Innervation of the proximal urethra of ovariectomized and estrogen-treated female rats

P.G. Smith and S. Bradshaw

R. L. Smith Mental Retardation and Developmental Disabilities Research Center, and

Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas, USA

Summary. The proximal urethra plays a central role in maintaining urinary continence, and sympathetic excitatory innervation to urethral smooth muscle is a major factor in promoting tonic contraction of this organ. Elevated estrogen levels are often associated with incontinence in humans. Because elevated estrogen levels result in degeneration of sympathetic nerves from the closely related uterine smooth muscle, we examined the effects of chronic estrogen administration on proximal urethral innervation. Ovariectomized virgin female rats received either vehicle or 17 ß-estradiol for 1 week, and smooth muscle size and parasympathetic, sensory and sympathetic nerve densities were assessed quantitatively throughout the first 3 mm of the proximal urethral smooth muscle. In vehicle-infused ovariectomized rats, parasympathetic nerves immunoreactive for vesicular acetylcholine transporter were most abundant, while calcitonin gene-related peptide-immunoreactive sensory nerves and tyrosine hydroxylase-immunoreactive sympathetic nerves were less numerous. The densities of parasympathetic and sensory nerves remained constant along the proximal urethra, while sympathetic nerves showed a significant increase along a proximal-distal gradient. Administration of 17ß-estradiol for 7 days via subcutaneous osmotic pump did not change smooth muscle area in sections, and neither densities nor total innervation of any nerve population was altered.

These findings reveal a rich cholinergic innervation of the proximal urethra, and a pronounced gradient in sympathetic innervation. Unlike the embryologically similar uterine smooth muscle, estrogen does not influence muscle size or composition of innervation, indicating that estrogen's actions on innervation are highly target-specific. Thus, estrogen's effects on urinary continence apparently occur independently of any significant remodeling of smooth muscle or resident innervation.

Key words: Urethra, Autonomic innervation, Estrogen

Offprint requests to: Peter G. Smith, Ph.D., Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KS, 66160-7401. Fax: (913) 588-5677. e-mail: psmith@kumc.edu

Introduction

The relationship between gonadal steroidal hormones and visceral smooth muscle innervation remains poorly understood. Smooth muscle of the uterus is significantly affected by serum estrogen (E2) levels, such that myometrial size and contractile properties are increased. Moreover, uterine innervation is profoundly altered by E2. Increasing plasma E2 in rodents leads to massive sympathetic nerve degeneration, while other populations of uterine nerves apparently are unaffected (Zoubina et al., 1998, 2001). Therefore, in at least some visceral targets, E2 elicits marked plasticity of smooth muscle innervation.

It is not clear whether E2 affects sympathetic innervation of other smooth muscle targets, but there is some evidence that it may. One possible candidate is the urethra. Urethral smooth muscle normally is tonically contracted by sympathetic innervation, which is essential in maintaining urinary continence (van Geelen et al., 1982; Dubrovsky and Filipini, 1990). Like the uterus, the urethra originates embryologically from the urogenital ridge of the intermediate mesoderm, and these structures are continuous as the cloaca prior to their separation by the formation of the urorectal septum (Smythe and Jacoby, 1953; Sadler, 1990). During pregnancy when E2 levels are high, stress urinary incontinence is common (Stanton et al., 1980; Iosif and Ulmsten, 1981), and can also occur in association with E2 fluctuations during the menstrual cycle (Miodrag et al., 1988). Although the relationship between E2 and urethral sphincter innervation apparently has not been examined, pregnancy in rats is associated with degeneration of bladder sympathetic nerves (Qayyum et al., 1989). Because structural changes in urethral innervation could underlie some forms of incontinence, this represents a potentially important area of investigation.

In the present study, we treated ovariectomized (OVX) rats chronically with E2 and compared urethral innervation with that of untreated OVX rats. As E2 could affect not only sympathetic but also sensory and parasympathetic innervation, nerve densities of all these axonal populations were assessed using highly selective markers. Because E2 also may be affecting smooth

muscle size, this too was measured. Finally, since innervation density may vary regionally, quantitative analyses from rigidly standardized locations of the proximal urethra were performed.

Materials and methods

Animals and tissue preparation

Experiments were conducted on Sprague-Dawley rats (Harlan). National Institutes of Health (NIH) guidelines on laboratory animal care were followed, and all experimental protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee. Twenty female virgin 2-3-month-old rats were kept two to three per cage in a light- and climate-controlled room with a 12-hour light-dark cycle starting at 6 AM; they received food and water ad libitum.

Rats were anesthetized with a mixture of ketamine hydrochloride (60 mg kg^{-1} i.p., Lloyd Laboratories, Shenandoah Iowa, USA), xylazine hydrochloride (8 mg kg^{-1}, Rompun, Bayer, Shawnee Mission, KS, USA), and atropine sulfate (0.4 mg kg^{-1}, Vedco, St. Joseph, MO, USA). Bilateral ovariectomies were performed on the rats according to previously described procedures (Zoubina et al., 2001). One week later, pellets containing 17ß-estradiol (21-day release, 0.1 mg/tablet; Innovative Research of America, Sarasota, Florida, USA) or placebo were implanted subcutaneously. Previous studies have shown that these procedures result in sustained E2 plasma levels of 220±33 pg/mL (Zoubina et al., 2001).

