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Inhibition of Steroid-Induced Galanin mRNA Expression in GnRH Neurons by Specific NMDA-Receptor Blockade

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Abstract

Galanin mRNA levels in GnRH neurons increase in association with a steroid-induced LH surge in female rats. Both the steroid-induced LH surge and the concomitant increase of galanin mRNA in GnRH neurons are blocked by non-specific inhibition of central nervous system activity imposed by pentobarbital and specific central alpha-adrenergic receptor blockade. Based on these observations, we hypothesized that galanin gene expression in GnRH neurons is induced whenever GnRH neurons become activated to generate an LH surge. If this were the case, then *any* neurotransmitter receptor blocking agent that inhibits the LH surge by central mechanisms would likewise block the associated increase in galanin mRNA in GnRH neurons. We tested this hypothesis by examining the effects of an N-methyl-D-aspartate (NMDA) receptor antagonist on the steroid-induced LH surge and on levels of galanin mRNA in GnRH neurons. Three groups of ovariectomized rats were used: Group 1-treated with estradiol and progesterone (E/P) and sacrificed at the peak of the LH surge; Group 2-treated the same as Group 1 except that dizocilpine (MK801, an NMDA receptor antagonist) was used to block the LH surge; and Group 3-treated the same as Group 1 except they received vehicle instead of E/P. Double-and single-label *in situ* hybridization followed by computerized image analysis were used to measure levels of galanin mRNA and GnRH mRNA in GnRH neurons [as grains/cell (g/c)]. E/P treatment induced a 3-fold increase in LH levels and a 5-fold increase in the galanin mRNA signal content of GnRH neurons. Treatment with MK801 completely prevented the LH surge in all animals and also blocked the steroid-induced increase in galanin mRNA in GnRH neurons. As assessed by 2 independent GnRH single-labeled assays, neither GnRH message content nor the number of identifiable GnRH neurons differed among the experimental groups. We conclude that the increase in galanin mRNA levels in GnRH neurons is tightly coupled to the occurrence of a steroid-evoked LH surge, and we infer that induction of galanin gene expression in GnRH neurons is induced as a consequence of synaptic activation of GnRH neurons.

Rising plasma levels of ovarian steroids in the female rat lead to an activation of gonadotropin-releasing hormone (GnRH) neurons whose amplified secretory activity results in the preovulatory release of LH (1). Several neurotransmitters are thought to be involved in the process of generating an LH surge, including neuropeptide Y, norepinephrine, and galanin (2). Galanin is expressed in a variety of neurons throughout the brain, including GnRH neurons (3–5). While the specific role that galanin plays in the generation of an LH surge is unknown, its importance is underscored by the observation that galanin antibodies and the galanin R1 receptor antagonist, galantide, inhibit the GnRH/LH surge (6, 7). Furthermore, levels of galanin mRNA in GnRH neurons are increased in association with the occurrence of an LH surge both during proestrus and in ovariectomized rats treated with estrogen and progesterone (E/P) (8, 9).

We have previously shown that a blockade of the LH surge by either a general anesthetic (pentobarbital) or a specific α-adrenergic blocking agent (phenoxybenzamine) prevents the induction of galanin gene expression in GnRH neurons, even in the presence high levels of ovarian steroids (9). Based on these observations, we hypothesized that galanin mRNA induction in GnRH neurons is tightly coupled to the occurrence of an LH surge; therefore, *any* agent that blocks the LH surge should also prevent the induction of galanin mRNA in GnRH neurons. We tested this hypothesis by investigating the effects of MK801, an N-methyl-D-aspartate (NMDA) receptor antagonist, on the induction of galanin mRNA in GnRH neurons. MK801 has previously been shown to block the LH surge in both proestrus and E/P-primed ovariectomized rats. Based on our hypothesis, we predicted that MK801 would block the induction of galanin message in GnRH neurons of E/P-treated ovariectomized rats, and present evidence to support this supposition.

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Results

Serum hormone concentrations

The mean LH concentration in the ovariectomized control group was 21 ± 2 ng/ml. E/P treatment induced LH surges in all of the animals in the surge-activated group, resulting in a mean LH concentration of 68 ± 18 ng/ml at the time of sacrifice ($P < 0.001$ *vs* the control group). The LH surge was effectively blocked in each of the animals that were treated with MK801. Serum LH concentrations in the surge-blocked group were 5 ± 1 ng/ml ($P < 0.001$ *vs* the surge-activated group).

