Cell Tissue Res (2003) 314:191–205
DOI 10.1007/s00441-003-0799-9

REGULAR ARTICLE

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Plasticity in developing rat uterine sensory nerves: the role of NGF and TrkA

Received: 20 June 2003 / Accepted: 13 August 2003 / Published online: 13 September 2003

Abstract In the present study we investigated the effects of infantile/prepubertal chronic oestrogen treatment, chemical sympathectomy with guanethidine and combined sympathectomy and chronic oestrogen treatment on developing sensory nerves of the rat uterus. Changes in sensory innervation were assessed quantitatively on uterine cryostat tissue sections stained for calcitonin gene-related peptide (CGRP). Uterine levels of NGF protein, using immunohistochemistry and ELISA, and mRNA, using Northern blots and in situ hybridization, were also measured. Finally, levels of TrkA NGF receptor in sensory neurons of T13 and L1 dorsal root ganglia (DRG), which supply the uterus, were assessed using densitometric immunohistochemistry. These studies showed that: (1) chronic oestrogen treatment led to an 83% reduction in the intercept density of CGRP-immunoreactive nerves; (2) sympathectomy had no effect on the density of uterine sensory nerves or on the pattern of oestrogen-induced changes; (3) NGF mRNA and protein increased following sympathectomy or chronic oestrogen treatment; and (4) oestrogen produced increased intensity of labelling (28%) for TrkA receptors in small-diameter sensory neurons, but decreased labelling (13%) in medium-sized neurons, which represent the large majority of the DRG neurons supplying the upper part of the uterine horn. Contrary to expectations, increased levels of NGF after sympathectomy and oestrogen treatment did not lead to increased sensory innervation of the uterus. The possibility that alterations in neuronal levels of TrkA contribute to the lack of response of uterine sensory nerves to the oestrogen-induced increase in NGF levels is discussed.

Keywords Uterus · Sensory nerves · CGRP · Oestrogen · Plasticity · NGF · TrkA · Rat (Wistar)

This work was supported by The Wellcome Trust, UK (CRIG Grant 058122/Z/99/Z/JC/KO), and PEDECIBA, Universidad de la República, Montevideo, Uruguay

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Introduction

The uterus is supplied by sympathetic, parasympathetic and sensory nerves, which show considerable species variation with regard to their presence, distribution and relative density (Owman and Stjernquist 1988; Papka and Traurig 1992). Uterine sympathetic nerves undergo a profound remodelling in response to physiological and experimental changes in the circulating levels of sex hormones (Owman and Stjernquist 1988; Brauer et al. 1992, 1995; Zoubina et al. 1998, 2001); however, the influence of sex hormones on uterine parasympathetic and sensory nerves is less well established (Stjernquist et al. 1985; Alm and Lundberg 1988; Owman and Stjernquist 1988; Amira et al. 1995; Haase et al. 1997; Zoubina et al. 1998).

In previous studies we showed that sympathetic nerves to the uterus are particularly susceptible to oestrogen during postnatal development (Chávez-Genaro et al. 2002). Chronic administration of oestrogen to rats during the infantile/prepubertal period provokes, at 28 days of age, complete loss of intrauterine sympathetic nerves, comparable to that observed at late pregnancy (Haase et al. 1998). More recently, we showed that developing uterine cholinergic nerves are less susceptible to oestrogen than sympathetic nerves, and, following infantile/prepubertal chronic oestrogen treatment, a well-developed plexus of cholinergic nerves was observed in the rat uterus (Richeri et al. 2002). To further elucidate the effects of oestrogen on the postnatal development of uterine nerves, we have assessed the effects of infantile/prepubertal chronic oestrogen treatment on the peptidergic-sensory innervation of the rat uterus using immunohistochemical methods. In order to disclose to what extent oestrogen-induced changes in sensory nerves were related to the absence/impairment of sympathetic nerves, we compared the effects of chronic oestrogen treatment with those provoked by guanethidine-induced sympathectomy and combined sympathectomy and chronic oestrogen treatment.

Taking into account that nerve growth factor (NGF) has been shown to play an important physiological role in the development and maintenance of neural crest-derived sensory neurons (Levi-Montalcini 1987), and considering that in the periphery, sensory nerves compete with sympathetic nerves for NGF (Korsching and Thoenen 1985), we assessed the effects of chronic chemical sympathectomy with guanethidine and chronic oestrogen treatment on the uterine levels of NGF protein and messenger RNA. In addition, the pattern of distribution of NGF protein and mRNA was assessed on cryostat tissue sections using immunohistochemistry and mRNA in situ hybridization.

The recent demonstration that uterine-projecting sensory neurons in the dorsal root ganglia (DRG) express oestrogen receptor alpha and beta immunoreactivity and mRNA (Papka et al. 2001) raises the possibility that in addition to changes in the target levels of NGF, oestrogen could affect the uterine sensory innervation through changes in the responsiveness of neurons to neurotrophins. This possibility is reinforced by studies showing that oestrogen leads to alterations in the expression of NGF receptors in adult sensory neurons (Sohrabji et al. 1994; Liuzzi et al. 1999). In addition, sex hormone-induced upregulation of CGRP synthesis in female DRG neurons is mediated by NGF (Gangula et al. 2000) and involves alterations in the neuronal receptivity to neurotrophins (Lanlua et al. 2001a, 2001b). Neuronal responsiveness to NGF is mediated by two receptors: TrkA, a member of the family of tyrosine kinase receptors, and $p75^{NTR}$, a member of the tumour-necrosis factor receptor superfamily (Frade and Barde 1998). In this context, we carried out studies on the effects of chronic oestrogen treatment on the levels of the TrkA in retrograde-labelled uterine-projecting neurons of DRG using densitometric immunohistochemistry.

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) were used for this study. Animals were sexed at birth, weaned at 3 weeks and maintained under controlled conditions of temperature and illumination, with water and food ad libitum.

Sympathectomy

Females were injected daily (Monday to Friday) with 50 mg/kg of guanethidine monosulphate (Sigma, USA) beginning the 8th day after birth and continuing for 3 weeks (Brauer et al. 1994a; Richeri et al. 2002). Guanethidine was dissolved to appropriate doses with 0.1 M phosphate buffer saline (PBS) and administrated subcutaneously. Control animals from matched litters were injected with the vehicle.

Oestrogen treatment

Chronic oestrogen treatment was performed with β-oestradiol 17-cypionate (Laboratorios Köning, Argentina), diluted to appropriate doses with peanut oil (Sigma) to a final volume of 0.1 ml per dose. Intact and sympathectomized animals were injected subcutaneously with four doses of 10 μg oestrogen on days 10, 15, 20 and 25 of postnatal development (Brauer et al. 1995). Control animals from matched litters were injected with 0.1 ml of peanut oil per dose. All animals were killed at 28 days of age.

Retrograde tracing with Fluorogold and oestrogen treatment

Twenty-day-old rats (n=6) were anaesthetized with ether and the left uterine horn exposed through a small dorsal incision. Surgical procedures were conducted in accordance with the international guidelines for animal care approved by the IIBCE. Approximately 3 μl of a 4% solution of Fluorogold in PBS (FG, Fluorochrome, USA) was injected under aseptic conditions at one site close to the tubal end of the uterine horn using a 10-μl Hamilton syringe. After completion of injection, the area was dried and rinsed with sterile physiological saline solution to remove dye, which might have leaked from the injection site. The muscle and skin were sutured and the animals allowed to recover. Rats were injected with three doses of 20 μg of oestrogen 5, 7 and 9 days following FG administration and killed on day 10 (at 30 days of age).

