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Sexual Differentiation of Galanin Gene Expression in Gonadotropin-Releasing Hormone Neurons*

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ABSTRACT

In adult rats, only females are capable of generating an LH surge in response to high levels of ovarian steroids. This is because the ability to generate LH surges in adulthood in response to ovarian steroids is suppressed by high levels of testosterone during neonatal life. We have previously shown that galanin gene expression in GnRH neurons is induced by ovarian steroids in association with an LH surge, suggesting that galanin plays a role in the generation of the LH surge. If this is the case, then the induction of galanin gene expression in GnRH neurons in response to ovarian steroids might, like the LH surge itself, be sexually differentiated during the neonatal period. To test this hypothesis, we manipulated gonadal steroid levels in four groups of rat pups on the first day of life and looked at the ability of ovarian steroids to induce an LH surge and stimulate galanin gene expression in GnRH neurons of adult animals. The four groups consisted of female pups that were injected with either 10 μg of testosterone propionate (n = 6; testosterone-treated females) or vehicle (n = 6; control females) and male pups that were either castrated (n = 6; castrated males) or sham-castrated (n = 6; sham-castrated males). All animals were then gonadectomized at 59 or 60 days of age, except for the males that had been castrated neonatally. Seven weeks later, the animals were challenged with estradiol (E_2) benzoate and progesterone (P) in a paradigm that reliably produces an LH surge. (The rats were injected sc with 15–30 μg of E_2 on day 0 at 1030 h and 5 mg of P on day 2 at 1200 h.) The animals were then killed between 1800 h and 1840 h on day 2 at the time of the expected LH surge, and their brains were processed for double-label *in situ* hybridization to estimate cellular levels of galanin messenger RNA (mRNA) in GnRH neurons. We reconfirmed the classical observation that the ability to generate an LH surge in response to E_2/P is suppressed by neonatal exposure to testosterone. Galanin mRNA levels in GnRH neurons showed a similar pattern: after E_2/P priming, galanin mRNA levels in GnRH neurons were significantly higher in normal females and in males castrated neonatally than in normal males and in females treated with testosterone neonatally. These results demonstrate that the E_2/P induction of galanin gene expression in GnRH neurons at the time of the LH surge is sexually differentiated during the neonatal period and suggest that the increase in galanin gene expression in GnRH neurons could be one of the mechanisms underlying the sex difference in gonadotropin release seen in rats. (*Endocrinology* **137:** 4767–4772, 1996)

IN FEMALE RATS, the rising tide of estradiol (E_2) on diestrus and early proestrus acts both at the brain to induce the release of GnRH and at the pituitary to increase its sensitivity to GnRH resulting in the generation of a preovulatory gonadotropin surge (for review see Ref. 1). This cyclic or sex steroid-dependent release of the gonadotropins, LH and FSH, is sexually differentiated (for review see Refs. 2–4). The classical study of Caroll Pfeiffer (5) was the first to demonstrate that the neuroendocrine reproductive axis of the rat differentiates under the control of gonadal steroids during the first few days of life. It is now firmly established that in many mammalian species exposure to testosterone during a certain period of development, often referred to as the critical period, blocks the ability of ovarian steroids to induce an LH surge in adulthood (for review see Refs 2–4). Thus, neonatal exposure to testosterone appears to permanently alter, or organize, the neural substrates mediating cyclic release of LH; however, the cellular mechanisms by which this occurs remain to be elucidated.

In adult rats, a subset of GnRH neurons express and synthesize the neuropeptide, galanin (6–8). The distribution and concentration of galanin in GnRH neurons is sexually differentiated with females having a greater number of GnRH neurons containing detectable levels of galanin and galanin messenger RNA (mRNA) than males (6, 9), and this sex difference is determined by the presence of the testes during the neonatal critical period (10). Although the physiological role of galanin derived from GnRH neurons is presently unknown, several lines of evidence suggest that it plays a role in the generation of the LH surge. First, in female rats, LH secretion is increased by central infusions of galanin (11, 12), and these effects are blocked by prior treatment with galantide (12), a galanin antagonist. Second, the LH surge is attenuated by systemic infusions of galanin antiserum (13) or by central or systemic infusions of galantide (12). Third, galanin stimulates GnRH release *in vitro* from arcuate/median eminence fragments obtained from male or female rats (12, 14). Fourth, galanin mRNA levels in GnRH neurons increase coincidentally with a preovulatory or steroid-induced LH surge (15–17). Fifth, pharmacological blockade of the LH surge blocks the induction of galanin gene expression in GnRH neurons (16, 18). If the induction of galanin in