One week after pellet implantation, rats were deeply anesthetized with urethane (1.5 g kg^{-1}) and the bladder with urethra was exposed through a ventral incision by trephining the pubic symphysis. The trigonal region of the bladder together with approximately 5 mm of attached urethra was excised as a single block. Tissues from 5 estrogen-treated and 5 vehicle-infused OVX rats were immersed in TBS™ Tissue Freezing Medium (Triangle Biomedical Sciences, Durham NC, USA) and fresh-frozen on dry ice. Tissues from an additional 5 E2-treated and 5 OVX rats were also removed and placed overnight in picric acid-paraformaldehyde Zamboni's fixative at room temperature (Zoubina et al., 1998). Tissues were rinsed in PBS daily for 1 week at 4 °C and frozen in TBS medium as described above. Blocks were stored at -80 °C until sectioned.

The proximal urethra together with a portion of the trigone muscle was cryosectioned serially at 10 µm thickness and sections were thaw-mounted onto silane-coated slides as 6 sets, with 200 µm interposed between adjacent sections in a set. Sections were collected beginning central to the intersection of the ureters with the trigone muscle.

Immunofluorescence

To visualize smooth muscle in different regions of the urinary tract, tissue sections were fixed for 5 minutes in 4% paraformaldehyde, washed in PBST, blocked (5% goat serum, with 10mg/ml bovine serum albumin in PBST) for 20 min, and stained for 90 min at room temperature with Cy3-conjugated antibody to α-smooth muscle actin (α-SMA) (1:400, mouse monoclonal IgG, Sigma). Sections were washed in PBST and coverslipped in Fluoromount (Southern Biotechnology Associates, Birmingham, Alabama).

The pan-neuronal marker PGP 9.5 was used to visualize total innervation. Preliminary studies showed that this antiserum worked optimally in Zamboni-fixed urethra. Sections were stained overnight at 4 °C with rabbit IgG directed to PGP 9.5 (1:400,Biogenesis, Brentwood, NH), followed by application for 90 min of Cy3-conjugated goat-anti-rabbit secondary antibody (1:400; Jackson ImmunoResearch, West Grove, PA).

To assess sensory nociceptor innervation, sections were stained with an antibody to calcitonin gene-related peptide (CGRP). Optimal staining for this antigen was obtained in Zamboni-fixed tissue, and sections prepared in this manner were incubated overnight at 4 °C with rabbit antisera to CGRP (1:400; Chemicon, Temecula, CA), followed by Cy3-conjugated goat-anti-rabbit secondary antibody (1:400, Jackson Immunoresearch, West Grove, PA).

Sympathetic innervation was visualized by immunostaining sections for the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH). Preliminary investigations showed staining was optimal in fresh-frozen tissue. Sections were fixed for 5 min in 4% buffered formalin and stained overnight with a mouse monoclonal antibody to TH (1:100, DiaSorin, Stillwater, MN) followed by a 90 min incubation with Cy3 -conjugated goat-anti-mouse secondary antibodies.

To identify parasympathetic nerves, fresh-frozen sections, which provided optimal staining, were fixed in PFA and incubated overnight with an antibody to vesicular acetylcholine transporter (donkey IgG, 1:200, Chemicon, Temecula CA), followed by Cy3 donkey-anti-goat secondary antibody (1:100; Jackson Immunoresearch, West Grove, PA) for 90 minutes.

Quantitation

One stepped series from each set of sections was immunostained with α-SMA and viewed. The last section containing ureters in contact with bladder smooth muscle was taken as a fixed reference point (the vesico-ureteral junction, VUJ), which in specimens oriented for sectioning corresponded approximately to the beginning of the urethra. Sections were then selected for analysis at 1-mm intervals beginning at the VUJ and continuing through 2 mm distal. To assess smooth muscle sectional area, images of α-SMA immunostained sections were captured (Nikon Eclipse TE300 microscope with Optronics MagnaFire camera) and smooth muscle area was determined using a thresholding function (NIH image).

To provide an index of smooth muscle innervation,

nerve density was quantified in 3 randomly selected fields from each of the sections selected stained for neuronal antigens. A stereology grid with intersections at 20 µm intervals (Scion Image) was superimposed over a captured image. The number of intersections overlying stained nerves was counted, and divided by the total number of intersections overlying urethral smooth muscle to provide an index of the density of urethral smooth muscle innervation (% area). To provide an index of the total innervation present within smooth muscle at a given level, the average nerve density was multiplied by the smooth muscle area in mm2 measured from adjacent α-SMA-stained sections. Negative controls were conducted for all antibody staining protocols, which included primary antibody omission and preadsorbtions with the relevant antigen. Data were analyzed by two-way ANOVA with post-hoc comparisons by the Student- Newman-Keuls test (P<0.05 taken as a significant difference).

Results

Innervation of the proximal urethra in ovariectomized rats

PGP 9.5-immunoreactive innervation

Immunostaining of all intact nerves using antisera to PGP 9.5 revealed a dense ground plexus, which was restricted primarily to smooth muscle with fewer numbers in adjacent connective tissue (Fig. 1). PGP 9.5-ir innervation density did not vary significantly from the level of the VUJ through a distance 2 mm distal (Fig. 2). When normalized for changes in smooth muscle volume (Table 1), the total apparent sectional area occupied by PGP 9.5-ir nerves showed a significant decline in conjunction with the decrease in smooth muscle size between 0 and 1 mm distal to the VUJ (P=0.042).