Immunohistochemistry for CGRP, TH and TrkA

Animals were terminally anaesthetized with 50 mg/kg of sodium pentobarbital (Sigma) and perfused transcardially with 25 ml of heparinized saline solution (NaCl 0.9% + 50 IU/ml of Liquemine, Roche, Uruguay) followed by 25 ml of 4% paraformaldehyde (PFA) (Sigma). The uterine horns and T13 and L1 dorsal root ganglia (Nance et al. 1988) ipsilateral to the site of FG injection were dissected and fixed by immersion in 4% PFA for 1.5 h at 4°C. Tissues were washed in PBS, stored in 20% sucrose in PBS overnight at 4°C and embedded in tissue freezing medium (Shandon, USA). For the demonstration of sensory nerves cryostat sections (12 μm thick) were labelled with rabbit anti-calcitonin gene-related peptide (CGRP 1:800, Affinity Research Products, UK). In order to assess the extent of sympathectomy, some uterine

tissue sections were labelled with rabbit anti-tyrosine hydroxylase (TH 1:200, Affinity Research Products). For the demonstration of TrkA, ganglion tissue sections were labelled with rabbit anti-TrkA (1:400, Chemicon, USA). All incubations were carried out overnight in a humidified chamber and at room temperature. The specificity of the immunolabelling was checked by omitting the primary antibodies. At the end of the incubation period, sections were washed in PBS, incubated with goat anti-rabbit conjugated with Alexa-Fluor 568 (final dilution 1:400, Molecular Probes, USA) for 1.5 h at room temperature, washed in PBS and mounted in antifade mounting medium (Citifluor, London, UK). Sections were examined with a Nikon E800 microscope equipped with epifluorescence and fitted with the appropriate filters.

Quantitation of CGRP-I nerve fibres

In the uterine horn, the density of CGRP-immunoreactive (CGRP-I) myometrial fibres was assessed in the longitudinal myometrial layer (Chávez-Genaro et al. 2002). Nerve counting was carried out on three transverse cryostat sections of the cephalic region of the uterine horn per animal. Values recorded from each section and animal were averaged and used to calculate mean values. Under the fluorescence microscope and using a ×20 objective lens, five different myometrial areas per section were captured digitally using a CoolSNAP-Pro monochrome digital kit (Media Cybernetics, USA). Areas selected for nerve density estimations included: the antimesometrial border (one area), the mesometrial border (two areas) and the intermesometrial border (two areas). In order to obtain an estimate of the percentage area occupied by nerve fibres a stereological grid with an area of 0.18 mm^2 and line intersects at 17-μm intervals was superimposed over these myometrial areas and all the grid transects overlying nerve profiles were counted. The resulting number was multiplied by 100 and divided by the total number of grid squares occupied by the smooth muscle. Considering that oestrogen treatment provokes major changes in the size of the uterus, corrections for changes in the size of the target were carried out in order to estimate the total nerve area. To achieve this, the percentage area occupied by nerves was multiplied by the total area of the longitudinal myometrial layer and divided by 100. Estimations of the longitudinal myometrial layer area were performed on adjacent frozen tissue sections, mounted on gelatine-subbed glass slides, fixed by immersion in 4% PFA and stained with haematoxylin and eosin. Quantitative results are expressed as the mean ± SEM. Data were compared using a one-way ANOVA test followed by the Tukey-Kramer multiple comparisons test. Values of $P \leq 0.05$ were considered statistically significant.

Densitometric measurement of TrkA

Digital images of TrkA-labelled uterine-projecting neurons in the DRG were captured under identical conditions within the first 24 h after completing the immunostaining (Cowen and Thrasivoulou 1992) using a CoolSNAP-Pro monochrome digital camera and used for densitometric studies without any contrast or brightness corrections. Densitometric assessment of cytoplasmic fluorescence intensity (grey value) and neuronal size measurements were carried out using the Image Pro Plus software by Media Cybernetics (USA) (Gatzinsky et al. 2001). Grey values are expressed as the mean ± SEM. Data were compared using the unpaired Student's t test. Values of $P \leq 0.05$ were considered statistically significant.

ELISA for NGF

Tissue levels of NGF were measured in the whole uterine horn and isolated myometrium using a two-site enzyme-linked immunosorbent assay (ELISA) as described in detail in previous studies (Brauer et al. 2000b; Chávez-Genero et al. 2002). In view of the marked changes in the weight of the uterus provoked by oestrogen treatment, NGF protein levels are expressed as both total content of NGF per uterine horn and isolated myometrium and concentration per milligram of wet weight tissue.

Immunohistochemistry for NGF

Animals were anaesthetized with 50 mg/kg of sodium pentobarbital and perfused transcardially with 25 ml of heparinized saline solution followed by 25 ml of 2% PFA and 0.2% parabenzoquinone (Sigma) in 0.075 M phosphate buffer (Conner et al. 1992). The uterine horns were removed and fixed by immersion for 2 h at 4°C and cryoprotected in 30% sucrose in 0.1 M phosphate buffer overnight at 4°C. In order to improve the quality of comparisons, one cephalic portion of the uterine horn per animal group was embedded together in individual tissue blocks. For comparative purposes NGF immunoreactivity was assessed in both sympathetic and sensory neurons. Cryostat sections (20 μm thick) were mounted onto gelatine-subbed slides, washed for 10 min in TRIS-buffered saline (TBS), incubated for 5 min in TBS containing 0.3% hydrogen peroxide plus 3% normal goat serum, immersed for 20 min in TBS containing 0.25% Triton X-100 (Sigma) and blocked for 60 min in TBS plus 5% normal goat serum. Sections were then incubated overnight at room temperature in a humidified chamber with an affinity purified rabbit polyclonal antibody to β-NGF (final dilution 1:1,000), which was supplied by Dr. J. Conner (Conner et al. 1992). At the end of the incubation period, sections were washed in TBS, incubated in 1.5 μg/ml biotinylated goat anti-rabbit antibody (ABC Elite Kit, Vector Labs., USA) for 3 h at room temperature, washed in PBS, and incubated with an avidin-biotin-peroxidase reagent (ABC Elite Kit, Vector Labs., USA) for 90 min at room temperature. Sections were then reacted with a solution containing 0.04% diaminobenzidine (Sigma), 0.06% nickel chloride and 0.6% hydrogen peroxide for 3 min, washed in tap water and enhanced in osmium tetroxide vapours for 30 s (Rodrigo et al. 1996). Tissues were dehydrated and mounted in Entellan (Merck, Germany). The specificity of the immunolabelling was checked by omitting the incubation in the β-NGF antibody. Due to the limited amounts of antibody available, no preabsorption experiments were attempted. However, previous studies have demonstrated the specificity of this antibody (Conner et al. 1992; Chengsi and Crutcher 1995). Digital images of NGF-labelled tissues were captured under identical conditions using a CoolSNAP-Pro monochrome digital kit and submitted to identical contrast and brightness corrections using the Image Pro Plus software. Images were printed on Hewlett Packard HP Premium Inkjet glossy paper using a Hewlett Packard DeskJet 840C printer.

Isolation of cDNAs encoding rat NGF and GAPDH

Total RNA was extracted from adult male rat submandibular gland using Trizol reagent (Life Technologies), treated with DNase (Promega, USA) to remove possible contaminating genomic DNA and reverse transcribed with an oligo-dT primer and AMV reverse transcriptase (Promega) following the manufacturer's instructions. The cDNA obtained was subsequently used as a template for PCR reactions. PCR primers for amplification of rat NGF and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed corresponding to the coding region of these genes according to Zhou et al. (1999): NGF primers, sense 5'-TCAGCATTCCCTTGACACAGC-3' and antisense 5'-TCCAGTGCTTGGAGTCAATGC-3'; GAPDH, sense 5'-TGCTGGTGCTGAGTATGTCG-3' and antisense 5'-GCATGTCAGATCCACAACGG-3'. These primers were used in the PCR reactions following standard techniques. PCR products of the expected size were isolated (Concert Gel Extraction System, Life Technologies), cloned into pGEM-T-Easy (Promega) and sequenced using standard protocols on an ABI Prism 377 DNA sequencer. The recombinant plasmids obtained were named rRngf and rRgapdh.

