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Activation-Dependent Regulation of Galanin Gene Expression in Gonadotropin-Releasing Hormone Neurons in the Female Rat*

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ABSTRACT

In rats, galanin is colocalized in GnRH neurons, and galanin mRNA in GnRH neurons is increased coincidentally with the preovulatory gonadotropin surge. Whether the induction of galanin mRNA in GnRH neurons at proestrus reflects the action of sex steroids is unknown. We tested this hypothesis by challenging ovariectomized rats (n = 7) with estrogen and progesterone (E/P) to induce a LH surge and measuring galanin mRNA in GnRH neurons to determine whether there was an associated induction of galanin message in these cells. We used single and double label *in situ* hybridization and image analysis to compare among groups the levels of both galanin mRNA and GnRH mRNA in GnRH neurons. We found that steroid-primed animals showed an approximately 400% induction of galanin mRNA signal in GnRH neurons over that in vehicle-treated animals. Second, we hypothesized that steroid-dependent events which induce the expression of galanin mRNA in GnRH neurons depend on transsynaptic input to GnRH neurons. We tested this hypothesis by examining the effect of a pharmacological blockade of the steroid-induced activation of GnRH neurons on levels of galanin mRNA in these cells. We killed groups of ovariectomized adult female rats at the peak of a E/P-primed LH surge (n = 7) and after steroid priming followed by blockade of the LH surge with either the general anesthetic pentobarbital (n = 7) or the specific α-adrenergic receptor blocker phenoxybenzamine (n = 7). When we examined signal levels representing galanin mRNA content in GnRH neurons, we observed a 4-fold increase in signal for galanin mRNA in the GnRH neurons of steroid-primed (E/P surge) animals compared with that in oil-treated controls ($P < 0.0004$). This increase in galanin mRNA was prevented when the LH surge was blocked by treatment with either pentobarbital or phenoxybenzamine ($P < 0.03$ and $P < 0.0001$ *vs.* E/P surge controls, respectively). Cellular levels of GnRH mRNA were not different among control, E/P, and E/P plus pentobarbital groups ($P > 0.2$). These observations suggest that an increase in galanin mRNA levels in GnRH neurons is tightly coupled to the occurrence of a LH surge. By inference, induction of galanin mRNA in GnRH neurons reflects their activation, possibly via afferent neurons that transduce the steroid signal to GnRH neurons. (*Endocrinology* **134:** 1991–1998, 1994)

EVERY 4 days in the female rat, rising plasma concentrations of estradiol produced by developing follicles lead to an activation of hypothalamic GnRH neurons, which, in turn, induces a preovulatory release of gonadotropins from the pituitary. Although we know that on proestrus, activation of GnRH neurons by estradiol leads to increased secretion of GnRH (1, 2), the molecular events associated with this transduction process are uncertain. On the afternoon of proestrus, GnRH neurons apparently exhibit an increase in gene transcription, as indicated by the expression of the immediate early genes c-*fos* and c-*jun* (3–6). Heterodimers of *Fos* and *Jun* form a transcription factor, AP-1, which is known to activate the transcription of several other genes (7). Although the presence of *Fos* and *Jun* in GnRH neurons indicates that a stimulation of gene transcription is occurring, it provides no clue about the identity of other genes that may be induced through an AP-1-dependent mechanism. A plausible candidate would be the GnRH gene itself, because increased GnRH release is thought to accompany activation of the GnRH neuron, and the GnRH gene apparently contains a specific binding site for the AP-1 factor in its up-stream regulatory region (8). However, attempts to demonstrate changes in GnRH message levels associated with either the proestrous gonadotropin surge or with steroid treatments have produced contradictory results (9–18).

In the female rat, a subset of GnRH neurons synthesizes and secretes galanin, a neuropeptide that may play an important physiological function as a cotransmitter in these cells (19). Galanin's involvement in the generation of a gonadotropin surge was suggested first by the observation that administration of either galanin antiserum (20) or a specific galanin antagonist (21) attenuates the normal proestrous release of LH. Second, galanin mRNA as well as the galanin peptide content of GnRH neurons increase on the afternoon of proestrus in association with the proestrous gonadotropin surge (17, 22). Although it would seem probable that the induction of galanin mRNA on proestrus reflects the action of rising concentrations of estradiol and progesterone that precede the LH surge, this remains an inference. We tested this hypothesis by challenging ovariectomized (OVX) animals with exogenous steroids to induce a LH surge and determining whether there was an induction of galanin mRNA in the GnRH neurons of these animals. We report that steroid priming induces galanin message in GnRH neurons.

