Morphological Effects of Estradiol on the Neurons of the Major Pelvic Ganglia and the Urinary Bladders of Female Rats

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To elucidate the effect of estradiol (E_2) on the morphology of the neurons of the lower urinary tract during puberty, we carried out morphometric and immunohistochemical analyses of the major pelvic ganglia (MPG) in female rats. Four-week-old female Wistar rats were divided into the following groups: normal controls, ovariectomies, and ovariectomies receiving E_2 replacement. At the age of 12 weeks the MPG were stained with hematoxylin and eosin (H&E), and immunostained by the antibody against rat tyrosine hydroxylase (TH). Each section was evaluated by an image analysis system. In the ovariectomized rats, the size of neurons in the MPG was significantly reduced. Immunohistochemical analysis revealed that the size of TH-positive neurons was significantly increased compared with TH-negative neurons in the E_2-replaced rats. The results suggest that pubertal E_2 deficit reduces neuron size, yet induces a notable increase in the size of TH-positive (adrenergic) neurons in the MPG.

Key words: estradiol; major pelvic ganglia; ovariectomy; puberty

The sympathetic nervous system is thought to play a role in the control of bladder and urethral function, and estrogen has been used clinically for many years in the treatment of stress urinary incontinence. Based on studies of intraurethral pressure profiles of women, Schreiter and colleagues (1976) suggested that estrogen has a tonicising effect on urethral musculature, but they did not present any quantitative or dose-response data. Downie and coworkers (1975) showed that alpha-adrenoceptors of the bladder neck of estrous rabbits are more sensitive to adrenergic agonists than those of the detrusor.

The major pelvic ganglia (MPG) are located in the lateral ligament of the bladder, lateral to the prostate in male, and the vagina in female rats, and innervate the internal reproductive organs, lower bowel and lower urinary tract (Langworthy, 1965; Purinton et al., 1973; Tabatabai et al., 1986; Keast et al., 1989). Greenwood and colleagues (1985) showed that the significant differences in the number of neurons in the MPG correlated with the sex in rats. However, there have been no reports on the morphological effects of estradiol (E_2) on the lower urinary tract during puberty. In the present study, morphological effects of the MPG were investigated by observing changes in the E_2 level of rats.

Materials and Methods

Animals

Four-week-old female Wistar rats were divided into 3 groups. Group 1 remained intact and received no hormonal manipulation (control group; $n = 15$). Group 2 underwent bilateral ovariectomy at the age of 4 weeks and received no hormonal replacement (ovariectomized group, $n = 10$). Group 3 underwent bilateral ovariectomy at the age of 4 weeks, and daily from the age of 8 weeks subcutaneous replacement of 50 μg/body E_2 benzoate dissolved corn oil (E_2-replaced group, $n = 5$). Ovariectomies were performed using standard surgical procedures with the aid of ether anesthesia.

Abbreviations: ABC, avidin-biotin-peroxidase comples; DAB, 3,3-diaminobenzidine tetrahydrochloride; E_2, estradiol; H&E, hematoxylin and eosin; MPG, major pelvic ganglia; PBS, phosphate buffered saline; TH, tyrosine hydroxylase

Measurement of plasma E_2

Plasma E_2 concentrations were measured at 4 weeks in the control group, 8 weeks in the control and ovariectomized groups, and 12 weeks in each group. Since the highest values of plasma E_2 concentration were found in samples collected on the morning of pro-estrus, blood samples were collected in the control group in the morning of pro-estrus during the rats' estrous cycles, as determinated by vaginal smears.

Histopathology

Five rats at the age of 4 weeks from the control group, 5 rats at the age of 8 weeks from each of the control and ovariectomized groups, and 5 rats at the age of 12 weeks from all groups were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg), weighed and then perfused through the heart with physiological saline. The bladder of each rat in the 12-week age group was removed and weighed, and its MPG was removed and immediately frozen in an acetate/dry-ice bath. Using a serial section technique (10 μm section), hematoxylin and eosin (H&E) stain was used for routine observation and morphometric study, and tyrosine hydroxylase (TH) stain was used for immunohistochemical analysis.

Immunohistochemistry of MPG

The avidin-biotin-peroxidase complex (ABC) method was employed for TH detection (Klimaschewski et al., 1994). Cryostat sections were hydrated, and endogenous peroxidase activity was quenched by incubation for 30 min with 0.3% H_2O_2, followed by washing in a phosphate buffered saline (PBS, pH 7.2). Normal goat serum served as blocking agent. A rabbit antiserum against rat TH (Chemicon, Temecula, CA) was used at the recommended dilution of 1:250 in bovine serum albumin (1%)-containing PBS. The sections were incubated with the antiserum for 18 h at 4 ˚C. PBS was used to replace the antiserum in the controls. Goat anti-rabbit

IgG (Vectastein ABC kit, Burlingame, CA) was used to detect bound antibodies. 3,3-Diaminobenzidine tetrahydrochloride (DAB) was used as the final chromogen.