Galanin mRNA in GnRH neurons

Galanin mRNA signal levels in GnRH neurons of ovariectomized control rats were uniformly low in all animals (8 ± 3 grains/cell; Fig. 1). In contrast, E/P-priming led to a 5-fold increase in the overall galanin mRNA content of GnRH neurons in the surge-activated group (41 ± 6 grains/cell; $P < 0.001$ *vs* the ovariectomized control group). Increased galanin gene expression was mainly observed in GnRH neurons in the caudal section of the diagonal band of Broca (DBB) and the preoptic areas (data not shown). This increase of galanin mRNA levels in GnRH neurons was completely suppressed by MK801 (11 ± 5 grains/cell; $P < 0.001$ *vs* surge-activated animals; Fig. 2). Galanin mRNA levels in GnRH neurons of these surge-blocked animals was not significantly different from ovariectomized controls.

To assess the effects of E/P and MK801 treatment on galanin message levels in *non*-GnRH neurons, grains (reflecting galanin mRNA) were counted over selected neurons that did not co-label for GnRH mRNA. It should be pointed out that, although we attempted to obtain a random sample, subjectivity in the selection process is unavoidable. The galanin mRNA content in this population of non-GnRH neurons was not significantly different among the experimental groups (ovariectomized controls: 102 ± 12 *vs* surge-activated: 92 ± 7 *vs* surge-blocked: 88 ± 12 grains/cell).

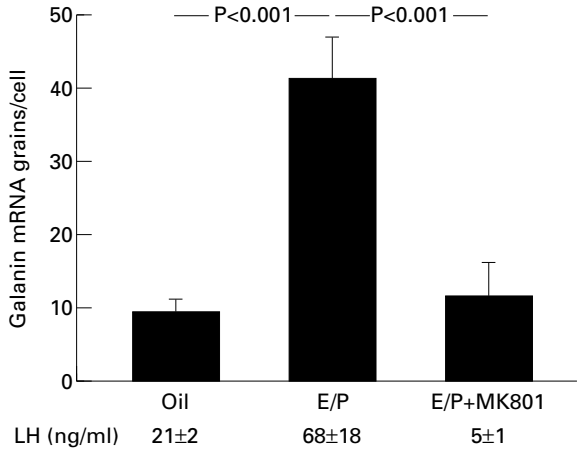

FIG. 1. Relative levels of galanin mRNA signal (grains per cell ± SEM) in GnRH neurons throughout the forebrain and rostral hypothalamus in groups of adult ovariectomized rats under different treatment regimens. The groups included control animals (castrated + oil-treated; n = 6), E/P-treated animals (castrated + estradiol/progesterone-primed (E/P); n = 7), MK801-treated (castrated + E/P-primed + MK801; n = 8).

GnRH mRNA content in GnRH Neurons

The experimental treatments did not alter either GnRH mRNA signal levels (ovariectomized controls: 129 ± 5 *vs* surge-activated: 130 ± 7 *vs* surge-blocked: 125 ± 4; Fig. 3A) or the number of GnRH mRNA-containing neurons (ovariectomized controls: 28 ± 10 *vs* surge-activated: 29 ± 6 *vs* surge-blocked: 28 ± 7; Fig. 3B). These findings were confirmed in a second GnRH single-label *in situ* hybridization assay run on the same set of animals.

Discussion

To analyze the role of cellular activation in the induction of galanin mRNA in GnRH neurons, we have blocked neuronal activity during the steroid-mediated LH surge with a pharmacological agent with defined receptor properties. The NMDA system was selected because of its known ability to influence the generation of the LH surge (10). We found that the NMDA receptor antagonist MK801 completely blocked the steroid-induced LH surge in ovariectomized animals. This observation is consistent with previous reports that NMDA receptor blockade inhibits both the proestrous and E/P-induced LH surges (11–13). Since NMDA does not appear to have an effect on the pituitary, the blockade of LH must result from an inhibition GnRH neuronal activity (14). In agreement with previous reports from this laboratory, we found increased galanin mRNA levels in GnRH neurons when E/P treatment was used to generate an LH surge (8, 9, 15). However, when the LH surge was blocked by MK801, E/P treatment failed to induce galanin mRNA expression in GnRH neurons. This observation is consistent with the hypothesis that the induction of galanin transcription is linked to cellular activation of GnRH neurons, regardless of the mechanism by which this activation is achieved. In fact, treatment with other pharmacological agents that block the LH surge, such as the general anesthetic pentobarbital or the specific alpha-receptor antagonist phenoxybenzamine, also prevents the induction of galanin mRNA in GnRH neurons (9).

