Androgen-Specific Critical Periods for the Organization of the Major Pelvic Ganglion

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Previous studies indicate that the major pelvic ganglion (PG) is dependent on testosterone for normal development. Tyrosine hydroxylase (T-OH), DOPA decarboxylase, and choline acetyltransferase (CAT) activities are significantly reduced by postnatal castration on day 10–11, while testosterone replacement therapy reversed all developmental enzyme activity deficits (Melvin and Hamill, 1987).

In the present studies castration on the day of birth combined with various testosterone-replacement paradigms produced effects demonstrating that the PG is sensitive to testosterone dosage and time of administration during early postnatal development. Gonadal hormone replacement experiments show that the androgens testosterone and dihydrotestosterone were effective in restoring T-OH and CAT activity deficits produced by neonatal castration. Estrogen therapy reversed the deficits in CAT activity, but was ineffective in reversing the alterations in T-OH activity. Treatment of pregnant dams with the anti-androgen flutamide altered the ontogeny of T-OH and CAT activities in pups despite replacement therapy on the day of birth. Thus, androgen-critical periods exist prenatally as well as postnatally.

These studies suggest that the organization of PG development is critically dependent on both the time of exposure and dose of testosterone. Prenatal and postnatal critical periods appear to exist. In addition, the lack of an effect of estradiol on tyrosine hydroxylase activity suggests that androgens are specifically responsible for organizing the noradrenergic development of the PG.

The major pelvic ganglion (PG) is a mixed autonomic ganglion composed of both parasympathetic and sympathetic elements (Dail et al., 1975; Dail and Evans, 1978) and is sexually dimorphic (Greenwood et al., 1985). The PG innervates androgen-sensitive accessory sex organs of male rats as well as structures such as the bladder and rectum (Langworthy, 1965; Purington et al., 1973). Previous studies indicate that the PG is sensitive

to hormonal manipulations. Tyrosine hydroxylase (T-OH) activity, an index of noradrenergic sympathetic development (Black and Geen, 1973), DOPA decarboxylase (DDC) activity, and choline acetyltransferase (CAT) activity, a cholinergic developmental marker, are significantly reduced by postnatal castration at day 10–11 (Melvin and Hamill, 1987). Testosterone replacement completely restored all maturational deficits produced by postnatal castration (Melvin and Hamill, 1987). Postnatal testosterone treatment increases cell size and catecholamine histochemistry in the PG of male but not female rats (Hervonen et al., 1972; Partanen and Hervonen, 1979a). Prepubertal orchectomy decreases cell size and catecholamine histochemistry, while testosterone replacement reverses the effects of castration (Partanen and Hervonen, 1979b).

The above-mentioned studies all support an activational role for testosterone in the PG. The activational effects of gonadal hormones on the nervous system are transient effects that persist only as long as the hormonal stimulus is present. Sex steroids acting during early critical periods of development can also produce permanent organizational effects on hormone-sensitive systems. Examinations of plasma testosterone during pre- and postnatal development have characterized the hormonal milieu during these critical organizational periods for sexual differentiation (Resko et al., 1968; Pang et al., 1979; Weisz and Ward, 1980; Corpechot et al., 1981). During the last week of embryonic life, male rats have significantly higher testosterone levels than females, with a major surge in testosterone occurring in males on embryonic days 18 and 19 (Weisz and Ward, 1980). At parturition, another rise in plasma testosterone occurs, and male levels exceed female levels throughout the first week of life (Pang et al., 1979; Weisz and Ward, 1980). Testosterone levels in males decline after the first week of life and do not exceed the perinatal and immediate postnatal levels until between 40 and 50 d of age, when male sexual behavior emerges (Beach, 1942; Resko et al., 1968).

Testosterone (T) easily diffuses across lipid membranes and thus directly enters the CNS and influences neural elements sensitive to gonadal steroids. Since T is metabolized in the brain to dihydrotestosterone (DHT) via 5- α reductase and to estradiol (E_2) via aromatase, and since hormonally responsive neural elements harbor androgen and estrogen receptors, T may regulate sexual differentiation of target neurons via both androgenic and estrogenic mechanisms (see MacLusky and Naftolin, 1981; McEwen, 1981, for reviews).