Morphometry of MPG

Each section was viewed under a light microscope, and the MPG's TH-positive and TH-negative neurons were counted in the 3 different sections from each rat. The size of the neurons in the sections stained with H&E was determined by means of an image analysis system. After routine light microscopic study, photomicrographs ($\times$ 400) were taken randomly and scanned into the National Institutes of Health image analysis system (Macintosh, Cupertino, CA). Outlines of neuron profiles were traced by a digital mouse and the outlined areas were then analyzed. Only neurons showing nuclei were counted into the calculation, and neuron profiles with an area < 50 mm^2 were excluded. For each group, the MPG neurons were counted in each section from each of 10 animals.

Statistical analysis

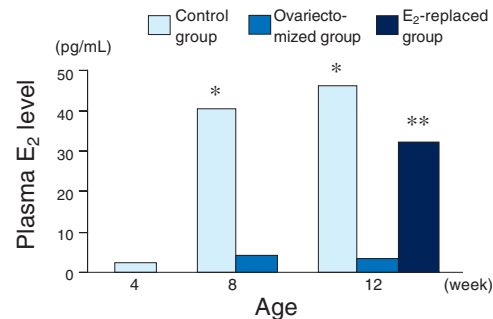All results were expressed as the mean ± SD and analyzed using the Mann-Whitney test.

Fig. 1. Plasma E_2 levels (pg/mL). A striking rise of plasma E_2 levels is observed at the age of 8 weeks in the control rats. E_2 replacement rises plasma E_2 level 10 times that of the subjects with the ovariectomized group. *Significant differences from values at 4 weeks for the control group; $P < 0.01$. **Significant differences from values for the ovariectomized group; $P < 0.01$.

Results

Plasma E_2 levels

In the control rats, a striking rise of plasma E_2 levels was observed at the age of 8 weeks, reaching a plateau that lasted until week 12. The ovariectomized group exhibited no rise in plasma E_2 levels. In the E_2-replaced group at the age of 12 weeks, the mean plasma E_2 level rose to 32.5 ± 5.1 pg/mL, a level significantly higher than that of the ovariectomized group (Fig. 1).

Bladder and body weights

Body weights were significantly heavier in the ovariectomized group than in the control and E_2-replaced groups. On the other hand, the bladder weights were significantly lower in the ovariectomized group than in the control and E_2-replaced groups. No significant differences were observed between the control and E_2-replaced groups in regard to both body weight and bladder weight (Table 1).

Morphological study of the MPG

In the 4-, 8- and 12-week-old control rats, most neurons in the MPG stained with H&E were either round or slightly ovoid in shape and varied in size.

Table 1. Body weight and bladder weight in 12-week-old rats

Group	Body weight (g)	Bladder weight (mg)
Control	212.7 ± 17.9	167.3 ± 19.2
Ovariectomized	241.6 ± 14.6	130.0 ± 21.5
E_2-replaced	218.3 ± 21.6	185.6 ± 43.0

**Significant differences; $P < 0.001$.

The pattern of TH-immunostaining was heterogeneous in the MPG neurons. Positive neurons showed a homogeneous cytoplasmic staining (Fig. 2).

The size distributions of the neurons in the MPG were shown in Fig. 3. Neuron size was measured in 1,167 to 1,361 cells in each group. The percentage of neurons more than 300 μm^2 were 4.5, 27.5 and 23.2 in 4-, 8- and 12-week-old control rats, respectively, and 3.9 and 4.5 in 8- and 12-week-old ovariectomized rats, and 29.0 in 12-week-old E_2-replaced rats. The size distributions in the 8- and 12-week-old ovariectomized rats did not disclose an increase in the percentage of large neurons, but in the E_2-replaced rats the distribution was similar to that of the control rats.

The number of counted immunostained neurons ranged from 1,235 to 1,648. The mean areas of TH-positive neurons (adrenergic neurons) and TH-negative neurons (non-adrenergic neurons) significantly increased in 8- and 12-week-old rats compared with those of
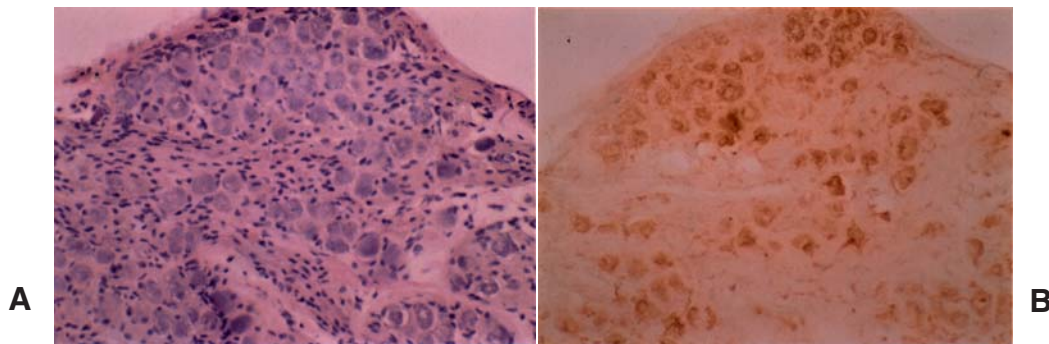