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GnRH neurons is necessary for the generation of the LH surge and constitutes one of the cellular mechanisms underlying the difference in LH secretion patterns between males and females, then the ability of ovarian steroids to induce galanin gene expression in GnRH neurons coincident with the LH surge should, like the pattern of LH secretion, be sexually differentiated during the neonatal period. In this study, we used double-label *in situ* hybridization histochemistry to determine if neonatal exposure to testosterone precludes the induction of galanin gene expression in GnRH neurons and the associated LH surge in adult, ovarian steroid-primed rats.

Materials and Methods

Animals and accommodations

Pregnant Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA). The pups from these rats were used in this experiment. All rats were housed in the animal-care facilities of the University of Washington Department of Comparative Medicine. The animals were maintained under constant temperature and a 14-h light, 10-h dark cycle with lights on at 0700 h. They were given free access to tap water and rat chow. After weaning, the rats were caged 2–4 animals/cage, and the animals that had received similar neonatal manipulations were housed together. All procedures were approved by the Animal Care Committee of the School of Medicine at the University of Washington in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Experimental design

To test the hypothesis that the ability of ovarian steroids to induce galanin mRNA expression in GnRH neurons is blocked by neonatal exposure to testosterone, rat pups were divided on the day of birth into four groups based on sex and neonatal sex steroid manipulation. Two of the groups consisted of female pups. The females in one group received an sc injection of testosterone propionate (10 μg in 0.03 ml of peanut oil). This treatment reliably produces sterility in female rats (19). The females in the other group received an sc injection of vehicle alone (0.03 ml of peanut oil). The two other groups consisted of male pups. The males in one of these groups were castrated, and the males in the other group were sham-operated. Neonatal castrations and sham-castrations were done under hypothermic anesthesia. The pup was placed on its dorsal surface and a small rostral-caudal incision was made on its ventral surface about 3 mm lateral and 3 mm rostral to the genital papilla. After carefully opening the abdominal cavity, the testis was located and removed. The incision was sealed with cyanoacrylic cement. The same procedure was repeated on the other side. After surgery, the animals were warmed on a heating pad and then returned to their mothers. Sham-castrations were performed as described above except that the testes were not removed. The litters were culled to 8 pups. All treatments (injections and operations) were done within 4–19 h postpartum. At 59 or 60 days of age, all of the animals in the four treatment groups were gonadectomized, except for the males that were gonadectomized as pups. These males were sham-operated.

Seven weeks later, all rats received a regimen of ovarian steroid treatments designed to elicit an LH surge. On day 0 at 1030 h, all animals were injected sc with E_2 benzoate (15–30 μg in 0.1–0.2 ml of peanut oil). On day 2 at 1200 h, the animals were injected sc with progesterone (P; Steris Laboratories, Inc., Phoenix, AZ; 5 mg/0.1 ml). All rats were then killed between 1800–1840 h, the time at which this steroid paradigm reliably produces an LH surge in normal, ovariectomized female rats (16).

Tissue preparation

After asphyxiation with CO_2, the rats were immediately decapitated. Their brains were rapidly removed, frozen on dry ice, and stored at −80 C. Trunk blood was collected and serum was separated and stored at −20 C until assayed for LH contents. Twenty-micron thick, coronal brain sections were cut on a cryostat and thaw-mounted onto siline-coated slides (Fisher Scientific, Fair Lawn, NJ). The slides were stored in air-tight boxes at −80 C until used in the assay. Sections were collected beginning at the level of Plate 16, according to the rat atlas of Paxinos and Watson (20), and ending at the level of the caudal aspect of the decussation of the anterior commissure. The tissue sections were collected onto four sets of slides, each one representing a one-in-four series of sections. One of these sets was processed for double-label *in situ* hybridization.

