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The Effects of Estradiol on Gonadotropin-Releasing Hormone Neurons in the Developing Mouse Brain

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The hypothalamic–pituitary–gonadal (HPG) axis plays a critical role in the control of reproduction. Two key hormonal components of the HPG axis are gonadal steroids and gonadotropin-releasing hormone (GnRH). Gonadal steroids are known to organize the development of neural substrates which control adult reproductive behavior; GnRH is required for normal reproductive structure and function. The possibility that gonadal steroids may produce organizational changes in the pattern of GnRH staining observed in the brain is investigated through the use of injections of estradiol to neonatal mice and subsequent GnRH immunocytochemistry at 2 months of age. Our results indicate that the number of GnRH-immunoreactive (GnRH-ir) cells is normally lower in females than males. Estradiol did not affect the number of GnRH-ir cells in females, but significantly increased the number of GnRH-ir cells in males, suggesting that early exposure to estradiol results in masculinization of the GnRH axis of males.

Key Words: GnRH; sexual differentiation; hypothalamic–preoptic area; neonates; estradiol; immunocytochemistry; mouse; organization.

The perinatal organization of the vertebrate brain plays a major role in determining adult reproductive function. Two brain regions that are involved in the control of reproductive behavior and physiology are the hypothalamus and the neurohypophysis, which, in conjunction with the gonads, constitute the hypothalamic–pituitary–gonadal axis (HPG axis). Two important hormonal components of the HPG axis are gonadal steroids and gonadotropin-releasing hormone (GnRH).

Gonadal steroids mediate developmental organization of the neural and nonneural substrates that are critical for adult reproductive function (Phoenix *et al.*, 1959). Experimental manipulation of testosterone results in changes in the expression of male-like external genitalia and frequency of mounting behavior by female offspring (Phoenix *et al.*, 1959), the number of neurons in the spinal nucleus of the bulbocavernosus in both males and females (Breedlove and Arnold, 1983), and anogenital distance (AGD) (Clemens *et al.*, 1978). The sexually dimorphic nucleus of the preoptic area (SDN-POA) is one of several regions of the rodent brain that is influenced during development by circulating gonadal steroids (Gorski *et al.*, 1978). The SDN-POA is larger in males than females (Gorski *et al.*, 1980), and injection with testosterone in early life causes this region to increase in size in both gonadectomized males and normal females (Rhees *et al.*, 1990). The estrogen antagonist tamoxifen and the androgen antagonist cyproterone acetate have been used to show the estrogenic mediation of sexual dimorphism in the

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SDN-POA (Döhler *et al.*, 1984, 1986). More recently, antisense oligonucleotides which block the production of the estrogen receptor have been used to show that in female rats SDN-POA size and parastrial nuclear size are mediated by estrogen and that estrogen influences the development of both lordosis and open field behavior (McCarthy *et al.*, 1993). These data suggest that aromatization of testosterone to estradiol is responsible for several key components of the masculinization process. Thus, gonadal steroids have dramatic organizational effects on areas of the hypothalamus and preoptic area that play a key role in the control of reproduction. It is possible that the organizational effects of gonadal steroids may also influence the development of the hypothalamic nuclei responsible for GnRH release.

The gonadotropin-releasing hormones are a family of decapeptide hormones found in all vertebrates examined thus far and which represents an important link between the brain and reproduction. In rodents, the majority of the GnRH-producing cells are in the preoptic region of the hypothalamus (reviewed in Silverman *et al.*, 1994). Most of the GnRH cells are located amidst the diagonal band of Broca, the bed nucleus of the stria terminalis, the preoptic region (periventricular, medial, and lateral), and the anterior hypothalamus (Silverman *et al.*, 1994). The majority of GnRH cells located in the preoptic region project to the median eminence and are associated with the hypophyseal portal system (Silverman *et al.*, 1994). Gonadotropin-releasing hormone is primarily responsible for causing the release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. It is also involved in the activation of lordosis in the female (Moser and Mathiesen, 1996; Pfaff, 1973; Sakuma and Pfaff, 1980). Moreover, in both mice and humans (reviewed in Silverman *et al.*, 1994), improper development and or molecular regulation of the GnRH forebrain nuclei results in hypogonadism and subsequent infertility. Thus, early and proper development of the forebrain GnRH axis is a necessary requirement for normal adult reproductive function in several mammalian species.