Vesicular acetylcholine transporter (VAChT)-immunoreactive innervation

Nerves immunostained by VAChT antisera formed a plexus throughout the smooth muscle with a density that appeared to approximate that of PGP 9.5-ir nerves (Fig. 3). There was no detectable regional variation in density either above or below the VUJ (Fig. 4). When total innervation was estimated by normalizing for differences in smooth muscle mass, VAChT-ir area decreased between the VUJ and 1 mm distally (P=0.02) but did not decrease further (Fig. 4).

Calcitonin Gene-Related Peptide (CGRP)-immunoreactive innervation

CGRP-ir nerves were present within smooth muscle, connective tissue, and occasionally within the
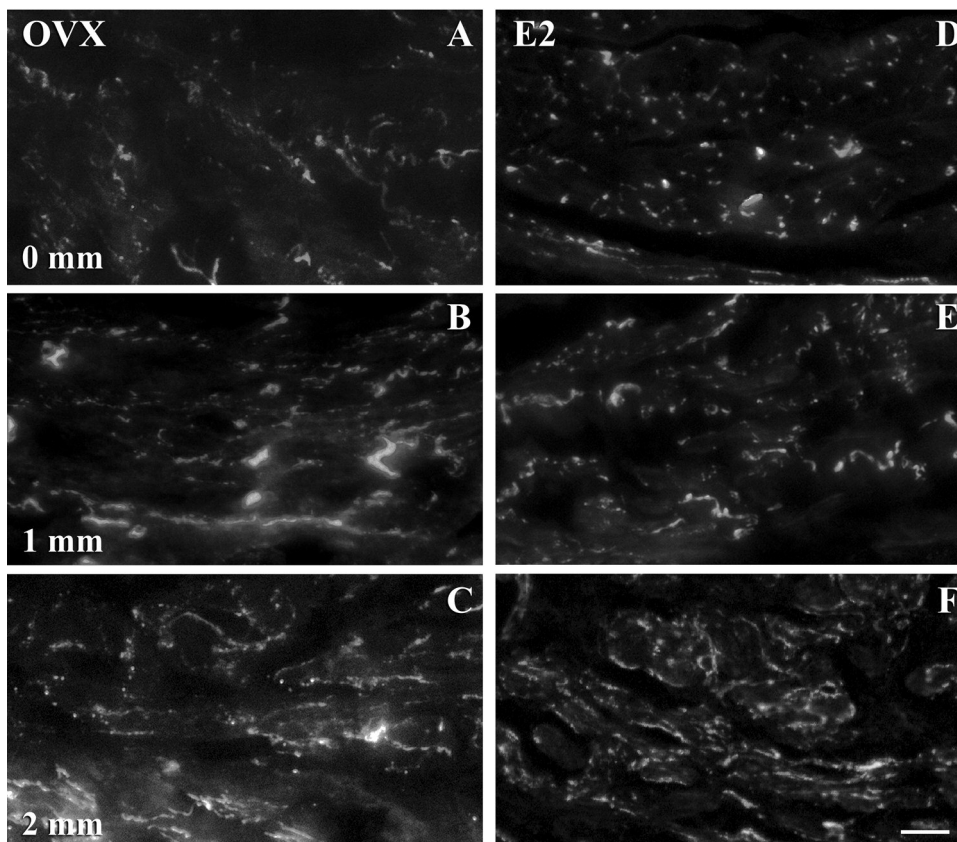

Fig. 1. Total proximal urethral innervation as visualized by immuno-staining for PGP 9.5 immunoreactivity. Images were obtained from the level of the vesico-ureteral junction (0 mm, **A and D**), 1 mm **(B and E)** and 2 mm (**C and F**) distal. **A, B, and C** were obtained from ovariectomized rats (OVX), and **D, E, and F** from ovariectomized rats treated for 1 week by continuous estrogen administration (E2). Scale bar in F: 25 µm for all pictures.

PGP 9.5
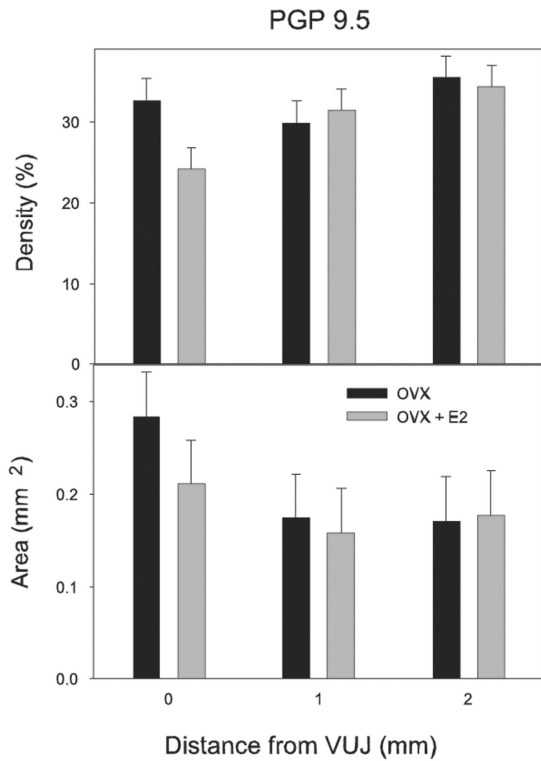
urothelium (Fig. 5). Within the smooth muscle, innervation density remained constant along the length of urethra studied, while total CGRP-ir axon area decreased between 0 and 1 mm distal to the VUJ (P=0.043, Fig 6). CGRP innervation density and total innervation area both were lower than that of PGP 9.5- and VAChT-ir innervation (p<0.01).