Riboprobe preparation

^{35}S-labeled galanin complementary RNA (cRNA) probe. The plasmid vector Bluescript (-) 13 (Stratagene, La Jolla, CA) containing a complementary DNA (cDNA) to rat galanin mRNA was provided by Drs. Henry Friesen and Maria Vrontakis (University of Manitoba, Winnipeg, Canada) (21). This plasmid contained a 680-bp insert that was complementary to 124 bases of 5′ untranslated message, the entire 372 bases of open reading frame, and 184 bases of 3′ untranslated message. *Hin*dIII was used to linearize the cDNA. The ^{35}S-labeled antisense riboprobe was synthesized *in vitro* using an Ambion MAXIscript Kit (Ambion, Austin, TX). The reaction contained the following ingredients: 50 μM α-thio-UTP of which 25% was ^{35}S-labeled and the remaining 75% was unlabeled; linearized cDNA (0.1 μg/μl); T7 RNA polymerase (0.5 U/μl); 1 × transcription buffer; 500 μM ATP, CTP, and GTP; RNAse inhibitor (0.625 U/μl); and 10 mM dithiothreitol (DTT). Residual DNA was digested with DNAse (0.5 U/μl) and the DNAse reaction was stopped by adding 80 mM EDTA. Finally, 1 μl of yeast transfer RNA (10 mg/ml) was added as carrier. The cRNA was separated from unincorporated nucleotides on a Sephadex G-50 column (Boehringer Mannheim, Indianapolis, IN). The final double-label hybridization solution contained a galanin cRNA probe concentration of 0.25 μg/ml·kilobase.

Digoxigenin-labeled GnRH cRNA probe. A 462-bp digoxigenin-labeled cRNA probe complementary to rat preproGnRH mRNA was used. The original plasmid containing the preproGnRH insert, previously described in detail (22), was generously provided by Dr. Anthony Mason (Genetech, South San Francisco, CA) and subcloned into a new plasmid vector (6). The cDNA was complementary to 170 bases of 5′ untranslated message, the entire 276 bases of open reading frame, and the first 16 bases of 3′ untranslated message. *Sal*I was used to linearize the cDNA. The riboprobe was synthesized *in vitro* in a transcription reaction containing the following ingredients: linearized cDNA (0.1 μg/μl); 1 × dig DNA labeling mixture (10 × solution contains 3.5 mM digoxigenin UTP, 6.5 mM unlabeled UTP, 10 mM GTP, ATP, and CTP; pH 6.5; Boehringer Mannheim, Indianapolis, IN); SP6 RNA polymerase (2 U/μl; Boehringer Mannheim, Indianapolis, IN); 1 × transcription buffer (provided with polymerase); RNAse inhibitor (2 U/μl); and 10 mM DTT. Residual DNA was digested with DNAse (0.5 U/μl). The DNAse reaction was stopped by adding 80 mM EDTA. The cRNA was separated from unincorporated nucleotides on a Sephadex G-50 column. The purified probe was diluted 1:1200 in hybridization buffer for double-label *in situ* hybridization. This concentration was determined to be optimal based on a test *in situ* hybridization assay.

The control experiments used to validate the integrity, binding kinetics, and specificity of the GnRH and galanin cRNA probes have been previously described (6, 23).

Double-label in situ hybridization

The method used for double-label *in situ* hybridization is based on the procedure used by Marks *et al.* (6) with slight modifications. In brief, sections were fixed in 4% paraformaldehyde for 5 min, rinsed in phosphate buffer, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then rinsed in 2 × SSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate), dehydrated in ethanol, delipidated in chloroform, rehydrated in a second ethanol series, and air-dried. The hybridization solution [freshly denatured ^{35}S-labeled galanin probe, digoxigenin-labeled GnRH probe, and yeast transfer RNA (1.8 mg/ml) in hybridization buffer consisting of 52% deionized formamide; 10% dextran sulfate; 0.3 M NaCl; 8 mM Tris, pH 8.0; 0.08 mM EDTA; 0.02% BSA; 0.02% Ficoll; 0.02% polyvinylpyrrolidone; and 200 mM DTT] was applied to the tissue (50 μl/slide). The slides were then