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How galanin is involved in the generation of a steroid-induced LH surge is currently unknown. The induction of galanin expression in GnRH neurons at proestrus may be a requisite step in the process leading to activation of these neurons and enhanced GnRH release. Alternatively, galanin induction may only be an incidental reflection of the activational process. All current evidence suggests that GnRH neurons themselves do not express classical estrogen receptors (23), and by inference, estrogen's action on GnRH neurons must occur indirectly through intermediary systems, such as the endogenous opioids (24, 25). This would imply that the estrogen-dependent induction of galanin mRNA in GnRH neurons must involve transsynaptic activation of GnRH neurons. A corollary to this reasoning would posit that blockade of the LH surge through interruption of afferent input to the GnRH neuron would likewise block the steroid-dependent increase in galanin mRNA in GnRH neurons. We tested this hypothesis by examining the effect of a pharmacological blockade of the steroid-induced LH surge on galanin mRNA levels in GnRH neurons. Two different pharmacological agents were used to block the LH surge. The first was a general anesthetic, pentobarbital. Pentobarbital blocks the LH surge by either effecting a direct inhibition GnRH neurons or altering the activity of their afferent input. Therefore, in a second group of animals, we used a more specific blocking agent, the α-adrenergic blocker phenoxybenzamine, to block only one of the many inputs to the GnRH system. We report here that both general and specific blockade of the LH surge prevented the increase in galanin mRNA in GnRH neurons that is associated with the steroid challenge. In contrast, the cellular content of GnRH mRNA was altered by neither the steroid priming regimen nor the pharmacological manipulation.

Materials and Methods

Animals

Adult (60-day-old) female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) and housed under pathogen-free, constant temperature conditions, with an alternating light-dark cycle (14 h of light, 10 h of darkness, with lights on at 0700 h). The animals were given free access to tap water and rat chow. The rats were handled daily for 1 week before ovariectomy and the steroid treatments. Ovariectomy was performed under ether anesthesia, and the animals were allowed to recover for 21 days before initiation of the steroid and drug injections.

Experimental design

Exp 1. We tested the hypothesis that galanin mRNA expression in GnRH neurons is induced by ovarian steroids in association with a LH surge, and we investigated the possibility that the activation of GnRH neurons after exposure to ovarian steroids is required for the induction of galanin mRNA levels in these cells. This was accomplished by blocking neuronal activation with a general anesthetic, sodium pentobarbital. Three groups of animals were used. The control group (n = 6) received a sc injection of peanut oil (0.2 ml) on day 0 at 1030 h and on day 2 at 1200 h. At 1400 h on day 2, each animal was given an ip injection of saline (0.2 ml). The surge-activated and surge-blocked groups were primed with estrogen and progesterone (E/P) as follows. On day 0 at 1030 h, animals were injected sc with estrogen (30 μg in 0.2 ml, dissolved in peanut oil). On day 2 at 1200 h, the animals received a sc injection of progesterone (Eli Lilly Co., Indianapolis, IN; 5 mg in 0.1 ml in peanut oil). At 1400 h on day 2, the surge-activated group was given an ip injection of saline (n = 7), and the surge-blocked group was given an ip injection of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL; 40 mg/kg; n = 6). Levels of galanin mRNA in GnRH cells were compared among these groups by double label *in situ* hybridization. The rats of all groups were killed by CO_2 asphyxiation between 1730–1800 h on day 2.

Exp 2. We tested the hypothesis that the blockade of GnRH neuronal activity (and thereby the LH surge) by a more specific neurotransmitter receptor blocker would prevent the increase in galanin mRNA levels in GnRH neurons. Of the possible choices of agents known to block the steroid-induced LH surge, we chose phenoxybenzamine, an α-adrenergic receptor blocker. The experiment was performed as outlined in the preceding paragraph, except that the surge-blocked group received phenoxybenzamine (Dibenzyline, SmithKline French, Philadelphia, PA; 20 mg/kg; n = 7) instead of pentobarbital. Tissue from the surge-activated and oil-treated control groups described in Exp 1 was used in this experiment as well, but was processed in a separate *in situ* hybridization assay with the tissue from the phenoxybenzamine surge-blocked group.

Exp 3. We used a separate set of the tissue sections prepared in Exp 1 in a single label *in situ* hybridization experiment to assess the number of GnRH cells as well as the level of cellular GnRH mRNA content in neurons throughout the basal forebrain and rostral hypothalamus.