Since the developing fetus is exposed to elevated levels of estrogen from maternal and placental circulation, and since estrogen formation within the brain plays a critical role in sexual differentiation during development, the brain must be protected

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from circulating estrogen. A plasma estrogen binding system (α -fetoproteins) exists that effectively binds circulating estrogen and precludes its entry into the CNS. Since α -fetoprotein does not bind testosterone, testosterone freely enters the CNS and executes its effects directly or indirectly via androgenic and estrogenic metabolites. Investigation of the organizational effects of gonadal steroids have focused on CNS structures, primarily the preoptic anterior hypothalamus and the spinal nucleus of the bulbocavernosus (for reviews, see MacLuskey and Naftolin, 1981; McEwen, 1981).

In the peripheral autonomic nervous system, cell number, synaptic input (Wright and Smolen, 1983a, b), and plasticity (Milner and Loy, 1980) of the superior cervical ganglion are organized by gonadal hormones. In addition, androgens are apparently responsible for organizing cell number and T-OH and CAT activities in the sexually dimorphic sympathetic hypogastric ganglion (Melvin et al., in press). Since gonadal steroids apparently organize neuron number within the sexually dimorphic PG, males have 2.5-fold the number of neurons of females (Greenwood et al., 1985), and since PG transmitter enzymes are influenced by testosterone (Melvin and Hamill, 1987), we hypothesized that PG neurotransmitter characteristics are also organized by gonadal hormones. In order to test this notion, the present studies were designed (1) to determine if a dose- and time-dependent hormonal organizational period exists during postnatal development in the PG, (2) to examine the prenatal role of androgens on the development of neurotransmitter indices in the PG, and (3) to assess whether androgens or the aromatized metabolic product of testosterone, estradiol, is responsible for organizing the PG.

Materials and Methods

Experimental animals. Timed-pregnant Sprague-Dawley rats were obtained 1 week prior to giving birth from Charles River Laboratories (Boston). Neonatal males were subjected to either bilateral castration or sham operation under halothane anesthesia within 12 hr of birth. At 21 d of age, they were weaned and housed 3–5 rats per cage with free access to food and water. The rats were maintained in a temperature-controlled environment ($24 \pm 1^\circ\text{C}$) and exposed to 12 hr of light daily. The gonadal hormone replacement studies followed those previously described for organizational and activational studies of sexually dimorphic neural structures (Breedlove and Arnold, 1983a, b; Hamill and Guernsey, 1983; Hamill et al., 1984; Melvin and Hamill, 1986; Melvin et al., 1988). The protocols outlined below utilize testosterone propionate (TP) in doses similar to those utilized by Breedlove and Arnold to examine organizational issues of sexual dimorphism. The long-acting preparation of testosterone decanoate (TD) and testosterone enanthate (TE) were utilized to maintain androgenic effects during adolescence and maturity. Our original studies utilized TD (Hamill and Guernsey, 1983; Hamill et al., 1984), but we changed to TE when TD became unavailable. The dose of TE, like TD, provides physiological levels of testosterone, suppresses luteinizing hormone to normal levels, and increases accessory gland weight in castrated animals (Gerrity et al., 1982). The specific protocols are described below.

In *Study 1*, neonates were injected with either 0.1 ml of vehicle (soybean oil) or TD (20 mg/kg) on the day of surgery; delayed-treatment group received TD on postnatal day 10. TD (20 mg/kg/2 wk) was administered to maintain androgenic effects.

In *Study 2*, vehicle or TP (1 mg/rat) with TE (10 mg/kg) was administered on the day of surgery; delayed TP/TE treatment was given on day 10. Testosterone levels were then maintained with TE (10 mg/kg/wk).

