Neonatal imprinting predetermines the sexually dimorphic, estrogen-dependent expression of galanin in luteinizing hormone-releasing hormone neurons

(colarization/double labeling immunocytochemistry/hypothalamic sexual differentiation/reproduction)

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ABSTRACT The incidence of colocalization of galanin (GAL) in luteinizing hormone-releasing hormone (LHRH) neurons is 4-5-fold higher in female than male rats. This fact and the finding that the degree of colocalization parallels estradiol levels during the estrous cycle suggest that GAL is an estrogen-inducible product in a subset of LHRH neurons. To analyze further this paradigm we evaluated the effects of gonadectomy and steroid replacement therapy in male and female rats. Ovariectomy resulted in a significant decrease in the number of cells colocalizing LHRH and GAL, whereas estradiol replacement to such animals restored the incidence of colocalization to that observed in controls. In males, however, estradiol treatment failed to enhance the incidence of colocalization of GAL and LHRH, indicating, therefore, that the colocalization of these peptides is gender-determined. This possibility-i.e., gender-specific determination of LHRH neurons coexpressing GAL—was evaluated by neonatal manipulation of hypothalamic steroid imprinting. As mentioned above, male rats did not respond to estrogen or testosterone by increasing GAL/LHRH colocalization as females did. Neonatally orchidectomized rats, whose hypothalami have not been exposed to testosterone during the critical period, when treated with estrogen in adulthood showed an increase in colocalization of GAL and LHRH similar to that seen in female animals. These observations indicate that the colocalization of GAL is neonatally determined by an epigenetic mechanism that involves the testis. In summary, this sex difference in the incidence of colocalization of GAL and LHRH represents a unique aspect of sexual differentiation in that only certain ontotypic characteristics of a certain cellular lineage are dimorphic. The subpopulation of LHRH neurons that also produces GAL represents a portion of the LHRH neuronal system that is sexually differentiated and programed to integrate, under steroid control, a network of LHRH neurons that could synchronize their activity to control the estrous cycle in rats.

Recent observations from our laboratory indicate that a subpopulation of luteinizing hormone-releasing hormone (LHRH) neurons in the rat brain also expresses galanin (GAL) (1). The incidence of colocalization exhibits a striking sex difference—i.e., in females ~80% of the LHRH neurons contain GAL, whereas in males only 10-15% of these neurons synthesize this peptide (2). In situ hybridization studies have corroborated our earlier findings since GAL mRNA is colocalized with the decapetide LHRH in LHRH neurons (3). It has been reported that estradiol has a profound effect on GAL gene expression in the pituitary and the hypothalamus (see ref. 4 for a review). In this respect, we have observed differential coexpression of GAL in LHRH neurons during the estrous cycle (5), suggesting that GAL expression in LHRH-immunoreactive (LHRH-i) neurons is dependent on the steroid imprinting.

In the rat, the determination of gender-dependent brain differentiation occurs during the last 4 to 5 days of fetal life and the first 5-10 days of the neonatal period (6, 7). Many of these changes are thought to be due to the epigenetic action of gonadal steroids during the critical period of development (see refs. 8-10 for reviews). Since GAL and LHRH coexpression is more predominant in females than males, we hypothesized that the colocalization of GAL and LHRH could be neonatally determined. Considering that female LHRH neurons in adulthood respond to estradiol input with a dramatic increase in GAL synthesis, we postulated that, if the hypothalamus of neonatal male rats is not exposed to androgens during the critical differentiation period, LHRH neurons would be feminized. Therefore, these LHRH neurons would respond in adulthood to estrogen as typical female LHRH neurons—i.e., synthesize GAL.

The present experiments were designed to examine whether neonatal imprinting of brain structures by gonadal steroids determines sexually dimorphic expression of GAL in LHRH neurons. For this purpose, we used a short-term exposure to estradiol as the hallmark for distinguishing between feminized and masculinized hypothalami—i.e., high or low incidence of coexpression of GAL in a subpopulation of LHRH neurons in response to steroids. Parts of these studies have been reported in an abstract (11).