Neonatal treatment of rats with estradiol decreases the number of cells stained for GnRH in the male and increases the number of cells in the female within the first 10 days of life (Elkind-Hirsch *et al.*, 1981). However, this analysis was qualitative and thus did not examine quantitative differences in the effect of estradiol on the number of GnRH cells. Data from radioimmunoassays on brain tissue from neonatally estrogenized rats suggest that early estrogen treatment modifies the hypothalamic mechanism involved in the release of LHRH (Hayashi *et al.*, 1991). Two previous studies have quantitatively examined GnRH cells in mice (Hoffman and Finch, 1986; Wray *et al.*, 1989). Wray *et al.* (1989) examined the progenitor cells that give rise to forebrain GnRH cells and provided counts of GnRH cells from embryonic day 10.5 through adulthood. Hoffman and Finch (1986) looked at GnRH cells during aging in a different strain of mice and found that onset of reproductive dysfunction did not correlate with a loss of GnRH forebrain cells. Presently, no data are available on the effects of estradiol on the development of the GnRH axis in mice. This study tests the hypothesis that early postnatal estradiol treatment affects the number of GnRH-immunoreactive (GnRH-ir) neurons in the preoptic area of mice of both sexes during the first 2 months of development.

MATERIALS AND METHODS

Animals

Adult mice of ND4 Swiss Webster strain were purchased from Simonsen Labs (Gilroy, CA), given food and water *ad libitum*, kept on a 12-h light–12-h dark regimen, and allowed to breed. Within 24 h after birth, the dams with their litters (housed together) were randomly assigned to different experimental groups. Immediately after group assignment, neonatal pups received the first of 14 daily intraperitoneal injections (0.05 ml) of either sesame oil (control) or 10.0 mg of 17β-estradiol in sesame oil. All injections were administered with a 27-gauge needle using filtered sesame oil as a vehicle. The choice of estradiol dosage was based on previous experiments where this dose induced significant morphological and biochemical alterations in the reproductive organs of immature female mice (Eroschenko *et al.*, 1995). We were notified as to the availability of brain tissue from these animals after the study was completed, and the established

experimental design places some limitations on our methods and interpretations.

At 2 months of age (56–60 days), all treated mice were anesthetized, injected with 100 ml heparin (1000 units/ml), and perfused with 0.9% sodium chloride and then with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Anogenital distance and body weight were recorded before perfusion, whereas ovarian weight was recorded after perfusion (testes weight was not recorded). The brains were removed, blocked, sunk in 30% sucrose overnight at 4° C for cryoprotection, sectioned at 50 μm in the coronal plane on a cryostat, and then stored in 0.1 M phosphate buffer (PB) at 4°C until the immunostaining was performed.

Immunocytochemistry

Immunocytochemistry was carried out on free-floating sections using culture plates. Briefly, the sections were rinsed twice in PB with 0.4% Triton X-100 (PBX) for 5 min. The sections were then incubated for 1 h in the presoak solution (3% normal goat serum in PBX) at room temperature on a shaker. The sections were then incubated with the primary antibody (monoclonal antibody to GnRH, LR 132 (Park and Wakabayshi, 1986), diluted 1:1000 in the presoak solution) overnight (at least 16 h) at room temperature on a shaker. The sections were rinsed twice in PBX for 5 min. Biotinylated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD; KPL) was then applied for 1 h at room temperature on a shaker. The sections were rinsed twice with PBX for 5 min. Incubation of the sections with streptavidin–peroxidase (KPL) was at room temperature for 1 h. The sections were rinsed twice with PBX for 5 min. DAB (diaminobenzidine), diluted according to the manufacturer's instructions (KPL), was applied to the sections for 10 min. The sections were rinsed in PB to stop the DAB reaction, mounted on slides, air dried, dehydrated, and coverslipped.