Northern blot analysis on NGF mRNA

Total RNA from freshly isolated rat uterine horns of prepubertal controls, chronic oestrogen-treated and guanethidine-treated, was extracted using Trizol reagent (Life Technologies) and quantified by optical density reading at 260 nm. Integrity was assessed by visualization under ultraviolet light following electrophoresis on agarose gels and staining with ethidium bromide (ratio of 28S/18S RNA species $\geq$2). Equivalent amounts of total RNAs (40 μg per lane) were fractionated on a 1.5% agarose/formaldehyde gel and transferred by capillary action to Hybond-N^+ membranes (Amersham) and fixed by baking for 2 h at 80°C. The insert of rRngf used for hybridization was radiolabelled by the random priming method using Klenow polymerase. The blot was pre-hybridized for 2 h with hybridization buffer (50% formamide, 0.8 M NaCl, 50 mM phosphate buffer, 5 mM EDTA, 0.1% SDS, ×10 Denhardt's solution, 100 μg/ml salmon sperm DNA, 5% dextran sulphate). Hybridizations were performed overnight at 42°C with 2×10^6 cpm/ml probe in hybridization buffer. In order to reduce non-specific binding, high stringency washes ranging from 2×SSC (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS (sodium dodecyl sulphate), at 42°C for 30 min, to 0.2× SSC, 0.1% SDS at 65°C for 20 min were employed. Filters were exposed for 2 days at −80°C. The filters were then stripped and re-probed with radiolabelled insert of rRgapdh under the same conditions. Signals from non-saturated autoradiograms were captured digitally at 800 dpi using a Genius ColorPage Vivid Plus scanner. Digital images were printed on Hewlett Packard HP Premium Inkjet glossy paper using a Hewlett Packard DeskJet 840C printer.

The mean linear optical density (grey value) of each band and surrounding background was measured from digital images using the Image Pro Plus software. Following background subtraction, the optical densities of GAPDH bands of treated animals were normalized to GAPDH values measured in controls (considered 100%), and the value obtained used to correct measurements of NGF. Considering that a very recent study has shown that oestrogen upregulates GAPDH mRNA in both immortalized sheep endometrial stroma cells and human endometrial adenocarcinoma cells (Farnell and Ing 2003), NGF transcripts were also normalized to the 28S ribosomal RNA bands, visualized after ethidium bromide staining. These bands showed a consistently uniform optical density. Similarly, GAPDH transcripts were normalized to the 28S ribosomal RNA signal. Quantitative data were compared using a one-way ANOVA test followed by the Tukey-Kramer multiple comparisons test. Values of $P\leq0.05$ were considered statistically significant.

In situ hybridization of NGF mRNA

Sense and antisense digoxigenin-labelled riboprobes were generated from the rRngf using T7 and Sp6 polymerases (RNA labelling kit, Boehringer Mannheim, Germany). Unfixed fresh uterine horns from the three different animal groups were frozen in embedding tissue freezing medium at −20°C. Cryostat tissue sections (12 μm) were mounted on silane-coated slides (Silane-Prep slides, Sigma), washed in PBST (1×PBS, 0.1% Tween-20) and incubated for 15 min at room temperature in 1 μg/ml proteinase K in PBST. Slides were washed twice (5 min each) in PBST, and fixed for 20 min in 4% paraformaldehyde in PBST containing 0.1% glutaraldehyde. After fixation, sections were washed twice in PBST, incubated at 65°C for 10 min in order to inactivate endogenous alkaline phosphatases and pre-hybridized for 2 h at 50°C with hybridization solution [50% deionized formamide, 1.3×SSC (sodium saline citrate), pH 5.3, 5 mM EDTA, 50 μg/ml yeast tRNA, 0.2% Tween-20, 0.5% CHAPS (Sigma), 100 μg/ml heparin]. The hybridization was carried out in a humidified chamber overnight at 50°C with 50 μl of the hybridization solution containing 2 ng/μl of previously denatured (95°C, 3 min) digoxigenin-labelled RNA probe. Posthybridization washes were carried out with 2×SSC at 50°C (two washes of 20 min each), 1×SSC at 50°C (two washes of 20 min each), and 1×SSC at room temperature (two washes of 15 min each). The sections were equilibrated with MAB solution (100 mM maleic acid, pH 7.5, 150 mM NaCl) containing 0.1% Tween-20 (MAB-T), at room temperature (two washes of 10 min each) and pre-blocked with MAB containing 1% BSA (MAB-B) for 30 min at room temperature and blocked with 5% heat-inactivated sheep serum in MAB-B (blocking solution) for 2 h at room temperature. Sections were incubated overnight at 4°C with 1:2,000 alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody (Boehringer) in blocking solution. After washing with MAB-T, sections were rinsed twice with developing solution (100 mM NaCl, 100 mM TRIS-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20) and incubated with 4.5 μl NBT (nitroblue tetrazolium) and 3.5 μl BCIP (5-bromo-1-chloro-3-indolyl phosphate) per millilitre of developing solution. After colour development, sections were washed twice with PBST, fixed in 4% paraformaldehyde in PBS for 30 min and mounted with 70% glycerol. Digital images of NGF-labelled tissue were captured under identical conditions using a CoolSNAP-Pro monochrome digital kit and submitted to identical contrast and brightness corrections using the Image Pro Plus software. Images were printed on Hewlett Packard HP Premium Inkjet glossy paper using a Hewlett Packard DeskJet 840C printer.

Results

Effects of oestrogen and sympathectomy on uterine CGRP-I nerves

In prepubertal controls, CGRP-I fibres penetrated the uterus through the mesometrium, accompanying blood vessels or as free bundles and isolated fibres (Fig. 1A). Within the uterus, CGRP-I nerves were distributed in both the circular and longitudinal myometrial layer and around blood vessels located in the intramyometrial vascular space (Fig. 1B, C). Some nerve fibres were seen in the endometrium associated with the vasculature as well as with endometrial epithelium and glands (Fig. 1B, C). This pattern of innervation showed, however, considerable regional variation. Most of the fibres associated with the longitudinal myometrial layer were seen in the cephalic region of the uterine horn (Fig. 1B), whereas most of the innervation associated with the circular myometrial layer was restricted to the caudal region (Fig. 1C).

The pattern of CGRP-I fibres was unaffected by chronic oestrogen treatment, but the innervation was considerably sparser. Several well-preserved perivascular and non-vascular CGRP-I fibres were seen in the mesometrium and mesometrial triangle (Fig. 1D). Within the uterus, several varicose nerve fibres and preterminal bundles were recognized in both the longitudinal (Fig. 1E) and circular (Fig. 1F) myometrial layers and around blood vessels. Quantitative studies showed that the percentage area occupied by CGRP-I nerves in the longitudinal myometrial layer was reduced to 17% of control values following chronic oestrogen treatment (Table 1), but the reduction could be accounted for by changes in the area of the longitudinal myometrial layer (Table 1).

