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Regulation of Gonadotropin-Releasing Hormone (GnRH) and Galanin Gene Expression in GnRH Neurons during Lactation in the Rat*

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ABSTRACT

Galanin is colocalized with GnRH, and its expression in these neurons is enhanced at proestrus, a time of activation of GnRH neurons. We tested the hypothesis that the expression of both the GnRH and galanin mRNAs in GnRH neurons decrease during lactation in the rat, a reproductive state characterized by reduced gonadotropin secretion. For double label *in situ* hybridization, GnRH mRNA was detected with an antisense cRNA probe labeled with the hapten digoxigenin, whereas galanin mRNA was detected with a cRNA probe labeled with ^{35}S. The number of silver grains deposited over a digoxigenin-labeled cell body provided an index of galanin mRNA levels in GnRH cells. We observed a 60% reduction in signal (grains per cell) for galanin mRNA in GnRH neurons of lactating animals compared with those of diestrus animals ($P < 0.004$), with no difference in the number of GnRH neurons between groups. To compare cellular GnRH mRNA content between groups, we used single label *in situ* hybridization and image analysis. Signal levels (grains per cell) for GnRH mRNA were not different between diestrous and lactating animals in either an initial (diestrus, 121.4 ± 5.9; lactation, 117.3 ± 8.0; $P > 0.7$) or in a subsequent trial (diestrus, 184.0 ± 10.4; lactation, 197.5 ± 13.0; $P > 0.7$). To confirm and extend these findings, we used a RNAse protection assay to measure and compare the content of GnRH mRNA in hypothalamic fragments between diestrous and lactating animals. The concentration of GnRH mRNA (picograms of mRNA per 25 μg total RNA) was not different between the two groups (diestrus, 1.21 ± 0.25; lactation, 1.25 ± 0.13; $P > 0.7$). A determination of the total GnRH peptide content by RIA in a separate set of hypothalamic dissections revealed no difference between groups in the level of GnRH content (nanograms) per hypothalamus (diestrus, 6.0 ± 0.6; lactation, 5.7 ± 0.4; $P > 0.4$). We conclude that galanin mRNA expression in GnRH neurons of the rat is diminished during lactation, whereas GnRH expression continues unabated. This decrease in galanin gene expression associated with lactation may lead to decreased synthesis and secretion of galanin, which, in turn, could diminish the pulsatile secretion of GnRH or reduce its activity at the pituitary. (*Endocrinology* **133:** 1450–1458, 1993)

DURING lactation in the rat, there is an absence of pulsatile LH secretion (1–5) and a greatly decreased content of pituitary GnRH receptors (5, 6), which have been attributed to diminished GnRH release from the hypothalamus (5–8). Lactating rats do not secrete GnRH in response to depolarizing challenges by *N*-methyl-D,L-aspartate, indicating that their GnRH neurons have become refractory to exogenous stimulation (8, 9). This implies that during lactation, some aspect of the GnRH neuron is altered, such that pulsatile GnRH release is inhibited. One plausible mechanism accounting for this disruption in the activity of GnRH neurons is a compromise in the synthesis of GnRH itself. Although the total hypothalamic content of GnRH appears to be unaltered during lactation (1, 7, 10), it is conceivable that the synthesis and secretion of GnRH are diminished concomitantly, leaving steady state levels of GnRH unchanged. If this were the case, one would expect that reduced synthesis of GnRH would be reflected in reduced levels of cellular GnRH mRNA. We tested the hypothesis that GnRH synthesis is decreased during lactation by measuring cellular levels and total hypothalamic content of GnRH mRNA by *in situ* and solution hybridization, respectively, and comparing these values between groups of lactating and cycling female rats.

In addition to examining possible limitations in the synthesis of GnRH, we considered whether alterations in the coexpression of other products of GnRH neurons might account for their diminished activity during lactation. Galanin is a gut-brain peptide produced in a large number of hypothalamic neurons, including a subset of GnRH neurons (11, 12). Although its functional role in GnRH neurons is uncertain, galanin administered into the brain induces LH release (13), possibly through an induction of GnRH secretion (14, 15). We have previously demonstrated that expression of galanin mRNA in GnRH neurons is increased at the time of the proestrous surge of gonadotropin secretion, presumably coincident with activation of GnRH neurons (16). Given the association of an increase in galanin mRNA in GnRH neurons and the concomitant activation of these cells, we asked whether the diminished activity of GnRH neurons associated with lactation would, likewise, be accompanied by a reduction in galanin mRNA coexpression by GnRH neurons. We tested this hypothesis by performing double labeling *in situ* hybridization to identify GnRH mRNA-expressing neurons, to estimate cellular levels of galanin mRNA

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coexpressed within them, and, finally, to compare these values between groups of lactating and cycling female rats (12).

Materials and Methods

Animals

Pregnant female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA) and housed under constant light-dark (14 h of light, 10 h of darkness) conditions, with lights on at 0600 h. The animals had free access to rat chow and tap water. They were placed into individual maternity cages on day 20 of pregnancy. One or 2 days after parturition (days 1 and 2 of lactation), litter size was adjusted to eight pups for all lactating animals. Control females had their estrous cycle monitored by daily vaginal lavage, and were killed at 1000 h during diestrous day 1 (diestrus-1). Only females that had completed at least two consecutive 4-day estrous cycles were included in the study.

Experimental design

Exp 1. We examined the influence of lactation on expression of the galanin mRNA in GnRH neurons using a double *in situ* hybridization protocol to identify GnRH mRNA-positive cells for analysis of their relative galanin mRNA content (16). In addition, we counted the total number of GnRH neurons identified in the sections examined. Experimental groups included animals that were killed on diestrus-1 at 1000 h (n = 5) and on the tenth day of lactation with eight pups (1000 h; n = 4).

Exp 2. To confirm and extend the findings of previous investigations (1, 7, 10), we measured GnRH content in the brains of lactating and cycling rats. Animals were killed on the tenth day of lactation with an eight-pup litter (1000 h; n = 18) and on day 1 of diestrus (1000 h; n = 18). Whole brains were removed and placed ventral side up in a cold brain slicer (Zivic-Miller, Allison Park, PA) with slots at 1-mm intervals. The following areas were dissected out: median eminence (ME; n = 6), ME plus arcuate nucleus (ME-ARC; n = 6), or the entire preoptic area-medial basal hypothalamus-ME (POA-MBH-ME; n = 6). The hypothalamus was dissected by placing one razor blade at the anterior border of the optic chiasm and another at the anterior border of the mammillary bodies. The lateral borders were the hypothalamic sulci, and the depth was 3 mm.