Cell Count Analysis

We counted all stained cells in which a nucleus and one or more cell processes were visible. We included the second criterion to avoid double counting cells that were cut through the plane of the nucleus and thus would exhibit a nucleus on more than one section. The forebrain region examined was delineated using specific neuroanatomical landmarks including the merger of the right and left radiations of the frontal aspect of the corpus callosum, anteriorly, to the appearance of the fornix and the disappearance of the anterior commissure, posteriorly. This forebrain area includes the medial and lateral preoptic areas, the medial and lateral septal nuclei, and the diagonal band of Broca. Total cell counts in the forebrain for each animal were tabulated. The GnRH-ir cells in some brain sections were not visible because of poor staining. It is not clear why some sections did not stain well, but poorly stained sections were distributed haphazardly among brains and no brains were composed entirely of poorly stained sections. Thus, GnRH-ir cell number in brains with poorly stained sections would not be a reliable indicator of the total number of cells in those brains. In order to exclude brains with poorly stained sections, group averages were calculated from the four highest values in each group. The top four brains were chosen for two reasons: (1) one treatment group only had four brains that showed robust staining for all sections and (2) to maintain equal sample sizes across all treatments. This ensured that the staining quality was robust in all brains used in the analyses (Fig. 1). Differences between the means for all groups were analyzed using analysis of variance (ANOVA). The statistical significance of the differences among the individual groups was analyzed using Fisher's LSD. Linear regression was used to evaluate the relationship between gonad size and body size (StatView 4.01 for the Macintosh, Abacus Concepts, Inc.). All data are presented as the mean ± standard error of the mean.

RESULTS

Localization of GnRH-Producing Neurons

Our identification of GnRH-ir cells in the mouse forebrain (Fig. 1) is similar to previously published work (reviewed in Silverman *et al.*, 1994). All GnRH-ir neurons were located in forebrain regions that were included in the quantitative analyses.

Cell Counts

There were significant differences in the number of GnRH-ir cells among the four treatment groups (Fig. 2;