The mechanisms by which ovarian steroids act upon GnRH neurons to activate the release of GnRH are unknown. Because the GnRH neuron itself does not appear to contain steroid receptors, it is likely that other neurons act as transducers, sensing steroid levels and transmitting this information to the GnRH neuron transynaptically. Because NMDA blockers such as MK801 prevent the steroid-induced activation of GnRH neurons (11–13), it is tempting to speculate that excitatory amino acids are involved in transducing the steroid signal that activates GnRH neurons during an LH surge. However, this simple explanation does not account for the fact that the specific blockade of any one of several other neurotransmitters also prevents steroid-induced LH surges (16–19). There are at least 2 alternative hypotheses that would account for the apparent involvement of multiple neurotransmitter systems. One would be that the steroid signal is sensed by steroid transducer neurons and relayed through a series of different neurotransmitter systems before arriving at the GnRH neuron. An inhibition of either the transducer neurons or any one of the relay neurons would prevent the signal from arriving at its destination. Another possibility is that the steroid-transducing neurons act directly on the GnRH system, but additional tonic input from other neurotransmitter systems is required before the GnRH neuron can respond to the steroid

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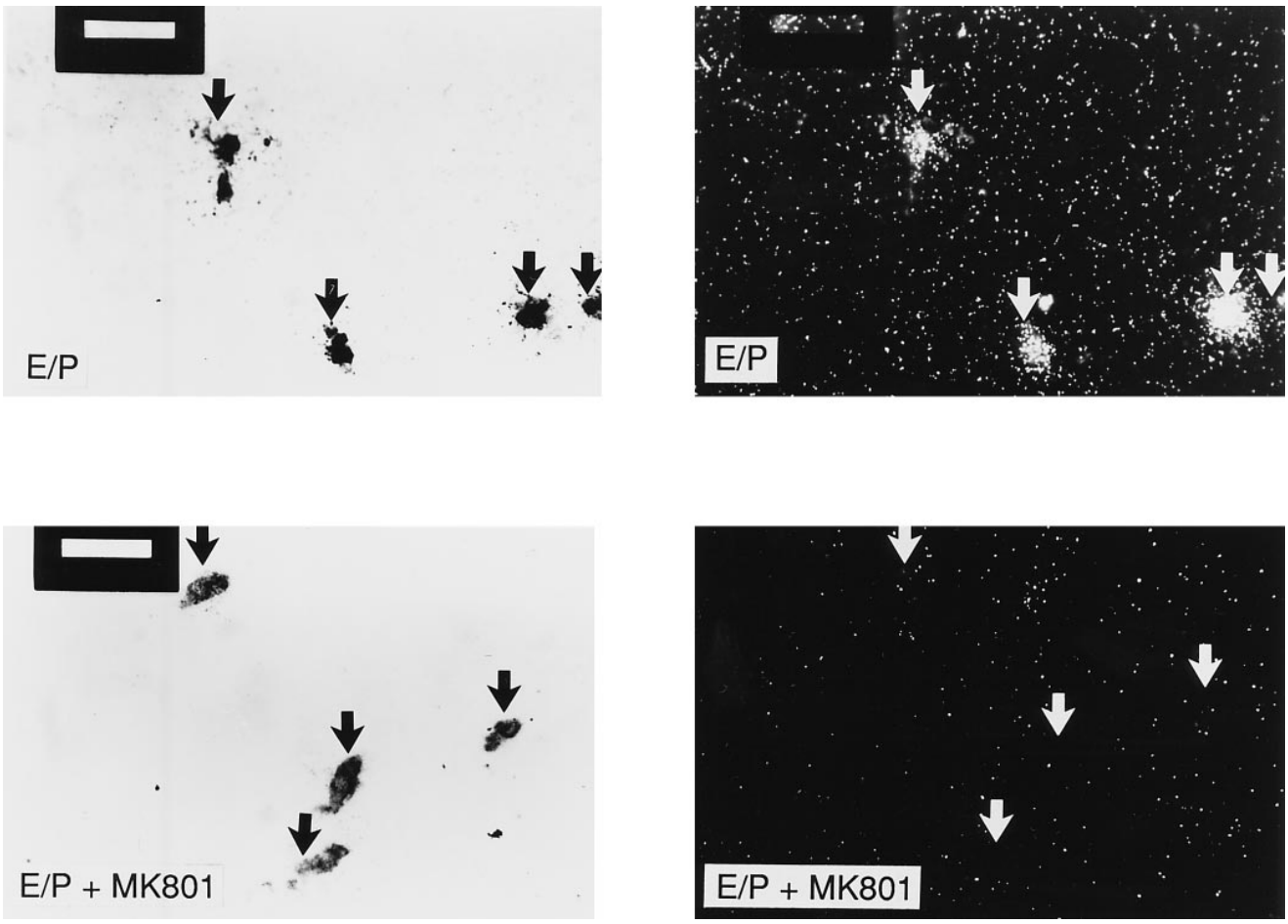