Tissue preparation

Upon asphyxiation, the rats were immediately decapitated; their brains were rapidly removed, frozen on dry ice, and stored at −80 C. Trunk blood was collected, from which the serum was separated and stored at −20 C until assayed for LH and estradiol contents. Coronal brain slices (20 μm) were cut with a cryostat, thaw-mounted onto saline-coated slides (Fischer Scientific, Fair Lawn, NJ), and stored in air-tight boxes at −80 C until needed. Tissue was collected according to the rat atlas of Paxinos and Watson (26), starting rostrally at the genu of the corpus callosum and continuing caudally 60 μm beyond the decussation of the anterior commissure. The tissue slices were collected on four sets of slides, each set representing a one in four series of sections.

Riboprobe preparation

^{35}S-Labeled galanin cRNA probe. The plasmid vector Bluescript containing a cDNA complimentary to rat galanin mRNA (27) was provided by Drs. Henry Friesen and Maria Vrontakis (University of Manitoba, Winnipeg, Canada). This plasmid was used for *in vitro* transcription of a 680-basepair ^{35}S-labeled cRNA probe for galanin mRNA. The galanin probe transcription reactions had 25% the total UTP as [^{35}S]UTP. The final double label hybridization solution contained a galanin cRNA probe concentration of 0.25 μg/ml·kilobase. The control experiments used to validate the integrity, binding kinetics, and specificity of the galanin cRNA probe have been previously described (17).

Digoxigenin-labeled GnRH cRNA probe. A 462-basepair digoxigenin-labeled cRNA probe complementary to rat GnRH mRNA was used. The original plasmid containing the GnRH insert, previously described in detail (28), was generously provided by Dr. Anthony Mason (Genentech, South San Francisco, CA). The probe was synthesized *in vitro* from linearized DNA with 400 μM digoxigenin-11 UTP (Boehringer Mannheim, Indianapolis, IN), 100 μM unlabeled UTP, 500 μM GTP, ATP, and CTP with SP6 RNA polymerase. Residual DNA was digested with DNAse, and the cRNA probe was separated from unincorporated nucleotides on a Sephadex G-50 column. The purified probe was diluted 1:50 in hybridization buffer for double label *in situ* hybridization. This concentration was determined to be optimal by a test *in situ* hybridization assay.

^{35}S-Labeled GnRH cRNA probe. The probe used in Exp 5 was synthesized from the same plasmid used for the synthesis of the digoxigenin-labeled probe. The probe was synthesized *in vitro* from linearized DNA, with 14% of the total UTP replaced with [^{35}S]UTP. The final concentration of

the GnRH cRNA probe in this experiment was 0.25 μg/ml·kilobase.

Control experiments used to validate the integrity, binding kinetics, and specificity of both the digoxigenin- and ^{35}S-labeled GnRH probes have been previously described (28, 29). Both probes were heat denatured before they were added to the final hybridization buffer.

Double label in situ hybridization

The method used for double label *in situ* hybridization has been reported previously (29) and is repeated here in an abbreviated form. Sections were fixed in 4% paraformaldehyde and treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The slides were rinsed in 2 × SSC (1 × SSC = 150 mM NaCl and 15 mM Na citrate), dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated in a second ethanol series, and air dried. The tissue sections were prehybridized for 2 h at 60 C with hybridization buffer containing 2 mg/ml denatured total yeast RNA, rinsed in 2 × SSC, dehydrated briefly in 70% ethanol, and air dried. The final hybridization buffer containing both probes was applied (60 μl/slide) to the tissue, covered with a Parafilm coverslip, and sealed with rubber cement. The slides were 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 wash at 65 C in 0.1 × SSC. After this, the slides were placed in 2 × SSC plus 0.05% Triton X-100 containing 2% normal sheep serum for 1 h, washed in buffer 1 (100 mM Tris-HCl, pH 7.5, and 150 mM NaCl), and incubated for 4 h at 37 C with antidigoxigenin antibody fragments conjugated to alkaline phosphatase (Boehringer Mannheim), diluted 1:1000 in buffer 1 containing 1% normal sheep serum and 0.3% Triton X-100. After washing, the slides were incubated in a Chromagen solution for 6 h at 37 C. The reaction was stopped, and the slides were air dried and stored in the dark. The next day, the slides were dipped in a 3% solution of parlodion (Fisher Scientific) in isoamyl acetate, allowed to dry, and dipped in Kodak NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY). For Exp 1 and 2, the slides were exposed for 10 days. For Exp 3, the assay was terminated after the stringent washes on the second day, and the emulsion-coated slides were exposed for 7 days. These slides were counterstained with cresyl violet to aid in identification of cells for final analysis.

Semiquantitative analysis of cellular mRNA

Tissue sections were viewed under darkfield illumination on a Zeiss Axioskop (Zeiss, New York, NY) equipped with a ×40 objective and a 100-watt mercury vapor epiillumination light source. We determined the number of silver grains per cell using a grain-counting program, as previously described (29). This system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer. Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN) attached to the Zeiss Axioskop.

