Expression of Estrogen Receptor α in the Anteroventral Periventricular Nucleus of Hypogonadal Mice

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Gonadotropin-releasing hormone-1 (GnRH-1) neurons play critical roles in the development and maintenance of reproductive function in all vertebrates. Due to a truncation in the *GnRH-1* gene, hypogonadal (hpg) mice are unable to synthesize GnRH-1 and are infertile. These animals develop in the complete absence of exposure to gonadal steroid hormones, making them an interesting model for understanding brain sexual differentiation and dimorphism. We studied expression of the estrogen receptors (ERs) α and β in the medial anteroventral periventricular nucleus (mAVPV), an important reproductive neuroendocrine brain region, in wild-type and hpg mice of both sexes. Adult wild-type and hpg mice of the same genetic background were used to quantify numbers of ERα and ERβ immunoreactive cells in the mAVPV using a stereologic approach. Quantitative analyses showed that ERα cell numbers were significantly higher in hpg than wild-type mice, irrespective of sex. Qualitatively, ERα-immunoreactive cells were concentrated more densely along the ventricular zone of the AVPV of wild-type female mice compared with wild-type male mice or hpg male and female mice. No ERβ-immunoreactive cells were detected in the mAVPV of any mice, a result that was surprising because ERβ expression is abundant in the mAVPV of rats. These results on ERα provide additional evidence that the female brain is not the "default" organizational pattern, because neither ERα cell number nor its distribution in hpg mice, which develops with a deficiency of reproductive hormones, resembles that of the wild-type female mouse. Differences in ERα expression may be due in part to the absence of gonadal steroid hormones, although they more likely to also involve othe factors, potentially GnRH itself. Exp Biol Med 230:49–56, 2005

Key words: estrogen receptor; hypothalamus; preoptic; AVPV; hypogonadal; GnRH; stereology

Gonadotropin-releasing hormone (GnRH) neuron play a critical role in the development and main tenance of reproductive function in all vertebrate (1). A spontaneous autosomal recessive deletion in th *GnRH-1* gene (encoding the hypophysiotropic GnRH-decapeptide) resulted in hypogonadal (hpg) mice, which ar unable to synthesize the mature GnRH-1 peptide and ar infertile (2). As a consequence of this absence of GnRH hpg mice exhibit extremely low levels of pituitary an serum gonadotropins and infantile gonads. The uterus i female and accessory sex glands in male hpg mice ar extremely small, indicating the presence of minima amounts, if any, of sex steroid hormones in these mic (2). Therefore, this hpg animal model provides an excellen opportunity to study brain morphology and recepto expression in the absence of any gonadal sex steroi hormone and other reproductive hormone actions through out development.

The effects of the sex steroid hormone estrogen ar mediated in large part through the genomic estroge receptors (ERs) α and β (3). With respect to its feedbac actions on the hypothalamic-pituitary-gonadal axis, estroge may act on GnRH neurons directly, most probably via th ERβ, as shown in rats and mice (4–6) or possibly throug membrane ERs (7). In addition, estrogen may also affec GnRH neurons indirectly, including through the ERα that i abundant in the hypothalamus but probably absent in GnRI neurons (8).

Both ERα and ERβ are differentially distributed in th hypothalamus and other brain regions (9, 10). In rats, ERα i highly expressed in regions that regulate reproductiv physiology and behavior, including anteroventral periven

This work was supported by the National Institutes of Health grants PO1 AG16765 (A.C.G.) and RO1 MH59824 (G.V.R.).

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Received July 14, 2004.
Accepted September 14, 2004.

1535-3702/05/2301-0049$15.00

tricular nucleus (AVPV), medial preoptic nucleus, arcuate nucleus, ventromedial nucleus, and amygdala (9, 11, 12). The ERβ is more abundantly expressed in regions of the brain not traditionally considered to be involved in reproductive physiology, including the cerebral cortex, hippocampus, paraventricular nucleus, and supraoptic nucleus, although it is also found in the preoptic nucleus, bed nucleus of the stria terminalis (BST), amygdala, and AVPV of rats (13–15). A recent study (10) on ER expression in ovariectomized female mice demonstrated several species differences in distribution compared with the ovariectomized rat, although in general the distribution pattern is largely similar between these two rodent species.