In *Study 3*, either TP (1 mg/rat), dihydrotestosterone benzoate (DHTB, 1 mg/rat), estradiol benzoate (E₂B, 0.1 mg/rat), or vehicle treatment was given on the day of surgery and every other day until day 10, at which time TP (1 mg/rat) and TE (10 mg/kg) were administered to all castrated groups. Testosterone levels were maintained with TE (10 mg/kg/wk).

In *Study 4*, timed-pregnant rats were treated with an anti-androgen, flutamide (5 mg/rat/day), in 0.1 ml of propylene glycol, beginning on embryonic day 15 until the day before parturition. Subsequent to castration on the day of birth, males (group 1) were given TP (1 mg/rat) or vehicle (group 2) every other day until day 10, at which time TP (1 mg/rat) and TE (10 mg/kg) were administered to group 1 and group 2, the delayed-treatment group. Testosterone effects were maintained with TE (10 mg/kg/wk). Prior to assay, animals were killed by exposure to ether vapor.

Surgical procedures. All castrations were performed within 12 hr of birth. Animals were anesthetized with 3.0% halothane in 100% oxygen and received either sham operation or bilateral castration preserving the vas deferens and epididymes. The surgical approach required bilateral abdominal incisions inferior and lateral to the umbilicus. Surgical wounds were closed with flexible collodion. At the time of assay, the major PG were removed from their location posterior and lateral to the prostate with the aid of a dissecting microscope. Ganglia were homogenized in distilled water prior to assay.

Biochemical procedures. CAT activity was assayed using minor modifications (Melvin and Hamill, 1986) of the methods of Fonnum (1969, 1975). Tyrosine hydroxylase activity was assayed utilizing modifications (Melvin and Hamill, 1986) of previously described methods (Black et al., 1971; Black, 1975). Total protein estimates were performed according to Lowry et al. (1951), with BSA as standard.

Statistics. The significance of measured differences between treatment groups were analyzed with the 1-way analysis of variance and the Newman-Keuls test. For each data set, the *F*-statistic (*F* value) and *p* value for the ANOVA are provided, as well as the *p* value for the individual comparisons between means as determined by the Newman-Keuls test.

Results

Effects of testosterone on the postnatal development of the PG

To determine if the development of enzyme activities in the PG is either sensitive to the dose of testosterone or the time of administration, newborn male rats received either bilateral castration or sham operation within 12 hr of birth. In *Study 1*, castrated males were divided into 3 groups. Group 1 received TD (20 mg/kg/2 wk) immediately following surgery, while group 2 received vehicle and then testosterone decanoate replacement therapy at day 10. Sham-operated and castrated males (group 3) received vehicle only. Preliminary studies determined that this level of TD (20 mg/kg/2 wk) maintains enzyme activities and target-tissue mass throughout development and in adult rats. After 12 postoperative weeks groups of 8 animals each were sacrificed and the PG assayed for T-OH and CAT activities. Neonatal castration dramatically reduced T-OH and CAT activities to less than 3 and 25%, respectively, compared with controls (Fig. 1). Testosterone replacement at the time of surgery restored T-OH and CAT activities to normal, while delayed replacement therapy beginning at day 10 was not effective in restoring enzyme activities to normal levels (Fig. 1).

In a second study, a testosterone dose representative of other organizational studies (Gorski et al., 1978; Breedlove et al., 1982; Breedlove and Arnold, 1983b) was employed to examine the effects of increased levels of testosterone on enzyme activities in castrated neonates. All males were either castrated within 12 hr of birth or received sham operations. Castrated group 1 received TP (1 mg/rat) with TE (10 mg/kg) immediately following surgery, and group 2 received vehicle until day 10, at which time they received the TP/TE replacement therapy. Sham-operated and castrated group 3 received vehicle only. TE (10 mg/kg/wk) was used to maintain testosterone levels. Preliminary studies demonstrated that TE (10 mg/kg/wk) maintained enzyme activities and target-tissue weight throughout development and in adult rats. After 12 postoperative weeks, groups of 8 animals were killed and the PG assayed for T-OH and CAT

