Estrogen Increases Proenkephalin Messenger Ribonucleic Acid Levels in the Ventromedial Hypothalamus of the Rat

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The effects of estrogen on proenkephalin (PE) gene expression were measured in neurons of the ventromedial hypothalamus. Slot blot hybridization analysis indicates that the levels of PE mRNA in the ventromedial hypothalamus of ovariectomized rats increase 3.1-fold after 2 weeks of estrogen replacement. *In situ* hybridization reveals that the estrogen-inducible enkephalinergic neurons are located in the ventrolateral aspect of the ventromedial nucleus, a subnucleus known to contain many estrogen-concentrating neurons. The increase in PE mRNA levels is due to both a 63% increase in the number of detectable PE mRNA-containing neurons and a 2.0-fold increase in the levels of PE mRNA per enkephalinergic neuron (1.63 × 2.0 = 3.3-fold overall induction). This estrogen-regulated enkephalinergic cell group may represent part of the neural network mediating estrogen's effects on reproductive behavior and/or other neuroendocrine processes. (Molecular Endocrinology 2: 1320–1328, 1988)

INTRODUCTION

Neurons in the mediobasal hypothalamus (MBH) which contain estrogen receptors mediate the effects of estrogen on neuroendocrine processes and reproductive behavior in the female rat (1). Ultrastructural studies (2–6) and experiments using protein synthesis inhibitors (7, 8) indicate that estrogen dramatically alters RNA and protein synthesis in these neurons. Therefore, as in its peripheral sites of action, estrogen is thought to act in the brain, in part, by regulating gene expression. By inducing the synthesis of specific neuronal gene products, estrogens may alter neurotransmission. We are interested in learning whether estrogen regulates the synthesis of neuropeptides in hypothalamic neuronal groups that contain estrogen receptors. By doing so, estrogen may affect how these hypothalamic neurons communicate with other nerve cells to alter pituitary function and behavior.

Evidence has been accumulating that hypothalamic opioidergic systems are involved in the regulation of a number of neuroendocrine processes and that the activity of these opioidergic systems may be influenced by gonadal steroids. Although all three families of opioid peptides are present in neurons of the MBH (9), we have previously demonstrated that the majority of neurons in the ventrolateral aspect of the ventromedial nucleus (VL-VM) contain proenkephalin (PE) mRNA (10). Since this nucleus contains many estrogen-concentrating neurons (1, 11) we investigated whether estrogen regulates the synthesis of PE mRNA in VL-VM neurons.

To address this question, we have employed slot-blot hybridization analysis to examine the effect of estrogen on the total levels of PE mRNA in the VL-VM. We have also used *in situ* hybridization to determine the effects of estrogen on PE mRNA levels in individual VL-VM neurons.

RESULTS

Slot-Blot Hybridization

For slot-blot hybridization analysis, total RNA was isolated from individual MBH tissue blocks. Although the dissected tissue included both the ventromedial nucleus (VMN) and the arcuate nucleus, the PE mRNA levels in this tissue are reflective of VMN levels, since there are very few PE mRNA-containing cells in the arcuate nucleus (see Figs. 4 and 5) (10). Individual RNA samples, isolated from six ovariectomized rats and five ovariectomized, estrogen-replaced rats, were used for this experiment. After correcting for minor differences in the amount of RNA from each sample bound to the filter, (see *Materials and Methods* for details), it was found that the average PE mRNA levels of estrogen-replaced

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rats were 3.1 times greater than those of the ovariectomized rats (Figs. 1 and 2).

As controls, total RNA was also isolated from the medial amygdala, another nucleus which contains both estrogen receptors and PE mRNA, and the caudate-putamen, which contains PE mRNA but not estrogen receptors. In contrast to our findings in the VL-VM, estrogen treatment did not significantly alter PE mRNA levels in either of these tissues (medial amygdala = 101% of ovariectomized control; caudate-putamen = 105% of ovariectomized control). Samples of total RNA isolated from liver tissue, which does not express the PE gene, did not contain hybridizable PE mRNA.

In Situ Hybridization

The VL-VM consists of a heterogeneous population of neurons, both morphologically and chemically (12, 13). Therefore, it is possible that estrogen may effect changes in the expression of a gene in a discrete subpopulation of VL-VM neurons. To examine this possibility, we performed *in situ* hybridization to characterize the estrogen-induced increase in PE mRNA levels at the cellular level.