Following guanethidine sympathectomy, a complete loss of sympathetic nerves in both intact and oestrogen-treated animals was confirmed (not shown). Sympathectomy had no obvious effects on the pattern and density of

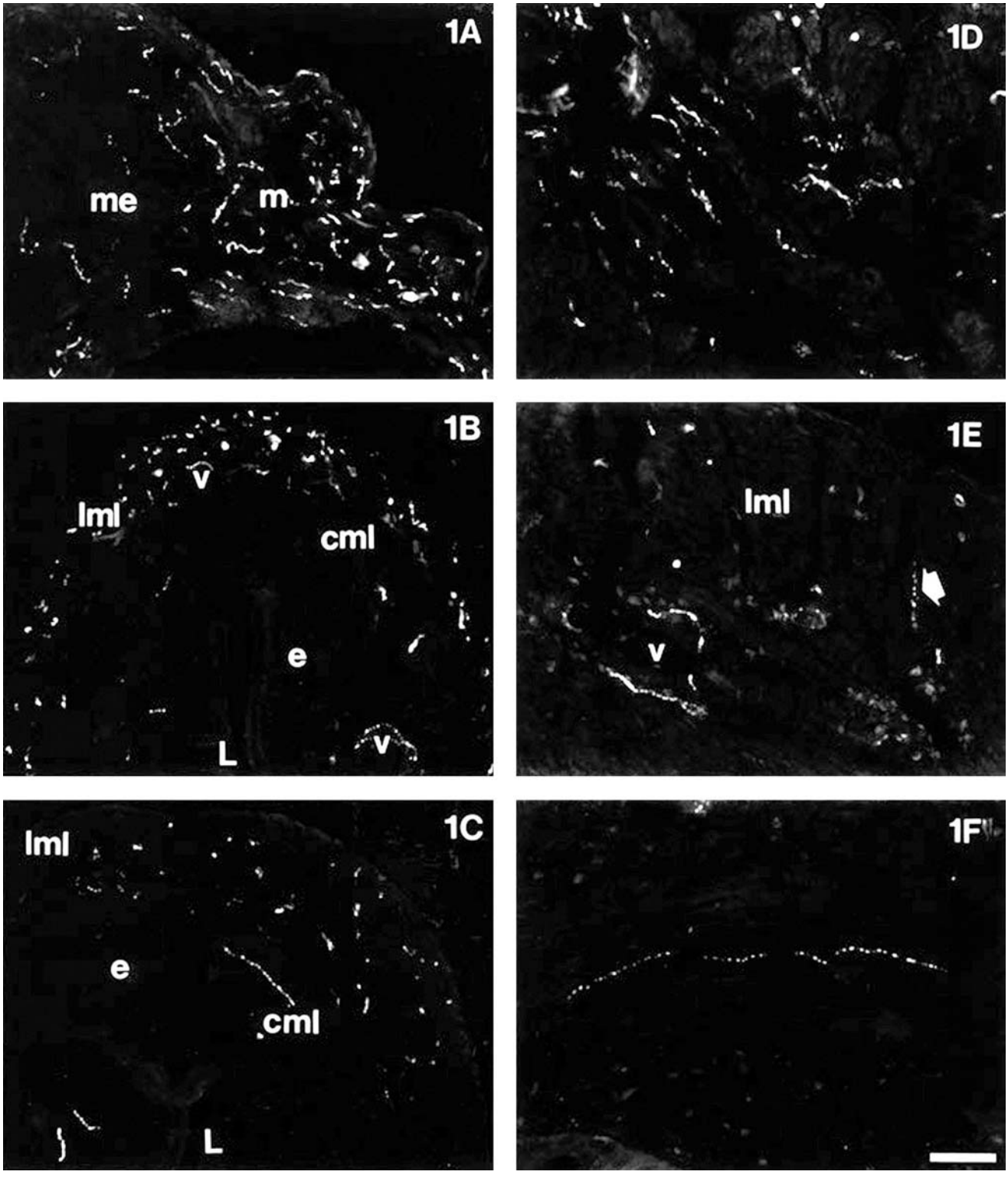


Fig. 1 Transverse cryostat tissue sections of the rat uterine horn showing the pattern of distribution of calcitonin gene-related-immunoreactive (CGRP-I) nerves in prepubertal controls **(A–C)** and following infantile/prepubertal chronic oestrogen treatment **(D–F)**. **A** illustrates the innervation of the mesometrium (*m*) and mesometrial entrance (*me*). **B** and **C** show, respectively, the differential pattern of distribution and density of CGRP-I nerve fibres in the cephalic and caudal region of the uterine horn. **D, E** and **F** illustrate, respectively, "oestrogen-resistant" CGRP-I nerve fibres in the mesometrium, longitudinal myometrial layer (*arrow*) and circular myometrial layer (*cml* circular myometrial layer, *e* endometrium, *L* uterine lumen, *lml* longitudinal myometrial layer). *Scale bar in* **F** 100 μm

Table 1 Quantitative assessment of the effects of chemical sympathectomy using guanethidine (*GTD*), infantile/prepubertal chronic oestrogen treatment (*COT*) and combined sympathectomy and infantile/prepubertal chronic oestrogen treatment (*GTD+COT*) on the area of the longitudinal myometrial layer (*LML*), the percentage area and total nerve area occupied by CGRP-immunoreactive nerves in the LML of the cephalic region of the rat uterine horn. Results are expressed as the mean ± SEM (*n*). Data were compared using a one-way ANOVA test followed by the Tukey-Kramer multiple comparisons test

	Area LML (mm^2)	Nerve area (%)	Nerve area (mm^2)
Control	0.06±0.003 (6)	14.9±0.7	0.009±0.002
GTD	0.05±0.004 (6) ns	17.3±0.9 ns	0.009±0.002 ns
COT	0.50±0.04 (6) a, b	2.5±0.2 a, b	0.013±0.003 ns
GTD+COT	0.52±0.01 (6) a, b	2.1±0.2 a, b	0.011±0.003 ns

Values of $P \leq 0.05$ were considered statistically significant: *a* significant difference with control, *b* significant difference with GTD, *ns* non-significant

Table 2 Effects of chemical sympathectomy with guanethidine (*GTD*) and chronic oestrogen treatment (*COT*) on the wet weight (mg), NGF protein total content (pg) and NGF protein concentration (pg/mg wet weight tissue) in the rat uterus. Results are expressed as the mean ± SEM (*n*). Data were compared using a one-way ANOVA followed by the Tukey-Kramer multiple comparison test

Treatment	Tissue weight	NGF protein total content	NGF protein concentration
Total uterine horn			
Control	20.7±0.9 (8)	11.2±1.2	0.55±0.07
GTD	17.5±1.0 (8)	43.9±8.1 a	2.71±0.64 a
COT	87.1±3.3 (13) a, b	30.6±4.2 a	0.36±0.06 b
Isolated myometrium			
Control	14.7±0.9 (5)	10.8±0.9	0.74±0.05
GTD	8.9±1.2 (6)	31.3±5.8 a	3.68±0.58 a
COT	31.9±7.1 (6) a, b	36.4±11.9 a	1.33±0.33 b

Values of $P \leq 0.05$ were considered statistically significant: *a* significant difference with control, *b* significant difference with GTD

CGRP-I nerves. Quantitative measurements (Table 1) showed that neither the percentage area nor the total area occupied by CGRP-I nerves in the longitudinal myometrial layer was affected by guanethidine treatment. Similarly, the pattern of oestrogen-induced changes was unaffected by sympathectomy (Table 1).

