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Estrogen Promotes Sympathetic Nerve Regeneration in the Rat Proximal Urethra

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Abstract

Objectives: Sympathetic innervation mediates tonic contraction of proximal urethral smooth muscle, thus contributing to urinary continence. Urethral innervation is particularly susceptible to damage during vaginal delivery, a time characterized by decreasing estrogen levels. Because regeneration of other nerves types can be influenced by estrogen, the present study was conducted to assess whether sympathetic reinnervation of the rat proximal urethra is affected by differences in estrogen levels.

Methods: Adult female rats were ovariectomized and implanted with pellets containing vehicle or estrogen to achieve serum levels similar to rodent pregnancy. Rats were injected intravenously with vehicle or the selective sympathetic neurotoxin 6-hydroxydopamine, which produces uniform and complete destruction of terminal sympathetic axons. At 1, 4, 12 and 25 days, tyrosine hydroxylase-immunoreactive sympathetic innervation of the proximal urethral smooth muscle was assessed quantitatively.

Results: In rats with intact innervation, the proximal urethra is densely innervated, and nerve density is comparable irrespective of estrogen status. 6-Hydroxydopamine induced marked sympathetic axon disruption by 1 day and complete denervation by 4 days post-injection in ovariectomized rats receiving vehicle or estrogen. In vehicle-treated rats, few nerves were present at 12 days post-sympathectomy, and innervation remained substantially below normal levels at 25 days. In estrogen-treated rats, sympathetic reinnervation was 2-fold greater at 12 days, and by 25 days was comparable to controls.

Conclusion: Estrogen improves sympathetic reinnervation of the proximal urethra. Estrogen titers in individuals with urethral sympathetic nerve damage may therefore influence the rate and extent of urethral smooth muscle reinnervation.

Keywords

Noradrenergic; urethral sphincter; regeneration; 17 β -estradiol

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Smooth muscle of the proximal urethra plays an important role in maintaining urinary continence. This muscle is contracted tonically by the excitatory actions of norepinephrine released from sympathetic nerves ¹, which normally are abundant in the sphincter region of the urethra ^{2,3}. Conditions in which this urethral sympathetic innervation is compromised can therefore lead to urinary incontinence.

Because of its close proximity to the uterus and vaginal canal, trauma occurring during vaginal childbirth can result in significant compression of the urethra and its associated nerves, thus potentially disrupting this resident innervation. Indeed, 42% of women after a single birth report urinary incontinence, as compared to 6-17% of nulliparous women, and this rises to 53% with 3 or more births ^{4,5}. Incontinence after childbirth has been strongly associated with damage to proximal urethral sympathetic innervation ⁶. Therefore, factors affecting sympathetic nerve regeneration to the urethra may impact quality of life for large numbers of individuals.

One factor that may influence outcome following sympathetic nerve damage is the level of serum estrogen (17 β -estradiol, E2) during the period of damage and regeneration. E2 levels rise at about the third month of pregnancy and, while declining after parturition, remain significantly elevated through 2 weeks post-partum relative to nulliparous women in the luteal phase of the menstrual cycle ^{7,8}. Recent studies show that E2 can promote somatic motor axon regeneration following crush lesion ⁹⁻¹². However, elevated E2 can also elicit degeneration of sympathetic axon terminals innervating the adult rodent uterus ¹³. Accordingly, there is reason to suggest that E2 may influence sympathetic reinnervation of urethral smooth muscle after injury.

In the present study, we used the highly selective noradrenergic neurotoxin 6-hydroxydopamine (6-OHDA) to induce a complete and uniform lesion of urethral sympathetic innervation ¹⁴. We then used quantitative morphometry to compare the extent of sympathetic reinnervation of urethral smooth muscle under low- and high-E2 conditions.

Materials and Methods

Animals and Tissue Preparation

Experiments were conducted on 39 female 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. Female virgin 2-3-month-old rats were housed 2-3 per cage in a light- and climate-controlled room with a 12-hour light-dark cycle starting at 6 AM with food and water ad libitum.