Tyrosine Hydroxylase (TH) immunoreactive innervation

Nerves immunoreactive for TH were localized to the circular smooth muscle (Fig. 7). Innervation density remained constant from the VUJ to a distance 1 mm distal, but increased significantly between 1 and 2 mm distal to the VUJ (Fig. 8, P=0.021). Despite the reduction in smooth muscle mass distally, the total area occupied by TH-ir nerves did not change (Fig. 8).

Fig. 2. Quantitative analyses of the PGP 9.5-immunoreactive innervation of the proximal urethra at the level of the vesico-ureteral junction (0) and 1 and 2 mm distal in ovariectomized (OVX) and ovariectomized rats receiving 1 week of continuous estrogen administration (OVX + E2).
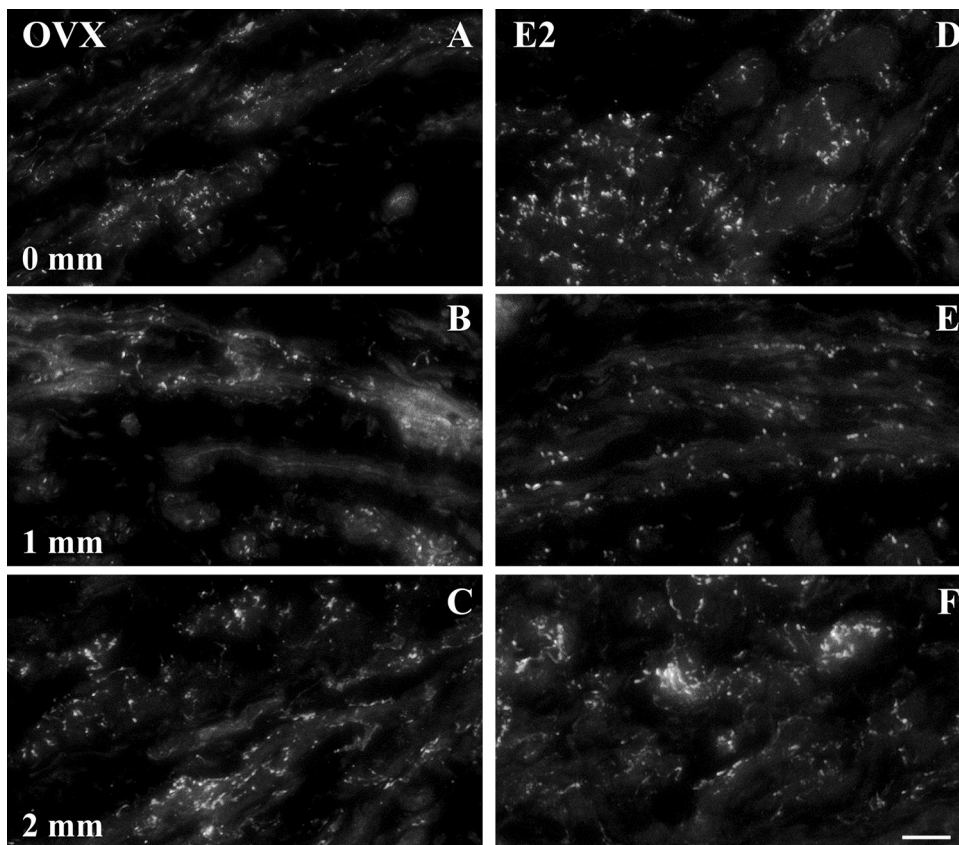

Fig. 3. Proximal urethral innervation as visualized by immunostaining for vesicular acetylcholine transporter immunoreactivity. Images were obtained from the level of the vesico-ureteral junction (0 mm, **A and D**), 1 mm **(B and E)** and 2 mm **(C and F)** distal. **A, B, and C** were obtained from ovariectomized rats (OVX), and **D, E, and F** from ovariectomized rats treated for 1 week by continuous estrogen administration (E2). Scale bar in F: 50 µm for all pictures.

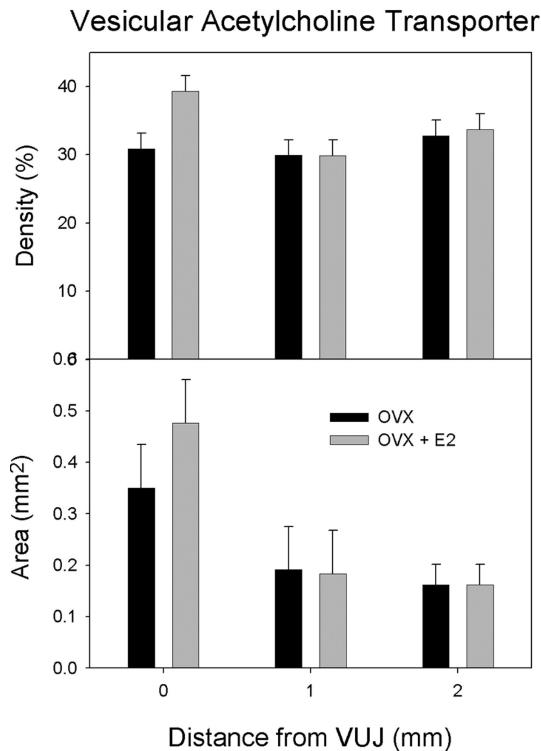## Vesicular Acetylcholine Transporter

Fig. 4. Quantitative analyses of the vesicular acetylcholine-immunoreactive innervation of the proximal urethra at the level of the vesico-ureteral junction (0) and 1 and 2 mm distal in ovariectomized (OVX) and ovariectomized rats receiving 1 week of continuous estrogen administration (OVX + E2).