MATERIALS AND METHODS

Animals. Eighty-four CD/Sprague-Dawley rats (Charles River Breeding Laboratories) were used in these studies. Twenty-four hours before sacrifice, each animal received an intraventricular injection of colchicine as described (12). Animals were subsequently perfused with 1% and then 4% paraformaldehyde in 0.05 M phosphate-buffered saline (PBS) under deep tribromoethanol anesthesia (25 mg/100 g of body weight, i.p.).

Newborn male and female rats were neonatally castrated under hypothermic anesthesia. A small horizontal cut was made in the abdominal wall below the umbilicus through which the testes or the ovaries were removed. The incision was sutured with silk and covered with Collodion (Fisher Scientific) to prevent cannibalization by the mothers. Sham operations were performed as described above except that the gonads were not removed. The animals were placed

Abbreviations: GAL, galanin; GAL-i, GAL-immunoreactive; LHRH, luteinizing hormone-releasing hormone; LHRH-i, LHRH-immunoreactive; nOVX, neonatally ovariectomized; nORDX, neonatally orchidectomized; DAB, 3,3'-diaminobenzidine hydrochloride; MPA/DBB, medial preoptic area/diagonal band of Broca; OVLT, organum vasculosum of the lamina terminalis.

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under a heat lamp and, after recovery from anesthesia, returned to their mothers and control littersmates.

Seventy-day-old neonatally ovariectomized (nOVX), orchidectomized (nORDX), or sham-operated littersmates were subdivided into two groups. Half of the animals in adulthood received estradiol-containing capsules (250 μg/ml of oil; 20 mm long) s.c. for 7 days, whereas the other half received capsules containing crystalline testosterone, and the results were compared to those seen in oil-treated control male and female rats.

**Immunocytochemistry. Single staining.** Free-floating vibratome sections were washed in 0.01 M PBS and then exposed to an alcohol shock (12). Following a 10-min rinse in PBS, sections were incubated in 2% normal donkey serum (NDS) to block nonspecific staining. Subsequently, sections were incubated in the primary antiserum (sheep anti-rat GAL, FMS-FJL 17-3, 1:100,000 dilution) for 24 hr at 4°C. After rinsing the sections in PBS and 2% NDS for 10 min each, they were exposed to secondary antiserum (biotinylated donkey anti-sheep IgG, 1:500; Jackson ImmunoResearch) for 1 hr at room temperature. Following a 10-min rinse in PBS, sections were incubated in peroxidase-labeled streptavidin (Jackson ImmunoResearch) at a 1:800 dilution for 1 hr at room temperature. After a 10-min rinse in PBS, sections were exposed to 3,3′-diaminobenzidine hydrochlo-

<table>
<thead>
<tr>
<th>Group</th>
<th>Female</th>
<th>Male</th>
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<tr>
<td>Intact*</td>
<td>100.60 ± 2.62</td>
<td>13.40 ± 1.40</td>
</tr>
<tr>
<td>Gonadectomy</td>
<td>10.20 ± 1.39</td>
<td>8.20 ± 0.86</td>
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<tr>
<td>+E₂</td>
<td>112.40 ± 7.68</td>
<td>12.20 ± 1.24</td>
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<tr>
<td>+T</td>
<td>106.40 ± 5.50</td>
<td>ND</td>
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<tr>
<td>Intact + E₂</td>
<td>ND</td>
<td>12.40 ± 1.12</td>
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E₂, estradiol; T, testosterone; ND, not determined. n = five animals in each group.

*Intact female rats were used in estrus.

\*P < 0.05 vs. intact female rats (Kruskal–Wallis’ test followed by the nonparametric Student–Newman–Keul’s test).

\*P < 0.05 vs. intact or steroid-treated groups (Kruskal–Wallis’ test followed by the nonparametric Student–Newman–Keul’s test).

ride (DAB; 0.4 mg/ml in 0.05 M Tris buffer, pH 7.6) containing 8 μl of 30% H₂O₂. Following a 10-min rinse in Tris buffer and PBS, sections were mounted on glass slides, air-dried, placed in absolute alcohol, transferred to xylene, and coverslipped with Permount (see ref. 12 for details).

