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# Estrogen-induced collagen reorientation correlates with sympathetic denervation of the rat myometrium

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ABSTRACT

Estrogen inhibits the growth and causes the degeneration (pruning) of sympathetic nerves supplying the rat myometrium. Previous cryoculture studies evidenced that substrate-bound signals contribute to diminish the ability of the estrogenized myometrium to support sympathetic nerve growth. Using electron microscopy, here we examined neurite-substrate interactions in myometrial cryocultures, observing that neurites grew associated to collagen fibrils present in the surface of the underlying cryosection. In addition, we assessed quantitatively the effects of estrogen on myometrial collagen organization in situ, using ovariectomized rats treated with estrogen and immature females undergoing puberty. Under low estrogen levels, most collagen fibrils were oriented in parallel to the muscle long axis (83% and 85%, respectively). Following estrogen treatment, 89% of fibrils was oriented perpendicularly to the muscle main axis; while after puberty, 57% of fibrils acquired this orientation. Immunohistochemistry combined with histology revealed that the vast majority of fine sympathetic nerve fibers supplying the myometrium courses within the areas where collagen realignment was observed. Finally, to assess whether depending on their orientation collagen fibrils can promote or inhibit neurite outgrowth, we employed cryocultures, now using as substrate tissue sections of rat-tail tendon. We observed that neurites grew extensively in the direction of the parallel-aligned collagen fibrils in the tendon main axis but were inhibited to grow perpendicularly to this axis. Collectively, these findings support the hypothesis that collagen reorientation may be one of the factors contributing to diminish the neuritogenic capacity of the estrogen-primed myometrial substrate.



## 1. Introduction

Estrogen inhibits the growth and causes the degeneration (pruning) of sympathetic nerve fibers supplying the uterine smooth muscle (myometrium). These effects of estrogen are driven by changes in the ability of myometrium to support its sympathetic innervation. Moreover, evidences indicate that this plasticity is regulated by a complex multifactorial mechanism, involving a range of target-derived molecular signals with negative effects on sympathetic nerves (Brauer and Smith, 2015; Brauer, 2016).

In a previous study, we assessed the contribution of substrate-bound signals to the reduced neuritogenic capacity of the estrogenized myometrium using the tissue section culture method (cryoculture), (reviewed in Crutcher, 1993). In our studies, cryocultures consisted in culturing rat sympathetic ganglion explants on frozen tissue sections of rat myometrial longitudinal layer (Richeri et al., 2010; Brauer and Smith, 2015). The main outcomes of this investigation were that: (a) growing neurites preferentially followed the orientation of the main axis of the longitudinally sectioned smooth muscle; (b) neurites grew closely associated to the muscle bundles and generally avoided the areas of the section occupied by the connective tissue separating bundles; and (c) neurite outgrowth was markedly inhibited on myometrial sections from rats treated with estrogen. These results were interpreted to suggest that neurite growth was deeply influenced by features of the underlying tissue section; and that estrogen modifies myometrial substrate properties, so that it is less supportive for sympathetic neurite growth.

The mechanisms underlying the stereotyped growth of neurites on myometrial tissue sections and the reduced neuritogenic capacity of the estrogenized myometrial substrate are largely unknown. Preliminary observations, led us to the hypothesis that alterations in the spatial orientation of collagen fibrils might be involved (Martínez et al., 2009; Richeri et al., 2010). To further explore this hypothesis; in the present study we analyzed: (a) the nature of neurite-myometrial substrate interactions in cryoculture using transmission electron microscopy; (b) the extent of collagen rearrangements in situ, using as models

*Abbreviations:* ECM, extracellular matrix; LML, longitudinal myometrial layer; OVX, ovariectomized; SMCs, smooth muscle cells.

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adult OVX rats treated with estrogen (Zoubina et al., 2001) and immature rats undergoing natural puberty (Brauer et al., 1992); (c) whether in situ, changes in collagen orientation occurs in the areas of myometrium were sympathetic nerves distribute; and (d) the relevance of collagen alignment on the patterning and extent of sympathetic neurite outgrowth employing cryocultures where frozen tissue sections of rat-tail tendon were used as substrate.

We believe that these studies will add to the understanding of the nature of target-derived signals regulating plasticity in uterine sympathetic nerves. A better understanding of these mechanisms has gained relevance due to increasing evidences showing alterations in uterine nerves in different estrogen-regulated gynecological diseases (Brauer and Smith, 2015; Brauer, 2016).

## 2. Materials and methods

### 2.1. Animals and treatments

Studies were conducted on female Wistar-derived albino rats from the breeding colony held at the Instituto de Investigaciones Biológicas Clemente Estable (IIBCE, Montevideo, Uruguay). Animals were sexed at birth, weaned at 3 weeks and maintained under controlled conditions of temperature (21–22 °C) and illumination (12 h light/dark cycles) with water and food provided ad libitum. All animal procedures were conducted in accordance with standards of the Ethics Committee on the Use of Laboratory Animals (CEUA-IIBCE) regulated by the National Law No. 18611.

#### 2.1.1. Ovariectomy and estrogen treatment

Adult rats (10 weeks old) were anesthetized with 90 mg/kg ketamine (Unimedical, Uruguay) plus 10 mg/kg xylazine (Unimedical) and bilaterally ovariectomized through two small incisions in the lumbar region. Ovariectomized rats were treated with three subcutaneous injections of 50 μg of β-estradiol 17-cypionate (Laboratorios König, Argentina) given on days 5, 7, and 9 following surgery and euthanized on day 10. Control animals were treated with vehicle (peanut oil; Sigma-Aldrich, USA).

