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22. Brief exposure to excitatory amino acids was carried out in room air, in an exposure solution of 130 m*M* Na^+, 5.4 m*M* K^+, 0.8 m*M* Mg^{2+}, 1.8 m*M* Ca^{2+}, 130.6 m*M* Cl^-, 20 m*M* Hepes (*p*H 7.4 at 25°C), and 15 m*M* glucose. After 5 min, the exposure solution was washed out and replaced with MEM plus glucose (25 m*M*), and the cultures were returned to the 37°C, CO_2 incubator. The 24-hour exposure to excitatory amino acids was carried out in the incubator in MEM plus 25 m*M* glucose.
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24. In other experiments, 5-min exposure to either 500 μ*M* glutamate or 500 μ*M* NMDA was carried out in the medium used for 24-hour exposures (MEM plus glucose). The medium was warmed to 37°C and gassed with CO_2 to *p*H 7.2; after brief room-air manipulation, cultures were placed in the 37°C incubator for most of the 5-min exposure period. This change in procedure did not affect outcome: 100 μ*M* nifedipine still produced little improvement in neuronal survival.
25. Whole-cell recordings were achieved with patch electrodes as described by O. P. Hamill *et al.*, *Pfluegers Arch.* **391**, 85 (1981). Before an experiment, the culture medium was replaced by a salt solution containing 140 m*M* NaCl, 2.8 m*M* KCl, 1 m*M* $CaCl_2$, 10 m*M* Hepes, and 5 m*M* glucose. The patch pipette was filled with a solution containing 140 m*M* CsCl, 0.5 m*M* $CaCl_2$, 5 m*M* EGTA (*p*Ca ≈ 7.3), 10 m*M* Hepes, and 5 m*M* Mg^{2+}–adenosine triphosphate. The perfusion solution was the same as the extracellular solution, plus 5 μ*M* glycine, 1 μ*M* tetrodotoxin, and any indicated drug; delivery was from a large-bore pipette fed by a rotary selector valve.
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35. The absolute value of the LDH efflux produced by a given toxic exposure was consistent among sister cultures from a single plating but differed among different platings, largely as a function of neuronal density. Therefore, in each experiment LDH values were scaled to the near maximal levels obtained by standard exposures to 500 μ*M* NMDA for 5 min or 300 μ*M* NMDA for 24 hours.
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HIP-70: A Protein Induced by Estrogen in the Brain and LH-RH in the Pituitary

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Estrogen and luteinizing hormone–releasing hormone (LH-RH) interact to influence both behavior and gonadotropin release. However, little is known about the biochemical mechanisms that mediate the effects of these hormones or their interactions. The most prominent protein induced by estrogen in the ventromedial hypothalamus has the same amino-terminal sequence as the most prominent protein induced by LH-RH in the pituitary in vitro and in vivo; these proteins comigrate on two-dimensional gels. Furthermore, the hormonal induction may be caused by modification of a constitutive protein with the same molecular weight (70,000) but a slightly more acidic isoelectric point, whose level is inversely related to the level of the induced form after estrogen treatment. Thus estrogen and LH-RH may interact by additively or synergistically inducing this protein, which is called HIP-70.

ESTROGEN ACTS ON HYPOTHALAMIC neurons to regulate sexual behavior and gonadotropin secretion (*1*). LH-RH acts on neurons in the hypothalamus and central gray area to facilitate female sexual behavior, but only in the presence of estrogen (*1, 2*). Similarly, LH-RH acts on the pituitary to facilitate gonadotropin secretion both in vivo and in vitro (*3, 4*); this facilitatory effect of LH-RH is enhanced by estrogen in vivo and in vitro (*3, 5*). However, the biochemical mechanisms by which estrogen and LH-RH facilitate behavior and gonadotropin secretion are unknown, as are the mechanisms by which these hormones synergize.