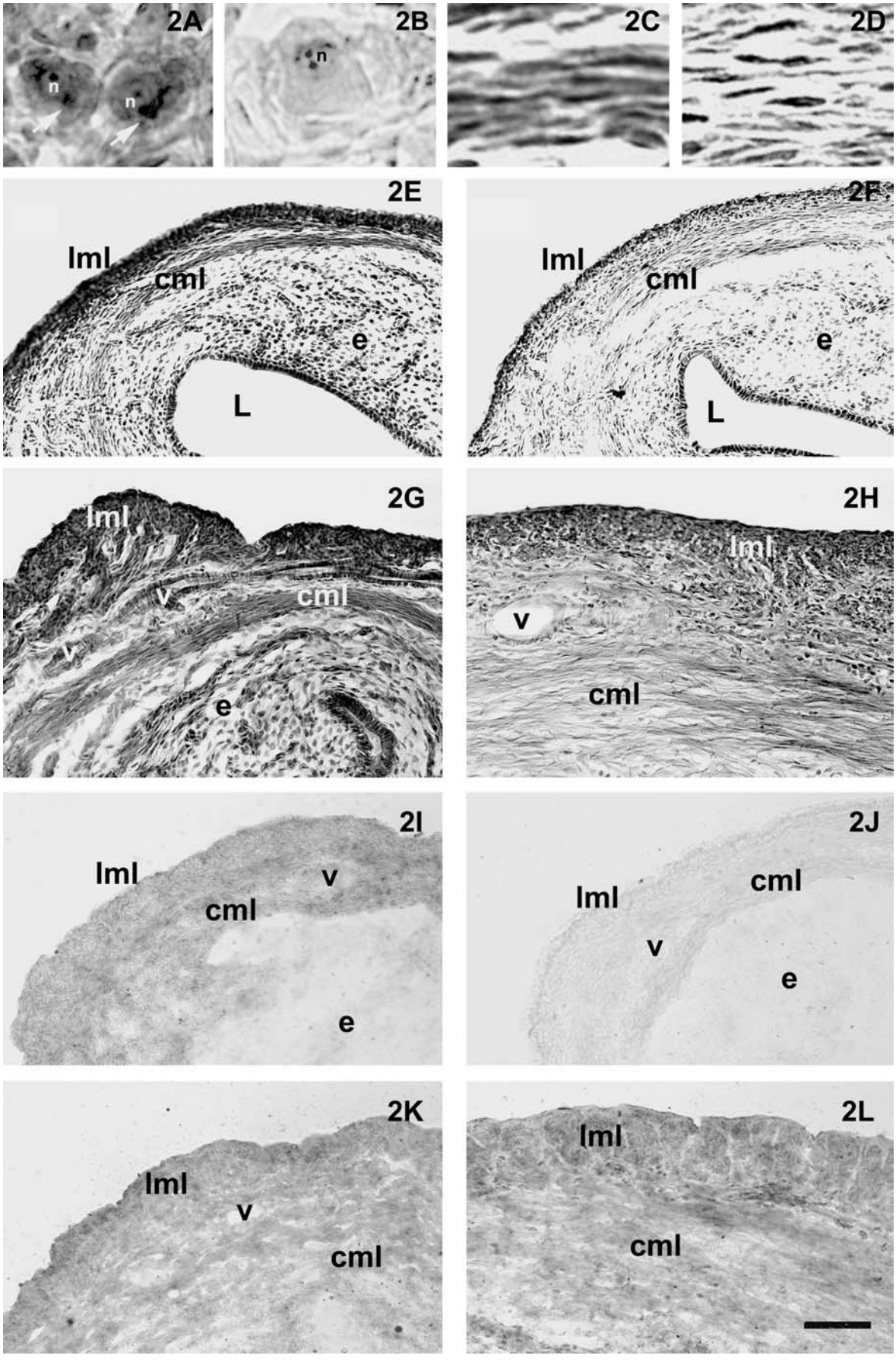
Morphological localization of NGF

In neurons, NGF immunoreactivity was observed as a dark cytoplasmic perinuclear precipitate (Fig. 2A). This labelling was specific, as demonstrated by omitting the primary antibody (Fig. 2B). In the uterine horn of control animals, most of the NGF-like specific immunostaining was seen in the myometrial compartment (Fig. 2E, F), labelling the cytoplasm of myometrial smooth muscle cells (Fig. 2C, D). No nervous structures were revealed by NGF immunostaining. Sympathectomy (Fig. 2G) did not alter the pattern of distribution of NGF immunolabelling; however, immunostaining appeared to be slightly more intense in guanethidine-treated animals than in controls. The pattern and density of NGF immunostaining was unaffected by both infantile/prepubertal chronic oestrogen treatment (Fig. 2H). In situ hybridization studies showed a similar pattern of uterine NGF mRNA localization (Fig. 2I–L).

Effects of oestrogen and sympathectomy on levels of NGF protein and mRNA

The results of the NGF protein assays are summarized in Table 2. In all animal groups, most of the NGF protein was confined to the myometrial compartment. Following sympathectomy, a marked increase in the total content of NGF protein was observed in both the uterine horn and isolated myometrium. Since sympathectomy did not alter the wet weight of these tissues, this represents a fivefold increase in NGF protein concentration. Following chronic oestrogen treatment, NGF content in the uterine horn showed a threefold increase. Because this change

Fig. 2A–L Histological localization of NGF protein and mRNA. **A** shows the distribution of NGF immunoreactivity in sympathetic neurons of the rat suprarenal ganglion. Immunoreactivity appears as a dark precipitate in the neuronal cytoplasm (*arrows*). This labelling is absent when the first antibody is omitted **(B)**. Nucleoli (*n*) are seen in both preparations. **C** illustrates the distribution of NGF-like immunoreactivity in myometrial smooth muscle cells. Specific immunoreactivity labels the smooth muscle cell cytoplasm. Note that when the first antibody was omitted **(D)** only cell nuclei appear stained. **E, G, H** show, respectively, the distribution of NGF-like immunoreactivity in the uterine horn of a prepubertal control rat, and following guanethidine sympathectomy and chronic oestrogen treatment. In **F**, the first antibody was omitted. **I, K** and **L** illustrate, respectively, transverse cryostat tissue sections of the rat uterine horn hybridized with the NGF antisense RNA probe in a prepubertal control and following guanethidine sympathectomy and chronic oestrogen treatment. **J** shows a tissue section from a prepubertal control hybridized with the sense RNA probe (*cml* circular myometrial layer, *e* endometrium, *lml* longitudinal myometrial layer, *L* lumen, *v* blood vessel). *Scale bars* 10 μm **(A–D)**, 100 μm **(E–L)**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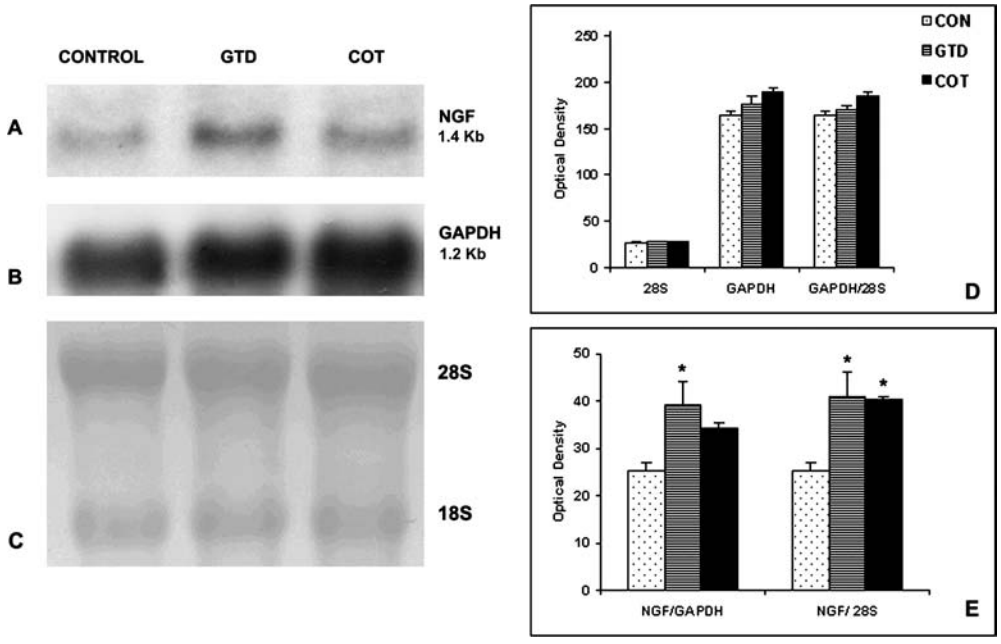