FIG. 2. Upper left panel: A brightfield photomicrograph of 4 GnRH neurons (black arrows), labeled with a digoxigenin-labeled probe for GnRH mRNA in an animal killed during an estradiol/progesterone (E/P)-induced LH surge (1800 h). Upper right panel: A darkfield photomicrograph of the same view as the upper left panel showing the same 2 GnRH neurons simultaneously labeled with an ^{35}S-labeled probe for galanin mRNA (open arrows). Note abundance of silver grains in each cluster, indicating a high level of galanin message expression in these cells. Lower left panel: A brightfield view of 4 GnRH neurons (black arrows) labeled with a digoxigenin-labeled probe for GnRH mRNA, in an E/P-primed animal which was concomitantly treated with MK801 and killed at 1800 h. Panel lower right: A dark field photomicrograph of the same view as lower left panel, showing the same GnRH neurons simultaneously labeled with an ^{35}S-labeled probe for galanin mRNA (open arrows). Note relatively few silver grains over each cell, indicating a low level of galanin message expression. Also note the silver grain clusters not associated digoxigenin-labeled cells, indicating the presence of galanin mRNA-containing cells in an unidentified population of non-GnRH neurons. The bar indicates a length of 50 μm.

signal. According to this scenario, only 1 or maybe 2 neurotransmitter systems actually mediate the steroid signal, while the others play a permissive role. [See (19) for a more detailed discussion of how a model like this might work.] In any case, it is impossible on the basis of this study, either to identify the neurotransmitter pathways through which steroids activate GnRH neurons or to determine the specific role that NMDA neurons play in this process.

Lee and coworkers have observed that the immediate-early genes c-fos and c-jun are induced in GnRH neurons during the proestrous LH surge (20–22). The presence of these markers of neuronal activity indicates that the transcription factor AP-1, a heterodimer of the Fos and Jun proteins, regulates the expression of a number of genes in the GnRH neuron during the LH surge. Galanin is a probable target for AP-1's action, since there appears to be an AP-1 site in 5′ flanking region of the galanin gene (23). Therefore, the induction of galanin mRNA in GnRH neurons during an LH surge is likely dependent on the expression of c-fos and c-jun. This being the case, one would expect galanin mRNA expression in GnRH neurons to be suppressed by the same factors that suppress the induction of c-fos in these cells. In fact, we now know that several pharmacological agents, including pentobarbital and MK801 block the LH surge-related induction of both c-fos and galanin mRNA in GnRH neurons (9, 12, 24). Therefore, rather than demonstrating the direct activation of c-fos by any particular receptor type, these observations serve to strengthen the concepts that the galanin gene expression in GnRH neurons is dependent on the induction of c-fos and that like the immediate early genes, galanin mRNA induction is associated with GnRH neuronal activation.