**Double labeling.** Representative sections from each brain were incubated with a mixture of sheep anti-rat GAL and rabbit anti-LHRH (H16, 1:30,000) sera for 24 hr at 4°C.

**Fig. 1.** Immunocytochemical localization of LHRH-like GAL-i perikarya in the MPA/DBB of the forebrain. Note the high number and staining intensity of fusiform LHRH-like GAL-i perikarya at the level of the OVLT (star) in an estrus female (A), an adult OVX estradiol-treated female (C), and a nORDX male rat treated with estradiol in adulthood (D). Conversely, the number of immunoreactive perikarya (arrows) and the intensity of their staining in a control male rat (B) are very low. (×80.)
Following a 10-min rinse in PBS, sections were exposed to a mixture of Texas Red-labeled donkey anti-sheep IgG (Jackson ImmunoResearch) and fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch), at final dilutions of 1:1,000, for 2 hr at room temperature. Sections were then washed in PBS and mounted on glass slides. The air-dried slides were placed in absolute alcohol and coverslipped from xylene with DPX mountant (Fluka).

**Microscopic Analysis.** DAB- or fluorescent dye-stained sections were examined with a Zeiss Axioshot microscope equipped with the necessary filter combinations. Ektachrome 64 color and Tmax 100 and 3200 black-and-white films (Kodak) were used for photography. GAL-containing LHRH perikarya were identified by their peculiar morphology and location—i.e., they are small, fusiform, and distributed in the medial preoptic area/diagonal band of Broca (MPA/DBB) in a tent-like manner, whereas typical GAL-immunoreactive (GAL-i) perikarya that do not contain LHRH are larger, multipolar, and located in more lateral and basal regions of the preoptic area. These “LHRH-like” GAL-i (LHRH/GAL) perikarya were counted in five consecutive 30-μm vibratome sections [150-μm segment of the brain containing the organum vasculosum of the lamina terminalis (OVLT)]. The results are expressed as the mean ± SEM. To confirm that these fusiform LHRH-like GAL-i perikarya contained LHRH, representative sections were double-stained using procedures outlined above. A total of five brains per group was analyzed for all quantitative studies.

Immunoreactive cells were assigned to one of the following categories of staining intensity: weak, medium, and intense.

**Statistical Analysis.** Data were evaluated for significant differences using the SAS statistical package (SAS Institute, Cary, NC). A Kruskal–Wallis’ or Friedman’s test was performed when appropriate (13). As post-hoc evaluation, the nonparametric Student–Newman–Keul’s test was utilized to determine specific group-to-group differences.

**RESULTS**

**Estradiol as a Marker for Gender-Specific Coexpression of LHRH and GAL.** As previously reported by our group (2), ~75% of LHRH neurons in the MPA/DBB region contain GAL in female rats. In these animals, an average of 100 LHRH/GAL perikarya was observed (30–35 cells per section, Table 1; Figs. 1 A and 2). In contrast, the same segment of the male brain contained only an average of 13 colocalizing perikarya (0–3 cells per section; Table 1; Figs. 1 B and 2), with weak staining intensity. Ovarioectomy resulted in a statistically significant decrease in the number of LHRH/GAL perikarya to an incidence similar to that observed in male rats (Table 1). In OVX animals, however, those that were treated with either estradiol (Fig. 1 C) or testosterone (not shown) the number (Table 1) and the staining intensity of LHRH/GAL cell bodies dramatically increased (Fig. 1 C) and was similar to that observed in intact female animals. Orchidectomy significantly reduced the low incidence of colocalization of LHRH and GAL, whereas, estradiol treatment restored the expression of GAL in LHRH neurons to intact male levels (Table 1). These results clearly demonstrate that the expression of GAL in a subpopulation of LHRH neurons is estrogen dependent.

