**Physiology.** In the article “Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrus surge of luteinizing hormone” by Wen-Sen Lee, M. Susan Smith, and Gloria E. Hoffman, which appeared in number 13, July 1990, of *Proc. Natl. Acad. Sci. USA* (87, 5163–5167), Figs. 3 and 4 were interchanged due to a printer’s error that occurred after the authors had checked the page proofs. The figures and their legends are shown below.

**Evolution.** In the article “A clonal theory of parasitic protozoa: The population structures of Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas, and Trypanosoma and their medical and taxonomical consequences” by Michel Tibayrenc, Finn Kjellberg, and Francisco J. Ayala, which appeared in number 7, April 1990, of *Proc. Natl. Acad. Sci. USA* (87, 2414–2418), the authors request that the following correction be noted. The list of references in the second column of Table 2, on p. 2416, should read, from top to bottom: 32, 33, 35, 25, 26, 27, 28, 30, 30, 8, 8, 8, 19, and 20.

**Neurobiology.** In the article “Number of ‘blobs’ in the primary visual cortex of neonatal and adult monkeys” by Dale Purves and Anthony-Samuel LaMantia, which appeared in number 15, August 1990, of *Proc. Natl. Acad. Sci. USA* (87, 5764–5767), the following correction should be noted. On p. 5766, the sentence beginning on line 21 should read as follows. Apparently, the number of neural circuits increases in this part of the growing brain, rather than remaining stable (or declining because of selective or regressive processes).
Luteinizing hormone-releasing hormone neurons express Fos protein during the proestrous surge of luteinizing hormone
(oncogene product/estrous cycle/immunocytochemistry/hypothalamus/preoptic area)

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ABSTRACT The ability of luteinizing hormone-releasing hormone (LHRH) neurons to express the oncogene c-fos was examined during the estrous cycle in rats. The immunocytochemical localization of the c-fos-encoded antigen, Fos, was coupled with the immunocytochemical localization of LHRH. LHRH neurons showed no Fos immunoreactivity during diestrus-1, diestrus-2, estrus, or the morning of proestrus. However, Fos was expressed in LHRH neurons from 1600 to 2200 hours during proestrus. The timing of onset of Fos expression in LHRH neurons during proestrus suggests a strong correlation with increased LH secretion. Pentobarbital, which blocks the preovulatory LH surge, blocked Fos expression in LHRH neurons, but the LHRH neurons expressed Fos on the following afternoon at the time of the expected delayed LH surge. Not all LHRH neurons expressed Fos during the LH surge. Approximately half of the LHRH neurons were activated in the preoptic area and anterior hypothalamus; more anteriorly positioned LHRH neurons did not express Fos, resulting in an overall stimulation of 40% of the LHRH neurons. These data provide direct evidence that stimulation of LHRH neurons during proestrus takes place at the LH surge, and identify the specific population of LHRH neurons which are activated.

The central nervous system is essential for the regulation of the pituitary–gonadal axis. Although it was first thought that the central nervous system directly innervated the anterior pituitary, later studies revealed that a neuroendocrine hormone system, the luteinizing hormone-releasing hormone (LHRH) system, provided the final common pathway for regulation of gonadotropin secretion. The importance of LHRH for the induction of the preovulatory LH surge is unquestioned. Systemic injection of an antiserum against LHRH completely blocks LH release, including the preovulatory surge of LH (1, 2). Yet despite the vast literature addressing the regulation of LH release, we still know little about the cellular activity of individual LHRH neurons or the mechanism of their activation.

LHRH release in the rat changes during the estrous cycle. LHRH output, estimated by sampling of hypophysial portal blood (3) or by push–pull perfusion (4, 5), is increased at the time of the LH surge but not at other times during the estrous cycle. While it is tempting to associate increased output of LHRH during the estrous cycle with increased stimulation of LHRH cells, direct evidence is lacking. The ability of various neurotransmitters to effect LHRH release from the median eminence or from synaptosome preparations (6–8), both of which lack LHRH soma, raises the question of the site of LHRH activation during an LH surge.