In order to determine the levels of PE mRNA in individual VL-VM neurons, MBH cryostat sections were hybridized with a ^{3}H-labeled PE complementary DNA, and the resulting hybrids localized by emulsion autoradiography. Sections from 5 ovariectomized and 5 ovariectomized, estrogen-replaced rats were included in the experiment. Estrogen treatment resulted in an increase in both the number of labeled neurons per VL-VM and the number of grains per labeled neuron (Table 1 and Figs. 3–5). The average number of labeled neurons per section in the VL-VM of ovariectomized rats was 127 ± 14 (mean ± SEM); the average for estrogen-replaced rats was 207.2 ± 24. This represents a 63% increase in the number of PE mRNA expressing-neurons in the VL-VM after estrogen replacement. The number of grains per labeled neuron in the VL-VM increased 2.0-fold from 16.8 ± 0.5 (mean ± SEM) for the ovariectomized rats to 33.7 ± 0.7 for the estrogen-replaced rats. Therefore, as measured by *in situ* hybridization, estrogen replacement results in a 3.3-fold overall increase in PE mRNA levels per VL-VM (1.63 × 2.0 = 3.3) (Table 1).

The increased PE mRNA levels were not restricted to any morphologically discernible subpopulation of VL-VM neurons, nor were there any obvious regional differences in message levels throughout the VL-VM. Consistent with this observation, the lack of a biphasic distribution of PE mRNA-containing cells in the estrogen-replaced rats (Fig. 6) suggests that the estrogen induction was not limited to a specific subpopulation of enkephalinergic neurons.

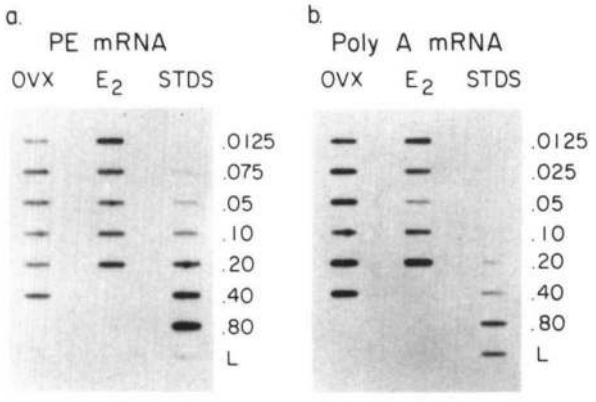

Fig. 1. Autoradiograms of Slot Blots of 1.0 μg Aliquots of Total RNA Isolated from Individual MBH of Ovariectomized (OVX) and Ovariectomized, Estrogen-Replaced (E_2) Rats

Each slot represents RNA from one rat. The filter was first hybridized with the [^{32}P]PE cDNA to determine the relative amounts of PE mRNA among samples (*left*). The same filter was stripped of the PE cDNA and rehybridized with the [^{32}P]-oligo-dT oligomer to determine the relative amounts of poly A RNA (*right*). STDS, Serial dilution of caudate-putamen total RNA (0.0125–0.8 μg). L, Liver total RNA (1.0 μg).

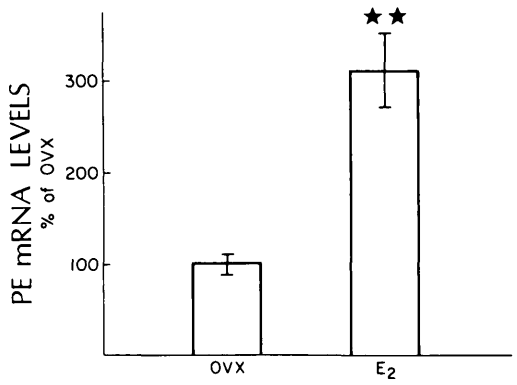

Fig. 2. Relative Amounts of PE mRNA in the MBH of Ovariectomized (OVX) and Estrogen-Replaced (E_2) Rats as Assessed by Filter Hybridization and Densitometry

Values were normalized for differences in the amount of poly A RNA present in each sample. *Bars* represent the mean ± SEM of n rats. **, Significantly different from OVX, $P < 0.005$ by *t* test.

Table 1. *In Situ* Hybridization, PE mRNA

	Grains/Cell	Labeled Cells/VL-VM	Total Grains/ VL-VM (% of OVX)
Ovariectomized (OVX) rats	16.8 ± 0.5	127 ± 14	100
Estrogen-replaced rats	33.7 ± 0.7[a]	207 ± 24[a]	334[a]

Values for grains per cell and labeled cells per VL-VM are expressed as mean ± SEM.
[a] $P < 0.005$ by *t* test.

