Rapid photoperiod-induced increase in detectable GnRH mRNA-containing cells in Siberian hamster

TARJA PORKKA-HEISKANEN, NAHERIN KHOSHABA, KATHRYN SCARBROUGH, JANICE H. URBAN, MARTHA H. VITATERNA, JON E. LEVINE, FRED W. TUREK, AND TERESA H. HORTON

Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208

Siberian hamsters are long day (LD) breeders; both puberty and adult reproductive function are inhibited by exposure to short photoperiods. In male hamsters housed in long photoperiods, puberty begins at ~20 days of age, with the gonadotropic and steroid hormones attaining an adult profile at ~40 days of age (32). Exposure of young hamsters to short photoperiods delays puberty, as evidenced by the inhibition of the pubertal rise in serum gonadotropins and the delay of testicular growth (8, 32). In adult male Siberian hamsters, exposure to short photoperiods leads to a reduction in serum luteinizing hormone and follicle-stimulating hormone (FSH) within 1 wk and is followed by testicular regression within 3–4 wk (8, 33).

After the hamsters have spent several weeks in short days, the pituitary content and serum concentrations of gonadotropins have been significantly suppressed (26). The size of GnRH perikarya in the hamsters is brought about by a change in the hypothalamic-pituitary-gonadal axis. In contrast, hypothalamic content of GnRH has been observed both to increase or remain unchanged in hamsters chronically exposed to short days relative to LD hamsters (9, 14, 22, 24, 25). In Syrian hamsters, the number of GnRH-immunoreactive neurons is observed to increase (19) or to remain unchanged (30) in short photoperiods. Similarly, the size of GnRH perikarya in hamsters chronically exposed to short days increases, suggesting that synthesis is continuing and GnRH is accumulating despite reduced rates of secretion (30). The number of GnRH-immunoreactive neurons in adult Siberian hamsters is unchanged after chronic exposure to short photoperiods, again suggesting that synthesis is not suppressed in short photoperiods (30, 31). However, when puberty is inhibited by chronic exposure to short photoperiods or melatonin in Siberian hamsters, the number of GnRH-immunoreactive cells decreases (2). Finally, the amount of mRNA encoding GnRH does not differ among Syrian hamsters chronically exposed to long or short photoperiods (18). Given that the cellular content of a peptide reflects the combined contributions of synthesis and secretion, this accumulated evidence has led to the hypothesis that suppression of the hypothalamic-gonadal axis during chronic exposure to short photoperiods is brought about by a change in the pattern of GnRH secretion and not simply by the inhibition of synthesis. Thus the mechanism responsible for the continued suppression of the hypothalamic-pituitary-gonadal axis during chronic exposure to short photoperiods may be mediated primarily by factors influencing the secretory activity of GnRH neurons.

In contrast to the stable endocrine states attained after chronic exposure to long or short photoperiods, an acute change in photoperiod induces a rapid change in the endocrine state of hamsters. In Siberian hamsters, serum FSH increases significantly within 3–5 days of exposure to long days (21, 27). Increases in FSH secretion are GnRH dependent; administration of a GnRH antagonist attenuates LD-induced FSH secretion and gonadal growth (28). In Syrian hamsters, transfer to long days provokes a decrease in hypothalamic content of GnRH within 24 h of the change, suggesting that stored GnRH is released rapidly after photostimulation (24). These observations suggest that acute photostimulation rapidly activates the hypothalamic-gonadal axis.

Our long-term objective is to elucidate the neuroendocrine mechanism by which an acute change in photoperiod influences the GnRH neuronal system and evokes the transition from the inactive to the reproductively active state. To date, increased FSH secretion occurring 3–5 days after photostimulation is the earliest marker
control animals were not returned to long days but were maintained in 16:8-h light-dark. After 3 wk in short photoperiod (i.e., at 10 wk of age), hamsters were transferred back to 16:8-h light-dark (0700) or 6:18-h light-dark (0900) of their previous photoperiod. All transfers to 16:8-h light-dark were effected so that the onset of light (lights on) of the first day of short day (SD) exposure, hamsters were transferred back to 6:18-h light-dark (0900) at 6 wk of age. There were no significant differences in the parameters measured at these ages, and these results are included in the main text. Experiment being a complete long day. Hamsters euthanized after chronic exposure to long or short days. In this experiment, data were collected in two replicates. SD control hamsters were euthanized 2 h after lights on in either 16:8-h light-dark (0700) or 6:18-h light-dark (0900) of their respective photoperiod. Details of this experiment follow.