**Epigenetic Regulation of GAL Expression in a Subset of LHRH Neurons.** The above-mentioned observations clearly show that short-term estradiol treatment can be utilized as a marker of feminized or masculinized LHRH neurons. Using this model we observed a sexual dimorphism in LHRH neurons. In intact, oil-treated females, an average of 100 LHRH/GAL perikarya was observed in the MPA/DBB region (Fig. 2 Upper). In contrast, in intact, oil-treated males, only 13 cells were counted in a similar area of the brain (Fig. 2). Neonatal OVX significantly reduced GAL/LHRH coex-

**DISCUSSION**

The present data provide direct evidence for the key role of estradiol in GAL expression within a subpopulation of LHRH neurons. More importantly, our results indicate that GAL synthesis in LHRH neurons is sexually dimorphic and neonatally determined depending on steroid imprinting. Furthermore, these studies provide clear evidence supporting the viability of the “androgen theory” in sexual differentiation. In adult males, LHRH neurons do not respond to estradiol or testosterone with as dramatic an increase in LHRH/GAL coexpression as female rats after a challenge with steroids. When males rats are nORDX and challenged with steroids in

**FIG. 2.** Feminization of the male MPA/DBB region after neonatal orchidectomy. LHRH neurons were considered feminized, if in adulthood the incidence of colocalization with GAL, in response to estradiol (E2) or testosterone (T), was similar to that observed in female rats treated with steroid. nGX, neonatal gonadectomy. *P 0.05 vs. all groups; †P 0.05 vs. intact male, nGX and NS vs. female, nGX + E2 or T.
adulthood, they depict a response to steroids similar to that observed in females. (Notice the identical increase in the incidence of LHRH/GAL coexpression in nORDX males, intact females, ovariectomized, or nOVX females in response to a steroid challenge in adulthood by comparing the shaded areas in Fig. 3.) These observations clearly indicate that such male animals after orchidectomy neonatally exhibit feminized hypothalami during their entire lifetime. In female rats, and following the androgen treatment, in the absence of neonatal treatment with steroids their hypothalami are feminized. This is reflected by the fact that ovariectomy neonatally does not alter the ability of LHRH neurons to express GAL in response to a steroid challenge in adulthood. (Compare the left side of Fig. 3A with the right side of Fig. 3B.)

The above-mentioned observations indicate that neonatal exposure to steroids in male rats provides an imprinting that prevents estrogen-induced GAL expression in LHRH neurons in adulthood. The colocalization of LHRH and GAL as an indicator of sexual dimorphism provides an example of the “organizational” and “activational” effects of estrogens (see ref. 14 for a review). The organizational events determined by the presence or absence of gonadal steroids during the neonatal differentiation period will determine whether LHRH neurons can be activated by estrogens to produce GAL during adult life, which itself is intimately involved in the regulation of LHRH secretion and action (5). Our studies provide further evidence that the developmental influences of hormones, in this case steroids, can actually alter the occurrence of these events for the entire life of the animal. Sexual functions controlled by the brain differ in male rats and females—among others, gonadotropin hormone release from the anterior pituitary, male and female sexual and social behavior, learning and motivation, vocalization, regulation of food intake, and body weight (15, 16). One of the most intensely studied functional differences between the female and male relates to hypothalamic regulation of reproductive physiology and behavior. The MPA, a key area in the regulation of reproduction in the rat (17–19), displays size differences in males and females (20, 21). This sexual dimorphism is restricted to a smaller portion of the MPA, called the sexually dimorphic nucleus of the MPA, which is larger in adult male rats than in females (20, 22). Brain sexual dimorphism, however, is not restricted to the MPA but exists also among others, in the bed nucleus of the stria terminalis, the locus ceruleus, the amygdala, and the spinal cord. In addition, sexual dimorphism has been reported in dendritic structures of the MPA, in hypothalamic [³H]estradiol uptake, distribution of LHRH neurons, synaptic input to LHRH neurons, and distribution of catecholaminergic and peptidergic receptors (for a recent review see ref. 9). In all of these cases sex differences proved to be dependent upon the degree of androgen exposure during the perinatal period of life.