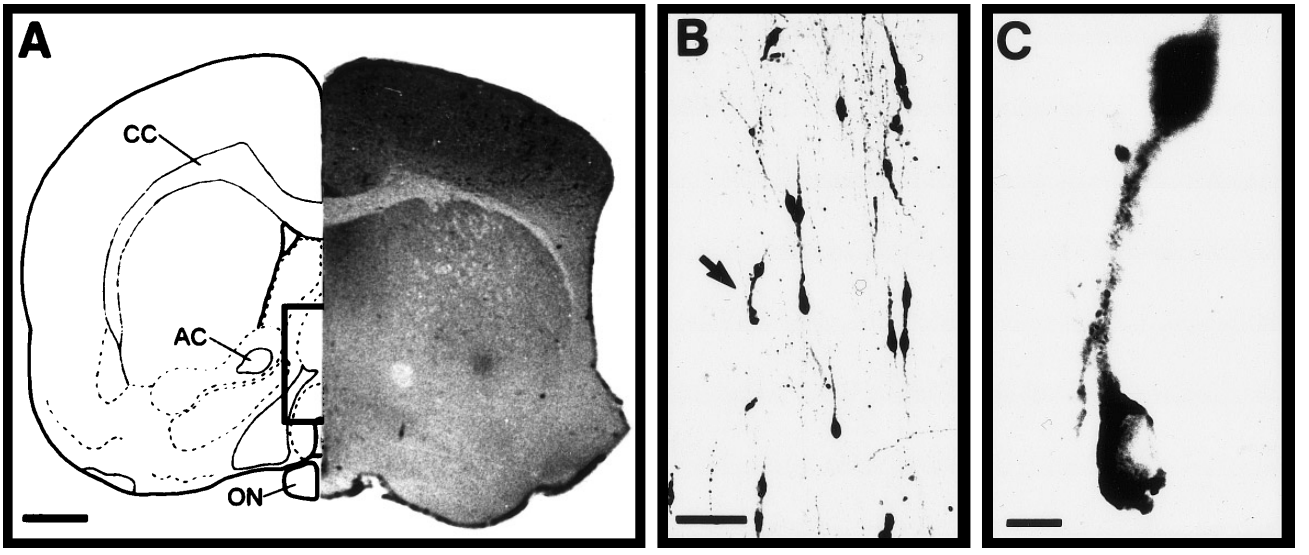

FIG. 1. Characteristics of the mouse GnRH forebrain system. (A) Cross-section of the mouse brain. CC, corpus callosum; AC, anterior commissure; ON, optic nerve. Bar, 700 μm. The box indicates the area of B (not to scale). (B) A photomicrograph of representative GnRH-ir neurons in the preoptic area of the mouse brain at 2 months of age. Bar, 100 μm. Arrow, GnRH-ir neurons shown in C. (C) Higher magnification photomicrograph of representative GnRH-ir neurons. Bar, 10 μm.

ANOVA, $F = 10.47$, $df = 3$, $P < 0.01$). The number of GnRH-ir cells in control males was 17% greater than in control females (statistically significant at $P = 0.057$, LSD). There were no significant differences in the number of GnRH cells between control females (307.5 ± 13.88 cells per brain) and estradiol-treated females (296.5 ± 28.37; $P = 0.72$) (Fig. 2). In contrast, estradiol-treated males have significantly more GnRH-ir neurons (443.0 ± 21.53; $P = 0.03$) than control males (369.5 ± 16.56). Similarly, estradiol-treated males exhibited a significantly greater number of cells than estradiol-treated females ($P < 0.001$).

Somatic Characteristics

The body weight of the female mice was significantly increased by estradiol treatment ($P < 0.01$, Table 1). The male controls were, as expected, heavier than

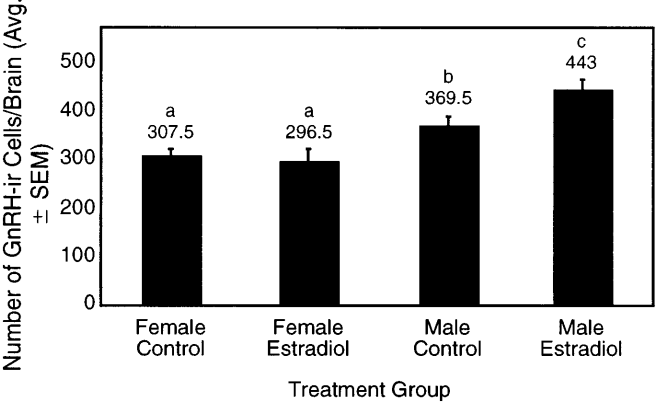

FIG. 2. The effects of estradiol treatment on GnRH immunoreactivity in male and female mouse brains at 2 months of age. The columns and error bars represent the means and standard errors of the number of GnRH-ir neurons per brain ($n = 4$), respectively. Different letters (a, b, c) indicate significant differences between the groups as determined using Fisher's LSD test ($P < 0.05$).

TABLE 1
Effects of Estradiol on Morphological Characteristics of Two-Month-Old Mice

	Body weight (g)		Anogenital distance (mm)		
Group	Males	Females*	Males	Females	Ovarian weight (mg)
Control	30.9 ± 0.92	23.3 ± 0.36	11.4 ± 0.52	4.9 ± 0.36	7.0 ± 0.92
Estradiol	31.8 ± 1.67	26.3 ± 0.64	11.0 ± 0.41	5.0 ± 0.41	5.1 ± 0.68

*Significant difference ($P < 0.05$) between control and estradiol treated mice. For all cells in the table, $n = 4$.

the female controls ($P < 0.001$, Table 1). Anogenital distance was not affected by estradiol treatment in either males or females (Table 1). However, the AGD data show the expected sexual dimorphism and are in agreement with previously published values showing males to have a greater AGD than females (Simon and Cologer-Clifford, 1991). Ovarian weight showed a nonsignificant decrease in estradiol-treated females relative to control females ($P = 0.16$, Table 1); however, only estradiol-treated females had cystic ovaries.

DISCUSSION

Gonadal steroids affect the development and sexual differentiation of all vertebrates that have been examined. Our results indicate that the number of preoptic GnRH-ir cells in adult mice may be dependent upon the steroidal milieu during development. Since several brain areas, including the suprachiasmatic nucleus, are not affected by estrogens (Döhler *et al.*, 1986), it is likely that the changes we observed in the number of GnRH-ir cells are not a result of nonspecific effects of estradiol on the brain.