Fig. 4. All animals were euthanized using carbon dioxide.

MATERIALS AND METHODS

Creases in FSH section.

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laboratory mouse GCG Package accession no. M14872) and human (1) and the partial sequence available for the domestic sheep (GCG Package accession no. U02517) (Fig. 1). When discrepancies between the sequences for the rat and mouse were found, the sheep and human sequences were used for comparison to estimate the most evolutionarily conservative sequence. The oligonucleotides are fundamentally derived from the rat sequence; only a few bases were changed to correct for differences between the rat and mouse sequences. An example of the hybridization using this cocktail is shown in Fig. 2. The oligonucleotides bound only to cells in regions of the brain where GnRH neurons are found.

Tissue preparation and in situ hybridization procedures. Frozen tissue was cut in 20-µm sections from the anterior hypothalamus through the diagonal band of Broca. Sections were collected at 60-µm intervals.

The hybridization protocol has been previously described in detail (15). Briefly, the sections were fixed for 5 min using 4% paraformaldehyde at 4°C, acetylated for 10 min in 0.1% triethanolamine containing 0.25% acetic anhydride, dehydrated with graded alcohols, and delipidated for 5 min in chloroform and 95% ethanol.

The oligonucleotide(s) were labeled at the 3' end using either [35S]dATP (experiment 1) or [33P]dATP (experiment 2) (DuPont-NEN, Boston, MA). The labeled oligonucleotides were separated from unincorporated nucleotides using NENsorb 20 Nucleic Acid Purification Cartridges (DuPont-NEN) and mixed with 1% tRNA as previously described (15). After heating at 80°C for 3 min and cooling on ice, the solution was mixed with the hybridization mixture (10% dextran sulfate, 0.3 M NaCl, 10 mM tris(hydroxymethyl)aminomethane, 1 mM EDTA, 1× Denhardt’s solution, and 10 mM dithiothreitol). The final concentration of oligonucleotide in experiment 1 was 3 pmol/ml. The four oligonucleotides used in experiment 2 were combined in solution and labeled as a cocktail. The final concentration of each oligonucleotide in the cocktail was also 3 pmol/ml, yielding a total mass for the four oligonucleotides of 12 pmol/ml. The specific activity of the [35S]labeled probes for the two assays run in experiment 1, were 7,700 and 8,360 Ci/mmol. The specific activity of each [33P]labeled oligonucleotide used in experiment 2, calculated from the activity of the four-oligonucleotide cocktail, was 3,806 Ci/mmol.

Sections were covered with the hybridization mixture, incubated overnight at 31°C, and washed in 1× saline sodium citrate (1.5 M NaCl, 0.015 M sodium citrate, pH 7.0) four times for 15 min at 51°C and two times for 1 h at room temperature. After dehybridization through graded alcohols, the slides were dipped in photographic emulsion and were exposed for 2 wk ([35S]dATP-labeled oligonucleotides) or 11 days ([33P]dATP-labeled oligonucleotides). After development, the slides were counterstained using either 0.1% cresyl violet (experiment 1) or 0.1% toluidine blue (experiment 2).

In preliminary experiments, the number of cells identified using the in situ hybridization procedures was considerably less than the number of GnRH cells reported in studies using immunocytochemistry (34). Because of this disparity, the in situ hybridization procedure was tested using several different assay conditions to improve the sensitivity of the procedure. These modifications included changing the temperature at which the hybridization was carried out, changing the concentration of formamide in the hybridization buffer, and changing the stringency of the washes by altering the wash temperatures. The assay conditions selected represent the optimum conditions as determined in our laboratory.

Analysis of hybridization results. No brain atlas exists for the Siberian hamster; however, the regions examined correspond to the coordinates bregma −1.80 mm through +0.70 mm in the rat brain (13). This region contains the majority of GnRH cells as identified by immunohistochemistry in this species (2). Sections were compared with the rat atlas to ensure that equivalent regions were examined in all hamsters. The separation of sections by 60-µm intervals corresponds to one-third of the total tissue in the region examined; thus the results are reported as the relative number of cells per one-third brain.