Exp 3. We examined the influence of lactation on the expression of GnRH mRNA in individual neurons and on the number of GnRH mRNA-positive neurons throughout the basal forebrain and rostral hypothalamus of adult female rats. Lactating (n = 6) and diestrus-1 (n = 6) animals were used in this analysis. Using a radiolabeled cRNA probe and *in situ* hybridization, we compared the cellular GnRH mRNA content in individual neurons and counted the total number of GnRH neurons in the tissue sections examined.

Exp 4. In this experiment, we used a second set of tissue sections from the animals in Exp 3 and repeated the *in situ* hybridization and grain-counting analysis. In this case, we were led by the findings of Malik *et al.* (17) to increase the duration of exposure of the slides so as to increase the total number of grains per cell and, potentially, to highlight subtle differences between the experimental groups.

Exp 5. We sought to confirm or refute the findings of Exp 3 and 4 with a RNase protection assay. The GnRH mRNA content of the POA-MBH was compared between groups of lactating (day 10, eight pups; n = 6) and diestrus-1 (n = 6) animals, with cyclophilin mRNA serving as an internal control standard, as described previously (18). This experiment was repeated with different groups of lactating (day 10, eight pups; n = 5) and diestrus-1 (n = 5) animals to confirm the findings of the first assay.

In situ hybridization histochemistry

Tissue preparation. For Exp 1, 3, and 4, animals were killed by asphyxiation with carbon dioxide and immediately decapitated. The brains were rapidly removed, frozen on dry ice, and stored at −80 C. Coronal brain slices (20 μm) were cut with a cryostat, thaw mounted onto poly-L-lysine (50 mg/ml)-coated slides, and stored at −80 C in air-tight boxes. Using the rat atlas of Paxinos and Watson (19) as an anatomical guide, we collected coronal slices beginning rostrally at the genu of the corpus callosum and continuing caudally through the decussation of the anterior commissure. The sections were collected on three sets of slides, with each set representing a one in three series of sections over the entire rostral to caudal extent of sectioning.

Probe preparation. A detailed methodology for single and double label *in situ* hybridization has been reported previously (16), and the outline that follows briefly describes this methodology and identifies changes specific to this group of experiments. A 462-basepair (bp) ^{35}S-labeled cRNA probe, complementary to rat GnRH mRNA, was used for single label hybridization histochemistry. The original plasmid containing the GnRH insert, previously described in detail (12, 20), was generously provided by Dr. Tony Mason (Genentech, South San Fransisco, CA). After *in vitro* probe synthesis with 14% of the total UTP replaced with [^{35}S]UTP, the probe was added to the hybridization buffer to obtain a final concentration of 0.25 μg/ml·kilobase. The control experiments used to validate the integrity, binding kinetics, and specificity of the GnRH cRNA probe have been previously described (21, 22).

A digoxigenin-labeled cRNA probe for GnRH mRNA was made from the same plasmid used for synthesis of the ^{35}S-labeled probe. The probe was synthesized *in vitro* from linearized DNA with 400 mM digoxigenin-11-uridine-5′-triphosphate (Boehringer Mannheim, Indianapolis, IN), 100 mM unlabeled UTP, 500 mM GTP, ATP, and CTP, and SP6 polymerase. Residual DNA was digested with DNAase, and the cRNA probe was separated from unincorporated nucleotides on a Sephadex G-50 column. The purified probe was diluted 1:40 in hybridization buffer for double label hybridization histochemistry.

The plasmid vector Bluescript containing a cDNA complimentary to rat galanin mRNA (23) was used to generate the galanin cRNA probe. This plasmid was used for *in vitro* transcription of a 680-bp ^{35}S-labeled cRNA probe for galanin mRNA and to generate control unlabeled cRNA. The galanin probe transcription reactions had 25% of their total UTP as [^{35}S]UTP. The final double label hybridization solution contained a galanin cRNA probe concentration of 0.30 μg/ml·kilobase.

Before the double label hybridization procedure, both the ^{35}S-labeled galanin cRNA probe and the digoxigenin-labeled GnRH cRNA probe were heat denatured and added to hybridization buffer. Because the exact yield of the transcription reaction with digoxigenin-UTP for the GnRH cRNA probe cannot be deduced, a test *in situ* hybridization was performed to determine empirically the optimal concentration for this probe.

Single label in situ hybridization for GnRH mRNA. Using the probe complementary to GnRH mRNA, we performed single label, *in situ* hybridization as previously described (16, 22). Briefly, 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 in a series of alcohols, delipidated in chloroform, and rehydrated in a second series of alcohols. The GnRH cRNA probe was diluted in hybridization buffer and placed onto each slide (60 μl/slide). Individual slides were covered with Parafilm, sealed with rubber cement, and incubated overnight in moist chambers at 60 C. On the following day, the slides received a RNAse-A treatment and a series of salt washes of increasing stringency (4 × SSC-0.1 × SSC). The slides were dehydrated in alcohols and air dried.

Autoradiography and histological staining of single label slides. Slides were dipped in Kodak NTB-2 emulsion (42 C; Eastman Kodak, Rochester, NY), diluted 1:1 with 600 mM ammonium acetate. The tissue sections were allowed to air dry for 2 h and then stored with desiccant in light-tight boxes at 4 C for 4 days (Exp 3) or 6 days (Exp 4). The slides were developed in Kodak D-19 developer and counterstained with cresyl violet before application of coverslips.