The comparison between untreated males and females suggests that the number of GnRH-ir neurons in mice may be sexually dimorphic. One explanation for our result is that gonadal steroids present in the developing male and female differ, resulting in different numbers of GnRH-ir neurons at 2 months of age. An alternative explanation is that because male mice are on average larger than female mice (Table 1), they may have a larger brain and hence a larger number of GnRH-ir neurons. However, regression analysis between body weight and the number of GnRH-ir neurons, for the animals in our study, shows that there is no statistically significant relationship. Thus, our data suggest a sexual dimorphism in the number of GnRH-ir neurons in the preoptic area of the mouse brain. This pattern resembles observations in the rat, where males were reported to have a greater number of GnRH-ir neurons than females; however, this difference was not quantified (Elkind-Hirsch *et al.*, 1981). A second study on rats did not find a difference in the number of GnRH cells between males and females, but this study did not indicate the estrous state of the female animals (see discussion below) (Silverman *et al.*, 1994).

Our data differ from two previous studies on mice in several ways. We counted fewer cells (300–400) compared to total GnRH cell numbers in Wray *et al.* (1989) (about 800) and Hoffman and Finch (1986) (about 650). While this is a dramatic difference, several sources of variation may account for these differences. All three studies used sections of different thickness (present study, 50μm; Wray *et al.*, 12–14 μm; Hoffman and Finch, 30μm) and employed different criteria for counting cells (we required a nucleus and at least one neurite, Hoffman and Finch required a nucleus, and Wray *et al.* did not provide any specific criteria). The use of thinner sections and less conservative criteria for cell counting would have increased the probability of multiply counting cells, resulting in the higher cell numbers relative to our data.

Each of the studies utilized different strains of mice which have been genetically isolated for many generations. Given that normal reproductive function can be maintained with a very small number of GnRH cells (Silverman *et al.*, 1994), there is no reason to expect different strains to maintain similar cell numbers. An additional difference between the studies involves gonadal influences. Hoffman and Finch ovariectomized their mice, while Wray *et al.* did not. We were

unable to include gonadectomized controls in this study. However, Hoffman and Finch found that ovariectomy did not significantly affect cell numbers in adult females.

There are a number of factors that can affect the amount of GnRH immunostaining observed in adult rodent brains. Changing gonadal hormone titers over the estrous cycle of the rat are correlated with GnRH content of the hypothalamus (Araki *et al.,* 1975; Freeman, 1988), and GnRH mRNA expression increases in late proestrus (Silverman *et al.,* 1994). The number of GnRH neurons that are stained in the brain of the rat is significantly lower in late estrus compared with the remainder of the cycle (Ronnekleiv and Kelly, 1986; Silverman and Witkin, 1994), although at least one study has shown that the number of neurons stained for GnRH may not change during the estrous cycle of the rat (Marks *et al.,* 1993). Low estradiol levels reduce the release of GnRH in the female rat (Silverman *et al.,* 1994), while a surge of estradiol precipitates the release of GnRH and subsequently the release of luteinizing hormone from the pituitary (Kaynard *et al.,* 1988; Krey and Parsons, 1982). Thus, variation in GnRH cell number may, or may not, occur over the course of the estrous cycle. Since the estradiol-treated female mice in our study were presumed to be in a state of persistent vaginal estrus or diestrus, this variation should have a minimal effect on the variation in GnRH cell number that we observed.

Neonatal treatment with estradiol affects the uterus by causing a decrease in the number of estrogen receptors in the 2-month-old rat (Csaba and Incezefi-Gonda, 1992). A similar effect may occur in the brain, decreasing the estrogen receptor-binding capacity and hence decreasing the adult sensitivity of GnRH neurons to estradiol. Thus estradiol may have an activational effect, but this would be masked by the decreased sensitivity of these neurons. Unfortunately, the design of this study prevents the testing of this hypothesis. Regardless, the lack of difference between the control and estradiol-treated females indicates that neonatal estradiol treatment does not appear to have an effect on the number of GnRH-producing neurons in the female mouse.