To estimate the level of galanin mRNA content in each GnRH neuron, a purple-stained digoxigenin-labeled GnRH mRNA-containing cell was first isolated under brightfield illumination. The silver grains overlying these cells were then analyzed under darkfield illumination by the image processor. Twenty-one sections per brain, equally spaced throughout the diagonal band of Broca and preoptic area, with the most caudal slice at the level of the suprachiasmatic nucleus and the rostral aspect of the lateral hypothalamus, were analyzed for the number of grains per cell, representing relative galanin mRNA content in GnRH neurons. Areas examined for cellular galanin mRNA content in single labeled cells included the preoptic area, diagonal band of Broca, and median preoptic nucleus. In these experiments, the sections were anatomically matched between groups, and the analysis was performed by an operator unaware of the animal's experimental group.

GnRH cells were identified under brightfield illumination by the presence of a dark purple-stained cell body. Although the operator was able to obtain an estimate of the number of cells that appeared to be unambiguously double labeled, grain count analysis for galanin mRNA signal in all GnRH neurons avoids subjective determination of whether a particular cell is double labeled. Single labeled GnRH mRNA-expressing neurons (Exp 3) were identified by the presence of a discrete grain cluster associated with a cresyl violet-stained nucleus (30).

Statistical analysis

For these experiments, n refers to the number of experimental animals within a group, and this was the n used in the analysis. For cellular GnRH mRNA or galanin mRNA content determinations, the mean grains per cell from individual animals (and anatomical areas within animals) were used to calculate the mean ± SEM for each group. The differences between groups were assessed by analysis of variance. The rejection level for statistical tests was set at $\alpha = 0.05$. When the analysis of variance indicated a significant difference between groups within an experiment, Duncan's new multiple range test was used to identify significant differences between pairs of groups.

Serum LH measurements

Serum LH levels were measured by RIA in the laboratory of Dr. William Bremner (Director, Radioimmunoassay Core of the Population Center for Research in Reproduction, Seattle, WA). LH was measured with a RP-3 standard by a double antibody RIA, with reagents obtained from the National Hormone and Pituitary Program. The intra- and interassay coefficients of variation were 6.4% and 10.2%, respectively.

Results

Hormone levels

Table 1 comprises the serum hormone data for all experimental groups. E/P priming induced large amplitude LH surges in the EP- and saline-treated animals. There was no evidence of a LH surge in either the oil plus saline or the EP plus pentobarbital animals. In three of the seven animals treated with phenoxybenzamine, the LH surge was completely blocked (LH, 3 ± 1 ng/ml). The remaining four animals had much higher LH levels (61 ± 16 ng/ml) and were considered to have had a surge. These subgroups were considered separately for all subsequent analyses (E/P+phbz/blocked and E/P+phbz/surge, respectively). Although the cause of this incomplete response to the α-receptor blockade in this experimental group remains unresolved, it has been observed by other investigators who used similar treatment paradigms (31).

Galanin and GnRH mRNA content in GnRH neurons

Exp 1. E/P treatment increased galanin mRNA signal levels in GnRH neurons by nearly 400% compared with those in oil-treated controls (oil plus saline, 16 ± 2 grains/cell; E/P plus saline, 63 ± 8 grains/cell; $P < 0.0005$; Fig. 1). This increase was blocked by pentobarbital (E/P plus pentobarbital, 26 ± 3 grains/cell; E/P plus saline, 63 ± 8 grains/cell; $P < 0.005$). However, the galanin mRNA signal level was significantly higher in the pentobarbital-treated group than

TABLE 1. Mean serum LH levels for all experimental groups

Group	LH (ng/ml)
Oil + saline	12 ± 1
EP + saline	78 ± 12
EP + pentobarbital	4 ± 1
EP + phenoxybenzamine (blocked; n = 3)	3 ± 1
EP + phenoxybenzamine (not blocked; n = 4)	61 ± 16