Double label in situ hybridization for GnRH and galanin mRNAs. We performed a double label *in situ* hybridization to identify cells containing both GnRH mRNA and galanin mRNA following a protocol similar to that described for the single label *in situ* hybridization. In this case, the tissue sections were prehybridized for 2 h at 60 C with cRNA hybridization buffer containing 2.0 mg/ml freshly denatured total yeast RNA and then rinsed in 2 × SSC for 5 min before application of the probe mixture containing both the ^{35}S-labeled galanin cRNA probe and the digoxigenin-labeled GnRH cRNA probe in hybridization buffer. After the stringent washes of the second day, the slides were placed in 2 × SSC-0.05% Triton X-100 containing 2% normal sheep serum for 30 min. The slides were washed in buffer 1 [100 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and then incubated for 3 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. The slides were washed and incubated in a chromagen solution for 4 h at 37 C. The reaction was stopped, and the slides were air dried. Before being dipped in emulsion, the slides were dipped in 3% parlodion (Fisher Scientific, Fair Lawn, NJ) dissolved in isoamyl acetate. For double label analysis, 8 days of exposure (Exp 1) were allowed for visualization of silver grain clusters representing galanin mRNA-positive cell bodies.

Semiquantitative analysis of cellular mRNA signal levels. Tissue sections were viewed under a Zeiss Axioskop (Zeiss, New York, NY), equipped with a ×40 epiillumination darkfield objective. We determined the number of silver grains per cell using a grain-counting program previously described (12, 16). This system consisted of a PixelGrabber video acquisition board (Perceptics Corp., Knoxville, TE) attached to a Macintosh IIfx computer (Apple Computer Corp., Cupertino, CA). 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 expression in each GnRH neuron, a purple-stained digoxigenin-labeled GnRH mRNA-containing cell was first isolated under brightfield illumination. The silver grains overlying each cell were then visualized and counted under darkfield epiillumination by the image analyzer. Seventeen tissue sections per animal, equally spaced throughout the diagonal band of Broca and POA, 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. In all 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, as previously described (12, 16). To avoid the subjective decision of whether a GnRH neuron was double labeled, all GnRH neurons were analyzed for galanin mRNA content by grain count measurements.

Control experiments. Control experiments were performed to assess the specific tissue binding of the galanin ^{35}S-labeled cRNA probe as well as both ^{35}S- and digoxigenin-labeled GnRH cRNA probes, as described previously (12, 22, 24). The control experiments included hybridization with a labeled sense probe and incubation with a 100-fold excess concentration of unlabeled antisense probe in the presence of ^{35}S-labeled antisense probe. The tissue was processed for hybridization histochemistry as described above. Both experiments resulted in complete loss of specific labeling, as evidenced by a lack of either photographic grain clusters in tissues incubated with the ^{35}S-labeled probes or purple-stained cells in tissues incubated with the digoxigenin probe (data not shown).

GnRH peptide content analysis

Tissue preparation. The tissue samples from Exp 2 were sonicated in 100 ml 0.1 N acetic acid (ice-cold). An additional 400 ml 0.1 N acetic acid were added to rinse the sonication probe. The samples were centrifuged, and the supernatant was removed and frozen until analysis of GnRH content by RIA.

RIA for GnRH. The RIA for GnRH was performed as described previously (4). Briefly, the samples were diluted 1:10 in assay buffer (2% BSA in PBS, pH 7.4) and assayed in 5- and 10-ml duplicates. The GnRH antiserum was provided by Dr. Robert Kelch (University of Michigan) and used at an assay dilution of 1:300,000. The sensitivity of the assay was 0.2–0.3 pg/tube, and the ED_{50} of the assay was 5.2 ± 0.3 pg.

Solution hybridization-RNase protection assay for GnRH mRNA

Tissue preparation. For Exp 5, rats were killed by decapitation, and brains were quickly removed and placed ventral side up in the cold brain slicer. The dissection boundaries of the POA-MBH were similar to those described above, except that the caudal border extended only 3 mm from the optic chiasm. The tissue was snap-frozen on dry ice and stored at −80 C until processed for RNA extraction.

Isolation and quantification of total cytoplasmic RNA. Total RNA was extracted using the RNAzol method developed by Chomczynski and Sacchi (25). To the thawed tissue, 1 ml RNAzol (Cinna-Biotec Laboratories, Houston, TX), which contains guanidinium-thiocyanate, sodium citrate, lauryl sarcosine, mercaptoethanol, and acid phenol, was added per 50 mg tissue. After homogenization, 0.1 vol choloroform was added, and the samples were mixed and then incubated on ice for 15 min. After centrifugation, the supernatant was precipitated with ice-cold ethanol. The pellet was washed with 70% ethanol, dried, and dissolved in pure water. Two- to 5-ml aliquots were removed from each sample to measure total RNA content by use of A260 readings. The remainder of the sample was further purified by precipitation with 0.2 M NaCl and 1 vol ethanol. The samples were centrifuged and stored at −80 C until use in the RNase protection assay.

Synthesis of GnRH cRNA probe and reference RNA. The protocols for labeling of GnRH riboprobes were similar to those developed by Roberts *et al.* (26, 27). The template for the *in vitro* synthesis of GnRH riboprobes was a 360-bp *Eco*RI-*Bam*HI fragment of a rat cDNA (20) containing about two thirds of the coding sequence of the pro-GnRH-GAP mRNA (kindly provided by J. L. Roberts) (26). The cDNA was cloned into bluescript vector; linearization with *Eco*RI and transcription with T3 RNA polymerase yielded antisense GnRH cRNA probe, whereas linearization with *Bam*HI and transcription with T7 RNA polymerase yielded sense GnRH mRNA. For the antisense probe, 200 ng template DNA were combined with 150 mCi [^{32}P]CTP (New England Nuclear, Boston, MA; 800 Ci/mmol) and 500 mM ATP/UTP/GTP to yield high specific activity probe (1–2 × 10^9 cpm/mg). An aliquot of the probe was electrophoresed through a 4% polyacrylamide-7 M urea denaturing gel to assess whether most of the probe consisted of full-length transcripts. The unincorporated [^{32}P]CTP was removed by ammonium acetate-ethanol precipitation, and the pellet was dissolved into hybridization buffer [80% formamide containing 0.04 M PIPES (pH 6.4), 0.4 M NaCl, and 1.0 mM EDTA] to produce 300,000 cpm/5 ml.