Overall, TR-ir innervation was significantly lower than that of PGP 9.5- and VAChT-ir innervation (P<0.01) and comparable to that of CGRP-ir innervation.

Estrogen administration to ovariectomized female rats

Estrogen administration did not significantly affect the size of the urethral smooth muscle (Table 1), or distributions or numbers of urethral smooth muscle nerves immunoreactive for PGP 9.5 (Figs. 1, 2), VAChT (Figs. 3, 4), CGRP (Figs. 5, 6) or TH (Figs. 7,8).

Discussion

Innervation of the female urethra in ovariectomized rats

This study confirms a rich and varied innervation within the proximal urethra of the female rat.
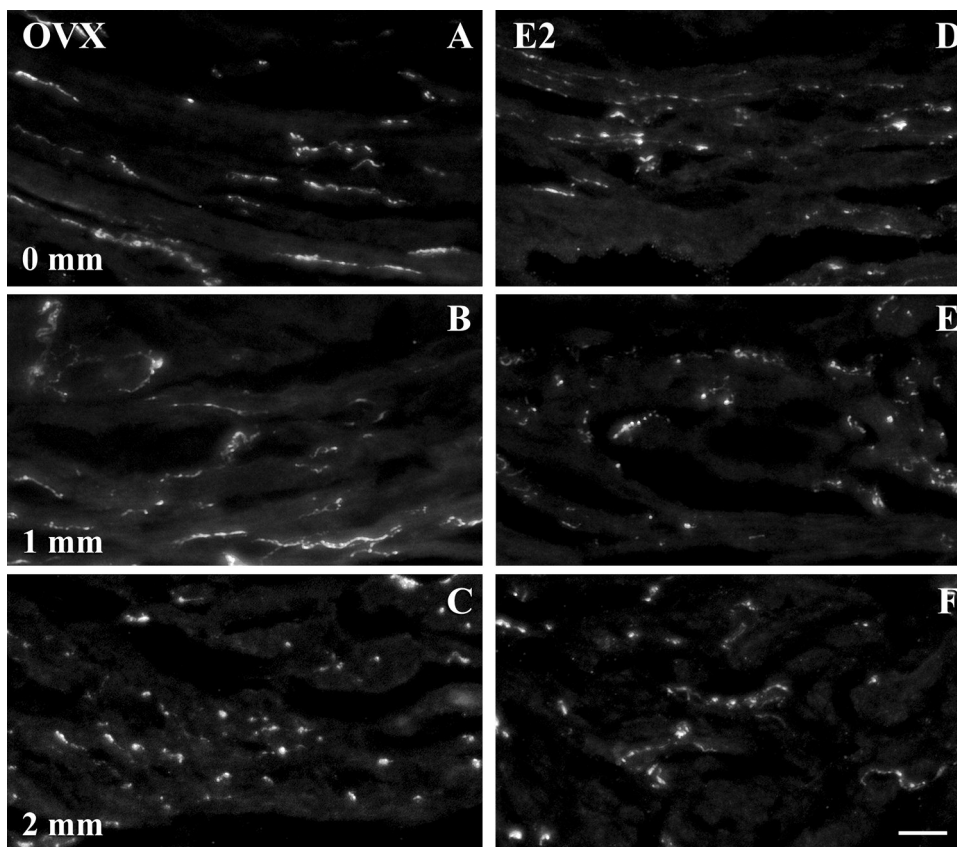

Fig. 5. Proximal urethral innervation as visualized by immunostaining for calcitonin gene-related peptide immunoreactivity. Images were obtained from the level of the vesico-ureteral junction (0 mm, **A and D**), 1 mm **(B and E)** and 2 mm **(C and F)** distal. **A, B, and C** were obtained from ovariectomized rats (OVX), and **D, E, and F** from ovariectomized rats treated for 1 week by continuous estrogen administration (E2). Scale bar in **F**: 50 μm for all pictures.

CGRP

Immunostaining for PGP 9.5 revealed a dense plexus of peripheral nerves within and around smooth muscle, confirming earlier studies (Alm et al., 1995). Our quantitative data show that overall nerve density remains
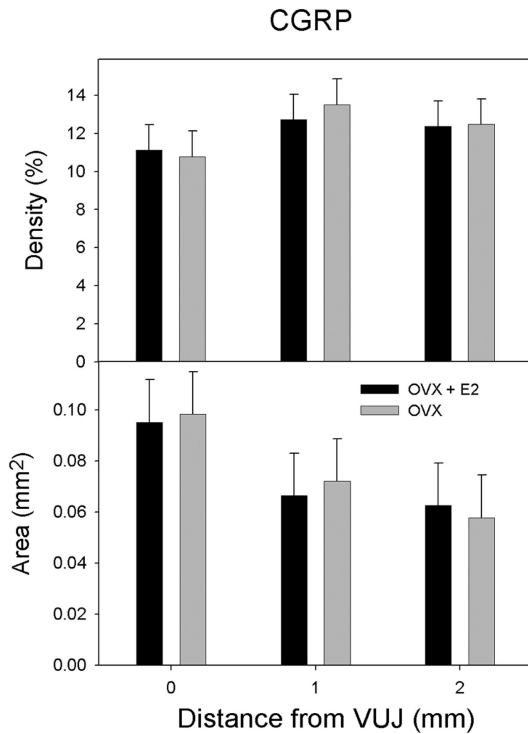
Table 1. Areas of smooth muscle (in mm^2) measured from α-smooth muscle actin stained sections taken from the proximal urethra at different distances from the vesico-ureteral junction.