We performed a quantitative stereologic study of the number of cells that express nuclear ERα or ERβ in male and female mice and compared differences between wild-type and hpg mice. This latter comparison enabled us to determine whether ER cell number in the brain was altered in the absence of GnRH and sex steroid regulation throughout the life of the organism. We focused our analysis on the AVPV, which is sexually dimorphic (16), expresses both ERs in the rat (11–15), and plays critical roles in reproduction (16, 17).

Material and Methods

Animals. The hpg and wild-type mice of the same genetic background (C3H/HeH x 101H) were housed in a colony room, with a partially reversed light cycle of 14:10 (lights on at 2300 hrs and lights off at 1300 hrs). Because we were interested in the expression of ERs in the AVPV under the influence of the normal physiologic hormonal environment for both age and sex, the mice analyzed in this study were gonadally intact. Female wild-type mice were not subjected to vaginal smears and were therefore used at random times during their estrous cycles. Food and water were available *ad libitum*. Animals were 12 weeks of age at the time of experimentation. All animal studies were conducted in accordance with the Guide for the Care and Use of Experimental Animals, using protocols approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine.

Immunocytochemistry for Stereologic Analyses. Mice (n = 5, 4, 5, and 5 for wild-type female, wild-type male, hpg female, and hpg male mice, respectively) were deeply anesthetized by an overdose of chloral hydrate (760 mg/kg) and were perfused transcardially with isotonic sodium chloride solution for 2 mins followed by 4% paraformaldehyde for 20 mins (flow rate, 3 ml/min). The brains were dissected and stored overnight in 4% paraformaldehyde at 4°C. Coronal sections, 50 μm thick, were obtained using a Vibratome (Ted Pella, Redding, CA). Endogenous peroxidase was blocked by treating the sections with 0.05% H_2O_2 followed by washes in phosphate-buffered saline (PBS) (pH 7.4). Nonspecific binding of the antibody was blocked by incubating free-floating sections for an hour in PBS that contained 2% normal donkey serum (NDS) and 0.2% Triton X-100. This was followed by incubation of the sections in primary antibody for ERα (C1355, Upstate Biotechnology, Lake Placid, NY; 1:20,000) or ERβ (ZP8, Zymed Laboratories, San Francisco, CA; 0.5 μg/ml) in PBS that contained NDS and Triton X-100 for 48 hrs. The specificities of the antibodies used were tested, and the dilutions determined by extensive pilot studies. Both antibodies are extremely well validated (4, 18–20) and have previously been used in our own laboratory for detection of nuclear ERα and ERβ in the rat (11, 12, 15, 21). A series of 1:3 sections through the AVPV was used; the first of each series was used to determine immunolabeling of ERα and the second for ERβ. Every third section was used for Nissl staining with neutral red to assess the contour of the AVPV. For ER immunostaining, after washes in PBS, the sections were incubated with a dilute anti-rabbit IgG-biotin (1:200 in PBS that contained 1% NDS and 0.1% Triton X-100) raised in donkey (Jackson Immuno Research, West Grove, PA) for 2 hrs, followed by further processing using the ABC kit (Vector Labs, Burlingame, CA). The ER immunoreactivity was visualized using diaminobenzidine (DAB) as the chromogen. The sections were floated onto gelatin-coated slides and coverslipped using Permount.

Stereologic Analysis. A modified stereologic analysis was performed. Each immunostained section throughout the AVPV was outlined at low magnification (X10) on the live computer image as described previously (11, 12, 15, 21). The sections were carefully matched among all the animals and the AVPV identified using adjacent Nissl-stained sections and anatomic landmarks with the assistance of a mouse brain atlas (22). The borders of the AVPV were demarcated most rostrally by the caudal aspect of the organum vasculosum of the lamina terminalis, where the anterior commissure first began to cross, and most caudally by where the anterior commissure was first fully crossed, at the level of the rostral medial preoptic nucleus (11, 15). There were no differences in the location of the median AVPV (mAVPV) between wild-type and hpg mice, and this region could be identified in our Nissl-counterstained tissues. Following contouring of the AVPV, a boundary was drawn, and then a vertical boundary 60 μm from the ependyma of the third ventricle was drawn. Cells falling within this 60-μm wide region were quantified. This region is henceforward referred to as the mAVPV. The 60-μm boundary was used based on our pilot observations of heterogeneity of ER expression in the AVPV; the use of this boundary in our modified stereologic analysis made the analyses more accurate and less heterogeneous and diminished manual error. The StereoInvestigator software (MicroBrightField Inc., Wiliston, VT) placed disector frames using a systematic random design within each contour that outlined a 50 x 50-μm grid, and the DAB-stained ER-labeled nuclei were counted within 50 x 50-μm optical dissectors in the *x-y* axis. The final postprocessing thickness of the sections was measured by the microcator.