Fig. 3A–E Northern blot analysis of rat uterus total RNA (40 μg per lane), showing the effects of chemical sympathectomy with guanethidine (*GTD*) and chronic oestrogen treatment (*COT*) on levels of uterine NGF mRNA. **A** shows the 1.4-kb signal detected by the 473-bp cDNA NGF probe. **B** shows the 1.2-kb signal detected by the 475-bp cDNA GAPDH probe. **C** illustrates the 28S and 18S ribosomal RNA bands in an ethidium bromide-stained gel (inverted contrast). **D** summarizes the effects of GTD and oestrogen (*COT*) on the mean grey level (optical density) of the 28S ribosomal RNA, GAPDH and GAPDH normalized to the 28S signal. **E** shows the effects of sympathectomy (*GTD*) and oestrogen (*COT*) on the levels of NGF mRNA normalized to GAPDH (*NGF/GAPDH*) and to the 28S ribosomal RNA (*NGF/28S*). Mean data (n=3) were compared using a one-way ANOVA test followed by the Tukey-Kramer multiple comparisons test. Values of $P \leq 0.05$ were considered statistically significant

matched the increase in the wet weight of the uterus, our results indicate that no significant changes in protein concentration occurred. Similar effects were seen in the isolated myometrium following chronic oestrogen treatment.

Results of Northern blot assays are summarized in Fig. 3. The GAPDH signal was slightly increased following both guanethidine sympathectomy and chronic oestrogen treatment (Fig. 3B). However, optical density measurements showed that this increase was not statistically significant (Fig. 3D). Similarly, no significant changes in GAPDH levels were detected following normalization to the 28S RNA signal (Fig. 3C, D). Uterine levels of NGF mRNA were significantly increased following guanethidine sympathectomy (Fig. 3A, E). In oestrogen-treated animals, uterine levels of NGF mRNA—normalized to GAPDH—showed a non-significant 39.5% increase. Following normalization to the 28S RNA signal, the oestrogen-induced increase in NGF mRNA levels was 58.5% of control values and was significant.

Effects of oestrogen on the levels of TrkA in uterine-projecting neurons of the dorsal root ganglia

In controls, the diameter of FG-labelled neurons in T13 and L1 dorsal root ganglia ranged between 11 and 43 μm and fell into the three established size categories (Papka et al. 2001). Small neurons (less than 20 μm) represented 16.1% of FG-labelled neurons; medium-sized neurons (20–40 μm) comprised 83.8% and large neurons (more than 40 μm) represented less than 1% of the total. The overall size and relative proportion of these DRG neurons were unaffected by chronic oestrogen treatment.

The effect of oestrogen on TrkA fluorescence intensity is shown in Table 3. In small neurons, a 28% increase in the mean grey value was observed following chronic oestrogen treatment. In contrast, TrkA fluorescence intensity showed a 13% decrease in medium-sized DRG neurons of oestrogen-treated animals. Control FG-labelled medium-sized neurons showed considerable variations in TrkA fluorescence intensity (Fig. 4A–D) and measurement studies detected grey values ranging from 30 to 190 linear optical density units (LODU). Quantitative studies (Table 3) showed that in controls, 27% of medium-sized FG-labelled DRG neurons displayed low

Fig. 4 Cryostat sections of dorsal root ganglia of prepubertal rat controls **(A–D)** and following chronic oestrogen treatment **(F, G)** showing Fluorogold-traced medium-sized sensory neurons labelled for TrkA. **B** illustrates a control uterus-projecting neuron (*a*) showing low levels of TrkA. In **D** a control uterus-projecting neuron (*b*) displaying high levels of TrkA is observed. **G** shows three oestrogen-treated medium-sized Fluorogold-traced neurons. One of them (*a*) shows very low levels of TrkA, whereas the other two (*b*) show high levels. *Scale bar* 20 μm

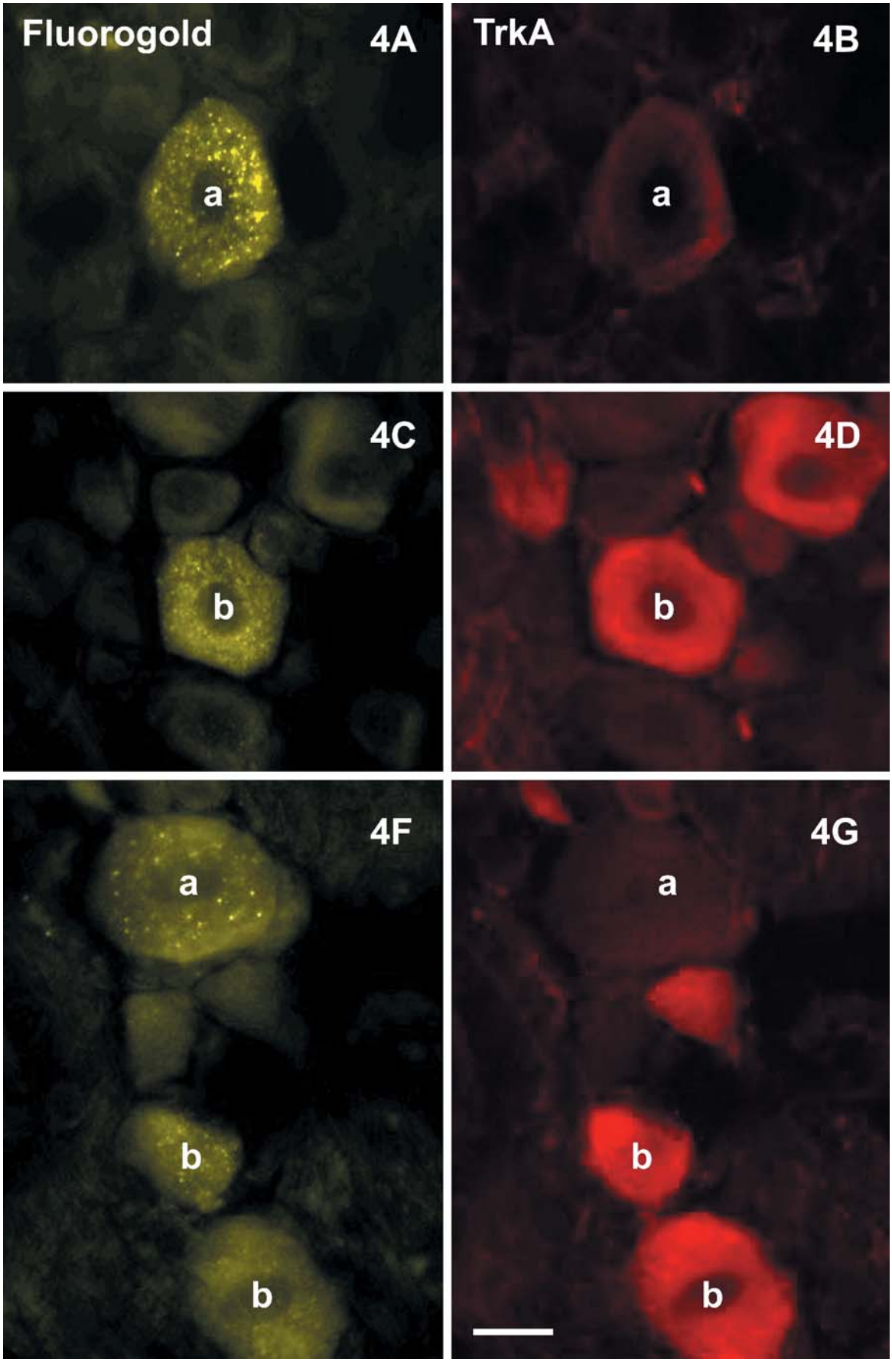
Fluorogold
4A
a
TrkA
4B
a
4C
b
4D
b
4F
a
b
b
4G
a
b
b