Figure 1. Effects of castration and testosterone replacement (TD) on the development of tyrosine hydroxylase and choline acetyltransferase activities in the major pelvic ganglion. Neonatal rats were castrated within 12 hr of birth or received sham operations. Castrated group 1 received 20 mg testosterone decanoate/kg body weight immediately following surgery, while castrated group 2 received the same dosage, but treatment was delayed until day 10. Testosterone levels were maintained with TD, 20 mg/kg/2 wk. Control and castrated control (group 3) animals received vehicle only. After 12 postoperative weeks, groups of 8 animals each were sacrificed and the PG assayed for T-OH and CAT activity. Tyrosine hydroxylase activity is expressed as pmol/ganglion hr $\pm$ SEM. Choline acetyltransferase activity is expressed as nmol/ganglion hr $\pm$ SEM. Data analysis for tyrosine hydroxylase activity reveals an F value of >8.52 ($p < 0.0005$); castrated groups vs. control and day 0 TD, $p < 0.01$; castrated vs. day 10 TD, no significant difference ($0.05 < p < 0.10$); day 10 TD group vs. control and day 0 TD, $p < 0.10$. Control vs. day 0 TD, no significant difference. Data analysis for choline acetyltransferase activity reveals an F value of 8.28 ($p < 0.001$); castrated group vs. control, day 0 TD, and day 10 TD, $p < 0.01$; day 10 TD vs. control and day 0 TD, $p < 0.01$; day 0 TD vs. control, no significant difference. * Differs from control at $p < 0.01$.

activities. As observed in Study 1, enzyme activities in castrated controls were significantly less than all other groups (Fig. 2). Immediate replacement with TP/TE following castration completely reversed the loss of CAT enzyme activity and, in fact, resulted in a significant increase in PG T-OH activity. As seen in the first study, delayed replacement therapy was unable to completely reverse the reduced levels of enzyme activities (Fig. 2).

Prenatal role of androgens on development

To examine the role of prenatal androgens on the development of enzyme activities, flutamide, a nonsteroidal anti-androgen,

Figure 2. Effects of castration and testosterone replacement (TP/TE) on the development of tyrosine hydroxylase and choline acetyltransferase activities in the major pelvic ganglion. Neonatal rats were castrated within 12 hr of birth or received sham operations. Castrated group 1 received 10 mg testosterone enanthate/kg body weight and 1 mg testosterone propionate/rat immediately following surgery, while castrated group 2 received the same dosage, but treatment was delayed until day 10. Testosterone levels were maintained with 10 mg testosterone enanthate/kg/wk. Control and castrated control (group 3) animals received vehicle only. After 12 postoperative weeks, groups of 8 animals were sacrificed and the PG assayed for T-OH and CAT activity. Tyrosine hydroxylase activity is expressed as pmol/ganglion hr $\pm$ SEM. Choline acetyltransferase activity is expressed as nmol/ganglion hr $\pm$ SEM. Data analysis for tyrosine hydroxylase activity reveals an F value of >8.1 ($p < 0.0005$); castrated group vs. control, day 0 TD and day 10 TD, $p < 0.01$; day 10 TD vs. control and day 0 TD, $p < 0.01$; day 0 TD vs. control, $p < 0.01$. Data analysis for choline acetyltransferase reveals an F value of >8.1 ($p < 0.0005$); castrated group vs. day 10 TD, day 0 TD, and control, $p < 0.01$; day 10 TD, day 0 TD, and control, $p < 0.01$; day 0 TD vs. control, no significant difference. * Differs from control at $p < 0.01$.

was given subcutaneously to pregnant dams (5 mg/rat/d in 0.1 ml of propylene glycol) from E15 to E21 (day of breeding = E0, date of birth = E22.5). Within 12 hr of birth, male pups from flutamide-treated dams were subjected to bilateral castration. Pups from control dams were sham-operated only. To control for possible postnatal effects of flutamide on maternal behavior or to control for the presence of the anti-androgen in the dams' milk following delivery, one group of sham-operated control pups was raised by prenatally flutamide-treated dams. Castrated, flutamide-treated pups were randomly separated into 3 groups. Group 1 was given TP (1 mg/rat) immediately after