The most prominent estrogen-induced protein synthesized in the rat ventromedial hypothalamus (VMH) is a 70-kD protein (EI70A) that is transported to the midbrain central gray (MCG) (*6*). Estrogen appears to influence lordosis by inducing a polypeptide in the VMH that is transported to the MCG (*1, 7*). Although several proteins are induced by estrogen in the ventromedial nucleus of the hypothalamus and may play a role in lordosis, EI70A is the only induced protein known to be transported to the MCG. The most prominent LH-RH–induced protein synthesized in the rat anterior pituitary gland is a protein (LHRH70A) originally reported to have a molecular size of 69 kD (*4*). This protein represents about 0.1% of the total protein from pituitaries treated with LH-RH in vitro, but it is not detected in pituitaries not treated with LH-RH (*4, 8*). The physical similarity of the proteins induced by the pituitary and hypothalamus and the similar induction kinetics [1 to 4 hours after hormone treatment (*6, 8*)] suggested that the same protein might mediate some effects of both hormones. This could provide a basis for interaction; for example, each hormone could independently cause a 10-fold induction of the protein, leading to a potential multiplicative 100-fold induction and a magnification of the effects of both hormones.

The induction of EI70A by estrogen tends to be correlated with a reduction of a protein (EI70B) which, on two-dimensional gels, migrates to a slightly more acidic isoelectric point (*p*I) than EI70A (*6, 9*). This finding suggests that estrogen induces EI70A by modifying its acidic isoform. There has been no clear evidence that LH-RH induces LHRH70 by such a mechanism, since the pituitary protein equivalent to EI70B (LHRH70B) has not been reported to be influenced by LH-RH. To examine if EI70A is the same protein as LHRH70A, and to examine if EI70A and LHRH70A are modifications of the slightly more acidic proteins EI70B and LHRH70B, we used recently developed techniques (*10*) to determine the amino-terminal sequences of EI70A and LHRH70A, and their corresponding acidic neighbors, directly after separation on two-dimensional gels.

By means of two-dimensional gel electrophoresis we separated proteins from the VMH of ovariectomized rats that had been implanted with estrogen-filled silicone elastomer capsules 14 hours before they were killed by decapitation (after carbon dioxide anesthesia), and proteins from pituitary glands from intact rats anesthetized with sodium pentobarbitone and injected intravenously with LH-RH (50 ng per 100 g of body weight) at 1230 hours on proestrus. The proteins were then transferred to a polyvinyldifluoride membrane and stained with 0.1% Coomassie blue in 50% MeOH (Fig. 1). Spots corresponding to EI70A,

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EI70B, LHRH70A, and LHRH70B were determined on the basis of position (with respect to common protein landmarks) and visible hormone inducibility. The brain and the pituitary gland have different protein patterns, although some common proteins can be identified (Fig. 1). In the region of 70-kD proteins with a *p*I of 5.9, estrogen induced a protein in VMH, and LH-RH induced a protein of similar mobility in the pituitary gland (Fig. 1). The estrogen-induced VMH 70-kD protein was EI70A reported in (*6*), as indicated by its relation to internal markers and by the usual absence of this protein in tissue from ovariectomized rats. The LH-RH–induced protein (LHRH70A) also migrated to the same relative position in the gel as the LH-RH–induced protein reported previously (*4, 8*). To determine whether EI70A comigrated precisely with the LH-RH–induced protein, the same internal markers were identified on gels from VMH and pituitary. When internal markers close to the induced proteins were superimposed, EI70A was precisely aligned with LHRH70A (Fig. 1). Similarly, the proteins acidic to EI70A and LHRH70A, designated EI70B and LHRH70B, were also precisely aligned (Fig. 1).