While a robust and rapid induction of the galanin gene was observed in GnRH neurons, this was *not* found in the galanin mRNA-containing neurons surrounding GnRH neurons. Analysis of these cells did not reveal any discernible alterations in galanin mRNA content either during surge-activation or during surge-blockade. These findings suggest that only a subset of

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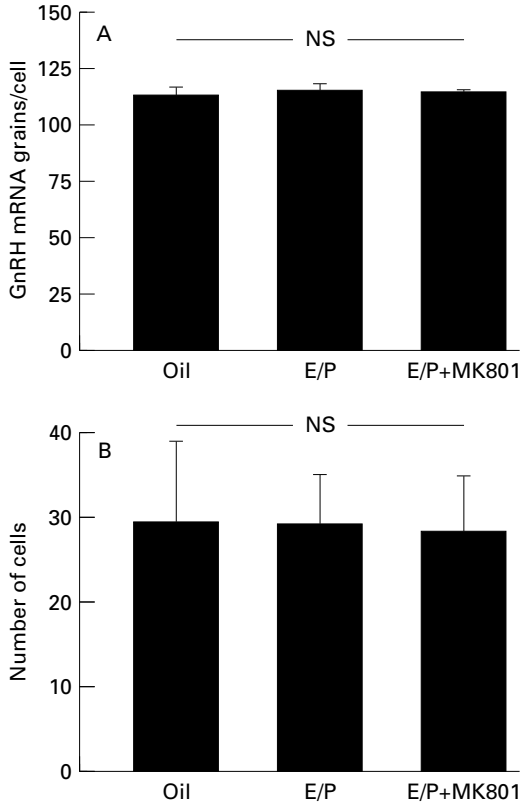

FIG. 3. A. Relative levels of GnRH mRNA signal (grains per cell ± SEM) in cells throughout the forebrain and rostral hypothalamus in groups of adult, ovariectomized rats under different treatment regimens. The groups included control animals (castrated + oil-treated; n = 8), E/P-treated animals (castrated + estradiol/progesterone-primed; n = 7), MK801-treated (castrated + E/P-primed + MK801; n = 8). B. Relative number of GnRH mRNA-containing neurons throughout the forebrain and rostral hypothalamus in groups of adult, ovariectomized rats under identical treatment conditions as described above.

hypothalamic galanin neurons, i.e., those coexpressing GnRH mRNA, are regulated as a function of neuronal activation. However, caution should be exercised with respect to this interpretation. Galanin message levels were measured in only a small sample of non-GnRH neurons, and while an attempt was made to make this an unbiased, random sample, the selection of the cells was a subjective process. Because there is a tendency for the operator to pick out large, well-formed clusters of silver grains, it is possible that there were true differences between groups, but they were masked by a selection bias. Nevertheless, this provisional finding does suggest that there is a heterogeneity within the population of hypothalamic galanin mRNA-containing neurons.

In this study, we have been unable to demonstrate a change in either the content of GnRH mRNA in individual neurons or the number of neurons expressing GnRH mRNA in response to the generation of a steroid-induced LH surge. Similarly, inhibition of this surge with MK801 produced no discernible effects on GnRH mRNA signal levels in GnRH neurons. Previous investigations on the steroid regulation of GnRH gene expression have yielded contradictory and equivocal results (25–28). Our finding of no changes in GnRH mRNA levels in response to steroid modulation are preceded by previous studies in our laboratory employing similar methodologies and comparable experimental designs (8, 9, 29). However, our results disagree with a report suggesting an augmentation of intracellular GnRH mRNA content following a steroid-primed LH surge (30). In the current study, we also found no detectable changes in GnRH mRNA levels following blockade of the LH surge by MK801. These results agree with our previous findings of unaltered GnRH gene expression during blockade of the steroid-primed LH surge by the general anesthetic pentobarbital or the alpha-adrenergic receptor antagonist phenoxybenzamine (9). However, in a recent study, NDMA receptor blockade was reported to reduce GnRH mRNA levels in the rat (31). The reasons for these discrepancies are unknown, but some differences may be reconciled by variances in the employed methodologies (*in situ* hybridization *vs* RNA-blot hybridization), use of different experimental models (immature *vs* adults rats, chronically *vs* acutely ovariectomized rats) or various means to assess the intracellular GnRH message (grain counting *vs* optical density measures). Furthermore, our results certainly do not prove that GnRH mRNA levels remain unchanged in the face of the experimental manipulations we performed. However, if the activation of GnRH neurons results in a change in cellular GnRH mRNA content, this change must be relatively small compared to the striking alterations in the galanin mRNA signal levels found in the same cells.