Rats were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (60 mg/kg i.p., Lloyd Laboratories, Shenandoah, Iowa), xylazine hydrochloride (8mg/kg, Bayer, Shawnee Mission, Kansas), and atropine sulfate (0.4 mg/kg, Vedco, St. Joseph, Missouri). Bilateral ovariectomies (OVX) were performed as described previously ¹³. Two weeks following OVX, pellets containing placebo or 17 β -estradiol (E2, 60-day release, 0.25 mg/pellet; Innovative Research of America, Sarasota, Florida) were implanted subcutaneously at an intrascapular location. OVX reduces plasma estradiol to undetectable levels, and this pellet regimen restores it to levels similar to those seen during pregnancy ^{13,15}.

Three days after pellet implantation, 31 rats were re-anesthetized with ketamine-xylazine-atropine and injected through the tail vein with 6-hydroxydopamine hydrochloride (100 mg/kg, Aldrich; St. Louis, MO) or its vehicle (a degassed solution of 1 mg/ml ascorbic acid and normal saline, 10 μ l/g body weight) ¹⁶.

Following 6-OHDA injection, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p., Abbot Laboratories, North Chicago, Illinois) at intervals of 24 hours (N=4 OVX and 4 E2-treated), 4 days (N=4 OVX and 3 E2-treated), 12 days (N=4 OVX and 4 E2-treated), or 25 days (N=4 OVX and 4 E2-treated). Additionally, OVX rats not treated with 6-OHDA were implanted with placebo pellets (N=4) or E2-containing pellets (N=4) and maintained for 28 days to serve as innervated control tissue under low and high E2 conditions.

The trigone and proximal urethra were removed as a single block through a ventral incision, and animals were killed by asphyxiation. Tissue was snap-frozen in TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham North Carolina) and stored at -80°C . Cryosections at 10 μm thickness were obtained perpendicular to the long axis of the urethra and collected onto silanecoated slides in stepped series with 200 μm intervals between adjacent sections. Sections were collected beginning from the vesico-ureteral junction to a level $>2\text{mm}$ distal to the junction.

Immunofluorescence

Tissue sections were fixed on slides for 5 minutes in 4% paraformaldehyde, washed twice for 10 minutes in PBST, blocked (5% goat serum with 10mg/ml bovine serum albumin in PBST) for 20 minutes, and incubated overnight with a mouse monoclonal antibody to tyrosine hydroxylase (TH, 1:100, ImmunoStar, Hudson, Wisconsin) followed by a 90 minute incubation with a Cy3-conjugated goat-anti-mouse secondary antibody (1:200, Jackson ImmunoResearch, West Grove, Pennsylvania). Sections were coverslipped with Fluoromount (Southern Biotechnology Associates, Birmingham, Alabama), and viewed a Nikon Eclipse TE300 microscope equipped with an Optronics MagnaFire camera.

Analysis of urethral innervation

To ensure that comparable regions of the proximal urethra were analyzed in all animals, each series of sections was previewed. The last section in the series that contained trigone muscle with contiguous ureters was designated as the vesico-ureteral junction, corresponding roughly to the beginning of the urethra. A region of urethra located exactly 2 mm (i.e., 200 sections) distal to the vesico-ureteral junction was then selected for analysis; we have previously found this region of the proximal urethra to contain the highest sympathetic nerve density³.

To assess the completeness of sympathetic nerve destruction, tissue at 1 and 4 days following 6-OHDA injection was inspected visually. To quantify sympathetic axon regeneration of the proximal urethra, tissue from 6-OHDA-treated rats at 12 and 25 days and from control rats was analyzed quantitatively. Digital images encompassing both the longitudinal and smooth muscle layers were captured from 3 randomly selected fields spaced roughly equidistantly (i.e., 12, 4 and 8 o'clock) around the circumference of the urethra in each rat. A stereology grid (Scion Image) was superimposed upon the image and numbers of intersections overlying immunoreactive (ir) nerves were counted, as were total numbers of intersections over smooth muscle. Nerve counts were divided by smooth muscle counts for each of the three areas to obtain apparent percentage of smooth muscle area occupied by immunoreactive nerves, and these were averaged for each section¹³. Statistical significance was assessed using one-way or two-way ANOVA. $P \leq 0.05$ was accepted as a statistically significant difference.

