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ESTROGEN INCREASES THE NEURO-REGENERATIVE RESPONSE OF PUDENDAL MOTONEURONS AFTER A PUDENDAL NERVE CRUSH INJURY IN FEMALE RATS

Aims of Study

Along with the other tissues and pelvic floor structures responsible for continence, the pudendal nerve, which innervates the external urethral sphincter (EUS), can be damaged during childbirth [1]. Stress urinary incontinence (SUI) has been correlated with both childbirth injuries and pudendal nerve injury [2,3]. However, symptoms often do not occur until menopause, suggesting that hormonal factors, particularly the decreased estrogen levels of menopause, may play an important role [4,5]. In addition, several lines of research suggest that estrogen improves neuro-regeneration of injured nerves [6,7]. It has been shown previously that a bilateral pudendal nerve crush injury induces symptoms of SUI in female rats, probably due to denervation of the EUS and resultant decreased urethral resistance [8,9]. The purpose of this study was to determine the effect of estrogen on urethral function and the neuro-regenerative response of pudendal motoneurons after pudendal nerve crush.

Methods

Thirty female virgin Sprague-Dawley rats were ovariectomized (OVX). They were anesthetized, the Fallopian tubes were sutured, and the ovaries removed. Three days later, they were placed in one of 3 groups: (1) bilateral pudendal nerve crush plus implant of a subcutaneous estrogen-containing capsule (NC+E); (2) bilateral pudendal nerve crush plus implant of a sham saline-containing capsule (NC+S); (3) control group that did not undergo nerve crush but did receive OVX and an estrogen capsule (C). Unoperated controls were used since even a sham surgery can injure the pudendal nerve [9].

Pudendal Nerve Crush and Estrogen Capsule Implantation. Those rats undergoing nerve crush were anesthetized with a mixture of ketamine and xylazine, and the pudendal nerve was located in the ischiorectal fossa. The entire pudendal nerve was crushed twice for 30 seconds, just proximal to the branch point of the obturator nerve [9]. Nerve injury was observed immediately afterward by transparency of the nerve sheet at the crush site. Either an estrogen or a sham saline-containing capsule was then implanted subcutaneously prior to closing the skin incision. Control animals in the C group received the identical anesthesia and a subcutaneous implant but no nerve crush. To create each estrogen capsule, 100% crystalline estradiol was packed into a 10mm length of silastic tubing, which was plugged at both ends and incubated in 0.1% phosphate-buffered saline for 24 hours prior to experimental use. Sham capsules contained only sterile physiological saline.

Bladder Catheter Implantation. Two days after pudendal nerve crush, the rats were anesthetized as above, and a circular purse-string suture was placed on the bladder wall. A small incision was made and the catheter (PE-50 tubing with a flared tip) was implanted. The catheter was tunneled subcutaneously to the neck where it exited the skin.

Leak Point Pressure (LPP) Testing. Four days after nerve crush, the animals were anesthetized with urethane and placed supine for LPP testing [10]. The bladder was palpated to empty and filled with saline at 5 ml/hr. Gentle pressure was applied to each rat's abdomen and was slowly increased until the first leakage of saline through the urethra, when the pressure was rapidly removed. Peak pressure was taken as LPP. The procedure was repeated at least 3 times and mean values of LPP and the external abdominal pressure, P_{diff}, were calculated for each rat and for each group.

Tissue Harvest and Preparation for In Situ Hybridization. Immediately after the LPP study, the rats underwent cardiac perfusion with saline. The spinal cord was exposed and frozen with liquid nitrogen in situ. The lumbar and sacral segments were dissected and sectioned transversely (15 μm) on a cryostat through both the dorsal motoneurons (DLM) of Onuf's nucleus, which innervate the external urethral sphincter, and the retrodorsolateral motoneurons (RDLM), which course in the sciatic nerve. RDLM cells are not affected by pudendal nerve crush [8] and serve as an uninjured control.

In Situ Hybridization. Part of a neuroregenerative response to injury involves upregulation of β_{II} tubulin, a cytoskeletal protein involved in pudendal nerve regrowth [8]. We used i*n situ* hybridization to determine β_{II} tubulin mRNA upregulation as a measure of the neuro-regenerative response of the pudendal nerve. A rat cDNA insert specific for β_{II} tubulin, was prepared and labeled with ^{33}P-dATP using a nick translation kit. Hybridization of the spinal cord tissue with labeled probe was done using a standard procedure [8]. Slides were dipped in emulsion, exposed in light tight boxes at 4°C, and developed after determining proper exposure time through similarly treated test slides. Net grain density in 3 DLM and 3 RDLM cells from each animal were taken by subtracting background density from cellular grain density and a mean for each animal was calculated. Mean DLM grain density for each animal was normalized to mean RDLM grain density to account for different hybridization levels of different slides.

Serum Estrogen Analysis. A 2ml sample of blood was taken at the time of euthanasia and serum was stored at 20°C for estrogen analysis. Radio-immunoassay was used to determine the level of estrogen in serum of the rats.

Data Analysis. Data is presented as mean ± standard error of the mean for each experimental group. Statistical comparisons were made using a one-way ANOVA followed by a Student-Newman-Keuls posthoc test with $p<0.05$ indicating a significant difference.

Results

Serum Estrogen Level. Rats that received a sham implant (NC+S) had significantly lower serum estrogen levels (37 ± 9 pg/ml) than either C rats (77 ± 15 pg/ml) or NC+E rats (91 ± 10 pg/ml), demonstrating that the subcutaneous method of delivering estrogen was sufficient to significantly raise serum estrogen levels.

LPP. LPP and P_{diff} were significantly decreased 4 days after pudendal nerve crush compared to the C group (36 ± 5 and 32 ± 5 cmH_2O, respectively). The differences were significant regardless of treatment: LPP and P_{diff} were significantly decreased in both the NC+E group (25 ± 2 and 18 ± 2 cmH_2O, respectively) and in the NC+S group (25 ± 2 and 19 ± 2 cmH_2O, respectively). The differences in LPP and P_{diff} between the NC+E and NC+S groups were not significant.

Neuro-regenerative Response. Normalized DLM grain density was increased in the NC+S group (1.08 ± 0.23) compared to the C group (0.89 ± 0.06), but not significantly. In contrast, the NC+E group had significantly increased normalized DLM grain density (1.55 ± 0.13) compared to the C group, indicating a significantly upregulated neuroregenerative response to injury with estrogen.

Conclusions

When administered at the time of injury, estrogen significantly increases the neuro-regenerative response to injury in the pudendal nerve. However, 4 days after injury no significant functional outcome of this effect is observable. It may be that at later time points after injury, a functional difference will be observable between estrogen and sham treated animals. Future research will be aimed at investigating both the molecular mechanism and the functional outcome of this improved neuroregenerative response with estrogen. This well-characterized animal model could be useful for preclinical testing of agents designed to facilitate pudendal nerve regeneration after injury.

References

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