The mounted section thickness was on average 12 μm, and therefore the counting frame height was kept at 10 μm for all sections studied. As we have described previously (12), because the neuronal number estimates were made using with the optical fractionator and do not depend on a direct measurement of the volume of reference of the region considered, the shrinkage of the tissue during histologic processing does not influence the precision of these estimates. For these calculations, the average postshrinkage value for the *z* axis was used, although volume estimates are based on postprocessing materials that have shrunk in all three dimensions. No attempt to introduce correction factors for shrinkage was made, because it likely differs in the *z* and *x-y* directions. Six to 10 sections were available for analysis. Only animals from which an intact series of sections was available were retained for analysis.

The optical fractionator was used to estimate the total number of ER-immunoreactive nuclei in the mAVPV, and stereologic analyses were performed using a 63X immersion oil with a 1.4 numerical aperture objective on a Axioplan 2 photomicroscope (Zeiss, Jena, Germany). Further stereologic procedures and analyses were performed identically to our previously published work and are described in detail in previous reports (11, 12, 15, 21).

Statistical Analysis. Differences in the number of ERα- and ERβ-immunoreactive nuclei were determined in the mAVPV of wild-type and hpg male and female mice using Statview 5.0 statistical software (SAS Institute Inc., Cary, NC) for the Macintosh computer. In each case, two-way analysis of variance was performed (variables: genotype and sex). When indicated, post hoc analysis was performed using the Fisher protected least significant difference test. Effects were considered significant at $P < 0.05$.

Results

Distribution of ERα in Wild-type and hpg Mice. Nuclear ERα immunostaining was abundant in the mAVPV and surrounding hypothalamic and preoptic regions of both wild-type and hpg mice. Representative photomicrographs of ERα-immunoreactive cells in the mAVPV of wild-type and hpg male and female mice are shown in Figure 1. The area of the AVPV as indicated by Nissl staining and on a mouse brain map (22) are shown in Figure 1C and F, respectively. Qualitatively, there appeared to be higher numbers and greater density of ERα-immunoreactive cells in the hpg than wild-type mice for both males and females. Moreover, the distribution of ERα in the AVPV differed qualitatively, depending on sex and genotype. In the wild-type female mice, ERα appeared to be concentrated most densely near the third ventricle (Fig. 1A), whereas the expression in hpg female mice was more diffusely distributed throughout the AVPV (Fig. 1B). Male mice of both genotypes showed a fairly even distribution of ERα throughout the AVPV, similar to that in hpg female mice (Fig. 1D for wild-type male mice and Fig. 1E for hpg male mice). Based on these qualitative observations, we performed a quantitative stereologic analysis of the number of ERα-expressing cells in the mAVPV of the four groups of mice.

Stereologic Analysis of the Number of ERα Cells in the mAVPV. The number of ERα-immunoreactive cells in the mAVPV of hpg and wild-type mice was quantified by unbiased stereologic methods. There was a significant effect of genotype ($P < 0.001$) but not sex ($P = 0.17$) and no interaction of genotype and sex ($P = 0.85$) on the number of ERα-expressing cells in the mAVPV (Fig. 2). Post hoc analysis indicated that the number of ERα-immunoreactive cells was significantly higher in the hpg than the wild-type mice irrespective of sex ($P < 0.001$).