Males treated with estradiol show a significant increase in the number of GnRH cells compared to control males. At the same time, our data show no change in the AGD indicating no effects on androgen-mediated characteristics. The increase in the number of GnRH-ir neurons contrasts with prior data from the rat which showed a decrease in the number of GnRH-ir cells after treatment with estradiol in male rats (Elkind-Hirsch *et al.,* 1981). However, our data were collected at 2 months of age while the data from the rat were collected before 10 days of age. After neonatal treatment with estradiol, hypothalamic concentrations of GnRH are lower in early neonatal life (5–7 days) and higher later in development (60 days) when compared with those of the untreated control rat (Goomer *et al.,* 1977). This could explain the differences between our results and those of Elkind-Hirsch *et al.* (1981).

Since the male mouse brain contains active aromatase (Wozniak *et al.,* 1992), it is possible that testosterone in normal males is aromatized to estradiol causing an increase in the number of GnRH-ir neurons above that of normal females. This idea is consistent with our data (and prior data, Elkind-Hirsch *et al.,* 1981) showing a greater number of GnRH-ir neurons in males. Taken together, these results suggest that this sexual dimorphism may be mediated by the aromatization of testosterone to estradiol. However, it is known that both 5α-dihydrotestosterone and estradiol can affect the number of GnRH-ir neurons in rats (Silverman *et al.,* 1994) and frogs (Iela *et al.,* 1994). Thus, it is probably unlikely that aromatase alone is responsible for the difference in GnRH-ir neuron numbers.

The exact mechanisms whereby estradiol mediates GnRH cell function in the mammal brain are not entirely clear. Several studies indicate that GnRH-producing neurons in a variety of mammalian species do not possess estrogen receptors as adults (Herbison and Theodosis, 1992; Lehman and Karsch, 1993; Silverman *et al.,* 1994). To our knowledge, there are no existing studies that examine potential colocalization of estrogen receptors in GnRH cells of preadult mammals. Given that the GnRH gene in mice (Radovick *et al.,* 1992) contains a region that binds the estrogen receptor (estrogen response elements), it is possible that GnRH-producing neurons do express the estrogen receptor during a specific developmental period. Transient expression of estrogen receptors during development has been shown in the rat cortex (Yokosuka *et al.,* 1995). Although the estrogen receptor has not been identified *in vivo* in adult GnRH cells, there are cell

lines that do produce GnRH and may express the estrogen receptor (Poletti *et al.,* 1994). An alternative hypothesis is that a more complex mechanism is involved that does not require the GnRH-producing neurons to respond directly to estrogen (Silverman *et al.*, 1994).

While our data do not show a significant decrease in ovarian weight in estradiol-treated females (Table 1) this trend is consistent with earlier data in the adult rat (Brawer *et al.,* 1986; Kikuyama, 1962) and mouse (Eroschenko *et al.*, 1995; Martinez and Swartz, 1991). Only the estradiol-treated animals in our study had cystic ovaries, which is consistent with a state of estradiol-induced persistent vaginal estrus or diestrus (Aihara and Hayashi, 1989; Kikuyama, 1962), as shown in earlier studies (Aihara and Hayashi, 1989; Walters, *et al.*, 1993).

In conclusion, we have shown that male mice have more preoptic GnRH-ir neurons than females, and estradiol treatment of neonatal mice causes sex-specific increases in the number of GnRH-ir neurons; males respond and females do not. This is consistent with the notion of sexually dimorphic neural substrates developing at an early age. In response to high levels of exogenous estradiol, males show an increase in GnRH-producing neurons in the brain. It is possible that these changes in the number of GnRH-ir neurons are normally mediated through the aromatization of testosterone to estradiol in the brain. Finally, it is known that by changing the neonatal steroidal milieu, the organization of the brain can be altered in ways that have a significant impact on adult reproduction. In summary, one interpretation, consistent with our results, is an organizational effect of estradiol on the number of GnRH-ir neurons in the male mouse. A putative mechanism for this effect is suggested by the higher number of cells in estradiol-treated males.

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