Fig. 2. Morphological study of MPG. **A:** H&E stain of MPG. The neuron cell bodies are round or oval and contain a prominent pale nucleus (× 200). **B:** TH stain of MPG. TH-positive neurons are stained brown. TH-negative neurons are not stained (× 200).
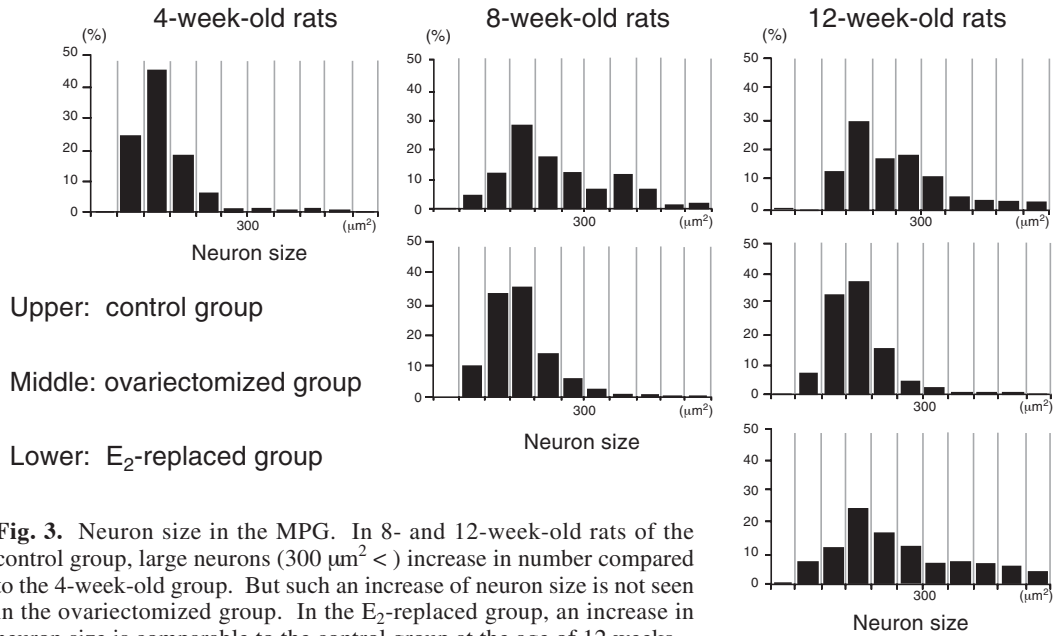
4-week-old rats

8-week-old rats

12-week-old rats

Neuron size

Upper: control group

Middle: ovariectomized group

Lower: E$_2$-replaced group

Neuron size

Neuron size

Fig. 3. Neuron size in the MPG. In 8- and 12-week-old rats of the control group, large neurons (300 μm^2 <) increase in number compared to the 4-week-old group. But such an increase of neuron size is not seen in the ovariectomized group. In the E$_2$-replaced group, an increase in neuron size is comparable to the control group at the age of 12 weeks.

the 4-week-old rats in the control group. However, such an increase of mean areas of both TH-positive and TH-negative neurons was not found in the ovariectomized group. In the 12-week-old E$_2$-replaced rats, the mean areas of both TH-positive and TH-negative neurons were significantly larger than that of 8- and 12-week-old ovariectomized rats, with a significantly larger increase in TH-positive neurons than in TH-negative neurons. The ratios of TH-positive neurons to all neurons obtained showed no differences between each group (Table 2).

Table 2. The mean areas (μm^2) of TH-positive neurons and TH-negative neurons, and the percentage of TH-positive neurons to all neurons

Group	Rats		
	4-week-old	8-week-old	12-week-old
Control group			
TH-positive neurons	138.4 ± 102.6	259.5 ± 72.5	258.7 ± 109.6
TH-negative neurons	136.9 ± 52.3	229.9 ± 42.7	243.6 ± 74.8
TH+%	27.3	29.0	31.3
Ovariectomized group			
TH-positive neurons		150.9 ± 55.5	159.6 ± 56.8
TH-negative neurons		166.5 ± 67.5	176.5 ± 71.7
TH+%		30.5	28.3
E$_2$-replaced group			
TH-positive neurons			303.3 ± 123.9
TH-negative neurons			233.7 ± 128.1
TH+%			29.6

TH+%, percentage of TH-positive neurons to all neurons.
 *Significant differences; *P* < 0.01.
**Significant differences; *P* < 0.001.