#### 2.1.2. Pubertal transition

Prepubertal animals were euthanized before the occurrence of vaginal canalization. Pubertal rats were euthanized at the first estrus following vaginal canalization, as determined by vaginal cytology (Brauer et al., 1992).

#### 2.1.3. Treatment with 5-hydroxydopamine

Prepubertal females were injected intraperitoneally with 100 mg/Kg of 5-hydroxy-dopamine (Sigma-Aldrich) diluted in saline solution containing 0.2% ascorbic acid and euthanized 20 h after treatment (Tranzer and Thoenen 1967; Knight 1980).

### 2.2. Cryocultures

Cryocultures were carried out as previously described (Richeri et al., 2010), using as substrate frozen tissue sections of the longitudinal myometrial layer (LML) or rat-tail tendon, both from adult OVX rats. Briefly, the uterine horns were removed under aseptic conditions, opened longitudinally and flat-mounted onto pre-cooled cryostat chucks. For the preparation of tendon tissue blocks, isolated rat-tail tendons were cut in 1-cm-long fragments and placed parallel to each other in flat bullet-shaped embedding molds (Sigma-Aldrich, USA: Cat. N° E4390) filled with Dulbecco's modified Eagle's medium (DMEM/F12, Sigma-Aldrich). When the mold cavity was filled with tendon fragments, the excess of liquid was removed and tissues allowed freezing at −20 °C. Cryostat tissue sections (14 μm thick) of myometrium or tendon blocks were thaw-mounted onto the bottom of 35-mm untreated plastic tissue-culture dishes (Falcon BD, USA).

Superior cervical ganglia from neonatal female rats (3–5 days old) were removed, freed from connective tissue and dissected into explants (500–600 μm in diameter). Each explant was placed on top of a tissue sections (Fig. 1a). Cultures were grown for three days in serum-free Neurobasal medium (Gibco, Invitrogen, USA) supplemented with 2% B-27 (Gibco, Invitrogen), 0.5 mM L-glutamine (Sigma-Aldrich), 1% antibiotics (Gibco, Invitrogen) and 0.5 ng/ml NGF (Harlan Bioproducts for Science, USA).

In rat-tail tendon cryocultures, neurites were demonstrated with a fluorescent vital dye (5-carboxyfluorescein diacetate, acetoxymethyl ester; Molecular Probes, Invitrogen) for 1.5 h and examined under a Nikon E800 fluorescence microscope. Images were captured using a CoolSNAP-Pro Monochrome Digital camera with Image ProPlus software (Media Cybernetics, USA), (Richeri et al., 2010). In cryocultures carried out on myometrial tissue sections, neurite outgrowth was assessed by contrast phase microscopy and cultures fixed and processed for transmission electron microscopy (TEM) as detailed below.

### 2.3. Electron microscopy

#### 2.3.1. Studies on myometrial cryocultures

Cultures were fixed for 5 min in 0.5% glutaraldehyde (Sigma-Aldrich) diluted in 0.1 M phosphate buffer (PB; pH 7.2) at room temperature (to reduce neurite retraction) followed by 2.5% glutaraldehyde for 1 h at 4 °C. After washing in PB, tissues were post-fixed in 1% osmium tetroxide (Sigma-Aldrich), dehydrated in alcohol series and embedded in Epon (Fluka, USA). Ultrathin sections, transverse to the direction of both the smooth muscle main axis and growing neurites were obtained (Fig. 1b, c), stained with uranyl acetate and lead citrate and examined under a Jeol 100CXII TEM operated at 60 kV.

#### 2.3.2. Studies on myometrium in situ

Uteri of adult OVX rats treated with estrogen or vehicle; and prepubertal and pubertal females were removed and freed from fat and connective tissue. From each uterine horn, two pieces of the middle region were dissected and fixed by immersion in 2.5% glutaraldehyde diluted in PB for 24 h at 4 °C. After washing, tissues were post-fixed in 1% osmium tetroxide for 1.5 h at 4 °C, dehydrated in alcohol series and absolute acetone and embedded in Durcupam ACM (Fluka). Ultrathin sections, transverse to the longitudinal myometrial layer main axis, were stained with uranyl acetate and lead citrate and examined under the TEM.

#### 2.3.3. Quantitative assessment of collagen orientation

Changes in collagen organization were quantitatively evaluated on images of the interstitial ECM surrounding transversely-sectioned smooth muscle cells (SMCs) of the LML. Measurements were carried out on five images obtained from four different regions (depths) of each uterine horn (20 images per horn). All data obtained from each horn ($n = 6$) were averaged and used for statistical comparison. As illustrated in Fig. 2, the following parameters were measured: (a) percentage area of the total image occupied by smooth muscle cells and interstitial ECM, and further calculation of the ECM/smooth muscle ratio; (b) percentage area of the interstitial ECM occupied by collagen; and (c) percentage of the total collagen area represented by fibrils oriented parallel and non-parallel to the smooth muscle cells long axis.

### 2.4. Immunohistochemistry for sympathetic nerves and histology

Uterine horns from prepubertal rats ($n = 3$) were fixed by immersion in 4% PFA for 1.5 h at 4 °C, washed in phosphate-buffered saline (PBS), stored in 12% sucrose in PBS overnight at 4 °C and embedded in tissue freezing medium (Shandon, USA). Cryostat tissue sections (8 μm) were obtained at two different depths of the middle region of the uterine horns, separated for at least 2 mm. For the demonstration of sympathetic nerves, sections were thaw-mounted onto gelatin-coated glass slides and incubated overnight at room temperature in a humid chamber with