VUJ DISTANCE	OVX FIXED	OVX FRESH	E2 FIXED	E2 FRESH
0 mm	0.87±0.13	1.07±0.21	0.88±0.1	1.17±0.24
1 mm	0.58±0.13	0.62±0.08	0.58±0.03	0.62±0.08
2 mm	0.49±0.05	0.50±0.05	0.49±0.04	0.66±0.12

No significant differences were detected between fresh-frozen and fixed smooth muscle areas, or in ovariectomized (OVX) and estrogen (E2) treated smooth muscle areas. Smooth muscle area decreased significantly between the VUJ and 1 mm distal (P=0.002 in fresh-frozen specimens, P=0.001 in fixed specimens), but not between 1 and 2 mm distally.

Fig. 6. Quantitative analyses of calcitonin gene-related peptide-immunoreactive innervation of the proximal urethra at the level of the vesico-ureteral junction (0) and 1 and 2 mm distal in ovariectomized (OVX) and ovariectomized rats receiving 1 week of continuous estrogen administration (OVX + E2).
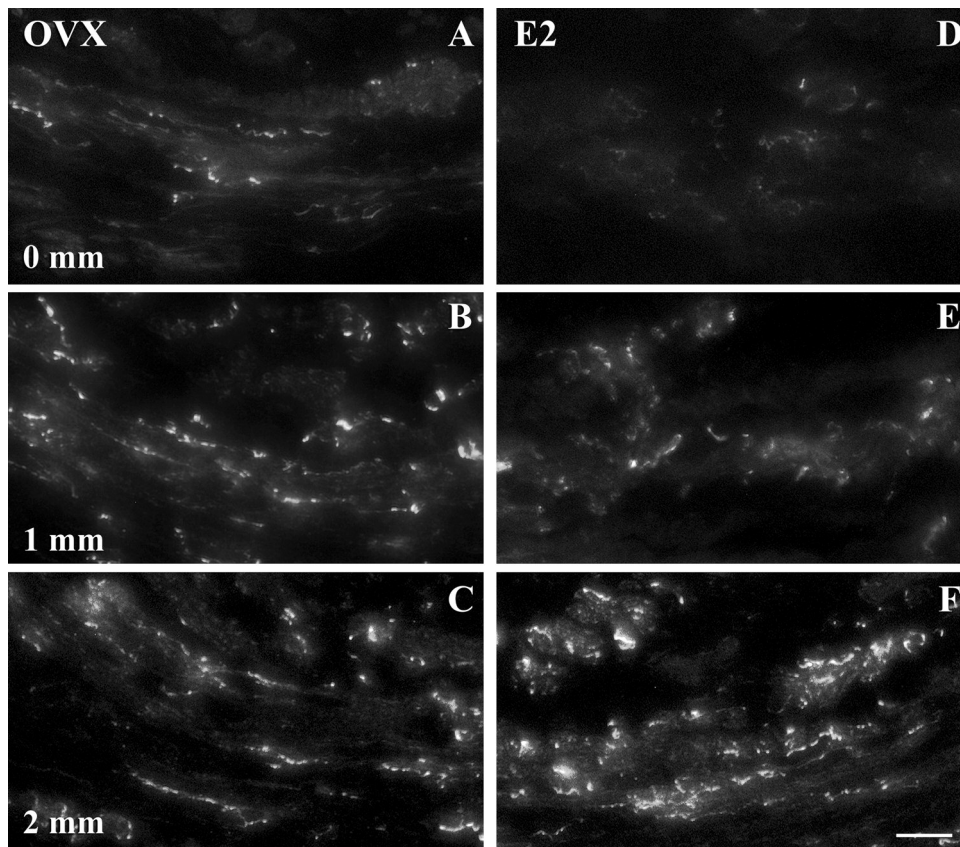

Fig. 7. Proximal urethral innervation as visualized by immunostaining for tyrosine hydroxylase immunoreactivity. Images were obtained from the level of the vesico-ureteral junction (0 mm, **A and D**), 1 mm **(B and E)** and 2 mm **(C and F)** distal. **A, B, and C** were obtained from ovariectomized rats (OVX), and **D, E, and F** from ovariectomized rats treated for 1 week by continuous estrogen administration (E2). Scale bar in F: 50 µm for all pictures.

relatively constant within this region, while total innervation decreases between 0 and 1 mm distal to the VUJ in concert with a reduction in total muscle mass. The smooth muscle geometry within this portion of the urethra is believed to be consistent with sphincter function required for urinary retention (Gosling and Dixon, 1975), and these findings support a role of innervation in regulating contractile status.