Lack of ERβ in Wild-type and hpg Mice. No ERβ-immunoreactive cells could be detected in the mAVPV of male and female mice of both genotypes (Fig. 3A–D). However, nuclear ERβ expression was easily detectable in other parts of the brain, such as the BST (Fig. 3E and F), indicating that this absence in the mAVPV was not due to a failure of the antibody to detect ERβ-positive nuclei in the mouse brain. The ERβ was detectable in the BST of all four groups, and no qualitative differences were noted. In addition, the identical mice were used to provide alternate series of sections for the detection of ERα and ERβ immunoreactivity, indicating that the lack of ERβ in the AVPV was also not due to inadequate perfusion or tissue preparation. Since no ERβ-immunoreactive cells could be detected in the AVPV of any of the mice examined, no stereologic analysis could be performed on this molecule.

Discussion

In the present study, we analyzed the expression of the two nuclear ERs found in mammals, ERα and ERβ in the murine AVPV. We performed our analyses on the basis of genotype (wild-type vs. hpg mice) and sex (male vs. female), the latter because the expression of ERs in AVPV of rats has been reported to be sexually dimorphic (14, 23) and the former because hpg animals develop in the complete absence of GnRH and the organizing effects of gonadal steroid hormones (2). We found that ERα but not ERβ immunoreactivity is detectable in the murine mAVPV. Moreover, quantitative stereologic analysis indicated that ERα cell number does not differ between male and female mice, irrespective of genotype. However, ERα cell numbers are significantly greater in hpg than wild-type mice, independent of sex. Thus, ERα cell numbers are more abundant in animals that lack exposure to GnRH and sex steroid hormones throughout their development.

Effects of Sex on ER Expression in Wild-type Mice. The sexual dimorphism in the size and neurochemical makeup of the AVPV in rats is due to the organizing effects of prenatal and neonatal testicular hormones (16, 23). In that species, the AVPV is larger in female than males, and ERα and ERβ expression in the AVPV of rats is also sexually dimorphic, again being