The reaction mixture for the reference RNA contained 1 mg template DNA and 500 mM ATP/GTP/CTP/UTP (27). The reference RNA was purified by phenol extraction, followed by chloroform extraction, and then precipitated with ammonium acetate-ethanol and dissolved in water. The purity of the preparation was checked by electrophoresing an aliquot of the preparation along with a RNA size marker through a 4% polyacrylamine-7 M urea denaturing gel, followed by staining with ethidium bromide. Preparations were used if they showed only one band at the appropriate size for full-length transcripts. The reference preparation was quantified with A260 readings. A check on this measurement was made by spotting increasing amounts of standard RNA and the reference preparation onto a 1% agarose gel containing ethidium bromide. The preparation was stored at a concentration of 100 ng/ml. Typical yields were 5–10 mg RNA.

A 111-bp fragment of cyclophilin cDNA, obtained from the ~approximately 680-bp clone (provided by Dr. J. Douglass) and representing the first 104 coding nucleotides between restriction sites *Pst*I and *Xmn*I, was subcloned into pGEM 3Z vector. Linearization with *Hin*dIII and transcription with T7 yielded antisense cyclophilin cRNA probe. A protocol similar to that described above was used to synthesize the [^{32}P]CTP-labeled cyclophilin probe, except that only 10 mCi [^{32}P]CTP (New England Nuclear; 800 Ci/mmol) was added, resulting in a specific activity of 5–6 × 10^7 cpm/mg. The final dilution of the cyclophilin probe in hybridization buffer was 5000 cpm/5 ml.

Measurement of pro-GnRH mRNA. The protocol used for the RNase protection assay has been described previously (26, 27). For the standard curve, duplicate samples were run containing 100, 200, 400, 1000, 2000, or 4000 μg GnRH reference RNA and the probes (300,000 cpm GnRH cRNA plus 5,000 cpm cyclophilin cRNA; total volume, 25 ml hybridization buffer). For the tissue RNA samples, the same amount of probe was mixed with 25 mg total hypothalamic RNA extracted from each animal, with a total volume of 25 ml hybridization buffer. After hybridization overnight at 45 C, the samples were treated with 300 ml RNase mix containing 40 mg/ml RNase-A and 2 mg/ml RNase-T1 for 1 h at 30 C. This was followed by proteinase-K treatment (40 mg/ml) for 15 min at 37 C. The samples were phenol-chloroform extracted, and the RNA was precipitated with ammonium acetate-ethanol. The pellets were dried and dissolved in 5 ml dye mix. After denaturing at 85 C, the samples were electrophoresed through a 0.4-mm 4% polyacrylamide denaturing gel. After drying the gel, it was exposed overnight to XAR-5 film. The film was used to identify the protected bands on the gel, which were cut out, placed in scintillation fluid, and counted. For the standard curve, the counts per min were plotted against the amounts of reference RNA, and a regression line was calculated (see Fig. 5) and used to determine the amount of protected GnRH mRNA in each sample containing 25 mg total RNA. The results were normalized against the cyclophilin signal, which served as the internal control.

Statistical analysis for all experiments

For all experiments, n refers to the number of experimental animals within a group, and this was the n used in each analysis. For cellular GnRH or galanin mRNA content determinations by *in situ* hybridization, the mean grains per cell from individual animals (and anatomical areas within animals) were used to calculate the mean ± SEM for each group. In each experiment, the differences between groups were assessed by Student's *t* test or analysis of variance. The rejection level was set at $\alpha = 0.05$.

Results

Galanin mRNA content in GnRH neurons: lactation vs. diestrus-1

Exp 1. Animals killed at 1000 h on day 10 of lactation had greater than 2-fold lower galanin mRNA signal levels in GnRH neurons compared with those killed at 1000 h on diestrus-1 ($P < 0.004$; Fig. 1), which represents a nadir of expression relative to other stages of the estrous cycle (16). Visual inspection indicated that there were approximately 3 times as many double labeled GnRH neurons in diestrous animals as in lactating animals (diestrus-1, 39 ± 6%; lactation, 12 ± 3%; $P < 0.005$). There was no significant difference among groups in the overall galanin mRNA signal levels in non-GnRH-expressing galanin neurons sampled from throughout the rostral to caudal extent of the GnRH neuronal population (diestrus-1, 102 ± 7; lactation, 107 ± 2 grains/cell; $P > 0.50$). Also, there was no significant difference among groups in the total number of GnRH neurons counted (diestrus-1, 61 ± 8; lactation, 60 ± 13; $P > 0.90$). Simultaneous bright- and darkfield images of representative cells from this experiment are shown in Fig. 2.

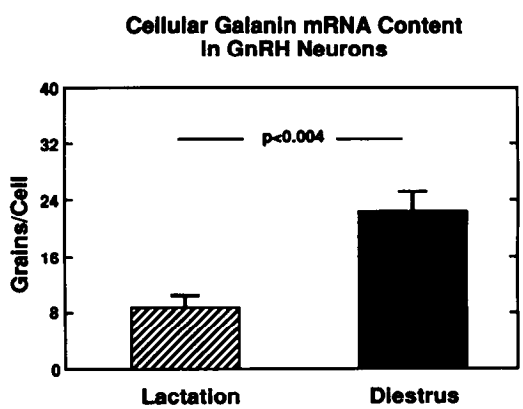

FIG. 1. A comparison between adult female rats on diestrus-1 (n = 5) and during lactation (day 10, 8 pups; n = 4) in galanin mRNA signal levels (grains per cell ± SEM) in GnRH mRNA-positive cells throughout the forebrain and rostral hypothalamus. In each case, 15 sections/animal were analyzed.

GnRH peptide and GnRH mRNA content: lactation vs. diestrus-1

Exp 2. There was no difference between lactating and diestrous rats in hypothalamic GnRH content (nanograms per area) in any of the anatomical areas of dissection (ME: lactation, 5.2 ± 0.5; diestrus, 4.8 ± 0.5; ME-ARC: lactation, 6.3 ± 0.6; diestrus, 5.4 ± 0.5; POA-MBH-ME: lactation, 5.7 ± 0.4; diestrus, 6.0 ± 6; $P > 0.71$; Fig. 3).