A predominant axonal population comprising this innervation was revealed following immunostaining for VAChT. Immunofluorescent fibers were observed at densities that approximated those of PGP 9.5, implying that these nerves comprise a major constituent of the urethral smooth muscle plexus. While denervations were not performed in the present study, VAChT is recognized as a highly selective marker of cholinergic neurons (Schafer et al., 1998). Previous studies have shown rich urethral innervation by cholinesterase positive nerves (Gosling and Dixon, 1975; Watanabe and Yamamoto, 1979; Alm et al., 1995), and although acetylcholinesterase is not specific for cholinergic nerves, this is consistent with dense cholinergic innervation. Similarly, nitrergic nerves in smaller numbers have also been identified in the proximal urethra (Alm et al., 1995; Persson et al., 1995, 1997, 1998), and nitric oxide synthase colocalizes with choline

acetyltransferase in neurons of the major pelvic ganglion (Persson et al., 1998). Accordingly, our findings of VAChT-ir nerves are consistent with the presence of highly abundant cholinergic parasympathetic innervation within proximal urethral smooth muscle. These nerves are likely to participate in urethral smooth muscle relaxation which is mediated by both cholinergic and nitrergic mechanisms (Persson et al., 1998; le Feber and van Asselt, 1999). Thus, our findings imply a dense and uniform cholinergic parasympathetic inhibitory innervation to the proximal urethra.

CGRP-ir nerves also provide a substantial contribution to the proximal urethral smooth muscle ground plexus, though they are considerably less abundant than cholinergic parasympathetic nerves. These findings are consistent with previous reports showing a moderate CGRP-ir innervation to the bladder neck and proximal urethra (Ghatei et al., 1985; Alm et al., 1995; Persson et al., 1997; Gabella and Davis, 1998). These axons project from neurons located in the lower thoracic and lumbar dorsal root ganglia, and therefore apparently represent sensory nociceptor innervation to the proximal urethra (Su et al., 1986).

Sympathetic innervation to the proximal urethra is well documented (Gosling and Dixon, 1975; Warburton and Santer, 1994; Kihara and de Groat, 1997), and is known to play an important role in maintaining tonic contraction of the sphincteral smooth muscle (Kiruluta et al., 1981; Willette et al., 1989). A question that remains is the relative contribution of the excitatory sympathetic innervation with respect to inhibitory parasympathetic innervation. To the extent that this has been examined qualitatively or semi-quantitatively, similar numbers of nerves have been reported within urethral smooth muscle using electron microscopy (Gosling and Dixon, 1975) or TH-ir and acetylcholinesterase histochemistry (Alm et al., 1995). The present quantitative analyses support the idea that the cholinergic innervation is more abundant in the regions of the proximal urethra examined in this study. However, it is noteworthy that there was a gradient in sympathetic innervation, with numbers of fibers increasing in the transitional zone between bladder neck and urethra, implying greater sympathetic control in the more distal parts of the proximal urethra.

Chronic estrogen elevation and urethral innervation

An effect of E2 on urethral function has long been inferred. There is strong clinical evidence for a relationship between E2 plasma levels and urinary continence, and estrogen treatment has been advocated as a palliative strategy for treating incontinence in postmenopausal women for over half a century (Thom and Brown, 1998). The urethra possesses E2 receptors and E2 administration is reported to increase wet weight of the rabbit urethra (Batra and Iosif, 1983). Moreover, because estrogen has been reported to increase sympathetic nerve density of the rabbit bladder (Levin et al., 1981), a thorough assessment of the proximal
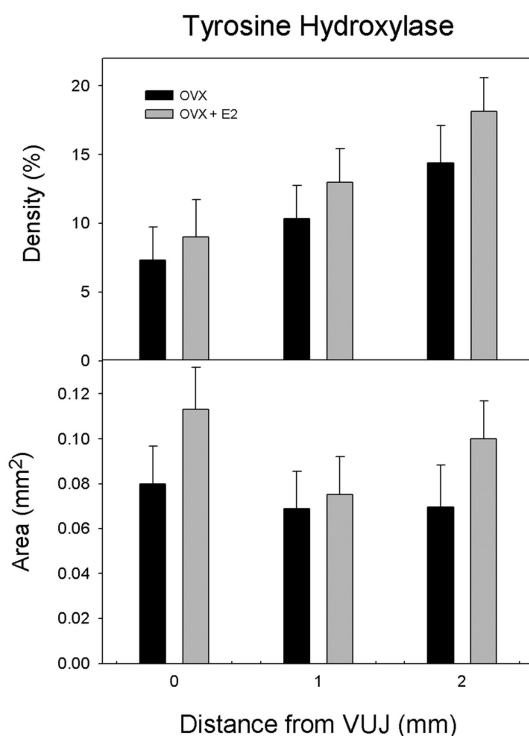

Fig. 8. Quantitative analyses of tyrosine hydroxylase-immunoreactive innervation of the proximal urethra at the level of the vesico-ureteral junction (0) and 1 and 2 mm distal in ovariectomized (OVX) and ovariectomized rats receiving 1 week of continuous estrogen administration (OVX + E2).

urethral urinary sphincter smooth muscle is warranted.

Our findings show that structural features of the urethral smooth muscle and its innervation remain remarkably stable despite estrogen treatment. Hence, although urethral weight is reportedly increased by E2 (Batra and Iosif, 1983), we observed no change in smooth muscle mass. Further, while E2 treatment identical to that used in the present study leads to a marked depletion of sympathetic nerves innervating closely related uterine smooth muscle (Zoubina et al., 2001), no change in density or in total area occupied by sympathetic nerves (or other neural populations) could be demonstrated in the proximal urethra. These findings indicate that the effects of estrogen on peripheral target innervation are highly tissue specific. Although we cannot discount possible effects on transmitter release or postjunctional contractile properties, the present study provides evidence that E2 does not influence urethral function as a result of changes in smooth muscle mass or composition of peripheral innervation.

References

Alm P., Zygmunt P.K., Iselin C., Larsson B., Uvelius B., Wegner S. and Andersson K.E. (1995) Nitric oxide synthase-immunoreactive, adrenergic, cholinergic, and peptidergic nerves of the female rat urinary tract: a comparative study. J. Auton. Nerv. Syst. 56, 105-114.