Figure 3. Effects of prenatal anti-androgen administration on the development of tyrosine hydroxylase and choline acetyltransferase activities in the major pelvic ganglion. Pregnant dams received anti-androgen (5 mg flutamide/rat/d) or vehicle for 7 d (E15–E21). Within 12 hr of birth male pups were castrated or sham-operated. Castrated flutamide-treated males were divided into 3 groups. Group 1 received testosterone propionate (TP, 1 mg/rat) immediately after surgery and every other day until day 10, at which time groups 1 and 2 (vehicle-treated until day 10) received TP (1 mg/rat) plus testosterone enanthate (TE, 10 mg/kg). TE (10 mg/kg/wk) was used to maintain testosterone levels. Group 3 and control animals received vehicle only. Control animals were raised by 2 groups of mothers, either vehicle- or flutamide-treated during pregnancy. After 10 postoperative weeks, 8 animals in each group were assayed for T-OH and CAT activities. Tyrosine hydroxylase activity is expressed as pmol/ganglion hr $\pm$ SEM. Choline acetyltransferase activity is expressed as nmol/ganglion hr $\pm$ SEM. Data analysis for tyrosine hydroxylase activity reveals an F value of 5.7 ($p < 0.0005$); no TP group vs. day 10 TP, no significant difference; no TP vs. TP day 0, $p < 0.05$; no TP vs. both control groups, $p < 0.01$; TP day 10 group vs. day 0 TP, $p < 0.05$; TP day 10 vs. both controls, $p < 0.01$; TP day 0 group vs. both controls, $p < 0.01$; control vs. flutamide control, no significant difference. Data analysis for choline acetyltransferase activity reveals an F value of 6.79 ($p < 0.0005$); no TP group vs. day 10 TP, no significant difference; no TP vs. day 0 TP and both control groups, $p < 0.01$; TP day 10 group vs. day 0 TP, and both control groups, $p < 0.01$; TP day 0 group vs. controls, $p < 0.01$; control vs. flutamide control, no significant difference. * Differs from control at $p < 0.01$; † differs from control at $p < 0.05$.

surgery and every other day until day 10, at which time TP (1 mg/rat) plus TE (10 mg/kg body weight) were given. Group 2 received vehicle only until day 10, at which time TP (1 mg/rat) plus TE (10 mg/kg) were administered. TE (10 mg/kg/wk) maintained testosterone levels in the hormone-treated groups. Controls and the castrated, flutamide-treated group 3 received ve-

Figure 4. Effects of androgens and estradiol on the development of tyrosine hydroxylase and choline acetyltransferase activities in the major pelvic ganglion. Neonatal rats were castrated within 12 hr of birth or received sham operations. Castrated groups received either testosterone propionate (TP, 1 mg/rat), dihydrotestosterone benzoate (1 mg/rat), estradiol benzoate (0.1 mg/rat), or vehicle immediately following castration and every other day until day 10, at which time all castrated groups received TP (1 mg/rat) plus testosterone enanthate (TE, 10 mg/kg). Hormone-treated groups were maintained on TE (10 mg/kg/wk). After 12 postoperative weeks, groups of 8 animals each were sacrificed and the PG was assayed for T-OH and CAT activities. Tyrosine hydroxylase activity is expressed as pmol/ganglion hr $\pm$ SEM. Choline acetyltransferase activity is expressed as nmol/ganglion hr $\pm$ SEM. Data analysis for tyrosine hydroxylase reveals an F value of 5.7 ($p < 0.0005$); TP day 10 group vs. E2B, no significant difference; TP day 10 vs. DHTB, TP day 0, and control groups, $p < 0.01$; E2B group vs. DHTB, TP day 0, and control, $p < 0.01$; DHTB group vs. control, no significant difference; DHTB vs. TP day 0, $p < 0.01$; TP day 0 group vs. control, $p < 0.05$; TP day 0 vs. DHTB group, $p < 0.01$; TP day 0 vs. E2B and TP day 10, $p < 0.01$. Data analysis for choline acetyltransferase activity reveals an F value of 5.7 ($p < 0.0005$); TP day 120 group vs. E2B and DHTB groups, $p < 0.05$; TP 10 groups vs. control and TP day 0 groups, $p < 0.01$; E2B vs. TP day 0 group, $p < 0.01$; E2B group vs. DHTB and control, no significant differences; E2B group vs. TP day 10, $p < 0.05$; DHTB group vs. TP day 0 group, $p < 0.01$; DHTB group vs. E2B and control groups, no significant differences; DHTB group vs. TP day 10, $p < 0.05$; TP day 0 group vs. all other groups, including control, at $p < 0.01$. * Differs from control at $p < 0.01$; † differs from control at $p < 0.05$.