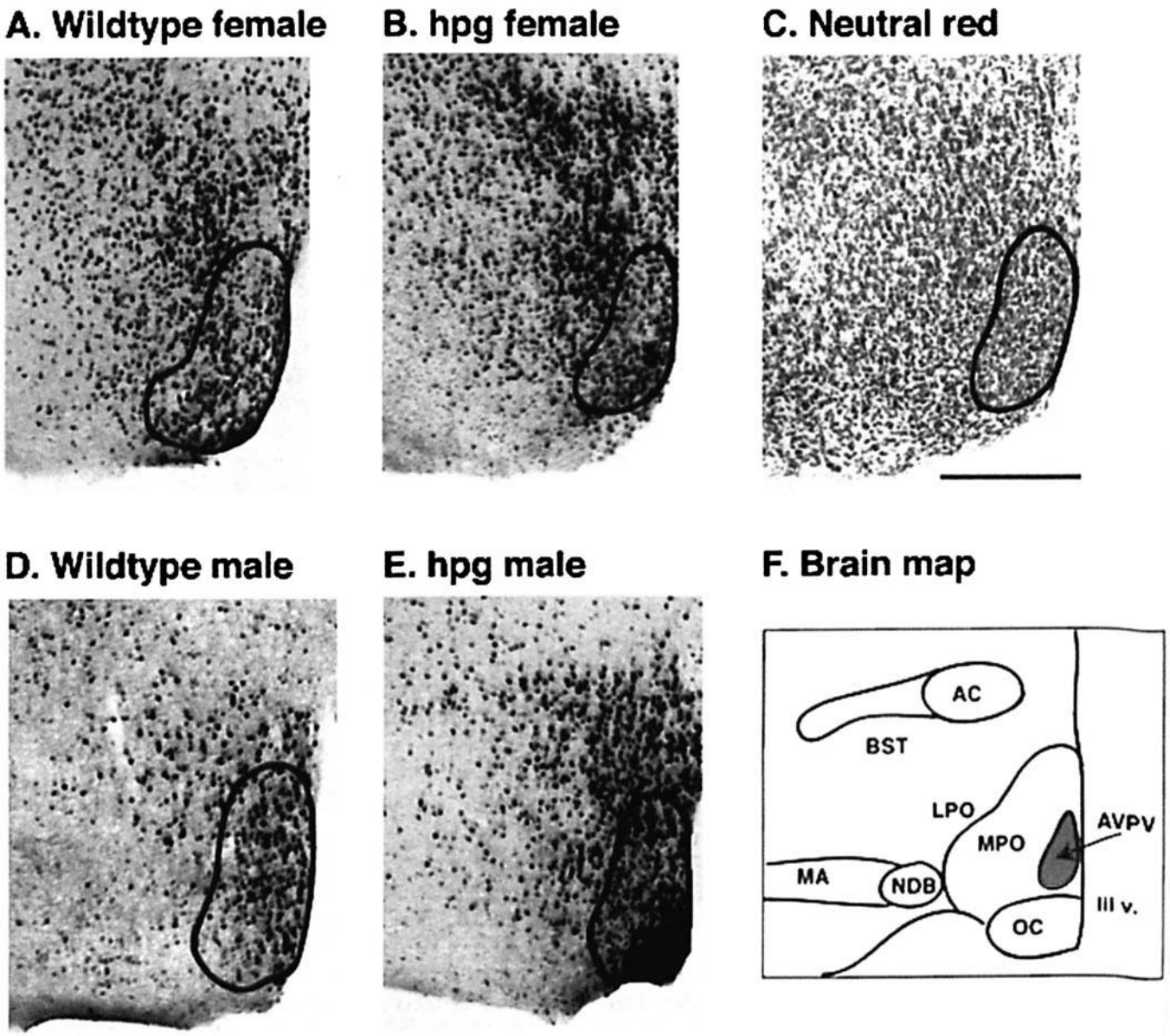

Figure 1. Photomicrographs of estrogen receptor α (ERα)–immunoreactive cells in the AVPV of female and male wild-type and hpg mice. The anteroventral periventricular nucleus (AVPV) (indicated by the contour) and surrounding preoptic regions were immunolabeled with ERα, seen as dark brown nuclear staining. Representative photomicrographs are shown for a representative wild-type female (A), hypogonadal (hpg) female (B), wild-type male (D), and hpg male (E) mice. (C) A Nissl-counterstained section from a wild-type male mouse. (F) The position of the AVPV in a mouse brain atlas is shown in green (modified from Ref. 22 at the level of Bregma = 0.10 mm). Scale bar, 100 μm. AC, anterior commissure; BST, bed nucleus of the stria terminalis; LPO, lateral preoptic area; MA, magnocellular preoptic nucleus; MPO, medial preoptic area; NDB, nucleus of the diagonal band; OC, optic chiasm; iii v, third ventricle.

higher in females than males (13, 14). The ERβ messenger RNA (mRNA)–expressing cells are also dimorphic in their distribution in the rat AVPV, with ERβ-expressing cells being more medially concentrated in the AVPV of females and more evenly distributed throughout the AVPV of males (14).

These previous findings in rats are considerably different from our present quantitative results in mice, which demonstrate no sex difference in ERα cell number in the mAVPV. However, our study focused on the most medial 60 μm of the AVPV, representing approximately 75%–80% of this region, whereas others in rats studied the entire AVPV. Herein, despite the lack of a quantitative sexual dimorphism in ERα cell number between male and female mice, we observed a qualitative difference in the distribution of ERα between the sexes. In female mice, ERα appeared to be more concentrated in more medial parts of the mAVPV, whereas in male mice ERα was distributed more evenly throughout the mAVPV. In fact, this dimorphic distributional pattern of ERα in mouse mAVPV is similar to that reported for ERβ in the AVPV of rats (14, 21). However, unlike mice, ERα in female rats is distributed