It has been found in previous studies in the rat (15) and in preliminary analysis of the current samples that, in our laboratory, changes in cell number are an equal or more-sensitive indicator of the presence of GnRH mRNA than cell brightness (corresponding to the number of silver grains). Therefore, cell number was used as the indicator of GnRH mRNA content in the present studies. Dark-field and bright-field microscopy were used to identify labeled cells; the observation of a silver grain cluster over a stained nucleus was taken to indicate the presence of a GnRH mRNA-containing cell.

The tissues from the two replicates of experiment 1 were assayed separately. Each assay contained tissue from LD and SD control groups collected for the respective replicate. There was a distinct difference in the sensitivity of the two assays. For example, the average numbers of cells in the two assays were 3.6 and 23.2 for the LD controls and 8.4 and 39.2 for the SD controls. Although the pattern of differences was the same, the differences in raw cell numbers distort the overall picture of the results; therefore, all results are reported in terms of the normalized cell distributions. Data from tissues within each assay were normalized using the mean and

Fig. 2. Example of in situ hybridization using oligonucleotide cocktail described in materials and methods. Tissue is from a short day-to-long day (SD>LD) animal in experiment 2. A: cluster of four cells in diagonal band (×10). White arrows indicate cells. B: 1 of 4 cells from cluster in A (×50).
where \( z \) is the normalized value, \( x \) is cell number for an individual animal, \( m \) is average number of cells for SD controls in the same assay, and \( \sigma \) is variance of cell number for SD controls in the same assay. This manipulation converts data to the standard normal distribution, which has a mean of 0 and a variance of 1 (12). If groups do not differ from their control, they will have a mean of zero. A difference among groups can be detected as displacement from zero.

Statistical analysis. Because two assays were required to accommodate the two replicates in experiment 1, the values are normalized to the SD control animals assayed in the same assay. The results of experiment 2 are presented both in terms of the absolute number of cells and the normalized distribution of cells to permit comparison to experiment 1. Before statistical analysis, data were tested for normality using the modified Levene equal variance test (7). Log transformation was used to normalize data when necessary. The use of log-transformed data is reported for the specific data in RESULTS. Groups were subsequently compared using one-way analysis of variance (ANOVA). When log transformation was used, the results of ANOVAs using transformed and untransformed data were compared. In all cases, the conclusions derived from the analyses were the same. If significant differences were revealed by the ANOVA, post hoc comparisons were made using Tukey's honest significant difference (HSD) test in experiment 1 (7). Because of the smaller number of groups and smaller sample sizes, Fisher's least-significant difference (LSD) test was used to make post hoc comparisons in experiment 2 (7). Groups were judged to be significantly different if \( P < 0.05 \).

RESULTS

Experiment 1. A shift in the normalized distribution of cells, indicative of an increase in the number of cells containing GnRH mRNA as determined by in situ hybridization, was detected within the first 2 days of photostimulation (Fig. 3). Significant treatment effects were observed within each replicate. Similar patterns of change were seen in the raw cell numbers from the two replicates, despite the fact that the absolute numbers of cells detected by the two assays were very different (e.g., average number of cells in SD controls; replicate 1 = 8.4, replicate 2 = 39.3). This shift in the distribution of detectable cells preceded increases in serum FSH concentrations and body and paired testes weights by several days.

The number of detectable GnRH mRNA-containing cells per one-third brain varied from 5 to 68 per animal. The maximum number of cells was observed on the second day of photostimulation in replicate 1 (\( x = 24.8 \)) and on the first day in replicate 2 (\( x = 50 \)). When the variance of the SD controls in the same assay according to the following equation

\[
z = \frac{(x - m)}{\sigma}
\]

was used to normalize data when necessary. The use of log-transformed data is reported for the specific data in RESULTS. Groups were subsequently compared using one-way analysis of variance (ANOVA). When log transformation was used, the results of ANOVAs using transformed and untransformed data were compared. In all cases, the conclusions derived from the analyses were the same. If significant differences were revealed by the ANOVA, post hoc comparisons were made using Tukey's honest significant difference (HSD) test in experiment 1 (7). Because of the smaller number of groups and smaller sample sizes, Fisher's least-significant difference (LSD) test was used to make post hoc comparisons in experiment 2 (7). Groups were judged to be significantly different if \( P < 0.05 \).