DISCUSSION

It has been demonstrated previously that estrogen treatment increases the rate of protein synthesis in

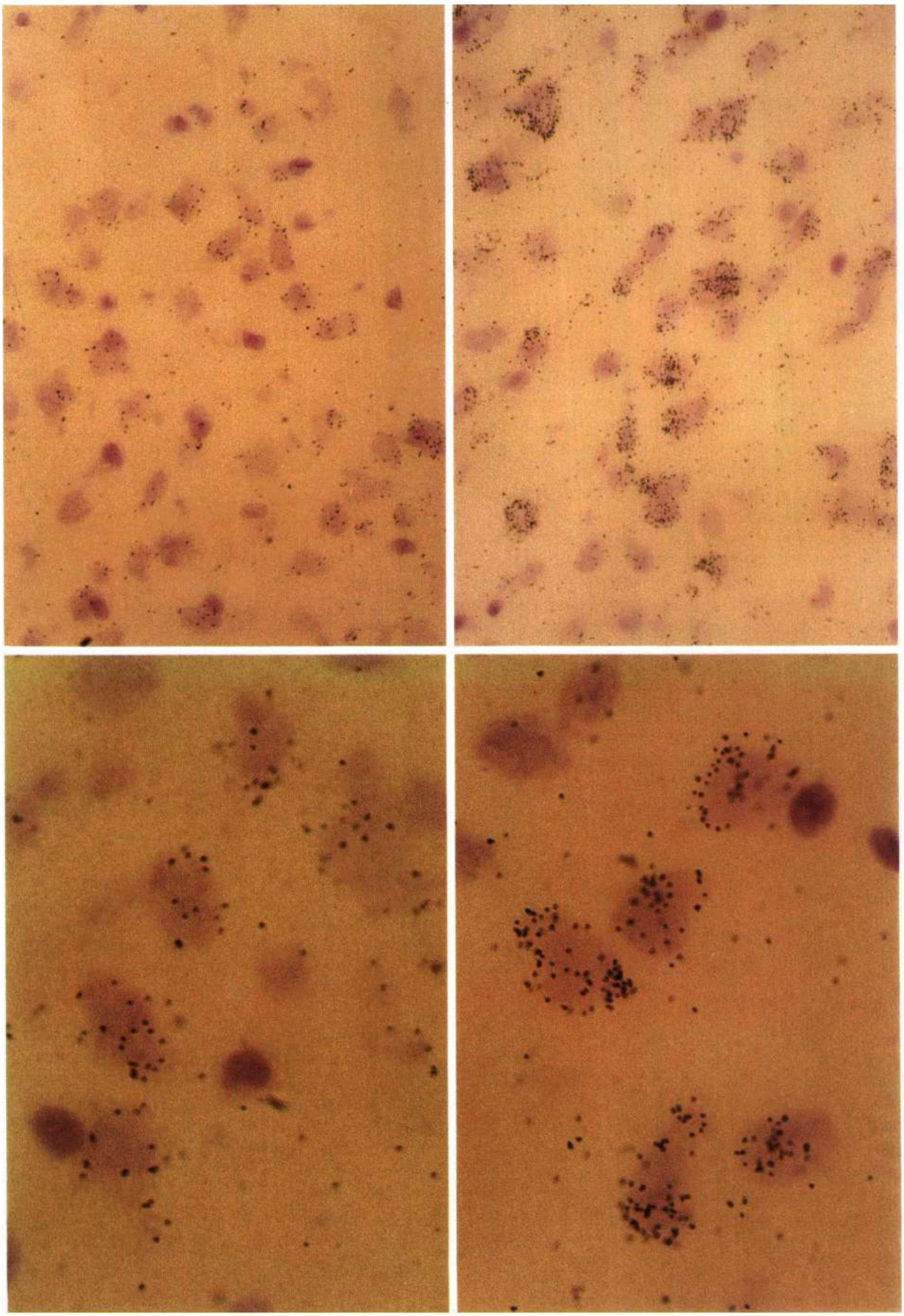

Fig. 3. Bright Field Photomicrographs of Representative Fields of VL-VM Neurons Hybridized with the [^{3}H]PE cDNA

A, Ovariectomized rat. B, Estrogen-replaced rat. C, Ovariectomized rat, high magnification. D, Estrogen-replaced rat, high magnification. Scale bars = 10 μm. Panels A and B are of the same magnification, as are panels C and D.

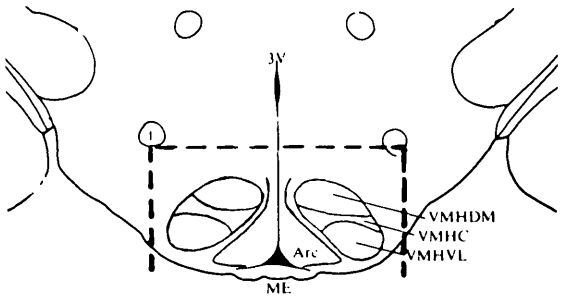

Fig. 4. Drawing of the Ventral Portion of the Rat Forebrain at the Level of the VL-VM

The *dashed lines* surround the region of the MBH which was dissected and used for RNA isolation. VMHDM, VMHC, and VMHVL, Dorsomedial, central, and ventrolateral aspects of the VMH nucleus. Arc, arcuate nucleus; ME, median eminence; 3V, third ventricle, f, fornix.