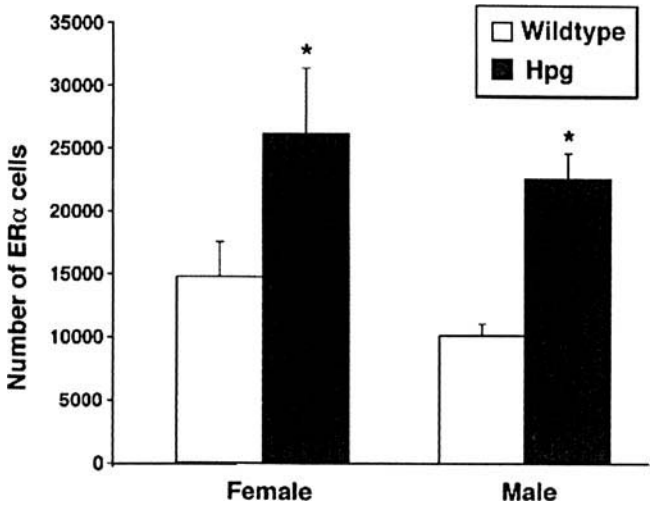

Figure 2. Stereologic quantification of the total number of estrogen receptor α (ERα)–immunoreactive neurons in the anteroventral periventricular nucleus of female and male wild-type and hypogonadal (hpg) mice. The white bar shows data for wild-type animals, and the black bar shows data for hpg mice. In both female and male mice, there was a significant effect of genotype on ERα cell numbers (*$P <$ 0.001). No effect of sex was observed.

more evenly throughout the AVPV (11, 12). It is noteworthy that Simerly *et al.* (24) reported that the AVPV of mice has a sexually dimorphic expression of tyrosine hydroxylase immunoreactivity but not preproenkephalon mRNA. That study, together with our current report, suggests that the phenotype of some (tyrosine hydroxylase) but not all (ERα, preproenkephalon) neurons in the AVPV can vary with sex in the mouse. It is also currently unknown whether the murine AVPV is sexually dimorphic, and this merits further investigation.

To our surprise, we found that ERβ is undetectable in the AVPV of mice. This is in contrast to results of previous reports in rats, including two from our own laboratory that showed abundant expression of this molecule in AVPV (14, 21). We were able to detect ERβ in other brain regions of mice, such as the BST, similar to a recent report (10), indicating that the absence of ERβ in the AVPV in our present study is not due to a failure of our experimental procedure to detect the antigen in mouse neural tissue. Nevertheless, a study by Mitra *et al.* (10) reported low numbers of ERβ-immunoreactive cells in the AVPV of ovariectomized female C57BL/6J x 129 mice. Their animals differed from ours in the strain (herein we used C3H/HeH x 101H mice), the ovarian status (the mice of Mitra *et al.* were ovariectomized, whereas ours were intact), and the fixation method (their animals were perfused with a fixative that contained acrolein). In addition, the studies used different antibodies (the current study used a Zymed antibody that recognizes the C-terminus 18 amino acids of the mouse ERβ, whereas the antibody used by Mitra *et al.* recognizes amino acid residues 64–82 of a region of ERβ that is conserved in rat, mouse, and human) and different detection methods (we used a DAB system, and Mitra *et al.* used a tyramine signal amplification system with nickel-DAB; this latter system would increase detectability of low-level expression of ERβ). Because of all these differences, it is impossible for us to infer whether one or all of these differences may account for the finding of Mitra *et al.* of low expression of ERβ, and our total absence of ERβ expression, in the murine AVPV. In a pilot study, we had perfused some of our C3H/HeH x 101H control mice with the fixative that contained acrolein, similar to that used by Mitra *et al.* (10), and also failed to detect any ERβ immunoreactivity in the AVPV of our animals, suggesting the difference is more likely attributable to one or more of the other differences described herein. Nevertheless, a more salient point is that in both their study (10) and ours there was low or no expression of ERβ in murine AVPV, which is far different from previous reports in rats that show high ERβ expression in this region (14, 15, 21).

Effects of Genotype (hpg vs. Wild-type) on ER Expression in the AVPV. Gonadotropin-releasing hormone is required for reproductive function in all vertebrates (1). The hpg mouse, which does not synthesize the mature GnRH-1 decapeptide (25), has proven to be extremely useful in understanding brain function in the absence of GnRH and the resulting lack of development of the pituitary and gonadal components of the reproductive axis (2). When we compared ERα-immunoreactive cell numbers in the mAVPV between wild-type and hpg mice, we found an effect of genotype, with significantly higher numbers of ERα-positive cells in hpg than wild-type mice. We did not observe any sex difference in ERα expression in the mAVPV of either hpg or wild-type mice. As was the case for wild-type mice, we also did not detect any ERβ immunoreactivity in the mAVPV of hpg mice.