Exp 3. We observed no significant difference among groups in either the overall cellular levels of GnRH mRNA signal (Fig. 4) or the overall number of GnRH mRNA-containing neurons analyzed per animal (diestrus, 82 ± 9; lactation, 72 ± 9; $P > 0.45$). Moreover, there was no significant difference in either the number of GnRH neurons or the cellular content of GnRH mRNA when the cells were analyzed by discrete anatomical areas, as illustrated by the data from the organnum vasculosom of the lamina terminalis (OVLT)-POA shown in Fig. 4. The areas examined and compared between groups included the medial septum, diagonal band of Broca, OVLT, POA, and anterior hypothalamus.

Exp 4. Increasing the length of exposure of the slides to emulsion from 4 to 6 days did increase the average number of grains per cell observed. However, there were still no significant differences among groups in either the overall cellular levels of GnRH mRNA (diestrus-1, 182.7 ± 11.3; lactation, 196.7 ± 10.1 grains/cell) or when the cells were analyzed by discrete anatomical areas (OVLT-POA: diestrus, 186.5 ± 12.1; lactation, 199.1 ± 10.1).

Exp 5. A standard curve for the solution hybridization-RNase protection assay is shown in Fig. 5A. The regression line was used to quantify GnRH mRNA. A photograph of the gel from the first assay is shown in Fig. 5B. Each band was counted and normalized with respect to the cyclophilin signal. The results from the first group of animals studied (n = 6 for each group) showed that GnRH mRNA (expressed as picograms per 25 mg total RNA) did not differ between lactation and diestrus (Fig. 5C). Also shown is the GnRH mRNA content of a POA mRNA pool that is run in duplicate in each assay. The RNA samples from the second group of animals (n = 5 for each group) were analyzed in the same manner. Because the value for the POA pool differed between the two assays, the data were expressed as a percentage of the POA mRNA pool to permit analysis of the com-

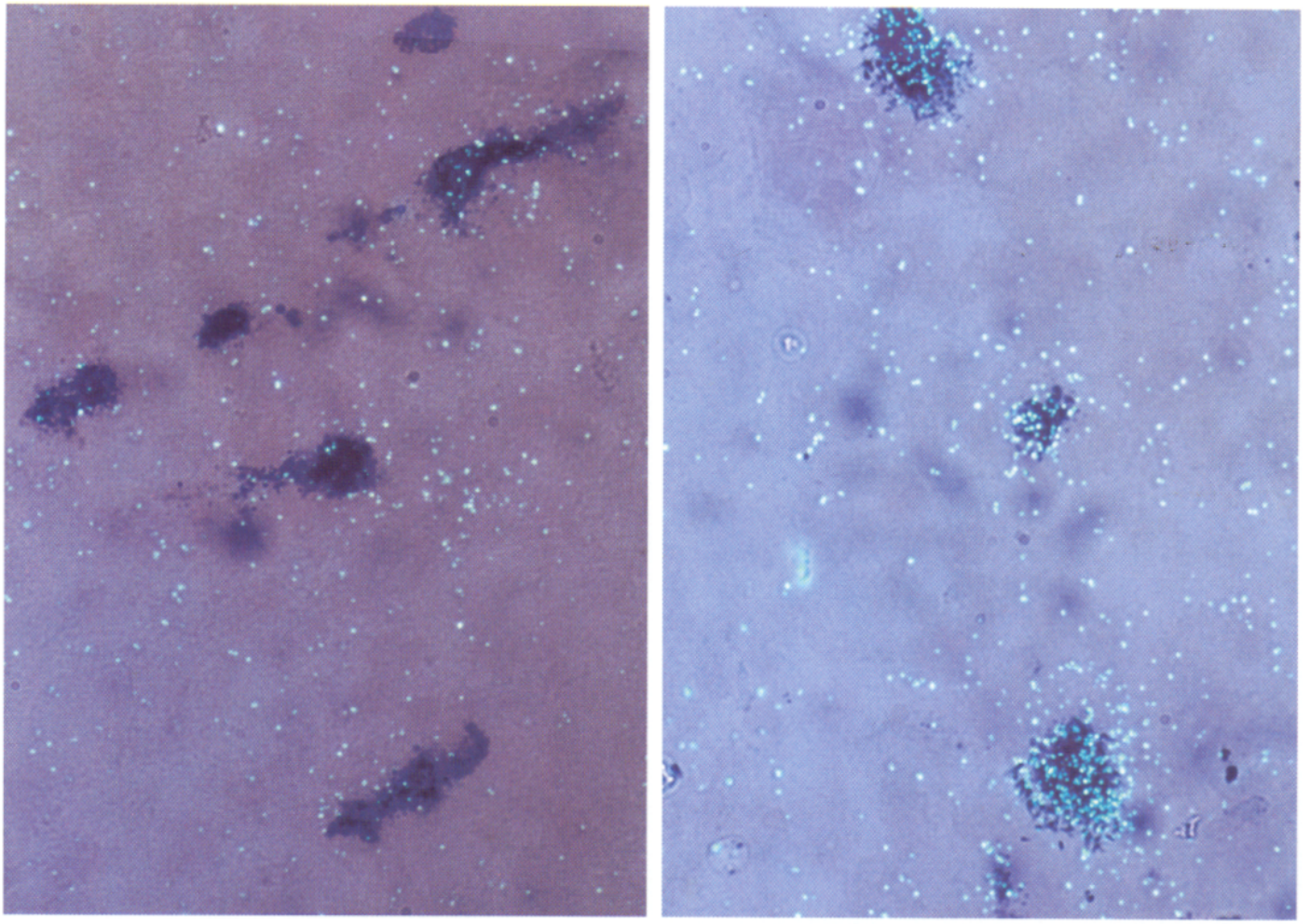

FIG. 2. Simultaneous bright- and darkfield photomicrographs (×40 objective) of the medial POA of adult female rats, showing cells labeled with a digoxigenin-conjugated cRNA probe for GnRH mRNA and a cRNA probe for galanin mRNA labeled with ^{35}S in sections obtained from animals killed at 1000 h on diestrus-1 (*left panel*) and on day 10 of lactation with eight pups (*right panel*).