hicle only. After 10 postoperative weeks, 8 animals in each group were sacrificed and T-OH and CAT activities determined. No differences were noted between control animals raised by untreated or flutamide-treated dams (Fig. 3). Testosterone treatment initiated immediately following birth only partially restored T-OH and CAT activities. However, delayed replacement

therapy at day 10 was unable to significantly increase T-OH or CAT activities relative to the castrated, non-testosterone-treated group (Fig. 3).

Androgen-specific organization

To determine whether testosterone or one of its metabolites is responsible for the organization of the PG, male pups received castration or sham operation within 12 hr of birth. Castrated groups received either TP (1 mg/rat), DHTB (1 mg/rat), E₂B (0.1 mg/rat), or vehicle immediately following castration and every other day until day 10, at which time all castrated groups, including the delayed-treatment group, received TP (1 mg/rat) plus TE (10 mg/kg). Hormone-treated groups were maintained on TE (10 mg/kg/wk), while controls received vehicle treatment throughout. After 12 postoperative weeks, groups of 8 animals each were sacrificed and the PG assayed for T-OH and CAT activities. DHTB therapy restored the deficits in T-OH and CAT activities observed in the delayed-treatment group. TP not only reversed the enzyme deficits but produced a significant increase in T-OH and CAT activities relative to controls (Fig. 4). E₂B, on the other hand, was ineffective in restoring the T-OH activity deficit relative to the delayed treatment group; however, E₂B was able to restore the CAT activity deficit (Fig. 4).

Discussion

Organizational versus activational effects

Gonadal hormones act during early critical periods to permanently organize morphological and biochemical characteristics of neurons associated with sexual function. This critical period occurs perinatally; thus, gonadal hormone manipulations after approximately postnatal day 10 are typically unable to affect the organization of hormone-sensitive systems. In contrast to permanent organizational effect, the action of gonadal hormones subsequent to these critical periods produces transient activational effects that persist only as long as the hormonal stimulus is present (for reviews, see McEwen and Parsons, 1982; Arnold and Gorski, 1984; McEwen et al., 1984; Arnold and Breedlove, 1985). Previous studies of the PG demonstrated that postnatal castration decreases the levels of T-OH and CAT activities (Melvin and Hamill, 1987) and reduces the size and catecholamine histofluorescence of neurons in the PG (Partanen and Hervonen, 1979b). However, these prior studies did not determine if the influence of gonadal hormones is permanent or transitory in nature.

In the present studies, immediate testosterone replacement following castration (Fig. 1) completely reversed the loss of T-OH and CAT activities following castration. In contrast, delayed testosterone replacement starting on day 10 only partially restored enzyme activity deficits to the adult levels. Thus, it appears that during the first 10 d, gonadal hormones influence the eventual adult levels of enzyme activity for both CAT and T-OH by organizing the neurochemical development of the PG. Thus, this report is the first to demonstrate an organizational influence on the neurochemical development of the PG.