In rodents, perinatal gonadal steroid hormone exposure is important for organizing the brain to result in the appropriate sex-typical physiology and behavior in adulthood (26). Therefore, the low or absent sex steroid hormone levels in the hpg male mouse result in insufficient exposure to androgens and estrogens during the critical period of organization of the brain. This is likely to be responsible for the greater abundance of ERα-immunoreactive cells in hpg male mice compared with wild-type male mice. The increased number of ERα-expressing cells in the AVPV of female hpg mice compared with wild-type mice is more puzzling, because normally female rodents have relatively little exposure to perinatal sex steroid hormones, and the absence of estrogen and/or testosterone that occurs in hpg female mice would not be anticipated to cause such a significant elevation in ERα-immunoreactive cells compared with their wild-type female counterparts. There are several alternative possibilities that could explain our observations. First, even the low circulating estradiol and testosterone levels in neonatal wild-type mice may be sufficient to downregulate ERs compared with hpg mice, which have no estrogen to exert this effect. Second, factors

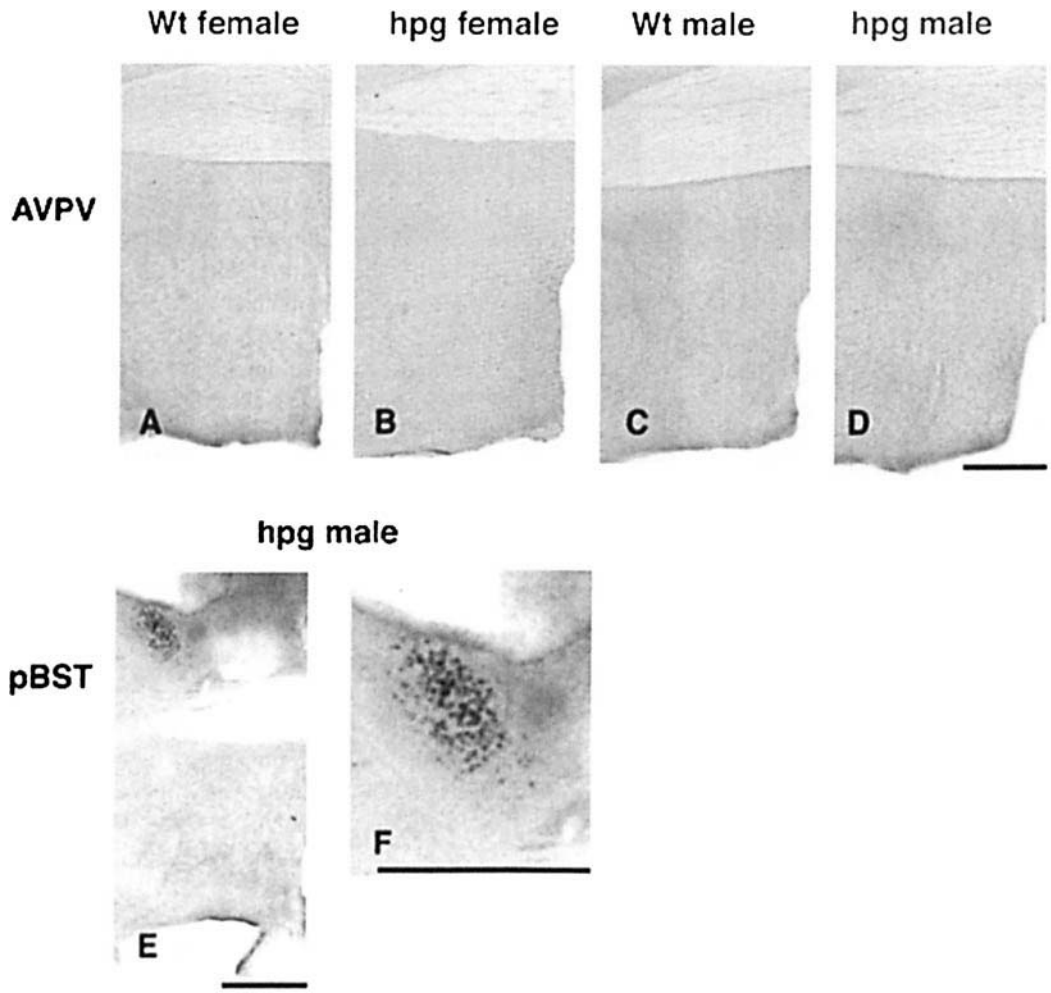

Figure 3. Photomicrographs of estrogen receptor β (ERβ) immunoreactivity in the anteroventral periventricular nucleus (AVPV) and principal nucleus of the BST (pBST) of representative female and male wild-type (wt) and hypogonadal (hpg) mice. (A–D) Lack of ERβ immunoreactivity in the AVPV and surrounding preoptic regions of representative wild-type female (A), hpg female (B), wild-type male (C), and hpg male (D) mice. (E) pBST of a representative male hpg mouse (upper left in panel), demonstrating the presence of ERβ immunoreactivity. (F) The pBST region of panel E at higher magnification. Scale bar, 100 μm.

other than sex steroid hormones that differ between hpg and wild-type animals may be responsible for the increased numbers of ERα-expressing cells in the AVPV of hpg mice. For example, GnRH is absent and gonadotropin levels are suppressed (2) in hpg mice of both sexes. Although none of these hormones has typically been considered to exert organizing influences, perhaps they play a more important role in organizing and/or maintaining levels of ERs than has previously been suspected. Third, gonadal steroid hormones in developing wild-type female mice may play a more important and active organizational role in brain development than previously suspected. This is an intriguing possibility, providing further evidence that the female neural sexual differentiation pattern is not a "default" pathway (27, 28). Future studies on genotypic differences in ERα expression in the AVPV of hpg mice will help to differentiate the mechanisms responsible, such as decreased apoptotic cell death or increased cell survival of those neurons or glia that express ERα.