neurons of the VL-VM. Ultrastructural studies reveal an increase in nuclear and nucleolar size and an increase in the amount of stacked rough endoplasmic reticulum in the cytoplasm of these neurons (2–6). *In situ* hybridization studies indicate that the levels of ribosomal RNA are also increased (14). In addition, recent studies using two-dimensional gel electrophoresis of proteins synthesized in the VMN demonstrate that certain, as yet unidentified, proteins are induced by estrogen (15, 16). It is thought that estrogen-induced gene products are involved in altering neurotransmission between VL-VM neurons and their projection areas as well as communication into and within the VL-VM itself (5, 17, 18). Although estrogen treatment has been shown to affect several neurotransmitter systems in the VMN (19–22), we report here the first demonstration that estrogen increases the expression of a neuropeptide gene in the VL-VM.

Although we do not know whether PE gene expression is directly induced by estrogen in the VL-VM, the anatomical substrate necessary to support such a mechanism does exist. At least 40% of VL-VM neurons contain estrogen receptors (12). In the present study, we have found that approximately 80–90% of VL-VM neurons contain PE mRNA in the estrogen-replaced rat. Therefore, some of the PE-expressing cells must contain estrogen receptors and may respond directly to the hormone. The elevation in PE mRNA levels could result from an increased rate of transcription of the PE gene, an increased rate of processing of nuclear transcripts and/or an increase in the stability of the message. Experiments are in progress to distinguish between these possible mechanisms.

The increased number of detectable PE mRNA-containing neurons in the VL-VM could result from either the recruitment of neurons which previously did not express the PE gene or an increase in the levels of PE mRNA in neurons which previously had very low levels of the message. Although we are unable to distinguish between these two possibilities, it is interesting to note that the distribution curve does not simply shift to the right after estrogen treatment, as would be expected if there were a uniform, incremental increase in the PE mRNA levels of all enkephalinergic cells (Fig. 6). Rather, the distribution curve from the estrogen-replaced rats has a different shape, with a larger and extended right side. One possible explanation for this is that the 63% increase in the number of labeled cells may be due to a quantal increase in PE mRNA levels in some neurons from either zero, or low, undetectable levels to much higher levels.

It is interesting to contrast the results of this study, in which we have found an increase in both the amount of message per cell and the number of labeled cells, to those of a previous study in which we examined the effect of haloperidol treatment on PE mRNA levels in the caudate-putamen (23). In that study we found that haloperidol treatment resulted in a 3.0-fold increase in PE mRNA levels in the caudate-putamen. However, in that case the increase was entirely due to increased levels of the message per neuron, as there was no increase in the number of labeled neurons. It appears, therefore, that PE message levels may be regulated differently in these separate brain regions. This may reflect differences between steroidal inputs and classical synaptic inputs.

The increase in PE mRNA levels in the VL-VM suggests that these neurons are synthesizing more PE peptides. This is supported by the work of other investigators who have reported that estrogen increases enkephalin peptide levels in VL-VM projection areas. For example, several groups have found that enkephalin peptide levels in the medial preoptic area decrease after ovariectomy and are restored by estrogen administration (24, 25). In addition, met-enkephalin levels in the MBH and the preoptic area are greatest during proestrus, when serum estrogen levels are maximal (26). Our findings also comport with a recent report that enkephalin immunoreactivity in fibers of the periventricular preoptic area is positively regulated by estrogen (27). This report is especially relevant to the present study, as these fibers have been shown to originate in the VL-VM (28). This preoptic enkephalinergic fiber plexus is sexually dimorphic (27) and terminates in close proximity to preoptic area cell groups involved in the control of gonadotropin release (29). This may be of functional significance since there is evidence that opioid peptides are involved in the regulation of gonadotropin secretion by gonadal steroids (30).

Approximately 30% of the VL-VM neurons which contain estrogen receptors project to the dorsal midbrain (31), and these neurons are thought to be involved in the control of estrogen-dependent sexual behaviors. A recent study indicates that many of these neurons are enkephalinergic (32). Although most of the evidence to date suggests that β-endorphin may have an inhibitory effect on sexual behavior (33, 34), there is recent evidence that opioid δ-receptor-specific agonists facilitate lordosis behavior in rats (35). Since met-enkephalin selectively binds to δ-receptors, it could act to facilitate lordosis by binding to this receptor subtype. Consistent