To determine whether the critical period for PG organization extends into prenatal development, pregnant dams were given injections of flutamide, an anti-androgen (Neri et al., 1972), during their last week of pregnancy. The results suggest that organization occurs both pre- and postnatally. Testosterone replacement initiated immediately after neonatal castration did not restore T-OH or CAT activities to control levels, implying that prenatal androgens influence PG organization. Breedlove

et al. (1983a) and Dohler et al. (1982) have previously demonstrated that prenatal organization in 2 separate nuclei of the CNS is produced by gonadal hormones. Therefore, it appears that a prenatal component for sex steroid organization may be a general rule for neurons in hormone-sensitive systems. Additionally, in the flutamide-treated group, delayed testosterone replacement was unable to significantly alter the levels of T-OH and CAT activities compared with castrated, vehicle-treated males (Fig. 3). It appears, then, that prenatal exposure to an androgen influence must also permit the partial response to delayed administration of testosterone we observed in our first 2 study paradigms (Figs. 1, 2). Accordingly, androgenic signals must occur along a continuum of developmental time points from at least E15 until day 10 of postnatal life. Additionally, signals at an earlier developmental stage appear to determine responses later in development, and during adulthood. This expanded continuum of hormonal influences is in keeping with recent discussions regarding the blur between organizational and activational effects of sex steroids (Arnold and Breedlove, 1985). For instance, neonatal steroids permanently alter the accumulation of testosterone or its metabolites during adulthood. Additionally, there is clear evidence that both during development and adulthood neuron number, neurite growth, neuron size and form, and neurochemical processes, as well as steroid binding and accumulation, may all be altered by gonadal steroids (see review by Arnold and Breedlove, 1985).

Specificity of hormonal responses

To examine the hormone specificity of the organizational actions observed on the PG, restoration of enzyme activities was attempted with testosterone, DHT, or estradiol E₂. The testosterone metabolites, DHT and E₂, produce organizational effects apparently via separate receptor-mediated mechanisms (Lieberburg et al., 1977; Breedlove et al., 1982; Dohler et al., 1984); thus, our studies sought to determine if androgenic and/or estrogenic influences regulate organization of the PG. Neonatal castration followed by testosterone therapy every other day until day 10 produced an elevated level of T-OH activity (Study 4, Fig. 4). In contrast to Study 2, this paradigm also increases the adult level of CAT activity. The hormone-specificity paradigm utilized high testosterone levels throughout the first 10 postnatal days instead of just the first few days as in Study 2. Thus, the organization of CAT activity (Study 4) may respond to higher levels of testosterone or occur over a longer time frame. The observed increases of T-OH activity were of similar magnitude in Studies 2 and 4, suggesting that the early high dose of testosterone may be sufficient to organize T-OH. DHT also reversed the loss of enzyme activity produced by neonatal castration, but, unlike testosterone, did not increase the levels of T-OH or CAT activities above those in controls. Testosterone is reduced to DHT by 5 α -reductase in neural tissues (Denef et al., 1973); thus, the ability of testosterone to increase activity beyond control levels may be the result of the combined actions of testosterone and DHT. E₂, on the other hand, had no effect on T-OH activity, suggesting that estrogens do not regulate noradrenergic ontogeny in the PG. Conversely, E₂ did restore CAT activity to normal.

Potential mechanisms

The exact mechanisms by which gonadal steroids exert their organizational effects in the PG are not entirely clear. On the one hand, testosterone appears to influence both noradrenergic