Table 3 Summary of (*a*) the effects of chronic oestrogen treatment (*COT*) on TrkA fluorescence intensity (grey level) in small and medium-sized Fluorogold-labelled uterine-projecting neurons of T13 and L1 dorsal root ganglia and (b) oestrogen-induced changes in the number of medium-sized neurons displaying low (less than 80 linear optical density units, LODU) and high (more than 80 LODU) TrkA fluorescence intensity. Fluorescence intensity is expressed as the mean ± SEM (*n* number of neurons). Single comparisons between groups were done using the unpaired Student's *t* test

	(a) Mean linear optical density		(b) Number of medium-sized neurons	
	Small	Medium	<80 LODU	>80 LODU
Control (three rats)	65.7±5.8 (17)	99.2±3.8 (88)	24/88 (27%)	64/88 (73%)
COT (three rats)	83.8±3.6 (63)*	86.0±2.8 (180)**	90/180 (50%)	90/180 (50%)

*$P \leq 0.05$, **$P \leq 0.01$

grey levels (less than 80 LODU), whereas the remaining 73% showed higher TrkA fluorescence intensity (more than 80 LODU). Following chronic oestrogen treatment (Fig. 4E, F), the range of grey values was similar to controls (32–182 LODU); however, the number of medium-sized neurons showing low TrkA fluorescence intensity (less than 80 LODU) increased from 27% to 50% while the number of neurons displaying high fluorescence intensity (more than 80 LODU) decreased from 73% to 50%.

Discussion

Effects of oestrogen and sympathectomy on uterine sensory nerves

The results reported in this study regarding the pattern of distribution and relative density of CGRP-immunoreactive nerves in the prepubertal rat uterus are in agreement with previous findings on the cycling animal (Papka and Traurig 1992; Zoubina et al. 1998). Following infantile/prepubertal chronic oestrogen treatment, several myometrial and perivascular CGRP-I nerve fibres were seen in the rat uterus, but the myometrial innervation was considerably sparser. Consistently, nerve density measurements showed that oestrogen induced a marked reduction in the percentage area occupied by CGRP-I nerves in the longitudinal myometrial layer. However, this change was not evident after correction for changes in the size of the myometrial layer, suggesting that early exposure to oestrogen prevents the matching of sensory nerves as the uterus enlarges. These results indicate that while developing sensory nerves are inhibited by oestrogen from growing in response to target expansion, this inhibition is less than that seen in developing sympathetic nerves (Brauer et al. 1995; Chávez-Genero et al. 2002). This contention is supported by the observation that, unlike sympathetic nerves, the density of CGRP-I nerves is unaffected by the endocrine changes accompanying the natural oestrous cycle (Zoubina et al. 1998). The pattern of oestrogen-induced changes observed in CGRP-I nerves was also different from that of cholinergic nerves, whose development appears to be promoted by oestrogen (Richeri et al. 2002).

The factors determining differential susceptibility to oestrogen of sympathetic, sensory and parasympathetic uterine nerves still remain unclear; however, some explanations can be advanced. For example, alpha- and beta-oestrogen receptors have been shown to be present in uterine-projecting sensory, parasympathetic and sympathetic neurons (Williams and Papka 1996; Williams et al. 1997; Papka et al. 1997, 2001; Zoubina et al. 2002). It is therefore possible that differential responses to oestrogen in these three neuronal populations could be related to differences in the pattern and/or timing of expression and co-expression of alpha- and beta-oestrogen receptors (Papka and Storey-Workley 2002). On the other hand, recent studies have shown that, in addition to direct effects on neurons, some of the effects of oestrogen on uterine sympathetic nerves are mediated through changes in the target uterine tissue (Brauer et al. 2000a, 2002; Chávez-Genaro et al. 2002; Krizan-Agbas and Smith 2002). It is therefore possible that some of the differential responses to oestrogen in uterine-projecting sympathetic, parasympathetic and sensory nerves could be initiated by different signals provided by the target and/or by differences in the responsiveness of neurons to these signals.

Several studies have led to the concept that removal of a population of nerve fibres induces changes in the density of innervation and/or in neurotransmitter expression in the remaining nerves supplying these target tissues (Kessler et al. 1983). For instance, increased density of sympathetic nerves and noradrenaline levels has been shown following removal of sensory nerves in different peripheral targets, including the uterus (Terenghi et al. 1986; Luthman et al. 1989; Brauer et al. 1994b; Sann et al. 1995). Similarly, sympathectomy results in an increase in the density of sensory nerves and neuropeptide levels (Allen et al. 1985; Van Ranst and Lauweryns 1990; Aberdeen et al. 1990; Mione et al. 1992). Our current studies show that neither the pattern nor the density of uterine CGRP-I nerves is affected by sympathectomy, thus confirming previous findings showing that sympathectomy did not increase the levels of CGRP and substance P in the uterus (Brauer et al. 1994b). Even more relevant, the current results indicate that sympathectomy does not increase the growth rate of uterine sensory nerves following chronic oestrogen treatment, indicating that uterine sensory nerves are less responsive to sympa-

thetic denervation than those supplying other peripheral organs.

Effects of oestrogen and sympathectomy on uterine NGF

Our current immunohistochemical data show that NGF-like immunoreactivity is confined to the neuronal cytoplasm, thus confirming previous observations (Conner et al. 1992; Zhou et al. 1994; Chengsi and Crutcher 1995). In the uterus, most of the NGF was restricted to the myometrial compartment, thus matching the distribution of sensory and sympathetic nerves (Korsching and Thoenen 1983; Harper and Davis 1990). Several studies demonstrate that NGF is synthesized by a variety of cells present in different projection fields of NGF-responsive neurons, including epithelial cells, smooth muscle cells, fibroblasts and Schwann cells (Bandtlow et al. 1987; Thoenen et al. 1988). In contrast to observations in the iris (Rush 1984; Finn et al. 1986), no nervous structures were immunoreactive for NGF in the uterus. A similar pattern of NGF-like immunoreactivity was recently reported for the mouse uterus (Bjorling et al. 2002); however, we did not observe strong epithelial NGF-like immunostaining as reported for the mouse.

Reciprocal relationships between sympathetic and sensory nerves are thought to be mediated by changes in the availability of NGF in the target (Kessler et al. 1983; Korsching and Thoenen 1985; Hill et al. 1988; Schicho et al. 1998), and several studies have shown that pharmacological and surgical denervation as well as nerve lesions and tissue culture elicit an increase in NGF protein levels in the targets of NGF-responsive neurons (reviewed by Rush et al. 1995). Sympathectomy-induced increases in NGF protein are thought to result from altered synthesis and/or protein accumulation, due to the absence of uptake by nerves. However, the relative contribution of these two mechanisms appears to be affected by the denervation procedure (reviewed by Rush et al. 1995). Results reported in this paper show that uterine levels of NGF protein and mRNA coordinately increase following chemical sympathectomy with guanethidine, suggesting that removal of developing sympathetic nerves by this method induces the synthesis of NGF in the rat uterus. However, since NGF protein increases more than mRNA, it is possible that other mechanisms such as protein accumulation and/or alterations in protein (Vizzard et al. 2000) and mRNA stability (Farnell and Ing 2003) contribute to the increased NGF protein levels.