Although we noted a qualitative difference in the distribution of ERα cells between male and female wild-type mice, this was not observed between male and female hpg mice. In wild-type female mice, ERα-expressing cells were concentrated in greater proximity to the third ventricle, whereas wild-type male mice and hpg male and female mice had ERα cells spread more evenly throughout the mAVPV. The mechanism for this difference in ERα distribution in the mAVPV, which differs between male and female wild-type mice, must not be the same as the mechanism that determines absolute ERα cell numbers, which are similar between male and female wild-type mice. The sex difference in ERα distribution in the mAVPV of wild-type male compared with female mice may be due to an ovarian (potentially nonsteroidal) factor in wild-type female mice that organizes the heterogeneous ERα expression pattern and is absent in wild-type male mice and hpg mice of both sexes.

The hpg mice provide an interesting comparison to other experimental models. For example, the hpg mouse is

different in hormonal exposures and sensitivity compared with ERα knockout (ERαKO) and ERβ knockout (ERβKO) mice. Female ERαKO mice have elevated gonadotropin protein and mRNA levels and greatly increased circulating estradiol levels (29, 30), and male ERαKO mice have higher testosterone and luteinizing hormone levels (29, 31), presumably as a result of reduced sex steroid feedback on the hypothalamic-pituitary-gonadal axis. By contrast, in hpg mice, all of these hormones are undetectable or suppressed (2, 32). In addition, hpg mice differ from another experimental model, neonatally castrated animals, because the latter have elevated GnRH and gonadotropin levels (33), whereas hpg mice do not. It is also relevant to compare our observation of an increase in ERα cells in the AVPV of hpg mice with an earlier study (34) that showed significantly lower androgen receptor immunostaining in several structures in the basal forebrain of hpg than wild-type male mice. These findings, taken together, indicate that the regulation of neural expression of sex steroid hormone receptors involves distinct mechanisms that are differentially affected by circulating sex steroid hormone levels.

In summary, we found a significant increase in the number of cells that express ERα in the mAVPV of hpg mice compared with wild-type mice. No sex difference in the number of ERα cells was observed in either genotype, although the distribution of ERα-expressing cells was qualitatively different in wild-type female mice compared with all other groups. The ERβ was not detectable in the murine AVPV, which was surprising because it is abundantly expressed in this region in the rat (14, 15, 21). Taken together, our results indicate that the hormonal milieu that governs the expression of ERs are altered in mice that lack GnRH neurons and suggest that the expression of these receptors potentially involves nonsteroidal factors.

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