covered with siline-coated glass coverslips and incubated in humid chambers overnight at 60 C. The next day the tissue was treated with RNAse-A and washed under conditions of increasing stringency, including a 30-min wash at 65 C in 0.1 × SSC. The sections were then incubated for 60 min in blocking buffer [0.05% Triton X-100 and 2% normal sheep serum in 2 × SSC]. Next, the sections were washed 2 × 10 min in Buffer 1 (100 mM Tris-HCl and 150 mM NaCl; pH 7.4), incubated for 3 h at 37 C in Buffer 1 containing anti-digoxigenin fragments conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) diluted 1:1000, 1% normal sheep serum, and 0.3% Triton X-100. After rinsing the sections 2 × 10 min in Buffer 1 and 1 × 10 min in Buffer 2 (100 mM Tris-HCl, 50 mM $MgCl_2$ and 100 mM NaCl, pH 9.5), they were incubated in chromagen solution [Buffer 2 containing nitro blue tetrazolium-chloride (340 μg/ml), 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml), and levamisole (240 μg/ml)]. The reaction was stopped by rinsing the sections 2 × 15 min in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) when digoxigenin-labeled cells were clearly visible at the light microscopic level. Next, the slides were dipped in 70% ethanol, air-dried and, coated in 3% parlodian dissolved in isoamyl acetate. After air-drying, the slides were dipped in Kodak NTB-2 emulsion that was diluted 1:1 in 600 mM ammonium acetate and heated to 44 C, exposed for 9 days, and developed.

Semiquantitative analysis of cellular galanin mRNA levels in GnRH neurons

Slides were assigned a random three-letter code, alphabetized, and read in order with an automated image-processing system by an operator unaware of the animal's experimental group. The number of silver grains per cell was determined by a grain-counting program, as previously described (6). The image processing system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer (Apple Computer, Cupertino, CA). Video images were obtained by a Dage model 65 camera (Dage-MTI, Michigan City, IN) attached to a Zeiss Axioskop (Zeiss, New York, NY). The microscope was equipped with a 40× objective and a 100-watt mercury vapor epiillumination light source with polarizing cube.

GnRH neurons were identified under brightfield illumination by the presence of purple precipitate in the cytoplasm of a cell, indicating the presence of the digoxigenin-labeled cRNA probe for GnRH mRNA. Clusters of silver grains overlying these GnRH neurons, indicating the presence of the ^{35}S-labeled cRNA for galanin mRNA and its coexpression with GnRH mRNA, were counted under darkfield illumination by the computerized image processor. To minimize differences among experimental groups and make conservative estimates of the changes in galanin mRNA levels, all identifiable GnRH neurons were analyzed for silver grain counts and included in the totals. This avoided making subjective decisions on whether a particular GnRH cell was double-labeled for galanin mRNA. Thus, the grain count estimates may underestimate the actual galanin mRNA levels in a subset of galanin mRNA-expressing GnRH neurons. Fifteen sections per animal, equally spaced from the diagonal band of Broca to the caudal preoptic area, were analyzed for the number of grains per cell.

LH assay

Serum levels of LH were measured under the auspices of the RIA core of the Population Center for Research in Reproduction at the University of Washington (Dr. William Bremner, Director) with reagents provided by NIH. The standard was rLH-RP3, and the antiserum was anti-rLH-S11 (NIDDK, Bethesda, MD). The tracer was purchased from Corning Hazelton, Inc. (Vienna, VA). All samples were measured in a single assay. Based on the duplicate values of the samples run in the assay the intraassay coefficient of variation was 15%.

Statistical analysis

For the experiment, "n" refers to the number of experimental animals within a group, and this "n" was used for the analysis. For galanin mRNA content determinations, the mean grains per cell from individual animals were used to calculate the mean ± SEM for each group. Differences among groups in mean levels of galanin mRNA and the number of GnRH mRNA-containing cells counted were assessed by ANOVA. When the ANOVA indicated a significant difference, Fisher's protected least significant test was used to test for differences between groups. The rejection level for statistical tests was set at $\alpha = 0.05$. Several of the LH values exceeded the upper limit of the assay and were assigned a maximum value. The Kruskal-Wallis test was used to assess differences among groups in LH levels. When a significant difference among groups was found, differences between groups were assessed using the Mann-Whitney *U* test.

Results

Serum LH levels

Table 1 shows the serum LH data for all experimental groups. The groups differed overall ($P < 0.005$) in their serum LH response to E_2/P. Mean serum LH levels were 5- to 10-fold higher in the two groups of rats that had little or no exposure to testosterone neonatally. Mean LH levels were higher in control females than in testosterone-treated females ($P < 0.005$) or control males ($P < 0.005$). In castrated males, mean LH levels were higher than in control males ($P < 0.01$) but lower than in control females ($P < 0.005$). All of the control females and all but one of the neonatally castrated males appeared to have an LH surge (*i.e.* for each animal the LH level was at least three times greater than the mean LH level in control males). The one neonatally castrated male that did not show an LH surge in response to E_2/P (LH = 11 ng/ml) was excluded from the analysis of the castrated male group; however, the brain from this animal was processed by double-label *in situ* hybridization. The results from this animal are noted separately in the following section.