Fig. 3. Results of experiment 1. First 2 bars of each graph present data for SD and LD control animals, respectively. Remaining bars are for animals transferred from short to long photoperiod for indicated number of days before euthanasia. A: normalized distributions of GnRH mRNA-containing cells. Cells were counted per 1/3 brain. B: body weights (means ± SE) of juvenile Siberian hamsters. C: paired testes weights (means ± SE). D: serum follicle-stimulating hormone (FSH) concentrations (means ± SE). SD > SD (\( n = 10 \) except FSH, where \( n = 5 \)), animals maintained under short day; LD > LD (\( n = 10 \) except FSH, where \( n = 5 \)), animals maintained under long day; SD > LD, animals transferred from short to long days after 3-wk exposure to short days. SD > LD animals were kept under long days for either 1 (+1, \( n = 17 \) except FSH, where \( n = 3 \)), 2 (+2, \( n = 24 \) except FSH, where \( n = 6 \)), 3 (+3, \( n = 8 \)), 4 (+4, \( n = 6 \)), 10 (+10, \( n = 5 \)) or 21 (+21, \( n = 7 \)) days. 16:8D, 16 h light:8 h dark. *Differs from LD > LD; †differs from SD > SD; \( P < 0.05 \).
A 1-way ANOVA of normalized data, degrees of freedom (df) = 7(71), F = 2.82, P = 0.01; Tukey's HSD, P < 0.05. The shift in the distribution of cells on day 1 was not significant when the data from the two replicates were combined. Sixteen of twenty-five animals (64%) on the second morning of LD exposure had normalized cell values >0, indicative of an increase in cell number. On the first morning of LD exposure, 9 of 17 animals (53%) had positively shifted values. No other groups showed similar positively shifted distributions.

LD control animals had heavier body weights than either SD controls or animals that had been transferred to long days for 1–4 days (Fig. 3B). Transferred animals attained body weights equivalent to those of the LD control group by 10 days after the transfer to long days. Twenty-one days after the transfer to long days, body weight was higher than in any other group [1-way ANOVA, df = 7(80), F = 27.52, P < 0.001; Tukey's HSD, P < 0.05]. Paired testes weights were heavier in the LD control and in transferred hamsters 21 days after the transfer than in other groups (Fig. 3C) [1-way ANOVA, df = 7(80), F = 205.17, P = 0.001. Tukey's HSD, P < 0.05]. Paired testes weights of the SD control group were very small (average 21.2 ± 1.7 mg). Paired testes weights of animals transferred to long photoperiod for 1–4 days were slightly larger but still small (averages range from 30.2 to 34.3 mg). The paired testes weights of the SD control animals were significantly smaller than the paired testes weights of males exposed to long photoperiod for 2 (average = 32.9 ± 2.3 mg) or 3 (average = 34.3 ± 2.3 mg) days but did not differ from those of animals exposed for 1 (average = 30.2 ± 3.8 mg) or 4 (average = 30.3 ± 3.6 mg) days. Serum FSH concentrations were highest 10 and 21 days after transfer to long photoperiod (Fig. 3D) [1-way ANOVA, df = 7(37), F = 6.75, P < 0.001; Tukey's HSD, P < 0.05].

Experiment 2: The number of detectable cells per one-third brain ranged from 14 to 101 per animal. Hamsters transferred from short to long days had significantly more labeled cells than did either the long or SD control animals (Fig. 4A) [1-way ANOVA, df = 2(17), F = 4.03, P = 0.04; Fisher's LSD, P < 0.05]. To facilitate comparison of the results of experiment 2 with those of experiment 1, the number of cells was normalized to the SD control group (Fig. 4B). When compared using the standard normal distribution, the distribution of cells in hamsters transferred from short to long days also was shifted significantly, indicating an increase in the number of labeled cells (Fig. 4B) [1-way ANOVA, df = 2(17), F = 4.03, P = 0.04. Fisher's LSD, P < 0.05]. The largest numbers of cells were located in the diagonal band of Broca, the medial preoptic nucleus, and the medial preoptic area (Fig. 4C). The hamsters that were transferred from short to long days tended to have more detectable cells in the diagonal band of Broca, medial preoptic nucleus and preoptic area, and lateral hypothalamic area than did hamsters in either...
of the control groups. However, these regional differences in distribution were not statistically significant.