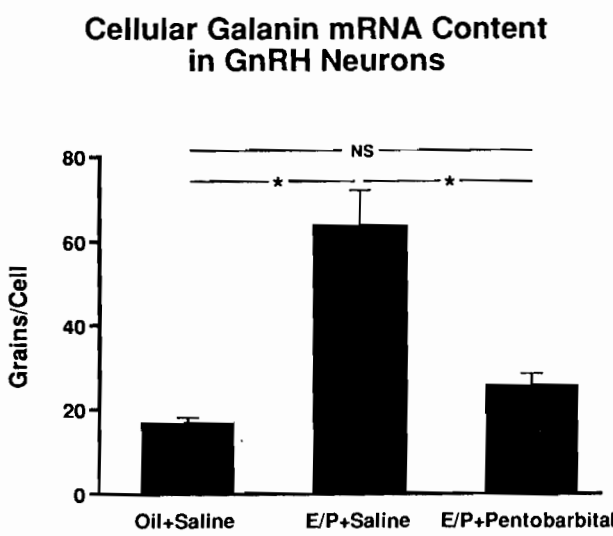

FIG. 1. Relative levels of galanin mRNA signal (grains per cell ± SEM) in GnRH neurons throughout the forebrain and rostral hypothalamus in groups of adult OVX rats under different treatment regimens. The groups included control animals (oil plus saline; n = 6), steroid-primed animals (EP plus saline; n = 7), and steroid-primed pentobarbital-treated animals (EP plus pentobarbital; n = 6).

in the oil/saline control group (oil plus saline, 16 ± 2 grains/cell; E/P plus pentobarbital, 26 ± 3 grains/cell; $P < 0.05$). The overall distribution and intensity of grain clusters representing galanin message in non-GnRH neurons was not subjectively different among groups (data not shown). Simultaneous bright- and darkfield images of representative cells from this experiment are shown in Fig. 2.

Exp 2. Galanin mRNA levels in GnRH neurons were significantly higher in the E/P+phbz/surge group than in the E/P+phbz/block group (31 ± 3 *vs.* 7 ± 2 grains/cell; $P < 0.003$; Fig. 3), and these levels paralleled those in the E/P plus saline and oil plus saline groups (42 ± 5 *vs.* 5 ± 1 grains/cell, respectively; $P < 0.0001$). There was no significant difference in the amount of galanin mRNA between the E/P plus saline and the E/P+phbz/surge groups (42 ± 5 *vs.* 31 ± 3 grains/cell, respectively; $P = 0.18$). The overall distribution and intensity of grain clusters representing non-GnRH galanin neurons were not subjectively different among groups (data not shown).

Exp 3. We observed no significant difference among groups in either cellular levels of GnRH mRNA signal (oil plus saline, 153 ± 5; E/P plus saline, 136 ± 6; E/P plus pentobarbital, 140 ± 10 grains/cell; $P = 0.2$; Fig. 4A) or the number of GnRH mRNA-containing neurons (oil plus saline, 110 ± 18; E/P plus saline, 125 ± 25; E/P plus pentobarbital, 115 ± 18 total cells; Fig. 4B).

Discussion

We previously reported that the galanin mRNA content in GnRH neurons varies across the estrous cycle of the rat, and on the day of proestrus, the galanin message content in GnRH neurons increases between 1200–1800 h coincident with the midcycle LH surge (17). In this report we demonstrate that a steroid-induced LH surge in OVX rats is associated with an increase in galanin mRNA signal levels in GnRH neurons. The observation that E/P priming produces both a LH surge and an increase in galanin mRNA content in GnRH neurons suggests that during proestrus these same events are mediated by sex steroids. The mechanism for regulation of the galanin gene in GnRH neurons by ovarian steroids remains unknown. However, because GnRH neurons are not thought to express estrogen receptors and apparently do not concentrate estradiol (23), it is likely that the stimulatory effects of ovarian steroids are mediated by other neural systems, such as neurotensin (32, 33) or neuropeptide-Y (34, 35). Alternatively, the steroid signal may reach GnRH neurons directly via cell surface steroid receptors (36–38) in a manner analogous to that which allows olfactory neurons to respond to steroidal odorants (39).

Our demonstration of an increase in galanin mRNA in GnRH neurons of OVX steroid-primed rats was presaged by the earlier work of Lee *et al.* (3–6), showing the expression of the immediate early genes c-*fos* and c-*jun* in GnRH neurons under similar experimental conditions. These investigators demonstrated that the expression of c-*fos* in GnRH neurons is stimulated during the proestrous LH surge, and that this induction could be reproduced in steroid-primed OVX animals (3–6). The expression of c-*fos* and c-*jun* in GnRH neurons indicates that the transcription factor AP-1, a heterodimer of the *Fos* and *Jun* proteins, is available to regulate the expression of a number of genes by binding to a region of DNA known as the AP-1-binding site (7). There are many possible targets within the GnRH neuron for transcriptional regulation by AP-1, including the GnRH gene itself (8). The galanin gene has been sequenced (40), and an AP-1 site was found in the 5′-flanking region near other sites important for transcriptional regulation, indicating that the rate of transcription of the galanin gene is likely to be regulated by alterations in cellular levels of the AP-1 transcription factor. The link between c-*fos* expression and galanin expression in GnRH neurons is strengthened by the observation that the distribution of cells colocalizing GnRH and *Fos* during the proestrous LH surge is virtually identical to the distribution of GnRH neurons that colocalize galanin mRNA and peptide during this same period (4, 17, 19, 22). We hypothesize that as a result of recruitment and activation of GnRH neurons during the ascending phase of the LH surge (6), the immediate early genes c-*fos* and c-*jun* are rapidly expressed, thereby leading indirectly to an induction of galanin gene transcription. This model predicts that the galanin mRNA content in GnRH neurons will be increased under conditions that lead to activation of these cells and to a subsequent LH surge, including a LH surge brought about by the administration of estradiol and progesterone to OVX animals.