Batra S.C. and Iosif C.S. (1983) Female urethra: a target for estrogen action. J.Urol. 129, 418-420.

Dubrovsky B. and Filipini D. (1990) Neurobiological aspects of the pelvic floor muscles involved in defecation. Neurosci. Biobehav. Rev. 14, 157-168.

Gabella G. and Davis C. (1998) Distribution of afferent axons in the bladder of rats. J. Neurocytol. 27, 141-155.

Ghatei M.A., Gu J., Mulderry P.K., Blank M.A., Allen J.M., Morrison J.F., Polak J.M. and Bloom S.R. (1985). Calcitonin gene-related peptide (CGRP) in the female rat urogenital tract. Peptides 6, 809-815.

Gosling J.A. and Dixon J.S. (1975). The structure and innervation of smooth muscle in the wall of the bladder neck and proximal urethra. Br. J. Urol. 47, 549-558.

Iosif S. and Ulmsten U. (1981) Comparative urodynamic studies of continent and stress incontinent women in pregnancy and in the puerperium. Am. J. Obstet. Gynecol. 140, 645-650.

Kihara K. and de Graot W.C. (1997). Sympathetic efferent pathways projecting to the bladder neck and proximal urethra in the rat. J. Auton. Nerv. Syst. 62, 134-142.

Kihara K. and de Groat W.C. (1997). Sympatehtic efferent pathways projecting to the bladder neck and proximal urethra in the rat. Invest. Urol. 18, 460-465-

leFeber J. and van Asselt E. (1999). Prudendal nerve stimulation induces urethral contraction and relaxation. Am. J. Physiol. 277, R1368-1375.

Levin R.M., Jacobowitz D. and Wein A.J. (1981). Autonomic innervation of rabbit urinary bladder following estrogen administration. Urology

17, 449-453.

Miodrag A., Castleden C.M. and Vallance T.R. (1988). Sex hormones and the female urinary tract. Drugs 36, 491-504.

Persson K., Alm P., Johansson K., Larsson B. and Andersson K.E. (1995). Co-existence of nitrergic peptidergic and acetylcholine esterase-positive nerves in the pig lower urinary tract. J. Auton. Nerv. Syst. 52, 225-236.

Persson K, Johansson K., Alm P., Larsson B. and Anderson K.E. (1997). Moprphological and functional evidence against a sensory and sympathetic origin of nitric oxide syntashe-containing nerves in the rat lower urinary tract. Neuroscience 77, 271-281

Persson K., Alm P., Uvelius B. and Andersson K.E. (1998). Nitrergic and cholinergic innervation of the rat lower urinary tract after pelvic ganglionectomy. Am. J. Physiol. 274, R389-R397.

Qayyum M.A., Fatani J.A. and Abbas M.O. (1989). Degeneration of adrenergic nervers in the urinary bladder during pregnancy. Acta Anat. 136, 303-305.

Sadler T.W. (1990). Langma´s Medical Embryology. Williams & Wilkins. Baltimore.

Schafer M.K., Eiden L.E. and Weihe E. (1998). Cholinergic neurons and terminal fields revealed by immunocytochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. Neuroscience 84, 361-376.

Smythe G.A. and Jacoby R.A. (1953). Gynecological and obstetrical anatomy and function histology. Williams & Wilkins. Baltimore.

Stanton S.L., Kerr-Wilson R. and Harris V.G. (1980). The incidence of urological symptoms in normal pregnancy. Br. J. Obstet. Gynaecol. 87, 897-900.

Su H.C., Wharton J., Polak J.M., Mulderry P.K., Ghatei M.A., Gibson S.J., Terenghi G., Morrison J.F., Ballesta J. and Bloom S.R. (1996). Calcitonin gene-related peptide immunroeactivity in afferent neurons supplying the urinary tract: combined retrograde tracing and immunohistochemistry. Neuroscience 18, 727-747

Thom D.H. and Brown J.S. (1998). Reproductive and hormonal risk factors for urinary incotinence in later life: a review of the clinical dnd epidemiologic literature. J. Am. Geriatr. Soc. 46, 1411-1417.

van Geelen J.M., Lemmens W.A., Eskes T.K. and Martin J.C.B. (1982). The ruetrhal pressure profile in pregnancy and after delivery in healthy nulliparous women.

Warburton A.L. and Santer R.M. (1994). Sympathetic and sensory innervation of the urinary tract in young adult and aged rats: a semi-quantitative histochemical and immunohistochemical study. Histochem. J. 26, 127-133.

Watanabe A.L. and Yamamoto Y.T. (1979). Autonomic innervation of the muscles in the wall of the bladder and proximal urethra of male rats. J. Anat. 128, 873-886.

Willette R.N., Sauermelch C. and Hibe J.P. (1989). Stimulation of postjunctional alpha-1 and alpha-2-adrenoreceptors increases proximal urethral perfusion presure in the pithed rat. J. Auton. Pharmacol. 9, 63-70.

Zoubina E.V., Fan Q. and Smith P.G. (1998). Variations in uterine innervation during the estrous cycle in rat. J. Comp. Neurol. 397, 561-571.

Zoubina E.V., Mize A.L., Alper R.H. and Smight P.G. (2001). Acute and chronic estrogen supplementation decreases uterine smpathetic innervation in ovariectomized adult virgin rats. Histol. Histopthol. 16, 989-996.