Direct evidence for activation of LHRH neurons at their perikarya at the time of an LH surge has come from the application of the molecular biology of the c-fos oncogene to the study of LHRH neurons. The c-fos product, Fos, is expressed in a number of neuron systems after stimulation. Since most neurons express little or no Fos until they are stimulated, the presence of the oncogene product has been used as a marker for activity. Fos concentrates within the cell’s nucleus and is stained with immunocytochemical techniques, providing an anatomical marker of stimulation compatible with techniques for phenotype identity. Seizure activity (9, 10), electrical stimulation (11–13), noxious stimuli (14), photostimulation (15), osmotic stress (16, 17), and selected neurotransmitter agonists [cholecystokinin (18); nicotine (15); excitatory amino acids (19)] all have elicited Fos expression in appropriate neuron systems. In the immature female rat, induction of an LH surge by exogenous steroid administration induces Fos expression in LHRH neurons (20). The dependence of the natural LH surge on gonadal steroids (21) strongly suggests that LHRH neurons would similarly express Fos at the time of the LH surge on the day of proestrus. Thus, in the present study, we used Fos expression to identify the neurons activated during the preovulatory LH surge as a means of defining the population of LHRH neurons that stimulate the LH surge.

MATERIALS AND METHODS

Animals. Adult female Sprague–Dawley rats were maintained on a 12 hr light/12 hr dark schedule (lights on 0600–1800) and given free access to food and water. All rats showed two or more consecutive 4-day estrous cycles (by daily vaginal smear) before sacrifice. At designated times during the estrous cycle (Table 1) representing defined stages of LH secretion, rats were killed by an overdose of pentobarbital and prepared for immunocytochemical localization of Fos and LHRH (20).

Pentobarbital prevents the LH surge when administered prior to the anticipated LH surge on proestrus (22). On the day following pentobarbital anesthesia, animals will manifest a delayed LH surge at the same time of day that the normal LH surge would have appeared. To study the effect of pentobarbital on Fos expression in LHRH neurons, seven animals were given pentobarbital (75 mg/kg, i.p.) at 1230 on proestrus to block the preovulatory LH surge. Four of these animals were killed between 1600 and 1630 on the day of pentobarbital treatment and the three remaining animals were killed between 1600 and 1630 on the following day, at the time of the anticipated delayed LH surge.

Immunocytochemical Procedures. Rats were killed by an overdose of pentobarbital (100 mg/kg, i.p.) and perfused transcardially, first with normal saline (0.9% NaCl) containing 2% sodium nitrite and then with 2.5% acrolein (EM grade, Polysciences) in phosphate-buffered 4% paraformaldehyde.
(pH 7.0). After fixation for 3–10 min, each animal was again perfused with saline to flush any residual acrolein from the vasculature. Any acrolein present in the effluent from the animal was neutralized with aqueous 10% sodium bisulfite. The brain was removed, blocked, and immersed in 25% sucrose. With a freezing microtome, each brain was cut into 12 series of 25-μm sections. The sections were stored in tissue culture dishes containing cryoprotectant (23) until staining for Fos and LHRH was initiated.

Immunocytochemistry of Fos and LHRH was performed as described (20). A dual immunoperoxidase protocol was used to obtain permanent records of the response (since neither chromogen fades). An immunoperoxidase/immunofluorescence method (24) was used to obtain photomicrographs for publication, to obviate the need for color plates. Streptavidin-Texas Red served as the fluorophore-linked probe.