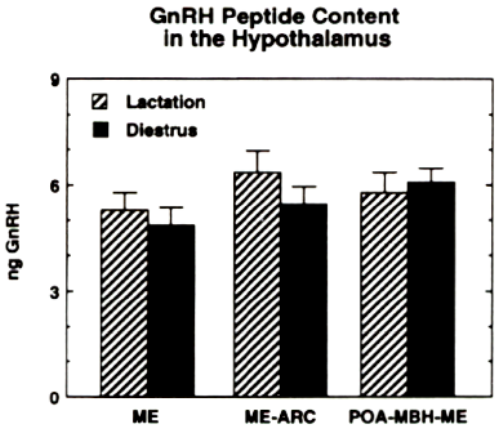

FIG. 3. Effect of lactation on GnRH peptide content in the hypothalamus, determined by RIA. The data represent the mean ± SEM; there were six animals per area per group.

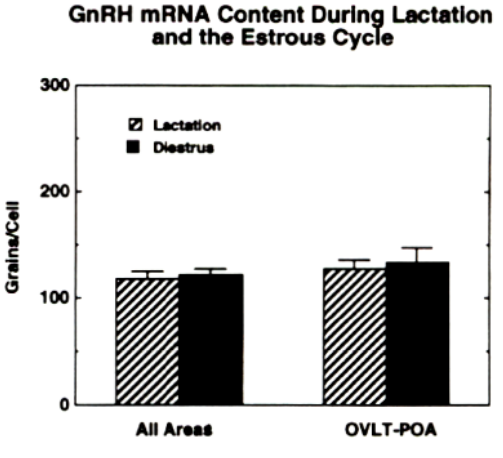

FIG. 4. A comparison between lactating and diestrous animals in GnRH mRNA content (grains per cell ± SEM) in cells labeled by *in situ* hybridization throughout all areas of the forebrain and rostral hypothalamus and in the OVLT-POA. n = 6 for each group.

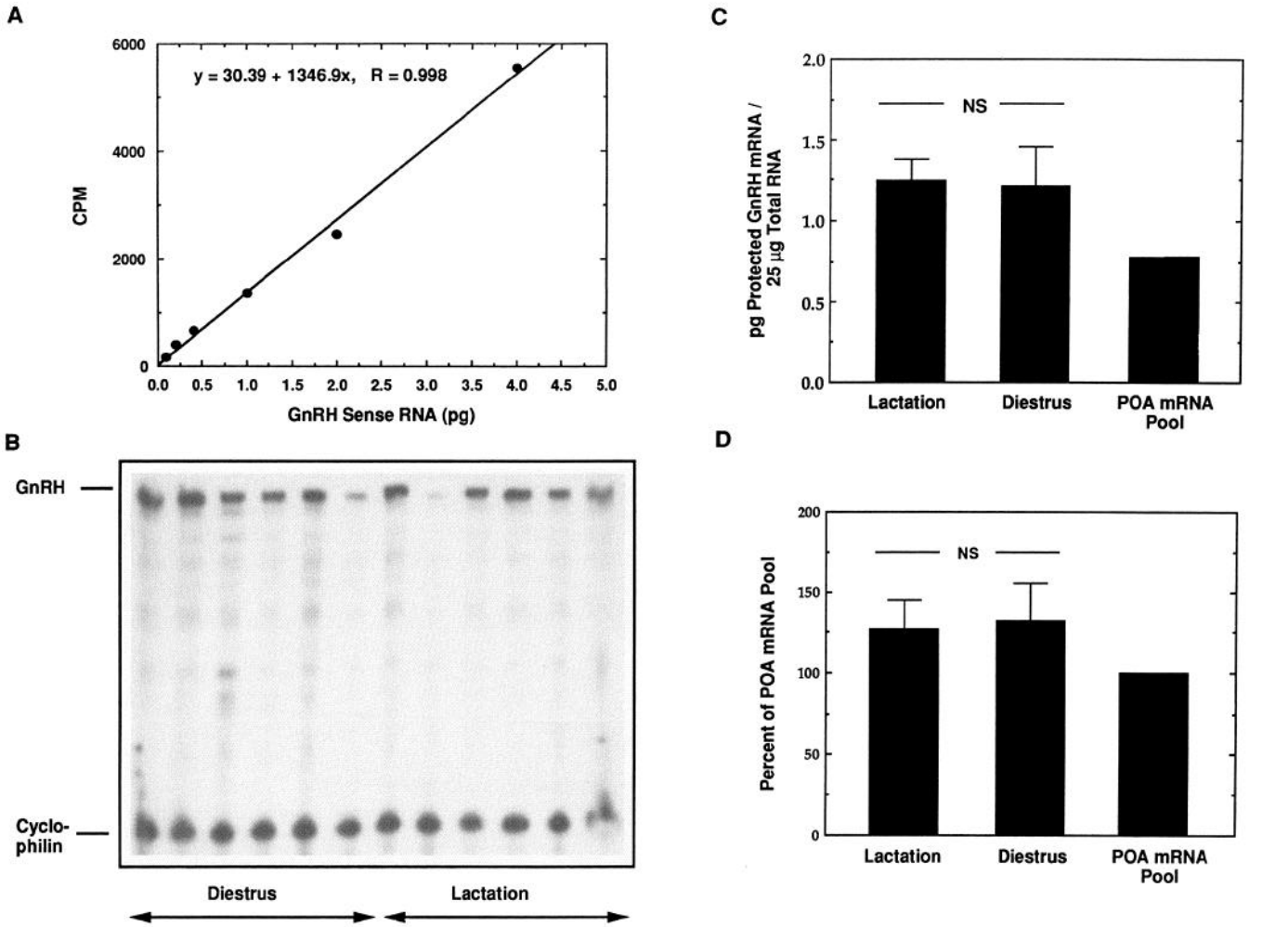

FIG. 5. A, A standard curve used to quantify GnRH mRNA, obtained by hybridizing reference GnRH with a ^{32}P-labeled GnRH cRNA probe. The regression line was produced by plotting the known amounts of reference GnRH against the counts per min obtained from the excised protected bands on the gel. B, Autoradiogram of gel electrophoresis of protected GnRH mRNA (*top bands*) and cyclophilin mRNA (*bottom bands*). Twenty-five milligrams of total RNA from individual POA-AH dissections were hybridized with ^{32}P-labeled GnRH cRNA probe (1–2 × 10^9 cpm/mg) and ^{32}P-labeled cyclophilin cRNA probe (5–6 × 10^7 cpm/mg). Lanes 1–6, Diestrous animals; lanes 7–12, lactating animals. C, Measurement of GnRH mRNA (picograms of protected GnRH mRNA per 25 mg total RNA) in the protected bands shown in B, using the standard curve shown in A. Data represent mean ± SEM. D, A comparison between GnRH mRNA in lactating and diestrous animals. Data were combined from both assays (n = 11 for each group) and normalized to the standard POA mRNA pool.

bined groups (Fig. 5D; n = 11). Again, there was no difference in GnRH mRNA content between lactating and diestrous animals.