The brain is thought to be inherently differentiated toward female unless male differentiation is superimposed by androgen exposure during the critical period of development (ref. 23; see also refs. 8–10 for reviews). The effect of androgens on neonatal hypothalamic differentiation is believed to be mediated by intracellular conversion of this steroid into certain brain areas to estrogens (24–26). The assumption that female sexual differentiation in the rat brain would proceed in the absence of gonadal steroids is based on the early observation that ovariectomy of newborn female rats did not interfere with female differentiation of the brain (23). The organizational and functional aspects of sexual differentiation, however, are more complex. An interaction between estrogens, aromatizable androgens, and neurotransmitters through epigenetic mechanisms seems to play a key role in this phenomenon; however, it is possible that sexual dimorphism in certain regions of the central nervous system may develop primarily under genetic control, in the absence of sex steroids (see ref. 8 for a review).

A significant finding of these experiments is the observation that the profound gender-specific differences observed in the differentiation of LHRH neurons were only apparent when GAL was used as a marker. The number and the distribution of LHRH neurons, as well as expression of the decapeptide and its message, are not sexually dimorphic. The partial colocalization of GAL and LHRH represents a unique situation in which a primary phenotype (i.e., LHRH) is expressed in all neurons and is not subjected to sexual dimorphism, whereas a secondary phenotype (i.e., GAL expression) is clearly sexually dimorphic and becomes, therefore, a candidate for influencing sex-related differences in neuronal function. These characteristics, however, may emphasize key functional differences in the role of these LHRH/GAL-producing neurons. Indeed, these could represent neurons that are recruited by estrogen during proestrus

Fig. 3. Schematic representation of the effect of estradiol (E₂) or testosterone (T) treatment on the incidence of colocalization of GAL and LHRH in the MPA/DBB region in female (Fig. 3A, left) and male (Fig. 3A, right) rats. Note that the number of LHRH/GAL-containing perikarya is high in the female brain but low in the male brain. In neonatally gonadectomized (nGX) females the incidence of colocalization is low, similar to that observed in intact or steroid-treated males (compare the shaded areas in A and B). In nORDX male rats that were treated with E₂ or T in adulthood, the feminized hypothalami contain large numbers of LHRH/GAL-i perikarya. The image is similar to that observed in intact or steroid-treated females (compare the left side of A with the right side of B).
in the rat to participate in the events leading to the preovulatory LHRH and luteinizing hormone (LH) surge. Recently, we have provided compelling evidence to indicate that endogenous GAL modulates the preovulatory surge of LH (27). Thus, this LHRH/GAL neuronal subpopulation could represent a portion of the LHRH neuronal system that is sexually differentiated and programed to integrate, under sex steroid modulation, a network of neurons that would synchronize their activity to control the estrous cycle.

Since it is well accepted that the LHRH cells themselves do not contain a significant number of estradiol receptors (28), it is likely that other neuronal systems that are in direct contact with LHRH neurons may be involved in this phenomenon (see ref. 29 for a review). It is possible that the actions of estrogens are mediated through complex connections among LHRH neurons with other peptidergic or aminergic systems. However, similar to other peptidergic neurons in the hypothalamus (30), estradiol may exert membrane effects on LHRH neurons that may lead to changes in membrane conductivity, the activation of second messenger systems, etc., resulting in changes in LHRH synthesis and secretion. In fact, it has recently been reported that, in chicken granulosa cells, estradiol is capable of releasing intracellular calcium through a nongenomic mechanism (31).

In addition, neurosteroids have been shown to interact with GABAergic mechanisms at the receptor membrane level. Since GABAergic (GABA = γ-aminobutyric acid) inputs, interacting with excitatory amino acids, control the estradiol-induced LH surge (32, 33) and LHRH secretion (32), it is likely that neurosteroids may represent a nongenomic regulatory mechanism of steroids influencing LHRH neurons.

The results of these experiments clearly show that estradiol plays a key role in the production of GAL in LHRH-i neurons. Estradiol (or testosterone) always increases the number of colocalizing perikarya in female rats, in adult, OVX, and nOVX rats. Conversely, among male rats, only nORDX animals responded to such treatment.