Specifically, after removal of the sections from cryoprotectant and rinsing in phosphate-buffered saline (PBS), the tissue was treated with a 1% NaBH4 solution (Sigma) for 15–20 min and then rinsed in PBS until bubbling stopped. For both methods, incubation with the anti-Fos (in 0.4% Triton X-100 solution made in PBS) proceeded at 4°C for 48 hr. Sheep anti-c-Fos (amino acids 1–14; lot OA-11-821, Cambridge Research) was used at a concentration of 1:45,000. After rinsing in PBS, the tissue was incubated in biotinylated rabbit anti-sheep IgG (heavy and light chains; Vector Laboratories, 1:600 in PBS with 0.4% Triton X-100) for 1 hr at room temperature, rinsed, and incubated for 1 hr in avidin-biotin complex fluid [4.5 μl per ml of incubation mixture (PBS with 0.4% Triton X-100), Vector Laboratories, “elite” kit]. After rinsing in 0.175 M NaOAc, the Fos antibody-peroxidase complex was stained with a solution of NiSO4 (25 mg/ml), 3,3'-diaminobenzidine (0.2 mg/ml), and H2O2 (0.83 μl of a 3% solution per ml of reaction solution) in aqueous 0.175 M NaOAc. After 40–50 min, the tissue was transferred into acetate solution to stop the reaction, rinsed in PBS, and then incubated in the anti-LHRH for 24 hr at 4°C. Rabbit anti-LHRH (gift of Robert Benoit and Roger Guillemin, Salk Institute, La Jolla, CA) was used at 1:100,000 for the dual peroxidase method or 1:30,000 for the immunofluorescence component of the peroxidase/immunofluorescence method. After rinsing, the tissue was incubated in biotinylated goat anti-rabbit serum (Vector Laboratories, 1:600) for 1 hr at room temperature, rinsed, and incubated with the avidin-biotin complex reagent (as above). Then staining of the LHRH structures in the dual peroxidase method was initiated. The tissue was rinsed once with PBS and then twice with 50 mM Tris buffer (pH 7.2); the attached peroxidase was stained with a mixture of H2O2 (0.83 μl of a 3% solution per ml of reaction solution) and 3,3'-diaminobenzidine (0.2 mg/ml) for 4–10 min. For immunofluorescence visualization of LHRH, the sections were rinsed and incubated in streptavidin-Texas Red (10 μl/ml) for 1 hr at 37°C following incubation with biotinylated anti-rabbit serum (1:600). Following staining with diaminobenzidine or incubation with the streptavidin-Texas Red probe, the tissue was rinsed in saline, and then fixed in 4% paraformaldehyde.

Table 1. Fos expression in LHRH neurons during the estrous cycle

<table>
<thead>
<tr>
<th>Day of sacrifice</th>
<th>Time</th>
<th>n</th>
<th>Fos staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrus-1</td>
<td>0900–1100</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1600–1700</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Diestrus-2</td>
<td>0900–1100</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1600–1700</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Proestrus</td>
<td>0900–1100</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1400–1530</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1530–1700</td>
<td>4</td>
<td>+</td>
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<tr>
<td></td>
<td>1700–1800</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1800–2200</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Estrus</td>
<td>0900–1100</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>1600–1700</td>
<td>3</td>
<td>–</td>
</tr>
</tbody>
</table>
mounted on gelatin/chrom alum-coated glass slides, dehydrated through graded ethanol solutions, cleared in xylenes or Histoclear, and coverslipped with Histomount (VWR Scientific).

The presence of Fos was evident as blue-black reaction product in cell nuclei; LHRH immunoreactivity within the cell cytoplasm was stained either brown (dual peroxidase) or bright red (peroxidase/fluorescence). The pattern of Fos expression among LHRH neurons was plotted with the aid of stage-mounted potentiometers linked to a Macintosh II computer. The spreadsheet in which the cell plots were stored was used to obtain cell counts. Controls for the specificity of the antisera consisted of incubation of tissue in antisera that had been preabsorbed with antigen (10–20 μg of LHRH or 10 μg of Fos per ml of diluted antiserum); this procedure blocked all staining.

RESULTS

Fos Expression in LHRH Neurons During the Estrous Cycle. During proestrus, LHRH neurons expressed Fos at the time of the expected LH surge (Fig. 1) and the Fos immunoreactivity persisted until 2200 but was gone by the following morning (Table 1). At other times of the cycle LHRH neurons were devoid of Fos immunoreactivity (Table 1, Fig. 2). The time of onset of Fos expression was abrupt (between 1500 and 1600). Prior to 1500 on proestrus, none of the LHRH neurons expressed Fos. By 1600, Fos expression in LHRH neurons was present in all animals. At 1800–2200, Fos was still expressed in LHRH cells but the intensity of the Fos staining was attenuated.

Effect of Pentobarbital on Fos Expression in LHRH Neurons. Administration of an anesthetic dose of pentobarbital at 1230 on proestrus completely blocked Fos expression in LHRH neurons on the afternoon of proestrus (Fig. 3), but the LHRH neurons expressed Fos on the following afternoon at the time of the expected delayed LH surge (Fig. 4).