Exposure to short photoperiod resulted in lower body weights (means for SD exposed animals were 29 and 32 g vs. 39 g for LD exposed animals) and paired testes weights (means of 52 and 60 mg/pair vs. 736 mg/pair). Animals were examined only on day 2 after the transfer to long photoperiod in this experiment; this was insufficient time to detect any changes in body weight or paired testes weights.

**DISCUSSION**

The first long day to which the experimental hamsters were exposed began with an advance in the time at which lights came on in the morning (lights on at 0500 instead of 0900 CST). The present results show that the number of cells expressing detectable levels of GnRH mRNA begins to increase within a few hours of the initial lengthening of the photoperiod. In hamsters that were placed in short photoperiod at 21 days of age (experiment 1) to delay the progress of sexual maturation, the positive shift in the distribution of detectable cells, indicative of an increase in the number of detectable cells, reached statistical significance by the morning of the second long day then disappeared by the morning of the third day. The transient nature of the increase may explain why previous studies have not detected a change in GnRH cell number after photostimulation or treatment with melatonin (6). Sexually mature hamsters that were placed in short photoperiod to cause gonadal regression (experiment 2) also show a near doubling of the number of detectable GnRH mRNA-containing neurons by the morning of the second day of photostimulation, the only time point examined. The present data demonstrate that activation of GnRH neurons is an early step in the photostimulation of the hypothalamic-pituitary-gonadal axis in Siberian hamsters in which the process of gonadal maturation has been delayed (experiment 1) or reversed (experiment 2) by exposure to short photoperiods.

There is a common perception that the hypothalamic-pituitary-gonadal axis of Siberian hamsters in which sexual maturation has been delayed by short days responds more rapidly to a change in photoperiod than does that of animals that have undergone gonadal regression after the onset of sexual maturity. The current data suggest that the neuroendocrine system of hamsters in which puberty had been inhibited and those in which mature gonads had undergone regression responded equally rapidly to the increase in photoperiod. It is worth noting that serum FSH concentrations have been observed to increase as early as 3 days after photostimulation in Siberian hamsters in which gonadal development has been delayed or reversed by exposure to short photoperiods (21, 27).

The transient increase in the number of labeled cells may represent the activation of the hypothalamic-pituitary-gonadal axis and accompanying changes in the relationship between gene transcription, synthesis, and secretion of GnRH after photostimulation. Whether the increase in the number of detectable cells is a result of increased GnRH gene transcription or increases in mRNA stability remains to be determined in future experiments. The transient increase in cell number coding for GnRH mRNA after photostimulation suggests that the change in photoperiod may drive a transient increase in GnRH synthetic capacity in a population of cells that had previously expressed GnRH mRNA at undetectable levels.

In experiment 1, in which the hamsters entered the experiment at 21 days of age and were killed between 42 and 63 days of age, we estimated that the entire brain would contain between 15 and 204 GnRH cells. In experiment 2, in which the animals entered the experiment at 42 days of age and were killed at 72 days of age, the total number of labeled cells ranged from 42 to 303 per brain. The number of cells detected by in situ hybridization was highly variable and low relative to the numbers detected in this species by immunocytochemistry (34). Immunocytochemical studies indicate that the number of cells containing GnRH increases between 15 and 25 days of age from ~200 cells to ~350 cells; the number of cells is known to remain constant through 40 days of age (34). It seems plausible that the total cell number measured by immunohistochemistry reveals all or a large proportion of GnRH cells, because the amplification inherent in translation and storage of the neuropeptide aids in its detection by immunohistochemistry. In experiment 2, the complexity of the hybridization probe was increased fourfold by use of a cocktail of oligonucleotides, and 33P was used instead of 35S to enhance the sensitivity of the assay. There was a slight increase in the number of cells detected in experiment 2; however, the increase did not account for the differences in cell numbers observed in this study and immunocytochemical studies.

A discrepancy between the number of cells detected with in situ hybridization and immunocytochemistry has also been noted in the Syrian hamster (18). The oligonucleotide used to label the Syrian hamster tissue was derived from the human GnRH gene. The high degree of homology among the Syrian hamster gene and that of a wide number of species (human, rat, and mouse) suggests that it is unlikely that the sequence of the Syrian hamster gene will differ significantly (10). Until the gene for Siberian hamster GnRH is cloned and sequenced, it will not be possible to rule out the possibility that sequence mismatches are responsible for the low detection rate. It is also necessary to consider the possibility that neurons may be able to make adequate amounts of peptide from very little mRNA, resulting in the lower total number and greater variability in number of cells detected by in situ hybridization. Ronchi et al. (18) suggest that the differences in cell numbers detected by in situ hybridization and immunocytochemistry may also be the result of factors such as section thickness, assay sensitivity, or differential stability of mRNA and peptides.