The finding that GnRH neurons do not concentrate estradiol indicates that the ability of sex steroids to trigger transcriptional events in GnRH neurons requires a transsynaptic event (23). This idea is strengthened by the observation that pharmacological blockade of the LH surge with the anes-

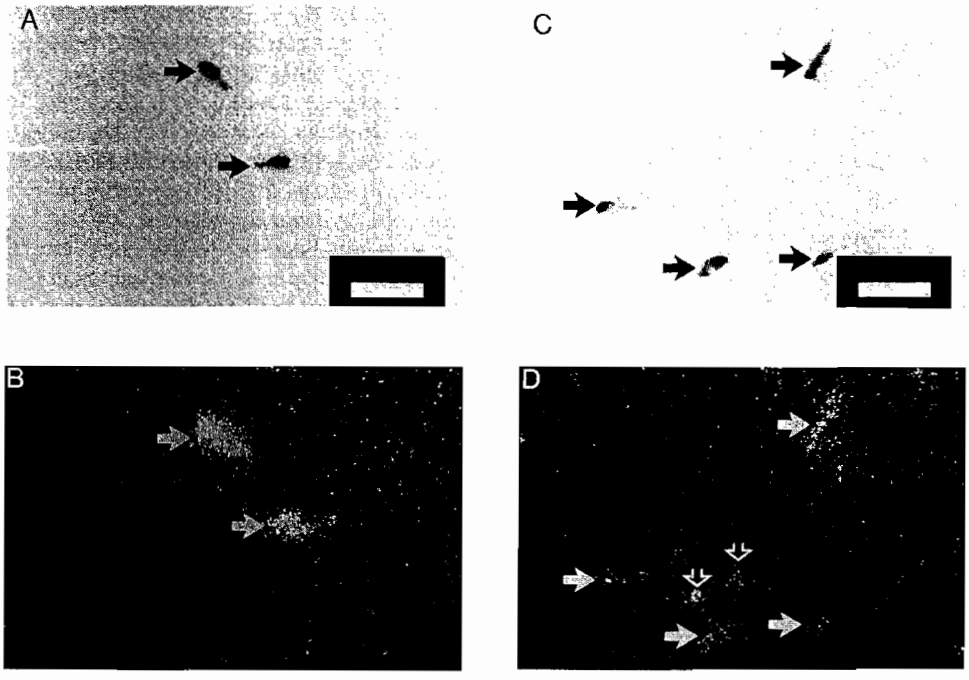

FIG. 2. A, A brightfield view of two GnRH neurons (*solid arrows*), labeled with a digoxigenin-labeled probe for GnRH mRNA, in an animal killed during a steroid-induced LH surge (1800 h). B, A darkfield photomicrograph of the same view as that in A, showing the same two GnRH neurons simultaneously labeled with an ^{35}S-labeled probe for galanin mRNA (*solid arrows*). Note the abundance of silver grains in each cluster, indicating a high level of galanin message expression in these cells. C, A brightfield view of four GnRH neurons (*solid arrows*), labeled with a digoxigenin-labeled probe for GnRH mRNA, in a steroid-primed animal killed subsequent to a pentobarbital-induced blockade of the LH surge (1800 h). D, A darkfield photomicrograph of the same view as that in C, showing the same four GnRH neurons simultaneously labeled with an ^{35}S-labeled probe for galanin mRNA (*solid arrows*). Note the relatively few silver grains in each cluster, indicating a low level of galanin message expression in these cells. Also note the silver grain clusters not associated with digoxigenin-labeled cells (*open arrows*), indicating the presence of galanin mRNA-containing cells in an unidentified population of non-GnRH neurons. The reticle delineates a length of 50 μm.