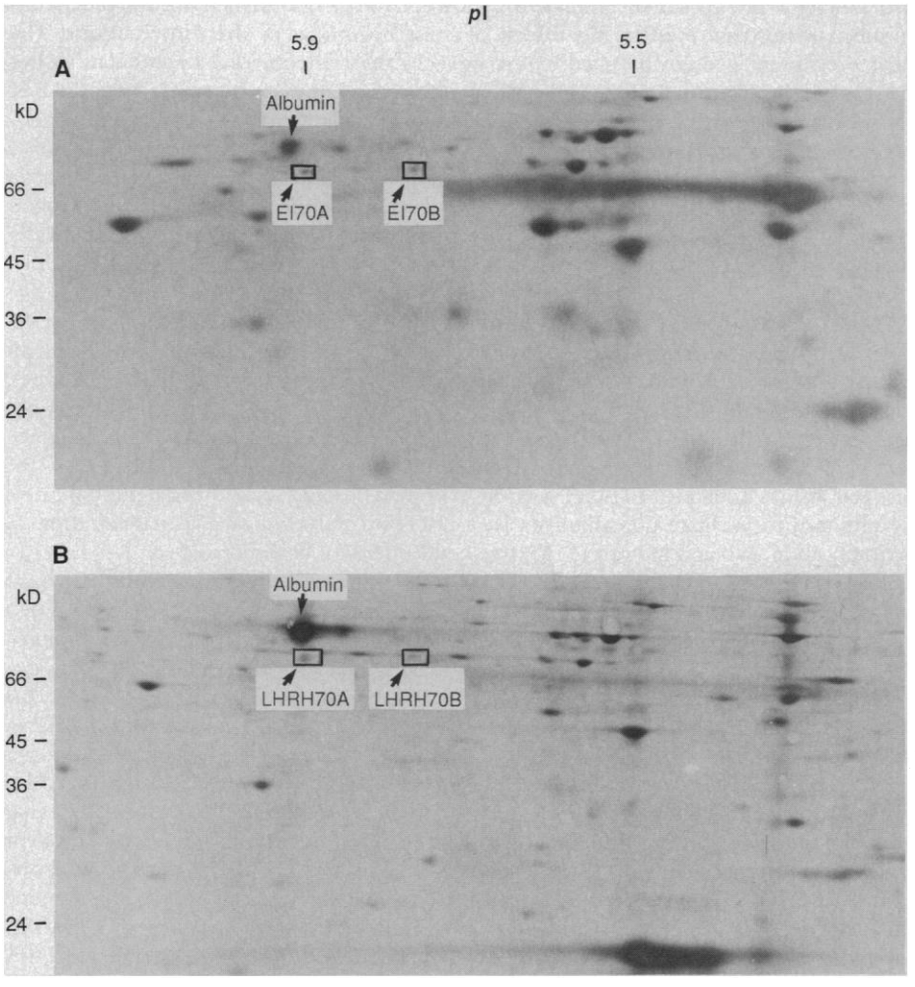

Fig. 1. Visualization of (**A**) estrogen-induced protein (EI70A) and its acidic isoform (EI70B) from VMH and (**B**) LH-RH–induced protein (LHRH70A) and its acidic isoform (LHRH70B) from the pituitary after two-dimensional gel electrophoresis, followed by transfer to polyvinyldifluoride (Immobilon, Millipore) and visualization by staining with Coomassie blue. The left-right orientation corresponds to a basic-acidic *p*I gradient, so proteins on the left are more basic. (A) Induction and detection of estrogen-induced hypothalamic EI70A was as in (*6, 8*). Female Sprague-Dawley–derived rats (from Charles River, Boston, Massachusetts; maintained at Rockefeller University, New York) were ovariectomized by the supplier and maintained under controlled lighting (lights on 0600 to 1800 hours) and temperature (23°C). The rats were 3 to 4 months old and were given free access to Purina rat chow and tap water. Rats were given a single 5-mm subcutaneous implant containing crystalline 17β-estradiol (Sigma) and were killed 14 hours later. The VMH was removed by microdissection (*6, 8*) and stored frozen. (B) Induction and detection of LH-RH–induced pituitary LHRH70A was as in (*4, 8*). Adult female Wistar rats (200 to 250 g; from Charles River UK, Margate, Kent; maintained at Edinburgh University, Edinburgh) were maintained under controlled lighting (lights on 0500 to 1900 hours) and temperature (22°C) and allowed free access to rat chow (Labsure, Hanea, Cambridgeshire) and tap water. Estrous cycles were followed by the daily inspection of vaginal smears, and only those animals that had shown at least two consecutive 4-day cycles immediately before experimentation were used. On the morning of proestrus, female rats were given an intravenous injection of LH-RH in saline (50 ng per 100 g of body weight) and anesthetized with pentobarbitone (36 mg/kg, intraperitoneally) between 1230 and 1330 hours. One hour later, by which time LH-RH had dramatically increased pituitary sensitivity to itself (*3*), rats were killed by decapitation and the pituitaries were frozen. Proteins were solubilized and subjected to two-dimensional gel electrophoresis (*6, 8*) and transferred to a polyvinyldifluoride membrane for 4 hours at 4°C in 25 m*M* tris-base, 192 m*M* glycine, *p*H 8.3, and 20% MeOH. The filter was dried for 1 hour, then wet in MeOH for 5 s, washed for 5 min in water, stained in 0.1% Coomassie blue R-250 in 50% MeOH for 5 min, destained in 50% MeOH and 10% acetic acid for 5 min, washed in water for 10 min, and then air-dried for 30 min.

