgin or pregnant rats killed in different periods of gestation or post partum

Group	Density of innervation (%)	Range
Virgin	17.5 $\pm$ 8.5	7–36
Pregnant 7 days	28.1 $\pm$ 7.4	17–37
Pregnant 19 days	6.8 $\pm$ 2.7 <sup>A</sup>	0–24
Post partum	61.8 $\pm$ 28.8 <sup>A,B</sup>	30–74

<sup>A</sup> $P < 0.05$  v. virgin and 7 days of pregnancy. <sup>B</sup> $P < 0.05$  v. virgin, 7 and 19 days of pregnancy.

noradrenalin in different species in a similar period of gestation are in accordance with these observations, because no changes in the amine concentration have been found (Thorbert 1978; Brauer *et al.* 2000a). The reduced changes observed in early pregnancy appear to be unrelated to alterations in uterine innervation but may be related to changes associated with implantation (Klukovits *et al.* 2002).

At the end of the gestational period, sympathetic innervation of the longitudinal myometrial layer of the occupied horn disappeared, whereas no significant changes in the empty horn of pregnant rats with unilateral ligation of the Fallopian tube were observed. These results could further support the idea of denervation by stretching factors more than by hormonal effects. It has been proposed that the enlargement and stretching of the uterus at term may result in a dilution of trophic factors, and finally in a neuronal atrophy (Haase *et al.* 1997). This is in agreement with biochemical and immunohistochemical studies in the human uterine artery (Naves *et al.* 1998) or in the horn of the gravid uterus of the rat (Varol *et al.* 2000) where the nerve growth factor (NGF), its mRNA expression and its mature isoform  $\beta$  (Lobos *et al.* 2005) were decreased during the middle and late stages, at a time when the size of the uterus is increased and the noradrenergic nerves appeared structurally damaged. Electric-field-stimulation studies in isolate uterine rings of pregnant animals show a degeneration and loss of adrenergic nerve fibres apparent by Day 15, with a reduced responsiveness of uterine smooth muscle in early pregnancy.

A few days after delivery, the density of sympathetic innervation of the longitudinal myometrial layer increased, showing a similar pattern to that observed in early pregnant animals. Our results showed that in the non-fetus-bearing horn, the size of the myometrial layer was rapidly recovered without changes in the density of innervation. We interpreted these results as indicative that remodelling of the sympathetic uterine innervation during pregnancy has an important mechanical component. However, the participation of steroid hormones as modulators of sympathetic innervation could not be denied because some reports have indicated that oestrogen is able to deplete uterine sympathetic innervation by acting as a signal (Zoubina *et al.* 2001).

Furthermore, our results obtained by *in oculo* myometrial transplants, where the proportion of sympathetic innervation showed a dramatic reduction at the end of the gestational period and a significant increase after delivery, are in contrast with the idea of mechanical factors associated with denervation as the only component of this process. In this case, no stretching forces were participating in the response of the tissue, and the loosening of sympathetic fibres was observed in the same magnitude as in the longitudinal myometrial layer of the occupied horn in the pregnant animal and not in the empty horn. The differences observed in both models (prepubertal myometrial transplant v. adult tissue) could be associated with the maturity of uterine sympathetic nerve fibres and their vulnerability to the effects of gestational and post partum milieu. Previous studies have shown that the mature uterine sympathetic fibres are less susceptible to the effects of oestrogen than developing ones (Chávez-Genaro *et al.* 2002). In the same way, the *in oculo* transplants of myometrium from post partum guinea-pigs failed to support

sympathetic reinnervation in the myometrium of immature animals (Brauer *et al.* 1998). Moreover, in pregnant animals, the gestational milieu involves a major number of hormonal and local factors, and their effects on the degeneration and loss of sympathetic fibres (Thorbert 1978; Sporong *et al.* 1981; Alm *et al.* 1988) are accompanied by a progressive reduced responsiveness of uterine smooth muscle to electric field stimulation (Klukovits *et al.* 2002). The effects of gestational hormone milieu are also observed at central nervous system level. Wiesel *et al.* (2004) used a transneuronal viral tracing method to show a significant reduction of viral-labelled neurones in pregnant compared with virgin rats and with highest frequency of labelling occurring in lactating animals.

Our results support the idea that in the rat, uterine sympathetic innervation disappears at the end of the gestational period as a result of the interaction of hormonal and mechanical influences derived from the fetus-placenta complex, as has been reported in the guinea-pig model (Owman 1981; Alm *et al.* 1988). On the other hand, these results are in agreement with previous observations that immature sympathetic innervation is more sensitive to hormonal milieu than mature fibres (Chávez-Genaro *et al.* 2002).

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