and cholinergic components of the PG, whereas estrogen regulates only cholinergic organization. Two potential mechanisms appear to exist for the organization of noradrenergic components of the PG. First, testosterone may influence neuronal survival in the PG. A prior study in the HG demonstrated that reduced levels of enzyme activities following castration at birth are related to the loss of neurons (Melvin et al., in press). Therefore, castrations on the day of birth and delayed testosterone treatment, or prenatal flutamide therapy, castration, and delayed testosterone treatment may result in a substantial loss of noradrenergic principal cells in the major PG. This hypothesis that testosterone is critical for neuronal survival is consistent with the observation that the PG is sexually dimorphic: The male PG has 2.5-fold more neurons ($14,654 \pm 936$ vs. 5892 ± 79 ; mean $\pm$ SD) than the female ganglion (Greenwood et al., 1985). Similarly, testosterone is also responsible for the sexual dimorphism that exists in the noradrenergic superior cervical ganglion: Neuron number (Dibner and Black, 1978; Wright and Smolen, 1983a) and synaptic input are increased by testosterone (Wright and Smolen, 1983a). Alternatively, testosterone may encode the noradrenergic neuron's response to gonadal steroids. For instance, the hormone-replacement paradigm utilized in Study 2, a program representative of other organizational studies (Gorski et al., 1978; Breedlove et al., 1982), also reversed the deficits in enzyme activities produced by castration. However, the use of TP actually resulted in an elevated level of T-OH activity when examined at adulthood. In a similar experiment utilizing the sympathetic hypogastric ganglion, the increased level of T-OH activity was not accompanied by an increase in the number of principal cells (Melvin et al., in press), suggesting that the increase represents an elevation of enzyme activity per cell. Thus, in the PG the increase in T-OH activity in testosterone-treated rats may also represent an increase in enzyme activity per cell. As observed in the initial replacement study, delayed testosterone replacement with TP/TE was unable to completely restore enzyme activity levels, implying an early organizational action by gonadal hormones.

Therefore, pre- and perinatal androgen deprivation may result in a substantial loss of noradrenergic principal cells in the major PG. Alternatively, hormonal exposure at these early developmental periods may be critical in encoding the neuron's response to gonadal steroids. In either case, the neuron might fail to respond to testosterone at day 10. Thus, prenatal and immediate postnatal testosterone must organize the PG's noradrenergic responses to its hormonal milieu.

In all of our studies, hormonal alterations did not influence CAT activity to the same extent as T-OH activity. The mixed sympathetic-parasympathetic nature (Dail et al., 1975; Dail and Evans, 1978) of the PG makes interpretation of the cholinergic alterations problematic. For instance, do the CAT changes occur in sympathetic or parasympathetic presynaptic cholinergic components, in postsynaptic parasympathetic neurons in the PG, or in all elements? Our previous studies in the PG suggested that alterations in CAT enzyme specific activity occurred and might indicate hormonal control of parasympathetic neurons (Melvin and Hamill, 1987). Although the exact locus of the estrogen effect is not entirely clear, the cholinergic parasympathetic component of the PG appears to be sensitive to estrogen levels; thus, restoration of CAT activity occurs. Similarly, estrogens have an effect on the regulation of CAT activity in neurons of the CNS (Luine et al., 1980a, b). Another possible explanation for the estrogen effect involves recognizing that dif-

ferent hormones alter the phenotypic expression of sympathetic neurons. Epidermal growth factor reduces the noradrenergic/cholinergic ratio (Fukada, 1980), while glucocorticoids increase the noradrenergic/cholinergic ratio in sympathetic ganglia (McLennan et al., 1980). Therefore, estrogens may be able to alter the phenotypic expression of the PG during this early critical period. Correlative morphological studies would be required to distinguish these possibilities.

Overall, these observations complement previous studies of the roles of hormonal influences on the development of peripheral (Wright and Smolen, 1983a, b) and central (Raisman and Field, 1973; Gorski et al., 1978; Breedlove and Arnold, 1983a, b) neuronal systems. The mixed PG appears to have noradrenergic components that are exquisitely sensitive to androgen control and cholinergic components that respond to both androgens and estrogens. Additionally, since the sensitive period for gonadal steroids exists prenatally, as well as postnatally, organizational control exists within the embryo, a phenomenon common to other hormone-sensitive systems (Dohler et al., 1982; Breedlove et al., 1983a, b).

As mentioned, these perinatal (day 1 of life) and prenatal (embryonic day 15 to birth) organizational periods are associated with specific testosterone profiles: Plasma levels of testosterone in E18 embryos and during early perinatal periods (birth to 3 hr) exceed levels occurring subsequently during the first month of development (Corbier et al., 1978), and levels in males exceed those in females. These testosterone surges are thus critical for organizing neuronal systems destined to be involved in central and peripheral neuroendocrine functions. The present studies, viewed together with our previous observations in the PG (Melvin and Hamill, 1987), extend the interaction of organizational and activational effects of hormones to the peripheral parasympathetic nervous system.

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