Galanin mRNA content in GnRH neurons

A one-way ANOVA indicated galanin mRNA levels in GnRH neurons differed significantly among groups ($P < 0.0001$). As seen in Fig. 1, E_2/P priming increased galanin mRNA levels in GnRH neurons to a significantly greater extent in the two groups of rats that had little or no exposure to testosterone neonatally (control females: 37 ± 1 grains/cell; castrated males: 41 ± 4 grains/cell) than in the two groups of rats that were exposed to testosterone neonatally (control males: 22 ± 2 grains/cell; testosterone-treated females: 21 ± 2 grains/cell). The photomicrograph in Fig. 2 illustrates the higher galanin mRNA expression in GnRH neurons of a neonatally castrated male compared with a neonatally testosterone-treated female. Mean levels of galanin mRNA in GnRH neurons did not differ significantly between the castrated males and the control females nor between the control males and the testosterone-treated females. In the one neonatally castrated male that did not show an LH surge in response to E_2/P in adulthood, galanin

TABLE 1. Mean levels of serum LH for all treatment groups

Treatment group (Sex and neonatal treatment)	LH (ng/ml)[a]
Female:oil (n = 6)	>200[b]
Female:testosterone (n = 6)	17 ± 5
Male:sham castration (n = 6)	19 ± 5
Male:castration (n = 5)	108 ± 18

[a] Values are expressed as mean ± SEM.

[b] LH values for each of the animals in this group exceeded the limits of the assay; therefore, each animal was assigned a value of 200 ng/ml, which was the upper limit of the assay.

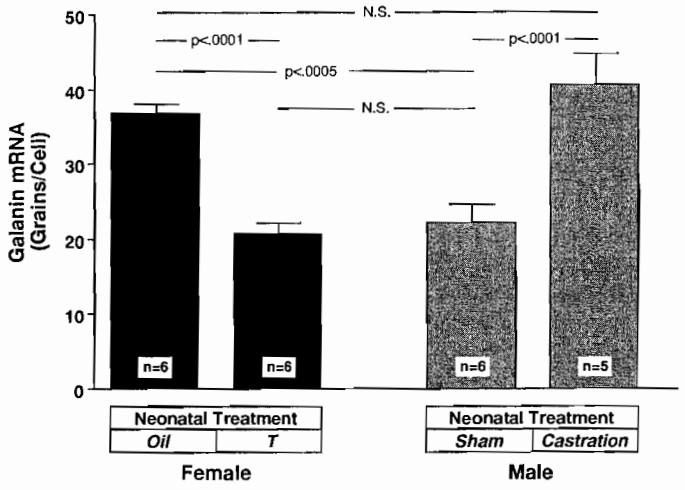

FIG. 1. Relative levels of galanin mRNA (grains per cell ± SEM) in GnRH neurons throughout the forebrain in groups of adult, gonadectomized, estradiol- and progesterone-primed male and female rats under different neonatal treatment regimens. The two female groups consisted of control animals (adult females injected with oil vehicle neonatally: oil) and experimental animals [adult females injected with testosterone (T) propionate neonatally: T]. The two groups of males consisted of control animals (adult males neonatally sham-castrated; sham) and experimental animals (adult males neonatally castrated: castration).

mRNA expression in GnRH neurons was low (12 grains/cell) and comparable with that found in rats neonatally exposed to testosterone.

There were no significant differences among groups in the number of GnRH mRNA-containing neurons counted (control females, 135 ± 8; testosterone-treated females, 126 ± 11; control males 101 ± 14; castrated males 118 ± 24 total cells).

Discussion

We have shown here that both the steroid-induced LH surge mechanism and the coincident induction of galanin mRNA in GnRH neurons are sexually differentiated during the neonatal critical period, and that neonatal exposure to testosterone, or lack thereof, is crucial in determining the adult's ability to display both phenomena. Previously, Merchenthaler and his colleagues showed that in the adult rat the number of GnRH neurons that contain galanin after E_2 treatment is dependent on whether the testes are present during the neonatal critical period (10). The present results extend these observations by showing that the induction of galanin mRNA in GnRH neurons at the time of a steroid-induced LH surge is sexually differentiated during the neonatal period and that the testicular compound responsible for these effects is testosterone. Furthermore, the present results confirm our previous observations that there is a tight association between the induction of galanin gene expression in GnRH neurons and the LH surge. To date, attempts to disassociate these two event have failed; we have tested a number of pharmacological agents known to block the LH surge and found that they also block the coincident induction of galanin gene expression in GnRH neurons (16, 18). Taken together, these observations suggest that the induction of galanin in GnRH neurons could be one of the events underlying the sex difference in the LH surge mechanism in the rat.