To determine the amino-terminal sequences of EI70A and LHRH70A, and their acidic neighbors, hypothalamic proteins from rats given estrogen and pituitaries from rats given LH-RH were subjected to two-dimensional gel electrophoresis, transferred to polyvinyldifluoride membranes, and stained with Coomassie blue. The amino-terminal sequences of these proteins are given in Table 1. The sequences of EI70A, LHRH70A, EI70B, and LHRH70B are identical. The sequence did not match the sequence of any known protein in GenBank.

These results demonstrate that the most prominent protein induced by estrogen in the rat hypothalamus (EI70A) and the most prominent protein induced by LH-RH in the rat pituitary (LHRH70A) share an identical amino-terminal sequence. Furthermore, these proteins comigrate during two-dimensional gel electrophoresis with a molecular size of 70 kD and a *p*I of 5.9. Because our gels are reproducible and even a single charge difference will alter migration patterns of proteins on two-dimensional gels (*11*), and because the amino-terminal sequences are identical, we provisionally conclude that EI70A and LHRH70A are the same protein, which we have called HIP-70 (hormone-induced protein–70 kD). Because the incorporation of radioactive amino acids into HIP-70 has been demonstrated in the hypothalamus (*6*) and in the pituitary in vitro (*4*), it is clear that HIP-70 is induced independently in both tissues. EI70B and LHRH70B also comigrate with each other, and the amino-terminal sequences of these proteins are also identical to each other and to EI70A and LHRH70A.

The hormone-induced modification that leads to the appearance of EI70A and LHRH70A is unknown, but the apparent shift in *p*I from the acidic isoforms is consistent with a single dephosphorylation event (*11*). Although estrogen and LH-RH have different primary mechanisms of action, their mechanisms of action could converge

Table 1. Amino-terminal sequence of LH-RH–induced pituitary protein (LHRH70A) and its acidic isoform (LHRH70B) and estrogen-induced brain protein (EI70A) and its acidic isoform (EI70B). For sequencing, the proteins were induced as in Fig. 1 and text. The VMH proteins from eight rats were separated on eight two-dimensional gels, transferred from two-dimensional gels to a polyvinyldifluoride membrane (Millipore), and stained as in Fig. 1. These proteins were then cut out of the membrane (boxed in Fig. 1), and spots from eight membranes were pooled and subjected to microsequencing by sequential Edman degradation on a gas-phase sequencer (Applied Biosystems, model 470A). Edman degradation cycles were continued at least five times or until cycle-specific amino acid peaks became ambiguous. X indicates an ambiguous amino acid peak.