Discussion

This study demonstrates that the galanin mRNA content in GnRH neurons is greatly diminished during lactation in the rat. These data can be interpreted in the context of other observations to derive a working hypothesis for the function of galanin in the regulation of GnRH release. First, the lactation-induced suppression of galanin mRNA content in GnRH neurons is not apparently a general phenomenon in the hypothalamus, as the overall distribution and level of silver grains representing galanin mRNA expression in non-GnRH neurons are not significantly different between cycling and lactating animals. These data regarding overall galanin expression in non-GnRH neurons were obtained from an anatomically restricted (*i.e.* rostral hypothalamus), random sampling of the many galanin neurons found throughout the entire brain and should be interpreted with caution. It is apparent that many galanin/non-GnRH neurons in the immediate vicinity of GnRH neurons do not apparently alter their level of expression of galanin mRNA during lactation. Second, preliminary evidence adduced by Merchenthaler (28) suggests that the galanin peptide content in GnRH neurons is also reduced during lactation, as reflected by a loss of colocalized galanin immunoreactivity in this same population of cells. Finally, galanin peptide enhances GnRH release from the isolated ME *in vitro* (15), and intracerebro-

ventricular injections of galanin can trigger LH release *in vivo* (13). Thus, it is conceivable that the loss of galanin expression in GnRH neurons accounts for the inhibition of gonadotropin release observed during lactation (1–5) and that, conversely, the enhanced expression of galanin mRNA (16) and peptide (11) in GnRH neurons observed on the day of proestrus facilitates the preovulatory gonadotropin surge (29–31). These effects would presumably be accomplished presynaptically via galanin receptors known to be localized in the ME (14), thereby providing a site for immediate ultrashortloop feedback action of galanin at the level of the GnRH nerve terminal (15). This proposed mechanism would be analogous to that which is thought to account for galanin-mediated inhibition of acetylcholine release in the ventral hippocampus (32). In this system, galanin is colocalized with acetylcholine in neurons located in the medial septum and diagonal band (33). These neurons project primarily to the ventral hippocampus, where galanin is thought to limit acetylcholine release via an inhibitory presynaptic G-protein-coupled galanin receptor (32, 34).

Lactation is characterized by alterations in the circulating levels of several hormones known to effect the release of GnRH from the hypothalamus, any one of which could also have an effect on galanin gene expression. Plasma estrogen levels are greatly suppressed during lactation, and conversely, administration of exogenous estradiol can induce LH release, indicating that the inhibition of LH release during lactation may be due in part to the loss of the stimulatory effects of estradiol (35–37). Galanin mRNA expression in GnRH neurons is also supported by circulating estradiol (16), and the low levels of galanin mRNA expression in GnRH neurons during lactation may reflect a response to the diminished plasma levels of estradiol. Other factors may be involved in the suppression of galanin mRNA expression in GnRH neurons. These include factors known to inhibit gonadotropin secretion, such as the neural stimulus of suckling and high circulating levels of progesterone and PRL (37–39). For example, the hypothalamic response to exogenous estradiol administration is significantly decreased during lactation, which is attributable to the presence of either a suckling stimulus or high levels of PRL (35–38). Although the effect that these stimuli may have on galanin mRNA expression in GnRH neurons remains undefined, it is likely that regulation of this peptide is multifactorial.

Our findings of lactation-induced changes in the expression of galanin mRNA in GnRH neurons contrast with the results related to GnRH mRNA. In repeated trials using single label *in situ* hybridization, we have shown that neither the number of cells expressing GnRH mRNA nor the cellular content of GnRH mRNA is different in cycling compared with lactating animals. These results were confirmed in a separate experiment, also run in repeated trials, in which we measured hypothalamic GnRH mRNA content with a RNase protection assay. The results of this assay are in good agreement with others concerning the GnRH mRNA content in the hypothalamus, as assessed by solution hybridization assay (26, 40). These data demonstrate that transcriptional regulation of the GnRH gene is unlikely to play an important role in the regulation of the GnRH neuron and does not account in any significant manner for the apparent decline in GnRH release associated with lactation. The relative constancy of the cellular levels of GnRH mRNA in the face of dramatic alterations in the hormone milieu during lactation is analogous to our previous findings in male (22, 41) and female (16) rats, in which we have demonstrated steady state GnRH mRNA levels in situations where the pattern of secretion of GnRH is clearly altered.

Changes in GnRH levels may be accomplished by mechanisms that are independent of changes in cellular mRNA content. Thus, in parallel with our studies on GnRH mRNA content, we examined the effects of lactation on hypothalamic GnRH peptide content. Our measurements are consistent with previous results indicating that the hypothalamic content of GnRH peptide is not dramatically altered by lactation (1, 7, 10). Together, these data demonstrate that the overall biosynthetic capacity for GnRH production by the hypothalamus remains unaltered during lactation, indicating that the primary level of restraint on the release of gonadotropins is an inhibition of the pulsatile release of GnRH. This inhibition appears to be robust, as treatment with an excitatory amino acid fails to elicit LH release in lactating animals (9), with more intense electrochemical stimulation of the hypothalamus being required to elicit any substantial release of LH (8).