The current study demonstrates that exposure to testosterone neonatally permanently disrupts the ability of the adult to display an induction of galanin gene expression in GnRH neurons in response to E_2/P priming, but whether testosterone itself, or some other active metabolite (*e.g.* dihydrotestosterone or E_2), is actually responsible for this action is currently unknown. The conversion of testosterone to E_2 appears to be necessary for the defeminization of cyclic gonadotropin release by androgens (for review see Refs. 4, 24, 25) and the same (or similar) process may also be necessary for differentiating the ability of GnRH neurons to express galanin mRNA in response to E_2/P. If this were the case, E_2 would most likely defeminize cyclic LH release by acting on estrogen-sensitive inputs to GnRH neurons, rather than acting directly on these cells, because GnRH neurons in rats do not appear either to concentrate E_2 or to express estrogen receptors (26, 27). Neuropeptide Y (NPY), β-endorphin, GABA, or norepinephrine (NE) neural systems could mediate these effects because neurons containing these neurotransmitters/neuropeptides are known to influence LH secretion (28), provide synaptic contacts to GnRH neurons (29–33), and concentrate E_2 (34–37). Alternatively, there is the possibility that GnRH neurons transiently express classical estrogen receptors during early development or that E_2 has direct effects on GnRH neurons that are not mediated through classical estrogen receptors. For example, E_2 can specifically bind to rat synaptosomal membranes and can exhibit rapid effects on brain tissue that are consistent with a nongenomic mode of action (for review see Ref. 38).

Although animals in both of the groups that had little or no exposure to testosterone neonatally (*i.e.* neonatally castrated males and normal females) showed LH surges in response to E_2/P priming, the control females had significantly higher LH values than the neonatally castrated males. The difference in LH surge amplitude between control females and neonatally castrated males seen in this study has also been observed in other studies in which the rats were primed with either E_2 alone (39) or E_2 plus P (40). This difference appears to be attributable to the fact that the males have more exposure to testosterone perinatally and are gonadectomized for a considerably longer time than the females (39, 40). In contrast to LH levels, galanin mRNA levels in GnRH neurons did not significantly differ between control females and neonatally castrated males. Thus, it is likely that there are other factors besides galanin mRNA levels in GnRH neurons that determine the amplitude of the LH surge, and these factors remain differentiated despite attempts to remove the neonatal influence of testosterone.

Several lines of evidence suggest that other factors, besides galanin induction in GnRH neurons, may underlie the sexual differentiation of the LH surge mechanism. For example, noradrenergic neuronal activity, as measured by an increase in NE turnover, increases in the median eminence before the LH surge (41–44). Similarly, NPY levels in the median eminence increase before the LH surge (45–47). Although neonatal exposure to either testosterone or E_2 blocks the ability of E_2/P priming to elicit an LH surge in adult rats (46, 48), the latter treatment does not block the E_2/P-stimulated in-