Protein	Sequence
LHRH70A	NH_2-DVLELTDENFESRVSDTG
EI70A	NH_2-XVLELTDENFESXVSXTG
LHRH70B	NH_2-XVLELTDENXESRVSXXX
EI70B	NH_2-XVLELTXENFEXXXXXXX

by increasing the transcription or activity, respectively, of a common phosphatase (*12*) that would dephosphorylate the acidic isoforms EI70B and LHRH70B. For example, estrogen decreases the phosphorylation of a uterine protein whose phosphorylation is also regulated by adenosine 3′,5′-monophosphate (cAMP) (*13*), LH-RH–stimulated LH release may be potentiated by cAMP (*14*), peptide hormones can cause dephosphorylation through a cAMP-dependent mechanism (*15*), and there is a direct quantitative relation between the dephosphorylation of a specific protein, myosin, and the release of the neurotransmitter serotonin (*16*). The HIP-70 may represent the first example of a steroid hormone and a peptide hormone acting to decrease phosphorylation of the same protein in a common target tissue. Thus estrogen and LH-RH could each contribute to the dephosphorylation of EI70B or LHRH70B (leading to increased HIP-70) and synergistically amplify each other's ability to enhance neuroendocrine secretion. In this study HIP-70 was induced by LH-RH in pituitaries of intact proestrous rats, in which ovarian estrogen was rising but endogenous LH-RH was still too low to induce the LH surge or pituitary sensitization to LH-RH (self-priming) (*3*). It remains to be determined if LH-RH could induce HIP-70 in the absence of estrogen.

HIP-70 appears to be a novel protein. Further work will be necessary to determine the physiological function of HIP-70 and whether dephosphorylation of EI70B and LHRH70B leads to enhanced neuroendocrine secretion, but our studies have indicated the utility of combining two-dimensional gel electrophoresis with a microsequencing procedure to analyze hormone regulation in complex protein mixtures (*10*).

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Isolation of a G Protein That Is Modified by Learning and Reduces Potassium Currents in *Hermissenda*

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In *Hermissenda crassicornis* conditioned to associate light and rotation, type B photoreceptor neurons exhibit pairing-specific decreases in the potassium currents I_A and $I_{K\text{-}Ca}$, which account for many of the behavioral changes elicited by associative conditioning. To determine which proteins are involved in storage of this memory, high-performance liquid chromatography was used to examine proteins from *Hermissenda* eyes. Conditioning-specific changes in four phosphoproteins were observed 24 hours after conditioning. One of these proteins, cp20, was purified to apparent homogeneity and found to be a G protein. When injected back into *Hermissenda* type B cells, cp20 reduced I_K and $I_{K\text{-}Ca}$ in a manner indistinguishable from the reduction caused by conditioning, suggesting that this protein may play a crucial role in memory acquisition or retention.

IN THE TYPE B PHOTORECEPTOR NEURONS in the eye, associative conditioning of the marine nudibranch mollusk *Hermissenda crassicornis* with paired light and rotation causes a decrease in early K^+ (I_A) and Ca^{2+}-dependent K^+ currents ($I_{K\text{-}Ca}$) (*1*) and changes in the branching volumes of the terminal endings (*2*). Protein kinases and phosphoproteins may be involved at an early stage of these processes (*3*). For example, phosphorylation of a 20- to 21-kD protein from the *Hermissenda* eye is increased 3 hours after associative conditioning (*4*), perhaps mediated by protein kinase C (PKC) (*3, 5*). PKC, possibly together with Ca^{2+}-calmodulin–dependent kinase II, might phosphorylate this 20-kD protein, in turn producing the K^+ current changes induced by conditioning (*3, 5, 6*).

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To test these possibilities, *Hermissenda* were trained to associate light and rotation on a circular turntable (*7*), a procedure that induced a change in the phototactic response of the group exposed to paired light and rotation [latency ratio (latency in minutes after training/latency before training), 6.37 ± 1.56 (mean ± SEM, $n = 14$; $P < 0.001$; two-tailed t test)]. Animals receiving the same stimuli as the paired group, presented in a random temporal relationship, or animals receiving no light or rotation did not show a significant change [latency ratios, 1.33 ± 0.48 ($n = 12$) and 1.76 ± 1.47 ($n = 12$), respectively].

To examine how the proteins in *Hermissenda* neurons were affected by conditioning, individual eyes or circumesophageal ganglia (CNS) were isolated from trained, random, and naïve *Hermissenda* 24 hours after the end of training, and the proteins were analyzed by anion-exchange high-performance liquid chromatography (HPLC) (Fig. 1A).

The area (absorbance at 280 nm) of only