Based on these observations, we propose a model for the suppression of gonadotropin secretion during lactation that emphasizes alterations in the release of GnRH, rather than an inhibition of its cellular biosynthesis. This model predicts that galanin expression in GnRH neurons is requisite for producing the distinctly pulsatile pattern of GnRH release that is capable of stimulating the pituitary gonadotrope in a robust fashion. Galanin could most effectively alter GnRH secretion by acting directly on the GnRH neurons from which it is released. The degree of influence galanin has on GnRH release would be related to its concentration at the nerve terminal, which, in turn, may depend on its relative rate of biosynthesis. Factors controlling this biosynthetic rate would include ovarian steroids, which may act either directly on the GnRH neuron or indirectly on other neurons that provide afferent input to GnRH neurons. Although this model focuses on the effect of galanin expression in GnRH neurons on GnRH release, other mechanisms may contribute to the processes by which LH release is inhibited during lactation. For example, changes in inhibitory neural inputs from endogenous opioid peptide (42) and γ-aminobutyric acid (43) neurons have been proposed as inhibitors of GnRH neuronal activity during suckling, indicating that the suppression of gonadotropin release involves a multifaceted neural response.

In summary, we have shown that the expression of galanin mRNA in GnRH neurons is inhibited during lactation, whereas the expression of GnRH mRNA is unaltered in this state. Although the impact of a loss of galanin gene expression in GnRH neurons on the reproductive neuroendocrine axis remains unknown, the preponderance of experimental data suggests that it may in part be responsible for the loss

of pulsatile GnRH secretion from the hypothalamus during lactation. Despite this uncertainty, our data demonstrate that the inhibition of GnRH release during lactation does not result from a decrease in the biosynthesis of this peptide. We infer that an alteration in the synaptic input to GnRH neurons coupled with depletion of colocalized galanin peptide are responsible for the suppression of pulsatile LH secretion in lactating rats.

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Erratum

In the article, "Regulation of gonadotropin-releasing hormone (GnRH) and galanin gene expression in GnRH neurons during lactation in the rat," by Daniel L. Marks, M. Susan Smith, Donald K. Clifton, and Robert A. Steiner (*Endocrinology* **133:** 1450–1458, 1993), on page 1454 Fig. 2 was incorrectly positioned and should be as shown below. The printer regrets the error.

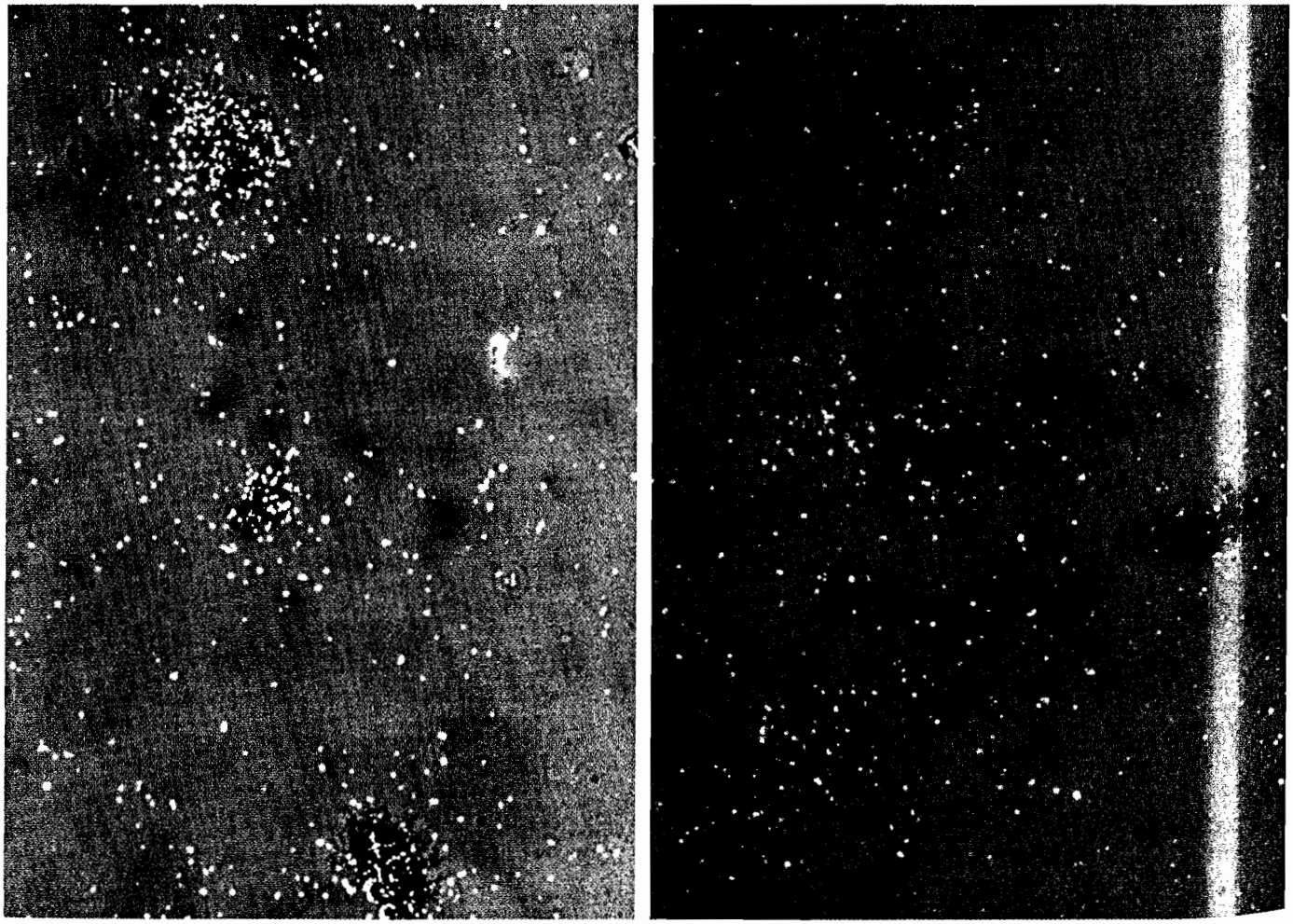

FIG. 2. Simultaneous bright- and darkfield photomicrographs (×40 objective) of the medial POA of adult female rats, showing cells labeled with a digoxigenin-conjugated cRNA probe for GnRH mRNA and a cRNA probe for galanin mRNA labeled with ^{35}S in sections obtained from animals killed at 1000 h on diestrus-1 (*left panel*) and on day 10 of lactation with eight pups (*right panel*).