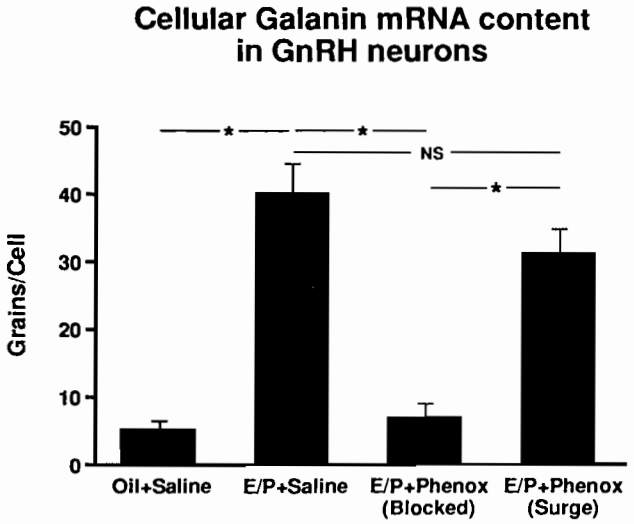

FIG. 3. Relative levels of galanin mRNA signal (grains per cell ± SEM) in GnRH neurons throughout the forebrain and rostral hypothalamus in groups of adult OVX rats under different treatment regimens. The groups included control animals (oil plus saline; n = 6), steroid-primed animals (EP plus saline; n = 7), steroid-primed phenoxybenzamine-treated animals with blocked (Blocked; n = 3), and unblocked (Surge; n = 4) LH surges.

thetic sodium pentobarbital eliminates the expression of *Fos* in GnRH neurons at proestrus (4). We now demonstrate that after steroidal priming, administration of this general anesthetic that effects a blockade of the LH surge also blocks the induction of galanin mRNA in GnRH neurons. Thus, the transduction pathway that leads from alterations in sex steroid levels to an induction of galanin gene expression in GnRH neurons must involve an excitation-dependent neural event. It is conceivable that pentobarbital acts to depress the activity of neurons that express estrogen receptors and provide a stimulatory input to GnRH neurons, thereby effectively blocking their transcriptional activation. However, because pentobarbital is likely to have a direct effect on the excitation threshold of GnRH neurons, it is possible that a direct action of sex steroids on GnRH neurons was masked by this manipulation.

To further analyze the role of cellular activation in the induction of galanin mRNA in GnRH neurons, we blocked this activation with a second, more specific, pharmacological agent with defined receptor properties. The α-adrenergic system was selected because it is known to modulate the character of the LH surge, presumably via direct synaptic contact with GnRH neurons (41, 42). The administration of norepinephrine to OVX steroid-primed rats elicits LH release, whereas interference with catecholamine synthesis or treatment with receptor antagonists blunts the LH surge (43–48).

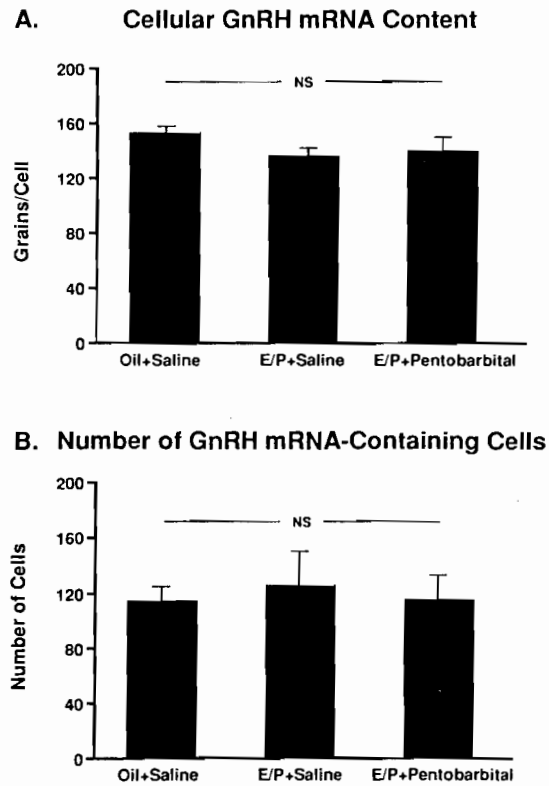

FIG. 4. A, Relative levels of GnRH mRNA signal (grains per cell ± SEM) in cells throughout the forebrain and rostral hypothalamus in groups of adult OVX rats under different treatment regimens. The groups included control animals (oil plus saline; n = 6), steroid-primed animals (EP plus saline; n = 7), and steroid-primed pentobarbital-treated animals (EP plus pentobarbital; n = 6). B, Relative number of GnRH mRNA-containing neurons throughout the forebrain and rostral hypothalamus in groups of adult OVX rats under the same treatment conditions described for A.