Results

Intact innervation

Control OVX rats receiving vehicle injections exhibited intact innervation similar to that described previously³. The longitudinal and circular urethral smooth muscle layers contain circumferentially oriented TH-ir fibers, with frequent varicosities (Fig. 1.A). Quantitative

analysis documented a highly dense sympathetic innervation of this region of the urethra (Fig. 2).

OVX rats treated for 28 days with E2 showed a pattern of urethral innervation that was fully comparable to that of untreated OVX rats (Fig. 1.B). Quantitative analysis revealed that long-term E2 treatment did not affect proximal urethral innervation density (Fig. 2).

6-OHDA-induced sympathetic axon degeneration in OVX and E2-treated rats

In OVX rats, 6-OHDA injection elicited rapid disruption of TH-ir fibers. At 1 day following 6-OHDA administration, TH-ir was present as punctate material rather than as continuous fibers (Fig. 1.C). Only rarely were intact axons observed. The pattern of TH-ir in E2-treated rats was indistinguishable from that of OVX rats receiving placebo pellets (Fig. 1.D).

At 4 days following 6-OHDA injection of OVX rats, all TH-ir axons had degenerated, and only occasional debris was observed (Fig. 1.E). In E2-treated rats, degeneration of fibers and removal of debris appeared to be fully comparable to that observed in OVX rats (Fig. 1.F).

Effect of E2 on sympathetic axon regeneration

In OVX rats at 12 days following 6-OHDA injections, TH-ir fibers were present in smooth muscle of the proximal urethra (Fig. 1.G). These fibers occasionally had enlarged terminal regions, suggestive of growth cones of regenerating fibers. In contrast to intact innervation, axons were not uniformly distributed throughout the urethral smooth muscle, but tended to be clustered in some regions and absent in others. Quantitative analysis revealed that innervation density was significantly below that of OVX urethras of rats not treated with 6-OHDA ($p=0.003$, Fig. 2). In OVX rats at 25 days post-6-OHDA, the pattern of reinnervation was similar to that at 12 days, although varicose regions of the axons appeared to occur with greater frequency (Fig. 1I). Quantitative analysis showed no significant increase in fiber density between 12 and 25 days in 6-OHDA treated OVX rats, and innervation density remained depressed relative to control OVX urethras ($p=0.007$, Fig. 2).

E2-treatment resulted in increased TH-ir sympathetic nerve density relative to OVX rats at both 12 and 25 days post-6-OHDA administration ($p=0.005$, Fig. 2). At 12 days after lesioning, innervation density remained depressed relative to E2-treated rats with intact innervation ($p=0.013$, Fig. 2). However, at 25 days, innervation density of E2-treated 6-OHDA-lesioned rats was statistically indistinguishable from that of E2-treated controls (Fig. 2).

Discussion

Our findings show that E2 increases proximal urethra sympathetic innervation at 12 to 25 days following injury. In principle, this could be due to increased pre-injury innervation density, axonal protection from injury, or enhanced axonal regeneration. If E2 increases resident innervation, then loss may be less extensive with greater numbers of parent axons from which sprouting could occur. E2 treatment for 3 days prior to 6-OHDA administration may be sufficient to increase sympathetic axon sprouting, as occurs with cultured sensory dorsal root ganglion neurons¹⁷, and E2 does increase intact innervation of some tissues including sensory nociceptor innervation of the rat mammary gland and vasculature^{18,19}. However, E2 elevations for 1 week in a previous study³ and 28 days in the present study failed to alter uninjured proximal urethral sympathetic nerve density. E2 effects on sympathetic nerve density after nerve injury therefore cannot be attributed to increases resident innervation.

E2 may also protect sympathetic axons from injury. 6-OHDA is selectively taken up by the norepinephrine transporter and concentrated in noradrenergic axons, producing reactive intermediates that are toxic to distal noradrenergic axons²⁰. Factors that ameliorate this damage

could improve outcome, and E2 protects central neurons from a variety of injurious stimuli²¹ including 6-OHDA²². However, the present study suggests that E2 is not protective to urethral sympathetic nerves. Sympathetic axons were extensively disrupted at 1 day post- 6-OHDA injection, and intact sympathetic nerves were essentially absent at 4 days following 6-OHDA administration in OVX rats, and E2-treated rats showed comparable damage. Thus the extent of damage induced by 6-OHDA appeared to be independent of E2 levels at the time of injury. These findings argue strongly against a neuroprotective effect of E2 in preventing 6-OHDA-induced damage to peripheral urethral sympathetic axons, suggesting that neuroprotection does not contribute to urethral sympathetic reinnervation after 6-OHDA-induced degeneration.