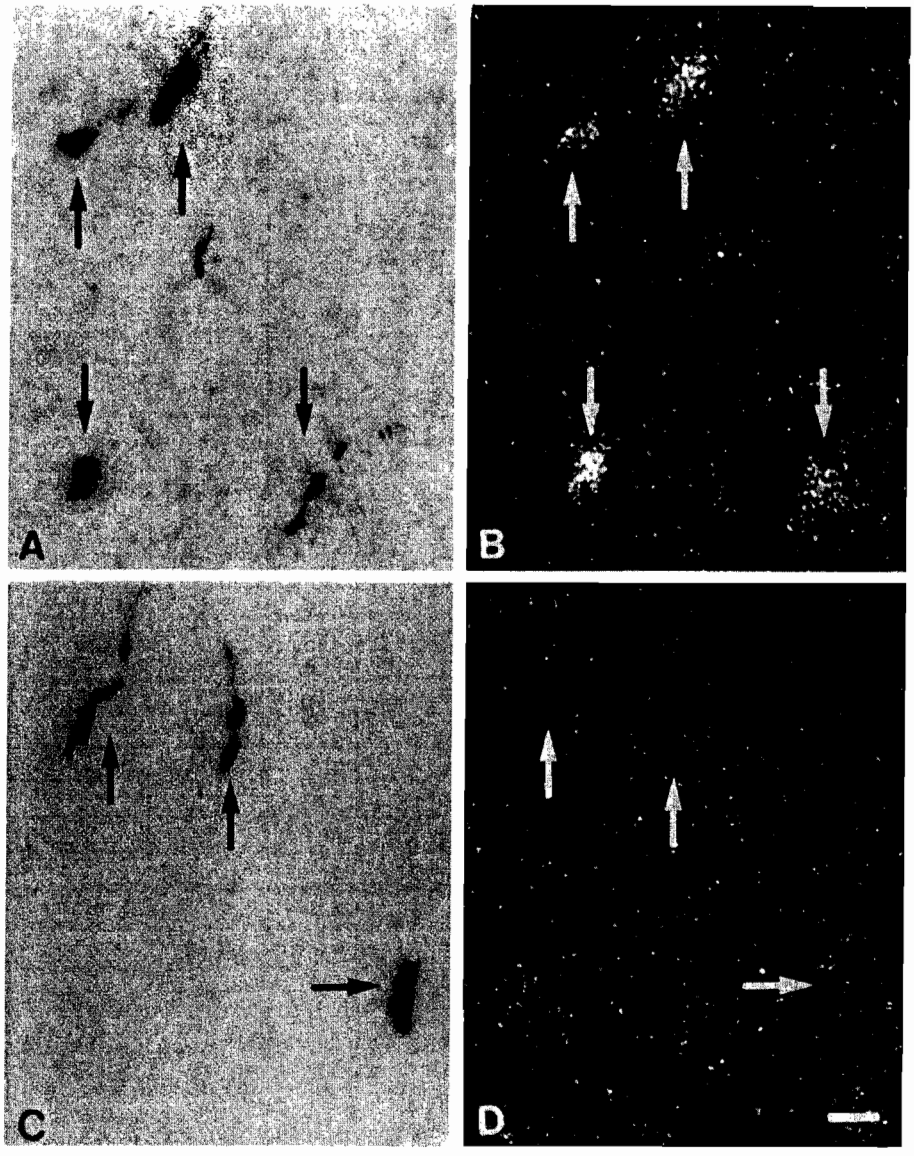

FIG. 2. A, Brightfield photomicrograph of GnRH neurons (*black arrows*), labeled with a digoxigenin-labeled probe for GnRH mRNA, in an adult, male rat that was castrated neonatally and killed at the time of an estradiol (E_2)- and progesterone (P)-induced LH surge (1800 h). B, Darkfield photomicrograph of the same view as in A, showing the same four GnRH neurons labeled with a ^{35}S-labeled probe for galanin mRNA (*white arrows*). Note the abundance of silver grains (*white dots*) over each cell, indicating the high level of galanin mRNA expression in these neurons. C, Brightfield photomicrograph of GnRH neurons (*black arrows*) labeled with a digoxigenin-labeled cRNA probe for GnRH mRNA in an adult, gonadectomized female treated with testosterone propionate neonatally and killed at 1800 h after E_2/P priming. D, Darkfield photomicrograph of the same view as panel C, showing the same three GnRH neurons labeled with a ^{35}S-labeled probe for galanin mRNA (*white arrows*). Note the relative absence of silver grains indicating the low level of galanin mRNA expression in these neurons. *Scale bar* = 20 μm.

crease in median eminence NE turnover or NPY levels (46). The fact that these two events can be disassociated from the LH surge suggests that neither is sufficient for the generation of an LH surge, although it is clear that either may influence the expression of an LH surge when it does occur. Whether the induction of galanin gene expression in GnRH neurons at the time of an LH surge could be similarly disassociated from the LH surge is unknown; however, we have tried several other ways to establish a disassociation and have been consistently unable to do so (16, 18).

In summary, our work has shown that the induction of galanin gene expression in GnRH neurons in response to E_2/P in adults rats is, like the LH surge itself, sexually differentiated by the endocrine milieu of the neonatal critical period. Furthermore, these results demonstrate a tight coupling between the LH surge and the induction of galanin gene expression in GnRH neurons, suggesting that the increase in galanin gene expression in GnRH neurons could be one of the mechanisms underlying the sex difference in gonadotropin release seen in rats.

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