Like sympathectomy, infantile/prepubertal chronic oestrogen treatment also leads to a marked increase in the total content of NGF protein in the uterus, thus confirming previous findings (Chávez-Genaro et al. 2002; Bjorling et al. 2002). Our Northern blot assays showed that the analysis of results of oestrogen treatment on uterine NGF mRNA levels is influenced by the gene used for normalization. Considering that previous studies showed that oestrogen could enhance expression of some commonly used controls such as beta-actin (Guo and Gorski 1988), we employed the widely used housekeeping gene GAPDH as a control. Following chronic oestrogen treatment, uterine NGF mRNA levels showed a 39.5% increase compared with the GAPDH signal. However, this increase was not statistically significant. This result could be related to the observation that the GAPDH signal was also slightly increased in oestrogen-treated animals. Although this increase was not statistically significant, this observation supports the recent observation that GAPDH expression is increased by oestrogen in endometrial cells (Farnell and Ing 2003). To overcome this possibility, we also normalized our NGF mRNA data to the signal of the 28S ribosomal subunit, which was consistently stable in our assays. Based on this comparison, the increase in NGF mRNA levels following oestrogen treatment was more evident (58.5%) and statistically significant. These results emphasize the importance of selecting appropriate controls for Northern blot assays in oestrogen-susceptible targets.

Coordinated changes in uterine NGF protein and message have been reported in the rat uterus during middle and late pregnancy (Varol et al. 2000) as well as following ovariectomy and sex hormone administration to ovariectomized mice (Bjorling et al. 2002). Sex hormone-induced increases in NGF mRNA levels could result from a direct effect of oestrogen on uterine NGF-producing cells. This possibility is consistent with the presence of oestrogen receptors in the uterus (Ennis and Stumpf 1989), and supported by the observation that sequences similar to the classical oestrogen response element mediate oestrogen-induced upregulation of BDNF in the developing brain (Sohrabji et al. 1995; Toran-Allerand 2000). The possibility that induction of NGF synthesis is related to an oestrogen-induced inflammatory-like response in the uterus should not be disregarded (Heumann et al. 1987).

Possible factors preventing the growth of sensory nerves in response to increases in the uterine levels of NGF

Factors preventing the growth of sensory nerves in response to increases in the uterine levels of NGF still remain unclear. Although it is becoming clear from in vivo and in vitro studies that postnatal NGF-dependent sensory and sympathetic neurons lose their survival dependence on neurotrophins during the first weeks of postnatal life (Schwartz et al. 1982; Easton et al. 1997; Orike et al. 2001a, 2001b), it is known that other aspects of neuronal physiology, such as axonal growth, remain under the influence of neurotrophic factors during the adult life and in ageing.

In addition to changes in neurotrophic factor availability, it has been demonstrated that neurotrophin receptor expression or its regulation might contribute to neuronal plasticity (Cowen et al. 2003). In this context, we conducted preliminary studies to evaluate the possibility that oestrogen could alter the neuronal responsiveness to NGF in uterine-projecting neurons of the dorsal

root ganglia. These studies show that oestrogen provokes contrasting effects in levels of TrkA in different populations of uterine-projecting DRG neurons. Optical density of TrkA immunofluorescence increased by 28% in small-diameter neurons, but decreased by 13% in medium-sized neurons, which represent the large majority of the DRG neurons projecting to the upper part of the uterine horn. However, within this population there may be differential changes. The reduction of immunofluorescence appears to be the result of the effect of oestrogen on a subpopulation of medium-sized uterine-projecting neurons rather than a general decrease in the levels of TrkA in the whole population of uterine-projecting medium-sized neurons. These results suggest that oestrogen may regulate plasticity in uterine sensory nerves through changes in the neuronal responsiveness to NGF. However, factors controlling differential oestrogen-induced changes in the expression of TrkA in uterine-projecting DRG neurons remain unclear. Differences in the pattern of oestrogen receptor expression (Papka et al. 2001) and/or differential interactions with selected regions or tissues in target uterus (i.e., blood vessels, endometrium and myometrium) could be potential explanations.

The effect of oestrogen on TrkA levels in DRG neurons in general still remains controversial. Some studies have shown that acute oestrogen treatment increases levels of TrkA mRNA (Sohrabji et al. 1994) and protein (Lanlua et al. 2001a) in DRG neurons. In contrast, long-term oestrogen replacement has been shown to decrease TrkA mRNA in these sensory neurons (Liuzzi et al. 1999). However, it is important to point out that these studies assessed whole ganglia by biochemical methods and could not, therefore, detect differences in the behaviour of specific neuronal populations or subpopulations.

It is known that dorsal root ganglion neurons are remarkably diverse and several classifications have been employed to divide these neurons into subpopulations, including size, and cytological, chemical and physiological properties. For instance, primary sensory neurons (Bergman et al. 1999), including those projecting to the uterus, synthesize a variety of neuromodulators, such as CGRP, substance P, galanin, nitric oxide, neurokinin A and calretinin (Gibbins et al. 1985; Alm and Lundberg 1988; Papka 1990; Papka and McNeil 1992a, 1992b; Papka et al. 1999). In addition, sensory neuron subpopulations can also be identified by their expression and co-expression of different Trk isoforms (i.e. TrkA, TrkB and TrkC) (Mu et al. 1993; McMahon et al. 1994; Wright and Snider 1995; Kashiba et al. 1995), and consequently by their growth responses to different neurotrophic factors (Gavazzi et al. 1999).

During prenatal development, 70–80% of DRG neurons express TrkA and require NGF for survival. However, in the first 2 weeks of postnatal life, the percentage of TrkA-positive DRG neurons decreases markedly (Bennett et al. 1996a; Molliver and Snider 1997), and this phenomenon is independent of NGF levels (Molliver and Snider 1997). In the rat L4/5 DRG, the adult proportion of NGF-responsive neurons (40%) is attained at 14 days of age (Bennett et al. 1996a), and 92% of these neurons express CGRP (Averill et al. 1995; Bennett et al. 1996b). Over the same period (1–14 days after birth), there is a marked increase in the proportion of L4/5 DRG neurons which bind the lectin IB4 (Bennett et al. 1996b). These neurons are dependent on NGF for survival during prenatal development. However, early in the postnatal period, these neurons downregulate TrkA expression and become responsive to glial-derived neurotrophic factor (GDNF) (Bennett et al. 1998; Gavazzi et al. 1999). Neurons labelled by IB4 were often regarded as non-peptidergic; however, it has been recently shown that many IB4-positive neurons in DRG express CGRP and remain dependent on NGF (Kashiba et al. 2001). Although it is not known whether uterine-projecting sensory neurons undergo a similar pattern of changes, it is possible that infantile/prepubertal chronic oestrogen treatment increases the proportion of DRG neurons that naturally downregulate TrkA expression during the early postnatal development. Additional studies will be necessary to analyse this possibility.

Acknowledgements The authors gratefully acknowledge the generous donation of NGF antibody by Dr. J.M. Conner. The technical assistance of Mrs. M.M. Portela, K. Shockley and N. Cuña is greatly appreciated.

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