Characterization of Fos Stimulation in LHRH Neurons. As has been described (25), LHRH neurons were organized into a diffuse, scattered population of cells that extends from the medial surface of the olfactory bulb, through the septum and preoptic area into the anterior hypothalamus. Fos expression in LHRH neurons was limited to a subpopulation encompassing at most 40% of the overall LHRH population (Table 2). The Fos-immunoreactive LHRH neurons were most numerous close to the organum vasculosum of the lamina terminalis (OVLT), below the anterior commissure and extending caudalward into the anterior hypothalamus (Fig. 5). Rarely were the LHRH neurons that expressed Fos found above the anterior commissure or rostral to the OVLT. Thus, the LHRH neurons lying within the nervous terminals and septum remained "silent" at the time of an LH surge and could be distinguished from the more caudal LHRH cell population.

Fos Expression in Other Neurons of the Forebrain. In all of the rats examined, irrespective of the stage of the estrous cycle, a small number of neurons contained Fos immunoreactivity in the midline thalamus and paraflocculcoptic cortex as well as scattered in the preoptic area and hypothalamus. This observation indicates that the sensitivity of the staining procedure remained comparable across all of the experiments and that these neuronal populations are not likely to play a selective role in regulating LH secretion. No discrete populations of neurons (other than LHRH cells) in the preoptic area or other regions of the forebrain conspicuously expressed Fos only at the time of the LH surge, suggesting that afferents to the LHRH system lie outside the forebrain or do not express Fos-related antigens with a conserved N terminus when stimulated.

DISCUSSION

Our results show that expression of Fos in LHRH neurons occurs specifically on the afternoon of proestrus and not during other times of the estrous cycle. The absence of Fos in LHRH neurons during other days of the estrous cycle demonstrates that the stress involved with handling the animals and induction of anesthesia did not induce Fos. This
is most likely because these procedures were executed within 15 min of tissue fixation, which is insufficient time for synthesis of Fos (10). The absence of Fos in LHRH neurons except during the afternoon of proestrus does not mean that LHRH neurons are completely inactive during the remainder of the cycle. Previous studies indicate that LHRH-dependent LH pulses occur during other days of the estrous cycle (27). It is possible that Fos is not expressed during basal pulsatile activity of LHRH neurons or that our methods are not sensitive enough to detect very low levels of Fos activity. In either case, the specific expression of Fos demonstrates that LHRH neurons are activated on the afternoon of proestrus in association with the preovulatory LH surge.

To our knowledge, this is the first demonstration that a large population of LHRH cell bodies is activated under physiological conditions. Fos expression in LHRH neurons first appeared at 1600 on the day of proestrus, a time when the preovulatory surge has already begun. For LHRH neurons, a minimum of 45 min is required for Fos protein to be detected after the initiation of a strong stimulus such as electrochemical stimulation (W.-S.L., unpublished observations). Thus, it seems reasonable to speculate that the activation of LHRH neurons on the afternoon of proestrus may have begun at 1500-1530. In hippocampus and cortex, Fos mRNA reaches a peak within 30 min after stimulation, and Fos protein peaks 1-2 hr later (10). Therefore, our data are consistent with a physiological stimulus for induction of Fos that coincides with the rising phase of LH secretion, suggesting that the same signal releases LHRH and induces Fos expression.

In female rats, the proestrous LH surge is preceded by marked increases in LHRH-pulse amplitude and LHRH output that persist for the duration of the LH surge (4). While increases in the afferent stimulation of LHRH neurons at the perikaryon or dendrites could explain these data, many regulators of LHRH release are effective in vitro with preparations containing only nerve terminals (6-8), suggesting that LHRH release may be locally regulated at the terminals. Preliminary data suggest that only one of the two mechanisms induces Fos expression. The excitatory amino acid N-methyl-d-aspartate can stimulate LHRH secretion from median eminence in vitro (28, 29). Intravenous administration of N-methyl-d-aspartate increases LH secretion in female rats but does not induce Fos expression in LHRH neurons, whereas electrochemical stimulation of the preoptic area does. These data suggest that perikaryal activation, but not terminal stimulation, induces Fos expression. Consequently, the expression of Fos during an LH surge appears to reflect stimulation of LHRH neurons initiated at the perikaryon.