Systemic 6-OHDA administration to adult mammals selectively destroys sympathetic axon terminals while leaving the parent axons (which lack significant neurotoxin uptake) intact¹⁴. Target reinnervation therefore occurs relatively rapidly. In OVX rats, substantial numbers of sympathetic axons were present by 12 days after lesion, whereas the rate of reinnervation appeared to slow thereafter and no further increase was observed through 25 days. These findings compare favorably with reports from other peripheral targets with regard to time course for reinnervation and their failure to attain their pre-lesion innervation densities^{23,24}. Thus, only partial reinnervation of the proximal urethra is achieved in OVX rats. In rats whose E2 titers were persistently elevated, reinnervation was significantly improved. Sympathetic nerve density in E2-treated rats was more than 2-fold greater than in OVX rats at 12 days post-lesion, and this difference persisted through 25 days. Moreover, urethral innervation density in E2-treated rats at 25 days post-lesion was statistically comparable to that of intact rats. Morphologically, these regenerated axons exhibited varicosities adjacent to smooth muscle cells, which is characteristic of sites of sympathetic neuroeffector transmission. Therefore, E2 exerts substantial effects on the rate and extent of sympathetic nerve reinnervation of the proximal urethra.

While the effects of E2 on peripheral nerve regeneration are not well understood, evidence suggests that E2 may affect regeneration of multiple types of nerve pathways. E2 enhances regeneration of motor axons of the hamster facial nerve⁹ and the mouse sciatic nerve¹⁰. Of particular relevance to the present study, the pudendal nerve, which provides motor innervation to skeletal muscle of the external urethral sphincter, also showed improved regeneration in the presence of elevated E2^{11,12}. Therefore, E2 may play an especially prominent role in determining the extent of functional recovery of urinary continence after nerve injury associated with childbirth. Moreover, because post-partum levels of maternal estrogen are influenced by a number of factors (e.g., depressed as a result of breast feeding)⁸, differences in estrogen levels may be a significant variable in determining outcomes of urethral sympathetic injury following vaginal delivery.

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Figure 1.

Fluorescence photomicrographs of the smooth muscle layers of the proximal urethra 2 mm distal to the vesico-ureteral junction immunostained for the sympathetic axon marker tyrosine hydroxylase. A) Control tissue with intact sympathetic innervation from an ovariectomized (OVX) rat implanted with a placebo-releasing pellet for 28 days. B) Tissue from an OVX rat with intact sympathetic innervation 28 days following implantation of a pellet releasing 17 β -estradiol (E2). C) Tissue from an OVX rat 1 day following administration of 6-hydroxydopamine (6-OHDA). D) Tissue from an OVX rat receiving E2 at 1 day following 6-OHDA injection. E) Tissue from an OVX rat 4 days following administration of 6-OHDA. F) Tissue from an OVX rat receiving E2 at 4 days following 6-OHDA injection. G) Tissue from

an OVX rat 12 days following administration of 6-OHDA. H) Tissue from an OVX rat receiving E2 at 12 days following 6-OHDA injection. I) Tissue from an OVX rat 25 days following administration of 6-OHDA. J) Tissue from an OVX rat receiving E2 at 25 days following 6-OHDA injection. Scale bar in J = 20 μ m.

Figure 2.

Quantitative analysis of sympathetic innervation of urethral smooth muscle 2 mm distal to the vesico-ureteral junction. Sympathetic axon density was determined by measuring apparent axon area expressed as function of total smooth muscle (% Area). Measurements were obtained from ovariectomized rats with intact innervation (Control) implanted for 28 days with a placebo-containing pellet (OVX) or a pellet containing 17β -estradiol (Estrogen). Measurements were also obtained from OVX and estrogen-treated rats at 12 days and 25 days following 6-OHDA injection.