We used the α-adrenergic receptor antagonist phenoxybenzamine to block the steroid-primed LH surge and assessed the effect of this treatment on galanin mRNA expression in GnRH neurons. We report that in all the cases in which GnRH neuronal activity was blocked, as reflected by the failure of the LH surge, the increase in galanin mRNA levels in GnRH neurons was commensurately blocked. In contrast, all animals in which phenoxybenzamine treatment failed to block the steroid-primed LH surge had dramatic increases in galanin mRNA content in their GnRH neurons, coincident with the LH surge. The failure of phenoxybenzamine treatment to abolish the LH surge in the entire group of animals is not unprecedented, as Clifton and Sawyer (31) also reported an incomplete blockade of the LH surge in rats after treatment with the same drug. This is consistent with the proposed role of the α-adrenergic system as a potent modulator of LH secretion, rather than as a mandatory system for generation of a LH surge (31, 49–51). It is important to note that our experiments were not designed to demonstrate that the stimulation of galanin mRNA expression in GnRH neurons is transduced exclusively by the α-adrenergic system. Rather, these results serve to emphasize our hypothesis that the induction of galanin transcription is linked to cellular activation, regardless of the mechanism by which this activation is achieved. Indeed, treatments with phenoxybenzamine or pentobarbital may serve only to increase the threshold of excitability of the GnRH neuron by removing tonic stimulatory inputs.

Our findings regarding the regulation of galanin mRNA content in GnRH neurons contrast with our studies of GnRH mRNA levels in the same experimental groups. We demonstrate that neither the content of GnRH mRNA in individual neurons nor the number of neurons expressing GnRH mRNA changes in response to the generation of a steroid-primed LH surge. Similarly, inhibition of this surge with pentobarbital produces no discernible effect on GnRH mRNA signal levels. Although experiments analyzing GnRH mRNA levels in response to ovarian steroids have yielded equivocal and contradictory results (11–15), our results are in agreement with those of Park *et al.* (10), who employed a similar methodology and experimental design. However, the results of Kim *et al.* (52) indicate that GnRH mRNA levels are augmented in response to an E/P-primed LH surge. Because these researchers employed a different methodology (RNA blot hybridization) and a different experimental preparation (immature rats), it is possible that these contradictions reflect differences in the protocols rather than differences in the physiological responses. In any case, our data indicate that if activation of GnRH neurons results in alterations in GnRH mRNA content, these changes are small relative to the changes in galanin mRNA in these same cells.

Although the data from these studies and previously published reports (17, 29, 53, 54) are helping to define the mechanisms by which expression of the galanin gene in GnRH neurons is regulated, the possible physiological significance of galanin coexpression in GnRH neurons remains unknown. Although we (17, 54) and others (19, 22) have proposed that galanin may play a direct role in stimulating GnRH release via galanin receptors in the median eminence (55), evidence from other neural systems indicates that galanin may be playing an inhibitory role as well. Galanin is a cotransmitter in cholinergic neurons projecting from the medial septum to the ventral hippocampus (56, 57), where galanin is thought to limit the release of acetylcholine via an autoinhibitory presynaptic mechanism (58, 59). Because galanin content as well as the expression of galanin receptors are greatly diminished after lesions of the medial septum, this feedback inhibition is thought to be due to galanin that is coreleased from the cholinergic neurons, rather than from galanin-containing afferents from another brain region (58). If galanin functions in a similar manner in GnRH neurons, then galanin would act at the GnRH nerve terminal from which it is released to limit further GnRH secretion. This type of feedback could facilitate the shaping of GnRH secretion into distinct pulses by minimizing the interpulse release of GnRH. When the secretory drive on GnRH neurons is high (at proestrus), large distinct pulses would prevail. In contrast, in physiological states such as lactation, where galanin expression in GnRH neurons is minimal (54), GnRH secretion would proceed in a continuous nonpulsatile fashion, thereby providing minimal stimulus to the pituitary gonadotrope (60). The hypothesis that galanin may shape

hormone secretion into a pulsatile mode with low nadir levels may be relevant to other neuronal systems that regulate hormone secretion from the anterior pituitary. Indeed, galanin is expressed in GH-releasing hormone neurons of the male rat at levels significantly greater than those found in the female (61), and the male rat secretes GH in much more distinct pulses than the female (62). Furthermore, treatment of a male rat with an antibody directed against galanin feminizes the pattern of GH secretion, primarily by increasing the baseline interpulse secretion (63).

In summary, we have shown that the expression of galanin mRNA in GnRH neurons is stimulated under conditions that lead to activation of these cells and thereby to a LH surge. The induction of galanin mRNA in these cells by ovarian steroids requires that the steroid signal be transduced, via a pathway that includes GnRH neurons, into a LH surge. Although the physiological impact of galanin gene expression in GnRH neurons remains unknown, the available experimental data indicate that galanin plays a role in the regulation of gonadotropin secretion, perhaps by acting as a pulse-shaping neurotransmitter at the level of the GnRH nerve terminal.

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