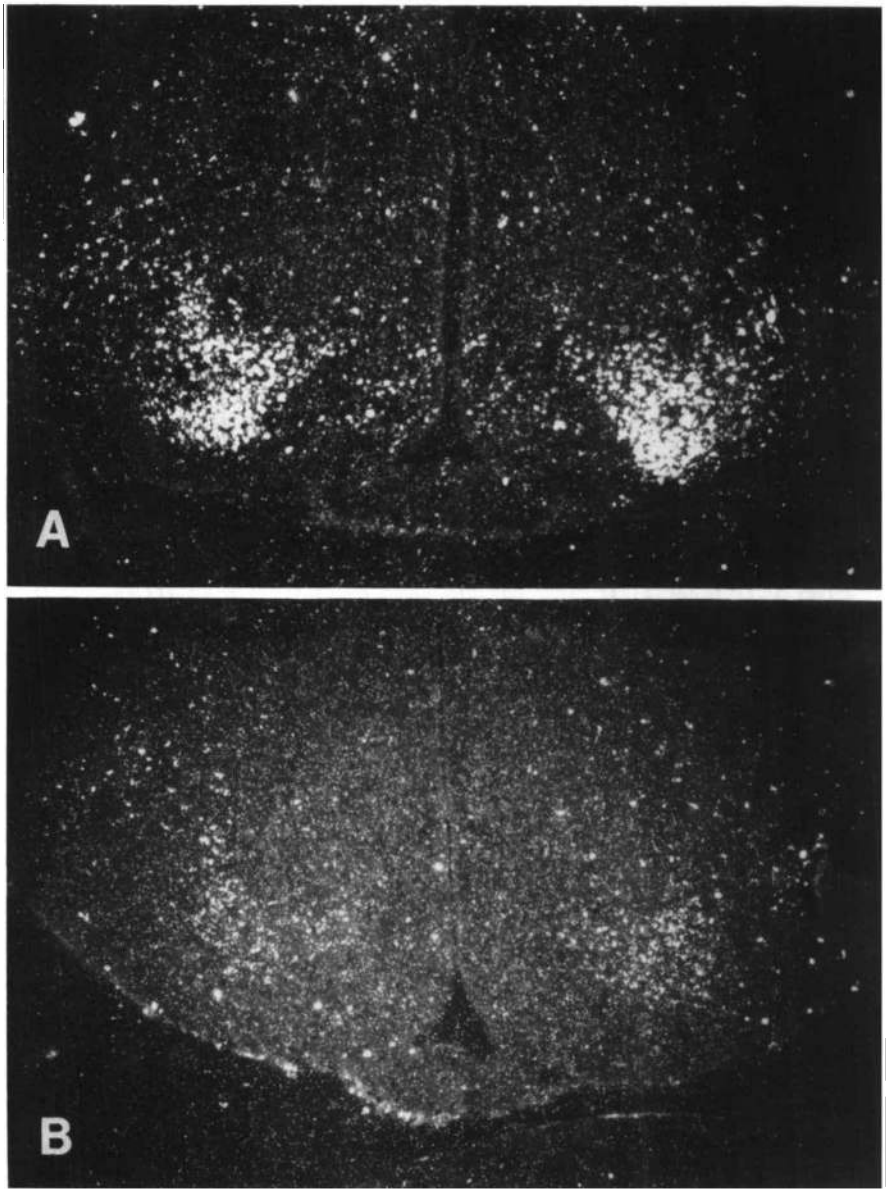

Fig. 5. Low magnification Dark Field Photomicrograph of the MBH Showing the Distribution of PE mRNA-Containing Neurons by *in Situ* Hybridization Using a [^{3}H]PE cDNA Probe

A, Representative section of an estrogen-replaced rat. B, Representative section of an ovariectomized rat.

with this is the finding that estrogen increases enkephalin levels in the midbrain (24).

The estrogen-induced increase in PE gene expression in the VL-VM may also be relevant to the finding that the release of GH from the pituitary is influenced by both hypothalamic opioid systems and gonadal steroids. Enkephalin analogs stimulate GH release (36), and this regulation is thought to occur partly at the hypothalamic level, where enkephalins have been shown to act by increasing the release of GH-releasing hormone (37) and decreasing the release of somatostatin (38). Although the role of estrogens in the control of GH secretion is not completely understood, estrogens are capable of stimulating GH release through actions at the level of the hypothalamus as well as the pituitary (39).

The excellent quantitative agreement between the results of *in situ* hybridization and slot-blot hybridization (3.3-fold *vs.* 3.1-fold) in this study is consistent with our previous findings (23) and further validates the use of *in situ* hybridization for the precise estimation of the relative levels of neuropeptide mRNAs in the brain.