One theory of LHRH function is that the LH surge arises from the synchronization of LHRH cell firing (30) and not from increased activity. If this is true, then at all times during the estrous cycle, some LHRH neurons should be highly active and express Fos while others would not. The presence of Fos immunoreactivity in LHRH neurons only on the afternoon of proestrus supports the theory that increased afferent neuronal stimulation of the LHRH neurons, not synchronization of ongoing activity, dictates the LH surge.

The expression of Fos in LHRH neurons not only indicates that activation of LHRH neurons has occurred but defines which LHRH neurons are involved in stimulation of the preovulatory LH surge. Approximately half of the LHRH neurons located below the anterior commissure at the level of the OVLT extending through the preoptic area and more caudally into the anterior hypothalamus were activated during the proestrous LH surge. The activation of a portion of LHRH neurons during the LH surge was anticipated by tract-tracing studies; only 50-70% of LHRH neurons project to the median eminence or other circumventricular organs (31-33). Neuroendocrine LHRH neurons are evenly spread throughout the rostral-caudal extent of the LHRH population including the septal area and rostral forebrain (33). Activation of only a portion of the LHRH cells capable of projecting to the median eminence suggests that stimulatory input does not reach all the LHRH neurons that project to the median eminence.

The pattern of Fos induction in LHRH neurons during the proestrous LH surge was not qualitatively different from that observed after an LH surge induced in immature female rats by exogenous steroid administration (20). Whether 40% of the LHRH neurons are necessary to elicit an LH surge is uncertain. Ensurance of gonadal function by redundancy within the LHRH system is more likely; relatively few LHRH neurons are needed to maintain reproductive function. Following anterior deafferentation, male reproductive function is maintained with only a fraction of the LHRH tracts intact (34). Moreover, lesions and knife cuts that sever the principal tract of LHRH neurons to the median eminence reduce the LH surge but do not block ovulation in female rats (35). Thus

Table 2. Proportion of LHRH neurons stimulated during proestrus

<table>
<thead>
<tr>
<th>Time during proestrus</th>
<th>LHRH cells</th>
<th>LHRH/Fos cells</th>
<th>Estimated total LHRH population</th>
<th>% Fos-stimulated LHRH cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1530-1700</td>
<td>122.3 ± 16.5</td>
<td>30.8 ± 9.5</td>
<td>1128.5 ± 152.4</td>
<td>25.3 ± 6.5</td>
</tr>
<tr>
<td>1700-1800</td>
<td>131.5 ± 12.8</td>
<td>51.5 ± 4.9</td>
<td>1213.8 ± 118.6</td>
<td>40.3 ± 6.0</td>
</tr>
<tr>
<td>1800-2200</td>
<td>103.8 ± 10.2</td>
<td>30.8 ± 11.8</td>
<td>957.7 ± 94.1</td>
<td>31.3 ± 11.9</td>
</tr>
</tbody>
</table>

The data (mean ± SEM) represent cell counts from a 1-in-12 series of sections, obtained from four brains per group. The estimated total LHRH population was obtained by multiplying the number of LHRH cells by 12, and then correcting for split nuclei (26).
it appears that endogenous and exogenous steroid stimulation of LHRH neurons activates a greater number of LHRH neurons than are essential for the LH surge.

Whereas the absence of Fos immunoreactivity within LHRH neurons is not commensurate with the absence of activity, there is little question that Fos should be viewed as a marker for increased activity and the resultant synthetic demand. Fos is one of a number of transcription factors regulating gene expression. Although unable to bind DNA directly, dimerization of Fos with another oncogene product, Jun, permits binding to a specific region of DNA, known as the AP-1 binding site (TGACTCA). The Fos/Jun heterodimer may also bind to AMP-responsive gene elements that regulate neuropeptide synthesis (36). The rat gene that regulates LHRH synthesis has been sequenced (37), and an AP-1 binding site is found close to the 5′ flanking region near the promotor sites. Its presence supports the hypothesis that LHRH synthesis may be one important target of Fos action in the LH system. While the function of Fos is the subject of intense investigation, the use of Fos immunoreactivity as a marker for stimulated neuronal activity (irrespective of the function of Fos) offers promise for characterizing LHRH stimulation and the functional pathways for its activation.

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