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Discussion

The present study demonstrated a rapid increase of plasma E_2 levels between the ages of 4 weeks and 8 weeks in the control rats. Due to the ovariectomies, plasma E_2 levels were kept low until the age of 4 weeks, but after E_2 replacement, plasma E_2 levels rose about 10 times that of the subjects with no E_2 replacement. In the E_2-replaced group, mean plasma E_2 levels reached 32.5 ± 5.1 pg/mL at the age of 12 weeks. This level was over the mean level of E_2 through the estrous cycle, or about two-thirds of the maximum value reported during the estrous cycle (Yoshinaga et al., 1969; Brown-Grant et al., 1970; Dupon and Kim, 1973; Handa et al., 1987; Shulman et al., 1987; Albert et al., 1991). Albert and workers (1991) showed that a plasma E_2 level of 15 pg/mL was slightly below the mean level of E_2 present through the estrous cycle in rats maintaining normal body weights. Therefore, our method of E_2 replacement was thought to be sufficient for the study of the effect of E_2 on the MPG of rats.

A previous study demonstrated that ovariectomized rats showed an increase in body weight and a decrease in bladder weight in comparison to control rats (Tanaka, 1959). In the present study, the body weights of the ovariectomized rats significantly increased in comparison with those of the normal and the E_2-replaced rats, while the bladder weights of the ovariectomized rats significantly decreased in comparison with those of the normal and the E_2-replaced rats. These results suggest that E_2 increases bladder weight while decreasing body weight, and that an E_2 deficit causes the opposite reaction. The reason for this is unclear, but it is suspected that E_2 may increase the bladder's cellular mass and capacity (Shapiro, 1986).

Our morphometric and immunohistochemical studies have demonstrated that prepubertal ovariectomy reduces the size of both TH-positive (adrenergic) and TH-negative (non-adrenergic) neurons in the MPG, and E_2 replacement recovered the size of both types of neurons in the MPG following prepubertal ovariectomy, yet the size of adrenergic neurons showed a significant increase in comparison with that of non-adrenergic neurons. This result suggests that adrenergic neurons are more sensitive to E_2 replacement than are non-adrenergic neurons.

Levin and colleagues (1980, 1981) showed that estrogen induced a marked increase in the response to alpha-adrenergic and muscarinic cholinergic agonists in immature rabbit urinary bladders, but no alterations were noted in their response to beta-adrenergic agonists. In addition, it was observed that estrogen administration induced a moderate increase in the adrenergic innervation of the immature rabbit bladder detrusor, whereas no changes could be observed in cholinergic innervation. Hodgson and coworkers (1978) found that in mature female rabbits, the annular urethral alpha-adrenergic response was decreased by ovariectomy, but the bladder alpha-adrenergic response was not influenced. Miodrag and others (1988) reported that pretreatment of immature female rabbits with E_2 significantly increased the density of the alpha-adrenoceptors in the bladder body but not in the bladder base, but showed no significant changes in beta-adrenoceptor density in any part of the bladder. Shapiro (1986) has shown that estrogens increase the weight and modulate the muscarinic cholinergic receptor density of the mature female rabbit bladder body, and this may result in a reduction of detrusor tone and an increase in bladder capacity. Batra and Andersson (1989) showed that estrogen treatment of mature ovariectomized rabbits caused a marked reduction in the density of muscarinic receptors. These studies suggest that in the bladders of immature animals estrogen administration induces a significant increase in adrenergic and cholinergic responses, which results in an increase in adrenergic innervation and an increase in adrenergic and muscarinic receptor densities. Yet in the bladders of mature animals, estrogen administration results in no such increase in adrenergic response. Our study also showed that the size of both adrenergic and non-adrenergic neurons in the MPG of rats increased the percentage of the small neurons and decreased the percentage of large neurons by ovariectomy at 4 weeks,

and the size of the neurons, particulary adrenergic neurons, increased by E_2 administration from 8 weeks. These results suggest that the growth of both adrenergic and non-adrenergic neurons in the MPG is stimulated by estrogen in immature rats, but adrenergic neurons are more susceptible to later estrogen stimulation. Postganglionic branches from the MPG reach not only to the bladder but also to the rectum, clitoris, proximal urethra, and the dorsal surface of the bladder (Langworthy, 1965; Purinton et al., 1973). Therefore our study may show that estrogen administration influences the response of the urinary bladder by stimulating the growth of the MPG surrounding it.

In summary, the present study suggests that pubertal estrogen deficit reduces the size of neurons in the MPG and that estrogen replacement increases neuron size, especially the adrenergic neurons in the MPG.

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