MATERIALS AND METHODS

Treatment of Animals

Female Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, 200–250 g BW) were housed with

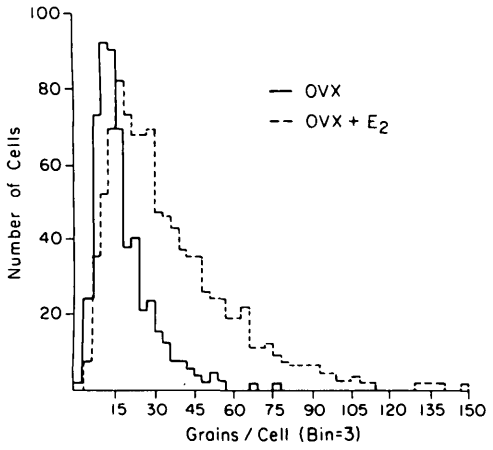

Fig. 6. Histogram Demonstrating the Frequency Distribution of PE mRNA-Containing Neurons in the VL-VM of Ovariectomized and Estrogen-Replaced Rats

Each bin represents three consecutive integral values for grains per cell.

a 12-h light, 12-h dark cycle and given food and water *ad libitum*. Rats were ovariectomized under methoxyflurane anesthesia. One week after ovariectomy the rats were anesthetized again with methoxyflurane and implanted sc with a 5-mm Silastic capsule (id, 0.059 in.; od 0.077 in.; Dow Corning Corp., Midland, MI) which was either filled with crystalline 17β-estradiol (Sigma Chemical Co., St. Louis, MO) or empty.

Brain Dissection and Tissue Preparation

Two weeks after receiving the capsules, the rats were killed by decapitation. For *in situ* hybridization, brains were removed and a coronal slice which included the entire ventromedial hypothalamus (VMH) was frozen on dry ice, wrapped in aluminum foil, and stored at −70 C until sectioning. The tissue was transferred from the freezer to a cryostat and allowed to equilibrate to −20 C before cutting. Cryostat sections (10 μm) were thaw-mounted onto glass microscope slides which had been coated with (poly) L-lysine (Sigma, 50 μg/ml). The mounted sections were then fixed in 3% buffered paraformaldehyde containing 0.02% diethylpyrocarbanate for 5 min, rinsed in PBS, dehydrated in an alcohol series, and stored at −70 C.

For RNA isolation and slot-blot analysis, the brains were removed, submerged in ice-cold saline for 30 sec, and then placed in a Jacobowitz brain slicer (Zivic-Miller) which was mounted on ice. A 2 mm slice with a rostral border at the level of the supraoptic commissure was removed and placed on a glass slide. While the tissue was being viewed with a dissecting microscope (Zeiss, New York, NY) the VMH was dissected with a razor blade. Saggital cuts were made from the fornix to the base of the brain, and a horizontal cut was made across the brain at the level of the fornix. The dissected tissue included the entire rostral-caudal extent of the VMN and the rostral portion of the arcuate nucleus that borders it (Fig. 4). The medial amygdala was dissected from this same tissue slice by cutting along the ventral border of the optic tracts and then saggitally from the optic tract to the base of the brain. Caudate-putamen tissue was dissected from a more rostral tissue slice as described previously (23). The tissue was frozen on dry ice and stored at −70 C until homogenization.

Preparation of the Rat PE cDNA Probe

The PE probe was a 435 base pair cDNA complementary to the mRNA encoding amino acids 56–200 of rat PE (40). This includes a region of the precursor that contains three Met-enkephalin peptides and the octapeptide, Met-enkephalin-Arg_6-Gly_7-Leu_8. The PE cDNA probe was excised from plasmid pRPE-1 using *Pvu*II restriction endonuclease and purified by agarose gel electrophoresis. The cDNA was nick translated using [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, 6000 Ci/mmol) to a specific activity of about 1×10^8 cpm/μg, or using all four [^{3}H]dNTPs (Amersham, 25–96 Ci/mmol) to a specific activity of approximately 10^7 cpm/μg. For *in situ* hybridization, the DNase conditions employed yielded fragments which were approximately 50–100 base pairs in length.

In Situ Hybridization

The method used for *in situ* hybridization has been previously described in detail (41). In brief, prehybridization was carried out at 37 C for 2 h and hybridization at 37 C for 48 h. For hybridization, sections were incubated with 20 μl hybridization buffer containing either 51,000 or 37,000 cpm heat-denatured [^{3}H]PE cDNA. After hybridization, the sections were rinsed twice in 2× sodium chloride, sodium citrate buffer (2× SSC, 1× SSC = 150 mM sodium chloride, 15 mM sodium citrate) containing 0.05% sodium pyrophosphate for 10 min each, and then washed in two changes of 0.5× SSC, 0.05% sodium pyrophosphate for 48 h at room temperature. After washing, the sections were dehydrated in an alcohol series in which 300 mM ammonium acetate was substituted for water and allowed to dry at room temperature. The slides were dipped in Kodak NTB2 emulsion and exposed at 4 C, desiccated, and in the dark, for 40 days. The emulsion autoradiograms were photodeveloped using Kodak D19 developer and Kodak Fix (Eastman-Kodak, Rochester, NY). Sections were stained with cresyl violet, dehydrated, cleared with Histoclear (National Diagnostics, Somerville, NJ), and coverslipped with Permount.