Research reports from the late 1970s document an increase in gonadotropin secretion in an avian species, the Japanese quail, during the first day of long photoperiod exposure (4, 5). Increases in gonadotropin secre-
tion in mammals appear to be more sluggish; most reports detect the earliest rise in gonadotropin secretion 3 days after transfer to long photoperiod (20, 21, 26, 27). A single report on Syrian hamsters indicated that transfer to long days provoked an increase in FSH secretion and a decrease in hypothalamic GnRH content, indicative of GnRH release, within 24 h of photostimulation (24). The present data indicated a significant increase in FSH levels by day 10 relative to the SD controls. Although this delay is longer than those reported previously, it is consistent with the observation that the secretion of FSH is delayed for a few days after the onset of photostimulation. The failure to detect an earlier rise may be because of the fact that serum for the measurement of FSH was collected only in the second replicate of the experiment. The serum hormone levels were showing evidence of an increase by day 3 (from 2.43 ± 0.15 ng/ml in SD controls to 3.42 ± 0.93 ng/ml on day 3); however, the sample sizes may have been too small to detect a difference among SD control animals and animals exposed to long photoperiods for only 3 or 4 days. This may also explain why the difference between the LD and SD controls (3.85 ± 0.62 vs. 2.43 ± 0.15 ng/ml) was not statistically significant (Fig. 3D).

A clear and statistically significantly increase in testes size was seen after 10 days of exposure to long photoperiod (Fig. 3C). Significant differences in average testes weights were observed among SD controls and animals exposed to long days for 2 and 3 days (Fig. 3C); however, the small differences and the absence of a difference on day 4 suggests that these differences do not result from the effects of photostimulation. When the data were reanalyzed using an analysis of covariance to correct for the effect of body weight, there were no differences among the SD control groups and the animals exposed to long photoperiod for 1–4 days (data not shown). These results suggest that there is no biologically significant increase in testes weight within the first 4 days of photostimulation.

The present data support the hypothesis that the activity of the GnRH neuronal system changes rapidly after photostimulation. These data are the first to demonstrate that a single long day can stimulate a change in molecular events associated with GnRH synthesis in a mammalian species. We conclude that the increase in the number detectable GnRH mRNA-containing cells is an early sign of the activation of the gonadal axis in photostimulated Siberian hamsters. It is possible that an increase in GnRH cell activity, e.g., increased GnRH synthesis and release, is reflected by the increase in the detectable number of cells containing GnRH mRNA. With the use of this early marker for the activation of the hypothalamic-pituitary-gonadal axis, future studies can attempt to identify the neuroendocrine cascade connecting the exposure to lengthened photoperiod to the activation of the hypothalamic-pituitary-gonadal axis.

Perspectives

Seasonal changes in reproductive condition represent the physiological conversion of an animal from one relatively stable state (i.e., reproductively inactive) to another (i.e., reproductively active). These relatively stable physiological states are linked by transition periods, during which the animal undergoes major endocrine changes. Our understanding of the mechanism by which photoperiod controls physiological function, reproductive function in particular, will depend on recognizing the physiological differences between the stable and transitional periods. The cascade of neuroendocrine events by which transfer to a long photoperiod activates the hypothalamic-gonadal axis is unknown. Part of the difficulty of elucidating the cellular events in this cascade has been the lengthy delay between the application of a stimulus (change in photoperiod) and an observable response (change in serum hormone concentrations or onset of gonadal growth). Thus events responsible for the transition may be missed by examining animals that have already moved to the next physiological state. The development of a model system in which there is an endpoint that can be observed soon after the change in photoperiod should help elucidate the neuroendocrine cascade responsible for the transition of the animal from the reproductively inactive to the reproductively active state.

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Current address for T. Porkka-Heiskanen: Institute of Biomedicine, University of Helsinki, Helsinki, Finland.

Address for reprint requests: T. H. Horton, Dept. of Neurobiology and Physiology, Northwestern Univ., 2153 N. Campus Dr., Evanston, IL 60208.

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