In summary, we have confirmed that the expression of the galanin gene in GnRH neurons is markedly induced under conditions which activate these cells leading to an LH surge. The induction of galanin mRNA in GnRH neurons is steroid-dependent but requires that the steroid signal be transduced into an additional activational process which, in turn, induces galanin gene expression in GnRH neurons. As a corollary, *any* interruption of this activational process in GnRH neurons will preclude induction of galanin mRNA in these cells.

Materials and methods

Animals

Adult (70 day old) female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA, USA). They were housed under pathogen-free, temperature (20 °C) and light-controlled (alternating light-dark cycle with 14 h light, 10 h dark, lights on at 07.00 h) conditions. The animals were given free access to tap water and rat chow. The rats were ovariectomized under ether anesthesia and were allowed to recover for 21 days before start of the steroid injections. During the week before ovariectomy and for three weeks afterwards, the animals were handled daily.

Experimental protocol

We tested whether a specific blockade of NMDA receptors would prevent the increased expression of galanin mRNA in GnRH neurons that normally accompanies a steroid-induced LH surge. Three groups of ovariectomized animals were studied: control (n = 8), LH surge-activated (n = 7), and LH surge-blocked (n = 8). The surge-blocked group was primed with estradiol and progesterone (E/P) and then given MK801 to block the LH surge that is normally induced by E/P treatment. The surge-activated group was treated in a similar manner except they received saline (vehicle) instead of MK801. The control group was treated with oil rather than E/P and also received saline instead of MK801. All rats were killed at or near the peak of the LH surge (between 18.00 and 19.00 h in our laboratory) by CO_2 asphyxiation immediately followed by decapitation. Trunk blood was collected, centrifuged and stored at −20 °C until assayed for serum LH levels. The brains were rapidly removed, frozen on dry ice, and stored whole at −80 °C.

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Drug treatments

E/P treatment consisted of an s.c. injection of 50 μg estradiol benzoate in 0.2 ml of peanut oil at 10.30 h on day 0 followed by 5 mg of progesterone in 0.1 ml of peanut oil injected s.c. on day 2 at 12.00 h. An equivalent volume of oil alone injected at the same times was used in the control group. The surge-blocked group received 0.2 mg/kg s.c. injections of MK801 in 0.2 ml saline at 12.00 h and 15.00 h on day 2, while the surge-activated group received injections saline only at these 2 times.

Tissue preparation

Shortly before slicing, the brains were allowed to equilibrate in the cryostat chamber at −20 °C. Coronal brain slices (20 μm) were cut with a cryostat, thaw-mounted onto polylysine-coated slides (Fischer Scientific, Fair Lawn, NJ, USA), and stored in air-tight boxes at −80 °C until needed. Tissue was collected according to the rat atlas of Paxinos and Watson (32). beginning rostrally at the genu of the corpus calossum and continuing caudally 60 μm beyond the decussation of the anterior commissure. The tissue slices were collected on 4 sets of slides, placing every fourth slice into a given set.

Riboprobe preparation

^{35}S-labeled galanin cRNA probe. The plasmid vector Bluescript containing a cDNA complimentary to rat galanin mRNA (33) was provided by Dr M. Vrontakis (University of Manitoba, Winnipeg, Canada). The plasmid consisted of a 680 bp segment of rat galanin cDNA inserted into the EcoR1 site of Bluescript (Stratagene, San Diego, CA, USA). Hind III was used to linearize the cDNA, and the ^{35}S-labeled antisense riboprobe was generated by use of T7 RNA polymerase. The galanin probe transcription reactions had 25% of 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.

Digoxigenin-labeled GnRH cRNA probe. A 462-base pair digoxigenin-labeled cRNA probe complementary to rat GnRH mRNA was used. The original plasmid containing the GnRH insert (34) was generously provided by Dr A. Mason (Genentech, South San Francisco, CA, USA). The probe was synthesized *in vitro* from linearized DNA with 400 μM digoxigenin-11 uridine-5′-triphosphate (dig-11-UTP, Boehringer Mannheim, Indianapolis, IN, USA), 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 G-50 Sephadex column (Boehringer Mannheim, Indianapolis, IN, USA). The purified probe was diluted 1:40 in hybridization buffer for double-label *in situ* hybridization. This concentration had been determined by a test *in situ* hybridization assay to yield optimal binding conditions. Both probes were heat-denatured before they were added to the final hybridization buffer.