In Situ Hybridization Specificity Controls

To control for positive chemographic artifacts, some sections were hybridized with probe diluent alone. No labeled cells were found in these sections. Pretreatment of the sections with RNase A (Sigma, 20 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, for 30 min at 37 C before hybridization also resulted in the absence of labeled cells (10). Hybridization of the sections with a 25-fold excess of unlabeled PE cDNA before addition of the labeled probe greatly reduced the hybridization signal. In a previous study, hybridization of sections with a [^{3}H]POMC cRNA did not yield a hybridization signal in the VL-VM (41).

Grain Counting

In a quantitative study of this nature it is critically important that the concentration of probe should not be a limiting factor in the hybridization reaction. If it were, differences between treatment groups could be underestimated (see Ref. 23 for discussion). In the present study we demonstrated that the reaction was not limited by probe availability, since no differences in autoradiographic signal were observed between the sections that were hybridized with either 51,000 cpm or 37,000 cpm cDNA. The sections which received the higher probe concentration were used for grain counting analysis.

To compare accurately sections from the two treatment groups, it was necessary to choose sections from each brain that were cut through the same coronal plane, providing the same sampling of VL-VM neurons. Therefore, we used only sections that were cut in the plane perpendicular to the rostral-caudal axis of the brain at the level of the posterior VL-VM. This corresponds to levels A3990–A4110 of the rat brain stereotaxic atlas of Konig and Klippel (42). This level was chosen because a high concentration of estrogen-concentrating neurons and PE mRNA-containing neurons are present in the posterior VL-VM.

To quantitate the signal from the emulsion autoradiograms,

we determined the number of labeled neurons per VL-VM and the number of grains per labeled cell. A light microscope (Zeiss) was used to view the sections, and labeled cells and grains were counted manually. This procedure was performed on two sections per brain. When possible, neurons and grains were counted on the left side of one section and on the right side of the second section. The number of labeled neurons in the VL-VM was determined by counting all of the labeled neurons within the borders of the VL-VM. The recording of neurons was facilitated by positioning an ocular grid over the VL-VM and systematically counting the labeled neurons within each row of the grid. Since the number of labeled cells in the VL-VM was too great to include every cell in the grain-counting analysis, the following sampling procedure was employed to ensure unbiased selection of the neurons to be included for grain counting. While viewing the sections under high magnification (400×), an ocular grid (5 mm × 5 mm) was positioned over neurons in the VL-VM. The section was then moved in the dorsal-ventral direction, and the number of silver grains overlying each labeled neuron was recorded for each labeled neuron viewed within the two center rows of the grid. This was repeated two additional times for each section, repositioning the grid so that neurons were included from all dorsoventral and mediolateral portions of the VL-VM.

Background values were determined by positioning the ocular grid over a region of cortex known to be devoid of enkephalinergic cells (10) and counting the number of grains per neuron in that area. This procedure was performed for each section, and the background values were expressed as the average number of grains per neuron in this region. (Mean background levels for all rats were approximately one grain per cell.) A neuron was considered to be labeled only if the number of grains overlying it was greater than 3× background. Only those cells whose stained nuclei were visible were included in the analysis. To confirm that we were operating within the linear response range of the emulsion, plastic strips containing a serial dilution of ^{3}H (Amersham) were mounted on glass slides and dipped in emulsion with the tissue sections. These slides were developed with the tissue autoradiograms, and the linear response range of the emulsion was determined by plotting grain densities *vs.* ^{3}H concentration. The grain density of the emulsion overlying all labeled VL-VM neurons fell within the linear response range of the emulsion.

RNA Isolation

Total RNA was isolated from the VMH tissue samples by a modification of the method of Chirgwin *et al.* (43). Tissue was removed from the freezer (−70 C) and immediately placed in 2.0 ml guanidine isothiocyanate homogenization buffer (4 M guanidine isothiocyanate, 0.5% sodium *N*-laurylsarcosine, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, and 0.1% Sigma Antifoam A emulsion), broken apart by aspiration through a pipet tip, and then sonicated briefly to dissolve any remaining tissue fragments. This suspension was centrifuged at 2,300 × *g* for 1 h to pellet insoluble material, aspirated through a 18-gauge needle to shear DNA, and then layered onto a 1.0 ml bed of cesium chloride (5.7 M CsCl, 0.1 M EDTA, pH 7.0). The samples were centrifuged in a Sorvall ultracentrifuge at 28,000 rpm in a TST 41.14 rotor for 24 h at 20 C, and RNA pellets were recovered. The A_{260}/A_{280} ratio was greater than 1.8 for all samples. Total RNA concentrations were calculated from A_{260} values. Individual VMH samples, whose wet weight was approximately 10 mg, yielded 5–10 μg total RNA. RNA was isolated from the medial amygdala tissue in the same manner. The procedure for isolating RNA from the larger samples of caudate-putamen and liver has been described previously (23).