^{35}S-labeled GnRH cRNA probe. This riboprobe was transcribed from the same plasmid used for the synthesis of the digoxigenin-labeled GnRH probe. The antisense riboprobe was transcribed *in vitro* under the control of the SP6 polymerase 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.

The control experiments used to validate the integrity, binding kinetics, and specificity of the digoxigenin-labeled and ^{35}S-labeled galanin probes have been previously described (5, 34). The specificity of the riboprobes was tested by application of sense probes, competitive administration of an excess of unlabeled probes with labeled probes, and pretreatment of tissue with RNAse.

In situ *hybridization*

The method used for double-label *in situ* hybridization has been published previously (5, 35). In brief, 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 × salt-sodium citrate solution = 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 with increasing stringency, including a wash at 65 °C in 0.1 × SSC. For single-label *in situ* hybridization assays, the tissue was dehydrated in ethanol, dipped in emulsion, and exposed for 6 days. These slides were counterstained with cresyl violet to help in identifying cells for final analysis (34).

For double-label assays, the slides were placed in 2 × SSC plus 0.05% Triton X-100 containing 2% normal sheep serum (NSS) for 1 h. They were washed in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated for 4 h at 37 °C with anti-digoxigenin antibody fragments conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA) diluted 1 : 1000 in buffer 1 containing 1% NSS 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. After being dried overnight, the slides were dipped in a 3% solution of parlodion (Fisher Scientific, Fair Lawn, NJ, USA) in isoamyl acetate, to prevent chemographic artifacts in the autoradiographic steps. The slides were air-dried, dipped in Kodak NTB-2 photographic emulsion and then stored in light-tight boxes at 4 °C. The slides were allowed to expose for 10 days, then developed and cover-slipped.

Semiquantitative analysis of cellular mRNA

Slides were assigned a 3 letter code, and then read in random order with an automated image processing system by an operator unaware of the animal's experimental group. We determined the number of silver grains per cell using a grain counting program as previously described (5). This system consisted of a Pixel-Grabber video acquisition board (Perceptics Corp, Knoxville, TN, USA) attached to a Macintosh II*fx* computer. Video images were obtained by a Dage model 65 camera (Dage-MTI, Inc., Michigan City, IN, USA) linked to a Zeiss Axioskop (Zeiss, New York, NY, USA) equipped with a 40 × objective and a 100 watt mercury-vapor epi-illumination light source with a polarizing cube.

The number of grains per cell, representing the relative galanin content in GnRH neurons, were counted in twenty-one sections per brain. These sections were equally spaced throughout the diagonal band of Broca (DBB) and preoptic area (POA), with the most caudal slice at the level of the suprachiasmatic nucleus and the rostral aspect of the lateral hypothalamus. Single-labeled GnRH mRNA-expressing neurons were identified by a discrete grain-cluster associated with a cresyl violet stained nucleus. In double-labeled sections, GnRH cells were identified under brightfield illumination by the presence of a dark purple-stained cell body. The silver grains overlying these cells were analyzed under darkfield illumination by the image processor. Although the operator could subjectively estimate the number of cells that appeared to be double-labeled, performing the grain count analysis for galanin mRNA signal in *all* GnRH neurons provided a more objective measure, because this avoided the subjective decision of whether a particular cell was single- or double-labeled. Therefore, for the double-labeled assays, we counted silver grains over *all* identifiable digoxigenin-labeled GnRH cells and recorded the anatomical location of each of these cells. Similarly, grains over *all* GnRH neurons in the single-labeled assay were counted, to avoid potentially biased decisions. The number of grains per cell is referred to here as the relative mRNA signal level.

Serum LH determinations

Serum LH levels were measured with a RP-3 standard by a double-antibody radioimmunoassay with reagents obtained from the National Hormone and Pituitary Program. The intra- and inter-assay coefficients of variation were 6.4% and 10.2%, respectively.

Statistical analysis

For all experiments, 'n' refers to the number of experimental animals within a group, and this was the 'n' used in the statistical analyses. For estimating cellular levels of GnRH mRNA or galanin mRNA, the mean number of grains per cell from individual animals were used to calculate the mean ± SEM for each group. Differences between groups were assessed by analysis of variance (ANOVA). When the ANOVA indicated a significant difference between groups within an experiment, Fisher's PLSD test was used to identify significant differences between groups. The rejection level for statistical tests was set at alpha = 0.05.

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