Slot-Blot Hybridization

The relative amount of PE mRNA in each of the RNA samples was determined by slot-blot analysis. Aliquots of each sample, containing 1.0 μg total RNA, were transferred to a nitrocellulose filter (pore size 0.45 μm, Schleicher & Scheull, Keene, NH), with the aid of a slot-blot filtration manifold (Schleicher & Scheull) using gentle vacuum. The nitrocellulose filter was dried under a heat lamp and then baked at 80 C under vacuum for 2 h.

The resulting slot blots were prehybridized, hybridized, and washed according to the method of Thomas (44). Briefly, the filters were incubated in 1.0 ml/10 cm^2 prehybridization buffer [50% (vol/vol) deionized formamide, 5× SSC, 50 mM sodium phosphate, pH 6.5, 0.02% each BSA, Ficoll, and polyvinylpyrolidine and 250 mg/ml sheared, sonicated, heat-denatured herring sperm DNA) for 18–20 h at 42 C. After prehybridization, the buffer was replaced with hybridization buffer [prehybridization buffer and 50% (wt/vol) dextran sulfate (4:1)] containing 5×10^{6} cpm/ml heat-denatured [^{32}P]PE cDNA. The blots were then hybridized for 18–20 h at 42 C. After hybridization, the blot was washed with agitation in 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature four times for 5 min each, and then in 0.1× SSC/0.1% SDS at 50 C, two times for 15 min each. The filters were then dried under a heat lamp and exposed to Kodak XAR-5 x-ray film using two intensifying screens (Cronex) at −80 C for variable times (hours to days).

To control for minor differences in the amount of RNA bound to the filter from each sample, the blots were reprobed with a [^{32}P]oligo-dT oligomer to determine the relative amounts of poly A^{+} RNA present in each band (23, 45). The filters were stripped of the PE cDNA by washing in 0.1% SDS at 100 C, two times for 15 min each. Autoradiograms of the stripped blots were examined to ensure complete removal of the PE probe. The oligo-dT oligomer (12–18 nucleotides in length, P. L. Pharmacia, Piscataway, NJ) was labeled with ^{32}P and T_4 polynucleotide kinase (Bethesda Research Laboratory, Bethesda, MD) to a specific activity of 1.2×10^{8} cpm/μg. Prehybridization and hybridization were carried out as described for the PE cDNA except that formamide was excluded from the hybridization buffers. The reprobed blots were washed with 2× SSC-0.1% SDS at room temperature two times for 15 min each, and then exposed to x-ray film for autoradiography as described above.

Separate filters were used for the VMH, medial amygdala, and caudate-putamen RNA samples. Liver RNA, which served as a negative control tissue for PE mRNA, was applied to each blot. In addition, transfer RNA was applied to each filter to serve as a negative control for the oligo-dT hybridization. Each filter also contained a serial dilution curve of caudate-putamen RNA which was used to confirm the linearity of the autoradiographic signal.

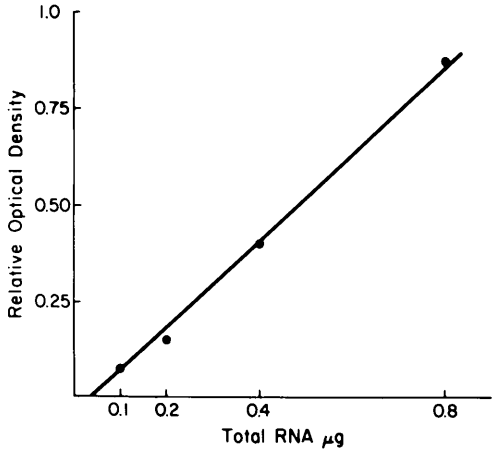

Fig. 7. Plot of the Amount of Caudate-Putamen RNA Applied to the Slot Blot *vs.* Absorbance Values of the Corresponding Bands on the Autoradiogram after Hybridization with PE cDNA

The relative absorbance values of the bands on the autoradiograms were determined using a flat-bed laser scanning densitometer. The absorbance values for individual bands on the autoradiograms of PE-probed blots were normalized to the absorbance values of the corresponding band on the autoradiogram of the oligo-dT probed blot. The corrected values were then expressed as the ratio: relative absorbance units PE mRNA per relative absorbance units poly A^+ mRNA. Comparisons of RNA levels were made only between bands which were on the same filter. Autoradiogram exposure times were chosen so that the absorbance values of all bands were within the linear response range of the x-ray film and the densitometer (Fig. 7).

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